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Original Research

Nephroprotective, diuretic and antioxidant effects of some medicinal herbs in gentamicin-nephrotoxic rats

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Abstract

Aim: To investigate the nephroprotective, diuretic and antioxidant effects of extracts of Petroselinum sativum, Eruca sativa and Curcuma longa herbs in gentamicin (GM)-nephrotoxic rats.

Material and Methods: Forty two adult male Sprague Dawley rats were randomly distributed into six equal groups. Group 1 was given sterile saline solution by intraperitoneal (i.p.) injection (negative control). Group 2 (nephrotoxic) was injected with GM (80 mg/kg, i.p.) for 8 days during the last week of the experiment. Groups 3, 4, 5 and 6 were orally pretreated with herbs extracts, alone and in combination, for 6 weeks along with GM during the last week. Blood and urine samples were collected for biochemical analyses. Kidney specimens were taken for estimating oxidant/antioxidant parameters and for histopathology.

Results: GM induced nephrotoxicity characterized by biochemical and histopathological alterations, increased lipid peroxidation and reduced activity of antioxidant enzymes in kidney tissues. Aqueous extracts of Petroselinum sativum, Eruca sativa and Curcuma longa herbs caused nephroprotective effect as it decreased in the elevated serum urea, creatinine and alkaline phosphatase (ALP) activity and normalized serum levels of Na+ and K+ electrolytes in GM-intoxicated rats. These extracts also increased the urine volume and urinary excretion of Na+ and K+, ameliorated renal tubular necrosis and increased activities of renal antioxidant enzymes in GM-intoxicated rats.

Conclusion: Aqueous extracts of Petroselinum sativum, Eruca sativa and Curcuma longa produce nephroprotective, diuretic and antioxidant effects in GM - nephrotoxic rats. These herbs may be beneficial for patients who suffer from kidney diseases and those on GM therapy.

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INTRODUCTION

Nephrotoxicity induced by several synthetic drugs represents a serious problem for many populations in the world. Gentamicin (GM) is one of aminoglycoside antibiotics commonly used for the treatment of Gram negative bacterial infection in man. It is an effective drug against resistant bacterial strains to other antibiotics, but its nephrotoxic side effect has limited its therapeutic use [1]. Nowadays, the incidence of aminoglycosides-induced nephrotoxicity had increased and about 30% of patients treated with GM for more than 7 days showed signs of nephrotoxicity and neurotoxicity [2, 3]. Nephrotoxicity caused by GM seemed to be attributed to the oxidative stress caused by generation of reactive oxygen species [4, 5]. Aminoglycoside antibiotics were suggested to stimulate formation of reactive oxygen species (ROS) and cause renal oxidative stress [6]. On the other side, ROS scavengers and natural antioxidants can be used to alleviate nephrotoxicity induced by GM [7, 8]. Gentamicin was suggested to induce nephrotoxicity by inhibiting protein synthesis in renal cells. This mechanism specifically induced necrosis of cells in the proximal tubule, resulting in acute tubular necrosis which can lead to acute renal failure [9].

Medicinal plants and herbs have played an important

role in the prevention and treatment of kidney diseases. In this concern, *Petroselinum sativum* (parsley) aqueous seed extract was reported to produce a diuretic effect in rats. The mechanism of action of parsley seems to be mediated through an inhibition of the Na+/K+ pump that would lead to a reduction in Na+ and K+ reabsorption leading thus to an osmotic water flow into the lumen, and diuresis [10]. Moreover, *Petroselinum sativum* prevented formation of calcium oxalate stones in rats with nephrolithiasis and reduced the number of calcium oxalate deposits [11]. *Eruca sativa* is widely used in folklore medicine as a remedy of renal ailments. *Eruca sativa* produced potent antioxidant and renal protective activities and also precluded oxidative damage inflicted to the kidney by mercuric chloride in rats [12]. Curcumin, the active principle of turmeric (*Curcuma longa*) ameliorated diabetic nephropathy in rats [13]. The antioxidative activity was found to be responsible for the nephroprotective action of curcumin. The ethanol extract of *Curcuma comosa* exhibited an effective protection against cisplatin-induced nephrotoxicity in mice that mediated through its antioxidant activity [14]. *Curcuma longa* (turmeric) extract was found to possess multiple therapeutic activities that block the cardiac, hepatic, and renal toxicities induced by doxorubicin and had antioxidant activity [15]. It was concluded that curcumin might be potentially useful in some kidney diseases by preventing renal inflammation [16].

The present study was carried out to investigate the nephroprotective, diuretic and antioxidant effects of *Petroselinum sativum*, *Eruca sativa* and *Curcuma longa* medicinal herbs, alone and in combination, in gentamicin- nephrotoxic rats.

MATERIALS AND METHODS

Herbs

Petroselinum sativum (Parsley, Family *Apiaceae*) seeds; *Eruca sativa* (Rocket, Family *Brassicaceae*) seeds and *Curcuma longa* (Turmeric, Family *Zingiberacae*) rhizomes were purchased from the Agricultural Seeds, Herbs and Medicinal Plants Company, Cairo, Egypt. The dry seeds and rhizomes of the herbs were finely grinded into fine powders and used for the preparation of aqueous extracts.

Gentamicin

Gentamicin (Garamycin® injection), an aminoglycoside antibiotic, was obtained from Memphis Company for Pharmaceutical and Chemical Industries, Cairo, Egypt. It is dispensed in the form of ampoules, each containing 40 mg/ml of gentamicin sulphate. The injected dose of gentamicin 80 mg/kg b.wt.to rats was selected to induce acute nephrotoxicity [17].

Rats and feeding

Forty two adult male rats of Sprague Dawley strain weighing 150-155 g body weight and 8-10 weeks old were used in this study. The rats were purchased from the Laboratory Animal Colony, Helwan, Egypt. The animals were housed under hygienic conditions at a room temperature of 25 ± 2 °C with relative humidity of 50–55% and on 12 hr light/12 hr dark cycles. Rats were fed on commercial rat pellets which composed of 10% wheat bran, 44% soy bean powder, 20 % net protein, 5 % fats, 3.3%, fibers and fish meal, molasses, salts (sodium chloride, calcium carbonate, calcium phosphate) and methionine. These pellets are manufactured by Cairo Agriculture Development Company, Giza, Egypt.

Preparation of aqueous extracts of herbs

One hundred and fifty grams of fine powder of each herb were soaked in one liter of hot water (to obtain 15 % concentration) at 50°C for 2 hours and thereafter kept in a refrigerator with daily shaking for 5 days. The aqueous extract was obtained in by filtration with double layers of gauze to get rid of herb debris. For preparing the herb mixture, fifty grams of each herb powder were thoroughly mixed together, soaked in one liter of hot water at 50°C for 2 hours and processed as previously mentioned. The prepared aqueous extracts were kept in a refrigerator pending for further use.

Design of experiment

Forty two adult male Sprague Dawley rats were randomly divided into six equal groups, each of 7 animals. Group 1 was injected intraperitoneally (i.p.) with sterile saline (0.2ml/rat) and kept as normal control. Group 2 was injected i.p. with gentamicin in a dose 80 mg/kg for 8 consecutive days during the last week of the experiment to induce acute nephrotoxicity [17] and kept as nephrotoxic control. Groups 3, 4, 5 and 6 were pretreated with one of the aqueous extracts of the three herbs each at 5% and their mixture at 15% concentration (1ml/rat), respectively, along with gentamicin during the last week. Twenty four hours after the last administration, animals were placed in separate metabolic cages for 24 hr and total urinary volume was measured. A drop of concentrated hydrochloric acid was added to urine before being stored at 4°C. Urine samples were analyzed for sodium and potassium levels. Blood samples were collected and used for serum separation. Serum samples were used for estimation of blood urea, uric acid, creatinine and alkaline phosphates as well as serum sodium and potassium levels. Kidney tissue specimens were collected from right kidneys and stored at -18°C for estimation of oxidant/antioxidant status. The left kidney specimens were preserved in 10% neutral

formalin for histopathology.

Serum and urine analyses

Concentrations of blood urea nitrogen [18]; uric acid [19] and creatinine [20] were estimated using specific diagnostic kits (Sigma Aldrich, St. Louis, USA). The activity of serum alkaline phosphates (ALP) enzyme was estimated [21] using standard reagent kits (Sigma Aldrich, St. Louis, USA). Serum and urine levels of sodium and potassium electrolytes were determined using flame photometer (Model FP 20 seas, Seag Radim Company, Italy) with specific diagnostic kit (BioMérieux, France) as described by Ali [22].

Preparation of kidney homogenate

One gram of the right kidney tissue was collected, washed in ice-cooled 0.9% NaCl and homogenized in ice-cooled 1.15% potassium chloride solution and 50 mMol potassium phosphate buffer solution (pH 7.4) to yield 10% homogenate (W/V). Homogenization was performed using ultrasonic homogenizer. The homogenate was then centrifuged at 4000 rpm for 5 minutes at 4°C. The supernatant was collected and kept for further use.

Assessment of oxidant / antioxidant activity

Reduced glutathione (GSH) content of kidney tissue was determined using chemical method [23]. The method is based on the reduction of 5, 5´dithiobis (2-nitrobenzoic acid) with glutathione producing a yellow compound. The reduced chromogen was directly proportional to GSH concentration and its absorbance was measured at wave length 412 nm.

Determination of lipid peroxidation (LPX)

LPX in renal tissue was measured according to Ohkawa *et al.* [24]. The technique is based on the reaction of thiobarbituric acid with lipid peroxides malondialdehyde (MDA) in acidic medium at 95°C for 45 minutes to form thiobarbituric acid reactive substance (TBARS). The resulting pink color was extracted with n-butanol and its absorbance was determined spectrophotometrically at wave length 530nm.

Determination of superoxide dismutase (SOD)

The renal SOD activity was measured according to Nishikimi *et al.* [25]. This assay relies on the ability of SOD enzyme to inhibit the phenazine methosulphate-mediated reduction of nitroblue tetrazolium dye.

Determination of glutathione peroxidase (GPx)

Renal GPx activity was measured by the method of Paglia and Valentine [26]. This assay is an indirect measurement of the activity of GPx. The oxidized glutathione (GSSG), produced upon reduction of organic peroxide by GPx, was recycled to its reduced state by the enzyme glutathione reductase (GHR). The reaction was initiated by the addition of hydrogen peroxide, and the oxidation of NADPH to NADP+ is accompanied by a decrease in the absorbance at wave length 340 nm.

Determination of catalase (CAT)

Renal CAT activity was measured in tissue homogenate according to Aebi [27]. The assay is based on that catalase reacts with a known quantity of hydrogen peroxide. This reaction is stopped after exactly one minute with catalase inhibitor. In the presence of peroxidase, the remaining hydrogen peroxide reacts with 3, 5-Dichloro-2-hydroxybenzenesulfonic acid and 4-aminophenazone to form a chromophore with a colour intensity inversely proportional to the amount of catalase.

Histological procedure

Kidney specimens were taken and fixed in 10 % neutral formalin solution. The fixed specimens were trimmed, dehydrated in ascending grades of alcohol, cleared in xylene. They were embedded in paraffin boxes, sectioned at 4-6 microns thickness, stained with Hematoxylen and Eosin (H&E) and examined microscopically according to Carleton [28].

Statistical analysis

Data were expressed as mean ± standard error (SE). Differences between control and treated groups were tested for significance using one-way analysis of variance (ANOVA) followed by Duncan's multiple range test [29]. Statistical analyses were performed using the SPSS (Statistical Package for the Social Sciences), software program.

RESULTS

Intraperitoneal injection of gentamicin (GM) in a dose of 80 mg/kg for 8 consecutive days to rats caused nephrotoxicity manifested by significant ($P < 0.05$) increases in serum levels of blood urea nitrogen, creatinine and activity of alkaline phosphatase (ALP) enzyme when compared with healthy control rats. Oral administration of aqueous extracts of *Petroselinum sativum*, *Eruca sativa* and *Curcuma longa* herbs, alone and in combination, along with GM induced significant ($P < 0.05$) decreases in the elevated levels of blood urea nitrogen, creatinine and activity of ALP when compared with GM-intoxicated rats as recoded in Table (1). Non significant changes were reported in serum levels of uric acid between the different experimental groups (healthy control, GM-intoxicated and herb-treated).

Table 1. Effect of aqueous extracts of *Petroselinum sativum* (PS), *Eruca sativa* (ES) and *Curcuma longa* (CL) herbs on serum urea and creatinine and alkaline phosphatase enzyme (ALP) in gentamicin-nephrotoxic rats.

Parameters Groups	Urea (mg/dL)	Creatinine (mg/dL)	ALP (U/L)
Group1 Healthy control	31.95±2.44^{d}	0.58±0.02^{d}	58.4±4.52^{d}
Group 2 Nephrotoxic control	70.46±3.54^{a}	0.97±0.04^{a}	77.6±4.21^{a}
Group 3 PS 5 %	39.35±2.10^{b}	0.68±0.02^{b}	65.4±5.32^{b}
Group 4 ES 5 %	40.42±2.24^{b}	0.65±0.01^{b}	64.8±4.22^{b}
Group 5 CL 5 %	38.63±3.30^{b}	0.67±0.03^{b}	63.5±5.03^{b}
Group 6 Mixture 15 %	36.46±3.70^{c}	0.56±0.04^{c}	62.3±3.44^{c}

Means ± SE with different superscripts in the same column are significant at $P < 0.05$
using one way ANOVA test.
n=7 rats.

Daily intraperitoneal injection of GM to rats for 8 days caused significant decreases in serum levels of sodium (Na^+) and potassium (K^+) electrolytes when compared with the healthy control group. Oral administration of aqueous extracts of *Petroselinum sativum*, *Eruca sativa* and *Curcuma longa* herbs and their mixture concomitantly with GM normalized the decreased levels of Na^+ and K^+ electrolytes in the serum when compared with GM-intoxicated rats as illustrated in Fig. (1).

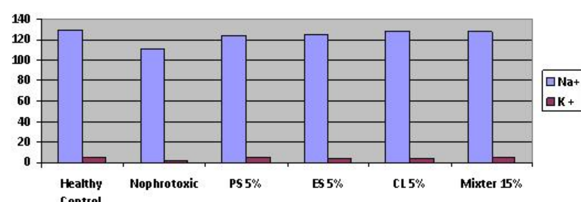

Fig.1. Effect aqueous extracts of Petroselinum sotiyum, (PS), Eruca sativa (ES) and Crcuma longa (CL) herbs on serum sodium Na+ and potassium levels in gentamicin-nephrotoxic rats.

Intraperitoneal injection of GM to rats for 8 days caused significant ($P < 0.05$) decreases in urine volume and concentrations of urinary Na^+ and K^+ electrolytes as compared to the healthy control group. Oral administration of aqueous extracts of *Petroselinum sativum*, *Eruca sativa*, *Curcuma longa* herbs and their mixture along with GM significantly ($P < 0.05$) increased urine volume and urinary levels of Na^+ and K^+ electrolytes as compared to GM-intoxicated rats as recorded in Table (2).

Rats injected daily with GM for 8 consecutive days had a significant ($P < 0.05$) decrease in the content of reduced glutathione (GSH) and an increase in the level of lipid peroxidation product, malondialdehyde (MDA), in kidney tissues when compared with the healthy control group. Oral administration of aqueous extracts of *Petroselinum sativum*, *Eruca sativa* and *Curcuma longa* herbs and their mixture concomitantly with GM caused a significant ($P < 0.05$) increase in GSH and a decrease in MDA contents in renal tissue when compared with GM-intoxicated rats as shown in Fig. (2).

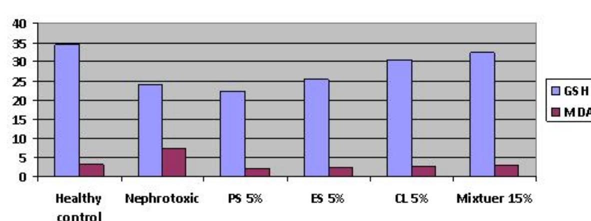

Fig.2. Effect aqueous extracts of Petroselinum sotiyum, (PS), Eruca sativa (ES) and Crcuma longa (CL) herbs on kidney levels of reduced glutathione (GSH and malondialdehyde (MDA) in gentamicin-nephrotoxic rats

Intraperitoneal injection of GM to rats for 8 consecutive days induced significant ($P < 0.05$) decreases in the activity of renal superoxide dismutase (SOD), glutathione peroxidase (GPx) and catalase (CAT) antioxidant enzymes when compared with the healthy control group. Oral administration of aqueous extracts of *Petroselinum sativum*, *Eruca sativa* and *Curcuma longa* herbs and their mixture concomitantly with GM significantly ($P < 0.05$) increased the activity of SOD, GPx and CAT enzymes when compared with GM-intoxicated rats (Table 3).

