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Original Research

Anti-diabetic and antioxidant effect of cinnamon in poorly controlled type-2 diabetic Iraqi patients: A randomized, placebo-controlled clinical trial

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ABSTRACT

Aim: To determine the effect of cinnamon on fasting blood glucose, hemoglobin (Hb) A1c, and oxidative stress markers in poorly controlled type 2 diabetes. **Patients and Methods:** A total of 25 type 2 diabetic patients of both sexes, aged 49.1 ± 6.0, treated only with hypoglycemic agent sulfonylurea (glibenclamide) were randomly assigned to receive either 1 g of cinnamon or placebo daily for 12 weeks. **Results:** A highly significant ($P \leq 0.001$) reduction (10.12%) of fasting blood glucose level after 6 and 12 weeks of treatment 10.12% and 17.4%, respectively, compared to baseline value and to placebo group at corresponding duration. Meanwhile, the value of glycosylated Hb reduced in cinnamon treated group by (2.625%) and (8.25%) after 6 and 12 weeks, respectively, although this reduction was non-significant compared to baseline value. Concerning the oxidative stress markers, the level of serum glutathione showed highly significant ($P \leq 0.001$) elevation after 12 weeks as compared to baseline value and placebo group at corresponding duration, malondialdehyde serum level decreased after treatment of diabetic patients with cinnamon resulted in highly significant ($P \leq 0.001$) reduction after 6 and 12 weeks compared to placebo group, but when compared to baseline value, there is a (15%) reduction only after 12 weeks of treatment which was considered highly significant ($P \leq 0.001$) change, Finally, administration of cinnamon to diabetic patients for 12 weeks resulted in significant ($P \leq 0.05$) elevation of superoxide dismutase level. **Conclusion:** Intake of 1 g of cinnamon for 12 weeks reduces fasting blood glucose and glycosylated Hb among poorly controlled type 2 diabetes patients, as well as, there is improvement in the oxidative stress markers, indicating the beneficial effect of adjuvant cinnamon as anti-diabetic and antioxidant along with conventional medications to treat poorly controlled type 2 diabetes mellitus.

KEY WORDS: Antioxidants, cinnamon, poorly controlled diabetes mellitus, type 2 diabetes

INTRODUCTION

Type 2 diabetes mellitus (T2DM) is one of the most prevalent diseases worldwide and in Iraq, The WHO estimates a prevalence of 347 million people with diabetes worldwide in 2013 [1]. The prevalence is expected to double between 2005 and 2030 and the greater proportion of this increase would be in the low to middle-income countries of Asia, Africa and South America [2]. Despite advances in methods of diagnosis and treatment protocols, poorly controlled T2DM responsible for high incidence of morbidity that extensively disturbed the quality of life in addition to high expenditures each year [3]. Understanding of mechanisms underlies the pathogenesis of disease enable better targeting of changes that led to good glycemic control and improvement in overall outcomes.

Oxidative stress is the loss of the normal balance between prooxidants and antioxidants represent important implicated mechanism in the pathogenesis of diabetic complications [4], patients with type 2 diabetes are vulnerable to increase oxidative stress due to the excessive production of free radicals especially reactive oxygen species (ROS) and impaired antioxidant defense mechanism [5]. Poorly glycemic control leads to hyperglycemia which results in increased production of ROS which causes membrane damage due to peroxidation of membrane lipids and protein glycation [6]; for this reason, the targeting of oxidative stress is necessary in diabetic patients. Many studies have shown that the use of antioxidants, as well as herbal agents, might help control the oxidative stress [7]. The use of herbal medicinal plants especially those used in folk medicine for the treatment of diabetes is common in Iraqi diabetic patients, among these, the use of cinnamon [8].

Cinnamon is one of the most widely used spices in the food and beverage industry, worldwide has also been well recognized for its medicinal properties for a long time. Traditional medicine has used cinnamon extracts for ailments such as arthritis, diarrhea, and menstrual irregularities. The dry bark of cinnamon trees is

rich in botanical source of polyphenolics and has been used to improve general health and treat a variety of disease conditions including diabetes [9]. In addition to anti-diabetic properties, cinnamon is known to have anti-inflammatory, antibacterial, and antioxidant properties [10]. Cinnamon lowering effect of glucose level may be due to many mechanisms; many *in vitro* studies have showed that cinnamon increases glucose entry into cells by enhanced insulin receptor phosphorylation and the translocation of glucose transporter glucose transporter-4 (GLUT4) to the plasma membrane [11]. The active compound responsible is believed to be poly-phenolic compound [12]. Another possible mechanism that explains the hypoglycemic effect of cinnamon is an increase in the expression of peroxisome proliferator-activated receptor (PPAR) (alpha) and (gamma) receptors thereby increasing insulin sensitivity [13]. Furthermore, it has also been demonstrated that cinnamon possesses an inhibitory effect on intestinal glucosidases and pancreatic amylase. Ceylon cinnamon is the most potent inhibitor of pancreatic amylase and intestinal sucrase [14]. A clinical study has demonstrated its ability to delay gastric emptying as well as lowering the postprandial glucose level [15].

Thus, cinnamon considered an important anti-diabetic spice, and different studies involving cinnamon administration have produced contrasting results. The aim of this randomized, double-blinded clinical trial was to analyze the effect of cinnamon powder on fasting blood glucose, glycosylated hemoglobin A1c (HbA1c) and oxidative state in Iraqi patients with type 2 diabetes.

PATIENTS AND METHODS

A prospective, placebo-controlled randomized clinical trial was carried out on 26 patients with T2DM who attend the Specialized Center for Endocrinology and Diabetes-AL-Risafa, Directoratc of Health-Baghdad were enrolled in this study. The inclusion criteria: Patients with T2DM of both sexes on sulfonylurea (glibenclamide), with age range 40-65 years (49.1 ± 6.0), and have disease duration of 5-10 years. The exclusion criteria: They should not have other associated chronic diseases like liver and kidney disorders and cardiovascular complications. Patients who are pregnant and breast feeding are excluded. They should not be on insulin therapy or other anti-diabetic drugs, or on antioxidant drugs like aspirin, and any associated drugs should be considered. They should not taking other hypolipidemic agent; anti-inflammatory or non-steroidal anti-inflammatory drugs.

The patients treated previously with full maximum dose of sulfonylurea (glibenclamide) (15 mg/day) and kept on dietary control, but with poor glycemic control as evidenced by abnormal values of fasting plasma glucose (FPG) and glycated Hb; those patients are carefully evaluated while they are on their already established treatment program for DM control for 2 weeks before the randomization:

1. Group A includes 12 patients treated with placebo in capsule dosage form in addition to the already given oral hypoglycemic agent (glibenclamide) and dietary control, for 12 weeks.
2. Group B includes 13 patients treated with cinnamon powder 500 mg in hard gelatin capsule twice daily (1000 mg/day) in addition to the already given oral hypoglycemic agent (glibenclamide) and dietary control for 12 weeks. The cinnamon powder was obtained from local market and approved by Medicinal Plant Center-Baghdad, Iraq.

Sample Collection and Preparation

After 12 h fasting, blood samples were collected from all subjects by venepunture (10 ml), before starting drug treatment (as baseline samples) and then after 6 and 12 weeks of treatment to follow the changes in the studied parameters.

Blood samples were divided into two tubes, one heparinized tube (1 ml of whole blood used for HbAlc determination) and the other part was collected in plain tube, then centrifuged at 3000 rpm for 10 min at 4°C. after centrifugation and isolation of cellular fraction; the obtained plasma fraction was divided into three parts in Ependorff tubes and stored frozen until analysis performed.

Biochemical Assay Methods

Measurement of serum glucose level (FPG)

Serum glucose level was evaluated using a ready- made kit for this purpose, according to the method of Barham and Trendoer [16], which is based on enzymatic oxidation of glucose to form glucuronic acid and hydrogen peroxide, and the reaction of the later with phenol and formation of quinonimine was followed spectrophotomertically at 505 nm. Results were expressed as mmol glucose/l, based on comparison with a standard glucose solution treated with the same method.

Determination of glycated HbAlc

The variant HbAlc program utilizes the principles of ion exchange high-performance liquid chromatography for the automatic and accurate separation of HbAlc. Prepared samples are automatically injected into analytical flow path and applied to the cation exchange column, where the Hb is separated, based on the attraction of Hb to the column material. The separated Hb then passes through the flow cell of the filter photometer, where changes in the absorbance at 415 nm are measured. Samples are required to hemolyze the blood and remove Schiff base. Samples are first diluted with hemolysis reagent and then incubated at 18-28°C for a minimum of 15 min [17].

Measurement of serum Malondialdehyde (MDA)

MDA is a by-product of lipid peroxidation and its measurement is based on the reaction of thiobarbituric acid (TBA) with MDA forming TBA-2 MDA adducts. According to the standard method of Stocks and Dormandy [18], which is modified by Gilbert *et al.* [19]. The method included the addition of 1.75 ml of saline azide to 0.25 ml plasma. Then, the mixture was centrifuged and 2 ml of supernatant was mixed with 0.5 ml of

H_2O and 0.5 ml of 1% TBA in 0.05 M NaOH. The mixture was incubated in a boiling water bath for 15 min to achieve color development. The tubes were cooled under tap water and the extent of MDA production was estimated from the absorbance at 532 nm. MDA concentration was calculated using a molar absortivity coefficient of 1.56×10^5/M/cm and the results were expressed as μmol MDA/l.

Measurement of serum glutathione (GSH) levels

GSH contents (measured as total sulfhydryl groups) were measured according to the method of Godin *et al*. [20]. 0.5 ml of serum was mixed with 206 ml of 3 mM DTNB prepared in 0.1 M phosphate buffer (pH 8). The yellowish color chromophor formed was measured spectrophotometrically at 412 nm during 2 min, and the concentration of GSH was calculated using a standard curve use for this purpose.

Measurement of serum superoxide dismutase (SOD) levels

SOD is one of the most important anti-oxidative enzymes. It catalyzes the dismutation of the superoxide anion into hydrogen peroxide and molecular oxygen. The sensitive SOD assay kit utilizes WST-1 that produces a water-soluble Formazan dye upon reduction with superoxide anion. The rate of the reduction with a superoxide anion is linearly related to the xanthine oxidase activity and is inhibited by SOD. Therefore, the inhibition activity of SOD can be determined by a colorimetric method. Detection method-absorbance (450 nm).

Statistical Analysis

The results were expressed as a mean±standard deviation. Student's *t*-test for paired and unpaired sample and ANOVA test was used to examine the degree of significance, $P < 0.05$ considered significant and less than 0.001 considered highly significant.

RESULTS

Administration of cinnamon 1000 mg to diabetic patients resulted in highly significant ($P \leq 0.001$) reduction (10.12%) of fasting blood glucose level after 6 weeks of treatment compared to baseline value; the reduction in fasting blood glucose level was (17.4%) after 12 weeks of treatment which was also highly significantly ($P \leq 0.001$) compared to baseline value and to placebo group at corresponding duration [Figure 1].

At the same time periods, the value of glycosylated Hb reduced in cinnamon treated group by (2.625%) and (8.25%) after 6 and 12 weeks respectively, although this reduction was non-significant compared to baseline value, but it was in line with that of fasting blood glucose [Figure 2]; and both changes give clear indication about the glucose lowering effect of cinnamon in type 2 diabetic patients.

The level of serum glutathione, the natural antioxidant, increased significantly ($P \leq 0.05$) after 6 weeks in both placebo and cinnamon treated groups compared to baseline value, while only cinnamon treated group showed highly significant ($P \leq 0.001$) elevation after 12 weeks as compared to baseline value and in comparison to placebo group at corresponding duration [Figure 3].

Concerning the MDA serum level, treatment of diabetic patients with cinnamon resulted in highly significant ($P \leq 0.001$)

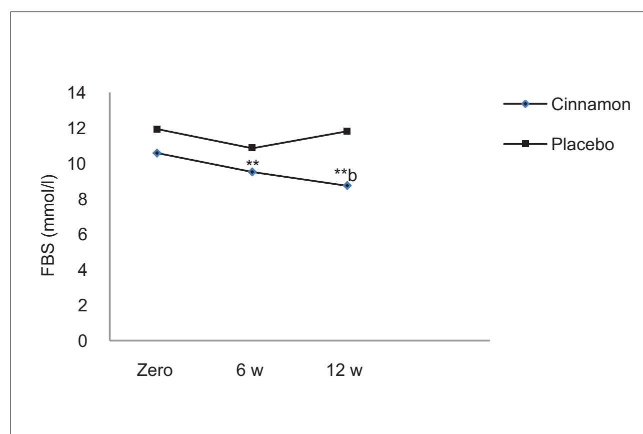

Figure 1: Effect of 1 g cinnamon powder on fasting blood glucose in diabetic patients. **Highly significant difference from baseline ($P < 0.001$). [b]Highly significant difference ($P < 0.001$) between cinnamon group and placebo group at corresponding duration

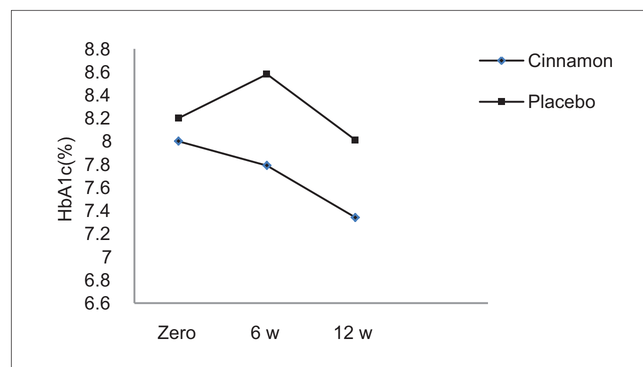

Figure 2: Effect of 1 g cinnamon powder on hemoglobin A1c % in diabetic patients

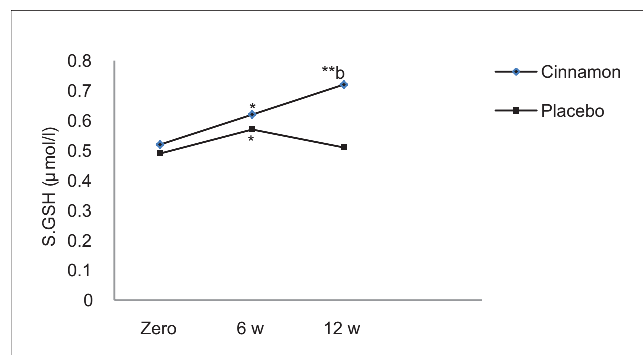

Figure 3: Effect of 1 g cinnamon powder on reduced glutathione serum level in diabetic patients. *Significant difference from baseline ($P < 0.05$), **Highly significant difference from baseline ($P < 0.001$). [b]Highly significant difference ($P < 0.001$) between cinnamon group and placebo group at corresponding duration

reduction after 6 and 12 weeks compared to placebo group, but when compared to baseline value, there is a (15%) reduction only after 12 weeks of treatment which was considered highly significant ($P \leq 0.001$) change [Figure 4].

Finally, results of this study showed that there was significant ($P \leq 0.05$) difference in the baseline level of serum SOD between placebo and cinnamon treated groups, in spite of that, administration of cinnamon to diabetic patients for 12 weeks resulted in significant ($P \leq 0.05$) elevation of SOD level [Figure 5].

DISCUSSION

This clinical trial demonstrates the hypoglycemic and antioxidant effects of 500 mg cinnamon powder twice daily in type 2 diabetic patients, the used cinnamon dose was tolerated well by patients, and there was no compliance. Khan *et al.* (2003) showed that administration of 1, 3 and 6 g per day of cinnamon improve blood glucose and lipid profile in type 2 diabetic people [21], and suggest that using cinnamon with a diet of diabetics may reduce incidence of risk factors associated with diabetes and cardiovascular diseases. Mang *et al.* demonstrated that administration of 3 g of cinnamon aqueous extract a day to type 2 diabetic

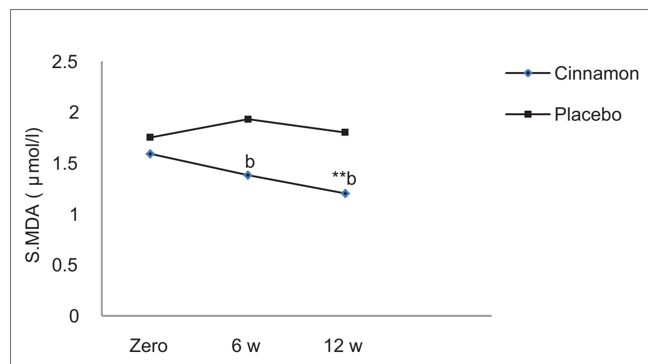

Figure 4: Effect of 1 g cinnamon powder on malondialdehyde serum level in diabetic patients. **Highly significant difference from baseline ($P < 0.001$). [b]Highly significant difference ($P < 0.001$) between cinnamon group and placebo group at corresponding duration

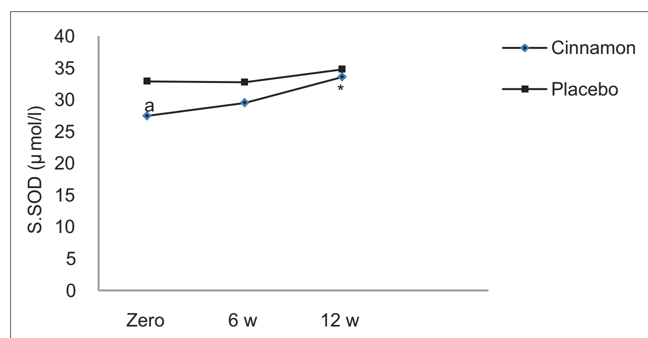

Figure 5: Effect of 1 g cinnamon powder on superoxide dismutase serum level in diabetic patients. *Significant difference from baseline ($P < 0.05$), [a]Significant difference ($P < 0.05$) between cinnamon group and placebo group at corresponding duration

patients on oral hypoglycaemic treatment resulted in significant reduction of the initial FPG values. However, the trial failed to demonstrate a significant lowering of HbA1C or plasma lipids [9]. Crawford showed that administration of 1g of cinnamon to diabetic patients for 90 days lowered the HbA1c by 0.83% as compared to 0.37% reduction in patients receiving usual care alone [22]. In a randomized, placebo-controlled double-blind clinical trial Akilen *et al.* studied the effect of cinnamon on diabetics on oral hypoglycemics by administering 2 g of cinnamon daily over a period of 12-week. The results demonstrated a significant reduction in HbA1c level. The study also demonstrated a significant reduction in blood pressure, FPG, body mass index and waist circumference at 12 weeks of treatment [23]. Other study done by Suppapitiporn showed the effect of cinnamon *cassia* powder in type 2 diabetics on oral therapy consisting of metformin or sulphonylurea in randomized, placebo-controlled clinical trial. After a 12-week period, HbA1c was decreased nonsignificantly in treated patients [24]. Recently, Anderson *et al.* found that diabetic individuals received cinnamon 500 mg/day for 2 months showing a significant decrease of both FPG compared the placebo control group, insulin sensitivity, assessed by homeostasis model assessment-insulin resistance, was also significantly improved by administration of cinnamon extract [25].

In this study, there is a highly significant reduction in fasting glucose level after 6 and 12 weeks of cinnamon use indicating the good glucose lowering effect of cinnamon powder while in placebo group there is no reduction a result which corroborate cinnamon effect. Many studies investigated the mechanism(s) of cinnamon as hypoglycemic agents, as reviewed by Medagama (2015); it may be due to its action at different levels of the insulin-signaling pathway [26]. Besides its action on insulin receptor phosphorylation and translocation of glucose transporter GLUT4 to the plasma membrane as mentioned above, studies showed that cinnamon administration increases the level glucagon-like peptide 1 [27,28]. Furthermore, cinnamon may increase the expression of PPAR and increasing insulin sensitivity [13]; Adisakwattana *et al.*, showed that cinnamon may be useful for the control of blood glucose level in diabetic patients through inhibition of intestinal α-glucosidase [14]; Anand *et al.* demonstrated that cinnamon inhibits gluconeogenesis and stimulate glycogen synthesis thus, improving glucose metabolism [29]; finally, cinnamon may delay the gastric emptying and caused reduction in post-prandial blood glucose [15].

Oxidative stress is one of the important factors in diabetes that plays an important role in vascular complications [30], evaluation of such changes markers may enable to determine the optimum time of targeting these changes. Nowadays, natural antioxidants are considered the preferred choice for the replacement of synthetic ones; these natural antioxidants can be formulated as food stuffs and can help prevent oxidative damage occur due to many diseases including type 2 DM [31]. It has been found that polyphenols, the natural dietary antioxidants found in cinnamon have been shown to reduce oxidative stress in a dose-dependent manner. Specific antioxidant

phytochemicals that have been identified in cinnamon include epicatechin, camphene, eugenol, gamma-terpinene, phenol, salicylic acid, and tannins [10].

Rao and Gan reviewed the antioxidant effects of cinnamon; they reported that cinnamon increased GSH level, also increase the activity of SOD [32]. In recent study, and Saifan showed that administration of cinnamon aqueous extract to obese diabetic rat resulted in improvement of activity of tissue antioxidant enzymes [33]. Finally, Roussel *et al*. show that administration of 250 mg of an aqueous extract of cinnamon 2 times per day for 12 weeks to subjects with impaired fasting blood glucose resulted in increasing the level of plasma thiol group while plasma MDA level decreased compared to that of placebo, they found that antioxidant effects were larger after 12 than 6 weeks [34]. The results obtained in this study showed that there is significant improvement in oxidative stress markers after 6 weeks, while highly significant increase in GSH level of diabetic patients after 12 weeks of cinnamon administration, at the same time, plasma MDA level highly significantly decreased after 12 weeks indicating the antioxidant effect of cinnamon in time-dependent manner.

CONCLUSION

In conclusion, administration of 1 g of cinnamon powder for 12 weeks reduces fasting blood glucose and glycosylated Hb among poorly controlled type 2 diabetes patients, as well as, increase the level of serum glutathione and SOD while reduces serum level of MDA, indicating the beneficial effect of adjuvant cinnamon as antidiabetic and antioxidant along with conventional medications to treat poorly controlled T2DM.

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Original Research

Nematicidal and antimicrobial activities of methanol extracts of 17 plants, of importance in ethnopharmacology, obtained from the Arabian Peninsula

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ABSTRACT

Aim/Background: The development of resistance to synthetic drugs by target organisms is a major challenge facing medicine, yet locked within plants are phytochemicals used in herbal medicine (especially in the Arabian Peninsula) that may find application in this regard. In pursuit of unlocking these "hidden treasures," the methanol extracts of leaves, aerial parts, fruits, and resins of 17 plants used in the Arabian Peninsula were screened for antimicrobial activities. **Materials and Methods:** The nematicidal, antibacterial, and antifungal activities were determined using appropriate assays. *Steinernema feltiae*, *Staphylococcus carnosus*, *Escherichia coli*, and *Saccharomyces cerevisiae* were used as test organisms. Concentrations of the extracts ranging from 0.5 to 20 mg/ml were tested and appropriate statistical tests performed on the data generated. **Results:** The results show that extracts from *Solanum incanum*, *Chenopodium murale*, *Commiphora myrrha*, *Anthemis nobilis*, and *Achillea biebersteinii* were the most active and had very high activities against two or more of the test organisms at low concentrations. Extracts of the leaves of *S. incanum* and resins of *Ferula asafoetida* were the most active nematicides, with significant activity at 0.5 mg/ml. Extracts of *C. myrrha* and *C. murale* had the most active antibacterial activity with inhibition zones of 12-15 mm and minimum inhibitory concentrations (MICs) of 2.5 mg/ml for both bacteria. Extracts of the leaves of *A. biebersteinii* were the most active fungicide, giving an MIC of 1.5 mg/ml. **Conclusion:** The results validate the use of these plants in ethnopharmacology, and open new vistas of opportunities for the development of cheap but effective agents that may be useful against infectious diseases.

KEY WORDS: Antimicrobials, *Commiphora myrrha*, medicinal plants, nematicides, phytochemicals, *Solanum incanum*

INTRODUCTION

Although significant progress has been achieved during the last 50 years in fighting infectious diseases, they still remain an important cause of morbidity and mortality globally [1]. Infections cause an estimated 50% of all deaths in tropical countries, where as much as three million preschool children die each year solely due to infections of the gastrointestinal tract [2].

Besides bacteria and fungi, nematodes transmitted from the soil cause diseases which affect 25% of the world's population, again mostly in the tropics. They are known to lead to anemia and to cause retarded physical and mental growth [3,4]. The negative effects of nematodes on agricultural livestock are also well-documented [5,6]. As for bacteria enteropathogenic strains of Gram-negative *Escherichia coli* are known to cause acute and chronic diarrhea, vomiting, and fever in infants [7]. The Gram-positive bacterium *Staphylococcus aureus* can multiply and spread widely in tissues resulting in enteric infections, boils, skin sepsis, endocarditis, and pneumonia. Their heat-stable endotoxins cause diarrhea, fever, abdominal cramps, and vomiting with an attendant electrolyte imbalance [8]. Owing to their ability to thrive better in warm, humid environments, fungal infections are equally problematic and more rampant in the tropics and sub-tropics than any other place in the world. They cause diseases ranging from superficial mycoses, cutaneous mycoses, sub-cutaneous mycoses, and systemic mycoses; and are usually very difficult to treat [9]. These organisms also cause diseases in domestic and farm animals resulting in massive economic losses.

Unfortunately, many drugs currently available for the treatment of infections are expensive and often not readily available or are easily counterfeited. Furthermore, the development of resistances to these drugs is a major setback to their continued use in humans and livestock [10-12].

Interestingly, the tropics where most of these infections are rampant are also amazingly rich in a diversity of plants and fungi. Given the WHO report that medicines derived from plants serve the health needs of approximately 80% of people globally [13], it is important to screen plants that are used in ethnopharmacology and ethnomedicine for activities against nematodes, bacteria, and fungi. Such plants may provide new and, above all, inexpensive and locally available drugs and improve the health of people in economically under-developed or developing countries.

In the light of the above, the nematicidal and antimicrobial properties of methanol extracts of 17 plants used in ethnopharmacology and ethnomedicine around the tropics and sub-tropics, and particularly in Saudi Arabia and Yemen were investigated [Figure 1]. The primary aim of this investigation has been to uncover phytochemical products that can be produced locally and in sufficient commercial quantities to be used in improving Medicine and Agriculture, especially in some of the developing economies of the world. Details of the plants, the parts harvested and their uses in folk medicine have been obtained from published literature, and traditional users of the plants [14-16] are summarized in Table 1.

MATERIALS AND METHODS

Plant Materials

The plant materials were collected between the months of March and April 2014 at different locations in Al Baha town, and its outskirts, Saudi Arabia. *Dendrosicyos socotrana* and *Dracaeana cinnabari* were collected from the island of Socotra between November and December 2014. Those plants were identified taxonomically at the Department of Botany, Faculty of Science, Aden University, Republic of Yemen. Voucher specimens of the plant materials were deposited at the Pharmacognosy Department, Faculty of Clinical Pharmacy, Al Baha University, Saudi Arabia for the Saudi plants and at the Department of Botany, Faculty of Science, Aden University, Yemen for *D. socotrana* and *D. cinnabari*.

Preparation of Plant Extracts

The plant parts harvested were air-dried under the shade at ambient temperature and powdered with a blender. The powdered plant material (10 g) was extracted with absolute methanol (4 × 100 ml). The extractions were carried out at room temperature with the constant shaking of the extraction set-up. Thereafter, the mixtures were filtered, and the filtrate

Figure 1: Map of Arabian Peninsula indicating the regions of plant collection

Table 1: Medicinal plants selected as part of this study, ethnobotanical information and relevant characteristics

Species	Plant family (voucher no)	Part tested (yield in %)	Local name	Traditional uses
Achillea biebersteinii Afan.	Asteraceae (CP-101)	Fl (1.5)	Thafra	Antispasmodic, and for[1] kidney inflammation
Achillea biebersteinii Afan.	Asteraceae (CP-102)	L (2.6)	Thafra	As antispasmodic, and for inflammation
Calotropis procera (Aiton) W.T. Aiton	Asclepiadaceae (CP-091)	L (4.2)	Alashur	For treating leprosy and filariasis
Chenopodium murale L.	Amaranthaceae (CP-081)	F (7.3)	Jkheara	Leishmaniasis[1]
Dendrosicyos socotrana Balf.f.	Cucurbitaceae (CP-14-1)	L (4.3)	Al-kheyar	Severe constipation[2]
Dodonaea viscosa Jacq.	Sapindaceae (CP-061)	L (3.5)	Shath	For treating chronic ulcer, burns, leishmaniasis[2]
Dracaena cinnabari Balf.f.	Asparagaceae (CP-17-1)	Re (3.5)	Dam Al-akhween	Antispasmodic, wound healing[2]
Euphorbia helioscopia L.	Euphorbiaceae (CP051)	AP (4.2)	Al-dehin	Antiseptic
Lavandula dentata L.	Lamiaceae (CP-041)	AP (2.9)	Al-shiah	As antispasmodic, antiseptic when the leaves are chewed[1]
Pulicaria crispa SCH.BIP	Asteraceae (CP-102)	AP (3.1)	Arararabi	Antimalarial, stomach disorders[2]
Punica granatum L.	Punicaceae (CP-011)	Fl (2.5)	Al-roman	Anthelmintic, antiseptic[2]
Ruta chalepensis L.	Rutaceae (CP-121)	L (5.2)	Al-shathab	Antimicrobial[2]
Solanum incanum L.	Solanaceae (CP-131)	F (7.6)	al-hadak	Antiseptic[2]
Solanum incanum L.	Solanaceae (CP-132)	L (3.9)	Al-hadak	Wounds, paste from fruits for treating leishmaniasis[2]
Verbesina encelioides (Cav.) Benth and Hook. F. ex a. Gray	Asteraceae (CP-021)	L (3.7)	Safeara	Wounds, skin diseases[2]
Withania somnifera (L.) Dunal	Solanaceae (CP-011)	F (4.6)	Alobeb	Chronic dermatitis[2]
Withania somnifera	Solanaceae (CP 012)	L (8.5)	Alobeb	Chronic dermatitis[2]

AP, aerial parts, F: fruits, L: leaves, Re: resins, Fl: Flowers, [1]Information obtained from Ali *et al.*,1999, Ali *et al.*, 2001, Fatimi *et al.*, 2005, Gashash 2012 and [2]interviewing with local people

evaporated to dryness *in vacuo* at 40°C to yield the methanol extracts subsequently used as a part of our studies. The yields of each dried extract were calculated in %. The resulting dried crude extracts were stored at 4°C until they were analyzed for nematicidal and antimicrobial properties.

Nematicidal Activity

Steinernema feltiae was purchased from Sautter and Stepper GmbH (Ammerbuch, Germany), as a powder cake product and stored in the dark at 4°C. Fresh samples were ordered before each experiment, and each opened batch was discarded after 6 days. Prior to each experiment, a homogeneous mixture of nematodes was prepared by suspending 200 mg of powder cake in 50 ml of distilled water at 27°C to revive the nematodes. Contained therein, the suspension was allowed to stand at room temperature though with occasional rocking and in moderate light for 30 min. Thereafter, the viability of the nematodes in suspension was determined with a microscope at four-fold magnification (TR 200, VWR International, Belgium). A viability of more than 80% was considered optimal and seen as a prerequisite for each experiment.

Each plant extract (100 mg) was dissolved in 5 ml of 2% DMSO to yield a 20 mg/ml stock solution. From this stock solution, a series of dilutions in water was prepared with 0.5, 1, 3, 5, 10, and 15 mg/ml solutions which were then used for the experiments. To each well in the 96-well plate, 10 µl of the nematode suspension was added (which usually contains 30-40 nematodes per well). Thereafter, 100 µl of each concentration of the plant extracts was added to each well. The control experiment was performed with the DMSO/water vehicle in place of the extracts. The well plates were then assessed immediately for viability under the microscope before incubation in the dark at room temperature for 24 h. After 24 h, 50 µl of distilled water at 50°C was added to each well to stimulate the movement of the nematodes. Thereafter, live and dead nematodes were counted under the microscope (magnification × 4). Each concentration was tested in three different wells per experiment, and each experiment was repeated three times to yield a total of nine repeats per individual experiment.

The viability of the nematodes was expressed as percentages. The viability values were calculated using the equation:

$$\text{Viability } (\%) = \left[\frac{V_{24h}}{V_{0h}}\right] \times 100$$

Where, $V_{24\,h}$ is number of live nematodes after 24 h and $V_{0\,h}$ is the number of live nematodes at 0 h.

Antimicrobial Activity

Two bacterial strains, *Staphylococcus carnosus* TM 300 and *E. coli* K2 (representing Gram-positive and Gram-negative bacteria species, respectively) as well as the fungus *Saccharomyces cerevisiae* were used as representative model organisms for the antimicrobial investigations.

The disc diffusion assay [17] was used to determine antimicrobial activities of the extracts investigated. Nutrient Luria-Bertani and Yeast Extract-Peptone-Dextrose (YPD) (Sigma-Aldrich, Steinheim, Germany) were used as media. Sterile plates were inoculated evenly using sterile swab sticks. Sterile qualitative filter paper discs of 6 mm diameter (VWR International GmbH-Darmstadt, Deutschland, ref. No. 601110, lot. 06513) were impregnated with 20 µl of each extract solution (equivalent to 4 mg/disc). The paper discs were allowed to dry

before being gently placed on the surface of the inoculated agar plates, at positions that were equidistant from each disc. The plates were kept for 3 h in a refrigerator to enable pre-diffusion of the substances into the media. A mixture of Penicillin-Streptomycin-Amphotericin-B was used as positive control while the solvent (methanol) was used as negative control. Plates inoculated with bacteria and yeasts were incubated for 18-24 h at 37°C. Inhibition zone diameters around each disc (diameter of inhibition zone plus diameter of the disc) were measured and recorded at the end of the incubation time [18]. An average zone of inhibition was calculated for the three replicates.

The MIC was determined using the broth microdilution method of Mann and Markham [19], with slight modifications. Fresh cultures of bacteria on LB agar and yeast on YPD agar were prepared and incubated for 18-24 h. From these cultures, inocula were prepared by suspending colonies of the respective organisms in sterile 0.85% NaCl solution and then adjusted to 0.5 of the McFarland standard (1.5×10^8 CFU/ml for bacteria and 1.5×10^6 CFU/ml for yeast). Different concentrations (0.5, 1.0, 2.5, 5.0, and 10 mg/ml) of the plant extracts were added to the LB or YPD broth in 96-well plates, and the inocula were subsequently added to each well. Thereafter, the plates were incubated at 37°C for 18-24 h. Antibacterial activity was detected by adding 20 µL of 0.01% sodium resazurin (Sigma) and incubating the plates for 1 h. A change from blue to pink indicates a reduction of resazurin and, therefore, bacterial growth. The assay was conducted in triplicate, and three independent experiments were performed on different occasions. The MIC value was defined as the minimum concentration of test sample that inhibited the selected organism's growth, i.e., at which no growth was observed. The reference antibiotics used were penicillin-streptomycin-amphotericin B (Sigma-Aldrich, Steinheim, Germany).

Statistics

The data generated was subjected to descriptive statistical analysis and the results are presented as mean ± standard error of mean. Differences between the means (test versus control) were assessed for statistical significance using the one-way ANOVA test with the significance threshold fixed at $P < 0.05$. The GraphPad Prism software (GraphPad Inc., USA) was used for all statistical analyzes. The results are presented in Tables 1-3 and Figures 1-3 and statistical significances in the Figures 1-3 are marked as *, **, or *** when the $P < 0.05$, < 0.01 or < 0.001, respectively.

RESULTS

For all plant materials under investigation, suitable extracts could be obtained in good quality and yield. Table 1 briefly summarizes the individual yields of extraction for the different extracts. It should be emphasized that the fruits of *Withania somnifera* yielded the most extract (8.5%) while the leaves of *Achillea biebersteinii* yielded the least (2.6%) [Table 1]. The average yield was approximately 5%. The extracts obtained were subsequently investigated for biological activity, first against the nematode *S. feltiae*, and subsequently against different bacteria and the fungus *S. cerevisiae*.

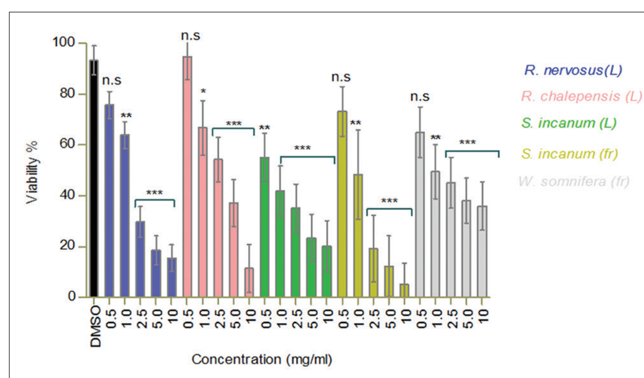

Figure 2: Nematicidal activity of the most active plant extracts against the model nematode *S. feltiae*

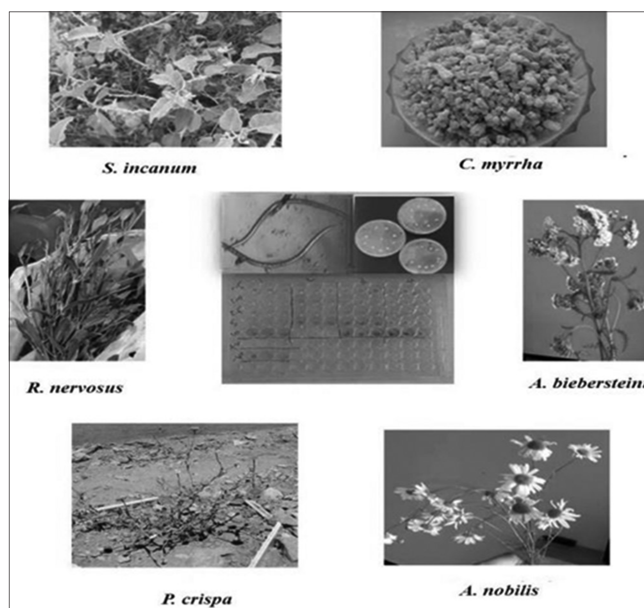

Figure 3: The plants which exhibited the highest toxicity against the selected microorganisms

Nematicidal Activity

In the case of nematodes, a distinctively different activity could be observed for the methanol extracts of aerial parts of *Chenopodium murale*, *Pulicaria* crispa, *Euphorbia helioscopia*, and *Lavandula dentata*, leaves of *Dodonaea* viscosa, *Verbesina encelioides*, and *A. biebersteinii*; leaves of *W. somnifera*, *Calotropis procera*, *D. socotrana*, *Rumex nervosus*, *Ruta chalepensis*, and *Solanum incanum*; resins of *C. myrrha* and *D. cinnabari*; fruits of *S. incanum* and *W. somnifera*; flowers of *Punica granatum*, *A. biebersteinii*, and *Anthemis nobilis*. These activities are summarized in Table 2.

While some extracts showed considerable activity against *S. feltiae*, others were hardly active. The extract from the leaves of *S. incanum* was the most active as it resulted in statistically significant mortality of the nematodes at the

lowest concentration tested (0.5 mg/ml) [Figure 2]. Purely for comparison: this concentration corresponds to 2 mM of a chemically pure compound with a molecular weight of 250 g/mol. The next most active extracts in order of activity were those from *S. incanum* and *W. somnifera* fruits, *R. nervosus* leaves, *P. crispa* aerial parts, and resins of *C. myrrha*, each showing statistically significant nematicidal activity at a concentration of 1 mg/ml. This was followed by extracts from *E. helioscopia*, *D. viscosa*, *A. biebersteinii*, *P. granatum*, *D. socotrana*, and *D. cinnabari*, each with statistically significant nematicidal activity at a concentration of 2.5 mg/ml. In contrast, extracts from *C. murale* (10 mg/ml), *L. dentata* (10 mg/ml), and *C. procera* (20 mg/ml) were hardly effective as nematicides.

Table 2: Nematicidal activity of plant extracts against ***S. feltiae***

Species	Concentration of extract (mg/ml)				
	0.5	1.0	2.5	5.0	10.0
Achillea biebersteinii	-	-	**	**	***
Anthemis nobilis	-	-	-	*	***
Calotropis procera	-	-	-	-	-
Chenopodium murale	-	-	-	-	**
Commiphora myrrh	-	*	**	***	***
Dendrosicyos socotrana	-	-	-	*	**
Dodonaea viscosa	-	-	**	***	***
Dracaena cinnabari	-	-	*	**	***
Euphorbia helioscopia	-	-	***	***	***
Lavandula dentata	-	-	-	-	***
Pulicaria crispa	-	*	**	***	***
Punica granatum	-	-	*	**	***
Rumex nervosus	-	**	***	***	***
Ruta chalepensis	-	*	***	***	***
Solanum incanum	***	***	***	***	***
Solanum incanum (fruits)	-	**	***	***	***
Verbesina encelioides	-	-	-	**	**
Withania somnifera	nt	-	-	*	***
Withania somnifera (fruits)	-	**	***	***	***

nt stands for "not tested;" *, **, and *** show significant differences at $P<0.05$, <0.01 and <0.001, respectively *S. feltiae: Steinernema feltiae*

Antimicrobial Activity

The extracts were then tested for their activity against two bacteria, *S. carnosus* and *E. coli*, which are representative of Gram-positive and Gram-negative bacteria, respectively. An activity profile similar to one in nematodes could be observed. In the case of *S. carnosus*, the extract from the fruits of *S. incanum*, but also extracts of the resins of *D. cinnabari* and *C. myrrha* and the aerial parts of *E. helioscopia* resulted in high zones of inhibition and low MIC values against this strain of *Staphylococcus*. *E. coli* appeared to be even more sensitive against a wider range of extracts, such as extracts obtained from the leaves of *C. murale*, *S. incanum*, *D. socotrana*, *A. biebersteinii*, and *V. encelioides*, from the flowers of *P. granatum* and *A. nobilis*, the resins of *C. myrrha*, and the aerial parts of *P. crispa*. All of these extracts resulted in high zones of inhibition and low MIC values against *E. coli*. Ultimately, extracts from the resins *C. myrrha*, the leaves of *D. socotrana* and *S. incanum*, and from the flowers of *A. nobilis*, provided the best antibacterial activity for both Gram-positive and negative bacteria [Table 3].

A very similar picture also emerged with the single-cell fungus *S. cerevisiae* [Table 3]. Since the broth microdilution method is known to be more reliable and useful in testing plant extracts for activity [19], we have collected data via both, the disc diffusion and the microdilution method. Indeed, in our hands, the latter was also more reliable with regard to toxicity against *S. cerevisiae*. Extracts of the leaves of *A. biebersteinii*, *C. murale*, and *R. chalepensis*, of the aerial parts of *P. crispa*, of the flowers of *A. nobilis* and of the fruits of *S. incanum* inhibited the growth of *S. cerevisiae* at low concentrations. Other extracts such as the

Table 3: Antimicrobial activity of plant extracts against ***S. carnosus*** and ***E. coli***

Botanical name	Inhibition zones (mm)			MIC (mg/ml)		
	S. carnosus	*E. coli*	*S. carnosus*	*S. carnosus*	*E. coli*	*S. carnosus*
Achillea biebersteinii	8±2	13±4	-	4.0	2.5	1.5
Anthemis nobilis	8±3	15±4	-	4.0	2.5	2.5
Calotropis procera	-	-	-	-	-	-
Chenopodium murale	15±5	13±4	-	2.5	2.5	2.5
Commiphora myrrha	12±3	13±6	-	2.5	2.5	-
Dendrosicyos socotrana	8±2	12±2	-	4.0	2.5	5.0
Dodonaea viscosa	-	-	-	-	-	-
Dracaena cinnabari	13±4	15±2	-	2.5	4.0	-
Euphorbia helioscopia	12±2	15±3	-	2.5	2.5	-
Lavandula dentata	-	-	-	-	-	10.0
Pulicaria crispa	—	11±4	-	-	2.5	2.5
Punica granatum	8±2	6±1	-	4.0	2.5	5.0
Rumex nervosus	-	5±1	-	-	4	5.0
Ruta chalepensis	-	15±3	-	-	2.5	2.5
Solanum incanum	8±2	13±4	-	4.0	2.5	10.0
Solanum incanum (fruits)	12±2	15±4	-	2.5	2.5	2.5
Verbesina encelioides	-	11±2	-	-	2.5	-
Withania somnifera	-	10±2	-	-	4.0	5.0
Withania somnifera (fruits)	-	10±3	-	-	4.0	-
6 mg penicillin, 6 mg streptomycin and 25 μg amphotericin B	30±1	30±2	-	0.01	0.01	0.03

S. cerevisiae, E. coli and *S. cerevisiae* represent *Staphylococcus carnosus* TM 300; *Escherichia coli* K2; and *Saccharomyces cerevisiae*; Negative control did not show any activity

ones of the leaves of *R. nervosus* and *W. somnifera* and the flowers of *P. granatum* also inhibited the growth of *S. cerevisiae*, yet to a lesser extent. The extracts from the other plants showed little or no effect with respect to the growth of yeast.

Overall, extracts from 14 of the 17 plants under investigation exhibited high activity against at least one organism tested while the other three plants, *D. viscosa*, *C. procera*, and *L. dentata* showed only limited activities against all of the organisms tested. Among the active plants, *S. incanum* clearly attracted the most attention. Intriguingly, extracts of the fruits of *S. incanum* appeared to possess high activity against all four organisms tested, while the leaves of this plant had limited activity against *S. cerevisiae* and *S. carnosus*. Preparations from other plants were also rather active such as extracts of the aerial parts of *P. crispa* and extracts of the resins of *C. myrrha*, which yielded high activities against three of the four organisms tested. Less active but still of interest due to possible harvesting and processing where the flowers of *A. nobilis* and leaves of *A. biebersteinii*, which showed high activities against two of the four organisms tested.

DISCUSSION

Although synthetic nematicides and antimicrobials used in Medicine and Agriculture are effective and rapid-acting, the challenges of resistance to these agents by microorganisms, and the concerns to human health and the environment raised by their use in Agriculture, have spurred research efforts at developing "green" plant-based or plant derived alternatives. In fact, various phytochemicals are known to be safe to both humans and the environment. When used in Agriculture, as in the case of nematicides, they are "biodegradable" and usually do not persist in the fields for longer periods of time than is really necessary [20]. Naturally, therefore, research efforts have been geared toward plants that are used in Folk Medicine and Agriculture from different cultures, with a view to identifying those that can be used to develop "green" phyto-protectants, antimicrobials, and pesticides. Many of these plants contain a cocktail of phytochemicals, true treasure chests for bio-activity against the myriad of microorganisms that pose challenges to Medicine and Agriculture, especially in developing countries of the tropics.

Our study has, therefore, investigated the nematicidal and antimicrobial properties of methanol extracts of leaves, aerial parts, and resins from 17 plants used in traditional ethnopharmacology and ethnomedicine in Saudi Arabia, Yemen and neighboring countries of the Arabian Peninsula. Deliberately, methanol was used as the solvent of extraction based on our experience over the years in working with plant products and reports from other researchers working on similar subjects [21]. Indeed, several studies have shown that methanol is the solvent of choice for the extraction of antimicrobial constituents of plants [22-25]. Compared to ethanol, methanol is also less controversial culturally.

Interestingly, extracts from five of the plants studied [Figure 3] - namely *S. incaum*, *P. crispa*, *C. myrrha*, *A. biestersteinii*, and *A. nobilis* - exhibited high activities at low concentrations against two or more of the organisms tested. *S. incanum*, in particular, which is also known as Jericho tomato, attracted our particular interest, as several parts of this plant seem to be extraordinarily toxic. Though there are reports of toxicity of the plant, such reports should spur on research especially as the plant is particularly promising for several reasons. First, it grows readily, widely and requires little care. Second, it can be harvested and processed easily. Third, it is not used for any other purposes, hence has little "value." Finally, its extract is amenable to further purification and/or modification, which may improve activity significantly. Indeed, the literature available to date on the most active plants show that *S. incanum* is rich in phytochemicals such as incanumine, solasodine, carpesterol, β-sitosterol, stigmasterol, and khasianine [22]. Lin *et al*. [26] also reported the presence of quercetin, kaempferol, and astragalin in parts of the plant. Besides, members of the *Solanaceae* family have been known for a long time to possess antibiotic activity, which is likely due to the presence of glycosides and alkaloids [27].

Ultimately, a partially purified, stabilized, and appropriately conserved extract of the fruits and/or leaves of the Jericho tomato may well be suitable for applications in the field of Agriculture and possibly also Medicine. Within this context, possible toxic effects on humans and higher animals need to be considered and addressed in earnest, and the possibility of synergism in the action of the individual chemical components contained within the different extracts may have to be accounted for.

Indeed, the activity observed for extracts of *S. incanum*, and the various other extracts may arise from a variety of chemical components and biochemical mechanisms. Though we do not currently possess data on the mechanisms of action, it is known, for instance, that plant extracts often exert their lethal effects through the disruption of cell membrane permeability in organisms that come in contact with them [28]. Within this context, variations observed in the effectiveness of the extracts in killing the nematodes, bacteria, or fungi may indeed be explained by the biological differences which exist between the organisms, for instance, differences in cell wall structure and composition. In fact, it has been reported in other studies, and corroborated by this study, that plant extracts often show a higher activity against bacteria compared to fungi, and this may, in part, be due to differences in the cell wall synthesis and structure [25,29,30]. Specific phytochemicals, such as tannins, furthermore have the capacity to bind to and subsequently denature or disrupt proteins, and if such proteins are vital structural or catabolic proteins, would result in the death of the organism [31-33]. There are obviously many other possible mechanisms and mode(s) of action associated with the plethora of phytochemicals found in those plants.

