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Original Research

# A Study of Coagulation Profile in Cases of Snake Bites

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## ABSTRACT

In the present cross sectional study we have studied coagulation profile and clinical manifestations in cases of snake bite and correlated the severity of clinical findings with the coagulation profile. A total of 158 cases of snake bite were included for analysis of coagulation profile in this analytical study by excluding the neurotoxic cases. Of these, 67 (40.6%) cases were non-venomous, whereas 98 (59.4%) cases were venomous snake bite cases. Out of the 98 venomous snake bite cases, 91 (55.4%) cases were vasculotoxic snake bite and 07 (4.2%) cases were neurotoxic snake bite. We observed a positive result of 20 minute whole blood clotting test (WBCT) in 53 (58.2%) vasculotoxic snake bite cases. The sensitivity of 20 minute WBCT was 66.2% and specificity was 97.5%. Local pain was the most common local symptom observed in 133 (84.2%) cases followed by swelling at the site of bite which comprised of 127 (80.4%) cases. All the investigative steps were carried out and classified according to ICD-10 standards.

KEY WORDS: *Non-venomous snake bites, Vasculotoxic, Venomous snake bites, Coagulation profile, Bleeding time, Clotting time*

## 1. INTRODUCTION

### *1.1 Background*

Snake Bite is a major industrial and rural possibility, since the country of Exotic snakes has always been India. More than 60 venomous snakes are populated in India. Spectacular cobra (Naja Naja), common krait (Bungarus caeruleus), saw-scaled viper (Echis carinatus) and vipers of Russell (Daboia russelii) are amongst the most frequent species found in India [1-3].

A reliable longitudinal estimate of the mortality of snake bite is quite complicated as most of the instances arise in remote areas. Throughout tropical and subtropical regions with heavy rain as well as hot atmosphere, Snakebite is a general urgent medical problem for rural populations. Very few medical trials on snake envenomation are currently available, particularly on hematological envenomation problems [3,4].

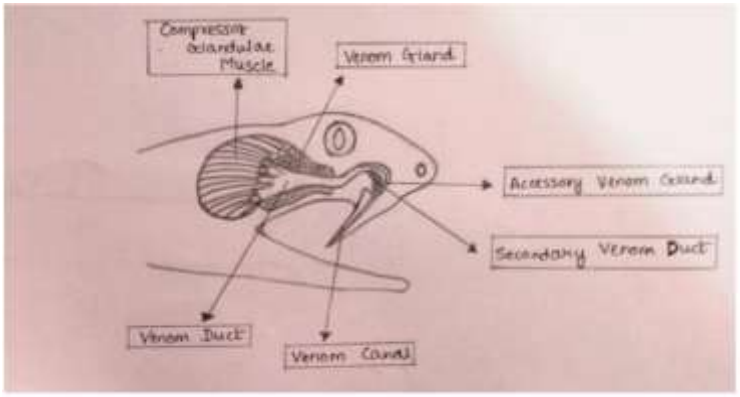


**Figure 1: Venom apparatus of snake**

Most contaminants in snake venom combine and induce coagulopathy with the coagulation process and fibrinolytic method. Regional and neurological effects consistent with snakebite correlated with snake venom toxins [5].

The precise epidemiological causes or even fatalities in shark bite cases in Maharashtra are not reported correctly. There are an approximate 10 000-50 000 annual deaths in India , notably after cobra bite, common krait, Russell's viper, as well as a vipere on a range [6].

Study of coagulation profile helps in early diagnosis of DIC and prevention of such complications. Considering the above facts, the present study of coagulation profile in cases of snakebite was undertaken.

*1.2 Aims and Objectives*

1. To detect changes in Coagulation profile in cases of snake bites.
2. To study the coagulation profile in cases of snake bites.
3. To study different clinical manifestation in cases of snake bites.
4. To correlate severity of clinical findings with the results of coagulation
5. Profile.

## 2. LITERATURE REVIEW

Fontana et al (1781) was the first to describe snake venom & blood coagulants interactions. Mitchell & Richert (1886) proved that altered coagulation was associated with globulin fraction of venom. Most of the venom show exhibit procoagulant and anticoagulant activities [7].

According to the study done by Kumar et al. [8], Snake-bite is an environmental, occupational and climatic hazard in rural area. A conducive ecology is available more in the rural areas for this reptiles to inhabit. In this study, the number of cases from rural area was 92.4% out of the total snake bite cases. Among these cases, 86% were vasculotoxic snake bite.

According to [9] thrombin like enzymes also have anticoagulant activity involving two separate enzymes one activating fibrinogen & other activating plasminogen. This accounts for increased levels of FDP. The effect of Snake venom on blood clotters is summation of 3 mechanisms i.e. pro-coagulant, anticoagulant and fibrinolytic activities presenting as DIC coagulopathy.

The male to female ratio was recorded as 1.6:1 in a the study done by Chaudhari et al., [10]. Males showed a higher incidence as compared to females which may be because predominantly males were involved in farming.

## 3. MATERIALS AND METHOD

The present study was a two-year cross-sectional study, carried out in the Department of Pathology in our KIMS, Karad. Our study includes snake bite coming to our tertiary care hospital during the period of 2 years. A written informed consent was taken from all the cases included in the study for the treatment, investigations and research purpose.

After the probable clinical diagnosis of snake bite, the blood sample was drawn from all the snake bite patient under

aseptic precautions to perform 20 minutes Whole Blood Clotting Test (WBCT), hematological tests, coagulation tests, serological tests and other additional investigation if required. The patients with positive result of 20 minutes WBCT and clinical picture of poisonous snake bite were started with polyvalent ASV immediately.

The results of hematological and coagulation investigations of the blood sample taken just after admission in the casualty department were included for analysis purpose. Certain tests were repeated by the clinicians as and when required by them, but the findings of the repeat test were not included in the present study.

The patient was followed up till the discharge. The treatment and outcome data was noted in the proforma along with the results of all the investigations. The categorisation of snake bite into non-venomous, vasculotoxic and neurotoxic bite was done after considering the clinical data and all the investigations result and classified according to ICD-10.

The ICD-10 code is as follow:

- Snake bite -T63.0
- Non-venomous W59
- Venomous X20

### 3.1 Investigative Analysis

*Complete blood count*

It included Haemoglobin, Total leucocytes count, Differential leucocytes count and platelet count. For complete blood count the blood sample was taken in EDTA vacutainer. The blood collected in the vacutainer is then tested in 5 part Sysmex XT 1800i automated haematology analyser. The count is then noted down.

*Erythrocyte Sedimentation Rate (ESR)*

Done by Westergren method. The complete blood count and platelet counts were confirmed by peripheral examination of smears.

*Coagulation tests*

For all the coagulation tests, the blood was collected in Trisodium citrate vacutainer.

*D-Dimer assay*

Done on Transasia semiautomated Erba CHEM-5 plus analyser using the Quantia Quantitative Turbidimetric Immunoassay DDimer kit from Tulip Diagnostics.

*Bleeding time*

Bleeding time of patient was done by Dukes method.

*Clotting*

Clotting time is done by capillary method.

*Biochemical tests*

For all Biochemical tests the blood was collected in plain vacutainer.

*Serum Creatinine*

Done on the automated analyzer using the principle of Jaffe method.

*Blood Urea*

Done on the automated analyzer using the principle of modified Fawcett and Scott method.

*Blood sugar*

Done on the semiautomated analyzer.

*Liver Function Test*

Done on the automated biochemistry analyzer.

*Other investigations*

Urine, CPK-MB, ECG, Chest X ray and USG. The statistical data was done by using Graphpad Instat software.

### 3.2 Complete Blood Count

Done on Sysmex XT 1800i Automated haematology analyser with 5 parts differential function for invitro diagnostics. XT 800i can analyse 24 parameters of blood sample.

*Other Parameters analysed*

Prothrombin Time, Activated Partial Thromboplastin Time, Clotting factors and Fibrinogen.

## 4. OBSERVATIONS AND RESULTS

*4.1 Observations*

The research paper is a two-year cross-sectional study, carried out in the Department of Pathology in our institute. Our study includes cases of snake bite admitted in our tertiary care hospital during the period of 2 years.

**Table 1: Distribution of snake bite cases according to clinical presentation.**

| Clinical diagnosis | | Total cases | Percentage(%) |
|---|---|---|---|
| Non-Venomous Snake Bite | | 67 | 40.6 |
| Venomous Snake Bite | Vasculotoxic snake bite | 91 | 55.2 |
| | Neurotoxic snake bite | 07 | 4.2 |
| Total | | 165 | 100 |

During the study period, a total of 165 snake bite cases were admitted in our hospital. Of these, 67 (40.6%) were non-venomous and 98 (59.4%) were venomous snake bite cases. Out of the 98 venomous snake bite cases, 91 (55.2%) were vasculotoxic and 07 (4.2%) were neurotoxic cases.

In findings a total of 158 cases of snake bite were included for analysis of coagulation profile in the present study by excluding the 07 neurotoxic cases. So hereafter, 158 cases was considered as 100 % for further study.

Out of the 158 snake bite cases included in this study, maximum number of cases were observed in third and fourth decades which comprised of 37 (23.4%) and 32 (20.2%) cases respectively. The youngest patient in the present study was a one year old child; while oldest were two patients of 75 years.

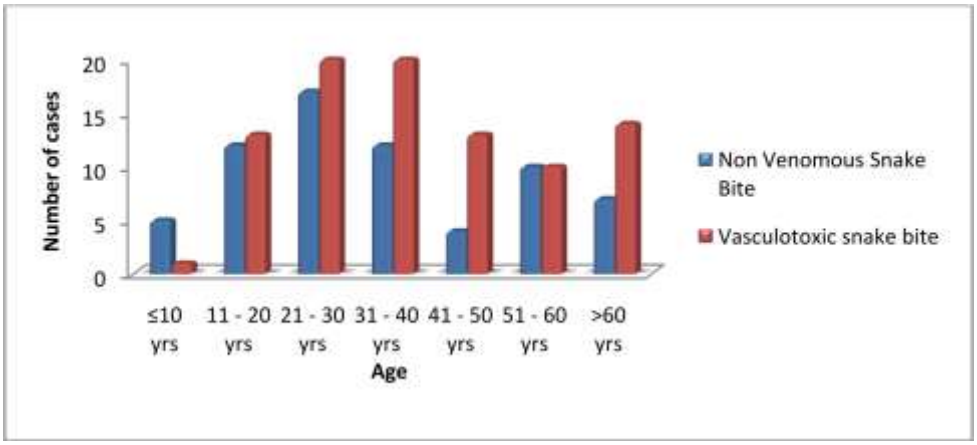


**Figure 2: Cases of snake bites in relation to age**

**Table 2: Distribution of cases of snake bites based on gender**

| Sex | Non-Venomous Snake Bite | Vasculotoxic snake bite | Total cases |
|---|---|---|---|
| Female | 27 | 34 | 61 (38.6%) |
| Male | 40 | 57 | 97 (61.4%) |
| Total | 67 | 91 | 158 (100%) |

In the present study, male preponderance was noted with 97 (61.4%) cases, whereas 61 (38.6%) cases were seen in females.

**Table 3: Distribution of snake bite cases based on occupations**

| Occupation | Non-Venomous Snake Bite | Vasculotoxic snake bite | Total cases |
|---|---|---|---|
| Children | 04 | 00 | 04 (2.5%) |
| Contractor | 01 | 00 | 01 (0.6%) |
| Driver | 00 | 01 | 01 (0.6%) |
| Farmer | 40 | 71 | 111 (70.3%) |
| Housewife | 07 | 02 | 09 (5.7%) |
| Shopkeeper | 00 | 01 | 01 (0.6%) |
| Student | 15 | 15 | 30 (19.1%) |
| Sugar factory worker | 00 | 01 | 01 (0.6%) |
| Total | 67 | 91 | 158 (100%) |

Our findings show that out of 158 total patients, 111(70.2%) snake bite cases were of farmers.

*Other finding*

In this study, tourniquet application was the most commonest primary treatment received before admission to our tertiary care centre which comprised of 80 (50.6%) out of 158 snake bite cases.

ASV was received by 18 (11.4%) out of 158 snake bite cases at primary health centre before admission to our tertiary care centre. Herbal medicine was applied at the diseased site by 05 (3.2%) out of 158 snake bite cases. Incision was made at the diseased site by 08 (5.1%) out of 158 snake bite cases.

Primary treatment was not received by 47 (29.7%) out of 158 snake bite cases before admission to our hospital.

*4.2 Haemotoxic/vasculotoxic manifestations*

In the present study, non-venomous snake bite presented only with local symptoms. However, vasculotoxic snake bite cases showed Gum bleed, hematuria, hematemesis, hemoptysis, Epistaxis, ecchymosis, Oliguria, local bleed, abdominal pain, vomiting and cellulitis on admission to our hospital. Out of these presentations, local bleeding was the most common presentation which comprised of 60 (65.9%) cases followed by cellulitis in 23 (25.3%) cases among the 91 vasculotoxic snake bite.

**Table 4: 20 minuts WBCT test results**

| 20 minute WBCT | Non-Venomous | Vasculotoxic | Total |
|---|---|---|---|
| Blood Clotted | 67 | 38 | 105(66.5%) |
| Blood not clotted | 00 | 53 | 53(33.5%) |
| Total | 67 | 91 | 158(100%) |

Out of the 53 cases with positive WBCT, 51 cases had INR more than 1.4 and remaining 02 cases had INR less than 1.4. Out of the 105 cases with Negative result of WBCT, 26 cases had INR value of more than 1.4 and 79 cases had INR value less than 1.4. The sensitivity of 20 minute WBCT was 66.2% and Specificity was 97.5%.

**Table 5: Observations based on INR parameters**

| INR | Non-Venomous bite | Vasculotoxic bite | Total Cases |
|---|---|---|---|
| 1 - 1.4 | 67 | 14 | 81 (51.3%) |
| 1.5 – 3.5 | 00 | 40 | 40 (25.3%) |
| >3.5 | 00 | 37 | 37 (23.4%) |
| Total | 67 | 91 | 158 (100%) |

In the present study, INR was increased in 77 (48.7%) cases and INR was within normal range in 81 (51.3%) cases out of the 158 snake bite cases. Among the cases with increased INR, 37 (23.4%) cases had INR value of more than 3.5. All the cases with increased INR belonged to the vasculotoxic snake bite.

**Table 6: PT analysis**

| PT(in Seconds) | Non-Venomous | Vasculotoxic | Total Cases |
|---|---|---|---|
| 12-16sec | 67 | 14 | 81(51.3%) |
| 17-180sec | 00 | 54 | 54(34.2%) |
| >180sec | 00 | 23 | 23(14.5%) |
| Total | 67 | 91 | 158(100%) |

In the present study, PT was increased in 77 (48.7%) cases and PT was within normal range in 81 (51.3%) cases out of the 158 snake bite cases. Among the cases with increased PT, 23 (14.5%) cases had a PT value of more than 180 seconds. All the cases with increased PT belonged to vasculotoxic snake bite.

**Table 7: APTT analysis of snake bites**

| APTT(in seconds) | Non-Venomous | Vasculotoxic | Total cases |
|---|---|---|---|
| 25 - 40 sec | 67 | 24 | 91 (57.6%) |
| 41- 200 sec | 00 | 42 | 42 (26.6%) |
| >200 sec | 00 | 25 | 25 (15.8%) |
| Total | 67 | 91 | 158 (100%) |

In the present study APTT values were within normal range in all 67 cases of non-venomous snake bite. However, out of 91 vasculotoxic snake bite cases, (42+25) patients indicated increased values of APTT.

**Table 8: D-Dimer analysis of snake bites**

| D-Dimer(ng/ml) | Non-Venomous | Vasculotoxic | Total cases |
|---|---|---|---|
| 200-250 | 67 | 59 | 126 (79.8%) |
| >250 | 00 | 32 | 32 (20.2%) |
| Total | 67 | 91 | 158 (100%) |

In the present study, values of D-Dimer Assay were seen increased above normal limits in 32 (20.2%) cases out of 158 snake bite cases.

**Table 9: Bleeding time analysis of snake bites**

| Bleeding Time (Minutes) | Non-Venomous | Vasculotoxic | Total cases |
|---|---|---|---|
| <2 min | 05 | 03 | 08 (5.1%) |
| 2-7 min | 62 | 75 | 137 (86.7%) |
| >7 min | 00 | 13 | 13 (8.2%) |
| Total | 67 | 91 | 158 (100%) |

In the present study, Bleeding time was increased in 13(8.2%) cases out of 158 snake bite cases, while all the remaining snake bite cases had bleeding time within normal range.

**Table 10: Clotting time analysis of snake bites**

| Clotting time(minutes) | Non-Venomous | Vasculotoxic | Total cases |
|---|---|---|---|
| 4 - 9 min | 67 | 15 | 82 (51.9%) |
| >9 min | 00 | 76 | 76 (48.1%) |
| Total | 67 | 91 | 158 (100%) |

In the present study, Clotting time was increased in 76 (48.1%) cases out of the 158 snake bite cases. All the patients with increased Clotting time belonged to vasculotoxic snake bite category. All 67 non venomous snake bite cases and 15 out of 91 vasculotoxic snake bite cases showed Clotting time within normal range.

**Table 11: Vasculotoxic snake bite cases based on altered coagulation profile and platelets**

| Parameters | Altered status | Number of patients (Total = 91 cases) |
|---|---|---|
| Prothrombin time | Increased | 77 (84.6%) |
| APTT | Increased | 67 (73.6%) |
| D-Dimer assay | Increased | 32 (35.2%) |
| Clotting time | Increased | 76 (83.5%) |
| Bleeding time | Increased | 13 (14.3%) |
| Platelet count | Decreased | 26 (28.6%) |

In the present study, Prothrombin time was increased in 77 (84.6%) cases, APTT was increased in 67 (73.6%) cases, value of D-Dimer assay was increased in 32 (35.2%) cases, clotting time was increased in 76 (83.5%), bleeding time was increased in 13 (14.3%) cases and thrombocytopenia was noted in 26 (28.6%) cases out of the 91 vasculotoxic snake bite cases.

All the vasculotoxic snake bite cases received ASV. Fresh Frozen Plasma was given to the 49 snake bite cases showing bleeding diathesis. PCV was given to 07 snake bite cases with haemoglobin level less than 8g/dl. All the 11 cases of acute renal failure received hemodialysis. Surgical intervention was done in 14 out of 23 cases of cellulitis. Antibiotics were given to 71(44.9%) cases out of 158 snake bite, while all snake bite cases received tetanus toxoid prophylacticaly.

## 5. CONCLUSION

Results from this study showed that vasculotoxic snake bite causes coagulopathy, spreading cellulitis and ARF. Laboratory tests like PT, INR, APTT and D-Dimer assay proved to be very sensitive and specific in early detection of derangement in coagulation. Results of these tests correlated well with the severity of clinical features and complications. Timely intervention with hemodialysis and support with appropriate use of blood components helped to reduce severity of complications. Though, 20 minute WBC test as marker of coagulation abnormality showed low sensitivity, it had high specificity. Thus, it can be used as an initial investigation which can be carried out at bedside for detecting coagulopathy among the snake bite cases

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Original Research

# Congenital Malformations in Fetal Andearly Neonatal Autopsies in a Tertiarycare Hospital

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## ABSTRACT

The awareness regarding fetal and neonatal (perinatal) mortality and its preventable causes is increasing. It has led to increase in finding the preventable causes of death in perinatal period. Out of 223 perinatal autopsies performed 57 (25.56%) had congenital Malformations. On comparing the sex ratio, the congenital malformations were slightly more in males (52.63%) with male to female ratio being 1.25:1. In the present study, there were 3 cases (5.26%) where sex of the fetus was ambiguous. Most congenital fetuses ranged from 20 to 24 weeks (63.15%) of their gestational ages. This could be attributed to therapeutic termination done in this period after detecting malformation on Ultrasonography. In this study the 13/57 (22.80%) cases showed multiple malformations occurring in single fetus or neonate. Variety of syndromes have been identified in the study like Down's syndrome, Potter's syndrome etc.

KEY WORDS: *Down's syndrome, neonatal, malformations*

## 1. INTRODUCTION

Antenatal care in India is rising due to improvement in awareness; still few congenital malformations can be missed in routine check-ups. Few congenital malformations can be diagnosed prenatally with ultrasonography techniques, various maternal serum assays, confirmation relies on actual examination of the fetus or neonate [1].

For quite some time, congenital malformations have been reported to be a major cause of mortality and morbidity in children in the developed countries [2]. In the recent years, congenital disorders are becoming to be public health issue in developing countries, due to an epidemiological transition,which involves significant decline in infant mortality rates due to reduction of infections and malnutrition and relative increase of morbidity and mortality due to congenital malformations [2-4].

Antenatal investigations, such as ultrasonography (USG), maternal serum enzyme hormone assays cannot determine significant number of congenital malformations, for which autopsies are must [5-6]. Fetal and early neonatal autopsy in cases of congenital malformations not only confirms but also provides additional information and is helpful in counseling the parents regarding prevention of similar congenital malformations in future pregnancies [7].

*Risk factors and associated Factors for congenital malformations*

Congenital malformations can present in a single organ, system, or may involve multiple organs of body. Major congenital malformation can lead to fetal or early neonatal

loss due to incompatibility to cope up with life [8].

*Neonate Death*

When baby is born alive and death occurs between birth to the 28th day of life, it is defined as neonatal death. Type: Early (upto 7 days after birth), Late (8th 28th day after birth) [9].

This study is therefore undertaken to ascertain various congenital malformations and to classify those according to organ system involved and measure the utility of autopsy in final diagnosis.

*1.1 Aims and Objectives*

1. To study congenital malformations in fetal and early neonatal autopsies
2. To detect congenital malformations in fetal and early neonatal autopsies.
3. To diagnose visceral malformations in fetal and early neonatal autopsies and categorize those according to organ system.
4. To compare antenatal ultrasonography findings with those of autopsy findings.
5. To perform cytogenetic studies to confirm morphological diagnosis wherever required.

## 2. LITERATURE REVIEW

Maternal age is an important parameter in the birth of congenitally malformed fetuses.21 Maximum number of fetuses 39 (68.42%) were born to mothers in age group of 20-24 years. This finding was similar with study done by Subhashini et al., (41.8%) [10] and Kapoor K et al. (60%) [11].

The exact reason for malformations in mothers of younger age group could not be found. However, it may be attributed to multifactorial etiologies like viz. genetic causes or infections.

Some authors have noticed that certain abnormalities appear more often in the first-born than in subsequent pregnancies. The incidence of malformations declines with increasing birth order [12-14]. Among the malformed fetuses or neonates, 50.87% are born to primigravida. Similar finding was seen by Kapoor K et al. (50%) [11], Subhashini et al., (40.1%) [10], and Parmar et al (42%) [15].

## 3. METHODOLOGY

*1. Sample Study*

This descriptive analytical study was carried out in Department of Pathology of a tertiary care hospital for detection of congenital malformations in fetal and early neonatal autopsies. Perinatal autopsies done in Department of Pathology between the period of 2 + 2(previous) = 4 years were studied and analyzed.

*2. Investigative Steps*

1. Relevant clinical data (maternal history and antenatal investigations)was collected from the case sheets.
2. During autopsy procedure, photographs were taken for unusual gross findings.
3. Postmortem radiographs of abnormal features were taken whenever required.
4. For each fetus or neonate, morphometric examination was done.
5. Autopsies were carried out by the standard technique (EDITH. L. POTTER).
6. Sections from each organ were submitted for histo-pathological examination. Following routine tissue processing, paraffin embedding, section cutting, staining was performed by routine Haematoxylin and Eosin stains.
7. Clinical data and the pathological findings were recorded in proforma.
8. Autopsy findings were compared with antenatal ultrasound findings.
9. Microphotographs were taken in cases with congenital malformations diagnosed on histopathological study (microscopy).

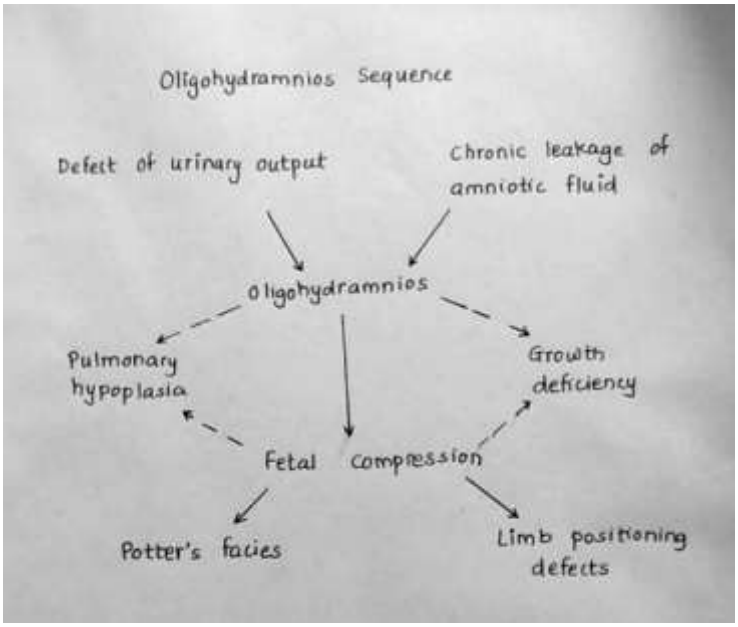


**Figure 1: In the fetus, oligohydramnios sequence can be noted morphologically on autopsy.**

## RESULTS

The present study of Congenital Malformations in fetal and early neonatal (Perinatal) autopsies carried out in Department of Pathology of a tertiary care hospital. Perinatal autopsies done during the period of 5 years were studied and data analyzed.

During the period of 5 years, the total perinatal deaths in the said hospital were 678 and total number of perinatal autopsies performed were 223. Out of these, 57 cases showed congenital malformations, which were further analyzed (Table 1). Table 1 shows fetal autopsies 94.74% and early neonates within 7 days of life were 5.26%. The ratio being 18:1.

**Table 1: Distribution of malformed fetuses and neonates**

| | Number | Percentage (%) |
|---|---|---|
| Fetuses | 54 | 94.74 |
| Neonates | 03 | 5.26 |
| Total | 57 | 100 |

**Table 2: Gender wise distribution of malformed fetuses & neonates**

| Gender | Male | Female | Ambiguous-genitalia | Total |
|---|---|---|---|---|
| Fetuses | 29 | 22 | 3 | 54 |
| Neonates | 1 | 2 | 0 | 3 |
| Total | 30 | 24 | 3 | 57 |

Table 2 shows gender wise distribution of malformed fetuses and neonates in the study. Out of 54 fetuses showing congenital malformations 29 were male, 22were female while 3 were having ambiguous genitalia.

In 3 cases with ambiguous genitalia, final diagnoses given after complete autopsy were Edward's syndrome, Sirenomelia (Mermaid syndrome) and acephalic acardiac fetus each. In 3 cases of neonates, 2 were female and 1 was a male.

**Table 3: Gestational age of fetuses and neonates**

| Gestational-age | Number | Percentage (%) |
|---|---|---|
| 20-24 weeks | 36 | 63.15 |
| 25-29 weeks | 13 | 22.81 |

| | | |
|---|---|---|
| 30-34 weeks | 03 | 5.27 |
| 35-39 weeks | 02 | 3.50 |
| Full term born (within 7 days) | 03 | 5.27 |
| Total | 57 | 100 |

Table 3 above shows gestational age of fetuses on early neonates affected by congenital malformations. Fetuses and neonates with malformations were divided in groups (age in weeks) based on their gestational age. Mostly the malformations were detected in the gestational age group of 20-24 weeks (63.15%).

Cases presented after birth were 3 (5.27%). All of them were early neonates within 7 days of age, with full term maturity.

**Table 4: Distribution of malformed Fetuses and Neonates according to weight**

| Weight in Grams | Number | Percentage (%) |
|---|---|---|
| 350-1000 | 41 | 71.92 |
| 1001-2000 | 12 | 21.06 |
| 2001-3000 | 03 | 5.27 |
| 3000-4000 | 00 | 00 |
| >4000 | 01 | 1.75 |

Above Table 4 shows weight wise distributions of malformed fetuses and neonates. The malformed perinatal autopsy cases were divided according to weight in grams. Maximum cases 41 (71.92%) were in the group of 350-1000 grams weight, followed by 12 cases (21.06%) in weight group 1001 to 2000 grams.

There was a single case above the weight 4000 grams, which was a neonate (Table 4).

Table 5 below shows various maternal factors noted in malformed fetuses and neonates.

**Table 5: Maternal Factors seen in malformed fetuses & neonates**

| Character | Number (out of 57) | Percentage (%) |
|---|---|---|
| Maternal Age in years | | |
| <20 | 00 | 00 |
| 20-24 | 39 | 68.42 |
| 25-29 | 14 | 24.56 |
| >30 | 4 | 7.02 |
| Parity | | |
| Primipara | 29 | 50.87 |
| Multipara | 27 | 47.37 |
| Grand-multipara | 01 | 1.76 |
| Consanguinity | | |
| Yes | 04 | 7.02 |
| No | 53 | 92.98 |
| H/o Previous Abortion | | |
| Yes | 14 | 24.56 |
| No | 43 | 75.44 |

Maternal factors of the malformed fetuses and neonates were analyzed under various headings (Table 5).

The most common age group of mothers with affected babies was 20 to 24 years in 39 (68.42%) cases, while in only 4 (7.02%) cases, mothers were above the age of 30 years.

Out of these 4, one case was the neonate having Down's syndrome whose mother was 36 years of age.

In 50.87% of malformed fetuses and neonates mothers were primiparama, 47.37% of mothers were multipara and there was only one 1.76% grand multipara mother.

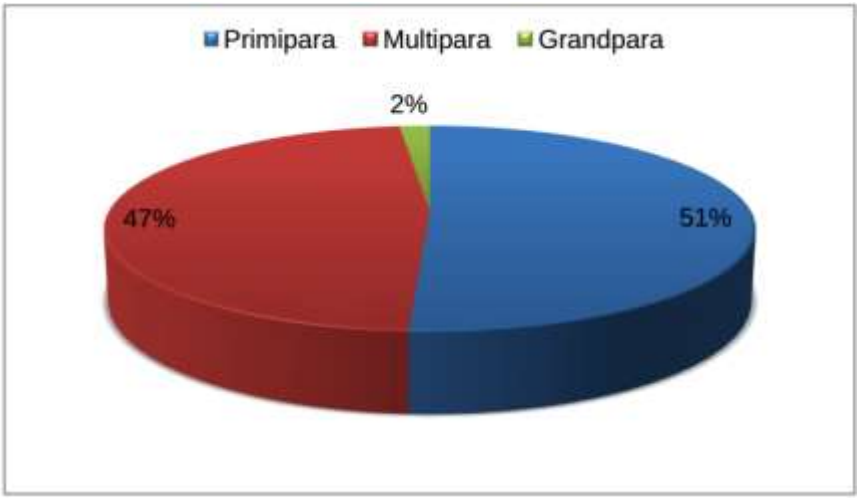


**Figure 2: Pie chart showing distribution of parity amongst mothers**

History of previous abortion was present in 14 mothers (24.56%). Two out of 14 mothers had history of repeated abortions, with one mother had history of 4 abortions.

History of consanguinity was present in 4 mothers (7.02%), amongst these only one mother was having history of previous abortion. For all 14 mothers having bad obstetric history, serology testing for TORCH complex was performed. 2 out of these 14 cases, titre positivity for TORCH complex is noted.

**Table 6: Correlations of Antenatal Autopsy and USG findings**

| Autopsy and USG findings (correlation) | Cases | Percentage (%) |
|---|---|---|
| USG findings confirmed on autopsy | 54 | 94.74 |
| No change in diagnosis | 27 | 47.37 |
| Additional findings noted in the autopsy | 27 | 47.37 |
| Changes in Diagnosis | 03 | 5.26 |
| Total | 57 | 100 |

Table given above shows a correlation of antenatal USG findings with autopsy findings.

Correlation of antenatal ultra-sonography findings with final autopsy findings in all the cases was done.

The autopsy findings in 27 (47.37%) cases showed additional information than ultrasonography. Ultrasonography findings were same as autopsy in 27 (47.37%) cases.

On autopsy, there was change in diagnosis in 3 (5.26%) cases, as shown in Figure 3.

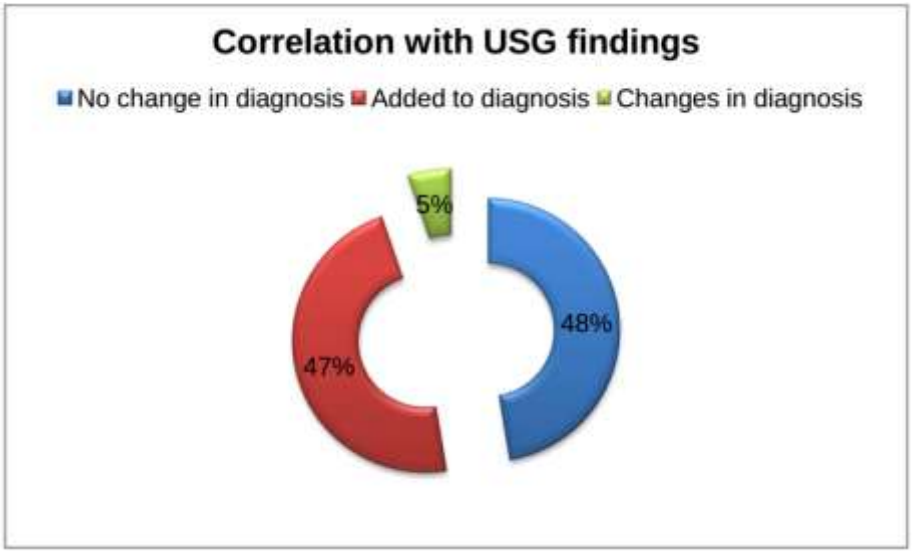


**Figure 3: Pie diagram showing correlation of USG and autopsy findings**

- Various systems involved in malformations in all cases were evaluated for their frequency.
- Most common system involved was musculo-skeletal in 30 cases, followed by central nervous system in 17 cases.
- Musculo-skeletal system malformations ranged from simply club foot to lethal malformation such as limb body wall complex.
- Central nervous system was affected in 17 cases, with mostly lethal malformations like craniospinal rashischisis.
- Cardiovascular system was affected in 10 cases, and gastrointestinal in 9 cases.
- Gastrointestinal system malformations were always associated with malformations of different other systems indicating its longer duration of embryogenesis.

Spectrum of congenital malformations observed in all the perinatal autopsies. Various congenital malformations in the study were classified according to organ systems involved as per WHO recommendations (Figure 4).

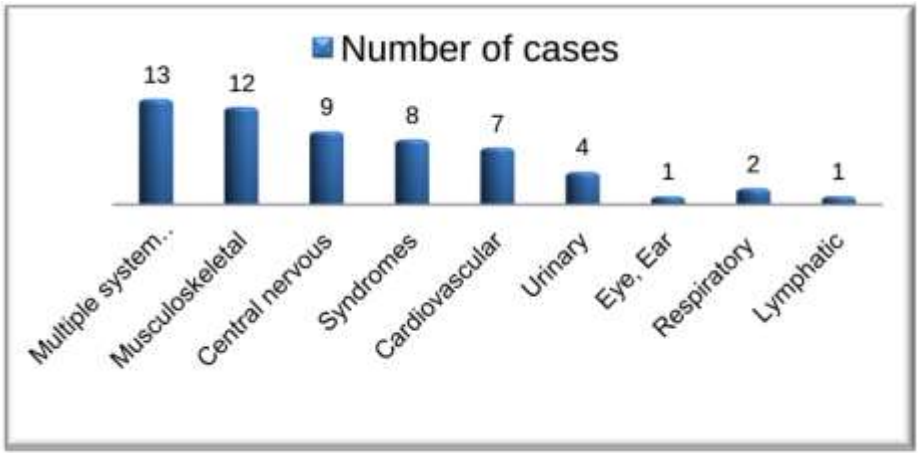


**Figure 4: Bar diagram showing system wise distribution of cases of congenital malformations**

Table 7 below shows various musculo-skeletal malformations noted during the study.

**Table 7: Distribution of congenital malformations involving musculoskeletal system**

| Congenital Malformations | Associated malformations | Number of cases |
|---|---|---|
| Diaphragmatic hernia | Hypoplastic lungs | 03 |
| Omphalocele | Hypoplastic lungs | 01 |
| CTEV | | 02 |
| CTEV, Rocker Bottom foot | | 01 |
| Radial Ray anomaly | | 02 |
| Limb deformity | | 01 |
| Hypoplastic Nasal Bone | Premaxillary protrusion | 01 |
| Arthropogyrosis | | 01 |
| Total | | 12 |

Most common system involved was musculoskeletal with 12 cases (21.05%). Diaphragmatic hernia was observed in three cases. Three cases of diaphragmatic hernia also had hypoplastic lungs as secondary involvement.

Limb deformities, congenital talipes equinovarus constituted other cases of musculoskeletal system. One case was diagnosed as Arthropogyrosis due to involvement of multiple joints in malformation.

**Table 8: Distribution of congenital malformations involving CNS**

| Congenital Malformations | Associated malformations | Number of cases |
|---|---|---|
| Spina Bifida | | 02 |
| Spina Bifida | Single umbilical artery | 01 |
| Spina Bifida, anencephaly, Craniofacial Rashischisis | | 01 |
| Spina Bifida, Hydrocephalus | | 01 |
| Anencephaly | | 01 |
| Craniospinal Rashischisis | | 01 |
| Holoprocencephaly | | 01 |
| Meningomyelocele, Hydrocephalus | | 01 |
| Total | | 09 |

Above table (Table 8) shows central nervous system malformations seen in the study. Central nervous system malformations were seen in 9 cases (15.79%) with common malformation being spina bifida.

Spina bifida has also been related to many CNS system malformations suggesting its similar embryological period of development.

Out of all 57 cases, maximum number of cases showed multisystem involvement of malformations. Respiratory system was affected in 2 cases (3.51%) with one having bilateral hypoplastic lungs and other being congenital adenomatoid malformation. 13 cases (22.81%) out of 57, had multiple malformations affecting various systems. Out of these cases, commonest system affected was musculoskeletal in association to systems like central nervous system and/ or cardiovascular system.

Gastrointestinal system malformations were always associated with malformations of different other systems indicating its longer duration of embryogenesis.

## 4. CONCLUSION

Following conclusions were drawn from the results of the present study. Perinatal autopsy is useful in detecting and confirming congenital malformations in fetuses and neonates. Identification and classification of the visceral congenital malformations can be achieved with good autopsy technique.In the present study, the commonest system affected by congenital malformations was musculo-skeletal system (19.30%).

Despite advances in imaging such as antenatal ultrasonography and serology, perinatal autopsy is superior and continues to play an important role in diagnosing congenital malformations. The findings of autopsy are not only of theoretical importance but also of practical significance to clinicians in the form of estimating the risk of recurrence and in genetic counseling.

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Original Research

# Pattern of Utilization of Blood and Blood Components in a Teaching Hospital

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## ABSTRACT

The present study was observational analytical study carried out in a teaching hospital from July 2014 to December 2015. Total transfusions carried out were 10358 in this study period of 18 months. These total transfusions were further analyzed into whole blood and component use. PCV was the most commonly used component for anemia patients as well as for thalassemic patients. All cryoprecipitate transfused during study were utilized by Medicine department for hemophiliac patients. Total 3619 transfusions, were carried out in medicine department during study period. No whole blood was transfused in radiotherapy department during study period. Along with decrease in use of whole blood, PCV use was significantly increased for transfusions. Significant finding noted during study period, Platelet use for transfusion was slightly reduced with increase in FFP transfusions.

KEY WORDS: *Blood components, Blood transfusion, Blood transfusion, Immunization*

## INTRODUCTION

Blood transfusion has come a long way from early 20th century when it was a complex and risky procedure. Currently transfusion medicine is a speciality in its own right. Blood is an amazing fluid. Blood is the most precious and unique gift that one human being can give to another human being. In addition, not many studies from India have evaluated the use of component therapy partially because components are not made available by most blood banks. Services used in a broad range of hospital procedures, accidents, emergency obstetric services, and other surgeries. The demand for blood is increasing day by day because of urbanization and industrialization, road traffic accidents, advancement of medical science, advancement of surgical procedure like cardiac surgery, transplant surgery. The emphasis has shifted from the use of whole blood to component therapy, as blood is a scarce and precious resource. Currently Good Clinical Practice (GCP) guidelines mandate transfusion therapy for specific well established indications and use of blood components rather than whole blood.

## AIM AND OBJECTIVES

### *AIM*

Pattern of utilization of blood and blood components in a teaching hospital.

### *OBJECTIVES*

To study the utilization pattern of blood and blood components in a teaching hospital. To study the indications

for transfusions for different components during the study period.

## REVIEW OF LITERATURE

In 1869 Braxton Hicks suggested sodium phosphate as nontoxic anticoagulant as clotting was a major obstacle to the transfusions which were being carried out.[1] Karl Landsteiner discovered ABO group and explained serious reactions of incompatible transfusions in 1901 [2] and this revolutionized blood transfusions.

A sterile, anticoagulant or preservative solution provides the necessary metabolic nutrients to maintain intracellular energy and decreases the possibility of bacterial contamination. Refrigeration storage temperature slows the metabolism down and minimizes bacterial proliferation.[3]

Hemolytic reactions occur most commonly in women due to prior RBC immunization during pregnancy. Varying incidence of 1 in 6200 to 1 in1400 transfusions is observed.[4] One unit of packed cell contains CPD or CPDA-1 as preservative- anticoagulant. It is prepared by removing 200-250 ml plasma from whole blood and it has a haematocrit of 70% to 80%.[5] If 100 ml additive solution is added, then haematocrit drops to 50 to 60%.

A unit of RBCs contains approximately 250 mg of iron. As red cells are destroyed, the majority of the released iron cannot be excreted and is stored in the body as hemosiderin and ferritin. Transferrin becomes saturated after the administration of 10-15 units of RBCs to a non-bleeding patient and iron accumulates in the reticuloendothelial system, liver, heart, spleen, and endocrine organs causing tissue damage leading to heart failure, liver failure, diabetes and hypothyroidism. Patients on long term treatment therapy needs iron chelation prophylactically.[6] After light spin, platelet rich plasma (PRP) is emptied into one bag and additive from other bag is added to RBCs. PRP is spun again in high spin to get platelet concentrate and platelet poor plasma. Plasma is either frozen and stored as fresh frozen plasma (FFP) or frozen and thawed at 4ºC to get plasma and cryoprecipitate. Platelet concentrate has to be prepared within 6 hours of collection of whole blood. Supernatant from cryoprecipitate preparation depleted in Factor VIII and Fibrinogen is called cryo-poor or cryo-supernatant plasma.[7]

Surgery based on the judgment of surgeon and anesthetist. Usually not necessary if hemoglobin is more than 10 gm% pre-op and advisable if hemoglobin is less than 7.0 pre-operative 16. Patient approaching delivery and has Hb less than 7g/dl. Anemia Transfusion should be done on clinical indication and not solely on hematological indications. Hemoglobin of less than 7g/dl is generally considered an indication for transfusion while hemoglobin more than 10 does not warrant transfusion. Values between 7g/dl and 10g/dl should be considered with background clinical setup. In deficiency anemia, transfusion done only to tide over hemodynamic compromise or presence of active bleeds in presence of deficiency status. In hemolytic anemia, transfusion is usually required. In hypo proliferative disorders, patient is usually dependent on transfusion.

Post transfusion increment after transfusion of 1 unit platelet is 5-8 x 109/L. Average adult dose is 1 unit / 10 kg body wt. / transfusion episode. Normal platelet survives 8 days in circulation. Transfused platelet survives 5-6 days. Patient‟s response to platelet transfusion should be determined by checking platelet count within 1 hour of transfusion and also in 16-24 hours. Quality assurance scheme in a blood transfusion service is basically intended to ensure safe and effective blood and blood components. Quality Control is an integral part of Quality assurance as it provides the means to measure and monitor the quality procedure, reagents and instruments. The two most commonly used types of water are pyrogen-free distilled water and pyrogen-free deionized water, each of which should be maintained at 80ºC. Water preparation and delivery systems should be tested at regular intervals for pyrogenicity and conductance. The water system should be continuously circulating and should have no dead ends. An adequate supply of steam should be provided for the cleaning of equipment and the operation of apparatus used to sterilize the equipment and containers. The steam should be maintained to a standard of cleanliness such that it does not cause or leave a contaminating deposit on the equipment and containers.

## MATERIALS AND METHODS

This observational descriptive study for 18 months (July 2014 - December2015) on pattern of utilization of blood and

blood components was carried out in the Blood Bank of Department of Pathology in a teaching hospital. Different components prepared in the blood bank were noted. Data regarding sex of the patient, indication of transfusion, blood or blood component to be used, different blood groups used, department and units where transfusion to be carried out etc. were noted down from the daily records of blood bank. The transfusions were also categorized according to unit of department using the blood or blood components.

*Inclusion Criteria*

All the transfusions of blood or blood components during the study period in our Hospital.

*Exclusion Criteria*

Units issued out-side hospitals other than our hospital for transfusion. The data was analyzed for the pattern of blood and blood component usage by different specialties, for different indications in different patients.

The results obtained were tabulated and pattern of utilization was noted.

## OBSERVATIONS AND RESULTS

**Table 1: Utilization of blood & blood components in 18 months**

| Blood and Blood Component | Number | Percentage |
|---|---|---|
| WB | 139 | 1.35 |
| PCV | 6871 | 66.33 |
| FFP | 1674 | 16.17 |
| PLT | 1670 | 16.12 |
| CRYO | 4 | 0.03 |
| TOTAL | 10358 | 100 |

There were total 10358 transfusions which were carried out during the study period of 18 months. Whole blood and component utilization was calculated in all these transfusions. Out of all transfusions,139 were whole blood transfusions, 6871 were packed cell transfusions, 1674 fresh frozen plasma transfusions, 1670 platelet transfusions, and 4 were cryo-precipitate. According to the observation maximum component use was attributed to PCV (66.33%), followed by almost equal percentage by fresh frozen plasma (16.17%) and platelets (16.12%). Rarely used component was cryo-precipitate (0.03%). (Table No.1)

**Table 2: Gender wise distribution of utilization of total transfusions**

| Gender | Number of transfusions | Percentage |
|---|---|---|
| Male | 5756 | 55.56 |
| Female | 4602 | 44.44 |
| Total | 10358 | 100 |

Total transfusions were divided according to gender of recipient. There were 5756 male and 4602 female recipient patients. The male to female ratio was 1.25:1. (Table No. 2)

The pattern of utilization of different components according to the specialized departments was also analyzed.

**Table 3: Department wise distribution of total transfusions**

| Department | Total Transfusions | Percentage |
|---|---|---|
| Medicine | 3619 | 34.94 |
| Surgery | 2518 | 24.30 |
| Obstetrics- Gynecology | 1566 | 15.12 |
| Orthopedics | 632 | 6.10 |

| | | |
|---|---|---|
| Radiotherapy | 1030 | 9.95 |
| Pediatrics | 993 | 9.59 |
| Total | 10358 | 100 |

The maximum use of blood and components was noted in Medicine department (34.94%), followed by Surgery department (24.30%). Minimum transfusions were carried out in Orthopedics department (6.10%). (Table No. 3)

**Table 4: Common indications for transfusions observed in the study**

| Indication of Transfusion | WB | PCV | FFP | PLT | CRYO | Number | % |
|---|---|---|---|---|---|---|---|
| Anemia | 47 | 3461 | - | - | - | 3508 | 33.87 |
| Thalassemia | - | 478 | - | - | - | 478 | 4.61 |
| Malignancy | - | 594 | 50 | 386 | - | 1030 | 9.95 |
| Bleeding | - | - | 1150 | 868 | 4 | 2022 | 19.52 |
| Operative | 77 | 1916 | 74 | 416 | - | 2483 | 23.97 |
| Shock | 15 | 317 | 400 | - | - | 732 | 7.07 |
| Dialysis | - | 105 | - | - | - | 105 | 1.01 |
| Total | 139 | 6871 | 1674 | 1670 | 4 | 10358 | 100 |

During the study period, the data was analyzed for blood group of ordered blood and blood group of issued blood. There was no compatible blood group transfusion for blood due to sufficient amount of availability of all the blood groups in the blood bank. Total transfusions were analyzed for the indications of transfusion. The most common indication was anemia (33.87%). For anemia commonly used component was PCV. Few times even whole blood was used for anemic patients. Another indication for transfusion was operative purpose (23.82%). Most common component used for operative purpose was PCV followed by Platelets. Transfusion for bleeding were 2022, and FFP was commonest component used followed by Platelets. (Table No.4).

## DISCUSSION

The availability of donated blood and the demand for blood components must be balanced to provide adequate supply. At present the supply of donated blood is unable to keep up with demand. Considering this, in many nations whole blood transfusion is reduced and instead whole blood is separated in to components. These components are then used separately, as indicated in according to patient's need. The present study was conducted over 18 months period. Total number of transfusions observed during study period was 10358. The most common component use was attributed to PCV (66.33%), followed by platelets (16.12%) and fresh frozen plasma (16.17%). Similar findings were noted by studies done by Richa Gerg et al[8], and Mohammad Z Q et al.[9] Rarely used component was cryoprecipitate (0.03%). This is comparable with study done by Mohammad Z Q et al[9] in 2015. In our study the whole blood use was only 1.35% out of whole transfusions. Similar finding was noted by Venkatachalapathy et al [10](8.55%) in 2012.

The whole blood utilization was limited to few indications such as heavy blood loss and emergency. Also noted was in given period the use of whole blood reduced gradually indicating the impact of transfusion medicine in teaching. However, this was not in concordance with study done by Guar DS et al[11], indicating lack of awareness. A very striking difference was seen in the use of blood and blood components by the speciality departments. In our study, the maximum transfusions were seen in Medicine department (34.94%). Study done by Agarwal et al also shows similar results (44.13%) for medicine department .[12]

## CONCLUSION

The present observational descriptive study was conducted in a teaching hospital from July 2014 to Dec 2015. From the present study, following conclusions can be drawn. The study provides data regarding requirement of blood and blood component use in this teaching hospital. It is necessary to study different component requirement so as to improve component separation to avoid wastage and shortage. Regular clinical meetings on transfusion medicine for indication of different components are necessary to achieve

judicial use of components. More such studies are needed to standardize the component utilization to improve patient care.

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Original Research

# Medicinal plants used by traditional healers from South-West Algeria: An ethnobotanical study

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## ABSTRACT

**Background/Aim:** This study aimed to document and analyzes the local knowledge of medicinal plants' use by traditional healers in South-west Algeria. **Methods:** The ethnobotanical survey was conducted in two Saharian regions of South-west of Algeria: Adrar and Bechar. In total, 22 local traditional healers were interviewed using semi-structured questionnaire and open questions. Use value (UV), fidelity level (FL), and informant consensus factor (FIC) were used to analyze the obtained data. **Results:** Our results showed that 83 medicinal plants species belonging to 38 families are used by traditional healers from South-west of Algeria to treat several ailments. Lamiaceae, Asteraceae, Apiaceae, and Fabaceae were the most dominant families with 13, 8, 6, and 4 species, respectively. Leaves were the plant parts mostly used (36%), followed by seeds (18%), aerial parts (17%) and roots (12%). Furthermore, a decoction was the major mode of preparation (49%), and oral administration was the most preferred (80%). Thymus vulgaris L. (UV = 1.045), Zingiber officinale Roscoe (UV = 0.863), Trigonella foenum-graecum L. (UV=0.590), Rosmarinus officinalis L. (UV = 0.545), and Ruta chalepensis L. (UV = 0.5) were the most frequently species used by local healers. A great informant consensus has been demonstrated for kidney (0.727), cancer (0.687), digestive (0.603), and respiratory diseases. **Conclusion:** This study revealed rich ethnomedicinal knowledge in South-west Algeria. The reported species with high UV, FL, and FIC could be of great interest for further pharmacological studies.

KEY WORDS: Algeria, ethnobotanical, medicinal plants, phytotherapy, traditional healers, use-value

## INTRODUCTION

According to the WHO statistics, about 80% of African populations use traditional medicine for their primary health care. In recent years, there has been a remarkable rise of medicinal plant's use, probably due to their local abundance, cultural significance and inexpensive procurement [1]. An urgent need to develop national pharmacopoeia, monographs of medicinal plants, and national standards and guidelines has been emphasized [2]. It has been reported that of 121 anticancer drugs used today, 90 are derived from plants. In addition, 60% of new drugs introduced between 1981 and 2002 are plants derived [3]. Although, the development of new active natural drugs requires integration of several sciences such as botany, chemistry and pharmacology, recording how a plant is used in folk medicine by an ethnic group is the major common strategy [4]. In addition, ethnobotanical studies play an important role for the conservation and valorization of biological resources [5].

Medicinal plants have been used in Algeria for centuries to treat different ailments. Although Algeria is one of the richest Arab countries with 3164 plant species [6], few ethnobotanical studies have been carried out in the country [7,8]. In South of Algeria, the Sahara, one of the world-largest deserts, local populations still relay on traditional healers for their health care. Thus, the aim of this study was to document and analyze the local knowledge of medicinal plants' use by traditional healers in South-west Algeria.

## MATERIALS AND METHODS

### Study Area

Sahara, the world's largest non-polar desert covers 84% of the total Algerian area (2.381.741 km$^2$). The ethnobotanical survey was conducted in two Saharian regions of South-west of Algeria: Adrar and Bechar, both located on the borders between Algeria and Morocco [Figure 1]. Adrar (27°52′N, 0°17′W) is the second-largest department of the country covering about 427,368 km$^2$ [9]. Bechar (31°37' N, 2°13' W) covering an area of 161,400 km$^2$ is the sixth-largest department in the country. Climate is hot and dry in summer and very cold in winter with 100 mm rainfall per year [10].

### Data Collection

This study has been carried out between 2010 and 2014, in several times. We interviewed individually 22 traditional healers

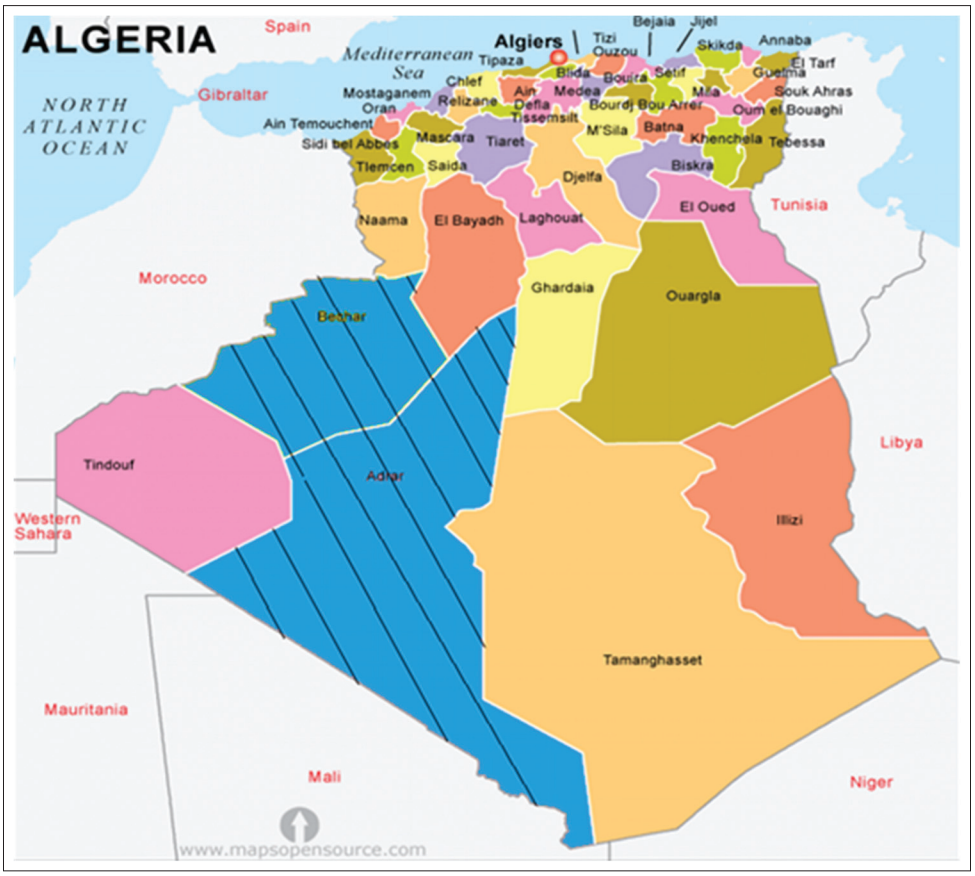


**Figure 1:** Location of the study area (Adrar and Bechar, South-West Algeria)

practicing in the study area, after obtaining their consent. Semi-structured questionnaire and open questions were used to record the use of medicinal plants (vernacular names, ailments treated, parts used, modes of preparation/administration, and ingredients). Local names were given in Arabic and/or in Amazigh or Tergui languages. Botanical identification and authentication were done by Dr. Kada Righi (Department of Agriculture, Faculty of Nature and Life sciences, Mascara University, Algeria). The voucher specimens were prepared and submitted to the LRSBG herbarium (Department of Biology, Faculty of Nature and Life Sciences, Mascara University, Algeria). All the informants were men and their age was 37 ± 11 years.

The ailments reported to be treated using the cited species were grouped into 12 categories [Table 1]. Each citation of a particular part of a particular plant was recorded as one use report. If one informant used a plant to treat more than one disease in the same category, it was considered as a single use-report [11].

### Quantitative Analysis

Use-value (UV), fidelity level (FL), and informant consensus factor (FIC) were calculated using the following standard formulas [12]:

Use-value: $UV = \Sigma U/n$

U: Number of use reports cited by each informant for a given plant species,

n: Total number of informants interviewed for a given plant.

Fidelity level (FL): $FL\ (\%) = (Np/N)*100$

Np: Number of use reports for a given species reported to be used for a particular ailment category,

N: Total number of use reports cited for any given species.

Informant Consensus Factor: $FIC = (Nur–Nt)/(Nur–1)$

Nur: Number of use citations in each category,

Nt: Number of species reported in each category.

## RESULTS

### Botanical Data, Used Parts, Mode of Preparation, Routes of Administration and Ailments Treated

In this study, 83 medicinal plants species belonging to 38 families [Figure 2] were reported to be used by traditional healers from South-west of Algeria to treat several ailments [Table 2]. In consistence with most of ethnobotanical studies around the world, leaves were the plant parts mostly used (36%) by local healers in South-west of Algeria. In addition, seeds (18%), aerial parts (17%), and roots (12%) were also the most used parts [Figure 3]. We found that a decoction was the major mode of preparation (49%). In addition, different medicinal plants are used as raw (32%), infused (16%), or macerated (3%) [Figure 4]. Oral, topical, inhalation, and nasal routes were the reported ways of administration in the study area. As shown in Figure 5, most herbal remedies in South-west Algeria were

Table 1: Ailments grouped by different ailment categories

| Category | Ailments/disorders | Abbreviation |
|---|---|---|
| Kidneys diseases | Stone, infections | KD |
| Gastro-intestinal diseases | Hemorrhoids, stomach ulcer, stomach-ache, dysentery, colic, gases, constipation, colitis, parasites, hydatic cyst, liver problems, hepatitis, biliary problems, anemia, diarrhoea, toothache | GISD |
| Skin diseases | Skin diseases, fungal infections, burns | SD |
| Cancer | Tumors, cancers, metastases | Can |
| Endocrine system diseases | Diabetes, goitre, weight loss | ESD |
| Respiratory tract diseases | Cold, cough, asthma, bronchitis, flu , allergy | RTD |
| Skeleto-muscular system disorder | Rheumatism, arthritis, inflammation, body pain | SMSD |
| Cardiovascular system diseases | Cholesterol, high blood pressure, heart problems | CSD |
| General health | Blood purification, body pain, tonic, psychopathic disorders, systemic healing, systemic problems | GH |
| Hair care | Hair loss, hair growth | HC |
| Nervous system | Depression, anxiety, vertigo, migraine, dementia, depression | NS |
| Sexual-reproductive problems | Menstrual cramps, infertility, sexual impotence, genycological problems | SRP |

Table 2: List of medicinal plants used by traditional healers in South west-Algeria

| Botanical name | Part used | Ailment category: N of use reports | Preparation method | Administration | UV |
|---|---|---|---|---|---|
| *Acacia gummifera* Willd. Mimosaceae | Roots | RTSD: 6 (cough, bronchitis) | Infusion | Oral | 0.318 |
| | | CSD: 1 (hypertension) | Raw | Oral | |
| *Ajuga iva* (L.) Schreb. *Lamiaceae* | Aerial parts | GISD: 2 (digestive disorders) | Decoction | Oral | 0.136 |
| | | ESD: 1 (diabetes) | Decoction | Oral | |
| *Ammoides pusilla* (Brot.) Breistr. *Apiaceae* | Fruit | CSD: 2 (hypertension) | Decoction | Oral | 0.227 |
| | | RTD: 1 (flu) | Infusion | | |
| | | NS: 1 (vertigo) | Decoction | | |
| | | GH: 1 (tonic) | | | |
| *Anacyclus pyrethrum* (L.) Lag. Asteraceae/Compositae | Roots | SRP: 2 (female sterility) | Decoction | Oral | 0.136 |
| | | Can: 1 | Raw | Oral | |
| *Artemisia absinthium* L. Asteraceae | Aerial parts | GISD: 2 (intestine problems) | Decoction | Oral | 0.136 |
| | | RTD: 1 (asthma) | | | |
| *Artemisia campestris* L. Asteraceae | Aerial parts | RTD: 2 (bronchitis) | Infusion | Oral | 0.139 |
| | | GISD: 1 (stomach pain) | | | |
| *Artemisia herba-alba* Asso Asteraceae | Aerial parts | GISD: 7 (stomachache, ulcer) | Decoction | Oral | 0.454 |
| | | CSD: 2 (hypertension) | | | |
| | | RTD: 1 (asthma) | | | |
| *Atriplex halimus* L. Chenopodiaceae | Seeds | Can: 4 (cancers) | Decoction | Oral | 0.454 |
| | Leaves | CSD: 3 (hypercholesterolemia) | | | |
| | | GISD: 3 (hydatic cyst) | | | |
| *Berberis vulgaris* L. Berberidaceae | Roots | Can: 4 (cancers) | Decoction | Oral | 0.318 |
| | | CSD: 2 (hypercholesterolemia) | | | |
| | | GISD: 1 (stomachache) | | | |
| *Borago officinalis* L. Boraginaceae | Aerial parts | GISD: 2 (stomachache) | Decoction | Oral | 0.136 |
| | | RTD: 1 (cold) | Raw | oral | |
| *Carex arenaria* L. Cyperaceae | Roots | GISD: 1 (digestive disorders) | Decoction | Oral | 0.090 |
| | | ESD :1 (hypertension) | Decoction | Oral | |
| *Carum carvi* L. Apiaceae | Seeds | GISD: 4 (stomach, appetite, kids colitis) | Decoction | Oral | 0.272 |
| | | CSD: 1 (hypertension) | | | |
| | | RTD: 1 (kids cough) | | | |
| *Cassia angustifolia* VahlFabaceae | Leaves | GISD: 7 (stomachache, constipation, gases) | Infusion | Oral | 0.363 |
| | | GH: 1 (psychopathic disorders) | Decoction | | |
| *Cinnamomum camphora* (L.) J.Presl Lauraceae | Leaves | GISD: 2 (hemorrhoids) | Raw | Topical | 0.136 |
| | | RTD: 1 (cough) | Vapor | Nasal | |
| *Cinnamomum cassia* (L.) J.Presl Lauraceae | Bark | RTD: 1 (respiratory affections) | Raw | Oral | 0.136 |
| | | GISD: 1 (stomachache) | Decoction | | |
| | | SRP: 1 (menstrual pains) | | | |

*(Contd...)*

Table 2: *(Continued)*

| Botanical name | Part used | Ailment category: N of use reports | Preparation method | Administration | UV |
|---|---|---|---|---|---|
| *Citrullus colocynthis* (L.) Schrad. Cucurbitaceae | Fruits | GISD: 1 (haemorrhoids) | Raw | Topical | 0.045 |
| *Cotula cinerea* Delile Asteraceae | Whole | RTD: 1 (pharyngitis) | Raw | Oral | 0.181 |
| | | GH: 1 (systemic healing) | | | |
| | | NS: 1 (migraine) | | | |
| | | GISD: 1 (stomachache) | Decoction | Oral | |
| *Cucurbita maxima* Duchesne Cucurbitaceae | Seeds | NS: 1 (migraine) | Raw | Vapor | 0.045 |
| *Cuminum cyminum* L. Apiaceae | Seeds | GISD: 4 (stomachache, gases, constipation) | Decoction/ Raw | Oral | 0.272 |
| | | RTD: 1 (kids cough) | Decoction | | |
| | | SRP: 1 (menstrual pain) | Decoction | | |
| *Cupressus sempervirens* L. Cupressaceae | Aerial parts | GISD: 2 (bad digestion, intestine disorders) | Maceration | Oral | 0.090 |
| *Curcuma longa* L. Zingiberaceae | Rizhomes | GISD: 1 (liver diseases) | Decoction | Oral | 0.067 |
| *Cyperus esculentus* L. Cyperaceae | Tuber | GISD: 1 (kids appetite) | Raw | Oral | 0.045 |
| *Daphne gnidium* L. Thymelaeaceae | Leaves | HC: 1 (hair loss) | Raw | Topical | 0.046 |
| *Eucalyptus globulus* Labill. Myrtaceae | Leaves | RTD: 3 (flu, cough) | Vapor | Inhalation | 0.136 |
| *Ferula communis* L.Umbelliferae/ Apiaceae | Aerial parts | SMSD: 1 (fractures) | Decoction | Topical | 0.045 |
| *Foeniculum vulgare* Mill. Umbelliferae/Apiaceae | Seeds | GISD: 5 (stomchache, colitis, gases) | Infusion | Oral | 0.363 |
| | | CSD: 2 (hypertension) | Raw | | |
| | | RTD: 1 (cough) | Decoction | | |
| *Fraxinus angustifolia* Vahl Oleaceae | Leaves | RTD :1 (allergy) | Infusion | Oral | 0.045 |
| *Globularia alypum* L. Globulariaceae | Leaves | GISD: 2 (hepatitis, anaemia) | Decoction | Oral | 0.181 |
| | | GH: 1 (body purification) | | | |
| | | HC: 1 (hair loss) | Raw | Topical | |
| *Glycyrrhiza glabra* L. Papilionaceae/Fabaceae | Roots | RTD: 10 (laryngitis, bronchitis, cough) | Infusion/ Raw | Oral | 0.500 |
| | | GISD: 1 (appetite) | Raw | Oral | |
| *Haloxylon salicornicum* (Moq.) Bunge ex Boiss. Chenopodiaceae | Aerial parts | SD: 3 (wound, infections) | Raw | Topical | 0.181 |
| | | GH: 1 (pain) | | | |
| *Hibiscus sabdariffa* L. Malvaceae | Aerial parts | CSD: 1 (hypertension) | Decoction | Oral | 0.045 |
| *Hyoscyamus niger* L. Solanaceae | Leaves | SD: 1 (eczema) | Raw | Topical | 0.045 |
| *Juglans regia* L. Juglandaceae | Fruits | GISD: 1 (obesity) | Infusion | Oral | 0.045 |
| *Juniperus phoenicea* L. Cupressaceae | Leaves | GISD: 9 (stomach pain, gases) | Decoction | Oral | 0.500 |
| | | CSD: 1 (heart disease) | Raw | | |
| | | RTD: 1 (apnoea) | Decoction | | |
| *Laurus nobilis* L. Lauraceae | Leaves | GISD: 2 (digestive disorders) | Infusion | Oral | 0.181 |
| | | CSD: 2 (hypertension) | Decoction | | |
| *Lavandula latifolia* Medik. Lamiaceae | Flowers | SRP: 3 (female sterility, lactogene) | Raw/ Decoction | Oral | 0.272 |
| | | RTD: 2 (kids cough) | Decoction | Oral | |
| | | GISD: 1 (gases) | | | |
| *Lavandula stoechas* L. Lamiaceae | Leaves | ESD: 1 (diabetes) | Infusion | Oral | 0.090 |
| | | CSD: 1 (hypercholesterlemia) | | | |
| *Lawsonia inermis* L. Lythraceae | Leaves | HC: 3 (hair loss) | Raw | Topical | 0.272 |
| | | KD: 1 (cystitis) | Decoction | Oral | |
| | | SD: 1 (infections) | | | |
| | | GH: 1 (general pain) | Infusion | Oral | |
| *Lepidium sativum* L. Cruciferae/Brassicaceae | Seeds | RSD: 2 (bronchitis) | Raw | Oral | 0.363 |
| | | ESD: 1 (diabetes) | | | |
| | | CSD: 1 (hypertension) | | | |
| | | SMSD: 1 (rheumatism) | Decoction | Topical | |

*(Contd...)*

Table 2: *(Continued)*

| Botanical name | Part used | Ailment category: N of use reports | Preparation method | Administration | UV |
|---|---|---|---|---|---|
| | Whole | Can: 1 | Raw | Oral | |
| | | RTD: 1 (cold) | | | |
| | | SRP: 1 (genycological problems) | | | |
| *Linum usitatissimum* L. Linaceae | Seeds | GISD: 3 (digestive disorders) | Raw | Oral | 0.363 |
| | | CSD: 2 (hypertension) | | | |
| | | RTD: 2 (allergy) | | | |
| | | ESD: 1 (diabetes) | | | |
| *Lippia citriodora* (Palau) Kunth Verbenaceae | Leaves | GISD: 1 (colitis) | Infusion | Oral | 0.045 |
| *Lupinus albus* L. Fabaceae/Leguminoseae | Seeds | ESD: 4 (diabetes) | Raw | Oral | 0.181 |
| *Marrubium vulgare* L. Lamiaceae | Aerial parts | SMSD: 1 (arthritis) | Decoction | Topical | 0.045 |
| *Matricaria discoidea* DC. Asteraceae/compositae | Aerial parts | RTD: 1 (cold) | Decoction | Oral | 0.046 |
| *Mentha pulegium* L. Lamiaceae | Aerial parts | RTD: 3 (cough, Flu) | Infusion | Oral | 0.227 |
| | | GISD: 1 (appetite) | | | |
| | | CSD: 1 (cardiac diseases) | Decoction | | |
| *Myrtus communis* L.Myrtaceae | Seeds | GISD: 4 (gases, parasites) | Decoction | Oral | 0.227 |
| | | RTD: 1 (flu) | Decoction | Oral | |
| *Nerium oleander* L.Apocynaceae | Leaves | SD: 1 (furuncle) | Raw | Topical | 0.045 |
| *Nigella sativa* L. Ranunculaceae | Seeds | RTD: 4 (cough, bronchitis) | Raw | Oral | 0.181 |
| | | GH: 2 (systemic healing) | Decoction | | |
| *Ocimum basilicum* L. Lamiaceae | Leaves | RTD: 4 (allergy, cough) | Decoction | Oral | 0.318 |
| | | NS: 2 (sedative) | | | |
| | | ESD: 1 (goitre) | | | |
| *Origanum majorana* L. Lamiaceae | Leaves | NS: 3 (sedative, migraine) | Decoction | Oral | 0.363 |
| | | RTD: 2 (allergy, cough, flu) | | | |
| | | GISD: 2 (obesity) | Raw | | |
| *Ormenis nobilis* (L.) J.Gay ex Coss. & Germ. Asteraceae | Flowers | SD: 3 (wounds) | Decoction | Oral | 0227 |
| | | SMDS: 2 (rheumatism) | | | |
| *Parietaria officinalis* L. Urticaceae | Leaves | KD: 4 (kidney stones) | Decoction/ | Oral | 0.181 |
| *Peganum harmala* L. Zygophyllaceae | Roots | NS : 2 (dementia, depression) | Vapor | Inhalation | 0.136 |
| | Seeds | GISD: 1 (parasites) | Decoction | Oral | |
| *Pimpinella anisum* L. Apiaceae | Seeds | GISD: 4 (gases, colitis) | Raw | Oral | 0.410 |
| | | RTD: 3 (kids bronchitis, cough) | Decoction | | |
| | | CSD: 2 (hypertension) | Raw | | |
| *Pinus halepensis* Mill. Pinaceae | Leaves | GISD: 2 (stomachache) | Decoction | Oral | 0.093 |
| | | CSD: 1 (hypertension) | Decoction | Oral | |
| *Pinus maritima* Mill. Pinaceae | Leaves | RTD: 1 (flu) | Raw | Inhalation | 0.046 |
| *Pistacia lentiscus* L.Anacardiaceae | Leaves | GISD: 5 (stomachache, colitis) | Raw/ decoction | Oral | 0.363 |
| | | SD: 2 (skin diseases) | Raw | Topical | |
| | | RTD: 1 (bronchitis) | Infusion | Oral | |
| *Prunus persica* (L.) Batsch Rosaceae | Leaves | Can: 4 (cancers) | Raw/ decoction | Oral | 0.227 |
| | | GISD: 1 (colon) | Decoction | | |
| *Quercus infectoria* G.OlivierFagaceae | Aerial parts | GISD: 4 (ulcer, hemorrhoids) | Raw | Oral | 0.181 |
| | | SD: 1 (wounds) | Maceration | Topical | |
| *Rhamnus alaternus* L.Rhamnaceae | Leaves | GISD :3 (hepatitis) | Decoction | Oral | 0.136 |
| *Rhamnus purshiana* DC. Rhamnaceae | Barks | RTD: 2 (pharyngitis) | Raw | Topical | 0.093 |
| *Rosa canina L.*Rosaceae | Flowers | RTD: 1 (cough) | Decoction | Oral | 0.045 |
| *Rosmarinus officinalis* L.Lamiaceae | Leaves | SRP: 5 (menstrual problems) | Decoction | Oral | 0.545 |
| | | CSD: 3 (hypertension) | Infusion | Oral | |
| | | NS: 2 (memory) | | | |
| | | Fev: 1 (fever) | Decoction | Oral | |
| | | GISD: 1 (stomachache) | | | |
| *Rubia tinctoria Salisb.*Rubiaceae | Roots | GISD: 1 (Anaemia) | Decoction | Oral | 0.045 |

