Review Article

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Studies with *Myrtus communis* L.: Anticancer properties

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ABSTRACT

Myrtus communis (MC) L. is a well-known Mediterranean plant with important cultural significance in this region. In ancient times, MC was accepted as a symbol of immortality. Maybe due to this belief, it is used during cemetery visits in some regions. Although it is a well-known plant in cosmetics, and there is a lot of studies about its different medical properties, anticancer studies performed using its different extracts or oils are not so much, but increasing. We collected these anticancer property-related studies in this review.

KEY WORDS: Myrtucommulone, *Myrtus communis*, new drug development

Cancer is a group of diseases which are characterized by uncontrolled growth and spread of abnormal immortal cells, and its causes may be both external and internal factors. Cancer is a very important health problem, because its frequency is increasing every day, and there is no satisfying medical treatment method available today [1]. Although cancer is one of the most-studied diseases all around the world, it looks like that the main source of hope is natural, and especially plant derived, products. Because natural or natural-derived products have been the most significant source of drugs in modern medicine and dominant role of these natural products in cancer is obvious with about 74% of anticancer compounds being natural products, or natural product-derived products [2]. Probably, heavy screening and goal-oriented detailed scientific research would be the most distinctive steps in determining and developing potentially "druggable" targets in cancer research [2,3].

Myrtus communis L. (MC) is an evergreen shrub with a height of about 1-5 m, and probably the only myrtle variety that has an important cultural significance in Mediterranean region and Anatolia [4]. Besides its cultural importance, MC has a lot of medical usages in different indications such as diarrhea, peptic ulcers, hemorrhoids, inflammation, bleeding, headache, palpitation, leucorrhoea, urethritis, epistaxis, conjunctivitis, excessive perspiration, cough, pulmonary and skin diseases, diabetes mellitus, antiseptic, pain, heartburn, swelling, stiffness of the limbs, to remove mucus from the chest [4-7]. There are also some information about the usage of MC for anticancer purposes in traditional medicine [3,6,8]. Anticancer properties of MC were previously investigated in a number of studies, and in some of these studies myrtucommulone which is a unique, nonprenylated acylphloroglucinol, was found to be an active compound [9]. Myrtucommulone has antioxidant, antibacterial, anti-inflammatory, anti-diabetes, and anticancer properties, and it is found in the leaves of MC [5-7,9-20] [Table 1].

STUDIES ABOUT ANTICANCER PROPERTIES OF MC L

Alwan *et al*. showed that ethanolic extract of MC inhibited aryl hydrocarbon hydroxylase (AHH) activity and ^{3}H-benzo(a) pyrene (^{3}H-BP) binding to rat liver microsomal protein, effectively. In the same study, no inhibitory effect was shown with aqueous extracts [21].

In another study of the same author, different organic extracts of eight plants were tested against AHH activity and ^{3}H-BP binding to DNA *in vitro*. The obtained *in vitro* effects of plants are similar with the *in vivo* effects from the previous study, and MC showed significant inhibitory effect when n-butanol extract was used. The n-butanol extract was more effective than the extracts of chloroform and petroleum-ether, respectively. None of the aqueous extracts showed any inhibitory effects on both AHH activity and ^{3}H-BP binding to DNA [22].

In a screening study performed at UNLV Cancer Research Institute at Brigham Young University, essential oils of various plants were tested against different cancer cell lines at 2005. At 100 μg/ml concentration, MC essential oil showed 81.4% cell line inhibition at breast cancer cell line, while at 200 μg/ml concentration the inhibition percent was 67 and 95.2 for prostate and breast cancer cell lines, respectively. Probably, the most exciting and hopeful result of this study is the inhibition value of essential oil of MC on 3T3 fibroblast cell line is 3.7% and 6.5% for the dosages of 100 μg/ml and 200 μg/ml, respectively. We may talk about a selective anticancer effect

Table 1: Summary of studies about anticancer properties of *Myrtus communis* L.

Key findings	Used part(s)	Reference
Ethanolic extract of MC inhibited AHH activity and ^{3}H-BP binding to rat liver microsomal protein	Ethanolic, water extracts	[21]
n-butanol extract of MC inhibited AHH activity and ^{3}H-BP binding to DNA *in vitro*	n-butanol, chloroform and petroleum-ether extracts	[22]
At 200 μg/ml concentration the inhibition percent was 67, 95.2 and 6.5 for prostate and breast cancer cell lines and 3T3 fibroblast cell line respectively	Essential oil	[23]
Authors tested of MC essential oil increased survival time on Ehrlich tumour of injected CD1 mice	Essential oil	[24]
MC water extract provides almost complete cure in Erlich ascites tumour injected mice	Water extract	[25]
Methanol and hot water extracts of MC were tested for their anticancer activities against two cancer cell lines (5637 and MCF-7): IC_{50} values for anticancer activity test was calculated as >50 μg/mL	Methanol and hot water extracts	[26]
Myrtucommulone-A induced apoptosis in cancer cell lines, with marginal cytotoxicity for non-transformed cells, via the mitochondrial cytochrome c/ Apaf-1/caspase-9	Myrtucommulone-A	[5]
Aromatic phloroglucinol core is essential for the cytotoxic activity of myrtucommulone	Myrtucommulone A, J, K, L	[12]

AHH: Aryl hydrocarbon hydroxylase, ^{3}H-BP: ^{3}H-benzo(a)pyrene, MC: *Myrtus communis*

amlording to these results. This kind of screening studies are fast, inexpensive, and very useful for providing well-directed leads for further studies [23].

In another study, authors tested MC for its anticancer effects on Ehrlich tumor of injected CD1 mice. In this study, authors postulated that MC distilled oil has cancer preventive properties, and they determined maximum tolerance dose (MTD) for MC distilled oil first. At the CD1 race mice, MTD is found 2 ml/kg and the LD50 is 2.5 ml/kg. From the four group of animals into which Ehrlich tumor is injected, one group is used as a control group, the other three groups are given different doses of MC L. (Doses are 0.1, 0.2, 0.4 ml/kg for the 2, 3, 4, experiment groups, respectively). Survival time increased in the experiment groups significantly [24].

Furthermore, we performed a similar study with Erlich ascites tumor injected mice and found that MC water extract provides almost complete cure in these animals when injections for extract and ascites cells had been started to apply together. In our study, animals with Erlich ascites tumor died about 19 days after injection, but extract treated animals continue to live and we terminated the study at 29th day of the experiment and showed that there is also histopathologically sumlessful cure [25].

Methanol and hot water extracts of MC were tested for their anticancer activities against two cancer cell lines (5637 and MCF-7) by Yemeni researchers. Authors also tested plant extracts for their antimicrobial and antioxidant activities. Although antimicrobial activity with MIC values ≤125 μg/mL against Gram-positive bacteria and a significant antioxidant activity were determined for MC, IC_{50} values for anticancer activity test were calculated as >50 μg/mL [26].

Myrtucommulone, a nonprenylated acylphloroglucinol, is probably one of the most attracting molecules in MC. Besides its other properties, recently it was shown that myrtucommulone-A induced apoptosis in cancer cell lines, with marginal cytotoxicity for non-transformed cells, via the mitochondrial cytochrome c/Apaf-1/caspase-9 [5]. In the mentioned study, authors showed that cell death had been caused via apoptosis and they found it to be much less cytotoxic for non-transformed human peripheral blood mononuclear cells (PBMC) or foreskin fibroblasts (EC_{50} cell death = 20-50 μM), and myrtucommulone up to 30 μM hardly caused processing of poly(ADP-ribose)polymerase (PARP), caspase-3, -8, and -9 in human PBMC. They concluded that the myrtucommulone -induced apoptosis was mediated by the intrinsic rather than the extrinsic death pathway; hence, myrtucommulone caused loss of the mitochondrial membrane potential in MM6 cells and evoked release of cytochrome c from mitochondria. Furthermore, it was found that Jurkat cells deficient in caspase-9 were resistant to myrtucommulone-induced cell death and no processing of PARP or caspase-8 was evident. In cell lines deficient in either CD95 (Fas, APO-1) signaling, FADD or caspase-8, myrtucommulone was still able to potently induce cell death and PARP cleavage [5].

When different myrtucommulones were tested against different cancer cell lines, it was shown that aromatic phloroglucinol core is essential for the cytotoxic activity; myrtucommulone types without phloroglucinol core do not have cytotoxic effects against some cancer cell lines while myrtucommulones with phloroglucinol core have cytotoxic effects against the same cancer cell lines [12].

These results are so important and exciting, because all of these findings look like that sound of footsteps of the pioneer of an ideal and selective anti-cancer drug in the near future [27]. But we need to perform new studies in order to support and improve the results for developing long-awaited cancer drug.

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Original Research

Extracts of *Cordia gilletii* de wild (Boraginaceae) quench the quorum sensing of *Pseudomonas aeruginosa* PAO1

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ABSTRACT

Aim: The fight against infectious diseases and antimicrobial resistances needs the exploration of new active compounds with new proprieties like disrupting quorum sensing (QS) mechanisms, which is a cell-to-cell communication that regulates bacterial virulence factors. In this work, leaves and root barks extracts of a Congolese medicinal plant, *Cordia gilletii*, were investigated for their effect on the production of *Pseudomonas aeruginosa* major virulence factors regulated by QS. **Materials and Methods:** The effect of *C. gilletii* extracts on virulence factors of *P. aeruginosa* PAO1 was studied by the evaluation of the production of pyocyanine, elastase and biofilm; and by the measurement of the expression of QS-related genes. **Results:** The dichloromethane extract from root barks was found to quench the production of pyocyanin, a QS-dependent virulence factor in *P. aeruginosa* PAO1. Moreover, this extract specifically inhibits the expression of several QS-regulated genes (i.e. *lasB*, *rhlA*, *lasI, lasR, rhlI,* and *rhlR*) and reduces biofilm formation by PAO1. **Conclusion:** This study contributes to explain the efficacy of *C. gilletii* in the traditional treatment of infectious diseases caused by *P. aeruginosa*.

KEY WORDS: Antimicrobial, *Cordia gilletii*, quorum-sensing

INTRODUCTION

Pseudomonas aeruginosa is one of the major causes of nosocomial diseases; it can secrete a diversity of virulence factors and in parallel forms biofilms to ensure the infection success. The production of key virulence factors in *P. aeruginosa* and other important pathogenic bacteria is regulated by a cell-to-cell communication mechanism known as quorum sensing (QS). This mechanism enables bacteria to detect their population density through the production, release, and perception of small diffusible molecules called autoinducers and to coordinate gene expression accordingly [1]. In *P. aeruginosa*, two QS systems (*las* and *rhl*) drive the production (by the synthetases LasI and RhlI) and the perception (by the transcription factors LasR and RhlR) of the acyl-homoserine lactones (AHLs) *N*-(3-oxododecanoyl)-L-homoserinelactone (3-oxo-C12-HSL) and *N*-butanoyl-L-homoserine lactone (C4-HSL), respectively [2]. Once LasR interacts with 3-oxo-C12-HSL, it induces the *las* system (by increasing *lasI* expression) and triggers the production of LasB elastase, LasA protease, Apr alkaline protease, and exotoxin A [3]. RhlR interacts with C4-HSL, resulting in an enhancement

of the production of rhamnolipids, pyocyanin, LasB elastase, hydrogen cyanide, and cytotoxic lectins [3-5]. In addition, biofilm formation and maturation is also regulated by *las* system [6,7] and indirectly by *rhl* system under nutritional condition [8,9]. Indeed, some studies demonstrated the role of rhamnolipids in biofilm architecture and maintenance [10-12]. The *las* and the *rhl* systems are organized in a hierarchical manner where the *las* system regulates the *rhl* system at the transcriptional and posttranscriptional levels [2,13,14]. In addition, *P. aeruginosa* releases a third intercellular signal, 2-heptyl-hydroxy-4-quinolone (designated the *Pseudomonas* quinolone signal), which interacts with the AHL systems in an intricate way [15] and acts as a link between the *las* and *rhl* quorum-sensing systems [16].

Since fundamental virulence processes in many pathogenic bacteria are regulated by QS systems, an interesting strategy to overcome the emergence of antibiotic-resistant microorganisms is to interfere with this cell-to-cell communication mechanism in order to attenuate their virulence [17]. Thus, medicinal plants traditionally used to treat infectious diseases should be screened, not only for their antimicrobial properties, but also for their capacity to inhibit QS mechanisms in bacteria.

In this study, we investigated the QS inhibitory (QSI) effects of extracts from a Congolese medicinal plant, *Cordia gilletii* De Wild. The root barks extracts from this plant species are used for the treatment of malaria and diarrhea (decoction), for wounds and skin diseases (topical application), whereas leaves decoction is used against malaria [18].

MATERIALS AND METHODS

Plant Material and Extracts Preparation

Root barks and leaves of *C. gilletii* were collected in Kisantu area the (Democratic Republic of Congo) in January 2005, and voucher specimen has been deposited under the number BR-SP.627986 at the National Botanical Garden of Meise, Belgium. Powders of the two-plant parts were exhaustively and successively extracted with solvents of increasing polarity (*n*-hexane, dicholoromethane, ethyl acetate and methanol). The evaporation of solvents in Buchi® rotavapor yielded crude extracts, which were dissolved in dimethyl sulfoxide (DMSO) at a final concentration of 10 mg/ml.

Bacterial Strains and Oulture Conditions

P. aeruginosa PAO1 wild-type and reporter strains were grown in liquid LB cultures (5 ml) supplemented with 50 mM 3-(*N*-morpholino) propanesulfonic acid (MOPS; pH 7.0) at 37°C supplemented with carbenicillin (300 μg/ml) when appropriate as described previously [19,20]. For the detection of anti-QS activity, we used reporter strains including six PAO1-derived strains harboring QS-related promoter-*lacZ* fusions (*lasB-lacZ*; *rhlA-lacZ*; *rhlI-lacZ*, *rhlR-lacZ*; *lasI-lacZ* and *lasR-lacZ)*, and PAO1-derived strains harboring QS-independent *ace*A gene *(ace*A-*lacZ)*, described previously [19,20].

Quantitative Analysis of Pyocyanin and Elastase Production in *P. aeruginosa* PAO1

Inhibition of pyocyanin and elastase production in *P. aeruginosa* PAO1 wild type was assessed according to previously described procedures [21,22]. Briefly, *P. aeruginosa* PAO1 were grown overnight polystyrene tube containing 5 ml of LB-MOPS medium (37°C and agitation at 175 r.p.m). The cells were washed twice in fresh LB-MOPS medium, and the pellets were suspended in LB-MOPS medium. Then, 50 μl portions of the cell suspension were added to 940 ml of LB-MOPS, spectrometrically evaluated at 600 nm (in order to obtain a A_{600} ranging between 0.020 and 0.025, corresponding to ~10^7 CFU/ml) using a SpectraMax M2 device (Molecular Devices, California, USA) and supplemented with 10 μl of DMSO (1% [vol/vol], final concentration) or 10 μl of plant extract dissolved in DMSO (100 μg/ml, final concentration). After 18 h of growth, samples were taken to assess the growth (A_{600}). After centrifugation (16,000 ×*g*, 5 min), 900 μl of supernatant were mixed with 500 μl of chloroform in eppendorf tube. The organic phase was transferred in a new eppendorf tube and pyocyanin was extracted with 300 μl of HCl 0.2 N and quantified spectrometrically at 380 nm [19]. LasB elastase production was assessed through the measurement of elastase activity using elastin-Congo Red ($A_{495\,nm}$). The statistical significance of each test ($n = 6$) was evaluated by conducting Student's *t*-tests using the GraphPad Prism software (GraphPad software Inc., CA, USA), and a $P \leq 0.01$ was considered significant.

Gene Expression and Beta-galactosidase Measurements

PAO1 reporter strains were prepared as described for pyocyanin quantification (see previous section). PAO1 strains (50 μl) were grown in 940 μl of LB medium at 37°C under agitation (175 r.p.m), supplemented with 10 μl of plant extract or naringenin (4 mM, final concentration) or DMSO (1% [vol/vol], final concentration) and incubated for 18 h. After incubation, the cell growth was assessed as previously and the absorbance of the medium after centrifugation of the bacteria (16,000 ×*g*, 5 min) was used as a blank. The sample used for cell growth assessment was used to perform the β-galactosidase assay with *o*-nitrophenyl-β-D-galactopyranoside as previously described [11]. Promoterless *lacZ* fusion strains were used as controls. The A_{600} values were measured to account for the differences in cell density [19]. All experiments were performed in six replicates.

Biofilm Quantification

Quantification of biofilm formation by *P. aeruginosa* PAO1 was assessed according to previously described procedures [23]. PAO1 cells were incubated statically for 24 h at 37°C in 24-well polystyrene plates containing biofilm broth medium (Na_2HPO_4 1.25 g/L, $FeSO_4.7H_2O$ 0.0005 g/L, glucose 0.05 g/L, $(NH_4)_2SO_4$ 0.1 g/L, $MgSO_4.{}_2H_2O$ 0.2 g/L, KH_2PO_4 0.5 g/L) supplemented with plant extract (100 μg/ml) or DMSO 1% or naringenin (4 mM). After 24 h of incubation, biofilm biomass was quantified *via* crystal violet staining. All experiments were performed in six replicates.

RESULTS

C. gillettii Root Barks and Leaves Extracts Reduce Pyocyanin and Elastase Production in *P. aeruginosa* PAO1

C. gilletii root barks and leaves extracts were investigated for their effect on pyocyanin production. As shown in Figure 1a, all tested extracts decreased drastically pyocyanin production with no significant effect on *P. aeruginosa* PAO1 growth when compared to the DMSO control. Besides, all extracts (except root barks methanol and leaves dichloromethane extracts) decrease significantly elastase production in PAO1 although less spectacular compared to pyocyanin reduction [Figure 1b]. In addition, no elastase-like activities (which could interfere with the tests) were observed when the extracts were used in bacteria-free control tests (data not shown).

Root Barks Dichloromethane (RBDCM) Extract and Leaves Methanol Extract Reduce *lasB* and *rhlA* Gene Expression in *P. aeruginosa* PAO1

In the case of the decrease of pyocyanin and elastase production was due to the interference of *C. gilletii* extracts with QS mechanisms, we assessed the impact of *C. gilletii* extracts on QS-regulated genes *lasB* and *rhlA* genes (coding for lasB elastase and rhamnolipid, respectively) expression. Therefore, the effect of *C. gilletii* extracts on *lasB* and *rhlA* genes expression was monitored by using two PAO1 reporter strains harboring QS-related (*lasB* and *rhlA*) promoter-*lacZ* fusions. PAO1 reporter strain harboring QS-independent *aceA* gene (coding for isocitrate lyase) was used to verify that the drop in β-galactosidase activity was indeed associated with a reduction in QS-related gene expression rather to a general effect on transcription/translation mechanisms. Naringenin, a flavanone, which is known to affect QS signaling in *P. aeruginosa* PAO1 without affecting bacterial growth [20], was used as a positive control. As shown in Figure 2, the results highlight that RBDCM extract and leaves methanol extract, at final concentration of 100 μg/ml reduce QS-regulated *lasB* and *rhlA* genes expression without affecting PAO1 cells growth. Indeed, colony-forming unit of *P. aeruginosa* PAO1 wild-type and reporter strains grown in the presence of extracts for 18 h were similar to those of DMSO-treated cells (data not shown). More interesting, RBDCM extract does not affect the expression of the control gene *aceA* [Figure 2c], contrarily to the leaves methanol extract. However, effects of leaves methanol extract on QS-independent *aceA* gene and QS-regulated (*lasB* and *rhlA*) genes may be the results of two or more different active compounds. Indeed, some compounds could do affect specifically the expression of QS-related genes and others the expression of QS-independent *aceA* gene and/or the transcription machinery without affecting PAO1 cells growth.

RBDCM Extract Affects the Expression of QS Regulator Genes in *P. aeruginosa*

Since QS-regulated (*lasB* and *rhlA*) genes expression is impaired by RBDCM extract of *C. gillettii*, we were interested in its effect on QS systems (*lasRI* and *rhlRI*) in *P. aeruginosa* PAO1. Therefore, the effect of root barks extract was further characterized by evaluating the expression of the AHL synthetase genes *lasI* and *rhlI* and the QS regulator genes *lasR* and *rhlR*. The results highlight that the RBDCM affects both QS systems (*lasRI* and *rhlRI*) [Figure 3]. Indeed, RBDCM inhibits significantly the expression of AHL synthetase genes *lasI* (24% ± 5% of inhibition) and *rhlI* (52% ± 5% of inhibition), and of the QS regulator genes *lasR* (25% ± 3% of inhibition) and *rhlR* (23% ± 4% of inhibition).

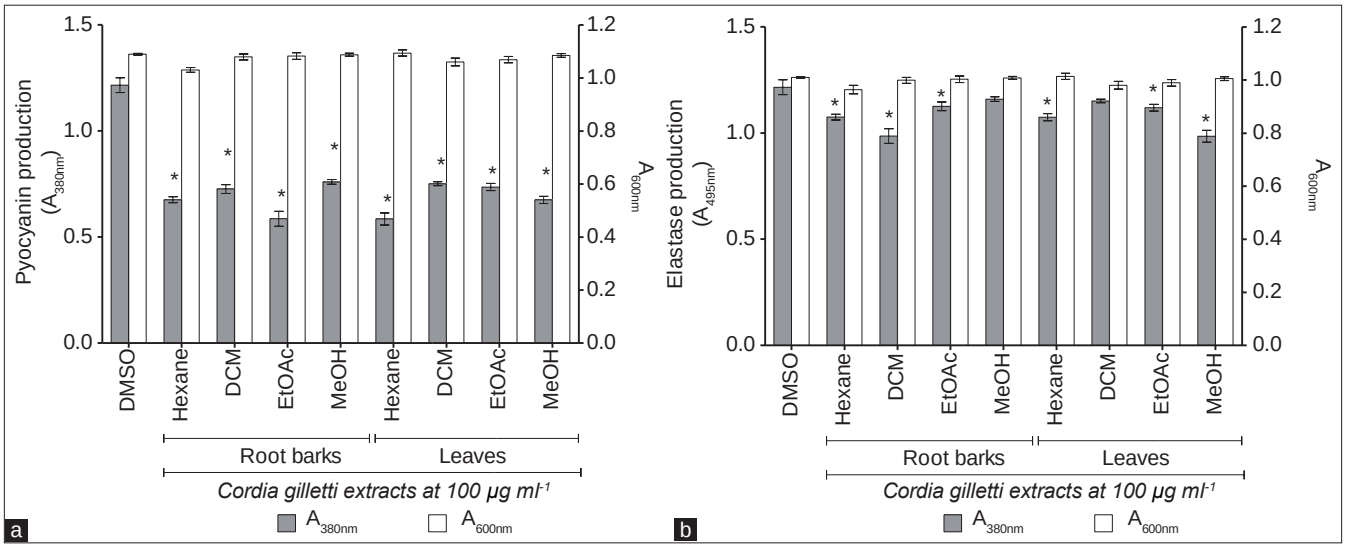

Figure 1: Effect of *Cordia gilletii* root barks and leaves extracts on pyocyanin and elastase production in *Pseudomonas aeruginosa* PAO1. (a) Effect of *C. gilletii* root barks and leaves extracts on pyocyanin production in P. aeruginosa PAO1. (b) Effect of *C. gilletii* root barks and leaves extracts on elastase production in *P. aeruginosa* PAO1 (Hexane: n-hexane, DCM: Dichloromethane, EtOAc: Ethyl acetate, MeOH: Methanol). Dimethyl sulfoxide (DMSO): control. *Significance at $P < 0.001$. All experiments were performed in six replicates

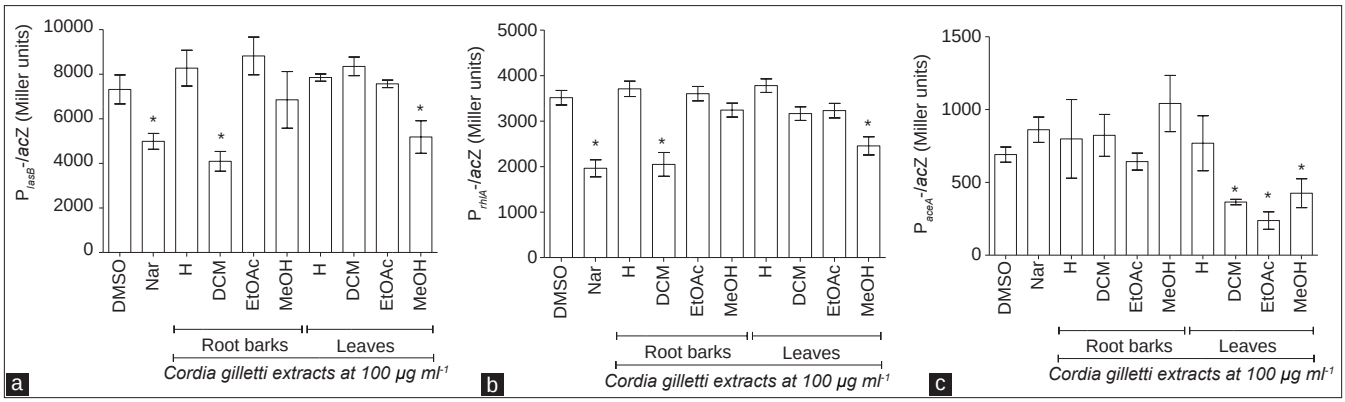

Figure 2: Effect of *Cordia gilletii* extracts on quorum sensing (QS)-regulated genes (a: lasB; b: rhlA) expressions, and QS-independent aceA gene (c) in *Pseudomonas aeruginosa* PAO1. Gene expression was measured as the β-galactosidase activity of the lacZ gene fusions expressed in Miller units. Root barks and leaves extracts were tested at 100 µg/ml (Hexane: n-hexane, DCM: Dichloromethane, EtOAc: Ethyl acetate, MeOH: Methanol). Dimethyl sulfoxide (DMSO) (1% [vol/vol], final concentration) was used as solvent control and naringenin (Nar: 4 mM, final concentration) as QS inhibitory control. *Significance at $P < 0.05$. All experiments were performed in six replicates

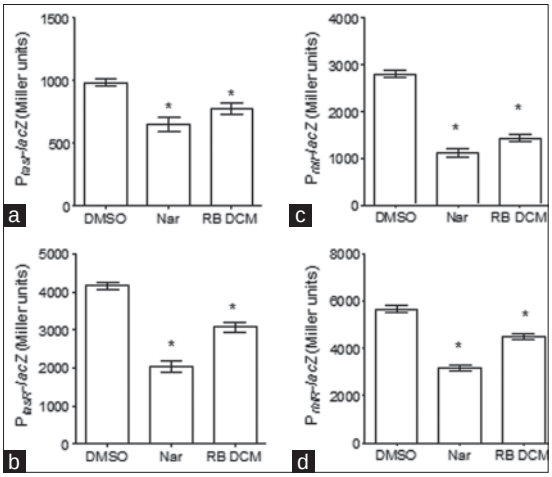

Figure 3: Effect of root barks dichloromethane (RBDCM) extract in *Pseudomonas aeruginosa* quorum sensing (QS) regulator genes (a: *lasI*; b: *lasR*; c: *rhlI*; d: *rhlR*). RBDCM extract was tested at 100 µg/ml. Dimethyl sulfoxide (1% [vol/vol], final concentration) was used as solvent control and naringenin (Nar: 4 mM, final concentration) as QS inhibitory control. *Significance at $P < 0.05$. All experiments were performed in six replicates

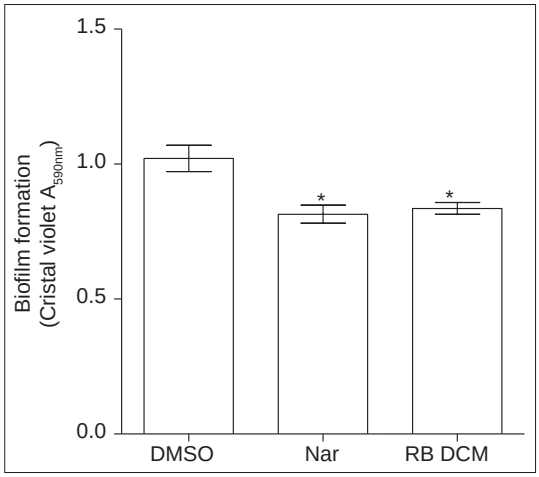

Figure 4: Effect of root barks dichloromethane (RBDCM) extract in biofilm formation by *Pseudomonas aeruginosa* PAO1. After 24 h of static incubation, biofilm biomass was quantified by using crystal violet staining. RBDCM extract was tested at 100 µg/ml. Dimethyl sulfoxide (1% [vol/vol], final concentration) was used as solvent control and naringenin (Nar: 4 mM, final concentration) as quorum sensing inhibitory control. *Significance at $P < 0.05$. All experiments were performed in six replicates

RBDCM Extract Inhibit in Biofilm Formation by *P. aeruginosa* PAO1

Since biofilm formation is partially controlled by QS mechanisms [6,7], the effect of RBDCM extract on *P. aeruginosa* PAO1 biofilm formation was assessed after 24 h. Noticeably, there were a significant decrease (21% ± 5% of inhibition) in biofilm formation when strain PAO1 was grown in the presence of RBDCM extract (100 μg/ml) compared with that of the negative control (DMSO) [Figure 4].

DISCUSSION

Few studies have already been reported the anti-QS effects of plants traditionally used in the treatment of infectious diseases [23-26]. *C. gilletii* belongs to the family of Boraginaceae and it is used in Congolese traditional medicine. Previously we have shown direct and indirect antimicrobial activities against pathogenic microorganisms [27]. To the best of our knowledge, none of the members of this plant family has been screened so far for inhibitory effects on QS, except for an interfering effect in the *Vibrio fischerii* bioluminescence [28].

In the present investigation, we have shown that *P. aeruginosa* PAO1 growth is not affected by any of the tested *C. gilletii* root barks or leaves extracts (at 100 μg/ml final concentration). Besides, by using a reporter strain coupled to *aceA* gene to evaluate the effect of the *C. gilletii* extracts on gene transcription machinery, we have discarded all leaves extracts as well as hexane, ethyl-acetate and methanol root barks

extracts. Accordingly, only dichloromethane root barks extract was found to specifically reduce in the same time QS-dependent virulence factors (pyocyanin and elastase) production, QS-regulated genes (*lasB* and *rhlA*) expression as well as QS-regulatory genes, suggesting the occurrence of a tissue specific compound(s) in *C. gilletii* that affect QS machinery in *P. aeruginosa* PAO1. However, we cannot exclude that other tested extract contain QSI compounds, particularly for leaves methanol extract which inhibits transcription of the QS-regulated *lasB* and *rhlA* genes and QS-independent *aceA* gene. Moreover, inhibition kinetic analysis should be led in order to detect time points in which the highest inhibition level of the QS phenotype and the QS genes could be recorded.

Biofilm formation in *P. aeruginosa* represents a protective mode of growth which may enhance bacterial survival under conditions of environmental stress [29]. Interestingly, RBDCM extract was found to specifically reduce biofilm formation by *P. aeruginosa* PAO1, which could be attributed to its QSI propriety. However, Shrout *et al*. [9] demonstrated that the QS dependence of biofilm formation is nutritionally conditional (i.e., QS systems are needed for biofilm formation in growth media with succinate as the sole carbon source but not glucose). Accordingly, as we used glucose as the sole carbon source, we cannot amputate biofilm reduction in the presence of RBDCM extract to the sole QS systems disruption.

Since root barks of *C. gilletii* are known to contain phenolic compounds [27], this class of molecules could represent one of the putative active compounds as some of them, catechin [19], naringenin [20] and perbergin [30] have already demonstrated anti-QS effect. However, QSI compounds from *C. gilletii* can be a new class of chemical structure compared with those flavonoids reported elsewhere and may show a different mechanism of inhibition. Besides, at this stage we do not have sufficient data to speculate the quorum inhibitory mechanism and the transcriptional and/or post-transcriptional level of interference of root barks of *C. gilletii*.

CONCLUSION

This study highlights anti-virulence propriety of *C. gilletii*, which could contribute to explain its efficacy in the traditional treatment of infectious diseases caused by *P. aeruginosa*. Further investigations are needed in order to identify the chemical nature of compound(s) responsible for these observed effects. Isolated compounds will have a greater advantage for human use and search for such compounds may contribute to the prevention of bacterial diseases without the concern of antibiotic resistance. Finally, we must point out that *C. gilletii*, although belonging to the family of Boraginaceae, one of the most important botanical families of plants producing pyrrolizidine alkaloids (PAs), does not harbor these alkaloids in investigated root barks and leaves samples (detection limit, 2 μg of PAs per gram of plant material) [31], excluding thus toxicological risk due to PAs and ensuring a probable safe use of this plant.

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Original Research

Some pharmacological effects of cinnamon and ginger herbs in obese diabetic rats

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ABSTRACT

Aims: The present study was designed to assess some pharmacological effects of cinnamon (CAE) and ginger (GAE) aqueous extracts in obese diabetic rats, and to elucidate the potential mechanisms. **Materials and Methods:** Forty-two Sprague-Dawley rats were randomized into 6 equal groups. Group 1 was a negative control and the other groups were rendered obese by feeding rats on high-fat diet for 4 weeks. The obese rats were subcutaneously injected with alloxan for 5 days to induce diabetes. Group 2 was a positive control, and Groups 3, 4, 5 and 6 were orally given CAE in doses 200 and 400 mg/kg and GAE in the same doses, respectively for 6 weeks. Blood samples were collected for serum biochemical analyses. Kidneys were dissected out to assay activity of tissue antioxidant enzymes: Superoxide dismutase, glutathione peroxidase and catalase. **Results:** CAE and GAE significantly reduced body weight and body fat mass; normalized serum levels of liver enzymes; improved lipid profile; decreased blood glucose and leptin and increased insulin serum levels in obese diabetic rats. Both extracts also increased activity of kidney antioxidant enzymes. **Conclusion:** CAE and GAE exhibit anti-obesity, hepatoprotective, hypolipidemic, antidiabetic and anti-oxidant effects in obese diabetic rats. These results confirm the previous reports on both extracts. The potential mechanisms underlying these effects are fully discussed and clarified. Our results affirm the traditional use of cinnamon and ginger for treating patients suffering from obesity and diabetes. The obese diabetic rat model used in this study is a novel animal model used in pharmacology researches.

KEY WORDS: Anti-obesity, anti-diabetic, anti-oxidant, biochemical analyses, cinnamon, ginger, hepatoprotective, hypolipidemic

INTRODUCTION

Obesity and diabetes are among the most challenging global health problems. Obesity is an excessive fat accumulation in the body that results from an imbalance between energy intake and energy expenditure. It is associated with genetic, metabolic, and behavioral components, and the rapid development of obesity might reflect other risk factors such as dietary fat intake, fat storage and metabolism, and lifestyle [1]. Obesity increases the risk for many diseases including diabetes mellitus, and there is a positive association between obesity and insulin resistance and infiltration of adipose tissues by inflammatory cells [2,3]. Insulin resistance that commonly accompanies obesity is a major risk factor for the incidence of diabetes mellitus [4].

Diabetes mellitus is a metabolic disease characterized by hyperglycemia due to insulin deficiency, insulin resistance, or both. The increased extracellular and intracellular glucose concentration results in oxidative stress that plays a key role in the onset and development of serious diabetes complications, notably diabetic nephropathy [4,5].

Cinnamon (*Cinnamomum zeylanicum* L.) is one of the most important spices used to flavor most foods in both Arabian and European countries. In animal studies, the aqueous extract of cinnamon potentiated insulin-regulated glucose utilization via enhancing insulin signaling pathway [6,7] and prevented insulin resistance induced by a high-fructose diet [8]. Cinnamon is used in folk medicine for its hepatoprotective [9], anti-oxidant [10], anti-obesity [11], antihyperlipidemic [12] and antidiabetic [8,13] activities.

Ginger (*Zingiber officinale*) is widely used as a culinary spice and has a long history for its health benefits. Ginger is used medicinally for its hepatoprotective and anti-oxidant [14], antidiabetic and antihyperlipidemic [15,16] and anti-obesity [17] effects.

The present study aimed to assess some pharmacological effects of cinnamon (CAE) and ginger aqueous extracts (GAE) in obese diabetic rat model and to elucidate the potential mechanisms.

MATERIALS AND METHODS

Plant Materials

Dried cinnamon (*C. zeylanicum* L., Family Lauraceae) barks and ginger rhizomes (*Z. officinale*, Family Zingiberaceae) were purchased from the local market of Agricultural Herbs, Spices and Medicinal plants, Cairo, Egypt. The dried plant materials were grinded into a fine powder and kept till the preparation of aqueous extracts.

Alloxan and Biochemical Kits

Alloxan was purchased from El-Gomhoryia Company for Chemicals; Cairo, Egypt as a white powder packed in brown bottles each containing 25 g alloxan monohydrate. Glucose enzymatic kits for estimating blood glucose (BG) and radioimmunoassay kits for leptin and insulin hormones were procured from Gamma Trade Company, Egypt. The other biochemical kits were obtained from Biodiagnostics Company, Dokki, Egypt.

Rats

Forty-two adult male Sprague-Dawley rats weighing 200-210 g body weight (B.wt) and 10-12 weeks old were used in this study. Animals were obtained from Laboratory Animal Colony, Agricultural Research Center, Giza, Egypt. Rats were housed in a well-ventilated animal room under controlled hygienic conditions of 24°C temperature, 50% relative humidity and 12 h light/12 h dark cycles. Basal diet and water were provided ad libitum. The experiment on rats was carried out in accordance with the recommendations of the National regulations on animal welfare and Institutional Animal Ethical Committee (IAEC).

Preparation of Basal Diet

The dietary supply of protein, fat, carbohydrates, vitamins and minerals were in accordance to the recommended dietary allowances for rats [18]. Basal diet was consisted of 20% protein, 10% sucrose, 5% corn oil, 2% choline chloride, 1% vitamin mixture, 3.5% salt mixture and 5% fibers. The remainder was corn starch up to 100%.

Preparation of Aqueous Extracts

A total of 200 g of each powder of cinnamon barks and ginger rhizomes were dissolved in 1 L of distilled water and boiled for 10 min, cooled and filtered using double layers of gauze to obtain 20% aqueous extracts [19].

Induction of Obesity and Diabetes

Obesity and acute hyperlipidemia were induced by feeding rats on high-fat diet (HFD) which supplies 45% calories from hog fat (lard) for 4 weeks. A 3-4 weeks HFD feeding is sufficient to induce obesity, and this model of obese rats is closely resembled the reality of obesity in humans [20]. The obese rats were then rendered diabetic by subcutaneous injection of alloxan (120 mg/kg) for 5 days to induce acute diabetes [21].

Design of Experiment

Forty-two mature male Sprague-Dawley rats were randomized into 6 groups of 7 rats each. Group 1 was negative (normal) control, and the other 5 groups were fed on HFD for 4 weeks to induce obesity. The obese rats were then rendered diabetic by subcutaneous injection of alloxan (120 mg/kg) for 5 days. Group 2 was kept obese diabetic (positive control) and groups 3,4,5 and 6 were orally given CAE in doses 200 and 400 mg/kg and GAE in the same doses respectively for 6 weeks. At the end of the experiment, B.wts of rats were recorded, and rats were then euthanized by prolonged exposure to ether anesthetic. The abdomen was opened, and body fats, including mesenteric, visceral, epididymal and retroperitoneal fats were carefully dissected out and total fat mass was weighed. The adiposity index (Ad. I) was calculated by dividing total body fat mass by B.wt and multiplied by 100 (Ad. I = fat weight [F.wt]/B.wt × 100) as described by Pichon *et al*. [22]. Blood samples were collected for serum biochemical analyses and kidneys were dissected out to assay the activity of tissue antioxidant enzymes.