While we cannot list all of the ingredients contained within our most active extracts and their suspected mode(s) of action, it is worth mentioning a few. Many potent phytochemicals have been found in *P. crispa*, especially sesquiterpene lactones and guaianolide sesquiterpenes [32,33]. Possibly, the *in vitro* antimicrobial activity and known anti-leishmanicidal activities

of the methanol extract of this particular plant are due to the presence of these phytochemicals [34,35]. Similarly, the genus *Commiphora* in general and *C. myrrha*, in particular, is a true hot(s) pot of biologically active secondary metabolites, with more than 300 of them identified and many of them associated with a pronounced activity against a variety of different microorganisms [36,37]. Those include flavonoids, alkaloids, tannins, glycosides, steroids, saponins, and terpenoids, and among them biologically highly active molecules such as myrracadinol A, B, and C, and myrracalamene A, B, and C, and triacont-1-ene [37]. Similarly, flowers of *A. nobilis*, a plant referred to in German language as "Alles zutraut," meaning "capable of anything" [38], have been used for a long time and are documented in more than 27 national pharmacopoeias. Indeed, this Chuck Norris of medicinal plants has been studied for centuries, and over a century ago, in 1914, Power and Jun [39]reported that the flowers contain essential oils, anthemene, anthemol, and anthesterol. More recently, the terpenoids: bisabolol, chamazulene, and sesquiterpenes; the flavonoids: apigenin, luteolin, and quercetin; and the coumarins: umbelliferone and scopoletin-7-glucoside, have been described as biologically active constituents of *A. nobilis*. Other active substances contained within that particular plant include angelic and tiglic acid esters, anthemic acid, choline, tannin, polysaccharides, phenolic, and fatty acids [40,41]. Finally, the extracts of *A. biebersteinii* contain large quantities of β-sitosterol, stigmasterol, sesquiterpene lactones, guaianolide, germacranolide, and flavonoids [42]. This list of phytochemicals for the five most active plants is clearly not exhaustive and is without prejudice to the apparent rich phytochemical constitution of the other ten plants that did show at least some activity against at least one of the organisms studied. Ultimately, it is, therefore, permissible to speculate that the nematicidal and antimicrobial activities observed in this study derive from the rich milieu of phytochemicals found in these very active plant extracts. These aspects now require further attention.

This study is limited by our inability to access a wider spectrum of organisms and test the activities of these extracts on them. It is hoped that these results will spur interest in these plants and provide the impetus to widen the screening and identification of the active constituents. Work on the latter is currently ongoing in our laboratory. Again, the nematicidal assays would have benefitted from a positive control. This would have afforded us a window to compare the data appropriately. We, however, feel that given the activity observed at concentrations as low as 1 mg/mL in these crude extracts, the purified extracts/fractions may have profound activity and testing them will necessarily require a positive control. In making the above assertion, we are mindful of interactions between individual constituents of plant extracts which may attenuate or accentuate the activity of their parent crude extract.

CONCLUSIONS

A future search for potent phytochemicals as an integral part of future "green" Medicine and Agriculture should consider the five plants identified by us as most active in more detail, with a particular focus on *S. incanum*. Isolating and characterizing the active compounds contained therein and elucidating the mode(s) of action will indeed be very important yet also challenging. It may also be useful to develop agents based on combinations of different extracts such as combined extracts of the fruits and leaves of *S. incanum*. Eventually, it may also be feasible to blend active phytochemicals from different plants such as *S. incanum* with *A. biebersteinii* and *R. nervosus* with *C. myrrha*. Moving on from natural products to synthetic chemistry, one may also envisage the design and development of synthetic analogues of the natural compounds, yet this task will be more challenging scientifically and also does not address the matter of local availability at a low cost. After all, the plants selected by us are readily available, easy to cultivate and harvest which renders their utilization cost effective in the Arabian Peninsula and parts of Africa.

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Original Research

Guava leaves polyphenolics-rich extract inhibits vital enzymes implicated in gout and hypertension *in vitro*

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ABSTRACT

Background/Aim: Elevated uric acid level, an index of gout resulting from the over-activity of xanthine oxidase (XO), increases the risk of developing hypertension. However, research has shown that plant-derived inhibitors of XO and angiotensin 1-converting enzyme (ACE), two enzymes implicated in gout and hypertension, respectively, can prevent or ameliorate both diseases, without noticeable side effects. Hence, this study characterized the polyphenolics composition of guava leaves extract and evaluated its inhibitory effect on XO and ACE *in vitro.* **Materials and Methods:** The polyphenolics (flavonoids and phenolic acids) were characterized using high-performance liquid chromatography (HPLC) coupled with diode array detection (DAD). The XO, ACE, and Fe^{2+}-induced lipid peroxidation inhibitory activities, and free radicals (2,2-diphenylpicrylhydrazyl [DPPH]* and 2,2´-azino-bis-3-ethylbenzthiazoline-6-sulphonic [ABTS]*+) scavenging activities of the extract were determined using spectrophotometric methods. **Results:** Flavonoids were present in the extract in the order of quercetin > kaempferol > catechin > quercitrin > rutin > luteolin > epicatechin; while phenolic acids were in the order of caffeic acid > chlorogenic acid > gallic acids. The extract effectively inhibited XO, ACE and Fe^{2+}-induced lipid peroxidation in a dose-dependent manner; having half-maximal inhibitory concentrations (IC_{50}) of 38.24 ± 2.32 μg/mL, 21.06 ± 2.04 μg/mL and 27.52 ± 1.72 μg/mL against XO, ACE and Fe^{2+}-induced lipid peroxidation, respectively. The extract also strongly scavenged DPPH* and ABTS*+. **Conclusion:** Guava leaves extract could serve as functional food for managing gout and hypertension and attenuating the oxidative stress associated with both diseases.

KEY WORDS: Angiotensin I-converting enzyme, Fe^{2+}-induced lipid peroxidation, oxidative stress, xanthine oxidase

INTRODUCTION

Gout and hypertension are two degenerative diseases that are capable of reducing the quality of life of individuals suffering from them. Research has shown that elevated uric acid level, an index of gout resulting from the over-activity of xanthine oxidase (XO), increases the risk of developing hypertension [1], indicating a link between gout and hypertension. In fact, hypertension is an established predictor of gout, in addition to other risk factors such as serum uric acid level and obesity [2]. The incidences of both diseases are increasing globally and both increases with advancing age. Whereas gout is a more prevalent in men over 30 years of age and in women above 50 years [3], hypertension is estimated to affect about 65% of the population in developed countries within the age group of 65-74 years [4]. It is projected that the number of adults with hypertension will increase to a total of 1.56 billion by the year 2025, which translates to about 60% increase [5]. The two diseases are degenerative in nature, being capable of reducing the quality of life of individuals suffering from them [5,6]. Hypertension is also known risk factor for other diseases, including arteriosclerosis, myocardial infarction, stroke, and end-stage renal disease, that also contribute to reduce the quality of life [5].

The level of uric acid in the cell depends on the activity of XO. XO catalyzes the terminal steps in the catabolism of purines in which hypoxanthine is converted to xanthine, and eventually to uric acid, during which molecular oxygen is reduced to superoxide, a reactive oxygen species (ROS) [1]. The over-activity of XO leads to hyperuricemia (increased concentration of uric acid), and the subsequent deposition of monosodium urate monohydrate crystals in tissue, especially joints, thereby resulting in gouty arthritis, or uric acid nephrolithiasis [3,7]. Hence, gout is a chronic inflammatory arthritis in which there is a high concentration of uric acid in body fluids, due to the over-activity of XO [8].

The activity of XO is linked with elevated arteriolar tone and consequently, hypertension [9]. Indeed, the activity of endothelial-bound XO rises by over 200% in patients with chronic heart failure [10]. The ROS produced by XO-catalyzed reactions are known to promote cardiovascular pathologies such as endothelial dysfunction, atherosclerosis, and hypertension [11]. The oxidative stress on endothelial cells resulting from the intracellular production of uric acid-derived radicals has the potential to activate the renin/angiotensin pathway, and consequently, the development of renal arteriolar disease [12]. In the rennin/angiotensin pathway, angiotensin 1-converting enzyme (ACE) (EC: 3.4.15.1) catalyzes the conversion of angiotensin I to angiotensin II, a known vasoconstrictor that increases blood pressure, by activating aldosterone secretion, and inactivating bradykinin, a vasodilator and hypotensive peptide [13]. ACE therefore plays a vital role in the regulation of blood pressure and normal cardiovascular function.

Thus, the inhibition of XO and ACE is an important strategy for the treatment and management of gout and hypertension, and this informs why chemically synthesized inhibitors of XO (including allopurinol) and ACE (including captopril, enalapril, and ramipril), are used clinically for the treatment of gout and hypertension, respectively [7,14]. Incidentally, synthetic inhibitors of XO and ACE both present with some adverse effects. The XO inhibitors are associated with the risk of developing hypersensitivity syndrome, characterized by side effects such as hepatic dysfunction, renal impairment, fever, rashes, and leukocytosis [15], whereas the ACE inhibitors have such adverse effects as skin rashes, cough, proteinuria, and hypotension [16]. However, research has shown that plant-derived inhibitors of both enzymes, including the polyphenolics, could be relatively safe and effective. Previous studies have also shown that several food and medicinal plants with a high level of flavonoids and other phenolic compounds are able to inhibit XO [17,18]; and ACE [19].

Guava (*Psidium guajava* Linn.), belonging to the family Myrtaceae, is found in the tropical and subtropical regions of the world including Nigeria. The plant has versatile applications ranging from food to folk medicine. In fact, it has been regarded as "a plant of multipurpose medicinal applications" [20], as different parts of it, including the roots, leaf and stem bark, have plenty of medicinal values. In particular, the leaf aqueous extracts were reported to possess anti-inflammatory and analgesic effects [21]; as well as anti-diabetic and anti-hypertensive activities [22] in rats. Other biological activities of the leaf extract include anti-microbial [23]; antioxidant, antibacterial and anti-tumor [24]; and several others as reviewed by Barbalho *et al.* [20]. In this study, we characterized the polyphenolics (flavonoids and phenolic acids) of guava leaves, and evaluated its inhibitory effect on XO and ACE; and it antioxidant effects *in vitro*, with a view to elucidating the possible mechanism of its anti-gout and antihypertensive activities.

MATERIALS AND METHODS

Samples Collection and Preparation

About 800 g of fresh leaves sample was collected from a guava plant in Akingbile, Moniya, Ibadan, Nigeria. The sample was botanically identified and authenticated at the herbarium of the Department of Botany, University of Ibadan, Nigeria. Thereafter, the sample was air-dried for 7 days and later ground finely to a particle size of 0.5 mm. The powdery sample was stored in air-tight plastic vials at –4°C until analysis.

Chemicals and Reagents

Methanol, formic acid, gallic acid, chlorogenic acid, caffeic acid, and ellagic acid purchased from Merck (Darmstadt, Germany). Catechin, epicatechin, quercetin, rutin, apigenin, and luteolin; porcine pancreatic lipase, Hippuryl-histidyl- leucine (Bz-Gly-His-Leu), Rabbit lung ACE, Xanthine, Allopurinol, 2,2-diphenylpicrylhydrazyl (DPPH), 2,2´-azino-bis-3-ethylbenzthiazoline-6-sulphoni (ABTS), and Trolox, L-ascorbic acid (Vitamin C) were acquired from Sigma Chemical Co. (St. Louis, MO, USA). All other chemicals used for analysis were of analytical grade.

Preparation of Polyphenolics-rich Extract

Polyphenolics-rich extract of guava leaves was prepared as described by Kuo *et al*. [25]. A portion of the leaves powder (100 g) was extracted three successive times with 300 mL of methanol at 50°C for 3 h, and the sample was filtered after each extraction with Whatman (No. 2) filter paper. The combined extract was partitioned with 200 mL hexane in a separatory funnel to get rid of the lipids and some pigments. The aqueous phase was extracted 3 times with 180 mL ethyl acetate and evaporated to dryness at 45°C under reduced pressure in a rotary evaporator. The residue obtained was used for the assays.

Quantification of Flavonoids and Phenolic Acids by High Performance Liquid Chromatography Diode Array Detection (HPLC-DAD)

The guava leaves extract was injected by means of Auto-sampler (Shimadzu, model SIL-20A) at a concentration of 15 mg/mL. Separations of phenolics were carried out using Phenomenex C_{18} column (4.6 mm × 250 mm × 5 μm particle size). The mobile phase comprised solvent A (water: formic acid [98:2, v/v]) and solvent B (acetonitrile), at a flow rate of 0.6 mL/min and injection volume of 40 μL. Gradient program was started with 95% of A and 5% of B until 2 min and changed to obtain 25%, 40%, 50%, 70% and 80% B at 10, 20, 30, 50 and 70 min, respectively, following the method described by Boligon *et al*. [26], with slight modifications. The extract and mobile phase were filtered through a 0.45 μm membrane filter (Millipore) and then degassed in an ultrasonic bath before use. Standards references stock solutions were prepared in the HPLC mobile phase at a concentration range of 0.025-0.300 mg/mL. Quantifications of the flavonoids and phenolic acids in the extract were carried out by integration of the peaks using the external standard method at the following wavelengths: 254 nm for gallic acid and ellagic acid; 280 nm for catechin and epicatechin; 325 nm for caffeic acid and chlorogenic acid; and 366 nm for quercetin, quercitrin, kaempferol, luteolin and rutin. The identification of the individual chromatography peaks and quantification of the corresponding phenolic compounds were based on a combination of retention time and spectral matching with those of reference standards. The chromatography analysis was carried out at ambient temperature and in triplicate.

Limit of Detection (LOD) and Limit of Quantification (LOQ) of Flavonoids and Phenolic Acids

LOD and LOQ were calculated based on the standard deviation (SD) of the responses and the slope using three independent analytical curves, as previously defined by Khaliq *et al*. [27]. LOD and LOQ were calculated as 3.3 and 10 σ/S, respectively, where σ is the SD of the response and S is the slope of the calibration curve.

Handling of Experimental Animal

Adult male Wister strain albino rats weighing 200-250 g were procured from the experimental animal breeding unit of Department of Veterinary Medicine, University of Ibadan, Nigeria. To ensure the protection of animals' welfare during experiments, the guidelines outlined in the Guide for the Care and Use of Laboratory Animals prepared by the National Academy of Science and published by the National Institute of Health (USA) [28], were followed. The rats were acclimatized in cages under ambient laboratory conditions for 7 days, during which they had free access to food and water.

Preparation of Liver Homogenates for XO and Lipid Peroxidation Inhibition Assays

The method described by Nakamura *et al*. [29] was followed to prepare the liver tissue homogenates used for XO and lipid peroxidation inhibition assays. The liver tissue was rapidly excised after decapitation of the rats under mild ether anesthesia. The tissue was washed in cold 0.15 M KCl, and blotted dry. Then, 1 g of it was homogenized in 9 volumes of ice-cold 50 mM Tris-HCl buffer (pH 7.4) containing 1 mM ethylenediaminetetraacetic acid. Thereafter, a portion of the homogenate was centrifuged for 10 min at 1400 × g to yield a low-speed supernatant that was used for the lipid peroxide assay. For the XO assay, another portion of the homogenate was sonicated twice on ice for 30 s and then centrifuged at 10,000 × g for 20 min at 4°C to obtain the supernatant fraction used.

XO Inhibition Assay

The ability of the extract to inhibit XO was tested according to the method reported by Umamaheswari *et al*. [18] with slight modification. The reaction mixture contained 300 μL of 50 mM sodium phosphate buffer (pH 7.5), 100 μL of the extract at different concentrations (15, 30, 45 and 60 μg/mL) in dimethyl sulfoxide (DMSO), 100 μL of freshly prepared tissue enzyme preparation (liver homogenate) and 100 μL of distilled water. The test mixture was pre-incubated at 37°C for 15 min. Thereafter, 200 μL of 0.15 mM of xanthine solution (substrate) was added to the mixture and it was further incubated at 37°C for 30 min. Next, 200 μL of 0.5 M HCl was added to terminate the reaction. Allopurinol was used as a positive control for the assay; a reference test containing 100 μL of DMSO instead of the extract was also carried out so as to obtain the maximum uric acid formed. The absorbance was measured at 295 nm on a UV/VIS spectrophotometer against a blank prepared in the same way except that the liver homogenate was replaced with the phosphate buffer. One unit (U) of this enzyme is defined as the amount of enzyme required to form 1 mmol of uric acid per min at the reaction conditions. The XO inhibitory ability of the extract was calculated as percentage inhibition as follows:

$$\%\ \text{XO inhibition} = ([A295_{reference} - A295_{sample}] \div A295_{reference}) \times 100.$$

Where $A295_{reference}$ is the reference without the extract, and $A295_{sample}$ is the absorbance of test containing the extract.

Lipid Peroxidation Inhibition Assay

The ability of the extract to inhibit Fe^{2+}-induced lipid peroxidation was tested according to the modified method of Ohkawa *et al*. [30]. Briefly, to a reaction mixture containing 100 μL of the homogenate supernatant, 30 μL of 0.1 M Tris-HCl buffer (pH 7.4) and different concentrations (15, 30, 45 and 60 μg/mL) of the extract, 30 μL of freshly prepared 25 μM solution of ferric sulfate was added to initiate lipid peroxidation. The volume was made up to 300 μL with deionized water before incubation at 37°C for 1 h. The color reaction was initiated by adding 300 μL of 81 g/L sodium duodecyl sulfate to the reaction mixture, followed by the addition of 600 μL of acetic acid/HCl (pH 3.4) and 600 μL of 0.8% (v/v) TBA (thiobarbituric acid). This mixture was incubated at 100°C for 1 h. The absorbance of thiobarbituric acid reactive species (TBARS) produced were measured at 532 nm in a UV-visible spectrophotometer. A reference test without the plant extract was carried out to obtain the maximum TBARS formation. The ability of the extract to inhibit Fe^{2+}-induced lipid peroxidation was expressed as percentage inhibition thus:

$$\% \text{ Inhibition} = ([A532_{reference} - A532_{sample}] \div A532_{reference}) \times 100$$

Where $A532_{reference}$ is the absorbance of the reference without the extract, and $A532_{sample}$ is the absorbance of test containing the extract.

ACE Inhibition Assay

ACE inhibition was assayed using a spectrophotometric method described by Cushman and Cheung [31]. ACE from rabbit lung (EC 3.4.15.1) and the substrate (Hippuryl-histidyl-leucine [Bz-Gly-His-Leu]) were used. In this assay, the Bz-Gly-His-Leu is cleaved by ACE to form hippuric acid, which is measured spectrophotometrically. Different dilutions (15, 30, 45 and 60 μg/mL) of the extract amounting to 50 μL and 50 μL ACE solutions (4 mU/mL) were pre-incubated at 37°C for 15 min. After pre-incubation, 150 μL of 8.33 mM of the Bz-Gly-His-Leu in 125 mM Tris-HCl buffer (pH 8.3) was added to the mixture to initiate the enzymatic reaction, and this was incubated at 37°C for 30 min. Next, the reaction was terminated by adding 250 μL of 1 M HCl. The hippuric acid produced by the reaction was extracted with 1.5 mL ethyl acetate. Subsequently, the ethyl acetate layer was separated from the mixture by centrifugation, and 1 mL of it was transferred to a clean test tube and evaporated to dryness in a hot-air oven. The resulting residue was redissolved in distilled water and its absorbance was measured at 228 nm. A reference test (without the extract), and a positive control test (containing 64 nmol/L of captopril) were carried out simultaneously with the test extract. 1 unit (U) of ACE activity is defined as the amount of enzyme required to catalyze the formation of 1 μmol of hippuric acid from hippuryl-histidyl-leucine per minute at 37°C. The ability of the extract to inhibit ACE activity was expressed as percentage inhibition thus:

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

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

Estimation of ABTS*+ Scavenging Ability

The ABTS*+ scavenging ability of the extract was determined according to the method described by Re *et al.* [32]. To generate the ABTS*+, an equal volume of 7 mM ABTS*+ aqueous solution was incubated with 2.45 mM $K_2S_2O_8$ for 16 h at room temperature in the dark; then its absorbance (at 734 nm) was adjusted to 0.7 ± 0.02 with 95% ethanol. Thereafter, appropriate dilution of the extract amounting to 0.2 mL was mixed with 2.0 mL ABTS*+ solution. The test mixture was kept in the dark for 15 min, after which its absorbance was measured at 734 nm. The ABTS*+ scavenging ability of the extract was subsequently calculated from a standard curve prepared using Trolox and expressed in Trolox equivalent (TE).

Determination of DPPH free Radical Scavenging Ability

The DPPH* scavenging ability of the extract was determined as described by Cervato *et al.* [33]. Appropriate dilutions (10, 20, 30 and 40 μg/mL) of the extract, amounting to 1.0 mL was mixed with 3.0 mL of DPPH* (60 μM). The test mixture was kept in the dark for 30 min, after which the absorbance was measured at 517 nm. A reference test (containing the DPPH* solution without the extract), and a reference standard (containing the DPPH* solution and ascorbic acid) were included in the assay. The DPPH* percentage scavenging ability of the extract was calculated as follows:

$$\% \text{ scavenging ability} = ([A517_{reference} - A517_{sample}] \div A517\text{control}) \times 100$$

Where $A517_{reference}$ is the absorbance of the reference test; and $A517_{sample}$ is the absorbance of the test containing the extract.

Statistical Analysis

Results of replicate experiments were expressed as mean ± SD. Independent samples *t*-test was performed on the result data at 95% confidence level using SPSS statistical software package, version 17. IC_{50} was calculated from the % inhibition versus extract concentration non-linear regression curve of the extract.

RESULTS

Characterization of the phenolics composition of the guava leaf extract using HPLC-DAD showed that the extract was rich in flavonoids and phenolic acid, and the result is presented in Table 1. Flavonoids were present in the extract in the order of quercetin > kaempferol > catechin > quercitrin > rutin > luteolin > epicatechin; while phenolic acids were in the order of caffeic acid > chlorogenic acid > gallic acids. A representative HPLC chromatogram of the extract is shown in Figure 1.

The inhibitory effects of the extract on the activities of XO and ACE, and Fe^{2+}-induced lipid peroxidation, expressed

as IC_{50}, are presented in Table 2. The extract inhibited XO, having IC_{50} of 38.24 ± 2.32 μg/mL, in comparison with the IC_{50} of 5.78 ± 0.25 μg/mL, observed for the standard reference XO inhibitor (allopurinol). The extract also inhibited ACE, with IC_{50} of 21.06 ± 2.04 μg/mL in relation to the IC_{50} of 4.96 ± 0.18 μg/mL, recorded for the captopril that was used as the standard reference ACE inhibitor. The result showed that the extract had an IC_{50} of 27.52 ± 1.72 μg/mL against Fe^{2+}-induced lipid peroxidation. The patterns of the inhibitory effects of the extract on XO, ACE and Fe^{2+}-induced lipid peroxidation were dose-dependent as depicted in Figure 2.

The ability of the extract to scavenge free radicals was tested using DPPH* and ABTS*+ assays, and the results are presented in Table 3. The extract strongly scavenged DPPH*, having half-maximal scavenging concentration (SC_{50}) of 13.38 ± 0.86 μg/mL, relative to that of ascorbic acid (7.38 ± 0.27 μg/mL), the standard reference antioxidant. In addition, the extract scavenged DPPH* in a dose-dependent manner [Figure 3]. Similarly, the extract strongly scavenged ABTS*+ as shown by its TE antioxidant capacity (TEAC) value of 3.20 ± 0.14 mmol TEAC/g.

Table 1: Flavonoids and phenolics acids composition of guava leaves extract

Compounds	Guava leaves extract (mg/g)	LOD (μg/mL)	LOQ (μg/mL)
Flavonoids			
Quercetin	5.11±0.01	0.027	0.089
Kaempferol	3.40±0.01	0.008	0.026
Catechin	2.39±0.01	0.013	0.042
Quercitrin	2.37±0.01	0.020	0.066
Rutin	2.26±0.03	0.014	0.047
Luteolin	2.23±0.01	0.017	0.056
Epicatechin	1.64±0.02	0.011	0.034
Phenolic acids			
Caffeic acid	6.28±0.03	0.019	0.061
Chlorogenic acid	2.31±0.04	0.008	0.026
Gallic acid	1.63±0.03	0.024	0.079

Results are expressed as mean±SD of triplicate determinations, LOD: Limit of detection, LOQ: Limit of quantification, SD: Standard deviations

Table 2: IC_{50} of guava leaves extract against XO, ACE activities, and Fe^{2+} -induced lipid peroxidation

	XO IC_{50} (μg/mL)	ACE IC_{50} (μg/mL)	Fe^{2+} -induced lipid peroxidation IC_{50} (μg/mL)
Guava	38.24±2.32[a]	21.06±2.04[a]	27.52±1.72
Allopurinol	5.78±0.25[b]	NA	NA
Captopril	NA	4.96±0.18[b]	NA

Results are expressed as mean±SD of replicate analysis. Values followed by different superscript letters along the same column vary significantly at $P<0.05$. NA: Not applicable, SD: Standard deviations, XO: Xanthine oxidase, ACE: Angiotensin 1-converting enzyme

Table 3: DPPH* IC_{50} and ABTS*+ scavenging ability of guava leaves extract

Antioxidant activity	Guava leaves extract	Ascorbic acid
DPPH* IC_{50} (μg/mL)	13.38±0.86[a]	7.38±0.27[b]
ABTS*+ scavenging ability (mmol TEAC/g)	3.20±0.14	NA

Results are expressed as mean±SD of triplicate analysis. Values followed by different superscript letters along the same row vary significantly at $P<0.05$. NA: Not applicable, SD: Standard deviations, DPPH: 2,2-diphenylpicrylhydrazyl, ABTS: 2,2′-azino-bis-3-ethylbenzthiazoline-6-sulphonic, The "*" on DPPH and "*+" on ABTS mean "radical" and "radical cation", respectively

DISCUSSION

Despite advances in medicine, the incidences of gout and hypertension continue to increase, partly due to the high cost of the available synthetic inhibitors of the enzymes (including XO and ACE) associated with both diseases, and their attendant side effects. Unfortunately, both diseases are degenerative, and are so, capable of reducing the quality of life of those suffering from them. This development has aroused research for natural inhibitors of these enzymes that could be effective, safe and more affordable. In this regard plant phenolics, particularly flavonoids and phenolic acids, have emerged to be promising

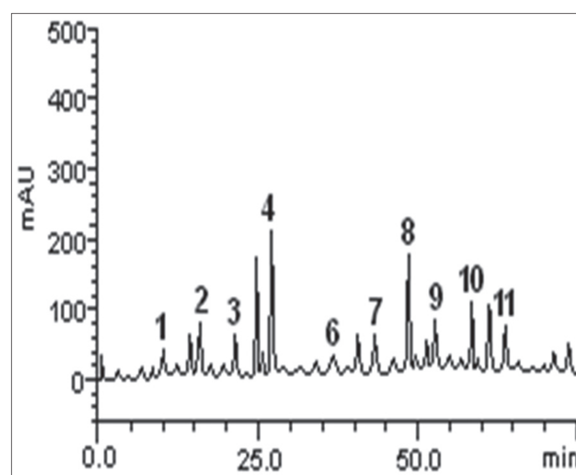

Figure 1: Representative high performance liquid chromatography profile of guava leaves extract. Detection UV was at 325 nm. Gallic acid (peak 1), catechin (peak 2), chlorogenic acid (peak 3), caffeic acid (peak 4), ellagic acid (peak 5), epicatechin (peak 6), rutin (peak 7), quercitrin (peak 8), quercetin (peak 9), kaempferol (peak 10) and luteolin (peak 11)

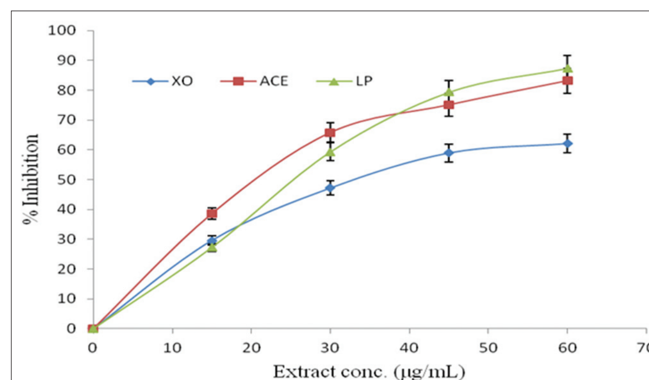

Figure 2: % inhibition-extract concentration curves showing the dose-dependent inhibition of xanthine oxidase (XO), angiotensin 1-converting enzyme (ACE) and Fe^{2+}-induced lipid peroxidation (LP) by guava leaves extract

candidates. Recent studies have revealed that various plants extracts rich in flavonoids and phenolic acids can inhibit one of these two enzymes [17,19]. Existing literature also indicated that guava leaves extracts possessed anti-inflammatory [21], and anti-hypertensive [22] activities in rats. Hence, we characterized the flavonoids and phenolics acids of guava leaves extract, and evaluated its XO and ACE inhibitory effects, and antioxidant activity; with a view to elucidating the possible mechanism(s) of its anti-gout and anti-hypertensive effects.

The phenolics composition of the guava leaves extract revealed that it contained the flavonoids: Quercetin, kaempferol, catechin, quercitrin, rutin, luteolin, and epicatechin; with quercetin being the most abundant flavonoid, followed by kaempferol. The result further revealed that the extract contained the phenolic acids: Caffeic acid, chlorogenic acid, and gallic acids; with caffeic acid, followed by chlorogenic acid as the most abundant. This result is in conformity with the report of Jang *et al.* [34], who identified the presence of gallic acid, catechin, chlorogenic acid, catechin, caffeic acid, rutin, and other phenolic compounds in guava leaf extract using HPLC. However, there were differences in the quantities of the phenolic compounds we detected and those they earlier reported. For instance, the quantities we observed for gallic acid, catechin, chlorogenic acid, caffeic acid and rutin (1.63 ± 0.03, 2.39 ± 0.01, 2.31 ± 0.04, 6.28 ± 0.03 and 2.26 ± 0.03 mg/g, respectively), were generally higher than the values (gallic acid: 0.09 ± 0.00 mg/g; catechin: 0.72 ± 0.04 mg/g; chlorogenic acid: 0.19 ± 0.01 mg/g; caffeic acid: 0.14 ± 0.00 mg/g; rutin: 0.34 mg/g) they reported [34]. These differences may partly be due to the variation in the detection system of the HPLC used. Whereas we used HPLC coupled with diode array detector (HPLC-DAD), Jang *et al.* [34] used HPLC coupled with a UV-vis multi-wavelength detector (HPLC-UV-vis). In addition, variations in biotic and abiotic factors relative to the regions where plants were grown; as well as variations in the period of sample collection, are also known to contribute to differences in the levels of phytochemicals in plants [24].

Previous reports have shown that various plant extracts rich in flavonoids and phenolic acids were potent in inhibiting some enzymes implicated in certain pathological conditions, including XO for gout [17], ACE for hypertension [19], α-amylase and α-glucosidase for Type 2 diabetics [35], in addition to possessing antioxidant activity, and other health benefits. The inhibition of XO is an important clinical strategy for treating hyperuricemia and gouty arthritis; as it helps in decreasing the amount of uric acid in circulation, and vascular oxidative stress [36]. In this respect, the plant-derived polyphenolics have been shown to be effective in inhibiting XO and alleviating the resultant hyperuricemia. As natural components of plant foods, they are considered to be safer than synthetic XO inhibitors including allopurinol [37]. The structure of the flavonoids promotes their ability to inhibit XO. In particular, the C-5 and C-7 hydroxyl groups of flavones and flavonols can replace the C-2 and C-6 ones of xanthine in the XO active site [38,39]. This effect is made possible by the mutual inter-convertibility of the carboxyl structures of xanthine to hydroxyl groups [40]. The IC_{50} of the guava leaves extract against XO in this study (38.24 ± 2.32 μg/mL) is lower than the 45.71 ± 1.44 μg/mL we recently reported for phenolics extract of *Tetrapleura tetraptera* fruit [17], and the 42 μg/mL reported for *Olea europaea* leaf extract [41]. The lower IC_{50} of the guava leaves extract indicates a stronger XO inhibitory activity than *T. tetraptera* fruit and *O. europaea* leaf extracts. By inhibiting XO, the guava leaves extract could be useful in managing hyperuricemia, the index of gout, and preventing the downstream events, including increased production of ROS, activation of the renin/angiotensin pathway and inactivation of bradykinin, that link hyperuricemia to hypertension.

The inhibition of ACE, the enzyme that catalyzes the conversion of angiotensin I to angiotensin II in the renin–angiotensin–aldosterone system, has become a strategic target for the treatment of hypertension and other cardiovascular diseases [42]. Angiotensin II is a known vasoconstrictor that activates the aldosterone secretion, and inactivates bradykinin, a vasodilator and hypotensive peptide [13]; thereby increasing the blood pressure. The angiotensin II is also capable of increasing the superoxide production activity of the endothelial cells. However, research has shown that plant-derived polyphenolics can inhibit ACE. Among the polyphenolic compounds, flavonoids and phenolic acids are prominent for their potent anti-hypertensive activity, and are therefore promising active principles for non-pharmacological nutraceutical intervention in hypertension [43]. The inhibition of ACE is a striking anti-hypertensive mechanism of flavonoids. The flavonoids have a combination of sub-structures on their skeleton that favors their ACE inhibitory effect. These sub-structures include the catechol group in the B-ring, the double bond between C2 and C3 at the C-ring, and the cetone group in C4 at the C-ring of the flavonoids [44]. Other mechanisms of their anti-hypertensive activity include improvement of endothelial function, modulation of vascular smooth muscle, cell signaling, and gene expression; and their antioxidant effect [45]. Interestingly, quercetin, the most abundant flavonoid in the guava leaves extract, has been reported to have anti-hypertensive activity which it mediates via several mechanisms including ACE inhibition, which is considered as the most important mechanism [46]. The salient inhibitory effect of quercetin on ACE activity could be attributed to its optimum binding affinity with the ACE, as it has a binding energy of –8.5 kcal/mol relative to the standard value of –7.0 kcal/mol, as revealed by its *in silico* analysis [45].

Phenolic acids also mediate their anti-hypertensive effect by inhibiting ACE and maintaining vascular endothelial function [47]. The ACE inhibitory effect of phenolic acids is due to the net contribution of their functional groups (COO^- and OH^-); the ability of the oxygen atom of their carboxylate moiety to form charge-charge interactions with the Zn^{2+} present in the ACE active site; and their ability to form a stable complex with ACE, through their interaction with the amino acids residues at the active site of ACE [46]. It is also noteworthy that caffeic acid, the most abundant phenolic acid in the guava leaves extract in this study, was reported to exhibit strong anti-hypertensive effect in both *in vitro* [48] and *in vivo* [49] studies. Bhullar *et al.* [50] further demonstrated that caffeic acid and

its derivatives exhibit a strong anti-hypertensive effect through the inhibition of ACE.

Vascular oxidative stress is a common feature of both gout and hypertension. Oxidative stress sets in when the oxidant burden (free radicals, ROS and reactive nitrogen species) of the body cells outweighs the available antioxidant defense system, both enzymic and non-enzymic. Hence, improving the antioxidant status of the body is important for the management of gout and hypertension. The guava leaves extract exhibited antioxidant activity by inhibiting Fe^{2+}-induced lipid peroxidation and scavenging free radicals (DPPH* and ABTS*+). Previous reports have indicated that the activities of XO [51] and angiotensin II [52] enhance the peroxidation of lipids. Oxidative damage to the membrane lipids produces an array of cytotoxic products, especially aldehydes such as malondialdehyde (MDA) [53]. These cytotoxic aldehydes play a role in a number of oxidative stress-induced inflammatory diseases [54], including gouty arthritis. Oxidative damage to the cell membrane caused by the peroxidation of membrane lipids has the tendency to disrupt the functions of membrane transport proteins and ionic channels; deactivate membrane-bound enzymes; and increase the permeability of the membrane lipid bilayer [37], to ions and other molecules that may be toxic to the cell. The ability of the guava leaves extract to inhibit Fe^{2+}-induced lipid peroxidation indicates that it could mitigate the oxidative damage to cell membrane lipids caused by ROS produced by the over-activity of XO in gout patience, and by angiotensin II in hypertensive patients. Interestingly, the extract had a lower IC_{50} (27.52 ± 1.72 μg/mL) against Fe^{2+}-induced lipid peroxidation, in comparison with the 36.97 ± 2.06 μg/mL (in rat liver homogenate) reported for *T. tetraptera* fruit extract [17].

The DPPH* SC_{50} (13.38 ± 0.86 μg/mL) observed in this study for the guava leaves extract is about twice higher than the IC_{50} of 6.25 μg/ml of its ethanolic extract earlier reported by Thephinlap *et al.* [55]. On the other hand, the ABTS*+ scavenging ability (3.20 ± 0.14 mmol TEAC/g) of the extract in this study is lower than the TEAC value (4.908 ± 0.050 mM/mg) of its ethanol extract reported by Tachakittirungrod *et al.* [56]. These variations could be attributed to differences in biotic and abiotic factors affecting the phytochemical level and bioactivities of the plant in the different regions where the leaves sample was collected, and variations in the period of sample collection [24] as earlier stated. The antioxidant activities of the guava leave extract could be attributed to the flavonoids and phenolic acids. These polyphenols have redox properties, which enable them to function as reducing agents, hydrogen donors, and singlet oxygen quenchers [57]; hence, their antioxidant activity. Thus, the guava leaves extract could help in scavenging the free radicals and ROS generated by the activities of XO and angiotensin II. Interestingly, using XO inhibitors is suggested to be a viable antioxidant approach in pathophysiologic conditions such as hypertension in which ROS production is exacerbated [11]. Our results confirm the report of Braga *et al.* [24], who observed high levels of antioxidant properties in guava leaves ethanol extract, and inferred that it could be used as functional food. It has earlier been stated that the activity of XO is linked with elevated arteriolar tone and consequently, hypertension [9]. One of the ways by which this occurs is through the formation of peroxynitrite ($ONOO^-$) by the reaction of the $O_2^{\bullet-}$ generated by the activity of XO in the endothelial cells, with endothelial nitric oxide (NO); thereby lowering the vasorelaxant effect of NO [11]. It is important to recall that the NO is produced from L-arginine by the catalytic function of endothelial nitric oxide synthase (eNOS) [58]. The $ONOO^-$ so-formed is a much more potent oxidant and cytotoxic agent, as experimental evidence suggests that its formation and the concomitant activation of downstream signaling pathways eventually result in injury of the endothelial cell [59,60]. The oxidative stress on endothelial cells resulting from the intracellular production of uric acid-derived radicals has the potential to activate the renin/angiotensin pathway [12], and consequently, elevate the blood pressure.

Thus, the mechanism of the anti-hypertensive activity of guava leaves extract could be viewed to be tripartite; the first is the inhibition of XO with a concomitant decrease in ROS generation; the second is the inhibition of ACE resulting in the decreased production of angiotensin II (a vasoconstrictor), with a concomitant activation of bradykinin (a vasodilator); the third is the scavenging of the ROS generated by the two pathways (XO- and ACE-catalyzed pathways), thereby attenuating the formation of the cytotoxic $ONOO^-$ from the reaction of the $O_2^{\bullet-}$ with NO, and maintaining the vasorelaxant effect of the NO. A proposed scheme of this tripartite mechanism of action is shown in Figure 4.

CONCLUSION

The inhibition of XO and angiotensin 1-converting; and scavenging of free radicals might be the possible mechanisms of the anti-gout and anti-hypertensive effects of guava leave extract. These effects could be attributed to the combined effect of the flavonoids and phenolic acids present in the extract. Guava leaves extract may, therefore, serve as a functional food for managing gout and hypertension and attenuating the oxidative stress associated with both diseases.

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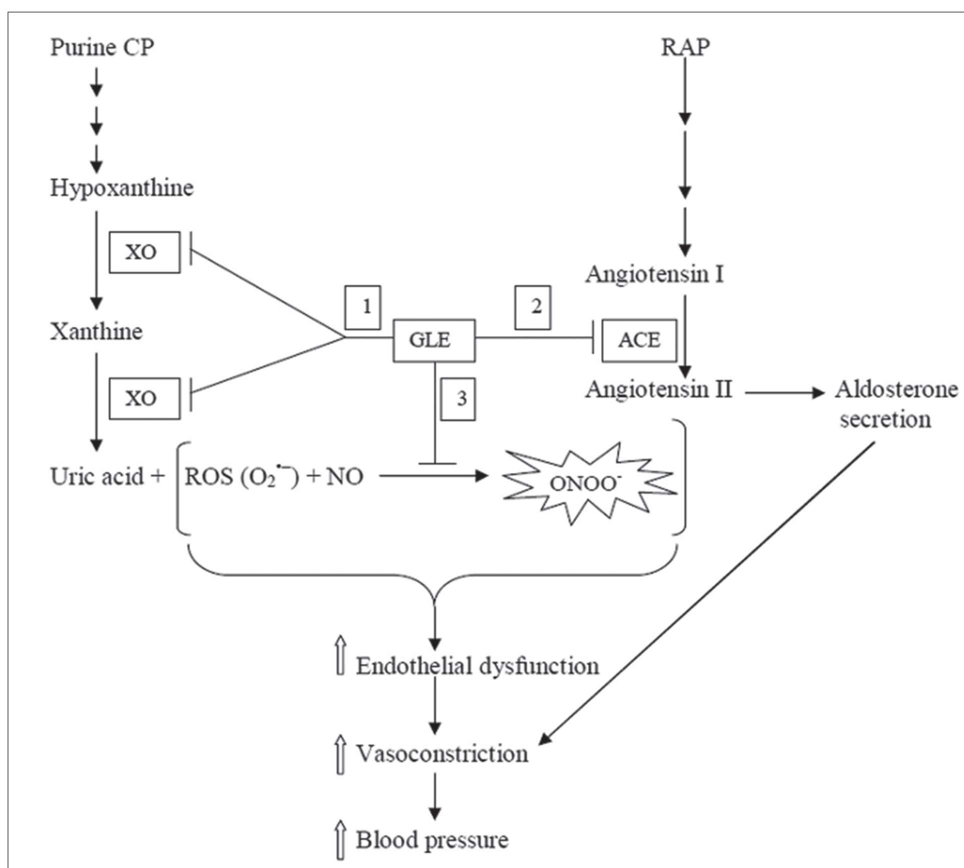

Figure 4: Schematic diagram of proposed tripartite mechanism of anti-hypertensive effect of guava leaves extract. Purine CP: Purine catabolic pathway, XO: Xanthine oxidase, RAP: Rennin-angiotensin pathway, ACE: Angiotensin I-converting enzyme, GLE: Guava leaves extract, $O_2^{\bullet-}$: superoxide anion radical, $ONOO^-$: Peroxynitrite, ↑ : Increase. The numbers: 1, 2 and 3 in rectangular boxes indicate the XO inhibition, ACE inhibition; and scavenging of reactive oxygen specie (respectively) by guava leaves extract

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Original Research

Investigation on hypoglycemic effects of ethanol extract of *Alpinia nigra* (Gaertn.) in animal model

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ABSTRACT

Background: Our study aims at exploring the hypoglycemic effect, efficacy, and possible mode of action of ethanol extract of *Alpinia nigra* (EEAN) as an antidiabetic agent in an animal model. **Methods:** Oral glucose tolerance test (OGTT) was used to identify primary hypoglycemic effect in mice. Three tests (glucose absorption, sucrose absorption, and disaccharidase activity) were carried out by gut perfusion and six segments studies to assess carbohydrate absorption and glucose utilization. **Results:** In OGTT, at 400 mg/kg and 800 mg/kg dose of EEAN extract significantly improved oral glucose tolerance among normal mice at 60 min and 90 min with compared to control. Both doses of extract significantly ($P < 0.01$) reduced blood glucose level and showed the hypoglycemic effect by retarding 11.43% and 20.82% of blood glucose level after 2 h of administration in glucose-induced mice, respectively. *In situ* perfused rat intestinal model demonstrated reduced glucose absorption at a 500 mg/kg dose. Inhibition of intestinal disaccharidase was also found by the extract. This was confirmed, yet again, via the six segment study. Throughout the length of the gastrointestinal tract, sucrose digestion was found to be inhibited which is also evident in the six segment study. **Conclusions:** This study suggests that the EEAN has hypoglycemic effects in a dose-dependent manner by inhibiting intestinal glucose absorption, and these may be effective in the treatment of diabetes. Further study is required to explicate the effect this extract or the active compounds have on the individual glucose transporters and the precise mechanism.

KEY WORDS: Alpinia nigra, blood glucose, glucose tolerance, hypoglycemic effect, oral glucose tolerance test

INTRODUCTION

Diabetes, a chronic metabolic disorder, is a major threat to global public health that is rapidly getting worse and the biggest impact on an adult of working age in developing countries. There is an estimation of 246 million people with diabetes in the world, of whom about 80% reside in developing countries [1]. Among two types of diabetes, Type 1 causes the immunological destruction of pancreatic β cells resulting in insulin deficiency [2]. Type 2 diabetes mellitus (DM), more prevalent form of the disease, is associated with both impaired insulin secretion and insulin resistance. It is often associated with obesity and hereditary disposition [3]. Multiple lines of therapeutic options have been so far designed and applied to get cure of diabetic ailments. However, the synthetic antidiabetic agents themselves are making numbers of inconveniences due to their side effects along with their higher costs [4]. As a result, alternative therapeutic ways are still in search to shunt those adverse effects caused by synthetic antidiabetic agents.

Traditional preparations from plant sources have recently and widely been used almost every corner of the world as an alternative medication for diabetes due to their less harmful effects and lower prices. The World Health Organization Study Group of DM has also acknowledged the therapeutic advantages of plants medicines in diabetic management as the plants were the first option as antidiabetic therapy before the advent of insulin and oral hypoglycemic drugs this issue [5]. Last two decades, plant materials are progressively formulated and marketed as herbal drugs [6]. It has been estimated that in the U.S. 25% of all prescription dispensed from community pharmacies contain plant extracts [7].

Although the succession of synthetic drugs, to certain extend, has raised the health care of people, until now the use and importance of phytomedicines for the same has never been neglected, and a large number of plants are screened for their efficacy against diabetic and hyperglycemic diseases [8,9]. *Alpinia nigra* (Gaertn.) B.L. Burtt, which belongs to the Zingiberaceae family, is known as Jongly Ada or Tara in Bengali. This aromatic and rhizomatous herb is also referred to as Galangal, False galangal, Greater galangal, Black-Fruited, or Kala. A. *nigra* it is used as traditional medicine for DM. Diabetic patients use it in various forms, e.g. juice of A. *nigra* is a natural cure against DM. A. *nigra*, which is widely cultivated in Asia, Africa, and South America, is a diverse medicinal plant which has also been therapeutically used in the treatment of various diseases. Various therapeutic activities of this plant which has been reported are anti-inflammatory [10], analgesic, antibacterial, cytotoxic [11], anthelmintic [12], anxiolytic-sedative [13], etc. Research showed that isolated compounds from A. *nigra* had well inhibition of α-glucosidase activity [14]. Diabetic patients use it in various forms, e.g. juice of A. *nigra* as a home remedy against DM. The hypoglycemic effect of A. *nigra* was not evaluated by the established methods.

In the present study, we first tried to find out the hypoglycemic effect of A. *nigra* by OGTT. We also tried to establish an indigenous system of medicine (herbal therapy) as antidiabetic drugs instead of chemical drugs. The mode of action of A. *nigra* leaf extract in the treatment of diabetes was also investigated.

MATERIALS AND METHODS

Chemical and Reagents

Reagents of analytical grades and deionized water (Purite, Oxon, UK) were used for the study. Sodium pentobarbital was purchased from Sigma-Aldrich (St Louis, MO, USA). Sodium chloride, D-glucose, sucrose, ethanol, calcium chloride, potassium chloride, and sodium hydrogen carbonate were obtained from BDH Chemical Ltd (Poole, Dorset, UK). All kits were purchased from Boehringer Mannheim GmbH, Germany. Wallac 1409 scintillation counter was supplied by Wallac, Turke, Finland while the microwell plate ELISA reader was obtained from Bio-Tek, USA. Rapid View™ (Blood glucose monitoring system, Model: BIO-M1, BIOUSA Inc, California, USA) with strips were purchased from Anderkilla, Chittagong. Glucose was purchased from the local scientific market, Chowkbazar, Chittagong. Glibenclamide was obtained from Square Pharmaceutical Ltd., Bangladesh.

Collection and Identification

Leaves of A. *nigra* were collected from the Bangladesh Centre for Scientific and Industrial Research (BCSIR), Chittagong, Bangladesh, in the month of April 2014. It was identified and authenticated by the standard taxonomical method at BCSIR.

Preparation of Plant Extract

The collected leaves (5 kg) were washed with fresh water and dried in the shade at room temperature (25°C). The dried leaves were grounded into fine powder by an electrical grinder (Wiley mill) and mesh (mesh number 50) was used to sieve the sample. Then, the powder of leaves of A. *nigra* was pasted by homogenizing with mortar and was suspended with water for preparing the ethanol extract. About 900 g of the leaves were dissolved in absolute ethanol (99% ethanol, source) for 7 days and then filtered. Collected supernatant was dried using a rotary vacuum evaporator (BUCHI Rotavapor R-114). Semisolid crude extracts were again dried with water bath at 80°C. The dried extracts (yield, 12%) were kept in the freezer (4°C) and utilized for biological screening.

Experimental Animals

6-7 weeks old Long-Evans male rats (approximately weighing 110 ± 15 g) and Swiss albino mice were chosen for the study. The animals were bred at BCSIR (Chittagong, Bangladesh). The animals were acclimatized under standard conditions (temperature 23 ± 2°C, relative humidity 55%) and were maintained on 12 h light-dark cycle. A standard pellet diet and *ad libitum* were supplied freely unless otherwise indicated. The overall nutrient composition of the diet was 36.2% carbohydrate, 20.9% protein, 4.4% fat, and 38.5% fiber with a metabolisable energy content of 1.18 MJ/100 g (282 Kcal/100 g). The animals were maintained in the laboratory, and the treatment was in the schedule. The animals described as fasted were deprived of food for at least 12 h but allowed free access to drinking water.