*(Contd...)*

Table 2: *(Continued)*

| Botanical name | Part used | Ailment category: N of use reports | Preparation method | Administration | UV |
|---|---|---|---|---|---|
| *Ruta chalepensis* L.Rutaceae | Leaves | GISD: 4 (intestine disorders, liver problems) | Infusion | Oral | 0.409 |
| | | NS: 2 (vertigo) | Decoction | Oral | |
| | | ESD: 2 (diabetes) | Infusion | Oral | |
| | | SMSD: 1 (gout) | infusion | Oral | |
| *Salvia officinalis* L.Lamiaceae | Flowers | GISD: 3 (digestive disorders) SRP: 2 (ovary inflammation, menstrual problems) CSD: 1 (hypertension) | Decoction | Oral | 0.272 |
| *Satureja calamintha* (L.) Scheele Lamiaceae | Leaves | Fev: 1 (fever) | Maceration | Oral | 0.090 |
| | | GISD: 1 (stomachache) | Decoction | Oral | |
| *Sesamum indicum* L.Pedaliaceae | Seeds | NS: 1 (memory strength) | Raw | Oral | 0.045 |
| *Stipa tenacissima* L.Poaceae/ Graminaceae | Whole | GISD: 1 (weight loss) | Decoction | Oral | 0.045 |
| *Teucrium polium* L.Lamiaceae | Leaves | GISD : 1 (gases) | Decoction | Oral | 0.045 |
| *Thymelaea hirsuta* (L.) Endl. Thymelaeaceae | Aerial parts | SRP : 2 (female sterility) | Vapor | Topical | 0.090 |
| *Thymus vulgaris* L.Lamiaceae | Whole | RTD :16 (bronchitis, laryngitis, allergy, flu, cough) | Decoction | Oral | 1.045 |
| | | GISD: 4 (colon disorders, diarrhoea) | Infusion | Oral | |
| | | CSD: 2 (hypertension) | Decoction | Oral | |
| | | ESD: 1 (diabetes) | | | |
| *Thypha angustifolia* L.Typhaceae | Seeds | GISD: 2 (haemorrhoids) | Raw | Topical | 0.136 |
| | | Fev: 1 (fever) | Decoction | Oral | |
| *Trigonella foenum*-graecum L.Fabaceae/Leguminoseae | Seeds | GISD: 5 (appetite, hepatitis) | Decoction | Oral | 0.590 |
| | | GH: 4 (tonic) | Raw | | |
| | | ESD: 2 (diabetes) | Infusion | | |
| | | CSD: 1 (hypertension) | Raw | | |
| | | IS: 1 (increasing immunity) | Decoction | | |
| *Triticum durum* Desf. Poaceae/ Graminaceae | Seeds | GISD: 1 (colon) | Raw | Oral | 0.045 |
| *Triticum repens* L.Poaceae/ Graminaceae | Roots | KD: 3 (diuretic) | Decoction | Oral | 0.318 |
| | | Can: 3 (cancer) | | | |
| | | GH: 1 (tonic) | | | |
| *Tussilago farfara* L.Asteraceae | Leaves | RTD :1 (cough) | Decoction | Oral | 0.045 |
| *Urtica dioica* L. Urticaceae | Aerial parts | GISD: 3 (weight gain, anemia) ESD: 2 (diabetes) | Decoction | Oral | 0.227 |
| *Viscum album* L. *Loranthaceae* | Leaves | SRP: 2 (breast milk secretion) SMSD: 2 (fractures) | Raw | Oral | 0.181 |
| *Vitex agnus-castus* L. Lamiaceae | Leaves Stems | SRP: 2 (internal uterine cold) | Raw | Oral | 0.090 |
| *Zingiber officinale* Roscoe Zingiberaceae | Roots | RTD: 9 (cough, flu, allergies) | Infusion/ Mac | Oral | 0.863 |
| | | GH: 4 (systemic problems) | Maceration | Oral | |
| | | GISD: 2 (digestive disorders, liver diseases) | Decoction | Oral | |
| | | CSD: 2 (cardiac diseases) | Raw | Oral | |
| | | SRP: 2 (aphrodisiac) | | | |
| *Ziziphus lotus* (L.) Lam. Rhamnaceae | Roots | ESD: 1 (diabetes) | Infusion | Oral | 0.318 |
| | | HC: 1 (hair loss) | Raw | Topical | |
| | Fruits | KD: 4 (renal disorders, renal calculi) | Raw/ decoction | Oral | |
| | | SRP: 1 (infections) | Decoction | Topical | |
| *Zygophyllum cornutum* Coss. Zygophylaceae | Leaves | GISD: 1 (stomachache) ESD: 1 (diabetes) | Decoction | Oral | 0.090 |

KD: Kidney diseases, GISD: Gatsrointestinal system diseases, SD: Skin diseases, ESD: Endocrine system diseases, RTD: Respiratory tract diseases, SMSD: Skeleto-muscular system disorders, CSD: Cardiovascular system diseases, GH: General health, HC: Health care, NS: Nervous system, SRP: Sexual-reproductive problems

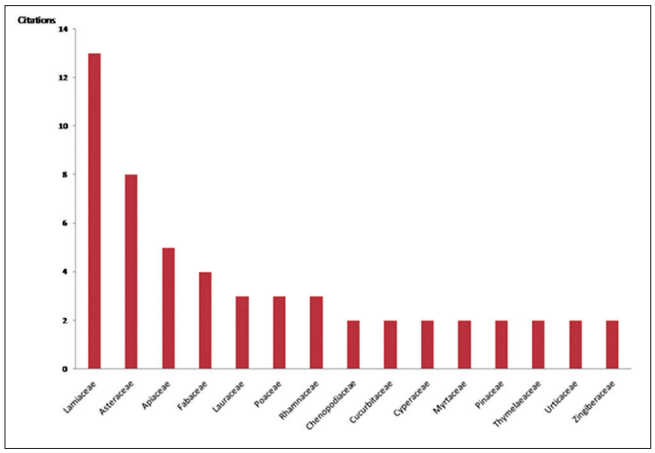


**Figure 2:** Distribution of reported species among the botanical families

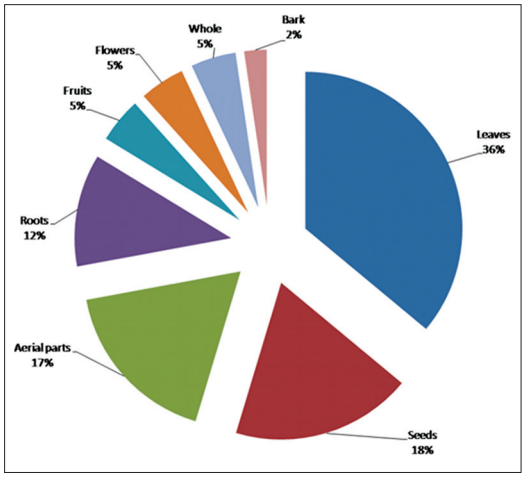


**Figure 3:** Plant parts used by traditional healers

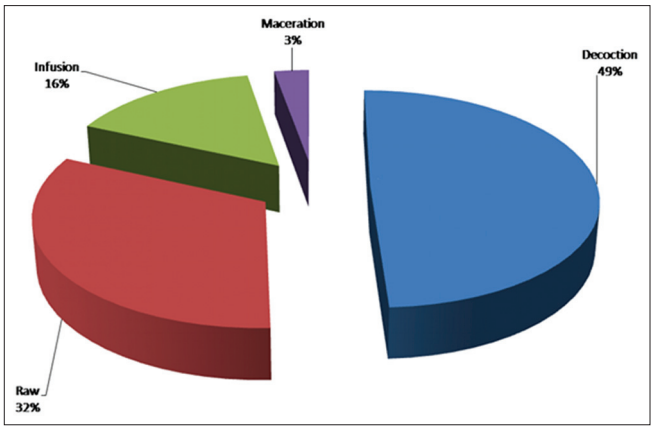


**Figure 4:** Modes of preparation used by traditional healers

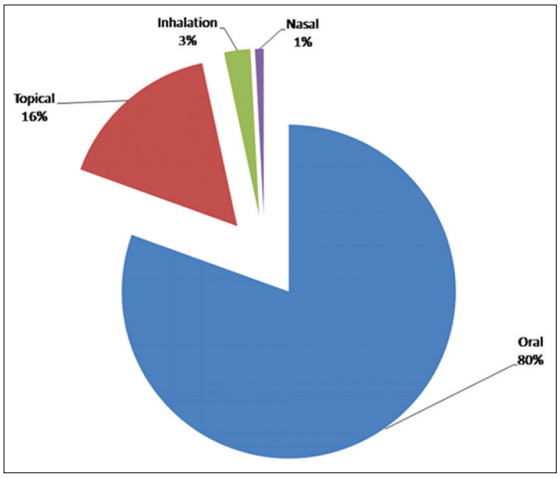


**Figure 5:** Routes of administration

administered orally (80%). Furthermore, as shown in Table 3, out of the 83 cited plants, 45 species are administered with other ingredients such as other plants (66%) or non-plant-aduvants (34%) such as olive oil, honey, milk, sugar, yogurt, or eggs. Honey is the adjuvant most added to different herbal remedies in South-west of Algeria (53%). Regarding the treated ailments, 35 species are reported to be used to treat more than one disease. According to our results [Figure 6], gastrointestinal disorders were the most commonly treated ailments with medicinal plants in south-west Algeria (33.6%), they were followed by respiratory diseases (23%) and cardiovascular diseases (9%).

## Quantitaive Analysis

UV of cited plants ranged from 0.045 to 1.045. The most commonly used species were *Thymus vulgaris* L. (UV = 1.045), *Zingiber officinale* Roscoe (UV = 0.863), *Trigonella foenum-graecum* L. (UV = 0.590), *Rosmarinus officinalis* L. (UV = 0.545), *Ruta chalepensis* L. (UV = 0.5), *Glycyrrhiza glabra* L. (UV = 0.5), A. *herba-alba* Asso (UV = 0.545), *Atriplex halimus* L. (UV = 0.545), and *Pimpinella anisum* L. (UV = 0.41).

The FIC reflects homogeneity of information provided by different informants regarding medicinal species used to treat a category of ailments. High FIC is correlated to species could be efficient in treating particular ailment [13]. Therefore, species with high FIC are to be prioritized for further pharmacological and phytochemical studies. As shown in Table 4, the highest FIC were found for kidney (0.727), cancer (0.687), digestive (0.603) and respiratory diseases (0.627). Four species are used to treat kidney diseases (KD) by local healers in South-west Algeria: *Lawsonia inermis* L. (topical use of leaves to treat cystitis), *Parietaria officinalis* L. (decoction of leaves is taken orally to treat kidney stones), *Triticum repens* L. (decoction of roots is used orally as diueretic) and *Ziziphus lotus* (L.) Lam. (fruits taken orally).

Cancer is ranked second regarding the FIC, demonstrating that local pharmacopeia could provide species with promising anticancer activities. Six species are used to treat different cancers: Roots of *Anacyclus pyrethrum* (L.) Lag., *T. repens* L. and *Berberis vulgaris* L., the whole *Lepidium sativum* L., seeds of A. *halimus* L. and leaves of *Prunus persica* (L.) Batsch.

To determine the most frequent species used for each ailment category, we calculated the FL. According to our results [Table 5], four species had the highest FL of 100%: *Eucalyptus globulus* Labill. (leave's vapor is inhaled for a cough and

Table 3: Ingredients added for the preparation of herbal medicines by the local traditional healers

| Botanical name | Other plants added in medicinal preparation | Other ingredients added |
|---|---|---|
| *Acacia gummifera* Willd. | *Anacyclus pyrethrum* (L.) Lag.<br>*Glycyrrhiza glabra* L.<br>*Lepidium sativum* L.<br>*Nigella sativa* L.<br>*Vitex agnus-castus* L.<br>*Zingiber officinale* Roscoe | Honey |
| *Ammoides pusilla (Brot.) Breistr.* L. | *Citrus limon* (L.) Osbeck | Yoghurt |
| *Anacyclus pyrethrum* (L.) Lag. | *Acacia gummifera* Willd.<br>*Aristolochia longa* L.<br>*Berberis vulgaris* L.<br>*Lepidium sativum* L.<br>*Vitex agnus-castus* L.<br>*Zingiber officinale* Roscoe | Milk<br>Honey |
| *Aristolochia longa* L. | *Berberis vulgaris* L.<br>*Haloxylon salicornicum* (Moq.) Bunge ex Boiss.<br>*Prunus persica* (L.) Batsch | Honey |
| *Berberis vulgaris* L. | *Aristolochia longa* L.<br>*Prunus persica* (L.) Batsch | Honey |
| *Carex arenaria* L.<br>*Carum carvi* L. | *Pinus halepensis* Mill.<br>*Foeniculum vulgare* Mill.<br>*Lavandula latifolia* Medik.<br>Pimpinella anisum L. | |
| *Cinnamomum camphora* (L.) J.Presl | | Olive oil |
| *Cinnamomum cassia* (L.) J.Presl | | Milk honey |
| *Citrullus colocynthis* (L.) Schrad. | | Olive oil |
| *Cuminum cyminum* L. | *Carum carvi* L.<br>*Foeniculum vulgare* Mill.<br>*Lavandula latifolia* Medik.<br>*Pimpinella anisum* L.<br>*Ruta chalepensis* L. | |
| *Cyperus esculentus* L. | Linum usitatissimum L. | Honey<br>Milk |
| *Daphne gnidium* L. | *Lawsonia inermis* L. | |
| *Foeniculum vulgare* Mill. | *Carum carvi* L.<br>*Cuminum cyminum* L.<br>*Lavandula latifolia* Medik.<br>*Linum usitatissimum* L.<br>*Pimpinella anisum* L.<br>*Teucrium polium* L.<br>*Trigonella foenum-graecum* L. | Honey |
| *Glycyrrhiza glabra* L. | *Acacia gummifera* Willd.<br>*Linum usitatissimum* L.<br>*Nigella sativa* L. | Honey<br>Milk |
| *Haloxylon salicornicum* (Moq.) Bunge ex Boiss. | *Lawsonia inermis* L. | Olive oil |
| *Hyoscyamus niger* L. | *Lawsonia inermis* L. | |
| *Juniperus phoenicea* L. | *Allium cepa* L.<br>*Lawsonia inermis* L.<br>*Punica granatum* L. | Honey |
| *Lavandula latifolia* Medik. | *Anacyclus pyrethrum* (L.) Lag.<br>*Carum carvi* L.<br>*Cuminum cyminum* L.<br>*Foeniculum vulgare* Mill.<br>*Teucrium polium* L.<br>*Origanum majorana* L. | Honey |

(Contd...)

Table 3: *(Continued)*

| Botanical name | Other plants added in medicinal preparation | Other ingredients added |
|---|---|---|
| *Lepidium sativum* L. | *Lawsonia inermis* L.<br>*Linum usitatissimum* L.<br>*Zingiber officinale* Roscoe | Honey<br>Milk |
| *Linum usitatissimum* L. | *Cyperus esculentus* L.<br>*Foeniculum vulgare* Mill.<br>*Glycyrrhiza glabra* L.<br>*Lepidium sativum* L.<br>*Pimpinella anisum* L.<br>*Trigonella foenum-graecum* L.<br>*Zingiber officinale* Roscoe | Honey<br>Sugar |
| *Lupinus albus* L.<br>*Marrubium vulgare* L. | | Honey<br>Olive oil |
| *Mentha pulegium* L. | *Artemisia herba-alba* Asso<br>*Citrus limon* L. | Milk |
| *Nigella sativa* L. | *Acacia gummifera* Willd.<br>*Glycyrrhiza glabra* L. | Honey |
| *Origanum majorana* L. | *Lavandula latifolia* Medik. | Honey<br>Olive oil |
| *Parietaria officinalis* L. | | Honey |
| *Pimpinella anisum* L. | *Carum carvi* L.<br>*Cuminum cyminum* L.<br>*Foeniculum vulgare* Mill.<br>*Lavandula latifolia* Medik.<br>*Linum usitatissimum* L. | |
| *Pinus halepensis* Mill. | *Carex arenaria* L. | |
| *Pinus maritima* Mill. | | Honey<br>Olive oil |
| *Pistacia lentiscus* L. | *Juniperus phoenicea* L. | Honey<br>Milk<br>Olive oil |
| *Prunus persica (L.)* Batsch | *Aristolochia longa* L.<br>*Berberis vulgaris* L.<br>*Haloxylon salicornicum (*Moq.*)* Bunge ex Boiss. | Honey |
| *Quercus infectoria G.Olivier* | | Honey |
| *Rhamnus alaternus* L. | | Honey |
| *Rosa canina* L. | | Honey |
| *Rubia tinctoria Salisb.* | | Honey |
| *Ruta chalepensis* L.<br>*Satureja calamintha (L.) Scheele* | *Cuminum cyminum* L.<br>*Mentha pulegium* L. | |
| *Sesamum indicum* L. | | Honey |
| *Teucrium polium* L. | *Foeniculum vulgare* Mill.<br>*Lavandula latifolia* Medik. | |
| *Thymus vulgaris* L. | *Mentha pulegium* L.<br>*Punica granatum* L. | Eggs<br>Milk |
| *Trigonella foenum-graecum* L. | *Foeniculum vulgare* Mill.<br>*Linum usitatissimum* L.<br>*Pimpinella anisum* L. | Honey<br>Milk |
| *Viscum album* L. | | Honey<br>Milk<br>Sugar |
| *Vitex agnus-castus* L. | *Acacia gummifera* Willd.<br>*Lepidium sativum* L.<br>*Zingiber officinale* Roscoe | |
| *Ziziphus lotus* (L.) Lam. | | Olive oil |

flu), *Lupinus albus* L. (seeds are taken orally for diabetes), *P. officinalis* L. (oral administration of leave's decoction for kidney stones), and *Rhamnus alaternus* L. (leave's decoction taken orally for the treatment of hepatitis). As shown in our

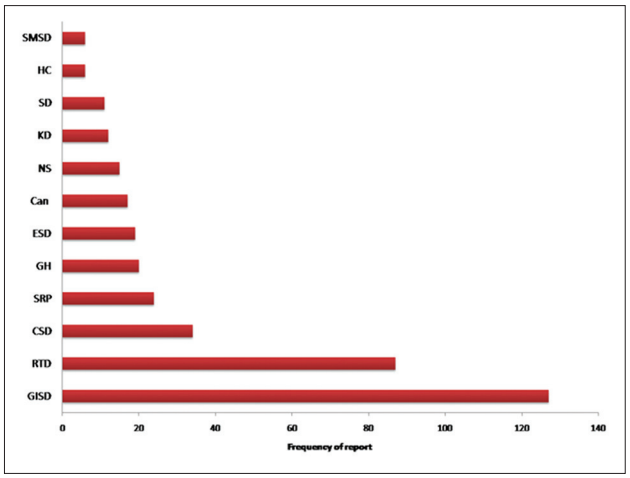


**Figure 6:** Ailments treated by the reported species. KD: Kidney diseases, GISD: Gatsro-intestinal system diseases, SD: Skin diseases, Can: Cancer, ESD: Endocrine system diseases, RTD: Respiratory tract diseases, SMSD: Skeleto-muscular system disorders, CSD: Cardiovascular system diseases, GH: General health, HC: Health care, NS: Nervous system, SRP: Sexual-reproductive problems

results, seeds of *L. albus* L. are commonly used (as raw) to treat diabetes.

## DISCUSSION

In this study, we report the use of 83 medicinal species belonging to 38 families. These findings are in line with those we published recently [7]. Local healers in both North-west and South-West of Algeria reflect that ancestral knowledge is very important with regard to the use of medicinal plants as complementary or alternative medicine. Our results showed that the most predominant families were Lamiaceae, Asteraceae, Apiaceae, and Fabaceae. Same results were reported in oriental Morocco, a region sharing with the study area most of climatic, demographic and geographical characteristics [14]. Furthermore, the predominance of Lamiaceae and Asteraceae is well documented in most of the ethnobotanical studies carried out in North African regions such as Algeria [15,16], Morocco [17], or Egypt [18]. Recently, Ramdane *et al.* [8] found that Lamiaceae followed by Asteraceae were the most predominant families of medicinal species used by the *Touareg* called "blue men of the Sahara" in extreme South of Algeria. Furthermore, leaves were the most frequent used plant parts. Recently, Benderradji *et al.* [19] demonstrated that in South-east of Algeria, leaves were the most commonly used parts in the treatment of different ailments. The predominance of leaves in herbal therapies may be attributed to their abundance in the region, and their richness in secondary metabolites produced by photosynthesis. On the other hand, a collection of leaves would be much easier and sustainable than that of roots or flowers [20].

According to our results, the decoction was found to be the major mode of preparation of the reported medicinal species. Similar findings were recently reported in South-east of Algeria (region of Ouargla) [21]. Decoction and infusion are highly valued and often preferred by local healers in Africa [22].

Table 4: FIC for commonly used medicinal plants

| Ailment | Nur | Nt | FIC |
|---|---|---|---|
| KD | 12 | 4 | 0.727 |
| Cancer | 17 | 6 | 0.687 |
| RTD | 87 | 33 | 0.627 |
| GISD | 127 | 51 | 0.603 |
| SRP | 24 | 12 | 0.521 |
| SD | 11 | 6 | 0.5 |
| NS | 15 | 9 | 0.428 |
| GH | 20 | 12 | 0.421 |
| HC | 6 | 4 | 0.4 |
| CSD | 34 | 21 | 0.393 |
| ESD | 19 | 13 | 0.333 |
| SMSD | 6 | 5 | 0.2 |

KD: Kidney diseases, GISD: Gatsro-intestinal system diseases, SD: Skin diseases, ESD: Endocrine system diseases, RTD: Respiratory tract diseases, SMSD: Skeleto-muscular system disorders, CSD: Cardiovascular system diseases, GH: General health, HC: Health care, NS: Nervous system, SRP: Sexual-reproductive problems

Table 5: FL values for common medicinal plants used

| Ailment category | Plants | FL (%) |
|---|---|---|
| RTD | *Eucalyptus globulus* Labill. | 100 |
| | *Glycyrrhiza glabra* L. | 91 |
| | *Acacia gummifera Willd.* | 85.71 |
| | *Nigella sativa* L. | 66.66 |
| | *Thymus vulgaris* L. | 69.56 |
| | *Mentha pulegium* L. | 60 |
| | *Ocimum basilicum* L. | 57 |
| SD | *Haloxylon salicornicum* (Moq.) Bunge ex Boiss. | 75 |
| | *Ormenis nobilis* (L.) J. Gay ex Coss. & Germ. | 60 |
| SRP | *Anacyclus pyrethrum (L.)* Lag. | 66.66 |
| ESD | *Lupinus albus* L. | 100 |
| NS | *Peganum harmala* L. | 66.66 |
| KDs | *Parietaria officinalis* L. | 100 |
| | *Zygophyllum cornutum Coss.* | 80 |
| GISD | *Rhamnus alaternus* L. | 100 |
| | *Cassia angustifolia* Vahl | 87.5 |
| | *Juniperus phoenicea* L. | 81.81 |
| | *Myrtus communis* L. | 80 |
| | *Artemisia herba-alba* Asso | 70 |
| | *Ajuga iva* (L.) Schreb. | 66.66 |
| | *Borago officinalis* L. | 66.66 |
| | *Carum carvi* L. | 66.66 |
| | *Cinnamomum camphora* (L.) J. Presl | 66.66 |
| | *Cuminum cyminum* L. | 66.66 |
| | *Pinus halepensis* Mill. | 66.66 |
| | *Thypha angustifolia* L. | 66.66 |
| | *Foeniculum vulgare* Mill. | 62.5 |
| | *Pistacia lentiscus* L. | 62.5 |
| | *Urtica dioica* L. | 60 |
| Cancer | *Prunus persica (L.)* Batsch | 80 |
| | *Berberis vulgaris* L. | 57.14 |

KD: Kidney diseases, GISD: Gatsrointestinal system diseases, SD: Skin diseases, ESD: Endocrine system diseases, RTD: Respiratory tract diseases, NS: Nervous system, SRP: Sexual-reproductive problems

Although our results are consistent with those we found in North-west of Algeria [7] and those reported in neighboring countries such as Morocco [23], we noticed that medicinal plants are never used as a paste in the region. In line with this, Moussaoui *et al.* [24] reported that in Mekenes (Morocco), paste was never used in administration of different herbal

formulations. The predominance of oral administration of the different medicinal plants in South-west Algeria is in total agreement with most of the carried out ethnobotanical studies in the country [25,26]. The predominance of oral administration may be explained by a high incidence of internal ailments in the region [5]. On the other hand, it's thought that oral route is the most acceptable for the patient. 45 species are administered with other plants - (66%) or nonplants-adjuvants. Honey was added in 53% of herbal formulations. Indeed, honey is considered sacred to Muslims and occupies an important place in Islamic medicine [27]. Furthermore, honey is considered as an instant energy source and is often used in Algeria to improve the acceptability of plants having a bitter taste unbearable [7]. In addition, we found that digestive and respiratory diseases were the most commonly treated ailments with medicinal plants. Our results corroborate those reported by Meddour *et al*. [28] showing that digestive and respiratory diseases were the predominant ailments treated by local populations using medicinal plants of Kabylia (North-west of Algeria). Similar findings were reported in Beni-Souif (Egypt) [29].

Our quantitative analysis showed that *T. vulgaris* L., *Z. officinale* Roscoe, *T. foenum-graecum* L., and *R. officinalis* L. were the most commonly used species with the highest UVs. *T. vulgaris* L., *Z. officinale* Roscoe, and *T. foenum-graecum* L. were found to be the most used species in North-west Algeria [7]. Our results demonstrate that both North and South regions of West Algeria present high level of similarities regarding the ethnomedicinal knowledge. The two regions share some social and environmental characteristics. Indeed, most of the local healers working in North-west Algeria are from the South-west. Recently, Mikou *et al*. found that *T. vulgaris* L., *R. officinalis* L., and *Artemisia herba-alba* Asso were the species most commonly used by local populations in Fes (Morocco) [30]. In the current study, the decoction of *T. vulgaris* L. is reported to be mainly (70%) used in the treatment of respiratory diseases such as bronchitis, laryngitis, allergy, flu, and cough. The plant is considered one of the most important antitussive herbal treatments in North Algeria [31]. The pharmacological properties of the plant have been attributed to a variety of active metabolites such as apigenin, luteolin, p-cymene, borneol, carvacrol, cymol, linalool, thymol, and triterpenic acids [32].

The high UV of *Z. officinale* Roscoe was reported in most of the ethnobotanical studies in muslim communities and may be explained by the influence of Islamic traditional medicine since the plant is mentioned in Holy Quran [33].

According to the calculated FIC, cancer is ranked second and is reported to be treated using six species: *A. pyrethrum* (L.) Lag., *T. repens* L., *Berberis vulgaris* L., *L. sativum* L., *A. halimus* L., and *P. persica* (L.) Batsch. Increasing incidence of different cancers in Algeria is well documented [34]. We have recently demonstrated that about 50% of Algerian cancer patients use different medicinal plants to treat and/or manage their illness [25,35].

FL is a useful indicator for identifying the informants' most preferred species in use for treating different disorders [36]. *E. globulus* Labill., *L. albus* L., *P. officinalis* L., and *R. alaternus* L. had the highest FL values of 100%. In line with our results, *E. globulus* Labill. has been reported to possess higher FL for respiratory diseases [37,38]. Furthermore, seeds of *L. albus* L. are used to treat diabetes.

Indeed, Knecht *et al*. demonstrated that extracts of the whole seeds resulted in a significant increasing of tolerance to an oral glucose bolus. Furthermore, the extract exhibited a marked antihyperglycemic activity [39]. The antidiabetic effect of the plant may be attributed to the presence of an active protein: Conglutin-γ. The latter has shown *in vitro* insulin-mimetic effects [40,41].

## CONCLUSION

In total, 83 medicinal plants species belonging to 38 families were reported to be used by traditional healers from South-west of Algeria. Our results showed important similarities with findings we previously reported from North-west of Algeria. Plants with high UV could be a promising source of active compounds against several ailments. Similarly, the plants with highest FL were identified and should be further studied regarding their phytochemicals and their biological activities. Furthermore, local healers from South-west Algeria demonstrated high consensus regarding treatment of KD and cancer.

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Original Research

ScopeMed

# DNA protective effect of ginseng and the antagonistic effect of Chinese turnip: A supplementation study

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## ABSTRACT

**Aim**: The aim of this clinical study is to provide scientific evidence for supporting traditional Chinese application and usage to the patients. For this purpose, we tested the ability if *Panax ginseng* extract to lower oxidative damage to nuclear DNA in human lymphocytes by comparing the effect of cooked Chinese turnip on this effect. **Materials and Methods:** Seven healthy subjects (4 males and 3 females from 37 to 60 years) participated two occasions which were at least 2 weeks apart. About 2 mL of fasting blood sample for baseline measurement was taken on arrival. They were requested to ingest the content of 5 ginseng capsules in 200 mL water. The subject remained fasting for 2 h until the second blood sample taken. In the other occasion, the experiment was repeated except a piece of cooked turnip (10 g) was taken with the ginseng extract. The two occasions could be interchanged. Comet assay was performed on two specimens on the same day for the evaluation of lymphocytic DNA damage with or without oxidative stress. **Results:** For the group with ginseng supplementation, there was a significant decrease in comet score for hydrogen peroxide ($H_2O_2$) treatment over the 2-h period while no change in DNA damage for unstressed sample. For the group with ginseng together with turnip supplementation, there was no significant difference in comet score for both $H_2O_2$ treatment and phosphate-buffered saline treatment. Ginseng extract could reduce DNA damage mediated by $H_2O_2$ effectively, but this protection effect was antagonized by the ingestion of cooked turnip at the same time. **Conclusion:** In the current study, commercial ginseng extract was used for supplementing volunteers. Ginseng extract could protect DNA from oxidative stress *in vivo* while turnip diminished the protection.

KEY WORDS: Antioxidant, DNA, ginseng, protection, turnip

## INTRODUCTION

DNA damage has been considered as an important cause of cancer and many diseases related to aging [1]. The main source of DNA damage is reactive oxygen species (ROS) which can be produced from endogenous sources such as mitochondria, peroxisomes, and inflammatory cell activation as well as exogenous sources including environmental pollutants, pharmaceuticals, and industrial chemicals. The oxidative stress may cause DNA damage and resulting the change in chromosome stability, genetic mutation, and altered gene expression that may lead to cancer development [2]. Reduction of DNA damage has been found to be possible by the antioxidant rich diet or supplement while negative correlation has been found between antioxidant level and DNA damage [3]. Numerous traditional Chinese medicinal herbs are recognized for their antioxidant properties [4-6]. Herbal decoctions may increase the activity of certain antioxidant enzymes such as superoxide dismutase (SOD) and glutathione peroxidase [7]. *Panax ginseng*, which is the most famous herb, has been used for curative and restorative functions for thousands of years. It has also been used to regulate human's physical, mental, and sexual ability [8]. Its *in vivo* antioxidant activity or protecting effect on DNA has been revealed [9]. For the convenience of intake, capsules containing ginseng extract are available commercially and are widely accepted in the market. In accordance with traditional Chinese medicine beliefs, Chinese turnip counteracts the beneficial effect of ginseng. Ginseng and Chinese turnip should not be taken together. In terms of DNA protecting activity of ginseng, this phenomenon has

been shown *in vitro* [10]. To provide the scientific evidence for supporting traditional Chinese medicine application and usage, the captioned common belief would be tested *in vivo* in the current study. The ability of ginseng extract to lower oxidative damage to nuclear DNA in human lymphocytes would be compared with the presence of cooked Chinese turnip. The evaluation of DNA damage would be achieved by comet assay. Damaged DNA of individual cells would be visualized under fluorescence microscope [11].

## MATERIALS AND METHODS

Ethical approval was obtained from the Human Subject Ethics Panel, Research Ethics Subcommittee, and Vocational Training Council. The trial was a cross-over study. Inclusion criteria were subjects not under long-term medication nor pregnant and at the age above 18. Physician-assessed the subjects and no acute or chronic diseases were reported. Seven healthy subjects (4 men and 3 women; 37-60 years old) entered the study with no dropout. The number of subjects tested based on the previous study which considers to detect the change of comet score of 45 (standard deviation = 15) with 80% power [9].

Written informed consent was obtained from the subject prior experiment. All subjects were requested to participate in two sampling occasions. They were requested to fast overnight before the experiment. At 09:00, 2 mL of venous blood was taken from the subject as the baseline followed by ingesting the content of 5 ginseng capsules suspended in 200 mL water. The subject remained fasting for 2 h and second blood sample was collected. Another occasion of the experiment was arranged at least 2 weeks after the first visit. The procedures of sampling were almost the same except the subject ingested a piece of cooked turnip (10 g) together with the ginseng capsules content. The two occasions could be interchanged which the subject could take turnip for the first part but no turnip intake for the second part. This could rule out the bias from carry-over effect. The comet assay was started in the afternoon of the same day of supplementation.

Harvesting of blood sample and comet assay was performed as described in the previous study in detail [12]. The gels were stained with 40 mL of ethidium bromide (2 mg/L) and then visualized at ×400 magnification using fluorescence microscope (Nikon Eclipse Ni with TRITC filter: Ex 540/25, Nikon, Tokyo, Japan). Damaged nucleus exhibited migration of the DNA toward the anode. The resulting image looked like a "comet" with the intensity of the comet tail relative to the head reflects the number of DNA breaks. The reason for this is that loops containing a break lose their supercoiling and become free to extend toward the anode. Quantification of DNA damage was done by visual scoring. Five classes (from 0 to 4) of damaged cells were classified according to different degrees of damage [13]. 100 comets per gel were counted, and two gels were prepared per slide. The score of a gel ranged from 0 to 400 in arbitrary unit which was positively associated to the DNA damage.

Equation for scoring:

Score = [(Grade 0×0) + (Grade 1×1) + (Grade 2×2) + (Grade 3×3) + (Grade 4×4)]/100

Grade 0+Grade 1+Grade 2+Grade 3+Grade 4=100

### Statistical Analysis

Prism 5.0 (GraphPad software, USA) was used with Wilcoxon signed rank test to evaluate the DNA protective effect for different groups. A $P < 0.05$ was considered statistically significant.

## RESULTS

The comet scores before and 2 h after ingestion were compared with ginseng group and ginseng+turnip groups. For the group with ginseng extract supplementation, there was a statistically significant decrease ($P = 0.0223$) in comet score in hydrogen peroxide ($H_2O_2$) treatment over the 2 h period while there was no significant difference ($P = 0.8125$) in score for unstressed phosphate-buffered saline (PBS) treatment (Figure 2a and b). There was no statistically significant change in comet score for both $H_2O_2$ treatment ($P = 0.2188$) and PBS treatment ($P = 0.5992$) over 2 h in ginseng+turnip group (Figure 2c and d). The results indicated that ginseng extract could lower DNA damage mediated by $H_2O_2$ effectively within a very short period of time (2 h). However, this protection effect was antagonized by the simultaneous ingestion of boiled turnip.

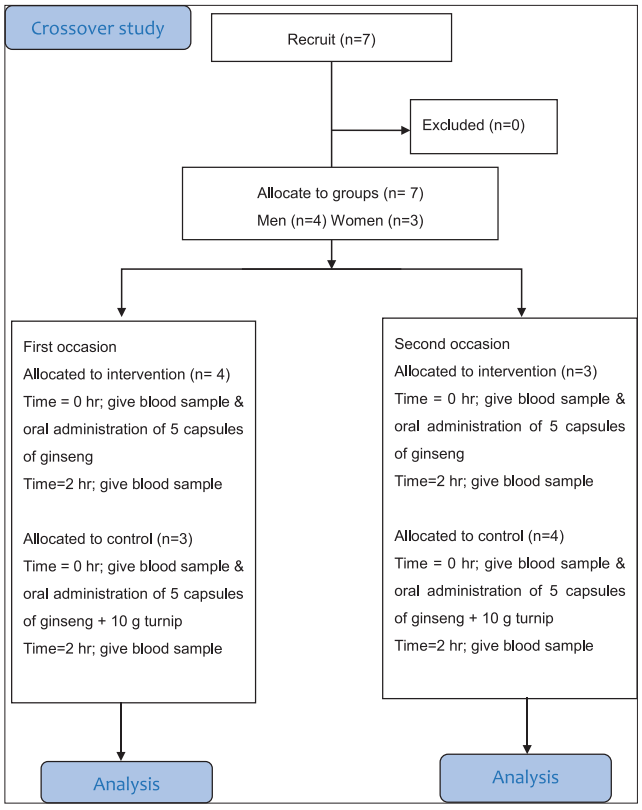


**Figure 1:** The flow diagram of the cross over study

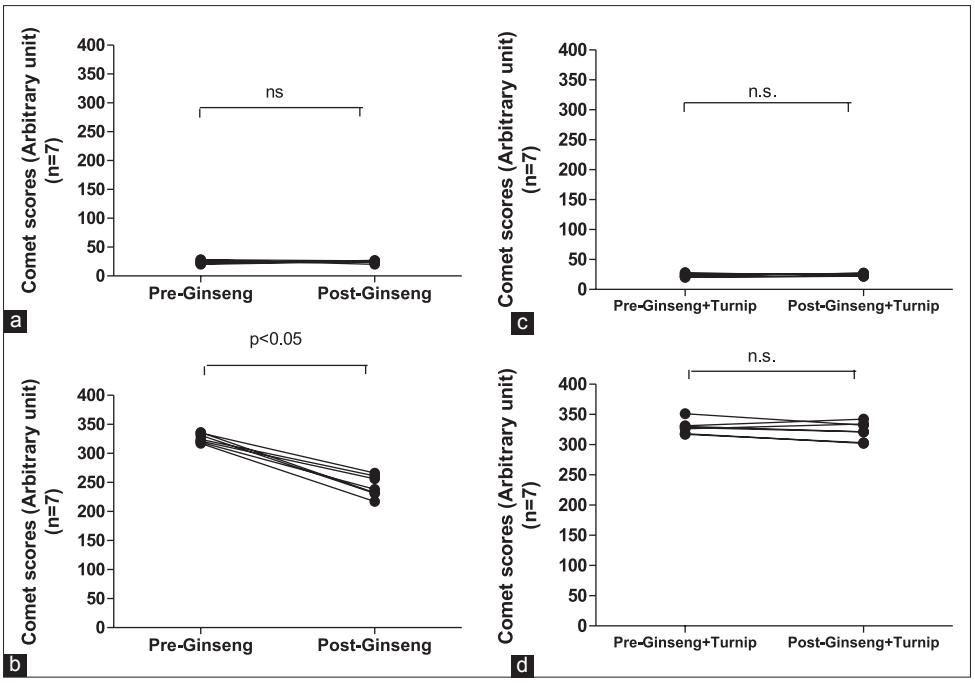


**Figure 2:** DNA damage before and 2 h after ginseng only supplementation. There was a significant decrease in comet score ($P$=0.0223) for, (a) hydrogen peroxide ($H_2O_2$) treated lymphocytes. No significant change in DNA damage ($P$=0.8125) for, (b) the control group. For DNA damage before and 2 h after ginseng and turnip supplementation. There was no significant decrease in comet score of lymphocytes for both, (c) $H_2O_2$ treated ($P$=0.2188) nor, (d) phosphate-buffered saline treated ($P$=0.5992)

## DISCUSSION

Antioxidants are substances that neutralize oxidative species that associated with DNA damage. Natural occurring antioxidants can be found in diet and the protective effects of dietary antioxidants on DNA damage have been extensively studies [14]. DNA damage is the critical cause of aging and it directs to diseases and cancer development [1]. For preventing the development of cancer and delaying aging process, minimizing DNA damage is essential.

Traditional Chinese herb such as ginseng has been extensively used for curative and restorative functions. Commercial ginseng extract can now be obtained in capsule form for convenient intake. A number of studies have reported the antioxidant action of ginseng [6,10,15]. Their antioxidant properties have been correlated with the properties of Chinese herbs according to the yin/yang ideology of TCM although not conclusive [5,16].

Studies in animals demonstrate protection against oxidative damage from cadmium chloride [17] and carbon tetrachloride [18,19]. Ginseng has been found to decrease generation of ROS and effectively decreased serum malondialdehyde (MDA) levels in healthy subjects [20]. Hypolipidemic effect of ginseng has been observed along with increased SOD and catalase activities [21]. It also has the supportive effect on exhaustive exercise with lowering MDA level [22].

In animal model, DNA damage and reproductive toxicity were evaluated in testis of rats exposed to 2,3,7,8-tetrachlorodibenzo-p-dioxinthe. A significantly decreased level of DNA damage and reduced pathological effects were observed in the ginseng extracts treated group. The effect of ginseng on DNA damage has also been shown in human in our previous study [9]. Comet assay shows decreased lymphocyte DNA damage and increased antioxidative enzymes after 8 weeks ginseng supplementation [23].

Results of this study showed that ingestion of ginseng extract could significantly increase lymphocytic DNA resistance to oxidative stress in a very short period of time as fast as 2 h. This could be reflected from the significantly reduction in comet scores for $H_2O_2$ treated lymphocytes in post-ginseng ingestion blood samples as compared with pre-ingestion. This agreed with our previous *in vitro* and supplementation study [6,9]. Effect of Chinese turnip on the effect of ginseng was also demonstrated in the current study. According to the rules and beliefs in the use of traditional Chinese medicine, there may be existence of incompatibility or antagonism between herbs or some foods. We have demonstrated that incubating ginseng along with turnip juice abolished the DNA protective effect of both American and Asian ginseng [10]. Both unboiled and boiled turnip juices are able to abolish protective effect offered by ginseng which implies, the effect of turnip juice is unlikely to be enzymatic [10]. No significant improvement in DNA protection 2 h after ingestion of ginseng extract together with cooked turnip confirmed turnip could diminish the protective effect of ginseng *in vivo* as well. This provided evidence to support the ideology of tradition Chinese medicine application regarding incompatibility.

In conclusion, this study showed that the ingestion of content of five capsules of commercial ginseng extract could

offer a significant decrease of DNA damage of lymphocytes demonstrated in comet assay. But with the ingestion of cooked turnip together with the ginseng extract content, the protection diminished. This supported our previous study and the traditional ideology that the incompatibility natures of ginseng and Chinese turnip in the sense of DNA protection.

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Original Research

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# Treatment of hemorrhoids with individualized homeopathy: An open observational pilot study

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## ABSTRACT

**Aim:** Controversies and disagreement exist on conventional treatment strategies of hemorrhoids due to relapse, inefficacy, and complications. We intend to evaluate the role of individualized homeopathic treatment in hemorrhoids. **Materials and Methods:** In this prospective, open, observational trial, hemorrhoids patients were treated using five standardized scales measuring complaints severity and anoscopic score. It was conducted at two homeopathic hospitals in India, during from mid-July 2014 to mid-July 2015. Patients were intervened as per individualized homeopathic principles and followed up every month up to 6 months. **Results:** Total 73 were screened, 52 enrolled, 38 completed, 14 dropped out. Intention to treat population ($n = 52$) was analyzed in the end. Statistically significant reductions of mean bleeding (month 3: −21.8, 95% confidence interval [CI]: −30.3, −13.3, $P < 0.00001$, d = 0.787; month 6: −25.5, 95% CI −35.4, −15.6, $P < 0.00001$, d = 0.775), pain (month 3: −21.3, 95% CI −28.6, −14.0, $P < 0.00001$, d = 0.851; month 6: −27.6, 95% CI −35.6, −19.6, $P < 0.00001$, d = 1.003), heaviness visual analog scales (VASs) (month 3: −8.1, 95% CI −13.9, −2.3, $P = 0.008$, d = 0.609; month 6: −12.1, 95% CI −19.1, −5.1, $P = 0.001$, d = 0.693), and anoscopic score (month 3: −0.4, 95% CI −0.6, −0.2, $P < 0.0001$, d = 0.760; month 6: −0.5, 95% CI −0.7, −0.3, $P < 0.0001$, d = 0.703) were achieved. Itching VASs reduced significantly only after 6 months (−8.1, 95% CI −14.6, −1.6, $P = 0.017$, d = 0.586). No significant lowering of discharge VASs was achieved after 3 and 6 months. **Conclusion:** Under classical homeopathic treatment, hemorrhoids patients improved considerably in symptoms severity and anoscopic scores. However, being observational trial, our study cannot provide efficacy data. Controlled studies are required. Trial Reg. CTRI/2015/07/005958.

KEY WORDS: Hemorrhoids, homeopathy, observational trial, visual analog scales

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## INTRODUCTION

Hemorrhoids are distal displacement and prolapse of the hemorrhoidal cushions, distension of the hemorrhoidal arteriovenous anastomoses, or dilation of the veins of the internal hemorrhoidal venous plexus resulting from deterioration of anchoring connective tissue [1]. Common symptoms include pain, itching, swelling, anal discomfort, and rectal bleeding; all

of which severely affect patient quality of life [2]. The condition affects 39-52% of adults [3,4]. The prevalence of hemorrhoids is extremely high in Western and other industrialized societies, with millions affected worldwide [3,5]. However, the true burden of disease is difficult to capture as many patients are reluctant to seek medical suggestions for various personal, cultural, and socio-economic reasons [2,6,7]. The prevalence of hemorrhoids in India according to recent surveys is around 40 million [8]. Several risk factors have been claimed to be the etiologies of hemorrhoid development including aging, obesity, depression, pregnancy, chronic constipation and diarrhea, low-fiber diet, spicy foods, and alcohol intake [9]. Hemorrhoids are generally classified by their location; internal (originates above the dentate line and covered by anal mucosa), external (originates below the dentate line and covered by anoderm), and mixed type. Internal hemorrhoids are further graded based on their appearance and degree of prolapse [10]:

- Grade I: Non-prolapsing hemorrhoids;
- Grade II: Prolapsing hemorrhoids on straining but reduce spontaneously;
- Grade III: Prolapsing hemorrhoids requiring manual reduction; and
- Grade IV: Non-reducible prolapsing hemorrhoids which include acutely thrombosed, incarcerated hemorrhoids.

Treatment options mainly depend on the type and severity of hemorrhoids, patient's preference, and the expertise of physicians. The current therapies can be grouped into conservative management, office-based procedures, and surgical treatment [5]. Increased fiber intake, medical therapies, and lifestyle changes are included in the conservative treatment options for non-thrombosed hemorrhoids [5,11,12]. The main goal of medical treatment is to control acute symptoms of hemorrhoids rather than to cure the underlying hemorrhoids [13]. Office-based modalities include rubber-band ligation, injection sclerotherapy, laser photocoagulation, bipolar diathermy, cryotherapy, Doppler-guided hemorrhoidal artery ligation and infrared coagulation [14]; still, they are not suitable for all grades of hemorrhoids and have recognized complications. When an office-based therapy is still ineffective, patients may consider further intervention such as hemorrhoidectomy, thrombectomy of external hemorrhoids, and stapled hemorrhoidectomy; however, no single technique has been universally accepted as superior [15]. Based on clinical practice, it is assumed that surgery is effective for severe prolapsing hemorrhoids, but it is difficult to deal with the post-operative complications such as relapse, pain, prolonged convalescence, fecal urgency, and anal stenosis [16,17]. Thus, controversies and lack of agreement still exist on treatment strategies.

Homeopathy ranks the most popular among the traditional, complementary, or alternative medicines. Homeopathy, according to its "law of similar," treats patients with a remedy that, in a healthy human, causes similar symptoms. Thus, the same diagnosis can be treated with different remedies in different patients ("individualization"), depending on "totality of symptoms," and consideration of different complex issues (e.g. "susceptibility," "miasm," etc.) [18,19]. Homeopathic literature shows anecdotal data on the usefulness of homeopathic medicines in hemorrhoids. Although remarkable cure of hemorrhoids with homeopathic medicines in casual clinical experiences has been noted, research evidence remains seriously compromised [20,21]. Only until recently, there is a single-blind, randomized, placebo-controlled trial of the homeopathic use of 50 millesimal potencies in acute attacks of hemorrhoidal disorders has been published substantiating efficacy of individualized homeopathic treatment [22]. Another recent paper reported hemorrhoids as one of the most frequently encountered clinical conditions in a homeopathic hospital surgery and medicine outpatient in West Bengal, India and is treated with a considerable success rate of 60.3-82.3% [23]. The present study was aimed at investigating the role of individualized homeopathic medicines in the treatment of hemorrhoids in an open observational study in real practice settings.

## MATERIALS AND METHODS

This study was conducted in the medicine and surgery outpatients of two Government homeopathic hospitals in West Bengal, India – Midnapore Homeopathic Medical College and Hospital (MHMCH) and Mahesh Bhattacharyya Homoeopathic Medical College and Hospital (MBHMCH). The study was started in mid-July 2014, enrollment continued until December 2014 and ended in mid-July 2015. In this prospective multicenter, open observational trial, patients were included consecutively on their first consultation with the participating physicians and followed up for 6 months using standardized scales. Before enrollment, written informed consent and approval by institutional ethics committees were obtained (IEC no. MHMCH/19/2015-16 and MBHMCH/370), and each patient was provided with a patient information sheet in local vernacular Bengali detailing the objectives, methods, risks and benefits of participating, and confidentiality issues. The study protocol (MHMCH/19/2015-16 and MBHMCH/70; version 1.0; date 28.05.2014) was registered with the Clinical Trials Registry, India, vide CTRI/2015/07/005958 on July 02, 2015, and has Universal Trial No. (UTN) U1111-1169-7497. The study was performed under the constant supervision of the independent ethics committees of the respective institutions.

Inclusion criteria were male and female patients between 25 and 60 years suffering from internal idiopathic/primary hemorrhoids presenting with any of the symptoms, namely, bleeding, pain (including discomfort and tenesmus during defecation or any other time), heaviness, pruritus, and mucus discharge with or without anitis. Patients with known but controlled diabetes (glycated hemoglobin <8%) and controlled hypertension and thyroid disorders were also eligible for the study. Patients using topical agents for hemorrhoids were included after a washout period of 1-week, subject to persistence of symptoms and signs of hemorrhoids.

Exclusion criteria were Grade IV piles, anal fissure and fistula, hereditary piles, inflammatory bowel disease, enlarged prostate, chronic alcoholism, recreational drug abuse, coagulation disorders, external hemorrhoids, previous history of surgery for hemorrhoids, hypertrophic anal papillae, rectal malignancies,

history of leukemic disorders, patients with obstruction of portal circulation, pregnancy leading to obstruction of the portal circulation, lactating mothers, patients with psychiatric diseases.

Before treatment (at baseline), patients independently rated their severity on five 100 mm visual analog scales (VAS; 0 = no complaints; 100 = maximum severity) measuring intensity of symptoms of hemorrhoids – bleeding, pain, heaviness, discharge, and itching; and anoscopic examination by the treating physicians on a scale of 0 to 2 as follows: 0 = No signs of inflammation, 1 = a rather active grade, hemorrhoids without overt inflammatory findings (mild anitis), and 2 = an actively or easily bleeding hemorrhoids with overt signs of inflammation and edema (severe anitis). We planned to measure the outcomes at baseline, every month, up to 6 months. Two conventionally trained surgeons performed the anoscopic assessments for each patient at each time point.

We postulated the null hypothesis ($H_0$) as pre-treatment score = post-treatment score; and alternative hypothesis ($H_A$) was pre-treatment score ≠ post-treatment score. The study design was open-label, observational, single arm, non-randomized, non-controlled, and interventional. We targeted to achieve a sample size around 50 conveniently within the stipulated time frame of 1 year, from mid-July 2014 to mid-July 2015.

Interventions were planned as administering indicated remedies in centesimal or 50 millesimal potencies as appropriate. In centesimal scale, each dose consisted of four cane sugar globules medicated with a single drop of the indicated medicine, preserved in 88% v/v ethanol. In 50 millesimal scale, a single medicated cane sugar globules of poppy seed size (no.10) dissolved in 50 ml distilled water with addition of 2 drops of 88% v/v ethanol, 10 doses marked on the vial, each dose of 5 ml to be taken after 10 uniformly forceful downward strokes to the vial in 45 ml normal water in a clean cup, to stir well, to take 5 ml of this liquid orally, and to discard rest of the liquid in the cup. Repetition 24, 12, or 8 hourly or even oftener, depended on the individual requirement of the case. All medicines were procured from a Good Manufacturing Practice-certified firm.

Following recruitment, selection of the single individualized medicine was based on the presenting symptom totality, repertorization and consultation with Materia Medica, and individualized dose on the judgment of susceptibility of the patients. As per individual requirement of the cases, aid of different repertories (RADAR® software, Archibel, Belgium) was taken with due consultation of Materia Medica. Overall decision making was influenced by consensus among the physicians. Subsequent prescriptions were generated according to Kent's observations and second prescription. Thus results of this study adhered to the criteria for reporting individualization in homeopathy [24].

All the participants were encouraged to have sitz bath as and when needed, maintain local hygiene, correct constipation, and unhealthy defecation habits such as ignoring the need to pass stools, irregular meals, spending a long time in the lavatory, straining, and lack of exercise. They were also advised about the importance of a fiber-rich diet in health and encouraged to consume food rich in natural fiber such as unpeeled fruits, vegetables, and whole-grain bread and will be prohibited from high consumption of spices.

Statistical analysis followed the intention-to-treat (ITT) approach; i.e., every included patient entered final analyses. Descriptive data (categorical and continuous) were presented in terms of absolute values, percentages, mean, and standard deviations (SD) as appropriate. Missing values were replaced by the last value carried forward method. To compare longitudinally obtained data measured repeatedly in the same patients at different points of time, paired *t*-test was performed comparing baseline data with that recorded after 3rd and 6th month. The significance level was set at $P < 0.05$ two-tailed. For within-subjects studies, dependence among means was corrected to make direct comparisons to effect sizes from between-subjects studies. To do this, correlations (Pearson's *r*) between the two means were calculated first, and then Cohen's d was estimated by the Morris and DeShon's (2002) Equation (8) [25]. Effect sizes were classified: As $|d|>0.8$, large; $|d|>0.5$, medium; $|d|>0.2$, small [26].

Adverse or serious adverse event(s), if any, was planned to be treated accordingly as per homeopathic principles, or if non-responding, then the patient should be referred for surgery.

## RESULTS

Total 73 patients suffering from hemorrhoids were screened for the project; 52 satisfied the pre-specified eligibility criteria; 21 were excluded for varied reasons. Since enrollment, each patient received classical homeopathic treatment for a period of 6-month. Total 38 continued till the end, 14 dropped out. Finally, the ITT population, i.e. 52 patients entered the analysis [Figure 1].

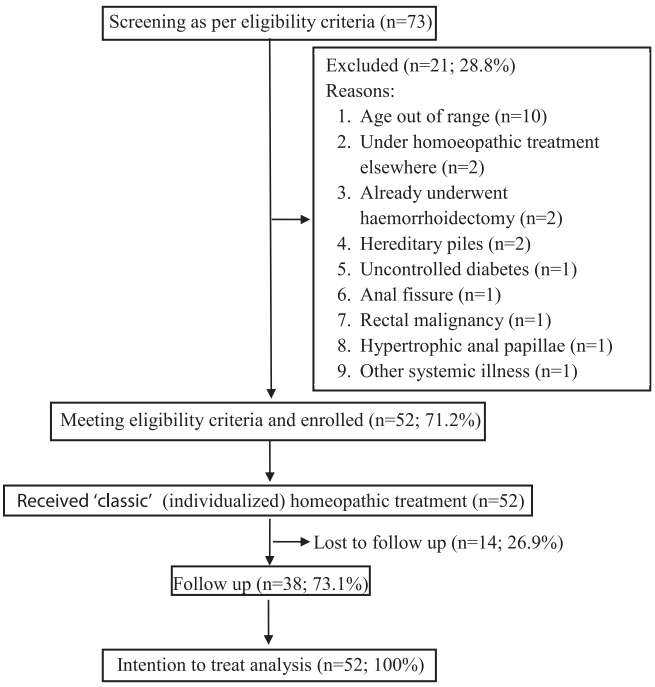


**Figure 1:** Study flow diagram

## Baseline Characteristics

Mean age of the patients was 42.2 years (SD 12.2); spanned all age groups, but majority belonged to the group of 46-65 years ($n = 25$; 48.1%). Most of the patients were male ($n = 32$; 61.5%). Mean body mass index was 24.3 (SD 5.0). Non-vegetarian food habit was mostly prevalent ($n = 50$; 96.2%); constipation ($n = 15$; 28.9%), and spicy food habit ($n = 5$; 9.6%) as probable risk factors. Homeopathy treatment alone was already availed for treatment of hemorrhoids by 26 (50%) patients and along with other therapies by 5 (9.6%) patients. Associated comorbidities identified were hypertension, bronchial asthma, migraine, polycystic ovaries, and osteoarthritis; each condition was found in single patients. Majority of the patients were married ($n = 44$; 84.6%); had qualification of graduate and above ($n = 24$; 46.2%); employment status of students and dependents ($n = 19$; 36.5%); but having monthly household income of Rs. 10,000 or less ($n = 19$; 36.5%) [Table 1].

**Table 1: Socio-demographics and baseline status ($n$=52)**

| Features | Description |
|---|---|
| Age mean±SD | 42.2±12.2 |
| Age groups (years) *n* (%) | |
| 18-30 | 11 (21.2) |
| 31-45 | 16 (30.8) |
| 46-65 | 25 (48.1) |
| Gender *n* (%) | |
| Male | 32 (61.5) |
| Weight (kg) mean±SD | 59.9±11.5 |
| Height (m) mean±SD | 1.6±0.1 |
| BMI mean±SD | 24.3±5.0 |
| Duration of complaints (years) mean±SD | 6.4±7.6 |
| Food habit *n* (%) | |
| Non-veg | 50 (96.2) |
| Risk factors *n* (%) | |
| Constipation | 15 (28.9) |
| Spicy food habit | 5 (9.6) |
| Treatment taken already *n* (%) | |
| Homeopathy alone | 26 (50) |
| Homeopathy with others | 5 (9.6) |
| Standard therapy | 6 (11.5) |
| Standard therapy with others | 4 (7.7) |
| Ayurveda | 3 (5.8) |
| Ayurveda with others | 3 (5.8) |
| Comorbidities *n* (%) | |
| Hypertension, bronchial asthma, migraine, polycystic ovaries, osteoarthritis | 1 each (1.9) |
| Marital status *n* (%) | |
| Married | 44 (84.6) |
| Others | 8 (15.4) |
| Education *n* (%) | |
| Secondary or less | 22 (42.3) |
| Higher secondary | 6 (11.5) |
| Graduate or above | 24 (46.2) |
| Employment *n* (%) | |
| Students and dependents | 19 (36.5) |
| Service | 16 (30.8) |
| Business | 9 (17.3) |
| Monthly household income *n* (%) | |
| <10,000 | 19 (36.5) |
| 10,000-30,000 | 18 (34.6) |
| >30,000 | 15 (28.9) |

SD: Standard deviation, BMI: Body mass index

## Bleeding VASs (mm)

The reductions achieved with time were as follows: Baseline 42.9 ± 28.9, 1st month 31.1 ± 20.4, 2nd month 25.8 ± 20.2, 3rd month 21.1 ± 20.8, 4th month 18.4 ± 21.5, 5th month 16.4 ± 20.6, and 6th month 17.4 ± 20.6. Mean reductions (mm) from baseline over month 3 (−21.8, 95% confidence interval [CI]: −30.3, −13.3; $t = -5.141$, $P < 0.00001$; paired *t*-test) and month 6 (−25.5, 95% CI −35.4, −15.6; $t = -5.120$, $P < 0.00001$; paired *t*-test) were statistically significant with almost large effect size (Cohen's d) of 0.787 (Pearson's $r = 0.379$) and 0.775 (Pearson's $r = 0.117$), respectively [Table 2 and Figure 2].

## Pain VASs (mm)

The decrease achieved with time were as follows: Baseline 45.7 ± 26.5, 1st month 32.7 ± 21.0, 2nd month 28.7 ± 22.1, 3rd month 24.5 ± 21.2, 4th month 23.3 ± 20.8, 5th month 21.1 ± 20.2, and 6th month 18.2 ± 20.1. Mean decrease (mm) from baseline over month 3 (−21.3, 95% CI: −28.6, −14.0; $t = -5.815$, $P < 0.00001$; paired *t*-test) and month 6 (−27.6, 95% CI: −35.6, −19.6; $t = -6.839$, $P < 0.00001$; paired *t*-test) were statistically significant with definitely large effect size (Cohen's d) of 0.851 (Pearson's $r = 0.455$) and 1.003 (Pearson's $r = 0.307$), respectively [Table 2 and Figure 2].

## Heaviness VASs (mm)

It declined with time as follows: Baseline 33.6 ± 23.5, 1st month 30.9 ± 22.4, 2nd month 26.2 ± 22.4, 3rd month 23.6 ± 19.6, 4th month 21.9 ± 19.4, 5th month 19.3 ± 19.7, and 6th month 19.6 ± 20.6. Mean decline (mm) from baseline over month 3 (−8.1, 95% CI: −13.9, −2.3; $t = -2.794$, $P = 0.008$; paired *t*-test) and month 6 (−12.1, 95% CI: −19.1, −5.1; $t = -3.459$, $P = 0.001$; paired *t*-test) were statistically significant with medium effect size (Cohen's d) of 0.609 (Pearson's $r = 0.710$) and 0.693 (Pearson's $r = 0.581$), respectively [Table 2 and Figure 2].

## Discharge VASs (mm)

It was reduced with time as follows: Baseline 14.9 ± 22.2, 1st month 13.7 ± 18.4, 2nd month 11.1 ± 15.4, 3rd month 10.5 ± 13.9, 4th month 10 ± 13.6, 5th month 8.7 ± 12.1, and 6th month 7.3 ± 11.6; however, mean reduction (mm) achieved from baseline over month 3 (−4.0, 95% CI: −10.9, 2.9; $t = -1.181$, $P = 0.250$; paired *t*-test) and month 6 (−6.3, 95% CI: −15.4, 2.8; $t = -1.403$, $P = 0.174$; paired *t*-test) were statistically non-significant with medium effect size (Cohen's d) of 0.293 (Pearson's $r = 0.654$) and 0.372 (Pearson's $r = 0.269$), respectively [Table 2 and Figure 2].

## Itching VASs (mm)

It declined with time as follows: Baseline 23.9 ± 20.1, 1st month 21 ± 17.8, 2nd month 18.9 ± 18.1, 3rd month 17.5 ± 17.5, 4th month 15.7 ± 18.0, 5th month 14.1 ± 18.7, and 6th month 12.6 ± 17.8. Mean reduction (mm) achieved from baseline over month 3 (−4.2, 95% CI: −8.9, 0.5; $t = -1.764$, $P = 0.086$; paired

Table 2: Changes in outcomes over 3 and 6 months ($n$=52)

| Outcomes | Baseline | Month 3 | Month 6 | Mean change (0-3) (95% CI) | Mean change (0-6) (95% CI) | Cohen's d (0-3) | Cohen's d (0-6) |
|---|---|---|---|---|---|---|---|
| VASs (mm) | | | | | | | |
| Bleeding ($n$=46) | 42.9 (28.9) | 21.1 (20.8) | 17.4 (20.6) | −21.8 (−30.3, −13.3)* | −25.5 (−35.4, −15.6 )* | 0.787 | 0.775 |
| Pain ($n$=49) | 45.7 (26.5) | 24.5 (21.2) | 18.2 (20.1) | −21.3 (−28.6, −14.0)* | −27.6 (−35.6, −19.6)* | 0.851 | 1.003 |
| Heaviness ($n$=34) | 33.6 (23.5) | 23.6 (19.6) | 19.6 (20.6) | −8.1 (−13.9, −2.3)* | −12.1 (−19.1, −5.1)* | 0.609 | 0.693 |
| Discharge ($n$=22) | 14.9 (22.2) | 10.5 (13.9) | 7.3 (11.6) | −4.0 (−10.9, 2.9) | −6.3 (−15.4, 2.8) | 0.293 | 0.372 |
| Itching ($n$=32) | 23.9 (20.1) | 17.5 (17.5) | 12.6 (17.8) | −4.2 (−8.9, 0.5) | −8.1 (−14.6, −1.6)* | 0.465 | 0.586 |
| Anoscopic score | 0.9 (0.6) | 0.5 (0.6) | 0.5 (0.6) | −0.4 (−0.6, −0.2)* | −0.5 (−0.7, −0.3)* | 0.760 | 0.703 |

CI: Confidence interval

$t$-test) was non-significant with medium effect size (Cohen's d) of 0.465 (Pearson's $r$ = 0.732). Reduction over month 6 (−8.1, 95% CI: −14.6, −1.6; $t$ = −2.499, $P$ = 0.017; paired $t$-test) was statistically significant with medium effect size of 0.586 (Pearson's $r$ = 0.483) [Table 2 and Figure 2].

### Anoscopic Score

The scores obtained at baseline, and every month up to 6 months were 0.9 ± 0.6, 0.8 ± 0.5, 0.6 ± 0.6, 0.5 ± 0.6, 0.5 ± 0.6, 0.4 ± 0.6, and 0.5 ± 0.6, respectively. When the score of baseline was compared with that of month 3 and month 6, the reductions achieved of −0.4 (95% CI: −0.6, −0.2; $t$ = −4.655, $P$ < 0.0001; paired $t$-test) and −0.5 (95% CI: −0.7, −0.3; $t$ = −4.728, $P$ < 0.0001; paired $t$-test) were statistically significant with almost large effect sizes of 0.760 (Pearson's $r$ = 0.615) and 0.703 (Pearson's $r$ = 0.550), respectively [Table 2 and Figure 3].

### Medicines Prescribed

The most frequently prescribed medicines were sulfur ($n$ = 14; 26.9%), nux vomica ($n$ = 9; 17.3%), calcarea phosphorica and natrum muriaticum ($n$ = 4 each; 7.7%), and causticum ($n$ = 3; 5.8%). Other prescribed medicines and their indications are enlisted in Tables 3 and 4. *Aesculus hippocastanum*, *Collinsonia canadensis*, muriatic acid, ratanhia, millefolium, and *Hamamelis virginiana* were used in lower centesimal potencies (6C, 30C) and mother tinctures as "acute" (rescue) remedies as and when required. Baseline prescriptions contained both centesimal ($n$ = 44; 84.6%) and 50 millesimal ($n$ = 6; 11.5%) potencies.

To detect medicine-specific treatment effects, if any, independent $t$-test was run to compare the reductions achieved on VASs and anoscopic scores over 3rd and 6th months by the two most frequently prescribed medicines – Sulfur and nux vomica. Analyses revealed significant reductions of pain VASs by sulfur only, both after 3 months (sulfur: 42.1 ± 32.2 vs. others: 12.9 ± 15.5; $t$ = 4.197; $P$ = 0.0001 two-tailed) and 6 months (sulfur: 49.6 ± 32.4 vs. others: 18.7 ± 19.9; $t$ = 3.964; $P$ = 0.0002 two-tailed) in comparison with other medicines. No other significant differences were observed under any occasion [Tables 5 and 6].

Some minor intercurrent illnesses were encountered, e.g., fever, indigestion, cough and cold, vertigo, frozen shoulder, diarrhea, dysentery, sore throat, hyperacidity, rheumatic complaints, and trauma; and all were treated successfully with homeopathic medicines.

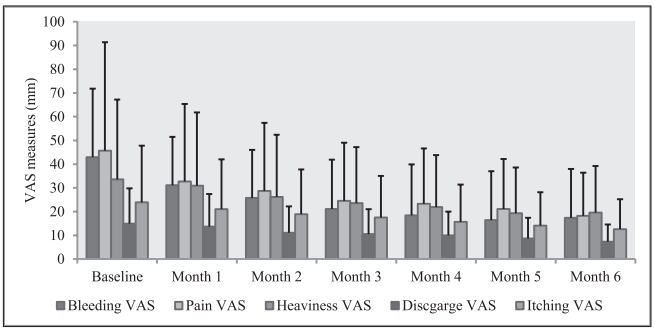


**Figure 2:** Changes in visual analog scales measures (mm) over months

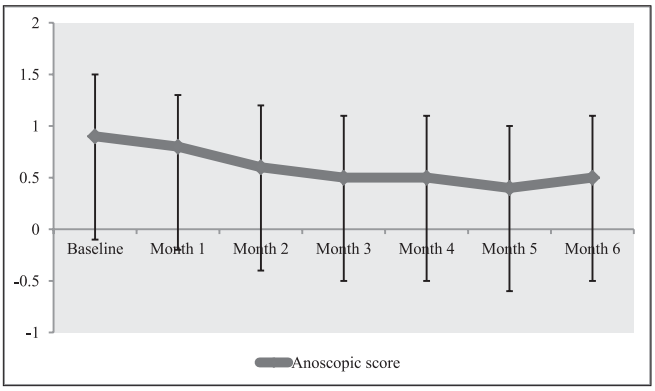


**Figure 3:** Changes in anoscopic score over months

## DISCUSSION

After individualized homeopathic treatment of the patients suffering from symptomatic hemorrhoids, there was statistically significant lowering of mean VAS intensity measures of bleeding, pain, and heaviness and anoscopic score over 3rd and 6th months. The discharge VAS also reduced but that was statistically non-significant. Improvement was also observed in itching VAS but over a 6th month only. The effect sizes of the severity ratings after 3 and 6 months were moderate to almost large (except discharge VASs). Assessments of disease severity consistently showed substantial improvements, although the disease was long-standing, chronic, and mostly pre-treated. This may be partly explained by placebo and/or regression to the mean effects that our study was not designed to control. We also cannot rule out overestimation of the

treatment effect and undisclosed use of concurrent therapeutic modalities, if any.

This prospective multicenter observational study was aimed to reflect the contemporary homeopathic health care in real practice settings and its outcome in 52 adult hemorrhoids patients. The methodological strengths of our study include the consecutive patient enrollment, the participation of qualified and experienced homeopathic physicians schooled in and practicing "classical" homeopathy, and use of standardized and already validated outcome scales. Our study is representative of individualizing ("classical") homeopathy only. In a broader interpretation of the law of similar, remedies are selected for symptoms both typical of the diagnoses and outside the predominating pathologies ("constitutional"). In contrast to randomized trials, our study describes patients from everyday practice with multiple morbidities and a large variety of life styles. This ensures a high degree of external validity that allows extrapolation to usual medical care. We used VASs that are validated, often used and allowed for assessments of a specific complaint as well as for generalization and interpretation across various diagnoses.