Biochemical Analyses

Serum aspartate aminotransferase (AST) and alanine aminotransferase (ALT) [23], alkaline phosphatase (ALP) [24], total cholesterol (TC) [25], triglycerides (TG) [26] and high-density lipoprotein cholesterol (HDL) [25] were chemically determined using specific diagnostic kits and measured using a spectrophotometer. Low-density lipoprotein (LDL) cholesterol was calculated according to Friedewald [26]. BG was determined using glucose enzymatic kits according to Siest *et al.*, [27]. Serum insulin was estimated using specific antibody radioimmunoassay kit [28] and leptin hormone was determined using enzyme-linked immunosorbent assay [29].

Preparation of Kidney Homogenate and Enzymes Assay

One gram of kidney tissue was washed with ice-cooled 0.9% NaCl solution and homogenized in 100 ml of ice-cooled 1.5% solution of potassium chloride and 50 mM potassium phosphate buffer solutions (pH 7.4) to yield 10% homogenate (w/v). Kidney homogenates were centrifuged at 4000 rpm for 10 min. at 4°C and the supernatants were used to assay the activity of antioxidant enzymes GPX, SOD and catalase (CAT) according to Paglia and Valentine [30], Spitz and Oberley [31] and Sinha [32], respectively.

Statistical Analysis

Data were presented as means ± standard error. Differences between control and treated groups were tested for significance using one-way analysis of variance followed by Duncan's

multiple range test [33]. Statistical analysis was performed using computerized software program Statistical Package for Social Sciences version 15 (Chicago, IL 60606-6412,USA).

RESULTS

Feeding of rats on HFD for 4 weeks significantly ($P < 0.05$) increased B.wt, body fat mass weight (F.wt) and Ad. I when compared to negative control rats fed on the basal diet. Oral administration CAE and GAE in doses 200 and 400 mg/kg given to obese diabetic rats for 6 weeks induced significant decreases in B.wt, F.wt and Ad. I when compared to the positive control group, in a dose-dependent manner as shown in Table 1.

Rats fed on HFD for 4 weeks had significant ($P < 0.05$) increases in serum levels of liver enzymes AST, ALT and ALP when compared with negative control rats. CAE and GAE in doses 200 and 400 mg/kg when orally given to obese diabetic rats significantly ($P < 0.05$) lowered the high serum levels of AST, ALT and ALP enzymes when compared to the positive control group, in a dose-dependent fashion, as illustrated in Figure 1.

As demonstrated in Figure 2, feeding of rats on HFD for 4 weeks produced significant ($P < 0.05$) increases in serum levels of TC and TG when compared to rats fed on the basal diet. CAE and GAE in doses 200 and 400 mg kg when given orally to obese diabetic rats significantly lowered the high levels of serum TC and TG in a dose-dependent manner, when compared with the positive control group.

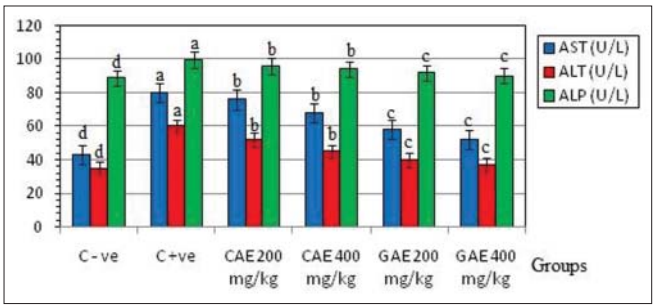

Figure 1: Effect of cinnamon aqueous extract and ginger aqueous extract on serum levels of aspartate aminotransferase, alanine aminotransferase and alkaline phosphatase liver enzymes in obse diabetic rats

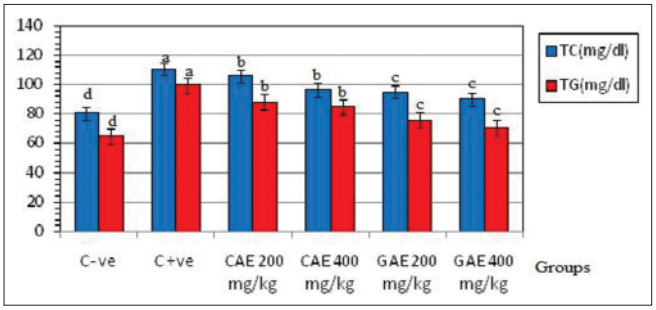

Figure 2: Effect of cinnamon aqueous extract and ginger aqueous extract on serum total cholesterol and triglycerides on obese diabetic rats

Feeding of rats on HFD for 4 weeks significantly ($P < 0.05$) decreased serum HDL and increased both LDL and At. I when compared to negative control rats. Oral administration of CAE and GAE in doses of 200 and 400 mg/kg to obese diabetic rats induced a significant ($P < 0.05$) increase in serum HDL and decreased in both LDL and At. I when compared with the positive control groups as depicted in Table 2.

Data in Table 3 showed that rats fed on HFD had significant ($P < 0.05$) increases in BG and leptin hormone and a decrease in insulin serum levels when compared to the negative control group. CAE and GAE when given orally in doses 200 and 400 mg/kg to obese diabetic rats for 6 weeks significantly ($P < 0.05$) decreased BG and leptin hormone and increased insulin serum levels when compared with the positive control group, in a dose-dependent manner.

Table 1: Effect of CAE and GAE on B.wt, F.wt and Ad. I in obese diabetic rats (n=7)

Parameters	B.wt (g)	F.wt (g)	Ad. I (%)
Group (1): Negative control	245.0±4.4^{d}	6.55±0.12^{d}	2.67±0.10^{d}
Group (2): Positive control	305.0±4.5^{a}	14.50±0.22^{a}	4.75±0.15^{a}
Group (3): CAE (200 mg/kg)	280.0±2.6^{b}	10.80±0.28^{b}	3.85±0.14^{b}
Group (4): CAE (400 mg/kg)	278.0±1.2^{b}	10.20±0.37^{b}	3.66±0.12^{b}
Group (5): GAE (200 mg/kg)	275.0±2.5^{c}	8.90±0.10^{c}	3.23±0.18^{b}
Group (6): GAE (400 mg/kg)	273.0±1.2^{c}	8.10±0.13^{c}	2.96±0.12^{c}

Means±SE with different letters superscripts (a, b, c, d) in the same column are significant at $P<0.05$ using one-way ANOVA test. CAE: Cinnamon aqueous extract, GAE: Ginger aqueous extract, B.wt: Body weight, F.wt: Fat weight, Ad. I: Adiposity index, SE: Standard error, ANOVA: Analysis of variance

Table 2: Effect of CAE and GAE on HDL, LDL and At. I in obese diabetic rats (n=7 rats)

Parameters	HDL (md/dl)	LDL (md/dl)	At. I LDL/HDL
Group (1): Negative control	69.20±2.11^{a}	18.20±2.31^{d}	0.263
Group (2): Positive control	54.34±2.55^{c}	52.40±2.18^{a}	0.964
Group (3): CAE (200 mg/kg)	62.66±3.22^{b}	44.80±2.15^{b}	0.714
Group (4): CAE (400 mg/kg)	63.45±3.12^{b}	43.20±2.27^{b}	0.680
Group (5): GAE (200 mg/kg)	64.50±2.76^{b}	30.45±3.19^{c}	0.472
Group (6): GAE (400 mg/kg)	65.00±3.22^{b}	28.22±2.16^{c}	0.434

Means±SE with different letters superscripts (a, b, c, d) in the same column are significant at $P<0.05$ using one way ANOVA test. CAE: Cinnamon aqueous extract, GAE: Ginger aqueous extract, HDL: High density lipoprotein, LDL: Low density lipoprotein, At. I: Atherogenic index, SE: Standard error, ANOVA: Analysis of variance

Table 3: Effect of CAE and GAE on BG and leptin and insulin hormones levels in obese diabetic rats (n=7 rats)

Parameters	BG (mg/dl)	Leptin (mg/dl)	Insulin (ng/ml)
Group (1): Negative control	195±3.2^{d}	6.60±0.15^{d}	1.95±0.15^{a}
Group (2): Positive control	225±4.1^{a}	9.99±0.11^{a}	0.82±0.13^{d}
Group (3): CAE (200 mg/kg)	186±3.2^{b}	5.35±0.18^{b}	0.99±0.24^{b}
Group (4): CAE (400 mg/kg)	184±2.8^{b}	5.85±0.17^{b}	1.10±0.12^{b}
Group (5): GAE (200 mg/kg)	157±3.2^{c}	2.40±0.19^{c}	1.72±0.14^{c}
Group (6): GAE (400 mg/kg)	155±2.5^{c}	2.45±0.18^{c}	1.82±0.16^{c}

Means±SE with different letters superscripts (a, b, c, d) in the same column are significant at $P<0.05$ using one-way ANOVA test. CAE: Cinnamon aqueous extract, GAE: Ginger aqueous extract, BG: Blood glucose, SE: Standard error, ANOVA: Analysis of variance

Feeding of HFD to rats for 4 weeks significantly ($P < 0.05$) decreased the activity of renal tissue levels of SOD, GPX and CAT antioxidant enzymes as compared to negative control rats. CAE and GAE when given in doses of 200 and 400 mg/kg to obese diabetic rats normalized the elevated renal tissue levels of SOD, GPX and CAT enzymes when compared with the positive control group, in a dose-dependent manner as recorded in Table 4.

DISCUSSION

The present study aimed to assess some pharmacological effects of CAE and GAE herbs in obese diabetic rats, and to elucidate the potential mechanisms.

Obesity is a major health problem that enhances the incidence of some diseases such as diabetes [2]. The oxidative stress plays an important role in the development of diabetes in obese individuals [3]. Both oxidative stress and the decreased antioxidant body defense mechanisms contribute to decreased insulin sensitivity and impaired insulin secretory response in obese diabetic rats [34,35]. In this study, obesity was induced by feeding rats on HFD for 4 weeks. This obese rat model closely resembles the reality of obesity in humans [20]. However, the experimental obesity could be also induced in rats and mice by other methods such as feeding on HFD [8], damage in the anterior hypothalamus and genetically induced-obesity. The obese diabetic rat model used in this study a novel animal model in pharmacology researches.

Results of the present study showed that CAE when given orally in 200 and 400 mg/kg to obese diabetic rats for 6 weeks produced a dose-dependent antiobesity effect. This effect was closely similar to the previous reports [11,12]. Similarly, GAE induced an antiobesity activity in obese diabetic rats and this effect agreed with the previous reports [17,36].

The potential mechanism underlying the antiobesity effect of CAE and GAE could be possibly attributed to their hyperinsulinimic effect that evident in the present study. Previous studies showed that hyperinsulinemia and insulin resistance are common features of obesity in rats [37] and in humans [38]. A second possible mechanism of the antiobesity activity of CAE and GAE could be due to the low serum level of leptin hormone reported in the current study. In this concern, Friedman [39] mentioned that leptin is a peptide hormone secreted by adipose tissues in proportion to body fat mass. When leptin circulates in the blood it acts on the brain to regulate food intake (appetite) and energy expenditure. When body fat mass decreases the serum leptin level decreases so stimulating appetite and suppressing energy expenditure till fat mass is restored. On this basis, the antiobesity activity and decreased Ad. I of CAE and GAE when given obese diabetic rats could be possibly attributed to the low serum leptin level that reported in the study. Conclusively, the antiobesity effect of CAE and GAE could be explained by their hyperinsulinimic activity and decreased serum leptin levels.

Table 4: Effect of CAE and GAE on activities of kidney SOD, GPx and CAT anti-oxidant enzymes in obese diabetic rats (n=7 rats)

Parameters	SOD (U/mg protein)	GPx (mM/min/mg protein)	CAT (mM/min/mg protein)
Group (1): Negative control	58.70±2.2[a]	69.0±2.1[a]	18.5±0.1[a]
Group (2): Positive control	36.50±2.3[d]	38.0±1.4[d]	13.8±0.2[d]
Group (3): CAE (200 mg/kg)	47.24±3.4[c]	22.0±1.3[b]	14.5±0.1[b]
Group (4): CAE (400 mg/kg)	48.15±2.5[c]	23.0±2.1[b]	15.8±0.1[b]
Group (5): GAE (200 mg/kg)	55.15±2.6[b]	0.49±1.5[c]	17.5±0.2[c]
Group (6): GAE (400 mg/kg)	57.17±2.3[b]	0.47±1.6[c]	17.8±0.2[c]

Means±SE with different letters superscripts (a, b, c, d) in the same column are significant at $P<0.05$ using one-way ANOVA test. Unit of GPx=mM of GSH utilized/min/mg protein. Unit of CAT=mM of H_2O_2 utilized/min/mg protein, CAE: Cinnamon aqueous extract, GAE: Ginger aqueous extract, SOD: Superoxide dismutase, GPx: Glutathione peroxidase, CAT: Catalase, SE: Standard error, ANOVA: Analysis of variance

The hepatoprotective effect of CAE and GAE reported in this study was evident from the significant decrease in serum levels of liver enzymes (AST, ALT and ALP) in obese diabetic rats. This effect was in accordance with the previous reports for cinnamon extract and its polyphenols [11,12] and for ginger [14] extract. The potential mechanism underlying the hepatoprotective effect of CAE and GAE could be attributed to the antioxidant activity of cinnamon [40] and of ginger [14]. The oxidative stress was reported to increase serum levels of liver enzyme (AST, ALT and ALP) in diabetic rats because hyperglycemia increases the production of ROS, which induces oxidative stress [4,5].

The hypolipidemic effects of CAE and GAE reported in this study were similar to the previous reports for cinnamon [11,12] and for ginger [16] extracts. The previous authors concluded that both extracts can lower the elevated levels of TC, TG and LDL in man and rats. The possible potential mechanism underlying the hypolipidemic effect of CAE and GAE could be due to their high contents of polyphenols (cinnamon) and of gingerols and shogaols (ginger) which inhibit the intestinal absorption of cholesterol with subsequent hypocholesterolemic activity.

Oral administration of CAE and GAE to obese diabetic rats caused hyperinsulinemia. This effect was also reported in previous studies [13,16] in rats. Some previous studies revealed that obesity is commonly associated with hyperinsulinemia and insulin resistance in rats [37] and in humans [38]. The hyperinsulinimic effect of CAE and GAE could be possibly explains their antiobesity effect, which was evident in this study, as obesity is commonly linked with hyperinsulinemia.

Leptin hormone plays a key role in regulating energy intake and energy expenditure. The level of circulating leptin is proportional to the total body fat mass. Oral administration of CAE and GAE to obese diabetic rats significantly decreased serum leptin levels in obese diabetic rats. This result agreed with the results of previous studies that revealed CAE and GAE decreased serum leptin level and depressed appetite in obese rats fed HFD [12] and human volunteers [41].

In obese diabetic rats, alloxan injection to rats induced hyperglycemia that caused renal oxidative stress which plays a

key role in the onset and development of diabetes [4,5]. Oral administration of CAE and GAE produced a dose-dependent antioxidant effect. The mechanism underlying the antioxidant effect of CAE and GAE could be attributed to their antidiabetic activity, which reported in this study and previous studies on cinnamon [8,13] and on ginger [15,16].

CONCLUSION

CAE and GAE herbs exhibit significant anti-obesity, hepatoprotective, hypolipidemic, antidiabetic and anti-oxidant effects in obese diabetic rats. These effects confirm the previous reports on cinnamon and ginger extracts. The mechanisms underlying these effects are fully discussed and clarified. This study provides a scientific evidence to substantiate the traditional use of cinnamon and ginger herbs in folk medicine for treating obesity and diabetes. The obese diabetic rat model used in this study is a novel animal model in pharmacology researches and it closely resembles the reality of obesity in humans.

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Original Research

Ethnobotanical study, antifungal activity, phytochemical screening and total phenolic content of Algerian *Aristolochia longa*

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ABSTRACT

Aim: *Aristolochia longa* (from the family *Aristolochiaceae*) is widely used in Algerian traditional medicine. Here, we document ethnomedicinal uses by local population of Mascara province (West Algeria) and we evaluate the antifungal activity, the phytochemical composition and total phenolic content of aqueous extract (decoction) of the roots of *A. longa* from Algeria. **Materials and Methods:** The ethnobotanical investigation was carried out in Mascara Province (West Algeria). Antifungal activity was assessed against *Saccharomyces cerevisiae*. Total phenolic content was measured using the Folin–Ciocalteu's reagent. **Results:** Our results showed that *A. longa* is widely used to treat several ailments such as cancer (38%), skin infections (14%), and diabetes (11%). Crushed roots are commonly used (89%) mixed with honey, milk, water or other medicinal plants. *A. longa* aqueous extract induced growth inhibition of *S. cerevisiae* cells in a dose - and time - dependent manner. An effective suppression of *S. cerevisiae* (97.06% inhibition of proliferation) was obtained at the 500 μg/mL after 72 h. Results of the phytochemical screening revealed that *A. longa* aqueous extract contained various bioactive compounds, including polyphenols and flavonoids. Total phenolic content in *A. longa* aqueous extract was found to be 6.07 $\pm$ 0.12 mg (gallic acid equivalents)/g. **Conclusion:** *A. longa* may be considered as a promising source of new drugs for treating cancer and as a good antifungal agent.

KEY WORDS: Algeria, antifungal, *Aristolochia longa*, ethnobotany, Mascara, phenolic, phytochemistry

INTRODUCTION

Medicinal plants are one of major resources of therapeutic agents. They are used by 80% of the world population in health care [1]. Medicinal plant use knowledge developed by local populations in Africa is now disappearing. There is, therefore, an urgent need to document this traditional knowledge [2].

Aristolochia, Family *Aristolochiaceae*, is a large genus of herbs and twining plants, found in the tropical and temperate regions of the world [3]. *Aristolochia* species have well known beneficial effects. Some of them were found to possess anticancer activity in several bioscreening studies [4]. The *Aristolochia* species are cultivated as ornamentals and popularly used as sources of abortifacient, emmenagogue, sedative, analgesic, anti-inflammatory, anti-feedant, muscle relaxant, antihistaminic, and anti-allergic drugs [5]. We have recently published a review on biological activities of *Aristolochia* plants [6]. Phytochemical studies on plants of the *Aristolochia* genus have yielded various types of compounds with antitumor, antiplatelet aggregation, immunomodulating and antifertility activities [7]. *Aristolochia longa*, commonly known as "Berrostom" to the local population in Algeria, is widely used in traditional medicine. In our previous study, we demonstrated that the aqueous extract of A. *longa* induced cell growth inhibition of Burkitt's lymphoma (BL41) cells in a dose-dependent manner. The extract induced apoptosis, loss of mitochondrial membrane potential and the activation of caspases-9 and -3 followed by poly(adenosine diphosphate-ribose) polymerase cleavage [8].

As part of our continuing work to investigate and biologically evaluate Algerian medicinal plants used in cancer treatment, the present study aimed to document ethnomedicinal uses, and to evaluate phytochemical composition and antifungal activity of A. *longa*.

MATERIALS AND METHODS

Ethnobotanical Study

The ethnobotanical investigation was carried out in Mascara Province. Mascara (5941 km^2) is located in the north-west of Algeria, (at 360 km of Algiers) with Mediterranean climate and mean annual precipitations of about 450 mm. In 2010, the total population was 826,334, with male/female ratio of about 1.04. Ethnomedicinal uses of A. *longa* information were collected and

documented through casual conversations and semi-structured interview technique [9] with local herbal practitioners and knowledgeable residents of the study area. The main focus was to collect the oral information about the ethnomedicinal uses of A. *longa* by local population.

Preparation of *A. longa* Aqueous Extract

Roots of A. *longa* were collected in March 2009; in "Tissemssilet," an administrative region located in western Algeria. Botanic identification and authentication were made by Dr. Kada Righi (Department of Agriculture, Faculty of Nature and Life Sciences, Mascara University, Algeria). The roots were dried, pulverized and finely sieved. The aqueous extract of A. *longa* was prepared as follows: The dried roots were boiled for 20 min at 100°C, cooled to room temperature, and then filtered. The solution passing through the filter was collected, concentrated, lyophilized and stored in a desiccator at +4°C until use.

Antifungal Activity Evaluation

Antifungal activity was evaluated using *Saccharomyces cerevisiae* from our laboratory collection (Département de Biologie, Faculté SNV, Université de Mascara). Sabouraud's dextrose broth was used for the preparation of fungal cultures and for the antifungal activity evaluation. A volume of 50 μl of overnight liquid cultures of yeast (density of 2×10^8 cells/mL) was added to each well of 96-well plates containing 50 μl media and various concentrations (0-500 μg/mL) of the extract in triplicate. The plates were incubated at 37°C. Absorbance was measured at 620 nm using a spectrophotometer (Shimadzu, Japan).

Phytochemical Screening

A. *longa* roots were screened for the presence of phytochemical constituents, such as alkaloids, terpenoids, anthraquinones, flavonoids, tannins, saponins, steroids and glycosides, with the standard qualitative phytochemical procedures described [10].

Determination of Total Phenolic Content

Total phenolic content was measured using the Folin–Ciocalteu's reagent as described [11]. Absorbance was measured at 760 nm using an ultraviolet spectrophotometer (Shimadzu, Japan). Gallic acid was used as a standard. A dose response linear regression was generated using the gallic acid standard absorbance, and the total phenolic content was expressed as mg gallic acid equivalents (GAE)/g dry weight of extract. Values were determined in triplicate.

Statistics Analysis

Mean data values are presented, with their standard deviations (mean ± SD). All statistical comparisons were made by Student's *t*-test, and statistical significance was defined as $P < 0.05$.

RESULTS

Ethnobotanical Study

In the present study, we interviewed 100 informants (female: 92; male: 8) in different locations of Mascara province (West Algeria). Almost all of the respondents knew and/or used A. *longa* for medicinal purposes. According to our results [Figure 1], A. *longa* is recognized to treat several ailments and health disorders. Cancer is the first ailment treated with the plant (39%), followed by skin infections (14%), diabetes (11%) and gastro-intestinal ailments (9%). Moreover, infertility and gynecological troubles are also treated with A. *longa*.

As shown in Figure 2, roots are the most frequently used part by local populations of Mascara (89%). Leaves (9%) and the entire plant (2%) are also used. The most usually method of preparation used is the mixture crushed roots - honey (63%), taken orally. Mixture with milk (13%) or other medicinal plants (12%) and decoction in water (11%) are also used to treat internal ailments. The paste (plant grinded with olive oil or water) is applied externally for skin infections and rheumatism [Figure 3].

Antifungal Activity of *A. longa* Aqueous Extract

To investigate the antifungal activity of the A. *longa* aqueous extract, *S. cerevisiae* cells were incubated with increasing

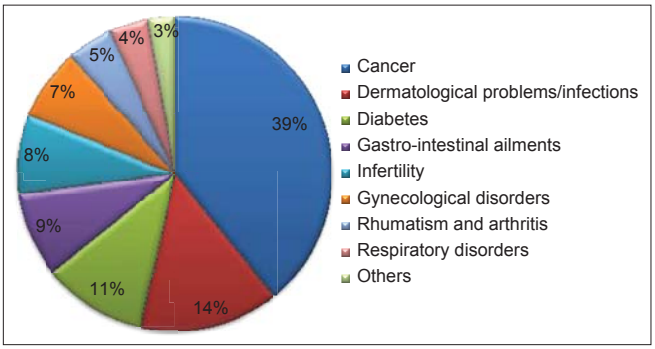

Figure 1: Ailments treatd with *Aristolochia longa*

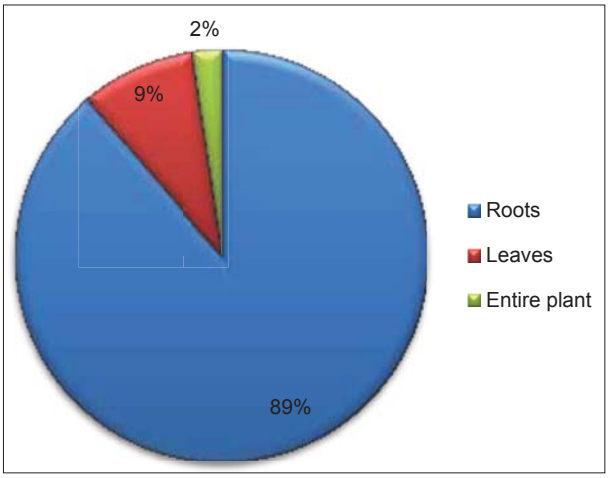

Figure 2: Plant parts used

concentrations (0-500 μg/mL). After 2, 48 and 72 h, cell viability was determined. Percent survival was determined when compared to untreated cells.

Cells were treated with increasing concentrations (from 0.00 to 500 μg/mL) of A. *longa* aqueous extract for 72 h and cell growth was measured as described in M and M. Percent survival was determined when compared to untreated cells. The difference in cell growth between untreated and A. *longa* aqueous extract-treated cells was found to be significant ($P < 0.001$). Data are represented as the mean ± standard deviation of three experiments per treatment group.

According to our results [Figure 4], A. *longa* aqueous extract induced growth inhibition of *S. cerevisiae* cells in a dose- and time- dependent manner. At 250 μg/mL, A. *longa* aqueous extract resulted in 30% and 67.65% inhibition of cell viability of *S. cerevisiae* cells after 48 and 72 h, respectively. At 500 μg/mL, the maximal inhibitory dose on the cell growth, the extract suppressed effectively the growth of *S. cerevisiae* cells after 72 h of treatment (97.06% inhibition of proliferation). We demonstrate a strong antifungal effect of A. *longa* aqueous extract as assessed against *S. cerevisiae* cells.

Phytochemical Screening of *A. longa*

Phytochemical screening of A. *longa* aqueous extract showed the presence polyphenols, flavonoids, tannins, C-heterosides, carbohydrates, and saponins. However, alkaloids, coumarins and O-heterosides were not detected [Table 1].

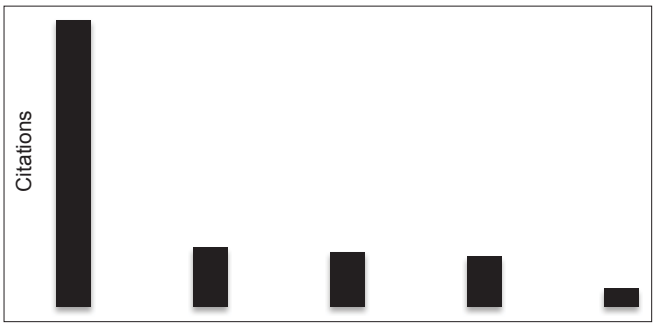

Figure 3: Preparation methods

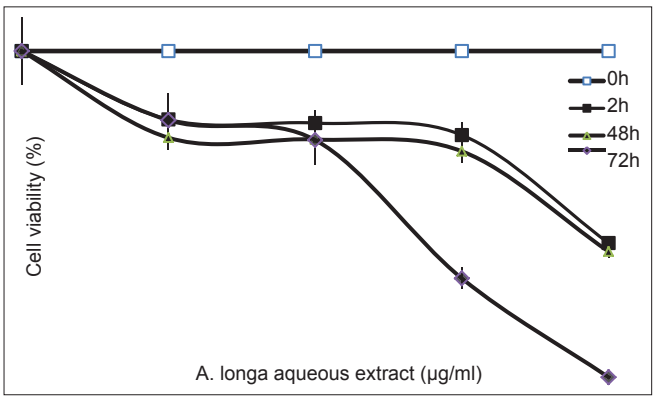

Figure 4: Effect of *Aristolochia longa* aqueous extract on growth *Saccharomyces cerevisiae* cells

Total Phenolic Content

Total phenolic content was estimated by using Folin–Ciocalteu reagent. Folin–Ciocalteu is a widely used and reported method for a simple and rapid estimation of total phenolics [12]. A standard curve [Figure 5] was obtained using gallic acid as a standard.

Total phenolic content in A. *longa* roots was found to be 6.07 ± 0.12 mg (GAE)/g.

DISCUSSION

Plants have played a vital role in the prevention and treatment of disease since prehistoric times [13]. Although local populations of Mascara have a long history of traditional use of medicinal plants, no studies have documented the traditional knowledge, which it is disappearing. The present study aimed to record the ethnomedicinal uses and to investigate the antifungal and the phytochemical profile of A. *longa*. The latter is widely used in Algeria.

Table 1: Preliminary phytochemical screening of *A. longa* roots

Constituents	Result
Phenolic compounds	+++
Flavonoids	+++
Gallic tannins	-
Catechic tannins	+++
Sterols and triterpenes	-
Alkaloids	-
Quinones	-
Carotenoids	-
Anthocyanins	-
Coumarins	-
Anthraquinones	-
Carbohydrates	+
C-heterosides	+
O-heterosides	-
Saponins	++

+: Presence, -: Absence, *A. longa: Aristolochia longa*

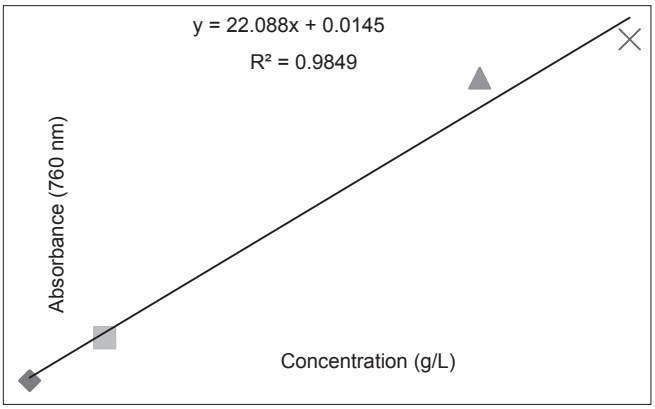

Figure 5: Calibration curve of gallic acid

Our results showed that the first ailment treated by local population using A. *longa* is cancer. Our findings are in consistence with previous studies. It has been reported that the most widely uses of A. *longa* in Algeria are in cancer treatment [14-16]. The use of this plant as an anti-cancer has been also reported in Morocco [17]. A*rthroleptis brevipes*, A*rachis monticola* and A*grimonia pilosa* are used as anticancer plants in Mexico [18]. We have recently demonstrated that an aqueous extract of A. *longa* induced apoptosis of BL41 cell line in a dose-dependent manner, by triggering the mitochondrial pathway [8]. Furthermore, our results revealed that skin infections are the second ailment group treated with A. *longa* in Mascara. Similar findings have been reported for A*ristolochia bracteolate* and A*cacia albida* used in Nigeria [19]. Current antifungal drugs are insufficient due to the limited selection of medications, their adverse side-effects and the emergence of resistance to them by fungal pathogens [20]. According to our results, A. *longa* aqueous extract induced growth inhibition of *S. cerevisiae* cells in a dose- and time- dependent manner. At 500 μg/mL, the maximal inhibitory dose on the cell growth, the extract almost abolished the proliferation of *S. cerevisiae* cells after 72 h of treatment (97.06% inhibition of proliferation). A. *longa* is considered as good antimicrobial drug [21]. Aristolochic acids, isolated from this plant inhibited *Escherichia coli*, *Pseudomonas aeruginosa*, *Streptococcus faecalis*, *Staphylococcus aureus* and *Staphylococcus epidermidis* [22]. Several plant extracts from the genus A*ristolochia* have been shown to possess strong *in vitro* antimicrobial activity [23-25].

Secondary metabolites produced by plants possess several interesting biological activities, and are a source of pharmacologically active principles against pathogenic microorganisms [26]. Since biological activities of medicinal plants are closely related to their chemical compounds [27], thus strong antifungal activity of the A. *longa* aqueous extract may be due to the detected compounds. Phytochemical screening of A. *longa* aqueous extract revealed the presence of polyphenols, flavonoids, tannins, c-heterosides, carbohydrates, and saponins. Phenolic compounds, flavonoids and saponins may be responsible for the antifungal activity. The mechanisms thought to be responsible for phenolic toxicity to microorganisms include enzyme inhibition by the oxidized compounds, possibly through reaction with sulfhydryl groups or through more nonspecific interactions with the proteins. On the other hand, saponins appear to act by disrupting the membrane integrity of fungal cells [28]. In the current study, total phenolic content of the A. *longa* aqueous extract was measured and found to be 6.07 ± 0.12 mg (GAE)/g. In A. *longa* total phenolics and flavonoids were estimated as 1.47 mg/g and 0.8 mg/g, respectively [29].

CONCLUSION

We present evidence that A. *longa* is widely used by local populations of Mascara province (West Algeria) to treat several ailments such as cancer. Crushed roots are commonly used mixed with honey, milk, water or other medicinal plants. Moreover, a strong antifungal effect (dose- and time- dependent manner) of A. *longa* aqueous extract as assessed against *S. cerevisiae* cells was demonstrated. Phytochemical screening revealed the presence of bioactive compounds that may contribute to the antifungal activity of the A. *longa* aqueous extract, such as flavonoids or saponins. Thus, A. *longa* may be considered as a promising source of new drugs for treating cancer and as a good antifungal agent.

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Original Article

Rapid but mild genoprotective effect on lymphocytic DNA with *Panax notoginseng* extract supplementation

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ABSTRACT

Background: Panax notoginseng (PN) is a well-known Chinese medicinal herb traditionally used as a hemostatic agent that strengthens and builds blood. The free radicals scavenging and antioxidant property of PN have been demonstrated in various studies either *in vitro* or in animal models, however, the genoprotective effect at human cellular level remains to be elucidated. **Aim:** The current supplementation study aimed to investigate the genoprotective effect of PN. The study explored the DNA protection effect after a single dose of 2500 mg commercial notoginseng extract in water. **Materials and Methods:** Six subjects, (3 males, 3 females) were recruited and each attended two trials. In the first trial, pre-ingestion and 2 hour-post-ingestion blood samples were collected and they were challenged with 50 μM H2O2. In the second trial, water was taken instead as control. Lymphocytes with or without challenge were then subjected to comet assay. DNA damage was assessed under fluorescent microscope. **Results:** Results showed a significant ($P < 0.05$) but mild decrease (3%) in the comet score after PN supplementation, indicating that PN supplementation reduces the H2O2-induced DNA damage in the lymphocytes and enhanced their resistance to oxidative damage giving a mild acute genoprotective effect against oxidant challenge. No change of comet score was observed in control trial.

KEY WORDS: Antioxidant, comet assay, hydrogen peroxide, pseudoginseng, supplementation

INTRODUCTION

Panax notoginseng (PN) or Sanqi in Chinese, is the root of the perennial herbaceous plant PN (Burk.) F. H. Chen. The plant belongs to the araliaceae family and is mainly grown naturally in the southwest provinces of China such as Yunnan and Guangxi, though they are also found in the southwest of Nepal and Burma. The root of notoginseng is usually collected in autumn before flowering or after the seed has ripened and sun-dried for use as medicine. Notoginseng was first described in about 600 years ago in the literature of Chinese herbs, "compendium of materia medica." Notoginseng is described as "a precious Chinese herbal medicine for the blood." This traditional herbal medicine is widely used for the treatment of blood disorders such as blood stasis, bleeding and blood deficiency. It is also used to heal traumatic injuries, to alleviate inflammation, swelling and pain, to stimulate blood circulation, and to speed up healing.

Many studies have revealed the physiological functions of notoginseng on the cardiovascular system, cerebrovascular system, blood clotting system, central nervous system, and endocrine system. Studies of the functions on blood clotting system have shown the anticoagulant and the anti-platelet activation, adhesion and aggregation effects of notoginseng [1,2] and studies of the functions on central nervous system have demonstrated that notoginseng saponins enhance antioxidant activity and protect neuron from oxidative stress and apoptosis [3,4]. Notoginseng has also demonstrated the antioxidative and the free radicals scavenging effect of notoginseng saponins on reactive oxygen species (ROS) [5-7]. These studies were performed *in vitro* by adding different free radicals to the notoginseng extract, measuring the radical scavenging activities, thus the antioxidant capacity of the extract. Most commonly used radicals are peroxyl radicals, hydroxyl radical, superoxide radical, and singlet oxygen because these are the most common free radical present in the human

biological system. However, *in vitro* studies could not be directly translated to *in vivo* interpretation because of the diverging test conditions [8]. Thus, the study on the genoprotective activity of notoginseng in human cells would be of research interest to provide useful information on the antioxidant property and genoprotective action of notoginseng at the human cellular level. Perhaps this may extend the pharmacological usage of notoginseng beyond the areas of cardiovascular and cardio-cerebrovascular diseases

MATERIALS AND METHODS

Chemicals

Sodium chloride (NaCl) and sodium dihydrogen phosphate ($NaH_2PO_4.2H_2O$) for PBS were from Panreac Quimica SA (Barcelona, Spain); disodium hydrogen phosphate (Na_2HPO_4) was from Sigma; Type VII low melting point (LMP) agarose, disodium ethylenediaminetetraacetic acid (EDTA) dehydrate; Triton X-100, sodium chloride for lysis solution; ethidium bromide were from Sigma (Steinheim, Germany). standard agarose (agarose 3:1) SA from Amresco (Solon, OH, USA). Tris (hydroxymethyl) aminomethane (Tris) was from Gibco (Carlsbad, CA, USA); sodium hydroxide were from Merck (Darmstadt, Germany). Hydrogen peroxide solution was from International Laboratory (South San Francisco, CA, USA).

Commercial notoginseng vege-capsules were purchased from Watson (group) Ltd., Hong Kong. Each capsule contained 500 mg notoginseng powder and content from 5 capsules (2500 mg) was used for each dose. The PN powder inside the 5 capsules was emptied into a glass, 100 mL of hot water was added and well-mixed. Another 100 mL of cold water was added and mixed to cool down the PN solution before taken.

Sample Collection

The study was approved by the Ethics Committee of Macao Society for the Study of Women's Health. All procedures involving human subjects complied with the Declaration of Helsinki. Six healthy subjects, 3 males and 3 females, aged from 27 to 57 (mean = 37); with no history of chronic disease and not on any medication or health supplements were recruited. A single dose of 200 mL of PN suspension was taken by subject 1 while 200 mL of water was taken by subject 2 in the same occasion. Venous blood samples were taken at 0 h and 2 h, respectively, and collected in EDTA tubes. The same procedure was repeated after 7 days with subject 1 taking water and subject 2 taking PN solution. A maximum of two subjects participated the study on a single day.

Oxidative Challenge

Lymphocytes were harvested as previously described [9]. 1 mL of 50 μM H_2O_2 was used to challenge pre- and post-supplementation lymphocytes sample for 5 min. Stressed and unstressed cells were then subjected to comet assay.

Comet Assay

Clean microscope slides were pre-coated with 1% SA. Two aspirations, each of 85 μL of 1% SA, were layered onto the dried pre-coated slides. Cover-slip was used to flatten the agarose. The coverslips were removed after the agarose was set at 4°C for 5 min. 170 μL of 1% LMP agarose was added to each tube, followed by quick layering of two aspirations, each of 85 μL of the LMP embedded lymphocytes onto the SA platform. Cover-slip was used to flatten the agarose. The slides were then put at 4°C for 5 min to facilitate gelling. The cover-slip was removed, and the slides were put into a Coplin jar containing 40 mL cold lysis solution for 1 h at 4°C. The slides were transferred to another Coplin jar containing 40 mL cold electrophoresis solution of pH >13 at 4°C for 20 min. The slides were put under alkaline electrophoresis solution and electrophoresed 20 V for 30 min. Alkaline electrophoresis solution was removed by 3 changes of distilled water at 5 min each. The slides were left to dry at room temperature overnight.