Hypoglycemic Effect in Glucose-Induced Hyperglycemic Mice

Oral glucose tolerance test (OGTT) was performed according to the standard method [15] with minor modification. Group I was treated as a normal control group, Group II treated with glibenclamide (5 mg/kg body weight), and Groups III and IV were treated with ethanol extract of A. *nigra* leaves at 400 mg/kg and 800 mg/kg body weight, respectively. Glucose solution (1 g/kg body weight) was administered at first. Then, drug and extract solutions were administered to the glucose fed. Serum glucose level of a blood sample from tail vein was estimated using glucometer at 0, 30, 60, 90, and 120 min. Percent decrease of blood glucose level after 120 min measured by the following equation,

$$\% \text{ decrease} = \frac{GL_{0\,\text{min}} - GL_{120\,\text{min}}}{GL_{0\,\text{min}}} \times 100$$

$GL_{0\text{min}}$ = Blood Glucose level at 0 min, $GL_{120\text{min}}$ = Blood Glucose level at 120 min

Sucrose Absorption from Gastrointestinal (GI) Tract

Rats were fasted for 12 h before receiving 50% sucrose solution by gavage (2·5 g/kg body weight) with (for experimental cases) or without (for control cases) ethanolic extract of A. *nigra* (500 mg/kg

body weight). Some of the rats were killed at these timing. The GI tract was excised and divided into six segments: The stomach; the upper 20 cm, middle and lower 20 cm of the small intestine; the cecum; the large intestine. Each segment was washed out with acidified ice-cold saline and centrifuged at 3000 rpm (1000 g) for 10 min. The resulting supernatant was boiled for 2 h to hydrolyze the sucrose followed by neutralization with NaOH. Blood glucose and the amount of glucose liberated from residual sucrose in the GI tract were measured. The GI sucrose content was calculated from the amount of liberated glucose [16].

Intestinal Glucose Absorption

An intestinal perfusion technique [17] was used to study the effect of A. *nigra* on intestinal absorption of glucose in 36 h fasted non-diabetic rats anesthetized using sodium pentobarbital (50 g/kg). The ethanolic extract of A. *nigra* (10 mg/ml, equivalent to 500 mg/kg) suspended in Krebs-Ringer buffer supplemented with glucose (54 g/L) was passed through pyloric, and the perfusate was collected from a catheter inserted at the end of the ileum. The control group was perfused with Krebs-Ringer buffer supplemented with only glucose. Perfusion was carried out at the rate of 0·5 ml/min for 30 min at 37°C, with perfusate being separated by every 5 min. The results were expressed as the percentage of absorbed glucose, calculated from the amount of glucose in solution before and after the perfusion.

Intestinal Disaccharidase Activity

A 20 h fasted rats were killed and the small intestines were isolated, cut longitudinally, rinsed with ice-cold saline and homogenized with 10 ml saline (0·9% NaCl) and centrifuged at 3000 rpm (1000 g) for 3 min. Aliquots (20 μl) of the supernatant from mucosal homogenate were mixed with 1 ml sucrose (40 mmol/L sucrose) in Eppendorf tubes. For the control group, aliquots (20 μl) of distilled water were further added to the Eppendorf tubes. For treatment group, aliquots (20 μl) of A. *nigra* extract of 0.5 mg/ml, 1.0 mg/ml, 2.0 mg/ml, and 5.0 mg/mL were mixed, respectively, in the Eppendorf tubes. These Eppendorf tubes were then incubated with at 37°C for 1 h. Disaccharidase activity was calculated by glucose concentration converted from sucrose as μmol/mg glucose per protein per h [18].

Statistical Analysis

Data were expressed as mean ± standard deviation (SD), $n = 6$ for all experiments. Analyzes were performed by one-way analysis of variance (ANOVA) using statistical software (Statistical Package for Social Science, version 19.0, IBM corporation NY) followed by Dunnett's *t*-test for comparisons. $P = 0.05$ or less were considered as significant.

RESULTS

Hypoglycemic Effect in Glucose-Induced Hyperglycemic Mice

Investigational induction of hyperglycemia resulted in increased glucose level in blood on mice, which is shown in Table 1. Both doses of leaf extract did not manifest any significant reduction in 30 min after administration. Most significant reduction ($P < 0.05$) was observed for 800 mg/kg dose of ethanol extract of A. *nigra* at 120 min. At 120 min, this dose also showed a significant reduction (20.82%) of blood glucose level. Standard glibenclamide (5 mg/kg) showed a significant reduction in 30, 60, 90, and 120 min, which decrease 40.82% blood glucose level of its initial (0 min). Time interaction with each specific hour in this experiment was also found significant ($P < 0.05$). Percentage of decrease of blood glucose level in glucose-induced mice after 2 hours with different treatment are also showed in Table 1.

Effects on Sucrose Absorption from GI Tract

Results were expressed as (mean value ± SD) in mg. Administration of extract of A. *nigra* (500 mg/kg) with the sucrose load in rats increased the residual intestinal sucrose content (mg) significantly ($P < 0{\cdot}05$) at 30 min in the stomach (15.7 ± 2.5*), upper 20 cm small intestine (15.1 ± 2.12*), middle small intestine (21.2 ± 2.86* mg), lower 20 cm small intestine (19.2 ± 2.52* mg), the control rats with the stomach (11.1 ± 2.04 mg), upper 20 cm small intestine (10.2 ± 1.93 mg), middle small intestine (6.5 ± 1.62 mg), lower 20 cm small intestine (3.6 ± 1.21 mg). Residual intestinal sucrose also increased significantly at 60 min in the stomach (7.5 ± 1.86* mg), upper 20 cm small intestine (6.4 ± 1.68* mg), middle small intestine (7.1 ± 1.84* mg), lower 20 cm small intestine (7.8 ± 2.01* mg), the control rats with the stomach (2.1 ± 1.21 mg), upper 20 cm small intestine (1.8 ± 0.76 mg), middle small intestine (2.2 ± 1.32 mg), lower 20 cm small intestine (2.3 ± 1.25 mg). The total sucrose content remaining in the GI tract was increased in A. *nigra* treated rats compared with normal controls [Figure 1].

Effects on Intestinal Glucose Absorption

As shown in Figure 2, intestinal glucose absorption (%) in non-diabetic rats was almost constant during 30 min of perfusion.

Table 1: Effect of *A. nigra* leaves extract on glucose-induced hyperglycemia (mmol/L) in normal mice

Group	0 min	30 min	60 min	90 min	120 min	% decrease of glucose level after 120 min
Control	8.02±2.11	8.37±2.18	7.98±2.11	7.04±1.79	8.29±3.31	-
Glibenclamide (5 mg/kg)	7.3±0.76	6.7±2.06*	5.32±1.67*	4.61±1.18*	4.32±1.01*	40.82
EEAN (400 mg/kg)	6.91±2.25	7.35±1.94*	6.78±1.74*	6.58±2.11*	6.12±2.03*	11.43
EEAN (800 mg/kg)	7.54±1.15	7.86±1.49*	7.21±1.13*	6.67±0.93*	5.97±1.64*	20.82

Values are presented in mean±SD (n=6). EEAN=Ethanol extract of *A. nigra* leave. Values with different superscripts in the same column are significantly different from control at each specific hour after the administration of standard and different doses of the extract. For $*P>0.05$ and One-way ANOVA followed by Dunnett's multiple comparison was performed to analyze this comparison, *A. nigra: Alpinia nigra*, SD: Standard deviation

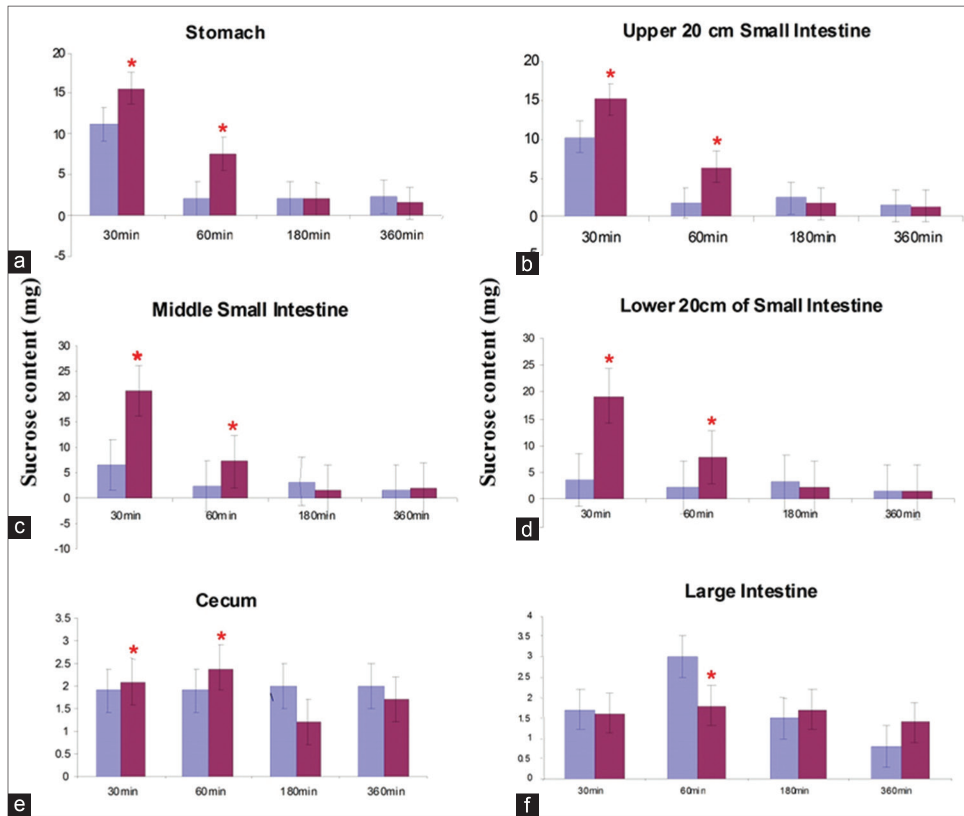

Figure 1: Graph comparing the total sucrose content (a-f) in the whole gastrointestinal tract at 30, 60, 180, and 360 min in a group of control rats versus rats given gavage with *A. nigra* extract. EEAN: Ethanol extract of *A. nigra* leave

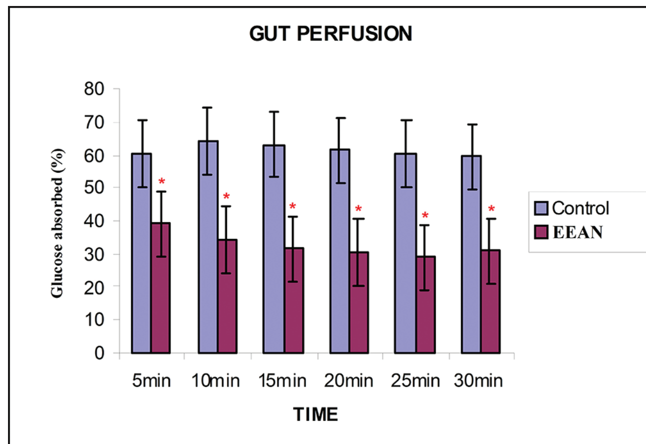

Figure 2: Graph showing the percentage of glucose absorbed in small intestine for 30 min at 5 min intervals. EEAN: Ethanol extract of *A. nigra* leave

The addition of A. *nigra* to the glucose perfusate resulted in a substantial decrease in intestinal glucose absorption during the whole experimental period ($P < 0.05$).

Effects on Intestinal Disaccharidase Activity

Results are expressed in mean ± SD. The intestinal disaccharidase activity decreased significantly in the disaccharide enzymes treated with A. *nigra* at all four concentrations of 0.5 mg/ml (0.86 ± 0.06 * µmol/mg/h), 1.0 mg/ml (0.78 ± 0.05*), 2.0 mg/ml (0.71 ± 0.05*), and 5.0 mg/ml (0.66 ± 0.07*), control enzymes (1.39 ± 0.28). The results are depicted in Figure 3.

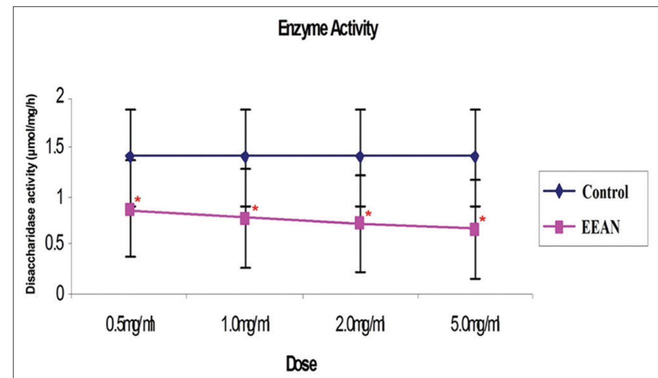

Figure 3: Graph comparing the enzyme activity of the control enzymes extracted from the lumen of rat versus those same enzymes treated with *A.nigra* extract. EEAN: Ethanol extract of *A. nigra* leave

DISCUSSION

There several tests offered for screening the hypoglycemic result of any sample or drug. However, the OGTT is usually thought of as additional inclined for the screening of impaired glycemia, as a result of it distinguishes the changes in post-prandial glycemia that tend to precede changes in abstinence

aldohexose. All the present established diagnostic processes for polygenic disease rely on a threshold price forced on never-ending delivery of blood sugar levels. Yet, the right glycemic threshold that discriminates "normal" from diabetic is not obvious. That's why choice for unknown sort two polygenic disease leftovers a polemic issue, there is clear proof that after it is known, complications are often prevented in several diabetic patients [19,20]. OGTT measures the body's ability to use aldohexose, the body's main supply of energy. OGTT are often accustomed diagnose pre polygenic disease and polygenic disease. The ethanol extract of leaves of A. *nigra* (EEAN) showed vital ability to scale back the elevated aldohexose level in traditional mice compared to the quality drug glibenclamide. At a dose of 800 mg/kg of EEAN showed the highest hypoglycemic effect and it decreased 20.82% of blood glucose level after 2 h of administration in glucose-induced mice, where glibenclamide (5 mg/kg) decreased 40.82%. At dose 400 mg/kg, EEAN decreased 20.82% of blood glucose level after 2 h of administration in glucose-induced mice.

The activity of A. *nigra* extract as antidiabetic drug agent and its doable mechanism was investigated in non-diabetic rats. The post-prandial symptom is undesirable because it will increase glycosylation merchandise, like methylglyoxal, that play a task within the development of diabetic tube disease [21]. Acute elevation of aldohexose conjointly will increase coagulation [22] and leads to multiple disturbances in epithelial tissue cell function [23]. It's renowned that high-fiber diets improve aldohexose tolerance in diabetes [24]. This result could also be attributable to backward stomach removal, increased viscus transit, or modification of the secretion and action of biological process enzymes [25]. The hypoglycemic activity that is found once given with a synchronal aldohexose load in diabetic rats indicates that the extracts could interfere with the viscus aldohexose absorption within the gut by varied mechanisms [26]. In the present study, the various effect of A. *nigra* extract on carbohydrate digestion and absorption in the gut was assessed. This was investigated by gut perfusion experiment where the ethanol extracts showed a gradual decrease in glucose absorption.

Since aldohexose lowering result of genus A. *nigra* was clearly evident from previous study reports, aldohexose absorption inhibition may are a doable mechanism accountable for the hypoglycemic effect [27]. Our study confirms this result similarly as a result of once genus A. *nigra* ethanolic extract was given beside saccharose answer; it considerably increased saccharose retention within the gut compared with solely the saccharose answer au fait cluster of rats. Similar *in vitro* studies dispensed with high concentrations of Glucophage conjointly showed such inhibition of aldohexose absorption [28]. The flavonoids and tannins are reportable to provide antidiabetic activity [29]. This antidiabetic drug property has been connected with the flexibility of the polyphenolic tannins and flavonoids and to inhibit α-glucosidase enzyme [30]. Our study confirmed the claim mentioned higher than since enzyme enzymes of rats treated with A. *nigra* ethanolic extract showed vital dose-dependent inhibition in activity compared with the controls.

CONCLUSION

The present study demonstrates that the ethanol extract of A. *nigra* showed well decrease of blood glucose level after 2 h of administration in glucose-induced mice and significant inhibition of carbohydrate digestion and absorption, which has resulted in the well-known hypoglycemic effects of A. *nigra*. Thus, A. *nigra* may be a useful dietary adjunct for the treatment of diabetes. Further study is necessary to investigate its pancreatic action.

AUTHORS' CONTRIBUTIONS

MSHK and MMNU carried out the study design, data collection, data interpretation, manuscript preparation, statistical analysis, and research grant collection. SMZH collected the plants and participated in experiments, data collection, literature search, and manuscript preparation. MMNU also acted as correspondence. All authors read and approved the final version of the manuscript.

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Original Research

Imbalance of the antioxidative system by plumbagin and *Plumbago indica* L. extract induces hepatotoxicity in mice

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ABSTRACT

Background/Aim: *Plumbago indica* (PI) L. and its active constituent, plumbagin, has been traditionally claimed for several pharmacological activities; however, there is little information regarding their toxicity. The present study aims to examine the effects of plumbagin and PI extract (PI) on hepatic histomorphology and antioxidative system in mice. **Materials and Methods:** Adult male intelligent character recognition mice were intragastrically administered plumbagin (1, 5, and 15 mg/kg/day) or PI (20, 200, and 1,000 mg/kg/day) consecutively for 14 days. Hepatic histomorphology was examined. Plasma alanine transaminase (ALT) and aspartate transaminase (AST) levels, hepatic lipid peroxidation, superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPx) activities, and the ratio of reduced to oxidized glutathione (GSH/GSSG) were determined. **Results:** Plumbagin and PI concentration-dependently induced hepatic injury based on histopathological changes via imbalance of antioxidative system. Plumbagin and PI significantly increased plasma ALT and AST levels, hepatic lipid peroxidation, and GPx activity but significantly decreased hepatic SOD and CAT activities. The GSH/GSSG ratio was significantly reduced by plumbagin. **Conclusion:** Plumbagin and PI caused hepatotoxic effects in the mice by unbalancing of the redox defense system. Therefore, plumbagin and PI-containing supplements should be used cautiously, especially when consumed in high quantities or for long periods.

KEY WORDS: Antioxidative system, glutathione, hepatotoxicity, plumbagin, *Plumbago indica*, transaminase

INTRODUCTION

Plumbago indica (PI) L., a shrub belonging to the family Plumbaginaceae, is widely distributed in tropical and subtropical regions of Africa, Australia, and Asia including Thailand [1]. PI root has been used as an ingredient in ayurvedic remedies for diarrhea, indigestion, and several skin diseases and has also been applied as an anthelmintic, appetite stimulant, and rubefacient [2-4]. In addition, PI extract has been reported to possess antibacterial activity [5-7]. While all *Plumbago* species have been claimed to have antifertility properties [8], PI was exceptionally used in the ancient remedies for abortion [2].

Plumbagin (5-hydroxy-2-methyl-1,4-naphthoquinone), a yellowish quinonoid compound, is the major constituent that contributes to the medicinal properties of PI [3,9]. The root contains the highest amount of plumbagin (0.17% w/w) among *Plumbago* species and is the part mostly contained in the medicinal remedies [10]. Plumbagin has been claimed for various biological activities, i.e., anthelmintic [4,9,11], antimalarial [12], antibacterial [6], antifungal [13], anti-inflammatory [14,15], immunosuppressive [16], abortifacient [8], anticancer [14,17-20], and antidiabetic activities [21].

Nowadays, traditional herbal remedies are often perceived as harmless due to the belief that they are natural [22]. Potential toxicities in these remedies are often overlooked due to a lack of scientific studies regarding safe-dosage range, safety windows of effective and toxic doses [23]. Recent evidence indicates that herbal supplements are regularly consumed as alternative medicines by approximately 20% of the adults in the United States [24]. Hence, any scientific information regarding undesirable effects and toxicity of these herbal supplements would be of great significance.

Plumbagin has been reported to exhibit several side effects including diarrhea, skin rashes, leukocytosis, and increased serum phosphatase levels [25]. In addition, there are reports of plumbagin-induced hepatotoxicity [3,25,26], cardiotoxicity [27], cytotoxicity toward human keratinocytes [26], and radiomimetic nucleotoxic and cytotoxic effects [3]. These effects are possibly due to the activity of plumbagin as a strong inducer of reactive oxygen species (ROS) and a depleting agent of cellular glutathione (GSH) [26,27].

Antioxidative enzymes, such as superoxide dismutase (SOD), catalase (CAT), and GSH peroxidase (GPx), are essential for regulating and balancing ROS [28-30]. Therefore, they can be employed as biomarkers to determine the levels of ROS production and oxidative stress [30]. In addition, drug-induced liver injury subsequent to long-term use of drugs or herbal supplements can be evaluated via histomorphology. Common histopathological patterns for drug-induced liver injury include acute hepatitis, with or without cholestasis [31].

In the current study, plumbagin and the methanolic crude extract of PI were examined for their influences on the antioxidative enzymes and GSH profile along with the plasma transaminase levels and hepatic histomorphology to assess hepatotoxic potential and their possible adverse effects in mice.

MATERIALS AND METHODS

Chemicals and Reagents

Plumbagin was obtained from the LKT Laboratories (St. Paul, Minnesota, USA). Bradford solution was a product of Bio-Rad (Hercules, CA, USA). Eosin Y 1% aqueous solution and Mayer's hematoxylin were from Bio Optica (Milan, Italy). Xylene and Permount® were bought from Fisher Scientific (Loughborough, UK). Alanine transaminase (ALT) and aspartate transaminase (AST), bovine serum albumin, β-nicotinamide adenine dinucleotide phosphate reduced form (NADPH), xanthine oxidase, nitroblue tetrazolium (NBT), 5,5-dithio-bis-(2-nitrobenzoic acid) (DTNB), SOD, CAT, GSH reductase, malondialdehyde (MDA), thiobarbituric acid (TBA), and 4-vinylpyridine (4-VP) were supplied by Sigma-Aldrich Chemical (St. Louis, Missouri, USA). Ammonium molybdate and hydrogen peroxide (H_2O_2) were purchased from Ajax Finechem (Melbourne, Australia). Trichloroacetic acid (TCA) was a product of Loba Chemie (Mumbai, India). All other laboratory chemicals were of the highest purity from commercial suppliers.

Preparation of the PI Methanolic Crude Extract

The root of PI was purchased from Mor Tong-In Thai Traditional Medicine (Mahasarakham, Thailand) in June 2014. Plant materials were identified by Dr. Waraporn Putalun, Faculty of Pharmaceutical Sciences, Khon Kaen University, Khon Kaen, Thailand. Reference specimens (PANPB-PI 2014-002) were deposited at the Herbarium of the Faculty of Pharmaceutical Sciences, Khon Kaen University. It was dried at 50°C in an oven then shredded and extracted with methanol for 3 h using a Soxhlet apparatus. The extract was then evaporated and freeze-dried into powder. The percentage yield of the PI extract was 33.40%.

Determination of the Plumbagin Content in the PI Extract

The PI extract was analyzed for the plumbagin content using high-performance liquid chromatography (HPLC) which was carried out on a Hypersil ODS column (Agilent Technologies, CA, USA, particle size 5 μm, 250 × 4 mm) coupled with an Agilent 1260 Infinity system and a UV detector (Agilent Technologies), before being eluted by an isocratic linear solvent system of acetonitrile and water (50:50 by volume) with a flow rate of 1 mL/min. The chromatogram was monitored at a wavelength of 410 nm and analyzed by the ChemStation software (Agilent Technologies). Identification and quantification of plumbagin were performed on the basis of retention time and peak area of the authentic standard of plumbagin (LKT Laboratories).

Animal Treatments

7-week-old male intelligent character recognition (ICR) mice were obtained from the National Laboratory Animal Center (Mahidol University, Nakhon Pathom, Thailand) and housed in the Animal Unit of Faculty of Pharmaceutical Sciences, Khon Kaen University (Khon Kaen, Thailand) under a controlled temperature (23 ± 2°C) and humidity (45 ± 2%). The protocol for animal handling and treatment was approved by the Animal Ethics Committee for Use and Care of Khon Kaen University (Approval No. AEKKU04/2558). At all times, the mice were housed on wood shaved bedding in polysulfone cages with *ad libitum* access to water and commercial rodent diet (SmartHeart® from Perfect Companion Pet Care Company, Bangkok, Thailand). The mice were intragastrically administered plumbagin (1, 5, and 15 mg/kg/day) or the PI extract (20, 200, and 1,000 mg/kg/day) in a 0.5% carboxymethyl cellulose solution (CMC) consecutively for 14 days ($n = 5$ for each group). The control group was daily given the vehicle (0.5% CMC) for the same period. At 24 h after the last treatment, the mice were sacrificed. The mouse plasma and livers were collected and immediately stored at –20°C for further analysis.

Hepatic Histomorphological Examination

Liver tissues were collected and examined their histomorphology under a light microscope. The tissues were fixed by immersion in 10% neutral buffered formalin overnight before being dehydrated by ethanol then embedded in paraffin. The paraffin pieces were sectioned using a microtome before being placed on microscopic slides. The slides with embedded liver tissue were stained with hematoxylin and eosin (H&E) and further evaluated for their hepatic histomorphological patterns at ×20 magnification using Nikon Eclipse TS100 inverted microscope with ELWD condenser: N.A. 0.3 (W.D. 75 mm). The image was analyzed and displayed on the screen using NIS-Elements D software [32].

Assessment of Plasma ALT and AST

The blood samples were centrifuged at 5,000×*g* at 4°C for 10 min. The supernatant was collected and incubated with ALT or AST substrates at 37°C for 5 min before further incubation with a 2,4-dinitrophenylhydrazine for 20 min. The reaction was stopped by adding 4 N NaOH before measuring the absorbance at a wavelength of 505 nm. The ALT and AST levels were determined using pyruvate as a standard [32].

Determination of Protein Content

The liver homogenate was diluted 50 folds with distilled water before determination of protein content using the Bradford method [33]. The absorbance of the mixture of the liver homogenate and Bradford solution was measured at a wavelength of 595 nm. The protein content was calculated using bovine serum albumin as a standard.

Assessment of SOD Activity

SOD activity was assessed as previously described [34]. Briefly, a mixture containing liver homogenate, chloroform, and ethanol was centrifuged at 13,000×*g*, 4°C for 30 min. The supernatant was collected and incubated with a mixture of xanthine, xanthine oxidase, and NBT at 25°C for 20 min. The reaction was stopped by adding $CuCl_2$ and the absorbance was measured at a wavelength of 550 nm. The SOD activity was calculated compared to the SOD standard.

Assessment of CAT Activity

CAT activity was assessed by the colorimetric method of Goth (1991) with some modifications [34]. The liver homogenate was incubated with H_2O_2 at 37°C for 1 min before adding ammonium molybdate to stop the reaction. The absorbance was measured at a wavelength of 405 nm. The CAT activity was calculated compared to the CAT standard.

Determination of the Lipid Peroxidation

The lipid peroxidation was determined by measuring the level of MDA formation [34]. In brief, TCA and TBA were added to the liver homogenate pre-incubated at 37°C for 1 h, followed by boiling at 100°C for 15 min. The reaction was immediately cooled down by immersion in an ice-bath before adding excess TCA to stop the reaction. The reaction mixture was then centrifuged at 1,123×*g*, 4°C for 5 min. The spectrofluorometric intensity of the supernatant was measured with excitation and emission wavelengths of 520 and 590 nm, respectively. The thiobarbituric acid-reactive substances (TBARS) content was calculated compared with the standard MDA.

Determination of Total GSH Content, the Ratio of Reduced to Oxidized GSH, and GSH Peroxidase Activity

The determination of total GSH content was performed as previously described [34]. Briefly, the liver homogenate was deproteinized by 5-sulfosalicylic acid (SSA) and kept at 2-8°C for 10 min before centrifugation at 10 000×*g*, 4°C for 10 min. The supernatant was then mixed with GSH reductase and DTNB before adding NADPH to start the reaction. The absorbance was measured at a wavelength of 405 nm every 60 s for 5 min. The slope of absorbance per min was plotted and compared to the standard GSH to calculate the total GSH content.

The oxidized GSH (GSSG) content was determined by the same procedure used for the determination of total GSH content after treatment of the sample with 4-vinylpyridine [34]. The reduced GSH content was calculated by subtracting the GSSG content from the total GSH content.

The GPx activity was assessed as described previously [34]. The liver homogenate was incubated with sodium azide at 30°C for 10 min before starting the reaction by adding the GSH substrate and H_2O_2. The reaction was stopped with SSA before centrifugation at 330 × *g* for 15 min. The GPx activity was measured as μmol of GSSG produced per min at 30°C, pH 7.4.

Statistical Analysis

All data were expressed as means ± SD and analyzed by one-way ANOVA followed by least significant difference (LSD) *post hoc* test using the Statistical Package for Social Sciences statistical program. $P < 0.05$ was considered to be significant.

RESULTS

The Content of Plumbagin in the PI Extract

The PI extract was quantitatively determined for the content of plumbagin using the HPLC method, in which the validation of the method was achieved within a linearity range of 1-100 mg/mL ($R^2 = 0.9990$). The precision of the method expressed as the coefficient of variation within the linearity range were 0.69 ± 0.29% (for within-day) and 0.87 ± 0.42% (for between-day). The accuracy was 102.16 ± 1.40% with the limit of determination and the limit of quantification of 10 and 34 ng/mL, respectively. The chromatograms of the plumbagin standard (retention time of 5.538 min, Figure 1a) and the PI extract (retention time of 5.494 min, Figure 1b) revealed that the major constituent in the PI extract was plumbagin. The content of plumbagin in the PI extract was 0.18 ± 0.01% dry weight extract.

Effect of Plumbagin and the PI extract on Hepatic Histomorphology

The histomorphological findings of the H&E-stained livers were shown in Figure 2. The control group demonstrated normal hepatic histomorphology [Figure 2a]. Mice fed with the lowest dose of plumbagin (1 mg/kg/day) [Figure 2b] and the PI extract (20 mg/kg/day) [Figure 2e] were not found any change in hepatic histomorphological anatomy compared to the control group. However, at the higher doses of both plumbagin and the PI extract, the evidence of liver injury was observed. The livers

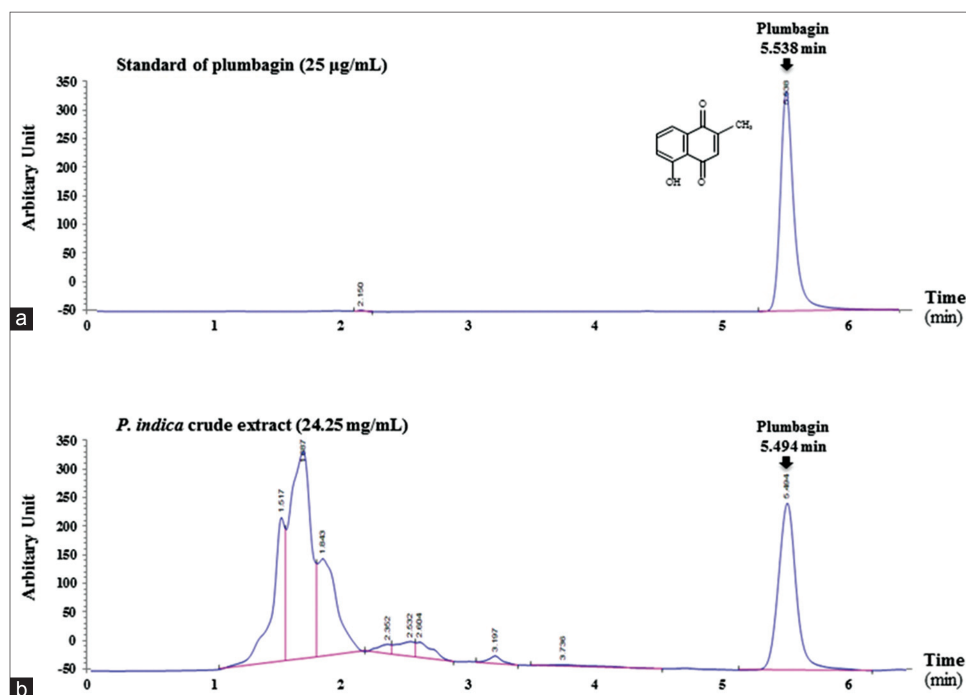

Figure 1: High-performance liquid chromatography chromatogram of the plumbagin standard and the *Plumbago indica* extract. (a) Chromatogram of the plumbagin standard at a concentration of 25 mg/mL, (b) Chromatogram of the *P. indica* extract at a concentration of 24.25 mg/mL

of mice received plumbagin (5 and 15 mg/kg/day) [Figure 2c and d] and the PI extract (200 and 1,000 mg/kg/day) [Figure 2f and g] were presented the nuclear shrinkage, where the nuclear chromatin materials were condensed and clumped together. In addition, at the highest doses of plumbagin (15 mg/kg/day) [Figure 2d] and the PI extract (1,000 mg/kg/day) [Figure 2g], the hepatic hydropic cell swelling, where the cytoplasm of hepatocyte was filled with vacuoles, and nuclear fading along with some hepatocyte nuclei disappearance were extensively observed. These hepatic histopathological findings revealed that plumbagin and the PI extract induced hepatotoxicity in the dose-dependent pattern.

Effect of Plumbagin and the PI Extract on ALT and AST Levels

Plumbagin and the PI extract had no significant influence on the profile of mouse body weight [Figure 3]. However, the treatments with plumbagin and the PI extract significantly elevated the levels of ALT and AST, compared with the control mice [Table 1]. While plumbagin significantly increased both plasma ALT and AST levels in a dose-dependent manner, the PI extract only increased the AST levels dose-dependently; the ALT levels were increased in a dose-independent manner.

Effect of Plumbagin and the PI Extract on Lipid Peroxidation in the Mouse Livers

The levels of MDA formation were significantly increased in a dose-dependent pattern by both plumbagin and the PI extract [Figure 4], corresponding with the increases of ALT and AST levels.

Effect of Plumbagin and the PI Extract on SOD and CAT Activities in the Mouse Livers

Both of plumbagin and the PI extract significantly lowered SOD activity in the mouse livers to at least 2 times lesser than the control group [Figure 5]. The CAT activity was significantly decreased by both plumbagin and the PI extract in a dose-dependent pattern [Figure 6].

Effect of Plumbagin and the PI Extract on the GSH Profile and GPx Activity in the Mouse Livers

Either an increase or decrease in the total GSH, GSH, and GSSG contents was noted [Table 2] in accord with the reduction of SOD and CAT activities [Figures 5 and 6]. Total GSH and GSH contents were significantly reduced or even unchanged by plumbagin, whereas those levels were modified, increased, decreased, or unchanged by the PI extract. Regarding the GSSG content, increase or a decrease was observed by either plumbagin or the PI extract. The ratio of GSH to GSSG was significantly reduced by plumbagin but unchanged by the PI extract.

The GPx activity was significantly induced by plumbagin at the dose of 5 mg/kg/day and the PI extract at the dose of 1,000 mg/kg/day [Figure 7]. These findings corresponded with the increases in the GSSG contents [Table 2].

DISCUSSION

In our study, the yield of plumbagin in the PI extract was 0.18 ± 0.01% dry weight which well correlated with the previous

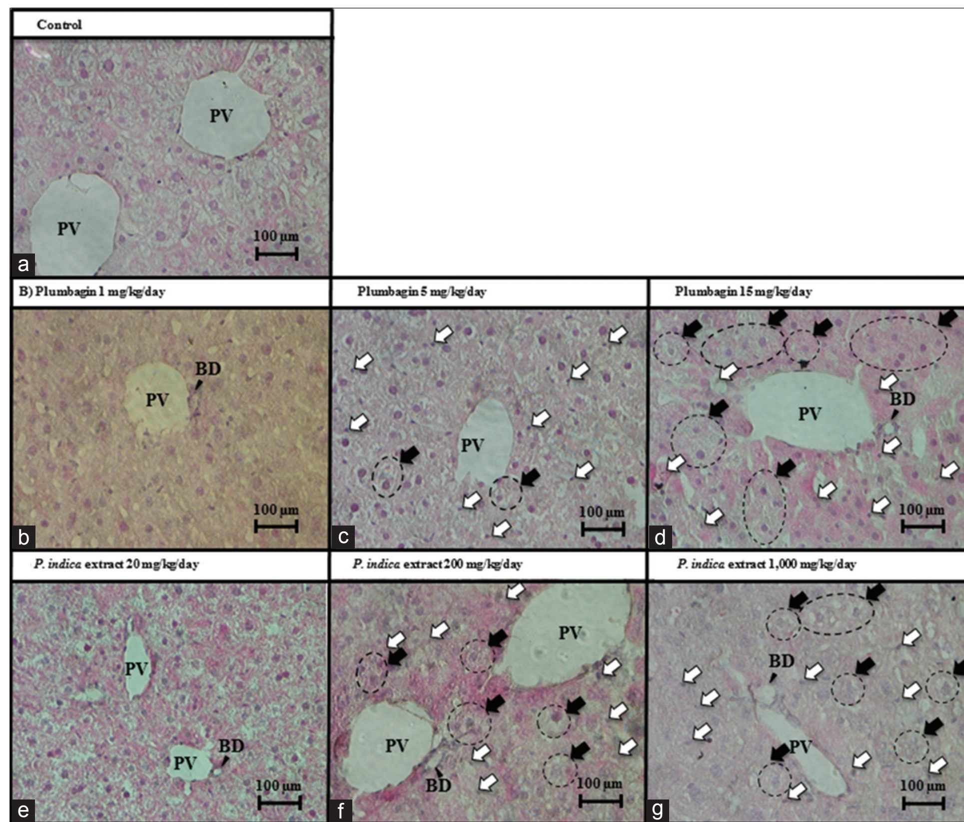

Figure 2: Liver histomorphology. Hepatic histomorphology of the mice treated with (a) control, 0.5% carboxymethyl cellulose (CMC), i.g., for 14 days, (b-d) Plumbagin in 0.5% CMC at the doses of 1, 5, and 15 mg/kg/day, i.g., for 14 days, (e-g) *P. indica* extract in 0.5% CMC at the doses of 20, 200, and 1,000 mg/kg/day, i.g., for 14 days. BD: Bile duct; PV: Portal vein. Open arrows indicate nuclear shrinkage. Closed arrows and circles indicate hydropic cell swelling

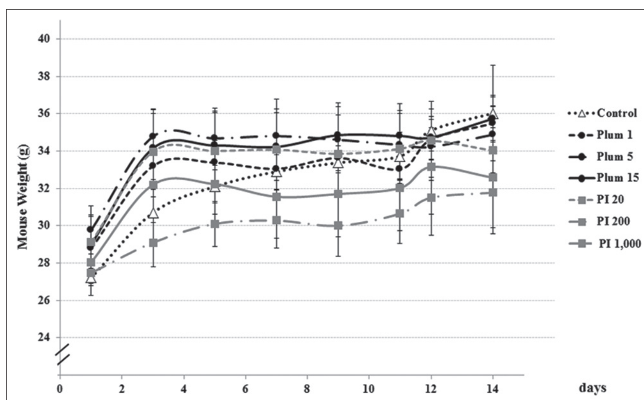

Figure 3: The weight profiles of mice during the treatments with plumbagin and the *Plumbago indica* extract. Mice were treated as described in the method ($n = 5$). Plum 1, 5, and 15, plumbagin in 0.5% carboxymethyl cellulose (CMC) at the doses of 1, 5, and 15 mg/kg/day, i.g., for 14 days; *P. indica* 20, 200, and 1,000, *P. indica* extract in 0.5% CMC at the doses of 20, 200, and 1,000 mg/kg/day, i.g., for 14 days

Table 1: The levels of ALT and AST in the mice after the treatments of plumbagin or the *P. indica* extract for 14 days

Treatment	ALT[#] (mmole/mg)	AST[#] (mmole/mg)
Control	15.58±2.96	3.72±1.57
Plumbagin		
1 mg/kg/day	19.97±3.07*	9.03±3.16*
5 mg/kg/day	20.26±0.83**	15.04±2.89***
15 mg/kg/day	27.37±1.21***	18.51±4.66***
P. indica extract		
20 mg/kg/day	21.12±1.20*	13.97±5.15***
200 mg/kg/day	19.70±4.91*	15.90±2.79***
1000 mg/kg/day	17.11±2.36	20.36±1.71***

[#]The data is expressed as mean±SD (n=5). Control, 0.5% carboxymethyl cellulose (vehicle). *$P<0.05$, **$P<0.005$, ***$P<0.001$ versus control using one-way ANOVA followed by LSD *post hoc* test, SD: Standard deviation, LSD: Least significant difference, *P. indica: Plumbago indica*, ALT: Alanine transaminase, AST: Aspartate transaminase

studies of the chloroform extract of PI root of 0.17% [10] and the ethanolic extract of PI root of 0.20% [35].

PI is extensively used in Ayurvedic and Thai traditional remedies, especially as a hematinic agent and for post-partum women [2]. Besides, the numerous pharmacological properties, the active constituent of PI, plumbagin, have been reported to possess many undesirable effects [25] principally due to its capability to generate ROS, leading to an imbalance of the oxidant-antioxidant system [26,27]. The accumulation of ROS induces excessive lipid oxidation, further along with oxidative attack to lipids, proteins, and DNA materials. This results in damage of cellular components including cellular membranes, functional and structural proteins, and DNA components [29]. In addition to non-specific tissue damage, ROS, particularly H_2O_2, was able to activate transcription

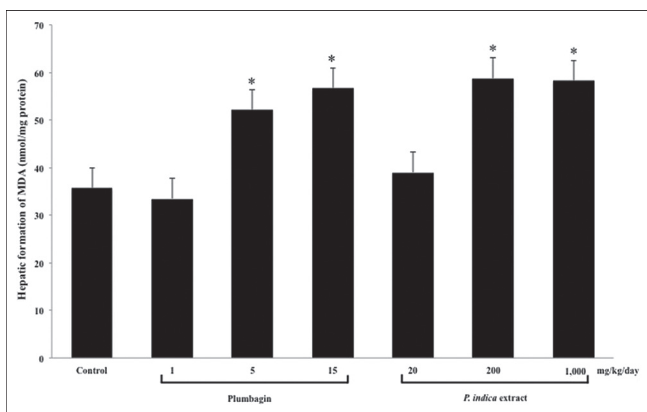

Figure 4: The hepatic lipid peroxidation in the mouse livers after the treatments with plumbagin and the *Plumbago indica* extract. Mice were treated, and the levels of malondialdehyde (MDA) formation were measured as described in the method ($n = 5$). *$P < 0.001$ versus Control using one-way ANOVA with least significant different *post hoc* test

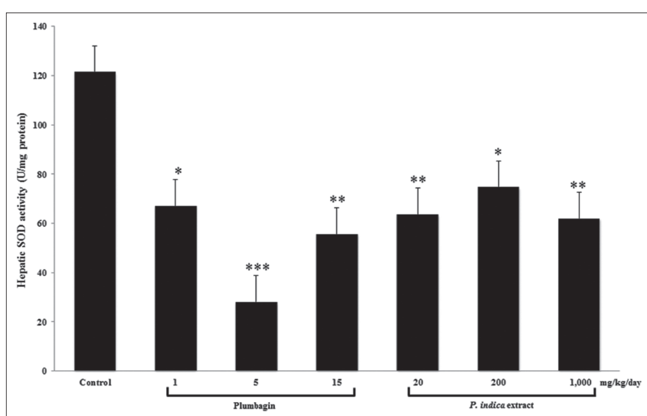

Figure 5: The superoxide dismutase (SOD) activity in the mouse livers after the treatments with plumbagin and the *Plumbago indica* extract. Mice were treated, and the SOD activity was measured as described in the method ($n = 5$). *$P<0.05$, **$P<0.01$, ***$P<0.001$ versus control using one-way ANOVA with least significant different *post hoc* test

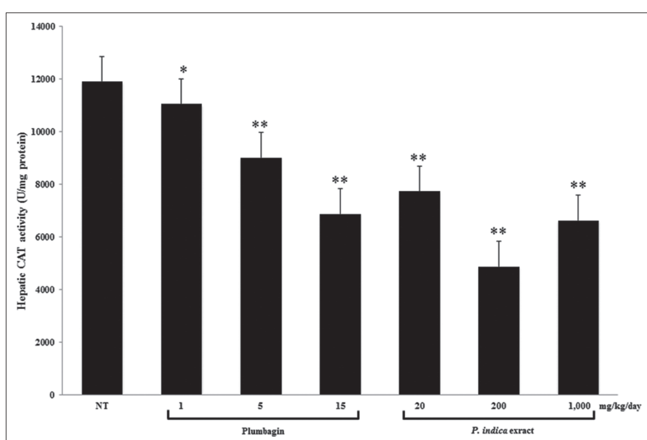

Figure 6: The catalase (CAT) activity in the mouse livers after the treatments with plumbagin and the *Plumbago indica* extract. Mice were treated, and the CAT activity was measured as described in the method ($n = 5$). *$P<0.05$, **$P<0.001$ versus control using one-way ANOVA with least significant different *post hoc* test

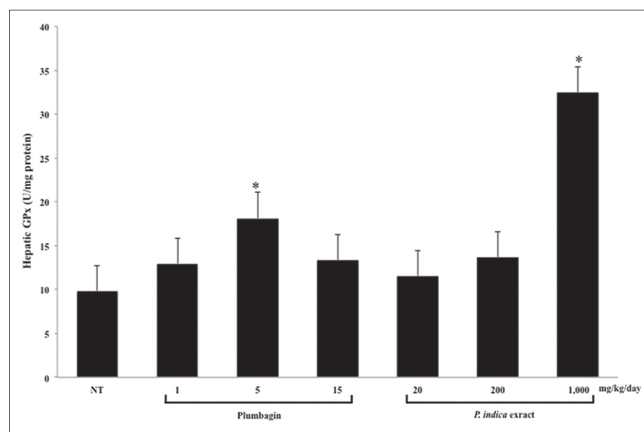

Figure 7: The glutathione peroxidase (GPx) activity in the mouse livers after the treatments with plumbagin and the *Plumbago indica* extract. Mice were treated, and the GPx activity was measured as described in the method ($n = 5$). *$P < 0.001$ versus control using one-way ANOVA with least significant difference *post hoc* test

factors of the nuclear factor kappa B family, which activates the inflammatory process [36,37]. Further study reported that H_2O_2 acted as a second messenger which activated protein kinase cascades coupled to inflammatory gene expression [36]. This information indicates that ROS not only produces non-specific damage to cellular components but also activates inflammation of the tissue. However, there is currently very little toxicological information regarding either plumbagin or the PI extract. In one study, Sumsakul *et al*. (2014) performed acute and subacute toxicity screening of plumbagin in ICR mice [12]. In the acute toxicity screening, a single dose of 100 mg/kg plumbagin showed no significant behavioral change, but a single dose of plumbagin at either 200 or 500 mg/kg led to anxiety and agitation and resulted in death within 24 h [12]. The subacute toxicity was investigated by oral administration of plumbagin (25, 50, and 100 mg/kg/day) for 14 days. The mice given with the two higher doses died within 8-11 and 4-8 days, respectively [12]. In another subacute toxicity study in rats, increases in serum alkaline phosphatase and ALT levels were noted following daily intraperitoneal administration of the PI root extract (50 mg/kg/day) for 30 days [38]. Our study is the first time to reveal the hepatotoxic impacts of the oral administration of plumbagin and the PI extract through the increased levels of transaminases in plasma and modification of antioxidative system in the mouse livers, including the increase in lipid peroxidation, the modulatory activities of antioxidative enzymes (SOD, CAT, and GPx), and related GSH profiles with lowering level of the GSH/GSSG ratio.

The LD_{50} of plumbagin in mice was reported at 16 mg/kg [25] with the oral bioavailability of 38.7% [39], and the LD_{50} of the ethanolic extract of PI root in mice was noted at 1,250 mg/kg [38,40]. Therefore, the doses of plumbagin of 1, 5, and 15 mg/kg/day and the PI extract of 20, 200, and 1,000 mg/kg/day were presently employed to examine their hepatotoxicity.

The mouse livers were collected and examined under a light microscope to evaluate hepatic histomorphological changes

Table 2: The glutathione profiles in the mouse livers after the treatments of plumbagin or the *P. indica* extract for 14 days

Treatment	Total glutathione# (mmole/mg)	GSH# (mmole/mg)	GSSG# (nmole/mg)	Ratio of GSH/GSSG#
Control	2.97±0.22	2.85±0.22	113.35±4.67	25.05±2.26
Plumbagin				
1 mg/kg/day	2.01±0.13**	1.91±0.13**	103.94±2.66**	18.38±1.14**
5 mg/kg/day	3.09±0.26	2.94±0.26	146.19±2.35**	20.03±1.80**
15 mg/kg/day	2.23±0.13**	2.10±0.13**	125.80±4.91**	16.77±1.58**
P. indica extract				
20 mg/kg/day	2.43±0.24**	2.34±0.23**	88.70±1.85**	26.39±2.34
200 mg/kg/day	2.89±0.18	2.78±0.17	107.94±2.57*	25.79±1.01
1000 mg/kg/day	4.30±0.27**	4.18±0.31**	171.31±2.63**	23.84±1.04

#The data is expressed as mean±SD (n=5). Control, 0.5% carboxymethyl cellulose (vehicle); GSH: Reduced glutathione, GSSG: Oxidized glutathione. *P<0.05, **P<0.001 versus control using one-way ANOVA followed by LSD post hoc test, SD: Standard deviation, LSD: Least significant difference, *P. indica: Plumbago indica*

after the administration of plumbagin and the PI extract. Liver injury induced by drugs or herbs can be presented with all kinds of patterns representing primary liver diseases [31]. The most common histopathological sign of drug-induced liver injury is acute hepatitis, with or without cholestasis. The hallmarks of acute hepatitis include portal and parenchymal inflammation and hepatocellular injury with or without necrosis [31]. In the present study, the lowest doses of either plumbagin (1 mg/kg/day) or the PI extract (20 mg/kg/day) did not change the histomorphological anatomy of the mouse liver while the higher doses of plumbagin and the PI extract underwent serious hepatic histomorphological changes. At the low concentrations of plumbagin (1 mg/kg/day) and PI extract (20 mg/kg/day), ROS production had only risen slightly which likely did not disturb the balance in the antioxidative system, a known cause of liver tissue damage. Nuclear shrinkage is a phenomenon where the nuclei are shrunken and dark, but still intact [Figure 2c and d, f and g]. This happens as an early stage of cellular degeneration as a sequence of chromatin clumping before the nuclear envelope is ruptured and the nucleus is disappeared, as known as nuclear fragmentation and nuclear fading, respectively [41]. The proposed mechanism behind these phenomena is the activation of caspase enzymes due to alterations of mitochondrial status, which, in turn, activates the intrinsic apoptotic pathway, resulting in DNA fragmentation and proteolysis of nuclear polypeptides [42,43]. In this study, the liver tissues of the mice receiving plumbagin of 5 mg/kg/day and the PI extract of 200 mg/kg/day were presented with hepatic nuclear shrinkage [Figure 2c and f] while those receiving the highest doses of both plumbagin and the PI extract demonstrated hepatic nuclear fading [Figure 2d and g], indicating an increase in cellular injury severity with the dose. Hepatic hydropic cell swelling was additionally observed [Figure 2d and g]. This was due to the accumulation of water in the cytoplasm, indicating a severe but reversible hepatic injury, which is not yet lethal but can result in liver lytic necrosis if the causative is continued [44]. To date, there is no study of the PI extract regarding its toxicological effects inducing hepatic histopathology. There is only one study claimed for the hepatoprotective effect of PI extract in Wistar albino rats induced hepatotoxicity using paracetamol. The PI extract at 200 and 400 mg/kg/day for 14 days were reported to prevent hepatic histopathological injury caused by paracetamol [45]. However, in the present study, the hepatic histopathology caused by the PI extract was indicated from the dose of 200 up to 1,000 mg/kg/day. The differences in experimental species and strains possibly affect the bioavailability of the PI extract including their susceptibility to toxicity. In addition, the solvent and extraction condition which were different between the studies of Rajasekaran *et al*. [45] (Soxhlet extraction using ethanol at 68°C for 72 h) and our study (soxhlet extraction using methanol at its boiling temperature for 3 h) might influence on the amounts of constituents in the extracts. Hence, the outcomes are possibly not the same.

