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Original Research

Antifungal effect of eugenol and carvacrol against foodborne pathogens *Aspergillus carbonarius* and *Penicillium roqueforti* in improving safety of fresh-cut watermelon

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ABSTRACT

Background: Essential oil components eugenol and carvacrol (ranging between 100 and 200 ppm for carvacrol and between 250 and 750 ppm for eugenol) were tested for antifungal activity against foodborne pathogenic fungal species *Aspergillus carbonarius* A1102 and *Penicillium roqueforti* PTFKK29 in *in vitro* and *in situ* conditions. **Materials and Methods:** *In vitro* antifungal activity of eugenol and carvacrol was evaluated by macrobroth method, while watermelon *Citrullus lanatus* L. Sorento slices were used for antifungal assays *in situ*. **Results:** Selected components, eugenol and carvacrol showed significant inhibitory effect against tested fungi (*A. carbonarius* A1102 and *P. roqueforti* PTFKK29) in yeast extract sucrose broth, as well as in *in situ* conditions. The minimal inhibitory concentration (MIC) of eugenol against *A. carbonarius* A1102 determined by macrobroth method was 2000 ppm, while against *P. roqueforti* PTFKK29 determined MIC was 1000 ppm. Carvacrol inhibited growth of *A. carbonarius* A1102 at minimal concentration of 500 ppm, while against *P. roqueforti* PTFKK29, MIC was 250 ppm. The assays in real food system watermelon slices for eugenol and carvacrol show that the inhibitory effect against both selected fungal species was concentration dependent. Furthermore, our results showed that antifungal effect of carvacrol as well as eugenol applied on watermelon slices in all concentrations was a result of effective synergy between an active antifungal compound and lower incubation temperature (15°C) in inhibition of *A. carbonarius* A1102. **Conclusion:** The present study suggests that the use of eugenol and carvacrol is promising natural alternative to the use of food chemical preservatives, in order to improve safety and quality of fresh-cut and ready-to-eat fruits.

KEY WORDS: Antifungal effect, *Aspergillus carbonarius* A1102, carvacrol, eugenol, *Penicillium roqueforti* PTFKK29, watermelon

INTRODUCTION

In recent years, consumers' demands regarding food production and food safety have changed dramatically. Consumers are more aware of the influence food has on their health [1]. Fruits and vegetables represent an important part in the human diet providing a significant amount of essential vitamins, minerals and dietary fibers. Subsequently, minimally processed food like fresh-cut fruits and vegetables has become an important part of the diet due to its high nutritional value, freshness and practical use [2]. While most food processing technologies involve the stabilization of products and methods for prolonging their shelf life, minimal processing actually reduces the shelf life of food, which represents a

potential microbiological risk for consumers, especially in case of improper hygienic conditions during distribution and processing [3].

Western society is facing a trend of "green" consumerism that sets a demand for food products preserved without synthetic additives, salt, as well as environmentally friendly food production technologies [4].

In order to follow this trend, one of the possible solutions in assuring food safety is the use of essential oils and their constituents as antimicrobial additives. Essential oils and their constituents like carvacrol and eugenol show antimicrobial activity against different toxicogenic and pathogenic microorganisms [5]. A number of studies have shown that they possess antibacterial [6,7], antiviral [8], antifungal [9-14], antiparasitic, insecticidal [15,16] and anticarcinogenic activity [17].

Constituents of essential oils like carvacrol and eugenol show antifungal activity against a wide range of fungi [18-20]. They could be used as a safer alternative to chemical fungicides in the food industry.

Carvacrol, (2-methyl-5-[1-methylethyl]-phenol), is a monoterpenoid phenol, a hydrophobic compound, soluble in ethanol, diethyl ether, carbon tetrachloride and acetone with a melting point at 1°C and boiling point at 237.7°C. It has been identified as the active constituent of essential oil of *Origanum vulgare* L. (oregano) and essential oil of *Thymus vulgaris* L. (thyme) that exhibits high antimicrobial and antioxidant activities [4].

Carvacrol has been shown to increase membrane fluidity and cause leakage of protons and potassium ions, resulting in a collapse of membrane potential and inhibition of adenosine triphosphate (ATP) synthesis [4]. Aside from the inhibition of the growth of vegetative bacterial cells, carvacrol is able to inhibit the production of diarrheal toxin by *Bacillus cereus* in broth and in real systems. Mode of action of toxin limitation includes two theories: If toxin excretion is an active process, there may be insufficient ATP or proton-motive force to export it from the cell. Alternatively, the lower specific growth rate may mean that the cells use all the available energy to sustain viability, leaving little over for toxin production [21].

Eugenol (2-methoxy-4-[2-propenyl]phenol) is an allyl chain substituted guaiacol, a major component of *Syzygium aromaticum* (clove) essential oil (approximately 85%), but also extracted from essential oils of *Myristica fragrans* Houtt. (nutmeg), *Cinnamomum cassia* Blume (cinnamon), *Ocimum basilicum* L. (basil) and *Laurus nobilis* L. (bay leaf). It is a clear to pale yellow oily liquid with a melting point at −7.5°C and boiling point at 254°C.

Sub-lethal concentrations of eugenol have been found to inhibit the production of amylase and proteases by *B. cereus*. Cell wall deterioration and a high-degree of cell lysis were also noted. The hydroxyl group on eugenol is thought to bind to proteins, preventing enzyme action in *Enterobacter aerogenes* [4].

Aspergillus carbonarius optimally grows at a temperature of 30°C, but it can grow even at 10°C. Water activity growth range is between 0.96 and 0.98, while the pH growth range is from 2 to 10 [22]. It is usually found in grapes, grape juice, wine, raisins, and sometimes on raw coffee beans [23]. Grape contamination can occur before harvest, during harvest or during processing [24].

A. *carbonarius* is the main producer of ochratoxin A, a nephrotoxin which afflicts all tested animal species, while its impact on human health is not easily determined [25]. The connection between ochratoxin A and Balcan endemic nephropathy has been the subject of numerous studies but is, as yet, not completely established [26]. Food products that could potentially be contaminated with ochratoxin A are jams and grape vinegar [24].

Penicillium roqueforti is a psychrothrophic mold that can grow well in cold temperatures and cause spoilage of refrigerated food products. However, the optimal growth temperature of *P. roqueforti* lies between 20°C and 30°C for water activity of 0.89-0.92. It can grow in a wide range of pH values (3-10) [22]. Furthermore, *P. roqueforti* is not susceptible to inhibiting activity of weak acids which are usually used as preservatives in the food industry [27].

Although known as a starter culture in "Roquefort" cheese production, *P. roqueforti* also produces certain toxins such as *P. roqueforti*toxin, roquefortin C, and mycophenolic acid. The latter two compounds have only limited toxicity [28].

The aim of this study was to determine the antifungal effect of essential oil constituents carvacrol and eugenol against the foodborne pathogenic molds A. *carbonarius* A1102 and *P. roqueforti* PTFKK29 in *in vitro* and *in situ* conditions on slices of watermelon *Citrullus lanatus* L. Sorento at different incubation temperatures.

MATERIALS AND METHODS

Fungal Cultures

Fungal cultures of A. *carbonarius* A1102 and *P. roqueforti* PTFKK29 species were obtained from the collection of fungi of the Faculty of Food Technology Osijek. Strains were maintained on slants of potato dextrose agar (PDA) (Biolife, Italy) at 4°C. Before experiments, cultures were grown on PDA slants at 25°C for 5 days. Spores were harvested and suspended homogeneously in sterile distilled water with 0.05% Tween 80. Afterwards, the spores in the suspension were counted (Bürker-Türk counting chamber) and their number was adjusted to 1×10^5 spores mL^{-1}.

In vitro Antifungal Activity of Eugenol and Carvacrol

In vitro antifungal activity of eugenol and carvacrol was evaluated by macrobroth method [29] on fungal species of A. *carbonarius* A1102 and *P. roqueforti* PTFKK29.

Eugenol (concentrations applied were: 250, 500, and 750 ppm) and carvacrol (concentrations applied were: 100, 150 and 200 ppm) (Sigma, Germany) were dissolved in solution of 10% Tween 80 (Biolife, Italy), 96% ethanol (Kemika, Croatia) and distilled water, sterilized by filtration and used immediately.

Yeast extract sucrose broth (YESB) was used as growth medium for measurement of the inhibitory effect of eugenol and carvacrol. Medium was sterilized at 121°C for 15 min followed by cooling to 50°C in a water bath. After cooling, different concentrations of eugenol and carvacrol were added to sterile broth, homogenized and 5 mL of growth medium was dispensed in test tubes. Fungal spores were inoculated (10^5 CFU/mL) in YESB and incubated at 25 ± 1°C, and the minimal inhibitory concentration (MIC μg/mL) was recorded after 72 h of incubation. Suitable controls: Broth control (without fungal spores), growth controls (with fungal spores), solvent (Tween 80 and 96% ethanol) and eugenol or carvacrol controls were set under identical conditions. The last tube with no apparent growth of the organism represented the MIC of the compound. To test minimal fungicidal concentration (MFC), from last tube 100 μL was transferred to YESB without antifungal compounds. After 72 h of incubation at 25°C if no growth was observed, MFC was detected. The experiment was performed in duplicates and repeated twice.

In situ Antifungal Activity of Eugenol and Carvacrol

Antifungal activity of eugenol and carvacrol was tested in *in situ* conditions on watermelon *C. lanatus* L. Sorento. Watermelon was washed in tap water and dried with paper towels followed by surface sterilization with 70% ethanol. Fruit was cut (with sterile knife) on 5 mm thick slices, and additionally slices of ϕ = 20 mm, were cut with sterile tube cap. Slices were transferred in Petri plates (5 slices/dish) and left in laminar hood with ultraviolet lamps turned on for 20 min. Each watermelon slice was inoculated with 5 μL of fungal spore suspension (1×10^5/mL) followed by 5 μL of tested component solution. Slices were incubated at 25°C and 15°C. Every second day, fungal colony radii were measured, in 2 perpendicular directions, until 6th day of the incubation period. Experiment was performed in duplicates in 2 independent replications. Experiments were performed in July 2012 and July 2013.

Statistical Analyses

Results were analyzed by Microsoft® Office Excel 2003 (Microsoft Corporation, Redmond, USA) and GraphPad Prism version 5.0 for Windows (two-way ANOVA with multiple comparison and Bonferroni *post-hoc* test) (GraphPad Software, San Diego, USA).

RESULTS

The antifungal activity of eugenol and carvacrol against fungal species *A. carbonarius* A1102 and *P. roqueforti* PTFKK29 expressed as MIC and MFC concentrations were presented in Table 1. Antifungal activity of tested compounds ranged from 1000 (MIC) to >2000 ppm (MFC) of eugenol or 250 (MIC) to 2000 ppm (MFC) of carvacrol.

The activity of eugenol and carvacrol on watermelon slices (*in situ* conditions) at 25°C and 15°C was presented in Figures 1-4.

DISCUSSION

Essential oils or their components like eugenol or carvacrol used for antifungal testing are becoming more interesting in modern food technology, although their effect is well-known. Nowadays, consumers concerned about their health are more interested in food produced with minimal processing

Table 1: Antifungal activity of eugenol and carvacrol on *A. carbonarius* and *P. roqueforti*

Species	Eugenol		Carvacrol	
	MIC	MFC	MIC	MFC
A. carbonarius	2000	>2000	500	2000
P. roqueforti	1000	2000	250	1000

MIC: Minimal inhibitory concentration (ppm), MFC: Minimal fungicidal concentration (ppm), *A. carbonarius: Aspergillus carbonarius*

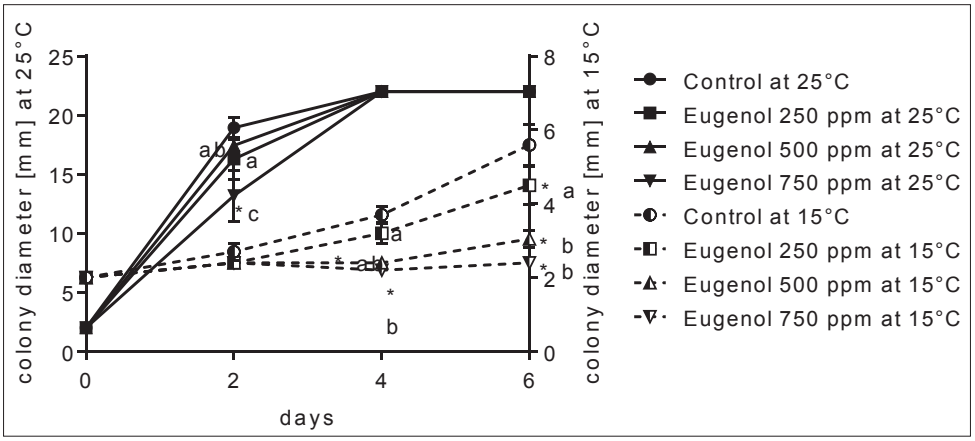

Figure 1: *Aspergillus carbonarius* colony growth inhibition by eugenol on watermelon slices at 25 and 15°C. *Significant difference compared to control ($P \leq 0.05$). Letters (a, b, and ab): Significant difference between tested treatments ($P \leq 0.05$)

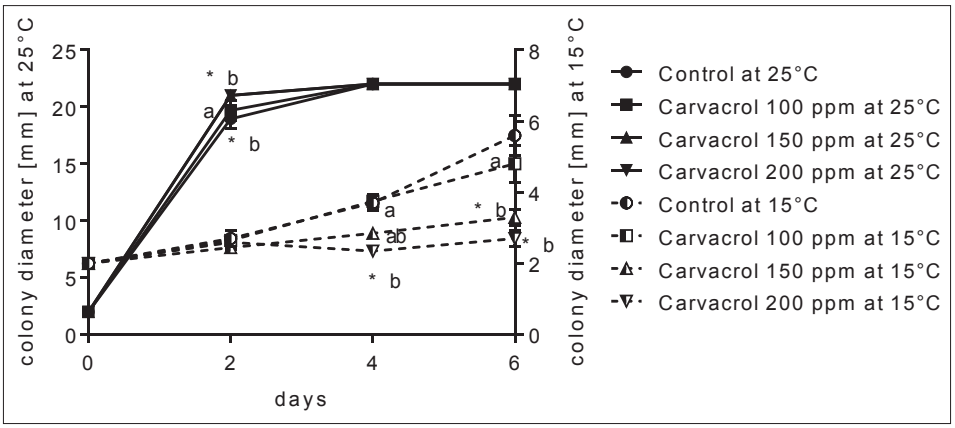

Figure 2: *Aspergillus carbonarius* colony growth inhibition by carvacrol on watermelon slices at 25 and 15°C. *Significant difference compared to control ($P \leq 0.05$). Letters (a, b, and ab): Significant difference between tested treatments ($P \leq 0.05$)

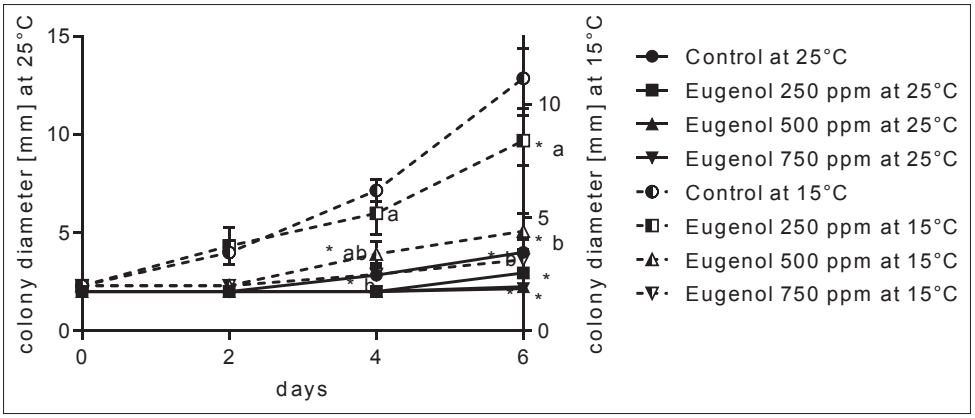

Figure 3: *Penicillium roqueforti* colony growth inhibition by eugenol on watermelon slices at 25 and 15°C. *Significant difference compared to control ($P \leq 0.05$). Letters (a, b, and ab): Significant difference between tested treatments ($P \leq 0.05$)

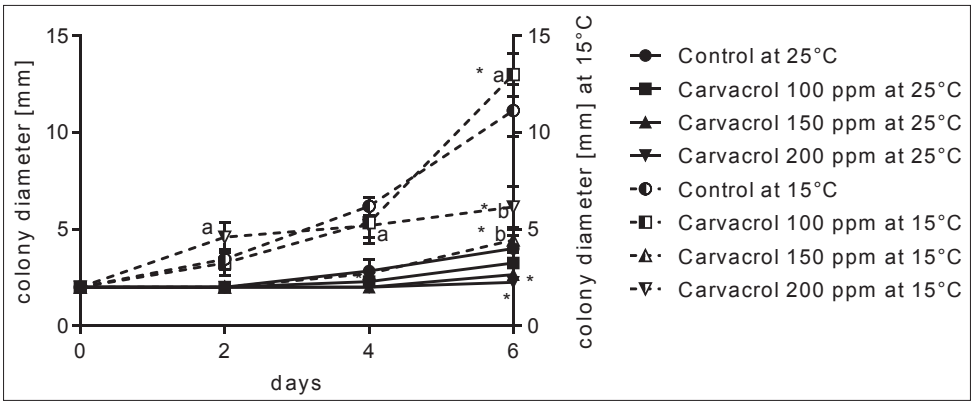

Figure 4: *Penicillium roqueforti* colony growth inhibition by carvacrol on watermelon slices at 25 and 15°C. *Significant difference compared to control ($P \leq 0.05$). Letters (a, b, and ab): Significant difference between tested treatments ($P \leq 0.05$)

or preservatives added. This represents a problem in food technology since food safety during the production process or storage is not easily attained. One of the most affected products is minimally processed food-fruit or vegetable salads. During processing minimally processed food, washing (with or without disinfectants), cutting and packaging are allowed. High temperature treatments are usually not permitted. These factors, together with cutting that cause cellular juice leakage, facilitate microbial growth. Fungi selected for this experimental study are important in the food industry for several reasons: *A. carbonarius* is widespread on fruits, produces a huge amount of contaminant conidia while *P. roqueforti*, although not common as a fruit detrimental fungus, is capable for growth at lower temperatures. Therefore, *P. roqueforti* goes in the group of important cold storage fungi. Watermelon, as well as other vegetables (melons, cantaloupes) and fruits

(strawberries, apples, etc.) have become very popular ready-to-eat minimally processed salads for consumers worldwide. However, the absence of more intensive preservation methods increases their susceptibility to fungal (or bacterial) growth and besides spoilage, minimally processed salads are widely known as serious safety issue.

The most sensitive fungal species tested was *P. roqueforti* PTFKK29 [Table 1], since eugenol acted fungicidally at 2000 ppm on its growth. Carvacrol, at a concentration of 1000 ppm, was even more effective in fungicidal activity on tested fungi. Furthermore, MIC of carvacrol was, four times lower compared to eugenol. Since fungicidal effect of carvacrol is well-known, compound, as well as oregano or thyme essential oils, can be used as efficient preservatives in food processing technology. Similarly, A. *carbonarius* A1102 was more susceptible to carvacrol (MIC value of 500 ppm compared to 2000 ppm of eugenol) although this species, compared to *P. roqueforti* PTFKK29 is more resistant to tested chemicals. Interpretation of results obtained from antimicrobial assays is demanding, due to different methodology or different strains applied. In a similar experiment [30] authors observed MIC activity of carvacrol and eugenol on *Penicillium expansum* at a concentration of 262 and 500 ppm, respectively, while in our experiment (unpublished data) the same species was inhibited by 500 and 2000 ppm of antifungal compounds tested. Selection of different strains of the same species in antifungal assays is suggestible, since quite different results can be obtained.

The results of MIC activity of eugenol and carvacrol were used in *in situ* testing of antifungal efficacy of compounds against same species on watermelon slices incubated at 25°C and 15°C. The main idea of performing this assay on selected temperature regimes came from improper cold storage conditions, often observed in markets. Concentrations of compounds applied were ¼, ½, and ¾ of MIC of eugenol and carvacrol.

At a temperature of 25°C, A. *carbonarius* A1102 grew rapidly and at 4th day of incubation, all tested concentrations of eugenol, as well as control samples, reached 22 mm (diameter of watermelon slices). This species showed rapid growth on tested fruit slices, especially at higher storage temperature applied. Statistically significant difference, compared to control, was observed only at the highest concentration (750 ppm) applied at 2nd incubation day while among concentration of eugenol, 250 and 750 ppm were significantly different [Figure 1]. Lower growth rate at 15°C was observed where lag phase of fungal growth occurred even in the control sample until 2nd day, while 500 and 750 ppm prolonged this phase until 6th day (possibly even further, although this day was the final day of incubation). Higher concentrations applied, at 4th and 6th day were statistically significant. Carvacrol affected A. *carbonarius* growth in a similar way [Figure 2] where, at 25°C almost all samples reached the end of fruit slices. However, significant difference was observed between 200 ppm and 150 ppm, compared to control sample (2nd incubation day). At 15°C fungal growth was slower, reaching <6 mm at the end of the incubation period (control sample). Although almost no difference was observed at the start of incubation time, incubation at 4th and 6th day indicated a significant difference in activity of carvacrol, where 200 and 150 ppm were different compared to results obtained by 100 ppm, as well as a control sample. Both eugenol and carvacrol acted similarly on A. *carbonarius* during *in situ* experiments on watermelon slices.

