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Original Research

Protective Effect against Hydroxyl-induced DNA Damage and Antioxidant Activity of Radix *Codonopsis*

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Keywords: Radix Codonopsis, µ³²Î, antioxidant activity, DNA oxidative damage, phenolic acid, flavonoids.

Abstract

Aim: As a typical Chinese herbal medicine, Radix *Codonopsis* has been used in traditional Chinese medicine for about 250 years. The study tried to investigate its antioxidant activity, then to discuss the antioxidant mechanism.

Methods: Radix *Codonopsis* was extracted by ethanol to obtain ethanolic extract of Radix *Codonopsis*. The extract was then determined by various antioxidant methods, including DNA damage assay, DPPH (1,1-diphenyl-2-picryl-hydrazl radical), ABTS [2,2′-azino-bis(3-ethylbenzo- thiazoline-6-sulfonic acid) radical] assay, Fe^{3+}-reducing assay and Cu^{2+}-reducing assay. Finally, the contents of total phenolics and flavonoids in the extract were determined by spectrophotometric method.

Results: The ethanolic extract of Radix *Codonopsis* showed protective effect against hydroxyl-induced DNA damage (IC_{50} 1180.28±137.73 µg/mL) and exhibited DPPH· scavenging, $ABTS^{+}$• scavenging, Fe^{3+} reducing, and Cu^{2+} reducing abilities, and the IC_{50} values were 3857.79±35.51, 271.82±5.66, 759.99±31.65, and 733.02±9.67 µg/mL, respectively. The contents of total phenolics and flavonoids in the extract were calculated as 12.56±0.56 and 11.95±0.52 mg quercetin/g, respectively.

Conclusion: Radix *Codonopsis* can effectively protect against hydroxyl-induced DNA damage. One mechanism of protective effect may be radical-scavenging which is via donating hydrogen atom (H·), donating electron (e). Its antioxidant ability can be mainly attributed to the existences of flavonoids or phenolic acids.

INTRODUCTION

It is well known that reactive oxygen species (ROS) are various forms of activated oxygen including free radicals and non-free-radical species. ROS, particularly hydroxyl radical (•OH) with high reactivity, can oxidatively damage DNA then lead to severe biological consequences including mutation, cell death, carcinogenesis, and aging [1].

Therefore, it is critical to search for potential therapeutic agents for DNA oxidative damage. In recent years, medicinal plants especially Chinese medicinal herbals have attracted much attention.

Radix *Codonopsis* (RC) (党参 in Chinese, Figure 1A) which comes from dried radixs of *Codonopsis pilosula* (Franch.) Nannf. (Figure 1B), *Codonopsis pilosula* Nannf. var. *modesta* (Nannf.) L.T. Shen, or *Codonopsis tangshen* Oliv., has been used as a Chinese herbal medicine for about 250 years [2,3]. In traditional Chinese medicine (TCM), it can tonify spleen to replenish *qi*.

According to free radical biology & medicine [4], its curative effects can partly be attributed to the antioxidant effect. However, its antioxidant effect has not been explored so far.

Therefore, the aim of the study was to investigate the antioxidant ability, then further discuss the antioxidant mechanism.

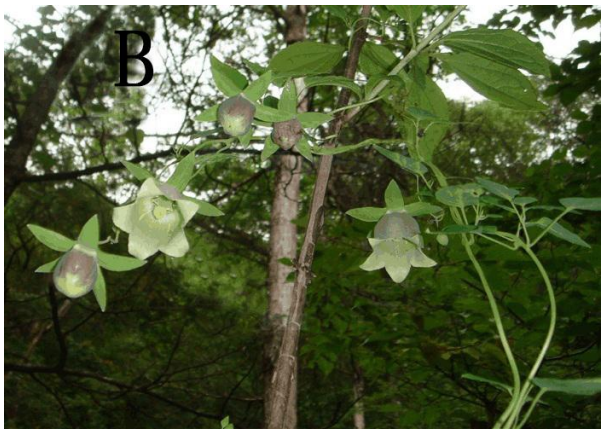


Figure 1. *Rhizoma Codonopsis* (A) and the plant *Codonopsis pilosula* (Franch.) Nannf. (B)

Figure 1A was contributed by Weitao Chen, Oct., 2012; Figure 1B was contributed by Zhijun Guo, Aug., 2011.

MATERIAL AND METHODS

Plant material

Radix *Codonopsis* was purchased from Caizhilin Pharmacy of Guangzhou University of Chinese Medicine (Guangzhou, China), and authenticated by Professor Shuhui Tan. A voucher specimen was deposited in our laboratory.

Chemicals

DPPH• (1,1-diphenyl-2-picryl-hydrazl radical), ABTS [2,2′-azino-bis(3-ethylbenzo- thiazoline-6-sulfonic acid diammonium salt)], BHA (butylated hydroxyanisole), Trolox [(±)-6- hydroxyl-2,5,7,8-tetramethlychromane-2-carboxylic acid], DNA sodium salt (fish sperm), neocuproine (2,9-dimethyl-1,10-phenanthroline), and Folin-Ciocalteu reagent were purchased from Sigma Co. (Sigma-Aldrich Shanghai Trading Co., China). Other chemicals used in this study were of analytical grade.

Preparation of extracts from Radix Codonopsis

Radix *Codonopsis* was powdered then extracted by absolute ethanol using a Soxhlet extractor for 6 hr. Extract was filtered using a Buckner funnel and Whatman No 1 filter paper. Filtrate was then concentrated to dryness under reduced pressure to yield ERC (ethanol extract of Radix *Codonopsis*). It was stored at 4°C for analysis.

Protective effect against hydroxyl-induced DNA damage

The experiment was conducted as described in previous report [5]. However, deoxyribose was replaced by DNA sodium. Briefly, sample was dissolved in methanol to prepare the sample solution at 8 mg/mL. Various amounts (20-100 μL) of sample solutions were then separately taken into mini tubes. After evaporating the sample solution in tube to dryness, 400 μL phosphate buffer (0.2 mol/L, pH 7.4) was brought to the sample residue. Then, 50 μL DNA sodium (10.0 mg/mL), 50 μL H_2O_2 (50 mmol/L), 50 μL $FeCl_3$ (3.2 mmol/L) and 50 μL Na_2EDTA (1 mmol/L) were added. The reaction was initiated by mixing 50 μL ascorbic acid (18 mmol/L) and the total volume of the reaction mixture was adjusted to 800 μL with buffer. After incubation in a water bath at 55 °C for 20 min, the reaction was terminated by 250 μL trichloroacetic acid (10g/100mL water). The color was then developed by addition of 150 μL 2-thiobarbituric acid (TBA) (0.4 mol/L, in 1.25% NaOH aqueous solution) and heated in an oven at 105 °C for 15 min. The mixture was cooled and measured using a spectrophotometer (Unico 2100, Shanghai, China) at 530 nm against the buffer (as blank). The percent of protection of DNA is expressed as follows:

$$Protective\ effect\ \% = \frac{A_0 - A}{A_0} \times 100\%$$

Where A_0 is the absorbance of the control without sample, and A is the absorbance of the reaction mixture with sample.

DPPH• radical-scavenging assay

DPPH• radical-scavenging activity was determined as described [6]. Briefly, 1 mL DPPH• ethanolic solution (0.1 mM) was mixed with 0.5 mL sample alcoholic solution (20 mg/mL). The mixture was kept at room temperature for 30 min, and then measured with a spectrophotometer (Unico 2100, Shanghai, China) at 519 nm. The DPPH• inhibition percentage was

calculated as:

$$Inhibition\ \% = \frac{A_0 - A}{A_0} \times 100\%$$

Where *A* is the absorbance with samples, while A_0 is the absorbance without samples.

$ABTS^+$• radical-scavenging assay

The $ABTS^+$• scavenging activity was measured as described [7]. Briefly, the $ABTS^+$• was produced by mixing ABTS diammonium salt (0.35 mL, 7.4 mmol/L) with potassium persulfate (0.35 mL, 2.6 mmol/L), kept in the dark at room temperature for 12 h to allow completion of radical generation. Before usage, the mixture was diluted with 95% ethanol (about 1:50) so that its absorbance at 734 nm was 0.70 ± 0.02. A 1.2 mL aliquot of diluted $ABTS^+$• reagent was brought to 0.3 mL sample ethanolic solution (8 mg/mL). After incubation for 6 min, the absorbance at 734 nm was read on a spectrophotometer (Unico 2100, Shanghai, China). The percentage inhibition was calculated as:

$$Inhibition\ \% = \frac{A_0 - A}{A_0} \times 100\%$$

Here, A_0 is the absorbance of the mixture without sample, *A* is the absorbance of the mixture with sample (or positive control).

Fe^{3+}-reducing power assay

Ferric cyanide (Fe^{3+}) reducing power was determined according to the method of [8] as described by Li [9]. In brief, sample solution *x* μL (20 mg/mL, *x* = 10, 30, 50, 70, and 90) was mixed with (350-*x*) μL Na_2HPO_4/KH_2PO_4 buffer (0.2 mol/L, pH 6.6) and 250 μL $K_3Fe(CN)_6$ aqueous solution (1 g/100 mL). The mixture was incubated at 50ºC for 20 min, 250 μL of trichloroacetic acid (10 g/100 mL) was added, and the mixture was centrifuged at 3500 r/min for 10 min. As soon as 400 μL supernatant was mixed with 400 μL $FeCl_3$ (0.1 g/100 mL in distilled water), the timer was started. At 90 s, absorbance of the mixture was read at 700 nm (Unico 2100, Shanghai, China). Samples were analyzed in groups of three, and when the analysis of one group has finished, the next group of three samples were mixed with $FeCl_3$ to avoid oxidization by air. The relative reducing ability of the sample was calculated by using the formula:

$$Relative\ reducing\ effect\ \% = \frac{A - A_{\min}}{A_{\max} - A_{\min}} \times 100\%$$

Here, Error! Reference source not found.A_{max} is the maximum absorbance and A_{min} is the minimum absorbance in the test. *A* is the absorbance of sample.

Cu^{2+}-reducing power assay

The cupric ions (Cu^{2+}) reducing capacity was determined by the method [10], with minor modifications. Briefly, 125 μL $CuSO_4$ aqueous solution (0.01 mol/L), 125 μL neocuproine ethanolic solution (7.5 mmol/L) and (750-*x*) μL CH_3COONH_4 buffer solution (0.1 mol/L, pH 7.5) were brought to test tubes. Then, different volumes of samples (4 mg/mL, *x* = 50-170 μL) were added to the tubes and mixed vigorously. The total volume of reaction mixture was adjusted to 1000 μL with the buffer. After acubation for 30 min, the mixture was measured at 450 nm (Unico 2100, Shanghai, China). The relative reducing power of the sample as compared with the maximum absorbance, was calculated by the formula:

$$Relative\ reducing\ effect\ \% = \frac{A - A_{\min}}{A_{\max} - A_{\min}} \times 100\%$$

Here, Error! Reference source not found.A_{max} is the maximum absorbance at 450 nm and A_{min} is the minimum absorbance in the test. *A* is the absorbance of sample.

Determination of total phenolics

Total phenolic content was determined using the Folin-Ciocalteu method [9]. Briefly, 0.5 mL sample methanolic solution (2 mg/mL) was added to 0.5 mL Folin-Ciocalteu reagent (2 mol/L). The mixture was stood for 3 min, followed by addition of 1.0 mL Na_2CO_3 aqueous solution (15 %, w/w). After incubation at ambient temperature for 30 min, the mixture was centrifuged at 3500 r/min for 3 min. The supernatant was measured using a spectrophotometer (Unico 2100, Shanghai, China) at 760 nm. The standard curve was prepared using different concentrations of quercetin and the result was expressed as quercetin equivalents in milligrams per gram extract.

Determination of total flavonoids

Total flavonoid content was measured using the $NaNO_2$ -Al $(NO_3)_3$ method [11]. Briefly, 1 mL sample methanolic solution (25 mg/mL) was mixed with 0.15 mL $NaNO_2$ aqueous solution (5%, w/w). The mixture stood for 6 min, followed by the addition of 0.15 mL Al $(NO_3)_3$ aqueous solution (10%, w/w). After incubation for another 6 min, the mixture was added by 2 mL NaOH aqueous solution (4%, w/w) then adjusted to 5 mL with distilled water. The $A_{508\ nm}$ value was read on a spectrophotometer (Unico 2100, Shanghai, China). The standard curve was obtained using standard quercetin and the result was also expressed as quercetin

in milligrams per gram extract.

Statistical analysis

Data are given as the mean ± SD of three measurements. The IC_{50} values were calculated by linear regression analysis. All linear regression in this paper was analyzed by Origin 6.0 professional software. Significant differences were performed using the *T*-test ($p < 0.05$). The analysis was performed using SPSS software (v.12, SPSS, USA).

Determination of total phenolics

The calculation of total phenolics was based on a calibration curve obtained with quercetin (not shown) and the result was expressed as quercetin equivalents in milligrams per gram of extract. According to the regression equation ($y = 74.23137x + 0.27967$), the content of total phenolics in ERC was calculated as 10.56±0.56 mg quercetin /g.

RESULTS

Protective effect against hydroxyl-induced DNA damage

Our data revealed that ERC along with the positive controls increased the percentages of protection in a dose-dependent manner (Figure 2A) and the IC_{50} value of ERC was 1180.28±137.73 μg/mL (Table 1).

DPPH• and $ABTS^{+}$• radical-scavenging assay

DPPH and ABTS assays have been widely used to determine the free radical-scavenging activity of various pure compounds or extracts. Both DPPH• and $ABTS^{+}$• are stable free radicals which dissolve in methanol or ethanol, and their colors show characteristic absorptions at 519 nm or 734nm, respectively. When an antioxidant scavenges the free radicals by hydrogen donation, the colors in the DPPH and ABTS assay solutions become lighter. The DPPH assay revealed that ERC can effectively inhibit DPPH• (Figure 2B) and its IC_{50} was 3857.79±35.51 μg/mL (Table 1). The ABTS assay indicated that ERC can also scavenge $ABTS^{+}$• in a concentration-dependent manner (Figure 2C) and its IC_{50} was 271.82±5.66 μg/mL (Table 1).

Fe^{3+} & Cu^{2+} reducing power assays

The dose-response curves in Figure 2D suggested that ERC exhibited Fe^{3+}-reducing power and IC_{50} value was 759.99±31.65 μg/mL (Table 1); Similar results (Figure 2E) could be observed in Cu^{2+}-reducing power assay, in which ERC also exhibited effective Cu^{2+}-reducing and its IC_{50} value was calculated as 733.02±9.67 μg/mL (Table 1).

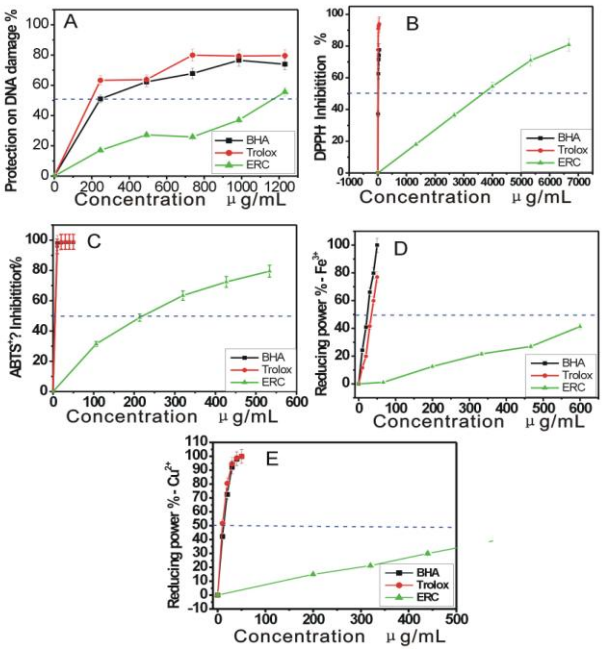


Figure 2. The dose response curves of ERC in the antioxidant assays: (A) protective effect on DNA damage; (B) DPPH· scavenging; (C) $ABTS^{+}$· scavenging (D) Fe^{3+}-reducing; (E) Cu^{2+}-reducing. Each value is expressed as Mean±SD (*n*=3). ERC, absolute ethanol extract of Radix *Codonopsis*. Trolox and BHA (butylated hydroxyanisole) were used as the positive controls

Table 1. The IC_{50} values of ethanol extract from Radix *Codonopsis* (ERC) (μg/mL)

	ERC	Positive controls	
		Trolox	BHA
Protecting DNA damage	1180.28±137.73[c]	306.13±26.11[a]	344.89±30.28[b]
DPPH• scavenging	3857.79±35.51[c]	9.75±0.06[a]	22.35±0.58[b]
$ABTS^{+}$• scavenging	271.82±5.66[b]	5.09±0.02[a]	5.21±0.25[a]
Fe^{3+}-reducing	759.99±31.65[c]	34.58±1.45[b]	22.88±1.03[a]
Cu^{2+}-reducing	733.02±9.67[c]	13.82±0.30[a]	16.09±0.47[b]

IC_{50} value is defined as the concentration of 50% effect percentage and expressed as Mean±SD (*n*=3). Means values with different superscripts in the same row are significantly different ($p<0.05$), while with same superscripts are not significantly different ($p<0.05$). BHA, butylated hydroxyanisole.

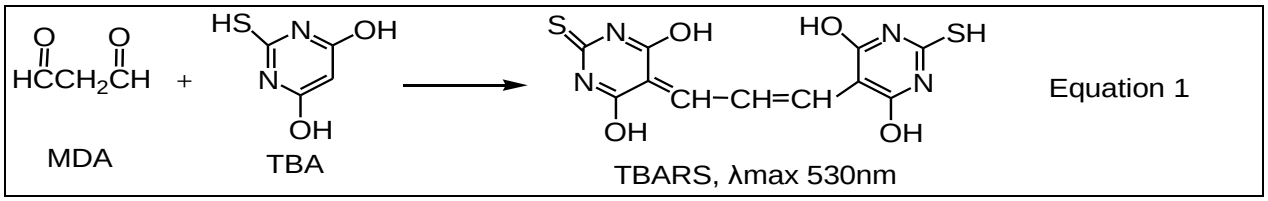
Determination of total flavonoids

The calculation of total flavonoids was also based on a calibration curve obtained with quercetin and the result was expressed as quercetin equivalents in milligrams per gram of extract. According to the regression equation ($y = 1.10239x + 0.02173$), the content of total flavonoids in ERB was calculated as 11.95±0.52 mg quercetin/g.

DISCUSSION

In the study, hydroxyl radical (•OH) is generated via Fenton reaction. Since •OH radical possesses extreme reactivity, it can easily damage DNA to generate malondialdehyde (MDA) and various oxidative lesions (Figure 3) [12,13].

As can be seen in Figure 3, these oxidative lesions don't contain conjugative system in the molecules and cannot be easily detected by a spectrophotometer. However, another product MDA can be easily detected by a spectrophotometric method. Because MDA can combine TBA (2-thiobarbituric acid) to yield TBARS (thiobarbituric acid reactive substances) which presents a maximum absorbance at 530 nm (Equation 1) [14].



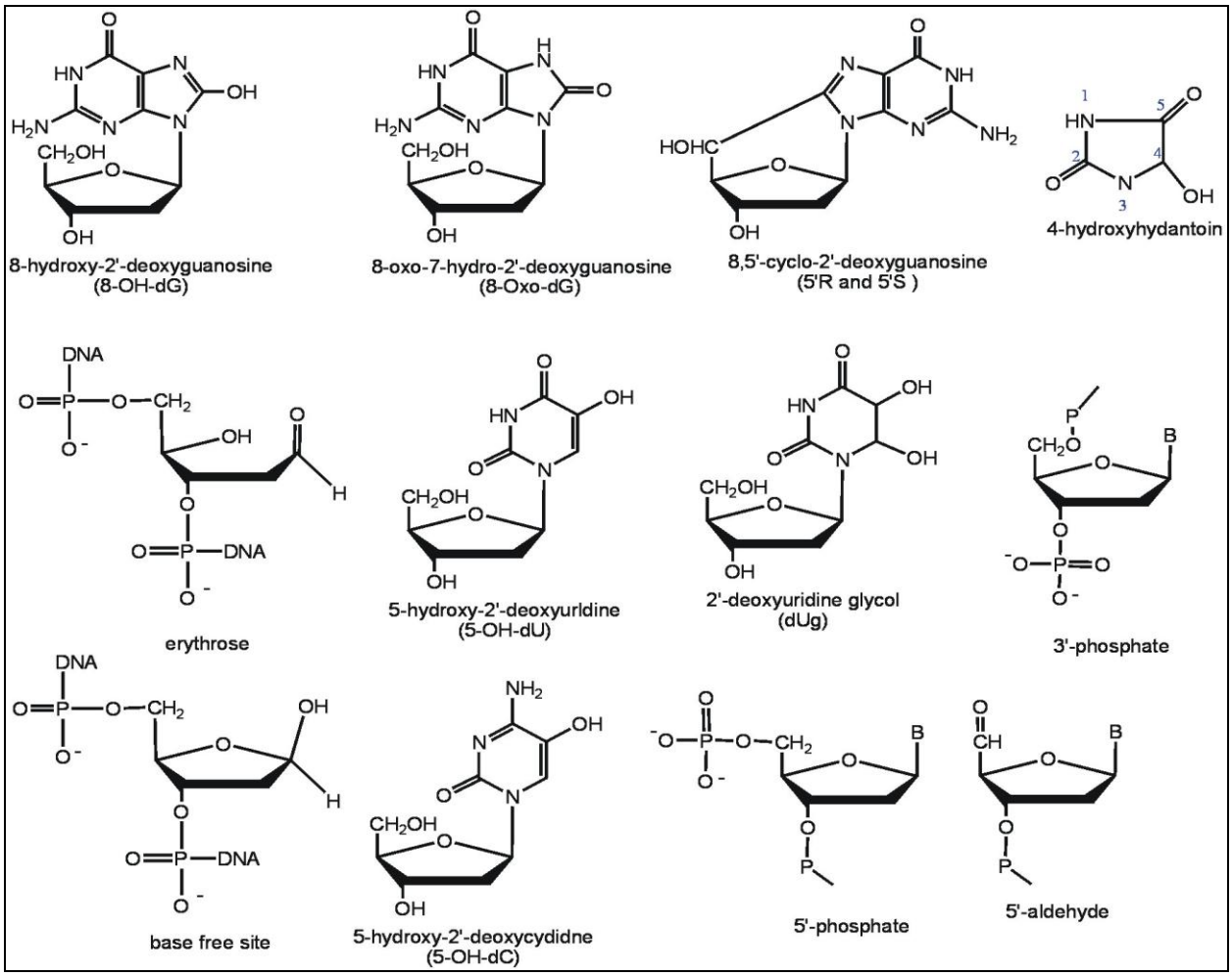


Figure 3. The structures of some oxidative lesions.

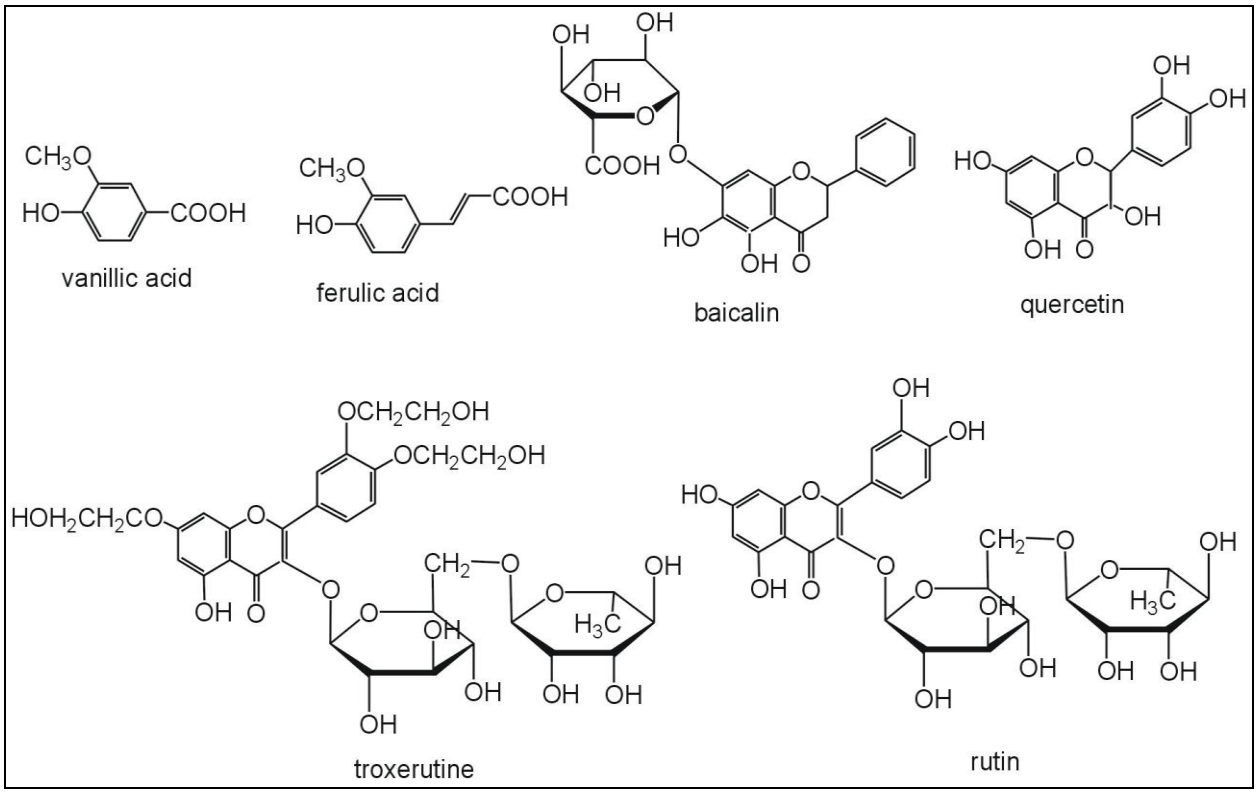


Figure 4. The structures of phenolic acids and flavonoids in Radix *Codonopsis*.