40 μL ethidium bromide was added to each gel for DNA staining to enable cell scoring under the fluorescence microscope (Eclipse 80i with Epi-fluorescence attachment, excitation filter G: Ex 510-560, Nikon, Tokyo, Japan). Each gel was covered with coverslip, and the slides were kept in the dark until counting. A total of 100 randomly selected cells was scored per slides (50 on each gel) using the visual scoring system from score 0 to score 4 according to the intensity of the comet head and the length of the comet tail. Score 0 indicated no tail and score 4 indicated almost all DNA is present in the tail. The degree of oxidative damage was measured by the amount of DNA liberated from the head of the comet, which meant the lower the score, the lesser damage by the hydrogen peroxide.

Statistical Analysis

Wilcoxon signed rank test was used to investigate for any significant difference between the pre- and post-notoginseng intake score as well as pre- and post-water intake (Prism 5.0, GraphPad Software, CA, USA).

RESULTS

The comet scores before and after 2 h of intake of 2500 mg commercial notoginseng powder in 100 mL water were shown in Figures 1 and 2. For all of the 6 subjects, there was varying degree of increase resistance to oxidative damage (decrease DNA damage/comet score), when challenged *in vitro* by H_2O_2 after notoginseng supplementation. There was a statistical significant reduction in comet score, but the effect was very mild (3%). There was no significant change in DNA damage ($P > 0.05$) when challenged by H_2O_2 in control (water only) trial [Figure 3].

DISCUSSION

Being a traditional Chinese herbal medicine, PN has been used for hundreds of years for health promotion or treatment of various blood diseases, especially the cardiovascular and cardio-

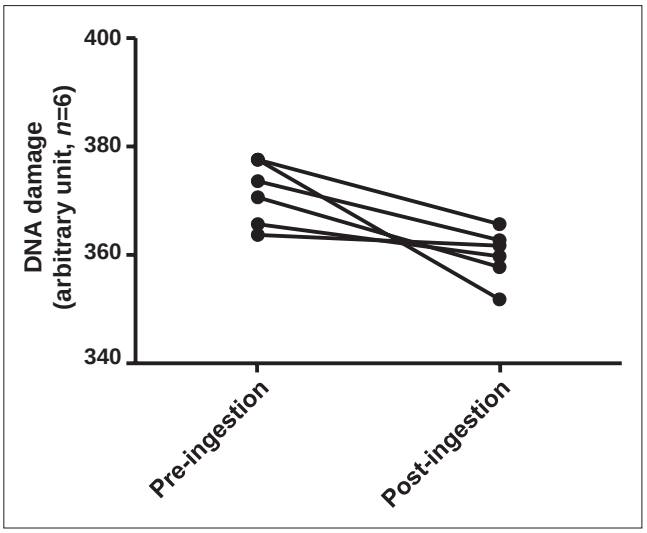

Figure 1: Acute effect of *Panax notoginseng* supplementation on DNA of human lymphocytes using the comet assay. Data expressed as the mean ± standard deviation of comet scores of data obtained from 6 subjects. Lymphocytes of the subjects after ingestion of 2500 mg commercial notoginseng extract were challenged *in vitro* by 50 μM H_2O_2. Lymphocytes of the subjects before ingestion were also challenged *in vitro* by 50 μM H_2O_2 and act as control. $P < 0.05$, compared to the control, the Wilcoxon signed rank test

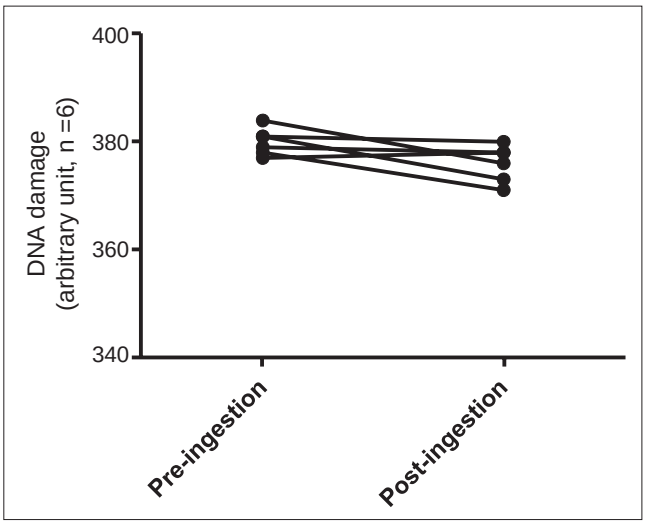

Figure 2: Acute effect of water intake on DNA of human lymphocytes using the comet assay. Data expressed as the mean ± standard deviation of comet scores of data obtained from 6 subjects. Lymphocytes of the subjects after 200 mL water intake were challenged *in vitro* by 50 μM H_2O_2. Lymphocytes of the subjects before intake were also challenged *in vitro* by 50 μM H_2O_2 and act as a control. $P > 0.05$, compared to the control, the Wilcoxon signed rank test

cerebrovascular disorders. Various studies have demonstrated the effectiveness of notoginseng to treat cardiovascular diseases in humans and myocardial infarct in animal models. It is suggested that the biological functions of notoginseng are partly due to their protective effects against ROS by suppressing the attack of •OH on membrane lipids, inhibiting lipid peroxidation, and thus protecting the cell membrane from oxidative injury. The inhibitory effects of notoginseng on lipid peroxidation have been

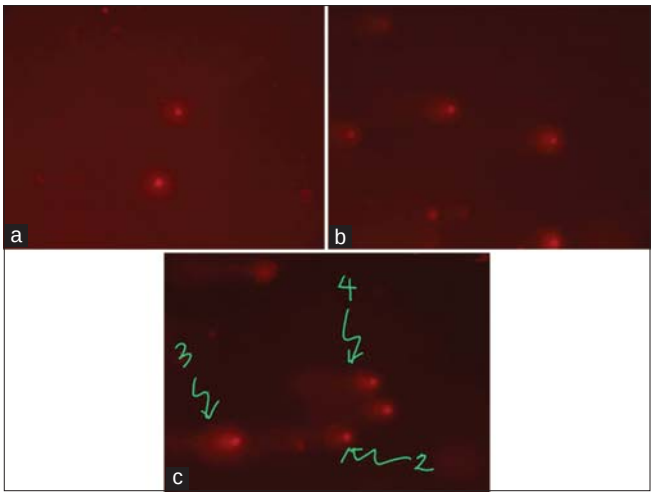

Figure 3: Comet images showing visual comet score (a) 0, undamaged DNA; (b) 1, mild DNA damage; (c) 2-4; moderate to severe DNA damage

reported in various animal studies [10,11]. Besides, some studies on notoginseng antioxidant activities have also demonstrated the *in vitro* antioxidative and the *in vitro* ROS scavenging effects of notoginseng saponins, showing strong scavenging activity toward •OH, H_2O_2 and O_2•$^-$ [6,7]. Dammarane saponins are the major bioactive components of notoginseng, in which the major constituents are notoginsenoside R1, ginsenosides Rg1, Re, Rb1, Rg2, Rh1, and Rd [12].

Although the antioxidant power of notoginseng has been confirmed in various studies, the DNA protective effect of notoginseng in human cells and the effective concentrations have yet to be explored. Our previous study demonstrated *in vitro* study DNA protective effect of notoginseng extract [13]. It has been found that pre-treatment with aqueous but not ethanolic extract of notoginseng is able to diminish H_2O_2-induced DNA damage in human lymphocytes. A wide range of concentrations of notoginseng extract has been used to investigate the effective level of notoginseng for this genoprotective effect. The current supplementation study explored the genoprotective effect of notoginseng after 2 h intake. Lymphocytes of subjects after drinking water or notoginseng supplement were challenged with H_2O_2 *in vitro* and DNA damage was investigated. The comet assay was used to detect DNA strand breaks, and the comet score was used to assess the DNA damage in the supplementation setting.

Results showed a statistical significant decrease ($P < 0.05$) in the comet score of the lymphocytes 2 h after ingestion. These results indicated that a single dose of notoginseng supplement of 2500 mg slightly reduced the H_2O_2-induced DNA damage in the lymphocytes and enhances their resistance to oxidative damage. However, the effect was mild which leaded to only ~3% decrease in comet score after treatment. This result contrasted the *in vitro* findings which showed an average of more than 20% reduction of the comet score, demonstrating a marked enhancement of genoprotection in the lymphocytes after notoginseng pretreatment [13]. The

difference in the extent of effectiveness between *in vitro* pretreatment and supplementation might be due to the fact that the latter involves a complicated process of absorption and metabolism before they can reach the lymphocytes in the systemic circulation to exhibit their effects. Our previous studies demonstrate supplementation with antioxidant rich dietary agents can significantly lowering of H_2O_2-induced DNA damage in lymphocytes within 2-2.5 h. Orange juice [14], grape seed extract, and ginseng extract (our unpublished data) are able to diminish 19-77% of comet score. Study on rat models and Caco-2 cells have proved that Rb1 and Rg1, two of the seven major saponins present in the notoginseng extract, are readily eliminated in stomach and degraded significantly in the large intestine. It is also reported that their absorption are mainly passive and the extent of absorption may be dominated by the low membrane permeability [15]. This assumption is supported by another study which reported the two main factors limiting the absorption of most ginsenosides and their deglycosylated metabolites to the systemic circulation are poor membrane permeability and active biliary excretion [16]. These studies have demonstrated low bioavailability of notoginseng in animals but that in humans remains to be elucidated. Since the bioavailability and metabolism of notoginseng have a great influence on the *ex vivo* antioxidant and genoprotective response, more data in these areas are needed before any conclusion can be drawn on the effectiveness of notoginseng as a genoprotective herb.

It is well-known that ROS damage on DNA results in single or double strand breaks; results in different forms of base damage producing products such as thymine glycol, 8-hydroxyguanosine, or abasic sites; and results in damage to deoxyribose sugar and DNA-protein cross-links. These damages, if they cannot be effectively repaired, can result in cell death or mutations and carcinogenesis. The antioxidative property of PN has already been shown in various studies. In the current study, we have further shown that this traditional herbal medicine which is intended to treat cardiovascular and cardio-cerebrovascular disorders, also possesses genoprotective effect in human lymphocytes. However, studies on the anti-cancer property of PN are scarce and whether PN behaves in the same way as the other herbs yet need to be explored. Validation of this concept requires some long-term prospective studies and clinical trials.

In conclude, the current study demonstrated that PN, which are considered to be antioxidative, also exhibited direct DNA protection against oxidant challenge in the comet assay *in vivo* with a mild effect. It could be absorbed, passed into the plasma, and offered genoprotection to the lymphocytes. Extended blood collection time point and/or supplementation may be of further research interest to explore the long-term accumulated effects and the integrated effects with other naturally produced free radicals and antioxidants in the complex "redox signaling" system in the body.

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Original Research

Anti-fungal activities of medicinal plants extracts of Ivorian pharmacopoeia

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ABSTRACT

Aim: This study was to evaluate *in vitro* anti-fungal activity of aqueous and hydroethanolic from medicinal plants extracts collected in Côte d'Ivoire. **Materials and Methods:** Plants extracts were prepared by homogenization and separately incorporated to Sabouraud agar using the agar slanted double dilution method. Ketoconazole was used as standards for anti-fungal assay. The anti-fungal tests were performed by sowing 1000 cells of *Candida albicans* on the previously prepared medium culture. Anti-fungal activity was determined by evaluating anti-fungal parameters values (minimal fungicidal concentrations [MFC] and IC50). **Results:** The results showed that all extracts possessed anti-fungal activities whose levels vary from plant species to another. Eight of them had a satisfactory anti-candidosic activity and extracts from *Terminalia* species were the most active. Among them the *Terminalia superba* extracts generated the strongest activities (MFC = 0.0975 mg/mL). Compared with ketoconazole (MFC = 0.390 mg/mL), the *T. superba* extracts, aqueous (MFC = 0.195 mg/mL) and hydroethanolic (0.0975 mg/mL) were successively twice and four times more active. The worst anti-fungal activity (MFC = 1600 mg/mL) was obtained with the *Guarea cedrata* aqueous extract. **Conclusion:** All medicinal plants extracts produced anti-fungal activities, and *T. superba* was the most active.

KEY WORDS: Anti-candidosic, anti-fungal, ketoconazole, plant extracts

INTRODUCTION

At the end of an ethnobotanical survey conducted in 1991 in the area of Issia (Cote d'Ivoire) several medicinal plants were collected and identified by our research team. These plants are commonly used in traditional circles by the healers for their curative virtues. A large number of plants among the cataloged species are granted anti-infectious properties [1]. The *in vitro* anti-microbial properties of some plants have already been assessed by some members of our research team. This study was initiated to verify the anti-microbial virtues which some plants are granted in order to continue the studies initiated by our team. In this report, we summarize the results obtained after some *in vitro* tests with aqueous and hydro-ethanolic extracts from each plant against *Candida albicans* one of the most pathogenic fungi species to human.

MATERIALS AND METHODS

Plant Material

Different parts of plants were collected in the area of Issia (Cote d'Ivoire) during the ethnobotanical investigations. The

collected parts were the steam barks of the trees species and the whole aerial parts for the herbaceous species. The studied plants species are the following:

- *Ceiba pentandra* (L.) Gaertn. (Harvested in Soubakaniédougou January 1, 1965, L. Aké-Assi Number 7601);
- *Entandrophragma angolense* (Welw.) C.D.C. (Côte d'Ivoire: Yakassé August 22, 1978, L. Aké-Assi Number 14198);
- *Entandrophragma cylindricum* (Sprague) (Mopri Forest, December 17, 1965, L. Aké-Assi Number 8348);
- *Guarea cedrata* (A. Chev.) Pellegr. (Côte d'Ivoire: National Parc of Azagny, January 22, 1986 L. Aké-Assi Number 17242);
- *Hunteria eburnea* Pichon (Abouabou forest, November 13, 1948, Mangenot and Aké-Assi Number 748);
- *Khaya ivorensis* A. Chev. (Côte d'Ivoire: Floristic National Center, Abidjan February 20, 1991, E. Aké-Assi Number 77);
- *Milicia excelsa* (Welw.) C.C. Berg (Agbo forest, September 25, 1957, Adjanohoun Number 1911B);
- *Mitracarpus villosus* (SW).DC. (Biankouma, August 11, 1975, L. Aké-Assi Number 12926);
- *Nesogordonia papaverifera* (A. Chev.) Cap. (Gabia, November 30, 1966, L. Aké-Assi Number 9320);
- *Physalis angulata* L. (Dabou, January 18, 1964, L. Aké Assi Number 7303);
- *Solenomostemon monostachyus* (P. Beauv.) Briq. (Man, November 15, 1953, L. Aké-Assi Number 2815);
- *Terminalia catappa* Linn. (Côte d'Ivoire: Azuretti, July 15, 1964, G. Cremers Number 266);
- *Terminalia ivorensis* A. Chev (Adiopodoumé, May 17, 1966, L. Aké-Assi Number 8855);
- *Terminalia mantaly* H. Perrier (Floristic National Center of Cocody University, Abidjan, May 23, 1995, E. Aké-Assi Number 217);
- *Terminalia superba* Engl. & Diels (Tonkoui Mountain February 26, 1969, L. Aké Assi Number 10477).

In the first step of the study some samples of all the plants species were identified and taxonomically authenticated by professor Aké-Assi at the Herbarium of the floristic national center of the Felix Houphouët-Boigny University (Abidjan, Côte d'Ivoire). In the second step, some great quantities of plant parts of each species were collected separately. The parts were carefully checked to take out all undesired specimen parts. The parts were then cut into small fragments and carefully air-dried for 2 weeks at ambient temperature in the laboratory, under continuous ventilation, away from sunlight and dust. After that step all, the vegetal pieces were crushed to a fine powder with an electrical grinder. Finally, the different types of powder were hermetically sealed in polyethylene bags and stored away from light and moisture until the time of extraction.

Extraction

Two sorts of extracts (aqueous and hydroethanolic) were prepared from the powder of each species. For the aqueous extract, 100 g of each type of powder were extracted in a solvent kept with 1 L of distilled water by homogenization in a blender. After six cycles of homogenization, the homogenates obtained were first wrung out in a fabric square and then filtered twice successively with absorbent cotton and once with Whatman 3 mm filter paper. The resulting filtrates were concentrated under vacuum using a Büchi rotary evaporator at 60°C [2]. Dark powder obtained is the aqueous crude extract. Hydroethanolic extracts were prepared following the same process by using a mixture of solvent ethanol 70% and water 30%.

Microorganism Studied

The tested fungi consist of a *C. albicans* (n 896/AB of 10.01.2000) clinical strain. It was both clinical isolate as well as authenticated and identified strain. This strain was provided by the Laboratory of Mycology of the Medical Sciences, Faculty of the Felix Houphouët-Boigny University (Abidjan Côte d'Ivoire).

C. albicans is an opportunistic fungus. It is a human normal communalist, and it belongs to the normal flora, but it becomes pathogenic when the immune system fails. It causes most of 83% of yeast infections [3,4]. The generalized infection caused by this fungus in individuals severely immuno-compromised often leads to death [5-9].

Anti-microbial Test

The anti-fungal activities were assessed by determining anti-fungal parameters values which are minimal fungicidal concentration (MFC); concentration that inhibits 99.99% of growth in the experimental tube compared with the growth control tube and concentration for 50% of inhibition (IC_{50}); graphically determined around each assay. For each extract, six replicate trials were conducted against *C. albicans* and ketoconazole was used as standards for anti-fungal assay. Ketoconazole was selected because it is an anti-fungal molecule usually prescribed to patients in the treatment of mycosis.

The anti-fungal tests were carried out on culture medium Sabouraud (Biomerieux, 51078 Ref: 777666501). The incorporation of plant extracts into the agar was made using the agar slanted double dilution method [2,10-12]. After this, all 10 tubes of each series were sterilized in an autoclave at 121°C for 15 min and then inclined with a small base at room temperature to allow their cooling and solidification of the agar. Both aqueous and hydroethanolic extracts were tested separately.

Fungal germs culture on slanted agar previously prepared was made by sowing 1000 cells of *C. albicans* [11]. These cultures were incubated at 30°C for 48 h. At the end of the incubation time, colonies were counted out by direct counting with a colony counter pen (Ceinceware, number 23382). The growth in the 10 experimental tubes was expressed as survival percentage, calculated, compared to 100% of growth in the growth control tube. The formula to calculate this is shown below. For the test tubes, concentrations of plant extracts ranged from 1600 to 0.024375 mg/mL with a ½ reason geometrical connection.

The processing of these data permitted to calculate the MFC values. It also made it possible to plot the curves of

activity of the extracts and the graphically determination of the IC_{50} values.

Formula to calculate the survival percentage:

$$S = \frac{n}{N} \times 100$$

S: Survival (%)
n: Number of colony in one experimental tube
N: Number of colony in the growth control tube.

Activity Classification Scale

The anti-fungal activities of the plant extracts were classified into the category following the scale below. The activity scale is divided into five levels on the basis of the MFC values.

Activity	Values
Very low activity	MFC values>50 mg/mL
Low activity	MFC value=50 mg/mL
Average activity	50 mg/mL>MFC values≥6.25 mg/mL
High activity	6.25 mg/mL>MFC values≥0.780 mg/mL
Very high activity	0.780 mg/mL>MFC values≥0.001526 mg/mL

RESULTS

The results were summarized in the form of curves of sensitivity and a table. The curves illustrate the evolution of the survival of *C. albicans* depending on the variation of the concentrations of the extracts [Figures 1 and 2]. In general, all sensitivity curves showed a progressively decreasing pace with slopes that are stronger or not as strong according to the extracts. The curves whose slopes are strong illustrate high anti-fungal potency and those that have a weak slope reveal low anti-fungal potency. They intersect the horizontal axis at different levels according to the extracts. The slopes of the curves of the extracts from the *Terminalia* species are the strongest. While the weakest slopes are observed with curve of extracts from *G. cedrata* (BOSS) and *E. cylindricum* (ECYL) [Figures 1 and 2]. The extracts from the other species have curves whose slopes are average. The decreasing shape of the activity curves shows that the 30 extracts acted according to a relation amount-effect.

Table 1 contains all the values of the anti-fungal parameters (MFC and the IC_{50}). All the extracts tested were active against *C. albicans*. The highest MFC values were produced by *G. cedrata*, while the lowest ones were produced by *T. superba*. All the hydroethanolic extracts from the four *Terminalia* species produced MFC values that are lower than the MFC value of ketoconazole.

In addition to the four *Terminalia* species, *E. angolense* (ETAN), *H. eburnea* (HUNT), *M. excelsa* (EMI) and *M. villosus* (MVS) are among those whose hydroethanolic extracts generated MFC values that are lowest than 50 mg/mL.

DISCUSSION

Analysis of the whole results shows that *C. albicans* are sensitive to all the extracts tested. The extracts were all capable of inhibiting the *in vitro* growth of the fungal germs. However, the levels of these anti-fungal activities are variable from one extract to another. As a matter of fact, the analysis of MFC values [Table 1] reveals that while some extracts resulted in very low MFC values, demonstrating their high efficiency, conversely extracts from some other species generated very high values of MFC revealing that they are not very efficient inhibiting the *C. albicans in vitro* growth. Among them the aqueous extract BOSS-X_{Aq} from *G. cedrata* (MFC = 1600 mg/mL) possesses the weakest anti-candidosic activity. On the other hand, the hydroethanolic extract TEKAM$_4$-X_0 from *T. superba*

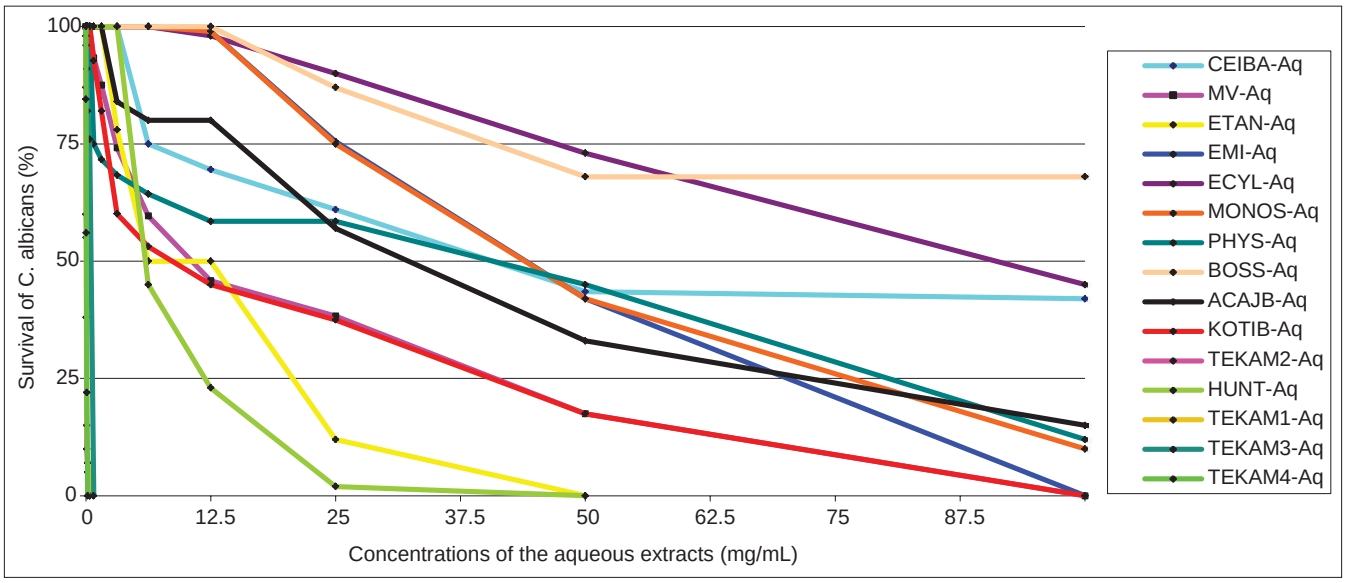

Figure 1: Antifungal activities of the crude extracts from 15 plants species. Sensitivity of *Candida albicans* to the aqueous extracts

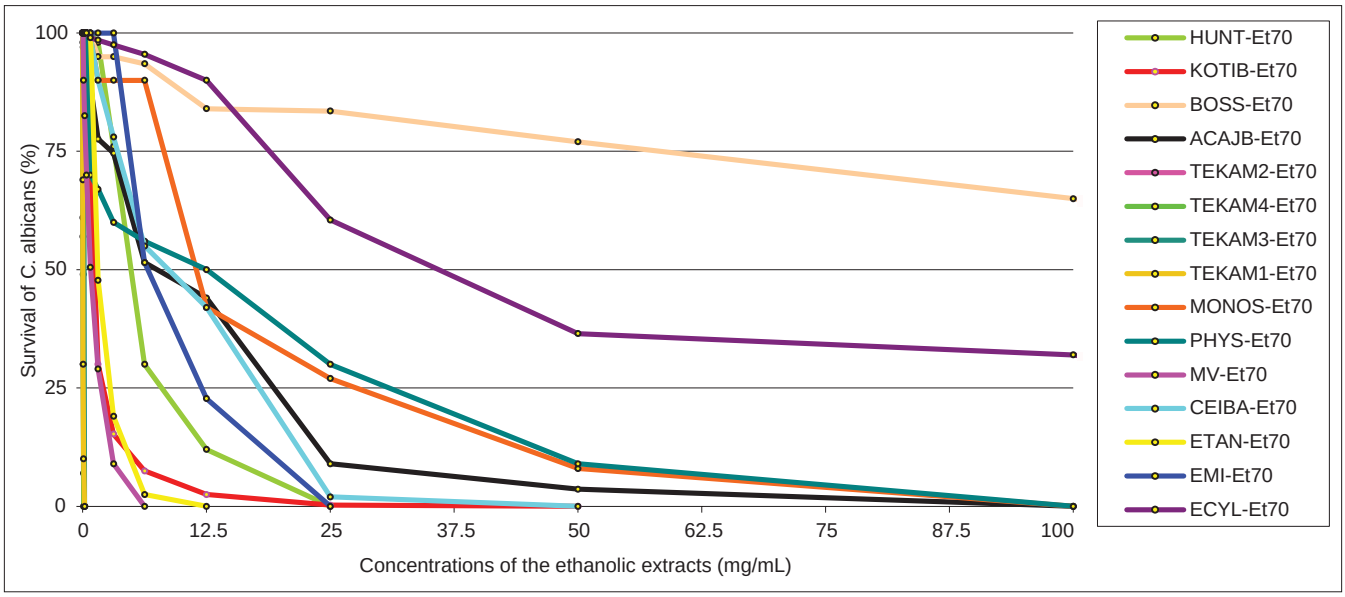

Figure 2: Sensitivity of *Candida albicans* to the hydroethanolic extracts

Table 1: Values of anti-fungal parameters of the extracts and reference anti-fungal drug

plants species and their code name	Values of anti-fungal parameters				Ratio of MFC values X_{Aq}/X_0 of each species
	IC_{50} (mg/mL)		MFC (mg/mL)		
	Aqueous extracts (X_{Aq})	Hydro-ethanolic extracts (X_0)	Aqueous extracts (X_{Aq})	Hydro-ethanolic extracts (X_0)	
C. pentandra (CEIBA)	40.625	8.571	200	50	4
E. angolense (ETAN)	6.25	1.524	50	12.5	4
E. cylindricum (ECYL)	91.071	36.071	400	200	2
G. cedrata (BOSS)	325.714	165	1600	800	2
H. eburnea (HUNT)	5.960	4.888	50	25	2
K. ivorensis (ACAJB)	8.705	7.589	200	100	2
M. excelsa (EMI)	8.705	6.563	100	25	4
M. villosus (MVS)	10.536	0.547	100	6.25	16
N. papaverifera (KOTIB)	8.705	1.181	200	50	4
P. angulata (PHYS)	40.179	12.5	200	100	2
S. monostachyus (MONOS)	43.75	11.473	200	100	2
T. catappa (TEKAM$_3$)	0.523	0.145	0.78	0.195	4
T. mantaly (TEKAM$_1$)	0.0423	0.03604	0.390	0.195	2
T. ivorensis (TEKAM$_2$)	0.054	0.0350	0.390	0.195	2
T. superba (TEKAM$_4$)	0.05746	0.03008	0.195	0.0975	2
Reference anti-fungal drug ketoconazole (KETO)	CI_{50} (mg/mL)		MFC (mg/mL)		
	0.01846		0.390		

IC_{50}: Inhibition concentration at 50%, MFC: Minimal fungicidal concentrations, *C. pentandra: Ceiba pentandra, E. angolense: Entandrophragma angolense, E. cylindricum: Entandrophragma cylindricum, G. cedrata: Guarea cedrata, H. eburnea: Hunteria eburnea, K. ivorensis: Khaya ivorensis, M. excelsa: Milicia excelsa, M. villosus: Mitracarpus villosus, N. papaverifera: Nesogordonia papaverifera, P. angulata: Physalis angulata, S. monostachyus: Solenomostemon monostachyus, T. catappa: Terminalia catappa, T. mantaly: Terminalia mantaly, T. ivorensis: Terminalia ivorensis, T. superba: Terminalia superba*

(CMF = 0.0975 mg/mL) exhibited the strongest anti-candidosic activity [Table 1].

On the basis of the classification scale of the levels of the activities, the analysis of the data shows that among the aqueous extracts, nine plant species produced extracts whose MFC values range from 100 mg/mL to 1600 mg/mL. This led to classify them as plants whose extracts possess some very low levels of anti-fungal activities. These species are the following: *C. pentandra*, *E. cylindricum*, *K. ivorensis*, *G. cedrata*, *M. excelsa*, *M. villosus*, *N. papaverifera*, *P. angulata*, *S. monostachyus*. For this reason, these species are not recommended to treat anti-fungal infections in traditional circles. Unless an extraction method permitting a better concentration of their active principles as well as the improvement of their anti-fungal activities was applied.

The results also show that the aqueous extracts from *E. angolense* and *H. eburnea* have an MFC value of 50 mg/mL so they have a low activity level (Table 1 and anti-fungal activity classification scale). In addition, the aqueous extract TEKAM$_3$-X_{Aq} from *T. catappa* inhibited the growth of *C. albicans* with a MFC value of 0.780 mg/mL. According to the anti-fungal activity classification scale, its anti-candidosic activity is classified as a high level of activity. Finally, the other three *Terminalia* species generated extracts whose MFC value are respectively 0.39 mg/mL for *T. ivorensis* and *T. mantaly*, 0.195 mg/mL for *T. superba*. This led to catalogue them in the category of those that have a very high level of anti-fungal activity (Table 1 and activity classification scale).

Otherwise, the comparison with previous works shows that the aqueous extract they got from *T. mantaly* was four times more active on *C. albicans* [13] than the same kind of extract we tested in this study. The MFC value obtained in their study was 0.0975 mg/mL for the aqueous extracts TEKAM$_1$-X_{Aq}, while the MFC value is 0.390 mg/mL in this present study for the same type of extract. On the other hand, the comparison with works on *T. superba* [14] reveals that TEKAM$_4$-X_{Aq} (MFC = 0.195 mg/mL) is two times more active than the

aqueous extract (MFC = 0.390 mg/mL) from *T. superba* tested by these authors. These differences of performance of these extracts could be explained by the fact that we did not collect the barks in the same area, and we did not test the extracts on the same *C. albicans* strain. The trees from which they collected the barks could contain low concentrations of active principles. And each fungal strain has its own sensitivity to anti-fungal drugs.

Meanwhile, the results from this study are in accordance with previous reports on the genus *Terminalia*, i.e. the works on *T. ivorensis* [15] and on *T. catappa* [16]. Because we obtained the same anti-fungal parameters values (MFC = 0.390 mg/mL and IC_{50} = 0.054 mg/mL for *T. ivorensis* and MFC = 0.780 mg/mL and IC_{50} = 0.523 mg/mL for *T. catappa*).

On the other hand, on the basis of MFC values, the comparison of the anti-fungal activities of all aqueous extracts shows that the aqueous extract from *T. superba* $TEKAM_4$-X_{Aq} (CMF = 0.195 mg/mL) is the most active of all. As a matter of fact, the MFC value ratios reveal that $TEKAM_4$-X_{Aq} is respectively two times more active than $TEKAM_1$-X_{Aq} (from *T. mantaly*) and $TEKAM_2$-X_{Aq} (*T. ivorensis*), it is also four times more active than $TEKAM_3$-X_{Aq} (*T. catappa*) and 256 times more active than ETAN-X_{Aq} (*E. angolense*) and HUNT-X_{Aq} (*H. eburnea*). In addition, the anti-candidosic activity of $TEKAM_4$-X_{Aq} is 512 times stronger than that of EMI-X_{Aq} (*M. excelsa*). $TEKAM_4$-X_{Aq} is also 1025 times more active than CEIBA-X_{Aq} (*C. pentandra*), ACAJB-X_{Aq} (*K. ivorensis*), PHYS-X_{Aq} (*P. angulata*), MONOS-X_{Aq} (*S. monostachyus*) and KOTIB-X_{Aq} (*N. papaverifera*). Lastly $TEKAM_4$-X_{Aq} is respectively 2051 times and 8205 times more active than ECYL-X_{Aq} (*E. cylindricum*) and BOSS-X_{Aq} (*G. cedrata*).

For the hydroethanolic extracts, the comparison of their performances on the basis of the MFC values highlights that for this kind of extracts, the four *Terminalia* species once more resulted in the lowest value of MFC [Table 1]. As a matter of fact, their MFC values range from 0.195 mg/mL to 0.0975 mg/mL [Table 1]. Among them, the hydroethanolic extract $TEKAM_4$-X_0 of *T. superba* possesses the lowest value of MFC (0.0975 mg/mL) meaning the strongest anti-candidosic activity. Inside this group of extracts possessing remarkable performances, $TEKAM_4$-X_0 is two times more active than $TEKAM_1$-X_0, $TEKAM_2$-X_0, and $TEKAM_3$-X_0. The last three extracts gave the same MFC value. However, the comparison of their IC_{50} values shows that $TEKAM_2$-X_0 (IC_{50} = 0.035 mg/mL) is the one that has the lowest IC_{50} value. So after $TEKAM_4$-X_0, $TEKAM_2$-X_0 is the second extract to be very active.

The results also show that four species (*E. angolense*, *H. eburnea*, *M. excelsa* and *M. villosus* produced hydroethanolic extracts whose MFC values range from 25 to 6.25 mg/mL [Table 1]. For this reason, their anti-candidosic activities are classified as the average level of activity. In this group, *M. villosus* is the species whose hydroethanolic extract is the most effective because it engendered the lowest MFC value (6.25 mg/mL).

Otherwise, from the comparison of the whole data of the hydroethanolic extracts the result is that BOSS-X_0 (*G. cedrata*) that produced the highest MFC value of 800 mg/mL, is the least active of all. In fact, it is 16,410 times less active than $TEKAM_4$-X_0 [Table 1]. So eventually, among all aqueous and hydroethanolic extracts, the extracts from *G. cedrata* produced the lowest anti-candidosic activities because their MFC values were the highest of all. On the other hand, extracts from the four *Terminalia* species were the most active; because they generated the lowest MFC and IC_{50} values against *C. albicans*. And particularly extracts from *T. superba* exhibited the best performances of all.

This strong anti-microbial potency of *T. superba* was also demonstrated by works on its methanolic extract which showed a broad spectrum of both anti-fungal and anti-bacterial activities [17]. This methanolic extract inhibited the *in vitro* growth of several strains of bacteria (*Mycobacterium smegmatis*, *Mycobacterium tuberculosis*, *Staphylococcus aureus*, *Escherichia coli*, *Shigella dysenteriae*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *Salmonella typhi*, *Citrobacter freundii*) and fungi (*C. albicans*, *Candida glabrata*, *Microsporum audouinii*, *Trichophyton rubrum*) [17].

Finally, the comparison of the performances of all extracts tested with that of ketoconazole, reveals that this reference anti-fungal drug is more active than most of extracts tested excepted few extracts from *Terminalia* species. As a matter of fact, KETO is 256 times more active than MONOS-X_0, PHYS-X_0 and ACAJB-X_0; 128 times more active than CEIBA-X_0 and KOTIB-X_0; 64 times more active than HUNT-X_0 and EMI-X_0; 32 times more active than ETAN-X_0; 16 times more active than MVS-X_0. Ketoconazole is also twice more active than $TEKAM_3$-X_{Aq}. On the other hand, KETO produced the same MFC value (0.390 mg/mL) as $TEKAM_1$-X_{Aq} and $TEKAM_2$-X_{Aq}. However, on the basis of their IC_{50} values, the comparison shows that Keto is respectively twice and three times more active than $TEKAM_1$-X_{Aq} and $TEKAM_2$-X_{Aq}.

However, compared with $TEKAM_1$-X_0, $TEKAM_2$-X_0, $TEKAM_3$-X_0 and $TEKAM_4$-X_{Aq} on the basis of their MFC values, KETO is respectively two times less active than these extracts. Finally, on the same basis of this comparison, $TEKAM_4$-X_0 (MFC = 0.0975 mg/mL), is clearly four times more active than ketoconazole [Table 1].

On the whole, the obtained data from this study highlighted the significant anti-fungal potency of plants of *Terminalia* genus. Otherwise, several investigations on plants of the genus *Terminalia* have already been conducted by some research teams. And many results of these previous reports share the results from the present study concerning their anti-microbial activities. Indeed, these works also confirmed the strong anti-microbial activities of extracts from a number of *Terminalia* species such as *T. catappa* [16,18,19], *T. chebula* [20], *T. glaucescens* [21], *T. ivorensis* [15,22-24], *T. macroptera* [25], *T. sericea* [26,27], and *T. superba* [14,17,28].

In addition, *M. villosus* was also reported for its broad spectrum of anti-fungal activities on *C. albicans*, *C. glabrata*, *C. tropicalis* and *A. fumigatus*, *A. flavus*, *C. neoformans*, *T. rubrum* and *T. mentagrophytes* [29-32]. The comparison reveals that on *C. albicans*, the *M. villosus* extracts [30] produced the same MFC values as the present report. However, on the basis of their IC_{50} values, they are two times less active than extracts from this study.

With other plant species, the comparison shows that $TEKAM_4$-X_{Aq} and $TEKAM_4$-X_0 of *T. superba* are respectively 256 times and 125 times more active than aqueous extract and hydroethanolic extract from *Microglossa pyrifolia* tested on *C. albicans* [2].

Moreover, the comparison of the whole data shows that for all plant species, hydroethanolic extracts are more active than the aqueous ones. As a matter of fact, for each case, hydroethanolic extracts produced MFC values that are 2-16 times lower than the MFC values of their aqueous equivalents [Table 1] if one establishes a comparison between extracts from the same plant species. This shows that for all plant species, hydro-ethanolic extracts concentrate a greater proportion of active principles than their aqueous equivalents. This assertion is similar to former investigation reports [2,13,15,16,29-31,33]. Indeed, the results of these previous investigations revealed that all the hydro-ethanolic extracts tested were 2-16 times more active than the equivalent aqueous extracts.

So we can deduce that between the two solvents used for extraction, the mixture ethanol-distilled water (70/30; v/v) is the solvent that permits the activity optimization and concentrates better the active principles from the different plants species. The active principles of all the plant species studied are more soluble in the mixture ethanol-distilled water (70/30; v/v). Considered under the chemical aspect, hydro-ethanolic extracts contain active principles that are of lipid nature and also polar molecules containing one or many oxygen atoms [2,34].

In addition, the active principles could be groups constituted by molecules of small sizes and average sizes (terpenoids, polyphenols, quinons, alkaloids, etc.) containing very low proportions of vegetal oil and chlorophyll [2,33]. Even if water can extract all type of molecules, water extracts contain a great content of macromolecules (polysaccharids, proteins and glycoproteins). They also contain a few species of polar lipids of small size, whose structures are simples. This high proportion of polysaccharids, glycoproteins and proteins could explain why aqueous extracts are always less active.