Compared to A. *carbonarius*, *P. roqueforti* showed slower growth rate at both selected temperature intervals [Figures 3 and 4]. Interestingly, this species grow faster at a lower temperature (15°C) which shows its psychrotrophic nature. Lag phase of fungal growth lasted until 4th incubation day for the control sample, while treatments prolonged this phase further to 6th day. Since the growth at 25°C was slow, differences between treatments and control are visible at 4th and 6th incubation day, although only difference between control and treatments was noticed. Further incubation would show differentiation between control and concentration of eugenol applied in this experimental setup. Higher growth rate with strong differentiation between concentrations applied was observed at 15°C incubation temperature where all treatments applied were significantly different compared to control sample. Although, considerably lower concentrations of carvacrol on slices were applied, similar results of fungal growth inhibition occurred at both temperatures applied [Figure 4]. Lag phase of *P. roqueforti* growth was shorter for 2 days compared to eugenol [Figure 3]. Although the growth rate of *P. roqueforti* treated with 100 ppm of carvacrol was faster, compared to control, as suspected since, if fungi are treated with chemicals applied in concentrations below their inhibitory concentration, their growth rate (and even mycotoxin production) can be even more pronounced. Differences are also possible due to fruit surface structure. Watermelon surface has pronounced hollows that make colony diameter assessment more complicated. This problem can be successfully resolved by an increasing number of fruit slices tested and with experiment repetitions.

Potential disadvantage in the application of essential oils or their components in food systems is a modification of characteristic sensory profile of the food. In this experimental study, this specific issue was not detected, since small volume of tested compounds was applied (5 μL).

CONCLUSION

Both selected essential oil components, eugenol and carvacrol inhibited tested fungi (A. *carbonarius* A1102 and *P. roqueforti* PTFKK29) in YESB, as well as in *in situ* conditions (watermelon slices). Lag phase of the fungal colony growth on watermelon was prolonged in response to higher concentrations of components applied, for a longer period of time. Lower temperature (15°C) retarded the growth of A. *carbonarius* A1102, while this effect was not observed during the growth of *P. roqueforti* PTFKK29. Inhibitory effect of both tested compounds against fungal growth of selected fungal species on watermelon slices is concentration dependent.

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Original Research

Low anti-ulcerogenic potentials of essential oils and methanolic extract of *Croton zambesicus* leaves

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ABSTRACT

Objective: This study evaluated the anti-ulcer properties of *Croton zambesicus* leaves. **Materials and Methods:** Group I was control. 40 mg/kg/bodyweight of indomethacin (the ulcerogen) was administered to rats of Groups II-VII. 4 h after administrations of ulcerogen; rats of Groups III-VII were treated daily with oral administrations of 40 mg/kg/bodyweight of omeprazole, 5 and 10 mg/kg/bodyweight of essential oils, 250 and 500 mg/kg bodyweight of methanolic extract of *C. zambesicus* leaves respectively for 4 days. Rats were euthanized on day 1 (Group II) and day 5 (Groups I and III-VII); thereafter, stomach and liver samples were removed for evaluations of gastric acidity, histopathological and alanine aminotransferase (ALT) status. **Results:** Analyses of gastric acid assays and histopathological examinations showed dose-dependent statistically significant higher levels ($P \leq 0.05$) of gastric acidity and non-restorations of the gastric mucosa layer to pre-ulceration states in rats of Groups IV-VII treated with extract doses when compared to Group III. Statistically non-significant (Group IV) or significant (Groups V-VII) higher ALT levels ($P \leq 0.05$) were observed in liver samples of rats treated with doses of essential oils and methanolic extract of *C. zambesicus* leaves when compared to Group II. **Conclusion:** Our study observed low anti-ulcerogenic potentials of doses of essential oils and methanolic extracts of *C. zambesicus* leaves.

KEY WORDS: *Croton zambesicus*, indomethacin, ulceration

INTRODUCTION

Ulceration refers to any break in the skin or mucus membrane and is classified according to the part of the digestive system in which it occurs [1,2]. Gastric ulcer occurs in the stomach while peptic ulcer occurs in sections of the gastro intestinal tract exposed to gastric acid and pepsin such as the stomach and duodenum [1,2]. The etiology of gastric ulceration is not clearly known. It results probably from an imbalance between aggressive (acid, pepsin and *Helicobacter pylori* infection) and defensive (gastric mucus and bicarbonate secretion, prostaglandins (PGs), cyclooxygenases, nitric oxide, innate resistance of the mucosal cells) factors; as well as factors such as genetic, psychosomatic, humoral and vascular derangements [1]. Pathological examination of gastric ulcer could be evaluated in any part of the stomach, but is most commonly obtained from the lesser curvature. Histologically, there is a break in the superficial epithelium penetrating down to the muscularis mucosa with a fibrous base accompanied with increase in inflammatory cells [1].

Croton zambesicus is a tree native to West and Central Africa. It grows up to 16 m in height and is traditionally used to treat fever, dysentery, hypertension, convulsions and bacterial infections [3-5]. Amongst the Yorubas of South West, Nigeria; it is locally called "ajekobale"' which means "witches do not dare to perch on it" and is, therefore, believed to possess spiritual properties that can be used to counter the forces of witchcrafts [3]. Scientific studies have observed antimicrobial properties of the bark of *C. zambesicus* [6], antiplasmodial [7] and anti-ulcer potentials of the ethanolic extracts of its roots [8] and anti-coagulant properties of dichloro methane and aqueous extracts of *C. zambesicus* leaves [9]. This study, therefore, compared the anti-ulcer properties of doses of methanolic extract and essential oils of the leaves of *C. zambesicus* to determine which of the extraction products would better improve the status of alanine aminotransferase (ALT) concentrations in the liver; gastric acidity and histopathological restoration of the stomach of adult Wistar rats in indomethacin-induced gastric mucosa ulceration.

MATERIALS AND METHODS

Collection, Authentication and Preparation of Plant Extract

Methanolic extraction of C. zambesicus leaves

C. zambesicus leaves were purchased from local traders at Oja-Tuntun market of Ilorin, Kwara State, Nigeria. The collected

samples were identified at the Department of Plant Biology of the University of Ilorin, Kwara State, Nigeria and deposited at the herbarium. Air-dried samples of *C. zambesicus* leaves were pulverized and 200 g of the plant material was extracted with 70% methanol for 24 h. The plant materials were re-soaked in 70% methanol for 2 weeks for maximum extraction. The extract was filtered, concentrated with rotary evaporator and further dried on a water bath to yield 5.4 g of the extract.

Extraction of essential oils of C. zambesicus leaves

C. zambesicus leaves weighing about 0.75 kg were placed in a Clevenger (distillation apparatus) overlying water. As the water got heated, the steam passed through the plant material, vaporizing the volatile compounds. The vapors flowed through a coil where they condensed back to liquid, which was then collected in the receiving vessel. This procedure yielded 1 ml of oil which was dissolved in 2.5 ml of dimethylsulphoxide and normal saline up to 100 ml.

Ethical Approval, Care and Feeding of Animals

Thirty-five adult female wistar rats weighing between 100 and 170 g were obtained from the colony bred of the Physiology Department of University of Ibadan, Ibadan, Oyo State, Nigeria. Animals were fed during the experiment with growers feed from Bendel Feed and Flour Mill Ltd., Nigeria. The animals were caged under standard condition in the well ventilated animal house of the Faculty of Basic Medical Sciences of University of Ilorin, Ilorin, Kwara State, Nigeria at room temperature of 25°C. Water was supplied *ad libitum* to the rats. Ethical approval was sought and received from the ethical committee of the Faculty of Basic Medical Sciences of University of Ilorin, Ilorin, Kwara State, Nigeria on the usage of animals for experimental studies.

Chemicals, Reagents and Laboratory Equipments

Indomethacin (Hovid, Nigeria), Omeprazole (Eprazole, China), sodium dihydrogen phosphate (NaH_2PO_4), disodium hydrogen phosphate (Na_2HPO_4), hydrogen peroxide (H_2O_2) and trichloroacetic acid were products of Aldrich Chemicals; sulphuric (VI) acid (H_2SO_4) and hydrochloric acid were products of BDH Chemical Limited, Poole, England; Tris buffers, phosphoric acid and pyrogallol were products of Sigma Chemicals, St. Louis USA and ALT assay kits of Randox Laboratories, United Kingdom. Spectrophotometer (Jenway Model 6405, UV/visible), centrifuge, pH meter (Rex model pH 25), Norm-jet needles and syringes (Norm-jet Inc. Tuttlinger, Germany) and anti-coagulant tubes (Sterling products, England).

Administrations of Doses of Drugs/Extracts to Animals

Doses of drugs (Normal Saline, Indomethacin and Omeprazole), methanolic extract and essential oils were administered orally to Wistar rats according to the earlier described protocol on animal models of gastric ulceration experimentation [10,11]. Oral administrations of drugs were carried out using 3-ml syringe with a 16-G, 3-inches needle attached. The thirty-five rats were divided into seven groups with five rats per group. Rats of Control Group I received physiological saline daily for 5 days (days 1-5). On day 1, a single oral dose of 80 mg/kg bodyweight of indomethacin administered to rats of Group II-VII that have been deprived of food for the previous 18-24 h, produced erosive lesions in the gastric mucosa within 4-6 h. Rats of Group II were euthanized after induction of gastric ulceration on day 1 for scoring of ulceration, gastric acidity assay, histological and biochemical evaluations.

Treatments of gastric ulceration was started on day 1 with a single oral administrations of 40 mg/kg bodyweight of omeprazole, 5 and 10 mg/kg bodyweight of essential oils of *C. zambesicus* leaves, 250 and 500 mg/kg bodyweight of methanolic extract of *C. zambesicus* leaves to rats of Groups III-VII respectively, 4 h after administration of 80 mg/kg bodyweight of indomethacin. The treatment procedures were continued daily for another 3 days (days 2-4). Administered drugs or extract doses were freshly prepared daily. Upon completion of treatment procedures on day 4, the animals were left without food but provided with water *ad libitum* 14 h prior to euthanasia. 30 min prior to euthanasia, 1 ml of 1% Evan's blue in saline was injected intravenously into the tail vein of rats of Group II (on day 1); Groups I and III-VII (on day 5) using a 1 ml 25-G, 5/8-inch needle to aid identification and evaluation of lesions and ulcerations.

Scoring System for Gastrointestinal Lesions in the Rats

Score	Characteristics
0	No ulcerations or mucosal damage
1	Up to 15 small mucosal ulcerations (<1 mm in diameter) observable only as slight depressions in reflected light
2	Small and medium mucosal ulcerations (1-4 mm in diameter); no ulcerations >4 mm in diameter
3	Predominantly medium and large ulcerations; ulcerations >4 mm in diameter; no intestinal adhesions
4	Large ulcerations; exhibit signs of perforations and adhesions which make it difficult to remove the intestinal tracts
5	Necropsy of dead or euthanized animals reveals evidence of massive peritonitis resulting from intestinal perforations

Evaluations of Gastric Acidity in Stomach Tissues

The stomach contents were collected into a centrifuge bottle, mixed properly with Normal Saline and the mixture centrifuged at 2000 revolutions/min for 10 min. One drop of phenolphthalein indicator was then added to the supernatant. 1 ml of the supernatant (volume of acid-V_A) was pipetted and titrated against 0.01M NaOH (concentration of the base-C_B). The color change was noted (end point) and the volume of base (V_B) used was recorded. Gastric acidity was calculated using $C_AV_A = C_BV_B$.

Evaluations of ALT Levels in Liver Tissues of Rats of Groups I-VII

The liver was excised and removed from each rat of Groups I-VII, cut into small pieces, placed in a mortar to which 0.1M phosphate buffer (extracting solution) of at least four times the

volume of the organ was added. The organ was homogenized into fine solution with the use of mortar and pestle. The homogenate was poured into a test tube and centrifuged at 5000 revolutions/min for 5 min. The supernatant was carefully removed and the residue was discarded. The supernatant served as the sample for the estimation of ALT levels which were determined in liver samples of rats of Groups I-VII based on the protocols described in assay kits of Randox Laboratories, United Kingdom.

Histological Analyses

The stomach samples of rats of Groups I-VII were excised and removed for histopathological evaluations as earlier described [11].

Statistical Analyses

The mean ± standard error of mean value of each of the measured parameters of gastric acidity and ALT assays in rats of Control Group I (which received physiological saline) and Group II (which received indomethacin only) were compared with those of Groups III-VII (indomethacin plus omeprazole, Indomethacin plus methanolic extract doses or indomethacin plus essential oils of *C. zambesicus* leaves) for any significant difference using the Student's *t*-test for unpaired samples. $P = 0.05$ (or less) were taken as statistically significant.

RESULTS

Analyses of gastric acid assays and histopathological examinations showed dose-dependent statistically significant higher levels ($P \leq 0.05$) of gastric acidity and non-restorations of the gastric mucosa layer to pre-ulceration states in rats of Groups IV-VII treated with extract doses of *C. zambesicus* leaves when compared to rats of Group III treated with 40 mg/kg bodyweight omeprazole [Table 1 and Figures 1-7]. Specifically, the cytoarchitectural components of the stomach of rats of Control Group I appeared normal while it appeared disrupted in rats of Group II which received only 80 mg/kg bodyweight Indomethacin without further treatment. In rats of Group II, the gastric mucosa components were eroded with multi-focal cellular necrosis, total degeneration of mucus secreting cells and excessive hemorrhage of the mucosa. However, the cytoarchitectural components of the stomach of rats of Group III treated with 40 mg/kg bodyweight omeprazole appeared normal though with mild hemorrhage of the gastric mucosa and few ulcerated sites. There was gradual regeneration of disrupted mucosa following indomethacin-induced gastric ulceration.

The cytoarchitectural components of the stomach of rats of Group IV treated with 5 mg/kg bodyweight of essential oils of *C. Zambesicus* leaves appeared disrupted. Large parts of the mucosa were eroded with multi-focal cellular necrosis, degeneration of mucus secreting cells and excessive hemorrhage of the mucosa. The cytoarchitectural components of the stomach of rats of Group V treated with 10 mg/kg bodyweight of essential oils of *C. Zambesicus* leaves appeared disrupted. The mucosa was eroded with multi-focal cellular necrosis, degeneration of mucus secreting cells and excessive hemorrhage of the mucosa. The cytoarchitectural components of the stomach of rats of Group VI treated with 250 mg/kg bodyweight of methanolic extracts of *C. Zambesicus* leaves appeared disrupted. The mucosa was eroded with multi-focal cellular necrosis, degeneration of mucus secreting cells and excessive hemorrhage of the mucosa. The cytoarchitectural components of the stomach of rats of Group VII 500 mg/kg bodyweight of methanolic extracts of *C. Zambesicus* leaves appeared disrupted. The mucosa was eroded with multi-focal cellular necrosis, degeneration of mucus secreting cells and excessive hemorrhage of the mucosa. However, some parts of the gastric mucosa showed normal cytoarchitectural components indicating possible gradual restoration of the gastric mucosa to pre-ulceration state.

Table 1: Analyses of the effects of extracts of *C. zambesicus* leaves on ulcer index and gastric acidity

Groups of rats	Dose of drugs/ extract	Ulcer index±S.E.M.	Gastric acidity (meq/l)	Statistical significance at $P \leq 0.05$ (Groups IV-VII vs. III)
II	80 mg/kg b.w. indomethacin	3.25±0.25	0.06	Significant increase
III	40 mg/kg b.w. omeprazole	0.36±0.23	0.009	Nil
IV	5 mg/kg b.w. essential oils of *C. zambesicus* leaves	3.25±0.48	0.02	Significant increase
V	10 mg/kg b.w. essential oils of *C. zambesicus* leaves	3.75±0.25	0.02	Significant increase
VI	250 mg/kg b.w. methanolic extract of *C. zambesicus* leaves	1.5±0.29	0.003	Significant increase
VII	500 mg/kg b.w. methanolic extract of *C. zambesicus* leaves	2.75±0.48	0.02	Significant increase

b.w.: Bodyweight, *C. zambesicus: Croton zambesicus*, S.E.M.: Standard error of mean of five determinations, $P \leq 0.05$

Figure 1: Photomicrograph sample of the stomach of rats of Group I which received physiological saline only (H and E, ×100). Up-down arrow extends through the length of gastric mucosa layer, bent arrow points to gastric pits and glands, arrow points to muscularis mucosae and the lightning bolt points to peptic cells. All identification shapes are in green color. The cytoarchitectural components of the stomach appeared normal

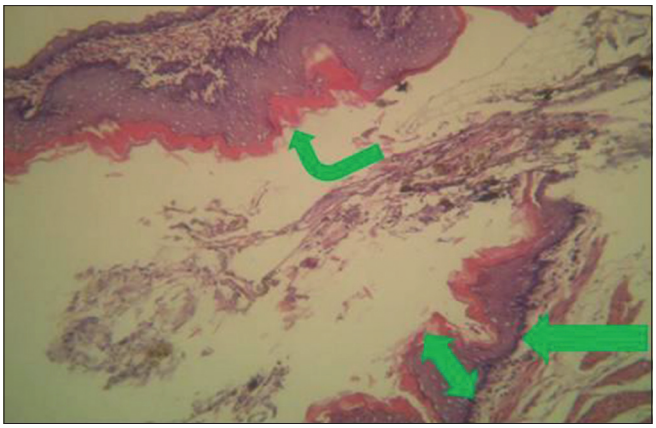

Figure 2: Photomicrograph sample of the stomach of rats of Group II which received 40 mg/kg/bodyweight of indomethacin only (H and E, ×100). Up-down arrow extends through the length of gastric mucosa layer, bent arrow points to gastric pits and glands and the arrow points to muscularis mucosae. All identification shapes are in green color. The cytoarchitectural components of the stomach appeared disrupted. The mucosa components were eroded with multi-focal cellular necrosis, total degeneration of mucus secreting cells and excessive hemorrhage of the mucosa

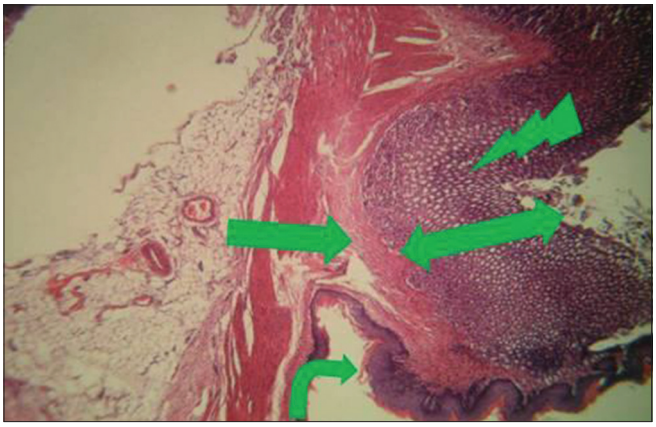

Figure 3: Photomicrograph sample of the liver of rats of Group VI treated with 40 mg/kg/bodyweight of omeprazole (H and E, ×100). Up-down arrow extends through the length of gastric mucosa layer, bent arrow points to gastric pits and glands, arrow points to muscularis mucosae and the lightning bolt points to peptic cells. All identification shapes are in green color. The cytoarchitectural components of the stomach appeared normal though with mild hemorrhage of the gastric mucosa and few ulcerated sites. There was gradual regeneration of disrupted mucosa following indomethacin-induced gastric ulceration

Statistically non-significant (Group IV) or significant (Groups V-VII) higher ALT levels ($P \leq 0.05$) were observed in liver samples of rats treated with doses of essential oils and methanolic extract of *C. zambesicus* leaves when compared to Group II [Table 2].

DISCUSSIONS

Indomethacin is a potent ulcerogen, especially in an empty stomach [10,11]. It induces ulceration mostly in the glandular (mucosal) part of the stomach [1,12] possibly through the inhibition of the release of protective factors like cyclooxygenases, PGE2, bicarbonate, mucus and antioxidants;

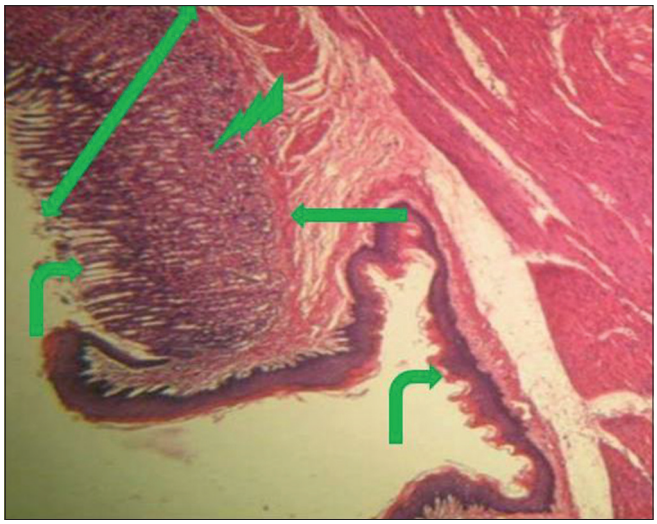

Figure 4: Photomicrograph sample of the stomach of rats of Group IV treated with 5 mg/kg/bodyweight of essential oils of *Croton zambesicus* (H and E, ×100). Up-down arrow extends through the length of gastric mucosa layer, bent arrow points to gastric pits and glands and the arrow points to muscularis mucosae. All identification shapes are in green color. The cytoarchitectural components of the stomach appeared disrupted. Large parts of the mucosa were eroded with multi-focal cellular necrosis, degeneration of mucus secreting cells and excessive hemorrhage of the mucosa

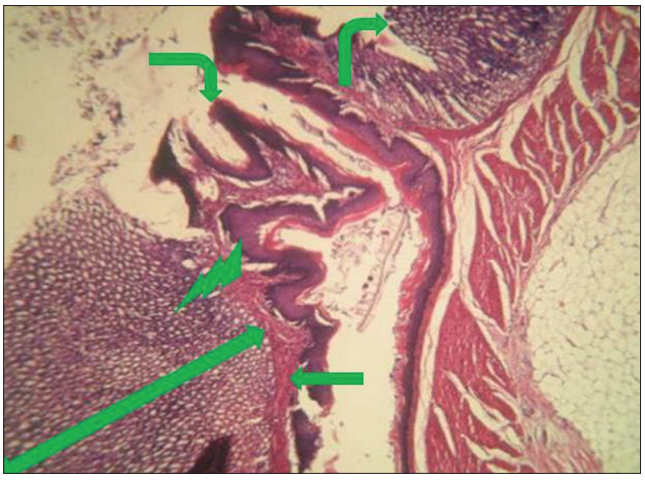

Figure 5: Photomicrograph sample of the stomach of rats of Group V treated with 10 mg/kg/bodyweight of essential oils of *Croton zambesicus* (H and E, ×100). Up-down arrow extends through the length of gastric mucosa layer, bent arrow points to gastric pits and glands and the Arrow points to muscularis mucosae. All identification shapes are in green color. The cytoarchitectural components of the stomach appeared disrupted. The mucosa was eroded with multi-focal cellular necrosis, degeneration of mucus secreting cells and excessive hemorrhage of the mucosa

while aiding vasoconstriction [13] and the increase of aggressive factors such as acid and oxidants [2,12,14]. PGs are potent anti-secretory and anti-ulcer agents which serve protective functions in the stomach by maintaining gastric micro circulation via mucus and bi carbonate stimulation [1,2,12].