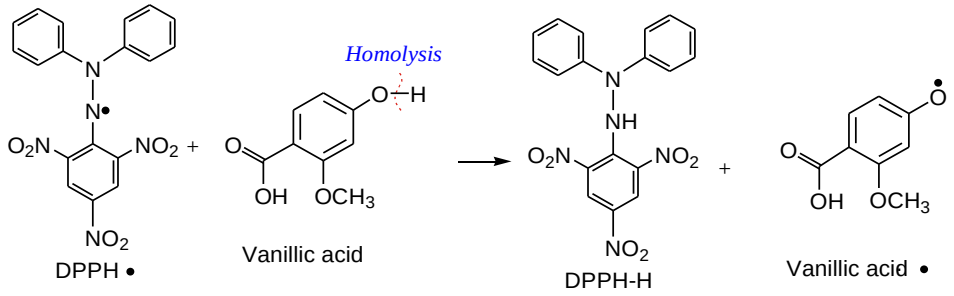
The value of A_{532nm} can therefore reflect the amount of MDA, and ultimately reflect the extent of DNA damage. The decrease of A_{532nm} value indicated a protective effect against DNA oxidative damage. Our results revealed that ERC can effectively protect against hydroxyl-induced DNA damage.

Previous studies showed that there are two approaches for natural antioxidant to protect DNA oxidative damage: one is to scavenge the •OH radicals then to reduce its attack; one is to fast repair the deoxynucleotide radical cations which were damaged by •OH radicals [15]. In order to further confirm whether the protective effect of extracts against DNA oxidative damage was relevant to its scavenging ability, we measured the scavenging abilities of ERC on DPPH• and ABTS+•.

The DPPH assay revealed that ERC can effectively eliminate DPPH•. On the other hand, the previous works suggested that DPPH• may be scavenged by an antioxidant through donation of hydrogen atom (H·) to form a stable DPPH-H molecule which does not absorb at 519 nm [16]. For example, vanillic acid which occurred in Radix *Codonopsis* [17,18], may scavenge DPPH• via the following proposed mechanism [19,20] (Equation 2).

In addition, ERC was proved to be of the ability of ABTS·+-scavenging which is regarded as an electron (e) transfer reaction [21].

The fact that ERC can effectively scavenge both DPPH• and ABTS+• radicals, suggests that ERC exerted radical-scavenging action maybe by donating hydrogen atom (H•) and electron (e).



Equation **2**

Although a reductant is not necessarily an antioxidant, an antioxidant is commonly a reductant [22]. The reducing power of an antioxidant may therefore serve as a significant indicator of its potential antioxidant activity [23]. Obviously, the results of Fe3+-reducing and Cu2+-reducing assays supported the results that ERG has antioxidant ability.

Since total phenolics and flavonoids are usually responsible for the antioxidant ability in plants, we determined the total phenolic and flavonoids contents. As mentioned above, ERC contained high amounts of total phenolics and flavonoids. In fact, at least four flavonoids and two phenolic acids (Figure 4) in Radix *Codonopsis* have been determined by HPLC, such as baicalin (3.9l μg/g), quercetin (2.12 μg/g), troxerutine (1.10 μg/g), and rutin (0.27 μg/g) [24].

CONCLUSION

As a typical Chinese herbal medicine, Radix *Codonopsis* can effectively protect against hydroxyl-induced DNA damage. One mechanism of protective effect may be radical-scavenging which is via donating hydrogen atom (H·), donating electron (e). Its antioxidant ability can be mainly attributed to the existences of flavonoids or total phenolics.

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Original Research

Anticancer activity of *Drosera indica* L., on Dalton's lymphoma ascites (DLA) bearing mice

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Keywords: 5-Fluorouracil, Antitumor, Drosera indica L., Heamatological changes, Packed cell volume.

Abstract

Aim: Drosera species are used as vital components in an Ayurvedic preparation called 'Swarnabhasma' (Golden ash) are used for the treatment of different diseases like bronchial asthma, rheumatoid arthritis, diabetes mellitus, nervous disorders In the present study was carried out to evaluate the antitumor potentials against Dalton's lymphoma ascites (DLA)-induced tumor.

Material and Method: The ethanol and aqueous extract of *Drosera indica* L., was given orally to mice at the dose of 250, 500 mg/kg body weight for 14 days to DLA bearing mice (4 Groups n=10) and 20mg/kg of 5-Fluorouracil (1 group as standard).

Result: Treatment caused significant reduction in body weight, packed cell volume (PCV) and viable tumor cell count when compared to the mice of the DLA control group. Restoration of hematological parameters towards normal was also observed. The dose at 250,500mg/kg of ethanol extracts and 500mg/kg of aqueous extract showed significant ($p<0.001$) result when compared with 250mg/kg of aqueous extract dose.

Conclusion: The results suggest that the ethanol and aqueous extracts of *D.indica* L., exhibit significant antitumor activity in DLA- bearing mice that is comparable to that of the reference standard, 5- fluorouracil.

INTRODUCTION

The plant-derived compounds have always been an important source of medicines for various diseases and have received considerable attention in recent years due to their diverse pharmacological properties including cytotoxic and cancer chemopreventive effects [1]. Cancer, characterized by uncontrolled growth and spread of abnormal cells, is caused by both external and internal factors. Most of the current anticancer drugs are derived from plant sources, which act through different pathways converging ultimately into activation of apoptosis in cancer cells leading cell death. Developments of anticancer drug is always a fascinating challenge and are classified in various ways according to their nature, sources, mechanisms of action, etc, which may be of synthetic chemicals, microbial products, plant or animal extracts [2].

Drosera is a cosmopolitan genus of insectivorous plants and consists of approximately 170 species. In India, *Drosera indica* L., *D. burmanii* and *D. peltata* J.E.Sm. ex Wild have been reported from many different locations. These species are used as vital components in an Ayurvedic preparation called 'Swarnabhasma' (Golden ash). Macerated *D. indica* is used to remove corns and this species has been categorized under the vulnerable medicinal plants list [3, 4]. Swarnabhasma (gold ash) has been used in several clinical manifestations including loss of memory, defective eyesight, infertility, overall body weakness and incidence of early aging. Swarnabhasma has been used by Ayurvedic physicians to treat different diseases like bronchial asthma, rheumatoid arthritis, diabetes

mellitus, nervous disorders, etc [5, 6]. This present study was carried out to evaluate the in vivo anti-tumour activity of ethanol and aqueous extract of *Drosera indica* L., against Dalton lymphoma ascites (DLA) in mice.

MATERIALS AND METHODS

Collection and Extraction

The plants of *D. indica* L., were collected from the foot hills of Svanadurga, Karnataka, India in the month of December 2010 and it was identified and authenticated by Dr. S. N. Yoganarasimhan,Taxonomist and Research Co-ordinator at M. S.Ramaiah College of Pharmacy, Bangalore, Karnataka, India. The plants were air dried and powdered, and then it was treated with petroleum ether, chloroform and ethanol successively in a Soxhlet apparatus for extraction. The plant material marc was soaked in the water-chloroform for 72 hr. The solvents were removed by distillation on a water bath at atmospheric pressure and the last traces were removed under reduced pressure using rotary evaporator. The ethanol extract of *D.indica* (EEDI) and aqueous extract of *D.indica* (AEDI) were completely dried and used for the *in vitro* and *in vivo* antitumor activities.

Tumor Cell lines

Dalton lymphoma ascites (DLA) cells were obtained under the courtesy of Amala Cancer Research Center, Thrissur, India, They were maintained by weekly intra-peritoneal inoculation of $2x10^6$ cells/per mouse.

Animals

Male and female adult Swiss Albino mice (20-25 g) were procured from KM College of Pharmacy, Madurai, Tamil Nadu. They were housed in microloan boxes in a controlled environment (temperature 25±2 °C) and 12 hr dark/light cycle) with standard laboratory diet (Sai Durga feeds and Foods, Bangalore) and water *ad libitum*. The study was conducted after obtaining Institutional animal ethical committee's clearance (Protocol.No.A.Raju 0903PH2254/JNTUH 2009). As per the standard practice, the mice were segregated based on their gender and quarantined for 15 days before the commencement of the experiment.

Study design

In anticancer activity study[7, 8], Animals were divided into seven groups (n=10) viz. Normal group (G1), DLA control group (G2), DLA+ 20mg/kg of 5-Fluorouracil treated group (G3), 250,500mg/kg of EEDI (G4 and G5) and 250, 500mg/kg AEDI (G6 and G7) of ten each and used for the study. The DLA cells were injected intraperitoneally ($2X10^6$ cells/ mouse) to all groups of animals except G1. On the second day the animals of G3 with 5- fluorouracil (20 mg/kg, i.p), G4 and G5 were treated with 250,500 mg/kg of EEDB and G6 & G7 with 250,500mg/kg of AEDI orally. The treatment was continued for next 14 days. G1 was treated with vehicle.

On day 15, the mice were sacrificed; blood was withdrawn by retro orbital plexus method and the following parameters were checked. The effect of EEDB and AEDB on tumor growth were examined by measuring viable tumor cell count, packed cell volume (PCV), body weight, mean survival time (MST) and percentage increase in life span (%ILS) .

Determination of tumor cell count

The ascitic fluid was taken in a RBC pipette and diluted 1000 times. Then a drop of the diluted cell suspension was placed on the Neubauer counting chamber and the number of cells in 64 small squares was counted.

Estimation of viable tumor cell count

The cells were then stained with Trypan blue (0.4% in normal saline) dye. These viable cells were counted by Cell count = (No. of cells x Dilution) / (Area x Thickness of liquid film)

Percentage Increase in Life Span (ILS %)

The effect of the EEDI and AEDI on percentage increase life span recording the mortality monitored and were calculated by

ILS (%) = [(Mean survival of treated group/ Mean survival of control group)-1] x100

Mean survival time (MST) = [1st Death + Last Death] / 2

Hematological studies

On day 15, the mice were sacrificed; blood was withdrawn by retro orbital plexus method and the following parameters were checked, Hemoglobin, Hematocrit, RBC, WBC, Neutrophil, Monocyte and Lymphocyte counts.

Statistical analysis

The experimental results were expressed as the mean ± S.E.M. Data were assessed by the method of One way ANOVA followed by Newman-Keul's multiple comparison test; $p< 0.05$ implied significance.

RESULTS

Acute toxicity

The both extracts of *D.indica* L., did not provoke any behavioral changes or manifestations of toxic symptoms in the animals, such as weight loss, increased motor activity, tremors, spasticity, loss of

right reflex, decreased motor activity, ataxia, sedation, muscle relaxation, hypnosis, arching and rolling, lacrimation, salivation, watery diarrheoa, writhing and urination, over an observation period of 24 h. The extracts were non-lethal even at the maximum single oral dose of 3000 mg/kg.

Antitumor activity

The extracts evoked tumor growth response showed in Table 1 with respect to packed cell volume, viable and non-viable cell counts and increase in life span. At the doses of 250,500mg/kg of both the extracts, as well as the reference standard drug, 5- fluorouracil, significantly ($p < 0.001$) normalized the body weight and viable cell count when compared with the DLA control group. A similar finding was made for significant increased in % life span and mean survival time when compared with DLA control group. There was a increased in PCV observed in DLA control mice, upon extract treatment , 500 mg of EEDI showed more significant ($p < 0.001$)than 250 mg/kg of EEDI and 250, 500mg/g of AEDI , where were less significant ($p < 0.01$) in control of PCV. These results indicating that the extracts exhibited a remarkable capacity to inhibit the growth of tumor induced by DLA cell line in a dose-dependent manner in experimental animals.

Hematological effects of the both extracts were shown in Table 2 ,after 14 days of treatment, the hematological parameters of the mice were significantly ($p < 0.001$) altered, compared to the DLA control group. Total WBC count and Neutrophil count was increased in DLA control group whereas Hb content, RBC count, Hematocrit, monocyte and lymphocyte count were decreased in the DLA control group. 14 days treatment of both the extracts treatment normalized the altered parameters into more or less normal at the dose of 250,500 mg/kg. in which 250mg/kg of AEDI showed less significant ($p<0.05$) than 250mg/kg of EEDI but in certain parameters 250mg/kg of EEDI and 500mg/kg of AEDI were equal significant ($p < 0.01$) in certain parameters which were shown in table.

Effect of EEDI and AEDI on peritoneal fluid assay shown in Table 3, increased in DNA, RNA and Total protein and decreased in Caspase-3 level was found in DLA control mice. Treatment with 250,500mg/kg of EEDI, significantly ($p<0.001$) reduced the DNA, RNA and Total protein and increased in Caspase-3 level.

Table 1. Effect of EEDI and AEDI on body weight, MST, % ILS, PCV, and viable tumor cell count of DLA-bearing mice

Parameters	Normal	DLA control	DLA+5FU (20mg/kg)	DLA+EEDI (250mg/kg)	DLA+EEDI (500mg/kg)	DLA+AEDI (250mg/kg)	DLA+AEDI (500mg/kg)
Body Weight (g)	22.6± 0.96	34.53±1.76	22.83±0.7	25.85±0.27[a]	23.4±1.03[a]	24.85±0.59[a]	25.43±1.4[a]
MST (Days)	40	11.5±0.65	32.25±1.65	34±0.91[a]	38±0.71[a]	27.5±0.65[a]	32±0.91[a]
ILS (%)	100	34.37±4.37	80.63±4.38	78.75±1.6[a]	98.13±1.2[a]	73.75±1.6[a]	85.63±2.14[a]
PCV(ml)	-	29.6±0.5	24.93±2.68	20.73±1.1[b]	13.35±0.34[a]	21.53±0.68[b]	20.35±0.57[b]
Viable cell Count (10^6cells / mouse)	0	17.08±0.64	0.93±0.2	3.88±0.7[a]	1.3±0.4[a]	11.88±0.6[a]	3.67±0.5[a]

The data were expressed as mean± SEM. n = 10. The data analyzed by one-way analysis of variance (ANOVA) followed by Newman-Keul's multiple comparison test.

a- $p<0.001$, compared to the DLA control group

b- $p<0.01$, compared to the DLA control group

Table 2. Effect of EEDI and AEDI on hematological parameters of DLA-bearing mice

Parameters	Normal	DLA control	DLA+5FU (20mg/kg)	DLA+EEDI (250mg/kg)	DLA+EEDI (500mg/kg)	DLA+AEDI (250mg/kg)	DLA+AEDI (500mg/kg)
Hemoglobin (g%)	14.08±0.38	8.98±0.31	12.95±0.64	13.55±0.2 [a]	14.25±0.2 [a]	10.63±0.47 [b]	13.1±0.35 [a]
Hematocrit (%)	41.18±0.54	24.05±1.03	38.3±0.84	37.58±0.89[a]	39±0.79 [a]	35.23±0.96 [a]	34.78±0.82 [a]
RBC Count (10^6/mm^3)	6.11±0.14	2.8±0.09	4.52±0.28	4.23±0.49[b]	5.78±0.15 [a]	3.57±0.08 [c]	4.12±0.13 [b]
WBC Count (10^3/mm^3)	4.66±0.17	11.15±0.31	4.87±0.41	5.75±0.31 [a]	4.63±0.28 [a]	6.55±0.27 [a]	5.6±0.57 [a]
Neutrophil (%)	17.27±0.19	57.35±0.9	21.25±0.95	23.05±1.02 [a]	19.97±0.9 [a]	35.22±2.08 [a]	22.3±1.02 [a]
Monocyte (%)	1.75±0.06	0.82±0.08	1.27±0.11	1.32±0.1 [b]	1.55±0.06 [a]	1.2±0.08[c]	1.45±0.13 [b]
Lymphocyte (%)	75.9±0.32	27.32±0.98	69.55±2.09	48.15±3.78 [a]	72.82±1.4 [a]	38.65±3.87 [b]	48.27±2.69 [a]

The data were expressed as mean± SEM. n = 10. The data analyzed by one-way analysis of variance (ANOVA) followed by Newman-Keul's multiple comparison test

a- $p<0.001$, compared to the DLA control group

b- $p<0.01$, compared to the DLA control group

c- $p<0.05$, compared to the DLA control group

Table 3. Effect of EEDI and AEDI on peritoneal fluid analysis

Parameters	DNA(mcg 10^{-6} cells)	RNA (mcg 10^{-6} cells)	Caspase-3(μmol pNA min^{-1} mL^{-1})	Total protein (mcg 10^{-6} cells)
Normal	1.55±0.06	3.25±0.08	1.88±0.05	32.85±0.31
DLA control	6.4±0.11	11.93±0.8	0.13±0.01	112.33±0.92
DAL+5FU (20mg/kg)	2.47±0.17	1.15±0.25	0.8±0.07	39.43±0.38
DLA+EEDI (250mg/kg)	2.3±0.12[a]	4.65±0.13 [a]	1.13±0.09 [a]	37.9±0.27 [a]
DLA+EEDI (500mg/kg)	1.67±0.23 [a]	3.33±0.09 [a]	2.18±0.08 [a]	32.9±0.29 [a]
DLA+AEDI (250mg/kg)	3.53±0.21 [a]	5.53±0.19 [a]	0.38±0.05[b]	64.48±2.23 [a]
DLA+AEDI (500mg/kg)	2.97±0.08 [a]	4.6±0.15 [a]	0.8±0.04 [a]	38.95±0.55 [a]

The data were expressed as mean± SEM. n = 10. The data analyzed by one-way analysis of variance (ANOVA) followed by Newman-Keul's multiple comparison test.

a- $p<0.001$, compared to the DLA control group

b- $p<0.01$, compared to the DLA control group

DISCUSSION

Cancer is a disease characterized by uncontrolled cellular growth, local tissue invasion and distant metastases and the free radicals have been implicated in carcinogenesis [9]. Supportive to this, many plant extracts containing antioxidant principles have been reported to possess anti tumor activity. In the present study, intraperitoneal inoculation of DLA cells in the mice produced an enormous increase in the cancer cell count, which indicated that there is progression of cancer in the animals. The reliable criterion for judging the anticancer effect of plant extract is reduction in viable cell count towards normal. It may be due to the extracts stimulate the growth and activity of immune cells by the production of Interleukins, which target tumor cells and cause lysis of the tumor cells by indirect cytotoxic mechanism. Furthermore, the reduced PCV and increased survival time of the mice suggest that the extracts might have exerted a delay in vascular permeability to the cells [10].

The reliable condition for judging the value of any anticancer drug is the prolongation of lifespan of the

animal and decreased WBC count from blood. The reduction in RBC or hemoglobin percentage in tumor bearing mice may be due to iron deficiency (anaemia) or due to hemolytic or myelopathic conditions [11]. Usually, myelosuppression and anaemia are the major problems encountered in cancer chemotherapy [12]. The results showed that both the doses of EEDB and AEDB has not only brought back hemoglobin content to normal but also the RBC count to normal. Analysis of the other hematological parameters such as WBC Count, differential cell count showed changes in the EAC-bearing mice. After 14 days of transplantation, daily oral extracts treated groups were able to reverse the changes in the hematological parameters following to tumor inoculation. This indicates that both the extracts posses protective action on the hemopoietic system. The hematocrit is the blood that consists of red blood cells. The hematocrit (hct) is expressed as a percentage. A low hematocrit is indicated in condition such as anemia, blood loss (traumatic injury, surgery, bleeding colon cancer), nutritional deficiency (iron, vitamin B_{12}, folate) and bone marrow problems. There is a decreased hematocrit percent in EAC-bearing mice and extract treatment retained the values more or less to normal.

In order to understand the mechanism of antitumor effect of 250, 500mg/kg of EEDB and AEDB, the main apoptotic marker, Caspase-3 was estimated in the peritoneal cells. Caspases are the central executioners of the apoptotic pathway [13]. They bring about most of the visible changes like cell shrinkage, condensation, margination and fragmentation of chromatin. It also summed up as retention of cytoplasmic organelle structure, but loss of positional interrelationships of organelles [14]. Caspase-3 is particularly activated during apoptosis and its activity was higher in extract treatment groups when compared with EAC control mice. According to willey AH., 1980 [15] during apoptosis a specific nuclease cuts the genomic DNA between nucleosomes to generate DNA fragments and the presence of this ladder has been extensively used as marker of apoptotic cell death. Hence present study showed that increased Caspase-3 activity, decreased DNA, RNA, protein content in the extracts treatment groups. Its strongly propose that extracts activate apoptotic pathways and implements the antitumor activity on EAC cells. A wide variety of natural compounds appear to possess significant cytotoxic as well as chemopreventive activity. Many of these agents act via apoptosis. Extracts of plants used in traditional medicine also have a similar property. Generally the major naphthoquinone found in D. indica is plumbagin (2-methyl-5-hydroxy-1,4-naphthoquinone) which increase of apoptotic cells by the activation of caspase-3, which plays a central role in apoptotic process. The DNA fragmentation induced by EEDI and AEDI was completely analyzed by the assay of peritoneal fluid assay. The main mechanism of action of flavanoids is release of cytochrome C to cytosol, processing of procaspase- 9 and activation of caspase- 3[16]. Based on our study and literature, mechanism of action of plumbagin is that involves mobilization of endogenous copper ions and the consequent DNA degradation through the generation of ROS [17]. In the present study the plant extracts contain chemical constituents (Plumbagin) showed cell growth inhibition through the antioxidant property.

CONCLUSION

The ethanol and aqueous extracts of *D. indica* L., possess significant antitumor activity against DLA cell line bearing mice.

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Original Research

Safety evaluation of *Morinda citrifolia* (noni) leaves extract: assessment of genotoxicity, oral short term and subchronic toxicity.

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Abstract

Morinda citrifolia L (noni) is an evergreen or small tree that grows in many tropical regions of the world. The use of the noni leaves has not been so studied however; there are reports of its pharmacological benefits.

Aims: The objective of this investigation was to assess the genotoxicity, short-term, and subchronic oral toxicity of *Morinda citrifolia* L leaves aqueous extract.

Methods: The genotoxicity of the *M. citrifolia* extract was investigated by measuring the frequency of micronuclei in mice bone marrow cells. The animals were treated with three doses of the extract (500, 1000, and 2000 mg/kg). For short-term toxicity, both sexes Wistar rats received 1000 mg/kg/day for 28 days. Animals were sacrificed for hematological and biochemical evaluation. For the subchronic study, Wistar rats were administered with three doses of *M. citrifolia* extract (100, 300, and 1000 mg/kg) by oral route for 90 days. Mortalities, clinical signs, body weight changes, food and water consumption, hematological and biochemical parameters, gross findings, organ weights, and histological examination were monitored during the study period.

Results: Genotoxicity and short-term toxicity test resulted in absence of toxicity at doses between 500 and 2000 mg/kg. Significant differences were observed in hemoglobin, and differential leukocyte count after subchronic dosing of the extract. Histology evaluation did not reveal treatment-related abnormalities. Variations observed were within to normal range and reversible.

Conclusions: In summary, 1000 mg/kg orally was the NOAEL for *M. citrifolia* extract for effects other than transient variations in some hematological parameters within normal range.

INTRODUCTION

Morinda citrifolia L. (noni) is an evergreen or small tree that grows in many tropical regions of the world. The fruit of this tree has a history of use in the pharmacopoeias of Pacific Islanders and Southeast Asia. In the past decade, the global popularity of noni fruit juice has increased dramatically [1,2]. While there are several publications describing various potential health benefits of noni fruit [3], the leaf extract has not been so studied.

The *M. citrifolia* leaf extract has shown pharmacological properties. Leaves of *M. citrifolia* showed good *in vitro* anthelmintic activity against human *Ascaris lumbricoides* [4]. Leaf methanol extract had potential antibacterial activities to both gram positive *S. aureus* and Methicillin Resistant *S. aureus* [5]. Wound-healing activity of ethanolic extract of *M. citrifolia* was observed in rats using excision and dead

space wound models [6]. Anti-inflammatory and analgesic effects were observed with the *M. citrifolia* leaf extract used in this study.

Information about toxicological potential of the leaf extract of *M. citrifolia* is very limited and is insufficient to support the safety of them. The LD_{50} of the methanol extract of fruit and leaf was found to be greater than 1000 mg/kg when injected intraperitoneally in mice [7].

The overall objective of this investigation was to characterize the genotoxicity, short-term and subchronic oral toxicity of *M. citrifolia* aqueous extract by the oral route. An additional aim was to identify no-observed-adverse-effect level (NOAEL) for short-term and subchronic *M. citrifolia* extract exposure.

MATERIALS AND METHODS.

Test substances.

Leaves of *M. citrifolia* were collected in April in the Medicinal Plant Experimental Station "Dr. Juan Tomás Roig" (Güira de Melena, Artemisa, Cuba). Voucher specimen (Nº 4741) was deposited at the "Dr. Juan Tomás Roig" herbarium in the cited Experimental Station. The leaves were dried in a recycled air stove at 45ºC for two days. Dried *M. citrifolia* leaves were extracted with demineralized water at 100ºC for one hour with agitation. The extract obtained was dried with spray drier equipment as described previously [8]. The dry powder obtained was used for the studies.

To detect the presence of various chemical constituents in *M. citrifolia* extract, phytochemical screening was performed according to the method described by García *et al* [9]. The extract was qualitatively analyzed for the presence of essential oils, terpenoids, flavonoids, glycosides, amines, aminoacids, oligosaccharides, alkaloids, anthraquinone compounds, and coumarins. The phytochemical screening of the extract showed the presence of terpenoids, flavonoids, amines, aminoacids, and anthraquinone compounds.

The extract was standardized in accordance with the content of anthraquinone compounds and total anthracen-derived. Anthraquinone compounds were performed by quantification of colored phenols obtained by chemical reaction of alkali and anthracen-derived. Total anthracen-derived content was determined by quantification of colored phenols obtained by anthracen-derived phenols oxidation with ferric chloride in acid medium. Quantification was performed by using a spectrophotometer at 525 nm. Reference substance used was cobalt chloride 1% in ammonium alkaline solution equivalent to 0.43 mg of oxianthraquinone. Results were express as % w/v from calibration curve (r^2=0.999) [10]. *M. citrifolia* total extract with 2.09% of anthraquinone compounds and 11.21% of total anthracen-derived was used in the studies [8].

Animals.

Animal care was performed in conformity with Canadian Council for Animal Care guidelines [11]. Healthy Wistar (Cenp:Wistar) rats of both sexes and NMRI albino mice were used in the studies. Rats used in short-term and subchronic toxicity tests were 6 weeks old at the onset of dosing. Mice weighing 22 ± 2 g were used in genotoxicity evaluation. Animals were obtained from the Laboratory Animal National Centre (CENPALAB), Havana, Cuba and were randomly assigned to dosage groups. Each group of 5 to 10 animals was housed together by sex in polycarbonate cages in a light- and humidity-controlled biohazard suite (24 ± 2 °C; 55 ± 5% relative humidity), with a 12-hour light-dark cycle, and free access to drinking water and a standard laboratory diet CMO1000 (CENPALAB). Experiments were conducted in accordance with the Guiding Principles in the Use of Animals in Toxicology [12]. The experimental protocols were approved by the Institutional Ethical Committee.