Randomized controlled trials (RCTs) are known to be the gold standard for medical research investigating the efficacy of new interventions. However, this is also not an unquestionable truth. Whereas RCTs unquestionably hold many advantages over observational studies, it should be recognized that they also have many flaws that render them fallible under certain

**Table 3: Medicines prescribed at baseline**

| Medicines | Number of prescriptions* *n* (%) |
|---|---|
| Sulfur | 14 (26.9) |
| Nux vomica | 9 (17.3) |
| Calcarea phosphorica | 4 (7.7) |
| Natrum muriaticum | 4 (7.7) |
| Causticum | 3 (5.8) |
| Calcarea carbonica | 2 (3.9) |
| Natrum sulfuricum | 2 (3.9) |
| Nitric acid | 2 (3.9) |
| Pulsatilla nigricans | 2 (3.9) |
| Ratanhia | 2 (3.9) |
| Aloe socotrina | 1 (1.9) |
| Apis mellifica | 1 (1.9) |
| Arnica montana | 1 (1.9) |
| Lycopodium clavatum | 1 (1.9) |
| Mercurius solubilis | 1 (1.9) |
| Phosphorus | 1 (1.9) |

*Placebo was prescribed at baseline in two occasions

**Table 4: Indications of the most frequently prescribed medicines**

| | |
|---|---|
| Sulfur | |
| | Bleeding piles with constipation, hard stool, burning pain in rectum during and after stool; burning and itching in rectum and piles |
| | Weeping disposition, weak memory, easily excited and irritated, quick motioned, quick tempered; lazy, indolent |
| | Thermally hot; catches cold readily; scrofulous diathesis; skin extremely sensitive to atmospheric changes |
| | Desire for sweet and meat |
| | Averse to washing; offensive odor from body even after washing; stoop-shouldered and bright red lips |
| Nux vomica | |
| | Painful bleeding piles with ineffectual urging and constipation; alternate constipation and diarrhea |
| | Colic after meals; sour eructations, nausea, and vomiting, agg. morning |
| | Thermally chilly; oversensitive to external impressions |
| | Patients who are anxious, hypochondriac, and leading a sedentary life |
| | Persons very particular, careful, irritable, impatient, easily excited and angered |
| Calcarea phosphorica | |
| | Painful piles; agg. from thinking of ailment, amel. hot application |
| | At every attempt to eat, colicky pain in abdomen |
| | Thermally chilly |
| Natrum muriaticum | |
| | Bleeding piles; burning and stitching after stool; stool dry, hard, crumbling; anus seems contracted |
| | Irritable, nervous patients; weeping disposition; suppressed and silent grief |
| | Tendency to catch cold easily |
| | Mapped tongue, indented edges, red insular patches |
| | Bad effects of excessive use of salt; craving for salt, aversion to bread |
| | Better while fasting |
| Causticum | |
| | Piles swollen, itching, burning, soreness, with constipation, and causing obstruction in the passage of stool; pain in piles while walking |
| | Intense sympathy for suffering of others; melancholy, sad, hopeless; ailments from long lasting grief or sorrow, severe mental shock |
| | Thermally chilly |

**Table 5: Medicine-specific treatment effects observed after 3 months**

| Groups | Reduction of bleeding VAS | *P* value | Reduction of pain VAS | *P* value | Reduction of heaviness VAS | *P* value | Reduction of discharge VAS | *P* value | Reduction of itching VAS | *P* value | Reduction of anoscopic score | *P* value |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Sulfur | 29.5 (31.1) | 0.234 | 42.1 (32.2) | 0.0001* | 6.2 (19.1) | 0.619 | 6.7 (17.7) | 0.627 | 3.1 (9.3) | 0.740 | 0.39 (0.49) | 0.749 |
| Others | 18.4 (26.5) | | 12.9 (15.5) | | 9.2 (15.9) | | 2.9 (15.7) | | 4.8 (16.2) | | 0.44 (0.50) | |
| Nux vomica | 32.8 (34.2) | 0.205 | 24.4 (17.7) | 0.682 | 9.9 (15.3) | 0.750 | 0 (0) | 0.416 | 12.3 (17.8) | 0.071 | 0.38 (0.48) | 0.777 |
| Others | 19.1 (26.2) | | 20.5 (26.7) | | 7.6 (17.6) | | 6.1 (19.8) | | 1.9 (12.1) | | 0.44 (0.50) | |

Independent *t*-test; *$P<0.05$ two-tailed considered as statistically significant. VAS: Visual analog scales

Table 6: Medicine-specific treatment effects observed after 6 months

| Groups | Reduction of bleeding VAS | *P* value | Reduction of pain VAS | *P* value | Reduction of heaviness VAS | *P* value | Reduction of discharge VAS | *P* value | Reduction of itching VAS | *P* value | Reduction of anoscopic score | *P* value |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Sulfur | 32.7 (35.5) | 0.344 | 49.6 (32.4) | 0.0002* | 8.8 (17.3) | 0.491 | 6 (18.3) | 0.963 | 5.4 (18.7) | 0.542 | 0.5 (0.5) | 0.658 |
| Others | 22.3 (31.9) | | 18.7 (19.9) | | 13.9 (22.2) | | 6.5 (22.9) | | 9.6 (19.8) | | 0.4 (0.6) | |
| Nux vomica | 35 (39.3) | 0.353 | 32.8 (17.3) | 0.544 | 20.5 (21.1) | 0.205 | −3.1 (8.3) | 0.142 | 18.8 (17.8) | 0.087 | 0.5 (0.5) | 0.928 |
| Others | 23.2 (31.4) | | 26.4 (29.7) | | 9.7 (20.0) | | 11.1 (24.5) | | 5.2 (18.9) | | 0.48 (0.58) | |

Independent *t*-test; *$P<0.05$ two-tailed considered as statistically significant. VAS: Visual analog scales

circumstances. RCTs may suffer from low external validity, i.e., rigid design control could reduce the ability to generalize the results. Another issue relating to RCTs is the fact that recruitment, randomization, and blinding are not always possible because of technical or ethical issues. So, a well-designed observational trial can be a better alternative in this situation, and they, in fact, do not systematically overestimate the magnitude of treatment effects as compared with those in RCTs [27,28]. So, RCTs and observational trials can be used synergistically to obtain more and better information. For example, observational trials can be used to test the external validity of RCTs by expanding the settings to a more representative population and formulate hypotheses for RCTs to test [29]; identify structures, processes, and outcomes to study and help establish the appropriate sample size for an RCT [30]. The main strength of observational studies is their greater proximity to "real life situations" by capturing large amount of uneven data since RCTs have stricter inclusion criteria and rigid protocols that may not reflect clinical practice. Other advantages of observational trials are that they are usually cheaper than RCTs, can be used to investigate rare outcomes, and easy to perform. Observational trials are also important for creating new hypotheses, proving the external validity of RCTs already performed, and establishing the sample size for RCT. In this way, observational trials can be complementary to RCTs in spite of their evidence level being lower than that of RCTs. This kind of investigation is crucial for elucidating many scientific questions [31]. Observational trials cannot replace RCTs nor do RCTs make non-experimental studies unnecessary or undesirable. When both experimental and non-experimental studies address the same question, both can contribute usefully to answering the question. Thorough consideration of both RCTs and observational trials will enhance the interpretation of the totality of the evidence and lead to stronger overall inferences about clinical questions and decision-making [32]. The advantages of including both in a meta-analysis could outweigh the disadvantages in many situations and is consistent with an evidence-based approach [33]. "Every research strategy within a discipline contributes importantly relevant and complementary information to a totality of evidence on which rational clinical decision-making and public policy can be reliably based. In this context, observational evidence has provided and will continue to make unique and important contributions to this totality of evidence on which to support a judgment of proof beyond a reasonable doubt in the evaluation of interventions" [34].

Comparing to the efficacy study by Chakraborty *et al*. [22], noticeable drop of VAS intensity measures of pain, bleeding, heaviness, itching, and anoscopic score was observed; however, there was no significant reduction of discharge, a similar finding of the earlier study [22]. Thus, the exact role of individualized homeopathic treatment in controlling discharges of hemorrhoids becomes questionable in two different study designs and needs further evaluation.

Although specific statistical analyses were run to detect specific treatment effects of individual remedies (sulfur and nux vomica), the study was never designed to assess the same; rather only to assess the role of individualized mode of homeopathic treatment. It may be only a play of chance or mere coincidence that in the representative sample of hemorrhoids patients, higher symptom similarities were observed with sulfur or nux vomica symptoms, hence prescribed in higher frequencies than other medicines; or significant pain reduction was attributed by sulfur group of patients; but these findings, in no way, undermine the potential of other medicines in improving hemorrhoids. Rather, these findings should be subjected to controlled efficacy trials for confirmation in future.

Our study does not support conclusions as to the effectiveness of the homeopathic remedies because no methodology for this purpose (control group, randomization, blinding, etc.) was built into its design. The aim of the investigation was to explore the effects of homeopathic medical care in treating some forms of hemorrhoids. Any improvement observed in this study should not be extrapolated to other forms of hemorrhoids. The data may also be helpful in the planning of further research projects on homeopathy. It would require specific instruments for more detailed assessment and effect size comparability, and a longer observation period.

## CONCLUSION

Under "classical" or "individualized" homeopathic treatment of 6 months, the severity of hemorrhoidal symptoms – pain, bleeding, heaviness, and itching improved substantially; however, there was no significant lowering of discharge. Overall, homeopathic treatment appeared promising but needs further rigorous exploration in different designs for arriving at a confirmatory conclusion.

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Original Research

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# *In vitro* xanthine oxidase and albumin denaturation inhibition assay of *Barringtonia racemosa* L. and total phenolic content analysis for potential anti-inflammatory use in gouty arthritis

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## ABSTRACT

**Aim:** This study was conducted to evaluate the *in vitro* anti-inflammatory activities and total phenolic content (TPC) of methanolic extracts of infloresence axes, endosperms, leaves, and pericarps of *Barringtonia racemosa* L. **Methods:** The anti-inflammatory study was conducted by assessing the potential through xanthine oxidase (XO) and albumin denaturation inhibition assays. Meanwhile, the TPC in the extracts were assessed by Folin-Ciocalteu assay. **Results:** In the XO inhibition assay, the infloresence axes extract was found to exert the highest inhibition capacity at 0.1% (w/v) with 59.54 ± 0.001% inhibition followed by leaves (58.82 ± 0.001%), pericarps (57.99 ± 0.003%), and endosperms (57.20 ± 0.003%) extracts. Similarly in the albumin denaturation inhibition assay, the infloresence axes extract had shown the greatest inhibition capacity with 70.58 ± 0.004% inhibition followed by endosperms (66.80 ± 0.024%), leaves (65.29 ± 0.006%), and pericarps extracts (43.33 ± 0.002%). Meanwhile, for TPC analysis, leaves extract was found to have the highest phenolic content (53.94 ± 0.000 mg gallic acid equivalent [GAE]/g DW) followed by infloresence axes (31.54 ± 0.001 mg GAE/g DW), endosperms (22.63 ± 0.001 mg GAE/g DW), and the least was found in pericarps (15.54 ± 0.001 mg GAE/g DW). **Conclusion:** The results indeed verified the *in vitro* anti-inflammatory activities of *B. racemosa* and supported its potential to be used in alleviating gouty arthritis and XO-related diseases.

KEY WORDS: Anti-inflammatory activity, *Barringtonia racemosa*, protein denaturation inhibition, total phenolic content, xanthine oxidase inhibition

## INTRODUCTION

According to the data from the World Health Organization, the value of worldwide annual market for herbal medicinal products approaches US$60 billion [1]. Modern science has proven that the association of plants in medicinal approach is scientifically sound due to the bioactive compound constituents in plants which later paved the way for further discovery of drug developments from plants.

*Barringtonia racemosa* (L). which is also known as Putat, "fish poison" or "powder puff" is a type of plant species found to be widely distributed from Eastern Africa and Madagascar to Micronesian and Polynesian Island. Due to such diverse distribution, this species has been very well associated ethnobotanically in various tribes around the world. Pharmacologically, this species had been scientifically proven to possess various medicinal benefits [2], among which are antibacterial [3-5], anti-mycobacterial [6], and anti-fungal [4]. Apart from being proven to have anti-infective activities, *B. racemosa* also successfully showed promising anti-tumor [7] and anti-arthritic activities [8] with excellent analgesic [5,9,10] and antioxidant properties [11-13].

One of the most common and essential properties of secondary metabolites is anti-inflammatory activity. Inflammation is caused by release of chemicals from tissues and migrating cells [14]. The occurrence of inflammation has been regarded to be associated in a number of disorders and prominently related to the painful condition. Gouty arthritis has been

identified to be the most common inflammatory arthritis in general practice and may significantly impair quality of life due to restricted mobility [15]. This study was carried out to evaluate the potential of *B. racemosa* to be used as a natural remedy for the treatment of inflammatory diseases. Particular focus on inflammatory gouty arthritis was emphasized since xanthine oxidase (XO) inhibitory activity assay was being scrutinized; wherein the XO has been identified as the culprit in gouty arthritis pathogenesis due to the formation of uric acid deposition in gouty patients.

## MATERIALS AND METHODS

### Sample Preparation and Extraction

The plant materials of *B. racemosa* were collected from Nasuha Herbal Farm, Johor, Malaysia. The determination of botanical term for plant parts used was made by referring to glossary of botanical terms [16] and Prance [17]. Four plant parts were studied which were infloresence axis, leaf [Figure 1], endosperm, and pericarp part of fruits [Figure 2]. The samples were air dried under the shade at ambient temperature ranging from 28°C to 32°C. The dried plant samples were further crushed into coarse powder using a domestic electric grinder (Philips, Netherlands). The extracts were prepared by soaking 5 g of samples in 200 ml of 70% methanol (Merck, Germany) diluted with 30% deionized water (Arium® pro Sartorius, Germany). The macerated samples were filtered using Whatman filter paper Number 1 (70 mm) (GE Healthcare, UK) placed on Buchner funnel whereby the filtration was assisted by a vacuum pump (GAST, USA). The resulting filtrates were then concentrated under vacuum using rotary evaporator system (Buchi, Switzerland) to yield concentrated extracts of samples. The resulting extracts were kept in glass vials and refrigerated at 4°C until further use.

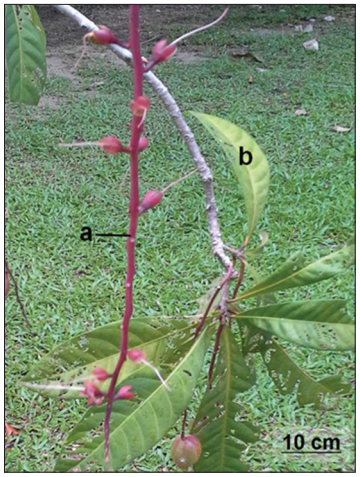


**Figure 1:** The (a) infloresence axis and (b) leaf of *Barringtonia racemosa*

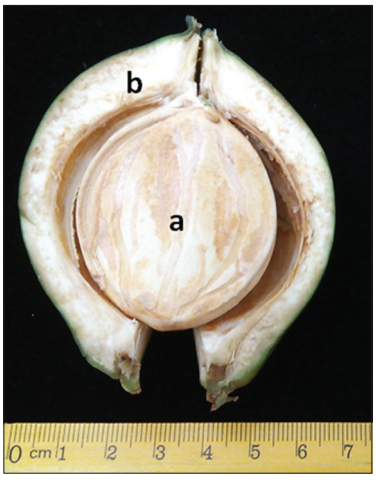


**Figure 2:** The (a) endosperm and (b) pericarp part of *Barringtonia racemosa* fruit

### Anti-inflammatory Activity Assays

*XO inhibitory activity of B. racemosa*

The assay method was carried out according to Azmi *et al.* [18] with some modifications in which the positive standard used in the current study was oxypurinol. The concentrations of samples and standards used in the study were expressed in the form of a percentage (%). The plant extracts were prepared at a concentration of 0.1% each (1.0 mg/ml). The assay mixtures were prepared by adding 300 $\mu$l of 50 mM potassium phosphate buffer (pH 7.5), 100 $\mu$l sample solutions, 100 $\mu$l of freshly prepared XO enzyme solution (0.2 U/ml in phosphate buffer), and 100 $\mu$l of deionized water. The mixtures were incubated at 37°C for 15 min. Afterward, 200 $\mu$l substrate solution (0.15 mM of xanthine) will be added into the assay mixture and further incubated at 37°C for 30 min. The reaction was stopped by the addition of 200 $\mu$l of 0.5 M hydrochloric acid. The XO inhibitory activities were assayed spectrophotometrically at 295 nm (indication of uric acid formation at 25°C) using UV/vis spectrophotometer (Jasco V-630 Spectrophotometer, Japan), and the data were processed by Spectra Manager system. Oxypurinol (001 %) was used as a positive control. The assay mixture without sample extract served as a negative control. All assays were done in triplicate; thus, inhibition percentages are the mean of three observations. The XO inhibitory activities were expressed as the percentage of inhibition of XO, calculated as follows:

$$\% \text{ XO inhibition} = (1 - B/A) \times 100\%$$

Where B is the absorbance reading of the test sample, and A is the absorbance reading without test sample (negative control).

Various concentrations of 0.025%, 0.050%, 0.075%, and 0.100% of oxypurinol and the most optimum extract were evaluated for XO inhibitory activity. The dose-response graph was utilized to generate a linear equation to estimate the concentration at which maximal inhibition (100%) is obtained.

*Albumin denaturation inhibitory activity of B. racemosa*

The assay was carried out by adopting the methods described by Kumari *et al.* [19] with some modifications in which the volume of each component in the reaction mixtures was reduced

by half. The plant extracts and positive standards (ibuprofen and diclofenac) were prepared at a concentration of 0.1% each (1.0 mg/ml). A reaction vessel for each mixture was prepared consisted of 200 $\mu$l of egg albumin, 1400 $\mu$l of phosphate buffered saline, and 1000 $\mu$l of the test extract. Distilled water instead of extracts was used as a negative control. Afterward, the mixtures were incubated at 37°C for 15 min and then heated at 70°C for 5 min. After cooling, their absorbances were measured at 660 nm (Jasco V-630 Spectrophotometer, Japan) and the data were processed by Spectra Manager system. The inhibition percentage of protein denaturation was calculated using the following formula:

$$\% \text{ Denaturation inhibition} = (1-D/C)\times 100\%$$

Where D is the absorbance reading of the test sample, and C is the absorbance reading without test sample (negative control).

### Total Phenolic Content (TPC) Analysis of *B. racemosa*

The TPC analysis using Follin-Ciocalteau method was carried out according to Almey *et al.* [20] with minor modifications in the duration of incubation before absorbance measurement being recorded which was altered to be 1 h (60 min). Gallic acid was used as a positive reference standard. The plant extracts as well as the positive standard were prepared as stock solutions at a concentration of 0.1% each (1.0 mg/ml). Working standards of between 0.01 and 0.05% were prepared for TPC analysis. The reaction vessels were prepared by placing 100 $\mu$L plant extract added with 750 $\mu$L Folin-Ciocalteu reagent in each vessel. The mixture was incubated at room temperature (25°C) for 5 min. Afterward, 750 $\mu$L of 6.0% (w/v) sodium carbonate was added into each reaction vessel and mixed thoroughly. The mixtures were incubated at room temperature (25°C) for 60 min before the absorbance of each sample was read at 765 nm by spectrophotometer (Thermo GENESYS™ 20 Visible Spectrophotometer, USA). The assays were done in triplicate. The standard calibration curve of gallic acid was plotted and used for the determination of TPC in the test samples. A linear equation was generated from the curve to identify related gallic acid concentration by substituting the corresponding absorbance value as "y" values in the equation to find the resulting concentration of gallic acid (mg/ml) as the "x" values. Afterward, the TPC of each plant sample was calculated according to the following formula. The results were expressed as mg GAE/g DW (mg gallic acid equivalent/g dry weight of extract):

$$\text{TPC} = (C)\ V/M$$

Where, TPC = Total phenolic content (mg GAE/g DW)
C = Concentration of GA from calibration curve linear equation (mg/ml)
V = Volume of the extract solution (ml)
M = Weight of extract used (g).

### Statistical Analyses of Results

The results were expressed as mean ± standard deviation of triplicate readings and statistically analyzed by IBM SPSS Statistics version 17.0. The data were subjected to one-way analysis of variance. Tukey's honest significant difference (HSD) test ($P < 0.01$) was performed to determine the significance of the difference between means of different plant part extracts.

## RESULTS

### Anti-inflammatory Activitiy Assays

*XO inhibitory activity of B. racemosa*

All extracts had shown more than 50% inhibition against XO activities [Table 1]. This signified that the extracts effectively inhibited XO from catalyzing the action of converting xanthine to uric acid at a considerably low concentration of 0.1%. The infloresence axes extract was found to exert the highest inhibition capacity at 0.1% (v/v) with 59.54 ± 0.001% inhibitory activity followed by leaves (58.82 ± 0.001%), pericarps (57.99 ± 0.003%), and endosperms (57.20 ± 0.003%) extracts. Meanwhile, the positive control, oxypurinol recorded 68.36% inhibition of XO activities at a relatively similar concentration.

*Albumin denaturation inhibitory activity of B. racemosa*

All extracts had given more than 50% inhibition [Table 1]. The infloresence axes extract had shown the greatest inhibition capacity with 70.58 ± 0.004% followed by endosperms (66.80 ± 0.024%), leaves (65.29 ± 0.006%), and pericarps extracts (43.33 ± 0.002%). Meanwhile, the non-steroidal anti-inflammatory drugs (NSAIDs) drugs used which were ibuprofen and diclofenac sodium exhibited relatively higher inhibition capacity of 83.53 ± 0.003% and 94.90 ± 0.004%, respectively.

*TPC Analysis of B. racemosa*

A calibration curve had been plotted using the absorbance data of gallic acid over serial concentrations [Figure 3]. An equation of $y = 6.2500x + 0.0429$ was obtained for its linear equation with the correlation value of $R^2 = 0.9944$.

Different plant parts had given different TPC values in *B. racemosa* [Table 1] with the leading phenolic content

**Table 1: The anti-inflammatory activities (in xanthine oxidase inhibitory and albumin denaturation assay) and TPC of different plant parts of *B. racemosa***

| Plant part | Assay | | |
|---|---|---|---|
| | Percentage±SD | | TPC (mg GAE/g DW±SD) |
| | Xanthine oxidase inhibition | Albumin denaturation inhibition | |
| Infloresence axis | 59.54±0.001[a] | 70.58±0.004[a] | 31.54±0.001[a] |
| Endosperm | 57.20±0.003[b] | 66.80±0.024[a] | 22.63±0.001[b] |
| Leaf | 58.82±0.001[c] | 65.29±0.006[a] | 53.94±0.000[c] |
| Pericarp | 57.99±0.003[d] | 43.33±0.002[b] | 15.54±0.001[d] |

Means in each assay followed by the same letter are not significantly different from each other according to Tukey HSD test at $P<0.01$.
SD: Standard deviation, *B. racemosa*: *Barringtonia racemosa,*
TPC: Total phenolic content

was found in leaves extract (53.94 ± 0.000 mg GAE/g DW). This was followed by infloresence axes (31.54 ± 0.001 mg GAE/g DW), endosperms (22.63 ± 0.001 mg GAE/g DW) and the least was found in the pericarp part of fruits extracts (15.54 ± 0.001 mg GAE/g DW).

## DISCUSSION

### *In Vitro* Anti-inflammatory Activities

*XO inhibitory activity of B. racemosa*

According to a review by Borges *et al.* [21], XO is a type of enzyme which is ubiquitously found among species and tissues of mammals and it catalyzes the oxidative hydroxylation of purine substrates and subsequent reduction of $O_2$ with generation of reactive oxygen species, either superoxide anion radical or hydrogen peroxide. In rheumatological diseases point of view, XO is an enzyme responsible for catalyzing the oxidation of hypoxanthine to xanthine and further lead to the formation of uric acid [22] in which the elevated beyond normal levels will be the underlying reason of gouty arthritis attack [23]. Even though XO is the factor which leads to the occurrence of gouty arthritis, there is an overwhelming acceptance that XO serum levels are significantly increased in various pathological states such as hepatitis, inflammation, ischemia-reperfusion, carcinogenesis, and aging [21].

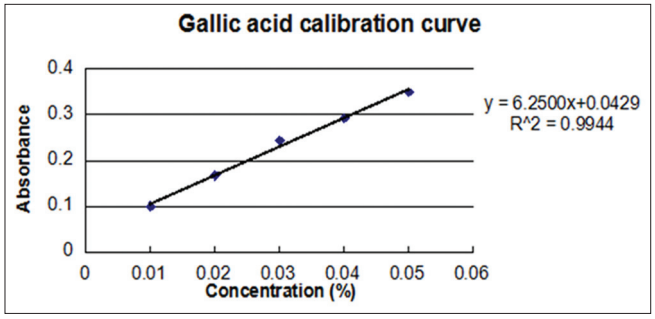


**Figure 3:** Gallic acid calibration curve for Folin-Ciocalteu total phenolic content assay of *Barringtonia racemosa*

The action of XO inhibitor is therefore required in the prevention and treatment of gout whereby it inhibits the biosynthesis of uric acid from purine [Figure 4] and allopurinol is the widely used synthetic XO inhibitor used in modern medicine in the treatment of gout [24]. Both allopurinol and its active metabolite, oxypurinol (isosteres of hypoxanthine and xanthine, respectively), inhibit XO. Their competitive inhibitions thereby limiting the biosynthesis of uric acid hence promoting renal clearance of hypoxanthine and xanthine [21,25].

Nevertheless, allopurinol may exert certain side effects due to allergy and may generate rashes [18]. In serious cases, allopurinol may also lead to fatality due to adverse drug reactions attributed to allopurinol hypersensitivity [18,26]. Due to such reason, the search for natural sources of plant-based medicines with profound effects of XO inhibition activities is seen to be worthwhile.

Considering the present study, it had been proven that *B. racemosa* effectively inhibits XO activity which resulted in great inhibition percentage in the most optimum extract from the infloresence axes part (59.54 ± 0.001%) at a concentration of as low as 0.10%. Oxypurinol was used as a positive standard for XO inhibition in this study whereby it is known as an active metabolite of allopurinol; hence functions as an inhibitor of XO. Oxypurinol had shown greater XO inhibition by exerting 68.36 ± 0.003% inhibitory activity at a concentration of 0.1%. The linear equations generated from the dose-response plot of infloresence axes extract and oxypurinol were utilized to estimate the effective concentration for maximal inhibition in both samples. On value substitution, maximal inhibition (100%) in infloresence axes extract was estimated to be at a concentration of 1.07% (10.7 mg/ml) while oxypurinol exhibited 0.41% (4.10 mg/ml) as its estimated effective concentration for maximal XO inhibition. According to *post-hoc* analysis of Tukey HSD, there were significant differences ($P < 0.01$) between means of each sample in the XO inhibition activity.

The previous studies done on *B. racemosa* showed its protective effects against adjuvant-induced arthritis in animal models [8], and therefore, corroborated the potential use of this species to

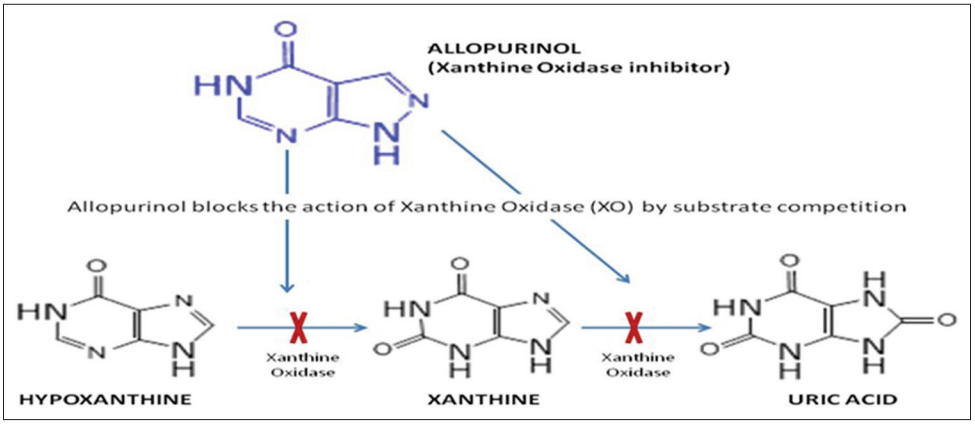


**Figure 4:** The reaction cascade of allopurinol in xanthine oxidase inhibition mechanism and uric acid biosynthesis in gouty arthritis pathogenesis

alleviate inflammatory symptoms in rheumatic-related diseases. In the study carried out by Patil *et al.* [8], the consumption of *B. racemosa*-derived bartogenic acid resulted in protection against primary and secondary arthritic lesions, body weight changes, and hematological pertubations. In addition, the serum markers of inflammation and arthritis in the arthritic rats were also reduced. On radiological analysis and pain score, the effectiveness of the species was found to be promising and protective against arthritis. Considering its effectiveness in rheumatological disorders, therefore in the current study the potential use of *B. racemosa* in alleviating gouty arthritis was being assessed since to date, there were neither *in vivo* nor *in vitro* studies had ever been recorded to evaluate its potential to be used as gouty remedies. In addition, according to toxicological studies done on *B. racemosa*, it had been verified that the species is devoid of toxicity [9,10] and may serve as a potential candidate to be further developed into a herbal-based formulation for gouty arthritis treatment.

*Albumin denaturation inhibitory activity of B. racemosa*

Inflammation is the reaction process of living tissues to stimuli evoked by inflammatory agents such as physical injuries, heat, microbial infections, and noxious chemical irritations. The response of cells toward inflammation will lead to certain pathological manifestations characterized by redness, heat, swelling, and pain with even impaired physiological functions. Inflammation has been implicated in the pathogenesis of many diseases including arthritis, stroke, and cancer [27]. Protein denaturation has been well correlated with the occurrence of the inflammatory response and leads to various inflammatory diseases including arthritis [28]. According to Opie [29], tissue injury during life might be referable to denaturation of the protein constituents of cells or of intercellular substance. Hence, the ability of a substance to inhibit the denaturation of protein signifies apparent potential for anti-inflammatory activity.

The capacity of different plant parts of *B. racemosa* to inhibit protein denaturation of albumin which was ranging from 43.33 ± 0.002% to 70.58 ± 0.004% inhibition in this assay had therefore provided another evidence for its promising anti-inflammatory properties. In the current study, ibuprofen and diclofenac sodium, the two routinely used NSAIDs for arthritis had been used as the reference compound anticipated to exert optimally positive inhibition percentage. A statistical analysis had shown that there were no significant differences ($P > 0.01$) between means of different plant parts except in pericarp, whereby the extract exhibited significant differences in multiple comparison analysis of *post-hoc* Tukey HSD test.

In clinical setting, major pharmacological agents used for the anti-inflammatory and pain-relief management are NSAIDs due to their capacity in inhibiting protein denaturation [30]. However, this type of drugs is associated with adverse effects on gastrointestinal tract leading to the formation of gastric ulcers and may result in cardiovascular complications as well [31-33]. Interestingly, *B. racemosa* had been documented to be used as a natural remedy for gastric ulcer as quoted by Hussin *et al.* [4] according to Deraniyagala *et al.* [9]. Indeed, this provides another added value for the species to be considered as a potential candidate for anti-inflammatory agent, deliberating the expectation that the risk of developing gastric ulcers could be minimized considering its ethnopharmacological use.

## TPC Analysis of *B. racemosa*

Phenolics are broadly distributed in the plant kingdom and are the most abundant secondary metabolites of plants [34]. Due to such reason, high intake of fruits and vegetables in daily diet are being recommended. Apart from being frequently associated with anti-oxidative properties [35], the presence of phenolic compounds in plants has been attributed to a number of significant pharmacological activities for instance as a cancer cell growth and development inhibitor [36-38], as natural antiulcer due to their gastroprotective nature [39] and providing pain relief for arthritis-related diseases [40].

In this study, the highest TPC was found in the extract of leaves followed in descending order by infloresence axes, endosperms, and pericarps. The *ex vitro* leaves extract of *B. racemosa* had demonstrated the greatest content of phenolic compounds (53.94 ± 0.000 mg GAE/g DW) and the least was found in the pericarp part of fruits (15.54 ± 0.001 mg GAE/g DW). The differences between means of plant parts in this TPC study were statistically significant ($P < 0.01$) according to *post-hoc* Tukey HSD test.

It has been observed that the values of TPC from the samples in the current research recorded greater phenolic content from those documented in the previous researches. Nurul-Mariam *et al.* [12] reported that the highest TPC was obtained from the methanolic extract of a stick of *B. racemosa* which recorded the value of 29.9 ± 0.02 mg GAE/g freeze dried weight. Meanwhile, Zawawi *et al.* [41] were investigated the value of TPC of *B. racemosa* in methanolic extract of young leaves, and the TPC value of the most optimum samples was found to be relatively low (0.34 mg GAE/g DW) as compared to that of TPC obtained from the current research. The differences in the findings could be due to many factors and may be affected by genotype, plant age, and developmental stages as well as sample preparation procedures.

Another point to be highlighted in the present study is the superiority of infloresence axis in its anti-inflammatory activities among other plant parts in both anti-inflammatory assays. It has been noted that even though the infloresence axis part was having the greatest anti-inflammatory activities; nevertheless in terms of plant phenolic content, its superiority was lower than leaves extract. Therefore, it could be suggested that the anti-inflammatory activities shown by the infloresence axes extract were possibly not solely due to the phenolic content of the species. The activities were, therefore, could be anticipated to be influenced by any other factors such as fatty acids compositions. The findings were almost similarly portrayed in *Jatropha curcas* L. in which the roots sample of *J. curcas* showed the highest anti-inflammatory activity but contained lower phenolic compounds than the leaves extract [42].

Nevertheless, more further studies are underway to investigate the distinctive presence of phenolic compounds in *B. racemosa* since it has been reported by Hussin *et al.* [4] that there was significant detection of phenolic compounds in the species (leaf, stick and bark parts) by using high performance liquid chromatography analysis. Therefore, this indeed requires further analyses to elucidate the compounds responsible for *B. racemosa*'s anti-inflammatory activities.

## CONCLUSION

This study showed promising properties of *B. racemosa* to be potentially used as a plant-derived anti-gouty arthritis remedy. The superior activities of infloresence axis had shown its potential to be optimally harnessed as a candidate in the mitigation of inflammatory diseases. Due to its XO inhibitory activity, this species would be useful in preventing the progress of other XO-related diseases as well. It is suggested that intense *in vivo* studies to be conducted to determine the amount recommended for consumption. Owing to its pharmacological importances, further studies and investigations are therefore required for this species to be optimally developed as pharmaceutical preparations in alleviating inflammation.

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# Hematological changes and nitric oxide levels accompanying high-dose artemether-lumefantrine administration in male guinea pigs: Effect of unsweetened natural cocoa powder

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## ABSTRACT

**Background:** Unsweetened natural cocoa powder (UNCP), prepared after removal of the cocoa butter, is a common beverage in Ghana. It possesses antimalarial prophylactic property and has a beneficial effect on blood components. **Aim:** The aim of this study was to determine whether regular dietary supplement of UNCP mitigates high-dose (HD) artemether-lumefantrine (A-L)-induced hematological disorders and to determine the effect on nitric oxide (NO) levels. **Materials and Methods:** Adult male guinea pigs (300 g - 350 g) were randomly divided into 5 groups of 6 guinea pigs each. Among the 5 groups, 3 groups were treated with UNCP (300, 900, and 1500 mg/kg body weight) for 14 days. A-L (75 mg/kg) was administered from the 12$^{th}$ to 14$^{th}$ day. One of the remaining 2 groups received distilled water only, i.e., vehicle control group (VCG) while the other received 75 mg/kg A-L only, i.e., negative control group (NCG). Blood samples from all groups were obtained by cardiac puncture (day 15) followed by hematological and NO analysis. **Results:** A-L reduced white blood cells (WBC) by 31.87%, lymphocyte count by 45.99%, hemoglobin by 11.72%, hematocrit by 18.56%, and platelet count by 33.08% in the NCG. Administration of various doses of UNCP increased WBC and lymphocyte count ($P > 0.05$) compared to the NCG. UNCP and A-L combination caused an increase in NO levels when compared to the VCG. **Conclusion:** Regular consumption of UNCP by guinea pigs increases plasma NO and restores some hematological disorders induced by a 3-day HD A-L administration.

KEY WORDS: Artemether-lumefantrine, hematology, nitric oxide, phytochemistry, unsweetened cocoa powder

## INTRODUCTION

Plants have been consumed or exploited for their medicinal purposes from time immemorial for as long as history has been recorded. Three categories of herbs are distinguished in Cherokee medicine, namely, food herbs, medicinal herbs, and poison herbs [1]. The food herbs are mild in action and are expected to have very low toxicity. One of such food herbs is the plant *Theobroma cacao*. The species *T. cacao* belongs to the class *Equisetopsida* and family *Malvaceae*. Cocoa is a major nutraceutical in Ghana and most parts of Africa. As a food herb, it is used by most indigenous people either in a raw state using the bean or the pod, as dark chocolate, and as a beverage in a powdered form obtained from the cocoa bean. Research

works are currently directed at gathering plant materials from the rain forests and other places for their potential medicinal values [2].

Cocoa contains water-soluble polyphenols (also called flavanols), which include catechins, epicatechin, procyanidins, anthocyanins, and leukoanthocyanins [3]. The quantitative analysis of unsweetened natural cocoa powder (UNCP) has also been well researched and proven that even flavanol contents are not likely to change during different manufacturing processes [4-6]. Several polyphenols such as 14 N-phenylpropenoyl-L-amino acids, N-[4'-hydroxy-(E)-cinnamoyl]-L-tryptophan, and N-[4'-hydroxy-3'-methoxy-(E)-cinnamoyl]-L-tyrosine have also been identified [7-9]. Pharmacologically, cocoa is known to exert antioxidant [10], anti-inflammatory [11,12], antimalarial, and anti-asthmatic properties [13-15]. Studies have also shown that UNCP could protect the liver, kidney, and heart against chemical injury in animals [2,3,10]. The antioxidant properties of these flavanols are ascribed partly to their structural characteristics, and it represents the molecular basis for their radical-scavenging property [3,10]. In spite of cocoa's antimalarial activity, its ability to increase nitric oxide (NO) production [16-18], white blood cell (WBC), and platelet counts (PLT) without changing hematocrit (HCT) and hemoglobin (HGB) levels could be of immense importance in malaria-infected individuals [19].

Artemether-lumefantrine (A-L) is a preferred artemisinin-based antimalarial in Ghana. Studies have shown that artemether can induce changes in hematological profiles in rats, which may aggravate anemia in malaria patients [20]. Besides, A-L has been found to reduce red blood cell count (RBC), HGB, and packed cell volume (PCV) in patients under treatment [21]. Though the WHO-recommended artemisinin-based combination therapy (ACT) [22,23] has impressive parasiticidal properties both *in vivo* and *in vitro*, there have been issues of treatment failures, resistance, and increasing cases of toxic effects [24,25]. Some countries have, therefore, considered increasing the dose of A-L used in the management of malaria to arrest the issue of resistance [26]. However, increase in dose implies that there will be increased side effects, adverse reactions, and other toxic effects [27]. A-L administration has been found to reduce NO levels. On the contrary, other studies have also shown that A-L increases NO levels as a compensatory mechanism in cases of reduced NO levels [16,26].

UNCP, prepared after removal of the cocoa butter from powdered cocoa beans, is taken several times on a daily basis as a beverage by patients while on treatment with A-L, a common practice in Ghana. Hematological parameters are one of the vital indices monitored during malaria treatment.

It is against this background that this study was conducted to investigate the effect of UNCP on the hematological parameters and NO levels during high-dose (HD) A-L administration.

The result of this study would serve as one of the scientific premises for discovering the beneficial effect of UNCP during its simultaneous consumption with A-L during malaria treatment.

## MATERIALS AND METHODS

### Preparation of UNCP Solution

The UNCP is a non-alkalized cocoa from Hords Company Ltd. Calculated amount (9.6 g) of Brown Gold Natural Cocoa Powder (Batch number BT620IT; FDA/DK 06-070) manufactured in Accra by Hords Company Ltd., Ghana, was dissolved in warm distilled water (40 ml) with stirring, making a concentration of 240 mg/ml (of the UNCP). This was administered to the guinea pigs in groups 3, 4, and 5 at their respective doses (i.e. 300, 900, and 1500 mg/kg) via oral gavage.

### Phytochemical Analysis

Phytochemical analysis was conducted to determine the various constituents in the unsweetened natural cocoa [28].

### Saponin Test

About 0.5 g of UNCP was added to water in a test tube. The test tube was shaken to observe foam formation.

### Tannins Test

About 0.5 g of UNCP was dissolved in 80% of aqueous methanol (10 $cm^3$). Freshly prepared iron (III) chloride solution was added and the color change was observed.

### Flavanoids Test

About 0.1 g of UNCP was added to 80% ethanol (15 $cm^3$). To the filtrate was added magnesium turnings followed by concentrated HCl (0.5 $cm^3$), and observed for color changes within 10 minutes.

### Cardiac Glycoside Test

About 0.5 g of UNCP was dissolved in chloroform (2 $cm^3$) in a test tube, after which concentrated sulfuric acid was carefully added down the side of the test tube to form a lower layer.

### Animal Experimentation

30 adult male guinea pigs weighing between 300 g and 350 g were purchased from the Animal Experimentation Department of the Noguchi Memorial Institute for Medical Research, University of Ghana, Legon. These animals were chosen according to their weight. The guinea pigs were acclimatized to laboratory environment (20-23°C) with a 12 h light-darkness cycle for 7 days prior to experimentation. The guinea pigs had access to standard laboratory diet and water *ad-libitum*. The study protocol was approved by the

departmental Ethical and Protocol Review Committee and the Noguchi Memorial Institute for Medical Research Institutional Animal Care and Use Committee with protocol approval number: 2013-01-3E.

### Experimental Design

The adult male guinea pigs were randomly assigned to 5 groups with each group containing 6 guinea pigs. Three groups received the UNCP at doses of 300, 900, and 1500 mg/kg per body weight, respectively, for 14 days, with two other groups serving as controls [15]. Animals in one of the two controls were given only A-L at a dose of 75 mg/kg body weight (negative control group [NCG]) whereas those in the other group were given distilled water (vehicle control group [VCG]) [20].

Nearly, 9.6 g of the UNCP was weighed and dissolved in 40 ml of hot distilled water and stirred continuously until homogeneous mixture was formed (everything went into solution). This was given to the animals daily for 14 days.

### Preparation of A-L Solution

A concentration of 20 mg/ml of the artemether was prepared and administered to the guinea pigs in the various groups at a dosage of 75 mg/kg body weight via oral route using the oral gavage for 3 days (i.e. from 12$^{th}$ to 14$^{th}$ day). Dosage was calculated with reference to the dose of artemether in the drug combination. To achieve this, 70 tablets of A-L (Novartis Coartem®) dispersible tablets (20/120 mg), which is equivalent to 1400 mg of artemether, was dissolved in 70 ml of distilled water and stirred until obtaining a completely homogeneous mixture.

In all cases, fresh solutions of UNCP and A-L were prepared before each dosing. The guinea pigs were, thus, observed daily for a total period of 14 days.

Group 1: Control (distilled water only)/vehicle control (CTRL)
Group 2: 75 mg/kg A-L (last 3 days)/negative control (COART)
Group 3: Cocoa 300 mg/kg (14 days) + 75 mg/kg A-L (last 3 days) (300)
Group 4: Cocoa 900 mg/kg (14 days) + 75 mg/kg A-L (last 3 days) (900)
Group 5: Cocoa 1500 mg/kg (14 days) + 75 mg/kg A-L (last 3 days) (1500).

### Calculation of Human Equivalent Doses (HED)

Conversion of animal doses to HED was based on basal surface area. HEDs were calculated according to a study by Reagan-Shaw *et al*., 2008, [29] using the formula:

$$\text{HED}\left(\text{mg}/\text{kg}\right)=\text{Animal dose}\left(\text{mg}/\text{kg}\right)\times\frac{\text{Animal km}}{\text{Human km}}$$

Taking note of the value of km factors (i.e., body weight, kg/surface area, m$^2$) for adult and guinea pigs to be 37 and 8, respectively, and an average weight of Ghanaian to be 70.0 kg, the HED and number of teaspoonful of UNCP at the various dose levels are calculated as follows:

300 mg/kg = 65 mg/kg, which is equivalent to 4540 mg for a 70 kg man = 4.54 g daily.

Assuming 1 teaspoonful of UNCP is 2.5 g, 300 mg/kg UNCP corresponds to approximately 2 teaspoonful of UNCP daily.

900 mg/kg = 194.6 mg/kg HED, which is equivalent to 13622 mg/70 kg man = 13.42 g daily (equivalent to 5.4 teaspoonful daily).

1500 mg/kg = 324.3 mg/kg HED, which is equivalent to 22701 mg/70 kg man = 22.7 g daily (equivalent to 9 teaspoonful daily).

75 mg/kg = 16.22 mg/kg HED, which is equivalent to 1135.4 mg/70 kg man = 1140 mg of A-L daily.

### Food and Water Intake and Body Weight

Food from Ghana Agro Food Company Ltd. and water consumption by the animals in the various groups was monitored both morning and evening throughout the study. Body weights were measured using and electronic balance daily before dosing of the animals, and any change was noted. Besides, the animals were monitored for any clinical observations.

### Hematological Studies

Guinea pigs were euthanized with 50 mg/kg chloroform by exsanguination and 2 ml of blood was sampled by cardiac puncture and transferred into EDTA-2k test tubes for immediate analysis. An automated hematology analyzer (KX-2IN, Sysmex Corporation, Japan) was used to estimate the counts of the various parameters considered in this study.

### NO Level Determination

The nitrite concentration in the plasma was measured as an index of NO levels by Griess reagent system (South Africa) according to the manufacturer's instruction.

In this measurement, the total NO kit by R&D Systems was used in this study. In this system, nitrate is converted to nitrite using nitrate reductase, after which the total nitrite is measured.

The principle of this assay determines NO concentration based on the enzymatic conversion of nitrate to nitrite by nitrate reductase. The reaction is followed by colorimetric detection of nitrite as an azo dye product of the Griess Reaction. The Griess Reaction is then based on the two-step diazotization reaction, in which acidified $NO_2^-$ produces a nitrosating agent, which reacts with sulfanilic acid to produce the diazonium ion. This ion is then coupled to *N*-(1-naphthyl) ethylenediamine to form the chromophoric azo-derivative, which absorbs light at 540-570 nm.

### Statistical Analysis and Data Evaluation

Statistical analysis of the data was done using GraphPad Prism Software version 5.0. Results were expressed as mean ± standard error of mean, $n = 6$. Statistical analysis was performed using one-way analysis of variance (ANOVA) followed by Student–Newman–Keuls *post-hoc* test. Statistical significance was set at $P < 0.05$.

With the NO experiment, data were expressed as ± standard deviation (SD), Dunnett's multiple comparison test was used in this *post-hoc* analysis. All statistical analyses were performed using GraphPad prism 5 software.

## RESULTS

### Phytochemical Analysis

Phytochemical analysis of UNCP showed the presence of saponins, flavonoids, tannins, and cardiac glycosides.

### Food and Water Intake and Body Weight

There was no increase in food consumption. Water consumption and urine output, however, increased except in the VCG and NCG. Changes in body weights were not significant. Animals that received 75 mg/kg A-L only showed signs of piloerection on day 13.

### Effects of A-L and UNCP on WBC Count

Figure 1a shows that A-L administration was accompanied by a reduction in WBC count in the NCG (Coartem®) by 31.87% as compared to the VCG ($P > 0.05$). Administration of UNCP at doses of 300, 900, and 1500 mg/kg body weight restored the WBC levels during concomitant administration with A-L ($P = 0.1158$).

### Effects of A-L and UNCP on Neutrophil Count

Figure 1b shows that A-L administration increased the neutrophil count of the NCG (Coartem®) by 30.20% as compared to the VCG ($P = 0.06$). Administration of UNCP at a dose of 300 mg/kg body weight decreased the neutrophil count by 90.38% as compared to the NCG (Coartem®). In addition, UNCP administration at doses of 900 and 1500 mg/kg restored these neutrophil levels during A-L administration ($P = 0.06$).

### Effects of A-L and UNCP on Monocyte Count

Figure 1c shows that A-L administration insignificantly caused a slight increase in monocyte count in the NCG (Coartem®) by 2.55% as compared to the control group ($P > 0.05$). Administration of UNCP at a dose of 300 mg/kg caused an insignificant decrease in monocyte count compared to the NCG. At a dose of 1500 mg/kg UNCP, however, levels of monocyte count during A-L administration increased by 24.03% ($P > 0.05$).

### Effects of A-L and UNCP on Lymphocyte Count

Figure 1d shows that A-L administration decreased the lymphocyte count of the NCG (Coartem®) by 45.99% as compared to the VCG ($P > 0.05$). Administration of UNCP at doses of 300, 900, and 1500 mg/kg body weight restored these lymphocyte count levels by 36.49%, 43.95%, and 56.19%, respectively ($P = 0.0014$), during A-L administration.

### Effects of A-L and UNCP on HGB Level

Figure 1e shows that A-L administration decreased HGB level of the NCG (Coartem®) by 11.72% as compared to the VCG ($P > 0.05$). Administration of UNCP at doses of 300, 900, and 1500 mg/kg body weight restored the HGB levels during A-L administration ($P > 0.05$).

### Effects of A-L and UNCP on Eosinophil Count

Figure 1f shows that administration of A-L and UNCP did not significantly influence eosinophil count although it increased by 6.03% in the NCG compared to the VCG ($P=0.1370$). Administration of UNCP at a dose of 300 mg/kg body weight increased eosinophil count by 25.64% compared to the NCG. In addition, comparing with eosinophil count of the NCG, UNCP at 900 mg/kg body weight caused a decrease of 110.81% while that of the 1500 mg/kg restored the eosinophil levels during the simultaneous administration of A-L by 16.58% ($P > 0.05$).

### Effects of A-L and UNCP on RBC

Figure 1g shows that A-L administration decreased RBC count of the NCG (Coartem®) by 6.43% as compared to the VCG ($P > 0.05$). Prophylactic administration of UNCP with A-L at doses of 300, 900, and 1500 mg/kg body weight restored the decreased levels of the RBC count by 4.17%, 5.55%, and 12.55%, respectively ($P > 0.05$).

### Effects of A-L and UNCP on Basophil Count

Figure 1h shows that the administration of A-L and UNCP did not significantly influence basophil count although it increased by 7.273% in the NCG compared to the NCG ($P > 0.05$). Administration of UNCP and A-L at a dose of 300 mg/kg increased basophil by 8.06% compared to the NCG. UNCP at 900 mg/kg caused a decrease at 34.14% while that of the 1500 mg/kg body weight restored the decreased levels of basophils by 39.56% ($P > 0.05$).

### Effects of A-L and UNCP on HCT Level

Figure 1i shows that A-L administration decreased HCT count in the NCG by 18.56% as compared to the VCG ($P < 0.05$). Administration of UNCP at doses of 300, 900, and 1500 mg/kg body weight restored HCT levels during A-L administration by 6.72%, 6.44%, and 11.37%, respectively ($P > 0.05$).

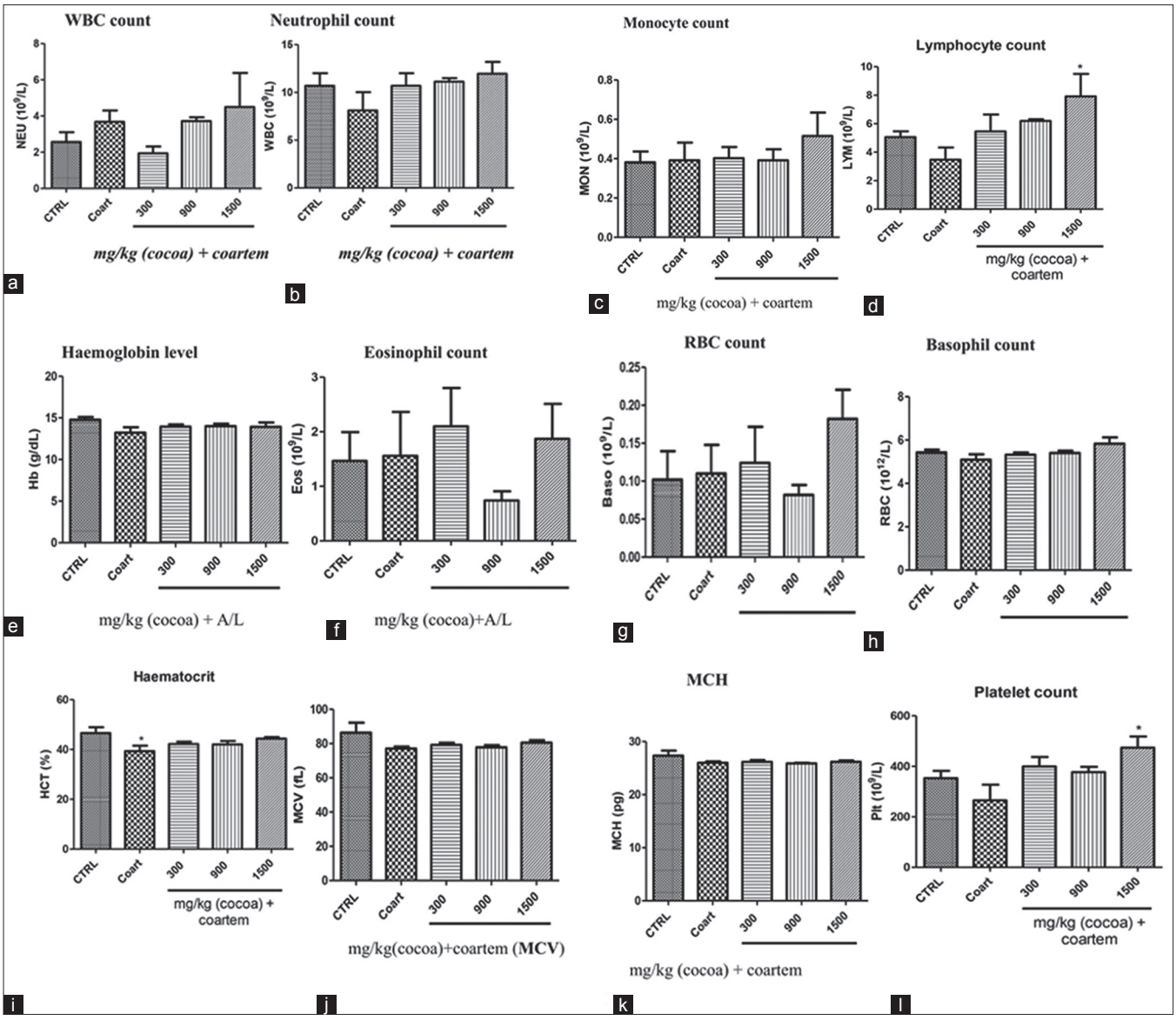


**Figure 1:** Effect of unsweetened natural cocoa powder (low dose [LD] = 300, medium dose [MD] = 900, and high dose [HD] = 1500 mg/kg) on some hematological parameters of guinea-pigs, (a) White blood cells. (b) Neutrophil count. (c) Monocyte count. (d) Lymphocyte count. (e) Hemoglobin level. (f) Eosinophil count. (g) Red blood cell count. (h) Basophil count. (i) Hematocrit. (j) Mean corpuscular volume. (k) Mean corpuscular hemoglobin. (l) Platelet count during 75 mg/kg artemether-lumefantrine administration. Values are mean ± standard error of mean (*N*=6). *means $P < 0.05$, **$P < 0.01$, and ***$P < 0.001$ compared to the vehicle control group (one-way analysis of variance, followed by a Dunnett's multiple comparison test)

## Effects of A-L and UNCP on Mean Corpuscular Volume (MCV)

Figure 1j shows that A-L administration insignificantly decreased MCV count of the NCG by 12.21% compared to the vehicle controls ($P > 0.05$). Administration of UNCP at doses of 300, 900, and 1500 mg/kg body weight restored the MCV levels ($P > 0.05$).

## Effects of A-L and UNCP on Mean Corpuscular Hemoglobin (MCH)

Figure 1k shows that the administration of A-L and UNCP did not significantly influence MCH although MCH decreased by 5.1% in the NCG compared to the VCG ($P > 0.05$). Administration of UNCP at a dose of 300 mg/kg body weight increased MCH by 0.61% compared to the NCG. In addition, comparing with MCH of the NCG, UNCP at 900 mg/kg body weight caused a reduction of 0.39% while that of the 1500 mg/kg body weight reversed these levels when administered with A-L ($P > 0.05$).

## Effects of A-L and UNCP on PLT

Figure 1l shows that A-L administration decreased PLT of the NCG by 33.08% as compared to the VCG ($P > 0.05$). Administration of UNCP with A-L at doses of 300, 900, and

1500 mg/kg body weight restored the decreased levels of the PLT by 33.62%, 29.70%, and 44.02%, respectively ($P < 0.05$ for UNCP administration at 1500 mg/kg body weight).

### Effects of A-L and UNCP on NO

Figure 2 shows the effect of UNCP (300, 900, and 1500 mg/kg) on nitrite (NO metabolite) concentrations in the plasma of guinea pigs during A-L administration. Values are mean ± SD ($n = 5$) and $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$ when compared to the A-L control (one-way ANOVA followed by a Dunnett's multiple comparison test). The low-dose UNCP (300 mg/kg) + A-L produced the greatest increase in NO followed by MD UNCP + A-L, which were both statistically significant, and then HD UNCP + A-L.

### NO Levels

The group administered with UNCP at a dose of 300 mg/kg recorded the highest increase of 149.71% in NO ($P < 0.05$) followed by the administration of UNCP at a dose of 900 mg/kg with 34.25% ($P < 0.05$), then group with UNCP at a dose of 1500 mg/kg group at 4.88% ($P < 0.05$) when compared to the VCG [Figure 2].

## DISCUSSION

Erythrocytic indices such as erythrocyte or RBC counts, HCT or PCV, and HGB are important indicators of the functional state of the erythron. Erythrocyte counts reflect the total number of RBC per unit volume of circulating blood whereas HGB determinations indicate the oxygen-carrying capacity of blood, and HCT determinations show the proportion of blood that is made up of cellular elements and the proportion that is plasma [30]. This study imitated the normal duration of Coartem used in Ghana (i.e. 3 days). UNCP at a dose of 75 mg/kg has also been used in previous studies as HD UNCP [19].

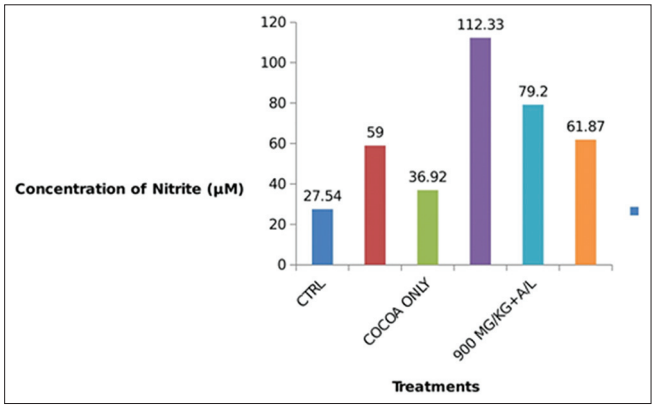


**Figure 2:** Effect of unsweetened natural cocoa powder (low dose [LD] = 300, medium dose [MD] = 900, and high dose [HD] = 1500 mg/kg) on nitrite concentration in plasma of guinea pigs during artemether-lumefantrine (A-L) administration. Values are mean ± standard deviation ($n = 5$) and $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$ compared to the control (one-way analysis of variance followed by a Dunnett's multiple comparison test). The LD unsweetened natural cocoa powder (300 mg/kg) + A-L produced the greatest increase (147.33 ± 117.78, $P < 0.05$, i.e., 149.71%) in nitric oxide followed by MD unsweetened natural cocoa powder + A-L (79.21 ± 36.24, $P < 0.05$, i.e., 34.25%) and then HD unsweetened natural cocoa powder + A-L (61.88 ± 3.83, $P < 0.05$, i.e., 4.88%) when compared to the A-L only group

From the results, A-L administration decreased the levels of WBC count, lymphocyte count, HGB, RBC count, and PLT, as observed in Figure 1a, c, e, h, and l, respectively, in the NCG (Coartem®). Normally, these reduced indices imply bone marrow depression, autoimmune hemolytic anemia, systemic lupus, or severe hemorrhage. The results concurred with similar results documented by the previous researchers that ACTs are known to cause embryonic erythrocyte depletion and delay erythroid differentiation [31].

The characteristic peroxide lactone structure of the artemether is indispensable for its antimalarial property, thus the splitting of this endoperoxide bridge by heme iron species in the guinea pigs may have resulted in the release of reactive oxygen that induced oxidative stress and caused the decreases that were observed with A-L administration. UNCP restored the decreased levels of HCT, HGB, and RBC, as observed in the A-L control group. Though not studied in this research, this might have been due to the antioxidant activity of UNCP, which is able to mop up the free radicals produced by the artemether moiety. The increased levels with UNCP (particularly with HCT levels) may be observed in dehydrated states as cocoa has been reported to possess diuretic effects. This was also confirmed in the general observations made, especially in the UNCP-treated groups as a general increase in diuresis and water consumption. The changes in body weight remained as an insignificant reduction. The decreased levels of WBC and lymphocyte count with A-L administration observed from the results concur with the previous work by Wang *et al.* [32] who also reported similar results, and that derivatives of artemisinin exhibited potent immunosuppressive activity by decreasing WBC and lymphocytes. However, UNCP restored the decreased levels of WBC ($P > 0.05$) and lymphocyte ($P < 0.05$) count as observed with A-L administration alone. The increase in WBC of the experimental guinea pigs meant that the administration of cocoa could boost the immune system, since these cells are important in protecting and fighting infections. This supports and explains the earlier observations that cocoa promotes superlative health by strengthening the immune system and prevents many viral diseases [19].

UNCP restored the decreased PLT as observed in A-L [Figure 1l]. The decreased PLT levels may be indicative of the presence of liver disease caused by the hepatotoxic effect of the HD A-L. Normally, PLT diminishes due to PLT trapping leading to enlarged spleen (splenomegaly). Splenomegaly due to artemether has been known since decades [33].

The mean corpuscular values (MCV and MCH), obtained from the RBC count, HCT, and HGB, are usually useful in elucidating and classifying anemia morphologically; they represent an estimation of the alterations in size and HGB content of individual RBC [30,34]. Results of this study showed that there were no significant differences in MCV and MCH during UNCP intake following A-L administration, and this may

be due to the absorption of iron from cocoa (non-heme iron source), which is not high, since the absorption of non-heme iron is less efficient as compared to heme iron sources [35]. Besides, the polyphenols contained in UNCP are known to decrease the absorption of non-heme iron [36,37]. The effect on the RBC notwithstanding HGB levels was not significantly affected. Perhaps, a more prolonged intake of cocoa could have had an effect that may be worth further investigating.

The differential leukocyte counts reflect the systemic status of an animal in relation to its response and adjustment to injurious agents, stress, and/or deprivation; the indices are of value in confirming or eliminating a tentative diagnosis, in making a prognosis and guiding therapy [30]. It could further provide information on the severity of an injurious agent, the virulence of an infecting organism, the susceptibility of a host, and the nature, severity, and duration of a disease process [38]. From the results, the administration of A-L and UNCP did not have any influence on neutrophil count, monocyte count, eosinophil count, and basophil count, as seen with Figure 1b, d, f, and g. This observation may be due to the resiliency of the bone marrow which is able to resist the effects of some chemical agents and also the short-term administration of both A-L and UNCP. The inconsistency in basophil numbers as observed in the results following the administration of A-L followed by UNCP, and even sometimes, their total absence in blood of rodents (guinea pigs) is a normal occurrence. The increase in lymphocyte observed with UNCP administration may also be due to the fact that lymphocytes can also originate from sites other than the bone marrow such as the thymus [14,30].

The UNCP and A-L combination increased the levels of NO as compared to the other groups [Figure 2]. These no increase in the animals that received both UNCP and A-L could be attributed to the flavonoid content of the unsweetened natural cocoa [14]. Flavonoid-rich chocolate and cocoa drinks have been found to increase NO levels. Other studies have shown that A-L increases the level of oxidants such as superoxides ($O^{2-}$) and peroxides ($H_2O_2$), leading to oxidative stress. This further leads to a reduction in NO levels, since superoxides and peroxides are considered as NO scavengers [14,16-18]. The rise in NO observed in the A-L administered group could be as a result of a compensatory mechanism trying to restore the NO level, which is in line with other findings [16]. The high increase in NO levels observed in the cocoa and A-L combinations, especially in the animals receiving 300 mg/kg and 900 mg/kg of UNCP, may be indicative of enhanced protective effects of UNCP at these dose levels. This is extremely important in view of the effect of A-L on hematology as observed by other researchers [20]. Other studies have also revealed that the highest antihypertensive effect (due to NO vasodilator effects) of an orally administered flavonol-rich cocoa powder to spontaneously hypertensive rats was at a dose of 300 mg/kg and 900 mg/kg [39,40]. From our calculations, optimum effect of UNCP in preventing potential A-L induced hematotoxicity lies between 300 and 900 mg/kg. This corresponds to between 2 teaspoonful and 6 teaspoonful of UNCP daily [29]. It is evident from the above that a daily dose of 300, 900, and 1500 mg/kg corresponds to 4.54 g, 13.42 g, and 22.70 g UNCP daily, respectively.

The normal recommended use of UNCP in Ghana as a beverage is 1-2 teaspoonful 3 times daily (i.e., 5 g - 15.0 g daily). The phytochemical components of UNCP are likely to play a major role, and since UNCP is a non-alkalized powder, it is likely to have a greater percentage of total polyphenols, increased epicatechin and proanthocyanidins, as compared to alkalized cocoa powder. Though comparatively this was not part of this study, other studies have proved these differences in phytochemical composition [3-7].

This study contributes much to the hematoprotective potential of UNCP during HD A-L administration, since in the pathogenesis of malarial infection, hematological values are also paramount. It is important to determine the mechanism of this activity and the quantitative components of UNCP in further studies. Besides, it would be more expedient to conduct further studies in malarious guinea pigs much more, as UNCP has been found to possess antimalarial activity [13,14,41]. Thus, this study may have a beneficial impact on therapeutic strategies.

## CONCLUSION

UNCP restored some hematological disorders induced by A-L in guinea pigs by causing a significant increase in lymphocyte and PLT levels at a dose of 1500 mg/kg that was otherwise decreased by the administration of HD A-L. There was also an increase in NO with different doses of UNCP administration as a sequel to A-L dosing, which makes them a safe and advantageous combination. This research indicates the potential of daily ingestion of UNCP to prevent deleterious effects of A-L for the management of malaria.

## RECOMMENDATION

Further studies to study into the quantitative analysis of UNCP and its mechanism behind this hematological effect are recommended.

## LIMITATIONS

Quantitative components of this cocoa powder would have to be analyzed in subsequent studies and to explore mechanisms of this action. Further, this effect of cocoa should be studied in malarious guinea pigs.

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Original Research

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# Antibacterial synergy between rosmarinic acid and antibiotics against methicillin-resistant *Staphylococcus aureus*

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## ABSTRACT

**Aim/Background:** Medicinal plants have ability to resist microorganisms by synthesizing secondary metabolites such as phenols. Rosmarinic acid (RA) is a phenylpropanoid widely distributed in plants and well known as therapeutic and cosmetic agent. Methicillin-resistant *Staphylococcus aureus* (MRSA) which is resistant to all kinds of β-lactams, threatens even most potent antibiotics. To improve the efficiency of antibiotics against multi-drug resistant bacteria and to reduce the antibiotic dose, the antibacterial activity and the synergistic effect of RA with standard antibiotics against *S. aureus* and MRSA was investigated. **Materials and Methods:** Antibacterial activity of RA against *S. aureus* and a clinical isolate of MRSA was evaluated by agar well diffusion method. Minimum inhibitory concentration (MIC) of RA was determined by broth dilution method. Synergism of RA with various antibiotics against *S. aureus* and MRSA was studied by broth checkerboard method and time-kill kinetic assay. Effect of RA on microbial surface components recognizing adhesive matrix molecules (MSCRAMM's) of *S. aureus* and MRSA was studied using sodium dodecyl sulfate - polyacrylamide gel electrophoresis. **Results:** MIC of RA was found to be 0.8 and 10 mg/ml against *S. aureus* and MRSA, respectively. RA was synergistic with vancomycin, ofloxacin, and amoxicillin against *S. aureus* and only with vancomycin against MRSA. The time-kill analysis revealed that synergistic combinations were a more effective than individual antibiotics. MSCRAMM's protein expression of *S. aureus* and MRSA was markedly suppressed by RA + vancomycin combination rather than RA alone. **Conclusion**: The synergistic effects of RA with antibiotics were observed against *S. aureus* and MRSA. RA showed inhibitory effect on the surface proteins MSCRAMM's. Even though RA was shown to exhibit a synergistic effect with antibiotics, the MIC was found to be higher. Thus, further studies on increasing the efficacy of RA can develop it as an adjuvant for antibiotics.

KEY WORDS: Antimicrobial resistance, methicillin-resistant *Staphylococcus aureus*, microbial surface components recognizing adhesive matrix molecules, rosmarinic acid, synergy

## INTRODUCTION

Antibiotic resistance of micro-organisms is a major challenge confronted by all scientists who are involved in antibiotic drug discovery. Bacteria widen its resistance to antibiotics by mutating existing genes (vertical evolution) or by acquiring new genes from other strains or species (horizontal gene transfer). The sharing of genes between bacteria by horizontal gene transfer occurs by many different mechanisms [1].

Methicillin-resistant *Staphylococcus aureus* (MRSA) is a nosocomial and community-acquired pathogen that has developed resistance to various antibiotics such as β-lactams, quinolones, aminoglycosides, vancomycin, oxazolidinone, and streptogramin type antibiotics [2-4]. Newly emerging community-associated MRSA (CA-MRSA) is transmissible from healthcare acquired MRSA (HA-MRSA). Some CA-MRSA strains display enhanced virulence, spreading more rapidly and causing illness much more severe than HA-MRSA infections, affecting vital organs which lead to sepsis, toxic shock syndrome, and necrotizing pneumonia [5]. One of the strategies employed to triumph over the bacterial resistance is the use of a combination of drugs. The secondary metabolites of the plants are the good sources for combination of drugs to act as multidrug resistant modifiers with varied mechanisms of action [6]. Polyphenols is a prominent class of plant metabolites possesses

efficient antimicrobial action. A number of reports are available on the synergistic interactions of polyphenols with antibiotics to overcome microbial resistance like epigallocatechin gallate from green tea [7], tellimagrandin I and rugosin B from rose red (*Rosa macdub*) [8], baicalein from *Scutellaria amoena* [9], and corilagin from *Arctostaphylos uva-ursi* [10].

Rosmarinic acid (RA) is a well-known phenylpropanoid and chemically it is a dimer of caffeic acid and 3, 4-dihydroxyphenyl lactic acid, bound by an ester linkage [Figure 1]. RA belongs to the group of polyphenols. It is known for its therapeutic and cosmetic properties. It is well accounted as an antioxidant [11,12], anti-inflammatory [13,14], and antimicrobial agent [11,15,16]. RA was described to possess antimicrobial activity against wild strains of *Bacillus subtilis*, *Micrococcus luteus* and *Escherichia coli* [17], *Pseudomonas aeruginosa*, *S. aureus*, *Shigella* sp., and *Enterobacter* [15], *Candida albicans*, and *Aspergillus niger* [16]. Further, it was reported that RA possesses bactericidal activity against acne causing pathogens such as *S. aureus*, *Staphylococcus epidermidis*, and *Propionibacterium acne* through its membrane-damaging effect [18]. Previously, the synergistic effect of *Rosmarinus officinalis* extract with cefuroxime against MRSA was reported [19]. Although RA is stated to be an outstanding antimicrobial agent, its effect on MRSA is not yet studied in detail. Furthermore, there is nil report on the synergistic effect of RA with antibiotics against *S. aureus*.

Thus, the current study was designed to evaluate the antibacterial activity and to determine the minimum inhibitory concentration (MIC) of RA against *S. aureus* and MRSA. To develop RA as an adjuvant to antibiotics, the synergistic effect of RA with antibiotics against *S. aureus* and MRSA was explored. Further to understand the mechanism of action, the role of the effective synergistic combination of RA on microbial surface components recognising adhesive matrix molecules (MSCRAMM's) – surface proteins of *S. aureus* and MRSA was also studied.

## MATERIALS AND METHODS

### RA

RA (90% pure) was obtained from Sami Labs, Bengaluru, India.