Analyses of gastric acid assays and histopathological examinations showed statistically significant higher levels ($P \leq 0.05$) of

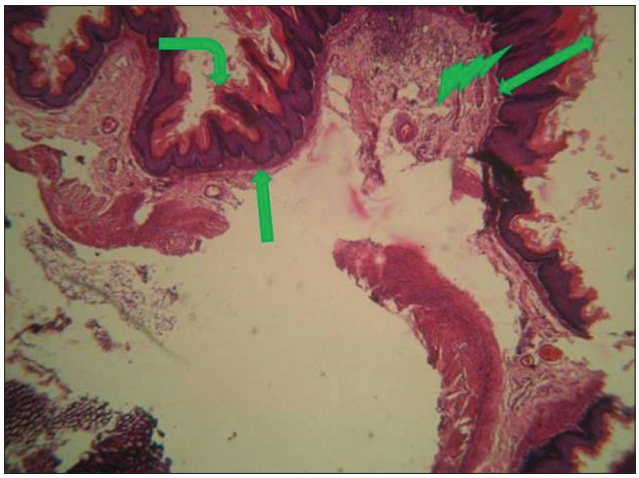

Figure 6: Photomicrograph sample of the stomach of rats of Group VI treated with 250 mg/kg/bodyweight of methanolic extract of *Croton zambesicus* (H and E, ×100). Up-down arrow extends through the length of gastric mucosa layer, Bent arrow points to gastric pits and glands, Arrow points to muscularis mucosae and the Lightning Bolt points to peptic cells. All identification shapes are in green color. The cytoarchitectural components of the stomach appeared disrupted. The mucosa was eroded with multi-focal cellular necrosis, degeneration of mucus secreting cells and excessive hemorrhage of the mucosa

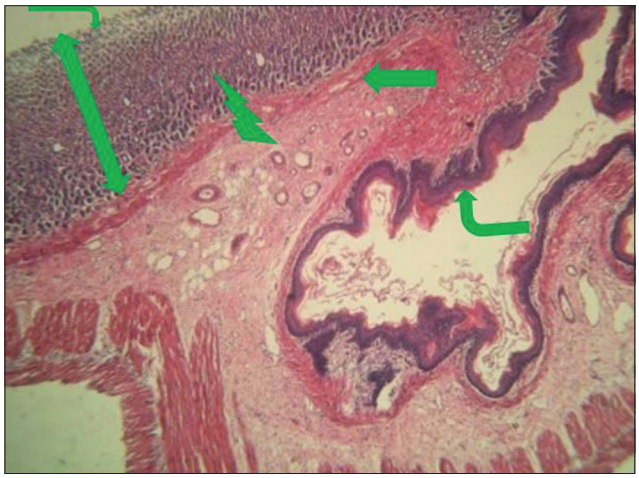

Figure 7: Photomicrograph sample of the stomach of rats of Group VII treated with 500 mg/kg/bodyweight of methanolic extract of *Croton zambesicus* (H and E, ×100). Up-down arrow extends through the length of gastric mucosa layer, bent arrow points to gastric pits and glands, arrow points to muscularis mucosae and the lightning bolt points to peptic cells. All identification shapes are in green color. The cytoarchitectural components of the stomach appeared disrupted. The mucosa was eroded with multi-focal cellular necrosis, degeneration of mucus secreting cells and excessive hemorrhage of the mucosa. However, some parts of the gastric mucosa showed normal cytoarchitectural components indicating possible gradual restoration of the gastric mucosa to pre-ulceration state

gastric acidity and disrupted cytoarchitectural components of the stomach and eroded gastric mucosa in rats of Group II that received only 40 mg/kg bodyweight of indomethacine when compared to rats of Control Group I (which received only physiological saline) or Group III that were treated with 40 mg/kg bodyweight of omeprazole. Histopathologic alanalyses

Table 2: Statistical analyses ALT levels in liver samples of rats of Groups II and I-VII

Groups of rats	Doses of drugs/ extract	ALT concentrations (mg/dl)±S.E.M.	Statistical significance at $P \leq 0.05$ (Groups IV-VII vs. II)
II	40 mg/kg b.w. indomethacin	46±14.04	NIL
IV	5 mg/kg b.w. Essential oils of *C. zambesicus* leaves	48.5±11.53	Non-significant increase
V	10 mg/kg b.w. essential oils of *C. zambesicus* leaves	91.5±11.53	Significant increase
VI	250 mg/kg b.w. methanolic extract of *C. zambesicus* leaves	70.0±5.015	Significant increase
VII	250 mg/kg b.w. methanolic extract of *C. zambesicus* leaves	77.5±2.51	Significant increase

ALT: Alanine aminotransferase, b.w.: Bodyweight, *C. zambesicus: Croton zambesicus*, S.E.M.: Standard error of mean of five determinations, $P \leq 0.05$

of the stomach of rats of Group II showed that the gastric mucosa components were eroded with multi-focal cellular necrosis, total degeneration of mucus secreting cells and excessive hemorrhage of the mucosa [Table 1 and Figures 1-3]. This implied that the administration of indomethacin possibly induced the generations of acids and reactive oxygen species which resulted in the disruption of the cytoarchitectural components of the gastric mucosa of rats of Group II. The observed pathological changes were further made possible with the inhibition of increased production of endogenous antioxidants and prostanglandins by Indomethacin actions; and in the absence of the administration of treatment drugs which could aid the production of protective factors such as PGs, mucous and antioxidants.

Evaluations of gastric acid assays and histopathological examinations showed dose-dependent statistically significant higher levels ($P \leq 0.05$) of gastric acidity and non-restorations of the gastric mucosa layer to pre-ulceration states in rats of Groups IV-VII treated with extract doses of *C. zambesicus* leaves when compared to Group III [Table 1 and Figures 1-7]. The gastric mucosae of rats of Groups IV-VII were eroded with multi-focal cellular necrosis, total degeneration of mucus secreting cells and excessive hemorrhage of the mucosa. This implied that the treatments of indomethacin-induced gastric ulceration in rats with extracts doses were not able to significantly reverse the adverse effects of indomethacin administration in treated rats. Furthermore, extracts doses of *C. zambesicus* leaves could possibly not induce the generation of adequate cyclooxygenases, PGs, bicarbonate, mucus and antioxidants in treated rats. However, some parts of the gastric mucosa in rats treated with 500 mg/kg bodyweight of the methanolic extract of *C. zambesicus* leaves (Group VII) showed normal cytoarchitectural components indicating possible gradual restoration of the gastric mucosa to pre-ulceration state. This noted observation is in agreement with a previous study [15], which opined that crude leaf extract of *C. zambesicus* (200-600 mg/kg) significantly ($P < 0.001$) inhibited chemically – induced ulcers in rats.

Statistically non-significant (Group IV) or significant (Groups V-VII) higher ALT levels ($P \leq 0.05$) were observed in liver samples of rats treated with doses of essential oils and methanolic extract of *C. zambesicus* leaves when compared to Group II [Table 2]. ALT is the enzyme produced within the cells of the liver and is the most sensitive marker for liver cell damage [16]. Increased ALT levels occur in conditions where cells of the liver have been inflamed or undergone cell death. As the cells are damaged, the ALT leaks into the bloodstream leading to a rise in the serum levels. However, ALT levels may or may not correlate with the degree of cell death or inflammation [16]. The significantly increased elevated levels of ALT in liver samples of rats of Groups II and V-VII could possibly indicate decreased functional capacity and cellular damage of the liver consequent to the adverse effects of indomethacin-induced generations of reactive oxygen species.

Phytochemical analyses of different parts of *C. zambesicus* showed the presence of flavonoids in its ethanolic root extract [5], diterpenes [17,18], triterpenes and trihydroxyflavone [18], cardiac glycosides and steroids in its stem bark [19]. Flavonoids such as flavones and glycosides promote mucosa PG content, decrease histamine production by mast cells, scavenge free radicals and are natural anti-ulcer agents [20]. Similarly, di-and tri-terpenes have been reported to possess gastroprotective effects in gastric ulceration [2].

The observed low anti-ulcerogenic potentials of administrations of 5 and 10 mg/kg bodyweight of essential oils and 250 mg/kg bodyweight of methanolic extract of *C. zambesicus* leaves in this study could possibly be due to the presence of flavonoids, di-and tri-terpenes in reduced non-potential quantities in *C. zambesicus* leaves. The observed anti-ulcerogenic potential of administration of 500 mg/kg bodyweight of methanolic extract of *C. zambesicus* leaves was lower than that of the standard drug (40 mg/kg bodyweight of omeprazole). This is in agreement with a previous study which observed lower anti-ulcerogenic potentials of ethanolic root extract of *C. zambesicus* when compared to the standard drug (Cimetidine) [8].

The observed low anti-ulcerogenic potentials of administrations of 5 and 10 mg/kg bodyweight of essential oils, 250 and 500 mg/kg bodyweight of methanolic extract of *C. zambesicus* leaves in this study could possibly be due to the presence of flavonoids, di-and tri-terpenes in reduced non-potential quantities in *C. zambesicus* leaves.

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Original Research

Evaluation of safety and protective effects of *Potentilla fulgens* root extract in experimentally induced diarrhea in mice

Vareishang Tangpu, Khirod Deori, Arun Kumar Yadav

ABSTRACT

Aim: The roots of *Potentilla fulgens* Wall. ex Hook. (Rosaceae) have been used in the indigenous system of medicine in Northeast India to treat diarrhea. The aim of this study was to investigate the safety and protective effects of *P. fulgens* root extract in experimentally induced diarrhea in mice. **Materials and Methods:** The protective effects of *P. fulgens* root extract was investigated against experimentally induced diarrhea in mice, using four experimental models, that is the measurement of fecal output, castor oil model, prostaglandin E2 (PGE2) enteropooling assay, and gastrointestinal transit test. The safety assessment of root extract was done in mice on the basis of general signs and symptoms of toxicity, food water intake and mortality of animals following their treatment with various doses of extract (100-3200 mg/kg). In addition, the serum glutamate oxaloacetate transaminase, serum glutamate pyruvate transaminase, cholesterol and total protein of experimental mice were also monitored to assess the toxicity of root extract. **Results:** In the safety assessment studies, *P. fulgens* root extract did not showed any visible signs of toxicity, but mortality was observed in a single animal at 3200 mg/kg dose of extract. The extract also did not showed any adverse effects on the studied serum parameters of experimental animals. In the antidiarrheal tests, administration of 800 mg/kg dose of extract to mice showed 50% protection from diarrhea evoked by castor oil. In addition, the extract also showed 29.27% reduction in PGE2-induced intestinal secretion as compared with 30.31% recorded for loperamide, a standard antidiarrheal drug. **Conclusions:** The results of this study indicate that *P. fulgens* root extract possesses significant antidiarrheal properties. Therefore, the roots of this plant can be an effective traditional medicine for protection from diarrhea.

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INTRODUCTION

Diarrhea is one of the principal causes of morbidity and mortality among children in developing countries [1]. Of India's more than 2.3 million annual deaths among children, about 334,000 alone are attributable to diarrheal diseases [2]. In order to combat the problems of diarrhea globally, the World Health Organization in its diarrheal disease control program has given a special emphasis on the use of traditional folklore medicines in control and management of diarrhea [3]. Medicinal herbs constitute an indispensable component of the traditional medicine practiced worldwide due to their easy accessibility. The use of herbal medicines in the treatment of diarrheal diseases is a common practice in many developing countries [4]. In India, the use of herbal medicines for the treatment of diarrheal diseases is particularly more common in the North-Eastern region, which is inhabited by several indigenous tribes [5-7].

Potentilla fulgens Wall. ex Hook. (Rosaceae) [Figure 1] is a yellow herb of 1-2 ft height. In India, it grows at altitudes of 1500-2000 msl in Sikkim, Assam, Meghalaya, Manipur and Nagaland states. Previous experimental studies on this plant have revealed that its root extract possesses antitumor [8], antioxidant [9], gastroprotective [10], and anthelmintic [11] activities. Whereas, the phytochemical studies on *P. fulgens* have revealed that it contains a few terpenes, namely potentene A, potentene B, and hyptadienic, tormentic and rosamultic acids, besides phenolic compounds viz. epicatechin and epiafzelchin, a flavonoid potifulgene and a glycoside viz. rutin [12,13].

During the course of our ethnopharmacological studies in Manipur, India, it came to our notice that the fresh roots of *P. fulgens* (vernacular name: *Ngarunri*) are either chewed or taken in the decoction form in the treatment of diarrheal disorders by the native people. A literature survey revealed no reports on antidiarrheal effects of this plant species. Therefore,

Figure 1: *Potentilla fulgens*: Whole plant and a flower

considering the ethnomedicinal use of this plant against diarrhea in Manipur, India, in this study, we were interested to investigate the safety and protective effects of *P. fulgens* root extract in experimentally induced diarrhea in mice.

MATERIALS AND METHODS

Preparation of Plant Extracts

The plant material for this study was collected from Manipur, India and authenticated by Dr. Gurung, Herbarium Curator, Department of Botany, North-Eastern Hill University (NEHU), Shillong. A herbarium record of the material was also prepared and assigned a voucher number, i.e., AKY 221, which has been deposited in the Department of Zoology, NEHU, Shillong. The roots were dried under shade and pulverized into powder. Known amounts of the powdered materials were suspended in methanol and engaged for refluxing using Soxhlet fractional distillation apparatus at 40-50°C for 4-6 h. The resulting suspension was decanted out discarding the remnants and the filtrate was further concentrated in a rotatory evaporator under reduced temperature and pressure for removal of the solvent. The percentage yield of final extract was 5.15% w/w.

Drugs and Chemicals Used

Loperamide (Lopax, Axar Pharmaceuticals, Baroda, India) was used as a standard antidiarrheal drug. Castor oil (Fine, Mumbai, India) and prostaglandin E2 (PGE2) (Sigma-Aldrich Chemical Pvt. Ltd., USA) as diarrhea-inducing agents, activated charcoal (Merck, India) as an intestinal transit marker, and Gum Acacia (Fine Chem, Boisar, India) and Tragacanth powder (Central Drug House Pvt. Ltd., Bombay, India) as suspension agents were used in this study.

Experimental Animals

Male and female Swiss albino mice of 6-8 weeks of age (20-30 g) were procured from Pasteur's Institute, Shillong, Meghalaya, and housed singly in polycarbonate cage with free access to standard rodent diet and tap water *ad libitum*. The animals were acclimatized to laboratory condition for 7 days prior to the experiments and maintained at 25 ± 3°C under a light/dark cycle of 12 h. All procedures in this study were performed according to the Institutional Ethics Committee (animal model) guidelines of the NEHU, Shillong and Committee for the Purpose of Control and Supervision of Experiments on Animals Guidelines of Indian Council of Medical Research, New Delhi.

Acute Oral Toxicity Test

Determination of median lethal dose (LD_{50})

Mice were selected randomly and divided into seven groups of six animals' in each. Group I served as control and II-VII were given a single oral dose of test extract with a dose progression factor of two at 100, 200, 400, 800, 1600, and 3200 mg/kg, respectively. Animals were fasted for 3 h from food, but not water, prior to administration of doses. The general signs and symptoms of toxicity, food water intake and mortality rates of animals were observed for 72 h post-treatment. From these observations, LD_{50} was calculated using SPSS software.

Serum Biochemical Tests

In another experiment, mice were divided into two groups of six animals in each for studying the effects of test extract on various serum biochemical profiles. Group I served as control and Group II was given 800 mg/kg single oral dose of extract (the highest dose tested for antidiarrheal activity in this study). Animals were fasted for 3 h from food, but not water, prior to the administration of doses. At the end of the experiment, i.e., 24 h post-treatment, animals were sacrificed by cervical dislocation. The blood samples were collected by cardiac puncture and kept for 30 min without disturbing and then centrifuged for 15-20 min at 2000 rpm to separate the serum. From this processed serum, levels of serum glutamate oxaloacetate transaminase (Enzyme Commission [EC], 2.6.1.1), serum glutamate pyruvate transaminase (EC 2.6.1.2), cholesterol and total protein were estimated by standard methods [14-16], using a semi-automated biochemical analyzer (Bayer).

Antidiarrheal Activity

The antidiarrheal efficacy of extract was assessed using the following four experimental models of diarrhea in mice.

Measurement of Fecal Output (FOP)

Fecal output was measured following methods of Bass [17] and Pillai [18] with modifications. Animals were divided into six groups ($n = 6$), Group I served as the control and received 2% gum acacia (0.5 ml); Groups II-V were treated with 100, 200, 400 and 800 mg/kg of plant extract, respectively, while Group VI received 0.5 ml of standard antidiarrheal drug loperamide at 5 mg/kg. After 8 h post-treatment, fecal materials were collected, dried in an incubator and their weights were recorded. The percentage FOP was calculated and expressed in terms of percentage reduction as follows:

$$\% \text{ FOP} = \frac{f_t \times 100}{f_c}$$

Where, f_t is the mean fecal weight of each treatment group, and f_c is that of the control group [19].

Castor Oil Model

The method was modified from Jacoby *et al.* [20], Otshudi *et al.* [21] and has been described previously by Tangpu and Yadav [6]. Overnight-fasted mice were randomly divided into six groups (n = 6). Group I received 0.5 ml of 2% gum acacia suspension; Groups II-V were treated with 100, 200, 400 and 800 mg/kg of plant extract, respectively; Group VI mice were given 0.5 ml of 5 mg/kg of loperamide. One hour later, diarrhea was induced in all groups by inoculating castor oil (0.5 ml/mouse, p.o.). The numbers of diarrheal episodes were recorded for each time, and cumulative values were calculated for 4 h post-induction of diarrhea and the numbers of animals devoid of diarrheal droppings at 4 h were considered as a percentage protection from diarrhea.

PGE2-Enteropooling Assay

The protocol was adopted from Robert *et al.* [22] with modifications as described previously by Tangpu and Yadav [6]. Overnight-fasted mice were randomized into seven groups (n = 6). The animals received PGE2 as diarrheal agent (0.5 ml of 100 μg/kg PGE2 in 5% ethanol in saline). Group I served as control (0.5 ml; 2% gum acacia); Group II served as a vehicle control (100 μg/kg PGE2 + 2% gum acacia); Groups III-VI received 100, 200, 400, and 800 mg/kg of plant extract, respectively; Group VII received 5 mg/kg dose of loperamide. All these treatments were done 1 h prior to PGE2-diarrheal induction. 30 min later, animals were sacrificed, and their small intestines were ligated from pyloric sphincter to ileocaecal junction and assessments of the accumulation of intestinal fluid secretion induced by PGE2 were made and calculated in terms of percentage reduction.

Gastrointestinal Transit Test

In this test, mice were divided into six groups (n = 6) and allowed to starve for 16 h prior to the experiment. Group I served as the control, Groups II-V were treated with test extract at 100, 200, 400, and 800 mg/kg oral dose, respectively. Group VI animals received 5 mg/kg loperamide. 5 min later, 0.5 ml of charcoal meal was orally inoculated to each mouse. All the mice were sacrificed 30 min later, their small intestines from the pylorus to caecum cut out and distance travelled by the charcoal marker was measured, and expressed as a percentage of the total length of small intestines. The percentage inhibition of the marker transit in the intestine was calculated as described by Akah and Offiah [23].

Analysis

All the data are represented as mean ± standard error of the mean. The significance of the difference between the control and treated groups were determined by the Student's t-test, and $P < 0.05$ was considered as significant.

RESULTS

Acute Toxicity Test

LD_{50}

Oral administration of plant extract starting from 100 to 3200 mg/kg caused mortality in single mice at the dose of 3200 mg/kg, observed within 72-h post-treatment; however, no visible clinical signs were observed in the rest of experimental animals. Using SPSS software, the LD_{50} of extract was found to be 5355.97 mg/kg in mice.