Genotoxicity evaluation

To assess *M. citrifolia* extract mutagenicity, doses of 500, 1000, and 2000 mg/kg body weight were administered by gastric intubation to groups of five animals for each sex and treatment. A positive control (cyclophosphamide 20 mg/kg) and a negative control (distilled water) were also included. Mice were euthanized by cervical dislocation 24 hours after two administrations of the *M. citrifolia* extract spaced by 24 hour intervals [13-14].

After treatment, mice femurs were dissected and bone marrow smears were obtained as described [15]. To detect MNPCE (micronucleated polychromatic erythrocyte) frequency, we fixed the smears with Giemsa (1:30), prepared two slides for each mouse, and scored 1000 polychromatic erythrocytes (PCE) per slide. The results were the average of two slides. To determine the cytotoxic activity, we simultaneously computed 1000 normochromatic erythrocytes and the polychromatic erythrocyte frequencies.

For mutagenic activity, we compared the MNPCE frequencies obtained for the treated groups and the negative control group. To evaluate cytotoxicity, the polychromatic erythrocytes/normochromatic erythrocytes ratio (PCE/NCE) of all treated groups were compared to the result obtained for the negative control group.

Short-term toxicity test

Rats were allocated to two groups of each sex (7-8

weeks of age, 120-160 g of body weight, n=5 per group). Animals were exposed to *M. citrifolia* extract in drinking water for a 28-day period as described Wilson *et al*. [16] considering the body weight and water consumption of two previous weeks. The concentration was calculated in mg of total extract /500 ml so that dose delivered was therefore 1000 mg/kg/day of total extract. Concentration of *M. citrifolia* extract in drinking water was adjusted every 7 days according to body weight and water consumption to achieve the targeted dose level. Another five rats of each sex were assessed for control. Animals were monitored weekly for body weight, food and water consumption. Both behavior and clinical signs were monitored daily. Blood samples were collected from abdominal vein under anesthesia of sodium pentobarbital (40 mg/kg) on day 28 of dosing for hematology and chemical biochemistry [17].

Subchronic toxicity test

Rats were allocated to four groups of each sex (7-8 weeks of age, 120-160 g of body weight, n=10 per group). Animals were exposed to *M. citrifolia* extract in drinking water for 90 days as described above. The dosages delivered were 0, 100, 300 and 1000 mg/kg/day. Animals were monitored for body weight, food and water consumption, and behavioral and clinical signs as described in the short-term toxicity study. At the completion of the subchronic study, blood samples were collected as described above and serum obtained for hematological and biochemical analyses [18].

Hematology included determination of hematocrit by microhematocrit capillaries, hemoglobin concentration by diagnostic kit produce by Biologic Products Inc. Havana, Cuba, erythrocyte and leukocyte count in Neubauer chamber, differential leukocyte count by extension in microscopy slides and blood clotting time by addition of calcium chloride to citrated blood. Clinical biochemistry included glucose, total cholesterol, creatinine, urea, aspartate aminotransferase (AST), alanine aminotransferase (ALT) and alkaline phosphatase were measured used diagnostic kit produced by Biologic Products Inc. Havana, Cuba. The absorbance values were determined in a spectrophotometer Spectronic Genesys 2.

After collection of blood samples, rats were euthanized by exsanguination. Selected organs for weight were liver, kidneys, adrenals, spleen, brain, heart, ovaries and testes. Selected organs (heart, kidneys, liver, spleen, brain, lungs, stomach, intestines, thymus, adrenals, thyroid, parathyroid, trachea, pancreas, salivary glands, cervical ganglion, gonads, prostate, ovaries and seminal bladder) were removed, fixed, sectioned, and stained for histopathological examination [18].

A satellite group of 10 animals per sex (5 control and 5 treated) was used in the top dose group for observation, after the treatment period, for reversibility or persistence of any toxic effects. In these groups blood and organs were taken 28 days after the end of the 90 day treatment period [18].

Statistics.

Results were expressed as the mean ± SEM. All statistical analysis was assessed using the GraphPad Prism Version 5 (GraphPad Software, San Diego, California, USA). Each test group was compared with control. Results of male and females animals were evaluated separately. One-way analysis of variance (ANOVA) and the Tukey-Kramer Multiple Comparisons Test were performed. Statistical significance was considered at $p<0.05$.

RESULTS.

Genotoxicity evaluation

The results obtained for mice treated with different concentrations of *M. citrifolia* extract are shown in Table 1. No significant difference in the frequency of MNPCE was observed between mice treated with *M. citrifolia* extract and the negative control ($p > 0.05$) for both sexes. A high increase in the frequency of MNPCE was detected in mice treated with cyclophosphamide compared to the negative control ($p < 0.01$). No significant differences in the PCE/NCE ratio were observed when comparing mice treated with *M. citrifolia* extract and the respective negative control.

Short-term toxicity test

Signs of toxicity were not observed during the experimental period in treated groups. Mean water, food consumption and body weight trends were not affected within 28-days exposure of *M. citrifolia* extract (Table 2). The biochemical and hematological parameters were not affected during the study (Table 3).

Subchronic toxicity test

Signs of toxicity and mortality were not observed during the 90 day experimental period in treated groups. No treatment-related variations in body weight trends, food and water consumption were observed during the study period (Table 4). From the hematological parameters tested hemoglobin levels were significantly decreased in males at dosages of 300 and 1000 mg/kg/day and female at dose of 1000 mg/kg/day (Table 5). In these goups, erythrocyte counts decreased without statistical significance. These effect were not dose related and within or close to

normal range. Differential leukocyte count showed a significant increase in lymphocytes while neutrophils decreased in treated male and female groups (Fig. 1). Any significant effect was not observed after 28-days recovery period for hemoglobin and differential leukocyte count in both sexes treated animals (Fig. 2).

No treatment-related changes were observed in biochemical parameters tested during the study period (Table 6). Any statistical variation was not observed in the relative organ weight (as % of total body weight) compared to control group (Table 7). There were no treatment related histological changes in animals treated with high dose and control group in both sexes.

Table 1. Frequency of MNPCE and PCE/NCE ratio in mice treated with *M. citrifolia* extract. Values represent the mean ± SEM (n=5), ** $p < 0.01$ significantly different from control.

Treatment	PCE/NCE		MN-PCE/1000	
Untreated	1.39 ± 0.35	1.32 ± 0.46	0.24 ± 0.20	0.43 ± 0.18
distilled water	1.13 ± 0.09	1.27 ± 0.25	0.18 ± 0.13	0.20 ± 0.07
M. citrifolia 500 mg/kg	1.33 ± 0.16	1.29 ± 0.08	0.22 ± 0.11	0.18 ± 0.08
M. citrifolia 1000 mg/kg	1.32 ± 0.14	1.36 ± 0.13	0.26 ± 0.13	0.29 ± 0.12
M. citrifolia 2000 mg/kg	1.39 ± 0.11	1.40 ± 0.11	0.12 ± 0.11	0.30 ± 0.10
Cyclophosphamide 20 mg/kg	0.53 ± 0.07**	0.63 ± 0.06**	5.38 ± 0.75**	7.62 ± 1.80**

Table 2. Body weights, food and water consumption of animals following 28-days exposure to *M. citrifolia* extract. Values represent the mean ± SEM (n=5).

Parameter	Male		Female	
	Control	1000 mg/kg/day	Control	1000 mg/kg/day
Initial weight (g)	135.0 ± 6.6	151.0 ± 8.0	134.0 ± 5.2	127.0 ± 4.0
Final weight (g)	319.0 ± 5.3	319.0 ± 7.4	214.0 ± 6.2	205.0 ± 8.0
Weight gain (g)	184.4 ± 8.4	168.0 ± 7.9	80.0 ± 8.8	78.4 ± 8.3
Food intake (g/animal/day)	24.5 ± 2.0	24.6 ± 2.2	18.2 ± 2.1	18.7 ± 1.8
Water consumption (ml/animal/day)	39.8 ± 5.6	48.4 ± 1.9	30.2 ± 3.1	38.0 ± 1.9

Table 3. Results of hematological and biochemical parameters following 28-days exposure to *M. citrifolia* extract. Values represent the mean ± SEM (n=5).

Parameter	Male		Female	
	Control	1000 mg/kg/day	Control	1000 mg/kg/day
Hemoglobin (mmol/l)	10.8 ± 1.7	11.3 ± 0.2	11.9 ± 0.5	10.2 ± 0.5
Erythrocyte count (cellx10^6/mm^3)	8.99 ± 0.7	9.14 ± 0.8	8.51 ± 0.5	9.22 ± 0.6
Leukocyte count (cellx10^3/mm^3)	8.75 ± 0.1	7.64 ± 0.5	5.87 ± 0.8	4.96 ± 0.8
Blood clotting time (sec)	98.2 ± 12.9	109.0 ± 8.3	135.6 ± 17.0	117.8 ± 20.5
Alkaline phosphatase (U/l)	167.1 ± 8.8	139.0 ± 25.9	135.9 ± 21.6	134.1 ± 15.9
Glucose (mmol/l)	5.7 ± 1.0	5.0 ± 0.8	6.8 ± 0.4	7.3 ± 0.3
Cholesterol (mmol/l)	1.1 ± 0.2	0.7 ± 0.2	1.2 ± 0.3	1.3 ± 0.1
Creatinine (µmol/l)	34.2 ± 1.2	37.8 ± 4.2	22.9 ± 2.6	24.8 ± 2.0

Table 4. Body weights, food and water consumption of animals following 90-days exposure to *M. citrifolia* extract. Values represent the mean ± SEM (n=10), † $p < 0.01$ significantly different from 100 mg/kg/day treated group.

Treatment	Control	*M. citrifolia*		
Doses (mg/kg/day)	0	100	300	1000
(Male)				
Initial weight (g)	143.1 ± 4.0	141.7 ± 2.3	153.2 ± 2.4	145.3 ± 2.4
Final weight (g)	400.4 ± 10.9	438.2 ± 7.5	412.9 ± 15.5	391.1 ± 15.0
Weight gain (g)	280.7 ± 12.7	299.4 ± 7.0	276.4 ± 18.3	251.5 ± 13.8
Food intake (g/animal/day)	23.37 ± 0.8	25.0 ± 1.0	24.0 ± 1.1	22.2 ± 0.7
Water consumption (ml/animal/day)	46.0 ± 2.0	46.8 ± 1.4	46.5 ± 1.8	45.5 ± 1.6
(Female)				
Initial weight (g)	130.5 ± 1.7	129.4 ± 3.0	128.1 ± 2.3	141.4 ± 2.6
Final weight (g)	261.9 ± 5.3	253.2 ± 7.6	272.6 ± 5.7	263.4 ± 4.4
Weight gain (g)	131.4 ± 6.2	123.8 ± 7.2	144.5 ± 5.9	122.0 ± 5.1
Food intake (g/animal/day)	19.7 ± 1.2	23.0 ± 0.9	19.6 ± 1.0	18.8 ± 1.0
Water consumption (ml/animal/day)	38.5 ± 1.7	35.2 ± 1.4	43.3 ± 1.5†	43.5 ± 1.1†

Table 5. Results of hematological parameters following 90-days exposure to *M. citrifolia* extract. Values represent the mean ± SEM (n=10), * $p < 0.05$, ** $p < 0.01$ significantly different from control.

Treatment	Control		M. citrifolia		Control Range
Doses (mg/kg/day)	0	100	300	1000	
(Male)					
Hemoglobin (mmol/l)	11.1 ± 1.0	11.9 ± 0.6	8.5 ± 0.4 **	8.8 ± 0.4 **	9 – 12
Hematocrit (%)	50.6 ± 0.5	50.4 ± 0.8	49.1 ± 1.0	50.1 ± 0.7	47 - 56
Erythrocyte count (cellx10^6/mm^3)	7.07 ± 0.6	7.25 ± 0.6	5.61 ± 0.6	5.81 ± 0.6	5.37 – 8.76
Leukocyte count (cellx10^3/mm^3)	5.29 ± 0.8	6.63 ± 0.5	6.09 ± 0.9	5.68 ± 0.5	4.00 – 6.55
Blood clotting time (sec)	97.3 ± 5.2	95.0 ± 6.9	83.0 ± 4.4	93.0 ± 4.8	82 – 111
(Female)					
Hemoglobin (mmol/l)	9.1 ± 0.6	8.6 ± 0.4	9.6 ± 0.5	7.2 ± 0.5 *	9 – 11
Hematocrit (%)	45.7 ± 0.5	48.5 ± 1.3	48.7 ± 0.6	47.2 ± 0.9	42 – 45
Erythrocyte count (cellx10^6/mm^3)	4.08 ± 0.2	4.23 ± 0.04	4.45 ± 0.7	3.03 ± 0.4	3.48 – 4.68
Leukocyte count (cellx10^3/mm^3)	4.55 ± 0.2	4.72 ± 0.2	5.43 ± 0.4	3.89 ± 0.2	3.86 – 7.70
Blood clotting time (sec)	96.3 ± 11.9	78.8 ± 12.3	91.0 ± 12.8	79.8 ± 5.4	84 - 118

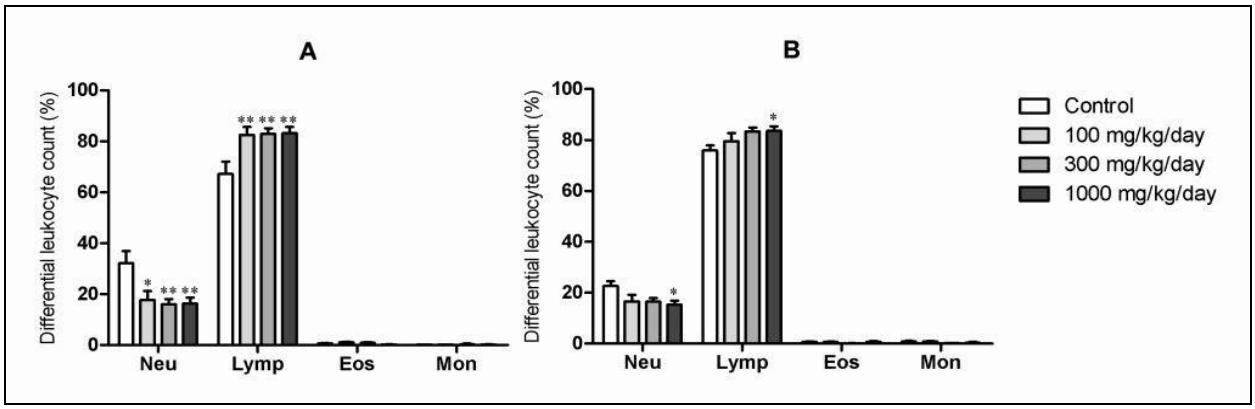

Figure 1. Differential leukocyte counts in rats exposed to subchronic oral doses of *M. citrifolia* extract. (A) Male, (B) Female. Values are mean ± SEM; n=10 animal/group; *$p < 0.05$, **$p < 0.01$ (significantly different from control).

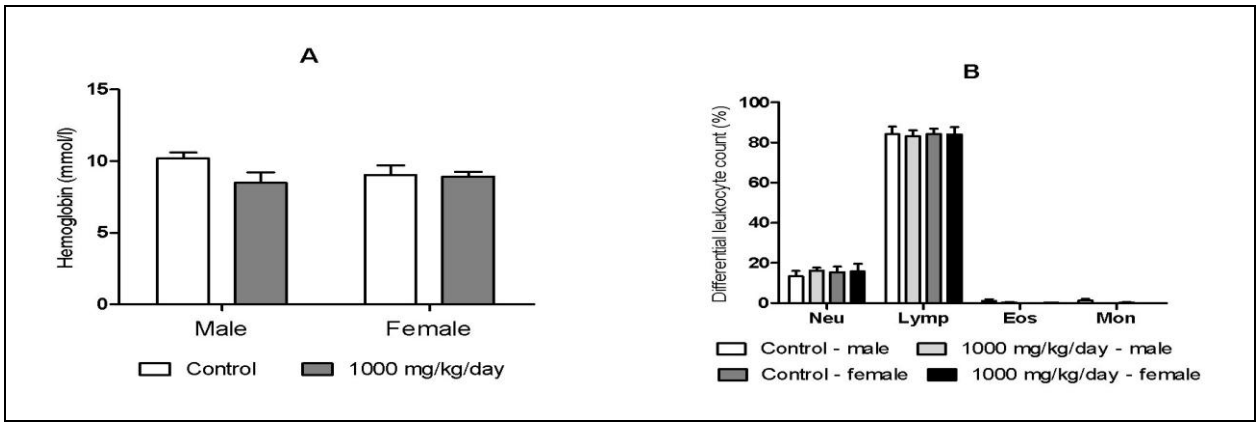

Figure 2. Hemoglobin (A) and differential leukocyte counts (B) in rats following 28-days recovery period of subchronic oral dosing of *M. citrifolia* extract. Values are mean ± SEM; n=5 animal/group.

Table 6. Results of biochemical parameters following 90-days exposure to *M. citrifolia* extract. Values represent the mean ± SEM (n=10).

Treatment	Control		M. citrifolia		Control Range
Doses (mg/kg/day)	0	100	300	1000	
(Male)					
Alkaline phosphatase (U/l)	69.4 ± 8.2	63.6 ± 3.9	76.1 ±10.4	89.5 ± 6.1	50 – 90
AST (U/l)	25.5 ± 2.9	30.2 ± 3.8	28.6 ± 2.9	28.5 ± 6.3	34 – 48
ALT (U/l)	14.3 ± 1.7	10.4 ± 1.4	10.6 ± 2.4	14.6 ± 5.8	10 – 18
Urea (mmol/l)	19.0 ± 2.8	24.8 ± 2.8	17.8 ± 1.6	19.5 ± 1.8	12 – 22
Creatinine (µmol/l)	21.2 ± 3.4	26.9 ± 3.2	26.9 ± 3.5	26.3 ± 2.9	12 – 30
Glucose (mmol/l)	4.7 ± 0.7	3.5 ± 0.3	5.9 ± 0.5	4.7 ± 0.4	2.9 – 6.5
Cholesterol (mmol/l)	1.3 ± 0.1	1.2 ± 0.1	1.2 ± 0.1	1.1 ± 0.1	1.1 – 1.5
(Female)					
Alkaline phosphatase (U/l)	45.1 ± 4.2	41.7 ± 6.6	38.5 ± 3.4	38.5 ± 2.5	35 – 55
AST (U/l)	26.6 ± 5.4	30.4 ± 6.1	31.6 ± 4.9	28.5 ± 5.2	14 – 37
ALT (U/l)	16.7 ± 1.1	20.2 ± 4.6	20.5 ± 3.3	18.5 ± 2.5	14 – 19
Urea (mmol/l)	18.2 ± 0.8	18.7 ± 1.2	19.3 ± 1.3	17.1 ± 1.1	16 – 20
Creatinine (µmol/l)	40.7 ± 4.2	38.9 ± 3.8	36.6 ± 4.2	32.6 ± 3.5	31 – 44
Glucose (mmol/l)	4.3 ± 0.2	4.9 ± 0.2	5.1 ± 0.3	5.3 ± 0.3	3.8 – 5.0
Cholesterol (mmol/l)	0.92 ± 0.17	1.42 ± 0.07	0.56 ± 0.06	0.81 ± 0.14	0.5 – 1.3

Table 7. Organ weights (% total body weight) of both sex rats following 90-days exposure to *M. citrifolia* extract. Values represent the mean ± SEM (n=10).

Treatment	Control		M. citrifolia	
Doses (mg/kg/day)	0	100	300	1000
(Male)				
Liver	2.5047 ± 0.11	2.5013 ± 0.13	2.4553 ± 0.04	2.4172 ± 0.04
Kidney	0.6634 ± 0.03	0.6693 ± 0.03	0.6464 ± 0.01	0.6643 ± 0.01
Adrenals	0.0108 ± 0.001	0.0138 ± 0.001	0.0130 ± 0.001	0.0143 ± 0.001
Testis	0.9292 ± 0.03	0.9474 ± 0.04	0.9101 ± 0.03	0.9836 ± 0.04
Thymus	0.0908 ± 0.02	0.0643 ± 0.01	0.0727 ± 0.01	0.0726 ± 0.01
Spleen	0.1708 ± 0.007	0.1609 ± 0.005	0.1831 ± 0.013	0.1865 ± 0.014
Brain	0.4293 ± 0.02	0.4146 ± 0.02	0.4386 ± 0.02	0.5017 ± 0.02
Heart	0.3154 ± 0.01	0.3155 ± 0.01	0.3468 ± 0.02	0.3388 ± 0.01
(Female)				
Liver	2.5731 ± 0.06	2.5188 ± 0.06	2.7747 ± 0.15	2.5345 ± 0.05
Kidney	0.6744 ± 0.02	0.6726 ± 0.02	0.6145 ± 0.02	0.5992 ± 0.02
Adrenals	0.0153 ± 0.002	0.0194 ± 0.002	0.0196 ± 0.002	0.0188 ± 0.002
Ovaries	0.0495 ± 0.003	0.0374 ± 0.005	0.0490 ± 0.003	0.0476 ± 0.006
Thymus	0.1306 ± 0.01	0.1437 ± 0.01	0.1171 ± 0.01	0.1404 ± 0.01
Spleen	0.2423 ± 0.01	0.1811 ± 0.01	0.2370 ± 0.01	0.2199 ± 0.01
Brain	0.6700 ± 0.02	0.5959 ± 0.04	0.6271 ± 0.02	0.6667 ± 0.02
Heart	0.3528 ± 0.01	0.3428 ± 0.01	0.3549 ±0.01	0.3537 ± 0.02

DISCUSSION

M. citrifolia is one of the most popular herbal formulas in the world; however, evidence-based information about leaves toxicity is limited. Many studies have reported pharmacological efficacies and benefits of *M. citrifolia* leaves [4-6,19], but there is few information on its risk and safety. To evaluate the genotoxicity, *M. citrifolia* extract was orally given at doses 0, 500, 1000 or 2000 mg/kg to male and female mice. No genotoxic effect was observed after oral doses of *M. citrifolia* extract in the mice bone marrow micronucleus test. No treatment-related toxicity was observed in the study carried out to evaluate the sub-acute 28-days repeated oral dose toxicity of *M. citrifolia* extract. These results are consistent with *in vivo* and *in vitro* toxicity tests of noni leaves and seed [19-21].

Some changes was observed in the study carried out to evaluate the subchronic 13-week repeated oral dose toxicity of *M. citrifolia* extract. Hemoglobin and differential leukocyte count were significantly affected and erythrocyte count was marginally affected after *M. citrifolia* subchronic exposure. Hematological varations were within or close to normal range and reversibly.

Two types of toxicities essentially affect red blood cells: competitive inhibition of oxygen binding to hemoglobin and chemically induced anemia in which the number of circulating erythrocytes is reduced in response to red blood cell damage [22]. In our study we observed erythrocyte and hemoglobin reduction probably caused by erythrocyte damage. A possible explanation for the erythrocyte and hemoglobin reduction in treated animals could be the induction of erythrocyte membrane damage. The damaged erythrocytes could be recognized by splenic macrophages, which remove and destroy them. Therefore, the number of red blood cells destroyed could be exceeding the bone marrow's capacity to replace them.

Oxidative stress has been suspected in several pathologies including intoxication, genotoxicity and cancer development [23-24]. In our study, reductions in the erythrocyte count of treated rats could be a consequence of oxidative stress complication which is incriminated to induce hemolysis by shortening RBC survival and increasing their fragilities. The possible mechanism through the erythrocyte damage occurs is being investigated.

The destruction of damaged erythrocytes could be induced antibodies formation against certain erythrocyte components. Differential leukocyte count show a significant lymphocyte increase while neutrophil decrease. It is know that lymphocytes involved in both humoral and cellular immunity. Previous study reported anemia in SOD1 deficiency mice due to increased erythrocyte vulnerability by the oxidative modification of proteins and lipids [25]. Since oxidized erythrocyte components are antigenic in regards to the formation of autoantibodies, a long-term exposure to oxidative stress causes an autoimmune response to oxidized erythrocytes. Increase in lymphocyte cells observed in our study could be cause by immune response against oxidized erythrocytes. These effects were reversible after 28-days of recovery period.

Previously studies reported the safe use of *M. citrifolia* leaf as a food [26]. In this study, absent of toxicity was observed after acute, subacute and subchronic oral dosing of *M. citrifolia* leaves extracts at doses between 200 and 20 mg per animal per day. In other report, noni seed extract was non-toxic in the 28 day oral toxicity test in rats at dose of 1000 mg/kg. The extract was non-cytotoxic, with an LC_{50} > 1 mg/ml, and non-genotoxic [21]. Our results show slight variations in few hematological parameters that were close or within to normal range and reversible after subchronic oral dosing of *M. citrifolia* leaves extract. The extract was non-toxic and non-genotoxic according to the results of sub-acute and genotoxicity assays.

In summary, 1000 mg/kg orally was the sub-acute NOAEL for *M. citrifolia* extract, for the absence of toxic response. For *M. citrifolia* subchronic exposure, the NOAEL was 1000 mg/kg for effects other than transient variations in some hematological parameters within normal range.

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Original Research

Toxicological study on aqueous extract of *Allanblackia froribunda* (Clusiaceae) on rats

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Keywords: Allanblackia floribunda; acute and subacute toxicity; oral and intraperitoneal administration, animal toxicology

Abstract

Aim: The acute and sub-acute toxic effects of aqueous extract of *Allanblackia froribunda* (Clusiaceae) were studied in rats.

Methods: The acute toxicity was carried out orally with 0, 14, 16, 18 and 20 g kg^{-1} b/w and intraperitoneally with 0, 50, 100, 150, 200 mg kg^{-1} body weight, while the subacute was only carried out orally with 0(distilled water), 400, 500 and 600 mg kg^{-1} b/w for four weeks.

Results: In acute test, the oral administration did not cause any death treatment related signs. The LD_{50} estimated to be 125 mg kg^{-1} (Intraperitoneal route). We noted a decrease in food, water consumption and body weight of treated animals. Diarrhea occurred in rats at dose 150 mg kg^{-1}. Analysis of serum showed an increase in ALT at dose 50 mg kg^{-1} ($p < 0.05$) and serum creatinine at doses 50 and 100 mg kg^{-1} ($p < 0.05$ and $p < 0.01$ respectively) while serum proteins decreased at dose 100 mg kg^{-1} ($p < 0.001$). The histological changes of the main target organs (liver and lung) were observed while section of kidney and gonads remained normal (data not shown). In subacute treatment, neither significant difference was observed on body weight, food and water consumption nor organs and haematological parameters. The biochemical analysis showed that the level of ALT dose dependently decreased ($p < 0.01$) at all doses in male and female while tissue creatinine decreased ($p < 0.05$) only in female.