Table 2. Effect of aqueous extracts of Petroselinum sativum (PS), Eruca sativa (ES) and Curcuma longa (CL) herbs on urine volume and urinary sodium (Na+) and potassium (K+) levels in gentamicin-nephrotoxic rats.

Parameters Groups	Urine volume (ml)	Na^+ (mEq/L)	K^+ (mEq/L)
Group 1 Healthy control	3.75± 0.23^d	93.12±4.86^d	20.70±1.11^b
Group 2 Nephrotoxic control	3.2± 0.25^d	90.55±2.27^c	18.12±0.07^c
Group 3 PS 5%	6.3± 0.15^b	120.75±0.24^b	42.85±0.02^a
Group 4 ES 5%	7.3± 0.15^b	155.79±0.14^b	41.76±0.03^a
Group 5 CL 5%	6.5±0.34^b	145.77±0.08^b	40.78±0.05^a
Group 6 Mixture 15%	7.5 ±0.24 ^{a}	167.95±0.04^a	40.88±0.03^a

Means ± SE with different superscripts in the same column are significant at $P < 0.05$

using one way ANOVA test.

n=7 rats.

Table 3. Effect of aqueous infusions of *Petroselinum sativum* (PS), *Eruca sativa* (ES) and *Curcuma longa* (CL) herbs on the activity of superoxide dismutase (SOD), glutathione peroxidase (GPx) and catalase (CAT) enzymes in kidney tissue of gentamicin-nephrotoxic rats.

Parameters Groups	SOD (U/mg protein)	GPx (nmol/min/mg protein)	CAT (nmol/min/mg protein)
Group 1 Healthy control	51.8±0.18^a	0.50±0.02^a	0.186±0.001^a
Group 2 Nephrotoxic control	36.00±2.3^c	0.13±0.03^c	0.144±0.003^c
Group 3 PS 5%	39.00±2.62^c	0.25±0.04^b	0.123±0.004^b
Group 4 ES 5%	42.25±2.42^b	0.28±0.01^b	0.135±0.005^b
Group 5 CL 5%	44.64±3.75^b	0.39±0.03^b	0.169±0.002^b
Group 6 Mixture 15%	48.77±2.43^c	0.44±0.02^b	0.177±0.001 ^{b}

Means ± SE with different superscripts in the same column are significant at $P < 0.05$

using one way ANOVA test.

n=7 rats.

Histological examination of kidneys of healthy rats showed normal histological structure of renal parenchyma (glumeruli and tubules) as illustrated in Fig. (3). Kidneys of rats intoxicated with GM (80 mg/kg, i.p.) for 8 successive days revealed marked necrosis of renal tubules associated with presence of protein casts in their lumens (Fig.4). Examination of kidneys of rats given orally the aqueous extract of *Petroselinum sativum* herb concomitantly with GM showed mild congestion of intertubular blood capillaries (Fig.5). In rats given the aqueous extract of *Eruca sativa* herb concomitantly with GM, the examination of kidney showed vacoulations of epithelial lining of renal tubules (Fig.6). In rats received the aqueous infusion of *Curcuma longa* herb along with GM, the microscopic examination of kidneys revealed little peritubular leukocytes infiltration (Fig.7). Concomitant administration of the mixture of *Petroselinum sativum*, *Eruca sativa* and *Curcuma longa* herbs along with GM showed almost normal histological architecture of renal parenchyma (Fig.8).

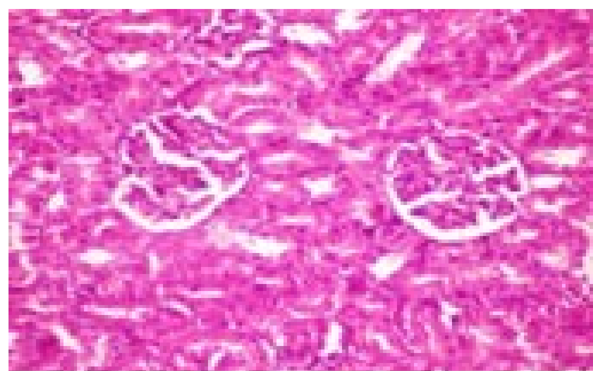

Fig 3. Kidney healthy control rat showing normal architecture of renal parenchyma (glumeruli and tubules)

(H & E, X 200)

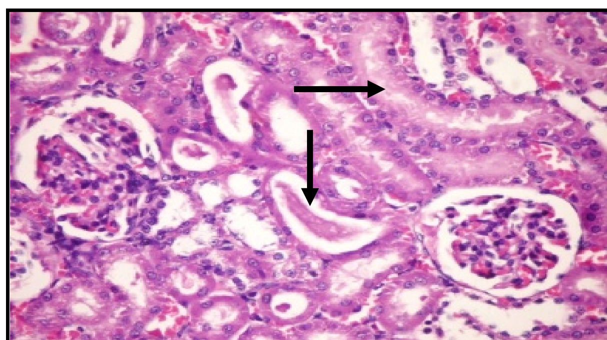

Fig 4. Kidney of a gentamicin-intoxicated rat showing marked necrosis of renal tubules (Arrow) with protein casts in their lumens (Arrow).H & E, X 200)

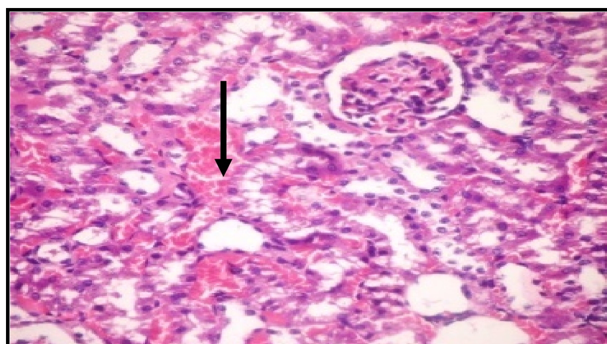

Fig. 5. Kidney of a gentamicin- intoxicated rat given orally aqueous extract of *Petroselinum sativum* herb showing mild congestion of intertubular blood capillaries (Arrow).
(H & E, X 200**)**

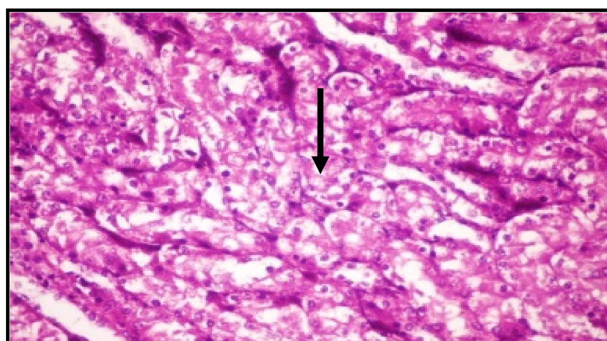

Fig. 6. Kidney of a gentamicin-intoxicated rat and given orally aqueous extract of *Eruca sativa* herb showing vacoulations of epithelial lining of renal tubules (Arrow). (H & E, X 200**)**

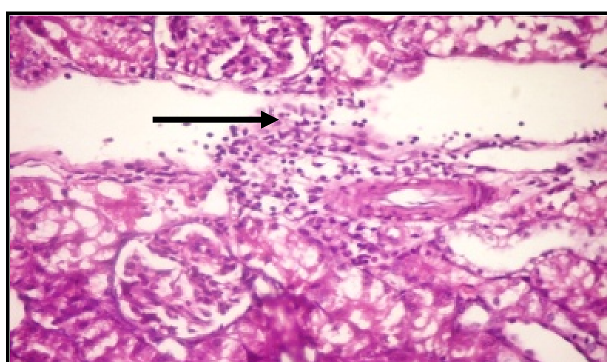

Fig. 7. Kidney of a gentamicin- intoxicated rat and given orally aqueous infusion of *Curcuma longa* herb showing peritubular leukocytes infiltration (Arrow). (H & E, X 200)

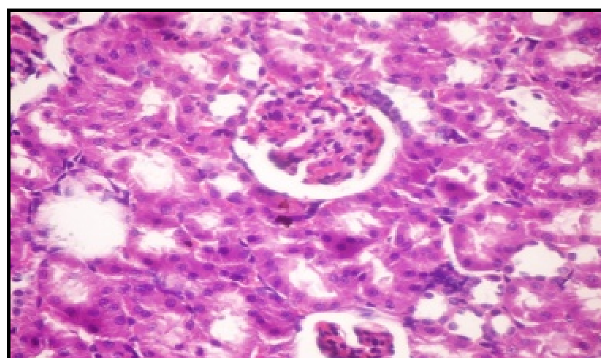

Fig. 8. kidney of a gentamicin- intoxicated rat and given orally aqueous extract of mixture of the 3 herbs showing almost normal histological structure of renal parenchyma.
(H & E, X 200**)**

DISSCUSION

The nephroprotective, diuretic and antioxidant activities of aqueous extracts of *Petroselinum sativum*, *Eruca sativa* and *Curcuma longa* herbs, alone and in combination, against gentamicin (GM) - induced nephrotoxicity in rats were investigated.

The results revealed that intraperitoneal injection of gentamicin (GM) to rats caused signs of nephrotoxicity manifested by significant increases in serum urea, creatinine and activity of ALP enzyme associated with decreases in serum levels of sodium and potassium electrolytes. Urine analysis showed significant decreases in urinary excretion of sodium and potassium in GM-intoxicated rats. In addition, lipid peroxidation in kidney tissues showed significant elevation of lipid peroxide malondialdehyde (MDA) and the antioxidant enzymes were markedly decreased in GM- intoxicated rats. Examination of kidney sections of GM**-**intoxicated rats revealed marked necrosis of renal tubules. These results were in agreement with findings of previous authors [1, 2, 3, 4, 5] who concluded that GM induces nephrotoxicity manifested by biochemical and histological changes in rats.

The mechanism of nephrotoxicity caused by GM was attributed to stimulation of generation of reactive oxygen species (ROS) causing tissue oxidative stress [4, 5, 6]. GM**-** nephrotoxicity associated with decreased serum levels of sodium and potassium suggested that the site of GM action is the distal convoluted tubules causing increased urinary excretion of sodium and potassium [3]. In addition, it was previously reported that high serum alkaline phosphatase (ALP) concentrations might be a marker of renal inflammation [30].

Oral administration of *Petroselinum sativum*, *Eruca sativa* and *Curcuma longa* herbs and their mixture caused nephroprotective and diuretic effects as they reversed the biochemical and histological alterations induced by GM in rats. These herbs also produced an

antioxidant activity as evident by decreasing lipid peroxidation byproduct (MDA), increasing content of reduced glutathione and restoring activities of antioxidant (SOD, GPx and CAT) enzymes in renal tissue.

The nephroprotective effect of *Petroselinum sativum* herb, reported in the present study, was similar to that reported by Afzal *et al.* [31] who found that a polyherbal formulation containing *Petroselinum sativum* (parsley) produced a nephroprotective effect in rats. This effect of *Petroselinum sativum* was attributed to its *in vitro* antioxidant activity as free radical scavenger or due to its high content of flavonoids [32]. The diuretic effect of *Petroselinum sativum* was reported by Kreydiyyeh and Usta [10] who found that parsley aqueous seeds extract produced a diuretic effect in rats. The previous authors concluded that the mechanism of action of parsley seems to be mediated through an inhibition of the Na+/K+ pump that would lead to a reduction in Na^+ and K^+ reabsorption thus leading to an osmotic water flow into the lumen, and diuresis.

Concerning *Eruca sativa* herb, it is widely used in folklore medicine and has a good reputation as a remedy of renal ailments. It was reported that *Eruca sativa* produced potent antioxidant and renal protective activities and precluded oxidative damage inflicted to the kidney by mercuric chloride in rats [12]. Recently, *Eruca sativa* L extract was reported to produce an antioxidant effect due to its free radicals scavenging activity *in vivo* [33] and *in vitro* [34]. Moreover, *Eruca sativa* was reported to protect the liver against CCl4-induced hepatic injury through its potent antioxidant activity in rats [35].

Regarding Curcuma *longa* herb, it was reported that curcumin derived from plant *Curcuma longa* ameliorated diabetic nephropathy in rats and the antioxidant mechanism being responsible for the nephroprotective action of curcumin [13]. Ademiluyi *et al.* [36] reported that dietary inclusion of ginger (*Zingiber officinale*) and turmeric (*Curcuma longa*) rhizomes attenuated gentamicin-induced nephrotoxicity in rats. The bioactive substance curcumin isolated from turmeric (*Curcuma longa*) rhizomes produced a renoprotective activity via its antioxidant effect [37]. The authors concluded that curcumin might be potentially useful in some kidney diseases by preventing renal inflammation. *Curcuma longa* (turmeric) extract was found to possess multiple therapeutic activities that block the cardiac, hepatic, and renal toxicities induced by doxorubicin [15] and by arsenic trioxide [38] and had as a free radical scavenger activity. In addition, the previous studies revealed that curcumin derived from *Curcuma longa* (turmeric) extract inhibited biofilm development of uropathogens [39]. Curcumin can protect the liver from the damage caused by N-nitrosodiethylamine in rats and has the potential to be used in a therapy of liver cancer [40]. It was suggested that curcumin might be potentially useful in kidney diseases by preventing renal inflammation [16].

The ameliorative effect of histopathological changes induced by GM in kidney of rats by the studied herbs was parallel with the reported biochemical alterations in the current study. The amelioration of renal tubular necrosis by the studied herbs in GM- intoxicated rats was similar to that reported by Afzal *et al.* [31] for *Petroselinum sativum*; by Sarwar *et al.* [12] for *Eruca sativa* and by Kheradpezhouh *et al.* [41] for *Curcuma longa*.

In conclusion, *Petroselinum sativum*, *Eruca sativa* and *Curcuma longa* herb extracts produce nephroprotective, diuretic and antioxidant effects in gentamicin (GM) - nephrotoxic rats. Therefore, intake of aqueous extract of these herbs and their mixture may be potentially useful for patients who suffer from kidney diseases and those on GM therapy.

CONFLICT OF INTERESTS

None.

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Original Research

Antinociceptive activity of flower buds extract of *Sophora japonica* and its main active ingredient quercetin in bee venom-induced rat model

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Abstract

Aim: *Sophora japonica* L. is one of the traditional medicines in the world for a long time. The major ingredients flavonoids are shown to various pharmacological and nutritional values. The study was performed to investigate the antinociceptive activity of methanolic extract (ME) and its main flavonoid quercetin from flower buds of *S. japonica* in the bee venom-induced rat model.

Methods: The antinociceptive activity of the ME of flower buds from *S. japonica* (50-500 mg/kg) and the isolated compound quercetin (5-50 mg/kg) were evaluated by using bee venom-induced rat model after administrating respectively.

Results: The administration of ME from *S. japonica* and quercetin significantly increased the tolerance to pain in SD rats in comparison to control. The ME of *S. japonica* and quercetin produced significant inhibition of both the early (neurogenic pain or acute pain, 0-10 min) and the late (inflammatory pain or tonic pain, 11-40 min) phases of bee venom-induced pain. Furthermore, the ME (100-500 mg/kg, i.p.) caused a significant increase in the latency to response in the hot-plate test. However, the quercetin (5-50 mg/kg, i.p.) had no significant effect in the hot-plate test.

Conclusion: These results suggest that the ME of *S. japonica* and its main active ingredient quercetin appear to contribute for the antinociceptive property in the bee venom-induced rats. The investigation gives an evidence of potential uses of the flower buds extract of *S. japonica* and quercetin as medicines of adjunctive therapy pain.

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INTRODUCTION

Dried flowers and buds of *Sophora japonica* L. are well-known herbal medicine which have been used in traditional Chinese medicine for a long time [1]. The flowers and buds have been used to cure the diseases including hemafecia, hemorrhoids blood, bloody flux, uterine bleeding, hematemesis, liver heat and red eyes, headache and dizziness, and so on [2,3]. Main components of *S. japonica* include flavones, isoflavones, triterpene glycosides, phospholipids and alkaloids. Moreover, *S. japonica* contains five main flavonoids of rutin, quercetin, isorhamnetin, genistein and kaempferol [4,5]. Quercetin is one of the more abundant flavonoids found in nature, and its biological effects have been extensively reviewed [6,7]. Many results have demonstrated that quercetin inhibits nociceptive responses in mice and rats models of nociception [7,8]. Clinically, quercetin was also effective in a pilot study against painful bladder syndrome cystitis [9]. However, the mechanisms related to its analgesic effects remain unknown.