Serum biochemical profile

The different serum parameters of mice following treatment with 800 mg/kg dose of plant extract are shown in Figure 2. There were no significant differences observed in the levels of any serum parameters from the extract-treated after 24-h post-treatment as compared with the control group.

Antidiarrheal Activity

Measurement of FOP

The plant extract moderately reduced the FOP as compared to standard drug loperamide in a dose-dependent fashion. At 800 mg/kg dose, the percentage reduction in FOP was found to be 26.37%, while loperamide at 5 mg/kg concentration showed 41.76% reduction in FOP [Figure 3].

Effect on Castor Oil Model

There were significant differences in percentage protection of diarrhea induced by castor oil as compared to the control. In

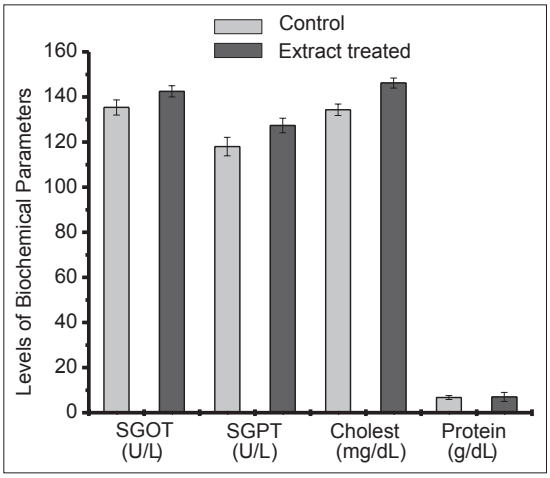

Figure 2: Effects of *Potentilla fulgens* root extract on the levels of SGOT, SGPT, cholesterol and protein after 24-h post-treatment of mice with 800 mg/kg dose of extract. SGOT: Serum glutamate oxaloacetate transaminase, SGPT: Serum glutamate pyruvate transaminase, Cholest: Cholesterol. Values are plotted as mean ± standard deviation, Student's t-test (n = 6). There was no significant change in serum biochemical parameters with the control mice during treatment

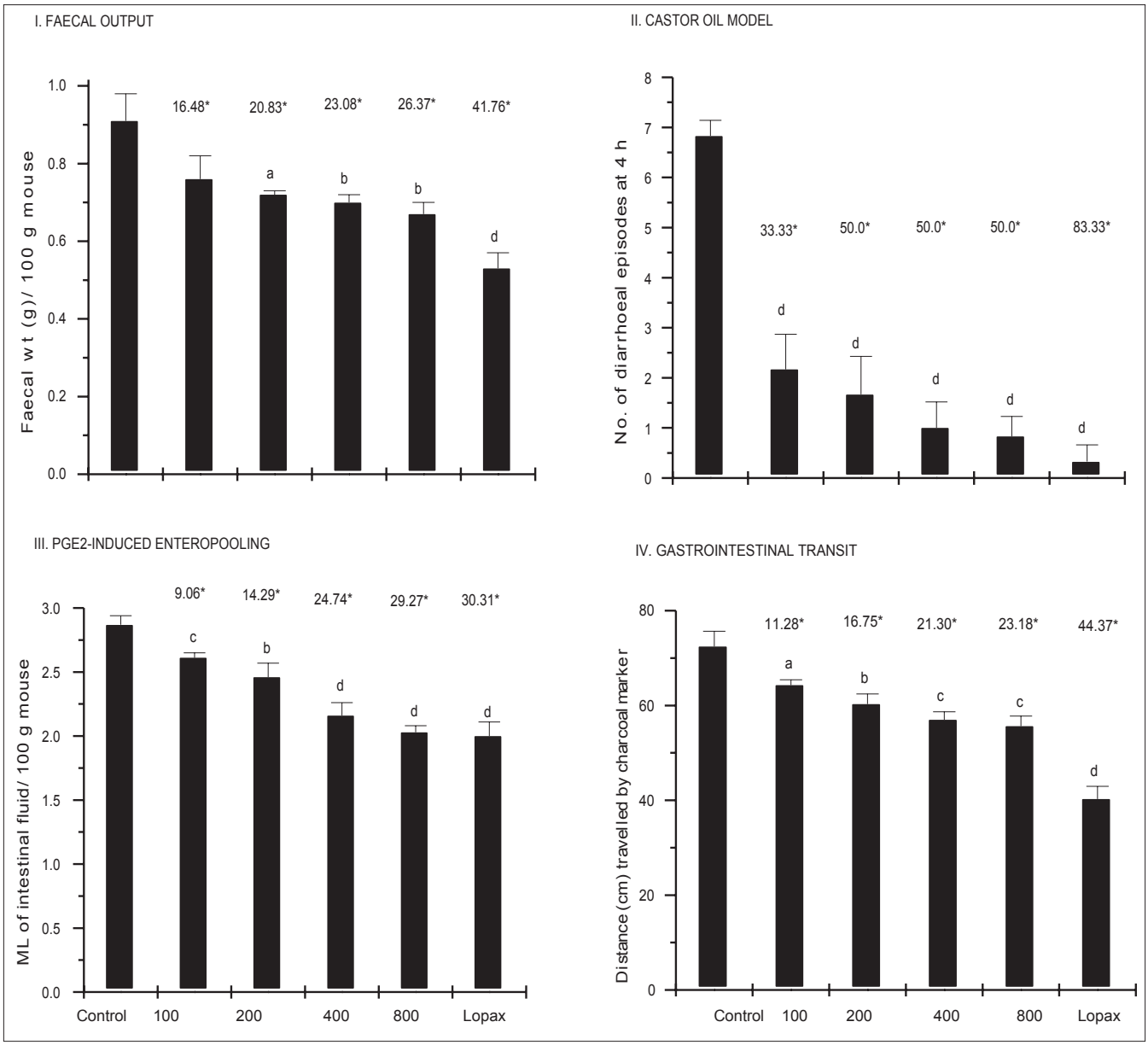

Figure 3: Antidiarrheal efficacy of *Potentilla fulgens* root extract, as represented by four different study parameters. Values are plotted as mean ± standard error of mean (n = 6). *% reduction, or % inhibition, or % animal protection from diarrhea. Treatment (mg/kg, p.o.): Extract at 100, 200, 400, and 800; lopax at 5. [a,b,c,d] P < 0.05, 0.02, 0.01, and 0.001, versus control, Student's t-test

this case, the 800 mg/kg dose of extract could protect 50.00% of the animals from diarrheas evoked by castor oil. In comparison, loperamide at 5 mg/kg dose showed 83.33% protection of diarrhea [Figure 3].

Effect on PGE2-Enteropooling Assay

The differences in reduction of PGE2-induced intestinal secretion by the plant extract was found to be weakly significant in a dose-dependent fashion as compared with the control. The extract reduced 29.27% intestinal secretion at 800 mg/kg dose. The percentage reduction by 5 mg/kg dose of loperamide was showed 30.31%, which was almost comparable with 800 mg/kg dose of extract [Figure 3].

Effect on Gastrointestinal Transit Test

The inhibition of charcoal meal transit along the small intestine in treated group was found to be 23.18% at 800 mg/kg dose of extract [Figure 3]. Although the plant extract showed statistically significant inhibition of charcoal meal transit as compared with control, it was observed that inhibition by the highest tested dose of 800 mg/kg was comparatively low as compared with 44.37% recorded for 5 mg/kg dose of loperamide.

DISCUSSION

In different parts of the world, medicinal plants have been used traditionally for the treatment of various ailments, including

diarrheal diseases. Although, they offer various benefits in the healthcare system, still not much is known about the alleged effects and safety of many medicinal plants that are used as traditional medicines. This may be because of either traditional healers offer such medicines without taking into account the toxicity aspects of herbs or people believe that herbal medicines are natural and therefore they are devoid of any harmful effects. It is therefore important to properly justify the safety and efficacy of medicinal plants that are used as traditional medicines. During the course of our ethnopharmacological studies in Manipur, India, we identified nine medicinal plants that are used traditionally for the treatment of diarrhea by various tribal communities. Of these, a highest (73.33%) user response was recorded for *Rhus javanica* (Anacardiaceae) and a lowest (35.00%) for *Galinsoga parviflora* (Asteraceae). Herein, the user response for *P. fulgens* was recorded to be 50.00% [24]. Therefore, keeping in view the popularity of this plant among tribal communities, the present study was aimed to evaluate the antidiarrheal efficacy and safety of *P. fulgens* root extract in experimental models of diarrhea in Swiss albino mice.

In the acute toxicity study, the plant extract did not show any visible signs of toxicity, but mortality was observed in a single animal at 3200 mg/kg dose of extract. We assume that the cause of mortality only in single mice might be due to other physiological factors rather than the adverse effect of extract, as no visible signs of toxicity were observed in the rest of animals. A recent study also tested the acute toxicity of *P. fulgens* root extract and did not find any mortality or symptoms of toxicity in animals up to 4000 mg/kg [10]. The present study also did not record any significant differences in any of the studied serum biochemical parameters between the extract-treated (800 mg/kg dose) and control group of animals. On the basis of these findings, it may be said that *P. fulgens* root extract does not possesses any toxic effects in experimental animals up to 3200 mg/kg dose.

In this study, the plant extract showed significant antidiarrheal activity in all four models of diarrheas tested. Diarrhea results from an imbalance between the absorptive and secretive mechanisms in the intestinal tract, accompanied by hypermotility and intestinal hurry, which results in an excess loss of fluid through the faces [25]. Therefore, most of the animal based antidiarrheal studies are mainly focused on absorptive, anti-secretory or anti-motility effects of the test substances [5,6].

This study revealed that the plant extract reduces the FOP of animals in a dose-dependent manner, which indicates the presence of an anti-secretory or pro-absorptive property in the extract. Laloo *et al.* [10] have also reported that 200 and 400 mg/kg, p.o. dose of *P. fulgens* root ethanolic extract significantly inhibits ethanol and pyloric ligation-induced gastric ulcers due to its anti-secretory properties.

The induction of diarrhea by castor oil is due to ricinoleic acid which is produced as a result of hydrolysis by lipases in the small bowel, which in turn stimulates PGE2 secretion and together acts primarily in the small intestine to stimulate secretion of fluids and electrolytes and speeds up the intestinal transit [26]. In castor oil model, the plant extract could protect up to 50% of animals from diarrheas. A similar pattern of diarrhea protection was reported for *Cymbopogon citratus* and *R. javanica* from our previous antidiarrheal studies [5,6]. This indicates that the secretion of intra-luminal fluid is perhaps blocked by the test extract, which slows down the intestinal transit. Loperamide, the standard antidiarrheal drug, which is known for its anti-motility and anti-secretory properties, also works on the similar mechanism of action [27]. PGE2 are known to be associated with changes in the bowel that stimulates diarrhea [28]. The extract also showed significant differences in reduction of intestinal secretion in PGE2-enteropooling assay in a dose-dependent manner. The reduction of intestinal secretion by plant extract was quite comparable with that of loperamide, which shows its potential in reducing bowel movements induced by PGE2. Further, a reduction in intestinal motility was observed in gastrointestinal transit test. The extract moderately inhibited the charcoal meal transition along the small intestine as compared with loperamide in a dose-dependent manner. Thus, it appears from these findings that the plant extract may mediate its effects more or less in a same way as the standard drug loperamide.

In our previous studies, we have reported some other antidiarrheal plants viz. *C. citratus* and *R. javanica* from the same region [5,6]. These two plants showed comparatively better antidiarrheal activities than what was observed for *P. fulgens* in the present study. Several antidiarrheal studies have shown that plant extracts containing tannins, flavonoids, alkaloids, saponins, and steroids usually possess significant antidiarrheal activity [29,30] and most frequently, tannin containing plants are used to treat diarrhea and dysentery in traditional medicines [31]. For example, Nsaka Lumpu *et al.* [32] evaluated the phytochemical and antidiarrheal property of *Alstonia congensis* and suggested that antidiarrheal effects of this plant were largely contributed by the presence of tannins. Tannins have also been known to be important constituents of *Potentilla* species and therefore it is most likely that the antidiarrheal effect of this plant is due to its tannins components [33].

CONCLUSION

The present study clearly suggests that the *P. fulgens* root extract possesses significant antidiarrheal activity and is safe to use as traditional medicine. Further studies, however, are necessary to isolate and identify the active ingredients of this plant to understand its precise mechanism of antidiarrheal action.

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Original Research

Antigiardial effect of *Anethum graveolens* aqueous extract in children

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ABSTRACT

Background: *Giardia lamblia* is the most common intestinal parasite of humans identified worldwide. In spite of metronidazole (Met) is the most commonly used drug for the treatment of giardiasis in humans, low patient compliance and side-effects, especially in children encourage efforts to look for new and safe agent; many plants used in folk medicine thought to have antigiardial effect, *Anethum graveolens* (AG) (dill) is an annual herb cultivated in Iraq used both as a medicinal agent and as food spice. The aim of this study was to investigate the effect of aqueous extract (AE) of AG leave in the treatment of giardiasis, compared with that of standard drug Met. **Patients and Methods:** A prospective randomized clinical trial was carried out on 28 pediatric patients of both sexes with age of <1 year ranging from 3 to 11 months, who attend to outpatient private clinic in Baghdad for a period of 6 months from June 2013 to December 2013, Patients participate in this study were allocated into two groups: Group A composed of 14 patients treated with Met 15 mg/kg 3 times a day for 5 days. Group B composed of 14 patients treated with AGAE 1 ml 3 times a day for 5 days. Stool samples were collected at 0 time before administration of treatment, after 5 days and after 14 days from starting the treatments to check the efficacy of treatment. **Results:** Administration of AGAE results in a significant decrease in incidence of *G. lamblia* after 5 days of treatment indicating the efficacy of AGAE in the treatment of giardiasis a result that is comparable to that of Met. **Conclusion:** This study showed that pediatric patients with giardiasis may benefit from 5 days treatment with AGAE administered as 1 ml 3 times daily, the improvement in the symptom with this herbal agent was comparable to the standard pharmacological agent Met; results showed that AG is safe and tolerable over treatment course.

KEY WORDS: *Anethum graveolens*, giardiasis, herbal medicine, metronidazole

INTRODUCTION

Giardia lamblia (also known as *Giardia intestinalis* and *Giardia duodenalis*) is the most common intestinal parasite of humans identified worldwide [1]. This flagellated protozoan causes a generally self-limited clinical illness (i.e. giardiasis) typically characterized by diarrhea, abdominal cramps, bloating, weight loss, and malabsorption; asymptomatic infection also occurs frequently [2].

Giardia infection is transmitted through the fecal-oral route and results from the ingestion of *Giardia* cysts through the consumption of fecally contaminated food or water or through person-to-person (or, to a lesser extent, animal-to-person) transmission [3]. The cysts are infectious immediately upon being excreted in feces. The infectious dose is low; ingestion of 10 cysts has been reported to cause infection [4]. The prevalence of infection is commonly between 2% and 5% in the developed world and 20-30% in the developing and underdeveloped countries [5]. Existing chemotherapy protocols recommend that patients should be treated if the parasite is found, irrespective of the presence or absence of acute symptoms [6]. However, some investigators question the usefulness of chemotherapy in infected people in endemic areas due to the extremely high rate of reinfection, as high as 90% in some studies [7]. Treatment preferences vary among clinicians and in different locations. The most widely used treatment protocols employ metronidazole (Met) given 3 times/day for 3-5 days [8]. Met is typically administered in doses of 250 mg 3 times a day for 5-7 days for adults and 15 mg/kg 3 times a day for 5-7 days in children. In recent years, therapeutic failure of Met, the first-line drug of choice in giardiasis in humans, has increasingly been reported from all around the world [9]. Met is prescribed widely for a wide range of nonparasitic infectious diseases. Low compliance of patients with the current Met therapy protocols, the emergence of the Met-resistant strains of the parasite and other pathogens, and rapid reinfection of treated patients in the endemic areas are additional reasons for considering alternative therapies [10]. Poor adherence to the prescribed frequency and duration of Met, especially in children less than 1 year, in addition to common adverse reactions frequently reported with Met include metallic taste, nausea, vomiting, diarrhea, and epigastric discomfort represent important causes for finding safer drugs with less toxicity and more effective therapeutic properties with low incidence of side-effects.

Research in herbal medicine has increased in the world as an alternative solution to health problems; furthermore, side-effects of drugs in current use shift the orientation toward herbal medicine, especially high percent of people thought that using of herbal medicine is free of side-effect [11].

Anethum graveolens (AG) Umbilliferae, known as dill, is an annual herb growing in the Mediterranean region, Europe, Central and Southern Asia; the plant is used both as a medicinal agent and as food spice. Dill has been used traditionally for gastrointestinal disturbances such as flatulence, indigestion, and colic [12]. In Iraq, dill was used as food spice and in Gripe Water for children as antiflatulent. The active constituents of AG have been classified as flavonoids, phenolic compounds, and essential oils, the phytochemical screening of plant showed that leaves, stems, and roots were rich in tannins, terpenoids, cardiac glycosides and flavonoids [13-15]. Pharmacological effects of AG include antibacterial [16], antifungal [17], antispasmodic [18], antisecretory, and mucosal protective effects [19].

The aim of this study was to investigate the effect of aqueous extract (AE) of AG leave in the treatment of giardiasis, compared with that of standard drug Met.

PATIENTS AND METHODS

A prospective randomized clinical trial was carried out on 28 pediatric patients of both sexes with age of <1 year ranging from 3 to 11 months, who attend to outpatient private clinic in Baghdad for a period of 6 months from June 2013 to December 2013; the study was approved by Scientific and Ethical Committee in Alkindy College of Medicine/University of Baghdad, an informed consent was taken from all the parents. To be included in the study, a child had mono-infection with *G. lamblia* proven by microscopic examination of fecal sample, with diarrhea and abdominal pain; the exclusion criteria were known history of sensitivity to Met, those receiving any antiparasitic or antibiotic chemotherapy within 3 weeks and patients having disease other than giardiasis. Patients participate in this study were allocated into two groups:

- Group A: Composed of 14 patients treated with Met 15 mg/kg 3 times a day for 5 days
- Group B: Composed of 14 patients treated with AGAE 1 ml 3 times a day for 5 days.

History was taking from parents; a special form was used to record clinical signs and symptoms before starting treatment and at the end of course, a physical examination and weight measurement was carried out for each child.

The evaluation of efficacy of the chemotherapy was based on parasitological response to therapy assessed by the same laboratory tests that were done initially. Parents of each child were asked to provide three fecal samples on day 0, 5, and 14 after treatment. Furthermore, they were encouraged to return to the clinic at any time, if they considered that his or her child was ill. A child was only considered to be cured, if no *Giardia* trophozoites or cysts could be found in any of the three fecal specimens.

AGAE Preparation

AG was obtained from local market; it is approved by Medicinal Plant Center-Baghdad, Iraq. Aqueous extraction was performed by adding 200 ml of water to 20 g of AG dried leaves then boiling for 10 min; wait for the solution to become cold; the extract filtered and leaved for evaporation until 100 ml.

Minitab software package was utilized for statistical analysis; results expressed as mean ± standard deviation; paired Student's *t*-test was used to evaluate significant changes; $P \leq 0.05$ considered being significant.

RESULTS

The results of this study showed that the incidence of *G. lamblia* was 100% in the stool samples of all pediatric patients before treatment, administration of Met to the group A decreased significantly $P \leq 0.05$ the incidence percent to 7.14% after 5 days of treatment, while after 14 days stool examination revealed nil incidence of parasite Figure 1; on the other hand, treatment with AGAE 1 ml 3 times a day for 5 days reduce significantly $P \leq 0.05$ the incidence percent in Group B to 14.28% where only two samples contain the parasite, and after 14 days the percent was zero, indicating the efficacy of AGAE in the treatment of giardiasis compared to Met, Figure 1.

Figure 2 showed that treatment with Met significantly $P \leq 0.05$ decrease the frequency of bowel motion in pediatric patients by 118.93% at day 1 of treatment, while at day 2 after starting treatment the frequency of bowel motion reduced by 54.35% compared with day 1, and at days 3, 4, and 5 the frequency of bowel motion become normal, Figure 2; in Group B, administration of AGAE 1 ml 3 times daily reduce the frequency of bowel motion significantly $P \leq 0.05$ at the first treatment day by 82.24%, while at 2nd day after starting treatment the reduction percent was 87.18% compared to day 1, at days 3, 4, and 5 the frequency of bowel motion return to normal, Figure 2; these data again indicating the efficacy of AGAE in the treatment of giardiasis compared to Met.