Conclusion: These results suggest that the extract does not present danger orally, but parenteral administration is not recommended

INTRODUCTION

The development in human exposure to natural products known to originate from folk medicine has conducted to a scientific interest in their biological effects. To improve the assessment of their pharmacological and toxicological profile, scientific evidence-based approaches have been undertaken to evaluate composition, quality, potential medicinal activity and safety of these natural products.

Allanblackia floribunda is a tree found in the equatorial forest of East part of Nigeria areas, to the Central African Republic and West of the Democratic Republic of Congo. In Cameroon, it is found in the region of the Centre specifically Obala and Okola; in the region of the Southwest in Limbe [1].

Previous phytochemical studies on the plant helped to isolate several metabolites. The bark of *Allanblackia floribunda* contain benzophenones (Guttiferone), Xanthones (1,2,5-trihydroxyxanthone and 4,5-dihydro-1,6,7- trihydroxy-4',4',5 - trimethoxyfurano - [2',3:3,4] Xanthone) and Biflavonoides (Morelloflavone and Volkensiflavone) [2]. The fruits of *A. floribunda* consist mostly of stearic and oleic fatty acid [3].

Allanblackia floribunda has been shown to display a wide spectrum of biological and pharmacological activities, which provide experimental support for the empiric ethno-pharmacological use of this plant in traditional medicine. The decoction of *Allanblackia floribunda* lumber is used in Gabon to treat dysentery and dental pain. In the Democratic Republic of the Congo, decoctions of leaves and bark are used to treat asthma, bronchitis and cough [3]. In Cameroon, the macerate of the bark is used to treat sexual impotence and male fertility in acute problems as well as in the treatment of hypertension [4]. Xanthones isolated from the bark of *Allanblackia floribunda* have in vitro moderate cytotoxicity on KB cancer cells [2].

Despite the extensive use of plants in traditional medicine, *Allanblackia floribunda* has not been subjected to adequate toxicological assessment. Therefore, motivated by this, we aimed at the present study to carry out basic toxicological studies and establish the safety of an aqueous extract of the bark of the plant, focusing on its acute and subacute toxicity in rats through oral and intraperitoneal route.

MATERIAL AND METHODS

Plant material

Mature stem and bark of *Allanblackia floribunda* were collected from South west region of Cameroon in the locality of Limbe on March and stored at room temperature in a dry place prior to use. The plants were authenticated by Mr LITONGA NDIVE Elias, botanist of the National garden of Cameroon and a voucher specimen was deposited at the national herbarium under the number SCA 4350

Preparation of the aqueous extract of *Allanblackia floribunda*

Stem and bark were cut and crushed with a roping. The resulting powder was used in preparing the aqueous extract. A 2000 g of the powdered stem and bark was suspended in 7 l of distilled water, heated and boiled for 45 min. After filtration by the Whatmann no. 3 papers, the resultant filtrate was eliminated by concentration in a rotor evaporator under reduced pressure to give a yield of 70 g (3.5 %) of aqueous extract. The obtained extract was further diluted to obtain different concentrations in one 1 ml, and then stored at -4°C until required for use.

Animals

Acute toxicity

The toxicity study was carried out using female and male Wistar rats weighing between (120-150 g). Animals were kept in a temperature-controlled environment (23 ± 2ºC) with a 12 h light-dark cycle and food and water were freely available except for a short fasting period before oral or intraperitoneal administration of single dose of the *Allanblackia floribunda* extract [5]. Rats in this experiment were carried out in accordance with the recommendation of the guidelines for care and the laboratory animals used was approved by the Institutional Animal Ethics Committee of the Faculty of Science-Section, University of Douala. According to the way of administration, the animals were divided into one control group and four treated ones, each group consisting of ten animals for oral and intraperitoneal route respectively.

The raw material of *Allanblackia floribunda* extract was dissolved/suspended in distilled water and administered by gavages at doses of 0, 14, 16, 18, 20 g kg^{-1} or by the intraperitoneal route at doses of 0, 50, 100, 150, 200 mg kg^{-1}. Treated animals were deprived of food and water for 2 h to assess the general behaviour of rats and thereafter during a period of 48 h for died animals [6]. During a 48-h period of observation, the body weight changes, food and water intake were recorded. Surviving animals were killed by cervical displacement after 7 days of observation for any signs of toxicity and deaths and the latency of death. Blood samples were collected from the orbital sinus under ether anaesthesia. The obtained blood samples were centrifuged at 3000 g at 4°C for 15 min to obtain the serum and stored at 20°C until analysis for biochemical and haematological analysis instead. After the collection of blood samples, the liver, kidneys, lungs, (testis and ovaries for further research) were removed for macroscopic and biochemical analysis. Portions of these organs collected from the control group and the *Allanblackia floribunda* treated groups were fixed in Bouin medium and were embedded in paraffin, then subjected to haematoxylin–eosin staining. The pathological observations of all organs were performed on gross and microscopic bases. The biochemical parameters evaluated included tissue and serum creatinine, serum protein, alanine aminotransferase (ALT), aspartate aminotransferase (AST), total cholesterol (TC), triglycerides (TG), and were assessed using commercial kits. Red blood cell (RBC) count, white blood cell (WBC) and haematocrit counts were determined concerning haematological parameters. The LD_{50} value was determined according to the method of Deichmann and Leblanc [7].

Subacute toxicity

Healthy rats of both sexes weighing between (151-234 g) were housed under the same conditions as described above for the acute toxicity. The *Allanblackia floribunda* extract was given only through the oral route. The animals were divided into one control group (distilled water) and three treated groups (400, 500,

600mg kg^{-1} of raw material as per the guidelines of World Health organization [5]. Each group consists of ten animals. Toxic manifestations, mortality, body weight changes, food and water intake were monitored daily during the 28 day period. At the end of the experiment, the biochemical, haematological and histopathological analysis was also assessed as described above for the acute toxicity.

Statistical analysis

Values are expressed as mean ± SEM Statistical analysis was performed using the Mann–Whitney test. P-values less than 0.05 were considered to be significant.

RESULTS

Acute toxicity

The single oral administration of the aqueous extract of *Allanblackia floribunda* at all given doses (up to 20 g kg-1) caused no noticeable change in the general behavior of the rats and there were no significant changes in body weight, food and water intake of the rats as compared to the control group. Both the control and treated groups appeared relatively healthy during the period of study. There were no deaths reported in any of the groups. Nevertheless, the intraperitoneal administration caused dose dependent lethal effects with diarrhea at dose 150 mg kg^{-1}. The dead animals are observed 24 hours after administration of the extract. Starting at dose 200 mg kg^{-1}, the aqueous extract of *Allanblackia floribunda* resulted in a total lethality. The relative weight of organs (liver, kidney, lungs, testis, and ovaries) of control and treated group did not change. However food and water intake highly decreased and consequently led to the decrease of body weight (Table1). The acute intraperitoneal toxicity (LD_{50}) of *Allanblackia floribunda* extract in rat was 125 mg kg^{-1}. Except kidney, the histology of lungs and liver of treated animals presented morphological changes (data not shown).

Table 2 summarizes the effect of the extract on the biochemical parameters used in this study: After 7 days of observation, all rats treated at dose 150 mg kg-1 died. Analysis of serum of survivors showed an increase in ALT at dose 50 mg kg^{-1} ($p < 0.05$), serum creatinine at doses 50 and 100 mg kg^{-1} ($p < 0.05$ and $p < 0.01$ respectively) while serum proteins decreased at dose 100 mg kg^{-1} ($p < 0.001$).

Subacute toxicity

In subacute treatment, neither significant difference was observed in food and water intake nor organs weight in both male and female (Table 3). The effect of *Allanblackia floribunda* aqueous plant extract on biochemical parameters is presented in Table 4. The liver marker (ALT) dose dependently decreased ($p < 0.01$) at all doses in males, at dose 400 mg kg^{-1} ($p < 0.05$), at dose 500 and 600 mg kg^{-1} ($p < 0.01$) in female, tissue creatinine at dose 500 and 600 mg kg^{-1} in female with ($p < 0.05$). The histopathology of liver, kidney, lung and gonads did not reveal treatment-related changes (data not shown). The haematological analysis (Table 5) showed no significant differences in any of the parameters examined in either the control or treated groups of the both sex. All the values remained within normal limits throughout the experimental period.

Table 1. Mean relative body weight in rats treated with *Allanblackia floribunda* before and 48h after the treatment

Period of treatment	Dose (g)			
	0	50	100	150
Before the treatment	140.23±5.22	172.09±4.20	157.02±4.55	154.03±3.91
48h after the treatment	148.09±8.56	164.75±5.02	141.48±4.41*	136.26±2.88 **

Values are Mean ± SEM, n=5 *p<0.05 ; **p<0.01 vs. control group

Table 2. Biochemical values of rats in acute treatment with *Allanblackia floribunda* aqueous extract after 7 days of observation

Biochemical parameters	Dose (mg kg^{-1})		
	0	50	100
ASAT (IU/L)	64.79±25.85	108.19±73.12	135.96±105.56
ALAT (IU/L)	200.60±51.33	310.61±66.31	698.24±200.92*
Serum Creatinine (mg/dL)	0.95±0.30	2.55±0.63*	3.68±0.39**
Serum Protein (mg/dL)	36.00±4.10	35.83±4.09	7.99±0.88***

Values are Mean ± SEM, n=5 *p<0.05; **p<0.01; ***p<0.001 vs. control group

Table 3. Effect of aqueous extract of *Allanblackia floribunda* on organs weight of rats after subacute treatment

Dose (mg kg^{-1})	Kidney	Liver	Lungs	Testis	Ovary
Male					
0	0.279±0.009	3.183±0.137	0.511±0.043	0.832±0.141	-
400	0.284±0.122	2.930±0.102	0.481±0.043	0.969±0.021	-
500	0.311±0.012	3.241±0.062	0.588±0.043	1.037±0.048	-
600	0.285±0.009	2.836±0.051	0.445±0.039	1.027±0.027	-
Female					
0	0.271±0.003	3.465±0.101	0.697±0.037	-	0.062±0.009
400	0.278±0.011	3.699±0.078	0.611±0.033	-	0.079±0.015
500	0.292±0.006	3.653±0.113	0.689±0.106	-	0.060±0.004
600	0.286±0.014	3.152±0.184	0.636±0.073	-	0.053±0.013

Values are Mean (g/100 g per body weight) ± SEM, n=5

Table 4. Biochemical values of rats in subacute treatment with *Allanblackia floribunda* aqueous extract

Biochemical parameters	Dose (mg kg^{-1})			
Males	0	400	500	600
ASAT (IU/L)	39.02 ± 3.61	59.66 ±12.58	70.25 ± 15.02	57.58±15.22
ALAT (IU/L)	187.55 ±46.21	45.36±16.18**	38.04± 22.42**	37.15± 5.43 **
Cholesterol (mmol/L)	1.35 ±0.16	1.29 ±0.42	1.08 ± 0 .15	1.35± 0.53
Triglycerides (mg/dL)	1.28 ± 0.11	0.71± 0.11	0.77 ±0.15	0.96 ± 0.23
Serum Creatinine (mg/dL)	0.94 ± 0.28	0.53 ± 0.22	0.43± 0.24	0.19 ± 0.16
Tissue Creatinine (mg/dL)	0.31 ±0.14	0.18 ±0.02	0.17 ± 0.08	0.22 ± 0.13
Serum Protein (mg/dL)	36.00 ± 4.05	35.66 ± 4.96	34.66 ± 7.39	38.16 ± 4.15
Females				
ASAT (IU/L)	54.79±25.85	40.68 ±71.91	38.73 ±15.86	33.47±10.92
ALAT (IU/L)	114.33±25.39	34.55±17.88*	27.35±5.04**	16.18±1.59**
Cholesterol (mmol/L)	0.72±0.16	1.27 ±0.21	0.87 ±0.19	1.61 ±0.65
Triglycerides (mg/dL)	0.85±0.33	1.14 ±0.25	1.26 ±0.13	1.23 ±0.13
Serum Creatinine (mg/dL)	0.89±0.18	0.69 ±0.16	0.31 ±0.13	0.33±0.06
Tissue Creatinine (mg/dL)	0.36±0.06	0.11±0.05	0.08±0.04*	0.06±0.08*
Serum Protein (mg/dL)	40.58 ±4.11	32.33 ±3.61	40.27 ±2.39	27.46 ±4.88

Values are Mean ± SEM, n=5 *p<0.05; **p<0.01 vs. control group

Table 5. Haematological values of rats in subacute treatment with *Allanblackia floribunda* aqueous extract

Haematological parameters	Dose (mg kg^{-1})			
Males	0	400	500	600
White Blood Cells (WBC) (10^3/µL)	7.0± 1.0	9.0± 2.0	9.0± 2.0	6.0± 1.0
Haematocrites(%)	44.7±2.9	46.0±6.0	39.0±2.0	41.2±6.3
Red Blood Cells (RBC), (10^6/µL)	9.8±0.76	9.6 ± 1.1	8.0±0.72	8.4 ± 1.2
Females				
White Blood Cells (WBC) (10^3/µL)	8.0 ± 2.0	7.7 ± 2.0	5.1 ± 0.88	9.0 ± 7.0
Haematocrites(%)	37.1 ± 1.2	40.3 ± 4.4	35.0±3.0	27.9± 11.9
Red Blood Cells (RBC), (10^6/µL)	8.0 ± 1.0	8.0± 0.836	7.0 ± 0.60	4.3 ± 2.1

Values are Mean ± SEM, n=5

DISCUSSION

Acute toxicity tests in animal may be used to satisfy requirements of classification of the danger of the LD_{50} value, and for the assessment of risks to human health and the environment [8]. It is on this basis that an assessment of the acute toxicity in the rat of the aqueous extract of *Allanblackia floribunda* was carried out.

The present study revealed that the administration of a single oral dose of *Allanblackia floribunda* aqueous extract caused no change on the studied behavioral parameters at dose up to 20 g kg^{-1}. Since the pharmacological properties of *Allanblackia floribunda* have been proven orally in rats with minimum active dose at 200 mg kg^{-1} [4], we can say that, the absence of adverse effects at high doses is due to the fact that the compounds are not toxic orally or may be digested or neutralized by some enzymes of the gastrointestinal tract before their passage to the blood. Similar observations have been reported earlier for the extract of Turraeanthus mannii [9]. There were no differences among groups in weight gain or final weight indicating that *Allanblackia*

floribunda extract did not alter protein, carbohydrate or fat metabolism. An estimated 20 g kg^{-1} LD_{50} value was obtained. This brings us to classify the aqueous extract of *Allanblackia floribunda* among the relatively harmless substances by oral route in rats [10]. However the acute intra peritoneal treatment caused changes in the Behavioral responses at doses from 50 to 150 mg kg^{-1}, characterized by a decrease of locomotion, aggressiveness and sensitivity to the touch. These behavioral changes indicated that some parts of the central nervous system were affected. Similar results were observed by Diezi, (1992) [11] with high dose of drugs which lead to the depression of the central nervous system. Animals treated at dose 150 mg kg^{-1} presented diarrhea, suggesting either a decrease in the intestinal absorption due to an acceleration of the intestinal transit causing the rapid elimination of the extract in the feces, or retention of water at the level of the intestine, thus increasing the volume and hydration of the feces. It is the dehydration of animals caused by diarrhea which could be the cause of the weight losses and even mortalities found in this study concerning the way of administration of extract.

Although both AST and ALT are common liver markers that are associated to the hepatocellular damage, only ALT is remarkably specific for liver function since AST is mostly present in the myocardium, skeletal muscle, brain and kidneys [12]. The increase of ALT level at the dose of 100 mg kg^{-1} in this study could result from liver's attacks such as cirrhosis, cytolysis [13] and are confirmed with tissue damage observed in the liver section characterized by the dilation of the capillaries, inflammation and leucocytes infiltration (data not shown).

The increase of the serum creatinine, which is an indicator of reduced glomerular filtration rate, is a sign of the decline in renal function [14]. In the present study, although the renal histology presented a normal architecture, serum analysis indicated a rise in serum creatinine at doses 50 to 100 mg kg^{-1}. It may therefore be suggested that the nature of the toxicological effect of *Allanblackia floribunda* at this stage would be more functional than structural. This may be due to the presence of flavonoids in extract of bark of *Allanblackia floribunda* [2]. These compounds have antioxidant properties that played protective role on organs [15]. Our results are similar to those found by Hind et *al*., (2011) [16].

This work also showed that the intra peritoneal administration of the aqueous extract of *Allanblackia floribunda* caused a significant decrease in the levels of serum proteins at dose of 100 mg kg^{-1}. This may be due either to the decrease in food intake or bad protein metabolism [17]. Since serum proteins are synthesized by the liver, their reduction would be the consequence of hepatic metabolism insufficiency in connection with impairment of hepatic cells indicated on the histological sections.

Pulmonary lesions characterized by dose-dependent inflammation in this study, may have originated from respiratory failure or poor oxygenation of the blood. Such results were obtained by Sreekanth et al. (2006) [18] in toxicological studies of the bark of calycopteris floribunda in rabbits and rats. It is then clear that acute intraperitoneal administration of the aqueous extract of *Allanblackia floribunda* is a potential danger of the vital organs. These results are in agreement with the fact that the toxicity of a pharmacological substance varies with the dose of the substance absorbed, the route of administration, the type and degree of damage and the duration of treatment [5].

In subacute treatment, neither significant difference was observed in food and water consumption nor organs (liver, lung, kidney, testis and ovaries) and body weight. No behavioral change and no signs of toxicity were observed during the period of administration. Since the changes of body weight are considered as indicators of the adverse effects of drugs and chemical compounds [19, 20], one could conclude that the repeated administration of the aqueous extract of *Allanblackia floribunda* after seven weeks is non-toxic in rats.

The haematological status after 28 days of oral administration of aqueous extract of *Allanblackia floribunda* was also assessed. It is known that hematological parameters provide vital information regarding the status of activity of the bone marrow and intra-vascular effects such as hemolysis and anemia [21]. In general the results showed that the values for the haematocrits, RBC and WBC did not changed in treated groups compared to the control.

Xanthones are among secondary metabolites found in *Allanblackia floribunda* [2]. They are known for their hepatoprotective properties [22]. We observed in both sexes a significant decrease at all doses of ALAT level. Our results are similar to those obtained by DIMO et al., (2010) [4] when studying the antihypertensive and antioxidant effects of the aqueous extract of the bark of *Allanblackia floribunda* in rats. This decline may be caused by the biological action of xanthones, indicating a proper functioning of the liver.

Allanblackia floribunda significantly lowered tissue creatinine levels in females. The significant ($P < 0.5$) reduction in creatinine concentration in all the extract-treated groups indicates that the extract does not exert deleterious effect on the renal function. The normal function of kidney of *Allanblackia floribunda* treated animals can be explained by the presence of flavonoids in the extract since Flavonoids also reduce cytotoxicity by their antioxidant activity [15]. It is why the

histological section of the kidney showed normal renal veins, renal tubules and glomerular structures after the long period of treatment on subacute toxicity in this study. The histological section of gonad also showed a normal architecture, showing that the extract did not altered the reproductive functions.

CONCLUSION

In conclusion, this study presents strong evidence of the nontoxic effect of the aqueous extract of *Allanblackia floribunda* in oral acute and subacute treatment. However this extract administered through the intra peritoneal route is very toxic and can lead to deleterious effect on some target organs and alterations in the behavioral responses. Then the use of extract of *Allanblackia floribunda* can be considered safe and confirm the extensive utilization of the plant in traditional medicine

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Original Research

In vitro control of Anthracnose disease of cowpea (*Vigna unguiculata* L. Walp) caused by *Colletotrichum destructivum* O'Gara with *Cyathula prostrata* L. and *Diodia scandens* SW leaf extracts

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Keywords: Benomyl fungicide, Colletotrichum destructivum, cowpea, Diodia scandens

Summary

Aim/Background: *Colletotrichum destructivum* is one of the most important causes of anthracnose disease of cowpea leading to a great reduction in their production and yield potentials. The constant application of chemicals to control these phytopathogens poses potential threats to human health and the environment. Potential non-chemical control strategy such as the use of botanicals would be a better alternative. Botanicals are readily available, safe, efficacious and eco-friendly. This study was undertaken to investigate the potentials of *Cyathula prostrata* and *Diodia scandens* leaf extracts to control the in vitro mycelial growth and sporulation of *C. destructivum*, causal agent of anthracnose disease of cowpea (*Vigna unguiculata* L. Walp).

Methods: The leaves of both plants were air dried, pulverized, and the fine powder extracted by conventional maceration techniques using aqueous solution. Prepared concentrations (40-100%) of the extracts were then tested against the in vitro mycelial growth and spore germination of the purified test pathogen (*C. destructivum*) isolated from infected cowpea pods. Benomyl (3%) was used as reference fungicide drug.

Results: It was observed that the extract treatments at 60, 80 and 100% significantly ($P<0.05$) inhibited the mycelial growth and sporulation of the fungal pathogen in a concentration dependent pattern. *D. scandens* leaf extract treatment was more effective than *C. porstrata* extract, and its effect on the pathogen's sporulation at 100% after 3hours was greater ($P> 0.05$) than that of benomyl fungicide (3%).

Conclusion: These findings suggest that *C. prostrata* and *D. scandens* leaf extract have the potentials as veritable control agents of anthracnose disease of cowpea in Africa.

INTRODUCTION

Cowpea (*Vigna unguiculata* L.Walp) is one of the most ancient crops known to man. It is widely cultivated by millions of people in the tropics as a major source of their livelihoods [1]. It is cultivated globally primarily as a vegetable, cover and cash crops [2, 3]. Cowpea is rich in quality protein and has energy content almost equivalent to that of cereal grains. Cowpea grain is consumed directly after cooking, or as a component of meals made from cereals or root crops [4]. Cowpea cakes (made from mashed and fried seed) are also sold as a fast food along roadsides in humid forest of South-western Nigeria [5].

Cowpea is widely cultivated in the humid tropics of South-western Nigeria. Report showed that Nigeria was regarded as the world's largest producer (with 2.1 million tonnes) of cowpea, followed by Niger (650,000 tonnes) and Mali (110,000 tonnes) in the year 2002 [6]. However, its cultivation is faced with several set-backs such as attack by pests and diseases in of South-western Nigeria [7]. Notably among them is the incessant attack by a host of many seed-borne

pathogens such as anthracnose disease which is characterized by black lesions typical of the fungal Genera *Colletotrichum.*

Plant diseases caused by the genus *Colletotrichum* are generally called anthracnose. Anthracnose, Greek-derived word meaning 'coal', is characterized by very dark, sunkun lesions, containing spores in infected plant [8]**.** Anthracnose disease of cowpea affects the aerial parts with the production of water-soaked lesions in all tissues of cowpea plant. It is induced by *Colletotrichum destructivum* O'Gara, a common anthracnose disease of cowpea in Nigeria **[**9]. Initial infection by *Collectotrichum* species involves a series of processes including the attachment of conidia to plant surfaces, germination of conidia, production of appresoria, penetration of plant epidermis, growth and colonization of plan tissue and production of acervuli and sporulation [8, 9]. It has wide host range affecting egg plants, *citrus*, cotton, tomato, coffee, banana, water melon, wheat, yam, onion, avocado and legumes with a great reduction in their productions and yield potentials. This seed-borne fungus is usually found on soil surface or plant debris surviving for at lease two years on diseased stem tissues either on the soil surface or buried [10,11]. Cowpea plants and seeds infected by *C. destructivum* have been reported to manifest necrotic flecks or lesions on the girdle stem, penducles and petioles [2].

Fungicides like benomyl, thiophanate-methyl, thiobendazole, strobilurin fungicide azoxystrobin (Quadris), trfloxystrobin (fint), and pyraclostrobin(Cabrio) are being applied as pre- and post-harvest sprays to effectively reduce the degree of infection by *C. destructivum*, and *C. gloeosporioides* [4, 12]. However, fungicide tolerance often arises quickly, if a single compound is relied upon too heavily [13]. Moreover, there are numerous reports of negative effects of using chemicals on farmers' income and health, and toxic contamination to the environment, particularly in the developing countries [14]. Besides, the excessive use of synthetic chemical fungicides has lead to reduction in effectiveness and development of resistance within the population of post-harvest pathogens [15].

The use of pesticides of plant origin have been suggested by some workers as alternatives to synthetic chemicals, in order to counter the potential hazards and pollution problems associated with the use of synthetic chemicals [16, 17]. Recent studies have confirmed the efficacies of plant extracts in the control of fungal diseases [18-20]. Presently, considerable efforts are directed at exploring the potentials of botanical as alternative or complementary to synthetic chemicals. Botanicals have the advantage of not only being readily available, affordable, safe, and efficacious but biodegradable, hence environmentally friendly [15, 21, 22]. Laboratory and field trials have shown that the crude extract from rhizome, leaves and creeping branches of sweeflag (*Acorus calamus* L.), palmorosa (*Cymbopogon martinii*) oil, *Ocimum sanctum* leaf extract, and neem (*Azadirachia indica*) oil could restrict growth of anthracnose fungus [23]. With these promising results, there is still the need to search for more botanicals so as to complement the available arsenal of synthetic chemical and ultimately, increase the array of choices required for effective management of anthracnose diseases caused by *C. destructivum*.

Cyathula prostrate L. Blume (Amaratheceae) is an annual, branched herb/shrub reaching up to 1m with stem trails on the ground and bears leaves which are rhomboid-oblong and adhesive fruits [24]. Its extract is applied to manage diverse human ailments in Nigeria and other African countries [24, 25-27]. Recently, it was documented to possess significant antibacterial and antifungal activities against some human pathogens [27].

Diodia scandens SW (Rubaceae) is a straggling perennial herb with slender angular stem up to 3m high, with opposite to alternate ovate lanceolate leaves and white clustered flowers [28]. In Nigeria, the leaf extracts are used to cure eczema, stop bleeding, manage bruises and as arbotifecient [29, 30]. It antimicrobial and anti-dermetophytic activities have been reported [31, 32]. Recently, the potential of *D scandens* leaf extract in the control of fungal rot of white yam was also demonstrated [33].