Hepatocytes contain intracellular ALT and AST enzymes [46]. AST is additionally found in skeletal muscles and erythrocytes. Therefore, AST is a biomarker indicating the damage of liver and other organs [47], whereas ALT is rather quite specific to liver injury [48]. Elevations of these two intracellular transaminase enzymes in the serum indicate leakage of hepatocytes, therefore suggesting liver injury [49]. Plumbagin and the PI extract significantly induced both ALT and AST in the mouse plasma suggesting the occurrence of liver injury. In addition, elevation of the plasma AST level was more prominent than that of the ALT, indicating a possibility of injury in extrahepatic organs.

TBARS assay measures formation of MDA, a product resulting from oxidative damage of lipid [50]. The dose-dependent increase in the MDA levels observed in the mouse livers after the treatment with either plumbagin or the PI extract suggested that these treatments lead to lipid peroxidation in the hepatic tissues. By contrast, a single intraperitoneal dose of plumbagin at <4 mg/kg was reported to decrease hepatic MDA levels, protecting against oxidative damage in adult male Wistar rats [51]. In addition, *Plumbago zeylanica* (white leadwort) extract provided a hepatoprotective effect in alcohol intoxicated Wistar albino rats [52]. It is likely that these different findings result from differences in species and strains of experimental animals, cultivation and harvesting of the herb, the method of extraction, and the dosage regimens employed in the different studies. Our investigation observed the effects of plumbagin and the PI extract in the ICR mice at a stronger dosage regimen with a longer period of administration, representing the Thai traditional regimens of PI.

The antioxidative enzymes, SOD, CAT, and GPx, provide the first line of defense against oxidative damage [53]. SOD is the first enzyme in the antioxidant armory, catalyzing superoxide

into H_2O_2, which is then converted into oxygen and water by GPx and CAT [53]. GPx is responsible for the detoxification process when low amounts of H_2O_2 are present, followed by CAT after saturation of GPx [54]. We observed an inhibitory effect of plumbagin and the PI extract on the hepatic SOD and CAT activities, whereas the GPx activity was provoked. Correspondingly, plumbagin was reported to modify the expression of genes involved in ROS metabolism; down-regulation of CAT and mitochondrial Mn-SOD expression while up-regulating the expression of GPx in LNCaP cells [55].

GSH is a non-enzymatic intracellular antioxidant present in all mammalian tissues; however, it is most abundant in the liver which is the main site of GSH synthesis [56]. Total GSH content consists of the reduced form (GSH) plus the oxidized form (GSSG). During oxidative stress, GSH is oxidized to GSSG, which is then converted back to its reduced form by GSH reductase (GR). Over-capacity of GR leads to the export of excess GSSG out of the cell [56]. In our hands, both plumbagin and the PI extract depleted the hepatic GSH content while increased the hepatic GSSG content, resulting in a decrease in the GSH/GSSG ratio and imbalance of the oxidant-antioxidant system.

This imbalance in the cellular redox status ultimately results in hepatic oxidative stress as demonstrated by the elevation of hepatic MDA levels. Prolong oxidative stress then results in chronic inflammation and cellular injury [29]. Hence, we propose a model for plumbagin- and the PI-induced hepatotoxicity; plumbagin and the PI extract initiated oxidative stress in the hepatic tissue through an imbalance of the hepatic antioxidative system: A decrease in SOD and CAT activity along with an increase in GPx activity and deterioration of the GSH antioxidant system. These findings resulted in increases in the hepatic lipid peroxidation and hepatocyte injury as demonstrated by the increases in plasma ALT and AST levels, including hepatic histopathological changes.

CONCLUSION

Herewith the specific hepatotoxic properties of plumbagin and the PI extract were underlined. The findings suggest that practitioners should be cautious for the use of plumbagin or PI-containing supplements, especially if they are consumed at high quantity or for a long period. In addition, it is worth for further study on the pharmacology and toxicology of plumbagin and the PI extract in depth at the molecular mechanism.

ACKNOWLEDGMENTS

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Original Research

Methanolic leaf extract of *Gymnema sylvestre* augments glucose uptake and ameliorates insulin resistance by upregulating glucose transporter-4, peroxisome proliferator-activated receptor-gamma, adiponectin, and leptin levels *in vitro*

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ABSTRACT

Aims: The present study was undertaken to evaluate the effect of methanolic leaf extract of *Gymnema sylvestre* (MLGS) on glucose transport (GLUT) and insulin resistance *in vitro*. **Materials and Methods:** Peroxisome proliferator-activated receptor-gamma (PPAR-γ) and GLUT-4 expression were assessed in L6 myotubes for concluding the GLUT activity, and adiponectin and leptin expression was studied in 3T3 L1 murine adipocyte cell line to determine the effect of MLGS (250-750 μg/ml) on insulin resistance. **Results:** The findings of the experiments have demonstrated a significant and dose-dependent increase in glucose uptake in all the tested concentrations of MLGS, further the glucose uptake activity of MLGS (750 μg/ml) was at par with rosiglitazone (50 μg/ml). Concomitantly, MLGS has shown enhanced GLUT-4 and PPAR-γ gene expressions in L6 myotubes. Furthermore, cycloheximide (CHX) had completely abolished the glucose uptake activity of MLGS when co-incubated, which further confirmed that glucose uptake activity of MLGS was linked to enhanced expression of GLUT-4 and PPAR-γ. In addition, in another experimental set, MLGS showed enhanced expression of adiponectin and leptin, thus confirms the ameliorative effect of MLGS on insulin resistance. **Conclusion:** These findings suggest that MLGS has an enhanced glucose uptake activity in L6 myotubes, and ameliorate the insulin resistance in 3T3 L1 murine adipocyte cell line *in vitro*.

KEY WORDS: Adiponectin, antidiabetic activity, diabetes, glucose transport, *Gymnema sylvestre*, leptin, L6 myotubes, peroxisome proliferator-activated receptor-gamma, 3T3 L1 murine adipocyte cell line

INTRODUCTION

Diabetes is a metabolic disorder of diverse etiology, manifested as hyperglycemia, polyuria, polydipsia, and polyphagia, characterized by disturbed carbohydrate, fat, and protein metabolism, due to defects in insulin secretion, and/or insulin action, or both [1]. Various classes of synthetic drugs are available for the treatment of diabetes; however, the inherent deleterious effects associated with these drugs have made a massive scope for discovering better and safe drug for the treatment of diabetes. In this perspective, plant-based medicines are believed to have great avenue over synthetic classes of drugs, and in recent times, many researchers have found numerous herbal medicines as highly promising in the treatment of diabetes [2]. In this context, *Gymnema sylvestre* is a very popular plant well known for its antidiabetic property, and it was scientifically validated for antidiabetic and hypoglycemic activity [3-6]. However, the molecular mechanism behind the hypoglycemic and antidiabetic activity of *G. sylvestre* remain uncertain, in this context we thought to evaluate the effect of *G. sylvestre* on glucose transporter-4 (GLUT-4), peroxisome proliferator-activated receptor-gamma (PPAR-γ), adiponectin, and leptin gene expression levels *in vitro*. GLUT-4 is one of the major GLUTs known to facilitate the glucose uptake in major tissues such as skeletal muscle, thus abnormal and/or decreased expression of GLUT-4 leads to diminished glucose uptake into the target tissues [7,8]. Further, PPAR-γ is a transcription factor belonging to the nuclear receptor superfamily, which is known to play a vital role in insulin signaling induced glucose uptake, and the role PPAR-γ in the development of insulin resistance has been scientifically well demonstrated, and therefore, the drugs that enhance PPAR-γ expression and/or acts as PPAR-γ agonists play a key role in reversing the insulin resistance [9-13].

In addition, adiponectin and leptin are the fundamental insulin-sensitizing adipokines secreted by adipocytes [14]. Adiponectin enhances insulin sensitivity and helps in controlling dyslipidemia through AMPK activation and increased fatty acid (FA) oxidation [15,16]. Scientifically, it is ascertained that abnormal and/or diminished expression of adiponectin leads to insulin resistance, dyslipidemia, and atherosclerosis in rodents and humans [17]. Similarly, leptin is an another adipocytokine produced by adipocytes, and it is known to play a remarkable role in controlling body weight, metabolism, and reproductive functions [18], Leptin presents in central nervous system acts as a checkpoint to control the food consumption, in short low levels of leptin leads to increased food consumption, and it is very clearly demonstrated in leptin knockout animals, and further leptin levels are observed to be significantly low in obese individuals compared to healthy volunteers [19,20]. Concisely, the available scientific literature suggests that GLUT-4, PPAR-γ, adiponectin, and leptin levels have a positive correlation in controlling the blood glucose levels and insulin resistance in patients suffering from diabetes and its complications.

Considering the potential anti-diabetic property of *G. sylvestre*, we thought to explore the possible correlation between the anti-diabetic activity of *G. sylvestre* and GLUT-4, PPAR-γ, adiponectin, and leptin gene expressions *in vitro*.

With the above hypothesis, the present study was designed to explore the possible mechanism behind the hypoglycemic and antidiabetic activity of *G. sylvestre*, through evaluating its effect on GLUT-4, PPAR-γ, adiponectin, and leptin gene expression *in vitro*.

MATERIALS AND METHODS

Reagents, Chemicals, and Cell Lines

Avian moloney leukemic virus (AMLV) reverse transcriptase (RT) and taq polymerase (dNTP) were purchased from Thermo Scientific, USA. Insulin, rosiglitazone, TRI reagent, cell culture supplements, and reagents used were of molecular biology grade and purchased from Sigma-Aldrich, St. Louis, USA.

Collection of Plant Material

The leaves of *G. sylvestre* were collected from Hospete (Bellary District, Karnataka, India) in the month of May-June 2013, considering the seasonal conditions for obtaining maximum phytoconstituents, and were authentified by Dr. K. P. Sreenath, Professor, Department of Botany, Bangalore University, Bengaluru, Karnataka, India. A voucher specimen of the plant material is preserved in the department with specimen no. 2013-14/GS/BT-01.

Plant Material Preparation

The shade dried leaf powder of *G. sylvestre* was subjected to extraction process as follows. Exactly 50 g of the plant material was extracted with various organic solvents successively in the ascending order of polarity (hexane, dichloromethane, ethylacetate, and methanol) in Soxhlet apparatus. In brief, 50 g of the plant material was initially extracted with 1000 ml hexane at 60°C for 24 h, the marc obtained was completely dried and extracted with 1 L of dichloromethane for 24 h, and subsequently, the marc obtained was extracted with 1 L of ethyl acetate and followed by 1 L of methanol.

Extracts were concentrated by Rotary evaporator (Rotavap-Remi Instrument) under reduced pressure at room temperature, and 4 mg of dried extract was reconstituted to 4 ml with respective solvents and diluted to attain the final concentration 750 μg/ml, 500 μg/ml, and 250 μg/ml for glucose uptake studies.

Propagation and Maintenance of L6 Cells and 3T3 L1

L6 cells (Rat skeletal muscle), cell culture was procured from National Centre for Cell Sciences (NCCS), Pune, Maharashtra, India. L6 cells were cultured and maintained in Dulbecco's modified Eagle's medium (DMEM) with 10% inactivated fetal bovine serum (FBS) along with penicillin (100 μg/ml), streptomycin (100 μg/ml), and amphotericin B (5 μg/ml) in an humidified atmosphere of 5% CO_2 at 37°C until confluent. The cells were dissociated with trypsin phosphate versene glucose solution (0.2% trypsin, 0.02% EDTA, and 0.05% glucose in phosphate buffered saline). The stock cultures were grown in 25 cm^2 culture flasks, and the experiments were carried out

in 60 mm petri dishes and 96 well microtiter plates (Tarsons India Pvt., Ltd., Kolkata, West Bengal, India) [21].

3T3 L1 adipocyte cell culture was procured from NCCS, Pune, Maharashtra, India. The cells were grown to confluence in DMEM containing glucose. The monolayer of the 3T3 L1 cells was trypsinized and resuspended at 1×10^4 cells/ml in DMEM with 10% FBS. 0.5 ml of cell suspension was seeded per well in 24 well plates (Tarsons India Pvt. Ltd., Kolkata, India); the plates were incubated and allowed to attain the confluent monolayer. Growth medium was removed and followed by the addition of adipogenesis initiation media containing 0.5 mm 3-iso butyl-1-methyl xanthine, 10% FBS and 1 μm dexamethasone in DMEM. Initially, the cell lines were incubated for 48 h at 5% CO_2 at 37°C, after incubation adipogenesis initiation media was replaced by adipogenesis progression media (DMEM with 10% FBS and 10 μm/ml insulin). Subsequently, the plate was incubated for 48 h at 5% CO_2 at 37°C, and then adipogenesis progression media was removed, and the cells were treated with various concentrations of test extracts along with controls in adipogenesis maintenance media (DMEM with 10% FBS). The test drugs samples were dissolved in DMEM media and incubated with adipogenesis maintenance media [22].

Glucose Uptake Assay

The fully differentiated L6 myotubes were serum starved overnight and washed with HEPES in KREB's Ringer phosphate solution (KRP buffer) and incubated with KRP buffer with 0.1% bovine serum albumin for 30 min at 37°C. Myotubes were treated with various concentrations of test drug, and standard along with vehicle controls in 60 mm Petri plates, D-glucose solution was added to all the plates and incubated for 30 min at 37°C. Subsequently, the liquid medium was aspirated from all the plates to terminate the glucose uptake, and the cells were washed thrice with ice-cold KRP buffer solution. Further, the cells were lysed with 0.1 M NaOH solution and an aliquot of the cell lysates were used to measure the cell-associated glucose. Glucose uptake was estimated by Biovision Kit Inc., USA. Three independent experimental values in duplicates were taken to determine the percentage enhancement of glucose uptake over controls [23,24].

RT-Polymerase Chain Reaction (RT-PCR)

RT-PCR was carried out as per Kumar *et al*., 2015 [21,23]. In short, after the completion of the incubation period, the cells were lysed in TRI reagent, proteins were extracted with chloroform, and the total RNA was precipitated with isopropanol. The RNA precipitate was washed with 70% ethanol and resuspended in 50 μl of DEPC-treated water. Reverse transcription was carried out using 200 units of avian RT and 200 ng/μl oligo d(T)18. The primers used were as follows, GLUT-4: Sense, 5'-CGG GAC GTG GAG CTG GCC GAG GAG-3'; anti-sense, 5'CCC CCT CAG CGA GTG A-3' (318-bp), [12]; PPAR-γ; sense, 5'-GGA TTC ATG ACC AGG GAG TTC CTC-3'; anti-sense, 5'-GCG GTC TCC ACT GAG AAT AAT GAC-3' (155-bp), [12]; glyceraldehydes-3-phosphate dehydrogenase (GAPDH); sense, 5'CCA CCC ATG GCA AAT TCC ATG GCA-3'; anti-sense, 5'-TCT AGA CGG CAG GTC AGG TCC ACC-3' (588-bp) [12]. For PCR reaction, 1 μl of cDNA mixture was added to a PCR reaction mix containing ×10 PCR buffer, 2 mM dNTP, 10 pM of paired primers, 2 units of dNTP. PCR products were run on 1.5% agarose gels, stained with ethidium bromide and photographed.

Total RNA was obtained from cultured 3T3-L1 adipocytes (Day 8) as per previously mentioned procedures. cDNA was generated from total RNA and synthesized using a high capacity cDNA reverse transcription kit. The PCR conditions were as follows: For GAPDH, 25 cycles of 95°C for 30 s, 55° C for 30 s.

The primers used are adiponectin: Sense, 5'-GTTCTACTGCAACATTCCGG-3'; antisense, 5'-TACACCTGGAGCCAGACTTG-3'; leptin: Sense, 5'-TGTGCTGCAGATAGCCAATG-3'; Antisense, 5'-AGGGAGCAGCTCTTGGAGAAG-3'. To facilitate comparisons between sample groups, quantities of all targets in test samples were normalized to GAPDH. PCR products were run on 1.5% agarose gels, stained with ethidium bromide and photographed [12].

Statistical Analysis

All the values were expressed as mean ± standard error of mean. The results were analyzed statistically using one-way ANOVA followed by Tukey's multiple comparison test using GraphPad Prism version 4.0 for Windows, GraphPad Software, San Diego California USA. The minimum level of significance was fixed at $P < 0.05$.

RESULTS

Extraction of Plant Material

The leaf powder of *G. sylvestre* was successively extracted with dichloromethane, hexane, ethyl acetate, and methanol. The extractive values of dichloromethane, hexane, ethylacetate, and methanol were found to be 0.52, 0.7, 2.5, and 3.3% w/w (gram per gram), respectively. In a pilot study, all the extracts were subjected for glucose uptake assay using L6 myotube and based on the findings methanolic extract was found to be promising; hence, only methanolic extract was subjected for further studies.

Evaluation of Glucose Uptake

Glucose uptake activity of MLGS was assessed using rosiglitazone (50 μg/ml) as the reference standard, and the dose of rosiglitazone was selected based on the pilot plant study performed in house (unpublished data).

The MLGS at concentrations ranging from 250 to 750 μg/ml showed dose-dependent stimulation of glucose uptake. Noteworthy, MLGS at 750 μg/ml concentration has offered approximately 2-fold increase in glucose uptake from basal glucose concentration, similarly, rosiglitazone also showed a substantial increase in glucose uptake. The results are given in Figure 1.

Further, the outcome of MLGS on glucose uptake was thought to be intervened through increased expression of GLUT-4 and PPAR-γ synthesis and thereby enhanced protein synthesis. To authenticate this hypothesis, another experiment was carried out connected to the glucose uptake activity of MLGS, in presence and absence of a protein synthesis inhibitor cycloheximide (CHX) in L6 myotubes. The findings have revealed complete blockade of glucose uptake activity of MLGS in the presence of CHX; these findings propose that the elevated glucose uptake activity of MLGS is due to enhanced protein synthesis [Figure 2].

Effect of MLGS on GLUT-4, PPAR-γ, Adiponectin, and Leptin Transcription Level

We determined the outcome of MLGS on GLUT-4, PPAR-γ, adiponectin, and leptin mRNA expression by semi-quantitative RT-PCR. MLGS has significantly elevated the GLUT-4 transcript level compared to control. A depictive image of agarose gel

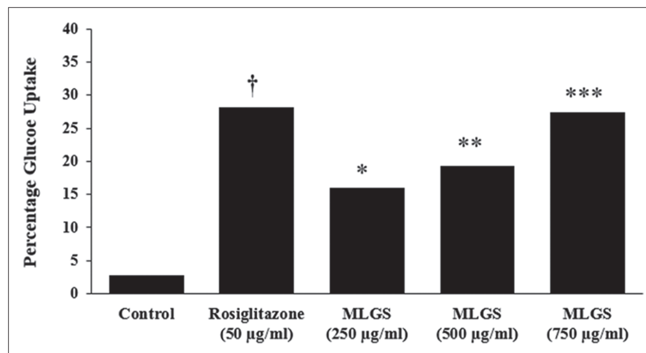

Figure 1: Glucose uptake activity of methanolic leaf extract of *Gymnema sylvestre* (MLGS) in L6 myotubes. All of the values are expressed as x ± standard error of mean (n = 3); means of various groups were statistically compared by ANOVA followed by Tukey's multiple comparison test using Graph Pad version 4.0. $^{\dagger}P$ < 0.001 corresponds to rosiglitazone versus control; $^{*}P$ < 0.05, $^{**}P$ < 0.01, $^{***}P$ < 0.01 corresponds to MLGS versus control

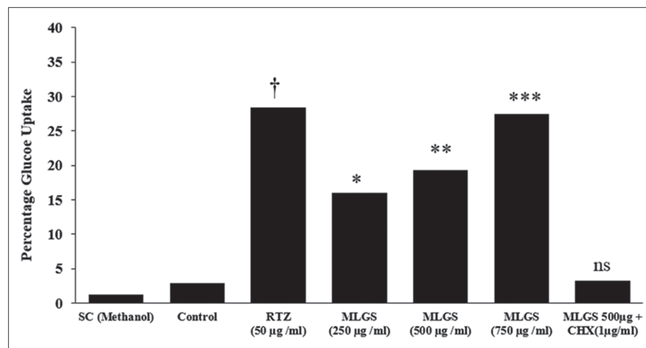

Figure 2: Effect of cycloheximide on methanolic leaf extract of *Gymnema sylvestre* (MLGS) intervening glucose uptake in L6 myotubes. ns: Not significant. The values are $\bar{x}$ ± Standard error of mean (n = 3) of independent experiments, means of various groups were statistically compared by ANOVA followed by Tukey's multiple comparison test using Graph Pad version 4.0. $^{\dagger}P$ < 0.001 corresponds to rosiglitazone versus control; $^{*}P$ < 0.05, $^{**}P$ < 0.01, $^{***}P$ < 0.01 corresponds to MLGS versus control. ^{ns}P > 0.05 corresponds to MLGS 500 µg/ml + cycloheximide versus MFMC 500 µg/ml

[Figure 3a and b] and The corresponding densitometry scanning [Figure 3c] confirms approximately 7.1-fold increase in GLUT-4 expression in MLGS (750 μg/ml) group and 4.6-fold increase in rosiglitazone group compared to control [Figures 3a-c].

As a second step, we studied the role of PPAR-γ in GLUT activity exhibited by MLGS. In findings, the MLGS has shown significant and dose-dependent upregulation of PPAR-γ compared to control, the higher dose of MLGS (750 μg/ml) showed approximately ~ 2.5-fold increase in PPAR-γ expression compared to control, similarly, the reference standard rosiglitazone has also shown a significant increase in PPAR-γ compared to control. The results are shown in Figure 4a-c.

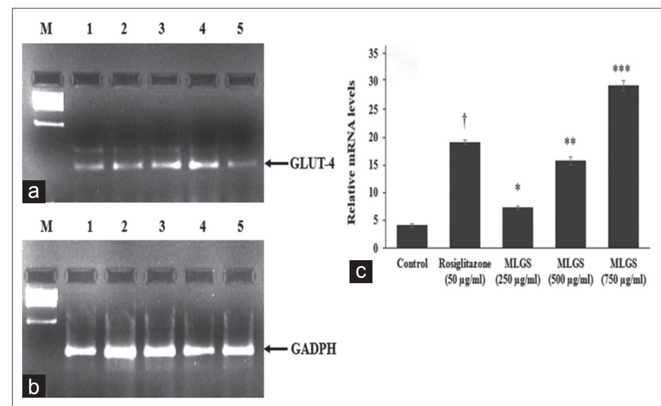

Figure 3: Effect of the methanolic leaf extract of *Gymnema sylvestre* (MLGS) on glucose transport-4 transcripts in L6 myotubes. (a and b) - M: Ikbp marker, Lane 1: 250 µg/ml MLGS, Lane 2: 500 µg/ml MLGS, Lane 3: 750 µg/ml MLGS, Lane 4: 50 µg/ml rosiglitazone, Lane 5: Control. (c) The values are x ± standard error of mean (n = 3) of independent experiments, means of various groups were statistically compared by ANOVA followed by Tukey's multiple comparison test using Graph Pad version 4.0. $^{\dagger}P$ < 0.001 corresponds to rosiglitazone versus control; $^{*}P$ < 0.05, $^{**}P$ < 0.01, $^{***}P$ < 0.01 corresponds to MLGS versus control

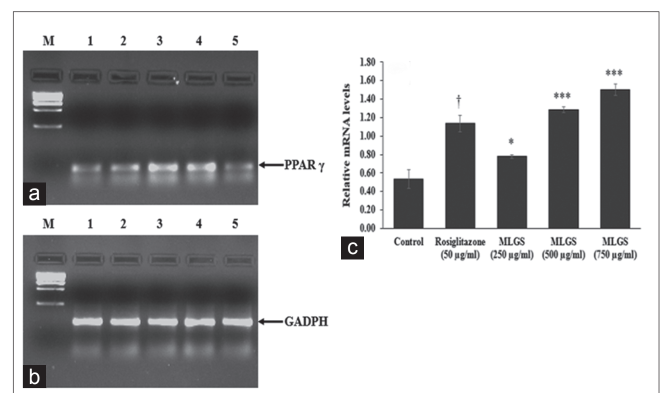

Figure 4: Effect of the methanolic leaf extract of *Gymnema sylvestre* (MLGS) on peroxisome proliferator-activated receptor-gamma transcripts in L6 myotubes. (a and b) - M: Ikbp marker, Lane 1: 250 µg/ml MLGS, Lane 2: 500 µg/ml MLGS, Lane 3: 750 µg/ml MLGS, Lane 4: 50 µg/ml rosiglitazone, Lane 5: Control. (c) The values are x ± standard error of mean (n = 3) of independent experiments, means of various groups were statistically compared by ANOVA followed by Tukey's multiple comparison test using Graph Pad version 4.0.$^{\dagger}P$ < 0.001 corresponds to rosiglitazone versus control; $^{*}P$ < 0.05, $^{***}P$ < 0.01 corresponds to MLGS versus control

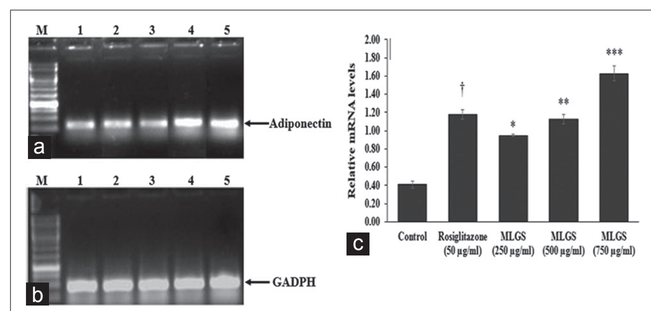

Figure 5: Effect of the methanolic leaf extract of *Gymnema sylvestre* (MLGS) on Adiponectin transcripts in 3T3 L1 cell lines. (a and b) - M: lkbp marker, Lane 1: Control, Lane 2: 50 µg/ml rosiglitazone, Lane 3: 250 µg/ml MLGS, Lane 4: 500 µg/ml MLGS, Lane 5: 750 µg/ml MLGS, (c) The values are x ± standard error of mean (n = 3) of independent experiments, means of various groups were statistically compared by ANOVA followed by Tukey's multiple comparison test using Graph Pad version 4.0. †P < 0.001 corresponds to rosiglitazone versus control; *P < 0.05, **P < 0.01, ***< 0.01 corresponds to MLGS versus control

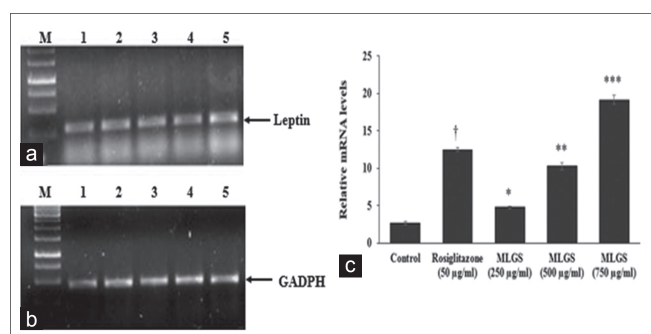

Figure 6: Effect of the methanolic leaf extract of *Gymnema sylvestre* (MLGS) on leptin transcripts in 3T3 L1 cell lines. (a and b) M: lkbp marker, Lane 1: Control, Lane 2: 50 µg/ml rosiglitazone, Lane 3: 250 µg/ml MLGS, Lane 4: 500 µg/ml MLGS, Lane 5: 750 µg/ml MLGS, (c) The values are x ± standard error of mean (n = 3) of independent experiments, means of various groups were statistically compared by ANOVA followed by Tukey's multiple comparison test using Graph Pad version 4.0.†P < 0.001 corresponds to rosiglitazone versus control; *P < 0.05, **P < 0.01, ***P < 0.01 corresponds to MLGS versus control

Based on the above findings, it was concluded that MLGS could upregulate the expression of GLUT-4 and PPAR-γ, and thereby increased the glucose uptake *in vitro*. Further, the glucose uptake activity of MLGS was completely abolished in the presence of CHX (1 µg/ml), which is a protein synthesis inhibitor, thus it was confirmed that glucose uptake activity of MLGS is mediating through enhanced expression of GLUT-4 and PPAR-γ. Furthermore, in another experimental set, MLGS has shown enhanced expression of adiponectin, leptin by 3.9-fold and 7.1-fold, respectively, compared to control [Figures 5a-c and 6a-c] Collectively, it can be concluded that MLGS shows hypoglycemic activity through enhanced expression of GLUT-4, and reverses insulin resistance by enhancing the expression of PPAR-γ, adiponectin, and leptin, respectively.

DISCUSSION

Diabetes is a metabolic disorder with varied etiology, many important biochemical entities involved in the development of abnormal GLUT. In this context, the markers such as GLUT-4, PPAR-γ, adiponectin, and leptin are well known to play a crucial role in insulin-dependent glucose transport [25]. The declined GLUT-4 translocation and defective insulin signaling cascade were demonstrated as one among the prevalent factors in type 2 diabetes and insulin resistance [25]. Thus, the agents that enhance the GLUT-4 expression could decrease the circulating blood glucose levels by enhancing cellular glucose uptake. In this context, L6 muscle cell line is a relevant *in vitro* model to study the GLUT activity; moreover, skeletal muscle is the main site for primary glucose clearance and glucose utilization [26]. Earlier reports of L6 myotubes manifested the maximum glucose uptake activity by troglitazone and rosiglitazone at 10 and 100 µM concentrations, respectively [10]. In addition, the enhanced glucose uptake in L6 cells is majorly intervened through increased GLUT-4 level [26,10]. In these lines, the findings in the present study are in compliance with the literature reports where there is a coincidental increase in glucose uptake along with enhanced GLUT-4 levels in L6 myotubes, the MLGS has shown increased glucose uptake, and facilitated the enhanced GLUT-4 expression. In literature, CHX was used as a protein synthesis inhibitor to completely abolish the troglitazone-mediated glucose uptake [26]. Similarly, in the present study, the glucose uptake activity of MLGS was completely blocked in the presence of CHX, which clearly confirmed the role of new protein synthesis compatible to GLUT.

Altogether, the above findings proved that glucose uptake is dependent on increased or decreased expression of GLUT-4, further enhanced glucose uptake in the present study was concomitant with the increased expression of GLUT-4 encoding mRNA, in L6 myotubes on incubation with MLGS [27].

Furthermore, PPAR-γ is a nuclear transcriptional factor known for its pivotal role in the insulin receptor signaling cascade in glucose uptake, also the levels of PPAR-γ is noted to be less in insulin resistance. In this context, the PPAR-γ agonists belong to the chemical class of thiazolidinedione's (TDZs) (such as rosiglitazone and pioglitazone) play a major role in reversing the insulin resistance [28]. Further, PPAR-γ activation leads to enhanced glucose-stimulated insulin secretion and increased insulin sensitivity [29].

In continuation, adiponectin is an abundantly expressed adipokine that exerts a potent insulin-sensitizing effect through binding to its receptors AdipoR1 and AdipoR2, leading to activation of AMPK, PPAR-α, and presumably other yet-unknown signaling pathways. Thus, the serum levels of adiponectin have a positive correlation with insulin sensitivity [30]. Adiponectin is known to increase insulin sensitivity by three major mechanisms, First, in skeletal muscle adiponectin increases the expression of CD36 and acyl-coenzyme-A oxidase molecules involved in transport and combustion of FA, and also increases uncoupling protein 2 required during energy dissipation. These changes led to decreased triglyceride content in skeletal muscle, which contributes to improved insulin signaling transduction [17]. Furthermore, adiponectin stimulates phosphorylation of acetyl coenzyme-A carboxylase, increases fatty-acid combustion,

glucose uptake, and lactate production in myocytes, through activation of AMPK. These changes reduce gluconeogenesis in the liver; thus, adiponectin shows acute glucose lowering effect [17]. Remarkably, the PPAR-γ agonist belongs to TZDs class are well known to raise the circulating adiponectin levels, this is well demonstrated by exposing 3T3-L1 cells to TZDs [31].

In line with the above reports, in the present study, the effect of MLGS on adiponectin levels was evaluated using 3T3-L1 cell lines, and the outcomes of the study showed a significant increase in expression of adiponectin in MLGS and rosiglitazone-treated groups.

In obesity-linked insulin resistance, both adiponectin and adiponectin receptors are downregulated. Apart from adiponectin, Leptin is another very important adipocytokine majorly secreted by adipocytes, the net action of leptin is to inhibit appetite, stimulate thermogenesis, decrease blood glucose, and reduce body weight and fat. Leptin also directly affects glucose metabolism in the liver, by inhibiting gluconeogenesis mediated through phosphatidylinositol 3-kinase (PI3K)-dependent activation of phosphodiesterase 3B [32], interferes with lipid metabolism mediated through activation of PI3K [33]. Further, many studies have confirmed a complex interaction between leptin and insulin-signaling pathways on glucose metabolism. The observations from *in vitro* and *in vivo* studies suggest that leptin promotes energy dissipation and decreases lipid deposition in adipose tissues [33].

Leptin receptor-mediated JAK–STAT signaling is essential for regulation of food intake and body weight. It also limits the accumulation of triglycerides in the liver and skeletal muscle through a combination of direct activation of AMPK and indirect actions mediated through central neural pathways, thereby improving insulin sensitivity [34]. In addition, leptin regulates pancreatic β-cells function through direct actions [35] and indirectly through central neural pathways [36].

On the whole, leptin enhances peripheral (hepatic and skeletal muscle) insulin sensitivity and modulates pancreatic β cell function. Leptin resistance/deficiency leads to reduced leptin-mediated JAK–STAT signaling and upregulation of suppressor of cytokine signaling-3 (SOC-3). In contrary, expression of leptin is directly related to insulin sensitization and activation of JAK-STAT signaling thereby reducing SOC-3. Altogether, expression of leptin has profound positive relation with the glucose metabolism and insulin signaling.

In this regard, in the present study, the effect of MLGS was examined on the expression of leptin in 3T3-L1 cells *in vitro* using rosiglitazone as the reference standard. A significant increase in leptin levels was observed in MLGS and rosiglitazone-treated groups. These findings observed are in constituent with the GLUT-4, PPAR-γ, adiponectin and leptin gene expressions to link with the hypoglycemic and antidiabetic activity of MLGS.

CONCLUSION

The role of GLUT-4, PPAR-γ, adiponectin, and leptin in insulin dependent glucose transport and insulin resistance is well understood with available scientific literature, and thus, these factors have a key role in etiology of diabetes and related complications.

In the present study, an attempt was made to investigate the mechanism involved in hypoglycemic and antidiabetic activity of methanolic leaf extract of G. *sylvestre*.

The findings of the study showed that MLGS could dose-dependently enhance the expression of GLUT-4, PPAR-γ, adiponectin, and leptin, and thereby shows potential antidiabetic activity. Interestingly, the actives responsible for hypoglycemic and antidiabetic activity of MLGS needs to be isolated, in this regard, further studies are in the pipeline to identify and isolate the phytoconstituent responsible for antidiabetic activity of MLGS.

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Original Research

Anticoagulant effect and safety assessment of an aqueous extract of *Pseudocedrela kotschyi* (Schweinf.) harms and *Adenia cissampeloides* (Planch. Ex Hook.) harms

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ABSTRACT

Background: Currently available therapeutic options for thromboembolic disorders are often very expensive and are associated with unfavorable side effects. **Aim:** To establish the anticoagulant effect and safety profile of an extract made from of the root bark of *Pseudocedrela kotschyi* (Schweinf.) Harms and the aerial part of *Adenia cissampeloides* (Planch. ex Hook.) Harms (PAE). **Materials and Methods:** PAE (0.5-2.0 g/L) effect on prothrombin time (PT) and activated partial thromboplastin time (aPTT) were evaluated on whole blood drawn from the marginal ear vein of New Zealand White rabbits. Effect of PAE (250-2000 mg/kg) on bleeding time (BT) and clotting time (CT) in Sprague-Dawley rats were also assessed. Histopathological, hematological, and liver function studies were also carried out to assess the safety for use of PAE (250-2000 mg/kg). **Results:** PAE had no significant effect ($P > 0.05$) on PT, but resulted in a significant increase ($P \leq 0.05$-0.0001) in aPTT. The PAE treatment resulted in a significant increase ($P \leq 0.05$-0.0001) in BT and CT *in vivo* compared with control. Safety studies indicated no deaths with PAE treatment with hematological and liver function tests being normal. Histological studies revealed pathological changes in the liver at a PAE treatment dose of 2000 mg/kg but all doses had no detrimental effect on kidney and stomach tissue. The no-observed-adverse-effect-level was <2000 mg/kg when given orally. **Conclusion:** PAE has anticoagulant effect *in vitro* and is safe to use at oral doses <2000 mg/kg.

KEY WORDS: Activated partial thromboplastin time, *Adenia cissampeloides,* bleeding time, clotting time, prothrombin time, *Pseudocedrela kotschyi*

INTRODUCTION

Thromboembolic disorders have become a leading cause of morbidity and mortality and an important challenge confronting physicians and surgeons, the world over [1]. The cause of thromboembolism is usually unknown but is by convention attributed to Virchow's triad: Hemodynamic changes (stasis and turbulence), hypercoagulability, and endothelial injury [2]. Despite it being a preventable condition, thromboembolism remains a leading cause of morbidity and mortality globally, and Ghana is no exception. Deep vein thrombosis (DVT) and pulmonary embolism (PE) occur in 1 in 1000 patients annually in adult patients with the frequency of occurrence increasing with old age [3]. About 6% and 10% of patients die within a month following an episode of DVT and PE, respectively [4]. The impact of DVT and PE goes beyond the increased risk of mortality. For patients who survive these conditions, they sometimes will have to live with the dilapidating effect, e.g., loss or impairment of bodily function due to stroke, and post-thrombotic syndromes such as ulcers due to months of being bed ridden and impaired blood circulation resulting in gangrene [5], just to mention a few.

Anticoagulants remain the mainstay in the pharmacological management of thromboembolic disorder and have proven over the years to be effective and safe [6]. Despite the effectiveness of conventional pharmacological agents in the management of thromboembolic disorders, they are often associated with unfavorable side effects such as increased risk of bleeding and gastric ulceration and also prohibitive high treatment cost [7].

With the high cost and unfavorable side effects of orthodox medicines, there is the need to search for alternative therapeutic agents and the plant kingdom continues to show huge promise.

Pseudocedrela kotschyi (Schweinf.) Harms of the family Meliaceae, and *Adenia cissampeloides* (Planch. ex Hook.) Harms of the family Passifloraceae have found numerous uses in traditional medicine [8-11], e.g., *P. kotschyi* traditionally has been used in the management of several diseases and ailments. Decoctions and macerations of the stem and root bark have found use in the management of ulcers, sores, rheumatism, leprosy, syphilis and gingivitis [12]. Crushed leafy twigs and leaf decoctions have also been employed in the management of edema, rash and compound fractures with young stems and roots used as chewing stick to keep the teeth healthy. Research into the medicinal properties of *P. kotschyi* such as antimicrobial, antiprotozoal, and antidiabetic effect of *P. kotschyi* and its constituents have shed more light and sought to justify the folkloric medicinal use of the plant [13-16].

Traditionally, A. *cissampeloides* has found several uses in folkloric medicinal practice in infusions and decoctions of the stem, leaves or root have been employed in the management of gastrointestinal disorders such as, constipation, stomach ache, diarrhea, and dysentery. Such infusions have also been used in the management of rheumatic pain, numbness, malaria, wound dressing and worm infestations [17]. Studies that have been carried out on the medicinal properties of A. *cissampeloides* include: the antiplasmodial activity, antimicrobial properties and effect on blood pressure and serum analytes [18].

In traditional medicinal practice in Ghana, mixtures of extracts of the root bark of *P. kotschyi* and the aerial part of A. *cissampeloides* are currently employed in the management of circulatory disorders such as numbness.

This current study, therefore, sought to investigate the anticoagulant properties and safety profile of an extract made from the root bark of *P. kotschyi* and the aerial part of A. *cissampeloides* using *in vitro* and *in vivo* experimental protocols.

MATERIALS AND METHODS

Plant Collection and Authentication

A. *cissampeloides* and *P. kotschyi* [Figure 1a and b] were collected from Tetrem near Offinso and Ayigya near KNUST, respectively; in the Ashanti Region of Ghana in February 2015. Plant materials were verified at the Department of Herbal Medicine, KNUST, where voucher specimen (numbers: KNUST/HM1/2013/S043 and KNUST/HM1/2013/S048) have been kept.

Preparation of *P. kotschyi* and A. *cissampeloides* extract (PAE)

PAE was prepared by boiling 1.5 kg of root bark of *P. kotschyi* and 1.0 kg of the aerial part of A. *cissampeloides* in 10 L of distilled water. The mixture was boiled for 45 min and cooled. The supernatant solution was decanted and dried, using a freeze drier (YK-118 Vacuum Freeze Drier, True Ten Industrial Company, Taiwan), to obtain solid pellets (percentage yield = 0.45%). The solid pellets were weighed and labeled PAE. Concentrations of PAE were prepared by weighing the required amount of pellets on an electronic balance (Sartorious AG, Germany) and dissolving the pellets in the required amount of distilled water.

Figure 1: (a) The root bark of *Pseudocedrela kotschyi* and (b) the aerial part of *Adenia cissampeloides*

Drugs and Chemicals

The drugs and chemicals used in this study included: Heparin sodium (Rotexmedica, Germany), soluble aspirin tablets 75 mg (Aspar Pharmaceuticals, London), rivaroxaban tablet 10 mg (Bayer, Germany), and dalteparin sodium (Pharmacia, USA).

Qualitative Phytochemical Analysis

The standard method for qualitative phytochemical screening described by Sofowora (1982) [19] was employed.

Animals

Adult New-Zealand White rabbits (weight: 1-2.6 kg), and Sprague-Dawley rats (weighing 225-300 g) were used in this study. Animals were housed in roomy cages with ambient day/night cycle, and room temperature (25±3°C). Animals were fed normal pellet chow and tap water for drinking water. They were allowed to acclimatize to the laboratory environment before use in experiments. All animals were kept according to the National Institute of Health Guidelines for the Care and Use of Laboratory Animals (NIH, Department of Health and Human Services publication no. 85-23, revised 1985) and was approved by the Departmental Ethics Committee (Committee on Animal Research, Publication and Ethics (CARPE); Ethics Reference No: FPPS/PCOL/0006/2013).

In vitro Clotting Profile

Briefly, 3.5 ml quantities of whole blood, drawn from the marginal ear vein of rabbits were added to 0.2 ml of 0.5, 1.0, 1.5 or 2.0 g/L PAE in sodium citrate tubes and analyzed by a semi-automated coagulation analyzer, to determine the effect of the extract on prothrombin time (PT) and activated partial thromboplastin time (aPTT). The tests were repeated using 0.2 ml of distilled water (negative control), reference standards rivaroxaban (0.1, and 0.01 mg/ml), aspirin (0.75, and 0.075 mg/ml), heparin (5, and 50 IU/L) and dalteparin (5, and 50 IU/L).

Effect of PAE on PT

Pre-warmed platelet poor plasma (prepared by centrifuging test samples at 1500 g for 15 minutes; 37°C), was added to thromboplastin (tissue factor)-calcium reagent which activates the coagulation cascade at Factor VII. The time taken for clot formation (PT) was determined using a semi-automated coagulation analyzer (CoaDATA 504, Labitec, Germany).

Effect of PAE on aPTT

Platelet-poor plasma (prepared by centrifuging test samples at 1500 g for 15 min) was added to an equal volume of partial thromboplastin reagent (phospholipid plus contact activator, e.g., Silica) and warmed to 37°C for an exact incubation time. Pre-warmed (37°C) calcium chloride reagent (0.025 M) was added to this mixture to activate the coagulation cascade. Time required for clot formation (aPTT) was determined using a semi-automated analyzer.

Bleeding Time (BT) Determination

The BT determination described by Gadi *et al.*, (2009) [20] was employed with slight modifications. Briefly, Sprague-Dawley rats (225-300 g) were divided into four groups of 5 animals each and labeled groups A to F. Groups A, B, C, and D were treated orally with 250, 500, 1000 or 2000 mg/kg daily dose of PAE by gastric lavage, respectively. Groups E and F were given orally 75 mg/kg acetylsalicylic acid (ASA) and 2 ml/kg distilled water as positive and negative controls respectively. Rats were slightly anesthetized, kept in a restrainer and tails disinfected. Very small pieces of (1-2 mm) from the tip of the tail was SNIPPED off by a pair of sterilized scissors and blotted with filter paper (at 15 s intervals) until no blood flow was observed. The period between the tail snip and the cessation of bleeding was taken as the BT (in seconds).

Determination of Clotting Time (CT)

Sprague-Dawley rats (225-300 g) were divided into four groups of 5 animals each and labeled Groups A to F. Groups A, B, C, and D were treated orally with PAE dose of 250, 500, 1000 and 2000 mg/kg daily, respectively. Groups E and F were treated with acetylsalicylic acid 75 mg/kg and 2 ml/kg distilled water orally, serving as positive and negative controls, respectively. Tails of rats were disinfected and a snip taken off the tail at 1-2 mm from the tip. A drop of blood from the tail was placed in the middle of a clean clear glass slide (CAT.No.7102, Meno Int., Dunstable, England) and the timer started. A clean hypodermic needle was passed through the drop of blood carefully every 30 s. This was repeated carefully observing for the formation of the first fibrin strand. The time that elapsed for the formation of the first fibrin strand was taken as the CT.

Safety Studies

The rats were grouped into four ($n = 6$) and given PAE at doses of 250, 500, 1000 or 2000 mg/kg daily by gastric gavage, for a period of 2-weeks. The animals were observed continuously during the first hour, and then every hour for 6 h, then after 12 and 24 h, and finally after every 24 h, up to 2 weeks, for any physical signs of toxicity such as writhing, gasping, bleeding, palpitation and respiratory rate, or mortality. A sample of blood was obtained for liver function test and hematological assessment. Histopathological studies on the liver, kidney and stomach were carried out.

Hematological Profile

Rats were sacrificed by decapitation, blood samples collected into MediPlus vacutainer K3 EDTA tubes (Sunphoria Co. Ltd., Taiwan) and analyzed using a hemato-analyzer (Sysmex XP – 300TM Automated Hematology Analyzer, USA) for the hematological profile.

Liver Function Test

Rats from the various groups were sacrificed by decapitation at the end of the treatment period and blood samples collected into serum separating gel (BD Vacutainer® Blood Collection Tube Product, USA). This was allowed to stand in test tube rack for 30 min for the blood to clot and then centrifuged at 1800 rpm for 10 min to obtain the serum. Serum was then analyzed using a clinical chemistry analyzer (Vital Scientific N. V, Netherlands) to determine levels of liver enzymes, i.e., alanine aminotransferase (ALT) and aspartate aminotransferase (AST) and total protein.

Histopathological Studies

At the end of the safety study, liver, kidney, and stomach from each sacrificed rat were dissected out and cleared of excess fat. Isolated organs were then fixed in 10% buffered formalin and processed for paraffin sectioning by dehydration in varying concentrations of alcohol, cleared with xylene and embedded in paraffin blocks. Sections of about 5 μm in thickness were stained with hematoxylin and eosin (H&E) for histological study.

Statistical Analysis

Data were expressed as a mean ± standard error of mean. Statistical analyses were performed using both one- and two-way Analysis of Variance (ANOVA) followed by Sidak *post-hoc* test with confidence interval of 95%.

RESULTS

Qualitative Phytochemical Analysis

The phytochemical analysis confirmed the presence of flavonoids, alkaloids, saponins, condensed tannins, phytosetrols, triterpenoids, and general glycosides.

In vitro clotting Profile

PT

Baseline PT was 10.51 ± 0.13 s. There was no significant difference in PT between treatment groups, i.e., aspirin, heparin,

dalteparin and PAE-treated groups compared to control. However, rivaroxaban (0.1 mg/ml) showed a significant increase ($P \leq 0.0001$) in PT as compared with control [Figure 2].

aPTT

Positive controls (rivaroxaban 0.01 mg/ml, aspirin 0.075 mg/ml, heparin 5 IU/L and dalteparin 5 IU/L) and PAE (0.5 g/L) showed no significant difference in aPTT compared with control, baseline aPTT was 85.91 ± 1.25 s. There was, however, an increase in aPTT resulting in a significant difference compared with control at treatment doses of 0.1 mg/ml rivaroxaban, 0.75 mg/ml aspirin, 50 IU/l heparin and 50 IU/L dalteparin. PAE at (1.0, 1.5 and 2.0 g/L) also showed a significant increase ($P \leq 0.05$ - 0.0001, respectively) compared with control [Figure 3].