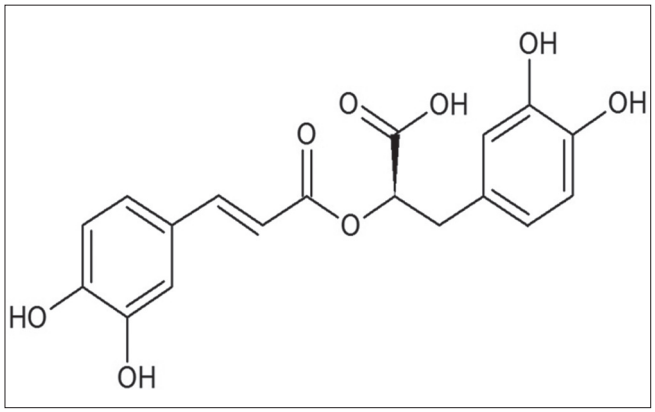


**Figure 1:** Structure of rosmarinic acid

### Bacterial Strains and Culture Medium

*S. aureus* ATCC 25923 was used in the study. Clinical isolate of MRSA was obtained from Doctors Diagnostic Centre, Trichy (India), and resistance against methicillin was observed by Kirby-Bauer method. Mueller-Hinton agar (MV173) and Mueller-Hinton broth (MV391) were purchased from Hi Media Laboratories Pvt. Ltd., Mumbai, India. The bacteria were rejuvenated in Mueller-Hinton broth at 37°C for 18 h and then stocked at 4°C in Mueller-Hinton agar. Subcultures were prepared as required. The inoculum size of the bacterial culture was standardized according to the National Committee for Clinical Laboratory Standards Guideline [20]. Further, they were analyzed by Mannitol salt agar test (+), catalase test (+), fermentation and oxidation of glucose (+), DNAse test (+), and tube coagulase test (−) for the confirmation of the strain. The antibiotics amoxicillin, ofloxacin, and vancomycin were obtained from Cipla Ltd., Mumbai, India.

### Evaluation of Antibacterial Potency of RA

*Agar well diffusion method*

Antibacterial activity was evaluated by agar well diffusion method according to the National Committee for Clinical Laboratory Standards [20]. Petri plates containing 20 ml Mueller-Hinton agar were seeded with 24 h culture of bacterial strains with 0.5 McFarland standard equivalent using spread plate method. The diameter wells of 6 mm were cut and 20 $\mu$l of RA at various concentrations were added. The plates were incubated at 37°C for 24 h and measured for antibacterial activity by observing the diameter of the zone of inhibition. The above experiment was done in triplicate to establish the statistical value.

*MIC*

MIC of RA was determined by microbroth dilution method as described by NCCLS, 2000 [20]. Increasing concentration of 1-10 mg/ml of RA was prepared using Mueller-Hinton broth as diluent and inoculated with 20 $\mu$l of bacterial inoculums with turbidity equivalent to 0.5 of McFarland scale. The mixture was incubated for 24 h at 37°C for the growth of bacteria. The lowest concentration at which there was no bacterial growth determined using ultraviolet spectrophotometer was taken as the MIC of RA [21].

### Evaluation of Synergy with Antibiotics

*Broth checkerboard method*

Broth checker board method was used to find the synergism of RA with the standard antibiotics amoxicillin, ofloxacin and vancomycin against MRSA [22]. RA was taken in the microfuge tube in ascending concentration from lowest inhibitory concentration to double the concentration of MIC and was arranged in a row. All the antibiotics were prepared in a similar manner and were arranged in a column. Using the checker board, various combinations of RA with antibiotics were obtained. The minimal concentration at which there

was no growth of organism was fixed as MIC. The fractional inhibitory concentration index (FIC index) was calculated for each combination by the formula:

$FIC_A + FIC_B = FIC_I$,

Where, $FIC_A$ = MIC of RA in combination/MIC of RA alone; $FIC_B$ = MIC of any antibiotic in combination/MIC of antibiotic alone. If FIC index ≤0.5 – synergy; FIC index >0.5 to <1 - additive; FIC index >1 to <4 - no interaction; FIC index >4 – antagonism.

### Time Kill Analysis

Time-kill kinetics was analyzed only for synergistic combinations of antibiotics and RA confirmed by checker board method [23]. Mueller-Hinton broth with combinations was inoculated with organism which was adjusted to 0.5 McFarlands standards and kept in a shaking incubator at 35°C. Samples were taken at 0, 6, 12, 24 and 30 h after inoculation. The extent of growth inhibition was analyzed at standardized $OD_{620\ nm}$.

### Effect of RA on Surface Protein Fraction

Mueller-Hinton broth was inoculated with the overnight bacterial culture of *S. aureus*, MRSA, and RA. The mixture was then incubated and centrifuged to form pellet. The pellet was resuspended in 1 M LiCl in about one-tenth of the media volume. The suspension was then incubated in a shaker at 42°C for 2 h. The bacteria were pelleted and the supernatant containing noncovalently attached surface proteins to the plasma membrane was removed. The proteins were quantified by recording the absorbance at 280 nm. 1 M LiCl was used as blank. The protein fraction was then run on sodium dodecyl sulfate - polyacrylamide gel electrophoresis (SDS-PAGE) and compared with the standard proteins [24].

## RESULTS

### Antibacterial Activity

The antibacterial activity of RA against *S. aureus* and MRSA was evaluated by agar well diffusion method by measuring the inhibition zones. RA showed inhibition zone with 12.4 mm diameter against *S. aureus* and 11.6 mm against MRSA. Inhibition zone for RA and antibiotics were compared and shown in Table 1. MIC of RA was evaluated by broth dilution method and shown in Table 2. MIC of RA was found to be 0.8 mg/ml against *S. aureus* and 10 mg/ml against MRSA.

### Synergistic Evaluation of RA and Antibiotics

To improve the antibiotic efficiency as well as to reduce the antibiotic dose, the antibacterial activity of the combinations of RA and standard antibiotics (amoxicillin, ofloxacin, and vancomycin) on susceptibility of *S. aureus* and MRSA by broth checker board method was investigated. The calculated FIC index is shown in Table 2. RA at ¼ × MIC value reduced the MIC of vancomycin, amoxicillin, and ofloxacin by ¼ times against *S. aureus*. However against MRSA, only vancomycin was found to be synergistic. All the synergistic combinations have shown FIC index value of 0.5. As MIC value for amoxicillin and ofloxacin was not observed against MRSA until the dose level of 80 μg/ml, further determinations for its MIC was not carried out and thus these two antibiotics were not studied for synergism by broth checker board method.

### Time-Kill Kinetic Assay

The effect of RA, antibiotics, and synergistic combinations were studied on the bacterial growth rate by time-kill kinetic

Table 1: Antibacterial activity of RA against *S. aureus* and MRSA strains (as inhibition zones in mm)

| Compounds | Diameter of inhibition zones (mm) against *S. aureus* | Concentration (volume – 20 μl) | Diameter of inhibition zones (mm) against MRSA | Concentration (volume – 20 μl) |
|---|---|---|---|---|
| RA | 12.4±0.4 | 0.2 mg/ml | 11.6±0.4 | 2 mg/ml |
| | 15.4±0.5 | 0.4 mg/ml | 14.2±0.2 | 4 mg/ml |
| | 16.4±0.4 | 0.6 mg/ml | 17.6±0.2 | 6 mg/ml |
| | 18.4±0.6 | 0.8 mg/ml | 20.0±0.4 | 8 mg/ml |
| Streptomycin | 27±0.1 | 10 μg/disc | 9.0±0.2 | 10 μg/disc |
| Ofloxacin | 14±0.4 | 5 μg/disc | - | 5 μg/disc |
| Ciprofloxacin | 22±0.2 | 5 μg/disc | 7.0±0.3 | 5 μg/disc |
| Chloramphenicol | 28±0.2 | 30 μg/disc | 20±0.5 | 30 μg/disc |

*S. aureus: Staphylococcus aureus,* MRSA: Methicillin-resistant *Staphylococcus aureus,* RA: Rosmarinic acid

Table 2: MIC values and synergism of RA with antibiotics

| Strains | Antibiotics | MIC of antibiotics | MIC of RA | FIC index | Interpretation |
|---|---|---|---|---|---|
| *S. aureus* (ATCC 25923) | Vancomycin | 20 μg/ml | 0.8 mg/ml | 0.5 | Synergy |
| | Ofloxacin | 20 μg/ml | | 0.5 | Synergy |
| | Amoxicillin | 30 μg/ml | | 0.5 | Synergy |
| MRSA (Clinical isolate) | Vancomycin | 40 μg/ml | 10 mg/ml | 0.5 | Synergy |
| | Ofloxacin | >80 μg/ml | | - | - |
| | Amoxicillin | >80 μg/ml | | - | - |

*S. aureus: Staphylococcus aureus,* MRSA: Methicillin-resistant *Staphylococcus aureus,* RA: Rosmarinic acid, MIC: Minimum inhibitory concentration

assay. The results are shown in Figures 2-5. It was observed that the synergistic combinations of RA and antibiotics have shown better time kill kinetics as compared to RA and antibiotics alone against *S. aureus*. The synergistic combinations retained a strong effect on decreasing bacterial growth in lag phase, log phase, and stationary phase when compared with individual amoxicillin and RA as shown in Figure 2. Amoxicillin and RA alone showed stronger effect only on the stationary phase and a lower effect in lag phase of *S. aureus*. The synergistic combination of RA + ofloxacin showed a much better inhibition in the log and stationary phase compared to RA and ofloxacin individually as shown in Figure 3. RA + vancomycin combination showed a better effect than individual compounds on log phase, while in lag and stationary phase showed effects similar to singular drugs as shown in Figure 4. Similarly, on synergistic combinations against MRSA, there was marked inhibition in lag and stationary phase on comparing with vancomycin and RA individually as shown in Figure 5. The antibacterial effects of all synergistic combinations were much potent in log phase compared to the individual effects.

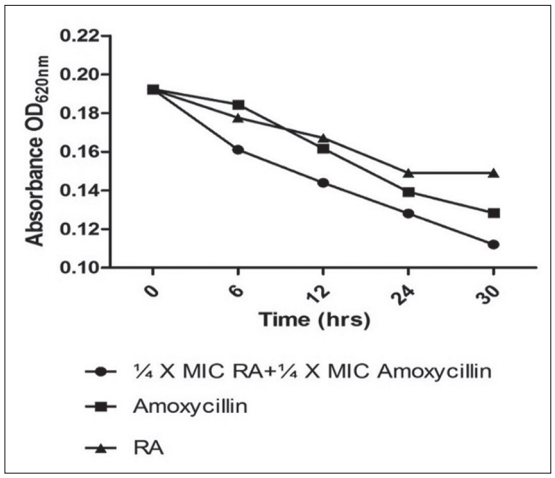


**Figure 2:** Time kill curve for rosmarinic acid, amoxicillin and its synergistic combination against *Staphylococcus aureus*

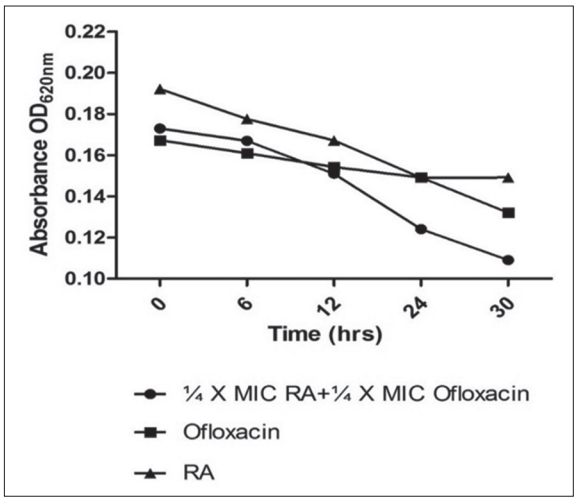


**Figure 3:** Time kill curve for rosmarinic acid, ofloxacin and its synergistic combination against *Staphylococcus aureus*

### Effect of RA on Surface Protein Fraction of MRSA

MSCRAMMs were isolated by protein precipitation method from *S. aureus* and MRSA and were then studied by running the protein fractions on SDS-PAGE. The gel picture [Figure 6] showed expression of proteins from 40 to 90 kDa in *S. aureus* control. A single band was expressed in the RA as well as vancomycin-treated sample, whereas the RA + vancomycin combination treated sample showed no protein expression. No bands were observed. Similarly, the protein expression in MRSA control showed intense bands, which were appeared to be suppressed in vancomycin and RA alone treated samples [Lane 2 and Lane 3 in Figure 7]. The RA + vancomycin combination have shown marked reduction in number and intensity of bands when compared to control lane.

## DISCUSSION

RA, a polyphenol found in higher concentration in *R. officinalis* is a well-known therapeutic and cosmetic secondary metabolite

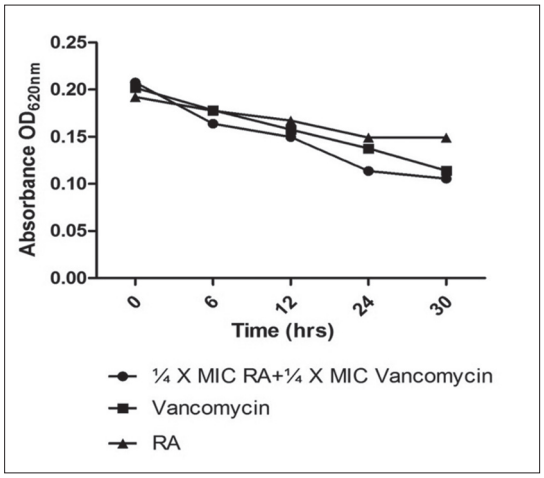


**Figure 4:** Time kill curve for rosmarinic acid, vancomycin and its synergistic combination against *Staphylococcus aureus*

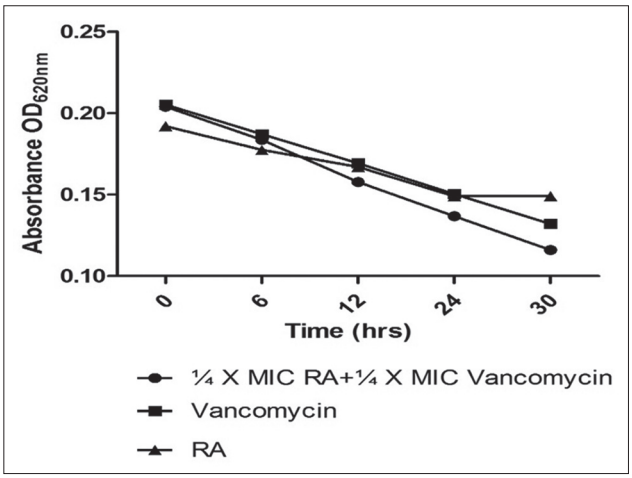


**Figure 5:** Time kill curve for rosmarinic acid, vancomycin and its synergistic combination against methicillin-resistant *Staphylococcus aureus*

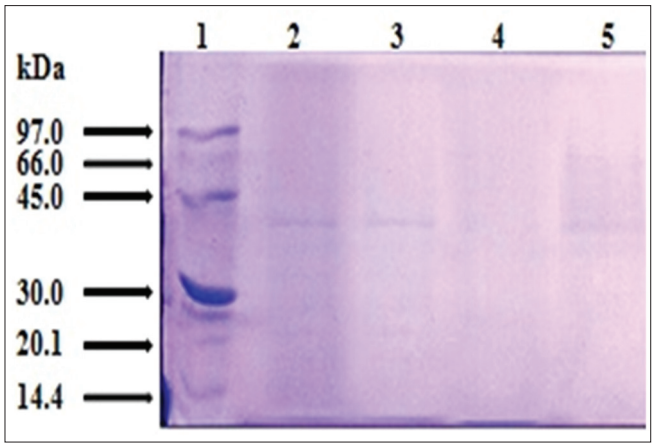


**Figure 6:** Sodium dodecyl sulfate - polyacrylamide gel electrophoresis analysis of microbial surface components recognizing adhesive matrix molecules in *Staphylococcus aureus*. Lane 1 - Protein marker; Lane 2 - Vancomycin; Lane 3 - RA; Lane 4 - Rosmarinic acid + Vancomycin; Lane 5 – *S. aureus* control

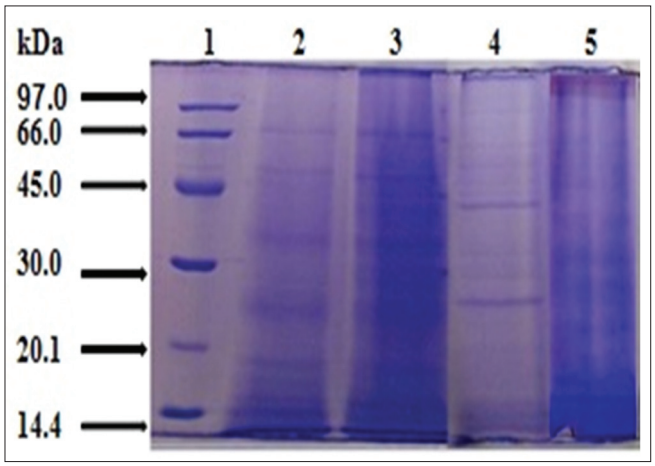


**Figure 7:** Sodium dodecyl sulfate - polyacrylamide gel electrophoresis analysis of microbial surface components recognizing adhesive matrix molecules in Methicillin-resistant *Staphylococcus aureus* (MRSA). Lane 1: Protein marker; Lane 2: Vancomycin; Lane 3: Rosmarinic acid (RA); Lane 4: RA + vancomycin; Lane 5: MRSA control

with antimicrobial [11,25-27] and antioxidant [11,28,29] properties. RA was reported to have antibacterial activity against *S. aureus* [11,18,30-33], whereas it was reported to be inactive up to 480 $\mu$g/ml against MRSA [26]. The efficacy of RA on MRSA was not addressed systematically. In an aim to know the actual MIC of RA against MRSA, this study evaluated the antibacterial potency of RA by measuring inhibition zones and also evaluated the MIC by broth dilution method. The MIC of RA was found to be 0.8 mg/ml against *S. aureus* and 10 mg/ml against MRSA. The MIC values were found to be on the higher side stating the lesser efficacy of RA against *S. aureus* and MRSA. There was an earlier report that the antimicrobial activity of phenolic compounds was mainly due to the inactivation of cellular enzymes which was highly dependent on the rate of penetration of the compounds into the cell or its ability to cause membrane permeability changes [34]. In the current study, the higher MIC value of RA might be due to its poor penetration capability into the bacterial cell wall. Further, as the MIC of RA against MRSA (10 mg/ml) was found to be higher in concentration, an effort was taken to study the synergistic possibilities of RA with antibiotics to develop RA as an adjuvant to the antibiotics. The results proved that RA was synergistic with the commercial antibiotics amoxicillin, ofloxacin and vancomycin against *S. aureus* and only with vancomycin against MRSA. The synergistic effect of RA was further confirmed by time-kill study, where the combinations have shown marked reduction of bacterial growth over time especially in the log phase of bacterial growth when compared with the individual antibiotics and RA against both *S. aureus* as well as MRSA. These results clearly show that RA combined with antibiotics could produce maximum bactericidal effect may be because of its higher efficacy in the log phase of bacterial growth. RA at the specified MIC was found to be synergistic with the antibiotics.

There are several virulence factors in the surface membrane proteins of bacteria. MSCRAMMs are a major virulence factor, which is present on the cell surface. They are covalently anchored transmembrane molecules in bacteria and have been shown to be a prominent antimicrobial target for antibiotics. MSCRAMMs are the major factors to cause infections as they mediate the initial host-bacterial interactions. It includes clumping factor A, protein A and fibronectin binding protein A, which are the major factor for initiating host-pathogen interactions [35,36]. Apart from MSCRAMMs cell surface proteins also contains penicillin-binding protein 2a and fmt B gene encoded protein, which is the major cause for resistance in MRSA. To study the possible mechanism of action of RA, the effect of RA on surface proteins was explored. The expression of MSCRAMM's in *S. aureus* was comparatively lesser in respect of MRSA, as resistant strains (MRSA) express a large number of surface proteins as its virulence factors. The combination of RA and vancomycin could able to suppress the expression of surface proteins (MSCRAMM's) completely in *S. aureus*, whereas its effect on MRSA was only moderate as the combination showed minimum number of intense bands when compared with its control. The results clearly indicate that the antibacterial activity of RA might be due to its action on the surface proteins MSCRAMM's of *S. aureus* and MRSA.

## CONCLUSION

To conclude, RA is an antibacterial agent against *S. aureus* and MRSA, but the MIC values are on the higher side. Even then, RA may act as an adjuvant or resistant modulating agent confirmed by its synergistic effect with antibiotics. The suppression of surface proteins MSCRAMM's by RA in *S. aureus* and MRSA might be one of the mechanism responsible for its antibacterial action. However, future studies on increasing the efficacy of RA and identification of the detailed mechanism for its synergism with antibiotics has to be studied.

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Original Research

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# Antidiabetic and antioxidant effects of *Croton lobatus* L. in alloxan-induced diabetic rats

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## ABSTRACT

**Background:** *Croton lobatus* contains a high amount of antioxidant phytochemicals that probably account for its wide use as food and medicine in the traditional communities of West Africa. **Methods:** The study evaluated the modulatory role of methanol extract of Croton lobatus leaf on alloxan-induced diabetes and associated cardiovascular complications. Male rats were randomly selected and assigned to one of six groups (A to F) of eight animals each: A (distilled water); B (corn oil); C (Alloxan); D (Alloxan + 100 mg kg-1 Croton lobatus); E: (Alloxan + 200 mg kg-1 C. lobatus); and F (Alloxan + 100 mg kg-1 glibenclamide). **Results:** Acute toxicity studies revealed no mortality of rats at the administration of different doses of extract up to the 5,000 mg kg-1 dose. Histology of the pancreas showed focal area of necrosis, and fatty infiltration in diabetic untreated rats, but these lesions were absent in pancreas of rats treated with *C. lobatus* extract. **Conclusion:** Methanol leaf extract of *C. lobatus* reduced arteriogenic risk factors, improved antioxidant status, restored the observable pathological lesions associated with experimental diabetes in rats, and thus offers a new therapeutic window as herbal therapy for the treatment of diabetes mellitus and associated cardiovascular complications.

KEY WORDS: Blood pressure, diabetes, electrocardiogram, oxidative stress

## INTRODUCTION

Diabetes mellitus ranked among the leading causes of death in developed countries and is one the most prevalent metabolic disorder in the world [1]. Although several etiologies have been implicated, defects in insulin secretion, insulin action, or both are often the primary characteristic of the disease [2]. Long-term complications of diabetes include coronary heart diseases, retinopathy, nephropathy, and foot ulceration [3]. Alloxan (2,4,5,6-tetraoxypyrimidine; 5,6-dioxyuracil), one of the commonly used drugs for the induction of experimental diabetes in rats, is a diabetogenic agent that selectively destroys pancreatic β-cells [4].

Oxidative stress, which reflects an imbalance between the systemic manifestation of reactive oxygen species (ROS) and a biological system's ability to readily detoxify the reactive intermediates [5], has been implicated in the pathogenesis of diabetes via the apoptosis of pancreatic β-cells, and insulin resistance respectively [6]. Hyperglycemia generates ROS which in turn cause damage to the cells in many ways [7]. The resulting oxidative stress mediated damage to the cells ultimately results in secondary complications associated with diabetes [8].

Many of the currently available therapeutic options for the management of diabetes mellitus have serious adverse effects including hypoglycemia, gastrointestinal disturbances,

liver toxicity, and heart failure [9]. Consequently, natural compounds, in recent time, have been exploited as feasible alternatives for the treatment of diabetes, and its complications, because they are generally considered to be less expensive and safe [10]. *Croton lobatus* (family Euphorbiaceae) is an erect, annual, herbaceous plant, often harvested from the wild for local use as food and medicine [11]. Phenolic substances including lignoids, proanthocyanidins, and flavonoids with highly potent antioxidant properties have been reported as the predominating phytochemicals of *C. lobatus* leaves [12]. Since cellular oxidative stress has been reported to play cardinal roles in the development of hyperglycemia-related tissue damage [13], this study was designed to evaluate the ameliorative effects of *C. lobatus* on alloxan-induced diabetes and associated cardiovascular derangements by determining the effects of the leaf extract on blood glucose level, blood pressure changes, electrocardiographic (ECG) abnormalities, lipid profile, and anti-oxidant status of alloxan-induced diabetic rats.

## MATERIALS AND METHODS

### Collection and Identification of Plant Material

Fresh mature leaves of *C. lobatus* were collected from Orogun area, Ibadan, and were identified and authenticated by a taxonomist at the herbarium in the Department of Botany, University of Ibadan, Ibadan. The voucher number UIH–22482 was assigned.

### Chemicals and Drugs

Alloxan was obtained from Sigma Chemical Co. (St. Louis, M.O., USA). Daonil® (glibenclamide) manufactured by Aventis Pharma Ltd. was purchased from Diadem pharmacy in Ojoo, Ibadan-Nigeria. Randox kits (Randox Laboratories Limited) for the determination of triglyceride (TG), cholesterol, and high-density lipoprotein (HDL) were purchased from Long Global Health Ltd., Lagos, Nigeria.

### Preparation of Methanol Leaf Extract of *C. lobatus*

The extraction of *C. lobatus* was carried out according to the method described by Iweala and Okeke [14]. The fresh leaves of the plant were air dried for 30 days and milled to powder using an electrical blender. 1 kg of the powdered leaves was macerated in 6 L of distilled methanol for 72 h with occasional stirring. The liquid extract obtained was filtered through a cotton wool and finally with Whatmann no.1 filter paper. The filtrate was passed through a pressure pump to ensure that no particles or residues were left in the filtrate. The filtrate was concentrated in a rotary evaporator (Stuart model RE300D) at a temperature of 45°C. The concentrated extracts were subsequently evaporated to dryness on a water bath at 50°C to obtain a sticky, dark green mass weighing 37.18 g which represents 3.72% yield, and kept refrigerated at 4°C until use.

### Experimental Animals

A total of 48 healthy male albino rats of the Wistar strain weighing between 100 and 160 g, obtained from the central animal house of the University of Ibadan, Ibadan, Nigeria, were used for the study. The animals were kept in groups of 8 per white plastic cage within the animal house to acclimatize for 2 weeks before the experiments with conditions of the animal housing facility, ambient temperature, standard environmental conditions of 12 h light and 12 h dark and adequate ventilation for 2 weeks. The rats were fed with standard rat diet (Ladokun Feeds, Ltd.) and clean water was provided *ad libitum*. All experimental protocols were conducted in compliance with the National Institute of Health Guide for Care and Use of Laboratory Animals.

### Acute Toxicity Test

Acute oral toxicity was carried out using the procedure described by the Organisation for Economic Cooperation and Development [15]. The test was performed using healthy albino rats of Wistar strain weighing between 83 and 118 g. The animals were divided into six groups of three rats each and administered 0, 50, 100, 200, 3000, 5000 mg/kg orally. Animals were observed after dosing at least once during the first 30 min, periodically during the first 24 h with special attention given during the first 4 h, and daily thereafter for a total of 14 consecutive days.

### Experimental Design

The animals were randomly selected and assigned into one of the six groups (A to F) of eight animals each: A (distilled water), B (corn oil), C (Alloxan), D (Alloxan + 100 mg/kg *C. lobatus*), E: (Alloxan + 200 mg/kg *C. lobatus*), and F (Alloxan + 100 mg/kg glibenclamide). Diabetes was induced in Groups C, D, E, and F by a single intraperitoneal injection of aqueous alloxan monohydrate at a dose of 100 mg/kg, after the rats were subjected to overnight fast, and fasting blood glucose was determined. 96 h after alloxan administration, blood glucose levels of the rats were determined with the aid of an Accu Chek® active digital glucometer using test strips. Animals with blood glucose level greater than or equal to 200 mg/dl were considered diabetic and selected for the study.

### Blood Pressure Measurement

Indirect blood pressure parameters (systolic, diastolic, and mean blood pressure) were determined in unanesthetized rats by tail plethysmography using an electrosphygnomanometer (CODA, Kent Scientific, USA). The average of at least nine readings, taken in the quiescent state, following acclimatization, was taken per animal.

### ECG

Standard lead II ECG was recorded in conscious rats using a 7-lead ECG machine (EDAN VE-1010, Shanghai, China) 24 h after the last administration of the extract. The machine was calibrated at 20 mm/mV paper speed and 50 mm/s paper speed. From the recorded ECGs, parameters such as heart rate (HR), P-wave duration, PR-interval, QRS duration, R-amplitude, QT segment, and Bazett's correction of the QT interval were determined.

### Blood Sample Collection and Preparation

At the end of the treatment period, blood was collected by retro–orbital venous puncture using micro-hematocrit capillary tubes into lithium heparinized bottles and taken to the laboratory for plasma preparation. Plasma was separated by centrifugation (G+M; Great Medical England centrifuge model 80-2) at 4000 revolutions per minutes for 15 min. The plasma obtained was collected by pipetting into a plain bottle using Pasteur pipette and refrigerated at 4°C until required for biochemical assays.

### Sacrifice of Animals and Relative Organ Weight Determination

At the end of the experimental period, 5 rats from each group were sacrificed by cervical dislocation. The abdomen was dissected using dissecting tools; the kidney, pancreas, liver, and heart were removed and weighed to determine their weight in relation to the total body weight of the animals [16].

### Histopathology of the Pancreas

The pancreas from each rat was removed immediately and preserved in a sample bottle containing 10% formalin solution. The pancreas was processed by the paraffin technique. Sections of 5 $\mu$m thickness were cut and stained by hematoxylin and eosin for histological examination.

### Assay for Plasma Lipid Profile

Cholesterol, TGs, and HDL tests were carried out using Randox kits. Very low-density lipoprotein cholesterol (VLDL-C) and LDL-C were estimated as described by Friedwald *et al*. [17].

### Determination of Markers of Oxidative Stress and Antioxidant in Plasma

Superoxide dismutase (SOD) was determined by measuring the inhibition of auto-oxidation of epinephrine at pH 10.2 at 30°C as described by Misra and Fridovich [18] with modification from Oyagbemi *et al*. [19]. Glutathione (GSH) Level was determined using Ellman's reagent. Lipid peroxidation was determined by measuring the formation of thiobarbituric acid reactive substances according to the method of Varshney and Kale [20]. The MDA level was calculated according to the method of Adams and Sergi [21]. Hydrogen peroxide was generated by the method described by Wolff [22]. Nitric oxide (NO) was determined using standard protocol [23].

### Statistical Analysis

Analysis of variance was performed using the Minitab 16.0 statistical software program. Duncan Multiple Range Test and Fischer's pairwise comparison test were used for separation of statistically significant means. All the data were expressed as a mean ± standard deviation. $P < 0.05$ was considered significant.

## RESULTS

The median lethal dose for the *C. lobatus* extract was estimated to be greater than 5000 mg/kg, since visible adverse effects and mortality were not recorded at this dose. The body weight of the diabetic untreated rats decreased significantly ($P < 0.05$) compared with the control and treated groups of rats [Table 1], significant differences were not recorded in the relative organ weight of rats [Table 2]. The blood glucose level of diabetic untreated rats significantly increased ($P < 0.05$) when compared with control rats, but administration of 100 mg/kg and 200 mg/kg *C. lobatus* extract, significantly decreased ($P < 0.05$) the blood glucose level from hyperglycemic to normoglycemic levels, similar to those of the glibenclamide-treated rats [Table 3].

There was no significant difference ($P > 0.05$) in the diastolic and mean arterial blood pressures of the diabetic untreated groups of rats when compared with the control group, but the systolic blood pressure significantly increased ($P < 0.05$) compared with the control [Table 4]. The extract at 100 mg/kg caused a significant decrease ($P < 0.05$) in the systolic, mean arterial blood pressure, and pulse pressure of rats when compared with those of the diabetic untreated group of rats. There was no significant difference in the HR, P-wave duration (P-Dur), PR interval (PR-Int), QRS, QTc and Ramp of diabetic untreated rats (Group C) when compared with the control, but there was a significant difference in QT duration of diabetic

Table 1. Percentage body weight gain of alloxan-induced diabetic rats

| Groups weeks | A | B | C | D | E | F |
|---|---|---|---|---|---|---|
| Week 0 | 123.70±7.09 | 124.30±3.21 | 108.50±2.12 | 107.50±3.54 | 100.00±11.31 | 113.00±4.24 |
| Week 2 | 151.00±10.58 | 144.00±10.58 | 95.00±4.04 | 104.00±3.21 | 118.50±4.95 | 136.50±6.36 |
| %age weight gain (%) | 18.1 | 13.88 | −14.2 | −3.4 | 15.61 | 17.2 |

Table 2. Relative organ weight of alloxan-induced diabetic rats

| Groups organ | A | B | C | D | E | F |
|---|---|---|---|---|---|---|
| Kidney | 0.65±0.19 | 0.72±0.09 | 1.05±0.11 | 0.94±0.06 | 0.85±0.08 | 0.79±0.08 |
| Liver | 4.17±0.52 | 4.00±0.99 | 5.00±0.39 | 3.34±2.61 | 3.94±0.55 | 5.51±2.32 |
| Pancreas | 0.23±0.12 | 0.29±0.09 | 0.32±0.03 | 0.27±0.10 | 0.19±0.06 | 0.23±0.09 |
| Heart | 0.22±0.06 | 0.37±0.07 | 0.38±0.01 | 0.37±0.09 | 0.41±0.06 | 0.38±0.16 |

Superscript (a) indicate significant increase compared with groups A or B at $P<0.05$

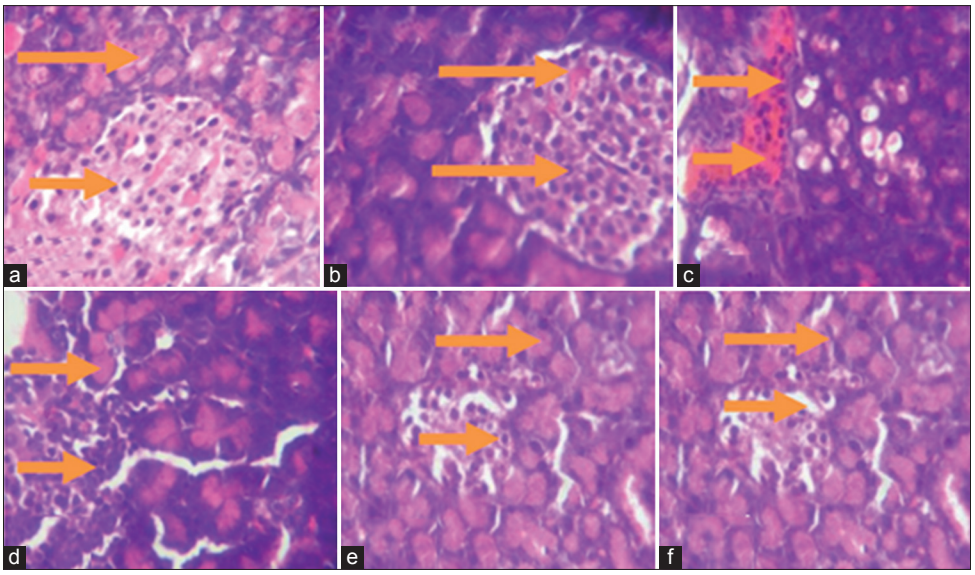


**Figure 1:** Effect of *Croton lobatus* leaf extract on pancreas in alloxan–induced diabetic rats. Histology of the pancreas following induction of diabetes mellitus and treatment with *C. lobatus* leaf extract in rats. (a and b) normal controls with normal Islet; (c) Untreated diabetic group with congestion of vessels, and focal area of necrosis, and fatty infiltration; (d) Diabetic treated with 100 mg/kg *C. lobatus* with moderate congestion of vessels, islets appear normal but few and small in size; (e) Diabetic treated with 200 mg/kg with mild congestion of vessels and normal islets, and normal exocrine acini containing zymogen granules; (f) Diabetic treated with gilbenclamide shows congestion of vessels, normal islets, and normal exocrine acini containing zymogen granules. Histologic slides were stained with Hematoxylin and Eosin (×400)

Table 3. Effect of *Croton lobatus* leaf extract on blood glucose of alloxan-induced diabetic rats

| Groups days | A | B | C | D | E | F |
|---|---|---|---|---|---|---|
| Day 1 | 45.60±8.85 | 44.60±5.81 | 231.50±11.39[a] | 156.20±7.50[b] | 165.00±4.90[b] | 170.00±4.26[b] |
| Day 2 | 65.80±10.06 | 62.00±7.07 | 242.00±1.81[a] | 140.50±3.67[b] | 171.60±2.75[b] | 126.80±1.63[b] |
| Day 3 | 69.40±7.50 | 81.69±11.30 | 255.00±2.05[a] | 134.00±1.89[b] | 70.50±3.10 [b] | 121.50±9.04[b] |
| Day 4 | 72.20±11.40 | 70.80±11.80 | 253.00±2.00[a] | 89.50±5.06 [b] | 129.80±4.78[b] | 79.80±10.90[b] |
| Day 5 | 63.80±4.87 | 61.40±4.39 | 255.05±2.00[a] | 145.00±10.48[b] | 147.80±4.69[b] | 62.00±12.52[b] |
| Day 6 | 58.00±5.61 | 57.60±8.02 | 250.00±2.05[a] | 177.50±5.37[b] | 131.00±1.25[b] | 60.80±1.73[b] |
| Day 7 | 68.00±11.20 | 62.20±15.40 | 256.00±2.23[a] | 169.80±4.56[b] | 142.00±4.32[b] | 11.30±4.38 [b] |
| Day 9 | 56.80±7.95 | 66.40±9.32 | 270.50±17.54[a] | 173.30±10.79[b] | 130.30±2.90[b] | 84.3±5.50 [b] |
| Day 11 | 102.60±14.86 | 94.00±15.43 | 279.30±4.81[a] | 179.80±4.58 [b] | 124.00±7.54[b] | 95.30±4.37[b] |
| Day 12 | 70.40±17.23 | 60.80±16.65 | 284.00±8.37[a] | 210.80±5.49 [b] | 115.00±5.47[b] | 93.80±4.90 [b] |
| Day 14 | 63.00±14.14 | 59.40±18.63 | 297.80±7.62[a,d] | 204.30±4.22[b,d] | 119.80±2.25[b,c] | 112.80±6.54[b,c] |

Superscript (a) indicate significant increase compared with groups A or B at $P<0.05$, Superscript (b) indicate significant decrease compared with group C at $P<0.05$, Superscript (c) indicate significant decrease compared with day 1 of treatment at $P<0.05$, Superscript (d) indicate significant increase compared with day 1 of treatment at $P<0.05$

Table 4. Effect of *Croton lobatus* leaf methanol extract on blood pressure of alloxan-induced diabetic rats

| Groups parameters | A | B | C | D | E | F |
|---|---|---|---|---|---|---|
| Systolic (mmHg) | 128.50±22.61 | 131.30±3.01 | 146.80±18.10[a] | 100.00±13.80[b] | 129.00±13.67[b] | 122.70±13.80[b] |
| Diastolic (mmHg) | 109.70±23.89 | 105.00±4.43 | 109.60±26.93 | 88.80±8.41[b] | 116.80±11.86[a] | 111.70±14.43[ab] |
| MAP (mmHg) | 115.50±22.40 | 113.50±3.73 | 121.60±24.03[a] | 92.00±5.10[b] | 120.50±10.41 | 86.70±19.30[b] |
| Pulse Pressure (mmHg) | 7.30±0.58 | 26.30±3.83 | 37.20±9.31[a] | 2.50±1.00[b] | 10.00±1.00[b] | 11.00±2.65[b] |

Superscript (a) indicate significant increase compared with groups A or B at $P<0.05$, Superscript (b) indicate significant decrease compared with group C at $P<0.05$

untreated rats when compared with when compared with the control [Table 5].

There was a significant increase ($P < 0.05$) in the levels of total cholesterol (TC), TG and VLDL-C, but low HDL-C in diabetic untreated rats when compared with the control rats. Rats treated with *C. lobatus* extract showed a significant decrease ($P < 0.05$) in TC, TG, and VLDL-C when compared with the diabetic untreated counterparts, but the levels of these parameters in the extract treated groups were similar to those of the control and glibenclamide treatment group [Table 6].

The extract of *C. lobatus* significantly increased ($P < 0.05$) the level of SOD in a dose-dependent manner, compared with the diabetic untreated group of rats [Table 7]. There was a significant decrease ($P < 0.05$) in serum NO level of diabetic

Table 5. Effect of *Croton lobatus* leaf extract on electrocardiographic parameters of alloxan-induced diabetic rats

| Groups parameters | A | B | C | D | E | F |
|---|---|---|---|---|---|---|
| H/R (/min) | 269.0±60.3 | 256.0±19.92 | 220.3±12.01[a] | 205.0±20.88[a] | 219.3±14.47[a] | 246.7±40.67 |
| P-Dur (ms) | 20.7±1.53[b] | 25.0±3.61 | 24.7±3.21 | 26.7±3.51 | 25.0±3.61 | 24.7±3.21 |
| PR-Int (ms) | 46.0±9.54 | 44.0±4.00 | 50.7±4.16[b] | 53.0±4.36[b] | 50.0±5.29[b] | 52.0±1.73[b] |
| QRS (ms) | 17.0±3.46 | 14.3±1.15 | 12.7±0.58 | 15.3±1.53 | 16.3±7.77 | 16.0±3.61 |
| QT-Dur (ms) | 58.7±11.7 | 62.0±4.58 | 82.0±5.57[b] | 79.0±12.8[c] | 103.0±24.5[c] | 88.3±7.57 |
| QTc (ms) | 125.0±37.5 | 128.0±6.43 | 157.0±15.1[b] | 146.0±26.6[d] | 196.0±42.1[c] | 177.0±8.02[c] |
| Ramp (mV) | 0.4±0.24 | 0.4±0.08 | 0.6±0.15 | 0.4±0.09 | 0.5±0.15 | 0.4±0.12 |

Superscript (a) indicate significant decrease compared with groups A or B at $P<0.05$, Superscript (b) indicate significant increase compared with groups A or B at $P<0.05$, Superscript (c) indicate significant increase compared with group C at $P<0.05$, Superscript (d) indicate significant decrease compared with group C at $P<0.05$

Table 6. Effect of *Croton lobatus* leaf extract on lipid profile in alloxan-induced diabetic rats

| Groups parameters | A | B | C | D | E | F |
|---|---|---|---|---|---|---|
| TC (mg/dL) | 28.00±2.45 | 21.90±11.70 | 52.30±13.50[a] | 23.10±7.28[b] | 11.00±3.60[b] | 27.40±9.48[b] |
| TG (mg/dL) | 39.60±23.60 | 48.40±24.00[c] | 128.00±34.70[a] | 55.60±29.70[b] | 80.50±19.60[b] | 74.10±25.80[b] |
| HDL (mg/dL) | 32.60±9.19 | 42.00±11.30 | 16.20±13.10[c] | 31.60±8.53[d] | 34.90±10.10[d] | 26.20±1.07[d] |
| VLDL (mg/dL) | 7.90±4.72 | 9.70±4.79 | 25.50±6.95[a] | 11.10±5.93[b] | 16.10±3.91[b] | 14.80±5.15[b] |
| LDL (mg/dL) | 12.50±10.51 | 29.70±19.98 | 14.10±16.68 | 19.60±10.09 | 39.90±10.73[d] | 13.60±12.71[b] |

Superscript (a) indicate significant increase compared with groups A or B at $P<0.05$, Superscript (b) indicate significant decrease compared with group C at $P<0.05$, Superscript (c) indicate significant decrease compared with groups A or B at $P<0.05$, Superscript (d) indicate significant increase compared with group C at $P<0.05$

Table 7. Effect of *Croton lobatus* leaf extract on oxidative stress markers in alloxan-induced diabetic rats

| Groups parameters | A | B | C | D | E | F |
|---|---|---|---|---|---|---|
| SOD units/mg protein | 107.00±4.24 | 82.70±15.50 | 96.90±9.47[a] | 89.70±0.99 | 122.00±2.53[b] | 112.00±5.18[b] |
| Nitric Oxide µmole/L | 1.04±0.04 | 0.70±0.26 | 0.40±0.02[c] | 0.40±0.17[c] | 0.80±0.23[d] | 0.80±0.03[d] |
| GSH µmole/mg protein | 64.00±0.35 | 63.50±0.35 | 13.10±2.30[a] | 63.90±2.30[b] | 64.10±0.18[b] | 66.10±0.88[c] |
| $H_2O_2$ µmole/mg protein | 12.00±0.71 | 10.50±0.35 | 12.50±0.35 | 11.90±0.18 | 11.90±0.53 | 10.80±0.35 |
| MDA µmole/mg protein[e] | 0.50±0.01 | 1.40±0.27 | 13.90±2.97[a] | 0.60±0.30[b] | 0.50±0.07[b] | 0.60±0.15[b] |

Superscript (a) indicate significant increase compared with groups A at $P<0.05$, Superscript ([b]) indicate significant decrease compared with group C at $P<0.05$, Superscript (c) indicate significant decrease compared with groups A or B at $P<0.05$, Superscript (d) indicate significant increase compared with group C at $P<0.05$

untreated animals when compared with the control, but *C. lobatus* extract at 200 mg/kg significantly increased the level of this parameter in rats. Similarly, the reduced GSH level in the diabetic untreated rats significantly decreased when compared the control group, but rats treated with the 100 and 200 mg/kg dose of *C. lobatus* had a significantly elevated level of GSH compared with the diabetic untreated group [Table 7]. There was no significant difference ($P > 0.05$) in $H_2O_2$ of the diabetic group of rats when compared with the control, and with the rats treated with *C. lobatus* extract and glibenclamide. However, the diabetic untreated rats showed a significant increase ($P < 0.05$) in the level of malondialdehyde (MDA) compared with control rats. Administration of the extract of *C. lobatus* at 100 and 200 mg/kg significantly decreased ($P < 0.05$) the MDA level compared with the diabetic untreated rats.

Histology of the pancreas showed focal area of necrosis, and fatty infiltration in diabetic untreated rats, but these lesions were absent in pancreas of rats treated with C. lobatus extract (Figure 1).

## DISCUSSION

In general, medicinal plants are believed to be safe, but a number of these therapeutic agents have been reported to cause specific organ damage in man and animals [24]. For instance, hepatotoxicity, nephrotoxicity, thrombocytopenia, and genotoxicity have been associated with the use of a number of medicinal plants [25]. The non-observance of mortality in rats even at the extremely high dose of 5000 mg/kg suggests a wide safety margin for the extract of *C. lobatus* used in this study.

In this study, the induction of diabetes mellitus in rats by alloxan was manifested by the significant reduction ($P < 0.05$) in the blood glucose level of rats administered 100 mg/kg alloxan monohydrate alone compared with those of the control group of rats [Table 3]. Further, the hyperglycemic state observed in rats became increasingly severe with increasing number of days as depicted by the significant elevation in the blood glucose level on the 14$^{th}$ day following induction of diabetes compared with the first day of therapy [Table 3]. However, the extract of *C. lobatus* showed a significant inhibition of alloxan-induced diabetes mellitus and exerts a potent anti-hyperglycemic effect as demonstrated by the significant decrease ($P < 0.05$) in blood glucose with the 100 and 200 mg/kg *C. lobatus* leaf extract treated groups, similar to those treated with the sulfonylurea glibenclamide, albeit, the 200 mg/kg dose showed higher effect over the 2-week treatment period. Observations in this study are similar to the reports of Okokon *et al.* [26] who reported

significant hypoglycemic activity for *Croton zambesicus* in alloxan-induced diabetes mellitus.

The antidiabetic activities of medicinal plants have been ascribed to their phytochemical constituents [27] that may delay the development of diabetic complications and regulate the associated metabolic abnormalities through a variety of mechanisms [28]. For instance, different polysaccharides of *Ganoderma lucidum* have been reported to increase plasma insulin levels in both normal and glucose-loaded mice, with accompanying decreased hepatic glycogen content due to a modulation of glucose metabolizing enzymes in the liver [29]. Moreover, the naturally occurring phenolic compounds, flavonoids, and alkaloids are reported to inhibit the activities of glucosidase, a key enzyme in the metabolism of glucose [30]. It is probable that the anti-diabetic effects of *C. lobatus* observed in this study is due to a stimulation of insulin secretion or improvement in digestion along with a reduction in blood sugar level, and inhibition of the breakdown of starch to glucose by inhibiting alpha-glucosidase activity [31]. Diabetes mellitus has been known to be associated with weight loss [32]. The weight loss in diabetic untreated rats, as observed in this study, may be due to dehydration and ineffective metabolism of carbohydrate, protein and fat [33]. However, the increase in body weight of diabetic rats treated with plant extract suggests a potent inhibition of diabetes by *C. lobatus* leaf extract.

Observation of a significantly elevated blood pressure in the untreated diabetic group of rats relative to the control and *C. lobatus* treated group [Table 4] probably is a manifestation of one of the complications of a persistent hyperglycemic state. Hyperglycemia causes changes in vascular structure and function by decreasing NO bioavailability and increasing the production of hydrogen peroxide in vascular endothelium [34]. From the result of this study, there was a significant decrease ($P < 0.05$) in NO levels in diabetic untreated rats, whereas the administration of *C. lobatus* extracts to diabetic rats increased the level of NO and in a dose-dependent manner [Table 7]. Since NO is a potent vasodilator, decreased bioavailability or biosynthesis of NO may potentiate the development of the hypertensive state [35].

Diabetes mellitus has been associated with cardiovascular complications of tachycardia, shortening of QT intervals and QRS as well as shortened activation time of ventricular myocardium [36]. Lee *et al.* [37] reported a prolongation of QT interval with hypoglycemia, and this was associated with increased risk of ventricular arrhythmias. Increased free radical (superoxide anion) activity can potentially elevate blood pressure by inactivating NO and consequently increase systemic vascular resistance [38]. Conversely, enhanced activity of NO has been found to play a key role in the reduction of vascular resistance and blood pressure that are elevated in hypertensive subjects [39]. Observations of this study on blood pressure changes of rats are similar to the findings of Paez *et al.* [40] who reported a prevention of the development of hypertensive state following the administration 200 mg/kgethanol leaf extract of *Croton schiedeanus* in NO deficit-induced hypertension, and attributed the effect to the flavonoid, diterpenoid, and fenibutanoid metabolites of *Croton* species that exert a vasodilatory effect by modulating the NO/cyclic guanosine monophosphate pathway. The blood pressure lowering effects of *C. lobatus* extracts, as observed in this study, may be due to direct or indirect action of constituent polyphenols that have been reported to decrease blood pressure by increasing endothelial NO bioavailability via their antioxidant action, and their capacity to activate vascular endothelial NO synthase [41].

The significantly elevated ($P < 0.05$) levels of TC, TG, VLDL-C, and low high HDL-cholesterol (HDL-C) observed in the diabetic untreated rats compared with those treated with *C. lobatus* suggest positive modulatory role of extract in lipid metabolism probably due to a rapid mobilization of TG and consequent increased plasma free fatty acids levels. Hypercholesterolemia is a common complication of diabetes mellitus and elevated VLDL-C TG reduces levels of cardioprotective HDL-C, with attending consequence of a reduction in antioxidant activities [42]. The results of this study corroborate earlier reports that most hypoglycemic plants have potentials of ameliorating diabetes-associated abnormalities of lipid metabolism *in vivo* [43]. The high level of HDL-C in diabetic rats treated with extract is advantageous because HDL-C transports fat molecules (such as cholesterol and TGs) out of arterial vasculature, and thus prevents cardiovascular diseases [44].

Furthermore, in this study, a significant decrease ($P < 0.05$) was observed in the activity of SOD of the diabetic untreated group of rats, compared with those of the control. The decreased SOD activity may be due to high level of free radicals with decreased antioxidant defense mechanisms [45]. The significantly increased SOD level in diabetic rats following the administration of *C. lobatus* extract suggests a positive modulatory role for the extract in the amelioration of the induced oxidative stress. Reduced GSH normally plays the role of a direct intracellular free-radical scavenger through interactions with free radicals [46]. The observed reduction in GSH level in diabetic untreated rats may be a result of the increased utilization of GSH due to oxidative stress [47]. MDA has been documented as a primary biomarker of free radical mediated lipid damage and oxidative stress [48]. The MDA level in diabetic rats treated with *C. lobatus* extract significantly decreased when compared with diabetic untreated rats [Table 7]. Medicinal plants with high flavonoid content have been reported to decrease MDA level in plasma, and thus provide protection against many chronic diseases by virtue of their free radical scavenging properties [49].

Pancreatic Islets are especially vulnerable to oxidative stress-mediated injuries because the antioxidant defense system of the pancreas is considerably weaker than those of other tissues [50]. In this study, histopathological evaluation revealed focal area of necrosis of islet cells in untreated diabetic rats. However, pancreatic islet cells of diabetic rats treated with *C. lobatus* leaf methanol extracts appear normal. This suggests an inhibition of the toxic mechanisms involved in the alloxan-induced, oxidative stress-mediated destruction of pancreatic Islet cells by the antioxidant rich, flavonoid-containing *C. lobatus*

extract inducing increased antioxidant enzyme activity, and consequently preserving Islet cells integrity [51].

## CONCLUSION

This study shows that *C. lobatus* possesses blood glucose lowering effects, antihypertensive effect, as well as antioxidant and free radical scavenging properties. Therefore, further investigation on the different phytochemical constituents may be beneficial for the treatment and management of diabetes mellitus and its complications, and provide a safer and cheaper alternative to the currently available anti-diabetic drugs.

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Original Research

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# Evaluation of the synergistic effect of *Allium sativum*, *Eugenia jambolana*, *Momordica charantia*, *Ocimum sanctum*, and *Psidium guajava* on hepatic and intestinal drug metabolizing enzymes in rats

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## ABSTRACT

**Aims/Background:** This study was to investigated the synergistic effect of polyherbal formulations (PHF) of *Allium sativum* L., *Eugenia jambolana* Lam., *Momordica charantia* L., *Ocimum sanctum* Linn., and *Psidium guajava* L. in the inhibition/induction of hepatic and intestinal cytochrome P450 (CYPs) and Phase-II conjugated drug metabolizing enzymes (DMEs). Consumption of these herbal remedy has been extensively documented for diabetes treatment in Ayurveda. **Methodology:** PHF of these five herbs was prepared, and different doses were orally administered to Sprague–Dawley rats of different groups except control group. Expression of mRNA and activity of DMEs were examined by real-time polymerase chain reaction and high performance liquid chromatography in isolated liver and intestine microsomes in PHF pretreated rats. **Results:** The activities of hepatic and intestinal Phase-II enzyme levels increased along with mRNA levels except CYP3A mRNA level. PHF administration increases the activity of hepatic and intestinal UDP-glucuronyltransferase and glutathione S-transferase in response to dose and time; however, the activity of hepatic sulfotransferase increased at higher doses. **Conclusions:** CYPs and Phase-II conjugated enzymes levels can be modulated in dose and time dependent manner. Observations suggest that polyherbal formulation might be a possible cause of herb-drug interaction, due to changes in pharmacokinetic of crucial CYPs and Phase-II substrate drug.

KEY WORDS: Cytochrome P450, herb-drug interaction, microsomes, sulfotransferase, UDP-glucuronyltransferase

## INTRODUCTION

Diabetes is a chronic endocrine disorder, characterized by absolute or relative insulin deficiency. The peoples of the world especially in India are facing major health problem such as diabetes. There were 366 million people with diabetes in 2011, a number projected to rise to 552 million by 2030 [1]. Herbal remedies remain one of the major approaches as complementary alternatives in the management of diabetes mellitus. This is because their benefits are well documented from a historical perspective in diabetic populated areas [2]. A huge proportion of Indian population relies on the use of traditional medicines for the use of their primary health care [3]. A major problem resulting from a combination of Ayurvedic medicine in present medical practices is a lack of clinical and scientific data to provide evidence for their efficacy and safety. It is a requirement to carry out research on herbal mixtures, to develop simple bioassays for biological standardization and pharmacological evaluation.

However, polyherbal formulations (PHF) are well documented to have the potential of inducing or inhibiting enzyme expression and their activities [4-6]. Several native products have been studied for the activity on drug metabolizing enzymes (DMEs) by several researchers [7,8] and North American botanicals [9]. Furthermore, most of the studies on herb-drug interactions deal

with induction or inhibition of enzymes, and less attention has been paid toward the recovery of these modulated enzymes back to normal levels following the discontinuation of respective herbal drug [10]. The major DMEs, the cytochrome P450 (CYP), consist of hemeproteins superfamily that catalyzes the oxidative metabolism of a wide variety of exogenous and indigenous chemicals. Several isoforms such as CYP1A1, CYP1A2, CYP2C9, CYP2B1, CYP2C1, CYP2D6, CY2E1, and CYP3A4 appear to be most relevant isozymes participated in the metabolism of clinically significant drugs [11].

Five plants, all native in India were used in PHF. *Eugenia jambolana* Lam. is known for its anti-diabetic activity [12]. Fruit of *Momordica charantia* L. is a known for anti-diabetic activity [13]. *Psidium guajava* L. and *Allium sativum* L. are known for their anti-diabetic activity [14,15]. *Ocimum* sanctum Linn. (Holy basil) has been mentioned in Indian system of traditional medicine to be of value in the treatment of diabetes mellitus [16]. To achieve the aim, the effect of PHF administration on the transcriptional level as well as the functional activity of Phase-I and II DME's in dose and time dependent manner. The effect of PHF treatment discontinuation after multiple weeks on Phase-I and II DMEs was evaluated to assess the time required for the recovery of PHF modulated enzymes back to control levels. With this aim, the present work was designed to study the effect of PHF administration on Phase-I and II DMEs of liver and intestine to assess its drug interaction potential.

## MATERIALS AND METHODS

### Chemicals and Reagents

Acetaminophen, bufurolol, 1'-hydroxybufurolol, caffeine, dexamethasone, ethoxyresorufin, glutathione (reduced), mephenytoin, 4-hydroxymephenytoin, pentoxyresorufin, resorufin, phenacetin, warfarin, 7-hydroxywarfarin, phenylmethanesulfonyl fluoride, p-nitrophenol (PNP), UDP-glucuronic acid (ammonium salt) (UDPGA), 1-chloro-2, 4-dinitrobenzene (CDNB), 2-mercaptoethanol, 3'-Phosphoadenosine-5'-phosphosulfate (PAPS), flavin adenine dinucleotide, dicoumarol, 2,6 dichlorophenolindophenol, 2-naphthylsulfate, high performance liquid chromatography (HPLC) grade acetonitrile, and methanol were purchased from Sigma–Aldrich (St. Louis, MI, USA). Nicotinamide adenine dinucleotide phosphate (NADPH) and dimethyl sulfoxide were purchased from SRL Pvt. Ltd (Mumbai, Maharashtra, India). Testosterone, 6β-hydroxytestosterone, chlorzoxazone, and 6-hydroxychlorzoxazone were purchased from Cayman chemical company (USA). Ultrapure water (18.2 M/Ω cm) was obtained from Milli-Q PLUS PF water. All other chemicals were commercially available or HPLC grade.

### Preparation of PHF

Five herbs *E. jambolana* Lam. (Jamun) seeds, *M. charantia* L. (Bitter gourd) fruits, *O. sanctum* Linn. (Holy Basil) leaves, A. *sativum* L. (garlic), and *P. guajava* L. (guava) were obtained from the local vegetable market in the city of Lucknow, India. The selected herbal materials were shade dried and grinded by mixer grinder. The prepared hydroalcohol extracts were concentrated using rotary evaporator at 40°C temperature. The concentrated extracts were freeze-dried at −20°C for 12 h then lyophilized using lyophilizer. The lyophilized extracted powders were stored in an airtight glass box and kept in the desiccator until used. PHF was prepared by mixing 200 mg powder of each herb in a single formulation.

### Animals

Male Sprague–Dawley rats of weight between 220 ± 20 g were provided by CSIR-IITR (India). Animals were maintained at 25°C temperature in steel cages with alternate 12 h of light and dark cycles and given a pallet diet and water. Before start of experiment, rats were acclimatized for 7 days then divided into two groups, PHF pre-treated ($n = 5$) and vehicle treated control ($n = 5$). Rats in pretreated group were gavaged (16-gauge gavage needle) with PHF (50, 100, and 200 mg/kg/day) for 7 days and multiples of weeks. The PHF suspension was made in 0.5% sodium carboxymethyl cellulose for oral administration. The control group was administered the same volume of vehicle for 7 days. Animals were allowed free access to food and water but before euthanasia, rats were overnight fasted to decrease the intestinal content. At the end of the experiment, rats were sacrificed by anesthetic ether inhalation. Experiments were carried out in accordance with current legislation on animal experiments as per Institutional Animal Ethical Committee at King George's Medical University Lucknow (IAEC approval no IAEC/2013/44).

### Assessment of PHF Pretreatment on Hepatic and Intestinal Phase-I DMEs

The effect of PHF administration of Phase-I enzymes (CYPs) was evaluated at transcriptional and assessment of functional activity level was performed by various enzyme assays.

### RNA Isolation, Primer Designing and Real-Time Polymerase Chain Reaction (RT-PCR) for CYPs

Rats were pretreated with PHF and vehicle for 7 days than rats were sacrificed with inhalation of anesthetic ether. A part of liver and intestine (10-50 mg) was homogenized with TRIzol (Invitrogen Life Technologies, USA) and RNA was isolated. Quantitation and purity check were accomplished using Nano drop (Thermo 6000, USA). Isolated RNA was instantly used for cyclic deoxyribose nucleic acid (cDNA) synthesis [17]. The cDNA sequences for various rat CYPs were obtained from Gene bank (gene bank as session number and sequences in [Table 1]. To make sure the specificity of primer sequences to target mRNA, each sequence was searched for homology with NCBI BLAST.

Effect of PHF pretreatment on the mRNA expression of CYPs was evaluated using RT-PCR. For quantitative RT-PCR, cDNA was amplified using Light Cycler 480 (Roche Molecular Biochemicals, USA) using SYBR green kit (Fermentas, USA).

Table 1: Oligonucleotide primer used for RT-PCR

| CYP | Gene accession no | Forward primer sequence | Reverse primer sequence |
|---|---|---|---|
| CYP1A1 | NM_012540.2 | TTCAGTTCAGTCCTTCCTCACA | GAAGGCTGGGAATCCATACA |
| CYP1A2 | NM_012541.3 | CTACAACTCTGCCAGTCTCCAG | CCTCTCAACACCCAGAACACT |
| CYP2B1 | NM_001134844.1 | TTCAGTTCAGTCCTTCCTCACA | GAAGGCTGGGAATCCATACA |
| CYP2B2 | NM_001198676.1 | GGGGAACCTCCTGCAGTT | GTGAACACATCTCCATATTTTTCG |
| CYP2C11 | U33173.1 | GGAGGAACTGAGGAAGAGCA | AATGGAGCATATCACATTGCAG |
| CYP2D1 | AB008422.1 | TCAGGATGGTGAAACTAGTGGA | TGGGAACGTGTTAAGAACCTC |
| CYP2D2 | NM_012730.1 | GAAGGAGAGCTTTGGAGAGGA | AGAATTGGGATTGCGTTCAG |
| CYP2E1 | AF061442.1 | CTGACTGTCTCCTCATAGAGATGG | TCACAGAAACATTTTCCATTGTGT |
| CYP3A1 | L24207.1 | GCAGGAGGAGATCGACAGG | CCAGGTATTCCATTTCCATCA |
| CYP3A2 | U09742.1 | ACC CGT CTG GAT TCT AAG CA | TGGAATTATTATGAGCGTTCAGC |
| GAPDH | M17701.1 | AGCTGGTCATCAATGGGAAA | ATTTGATGTTAGCGGGATCG |

CYP: Cytochrome P450, RT-PCR: Real time polymerase chain reaction

The reaction was performed in 96-well PCR plate. The reaction mixture was consisted of 1X SYBR green, 200-300 nM primers, and 2 $\mu$l of cDNA in final reaction volume of 20 $\mu$l. The thermal cycle condition was 95°C for 15 min to activate Amplitaq Gold DNA polymerase, 95°C for 15 s and anneal/extension at 60°C for 1 min (40 cycles). Glyceraldihyde-3-phasphate dehydrogenase (GAPDH) was used as internal loading control. Fold changes in mRNA level were resultant after normalizing with GAPDH mRNA levels [17].

## Evaluation of PHF Treatment on Function Activity of CYPs

To evaluate the effect of PHF pretreatment on CYPs functional activity various enzyme assays were performed with specific CYP probe substrates using microsomes structured from PHF treated and control animals.

## Preparation of Microsome and Cytosolic Fractions

Liver and intestine were quickly excised, snap frozen, and stored at −80°C until used after perfusion with ice-cold normal saline. Microsome and cytosolic fractions were prepared [18]. Microsomal protein and cytosolic protein were determined using Lowry method of protein assessment using BSA as standard [19]. CYP concentrations in rat liver microsomes (RLM) and rat intestine microsomes were quantified [20].

## Assessment of CYPs Mediated Phase-I Enzymatic Activity

Microsomes prepared from PHF pre-treated, recovery and control groups were used for assessing the effect of PHF pretreatment and discontinuation on the enzymatic activity of hepatic Phase-I DMEs. CYP-mediated Phase-I DMEs were analyzed with following enzymatic assays.

*CYP1A1-dependent ethoxyresorufin O-deethylase (EROD) and CYP2B-dependent pentoxyresorufin O-dealkylase (PROD) assay*

EROD and PROD assays [18] were performed with slight modifications. In brief, the reaction mixture consisted of a 100 mM Tris-HCl buffer (pH 7.4), 10 mM $MgCl_2$, 1 mg/ml microsomal protein with a final reaction volume of 0.2 ml. Ethoxyresorufin (5 $\mu$M) and pentoxyresorufin (10 $\mu$M) were used as probe substrate for CYP1A1 and CYP2B, respectively. The reaction mixture was pre-incubated for 5 min at 37°C. After pre-incubation, the reaction was initiated with addition of NADPH (2 mM), followed by incubation at 37°C for 15 min. An equal amount of buffer was added to the blank reaction. The reaction was terminated by addition of 300 $\mu$l ice cold methanol. The reaction was mixed and centrifuged at 12,000 rpm for 15 min at 4°C. The supernatant was used as HPLC sample. The amount of formed metabolite (resorufin) was determined using HPLC with fluorescence detector. The activity was expressed as pMol of product formed/min/mg.

*CYP1A2-dependent phenacetin O-deethylase (POD) assay*

POD assay [21] was performed with slight modifications. Phenacetin (50 $\mu$M) was incubated with RLM (1 mg/ml), 10 mM $MgCl_2$, in a total volume of 0.2 ml of potassium phosphate buffer (100 mM, pH 7.4). Reaction mixture was pre-incubated for 5 min at 37°C. Reaction was initiated with adding of NADPH (2 mM). After 20 min incubation at 37°C reaction was terminated by adding up equal volume of ice-cold acetonitrile (containing internal standard caffeine). The blank reaction consisted of equal amount of buffer. The mixture was vortexed and centrifuged at 12,000 rpm for 15 min at 4°C. The supernatant obtained was used as HPLC sample. Quantification of product (acetaminophen) was done from standard curve using HPLC-UV. Activity was expressed as nMol of product formed/min/mg.

*CYP2C19-dependent mephenytoin 4-hydroxylation assay*

Mephenytoin 4-hydroxylation assay [22] was carried out with slight modifications. Mephenytoin (50 $\mu$M) was incubated with RLM (1 mg/ml), 10 mM $MgCl_2$, in a total volume of 0.2 ml of 100 mM potassium phosphate buffer (pH 7.4). Reaction mixture was pre-incubated for 5 min at 37°C. The reaction was started with the addition of NADPH (2 mM) and terminated by adding up the equal volume of ice-cold acetonitrile (containing internal standard beta-naphthol) after 40 min incubation at 37°C. The blank reaction consisted equal amount of buffer. The mixture was vortexed and centrifuged at 12,000 rpm for 15 min at 4°C. The supernatant was used as HPLC sample. Quantification of product (4-hydroxymephenytoin) formed was

done using standard curve of metabolite using HPLC. Activity was expressed as nMol of product formed/min/mg.

*CYP2D-dependent bufurolol 1-hydroxylation assay*

Bufurolol (20 $\mu$M) was incubated with RLM (1 mg/ml), 10 mM $MgCl_2$, in a total volume of 0.2 ml of potassium phosphate buffer (100 mM, pH 7.4). Reaction mixture was pre-incubated for 5 min at 37°C. The reaction was started with the addition of NADPH (2 mM) and reaction was terminated by adding equal volume of ice cold acetonitrile after 20 min incubation at 37°C. The blank reaction contained equal amount of buffer. The mixture was then sturdily mixed, chilled on ice for 15 min, and centrifuged at 12,000 rpm for 15 min at 4°C. Supernatant obtained was used as HPLC sample. Quantification of product (1-hydroxybufurolol) formed was done from standard curve of metabolite using HPLC. Activity was expressed as nMol of product formed/min/mg.

*CYP2E1-dependent chlorzoxazone 6-hydroxylation assay*

Chlorzoxazone 6-hydroxylation assay [23] was performed with slight modifications. Chlorzoxazone (50 $\mu$M) was incubated with RLM (1 mg/ml), 10 mM $MgCl_2$, in a total volume of 0.2 ml of potassium phosphate buffer (100 mM, pH 7.4). Reaction mixture was pre-incubated for 5 min at 37°C. The reaction was started with the addition of NADPH (2 mM) and reaction was terminated by adding equal volume of ice cold acetonitrile (containing internal standard phenacetin) after 20 min incubation at 37°C. Blank reaction contained equal amount of buffer. The mixture was then vigorously mixed and centrifuged at 12,000 rpm for 15 min at 4°C. Supernatant obtained was used as HPLC sample. Quantification of product (6-hydroxychlorzoxazone) formed was done from standard curve of metabolite using HPLC. Activity was expressed as nMol of product formed/min/mg.

*CYP3A-dependent testosterone 6β-hydroxylation assay*

Testosterone 6β-hydroxylation assay was performed [24]. Testosterone (100 $\mu$M) was incubated with RLM (1 mg/ml), 5 mM $MgCl_2$, in a total volume of 0.2 ml of 100 mM potassium phosphate buffer, pH 7.4. Reaction mixture was pre-incubated for 5 min at 37°C. The reaction was started with addition of NADPH (2 mM) and reaction was terminated by adding of equal volume of ice-cold acetonitrile (containing internal standard dexamethasone) after 20 min of incubation at 37°C. Blank contains equal amount of buffer. The mixture was vigorously mixed and centrifuged at 12,000 rpm for 15 min at 4°C. Supernatant obtained was used as HPLC sample. Quantification of product (6β-hydroxytestosterone) formed was done from standard curve of metabolite using HPLC. Activity was expressed as nMol of product formed/min/mg.

## Assessment of PHF Pretreatment on Hepatic and Intestinal Phase-II DMEs

To examine the effect of PHF pretreatment on hepatic and intestinal Phase-II DMEs, microsomal and cytosolic conjugation enzyme activities were evaluated. Animals experiment and microsomal and cytosolic fractions were prepared as described. Phase-II DME's were analyzed with following enzymatic assays.

*UDP-glucuronyltransferase (UDPGT) assay*

UDPGT activity was measured by determining the PNP conjugation with UDPGA [25]. Activity was performed by determining amount of PNP consumed. The reaction mixture was consisted of 0.5 mM PNP, 4 mM $MgCl_2$, 50 mM TrisHCl (pH 7.4) and 1 mg/ml microsomal protein. The samples were preincubated in shaking water bath for 5 min at 37°C and the reaction was initiated with addition of UDPGA (2 mM). Blank samples were performed in the absence of UDPGA, but the equal volume of buffer was added. After taking 15 min for incubation, reactions were broken by the addition of trichloroacetic acid (5% final concentration). Samples were then centrifuged at 12,000 rpm for 15 min; supernatant was collected and NaOH was added to the reaction mixture to achieve final concentration of 0.5 M. Consumed PNP was calculated by subtracting value from blank. PNP concentration was determined at 410 nm using standard curve of PNP. Activity was expressed as nMol of conjugate/min/mg.

*Glutathione S-transferase (GST) assay*

Cytosolic GST activity to conjugate CDNB with reduced glutathione (GSH) was estimated [26]. Reaction mixture contained potassium phosphate buffer (0.3 M, pH 6.5) GSH (30 mM) and CDNB (30 mM) in 96 well plate. Reaction was initiated with addition of cytosolic enzyme. Blank contained an equal volume of buffer. The reaction was continuously monitored at 37°C in plate reader at 340 nm immediately after addition of cytosolic protein. Activity was expressed as nMol conjugate/min/mg.