Alternatively as regards this aspect, the analysis of the antifungal parameter values of Table 1 shows that among the aqueous extracts, only four plant species (*T. catappa*, *T. ivorensis*, *T. mantaly*, *T. superba*) gave extracts exhibiting a high anti-fungal activity level. But for the hydro-ethanolic extracts, eight plant species (*E. angolense*, *H. eburnea*, *M. excelsa*, *M. villosus*, *T. catappa*, *T. ivorensis*, *T. mantaly*, *T. superba*) gave satisfactory anti-fungal activities.

Furthermore, many authors explained that most of the plants synthesize various secondary metabolites which are useful for their normal biology and to fight pathogenic microorganisms (virus, bacteria, fungi and various parasites) attacks [35-37]. These anti-microbial secondary metabolites are found melted among diverse substances and compounds extracted from plants. This would explain why the extracts from most of these plants have anti-fungal activities. The variability of their efficiency would be not only connected to various secondary metabolites content (alkaloids, terpenoids, polyphenols, quinons, coumarins) that plants produce, but also with the toxic power of these biomolecules to microorganisms [35,36,38].

CONCLUSION

This study showed that *C. albicans* are sensitive to all the extracts in dose-response relationship. All the 15 plant species produced active extracts with more or less raised performance levels. It proves and allows the understanding of the foundation of their use in traditional recipes, against infections. However, among them, only eight species gave extracts possessing good anti-fungal properties, allowing them to neutralize pathogenic microorganisms involved in infections. These species are *E. angolense*, *H. eburnea*, *M. excelsa*, *M. villosus*, *T. catappa*, *T. mantaly*, *T. ivorensis* and *T. superba*. But the most useful plants, because their extracts are by far very active, are the four *Terminalia* species (*T. catappa*, *T. ivorensis*, *T. mantaly*, and *T. superba*). The levels of their anti-fungal activities ranged from high to very high. Among them, the *T. superba* extracts generated the most excellent anti-candidosic activities. Furthermore, for each plant species, the hydroethanolic extracts were always noticeably 2-16 times as active as their equivalent aqueous.

Otherwise, the anti-fungal drug ketoconazole is clearly more active than most of extracts tested excepted $TEKAM_1$-X_0, $TEKAM_2$-X_0, $TEKAM_3$-X_0, $TEKAM_4$-X_{Aq} and $TEKAM_4$-X_0 the extracts from the *Terminalia* species. In fact $TEKAM_1$-X_0, $TEKAM_2$-X_0, $TEKAM_3$-X_0 and $TEKAM_4$-X_{Aq} are twice as active as ketoconazole; while $TEKAM_4$-X_0 is four times as active. This study confirms the real and strong anti-fungal activities of extracts of extract from the *Terminalia* species. Among the two solvents used for the extractions, the mixture ethanol-distilled water (70/30; v/v) is the solvent that permits the best optimization of the extraction and concentration of the active principles from a different plant species.

It would be thus desirable to continue this work by investigating more the anti-fungal potency of extracts from the four *Terminalia* species. Specifically our further works will aim the search and the isolation of compounds responsible for the anti-fungal activities as well as the determination of the chemical structure of these active principles. Our team hopes that these investigations will help to isolate new active principles that may be used in human therapeutic or to isolate metabolites that could serve as templates for synthesizing new and more active

molecules. These new molecules (natural or semi-synthetic) could then afford to expand the therapeutic arsenal and make it more effective.

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Original Research

Effects of *Tapinanthus globiferus* and *Zanthoxylum zanthoxyloides* extracts on human leukocytes *in vitro*

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ABSTRACT

Objective: This study aimed at investigating the genotoxicity and cytotoxicity effect of *Tapinanthus globiferus* and *Zanthoxylum zanthoxyloides* to human leukocytes. In addition, the reductive potential and the chemical composition of the two plant extracts were also determined. **Materials and Methods:** Human leukocytes were obtained from healthy volunteer donors. The genotoxicity and cytotoxicity of *T. globiferus* and *Z. zanthoxyloides* were assessed using the comet assay and trypan blue exclusion, respectively. The antioxidant activity of the plant extracts was evaluated by the reducing power assay. Furthermore, high-performance liquid chromatography-diode array detector was used to characterize and quantify the constituents of these plants. **Results:** *T. globiferus* (10-150 μg/mL) was neither genotoxic nor cytotoxic at the concentrations tested, suggesting that it can be consumed safely at relatively high concentrations. However, *Z. zanthoxyloides* showed cytoxicity and genotoxicity to human leukocytes at the highest concentration tested (150 μg/mL). In addition, the total reducing power of *T. globiferus* was found higher than *Z. zanthoxyloides* in potassium ferricyanide reduction. Both plants extract contained flavonoids (rutin and quercetin) and phenolic acids (chlorogenic and caffeic). **Conclusion:** The results obtained support the fact that some caution should be paid regarding the dosage and the frequency of use of *Z. zanthoxyloides* extract.

KEY WORDS: Cytoxicity, genotoxicity, high performance liquid chromatography-diode array detector, *Tapinanthus globiferus, Zanthoxylum zanthoxyloides*

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INTRODUCTION

Reactive oxygen species (ROS) are oxidizing, highly reactive and unstable molecules are containing oxygen. They produced during normal cellular metabolism as by-products of respiration in the mitochondria. They include hydroxyl radical (OH•), superoxide anion ($O_2^{\bullet -}$), hydrogen peroxide (H_2O_2) and singlet oxygen [1]. Cumulative evidence suggests that ROS play important roles in signal transduction, sensing of oxygen tension and regulation of functions controlled by oxygen concentration [2]. They are also involved in boosting the immune system [3]. However, ROS can be harmful when its cellular levels exceed the level of cellular antioxidants, which results in oxidative stress. Oxidative stress would eventually cause injury to cellular macromolecules such as membrane lipids, proteins and nucleic acids, thereby affecting the normal functioning of cells.

DNA is one of the major targets of ROS in living cells and tissues. ROS induces DNA mutations that can cause or lead to

cancer and age-related disorders [4]. Hydroxyl radicals ($OH^{\bullet}$), an oxidant obtained from the breakdown of H_2O_2 is majorly responsible for DNA damage. It reacts with DNA molecule causing DNA protein cross-links, DNA strand breaks and alkali-labile sites [4,5], which may lead to permanent damages that can cause severe biological consequences [6]. Furthermore, Shi *et al.* [7] revealed that $O_2^{\bullet -}$ and H_2O_2 are capable of inducing strand-breaks and oxidation of DNA bases.

Further, studies have shown that DNA damage can be minimized or prevented by the use of natural antioxidants such as vitamin C, vitamin E, carotenoids, flavonoids, and other polyphenolic compounds, by scavenging or inactivating ROS. Particularly, natural compounds exhibit protective effects when used in oxidative stress-induced DNA damage [8]. Furthermore, plants rich in antioxidants have been shown to protect ROS-induced oxidative DNA damage [9].

Tapinanthus globiferus and *Zanthoxylum zanthoxyloides* are plants commonly used as folkoric medicine and highly consumed in the Nigeria and Cameroon. *T. globiferus* known as mistletoe (in English) belongs to the family Loranthaceae. It is a woody, spreading shrub with blackish, smooth stems made rough by the presence of lenticels. It is popularly called "afomo" in South Western Nigeria whereas, Z. *zanthoxyloides* (family, Rutaceae) is commonly known as candle wood. The root of Z. *zanthoxyloides* is used as antibacterial toothbrush in South Western Nigeria, and the decoction of its leaves and roots is used to wash wounds for healing. In addition, the bark of the plant is used in the treatment of intestinal worms and edema. Likewise, *T. globiferus* is commonly consumed for the treatment of hypertension, ulcers, diabetics, weakness of vision, and for promoting muscular relaxation before delivery. Recent studies revealed that the plants exhibit a variety of pharmacological activities including antitrypanosomal [10,11], antimicrobial [12], anti-inflammatory [13] activities, and are rich in antioxidants [14].

Human leukocytes are used to evaluate DNA damage, repair studies and genotoxicity using comet assay because leukocytes are obtained in a relatively non-invasive way and do not require tissue disaggregation [15]. Comet assay is highly sensitive for *in vitro* genotoxicity test methods on leukocytes [16] and is of particular importance for safety evaluation. For instance, genotoxicity can be a consequence of long-term exposure to very low levels of chemicals and have a hereditary and delayed-onset nature that may lead to major consequences at the population level [17].

Considering the growing interest in the use of medicinal plants to treat and/or prevent various diseases associated with free radicals, there is an urgent need to provide information on toxicity risk-assessment of plants extracts. Therefore, the present study aimed at investigating the possible genotoxic and cytotoxic potential of *T. globiferus* and Z. *zanthoxyloides* in human leukocytes. A further attempt was made to determine the reducing potential (conversion of Fe (III) to Fe (II)) of these plants as well as their chemical characterization.

MATERIALS AND METHODS

Chemicals

All chemicals used including solvents were of analytical grade.

Plants Collection and Extraction Procedure

The leaves of *T. globiferus* and stem bark of Z. *zanthoxyloides* were obtained from Ogbomoso, Nigeria in 2013 and were identified by Dr. Ogunkunle of the Botany Unit, Department of Pure and Applied Biology (Ladoke Akintola University of Technology, where the specimen was deposited). The dried leaves and stem bark were pulverized into a powdery form, after which 100 g of *T. globiferus* and 100 g of Z. *zanthoxyloides* were macerated at room temperature with ethanol (70%) and extracted for 3 days. The combined ethanolic extract of each sample was filtered on the 3rd day and the solvent was fully evaporated under reduced pressure to give a green solid for *T. globiferus* and yellow solid for Z. *zanthoxyloides*. The ethanolic extract of *T. globiferus* was then suspended in water, while, that of Z. *zanthoxyloides* was suspended in ethanol in order to prepare different concentrations (10-150 μg/mL) used in the experiments.

Quantification of Some Flavonoids and Phenolic Compounds by High Performance Liquid Chromatography-Diode Array Detector (HPLC)

Reverse phase chromatographic analyses were carried out under gradient conditions using C_{18} column (4.6 mm × 250 mm) packed with 5 μm diameter particles; the mobile phase was water containing 2% acetic acid (A) and methanol (B), and the composition gradient was: 5% of B until 2 min and changed to obtain 25%, 40%, 50%, 60%, 70% and 100% B at 10, 20, 30, 40, 50 and 65 min, respectively, following the method described by Laghari *et al.* [18] with slight modifications. The extracts of *T. globiferus and* Z. *zanthoxyloides* were analyzed, at a concentration of 5 mg/mL. The presence of six phenolics compounds was investigated, namely, gallic, chlorogenic and caffeic acids and the flavonoids quercetin, rutin and kaempferol. Identification of these compounds was performed by comparing their retention time and ultraviolet (UV) absorption spectrum with those of the commercial standards. The flow rate was 0.6 mL/min, injection volume 40 μL and the wavelength were 254 nm for gallic acid, 325 nm for caffeic and chlorogenic acids, and 365 nm for quercetin, rutin and kaempferol. All the samples and mobile phase were filtered through 0.45 μm membrane filter (Millipore) and then degassed by ultrasonic bath prior to use. Stock solutions of standards references were prepared in the HPLC mobile phase at a concentration range of 0.031-0.250 mg/mL for kaempferol, quercetin and rutin; and 0.006-0.250 mg/mL for gallic, caffeic and chlorogenic acids. All chromatography operations were carried out at ambient temperature and in triplicate. The limit of detection (LOD) and limit of quantification (LOQ) were calculated based on the standard deviation of the responses and the slope using three independent analytical curves as defined by ICH [19].

LOD and LOQ were calculated as 3.3 and 10 σ/S, respectively, where σ is the standard deviation of the response and S is the slope of the calibration curve.

Obtension of Human Leukocytes

Heparinized venous blood was obtained from healthy volunteer donors from the Hospital of the Federal University of Santa Maria (UFSM), Santa Maria, RS, Brazil (age 25 ± 10). This work was carried out in accordance with the Guidelines of the Ethical Committee of UFSM and approved by the Institutional Review Board of UFSM (0089.0.248.000-12). Differential erythrocyte sedimentation with dextran was used to separate leukocytes of the blood as previously described [20].

Genotoxicity evaluation of T. globiferus and Z. zanthoxyloides using comet assay

The comet assay was performed under alkaline conditions according to the method of Santos *et al.* [21]. Briefly, peripheral leukocytes were incubated for 3 h in the absence or presence of plant extract, at different concentrations (10-150 μg/mL). Hydrogen peroxide (100 μM) was used as a positive control, while water was used as negative control (NC). After incubation and electrophoresis, one hundred cells per sample were randomly selected and visually scored according to tail length into five classes: (1) Class 0: Undamaged, without a tail; (2) Class 1: With a tail shorter than the diameter of the head (nucleus); (3) Class 2: With a tail length 1-2 times the diameter of the head; (4) Class 3: With a tail longer than 2 times the diameter of the head and (5) Class 4: Comets with no heads. Comets with no heads and images with nearly all DNA in the tail or with a very wide tail were excluded from the evaluation because they probably represent dead cells. DNA damage was presented as DNA damage index (DI) and it is based on the length of migration. The DI was calculated from cells in different damage classes as follows: DI = n1 + 2n2 + 3n3 + 4n4. Where, n1-n4 represents the number of cells with level 1-4 of damage. The slides were analyzed under blind conditions by at least two individuals.

Cytotoxicity evaluation of T. globiferus and Z. zanthoxyloides by trypan blue

The toxic effects of *T. globiferus and Z. zanthoxyloides* toward leukocytes were determined as described by Mischell and Shiingi [22] with slight modifications. Briefly, 2.5 μL of different concentrations of the extracts (10-150 μg/mL) was added to leukocytes suspension (497.5 μL) and incubated in the presence or absence of hydrogen peroxide (2 mM) + azide (1 mM), for 3 h at 37°C in a water bath. Hydrogen peroxide (2 mM) + azide (1 mM) was used as a positive control whereas distilled water was used as NC. After the incubation, a volume of 50 μL of leukocytes suspension was mixed with 50 μL of 0.4% trypan blue solution and left for 5 min. The cell viability was determined microscopically (×400 magnification) using a hemocytometer and was calculated as the number of living cells (i.e., those not stained with trypan blue) divided by the total number of cells multiplied by 100.

Reducing Power Assay

The Fe^{3+} reducing power of the extracts was determined according to a modified method of Mathew and Abraham [23]. Various concentrations of *T. globiferus and Z. zanthoxyloides* (10-150 μg/mL) (200 μL) were mixed with 625 μL of potassium phosphate buffer solution (0.2 M, pH 6.6) and 625 μL of potassium ferricyanide (1%, w/v), followed by incubation at 50°C for 20 min. The reaction was stopped by adding 625 μL of trichloroacetic acid solution (10%, w/v) and then centrifuged at 5000 ×*g* for 10 min. A known volume (625 μL) of the upper layer solution (obtained after centrifugation) was taken in another test tube and mixed with 625 μL of distilled water, then, 250 μL of ferric chloride solution (0.1%, w/v) was added and mixed well. The absorbance was measured at 700 nm in a spectrophotometer. The blank was prepared by the same procedure without plant extracts. Ascorbic acid (10-150 μg/mL) was used as a positive control.

Statistical Analysis

Values were expressed as mean ± standard error of the mean. One-way ANOVA, followed by Benferroni post-test was used to evaluate the differences among the groups. The results were considered as statistically significant for $P < 0.05$.

RESULTS

Phytochemical Constituents

The HPLC analysis was used to identify and quantify the presence or absence of phenolic acids and flavonoids from the leaf extract and stem bark of *T. globiferus*, and *Z. zanthoxyloides* respectively. The results of HPLC profile indicate that both plant extracts contain chlorogenic and caffeic acids, rutin and quercetin [Figure 1]. However, gallic acid, present in the leaf extract of *T. globiferus*, was absent in the stem bark of *Z. zanthoxyloides*. Similarly, kaempferol, absent in the

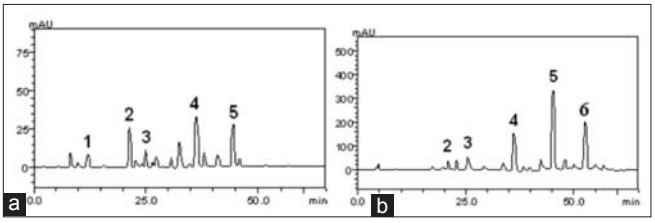

Figure 1: High performance liquid chromatography (HPLC) profile of the leaf extract of *Tapinanthus globiferus* (a) and *Zanthoxylum zanthoxyloides* stem bark extracts (b). Gallic acid (peak 1), chlorogenic acid (peak 2), caffeic acid (peak 3), rutin (peak 4), quercetin (peak 5) and kaempferol (peak 6). The chromatography peaks were confirmed by comparing its retention time with those of reference standards and by diode array detector spectra (200-500 nm). Calibration curve for gallic acid: Y = 11611x + 1468.8 (r = 0.9999); chlorogenic acid: Y = 14762x + 1257.5 (r = 0.9997); caffeic acid: Y = 11526x + 1293.1 (r = 0.9995); rutin: Y = 13035x – 1045.9 (r = 0.9998); quercetin: Y = 15105x – 1192.3 (r =0.9998) and kaempferol: Y = 15223x – 1303.9 (r = 0.9999). All chromatography operations were carried out at ambient temperature and in triplicate

leaf extract of *T. globiferus*, was present in the stem bark of *Z. zanthoxyloides* [Figure 1 and Table 1]. These compounds were identified by comparing their retention times and UV spectra to that of authentic standards analyzed under identical analytical conditions. Quantitative HPLC analysis showed that the rutin was the major component in *T. globiferus* (9.14 ± 0.1 mg/g) while caffeic acid was the minor (1.98 ± 0.03 mg/g). However, the major component found in *Z. zanthoxyloides* was quercetin (48.09 ± 0.03 mg/g), while chlorogenic acid (4.23 ± 0.01 mg/g) was the minor [Table 1].

Effects of *T. globiferus* and *Z. zanthoxyloides* on DNA Damage

Table 2 shows the comet assay results obtained after exposure of human leukocytes to various concentrations (10-150 μg/mL) of *T. globiferus and Z. zanthoxyloides*. H_2O_2 (positive control) induced a significant increase in DNA migration when compared to NC ($P < 0.001$), as evidenced by the DI [Table 2]. Ethanol used as a vehicle for *Z. zanthoxyloides* did not have any effect on DNA migration in comparison with the NC ($P > 0.05$). There was no significant difference in the DI when the cells were treated with *T. globiferus* (10-150 μg/mL) when compared to NC ($P > 0.05$). However, a statistically significant increase in DNA DI was observed at 150 μg/mL of *Z. zanthoxyloides*. Generally, when the human leukocytes were exposed to both plant extracts (10-150 μg/mL), the majority of leukocytes examined on slides were undamaged (Class 0). Few leukocytes showed minor DNA damage (Class 1) and very few showed a large amount of DNA damage (Class 2-4) [Table 2].

Table 1: Qualitative and quantitative analyses of some flavonoids and phenolic compounds from the leaf extract of *T. globiferus* and *Z. zanthoxyloides* stem bark extract by HPLC-DAD

Compounds	$t_{R\ (min)}$	*T. globiferus*		*Z. zanthoxyloides*		LOD	LOQ
		mg/g	%	mg/g	%	(μg/mL)	(μg/mL)
Rutin	40.25	9.14±0.1	0.83	18.25±0.06	2.01	0.022	0.074
Quercetin	50.11	7.08±0.02	0.7	48.09±0.03	4.82	0.028	0.092
Kaempferol	60.18	-	-	26.03±0.07	2.61	0.031	0.103
Gallic acid	11.92	2.35±0.13	0.23	-	-	0.017	0.056
Chlorogenic acid	23.86	6.83±0.1	0.61	4.23±0.01	0.42	0.036	0.119
Caffeic acid	25.09	1.98±0.03	0.19	9.02±0.08	0.92	0.009	0.028

Results are expressed as mean±standard deviations of three determinations. LOD: Limit of detection, LOQ: Limit of quantification, t_R: Retention time, *T. globiferus: Tapinanthus globiferus, Z. zanthoxyloides: Zanthoxylum zanthoxyloides*, HPLC-DAD: High performance liquid chromatography-diode array detector

Effects of *T. globiferus* and *Z. zanthoxyloides* on Leukocytes Viability

In order to assess the toxicity of *T. globiferus and Z. zanthoxyloides* on human leukocytes, cellular viability was evaluated following exposure, by using the trypan blue assay dye exclusion method. The H_2O_2 + azide were used to inhibit catalase activity in leukocytes and consequently detect the toxicity induced by H_2O_2. H_2O_2 + azide used as positive control, caused a significant decrease in cell viability (approximately 48% decrease) when compared to control [Figure 2a and b; $P < 0.05$]. *T. globiferus* at all the concentrations tested did not have any effect on cell viability [Figure 2a], whereas, *Z. zanthoxyloides* at the highest concentration (150 μg/mL) exhibited a significantly decrease [Figure 2b] when compared to control ($P < 0.05$). It should be noted that 150 μg/mL of *Z. zanthoxyloides* concentration was genotoxic and cytotoxic to human leukocytes.

Reducing Power Potential of *T. globiferus* and *Z. zanthoxyloides*

As depicted in Figure 3, *T. globiferus and Z. zanthoxyloides* showed increased absorbance with increased concentrations, which indicates increased ferric reducing power. However, the reducing potential of both extracts was lower than that of ascorbic acid used as standard antioxidant. The reducing power of the extracts and ascorbic acid decreased in the order ascorbic acid > *T. globiferus* > *Z. zanthoxyloides*.

Table 2: Effect of *T. globiferus* and *Z. zanthoxyloides* on human leukocytes

Treatment	Extract concentration (μg/mL)	Levels of DNA damage					DI
		0	1	2	3	4	
Control (H_2O, NC)	0	96.25±0.14	3.125±0.23	0.5±0	0.125±0.12	0±0	4.500±0.20
Etanol (vehicle)	-	95.91±0.19	3.52±0.23	0.48±0	0.141±0.12	0±0	4.480±0.32
H_2O_2 (PC)	100 μM	71.2±0.26	22.57±0.01	3.87±0.36	1.25±0.28	1.38±0.26	40.01±1.22*
T. globiferus	10	96.25±0.25	3.125±0.47	0.375±0.12	0.25±0.14	0±0	4.625±0.23
	25	95.75±0.14	3.875±0.31	0.375±0.23	0±0	0±0	4.625±0.23
	50	94.75±0.25	4.5±0.35	0.75±0.14	0±0	0±0	6.000±0.20
	100	94.5±0	5.25±0.25	0.375±0.23	0±0	0±0	6.000±0.28
	150	94±0.35	5.625±0.37	0.375±0.23	0±0	0±0	6.375±0.47
Z. zanthoxyloides	10	96.25±0.25	2.87±0.55	0.37±0.24	0.37±0.12	0.12±0.12	5.25±0.32
	25	95.87±0.24	2.62±0.43	1.25±0.24	0.37±0.12	0±0	6.00±0.2
	50	95.12±0.37	4.75±0.32	0.12±0.12	0±0	0±0	5.00±0.45
	100	94.87±0.12	4.37±0.4	0.75±0.32	0±0	0±0	5.87±0.42
	150	94.12±0.24	4.87±0.59	0.87±0.43	0±0	0±0	7.00±0.45#

The results are mean±SEM of $n=4$ independent experiments. Water was used as NC while hydrogen peroxide was used as PC. *$P<0.001$ versus control (H_2O), #$P<0.001$ versus PC. *T. globiferus* did not have any effect on DNA damage at the concentrations tested. DI: Damage index, *T. globiferus: Tapinanthus globiferus, Z. zanthoxyloides: Zanthoxylum zanthoxyloides*, SEM: Standard error of the mean, NC: Negative control, PC: Positive control

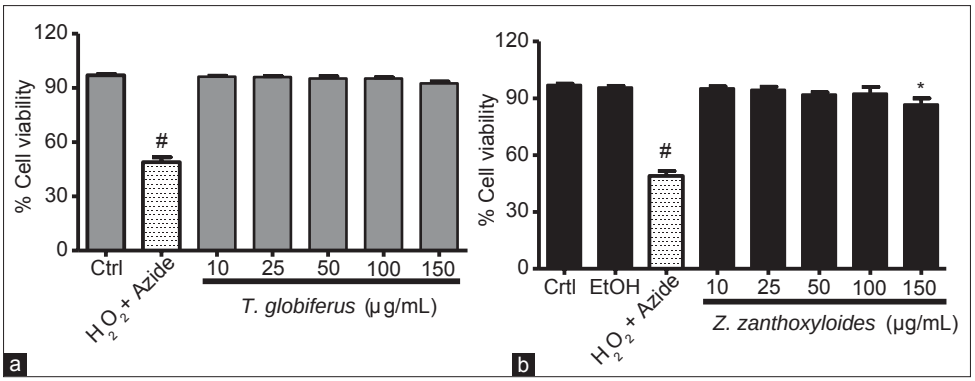

Figure 2: Survival of leukocytes treated with *Tapinanthus globiferus* (a) and *Zanthoxylum zanthoxyloides* (b) for 3 h. Results are expressed as mean ± standard error of the mean, $n = 4$. H_2O_2 (2 mM) + azide (1 mM) was used as positive control. #$P < 0.001$ versus control (Ctrl), *$P < 0.05$ versus Ctrl. *T. globiferus* was not cytotoxic to leukocytes at the concentrations tested, while, *Z. zanthoxyloides* does at the highest concentration

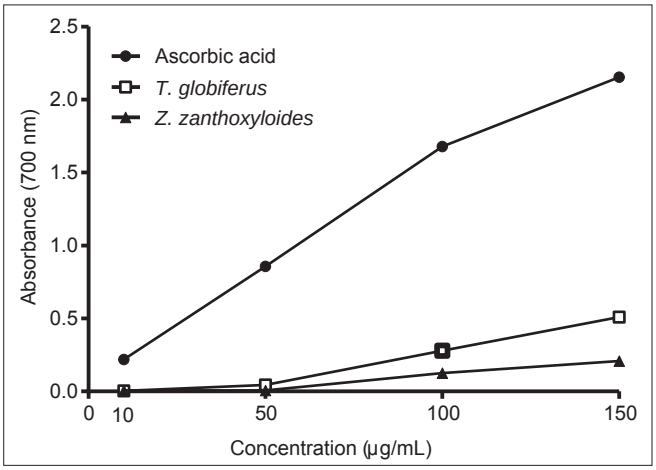

Figure 3: Reductive ability of the leaves extract of *Tapinanthus globiferus* and *Zanthoxylum zanthoxyloides* stem bark extract versus ascorbic acid. Values expressed in absorbance are the mean ± standard error of the mean of $n = 4$ performed in duplicates

DISCUSSION

Although medicinal plants are regarded as safe, there is increasing evidence that plant extracts and/or their chemical constituents can have toxic effects [24]. Therefore, the toxicity evaluation of plant extracts used in folk medicine is highly recommended. In the present study, the genotoxicity and cytotoxicity effects of *T. globiferus* and *Z. zanthoxyloides* were investigated in human leukocytes, as well as their reducing potential. The results demonstrated that *T. globiferus* was neither genotoxic nor cytotoxic to human leukocytes at all the concentrations tested. However, *Z. zanthoxyloides* was genotoxic and cytotoxic at the highest concentration tested (150 µg/mL). These results indicate that the use of *T. globiferus* at relatively high concentrations could be regarded as safe. The genotoxicity and cytotoxicity effects of *Z. zanthoxyloides* at the highest concentration tested leads to DNA damage, an indication of the presence of chemical constituents which interacted with DNA, leading to damage. Another explanation could be a synergistic interaction of compounds within the plant extracts resulting in the observed damage to DNA [25]. Although the comet assay has been criticized for the agarose concentration [15,26], it has become the most popular method for measuring DNA damage of various sorts, including oxidative damage inflicted by ROS [16,26].

Natural antioxidants found in plants and vegetables are extensively studied for their ability to protect the organism and cells from the deleterious effects induced by oxidative stress [27-29]. In previous studies, *T. globiferus* and *Z. zanthoxyloides* have shown antioxidant activity by the 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging and iron chelating activities [30,31]. In this study, the reductive potential of *T. globiferus* and *Z. zanthoxyloides* was determined on the basis that this assay has a different mechanism of action in relation to DPPH and iron chelating assays. In addition, reducing power of a compound is associated with antioxidant activity and may serve as a significant reflection of its potential antioxidant capacity [20,32]. This assay is based on the reduction of Fe^{3+}/ferricyanide complex to the Fe^{2+} form in the presence of antioxidant. The reduction is observed by the change of the yellow test solution to green or blue color depending on the reducing power of antioxidant samples. In addition, a higher absorbance indicates a higher ferric reducing power. Here, *T. globiferus* and *Z. zanthoxyloides* showed increased ferric reducing power with an increased concentration as ascorbic acid, indicating that both plant extracts have antioxidant activity. In the agreement to this, Amarowicz and Troszynska [33] demonstrated a direct relationship between reducing power and antioxidant activity. Consequently, the reducing power of these plant extracts may be associated with the antioxidant activity of phenolic acids and flavonoids found in these extracts.

CONCLUSION

The safety evaluation of *T. globiferus* and *Z. zanthoxyloides* revealed that *T. globiferus* (10-150 µg/mL) was neither genotoxic nor cytotoxic to human leukocytes following 3 h exposure. This indicates that its popular use in infusion might be considered safe for consumption. In contrast, *Z. zanthoxyloides* at the highest concentration tested (150 µg/mL) showed genotoxicity and cytotoxicity effects, therefore not safe for consumption. Both plants showed antioxidant activity as evidenced by their

reducing power potential, which can be attributed at least, in part, to their flavonoid and phenolic contents.

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Original Research

Role of fumaric acid in anti-inflammatory and analgesic activities of a *Fumaria indica* extracts

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ABSTRACT

Aim: The aim was to test whether the ethanolic extract of *Fumaria indica* (FI) possesses anti-inflammatory and analgesic activities, and fumaric acid (FA) could be one of its bioactive constituent involved in such activities of the extract. **Materials and Methods:** For anti-inflammatory activity, carrageenan-induced edema and cotton pellet induced granuloma tests in rats and for analgesic activity rat tail flick test and hot plate and acetic acid writhing tests in mice were used. All tests were performed after seven daily oral doses of the FI extract (100, 200, and 400 mg/kg/day) and pure FA (1.25, 2.50, and 5.00 mg/kg/day). **Results:** Anti-inflammatory activities of FI and FA were observed in carrageenan-induced edema and cotton pallet granuloma even after their lowest tested doses. No analgesic activity of lowest tested dose of FA was observed in the acetic acid writhing test, but likewise, all tested dose levels of FI, higher tested dose levels of FA were also possess significant analgesic activity in this test. Further, significant analgesic activities of both FI and FA in hot plate and tale flick tests were observed after all their tested doses. **Conclusions:** These observations are in agreement with our working hypothesis on the connection of FA in mode(s) of action(s) of FI, and reaffirm the conviction that FI could be an herbal alternative against fibromyalgia and other pathologies often associate with, or caused by, inflammatory processes.

KEY WORDS: Analgesic, anti-inflammatory, central sensitivity syndrome, central nervous system function *Fumaria indica*, fumaric acid

INTRODUCTION

Increased sensitivity of the central nervous system to painful stimuli is encountered in numerous chronic inflammatory diseases. A group of psychiatric and pathological symptoms that accompany increased central sensitivity are now often referred to as "central sensitivity syndrome" (CSS) [1]. Comorbidities of depression, anxiety, and other mental health problems are often encountered in almost all patients suffering from CSS. Available, psychoactive, analgesic, anti-inflammatory, and other drugs now commonly used for treatments of chronic diseases with CSS do not appropriately meet the therapeutic demands of patients, and as yet little concentrated efforts have been made to discover and develop drugs potentially useful for prevention or cure of enhanced central sensitivity to pain. Preclinical and therapeutic observations made with diverse types of extracts of several traditionally known medicinal plants have revealed that repeated daily doses of some of them alters central sensitivity to environmental and metabolic stress. Several such herbal preparations, now commonly referred to as herbal adaptogens, are some of the more popular herbal remedies often used not only in traditionally known medical practices, but also are now often recommended by practitioners and scholars of integrative medicine well-trained in modern medical sciences [2]. Despite extensive efforts though, many questions concerning their active principles and modes of action remain open, and their therapeutic potentials for treatments of CSS associated hyperalgesia still remain at the best speculative only.

Fumaria indica (FI) Linn., a wildly growing weed of the fumariaceae family, is one such traditionally known medicinal plant widely used for diverse therapeutic purposes in ayurvedic and other traditionally known medical systems of India and other Asiatic countries. Extensive efforts made by medicinal phytochemists and pharmacologists during several more recent decades have revealed diverse therapeutically interesting bioactivities of different types of extracts of the plant, and numerous structurally and functionally diverse secondary metabolites of the plant are now known also [3]. More recent observations made with a hydro alcoholic extract of FI have revealed that repeated daily oral doses of the extract effectively suppresses central sensitivity to metabolic as well as environmental stress, and that fumaric acid (FA) and its conjugates could as well be its quantitatively major bioactive constituents involved in its anti-stress or adaptogenic activities [4,5].

It had earlier been suggested that monomethyl-fumarate is one of the hepatoprotective constituent of FI extracts [6], and a more recent report has revealed anti-inflammatory and analgesic activities of a hydro alcoholic extract of the plant in animal models [7]. However, in this later-mentioned report only acute oral doses of the tested extract were tested, and the fumarate contents of the extract were not quantified. Since acute dose effects of drugs and other bioactive agents are often not identical and can even be opposite [8,9], it was of interest to test whether anti-inflammatory and analgesic activities of FI can be detected after its repeated daily doses and whether FA is also its bioactive constituent involved in such activities of the extract. Results of the very first set of experiments conducted to verify such possibilities experimentally were described and discussed in this communication.

MATERIALS AND METHODS

Animals

Adult Charles Foster albino rats (150 ± 10 g) and Wistar mice (20 ± 5 g) were obtained from the Central Animal House, Institute of Medical Sciences, Banaras Hindu University, Varanasi, and were randomly distributed into different experimental groups of 6 animals each. The animals were housed in polypropylene cages at an ambient temperature of 25 ± 1°C and 45-55% relative humidity, with a 12:12 h light/dark cycle. Unless stated otherwise, the animals were always provided with commercial food pellets and water *ad libitum*. They were acclimatized to laboratory conditions for at least 1 week before using them for the experiments. Principles of laboratory animal care (NIH publication number #85-23, revised in 1985) guidelines were always followed, and prior approval of Central Animal Ethical Committee (Dean/11-12/CAEC/324) of Banaras Hindu University was obtained.

Plant Material and Extraction

A shade-dried and pulverized sample of FI was thoroughly extracted using a soxhlet apparatus and 50% ethanol (v/v) as the extraction solvent. The plant was collected locally in Varanasi, and it was botanically authenticated by Prof. N. K. Dubey, Department of Botany, Faculty of Science, Banaras Hindu University. An herbarium specimen of the plant has been preserved (specimen voucher, January 01, 2009) for reference purposes. Yield of the dried FI extract obtained from the dried plant sample was 10.17% w/w.

Contents of FA and its Conjugates in FI

FA is one of the quantitatively major extractable secondary metabolite of FI, and fairly high oral doses of its esters are now therapeutically used for treatments of inflammatory diseases such as psoriasis and multiple sclerosis [10-14]. Therefore, free and conjugated FA contents of FI were quantified by a well-standardized analytical procedure using high-performance thin layer chromatography and CAMAG TLC Scanner - III and Camag Linomat applicator IV. Commercially available FA and the di-methyl fumarate (Sigma-Aldrich, USA) were used as authentic markers. For quantifying free FA FI and pure FA were dissolved in methanol. For the estimation of FA conjugates, FI and pure di-methyl fumarate were dissolved in 50 mL of 5N HCL and refluxed for 2 h and the hydrolyzed samples were dried on a water bath and re-dissolved in methanol. The test samples and FA solutions of known concentration were applied to pre-coated silica gel plate (Merck $60F_{254}$) and developed up to 90 mm using a solvent system consisting of formic acid:chloroform:butanol:heptane (12:16:32:44). The developed plates were dried and scanned under absorbance mode (scanning wavelength λ 260 nm), and calculations were based on the area of peaks of the sample and corresponding authentic marker FA. Free FA content of FI was 0.45% (w/w), and that of its conjugates (calculated as the di-methyl fumarate) was 0.35% (w/w).

Drugs and Chemicals

Analytically pure FA and dimethyl fumarate were purchased from Sigma-Aldrich, USA. The reference anti-inflammatory agents aspirin (Alkem Laboratories Ltd., India) and indomethacin (Sun Pharmaceutical Industries Ltd., India); and a centrally acting analgesic pentazocine (Ranbaxy, India) were used. Other chemicals and reagents were of the purest grade available from local suppliers.

Treatments

All test agents and the reference drugs were suspended in 0.3% carboxymethyl cellulose for oral administrations. The control groups were always treated with the vehicle. Doses of FI (100, 200, and 400 mg/kg/day) or FA (1.25, 2.5 and 5.0 mg/kg/day) were administered once daily for 7 consecutive days, and their last doses were administered 1 h before the tests. The treatment regimen and doses of FI were based on our earlier observation dealing with psychopharmacological activity profile of the extract. Choice of the dose range of FA used was based on the total fumarate contents of FI (0.8%) and diverse reports on anti-inflammatory or immune function modulating effects of FA esters. Unless stated otherwise, the

reference drugs aspirin (100 mg/kg), indomethacin (5 mg/kg) and pentazocine (30 mg/kg) were orally administered only once (i.e. only 1 h before the tests).

Carrageenan Induced Edema Test

The test procedure described by Winter *et al.* [15] and well standardizes in our laboratories [16] was used. In short, 0.1 mL of a 1% carrageenan suspension in saline was injected in the sub-plantar region of the left hind paw of male rats, and the paw was marked with ink at the level the lateral malleous of the injected paw. The paw volume (mL) was measured before and 3 h after carrageenan injection using a mercury plethysmography. The edema volume was calculated.

Cotton Pellet Granuloma Tests

Sub-acute inflammation was induced in rats by implanting cotton pellets according to the method described by Winter and Potter [17]. In short, sterile cotton (50 ± 1 mg) soaked in 0.2 mL of distilled water containing penicillin (0.1 mg) and streptomycin (0.13 mg) was implanted bilaterally in axilla under the ether anesthesia. The animals were sacrificed on the 7th day. Resulting granulation tissues with a cotton pellet were dried at 60°C overnight and weighed. The weight (mg) of the cotton pellet before implantation was subtracted from the dried weight of dissected granuloma pellet. In this test, not only FI and FA, but also the reference drug, indomethacin, were administered for 7 consecutive days, beginning from the day of cotton pellet implant.

Tail Flick Test

The method described by Davies *et al.* [18] was followed. In short, a rat was placed in a holder, with its tail coming out through a slot in the lid. The tail was kept on the bridge of an analgesiometer (Techno, India) jacket with an electrically heated nichrome wire underneath. The tail received radiant heat from the wire, heated by passing current of 6 mA. Through the water jacket, cold water was continuously passed, so that the bridge did not get heated and tail could be conveniently placed over the bridge. The time taken for the withdrawal of the tail after switching on the current was taken as the latency period, in seconds, of "tail flicking" response. This latency period was considered as an index of nociception. The cut off time for determination of latency period was 30 s [19]. Tail flick latencies were assessed for each rat just before treatments on the test day and 1, 2, and 3 h thereafter, and the means of the tail-flick latencies were used for statistical analysis.