Bleeding and CT

PAE treatment (250, 500, 1000 and 2000 mg/kg) showed a dose-dependent increase ($P \leq 0.0001$) in bleeding and CT compared to the control. There were dose-dependent significant increases ($P \leq 0.0001$) in bleeding and CT between PAE and aspirin treatments [Figures 4 and 5]. There was a significant increase ($P \leq 0.0001$) in CT as compared with BT in both aspirin and PAE treated groups. This difference between bleeding and CT was significantly higher ($P \leq 0.0001$) in the treatment groups compared with the untreated group (control) [Figure 6].

Safety Studies

PAE was devoid of any toxic effects in rats in doses up to 2000 mg/kg when given orally; no deaths were also recorded in any of the treatment groups and control group. Cage-side

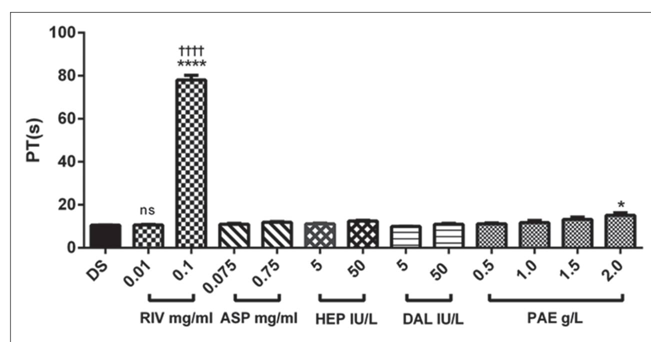

Figure 2: The effect of rivaroxaban, aspirin, heparin, dalteparin and PAE on prothrombin time. Values plotted are means ± standard error of the mean; (n = 5). ****$P \leq 0.0001$; *$P \leq 0.05$; ns $P > 0.05$ compared to control (One-way ANOVA followed by Sidak's *post hoc* test). ††††$P \leq 0.0001$ comparison between two doses of same treatment

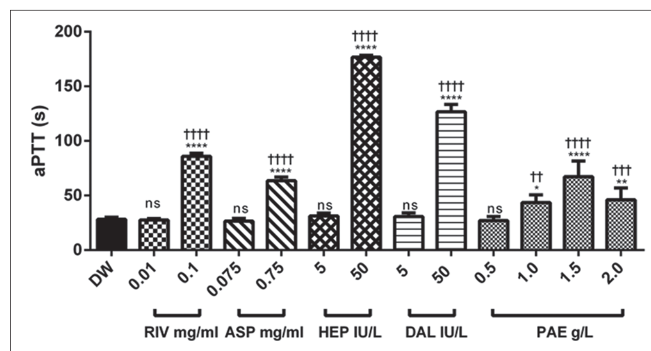

Figure 3: The effect of rivaroxaban, aspirin, heparin, dalteparin and PAE on activated partial thromboplastin time (aPTT). Values plotted are means ± standard error of the mean; (n = 5), ****$P \leq 0.0001$; **$P \leq 0.01$; *$P \leq 0.05$; ns $P > 0.05$ compared to control (One-way ANOVA followed by Sidak's *post hoc* test). ††††$P \leq 0.0001$; †††P = 0.0001; ††$P \leq 0.01$ comparison between the two doses of same treatment

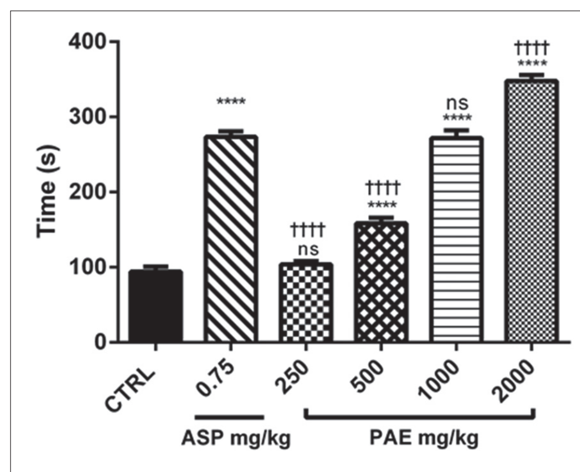

Figure 4: The effect of PAE and Aspirin on bleeding time in Sprague-Dawley rats. Values plotted are means ± standard error of the mean; (n = 5). ****$P \leq 0.0001$; ns $P > 0.05$ compared to control (One-way ANOVA followed by Sidak's *post-hoc* test). ††††$P \leq 0.0001$; ns $P > 0.05$ comparison between aspirin and the various doses of *Pseudocedrela kotschyi* and *Adenia cissampeloides* extract

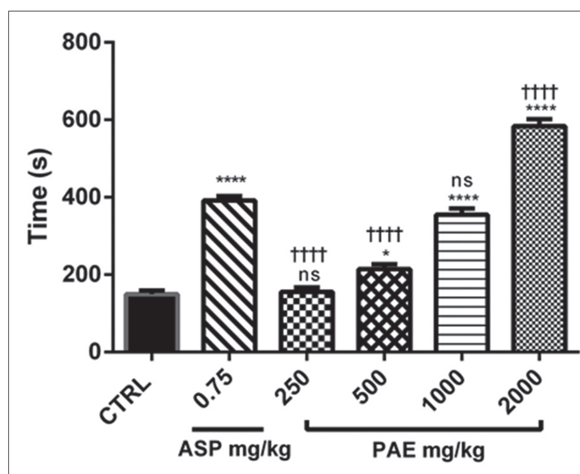

Figure 5: The effect of *Pseudocedrela kotschyi* and *Adenia cissampeloides* extract and aspirin on clotting time in Sprague-Dawley rats. Values plotted are means ± standard error of the mean; (n = 5). ****$P \leq 0.0001$; *$P \leq 0.05$; ns $P > 0.05$ compared to control. (One-way ANOVA followed by Sidak's *post hoc* test). ††††$P \leq 0.0001$; ns $P > 0.05$ comparison between aspirin and the various doses of *Pseudocedrela kotschyi* and *Adenia cissampeloides* extract

observations also revealed no change in grooming habits, consistency of stool and respiratory rate in the treatment groups compared with the control. Locomotor and lachrymatory activities appeared normal with the absence of piloerection in both control and treatment groups.

Liver Function Test

Liver function tests appeared normal with ALT being slightly elevated in the PAE-treated groups compared to control. There was a dose-dependent increase in ALT from 250 mg/kg to 1000 mg/kg of PAE. However at 2000 mg/kg of PAE, ALT levels dropped slightly but was still higher compared to the control [Table 1].

Hematological Profile

Hematological analysis of PAE-treated groups appeared normal compared with control except for platelet counts which were slightly higher in the PAE-treated groups (500, 1000, 2000 mg/kg) compared with the control [Table 2].

Histopathological Studies

The hepatic tissue of control group revealed normal architecture showing normal hepatocytes with nuclei, central and portal veins. Sinusoids appeared to radiate in a divergent fashion from a focal point towards the peripheries [Figure 7]. Treatment with 250 mg/kg PAE revealed no difference in the structural integrity of hepatic tissue compared with control [Figure 7]. PAE treatment at 500, 1000, and 2000 mg/kg also showed normal central and portal veins, hepatocytes, and nuclei; however, there was sinusoid dilatation, from minor to major, compared with the control. Portal veins also appeared dilated with 2000 mg/kg PAE treatment [Figure 7]. Renal tissue in control animals revealed normal tissue architecture, with normal sized glomerulus, urinary space, distal and proximal tubules with nuclei and Bowman's capsule [Figure 8]. PAE treatments at 250, 500, 1000, and 2000 mg/kg showed no significant change in renal tissue architecture compared with the control [Figure 8]. The stomach also showed normal tissue architecture with normal gastric

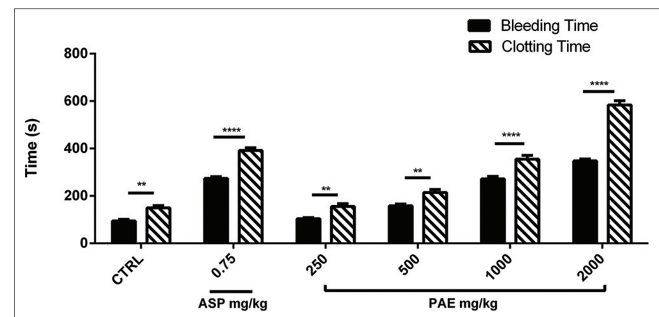

Figure 6: The effect of *Pseudocedrela kotschyi* and *Adenia cissampeloides* extract and Aspirin on Bleeding and clotting time in Sprague-Dawley rats. Values plotted are means ± standard error of the mean; (n = 5). ****$P \leq 0.0001$; **$P \leq 0.01$ compared to control (Two-way ANOVA followed by Sidak's *post hoc* test)

Table 1: Effect of a 14-day PAE treatment on serum liver enzymes in Sprague-Dawley rats

Parameter	Unit	Control	PAE 250 mg/kg	PAE 500 mg/kg	PAE 1000 mg/kg	PAE 2000 mg/kg
AST	U/L	263.57±19.38	315.00±4.18	285.57±8.78	279.97±10.52	283.90±16.44
ALT	U/L	98.20±18.63	144.27±16.34	153.87±40.64	154.80±21.64	150.77±20.79
ALP	U/L	85.92±3.82	92.65±3.34	98.58±5.24	108.38±2.75	112.49±2.04
GGT	U/L	1.83±0.49	3.833±1.79	3.67±1.73	8.40±3.65	3.13±1.57
TP	g/L	71.53±4.43	69.90±5.23	74.63±0.35	72.97±2.32	73.20±1.76
ALB	g/L	38.57±2.22	38.20±5.70	40.43±0.82	37.63±4.74	40.30±1.44
GLOB	g/L	36.53±5.00	31.70±4.25	34.20±1.11	35.33±4.78	32.83±3.04
TBIL	Umol/L	0.93±0.22	0.87±0.27	0.70±0.15	0.73±0.27	0.833±0.17
BIL-D	Umol/L	0.00±0.00	0.07±0.07	0.07±0.07	0.13±0.13	0.20±0.15
BIL-IND	Umol/L	1.00±0.40	0.83±0.19	1.17±0.48	1.03±0.42	0.73±0.15

Values quoted are means±SD. AST: Aspartate aminotransferase, ALT: Alanine aminotransferase, ALP: Alkaline phosphatase, GGT: Gamma - Glutamyl transpeptidase, TP: Total protein, ALB: Albumin, GLOB: Globulin, TBIL: Total bilirubin, BIL-D: Direct bilirubin, BIL-IND: Indirect bilirubin. Values obtained were not significantly different from the control (ANOVA follow by Sidak *post-hoc* test), PAE: *Pseudocedrela kotschyi* and *Adenia cissampeloides* extract, SD: Standard deviation

Table 2: Effect of a 14-day PAE treatment on hematological profile on Sprague-Dawley rats

Hematology	Unit	Control	PAE 250 mg/kg	PAE 500 mg/kg	PAE 1000 mg/kg	PAE 2000 mg/kg
WBC	$10^3/\mu L$	5.97±0.48	5.47±0.37	7.70±0.68	6.77±0.47	4.97±1.62
RBC	$10^6/\mu L$	6.87±0.22	6.61±1.13	7.79±0.18	6.57±0.56	6.70±0.17
HGB	g/dL	12.43±0.12	11.57±1.92	13.57±0.29	11.27±0.35	10.87±1.29
HCT	%	44.20±0.12	42.63±7.89	48.97±2.16	43.40±3.38	42.50±1.45
MCV	fL	62.70±1.07	64.13±1.31	62.77±1.36	66.13±1.17	63.40±1.82
MCH	Pg	17.83±0.38	17.53±0.15	17.40±0.26	17.43±1.74	16.30±2.20
MCHC	g/dL	28.37±0.12	27.37±0.66	27.77±0.93	26.27±2.22	25.60±3.16
PLT	$10^3/\mu L$	640.33±20.95	512.33±96.73	852.33±74.71	904.67±91.45	737.67±109.75

Data is presented as mean±SEM. WBC: White blood cells, RBC: Red blood cells, HBG: Hemoglobin, HCT: Hematocrit, MCV: Mean corpuscular volume, MCH: Mean corpuscular hemoglobin, MCHC: Mean corpuscular hemoglobin concentration, PLT: Platelet. values obtained were not significantly different from the control (ANOVA follow by Sidak *post-hoc* test), SEM: Standard error of mean

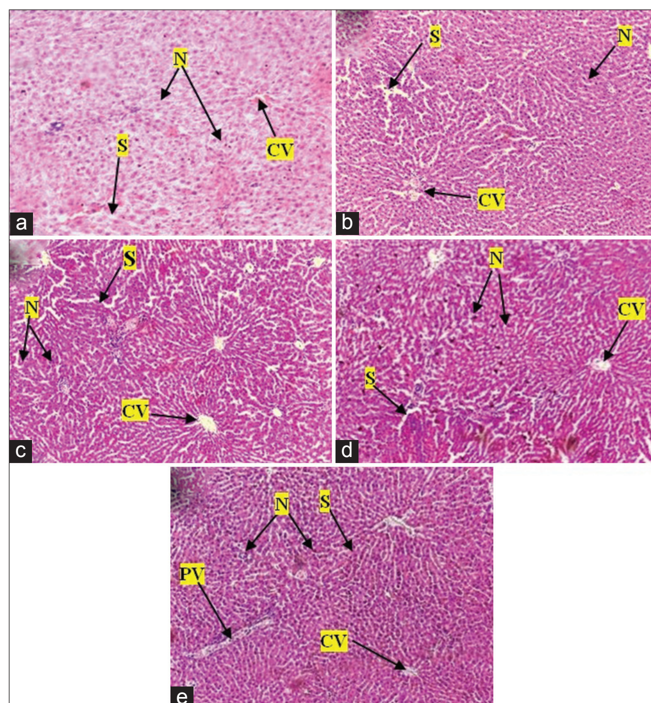

Figure 7: Photomicrographs of the liver of Sprague-Dawley rats after treatment with various doses of Pseudocedrela kotschyi and Adenia cissampeloides extract (a) control group showed normal hepatic architecture and hepatocytes, Nuclei (N), sinusoids (S) and central vein (CV), (b) 250 mg/kg PAE treatment showed hepatic tissue with normal hepatocytes, nuclei, sinusoids and central vein; (c) 500 mg/kg PAE treatment showed normal hepatocytes with nuclei, central vein and portal vein with slight dilatation of sinusoids (d) 1000 mg/kg PAE treatment showed normal hepatocytes with nuclei, central vein and portal vein. Sinusoids appear slightly dilated; (e) 2000 mg/kg PAE treatment showed normal hepatocytes with nuclei, central veins. Portal veins (PV) and sinusoids appear slightly dilated. H and E staining, Objective magnification: ×10

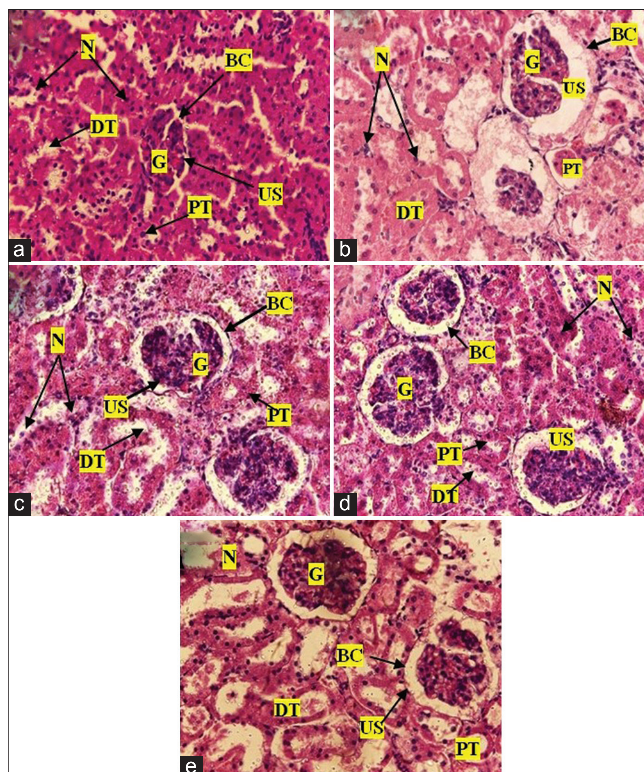

Figure 8: Photomicrographs of the kidney of Sprague-Dawley rats after treatment with various doses of Pseudocedrela kotschyi and Adenia cissampeloides extract: (a) the control showed normal appearance of glomerular (G), urinary space (US), Bowman's capsule (BC), proximal tubule (PT), distal tubules (DT) with their nuclei (N); (b) 250 mg/kg PAE treatment showed normal sized glomerulus, nuclei, Bowman's capsule, distal and proximal tubules. (c) 500 mg/kg PAE treatment showed normal renal architecture with normal cell distribution and cellular integrity; (d) 1000 mg/kg PAE treatment showed normal renal architecture i.e. renal corpuscles and renal tubules. Urinary space showing deposit of particles; (e) 2000 mg/kg PAE treatment, showed normal sized glomerulus, nuclei and renal tubules with little or no infiltration and deposits. H and E staining, Objective magnification: ×40

cells, mucosa and sub mucosa in all PAE treatments [Figure 9] compared with control [Figure 9]. Gastric epithelial cells appeared normal with no signs of erosion or tissue destruction and evenly spaced gastric pits.

DISCUSSION

Disturbances in the coagulation cascade often result in an increased rate of thrombus formation, i.e. venous or arterial thrombus and this has been associated with many cardiovascular diseases [21]. The anticoagulant effect of PAE was therefore evaluated by assessing its effect on PT, aPTT, BT, and CT. The safety profile of PAE was also assessed.

In the *in vitro* analysis PAE, heparin, aspirin, and dalteparin treatment did not have any significant effect on PT. However, rivaroxaban at 0.1 mg/ml and PAE treatment at 2.0 g/L resulted in an increase in the PT. PT, a measure of how long it takes the blood to clot, is an effective screening method for assessing the activity of the extrinsic coagulation pathway [22]. Rivaroxaban, a direct Factor Xa inhibitor [23], interferes with both the intrinsic and extrinsic pathway of the coagulation cascade (which could be as a result of direct inhibition or interference in synthesis of one or more clotting factors, i.e. factors I, II, V, VII, or X) thereby inhibiting the formation of thrombin and further development of thrombi [24].

Although lower concentrations of rivaroxaban, aspirin, heparin, dalteparin, and PAE did not show any significant effect on aPTT, higher concentrations increased aPTT significantly. aPTT is an important parameter in coagulation studies. Thromboplastin also known as thrombokinase is a plasma protein that affects blood coagulation by catalyzing the conversion of prothrombin to thrombin [25]. It is a combination of both phospholipids and tissue factor, both needed for the activation of the extrinsic pathway. The aPTT measures the activity of both the intrinsic pathway and the common pathways of the coagulation cascade by measuring the time taken for the formation of a fibrin clot [26]. It is often used to evaluate clotting abnormalities and as a monitoring parameter for the effect of anticoagulant treatments. The test is termed partial due to the absence of tissue factor in the reaction mixture.

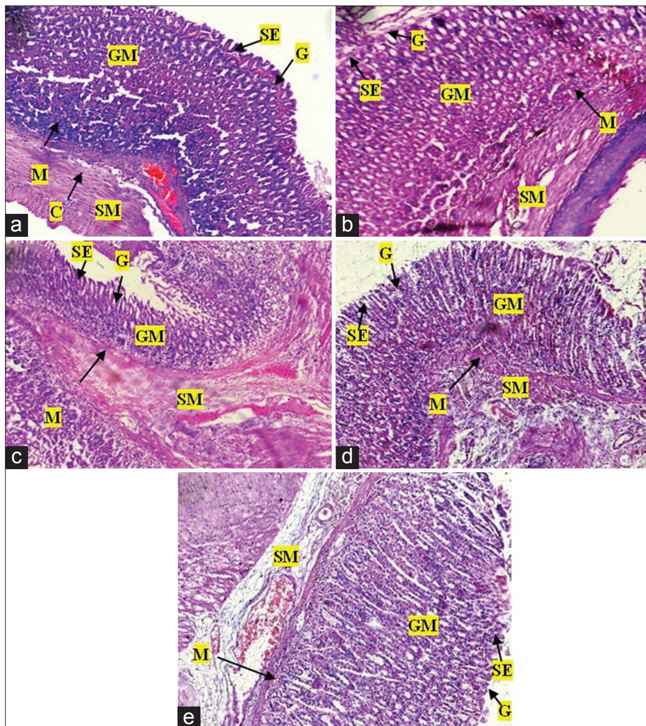

Figure 9: Photomicrographs of the stomach of Sprague-Dawley rats after treatment with various doses of Pseudocedrela kotschyi and Adenia cissampeloides extract. (a) Control showed normal architecture, intact gastric mucosa (GM), sub mucosa (SM), gastric pits (G) and surface epithelium (SE), and collagen fibres (C); (b) 250 mg/kg PAE treatment showed normal stomach architecture with no signs of erosion of epithelium or changes in gastric pits, intact mucosa and sub mucosa; (c) 500 mg/kg PAE treatment revealing normal stomach architecture with even distribution of cells and gastric pits. Intact epithelium, mucosa and sub mucosa; (d) 1000 mg/kg PAE treatment showed normal architecture with no superficial erosion. Gastric pits appeared normal with no unusual increase in depth or spacing, (e) 2000 mg/kg PAE treatment showed normal architecture with even distribution of cellular components and gastric pits, no visible signs of erosion of epithelium or changes in mucosa and sub mucosa. H and E staining, Objective magnification: ×10

The effects of PAE on PT and aPTT were similar to that observed for unfractionated heparin and the low-molecular-weight-heparin dalteparin. A prolonged aPTT with minimal effect on PT realized with PAE treatment, could be as result of PAE binding to the enzyme inhibitor antithrombin III (AT III) as seen with heparin. PAE binding to AT III could result in AT III activation thereby inactivating thrombin and Factor Xa; the end result being interference in the clotting ability of blood [27].

On safety, PAE was found not to be toxic in doses up to 2000 mg/kg when given orally with cage-side observations revealing no significant change in grooming habits and respiratory rate. No deaths were recorded in any of the groups. Acute toxicity is often depicted by reduced or increased activity, changes in grooming habits such as licking of fur or fur becoming unkempt and increased or slowed respiratory rate [28]. Such changes are often as a result toxic effects on normal physiological and biochemical processes, e.g., toxic effects on the parasympathetic system could be depicted by increased salivation and lacrimation symptoms [29]. The absence of these signs of toxicity in the safety model could mean the no-observed-adverse-effect-level of PAE is above 2000 mg/kg.

The liver function test results indicated a non-significant increase in ALT with 2000 mg/kg PAE treatment. Liver damage or injury is often characterized by an elevation of liver enzymes mainly ALT and AST [30]. ALT unlike AST, which is found in other organs like the heart and kidney, is mostly found in the liver and a good parameter for assessing liver injury [31]. PAE can, therefore, be said to be safe at lower doses of up to 2000 mg/kg. *A. cissampeloides* has been shown to contain substances that are hepatotoxic *in vivo* and excessive intake of it has been associated with liver complaints amongst the Zulu people in South Africa [32].

Hematological studies revealed a slight elevation of blood platelets albeit insignificant. An increase in platelet count after PAE treatment can be as a result of secondary thrombocytosis, which rarely results in an increased risk of thrombosis [33].

Histopathological studies of the liver, PAE treated groups at 2000 mg/kg showed slight dilatation of hepatic sinusoids and portal veins. Structural changes in the liver could be as a result of assault on the liver or inflammatory response. This could also account for the slight elevation in the ALT levels in PAE treated groups albeit insignificant. Histology of stomach tissue architecture also appeared normal with no visible signs of erosion of epithelium or disruption in the structural integrity of mucosa and submucosa. Use of conventional antithrombotic agents such as aspirin in thrombosis at risk populations has been associated with side effects such as gastric ulceration and bleeding. This has always proven a challenge of balancing benefit versus risk ratios in this category of patients. Gastric ulcerations are often depicted by erosion of the epithelium exposing the mucosa and sub-mucosa layers [34]. This was however notably absent after PAE treatment, and this could prove a significant advantage of PAE over conventional oral antithrombotic agents such as aspirin.

PAE treatment resulted in an increase in both bleeding and CTs compared with Aspirin. BT measures the time required for a clot to form and stop bleeding after a standardized skin incision, whilst CT, on the other hand, is the time required for a sample of blood to clot *in vitro* under standard conditions. BT albeit an old analytical parameter is one of the most important tests in coagulation studies and evaluates platelet function whilst CT gives an insight into the functioning or otherwise of the clotting cascade. Aspirin, an antiplatelet aggregating drug, irreversibly block the production of thromboxane A_2 in platelets thereby exhibiting an inhibitory effect to platelet aggregation [35]. Aspirin's antithrombotic action through its inhibitory effect on platelet aggregation is thought to be due to acetylation of platelet cyclooxygenase at the active site of the amino acid serine 529, thereby irreversibly inhibiting thromboxane A_2 formation [36]. The significant increase in clotting and BT s caused by PAE treatment could, therefore, be as a result of PAE interfering with the clotting cascade by inhibiting the synthesis

or function of clotting factors and also by inhibiting platelet aggregation thereby interfering with platelet plug formation (primary hemostasis), respectively. This effect could prove much beneficial in the management of thromboembolic disorders with reduced risk of bleeding.

Phytochemical screening of the aerial part of A. *cissampeloides* has confirmed the presence of tannins, saponins, flavonoids, phlobatannins, terpenoids, steroids, alkaloids, carbohydrates and glycosides. *P. kotschyi* root bark, on the other hand, has been found to contain tannins, coumarins, saponins and alkaloids. Several research has also focused on the possible effect of flavonoids on the circulatory system with a focus on the coagulation cascade and other factors affecting the process of hemostasis such as endothelial function and platelet aggregation [37]. The antiplatelet action of flavonoids has been thought to be receptor-mediated [38,39]. These receptor-mediated actions include inhibitory actions at adenosine receptors and also inhibition of von Willebrand binding to platelet glycoprotein Ibα. Some flavonoids have also been shown to inhibit thromboxane A2 (TXA2) mediated response [40,41]. The anticoagulant effect of PAE could, therefore, be as a result of an antiplatelet effect due to the flavonoid constituent. Compounds known to suppress the extent of the coagulation cascade are the coumarins. Coumarins have been found to interfere with coagulation cascade by inhibiting the vitamin K epoxide reductase multiprotein complex [42]. Naturally occurring coumarins have been found not to have anticoagulant properties, however, enzymatic metabolism and fermentation processes could result in the activation to metabolites that have anticoagulant properties [43]. *P. kotschyi* root bark is known to contain coumarins [44].

In comparing the effect of PAE on PT and aPTT, it could be realized that PAE has a significant effect on aPTT than on PT. This effect is very similar to that demonstrated by heparin. This suggests that the anticoagulant effect of PAE has a more significant effect on the intrinsic pathway of coagulation, relative to the extrinsic pathway.

CONCLUSION

The extract made from the root bark of *P. kotschyi* (Schweinf.) Harms and the aerial part of A. *cissampeloides* (Planch. ex Hook.) Harms has anticoagulant effect *in vitro*. It thus justifies the folkloric use of PAE in the management of circulatory disorders such as numbness and also recommended in the management of thromboembolic disorders such as DVT and varicose veins. The extract is safe at doses lower than 2000 mg/kg.

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Research Article

Anti-diabetic activity of traditional Indian gold containing preparation: *Shadguna Balijarita Makaradhwaja* on streptozotocin induced diabetic rats

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ABSTRACT

Background: *Makaradhwaja* a gold containing mercurial preparation used for diabetes mellitus in indigenous system of medicine. It is a popular aphrodisiac and rejuvenator traditional medicine. It is prepared by using processed gold, mercury and sulfur in different ratios by applying intermittent heating pattern in *Valuka Yantra*. **Objectives:** The aim of the study was to evaluate anti-diabetic effect of *Shadguna Balijarita Makaradhwaja* (SBM) on streptozotocin (STZ) induced diabetic rats. **Materials and Methods:** Diabetes was induced to normal rats by injecting STZ in dose 40 mg/kg. Powdered SBM and dried extract of *Tinospora cordifolia* were mixed with honey and administered orally for 20 days at dose 2.63 mg/kg and 42.34 mg/kg body weight, respectively. The effects of treatment on body weight changes and blood glucose levels were quantified on day 1, 5, 10, 15 and 21 of the experiments. On the 21st day, animals were sacrificed and gross histopathological changes in liver, kidney and pancreas were illustrated. Blood sugar level, glyacated hemoglobin, blood urea, serum cholesterol, serum creatinine, serum triglyceride and serum protein were estimated with standard methods. The study was conducted in the year 2011. **Results:** Test drug observed significant decrease ($P < 0.001$) in glyacated hemoglobin level compared to diabetic control rats. Blood sugar level of test drug group shown a significant decrease (279.11 $\pm$ 57.95) compared with diabetic rats. **Conclusion:** The present study demonstrates that SBM and dried extract of *T. cordifolia* with honey significantly reduces the blood glucose level and shows anti-diabetic effect.

KEY WORDS: Anti-diabetic activity, cinnabar gold, *Kupipakwa Rasayana*, mercury sulfide *Shadguna Balijarita Makaradhwaja*

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INTRODUCTION

Diabetes mellitus is threatening for the 21st century. It will be leading cause of morbidity and mortality in near future due to increasing incidence worldwide. Herbo-mineral-metallic drugs of Ayurveda having potential of decreasing blood sugar levels and found efficient on experimental animal models. *Makaradhwaja* [1] is such herbo-mineral-metallic composition.

Medieval classical texts of Ayurveda quoted that *Makaradhwaja* is one of the best rejuvenator and aphrodisiac [2] agent. It is prepared by using processed gold, mercury and sulfur in the ratio of 1:8:24 or 1:8:48 by sublimation in the traditional system of heating device *Valuka Yantra* [1,2]. Nowadays, it is prepared in modified vertical electrical muffle furnace (modified *Valuka Yantra*) [3]. It gives synergistic action with different herbs in the various disorders. It is therapeutically efficient in disorders such as *Madhumeha* (diabetes mellitus), *Jwara* (remittent fever), *Kushta* (skin disorders), and *Raktaja Vikara* (blood disorders) [2]. Previous studies claimed its safe use without any untoward effect and toxicity [4]. Specific action on cell-mediated immunity is proved by its immune-modulator

action [5]. In experimental and clinical studies, it is found effective in diabetes [6].

Guduchi (*Tinospora cordifolia* Linn.) is well-known *Madhumehahar* (anti - hyperglycemic) herb described throughout ayurvedic classics [7]. Most commonly, its stem is used for medicinal purposes. It is well-known anti-oxidant, anti-hyperglycemic, immune-modulatar, and rejuvenator [8]. Its different formulations are quoted in the ayurvedic classics like juice, decoction, powder and *Ghana* (dried extract) as per diseases.

Madhu (honey) is mentioned as *Madhumehahar* (~anti-diabetic) agent within classical texts of Ayurveda [9]. Its anti-diabetic activity is reported by Erejuwa *et al.* [10]. It is used as a vehicle drug (*Yogavahi*) in the numerous herbo mineral formulations. It is mentioned in the reference to *Makaradhwaja* as a vehicle drug [11]. In the present study, it was used as a vehicle drug as prescribed in texts of *Rasashastra*.

The present study was planned to evaluate anti-diabetic activity of *Shadguna Balijarita Makaradhwaja* (SBM) and *T. cordifolia* Linn with honey in streptozotocin (STZ) induced diabetic rats.

MATERIALS AND METHODS

Preparation of SBM and *Guduchi Ghana*

Test drug SBM was prepared as per the classical text reference in the Department of Rasashastra and Bhaishajya Kalpana, Institute of Post Graduate Teaching and Research in Ayurveda (IPGT and RA), Gujarat Ayurved University, Jamnagar in 2011 [1]. Raw Material *Swarna* (gold) was purchased from the local market, and *Hingula* (cinnabar) and *Gandhaka* (sulfur) were collected from Pharmacy of Gujarat Ayurved University, Jamnagar. Gold was subjected to *Shodhana* and after *Shodhana*, its foils were prepared. Cinnabar was processed to *Shodhana* (purification) and *Parada* (mercury) was procured from its sublimation by adopting *Nada Yantra* method [12]. *Gandhaka* (sulfur) was subjected to *Shodhana* by melting it and pouring in cow milk and continuously heated in same milk for 3 h [13]. Processed gold foils, mercury and sulfur were brought in ratio of 1:8:48 in weight. Amalgamation was done by adding gold foils to mercury. Fine lusterless powder *Kajjali* was prepared by triturating sulfur with above prepared amalgam. *Kajjali* was levigated with juice of *Kumari* (*Aloe barbadensis* Mill.) and *Japa* (*Hibiscus rosa-sinensis*) for 3 h consecutively. Levigated *Kajjali* was dried and powdered. The fine powder was filled in seven-layer mud smeared cotton cloth wrapped glass bottle (*Kacha Kupi*) and heated for 12 h in specially designed electrical muffle furnace. The heat was provided in controlled and gradually increasing temperature in modified electrical muffle furnace (modified *Valuka Yantra*). After the desired characteristic features of product preparation, the mouth of glass bottle was sealed; furnace was switched off and subjected for self-cooling. The highest recorded temperature during procedure was 600°C. Sublimed product was procured from neck of glass bottle, it was powdered and used for further analysis and study [14]. In analytical studies, it was observed that *Makaradhwaja* is consisted of red sulfide of mercury and having an empirical formula of HgS (mercury sulfide). Inductively coupled plasma optical emission spectrometry was observed that it contains 1.2% of gold with mercury and sulfur in finished product as a major element.

T. cordifolia's stems were collected from the herbal garden of Gujarat Ayurved University, Jamnagar. Stems of *T. cordifolia* were cut into small pieces and crushed. These crushed pieces were cooked with 4 times of potable water ant reduced at 1/4th of the same to prepare decoction. The decoction was sieved, cooked to semisolid consistency. The semisolid mass was dried in hot air oven at 45°C to prepare dry extract [15]. Average 5.21% yield was obtained from the stem extract. The *Guduchi Ghana* (dried extract) was powdered and stored [16].

Honey was collected from the local forest of Jamnagar.

Experimental Animals

Albino rats (160-220 g) of either sex were selected for this study. Animals were procured from the Animal house of Pharmacology laboratory of IPGT and RA, Gujarat Ayurved University, Jamnagar. Permission for the experiment was granted by Animal Ethical Committee of the Same Institute (IAEC-06/09-11/02). The animals were fed pellet diet and water. As per the guidelines of National Institute of Health's guide for the Care and Use of Laboratory Animals, the study was conducted [17].

Collection of Blood Samples

Tail veni-puncture method was applied for the collection of blood sample in the rats. For investigation Glucometer strip was used, and reading was noted down. By adopting this procedure blood glucose level of animals was estimated, prior injection of STZ, 5th, 10th, 15th, and 20th day during trials.

The sacrifice was carried out on the 21st day after completion of the study. Animals were anesthetized and stroked over tiles for sacrifice. The blood sample was collected by the dissection of jugular veins of animals. Biochemical parameters were investigated at pathology laboratory, IPGT, and RA, Jamnagar.

Experimental Induction of Diabetes

Diabetes was induced to rats by single intra peritoneal injection of STZ (40 mg/kg). STZ was weighed individually for each animal, according to its weight it was solubilized with 0.2 mL saline (154 mM NaCl) just prior to injection. After 72 h of STZ injection, rats with diabetic hyperglycemia (blood glucose more or equal to 250 mg/dL) were selected for experiment. Suspension of *Makaradhwaja* and *T. cordifolia* with honey was started fed to selected diabetic rats.

Anti-diabetic Activity

Anti-diabetic activity was evaluated by the effect of test drugs on the ponderal and biochemical parameters. Ponderal

parameters like gross body and different body organs weight were evaluated. Biochemical variables such as blood sugar, glycated hemoglobin (HbA1C), serum total cholesterol, serum high-density lipoprotein (HDL), serum triglyceride, serum creatinine, blood urea, serum glutamic-pyruvic transaminase (SGPT), serum glutamic oxaloacetic transaminase (SGOT), total protein, albumin, and globulin were estimated. Sacrificed animals gross and histological appearance of vital organs (liver, pancreas and kidney) were examined at the end of the study.

Experimental Design

Diabetic animals were divided into four groups. Food and water were provided to the animals.

- Group 1: Normal control (NC)
- Group 2: Diabetic control (DC)
- Group 3: DC + SBM and *Guduchi Ghana* with honey suspension (SBM)
- Group 4: DC + glibenclamide (reference standard [RS])

Dose Fixation

The dose of the drug was calculated by extrapolating the therapeutic dose to rat on the basis of body surface area ratio by referring to the table of Paget and Barnes (1964) [18].

Dose

Powdered SBM 2.63 mg/kg rat and dried extract of *T. cordifolia* 42.34 mg/kg rat were grinded with honey to prepare suspension. This suspension was administered orally to test drug group SBM diabetic animals. Glibenclamide, in the dose 0.45 mg/kg was administered to RS drug control group.

Statistical Analysis

All the values of were expressed as mean ± standard error mean. A statistical analysis was performed by using Student's *t*-test. It was calculated by using Microsoft excel programmer.

RESULTS

In NC rats, during the course of 21 days, 13.90% weight was increased. Insignificant decrease in body weight was observed in SBM and RS group in comparison to DC rats [Table 1]. In DC rats, weight of kidney and liver was increased up to significant extent. Treatment with test drug did not affect the weight of these organs to significant extent in comparison to the DC group [Table 2].

Initial blood sugar level was decreased by 44.27% and 44.04% in SBM and RS treated groups respectively [Table 3]. Raised levels of HbA1C were significantly attenuated by test drug SBM and RS [Table 4]. Increased blood sugar levels were insignificantly decreased by SBM and RS test drugs.

Non-significant rise in serum cholesterol, triglyceride and HDL were moderately decreased by administration of SBM. Blood urea levels decreased significantly in group SBM and moderately decreased in RS group. Elevated serum creatinine levels non-significantly decreased by administration of SBM which was non-significantly increased in RS group in comparison to DC group. Decreased SGOT parameter in DC group was significantly attenuated by SBM and RS drugs. Increased levels of SGPT were decreased up to significant extent by SBM and RS drugs [Table 5].

The accumulation of fat in liver was observed in histopathological study. SBM and RS treated animals restored the histological changes [Figure 1a-d]. Inflammation in blood vessels, increase in the thickness of bowman capsules, fat deposition, and change in size of the glomerulus were found in the kidney of

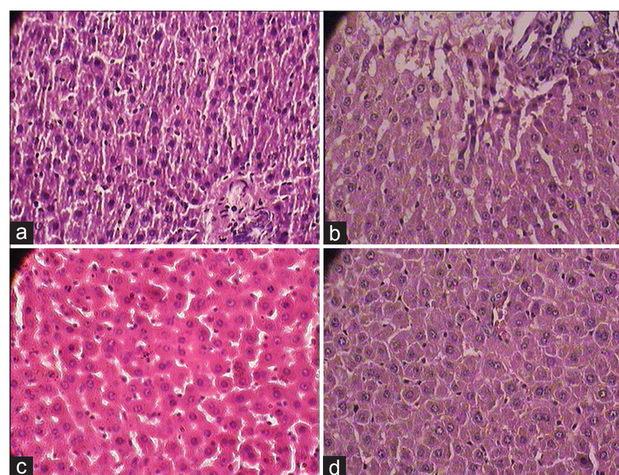

Figure 1: (a) Normal cytoarchitecture of liver in normal control group (1 × 400 magnification), (b) Photomicrographs of representative section of Liver. Macro and micro fatty changes, cell infiltration in almost all the sections streptozotocin control group (1 × 400 magnification) (c) *Shadguna Balijarita Makaradhwaja* treated rats shows almost normal cytoarchitecture of liver in comparison with streptozotocin control group diabetic rats (1 × 400 magnification), (d) Glibenclamide treated rat showed almost normal cytoarchitecture of liver sections in comparison with streptozotocin control group diabetic rats (1 × 400 magnification)

Table 1: Effect of test drug on body weight in STZ diabetic Wister strain albino rats

Group	0 day (g)	5th day (g)	10th day (g)	15th day (g)	21st day (g)	% change in comparison to 0 day
NC	187.00±10.95	192.00±11.27	196.33±11.03	205.67±10.28	213.00±10.57	13.90↑
DC (STZ)	170.67±11.73	165.33±12.49	165.67±14.00	167.00±13.61	166.67±15.85[α]	02.34↓
SBM	176.33±07.79	170.33±08.20	164.33±08.54	158.00±09.18	152.00±09.24	13.80↓
RS	172.67±04.15	162.00±09.62	158.33±14.04	161.17±14.38	161.33±17.15	06.57↓

Mean±SEM, NC: Normal control, DC: Diabetic control, SBM: *Shadguna Balijarita Makaradhwaja* control, RS: Reference standard control, STZ: Streptozotocin, ↑: Incease ↓: Decrease, SEM: Standard error of mean

diabetic rats. The treatment with SBM showed the normal histopathology of the kidney without any inflammatory vessels and fat deposition [Figure 2a-d]. Decreased Islets of Langerhans, smashed size of β cells and extensive necrosis, fibrosis and atrophy were observed in the pancreas of diabetic rats. STZ induced diabetic rat treated with SBM and glibenclamide restored the necrotic and fibrotic changes and up to moderate levels, raised the number of β cells. [Figure 3a-d].

DISCUSSION

Excessive breakdown of tissue proteins decreases body weight in diabetes. It was observed in DC rats in comparison to normal rats [19-21]. Treatment with SBM improved body weight to a certain extent, indicating that control over muscle wasting resulted from glycemic control.

Previous studies claimed that STZ destructs beta cells, which leads to cells less active that makes poor utilization by tissues [22,23]. This suggests that test drug may possess as insulin-like effect on peripheral tissues by inhibiting hepatic gluconeogenesis by promoting glucose uptake or metabolism [24]. Or it might be possible that test drug increases absorption of glucose into the muscles and adipose tissues [25]by stimulation of regeneration process and revitalization of remaining beta cells [26]. Hence, the hypoglycemic activity of SBM may be due to its protective action against damaged pancreatic beta cells and possibly because of increased insulin release or secretion or regeneration of damaged beta cell. *Makaradhwaja* is a well-known therapeutic medicine of rejuvenation in Indian system of medicine. Its immune-modulatory action was also established [5]. The observed effect may be attributed to the rejuvenation property of *Makaradhwaja*. The previous study supports its action too [27]. Moderate but insignificant decrease in blood sugar levels were also observed in test drug-treated animals.

Higher levels of HbA1C were observed in the diabetic rats compared with those in normal rats, it might be due to poor glycemic control. SBM treated diabetic rats significantly decreased the level of HbA1C, may be glucose metabolism was improved. This action represents that SBM has an ability to prevent the development of diabetes associated complications.

Elevated levels of SGPT indicating impaired liver function due to hepato-cellular necrosis. Due to elevated transaminase activities leads to diabetic complications like increased ketogenesis and glucogenesis [28], test drug significantly restored this parameter toward normal levels.

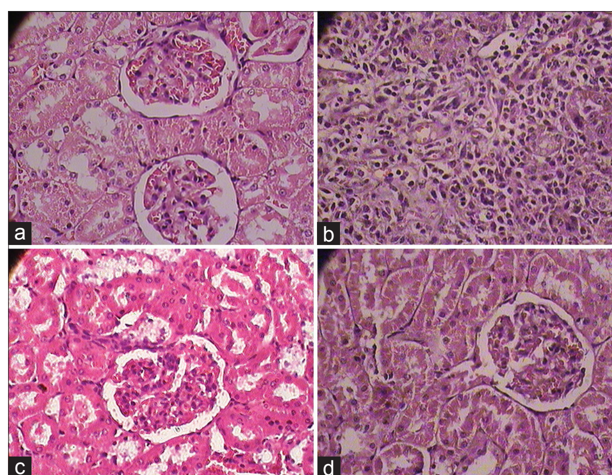

Figure 2: (a) Normal cytoarchitecture of sections of kidney in normal control group (b). (1 × 400 magnification), (b) Photomicrographs of representative section of kidney. Cell infiltration and micro-fatty changes in all the sections of streptozotocin control treated diabetic rats (1 × 400 magnification), (c) *Shadguna Balijarita Makaradhwaja* treated rat showed almost normal cytoarchitecture in comparison with streptozotocin control group diabetic rats (1 × 400 magnification), (d): Glibenclamide treated rat showed almost normal cytoarchitecture in comparison with streptozotocin control group diabetic rats (1 × 400 magnification)

Table 2: The effect of test drugs on weight of liver and kidney in streptozotocin induced diabetic Wistar strain albino rats

Organs	Kidney (g/100 g)	Liver (g/100 g)
NC	0.65±0.05	2.38±0.08
DC (STZ)	0.99±0.07$^{\alpha\alpha}$	3.49±0.19$^{\alpha\alpha\alpha}$
% change in comparison to NC	52.31↑	46.64↑
SBM	1.06±0.08	3.63±0.08
% change in comparison to DC	07.07↑	04.01↑
RS	0.92±0.08	3.36±0.17
% change in comparison to DC	07.07↓	03.73↓

Mean±SEM, $^{\alpha\alpha}P<0.01$, $^{\alpha\alpha\alpha}P<0.001$ (comparison to normal control group, unpaired '*t*' test) NC: Normal control, DC: Diabetic control, SBM: *Shadguna Balijarita Makaradhwaja* Control, RS: Reference standard control, SEM: Standard error mean, ↑: Incease ↓: Decrease

Table 4: The effect of test drugs on HbA1c in STZ induced diabetic Wistar strain albino rats

Parameter	NC	DC (STZ)	SBM	RS
HbA1c	5.62±0.06	12.12±0.70$^{\alpha\alpha\alpha}$	6.04±0.71***	7.68±0.92**

Data: Mean±SEM, $^{\alpha\alpha\alpha}P<0.001$, (comparison to normal control group, unpaired *t*-test) $^{**}P<0.01$, $^{***}P<0.001$ (comparison to DC group, unpaired *t*-test) NC: Normal control, DC: Diabetic control, SBM: *Shadguna Balijarita Makaradhwaja* control, RS: Reference standard control, HbA1c: Glycated hemoglobin, SEM: Standard error of mean

Table 3: The effect of test drugs on blood sugar level in STZ induced diabetic rats at various intervals

Group	Blood sugar level (mg/dl)					
	0 day	5th day	10th day	15th day	21st day	% change in comparison to 0 day
DC (STZ)	383.67±51.44	380.33±37.26	355.33±28.09	372.83±34.49	328.17±30.67	14.67↓
SBM	500.83±42.61	503.67±31.81	455.67±35.60	486.67±51.93	279.11±57.95*	44.27↓
RS	512.83±21.43	374.33±22.16	334.83±32.03	314.83±29.68	287.00±33.78*	44.04↓

Mean±SEM, $^{*}P<0.05$, $^{***}P<0.001$ (comparison to diabetic control, unpaired *t* test). NC: Normal control, DC: Diabetic control, SBM: *Shadguna Balijarita Makaradhwaja* control, RS: Reference standard control, SEM: Standard error mean, STZ: Streptozotocin, ↑: Incease ↓: Decrease

Table 5: The effect of test drugs on various serum biochemical parameters

Parameters	NC	DC (STZ)	SBM	RS
Blood sugar (mg/dl)	117.50±2.50	321.00±27.80$^{\alpha\alpha\alpha}$	261.22±60.01	286.33±29.52
Cholesterol (mg/dl)	57.17±4.72	62.00±4.03	45.67±4.46*	58.83±5.87
Triglyceride (mg/dl)	64.33±7.28	80.83±4.10	67.50±6.86	70.83±6.02
HDL (mg/dl)	25.00±3.22	34.17±2.60	29.50±3.77	28.33±3.26
Blood urea (mg/dl)	90.33±4.92	137.17±11.66$^{\alpha\alpha}$	57.00±5.49***	100.50±10.43*
Creatinine (mg/dl)	0.58±0.03	0.72±0.05$^{\alpha}$	0.65±0.03	0.75±0.07
SGPT (IU/L)	75.50±8.60	323.00±21.29	88.83±10.37***	90.33±3.10***
SGOT (IU/L)	223.00±12.84	120.83±11.67$^{\alpha\alpha\alpha}$	173.50±9.16**	304.67±20.66***
Total protein (g/dl)	7.35±0.11	6.70±0.23$^{\alpha}$	6.70±0.28	6.08±0.31
Albumin (g/dl)	3.38±0.11	3.08±0.09	3.45±0.07**	3.00±0.18
Globumin (g/dl)	3.93±0.16	3.67±0.25	3.25±0.25	3.13±0.27

Data: Mean±SEM, $^{\alpha}P<0.05$, $^{\alpha\alpha}P<0.01$, $^{\alpha\alpha\alpha}P<0.001$, (comparison to normal control group, unpaired *t*-test) $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$ (comparison to DC group, unpaired *t*-test), NC: Normal control, DC: Diabetic control, SBM: *Shadguna Balijarita Makaradhwaja* control, RS: Reference standard control, SEM: Standard error of mean, HDL: High-density lipoprotein

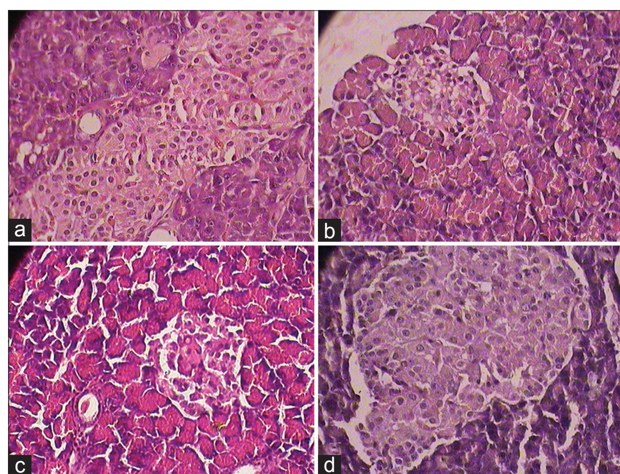

Figure 3: (a) Normal cytoarchitecture in normal control group, (b) Photomicrographs of representative section of Pancreas. Marked degeneration of Islets of Langerhans and degranulation of streptozotocin control treated diabetic rats (1 × 400 magnification), (c) SBM treated rats shows comparatively less degeneration of Islets of Langerhans with intact granules in comparison with streptozotocin control group diabetic rats (1 × 400 magnification), and (d) Glibenclamide treated rat showed normal cyto-architecture with intact granules in comparison with streptozotocin control group diabetic rats (1 × 400 magnification)

Elevation of urea and creatinine levels results due to renal dysfunction caused by free radical generation mediated stress in diabetes, persistent hyperglycemia, and hemo-dynamic changes within the kidney tissue [29-31]. Administration of SBM and glibenclamide to the diabetic rats significantly reduced the creatinine and urea levels, which represent the preventive action of SBM on kidney damages in diabetic condition perhaps due to the anti-oxidant properties. Blood urea level was significantly increase in diabetic rats compared to normal rats due to excessive breakdown of protein. This elevation was significantly decreased by test drug.