The bee venom (BV) model is a newly-developed experimental animal model of inflammatory pain [10]. Many behavioral experiments have demonstrated that the BV-inflamed animals show three major manifestations relevant to clinical pathological pain: persistent spontaneous nociception, primary thermal and mechanical hyperalgesia, secondary or mirror-

image thermal hyperalgesia [11]. The BV model is behaviorally characterized by an immediate persistent spontaneous pain-related paw flinching reflex lasting for more than 1 h, followed by 72-96 h of primary heat and mechanical hyper-sensitivity [12]. Thus, the aim of this present study was to evaluate the effect of the methanolic extract and its main flavonoid quercetin from flower buds of *S. japonica* in the bee venom-induced rat model and to elucidate the potential role in inflammatory pain.

MATERIALS AND METHODS

Preparation of plant extract

The flower buds of *S. japonica* were purchased from the local market. The flower buds were dried at 60°C for 1 day. The dried flower buds were milled to powder and soaked in 80% methanol using ultrasonic treatment for 15 min and repeated 3 times. And then the mixture was centrifuged at 5000 rpm for 10 min, the supernatant was taken and evaporated at 60°C to produce the methanolic extract (ME). The ME was stored in refrigerator at -20°C until use. The quercetin was separated and assayed as reported previously [13]. The ME and quercetin were dissolved in Tween 80/DMSO plus saline. The final concentration of Tween 80 and DMSO did not exceed 5%.

Experimental animals

The experiments were performed on male Sprague-Dawley rats weighing 180-250 g. The rats were housed in plastic boxes in groups of 4-6 with access to food and water and maintained on 12 h light/dark cycle at room temperature (22-26°C). All experimental procedures were carried out in accordance with ethical guidelines of the International Association for the Study of Pain for pain research in conscious animals.

Algesiometric assays

The BV lyophilized melittin (Sigma, USA) was dissolved in 0.9% sterile saline and the employed dose was 0.05 mg in 50 μl for all animals during the whole experiment. Injection of BV solution was administered into the posterior plantar surface of the rat hind paw as reported previously [14]. For the evaluation of effects of ME and quercetin, post-treatment with either drugs or vehicle (0.9% saline solution) was performed at 5 min after melittin injection (i.p.). For control group, each agent was injected into the hindpaw contralateral to the melittin treated side to exclude any systemic effects of the drugs. According to the method as described [15], a 30×30×30 cm transparent Plexiglas test box with a transparent glass floor was placed on a supporting frame of 30 cm high above the experimental table. The rat was placed in the test box for at least 30 min before administration of any chemical agents. After the acclimation period, i.p. injection of BV was made into the center of the plantar surface of one hind paw with slight restraint. Rats were returned to the boxes and immediately observed for bitings and lickings of the affected hind paw. The total time spent in biting and licking over the next 40 min was measured with a stopwatch and recorded to the nearest second in 5-min blocks during both phases as an indicator of nociception [16]. On the basis of pilot data and in keeping with the literature, the first phase of pain was defined as 0 to 10 min postinjection of BV, and the second phase of pain was defined as 11 to 40 min postinjection.

Hot-plate test

The hot-plate test was used to measure the response latencies according to the method described previously [17] with minor modifications. In these experiments, the TC-1 radiant heat stimulator (China) was maintained at 50 ± 1°C or 56 ± 1°C. The rats were placed on the surface of a 2 mm thick glass plate covered with a plastic chamber. Each rat was tested before administration of drugs in order to obtain the baseline. Animals were treated with ME (50-500 mg/kg, i.p.), quercetin (5-50 mg/kg, i.p.), morphine (5 mg/kg, i.p., positive control) or vehicle (0.9% saline, 10 ml/kg, i.p.) 1 h before testing, respectively. Five stimuli were repeated for each site and the latter three values were averaged as the mean withdrawal thermal latency. The inter-stimulus interval was more than 10 min. The thermal latency was defined as the duration from the onset of heat stimulus to the occurrence of hindpaw withdrawal reflex. The stimulus was stopped if the latency exceeded 30 s so as to avoid excessive tissue injury.

Statistical analysis

All results were reported as the mean ± SEM of three independent experiments, each was done with three replicates. The data were analyzed statistically by analysis of variance (ANOVA), and the difference between the means of samples was analyzed by the least significant difference (LSD) at probability level of 0.05.

RESULTS

We tested the antinociceptive activity of flower buds extract of *S. japonica* and its main active. The ME (50-500 mg/kg) and the purified compound quercetin (5-50 mg/kg) produced a dose related inhibition of BV-induced rats and caused significant inhibition of both neurogenic (0-10 min) and inflammatory (11-40 min) phases of BV-induced licking. As shown in Fig. 2, the administration of BV into the plantar surface of the

hind paw induced two typical phases of pain behavior. The first phase of pain, or acute pain, appears 0-10 min after BV injection, and the second phase of pain, or tonic pain, appears 11-40 min after a short-term quiescent interval (Fig.1A, C). Pretreatment of ME at a dose of 50 mg/kg in the first phase of pain and 100 mg/kg in the second phase of pain did not attenuate pain induced by BV. Quercetin relieves acute pain at a dose larger than 25 mg/kg and 10 mg/kg in the tonic pain (Fig.1B, D). Interestingly, pretreatment of ME or quercetin at a dose of 250 mg/ kg or 25 mg/ kg strongly inhibited the second phase of pain (p≤0.01), indicating that flower buds extract of *S. japonica* and quercetin exert their analgesic activity by inhibiting mainly tonic pain rather than acute pain in the BV test.

The results in Fig. 2A, C show that the ME of *S. japonica* (100-500 mg/kg, i.p.) produced significant increase in the pain latency in the hot-plate test at 50°C and 56°C (p≤0.05). In contrast, quercetin (5-50 mg/kg, i.p.) did not cause any significant increase in the pain latency in the hot-plate test against both temperatures studied (Fig. 2B, D).

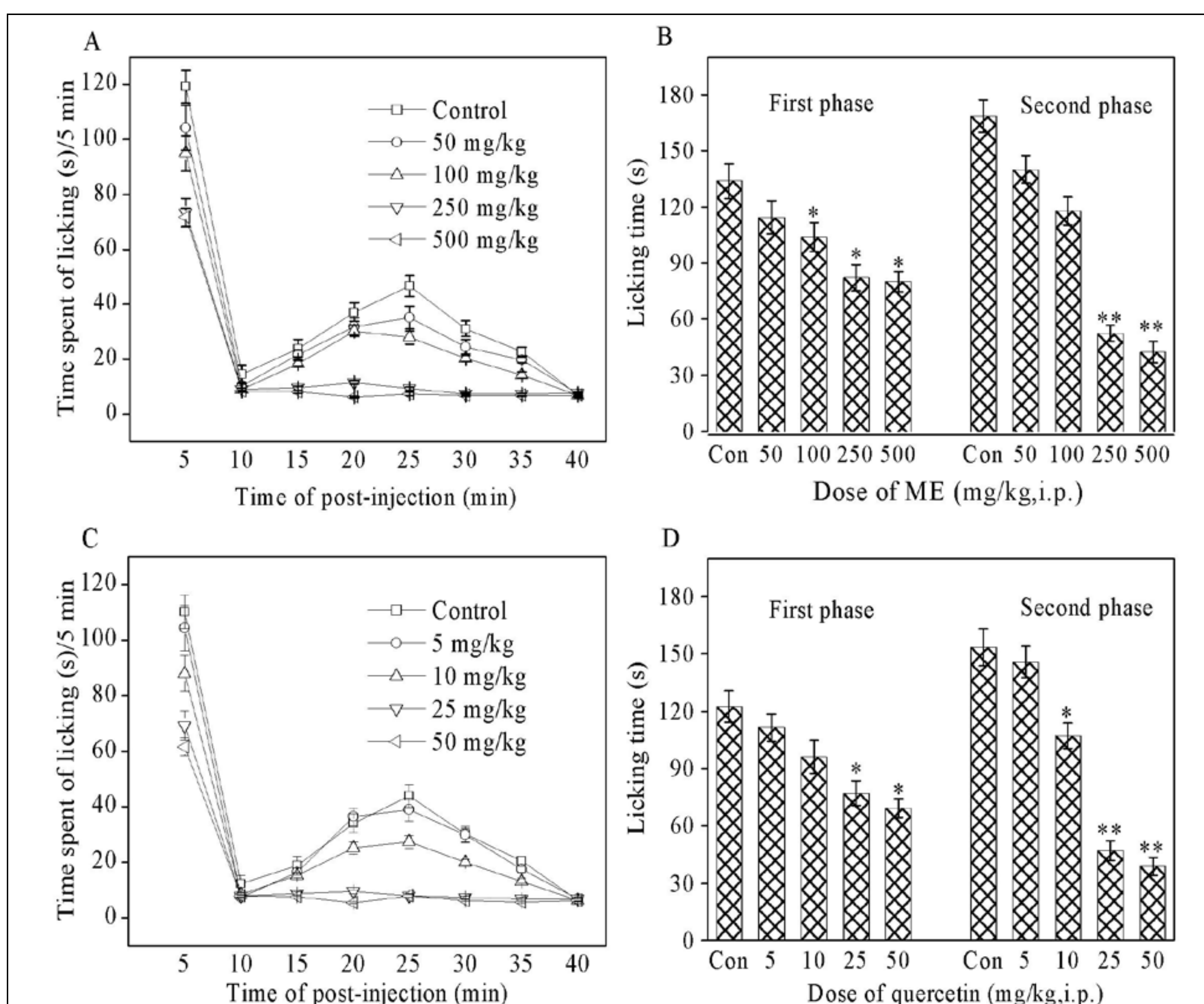

Fig. 1. The effect of the methanolic extract (ME) of flower buds from *S. japonica* and quercetin on pain induced by BV. Different dose of ME (A) and quercetin (C). Pain responses were measured immediately with 5-min block after intraplantar surface injection of 0.05 mg BV. Pain responses are the time spent licking. Each value represents mean ± SEM. The first phase (the acute pain: 0-10 min) and second phase (tonic pain: 11-40 min) of pain responses after the injection of BV subsequent to pretreatment with different doses of ME (B) and quercetin (D). *, p≤0.05 or ** p≤0.01 compared with 0.9% saline -treated controls (Con).

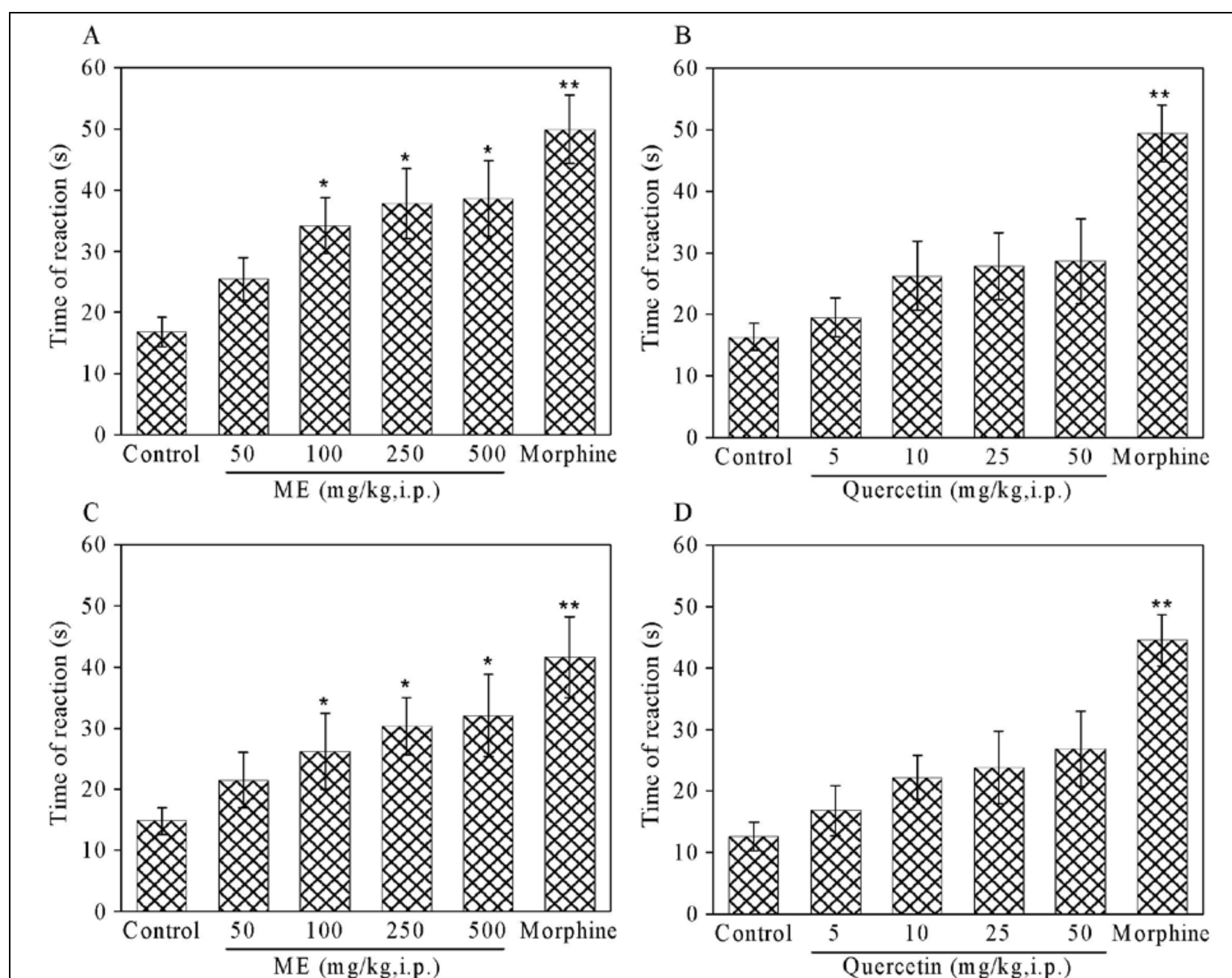

Fig. 2. Effect of methanolic extract (ME) of flower buds from *S. japonica* and quercetin in the hot-plate test at 50°C (A, B) and 56°C (C, D) in rats. Morphine (5 mg/kg, i.p.,) as positive control. Each value represents mean ± SEM. *, p≤0.05 or ** p≤0.01 compared with 0.9% saline -treated controls (Con).

DISCUSSION

Despite the great progress has really been made in the pain therapy in recent years, there is still a need for effective and potent analgesics, especially for the treatment of chronic pain [18]. In this regard, it has been widely shown that many plant-derived compounds present significant analgesic effects [19]. Thereby, they represent potential molecules for the development of new drugs, especially designed for the treatment or control of chronic inflammatory and painful states. These antinociceptive substances include alkaloids, terpenoids, flavonoids and others [20,21]. For this reason, plant-derived substances are particularly important in the development of new analgesic drugs. These natural products were known for their beneficial effects on health long before flavonoids were isolated as the effective compounds. Among these flavonoids, quercetin is a unique bioflavonoid that has been extensively studied by researchers over the past 30 years [22,24].

In this study, quercetin dose-dependently significantly inhibited (i.p.) both the neurogenic and inflammatory phases of BV-induced pain. Quercetin especially exhibits potent and consistent analgesic effects against inflammatory pain. These are consistent with reported results that quercetin had the similar analgesic effects in other pain model [25,26]. In addition, quercetin did not block responses in the hot-plate test, suggesting that the opioid system is not involved in analgesic action. However, the ME of flower buds from *S. japonica* dose-dependently (ME more than 100 mg/kg) inhibited nociceptive responses in the hot-plate test. This phenomenon suggests that other analgesic substances may work in the ME from *S. japonica* in the hot-plate test. The details need to be further investigated.

BV-induced pain is a screening tool for the assessment of analgesic or anti-inflammatory properties of natural and synthetic compounds. BV may act indirectly by inducing the release of endogenous mediators, which stimulate the nociceptive neurons sensitive to non-steroidal anti-inflammatory drugs and opioids [10,11]. In acetic acid-induced pain model, quercetin is a good antinociceptive agents and it is sensitive, but has poor specificity [27]. The BV-model is a complementation model for investigating the antinociception.