*Sulfotransferase (SULT) assay*

SULT activity in cytosolic fraction was measured using methylene blue extraction assay [27]. In brief, reaction mixture contains sodium acetate buffer pH 5.5, 7.5 $\mu$Mol 2-mercaptoethanol, 65 $\mu$Mol PAPS, 215 $\mu$Mol of 2-naphthol. After pre-incubation for 5 min at 37°C, reaction was initiated with addition of cytosolic enzyme. Control reaction also contained equal volume of enzymes solution but the reaction was instantly stopped by addition of equal volume of methylene blue reagent. Chloroform (2.0 ml) was added, and the reaction mixture was vortex vigorously. Mixture was centrifuged at 1000 × *g* for 15 min. Sodium sulfate was added to collected organic phase. The absorbance of organic phase was measured at 651 nm and specific activity (nMol product/min/mg protein) was calculated (10 nMol of 2-naphthylsulfate yields 0.3 absorbance at 651 nm). Activity was expressed as nMol of product formed/min/mg.

## Statistical Analysis

Statistical analyses were performed with help of Prism version 5.1. Student's *t*-test (non-parametric) and one-way analysis of

variance followed by the Student's Newman-Keuls multiple range tests were used as applicable. Data are obtained as mean with standard errors. The level of significance was set prior at $P < 0.05$.

## RESULTS

### Effect of PHF Administration on Hepatic Phase-I and Phase-II DMEs

To evaluate the effect of PHF administration on hepatic DMEs, dose and time dependent study was conducted. Similarly, the effect of PHF discontinuation after 1 week pretreatment was also evaluated to determine whether the modulated DMEs recovered back to normal level or not, when PHF administration was discontinued.

The treatment with PHF at lower dose (50 mg/kg/day) for 7 days only change mRNA expression of CYP1A1 (two-fold up-regulation) and CYP3A2 (1.9 fold down-regulation) as compared to vehicle treated control. However, the treatment with PHF (100 and 200 mg/kg/day dose) for 7 days significantly up-regulated the mRNA expression of CYP1A1 (5.1 and 5.9 fold), CYP1A2 (1.9 fold), CYP2D1 (2.2 and 2.7 fold), and CYP2D2 (3.5 and 3.9 fold), respectively compared to vehicle treated control. However, treatment with PHF (100 and 200 mg/kg/day dose), significantly down-regulation was found in the mRNA expression of CYP3A1 (1.9 fold) and CYP3A2 (2.6 and 2.4 fold), respectively compared to control. Interestingly, treatment with PHF (50, 100 and 200 mg/kg/day dose) for 7 days did not alter the mRNA expression level of CYP2B1, CYP2B2, CYP2C11 and CYP2E1 compared to vehicle-treated control [Figure 1].

Similarly, to validate the mRNA data, functional activity of CYPs were assessed after PHF treatment. The pretreatment with PHF (50, 100 and 200 mg/kg/day dose) for 7 days significantly increased the functional/catalytic activity of EROD (1.6, 3.7 and 5.4 fold), POD (1.5 fold), PROD (1.2, 1.4 and 1.5 fold) and bufurolol hydroxylase (2.9 fold), respectively, compared to control. However, pretreatment with PHF did not alter the enzymatic activity of, mephenytoin and chlorzoxazone hydroxylase enzymes. In addition, treatment with PHF (50, 100 and 200 mg/kg/day dose) for 7 days decreased the enzymatic activity of testosterone hydroxylase (2 fold), respectively, compared to control [Figure 2].

Likewise, when Phase-II conjugation activity was assessed in dose-response study, no alteration in UDPGT, GST and SULT was observed at lowest dose study (50 mg/kg/day for 1 week). However, increase in catalytic activity of UDPGT (1.6 and 2.3 fold) and GST (1.6 and 2.1 fold) was observed at 100 and 200 mg/kg/day, respectively, in comparison to control. Modulation in SULT functional activity (1.8 fold) was obtained at 200 mg/kg/day of PHF dose compared to control [Figure 3].

### Time Dependent Response of PHF Administration on Hepatic Phase-I and II DMEs

To assess the time-dependent response of DMEs rats were administered with PHF (100 mg/kg/day) up to 4 weeks and

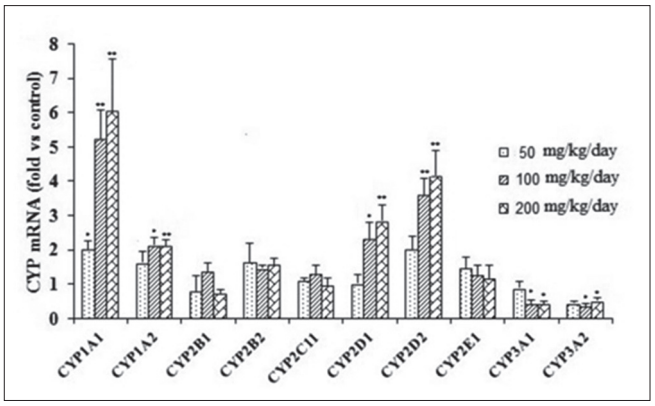


**Figure 1:** Dose response of polyherbal formulation (PHF) pretreatment on hepatic cytochrome P450 mRNA level. Rats were given 50, 100 and 200 mg/kg/day of PHF for 1 week. Control rats received same volume of blank formulation for same time duration. Each value is expressed as mean ± standard error from three independent experiments. Asterisks indicate significant differences (*$P < 0.05$, **$P < 0.01$) from control

activity was analyzed after every week. PHF pretreatment for 1, 2, 3 and 4 weeks increased the mRNA level of CYP1A1 (5.4, 5.5, 4.7 and 4.1 fold), CYP1A2 (1.7, 2, 1.9 and 2.9 fold), CYP2D1 (2, 2.1, 1.2 and 2.5 fold), and CYP2D1 (3.4, 2.9, 2.7 and 4.1 fold). Similarly, PHF treatment for 1, 2, 3 and 4 weeks decreased the mRNA levels of CYP3A1 (1.9, 2.8, 2.3 and 1.6 fold) and CYP3A2 (2, 2.5, 2 and 1.6 fold), respectively. No significant modulation in mRNA level of CYP2B1 and CYP2C11 was observed. However, CYP2E1 mRNA level was not altered by 2 weeks but after 3 and 4 weeks of PHF treatment mRNA level of CYP2E1 was increased by 1.9 fold in comparison to control [Figure 4].

In the same way, PHF treatment for 1, 2, 3 and 4 weeks increased the catalytic activities of CYP1A1 (3.5, 5, 7.2 and 8.6 fold), CYP1A2 (1.6, 1.5, 1.5 and 2.4 fold), and CYP2D (2.1, 2.1, 2.4 and 3 fold), respectively. Although increase in the mRNA level of CYP2B2 was observed, the CYP2B-dependent catalytic activity was increased significantly by 1.6 fold only after 4 weeks of PHF treatment. Similarly, CYP2E1-dependent catalytic activity was also increased by 1.4 fold after 3 weeks of PHF treatment. In addition, CYP3A-dependent catalytic activity significantly decreased by 1.8, 1.8, 2 and 2.1 fold after 1, 2, 3 and 4 weeks of PHF treatment [Figure 5]. However, no difference in CYP2C9 and CYP2C19-dependent catalytic activities was observed. When Phase-II conjugation enzymes activities were assessed, it was observed that UDPGT and GST activities were increased only by 1.5 fold after every 1 week of PHF treatment compared to control. No difference in catalytic activity of SULT was observed up to 2 weeks of PHF treatment. However, after 3 weeks of PHF treatment SULT activity was increased by 1.8 fold in comparison to control [Figure 6].

### Effect of PHF Discontinuation on Recovery of Modulated Hepatic DMEs

To assess the recovery of modulated hepatic CYP enzymes with discontinuation of PHF, rats were first treated for 1 week with PHF (100 mg/kg/day) for induction/inhibition and then PHF treatment

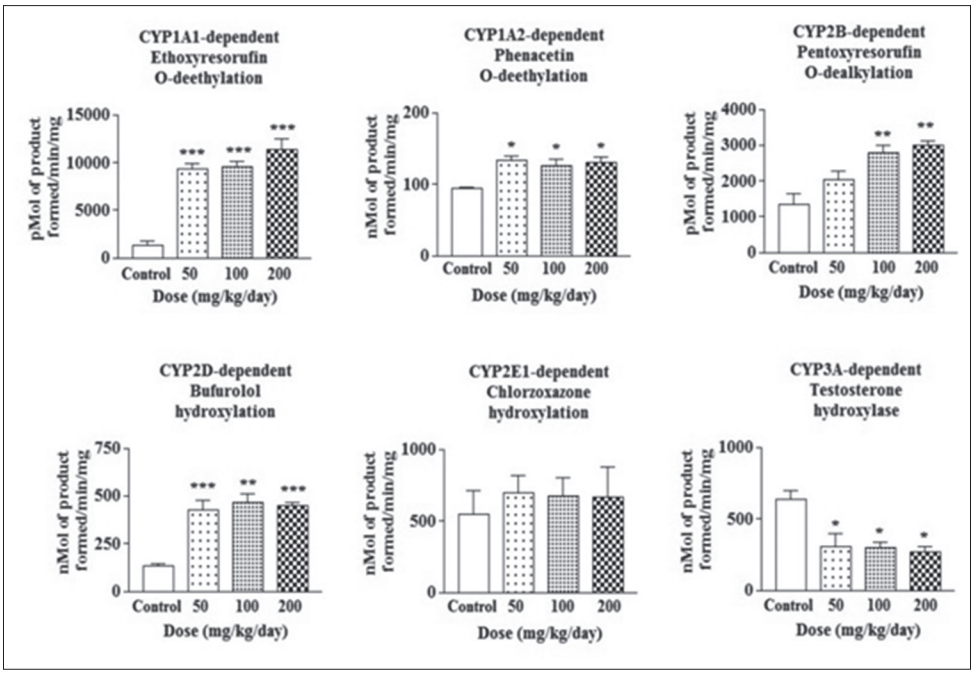


**Figure 2:** Dose response of polyherbal formulation (PHF) pretreatment on hepatic cytochrome P450 functional activity. Rats were given 50, 100, and 200 mg/kg/day of PHF for 1 week. Control rats received same volume of blank formulation for same time period of time. Each value is expressed as mean ± standard error from three independent experiments. Asterisks indicate significant differences (*$P < 0.05$, **$P < 0.01$) from control

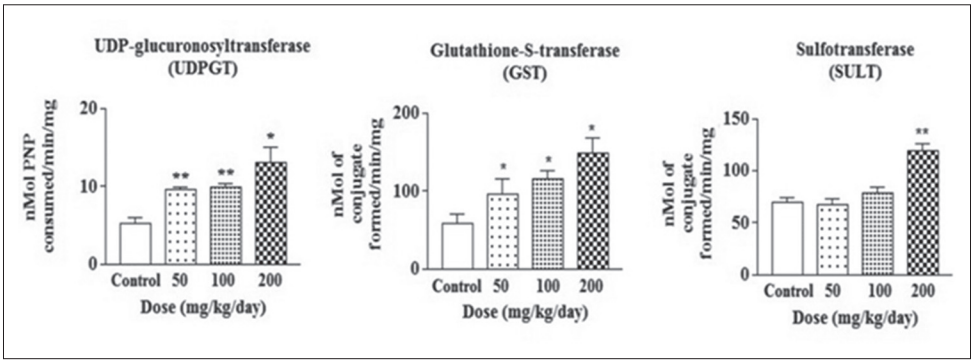


**Figure 3:** Dose response of polyherbal formulation (PHF) pretreatment on hepatic Phase II conjugation enzymes functional activity. Rats were given 50, 100 and 200 mg/kg/day of PHF for 1 week. Control rats received same volume of blank formulation for same period of time. Each value is expressed as mean ± standard error from three independent experiments. Asterisks indicate significant differences (*$P < 0.05$ and **$P < 0.01$) from control

was discontinued. Hepatic CYP mRNA expression level was assessed after the induction period and each week of the recovery period. Pretreatment with PHF (100 mg/kg/day) for seven days significantly increased the mRNA expression levels of CYP1A1 (5.2 fold), CYP1A2 (1.7 fold), CYP2B2 (3.1 fold), CYP2D1 (2.2 fold), and CYP2D2 (3.4 fold) in comparison to control. However, a significant decrease of 2 fold in mRNA expression of CYP3A1 and CYP3A2 was observed compared to control. However, no significant difference in mRNA level (CYP1A1, CYP1A2, CYP2D1, CYP2D1, CYP2D2, CYP3A1, and CYP3A2) was observed after 1 week of last PHF dose in comparison to control. No alteration in mRNA levels of CYP2B1, CYP2C11, and CYP2E1 was observed with PHF treatment [Figure 7].

Similarly, all CYP enzyme activities and Phase-II conjugation enzyme activities that were modulated with PHF treatment reverted back to control level after 1 week of PHF discontinuation except CYP1A1 and CYP2D-dependent enzymatic activities. The CYP1A1-dependent and CYP2D-dependent catalytic activities recovered back to control level after 2 weeks of BM discontinuation [Table 2].

## Dose-response of PHF Administration on Intestinal Phase-I and II DMEs

Pretreatment with PHF at 50, 100 and 200 mg/kg/day dose significantly induced the mRNA level of CYP1A1 by 3.2, 3 and 4.3 fold, respectively. Interestingly, PHF pretreatment for 1 week decreased the mRNA level of CYP3A2 (2, 1.9 and 2.2 fold) at 50, 100 and 200 mg/kg/day, respectively. However, the treatment with PHF (50, 100 and 200 mg/kg/day dose) for 1 week did not alter the mRNA expression level of CYP2C11, 2D1, 2D2 and 2E1 compared to vehicle-treated control [Figure 8].

To validate our mRNA data with active protein level, we measured the catalytic activity of respective CYPs with specific probe substrate and the result obtained supported the mRNA data. In dose response study a significant increase in CYP1A1-dependent catalytic activity by 1.4, 1.7 and 1.6 fold was observed at 50, 100 and 200 mg/kg/day, respectively. However, PHF pretreatment significantly decreased the CYP3A-dependent catalytic activity of testosterone 6β-hydroxylase by 1.5 fold at dose studied. No significant difference in the CYP2C9, CYP2C19, CYP2D, and CYP2E1-dependent catalytic activity was observed [Figure 9].

When Phase-II conjugation activity was assessed in dose-response study, no alteration in conjugation enzymes was observed at low PHF dose (50 mg/kg/day) when administered for 1 week. However, PHF pretreatment for one week significantly increased UDPGT (3.4 and 4.2 fold) and GST (1.4 and 1.9 fold) activity at 100 and 200 mg/kg/day, respectively. No alteration was observed in SULT activity with PHF treatment [Figure 10].

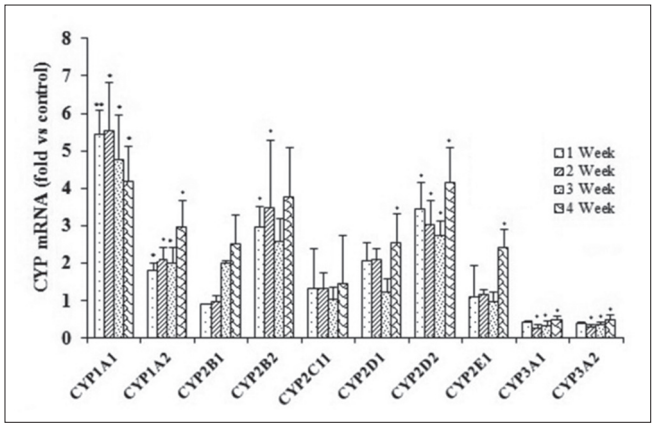


**Figure 4:** Time dependent effect of polyherbal formulation (PHF) pretreatment on hepatic cytochrome P450 mRNA level. Rats were given PHF (p.o 100 mg/kg/day) for 4 weeks and rats were sacrificed after every week. Control rats received the same volume of blank formulation for same time duration. Each value is expressed as mean ± standard error from three independent experiments. Asterisks indicate significant differences (*$P < 0.05$,**$P < 0.01$,***$P < 0.001$) from control

## Time-dependent Response of PHF Administration on Intestinal Phase-I and II DMEs

Similarly to evaluate the effect of different pretreatment duration on intestinal DMEs, rats were pretreated with PHF (100 mg/kg/day) for different time duration and any alteration in DMEs mRNA level and functional activity was measured and compared with control. In study, it was observed that mRNA level of CYP1A1 was increased by 3.3, 3.6, 3.5 and 5.4 fold after 1, 2, 3 and 4 weeks of treatment with PHF, respectively, compared to control. However, no significant difference was observed among the treatment duration. Similarly, no treatment time dependency was observed for CYP3A2 (2.2, 2.3, 2.1 and 2.1 fold decrease). In addition, PHF failed to alter the mRNA level of CYP2C11, 2D1, 2D2 and 2E1 compared to vehicle-treated control [Figure 11].

To confirm the RT-PCR results with functional activity of respective CYP enzymes, it was observed that CYP1A-mediated catalytic activity (EROD) was increased by 1.2, 1.2, 1.3 and 1.5 fold after 1, 2, 3 and 4 weeks of PHF treatment, respectively. The CYP3A-mediated testosterone hydroxylase catalytic activity was decreased by 1.7, 1.9, 2.4 and 2.2 fold after 1, 2, 3 and 4 weeks of PHF treatment, respectively. Similar to CYP1A-mediated catalytic activity no treatment duration dependency was observed. No significant difference was observed in CYP2E1, however, catalytic activity was significantly increased by 2.1 after 3 and 4 weeks of PHF treatment. Furthermore, no

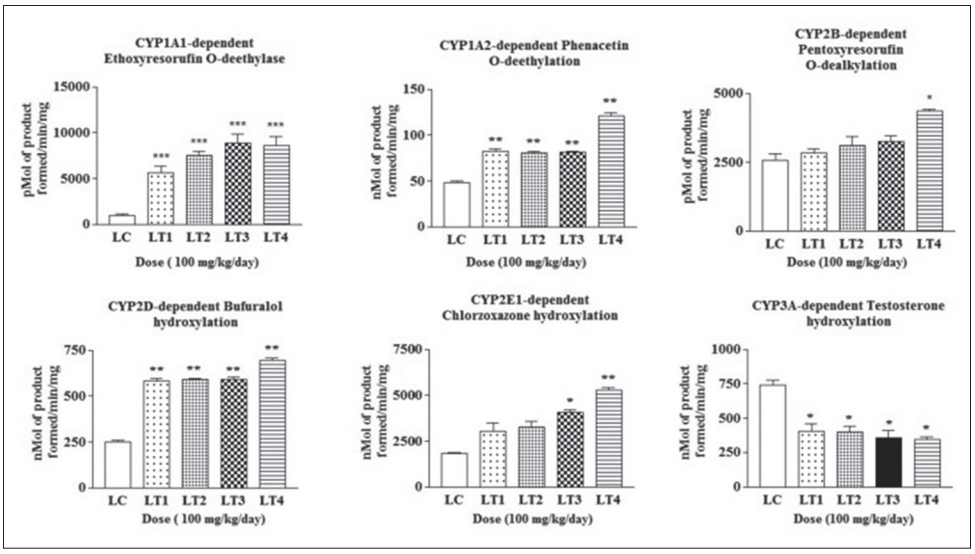


**Figure 5:** TTime dependent effect of polyherbal formulation (PHF) pretreatment on hepatic cytochrome P450 functional activity. Rats were given PHF (p.o 100 mg/kg/day) for 4 weeks and rats were sacrificed after every week. Control rats received the same volume of blank for same time duration. LC, LT1, LT2, LT3 and LT4 represents control group, 1 week pretreated group, 2 week pretreated group, 3 week pretreated group and 4 week pretreated group with PHF, respectively. Each value is expressed as mean ± standard error from three independent experiments. Asterisks indicate significant differences (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$) from control

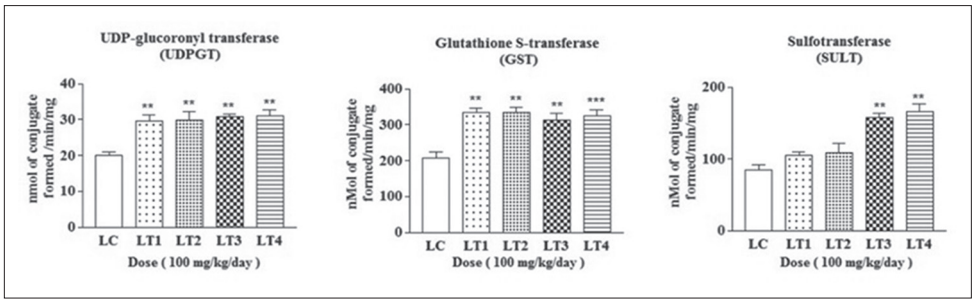


**Figure 6:** Time dependent effect of polyherbal formulation (PHF) pretreatment on hepatic Phase II conjugation enzyme functional activity. Rats were given PHF (p.o 100 mg/kg/day) for 4 weeks and rats were sacriced after every week. Control rats received the same volume of blank formulation for same time duration. LC, LT1, LT2, LT3 and LT4 represents control group, 1 week pretreated group, 2 week pretreated group, 3 week pretreated group and 4 week pretreated group with PHF, respectively. Each value is expressed as mean ± standard error from three independent experiments. Asterisks indicate significant differences (**P* < 0.05, ***P* < 0.01, ****P* < 0.001) from control

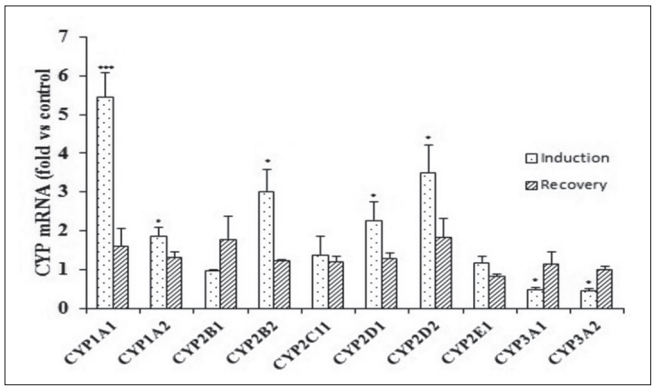


**Figure 7:** Effect of one week discontinuation of polyherbal formulation (PHF) treatment on hepatic CYPs mRNA level. Rats were treated with PHF (p.o 100 mg/kg/day) for one week and then PHF administration was discontinued for 1-week. Each value is expressed as mean ± standard error from three independent experiments. Asterisks indicate significant differences (**P* < 0.05, ****P* < 0.001) as compared to control

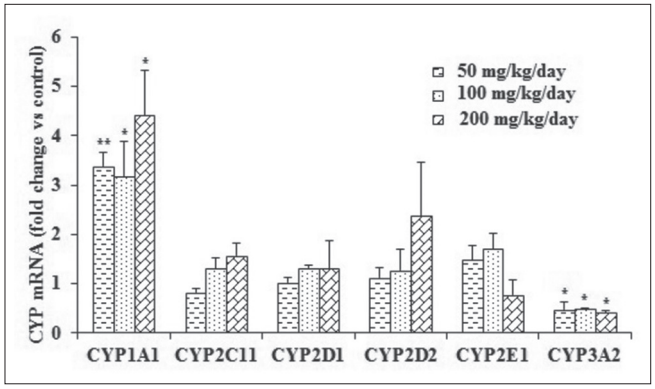


**Figure 8:** Dose response of polyherbal formulation (PHF) pretreatment on intestinal CYPs mRNA level. Rats were given 50, 100 and 200 mg/kg/day of standardized PHF extract for 1-week and sacrificed on 8th day. Each value is expressed as mean ± standard error from three independent experiments. Asterisks indicate significant differences (**P* < 0.05, ***P* < 0.01) from control

significant difference in CYP2C9, CYP2C19, and CYP2D-dependent catalytic activity was observed even after 4 weeks of PHF treatment [Figure 12].

## DISCUSSION

Recent studies have explained the importance and putative effect of anti-diabetic herbs in a single formulation on CYPs and conjugating enzymes. In this study, we used self-prepared polyherbal formulation to evaluate its effect on hepatic and intestinal CYP and conjugation enzymes. This herbal preparation was prepared by five herbs that have been traditionally used as an option for the treatment of diabetes. For effective and safe use of potential herbs, DMEs mediated herb-drug interactions must be studied. To the best of our knowledge, there is no such report available on hepatic and intestinal CYP and Phase-II (conjugation) enzymes for this type of polyherbal formulation. Therefore, the mechanism of herb-drug interactions that might occur with herbal consumption, alterations at transcriptional and/or translational level of DMEs must be studied. The true measure of enzyme response is their catalytic activity instead of total protein. Therefore, in the present study, we directly measured the enzymatic activities of individual CYP enzymes along with individual mRNA level instead of protein level to assess the fundamental mechanism of PHF mediated herb-drug interactions.

*In vivo* modulation of hepatic and intestinal DMEs was studied in a dose response manner using different doses, *viz*., 50, 100, and 200 mg/kg/day. Dose response experiment with PHF on CYP suggested that a lower dose (50 mg/kg/day) was adequate to produce effect on hepatic CYP enzymes. PHF pretreatment increased mRNA expression levels of hepatic CYPs to CYP1A1 > CYP2D2 > CYP2D1 > CYP2B2 > CYP1A2 that correspond well with catalytic activities of each CYP (CYP1A1-dependent EROD > CYP2D-dependent bufurolol hydroxylase > CYP1A2-depenedent phenacetin-O-dethylase). However, CYP1A1 is the only intestinal CYP that has been considerably upregulated with PHF treatment. Moreover, PHF pretreatment decreased the mRNA expression of hepatic and intestinal CYP3A1 and CYP3A2 genes and this decrease in mRNA level correspond well with CYP3A-dependent testosterone hydroxylase catalytic activity. Interestingly, no dose dependency was observed at mRNA level as well as the catalytic activity level of any CYPs. Alteration at mRNA level corresponds directly with the activity of respective

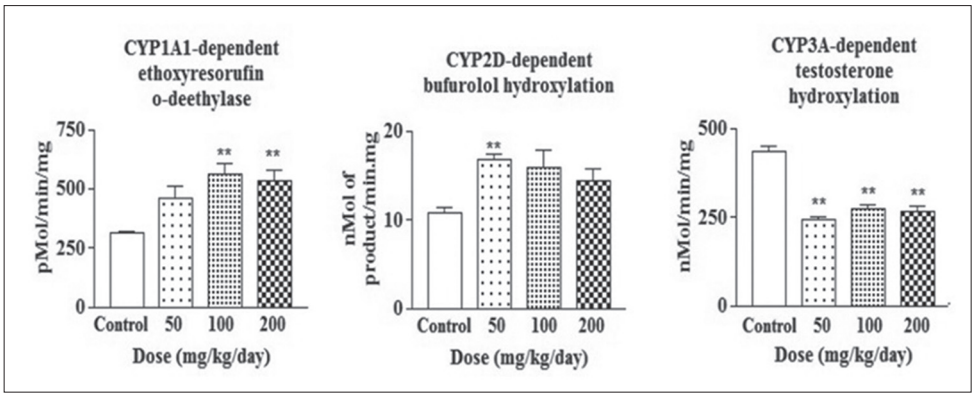


**Figure 9:** Dose dependent changes in intestinal cytochrome P450 activity in rats treated with standardized extract of polyherbal formulation (PHF). Rats were given 50, 100 and 200 mg/kg/day of standardized PHF extract for 1-week and sacrificed on 8th day. Control rats received same volume of blank formulation for same time duration. Each value is expressed as mean ± standard error from three independent experiments. Asterisks indicate significant differences (*$P < 0.05$, **$P < 0.01$) from control

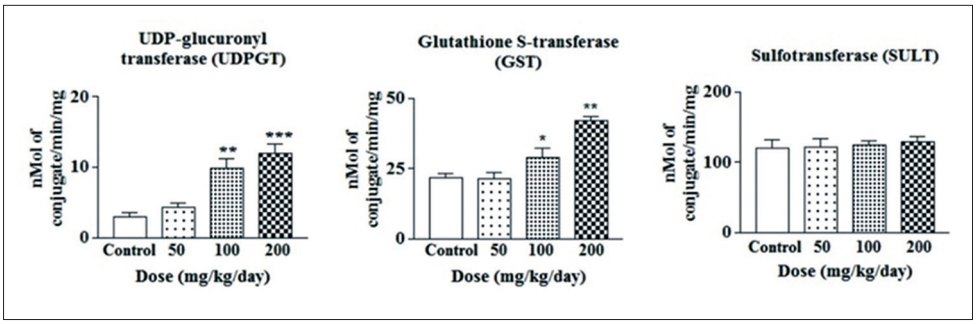


**Figure 10:** Dose dependent changes in intestinal conjugation Phase II enzyme activity in rats treated with extract of polyherbal formulation (PHF). Rats were given 50, 100 and 200 mg/kg/day of standardized PHF extract for 1 week and sacrificed on 8th day. Control rats received same volume of blank formulation for same time duration. Each value is expressed as mean ± standard error from three independent experiments. Asterisks indicate significant differences (*$P < 0.05$, **$P < 0.01$) from control

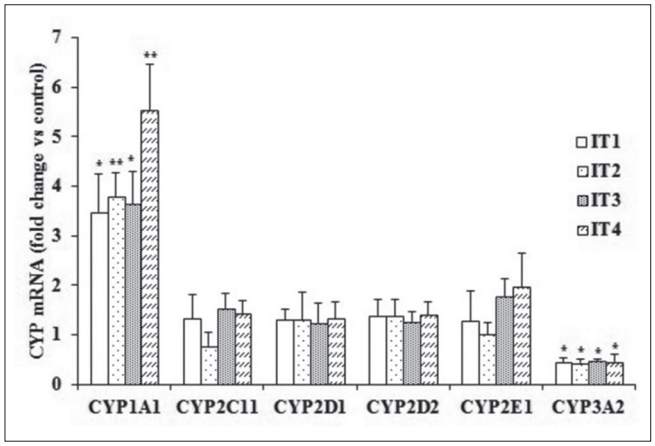


**Figure 11:** Time dependent effect of polyherbal formulation (PHF) pretreatment on intestinal cytochrome P450 mRNA level. Rats were given PHF (p.o 100 mg/kg/day) for 4 weeks and rats were sacrificed after every week. Control rats received the same volume of blank formulation for same time duration. IC, IT1, IT2, IT3 and IT4 represents control group, 1-week pretreated group, 2 week pretreated group, 3 week pretreated group and 4 week pretreated group with PHF, respectively. Each value is expressed as mean ± standard error from three independent experiments. Asterisks indicate significant differences (*$P < 0.05$, **$P < 0.01$) from control

CYP that may be attributed to alteration at the translational level. However, PHF at dose studied (50-200 mg/kg/day) failed to modulate the expression of hepatic and intestinal CYP2C11 and CYP2E1 activities at transcriptional and activity level. Besides, CYP2C11 and CYP2E1, intestinal CYP2D isoform was also unaffected both at transcriptional and catalytic activity level. Similarly, time response study with PHF (100 mg/kg/day) exhibited that 1 week pretreatment was sufficient to produce the alteration in CYPs at transcriptional and catalytic activity level, and no further modulation was observed with increased treatment duration. In addition, no dose and time dependency was observed (except CYP2E1, upregulated after 3 weeks of treatment) that suggests low dose and 1 week treatment was sufficient for absorption of PHF to concentration level and that too up to saturation level to put forth its effect. However, upregulation of CYP2E1 could be attributed to time taken by responsible PHF components to accumulate up to the level at which they can exert their effect. Even at high concentration pretreatment for 1 week, level that could alter the CYP2E1 was not achieved to exert its effect on CYP2E1. These alterations in the catalytic activity of various CYP enzymes with PHF administration may affect their own metabolism or drugs that are metabolized through these CYP's [28].

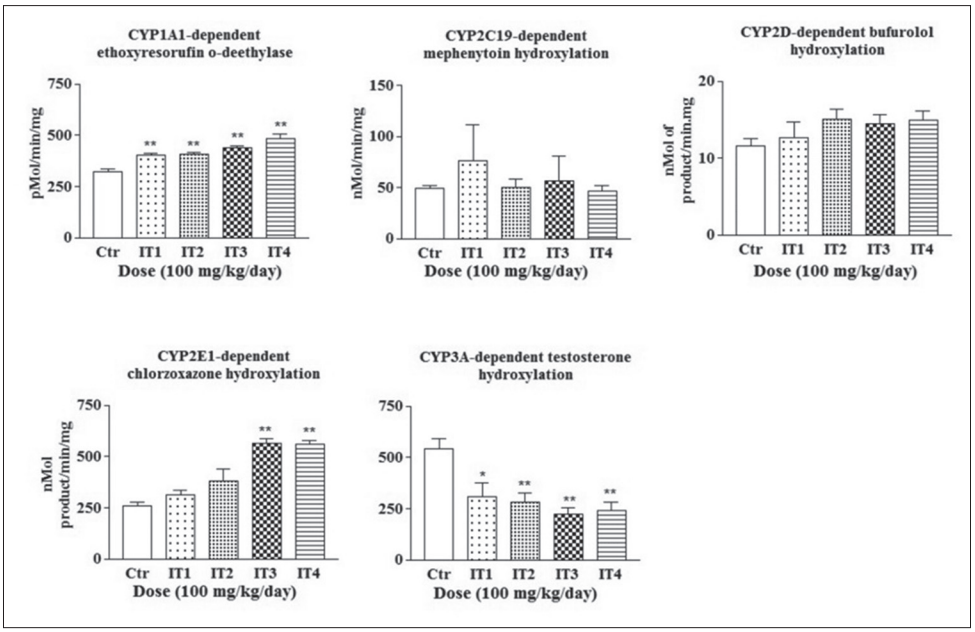


**Figure 12:** Time dependent effect of polyherbal formulation (PHF) pretreatment on intestinal cytochrome P450 functional activity. Rats were given PHF (100 mg/kg/day) for 4 weeks and rats were sacrificed after every week. Control rats received the same volume of blank formulation for same time duration. IC, IT1, IT2, IT3 and IT4 represents control group, 1 week pretreated group, 2 week pretreated group, 3 week pretreated group and 4 week pretreated group with BM, respectively. Each value is expressed as mean ± standard error from three independent experiments. Asterisks indicate significant differences (*$P < 0.05$, **$P < 0.01$) from control

**Table 2: Induction and recovery of hepatic CYP activities and Phase-II conjugation reactions in rats treated with PHF extract**

| DMEs | Untreated control | Treatment 1 week | Recovery (after 1 week oral administration of PHF) | | |
|---|---|---|---|---|---|
| | | | 1 week | 2 week | 3 week |
| CYP | | | | | |
| CYP1A1 (pmol/mg/min) | 926.56±412.76 | 6333.04±1230.25** | 4680.0±916.74* | 2590.09±323.29 | 1068.0±145.59 |
| CYP1A2 (nmol/mg/min) | 55.70±5.13 | 77.29±1.34 | 69.37±3.75 | 62.76±12.83 | 58.23±5.80 |
| CYP2B (pmol/mg/min) | 3002.29±117.16 | 1842.17±34.03 | 2791.59±345.22 | 2850.12±456.89 | 2919.29±567.65 |
| CYP2D (nmol/mg/min) | 249.66±12.66 | 582.66±21*** | 574.25±44.23* | 483.41±57.63 | 280.21±23.0 |
| CYP2E1 (nmol/mg/min) | 1830.06±106.8 | 3029.47±795.96 | 2031.8±133.35 | 1927.7±186.24 | 1930.90±198.87 |
| CYP3A (nmol/mg/min) | 743.48±63.053 | 403.22±111.84* | 450.14±49.06 | 510.91±30.45 | 650.45±19.98 |
| Conjugation assay | | | | | |
| UDPGT (nmol of PNP consumed/mg/min) | 20.03±2.56 | 29.52±3.78 | 24.25±2.76 | 25.23±4.23 | 21.94±5.23 |
| GST (nmol of conjuagte/mg/min) | 206.65±30.09 | 334.80±21.98* | 221.76±19.76 | 208.87±31.26 | 210.65±40.09 |
| SULT (nmol of conjuagte/mg/min) | 85.04±1.14 | 88.91±3.56 | 96.23±2.67 | 86.56±5.23 | |

Rats were given 100 mg/kg of PHF for 7 days and then PHF was discontinued. Intestinal CYP and conjugation enzymatic activities were determined after 1, 2 and 3 weeks of PHF discontinuation. Each value is expressed as mean±standard error from three independent experiments. Asterisks indicate significant differences (*$P<0.05$, **$P<0.01$, ***$P<0.001$) from control. CYP: Cytochrome P450, PHF: Polyherbal formulation, SULT: Sulfotransferase, GST: Glutathione S-transferase, PNP: p-nitrophenol, UDPGT: UDP-glucuronyltransferase

Similarly, a dose-dependent study with Phase-II conjugation activities revealed that PHF administration increased the hepatic and intestinal UDPGT and GST activity. No alteration was observed in intestinal SULT activity with various PHF doses and time-dependent durations. However, hepatic SULT activities were increased (1.8 fold) at higher doses (200 mg/kg/day). Conjugation enzymes (UDPGT and SULT) play a major role in drug metabolism. Therefore, an increase in activity of these enzymes might result in increased efficacy toward their role such as decreasing the risk of carcinogenicity and toxicity of many drugs, and their metabolites. Nevertheless, increased activity of the Phase-II DMEs (conjugation enzymes) might also affect the efficacy of many substrate drugs, e.g., Acetaminophen (UDPGT) 2-naphthol (SULT) [27].

We performed a recovery experiment to assess whether the altered level of DME's with PHF pretreatment recovered back to normal level or not after the discontinuation of PHF treatment. Although no considerable difference at mRNA and catalytic activity level of all CYP isoforms (hepatic) was observed in between control group and 1 week recovery group, the hepatic CYP1A1-dependent and hepatic CYP2D-dependent enzymatic activities were recovered back to control level after 2 weeks of PHF discontinuation. This late recovery could be attributed to increased amount of CYP1A1 and CYP2D protein that must be degraded to attain normal level. Similarly, to evaluate the effect of discontinuation of PHF after 1 week pre-treatment on Phase-II metabolizing enzymes, we observed a fast recovery of UDPGT, GST and SULT to control level with a recovery

period of 7 days. Such fast recovery from induced to control level ruled out the possibilities of Phase-II enzyme mediated herb-drug interaction following the PHF treatment. However, further investigation in human microsomes, human hepatocytes are required to validate their effect to clinical implementation.

## CONCLUSIONS

Findings from the present study suggest that the PHF formulation has both inductive and inhibitory effect on DMEs when orally administered. PHF are likely to induce DMEs except CYP3A but less likely to produce significant drug interactions. Certain major factors of metabolism such as competition between co-administered drugs, unspecific interactions with proteins and enzyme induction due to chronic intake are not addressed *in vitro* assay. However, data presented in this study clearly demonstrate that PHF might have the potential to inhibit or induce the metabolism of certain co-administered antidiabetic drugs which are metabolized by CYPs and Phase-II conjugated enzymes.

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Original Research

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# *In vitro* antileishmanial and antimalarial activity of selected plants of Nepal

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## ABSTRACT

**Background:** Nepal is very rich in biodiversity, and no extensive effort has yet been carried out to screen plants that are used by traditional healers against parasitic diseases. The aim of this study was to evaluate the *in vitro* antileishmanial and antimalarial activity of crude methanolic or ethanolic extracts of 29 plant species that are currently used by local people of Nepal for treating different ailments. **Methods:** Crude extracts of leaves, twigs, aerial parts, and/or roots of the selected plants were evaluated for *in vitro* inhibitory activity against intracellular amastigotes of *Leishmania infantum* and against erythrocytic stages of *Plasmodium falciparum*. To determine the selectivity index (SI), cytotoxicity was assessed on MRC-5 cells in parallel. **Results:** Three plant species, namely *Phragmites vallatoria* and *Ampelocissus tomentosa,* for which no antiprotozoal activity has previously been reported, and *Terminalia chebula* revealed antiprotozoal activity. The extract of *A. tomentosa* exhibited moderate activity against *L. infantum* with an inhibitory concentration 50% ($IC_{50}$) of 13.2 ± 4.3 μg/ml and SI >3, while *T. chebula* exhibited fairly good antiplasmodial activity with $IC_{50}$ values of 4.5 ± 2.4 μg/ml and SI values >5. **Conclusion:** In countries like Nepal, where the current health system is unable to combat the burden of endemic parasitic diseases, evaluation of local plants as a potential source of the drug can help in expanding the treatment options. The extent of untapped resources available in these countries provides an opportunity for future bioprospecting.

KEY WORDS: Crude plant extracts, *in vitro, Leishmania,* Nepal, *Plasmodium*

## INTRODUCTION

Leishmaniasis and malaria represent major public health problems with significant morbidity and mortality in Asia, Africa, and Latin America [1,2]. Lack of vaccines, emergence of drug resistance, and expensive chemotherapeutics are some of the major challenges for the control of these vector-borne diseases, in addition to disadvantages including hospitalization for (parenteral) treatment, occurrence of adverse effects, long-term therapy leading to poor compliance and poor availability of drugs, especially to economically weak populations residing in rural areas [3-6].

Given the limited number of novel drugs in the pipeline and the expanding resistance against current drugs, it remains imperative to explore alternative ways to find new drugs. Plants contain a broad diversity of secondary metabolites such as alkaloids, flavonoids, and phenolic derivatives that may have therapeutic value, and hence may represent an attractive source for novel drugs [7]. However, screening of each and every individual plant parts against wide range of pathogens is virtually impossible and plant selection based on ethnobotany and traditional practices, such as Ayurveda [8], Unani, Siddha, traditional Chinese medicine, and Japanese Kampo medicine increases the probability of finding "hit" molecules that can be subsequently developed toward "lead" development [9,10].

In Nepal, there is a huge variation in the number of medicinal and aromatic plants (MAP) [11,12]. For example, compilation of

the MAP database has listed 1624 medicinal plants in 2000 [13], rising to 1950 species in 2008 [14] clearly indicating that further exploration of the phytochemical and pharmacological properties of medicinal plants in Nepal should be continued. Up till now, very few indigenous Nepalese plants have been explored for their therapeutic potential against leishmaniasis and malaria. Starting from ethnobotanical literature and traditional use, the present study assessed the *in vitro* inhibitory activity potential of crude extracts of 29 selected Nepalese plants [Table 1], hence contributing to the medicinal knowledge of the local plant biodiversity.

## MATERIALS AND METHODS

### Plant Material

Leaves, twigs, aerial parts, and roots [Table 1] of selected plants were collected from different regions in Nepal [Figure 1] from December 2013 to April 2014. All the collected plant materials were identified in the Department of Plant Resources, Nepal, and Voucher specimens are deposited in Pharmacognosy Unit of Department of Plant Resources, Thapathali, Kathmandu, Nepal (http://www.dpr.gov.np).

### Extraction

The plant materials were washed thoroughly with water and shade dried at room temperature. Dried samples were crushed into powder by electric blending and subjected to Soxhlet extraction using polar solvents (ethanol and methanol). The extracts were evaporated on a rotary evaporator under vacuum till a solid mass was obtained. The extracts were kept at 4°C until analysis. All the extracts were kept in sealed vials, labeled properly, and transported to the Laboratory of Microbiology Parasitology and Hygiene, University of Antwerp, for integrated *in vitro* screening.

### Parasites and Cell Culture

Standard techniques **were used** as previously described [9]. Briefly, *ex vivo* amastigotes of *Leishmania infant*um (MHOM/MA(BE)/67) were used for the *in vitro* antileishmanial assay. The strain was routinely passed in Syrian Golden hamsters every 6-10 weeks. The chloroquine (CQ)-resistant Pl*asmodium falciparum* (K1 strain) was used for *in vitro* antiplasmodial activity testing. The human lung fibroblast cell line MRC-5 was cultured in minimum essential medium supplemented with 20 mM L-glutamine, 16.5 mM $NaHCO_3$, and 5% fetal calf serum.

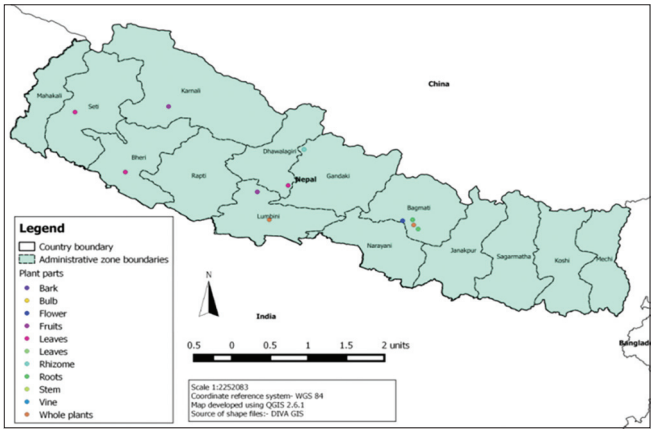


**Figure 1:** Sampling site in Nepal for the collection of plant species

**Table 1:** List of the selected plants for this study, their phytoconstituents, and traditional uses

| Plant species | Family | Voucher specimen | Part | Constituents | Reported traditional use | Reference |
|---|---|---|---|---|---|---|
| *Ageratum conyzoides* (L.) | Asteraceae | NPRL_01 | WP | Chromenes, benzofurans, flavonoids, farnesene derivatives daucanolides triterpenoids, sterols | In Asia, South America, and Africa, aqueous extract is used as a bactericide. It is also used to treat fever, rheumatism, and headache | [15-17] |
| *Swertia chirayita* (Roxb.) | Gentianaceae | NPRL_02 | S, L | Xanthones, amarogentin | In Indian tradition - used in treating bronchial asthma, liver disorders, chronic fever, anemia, stomachache and diarrhea | [18-20] |
| *Centella asiatica* (L.) urb. | Apiaceae | NPRL_03 | L | Centellin, centellicin, asiaticin | Used in the treatment of leprosy, skin disease and appetizer | [11] |
| *Drymaria diandra* Blume | Caryophyllaceae | NPRL_04 | WP | Drymariatin A, diandrines A-D, flavonoids, alkaloiods | Root juice is inhaled to treat sinusitis | [21] |
| *Syzygium aromaticum* (L.) | Myrtaceae | NPRL_05 | F, B | Eugenol, biflorin, kaempferol, rhamnocitrin, myricetin, gallic acid | Toothache and headache reliever, remedy against diarrhea, stomachache and bowel ailments, natural antihelminthic, antimalarial | [22] |
| *Zanthoxylum armatum* DC. | Rutaceae | NPRL_06 | F, L | Alkaloids, flavonoids, coumarins | Prevent tooth decay, fruit and seed used in asthma and blood purifier. used for the treatment of malaria, GI disorders, gonorrhea | [23] |
| *Cinnamomum zeylanicum* Blume | Lauraceae | NPRL_07 | B, L | Cinnamaldehyde, cinnamic acid, and cinnamate | Besides use as spice and flavoring agent, in Ayurvedic medicine - used for remedy for respiratory, digestive, and gynecological ailments | [24] |

*(Contd...)*

Table 1: *(Continued)*

| Plant species | Family | Voucher specimen | Part | Constituents | Reported traditional use | Reference |
|---|---|---|---|---|---|---|
| *Cuminum cyminum* L. | Apiaceae | NPRL_08 | S, F | Cuminaldehyde, Safranal, Sesquiterpenes | Used in treatment of fever, loss of appetite, diarrhea, vomiting, abdominal distension, edema | [25] |
| *Clerodendrum serratum* Moon | Lamiaceae | NPRL_09 | F, R | Triterpene, macrocyclic lactone, saponin, serratin, lupeol | Used to treat pain, inflammation, malaria fever in India | [26] |
| *Ehretia acuminata* R.Br. | Boranginaceae | NPRL_10 | L | Methyl and ethyl esters | The leaves and branches are used in Chinese medicine | [27] |
| *Oroxylum indicum* (L.), benth. Ex. Kurtz | Bigoniaceae | NPRL_11 | S, B, L | Chrysin, baicalein, baicalein-7-O-glucoside, baicalein-7-O-diglucoside | In treating Jaundice, diarrhea, malaria, arthritis, diabetes | [28] |
| *Phragmites vallatoria* (L.) Veldkamp | Poaceae | NPRL_12 | WP | NA | Used in wound healing, arthritis, antiemetics, febrifuges, rheumatism, and diabetes | [29] |
| *Pedilanthus tithymaloides* (L.) Poit | Euphorbiaceae | NPRL_13 | L | Anticancer-diterpene pedilstatin, octacosanol, cycloartenone and β-sitosterol | Traditionally used to heal wounds, burn, mouth ulcers, and venereal disease; found to be anticatarrhal, anti-inflammatory, antibiotic, antiseptic, antihemorrhagic, antiviral, antitumor | [30] |
| *Kalanchoe pinnata* Pers | Crassulaceae | NPRL_14 | L, S, R | Bufadienolides, α- and β-amyrins | Antibacterial (respiratory tract infection), antiparasitic, antidepressant, anticancer, anti-insecticidal, antiallergic, anti-inflammatory, antidiabetes | [31] |
| *Cirsium wallichii* DC. | Asteraceae | NPRL_15 | R | Thymol, β-linalool, eugenol | Used to treat fever, gastric problem, relieve burning sensation while urinating | [32] |
| *Arisaema griffithii* Schott. | Araceae | NPRL_16 | L, R | NA | Used to treat malaria, eat as vegetable | [11] |
| *Ampelocissus tomentosa* (Roth) Planch. | Vitaceae | NPRL_17 | F | NA | Used in menstrual bleeding, treating dysentery, fever, fistula, tuberculosis, and Insect bites | [33] |
| *Arisaema griffithii* Schott. | Araceae | NPRL_16 | L, R | NA | Used to treat malaria, eat as vegetable | [11] |
| *Dichrocephala integrifolia* (L.f.) Kuntze. | Asteraceae | NPRL_18 | L, F | Dichrocepholides A, B, C, parthenin | Used in treatment of malaria and hepatitis and wounds | [34] |
| *Boenninghausenia albiflora* (Hook.) Meisn. | Rutaceae | NPRL_19 | R | Acridone, komalin, albiflorin-2 and albiflorin-3 | Crushed plant placed inside nostrils in the treatment of malaria | [11,35] |
| *Cynoglossum zeylanicum* Thunb. ex. Lehm. | Boraginaceae | NPRL_20 | WP | n-Hexadecanoic acid, stigmasterol, oleic acid | Decoction prepared from the whole plant is used to arrest vomiting | [27] |
| *Sapindus mukorossi* Gaertn. | Sapindaceae | NPRL_21 | F | Triterpenoidal saponins | Used as expectorant, relieve joint pain, removing dandruff and the roots for treating gout and rheumatism | [36] |
| *Anacyclus pyrethrum* (L.) DC. | Asteraceae | NPRL_22 | R | Phenolics, chlorogenic acid | In Ayurveda "*rasayana*"-Plant with immune modulators and also used in treatment of epilepsy and seizure | [37] |
| *Adhatoda vasica* Nees | Acanthaceae | NPRL_23 | S | Alkaloids, tannins, saponins | Used to treat bronchitis, relieves cough and breathlessness, stop bleedings | [38] |
| *Boerhavia diffusa* L. | Nyctaginaceae | NPRL_25 | R | Boeravinones, Rotenoids | Used in jaundice, kidney problems, skin troubles, eye diseases, wounds, and inflammation | [39] |
| *Terminalia chebula* Retz. | Combretaceae | NPRL_26 | Fr | Chebulanin, punicalagin, terchebin, gallic acid, flavonoids, ursolic acid | Used in treatment of asthma, sore throat, vomiting, hiccough, diarrhea, dysentery, bleeding piles, ulcers, gout | [40] |
| *Rhododendron arboreum* Sm. | Ericaceae | NPRL_27 | F, L | Quercitrin, and coumaric acid | Used in vomiting, cough menstrual disorder, headache, throat ache, rheumatic pain | [41] |
| *Paris pollyphylla* Sm. | Trilliaceae | NPRL_28 | Rh | Diosgenin and pennogenin saponin | Gastric and menstrual problem, to remove worms | [42] |
| *Aleuritopteris anceps* (Blanf.) panigrahi | Pteridaceae | NPRL_29 | L, S | Chalcones, flavonols, flavonol-esters, kaempferol and quercetin | Used in preventing infection and inflammation | [43] |
| *Parthenium hysterophorus* L. | Asteraceae | NPRL_30 | WP | Sesquiterpene lactones, caffeic acid, chlorogenic acid, ferulic acid, sitosterol | Used to treat fever, diarrhea, neurologic disorders, UTI dysentery, and malaria | [44] |

L: Leaves, S: Stem, R: Root, B: Bark, F: Flower, WP: Whole plant, Rh: Rhizome, Fr: Fruit, NA: Not available, GI: Gastrointestinal

### Biological *In Vitro* Assays

The integrated panel of microbial screens and standard screening methodologies were adopted as previously described [9]. Plant extracts were tested at dilutions ranging from 128 to 0.25 $\mu$g/mL using automated robotics with a 10-fold serial dilution strategy. Initially, 2-fold serial dilutions were made in 100% dimethyl sulfoxide (DMSO) to ascertain complete solubility during the dilution process. An immediate dilution step was performed in Milli-Q water before transferring the respective compound dilutions to the test plates (1/20 dilution: 10 $\mu$L compound solution +190 $\mu$L cell medium and test system) so that the final in-test concentration of DMSO did not exceed 1%.

### Antileishmanial Activity

Mouse macrophages were stimulated by intraperitoneal injection of starch. 2 days after injection, macrophages were collected and seeded in each well ($3 \times 10^4$) of a 96-well plate. The plates were incubated at 37°C and 5% $CO_2$. After 2 days of outgrowth, *ex vivo* amastigotes were used to infect primary peritoneal mouse macrophages at a 10:1 infection ratio. The plates were further incubated for 2 h before the compound dilutions were added. After 5 days of incubation, cells were dried, fixed with methanol, and stained with 20% Giemsa to assess total intracellular amastigote burdens through microscopic reading. The results are expressed as the percentage reduction of amastigote burden compared to untreated control cultures and inhibitory concentration 50% ($IC_{50}$)-values were calculated.

### Antiplasmodial Assay

CQ-resistant *P. falciparum* 2/K 1-strain was cultured in human erythrocytes $O^+$ at 37°C under microaerophilic atmosphere (3% $O_2$, 4% $CO_2$, and 93% $N_2$) in RPMI-1640 supplemented with 10% human serum. 200 $\mu$L of infected red blood cells (1% parasitemia and 2% hematocrit) was added in each well of a 96 well plate containing prediluted extract. The test plates were kept in the modular incubator chamber for 72 h at 37°C, and subsequently, put at −20°C to lyse the red cells upon thawing. Next, 100 $\mu$L of Malstat™ reagent was put in new microtiter plate to which 20 $\mu$L of hemolyzed parasite suspension was added. After 15 min incubation at room temperature, 20 $\mu$l of nitro blue tetrazolium/polyethersulfone solution was added. The plate was incubated in the dark for another 2 h at room temperature and spectrophotometrically read at 655 nm. The $IC_{50}$ was calculated from the drug concentration - response curves. According to the WHO guidelines ([45]), antiplasmodial activity is very good with $IC_{50}$ <1 $\mu$g/ml; good to moderate if $IC_{50}$ of 1-10 $\mu$g/ml; weak if 15-50 $\mu$g/ml, and inactive if $IC_{50}$ >50 $\mu$g/ml, always taking into account a selectivity index (SI) higher than 10.

## RESULTS

### Antileishmanial Activity

Only one plant extract (*Ampelocissus tomentosa)* exhibited moderate activity against *L. infantum* with an $IC_{50}$ value of 13.2 ± 4.3 $\mu$g/ml and an SI value >3. *Paris polyphylla* also showed inhibitory activity but was also cytotoxic [Table 2].

### Antiplasmodial Activity

Three plant species, *Phragmites vallatoria*, *A. tomentosa*, and *Terminalia chebula* showed schizonticidal activity. Among them, *T. chebula* exhibited the best activity with $IC_{50}$ values of 4.5 ± 2.4 $\mu$g/ml and SI values >5.

### Cytotoxicity

*Kalanchoe pinnata*, *P. polyphylla*, and *Pedilanthus tithymaloides* were toxic to the MRC-5 cell line. *K. pinnata* was most toxic with cytotoxic concentration 50% value of 4.7 ± 1.8 $\mu$g/ml.

## DISCUSSION AND CONCLUSION

Leishmaniasis and malaria continue to be major public health problems, and the available drugs are generally expensive and not devoid of toxic side effects. Associated with poor compliance, the threat of drug resistance is also an emerging issue. Despite different strategies such as drug repurposing, identifying new therapeutic targets by chemoinformatics or screening diverse libraries of natural products, no new drugs have reached the market during the last decade. The present study was carried out to explore the potential of Nepalese medicinal plants that are used as part of traditional medicine. Nepal is very rich in biodiversity, which has not yet been explored satisfactorily due to the geopolitical situation, the lack of sophisticated labs, and the availability of trained manpower in industry and academics. The selected medicinal plants were screened against protozoal diseases using a "whole-cell based" approach, which can be considered more valid than enzyme-based subcellular approaches [9].

In the present study, *A. tomentosa* showed selective antileishmanial ($IC_{50}$ 13.2 ± 4.3 $\mu$g/ml) and antimalarial (11.7 ± 3.5 $\mu$g/ml) activity. To our knowledge, the antiprotozoal activity of this plant has never been investigated, and no active constituents have been documented in the literature. Further studies on bioassay-guided fractionation to identify the putative active constituents and to better understand the therapeutic targets will be necessary, including a screening of other species of *Ampelocissus* genus.

Likewise, good antimalarial activity was found for *T. chebula* and *P. vallatoria* with an $IC_{50}$ of 4.5 ± 2.4 and 12.0 ± 7.5, respectively, and SI of >5. This is the first observation that *P. vallatoria* showed potential activity against *Plasmodium*. The antiplasmodial activity of *T. chebula* has already been reported [22] with an $IC_{50}$ = 4.76 $\mu$g/mL against the CQ-sensitive (3D7) strain of *P. falciparum*, hence supporting its use in traditional medicine.

*P. tithymaloides* was also found to be active against *Leishmania* but was not totally devoid of cytotoxicity. In traditional medicine, *P. tithymaloides* is been used in treating multiple diseases (from antimicrobial to anticancer) related to the

Table 2: Antiprotozoal activity of extract of selected plants of Nepal and their cytotoxicity against MRC-5 cell lines

| Plant | Family | Solvent | Part used | *L. infantum* | SI | *P. falciparum* | | MRC-5 |
|---|---|---|---|---|---|---|---|---|
| | | | | $IC_{50}$ (μg/ml) | | $IC_{50}$ (μg/ml) | SI | $CC_{50}$ |
| *Ageratum conyzoides* | Asteraceae | Ethanol | WP | 96.5 | 0.6 | 72.4±28.3 | 0.8 | 62.7±3.3 |
| *Swertia chirayita* | Gentianaceae | Ethanol | L | >128 | nd | >128 | nd | >128 |
| *Centella asiatica* | Apiaceae | Ethanol | L | >128 | nd | >128 | nd | >128 |
| *Drymaria diandra* | Caryophyllaceae | Ethanol | WP | >128 | nd | >128 | nd | >128 |
| *Syzygium aromaticum* | Myrtaceae | Ethanol | L | 61.5±5.9 | 1.0 | 17.8±2.9 | 3.60 | 64.4±7.4 |
| *Zanthoxylum armatum* | Rutaceae | Ethanol | L | 18.8±3.7 | 3.1 | 23.5±1.5 | 2.7 | 63.8±2.5 |
| *Cinnamomum zeylanicum* | Lauraceae | Ethanol | L | 48.1 | 0.9 | 42.8 | 1.1 | 47.1 |
| *Cuminum cyminum* | Apiaceae | Ethanol | Fr | 64.4 | 1.3 | 18.8 | 4.3 | 81.7 |
| *Clerodendrum serratum* | Verbenaceae | Methanol | R | 65.4±11.1 | 0.4 | 65.4±11.1 | 0.3 | 25.3±6.8 |
| *Ehretia acuminata* | Boraginaceae | Methanol | L | 54.5 | 0.9 | 12.1 | 4.2 | 50.5 |
| *Oroxylum indicum* | Bignoniaceae | Methanol | B | 52.7±17.1 | nd | >128 | nd | >128 |
| *Phragmites vallatoria* | Poaceae | Methanol | WP | >128 | nd | 12.0±7.5 | >5 | 63.9±1.4 |
| *Pedilanthus tithymaloides* | Euphorbiaceae | Methanol | S | 11.8±2.4 | 1.1 | 30.6±1.9 | 0.4 | 12.8±2.3 |
| *Kalanchoe pinnata* | Crassulaceae | Methanol | L | 44.6±21.6 | 0.1 | >128 | nd | 4.7±1.8 |
| *Cirsium wallichii* | Asteraceae | Methanol | R | >128 | nd | 101.6 | nd | >128 |
| *Arisaema griffithii* | Araceae | Methanol | B | >128 | nd | >128 | nd | >128 |
| *Ampelocissus tomentosa* | Vitaceae | Methanol | V | 13.2±4.3 | 3.5 | 11.7±3.5 | 4.1 | 47.1±6.1 |
| *Dichrocephala integrifolia* | Asteraceae | Methanol | L | 64.9 | 1.0 | 52.2±13.4 | 1.2 | 65.5±0.2 |
| *Boenninghausenia albiflora* | Rutaceae | Methanol | L | 55.8 | 0.3 | 32.9±15.5 | 0.5 | 16.8±7.1 |
| *Cynoglossum zeylanicum* | Boraginaceae | Methanol | L | 64.9 | 0.3 | 79.6±4.5 | 0.6 | 49.1±16.5 |
| *Sapindus mukorossi* | Sapindaceae | Methanol | L | 63.4 | 0.4 | 47.5±0.6 | 0.4 | 23.7±3.2 |
| *Anacyclus pyrethrum* | Asteraceae | Methanol | Rh | 86.1 | nd | 52.8 | nd | >128 |
| *Adhatoda vasica* | Acanthaceae | Methanol | L | 64.9 | nd | 40.5 | nd | >128 |
| *Boerhavia diffusa* | Nyctaginaceae | Methanol | WP | 52.8 | 1.0 | 37.6±12.8 | 1.4 | 54.6±6.1 |
| *Terminalia chebula* | Combretacea | Methanol | Fr | 64.9 | 0.5 | 4.5±2.4 | >5 | 35.6±1.9 |
| *Rhododendron arboreum* | Ericaceae | Ethanol | F | 64 | nd | 42.9 | nd | >128 |
| *Paris polyphylla* | Trilliaceae | Ethanol | Rh | 8.8±6.7 | 1.4 | >128 | nd | 13.3±0.8 |
| *Aleuritopteris anceps* | Pteridaceae | Methanol | L | 101.6 | 0.8 | 48.7 | 1.7 | 85.5 |
| *Parthenium hysterophorus* | Asteraceae | Methanol | WP | 64.6 | 1.3 | 47.1 | 1.8 | 50.8 |

L: Leaves, S: Stem, R: Root, B: Bark, F: Flower, Fr: Fruit, WP: Whole plant, Rh: Rhizome, nd: Not determined, *L. infantum: Leishmania infantum, P. falciparum: Plasmodium falciparum,* SI: Selectivity index, $IC_{50}$: Inhibitory concentration 50%, $CC_{50}$: Cytotoxic concentration 50%

diverse phytoconstituents [Table 1]. The antiprotozoal activity of this plant might be due to the presence of a diterpene, as species belonging to the family Euphorbiaceae are rich in diterpenoids and triterpenoids [46]. In previous studies, various poly-O-acylated jatrophane diterpenoids have shown *in vitro* antiplasmodial activity with $IC_{50}$ values of 3.4-4.4 μg/ml, which has been confirmed *in vivo*, with 76% suppression of parasitemia in *P. berghei* infected mice [47,48]. Likewise, diterpenes such as jatrogrossidione and jatrophone have been found to have toxic effects against promastigotes of *L. braziliensis*, *L. amazonensis*, and *L. chagasi* with $IC_{50}$ in the range of 0.75-5 μg/ml [49]. The moderate cytotoxic nature of *P. tithymaloides* might be due to the presence of pedilstatin or eurphorbol, which have already been established as irritants and carcinogens [50].

Non-selective antileishmanial activity was shown for *P. polyphylla* and *K. pinnata*. *P. polyphylla* is known as "*satuwa*" and is traditionally used as anthelmintic and for reducing fever in the Himalayan region of Nepal. Our findings on cell toxicity of some plant extracts ($IC_{50}$ 15 μg/ml) warrants for some vigilance as sometimes misleading information like "natural products are always safe" could eventually lead to deleterious health if high doses of these plants are consumed for a long time. Quite a lot of published literature indeed lacks parallel cytotoxicity evaluation. For example, *P. polyphylla* diosgenin-type saponins revealed antileishmanial activity ($IC_{50}$ 1.6 μg/ml) but without parallel cytotoxicity evaluation [42]. In our study, *K. pinnata* was highly cytotoxic (4.7 ± 1.8 μg/ml) while published data support that *K. pinnata* may possess immunosuppressive effects and inhibit disease progression in *L. amazonensis*-infected individuals [31,51,52]. The same research group more recently reported that this plant possessed immunomodulatory activity and highlighted that oral dose of *K. pinnata* extract (400 mg/kg) is comparable to

Pentostam® (72 mg/kg) in reducing the hepatic and splenic parasitic burden [53].

Further research on these plants should now focus on the structural elucidation of the putative "active constituents," *in vitro* evaluation using preset $IC_{50}$ and SI cut-offs and *in vivo* evaluation in murine pharmacology models for pharmacokinetic and dynamic profiling.

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# Anthelmintic activity of a standardized extract from the rhizomes of *Acorus calamus* Linn. (Acoraceae) against experimentally induced cestodiasis in rats

**Purobi Nath, Arun K Yadav**

## ABSTRACT

**Background:** The rhizomes of a herb *Acorus calamus* Linn. (Acoraceae) have been widely used as a traditional medicine to cure intestinal-helminthic infections in India and South Africa. **Aim:** This study was undertaken to investigate the *in vivo* anthelmintic activity of a standardized methanolic extract obtained from the rhizomes *A. calamus* in a rodent model. **Materials and Methods:** A methanolic extract obtained from rhizomes of *A. calamus* was characterized for active principle using nuclear magnetic resonance $^{1}$H NMR, $^{13}$C NMR, mass and infrared spectroscopy. The amount of active principle in rhizome isolated active fraction of plant was assayed using high-performance liquid chromatography (HPLC). Later, the standardized rhizome extract of plant and its active principle were tested for *in vivo* anthelmintic efficacy against experimentally induced *Hymenolepis diminuta*, a zoonotic cestode, infections in rats. **Results:** The study revealed that β-asarone is the active principle of plant. The HPLC analysis of local variety of *A. calamus* revealed that active fraction contains 83.54% (w/w) of β-asarone. The *in vivo* study revealed that treatment of *H. diminuta* infected rats by a single 800 mg/kg dose of rhizome extract for 5 days results into 62.30% reduction in eggs per gram of feces counts and 83.25% reduction in worm counts of animals. These findings compared well with the efficacy of a reference drug, praziquantel. The active principle β-asarone showed slightly better anthelmintic effects than crude extract. In acute toxicity assay, a single oral 2000 mg/kg dose of extract did not reveal any signs of toxicity or mortality in mice, and the LD50 of the extract was noted to be >2000 mg/kg. **Conclusion:** Taken together, the results of this study indicate that rhizomes of *A. calamus* bear significant dose-dependent effects against intestinal helminths. Further, the Indian variety of *A. calamus* contains high β-asarone content. Therefore, there exists a great potential to develop some suitable anthelmintic herbal products from this plant.

KEY WORDS: Anthelmintic, *Hymenolepis diminuta*, intestinal helminthe, soil-transmitted helminths, traditional medicine

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## INTRODUCTION

Intestinal helminths affect more than 2 billion people worldwide and cause a range of adverse health problems, including anemia, diarrhea, and abdominal pain, impaired cognitive and physical development, particularly in developing countries [1]. Control is achieved by periodic deworming, and at present, two anthelmintics, i.e. albendazole and mebendazole are used in deworming programs in endemic regions. However, it has been recently reported that the global coverage of periodic deworming is still not sufficiently meeting target levels in all endemic regions [2]. As per the WHO latest data, in 2013, only 40% of children requiring treatment for intestinal helminths had access to anthelminthic medicines, and to the remaining 60% of children, these medicines were not accessible [3]. In many developing countries, people are still dependent on various herbal treatments to cure worm infections [4]. For example, in Asia, Africa, and some parts of Latin America, the herbal medicines, traditional treatments, and traditional practitioners constitute the main source of health care to treat various common ailments including intestinal worm infections [4]. Thus, these herbal medicines hold a great scope for not only new drug discoveries against parasitic diseases but also for further exploration for scientific evidence regarding the treatment and control of intestinal helminthiasis [5].

*Acorus calamus* Linn. (Araceae), commonly known as "sweet flag," is a perennial herb [Figure 1]. It is mostly found in swampy or marshy habitats in northern temperate and subtropical regions of Asia, North America, and Europe [6]. The rhizomes of this


**Figure 1:** *Acorus calamus*. (a) whole plant, (b) local medicine men collecting the tubers of plant, (c) partly-processed rhizomes

plant possess numerous medicinal properties and have been very widely used against several diseases and ailments, particularly in Indian, Chinese, Korean, and Thai medicines [6,7]. In the Indian ayurvedic medicine, the rhizomes of A. *calamus* are used to treat mental ailments such as epilepsy, schizophrenia, and memory disorders, as well as diarrhea, bronchial catarrh, intermittent fevers, cough, and asthma [8]. In Indian folk medicines, A. *calamus* rhizomes are considered useful against diseases of nervous system, throat, and as an antitumor, antipyretic, and antitussive agent [6]. In Thai medicine, A. *calamus* rhizomes have been used for blood purification and as an antipyretic [9]. In addition, experimental studies have also shown that A. *calamus* rhizomes possess anticonvulsant [10], antidiarrheal [11], antimicrobial [12], antibacterial [13], and anti-inflammatory activities [6]. Similarly, McGaw *et al*. [13] have also reported that A. *calamus* rhizomes are used in traditional medicine in KwaZulu-Natal, South Africa as an anthelmintic. The ß-asarone isolated from the rhizome of A. *calamus* has shown significant *in vitro* anthelmintic effects against *Caenorhabditis elegans* [14]. In another *in vitro* study, the active principle of this plant also showed a fast acting paralytic effect that was followed by mortality in the larvae of dog roundworm, *Toxocara canis* [15]. It would thus appear from aforesaid account that although few studies have established the *in vitro* anthelmintic effects of this plant, except one, no report is available regarding the *in vivo* anthelmintic effects of this plant against human intestinal helminths. The only *in vivo* study on anthelmintic effects of this plant was undertaken by Mägi *et al*. in Estonia [16]. In this study, the dried rhizome powder of A. *calamus* was tested for its nematicidal effects against *Oesophagostomum* spp. infections in pigs, and was found to significantly lower down the worm burden and eggs per gram (EPG) of feces counts of animals [16].

One of the special features of A. *calamus* is that it exhibits a polyploidy nature and as many as four karyotypes of this plant (i.e. diploid, triploid, tetraploid, and hexaploid) are known which have been reported to follow geographical patterns of distribution in various regions of the world [17]. Interestingly, he karyotypes of A. *calamus* also vary greatly in their qualitative and quantitative composition of essential oil which contains β-asarone, its anthelmintic active principle. The content of β-asarone in calamus oil has been reported to be high, i.e., 70-96% in tetraploids, low around 5-19% in triploids plants, and zero in diploid plants [18]. Kumar *et al*. tested the essential oils from A. *calamus* collected from different locations in the Himalayan region of India and observed that all the oils differed in their qualitative and quantitative makeup, although β-asarone was the major constituent of all of them which revealed a potent *in vitro* activity on poultry roundworm, *Ascaridia galli* [19]. In India, A. *calamus* grows mostly in wild in Jammu and Kashmir, Himachal Pradesh, Manipur, Tripura states, etc. In recent years, a significant body of literature has emerged that presents contradictory findings about the ploidy status and contents of β-asarone in Indian A. *calamus* populations. For example, Ogra *et al*. reported that most of the accessions of A. *calamus* from India are triploids (except only few which are diploids) with β-asarone content in their oil varies from 82% to 89.4% [18]. Conversely, Ahlawat *et al*. opined that most of the A. *calamus* accessions in India are predominantly tetraploids with the β-asarone content in their oil ranging between 73% and 88%, and triploid varieties are rather rare with β-asarone content in their oils ranging between 6.92% and 8.0% [17]. Thus, it would appear that β-asarone content in A. *calamus* oil is of paramount importance as it can affect the perceived therapeutic efficacy of this plant from one geographical region to another. As is evident none of the previous studies have standardized the amount of β-asarone in the rhizome oil of A. *calamus* with regard to its *in vivo* anthelmintic effects. The present study was, therefore, undertaken to evaluate the *in vivo* anthelmintic efficacy of a standardized methanol extract from the rhizomes of A. *calamus*, using experimentally induced *Hymenolepis diminuta* (a zoonotic tapeworm species) infections in Wistar rats.