STZ induced diabetic rat's increases the level of lipid peroxidation, as an indirect evidence of production of free radical [32]. Hyperlipidemia commonly associated with diabetes [33]. Test drug significantly attenuated serum lipid profiles in diabetic rats. STZ induced diabetic groups treated with glibenclamide and SBM brought back the increased level of total cholesterol and triglyceride near to the normal levels.

Histopathological changes in liver, kidney and pancreas were well restored by the test drug in comparison with DC group. The observed results show anti-diabetic potential of SBM. Observed results may be due to synergistic action of *Makaradhwaja* with adjuvant *T. cordifolia* and honey.

CONCLUSION

The present study demonstrates that SBM and dried extract of *T. cordifolia* with honey significantly reduces the blood glucose level and shows anti-diabetic effect. Restoration histopathological changes in different organs support safe and effective anti-diabetic action of test drug. A significant decrease in glycated hemoglobin shows effect of *Makaradhwaja* on diabetes-related complications.

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Original Research

A brief qualitative survey on the utilization of Yoga research resources by Yoga teachers

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ABSTRACT

Introduction: Yoga has become popular worldwide with increasing research done on its therapeutic potential. However, it remains to be determined whether such findings actually percolate down into teaching and practice of Yoga teachers/therapists. **Materials and Methods:** The aim of this survey was to document awareness of Yoga research findings in the Yoga community and find out how these were utilized. It was undertaken with a select group of 34 international Yoga teachers and therapists utilizing email and social media between August and December 2015. Majority of responders had well-established reputation in Yoga and were from diverse lineages with 30 of them having more than 5 years of experience in the field. A set of eight questions were sent to them related to essentiality of Yoga research, how they updated themselves on research findings and whether such studies influenced their teaching and practice. Responses were compiled and appropriate statistics determined for quantitative aspects while feedback, comments and suggestions were noted in detail. **Results and Discussion:** About 89% agreed that it was essential to be up-to-date on Yoga research but only 70% updated themselves regularly with average papers read fully per year being <10. Most accessed information through general news reports, emails from contacts, and articles on internet sites whereas only 7% were through PubMed. About 60% felt these studies helped them in general teaching whereas 20% said that such studies had not really influenced it in any way. **Conclusion:** This survey provides a basic picture of a general lack of awareness of Yoga research amongst practicing Yoga teachers and therapists. Though a majority agree research is important, few seriously update themselves on this through scientific channels. With regard to future studies, most wanted "proof" that could be used to convince potential clients and felt that more qualitative methods should be applied.

KEY WORDS: Health, survey, wellness, Yoga research

INTRODUCTION

Yoga has become increasingly popular worldwide with many taking it up professionally thus helping many more benefit from this health enhancing ancient art and science of humanity. A number of scientific studies have been done in the past five decades, and a recent extensive review has reiterated its therapeutic potential with scientific evidence for a wide range of psychosomatic conditions [1].

However, it remains to be determined as to how much such research findings actually percolate down into the day-to-day classroom teaching and clinical practice of the Yoga teachers/therapists.

Important questions that come to mind in this regard are:
1. Are Yoga teacher/therapists aware of recent updates in Yoga research?
2. Are these updates influencing their day to day teaching/clinical practice?
3. Are they satisfied with past and current research being done?
4. What are "priority needs" they wish from researchers?

This is important as a two-way dialog between them and researchers would enhance the focus, objectives as well as methods and ultimate direction of future research in the best productive manner.

In an attempt to determine answers for these pertinent questions, and foster understanding between "the lab and the field," this survey was done by this author amongst his international contacts in the field of Yoga and Yoga therapy.

Aim and Objectives

The aim of this survey was to understand the awareness of Yoga research findings in the Yoga teacher/therapist community and to find out how these findings were utilized by them in their day to day practice and teaching.

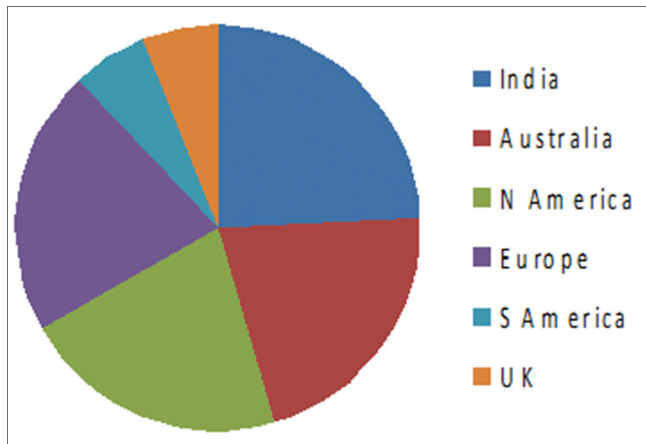
The secondary objective was to see what they felt would be more useful in the context of future research as this author feels such research efforts should be guided by needs of those who are to use it the most.

MATERIALS AND METHODS

This survey was undertaken in the months between August 2015 and December 2015. Emails were sent to potential participants and awareness of the survey carried out through social media including Twitter, Facebook, and SlideShare.

Survey Participants

This survey was conducted on a select group of 34 international Yoga teachers and therapists known to the author and included 8 from India, 7 from Australia, 8 from North America, 7 from Europe, and 2 each from South America and the UK.



The age of participants in the survey of which 18 were females and 16 males ranged from 28 to 77 (51.08 ± 12.84) years.

Majority of responders had well-established reputation as Yoga teachers/therapists and were from diverse lineages with 30 of them having more than 5 years of experience in the field. Four of them had more than 30 years of professional standing in the field of Yoga. Five of the other respondents who had <3 years of experience in the field were excluded from the survey.

Four of the respondents were medical doctors, 4 nursing professionals, 5 physical therapists, 3 psychologists, whereas the rest were all qualified Yoga teachers/therapists with diplomas/degrees in Yoga from eminent Yoga schools. Traditions represented included the Krishnamacharya-Desikachar tradition, Kaivalyadhama tradition, Gitananda tradition, Iyengar tradition, and Satyananda tradition.

How long have you been teaching Yoga?

<5 years	4
5-10 years	6
10-20 years	13
20-30 years	7
More than 30 years	4

Questions asked: The following questions were asked to the participants in the survey and responses obtained from them via email.

1. Do you think it is essential for Yoga teachers to be up-to-date on Yoga research?
2. Do you update yourself on research in Yoga regularly? If yes, how many papers on an average do you read fully per year?
3. Where/how do you access these studies?
4. Have these research studies influenced your teaching? If yes, how?
5. Is/are there any specific study/studies that has/have influenced you the most in the past 3-5 years? If yes, which one/s?
6. Are you satisfied with the quality of Yoga research at present?
7. What type of studies do you wish to see in the future?
8. Any other comments?

Compilation and analysis of responses: Responses from all participants were compiled and statistics (percentage and mean ± standard deviation) determined for quantitative aspects such as demographic parameters (country wised distribution, age, gender, and years of teaching/therapy). Qualitative analysis of important responses was done for other answers and important feedback, comments, and suggestions noted in detail.

RESULTS AND DISCUSSION

All the participants were eager to take part in this survey and expressed their support for such an endeavor. In this section, we shall take a look at the important responses given by them for each of the questions with appropriate details discussed in detail.

The first of the questions was as follows:

1. Do you think it is essential for Yoga teachers to be up-to-date on Yoga research?

About 89% of the teachers/therapists agreed that it was essential for Yoga teachers/therapist to be up-to-date on Yoga research. Some felt that it was not essential for teachers but that it was for therapists.

As a Yoga therapist trainer of 18 years from North America put it, "To move the profession forward into modern culture? Yes. To maintain the highest level of Ahimsa? Yes. Can someone support many people without doing so? Probably most of time."

Another Yoga therapist trainer of 15 years said, "My answer to this is twofold. In the realm of what Dr. Bhavanani calls Yogopathy [2], Yes I think it is essential. In today's world of "evidence-based medicine" being able to scientifically "prove" that Yoga Therapy is effective enables it to be more widely accepted as a valid treatment. In my experience, sharing this Yoga research as evidence is what opens both physicians and patients to trying it with respect versus skepticism. On the other hand, research whittles down factors to attempt to isolate that which is clinically relevant. As we know, Yoga is beyond the physical and is broad in its application and effects thus it is difficult to "prove" what exactly is happening through a research study. We can hypothesize; however, how the effects

are obtained may be beyond current scientific understanding. Thus, we do not want to fall into a trap of narrowing our focus of approach to that which is scientifically provable. All of this said, my thoughts are that Yoga Therapist should be up-to-date on Yoga research and have a clear understanding of its limitations.

The right means produces the right end, hence outcome of the Yoga practices become better “structured” said a senior Australian Yogacharya while another respondent from the UK suggested, “No, but it helps and is interesting.”

A senior Yoga therapist from Australia mentioned that this was most essential and that the Standards Committee in both International Association of Yoga Therapists (IAYT) and Australasian Association of Yoga Therapists (AAYT) had made it part of their educational standards for Yoga therapists for the same reason [3].

The next question was as follows:

2. Do you update yourself on research in Yoga regularly? If yes, how many papers on an average do you read fully per year?

Though a vast majority accepted the importance of research in Yoga, only 70% said they updated themselves on research in Yoga regularly with the average papers read fully per year being as follows. Only 9 of them read more than 10 papers a year, whereas 14 said they read less than 5 papers a year.

<5 papers per year	14
5-10 papers per year	10
10-20 papers per year	3
20-30 papers per year	2
More than 30 papers per year	4

3. Where/how do you access these studies?

It was found that most respondents were accessing their information on Yoga research through the internet and that general news reports, emails from their contacts, and articles on different internet sites were main sources. Only 7% were accessing their information through PubMed, whereas 10% still relied on various books on the subject.

General news reports, emails, and internet sites 72%
PubMed 7%
Google scholar 5%
Books 10%
Research Journals 4%
Research gate 2%

One has to be very careful with the information available on the net and this overreliance on unverified sources is something that needs correction as there is often a vast difference between what has been done in the actual study and what is popularized in the lay press.

An example is a recent study in the Journal of Neuroscience on how sleeping on the side influences clean-up of brain’s metabolic waste products and halts neurological diseases [4]. Many started to talk about it in social media mentioning possible benefits of humans sleeping on their side and how they did it or did not do it. It seemed to this author that most had not even bothered to go to the original source and read the full paper as rodent models were used in the study and not humans. Speak about how having access to more information may not actually result in our being better informed!

4. Have these research studies influenced your teaching? If yes, how?

About 60% felt that these studies had helped them in their general teaching while 55%, 45%, and 40% stated that it helped in their teaching of asanas, pranayama, and relaxation, respectively. About 20% said that such studies had not really influenced their teaching in any way.

General teaching 60%
Asana aspects 55%
Pranayama aspects 45%
Relaxation aspects 40%
Safety aspects 20%
Scientific understanding 20%
Confirmation of ancient wisdom 10%
Nutritional aspects 5%
Not really 20%

As one of the senior trainers noted, “When I can offer a patient a study that “shows” positive benefits of Yoga (cancer research, telomere research, etc.), they are much more open to following the recommendations I make to lifestyle changes and to our treatment approach [5-7].”

It was also noted that showing patients the positive effects of various techniques has encouraged them to be more compliant with what they are taught. A special mention was made of a recent study on the side plank pose (Vasisthasana), in idiopathic and degenerative scoliosis by Fishman and colleagues [8].

However, it was well brought out that one should look at the methodology when it comes to safety precautions as some of the studies on asanas to avoid have methodology concerns. It was noted that an article states that Sarvangasana causes strokes. However, when one reads the article fully we find that the person held it with his head rotated [9]. As the therapist responded, “I don’t know anyone who would do that so I disregard such studies.” She, however, continued, “Osteoporosis studies have influenced my approach with avoiding flexion [10].”

A senior Yogacharini from Berlin explained that the research findings enabled clearer thinking for the teacher and therapist and helped develop an ability to compare Yoga knowledge with allopathic medicine. It was also essential to understand and point out differences in the therapeutic principles.” She also felt that it enabled the teachers and therapists to be, “on the more safe side.”

An Indian Yoga teacher in the USA stressed the importance of being clear in the aim of one’s research. The focus should be

Yoga, and this should be applied as per the tradition involved. "When it is done in such a manner, it becomes easier for me as a teacher to serve 'ready material' to my clients when they have health problems" said she. "The bridge between Yoga and modern science as manifested through research and literary works of Swami Gitananda Giri and his successors strengthens our conviction. When such authentic knowledge is combined with the fact research has been done by medical doctors, it truly helps us convince prospective and current clients about benefits of Yoga" she continued.

A senior Yogacharya responded that he had personally applied concepts of Swarodaya Vigjnan (study of nasal cycle) learned about through his studies of research in Yoga and been motivated to do a verified analysis of these concepts in relation to staying alert while driving his motor vehicle [11,12]. He attached a detailed report of his personal study of the nostril dominance during different phases of the day and with regard to diverse activities, he was undertaking on a daily basis. He had also practiced the right or left uni-nostril breathing techniques and noted in detail the effects he felt immediately. This author is personally heartened to see such feedback "from the field" as this is actually the "spirit of research" that Yoga research findings should be motivating in all sadhakas. They should be motivated to "see for themselves" whether the findings of the research studies as "true or not" by applying the methodology in their own personal sadhana and life.

5. Is/are there any specific study/studies that has/have influenced you the most in the past 3-5 years? If yes, which one/s?

Most of the respondents did not point out any specific study/studies that has/have influenced them recently but gave more generalized replies. The few that were mentioned by name included recent publications from Kaivalyadhama (www.kdham.com), SVYASA (www.svyasa.edu.in), Patanjali Yogpeeth (www.divyaYoga.com), ICYER at Ananda Ashram (www.icyer.com), Krishnamacharya Yoga Mandiram (www.kym.org), ACYTER at JIPMER (www.jipmer.edu.in), and CYTER at Sri Balaji Vidyapeeth (www.sbvu.ac.in) along with specific mention of iRest in PTSD [13], GABA [14,15], neuroplasticity changes after mindfulness [16], and telomerase studies [6,7]. Studies on fast and slow Surya namaskar, uninostril pranayamas, cancer, depression, and dementia were also mentioned [15,17-19].

An Australian Yogacharya passed on the feedback from his student who said that the use of well researched and scientifically explained protocol [12] used when she was pregnant gave her a positive feeling of control up to and including the delivery of her baby.

As a Canadian Yogacharya responded, "hard to single one out, but studies on alternative nostril breathing [18,20-22], for instance, have influenced my own practice, and have allowed me to consider gradually incorporating these techniques, which I normally would have deemed too advanced, into some of my classes."

6. Are you satisfied with the quality of Yoga research at present?

The answers were equally divided between yes and no and a few actually said they were not sure. Some felt they did not have the required expertise to comment on it. Comments received included the noting of more prolific research coming out in recent times but at the same time bemoaning mediocre methodologies adopted. Though it was wholeheartedly accepted that research is a valuable tool, some felt that it was really only proving information handed down to us from the Yogis of yore. Over emphasis of quantitative rather than qualitative aspects of research were mentioned and a need felt for more studies combing both aspects. Longterm longitudinal studies were also suggested through it was at the same time mentioned that this may be highly ambitious.

As said by a senior Yoga therapist in the USA, "I'm satisfied with the rapid trajectory but much more work is needed to bring in mixed methods and participatory action research methodology to capture local, community change as well versus the Asmita of the individual "subject's" suffering.

An Indian Yoga therapist responded saying, "I am satisfied but there are many Gurus and many traditions of Yoga. Whenever we talk about Yoga education, therapy and especially research, a question always arises about 'which Yoga' was done? This leads to confusions in the minds of teachers, patients and students. We should try and research a model syllabus that brings the best of all traditions together" said she.

7. What type of studies do you wish to see in the future?

A senior leader in Yoga therapy from Australia responded "well-designed case studies that allow for individualizing of the protocol, and that can eventually be meta-analyzed."

A senior trainer from Canada suggested that studies should look at supporting evidence for Yoga as an aid to overcoming addictions, as well as the impact of physical postures in aiding peace of mind and emotional stability. Populations that meditate are said to have better grades, lower crime rates, and selfless behavior and this aspect needs further study. He also suggested orienting research around benefits of Seva and Karma Yoga.

One of the most senior Yoga therapist trainers in India remarked, "Studies should be oriented toward finding out the risks attended with the practice of Yoga." He went on to suggest that Yoga therapy research should be "individual specific" and look at the individual and their tendencies leading to illness rather than being focused on "illness" per se.

Another USA-based therapist mentioned, "With the increase in lifestyle diseases across the world and the positive effects Yoga has on reducing such diseases, producing more research to 'prove' what we know is critical for Yoga's acceptance as an effective and valid and necessary treatment for a majority of the population today."

Another Australian Yoga teacher suggested, “I'd like to see comparative studies between traditional classical Yoga (as a health and wellness practice) and say the gym, Pilates, cross-training, etc., and all the other new fads of ‘Yoga’ (Hot Yoga, power Yoga, and the plethora of new wave so-called Yoga fads). It would be interesting to see how effective other exercise modalities are in comparison to the ancient wisdom of Yoga: is the ancient wisdom holding its own in the modern world of technoexercise programs, gyms with so-called instructors, etc.? I would also like to see comparative analysis between Western medicine and Yoga therapy and Ayurveda.”

8. Any other comments?

A dedicated Yogacharya from Bangalore suggested Yoga teaching and therapy curricula should include studies on characterization of humans, ethical, and moral values, the study of Sanskrit as part of life, the study of yogic as well as spiritual textbooks written by the ancient Yogis and Rishis, as well as an overall spiritual education during adolescence.

A Swiss Yogacharini wisely commented, “If science really wants to understand Yoga and its relationship with the human being, it should not stick to just the pieces of the puzzle but attempt to see the bigger picture of Yoga and its holistic effects on humankind.”

A European Yoga teacher of 19 years mentioned an interesting meeting with her GP who is also a Homeopath and works with Chinese meridians. After a brief discussion on Yoga that she was doing, he said, “In all the thousands of people I see, you are the most balanced, do you understand Yin and Yang?” “We both confirmed the powerful results of the regular practice of Yoga and I felt very happy when I left his surgery because I felt like he was amazed and honest,” wrote she.

CONCLUSION

This survey provides a basic picture of the general lack of awareness about Yoga research among practicing Yoga teachers and therapists. Though a majority agree that research is important, few are seriously updating themselves on such research findings through scientific channels. Most are updating themselves through general articles on the net and such information seems to have minimal influence on their day-to-day teaching and practice in most cases. With regard to future studies, most wanted ‘proof’ that could be used to convince potential clients and felt that more qualitative methods should be applied. It was also felt that comparative studies should be done between traditional/classical methods of Yoga practice as compared with newer innovative styles.

A major limitation of the present work is that the sample size is small and that the author personally knows most of the respondents for a lengthy period of time. However, this direct connection and nonblinding is deemed essential in order to get a “first-hand grassroots” qualitative perspective that is otherwise lost in studies considering large samples and quantitative statistical analyses.

The overall picture was well summarized by a dedicated Yoga therapy trainer from the USA when she said, “I feel it is ideal for Yoga teachers to follow research however not essential. My experience in teaching Yoga Therapy to Yoga Teachers is that most have no idea how to read a study and how to discern valid information. Thus, they often draw incorrect conclusions from reading simply the abstract or from not understanding the bigger picture. In order for them to obtain value from reading Yoga research, they need training in how to read research.”

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Original Research

Mechanism of vasorelaxation induced by *Tridax procumbens* extract in rat thoracic aorta

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ABSTRACT

Background/Aim: *Tridax procumbens* (Linn) (Asteraceae) is one of the herbs widely distributed in many parts of the world. Its leaves have long been used for the treatment of hypertension in Nigeria. Previous studies have shown that aqueous leaves of *T. procumbens* extract (TPE) lowers blood pressure through endothelium-dependent and -independent mechanism in the aortic rings isolated from normotensive rats. The aim of the present study was to further investigate mechanisms of TPE-induced relaxation in the aortic artery by assessing its mechanistic interactions with nitric oxide (NO) synthase, cyclic guanosine monophosphate (cGMP), and cyclic adenosine monophosphate (cAMP). **Materials and Methods:** The aortic artery isolated from healthy, young adult normotensive Wistar albino rats (250-300 g) were pre-contracted with phenylephrine (PE) (10^{-7} M) and KCl (60 mM) and were treated with various concentrations of aqueous extract of TPE (0.5-9.0 mg/ml). The changes in arterial tension were recorded using Ugo Basile model 7004 coupled to data capsule acquisition system model 17400. The interaction between TPE with cAMP and cGMP inhibitors was also evaluated. **Results:** The results showed that the TPE (0.5-9.0 mg/ml) significantly ($P < 0.05$) reduced the contraction induced by PE in a concentration-dependent manner. The vasorelaxant effect caused by the TPE was significantly ($P < 0.05$) attenuated with pre-incubation of cGMP (Rp-8Br PET cGMPS) and cAMP (Rp-AMP) inhibitor, respectively. **Conclusion:** These results suggest that TPE causes vasodilatory effects in a concentration-dependent manner in the isolated rat aortic artery. The mechanism of action of TPE is complex. A part of its relaxing effect is mediated directly by blocking or modulating cGMP and cAMP.

KEY WORDS: Cyclic adenosine monophosphate inhibitor, cyclic guanosine monophosphate inhibitor, Rp-8Br PET cyclic guanosine monophosphate, *Tridax procumbens*, vasorelaxant effect

INTRODUCTION

Hypertension is one of the most prevalent and important health problems affecting millions of people in developing as well as developed countries [1]. Although effective synthetic drugs for the treatment of hypertension exist, there remains a great interest in the use of natural plant extracts to attenuate the risk of cardiovascular disorders especially hypertension [2]. Many traditional medicinal herbs such as *Crocus sativus* petals [3], *Berberis vulgaris* root extract [4], *Schisandra chinensis* fruits [5], and many others have been reportedly used for the control of hypertension. However, many of these herbs have not been fully subjected to standard scientific evaluations to verify their potency.

Tridax procumbens L. (Asteraceae) is a common weed that grows in open places, *coarse-textured* soils of tropical regions throughout the world [6]. *T. procumbens* extract (TPE) has been for many years used in Nigeria as an antihypertensive agent [7]. However, the exact mechanisms responsible for its antihypertensive activity are still not fully understood.

Previous studies reported that TPE caused bradycardia and hypotension in normotensive rats [7] and that aqueous leaf of TPE produced relaxation of isolated rat aorta [8]. It was suggested that the relaxation may be partly due to inhibition of Ca^{2+} influx through receptor-gated channels [9]. In the present study, we investigate the role of cGMP and cAMP on the vasodilatory effects of TPE. Furthermore, its effects on the nitric oxide (NO) release were also investigated.

MATERIALS AND METHODS

Ethical Considerations

Experimental protocols and procedures used in this study were approved by the Animal Ethics Committee of the Lagos State University College of Medicine and also conformed to the 1985

guidelines for laboratory animal care of the National Institute of Health (NIH).

Plant Material

Fresh leaves of *T. procumbens* were collected from open grassland of Lagos State University College of Medicine, Ikeja, Lagos, Nigeria. Identification of the plant was carried out by a Taxonomist of the Forestry Research Institute, Mr. K.A Adeniji. Following identification, a specimen voucher number FHI 1008876 of the plant was deposited in the herbarium of the Forestry Research Institute, Ibadan, Nigeria.

Preparation of Extract

The leaves were air-dried at room temperature for a week, the dried leaves were pulverized with a laboratory mortar and pestle and 500 g of the powdered leaves were transferred into a conical flask containing 500 ml of distilled water; the mixture was shaken and allowed to stand for 24 h before filtration using a dry Whatman filter paper into a measuring cylinder. The filtrate was then concentrated by evaporation in a water bath (35-40°C) and stored at 4°C until used.

Animals

Healthy, young adult, male Wistar albino rats weighing 250-300 g were used. The animals were kept and maintained under conventional laboratory conditions of temperature, humidity, and light. The animals were allowed free access to standard pellet diet (Live Stock Feeds Nig. Ikeja, Nigeria) and water *ad libitum*.

Vascular Ring Preparation and Pharmacological Studies

The rats were anesthetized with pentobarbital (60 mg/kg, i.p.). The descending thoracic aorta was excised by midline incision, cleaned of fat and connective tissues, with care taken not to stretch the vessel excessively or to disturb the luminal surface of the rings, to ensure the integrity of the endothelium. The aorta was then cut into small rings (3-5 mm in width) and suspended between two wire stirrups in a jacketed organ bath containing 50 ml of normal Krebs physiological solution of the following composition (mM): NaCl 118.2, KCl 4.7, $CaCl_2 \cdot 2H_2O$ 2.5, KH_2PO_4 1.2, $MgCl_2$ 1.2, glucose 11.7. The bathing solution was bubbled continuously with a mixture of 95% oxygen and 5% carbon dioxide at 37°C. The rings were suspended with a resting tension of 0.8 g for at least 60 min then reduced to 0.6 g with constant changing of Krebs solution (every 15 min) to prevent accumulation of metabolites that could otherwise lead to misinterpretation of results [10]. The isometric tension was recorded by the force-displacement transducer (Model 7004; Ugo Basil Varese, Italy) connected to Data capsule Model 17400 for the isometric contractions. The rings were then progressively stretched to an optimal tension of 1 g and allowed to equilibrate for 60 min. Following the equilibration period, the aortic rings were allowed to achieve maximal tension by repeated exposure (each for 5 min) to isotonic potassium chloride solution (high K^+ 60 mM). The rings that showed vasoconstriction response induced by phenylephrine (PE) (10^{-7} M) were studied. PE was then washed out, and the tension was returned to the baseline levels before the administration of the drugs. The aortic ring was denuded to remove the endothelial layer in some preparations by inserting a pair of forceps into the lumen of each artery and gently rotating it. The viability of each aortic ring was validated by pre-contraction of PE (10^{-7} M) and relaxed by ACh (10^{-5} M) just before the experiment commenced. Relaxation of ≥ 70% indicated the presence of a functional or intact endothelial layer while the lack of relaxation indicated the successful removal of the layer.

Effects of TPE on Endothelium - Intact and - Denuded Ring Pre-contracted with PE

The endothelium-intact and -denuded arteries were pre-contracted with PE (10^{-7} M). After the tonic responses or contractions became stable, increasing concentrations of TPE (0.3-1.8 mg/ml) were added cumulatively.

To characterize TPE-induced vasodilatation pharmacologically, specimens were pre-treated with various inhibitors including a selective cGMP inhibitor (Rp-8-Br-PET-cGMPS) and the selective cAMP inhibitor (KT-5720).

Measurement of Nitrite Production in Isolated Aorta

Nitrite/nitrate, which is the index of vascular NO production was measured according to the method of Raghavan and Dikshit [11]. Describe briefly, aortic rings (6mm in length) were washed in Krebs solution and divided into different experimental groups, which were treated with control or TPE (0.3-1.8 mg/ml) in the endothelium-intact and -denuded rings incubated at 37°C in a shaker for 30 min in a total volume of 1 ml. After ring, supernatants were obtained by centrifugation, equal volumes of Griess reagent (1% sufanilamide and 0.1% naphthylethylene diamine in 5% phosphoric acid) were added, and the mixture was incubated at 37°C for 30 min. Absorbance was measured at 545 nm to determine total nitrite content. The NO production was calculated by subtracting nitrite concentration seen in the presence of the extract from the control, and this is regarded as NO production from experimental rings. Measurement for nitrite production was expressed as μmole/mg dry weight of tissue.

Drugs

L-PE chloride, potassium chloride (KCL), sodium chloride (9R,10S,12S)-2,3,9,10,11,12-Hexahydro-10-hydroxy-9-methyl-1-oxo-9, 12-epoxy-1H-diindolo [1,2,3-fg:3', 2',1'-kl]pyrrolo [3, 4-i][1,6] benzodiazocine-10-carboxylic acid, hexyl ester (KT-5720), and 2-Bromo-3,4-dihydro-3-[3,5-O-[(R)-mercaptophos phinylidene]-β-D-ribofuranosyl]-6-phenyl-9H-Imidazo[1,2-a] purin-9-one sodium salt (Rp-8-Br-PET-cGMPS). All were purchased from Tocris, United Kingdom.

Statistical Analysis

Data were analyzed using GraphPad Prism version 5.0 statistical software, and the results were expressed as means ± standard error, where n equals the number of animals from which blood vessels were isolated. The data were analyzed using one-way ANOVA. Dunnett's multiple comparison test was used to identify differences between individual means. The confidence interval was set at 95% so that in all cases, results with a value of $P < 0.05$ were considered to indicate statistical significance.

RESULTS

Effects of Graded Doses of TPE on PE and KCl-Induced Contraction

Figure 1 shows the typical tracing of relaxation responses to TPE (0.3-1.8 mg/ml) recorded in aortic ring pre-contracted by PE [Figure 1a] or by KCl [Figure 1b]. The tension developed was significantly reduced by cumulative application of TPE.

Role of Endothelium in TPE-Induced Relaxation

TPE (0.3-1.8 mg/ml) showed a concentration-dependent relaxation effect in both endothelium-intact and endothelium-denuded aortic rings after pre-contraction by PE. However, the functional removal of endothelium did not significantly modify TPE-induced relaxation in PE - pre-contracted aortic rings [Figure 2].

Effect of TPE on the Cyclic Guanosine Monophosphate (cGMP) and Cyclic Adenosine Monophosphate (cAMP) Activities

To assess the role of cGMP and cAMP in the relaxation produced by TPE in aortic rings pre-contracted by PE, we used aortic rings pre-incubated with Rp-8-Br-PET-cGMPS (30 μM) or a selective PKG inhibitor KT-5720 (50 μM) the selective PKA inhibitor in both endothelium-intact and endothelium-denuded rings as pharmacological tools. Under these conditions, the maximum relaxation produced by TPE in endothelium-intact rings was 46.2 ± 4.5%. Pre-incubation for 30 min with the PKG inhibitor Rp-8-Br-PET-cGMPS produced a significant decrease ($P < 0.01$) from the maximal relaxation elicited by TPE to 12.8 ± 2.7 and 16.7 ± 4.8 % in endothelium-intact and endothelium-denuded, respectively [Figure 3a and b]. Furthermore, pre-incubation with the PKG inhibitor KT-5720 significantly ($P < 0.01$) inhibited the relaxation induced by TPE in both endothelium-intact and -denuded [Figure 4a and b].

Effect of TPE on NO Production

As shown in Figure 5, the production of nitrite in the incubation media of aortic rings were significantly ($P < 0.01$) increased by TPE treatment in a concentration-dependent manner (0.3-1.8 mg/ml), while functional removal of endothelium significantly reduced this effect [Figure 5].

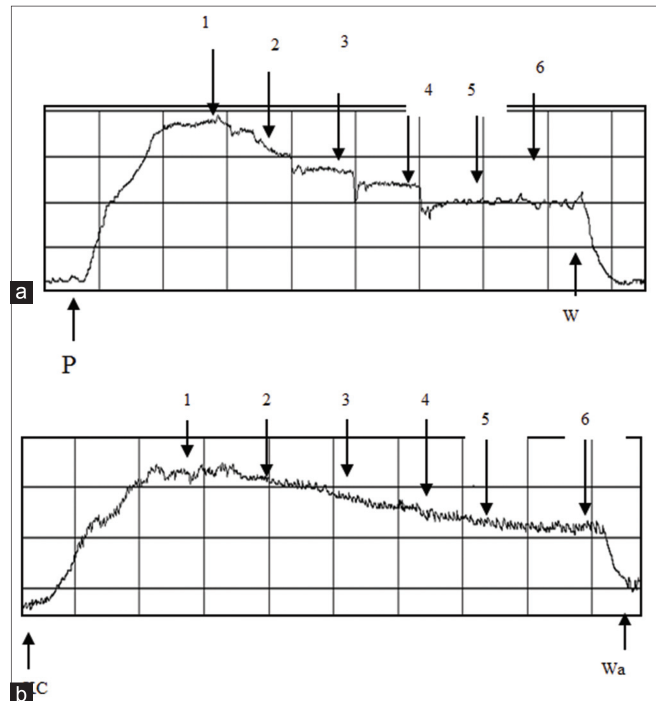

Figure 1: Typical tracing showing the vasorelaxant effects of graded concentration of TPE a (a) Phenylephrine (10^{-7} M) (PE)-induced and (b) (60 mM) KCl-induced contraction in the endothelium-intact aortic ring isolated from normotensive rat. Arrows 1-6 represent cumulatively administered TPE (0.3, 0.6, 0.9, 1.2, 1.5, and 1.8 mg/ml, respectively) administration of drup upward-arrow (P) and washed out at (W) downward-arrow

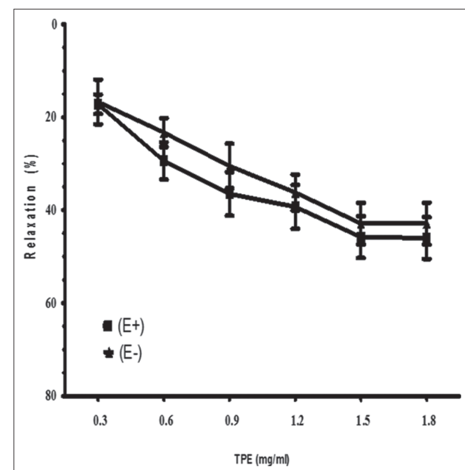

Figure 2: Concentration-response curve showing the vasorelaxant effect of TPE in endothelium-intact (E+) and -denuded (E–) rat aortic rings pre-contracted with phenylephrine (10^{-7} M). (E_{max} = maximal contraction considered as 100%). Each point represent the mean ± standard error (indicated by vertical line) of 6 experiments. Data were analyzed using one-way ANOVA followed by Dunnett's multiple comparison test

DISCUSSION

The data presented in this study provides strong evidence that cyclic GMP/cAMP play a major role in vasorelaxation induced

by TPE in rat aortic smooth muscle. This conclusion was based on the findings that the Rp-8-Br-PET-cGMPS (cGMP inhibitor) and KT-5720 (a cAMP inhibitor) significantly inhibit relaxation induced by TPE at all concentration tested.

Vasodilatation can be facilitated by inhibition of vasoconstriction and secretion of relaxant factors from vascular endothelium. The later is mediated by prostacyclin, bradykinin, and NO. Our previous observation in rats aortic smooth muscle showed that relaxation to TPE was markedly inhibited by blockers of NO and prostacyclin [12]. This would support the idea that cGMP-dependent pathways are involved in mediating relaxation to TPE in this tissue. Indeed, in the present study, TPE dose-dependently increases nitrite concentration in the rat aorta confirming that TPE relaxes the rat aorta via endothelium-dependent NO/cGMP pathways.

The mechanism by which cyclic GMP mediates the TPE-induced relaxation is not fully understood. However, it was suggested that cGMP may affect sequestration of intracellular Ca^{2+} by affecting Ca^{2+} extrusion pumps and hereby decrease the sensitivity for Ca^{2+} [13]. This mechanism may occur without a change in the membrane potential. Thus, the NO-cGMP role in the relaxation effect of TPE needs to be further investigated.

In this study, when the functional endothelium was removed the vasorelaxant action of TPE still persisted in denuded aortic strips.

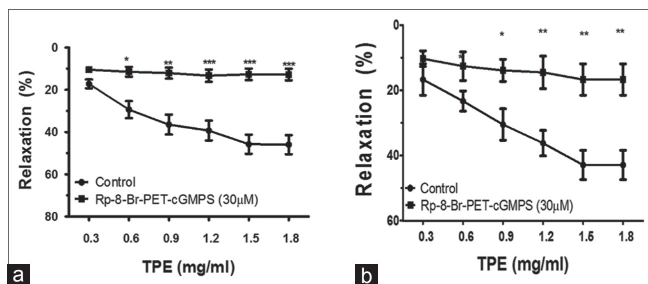

Figure 3: Line graph showing the effects of Rp-8-Br-PET-cGMPS (30 μM) on cumulative concentration response of *Tridax procumbens* extract (0.3 - 1.8 mg/ml) in (a) endothelium-intact (b) endothelium-denuded mesenteric artery pre-contracted with phenylephrine (10^{-7} M). Data were analyzed using one-way ANOVA followed by Dunnett's multiple comparison test. (*n* = 6) (**P* < 0.05; ***P* < 0.01; ****P* < 0.001)

This observation indicates that TPE may modulate vascular tone by acting directly on smooth muscle cells via another pathway. It is well known that contractile apparatus of vascular smooth muscle cells depends on the phosphorylation state of the myosin regulatory light chain (MLC) of myosin II, which is phosphorylated by Ca^{2+}/calmodulin-dependent MLC kinase. Phosphorylated MLC interacts with actin filaments, leading to contraction. Conversely, when MLC are dephosphorylated by MLC phosphatase, the interaction between actin-myosin ceases causing relaxation of the muscle [14]. This finding is consistent with results obtained from the previous study that vasorelaxant activity of TPE is mediated through calcium-dependent mechanisms [9].

The results of this study also showed that TPE-induced relaxation of the aortic artery was attenuated in the presence of cAMP inhibitor, and the TPE concentration-response curve was shifted significantly to the right. This indicates that cAMP is involved in the vascular relaxation induced by TPE. The mechanisms by which TPE relaxed the vascular smooth muscle through activation of cAMP signaling pathways are not known. However, reports from several studies indicated that increase in intracellular adenosine 3′, 5′-cyclic monophosphate (cAMP) levels ([cAMP]i), and the subsequent activation of cAMP-dependent signaling pathways leading to the relaxation of vascular smooth muscle can be activated through the following mechanisms; reduced entry of Ca^{2+} from extracellular space through ionic channels [15,16] reduced release of Ca^{2+} from intracellular stores [17]; and decrease of cytoplasmic Ca^{2+} concentration [18-20].

T. procumbens leaves have been reported to contain several active compounds such as alkaloids, flavonoids, quercitin, arachidic, and linoleic acid [21-23]. Earlier studies have reported the presence of dexamethasone, luteolin, and glucoluteolin [24,25], and recently, it was reported that TPE was very rich in linolenic acid [26].

Quercetin is a phytochemical belonging to the flavonoid family and is the most ubiquitous of the dietary flavonoids [27]. Reports indicated that quercetin decreases blood pressure (BP) and/or reduces the severity of hypertension in spontaneously hypertensive rats [28,29]. The flavonoid luteolin also

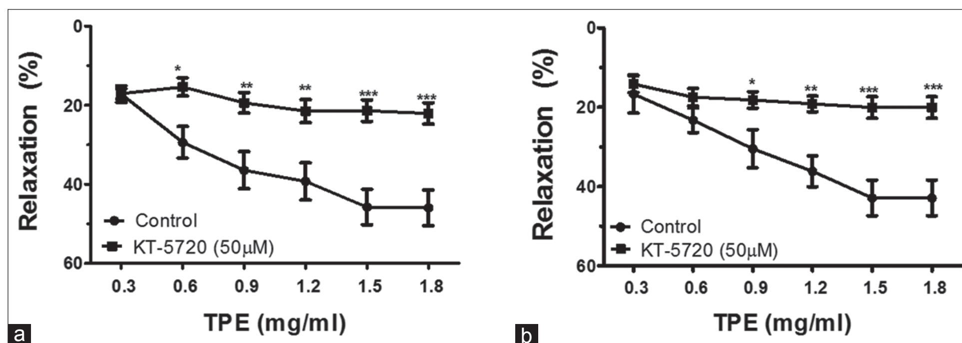

Figure 4: Line graph showing the effects of KT-5720 (50 μM) on cumulative concentration response of *Tridax procumbens* extract (0.3-1.8 mg/ml) in (a) endothelium-intact (b) endothelium-denuded mesenteric artery pre-contracted with phenylephrine (10^{-7} M). Data were analyzed using one-way ANOVA followed by Dunnett's multiple comparison test. (*n* = 6) (**P* < 0.05; ***P* < 0.01; ****P* < 0.001)

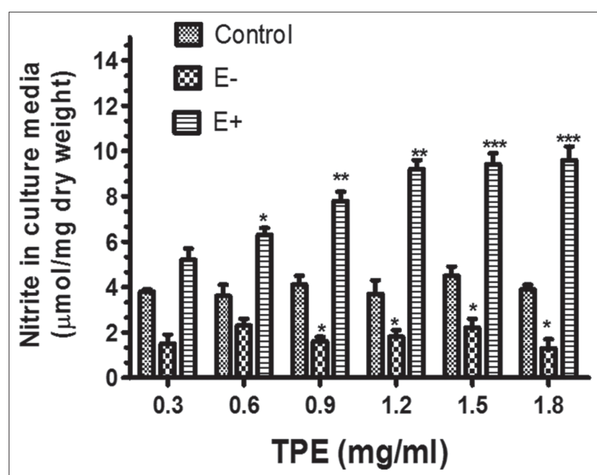

Figure 5: *Tridax procumbens* extract-induced nitrite production in (E+) endothelium-intact and (E–) endothelium-denuded of rat aorta expressed as µmole/mg dry weight. Data were analyzed using one-way ANOVA followed by Dunnett's multiple comparison test. (n = 6) (*P < 0.05; ** P < 0.01; *** P < 0.001)

induces NO production and arterial relaxation [30]. Thus, vasorelaxant activities of TPE might also result from these active compounds found in them. It has been established by studies that active compounds from the medicinal plant can act as vasodilators [31,32]. Plants showing propensity for NO production are promising candidates for vasodilatation and may have the potential for the prevention and treatment of cardiovascular diseases such as hypertension and atherosclerosis. Further work is necessary to isolate, identify, and characterize more active compound of *T. procumbens* and elucidates the molecular mechanisms of those acting on endothelial and smooth muscle cells.

CONCLUSION

This study investigated the vasorelaxant effect of TPE on isolated rat aorta and its possible mechanisms. The mechanism of its effect involves mainly activation of the NO-cGMP-cAMP pathways and partly by its direct action on the vascular smooth muscle via dephosphorylation of MLC, resulting in vasodilation. This study provides a mechanistic clue to the role of TPE, which has long been used for the treatment of high BP.

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Original Research

Antimalarial activity of fractions of aqueous extract of *Acacia nilotica* root

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ABSTRACT

Background: The problem of resistance of malarial parasites to available antimalarial drugs makes the development of new drugs imperative, with natural plant products providing an alternative source for discovering new drugs. **Aim:** To evaluate the antimalarial activity of eluted fractions of *Acacia nilotica* root extract and determine the phytochemicals responsible for its antimalarial activity. **Materials and Methods:** The extract was eluted successively in gradients of solvent mixture (hexane, ethyl acetate, and methanol) in multiples of 100 ml, and each fraction was collected separately. Eluates that showed similar thin layer chromatographic profiles and R_f values were combined to produce 4 main fractions (F-1, F-2, F-3, and F-4), which were tested separately for antimalarial activity using the curative test. Changes in body weight, temperature, and packed cell volume (PCV) were also recorded. **Results:** Fraction F-1 of *A. nilotica* at 50 and 100 mg/kg b/w produced significant and dose-dependent reduction in parasite count in *Plasmodium berghei* infected mice compared to the control, and also significantly increased the survival time of the mice compared to the control group. This fraction also ameliorated the malaria-induced anemia by improving PCV in treated mice. **Conclusion:** Antimalarial activity of extract of *A. nilotica* root is probably localized in the F-1 fraction of the extract, which was found to be rich in alkaloids and phenolics. Further study will provide information on the chemical properties of the active metabolites in this fraction.

KEY WORDS: *Acacia nilotica*, antimalarial, fractions, medicinal plant

INTRODUCTION

Malaria is a preventable and curable disease, yet it remains a devastating tropical disease, with a high infection and mortality statistics. It is the most prevalent parasitic disease and the most common cause of hospital visitation in Nigeria [1]. Globally, there are approximately 214 million malaria cases in 97 countries with ongoing malaria transmission and 80% of these cases were reported in the Sub-Saharan Africa. The disease caused approximately 438,000 deaths, 90% of which were in Sub-Saharan Africa, and 78% of these deaths occur in children under 5 years [2]. The main challenge to the effective management of diagnosed malaria cases is the resistance of the plasmodium parasite to commonly used antimalarial drugs, which results in the nonresolution of symptoms and in treatment failure [3,4]. Cases of resistance are currently extending to the artemisinin combination therapy with report of resistance to artemisinin drugs coming from Thailand, Vietnam, and Cambodia [2,5-7]. Furthermore, no new class of antimalarial drug has been introduced in clinical practice in the last 10 years [8,9]. This strongly supports the need for further research into new products that could complement the existing antimalarial, bearing in mind that it takes minimum of 10 years to develop a drug from discovery stage to completion of clinical trials [10]. The development of safe and effective anti-malarial preparations by simple procedures from locally grown medicinal plants, may offer new and complimentary drugs for malaria control, especially in remote geographical locations and in rural areas where modern antimalaria drugs are not readily available and malaria mortality is higher [11].

Acacia nilotica (Linn.) Willd. Ex Del. (also called Gum Arabic tree; Prickly tree, 'Gabaruwa' in *Hausa* and 'Igi Kasia/Booni' in *Yoruba*) is a scented thorny tree native to Africa (grows in Egypt, South Africa, Kenya, and Nigeria) and Indian subcontinents [12]. It is a nitrogen-fixing tree that grows to 14-17 m in height and 2-3 m in diameter.

African Zulu use the bark of A. *nilotica* to treat cough, diarrhea, dysentery, and leprosy [13]. The Massai (Kenya) use the bark and root decoction as aphrodisiac to cure impotence. The fruit is used to treat tuberculosis [14], whereas the powdered pods are consumed by Egyptians to treat diabetes mellitus [15]. In Northern Nigeria, the root is used for the treatment of malaria [16,17]. A. *nilotica* is rich in many secondary metabolites, such as tannins, terpenes, alkaloids, flavonoids, and phenolic [17], with known pharmacological properties, thus making it relevant in the treatment of various ailments.

This research aims at evaluating the antimalarial activity of fractions obtained from crude aqueous extract of A. *nilotica* root and investigates the active antimalarial phytochemical in the crude extract.

MATERIALS AND METHODS

Plant Collection, Identification, and Authentication

Fresh root samples of A. *nilotica* were collected around 8.45 am in Bamburu-Chaza, Suleja, North-central part of Nigeria. They were identified and authenticated as A. *nilotica* root at the herbarium of the National Institute for Pharmaceutical Research and Development (NIPRD), Abuja by a taxonomist, Mrs. Grace Ugbabe. Voucher specimen (NIPRD/H/6401) was prepared and deposited at the herbarium for referencing.

Experimental Animals

Swiss albino mice (*Mus musculus*, 25.00 ± 2 g) used for this study, were obtained from Animal Facility Center, NIPRD, Abuja. They were housed in well ventilated cages, fed with rat pellets and water *ad libitum*, and maintained under standard laboratory conditions (temperature of 25 ± 3°C and 12 h light/12 h dark cycle), in accordance with the guidelines for the care and use of laboratory animals by National Academy of Science (1996). Ethical approval for animal studies was obtained from the Animal Ethics Committee of NIPRD.

Malaria Parasites

Chloroquine-sensitive rodent *Plasmodium; P. berghei* NK 65 strain, was obtained from the National Institute for Medical Research, Lagos, Nigeria, and maintained alive by serial intraperitoneal passage in mice, every 5 days.

The reinfected mice were kept at the Animal Facility Center of NIPRD where this study was carried out.

Reagents and Chemicals

The chloroquine phosphate and silica gel (70-230 mesh) were obtained from Sigma-Aldrich Corporation, Missouri, 63103, USA. All other chemicals and reagents used were of analytical grade and prepared using distilled water.

Preparation of Aqueous Extract of *A. nilotica* root

The root sample of A. *nilotica* was air dried to constant weight and pulverized using grinding machine. The powder was stored in an airtight container and kept in a cool, dry place. Aqueous extraction was carried out following the cold maceration method. Four hundred grams of the powdered root of A. *nilotica* was soaked in 1 L of distilled water and kept for 24 h with intermittent shaking. The suspension was filtered after 24 h, with muslin cloth followed by filtration with Whatman filter paper (No.1). The filtrate was freeze-dried using AMSCO/FINN-AQUA GT2 Freeze dryer (Germany). This extraction procedure was carried out thrice to obtain sufficient quantity of extract for the entire study.

Column Fractionation of *A. nilotica*

Wet silica gel (70-230 mesh) was loaded into a column, and the crude extract was added on the upper layer [18]. The extract was eluted successively in gradients of solvent mixture (hexane, ethyl acetate, and methanol) in multiples of 100 ml and each fraction was collected separately. Eluates that showed similar thin layer chromatographic profiles and R_f values were combined to produce 4 main fractions, which were tested separately for antimalarial activity using the curative test.

Phytochemical Screening

The four fractions obtained were subjected to phytochemical screening following the procedures described by Sofowora [19], to determine the predominant secondary metabolite in each fraction.

Antimalarial Test

Parasite inoculation

Each mouse used in the experiment was inoculated intraperitoneally with 0.2 ml of infected blood (containing about 1×10^7 parasitized erythrocytes) obtained from a single donor mouse previously infected with chloroquine-sensitive *P. berghei* (containing 25.5% parasitemia). This was prepared by calculating the percentage parasitemia of donor mouse and diluting the blood with physiological saline so that 0.2 ml of diluted blood contained 1×10^7 infected erythrocytes [20].