In conclusion, the flower buds extract of *S. japonica* and quercetin exhibit potent analgesic effects against neurogenic and inflammatory pain. The potent antinociceptive action against the neurogenic pain response caused by BV (first phase) models strongly suggests that it may be acting through interaction with pain signal pathway. The further studies are required to determine the interaction with other pain systems.

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Original Research

Phytochemicals of *Chrysophyllum albidum*, *Dacryodes edulis*, *Garcinia kola* chloroform and ethanolic root extracts and their antimicrobial properties

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Keywords: Phytochemical, Antimicrobial, Chrysophyllum albidum, Dacryodes edulis, Garcinia kola, root extracts

Summary
Aim: The phytochemistry and antimicrobial qualities of the chloroform and ethanolic root extracts of *Chrysophyllum albidum* G. Don Holl, *Dacryodes edulis* H.J. Lam and *Garcinia kola* H were investigated.
Method: Routine methods were used. The test isolates utilized were; *Escherichia coli*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Bacillus subtilis*, *Aspergillus niger*, *Penicillium notatum*, *Mucor mucedo* and *Candida albicans*.
Results: Phytochemical screening revealed the presence of alkaloids, flavonoids, saponins and tannins. Both extracts showed broad spectrum of antimicrobial activities but chloroform extracts gave higher zones of inhibition compared to corresponding concentrations of ethanol extracts. Maximal inhibitory zones were shown by *P. aeruginosa*; 30.7 mm ± 0.01 against *G. kola* chloroform root extract. Chloroform root extracts of *G. kola* were comparatively more potent against the test isolates than *C. albidum* and *D. edulis* root extracts. All the respective root extracts exhibited a greater antibacterial activity in comparison with the antifungal attributes.
Conclusion: The presence of bioactive antimicrobial compounds in the examined extracts of the medicinal plants particularly *G. kola* could indicate the possibility of obtaining potentially valuable antimicrobial phytochemicals from the plants.

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INTRODUCTION

Man since ancient time has been dependent on plants for food, drinks, shelter, clothing, equipment, dental care and medicine [1]. Infections due to pathogenic bacteria and fungi represent a critical problem to human health [2]. Over 25% of the prescribed medicines in industrialized countries are derived directly from plants [3]. Benefits derived from using medicine obtained from plants are that they are relatively safer than synthetic alternative by offering profound therapeutic benefits and more affordable treatment [4]. Plants contain phytochemicals, which are natural bioactive compounds [5] and possess antimicrobial, anti-oxidant and physiological activities [6].

Chrysophyllum albidum G. Don Holl (Sapotaceae) tree is common throughout the tropical Central, East and West Africa region and is valued for its edible fruits and ethno-medical uses [7-8]. *C. albidum* fruits (known as African star apple) are widely eaten in southern Nigeria [9]. The bark is used as a remedy for yellow fever and malaria, while the leaves are used as emollients and for the treatment of skin eruptions, diarrhea and stomach ache, resulting from infections and inflammatory reactions [10]. *Dacryodes edulis* H.J. Lam (Burseraceae) commonly called African pear is a fruit tree native to Africa. The fruit tree is an ellipsoidal drupe which varies in length from 4-12 cm. The fruit of *D. edulis* can be eaten raw, cooked, boiled or roasted [11]. The bark or leaf decoction is used to treat toothache, gum problem, tonsillitis and earache [12].

The roots are known to treat beri-beri and rickets when boiled with other herbs and administered orally [11]. *Garcinia kola* H. (Guttiferaceae) is a tree that grows in rain forests and swamps of West Africa and grows as a medium sized tree up to a height of about 12 m high [5]. *G. kola* is believed to be an important source of new chemical substances with potential therapeutic benefits. From its roots to its leaves, the plant is known to contain several phytochemicals noted for their medicinal importance [13]. *G. kola* is also used in the treatment of liver disease and diarrhea [13-14].

This study was done to investigate the phytochemical constituents and anti-microbial activity of *Chrysophyllum albidum*, *Dacryodes edulis* and *Garcinia kola* chloroform and ethanolic root extracts with a view of validating their folk use in treating some microbial ailments.

MATERIALS AND METHODS

Plant collection, preparation and extraction

The root samples were collected from the Forestry Department; Faculty of Agriculture, University of Benin, Benin City, Edo State, Nigeria in April 2012. The plants (trees) were identified by Dr. G.Emelue of the same Department. Samples of all three plants were kept in our institutions herbarium with voucher numbers UBHs0257, UBHb0291, UBHg0281 respectively. The materials were washed and cut into small pieces and air dried before grinding. Then 50 g of each powdered plant was soaked separately in 500 ml chloroform and 95% ethanol at room temperature for 24 h. The extracts were then filtered through cheesecloth for 30 minutes. The filtrates were collected, filtered through Whatman No 1 filter paper and evaporated in water bath at 40°C. The extracts were collected and kept at 4°C for further investigation.

Phytochemistry

Qualitative tests for alkaloids, tannins, saponin, flavonoids, steroids and terpenoids were conducted using standard methods such as Mayer's reagent test for determination of alkaloids, frothing test for saponin determination and flavonoid determination [15-16]. Quantitative phytochemical constituents of the ethanolic extracts were determined using methods described by [16-19].

Test organisms

Several clinical test isolates were used in this study; *Pseudomonas aeruginosa, Staphylococcus aureus, Bacillus subtilis, Escherichia coli, Candida albicans, Penicillium notatum*, *Mucor mucedo* and *Aspergillus niger.* The bacterial and yeast pure cultures were sourced from the Medical microbiology laboratory, University of Benin Teaching Hospital. The filamentous fungal isolates were obtained from Edo Environmental Consults and Laboratory, Benin City. The identities of the bacterial pure cultures were confirmed by conducting several routine morphological and biochemical tests as described by [20] and [21]. The results of the tests were compared with reference tables as stated by [22].

Standardization of the microbial inocula

The procedures as described by [23] and [24] were adapted in the standardization of the microbial test pure cultures All the test bacterial and fungal isolates were sub-cultured on freshly prepared Nutrient agar and Sabouraud Dextrose agar plates and incubated for 24 h and 48 h respectively. Portions of the streaked bacterial and fungal colonies were transferred into test tubes containing 8ml of sterile nutrient broth and incubated for 12h and 48 h at 37 ^{0}C. The growth of bacterial and fungal suspension obtained was compared to that of freshly prepared Barium sulphate opacity standard {0.5 ml of 1% Barium in Chloride to 99.5 ml of 1% H_2SO_4 (0.36 Normal).The turbidity was adjusted by adding more sterile nutrient broth to match 0.5 McFarland standards (10^6 cfu/ml and 10^6 spores /ml).

Determination of antimicrobial activity

The agar-well diffusion assay as described by [25] was used to ascertain the inhibitory effects of the respective root extracts on the test isolates. The tests were carried out using a stock concentration of 100 mg/ ml prepared by dissolving 0.01 g of the respective ethanolic and chloroform root extracts into 10 ml of distilled water. Prepared and labeled Nutrient agar (NA) and Sabouraud Dextrose agar (SDA) plates were seeded with 2ml of standardized bacterial and fungal broth cultures. The microbial lawn was done using a sterile glass rod. The seeded plates were allowed to dry. A 4 mm sterile cork borer was used to punch 2 equidistant holes in the middle of the labeled inoculated agar plates. The holes were filled with 0.2 ml of the differing concentrations of the seed extracts. The bored agar plates were left at room temperature for 10 min, allowing the diffusion of the extracts into the agar. Then the plates inoculated with bacterial and yeast isolates were incubated at 35 ^{0}C for 24 h. The plates with the filamentous fungal cultures were kept at room temperature for 72 h. At the end of the incubation period, the plates were observed. The antibacterial activity of the seed extracts was assessed by an inhibition zone surrounding the well. The mean zones of inhibition was measured and expressed in millimeters. The process was repeated in the case of fungi however, inoculated plates were stored at room temperature for 72 h after which the zones of inhibition

were measured using a meter rule. However, commercially available antibiotic discs (Pefloxacin and Nystatin) were used as positive control for the bacterial and fungal test cultures.

Minimum Inhibitory Concentration (MIC)

The MIC of both the chloroform and ethanolic root extracts were determined by the broth dilution method [26]. The plant extracts were prepared to the highest concentration of 100 mg/ ml (stock concentration) in sterile distilled water and serially diluted to give concentrations ranging from 50 mg /ml to 3.125 mg/ ml. Zero point one (0.1) ml of the standardized microbial broth cultures were inoculated into the labeled tubes containing the diluted extracts. The tubes were incubated at room temperature for 24 h for bacteria and at room temperature for 48 h for fungi [27]. The least concentration of the extract which inhibited the growth of the inoculums was considered as the minimum inhibitory concentration.

Statistical analysis

Results are expressed as mean ± standard error of mean using SPSS 16.0 computer software package. The level of significance was determined at 0.05.

RESULTS AND DISCUSSION

Alkaloids, flavonoids, saponins and tannins were present in all the respective ethanolic root extracts (Table 1) whilst terpenoids and steroids were not detected. These bioactive compounds have been known to show medicinal activities as well as exhibited physiological activities useful to man [28]. Saponins have been reported to have antifungal properties while tannins prevent the development of microorganisms by precipitating microbial protein and making nutritional proteins unavailable to them [29]. The saponin and tannin content of the extracts ranged from 0.57% and 1.25% for *D. edulis* and *G. kola* to 2.34% and 1.97% for *G. kola* and *C. albidum* respectively (Table 2). The alkaloid content of the respective root ethanolic extracts ranged from 0.22% for *D. edulis* for 0.57% for *G. kola* (Table 2). Flavonoids have been shown to have a wide range of biological and pharmacological activities in in vitro studies. Examples include anti-allergic,[30] anti-inflammatory,[30,31] antioxidant,[31] anti-microbial (antibacterial, [32, 33] antifungal,[34, 35] and antiviral[34, 35]), anti-cancer,[36][31] and anti-diarrheal activities[37]. Flavonoids have also been shown to inhibit topoisomerase enzymes [38, 39] and to induce DNA mutations in the mixed-lineage leukemia (MLL) gene in vitro studies[40]. The flavonoid value varied from 0.29% for *C. albidum* to 1.86% for *G. kola* (Table 2). The presence of plant secondary metabolites has been implicated for most plants therapeutic activities [41]. Also, plants containing these metabolites (alkaloids, flavonoids, tannin, saponins etc) usually demonstrate stronger antimicrobial properties than others [42]. Ethno botanical reports by [11] showed that root extracts of *D. edulis* are used in the treatment of leprosy, hypertension, beri-beri and rickets. The stems resin exudate is applied to ecto-parasitic infestations and infections as well as dressing cuts, bruises and wounds [7, 43, and 44].The phytochemicals identified in the ethanolic extract of *C. albidum* (Table 1 and 2) are similar to the report of [45] . The results obtained for the quantitative phytochemical screening of *G. kola* are similar to a report by [5].

Table 1. Qualitative phytochemical constituents of *C. albidum, D. edulis* and *G. kola* ethanolic root extracts

Phytochemical	*C. albidum*	*G. kola*	*D. edulis*
Alkaloids	+	+	+
Flavonoids	+	+	+
Saponins	+	+	+
Tannins	+	+	+
Terpenoids	ND	ND	ND
Steroids	ND	ND	ND

Legend: +: Present, ND: Not Detected

Table 2. Quantitative phytochemical constituents of *C. albidum, D. edulis* and *G. kola* ethanolic root extracts.

Phytochemical	*C. albidum* (%)	*G. kola* (%)	*D. edulis* (%)
Alkaloids	0.24	0.57	0.22
Flavonoids	0.29	1.86	0.60
Saponins	2.04	2.34	0.57
Tannins	1.97	1.25	1.32
Terpenoids	0.00	0.00	0.00
Steroids	0.00	0.00	0.00

The zone of inhibition due to the antimicrobial activities of the chloroform and ethanolic extracts of *C. albidum*, *D. edulis* and *G.kola* are presented in Tables 3 and 4. The inhibitory zones elaborated by the test isolates exposed to *C. albidum* ranged from 5.3 mm ± 0.07 for *Mucor* sp. to 18 mm ± 0.03 for *E. coli* (Table 3). The microbial cultures exposed to *D. edulis* ethanolic root extract showed inhibitory zones which varied from 1.67 mm ± 0.20 for *P. notatum* to 12 mm ± 0.04 recorded for *E. coli* (Table 3). Zones of inhibition exhibited by the exposed isolates to *G. kola* alcoholic extract ranged from 2.7 mm± 0.20 for *P. notatum* to 19.3 mm ± 0.01 for *E. coli* (Table 3). The inhibitory zones observed in the bacterial isolates exposed to the control antibiotic; Pefloxacin and Nystatin ranged from 10 mm ± 0.04 and 5 mm ± 0.00 for *P. aeruginosa* and *A. niger* to 19.00 mm ± 0.01and 21 mm ± 0.18 for *S. aureus* and *C. albicans* (Table 3).

Table 3. Antimicrobial activity of *C. albidum, D .edulis* and *G. kola* ethanolic root extracts

Test organisms	Zone of inhibition (mm)			Positive control	
	C. albidum	*D. edulis*	*G. kola*	Pefloxacin (10μg)	Nystatin (100mg/ml)
E. coli	18 ±0.03	12 ±0.04	19.3 ±0.01	14 ±0.00	ND
P. aeruginosa	15.3 ±0.03	6 ±0.08	10 ±0.04	10 ±0.04	ND
S. aureus	8 ±0.06	17 ±0.03	17 ±0.03	19 ±0.01	ND
B. subtilis	6 ±0.08	1.67±0.23	NZI	16 ±0.02	ND
A. niger	NZI	5 ±0.00	7.3 ±0.09	ND	5 ±0.00
P. notatum	7.00±0.00	5.67±0.07	2.7 ±0.20	ND	8 ±0.29
M.mucedo	5.3 ±0.07	5.67±0.07	NZI	ND	11 ±0.25
C. albicans	9.6 ±0.06	6.67±0.06	7 ±0.07	ND	21 ±0.18

Legend: Values are means ± Std. Error, ND: Not Determined, NZI: No Zone of Inhibition

The inhibitory zones displayed by the test isolates exposed to *C. albidum* chloroform root extract ranged from 9 mm± 0.05 for *B. subtilis* to 26 mm ± 0.02 for *E. coli* (Table 4). The microbial cultures exposed to *D. edulis* extract showed inhibitory zones which varied from 5.00 mm± 0.00 for *A. niger* to 21 mm ± 0.02 for *E. coli* (Table 4). Zones of inhibition elicited by the exposed isolates to *G. kola* chloroform root extract ranged from 2.7 mm± 0.20 for *P. notatum* to 30.7 mm ± 0.01 for *P. aeruginosa* (Table 4). The inhibitory zones elaborated by the bacterial isolates exposed to the control antibiotics; Pefloxacin and Nystatin varied from 10 mm± 0.03and 5 mm± 0.00 for *P. aeruginosa* and *A. niger* to 19 mm± 0.01 and 21 mm ± 0.18 for *S. aureus* and *C. albicans* respectively (Table 4).

Comparatively, the chloroform root extracts were more potent against the test isolates than the ethanolic extracts (Tables 3 and 4). This could be indicative of the increased solubility of the phytochemicals in chloroform solvent. The observed antimicrobial activity of the respective root extracts might have been dependent on both the concentration as well as nature of the extraction solvent used. Comparatively, the *G. kola* chloroform root extract were more potent against the test isolates than the antibiotic control; Pefloxacin (Table 4). However the antifungal activities of the all the root extracts were lesser than that elicited by the antifungal drug; Nystatin (Table 3 and 4). The susceptibility of the test bacterial isolates to *G. kola* chloroform root extract might be reflective of the broad spectrum bactericidal activities of the respective phytochemicals present in the extract.

The highest MIC values were displayed by the ethanolic root extracts of *D. edulis* and *G. kola* (6.25 mg/ml) against *E. coli* (Table 5). *A. niger*, *B. subtilis* and *M. mucedo*. were resistant to the highest concentration (100 mg/ml) of *C. albidum*, *D. edulis* and *G. kola* ethanolic root extracts (Table 5).