## MATERIALS AND METHODS

### Plant Material

The rhizomes of A. *calamus* [Figure 1] were collected from North Tripura district of Tripura (24° 36' N latitude and 92° 19' E longitude) in October 2010. The plant specimen was identified by a Curator in the Department of Botany, North-Eastern Hill University (NEHU), Shillong, and a voucher specimen (No. AKY-11883) has been retained in the Department of Zoology, NEHU. The rhizomes were dried under shade and ground into fine powder form. Plant material was extracted with different solvents, n-hexane, n-butanol, chloroform, ethyl acetate, acetone, methanol, and water using Soxhlet extractor at 40°C for 4-5 h. The process was repeated thrice with fresh solvent. The ratio of sample to solvent was 1:10 (m/v). Each extract was subsequently filtered, and the filtrates were concentrated under reduced pressure in a vacuum rotary evaporator. The crude extracts were subjected to thin layer chromatography (TLC) plate, and the separation pattern of the extract was monitored in chloroform and methanol (9:1), which showed distinct separated spots. The methanol extract gave maximum number of spots in TLC, and therefore, it was selected for *in vivo* testing. The final yield of methanol extract was 15% (w/w).

### Characterization of Active Component

Sample preparation for column chromatography was done by adsorption of extract on activated (105°C, 30 min) silica gel (100-200 mesh) with ratio 1:10, respectively. Extract was kept for drying in a rotary evaporator, and finally, lyophilized until free flowing material was formed. The dried free flowing prepared sample was subjected to column chromatography using a 20 cm × 2.5 cm glass column filled with silica gel (mesh size: 100-200, SRL) in n-hexane. Prepared sample of methanol extract was added to the free volume at the head of the column. After settling down of the material, fractionation was conducted over silica gel with n-hexane/chloroform (98:2-90:10) solvent system. Fractions were collected, and the solvent was removed to reduce volume of fraction by evaporation in vacuum at 40°C. Dried fractions were suspended in chloroform:methanol (3:1) and diluted when necessary. In each solvent preparation, five fractions were collected and monitored by TLC method (n-hexane:chloroform [9:1]). Separation pattern of fractions on TLC plate was observed by iodine vapor. After concentrating and left standing for overnight, purity of the entire fraction was tested on TLC plate. Fraction no. 3-8 showed single spot on TLC plate. A pale yellow liquid of $R_f$ value 0.42 was obtained in n-hexane:chloroform (9:1) on precoated TLC plate (Aluchrosep Silica Gel 60/UV254, SD fine, size 5 cm × 20 cm and 0.2 mm thickness). To confirm the purity of isolated compound, parallel spot was run on TLC plate with standard β-asarone in n-hexane/chloroform (9:1). The purity of this compound, yellow oil, was confirmed by TLC using various solvent systems and $^1$H NMR.

The chemical structure of the calamus oil isolated fraction was predicted through detailed spectroscopic procedures- $^1$H NMR, $^{13}$C NMR, mass and infrared (IR) spectroscopy. IR spectrum was recorded in chloroform on a Fourier transform IR spectrophotometer (Nicolet Impact 1-410) calibrated against the polystyrene absorption at 1601 $cm^{-1}$. Mass spectrum was recorded by liquid chromatography (LC) - mass spectrometry using waters ZQ-4000 mass spectrometer. $^1$H NMR (300 MHz) and $^{13}$C NMR (75 MHz) spectra were recorded using $CDCl_3$ as solvent in a Bruker Avance II-400 NMR machine, considering tetramethylsilane (TMS) as an initial standard and chemical shift values were in δ ppm values. Spectra were referenced to TMS ($^1$H) or solvent ($^{13}$C) signals. Fraction containing active compound was further applied to reverse-phase high-performance LC (RP-HPLC) system (SHIMADZU, LCIOAT, Kyoto, Japan) equipped with Shimadzu SPD-10A ultraviolet-visible detector. RP-HPLC analysis was carried out in isocratic conditions using $C_{18}$ reverse phase column with a particle size of 5 μm, 250 mm × 4.6 mm. Samples were filtered through 0.45 μm ultra-membrane filters (Millipore, Germany). Running conditions included: Injection volume, 20 μl; mobile phase, methanol:0.5% acetic acid in water (75:25 v/v); flow rate, 1 ml/min; and detection wavelength at 210 nm. The calibration curves of β-asarone were linear from 0.01~100 μg/ml ($r$ = 0.994, $n$ = 6). The percentage of β-asarone in isolated fraction was determined by calculating the peak area of HPLC chromatograms.

### Experimental Animals

Wistar rats of either sex, weighing 180-200 g, were used. Animals were acclimatized for 15 days in the laboratory and had *ad libitum* access to standard rodent food and water. *H. diminuta* infection in rats was maintained by inoculating the cysticercoids obtained from experimentally infected flour beetle *Tribolium confusum*, the intermediate host [20]. All the experiments on rats were conducted after due approval by the Institutional Ethics Committee (animal models), NEHU, Shillong.

### Acute Toxicity Study

The rhizome extract was subjected to acute toxicity study according to the OECD guidelines [21]. The extract was tested at a limit dose of 2000 mg/kg by oral route using five female Swiss albino mice. Each animal was dosed individually with 2000 mg/kg dose of extract and observed for any adverse toxicity or mortality for 2 weeks. The LD50 was predicted to be above 2000 mg/kg if three or more animal survived in this experiment.

### Evaluation of *In Vivo* Anthelmintic Activity

The anthelmintic effects of extract were tested on adult *H. diminuta* infections in rats. Animals were divided into five groups, each comprising of 6 rats. Each animal was then orally infected with four cysticercoids and maintained in a separate cage. Group I animals served as the untreated controls and received water with a few drops of 1% dimethyl sulfoxide (vehicle), daily on days 21-25 post-inoculation (p.i.) of cysticercoids. Groups II, III, and IV of animals were treated with 200, 400, and 800 mg/kg, respectively, doses of plant extract on days 21-25 p.i. of cysticercoids. Group V of animals served as the positive controls and was given 5 mg/kg of praziquantel (distocide®), the reference drug for the same duration. The efficacy of extract was determined by percentage reduction in EPG counts and percentage reduction in worm counts during pre-and post-treatment periods [22]. Herein, the EPG counts of animals were estimated for 3 days (days 18-20 p.i.) before treatment and 3 days (days 26-28) after treatment. Finally, all the animals were sacrificed on day 39 p.i., and the worms in their intestine were recovered to work out the percentage reduction in worm counts.

### Statistical Analysis

Data from experiments are expressed as mean ± standard error of mean. The level of significance between treatment and control was analyzed by Student's *t*-test. The $P < 0.05$ was considered to be statistically significant.

## RESULTS

The TLC profile of methanolic rhizome extract of *A. calamus* showed six spots [Figure 2]. The $R_f$ value of isolated purified compound was recorded to be 0.42. The chemical structure of the calamus oil isolated fraction was predicted through $^1$H NMR, $^{13}$C NMR, mass and IR spectroscopy and found related

to β-asarone. The HPLC chromatogram of the isolated fraction, showing β-asarone peak, is depicted in Figure 3. The structure of isolated compound, β-asarone was confirmed with the literature [12,14] and showed similar spectral patterns.

Administration of 2000 mg/kg single limit dose of A. *calamus* rhizome extract to five mice did not reveal any signs of toxicity or mortality in any animals. All the treated animals were found to be healthy and normal in their behavior, breathing, posture, food and water consumption, etc., during the observation period of 14 days.

As monitored by EPG counts and percentage reduction in worm counts, A. *calamus* rhizome extract showed dose-dependent efficacy ($P < 0.001$) against adult *H. diminuta* infections in rats [Table 1]. Treatment of *H. diminuta* infected rats by a single 800 mg/kg dose of extract for 5 days (days 21-25 p.i. of cysticercoids) resulted into 62.30% reduction in EPG counts and 83.25% reduction in worm counts at necropsy of rats on day 39. This was well comparable with the effects of reference drug praziquantel which caused 85% reduction in EPG counts and 81% reduction in worm burden of animals. Herein, the control animals maintained an almost uniform trend in their EPG counts during the pre- and post-treatment periods. In a similar manner, the active principle of plant, β-asarone also revealed dose-dependent anthelmintic effects ($P < 0.001$). The treatment of animals by 40 mg/kg dose of β-asarone resulted in comparatively better efficacy and caused up to 92% of worm reductions and 79% EPG reductions in experimental animals [Table 2].

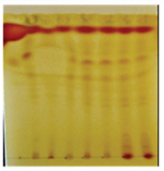
| Plant | Solvents | TLC profile | No. of spots |
|---|---|---|---|
| Acorus calamus | Chloroform and methanol (9:1) |  | n-hex: 3<br>n-but: 1<br>Chloroform: 4<br>Ethyl acetate: 5<br>Acetone: 5<br>Methanol: 6<br>Aquoeus: 5 |
| Beta-asarone | Chloroform and methanol (9:1) |  | $R_f$ value of β- asarone: 0.42<br>Methanol extract: 6<br>Standard beta-asarone: 1<br>Isolated beta-asarone: 1 |

**Figure 2:** Thin layer chromatography of rhizome extract of *Acorus calamus*, standard β-asarone and isolated compound, β-asarone

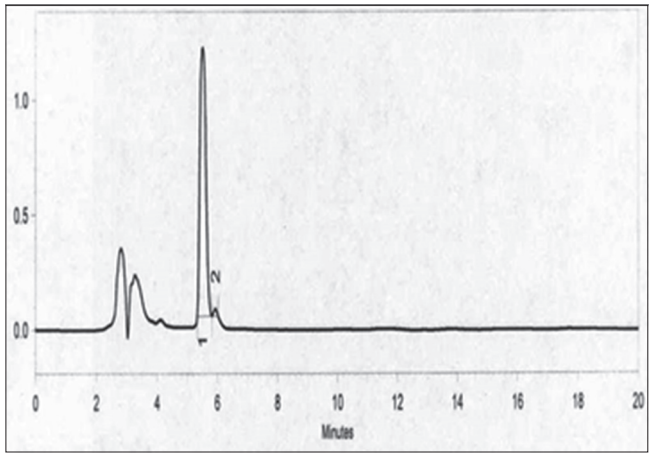



**Figure 3:** High-performance liquid chromatography chromatogram of *Acorus calamus* rhizome active fraction showing β-asarone peak (peak 1)

## DISCUSSION

India is considered as one of the most affected countries by intestinal helminths. According to latest available data, more than two-thirds of children aged 1-14 are in need of deworming in India [3]. As the anthelmintic drugs are yet to reach all endemic regions in the required quantities, traditional herbal medicines hold a great scope for the treatment of intestinal helminths.

During our on-going studies on documentation and scientific validation of traditional anthelmintic plants of India, it came to our notice that rhizomes of A. *calamus* have long been used by natives to cure intestinal worms. Although some previous workers have demonstrated that A. *calamus* rhizomes bear significant *in vitro* anthelmintic activity [14,15], so far no sufficient efforts have been made to systematically evaluate the *in vivo* anthelmintic activity of this plant. As it is well established, this plant exhibits a ploidy nature [17], and different varieties of this plant vary in its contents of active principle β-asarone [18], therefore, an evaluation of its *in vivo* anthelmintic effects with regard to the quantity of β-asarone present in its rhizomes seems desirable. Hence, this study was undertaken to evaluate the *in vivo* anthelmintic effects of a standardized methanol extract of A. *calamus* rhizomes against experimentally induced cestodiasis in rats.

The present study revealed that both the A. *calamus* rhizome extracts as well as its active principle, β-asarone possess dose-dependent effects ($P < 0.001$) against adult *H. diminuta* infections in rats. The study also indicated that treatment of rats by a single 800 mg/kg dose of extract for 5 days resulted into 62.30% reduction in EPG counts and 83.25% reduction in worm counts at necropsy of animals, which compared well with the efficacy of reference drug praziquantel. In recent years, researchers have shown increasing interest in studying the anthelmintic potentials of medicinal plants [5,23,24]. Several other studies have also employed *H. diminuta* - rat experimental model to validate the anthelmintic effects of various traditional anthelmintic plants [5,25]. These studies have mostly ascertained the anthelmintic potentials of various medicinal plants on adult or larval stages of *H. diminuta* and discussed their findings by reductions in EPG counts and worm burdens of experimental animals. The findings of our study are in agreement with the findings of two previous studies by Yadav and Tangpu and Yadav *et al*., wherein the treatment of *H. diminuta* infected rats by *Solanum myriacanthum* fruit extract revealed 60.49% reduction in the EPG counts and 56.60% reduction in worm counts, and by *Gynura angulosa* leaf extract showed 78.60% reduction in EPG counts and 70.75% reduction in worm counts of animals [25,26].

Table 1: Anthelmintic effects of *Acorus calamus* rhizome extract on adult *H. diminuta* worms in rats as assessed by reduction in EPG and worm counts ($n=6$)

| Groups | EPG (mean±SEM) | | Percentage difference in EPG counts (A-B) | Number of worms recovered/ rat (mean±SEM) | Percentage reduction in worm counts |
|---|---|---|---|---|---|
| | Pre-treatment (days 18-20) (A) | Post-treatment (days 26-28) (B) | | | |
| Control | 15.645±320 | 15.728±334 | 0.53 | 4.00±0.00 | 0.00 |
| Plant extract (mg/kg) | | | | | |
| 200 | 15.677±333 | 10.277±226* | −34.44 | 2.17±0.30** | 45.75 |
| 400 | 15.683±354 | 8.661±413* | −44.77 | 1.33±0.21** | 66.75 |
| 800 | 15.766±319 | 5.944±241* | −62.30 | 0.67±0.21** | 83.25 |
| Praziquantel (mg/kg) | | | | | |
| 5 | 15.883±352 | 2.370±24* | −85.08 | 0.75±0.34** | 81.25 |

Plant extract and praziquantel were administered on days 21-25 p.i. with four cysticercoids per rat. *$P<0.001$ versus pre-treatment value, Student's *t*-test; **$P<0.001$ versus control, Student's *t*-test, EPG: Egg per gram, SEM: Standard error of mean, *H. diminuta*: *Hymenolepis diminuta*, p.i.: Post-inoculation

Table 2: Anthelmintic effects of active principle, beta-asarone on adult *H. diminuta* worms in rats as assessed by reduction in EPG and worm counts ($n=6$)

| Groups | EPG (mean±SEM) | | Percentage difference in EPG counts (A-B) | Number of worms recovered/ rat (mean±SEM) | Percentage reduction in worm counts |
|---|---|---|---|---|---|
| | Pre-treatment (days 18-20) (A) | Post-treatment (days 26-28) (B) | | | |
| Control | 15.622±320 | 15.716±334 | 0.60 | 4.00±0.00 | 0.00 |
| Beta-asarone (mg/kg) | | | | | |
| 10 | 15.953±157 | 8.753±559* | −45.13 | 1.67±0.33** | 58.25 |
| 20 | 15.640±161 | 7.733±127* | −50.56 | 1.00±0.26** | 75.00 |
| 40 | 15.493±125 | 3.260±79* | −78.96 | 0.33±0.21** | 91.75 |
| Praziquantel (mg/kg) | | | | | |
| 5 | 15.892±352 | 2.380±24* | −85.02 | 0.70±0.22** | 82.50 |

Beta-asarone and praziquantel administered on days 21-25 p.i. with four cysticercoids per rat. *$P<0.001$ versus pre-treatment value, Student's *t*-test; **$P<0.001$ versus control, Student's *t*-test, EPG: Egg per gram, SEM: Standard error of mean, *H. diminuta*: *Hymenolepis diminuta*, p.i.: Post-inoculation

In this study, the anthelmintic effects of plant active principle, β-asarone were noted to be slightly better than crude extract and it caused up to 92% of worm reductions in experimental animals. In a related testing of root tuber, extract of *Carex baccans* (a traditional anthelmintic plant of India) and its active principle resveratrol revealed slightly lower anthelmintic efficacy [27]. This study revealed that treatment of rats by extract of *C. baccans* and resveratrol results into 56.01% and 46.05% EPG reductions, and 44.28% and 31.03% decrease in worm burden, respectively. In the study by Magi *et al*., the rhizome extract of *A. calamus* tested at 5 g/kg dose against pig-nodular worm *Oesophagostomum* spp. also showed 98% reduction of worm burden in pigs [16]. It thus appears from these studies that *A. calamus* rhizomes do possess potent *in vivo* anthelmintic effects.

In the current study, the chemical structure of isolated purified compound was predicted using various spectroscopic analytical procedures and found related to β-asarone. The spectral data of isolated purified when compared with the existing literature [12,14] showed similar spectral patterns and hence was identified as β-asarone. The yield of β-asarone was calculated to be 83.54% (w/w) in the isolated fraction, which indicates that local variety of *A. calamus* in this area is tetraploid in nature.

In acute toxicity assay, treatment of mice by a single oral dose of 2000 mg/kg of plant extract did not reveal any signs of toxicity or mortality within the 2-week observation period, and therefore, the LD50 of the extract was interpreted to be >2000 mg/kg. According to the globally harmonized system of classification and labeling of chemicals, substances having an LD50 value >2000 mg/kg are considered as relatively safe [28]. Therefore, it can be suggested that rhizome extract of *A. calamus* is practically devoid of any acute oral toxic effects in experimental animals.

## CONCLUSIONS

Taken together, the results of this study show that the rhizomes of *A. calamus* bear significant dose-dependent effects against intestinal helminths, and the local Indian variety of this plant contains high β-asarone content. Therefore, there exists a great potential to develop some suitable anthelmintic herbal products from this plant.

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# Inhibitory effect of leaves extracts of *Ocimum basilicum* and *Ocimum gratissimum* on two key enzymes involved in obesity and hypertension *in vitro*

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## ABSTRACT

**Aim:** To evaluate the phenolics composition and inhibitory effect of the leaves extracts of *Ocimum basilicum* and *Ocimum gratissimum* on two key enzymes (pancreatic lipase [PL] and angiotensin 1-converting enzyme [ACE]) involved in obesity and hypertension *in vitro*. **Materials and Methods:** The phenolics (flavonoids and phenolic acids) were quantified using high-performance liquid chromatography coupled with diode array detection. PL and ACE inhibitory effects; DPPH* and ABTS*+ scavenging activities of the extracts were tested using spectrophotometric methods. **Results:** *O. basilicum* had the following major phenolics: Rutin, quercetin, and quercitrin (flavonoids); caffeic, chlorogenic, and gallic acids (phenolic acids); while *O. gratissimum* had the following major phenolics: Rutin, quercitrin, and luteolin (flavonoids); ellagic and chlorogenic acids (phenolic acids). "Extracts of both plants inhibited PL and ACE; scavenged DPPH* in a dose-dependent manner". *O. gratissimum* extract was more potent in inhibiting PL ($IC_{50:}$ 20.69 μg/mL) and ACE ($IC_{50:}$ 29.44 μg/mL) than *O. basilicum* ($IC_{50:}$ 52.14 μg/mL and $IC_{50:}$ 64.99 μg/mL, against PL and ACE, respectively). *O. gratissimum* also scavenged DPPH* and ABTS*+ more than *O. basilicum*. **Conclusion:** *O. basilicum* and *O. gratissimum* leaves could be used as functional foods for the management of obesity and obesity-related hypertension. However, *O. gratissimum* may be more effective than *O. basilicum*.

KEY WORDS: Angiotensin I-converting enzyme, hypertension, obesity, *Ocimum* species, oxidative stress, pancreatic lipase

## INTRODUCTION

Obesity, especially visceral obesity, is the most important risk factor for the development of hypertension and other cardiovascular diseases [1]. Population-based risk estimates studies have indicated that no less than two-thirds of the prevalence of hypertension can be directly attributed to obesity [2]. Obesity and obesity-related hypertension have

become a global public health burden; their incidence being comparable with those of diabetes mellitus and chronic kidney diseases [3].

The pancreatic lipase (PL) (EC: 3.1.1.3) plays a pivotal role in the effective digestion of lipids, as it is responsible for the hydrolysis of the bulk (50-70%) of total dietary fats [4]. It is the key enzyme that hydrolyzes triglyceride to produce glycerol and fatty acids, thereby facilitating its uptake [5]. Thus, it is widely used as an index to evaluate the potential efficacy of natural products as anti-obesity agents [6]. Due to the vital catalytic function of PL, orlistat, currently used clinically for obesity management, has been designed to inhibit PL activity; thereby reducing the absorption of triglyceride. However, this drug like other PL inhibitors has some hepatic and gastrointestinal adverse effects [7,8].

The activation of the rennin-angiotensin system (RAS) has been reported as one of the possible mechanisms through which obesity could lead to hypertension and higher cardiovascular risk [3]. In fact, experimental evidence has shown that the RAS is activated in obesity; its involvement in the pathophysiology of obesity-related hypertension has also been reported [9]. In the RAS, angiotensin 1-converting enzyme (ACE) (EC: 3.4.15.1) plays a key role in the regulation of blood pressure and normal cardiovascular function. It catalyzes the conversion of angiotensin I to angiotensin II, which is known to increase the blood pressure. Hence, inhibition of ACE is an important strategy for the treatment and management of obesity-related hypertension [10,11]. Chemically synthesized ACE inhibitors including captopril, ramipril, enalapril and fosinopril, comprise a class of drugs frequently used clinically for the treatment of hypertension [12]. However, as with the PL inhibitors, these drugs have been reported to have some adverse effects such as cough, skin rashes, hypotension, and proteinuria [13,14].

Against the backdrop of the side effects associated with the synthetic inhibitors of both PL and ACE, research has shown that plant-derived inhibitors of these two enzymes have some potential for preventing or ameliorating obesity and hypertension, without noticeable side effects [15,16]. In this regard, plant polyphenolics have been reported by several studies to inhibit either one of these two enzymes (PL and ACE) or both [4,5,17,18].

The genus, *Ocimum* (Lamiaceae), comprises 65 aromatic species, distributed in tropical and subtropical regions of the world including Africa and Asia [19]. Various *Ocimum* species, including *Ocimum basilicum* and *Ocimum gratissimum*, have important culinary and pharmacological uses. Their culinary and pharmacological values are attributed mainly to their aromatic compounds [20]. Hence, the leaves are used as a fragrance and flavoring agent in a variety of products including food, beverages, condiments, and oral care products [21]. *O. gratissimum* leaves extract possesses anti-arthritic [22], hypoglycemic [19], anticonvulsant and anxiolytic activities [23]. On the other hand, *O. basilicum* leaves are used as anti-spasmodic, carminative, galactagogue, and stomachic in folk medicine [24]. To further explore the nutraceutical benefits of *O. basilicum* and *O. gratissimum*, this study evaluated the phenolics composition and inhibitory effects of their leaves extracts on two key enzymes (PL and ACE) involved in obesity and hypertension *in vitro*.

## MATERIALS AND METHODS

### Samples Collection and Preparation

About 500 g of fresh leaves samples of each of *O. basilicum* and *O. gratissimum* were collected from a local farm settlement in Akingbile area of Moniya, Ibadan, Nigeria. The samples were botanically identified and authenticated at the herbarium of the Department of Botany, University of Ibadan, Nigeria. Subsequently, they were air-dried for 7 days and later ground finely to a particle size of 0.5 mm. The powdery samples were used for the extracts preparation.

### Chemicals and Reagents

Methanol, formic acid, gallic acid, chlorogenic acid, caffeic acid, and ellagic acid were purchased from Merck (Darmstadt, Germany). Catechin, epicatechin, quercetin, rutin, apigenin, and luteolin; porcine PL, Hippuryl-histidyl-leucine, Rabbit lung ACE, DPPH, ABTS, and Trolox were acquired from Sigma Chemical Co. (St. Louis, MO, USA). All other chemicals used for the analysis were of analytical grade.

### Preparation of Polyphenolics Extract

Polyphenolics extracts of *O. basilicum* and *O. gratissimum* leaves were prepared as described by Kuo *et al*. [25]. A portion of the leaves powder (100 g) was extracted three successive times with 300 mL of methanol at 50°C for 3 h, and the samples were filtered after each extraction with Whatman (No. 2) filter paper. Lipids and some pigments were removed by partitioning the extract with 200 mL hexane in a separatory funnel. The aqueous phase was extracted three times with 180 mL ethyl acetate and evaporated to dryness at 45°C under reduced pressure in a rotary evaporator. The residues obtained were used for the assays.

### Quantification of Flavonoids and Phenolic Acids using High Performance Liquid Chromatography Coupled with Diode Array Detection (HPLC-DAD)

Extracts of the leaves were injected by means of auto-sampler (Shimadzu, model SIL-20A) at a concentration of 15 mg/mL. Separations were carried out using phenomenex $C_{18}$ column (4.6 mm × 250 mm × 5 mm particle size). The mobile phase comprised solvent A (water:formic acid [98:2, v/v]) and solvent B (acetonitrile), at a flow rate of 0.6 mL/min and an injection volume of 40 $\mu$L. Gradient program was started with 95% of A and 5% of B until 2 min and changed to obtain 25%, 40%, 50%, 70% and 80% B at 10, 20, 30, 50 and 70 min, respectively, according to the method described by Boligon *et al*. [26], with slight modifications. The extracts and mobile phase were

filtered through a 0.45 $\mu$m membrane filter (Millipore) and then degassed in ultrasonic bath before use. Standards references stock solutions were prepared in the HPLC mobile phase at a concentration range of 0.025-0.300 mg/mL. Quantifications of the flavonoids and phenolic acids in the extracts were carried out by integration of the peaks using the external standard method at the following wavelengths: 254 nm for gallic acid and ellagic acid; 280 nm for catechin and epicatechin; 325 nm for caffeic acid and chlorogenic acid; 366 nm for quercetin, quercitrin, kaempferol, luteolin and rutin. The individual chromatography peaks were confirmed by comparing their respective retention time with those of reference standards and by DAD spectra (200-600 nm). Calibration curves used were gallic acid: Y = 13581x + 1195.7 (r = 0.9997), catechin: Y = 12609x + 1187.4 (r = 0.9996), epicatechin: Y = 12719x + 1356.9 (r = 0.9995), caffeic acid: Y = 12750x + 1352.9 (r = 0.9999), chlorogenic acid: Y = 11825x + 1383.6 (r = 0.9998), ellagic acid: Y = 13192x + 1176.5 (r = 0.9997), quercitrin: Y = 12641x + 1295.7 (r = 0.9999), kaempferol: Y = 11846x + 1283.9 (r = 0.9994), rutin: Y = 11792x + 1305.8 (r = 0.9995), luteolin: Y = 13894x + 1267.1 (r = 0.9998), and quercetin: Y = 12618x + 1196.3 (r = 0.9996). All chromatography operations were carried out at ambient temperature and in triplicate.

The limit of detection (LOD) and limit of quantification (LOQ) of flavonoids and phenolic acids were calculated based on the standard deviation of the responses and the slope using three independent analytical curves, as previously defined by Khaliq *et al*. [27]. LOD and LOQ were calculated as 3.3 and 10 $\sigma$/S, respectively, where $\sigma$ is the standard deviation of the response and S is the slope of the calibration curve.

## PL Inhibition Assay

The ability of the extracts to inhibit porcine PL was determined using the method earlier reported by Eom *et al*. [28] with a slight modification. In this assay, PL hydrolyzes p-nitrophenyl butyrate to form p-nitrophenol which is measured spectrophotometrically. The enzyme solution was prepared by adding 30 $\mu$L (10 units) of porcine PL (Sigma, USA) in 10 mM morpholine propane sulfonic acid and 1 mM EDTA (pH 6.8) to 850 $\mu$L of Tris buffer (100 mM Tris-HC1 and 5 mM $CaCl_2$, pH 7.0). Thereafter, 100 $\mu$L of appropriate concentrations (20, 40, 60, 80 $\mu$g/mL) of the extracts or orlistat (positive control) were mixed with 880 $\mu$L of the enzyme solution and incubated for 10 min at 37°C. After incubation, 20 $\mu$L of the substrate solution (10 mM of p-nitrophenyl butyrate in dimethylformamide) was added to initiate the hydrolytic reaction, which was allowed to proceed for 20 min at 37°C. A reference test (without the extract) was included in the assay. The absorbance of the p-nitrophenol produced from the hydrolysis of p-nitrophenylbutyrate was measured at 405 nm. The inhibition of PL activity by the extracts was expressed as % inhibition thus:

$$\% \text{ Inhibition} = [(A405_{reference} - A405_{sample}) \div A405_{reference}] \times 100.$$

Where $A405_{reference}$ is the absorbance of the reference without the extract, and $A405_{sample}$ is the absorbance of test containing the extract.

## ACE Inhibition Assay

ACE inhibition was assayed by a spectrophotometric method described by Cushman and Cheung [29]. ACE from rabbit lung (EC: 3.4.15.1) and the substrate (Hippuryl-histidyl-leucine [Bz-Gly-His-Leu]) were used. In this assay, the Bz-Gly-His-Leu is cleaved by ACE to form hippuric acid, which is measured spectrophotometrically. A portion (50 $\mu$L) of appropriate concentrations of the extracts (20, 40, 60, 80 $\mu$g/mL) and 50 $\mu$L ACE solution (4 mU/mL) were pre-incubated at 37°C for 15 min. After pre-incubation, 150 $\mu$L of 8.33 mM of the Bz-Gly-His-Leu in 125 mM Tris-HCl buffer (pH8.3) was added to the mixture to initiate the enzymatic reaction, and this was incubated at 37°C for 30 min. Next, the reaction was terminated by adding 250 $\mu$L of 1 M HCl. The hippuric acid produced by the reaction was extracted with 1.5 mL ethyl acetate. Subsequently, the ethyl acetate layer was separated from the mixture by centrifugation, and 1 mL of it was transferred to a clean test tube and evaporated to dryness in a hot-air oven. The resulting residue was redissolved in distilled water and its absorbance was measured at 228 nm. A reference test (without the extract) and a positive control test (containing 64 nmol/L of captopril) were carried out simultaneously with the test extract. 1 unit (U) of ACE activity is defined as the amount of enzyme required to catalyze the formation of 1 $\mu$mol of hippuric acid from hippuryl-histidyl-leucine per minute at 37°C. The ability of the extracts to inhibit ACE activity was expressed as percentage inhibition thus:

$$\% \text{ Inhibition} = [(A228_{reference} - A228_{sample}) \div A228_{reference}] \times 100.$$

Where $A228_{reference}$ is the absorbance of the reference without the extract, and $A228_{sample}$ is the absorbance of test containing the extract.

## Determination of DPPH* Scavenging Ability

DPPH* scavenging ability of the extracts was determined as described by Cervato *et al*. [30]. Appropriate dilutions (30, 60, 90, 120 $\mu$g/mL) of the extracts amounting to 1.0 mL were mixed with 3.0 mL of DPPH* (60 $\mu$M). The test mixture was kept in the dark for 30 min, after which the absorbance was measured at 517 nm. A reference test containing the DPPH* solution without the extract was carried simultaneously. The DPPH* percentage scavenging ability of the extract was calculated as follows:

$$\% \text{ scavenging ability} = [(Abs517_{reference} - Abs517_{sample}) \div Abs517_{reference}] \times 100$$

Where $Abs517_{reference}$ is the absorbance of the reference test, and $Abs517_{sample}$ is the absorbance of the test containing the extract.

## Determination of ABTS*+ Scavenging Ability

ABTS*+ scavenging ability of the extracts was determined according to the method described by Re *et al*. [31]. To generate the ABTS*+, an equal volume of 7 mM ABTS*+ aqueous solution was incubated with 2.45 mM $K_2S_2O_8$ for 16 h at room

temperature in the dark; then its absorbance (at 734 nm) was adjusted to 0.7 ± 0.02 with 95% ethanol. Thereafter, appropriate dilution of the extract amounting to 0.2 mL was mixed with 2.0 mL ABTS$^{*+}$ solution. The test mixture was kept in the dark for 15 min, after which its absorbance was measured at 734 nm. The ABTS$^{*+}$ scavenging ability of the extracts was subsequently calculated and expressed in trolox equivalent (TE).

### Statistical Analysis

Results of replicate experiments were expressed as mean ± standard deviation (SD). *T*-test for independent samples was carried out on the result data at 95% confidence level using SPSS statistical software package, version 17. Half-maximal inhibitory and scavenging concentrations ($IC_{50}$ and $SC_{50}$) were calculated from the % inhibition versus extract concentration non-linear regression curve of each extract.

## RESULTS

The HPLC chromatograms of *O. basilicum* and *O. gratissimum* [Figure 1a and b] revealed the presence of gallic acid (retention time - Rt = 10.19 min; peak 1), chlorogenic acid (Rt = 22.35 min; peak 3), caffeic acid (Rt = 26.97 min; peak 4), epicatechin (Rt = 37.41 min; peak 6), rutin (Rt = 43.86 min; peak 7), quercitrin (Rt = 48.13 min; peak 8), quercetin (Rt = 52.01 min; peak 9), and kaempferol (Rt = 57.69 min; peak 10). In addition to these, catechin (Rt = 16.07 min; peak 2), ellagic acid (Rt = 29.83 min; peak 5), and luteolin (Rt = 64.15 min; peak 11) were also present in *O. gratissimum* [Figure 1b].

The quantitative composition of the phenolics (flavonoids and phenolic acids) is shown in Table 1. In *O. basilicum*, the flavonoids were in the order of rutin > quercetin > quercitrin > epicatechin > kaempferol, whereas the phenolic acids were in the order of caffeic acid > chlorogenic acid > gallic acid. In *O. gratissimum*, the flavonoids were in the order of rutin > quercitrin > luteolin > epicatechin > kaempferol > quercetin > catechin, while the phenolic acids were in the order of ellagic acid > chlorogenic acid > gallic acid > caffeic acid. Thus, the major flavonoids in *O. basilicum* were rutin, quercetin, and quercitrin; the major phenolic acids were caffeic, chlorogenic and gallic acids. But in *O. gratissimum* rutin, quercitrin and luteolin were the major flavonoids, whereas ellagic and chlorogenic acids were the major phenolic acids.

The abilities of the *O. basilicum* and *O. gratissimum* leaves extracts to inhibit porcine PL and ACE were expressed in terms of their half-maximal inhibitory concentrations ($IC_{50}$), and the results are presented in Table 2. The $IC_{50}$ of *O. gratissimum* against PL (20.69 ± 2. 14 μg/mL) was significantly ($P < 0.05$) lower than that of *O. basilicum* (52.14 ± 3.96 μg/mL). However, the $IC_{50}$ (3.48 ± 0.13 μmol/g) of orlistat, the reference PL inhibitor, was lower than those of *O. gratissimum* and *O. basilicum*. Similarly, the $IC_{50}$ of *O. gratissimum* against ACE (29.44 ± 3.98 μg/mL) was significantly ($P < 0.05$) lower than that of *O. basilicum* (64.99 ± 5.04 μg/mL) but that of captopril (19.32 ± 2.23 μmol/g), the reference ACE inhibitor, was lower than both. The patterns of PL and ACE inhibition by *O. basilicum* and *O. gratissimum* were both dose-dependent

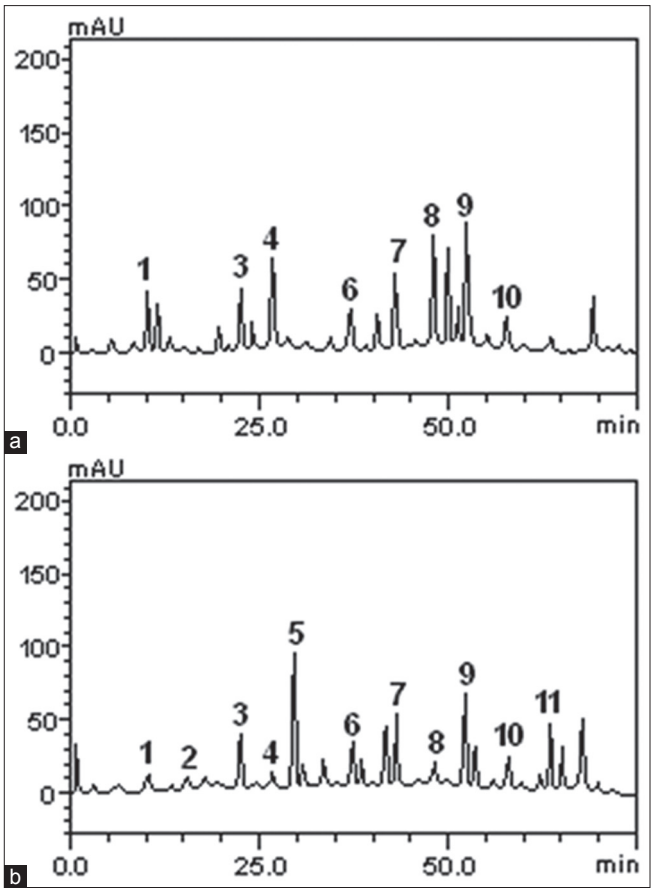


**Figure 1:** Representative high performance liquid chromatography chromatograms of *Ocimum basilicum* and *Ocimum gratissimum* extracts; detection ultraviolet was at 325 nm. Gallic acid (peak 1), catechin (peak 2), chlorogenic acid (peak 3), caffeic acid (peak 4), ellagic acid (peak 5), epicatechin (peak 6), rutin (peak 7), quercitrin (peak 8), quercetin (peak 9), kaempferol (peak 10), and luteolin (peak 11). (a) *O. basilicum,* (b) *O. gratissimum*

**Table 1: Flavonoids and phenolics acids composition of *O. basilicum* and *O. gratissimum* leaves extracts**

| Phenolic compounds (mg/g) | *O. basilicum* | *O. gratissimum* | LOD μg/mL | LOQ μg/mL |
|---|---|---|---|---|
| Gallic acid | 2.07±0.03$^{a}$ | 0.53±0.01$^{b}$ | 0.024 | 0.079 |
| Catechin | ND | 0.51±0.02 | 0.013 | 0.042 |
| Chlorogenic acid | 2.10±0.01$^{a}$ | 1.79±0.01$^{b}$ | 0.008 | 0.026 |
| Caffeic acid | 2.73±0.01$^{a}$ | 0.48±0.03$^{b}$ | 0.019 | 0.061 |
| Ellagic acid | ND | 3.96±0.02 | 0.026 | 0.085 |
| Epicatechin | 1.35±0.04$^{a}$ | 1.32±0.01$^{a}$ | 0.011 | 0.034 |
| Quercitrin | 2.49±0.01$^{a}$ | 2.07±0.01$^{b}$ | 0.020 | 0.066 |
| Quercetin | 3.18±0.01$^{a}$ | 0.54±0.03$^{b}$ | 0.027 | 0.089 |
| Rutin | 3.25±0.03$^{a}$ | 2.26±0.04$^{b}$ | 0.014 | 0.047 |
| Kaempferol | 1.06±0.01$^{a}$ | 1.03±0.01$^{a}$ | 0.008 | 0.026 |
| Luteolin | ND | 2.01±0.02 | 0.017 | 0.056 |

Results are expressed as mean±SD of three determinations. Values followed by different superscript letters along the same row differ significantly at $P<0.05$. LOD: Limit of detection, LOQ: Limit of quantification, *O. basilicum: Ocimum basilicum, O. gratissimum: Ocimum gratissimum,* SD: Standard deviation

as depicted by their % inhibition versus extract concentration curves [Figures 2 and 3] respectively.

The free radical scavenging activities of *O. basilicum* and *O. gratissimum* leaves extracts, as determined using DPPH* and ABTS*+ scavenging assays, are presented in Table 3. *O. gratissimum* had a significantly ($P < 0.05$) stronger DPPH* scavenging ability ($SC_{50:}$ 84.76 ± 1.06 μg/mL) than *O. basilicum* ($SC_{50:}$ 104.72 ± 1.85 μg/mL). However, ascorbic acid, the reference antioxidant, had much stronger DPPH* scavenging ability ($SC_{50:}$ 7.29 ± 0.34 μg/mL) than both *O. gratissimum* and *O. basilicum*. However, both leaves extracts scavenged DPPH* in a dose-dependent [Figure 4]. Similarly, *O. gratissimum* had a stronger ABTS*+ scavenging ability (1.82 ± 0.02 mmol TE/g) than *O. basilicum* extract (0.52 ± 0.01 mmol TE/g).

## DISCUSSION

The various health benefits of medicinal plants are attributed to the presence of secondary metabolites such as phenolic acids and flavonoids [32]. In this study, therefore, we quantified the flavonoids and phenolic acids constituents of *O. basilicum* and *O. gratissimum* leaves, and evaluated their inhibitory effects against PL and ACE, as well as their free radicals scavenging abilities *in vitro*. Flavonoids and phenolic acids are prominent for their diverse health benefits including anti-diabetic [33], anti-hypertensive and antioxidative [34]; hence, they are regarded as being pharmacologically important [35]. Interestingly, the level of caffeic acid (2.73 mg/g) we quantified in *O. basilicum* in this study is about 40% higher than the 1.65 mg/g reported earlier by Harnafi *et al.* [36].

The inhibition of PL, the most important enzyme in the digestion of dietary triglycerides, is one of the possible approaches to retard the uptake of fat, and consequently, reduce weight and obesity [37]. Polyphenols, including flavonoids and phenolic acids, from natural origin are regarded as a major class of the PL inhibitors [38,39]. However, flavonoids, especially the catechin-types, are presumed to possess more PL inhibitory and anti-obesity effect. The presence of galloyl moiety in the structural backbone of the polyphenolics plays an important role in their ability to inhibit PL. Possibly, the more the galloyl moieties, the stronger the PL inhibitory effect, as Nakai *et al.* [40] demonstrated that the PL $IC_{50}$ values of (−)-epigallocatechin 3,5-digallate with two galloyl groups, epigallocatechin gallate

**Table 2: $IC_{50}$ of *O. basilicum* and *O. gratissimum* leaves extracts against PL and ACE activities**

| Extract | PL $IC_{50}$ | ACE $IC_{50}$ |
|---|---|---|
| *O. basilicum* (μg/mL) | 52.14±3.96[a] | 64.99±5.04[a] |
| *O. gratissimum* (μg/mL) | 20.69±2.14[b] | 29.44±3.98[b] |
| Orlistat eq. (μmol/g) | 3.48±0.13 | NA |
| Captopril eq. (μmol/g) | NA | 19.32±2.23 |

Results are expressed as mean±SD of replicate analysis. Values followed by different superscript letters along the same column vary significantly at $P<0.05$. NA: Not applicable, *O. basilicum: Ocimum basilicum. O. gratissimum: Ocimum gratissimum*, SD: Standard deviation, PL: Pancreatic lipase, ACE: Angiotensin 1-converting enzyme

**Table 3: DPPH* $SC_{50}$ and ABTS*+ scavenging ability of *O. basilicum and O. gratissimum* leaves extracts**

| Antioxidant activity | *O. basilicum* | *O. gratissimum* | Ascorbic acid |
|---|---|---|---|
| DPPH* $SC_{50}$ (μg/mL) | 104.72±1.85[a] | 84.76±1.06[b] | 7.29±0.34[c] |
| ABTS*+ scavenging ability (mmol TE/g dw) | 0.52±0.01[b] | 1.82±0.02[a] | NA |

Results are presented as mean±SD of triplicate determinations. Values with different lowercase superscript letter along the same row vary significantly at $P<0.05$. NA: Not applicable, *O. basilicum: Ocimum basilicum. O. gratissimum: Ocimum gratissimum,* SD: Standard deviation, PL: Pancreatic lipase, ACE: Angiotensin 1 converting enzyme

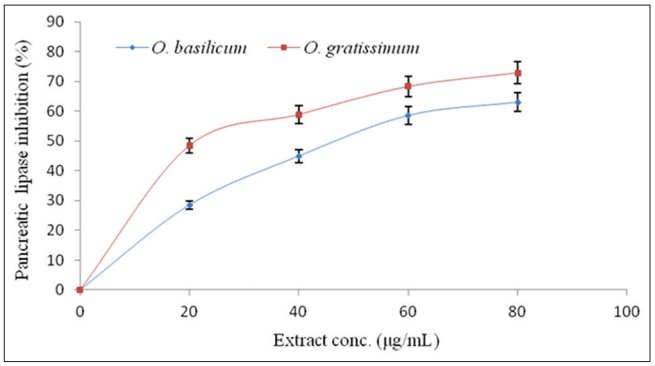


**Figure 2:** Pancreatic lipase % inhibition - extract concentration curve of *Ocimum basilicum* and *Ocimum gratissimum* leaves extracts

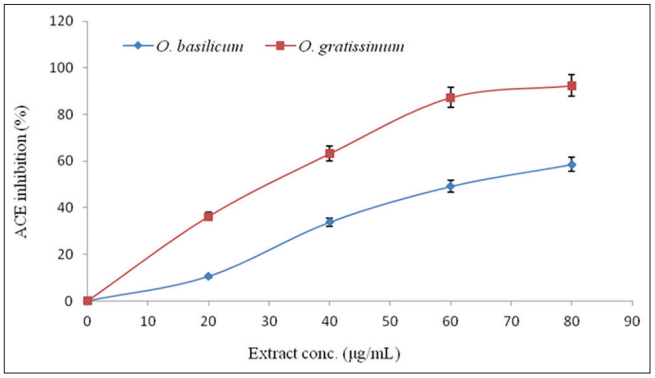


**Figure 3:** Angiotensin I-converting enzyme % inhibition - extract concentration curve of *Ocimum basilicum* and *Ocimum gratissimum* leaves extracts

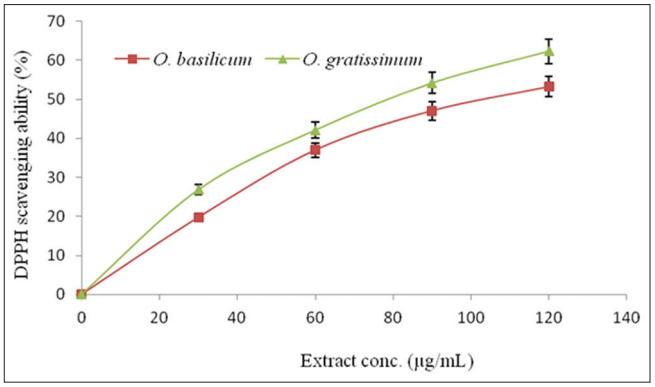


**Figure 4:** DPPH* % scavenging ability-extract concentration curve of *Ocimum basilicum* and *Ocimum gratissimum* leaves extracts

with one galloyl group and (−)-epigallocatechin with no galloyl group were 0.098, 0.349 and 20 $\mu$M, respectively, in their study in which they evaluated the inhibitory activities of 54 tea polyphenols against PL. The galloyl moieties may exert their inhibitory effect on PL by competing with its substrate for the active site, at least, at a substrate concentration below the $K_m$ value [38]. Therefore, it is possible that the presence of catechin in *O. gratissimum* accounted for its stronger ability to inhibit PL than *O. basilicum* that had no catechin [Table 1].

ACE catalyzes the cleavage of angiotensin I (a decapeptide) to angiotensin II (an octapeptide) and inactivates bradykinin, a vasodilator and hypotensive peptide [41]. Angiotensin II stimulates vasoconstriction, increases sodium and water reabsorption, and elevates blood pressure. Consequently, the excessive action of ACE leads to hypertension [42]; for this reason, inhibition of ACE has emerged as an important treatment and management strategy for hypertension [10,11]. Plant extracts rich in flavonoids and phenolic acids have been shown to inhibit ACE activity by previous studies [18,34]. The flavonoids are the largest group of polyphenolic compounds found in higher plants [43] and are regarded as an excellent source of functional anti-hypertensive products. Guerrero *et al*. [44] observed this in their study in which they evaluated the structure-activity relationship of inhibition of ACE activity by flavonoids. The combination of sub-structures on the flavonoid skeleton, including the catechol group in the B-ring, the double bond between C2 and C3 at the C-ring, and the cetone group in C4 at the C-ring, increases the ACE inhibitory activity of the flavonoids. Similarly, the phenolic acids are effective ACE inhibitors. Their ability to inhibit ACE has been attributed to the overall contribution of their functional groups, including carboxyl and hydroxyl groups; their ability to form charge-charge interactions with the zinc ion present in the active site of ACE, through the oxygen atom of their carboxylate moiety; and their interaction with the amino acids residues at the active site of ACE, to give a stable complex between the phenolic acid molecule and ACE. Thus, the ACE inhibitory activity of phenolic acids may be due the effect of this interaction with the zinc ion and the subsequent stabilization by other interactions with amino acids in the active site [45]. In this study, *O. gratissimum* had stronger ACE inhibitory activity than *O. basilicum* [Table 2]. This may be attributed to the presence of luteolin (flavonoid) in *O. gratissimum* (2.01 mg/g) and ellagic acid (phenolic acid), which were not detected in *O. basilicum* [Table 1]. This is supported by the findings of Guerrero *et al*. [44], who reported that luteolin had the highest ACE inhibitory activity, relative to other sixteen flavonoids in their study.

Systemic oxidative stress is a common feature of obesity [46], and by extension, obesity-related hypertension. Oxidative stress arises due to increased generation of free radicals and reactive oxygen species (ROS), with attendant reduction in the antioxidant defense system, both enzymic and non-enzymic, in the obese. Angiotensin II also induces oxidative stress, which plays a key role in the development of hypertension [47,48]. Free radicals and ROS are notorious for their ability to damage both intra- and extra-cellular macromolecules, including the DNA, lipids, and protein [49]. However, several previous studies have demonstrated that plant extracts rich in flavonoids and phenolic acids can scavenge free radicals [18,34]. The efficiency of flavonoids and phenolic acids as antioxidant polyphenols is attributable to their unique molecular structures. The hydroxyl groups of flavonoids, especially at the 3' OH and 4' OH of their three-carbon chain, make them potent electrons donors and terminators of chain reactions [50,51]; hence their antioxidant effect. On the other hand, the antioxidant activity of phenolic acids has been attributed to the ability of the phenolic ring in their structure to stabilize and delocalize unpaired electrons [52]. The ability of the *O. basilicum* and *O. gratissimum* leaves extracts to scavenge free radicals (DPPH* and ABTS*+) is an indication that they could help prevent and/or alleviate oxidative stress in obesity and obesity-related hypertension. This may complement the overall antihypertensive activity of the extracts, as Rodriguez-Rodriguez and Simonsen [53] reported that the anti-oxidative effects of ACE inhibitors contributed to their therapeutic effects in patients suffering from cardiovascular complications.

## CONCLUSION

The leaves extracts of *O. basilicum* and *O. gratissimum* inhibited PL and ACE; and scavenged DPPH* and ABTS*+. These bioactivities could be due to the combined effects of the flavonoids and phenolic acids present in the leaves. Hence, *O. basilicum* and *O. gratissimum* leaves could be used as functional foods for the management of obesity and obesity-related hypertension, and preventing the oxidative stress that characterizes both disorders. However, *O. gratissimum* may be more effective due to its stronger inhibitory effect on PL and ACE.

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Original Research

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# Comparative evaluation of anti-obesity effect of *Aloe vera* and *Gymnema sylvestre* supplementation in high-fat diet fed C57BL/6J mice

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## ABSTRACT

**Background:** The aim of the present study was to investigate, anti-obesity effect of *Aloe vera* (AV), and *Gymnema sylvestre* (GS) whole extract powders administration to high-fat diet (HFD) fed C57BL/6J mice for 12 weeks. **Materials and Methods:** At the end of experiment, different parameters such as body weight, feed intake, organ weights, fasting blood glucose, oral glucose tolerance test, plasma lipid levels, and expression analysis of adipocytokines were evaluated. **Results:** At the end of experimental period, oral administration of both herbs showed a significant ($P < 0.05$ and $P < 0.001$) decrease in the plasma glucose and lipid levels in HFD fed mice. In addition, increased in the epididymal fat (E. fat) weight in the HFD group was significantly ($P < 0.05$) reduced on GS administration alone. Finally, quantitative mRNA expression analysis of adiponectin gene was significantly up-regulated in AV supplementation. Further, no effect was observed with the both herbs on pro-inflammatory cytokines (interleukin 6 and tumor necrosis factor-α) in the E. fat tissue of HFD fed group. **Conclusions:** The anti-obesity and other metabolic studies depend on the type of diet, different parts of herbal extractions, and animal models used. Further studies are required in this area to strengthen the anti-obesity effects of herbs with active component, and it can be used a pro-drug instead of whole extract.

KEY WORDS: *Aloe vera*, *Gymnema sylvestre*, high-fat diet, inflammatory markers, obesity

## INTRODUCTION

Now-a-days, obesity is considered to be epidemic disease which leads toward a positive energy balance. It is associated with many health-related complications such as insulin resistance, Type-2 diabetes mellitus (T2DM), and other metabolic disorders. In obese individuals, the accumulation of more fat occurs in the adipose tissue and later stage fat deposition takes place other than non-adipose tissue (e.g., muscle and liver) [1]. Due to the accumulation of triglycerides (TG), the body does not respond to the action of insulin and results in insulin resistance, a hallmark of Type-2 diabetes [2-4]. Moreover, both familial and environmental factors play an significant part in obesity. The consumption of high-fat diet (HFD), an environmental factor is associated with the development of obesity and other metabolic diseases. Diet control, physical exercise, liposuction, and bariatric surgery are available to curb the obesity, but these are not effective with prolonged treatment duration. Hence, people are looking for alternative therapies such as herbal medicines and functional foods which are gaining the importance in treatment of obesity and its associated diseases. Nearly, 1200 plant species have been examined for their use in obesity and diabetes [5].

Herbs such as *Aloe vera* (AV) and *Gymnema sylvestre* (GS) showed antihyperglycemic and hypolipidemic effect in animal studies and clinical studies when administered through intragastric gavage [6,7]. *Aloe barbadensis* Miller (AV) belongs to the Liliaceae family, of which there are about 360 species and widely used in the manufacture of food and beverages, pharmaceuticals and cosmetics [8]. It has been used for centuries, treating obesity, diabetes, immunomodulator, anti-inflammatory, antiseptic, healing, and anti-tumor activities. Aloe gel contains predominantly polysaccharides such as glucomannan and acemannan possessing several medicinal values [9]. In a clinical trial, AV juice showed anti-diabetic activity performed by Mahidol University Research Group [10,11]. In a recent study, we demonstrated that AV gel powder (0.5% and 1% w/v) showed an increase in proteolysis and angiotensin I converting enzyme inhibitory activity in milk fermented with probiotics [12]. GS is another medicinal plant belonging to the Asclepiadaceaea family. In the Ayurvedic system, it is referred as "Meshasringa," and it has potent anti-obesity and anti-diabetic activities. It is also used in the treatment of asthma, eye complaints, inflammation, family planning, and snake bite [13]. Its leaves contain gymnemic acids which are a potent inhibitor of glucose absorption in the

intestine [14]. In addition, gymnemic acids bind to the receptors present in the tongue that prevent the glucose intake [15-18]. Studies showed that GS have been found to increase insulin secretion from the pancreatic β-cells and causes lowering of blood glucose levels in animals and T2DM patients [19,20]. No reports are available with the addition of whole extracts of AV and GS herbs to the HFD for studying its anti-obesity effects. In this study, we investigated the comparative evaluation of AV and GS whole extract powders role in anti-hyperglycemic, hypolipidemic effect, and obesity-related gene expression analysis in HFD fed C57BL/6J mice.

## MATERIALS AND METHODS

### Animals and Experimentation

Twenty-four male C57BL/6J mice (age 5-6 weeks old) were obtained from National Institute of Nutrition, Hyderabad (Andhra Pradesh, India). All the experimental animals were housed in a group ($n = 6$) and fed ad libitum water and food, under 12 h light and dark conditions. The Institutional Animal Ethics Committee guidelines were followed for handling animals in this study. After 1 week of acclimatization period, mice were divided into four groups, namely, control diet, HFD, HFD + AV (1% w/w) (HFD + AV) and HFD + GS (1% w/w) (HFD + GS), and fed for 12 weeks. The HFD contains carbohydrate 35%, protein 20%, and fat 35% (7% soya bean oil and 28% lard). The complete list of diets composition is present at Pothuraju *et al*. [21]. Body weight was measured at weekly interval. At the end of the experimental period, mice were sacrificed by cervical dislocation under diethyl ether anesthesia and blood was collected from heart to determine plasma insulin and lipid levels. Organs such as epididymal fat (E. fat), liver, kidney, and spleen were separated and weighed. A portion of E. fat tissue was immediately transferred into RNA later and stored at −20°C for further expression analysis studies.

### Fasting Blood Glucose Levels

The fasting blood glucose levels were determined by pricking the tail vein with needle gun and determined with GlucoDr® blood glucose meter at 0, 6, and 12 weeks in overnight fasting animals.

### Oral Glucose Tolerance Test (OGTT)

The OGTT was performed after 12 weeks for which mice were fasted for overnight (12 h) and 20% glucose solution (1 g/kg body weight) was administered via intra-gastric gavage. Blood was collected before and after 30, 60, 90, and 120 min of glucose administration.

### Plasma Insulin and Lipid Levels

Plasma insulin concentration was measured with sandwich ELISA kit (Crystal Chem. Inc., USA) while plasma total cholesterol (TC), TG, high-density-lipoprotein-cholesterol (HDL-C) levels were determined using enzymatic kit (M/s Span Diagnostics, India) and very low-density lipoprotein-cholesterol (VLDL-C) was calculated using Friedewald's equation, i.e., VLDL-C (mg/dl) = triglycerides/5.

### Isolation of Total RNA and Quantitative Analysis of Real-time Polymerase Chain Reaction (qRT-PCR)

Total RNA was isolated from E. fat using TRIzol (M/s Sigmal-Aldrich, USA) method. RNA purity was determined by spectroscopy using an ultraviolet spectrophotometer and the ratio of absorbance values at 260 and 280 nm was calculated (A260/A280), and integrity was measured by subjecting to agarose gel electrophoresis. The cDNA template was synthesized by reverse transcription of 500 ng of total RNA using first strand cDNA synthesis kit (M/s Fermentas, India). SYBR green was used for real-time PCR detection and the primers used for qRT-PCR are listed at Pothuraju *et al*. [21].

### Statistical Analysis

All the values are presented as mean ± standard error of mean. The data were statistically analyzed using GraphPad Software Version 5 (San Diego, CA, USA). Tukey's post-test was used to define statistically significant differences ($P < 0.05$) among the groups.

## RESULTS

### Effect of AV and GS on Body Weight Gain

At the end of experiment, no statistically significant difference was observed in the body weight gain between control and HFD fed groups. However, supplementation of both herbal ingredients (AV and GS extracts) showed reduction in the body weight gain but not statistically significant ($P > 0.05$, Table 1).

### Effect of AV and GS on Glucose and Insulin Levels

As shown in Figure 1, fasting blood glucose levels were determined at 0, 6, and 12 weeks. High-fat fed obese mice showed increase

Table 1: Body weight, plasma lipids, and insulin levels

| Parameters | Control | HFD | HFD+AV | HFD+GS |
|---|---|---|---|---|
| Body weight (g) | | | | |
| Initial | 26.71±0.78 | 26.25±0.77 | 26.05±1.52 | 26.05±0.77 |
| Final | 32.57±2.2 | 35.08±1.95 | 36.59±3.4 | 34.22±1.16 |
| TG (mg/dl) | 38.79±3.79 | 52.68±8.53 | 48.72±8.10 | 39.86±4.00 |
| TC (mg/dl) | 83.35±3.51[a] | 128.50±6.41[b] | 83.41±6.90[ac] | 67.78±5.31[ac] |
| HDL-C (mg/dl) | 67.21±1.45[a] | 65.19±1.45[a] | 77.32±4.98[a] | 87.59±3.27[b] |
| VLDL-C (mg/dl) | 7.75±0.76 | 10.53±1.70 | 9.74±1.62 | 7.96±0.80 |
| Insulin (ng/ml) | 0.48±0.14 | 1.44±0.85 | 0.54±0.21 | 0.53±0.08 |

[abc]Mean values within a row with at least one similar superscript letters do not differ significantly (P>0.05, Tukey's test). TG: Triglycerides, TC: Total cholesterol, HDL-C: High-density-lipoprotein-cholesterol, VLDL-C: Very low-density lipoprotein-cholesterol, HFD+AV: High-fat diet+*Aloe vera*, HFD+GS: High-fat diet+*Gymnema sylvestre*

in glucose levels at 6 weeks (153 ± 8.00 mg/dl) and 12 weeks (168.5 ± 7.90 mg/dl), respectively. Administration of both herbs such as AV (122.0 ± 7.79 and 124.0 ± 10.36 mg/dl, $P < 0.05$) and GS (131.3 ± 7.34, $P < 0.05$ and 104.2 ± 10.36 mg/dl, $P < 0.001$) to HFD fed mice, displayed a statistically lowering effect of blood glucose levels at the end of both 6 and 12 weeks, respectively. In OGTT levels were higher in HFD treatment and these glucose levels were lowered by both herbal extract supplementations without a significant difference (data not shown). On the other hand, HFD fed mice had higher levels of plasma insulin levels at the end of experiment. However, both AV and GS supplementation displayed lowering of plasma insulin concentrations without significant difference (Table 1). HFD fed mice exhibited increased fasting blood glucose and plasma insulin levels ($P < 0.05$). Both herbs showed significant decrease in the glucose levels; however, no effect was observed within insulin levels.

### Effect of AV and GS on Organ Weights

After 12 weeks, E. fat, liver, kidney, and spleen were weighed. The E. fat weight was significantly increased in HFD group (1.48 ± 0.24 g, $P < 0.05$) in comparison to control (0.96 ± 0.13 g) group. Supplementation of GS had a significant reduction in the E. fat mass (0.61 ± 0.14 g, $P < 0.05$), whereas no effect was observed with AV fed group (0.89 ± 0.14 g, $P > 0.05$). No significant difference was found in the rest of the organ weights among different groups. Interestingly, significantly reduced E. fat mass weight without a marked reduction in body weight gain was observed by GS supplementation to HFD fed obese mice.

### Effect of AV and GS on Plasma Lipid Profiles

The serum lipids (TC, TG, HDL-C, and VLDL-C) concentrations in a fasting condition of HFD fed obese mice were measured (Table 1). After 12 weeks, plasma TC levels were found to be higher in HFD compared to control group (128.5 ± 6.41 vs. 83.35 ± 3.51 mg/dl). Both herbal ingredients AV (83.41 ± 6.90) and GS (67.78 ± 5.31 mg/dl, $P < 0.05$) showed a significant reduction in the TC levels. Although in HFD (65.19 ± 1.45 mg/dl) group along with AV (77.32 ± 4.98, $P > 0.05$) and GS (87.59 ± 3.27 mg/dl, $P < 0.05$) fed groups, an increase in HDL-C levels was observed. However, a significant difference was observed only in HFD + GS group. No difference was observed in case of triglycerides and VLDL-C levels.

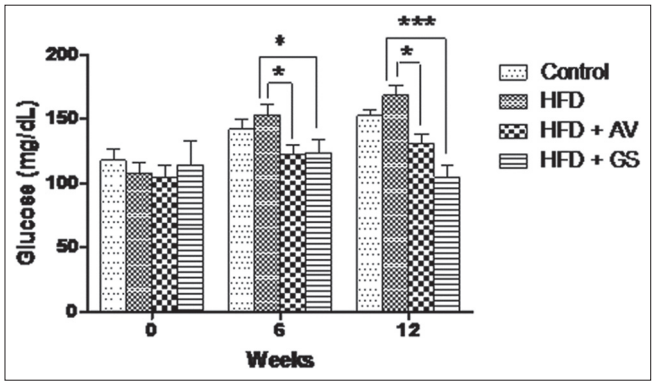


**Figure 1:** Effect of *Aloe vera* (AV) and *Gymnema sylvestre* (GS) on fasting blood glucose levels in mice fed with high-fat diet (HFD). Values are expressed as mean±standard error of mean ($n$=6). The bar over the histogram shows the significant difference of HFD+AV and HFD+GS with HFD group (*$P$<0.05, ***$P$<0.001)

### Effect of AV and GS on Expression Analysis

Expression analysis of genes such as adiponectin, leptin, and proinflammatory markers (interleukin 6 [IL-6] and tumor necrosis factor-α [TNF-α]) was done in E. fat tissue (Table 2). HFD fed mice showed downregulation of adiponectin expression levels. These adiponectin levels were significantly up-regulated by AV supplementation (4-fold change). No significant effect was observed in HFD + GS fed group. In addition, leptin and proinflammatory cytokines expression did not show any significant difference with the herbal ingredients.

## DISCUSSION

In the present investigation, comparative evaluation of both herbal ingredients was studied for their role in anti-obesity effect in HFD fed C57BL6/J mice for 12 weeks. Surprisingly, we did not observe any significant difference between control and HFD diet groups. The reason might be that the lard which is used in HFD preparation obtained from the local supplier. At the end of experiment, oral administration of AV and GS powders resulted in no significant difference in body weight and cumulative feed intake in comparison with the HFD fed group of mice (data not shown). Our results were consistently similar with Chihara *et al.* [22] who reported that AV gel extract did not show any significant difference in the reduction of body weight and food consumption after 7 weeks experimental period in HFD fed mice. Similarly, HFD supplementation of aloe formula such as processed AV gel (PAG), Aloe QDM, and Aloesin had no effect on body weight [23]. Administration of dried AV gel powder to diet-induced obesity rats did not display any significant effect on reduction in the body weight and food intake [24]. Misawa *et al.* [8,25] studied the oral ingestion of AV phytosterols (cycloartanol-Cy and lophenol-Lo) to Zucker diabetic fatty (ZDF) rats for 6 weeks and reported that no significant difference on body weight reduction and food intake. In contrary, administration of AV phytosterols (Cy and Lo) significantly reduced the body weight in diet induced obese (DIO) mice [26]. Few reports are available in relation to body weight and food intake by GS treatment. Our results are

**Table 2: Effect of *Aloe vera* and *Gymnema sylvestre* on analysis of gene expression in epididymal fat mass of mice fed with HFD**

| Gene | Control | HFD | HFD+AV | HFD+GS |
|---|---|---|---|---|
| Adiponectin | 1.03±0.15[a] | 0.63±0.14[a] | 5.5±2.04[b] | 0.61±0.05[a] |
| Leptin | 1.72±0.96[a] | 0.77±0.31[a] | 2.6±2.12[a] | 0.81±0.37[a] |
| TNF-α | 1.23±0.18[a] | 0.47±0.17[a] | 0.99±0.51[a] | 2.60±0.89[a] |
| IL-6 | 1.32±0.53[a] | 1.26±0.58[a] | 2.31±0.91[a] | 1.21±0.12[a] |

[a,b]Mean values within a row with different superscripts differ significantly ($P<0.05$). Values are (fold in change) expressed as mean±standard error of mean ($n=3$). HFD: High-fat diet, HFD+AV: High-fat diet+*Aloe vera,* HFD+GS: High-fat diet+*Gymnema sylvestre*

consistent with the observations of Shigematsu *et al.* [27] who reported that no significant effect on reduction in the body weight and food intake when GS administration to HFD fed Wistar rats. In contrary to our results, Kumar *et al.* [28] reported that GS extract in the HFD-induced obese Wistar rats showed a significant decrease in the body weight while no effect on food intake for 28 days. On the other hand, saponins rich aqueous extract of GS also showed reduce gain in body weight, and no effect in food consumption in HFD fed obese rats [29]. The different AV and GS extract preparations used by the researchers had different effects on reduction in the body weight gain and food intake. In our study, we speculated that the less body weight in herbal fed groups might be due to the satiety effect that results reduction in fat tissue (E. fat) weight which is related to body weight gain. However, we could not measure the fat and lean mass parameters in all treatment groups.

Fasting blood glucose results of the present study were significantly reduced at both 6 and 12 weeks, respectively by both herbal supplementations in comparison to HFD fed group. Our results were corroborated by Shin *et al.* [23] demonstrating that dietary aloe components showed significant reduction in the blood glucose levels in obese mice. Kim *et al*. [6] observed that the fasting blood glucose and insulin levels were significantly reduced in a dose-dependent manner when PAG was orally administered. On the other hand, AV phytosterols (Cy and Lo) administration to ZDF rats showed no effect on fasting blood glucose and serum insulin levels after 5 weeks [25]. Similarly, Pérez *et al.* [30] observed that polyphenol-rich extract from AV gel did not show any effect on insulin levels in insulin resistance mice. However, GS administration of HFD fed Wistar rats showed a significant reduction in the blood glucose and serum insulin levels [28]. The possible mechanism underlying the lowering of blood glucose levels might be due to inhibition of its absorption in the intestine. Hypoglycemic effect of AV might be associated with pancreatic insulin synthesis and its secretion responsible for the lowering of circulatory glucose levels [31] whereas, gymnemic acids present in the GS binds to the glucose receptors to prevent the intestinal glucose absorption [14].

Herbal administration showed no effect on organ weights (liver, kidney, and spleen) whereas, E. fat weight was decreased significantly in case of GS fed group alone as compared to HFD fed obese mice. Our results are in agreement with several reports which showed no significant effect on liver and E. fat mass on AV treatment [8,24,25,32]. Reddy *et al.* [29] reported that the weights of liver, kidney, heart, and adipose tissue (peritoneal and perirenal fat) were lowered upon GS supplementation in HFD fed Wistar rats. In contrast, Shigematsu *et al.* [27] reported that epididymal and mesentery fat weight was not decreased by GS administration. In obesity, the pattern of body fat accumulation is thought to play a part in disease risk and body fat accumulation was closely associated with E. fat mass. In our study, gymnemic acids present in the GS might have been involved in increasing the energy expenditure through uncoupling protein-2 (UCP-2-involved in thermogenesis) expression. Nevertheless, we could not the measure the expression of UCP-2 in adipose tissue and subcutaneous, mesenteric, retroperitoneal, and brown adipose tissue weights were also could not evaluate.

At the end of 12 weeks experimental period, plasma lipids were analyzed in HFD fed mice. Oral administration of both herbs showed a significant decrease in the plasma TC levels alone. Different results were observed by the researchers with the herbal extracts. Chihara *et al.* [22] reported that no significant effect on plasma TC and TG levels. Similarly, Misawa *et al.* [24] studied the oral administration of dried aloe gel (20 mg/kg body weight) and reported no effect on serum TC and TG levels in Sprague-Dawley rats. In contrary, serum TG levels were lowered by AV phytosterols (Cy and Lo) while no effect was observed in TC levels of ZDF rats [25]. Nomaguchi *et al.* [26] also reported that both serum TG and TC levels were significantly reduced by AV phytosterols in DIO C57BL/6J mice. In another study, reduction in the serum TC, TG, and LDL-C levels by GS administration in diabetic rats [7]. Reddy *et al.* [29] observed that decrease in the TG, LDL-C, VLDL-C, and increased in the HDL-C levels in HFD fed rats. Kumar *et al*. observed that GS extract showed a significant reduction in the serum total lipids (TC, TG, LDL-C, and VLDL-C) in HFD fed Wistar rats [28]. By considering above studies, it can be concluded that the differences in lipids levels depends on the type of animal models and extract preparations used. However, in our study, phytosterols present in the whole herbal extracts do not extensively absorb from the intestine rather it can bind to the cholesterol and prevent its absorption to show a hypolipidemic effect [33]. Furthermore, GS contains flavonoids, phenols, saponins, phenolic, and terpenoids might be responsible for lowering effect on lipids in high cholesterol fed rats [34].

Finally, expression analysis of adiponectin (role in regulating energy homeostasis) leptin, IL-6, and TNF-α genes were analyzed by qRT-PCR. In HFD fed mice, AV supplementation alone displayed up regulation of adiponectin mRNA levels. However, no significant difference was observed with the remaining genes. To the best of our knowledge, there are no such expression study reports available in this regard. However, further experiments are necessary to study the effect of AV and GS on the expression analysis of other obesity-related genes in animal models.