Grouping and Drug Administration

P. berghei-infected mice were randomly divided into five groups of 6 mice per group. Thirty mice were used for the antimalarial test of each of the four fractions. Group 1 was administered

10 ml/kg body weight distilled water (negative control), Groups 2, 3, and 4 were administered 25, 50, and 100 mg/kg b.w of each fraction. Group 5 was administered 5 mg/kg b/w chloroquine (positive control). Fractions of A. *nilotica* and chloroquine drug used in this study were administered orally using stainless metal oral cannula. Doses administered were calculated from the value of median lethal dose. Same procedure as above was repeated for the antimalarial test for fractions F-2, F-3, and F-4.

Evaluation of Antimalarial Activity of Fractions of *A. nilotica*

The Rane test was used to evaluate the schizonticidal activity of the fractions of A. *nilotica* in established malaria infection.

The evaluation of the curative potential of fractions obtained from A. *nilotica* root against established infection was carried out as described by Ryley and Peters [21]. Thirty mice were inoculated as earlier described on day 0, and left untreated for 72 h. The mice were weighed and randomized into five groups of six mice each. Group 1 was administered 10 ml/kg body weight of distilled water (negative control); Groups 2, 3, and 4 received fraction of A. *nilotica* at doses of 25, 50, and 100 mg/kg body weight/day orally, respectively, whereas mice in Group 5 (positive control) received 5 mg/kg body weight/day of chloroquine orally for 4 days (D_4-D_7). On day-8, each mouse was tail-bled, and a thin blood film was made on a microscope slide.

The films were stained with Giemsa stain and examined microscopically to monitor the parasitemia level [22,23]. The mean survival time of the mice in each treatment group, monitored over a period of 30 days was calculated using the expression:

$$\text{Mean survival time} = \frac{\text{Sum of survival of every animal in each group in days}}{\text{The number of animals in each group}}$$

Determination of Percentage Parasitemia

Thin blood films were made from the tail blood of each infected mouse, stained with Giemsa and examined under the microscope at ×100 magnification (oil immersion), to determine the level of parasitemia. Percentage parasitemia was calculated using the formula below:

$$\text{Percentage Parasitemia} = \frac{\text{Parasitized RBC}}{\text{Parasitized RBC} + \text{Non} - \text{Parasitized RBC}} \times 100$$

RBC = Red blood cells.

Determination of Packed Cell Volume (PCV)

PCV of the mice was measured before infecting them with parasite and after treatment. PCV was done to determine the effectiveness of the fractions in preventing hemolysis resulting from increasing parasitemia level associated with malaria. Heparinized capillary tubes were used for collection of blood from tail of each mouse. The capillary tubes were filled with blood up to 75% of their volume and sealed at the dry end with sealing clay. The tubes were placed in a microhematocrit centrifuge (Hawksley, England) with the sealed end outward and centrifuged for 5 min at 11,000 rpm [24]. The tubes were then taken out of the centrifuge and PCV was determined using a standard Micro-Hematocrit Reader (Hawksley, England).

Monitoring of Body Weight and Temperature Changes

Body weight of each mouse was measured before infection (day 0) and on day 8, using a sensitive digital weighing balance (OHAUS, USA). Rectal temperature was also measured with a digital thermometer before infection, and then daily. In order to evaluate the effect of the fractions on body weight and temperature, the fractions were administered to healthy (uninfected) mice at the doses of 25, 50, and 100 mg/kg b.w for 4 days.

Data Analysis

Graphpad prism Version 5.02 was used to analyze the data obtained and these were expressed as mean ± standard error of mean (SEM). The differences between means were compared using one-way Analysis of Variance, followed by Dunnet's test. $P < 0.05$ was considered statistically significant.

RESULTS

Phytochemical Screening

The summary of the phytochemical constituents of fractions of A. *nilotica* root extract, shown in Table 1, revealed that the fractions gave positive reactions to phenolic, tannins, alkaloids, anthraquinones, flavonoids, terpenes, and sterols. Fraction F-1 showed high amount of alkaloids compared with other three fractions [Table 1].

Antimalarial Activity of Fractions of A. *nilotica;* (F-1, F-2, F-3, and F-4)

Fractions F-1 and F-2 caused significant and dose-dependent reduction in the mean parasite count at doses of 50 and

Table 1: Phytochemical constituents of fractions (F-1, F-2, F-3, and F-4) of *Acacia nilotica* root

Phytochemicals	F-1	F-2	F-3	F-4
Phenolics	+	+	-	-
Tannins	-	+	+	-
Alkaloids	++	-	-	-
Saponins	-	+	+	-
Anthraquinones	-	-	+	+
Flavonoids	-	+	-	-
Terpenes	-	-	-	+
Sterols	-	-	-	+

-Not detected; +Present; ++High concentration

100 mg/kg b/w when compared with the control, although the reduction was less than chloroquine used as a standard drug [Table 2]. However, F-3 and F-4 did not produce significant reduction in parasite count at all the doses administered [Table 3]. Survival time was also prolonged by fraction 1 and 2, with fraction 1 producing a significantly prolonged survival time that was comparable to chloroquine [Table 2], unlike F-3 and F-4 that did not prolong the survival time.

Fraction F-1 produced moderate weight gain and increased PCV at the dose of 100 mg/kg b/w; however, it did not prevent temperature lowering effects of malaria infection in mice [Table 4]. Fraction F-2 did not prevent weight loss, reduction in rectal temperature, and PCV in the treated mice [Table 5].

DISCUSSION

P. berghei NK 65 was used in this study for inoculation and to predict antimalarial treatment outcome because of its ability to produce rodent model of malaria similar to human malaria infection [25,26]. Chloroquine, though no longer a first-line drug in the treatment of malaria, is used as control drug in this study because the *Plasmodium* parasite used for inoculation in this study is a chloroquine-sensitive strain. The Rane's test is commonly used in antimalarial screening for evaluating the curative capability of extracts/drugs on established infections. This screening test was previously employed for the antiplasmodial screening of the crude extract of A. *nilotica* [17]. This study was carried out *in-vivo*, in order to factor in the

Table 2: Curative effect of fractions F-1 and F-2 of *Acacia nilotica* in *Plasmodium berghei* infected mice

Treatment dose mg/kg b.w	Parasitemia count				Mean survival time (days)
	Pretreatment (D-3)	Posttreatment (D-8)	Posttreatment (D-14)	Posttreatment (D-21)	
F-1					
D/water 10	16.50±1.85	29.50±1.45	D	D	8±1.00
25	17.00±0.73	13.70±0.37*	18.00±0.65*	D	15±1.50*
50	17.50±0.85	6.70±0.35*	7.00±0.45*	D	18±1.00*
100	18.00±0.65	5.80±0.37*	4.50±0.75*	4.00±0.55*	21±1.00*
Chloroquine 5	17.50±0.75	2.50±0.25*	2.00±0.35*	1.50±0.35*	28±2.00*
F-2					
D/water 10	16.50±1.83	28.50±1.04	D	D	8±1.50
25	16.00±1.75	15.50±0.48*	25.00±1.55*	D	14±1.00
50	17.00±1.85	7.20±0.86*	9.50±0.75*	D	17±1.00*
100	18.00±1.65	6.50±0.75*	9.20±0.55*	D	17±1.00*
Chloroquine 5	17.50±1.75	2.50±0.35*	2.00±0.35*	1.50±0.25*	28±2.00*

D-3, 8, 14, and 21=Days 3, 8, 14, and 21. *Significantly different from control at $P<0.05$. $n=5\pm$SEM. D: Death, SEM: Standard error of mean

Table 3: Curative effect of fractions F-3 and F-4 of *Acacia nilotica* in *Plasmodium berghei* infected mice

Treatment dose mg/kg b.w	Parasitemia count				Mean survival time (days)
	Pretreatment (D-3)	Posttreatment (D-8)	Posttreatment (D-14)	Posttreatment (D-21)	
F-3					
D/water 10	16.00±0.83	28.50±1.04	D	D	8±2.00
25	18.00±0.73	27.70±0.86	D	D	9±1.50
50	18.00±0.95	24.20±0.71	D	D	12±1.00
100	17.00±0.75	20.50±0.48	24.50±1.45	D	14±1.50
Chloroquine 5	17.00±0.75	3.00±0.25*	2.50±0.35*	2.00±0.25*	28±2.00*
F-4					
10	16.00±1.83	29.50±1.04	D	D	8±2.05
25	16.00±1.83	26.70±1.86	D	D	9±1.65
50	18.00±1.95	25.20±1.71	D	D	10±1.34
100	17.00±1.75	24.00±1.48	D	D	11±1.50
Chloroquine 5	17.00±1.85	3.00±0.35*	2.50±0.25*	2.00±0.25*	28±2.10*

D-3, 8, 14, and 21=Days 3, 8, 14, and 21. *Significantly different from control at $P<0.05$. $n=5\pm$SEM. D: Death, SEM: Standard error of mean

Table 4: Temperature, weight, and PCV of *Plasmodium berghei*-infected mice treated with fraction F-1 of *Acacia nilotica*

Treatment dose	Temperature (°C)		% change	Weight (g)		% change	PCV (%)		% change
(mg/kg b.w)	D_0	D_8		D_0	D_8		D_0	D_8	
D/water 10	37.9±0.10	35.6±0.15	–6.1	18.6±1.55	16.5±1.35	–11.3	52.6±1.25	45.0±0.95	–14.4
25	37.8±0.11	37.4±0.08*	–1.0	18.2±1.75	17.5±1.55	–3.8	52.5±1.15	50.5±0.95	–3.8
50	37.7±0.05	37.5±0.10*	–0.5	19.2±1.45	19.0±1.25*	–1.0	51.5±1.05	51.7±1.15*	0.4
100	37.8±0.12	37.7±0.10*	–0.3	19.0±0.85	19.2±0.95*	1.0	51.6±1.35	52.5±1.65*	1.7
Chloroquine 5	37.9±0.13	38.0±0.12*	0.3	18.8±1.25	19.5±0.85*	3.7	52.8±1.55	53.9±1.45*	2.1

*Significantly different from control at $P<0.05$; $n=5\pm$SEM; D_0 and D_8: Days 0 and 8, SEM: Standard error of mean, PCV: Packed cell volume, SEM: Standard error of mean

Table 5: Temperature, weight, and PCV of *Plasmodium berghei*-infected mice treated with fraction F-2 of *Acacia nilotica*

Treatment dose	Temperature (°C)		% change	Weight (g)		% change	PCV (%)		% change
(mg/kg b.w)	D_0	D_8		D_0	D_8		D_0	D_8	
D/water 10	38.0±.25	35.4±.15	−6.8	19.1±.95	17.0±.35	−11.0	51.3±.35	45.2±	−11.9
25	37.9±.15	37.1±.25	−2.1	18.8±.20	17.4±.15	−7.4	52.2±.15	46.5±.25	−10.9
50	37.8±.10	37.2±.20*	−1.8	19.0±.35	18.0±.35	−5.3	52.1±.45	45.0±.55	−13.6
100	37.9±.35	37.6±.25*	−0.8	19.4±.20	18.4±.15	−5.1	51.4±.25	44.8±.35	−12.8
Chloroquine 5	38.0±.20	38.0±.15*	0.0	18.9±.95	21.1±.25*	1.1	52.5±.55	53.5±.45*	2.0

*Significantly different from control at $P<0.05$; $n=5$±SEM; D_0 and D_8: Days 0 and 8, PCV: Packed cell volume, SEM: Standard error of mean

possible effects of prodrug and involvement of immune system in the eradication of infection in a living host.

From the result of the antimalarial activity of the fractions in Table 2, fraction F-1 produced significant and dose-dependent antimalarial activity which was comparable to that of chloroquine, the control drug used. At the highest dose of 100 mg/kg b/w, F-1 was able to reduce parasite count from 18.0 to 4.0 (77.7% inhibition) compared to the standard drug which reduced the parasite count from 17.5 to 1.5 (91.4% inhibition). In the infected and untreated group, parasite count increased from 16.5 to 29.5. The percentage inhibition of the F-1, which is the closest (among the four fractions) to the standard drug, suggests that the active antimalarial compound in the crude extract of A. *nilotica* is localized in the F-1 fraction. Alkaloids and phenolic found in F-1 may possibly, individually or synergistically, account for its significant antimalarial activity, when compared with other three fractions. Many alkaloids derived from plants such as quinine and quinidine have been demonstrated to exhibit antimalarial activity [27,28]. Although fraction F-2 contain phenolic which may also account for some antimalarial activity observed (albeit lower than fraction F-1), the presence of saponin (phytodetergent) in this fraction, may account for the destruction of erythrocyte membrane manifesting as reduced PCV [29], which may also be responsible for the reduction in survival time of the F-2 infected mice [Table 2].

Anemia, change in body (rectal) temperature, and reduction in body weight are the general features of malarial infection in rodent (mice) and human malarial infection [30]. A potent antimalarial is expected to ameliorate anemia, prevent body weight loss, and stabilize temperature in infected mice with increasing parasitemia.

PCV was measured in this study to determine the effectiveness of fractions of A. *nilotica* in preventing malaria-induced hemolysis alongside its antimalarial activity. Malaria-induced hemolysis, either in human or rodents, may be due to rapid destruction/clearance of infected erythrocytes and/or sequestration of infected erythrocytes [30]. Fraction F-1 at doses of 50 and 100 mg/kg b/w produced significant increase in PCV [Table 4] when compared with the control, which suggests that this fraction of the extract could ameliorate anemia associated with malaria infection. The ability of this fraction to reverse reduction in PCV may be due to rapid clearance of parasite from infected erythrocytes before hemolysis occur or via enhanced erythropoiesis [29]. There was no reduction in PCV of mice at all the doses of fraction F-2 administered [Table 5]. The inability of fraction F-2 to improve the PCV of mice may be due to the presence of saponin in this fraction. Saponin is a phytodetergent known to destroy cell membrane by inducing cholesterol liberation from the cell membrane [31], resulting in erythrocyte hemolysis as manifested in the reduced PCV after administration of fraction F-2.

There was reduced rectal temperature with increasing parasitemia in the infected mice, in all the doses administered. The reduced rectal temperature could be attributed to reduced basal metabolic rate (observed during ongoing infection) or hypothermic effects of parasite multiplication. The highest dose of fraction F-1 at 100 mg/kg b.w, showed the best temperature stabilizing effect with the least reduction in rectal temperature in mice [Table 4]. Fraction F-2 could not stabilize the body temperature with increasing parasitemia as rectal temperature was significantly lower at all the doses of F-2 administered [Table 5].

Weight loss was recorded in all the doses of fractions F-1 and F-2 of A. *nilotica* administered to the mice except at 100 mg/kg b/w of F-2. This could either be due to reduced feed intake which may be attributed to appetite suppressing component of the extract such as saponin and tannins [32].

Our previous study showed that the crude aqueous extract of A. *nilotica* possess antimalarial activity [17]. Although the active antimalarial component of A. *nilotica* is not yet identified, the antimalarial activity of fractions F-1 of A. *nilotica* could be due to high proportion of alkaloid found in this fraction or synergistic effect of the alkaloids with the phenolic component. These phytochemicals have been documented to possess varying degree of antimalarial activity in plant extracts [10,16,32].

CONCLUSION

The results of this study showed that fraction F-1 of A. *nilotica* has significant antimalarial activity. This fraction was also able to improve the PCV of treated mice either by prevention of malaria-induce hemolysis or by enhancing erythropoiesis. However, F-1 could not reverse the reduced body temperature and weight loss associated with rodent malaria. The active antimalarial component of fraction F-1 of A. *nilotica* may be due to the alkaloid alone or combination of the alkaloid and the phenolic acting synergistically. This result also validate the antiplasmodial activity reported on the crude extract and the

use of the A. *nilotica* root extract for treatment of malaria by local communities in Northern Nigeria.

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Short Communication

Antibacterial efficiency of the Sudanese Roselle (*Hibiscus sabdariffa* L.), a famous beverage from Sudanese folk medicine

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ABSTRACT

Background: *Hibiscus sabdariffa* L. is a plant native to tropical Africa and intensively cultivated in Sudan. Its calyces are widely consumed with many uses in Sudanese folk medicine. **Materials and Methods:** The dried calyces of *H. sabdariffa* were subjected to soak in 80% v/v methanol to get the methanolic extract, which was tested against five Gram-negative and three Gram-positive referenced bacterial strains using disc diffusion method. Selected bioactive phytochemical compounds were also investigated using qualitative methods. **Results:** The results of the antibacterial test indicate that the methanol extract of *H. sabdariffa* calyces contained effective antibacterial agent(s), revealed a considerable zone of inhibition against all tested Gram-negative and Gram-positive bacteria, and it was a competitor to gentamicin and greatly higher than penicillin which showed weak or no effect. **Conclusion:** The results of current investigation support the folk medicine application of this plant against different microbial ailments and suggest it as a promising source for new antibacterial agents.

KEY WORDS: Antibacterial, disk diffusion, *Hibiscus sabdariffa*, methanol extract, phytochemical

INTRODUCTION

The battle with diseases started since the advent of man on earth and it will continue in an endless warfare. The discovery of antibiotics in the 1950s has turned the result of this war in favor of man, but few years later microbes returned with mutant strains, resistant to almost all inventive antibiotics. This forced scientists searching for new alternatives to be used against these adaptable microorganisms. Stupendous plants were the main renewable source for medications since times immemorial. Yet, many drugs from the modern medicine are originally derived from ancient herbal medicine. Currently, the dramatic increase in resistance of pathogens to current antibiotics leads to the requisite need for new antimicrobial agents [1,2].

Plants, particularly those prescribed against microbial infections since a long time in traditional and folk medicine from different societies could be promising sources for new antimicrobials [3]. In Sudan, the majority of Sudanese people, like many African countries, are still relying on traditional or folk medicine in treatment of diseases which are an integral part of an informal healthcare system, although this popular folk medicine has roots from Islamic and West African medicine [4]. Roselle (*Hibiscus sabdariffa* L) belongs to Malvaceae family, it is an annual tropical small shrub native to Africa and also distributed in Southeast Asia and Central America [5,6], it is Known locally as Karkadeh. The macerate of the red calyces of this plant [Figure 1] is one of the most famous public Sudanese beverages all over this country and all Sudanese people know and use, which are consumed as hot or cold drinks for the treatment of respiratory tract infections, hypertension, colds, and fever. It is also mixed with other plants to treat malaria [7,8].

Internationally, *H. sabdariffa* is well known, many parts of *Hibiscus sabdariffa* is employed and prescribed in traditional medicine in many countries such as African countries, India, Mexico, Brazil, China, and Iran [9]; leaves which eaten as vegetables are diuretic, antiseptic, digestive, purgative, sedative, demulcent, and astringent [10]; calyces are used for treating of hypertension, liver disorders, diuretic, digestive, and sedative [5,11,12]; seeds are rarely mentioned in traditional medicine compared to the other parts of *Hibiscus*, but seeds are roasted and consumed as food, also used traditionally as debility, diuretic, laxative, and tonic [13]. Although Sudan is the biggest African countries cultivated *H. sabdariffa* but scientific studies on this product is not adequate. The red *Hibiscus* calyces were found to have a pigment called anthocyanins; it is also rich in

Figure 1: The dried calyces of *Hibiscus sabdariffa*

polyphenolic compounds such as flavonoids and phenolic acids such as gallic and protocatechuic acid [14]. The objective of this study was to evaluate the antibacterial potential of the calyces of the Sudanese Roselle (*H. sabdariffa*) which intensively used in Sudanese folk medicine.

MATERIALS AND METHODS

Plant Material

Calyces of *H. sabdariffa* were purchased from local herbal markets in Khartoum, Sudan [Figure 1]; Dried calyces were crushed into fine powders, kept dry in a clean, well-tightened glass container until used.

Preparation of Methanol Extract

A total of 100 g of the dried powder of *H. sabdariffa* calyces was weighted and put in a sterile glass container, 500 ml of 80% methanol (to serve as hydroalcoholic solvent) was added gradually and soaked, and then the container was well tightened and kept in the refrigerator to avoid any microbial contamination or fungal growth. The closed container was subjected to frequent shaking (2-3 times a day) and macerated for up to 3 days. Then, the macerate was filtered using Whatman filter papers No.1; the filtrate was put in the incubator and allowed to evaporate at 45°C for up to 10 days to get a semi-solid extract. For antibacterial testing, the extract was reconstituted in absolute methanol to get a working concentration (500 mg/ml) and kept in a closed dark container until used.

Microorganisms

Eight referenced bacterial strains were used in testing the antibacterial activity of the methanol extract of *H. sabdariffa* calyces. These bacteria are representing five Gram-negatives (*Escherichia coli* ATCC 25922, *Salmonella enteric* ATCC 5174, *Klebsiella pneumonia* ATCC 27736, *Proteus vulgaris* ATCC 49132, and *Pseudomonas aeruginosa* ATCC 27853) and three Gram-positives (*Staphylococcus aureus* ATCC 25923, *Staphylococcus epidermidis* ATCC 49461, and *Bacillus cereus* ATCC 10876). Bacterial strains were purchased from Watin-Biolife, KSA.

Inoculum Preparation

Bacterial strains were cultured following the manufacturer's instructions. Bacterial cultures were identified microscopically and biochemically, then sub-cultured in sterile bottles containing Nutrient Broth (Scharlab, S.L., and Spain) and incubated overnight at 37°C. Prior of the antibacterial experiment, the working bacterial samples were adjusted to 0.5 McFarland to be equivalent to about 1-2 × 10^8 CFU/ml.

Antibacterial Assay

The antibacterial activity of methanol extract of *H. sabdariffa* calyces was evaluated by disc diffusion method as mentioned in Abdallah and Al-Harbi [15]; 100 μl from each working bacterial cultures were mixed with 20 ml warm autoclaved Mueller-Hinton agar (Watin-Biolife, KSA) in glass bottles size 50 ml, tighten and mixed well and then poured directly into 90 mm sterile plastic disposable plates (Jalil Medicals) and left to solidify at room temperature. 6 mm discs were previously prepared using Whatman No.1 filter paper, sterilized and saturated with 300 mg/ml of the methanol extract of *H. sabdariffa* calyces, the pre-experimental test showed that the blank disk (6 mm) could carry 20 μl of the extract at a concentration of 500 mg/ml, which equivalent to 10 mg/disc. Then, the wet, saturated disks were directly loaded on the cultured Mueller-Hinton Agar plate in aseptic conditions. On the same plate, 6 mm antibiotic discs; penicillin G 10 units and gentamicin 10 μg (Oxoid) were also loaded, which served as positive controls. Cultured plates with extract and antibiotic discs were incubated for about 24 h at 37°C. The mean zone of inhibition of two replicated disks on the same plate was taken in millimeter (mm) using a ruler, 6 mm zone diameter considered as no inhibition.

Preliminary Phytochemical Analysis

The phytochemical constituents of the same methanol extract which used in antibacterial testing were evaluated qualitatively as described elsewhere, for alkaloids (Mayer's test) [16], saponins (Foam test), tannins (Ferric chloride test), anthraquinones (Bornträger test) [17], phenolic compounds, and flavonoids were also investigated [18].

Statistical Analysis

All the statistical analyzes were performed with the SPSS 17.0 (SPSS Inc., Chicago, USA) statistical package, variables considered significant at $P < 0.05$.

RESULTS AND DISCUSSION

The current study revealed the considerable antibacterial activity of methanol extract of *H. sabdariffa* calyces against all tested bacteria, particularly the Gram-positives, as shown in Tables 1 and 2, respectively. Disc inhibition zones above 10 mm for the tested crude extract considered a good antibacterial activity (Blank disc diameter 6 mm). Interestingly, the investigated extract was found to inhibit

Table 1: The antibacterial activity of methanol extract of *H. sabdariffa* calyces by disc diffusion method (10 mg/disc) against Gram-positive bacteria

Test	Zone of inhibition (mm)*				
	P. aeruginosa	*E. coli*	*K. pneumonia*	*S. enterica*	*P. vulgaris*
80% v/v methanol extract 10 mg/disc	15.5±0.5	14.5±0.5	17.5±0.5	17.5±1.5	14.5±0.5
Penicillin G 10 units/disc	6.0±0.0	6.0±0.0	7.0±0.0	9.0±0.0	6.5±0.0
Gentamicin 10 μg/disc	20.0±0.0	18.0±0.0	21.0±0.0	12.0±0.0	20.0±0.0

*Inhibition zones are the mean including disc diameter (6 mm)±standard error of means, mm: millimeter, *P. aeruginosa: Pseudomonas aeruginosa* ATCC 27853, *E. coli: Escherichia coli* ATCC 25922, *K. pneumonia: Klebsiella pneumonia* ATCC 27736, *S. enterica: Salmonella enterica* ATCC 5174, *P. vulgaris: Proteus vulgaris* ATCC 49132, *H. sabdariffa: Hibiscus sabdariffa*

Table 2: The antibacterial activity of methanol extract of *H. sabdariffa* calyces by disc diffusion method (10 mg/disc) against Gram-negative bacteria

Test	Mean zone of inhibition (mm)*		
	S. aureus	*S. epidermidis*	*B. cereus*
80% v/v methanol extract 10 mg/disc	18.5±0.5	17.5±1.5	13.5±1.5
Penicillin G 10 units/disc	9.0±0.0	6.0±0.0	7.0±0.0
Gentamicin 10 μg/disc	15.0±0.0	6.5±0.0	10.0±0.0

*Inhibition zones are the mean including disc diameter (6 mm) ±standard error of means, mm: millimeter, *S. aureus: Staphylococcus aureus* ATCC 25923, *S. epidermidis: Staphylococcus epidermidis* ATCC 49461, *B. cereus: Bacillus cereus* ATCC 10876, *H. sabdariffa: Hibiscus sabdariffa*

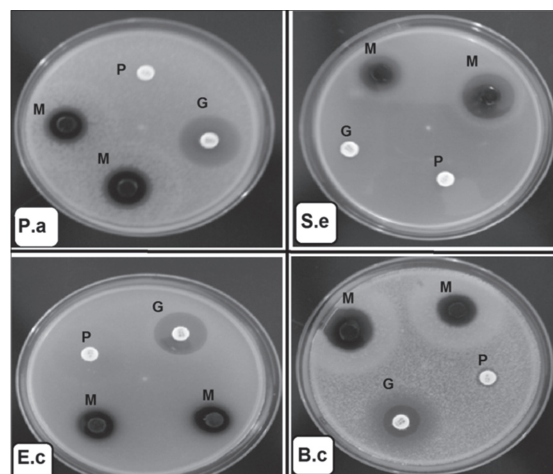

Figure 2: Representative photos showing the antibacterial activity of the methanol extract of *Hibiscus sabdariffa* calyces at concentration 500 mg/ml (10 mg/disc). P: Penicillin G 10 units/disc, G: Gentamicin 10 µg/disc, M: 80% Methanol extract of *Hibiscus sabdariffa* calyces 10 µg/disc, P.a: *Pseudomonas aeruginosa* ATCC 27853, S.e: *Staphylococcus epidermidis* ATCC 49461, E.c: *Escherichia coli* ATCC 25922, B.c: *Bacillus cereus* ATCC 10876

the growth of all tested Gram-positives and Gram-negatives, giving clear, obvious zones by disc diffusion method compared with the antibiotics [Figure 2], being statistically significant ($P < 0.05$). The highest antibacterial activity of *H. sabdariffa* calyces was recorded by *S. aureus* (18.5 ± 0.5 mm), followed by *S. epidermidis* (17.5 ± 1.5 mm), *S. enteric* (17.5 ± 1.5 mm), *K. pneumonia* (17.5 ± 0.5 mm), *P. aeruginosa* (15.5 ± 0.5 mm), *E. coli* (14.5±0.5 mm), *P. vulgaris* (14.5±0.5 mm), and *B. cereus* (13.5 ± 1.5 mm), respectively. Similar several previous studies were reported on the calyces of *H. sabdariffa* using different solvents, concentrations, and bacterial strains (clinical and referenced strains); Borrás-Linares *et al.* [19] published that the ethanol extract from 25 varieties of mexican *H. sabdariffa* calyces was effective against all Gram-negatives (*E. coli* and *S. enteritidis*) and Gram-positives (*S. aureus* and *Micrococcus luteus*), the greater effect was against the Gram-positive bacteria. Its ethanol extract was potent against bacteria isolated from wastewater, particularly *P. aeruginosa* [20]. Aqueous, ethanol, and methanol extracts *H. sabdariffa* calyxes' revealed good antibacterial activity against *Salmonella* cultures [6]. Its 80% methanol extract was also found active against Escherichia coli O157:H7, a major foodborne pathogen [21] and suggested as potential antibacterial in foods [22]. The current investigation and the majority of previous published studies unanimously agreed that *H. sabdariffa* calyces which collected from different localities around the world have effective antibacterial properties; this feature makes it a unique and promising antibacterial plant. It is known that the bioactivity of the extracts from the same plant species may vary according to the plant extraction process which affect greatly by many factors such as type of solvents, method of extraction, temperature (hot or cold liquids), age of the plant and season of harvesting [23], this stability of *H. sabdariffa* calyces as distinguished antibacterial agent is very interesting. Moreover, as represented in Figure 3, the antibacterial activity of the crude methanol extract of *H. sabdariffa* calyces (10 mg/disc) was significantly higher than penicillin G (10 units), and non-significant statistically when compared with the gentamicin (10 μg/disc), meaning that this plant extract is competitor to gentamicin. This is interesting since antibiotics are suffering from deterioration in its effectiveness around the globe [24], *S. aureus* is one of the most prevalent antibiotics resistant pathogens worldwide, particularly the methicillin-resistant *S. aureus* [25]. More antibacterial studies on the effects of the calyces of *H. sabdariffa* on this bacterium in particular are recommended. These considerable antibacterial properties of the methanol extract of *H. sabdariffa* calyces (80% v/v) are attributed to some bioactive phytochemical constituents present in this extract. Table 3 shows that the methanolic extract is rich in phytochemicals such as alkaloids, phenolic compounds, flavonoids, and saponins while tannins and anthraquinones did not detect. This is in agreement with Djeussi *et al.* [26], who found many phytochemicals in the methanol extract of *H. sabdariffa* calyces such as alkaloids, flavonoids, phenols, polyphenols, saponins, triterpenes, and

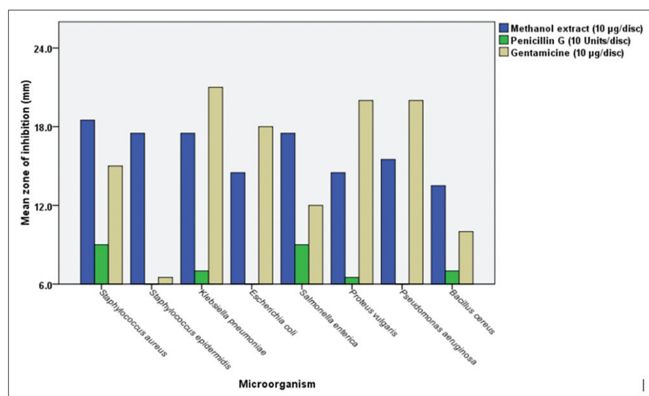

Figure 3: Comparison between the antibacterial effects of methanol extract *Hibiscus sabdariffa* and antibiotics

Table 3: The phytochemical analysis of 80% methanol extract (v/v) of *H. sabdariffa* calyces

Phytochemicals	80% methanol extract (v/v)
Alkaloids	+
Phenolic compounds	+
Flavonoids	+
Tannins	-
Saponins	+
Anthraquinones	-

+: Present, -: Absent, *H. sabdariffa: Hibiscus sabdariffa*

sterols but no anthraquinones, tannins, or anthocyanins. On the other side, Suliman *et al.* [27] figure out many bioactive phytochemicals in the aqueous extract of *H. sabdariffa* calyces such as saponins, phlobatannins, terpenoids, anthraquinones, tannins, steroids, and phenolic compounds. However, the antibacterial agent(s) in the calyces are not determined yet, it was believed that its antibacterial activity could be attributed to anthocyanins [28], but it was found that this antibacterial activity was high with *H. sabdariffa* calyces lacking totally these anthocyanins [6]. Accordingly, more future studies should be conducted to find out and isolate a single compound or group of compounds which could serve as an antibacterial drug. Certainly, additional pharmacological, microbiological, and toxicological studies are recommended.

CONCLUSION

The results of the current study support the widespread use of this popular plant in Sudanese folk medicine, particularly against some illnesses related to microbial infections. The output of this investigation also introduces the calyces of *H. sabdariffa* as one of the most promising sources for new natural and effective antibacterial drugs competitor to antibiotics.

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Review Article

Use and toxicity of complementary and alternative medicines among patients visiting emergency department: Systematic review

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ABSTRACT

Many studies have been conducted in health-care settings with regards to complementary and alternative medicine (CAM) use among patients. However, information regarding CAM use among patients in the emergency department (ED) is scarce. The aim of this article was to conduct a systematic review of published studies with regards to CAM use among the ED patients. A literature search of published studies from inception to September 2015 was conducted using PubMed, Scopus, and manual search of the reference list. 18 studies that met the inclusion criteria were reviewed. The prevalence rate of CAM use among ED patients across the studies ranged of 1.4-68.1%. Herbal therapy was the sub-modality of CAM most commonly used and frequently implicated in CAM-related ED visits. Higher education, age, female gender, religious affiliation, and chronic diseases were the most frequent factors associated with CAM use among the ED patients. Over 80% of the ED physicians did not ask the patients about the CAM therapy. Similarly, 80% of the ED patients were ready to disclose CAM therapy to the ED physician. The prevalence rate of CAM use among patients at ED is high and is growing with the current increasing popularity, and it has been a reason for some of the ED visits. There is a need for the health-care professionals to receive training and always ask patients about CAM therapy to enable them provide appropriate medical care and prevent CAM-related adverse events.

KEY WORDS: Alternative medicine, complementary medicine, emergency department, prevalence, traditional medicine

INTRODUCTION

The World Health Organization has reported the use of complementary and alternative medicine (CAM) therapy for disease prevention and maintenance of well-being to be increasing worldwide in the recent years [1]. The prevalence rate of CAM use among the general population in developed countries such as the US is 42%, Canada 50%, and France 75% [2]. Similarly, in developing countries, CAM utilization is becoming increasingly popular. Up to 90% population of Asian and African countries relied on CAM for primary health purposes [2]. The prevalence rate of CAM utilization among the general population in the developing countries such as Uganda is 60%, India 70%, and Ethiopia 90% [2]. In China, CAM accounts for around 40% of all health care delivered [3]. Furthermore, the global market sales of CAM increased from over US$ 700 million, in 1999, to over US$ 1,000 million, in 2001 [1]. In Australia, CAM use was responsible for an estimated national expenditure of $A2.3 billion in 2000 [4].

The reason for the current increased popularity may be related to the perceived CAM as a holistic approach to life, self-empowerment, "safer" low cost, greater accessibility, variety of practices, and philosophical beliefs [5]. Even though, CAM

use has made significant contributions in the healthcare management [3], the recent growth in the prevalence rate of CAM use among patients have raised concerns about the toxicity and the potential herbal-drug, herbal-disease, and herbal-herbal interactions [6,7]. However, some of the therapeutic potentials of some herbs have been established *in vitro* [8].

Extensive research on CAM utilization has been focused on patients receiving medical care at clinical settings. Clinical settings such as hospitals are the interface between CAM users and conventional therapies. Previous studies of ED patients have identified a higher prevalence rate of CAM usage among ED patients compared to the general population [9-12]. This is because, the ED comprises of many categories of patients with chronic underlying diseases such as diabetes mellitus, cardiovascular system disorder, central nervous system disorder, asthma, and cancer that were associated with utilization of CAM therapy [13,14]. Second, some of the ED visits due to toxicities or adverse events resulting from the use of CAM or non-adherence to conventional medication in preference to CAM therapy [15,16]. The ED physicians, on the other hand, do not always inquire about the use of CAM from patients, leading to wrong diagnosis and consequent deterioration of disease condition [17,18]. In spite of the high prevalence rate of CAM use among ED patients, only a few studies were conducted on CAM use among patients seeking care at the ED [19]. Furthermore, there is a paucity of systematic reviews on the studies on CAM use among ED patients.

The purpose of this systematic review is, therefore, to review published original articles regarding the prevalence of CAM use, CAM-related toxicities, and factors associated with CAM use among patients seeking care at ED.

LITERATURE SEARCH

The literature search and study selection were performed based on PRISMA for systematic review protocol [20]. An online search of literature from inception to September 2015 regarding the prevalence of CAM use among patients at ED was conducted in PubMed and Scopus using Medical Subject Heading (MeSH) and title abstract (tiab) terms. The keywords searched (including all MeSH, headings, subheadings, and tiab terms); "Complementary medicine" OR "alternative medicine" OR "traditional medicine" AND "prevalence" AND "emergency department (ED)." Only original research articles published in the English language were considered. Relevant articles were also identified from the reference list of the studies selected from PubMed and Scopus.

STUDY SELECTION CRITERIA

Studies were selected for inclusion in the current review based on the following criteria: (i) Studies conducted on CAM use among patients seeking medical care at ED. (ii) Studies on the knowledge, attitude, and perception of CAM use among physicians and patients in the ED. The exclusion criteria included (i) studies of CAM use among patients in other study settings (ii) Studies of CAM use among non-hospitalized patients.

OVERVIEW

The online search of literature generated 543 articles. A total of 18 studies that met the inclusion criteria were reviewed. The PRISMA flow in Figure 1 demonstrated the study flow selection. The key findings from these studies are summarized in Table 1.

The publications were based on studies conducted in six countries, with the majority of the studies from the United States of America (U.S) (12/18), Switzerland (2/18), Canada (1/18), New Zealand (1/18) China (1/18), and Australia (1/18). There were no published articles from the developing countries. 14 of the reviewed studies were conducted at tertiary hospitals while the remaining four were performed in secondary and primary health-care hospitals. Hence, the urgent need for more studies in other countries of the world, to have a comprehensive knowledge of CAM use among patients seeking care at ED. Having a wider knowledge of CAM will help conventional healthcare providers at all level of healthcare system to be aware and familiar with different modalities of CAM used as well as the adverse events related to the CAM use presented by the patients at the ED. These will eventually prevent CAM-related hospitalization and complication of an underlying illness.

DEFINITIONS OF CAM USED IN THE STUDIES REVIEWED

Different definitions of CAM have been reported among the studies. The variations may be because CAMs involved wide varieties of practices and beliefs that may be peculiar to a particular population. Moreover, the variation in the definition of CAM may have affected the estimation of the prevalence rates, generalization, and comparison of the findings in the studies reviewed. Therefore, standardization of the definition, terminology, and classification of CAM for comparison and generalization of the findings may not be possible [Table 2].

PREVALENCE RATE OF CAM USE AMONG ED PATIENTS

Due to the different definitions, practice, and prevalence rate of CAM use reported in the studies reviewed, general statement and comparison may not be possible. The prevalence rate of CAM use ranges from a low as 1.4% to as high as 68.1%. The studies reviewed reported different categories of prevalence rate of CAM use among patients at ED: (i) Prevalence rate of current CAM use, (ii) prevalence rate of CAM use in the past week, (iii) prevalence rate of CAM use in the past month, (iv) prevalence rate of CAM use in the past year, and (vi) prevalence rate of CAM use in the lifetime [Table 1]. Moreover, three studies reported a prevalence rate of 17%, 24%, and 25% of current CAM use for the management of the chief presenting complaints at the ED [22,24,27]. With regards to a prevalence rate of CAM use among gender, females were found to have higher proportions of CAM use among the studies reviewed, accounting for up to 68% of CAM users [17,11,13,22,24,28,33].

The previous study on CAM use among patients in other clinical settings have shown that the inclusion of prayer for

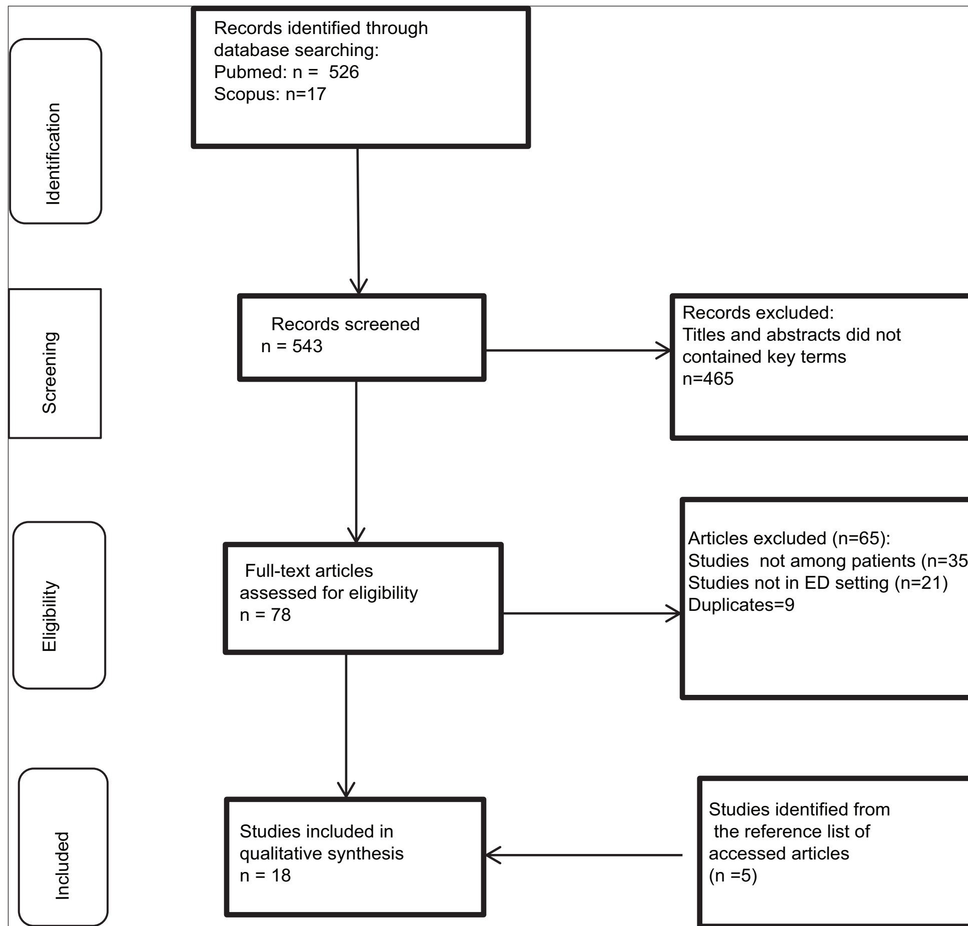

Figure 1: PRISMA flow of the study flow selection

healing (PFH) as a sub-modality of CAM has been attributed with inflation of prevalence rate of CAM use among the patients [36]. In the current review, the lower prevalence rate of CAM use reported by the studies that included PFH indicated that there was no overestimation in the prevalence rate [23,28]. This may be linked to the differences in religious belief of the study population and definitions of CAM adopted by the different studies.

SOURCE OF INFORMATION OF CAM THERAPY

Most of the CAM users learned about its use from family and friends, media, internet, physician, pharmacist, self-medication, and CAM practitioner. People are now more aware of CAM therapy because of the increased widespread information on the internet, media, the advent of online shopping and faster information dissemination through social networking among family and friends.

FREQUENTLY REPORTED CAM THERAPIES

Table 1 demonstrates the frequently used CAM across the studies. Herbal therapy was the frequently used CAM reported among the patients seeking medical care at ED. However, the majority of the herbal therapy reported in the reviewed studies also has the potentials to interact with conventional drugs leading to adverse events (Table 3 demonstrates the possible interactions and toxicities related to herbal medication use reported in the studies reviewed). Similarly, the most commonly used CAMs among children (≤18 years) were herbs, homeopathy, folk remedies, PFH, Ayurveda, traditional Chinese medicine, and anthroposophic medicine. The parents normally utilized the CAM on their children for wellness and management of infection [25,29]. Moreover, some of the CAM-users were using CAMs particularly to manage the symptoms associated with the presenting complaints at the ED, whereas, some were using it for wellness and other health conditions. With regards to the change in the pattern of CAM use among patients from the studies reviewed, there was no change in the pattern of CAM utilization among the CAM users from 1995 to 2010.

FREQUENTLY REPORTED CONDITIONS/DISEASES FOR USING CAM

The frequently reported conditions for which the patients reported using CAM included: Cold and flu, infections, pains, chronic diseases such as arthritis, hypertension, and diabetes, wellness, to aid digestion, sleep, energy, ecstasy, fever, abdominal pain, and respiratory disorder.

Table 1: Summary of the key findings

Author/year of publication	Setting/country	Sample size Duration	Prevalence of CAM use (%)	CAM frequently reported	Risk factors reported
Waterbrook, *et al.*, (2010)	Tertiary Referral Center, USA	75 1 week	54.7[a/d]	Herbal supplement, massage, Chinese medicine/acupuncture and homeopathy	Not reported
Zuzak, *et al.*, (2010)	University Children's Hospital, Switzerland	114 6 months	55[d]	Homeopathy, herbal medicine, anthroposophic medicine, traditional Chinese medicine, and Ayurveda	Not reported
Zuzak, *et al.* (2009)	University Children's Hospital, Switzerland	1143 (≤16 years) 6 months	25[a] 58[d]	Not reported	Caregivers with higher education level, and older age
Sawmi, *et al.*, (2007)	Urban Hospital, USA	602 (<18 years) 8 months	15[d]	Folk/home remedies Herbs, prayer for healing, and massage therapy	Age <5 years, religious affiliation, CAM use by caregiver
Tse and Lau (2007)	United Christian Hospital China	21, 475 31 days	1.4[b]	Chinese herbal medicine	Not reported
Nicholso, *et al.*, (2006)	Tertiary Referral and Trauma Center, New Zealand	1043 (>14 years) 2 months	11.9[d]	Herbal party pills	Age (14-25 years) Friend influence
Kim, *et al.*, (2005)	Two University Affiliated and Two Community Hospitals, USA	539 (>18 years) 1 month	37[c] 57[d]	Ginseng, Gingko, and ST. John's wort	Younger age, higher education level, and chronic pain
Losier, *et al.* (2004)	Tertiary Healthcare, Canada	620 (≤16 years) 8 months	12.8[d]	Homeopathy, spiritual/prayer, massage, play/music therapy, special diets, plant extracts, chiropractic	Higher education, age of the child >1 year
Li, *et al.* (2004)	University Medical Center, USA	356 15 months	55[c]	Chinese herb, herbal tea, Echinacea, ginseng, massage, mega-vitamins, spiritual healing	White Americans, High education, Christian, Buddhists
Taylor, *et al.* (2004)	Tertiary Referral and Trauma Center, Australia	404 12 months	12.4[a] 50.2[b] 68.1[c]	Herbs (Chamomile, green and peppermint teas, Echinacea, ginger, garlic/guarana	Younger age, higher education and Asian
Rolniak, *et al.* (2004)	Missionary Tertiary Hospital, USA	174 (>18 years) 2 months	47.1[d]	Spirituality/prayers, music therapy, meditation	Chronic disease condition, use of OTC pain relievers
Zun, *et al.*, (2002)	Level 1 Healthcare, USA	189 (≥18 years) 33 days	10[a] 43[d]	Massage, home remedies, prayer, chiropractor, and herbal medicines	Gender, type of CAM and means of administration
Rogers, *et al.*, (2001)	Rural Tertiary Care Center, Carolina, USA	944 36 days	14.3[d]	Ginseng, Ginkgo, and Dong quai	Old age
Weiss, *et al.*, (2001)	Tertiary Healthcare, Las Vegas, USA	350 (≥18 years) 6 months	24[a] 43[d]	Ginseng, *Ginkgo biloba*, Eucalyptus and St. John's wort	Not reported
Allen, *et al.*, (2000)	An Urban Hospital, New York, USA	50 (≥18 years) 1 month	28[c]	Prayer, Santeria and herb medicine	Female, longer stay in the US, history of Santeria use and religious practice
Gulla, *et al.*, (2000)	University Hospital, New York, USA	139 patients 7 days	56[d]	Massage, chiropractic, herbs, meditations, acupuncture	Nil
Hung, *et al.*, (1997)	Urban University Hospital, USA	626 (≥18 yeas) 1 month	21.7[e]	Goldenseal tea, garlic, ginger	Female, Asian
Pearl *et al.*, (1995)	Medical center, USA	84 (≥18 years) 12 months	43[b]	Ginseng, lycium, licorice, peony, tang-kuei, acupuncture, massage, external chi-gong	Affiliation with a practitioner of Chinese medicine

[a]On the day of ED visit, [b]in the past week of the ED visit, [c]in the past year, [d]ever used CAM, [e]in the past month, CAM: Complementary and alternative medicine, ED: Emergency department

FACTORS ASSOCIATED WITH CAM USE AMONG ED PATIENTS

Table 1 indicated several factors associated with CAM use among patients at ED. Four of the studies reviewed did not report factors associated with CAM use. Knowing the factors may help the conventional health-care professionals in further assessing the patients with the related factors, to prevent adverse events related to CAM use.

ED VISITS-RELATED TO CAM TOXICITIES

Herbal therapy has been the most frequently implicated CAM in the ED visit-related to toxicities and allergy from CAM use. From the studies reviewed, some of the chief presenting complaints at the ED were adverse effects related to an overdose and allergic reaction to herbal medications. A study conducted in Australia [17] reported an ED visit-related to poisoning with Jimsom weed (*Datura stramonium*) and an unidentified Chinese herbal medicine. The patients presented at the ED with thrombocytopenia and acute anticholinergic symptoms. The second study in New Zealand also reported an ED visit due to toxicities related to "herbal party pill" made from pepper plant (*Piper nigrum*) [34]. The CAM users took the herbal pill for the purpose of getting high, ecstasy, and energy. The patients presented at the ED with extreme alertness, agitation, palpitation, dizziness, and pruritus. In the other study in Hong Kong, China, 7.6% of the CAM users were presented at the ED

with CAM-related toxicities, including dermatitis and systemic allergic reaction from topical and oral use of Chinese herbal medicines, respectively [37].

Some of the herbs reported in the studies reviewed also have the potentials to cause adverse effects and interaction with conventional medicines. Some of the adverse effects, include palpitations, anxiety, tremor, nausea, vomiting, anticholinergic symptoms, and bleeding, were attributed to the use of guarana, ginseng, jimson weed, and gingko [17]. The physicians, nurses, and the pharmacists at the ED should be better informed and be familiar with the modalities of CAMs, to enable them to recognize the sign and symptoms of the CAM-related toxicities, and provide appropriated diagnosis and medical care. Table 3 demonstrates the frequently reported herbs by the ED patients and the related potential interactions/adverse effects.