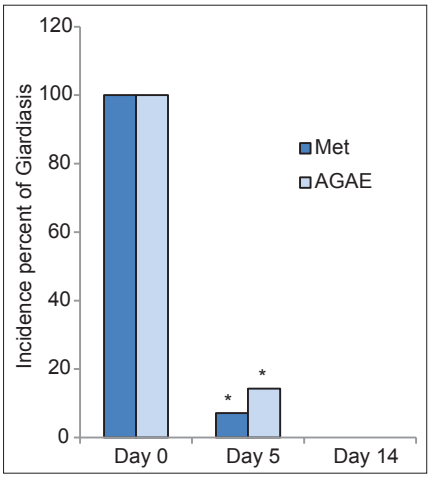

Figure 1: Antigiardial effect of *Anethum graveolens* aqueous extract compared to metronidazole in pediatric patients

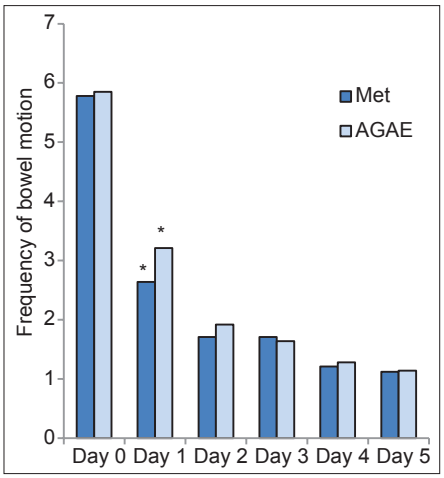

Figure 2: Frequency of bowel motion in pediatric patients during 5 days treatment with *Anethum graveolens* aqueous extract compared to metronidazole, results represents mean value

DISCUSSION

Because of the treatment failure and the adverse effects of medications used to treat giardiasis, many patients consider conventional treatment to be disappointing and often turn to complementary therapies. The choice to use natural rather than chemical therapies is attractive because many patients assume that natural products are safe and that they do not cause adverse effects, this have provided a continuous stimulus to search for other therapeutic alternatives [20].

Many natural materials have been shown to have antigiardial activity, the antigiardial activity of the phenol-rich essential oils of several plants had been examined, and their activity was evaluated based on the change in parasite growth, cell viability, adherence, and morphology [21]. Another *in vitro* study suggests that the crude extracts of *Citrullus lanatus* Var. were active against *G. lamblia* and may be recommended as a new source for the treatment of giardiasis [22]. Anthony *et al*. confirmed that berry polyphenols can influence *Giardia* survival *in vitro* and suggests that ellagitannins are most effective. Tannin-rich preparations may also have efficacious effects on diarrheal symptoms often associated with *Giardia* infection. Unlike many plant sources of antigiardial agents, berries are a natural and palatable foodstuff and therefore have few issues with toxicity, side-effects or acceptance [23]. Rahimi *et al*. Studied the antigiardial activity of *Sambucus ebulus in vitro*, he reported that there is excellent antigiardial of methanolic extract of *S. ebulus in vitro* against cyst of *G. lamblia* [24]. On the other hand, Al-masoudi studied the antigiardial activity of *Zingiber officinale* in combination with honey *in vivo*, she examined the effects of watery extracts against *G. lamblia* on the basis of killed trophozoite number, using experimental infections of *G. lamblia* in balb/c mice; she reported that extract of *Z. officinale* was more active specially when mixed with honey, so the percentage of dead trophozoite reach to 97.7% [25].

In this clinical trial, the antigiardial effect of AGAE was examined in pediatric patients; the results showed that the administration of 1 ml of the AE 3 times a day cause a significant reduction in the survival incidence of giardial trophozoite in stool samples a result that is comparable with that obtained from administration of the standard drug Met. There was no definite mechanism documented so far by which AG exert its effect; many studies had been shown multiple antimicrobial effects for this spice. It has been reported that AEs of AG showed a broad-spectrum antibacterial activity against *Staphylococcus aureus*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Salmonella typhimurium*, *Shigella flexneri* and *Salmonella typhii* [26]. The higher activity of extract can be explained on the basis of the chemical structure of their major constituents such as dillapiole and anethole, which have aromatic nucleus containing polar functional group that is known to form hydrogen bonds with active sites of the target enzyme [27]. Furthermore, it has been reported that compounds of dill when added to insecticides have increased the effectiveness of insecticides. Essential oil of AG is used as repellent and toxic to growing larvae and adults of *Tribolium castaneum*, wheat flour insect pest. In doses of 60 minims, anethole is a fairly potent vermicide for hookworm. All these activities against diverse microorganisms beside the safety profile of AG as edible herb encourage the use of its AE in the treatment of giardiasis. Another important point is the multi pharmacologic effects of AG like mucosal protective, antisecretory, antioxidant activity and the potent relaxant effect of contractions induced by a variety of spasmogens in rat ileum, which supports the use of dill in traditional medicine for gastrointestinal disorders [28]. These properties of dill may contribute effectively in rationalize the use of this herb in the treatment of giardiasis where many signs and symptoms like frequent bowel motion, abdominal pain and flatulence may be relieved in addition to its main use against giardiasis. However, determination of the exact mechanism by which dill exerts its antigiardial effect needs further deep investigation on molecular level, while large scale clinical trials with large sample size and multicenter studies are needed to clarify its role in the treatment of giardiasis. Another limitation in this study is the difficulties in monitoring the incidence of side-effects that may occur with Met such as headache, nausea, metallic taste, and abdominal pain compared to that when using AGAE since it was impossible to document such subjective side-effects in pediatric patients under 1 year of age, although according to pediatrics' mother the episodes of crying in pediatric patients on AGAE were less much than that on Met (data not shown) which may explained depending on the spasmolytic and antiflatulent properties of dill herb.

CONCLUSION

This study showed that pediatric patients with giardiasis may benefit from 5 days treatment with AGAE administered as 1 ml 3 times daily, the improvement in the symptom with this herbal agent was comparable to the standard pharmacological agent Met; results showed that AG is safe and tolerable over treatment course.

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Original Research

Assessment of protective and anti-oxidant properties of *Tribulus terrestris* fruits against testicular toxicity in rats

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ABSTRACT

Aims: This study was carried out to assess the protective and anti-oxidant activities of the methanolic extract of *Tribulus terrestris* fruits (METT) against sodium valproate (SVP)-induced testicular toxicity in rats. **Materials and Methods:** Fifty mature male rats were randomly divided into five equal groups ($n = 10$). Group 1 was used normal (negative) control, and the other four groups were intoxicated with SVP (500 mg/kg^{-1}, orally) during the last week of the experiment. Group 2 was kept intoxicated (positive) control, and Groups 3, 4 and 5 were orally pre-treated with METT in daily doses 2.5, 5.0, and 10.0 mg/kg^{-1} for 60 days, respectively. Weights of sexual organs, serum testosterone, follicle stimulating hormone (FSH) and luteinizing hormone (LH) levels, semen picture, testicular anti-oxidant capacity and histopathology of testes were the parameters used in this study. **Results:** Oral pre-treatment with METT significantly increased weights of testes and seminal vesicles; serum testosterone, FSH and LH levels and sperm motility, count and viability in SVP-intoxicated rats. METT enhanced the activity of testicular anti-oxidant enzymes and partially alleviated degenerative changes induced by SVP in testes. **Conclusion:** The pre-treatment with METT has protective and anti-oxidant effects in SVP-intoxicated rats. Mechanisms of this protective effect against testicular toxicity may be due to the increased release of testosterone, FSH and LH and the enhanced tissue anti-oxidant capacity. These results affirm the traditional use of *T. terrestris* fruits as an aphrodisiac for treating male sexual impotency and erectile dysfunction in patients. The study recommends that *T. terrestris* fruits may be beneficial for male patients suffering from infertility.

KEY WORDS: Anti-oxidant, histopathology, sperm, testis, testosterone, *Tribulus terrestris* fruits

INTRODUCTION

Infertility is one of the major health problems in life, and approximately about 30% of this problem is due to male factors [1]. Several factors can interfere with the process of spermatogenesis, reduce sperm quantity and quality and decrease male fertility. Many diseases such as coronary heart diseases, diabetes mellitus and chronic liver diseases as well as insufficient vitamins intake have deleterious effects on spermatogenesis and production of normal sperm [2]. Oxidative stress is a major predisposing risk factor for many chronic diseases, including male infertility problem [3,4]. On the other side, intake of natural anti-oxidant agents from plants together with vitamins E and C can protect sperm DNA from oxidative stress in the testis of rats [5], and antagonize testicular toxicity in rabbits [6]. Medicinal plants are a promising source for safe, natural anti-oxidant agents as they contain many bioactive constituents, especially anti-oxidant flavonoids and polyphenol compounds [7].

Tribulus terrestris plant, popularly known as puncture vine, is a perennial creeping herb with a worldwide distribution. Since ancient times, *T. terrestris* has a long history as a powerful aphrodisiac [8,9]. In traditional medicine, *T. terrestris* roots are used for treating male infertility [10,11]. Moreover, *T. terrestris* roots extract and total saponins extracted from the roots have beneficial effects on various ailments such as urinary tract infections, inflammations, leucorrhea, edema, and ascites [11,12]. *T. terrestris* fruits extract has been successfully used in Europe and Asia to treat sexual dysfunction in males [13]. The different pharmacological effects of *T. terrestris* plant were reviewed by Chhatre *et al.* [14]. The present study was designed to evaluate the protective and anti-oxidant activities of the methanolic extract of *T. terrestris* fruits (METT) against testicular toxicity and oxidative stress induced sodium valproate (SVP) in rats, and to examine the possible mechanisms.

MATERIALS AND METHODS

Plant Material

The fruits of *T. terrestris* (Family Zygophyllaceae) plant were obtained from the company of medicinal herbs, seeds, agricultural products, Cairo, Egypt. These fruits were botanically authenticated by Dr. Abdelhalim Mohamed, Flora and Taxonomy Department, Agricultural Research Centre, Giza, Egypt. Herbal

specimen was deposited at the Department of Pharmacology, Faculty of Veterinary medicine, Cairo University, Egypt.

Extraction of Plant Material

The dry fruits of *T. terrestris* plant were pulverized and freezing dried. Two hundred grams of powdered dried fruits were extracted with 2 l of 90% methanol (Sigma Aldrich for Chemicals, USA) by percolation for 72 h. The solvent was evaporated by vacuum distillation at 45°C using a rotatory evaporator (West Germany). The liquid extract yielded 10 g of gummy residue that used for preparing the different doses of the extract using tween 80 as a suspending agent.

Sodium Valproate (Depakin®)

It is one of products of Sanofi-Synthelabo Company, Paris, France. It was obtained as an oral solution packed in dark brown bottles each containing 40 ml. SVP is sold commercially under trade name Depakin® 200 mg/ml solution.

Rats and Husbandry

A total of 50 mature male Sprague Dawley rats with average body weight of 200-250 g and age of 10-13 weeks were used in this study. Rats were procured from Laboratory Animal Colony, Ministry of Public Health, Helwan, Egypt. The animals were housed in clean cages, kept under controlled hygienic conditions and maintained at room temperature at 25°C ± 2°C, relative humidity of 50% ± 5% and photoperiod of 12 h dark/12 h light cycles. Rats were fed on rat pellets, and free access of tap water was supplied. The experiment on animals was carried out according to the National Regulations on Animal Welfare and Institutional Animal Ethical Committee.

Experimental Design

The rats were randomized into five equal groups, of 10 animals each. Group 1 was normal (negative) control and administered diluted tween 80 (0.1 ml/rat). The other four groups were intoxicated by oral administration of SVP (500 mg/kg^{-1}) during the last week of the experiment period for induction of testicular toxicity [15]. Group 2 was kept intoxicated positive (control) and Groups 3, 4, and 5 were pre-treated with the METT in daily doses of 2.5, 5.0 and 10.0 mg/kg^{-1}, respectively. The extract was orally given once daily for 60 days to cover the period of the spermatogenic cycle in the rat [15]. Blood samples were withdrawn by puncture of retro-orbital plexus of veins in the eye using microcapillary tubes. The samples were kept standing for 15 min to clot then centrifuged at 10,000 rpm for 10 min to separate the serum which kept frozen at −70°C. The serum was used for estimation of testosterone, follicle stimulating hormone (FSH) and luteinizing hormone (LH) levels. Rats were anesthetized by prolonged exposure to ether, and a longitudinal incision was made in the skin of scrotum and both testes were exposed. Semen samples were collected from cauda epididymis by cutting the tail of epididymis and squeezed it into a clean watch glass. The semen samples were used for semen analysis. The testes and accessory sexual organs were dissected out and weighed, and relative weights of sexual organs were calculated. The right testes were immediately taken on ice cooled bags and kept frozen at −70°C until the assessment of the activity anti-oxidant enzymes superoxide dismutase (SOD), glutathione peroxidase (GPx) and catalase (CAT). The left testes were preserved in 10% neutral formalin solution till processed for histological examination.

Hormone Assay

Serum testosterone concentration was assayed according to the method described in the manufacturer's directions. The method is based on the enzyme-linked immune absorbent assay (ELISA) as described by Tietz [16]. The assay kit of testosterone was obtained from Immunometrics Limited, London, UK. Serum testosterone concentrations were obtained by correlating the absorbance of the test sample at 550 nm with the corresponding absorbance on the standard curve. Serum levels of FSH and LH hormones were determined using ELISA kits (Amersham, Buckinghamshire, UK) according to Ballester *et al.* [17]. Assay kits of FSH and LH were supplied by Diagnostic Automation Inc., Calabasas, USA.

Semen Analysis

The seminal content of epididymis was obtained by cutting of cauda epididymis using surgical blades and squeezed into a sterile clean watch glass. This content was diluted 10 times with 2.9% sodium citrate solution and thoroughly mixed to estimate the percentage of sperm progressive motility and sperm count as described by Bearden and Fluquary [18]. Thereafter, one drop of sperm suspension was withdrawn, smeared on a glass slide and stained by eosin-nigrosin stain. The stained seminal smears were examined microscopically to determine the percentage of sperm viability (alive/dead) and morphological abnormalities [19].

Anti-oxidant Enzymes Assay

Tissue specimens of the right testes after thawing was homogenized in nine volumes of ice cooled buffered 0.9% saline solution. The homogenate was then centrifuged at 4000 rpm for 15 min at 4°C and the supernatant was used for anti-oxidant enzymes assay. The activity of SOD was determined as described by Nishikimi *et al.* [20] and expressed as unit/mg protein. GPx activity was determined as described by Paglia and Valentine [21] and expressed as nmol of glutathione utilized/min/mg protein. CAT activity was estimated according to Sinha [22]. The activity of CAT was expressed as nmol of H_2O_2 utilized/min/mg protein.

Histological Procedure

Testes, seminal vesicle and prostate glands were fixed in 10% neutral formalin solution. The fixed specimens were trimmed, washed and dehydrated in ascending grades of alcohol, then cleared in xylene, embedded in paraffin, sectioned at 4-6 microns thickness and stained with hematoxylen and eosin stain, then examined microscopically [23].

Statistical Analysis

Data were presented as means ± standard errors. Differences between means in the experimental groups were tested for significance using Student's *t*-test. Differences were considered significant at $P < 0.05$ according to Snedecor and Cochran [24].

RESULTS

Oral administration of SVP to male rats in a dose of 500 mg/kg during the last week of the experiment induced significant ($P < 0.05$) decreases in weights of testes and seminal vesicles when compared to the normal control group. Rats pre-treated with oral administration of METT significantly normalized weights of testes and seminal vesicles, in a dose-dependent manner, when compared to SVP-intoxicated control group [Table 1].

Oral administration of SVP (500 mg/kg) to rats during the last week of the experimental period significantly decreased serum testosterone, FSH and LH levels when compared with the normal control group. Pre-treatments of SVP-intoxicated rats with METT significantly increased serum testosterone, FSH and LH levels when compared with the intoxicated control group, in a dose dependent fashion [Table 2].

SVP when given orally to male rats (500 mg/kg) during the last week of the experiment induced significant decreases in sperm motility, viability and count and increased percentages of sperm abnormalities when compared with the normal control group. Pre-treatment with METT significantly increased sperm motility, viability and count and decreased percentages of sperm abnormalities, in a dose dependent manner, as recorded in Table 3. The most frequently seen sperm abnormalities were detached, double and circular heads; and bent and coiled tails and a detached neck as demonstrated in Figure 1.

Data are shown in Table 4 revealed that intoxication of rats by SVP induced significant decreases in activities of testicular anti-oxidant enzymes SOD, GPx and CAT compared with normal control rats. The pre-treatment with METT with the three tested doses significantly increased the activity of

Table 1: Effect of methanolic extract of *Tribulus terrestris* fruits on weights of sexual organs in sodium valproate-intoxicated rats (n=10)

Groups	Testes (g)	Seminal vesicles (g)	Prostate gland (g)
Group (1): Negative control	2.55±0.25	1.25±0.16	0.80±0.10
Group (2): Positive control (SVP)	0.85±0.15***	0.43±0.22**	0.65±0.20
Group (3): METT (2.5 mg/kg)	1.15±0.25	0.55±0.12	0.66±0.12
Group (4): METT (5 mg/kg)	1.60±0.32*	0.95±0.23*	0.70±0.15
Group (5): METT (10 mg/kg)	2.27±0.43**	1.10±0.12*	0.77±0.16

Data were presented as means±SE. Differences between the groups were significant at *P<0.05, **P<0.01, ***P<0.001. METT: Methanolic extract of *tribulus terrestris* fruits, SVP: Sodium valproate, SE: Standard error

Table 2: Effect of METT on serum testosterone, FSH and LH levels in SVP-intoxicated rats (n=10)

Groups	T (ng/ml)	FSH (ng/ml)	LH (ng/ml)
Group (1): Negative control	24.2±2.6	149.7±6.2	4.2±0.1
Group (2): Positive control (SVP)	12.5±2.7**	90.86±3.3***	1.6±0.3**
Group (3): METT (2.5 mg/kg)	15.15±2.6*	95.59±5.4*	2.1±0.4*
Group (4): METT (5 mg/kg)	19.25±2.2**	103.75±3.4**	2.9±0.3*
Group (5): METT (10 mg/kg)	21.17±3.2***	111.82±4.4***	4.0±0.2**

Data were presented as means±SE. Differences between the groups were significant at *P<0.05, **P<0.01, ***P<0.001. METT: Methanolic extract of *Tribulus terrestris* fruits, SVP: Sodium valproate, FSH: Follicle stimulating hormone, LH: Luteinizing hormone, SE: Standard error

Table 3: Effect of METT fruits on sperm motility, viability, abnormality and count in SVP-intoxicated rats (n=10)

Groups	Sperm characters			
	Motility (%)	Viability (%)	Abnormality (%)	Count (10^6/ml)
Group (1): Negative control	92±2.15	90.0±3.3	3.5±1.05	72.0±4.13
Group (2): Positive control (SVP)	65.2±3.76***	40.6±4.2***	16.0±2.08**	55.5±3.17***
Group (3): METT (2.5 mg/kg)	69.2±3.17	50.6±3.4*	12.5±1.03*	60.55±3.34
Group (4): METT (5 mg/kg)	71.5±2.55*	60.6±2.3***	7.5±1.14**	65.82±3.15*
Group (5): METT (10 mg/kg)	77.5±3.15**	70.6±3.6***	5.5±1.15***	70.24±4.25**

Data were presented as means±SE, differences between the groups were significant at *P<0.05, **P<0.01, ***P<0.001. METT: Methanolic extract of *Tribulus terrestris* fruits, SVP: Sodium valproate, SE: Standard error

Table 4: Effect of METT on activity of testicular SOD, GPx and CAT in SVP-intoxicated rats (n=10)

Groups	SOD (U/mg protein)	GPx (nmol/min/mg protein)	CAT (nmol/min/mg protein)
Group (1): Negative control	22.09±1.5	244.5±5.6	372.9±9.5
Group (2): Positive control (SVP)	11.20±1.3***	136.6±8.5***	290.5±2.2***
Group (3): METT (2.5 mg/kg)	14.15±1.9*	148.2±6.4**	298.2±3.5**
Group (4): METT (5 mg/kg)	16.25±1.6*	152.5±7.2***	305.6±5.2***
Group (5): METT (10 mg/kg)	18.25±1.7**	165.8±9.6***	310.2±6.5***

Data were presented as means±SE, Differences between the groups were significant at *P<0.05, **P<0.01, ***P<0.001. METT: Methanolic extract of tribulus terrestris fruits, SOD: Superoxide dismutase, GPx: Glutathione peroxidase, CAT: Catalase, SVP: Sodium valproate, SE: Standard error

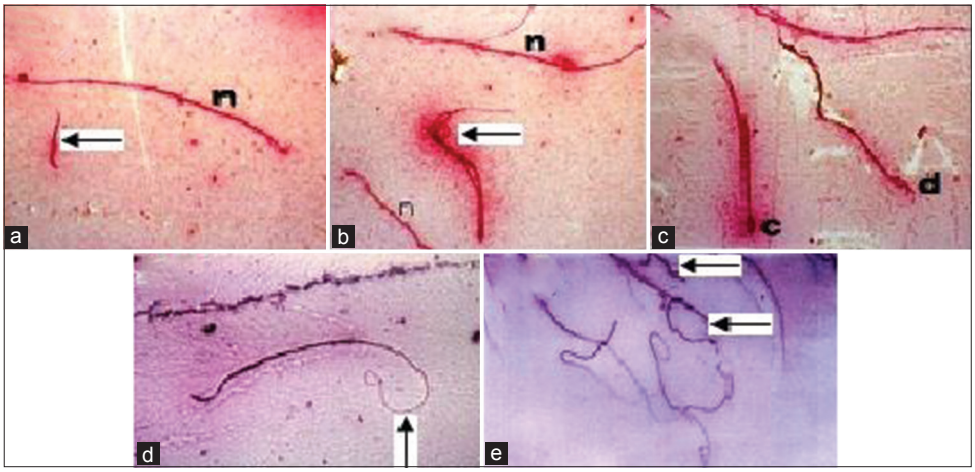

Figure 1: Seminal smears from cauda epididymis of the rat's testes: (a) Normal mature sperm (n) and detached head of sperm (arrow), (b) double head of sperm (arrow), (c) circular head (c) and deformed head (d), (d) bent tail (arrow), (e) coiled tail of sperm (arrow) and detached neck (arrow)

testicular SOD, GPx and CAT, in a dose dependent fashion, when compared with positive intoxicated rats.