Table 4. Antimicrobial activity of *C. albidum, D. edulis* and *G. kola* chloroform root extracts

Test organisms	Zone of inhibition(mm)			Positive control	
	C. albidum	*D. edulis*	*G. kola*	Pefloxacin (10μg)	Nystatin (100mg/ml)
E. coli	[a]26 ±0.02	21 ±0.02	23.7 ±0.02	14 ±0.00	ND
P. aeruginosa	20.3 ±0.02	25.3 ±0.02	30.7 ±0.01	10 ±0.03	ND
S. aureus	10.7 ±0.05	19.67±0.02	21 ±0.02	19 ±0.01	ND
B. subtilis	9 ±0.05	17.3 ±0.02	17 ±0.03	16 ±0.02	ND
A. niger	NZI	5 ±0.00	6.7 ±0.07	ND	5 ±0.00
P. notatum	7 ±0.00	5.7 ±0.07	2.7 ±0.20	ND	8 ±0.29
M.mucedo	5.7 ±0.07	5.7 ±0.07	3 ±0.16	ND	11 ±0.25
C. albicans	12 ±0.04	6.7 ±0.06	12.33±0.04	ND	21 ±0.18

Legend: Values are Means ± Std. Error, ND: Not Determined, NZI: No Zone of Inhibition

Table 5. Minimum Inhibitory Concentration (MIC) values of the ethanolic root extracts of *C. albidum, D. edulis* and *G. kola*.

Test Isolates	**Concentration of extracts (mg/ml)**		
	C. albidum	***D. edulis***	***G. kola***
E. coli	50	6.25	6.25
P. aeruginosa	50	50	50
S. aureus	100	50	50
B. subtilis	100	>100	>100
A. niger	>100	100	100
P. notatum	100	100	100
M. mucedo	100	>100	>100
C. albicans	100	100	100

Chloroform root extracts of *D. edulis* and *G. kola* elicited maximal MIC values against *E. coli* (3.125 mg/ml) (Table 6). *A. niger* exhibited the lowest MIC reading (>100 mg/ml) against *C. albidum* root extracts (Table 6).

Table 6. Minimum Inhibitory Concentration (MIC) of C. *albidum, D. edulis* and *G. kola* chloroform root extracts.

Test Isolates	Concentration of extracts (mg/ ml)		
	C. albidum	*D. edulis*	*G. kola*
E. coli	50	3.125	3.125
P. aeruginosa	50	12.5	12.5
S. aureus	50	25	25
B. subtilis	50	100	100
A. niger	>100	100	100
P. notatum	100	100	100
M. mucedo	100	100	100
C. albicans	100	50	50

CONCLUSION

Chloroform root extracts of *G. kola* were comparatively more potent against the test isolates than *C. albidum* and *D. edulis.* All the respective root extracts exhibited a greater antibacterial activity in comparison with the antifungal attributes. The presence of bioactive antimicrobial compounds in the examined alcoholic and chloroform extracts of the medicinal plants especially *G. kola* could indicate the possibility of obtaining potentially valuable antimicrobial phytochemicals from these plants. Further antimicrobial studies on the fractions of the solvent extracts of these plants are recommended.

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Original Research

Tricyclic sesquiterpene alpha-copaene prevents H_2O_2-induced neurotoxicity

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Abstract
Aim: Copaene (COP), a tricyclic sesquiterpene, is present in several essential oils of medicinal and aromatic plants and has antioxidant and anticarcinogenic features. But, very little information is known about the effects of COP on oxidative stress induced neurotoxicity.
Method: We used hydrogen peroxide (H2O2) exposure for 6 h to model oxidative stress. Therefore, this experimental design allowed us to explore the neuroprotective potential of COP in H2O2-induced toxicity in rat cerebral cortex cell cultures for the first time. For this purpose, methyl thiazolyl tetrazolium (MTT) and lactate dehydrogenase (LDH) release assays were carried out to evaluate cytotoxicity. Total antioxidant capacity (TAC) and total oxidative stress (TOS) parameters were used to evaluate oxidative changes. In addition to determining of 8-hydroxy-2-deoxyguanosine (8-OH-dG) levels, the single cell gel electrophoresis (SCGE or comet assay) was also performed for measuring the resistance of neuronal DNA to H2O2-induced challenge.
Result: The results of this study showed that survival and TAC levels of the cells decreased, while TOS, 8-OH-dG levels and the mean values of the total scores of cells showing DNA damage increased in the H2O2 alone treated cultures. But pre-treatment of COP suppressed the cytotoxicity, genotoxicity and oxidative stress which were increased by H2O2.
Conclusion: It is proposed that COP as a natural product with an antioxidant capacity in mitigating oxidative injuries in the field of neurodegenerative diseases.

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INTRODUCTION

Neurodegenerative diseases are defined as hereditary and sporadic conditions which are characterized by progressive nervous system dysfunction [1]. Epidemiological evidences showed that the most common neurodegenerative disorders such as Alzheimer's disease, Parkinson's disease, Huntington's disease, multiple sclerosis, stroke and brain trauma in which elevated levels of reactive oxygen species (ROS) and reactive nitrogen species (RNS) might induce severe cell damage through oxidative stress [2,3]. In studies to date, due to the efficacy of antioxidant natured compounds (such as ascorbic acid, isocampneoside II and β amyloid) in preventing oxidative damage in either cultured neuronal cells or in the brains of animals treated with various neurotoxic agents, it was proposed that antioxidants have significant potential therapeutic value as neuroprotective drugs in treatment of many neurodegenerative diseases. Thus, various antioxidants have been used to reduce oxidative stress or damage in cell culture models [4-6].

Alpha-copaene (α-COP, Fig.1.) is a tricyclic sesquiterpene derived from different plants; *Cedrelopsis grevei* leaves [7], *Xylopia Laevigata* [8], *Annona reticulate* [9] and *Ceratitis capitata* [10].

Previous studies α-COP -containing essential oils indicated anticarcinogenic, antioxidant, hepatoprotective and anti-inflammatory activities [11,12]. In addition, the limited numbers of recent investigations have revealed that α-COP possesses an important biological activities, including, anticarcinogenic activity and antioxidant activity [13,14]. However, there were no investigations about its protective effects against oxidative cytotoxicity in neuronal models as a result of its antioxidant property. Therefore, the aim of the present study was first to evaluate the cytotoxic, cytogenetic and oxidative effects of α-COP against H_2O_2-induced neuronal damage using rat cerebral cortex cell cultures to explore their neuroprotective potentials.

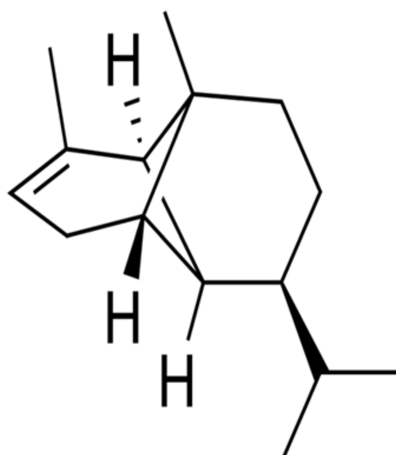

Figure 1. Chemical structure of α-COP

MATERIAL AND METHODS

Chemicals

Alpha-copaene (Cas: 3856-25-5, $C_{15}H_{24}$), Dulbecco modified eagles medium (DMEM), Hank's balanced salt solution (HBSS), neurobasal medium (NBM), sodium phosphate (NaH_2PO_4), potassium phosphate monobasic (KH_2PO_4), ethylenediaminetetraacetic acid (EDTA), phosphate buffer solution (PBS), dimethylsulfoxide (DMSO), Triton-X-100, DNase type 1, Tris, low melting point agarose, normal melting point agarose, ethidium bromide were purchased from Sigma-Aldrich® Germany Hydrogen peroxide was purchased from Merck® Germany. Fetal calf serum (FCS) and trypsin-EDTA were purchased from Biol Ind® Israel. All chemicals were of analytical grade.

Neuron Cell Culture

Primary rat cerebral cortex neuron cultures were prepared using rat fetuses as described previously [15]. A total of nine new-born Sprague-Dawley rats were used in the study. The rats were decapitated by making a cervical fracture in the cervical midline and the cerebral cortex was dissected and removed. The cerebral cortex was placed into 5 ml of HBSS, which had already been placed in a sterile petri dish and macromerotomy was performed with two lancets. This composition was pulled into a syringe and treated at 37°C for 25-30 min as 5 ml HBSS + 2 ml Trypsin-EDTA (% 0.25 trypsin- % 0.02 EDTA) and chemical decomposition was achieved. 8.5-9 µl of DNAse type 1 (120u/ml), was added to this solution and treated for 1-2 minutes, and centrifuged at 800 rpm for 3 min. After having thrown away the supernatant, 31.5 ml of NBM and 3.5 ml fetal calf serum were added to the residue. The single cell which was obtained after physical and chemical decomposition was divided into 3.5 ml samples in each of 10 flasks coated with poly-D-lysine formerly dissolved in PBS. The flasks were left in the incubator including 5% CO_2 at 37°C in the ventile position, then changed with a fresh medium of half of their volumes every 3 days until the cells were branched and reached until a certain maturity. *In vitro* neurotoxicity experiments were performed 8 days later. This study was conducted at the Medical Experimental Research Center in Ataturk University (Erzurum, Turkey). The Ethical Committee of Ataturk University approved the study protocol (B.30.2.ATA.0.23.85-73).

Treatments

Cytoprotective activities of α-COP on 0.5 mM H_2O_2-induced cell injury were investigated by MTT, LDH, TAC, TOS and Comet assays and 8-OH-dG analysis. For determining cytoprotectivity, the cells were seeded into 48-well plate at a density of 5×10^4 cells/well for 16 h and then exposed to medium in the presence of different concentrations of α-COP for 0.5 h before exposure to 0.5 mM H_2O_2 for 6 h. The cytotoxicity and genotoxicity of COP were also investigated. α-COP was dissolved in ethanol and ethanol was evaporated to dryness at ambient temperature. α-COP was applied into cultures at concentrations of 6.25, 12.5, 25, 50 and 100 µg/ml for 24 h. The concentrations were selected according to the works of Togar [13]. Cells incubated without α-COP and H_2O_2 was considered as control group. The cell viability, oxidative alterations and DNA damage analyses were carried out in four totally independent experiments.

MTT Assay

Viability of cells was studied using MTT Cell Proliferation Assay kit (Cayman Chemical Company, Ann Arbor, MI, USA) as described by the manufacturer. After incubation with compounds for 24 h, 10 µL of MTT solution was added to each well and re-incubated for 4 h at 37°C. After washing, the blue formazan was extracted from cells with

isopropanol/formic acid (95:5) and was photometrically determined at 560 nm. The density of formazan formed in control cells was taken as 100% viability.

LDH assay

LDH assay was carried out by the LDH-cytotoxicity assay kit (Cayman Chemical, USA), according to the manufacturer's protocol. A total of 100 µl of cell medium was used for LDH analysis. Released LDH catalyzed the oxidation of lactate to pyruvate with simultaneous reduction of NAD+ to NADH. The rate of NAD+ reduction was measured as an increase in absorbance at 490 nm. The rate of NAD+ reduction was directly proportional to LDH activity in the cell medium.

TAC and TOS analysis

The automated TAC and TOS assays were carried out by commercially available kits (Rel Assay Diagnostics, Turkey) on cell cultures of α-COP-treated cultures for 24 h [16,17].

SCGE assay

After the application of coverslips, the slides were allowed to gel at 4^0C for 30-60 min. The slides were immersed in freshly prepared cold lysing solution with 1% Triton X-100 and 10% DMSO added just before use for a minimum of 1 h at 4 °C) and refrigerated overnight followed by alkali treatment, electrophoresis (at 1.6 V cm for 20 min, 300 mA) and neutralization (0.4 M Tris, pH 7.5). The dried slides were then stained using ethidium bromide (20 µg/ml) after appropriate fixing for 10 min. The whole procedure was carried out in dim light to minimize artifact. DNA damage analysis was performed at a magnification of 100x using a fluorescence microscope (Nicon Eclips E6600, Japan) after coding the slides by one observer (Togar B). A total of 100 cells were screened per slide. A total damage score for each slide was derived by multiplying the number of cells assigned to each grade of damage by the numeric value of the grade and summing over all grades (giving a maximum possible score of 500, corresponding to 100 cells at grade 5).

8-OH-dG assay

8-hydroxy-2'-deoxyguanosine assay kits were purchased from Cayman Chemical® for determining 8-OH-dG levels in the cultures. Since it is a competitive assay that can be used for the quantification of 8-OHdG in homogenates and recognizes both free 8-OHdG and DNA-incorporated 8-OH-dG, many researches are being performed to use this protocol. This assay depends on the competition between 8-OHdG and 8-OHdG-acetylcholinesterase (AChE) conjugate (8-OH-dGTracer) for a limited amount of 8-OHdG monoclonal antibody [18]. All procedures were carried out in accordance with the provider manual.

Statistics

One-way analysis of variance (ANOVA) was used to determine the significant differences between the groups followed by a Dunnett's t-test for multiple comparisons. A probability <0.05 was considered as significant. All analyses were performed using SPSS version 18.0 (SPSS Inc®).

RESULTS

The observed cell viability and cytotoxicity are shown in Figures 2 and 3. Our results indicated that 6.25, 12.5, 25, 50 and 100 µg/ml concentrations of α-COP did not show any significant alterations in cell viability during 24 h as determined by MTT and LDH assays.

Cerebral cortical neurons were exposed to 0.5 mM H_2O_2. A significant reduction was shown in the cell viability and cell proliferation of the exposed neurons at the concentration of 0.5 mM H_2O_2. In fact, in cultures treated with 12.5 and 25 µg/ml of α-COP, H_2O_2-induced neuronal death was significantly reduced (Fig. 4).

H_2O_2-induced neuronal cell death was clearly evidenced by five folds increases in the activity of LDH compared with the observations of untreated controls. 12.5, 25 µg/ml of α-COP significantly blocked the H_2O_2-induced elevation of intracellular LDH release (Fig. 5).

Table 1. *In vitro* levels of TAC and TOS in cultured rat cortical neurons maintained in the presence of α-COP for 24 h.

	α-COP	
Concentrations (µg/ml)	**TAC (Trolox Equiv. / mmol L)**	**TOS (H_2O_2 Equiv. / µmol L)**
Control	28.6 ± 3.0[a]	1.7 ± 0.1[a]
6.25	28.8 ± 3.2[a]	1.7 ± 0.2[a]
12.5	32.0 ± 2.9[b]	1.5 ± 0.2[a]
25	34.6 ± 3.1[c]	1.7 ± 0.2[a]
50	28.4 ± 3.4[a]	1.6 ± 0.3[a]
100	28.0 ± 3.4[a]	1.7 ± 0.3[a]

[a,b,c] $p<0.05$

As shown from the results presented in Table 1, two concentrations of α-COP (12.5 and 25 µg/ml) caused a significant increase of TAC levels on cultured primary rat neurons compared with the controls. Therefore we have determined the most suitable application concentrations as 12.5, 25 µg/ml for investigations dealing with their *in vitro* protective effects in this study. On the other hand, α-COP did not change the TOS levels in cultured neuron cells at all concentrations.