Hot Plate Test

Mice were screened by placing them on a hot plate maintained at 55 ± 1°C and recording the reaction time in seconds for forepaw licking or jumping [19]. Only those mice reacting within 15 sec and did not show large variation when tested on four separate occasions (each 15 min apart) were taken for the test. The preselected animals were used for the test, whereupon FI and FA were administered for 7 consecutive days and the reference analgesic pentazocine only once on the 7th day of the test. The time (s) for forepaw licking or jumping on the heated plate of the analgesiometer was taken as an index of algesic state of the animals.

Acetic Acid Writhing Test

Acetic acid solution (15 mg/mL) at the dose of 300 mg/kg body weight was injected intraperitoneally, and the number of writhings in the following 30 min period were counted [20]. A significant reduction in a number of writhings (N) by treatment was considered as a positive analgesic response.

Statistical Analysis

All data are expressed as means ± standard deviation (SD) for each treatment group. Mean ± SD were calculated for the observed values in each experimental group. Statistical analysis was performed by one-way analysis of variance followed by Student-Newman-Keuls multiple comparison tests. GraphPad Prism 5 (GraphPad Software Inc., La Jolla, California, USA) was used for statistical analysis.

RESULTS

Carrageenan Induced Edema

Results summarized in Figure 1, revealed that FI 100 mg/kg/day significantly inhibits carrageenan-induced inflammatory response ($P < 0.05$), and that its maximal efficacy in this test is achieved after its 200 mg/kg daily doses. On the other hand, efficacy of FA in this test increased in dose-dependent manner. It must be noted that with 200 mg/kg, only 1.6 mg/kg FA and its conjugates were administered, and the efficacy of FI after this dose was much higher than those of the highest tested daily dose (5 mg/kg) of pure FA. Quantitatively, the efficacy of a single 100 mg/kg dose of aspirin observed was somewhat higher than that of the maximally observed effect of FI.

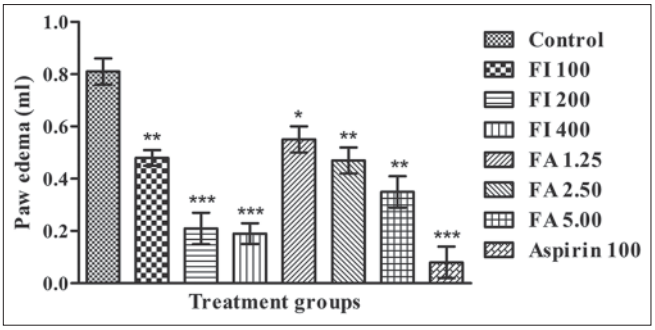

Figure 1: Effects of seven daily oral doses of FI extract and FA on carrageenan-induced paw edema in rats. FI: Ethanolic extract of *Fumaria indica*, FA: Fumaric acid, $n = 6$ animals in each group. Values are mean±standard deviation; *,** and ***$P < 0.05$, $P < 0.01$ and $P < 0.001$, respectively, in comparison to control

Cotton Pellet Induced Granuloma

Alike indomethacin, significant anti-inflammatory effects of both FI and FA were apparent in this test. Results summarized in Figure 2 reveal again that 200 mg/kg daily dose FI is almost the maximally effective one. The efficacy of this dose of FI was almost equal to that of 5 mg/kg daily doses of FA ($P < 0.01$).

Tail Flick Test

Results summarized in the Table 1 reveal that almost maximal possible analgesic activities of FI, as well as FA, were observed even after their lowest doses tested. Quantitatively, the efficacies of 100 mg/kg/day FI, or of 1.25 mg/kg/day FA were of the same order of magnitude as that of a single 30 mg/kg dose of the centrally acting analgesic pentazocine. Efficacies of all drug treatments were apparent 1 h after the treatments, which remained almost constant up to the last time point of measurements, i.e., 3 h ($P < 0.01$).

Hot Plate Test

It is apparent from Figure 3 that the mean reaction times of all test groups before treatments, or of the control Group 1 h thereafter, were almost equal and not significantly different from one another. Dose-dependent and pentazocine-like analgesic effect of FA was apparent on hour of the treatment, whereas such efficacies of FI after its all tested doses were almost equal.

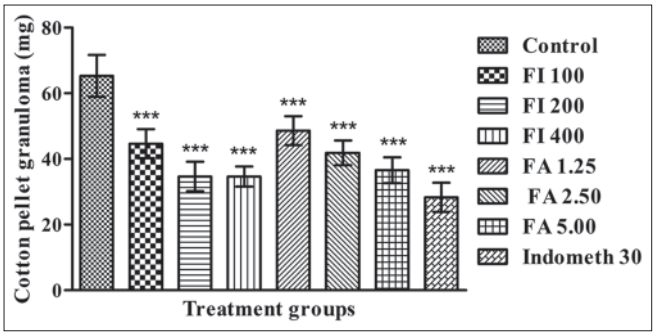

Figure 2: Effects of seven daily oral doses of FI extracts and those of FA in the rat cotton pellet granuloma test. FI: Ethanolic extract of *Fumaria indica*, FA: Fumaric acid, Indometh: Indomethacin, $n = 6$ animals in each group. Values are mean ± standard deviation, ***$P < 0.001$ in comparison to control

Analgesic efficacies of the highest tested doses of FI (200 and 400 mg/kg/day) and that of FA (5 mg/kg/day) were almost equal but were somewhat lower than that of a single oral dose of pentazocine (30 mg/kg).

Acetic acid writhing test: Statistically significant aspirin-like analgesic activity of FI was observed only after its 200 and 400 mg/kg daily doses, and that of FA after its highest dose (5 mg/kg/day) tested (Figure 4) ($P < 0.05$). Quantitatively, the observed effect of a single oral dose of aspirin was higher than those of the highest doses of FI or FA tested.

DISCUSSION

Observations reported in this communication not only add further experimental evidence in favor of to our conviction that repeated daily treatments with FI suppresses central sensitivity to environmental stimuli, but also strongly suggest that FA is quantitatively the major, but not the only, therapeutically interesting bioactive constituent of the extract. In addition, they reveal that daily treatment with fairly low oral doses of FA protects animal from peripheral inflammatory responses and that 200 mg/kg daily doses of FI is its maximally effective one for its anti-inflammatory and centrally acting analgesics like efficacies. However, quantitatively, the observed efficacies of FI cannot be explained by its analytically estimated FA contents only. Daily FA doses administered with 100 and 200 mg/kg/day doses of FI were 0.45 and 0.9 mg/kg, and the observed effects of 1.25 mg/kg/day doses of pure FA in both the models of inflammation were lower than that of 100 mg/kg/day doses

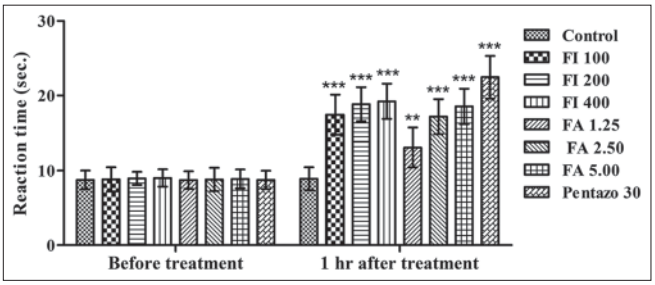

Figure 3: Effect of seven daily oral doses of FI extract and FA in mice hot plate test. FI: Ethanolic extract of *Fumaria indica*, FA: Fumaric acid, Pentazo: Pentazocine, $n = 6$ animals in each group. Values are mean ± standard deviation, ** and ***$P < 0.01$ and $P < 0.001$, respectively, in comparison to control

Table 1: Effects of FI extract and FA in rat tail flick test

Treatment	Dose (mg/kg)	Reaction time in seconds			
		0 h	1 h	2 h	3 h
Control	-	8.83±0.75	8.33±0.51	6.83±1.47	7.66±1.63
FI	100	9.16±0.75	17.5±1.87**,††	20.83±3.12**,††	19.5±1.87**,††
FI	200	7.83±0.73	16.16±1.60**,††	18.66±2.25**,††	19.16±2.7**,††
FI	400	10.16±1.16	18.66±0.80**,††	20.16±2.31**,††	19.16±2.22**,††
FA	1.25	8.78±0.82	16.24±1.38**,††	18.12±1.98**,††	18.04±1.62**,††
FA	2.50	9.06±0.74	17.58±1.65**,††	19.28±0.86**,††	18.16±1.64**,††
FA	5.00	8.82±0.90	17.85±1.52**,††	19.80±1.76**,††	19.54±2.18**,††
Pentazocine	30	7.66±1.21	18.83±3.60**,††	19.83±3.18**,††	21.16±3.86**,††

FI: Ethanolic extract of *Fumaria indica*, FA: Fumaric acid, n=6 animals in each group. Values are mean±SD, **P<0.01, respectively, in comparison to control, ††P<0.01 in comparison to 0 h. SD: Standard deviation

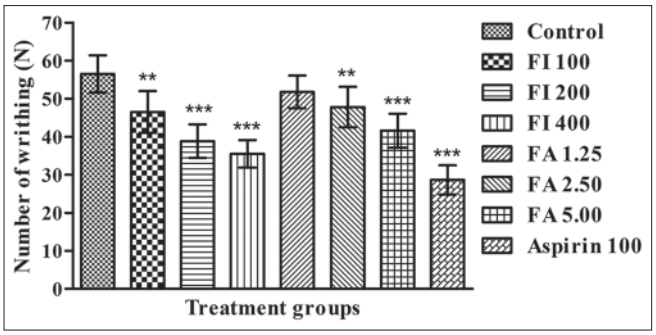

Figure 4: Effect of seven daily oral doses of FI and FA in mice writhing test. FI: Ethanolic extract of *Fumaria indica*, FA: Fumaric acid, $n = 6$ animals in each group. Values are mean ± standard deviation, ** and ***$P < 0.01$ and $P < 0.001$, respectively, in comparison to control

of FI. Analogous were also the observations made in both the rodent models for centrally acting analgesics used in this study.

Although clear dose-dependent and aspirin- or indomethacin-like anti-inflammatory activities of FA in carrageenan and granuloma tests were observed, only after its highest dose tested (5 mg/kg/day) significant aspirin-like analgesic activity of FA were detected in the acetic acid induced writhing test. Thus, it seems reasonable to assume that the biological mechanisms and processes involved in the observed anti-inflammatory activities of FA are not like those of non-steroidal anti-inflammatory drugs. Analogous were also the observations made with FI daily doses up to 200 mg/kg/day. However, some modest, but statistically significant, aspirin-like analgesic activity of FI was observed in the writhing test for peripheral analgesics. These observations could also indicate that apart from FA, FI contains some other bioactive components with mild anti-inflammatory and analgesic activities. Earlier observations in our laboratories have revealed that repeated daily administration of FI induces sedation in laboratory rodents [21,22]. Therefore, the possibility that it's observed analgesics like efficacies in this study could as well be due to behavioral alterations induced by its repeated daily doses. Further, more detailed studies will be necessary to clarify the situation. In any case, our results strongly indicate that FA is an anti-inflammatory constituent of FI.

FA was first isolated from another medicinal plant of the fumariaceae family, and therapeutic potentials of mixtures of FA and its conjugates for the treatment of psoriasis has also been known since long [10,11]. Numerous preclinical studies conducted during more recent decades have revealed that FA esters possess anti-inflammatory properties [12], and several properly controlled clinical trials have consistently demonstrated therapeutic efficacy of fairly high oral doses of methyl fumarates in patients with psoriasis and multiple scleroses [13,14]. However, the possibility that FA could as well be medicinally used as a safe and effective anti-inflammatory agent has never been experimentally verified. This is mainly because in comparison to its esters, oral bioavailability and cellular permeability of FA is almost negligible [23,24], and it is rapidly metabolized or catabolized after its oral intake.

In the light of these reports, the significant inhibitory effects of FA against peripheral inflammation and behavioral responses to thermal stimuli observed in this study after its lowest tested doses (1.25 mg/kg/day) seems to be due to its modulatory actions inside the gastrointestinal tract. Importance of gastrointestinal functions in regulating metabolic and mental processes are well-established and it is now becoming increasingly apparent that gut microbiota ecology and gut-brain axis plays crucial roles in dictating the delicate balance between health and diseases [25]. Therefore, it could as well be that modulating effects of FA on gut functions and its microbial population are also involved in the clinically observed beneficial effects of its esters. Since theoretically the FA conjugates present in FI could also be hydrolyzed to the parent acid, it is not impossible the observed quantitative discrepancies between the efficacy of FA and FI are mainly due to the presence of relatively high concentrations of FA conjugates [26,27]. Observations made during efforts to compare the efficacies of FA and its mono and dimethyl esters strongly suggest that such could indeed the case (unpublished data provided by the authors of this manuscript).

FA or its esters have often been reported to be the bioactive constituents of several medicinal plants [28-34], and it has since long been known that FA possesses antioxidant. Uses of FA enriched forage in veterinary medicine for promoting farm animal growth are also fairly common [35]. These facts taken together with the observations made to date with FI and FA in our laboratories, strongly suggest that efforts to identify the pharmacological targets and mechanisms involved in their therapeutically interesting pharmacological activity profiles could lead to the identification of novel non systemically acting therapeutic leads [4,5,21,22] potentially useful for prevention and cure of CSS in patients suffering from chronic inflammatory diseases. Moreover, since increased central sensitivity to metabolic stress is also often encountered in patients suffering from diabetes and other metabolic disorders and beneficial effects of FI has already been demonstrated in animal models [36]. A pharmacologically and analytically well-standardized FI extract could be more effective and cheaper herbal alternative for prevention and cure of such life-threatening medical conditions still spreading like epidemic in all countries.

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Original Research

Indigenous knowledge of plants in local healthcare management practices by tribal people of Malda district, India

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ABSTRACT

Aim: The present study was aimed at exploring the indigenous knowledge of native tribes on the utilization of wild plant species for local healthcare management in Malda district of West Bengal. **Materials and Methods:** Successive field surveys were carried out from July 2012 to August 2013 in search of traditional healers or practitioners who ceaselessly use their worthy knowledge to treat several ailments for human purposes. The information was collected by means of open-ended conversations, semi-structured questionnaire, group discussion, etc. Information obtained from the informants was also cross verified to check the authenticity. **Results:** The present study revealed that a total of 53 medicinal plants belonging to the 37 families are frequently used to treat 44 types of ailments with 88 herbal preparations. Of 53 plants, herbs possess the highest growth forms (32%) that were used in making traditional preparation, followed by shrubs (24%), trees (23%), climbers (17%), and parasites (4%). Roots comprised the major plant parts used (25%), followed by leaves (21%), seeds (17%), bark (13%), whole plant (8%) and fruits (6%) to prepare the medicinal formulations. The chief ailments treated in this province were azoospermia, diabetes, menstrual disorder, dysentery, rheumatism, etc. **Conclusion:** It can be concluded that the documentation of the ethnobotanical knowledge in management of local healthcare is the first step, which will open new door for the researchers in the field of modern drug development.

KEY WORDS: Ethnobotany, healthcare management, India, Malda district, tribal, West Bengal

INTRODUCTION

The knowledge of medicinal plants in India has been accumulated in course of many centuries based on several ancient medicinal systems, including ayurveda, unani and siddha [1]. According to the survey report of World Health Organization [2], 80% people of the developing world use plant remedies for several therapeutic purposes. India, one of the richest floristic regions of the world has diverse socio-economic, ethnic, linguistic and cultural areas. Therefore, the indigenous knowledge of medicinal plants and their use in treating several ailments might reasonably be expected in this country. Chandel *et al*. [3] have reported that nearly about 70% of tribal and rural inhabitants of India are to a large extent depended on medicinal plants for their primary healthcare management due to either insufficient or inaccessible or less availability of modern healthcare system. The information regarding the medicinal properties of plants came down traditionally generation after generation through traditional healers. Apart from the tribal groups, many other forest dwellers and rural people also possess unique knowledge regarding plant utilization.

Malda district of West Bengal, India [Figure 1] is situated between the latitude and longitude of 24°40′20″N to 25°32′08″N and 88°28′10″E to 87°45′50″E respectively with a total geographical area of 3455.66 sq km [4]. The district is characterised by its great archaeological relics such as Mourya Empire, Gupta Dynasty and Pala Dynasty. The region is covered with plentiful natural vegetation, which makes it verdant. River beds, ponds, marshy land etc. are good habitats for the wetland undergrowth. Most of the remote villages are covered by jungles, which consist chiefly of thorny scrub bush and large trees showing wide distribution of flora. The soil of the western region of the district is particularly suited to the growth of mulberry and mango, for which Malda has become famous. Various ethnic communities, including Santala, Rajbanshi, Namasudre, Polia, Oraon, Mundas, Malpaharias etc. are the inhabitants of this region. Of these Santala, Oraon is different from others due to their unique culture and tradition. They are quite popular to treat several types of local ailments of human and

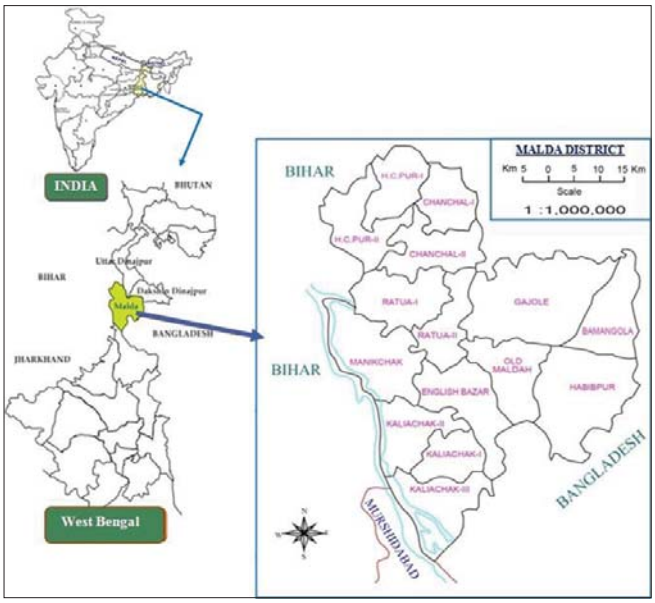

Figure 1: Map of study area (Malda district)

veterinary purposes [4]. Agriculture is the main source of income in the territory. Besides rearing of cattle, sheep, goats, fowls, etc. are the common practices among the tribal communities in this district. They also earn their livelihoods by selling milk, egg, flesh, etc., which plays a significant role in the rural economy of this district.

Preliminary floristic survey and a few numbers of folk usages of local plants had been studied for Malda district by Sur *et al.* [5,6], Pal and Das [7] and Chowdhury and Das [8], whereas Saha *et al.* [4] demonstrated a detailed picture regarding the ethnoveterinarian uses of plants. However, no detailed ethnomedicinal practices by local tribal communities had been done so far for this province. Hence, this is the first hand information on the ethnomedicinal usage by the ethnic people of Malda district as per author's best knowledge.

Now-a-days the traditional knowledge is in the way of erosion due to environmental degradation, deforestation, agricultural expansion and population pressure. Traditional knowledge of medicinal plants and their use by indigenous cultures are not only useful for conservation of cultural traditions and biodiversity but also for community healthcare and drug development at present and in the future. Therefore, recording of indigenous knowledge of medicinal plants is an urgent task. The objective of this study was to interact with local traditional healers and to document their knowledge on utilization of medicinal plants, their usage and the types of diseases treated, etc.

MATERIALS AND METHODS

Ethno Botanical Survey

The practice of medicinal plants is widespread among the tribal people of Malda district, and it is deeply rooted in their socioeconomic culture. However, the documentation of local medicinal practices is distinctly absent for the region. Considering the great cultural and ethnolinguistic diversity of the tribal people of the province, several field interviews were designed to cover as broad an area of the region as possible, in order to maximize the diversity of knowledge and the plant species employed in traditional remedy. The present survey was conducted during July 2012 to August 2013 in the district. Different interviewing procedures, including direct interview, group discussion, open-ended conversations, semi-structured questionnaire etc. were followed to get the information from the local traditional healers, known as Kavirajs, Baidyas or Ojhas and aged knowledgeable persons regarding the use of different medicinal plants curing several ailments. The purpose of this survey was explained to them in details, and prior informed consent was taken as per ethical guidelines of the International Society of Ethnobiology [9]. The villages were visited in different seasons to get the plant in its flowering condition. Plants were pointed out by the informants and their local names, used plant parts, formulation and dosages were also recorded.

Plant Collection and Identification

The plants were properly photographed, and herbarium was prepared for each specimen and deposited at Raiganj University College, Raiganj, India. The collected specimens were identified with the help of Central National Herbarium, Kolkata, India. The survey method followed in this study was that of the guided field-walk method as described by Jain [10] and the collection of voucher specimen, preservation, herbaria technique was followed as per Jain and Rao [11].

Total Key Informants

During the survey, we interacted with more than 100 informants and retained the information only from 74 informants. Among these, 55 were male (74.33%), and 19 were female (25.66%). More emphasis was given to the aged knowledgeable healers due to their vast experience in treating the local diseases and disorders. Kishori Barman (71 years), Uttam Kr. Mandal (53 years), Nargis Bibi (48 years), Farshed Ali (58 years), Fatema Begum (68 years), Basudeb Rajbanshi (55 years) Md. Subed Ali (44 years) etc. were the healers in the study area that we found.

Data Analysis

To analysis the data more clearly, obtaining from the informants, we set up our own database using Microsoft Access version 2007 and the parameters were name of the taxon, family name, voucher number, vernacular names, parts used, diseases treated, mode of administration or medicinal uses. We also analyzed the percentage between the used parts of plant species, growth forms of the species by putting them in the graph.

RESULTS

Plants Used

The present study revealed that a total of 53 medicinal plants belonging to 37 families were frequently used in the treatment

of 44 types of local ailments with 88 phytotherapeutic uses in the territory. The number of species most frequently used in the treatment of several disorders by each family was mentioned as Euphorbiaceae-6 species, Fabaceae-5 species, whereas Acanthaceae, Amaranthaceae, Vitaceae, Malvaceae, Solanaceae, Mimosaceae, and Zingiberaceae contributed 2 species to each family. The rest of 28 families were represented by 1 species in each. The scientific names of recorded species, their families, vernacular names, voucher number, used parts, mode of administration and local ethnic uses were illustrated in Table 1. Our study also exhibited that herbs were the most dominant growth forms with 17 species (32%), followed by 13 shrubs (24%), 12 trees (23%), 9 climbers (17%) and only 2 parasitic species (4%) treating different ailments as shown in Figure 2. *Andrographis paniculata*, *Amaranthus spinosus*, *Alstonia scholaris*, *Cuscuta reflexa*, *Jatropha gossypiifolia*, *Caesalpinia crista*, *Tamarindus indica*, *Sida rhombifolia* etc. were the most important plant used in the treatment of several diseases.

Parts of the Plant Used and Mode of Preparation

Various preparations of roots were used most number of occasions with 18 times (25%), followed by leaves with 15 times (21%), seeds with 12 times (17%), barks with 8 times (13%), whole plants with 6 times (8%), fruits with 4 times (6%), latex and gum with 3 times (4%) etc. as shown in Figure 3 in the treatment of several human disorders. A total of 88 types of formulations was being administrated to heal 44 types of ailments including azoospermia, diabetes, bone crack or ankle sprain, several types of pain, menstrual disorders, rheumatism, dysentery, etc. It had been observed that 20 types of diseases were healed by leaves, whereas 26 types of ailments cured by roots [Table 1]. A single plant part of same plant species was involved in treating different ailments and vice-versa.

The majority of remedies were prepared from fresh plant material in the form of a decoction, infusion or a paste. The most frequently used mode of remedy administration is oral ingestion, followed by external use. Most of the diseases and pains were usually treated either with a single plant or a mixture of plant parts. In some cases, ointments like mustered oil, ghee (a remedy from milk) etc. and other ingredients such as black peeper, ginger, curcuma, milk etc. were also used to make ethnic formulations along with the parts of plant species.

Diseases Treated and Medical Applications

A total of 44 types of diseases were reported to be cured in the present study. Azoospermia with 8 times was mostly healed disease in the study area, followed by different types of pains with 6 times, ankle sprain and diabetes with five occasions each whereas dysentery, inflammation, menstrual disorder, rheumatism, skin disorders, leucorrhea with 4 times each. Further, it can be concluded from Table 1 that the most of the preparations were oral except a few of external use. Various methods of preparation like crushing, grinding, direct use and homogenizing in water or with other plant extracts were used to prepare the traditional remedy. Mustered oil or ghee (a remedy from milk) was being utilized as an ointment at the time of external use such as itching, eczema, inflammation, pus, etc.

DISCUSSION

The prevalent diseases identified in the study area were azoospermia, ankle sprain, pain, diabetes, menstrual disorders, rheumatism, dysentery, skin disorders, etc. To expel ankle sprain or bone crack of local people, different plant parts like whole plant of *Cissus quadrangularis*, roots of *Tragia involucrata*, bark of *Litsea glutinosa*, bark of *Acacia catechu*, rhizome of *Alocasia macrorrhiza*, fruits of *Terminalia chebula* were administrated whereas eight plant species namely roots of *Bombax ceiba*, seeds of *C. reflexa*, *Ocimum kilimandscharicum* and *Abrus precatorius*, roots of *Curculigo orchioides* etc. were administrated to treat azoospermia [Table 1]. Diabetes was cured by means of leaf of *A. paniculata*, seeds of *Trigonella foenum-graecum*, seeds of *Syzygium cumini*, fruit of *Alpinia zerumbet* and whole parts of *Oxalis corniculata*. To treat menstrual disorders several plants had been utilized by the local traditional healers as explained in Table 1. There were few species used more than one occasion to prepare medicinal preparations curing different ailments, viz. *C. quadrangularis* known as harjora was used in bone crack and ankle sprain;

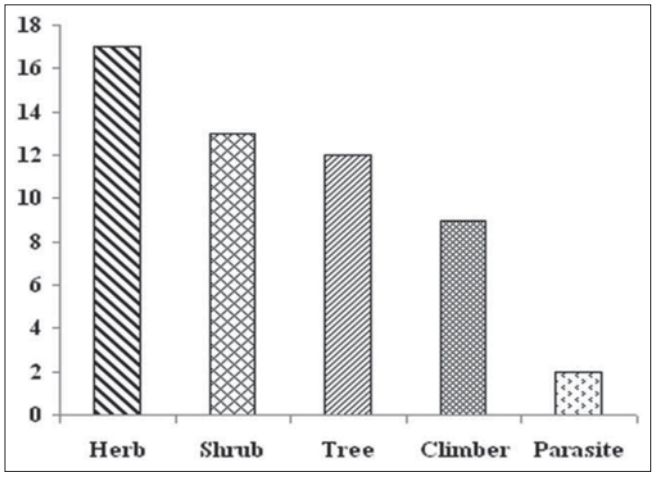

Figure 2: Growth forms of utilized species

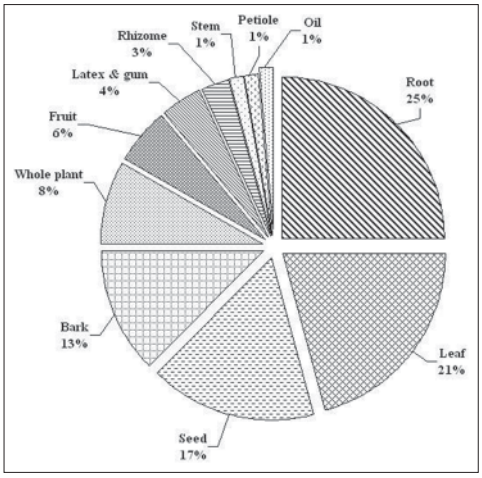

Figure 3: Pie chart of used plant parts

Table 1: List of medicinal plants investigated for local healthcare management with their ethnic use

Name of the plants/ voucher specimen number	Family	Local names	Parts used	Disease/formulation/administration
A. paniculata (Burm. f.) Wall. Ex Nees RUC/MLD-255	Acanthaceae	Kalmegh/ Mahatita/ Iswarnath	Leaf	Fever/Dysentry: The leaf is crushed and the juice is used to treat fever and chronic dysentery at early morning in empty stomach Diabetes: The leaf is grinded along with leaf of *S. chirata* to make a paste against diabetes. The formulation is taken twice a day for 2 months
H. hirta T. Ander. RUC/MLD-294	Acanthaceae	[Not Known]	Root	Bleeding piles: The root is crushed along with *Mentha* leaves and the paste is applied on rectum to stop bleeding piles for 2-3 weeks
A. spinosus L. RUC/MLD-251	Amaranthaceae	Kanta Khuria/ Kanta note	Root	Menstrual problem: The root is grinded and the decoction is mixed with milk and sugar to make a paste and used to treat irregular menstruation. The paste is taken twice in a day for 1 month Rheumatism: The root is grinded with sugar (slight), goat milk and mustered oil to make a paste and applied on affected area to treat rheumatism Cuts and wounds: The grinded root paste is used as an emollient on all types of cuts and wounds
A. aspera L. RUC/MLD-287	Amaranthaceae	Apang/Baro chirchiri	Root	Inflammation: The root is grinded with black pepper and the decoction is taken to treat inflammations in abdominal areas Pain: The roots are chewed with betel and lime to treat liver pain
F. vulgare Mill. RUC/MLD-286	Apiaceae	Mouri	Seed	Menstrual problem: The seeds with roots of *Ageratum conyzoides* and ginger are crushed and the juice is eaten to stop excessive blood discharge during menstruation Inflammation: The seeds along with the roots of *Sida rhombifolia* are crushed and the paste is applied to relief from inflammation of breast
A. scholaris (L.) R. Br. RUC/MLD-275	Apocynaceae	Chatim	Bark, leaf, latex	Anorexia: The bark decoction along with ginger is used to treat anorexia Pregnancy: The leaf decoction is feed to pregnant women to enhance delivery Pain: Latex is externally used in gum pain
A. macrorrhiza Schott. RUC/MLD-310	Araceae	Mankachu	Rhizome	Ankle sprain: The rhizomes are crushed along with ginger and mustered oil and slightly warmed. Finally, the paste is applied externally on ankle pain
C. rotang L. RUC/MLD-330	Aracaceae	Bet	Seed	Bronchitis: The seeds are dusted and mixed with cow-milk to treat bronchitis or cold and cough Skin disorders: Leaf paste along with seeds with *A. maxicana* is used externally in skin disorders
A. indica L. RUC/MLD-288	Aristolochiaceae	Iswarmul	Root	Impotency: The root is washed clearly and burned with the roots of *S. ovalifolia*. The ash is mixed with banana and taken in impotency of female
B. ceiba L. RUC/MLD-283	Bombacaceae	Simul (Beng.)	Root, gum	Azoospermia: The tender root (2-3-years-old tree) is crushed along with the roots of *C. orchioides* and used to induce sex and sperm production Laxative: Gum is used as an ingredient of laxative preparation
H. indicum L. RUC/MLD-277	Boraginaceae	Hatisur	Leaf	Dysentery: Leaf juice is used for curing dysentery and cough. Fresh leaf decoction is applied to wounds, boils and pruritus Conjunctivitis: The leaf juice is applied on eyes to cure eye disorders like inflammation, conjunctivitis etc.
T. chebula Retz. RUC/MLD-341	Combretaceae	Haritaki	Fruit	Bone crack: The *Cissus* stem (Harjora), *Litsea* stem (Daradmayda), *T. bellirica* fruit (Bahera) and an egg (white part) is crushed along with the Haritaki fruit, then the paste is applied externally on bone crack
C. reflexa Roxb. RUC/MLD-296	Convolvulaceae	Swarnalata/ Aloklata	Whole plant, seed	Nervous disorder: The plant is crushed with goat milk and the juice is feed to treat nervous disorder Azoospermia: The seed infusion is used to enhance sperm health and motility. The formulation is taken at night before sleep for 1-2 month
D. bulbifera L. RUC/MLD-411	Dioscoreaceae	Kham alu	Rhizome	Skin disorders: The rhizome is crushed along with leaf of *T. indica* and golmorich and the paste is applied as emollient in herpes, pusses and other skin diseases
D. montana Roxb. RUC/MLD-399	Ebenaceae	Choto gab/Ban gab	Bark, leaf	Leucoderma: The bark and leaves are together crushed and applied externally against leucoderma Diarrhoea: The decoction of bark is used against diarrhoea
J. gossypiifolia L. RUC/MLD-265	Euphorbiaceae	Varenda/ Jamalkota.	Leaf	Toothache: The leaves with salt and golmorich (2-3 pieces of seeds) are crushed and the paste used in toothache Abscesses: Leaf paste and latex are used as emollient on boils and abscesses Vomiting: Leaves juices are used to induce vomiting
P. reticulatus Poir. RUC/MLD-263	Euphorbiaceae	Panichitki	Root	Tumor: The root bark along with fruits of *Tamarindus* and zinger are crushed and slightly wormed then the paste is used as emollient on tumor
T. involucrata L. RUC/MLD-269	Euphorbiaceae	Bichatu/Bichuti	Root	Ankle sprain: The roots are crushed along with stem of harjora, curcuma and ginger to make paste and applied externally on broken leg and ankle sprain
E. tirucalli L. RUC/MLD-318	Euphorbiaceae	Shibjota	Stem	Galatogouge: The stem portion with leaf is crushed and the paste is taken orally to enhance milk production of women Pain: Stem is crushed along with zinger and the paste is applied as emollient on affected area to relief from pain

Contd...

Table 1: Contd.

Name of the plants/ voucher specimen number	Family	Local names	Parts used	Disease/formulation/administration
E. neriifolia L. RUC/MLD-305	Euphorbiaceae	Patsaij	Bark	Leucorrhea: Bark is crushed along with *P. betel* (3-5 pieces), lime and khoir (*A. catechu*) then the paste is taken orally to cure from leucorrhea
T. nudiflora L. RUC/MLD-343	Euphorbiaceae	Pithalu	Root	Enlargement of uterus: The roots are crushed and slightly warmed then it is applied externally until it cures
C. sophera L. RUC/MLD-279	Fabaceae	Kalkasunda/ Jhanjhane.	Root	Rheumatism: The root with ginger, garlic and black pepper are crushed and the paste is eaten to treat rheumatism
A. precatorius L. RUC/MLD-304	Fabaceae	Kunch (Beng.)	Seed	Pain: Seeds are crushed and paste is applied in stiffness of shoulder joint pain Azoospermia: Seeds are used to enhance sperm production
C. crista L. RUC/MLD-319	Fabaceae	Nata (Beng.)	Leaf, seed	Hydrocele: 3-4 pieces of apical leaf part are crushed with black pepper and taken to cure from hydrocele for 1 month Inflammation: Seed oil is applied externally against burning sensation of body
T. indica L. RUC/MLD-322	Fabaceae	Tetul (Beng.)	Fruit	Abdominal fat: Fresh fruits (1 kg) are boiled in water along with sugar (michri), and taken the juice twice to minimize abdominal fat Dysentery: Young fresh leaves are crushed along with sugar (michri), and the decoction is taken to treat dysentery
T. foenum-graecum L. RUC/MLD-333	Fabaceae	Methi	Seed	Kidney stone: The seeds are kept in a bowl of water then the decoction (1 glass) is taken at next morning in case of kidney stone for 15-20 days Diabetes: The seed powder is mixed with milk and taken at bed-time for 30 days against diabetes Dandruff: The seed paste is applied on head to prevent dandruff
C. orchioides Gaertn. RUC/MLD-335	Hypoxidaceae	Talmuli	Root	Azoospermia: The root (1-2 pieces) is chewed at every morning for 15-20 days to improve sperm production and motility
O. kilimandscharicum Guerke RUC/MLD-347	Lamiaceae	Dulal babu	Seed	Azoospermia: The seeds are taken in a bowl of water and left for whole night; then next morning it is crushed along with that water and taken to induce sperm production. The formulation is taken for 1 month
L. glutinosa (Lour.) C. B. Rob. RUC/MLD-259	Lauraceae	Daradmoyda	Bark, leaf	Bone crack: The stem bark is crushed along with harjora, curcuma to make a paste and applied as emollient on bone crack, ankle pain etc. Loose motion: Leaves are crushed and the juice is taken in case of loose motion
B. acutangula (L.) Gaertn. RUC/MLD-313	Lecythidaceae	Hizal (Beng.)	Bark, seed	Azoospermia: Bark is taken in a bowl of water and at the next morning the infusion is taken to condense watery semen for 30 days Sinus problem: The seeds are dusted and mixed with warm milk and then eaten at every evening for 1 month which effectively cure sinus problem
D. falcata (L.f.) Etting. RUC/MLD-340	Loranthaceae	Dharua	Bark	Menstrual problem: The bark is crushed along with bark of *S. indica*, fennel seeds and ginger and the juice is taken in case of irregular menstruation
S. rhombifolia L. RUC/MLD-310	Malvaceae	Peet Berala/ Bariala	Root	Abscesses: The roots are crushed with black pepper and areca nut and applied externally to cure from abscesses Inflammation: The roots and fennel seeds are crushed and the paste is used to relief from inflammation of breast
A. moschatus Medik. RUC/MLD-280	Malvaceae	Latakasturi (Beng.)	Seed, whole plant	Sex stimulant: Seeds are kept in a bowl for whole night. On the very next morning seeds are crushed along with roots of *C. orchioides* to make paste which act as sex stimulant and it enhances semen production
C. hirsutus (L.) Diels RUC/MLD-261	Menispermaceae	Jalkasha (Beng.)	Leaf	Azoospermia/late ejaculation: The leaf is crushed with water in a bowl and left for whole night and next morning the decoction is taken to induce semen production. It is also effective against late ejaculation
A. catechu Willd. RUC/MLD-326	Mimosaceae	Khoir	Bark	Ankle sprain: The bark is crushed along with harjora, curcuma, an egg and zinger to make a paste and applied externally on bone crack and ankle sprain Leucorrhea: The bark is crushed along with *P. betel*, lime and bark of *Euphorbia neriifolia* and then the paste is taken orally to cure from leucorrhea for 15-30 days
M. pudica L. RUC/MLD-307	Mimosaceae	Lajjabati (Beng.)	Root, leaf	Leucorrhea: Root decoction is used to treat leucorrhea for 20 days Breast Cancer: Leaves decoction is effectively used in breast cancer
F. benghalensis L. RUC/MLD-257	Moraceae	Bot	Latex, root	Nervous or body weakness: The latex mixed with sugar (batasa) are fed to induce semen production and in nervous or body weakness Rheumatism: The crushed apical prop root mixing with goat milk and sugar (batasa) are used to treat rheumatism
S. cumini (L.) Skeels RUC/MLD-337	Myrtaceae	Jam	Leaf, seed	Dysentery: Leaf is crushed along the leaf of *Tamarindus* sp. (tetul), michri (a type of sugar) and the roots of *Cephalandra* sp. (telakucha) and the paste is taken at empty stomach to prevent dysentery Diabetes: Seed powder is mixed with milk and taken twice a day in diabetes
O. corniculata L. RUC/MLD-320	Oxalidaceae	Amrul	Whole plant, root	Diabetes: The whole plant is crushed and juice is taken at early morning to prevent diabetes for 1-2 months Acidity/vomiting: The roots (3-4 pieces) are crushed with salt and taken to cure from acidity and vomiting

Contd...

Table 1: Contd.