Histopathological Examination

Histopathological examination of testes of normal control rats revealed normal architecture with normal germinal epithelium and fully mature sperms filled the lumen of seminiferous tubules as shown in Figure 2a. The testes of rats orally given SVP in a dose of 500 mg/kg during the last week of the experiment period showed histopathological lesions characterized by edema and necrosis of the germinal epithelium with severe atrophy of seminiferous tubules [Figure 2b]. Some testicular sections showed complete absence of mature sperms (azoospermia) as demonstrated in Figure 2c. The testes of rats given the large dose (10 mg/kg) of METT showed partial improvement in the germinal epithelium of seminiferous tubules, and some mature sperms appeared in them, but the size of seminiferous tubules still atrophied [Figure 2d].

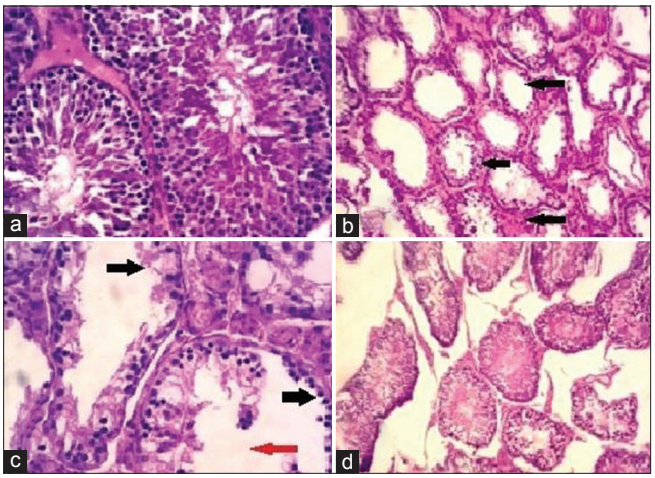

Figure 2: Photomicrographs of testes of: (a) Normal control rat showing normal histological structure of seminiferous tubules filled with mature sperms. Hematoxylen and eosin. (H and E × 400), (b) intoxicated rat by sodium valproate (SVP) showing atrophied seminiferous tubules and edema with absence of sperms (arrows). (H and E × 100), (c) intoxicated rat by SVP showing necrosis of the germinal epithelium of seminiferous tubules (black arrows) with absence of sperms (red arrow) (H and E × 400), (d) pre-treated rat by the large dose (10 mg/kg) of *Tribulus terrestris* fruits extract showing partial improvement of the germinal epithelium of seminiferous tubules and mature sperm were found in most of seminiferous tubules, but their sizes still atrophied. (H and E × 100)

DISSCUSION

The purposes of this study were to evaluate the protective and anti-oxidant activities of the METT against testicular toxicity induced by SVP in rats, and to examine its possible protective mechanisms.

Oral administration of SVP to rats induced male reproductive toxicity. The toxic effect of SPV characterized by decreased weights of the testes and seminal vesicles, low serum testosterone, FSH and LH levels, low semen quantity and quality, as well as incidence of testicular edema and necrosis with markedly atrophied seminiferous tubules. These effects were agreed with those previously reported [15,25-28]. These authors found that the weights of testes and epididymis as well as sperm count and viability were all decreased following SPV administration to rats and mice. In addition, serum levels of testosterone were dropped, and degeneration, edema necrosis and atrophy of most seminiferous tubules were seen upon histopathological examination of the testis. The previous authors attributed the toxic effect of SPV due to its direct cytotoxic effect on the testis and/or indirectly via decreasing serum testosterone level. Moreover, SVP is commonly used to induce male reproductive toxicity in rats and mice, and its toxic effect was found to a dose-dependent and of a reversible manner [27].

In this study, the oral pre-treatment with the METT at three dosage levels in SVP-intoxicated rats produced a protective effect against testicular toxicity. This protective effect characterized by increased weights of testes and seminal

vesicles, improved semen quality and quantity, increased serum testosterone, FSH and LH levels as well as partial amelioration of testicular histopathological lesions seen. This protective effect seemed to be a dose-dependent. These findings were partially in agreement with those previously reported [8-11,14,29,30]. The previous authors concluded that *T. terrestris* plant acts as a powerful aphrodisiac in rats, mice and humans; improves semen quality and quantity and increases weights of the testis and epididymis. The reported increase in serum testosterone levels following administration of METT in this study was previously recorded by El-Tantawy *et al.* [31] using both chloroformic and ethanolic extracts of *Tribulus alatus* fruits in rats. However, a limited number of animal studies displayed a significant increase in serum testosterone levels after administration of METT, but this effect was only noted in humans. Moreover, Qureshi *et al.* [11] concluded that the release of nitric oxide after administration of *T. terrestris* extract to rats may offer a possible explanation for its protective activity on male reproductive dysfunction, independent of the serum testosterone level. The authors concluded that *T. terrestris* plant is successfully used in the management of sexual dysfunction, including erectile dysfunction in patients. In addition, the reported anti-oxidant activity of METT in this study was evident from the enhancement of activities of testicular anti-oxidant enzymes. The anti-oxidant effect of *T. terrestris* extract was similar to that previously demonstrated [32,33]. The anti-oxidant activity of *T. terrestris* fruits was attributed to the presence of active derivatives of 4,5-di-p-coumaroylquinic acid, which were isolated from the fruits and reported to have potent anti-oxidant activity [34].

The mechanism(s) underlying the protective effect of METT against testicular toxicity induced by SVP in rats could be attributed to the increased release of testosterone, FSH and LH serum levels. It was previously reported that abnormalities in the synthesis and release of androgens or their depletion by medical or surgical castration may suppress libido and decline erectile and ejaculatory functions [35,36]. The other possible mechanism of the protective effect of METT could be due to its potent anti-oxidant property, so reducing oxidative stress in the testis and improving reproductive function. The anti-oxidant activity of METT was previously attributed to the presence of 4,5-di-p-coumaroylquinic acid that previously isolated from *T. terrestris* fruits and proved to exhibit a potent anti-oxidant effect [34].

CONCLUSION

SVP induces testicular toxicity in male rats. The METT has protective and anti-oxidant effects against SVP-induced testicular toxicity. The protective effect of METT against SVP toxicity might be due to the increased release of testosterone, FSH and LH and the enhancement of activity anti-oxidant enzymes in testicular tissue by METT. These results affirm the traditional use of *T. terrestris* fruits as a potent aphrodisiac for treating male sexual impotency and erectile dysfunction in patients. Therefore, *T. terrestris* fruits may be beneficial for male patients suffering from infertility due to oxidative stress. Moreover, isolation of bioactive constituents of *T. terrestris* fruits is necessary to search for safe, natural anti-oxidant agents to be developed for the prevention of infertility in males.

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Original Research

Evaluation of methanol extract of *Gongronema latifolium* leaves singly and in combination with glibenclamide for anti-hyperglycemic effects in alloxan-induced hyperglycemic rats

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ABSTRACT

Objective: This study evaluated the anti-hyperglycemic effect of the methanol extract of *Gongronema latifolium* leaves singly and in combination with an oral hypoglycemic agent; glibenclamide. **Materials and Methods:** The plant material was extracted with methanol for 48 h using cold maceration and concentrated *in vacuo* in a rotary evaporator. The methanol extract of *G. latifolium* at doses of 200, 300, 400, 500, and 800 mg/kg were studied for anti-hyperglycemic effect in alloxan-induced hyperglycemic rats. More so, the extract at doses of 400 mg/kg + 5 mg/kg glibenclamide and 500 mg/kg + 5 mg/kg glibenclamide were studied for possible additive effects. **Results:** The 300 mg/kg of the extract decreased blood glucose at 1 h post-treatment though not significantly ($P > 0.05$) compared with 5 ml/kg distilled water, but failed to lower the blood glucose at 3 and 6 h post-treatment. The 400 and 500 mg/kg decreased the blood glucose level from 1 to 6 h post-treatment. However, the decrease in blood glucose was only significant ($P < 0.05$) at 6 h post-treatment. The two combination protocol of the extract significantly ($P < 0.05$) decreased the blood glucose from 1 to 6 h post-treatment compared with 5 ml/kg distilled water. However, there was no significant ($P > 0.05$) difference between the effects of the combination protocol and glibenclamide 5 mg/kg alone though the effects of the combination protocol were better than that of glibenclmide 5 mg/kg alone. **Conclusion:** Our studies suggest that there is treatment benefit of combining extract of *G. latifolium* leaves and glibenclamide over *G. latifolium* or glibenclamide alone.

KEY WORDS: *Gongronema latifolium*, glibenclamide, combination, additive effect, treatment benefit

INTRODUCTION

Diabetes is a pathological and metabolic condition caused by inadequate insulin action with glucose intolerance being a common feature to all types [1]. Clinically, it is defined as a fasting plasma glucose level >7.8 mmol/l (140 mg/dl) or a 2 h post-prandial plasma glucose >11 mmol/l (200 mg/dl). Over 99% of cases of diabetes are caused by two diseases – Type 1 and Type 2 diabetes with Type 2 being 10 times more common than Type 1 [1]. Effective clinical management of diabetes relies on adequate control of blood glucose, which must take into consideration of the need to maintain adequate energy in the face of intermittent food intake along with variable exercise and thus variable demand [2]. The blood glucose concentration is controlled by a feedback system between liver, muscle, fat, and pancreatic islet cells and the overall pattern of control differing in basal and fed states. In insulin dependent diabetes mellitus (IDDM), insulin is the main form of treatment, while in the non-IDDM, diet is the cornerstone, often combined with oral hypoglycemic agent (metformin and the sulfonylureas) and/or insulin. Dietary management is essential in the management of both types of diabetes [2]. Various vegetables used for food in the Eastern part of Nigeria are known to have anti-hyperglycemic effect and therefore are consumed by diabetic patient even when on oral hypoglycemic agents or insulin. One of such vegetable is *Gongronema latifolium*. It is called amaranth globe in English; while it is known as *utazi* by the Igbo speaking parts of Eastern Nigeria. This is a herbaceous shrub of the tropical rain forest of the family *Asclepiadaceae*. It is a climbing shrub up to 5 m long. The leaves are commonly used either as vegetable or as a spice. Various pharmacological actions have been reported on this plant and include antioxidant potential [3], anti-asthmatic [4] antimalarial, anti-inflammatory and anti-sickling activities [5,6]. The plant has also been reported to cure cough, loss of appetite, and stomach disorders [7]. It is a popular remedy for diabetes [7]. This work was designed to evaluate the rationale for consumption *G. latifolium* leaves as hypoglycemic agent and again to verify if there is any treatment benefit or

order wise in combining the methanol extract of this plant with a standard oral hypoglycemic agent. This is because this plant is consumed by diabetic patients even when they are on an oral hypoglycemic agent.

MATERIALS AND METHODS

Animals

Albino rats between the ages of 10-12 weeks and weighing 120-140 g were used. They were kept in metal cages and fed and watered *ad libitum*. They were acclimatized for 14 days before the experiments. All animal experiments were in accordance with the guideline stipulated by the National Institute of Health for Care and use of laboratory animals (Pub. No. 85: 23 revised 1985).

Preparation of Plant Extract

The plant material was obtained from Orba in Udenu local Government Area of Enugu State Nigeria and identified as *G. latifolium* by a plant taxonomist at the Department of Botany University of Nigeria Nsukka. Fresh green leaves were dried under shed and ground to a coarse powder using hammer mill. A total of 400 g of the pulverized material was extracted in methanol using cold maceration for 48 h and filtered using Whatman No. 1 Filter Paper and concentrated *in vacuo* in a rotary evaporator.

Induction of Diabetes

The baseline 16 h fasting blood glucose level of each rat was determined using Accu-Chek Active Glucometer and Strips. Hyperglycemia was induced using a single intraperitoneal injection of alloxan monohydrate (160 mg/kg). Rats with fasting blood glucose >7.8 mmol/l or 140 mg/dl were considered hyperglycemic and were selected for the study.

Dose Response Effect of Methanol Extract of *G. latifolium* Leaves

Rats were grouped into 7 groups of 5. They were fasted for 16 h, but water was provided *ad libitum* before the experiment. The fasting blood glucose of each rat was determined before extract and drug were administered. Group 1 received 5 ml/kg distilled water and served as a negative control, Group 2 received 5 mg/kg glibenclamide and served as the positive control, while Groups 3-7 were treated with 200, 300, 400, 500, and 800 mg/kg, respectively. Change in blood glucose levels was accessed for each rat at 1, 3, and 6 h post-treatment. The percent change in blood glucose for each rat was calculated and average for each group determined.

Combination Effect of Methanol Extract of *G. latifolium* Leaves and Glibenclamide

20 rats grouped into 4 of 5 rats per group were used. They were fasted for 16 h before the experiment. The fasting blood glucose of each rat was determined before extract and drug administration. They were treated as follows: Group 1 (5 ml/kg distilled water), Group 2 (5 mg/kg glibenclamide), Group 3 (400 mg/kg extract + 5 mg/kg glibenclamide) and Group 5 (500 mg/kg extract + 5 mg/kg glibenclamide). Effects of these treatments on blood glucose were evaluated at 1, 3, and 6 h for each rat and average for each group determined.

Data Analysis

Data obtained were subjected to one-way analysis of variance and variant means separated *post-hoc* using least significant difference. Significance was accepted at $P < 0.05$.

RESULTS

Dose Response Effect

There was no reduction in blood glucose at extract doses 200 and 800 mg/kg. At 300 mg/kg of the extract, there was a reduction in blood glucose at 1 h post-treatment though not statistically significant ($P < 0.05$) compared with 5 ml/kg distilled water. The extract at doses of 400 mg/kg and 500 mg/kg caused a reduction in blood glucose at 1, 3, and 6 h post-treatment, but these effects were only significant at 6 h post-treatment when compared with 5 ml/kg distilled water. At 800 mg/kg, there was an increase in blood glucose (Figure 1).

Combination Effect of Methanol Extract of *G. latifolium* and Glibenclamide

There was significant ($P < 0.05$) reduction in blood glucose in the two combination protocol of the extract and glibenclamide at 1, 3, and 6 h post-treatment compared with 5 ml/kg distilled water. There was no significant ($P > 0.05$) difference between the two combination protocols, and between the protocol and glibenclamide alone, though the effects of the two combination protocol were higher than that of glibenclamide alone at 3 h post-treatment, while the combination of 400 mg/kg extract and 5 mg/kg glibenclamide was higher than glibenclamide (5 mg/kg) alone at 6 h post-treatment (Figure 2).

DISCUSSION

The anti-hyperglycemic effect of methanol extract of *G. latifolium* leaves was evaluated singly and in combination with glibenclamide to determine if there is a scientific basis for the traditional use of the plant as an anti-diabetic agent. More so to determine if there is potential herb-drug interaction (adverse effects) or treatment benefit of the combination (additive effect). This is because the leaves of *G. latifolium* are common component of most meals and diabetic patients on oral hypoglycemic agents equally consume it concurrently with these oral hypoglycemic agents. Alloxan-induced hyperglycemia is due to selective toxicity of alloxan on the pancreatic beta cells, generation of superoxide radicals and cytotoxic action mediated by generation of reactive oxygen species (ROS) [8-11]. The methanol extract of *G. latifolium* leaves did not decrease blood glucose of alloxan-

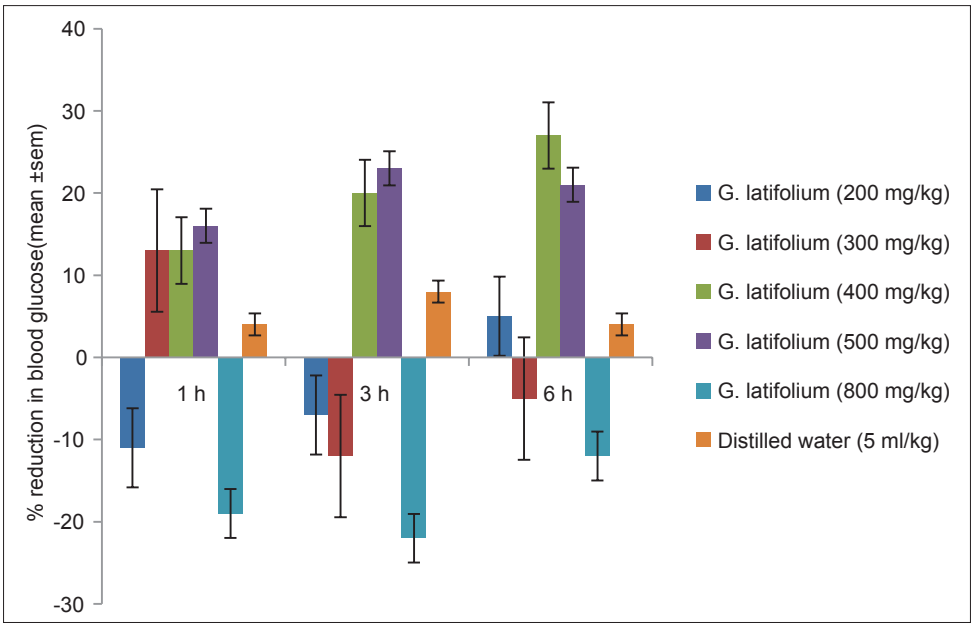

Figure 1: Dose response effect of methanolic leaf extract of *Gongronema latifolium* in alloxan-induced hyperglycemic rats

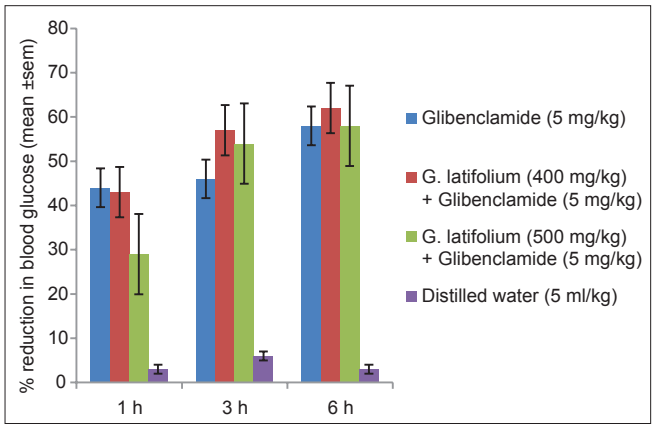

Figure 2: Effect of combination of different doses of methanolic leaf extract of *Gongronema latifolium* and glibenclamide in alloxan-induced hyperglycemic rats

induced hyperglycemic rats at doses of 200 and 300 mg/kg, but was able to decrease the blood glucose significantly ($P < 0.050$) at doses of 400 and 500 mg/kg 6 h post-treatment. This shows that the anti-hyperglycemic effect of the extract is dose-dependent. However, there was exacerbation of hyperglycemia at the dose of 800 mg/kg indicating that some phytochemical constituents of the extract or metabolic by-products of the extract contributed to elevated blood glucose. Some phytochemical constituents of methanol extract *G. latifolium* leaves include tannins, reducing sugars, flavonoids, saponins, and alkaloids [12]. These phytochemical constituents are known to have anti-hyperglycemic effects in other plants. Thus, could be responsible for the anti-hyperglycemic effect of *G. latifolium* leaves. These phytoconstituents are equally known to be antioxidant in other medicinal plant and therefore could have led to mopping up of ROS, therefore enhancing anti-hyperglycemic activity [13,14]. The combination of the methanol extract of *G. latifolium* and glibenclamide produced an anti-hyperglycemic effect that was higher than that of the extract or glibenclamide alone. However, this effect was not significantly better than that of glibenclamide alone. This effect of the combination protocol shows that there is treatment benefit in combining the extract and glibenclamide. This means that diabetic patients taking both *G. latifolium* leaves and glibenclamide simultaneously are likely going to have better glycemic control than those taking either glibenclamide or *G. latifolium* leaves alone. The combination protocol did not produce overt hypoglycemia showing that there is less risk of dangerous herb-drug interaction. In conclusion, our studies showed that there are basis for the use of *G. latifolium* as anti-diabetic agent traditionally and reveals a possible treatment benefit in combination of *G. latifolium* and glibenclamide in management of diabetes mellitus.

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Short Communication

Structure-activity relationships of antioxidant activity *in vitro* about flavonoids isolated from *Pyrethrum tatsienense*

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ABSTRACT

Aim: Antioxidant activity is one of the important indexes for estimating medicinal value for the traditional Chinese medicine. The aim of this study is to investigate the antioxidant activity of 11 flavonoids mainly revealing luteolin as mother nucleus isolated from *Pyrethrum tatsienense*. **Materials and Methods:** The antioxidant activity of 11 flavonoids was measured *in vitro* using the classical 1,1-diphenyl-2-picrylhydrazyl removal method. The percentages of scavenging activity of 11 flavonoids were analyzed by taking the choice of α-tocopherol as positive drugs, and the scavenging activity was plotted against the sample concentration to obtain the IC_{50} values. **Results:** Ten flavonoids containing phenolic hydroxyl groups have different levels of antioxidant activity. Antioxidant activity mainly depends on the numbers and the substitutional positions of phenolic hydroxyls in B ring. When C-3', 4' positions in B ring of flavonoids are replaced by hydroxyl groups, the antioxidant activity improved remarkably. Phenolic hydroxyl groups in A ring contribute some to antioxidant activity because of the electrophilic effect of C ring, and the numbers and substitutional positions of methoxyl and glycosyl have a little effect on the antioxidant activity. **Conclusion:** Structure-activity relationships of antioxidant activity about flavonoids isolated from *P. tatsienense* are concluded, which will be beneficial to deep understanding the pharmacological functions of this Tibetan medicine *in vivo* from the point of antioxidation.