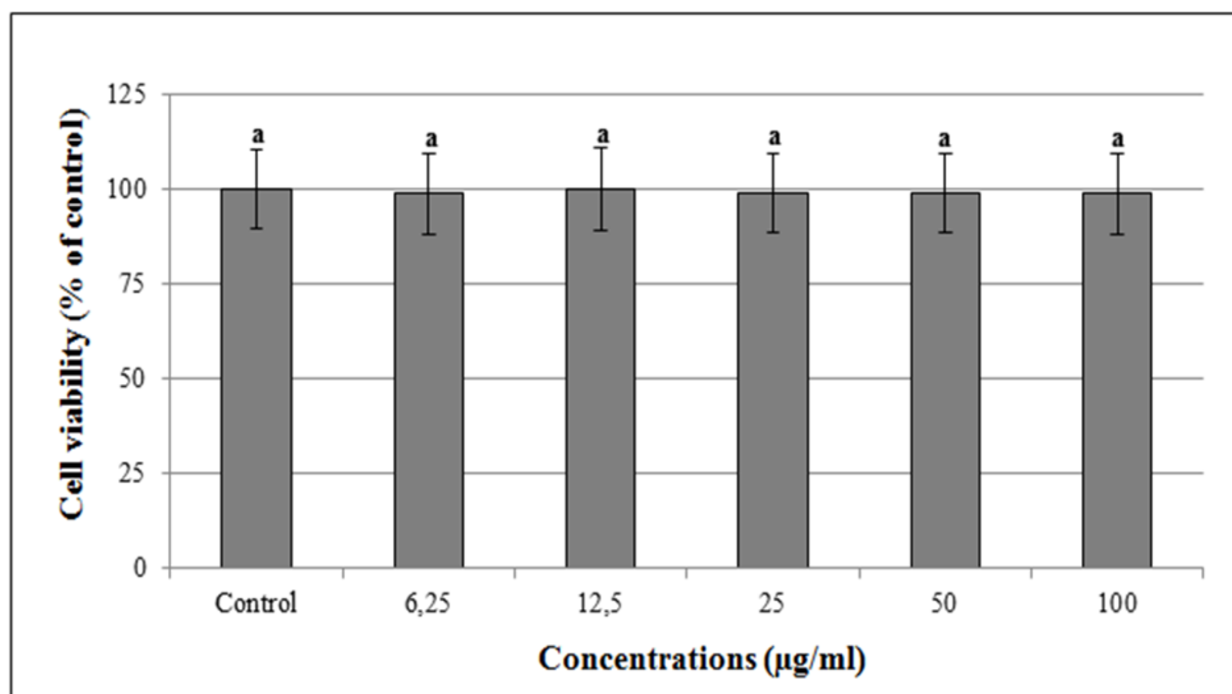

Figure 2. Viability of rat primary cortical neurons after 24 h exposure to α-COP

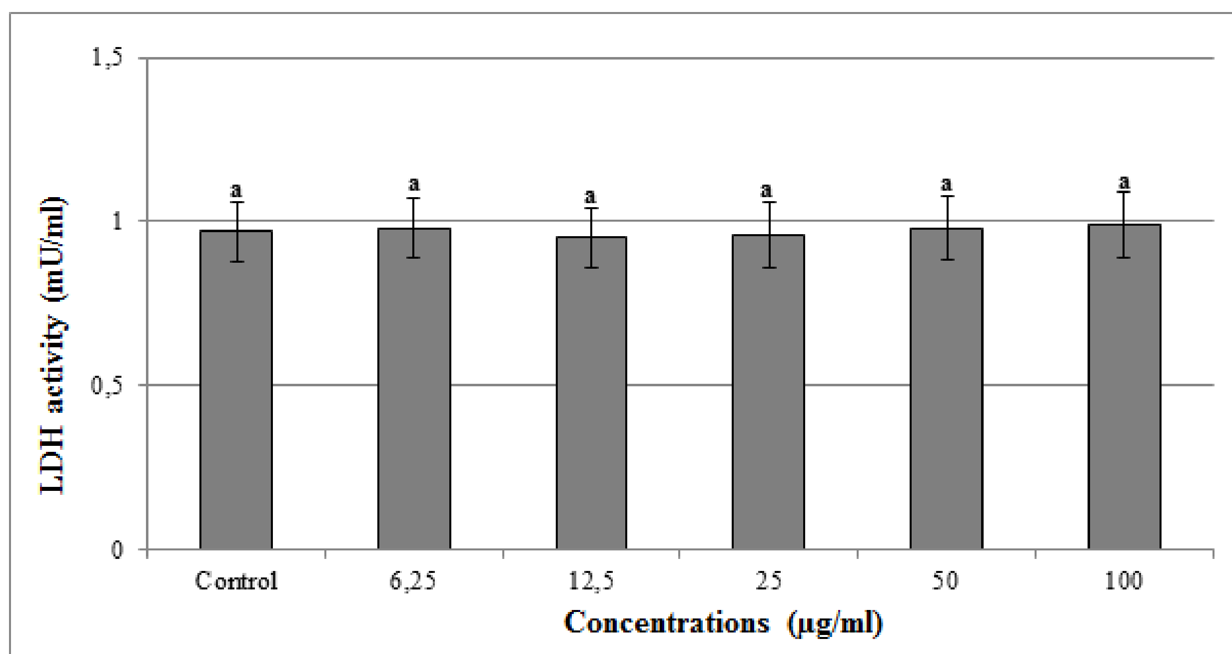

Figure 3. Extracellular level of LDH in cultured rat cortical neurons maintained in the presence of α-COP for 24 h. The abbreviations are as in Figure 2. Values are expressed as mean+SD for four cultures in each group. The bars are shown by different letter are significantly different from each other at a level of 5 %.

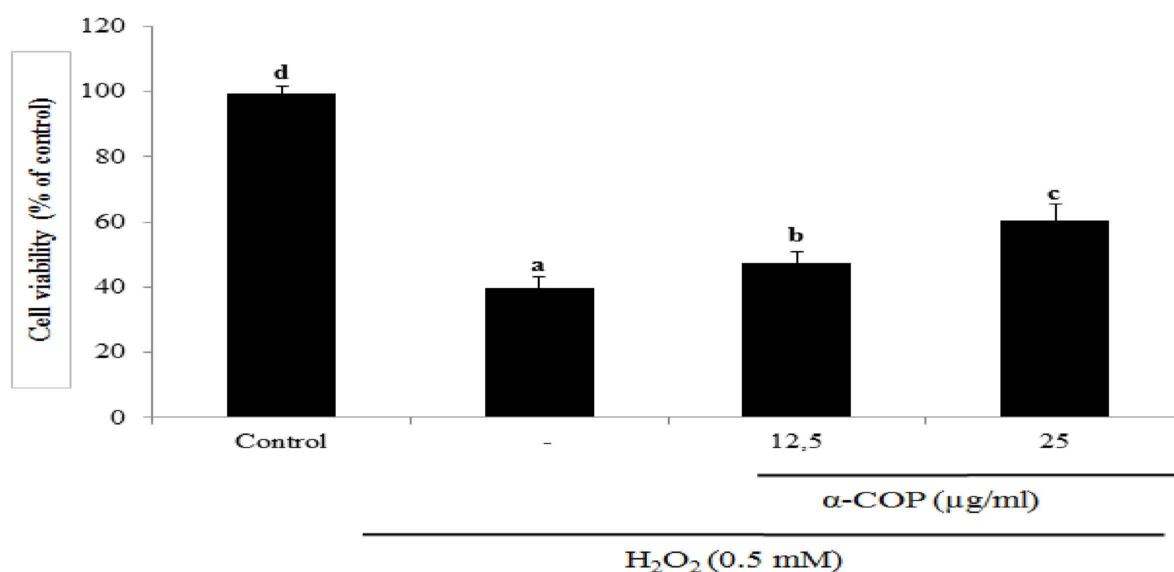

Figure 4. The effects of α-COP applications on cell viability against H_2O_2-induced cell death.

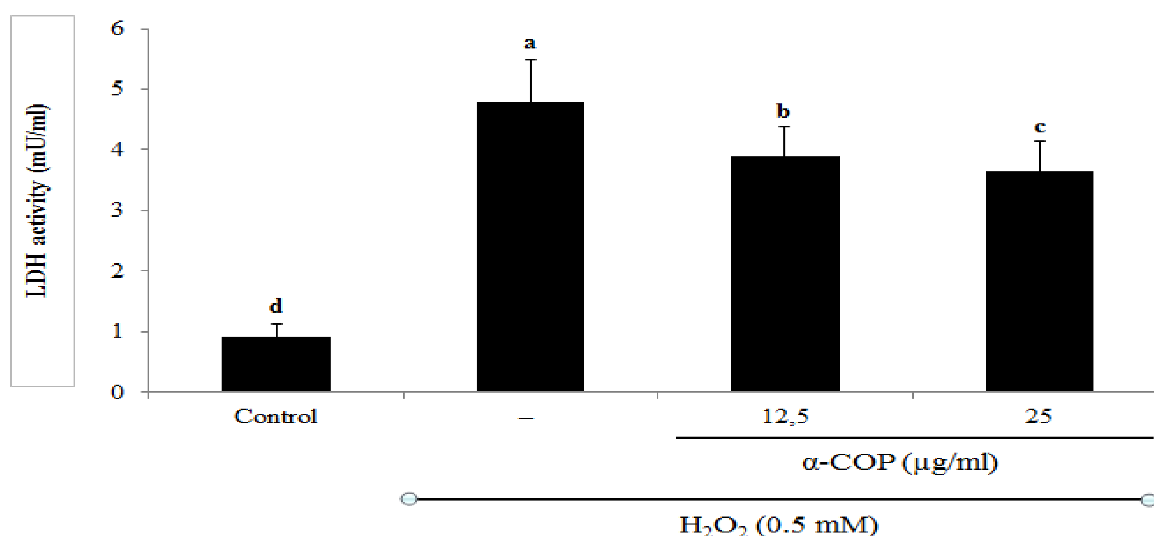

Figure 5. The effects of α-COP applications on H_2O_2-induced LDH. The abbreviations are as in Figure 4. The cells were pretreated with different α-COP concentrations for 30 min before exposure to 0.5 mM H_2O_2 for 6h. Values are expressed as mean+SD for four cultures in each group. The bars are shown by different letter are significantly different from each other at a level of 5 %.

Results reveal that exposure to H_2O_2 for 6 h resulted in significant decreases in TAC levels as compared to untreated group. The addition of H_2O_2 for 6 h led to a significant elevation in the level of TOS as compared to the control group (Table 2). Results of the present study demonstrate that level of TAC was significantly increased in cortical neurons pre-treated with α-COP as compared to those of H_2O_2-intoxicated cultures. Also, pre-exposure to α-COP led to a significant augmentation in the levels of TOS as compared to those of H_2O_2-intoxicated cultures (Table 2).

Table 3 shows the total damage score obtained in cultures treated with α-COP. α-COP at all tested concentrations did not alter the total damage scores as compared to the control group. However, DNA damage was significantly increased by H_2O_2-intoxication at 0.5 mM for 6 h when compared to the untreated group. Furthermore, α-COP pre-treatments significantly reduced DNA damage in cultures treated with the two concentrations of the compound (12.5, 25 µg/ml) plus H_2O_2 when compared to treatment with H_2O_2 alone.

Table 2. The effects of α-COP applications on oxidative alterations by H_2O_2 *in vitro*. The abbreviations are as in Table 1.

Treatments	α-COP	
	TAC level	TOS level
Control	28.6 ± 3.0[d]	1.7 ± 0.1[a]
H_2O_2 (0.5 mM)	13.6 ± 2.5[a]	4.8 ± 0.4[d]
12.5 µg/ml + H_2O_2	15.5 ± 2.7[b]	3.0 ± 0.3[b]
25 µg/ml + H_2O_2	17.8 ± 2.4[c]	2.2 ± 0.3[c]

Table 3. Total DNA damage score (Comet assay) and the levels of 8-OH-dG adducts (as pg/ml) in cultured rat cortical neurons maintained in the presence of different α-COP concentrations for 24 h. The abbreviations are as in Table 1.

Concentrations (µg/ml)	α-COP	
	Total DNA damage score	8-OH-dG level
Control	35.4 ± 5.3[a]	0.9 ± 0.1[a]
6.25	36.3 ± 5.1[a]	0.9 ± 0.2[a]
12.5	36.0 ± 5.4[a]	1.0 ± 0.1[a]
25	35.9 ± 5.5[a]	1.1 ± 0.3[a]
50	37.3 ± 4.6[a]	1.0 ± 0.1[a]
100	37.8 ± 5.1[a]	1.0 ± 0.2[a]

The levels of 8-OH-dG, a hallmark of oxidative stress-DNA base damage, were measured using an 8-OH-dG detection kit. There were no significant difference between the intracellular levels of 8-OH-dG in the control and all α-COP treated groups. On the contrary, the intracellular level of 8-OH-dG was significantly higher in H_2O_2-treated cultures in comparison with untreated cultures. But pre-treatment of α-COP decreased the 8-OH-dG formations which were increased by H_2O_2.

Table 4. The effect of α-COP pretreatments on DNA damage and 8-OH-dG levels (as pg/ml) generated by H_2O_2 treatment. The abbreviations are as in Table 1.

Treatments	α-COP	
	Total DNA damage score	8-OH-dG level
Control	35.4 ± 5.3[a]	0.9 ± 0.1[a]
H_2O_2 (0.5 mM)	196.3 ± 21.4[d]	4.3 ± 0.3[c]
12.5 µg/ml + H_2O_2	174.5 ± 24.5[c]	3.5 ± 0.3[bc]
25 µg/ml + H_2O_2	143.2 ± 26.8[b]	2.8 ± 0.3[b]

DISCUSSION

Neurons are in a perpetual state of oxidative stress, and this imbalance may lead to reduced levels of endogenous antioxidants. The increases in oxidative stress together with the decline in endogenous antioxidants are important underlying risk factors for older people to develop [19]. Accordingly, searching for neuroprotective drugs of natural origin against oxidative stress-induced neuronal death has thereby attracted increasing research interests. In particular our investigation has been focused on cortex cells since they were evaluated as vulnerable to Alzheimer's and Parkinson's pathologies via oxidative stress [20,21]. In comparison to more mature neurons in culture, immature neurons are particularly susceptible to cell damage induced by oxidative stress, for example, by H_2O_2 [22]. Thus, primary cultured cortical neurons are commonly used as a suitable *in vitro* model system for protection against H_2O_2- induced cellular damages.

MTT and LDH leakage assays, non-radioactive, fast and economical assays, are widely used to quantify cell viability and cytotoxicity [23]. In this study, the cultured primary rat neuron cells exposed to 6.25, 12.5, 25, 50 and100 µg/ml concentrations of α-COP did not show any significant changes in cell viability during 24 hours as determined by MTT and LDH assays (Fig 2, Fig 3). On the other hand, the cytotoxic effects of H_2O_2 on cultured rat cortical neuron cells were demonstrated by its strong inhibition on cell viability (Fig. 4) and elevated LDH leakage (Fig. 5). Our findings also revealed that α-COP pre-treatments reduced the cytotoxicity in a clearly dose-dependent manner. These results show that α-COP is capable of reducing H_2O_2-induced cytotoxicity and lipid peroxidation. Our findings were in line with previous reports. Tang et al. [24] demonstrated that huperzine A showed protective activity against H_2O_2-induced damage in SHSY5Y cells. In addition, linderagalactone E (5), linderane, hydroxylindestenolide, and linderalactone showed hepatoprotective activity against H_2O_2-induced oxidative damages on HepG2 cells [25]. Likewise α-COP showed hepatoprotective activity by reducing the malonaldehyde levels in liver homogenates from Wistar rats [11]. Moreover, Turkez et al. [26] found that α-FNS and β-FNS, naturel sesquiterpenic compounds, were capable of protecting against H_2O_2-induced cytotoxicity and oxidative DNA damage in cultured rat primary cortical neuron cells.

To explore the mechanism of the *in vitro* protective effect of α-COP on H_2O_2-induced oxidative damage in cortical neurons, TAS and TOS levels were also analyzed. As a matter of fact, TAC and TOS rapid and reliable automated colorimetric assay are frequently used to determine the oxidative alterations [27]. The results in Table 1 show that α-COP applications increased TAC levels of the cells without any alterations in TOS levels as compared to untreated cultures. The data further show that α-COP -treated

cells displayed modulating effect on H_2O_2-induced abnormalities in oxidative metabolism (Table 2). Although neurons were highly sensitive to H_2O_2, α-COP successfully protected cultured neurons from H_2O_2-induced oxidative stress. However recent studies suggested several mechanisms on not for the tested α-COP sesquiterpene compound but other natural antioxidants-mediated neuroprotection (such as sesamin, sesamolin, shenqi-wan, puerarin, agmatine and ganglioside) against H_2O_2 intoxication attributed to be via modulation of activity of tyrosine kinase of Trk receptors [28], activation of the PI3K/Akt signalling pathway [29], regulation of apoptosis by c-Myv Bcl-2 and Bax gene expressions [30] alpha 2-adrenergic receptor signalling pathway [31] inhibition of n-methyl-d-aspartic acid (NMDA) receptor [32], ttenuation of mitogen-activated protein kinases (MAPKs) and caspase-3 [33].

Comet test, oxidative DNA damage was also evaluated in cell cultures by measuring the 8-OH-dG level, since, in particular, this marker is most frequently measured as an indicator of oxidative DNA damage in neuronal degeneration models both *in vitro* and *in vivo* [34]. In this study, α-COP is neither genotoxic nor mutagenic on neuron cells using the comet and 8-OH-dG assays since the observed mean values of the total scores of cells showing DNA damage was not significantly different from control values (Table 3).