In view of the enormous potentials of these botanicals, it is pertinent to further explore their abilities as control agent of anthracnose diseases of cowpea. Research work in relation to anthracnose disease management of cowpea is yet to develop effective alternative/options. Hence, this study was undertaken with the aim of providing broader options by evaluating the potentials of leaf extracts of *Cyathula prostrata* and *Diodia scandens* against *Colletotrichum destructivum* O'Gara, the causal agent of anthracnose disease of cowpea in Nigeria**.**

MATERIALS AND METHODS

Collection of Samples

Fresh plant samples were collected from farm land around Novena University Campus in January 2012. They were identified and authenticated as *Cyathula prostrata* and *Diodia scandens* by Prof. J.M.O. Eze, a botanist in the Department of Biological Sciences, Novena University Herbarium, Amai campus, Delta State, Nigeria, where voucher specimens were deposited for future reference. Infected cowpea (V*igna*

unguiculata L. Walp) pods, with typical symptoms of anthracnose disease, were collected from the Research farm of Michael Okpara University of Agricultrure, Umudike, Nigeria.

Preparation and Extraction of Plant material

The leaves of the plant materials were separated, washed thoroughly in tap water and rinsed in sterile distilled water and allowed to air dry on laboratory bench for 14days. Thereafter, the dried leaves were ground into fine powder using an electric milling machine (Model Corona Lavesh 250) which was stored in air-tight bottle. Various grams (40-100g) of the powdered samples were weighed out and soaked separately in 100ml of sterile distilled water contained in 250ml conical flask, and allowed to stand for 24hours with intermittent manual shaken. The different extracts were subsequently filtered through four folds clean muslin cloth to obtain the following working concentrations; 40%, 60%, 80% and 100% (w/v).

Isolation and Identification of *Colletotrichum destructivum*.

The infected cowpea pods were cut into small pieces and surface sterilized for one minute in 1.0% sodium hypochlorite (NaOCl) solution and rinsed in three changes of sterile distilled water They were dried on sterilized filter paper and then plated on sterile Petri plates (9cm diameter) of Potato Dextrose Agar (PDA) and incubated at ambient temperature 28 ± 2°C for 1 week. After the emergence of mycelial growth, each of the fungal colonies was repeatedly sub-cultured to fresh sterile PDA plates to obtain a pure culture of the pathogen. Slides of fungal mycelia from fresh cultures were examined under the dissecting and compound microscope and confirm as *Colletotrichum destructivum* by comparing their morphological and cultural distinctiveness with images as described by [34].

***In vitro* Experiment**

The inhibitory effect of the extracts against the hyphal growth of *Colletotrichum destructivum* was determined using a growth rate method as described by [35], with slight medications. A volume of 16ml of molten PDA (prepared according to manufacturer instruction, and supplemented with Streptomycin sulphate (100mg/l) to prevent bacterial contamination) was thoroughly mixed with 4 ml aliquot each of the different extract concentrations (40-100%w/v) in a measuring cylinder. The final concentration of each plant extract in PDA was 20 mg/ml. The mixture was then distributed equally into sterile Petri dishes (9 cm diameter) to produce the toxic culture medium. The control media contained equal volume of sterile distilled water without any extract and or benomyl (3%w/v). Mycelial discs (5mm in diameter) from a 1-week-old culture of the fungus were collected using a sterile cork borer from the edge of an actively growing colony. Using sterile tweezers, one mycelial disc was placed at the centre of a Petri dish and incubated at 28±2°C. The mycelial growth was determined by measuring the colony diameter at 7 days after inoculation with a transparent ruler. Each treatment was replicated three times and the experiment was repeated twice. The percentage inhibition of mycelia growth was calculated using the formula of [36], as shown below:

$$\% \text{ Growth inhibition } = \frac{(DC - DT)\ X\,100}{DT}$$

Where DC = colony diameter of control and
DT = colony diameter of treated plates.

To determine the effect of the plant extracts on sporulation of *C. destructivum*, the method of [37], with slight modification, was adopted. A 5mm diameter discs was cut from advancing edges of 10 day-old pure cultures of *C. destructivum* O'Gara using a sterile cork borer. The mycelial disc was transferred into a sterile test tube and treated for a period of hourly with 5ml each of the extract concentration (40-100%). After each hour, the content of each test tube was centrifuged for 10 minutes and then filtered through four layers of sterilized cheese cloth to remove any adhering mycelia. 0.05ml of the suspension of the pathogen (adjusted to the desired level 1×10^5 spores/ml with the aid of a haemocytometer) was placed on sterile slides and incubated at 28°C for 24 hours in a humid chamber for spore germination. The controls set-up prepared along the test experiment included sterile distilled water or 3% benomyl (fungicide) instead of plant extracts in the test tubes. After 24 hours incubation, further spore germination on the slides was stopped by adding one drop of lactophenol in cotton blue to each suspension. The treatments were replicated three times. The effect of the treatments on the germination of spores of *C. destructivum* was evaluated by examining 100 randomly selected spores under a microscope field. Records were taken of the number of germinated spores per treatment per replicate. The fungi toxicity of the extracts was determined as the percentage inhibition of spore germination compared to the controls using the formula by [36]:

$$\% \text{ Spore inhibition } = \frac{(GC - GT)}{GC} X\,100$$

Where GC = Mean spore germination with Control
GT = Mean Spore germination with Treatment

Statistical analysis

Data were analyzed using analysis of variance(ANOVA) to determine the differences between treatment, and treatment means were separated with Duncan's Multiple Range Test (P = 0.05), using the SAS program [38].

RESULTS

The results from this study showed that leaf extracts of both plant demonstrated significant ($P < 0.05$) inhibitory activity on the mycelial growth and germination of the spores of *C. destructivum* especially at the higher concentrations of the extract. From Table 1, it shows that *D. scandens* leaf extract at concentrations of 60, 80 and 100% caused a marked mycelial growth inhibition diameter of 67.19, 78.10 and 85.55% respectively against the cowpea pathogen. However, only the 80% and 100% extract concentrations of *C. prostrata* gave a significant mycelial growth inhibition (60.25 and 68.25%) against the pathogen. No mycelial growth inhibition was recorded by the control (sterile distilled water) treatment, while a significant growth inhibition of 87.12% was observed from treatment of the pathogen with benomyl (3%).

The effect of the leaf extracts were concentration and time dependent (Table 2). The treatment of the pathogen's spore with 40% *C. prostrata* extract showed a gradual increase in the percentage inhibition of sporulation from 32.21-34.12% after 3hours. The percentage sporulation inhibition (45.01%) observed at 60% extract treatment after 3hours was however significantly higher ($P<0.05$) than those after 2hours. Moreover, extract treatment with 80 and 100%, were slightly higher after 1hour treatment period against the pathogens spores. With the leaf extract of *D. scandens*, treatment with 40% concentration after 3hours was significantly pronounced (48.31%). Treatments with the 60-100% generally displayed marked effect on the sporulation of the pathogen after 2hour contact speriod.

A comparison between the effect of both leaf extracts at 100% concentration and 3% benomyl (a standard fungicide), indicated that *D. scandens* achieved a greater ($P>0.05$) growth percentage inhibition of spore germination and mycelial growth of the pathogen than the fungicide (Table 1and 2). The percentage inhibition of sporulation observed from the leaf extract of *C. prostrata* and *D. scandens* were however significant when compared with the standard fungicide especially at 80 and 100%, and 60, 80 and 100% concentrations respectively.

DISCUSSION

The observed significant inhibition of mycelial growth and sporulation of *C. destructivum* by both aqueous *D. scandens* and *C. prostrata* in this study implies that the secondary metabolites from these plants possess antifungal properties. Previous study on the phytochemical analysis of aqueous leaf extracts revealed the presence of were saponins, tannins, flavonoids, cardiac glycosides and steroids in both plant [27, 33]. The presence of saponins and other phyto-compounds had been documented to exert antifungal activities [27, 32, 33, 39]. The differences in the potency of the leaf extracts on *C. destructivum* may probably suggest differences in type and amount of active ingredients in the extracting solvent. Hence, *D. scandens* compounds may be more soluble in water and may contain such substituent on the carbon skeleton as hydroxyl or carbonyl groups than *C. prostrata* compounds. Earlier researchers had reported similar observations [3, 33, 36, 37].

The observed significant in vitro concentration dependent inhibition of the mycelial growth of *C. destructivum* suggests that at relatively high concentration these extract could be effectively used to manage the incidence of this pathogen. Also, the significant inhibition of the spore germination at the various treatments especially after an hour treatment probably indicates that a relatively increase in the concentration and exposure time of extract-pathogen contact would produce a satisfactory management measure against pathogen of cowpea. The superior fungitoxic activity of *D. scandens* leaf extract over benomly fungicide at 100% concentration even after 3hours treatment further suggest the potency of this plant as a potential biological agents for the management of cowpea disease. This finding is however in disagreement with the work of [37], who reported that benomyl demonstrated superior activity over *Piper guineense* and *Carica papaya* aqueous leaf extracts at 100% concentrations. This could be attributed to the differences in the secondary metabolites of each plant.

The mechanisms of action of these plants though not determined could be due to the ability of the active ingredients of the extracts to either inhibite cellular spindle formation within the cell thereby preventing meiosis and mitosis, block the enzymes involved in appressoria formation or disrupt the cell membrane integrity thereby leading to leakage of radicals [42-44]. Although the selected concentration of tested plant species was unable to exhibit a 100% growth inhibition due to their crude nature, they could be purified and used alone or in combination with fungicides to minimize the application of fungicides.

Table 1: Effects of different extract concentrations of *C. prostrata* and *D. scandens* and 3% Benomyl on growth inhibition of *C. destructivum*

Treatment	Concentration % (w/v)	% Growth Inhibition
Sterile water	100	00.00
Benomyl	3	87.12 ± 0.02
C. prostrata	40	32.12 ± 0.03[b]
	60	40.75 ± 0.23[b]
	80	60.25 ± 0.15[a]
	100	68.25 ± 0.10[a]
D. scandens	40	44.18 ± 0.02[b]
	60	67.19 ± 0.16[a]
	80	78.10 ± 0.05[a]
	100	85.55 ± 0.15[a]

Data are means± standard deviation of triplicate determinations. Values with superscript (a or b) that are the same are not significantly different at P<0.05.

Table 2: Effects of different extract concentrations of *C. prostrata* and *D. scandens* and 3%Benomyl on inhibition of spore germination of *C. destructivum*

		Spore Germination inhibition (%)*		
		Exposure time (h)		
Treatment	Concentration %(w/v)	1	2	3
Sterile water	100	00.00	00.00	00.00
Benomyl	3	88.34 ± 0.01b	90.44 ± 0.00b	94.15 ± 0.10a
C. prostrata	40	32.21 ± 0.05b	33.76 ± 0.13b	34.12 ± 0.24b
	60	40.79 ± 0.11b	41.56 ± 0.15b	45.01 ± 0.12a
	80	63.23 ± 0.22b	65.33 ± 0.10a	66.02 ± 0.17a
	100	71.09 ± 0.05b	73.23 ± 0.15b	76.20 ± 0.11a
D. scandens	40	42.83 ± 0.10b	43.28 ± 0.08b	48.31± 0.10a
	60	52.21 ± 0.05b	54.57 ± 0.19ab	57.34 ± 0.20a
	80	72.15 ± 0.41b	74.09 ± 0.21b	77.05 ± 0.31a
	100	89.54 ± 0.04b	92.69 ± 0.16b	96.12 ± 0.01a

*Values are mean ± standard error. Means in along each row with similar letter(s) - (a or ab) are not significantly different at 0.05 level according to Duncan's Multiple Range Test.

The use of botanicals to control the incessant pathogens attack of edible crops of great nutritional value like cowpea is worth exploring. Apart from possessing antimicrobial properties, many of the phytochemicals in plant had been identified to be of health values to humans and animals. Flavonoids, saponins, tannins, cardiac glycosides among others were reported to exhibit anti-inflammatory, antiangionic anti-allergic effects, analgesic, anti-diarrhoeal and antioxidant properties [45-48]. Since plant species are noted to be abundantly available, biodegradable, eco-friendly and easy to prepare without handling hazard, their exploitation as potential bio-control agent against pathogen of cowpea is worthwhile.

CONCLUSION

The present study has shown that aqueous leaf extracts of *C. prostrate* and *D. scandens* at concentrations of 60-100% could significantly inhibit the *in vitro* sporulation and mycelia growth of *C. destructivum*, causal agent anthracnose disease of cowpea in a manner superior to benomyl, a standard fungicide. Since several species of *Colletotrichum* species have been reported to cause substantial losses and post-harvest damage to a wide range of crops, especially in the tropics, these plan extracts would probably serve as potential alternative or complementary agents to synthetic fungicides for use in the management species of *Colletotrichum.* Further study on the purified bioactive ingredients for potentiality under *in vivo* condition and possibly field applications is however recommended.

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Original Research

Ability of *Urtica chamaedroydes* pursh to restore hematopoiesis in anemic pregnant mice

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Summary

Aim/Background: Iron-deficiency anemia (IDA) is associated during pregnancy with increased mortality and morbidity maternal-infantile rate, malformations and fetal or neonatal death. To avoid the death or malformations of fetuses the Indian communities use *Urtica chamaedroydes*. The purpose of this study was to investigate the ability of the decoction of *U .chamaedroydes* (chichicastle) to restore the hematopoiesis in pregnant mice and to avoid malformations in fetuses.

Methods: Forty female mice CD_1, 8–12 weeks were distributed in groups A, B and C with ten animals each. Anemia was induced in groups A, B and C by courts in tail twice a week during 15 days. Hematological determinations were performed on days 0, 15 and 34. On day 15 the pregnancy was induced in all anemic mice and the healthy control group (D). During the gestation, groups A and D did not receive treatment. Group B was treated with a decoction of *Urtica chamaedroydes* (4 g/L) as drinking water and Group C with two sc administrations of 200 mg/kg of $FeSO_4$. On day 34 all groups were sacrificed.

Results: Groups treated with the decoction of *U. chamaedroydes* or $FeSO_4$ recovered the normal level of erythrocytes and platelets, none statistically significant differences were found between the concentration of these elements among days 0 and 34 within the same group. Neither the decoction nor $FeSO_4$ allowed recover the normal level of leukocytes. From the total fetuses, malformations were observed in 80 20, 5, 10 % of Groups A, B, D and C, respectively.

Conclusion: These results could support the traditional medicinal use of this plant in the treatment of the anemia and to prevent malformations.

INTRODUCTION

Iron deficiency is the most common nutritional disorder that affects at least one third of the world's population. It is a frequent cause of anemia during pregnancy, provoking a high risk of maternal-fetal mortality [1]. Generally 52% of pregnant women present anemia and more than 90% of them reside in developing countries [2].

In Mexico, some researchers [3] reported that anemia has higher incidence in pregnant women that inhabit indigenous communities (24.02%) that those not indigenous (14.67%), and pointed out that the prevalence of anemia during the first trimester of the pregnancy goes from 3.5 to 7.4% and reaches 15.6 to 55% in the third trimester. The importance of nutrition before and during pregnancy determines the risk late in

the intra-uterine growth, which is bigger when the women's state is unfavorable before the pregnancy [4]. Some studies have shown that when the women present Iron deficiency anemia (IDA) during the first and second trimesters of gestation, there is an increment in the rate of childbirth pre-term, low weight when being born, fetal deaths, delayed development motor and neural, in extreme cases, increase in the risk of maternal-fetal mortality [5].

Investigations concerning the iron-deficiency anemia in the embryonic period, particularly the formation of organs produce defects in the generation of cerebral cells, and several morphological malformations. During the gestation, anemia can also alters the cell proliferation by changes in the pattern of synthesis of diverse neurotransmitters, fatty acids, cholesterol and myelin, as well provokes a decrease in the synthesis of DNA since the ribonucleotide reductase requires iron as cofactor [6]. To avoid such effects in modern medicine, IDA is treated by oral or injected administration of $FeSO_4$. In Mexico, anemia in indigenous women is associated with malnutrition as a result of low resources and at the cost of the treatment. To counteract this suffering and the effects in pregnant women and over the gestated product, in indigenous communities far from hospitals and doctors, people use as alternative medicinal plants [7], among them the *Urtica dioica*, *Urtica chamaedroydes* and *Urtica urens*.

For the present study *Urtica chamaedroydes* Pursh was selected, because is empirically used in pregnant women with iron-deficiency anemia to counteract this syndrome and to avoid malformations in the fetus [8].

Urtica chamaedroydes Pursh. (Urticaceae), reaches 30 to 80 cm tall, branched and covered with stinging hairs. It has oval, or elongated leaves also covered with stinging hairs. The plant is widely distributed from the Center to the North of Mexico. The decoction is given in cases of anemia, asthma, and for purifying the blood. The plant is commonly known as white grass, chichicastle and ortiguilla [9].

In a preliminary study, our group reported as abstract, hematopoietic activity of *Urtica chamaedroydes* in anemic pregnant rats and the ability to reduce the number of malformations in their gestated products [10]. Although, this plants is used as antianemic, especially by indigenous groups in the country, no experimental information over its pharmacological action was found. And we aimed to determine the ability of the decoction of *Urtica chamaedroydes* Pursh to restore the hematopoiesis in anaemic pregnant mice and to avoid malformations in the fetuses.

MATERIAL AND METHODS

Plant material

Aerial parts of the plant were collected by one of the authors (Rodolfo Velasco) in Alto Lucero town, Veracruz in January 2009, and authenticated at the Herbario Metropolitano of the Universidad Autónoma Metropolitana (UAM) Iztapalapa, where a voucher specimen of the plant (70446) is stored.

Preparation of the decoction

The aerial parts were dried at room temperature, protected from dust and sunlight and grounded in a hand mill (Victoria, Colombia). 500 g of the ground material were boiled 15 min using 3 liters of distilled water. The decoction was filtered and evaporated to dryness under reduced pressure, at 35^{o}C in a Savant Speed Vac plus SC210A concentrator (Farmingdale, USA), then placed in vials and frozen at -20^{o}C until its administration to mice.

Phytochemical screening

A preliminary phytochemical study of the decoction was performed by coloring and precipitation assays as reported previously [11]. Total phenolic compounds were measured by Folin-Ciocalteau Method [12], Flavonoids were determined as described by Wolf, et al. [13].

Induction of iron-deficiency anemia

Forty female CD_1 mice between 8-12 weeks old were obtained from the animal facilities of the Facultad de Estudios Superiores Iztacala of the Universidad Nacional Autónoma de México. Animals were maintained with alternating 12 h periods of light and darkness and allowed free access to food and water according to the statutes of the CICUAL (Comité Institucional para el Uso y Cuidado de los Animales de Laboratorio) of the official Mexican norm for the production and maintenance of laboratory animals. NOM-062-200-1999 [14]. Mice were weighed and distributed in the following groups with ten mice each.

A. Induction of iron deficiency anemia without post-treatment (IDAw/o)

B. Induction of iron deficiency anemia post-treated with the aqueous *Urtica chamaedroydes* (IDAUch)

C. Induction of iron deficiency anemia post-treated with ferrous sulfate (IDAFe)

D. Healthy control (HC)

Iron-deficiency anemia was induced by cuts in the tail of mice on days 0, 4, 7, 11, 15. To evaluate the hematological conditions, animals were bleeding on days 0 and 15, the blood was collected in plastic tubes

containing dry heparin sodium (Pisa, Mex.). Hematological determinations were performed with an analyzer Sysmex KX-21N (Sysmex Corp. Japan). Also blood films were prepared and stained by the Wright's method (Hycel, Mex.). Once confirmed anemia, the pregnant was induced in all groups included the control group. During the gestation, group A (IDAw/o) anemic mice did not received any treatment, for group B (IDAUch) the decoction of aerial parts of *U. chamaedroydes* (4 g/L) was given as drinking water, group C (IDAFe) received two sc applications of 200 mg/kg of $FeSO_4$, (Nycomed, Mex.) and group D (HC) without treatment. On day 19 of gestation all mice were sacrificed in a CO_2 camera and the fetuses examined visually and under a stereoscopic microscope to detect the number and type of malformations. Malformations in fetuses from anemic mice from groups B and C, were compared against the healthy control group (group D), and those anemic mice without treatment (group A). Results are expressed as mean $\pm$ standard desviation. The comparison among Groups was made using variance analysis (ANOVA) and the LSD Fischer test. A p value less than or equal to 0.05 ($p<0.05$) was considered statistically significant.

RESULTS

From 500 g of ground material 49 g of dry decoction were obtained, a yield of 9.8%.

Phytochemical screening

Phenolic compounds and flavonoids were detected in the decoction, but no saponins or alkaloids were found. Total phenolic compounds and flavonoids in the dry decoction were 3.49% and 1.5%, respectively compared with their standards of gallic acid and catechin, respectively.

Induction of iron-deficiency anemia.

On day 15 mice from groups A, B and C presented signs of iron-deficiency anemia, manifested as lethargy, generalized pallor, 10% of them spent slept the most of the time. On this time no significant differences of corporal weight were found these groups, however such weight was 35% lower than mice from control group (D)**.**

Erythrocytes

Mice from groups, A (IDAw/o), B (IDAUch) and C (IDAFe) after induction of anemia (day 15) showed 54% average lower concentration of erythrocytes compared their own lecture on day 0, and the healthy control group at the same time. However, only group IDAw/o kept low concentration of erythrocytes until the end of experiment (day 34). Table 1.

Table 1. Concentration of erythrocytes and platelets in healthy mice and mice with iron deficiency anemia.

Group	Day	Erythrocytes $X^{10}12/l$	Platelets $X10^{12}/l$
HC	0	1.06 $\pm$ 0.10	1.35 $\pm$ 0.24
	15	1.12 $\pm$ 0.10	1.40 $\pm$ 0.18
	34	0.98 $\pm$ 0.20	1.18 $\pm$ 0.16
IDA	0	1.10 $\pm$ 0.12	1.43 $\pm$ 0.20
	15	*0.58 $\pm$ 0.08	0.89 $\pm$ 0.22
	34	*0.50 $\pm$ 0.06	*0.98 $\pm$ 0.14
IDAUch	0	1.20 $\pm$ 0.08	1.52 $\pm$ 0.15
	15	*0.63 $\pm$ 0.03	*1.10 $\pm$ 0.19
	34	0.92 $\pm$ 0.10	*1.57 $\pm$ 0.12
IDAFe	0	1.15 $\pm$ 0.10	129 $\pm$ 0.11
	15	*0.68 $\pm$ 0.10	*0.90 $\pm$ 0.10
	34	0.99 $\pm$ 0.05	119 $\pm$ 0.15

n=10, Mean $\pm$ Standard desviation, *p<0.05
HC= Healthy Control, IDA= Iron-deficiency anemia with out postreatment,
IDA= Iron-deficiency anemia postreated with *Urtica chamaedroydes*,

IDAFe= Iron-deficiency anemia postreated with Ferrous sulphate.

In group D (HC) the concentration of erythrocyte on day 34 was 18 % lower than their own lecture on day 0, change that is not statistically significant. Anemic mice treated with the decoction of *Urtica chamaedroydes* (IDAUch) or ferrous sulfate (IDAFe) at the end of the experiment recovered 76 % and 86%, of concentration of erythrocytes, respectively compared to their own concentration on day 0. In both cases the differences are statistically significant $p<0.05$, which means that treatment of pregnant anemic mice either with the decoction or $FeSO_4$ do not recover the normal level of erythrocytes at 34 day. Table 1. Examination of the stained blood films on day 15 for groups IDAw/o, IDAUch and IDAFe revealed morphological alterations in erythrocytes as; poikilocytosis; elliptocytosis and anysocytosis, besides hypochromic microcytes (small erythrocytes), morphology that it is usually in IDA [15].

Platelets

In control group (HC) the concentration of platelets at the end of the experiment was 87%, regarding day 0,

however the difference is not statistically significant. Although, platelets and leukocytes are usually in normal levels in patients with IDA, in these experiments, mice from group IDAw/o presented 31% of reduction of platelets, while in groups treated with the decoction of *U. chamaedroydes* or $FeSO_4$, the final concentration (day 34) was 103% and 92%, respectively compared to their own lectures on day 0. Both groups recovered the normal level of platelets, however in group treated with the decoction (IDAUch), such increase was significantly higher respect control group (HC) and group treated with $FeSO_4$, $p < 0.001$. Table 1.

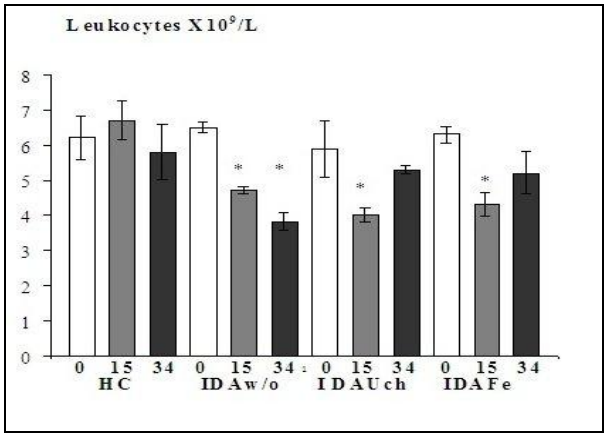

Figure 1. Concentration of leukocytes in pregnant anemic mice

n=10, Mean + S.D. , *p<0.05

HC =Healthy Control, IDA w/o = Iron-deficiency anemic mice without postreatment,

IDAUch = Iron-deficiency anemic mice treated with the decoction of U. chamaedroydes, IDAFe = = Iron-deficiency anemic mice treated with ferrous sulfate.

Leukocytes

In groups IDAFe, IDAUch and IDAw/o, the concentration of leukocytes diminished an average of 31% on day 15 of the experiment. Groups treated with the decoction of *U. chamaedroydes* or with $FeSO_4$ at the end of the experiment did not recover the normal levels of leukocytes regards day 0 (Figure 1).

Effect of Urtica chamaedroydes on malformations in fetuses

The results shown that 80% of fetuses from group of anemic mice without treatment (IDAw/o) presented malformations, meanwhile those treated with the decoction had 20%, fetuses from group treated with $FeSO_4$ 10%, and control group 5%. Figure 2. Being malformations of nasal graves and of palate the most observed.

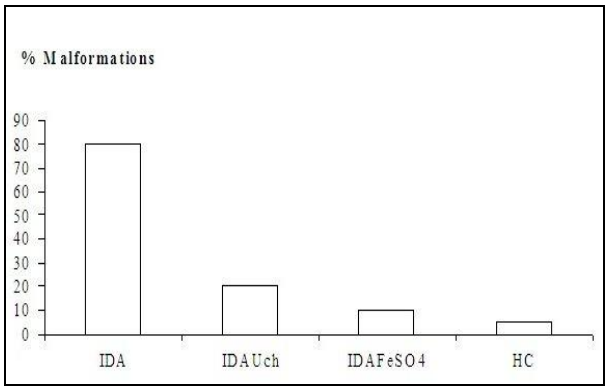

Figure 2. Malformations of fetuses from anemic pregnant mice.