Table 2: Definition of CAM adopted

Definition CAM	Number of studies	Country of study
"Medical interventions not taught widely at US medical school or generally available at US medical hospitals" Eisenberg, *et al.*, (1993) [21]	6 [12,13,22-25]	US, Canada, Switzerland
"Any group of diverse medical and health care systems, practices, and products that are not presently considered to be part of conventional medicine" National Centre for Complementary and Alternative Medicine, U.S [26]	4 [11,27-29]	US, Switzerland
"Any product including herbal remedies, vitamin, mineral and natural products that may be purchased without a prescription at a health food store, supermarket or from alternative medicine magazines and catalogs for the purpose of self-treatment" Anderson, *et al.*, (2000) [30]	1 [17]	Australia
"Medications that are considered as alternatives to prescription medicines" [31]	1 [31]	US
The remaining studies focused herbal therapy alone and did not report definitions of CAM	6 [18,32-35]	US, New Zealand, and China

CAM: Complementary and alternative medicine

DISCLOSURE OF CAM USE BY ED PATIENTS

9 studies have indicated that up to 70% of the ED patients do not inform their physician that they are using CAM therapies, 80% said they are willing to disclose to their ED physician the CAM they are using. Moreover, 82% claimed that the ED physicians do not ask them about CAM use. This is likely because some of the ED physicians may not have adequate knowledge and training of CAM therapy. The study on knowledge and usage of CAM among ED physicians shows that 46% of the ED physicians learned about CAM therapy from friends or family rather than medical school or other physicians. This finding also further buttresses this point [11]. Another study among pediatricians has indicated that most of the pediatricians did not ask about CAM use in children. This had led to many complications such as herbal-drug interactions [29]. The ED physician and other conventional healthcare providers should actively inquire about the use of CAM therapy in patients, to enable them provide appropriate care and prevent adverse events related to CAM use. Moreover, CAM should be included in the training of all health-care professionals, to have adequate knowledge of CAM therapies and CAM-related toxicities.

Limitations and Future Directions

All the studies reviewed were cross-sectional surveys that are associated with biases such as:

i. A sampling of subjects: Most of the studies employed convenience sampling. Thus, the subjects may not be a true representation of the population
ii. Non-response rates: The non-response rate of the respondents in the studies reviewed was up to 28%, and the majority of the studies did not mention the measures they took in avoiding the non-response bias. This may have affected the estimation of the prevalence rate in the studies

Table 3: Potential adverse effects/interactions associated with the commonly reported herbal medicines

Herbs	Possible herb-drug interaction	Potential toxicity
Ginseng (*Panax* spp.)	Morphine, aspirin, antihypertensives	Diminished analgesia, hypertension, ginseng abuse syndrome [18,35]
Ginkgo (*Ginkgo biloba*)	Aspirin, heparin	Bleeding [35,38]
Kava (*Piper methysticum*)	Risperidone, benzodiazepines	Hallucinations, behavioral changes, sedations, coma [35,39]
Dong quai (*Algelica sinensis*)	Warfarin, NSAIDs	Bleeding [35]
Goldenseal (*Hydrastis Canadensis*)	Cyclosporine, digoxin	Vomiting and diarrhea [33]
Garlic (*Allium sativum*)	Isoniazid, antiretrovirals	Hypotension, rash [33]
Peppermint (*Mentha piperita*)	Cyclosporine, magnesium sulfate, ketoconazole	Heartburn, reflux, water intoxication [33]
Pennyroyal (*Hedeoma pulegioides*)	Leflunomide, teriflunomide, lomitapide	Hepatotoxicity, renal failure, seizure [31]
ST John's wort (*Hypericum perforatum*)	Alprazolam, amitriptyline, digoxin, pentazocine	Rashes, photosensitivity [6]
Echinacea spp.	Caffeine, lovastatin, clozapine	Hepatotoxicity, anaphylaxis [40]
Guarana (*Paullinia cupana*)	Ephedrine, quinine, cimetidine	Palpitation, tachycardia, arrhythmias, high blood pressure [17,38]
Eucalyptus spp.	Glipizide, glyburide, pioglitazone, chlorpropamide, and ketoconazole	Hypoglycemic symptoms, seizure [41]
Chamomile (*Matricaria chamomilla*)	Sedatives	Allergic reactions, drowsiness [33]
Peony spp.	Warfarin, NSAIDs	Bleeding, skin rash [42]

iii. Recall bias: Inaccuracy and inability to recall information by respondents during the data collection using questionnaire, may also affect the accuracy of the findings.

Based on the findings from the articles reviewed, there is a need for: (i) more studies, especially in developing countries, and other healthcare settings such as secondary and primary hospitals, to better understand the CAM use among patients. (ii) Further studies on the knowledge, attitude, and perception of ED physicians to CAM therapy. (iii) Studies solely on CAM-related ED visit and evaluation of CAM use in studies of drug-related ED visits.

The findings of the studies reviewed have implications for ED physicians:

i. ED visit may be related to CAM use as such inquiring the patient about CAM therapy will prevent misdiagnosis and wrong medication at the ED.
ii. Some CAM therapies, herbal medicine, in particular, has the potentials to interact with the conventional treatments leading to adverse events.
iii. Adverse effects of some CAM therapies may mimic the clinical manifestations of many diseases leading to wrong diagnosis and complication of diseases.
iv. Three categories of CAM users may be found at the ED: Patients currently using CAM while on the ED visit (on the day of the visit), those using CAM for the management of the presenting complaints at the ED and those using CAM in the past week, month, or year.

The conventional health care providers should, therefore, be aware and familiar with CAM therapy the patient is using while on the ED visit.

CONCLUSION

There is growing prevalence rate of CAM use among ED patients. Herbal therapy was the sub-modality of CAM most commonly used by ED patients and was responsible for some of the ED visits. Moreover, the majority of the patients were not asked by the ED physicians regarding the CAM use, and most of the ED physicians seem to lack the knowledge of CAM therapy. Therefore, physicians and other health-care professionals at the ED should be better informed about CAM therapies, to improve patient safety.

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Review Article

Amarkand: A comprehensive review on its ethnopharmacology, nutritional aspects, and taxonomy

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ABSTRACT

In India, the term "*Amarkand*" is commonly used for around 30 different plant species belonging to genus *Eulophia* (Orchidaceae). This single local name *Amarkand* to different taxonomical species creates uncertainty about its ethnomedical and nutritional claims. In the present article, we have reviewed available literature regarding ethnopharmacology, phytochemistry, taxonomy, nutritional, and pharmacological studies of different *Amarkand* species. The literature was searched using Google Scholar, PubMed, Scopus, and Web of Science databases. Some textbooks and reference books were also used to collect information about traditional and ethnopharmacological records. *Amarkand* species have been used as a remedy for the treatment of various diseases such as diarrhea, stomach pain, rheumatoid arthritis, cancer, asthma, bronchitis, sexual impotency, tuberculosis, and so on. Nutritionally, *Amarkand* is considered as an excellent food for children and convalescents. Recent studies confirm antioxidant, anti-inflammatory, anti-diarrheal, and so forth activities to *Amarkand* species. These species are reported to possess various phytoconstituents such as flavonoids, terpenoids, and phenanthrene derivatives. The present review will help to understand overall ethnopharmacology, nutritional aspects, and taxonomy of *Amarkand* species.

KEY WORDS: *Amarkand*, ethnobotanical uses, pharmacology, phytochemistry

INTRODUCTION

Traditional medicines with therapeutic utility have been used since antiquity and are still contributing a significant role in the primary health-care system. It is estimated that 70-80% of the world's population relies on traditional herbal medicines for their primary health care [1]. Parallel to traditional medicines, several ethnobotanical medicinal plants have also been validated for their therapeutic efficacy with the help of modern scientific tests. Some of these ethnobotanical plants are receiving merits as both food and medicine [2], and *Amarkand* is one of the best examples of this.

The word *Amarkand* is composed of two different words "*Amar*" means immortal and "*kand*" stands for tubers. The word *Amarkand* is commonly used for 30 closely related plant species from genus *Eulophia* (Orchidaceae) and for one species from the genus *Dioscorea* (*Dioscorea bulbifera*, family: Dioscoreaceae). Since ancient times, *Amarkand* is believed to be an excellent health-promoting agent. Rizhomes/tubers of *Amarkand* are routinely consumed by the tribal parts of India as food as well as a therapeutic entity for better health and longevity [2-4]. In Ayurvedic medicine, *Amarkand* is generally prescribed as expectorant, anabolic, tonic, diuretic, astringent, digestive, and soft purgative [5]. Moreover, the usefulness of these species for the treatment of ear discharge, blood clotting, joint edema, and debility has also been highlighted in some ancient texts [5]. However, this single local name, *Amarkand* to different taxonomical species creates confusion about its ethnomedical and nutritional claims. In the present article, we have reviewed

the available literature regarding ancient therapeutic claims and recent chemical and pharmacological studies about Indian *Eulophia* species so as to link their ethnobotanical applications with recent scientific advances.

DISTRIBUTION

Genus *Eulophia* is highly diverse, occurs in a wide range of habitats, and belongs to family Orchidaceae. This plant produces two shoots, reproductive and vegetative, from their underground tubers. The genus *Eulophia* has a wide distribution and comprises over 230 species, which are widespread from tropical and Southern Africa, Madagascar and from neotropics to throughout tropical and subtropical parts of Asia and Australia. Among these, one species occurs in tropical America. In India, this genus is particularly distributed in tropical Himalaya and Deccan peninsula region. There are almost 723 records under *Eulophia* in International Plant Name Index. However, 500 are synonyms and many of them are ornamental [6]. Web of Science and Scopus showed about 247 and 80 documents, respectively, under the keyword "*Eulophia*" till October 2015.

Around 28 species are recorded from all over India, out of these, 20 species have medicinal importance. The medicinal properties of these species are documented in Table 1 [7-13]. *Eulophia* species are used for several therapeutic purposes in different parts of India [14]. *Amarkand* is the most prevailing name to all *Eulophia* species in India, however, these species are also known by several vernacular names such as *Balakand*, *Manakand*, *Munjatak*, *Amrita* (Sanskrit), *Ambarkand*, *Salam* (Hindi), *Budbar* (Bengali), *Salab* (Gujrati), *Amarkand, and Salibmisri* (Marathi).

MORPHOLOGY

Species under genus *Eulophia* are terrestrial herbs, autotrophic, or rarely heteromycotrophic [Figure 1a]. Perennating organs may be pseudobulbs or tuber like. These pseudobulbs are subterranean or born above ground, corm like, tuberous or rhizomatous, usually with several nodes and slender or thick fibrous roots at the base. *Eulophia* develops a chain of underground tubers [Figure 1b]. Leaves appear at or after anthesis, which are thin but tough, narrow, and grass like or lanceolate and plicate and are one to many, basal and having

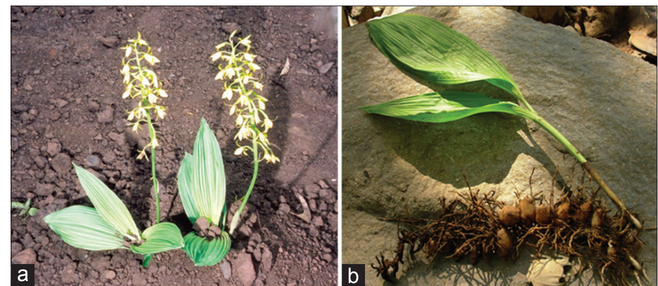

Figure 1: Representative photograph of (a) whole plant of *Eulophia* species and (b) chain of underground tubers of *Eulophia* species

Table 1: Distribution of *Eulophia* species throughout India

Species name	Synonymous	States*
Eulophia andamanensis Rchb. f.	-	Andaman and Nicobar
Eulophia bicallosa (D. Don.) Summerh.	-	Arunachal Pradesh
Eulophia dabia (D. Don.) Hochr.	*Eulophia campestris*	Arunachal Pradesh, Maharashtra
Eulophia densiflora Lindl.	-	Arunachal Pradesh, Assam
Eulophia dufossei Guillaumin.	-	Bihar
Eulophia epidendraea (J. Koenig ex. Retz.) C.E. C. Fisch.	-	Maharashtra
Eulophia explanata Lindl.	-	Gujarat, Goa
Eulophia flava (Lindl.) Hook. f.	-	Arunachal Pradesh, Himachal Pradesh
Eulophia graminae Lindl.	-	Tamil Nadu
Eulophia herbacea Lindl.	-	Maharashtra, Arunachal Pradesh
Eulophia kamarupa S. Chowdh.	-	Assam
Eulophia mackinnonii Duthie.	-	Arunachal Pradesh, Assam
Eulophia mannii (Rchb.f.) Hook. f.	-	Assam, Sikkim
Eulophia obtusa (Lindl.) Hook. f.	-	Uttar Pradesh, Himachal Pradesh
Eulophia ochreata Lindl.	-	Maharashtra
Eulophia pratensis Lindl.	*Eulophia ramentacea*	Maharashtra
Eulophia promensis Lindl.	-	West Bengal
Eulophia spectabilis (Dennst.) Suresh.	*Eulophia nuda*	Uttarakhand, Karnataka, Maharashtra
Eulophia tenella Rchb. f.	-	Arunachal Pradesh
Eulophia zollingeri (Reichb. f.) J.J. Sm.	-	Arunachal Pradesh
Eulophia pulchra (Thouars) Lindl.	-	Tamil Nadu, Kerala
Eulophia bracteosa Lindl.	-	Assam
Eulophia campanulata Duthie.	-	Himachal Pradesh, Uttarakhand
Eulophia campbellii Prain.		Assam, Sikkim
Eulophia emilianae C. J. Saldanha.	-	Karnataka
Eulophia tenella Rchb.f.		Uttarakhand
Eulophia macrobulbon (Par. et. Rchb. f.) hook. f.	-	Uttar Pradesh
Eulophia nicobarica N.P. Balkar and N.G. Nair.	-	Andaman and Nicobar
Eulophia pauciflora Guillaumin.	-	Bihar
Eulophia pulchra (Thouars) Lindl.	-	Tamil Nadu, Kerala

*States mentioned above are illustration purpose only

petiole-like leaf base, sometimes overlapping and forming a pseudostem. Some species lack green leaves and are saprophytic. The inflorescence is erect, lateral, racemose or rarely paniculate, laxly to sub-densely many flowered or occasionally reduced to a solitary flower. *Eulophia* species are mostly identified by their flowers. Two types of flowers occur within *Eulophia*. In the first type, the sepals and petals are similar in size, shape, and color while in the other, sepals are smaller than petals and often recurved. In both types, the lip extends into a spur which can be very diverse in shape [6,15].

ANATOMICAL STUDIES

Infrageneric classification of *Eulophia* R. Br. ex Lindl. was carried out based on methods of classical taxonomy, particularly the examination of generative and vegetative characters, followed by their comparison based on the data obtained from molecular studies and scanning electron microscopy [6]. Study on *Eulophia andamanensis* Rchb. f. found that 0.1% colchicine is effective to induce mutations to increase flower size [16]. *E. graminae* Lindl. was also studied for its unique storage structure of the rhizome, brief juvenile stage, *in vitro* flowering and autogamous mating system, which explains its strong colonization ability [17]. An anatomical study has been carried out on *Eulophia alta* to investigate the pollination biology, breeding system, nectar production, and floral scent composition of the plant. This study clearly showed that flowers of *E. alta* were self-compatible, partially autogamous and effectively pollinated by five bee species. The nectar sugar content was reported to be highest on the third day after flower opening. Floral fragrance analyzes revealed 42 compounds, of which monoterpenes and benzoids are predominant [18]. Studies on asymbiotic and symbiotic seed germination of the same plant revealed that the symbiotic seed culture is a more efficient way of propagation [19]. An optimized method was devised for asymbiotic *in vitro* seed germination, seedling development, and field establishment of *Eulophia nuda* [20], a similar study was carried out with *E. culleni* [21].

MYCORRHIZAL STUDY

Eulophia zollingeri (Rchb.f.) J J Sm. was tested for its mycorrhizal specificity along with mycorrhizal association pattern, which was examined using DNS-based fungal identification. Results revealed that it exclusively associates with the group of fungi belonging to *P. candolleana* group and provide evidence that mycoheterotrophic plants can achieve wide distributions, even though they have high mycorrhizal specificity, if its fungal partner is widely distributed [22]. Yan-Qiu *et al*. [23] identified the presence of endophytic fungi in the fresh roots of *Eulophia flava* Lindl. Total of 52 fungal strains of 17 genera were isolated from fresh root samples of plants growing in sugarcane fields and on the mountain. *Rhizoctonia* and *Fusarium* were the dominant endophytes in these specimens.

TAXONOMICAL AMBIGUITY

Genus *Eulophia* was formally described by John Lindley in 1823. In India, species from this genus were identified in the early 19th century. *Amarkand* is well known to Indians since *Vedic* period, as a medicinal and food species. However, until today, no authentic information is available with respect to the exact species of *Eulophia* which are termed as *Amarkand* in ancient texts. The existing botanical name was assigned to Indian *Eulophia* species in the 19th century, but these species do not match with the description of *Amarkand* in ancient texts. In addition, most of the species of *Eulophia* are morphologically closely similar. Initially, *E. nuda* Lindl. was identified as *Amarkand* [24]. It was later revealed that *Eulophia ochreata* Lindl. possesses higher medicinal and food value than *E. nuda* Lindl. [25,26]. Recently, it has been found that two newly described species of *Eulophia* have much better pharmacological activities than the earlier species. However, until today, no authentic information is available with respect to the exact species of *Eulophia* which could be considered as *Amarkand*.

TRADITIONAL AND ETHNOBOTANICAL USES

In Ayurvedic medicine, *Amarkand* is generally prescribed as expectorant, anabolic, tonic, diuretic, astringent, digestive, and soft purgative, and also recommended for the treatment of ear discharge, blood clotting, joint edema, and debility [5]. In addition, it is also considered as a general tonic to promote strength and alleviates all the three "doshas" [27]. These are also used in stomatitis, purulent cough; and in the heart problems, dyscrasia, and scrofulous diseases of the neck; bronchitis, blood diseases, and as a vermifuge [28].

Different *Eulophia* species have been extensively used in the traditional system of medicines in many countries [29,30]. In India, several ethnopharmacological uses/application have been reported for different species of *Eulophia* in different parts of the country, which are summarized in Table 2.

NUTRITIONAL STUDIES

Some of the *Eulophia* species have been studied for their nutritional properties. *E. campestris* Wall. is available as a *salep* (flour of starch) in Indian markets, as food for children and convalescents [50].

Balance between nutrients and anti-nutrients were studied in *E. ochreata* Lindl. It was found that tubers had low values of all free carbohydrates and had a low content of anti-nutrients such as phytic acid and trypsin inhibitors [51]. The proximate composition and mineral constituents indicated that these tubers are a good source of plant fibers, proteins, and carbohydrates [52].

Proximate analysis and minerals composition studies of tubers are also reported from India. Tubers of this wild edible plant are affluent of all nutrients such as starch, free sugars, oils, proteins, antioxidant phenols, and also a good source of almost all elements. Elemental profile of the same plant was checked using flame photometer and atomic absorption spectroscopy which revealed the presence of microelements such as iron and zinc in considerable amount. Jagtap *et al*. [26] studied the nutritive

Table 2: Ethnobotanical uses of *Eulophia* species

Botanical names	Part utilized	Form of drug	Uses
Eulophia campestris Wall.	Tubers	Fresh Juice	Gastro-intestinal disorders such as diarrhea, dysentery, stomach pain, laxative. Taken as an appetizer [31]
	Rhizome	Not mentioned	As a tonic. Stomach problem, as an aphrodisiac and for cough and cold [11]
	Tubers	Mucilage	Worm infestation and scrofula [12]
Eulophia dabia (D. Don.) Hochr.	Tubers	Not mentioned	Cough and cold [32]
Eulophia epidendrea (JKoen) Schltr.	Tubers	Paste	Applied externally on boils and on breast of feeding mother to control pain due to milk clotting [33]
	Tubers	Not mentioned	To treat tumor and Diarrhea [34]
	Tubers	Not mentioned	As an appetizer, anthelmintic, aphrodisiac, stomachic, alterative, worm infestation. Commonly give it to stimulate appetite and to purify blood in heart troubles [35]
Eulophia graminae Lindl.	Tubers	Extract	To treat ear problems as an ear drop [36]
Eulophia herbacea Lindl.	Tubers	Extract	To reduce liver swelling [37]
	Tubers	Roasted	To increase sperm count [37]
	Tubers	Crushed tubers fried in Mustered oil	Applied externally for rheumatism [38]
	Tubers	Not mentioned	Belly-ach [39]
	Tubers	Paste	To treat pimples [40]
	Seeds	Powder	Weakness (Fatigue) [41]
Eulophia nuda Lindl.	Tubers	Extract	Worm infestation and scrofula [12]
	Tubers	Not mentioned	To treat skin rash, acidity, piles, anorexia, anthrax, and stomach complaints [42]
	Tubers	Raw tubers	Rheumatoid arthritis [43]
	Tubers	Extract	Anticancer, antiasthmatic, and antibronchitis activity [44]
	Whole plant	Paste	Applied externally for boils and abscesses [27]
	Root	Root juice	To treat snakebite [38]
	Tubers	Extract	Anti-inflammatory activity [3]
	Tubers	Whole tuber	Abdominal pain due to non-menstruation, Spermatorrhea, Leukorrhea [45]
Eulophia ochreata Lindl	Tubers	*Salep*	Treatment of sexual impotency and male sterility [27,46]
	Tubers	Paste	Asthma and acute bronchitis [47,48]
	Tubers	Powder	To increase the stamina for physical activities [13]
	Tubers	Tonic	For restoring general health, strength, and vigor [25]
	Tubers	Decoction	Antinode in snakebite and to cure leukemia [43]
Eulophia pratensis Lindl.	Tubers	Paste	Applied externally and given internally to remove scrofulous gland in the neck [27]
Eulophia ramentaceae Lindl. Ex. Wight	Tubers	Not mentioned	Impotency related problems [49]

values of *E. ochreata* Lindl. (A*markand*) tubers with reference to its total protein content, fat content, reducing sugars, total carbohydrates, and Vitamin C, and reported that the tubers contain all nutritional factors in moderate quantity, except the maximum content of lipids (9 mg/g) among all the plants under the study. Authors suggested that these tubers could have potential worth in the diets of poor rural communities of India [53].

PHYTOCHEMISTRY

Medicinal plants produce thousands of patho-physiologically active principles that have been exploited over the years in the treatment of various ailments [54]. The qualitative and quantitative estimation of the phytochemical constituents of the medicinal plant is considered to be an important step in the herbal drug standardization [55]. Progress in phytochemistry has been aided enormously by the development of rapid and accurate methods of screening plants for particular bioactive chemicals.

Methanolic extracts of tubers of *E. epidendrea* (JKoen) Schltr. showed the presence of several classes of phytochemicals such as flavonoids, reducing sugars, cyanogenic glycosides terpenoids, and tannins [56]. Thin-layer chromatography (TLC) studies of the isolated fractions from leaves and tubers indicated the presence of flavonoids, sterols, and terpenoids [Table 3] [57].

Bhandari *et al*. [58] have detected the presence of phenanthrene nudol (2, 7-dihydroxy-3, 4-dimethoxyphenanthrene) in the fresh tubers of *E. nuda* Lindl. In the subsequent study, another six phenanthrene derivatives were also isolated from the same plant tubers [Table 2] [24]. Among these derivatives, the therapeutic potential was largely attributed to 9, 10-dihydro-2, 5-dimethoxyphenanthrene-1, 7-diol. Kshirsagar *et al*. [14] validated the ethnobotanical rejuvenating claim of *E. ochreata* Lindl. by studying its antioxidant activity. Two radical scavenging molecules were isolated from dichloromethane and ethyl acetate extracts of tubers of *E. ochreata* Lindl. [Table 2].

RECENT PHARMACOLOGICAL STUDIES

Pharmacology is the science of drug action on biological systems. Pharmacological characters can provide a better understanding of active principles in plants and their mode of action. Pharmacological trials are needed to investigate the unexploited potential of plants.

Table 3: List of biologically active compounds isolated from *Eulophia* species

Eulophia species	Plant part	Compound present
Eulophia epidendrea (JKoen) Schltr.	Leaves	Apigenin, luteolin, kaempferol, and quercetin
	Tuber fractions	β-sitosterol, β-sitosterolglucoside, β- amyrin and lupeol
Eulophia nuda Lindl.	Fresh tubers	2, 7-dihydroxy-3, 4-dimethoxyphenanathrene (Nudol)
		9,10-dihydro-2,5- dimethoxyphenanthrene-1,7-diol
		9,10-dihydro-4-methoxyphenanthrene-2,7- diol
		1,5-dimethoxyphenanthrene-2,7-diol
		1,5,7-trimethoxyphenanthrene-2, 6-diol
		5,7-dimethoxyphenanthrene-2,6-diol
		4,4′,8,8′-tetramethoxy-1, 1′-biphenanthrene-2,2′7,7′-tetrol
		4-Hydroxybenzaldehyde
		4-hydroxybenzyl alcohol
Eulophia ochreata Lindl.	Fresh tubers	9, 10-Dihydro-2, 5-Dimethoxyphenanthrene-1, 7-diol
		5, 7-Dimethoxyphenanthrene-2, 6-diol

Tubers of *E. campestris* Wall. are well known for its binding properties [23]. Ghule *et al*. [59] pointed out that large quantity of mucilage from tubers of this plant is used as binding agent in tablet formulation. This mucilage produces a sticky film of hydration on the surface of prepared tablets, which ultimately reduces drug release rate. Thick jelly of this mucilage is also reported to be highly nutritious [60]. Glycation inhibitory activity of *salep* (*E. campestris* Wall.) extract was assessed by trichloroacetic acid treatment. In this study, the formation of glycated products/AGEs was decreased at the highest concentration of *salep*, i.e., at 25 mg/ml [10]. Mucilage isolated from tubers of *E. herbacea* Lindl. has a potential as a suspending agent. It has a low rate of sedimentation, high viscosity, weak acidic pH and is easily re-dispersible. Thus, it can also be used as pharmaceutical adjuvant [61].

Methanol extract of tubers of *E. epidendrea* (JKoen) Schltr could significantly inhibit castor oil-induced diarrhea in rats, which was assessed by reduction in the frequency of defecation and the wetness of the fecal droppings compared to untreated control rats. The extract also significantly inhibited intestinal fluid accumulation (enteropooling). In addition, the extract appears to act on all parts of the intestine. Thus, it inhibited the propulsive movement of the intestinal contents in the charcoal meal treated model. These finding suggested that the methanol extract of the tubers of *E. epidendrea* (JKoen) Schltr may have an anti-diarrheal effect. This study validates the use of this plant as a non-specific anti-diarrheal agent in folk medicine [62].

The crude drug in the powder form prepared from tubers of *E. nuda* Lindl. has aphrodisiac potential [63]. Phenanthrene compounds, such as 1-phenanthrenecarboxylic acid, 1, 2, 3, 4, 4a, 9, 10, 10a-octahydro-1, 4a-dimethyl-, methyl ester, were isolated from *Eulophia herbacea* were found to have anticancer potential [64]. Pure compounds such as phenanthrene derivative, 9, 10-dihydro-2, 5-dimethoxyphenanthrene-1, 7-diol isolated from fresh tubers of *E. nuda* Lindl. showed good anti-proliferative activity against human breast cancer cell lines MCF-7 and MDA-MB-231 at concentration of 1000 μg/ml [42]. The same compound was isolated from tubers of *E. ochreata* Lindl. in the pure form and was analyzed for its anti-inflammatory activity using cell line and carrageenan-induced rat paw edema model. The compound inhibited the release of several pro-inflammatory mediators, particularly cytokines and could be a promising anti-inflammatory agent [65].

Similarly, anti-inflammatory and antioxidant activities were attributed to the methanolic extract of tubers of *E. ochreata* Lindl [25]. Moreover, different solvent extracts of this tuber were found to have potent antibacterial activity against *Bacillus subtilis*, *Staphylococcus Aureus*, and *Escherichia coli* [66]. Tubers are also reported to have promising antioxidant, antiglycation, and alpha-amylase inhibitory activity and may have potential in the treatment and management of the Type II diabetes [67]. Recently, we have studied seven *Amarkand* species for its phytochemical profile, polyphenolic content, and free radical scavenging activity and found that *D. bulbifera* and *E. ochreata* had the highest antioxidant potential [66,68]. Similarly, tubers of *E. ochreata* and bulbils of *D. bulbifera* have shown a high anti-fatigue potential among different *Amarkand* species [69]. Among different *Eulophia* species, *E. ochreata* has the highest score for biological activities [34].

PATENT

An Indian patent filed by Upadhyay *et al*. [70] focused on novel derivatives of phenanthrene, Eulophiol from *Eulophia* species, and its potential application in inhibition of immune stimulation involving Toll-like receptor ligands, especially TLR-4.

FUTURE PROSPECTIVES AND CONCLUSIONS

In India, traditional herbal medicines have a long history of practice and still are heavily practiced in rural and tribal populations. Around 2500 plants, out of 18,000 recorded plant species, are in medicinal use in the country [71]. Besides this, tribal communities of India have their own treasure of ethnomedicines based on their ecological and sociocultural background [72]. Most of the ethnomedicines have huge merits as potential medicines and functional foods [73]. However, single local name to different taxonomical species creates confusion about ethnomedical and nutritional efficacies. Therefore, there is need to identify exact and most bioactive ethnobotanical species, as well as validation of their ethnopharmacological and nutritional claims on the modern scientific ground.

Amarkand is a good example of having a strong background of regional ethnopharmacological and food uses. However, these species are not gaining expected regional and global attention due to the lack of scientific records about their biological and pharmacological activities. The ethnobotanical claims from the present review need to be subjected to pharmaco-chemical evaluation, which will help to discover true potential species of *Amarkand*. In addition, validation, standardization, and isolation of active ingredients from different *Amarkand* species are also important for their commercial exploitation.

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Letter to the Editor

Preventive effect of ascorbic acid against biological function of human immunodeficiency virus trans-activator of transcription

To elucidate the inhibition mechanism of human immunodeficiency virus type 1 (HIV-1) replication by ascorbic acid (AA), we have investigated and compared the effect of noncytoxic concentrations of AA on HIV-1 replication. Using trans-activator of transcription (TAT) expressing cells or non-expressing cells transfected HIV-1-long terminal repeat (LTR) chloramphenicol acetyl transferase (CAT) plasmid, we examined the action of AA on TAT dependent transcriptional activation of provirus *HIV-1* gene through enhancer/promoter of HIV-1-LTR. In TAT expressing cells, AA strongly reduced the levels of intracellular CAT activity in a dose-dependent manner. Alternatively in TAT non-expressing cells, CAT activity was reduced somewhat. Using other *in-vivo* and *in-vitro* experiments, AA inhibited the activity of TAT dependent *HIV-1* genome RNA elongation but did not inhibit the activity of basal transcriptional activation of provirus *HIV-1* gene. The intracellular *HIV-1* genome RNA pattern in AA treated cells infected with HIV-1 showed significant differences in the synthesis and the processing of individual viral RNA compared to the patterns of untreated controls. HIV-1 transcription was specifically reduced because in contrast to HIV-1 transcription, transcriptional activities through other viral promoters were not reduced by treatment of AA. Furthermore, the activation of transcription factors was not affected by treatment of AA. These results show that AA specifically inhibits the replication of HIV-1 on down-regulation of TAT dependent *HIV-1* genome RNA elongation.

Previous reports demonstrated the antiviral activity of AA against a broad spectrum of RNA and DNA viruses including polio virus, herpes virus, HIV-1 *in-vivo* and *in-vitro* [1-5]. Already, it has reported that the suppression of virus production and cell fusion in HIV-1 infected $CD4^+$ T-cells grown in the presence of non-toxic concentration of AA. Among the earliest studies on viral replication, it is reported that the growth of HIV-1, after the first replication cycle, was suppressed by the addition of AA, glutathione-SH (GSH), N-Acetyl L-Cysteine (NAC), butylated hydroxyanisole (BHA) or α-tocopherol/vitamin E to human diploid-cell culture [6-11]. There is increasing evidence that reactive oxygen intermediates (ROIs) play an important role in cellular processes such as signal transduction and the controlling gene expression. As actions of GSH and NAC such as thiol-containing antioxidants on the replication of HIV-1 is previously reported, GSH and NAC reduce the target DNA binding activities of nuclear factor κ-B (NF-κB), AP1 (Jun/Fos) or upstream stimulatory factor (USF), by oxidation-reduction (redox) regulation system [6,9,12-17]. It is reported that when these antioxidants such as GSH, NAC, and BHA are added into culture medium, they play such as radical scavenger in the cytsole of cells stimulated by tumor necrosis factor-alpha (TNF-α) or H_2O_2 and then the induction of NF-κB activity by these stimuli is blocked [9]. The suppression of the HIV-1 replication by GSH or NAC is caused by the inactivation of these transcriptional factors by redox system [9,12,14,18]. Furthermore, activation of NF-κB induced by TNF-α is reduced by treatment of vitamin E [9,11]. AA may be considered to play as antioxidant free radical scavenger such like GSH or NAC [19]. Thus, it is possible to regulate DNA binding activity of NF-κB by AA. However, the previous report shows that the life cycle of HIV-1 is suppressed by treatment of 100 μg of AA per ml (0.57 mM) [8], which is more low concentration than NAC as 30 mM (4.9 mg/ml) [18]. Furthermore, it was not established whether AA exerted a virus-specific effect or interacted directly with the activating substances. In several earliest reports, it is demonstrated that the inhibitory effect of AA is not directed inactivation of transcriptional factors such as NF-κB, USF [8,20]. The several research groups already reported that when HIV-1 infected $CD4^+$ T-cells was treated by AA with NAC, the release of HIV-1 particles was reduced about 2nd or 3rd fold than with AA alone or NAC alone [7]. It is speculated from these observations that AA inhibits the replication of HIV-1 by other system except redox system. Furthermore, the virus particle production and the cell fusion are reportedly suppressed in HIV-1 infected $CD4^+$ T-cells grown in the presence of non-toxic concentrations of AA, this report shows that the metabolism in cells is not affected by treatment of these concentrations of AA. AA may specifically block one or several points on the steps of the HIV-1 replication cycle. TAT, REV, VIF, VPR are reported to function as viral regulating proteins which specifically play on HIV-1 replication. In addition, the complex nature of the genome and the action of the two best-characterized viral trans-acting regulatory gene products, TAT and REV, indicate that HIV-1 has an efficient way of regulating its own gene expression during its infection cycle [21-29]. Actions of TAT and REV are so much important for regulation of HIV-1 life cycle [16-23,28,30]. The regulation mechanisms of HIV-1

replication by these viral proteins are not clearly revealed, but if regulation activities of TAT or REV are reduced by AA, this riddle of AA is solved. We have investigated the action of AA on HIV-1 life cycle under the controlled conditions *in-vivo* and *in-vitro*. AA specifically is demonstrated to inhibit TAT dependent *HIV-1* genome RNA elongation system using *in-vitro* and *in-vivo* experiments.

It was reported by several research groups that continuous exposure of HIV-1 infected CD4$^+$ T-cells to non-cytotoxic AA concentrations resulted in significant inhibition of both virus replication in chronically HIV-1 infected cells and multinucleated giant-cell formation in acutely HIV-1 infected CD4+ T-cells [4,7,8,20]. However, the molecular mechanism by which AA suppresses HIV-1 replication was not fully understood yet. There is increasing evidence that ROIs play an important role in cellular processes such as signal transduction and the control of gene expression [13,14]. The suppression of HIV-1 replication is caused by NF-κB, AP1 and USF, which are down-regulated by the redox system of antioxidants such as NAC, GSH, and BHA [7,9,18]. When AA was added together with NAC into the culture medium, extra viral reverse transcriptase (RT) was reduced to 20.0% of the control, compared with values of 30.0% and 50.0% seen, respectively, with AA alone and NAC alone [7]. This result indicates that there are the different target point between AA and NAC. Several data indicate that HIV-1 suppression by AA was not due to secondary effects resulting from inhibition of cellular growth or metabolic activity. In recent report and this study, it is demonstrated that activities of transcription factors are not reduced by AA treatment [20]. The experimental evidence presented in this study has demonstrated that AA could inhibit the HIV-1 replication by blocking the regulation on the step of TAT dependent *HIV-1* genome RNA elongation [Figure 1]. AA dose not inhibit the basal transcriptional activation of HIV-1, however as shown in *in-vitro* and *in-vivo* experiments, the TAT dependent transcriptional activation are strongly reduced by AA treatment [Figure 1]. The several experiments demonstrate that this suppression of *HIV-1* genome RNA expression was not caused by reducing activities of basal transcriptional factors containing RNA polymerase II and transcriptional factors, NF-κB, SP1, USF. Further, the earliest report shows that HIV-LTR-directed β-galactosidase expression in transiently transfected Jurkat cells is not inhibited by AA [20]. The *in-vivo* experimental evidence presented in this study has revealed that the inhibition of HIV-1 replication by treatment with AA is caused by inhibition of TAT dependent RNA elongation, but the basal transcriptional activation through HIV-1-LTR is not affected by treatment with AA. Furthermore, comparison of intracellular *HIV-1* genome RNA patterns in AA treated cells with corresponding patterns of untreated controls showed significant differences in the synthesis of viral genome RNAs. Importantly, the smallest *HIV-1* genome RNAs 2.0 kb were detected in cells treated by AA, TAT protein translated from smallest RNAs possibly was exists in cells, but other length RNAs were not detected by RNA blot. Thus, the results indicated in *in-vitro* experiments show that TAT dependent *HIV-1* genome RNA elongation system was strongly inhibited by AA. It is demonstrated in several reports that TAT could activate transcriptional activation and *HIV-1* genome RNA elongation after forming initiation complex with cellular cofactors [29,31-37]. Furthermore, the known species tropism of TAT protein appears to arise from the fact that not only TAT but also the cellular cofactor can markedly influence the RNA sequence specificity of the resultant protein complex [29,31]. In earlier studies, P-TEFb expressing in CD4$^+$ T-cells recognizes the loop structure in HIV-1 trans-activation response (TAR) and forms proteins/TAR complex, negative elongation factor, which directly interacts TAT protein [29,31,33,38], then the activation of transcription and *HIV-1* genome RNA elongation is activated by these proteins/TAT/TAR complexes [2,30]. In other result, Mammalian Suppresser of Sgv1 (MSS1), which strongly expresses in CD4$^+$ T-cells, activates with TAT the transcription through the promoter/enhancer of HIV-1-LTR, but activation mechanism by MSS1 is unclearly revealed [34,39]. It was demonstrated by RT-PCR with specific *MSS1* gene primer sets that expression of *MSS1* mRNA gene was not suppressed in cells treated by AA, expression of other cellular transcriptional cofactors have not been examined yet. TAT is demonstrated to recognize directory RNA polymerase II holoenzyme, which constructs with transcriptinal factor IID, transcriptinal factor IIB, then activates the transcription of *HIV-1* genome RNA as a mediator between TAR and basal transcriptional factors [40-44] [Figure 1]. There are possible two reasons why *HIV-1* genome RNA expression is downregulated by AA treatment. First, the expression of these cellular cofactors may be downregulated in cells treated by AA [Figure 1]. The second, the stereomatic conformation of TAT protein may be changed by the treatment of AA and be not able to play as the trans-activating mediator [Figure 1]. The previous report demonstrates that HIV-infected individuals have low levels of AA; however, this deficiency is not related to eating habits, since the intake of this nutrient was higher in this group than in the control group. HIV-infected individuals have specific characteristics that increase their oxidative stress, which is evidenced by increased C-reactive protein [45]. It is necessary to examine whether the TAT activity is downregulated in the cells treated by AA or not. Already, AA is used for the treatment of AIDS and AA at 90 μg/ml was attained in plasma in patients consuming oral AA to achieve urinary levels about 1 mg/ml [46]. Clinical facilities examined how AA interacts with antiretroviral therapy in individuals with HIV-1. AA usage appears to be associated with improved highly active antiretroviral therapy (HAART) adherence and HAART effectiveness as adjudicated by HIV viral loads and CD4$^+$ T-cell counts [2]. These findings are consistent with a high bowel tolerance reported for AIDS patients. Future AA studies should target specific HAART drugs and prospective clinical outcomes.

CONCLUSION

We conducted an analysis of HIV-1 life cycle examining the impact of AA usage. Significantly increased HAART adherence is demonstrated during periods of AA usage compared to when the patients were not consuming AA. Due to the potential impact this simple, inexpensive intervention may have on

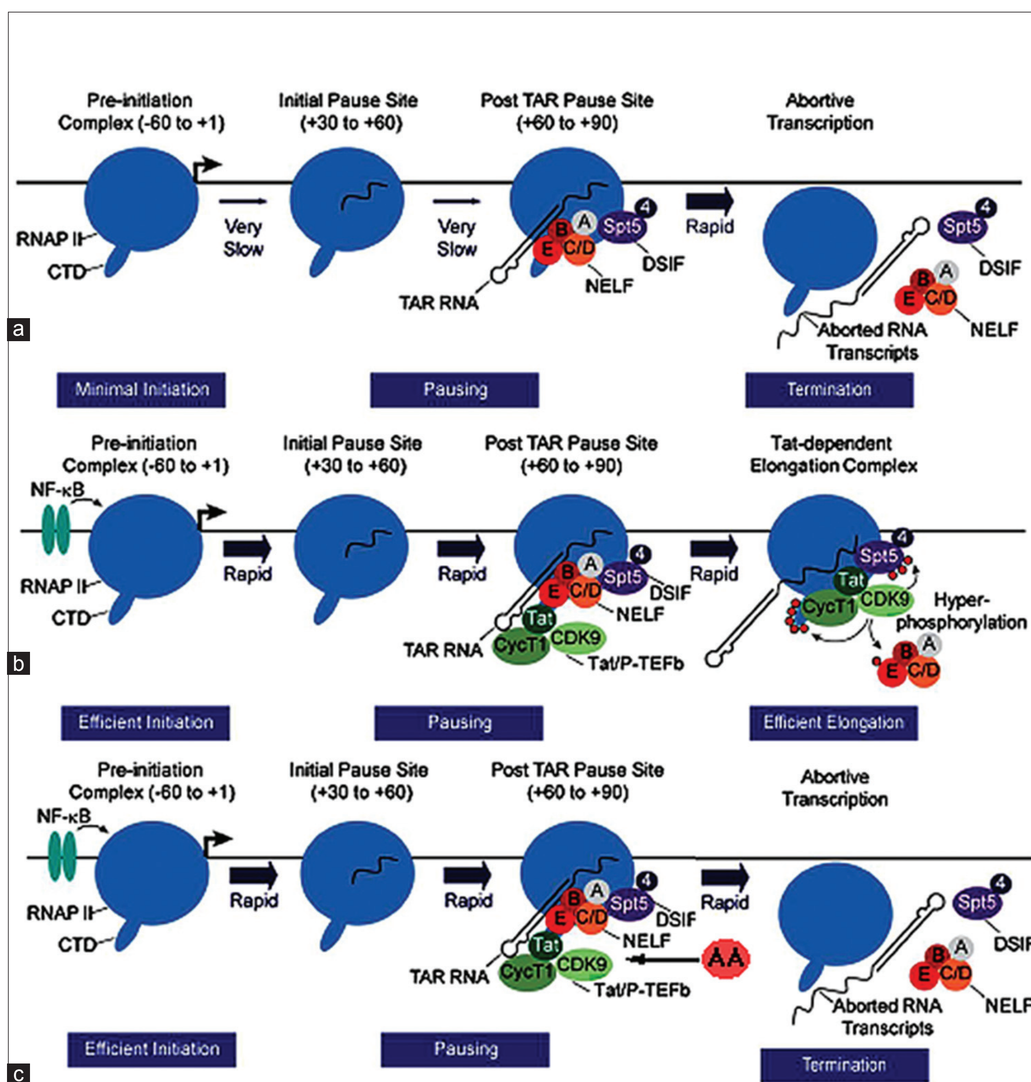

Figure 1: Preventive effect of ascorbic acid (AA) against trans-activator of transcription (TAT)-dependent *HIV-1* gene expression. (a) Latent human immunodeficiency virus type (HIV) provirus. In latent proviruses transcription elongation is very inefficient due to absence of the transcription elongation factor nuclear factor κ-B (NF-κB) as well as chromatin restrictions (not shown for simplicity). However, a significant number of proviruses carry RNAP II paused in the promoter-proximal region. The small number of transcription complexes that are able initiate and elongate through trans-activation response (TAR) are subject to additional elongation restrictions by negative elongation factor (NELF) which forces premature termination. (b) NF-κB and TAT-activated transcription. Initiation is strongly induced by NF-κB, which removes chromatin restrictions near the promoter through recruitment of histone acetyltransferases. Under these circumstances promoter clearance is also much more efficient, and there is an enhanced accumulation of elongation complexes in the promoter-proximal region. After the transcription through the TAR element, both NELF and the TAT/P-TEFb complex (the super elongation complex factors are not shown for simplicity) are recruited to the elongation complex via binding interactions with TAR RNA. This activates the CDK9 kinase and leads to hyperphosphorylation of the C-terminal domain of RNA polymerase II, Spt5, and NELF-E. The phosphorylation of NELF-E leads to its release. Although the promoter is transcribing more rapidly than in the latent condition, there is relatively little change in the amount of RNAP II that accumulates in the promoter-proximal region due to its rapid replacement by newly initiated transcription complexes. (c) There are possible two reasons why *HIV*-1 genome RNA elongation is downregulated by treatment with AA. First, the expression of these cellular cofactors may be downregulated in cells treated by AA. The second, the stereomatic conformation of TAT/cellular cofactors complex may be changed by the treatment of AA and be not able to play as the trans-activating mediator

HIV-positive patients, we believe a large community-based clinical trial is indicated.

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Letter to the Editor

Caffeic acid phenetyl ester and its ability to prevent viral infection - Some comments

A recent paper by Erdemli *et al*. on this journal reviewed the antiviral properties of caffeic acid phenetyl ester (CAPE) [1]. Recent literature showed that CAPE has also anti-inflammatory and immune-modulatory effects, as like as many other plant-derived phenolics [2] but its ability to exert an anti-inflammatory role has never been assessed in clinical trials, therefore, its activity on humans appears only presumptive. Yet, biomedical literature about CAPE effect on chronic inflammation and cancer appears quite promising and should encourage randomized controlled trials in patients. Its ability to affect *in-vitro* tumors is not so far than many other plant-derived phenolics, considered as anti-NF-κB agents [3], yet its antiviral potential might be attributed to independent pathways from interferon (IFN) induced by virus entry [4]. If CAPE is described only as an inhibitor of NF-κB activation, then some controversial issues would be raised. It is well-known that cells activate host-pathogen interactions, through the recognition of pathogen-associated molecular patterns by host sensors, which were defined as pattern recognition receptors. They include toll-like receptors (TLRs), RIG-I-like receptors (RLRs), NOD-like receptors (NLRs), and DNA receptors. Then, virus may trigger these innate immune receptors. In the cell response, the activation of the nuclear factor-κB (NF-κB) transcription pathway is crucial for the immediate early step of immune activation. However, there are a variety of viral effectors that have been shown to prevent NF-κB signalling, namely, they should act as polyphenols at least three different levels: (a) TLRs, IκB kinase complexes and at the transcriptional level [5]. The antiviral activity of CAPE might be more complex than expected, therefore, as it might interfere with innate immunity as like as viruses, if it should act only on NF-κB, even by preventing its activation [5]. Which are the major targets of CAPE within an infected or inflamed cell? The paper by Erdemli *et al*. report some interesting suggestions [1]. Further consideration should be made.

As many other phenol-bearing molecules from plant biochemistry, CAPE should exert a protective role for plants and a proto-toxic activity in animals. CAPE has been described in the past to possess a prooxidant activity [6], particularly in the range 1.0-0.5 μM, interestingly the same range reported for its antiviral potential [4]. This apparently contradictory evidence, where CAPE is both a pro-oxidant and an antiviral molecule, deserves further considerastion about the many targets of CAPE activity and its pleiotropism within a defined cell. The paper by Erdemli *et al*. did not fully address this concern but expanding the debate may be of major interest to shed a light on the role of CAPE in propolis. The relationship with the redox machinery should involve also mitochondria, which activate mitochondria antiviral signaling (MAVS) regulome during their mitochondrial dynamics [7]. Mild induction of oxidative redox species, triggered by xenobiotics such as plant phenolics, may impair MAVS regulome and then activate a stress response from infected cells: This speculative hypothesis would suggest that CAPE may act, even indirectly, on signaling molecules upstream of IFN regulatory factor 3 (IRF-3), which are modulated by redox-dependent processes, and include MAVS and the tumor necrosis factor receptor-associated factors adaptors, all of which are sensitive to redox regulation [8]. CAPE is yet able to suppress IRF-3 activation, following inflammation or innate immune response [9] but this activity may follow cell regulation of virus infection, which allows that the E3 ubiquitin ligase RBCC protein interacting with PKC1 (RBCK1) binds to IRF-3 and targets it for ubiquitination and subsequent degradation through a proteasome-dependent pathway, to dampen the overexpression of RBCK1 by virus and reduce side effects as autoimmune disorders due to the excessive activity of IFNs [10]. It is presumable that a fine regulation of ROS-response at the mitochondrial turnover and MAVS regulome, induced by sub-micromolar doses of CAPE, may induce initially an IRF-3 mediated signaling by a mild-oxidative stress, inducing intracellular IFN, which then rapidly disappears while CAPE still acting on the redox system to activate antioxidant scavenging systems. Incubation times settled in *in-vitro* experimental research may hamper the ability to gain insights about the first minutes of activity of the natural compound, of which we can retrieve evidence mainly regarding its anti-oxidant potential and its anti-NF-κB action, while it is possible that the early action exerted by these phenolics targets fine equilibria involving mitochondria dynamics and their relationship with oxidative stress, even stimulating early IFN action.

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