Name of the plants/ voucher specimen number	Family	Local names	Parts used	Disease/formulation/administration
A. mexicana L. RUC/MLD-311	Papaveraceae	Siyal kata/ Gandhila	Seed, leaf	Skin disorders: The seeds are fried and crushed and then this seed-dust are mixed with coconut oil and applied on body to prevent skin disorders like eczema, pus etc Conjunctivitis: The leaf juice is applied on eyes to cure eye disorders like inflammation, conjunctivitis etc
P. emblica L. RUC/MLD-270	Phyllanthaceae	Amlaki (Beng.)	Fruit	Late ejaculation: Dried fruits are dusted and eaten at morning and night after meal, which is very useful to prevent late ejaculation. The formulation is taken for 1-2 months Stungury: Boiled fresh fruits with slight salt are taken for 20-25 days to treat stungury
P. betel L. RUC/MLD-317	Piperaceae	Pan	Leaf	Leucorrhea: Leaf is crushed along with stem bark of *E. nerifolia*, lime and fruit of *A. catechu* (khoir), the paste is taken orally to cure from leucorrhea
P. zeylanica L. RUC/MLD-250	Plumbaginaceae	Sadachita/ Agrochita	Root	Appetizer/blood enhancer: The root is crushed and the decoction used as an appetizer and also acts as blood enhancer
S. munja Roxb. RUC/MLD-289	Poaceae	Siki ghas/ Biyana	Root, oil	Allergy/pain: The roots are crushed with curcuma and zinger and the paste is applied externally to cure from allergy and body pain Lumbago: The oil extracted from leaves, are used to treat from lumbago
H. cordata Thunb. RUC/MLD-335	Saururaceae	Anstagach	Leaf	Vomiting: The leaves are crushed along with zinger and golmorich to induce vomiting
S. ovalifolia Roxb. RUC/ MLD-271	Smilaceae	Bagnocha/ Kumarilata	Whole plant, root	Impotency: The root is washed clearly and burned with the roots of *A. indica*. The ash is mixed with banana and taken in impotency of female Rheumatism: The whole plants are crushed with the bark of *C. religiosa* and the juice is taken to treat rheumatism for 2 months
S. xanthocarpum Sch. and Weldl. RUC/MLD-293	Solanaceae	Kantikari (Beng.)	Whole plant, root, seed	Conjunctivitis: Whole plant is burned along with peyaj and used as emollient on eyes to cure from conjunctivitis Pain: Roots and seeds are crushed along with the stem of *E. tirucalli* to make paste and applied externally to treat chest pain
D. metel L. RUC/MLD-328	Solanaceae	Kalo Dhutura	Root, leaf	Paralysis: The roots are crushed along with mustered oil, ghee (a remedy from milk), black pepper, curcuma and sindur, and then used as an emollient on paralyzed area until it cures Hair growth: The leaves are crushed and applied on head for over night, and washed off by tea-liquor to promote new hair growth. It is applied for 10-12 days
A. augusta (L.) L. f RUC/MLD-300	Sterculiaceae	Ulatkambal	Petiole, bark	Azoospermia: The petiole is crushed and kept in a bowl of water for a whole night, then the infusion is taken at early morning at empty stomach as semen and sperm enhancer
C. quadrangularis L. RUC/MLD-312	Vitaceae	Harjora (Beng.)	Whole plant	Bone fracture/ankle sprain: The plant is crushed along with roots of *D. metel* (kalo dhutura), *Glycosmis* sp. (atiswar) leaves of *Tamarindus* sp. (tetul), ginger, salt and the pest is applied as emollient on bone fracture, ankle sprain (5-12 days)
C. trifolia (L.) Domin RUC/MLD-315	Vitaceae	Choto goaliarlata	Leaf	Menstrual disorder: The leaves are crushed along with roots of *A. aspera* (apang) and *Areca* fruit and the juice is taken on empty stomach at early morning to prevent irregular menstruation (20-30 days)
Z. cassumunar Roxb. RUC/MLD-323	Zingiberaceae	Ban ada/Bau ada	Rhizome	Ankle sprain: The rhizome is crushed along with ginger and roots of bichuti (*Tragia* sp.) and a paste is made which is used as emollient on broken bone and ankle sprain
A. zerumbet (Pers.) Burtt & Smith RUC/MLD-314	Zingiberaceae	Elach	Fruit	Diabetes: Fruit (10-12 pieces) is crushed along with *Musa* stem, (3-4 pieces; 10 cm each) *I. aquatica* (kalmi sag), leaf of *N. indicum* and pinch of michri (remedy of sugar) and then the extract juice is taken orally to treat diabetes (30-45 days)

A. paniculata: Andrographis paniculata, H. hirta: Hemigraphis hirta, A. spinosus: Amaranthus spinosus, A. aspera: Achyranthes aspera, F. vulgare: Foeniculum vulgare, A. scholaris: Alstonia scholaris, A. macrorrhiza: Alocasia macrorrhiza, C. rotang: Calamus rotang, A. indica: Aristolochia indica, B. ceiba: Bombax ceiba, H. indicum: Heliotropium indicum, T. chebula: Terminalia chebula, C. reflexa: Cuscuta reflexa, D. bulbifera: Dioscorea bulbifera, D. montana: Diospyros montana, J. gossypiifolia: Jatropha gossypiifolia, P. reticulatus: Phyllanthus reticulatus, T. involucrata: Tragia involucrata, E. tirucalli: Euphorbia tirucalli, E. neriifolia: Euphorbia neriifolia, T. nudiflora: Trewia nudiflora, C. sophera: Cassia sophera, A. precatorius: Abrus precatorius, C. crista: Caesalpinia crista, T. indica: Tamarindus indica, T. foenum-graecum: Trigonella foenum-graecum, C. orchioides: Curculigo orchioides, O. kilimandscharicum: Ocimum kilimandscharicum, L. glutinosa: Litsea glutinosa, B. acutangula: Barringtonia acutangula, D. falcata: Dendrophthoe falcata, S. rhombifolia: Sida rhombifolia, A. moschatus: Abelmoschus moschatus, C. hirsutus: Cocculus hirsutus, A. catechu: Acacia catechu, M. pudica: Mimosa pudica, F. benghalensis: Ficus benghalensis, S. cumini: Syzygium cumini, O. corniculata: Oxalis corniculata, A. mexicana: Argemone mexicana, P. emblica: Phyllanthus emblica, P. betel: Piper betel, P. zeylanica: Plumbago zeylanica, S. munja: Saccharum munja, H. cordata: Houttuynia cordata, S. ovalifolia: Smilax ovalifolia, S. xanthocarpum: Solanum xanthocarpum, D. metel: Datura metel, A. augusta: Abroma augusta, C. quadrangularis: Cissus quadrangularis, C. trifolia: Cayratia trifolia, Z. cassumunar: Zingiber cassumunar, A. zerumbet: Alpinia zerumbet, S. chirata: Swertia chirata, A. conyzoides: Ageratum conyzoides, A. maxicana: Argemone maxicana, T. bellirica: Terminalia bellirica, S. indica: Saraca indica, C. religiosa: Crateva religiosa, A. aspera: Achranthus aspera, I. aquatica: Ipomoea aquatica, N. indicum: Nerium indicum

A. spinosus was used to treat menstrual disorders, rheumatism, cuts and wounds; *T. foenum-graecum* was used against kidney stone, diabetes and dandruff problems.

As the tribal people remain busy throughout the year with their practice of livelihood from the agricultural sector, they rarely visit the hospitals in towns. Simultaneously, they cannot afford the cost of modern medicines. It has also been observed that some of the villages are in such remote areas where transportation facilities are inaccessible or sometimes become detached due to some natural calamities. Hence, the villagers cannot reach the nearby hospital. As a result, the ethnomedicinal practices are popular in the study area as it is more accessible, easy to prepare, low costs, and eco-friendly. Besides, the practice of medicinal plants treating the patients is an alternative source of income for the healers.

CONCLUSION

The present study exhibited that how different interviewing procedures helped to gather the information regarding the name of the diseases treated, plant resources and their usage, including their mode of administration. A total of 44 types of local ailments was treated with 88 phytotherapeutic uses in this district. The making procedure of herbal preparation is yet a secret and passed on generation after generation verbally. Proper analysis of herbal formulations and phytoconstituents of used plants can open new door for the researchers. However, ethnobotanical data is the basis of further validation of practices and plant uses in the context of a professional approach to develop new herbal drug [12].

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Original Research

Combination of lycopene, quercetin and poloxamer 188 alleviates anxiety and depression in 3-nitropropionic acid-induced Huntington's disease in rats

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ABSTRACT

Aim: The present study investigates synergistic effect of combination treatment of lycopene, quercetin and poloxamer188 in 3-nitropropionic acid (3-NP)-induced Huntington's disease (HD). **Materials and Methods:** Anxiety and depression were induced in male Wistar rat by intra-peritoneal administration of 3-NP (10 mg/kg) for 14 days. Body weight was assessed on day 1, 7 and 14, whereas locomotion, anxiety and depression were assessed at the end of the experiment. **Results:** Administration of 3-NP induces HD like symptoms and produced a significant decrease in body weight on day 7 ($P < 0.01$)and day 14 ($P < 0.001$), further decreased locomotion, time spent and number of entries in light area as well as increased immobility period were observed. The rats treated with lycopene and quercetin alone significantly restore the body weight and locomotion count as well as alleviate anxiety and depression. However, combination treatment of lycopene and quercetin with and without poloxamer 188 produced more significant effect on body weight compared to Huntington control rats, but no significant effect was found between the treated groups. However, significant increased on locomotion, time spent and number of entries in light area and decreased immobility period was observed in the combination treated groups when compared to single drug therapy. **Conclusion:** Combination treatment of lycopene and quercetin with and without poloxamer 188 in HD more effectively alleviate anxiety and depression than single drug therapy.

KEY WORDS: Huntington disease, 3-nitropropionic acid, lycopene, quercetin

INTRODUCTION

Huntington's disease (HD) is a neurodegenerative disorder characterized by progressive motor dysfunction, chorea, dystonia, emotional disturbances, memory and weight loss [1]. Today about 5-7 people/100,000 are affected by HD and in India pervasiveness of HD is somewhat higher [2]. Incidence of juvenile HD is in between 1% and 9.6% [3]. The symptoms of HD usually develop at the age of 30-50 and severity increases with age [4]. HD is caused by a CAG polyglutamine expansion, which is more than 35, produced malformed Huntington protein, results death of nerve cell in the basal ganglion [5-8]. The malformed protein also affects cellular pathways and hippocampal neurogenesis involved in mood disorders leads to depression and anxiety [9]. In caudate, putamen, and cingulate low glucose metabolism in HD results in dysfunctioning of paralimbic frontal lobes and basal ganglia due to energy impairment. This leads to depression as they are responsible for normal mood regulation [10].

7 (3-NP) is a well-versed experimental model to study HD in various animal models [11]. It produces mitochondrial dysfunction, oxidative stress and inhibits mitochondrial Complex II enzyme results in neuronal cell death due to energy impairment and apoptosis [12-14]. Further 3-NP increases the production of nitric oxide and depresses the spinal reflexes in a time-dependent manner that leads to depression [15]. Increased oxidative stress causes striatal damage and produce anxiety [16].

Currently, no treatment is available for HD, though some symptoms can be managed with medication such as antidepressant, antipsychotic and therapies such as physiotherapy and occupational therapy. Flavonoids (or bioflavonoid) are a class of plant secondary metabolites reported as antioxidant, anti-allergic, anti-cancer, antioxidant, anti-inflammatory and anti-viral [17]. Carotenoids are organic pigments found in the chloroplasts of bacteria and fungi. It decreases the risk of disease particularly certain cancers and eye disease due to its antioxidant potential [18].

Several studies reported synergistic effect of bioflavonoid and carotenoids combinations [19]. Further, lycopene and quercetin in combination with tyrosol were proved synergistic effect in the prevention of macrophage activation [20]. The treatment with lycopene significantly improved memory and restored glutathione system functioning and hence lycopene could be used to manage 3-NP induced behavioral and biochemical alterations [21]. Quercetin improved mitochondrial dysfunctions and antioxidant status and also ameliorate behavioral deficits along with histopathological changes [22]. Poloxamer 188, which is a polymer has ability to repair damaged cell membranes by increasing the lipid packing density as it provides mechanical sealing to neurons [23].

Therefore the present study designed to investigates synergistic effect of combinatorial treatment of lycopene, quercetin and poloxamer 188 on anxiety and depression in 3-NP-induced HD in Wistar rats.

MATERIALS AND METHODS

Drugs and Chemical

Lycopene was obtained as a gift sample from Zedip formulation (Ahemdabad, India). Quercetin and poloxamer 188 were procured from Research Lab Fine Chem. Industries (Mumbai, India). 3-NP was purchased from Sigma Aldrich (USA). All other reagents and chemicals were of analytical grade and purchased from local suppliers of Pune.

Animals

Male Wistar rats (200-250 g) were procured from National Institute of Biosciences, Pune. Rats were randomly placed separately in polypropylene cages with paddy husk as bedding. They were housed in environmentally controlled conditions (24 ± 2°C, 12 h light/12 h dark cycle), with free access to the standard diet (Nutrivet Lab., Pune) *ad libitum*. All the experimental procedures and protocols used in this study were reviewed and approved (SCOP/Institutional Animal Ethics Committee [IAEC]/2013/14/155) by the IAEC of Sinhgad College of Pharmacy, Pune, constituted under Committee for Purpose of Control and Supervision of Experiments on Animals by Ministry of Environment and Forests, Government of India, New Delhi, India. Ethical guidelines were strictly followed during all the experimental procedures. All efforts were made to minimize animal suffering and reduce the number of animals used.

Experimental Design

After 1 week of acclimatization, male Wistar rats were randomly divided into six groups ($n = 6$) and received treatment for 14 days. Group I served as control, received vehicle 1% gum acacia (w/v), Group II rats were injected with 3-NP served as Huntington control (HC), Group III-VI were injected with 3-NP and concomitantly treated orally with lycopene (25 mg/kg), quercetin (50 mg/kg), lycopene (25 mg/kg) and quercetin (50 mg/kg), and lycopene (25 mg/kg), quercetin (50 mg/kg) and poloxamer 188 (80 mg/kg) respectively. 3-NP was freshly prepared using distilled water and administered at a dose of 10 mg/kg body weight for 14 days. The lycopene, quercetin and poloxamer 188 were administered as a suspension prepared in 1% gum acacia (w/v) for 14 days. The behavioral observations were taken in between 9:00 A.M. and 11:00 A.M.

Evaluation of Body Weight

As the disease progresses swallowing become more difficult thereby decreased intake of calories results in body weight loss. Body weight was measured on 1st (before treatment), 7th, and 14th day post 3-NP administration using electronic weighing balance (Contech, C7-6K1)[24].

Evaluation of Locomotor Activity

As HD progresses, impairment in motor functions alters muscular movements and reduces the locomotor activity. Animals were placed in actophotometer where beam of light falls on photoelectric cells, and basal activity score was recorded over the period of 5 min that is recorded as no. of beams cut during locomotions [25].

Light and Dark Model

Neuropsychiatric problems are common in HD. Light-dark box is a potent and useful method for screening and detecting anxiolytics activity of a wide range of compounds with various modes of action. The instrument consists of 2 parts, 1/3 with opaque walls and covered (dark compartment), whereas the remaining 2/3 was open and illuminated (light compartment). The door between the two compartments permits rats to move from one side to another. Each rat was released in the light compartment and observed for 5 min. Time spent in light and dark compartment, and no. of entries in light, and dark compartment were recorded [26,27].

Forced Swim Test

Forced swim test is the most commonly used for assessment of depression in animal models. Rats were forced to swim in a cylinder from which they cannot escape. The water was deep enough, so the animal could not touch the bottom with its tail or feet. A depth of 30 cm is commonly recommended with temperature 24-30°C. Animals were observed continuously for 5 min during the swim test, and duration and time of immobility were measured. Any animal that sinks below the surface was removed from the water immediately [28,29].

Statistical Analysis

All the values are expressed as mean ± standard error of the mean ($n = 6$). The data were analyzed using one-way analysis of variance, followed by Tukey's multiple comparison tests. $P < 0.05$ were considered the minimum level of significance.

RESULTS

Body Weight

Intra-peritoneal administration of 3-NP for 14 days showed significant decrease in the body weight in HC rats when compared with control rats at day 7 and day 14 (13.24%, $P < 0.05$ and 26.37%, $P < 0.001$; respectively). Lycopene (25 mg/kg) and quercetin (50 mg/kg) as well as their combination with and without poloxamer 188 treatments in rats could not produce significant restoration in body weight on day 7. However, on 14th day significant restoration in the body weight was observed in rats treated with lycopene (23.16%) and quercetin (26.10%) when compared with HC rats ($P < 0.05$ and $P < 0.05$; respectively). Further, combination treatment of lycopene (25 mg/kg) and quercetin (50 mg/kg) with and without poloxamer 188 found to produce more significant restoration in the body weight compared with HC rats (29.68%; $P < 0.01$ and 32.88%; $P < 0.01$; respectively). However, no significant difference was found between the drug-treated groups [Table 1].

Locomotor Activity

Significant decrease in the number of locomotions was observed in 3-NP treated HC rats when compared with control rats ($P < 0.001$). Rats treated with quercetin (50 mg/kg) along with 3-NP restored the number of locomotion compared with HC rats ($P < 0.01$). Further, combination treatment of lycopene (25 mg/kg) and quercetin (50 mg/kg) with and without poloxamer188 more significantly restored the number of locomotions as compared to HC rats ($P < 0.001$ and $P < 0.001$; respectively). Rats treated with lycopene (25 mg/kg) could not produce significant change in the locomotion counts. However, rats treated with lycopene and quercetin with or without poloxamer showed a significant increase in locomotion count when compared with lycopene (25 mg/kg) treated rats ($P < 0.05$ and $P < 0.01$; respectively) [Figure 1].

Table 1: Effect of combination treatment of lycopene, quercetin and poloxamer 188 on body weight

Group	Body weight (g)		
	Day 1	Day 7	Day 14
Control	223.30±6.82	237.80±7.70	254.20±7.49
HC (10) [%]	230.35±6.31	206.30±3.23^{a} [13.24]	187.15±6.77^{c} [26.37]
L (25) (%)	233.05±5.61	235.37±8.95 (14.09)	230.50±9.75* (23.16)
Q (50) (%)	234.80±6.11	225.80±5.37 (9.45)	236.00±10.82* (26.10)
L (25)+Q (50) (%)	228.58±4.61	231.80±9.31 (12.36)	242.70±12.47$^{\#}$ (29.68)
L (25)+Q (50)+ P 188 (80) (%)	232.47±4.81	227.70±5.93 (10.37)	248.70±7.56$^{@}$ (32.88)

$^{a}P<0.05$ and $^{c}P<0.001$ versus control rats. $^{*}P<0.05$, $^{\#}P<0.01$ and $^{@}P<0.001$ versus HC rats. Values in parenthesis [ ] indicates percentage ↓ in comparison with control rats. Values in parenthesis ( ) indicates percentage ↑ in comparison with HC rats. HC: Huntington control, L: Lycopene, Q: Quercetin, P 188: Poloxamer-188

Light and Dark Model

Significantly decreased duration of time spent in light area and increased in the dark area was observed in HC rats injected with 3-NP for 14 days when compared with control rats ($P < 0.001$ and $P < 0.001$; respectively). Rats treated with lycopene and quercetin alone along with 3-NP increased the duration of time spent in light area ($P < 0.001$ and $P < 0.01$; respectively) and decreased in dark area ($P < 0.001$ and $P < 0.001$; respectively) when compared with HC rats. Further, combination treatment of lycopene (25 mg/kg) and quercetin (50 mg/kg) with and without poloxamer 188 more significantly increased the duration of time spent in light area ($P < 0.001$ and $P < 0.01$; respectively) and decrease in duration of time spent in dark area as compared to HC rats ($P < 0.001$ and $P < 0.001$; respectively). However the significant difference were observed in the rats treated with lycopene and quercetin combination with and without poloxamer 188 in the duration of time spent in light area ($P < 0.001$ and $P < 0.01$; respectively) and dark area ($P < 0.001$ and $P < 0.001$; respectively) when compared with quercetin treated rats. However, no significant change was observed in comparison with lycopene.

Further, a significant decrease in the number of entries in light area and increase in the dark area was observed in HC rats when compared with control rats ($P < 0.001$ and $P < 0.001$; respectively). Rats treated with lycopene and quercetin alone significantly increased the number of entries in light area ($P < 0.001$ and $P < 0.01$; respectively) and decreased in dark area ($P < 0.001$ and $P < 0.001$; respectively) when compared with HC rats. Further, combination treatment of lycopene (25 mg/kg) and quercetin (50 mg/kg) with and without poloxamer 188 more significantly increased the number of entries in light area ($P < 0.001$ and $P < 0.001$; respectively) and decrease in number of entries in dark area as compared to HC rats ($P < 0.001$ and $P < 0.001$; respectively). However, rats treated with lycopene, quercetin and poloxamer 188 combination produced significant decrease in the number of entries in the dark area when compared with lycopene, quercetin and their combination without poloxamer 188 treatment ($P < 0.001$, $P < 0.001$ and $P < 0.05$; respectively) [Figure 2].

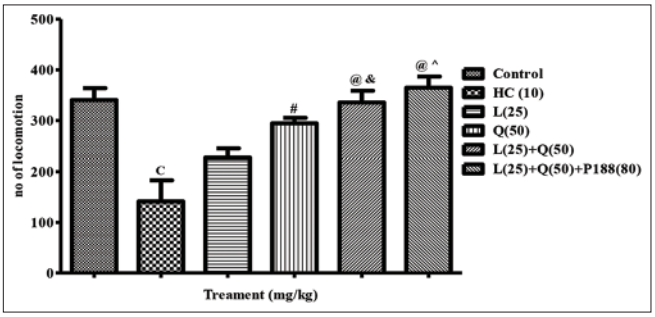

Figure 1: Effect of combination treatment of lycopene, quercetin and poloxamer188 on locomotor activity. $^{c}P < 0.001$ versus control; $^{\#}P < 0.01$, $^{@}P < 0.001$ versus HC; $^{\&}P < 0.05$, $^{\wedge}P < 0.01$ versus L (25), HC: Huntington control, P: Lycopene, Q: Quercetin, P 188: Poloxamer 188

Forced Swim Test

Significant decrease in the onset of immobility and increase duration of immobility was observed in HC rats when compared with control rats ($P < 0.001$ and $P < 0.001$; respectively). Rats treated with lycopene and quercetin alone along with 3-NP increase onset of immobility ($P < 0.001$ and $P < 0.05$; respectively) and decrease duration of immobility ($P < 0.001$ and $P < 0.01$; respectively) when compared with HC rats. Further, combination treatment of lycopene (25 mg/kg) and quercetin (50 mg/kg)with and without poloxamer 188 along with 3-NP more significantly increased onset of immobility ($P < 0.001$ and $P < 0.001$; respectively) and decreased duration of immobility compared with HC rats ($P < 0.001$ and $P < 0.001$; respectively). Further the significant difference was noted on the duration of immobility in lycopene and quercetin with or without poloxamer 188 treated rats, but on the onset of immobility significant difference was observed only with poloxamer188 combination when compared with quercetin treated rats ($P < 0.001$ and $P < 0.01$ and $P < 0.01$; respectively) [Figure 3].

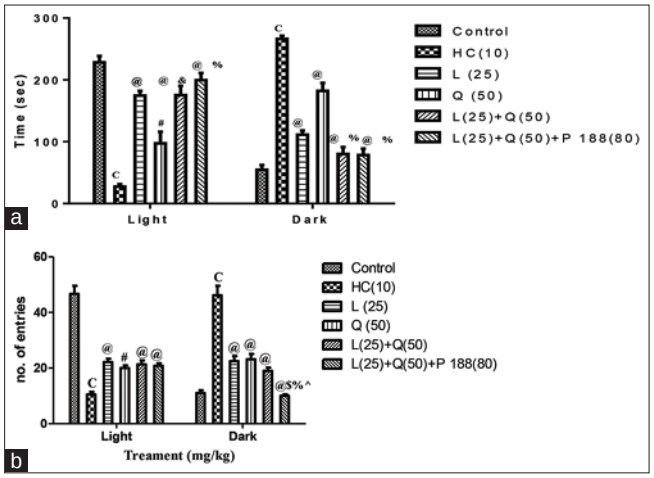

Figure 2: Effect of combination treatment of lycopene, quercetin and poloxamer188 on anxiety (a) time spent in light and dark area and (b) number of entries in light and dark area, $^{c}P < 0.001$ versus control; $^{\#}P < 0.01$ and $^{@}P < 0.001$ versus HC; $^{\&}P < 0.01$, $^{\%}P < 0.001$ versus Q (50); $^{\$}P < 0.001$ versus L (25); $^{\wedge}P < 0.05$ versus L (25) and Q (50) combination, HC: Huntington control, P: Lycopene, Q: Quercetin, P 188: Poloxamer 188

DISCUSSION

HD is a disorder in which nerve cells in certain parts of the brain waste away, or degenerate [30]. HD causes movement, psychiatric and cognitive difficulties [31]. In HD increase in oxidative stress and neuronal loss alters neurotransmitters that regulate mood results in depression and anxiety. In HD, there is a continual life change, which may be one of the sources of anxiety [32]. Strong evidences suggest involvement of energy impairment, excitotoxic processes, and apoptosis worsen the symptoms of HD. Striatum and hippocampus are more affected because nerve cells of the striatum are first to die as HD progresses [33]. HD is not a prevalent within any particular population, races, ethnic groups, sexes. Epidemiology of HD is less as compared to other diseases, but increasing prevalence suggests that there is a new scope for further research on HD [34].

Current medical therapies use pharmaceutical interventions with lifestyle modification to prevent or control HD. Various hypotheses including molecular genetics, oxidative stress, excitotoxicity, metabolic dysfunction, and mitochondrial impairment have been proposed to explain the pathogenesis HD despite to that there is no treatment available fully to stop the progression of the disease [35].

3-NP is known to produces behavioral, biochemical and morphologic changes in animals associated with HD similar to those occurring in human [36]. Intra-peritoneal administration of 3-NP increases oxidative stress, gait impairment and neurodegeneration [37]. Further, it produces depression and anxiety via energy impairment and striatal lesion. It is an irreversible inhibitor of succinic acid dehydrogenase (Complex II) and induces neuronal disorders in rats similar to those in patients with HD [13].

Bioflavonoid and carotenoid are upcoming molecules that can be used for a number of disease with less or no side-effect. They have excellent properties that help to reduce or cure the symptoms of many diseases [38]. Research proved that carotenoids and bioflavonoid have a complementary effect, making them both more effective when administer together rather than separately. The combinations of these are used to treat different diseases such as cancer, diabetes, asthma, coronary diseases, etc. [39-41]. Previous study

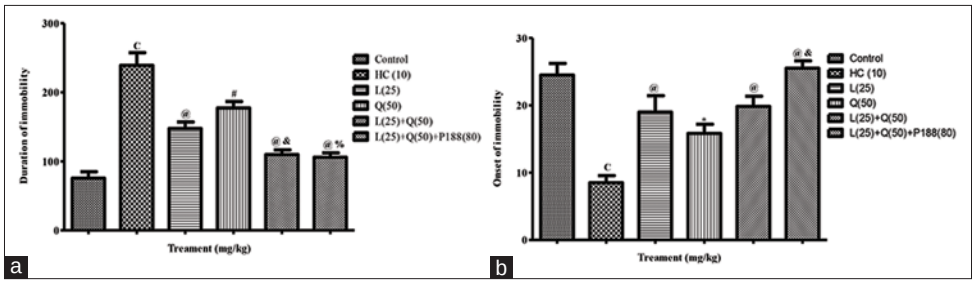

Figure 3: Effect of combination treatment of lycopene, quercetin and poloxamer188 on depression (a) duration of immobility (b) onset of immobility. $^{c}P < 0.001$ versus control; $^{*}P < 0.05$, $^{\#}P < 0.01$ and $^{@}P < 0.001$ versus HC; $^{\&}P < 0.01$, $^{\%}P < 0.001$ versus Q (50) HC: Huntington control, P: Lycopene, Q: Quercetin, P 188: Poloxamer 188

showed that lycopene has strong antioxidant properties, and quercetin has anti-inflammatory property with the addition of anticancer, anti-diabetes, anti-asthematic activity [42,43]. Lycopene treatment significantly attenuates the impairment in behavioral, biochemical and mitochondrial dysfunction as well as glutathione depletion that cures depression and anxiety [21]. Quercetin significantly decreases the concentration of malondialdehyde (an indicator of lipid peroxidation) and glutathione reductase activity [44]. In addition, poloxamer, 188 which is a polymer has the ability to repair damaged cell membranes by increasing the lipid packing density as it provides mechanical sealing to neurons [23]. Based on this evidences in our investigation we use the combinations of lycopene (caretonoid) and quercetin (bioflavonoid) with and without poloxamer 188 to study synergistic effect in alleviating anxiety and depression.

As HD progresses, there is strong impairment in motor functions that alter muscular movements and reduced locomotor activity [25]. As per the reports intra-peritoneal administration of 3-NP cause muscular atrophy which leads to decrease in locomotion counts. Reduced number of locomotion also indicates central nervous system (CNS) depression which is one of the symptoms of HD. In the present study, 14 days administration of 3-NP produced significant decreased in locomotions that are in agreement of the previous reports. Administration of lycopene and quercetin separately significantly increases the number of locomotion as compared to HC group, which may be due to as they reduce oxidative stress and inflammation. However, combinatorial treatment of quercetin and lycopene with or without poloxamer 188 restored a number of locomotion more significantly; further a significant difference was found as compared to lycopene treated rats, which showed a synergistic effect as they produce anti-oxidant and anti-inflammatory effect together.

3-NP produce CNS depression via oxidative stress and structural defects. Depression is one of the most common psychiatric disorders in the general population. Scientist investigate depression is a common feature of HD. Depression in HD is associated with basal ganglia abnormality and neurodegeneration which can be measured with the help of forced swim test. Due to depression animal will not try to escape a stressful stimulus (water tank) [28]. Administration of 3-NP in rats produced neurodegeneration and exhibit depression. In the present study, lycopene and quercetin significantly minimizes depression induced by 3-NP as compared to HD group. However combinations of these drugs are more effective as compared to individual administration as they work together. Addition of poloxamer 188 to this combination showed more significant effect on onset of immobility. There was more significant difference in the duration of immobility period in the rats treated with lycopene and quercetin with or without poloxamer 188 when compared with quercetin treated rats. On the onset of immobility only the rats treated with lycopene and quercetin with poloxamer 188 showed statistical significant difference when compared with quercetin treated rats.

Anxiety is one of the symptoms of HD. As per previous investigation anxiety in HD may be due to cholinergic hypofunction and increase oxidative stress [26]. 3-NP induces anxiety via increasing oxidative stress in experimental animal, which can be measure with the help of light and dark model as time spend in light or dark model. Light and dark model is one of the best animal models to measure the anxiety in animal. In our study, administration of 3-NP induced anxiety and reduced time spend in light area as compared to control rats. Co-administration of lycopene and quercetin alone along with 3-NP reduced anxiety and significantly increased the duration of time spent in light area. Further more significant reduction in anxiety and increase in the duration of time spent in light area were observed with combination of these drugs with or without poloxamer 188. Combinations of lycopene and quercetin with or without poloxamer188 exhibited significant increase in duration of time spent in light area and decrease in dark area. Further significant increase in time spent in light area and decrease in dark area was observed in rats treated with quercetin when compared with lycopene treated rats.

To sum up, lycopene and quercetin showed significant effect in the management of HD, moreover in our study we observed synergistic effect when lycopene and quercetin administered together with or without poloxamer 188. Further we have observed good results in rats treated with lycopene, quercetin and poloxamer 188 than lycopene and quercetin combination.

CONCLUSION

This study indicates that combination of lycopene and quercetin is an effective nutritional component to alleviate and/or prevent the complications of HD than single drug therapy, and these findings can be used as a basis for future studies.

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Monograph

Psiloxylon mauritianum (Bouton ex Hook.f.) Baillon (*Myrtaceae*): A promising traditional medicinal plant from the Mascarene Islands

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ABSTRACT

Psiloxylon mauritianum (PM) (Bouton ex Hook.f.) Baillon (*Myrtaceae*) is an evergreen endemic medicinal plant which has shown promising uses in traditional medicine from the Mascarene Islands (Mauritius and Réunion Islands). Folk use of this endemic plant in Mauritius and Réunion Islands has been geared toward the treatment and management of amenorrhea, dysentery and Type II diabetes mellitus. Recent findings from *in vitro* studies have led to the discovery of two potent acids namely corosolic acid and asiatic acid which have been shown to bear most inhibitory activities against *Staphylococcus aureus.* Such findings tend to appraise the therapeutic potential of this medicinal plant against infectious diseases. The present monograph has tried to establish the botanical description, traditional uses and the main constituents identified from PM (Bouton ex Hook.f.) Baillon. The limited documentation of *in vitro* assays of this plant demonstrates an urgent need for extensive research in order to validate other traditional uses and hence open new avenues for drug development.

KEY WORDS: Antimicrobial, medicinal plant, *Psiloxylon mauritianum*, *Staphylococcus aureus*

GENERAL INFORMATION

Psiloxylon mauritianum (PM) (Bouton ex Hook.f.) Baillon demarcates itself in the natural flora of the islands of Mauritius and Réunion as a rare endemic whilst also being indigenous to the Mascarene region in the Western Indian Ocean [Figure 1]. This species can be found on some of the mountains like Le Pouce and Deux Mamelles but can also be seen at piton, Grand Bassin, Trou Kanaka, Mon Vert and "Cascade 500 pieds" in Mauritius [1-3]. The endemic evergreen flowering plant which was formerly placed in the family Psiloxylaceae [4], now belongs to the *Myrtaceae* family [5] and has, fortunately, not been identified as being a critically threatened species in the Mascarene regions [2].

The genus name is composed of the Greek words "psilos-naked, glabrous, bare" and "xylon-wood" while the epithet "*mauritianum*" is derived from the name of the island Mauritius [3]. Common vernacular names include Bois Bigaignon or Bigaignon rouge in Mauritius and Bois de gouyave marron or Bois de pêche marron or Bois à grater in Réunion Island [2]. The vernacular name "bois a grater" has been attributed following the itching sensation arising from the dust of the crumbled bark [3].

BOTANICAL DESCRIPTION

PM is a small dioecious glabrous flowering plant of up to 10 m height assisted by a pale grey or white bark [2,6]. The stratified cork of the bark features anatomically crystalliferous wood fibers, a characteristic which is totally at odds with all *Myrtaceae* despite the fact that this family usually harbors poorly known wood [7]. The branches are relatively smooth and pinkish, while the petiole which is reddish on young leaves reaches a maximum length of 6 mm [3]. This evergreen plant bears simple, oblong and entire leaves which are spirally arranged [Figure 2]. Moreover, the coriaceous leaves with myrtaceous leaf venation, lack essential oils despite being lamina gland-dotted [1,2,6]. Inflorescence occurs as fascicles of three-seven flores which are scented and yellowish white in color, with calyx 5-6 mm in diameter, petals spreading and waxy [2,3]. Interestingly, each staminate flower bears 10-12 stamens in diplostemonous arrangement coupled with a non-functional gynoecium, while each pistillate flower usually contain the same number of sterile stamens (lacking pollen) as the staminate flower but having a normal trilocular gynoecium [7]. Furthermore, the short style and divided stigma of PM remains appealing organographic features which can hardly be compared to the normal features of most *Myrtaceae* [7]. Fruits are thin walled, 3-locular green berries crowned by

Figure 1: Mascarene Islands in the Western Indian Ocean

Figure 2: *Psiloxylon mauritianum*, (a) Whole plant, (b) Matured leaves and (c) Flowers and buds

persistent stigmas and appearing white upon maturation, while seeds with a scalariform-reticulate testa (lacking wings), straight embryo, flat cotyledons occur in large numbers [1-3].

ETHNOPHARMACOLOGICAL USES

PM has been extensively used in folk medicine among the local people of the Mascarene Islands in the Indian Ocean. Earlier clinical studies revealed the diuretic, antispasmodic and antiseptic properties of Bois de pêche marron [8]. A decoction of the leaves, taken in a cup twice daily has been reported to be used for Type II diabetes mellitus in Mauritius [9]. On the other hand, the twice-daily consumption of a cup of the decoction prepared from seven leaves of the plant, reinforced with the gizzard of a chicken and sweetened with some sugar is widely known to be useful against dysentery. Other folk data suggest its use in the treatment and management of common infectious diseases. In addition, the use of the wood decoction of PM in the treatment of amenorrhea and dysentery has been interestingly thrust into prominence among the local people [3,10]. In Réunion Island, the leaves were reported to be traditionally used to decrease the formation of uric acid in the body associated with gout as well as to help in the elimination of excess cholesterol from the body [11,12]. The folk people of Réunion Island also make use of the plant as an astringent [10]. Furthermore, a decoction of the barks of PM is still used against diarrhea [12]. A recent preliminary bio-guided assay fractionation performed on this species led to the discovery of two molecules which tend to appraise the folk use of PM as an antimicrobial [6] [Figure 3].

BIOLOGICAL ACTIVITIES OF SELECTED CONSTITUENTS

Preliminary phytochemical screening of the leaves indicate the presence of flavones, flavanes, flavonoids, phenols, terpenes and tannins [3,13,14]. The bacteriostatic properties of this endemic plant of Mauritius, documented from its traditional use, can be attributed to the presence of the high amount of triterpenoids identified as asiatic acid and corosolic acid [3,6] [Figure 4]. Interestingly, this can be compared to the isolation of tripenoid acids from the ethyl acetate and *n*-butanolic extracts of the leaves of *Syzygium guineese*, a plant from the *Myrtaceae* family in Cameroon, which was reported to exhibit potent antibacterial activity against various bacteria [15].

The biological activities of asiatic acid and corosolic acid were dependent upon the position of different substituent groups on the pentacyclic triterpinoid skeleton. The antimicrobial property of the compounds was related to the hydroxyl group at position 23 which when absent resulted in the loss of activity [6]. Asiatic acid and corosolic acid isolated from the ethyl acetate, and *n*-butanol extracts of the leaves of *S. guineese* (*Myrtaceae*) demonstrated potent antibacterial activities against *Escherichia coli*, *Bacillus subtilis* and *Shigella sonnei* whereas those isolated from the methanolic extracts of *Symplocos lancifolia* had anti-staphylococcal activity [15,16]. Asiatic acid isolated from extracts of *Syzygium claviflorum* was further identified as anti-HIV agent thus validating the broad spectrum of activities of asiatic acid isolated from the *Myrtaceae* family [15]. Likewise, the antimicrobial activities of asiatic acid and corosolic acid isolated from PM justify its use in the local folklore pharmacopeia as an anti-infective agent [14]. Crude acetone extracts of the leaves of PM had an inhibitory effect on gram-negative bacteria *Enterococcus faecalis*, *Staphylococcus aureus*, *E. coli* and *Pseudomonas aeruginosa* revealing the strong antibacterial activity of the extracts [6].