HC =Healthy Control, IDAw/o = Iron-deficiency anemic mice without postreatment,

IDAUch = Iron-deficiency anemic mice treated with the decoction of *U. chamaedroydes*, IDAFe = = Iron-deficiency anemic mice treated with ferrous sulfate.

DISCUSSION

On day 34, erythrocytes from groups HC, IDAUch and IDAFe had almost normal size and color (normocytic-normochromic), which means normal level of hemoglobin. Also erythrocyte indices were normal in groups IDAUch and IDAFe.

The almost normal counts of erythrocytes in groups treated with the decoction of *U. chamaedroydes* or $FeSO_4$ requires a previous synthesis of erythropoietin (EPO), and considering that this hormone shares 30% of similarity with thrombopoietin [16], hormone responsible for the production of platelets [17]. It is possible that the increase of platelets concentration seen could be due to an indirect stimulating effect of EPO on thrombopoiesis, triggered by the decoction or $FeSO_4$ as well, as a stimulating effect of EPO on the precursor cells of platelets (megakaryocyte), through a common cell, since both cell lines share the hematopoietic precursor cells BFU-EMeg [18].

The low concentration of leukocytes in these groups respect control group are statistically significant $p < 0.001$. Then the conclusion is that the decoction of the plant does not present leukopoietic activity, even some authors have reported that other member from the generous *Urtica* (*dioica*) stimulates in vitro the proliferation of lymphocytes [19]. Also a mitogenic activity on T lymphocytes is related with polysaccharides from the roots and leaves of *Urtica dioica* [20]. Besides, an immunomodulatory activity of this plant has been attributed to the flavonol glycosides isolated the leaves of *U. dioica* [21]. Both

activities are primarily dependent of the hematopoietic activity of the plant. However, such activities have not been described until now for *U. chamaedroydes*.

Generally, anemic mice without treatment had a lower number of fetuses but they were bigger and heavier than those from other groups. Fetuses from the healthy mice were smaller than those from other groups. Decoction of *U. chamaedroydes* contributes to restore the erythropoiesis and thrombopoieis, also reduces the number of malformations in the gestated products versus fetuses from pregnant anemic mice without treatment.

Remains to know, if the thrombopoietic effect seen with the decoction is direct through the stimulation on the line megakaryocyte-platelet, or indirect as result of its erythropoietic activity. Also it is necessary to determine the toxicity of this plant, since genotoxic, embryotoxic, mutagenic and abortive effects has been reported for *U. dioica* [22].

The decoction of *U. chamaedroydes* contributes to restore the erythropoiesis and to reduce the number of malformations in products of gestation. These results could explain and support the traditional medicinal use of this plant in the treatment of the anemia and to prevent malformations.

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Original Research

Effect of proanthocyanidin single oral dose on glucose tolerance in response to oral maltose load in healthy women

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Abstract

Aim: The present study was designed to assess the effect of standardized proanthocyanidin on postprandial blood glucose level after maltose load in non diabetic females.

Methods: Single oral dose (50 g) of proanthocyanidin tablet was administered by non diabetic adult females thirty minutes before oral ingestion of maltose load (50 g) using acarbose as comparator. Blood glucose was monitored before and 15, 30, 60, 120, and 180 min post ingestion of maltose.

Results: The results showed considerable decrease in 30 and 60 min postprandial hyperglycemia due to the use of proanthocyanidin, while non significant decrease of Area under the Curve (AUC) and delta AUC was obtained in both proanthocyanidin and acarbose groups compared with control. However, comparable results for 2 hr postprandial hyperglycemia were achieved by proanthocyanidin (41%) and acarbose (40%).

Conclusion: single oral dose of proanthocyanidin attenuates hyperglycemia after maltose challenge in non-diabetic females and such effect was comparable to that produced by acarbose.

INTRODUCTION

Diabetes mellitus a metabolic disorder of multiple etiology characterized by chronic hyperglycemia and glucose intolerance with impaired metabolism of glucose and other energy yielding fuels (proteins and lipids) resulting from defect in insulin secretion, impaired effectiveness of insulin's action, or combination of both [1-3].

Diabetic patients and those with untreated postprandial hyperglycemia are at increased risk of development long-term macro and micro vascular complications including atherosclerosis which may result in stroke and ischemic heart disease, retinopathy, neuropathy and nephropathy [3]. Therefore, effective blood glucose control is the key for preventing or reversing diabetic complications and improving quality of life [4]. Emerging evidence suggests that postprandial hyperglycemia induces glucose toxicity and deteriorates β-cells function [5]; in addition, it associates with elevated glycosylated hemoglobin and contributes to vascular complications [6]. As the postprandial hyperglycemia mostly related to the amount of digested and absorbed carbohydrates, therefore intervention by inhibiting enzymes involve in the digestion of poly and disaccharides like α- amylase and α- glucosidase, or by slowing the intestinal absorption of glucose represent an important approach in reducing the level of postprandial hyperglycemia [7,8].

Currently, although the available oral hypoglycemic drugs can alleviate diabetes, they have many limitations including secondary failure rates, improper pharmacokinetic properties and side effects [4]. Accordingly, complementary and alternative approaches are highly considered, including the adjuvant use of medicinal herbs and dietary

phytochemicals with anti-hyperglycemic activities to improve the quality of life in diabetic patients [9,10]. Polyphenols are a large and heterogenous group of phytochemicals of plant-based foods, such as tea, berries, wine and grape. Several studies indicated that consumption of plant foods is associated with lowered risk of major chronic diseases including diabetes and cardiovascular diseases [11,12]. Extensive research suggests that the proanthocyanidins isolated from grape seeds are beneficial in many health problems due to their antioxidant effects which found to be 20 times greater than vitamin E and 50 times greater than vitamin C [13,14]. Recently, *in vitro* studies have shown that procyanidins protect against free radical mediated cardiovascular and renal disorders [15,16]. Other studies indicated that various dietary polyphenols may influence carbohydrate metabolism and regulate glucose homeostasis in diabetic rats [17]. Moreover, it has been reported that flavonoids inhibit α-amylase and α-glucosidase activities *in vitro* [18]; however, no clear *in vivo* evidence available for the effect of proanthocyanidin on postprandial hyperglycemia in response to disaccharide load.

Therefore, the present study was designed to evaluate the effect of single oral dose of standardized proanthocyanidin on glucose tolerance after maltose challenge in non diabetic females.

MATERIALS AND METHODS

This study was carried out on six non diabetic females in the College of Pharmacy/ Baghdad University, with mean age 21.6 years, BMI 21.5 kg/m^2 and fasting blood glucose concentration of 106.2 mg/dL, in an open label, placebo controlled cross-over design. The study protocol was approved by the Research Ethics Committee, College of Pharmacy, University of Baghdad. Initially the health status of all subjects was reported, they do not have any previous or current disease, not use any medication or nutrient supplements known to influence blood glucose regulation and all have normal physical activity. Each female was enrolled thrice in 3 hr tolerance tests on separate days, with least 7 days washout period. The subjects were informed to avoid consuming flavonoids containing food as grapes, berries, tea, coffee, dark chocolate and soft drinks for at least three days before the day of the experiment. The maltose load test was performed in the morning after 12 hr overnight fasting. Blood samples were obtained from a fingertip using a lancing device, and glucose level was estimated representing zero time measurement (fasting glucose). A cross-over design of treatment was followed through which subjects were randomized into control and test (three subjects for each group). A washout period of 72 hr was followed before the next experiment; after an overnight fasting, either single 50mg tablet of proanthocyanidin, 100 mg tablet acarbose, or placebo was orally ingested. Thirty minutes following administration of doses, each individual consumed 50 g of maltose mixed in 250 ml water. Blood glucose level was estimated before and at 15, 30, 60, 90, 120 and 180 min using glucose oxidase-peroxidase reactive strips and a glucometer (ACCU-check, Germany). The total blood glucose over 3 hours was expressed as integrated area under curve for glucose (AUC_{0-180}), while the change in blood glucose from the basal level after maltose load was analyzed and represented as delta blood glucose expressed as (delta AUC).

Statistical Analysis

The data were expressed as mean±SD. The change in plasma glucose level with respect to baseline and time was estimated to represent area under the curve. Statistical significance was performed by one way factorial analysis of variance (ANOVA), followed by Benferroni's *post hoc* comparisons to compare means of AUC, using graph-pad prism 5 for windows software. P values less than 0.05 were considered to be statistically significant.

RESULTS

Ingestion of maltose (50 g) increases blood glucose level in normal control individuals and maximum concentration (C_{max}) of glucose (180.8 mg/dl) was achieved within 60 min (T_{max}) after ingestion of maltose, while pretreatment with both acarbose and proanthocyanidin resulted in C_{max} of 156.5 and 141.8 mg/dl respectively, 30 min post maltose load (Figure 2; table 1). In control group, the average increment of blood glucose was 62.5mg/dl after 30 min of maltose load, while acarbose produces 54.2 mg/dl increase at the same time. In proanthocyanidin group, the 30 min post-load glucose increased only by 36.8 mg/dl indicating that proanthocyanidin reduced the postprandial glucose elevation (Figure 3). Although proanthocyanidin-maltose load seems to be well tolerated, estimation of integrated AUC_{0-180} and delta AUC over 3 hr period revealed that proanthocyanidin did not show significant results compared to control; similar result was obtained with acarbose (Figures 4 and 5; table 2). The percent decrement of blood glucose produced by both proanthocyanidin and acarbose was found to increase with time to reach maximum (103.1 % and 97.5 % respectively) after 1 hr of maltose load, then return to about 41.31 % and 40.17% 2 hr postprandial compared to control group.

Table 1. Blood glucose levels in response to maltose load in normal subjects pretreated with proanthocyanidin or acarbose.

Group	Blood glucose level mg/dl					
	zero time	15 min	30 min	60 min	120 min	180 min
Placebo+Maltose	110.5±15.8	156±37.6	173±23.5	180.8±42.7	131.8±9.06	93.16±11.9
Acarbose+Maltose	102.3±10	141.3±12.5	156.5±19*	150.5±14.7	120.8±13.9	94.6±5.9
Proanthocanidin+Maltose	105.5±11.3	138.8±10.28	141.8±19.3*	140.5*±19.7	119.3±10.9	99.16±15.5

Values represent mean± SD; * significantly different *vs*. control (*P*< 0.05).

Table 2. Calculated AUC, C_{max}, T_{max} and delta AUC of different treatments

Group	AUC	C_{max}	T_{max}	Delta AUC
Placebo+Maltose	23629	180	60	5152
Acarbose+Maltose	23271	156.5	30	4956
Proanthocyanidin+Maltose	22522	141.8	30	3630

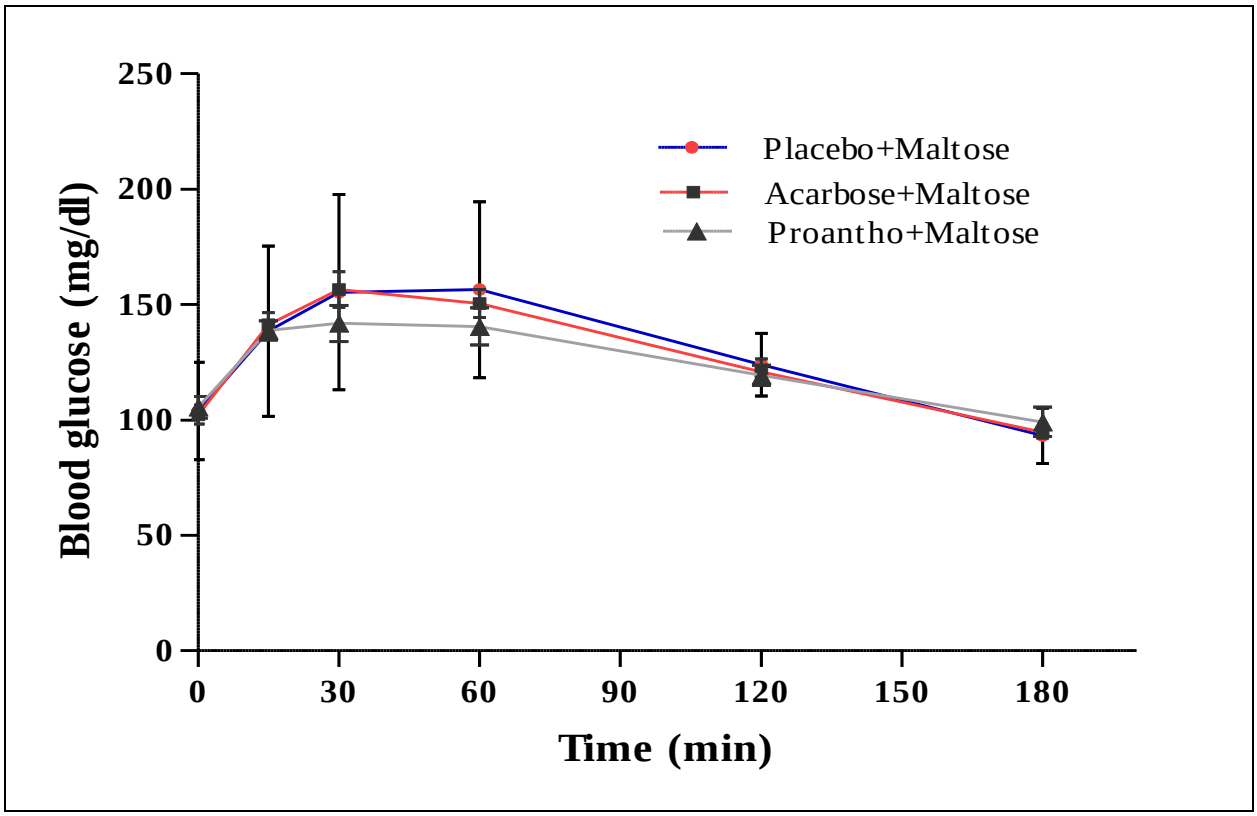

Figure 1. Blood glucose spikes in response to single oral dose of proanthocyanidin and acarbose relative to placebo-treated control after maltose challenge.

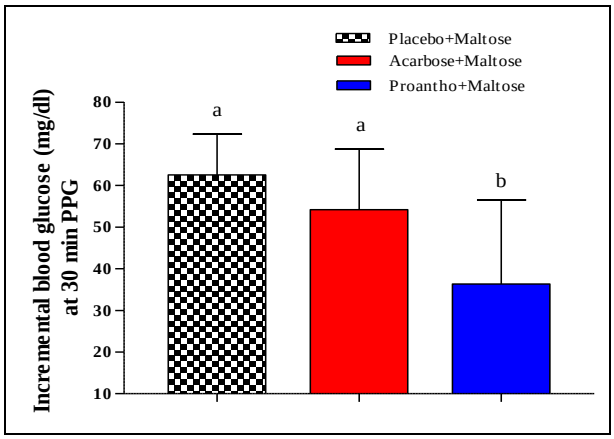

Figure 2. Effect of single oral doses of proanthocyanidin and acarbose on blood glucose level after 30 min from maltose load; values with non identical superscript (a,b) are considered significantly different (P< 0.05)

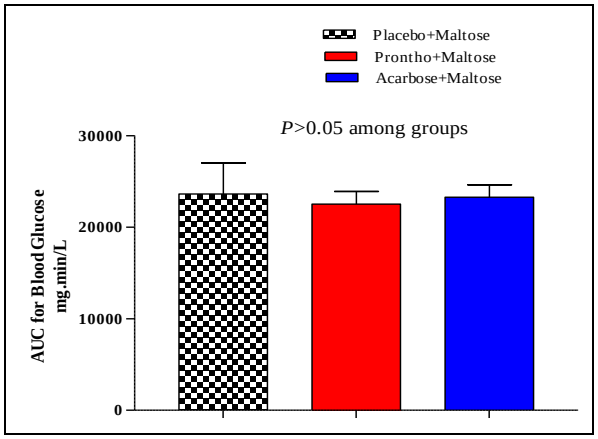

Figure 3. Effect of proanthocyanidin and acarbose on AUC_{0-180} min of blood glucose spikes in normal subjects after maltose load compared to placebo.

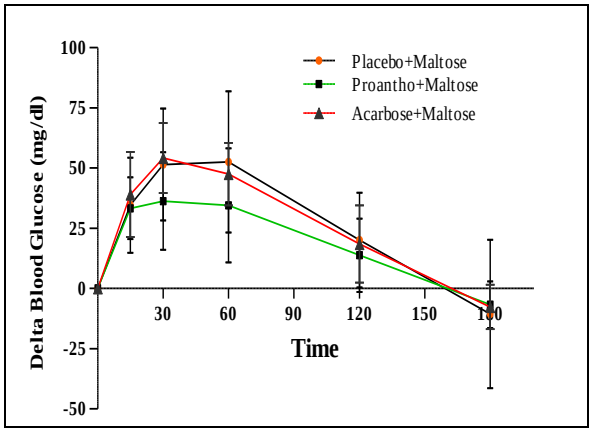

Figure 4. Effects of single oral doses of proanthocyanidin and acarbose on the change in blood glucose (Δ AUC_{0-180}) after maltose load compared to placebo.

DISCUSSION

Rapid absorption of glucose challenges the regulatory mechanisms of glucose homeostasis, and habitual consumption of high glycemic diets may therefore increase the risk of diabetic complications and cardiovascular diseases. Acute hyperglycemia was reported to induce endothelial dysfunction and result in microvascular damage through oxidation of low density lipoprotein (LDL) and other proatherogenic mechanisms [19]. Diet rich in carbohydrates produces sharp rise in the blood glucose level as the complex carbohydrates in the food is rapidly absorbed in the intestine aided by the effect of the digestive enzymes α-amylase and α-glucosidase which break dietary carbohydrates into absorbable glucose [20].

Actually, controlling postprandial plasma glucose level is critical in the early treatment of diabetes mellitus (DM) and in reducing chronic vascular complications. So, inhibition of enzymes that digest complex carbohydrates would reduce the rate of glucose release and absorption, and consequently suppress postprandial hyperglycemia [21]. Previous study reported the anti-postprandial hyperglycemic effect of grape flavonoids and phenolic compounds in diabetic mice, suggesting the valuable benefit of these bioactive products in management of impaired blood glucose levels in type II diabetes mediated by their capacity to inhibit α-glucosidase activity, the crucial enzyme for digestion of maltose into absorbable glucose [22]; so, we evaluate the effect of standardized grape seed extract (95% proanthocyanidin) on postprandial hyperglycemia after maltose load in healthy individuals. The reported data in the present study showed that proanthocyanidin provided significant tolerance for 30 and 60 min postprandial hyperglycemia relative to untreated control, while only 60 min tolerance was achieved by acarbose indicating the potency of proanthocyanidin to attenuate postprandial hyperglycemic spikes and alleviate oral glucose tolerance test (OGTT) better than acarbose. On the other hand, both treatments provided statistically non significant support to alter the disposal after an OGTT in healthy, non diabetic young women as indicated by the results of AUC_{0-180} and delta AUC_{0-180}. Considering that, it's possible that further study with older individuals and/or those with impaired glucose tolerance would provide more robust effects. Moreover, as subjects simply ingested a *single* dose 30 min before maltose load, it is possible that *chronic intake* for a period of weeks (as for most oral hypoglycemic agents) may provide additional benefit. Also further study is needed to investigate such hypotheses, possibly including both men and women. In fact, the phytochemical analysis reported on α-glucosidase inhibitors isolated from medicinal plants suggests that several potential inhibitors belong to dietary polyphenols has a characteristic structural features to inhibit the α-

glucosidase enzyme [23], which may be varied together with the antioxidant activity by the polyphenolic structure of proanthocyanidin [24]. In terms of overall effects, our data are not as strong as those obtained from the animal studies of Hogan *et al*, who reported favorable effects on glucose tolerance in response to treatment with grape pomace proanthocyanidin [18]. It is possible that differences in the usual response between animals and humans contributed to these mixed findings, coupled with the fact that our subjects were all healthy and non-diabetic. As stated above, while the condition effect for glucose was not of statistical significance, roughly all subjects responded to proanthocyanidin treatment better than acarbose, a well known clinically used α-glucosidase inhibitor, evidenced by lower glucose in response to the maltose load, especially within the first hr after load. In spite of the non-significant results, our findings may provide practical aid for selected individuals opting to use such dietary supplement for glucose disposal after carbohydrate-rich diet. Moreover, we believe that our findings for a response in non-diabetic individuals, with normal fasting glucose and normal glucose tolerance, provide justification for future studies inclusive of larger sample sizes of individuals with impaired glucose tolerance (i.e., diabetic, pre-diabetic). As has been noted for many dietary supplements, a relatively high degree of subject variability in response to treatment was observed which, when coupled with our relatively small sample size, limit the chances for detecting statistically significant findings. As this was a pilot study designed to determine whether proanthocyanidin has an application in larger trials involving diabetic and pre-diabetic patients, our small sample size is justified, and we believe that our objective was met. That is, our results merit attention in future to larger scale trials, in particular those involving individuals with pre-diabetes or untreated diabetes.

As conclusion, administration of single oral dose of proanthocyanidin results in a slight, albeit non-statistically significant lowering of blood glucose in response to an oral maltose load in healthy females.

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Original Research

Antifungal activities of *Terminalia ivorensis* A. Chev. bark extracts against *Candida albicans* and *Aspergillus fumigatus*

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Keywords: Terminalia ivorensis, antifungal activity, clinical strains, hydroalcoholic extract

Summary

Aim: The present study was undertaken to evaluate *in vitro* antifungal activity of aqueous and hydroacoholic extracts from bark of *Terminalia ivorensis* A. Chev. (Combretaceae).

Methods: *In vitro* antifungal activity of all the extracts was done by agar slant double dilution method. *Candida albicans* and *Aspergillus fumigatus* clinically important strains were used for the study. ketoconazole was used as standards for antifungal assay. Antifungal activity was determinated by evaluating of antifungal parameters values which are MCF (minimal concentration fungicide) and IC_{50} (Concentration for 50% of inhibition) around each assay.

Results: Result showed that the antifungal activity was more pronounced against *Aspergillus fumigatus* than *Candida albicans*. The hydroalcoholic extract showed best antifungal activity than ketoconazole.

Conclusion: Demonstration of antifungal activity of *T. ivorensis* provides the scientific basis for the use of this plant in the traditional treatment of diseases and may help to discover new chemical classes of antifungal substances that could serve as selective agents for infectious disease chemotherapy.

INTRODUCTION

Traditional medicine has been practiced for many centuries in many parts of the world, including Africa especially in rural areas to treat their diseases in low cost. Nature has provided a source of medicinal agents for thousands of years and an impressive number of modern drugs have been isolated from natural sources, many based on their use in traditional medicine [1]. In spite of the significant number of antifungal for the treatment of the mycosis, many cases of resistance were noted [2]. The development of microorganisms resistance to presently available commercial antifungal has necessitated the search for new antifungal agents. Many studies have been conducted with the extracts of various plants, screening antifungal activity as well as for the discovery of new antifungal compounds [3,4]. The efforts of scientists in establishing plants with promising antifungal property is yielding fruitful results as a number of plants with high antifungal property have been elucidated [5, 6].

Terminalia ivorensis A. Chev. belongs to the family Combretaceae. The plant is used by traditional faith healer to cure many skin troubles, mouth and teeth diseases, cough, diarrheas, diabetes and high blood pressure. The decoction or the bark's powder is used in traditional medicine to look after the wounds, the ulcers, the hemorrhoids, malaria and the yellow fever. The decoction is also used as analgesics in the event against rheumatism and muscular pains. The sheets juice applies to the cuts (wound) and is used inhaling against getting colds. It is also added with barks decoctions in rectal injection against gonorrhea and

kidney affections. It is also an aphrodisiac. In Côte d'ivoire, the roots of this plant are used much in the traditional pharmacopeia like toothpick against voices extinctions. The studies carried out in Nigeria showed that the plant is an anti-inflammatory drug and antiarthritic [7]. In Nigeria, in the veterinary medicine, the plant was proved having trypanocides and pesticides properties [8].

In This present work, antifungal activity of *Terminalia ivorensis* bark extracts were investigated against an array of clinically isolated as well as standard microbial cultures.

MATERIAL AND METHODS

Plant Material

The barks of *Terminalia ivorensis* were collected in May, 2006 from the campus site of Nangui-Abrogoua Abidjan, Côte d'ivoire Western Africa and identified by comparison with specimens : Forest of Adiopodoumé ,Côte d'ivoire, Western africa, May 17th 1966, Aké-Assi 8855 available at the Herbarium of the floristic national center, Félix Houphouët Boigny University, Abidjan ,Côte d'Ivoire,western Africa.

Microorganisms Studied

In vitro antifungal activity of all the extracts was done by agar slant double dilution method. *Candida albicans (*n° 896/AB du 10.01.2000) and *Aspergillus fumigatus (*n° 896/AB du 10.01.2000) clinically important strains were used for the study, which were both clinical isolates as well as identified strains. ketoconazole was used as standards for antifungal assay. Antifungal activity was determinated by evaluating of antifungal parameters values which are MCF (minimal concentration fungicide) and IC_{50} (Concentration for 50% of inhibition) around each assay. For each extract five replicate trials were conducted against each organism.

Extraction

The barks of *Terminalia ivorensis* were air dried and then powdered in a homogenizer and 100 g was used for different solvent extraction aqueous and hydroalcoholic extracts, the sample was extracted in solvent kept with one liter of water distilled by homogenisation in Blender. After six (6) cycles of homogenisation, homogenate was dried in a fabric square and filtered successively twice with absorbent cotton and once with paper whatman 3mm. Filtrate was concentrated thanks to a Büchi rotary evaporator at 60°C. Dark powder obtained is the aqueous total extract. Hydroalcoholic extract was prepared in the same process by using a mixture of solvent ethanol 70% and water 30%. That gave hydroalcoholic extract.

Antimicrobial Assay

Antifungal tests were carried out on culture medium Sabouraud (BioRAD /Réf:64494 ;Batch:7A2211). Vegetable extract incorporation to agar was made according double dilution method of tilted tubes. 11 test tubes were used including 9 test tubes containing vegetable extract and 2 pilot tubes. Among these two tubes, one without vegetable extract was used as witness of germs control growth while the other without germs and extract was used as witness of culture medium sterility control. Extract concentrations range in the tubes go from 390 to 1.52 µg/mL with geometrical connection of reason ½. All the tubes were pressure-sealed (121°C during 15 min), then tilted with small base at room temperature to allow their cooling and solidification of the agar [9,10]. Germs culture on agar slant previously prepared was made by sowing of 1000 cells of each stock of *C. albicans* and *A. fumigates* [11, 12]. Cultures were carried out and incubated with 30°C during 48 hours. After this time of incubation, germs were counted with pen of germs meter (CEINCEWARE number 23382) and growth in the 10 experimental tubes was evaluated expressed as survival percentage, calculated compared to 100% of pilot tube survival of growth control.The processing of these data made it possible not only to determine the fungicidal minimal concentrations (FMC), but also to plot the curves of activity of the extracts graphically determine the concentrations for 50% of Inhibition (CI_{50}).