## CONCLUSIONS

In conclusion, this study was carried out for comparative evaluation of anti-obesity effect and expression analysis of adiposity genes by AV gel and GS powders in high-fat fed C57BL/6J mice for 12 weeks. Both herbal extracts did not show any significant effect on body weight and food intake and further they displayed anti-hyperglycemic and hypolipidemic effects in HFD fed mice. In addition, E. fat pad was significantly decreased by GS administration alone. AV fed group alone showed a significant up regulation of adiponectin levels. Moreover, supplementation of both powder extracts did not show any effect on other genes. This study is the only preliminary experiment where we used 1% whole extract of herbs added to the HFD instead of active components. However, several researchers they did not show any significant effect on the supplementation of herbs using active component. For the better anti-obesity effects, using type of diet, herbal

extraction, treatment length, and animal models may be play crucial role.

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Original Research

# Effect of orlistat alone or in combination with *Garcinia cambogia* on visceral adiposity index in obese patients

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## ABSTRACT

**Aim:** The objective of this study was to estimate the effect of orlistat alone and in combination with *Garcinia cambogia* on visceral adiposity index (VAI) in obese patients. **Patients and Methods:** A total of 99 obese male patients were recruited with aged range between 37 and 46 years. They were randomized into three equal groups, first group treated with orlistat 120 mg/day, second group treated with *G. cambogia* 166 mg/day, and third group treated with orlistat 120 mg/day plus *G. cambogia* 166 mg/day. The duration of the treatments was three consecutive months. Body mass index (BMI), VAI, blood pressure, blood glucose, total lipid profile, atherogenic index, and cardiac risk ratio were recorded at baseline and after 3 months. **Results:** The treatment with *G. cambogia* leads to reduction in VAI $P < 0.05$, whereas orlistat has a beneficial effect on cardiometabolic profiles without a reduction in VAI $P > 0.05$. Combined therapy of *G. cambogia* plus orlistat showed the more significant effect in reduction of VAI $P < 0.05$, cardiometabolic profiles and anthropometric measures $P < 0.01$ compared to pretreatment period. **Conclusion:** Combination of *G. cambogia* with orlistat lead to more significant effect than orlistat alone in amelioration of cardiometabolic profile and VAI in obese patients.

KEY WORDS: *Garcinia cambogia*, obesity, orlistat

## INTRODUCTION

Obesity is a body fat excess as a result of positive energy balance or it is an excess of body fat mass that linked with frequent metabolic disorders such as ischemic heart diseases, dyslipidemia, type 2 diabetes mellitus, and hypertension [1,2].

Thus, the principal that been measures taken to manage the obesity like exercise and dietary restriction are often insufficient alone, so anti-obesity pharmacotherapy is needed as an adjuvant in the management of obesity [3].

Most of anti-obesity agents acting centrally leading to appetite suppression and reduction in food intake such as fenfluramine, sibutramine, and amphetamine which causes serious sympathetic activation and cardiovascular adverse effects that limit and restrict their uses chiefly in patients with cardiovascular disease [4]. Therefore, peripherally acting agents effects such as orlistat and *Garcinia cambogia* are recommended.

Orlistat is a potent and specific 47-91% of gastric lipase, 51-82% of pancreatic lipase) without any action on amylase, phospholipase and trypsin, this lead to inhibition of triglyceride (TG) hydrolysis, absorption of fatty acids, and monoglycerides from enterocytes consequently; the ingested fat absorption is diminished by about 36% [5].

Multicenter randomized trials on the efficacy of orlistat in weight reduction showed a favorable weight reduction and improvement in obesity profiles such as a reduction in insulin resistance, anthropometric measure, and improvement in lipid profiles in diabetic obese patients [6].

Orlistat was approved by Food and Drug Administration on 1999 as anti-obesity agent for long-term therapy; it is not absorbed so it's not produced any systemic manifestation, thus, it causes local gastrointestinal disorders which include reduction of fat soluble vitamin absorption (vitamin A, D, E, K), oily spot, flatulence, and increases frequency of defecations and rarely it may cause a severe hepatic damage [7].

On the other hand, *G. cambogia* contains an active constituent called hydroxycitric acid (HCA) that plays an important role in weight reduction, which is a competitive inhibitor of citrate-lyase enzyme that catalyze extra-mitochondrial citrate into acetyl coenzyme A and oxaloacetate causing a significant reduction of acetyl coenzyme A pools that limiting the availability of substrate for lipogenesis through activation of glycogenesis [8]. A study has been revealed that HCA inhibits *de novo* fatty acid and cholesterol biosynthesis leading to significant reduction of subcutaneous and visceral fat accumulations subsequently; long-term therapy with *G. cambogia* leading to significant weight reduction [9].

Regarding the cardiometabolic risk profile in obesity, visceral adiposity index (VAI) has been recognized as a clinical marker for adipose tissue dysfunction before it expands into an evident metabolic syndrome. VAI is highly associated with cardiovascular complications and insulin resistance [10], while body mass index (BMI) is incorrectly considered as a reasonable predictor for body fat percentage regardless of gender because of BMI is not always linked with cardiovascular mortalities [11]. Moreover, waist circumference (WC) has been regarded as an applicable index in the evaluation of adipose tissue distribution that is highly linked to visceral and abdominal obesity, but cannot distinguish between subcutaneous and visceral adipose tissue in the abdominal area [12].

Therefore, the objective of this study was evaluating the effect of orlistat alone or with *G. cambogia* on VAI in obese patients.

## PATIENTS AND METHODS

This randomized, population-based controlled study was done and carried out at Department of Clinical Pharmacology, College of Medicine, Al-Mustansiriya University, from June to September 2015, Baghdad-Iraq. The study was approved according to the guide of the Declaration of Helsinki and by the Institutional Review Board and Ethical Committee in College of Medicine. The procedures of the study were completely explained to all patients, and all patients provided a written consent before the contribution in this study according to the ethical resolution 33AT/2015.

### Study Design

In this study, 99 obese male patients were recruited; age range was 37-46 years. The patients were selected according to the European Society of Cardiology [13]. Appropriate patients, identified from a direct interview and according to the routine investigations while, obese patients with diabetes mellitus, renal failure, liver disorders, severe cardiovascular disorders, cerebrovascular disorders and severe anemia were excluded from this study. Obese patients were randomized into three equal groups, first group treated with orlistat 120 mg/day (xenical capsule, Roch), second group treated with *G. cambogia* 166 mg/day (Garcinia capsule, himalaya), and third group treated with both orlistat and *G. cambogia*. The duration of treatments was three consecutive months. The baseline data were recorded, then after the end of the duration of therapy; the biochemical and anthropometric measures were estimated. The patients were allocated to receive *G. cambogia* and orlistat in addition to their current treatment like antihypertensive agents. All medications were supplied free of charge. The patients were asked to avoid treatment with mineral and vitamins during the study. In addition, the patients have been advised for standard diet and avoidance of sedentary lifestyle.

### Assessment of Anthropometric and Cardio-Metabolic Measures

BMI = body weight (kg)/height ($m^2$) [14].

VAI is calculated according the specific formula which depends on WC, TG, and high-density lipoprotein (HDL) in mmol/L [15].

$$VAI = \left(\frac{1.31}{HDL}\right) \times \left(\frac{TG}{1.03}\right) \times \left(\frac{WC}{39.68 + (1.88 \times BMI)}\right)$$

### Assessment of Lipid Profile

TG, total cholesterol (TC), and HDL were assessed by specific ELISA kits; from this profile, we can measure the followings: Atherogenic index (AI) = log (TG/HDL), when TG and HDL measured in mmol/l., atherogenic coefficient (AC) = (TC-HDL)/HDL, low density lipoprotein (LDL) = (TC)-(HDL)-(TG)/5, very LDL (VLDL) = TG/5, and cardiac risk ratio (CRR) = TC/HDL [16].

Moreover, blood pressure measurements and other routine investigations such as fasting and random blood glucose were performed.

### Statistical Analysis

Statistical estimation of data was done through using the statistical package for social sciences software version 21.0 (SPSS). Data were expressed as mean ± standard error. Continuous variables were compared via analysis of variance (ANOVA), which also evaluated the significance between groups. Paired *t*-test was used to compare results before and after administration of treatments, *P* value for all statistical analysis regarded significant when it was $<0.05$.

## RESULTS

A total number of 99 obese male patients were enrolled in this study, of these 89 patients completed the study, 30 (26.7%) was allocated in orlistat group, 30 (26.7%) was allocated in *G. cambogia* and 29 (25.81%) was allocated in combined orlistat plus *G. cambogia* group.

A total number of 10 patients did not complete the study due to drug side effects, non-compliance and other reasons. At the end the duration of treatment, the study withdrawal rate was three (0.99%) [Figure 1].

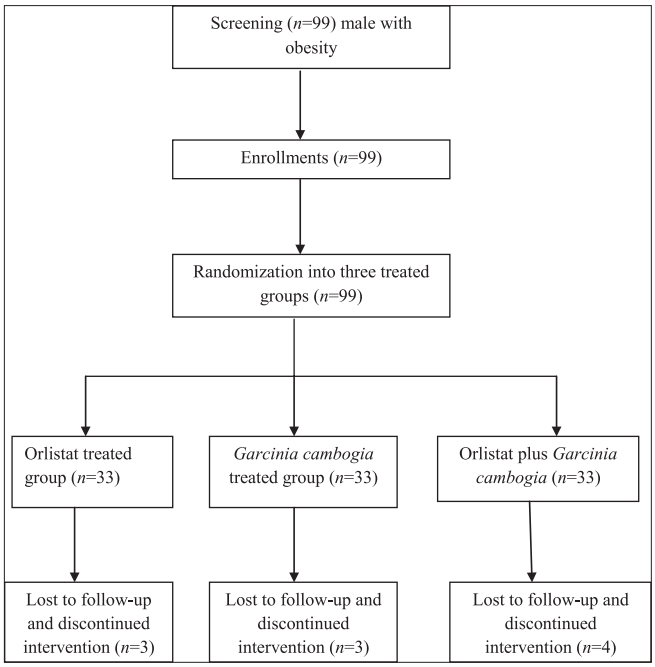


**Figure 1:** Flow chart of the present study

Most of the enrolled patients were hypertensive with dyslipidemia and other concomitant diseases that were currently treated with antihypertensive, antiplatelet, and hypolipidemic agents. The patient characteristics of the present study are shown in Table 1.

Orlistat produced significant reduction in body weight $P < 0.01$ and WC $P < 0.05$ compared to baseline parameters. Moreover, orlistat produced significant blood pressure lowering effect ($P < 0.01$). Regarding the effects of orlistat on lipid profile effect, it produced significant reductions in TC level $P < 0.0001$, VLDL $P < 0.0001$, LDL $P = 0.006$, AI $P = 0.0395$, AC $P = 0.040$, and CRR $P = 0.023$. The descriptive effects of orlistat showed in Table 2.

*G. cambogia* treatment led to significant body weight reduction ($P < 0.05$) and more significant reduction in WC ($P < 0.01$) compared with baseline data. *G. cambogia* therapy led to significant reduction in VAI $P = 0.0051$. Furthermore, *G. cambogia* produced a more significant effect on blood pressure, TC, and LDL ($P < 0.01$). Furthermore, AI ($P = 0.039$), AC ($P = 0.0381$), and CRR ($P = 0.0032$) are decreased significantly [Table 3].

Our study also revealed that combination of *G. cambogia* and orlistat showed a significant reduction in cardiometabolic indices; body weight and BMI ($P < 0.01$ and 0.05), respectively. Moreover, the combined effect demonstrated significant reductions in WC $P < 0.0001$, VAI $P = 0.023$, significant reduction in all lipid profile without significant elevation in HDL cholesterol ($P > 0.05$). Furthermore, combination treatment lowered blood pressure significantly ($P < 0.01$), [Table 4].

**Table 1: Characteristics of the study**

| Variables | Orlistat | *G. cambogia* | *G. cambogia* plus Orlistat |
|---|---|---|---|
| Number | 33 (33.33) | 33 (33.33) | 33 (33.33) |
| Age (years) | 41.22±4.64 | 42.48±7.03 | 39.73±5.83 |
| Gender (male) | 33 (33.33) | 33 (33.33) | 33 (33.33) |
| Body weight (kg) | 104.75±3.94 | 103.65±3.44 | 102.33±2.24 |
| Associated disease | | | |
| Dyslipidemia | 29 (29.29) | 30 (30.30) | 27 (27.27) |
| Hypertension | 22 (22.22) | 24 (24.24) | 25 (25.25) |
| Asthma | 2 (2.02) | 3 (3.03) | 4 (4.04) |
| Current therapy | | | |
| Statins | 27 (27.27) | 30 (30.03) | 25 (25.05) |
| Aspirin | 22 (22.02) | 29 (29.02) | 21 (21.02) |
| Clopidogrel | 11 (11.01) | 8 (8.08) | 9 (9.09) |
| Montelukast | 2 (2.02) | 3 (3.03) | 4 (4.04) |
| Theophylline | 2 (2.02) | 3 (3.03) | 4 (4.04) |
| Gemfibrozil | 15 (15.01) | 14 (14.01) | 5 (5.05) |
| Omega-3 fatty acid | 18 (18.01) | 16 (16.01) | 20 (20.62) |
| ACEI | 20 (20.02) | 20 (20.02) | 19 (19.01) |
| B-blockers | 2 (2.02) | 4 (4.04) | 6 (6.06) |

Data are presented as number, % and mean±SE; ACEI: Angiotensin-converting enzyme inhibitors. *G. cambogia: Garcinia cambogia*

**Table 2: Effects of orlistat on cardiometabolic indices in obese patients**

| Variables | Before (*n*=33) | After (*n*=30) | *P* |
|---|---|---|---|
| body weight (kg) | 104.75±3.95 | 90.55±1.87 | 0.002** |
| Height (cm) | 165.35±3.47 | 165.31±3.97 | 0.99 |
| BMI (kg/m$^2$) | 38.92±3.28 | 36.50±3.57 | 0.61 |
| WC (cm) | 101.11±3.46 | 91.76±2.11 | 0.0239* |
| HC (cm) | 116.55±5.14 | 110.88±6.99 | 0.512 |
| VAI | 4.05±0.64 | 3.32±0.21 | 0.28 |
| SBP (mmHg) | 158.73±1.34 | 143.12±1.09 | <0.0001** |
| DBP (mmHg) | 90.76±1.39 | 80.67±1.22 | <0.0001** |
| Pulse P (mmHg) | 67.96±0.05 | 62.45±0.13 | <0.0001** |
| Mean BP (mmHg) | 124.75±1.37 | 111.89±1.11 | <0.0001** |
| HDL (mg/dL) | 41.29±3.21 | 44.99±3.94 | 0.46 |
| VLDL (mg/dL) | 67.41±3.3 | 64.138±2.1 | <0.0001** |
| LDL (mg/dL) | 138.28±11.7 | 91.6±11.71 | 0.006** |
| TC (mg/dL) | 246.98±5.20 | 200.77±4.83 | <0.0001** |
| TG (mg/dl) | 337.05±16.52 | 320.69±12.76 | 0.432 |
| AI | 0.552±0.02 | 0.493±0.022 | 0.0395* |
| AC | 4.84±0.62 | 3.46±0.22 | 0.040* |
| CRR | 5.98±0.61 | 4.46±0.22 | 0.023* |
| PPG (mg/dL) | 138.77±4.62 | 139.95±5.62 | 0.87 |
| FBG (mg/dL) | 90.39±2.69 | 89.39±3.55 | 0.82 |

Data are expressed as mean±SE, *$P$<0.05, **$P$<0.01, BMI: Body mass index, WC: Waist circumference, HC: Hip circumference, VAI: Visceral adiposity index, BP: Blood pressure, SBP: Systolic blood pressure, DBP: Diastolic blood pressure, HDL: High density lipoprotein, VLDL: Very low density lipoprotein, LDL: Low density lipoprotein, TC: Total cholesterol, TG: Triglyceride, AI: Atherogenic index, AC: Atherogenic coefficient, CRR: Cardiac risk ratio, PPG: Postprandial glucose, FBG: Fasting blood glucose

One-way ANOVA test analysis, demonstrated significant differences in most cardiometabolic indices among orlistat, *G. cambogia* and orlistat plus *G. cambogia* except on height, LDL, HDL, CRR, fasting and postprandial blood glucose when $P > 0.05$, [Table 5].

**Table 3: Effects of *G. cambogia* on cardiometabolic indices in obese patients**

| Variables | Before ($n$=33) | After ($n$=30) | $P$ |
|---|---|---|---|
| body weight (kg) | 103.65±3.45 | 91.31±3.99 | 0.021* |
| Height (cm) | 165.35±2.18 | 165.31±4.97 | 0.99 |
| BMI (kg/m$^2$) | 37.91±2.29 | 33.41±1.61 | 0.110 |
| WC (cm) | 102.12±3.26 | 90.56±1.11 | 0.0016** |
| HC (cm) | 115.44±5.14 | 109.28±3.97 | 0.343 |
| VAI | 4.17±0.64 | 2.24±0.11 | 0.0051** |
| SBP (mmHg) | 151.72±3.34 | 133.12±2.09 | <0.0001** |
| DBP (mmHg) | 92.55±2.39 | 82.11±1.12 | 0.0002** |
| Pulse P (mmHg) | 59.17±1.04 | 51.01±0.97 | <0.0001** |
| Mean BP (mmHg) | 122.13±2.86 | 107.61±1.60 | <0.0001** |
| HDL (mg/dl) | 42.33±3.21 | 43.99±2.94 | 0.69 |
| VLDL (mg/dl) | 69.41±3.3 | 64.24±2.28 | 0.199 |
| LDL (mg/dL) | 139.20±6.21 | 94.16±3.22 | <0.0001** |
| TC (mg/dL) | 250.92±5.22 | 202.34±4.83 | <0.0001** |
| TG (mg/dL) | 347.05±6.52 | 321.22±5.39 | 0.0031** |
| AI | 0.554±0.01 | 0.503±0.022 | 0.039* |
| AC | 4.92±0.61 | 3.59±0.12 | 0.0381* |
| CRR | 5.92±0.41 | 4.59±0.11 | 0.0032** |
| PPG (mg/dL) | 132.77±4.62 | 135.17±3.83 | 0.68 |
| FBG (mg/dL) | 91.93±2.99 | 93.31±2.39 | 0.71 |

Data are expressed as mean±SE, *$P$<0.05, **$P$<0.01, BMI: Body mass index, WC: Waist circumference, HC: Hip circumference, VAI: Visceral adiposity index, BP: Blood pressure, SBP: Systolic blood pressure, DBP: Diastolic blood pressure, HDL: High density lipoprotein, VLDL: Very low density lipoprotein, LDL: Low density lipoprotein, TC: Total cholesterol, TG: triglyceride, AI: Atherogenic index, AC: Atherogenic coefficient, CRR: Cardiac risk ratio, PPG: Postprandial glucose, FBG: Fasting blood glucose. *G. cambogia: Garcinia cambogia*

**Table 4: Combined effect of *G. cambogia* plus orlistat on cardiometabolic indices in obese patients**

| Variables | Before ($n$=33) | After ($n$=29) | $P$ |
|---|---|---|---|
| body weight (kg) | 102.33±2.25 | 83.22±3.56 | <0.0001** |
| Height (cm) | 165.35±3.97 | 165.31±3.88 | 0.98 |
| BMI (kg/m$^2$) | 37.43±1.42 | 30.45±2.36 | 0.013* |
| WC (cm) | 103.17±3.63 | 84.16±1.54 | <0.0001** |
| HC (cm) | 114.11±5.18 | 102.17±3.11 | 0.041 |
| VAI | 3.01±0.22 | 2.17±0.17 | 0.023* |
| SBP (mmHg) | 152.93±3.28 | 133.10±2.03 | <0.0001** |
| DBP (mmHg) | 92.29±2.38 | 80.13±1.18 | <0.0001** |
| Pulse P (mmHg) | 60.64±0.9 | 52.97±1.69 | 0.0002** |
| Mean BP (mmHg) | 122.61±2.83 | 106.61±1.60 | <0.0001** |
| HDL (mg/dL) | 43.42±3.87 | 49.11±2.14 | 0.20 |
| VLDL (mg/dL) | 70.05±3.50 | 44.38±2.06 | <0.0001** |
| LDL (mg/dL) | 140.6±9.5 | 93.8±5.31 | <0.0001** |
| TC (mg/dL) | 254.12±5.83 | 187.31±2.53 | <0.0001** |
| TG (mg/dL) | 350.15±17.52 | 221.92±10.30 | <0.0001** |
| AI | 0.547±0.02 | 0.295±0.022 | <0.0001** |
| AC | 4.85±0.61 | 2.81±0.11 | 0.0022** |
| CRR | 5.85±0.11 | 3.81±0.01 | <0.0001** |
| PPG (mg/dL) | 131.62±4.11 | 131.17±3.49 | 0.933 |
| FBG (mg/dL) | 91.19±2.39 | 91.10±2.10 | 0.977 |

Data are expressed as mean±SE, *$P$<0.05, **$P$<0.01, BMI: Body mass index, WC: Waist circumference, HC: Hip circumference, VAI: Visceral adiposity index, BP: Blood pressure, SBP: Systolic blood pressure, DBP: Diastolic blood pressure, HDL: High density lipoprotein, VLDL: Very low density lipoprotein, LDL: Low density lipoprotein, TC: Total cholesterol, TG: Triglyceride, AI: Atherogenic index, AC: Atherogenic coefficient, CRR: Cardiac risk ratio, PPG: Postprandial glucose, FBG: Fasting blood glucose. *G. cambogia: Garcinia cambogia*

The compliance to the treatment was good, but some drug adverse effects were reported during the duration of the study among treated groups; fatty spot was the most reported side effect 7.92% in orlistat group which was decreased to the 0.99% in combined *G. cambogia* plus orlistat group, [Table 6].

## DISCUSSION

This study revealed a new modality in the treatment of obesity through combination of drug and herbal medicine. Orlistat therapy in the current study produced a significant reduction in BMI, TC, WC without significant effects on VAI compared to baseline values. These findings corresponded with other studies which demonstrated that orlistat therapy produced a significant body weight reductions in obese patients with additional amelioration on lipid profile, anthropometric measures, inflammatory markers (tumor necrosis factor α [TNF-α] and interleukin 6 [IL-6]), and cardiometabolic risk factors [17,18].

Orlistat leads to dose-dependent inhibition of intestinal fat absorption through suppression of intestinal lipase activity [19] which *per se* explains the improvement in TC levels, VLDL, LDL AI, AC, and CRR.

TG serum levels were not decreased during orlistat therapy in the present study since; TGs hydrolyzed before inhibition of intestinal lipase by orlistat [20] or due to small sample size that may affects the statistical results, patients non-compliance or small dose that may not affect high TG levels while; a reduction in serum cholesterol levels in orlistat-treated group may be through inhibition of 3-hydroxy-3-methylglutaryl coenzyme A reductase, decreasing in cholesterol intestinal absorption through decreasing of bilirubin blood concentration (via augmentations of bilirubin turnover and fecal excretion). Orlistat also decreases postprandial free fatty acid levels leading to the reduction in TG and cholesterol biosynthesis with a significant increment in insulin sensitivity since; free fatty acids interfere with insulin action [21]. Unfortunately, insulin resistance was not measured in the present study.

Furthermore, orlistat in the present study led to significant reduction in systolic and diastolic blood pressure, since treatment with orlistat in obese hypertensive patients, leads to the improvement in endothelial functions and significant vasodilatation that causing significant reductions in blood pressur [22].

As well, the presence of vascular endothelial dysfunction in obesity favor the development of cardiovascular disorders due to the ability of adipocytes for synthesis and secretion of adipocytokines - such as TNF-α, IL-6, and resistin - which play a potential role in induction of low-grade inflammatory changes at vascular endothelium causing obesity-induced vascular dysfunction [23]. Therefore, administration of orlistat leads to direct effect via reduction in adipocytes-induced inflammatory markers and indirect effect through augmentation of postprandial glucagon like peptide-1 that inhibits adipose tissue inflammatory mediators [24]. Moreover, orlistat produced significant homeostatic effects through reduction in plasma fibrinogen and P-selectin levels in obese patient [25]. All of these findings indicating a vascular protective effect of orlistat,

**Table 5: Variations of post-treatment effects among orlistat, *G. cambogia* and orlistat plus *G. cambogia* (intergroup comparisons)**

| Variables | Orlistat | *G. cambogia* | Combined | ANOVA | |
|---|---|---|---|---|---|
| | | | | F | P |
| body weight | 90.55±1.87 | 91.31±3.99 | 83.22±3.56**¶¶ | 9.62 | 0.0139$^{x}$ |
| Height (cm) | 165.31±3.97 | 165.31±4.97 | 165.31±3.88 | 0.000 | 1.00$^{x}$ |
| BMI (kg/m$^2$) | 36.50±3.57 | 33.41±1.61 | 30.45±2.36**¶ | 5.40 | 0.046$^{x}$ |
| WC (cm) | 91.76±2.11 | 90.56±1.11 | 84.16±1.54**¶¶ | 25.04 | 0.001$^{xx}$ |
| HC (cm) | 110.88±6.99 | 109.28±3.97 | 102.17±3.11**¶¶ | 3.58 | 0.095 |
| VAI | 3.32±0.21 | 2.24±0.11 | 2.17±0.17* | 726 | 0.0005$^{xx}$ |
| SBP (mmHg) | 143.12±1.09 | 133.12±2.09 | 133.10±2.03* | 33.4 | 0.001$^{xx}$ |
| DBP (mmHg) | 80.67±1.22 | 82.11±1.12 | 80.13±1.18¶ | 3.14 | 0.11 |
| Pulse P (mmHg) | 62.45±0.13 | 51.01±0.97 | 52.97±1.69** | 336.4 | 0.0005$^{xx}$ |
| Mean BP (mmHg) | 111.89±1.11 | 107.61±1.60 | 106.61±1.60* | 23.59 | 0.001$^{xx}$ |
| HDL (mg/dL) | 44.99±3.94 | 43.99±2.94 | 49.11±2.14*¶ | 3.89 | 0.08 |
| VLDL (mg/dL) | 64.138±2.1 | 64.24±2.28 | 44. 38±2.06**¶¶ | 102.07 | 0.0005$^{xx}$ |
| LDL (mg/dL) | 91.6±11.71 | 94.16±3.22 | 93.8±5.31 | 0.130 | 0.88 |
| TC (mg/dL) | 200.77±4.83 | 202.34±4.83 | 187.31±2.53**¶¶ | 17.06 | 0.003$^{xx}$ |
| TG (mg/dL) | 320.69±12.76 | 321.22±5.39 | 221.92±10.30**¶¶ | 33.51 | 0.001$^{xx}$ |
| AI | 0.493±0.022 | 0.503±0.022 | 0.295±0.022 | 416 | 0.0005$^{xx}$ |
| AC | 3.46±0.22 | 3.59±0.12 | 2.81±0.12 | 1999.6 | 0.0005$^{xx}$ |
| CRR | 4.46±0.22 | 4.59±0.11 | 3.81±1.11* | 4.27 | 0.07 |
| PPG (mg/dL) | 139.95±5.62 | 135.17±3.83 | 131.17±3.49**¶ | 4.04 | 0.07 |
| FBG (mg/dL) | 89.39±3.55 | 93.31±2.39 | 91.10±2.10** | 1.23 | 0.34 |

Data are expressed as mean±SE, *$P<0.05$, **$P<0.01$ combined versus orlistat group, ¶$P<0.05$, ¶¶$P<0.01$ combined versus *G. cambogia* group, $^{x}P<0.05$, $^{xx}P<0.01$ regarding *post-hoc* ANOVA test. BMI: Body mass index, WC: Waist circumference, HC: Hip circumference, VAI: Visceral adiposity index, SBP: Systolic blood pressure, DBP: Diastolic blood pressure, HDL: High density lipoprotein, VLDL: Very low density lipoprotein, LDL: Low density lipoprotein, TC: Total cholesterol, TG: Triglyceride, AI: Atherogenic index, AC: Athrogenic coefficient, CRR: Cardiac risk ratio, PPG: Postprandial glucose, FBG: Fasting blood glucose. *G. cambogia: Garcinia cambogia*

**Table 6: Side effects of treatment during the study**

| Side effects | *G. cambogia* | Orlistat | *G. cambogia*+Orlistat |
|---|---|---|---|
| Abdominal pain | 4 (1.32) | 12 (3.96) | 3 (0.99)** |
| Fatty spots | 0 | 24 (7.92) | 6 (1.98)**¶ |
| Flatulence | 2 (0.66) | 12 (3.96) | 3 (0.99)** |
| Diarrhea | 3 (0.99) | 9 (2.97) | 4 (1.32)* |
| Constipation | 5 (1.65) | 1 (0.33) | 3 (0.99)** |
| Headache | 13 (4.29) | 2 (0.66) | 2 (0.66)**¶ |
| Heartburn | 9 (2.97) | 5 (1.65) | 4 (1.32)*¶ |

Data are expressed as % and numbers. **$P<0.01$;*$P<0.05$ combined versus orlistat group. ¶$P<0.05$ combined versus *G. cambogia*.
*G. cambogia: Garcinia cambogia*

which *per se* explaining the blood pressure lowering effect in the current study. Unluckily, these biomarkers and endothelial function were not measured in the present study.

Therefore, in spite of weight reduction effects of orlistat on body weight it failed in the reduction of VAI, this finding is incompatible with findings of Smith *et al.* study that revealed a significant reduction in visceral adiposity following orlistat therapy [26].

Regarding the effect of G. *cambogia* on obesity indices, it reduced body weight, BMI, WC and VAI; these findings correspond with a study that showed a significant reduction in anthropometric indices with G. *cambogia* treatment [27] but other studies reported non-significant differences between *G. cambogia* and placebo during 12$^{th}$ weeks of treatment on anthropometric indices [28].

Furthermore, this study revealed a significant reduction in TC, total TG, AI, AC, CRR, and blood pressure lowering effect significantly due to the treatment with G. *cambogia* that led to a reduction in the visceral and subcutaneous fat accumulations regardless of gender effect [29]. This potential effect of G. *cambogia* was due to the effects of the active ingredient, HCA that inhibits lipogenesis and reduction in LDL levels that causing amelioration in vascular and visceral functions [30]. These observations may explain the significant reduction in VAI in the present study.

Several animal and human studies reported that treatment of obesity with G. *cambogia* led to significant body weight loss compared with placebo, but these studies were conducted on small groups and for short duration <12$^{th}$ weeks [31] while in the present study the duration of therapy reached the duration of 3 months with minimal adverse effects.

In addition to the peripheral effect, G. *cambogia* has a central appetite suppressant effect through upregulation of serotonin neurotransmission [32]. Therefore, Semwal *et al.* study pointed out that G. *cambogia* acts in the dual pathway through central and peripheral suppressant effects causing a more significant reduction in visceral adiposity compared to orlistat alone [33].

Furthermore, effects of combined treatment with G. *cambogia* plus orlistat on obesity indices demonstrated a significant reduction in most of the obesity and cardiometabolic risk scores mainly on hip circumference and VAI.

VAI is a mathematical model that depends on BMI, WC, HDL and TG serum levels, VAI is gender dependent that reflect fat distribution, it linked with cardiometabolic risk scores and visceral adiposity-induced complications such as hypertension and diabetes mellitus [34]. Thus, combined therapy of

*G. cambogia* plus orlistat led to a more significant reduction in VAI which pointed out to the potential additive effect of *G. cambogia* plus orlistat on weight reduction in obesity and obesity-induced complications.

Phytochemical analysis of *G. cambogia* detected polyphenolic flavonoids that inhibit pancreatic lipase activity which may explain the additive effect of *G. cambogia* with orlistat on weight reduction in obese patients [35]. Moreover, HCA isomer (2s, 3r) block pancreatic amylase and intestinal α-glucosidase leading to a reduction in the carbohydrate metabolism and absorption with significant reduction in blood glucose in diabetic patients [36]; this effect was not observed in our study since; all of the enrolled patients were non-diabetic. Consequently, *G. cambogia* potentiates the effect of orlistat in obese patients.

Regarding the reported side effects in the present study, combined treatment significantly showed less adverse effects compared to either *G. cambogia* or orlistat since, both drugs are well tolerated and *G. cambogia* induces feeling of satiety that decreases food intake and gastrointestinal disturbances [37,38].

## CONCLUSION

Combinations of *G. cambogia* and orlistat have a more favorable effect than orlistat alone on cardiometabolic profile and VAI in obese patients.

## LIMITATIONS OF THE STUDY

This study had many limitations. First, the total daily calorie intake was not determined and collected. Second, lifestyle and exercise were not documented. Finally, 3 months duration of treatment may be short for estimation the long-term effects of *G. cambogia* and orlistat.

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# Evaluation of anti-epileptic activity of leaf extracts of *Punica granatum* on experimental models of epilepsy in mice

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## ABSTRACT

**Objectives:** This study was aimed to examine the anti-epileptic activity of leaf extracts of *Punica granatum* in experimental models of epilepsy in Swiss albino mice. **Materials and Methods:** Petroleum ether leaf extract of *P. granatum* (PLPG), methanolic LPG (MLPG), and aqueous LPG (ALPG) extracts of *P. granatum* leaves was initially evaluated against 6-Hz-induced seizure model; the potent extract was further evaluated against maximal electroshock (MES) and pentylenetetrazole (PTZ)-induced convulsions. Further, the potent extract was evaluated for its influence on Gamma amino butyric acid (GABA) levels in brain, to explore the possible mechanism of action. In addition, the potent extract was subjected to actophotometer test to assess its possible locomotor activity deficit inducing action. **Results:** In 6-Hz seizure test, the MLPG has alleviated 6-Hz-induced seizures significantly and dose dependently at doses 50, 100, 200, and 400 mg/kg. In contrast, PLPG and ALPG did not show any protection, only high dose of ALPG (400 and 800 mg/kg, p.o.) showed very slight inhibition. Based on these observations, only MLPG was tested in MES and PTZ models. Interestingly, the MLPG (50, 100, 200 and 400 mg/kg) has offered significant and dose-dependent protection against MES ($P < 0.01$) and PTZ-induced ($P < 0.01$) seizures in mice. Further, MLPG showed a significant increase in brain GABA levels ($P < 0.01$) compared to control and showed insignificant change in locomotor activity in all tested doses (100, 200 and 400 mg/kg). Interestingly, higher dose of MLPG (400 mg/kg, p.o.) and Diazepam (5 mg/mg, p.o.) have completely abolished the convulsions in all the anticonvulsant tests. **Conclusion:** These findings suggest that MLPG possesses significant anticonvulsant property, and one of the possible mechanisms behind the anticonvulsant activity of MLPG may be through enhanced GABA levels in the brain.

KEY WORDS: 6-Hz seizures, anticonvulsant, diazepam, epilepsy, herbal medicine, maximal electroshock-induced seizures, pentylenetetrazole-induced seizures, *Punica granatum* leaves

## INTRODUCTION

Epilepsy is a serious neurological disease characterized by the transient occurrence of abnormal, excessive and/or synchronous neuronal activity in the brain, associated with various neurobiological, cognitive and psychological signs and/or symptoms [1]. Conventionally various chemical classes of drugs such as benzodiazepines (Diazepam), barbiturates (Phenobarbitone), gamma amino butyric acid (GABA) analogs, succinimides (ethosuximide), hydantoins (Phenytoin), carbamazepine, etc., were extensively used in the management of epilepsy [2]. Recently, many newer class of drugs such as vigabatrin, levetiracetam, topiramate, lamotrigine, zonisamide, lacosamide, rufinamide, and stiripentol have been developed, and they are considered to be comparatively safe [3]. However, despite of copious efforts, all the currently available drugs have one or more inherent side/adverse effects such as dizziness, mental slowing, ataxia, impaired concentration, mental confusion, sleep disturbance, anorexia, somnolence, and aggression [3]. Hence, there is great scope for safe and potent drug for the management of epilepsy, in his context herbal drugs are considered to have better edge over synthetic drugs, therefore many researchers are focusing on herbal remedies, to discover better and safe medicine for epilepsy. In this context, in literature, many plant-based medicines such as *Melanthera scanden* [4], *Myrtus communis* [5], *Abies webbiana* Lindl. [6], *Crocus sativus* [7], and *Dodonaea viscosa* (Linn.) [8] have been scientifically proved to possess potent anticonvulsant property in various experimental models of epilepsy. Similarly, in traditional system of medicine various parts of *Punica granatum* L. (belongs to family Punicaceae) has been used to treat varieties of ailments, and various parts of the plant have been scientifically proved for diverse biological activities such as antioxidant [9], hepatoprotective [10], antidiarrheal [11], antiulcer [12], anti-

inflammatory [13], antimalarial [14], atherosclerosis and thyroid dysfunction [15], antimutagenic [16], immunomodulatory [17], memory enhancing [18], wound healing [19], and anticancer [20] activities; thus, *P. granatum* is one of the most common and potent plant-based medicine in the management of various ailments. Considering the strong literature reports on antioxidant and neuroprotective actions of *P. granatum*, this study was undertaken to evaluate the anticonvulsant activity of leaf extracts of *P. granatum* in various experimental models of epilepsy.

## MATERIALS AND METHODS

### Drugs and Chemicals

Phenytoin was purchased from Sun Pharmaceutical Industries Ltd., Mumbai, India, diazepam was procured from Ranbaxy Laboratories, New Delhi, India, and pentylenetetrazole (PTZ) and GABA was purchased from Sigma-Aldrich, Bengaluru, India. All other solvents and chemicals used were of analytical grade purchased from Hi-Media Laboratories Pvt., Ltd., Bengaluru, India.

### Collection of Plant Material

The leaves of *P. granatum L.* were collected from Doddathekahalli, Sidlaghatta (T), Chikkaballapura (D) (Karnataka, India) in the month of May-June 2015 and it was identified and authenticated by Dr. K. Madhava Chetty, Professor of Botany, Sri Venkateshwara University, Tirupati, Andhra Pradesh, India. A voucher specimen of the plant material is preserved in the department with Voucher specimen number 1213.

### Plant Material Preparation

The shade dried leaves of *P. granatum* were powdered, sieved and subjected to extraction process as follows. The plant material was extracted with various organic solvents successively in the ascending order of their polarity (petroleum ether, methanol, and water). In brief, 100 g of the plant material was initially defatted with 1 L of petroleum ether in a round bottom flask at room temperature for 24 h; the marc obtained was completely dried and extracted with 1 L of methanol at 60°C for 48 h in Soxhlet apparatus, subsequently, the marc obtained was extracted with 1 L of double distilled water in a round bottom flask, at room temperature for 48 h. Extracts obtained were concentrated at room temperature under reduced pressure, using Rota-evaporator (Rotavap-Remi instruments).

### Formulation of Extracts for Administration

Various concentrations of petroleum ether leaf extract of *P. granatum* (PLPG), methanolic LPG (MLPG), and aqueous LPG (ALPG) were prepared in 3% tween 80 in distilled water. In short, required quantity of the extract was weighed and suspended in tween 80 and distilled water was added slowly with continuous shaking and volume was made up with distilled water, the concentration of tween 80 in the final formulation would be 3%. All the preparations were freshly prepared and administered by oral route at 10 ml/kg dose volume.

### Experimental Protocol

*Experimental animals*

Inbred Swiss albino mice (25-30 g) were used for the study. The animals were maintained in polypropylene cages at a temperature of 25°C ± 1°C and relative humidity of 45-55% in a clean environment under 12 h light/dark cycle. The animals had free access to standard rat/mice pellets (Pranav Agro Industry, Bengaluru, India) and purified water *ad libitum*.

All the experimental protocols were approved by Institutional Animal Ethics Committee (IAEC) (Protocol No. IAEC/KMC/10/2016) and were conducted according to the principles and guidelines of the Committee for the Purpose of Control and Supervision of Experimentation on Animals, India.

### Acute Oral Toxicity Studies

Acute oral toxicity of leaf extracts of *P. granatum* was determined by as per the OECD Guideline No. 425 - up and down procedure. In brief, nulliparous and non-pregnant female mice weighing between 25 and 30 g were fasted for 3 h and they were administered with single dose of extract, and the animals were observed for the toxic signs with special emphasis to changes in skin and fur, eyes and mucous membranes, and also respiratory, circulatory, autonomic and central nervous systems, and somatomotor activity and behavior pattern. Attention should be directed to observations of tremors, convulsions, salivation, diarrhea, lethargy, sleep and coma and mortality initially for 48 h (short term toxicity), animals found alive were further observed for 14 days (long-term outcomes). Lethal dose 50% was calculated using AOT425 stat program (OECD Guidelines No. 425, 2001) [21].

### 6 Hz Seizure Test

This test was carried out as per the previously described procedure [22,23]. In brief, mice were pretreated with extracts (doses ranging from 50 to 800 mg/kg, p.o.) for 3 days, the dose selection was done based on acute toxicity study, 100 mg/kg dose was selected and based on the response observed at 100 mg/kg, further dose levels were selected to establish dose-response correlation. Hence, in the process of establishing the dose-response correlation minimum dose was started with 50 mg/mg and saturation was observed after 200 mg/kg.

On Day-3, 1 h after the last dose, tetracaine ophthalmic solution (0.5% tetracaine hydrochloride) was applied, 30 min later the corneal electrodes were wetted with saline and electrical stimulation was given for 3 s (200 $\mu$s-duration, 32-mA mono polar rectangular pulses at 6 Hz) to all animal, using a constant current device ECT Unit 5780 (Ugo Basile, Comerio, Italy). In general, after the electrical stimulation, a stunned posture will be seen along with rearing and repeated movements that lasted

from 60 to 120 s in control animals. After the seizures, animals will be resumed to normal exploratory behavior. The end point in this model is defined as protection against the seizures, the animals which gain their normal exploratory behavior within 10 s of stimulation are considered as protected [24].

$$\%\text{Protection} = 100 - [(\frac{\text{Number of animal showed seizures}}{\text{Total number of animals used}}) \times 100]$$

### Maximal Electroshock (MES)-induced Convulsions

In bred, male Swiss albino mice (25-30 g weight) were randomized and assigned in six groups, each group consisting of eight animals ($n = 8$). The respective group animals were treated with vehicle (3% tween 80) or phenytoin (25 mg/kg) or MLPG (50, 100, 200 and 400 mg/kg) orally for 3 days. On the 3rd day, exactly 1 h after the assigned treatment, tetracaine ophthalmic solution (0.5% tetracaine hydrochloride) was applied to cornea. Immediately after the electrical stimulation, individually all the animals were observed in an open top plastic cage for 30 min. The parameters such as duration of hind limb flexion, hind limb extensor, stupor, and death/survival were recorded during the observation, and the percentage protection is calculated using survival rate using previously provided equation [25-27].

### PTZ-induced Convulsions

In bred, male Swiss albino mice (25-30 g) were divided into six groups each group consisting of eight animals ($n = 8$). The respective group animals were treated with either vehicle or diazepam (3 mg/kg) or MLPG (50, 100, 200 and 400 mg/kg) orally for 3 days. On the 3rd day, 1 h after the assigned treatment PTZ (80 mg/kg) was intraperitoneally administered to all the experimental animals, and they were individually observed in a plastic cage initially for 30 min and the animals survived were observed later up to 24 h. During the individual observation, the parameters such as onset of clonus, onset of tonic convulsions, and death/survival were recorded and expressed as percentage protection using the equation given previously [28].

### Effect of MLPG on GABA levels in the Brain

Male Swiss albino mice (25-30 g) were treated with MLPG (200 and 400 mg/kg, p.o.) and diazepam (3 mg/kg, p.o.) for 1 day (single dose), and 3 days (three doses) in two different experimental sets. The first experimental set animals were sacrificed on day 1, 1 h after the dosing, while the second set animals were sacrificed on day 3, 1 h after the assigned treatment. The brain was collected and freezed, and subsequently, the cerebellum and whole brain were separated using microtome blade. These tissues were weighed and transferred to 10 ml of ice-cold 0.1 M perchloric acid containing 15 $\mu$g/ml of valine (internal standard). Under ice-cold conditions the contents were homogenized and centrifuged at 5000 rpm for 10 min at 4°C, the supernatant obtained was subjected to GABA estimation as per Suher *et al.* [29,30]. In short, 100 $\mu$l of each supernatant of the samples or the standard GABA is added to a micro-tube containing 100 $\mu$l of 0.1 M potassium carbonate solution, to induced dansylation reaction. These solutions were vortexed thoroughly and centrifuged at 10000 rpm for 10 min. Later, 100 $\mu$l of supernatant from each sample was transferred into a Pyrex tube containing 100 $\mu$l of 0.1 M sodium hydrogen carbonate solution, to this mixture 400 $\mu$l of working dansyl chloride solution (1.25 mg/ml anhydrous acetone) was added. The tubes were vortexed for 30 s and incubated at 90°C in benchtop oven for 30 min, during the incubation period the tubes were not capped to allow most of the solvent to get evaporate. Subsequently, the tubes taken out of the oven, and allowed to cool to room temperature. The dansylated derivatives obtained were transferred to 1.5 ml microtubes and subjected to high-performance liquid chromatography (HPLC) analysis. For HPLC analysis, C8 reversed-phase HPLC columns (5 $\mu$m, 250 × 3.2 mm) was used, mobile phase consisting of deionized and helium degassed water: Acetonitrile (HPLC grade) mixture (65:35 v/v) containing 0.15% v/v phosphoric acid, and flow rate was kept at 0.5 ml/min. The fluorescence detector with excitation and emission wavelength of 333 nm and 532 nm were adjusted, respectively. Exactly, 25 $\mu$l of the dansylated GABA samples were transferred to HPLC micro-sample vials and injected into the column. The retention time of GABA and internal standard were determined, and peak ratios of the samples were calculated with reference to the internal standard. The quantification of GABA in the unknown biological sample is done using the calibration curve of standard GABA and expressed as ng/g of tissue [29]. The calibration curve for standard GABA is established by subjecting various concentrations (25-6400 ng/ml) of standard GABA stock solutions to dansylation process similar to unknown sample supernatants.

### Actophotometer Test

The actophotometer test was performed to evaluate the effect of MLPG on locomotor activity. In short, 48 male Swiss albino mice (25-30 g) were assigned into six groups ($n = 8$), and they were treated similar to previous tests. The locomotor activity was measured as a number of photocell counts by placing the animals individually in an actophotometer for 10 min, before and exactly at 60 min after the respective treatment [31].

The change in the locomotor activity was derived using photocell counts recorded before and after the assigned treatments, and reduction in locomotor activity is calculated using the following equation.

$$\%\text{Reduction in locomotor activity} = 100 - [(\frac{\text{Photocell counts after treatment}}{\text{Photocell counts before treatment}}) \times 100]$$

### Statistical Analysis

The data obtained was expressed as mean ± SEM. The locomotor activity results were statistically analyzed by paired *t*-test. Remaining all experimental findings were statistically analyzed by one-way analysis of variance followed by Dunnett's multiple comparison test using GraphPad Prism software version 5.0 for Windows (GraphPad Software, San Diego

California USA). The $P < 0.05$ was considered as statistically significant.

## RESULTS

### Extraction of Plant Material

The extractive values of PLPG, MLPG and ALPG extracts were found to be 0.69%, 12.52%, and 7.41% w/w (gram by gram), respectively.

### Acute Oral Toxicity Study

Acute oral toxicity study for the plant extracts was performed as per OECD Guidelines No. 425 up and down method. The outcome of the study showed that PLPG, MLPG, and ALPG were safe upto 2000 mg/kg, p.o. Further, no signs of toxicity were observed during short-term (48 h) and long-term (14 days) observation period.

### Anticonvulsant Activity

*6 Hz seizure test*

In 6 Hz seizure test, PLPG (100, 200 and 400 mg/kg), MLPG (50, 100, 200, 400 mg/kg), and ALPG (50, 100, 200, 400 and 800 mg/kg) were evaluated. In this model, the MLPG (50, 100, 200, 400 mg/kg) has alleviated 6-Hz-induced seizures significantly and dose dependently. In contrast, PLPG and ALPG did not show any protection, only high dose of ALPG (400 and 800 mg/kg, p.o.) showed very slight inhibition [Table 1].

### MES-induced Convulsions

Based on the outcomes of 6-Hz seizure test, only MLPG was further evaluated in MES and PTZ models. Interestingly, the MLPG (50, 100, 200 and 400 mg/kg) has offered significant and dose-dependent protection against MES-induced HLTF ($P < 0.01$), HLTE ($P < 0.01$) and stupor ($P < 0.01$) compared to control. Further, the high dose of MLPG has completely abolished hind limb tonic extensor and hind limb tonic flexion. Noteworthy, the reference phenytoin (25 mg/kg) has completely abolished MES-induced convulsions and mortality, and the effective dose ($ED_{50}$) value of MLPG was found to be 187.50 mg/kg [Table 2].

Table 1: Effect of *Punica granatum* leaf extracts on 6-Hz induced seizures in mice

| Treatment | Number of animals showed seizures/total animals used | % protection |
|---|---|---|
| Control (3% tween 80) | 8/8 | 0 |
| Diazepam 5 mg/kg, p.o. | 0/8 | 100 |
| PLPG 100 mg/kg, p.o. | 8/8 | 0 |
| PLPG 200 mg/kg, p.o. | 8/8 | 0 |
| PLPG 400 mg/kg, p.o. | 8/8 | 0 |
| MLPG 50 mg/kg, p.o. | 3/8 | 37.5 |
| MLPG 100 mg/kg, p.o. | 5/8 | 62.5 |
| MLPG 200 mg/kg, p.o. | 7/8 | 87.5 |
| MLPG 400 mg/kg, p.o. | 8/8 | 100 |
| ALPG 50 mg/kg, p.o. | 8/8 | 0 |
| ALPG 100 mg/kg, p.o. | 8/8 | 0 |
| ALPG 200 mg/kg, p.o. | 8/8 | 0 |
| ALPG 400 mg/kg, p.o. | 2/8 | 25 |
| ALPG 800 mg/kg, p.o. | 2/8 | 25 |

PLPG: Petroleum ether extract of *Punica granatum*, MLPG: Methanolic ether extract of *Punica granatum*, ALPG: Aqueous leaf extract of *Punica granatum*, *P. granatum: Punica granatum*

### PTZ-induced Convulsions

In continuation with MES model, MLPG (50, 100, 200 and 400 mg/kg) was evaluated against PTZ-induced seizures in mice. On administration of PTZ (80 mg/kg, i.p.) the control group animals have showed clonic and tonic convulsions and death. Remarkably, MLPG (50, 100, 200 and 400 mg/kg) has prolonged the PTZ-induced onset of clonus, onset of tonic, and reduced the mortality rate dose dependently compared to control. Exceptionally, a high dose of MLPG (400 mg/kg) has completely inhibited the PTZ-induced tonic convulsions and mortality, and the $ED_{50}$ value of MLPG was found to be 161.36 mg/kg [Table 3].

### Effect of MLPG on GABA Levels in the Brain

In this study, MLPG has evolved as a good anticonvulsant extract in all models (6 Hz Seizure Test, MES and PTZ models), further to explore the mechanism behind the anticonvulsant activity, various doses of MLPG (200 and 400 mg/kg) were evaluated for its influence on GABA levels in various parts of brain. The outcomes of the study showed significant elevation of GABA levels in cerebellum ($P < 0.01$) and whole brain other than cerebellum ($P < 0.01$), in both single dose (Day-1)and multiple doses (3 days) administration of MLPG compared to control. In similar lines, the reference standard diazepam (3 mg/kg) also showed significant elevation of GABA compared to control ($P < 0.01$) [Table 4].

### Actophotometer Test

Currently many potent anticonvulsant drugs are available in the market, however, most of these drugs have adverse effects related to musculoskeletal functions, with this hypothesis we thought to evaluate the MLPG for its influence on normal locomotor activity by actophotometer test, using diazepam (5 mg/kg) as a reference drug. Diazepam has significantly reduced the photocell counts and thereby decreased the locomotor activity (78.34%) significantly compared to before treatment. Furthermore, the MLPG has shown slight reduction in photocell counts compared to their basal values. However, no significant reduction in locomotor activity was observed in MLPG treatment compared to diazepam [Table 5].

## DISCUSSION

Epilepsy is one of the chronic and most common neurological disorders, affecting approximately 50 million people worldwide [32]. The basic and major mechanisms associated with epilepsy are increased synaptic connectivity of neurons

Table 2: Effect of methanolic leaf extract of *Punica granatum* on MES-induced convulsions in mice

| Treatment | Duration of HLTE[a] (s) | Duration of HLTF[a] (s) | Stupor[a] (s) | Number of animals survived/used | % protection | $ED_{50}$ value |
|---|---|---|---|---|---|---|
| Control (3% tween 80) | 17.63±0.78 | 7.13±0.4 | 127.38±4.9 | 0/8 | 0 | - |
| Phenytoin 25 mg/kg, p.o. | ND | ND | 49.75±2.7† | 8/8 | 100 | - |
| MLPG 50 mg/kg, p.o. | 12.25±0.9* | 5.38±0.5* | 109.38±5.4* | 1/8 | 12.5 | 187.50 mg/kg |
| MLPG 100 mg/kg, p.o. | 9.63±0.6† | 3.88±0.6† | 96.88±4.6† | 3/8 | 37.5 | |
| MLPG 200 mg/kg, p.o. | 4.63±0.4† | 1.13±0.3† | 88.13±5.9† | 5/8 | 62.5 | |
| MLPG 400 mg/kg, p.o. | ND | ND | 68.25±5.1† | 7/8 | 87.5 | |

[a]All of the values are expressed as mean±SEM ($n$=8); means of various groups were statistically compared by one way ANOVA followed by Dunnett's multiple comparison test using Graph Pad version 5.0. *$P$<0.01, †$P$<0.001 compared with control (3% tween 80). MLPG: Methanolic leaf extract of *Punica granatum*, HLTE: Hind limb tonic extensor, HLTF: Hind limb tonic flexion, ND: Not detected, *P. granatum: Punica granatum*

Table 3: Effect of methanolic leaf extract of *Punica granatum* on PTZ-induced convulsions in mice

| Treatment | Onset of clonus[a] (s) | Onset on tonic[a] (s) | Number of animals survived/used | % protection | $ED_{50}$ value |
|---|---|---|---|---|---|
| Control (3% tween 80) | 38.8±2.7 | 73.6±5.3 | 0/8 | 0 | - |
| Diazepam 5 mg/kg, p.o. | ND | ND | 8/8 | 100 | - |
| MLPG 50 mg/kg, p.o. | 48.3±3.3* | 83.7±5.3* | 1/8 | 12.5 | 161.36 mg/kg |
| MLPG 100 mg/kg, p.o. | 64.6±2.6† | 101.3±8.1† | 3/8 | 37.5 | |
| MLPG 200 mg/kg, p.o. | 148.2±5.7† | 187.8±11.5† | 5/8 | 62.5.0 | |
| MLPG 400 mg/kg, p.o. | 193.7±7.3† | ND | 8/8 | 100 | |

[a]All of the values are expressed as mean±SEM ($n$=8); means of various groups were statistically compared by one way ANOVA followed by Dunnett's multiple comparison test using Graph Pad version 5.0. *$P$<0.01, †$P$<0.001 compared with control (3% tween 80). MLPG: Methanolic leaf extract of *Punica granatum*, ND: Not detected, *P. granatum: Punica granatum*

Table 4: Effect of methanolic leaf extract of *Punica granatum* on GABA levels in brain

| Treatment | GABA levels (ng/g of tissue) | | | |
|---|---|---|---|---|
| | Cerebellum | | In whole brain other than cerebellum | |
| | 1 h after treatment | 1 h after 3 days treatment | 1 h after treatment | 1 h after 3 days treatment |
| Vehicle control (3% tween 80) | 411.8±29.3 | 425.2±26.8 | 2375.6±117.7 | 2491.3±124.5 |
| Diazepam 3 mg/kg, p.o. | 1363.2±37.3* | 2091.5±139.5* | 4831.5±115.8* | 6241.0±103.4* |
| MLPG 200 mg/kg, p.o. | 631.8±28.4* | 854.3±45.6* | 3221.1±115.6* | 3583.6±133.8* |
| MLPG 400 mg/kg, p.o. | 964.5±21.9* | 1427.7±45 0.5* | 4228.8±184.6* | 4727.5±145.5* |

All of the values are expressed as mean±SEM ($n$=6); means of various groups were statistically compared by ANOVA followed by Dunnett's multiple comparison test using Graph Pad version 5.0. *$P$<0.001 compared with Vehicle control. MLPG: Methanolic leaf extract of *Punica granatum*, GABA: Gamma amino butyric acid, *P. granatum: Punica granatum*

Table 5: Effect of methanolic leaf extract of *Punica granatum on locomotor activity (Actophotometer test)*

| Treatment | Photocell counts in 10 min | | % reduction in locomotor activity |
|---|---|---|---|
| | Basal | After treatment | |
| Vehicle control | 387.6±14.70 | 374.8±58.1[ns] | 3.28 |
| Diazepam 5 mg/kg, p.o. | 382.7±34.2 | 82.8±5.6* | 78.34 |
| MLPG 50 mg/kg, p.o. | 382.0±20.3 | 344.3±40.9[ns] | 9.84 |
| MLPG 100 mg/kg, p.o. | 386.1±16.9 | 340.2±60.7[ns] | 11.88 |
| MLPG 200 mg/kg, p.o. | 384.5±27.1 | 333.5±50.4[ns] | 13.26 |
| MLPG 400 mg/kg, p.o. | 388.8±18.2 | 343.6±59.3[ns] | 11.63 |

The photocell counts are expressed as mean±SEM ($n$=8). The basal values of the respective treatments were compared with post-treatment values by paired $t$-test using GraphPad version 5.0. *$P$<0.001 compared with their respective basal values, GABA: Gamma amino butyric acid, MLPG: Methanolic leaf extract of *Punica granatum*, NS: Not significant

(such as excitatory glutaminergic neurons), channelopathies (weekening of potassium channels and/or more persistent sodium channels, changes in voltage-gated ion channels), perturbance in synaptic receptors (suppressed GABAergic receptors, altered nicotinic receptors), decrease in inhibitory neurotransmission (decreased GABA levels), enhanced excitatory neurotransmission (enhanced glutamate levels) [33].

In this study, anticonvulsant activity of leaf extracts of *P. granatum* was evaluated against 6 Hz Seizure test, MES-induced convulsions and PTZ-induced convulsions in Swiss albino mice.

The 6 Hz seizure test is reported to involve a minimal, clonic phase, followed by stereotyped and automatistic behaviors which mimic the partial or limbic epilepsy in humans [34]. At present, the 6 Hz seizure test is preferred for early identification of anticonvulsant activity of new compounds which are effective against therapy-resistant epilepsy [35]. As per the recent antiepileptic discovery guidelines and recommendations, the test drugs which fail in conventional models (MES, PTZ, etc.,) are better to screen against 6 Hz seizure test, because 6 Hz seizure model is known as very sensitive and useful model, which is known to facilitate in identifying a very mild agent also against drug-resistant epilepsies [34,35]. Hence, this model is considered to be highly useful in identifying a new anticonvulsant drug, and also it is useful in avoiding false negatives. Therefore, all the extracts of *P. granatum* leaves were initially evaluated against 6 Hz seizure test. In this test, only MLPG (50, 100, 200, 400 mg/kg) has showed promising anticonvulsant effect. In contrast, PLPG and ALPG did

not show any protection, only high dose of ALPG (400 and 800 mg/kg, p.o.) showed very slight inhibition.

Based on the results obtained in 6 Hz seizure test, the MLPG was further evaluated against MES and PTZ-induced convulsions in Swiss albino mice.

In principle, the electroshock delivered in MES model is well known to potentiate the sodium influx through opening of sodium channels, and also increases glutamate levels, glutamate is an excitatory neurotransmitter, which binds with NMDA receptors and induces the symptoms that exactly mimic the petit mal epilepsy in humans [36]. Based on the underlying mechanism of MES convulsions, it can be understood that the agents which could block the voltage-dependent sodium channels (phenytoin, sodium valproate,) and/or the agents that decrease the levels of excitatory amino acids and/or antagonize their actions are proved to be effective in MES-induced epilepsy model (e.g., felbamate) [31].

Furthermore, PTZ is a potent GABA receptor antagonist, it is well known to decrease the GABA levels, and density of GABA-A receptors in various parts of the brain [37], this leads to continuous stimulation of cortical neurons and results in convulsions similar to absence seizures in humans [38]. Hence, it thought that the agents which enhance GABA levels, and/or enhances GABA-A receptor density, and/or GABA-A receptor agonists (like diazepam), and/or the agents behave like GABA are thought to be useful in abolishing PTZ-induced convulsions [31].

In both MES and PTZ models, the MLPG (50, 100, 200, and 400 mg/kg) showed significant and dose-dependent protection. Exceptionally, high dose of MLPG has completely abolished MES-induced Hind limb Tonic extensor and Hind limb Tonic flexion, also high dose (400 mg/kg) of MLPG has completely inhibited the PTZ-induced tonic convulsions and mortality. Further, the effect of MLPG was evaluated for its possible locomotor deficits; however, the MLPG did not show significant influence on locomotor activity.

With the above finding, it can be hypothesized that the MLPG is eliciting potent anticonvulsant activity by increasing the inhibitory neurotransmitters (GABA) and/or, decreasing the excitatory neurotransmission and/or, by blocking the sodium channels and/or by neutralizing the PTZ binding site. To explore the possible mechanism of action, the effect of MLPG was evaluated on GABA levels in brain. Interestingly pretreatment with MLPG showed a significant increase in GABA levels in cerebellum and whole brain other than cerebellum, in both single dose (Day-1) and multiple dose (3 days) administration of MLPG compared to control. Thus, we can conclude that one of the possible mechanisms behind the anticonvulsant effect of MLPG may be through enhanced GABA levels in the brain. All the findings in this study are in mutual relation and supports that MLPG possesses potent anticonvulsant activity and it could be a useful agent in treating both petit mal and grand mal epilepsy. Interestingly, many plant derived constituents such as aconitine, berberine, piperine, baicalin, vitexin, rutin, apigenin, chrysin, eugenol, ellagic acid, gallic acid, quercetin, kaemferol, abietic acid, and α-terpineol are well proved to possess anticonvulsant activity in various experimental models [39]. In these lines, some of the these phytocompounds such as gallic acid, ellagic acid, rutin, corilagin, kaempferol, luteolin, myricetin, quercetin, and quercimetrine were previously isolated from *P. granatum* [40]; based on these observations, we can conclude that the MLPG is eliciting its anticonvulsant activity by synergestic interaction of some these compounds, through enhanced GABA levels in the brain.

## CONCLUSION

These findings suggest that the MLPG possesses significant anticonvulsant property, further one of the possible mechanism/s behind the anticonvulsant activity of MLPG may be due to enhanced GABA levels in the brain. Indeed, there is a scope for further studies to explore molecular mechanism and identify the phytoconstituent responsible for the anticonvulsant activity. Hence, further studies are in the pipe line to explore the molecular mechanism of anticonvulsant action, and phytochemical studies are under progress to characterize and isolate the component responsible for the anticonvulsant activity of MLPG.

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Original Research

# Effects of herbal medicine on human uterine tumor-bearing nude mice

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## ABSTRACT

**Aim:** Uterine leiomyomas are the most common benign uterine neoplasms associated with significant morbidity. Herbal formulas capable of restoring yin-yang balance by dispersing blood stasis may be useful for managing fibroid symptoms. **Materials and Methods:** In this study, the antitumor properties of three herbs *viz.*, *Trogopterus xanthipes* Milen-Edwards, *Paeonia lactiflora* Pallas, and *Ulmus davidiana* Planch were evaluated in nude mice injected intravenously with human malignant myomas. Tumor fragments were xenografted subcutaneously through a flank incision in female mice. The mice entered the study for 8 weeks when their tumors reached the threshold volume (260 $mm^3$). The mice were randomly allocated to receive subcutaneous injections of normal saline (Group 1; negative control), *P. lactiflora* Pallas (Group 2), *U. davidiana* Planch (Group 3), *T. xanthipes* Milen-Edwards (Group 4), and intravenous injections of paclitaxel (Group 5; positive control). The weight and tumor volume were measured, followed by histopathology. **Results:** A few cases of abdominal distention and death were observed in the negative control group. Furthermore, a considerable enlargement of the liver and spleen was observed in the negative control group at autopsy with a gradual increase in body weight during the experiment. The mean tumor volume which increased in negative control mice reduced in mice treated with herbal remedies or paclitaxel from day 14 onwards ($P < 0.05$). The degree of necrosis and apoptosis induction from herbal treatments was similar to that of paclitaxel. **Conclusion:** Collectively, three herbs *viz.*, *T. xanthipes* Milen-Edwards, *P. lactiflora* Pallas, and *U. davidiana* Planch were able to induce necrosis and apoptosis of uterine leiomyoma cells, proving antitumor properties against uterine fibroids.

KEY WORDS: Leiomyomas, *Paeonia lactiflora* Pallas, *Trogopterus xanthipes* Milen-Edwards, tumor, *Ulmus davidiana* Planch

## INTRODUCTION

Uterine fibroids, also known as leiomyomas, myomas, or fibromyomas, are the most common benign uterine neoplasms associated with significant morbidity of nearly 40% in women during their reproductive years [1]. Typical symptoms related to uterine fibroids, including abnormal menstrual bleeding, dyspareunia, non-cyclic pelvic pain and pressure, infertility, and repeated miscarriages, can compromise the quality of life and may affect fertility and pregnancy outcomes. In fact, an increase in pregnancy loss and reduction in pregnancy and live birth rates have been reported in recent analyses of symptomatic women with intramural fibroids [2]. More than half of symptomatic women surveyed internationally reported a negative impact on their quality of life due to a general impairment in sexual life, work performance, family relationship, and housekeeping [3].

Although the etiology and biology of uterine fibroids are poorly understood, a few risk factors have been identified for the development and growth of leiomyomas, such as ethnicity, nulliparity, genetics, and hormonal factors [4]. The classical neoplastic transformation of myometrium to leiomyoma likely involves somatic mutations of normal myometrium and the complex interactions of steroid hormones with local factors, such as growth and apoptosis-related factors [5]. There is increasing evidence to suggest that estradiol upregulates epidermal growth factor (EGF) receptor, transforming growth factor-β (TGF-β), and platelet-derived growth factor but downregulates p53 protein in leiomyoma cells [6]. However, progesterone may contribute to leiomyoma cell growth by augmenting EGF, TGF-β, and Bcl-2 protein, and inhibiting insulin-like growth factor and tumor necrosis factor [6]. While the effects of progesterone on fibroid growth have been suggested to be dual (i.e., stimulatory and inhibitory), strong evidence supports the role of estradiol in promoting leiomyoma tumorigenesis and growth.

The management of symptomatic fibroids has been performed traditionally with surgeries such as myomectomy or hysterectomy. Another option of treatment is uterine artery embolization during which the blood vessels to the uterus are blocked for fibroid shrinkage [7]. The pharmacological choice of gonadotropin-releasing hormone agonists may also shrink fibroids and control abnormal bleeding [4]. However, alternative

herbal remedies have been proposed to control uterine fibroid-related symptoms.

Herbal remedies for fibroids are common in complementary medicine. In a 6-month pilot study, herb, acupuncture, and mind-body healing treatments were found to shrink fibroids, increase patient satisfaction, and reduce bothersome symptoms [8]. According to the traditional Chinese medicine theory, uterine fibroids are caused by yin-yang imbalance in the body, which can be interpreted as disturbances in the endocrine system and blood circulation. Herbal formulas classified as being capable of restoring yin-yang balance by dispersing blood stasis may be useful in the management of fibroid symptoms. The Hyul-Boo-Chook-Ur-Tang (Xue-Fu-Zhu-Yu-Tang in Chinese), which contains a decoction of 13 specific herbs in Korean medicine, has been used for a long time to treat uterine fibroids and has appeared in Dongui Bogam, a classic Korean medical book of Oriental medicine that was first published in 1613 [9].

The effects of individual herbs in the Hyul-Boo-Chook-Ur-Tang are not well studied; therefore, this current study was performed to determine reliable treatment alternatives for uterine fibroids. Three major effective herbs out of the 13 ingredients in the Hyul-Boo-Chook-Ur-Tang, according to Dongui Bogam, were selected to determine their effects in nude mice-bearing human fibroid tumors. *Paeonia lactiflora* Pallas (radix) was traditionally used to tonify and purify the blood. *Ulmus davidiana* Planch (radix) was used for purifying blood and has long been known to have anti-inflammatory properties. *Trogopterus xanthipes* Milen-Edwards (feces) has been prescribed for alleviating pain, promoting blood circulation, and resolving blood stasis to arrest bleeding [10].

The aim of our study was to determine whether *T. xanthipes* Milen-Edwards, *P. lactiflora* Pallas, and *U. davidiana* Planch have any antitumor properties against uterine fibroids. In the present controlled study, the nude mice were used as an *in vivo* tumor model for the transplantation and engraftment of leiomyomal cells to investigate the effect of herbs on clinical signs, body weight, tumor volume, and cell death.

## MATERIALS AND METHODS

Five-week-old female BALB/C nude mice ($n$ = 60, 14-19 g) were purchased from Central Lab. Animal, Inc. (Seoul, Korea). The mice were maintained under specific-pathogen-free conditions at the Experimental Animal Center at Biotoxtech Co., Ltd. (Ochang, Korea). The experiment was performed in strict accordance with the recommendations in the Guide for Care and Use of Laboratory Animals of the National Institutes of Health. The protocol was approved by the Animal Care and Use Committee at Biotoxtech Co., Ltd. (Ochang, Korea); the permit number was 101181.

The mice were held for 1 week after arrival to allow them to acclimate, and they had access to food and water *ad libitum*. The leiomyomal cells utilized in the current study were derived from human malignant myomas that were obtained at surgery from women (range, 25-55 years) undergoing myomectomy. The female nude mice were administered an intravenous injection of $1 \times 10^7$ leiomyomal cells and were euthanized 3 weeks later. Several well-isolated tumor colonies were harvested by excising the tumor-bearing tissues. Then, tumor colonies were aseptically dissected into 2 $mm^3$ sections.

For tumor xenograft, the mice were sedated by light isoflurane (Choongwae Pharma Corp., Seoul, Korea) anesthesia, and tumor colonies were implanted subcutaneously with an 11-gauge trocar inserted through a flank incision. The trocar was slowly withdrawn, and the incision was closed using an interrupted Vicryl suture for the skin. All manipulations were conducted under aseptic conditions using a laminar flow hood. All animals tolerated the procedure well.

After implantation, the mice were inspected daily for body weight loss, general clinical condition, and tumor formation. The tumor grew in the right flank area near the back of the mice. Animals were palpated for tumors twice weekly and entered into the study when the tumors reached an average volume of ~260 $mm^3$.

The mice were randomized into the following 5 groups (10 mice per group): Group 1, normal saline injected subcutaneously (negative control; 0.2 mL/day); Group 2, *P. lactiflora* Pallas injected subcutaneously (10 mg/kg/day); Group 3, *U. davidiana* Planch injected subcutaneously (10 mg/kg/day); Group 4, *T. xanthipes* Milen-Edwards injected subcutaneously (10 mg/kg/day); Group 5, paclitaxel injected intravenously (positive control; 5 mg/kg; Bristol-Myers Squibb Co, Princeton, NJ, USA). The concentration of paclitaxel used in this study has minimal effects on mouse morbidity [11], which was further adjusted considering the dose of each herb. Mice in Groups 1-4 were injected once per day for 8 weeks, whereas those animals in Group 5 were injected twice per week for 8 weeks.

On a weekly basis, the body weight and tumor size of the mice were measured. The maximal length (L) and perpendicular width (W) of the tumor were measured using a digital caliper, and the tumor volume was calculated using the following formula: Tumor volume ($mm^3$) = L (mm) × $W^2$ ($mm^2$) × ½. Changes in tumor volume were used as an overall indicator of antitumor efficacy. At the end of the experiment, autopsies were performed in the mice, and tumors were excised and weighed. In addition, the collected tumors were placed in phosphate-buffered 4% formalin for 16 hours at room temperature and embedded in paraffin. The obtained tissue sections (4.5 $\mu$m) underwent staining with hematoxylin-eosin and TUNEL for evaluating necrosis and apoptosis grade, respectively, before microscopic examinations.