RECENT FINDINGS

Rangasamy *et al.* [6] have recently investigated into crude acetone extracts and isolated constituents of leaves of PM for

Figure 3: *Psiloxylon mauritianum*, (a) Branchlet with flower buds, (b) Fruiting branchlet, (c) Flower bud, (d) Male flower, (e) Female flower, (f) Ovary sectioned, (g) Fruit sectioned, (h) Seed, (i) Embryo

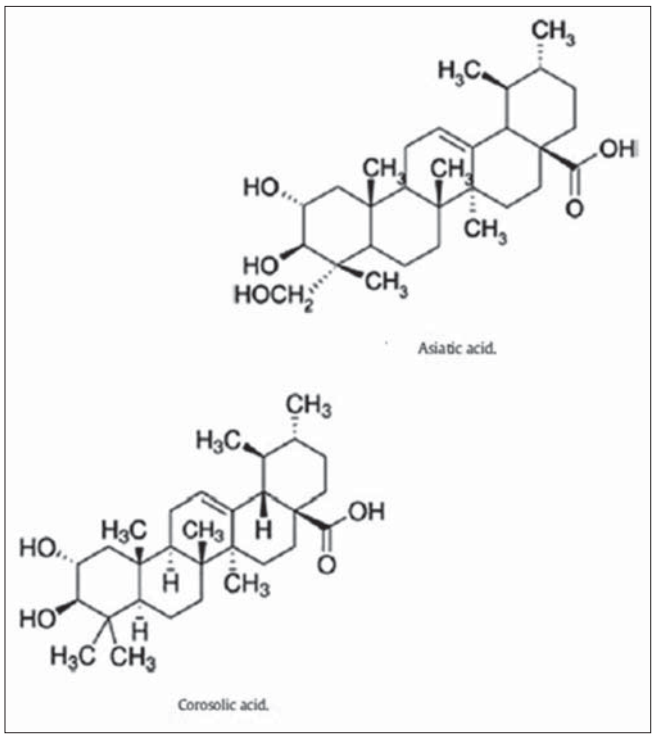

Figure 4: Asiatic acid and corosolic acid (adapted and modified from [6])

their antimicrobial, particularly, anti-staphylococcal activity, antioxidant properties and their potential cytotoxicity on Chinese hamster ovary (CHO) cells. PM was distinctly active against *S. aureus* (minimum inhibitory concentration [MIC] ≤ 51 μg/ml), *P. aeruginosa* (MIC ≤ 410 μg/ml) and *E. coli* (MIC 810 μg/ml) and was thus selected for bioassay guided fractionation among many other endemic plants [6]. PM was found to be the most markedly anti-staphylococcal plant both in terms of lowest MIC (≤ 19 μg/ml) recorded for its preliminary fractions and the highest number of intrinsic *S. aureus* growth inhibiting compounds revealed via bioautography [14]. The plant was found to possess no activity against *Candida albicans* and *Apergillus niger*. The PM crude acetone extracts exhibited dose-dependent radical scavenging activity in various antioxidant assays. Acetone leaf extract with an IC_{50} = 13.5 ± 1.1 μg/ml, demonstrated efficient 2,2-diphenyl-1-picrylhidrazyl radical scavenging activity which was significantly ($P < 0.05$) higher with respect to the control ascorbic acid (IC_{50} = 8.3 ± 0.3 μg/ml) and lower when α-tocopherol (IC_{50} = 18.2 ± 4.3) was used as control. Furthermore, the crude extract was markedly more effective at scavenging hypochlorous (HOCl) acid and hydroxyl radical than vitamin C. As a consequent, a solution of 500 μg/ml of the crude acetone extract of PM was found to be able to scavenge 97.0% of the total HOCl present in the reaction mixture as compared to only 49.5% for the same concentration of ascorbic acid ($P < 0.05$) [6,14]. On the other hand, the extract had a weak ferric reducing ability (3.9 ± 0.2 Trolox equivalent μm/g fresh plant) and low total phenolic content (14.9 ± 0.9 mg gallic acid equivalent/g dried plant). Cytotoxicity assays on CHO-wild type cells performed on different crude extracts and fractions, revealed the different samples to be moderately cytotoxic when compared to the reference standard cycloheximide, for which a solution of 0.41 ± 0.07 μg/ml was found to kill 50% of the CHO cells [6].

CONCLUSIONS

This monograph has endeavored to project some of the medicinal properties of the endemic plant PM, which has been documented in the traditional Mauritian pharmacopoeia as a plant having promising antimicrobial, antidiabetic and curative properties for the treatment of dysentery and amenorrhea. Preliminary study has evaluated the presence of corosolic acid and asiatic acid which appraise the folk uses of the plant as anti-infective. However, further research is required to explore and validate the therapeutic potential of the endemic plant with respect to new phytochemicals as lead molecules or new possible pharmacological effects of isolated molecules.

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Review Article

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Pharmacological effectiveness of the active phytochemicals contained in foods and herbs

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ABSTRACT

Food ingestions generally regulate many physiological functions to maintain a healthy life. Furthermore, herbal medicine is prescribed for the prevention and the treatment of various diseases. There are not a few herbal medicine-derived drugs (phytochemicals) clinically using now. The phytochemicals such as digitalis, curare, morphine, quinidine, atropine, and so on are so much important drugs for clinical treatments. Herbal medicine and foods are composed of many constituents. The pharmacological actions that contain phytochemicals are exerted each by each mediated through different receptors, ionic channels, and cellular signal transductions. Thus, they produce multiple pharmacological and pathophysiological functions mediated by the complex interactions with lots of the ingredients.

KEY WORDS: Anti-ageing effects, foods, herbal medicine, mixture, profitable effects, phytochemicals

INTRODUCTION

In plants, there are a lot of phytochemicals which contain many minerals and plenty nonsoluble dietary fibers to produce various physiological functions. Phytochemicals are in general classified by alkaloids, flavonoids, terpenoids, carotenoids polyketides, and phenylpropanoids. They exert anti-oxidative stress action [1], radical scavenging activity [2,3], antibacterial actions [4], and cardio-and neuro-vascular actions [5,6]. Moreover, the anti-ageing effects [7] and the improvement of poor blood circulation [8,9] have also been reported.

Herbal medicine is also composed of many herbs (from several to more than 10 constituents) and possesses plenty phytochemicals in each herb of the formulations for various clinical treatments [10]. In the phytochemicals of foods, as well as herbs, there are well-known numerous bioactive substances; i.e., lycopene in tomato, anthocyanin in blueberry and red wine, caffeine, theophylline and catechins in coffee and tea, capsaicin in paprika and red pepper, and quercetin in onion and many herbs. The pharmacological actions of some phytochemicals investigated so far in my laboratory and their important roles for cardiovascular and intestinal functions are discussed.

METHYLXANTHINES

Caffeine contained in coffee or tea is popularly in widespread use for a long time. Caffeine and theophylline are the well-known phytochemicals, as well using as a clinical drug. In cardiac Purkinje fibers, caffeine at 0.5-10 mM caused an initial transient increase and a subsequent decrease in the contractile force [11-13]. Caffeine's effects are modulated by the changes in the concentration of intracellular calcium $[Ca^{2+}]_i$. Under the conditions to elevate $[Ca^{2+}]_i$, the positive inotropic effect was reduced (or abolished), but the negative inotropic effect was further enhanced. Once $[Ca^{2+}]_i$ was declined, the positive inotropic effect was potentiated, and the negative inotropic effect was reduced or abolished. Therefore, the negative inotropic effect of caffeine is mostly related to the development of cellular calcium overload but not to Ca^{2+} depletion in sarcoplasmic reticulum (SR). The same responses had been shown in cardiac electrophysiological actions [14]. Although the mechanisms by which calcium overload decreases the force are not fully known, caffeine modifies a key factor to regulate Ca^{2+} homeostasis such as Ca^{2+} channel, ryanodine receptor on SR, and Ca^{2+} sensitivity to cardiac muscle.

Moreover, the arithmetic skill was enhanced by the intake of coffee (with 180 mg caffeine) at 40-60 min later under double-blind experiments [15], presumably resulting from the blockade of brain adenosine receptor [16,17]. Simultaneously, the systemic blood pressures (SBP) diastolic blood pressures (DBP) increased, but the heart rate (HR) decreased. In other methylxanthines, tea (mainly caffeine) decreased SBP and did not affect DBP. HR was markedly reduced. Cocoa (mainly

about 200 mg theobromine) reduced both SBP and DBP and also slightly decreased HR. Simultaneously, as indices of wave reflection; an augmentation index (AI), the ratio of ejection and reflection pressures from the radial artery was also measured [6]. The AI and the central systemic arterial blood pressure (CBP) significantly increased by 11.6% and 6.8% with coffee, and by 13.1% and 2.8% with tea, but decreased by 4.3% and 3.8% with cocoa, respectively. Cocoa contains theobromine, caffeine, cacao polyphenols, and theophylline (much lower content). In the cocoa, however, the content of theobromine or caffeine is not enough to elicit the significant effects by itself. Theobromine also antagonizes adenosine receptor-like caffeine. The methylxanthine derivatives cause the accumulation of Cyclic adenosine monophosphate (cAMP) by phosphodiesterase (PDE) inhibition, resulting in the productions of many physiological and pharmacological effects via protein kinase A (PK-A) activity. We, daily take some drinks containing several methylxanthines (and other phytochemicals). The resultant responses are exhibited as a net of the effects of the constituents and ingredients contained in a drink. A cup of coffee or tea increases human vascular wall tone, but a cup of cocoa decreases it, presumably due to the cardiovascular and central nervous modulation as a mixture.

Anthocyanins

Anthocyanins at 0.03-3 mg/ml from cassis and bilberry caused the potent vasorelaxation in rat aorta in a concentration-dependent manner [18]. Moreover, in 38 healthy paramedical students (averaged 26 years old), after an oral administration (100 mg/a tablet), the SBP and DBP had less or no effect; by −1.4% and −0.9% in cassis, and by −0.6% and −0.6% in bilberry anthocyanins, respectively. The HR decreased by 4.6% and 7.8%. Simultaneously, cassis and bilberry anthocyanins elevated AI by 5.0 ± 2.7% and 9.4 ± 2.4% ($P < 0.05$), and CBP by −1.1% and 1.3%, respectively. Thus, bilberry anthocyanin exhibited the stronger effects. The responses were lasted for approximately 1 h. Both anthocyanins increased human vascular wall tone and then, elevated the AI (in spite of the potent vasodilatation in isolated rat aorta), but had no or less effect on the HR, blood pressure, and CBP.

Capsaicin

The hemodynamic functions of 34 healthy students (approximately 24.3 years old) were also measured in comparison between a fiery noodle with so much hot flavor and non-hot noodle [19]. The indicator of hot taste is not clear yet, although Scoville heat unit is present. The Scoville scale of the hot and fiery noodle used in this study was never indicated anywhere. But it was not so easy for usual persons take all hot noodles. Both increased the blood pressure and the CBP. Hot noodle elevated especially the SBP by 9.2 ± 3.2% and the DBP by 29.5 ± 3.3% ($P < 0.05$), but non-hot noodle had less or no effect. The HR was reduced by 6-9% in both noodles. The AI increased and reached to maximal (by 12.7 ± 2.3%, $P < 0.05$) at 20 min later in hot noodle, whereas it had just a weak enhancement in non-hot noodle. These responses were lasted for 40-60 min. Capsaicin contained in the hot and fiery foods causes the acute hemodynamic effects, and may exert the multiple profitable actions as a hot medicine; the stimulation of energy metabolism, the enhancements of endothelial (NO)- and immune (immunoglobulin M)-activities, and potentiation of anti-oxidative stress action, as well as the elevation of body temperature.

Bilobalide

Most healthful foods are also derived from the bioactive substances in plants or animals. Bilobalide contained in *Ginkgo biloba* extract (GBE) at 1 μM enhanced the L-type Ca^{2+} current (I_{CaL}) by 40.0 ± 2.3% ($n = 6$, $P < 0.05$), and the delayed rectifier K^+ outward current (I_{Krec}) by 14.0 ± 2.3% ($n = 6$, $P < 0.05$), concentration-dependently. The inwardly rectifying K^+ current (I_{K1}) was unaffected. These responses were reversible (approximately 70-80% of control) after 10-20 min washout [5,20].

In general, the vasodilating actions decreased in accordance with ageing. The comparison between the vasodilating actions induced by GBE (as a health food in Japan) and the phytochemical, bilobalide, was examined [21-23]. The vasorelaxation induced by bilobalide at 30 μM significantly decreased from 11.8 ± 1.4% ($n = 4$) in 5-week-old rats to 2.3 ± 1.5% ($n = 5$, $P < 0.01$) in 25-week-old rats, and at 100 μM from 20.2 ± 3.4% ($n = 4$) to 5.6 ± 2.5% ($n = 5$, $P < 0.01$), respectively. On the other hand, GBE at 1 mg/ml decreased it from 28.4 ± 3.8% ($n = 5$) in 5-week-old rats to 23.7 ± 7.1 ($n = 7$) in 25-week-old rats, but not significantly. The maximum vasodilatation induced by GBE (3 mg/ml) was 73.7 ± 2.1% ($n = 4$, $P < 0.001$) in 10-week-old rats. Bilobalide (a phytochemical) elicited the age-dependent attenuation, but GBE (a mixer-maxter) maintained more vasodilatation even at elder ages.

Although the mechanisms are still unclear, there are several possibilities. The effect on the L-type Ca^{2+} channel in the fetal bovine is more marked than that in the adult, indicating an alteration of Ca^{2+} channel density. And the age-related modulation of Ca^{2+} channel inhibitor is not due to changes in affinity to drugs but is due to alterations in the population of the Ca^{2+} channel. Moreover, the vasodilatations induced by the bilobalide and GBE are produced mediated through the endothelium-dependent mechanisms (endothelium-derived relaxing factor [EDRF]) [21]. The endothelium-dependent vasorelaxation (via NO release) is slowly attenuated along with ageing. The vasodilations by both drugs would be also modulated by the other mechanisms, as well as the alterations of Ca^{2+} channel, cAMP, PGI_2, and PK-C. Besides bilobalide, GBE contains numerous constituents such as terpenoids (ginkgolides A, B, and C) and flavonoids (quercetin and rutin), which by themselves also produce vasodilating actions. Each contributes to the GBE-induced vasodilatation, and GBE as a whole produces the mixed responses [24]. The phenomena resemble the characteristics of Japanese herbal (Kampo) medicine as a mixture of herbal drugs; more effective for elder persons [10].

Quercetin

Quercetin, a kind of flavonoids, contains in many herbal medicines as well as onion. Quercetin causes lots of profitable physiological actions. In guinea pig ventricular cardiomyocytes, quercetin at 0.3-300 μM decreased the action potential duration and inhibited the underlying ionic currents (I_{CaL}, I_{Krec} and I_{K1}) [24]. In rat aorta, quercetin (0.1-100 μM) relaxed the contraction induced by 5 μM NE concentration-dependently [20,22]. NG-monomethyl-L-arginine acetate (L-NMMA) at 100 μM decreased the quercetin (100 μM)-induced vasorelaxation from 97.0 ± 3.7% (n = 10, P < 0.05) to 78.0 ± 11.6% (n = 5, P < 0.05). Endothelium removal as well attenuated the vasodilatation. In the presence of both L-NMMA (100 μM) and indomethacin (10 μM), the quercetin-induced vasorelaxation was further reduced by high K (30 mM) or 10 μM tetraethyl ammonium (TEA). Among K_{Ca} channel inhibitors, the quercetin-induced vasodilatation decreased at 0.3 μM apamin (sensitive to SK), but not at 30 nM charybdotoxin (sensitive to BK and IK) [25]. Under KCl-induced vasoconstriction, the quercetin-induced vasorelaxation was inhibited by PK-C inhibitors; Gö6983 (α-, β-, γ-, δ, and ζ-sensitive) dilated stronger than Ro-31-8425 (α-, β-, γ-, and ε-sensitive) [26].

Furthermore, the involvement with an endothelium-derived hyperpolarizing factor (EDHF) was investigated using rat mesenteric artery because EDHF is considered not contributed to the vasodilatation of aorta. The quercetin-induced vasodilatation was almost resistant to both L-NG-nitro arginine methyl ester (L-NAME, an NO-synthesis inhibitor) (100 μM) and indomethacin (100 μM), presumably related with EDHF. The candidates of EDHF are considered as K^+, epoxyeicosatrienoic acid and H_2O_2 from endothelium. The L-NAME/indomethacin-resistant quercetin-induced vasodilatation was inhibited by TEA (1 mM), and also by gap junction inhibitors of 18α- (100 μM) and 18β- (50 μM) glychrrhetinic acids [27,28]. Therefore, quercetin dilates the vascular smooth muscle mediated by endothelium-dependent (EDRF, EDHF, and gap junction) and-independent (the inhibitions of I_{CaL}, SK channel, and PK-Cδ, and PGI_2 production) mechanisms.

Sinomenin and Tetrandrine

Mokuboito (Mu-Fang-Yi-Tang) is composed of four herbal drugs; *Sinomenium acutum*, *Cinnamomi* cortex, *Ginseng* radix, and gypsum. S. *acutum* (a vine plant), the main constituent, possesses the phytochemicals such as sinomenine, tetrandrine, and magnoflorine. They are alkaloid. Sinomenine (1 mM) and tetrandrine (100 μM) inhibited the ionic currents to the same extent [29]. At 1 mM sinomenine inhibited the I_{CaL} at 0 mV by 18.2 ± 2.1% (n = 6, P < 0.05). The I_{Krec} at 60 mV was inhibited by 16.2 ± 2.6% (n = 6, P < 0.05), and the I_{K1} at −120 mV by 47.2 ± 3.8% (n = 6, P < 0.01). As a result, these phytochemicals simultaneously affected the action potential configurations. Sinomenine (300 μM) and tetrandrine (30 μM) had almost the similar effects, but magnoflorine (1 mM) had less or no effect. Interestingly, sinomenine abolished the dysrhythmias elicited by the cellular Ca^{2+} overload. Thus, these phytochemicals have the profitable electropharmacological and cardioprotective actions.

In the analyses of the age-related effects, sinomenine (100 μM) alone dilated NE-induced vasoconstriction by 68.8 ± 5.2% (n = 6, P < 0.01) in 10-weeks old rats, but only by 18.6 ± 1.5% (n = 6, P < 0.01) in 65-weeks old rats [30,31]. *S. acutum* caused the vasodilatations at 3 mg/ml by 96.7 ± 4.8% (n = 7, P < 0.01) in 10-weeks old rats, and by 46.0 ± 5.7% (n = 6, P < 0.01) in 65-weeks old rats. Mokuboito at 3 mg/ml dilated aorta by 98.9 ± 2.8% (n = 7, P < 0.01) in 10-weeks and by 97.5 ± 13.5% (n = 6, P < 0.01) in 65-weeks old rats. Thus, Mokuboito, *S. acutum* (multiple compounds) and sinomenine (single phytochemical) by themselves had the potent vasodilating actions. However, the pharmacological effects of just single phytochemical are strongly influenced in advance with ageing. In contrast, *S. acutum* (multiple compounds) suppresses the age-dependent attenuation of vasodilating action, and Mokuboito (as a mixture) maintains the marked action. In short, *S. acutum*- and sinomenine-induced vasodilatations decreased along with ageing, but Mokuboito has less or no effect on the age-dependent attenuation in any aged rats. The mixture is so much important against the age-dependent alterations of the effectiveness. Thus, herbal medicine produces more effective pharmacological stability [32]. Mokuboito exhibits as a net mediated by the complicated interactions among the contained ingredients.

Paeoniflorin and Glychrrhetic Acid

Shakuyakukanzoto (Shao-yao-Gan-chao-Tang), a traditional formulation of Kampo medicine, is composed of paeoniae radix and glycyrrhizae radix. Shakuyakukanzoto relaxed 0.3 μM carbachol (CCh)-induced contraction of rat ileum in a concentration-dependent manner [33]. Both components (paeoniae radix and glycyrrhizae radix) each dilated the CCh-induced contraction. Main ingredient is paeoniflorin in paeoniae radix and glycyrrhetic acid in glycyrrhizae radix. Both phytochemicals and the metabolic products (18-α- and 18-β-glycyrrhetinic acids) had almost the same actions. Under the conditions with spontaneous contractions, an application of Shakuyakukanzoto completely abolished the abnormal contractions. Thus, Shakuyakukanzoto caused the potent relaxant actions not only by the anti-cholinergic effect but also by Ca^{2+} channel and PDE inhibitory effects [34]. Furthermore, Shakuyakukanzoto produces an anti-spasmodic action. However, the whole effects induced by Shakuyakukanzoto are never equal with a sum of each effect of the phytochemicals in paeoniae radix and glycyrrhizae radix. The responses are exhibited as a net among the contained ingredients.

Phytochemicals in a Mixture

Phytochemicals by themselves generally produce anti-arteriosclerosis (or protection of ischemia and stroke) and improve poor blood circulation, resulting from the great vasodilating actions due to such multiple mechanisms as endothelium-dependent actions (EDRF and EDHF), and endothelium-independent actions (PGI_2 production, PDE inhibition, Ca^{2+}

channel inhibition, and PK-C inhibition) [35,36]. Moreover, phytochemicals have anti-oxidative stress action [1] and radical scavenging activity [37], produce anti-inflammation and immuno-modulation [38], and induce the mRNA leading to the production of some functional proteins.

There is a quite difference between single phytochemical and mixture drugs with a lot of ingredients (including phytochemicals). The effects of herbal medicine (a mixter-maxter) are never just a sum of each effect induced by all ingredients. The contained phytochemicals exhibit the pharmacological effects via the interaction with other contained ingredients, but never by themselves alone [39]. On the complex interactions, the effect of a phytochemical may be potentiated or attenuated (or blocked).

Mixture of plenty phytochemicals plays an important role for the anti-ageing effects and the potentiation of effective pharmacological and physiological responses. Advance in ages produces various pathophysiological deleterious changes such as plaque formation in vascular systems. Simultaneously, the age-dependent functional depressions of receptors, ion channels and cellular signal transduction pathways must be caused in the endothelium, and cardiac and smooth muscle cells. As a result, the age-related alterations would be responsible for physiological and anatomical reductions. Furthermore, ageing declines the sensitivity to drugs. However, herbal medicine generally modulates the age-dependent changes and can maintain the pharmacological effects (but not fully). In Kampo medicine, Rokumigan (Liu-Wei-Wan) possesses six herbs as a base, Hachimijiogan (Ba-Wei-Di-Huang-Wan), eight herbs (Rokumigan plus two herbs), and Goshajinkigan (Niu-Che-Shen-Qi-Wan), ten herbs (more two herbs are added to Hachimijiogan). These formulations increase in order to the number of contained ingredients in increment of two herbs. The larger the number of the contained ingredients of herbs become, the more potent vasodilatation is produced [39]. Furthermore, the addition of Gypsum (mainly calcium sulfate) produced stronger vasodilatation in Kampo medicine (Japanese herbal medicine) such as Mokuboito and Chotosan (Diao-Teng-San).

For elder persons, therefore, the mixture (such as health foods and herbal medicine) with lots of ingredients (and phytochemicals) plays a key role for much more effectiveness. The similar effectiveness might be also expected in general foods. Herbal medicine as a mixture keeps the balance and can produce either vasoconstriction or vasodilatation. Increasing the number of phytochemicals can maintain the effectiveness to some extent in elder rats, presumably due to compensation of the age-dependent decline of pharmacological sensitivity to the receptors.

CONCLUSION

Now, out of a number of residual phytochemicals which are not western drugs exit. From the modern in detail analyses, many phytochemicals have recently been discovered to possess the effective pharmacological activities including the modification of the genes of target; the hormone secretions for long life such as ghrelin, adiponectin and aquaporins, and switch-on of the long-life genes like sirtuins [40,41].

In the future, can some phytochemicals independently become a new drug? The development needs so much more clinical pharmacological tests, accompanied with long-term and huge cost. If so, it is not easy for any pharmaceutical company to start newly to develop a phytochemical as a new drug. Hence, it is doubtful that a phytochemical in herbs will be able to become independently as a clinical drug. Each phytochemical in herbs never plays an important role each by each, but exerts as a mixture of herbal medicine, leading to more effectiveness for the multiple diseases and the age-related disorders [10]. This is the most important role for the phytochemicals.

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Original Research

Protective effect of cornel iridoid glycoside in D-galactosamine/ tumor necrosis factor-α-injured L02 hepatocytes and its mechanism

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ABSTRACT

Aim: The aim was to determine the action mode of cornel iridoid glycoside (CIG) from *Fructus corni* on hepatoprotective activities, the effects of CIG on human hepatocyte cell line (L02) injured by D-galactosamine (GalN) and tumor necrosis factor-α (TNF-α) were examined. **Materials and Methods:** The percentage of cell viability was evaluated by cell counting kit-8 assay. Apoptosis was detected by flow cytometric analysis in human L02 hepatocytes. The expression levels of activating transcription factor-4 (ATF4), and C/EBP homologous protein (CHOP) were detected by western-blot analysis. In addition, the activity of caspase-3 was tested by enzyme-linked immunosorbent assay. **Results:** The results showed that CIG caused a significant increase in the viability of L02 cells injured by GalN/TNF-α, in accordance with a dose-dependent decrease of apoptotic cell death confirmed by flow cytometric analysis. Based on western blot and colorimetric assay, we found that GalN/TNF-α induced increased expression of ATF4, CHOP, and activation of caspase-3 while CIG pre-treatment had a dose-dependent suppression on them in this cell model. **Conclusion:** Overall, these findings demonstrate that CIG can effectively protect L02 hepatocytes against apoptosis induced by GalN/TNF-α, suggesting that it is a possible candidate target for liver disease therapy.

KEY WORDS: Apoptosis, cornel iridoid glycoside, D-galactosamine/tumor necrosis factor-α, L02 hepatocyte

INTRODUCTION

Fructus corni is derived from the dry ripe sarcocarp of *Cornus officinalis* Sieb. et Zucc. As an important traditional Chinese medicine, it is clinically used to treat vertigo, tinnitus, sweating, back pain, as well as soreness, and weakness of the waist and knees [1]. A number of chemical studies showed "*F. corni*" mainly contained loganin and morroniside, both belonged to iridoid glycosides, which we have usually named cornel iridoid glycoside (CIG) [2,3]. Recently, CIG has been found to possess a number of good biological activities, including blood glucose reduction, anti-tumor, anti-inflammatory, and protective effects on rat mesangial cell proliferation [2-4]. But so far, a few studies have been conducted on the hepatoprotective action of CIG.

The D-galactosamine (GalN)/tumor necrosis factor-α (TNF-α) model is a newly-developed experimental model used to trigger apoptosis of hepatocytes both *in vitro* and *in vivo* [5]. TNF-α, a pro-inflammatory cytokine, is identified as a key mediator of hepatocellular cell death in several hepatic diseases [5-7]. GalN, which has a broad impact on hepatocyte transcription and translation, could enhance sensitization to TNF-α [7]. In our previous study, we establish a model of hepatocyte injury in hepatocyte L02 cell line after 12 h combined administration of GalN (44 μg/ml) and TNF-α (100 ng/ml) [8]. Thus, in the present study, we have performed an experimental research on the protective effects of CIG using this cell model to explore its hepatoprotective potential.

MATERIALS AND METHODS

Reagents

CIG, with purity higher than 54%, mainly including morroniside and loganin [Figure 1], was provided by Jiangsu Zhongkang Fingerprint Development Co. Ltd. (Jiangsu, China). Dulbecco's modified eagle's medium (DMEM) and fetal bovine serum (FBS) were purchased from Gibco Co. (New York, USA). TNF-α was obtained from PeproTech Inc. (Rocky Hill, USA). Cell counting kit-8 (CCK-8) and the Annexin V-FITC apoptosis detection kit were from dojin laboratory (Kumamoto, Japan)

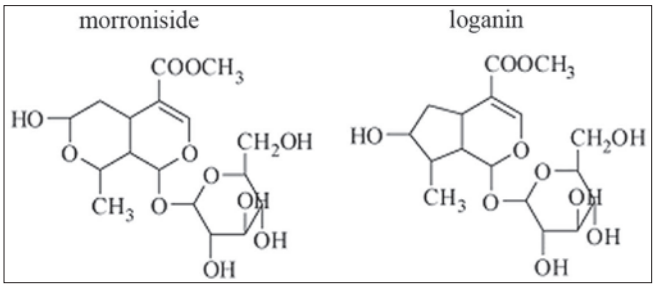

Figure 1: Chemical structure of cornel iridoid glycoside represented by morroniside and loganin

and Invitrogen Life Technologies (Carlsbad, USA), respectively. The antibodies against activating transcription factor-4 (ATF4) and C/EBP homologous protein (CHOP) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

Cell Culture

Human hepatocyte cell line L02 was provided by the Institute of Biochemistry and Cell Biology, Shanghai Institutes for Biological Sciences (Shanghai, China). Cells were cultured in a humidified 5% CO_2 incubator at 37°C in DMEM medium supplemented with 10% FBS. In subsequent experiments, the cells were divided into five groups: Normal control group, GalN/TNF-α group and three concentrations of CIG (10, 20, and 100 μg/ml) groups. The normal control group was incubated with medium alone; the GalN/TNF-α group were pre-incubated with vehicle phosphate buffer saline (PBS) for 24 h, and then apoptosis was induced by GalN (44 μg/ml) and TNF-α (100 ng/ml) for 12 h; the CIG groups were pre-treated with CIG at 10, 20 and 100 μg/ml for 24 h, and then were treated with GalN (44 μg/ml) and TNF-α (100 ng/ml) for 12 h.

Cell Viability Assay

Cell viability was determined using CCK-8 assay. Briefly, L02 hepatocytes (8×10^3) were seeded into 96-well plates and cultured for 24 h, followed by various designated treatments as described previously. Before terminating the cell culture, 5 mg/ml 2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium water-soluble tetrazolium-8, was added to each well and incubated at 37°C for 1 h. Then the optical density was measured at 450 nm using an enzyme-immunoassay instrument (BioRad, Richmond, USA). Cell viability rate = $(OD_{groups}/OD_{control}) \times 100\%$.

Flow Cytometric Analysis

Apoptotic cells were measured by flow cytometry as described elsewhere [9]. Briefly, the cultured cells were collected by centrifugation at 1000 rpm for 10 min, rinsed with ice-cold PBS twice, and stained in 200 μl of a working solution containing 5 μl of Annexin-V-FITC and 1 μl of 100 μg/ml PI in the dark for 15 min. The stained cells were then analyzed immediately on a fluorescence-activated cell sorter (Becton Dickinson, San Jose, CA, USA).

Western Blot Analysis

After various treatments, cell lysates were prepared in a lysis buffer containing 0.1% Triton X-100, 1 mM phenylmethylsulfonyl fluoride, 1 mg/ml aprotinin, and 10 mg/ml leupeptin. Following centrifugation at 12,000 rpm for 10 min, the protein concentrations of the supernatants were determined with a bicinchoninic acid protein assay kit (Bio-Rad, Hercules, CA, USA). For Western blot analysis, equal amounts of cellular protein (30 μg/lane) were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and blotted onto polyvinylidene difluoride membranes (Bio-Rad, Hercules, CA, USA). These membranes were subsequently blocked overnight with 5% skim milk in tris-buffered saline containing 0.1% Tris-buffered saline tween 20 (TBST). After three washes with TBST, membranes were incubated with primary antibodies overnight at 4°C and then further incubated with secondary horseradish peroxidase-conjugated antibodies (Amersham Pharmacia Biotech, Buckinghamshire, UK). Expression levels were normalized to β-actin (sigma, St Louis, MO, USA). The image capture and analysis were performed using an enhanced chemiluminescence system (Pierce, Rockford, USA).

Measurement of Caspase-3 Activity

Cell lysates were prepared after their respective treatment. The activity of caspase-3 was measured using a specific chromogenic enzymatic assay kit (Beyotime Institute of Biotechnology, Haimen, PR China) according to the manufacturer's protocol. The assay is based on spectrophotometric detection of the chromatophore ρ-nitroaniline (ρNA) after cleavage from the labeled substrate Ac-DEVD-ρNA by caspase-3 protease. The optical density of ρNA was measured at 405 nm using an enzyme-immunoassay instrument.

Statistical Analysis

Data were presented as mean ± standard deviation of three independent experiments. The analysis of variance and Student's *t*-test were used to test for significant differences, and $P < 0.05$ was considered statistically significant.

RESULTS

As shown in Figure 2, compared with normal control group, the survival of cultured L02 cells exposed to GalN/TNF-α was significantly inhibited ($P < 0.01$), while pretreatment of CIG (10, 20 and 100 μg/ml) dose-dependently increased the cell viability. There was no cytotoxicity on CIG at 10, 20, and 100 μg/ml (data not shown).

As shown in Figure 3, data analyzed by flow cytometry showed that after 12 h of exposure to GalN/TNF-α, the rate of cell apoptosis was markedly increased ($P < 0.01$) while CIG pretreatment decreased the apoptotic rate in a dose-dependent manner [Figure 3]. Collectively, these data indicate that CIG

protects L02 cells from GalN/TNF-α-induced cytotoxicity by attenuation of apoptosis.

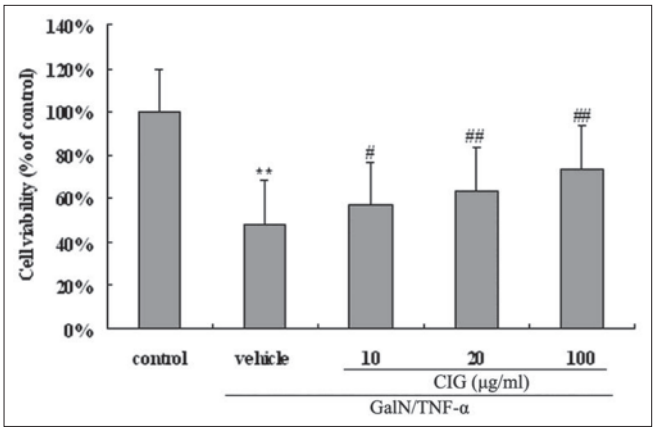

Figure 2: Effect of cornel iridoid glycoside on cell viability in D-galactosamine/tumor necrosis factor-α (GalN/TNF-α)-treated L02 cells. Data are presented as means ± standard deviation of values from triplicate samples. $^{**}P < 0.01$, versus control group; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, versus GalN/TNF-α group

To investigate the involved mechanism of CIG's inhibitory effect on hepatocyte apoptosis, we examined the expression of two signature endoplasmic reticulum (ER) stress marker proteins, ATF4 and CHOP, using western blotting analysis in L02 cells. The data in Figure 4 demonstrated that their expression was dramatically increased after 12 h-treatment by GalN/TNF-α. However, their up-regulation was significantly suppressed by CIG at different concentrations [Figure 4]. The result supports the involvement of ER stress in inhibition of apoptosis by CIG in GalN/TNF-α treated L02 cells.

The result in Figure 5 showed a significant increase in the activity of caspase-3 in GalN/TNF-α-injured L02 hepatocytes, while incubation of the cells with CIG (10, 20 and 100 μg/ml) for 24 h suppressed the activation of caspase-3 in a dose-dependent manner. The results suggested that CIG had a suppressive effect on the activation of caspase-3.

DISCUSSION

Initially, we assessed the effect of CIG on hepatocyte apoptosis using a human cell line L02 stimulated by GalN/TNF-α.

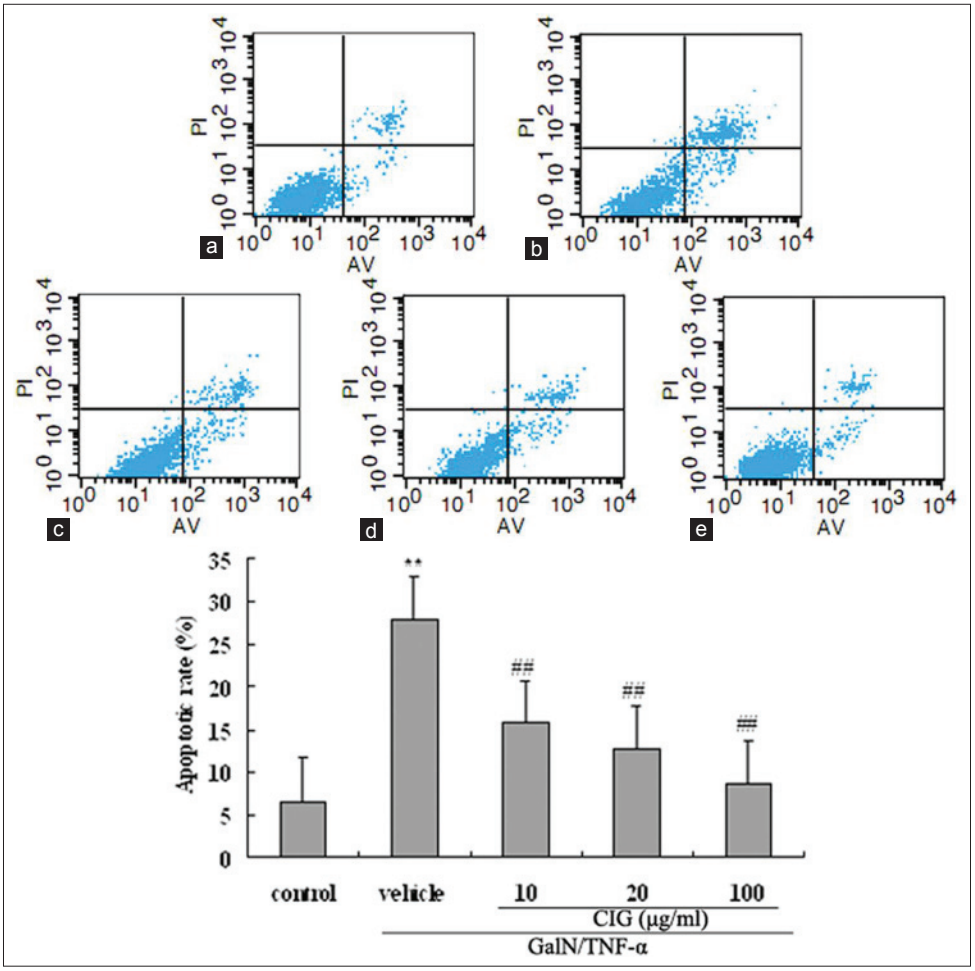

Figure 3: Effect of cornel iridoid glycoside (CIG) on D-galactosamine/tumor necrosis factor-α (GalN/TNF-α)-induced apoptosis detected by flow cytometric analysis in L02 hepatocytes. (a) control group; (b) GalN/TNF-α group; (c-e) CIG (10, 20, and 100 μg/ml) groups. Results are expressed as the percentages of early or late apoptotic cells. Data were presented as mean ± standard deviation from triplicate samples. $^{**}P < 0.01$, versus control group; $^{\#\#}P < 0.01$, versus GalN/TNF-α group

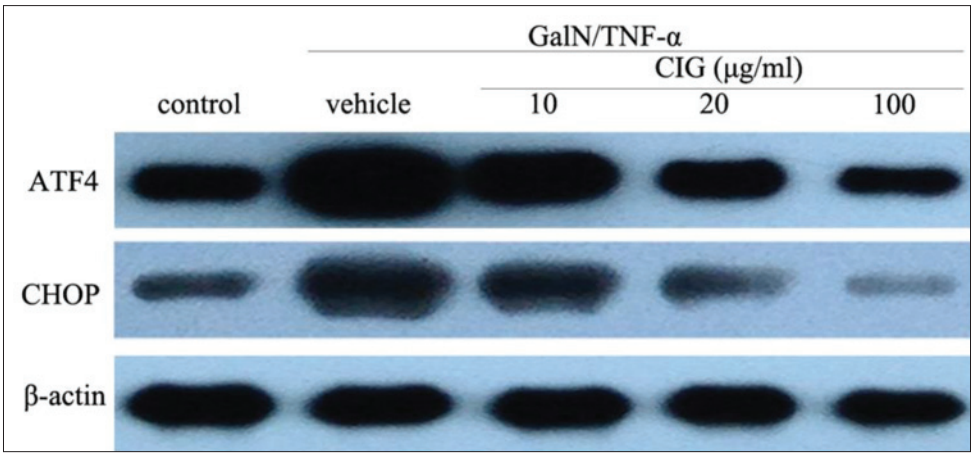

Figure 4: Effect of cornel iridoid glycoside on the expression of activating transcription factor-4 and C/EBP homologous protein after treatment with D-galactosamine/tumor necrosis factor-α

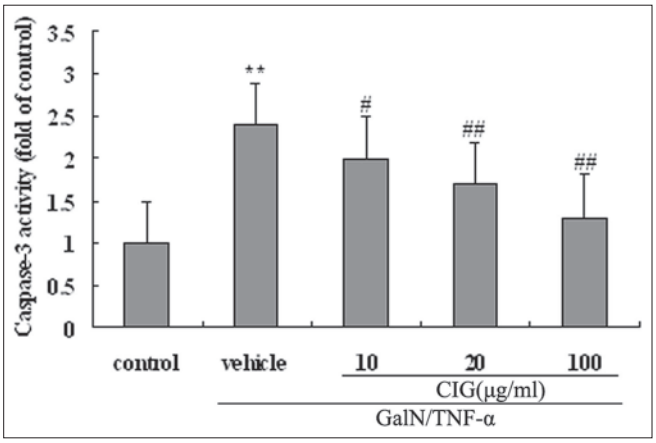

Figure 5: Effect of cornel iridoid glycoside on D-galactosamine/tumor necrosis factor-α (GalN/TNF-α) -induced caspase-3 activation. Values were expressed as the ratio of the caspase-3 activation levels to the control level, and the value of normal control was set to 1. Data are presented as means ± standard deviation of values from triplicate samples. $^{**}P < 0.01$, versus control group; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, versus GalN/TNF-α group

The result of CCK-8 assay showed that CIG pretreatment protected L02 cells from GalN/TNF-α-induced cytotoxicity in a dose-dependent manner [Figure 2]. Further flow cytometry examination revealed that the treatment with GalN/TNF-α resulted in obvious apoptosis of L02 cells while CIG decreased the apoptosis rate in a dose-dependent manner [Figure 3], indicating that the hepatoprotective effects of CIG might be related to resisting apoptosis under present *in vitro* conditions.