However, we observed significant elevation in 8-OH-dG release from neurons was observed after treatment with H_2O_2; in contrast, there was a significant decrease when we pre-treated with α-COP concentrations (Table 4). Our results indicated that α-COP treatment mitigated the genotoxicity induced by H_2O_2 in cortical neuron cells.

Collectively, data of this study provide direct evidence that α-COP is a potential antioxidant agent, which possesses good *in vitro* neuroprotective activity. But, further studies will be needed to clarify the mechanisms involved.

CONFLICT OF INTEREST

The authors declare that there are no conflicts of interest.

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Original Research

Nutritional and physicochemical profiles of some indigenous extracts used in alternative medicine

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Keywords: Alternative medicine, Aloe vera, Alma millsoni, Ganoderma and Archachatina maginata, Healthcare

Abstract
Background: Numerous drugs have entered the international market through exploration of ethnopharmacology and traditional medicine.
Aim: The need to document the beliefs and practice of our traditional healers, the wholesomeness and safety of some of indigenous extracts used in alternative medicine prompted this study.
Methods: Survey carried out between February 2011 and December 2011 in Ondo State and part of Oyo State on the usefulness of some local sources for alternative medicine showed that the people have strong belief in traditional medicine. A total of 200 respondents contacted filled-out the questionnaires.
Result: Majority of the studied population lived in the villages and semi urban areas of the States and within age range of 30 - 60. *Aloe vera, Alma millsoni, Ganoderma lucidum* and *Archachatina maginata* were reported to be useful for treatment of various ailments including labour pain, hypertension and diabetic. Though not without pockets of complications and mode of action not understood. Freshly prepared juice is safe and nutritious based on physicochemical profile but as storage progress at room temperature, bacterial contamination could be inimical to humans.
Conclusion: Therefore, careful thought must be made on the pros and coins of the effects of the local sources in alternative medicine or healthcare services. Governments at all level should encourage collaboration of western medicine and traditional medicine perhaps; there could be a way forward in treatment of problematic cases like HIV and cancer that have defiled current medical treatments.

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INTRODUCTION

Many medical therapies that are considered alternative in the United States are actually conventional medicine in other countries. For instance, Ayurvedic medicine is the indigenous, traditional medicine of India [1], Herbalism in Africa; in Nigeria, traditional medical care practitioners include the Wombi of the Hausa armies, a form of military Red Cross: the Barber-Surgeons or Gozan of the Nupe people: the Adahunse or Onisegun of the Yoruba and the Didia of the Igbos [2]. Naturopathy is a clinical specialty that emphasizes the use of natural product in both prevention and treatment of disease. Natural products extracts of therapeutic relevance are of paramount importance as reservoirs of structural and chemical diversity. A recent review on national pharmacopoeias from several countries reveals at least 120 distinct chemical substances from different plants that have utility as lifesaving drugs [3]. This has been achieved through chemical and pharmacological screening of only 6% of the total plant species. Untapped, hidden wealth in the flora needs to be unearthed and explored to cure diseases like AIDS, cancer, diabetes, etc [1, 3]. Pharmaceutical companies have renewed their strategies in favor of natural product drug development and discovery [4]. In fact, given the populations of China and India, the vast majority of people in the

world receive their medical care by interventions considered alternative in the United States. Moreover, the principle of homeopathy "like cures like" [5] should be the basis for science to go back to nature and seek solution to the ravaging and dilapidating effects of some chronic human diseases such as HIV (AIDS) and cancer that conventional medicine are unable to provide an effective treatment for.

Numerous drugs have entered the international market through exploration of ethnopharmacology and traditional medicine. Progress in genomics and proteomics has opened new gateways in therapeutics and drug discovery and development. Screening of different plants for novel anticancer compounds is also in progress with reference experiential data from traditional systems [6]. Botanical immune-drugs from traditional medicine can provide newer opportunities to bio-prospect diverse and synergistic chemical moieties, which in combination might act on multiple targets and improve the therapeutic spectrum [7]. Although, chromatographic techniques and chemical marker assisted characterization of the botanicals does not ensure consistent biological activity or stability [8]. Therefore, production of quality botanical medicines has become a challenge to regulatory authorities, scientific organizations and manufacturers. World Health Organization [9], USFDA [10], European Scientific Cooperative on Phytomedicine (ESCOP) [11] have published standard sets of guidelines to address the concerns. However, the increasing use of traditional therapies, demands more scientifically sound evidence for the principles behind therapies and for effectiveness of medicines.

In spite of the unevenness within the field of alternative medicine - from therapies that scientific studies had shown to hold great promise to unproved and possibly harmful remedies - the future looks bright for the continued growth of alternative medicine. The factors shaping its evolution, including the costs of high-tech, modern medicine and changing consumer philosophies, suggest that it is not a passing trend. Thus, the need to document the beliefs and practices of our native traditional medical care practitioner, the wholesomeness and safety of some of our ethno-botanic and bio-resources became necessary. This paper also aims at providing information on (African trado-medical practices) to the ongoing debate over alternative medicine in the United States.

MATERIALS AND METHODS

Ethno botanic and bio-source survey

Survey was carried out around Ondo State and Ibadan (part of Oyo State) of Nigeria between February 2011 and December 2011 by the use of questionnaire and interviewing herbal sellers and traditional medical care practitioners on the usefulness of *Aloe vera*, *A. maginata* serum, earthworm (*Alma millsoni*) and lingzhi- mushroom (*Ganoderma lucidum*) in ethno medicine in their communities and ways of preparing the extracts for used. A total of 200 respondents contacted, who gave their consent before filling the questionnaire were local people aged (30- 60 yrs) and reside in the villages and semi urban areas of the States with no standard educational background, their main occupation is farming, most of the females recruited hawk herbal product called "Agbo" around the villages and motor parks and market places.

Alma millsoni (ALM) extract

Samples of *Alma millsoni* was sourced from riverside of Okitipupa, Ondo State, washed with rain water and transported in a clean plastic bucket with sand and water to the laboratory for processing. The Alma tonic extraction was carried out according to the method described by Ang Lopez and Raelm [12].

Aloe vera (AV) extract

Samples of homegrown succulent leaves of aloe plant were harvested and washed with distilled water. Samples deposited in Biological Science Department, Achievers University, Owo, were identified and authenticated. *Aloe vera* juice was prepared following the method described by Wu *et al.* [13] with slight modification. The modification briefly stated included the following steps: Freshly harvested leaves of the plant were washed, drained and cut open. The inner pulp was scrapped into a clean beaker and warmed at 50 ^{o}C for 30 min. until the viscous light - yellow pulp became less viscous. The extract was filtered with a muslin cloth.

Ganoderma lucidum (GL) extract

Fruiting bodies of *G. lucidum* were obtained locally from open forest at Ipele in Ose Local Government Area of Ondo State, Nigeria. The fungal material was identified and authenticated in the Department of Biological Sciences, Joseph Ayo Babalola University, Ikeji-Arakeji, Osun State, Nigeria, where voucher specimen number 1103 has been deposited. Ganoderma extract was prepared using aqueous extraction method as described by Oluba *et al.* [14].

Archachatina maginata (AM) extract

The homemade giant land snails were transported to laboratory for processing. Samples deposited in Zoology Laboratory, Federal University of Agriculture, Abeokuta where it was identified and authenticated. The snails were washed thoroughly and rinsed with distilled water. With the aid of giant forceps, the shell were carefully cracked to access the sexual apparatus

and tapped out juice/fluid from the albumin gland into beaker. The fluid was then centrifuged at 5000 rpm for 10 min. thereby yielding supernatant, precipitated and gelatinous sludge. The bluish supernatant was carefully extracted into conical flask.

Bacteria isolation from the brews

Hygienically prepared samples of the four extracts were sent to Microbiology and Parasitology Laboratory Unit, Department of Medical Laboratory Science, Achievers University, Owo. All solid and liquid media used were products of Biotec Limited (code MM 1008s). A loop-full of each of the extracts was initially plated onto two 5% blood agar plates, one Cystine Lactose Electrolyte Deficient (CLED) agar plate, one Staphylococcus/Streptococcus selective agar plate and a section of Sabouraud agar. One of the blood agar plates was then incubated under anaerobic conditions (CO_2 jar) and the other four plates were incubated in air, at 37^0C for a maximum of 24 hrs. The plates were then examined and colonies types were identified. Further basic identification tests such as Gram staining reaction, oxidase reaction, indole test, motility and coagulase were carried out to confirm the growth. One (1.0 ml) of each of the extracts was inoculated on Potatoes Dextrose agar and nutrient agar to determine total bacteria count using double layer method as described by Baker and Breach [15].

Determination of moisture content

Thermal drying method was used in the determination of moisture content of the *G. lucidum*, *Aloe vare*, *A. millsoni* extract and *A. maginata serum* samples. One gram of dried sample was weighed in triplicate and placed in washed, dried and weighed crucible. This was placed in an oven and dried at 105^0C for 3 hr. The sample was allowed to cool in a desiccator and then re-weighed. The percentage moisture content was calculated

$$\% \text{ Moisture Content} = \frac{W_f}{Wi} \times 100$$

Where: W_f = Loss in weight on drying (g) (i.e. initial weight of sample – final weight of sample after drying to constant weight. W_i = initial weight of sample (g)

Titrate-able Acid (Acid-base titration)

The titration was carried out as described by Baker *et al*. [16]. At the equivalence point, the ions in solution are the same as if the pure salt of the base and acid had been dissolved, and the resultant pH is dependent on the extent to which the salt is hydrolyzed.

Plate 1. Giant land snails (*Archachatina maginata*)

Plate 2. *Aloe vera* plant

Plate 3. Earthworm (A. millsoni)

Plate 4. Lingzhi mushroom (*Ganoderma lucidum)*

Statistical Analysis

Results presented are means ± SEM of eight independent determinations. Results obtained from this study were statistically analyzed using one way analysis of variance (ANOVA). Significant differences between the treatment means were determined at 95% confidence level.

RESULTS

Ethno-botanic and bio-sources survey

From interaction with the studied population, it was establish that the extracts were prepared and administered to their clients under unhygienic conditions. Most of the traditional doctors claim that it was handed over to them from ancestor and had strong believe in it efficacy when preparation protocol are followed.

***Aloe vera* juice** are used for the treatment of ailments such as stomach pain, hyper level of cholesterol, hypertension, blood infection of unknown origin, wound healing and insomnia.

Ganoderma brew are use for the treatment of ailments such as hypertension, diabetes, hepatitis, stroke, cancer and nervous disorders.

Millsoni tonic are used for the treatment of ailments such as: viral infection - measles, labour pain - aid for faster contraction of the uterus and make birthing easier, stomach aches and bone problems.

***A. maginata* serum** are used for the treatment of ailments such as anemia, high blood pressure and other fat related ailments, reduce hemorrhoids and constipation, treatment of poor eye-sights, prevent heart problems and as suppressant in stroke treatment.

Physiochemical and Nutritional properties of AV, AM serum, ALM and GL, are shown on Table 1. Physicochemical and nutritional parameters were determined using standard methods as indicated on the table, water content of ALM (70%), AM serum (97%) and AV (97%) were very high except for fruiting bodies of Ganoderma which is naturally dry. The pH of ALM and AM serum were slightly alkaline 8.1 and 8.5 respectively, other species of earthworm encountered in the course of study had different pH, while AV and GL were slightly acidic between 5.0 and 6.2. It was observed in the course of this study that pH of AV varies with season. Titrate-able acidity (mol / dm^3) was higher in AV than GL and obviously not applicable to ALM and AM serum, dude to the pH which was slightly alkaline. Glucose a by- product of carbohydrate though detected in the four extracts, the concentration was higher in AV followed by ALM and least in AM serum. Cholesterol (lower density cholesterol (LDL), High density Cholesterol (HDL) and triglyceride) were detected in AM serum and ALM tonic but at a very low concentration when compared with cholesterol of red meat, it was completely absent in AV and GL which make them safer for consumption. Protein content of ALM and AM serum was high, a lower value was detected in AV and it was completely absent in GL (Figure 1). Extracts of ALM and GL stored at room temperature for 12 hr yielded bacteria count (<20 cfu / ml) as mixed growth of *E. coli* and *Staphylococcus* spp. AM serum yielded count (< 20 cfu / ml) of *Staphylococcus* spp. no yeast cell was isolated from any of these extracts and AV yielded no growth of microorganism.

Table 1. Physiochemical and Nutritional properties of *A. vera*, *A. maginata* serum, *Alma millsoni*

Parameters	*A. millsoni*	*maginata*	*Aloe vera*	*G. lucidum*	Method
Cultivation method	Natural	Natural	Natural	Natural	No chemical applied
Colour of raw extract	Brownish	Bluish	Yellowish	Brownish	
Titrate-able acidity (mol/dm^3)	N.A	N.A	5.0×10^{-6}	1.25×10^{-6}	Using 0.01N of sodium hydroxide
Appearance of dried extract	Brownish oily cake	Light brown crystal	Greenish powder	Brownish powder	
Moisture content of extract	< 7%	< 5%	< 8%	Negligible	Oven heat drying at 105^0C
Triglycerides (mmol/L)	2.4	0.9	Absent	Absent	
HDL (mmol/L)	1.1	0.1	Absent	Absent	
Aroma of dried powder	Bear meat	Fresh	Characteristic	Odourless	
Bacteria isolate Bacteria count (cfu/ml)	*E. coli, Staph.* spp.& no fungi growth (>20 cfu/ml)	*E. coli* and no fungi growth (>20 cfu/ml)	no growth	*E. coli* and *Staph.* spp no fungi growth (>20 cfu/ml)	1.0 ml pure extract (neat) on nutrient agar and PDS agar 12hr after storage at room temperature

TP= total protein, HDL=high density cholesterol, (Cholesterol was determined using Liebermenni Burchard method [17])

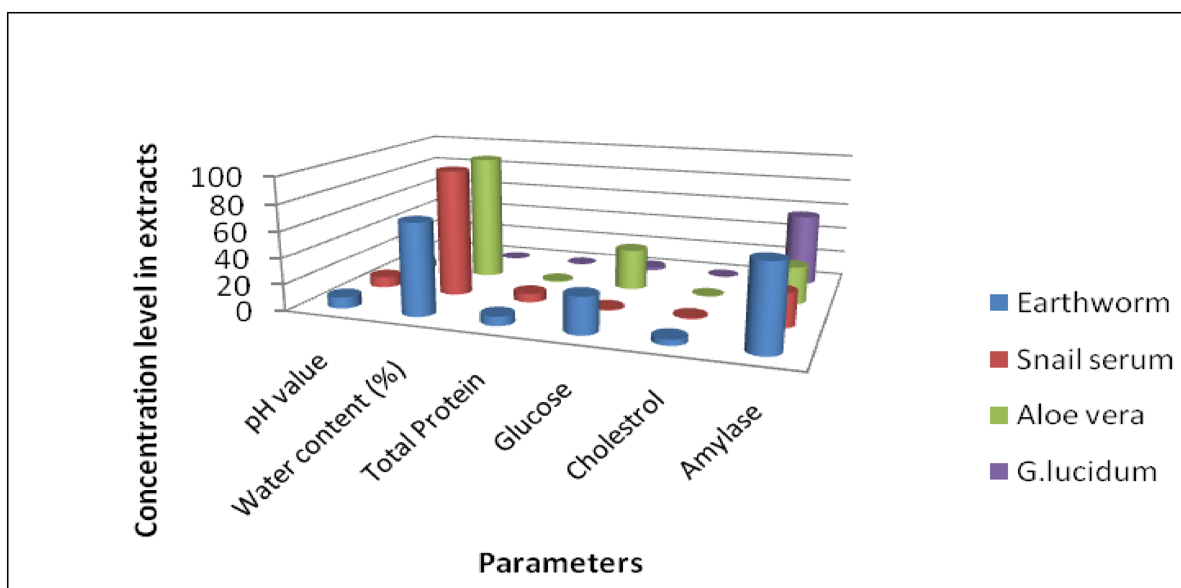

Figure 1. Concentration of Glucose, Protein, water content, pH Value, Amylase and cholesterol of *Aloe vera, A. millsoni Ganoderma lucidum and A. maginata*

DISCUSSION

In addition to the medical practices employed by traditional medical care practitioners, they have also evolved a profusion of folk healing practices that are not seen by either the folk healer or modern health practitioner. These home healing practices are handed down by word of mouth and are used to treat a variety of illnesses, including anxiety and depression, coughs and colds, burns and sunburns, bladder and kidney infections, bedwetting, bites and stings, asthma, arthritis, birthing problems, bleeding, diarrhoea, fever, infertility, insomnia, skin problems, and mouth and gum disorders. Almost every family has some specific home remedy that has been learned and passed down by older family members, and even in the face of more modern health beliefs they will continue to use these folk remedies before seeking for other western medical treatment methods.