KEY WORDS: Antioxidant activity, flavonoids, *Pyrethrum tatsienense*, structure-activity relationships

INTRODUCTION

Overproduced free radicals or a weakened natural antioxidant system can often lead to the oxidative stress that may eventually result in oxidative injury and diseases. Modern medical studies have shown that a variety of free radicals caused by oxidation in various tissues is one of the important factors of organic damage and pathological changes. Some serious diseases such as cardiovascular disease, diabetes, acquired immune deficiency syndrome, neurodegenerative diseases, inflammation, cancer, and aging process are related to oxidative damage caused by free radicals. It has been widely recognized nowadays that lowering oxidative stress can provide clinical benefits to a variety of pathological conditions, and antioxidant treatment as research focus has thus been regarded as a viable therapy to alleviate the oxidative injury in these disorders [1,2]. Under physiological conditions, a number of free radical scavengers in human body can effectively scavenge free radicals, which keeps the organisms healthy under the normal concentrations of free radicals. The free radical scavengers are usually divided into enzymes and non-enzymes. Enzymes scavengers comprise superoxide dismutase, catalase, glutathione peroxidase, and so on. Natural non-enzymatic scavengers contain Vitamin C, Vitamin E, tea polyphenols, and phenolic acid compounds, which exist widely in natural Chinese herbal medicines and foods [3,4].

Pyrethrum tatsienense (Bur. et Franch.) Ling [Figure 1], an herb of genus *Pyrethrum* in composite family, is mainly distributed in Qinghai, Sichuan, Yunnan and Tibet eastern part in China. *P. tatsienense* spends cold in nature and bitter taste, which has the functions of clearing away the heat-evil, expelling superficial evils, dispelling wind, eliminating dampness, anti-inflammatory and analgesia. Thus *P. tatsienense* has been widely used in folk and clinic in Tibet for the notable therapeutic effects of treating hepatitis, headaches, head injuries, ulcers, wounds, etc. [5]. The early phytochemical studies by our group showed that flavonoids mainly revealing luteolin as mother nucleus were present in considerable amounts in *P. tatsienense*, and most of flavonoids exist in the forms of glucoside. As we all know, therapeutic properties of flavonoids are diverse as antioxidant [6-9], anti-inflammatory, anti-neoplastic, hepatoprotective, neuroprotective activities [10]. Recently, ethanol extracts of *P. tatsienense* were found to have the hepatoprotective activity in D-galactosamine liver injury models [11], which reveals that flavonoids in *P. tatsienense* may be the active ingredients of hepatoprotective combined with the outcomes of phytochemical studies. The important clinical impacts of oxidative stress on liver disease mainly include changes in gene expression, inflammation trigger, liver fibrosis, cancer, and apoptosis. Reactive oxygen free radicals as the starting and regulating factors of liver cell damage may cause necrosis or apoptosis through direct or immune mechanism and hence the antioxidant activity is of great significance to protect liver cell damage.

Antioxidant activities of the 11 flavonoids isolated from *P. tatsienense* [Table 1], named as tricin (1), 4′-methoxy-tricin (2), apigenin (3), luteolin (4), luteolin-7-*O*-β-D-glucoside (5), apiolin-7-*O*-β-D-glucoside (6), apiolin-7-*O*-β-D-glucuronic acid methyl ester (7), quercetin-7-*O*-β-D-glucoside (8), 6-hydroxy-luteolin-7-*O*-β-D-glucoside (9), luteolin-7-*O*-β-D-glucoside aid (10), and apigenin-7-*O*-β-D-glucoside acid (11), were studied in some detail by the classic antioxidant method of 1,1-diphenyl-2-picrylhydrazyl (DPPH) assay *in vitro* in this experiment, and meaningful conclusions are drawn from the point of structure-activity relationships.

MATERIALS AND METHODS

Plant Material

The inflorescence of *P. tatsienense* (Bur. et Franch.) Ling were collected in Tibet Province in China, and authenticated by Prof. Chen-Chen Zhu (Institute of Clinical Pharmacology, Guangzhou University of Chinese Medicine). A voucher specimen (PT-201102) was deposited in Herbarium of Institute of Clinical Pharmacology, Guangzhou University of Chinese Medicine.

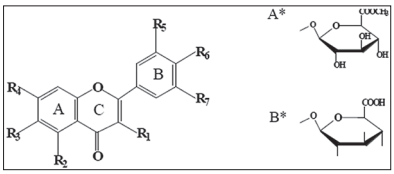
Materials and Apparatus

DPPH radical and α-tocopherol were obtained from Sigma Chemical Company (USA). The 11 flavonoids were isolated from ethanol extracts of *P. tatsienense* by the measures of normal phase, reversed phase, sephadex LH20 and molecular sieve column chromatograph. Their purities were over 98 % by normalization of the peak areas detected by high-performance liquid chromatography-ultra violet (UV) (Elite, China). All other reagents were of analytical grade.

Absorbance measurements were performed with a UV1000 UV-*vis* spectrophoto meter (Techcomp, China).

Figure 1: *Pyrethrum tatsienense* (Bur. et Franch.) ling

Table 1: The structures and antioxidant activity of 11 flavonoids isolated from *P. tatsienense*



Compounds	R_1	R_2	R_3	R_4	R_5	R_6	R_7	IC_{50} (mg/mL)
1	H	OH	H	OH	OCH_3	OH	OCH_3	9.13±0.31
2	H	OH	H	OH	OCH_3	OCH_3	OCH_3	--
3	H	OH	H	OH	H	OH	H	42.36±0.41
4	H	OH	H	OH	OH	OH	H	0.08±0.02
5	H	OH	H	*O*-glu	OH	OH	H	0.16±0.05
6	H	OH	H	*O*-glu	H	OH	H	38.77±0.37
7	H	OH	H	A*	H	OH	H	36.01±0.33
8	*O*-glu	OH	H	OH	OH	OH	H	0.14±0.03
9	H	OH	OH	*O*-glu	OH	OH	H	0.11±0.02
10	H	OH	H	B*	OH	OH	H	0.22±0.06
11	H	OH	H	B*	H	OH	H	11.30±0.31
α-tocopherol								0.12±0.03

All values were mean±SD (n=3) and the results were analyzed by ANOVA. "--"representing the scavenging activity of flavonoid can't be detected. SD: Standard deviation, *P. tatsienense*: *Pyrethrum tatsienense*

Sample Preparation

A total of 8.3 mg DPPH·was resolved in 200 mL of methanol to obtain 0.043 mg/mL stock solution. The 11 flavonoids and α-tocopherol were resolved in methanol to obtain 1.0 mg/mL stock solution, and then diluted by methanol to get 0.1, 0.3, 0.5, 0.8, and 1.0 mg/mL solutions, respectively.

Measurement of Antioxidant Activity

Each mixture contained 1.95 mL of DPPH solution and 50 μL of sample solutions, which reacted at room temperature for 20 min. Later, the absorbance values were measured at 517 nm. The absorbance value of methanol as blank control is A_0, and the absorbance value of reaction solution is A. Lower absorbance of the reaction mixture indicates higher radical scavenging activity. α-tocopherol was taken as positive control and the percentage DPPH· inhibition of the test samples was calculated as:

$$\text{Inhibition } \% = (1 - A/A_0) \times 100\ \%$$

Where A is the absorbance of the tested sample or positive control, A_0 is the absorbance of the reaction mixture without sample.

Each measurement was carried out three times in parallel.

Statistical Analyses

The measurements were performed in triplicate and the data were recorded as mean ± standard deviation. The IC_{50} value was defined as the final concentration of 50% free radical inhibition and was calculated by linear regression analysis. All values are shown with confidence interval and the $P < 0.05$. Statistical calculations were carried out using the SPSS (SPSS Inc., Chicago, IL, USA).

RESULTS AND DISCUSSION

DPPH· assay is the classics method to evaluate free radical scavenging activity of natural products. DPPH· are the stable purple free radicals with maximum absorption wavelength at 517 nm, which can accept an electron or hydrogen radical to become a stable diamagnetic molecule. The percentages of scavenging activity of 11 flavonoids at different concentrations were analyzed by taking α-tocopherol as the positive drug. The percentages of scavenging activity were plotted against the different sample concentration to obtain the IC_{50} values. The smaller the clearance rate IC_{50} value is, the stronger the antioxidant activity of flavonoid is.

Experimental results showed that DPPH free radical scavenging ability of flavonoids increased with the adding concentration of sample, which indicated that flavonoids could effectively scavenge free radicals with certain dose-effect relationships. As shown in Table 1, Comparing the IC_{50} values of flavonoid 1 with 3, antioxidant activity of the former was obviously larger than the latter although the numbers and substitutional positions of phenolic hydroxyls of these two compounds are the same. It is speculated that the ortho-positions of 4' hydroxyl are substituted by the two methoxyls as electron donors, which are favorable for the increase of conjugative effect of semi-quinonoid skeletons after hydrogen abstraction. In other word, methoxyl groups remaining at the ortho-positions of phenolic hydroxyl in aromatic ring play a synergistic effect on the antioxidant activity without considering its steric effect. The IC_{50} value of flavonoid 2 could not be detected, which showed this compound had no DPPH· scavenging activity owing to not containing phenolic hydroxyls in its structure. When C-7 position of flavonoid 4 is replaced by glucose, flavonoid 5 is obtained. The IC_{50} value of the latter was larger than that of the former, which indicated that antioxidant activity of flavonoid 5 decreased due to the reduced phenolic hydroxyls after glycosidation. Although glucose ring has several hydroxyls, it does not have the ability to delocalize electrons owing to not having conjugated system in its structure. Thus hydroxyls of glucose ring can not be viewed as active sites to scavenge DPPH free radicals. The antioxidant activity will decrease after phenolic hydroxyls glycosidation, the absorbance and distribution of flavonoids *in vivo* will change because of the increase of volume and polarity of the compounds. The same antioxidant effect will be obtained if flavonoid glycosides can form aglycone under the action of hydrolases *in vivo*.

On the other hand, free radicals clearance rate IC_{50} value of flavonoid 4, 5, 8, 9, and 10 are comparable with α-tocopherol, and the antioxidant activity of these five flavonoids are significantly stronger than the other six flavonoids. The reasons should be that the positions of C-3', 4' in B ring of these five flavonoids were all replaced by two hydroxyls, which lead to the significant increase of antioxidant activity. In other word, the catechol hydroxyls at C-3', 4' positions in flavonoids are the most important active sites. It is presumably that intramolecular hydrogen bond is formed by semi-quinonoid free radical with 4' hydroxyl after the first hydrogen abstraction, and further quinone is formed after the second hydrogen abstraction occurring at 4' hydroxyl [Figure 2][12]. Thus the *o*-dihydroxyls at C-3', 4' positions in flavonoids are more advantageous to the high delocalization effect of electrons. Although flavonoid 10 has two sets of *o*-dihydroxyls at C-3',4' positions in B ring and at C-5,6 positions in A ring respectively, the IC_{50} value of flavonoid 10 was a little lower than that of flavonoid 9. This result indicated that the catechol hydroxyls at C-5, 6 positions in A ring of flavonoid had an insignificant effect on the antioxidant activity. The reason probably is that the intramolecule hydrogen bond are formed by the hydroxyl at C-5 position in A ring with the carbonyl oxygen at C-4 position in C ring, which decrease the ability of hydrogen abstraction of *o*-dihydroxyls at C-5,6 positions in flavonoid 9. In addition, antioxidant activity of phenolic hydroxyls in A ring of flavonoid decreased significantly because of electrophilic effect of C ring.

CONCLUSIONS

The degree of antioxidant activity depends mainly on the ability of compounds providing protons to neutralize free radicals at the initial stage of oxidation or terminating the radical chain reaction [13]. The main process of flavonoids scavenging free radicals is that phenolic hydroxyls of flavonoids

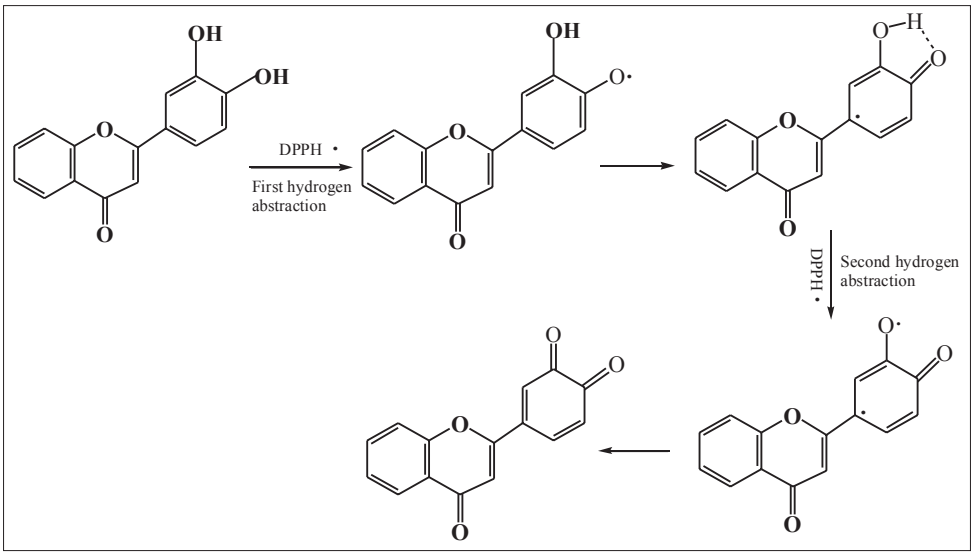


Figure 2: The possible scavenging 1,1-diphenyl-2-picrylhydrazyl mechanism occurring at C-3', 4' positions of flavonoids 4, 5, 8, 9, and 10

can react with free radicals to terminate chain reaction. Two related mechanisms to eliminate free radicals contain hydrogen abstraction reaction and electron transfer [14]. The ability of flavonoids scavenging free radicals depends mainly on the ease or complexity of the breakdown of phenolic hydroxyls and the stability of semi-quinonoid free radicals after hydrogen abstraction reaction. The bond dissociation energy of phenolic hydroxyl in the aromatic ring is a key mark to measure the strength of phenolic hydroxyl. The lower the dissociation energy is, the more active phenolic hydroxyl is. Thus the easier hydrogen abstraction reaction is, the stronger antioxidant activity is [15,16].

The experimental results of DPPH· methods show that flavonoids have strong antioxidant capacity. The relationships between the structures of flavonoid and antioxidant activity are as follows: (1) The increase of phenolic hydroxyls among flavonoids is favorable for the antioxidant activity, (2) antioxidant activity will increase remarkably when the C-3', 4' positions in B ring of flavonoids are replaced by hydroxyls, (3) the ortho position of phenolic hydroxyl containing methoxyl (electron donor group) will be favorable for the improvement of antioxidant capacity, (4) antioxidant activity decreases when flavonoids are glycosylated, which reflects that antioxidant capacity mainly depends on the number of phenolic hydroxyls and nearly has nothing to do with the hydroxyl in sugar ring, (5) the substitutional positions of phenolic hydroxyls have more effects on antioxidant activity than the numbers of phenolic hydroxyls have. This result is agreement with theoretical calculation results which combined phenolic bond dissociation enthalpy with radical scavenging ability [17].

The above studies indicate that flavonoids mainly revealing luteolin as mother nucleus isolated from *P. tatsienense* can effectively eliminate free radicals, which provide valuable clues for further clarifying the mechanism of preventing or treating hepatic injury induced by free radicals, especially for deeply analyzing the pharmacological functions of *P. tatsienense in vivo* from the point of antioxidation.

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Original Research

Proximate composition, mineral content and *in vitro* antioxidant activity of leaf and stem of *Costus afer* (Ginger lily)

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ABSTRACT

Aim: This study was designed to determine the proximate composition and mineral content of *Costus afer* leaf and stem, as well as to identify the most active antioxidant fraction. **Materials and Methods:** The proximate composition and mineral analysis of *C. afer* leaf and stem were performed using the standard methods described by Pearson and Association of Official Analytical Chemist while the 1,1 diphenyl 2 picryl hydrazyl (DPPH), thiobarbituric acid reactive species (TBARS), lipid peroxidation (LPO), and total antioxidant capacity (TAC) assays were used to determine the *in vitro* antioxidant activity of aqueous, *n*-butanol, ethyl acetate and hexane fractions of *C. afer* leaf and stem. **Results:** Proximate analysis revealed that the carbohydrate content was highest in the leaf (55.83 ± 3.71%) and stem (50.38 ± 1.27%) while crude fat content was lowest in the leaf (1.83 ± 0.43%) and stem (1.75 ± 0.48%). The minerals detected in appreciable quantity in both the leaf and stem samples were calcium, magnesium, potassium, sodium, chromium, lead, manganese, nickel, and copper. Further study showed that the aqueous leaf fraction exhibited a significantly ($P < 0.05$) high DPPH scavenging activity ($IC_{50} = 259.07\ \mu g/ml$) and TAC (7.95 ± 0.37 mg ascorbic acid equivalent/g) compared with the other test fractions while the aqueous stem fraction had the highest TBARS scavenging activity ($IC_{50} = 0.37\ \mu g/ml$) and inhibition of LPO ($IC_{50} = 41.15\ \mu g/ml$) compared with the other test fractions. **Conclusion:** The findings from this study indicate that *C. afer* could serve as a source of nutrient and minerals for animal nutrition and human metabolism. It also showed that the aqueous fractions of *C. afer* leaf and stem possess high antioxidant activity than the other fractions. In addition, this study may also explain the folkloric use of crude *C. afer* leaf or stem extracts in the treatment of oxidative stress associated diseases, including rheumatoid arthritis and hepatic disorder.

KEY WORDS: Antioxidant, *Costus afer,* mineral, plant, proximate

INTRODUCTION

Costus afer Ker Gawl of the family Zingiberaceae now known as Costaceae is a tall perennial herbaceous, unbranched medicinal plant with creeping rhizome [1]. It can be found in shady forest and riverbanks of Senegal, South Africa, Guinea, Nigeria, Ghana and Cameroon [2]. It is commonly called bush cane, ginger lily or spiral ginger, and in Nigeria it can be identified by several names such as Ireke omode-Western Nigeria, Okpete-Eastern Nigeria, Kakizawa-Northern Nigeria and Mbritem-Southern Nigeria, Akan asante in Ghana and Monkey sugar cane in Cameroun [2].

C. afer is a medicinal plant used traditionally for the treatment of rheumatoid arthritis, hepatic diseases, stomach ache, cough, measles, malaria, eye defects, and could also serve as an antidote for snake poisoning [2,3]. It is also important for other sociocultural purposes, including wrapping of indigenous food items, mat making, and feed for ruminants as well as an ornament for ritual purposes [4].

The major constituents of *C. afer* are steroidal sapogenins, saponins aferosides A-C, and dioscin, which are important biopharmaceuticals [3]. Phytochemical analysis of *C. afer* leaf and stem crude extracts has revealed the presence of flavonoids, saponins, alkaloids, tannins, phenols, and glycosides [5,6]. Antioxidant activity of the crude stem extracts has been reported [6]. *C. afer* leaf and stem crude extracts possess hepatoprotective activity [7-9].

Phytochemical compounds possess a wide range of nutritional, biological, and pharmacological effects [10,11]. Antioxidant property, anti-inflammatory activity, hormonal action, stimulation of enzymes, interference with deoxyribonucleic acid replication, anti-inflammatory effect, and physical action are some of the known possible effects of phytochemicals [2,10,11]. This study was designed to evaluate the proximate composition and mineral content of *C. afer* leaf and stem, as well as to identify the fraction of *C. afer* with the most active *in vitro* antioxidant activity.

MATERIALS AND METHODS

Collection of Plant Materials

C. afer plants were obtained from a farm land at Irolu in Ikenne Local Government Area, Ogun State, Nigeria. The plant was identified and authenticated by Professor O.A. Denton, a crop scientist in the Department of Agronomy and Landscape Design, School of Agriculture and Industrial Technology, Babcock University, Ilisan-Remo, Ogun State, Nigeria. A voucher sample with number of FHI-108001 has been deposited at Forestry Herbarium Ibadan (FHI).

Plant Processing, Extraction, and Solvent Partitioning

The leaves and stem were separated from the roots. The roots were discarded while the leaves and chopped stem pitches were air-dried under room temperature and pulverized using mechanical grinder. Three hundred grams powdered leaf and stem samples were extracted separately using 1800 ml of 70% methanol at 28°C with intermittent shaking for 48 h. The extracts were filtered using Whatman No.1 filter paper and the filtrates were subsequently concentrated using rotary evaporator at 30°C (Buchi Rotavapor RE; Switzerland). The concentrates were reconstituted with distilled water in a ratio of 1:2 (concentrate: distilled water) and partitioned by successive solvent fractionation method using separating funnel containing equal volume of reconstituted suspension and solvents in the following order: hexane, ethyl acetate, *n*-butanol and distilled water. The fractions obtained were concentrated again using rotary evaporator at 30°C and kept in the refrigerator at 4°C until further use a stock.

Proximate and Mineral Analysis

The proximate composition and mineral analysis were carried out on the plant stem and leaf for the quantitative determination of physicochemical constituents using standard procedures as described by Pearson [12] and Association of Official Analytical Chemist [13].