To explore the mechanism of the *in vitro* protective effect of CIG on GalN/TNF-α-induced apoptosis in L02 hepatocytes, a number of potential targets-including the mediators in ER stress signaling pathway, and the caspase family proteases were analyzed. There is increasing evidence that ER stress is a central initiator of liver cell injury and cell death in almost all acute, and chronic liver disease processes [10,11]. Previous reports have demonstrated that on cells exposed to agents that cause ER stress, general translation is suppressed, but selected ER stress-specific genes are translationally up-regulated, participating in ER-stress-corrective actions [12-14]. Among them, the two most studied signature markers are ATF4 and its downstream target, the CHOP, which are known to be required in the apoptotic response to ER stress [15,16]. Numerous studies have reported that during the later apoptotic phase of prolonged ER stress, the expression of ATF4 and CHOP, are usually upgraded to a constant high level in a variety of cell types [12-18]. It has been reported that some drugs could initiate cellular protection to enhance cell survival *in vitro* and *in vivo* models, targeting the ATF4-CHOP pathway of apoptosis [17-20]. Most evidence supports that selectively increased translation of ATF4, and subsequent induction of CHOP were able to cause growth arrest and apoptosis, which may all be associated with severe and prolonged ER stress [21,22].

Therefore, in subsequent experiments, we characterized the effects of CIG on the transcription factor ATF4 and the pro-apoptotic protein CHOP in L02 hepatocytes stimulated by GalN/TNF-α. Our results showed that the treatment with GalN/TNF-α induced a significant increase in ATF4 and CHOP expression. However, both the ER stress markers were significantly inhibited by CIG at a concentration-dependent manner [Figure 4]. These findings further indicate that the anti-apoptotic action of CIG could be associated with the ATF4-CHOP signaling cascade that mediates ER stress-induced apoptosis in L02 hepatocytes.

Furthermore, it is now well-established that proteases of the caspase family play a vital role in the effector mechanism of cell apoptosis [22,23]. Among them, caspase-3 is believed to be a key factor in apoptosis execution, and its activation represents a hallmark of apoptotic cell death [22,23]. In current research, as expected, we found that GalN/TNF-α resulted in a significant increase in the activation of caspase-3 in L02 cells, but in the presence of CIG of different concentrations, its activity was significantly reduced in a dose-dependent manner [Figure 5]. These observations suggest that inhibition of caspase-3 by CIG blocks L02 cell apoptosis.

In summary, data of this study provide direct evidence that CIG was able to protect L02 hepatocyte against apoptosis induced by GalN/TNF-α, suggesting that it is a potential hepatoprotective agent for liver disease therapy. The mechanisms may involve inhibition of ER stress-induced apoptosis via the ATF4-CHOP signaling pathway and related caspase-3 inactivation. Further studies will be needed to determine the exact mechanisms in the signal transduction pathways.

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Original Research

Acute and sub-chronic oral toxicity assessment of the aqueous extract leaves of *Ficus glumosa* Del. (Moraceae) in rodents

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ABSTRACT

Background: *Ficus glumosa* Del (Moraceae), a plant used in traditional medicine in Cameroon, Senegal, and East Africa for the treatment of edema, hemorrhoid, cardiovascular diseases especially hypertension. **Aim:** The present study evaluated the potential toxicity of the aqueous extract of the leaves of *F.glumosa* in acute and sub-chronic administration in rodents. **Methods:** Acute toxicity was evaluated on 3 months old mice of both sexes and weighing 20-30 g. A single dose (2-12 g/kg) of *F. glumosa* was administered orally to mice. Animal behavior, adverse effects, and mortality were determined for 14 days. In sub-chronic toxicity studied in both sexes of 9 weeks old rats and weighing 100-120 g at the start of the experiment, animals were treated orally with a daily dose of 300, 600 and 1200 mg/kg of the aqueous extract of the leaves of *F. glumosa* for 6 weeks. The body weight change, food, and water consumption, were determined throughout the experimental period, while the relative organ weights, the hematological and biochemical parameters of blood and urine, as well as the histology of tissues kidney and liver, were recorded at the end of the experiment. **Results:** For acute treatment, no dose used induced critical behavioral changes or death. In sub-chronic treatment, daily oral administration of *F. glumosa* at the dose of 300, 600, and 1200 mg/kg resulted in a significant increase in body weight relative to food and water consumption in the last week of treatment. The relative organ weights were not affected by treatment. No hematological changes were observed except the significant increase in platelets. Aspartate aminotransferase, alanine transaminase, alkaline phosphatase, total protein, increased while the total cholesterol, triacylglycerol, conjugated bilirubin, and total bilirubin significantly decreased. Index of renal function showed a decrease of creatinine, urea, uric acid and Na^+, Cl^- and Ca^{2+}, and inorganic phosphate. The histology of liver and kidney showed no significant alteration of tissue. **Conclusion:** These observations support the traditional use of *F. glumosa* in the treatment of hypertension. These results have shown that *F. glumosa* has a safety margin for therapeutic use.

KEY WORDS: Acute toxicity, *Ficus glumosa,* histopathology, leaf aqueous extract, sub-chronic toxicity

INTRODUCTION

Efforts of scientists and traditional therapists are growing in the direction of improvement and enhancement of the use of medicinal plants, to elucidate the pharmacological properties of these plants and possibly to extract active ingredients. Even when effective, preparations have to undergo toxicological tests. So far, population show no concern for the simple reason that they believe the preparation is free of toxicity. Many medicinal plants showed relative toxicity or were not toxic when administered in acute or subacute treatment in experimental animals. For instance *Ficus exasperata* (Moraceae) used in the treatment of asthma, bronchitis, and tuberculosis [1], *Celtis durandii* (Ulmaceae) used as effective antihypertensive plant [2,3], Ojeok-san used in the treatment of gastrointestinal diseases and fatigue [4], or *Erythrina senegalensis* (Fabaceae) used in the treatment of liver diseases, jaundice [5,6], and in the treatment of gastrointestinal diseases, malaria, infections were found to be moderately or non-toxic [7].

The toxicological evaluation of any medicinal plant preparation is important to ensure the safety of these phyto-medicines. *Ficus glumosa* Del (Moraceae) is a plant used in pharmacopeia of Cameroon, Senegal, and East Africa for the treatment of edema, hemorrhoid, cardiovascular diseases, especially hypertension. Originally from Ethiopia, it grows in many parts of tropical Africa and is typically found in dry country in the meadow and wooded bush [8,9]. *F. glumosa* grows on rocky outcrops, where it splits rocks; it could also be found along dried river beds. It reaches its maximum size (10 m) in the valleys. This species can also be found in the fringe forest, in the savannah, especially in the swamp forest in coastal areas. A decoction of the bark is used as a stimulant for milk production, for both women and animals [10,11]. The leaves are used in East Africa, Cameroon and Senegal for the treatment of skin diseases and diabetes [12]. Recently, acute toxicity of methanol extract of leaves of *F. glumosa* were carried out [9]. Phytochemical analysis of *F. glumosa* phytochemicals revealed the presence of flavonoids, saponins, carbohydrate, tannins, and triterpenes [9]. Effects of ethanol leaf extract of *F. glumosa* on fasting blood glucose and serum lipid profile in diabetic rats were also carried out [8]. The purpose of this study was to assess the acute and subacute toxicity of *F. glumosa* aqueous extract in rodents.

MATERIALS AND METHODS

Plant Material and Preparation of the Extract

The leaves of *F. glumosa* were harvested from Ngaoundéré, Adamawa region of Cameroon. Then, we proceeded to the identification by comparing the harvested plant to specimen N^0 60695/HNC deposited at the National Herbarium of Cameroon.

One thousand grams of fresh leaves of *F. glumosa* was steeped in 1 L of distilled water for 12 h at room temperature. The macerate was filtered through Whatman filter paper No. 3, and the filtrate concentrated in a rotary evaporator at 40°C for 24 h. This process repeated several times and yielded 112 g of concentrated of crude extract in the form of an oily paste. The extract was stored at −20°C.

Animals

Acute toxicity was evaluated on 3 months old mice (*Mus musculus*) of both sexes and weighing 20-30 g. Wistar rats of both sexes, of 9 weeks old and weighing 100-120 g at the start of the experiment were used to evaluate the subacute toxicity. Strains of animals were from Center Pasteur in Yaoundé. They were reared in the Department of Biological Sciences, Faculty of Sciences (University of Ngaoundéré). The animals were housed under controlled temperature (24 ± 2°C) and relative humidity (45 ± 10%). Moreover, they had free access to food (pellets from LANAVET [Laboratory NVS]) and filtered tap water. The animal handling was under the control of the veterinary surgeon of the Science Veterinary Surgeon and Medical School of the University Ngaoundéré. Experimental protocols and procedures were approved by the Institutional Animals Care and Use Committee, and the research was approved by the Animal Ethics Committee of the University of Ngaoundéré.

Acute Toxicity

Mice were divided into 6 groups of 10 each. Animals in each group were housed separately in Plexiglass cages. Mice were acclimatized in the laboratory environment 7 days before the start of the experiment. The mice were fasted for 12 h prior to the experiment with free access to water. Mice were orally administered; a single dose of *F. glumosa* aqueous extract (2-12 g/kg) or distilled water for the control group. Animals from the same batch received the same dose of extract once daily. The animals were observed during the first 2 h after administration of the extract and were supplied with food. Mortality was recorded after 24 h. Food and water intake and body weight of surviving animals were evaluated after 7 days. Dead animals were autopsied for macroscopic observation of internal organs [3].

Subacute Toxicity

Rats were divided into 4 groups of 10 each (5 males and 5 females). The control group was treated with distilled water, and the other 3 groups were administered the plant extract at the dose of 300, 600 and 1200 mg/kg. The doses were selected from the literature as appropriate doses to evaluate the hepato-protective activity [13,6]. The extract was administered by oral route once daily for 6 weeks. During this period, the behavior of the animals was observed and recorded. The weight, water, and food consumption were monitored at the end of each week. The last day of treatment, the animals were placed individually in metabolic cages for 24 h. Urine were collected; the pH was evaluated and stored at −20°C for biochemical analyzes. The survivors were anesthetized with chloroform and sacrificed. The arterio-venous blood was collected in heparinized tubes and centrifuged at 4900 rpm for 20 min. The collected plasma was stored at −20°C for biochemical analyzes. Liver, kidney, and

heart were removed, cleared of fat material, weighed and stored at −20°C for biochemical analyzes and a portion preserved in formalin for histological analysis.

Analysis

Urinary and plasma electrolyte concentrations were determined using a flame photometer (JENWAY PFP 7, Japan) according to standard methods described before [14]. Concentrations of creatinine, urea, glucose, albumin, and electrolytes in the plasma and urine samples were evaluated using a two-way digital spectrophotometer (SECOMAM RS 232C, Germany). Hematological and biochemical analyzes were performed by means of an automatic device type Toshiba 200 FR NEO (TOSHIBA Co., Japan). For hematological analysis, parameters like red blood cell, mean corpuscular volume, etc., were measured as described by Lahlou *et al*. [15]. Alanine transaminase (ALT), aspartate transaminase and alkaline phosphatase (ALP) were evaluated in serum and urine. Kidney functioning index was assessed by determination of the concentration of creatinine, urea, uric acid, Na^+, K^+, and Cl^-. The kidneys, liver and heart, were dissected out and fixed in 10% formalin fluid for hematoxylin and eosin staining.

Statistical Analyses

The results expressed are the mean ± standard error of the mean. Comparison of means was made using the Student's *t*-test and one-way ANOVA of Origin Graph software (Microcal Origin 6.0, Microcal, MA USA) software version 6.0. The difference was considered significant when $P < 0.05$.

RESULTS

Acute Toxicity Study

The extract at a dose 8000 mg/kg in single administration caused no death in mice during the 14 days of observation. But there were, however, a slight decrease in locomotion, aggression, sensitivity to noise and touch and a slight decrease in respiratory movement 2 h after administration of the extract of *F. glumosa*. The examination of organs did not show any signs of major pathology. The median lethal dose 50 (LD_{50}) of the *F. glumosa* aqueous extract should be above 12 g/kg. Generally, at this dose, the mice gain all their capacities within 48 h after administration of the extract. Animals that received the extract at this dose showed diarrhea. No animal had a convulsion after administration of the extract. Necropsy of sacrificed animals showed a digestive tract brownish aspect, probably due to the color of the extract. The extract had no negative impact on food and water consumption, mice showed a body weight gain [Table 1].

Subacute Toxicity

A single administration of *F. glumosa* aqueous extract (300, 600, and 1200 mg/kg) was able to provoke 24 h later a significant increase ($P < 0.05$) and dose-dependent volume of urinary excretion. Urine volume increased from 22.38 ± 3.13 ml/100 g/24 h in controls (distilled water) to 38.53 ± 4.17 ml/100 g/24 h at the dose of 300 mg/kg that represents an increase of 72.16%. With the dose of 600 mg/kg, urine volume increased to 46.81 ± 2.14 ml/kg/24 h representing an increase of 109.15%. For the highest dose (1200 mg/kg), the volume of urinary excretion went from 22.38 ± 3.13 in controls ml/100 g/24 h to 59.80 ± 2.65 to ml/100 g/24 h in the treated group [Figure 1], which represents an increase of 167.20%. The pH values of urine of animals treated with the extract of *F. glumosa* at the dose 300 mg/kg showed no significant change. The dose of 1200 mg/kg showed a nonsignificant ($P > 0.05$) pH values (7.12 ± 0.17). The pH value of the urine of animals treated with the extract at dose of 600 mg/kg was reduced (6.35 ± 0.67) than that of the control group [Figure 1].

Effect of F. glumosa on the relative organ weights

The aqueous extract of *F. glumosa* had no significant effect ($P < 0.05$) on the heart. However, the dose of 1200 mg/kg, increased significantly ($P < 0.05$) the relative weight of liver, kidney, testis, and epididymis in males whereas, in females, the weight of kidney, uterine, and ovarian was significantly increased 6 weeks after administration of a daily dose of the *F. glumosa* extract [Table 2].

Effect of F. glumosa on body weight change

The body weight of rats was not affected by the administration of a daily dose of the aqueous extract of *F. glumosa* for

Table 1: Effects of the aqueous extract of *F. glumosa* on animals behavior

Dose (g/kg)	% Mortality	Latency	Symptoms	% Weight change (g)	% Food consumption (g)	% Water consumption (mL)
0	0/10	-	None	16.88±12	14.51±11	27.00±03
2	0/10	-	None	24.67±11*	29.61±06*	12.50±02
4	0/10	-	None	28.98±07*	30.25±10*	21.59±06
6	0/10	-	None	20.44±09*	25.36±11*	25.45±10
8	0/10	>2h<4h	Light reduction in locomotion, aggressiveness, noise and touched sensitivity and respiratory movement	21.84±12*	36.29±06*	28.94±08
10	0/10	>2h<8h	Light reduction in locomotion, aggressiveness, noise and touched sensitivity and respiratory movement	16.55±13	17.50±12*	27.00±07
12	0/10	>2h<3h	Reduction in locomotion, aggressiveness, noise and touched sensitivity and respiratory movement	14.25±14	15.88±13	17.23±11

Values are means±SEM, $n=10$, $*P<0.05$, a significant difference compared to the control. SEM: standard error of mean, *F. glumosa: Ficus glumosa*

Table 2: Effects of the aqueous extract of *F. glumosa* on the relative organ weights

Organs	Male treatment (mg/kg)				Females treatment (mg/kg)			
	Control	300	600	1200	Control	300	600	1200
Liver	3.38±0.02	3.53±0.03	3.68±0.12	3.76±0.22*	3.12±0.02	3.15±0.01	3.13±0.02	3.24±0.11
Kidneys	0.71±0.04	0.72±0.02	0.73±0.02	0.75±0.04*	0.69±0.01	0.69±0.02	0.67±0.04	0.72±0.04*
Heart	0.30±0.01	0.31±0.02	0.31±0.02	0.32±0.04	0.34±0.02	0.35±0.03	0.35±0.03	0.36±0.04
Lung	0.72±0.01	0.73±0.04	0.75±0.02	0.74±0.03	0.70±0.03	0.71±0.04	0.75±0.02	0.73±0.03
Spleen	0.31±0.03	0.31±0.02	0.35±0.01	0.31±0.03	0.29±0.02	0.30±0.01	0.29±0.01	0.31±0.02
Testis	0.62±0.03	0.62±0.02	0.64±0.01	0.66±0.03*				
Epididymis	0.25±0.01	0.27±0.02	0.29±0.01	0.30±0.02*				
Uterus					0.27±0.03	0.31±0.02	0.35±0.01	0.38±0.03*
Ovary					0.031±0.003	0.032±0.002	0.035±0.001	0.037±0.003*

Values are means±SEM, n=5, *P<0.05, a significant difference compared to the control. SEM: Standard error of mean, *F. glumosa: Ficus glumosa*

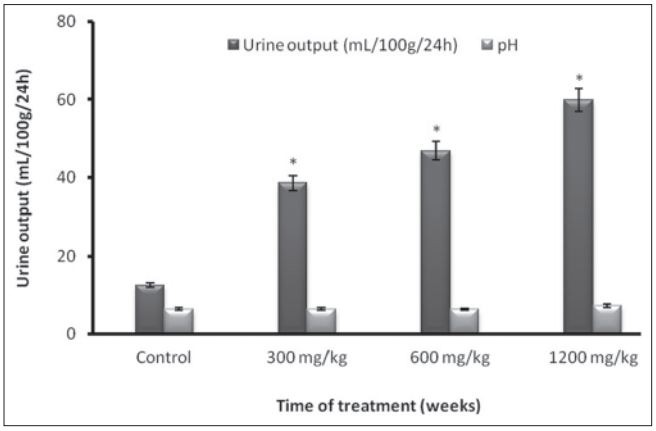

Figure 1: Effects of the aqueous extract of *Ficus glumosa* on the urinary excretion and pH for 100 g of body weight. Values are means ± standard error of mean, n = 5, *P < 0.05, significant difference compared to the control

6 weeks. At the doses of 300, 600, and 1200 mg/kg, the body weight of male rats varied, respectively, from 94.69 ± 0.85%, 87.87 ± 0.78%, and 79.12 ± 1.23% in week 6 of treatment, whereas in females the body weight change was from 83.12 ± 0.39%, 85.56 ± 0.58%, and 76.35 ± 1.28%, respectively, at the doses 300, 600 and 1200 mg/kg in week 6 of treatment. The body weight decreased in both male and female rats treated with the extract at the dose of 1200 mg/kg [Figure 2].

Effects of extract of F. glumosa on food and water intake

An increase of water consumption was observed in all groups. In animals treated with distilled water, the average water consumption increased by 83.12 ± 2.11% at week 6 of the experiment compared to week 1. In animals treated with the extract at dose of 300, 600, and 1200 mg/kg, consumption increased by 94.69 ± 2.68%, 89.87 ± 3.20%, and 74.12 ± 2.22% in males at week 6, respectively. The water consumption followed the same trend in females with increase of 90.69 ± 3.14%, 87.87 ± 3.11%, and 74.52 ± 4.18%, respectively, at doses of 300, 600, and 1200 mg/kg [Figure 3]. The oral administration of a daily dosage of the aqueous extract *F. glumosa* did not have effects in food consumption across treatments. However, there was an increase in food intake from week 1 to week 6 [Figure 4].

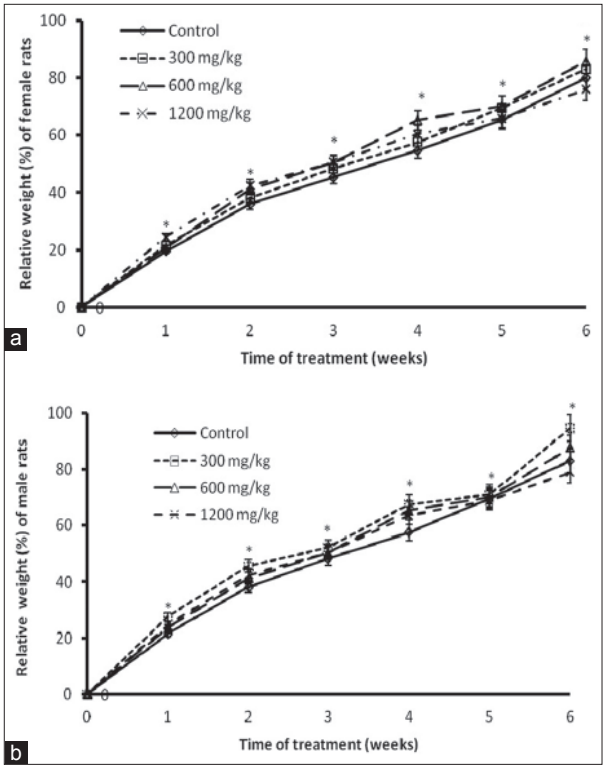

Figure 2: Effects of the aqueous extract of *Ficus glumosa* on body weight change in (a) females (b) males, 6 weeks after administration of a daily dose of extract. Values are means ± standard error of mean, n = 5, *P < 0.05, significant difference compared to the control

Effect of F. glumosa on hematological and biochemical parameters

Daily administration of *F. glumosa* aqueous extract for 6 weeks did not cause significant change in hematological parameters except the platelets which had been increased significantly by 67.79%, 65.97%, and 70.17%, respectively, at the doses of 300, 600, and 1200 mg/kg in males and 120.67%, 122.54%, and 126.85%, respectively, at doses 300 600 and 1200 mg/kg in females (P < 0.05). There were also a nonsignificant decrease in the percentage of basophiles, hematocrit, and a non-significant increase in the percentage of eosinophiles and hemoglobin in males as in females [Table 3].

The effect of the extract was evaluated in the index of liver function. It is clear that cholesterol decreased by 5.15%;

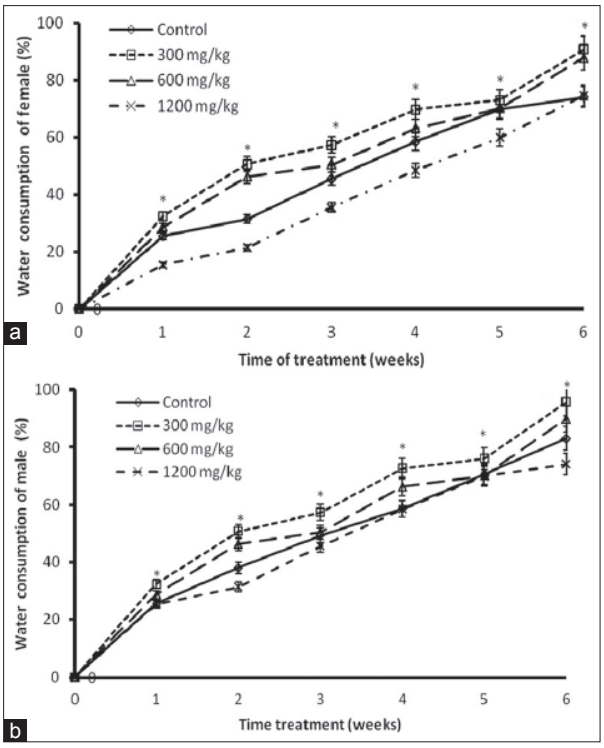

Figure 3: Effects of the aqueous extract of *Ficus glumosa* on water consumption in a) females, b) males, 6 weeks after administration of a daily dose of extract. Values are means ± standard error of mean, $n = 5$, $^*P < 0.05$, significant difference compared to the control

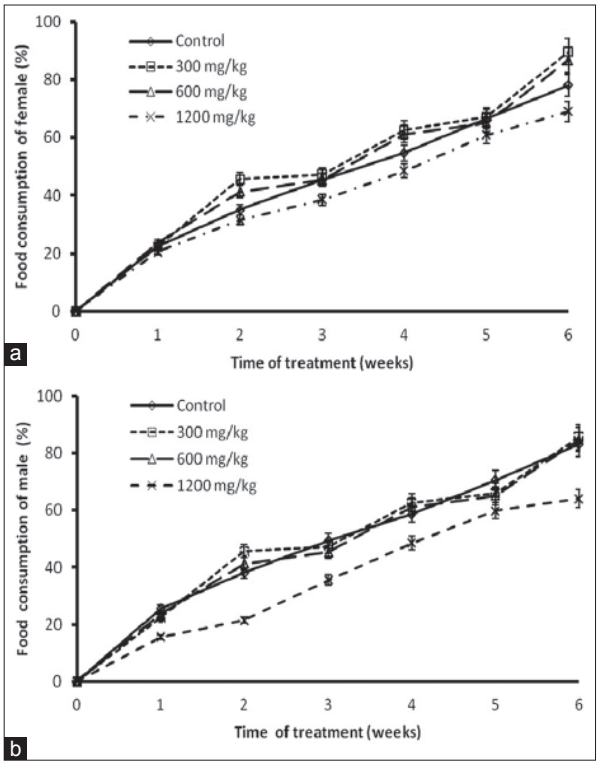

Figure 4: Effects of the aqueous extract of *Ficus glumosa* on food consumption in a) females, b) males, 6 weeks after administration of a daily dose of extract. Values are means ± standard error of mean, $n = 5$, $^*P < 0.05$, significant difference compared to the control

Table 3: Effects of the aqueous extract of *F. glumosa* on hematological parameters

Organs	Normal range	Male treatment				Females treatment			
		Control	300	600	1200	Control	300	600	1200
RBC ($\times 10^6/\mu L$)	5-10	9.12±0.12	9.13±0.23	9.11±0.12	8.76±0.22	8.12±0.22	8.15±0.21	8.43±0.12	8.24±0.11
WBC ($\times 10^3/\mu L$)	1-5	11.71±0.04	12.72±0.02	10.73±0.02	11.25±0.04	8.45±0.1	8.34±0.2	8.67±0.4	8.11±0.4
Platelets ($\times 10^3/\mu L$)	600-1100	456.30±56.61	765.61±86.52*	757.31±45.32*	776.52±34.44*	357.84±12.72	789.65±43.33*	796.35±81.43*	811.76±34.34*
Hemoglobin (g/dL)	11-19	11.22±0.41	11.73±0.54	11.79±0.62	12.74±0.73	11.70±0.53	11.71±0.44	12.75±0.72	11.73±0.63
Hematocrit (%)	35-57	38.31±2.13	41.31±2.32	39.35±2.11	42.31±2.13	36.29±2.08	41.30±2.11	43.29±2.07	41.31±2.22
RDW (%)	12-18	15.42±0.43	15.53±0.54	15.72±0.42	14.84±0.63	15.60±0.43	15.75±0.43	15.65±0.52	14.83±0.60
MCV (fL)	46-65	51.62±0.53	53.67±0.42	54.55±0.44	56.66±0.76	52.45±0.33	51.65±0.22	54.74±0.51	53.56±0.63
MCH (pg)	18-23	17.25±0.51	17.27±0.32	17.49±0.17	17.30±0.02	18.12±0.43	18.34±0.32	18.54±0.41	18.47±0.33
MCHC (g/dL)	31-40	32.12±0.3	32.22±0.2	32.14±0.1	32.26±0.3	33.11±0.3	33.12±0.2	33.24±0.1	33.16±0.3
Neutrophil (%)	2-20	18.62±2.13	19.56±4.22	20.68±3.44	18.56±3.57	19.27±2.33	20.36±3.52	20.85±3.31	19.38±4.43
Basophil (%)	0-7	1.62±0.13	0.92±0.12	0.94±0.11	0.96±0.13	0.83±0.13	0.7±0.12	0.6±0.11	0.7±0.13
Eosinophil (%)	0-1	1.62±0.23	2.22±0.12	2.34±0.24	1.69±0.03	1.60±0.21	2.02±0.32	3.14±0.21	1.78±0.23
Lymphocyte (%)	65-94	67.42±2.23	68.65±1.33	67.69±2.45	69.68±2.43	68.31±2.23	71.42±0.02	72.64±2.43	67.46±2.53
Monocyte (%)	0-6	4.82±0.63	5.61±0.22	6.54±0.43	4.36±0.63	4.77±0.33	6.62±0.32	6.14±0.41	5.16±0.46

Values are means±SEM, $n=5$, $^*P<0.05$, a significant difference compared to the control. SEM: Standard error of mean, *F. glumosa: Ficus glumosa*

10.16%; and 22.41%, respectively, at the doses of 300, 600, and 1200 mg/kg for male and 5.77%, 12.57%, and 21.14%, respectively, at the same doses in females. There was also a decrease in low-density lipoprotein and increase high-density lipoprotein in males and females. The extract did not cause variation in levels of blood glucose and albumin [Table 4].

The index of kidney function was also determined through the blood tests. It appeared from the analyzes that blood levels of creatinine, urea, uric acid, Cl^-, Na^+, K^+, inorganic phosphate, and Ca^{2+} significantly decreased when compared to the control ($P < 0.05$). Only the concentration of Mg^{2+} was significantly higher than that of the control ($P < 0.05$) [Table 5].

Histopathology

The examination of the histopathological cuts of liver, heart, and kidneys of animals treated with aqueous extract of the leaves of *F. glumosa* showed no abnormality.

DISCUSSION

The use of pharmacological properties of this plant in traditional medicine makes this toxicity studies fundamental. Oral administration of aqueous extract of *F. glumosa* in mice did not cause alteration in behavioral responses. No death occurred in any group. These results showed that a single dose of *F. glumosa* had no acute toxic effects, indicating that the medium LD_{50} is higher than 12 g/kg for mice. Therefore, oral administration of *F. glumosa* aqueous leaves extract is safe in mice. However, the aqueous extract of *F. glumosa* in the acute treatment caused at the dose 12 g/kg, depression resulting in a decrease in locomotion, sensitivity to noise and touch and movement and breathing. Acute diarrhea, which preceded the administration of the extract, could be attributed to a volume effect of the aqueous extract of the leaves of *F. glumosa* on intestines [9,16].

No deaths or no clinical signs of toxicity were recorded after daily administration of *F. glumosa* leaf extract for 6 weeks. The extract of *F. glumosa* did not have a significant effect on the heart of rats. However, the dose of 1200 mg/kg increased the relative weight of liver, kidney, testis, and epididymis in males significantly ($P < 0.05$). Whereas, in females, kidney, uterine, and ovarian weights were significantly increased 6 weeks after the treatment. This increase may be attributed to an adaptive response (inflammation or hyperactivity) to the accumulation of the extract or its metabolites within their body. Such results have been reported by Jimoh *et al.* [17], who found an increase in relative kidney weight and liver following subacute administration extract *Arctotis*

Table 4: Effects of the aqueous extract of *F. glumosa* on index of liver function

Organs	Normal range	Male treatment				Females treatment			
		Control	300	600	1200	Control	300	600	1200
Glucose (mg/dL)	70-119	87.53±2.97	89.23±2.11	88.83±2.01	89.35±2.34	90.12±3.39	91.58±2.3*	93.14±2.17*	93.56±3.21
ALT (IU/L)	10-50	39.15±3.9	41.25±2.5*	42.35±3.11*	43.02±3.15*	36.45±2.71	37.8±2.46*	39.49±2.94*	40.87±2.53*
AST (IU/L)	68-135	45.23±3.1	47.25±2.31*	48.35±2.49*	50.12±4.2*	39.27±2.64	40.1±2.65*	42.37±3.15*	43.52±2.17*
ALP (IU/L)	30-90	31.54±4.5	32.67±2.16*	34.26±4.56*	35.97±2.56*	26.31±2.15	27.34±3.14*	29.3±3.27*	30.97±4.26*
TP (g/dL)	4.8-9.2	6.49±0.26	6.98±0.22*	7.03±0.24*	7.12±0.28*	7.63±0.31	7.86±0.37*	8.07±0.36*	8.16±0.34*
Albumin	1.2-6	5.94±0.45	6.02±0.4	6.14±0.41	6.19±0.37	6.17±0.32	6.35±0.26	6.43±0.25	6.45±0.38
TB (mg/dL)	0-0.5	1.3±0.31	1.1±0.23	0.9±0.13*	0.7±0.15*	0.8±0.2	0.7±0.17	0.6±0.11*	0.4±0.09*
CB (mg/dL	0-1	1.15±0.21	1.79±0.11	1.9±0.22*	1.7±0.15*	1.8±0.31	1.7±0.14	1.6±0.21*	1.4±0.11*
TC (mg/dL)	38-96	61.35±3.14	58.34±2.16*	55.69±4.05*	50.12±3.62*	70.21±4.11	66.38±3.16*	62.37±3.6*	57.96±2.54*
TG (mg/dL)	60-140	87.35±3.54	90.01±3.14*	93.24±3.39*	94.21±3.26*	95.12±2.16	97.85±2.67*	99.6±2.64*	102.3±4.18*
LDL (mg/dL)	10-30	17.23±2.31	15.62±3.17*	14.11±3.29*	11.29±2.56*	22.03±3.14	19.36±2.54*	16.64±2.57*	13.89±3.27*
HDL (mg/dL)	15-35	21.35±1.37	22.01±2.04*	22.95±2.03*	23.15±3.01*	19.76±3.2	20.34±1.25*	20.96±2.17*	21.09±1.62*

Values are means±SEM, n=5, *P<0.05, a significant difference compared to the control. SEM: Standard error of the mean, *F. glumosa: Ficus glumosa*, ALT: Alanine transaminase, AST: Aspartate aminotransferase, ALP: Alkaline phosphatase, TP: Total protein, TB: Total bilirubin, CB: Conjugated bilirubin, TC: Total cholesterol, TG: Triacylglycerol, LDL: Low-density lipoprotein, HDL: High-density lipoprotein

Table 5: Effects of the aqueous extract of *F. glumosa* on index of kidney function

Organs	Male treatment				Females treatment			
	Control	300	600	1200	Control	300	600	1200
Creatinine (mg/L)	8.53±2.97	8.23±2.11*	7.83±3.01*	6.35±2.34*	8.12±2.39	8.58±2.3*	7.14±2.17*	6.56±3.21*
Urea (mg/L)	0.55±0.01	0.45±0.01*	0.35±0.02*	0.22±0.03*	0.45±0.01	0.41±0.02*	0.46±0.04*	0.38±0.03*
Uric acid (mg/L)	45.21±3.11	37.25±2.33*	28.35±2.42*	25.12±1.2*	42.20±2.64	40.1±2.65*	37.39±2.15*	26.32±1.16*
Cl^- (mEquiv./L)	83.53±4.31	72.65±2.26*	64.26±4.46*	57.93±2.56*	88.31±2.15	75.34±3.24*	69.3±3.22*	58.97±4.36*
Na^+ (mEquiv./L)	146.44±0.26	137.68±0.22*	141.03±0.24*	145.17±0.28*	142.62±0.71	145.56±0.57*	156.17±0.37*	162.36±0.64*
K^+ (mEquiv./L)	5.44±0.05	5.67±0.04*	5.76±0.01*	5.89±0.03*	5.24±0.02	5.44±0.03*	5.56±0.05*	5.89±0.03*
Ca^{2+} (mg/L)	81.32±0.31	77.11±0.43*	68.29±0.43*	63.71±0.45*	77.28±0.29	73.70±0.37*	68.62±0.31*	61.96±0.39*
Mg^{2+} (mg/L)	15.14±0.15	16.37±0.24*	20.16±0.31*	17.39±0.23*	15. 54±0.22	16.44±0.23*	18.56±0.25*	17.49±0.43*
Pi (mg/L)	31.35±0.24	28.34±0.36*	30.69±0.35*	28.32±0.62*	30.26±0.11	32.34±0.16*	29.37±0.22*	27.96±0.34*

Values are means±SEM, n=5, *P<0.05, significant difference compared to the control, mEquiv./L: Milli equivalent/L, SEM: Standard error of mean, *F. glumosa: Ficus glumosa*

arctotoides. A daily administration of a dose of *F. glumosa* aqueous extract did not affect the body weight after 6 weeks of treatment. The body weight of rats treated with the plant extract at the dose of 1200 mg/kg was, however, decreased in both males and females.

The hematological profile of treated rats showed no significant differences with the control group, except that platelets significantly ($P < 0.05$) increased by 67.79%; 65.97%, and 70.17%, respectively, at doses 300, 600, and 1200 mg/kg in males and 120.67%; 122.54%, and 126.85%, respectively, at doses 300, 600, and 1200 mg/kg in females. Analysis of blood parameters is relevant to assess the effect of plant extract on the bone narrow [18]. The increase of leukocytes may indicate strength of the defense mechanism of an organism [19,20] or an unknown subchronic inflammation. In addition, there were no inclusions in the red cells or white cells were observed from the cell morphology that supports the safety nature of the plant extract.

Total bilirubin was significantly increased ($P < 0.05$) in treated animals suggesting an increase of hemolysis. Biochemical analysis showed that daily administration of aqueous extract in subacute toxicity caused a significant increase ($P < 0.05$) in serum activity of aspartate aminotransferase (AST), ALP, and ALT in rats treated with an extract at doses of 300, 600, and 1200 mg/kg. The increases in AST and ALT may explain hepatocytes attack [21]. This toxic effect could be attributed to various secondary metabolites such as saponins, tannins, and flavonoids present in *F. glumosa* aqueous extract as suggested by Agbaje *et al*. [22] when studying the subacute toxicity of *Syzigium aromaticum* in rats.

The decrease in creatinine and cholesterol levels in rats treated with plant extract suggests beneficial effects of the extract on the kidney and liver [23]. The creatinine and urea levels increased in animals with renal dysfunction, particularly when the glomerular filtration rate is reduced. High cholesterol levels could be explained by the stimulation of lipid anabolism hepatocyte under the action of the extract or an exogenous supply of fatty compounds contained in the extract [24,25].

CONCLUSION

In conclusion, oral acute administration of *Ficus glumosa* aqueous extract produced no signs of toxicity in mice. No mortality was recorded in treated mice after 24 h. The autopsy of organs after 14 days of treatment showed no pathological abnormality in the internal organs. The LD_{50} was above of 12000 mg/kg. Therefore, oral acute administration of *F. glumosa* aqueous extract is safe in mice. Daily administration of the doses of 300, 600, and 1200 mg/kg of the aqueous extract of leaves of *F. glumosa* for 6 weeks in treatment was well-tolerated and did not cause lethal or toxic clinical symptoms in the rat of both sexes.

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