RESULTS

All the barks extracts of *T. ivorensis* were active against *C. albicans* and *A. fumigatus*. However the report/ratio of CMF on *Candida albicans* showed that the hydroalcoholic extract has been 4 times more active than the aqueous extract (Table 1).

Table 1. Antifungal activity of *Terminalia ivorensis* barks extracts against *Candida albicans* and *Aspergillus fumigatus*.

T. ivorensis extracts	*Candida albicans*		*Aspergillus fumigatus*	
	MCF (µg/mL)	IC_{50} (µg/mL)	MCF (µg/mL)	IC_{50} (µg/mL)
Aqueous extracts	390	39.60	195	8.28
Hydroalcoholic extracts	97.5	11.40	97.5	5.16

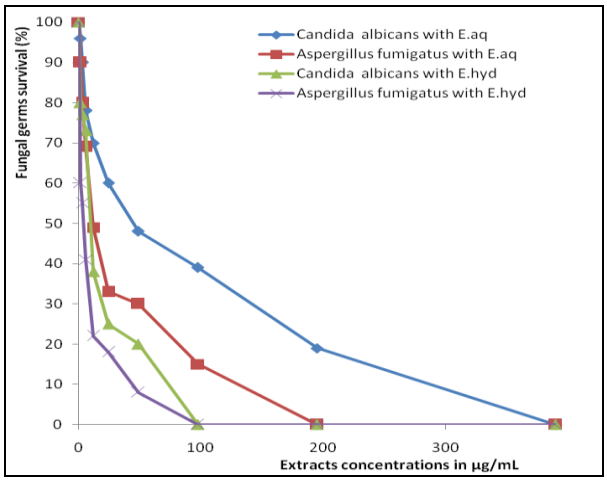

Figure 1. Antifungal of the aqueous extracts and hydroalcoholic activity from *Terminalia ivoriensis* against *Candida albicans* and *Aspergillus fumigatus*

DISCUSSION

Herbal medicine in developing countries is commonly used for the traditional treatment of health problems [13]. In spite of commercial antifungal drugs commonly employed in the treatment of infectious diseases, pathogenic microorganisms keep a resistance [2]. The searching for new antifungal agents is necessitated. In addition to this problem, pharmaceutical drugs (antibiotics) are sometimes associated with adverse effects on host including hypersensitivity, immunosuppression [14]. Therefore there is a need to develop alternative antifungal drugs for the treatment of infections obtained from various sources such as medicinal plants [15,16].

In the present study *T. ivorensis* barks extracted with water and hydroalcohol were investigated for it antifungal potentiality against *C. albicans* and *A. fumigatus* clinically important fungal strains. All the barks extracts of *T. ivorensis* were active against *C. albicans* and *A. fumigatus*. However the report/ratio of CMF on *Candida albicans* showed that the hydroalcoholic extract has been 4 times more active than the aqueous extract. In addition on *Aspergillus fumigatus*, the hydroalcoholic extract was 2 times more active than the aqueous extract. Compared to work of Kporou and al. [17,18] which found a value of CMF=10^5 µg/mL on *C albicans* with the aqueous extract of *Mitracarpus scaber* a rubiaceae, the aqueous extract of TEKAM 2 in this study is 256 times more active than that this plant extract. The hydroalcoolic extract of TEKAM 2 is 1,9 times more active than that of *Terminalia catappa* (CMF = 190 µg/mL) on *Candida albicans* [19].

The best antifungal activity was shown by the hydroalcohol extract on both fungal strains with low antifungal parameters values *C. albicans* (CMF = 97.5 µg/mL) and *A. fumigatus* (CMF = 97.5 µg/mL). The curves of activity with the two extracts against the two fungal strains are on the figure (Figure1).The decreasing shape of the activities curves showed that the 2 extracts have acted according to a relation amount-effect. Compared to the ketoconazale, the extracts tested of *Terminalia* had had a better antifungal activity. The most striking feature of the present findings is that many of the clinical isolates were resistant to the standard pharmaceutical drugs (antibiotics) used while the plant extracts showed moderate to good antifungal activity. The need of the hour is to find new drugs (antibiotics) because the microorganisms are getting resistant to the existing pharmaceutical drugs (antibiotics) [20, 21]. The persistent increase in multi drug resistant strains compels the search for more potent new antifungal. Thus there is a need for a continuous search for new effective and affordable antifungal drugs. The results of present study significated the potentiality of *T. ivorensis* barks as a source of therapeutic agents which may provide leads in the ongoing search for antifungal botanicals.

CONCLUSION

Present study showed that the *T. ivorensis* barks extracts possessed significant *in vitro* antifungal property against 02 clinical isolate as well as identified strains (*Candida albicans* and *Aspergillus fumigatus*). The hydroalcoholic extract exhibited strongest inhibitory effect on (*Candida albicans* and *Aspergillus fumigatus*) as compared to standard antibiotics (Ketoconazole) against the tested microorganisms. Extraction method as described [22,23] concentrated better one or more chemical species against aspergillar and candidosic activity. The present study justified the use in medicine traditional well of *T. ivorensis* to treat skin troubles and as solvents water and the koutoukou (drink extracted from the palm tree traditionally). It is necessary to carry out a bioassay guided fractionation of the extract in a bid to isolate and identify the compounds responsible for the antifungal activity. An elucidation of the mechanisms of action of these extract must be followed by toxicity and *in vivo* tests to determinate the therapeutic applicability of such compounds in combination therapy. These are subjects of ongoing investigation in our research group.

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Original Research

Potential effects of xanthone on inflammation status in atherosclerotic rats

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Keywords: Xanthone; inflammation; NF-κB p65; TNF-α; NO; hypercholesterol diet.

Summary
Aim: To clarify an effect of xanthones on inflammation status in hypercholesterolemic rats.
Method: A total of 32 Wistar rats were divided into four groups (n=8), including control, hypercholesterolemic diet groups, hypercholesterolemic diet + xanthone at dose 35; 70; and 140 mg/kg body weight (mg/kgBW). Control group received standard diet for 60 days. Hypercholesterolemic diet group received standard diet plus yellow egg, sheep oil, cholic acid, and pig oil for 60 days per oral. Analysis of nuclear factor-kappa beta p65/p50 distribution and tumor necrosis factor-alpha level, was done using enzyme linked immunosorbent assay technique. Analysis of nitric oxide level was done by colorimetric technique using spectrophotometer.
Results: Hypercholesterolemic diet significantly increased nuclear factor-kappa beta p65/p50 distribution, tumor necrosis factor-alpha level, and nitric oxide level compared with the control group ($p<0.05$). Xanthone decreased nuclear factor-kappa beta p65/p50 distribution in line with the distribution at standard diet at doses 70 and 140 mg/kg body weight. Xanthone significantly decreased tumor necrosis factor-alpha level compared with atherosclerotic diet group at all doses, although it did not reach the level at standard diet. Xanthone decreased nitric oxide level, reaching the level at standard diet in all doses.
Conclusion: Xanthone has antiinflammatory potentials by inhibiting distribution of nuclear factor-kappa beta p65/p50, decreasing tumor necrosis factor-alpha and nitric oxide level.

INTRODUCTION

The mangosteen fruit is known as the queen of the fruit due to its delicious taste and pleasant aroma. This plant originates from Southeast Asia and distributed in Thailand, India, Sri Lanka, Myanmar, Malaysia, the Philippines, China, Indonesia and other tropical countries [1]. In the United States, mangosteen product is now widely available due to the perception of the public as a supplement that can improve health. Mangosteen juice repredents a food supplement of the primary agricultural products, which tops the sales in 2005 [2].

Mangosteen rind has been used as a traditional medicine in Southeast Asia for many years, to cure diarrhea, dysentery, skin infections, chronic wounds, suppuration, leukorrhea, and gonorrhea [1, 3]. Various studies have revealed the potential effects of active ingredients from the mangosteen rind, among others, as antioxidants [4], antibacterial, antifungal, antimalarial, anti-inflammatory [5], and the cytotoxic activities of human immunodeficiency virus (HIV) inhibitor, aromatase inhibitors and quinone reductase activity inductor [6].

Anti-inflammatory potential of Garcinia mangostana (GM) was evidenced in the research by Yamakuni et al.,

[7] in which garsinon B (10μM) reduced the release of prostaglandind-E_2 (PGE_2) by 30%, which was induced by A23187 in C6 rat glioma cells. Garsinon B (20μM) also decreases the activation of nuclear factor-kappa beta p65/p50 (NF-κB p65/p50) which is induced by lipopolysaccharide (LPS) by 30%. Research by Chonmawang et al., [4] proved that GM can reduce the production of tumor necrosis factor-alpha (TNF-α) on inflammation caused by Propionibacterium acne.

The mangosteen fruit is a rich source of phenolic compounds. Various phenolic compounds in mangosteen include xanthone, tannins, and anthocyanins. Of these phenolic compounds, only xanthone is most frequently investigated [8]. Xanthone compounds are soluble in alcohol, ether, acetone, chloroform, and ethyl acetate, while flavonoids and polyphenols are soluble in water and other polar solvents [1,3]. Xanthone core is known as 9-xanthenone or dibenzo-γ-pyrone which is simetric. Xanthone is classified into five groups: (a) simple oxygenated xanton; (b) xanthone glycosides (c) prenylated xanthone*;* (d) xanthonolignoids and (e) other xanthones. Biological activities of xanthone is related to tricyclics but vary depending on the position of various substituents [9].

No various studies above revealed the potential effects of xanthone on inflammatory status in atherosclerotic mouse models. Therefore, this study will attempt to analyze the potential effects of xanthone on levels inflammation in atherosclerotic rats model. The hypothesis of this study is that ethanol extract of mangosteen rind can reduce levels of NF-κB p65/p50, TNF-α, and nitric oxide (NO) levels in atherosclerotic rats.

MATERIALS AND METHODS

Subjects

Subjects of the research were Rattus norvegicus obtained from the Laboratory of Pharmacology of the Faculty of Medicine, University of Brawijaya, Malang. The rats used here were male rats, aged 6-8 weeks, weight 100-150 grams, and kept in a cage with an open vent. Before the treatment, the mice were acclimatized for 2 weeks. Rats were kept in single system cages, each cage contains 1 rat. This cage is a plastic cage measuring 45 cm x 35.5 cm x 14.5 cm with a lid made of woven wires. The bottom of cage is rice husk, which was changed every three days. The research has been approved by the Research Ethics Committee of the Medical Faculty, University of Brawijaya, Malang.

Feed and xanthone supplementation

The feed given every day was feed for adult rats with composition of the Comfeed PAR-S, high protein flour, and water. This composition is the composition of the feed for standard diet. Weight of feed given per rat is ± 40 grams per day and replaced daily. For hypercholesterolemic diet, the composition of feed ingredients consist Comfeed PAR-S, flour, egg yolks, goat oil, cholic acid and pig oil. Xanthone used here is the xanthone isolates of Nacalai Tesque products.

The study groups consisted of a group of rats that received a standard diet, the rats that received the high-fat diet, as well as groups of rats that received the high-fat diet + xanthone diet (35, 70, and 140 mg/kg body weight (mg/kgBW)). The treatment was carried out for 60 days. Each treatment group consisted of eight rats for a total of rats from all groups were 32 rats.

Sampling

The sample was aorta after 60 days of treatment. Aortas were removed by putting rats in a jar containing cotton already soaked with ether for inhalation anesthesia. When the rats were already anesthetized, they were dissected to by opening the abdomen to the thorax. Once the heart was seen clearly, blood was collected by 5 mL syringe in their ventricles slowly. Once the blood was already removed, the aorta was cut and then stored in cold temperature and subject to imunohistochemistry (NF-κB p65/p50), enzyme linked immunosorbent (ELISA) assay (TNF-α) and spectrophotometric analysis (NO). All procedures were done according to instruction of kit manufactures.

Statistical analysis

Data are presented as mean ± standard deviation and differences between groups were analyzed using Analysis of Variance (ANOVA) test using Statistical Package for Social and Science (SPSS) 16.0 software. $p < 0.05$ was considered statistically significant.

RESULTS

ANOVA test of the distribution of NF-κB p65/p50 in the various treatment groups found significant differences ($p < 0.001$). Post Hoc Test found significant increase in the distribution of NF-κB p65/p50 between the standard diet groups compared with the hypercholesterolemic diet group ($p < 0.001$). Mann Whitney test showed significant decrease in distribution of NF-κB p65/p50 between the hypercholesterolemic diet group compared with hypercholesterolemic diet groups + xanthone isolates of 35 mg/kgBW ($p<0.001$); between hypercholesterolemic diet group compared with the hypercholesterolemic diet group + xanthone isolates of 70 mg/kgBW ($p<0.001$); between hypercholesterolemic diet group compared with hypercholesterolemic diet group + xanthone isolates of 140 mg/kgBW ($p<0.001$). Mann Whitney test found no significant differences

between standard diet groups compared with the hypercholesterolemic diet group + xanthone isolates of 70 mg/kgBW (p=0.183), and between standard diet groups compared with hypercholesterolemic diet group + xanthone isolates of 140 mg/kgBW (p=0.229).

Kruskall-Wallis test on TNF-α levels in the various treatment groups found significant differences ($p < 0.001$). Mann Whitney test found significant increase in the distribution of TNF-α between the standard diet groups compared with the hypercholesterolemic diet group (p=0.009). Mann Whitney test showed significant decreases in level of TNF-α between the hypercholesterolemic diet group compared with hypercholesterolemic diet groups + xanthone isolates of 35 mg/kgBW (p=0.009); between hypercholesterolemic diet group compared with the hypercholesterolemic diet group + xanthone isolates of 70 mg/kgBW (p=0.009); between hypercholesterolemic diet group compared with hypercholesterolemic diet group + xanthone isolates of 140 mg/kgBW (p=0.009).

Analysis of the levels of NO in the various treatment groups with the Kruskall-Wallis test found significant differences ($p < 0.001$). Mann Whitney test found significantly increase in NO levels between standard diet groups compared with the hypercholesterolemic diet (p=0.009); between standard diet groups compared with hypercholesterolemic diet + xanthone isolated of 35 mg/kgBW (p=0.009), between the standard diet group compared with the hypercholesterolemic diet + xanthone isolates of 70 mg/kgBW (p=0.009); between standard diet groups compared with the hypercholesterolemic diet + xanthone isolates of 140 mg/kgBW (p=0.009).

Mann Whitney U test showed significant decreases in NO level between the hypercholesterolemic diet group compared with hypercholesterolemic diet groups + xanthone isolates of 35 mg/kgBW ($p < 0.001$); between hypercholesterolemic diet group compared with the hypercholesterolemic diet group + xanthone isolates of 70 mg/kgBW (p=0.009); between hypercholesterolemic diet group compared with hypercholesterolemic diet group + xanthone isolates of 140 mg/kgBW (p=0.009). I was found no significant decrease in levels of NO between the cholesterolemic diet group + xanthone isolates of 35 mg/kgBW compared with hypercholesterolemic diet + xanthone isolates of 140 mg/kgBW (p=0.602); between cholesterolemic diet + xanthone isolates of 70 mg/kgBW compared with the hypercholesterolemic diet + xanthone isolates of 140 mg/kgBW (p=0.530).

DISCUSSION

Inflammation is a pathophysiological processes mediated by various signaling molecules produced mainly by leukocytes, macrophages, and plasma cells [10]. Inflammation is a predictor of cardiovascular disease and considered as precursors of metabolic syndrome. Various treatments were administered to manage inflammation. Steroid is the best medicine used to treat acute inflammation, but has side effects when used for long periods of time, such as lowering the resistance of infection. Non-steroidal anti-inflammatory drugs are also used to treat inflammation, but they bring about side effects such as gastrointestinal bleeding [11].

In this study, xanthone isolates are able to reduce the distribution of NF-κB p65/p50 starting at a dose of 35 mg/kgBW and reached a standard diet at dose levels of 70 mg/kgBW and 140 mg/kgBW. The mechanism of inhibition of NF-κB p65/p50 distribution is caused by the blockade of kappa-B kinase inhibitor activity. This is based on the research by Udani et al., [11] which has proved the potential effects of the mangosteen as anti-inflammatory substance through the inhibition mechanism of cyclooxygenase, changes in arachidonic acid into prostaglandin E_2, blockade of kappa-B kinase activity and inhibition of rat foot edema. Moreover, inhibition of NF-κB p65/p50 distribution will impede the genomic process of NF-κB p65/p50 which is characterized by a decrease in its protein product. This is evidenced by a decrease in production of TNF-α.

Table 1. Distribution of NF-κB p65/p50 and TNF-αNO levels in some groups

	Standard diets	Hypercholesterolemic diet + xanthone isolates			
Distribution & Levels		0 mg/kg BW	35 mg/kg BW	70 mg/kg BW	140 mg/kg BW
NF-κB p65/p50	5.40±2.88	19.20±1.92[a]	12.20±2.59[ab]	7.40±2.51[bc]	3.60±1.14[bcd]
TNF-α (ng/mL)	14.06±0.31	42.93±0.75[a]	23.55±1.13[ab]	25.84±4.15[ab]	17.55±1.03[abcd]
NO (mmol/L)	0.74±0.15	6.95±0.58[a]	1.47±0.19[ab]	1.09±0.11[abc]	1.59±0.60[ab]

NF-κB p65/p50: nuclear factor kappa beta p65/p50; TNF-α: tumor necrosis factor-alpha; mg/kg BW: miligram/kilogram body weight; ng/mL: nanogram/mililiter; mmol/L:milimol/liter;
[a]There was significant difference in standard diet group ($p<0.05$); [b]There was significant difference in hypercholesterolemic diet + xanthone of 0 mg/kg bw ($p<0.05$); [c]There was significant difference in hypercholesterolemic diet + xanthone of 35 mg/kg bw ($p<0.05$); [d]There was significant difference in hypercholesterolemic diet + xanthone of 70 mg/kg bw ($p<0.05$)

The mechanism of this decrease takes place through inhibition of NF-κB p65/p50 translocation to the nucleus characterized by a decrease in the distribution of NF-κB p65/p50. The decline in NF-κB p65/p50 distribution will cause a decrease in mRNA expression of TNF-α. The research by Chonmawang et al., [4] have shown that the GM extract at a dose of 50 mg/mL can inhibit the formation of TNF-α from peripheral blood mononuclear cells induced by Propionebacterium acne at 94.59%.

Macrophages play an important role in producing proinflammatory molecules of nitric oxide. Nitric oxide synthesized by the enzyme inducible nitric oxide synthase (iNOS) is an acute and chronic inflammatory mediators [10]. The study by Chen et al.[5] has proven mechanism of α-mangostin dan γ-mangostin as anti-inflammation in vitro. In RAW 264.7 cells exposed to LPS, α-mangostin dan γ-mangostin could inhibit the production of NO and PGE_2. The research by Tewtrakul et al., also found that extracts of mangosteen can lower levels of NO in RAW 264.7 cells exposed to LPS, which was better than α-mangostin dan γ-mangostin. In this study, reduced levels of NO is caused by the capability of xanthone to inhibit the expression of iNOS [12]. This is supported by Chen et al., saying that inhibition of NO production occurs through inhibition of iNOS expression rather than through inhibition of iNOS activity in RAW 264.7 cells exposed to LPS [5].

CONCLUSION

Xanthone has a good anti-inflammatory potential through the inhibition of NF-κB p65/p50 distribution, decreased levels of TNF-α and decreased levels of NO in aorta of atherosclerosis rats.

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Review Article

Recent advances in the multifunction of a natural occurring coumarin: osthole

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Abstract
Osthole, a natural occurring coumarin, an ingredient of a Traditional Chinese Medicine, possesses a range of pharmacological and biochemical functions, including hepatoprotection, neuroprotection, anti-cancer, anti-osteoporosis, and anti-inflammatory properties. Recently, osthole has attracted intense research interest. Of great interest is the possibility that osthole could be a promising lead compound for exploiting new drugs for therapy of several diseases. These include insight into strong cytotoxic activity on cancer cells by causing apoptotic bodies, DNA fragmentation, enhancing human poly(ADP-ribose) polymerase (PARP) degradation, inhibition of proliferation by suppressing fatty acid synthase (FASN), protection of liver by increasing metabolism of lipases, prevention of hepatitis by inhibiting the Fas-mediated apoptotic pathway, induction of differentiation by increasing bone morphogenetic protein (BMP)-2 production and activating SMAD1/5/8, p38, extracellular signal- regualted kinase (ERK) 1/2, and Wnt/β-catenin signaling, neuroprotectioin by a mechanism of inhibiting ionic currents, including Na+ and Ca2+ channels. It is therefore useful to build up some correlations with the data available in order to better explore the molecular and cellular mechanism of osthole actions in the treatment of diseases. This review will focus on recent advances in molecular and cellular mechanism of osthole actions.

INTRODUCTION

Osthole, 7-methoxy-8-(3-methyl-2-butenyl) coumarin (Figure 1), a naturally occurring compound, is known to have a variety of pharmacological and biochemical uses and considered to have potential therapeutic applications [1-2]. Osthole mainly exists in 14 species of Umbelliferae and 17 of Rutaceae. It has also been found to occur at a high percentage and be the effective compound in *Cnidium monnier* (L.) Cusson, which has been used in China for several hundred years as an herbal medicine to treat male sexual dysfunction by inhibiting phosphodiesterase type-5 (PDE-5), which degrades cyclic guanosine monophosphate (cGMP) that supports vasodilation to maintain rigidity by relaxing penile arteries and smooth muscle tissue [3].

Due to unique structural features such as methoxyl at C-7 position and 3-methyl-2-butenyl at C-8 position, which are elucidated to be essential groups for activities [4-5], osthole displays a wide spectrum of pharmacological and biological functions. Osthole has been shown to exhibit anti-cancer [6], anti-oxidant [1], anti-inflammatory and immunomodulatory [7-8] activities. It also has hepatoprotective [9] and neuroprotective [10] activities, suppresses postmenopausal osteoporosis [11], and improves lipid metabolism [12]. Thus, osthole has received considerable attentions recently because of its diverse uses, which make it a very promising natural lead compound for new drugs discovery.

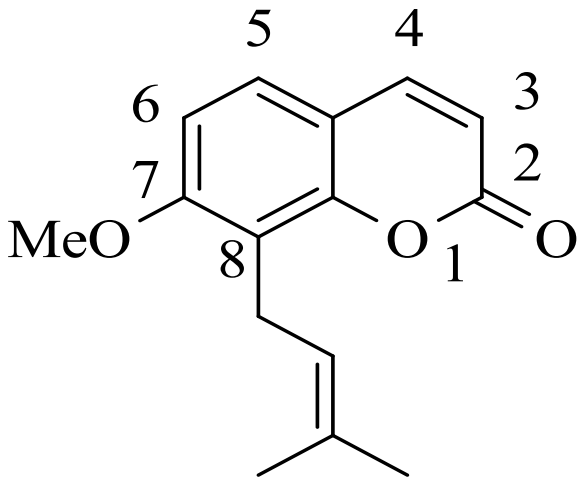

Figure 1 The chemical structure of osthole.

Anti-cancer

Osthole has been shown to have cancer-preventive and cancer-fighting properties. Fujioka (1999) had screened the antiproliferative constituents from Umbelliferae plants and found that the antiproliferative activities (ED_{50}, μg/ml) of osthole against human gastric adenocarcinoma (MK-1), human uterus carcinoma (HeLa), and murine melanoma (B16F10) cell lines *in vitro* were 82.7, 53.0, and 61.3, respectively [13]. Osthole (100 μM/L) showed a weak but significant antiproliferative activity against hormonoindependent PC3 and DU145 human prostate cell lines, 25.0% and 34.0% inhibition, respectively. However, osthole (100 μM/L) exhibited strong antiproliferative activity against mouse leukemia L1210 cell line with 65.4 ± 10.4% inhibition [14]. Antifibrotic activity of coumarins from *Cnidium monnier* fruits in hepatic stellate cells (HSC)-T6 had been screened. The results showed that osthole at a concentration of 100μM significantly inhibited proliferation of HSCs (cell viability was up to 31%) in a time- and concentration-dependent manner, which might be through the interference in cell proliferation not by cytotoxicity, and reduced collagen content to 24.5% [15].

Yang (2003) found that osthole exhibited strong cytotoxic activity on tumor cell lines, the IC_{50} values on human leukemia (HL-60), P-388, HeLa, and colorectal carcinoma (CoLo 205) were 14.9, 9.3, 31.7, and 29.9 μg/ml after 24 hour treatment. Osthole also caused apoptotic bodies, DNA fragmentation, and enhanced PARP degradation in HL-60 cells. The structure-activity relationship studies indicated that the prenyl group had an important role in the cytotoxic effects [16]. The *in vitro* studies with osthole showed that it inhibited the growth of HeLa, in a time- and concentration-dependent manner, with IC_{50} values of 77.96 and 64.94 μM after 24 and 48 h, respectively. Osthole had lower cytotoxic effects in primary cultured normal cervical fibroblasts. Moreover, osthole could induce apoptosis in HeLa by increasing DNA fragmentation and activation of PARP. The *in vivo* model showed that the survival days of the P-388 D1 tumor-bearing CDF1 mice were prolonged (ILS% = 37) after osthole (30mg/kg) was given once a day for 9 days. Based on these results, it was suggested that osthole could inhibit P-388 D1 *in vivo* and induce apoptosis in HeLa cells *in vitro* [17].

Recently, osthole has been reported to inhibit the migration and invasion of breast cancer cells (MCF-7 and MDA-MB 231 cell lines) by wound healing and transwell assays. Luciferase and zymography assays revealed that osthole effectively inhibited matrix metalloproteinase-2 (MMP-2) promoter and enzyme activity, which might be one of the causes that lead to the inhibition of migration and invasion by osthole [6]. While FASN has been shown to be expressed in many human solid tumors, FASN has also been identified in preneoplastic lesions. HER2 (alternatively known as *neu* or erbB2), which has also been identified in preneoplastic breast lesions, has been shown to upregulate FASN expression. Osthole was found to be effective in suppressing FASN expression, inhibit proliferation, and induce apoptosis in HER2-overexpressing breast cancer cells. Moreover, osthole inhibited the phosphorylation of Akt and mTOR. The use of Akt-overexpression revealed that the modulation of Akt and mTOR was required for osthole-induced FASN suppression [18].