The data were presented as the means ± standard deviations. The difference in the tumor growth rate among the 5 groups of nude mice was determined by repeated-measures analysis of variance. Differences with a $P < 0.05$ were considered significant.

## RESULTS

Some clinical abnormalities (i.e., abdominal distention and death) were observed in a few cases in the negative control group. In addition, a considerable enlargement of the liver and spleen was observed at autopsy. Several large grayish-white bulging nodules were visualized in the spleen. The liver appeared edematous with yellow discoloration. Coloring of the skin, skin induration, and abdominal distention were observed in mice injected with herbal remedies. Only abdominal distention was reported in the positive control group. The mice in the negative control group showed a gradual increase in body weight at least until 5 weeks of the experiment, which was probably due to an enlarged liver or spleen [Figure 1]. Afterward, the body weight of mice in both Groups 3 and 4 increased significantly compared to the negative control group. However, the positive control group and Group 2 (i.e., *P. lactiflora* Pallas-injected group) showed a completely opposite pattern. The metabolism inhibitory effects of *U. davidiana* Planch and *T. xanthipes* Milen-Edwards might have been associated with the declining effects of tumors during the later stage of the experiment. The treated animals showed significantly smaller tumors compared to the negative control group after 52 days ($P < 0.05$) [Figure 2]. The mean tumor volumes of Groups 1-5 animals were 2920 ± 1308 mm$^3$, 1765 ± 836 mm$^3$, 1807 ± 932 mm$^3$, 1717 ± 605 mm$^3$, and 1401 ± 745 mm$^3$, respectively. The mean tumor volume increased significantly from day 14 to day 52 in control mice but not treated mice. Furthermore, the tumor growth inhibition rates for Groups 1-5 were 0%, 20.6%, 21.7%, 20.3%, and 19.7%, respectively, confirming tumor regression in mice that were treated using herbs. When all tumors were further analyzed for necrosis and apoptosis using histopathological assay, the difference in tumor cell death was not significant among groups, but there was an uphill trend with treatment. The average percentage of apoptosis ([number of positive cells/number of total cells] × 100) for Groups 1-5 was 1.5%, 2.0%, 2.0%, 1.7%, and 1.7%, respectively [Figure 3]. The amount of apoptotic cell death varied with treatment; however, the percentage of apoptotic cells increased in tumors treated with herbs compared to the negative control tumors.

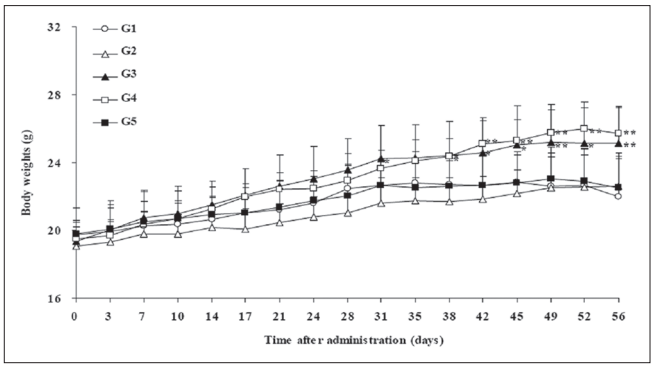


**Figure 1:** Effect of treatment on body weight (g) during the 8th week of experiment. Values at each point are presented as means ± standard deviations ($n = 10$). *$P < 0.05$ versus negative control, **$P < 0.01$ versus negative control. G1: Group 1, normal saline injected subcutaneously (negative control); G2: Group 2, *P. lactiflora* Pallas injected subcutaneously; G3: Group 3, *Ulmus davidiana* Planch injected subcutaneously; G4: Group 4, *T. xanthipes* Milen-Edwards injected subcutaneously; G5: Group 5, paclitaxel injected intravenously (positive control)

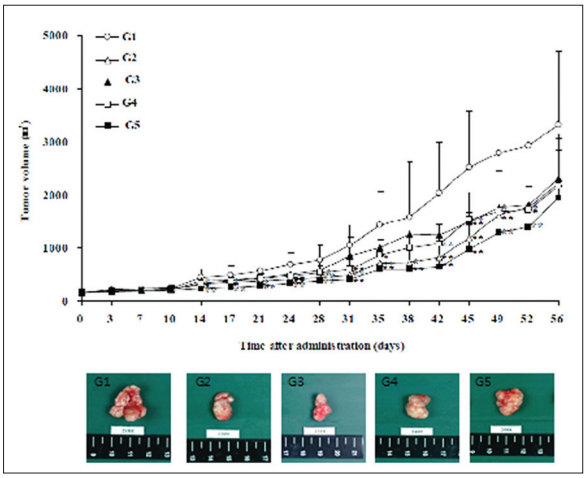


**Figure 2:** Effect of treatment on tumor volume (mm$^3$) during the 8th week of experiment. Each point in the curves represents the means ± standard deviations ($n = 10$). Representative tumor samples were obtained by implanting leiomyomal cells on the flank of the mice in each group. *$P < 0.05$ versus negative control, **$P < 0.01$ versus negative control. G1: Group 1, normal saline injected subcutaneously (negative control); G2: Group 2, *P. lactiflora* Pallas injected subcutaneously; G3: Group 3, *Ulmus davidiana* Planch injected subcutaneously; G4: Group 4, *T. xanthipes* Milen-Edwards injected subcutaneously; G5: Group 5, paclitaxel injected intravenously (positive control)

## DISCUSSION

Herbal medicine is defined as the use of herbs to treat a wide range of disorders and enhance well-being. Herbs act on the body, similar to pharmaceutical drugs, and therefore, herbs should be treated with care. In Korea, most herbs are administered as a form of decoction. The effects of decoction can be manifested after total and final reaction by the constituent compounds when administered to humans. Herbal treatment for fibroids is a medical tradition in many countries worldwide [12].

*Trogopterus* feces (the feces of *T. xanthipes* Milen-Edwards) are used for the treatment of amenorrhea, dysmenorrhea, menstruation-related pain, and retained lochia due to stasis by promoting blood circulation and removing stasis. These effects may be due to fatty acid esters [13] and flavonoid glycosides [14] extracted from *Trogopterus* feces, which have been proven to possess anticoagulative activity. The anticancer activities of *Trogopterus* feces have been determined by deferred proliferation and more obvious changes of leiomyomal cells, which is probably through downregulation of TGF-β receptor 2 [15]. As a key profibrotic cytokine, elevated expressions of TGF-βs, their receptors, and related signaling pathways are common characteristics in leiomyoma [16]. Although we did not investigate the effect of each herb on TGF-β-related signaling pathways, some anticancerous effects of herbs observed in the present study might have been mediated

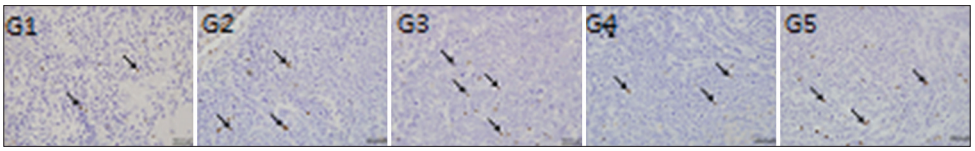


**Figure 3:** Representative micrograph showing terminal deoxytransferase-mediated dUTP nick-end labeling-stained apoptotic cells in each group. The arrows indicate terminal deoxytransferase-mediated dUTP nick-end labeling-positive apoptotic cells. G1: Group 1, normal saline injected subcutaneously (negative control); G2: Group 2, *P. lactiflora* Pallas injected subcutaneously; G3: Group 3, *Ulmus davidiana* Planch injected subcutaneously; G4: Group 4, *T. xanthipes* Milen-Edwards injected subcutaneously; G5: Group 5, paclitaxel injected intravenously (positive control)

through the downregulation of Smad and MAPK signaling pathways.

*P. lactiflora* Pallas combined with 4 other components has been reported as beneficial for reducing symptoms of uterine fibroids and shrinking uterine myomas [8,17]. Paeoniflorin, the main active constituent of *P. lactiflora* Pallas roots, has been proven to have potent antispasmodic, anticoagulative, analgesic, and anti-inflammatory activities [18]. Recently, another main monoterpene glycoside called albiflorin isolated from *P. lactiflora* Pallas was found to have similar anti-inflammatory effects to paeoniflorin [19].

*U. davidiana* Planch has been shown to have anti-inflammatory and anticancer activities based on its long history of clinical applications [20]. Its anti-inflammatory properties may be attributed to the inhibition of the cyclooxygenase pathway because prostaglandins are often highly produced in cancer and inflammation [21].

Histopathological analysis was performed to determine the induction of necrosis and apoptosis in each treatment and to further confirm the effects of herbal medicine at the cellular level. Interestingly, the level of the induction of necrosis and apoptosis from the herbal treatments was similar to that of paclitaxel. Paclitaxel has anticancer activity through a direct apoptotic effect, and it is commonly used to treat patients with gynecological cancer [22].

This study has several limitations. First, only three herbs of the Hyul-Boo-Chook-Ur-Tang have been examined for their potential antitumor properties against uterine fibroids. Therefore, the remaining herbs might have been overlooked despite considerable efforts to retrieve all relevant information of those herbs. Second, we did not explore any mechanisms to explain the potential antitumor properties of the herbs used. Another limitation of this study was the lack of a follow-up period, especially given that the tumor remained in the mice treated with herbs albeit the tumor growth was inhibited.

## CONCLUSION

Three herbs *viz*., *T. xanthipes* Milen-Edwards, *P. lactiflora* Pallas, and *U. davidiana* Planch were used to induce necrosis and apoptosis of uterine leiomyoma cells proving antitumor properties against uterine fibroids. We predict that these herbs must have balanced the yin-yang ratio by dispersing blood stagnation from the body and alleviating inflammation.

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Original Research

ScopeMed

# *In vitro* and *in vivo* anthelmintic effects of *Caesalpinia bonducella* (L.) Roxb. leaf extract on *Hymenolepis diminuta* (Cestoda) and *Syphacia obvelata* (Nematoda)

**Shyamalima Gogoi, Arun K. Yadav**

## ABSTRACT

**Background:** Leaves of *Caesalpinia bonducella* (L.) Roxb. have been traditionally used as an herbal remedy to treat the intestinal helminthic infections in traditional medicine of India. **Aim:** This study was undertaken to evaluate the potential *in vitro* and *in vivo* anthelmintic effects of *C. bonducella* leaf extract against *Syphacia obvelata* (Nematoda) and *Hymenolepis diminuta* (Cestoda). **Materials and Methods:** The *in vitro* anthelmintic activity of the extract was investigated on adult worms of *S. obvelata* (Nematoda) and *H. diminuta* (Cestoda) in terms of physical motility and mortality of parasites. The *in vivo* study was performed in *H. diminuta*-rat model and *S. obvelata*-mice model, by monitoring the egg per gram of feces count and worm count of animals following the treatment with different doses of plant extract. **Results:** The study recorded significant and dose-dependent anthelmintic effects of the extract on both the parasites. In the *in vitro* study, 30 mg/ml concentration of extract caused mortality of *H. diminuta* in 2.5 ± 0.2 h and *S. obvelata* in 3.57 ± 0.16 h. In the *in vivo* study, the extract showed a comparatively better efficacy on *S. obvelata*, where its 800 mg/kg dose revealed 93% reduction of worm load in mice, as compared to 85% worm load reduction of *H. diminuta* in rats. **Conclusions:** The findings suggest that leaf extract of *C. bonducella* possesses significant anthelmintic effects and supports its use as an anthelmintic in traditional medicine. This appears to be the first report of *in vivo* anthelmintic activity of *C. bonducella* against these parasites.

KEY WORDS: Anthelmintics, *Caesalpinia bonducella,* helminthiasis, *Hymenolepis diminuta*, soil-transmitted helminthiasis, *Syphacia obvelata*

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## INTRODUCTION

Intestinal helminths or soil-transmitted helminths (STH) are among the most widespread infections and affect more than 2 billion people worldwide [1]. India has the highest burden of STH in the world, with comparatively more prevalence in rural areas and infections encountered more often in children of school-age or younger age between 2 and 14 years at its highest rate. STH are mainly comprised the roundworm (*Ascaris lumbricoides*), the whipworm (*Trichuris trichiura*), and hookworms (*Necator americanus* and *Ancylostoma duodenale*). Although the intestinal helminth infections are relatively less pathogenic to human beings as compared to other infectious agents, cases of heavy worm burdens often lead to malnutrition, anemia, stunted growth, and other intestinal disorders in infected subjects [2]. The global strategy to control the STH is to control the morbidity due to worms applying mass drug administration (MDA) in endemic areas by two standard anthelmintic drugs: Albendazole (ABZ) and mebendazole [3]. However, despite the availability of safe and effective drugs, presently only about 40% of the people at risk of STH is under MDA coverage at global level [4]. In particular, in many African and Asian countries, including India, the anti-STH drugs do not reach in all endemic regions in a sufficient manner. In addition, since currently there are only limited number of drugs available against intestinal worms, the threat of drug resistance in STH is also posing a big challenge [5].

From ancient times, intestinal helminth infections have been treated by several herbal medicines, based on the traditional beliefs of different cultures. The herbal medicines are also easily available and affordable by a large section of the people in rural regions. In the recent past, a number of studies have been made to validate the anthelmintic effects of medicinal plants. For example, Abdel-Ghaffar *et al.* [6] reported that extracts prepared from coconut, onion, garlic, fig, date tree, chicory, ananas, and cistrose possess significant effects on cestodes, (*Hymenolepis diminuta*, *Hymenolepis microstoma*, and *Taenia taeniaeformis*)

and trematodes (*Fasciola hepatica and Echinostoma caproni*). Similarly, leaf extracts of *Adhatoda vasica* and *Clerodendrum colebrookianum* have also been found to be effective against *H. diminuta* infections in rats [7,8]. Recently, Deori and Yadav [9] have also reported the anthelmintic effects of stem bark extract of *Oroxylum indicum*, a traditional anthelmintic plant of India, on larval and mature worms of *H. diminuta*, a zoonotic tapeworm.

*Caesalpinia bonducella* (L.) Roxb., (Caesalpiniaceae), called as "*Letaguti/letaiguti*" in Assam, India, is a prickly shrub of about 20 m height [Figure 1a]. In addition to India, this plant is also distributed in other Southeast Asian countries such as China, Thailand, Philippines, Indonesia, and Malaysia, particularly in the waste ground and coastal areas and up to an altitude of 1000 m. The crude extracts prepared from different parts of *C. bonducella* have been widely used in folk medicines for treating various diseases such as, pneumonia [10], filarial infections [11] and have also been reported to possess hypoglycemic, antihyperglycemic, and hypolipidemic effects [12]. Interestingly, in Mishing tribe of Assam, India, *C. bonducella* leaves [Figure 1b] are taken as decoction, infusion or as vegetable to treat the intestinal helminth infections. In India, an anthelmintic herbal medicine prepared from the leaves of this plant is called as *Kusere* [Figure 1c]. In view of the extensive use of this plant in traditional medicine and considering the few significant scientific studies that has been carried out on this plant against intestinal helminths, this study was undertaken to evaluate the anthelmintic effects of *C. bonducella* leaf extract, using model parasites *Syphacia obvelata* (a rodent pinworm) and *H. diminuta* (a zoonotic cestode).

## MATERIALS AND METHODS

### Chemicals and Standard Drugs

All the chemicals used were of standard analytical grade, purchased from Merck, India. ABZ (Ambalal Sarabhai Enterprises Ltd., Vadodara) and praziquantel (PZQ) (Distocide, Chandrabhagat Pharma Pvt. Ltd., Mumbai, India) were used as reference drugs to compare the plant's anthelmintic efficacy.


**Figure 1:** *Caesalpinia bonducella* (a) Whole plant in its natural habitat, (b) close-up of plant leaves, (c) *Kusere*, the indigenous anthelmintic medicine prepared from plant leaves

### Plant Material and Preparation of Extract

The plant material was collected in October 2011 from Dhakuakhana, Lakhimpur district of Assam, and authenticated by a plant taxonomist. A voucher specimen (No. NEHU-12034) has been deposited in Ethnopharmacology and Parasitology Lab, Department of Zoology, NEHU, Shillong. The field-collected leaves were washed, dried under shade, and powdered to a fine grade for extraction with methanol in a Soxhlet extractor at 40°C. The final yield of extract was 3.61% (w/w), from the fresh material.

### Phytochemical Screening

The phytochemical analysis of *C. bonducella* methanol leaf extract was carried out to confirm the presence of various secondary metabolites, as described by Harborne [13], Trease and Evans [14], and Sofowra [15]. The presence of phytochemicals in trace or abundance was judged by the color intensity of the test results.

### Experimental Animal Models

Swiss albino mice of both sex weighing 20-25 g, and Wistar rats of both sex weighing 180-200 g, were used in the study. Experimental animals were kept at standard room temperature with 12 h light and dark cycle and fed standard rodent feed (Pranav Agro Industries Ltd., Delhi) and water *ad libitum*. Before the extract testing, all the animals were given 2 mg/kg dose of PZQ and 5 mg/kg dose of ABZ for 3 days, and their fecal samples were examined to confirm that they are free from any intestinal helminth infections. After acclimatization for 7 days, the natural infections of *S. obvelata* were identified in mice by the use of perianal cellophane tape, as described by Meade and Watson [16], with slight modifications. While infection of *H. diminuta* was maintained in Wistar rats as described by Tangpu *et al.* [17].

### Ethical Standards

All experiments were performed in accordance with Indian Animal Ethics Committee regulations. All the experiments on animals were performed following the approval from the Institutional Animal Ethics Committee (Animal Models) of North-Eastern Hill University, Shillong.

### Acute Oral Toxicity Study

The acute oral toxicity study was conducted according to the guidelines of Organization for Economic Cooperation and Development (OECD) revised up-and-down procedure for acute oral toxicity testing [18]. First, a limit dose of 2000 mg/kg body weight of plant extract was used in five female Swiss albino mice. Animals were dosed individually and observed for adverse clinical signs (e.g., tremors, convulsions, salivation, diarrhea, lethargy, sleep and coma, gait, and posture) or mortality during

first 30 min and then periodically during first 24 h. If the first animal survived, then four additional animals were given the same dose of extract at 48 h of interval, and all the animals were kept under observation for 14 days. The lethal dose, 50% ($LD_{50}$) was predicted to be above 2000 mg/kg if three or more animal survived in the experiment. Similarly, a limit dose of 5000 mg/kg was also tested using almost similar experimental protocols, except that herein the treatment of animals was done by 5000 mg/kg dose of extract, involving first only a single mouse and later two more additional animals, following which the dosing of further animals was terminated [18].

### *In Vitro* Anthelmintic Assay

The adult *S. obvelata* was collected from naturally infected mice, whereas the adult *H. diminuta* specimens were obtained from laboratory maintained infection in Wistar rats. The adult specimens of test parasites were washed several times in Hank's solution. The test worms ($n = 5$) were then kept in Petri dishes containing 10, 20, and 30 mg/ml concentrations of plant extract in Hank's solution and put inside an incubator at 37 ± 1°C. ABZ (5 mg/ml) and PZQ (1 mg/ml) were used as reference drugs. One set of worms ($n = 5$), maintained in Hank's solution, was kept as control. The anthelmintic efficacy of extract was determined in terms of physical motility of test worms, as evidenced by their paralysis or mortality [19]. The experiments were performed 3 times with five number of test worms per concentration of plant extract.

### *In Vivo* Anthelmintic Assays

For *S. obvelata*, infection in experimental mice was detected by the cellophane tape test. Infected animals were then divided into five groups, consisting of five animals in each group. Group I of mice served as control, whereas, Groups II, III, and IV of mice were treated with 200, 400, and 800 mg/kg doses of extract for 5 days. The Group V of mice was given 20 mg/kg dose of a reference drug, ABZ for the same duration. A cellophane tape preparation was obtained from each mouse, consecutively for 3 days before treatment and after treatment of plant extract to determine the eggs per gram of feces (EPG) count. For this, to each mouse, a single piece of cellophane tape was firmly applied to its anal region and sampling was done during 13:00-14:00 h. The length of tape varied between 25 and 30 mm. These tape samples were attached to the standard 25 mm × 75 mm microscope slides, and characteristics *S. obvelata* eggs were counted under a microscope [16]. Finally, the EPG of feces was calculated in all groups of animals to work out the reduction in EPG counts in treated groups [6]. On day 8 post-treatment, all the animals were sacrificed to determine their respective worm load.

Against *H. diminuta*, the efficacy of extract was evaluated against the larval and adult stages. Four cysticercoid larvae of *H. diminuta* were orally given to each Wistar rat to induce infection. For each stage, rats were divided into five groups, comprising of five animals per group. Against the larval stage of parasite, the first group of animals was used as control. The Groups II, III, and IV of animals were orally given 200, 400, and 800 mg/kg dose of extract, respectively, on day 2-4 post inoculation (p.i.) of cysticercoids. The animals of Group V received 5 mg/kg of reference drug, PZQ for the same duration. From day 18 p.i., fecal samples of rats were collected from each cage, and the EPG of feces counts was done for three consecutive days, i.e., days 18-20 p.i. On day 31 p.i., rats were sacrificed and the percentage reduction in worm count was also determined [17].

For the adult stages of parasite, almost similar methods were followed, except that there were some differences in days of treatments and EPG counts. Herein, extract treatment was done between day 21 and 23 p.i. of cysticercoids, and the EPG counts were undertaken between day 18 and 20 of p.i. (pre-treatment period) and during days 34-36 p.i. (post-treatment period).

### Statistical Analysis

All the results are represented as mean±standard error of the mean using GraphPad Prism (version 4.5) software. *In vitro* data were analyzed using Student's *t*-test and *in vivo* study; data were analyzed by one-way analysis of variance (ANOVA) followed by Tukey test with $P < 0.001$ being considered statistically significant.

## RESULTS

### Phytochemical Studies

The qualitative phytochemical tests revealed that methanol leaf extract of this plant contains terpenoids, tannins, alkaloids, flavonoids, glycosides and reducing sugars as major secondary metabolites. On the basis of color intensity of the test results, it was observed that tannins and flavonoids are present in abundance in the extract, whereas the others are present only in trace amounts.

### Acute Oral Toxicity

Oral administration of *C. bonducella* leaf extract at single limit doses of 2000 and 5000 mg/kg caused no signs of toxicity or morality in all the treated mice. During the observation period of 2-week, all the dosed animals were found to be healthy and normal without any apparent symptoms of adverse effects.

### *In Vitro* Studies

In the present study, *C. bonducella* leaf extract showed a dose-dependent activity against nematode *S. obvelata* and cestode *H. diminuta*. Exposure to varying concentrations of extract led to a significant reduction ($P < 0.001$) in paralysis and mortality time of test worms [Figure 2]. At 30 mg/ml concentrations of extract, the mean paralysis and mortality time of *S. obvelata* were observed to be 3.57 ± 0.16 h, which compared very well with the efficacy of a standard drug, ABZ, which showed a mean paralysis and mortality time of worms of 2.37 ± 0.21 h and 3.57 ± 0.16 h, respectively. While for *H. diminuta*, exposure to 30 mg/ml concentration of extract showed paralysis of worms in

4.86 ± 0.16 h and mortality in 2.5 ± 0.2 h. Herein, the reference drug PZQ revealed paralysis of worms in 1.46 ± 0.52 h and mortality in 3.46 ± 0.23 h. On the other hand, physical activity of nematode and cestode control worms was recorded till 36 ± 0.57 h and 32.44 ± 0.23 h, respectively. Thus, *in vitro* studies showed that exposure of test worms leads to an early paralysis, followed by mortality of parasites.

### *In Vivo* Studies

In the *in vivo* study against *S. obvelata* in mice, following treatment with different doses of plant extract a significant decrease was observed in the EPG counts of animals during the post-treatment periods [Table 1]. During the first 3 days of study, the pinworm eggs were detected in the cellophane-test of mice all groups. However, during consecutive 5 days administration of the highest dose of plant extract (800 mg/kg dose) to the animals, the number of eggs dropped significantly from 20.8 ± 2.14 to 5.1 ± 0.78 in animals. In comparison, in the ABZ treated animals, the number of eggs in cellophane-test reduced from 18.7 ± 0.88 to 2.4 ± 0.33 during the pre- and post-treatment periods. In contrast, no EPG reduction was observed in control animals and the same increased slightly from 25.8 ± 1.19 to 26.7 ± 1.41, during the pre- and post-treatment periods. In addition, the oral administration of 800 mg/kg dose of *C. bonducella* extract to *S. obvelata* naturally infected mice for 5 days also revealed a significant worm reduction of 93% as compared to control animals. Herein, the efficacy of extract was found to be quite comparable with the reference drug ABZ (20 mg/kg) which showed an EPG reduction of 97% [Table 1].

In the *in vivo* assay of plant extract against larval stages of *H. diminuta*, the highest dose of extract, i.e. 800 mg/kg, caused 84.38% reduction in EPG counts and 80% reduction in worm counts of animals [Table 2]. The effects of extract were almost similar with that of treatment of animals with 5 mg/kg dose of reference drug, PZQ, wherein reductions in EPG count and worm counts were recorded to be 82.60% and 85%, respectively. Further, the *in vivo* testing of extract against the adult stages of parasite also showed almost similar pattern, as in case of testing of extract against the larval stages of parasite. The animals treated with 800 mg/kg dose of extract showed 91.53% reduction in the EPG counts at the post-treatment

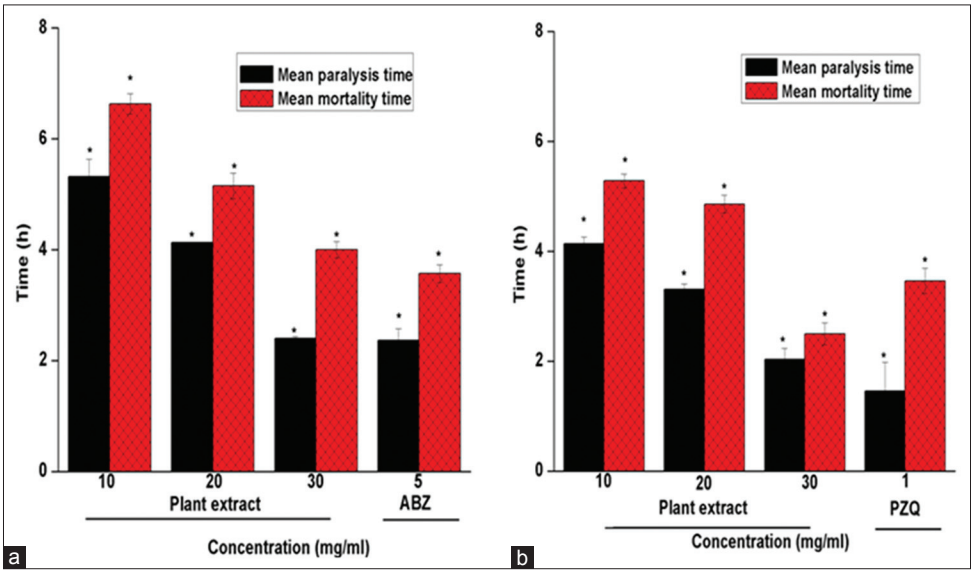


**Figure 2:** *In vitro* anthelmintic effects of *Caesalpinia bonducella* leaf extract investigated in terms of physical motility and mortality on adult parasites, (a) *Syphacia obvelata*, (b) *Hymenolepis diminuta*. Physical activity of control worms for respective experiment was recorded as 36 ± 0.57 h and 32.44 ± 0.23 h. Data are presented as mean ± SEM. **P* < 0.001, as compared to control, Student's *t*-test. SEM: Standard error of the mean

Table 1: Effects of *C. bonducella* leaf extract* on rodent pinworm, *S. obvelata* in naturally infected mice (*n*=5)

| Groups (mg/kg×day×dose) | EPG (mean±SEM) | | Worm count at necropsy | | Percentage reduction in worm count |
|---|---|---|---|---|---|
| | Pre-treatment (Days 1-3) | Post-treatment (Days 9-11) | Min-Max | Mean±SEM | |
| Control | 25.5±1.19 | 26.7±1.41 | 180-200 | 208.0±11.5 | 0 |
| Plant extract | | | | | |
| 200×1×5 | 18.5±0.87 | 12.0±0.85[a] | 100-180 | 146.0±12.8[b] | 29.8 |
| 400×1×5 | 22.5±1.09 | 11.2±0.66[a] | 100-200 | 136.0±22.2[b] | 34.6 |
| 800×1×5 | 20.8±2.14 | 5.1±0.78[a] | 12-20 | 15.4±1.6[b] | 92.6 |
| ABZ | | | | | |
| 20×1×5 | 18.7±0.88 | 2.4±0.33[a] | 1-7 | 7.0±1.90[b] | 96.6 |

Values are presented as the mean±SEM; One-way ANOVA followed by Tukey's test; *Administration of plant extract and reference drug ABZ on days 4-8 post EPG count; [a]*P*<0.05 compared to pre-treatment, [b]*P*<0.001, as compared to control. SEM: Standard error of the mean. ANOVA: Analysis of variance, EPG: Eggs per gram, ABZ: Albendazole¸ *C. bonducella*: *Caesalpinia bonducella, S. obvelata: Syphacia obvelata*

period, compared to 80.55% reduction by 5 mg/kg dose of PZQ [Table 3].

## DISCUSSION

This study was aimed at investigating the *in vitro* and *in vivo* anthelmintic effects of a leaf extract from *C. bonducella*, a traditional anthelmintic plant of India, against nematode, *S. obvelata* and cestode, *H. diminuta*. The acute toxicity study in mice revealed that the single limit doses of 2000 mg/kg and 5000 mg/kg of *C. bonducella* leaf extract did not cause any alteration in the animal's behavior, *viz*., breathing, posture, and food and water consumption or neither showed any toxic effects during the whole period of study. These preliminary observations indicate that the extract is non-toxic since $LD_{50}$ value of the extract is calculated to be <5000 mg/kg in mice, which is considered to be safe as per the OECD guidelines [18].

The *in vitro* testing of *C. bonducella* leaf extract against *H. diminuta* and *S. obveleta* showed that following exposure to different doses of extract the parasites reveal an early paralysis and complete loss of body movements as compared to control parasites. Some other similar kind of studies have also investigated the *in vitro* antiparasitic effects of different medicinal plants on adult helminth parasites by evaluating the effects of plant extracts on physical activity of test worms [20-22]. However, in many such studies very often the used concentrations of plant extracts have not always matched appropriately with their dosages used in the *in vivo* assays and thus this has caused some variations in estimating the efficacies of plant extracts in respective assays [23,24]. It is also worth mentioning here that many previous anthelmintic studies on medicinal plants have usually either employed *in vitro* assays [18], or in other cases only *in vivo* models [25] to assess the anthelmintic effects of plant extracts. However, it is only in few cases that an attempt has been made to employ both *in vitro* and *in vivo* tests to evaluate the anthelmintic properties of a specific medicinal plant [9]. Therefore, to achieve a more authenticated scientific data about the anthelmintic properties of plant extracts, it is more worthwhile that, as far as possible, the *in vitro* assays on these plants should also be supplemented by *in vivo* studies.

Our results of *in vitro* testing of extract showed marked effects on nematode parasite with a dose-dependent efficacy ($P < 0.001$) which are in agreement with the findings of Hussain *et al.* [26] against nematode, *Haemonchus contortus* where it has been reported that the leaves extract of *Trianthema portulacastrum* also caused dose and time-dependent anthelmintic effects on test parasites.

Similarly, the present findings of dose-dependent *in vivo* effects of *C. bonducella* extract on *S. obvelata* are in agreement with the findings of Camurça-Vasconcelos *et al.* [27], who investigated the antinematodal properties of *Croton zehntneri* and *Lippia sidoides* essential oils on intestinal nematodes of mice. Likewise, in a study by Kozan *et al.* [28], the highest dose of a Turkish folk medicinal plant, *Jasminum fruticans* also showed 74% reduction

**Table 2: Effects of *C. bonducella* leaf extract* on larval stages of *H. diminuta* infections in rats (*n*=5)**

| Groups (mg/kg×dose×day) | EPG (mean±SEM) Days 18-20 | Percentage reduction in EPG counts | Worms count/rat (mean±SEM) | Percentage reduction in worm counts |
|---|---|---|---|---|
| Control | 5866±464 | - | 3.8±0.20 | 5.00 |
| Plant extract | | | | |
| 200×1×3 | 1873±392[a] | −68.07 | 2.0±0.37[a] | 50.00 |
| 400×1×3 | 1790±335[a] | −69.48 | 1.4±0.24[a] | 60.00 |
| 800×1×3 | 916±123[a] | −84.38 | 0.8±0.37[a] | 80.00 |
| PZQ | | | | |
| 5×1×3 | 1094±348[a] | −82.60 | 0.6±0.24[a] | 85.00 |

Values are presented as the mean±SEM, one-way ANOVA followed by Tukey's test; *Administration of extract and reference drug PZQ on days 2-4 post-inoculation with four cysticercoids per rat; [a]$P<0.001$, as compared to control. SEM: Standard error of the mean, ANOVA: Analysis of variance, EPG: Eggs per gram, PZQ: Praziquantel, *C. bonducella: Caesalpinia bonducella, H. diminuta: Hymenolepis diminuta*

**Table 3: Effects of *C. bonducella* leaf extract* on mature stages of *H. diminuta* infections in rats (*n*=5)**

| Groups (mg/kg×dose×day) | EPG (mean±SEM) | | Percentage difference in EPG (A-B) | Worms count/rat (mean±SEM) | Percentage reduction in worm counts |
|---|---|---|---|---|---|
| | Pre-treatment Days 18-20 (A) | Post-treatment Days 28-30 (B) | | | |
| Control | 28992±4761 | 25233±1939 | −12.9 | 4.0±0.00 | 0.00 |
| Plant extract | | | | | |
| 200×1×3 | 22966±3885 | 7186±318[a] | −68.71 | 1.8±0.31[b] | 55.00 |
| 400×1×3 | 20700±2876 | 4510±428[a] | −78.21 | 1.2±0.37[b] | 70.00 |
| 800×1×3 | 20233±1354 | 1713±261[a] | −91.53 | 0.6±0.24[b] | 85.00 |
| PZQ | | | | | |
| 5×1×3 | 22125±3590 | 4303±471[a] | −80.55 | 0.8±0.20[b] | 80.00 |

Values are presented as the mean±SEM, One-way ANOVA followed by Tukey's test; *Administration of extract and reference drug PZQ on days 21-23 post-inoculation with four cysticercoids per rat; [a]$P<0.001$ compared to pre-treatment, [b]$P<0.001$, as compared to control. SEM: Standard error of the mean, ANOVA: Analysis of variance, PZQ: Praziquantel, *C. bonducella: Caesalpinia bonducella, H. diminuta: Hymenolepis diminuta,* EPG: Eggs per gram

in worm counts as against 93% observed in the present study. Furthermore, the findings of the present study are in agreement with the findings of Shariat *et al.* [29], who reported 95% reduction of *S. obvelata* worms following treatment of infected mice by *Pleurotus eryngii* extract.

Similar findings of dose-dependent anthelmintic effects of various other medicinal plants against cestodes have also been reported by Temjenmongla and Yadav [30] and Kundu *et al.* [31]. In our study, the highest dose of plant extract showed a decline in worm burden of animals by 85%, which was of almost alike to a decrease in worm burden by reference drug PZQ [Table 2]. In related studies by Sapaat *et al.* [32] and Gangwar *et al.* [33], more or less a similar kind of reduction in EPG counts of *H. diminuta* infections in rats was also observed following treatment with extracts from the seeds and fruits of papaya and *Mallotus philippinensis*. Interestingly, in the present study, *C. bonducella* extract showed a better efficacy not only against the adult stages but also almost similar effects on larval stages of the parasite. However, in most other related studies on *in vivo* anthelmintic effects of plants extracts on various developmental stages of *H. diminuta*, it has been reported that plant extracts possess rather more significant effects on adult stages than the larval stages of parasite [8,34].

The phytochemical screening of *C. bonducella* leaf extract revealed the presence of several major chemical constituents, but tannins and flavonoids were present in abundance. It is likely that the potent anthelmintic activity of its methanol extract may be due to the presence of either of these bioactive constituents such as tannins or flavonoids. It is apparent from literature that these plant secondary metabolites have also been used as a primary source of treatment against gastrointestinal helminths for many centuries, and they also exhibit significant anthelmintic activities [23,35]. For example, isoflavones, such as genistein and daidzein, which are found in a number of plants, including in *Flemingia vestita*, have shown significant anthelmintic effects on intestinal helminth parasites [36]. Similarly, condensed tannins, which offer important quality traits to plants, also possess potential anthelmintic effect against gastrointestinal helminth parasites [37]. There are several such studies on the secondary metabolites which suggest that it is very likely that *C. bonducella* leaf extract brings out its anthelmintic effects due to the presence of these secondary metabolites. However, only further studies on isolation and characterization of its active constituents may throw more light on same.

## CONCLUSION

From the overall study, it may be concluded that *C. bonducella* leaf extract possesses a significant *in vitro* and *in vivo* anthelmintic effects on both, the nematode *S. obvelata* and cestode *H. diminuta* parasites. However, the actual mode of action of the plant extract has to be determined to draw a complete picture of the active compounds that are responsible for its anthelmintic action. Nevertheless, these findings lend support to the traditional use of this plant as anthelmintic in indigenous medicine. It is hoped that refining of plant formulation in a holistic manner may contribute to the development of a suitable herbal medicine with a low risk of resistance to intestinal helminths.

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Original Research

ScopeMed

# Evaluation of wound healing activity of cow urine ark in diabetic Wistar albino rats

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## ABSTRACT

**Aim:** To evaluate wound healing activity of cow urine ark in diabetic rats. **Materials and Methods:** Streptozotocin-induced diabetic Wistar albino rats were randomly divided into six groups ($n = 6$). Three groups - diabetic control, active control (glibenclamide), and treatment (cow urine ark) were operated for excision wounds (EWs). Rats in these groups received distilled water 1 ml/day, glibenclamide 0.5 mg/kg body weight/day, and cow urine ark 5.5 ml/kg body weight/day orally till complete healing of the EWs. EWs were evaluated for wound contraction on 3rd, 7th, and 11th day and for reepithelization on 11th day. The other three groups were operated for incision wounds (IW) as well as dead space wounds (DW) in the same animal which received the above agents orally for 11 days. IWs were analyzed for wound breaking strength and DWs were analyzed for dry weight, hydroxyproline content, and histology of granulation tissue. **Results:** EWs showed significantly increased wound closure in the treatment group as compared to the diabetic as well as active control groups at 3rd ($P < 0.001$) and 11th ($P < 0.05$) post-wounding day and to the only diabetic control group at 7th ($P < 0.01$) post-wounding day. IWs showed significant improvement in wound breaking strength in the treatment as compared to diabetic ($P < 0.001$) and active control ($P < 0.01$) groups. DWs showed significant increase in hydroxyproline content of granulation tissue in the treatment as compared to diabetic control ($P < 0.001$) and active control ($P < 0.001$) groups. Wound breaking strength and hydroxyproline content also significantly increased in the active control group compared to diabetic control ($P < 0.001$ and $P < 0.05$, respectively). Granulation tissue dry weight was significantly increased in treatment and active control groups as compared to diabetic control ($P < 0.001$). **Conclusion:** Cow urine ark increases granulation tissue formation as well as collagen content. Wound contraction was also significantly improved. The cow urine ark could be potentially effective in promoting healing of diabetic wounds by increasing granulation tissue formation and collagen content, however, further studies are required for its clinical application.

KEY WORDS: Collagen, cow urine ark, granulation tissue, streptozotocin-induced diabetes

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## INTRODUCTION

Diabetes mellitus (DM) is part of a larger global epidemic of non-communicable diseases. India accounts for nearly 15% of the global diabetes burden with 40.9 million people affected [1]. The wound healing is a complex but highly dynamic process of cellular, physiological and biochemical events, which leads to the functional restoration of injured tissue. DM is one of the common causes of impaired wound healing, which imposes high morbidity and health care cost [2,3]. Chronic non-healing ulcers are the common complications associated with diabetes [4]. Diabetic patients often ignore lower extremity ulcers, which lead to wound infection and ultimately amputation. Causal mechanisms associated with impaired wound healing in diabetes are micro- and macro-vascular abnormalities, impaired epithelization, and reduced angiogenesis [5]. Besides, oxidative stress associated with hyperglycemia in wound environment has also been suggested to delay wound healing process in diabetic patients [6].

Because of lack of well-established medical treatment, diabetic wounds are conservatively treated by surgical debridement of necrotic tissue as well as by controlling blood sugar level. A variety of treatments had been evaluated for the management of diabetic wounds including herbal medicines. In ancient Indian Ayurvedic literature such as Charak Samhita and Sushruta Samhita, cow urine is mentioned as the most effective substance of animal origin with innumerable therapeutic value [7]. It is one of the important contents of "Panchgavya" containing five important substances obtained from cow, namely urine, dung, milk, ghee, and curd [8].

Medicinal properties of cow urine such as bioenhancer, antibiotic, antifungal, and anticancer have been patented

under US patent number 6,896,907 and 6,410,059 [9,10]. Several studies had suggested that cow urine has anti-diabetic potential probably due to its antioxidant properties [11,12]. Besides, external application of cow urine on excision wound (EW) has shown to hasten the wound healing [13]. Another study of histomorphological analysis of surgically created wounds in healthy goats showed significant infiltration of polymorphonuclear cells, neovascularization, and fibroblast proliferation in cow urine treated wounds [14]. Based on these observations, we designed this study to evaluate wound healing effect of cow urine ark in diabetic rats.

## MATERIALS AND METHODS

Institutional Animal Ethics Committee, Government Medical College, Bhavnagar, Gujarat (India) (Approval No. - 26/2012; Pharmacology No. - 24/2012) approval was taken before starting this study. Wistar albino rats of either sex with an approximate weight of 200-350 g were placed in individual polypropylene cages and acclimatized to the laboratory environment for 1 week. The rats were maintained on normal laboratory food and water *ad libitum*, under controlled room temperature (25°C ± 2°C, 60-70% humidity) and 12-12 h light-dark cycle.

### Streptozotocin (STZ) - Induced Diabetes

The rats were given a single intraperitoneal dose of STZ 50 mg/kg body weight (Alfa Aesar, A Johnson Matthey Company, MA/USA, CAS: 18883-66-4) to produce DM [15]. Random blood sugar (RBS) was measured from a blood drop drawn from the tail vein using glucometer (Accu - Chek Go, Roche Diagnostic, Germany) after 4 days of STZ injection. The rats with RBS >300 mg/dl were enrolled in the study. Insulin neutral protamine Hagedorn (5 IU/kg body weight; Wosulin, Wockhardt Limited, India) was given subcutaneously, once a day to maintain the RBS between 250 and 350 mg/dl.

### Experimental Design and Wound Models

The diabetic Wistar albino rats were divided into six groups (n = 6 in each group). Three groups - Group I (diabetic control), Group II (glibenclamide - active control), and Group III (Cow urine ark - treatment group) served as the EW groups. They received distilled water 1 ml/day, glibenclamide (SIGMA Life Science, New Delhi, India) 0.5 mg/kg body weight/day, and cow urine ark 5.5 ml/kg body weight/day orally till complete healing of the EWs. The cow urine ark was procured from Go-Vigyan Anushandhan Kendra, Nagpur, India. The remaining three groups - Group IV (Diabetic control), Group V (Glibenclamide - active control), and Group VI (Cow urine ark - treatment) served as incision and dead space wound (DW) groups and received the same doses of respective agents, as described above, for 11 days. All the wounds were inflicted under ketamine (75 mg/kg body weight) and xylazine (10 mg/kg body weight) anesthesia with proper aseptic precautions.

### EW

EWs were inflicted by excising a circular patch of full thickness skin (measuring approximately 500 $mm^2$) from the nape of the neck [16]. Wound margins were traced on transparent plastic sheets on day 0, 3, 7 and 11 to evaluate the wound contraction. Reepithelization was measured on $11^{th}$ post wounding day when epithelization was visible. The wound area was measured using a UTHSCA image analyzer (version 3.00, The University of Texas Health Science Center, San Antanio, USA) from the scanned transparent plastic sheets. Wound closure rate was calculated by the formula [15]:

$$\text{Wound closure rate } (\%) = \frac{\text{Area day } 0 - \text{Area day n}}{\text{Area day } 0} \times 100$$

Where area day 0 = Initial wound area at day 0,
Area day n = Area on $n^{th}$ post wounding day.

The wound re-epithelialization was calculated using the formula [15]:

Reepithelialization (%) on $11^{th}$ day = Total wound area (%) − Wound area not covered with epidermis (%).

### Re-sutured Incision and DW

About 5 cm long full thickness paravertebral incisions were made on either side of the vertebral column and sutured with black silk 4.0 sutures [17]. Sterile grass piths (measuring 2.5 cm × 0.3 cm) were inserted and sutured in the loose areolar tissue of the groins in the same rats to produce DWs. While in axillary region, sterile cotton pellet (weighing 10 mg) was inserted and sutured. The sutures of incision wound (IW) were removed on the $7^{th}$ day of wounding and of DWs on the $11^{th}$ day. The IW breaking strength was measured on $11^{th}$ post wounding day by constant water flow technique described by Lee under anesthesia [18]. The rats were then sacrificed with a high dose of ketamine and xylazine. The granulation tissue formed on grass piths were utilized for the hydroxyproline estimation and histological examination [19]. The hydroxyproline content was measured as $\mu$g/100 mg of granulation tissue. The cotton pellets with granulation tissue around it were excised from the axillary region and dried overnight at 60°C in hot air oven. The weight of overnight dried pellets was expressed as mg/100 g body weight [20].

Hematoxylin and Eosine (H and E) stained sections of granulation tissues were semi-quantitatively analyzed by a pathologist. Grades were given (grade 1-4) for the presence of polymorphonuclear cells, macrophages, fibroblasts, and neo-angiogenesis [21].

### Statistical Analysis

Analyses were performed using GraphPad InStat demo version number 3.0. The data were expressed as mean ± standard error of mean. One-way analysis of variance followed by Tukey–Kramer test for parametric variables and Kruskal–

Wallis followed by Dunn's multiple comparison test for non-parametric variables were used to compare mean differences between different groups. $P < 0.05$ was considered as statistically significant.

## RESULTS

### EW

The wound contraction was significantly higher in cow urine ark group than diabetic control group throughout healing period. Statistically significant wound healing was also observed in cow urine ark group as compared to glibenclamide group on day 3 and 11 (Table 1). The wound closure photographs of all the groups are shown in Figure 1. Re-epithelialization on the 11th post wounding day was not significantly affected in cow urine ark group (Table 1). The wound closure was a faster throughout healing period (owing to wound contraction) as shown in Figure 2. The wound closure was almost 100% on day 14 in cow urine ark group (Figure 2).

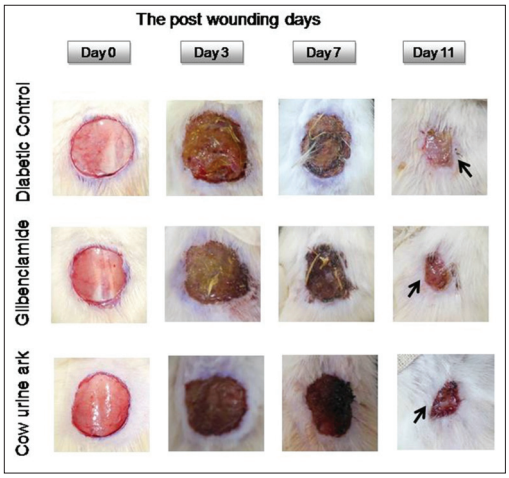


**Figure 1:** The excision wound healing time course noted on 0, 3, 7 and 11 post-wounding day

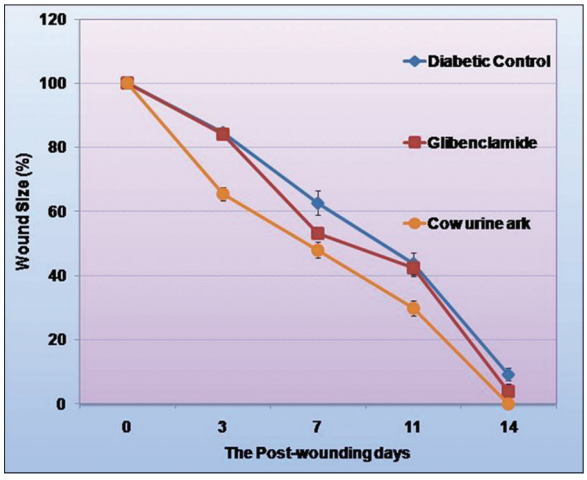


**Figure 2:** Wound healing curve. The graph shows the percentage of excision wound closure over the time period

### Resutured Incision and DW

Mean IW breaking strength on the 11th post wounding day in cow urine ark group was significantly higher as compared to diabetic control as well as glibenclamide group. The hydroxyproline content in granulation tissue from cow urine ark group was significantly higher than both the diabetic control and glibenclamide group (Table 2). Overnight dried cotton pellet weight (granulation tissue dry weight) was also significantly increased as compared to diabetic control group; however, it could not reach statistically significant level as compared to glibenclamide group.

A semi-quantitative histological analysis of granulation tissue obtained at 11th post-wounding day did not show any difference among the groups in any of the parameters examined (Table 3).

## DISCUSSION

The wound healing activity of cow urine ark in diabetic Wistar albino rats was evaluated in this study using several models

Table 1: Effect of cow urine ark on excision wound model

| Groups ($n$=6) | Wound closure (%) | | | Re-epithelialization (%) 11th Day |
|---|---|---|---|---|
| | 3rd Day | 7th Day | 11th Day | |
| Diabetic control | 15.3±1.4 | 37.3±3.8 | 56.3±3.4 | 56.01±2.5 |
| Glibenclamide | 15.7±1.2 | 46.7±1.8 | 57.6±2.5 | 63.2±1.8 |
| Cow urine ark | 34.5±2.1* | 52.0±2.5# | 70.2±2.4$ | 55.9±1.6 |

$n$=6 in each group, values are represented as mean±SEM, *$P$<0.001 as compared to diabetic control and glibenclamide; #$P$<0.01 as compared to diabetic control (Tukey Kramer multiple comparison test); $$P$<0.05 as compared to diabetic control and glibenclamide (Dunn's multiple comparisons test)

Table 2: Effect of cow urine ark on incision and dead space wound model

| Group | Incision wound breaking strength (g) | Granulation tissue dry weight (mg/100 g body weight) | Hydroxyproline (μg/100 mg granulation tissue) |
|---|---|---|---|
| Diabetic control | 265.8±10.4 | 26.1±0.6 | 19.3±0.5 |
| Glibenclamide | 369.2±8.9* | 37.5±0.9* | 24.4±0.9@ |
| Cow urine ark | 457.5±19.5*# | 39.1±1.4* | 36.5±1.9*$ |

$n$=6 in each group, values are represented as mean±SEM. *$P$<0.001 as compared to diabetic control; #$P$<0.01 as compared to glibenclamide; $$P$<0.001 as compared to glibenclamide; @$P$<0.05 as compared to diabetic control (Tukey–Kramer multiple comparison test)

Table 3: Semi-quantitative evaluation of histological changes in granulation tissue in dead space wound model

| Histological changes | Groups | | |
|---|---|---|---|
| | Diabetic control | Glibenclamide | Cow urine ark |
| Neutrophils | 1.0±0.36 | 1.5±0.55 | 1.1±0.1 |
| Macrophages | 0.3±0.21 | 0.8±0.3 | 0.6±0.21 |
| Fibroblasts | 2.5±0.42 | 1.4±0.2 | 2.3±0.21 |
| Neo-angiogenesis | 1.8±0.54 | 1.2±0.16 | 1.4±0.2 |

$n$=6 in each group, values are represented as mean±SEMs. (Dunn's multiple comparison test, $P$<0.05)

based on previous research, which demonstrated significant anti-diabetic, antimicrobial, and antioxidant effects [11,12,22]. Besides, volatile fatty acids present in cow urine ark probably contribute to its antioxidant properties which might reduce oxidative stress in diabetic wounds [23]. Cow urine has been studied for wound healing activity in non-diabetic wounds using EW model, however, at the best of our knowledge; this study is the first to evaluate the wound healing activity of cow urine ark in diabetic wounds using different wound models [13].

In this study, we used a well-established model of diabetic wound healing, i.e., STZ-induced diabetes model [24]. This model has been shown to exhibit increased superoxide levels [25]. Thus, it provides suitable opportunity to study effects of the study agent on diabetic wound healing. We used glibenclamide as active control as lowering of blood glucose in diabetes has shown to promote wound healing [26]. This active control group helps to differentiate if cow urine ark has any additional advantage in promoting diabetic wound healing over blood glucose lowering agents.

In EW group, cow urine ark significantly increased wound contraction as compared to the diabetic as well as active control groups. This is similar to the previous study which showed significant wound contraction in non-diabetic rats topically treated with cow urine [13]. Reepithelization on the $11^{th}$ post wounding day was not much affected by cow urine ark. Significant improvement in wound contraction as compared to glibenclamide in our study suggests that cow urine ark has an additional advantage over blood glucose lowering agents.

Cow urine ark had significantly increased the IW breaking strength, granulation tissue dry weight, and hydroxyproline contents compared to diabetic control. Furthermore, it had significantly increased IW breaking strength and granulation tissue hydroxyproline content as compared to glibenclamide group. Hyperglycemia associated with the DM is responsible for the generation of reactive oxygen species which in turn create excessive oxidative stress [27]. Increased oxidative stress leads to activation of matrix metalloproteinases (MMP) which increase collagen degradation and decreases collagen synthesis [28]. The antioxidant property of cow urine ark could decrease collagen degradation and increase collagen synthesis which can explain increased IW breaking strength and hydroxyproline content of granulation tissue in the treatment group.

Diabetic wounds are generally more susceptible to infection than non-diabetic wounds [29]. Wound infection is a common cause of wound chronicity. It prolongs the inflammatory phase by increasing the production of inflammatory cytokines which lead to additional tissue destruction as well as delays wound collagen synthesis [30]. Therefore, preventing wound infection would hasten wound healing process by promoting collagen synthesis. Various studies have demonstrated that cow urine, as well as its distillate, has antibacterial and antifungal activity against various clinical strains of these pathogens [8,22,31]. Thus, cow urine ark could have hastened the process of collagen formation by preventing the subtle wound infections which ultimately increased IW breaking strength, granulation tissue dry weight and hydroxyproline contents in this study.

Histological examination of granulation tissue obtained from DWs did not reveal any significant difference among the groups in polymorphonuclear cell infiltration, macrophage, fibroblast, or neo-angiogenesis in our study set up. This is in contrast to the previous study which showed maximum polymorphonuclear cell infiltration, neovascularization and fibroblast proliferation in surgically created wounds [14]. This could probably be explained by the method by which we obtained granulation tissue. In our study, granulation tissue was obtained from foreign body material inserted in dead space created in groins of the rats. While they analyzed tissue obtained from wound margin and healthy skin.

There are several limitations in our study. The methods used for estimation of re-epithelialization in EW and hydroxyproline content in granulation tissue are relatively crude and less sensitive; hence, limit interpretation of the results. More sophisticated methods might have been used for this analysis, however, due to limited resources, it could not be performed in our setup.

## CONCLUSION

This study demonstrated that cow urine ark significantly enhances the collagen content and granulation tissue formation in diabetic wounds. Thus, it can be useful in accelerating wound healing in diabetic patients because of its property of enhancing granulation tissue formation. Further clinical trials in diabetic patients would be more helpful for its clinical application.

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Short Communication

# Effects of a diet containing Brazilian propolis on lipopolysaccharide-induced increases in plasma plasminogen activator inhibitor-1 levels in mice

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## ABSTRACT

**Background:** Brazilian propolis has many biological activities including the ability to help prevent thrombotic diseases, but this particular effect has not been proven. Plasma levels of plasminogen activator inhibitor-1 (PAI-1), an inhibitor of fibrinolysis, increase under inflammatory conditions such as infection, obesity and atherosclerosis and such elevated levels predispose individuals to a risk of developing thrombotic diseases. **Aim:** This study aimed to determine the effects of a diet containing Brazilian propolis on lipopolysaccharide (LPS)-induced increases in plasma PAI-1 levels. **Materials and Methods:** Mice were fed with a diet containing 0.5% (w/w) Brazilian propolis for 8 weeks. Thereafter, the mice were subcutaneously injected with saline containing 0.015 mg/kg of LPS and sacrificed 4 h later. **Results:** Orally administered Brazilian propolis significantly suppressed the LPS-induced increase in PAI-1 antigen and its activity in mouse plasma. **Conclusion:** This study indicated that Brazilian propolis contains natural products that can decrease thrombotic tendencies in mice.

KEY WORDS: Dietary supplement, mouse, plasminogen activator inhibitor-1, thrombosis

## INTRODUCTION

Prothrombotic factors play a key role in the development of thrombotic diseases [1]. For instance, plasminogen activator inhibitor-1 (PAI-1) is the main physiological inhibitor of the fibrinolytic system, where it plays an important role in disease prevention by removing thrombi from the vascular system [2]. It is also the primary physiological inhibitor of tissue-type plasminogen activator, a key protease of the fibrinolytic system [2], through forming irreversible 1:1 complexes. An increase in plasma PAI-1 levels suppresses the normal fibrinolytic system and leads to prothrombotic states. Many factors such

as blood lipids, glucose, and insulin influence habitual PAI-1 levels. Therefore, elevated PAI-1 levels are intimately related to prothrombotic states such as hypertension, obesity, insulin resistance, and diabetes as well as aging [3-6]. Increased levels of plasma PAI-1 comprise a risk factor for thrombotic diseases and serve as a marker of the onset of such diseases [2]. Maintaining physiological plasma levels and activities of PAI-1 might thus represent a promising intervention for treating and preventing thrombotic diseases [7].

Many compounds that affect coagulation and platelet function are derived from natural sources such as garlic, ginger, gingko, and mushrooms [8,9]. Furthermore, the reports indicate that some products derived from natural sources inhibit PAI-1 production *in vitro* and in experimental animals *in vivo*. For example, Liu *et al*. reported that green tea polyphenols inhibit PAI-1 expression and secretion in endothelial cells [10], and Zhou *et al*. reported that salvianolic acid B attenuates PAI-1 production in human umbilical vein endothelial cells (HUVEC) incubated with tumor necrosis factor α (TNF-α) [11]. A citrus extract containing flavones represses PAI-1 expression in human colon fibroblasts [12] and xanthoangelol isolated from *Angelica keiskei* inhibits PAI-1 increases in mice plasma induced by lipopolysaccharide (LPS) and in culture media of HUVEC induced by TNF-α [13].

Propolis is a hive product comprising resinous materials collected by honey bees from plants and it includes over 300 chemical compounds [14]. Propolis is a feature of folk medicines and health supplements worldwide, and various biological activities have been indicated [14,15]. The composition and biological activities of propolis greatly depend on the location of the honey bees and the plant source from which it is derived [16]. Brazilian propolis contains various biologically active organic compounds in abundance such as artepillin C [17]. The effect of Brazilian propolis on various pathological conditions such as tumors, inflammation, diabetes, and immunocompromised patients have mainly been investigated *in vitro* and in experimental animals [18-22], inflammatory conditions alter the coagulation and fibrinolytic system, frequently leading to a procoagulant state [23]. Proinflammatory cytokines and endotoxins play a central role in the effects on the coagulation and fibrinolysis pathways [24]. Brazilian propolis inhibits increases in PAI-1 antigen induced by TNF-α in culture media of HUVEC [25]. Anti-inflammatory properties of Brazilian propolis have been demonstrated in mouse models of inflammation and in cultured activated macrophages [20,26,27]. This study aimed to determine the effects of a diet containing Brazilian propolis on LPS-induced plasma PAI-1 increases in model mice.

## MATERIALS AND METHODS

### Materials

Brazilian propolis (Institute for Bee products and Health Science, Yamada Bee Company Inc., Okayama, Japan) is an ethanol extract comprising 55% (w/w) solids. Samples of propolis were collected from colonies of Scaptotrigona bees between February 2007 and December 2008 in Minas Gerais, Brazil. Insoluble matter was removed by passage through diatomaceous earth and filter paper. LPSs from *Escherichia coli* 0111:B4 were obtained from Sigma Chemical Co. (St. Louis, MO, USA). All other materials were commercial products of the highest grade available.

### HPLC Analysis of Extract

The ethanol extract of Brazilian was analyzed by HPLC [28] using a Cosmosil 5C18-ARII column (Nacalai Tesque, Kyoto, Japan) and a gradient $CH_3CN$ in 0.1% trifluoroacetic acid at a flow rate of 1.0 mL/min. Compounds were detected at 260 nm.

### Animal Experiments

About 7-week-old male kwl ICR mice (Tokyo Laboratory Animals Science Co. Ltd., Tokyo, Japan) were housed at 24 ± 2°C and provided with water and the MF diet (Oriental yeast Co. Ltd., Tokyo, Japan) *ad libitum*. The mice were subcutaneously injected with saline containing 0.015 mg/kg of LPS, sacrificed at indicated times, and then, blood was collected into plastic tubes containing 3.2% sodium citrate. The effect of propolis was evaluated by providing the mice with water and the MF diet (Oriental yeast Co. Ltd., Tokyo, Japan) with or without 0.5% (w/w) of propolis ethanol extract for 2, 4 and 8 weeks *ad libitum*. The mice were then subcutaneously injected with saline containing 0.015 mg/kg of LPS. Blood specimens collected from the inferior vena cava using a plastic syringe and needle under pentobarbital (40 mg/kg i.p.) anesthesia 4 h later was mixed with 0.2 volumes of 3.2% sodium citrate. Platelet-poor plasma prepared by centrifugation at 7000 rpm (3800 ×g) for 10 min at 4°C in an MX-100 micro-centrifuge (TOMY, Tokyo, Japan) was stored at −80°C. All animal experiments proceeded in accordance with the Guide for the Care and Use of Laboratory Animal at Teikyo University and were approved by the Animal Care and Use Committee at Teikyo University (Permission No. 12-013).

### Measurement of Plasma PAI-1 Levels and Activity

Levels of total PAI-1 and PAI-1 activity in mouse plasma and corresponding active PAI-1 antigen levels were measured using relevant ELISA kits (Molecular Innovations Inc., Southfield, MI, USA) according to the manufacturer's instructions.

### Statistics

Data are expressed as means ± standard deviation. Statistical significance was determined using Mann–Whitney U tests. $P < 0.05$ was considered to represent significance.

## RESULTS

The effects of subcutaneous injection of LPS on plasma PAI-1 levels in mice were observed. The mice were subcutaneously injected with 0.015 mg/kg LPS, blood was collected at the indicated times, and then total PAI-I antigen in plasma

was determined. Figure 1 shows the time course of PAI-1 antigen levels in plasma after LPS injection. The LPS caused a significant increase in plasma PAI-1 levels that peaked 4 h after injection. The LPS-induced PAI-1 increase in plasma was statistically significant at 3 and 4 h compared with control mice (3 h control [means = 1.06, (95% confidence interval [CI], 0.43-1.69), $n = 4$] vs. 3 h LPS [means = 12.43, (95% CI, 7.87-17.00), $n = 3$], $P = 0.034$; 4 h control [means = 1.34, (95% CI, −0.26-2.94), $n = 4$] vs. 4 h LPS [means = 12.98, (95% CI, 8.12-17.84), $n = 3$], $P = 0.034$).

The inhibitory effects of dietary propolis on LPS-induced PAI-1 production were assessed in mice fed with a diet containing 0.5% (w/w) Brazilian propolis for 8 weeks. Thereafter, the mice were subcutaneously injected with saline containing 0.015 mg/kg of LPS and sacrificed 4 h later because PAI-1 levels peaked at this point [Figure 1]. Stimulation with LPS (LPS [+]) significantly increased levels of PAI-1 antigen in plasma compared with control LPS (−) mice (LPS [−] control [means = 1.49 (95% CI, 0.78-2, 21), $n = 8$] vs. LPS (+) control [means = 14.87 (95% CI, 12.36-17.38, $n = 11$], $P < 0.001$). Orally administered propolis significantly suppressed the LPS-induced increase of PAI-1 antigen in mouse plasma (LPS (+) control vs. LPS (+) propolis, [means = 7.74 (95% CI, 4.66-10.81, $n = 12$], $P = 0.002$) [Figure 2].

The plasma levels of PAI-1 activity were then measured using an ELISA that detects active PAI-1 antigen. Since the active form spontaneously converts to the latent form, understanding levels of the active form are important for evaluating plasma PAI-1 activity. Figure 3 shows that LPS also increased the level of active PAI-1 (PAI-1 activity) in plasma. Dietary propolis for 8 weeks significantly decreased the LPS-induced increase in PAI-1 activity (LPS [+] control [means = 13.07 (95% CI, 5.40-20.75), $n = 6$] vs. LPS (+) propolis, [means = 4.47 (95% CI, −0.09-6.40, $n = 6$], $P = 0.014$) [Figure 3]. The oral administration of propolis for 8 weeks did not affect plasma PAI-1 antigen levels in non-stimulated mice (LPS [−] control vs. LPS [−] propolis, [means = 1.16 (95% CI, 0.93-1.39), $n = 9$], $P = 0.643$) [Figure 2]. These results show that oral propolis administered for 8 weeks does not affect spontaneous plasma PAI-1 levels.

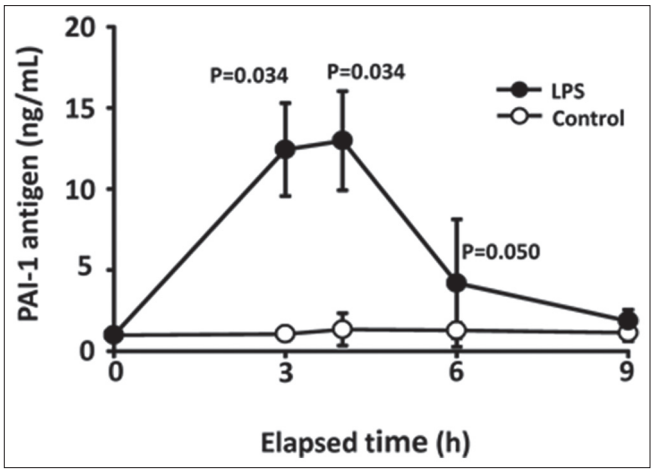


**Figure 1:** Time course of plasminogen activator inhibitor-1 (PAI-1) antigen levels in plasma after lipopolysaccharide (LPS) injection. Mice were subcutaneously injected with 0.015 mg/kg LPS, blood was collected at indicated times and then total PAI-1 antigen in plasma was determined using ELISA kits. Data are expressed as means ± standard deviation (LPS, $n = 3$; control $n = 4$)

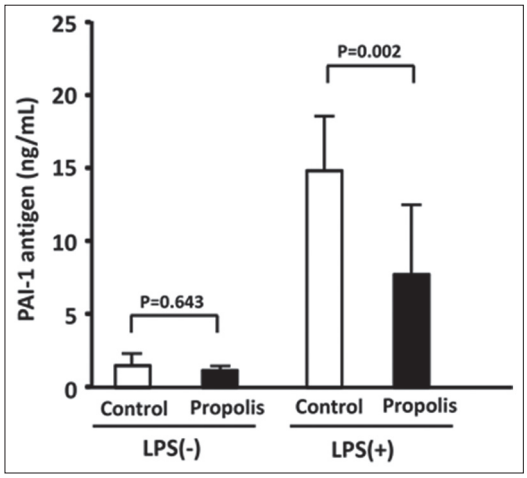


**Figure 2:** Effects of dietary propolis on lipopolysaccharide (LPS)-induced increase in plasminogen activator inhibitor-1 (PAI-1) antigen in mouse plasma. Mice were fed with a diet containing 0.5% (w/w) Brazilian propolis for 8 weeks. Thereafter, the mice were subcutaneously injected with saline containing 0.015 mg/kg of LPS and sacrificed 4 h later. Total PAI-1 antigen in plasma was determined using ELISA kits. Data are expressed as means ± standard deviation ($n = 8$~12)

## DISCUSSION

PAI-1 inhibits the normal fibrinolytic system and the increase in plasma are involved in the onset of thrombotic diseases. PAI-1 is a powerful acute-phase reactant, reflected by rapid, large increases in plasma levels during the acute-phase response [29]. Thus, the suppression of increased PAI-1 expression during the acute phase would improve prothrombotic conditions. This study aimed to determine the effects of diets containing Brazilian propolis on LPS-induced plasma PAI-1 increases in model mice. The amount of LPS used was considerably less than that applied in the LPS-induced disseminated intravascular coagulation model [30]. These findings confirmed that the present mouse model has a slight thrombotic tendency without clot formation, despite having increased plasma PAI-1 levels.

This study showed that orally administered Brazilian propolis significantly suppressed LPS-induced increases of PAI-1 antigen and activity in mice. PAI-1 production was significantly suppressed in mice that consumed Brazilian propolis for 8 weeks. This time frame is appropriate to explore anti-thrombotic and anti-inflammatory effects in mice [13,31,32].

Brazilian propolis contains many natural components [33,34] and Table 1 lists some of the components of the Brazilian propolis used in the present study. Among many natural compounds such as cinnamic acid derivatives (drupanin, artepillin C, and baccarin), benzoic acid derivatives (caffeic acid and coumaric acid), chlorogenic acid, and flavonoids (chrysin,

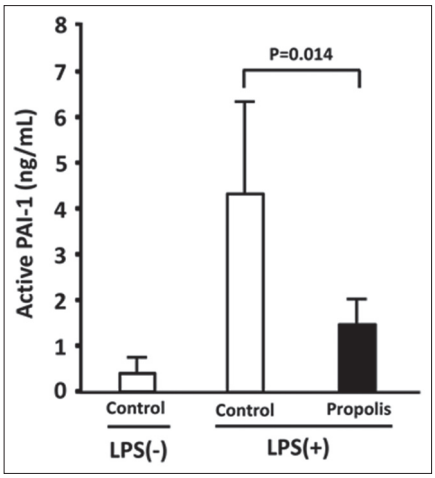


**Figure 3:** Effect of dietary propolis on lipopolysaccharide (LPS)-induced increase in plasminogen activator inhibitor-1 (PAI-1) activity in mouse plasma. Mice were fed with a diet containing 0.5% (w/w) Brazilian propolis for 8 weeks. Thereafter, the mice were subcutaneously injected with saline containing 0.015 mg/kg of LPS and sacrificed 4 h later. PAI-1 activity in plasma was determined using active PAI-1 ELISA kits. Data are expressed as means ± standard deviation ($n$ = 5~6)

**Table 1: Contents of cinnamic acid derivatives, benzoic acid derivatives, chlorogenic acid and flavonoids in ethanol extract of Brazilian propolis**

| Compound | Contents of ethanol extract of solid Brazilian propolis (mg/100 g) |
|---|---|
| Chlorogenic acid | 120 |
| Coumaric acid | 1200 |
| Caffeic acid | 140 |
| Drupanin | 1400 |
| Artepillin C | 9500 |
| Baccharin | 3500 |
| Chrysin | 2.9 |
| Naringenin | 1.9 |
| Pinocembrin | 37 |
| Kaempferide | 1700 |
| Kaempferol | 110 |

naringenin, pinocembrin, kaempferide, and kaempferol) [Table 1]; cinnamic acid derivatives are likely to have anti-thrombotic activities. The commercially-produced cinnamic acid derivative, artepillin C, the major cinnamic acid derivative in Brazilian propolis, tended to inhibit PAI-1 production in a previous study [25]. Konishi *et al*. have described the absorption and bioavailability of artepillin C in rats after oral administration [35]. The other cinnamic acid derivatives, drupanin and baccharin, are abundant in Brazilian propolis [Table 1] and have various biological activities [18,36-39]. Thus, one or more cinnamic acid derivatives in Brazilian propolis might contribute to the inhibition of PAI-1 production. Chrysin inhibits TNF-α -induced increases in PAI-1 antigen in culture media of human HUVEC, and LPS induces increases in PAI-1 antigen in mouse plasma [25]. Chrysin is abundant in European propolis [40], but the Brazilian propolis used in the present study contains only trace amounts (2.9 mg/100 g propolis extract). Thus, the contribution of chrysin in Brazilian propolis to inhibiting PAI-1 production might be minimal. Detailed information is needed about specific molecules in Brazilian propolis that suppress increases in PAI-1.

## CONCLUSION

The present findings indicated that Brazilian propolis contains some natural products that can decrease thrombotic tendencies in mice by suppressing increased plasma PAI-1 levels.

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