Determination of Antioxidant Activity using *In vitro* Methods

1,1-diphenyl 2-picryl hydrazyl (DPPH) assay

Free radical scavenging potentials of test fractions (aqueous, butanol, ethyl acetate, and hexane fractions), which is based on the capacity of the test fractions to reduced 1,1-diphenyl-2-picryl hydrazyl was adopted according to the procedure described by Mensor *et al*. [14]. 1 mm of 0.3 mM DPPH in methanol was added to 2.5 ml solution of varying concentrations of test fractions or standard Gallic acid at different concentrations of 50, 100, 250, and 500 μg/ml prepared using 10% dimethyl sulfuroxide (DMSO) and allowed to react at room temperature for 30 min. The absorbance of the resulting mixture was measured at 517 nm and converted to percentage antioxidant activity (AA %), using the formula:

$$AA\% = 100 - \frac{([\text{Abs sample} - \text{Abs blank}] \times 100)}{\text{Abs control}}$$

AA% indicates antioxidant activity of fractions; Abs sample indicates absorbance of sample; Abs blank indicates absorbance of blank (methanol and DPPH) and Abs control indicates absorbance of control. One milliliter of 0.3 mm DPPH plus methanol (2.5 ml) served as a control. This assay was carried out in triplicates for each concentration. The IC_{50} values were calculated for the samples. IC_{50} denotes the concentration of fractions required to scavenge 50% of DPPH radicals.

Thiobarbituric acid (TBA) assay

The thiobarbituric acid method of Ottolenghi [15] as modified by Kikuzaki and Nakatani [16] was used for the determination of antioxidant activity of the various leaf and stem fractions of *C. afer*. Gallic acid and ascorbic acid standards at concentrations of 0.1, 0.2, 0.4, 0.6, 0.8, and 1.0 μg/ml were prepared and made up to 1.0 ml in test tubes using 10% DMSO. Similarly, test leaf and stem fractions (aqueous, butanol, ethyl acetate and hexane fractions) concentrations of 0.1, 0.2, 0.4, 0.6, 0.8, and 1.0 μg/ml were also prepared and made up to 1.0 ml using DMSO. To these test solutions, 2 ml of 20% TCA and 2 ml of 0.67% of thiobarbituric acid solutions were added. Another set of test tubes containing the above reagents without any sample were used as control. The test tubes were placed in a boiling water bath (Uniscope, SM801A England) for 10 min. They were then cooled and centrifuged at 3000 rpm for 20 min. The absorbance of the supernatant was measured at 552 nm. This assay was carried out in triplicate.

Calculation

$$\text{Inhibition of TBA } (\%) = 1 - \frac{E}{C} \times 100$$

Where C = absorbance of fully oxidized control and E = absorbance in the presence of fraction/standard. The IC_{50} values were also computed for the standards and test fractions.

Inhibition of lipid peroxidation (LPO) assay

The inhibition of LPO by test samples and two standards (Gallic acid and ascorbic acid) was carried out by using egg

yolk homogenate as lipid-rich media according to the method described by Ruberto and Barrata [17]. A stock solution of 1 mg/ml was prepared for standards Gallic acid, ascorbic acid, and test fractions (aqueous, butanol, ethyl acetate and hexane leaf and stem). Egg yolk homogenate (0.5 ml, 10% v/v) was added to varying volumes of 10, 20, 50, and 100 μl of standards and test sample fractions and the volume made up to 1 ml with distilled water. Thereafter, 0.05 ml of $FeSO_4$ was added. The reaction mixture was subsequently incubated for 30 min at 37°C. After incubation, 1.5 ml of acetic acid was added to the reaction mixture, followed by 1.5 ml of 0.67% TBA in 20% sodium dodecyl sulfate. The resultant solutions were mixed in a vortex mixer and heated at 95°C for 60 min. After cooling, 5 ml of butan-1-ol was added, and the mixture centrifuged at 3000 rpm for 10 min. The absorbance of the organic upper layer was measured at 532 nm and converted to the percentage inhibition using the formula:

$$\text{Inhibition of lipid peroxidation } (\%) = \frac{1-E}{C} \times 100$$

Where C = absorbance of fully oxidized control and E = absorbance in the presence of test fraction/standard.

Total antioxidant capacity (TAC)

The TAC of the test fractions of *C. afer* leaf and stem was carried out using phosphomolybdenum method as described by Priesto *et al.* [18]. An aliquot of 0.1 ml of the various 1 mg/ml test fractions was combined with 1 ml of working reagent solution containing 0.6 M sulfuric acid, 28 mm sodium phosphate and 4 mm ammonium molybdate. The working reagent was prepared by dissolving 0.1092 g of 28 mm sodium phosphate and 0.1236 g of 4 mm ammonium molybdate in 25 ml of 0.6 M H_2SO_4. The standard consisted of 0.1 ml of varying concentrations of 1 mg/ml ascorbic acid (water soluble antioxidant) in methanol with 1 ml of the working reagent. The blank consisted of 0.1 ml methanol and 1 ml of the working reagent. The solutions were incubated in a water bath at 95°C for 90 min. After the samples had cooled, the absorbance values were determined using a spectrophotometer at a wavelength of 695 nm against blank. The antioxidant activity of the extracts was measured and expressed as ascorbic acid equivalents.

Statistical Analysis

Statistical analysis was carried out with the aid of SPSS for windows; SPSS Inc., Chicago, Standard version 17.0 to determine differences between the mean of the test fractions using Mann–Whitney U-test analysis. Linear regression analysis was performed to determine IC_{50} for the test fractions. *T*-test analysis was also performed. $P < 0.05$ was considered significant. All analyses were performed in triplicate. Data were reported as mean ± standard error of the mean.

RESULTS

Table 1 shows that the proximate composition of carbohydrate, crude protein, crude fat, and crude ash content in *C. afer* leaf sample were not significantly ($P > 0.05$) different from that of the stem. However, the moisture content of the dried leaf (18.63 ± 2.11%) was significantly ($P < 0.01$) higher than that of stem (6.76 ± 0.67%) while the crude fiber content of stem (27.28 ± 1.54%) was significantly ($P < 0.05$) higher than that of the leaf (21.16 ± 0.86%).

Table 2 shows that there were no significant differences ($P > 0.05$) between the amount of minerals detected in the leaf and stem. However, the amount of calcium, magnesium, and sodium were appreciably elevated in both stem and leaf samples compared with the other minerals detected. Cadmium was not detected in both samples.

The data in Figure 1 and Table 3 shows that all test fractions scavenged DPPH radical in a concentration dependent manner. However, the aqueous leaf fraction (IC_{50} = 259.07 μg/ml) exhibited a significantly ($P < 0.05$) high DPPH scavenging

Table 1: Proximate composition of *C. afer* leaf and stem

Parameters	Leaf (%)	Stem (%)
Moisture content	18.63±2.11	6.76±0.67
Crude fiber content	21.16±0.86	27.28±1.54
Crude ash content	11.47±1.47	10.91±0.50
Crude fat content	1.83±0.43	1.75±0.48
Crude protein content	2.75±0.56	2.93±1.16
Carbohydrate content	55.83±3.71	50.38±1.27

C. afer: Costus afer

Table 2: Mineral composition of *C. afer* leaf and stem

Mineral element	Leaf (mg/kg)	Stem (mg/kg)
Calcium	7.69±1.12	7.92±0.25
Magnesium	4.01±1.25	3.64±1.15
Potassium	1.02±0.34	0.95±0.03
Sodium	1.97±0.12	2.25±1.07
Chromium	0.07±0.01	0.10±0.05
Lead	0.01±0.00	0.02±0.00
Manganese	0.82±0.02	0.75±0.12
Nickel	0.17±0.01	0.12±0.05
Copper	0.44±0.02	0.52±0.11
Cadmium	ND	ND

ND: Not detected, *C. afer: Costus afer*

Table 3: Fifty percent inhibitory concentrations of different fractions of *C. afer* leaf and stem in different antioxidant system *in vitro*

Fractions of *C. afer*	IC_{50}		
	DPPH (50-500 μg/ml)	TBARS (0.1-1.0 μg/ml)	LPO (10-100 μg/ml)
Butanol stem	367.65	0.68	57.94
Butanol leaf	555.56	0.49	47.21
Hexane stem	537.63	0.49	51.02
Hexane leaf	333.33	0.48	52.19
Ethyl acetate stem	2500.0	0.40	60.39
Ethyl acetate leaf	1923.08	0.41	48.40
Aqueous stem	271.74	0.37	41.15
Aqueous leaf	259.07	0.38	43.90
Gallic acid	206.61	0.36	44.40
Ascorbic acid		0.40	44.09

DPPH: 1,1 diphenyl 2 picrylhydrazyl, TBARS: Thiobarbituric acid reactive species, LPO: Lipid peroxidation

activity compared with the other test fractions while ethyl acetate stem fraction (IC_{50} = 2500.00 μg/ml) had the least DPPH scavenging activity.

The data in Figures 2 and 3 and Table 3 shows that the test different leaf and stem fractions, Gallic acid and ascorbic acid scavenged thiobarbituric acid reactive species (TBARS) in a concentration dependent manner. The aqueous stem (IC_{50} = 0.37 μg/ml) had the highest TBARS scavenging activity while butanol stem had the lowest scavenging activity of TBARS. The LPO inhibitory activity of *C. afer* leaf and stem fractions showed that aqueous stem fraction (IC_{50} = 41.15 μg/ml) had the highest inhibition of LPO while ethyl acetate stem fraction (IC_{50} = 60.39 μg/ml) had the lowest inhibition of LPO activity [Figure 4 and Table 3].

TAC of the different fractions of *C. afer* leaf and stem showed that the aqueous leaf fraction had a significantly ($P < 0.05$) high TAC (7.95 ± 0.37 mg ascorbic acid equivalent/g); followed by hexane leaf fraction (7.07 ± 0.06 mg ascorbic acid equivalent/g)

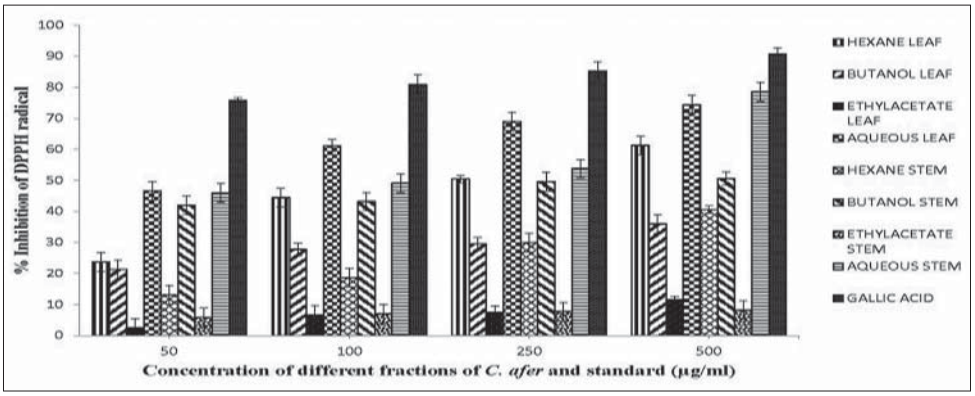

Figure 1: Percentage inhibition of 1,1 diphenyl 2 picrylhydrazyl radical by different concentration of *Costus afer* fractions

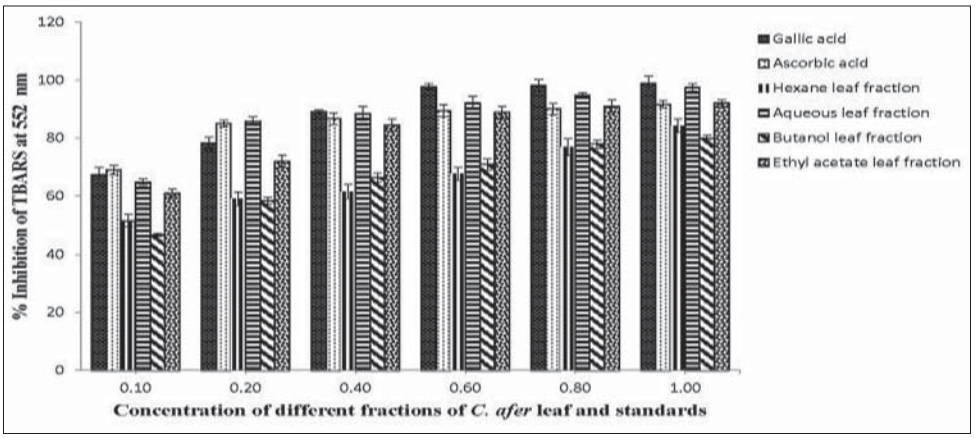

Figure 2: Percentage inhibition of thiobarbituric acid reactive species by different concentration of *Costus afer* leaf fractions

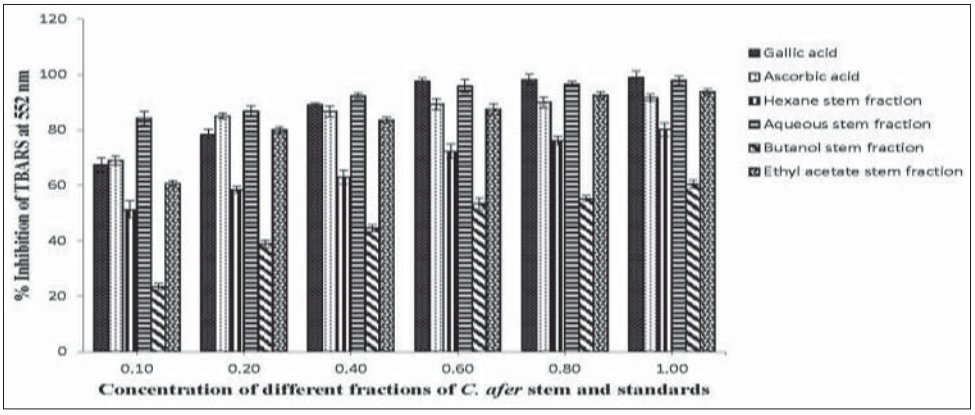

Figure 3: Percentage inhibition of thiobarbituric acid reactive species by different concentration of *Costus afer* stem fractions

and aqueous stem fraction (6.85 ± 0.36 mg ascorbic acid equivalent/g) when compared with other test fractions [Figure 5 and Table 3].

DISCUSSION

The proximate analysis data indicated that *C. afer* leaf and stem contained an appreciable amount of carbohydrate and crude fiber. These suggest that *C. afer* could serve as a source of energy and dietary fiber. Previous studies have shown that consumption of carbohydrates provides the body with the necessary energy required to drive cellular metabolism while dietary fiber could prevent the incidences of cardiovascular diseases, arteriosclerosis and increase intestinal transit time [19]. This may account for its use as folder in livestock production. The ash content also indicates that it could be a good source of minerals. The moisture content of the stem might be due to the succulent nature of the plant stem and could serve as a readily available source of fluid for quenching of thirst when dehydrated, especially in hot farm areas where water is not easily assessable to farmers, while the moisture content of the leaf could serve as a source of water to farm animals. However, the elevated moisture content of dried *C. afer* leaf compared to the dried stem indicates that the leaf may have reduced shelf life. The crude fat and protein contents of the leaf and stem suggested that they could be an important source for dietary fat and protein feed supplementation.

The mineral analysis revealed that the levels of calcium, magnesium, sodium, potassium, and manganese in *C. afer* leaf and stem were appreciably high compared to the other minerals detected. These minerals are essential in the body system for disease prevention and control [20]. The presence of these minerals may account for the ethnomedical use of *C. afer* in the treatment and management of inflammatory diseases. Calcium and potassium are important for growth and maintenance of strong bones, muscular function, synthesis of enzymes, and normal physiological function of the body [20]. Potassium and sodium also helps in the maintenance of acid-base balance in the body and osmotic pressure [21]. Magnesium serves as a cofactor for enzymes activation and biological structure promoter [22]. Manganese is an important modulator of cells functions and play vital role in the control of diabetes mellitus [23]. Chromium has been shown to participate in sugar metabolism and possible in the prevention of diabetes [24]. Nickel serves as a cofactor of important antioxidant enzymes such as superoxide dismutase [25]. Copper is a very powerful pro-oxidant and catalyzes the oxidation of unsaturated fats and oils as well as ascorbic acid [26]. The lead levels of 0.02 mg/kg stem and 0.01 mg/kg leaf may not lead to any health hazard in consumers since it is lower than the maximum permissible limit of 30 mg/kg lead for vegetables (FAO/WHO/2001)[20] and thus are within safe limits for the use of *C. afer* stem and leaf as herbal medicine.

Evaluation of the antioxidant activity of the different fractions of *C. afer* leaf and stem *in vitro* showed that they reduced DPPH to 1,1 diphenyl 2 picrylphenylhydrazine in a concentration dependent manner as evidenced by the decolorization of DPPH radical from purple to yellow color. Previous study had shown that when DPPH radicals react with suitable reducing agent

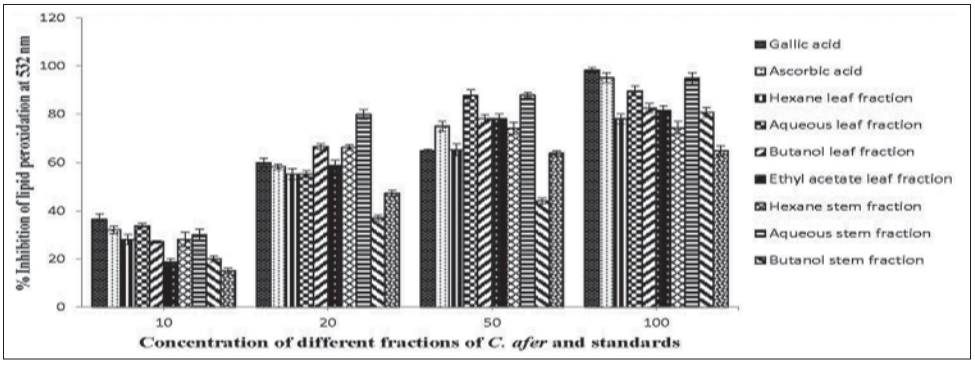

Figure 4: Percentage inhibition of lipid peroxidation by different concentration of *Costus afer* fractions

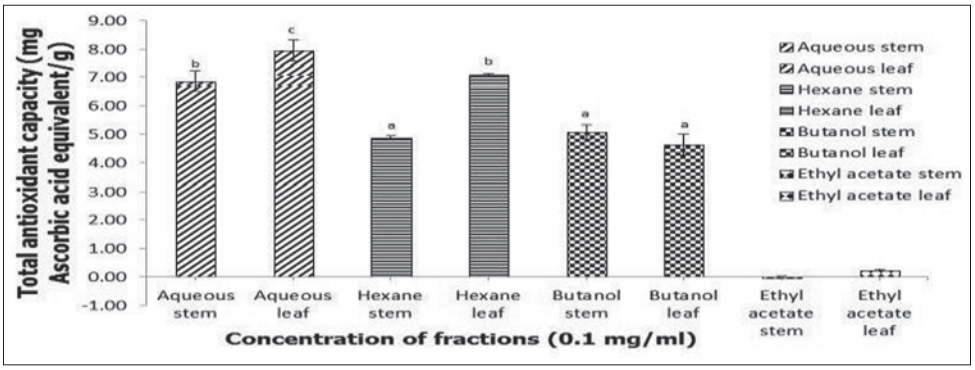

Figure 5: Total antioxidant capacity of *Costus afer* leaf and stem fractions

it loses color stoichiometrically depending on the number of electrons taken up [27]. The aqueous leaf fraction exhibited the highest DPPH scavenging activity when compared with the other test fractions. This indicates that the polar fraction of *C. afer* may contain higher antioxidant compounds than the non-polar fractions. Previous studies have shown that the majority of the antioxidant compounds are usually polar in nature. This is due to the presence of the hydroxyl groups present in the polyphenol and flavonoid ring structures of the antioxidant compounds [28].

In TBARS scavenging assay, the different test fractions inhibited the formation TBARS in a concentration dependent manner. The aqueous fractions of *C. afer* exhibited the highest TBARS scavenging activity when compared to the other test fractions. This also indicates that the polar fractions contained higher antioxidant activity than the non-polar fractions. Furthermore, the capacity of the various test fractions to break the chain reactions generated by propagating LPO was also in a concentration dependent manner. The aqueous fraction exhibited the highest inhibition of LPO when compared with the other test fractions. This further suggests that the polar fractions possess higher antioxidant activity than the non-polar fractions. LPO of membranes is part of the early events that takes place during the inflammatory response by stimulated polymorphonuclear cells and thus agents capable of preventing LPO of membranes could serve as a suitable candidate in anti-inflammatory drug discovery process [29].

Further study also demonstrated that the aqueous leaf fraction had a significantly ($P < 0.05$) high TAC when compared with the other test fractions using phosphate molybdenum assay, which is based on the reduction of molybdenum (VI) to molybdenum (V) with the subsequent formation of a green phosphate-molybdenum (V) complex in acidic condition. This further suggests that the aqueous fraction of *C. afer* leaf may possess a higher antioxidant activity *in vitro* when compared with the other test fractions. The reduction of molybdenum (VI) to molybdenum (V) may be attributed to the hydrogen and electron donating potentials of polyphenolic compounds present in *C. afer* [18]. This may also account for the higher antioxidant activity exhibited by the polar fractions in the various studied models as they tend to contain a lot of polyphenolic compounds [28].

CONCLUSION

The data from this study showed that the *C. afer* leaf and stem could serve as an important source nutrients and minerals. The leaf fractions of *C. afer* had more antioxidant activity than the stem fractions *in vitro*. More so, among the test fractions, the most active antioxidant fraction was the aqueous fraction followed by hexane, *n*-butanol and ethyl acetate fraction. These findings may explain the use of *C. afer* stem and leaf extracts in the ethnomedical practice as a therapeutic agent against most of the inflammatory and oxidative stress related diseases including rheumatoid arthritis and hepatic disorders.

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