Histone deacetylases (HDACs) are reported to be important in chromatin remolding and regulation of gene expression. Therefore, HDACs inhibitors are regarded as promising therapeutics for the treatment of cancer. The structures of all HDAC inhibitors reported so far can be divided into three functional subunits, each of which interacts with a discrete region of the HDAC active site. The three subunits include: a zinc-binding motif (such as hydroxamic acid), a hydrophobic cavity-binding linker with four to six carbon atoms, and a hydrophobic surface recognition cap that may confer class-specific recognition of the HDAC surface at the rim of the active-site cavity. Osthole is hydrophobic in nature with a Clog*P* value around 3.5 [19]. The hydrophobic nature of osthole is compatible with the chemical feature of the capping group in known HDAC inhibitors, which serves to form hydrophobic contact with the surface residues at the edge of the active site. Moreover, the branched aliphatic side chain in the chemical structure of osthole may be further exploited to provide specific interactions with different HDACs, which indicates that osthole could be a promising lead compound for anti-cancer drug [20].

Table 1. The biological activities of osthole on various cell lines.

Cell lines	Biological activity of osthole	Reference
MK-1	The value of ED_{50} was 82.7 μg/ml	[Fujioka *et al*, 13]
HeLa	The value of ED_{50} was 53.0 μg/ml	[Fujioka *et al*, 13]
	The value of IC_{50} was 31.7 μg/ml after 24 hr	[Yang *et al*, 16]
	The values of IC_{50} were 77.96 and 64.94 μg/ml after 24 and 48 hr	[Chou *et al*, 17]
B16F10	The value of ED_{50} was 61.3 μg/ml	[Fujioka *et al*, 13]
PC3	The inhibition rate of proliferative activity was 25.0%	[Riviere *et al*, 14]
DU145	The inhibition rate of proliferative activity was 34.0%	[Riviere *et al*, 14]
L1210	The inhibition rate of proliferative activity was 65.4 ± 10.4%	[Riviere *et al*, 14]
HSC-T6	cell viability was up to 31%	[Skin *et al*, 15]
HL-60	The value of IC_{50} was 24.4 μg/ml after 12 hr	[Yang *et al*, 16]
	The value of IC_{50} was 14.9 μg/ml after 24 hr	[Yang *et al*, 16]
PBMCs	The value of IC_{50} was 40.1 μg/ml after 12 hour treatment	[Yang *et al*, 16]
CoLo 205	The value of IC_{50} was 29.9 μg/ml after 24 hr	[Yang *et al*, 16]
P-388	The value of IC_{50} was 9.3 μg/ml after 24 hr	[Yang *et al*, 16]
MDA-MB 231	The inhibition rate of invasion activity was 97.6%	[Yang *et al*, 6]

Hepatoprotection

Alcohol is a major cause of fatty liver, which leads to inflammation, necrosis, fibrosis, and finally cirrhosis. There are currently no ideal pharmacological reagents that can prevent or reverse this disease. Osthole, however, appears to be a natural compound supported by several animal studies in regard to its liver-protective properties, including the prevention and treatment of fatty liver disease.

To evaluate the effects of osthole on fatty liver, a quail model with hyperlipidemic fatty liver and rat model with alcoholic fatty liver were set up by feeding high fat diet and alcohol, respectively. The results showed that after treatment with osthole the levels of serum total cholesterol (TC), triglyceride (TG), lower density lipoprotein-cholesterol (LDL-C), coefficient of hepatic weight, and the hepatic tissue contents of TC and TG were significantly decreased. The activity of superoxide dismutase (SOD) in liver was improved. In alcohol-induced fatty liver rats, the level of malondialdehyde (MDA) in liver was decreased. In high fat-induced fatty liver quails, glutathione peroxidase (GSH-PX) in liver was significantly improved. The histological evaluation of liver specimens demonstrated that the osthole dramatically decreased lipid accumulation [21]. A rat model with hyperlipidemic fatty liver liver was also successfully established. Similarly, the levels of rat serum TC, TG and LDL-C significantly were decreased as compared with those in the fatty liver model group after treatment with osthole [22]. In fatty milk-induced hyperlipidemic mice, the post-heparin plasma activities of lipoprotein lipase (LPL), hepatic lipase (HL), and total lipase (TL) were significantly increased after treatment with 10-20 mg/kg osthole for 3 weeks [22]. In addition, osthole could improve the insulin resistance induced by high-fat and high-sucrose emulsion in fatty liver rats, and its mechanism might be associated with increment of adiponectin release via activation of PPAR α/γ pathway [23].

To investigate the possible mechanism of osthole on the lipid-lowering effect, the BRL cells (rat hepatocyte line) were cultured and treated with osthole. The mRNA expressions of peroxisome proliferator-activated receptor (PPAR) α and its related target gene cholesterol 7a-hydroxylase (CYP7A) were increased in dose dependent manner, whereas the mRNA expressions of the other two target genes diacylglycerol acyltransferase (DGAT) and 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) reductase were decreased. However, these effects could be abrogated after pretreatment with specific inhibitor PPARα, MK886 [12].

During screening of Chinese herbal medicines for the activities against hepatitis B virus (HBV), osthole was found to inhibit the secretion of HBV surface antigens (HBsAg) *in vitro*. The secretion of HBsAg in culture medium of MS-G2 and HuH-7 cells transfected with HBV DNA decreased by 60% to 70% after osthole treatment, without any detectable cytotoxic effects. Osthole suppresses the secretion of HBV in cell culture probably because of its novel function in increasing the

glycosylation of HBsAg [24].

Counteracting the effects of hepatitis C virus (HCV) through the use of coumarins has proved challenging as only a small number, primarily osthole, are capable of inhibiting HCV replication and/or proliferation, counteracting the progression of hepatitis C into hepatocarcinoma [25].

Studies with hepatitis model mice revealed that osthole could prevent hepatitis by inhibiting the development of apoptosis, which indicated that osthole had some possibilities to become a hepatoprotective drug candidate for a wide variety of liver diseases. Fas (Apo-1/CD95) ligand, which is a type Ⅱ membrane protein, is a major inducer of apoptosis. Administration of osthole to mice at a dose of 10 mg/kg significantly inhibited the anti-Fas antibody-induced elevation of plasma alanine aminotransferase (ALT). Treatment of mice with anti-Fas antibody caused an elevation of caspase-3 activity at 3.5 and 6 hr. Pretreatment of mice with osthole (100 mg/kg) inhibited the elevation of caspase-3 activity caused by anti-Fas antibody. However, the addition of osthole (up to 10^{-4}M) to a liver cytosol fraction isolated from mice treatment with anti-Fas antibody did not inhibit caspase-3 activity *in vitro*. Thus, treatment of mice with osthole inhibited caspase-3 activity by an effect upstream of caspase-3 activation. Osthole attenuated the development of apoptosis and cell death; prevented anti-Fas antibody-induced hepatitis by inhibiting the Fas-mediated apoptotic pathway [9].

However, osthole exhibits low solubility in water and this property may lead to a decrease in the anti-hepatitis inhibitory effect upon oral administration. In a Con A-induced hepatitis mouse model, intraperitoneally administered osthole (100 mg/kg dose) resulted in 85% inhibition of Con A-induced elevation of ALT. However, oral administration of osthole at a 100 mg/kg dose caused only a 38% inhibition of Con A-induced elevation of plasma ALT [26]. Osthenol, an osthole derivative with substitution of a 7-methoxy group for 7-hydroxy of osthole, caused 32% inhibition of Con A-induced elevation of plasma ALT at the dose of 100 mg/kg (i.p.), whereas 7-HC caused only 9% inhibition [4]. These indicated that methoxy-group at position-7 and the 3-methyl-2-butenyl-group at position-8 are essential to lower plasma ALT in hepatitis.

Anti-osteoporosis

One of the most promising areas of potential benefits from the use of osthole clearly appears to be in the area of osteogenic (bone-building) activity. The compounds isolated from fruits of Cnidium monnieri (L.) Cuss (Umbelifera) were screened using the osteoblast-like UMR106 cells *in vitro*. Osthole, a major pharmacologically active constituent, significantly promoted the cells' activity [27]. To examine the effects of osthole on postmenopausal osteoporosis in ovariectomized (OVX) rat models, Li (2002) found that osthole might be just as effective as 17β-estradiol in suppressing bone loss due to ovariectomy but it perhaps did not work through the estrogen pathway [11]. In Traditional Chinese Medicine, the cause of weak bones or bone loss is generally regarded as a result of kidney deficiency. Osthole, the major effective compound of Fructus Cnidii which was a traditional herb that had been claimed to have kidney warming effects, was reported to be effective at inducing osteoblastic bone formation through the up-regulation of alkaline phosphatase (ALP) activity. Concentrations lower than 40 μg/mL, osthole seemed less effective, and cytotoxicity to osteoblasts was observed at concentrations higher that 320 μg/mL [28].

The survival of osteoblast cells is one of the determinants of the development of osteoporosis. Osthole exhibited a significant induction of differentiation in two human osteoblast-like cell lines, human osteosarcoma cell line (MG-63) and human fetal osteoblastic cell line (hFOB). Induction of differentiation by osthole was associated with increased BMP-2 production and the activations of SMAD1/5/8 and p38 and ERK1/2. Addition of purified BMP-2 protein did not increase the up-regulation of ALP activity and osteocalcin by osthole, whereas the BMP-2 antagonist noggin blocked both osthole and BMP-2-mediated ALP activity enhancement, indicating that BMP-2 production was required in osthole-mediated osteoblast maturation. However, pretreatment of osteoblast cells with noggin abrogated p38 activation, and partially decreased ERK1/2 activation, suggesting that BMP-2 signaling was required in p38 activation, and was partially involved in ERK1/2 activation in osthole-treated osteoblast cells. Cotreatment with either p38 inhibitor SB203580 [4-(4-fluorophenyl)-2-(4-methylsulfinyl-phenyl)-5-(4-pyridyl)-1H-imidazole] or p38 small interfering RNA (siRNA) expression inhibited osthole-mediated activation of ALP, but only slightly affected osteocalcin production. In contrast, the production of osteocalcin induced by osthole was inhibited by the mitogen activated protein kinase (MAPK) inhibitor PD98059 (2'-amino-3'-methoxyflavone) or by expression of an ERK2 siRNA. These data suggested that the BMP-2/p38 pathway linked to the early phase, whereas the ERK1/2 pathway was associated with the later phase in osthole-mediated differentiation of osteoblast cells [29].

Osteoporosis is defined as reduced bone mineral density with a high risk of fragile fracture. Current available treatment regimens include antiresorptive drugs such as estrogen receptor analogues and bisphosphates and anabolic agents such as parathyroid

hormone (PTH). However, neither option is completely satisfactory because of adverse effects. Local injection of osthole significantly increased new bone formation on the surface of mouse calvaria. Ovariectomy caused evident bone loss in rats, whereas osthole largely prevented such loss, improved bone microarchitecture, histomorphometric parameters, and biomechanical properties. *In vitro* studies demonstrated that osthole activated Wnt/β-catenin signaling, increased *Bmp2* expression, and stimulated osteoblast differentiation. Targeted deletion of the *β-catenin* and *Bmp2* genes abolished the stimulatory effect of osthole on osteoblast differentiation. Since deletion of the *Bmp2* gene did not affect osthole-induced *β-catenin* expression and the deletion of the *β-catenin* gene inhibited osthole-regulated *Bmp2* expression in osteoblasts. Thus, osthole might act through β-catenin-BMP signaling to promote osteoblast differentiation [30].

Neuroprotection

Osthole could inhibit voltage-gated Na^+ currents with state-dependence in mouse neuroblastoma N2A cells (IC_{50} = 12.3 mM and 31.5 mM at holding potentials of - 70 mV and - 100 mV, respectively). Current blockades were equally effective in both extracellular and intracellular applications of osthole. Osthole (18 mM) did not significantly affect the kinetics and voltage-dependence of Na^+ channel activation, but left-shifted the steady-state inactivation curve ($V_{1/2}$ = - 60.5 mV and - 78.7 mV in the absence and presence of osthole, respectively). Osthole also mildly but significantly retarded channel recovery from inactivation (recovery time constant = 19.9 ms and 35.6 ms in the absence and presence of osthole, respectively). In addition, osthole blocked Na^+ currents in a frequency-dependent fashion: blockades of 17 %, 34 % and 49 % when currents were triggered at 0.33 Hz, 1 Hz and 3.33 Hz, respectively. Taken together, osthole blocked voltage-gated Na^+ channels intracellularly with state- and frequency-dependence [31].

The effects of osthole on ionic currents in a mouse neuroblastoma and rat glima hybrid cell line, NG105-18, were investigated with the aid of the whole-cell voltage-clam technique. Osthole (0.3-100 μM) caused an inhibition of voltage-dependent L-type Ca^{2+} current ($I_{Ca,L}$) in a concentration-dependent manner. Osthole produced no change in the overall shape of the current-voltage relationship of $I_{Ca,L}$. The IC_{50} value of the osthole-induced inhibition of $I_{Ca,L}$ was 4μM. The presence of osthole (3μM) shifted the steady state inactivation curve of $I_{Ca,L}$ to a more negative potential by approximately -15mV. Osthole (3μM) also produced a prolongation in the recovery of $I_{Ca,L}$ inactivation. Although osthole might suppress phosophodiesterases to increase intracellular adenosine-3',5'-cyclic monophosphate (cyclic AMP) or guanosine-3',5'-cyclic monophosphate (cyclic GMP), sp-cAMPS did not affect $I_{Ca,L}$ and 8-bromo-cyclic GMP slightly suppressed it. Thus, osthole-mediated inhibition of $I_{Ca,L}$ was not associated with intracellular cyclic AMP or GMP. However, no effect of osthole on voltage-dependent K^+ outward current was observed [32]. Osthole significantly facilitated 4-aminopridine (4-AP)-evoked glutamate release in a concentration-dependent manner. The release facilitation by osthole results from an enhancement of vesicular exocytosis and not from an increase of Ca^{2+}-independent efflux via glutamate transporter. Examination of the effect of osthole on cytosolic [Ca^{2+}] revealed that the facilitation of glutamate release could be attributed to an increase in voltage-dependent Ca^{2+} influx. Consistent with this, ω-conotoxin MVIIC, a wide-spectrum blocker of the N- and P/Q-type Ca^{2+} channels, significantly suppressed the osthole-mediated facilitation of glutamate release, but intracellular Ca^{2+} release inhibitor dantrolene had no effect. Osthole did not alter the resting synaptosomal membrane potential or 4-AP-mediated depolarization; thus, the facilitation of 4-AP-evoked Ca^{2+} influx and glutamate release produced by osthole was not due to it decreasing synaptosomal excitability. In addition, osthole increased 4-AP-induced phosphorylation of PKC. These suggested that osthole effects a facilitation of glutamate release from nerve terminals by positively modulating N- and P/Q-type Ca^{2+} channel activation through a signaling cascade involving PKC [33]. Further investigation whether osthole possesses an action at the exocytotic machinery itself, downstream of a Ca^{2+} influx, Lin (2010) found that osthole-mediated facilitation of glutamate release involves modulation of downstream events controlling synaptic vesicle recruitment and exocytosis, possibly through an increase Ca^{2+}/calmodulin-dependent kinase II (CaMK II) activation and synapsin I phosphorylation, thereby increasing synaptic vesicle availability for exocytosis [34].

Considering the above-mentioned molecular mechanisms of action of osthole in preclinical studies, osthole was hypothesized to possess the antielectroshock action due to its effect on Ca^{2+} channels. The mouse maximal electroshock induced seizure model was built. Time course of anticonvulsant action of osthole revealed that it, in a dose-dependent manner, produced a clear-cut antielectroshock activity in mice and the experimentally- derived ED_{50} values for osthole ranged from 259 to 631 mg/kg [35]. Additionally, acute adverse-effect (neurotoxic) profile for osthole was determined in the chimney test. The results showed that the median toxic doses (TD_{50}) value ranged from 531 to 648 mg/kg. The protective index (as a ratio of TD_{50} and ED_{50} values) for osthole ranged from 0.83 to 2.44 [36]. Conclusively, osthole should

deserve more attentions from a preclinical point of view as a compound possessing potentially favorable activities in terms of suppression of seizures.

The 1-methyl-4-phenylpyridinium ion (MPP^+), an inhibitor of mitochondrial complex I, has been widely used as a neurotoxin because it causes a severe Parkinson's disease-like syndrome accompanied by increased levels of intracellular reactive oxygen species (ROS) and apoptotic death. To investigate the protective effects of osthole on MPP^+-induced cytotoxicity in cultured rat adrenal pheochromocytoma (PC12) cells, Liu (2010) reported that pretreatment with osthole on PC12 cells significantly reduced the loss of cell viability, the release of lactate dehydrogenase, the activity of caspase-3 and cytochrome *c*, the increase in Bax/Bcl-2 ratio and the generation of intracellular ROS induced by MPP^+. Moreover, the HPLC analysis of cell extracts confirmed that extracellular osthole did penetrate the cell membrane [10].

Recently, the neuroprotective effect of osthole against acute ischemic stroke was evaluated by neurological deficit score (NDS), dry-wet weight and 2,3,5-triphenyltetrazolium chloride (TTC) staining. The contents of MDA and glutathione (GSH), activity of myeloperoxidase (MPO) and the level of interleukin (IL)-1β and IL-8 after middle cerebral artery occlusion (MCAO) in rats were detected to investigate its anti-oxidative action and anti-inflammatory property. Pretreatment with osthole significantly increased in GSH, and decreased the volume of infarction, NDS, edema, MDA, MPO, IL-1β and IL-8, which might suggest that the anti-oxidative action and anti-inflammatory property of osthole contribute to a beneficial effect against stroke [37]. To investigate the effects of osthole on cognitive impairment and neuronal degeneration in hippocampus, models of occlusion of bilateral common carotid arteries (2VO) were established and found that osthole decreased the production of MDA, the ratio of bax/bcl-2, and the activation of caspase-3, and significantly increased the activities of glutathione peroxidase (GPx) and catalase. Additionally, oral administration of osthole for 3 weeks markedly attenuated cognitive deficits and neuronal damage [38]. The observed results suggested that osthole exhibits therapeutic potential for vascular dementia, which is most likely related, at least in part, to its anti-oxidation and anti-apoptotic actions.

Anti-inflammation

Osthole was tested in cyclooxygenase (COX) and 5-lipoxygenase (5-LOX) bioassays and turned out to be a moderate and selective a 5-lipoxygenase inhibitor (IC_{50} = 36.2 μM) [7]. In LPS-stimulated macrophages, osthole inhibited tumor necrosis factor-alpha (TNF-α), nitric oxide (NO) and COX-2 expression, without reducing the expression of IL-6. Furthermore, the phosphorylation of p38, c-Jun N-terminal kinase 1/2 (JNK1/2), protein kinase C (PKC)-α and PKC-ε induced by LPS was inhibited by osthole; however, the phosphorylation of ERK1/2 and PKC-δ was not reduced by osthole. Osthole also inhibited nuclear transcription factor kappa-B (NF-κB) activation and ROS release in LPS-stimulated macrophages [39].

Osthole showed significant inhibitory effects on ear swelling after dinitrofluorobenzene (DNFB) challenge, which exhibited the anti-allergic activity of osthole [40]. Asthma is an allergic inflammatory disease of the airways. Eotaxin, an eosinophil- specific C-C chemokine, is a potent chemo-attractant involved in the mobilization of eosinophils into the airway after allergic stimulation. The ability of osthole to regulate cytokine-induced eotaxin expression in the human bronchial epithelial cell line BEAS-2B was investigated. The results showed that IL-4 and TNF-α significantly induced eotaxin expression in BEAS-2B cells, expression of eotaxin was suppressed by osthole (0.1-10μM) in a dose-dependent manner; osthole did not suppress IL-4-induced p38, ERK or JNK expresseion, but suppress IL-4-induced STAT6 in a dose-dependent manner. This suggested that osthole suppressed IL-4-induced eotaxin in BEAS-2B cells via inhibition of STAT6 expression [41].

Multiple sclerosis (MS) is an inflammatory demyelinating disease of the central nervous system (CNS). Experimental autoimmune encephalomyelitis (EAE) is a well-established animal model for the study of the effects of osthole on the CNS demyelination. The results showed that osthole retarded the disease process when the therapy was initiated at subclinical periods, attenuated the clinical severity of EAE mice when the therapy was initiated at both subclinical and clinical periods, ameliorated inflammation and demyelination and improved the outcomes of magnetic resonance imaging. In addition, osthole blocked the reduction of nerve growth factor (NGF) and suppressed interferon gamma (IFN-γ) increase in EAE mice [42].

Table 2. The molecular targets of osthole

Biological functions	Molecular targets	Reference
Sexual performance enhancement	PDE-5	[Chen *et al*, 3]
Anti-cancer	MMP-2, MMP-9	[Yang *et al*, 6]
	FASN, Akt, mTOR	[Lin *et al*, 18]
	HDACs	[Huang *et al*, 20]
Hepatoprotection	TC, TG, LDL-C, SOD, MDA, GSH-PX	[Song *et al*, 21], [Zhang *et al*, 22]
	LPL, HL, TL, adiponectin, PPAR α/γ	[Zhang *et al*, 22]
	PPARα, CYP7A, DGAT, HMG-CoA reductase	[Sun *et al*, 12]
	HBsAg	[Huang *et al*, 24]
	ALT, caspase-3	[Okamoto *et al*, 4, 9, 26]
Anti-osteoporosis	ALP	[Zhang *et al*, 28]
	BMP-2, SMAD1/5/8, p38, ERK1/2	[Kuo *et al*, 29]
	Wnt/β-catenin, BMP-2	[Tang *et al*, 30]
Neuroprotection	voltage-gated Na^+ currents	[Leung *et al*, 31]
	voltage-dependent L-type Ca^{2+} current, sp-cAMPS phosophodiesterases, cyclic AMP, cyclic GMP	[Wu *et al*, 32]
	N- and P/Q-type Ca^{2+} channels, PKC	[Wang *et al*, 33]
	CaMK II, synapsin I	[Lin *et al*, 34]
	lactate dehydrogenase, caspase-3, cytochrome *c*, Bax/Bcl-2, ROS	[Liu *et al*, 10]
	MDA, GSH, MPO, IL-1β, IL-8	[Chao *et al*, 37]
	MDA, caspase-3, Bax/Bcl-2, GPx, catalase	[Ji *et al*, 38]
Anti-inflammation	COX, 5-LOX	[Resch *et al*, 7]
	TNF-α, NO, IL-6, p38, JNK1/2, PKC-α, PKC-ε, NF-κB, ROS	[Liao *et al*, 39]
	Eotaxin, STAT6	[Chiu *et al*, 41]
	NGF, IFN-γ	[Chen *et al*, 42]
Others	In glucose metabolism: PPARα, PPARγ, adipose fatty acid-binding protein 2, acyl-CoA synthetases, carnitine palmitoyltransferase-1A	[Liang *et al*, 44]
	MAPK	[Burns *et al*, 45]
	nitric oxide-cGMP	[Chan *et al*, 46]

Miscellaneous

PPARs are steroid transcription factors that affect the multiple target gene expressions involved in glucose metabolism and fatty acid storage [43]. Osthole significantly activated both PPARα and PPARγ in a dose-dependent manner based on the results of the transition transfection assay. The activation of PPARα and PPARγ by osthole also resulted in an increase in the expression of PPAR target genes such as PPAR itself, adipose fatty acid-binding protein 2, acyl-CoA synthetases, and carnitine palmitoyltransferase-1A [44]. Recent studies demonstrated that activated MAPK could phosphorylate and activate PPAR through agonist-independent manner [45]. osthole was able to increase the phosphorylation of MAPK and then activate MAPK. It was possible that osthole activated PPAR through agonist-independent and MAPK-dependent pathway. In addition, in the model of obese diabetic C57BKS.Cg-$m^{+/+}$Lepr^{db}/JNarl (*db*/*db*) mice, osthole was found to markedly reduce blood glucose level, which might be mediated through a PPAR-independent pathway [44].

Osthole also has been reported to possess anti-arrhythmic activity. Osthole induced relaxation in endothelium-intact rings with a lower threshold concentration (< 10 μM) than in endothelium-disrupted rings (> 10 μM). In the presence of L-NAME (a nitric oxide synthase inhibitor) or ODQ (a soluble guanylyl cyclase inhibitor), relaxation induced by osthole in endothelium-intact rings was similar to that in endothelium-disrupted rings. Osthole-induced relaxation was not inhibited by SQ22536 (an adenylyl

cyclase inhibitor). The endothelium-dependent relaxation of osthole was mediated through nitric oxide-cGMP signaling cascade [46].

CONCLUSION

The information provided above demonstrated that osthole had a diverse range of pharmacological and biochemical functions, including anti-cancer, hepatoprotection, anti-osteoporosis, neuroprotection, and anti-inflammatory properties. Due to its unique structural feature, osthole becomes a good candidate for the generation of lead compounds. Numerous studies confirmed osthole's potential role in animal models, yet further study is required to confirm its function in humans. The development of osthole-related drugs is very challenging due to the agent's poor absorption and low bioavailability; studies are still in their early stage. However, based on the compound's multifunctional actions, it appears that osthole will emerge as a promising therapeutic drug for the treatment and prevention of various chronic diseases.

Abbreviations

FASN = fatty acid synthase; BMP = bone morphogenetic protein

PBMCs = primary culture human peripheral blood mononuclear cells

ERK = extracellular signal- regualted kinase PDE-5 = phosphodiesterase type-5

cGMP = cyclic guanosine monophosphate HDACs = histone deacetylases

TC = total cholesterol TG = triglyceride

LDL-C = lower density lipoprotein-cholesterol SOD = superoxide dismutase

MDA = malondialdehyde GSH-PX = glutathione peroxidase

PPAR = peroxisome proliferator-activated receptor DGAT = diacylglycerol acyltransferase

HMG-CoA = 3-hydroxy-3-methylglutaryl-CoA HBsAg = hepatitis B surface antigen

ALT = plasma alanine aminotransferase OVX = ovariectomized

ALP = alkaline phosphatase PTH = parathyroid hormone

$I_{Ca,L}$ = voltage-dependent L-type Ca^{2+} current CaMK II = Ca^{2+}/calmodulin-dependent kinase II

MPO = myeloperoxidase MCAO = middle cerebral artery occlusion

GPx = glutathione peroxidase CNS = central nervous system

JNK1/2 = c-Jun N-terminal kinase 1/2 PKC = protein kinase C

EAE = Experimental autoimmune encephalomyelitis

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