For instance, in many families aloe leaf is used to treat burn, rather than a burn ointment or spray from the pharmacy. This finding on the use of *A. vera* among the studied groups supported the fact the plant juice have been used for various purposes from time past with valid result though not without pockets of complications and, do not understand the mode of action. However, according to Boudreau and Beland [18] the *Aloe barbadensis* (Miller) and *AV* has a long history of use as a topical and oral therapeutic. The plant is the source of two products; gel and latex, which are obtained from its fleshy leaves. *A. vera* products contain multiple constituents with potential biological and toxicological activities, yet the active components elude definition. Ingestion of *A. vera* has be associated with diarrhoea, electrolyte imbalance, kidney dysfunction, and conventional drug interactions; episodes of contact dermatitis, erythema, and photo-toxicity have been reported from topical applications [18]. However, it calls for caution among native user. Recently, the use of alternative treatment including *A. vera* and medicinal plant (extract) has been reported for the controlling avian coccidiosis [19 – 21].

The finding from the studied population indicates that the knowledge and use of *AM* serum or venom is popular and it is belief to be effective in their application with quick healing power. Land helix, or snail, has been used in medicine since antiquity and prepared according to several formulations. The finding is in tandem with the historical report of Bruno [22], who traced the understanding of *AM* properties from the time of Hippocrates to the 18th century, when various *AM* “preparations” were also recommended for external use with dermatological disorders and internally for symptoms associated with tuberculosis and nephritis. He also noted that 19th century saw a renewed interest in the pharmaceutical and medical use of snail venom with numerous indications for *AM* preparations. Not until recently, when U. S. Food Drug Administration (FDA) has also shown an interest in snails. Ziconotide (SNXIII), a synthetic peptide coming from *AM* venom, has been under FDA review since 1999. Pre-clinical and clinical studies of this new drug are promising [23].

Meanwhile, the wound healing property of *AM* mucin has been reported [24]. Similarly, its physiological and toxicological properties have been documented [25]. The use of the mucin in a muco-adhesive gel preparation and when fortified with honey and its effect on wound has also been reported [24].

Studies from other researchers have suggested that herbs such as shiitake and lingzhi (mushroom) can indeed stimulate the immune system [26], though the mode of action is complex and difficult to characterize. The report gathered from the studied population of trado-medical practitioners and herb sellers on the use of *GL* appear to be real, based on their claims and the report of the people in the communities especially the artisans that patronize them. However, there is therefore, the need for proper identification of the non-venomous type of the mushroom. The intake of the killer types can lead to untimely death, which calls for caution in it use among the populace. However, the edible type could be relied for health conditions such as hypertension and insomnia; again it should be used with caution, though there is no evidence of toxicity on tissues of laboratory rats as revealed by works of earlier scientists. Oluba *et al.* [14] reported that there was no significant difference in the absolute and relative weight of liver, heart, kidney, spleen of rats supplemented with 200 mg of Ganoderma for six weeks and the normal control rats.

The information gathered suggested that majority of the populace have the knowledge of it use and possibly have used but do not know the immunological and medical rational of it action in the system. Based on the claims of the trado-medical practitioner and communities studied, bioactive compounds for Pharmaceuticals to produce life saving medicines may be present in our native earthworms. This is consistent with the news for the world vermitech scientists who appeared in Philippines "News Today" on November 25, 2005 telling earthworm (Lumbricus) can help dissolve blood clots for stroke patients" [12]. In the last 30 years, a number of earthworm "clot-dissolving", "lytic" and "immune boosting" compounds have been isolated and tested clinically [27]. Current researches made in Canada, China, Japan and other countries on the identification, isolation and synthesis of some "bioactive compounds" from earthworm (Lumbricus and *Eisenia fetida*) with potential medicinal values for treatment of heart diseases have brought revolution in the vermiculture studies [28].

Oral intake of Alma tonic, *A. vera* jucie, Ganoderma brew and *AM* serum is safe, the pH of the extracts and titrate-able acid are low and within tolerable range. More so, lipid profile show that the cholesterol; triglyceride, HDL and LDL content are moderately low. Earthworm and *AM* serum are reach in protein, that makes the tonic nutritious and, freshly prepared tonic from these local sources may be used by protein deficiency individual to build-up proteins in the body especially immunocompromised and immunodeficient and elderly, since the cholesterol level is low and may pose little or no threat to the system, especially on vital organs such as the heart. Glucose content of *A. vera* juice was very high, it may be used for energy boost as well.

The bacterial count was significantly low, moreover, *Salmonella* spp., *Shigella* spp. and *Candida* spp. which are of more clinical significance were not isolated from the extracts but as storage progress bacteria and fungi contamination in the extracts could be inimical to human health. These extracts (*ALM*, *AM* serum and *GL*) may be used as probiotics or enrichment culture medium, since the brews supported bacteria growth (*Staphylococcus aureus* and *E. coli*). The sterility of *A. vera* suggest that it may possess antimicrobial potentials, however, the acidic pH may have influenced this action, further research are recommended to verify this observation. Therefore, careful thought should be made regarding the pros and coins of the effects of the extracts, when considering it for use in any case, as the mode of action of these extracts remains unknown and the claims of the communities studied have not been scientifically proven.

In support of World Health Organization, there is need to promote the use of traditional medicine for healthcare. Government should set up a board of council to facilitate the co-existing and co-practicing of all health service providers (both traditional and western doctors), the collaboration aims would be: Support and integrate traditional medicine into national health systems in combination with national policy and regulation for products, practices and providers to ensure safety and quality; ensure the use of safe, effective and quality products and practices, based on available evidence; Acknowledge traditional medicine as part of primary health care, to increase access to care and preserve knowledge and resources; and ensure patient safety by upgrading the skills and knowledge of traditional medical care practitioners.

CONCLUSION

Studied population revealed that the people have the knowledge of the practices of the trado-medical care practitioner and herbal seller on the uses of earthworms, *Aloe vera*, *A. maginata* serum and Ganoderma in the preparation of concoctions for various purposes. They belief herbs made from local

sources are more effective and opt for their use as first and last option. Even when they are placed on medication by Physicians, they often complement such treatment with herbal concoctions for effective treatment. The intake of the studied extracts is safe and nutritious when protein content, glucose level, cholesterol (triglyceride, LDL and HDL), amylase and pH of earthworm, *AV*, *AM* serum and *GL* are considered.

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CONFLICT OF INTERESTS

The authors do not have a direct financial relationship with the commercial identity mentioned in this paper.

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Original Research

Evaluation of the acute toxicity, phytochemical constituents and anti - ulcer properties of methanolic leaf extract of *Annona muricata* in mice

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Keywords: Acute, anti - ulcer, Annona muricata, cimetidine

Summary

Aim: This study investigated the acute toxicity, phytochemical constituents and anti - ulcer properties of methanolic leaf extract of *Annona muricata* in mice.
Method: The anti - ulcer activity was evaluated using absolute ethanol-induced ulcer and aspirin-induced ulcer models in mice. An LD_{50} of 354.8 ± 8 mg/kg body weight, bw of the extract was obtained on oral administration. Investigation of the phytochemical constituents of the plant extract revealed the presence of saponins, alkaloids and traces of tannins.
Results: All doses of the extract (50, 75 and 100 mg/kg) used for the study significantly reduced ($p<0.05$) the mean number of ulcers in both ulcer models when compared to the untreated group A (10 ml/kg distil water). Optimum antiulcer activity of the extract against absolute ethanol-induced ulcer was noted at 50 mg/kg bw. At this 50 mg/kg, the mean number of ulcers and mean ulcer index of the extract was significantly lower ($p<0.05$) than that of Cimetidine at 100 mg/kg (3.60 ± 0.51: 5.00 ± 0.32; 1.5±0.05: 0.98±0.03), the treated control group whereas the protective index of the extract was higher than that of cimetidine (50.51 %: 24.24 %).
Conclusion: The results obtained from this study strongly suggest that methanolic leaf extract of *Annona muricata* can be effectively used for the treatment of ulcer in low doses and can provide better therapeutic effect than cimetidine if used in ulcers caused by alcoholism and related agents.

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INTRODUCTION

Gastric ulcer is a breach in the mucosa of the stomach that extends through the muscularis mucosa into the sub-mucosa or deeper [1]. It is contrasted to erosions, in which there is loss of the superficial epithelium of the mucosa. Although ulcers may occur anywhere in the alimentary tract, none are as prevalent as the peptic ulcers that occur in the stomach and duodenum. Peptic ulcers are chronic, most often solitary lesions that occur in any portion of the gastrointestinal tract that are exposed to the aggressive action of acid-peptic juices. Peptic ulcer disease is a common illness, affecting about 10% of men and 5% of women during their lifetimes [2]. Common causes of peptic ulcer are factors that increase gastric acid production or impair mucosal barrier protection, such as use of salicylates and other non - steroidal anti-inflammatory drugs (NSAIDs), tobacco smoking, *Helicobacter pylori* infection of the upper gastrointestinal tract, pathologic hypersecretory disorders, consumption of alcohol and coffee, and severe physiological stress [2]. Management of gastric ulceration is aimed at treatment of the primary cause (if identified), inhibition of gastric acid secretion, and, if necessary, control of gastric haemorrhages [3]. Drugs utilized in the treatment of ulcers include: (a) Antacids – Examples are aluminum

hydroxide gel, magnesium hydroxide, sodium bicarbonate, magnesium trisilicate [3]. (b) H_2-receptor antagonists – Examples are cimetidine, ranitidine, famotidine, nizatidine [4]. (c) Proton pump inhibitors – Examples are omeprazole, pantoprazole, lansoprazole, rabeprazole, esomeprazole [4]. (d) M_1-receptor antagonists – Example is pirenzipine [5]. (e) Cytoprotective agents – Examples are colloidal bismuth subcitrate, tripotassium dicitratobismuthate, misoprostol, sucralfate [6]. In ulcers caused by *H. pylori*, antibiotics such as clarithromycin and amoxicillin together with antisecretory drugs like lansoprazole or omeprazole should be given especially to patients with duodenal ulcers. There are many unexplored drugs that might produce ulcer-healing effects and this advocated the search for antiulcer drugs that can enhance mucosal blood flow, epithelial restitution and mucosal alkaline secretion or inhibit luminal pepsin activity [7]. *Annona muricata* plant commonly called sour sop, is a tropical, flowering, evergreen tree in the family Annonaceae. It can reach heights of 10 metres and is low branching and bushy with upturned limbs keeping the tree slender. It does not tolerate cold and is susceptible to strong winds. The fruit is heart-shaped to oval and may be sometimes irregular, lopsided or curved due to improper carpel development or insect injury. The leaves, normally evergreen, are alternate, smooth, glossy, dark-green on the upper surface, lighter beneath; oblong, elliptic or narrow-obovate, pointed at both ends, 2½ to 8 inches long and 1 to 2½ inches wide; and highly aromatic when crushed. The flowers, which are borne singly, may emerge anywhere on the trunk, branches or twigs [8]. This present study thus seeks to determine the phytochemical constituents and find out if the methanolic leave extract of *Annona muricata* possesses acute toxicity and anti - ulcer activity using mice as a model.

MATERIALS AND METHOD

Plant materials

Fresh leaves of *Annona muricata* were collected from Achere, in Nsukka L.G.A of Enugu State, Nigeria in January, 2012 and were identified by Mr. Ozioko, a plant taxonomist in Botany Department of the University of Nigeria, where the leaves are deposited for further study.

Experimental animals

A total of 118 male adult mice weighing 18 ± 2 g were used for the experiment. They were obtained from the laboratory animal house of the Department of Veterinary Physiology and Pharmacology, University of Nigeria, Nsukka and were fed with standard commercial grower feed (Vital Feeds, GCOM Nig. Ltd). They were acclimatised for two weeks prior to the research work. They were fasted of food four hours prior to commencement of the experiment but portable water was however provided *ad libitum*. The animals were maintained under a cycle of 12 hours of light and 12 hours of darkness daily throughout the period of the experiment. They were housed in clean cages in fly-proof house at room temperature of 27 – 32°C and relative humidity of 65 – 70%. The standard guidelines for use of laboratory animals for experimental purposes were adhered to according to University of Nigeria laboratory animal handling rules.

Drugs and chemicals

Cimetidine (Mancare Pharmaceuticals, India), Aspirin (Sigma, Spain), Absolute methanol (Sigma-Aldrich®, Germany), distilled water, Tween 80, Olive oil, Ferric chloride, Lead acetate, Fehlings I solution, Fehlings II solution, Dragendorff's solution, Meyer's reagent, Wagner's reagent, 5% iodine solution, Molisch's reagent were used.

Preparation and extraction of plant material

The fresh leaves of *A. muricata* were dried under shade. The dried leaves were pulverized using manual grinding machine into fine particles. Cold extraction was performed. 300 grams of the powdered plant leaf was extracted in 2,500 millilitres of 80% methanol with intermittent shaking at intervals of 2 hours for 48 hours. The extract was filtered using Wattman size 1 filter paper and concentrated *in vacuo* using vacuum rotary evaporator connected to cold water circulator and pressure pump at 40ºC and 210mmHg pressure. The percentage yield was determined using the formula:

$$\text{Yield} = (\text{weight of material obtained} \div \text{weight of starting material}) \times 100 \div 1$$

The extract was then kept in the refrigerator at 4 ^{0}C for use in the experiment.

Experimental design

1. Acute toxicity test: The modified up and down procedure [9, 10] and log-probit graph methods [11] were used for the determination of median lethal dose (LD50). A dose of 175 mg/kg was the initial dose and increased by dose progression factor of 3.2 until a maximum limit dose was reached using 3 female mice in each dose. Thereafter four groups of three female mice weighing 18 ± 2 g were used for the determination of median lethal dose (LD50) of the extract. The groups were dosed as follows: 175, 313 and 550 mg/kg body weight, bw of the extract for the treatment groups and 10 ml/kg bw distilled water for the control group. The mice in the treatment and the control groups were observed for signs of toxicity and

death for 48hrs, and then for 14 days after which the animals were sacrificed for postmortem study.

2. Anti - ulcer effect of the extract using absolute ethanol-induced ulcer model:

Standard method [12] was adopted for this experiment. In this experiment, mice were distributed using simple random sampling method into five groups of ten animals each that and fasted of food for four hours but allowed free access to water prior to commencement of the experiment. Mice in group A, which served as the negative control, were given distilled water at the dosage of 10 ml/kg while group B mice, which served as positive control, were administered Cimetidine at 100 mg/kg. The remaining three groups, C, D, and E, received 50 mg/kg, 100 mg/kg and 200 mg/kg of the methanolic plant extract respectively. The extract was dissolved in 5% Tween 80 and distilled water after which drug concentration was determined prior to administration. Thirty to fifty minutes later, 0.3 ml of absolute ethanol was administered each to all the mice after which the animals were euthanized using mild ether anaesthesia 1 hour later and their stomachs were harvested for ulcer evaluation. All drugs were administered via the oral route using the intubation tube.

3: Anti-ulcer effect of the extract using aspirin-induced ulcer model

Standard method [13] was adopted for this experiment. In this experiment, mice were distributed using simple random sampling method into five groups of ten animals each and fasted of food for four hours but allowed free access to water prior to commencement of the experiment. Mice in group A, which served as the negative control, were given distilled water at the dosage of 10 ml/kg while group B mice, which served as positive control, received 100 mg/kg of Cimetidine. The remaining three groups, C, D, and E, received 25, 50 and 75 mg/kg of the methanolic plant extract respectively. The extract was dissolved in 5% Tween 80 and distilled water after which drug concentration was determined prior to administration. About 30 – 50 minutes later, 200 mg/kg of acetylsalicylate was administered each to all the mice after which the animals were euthanized using mild ether anaesthesia 1 hour later and their stomachs were harvested for ulcer evaluation. All drugs were administered via the oral route using the intubation tube.

Determination of ulcer parameters

The number and size of ulcers were determined with the aid of x 10 magnifying glass and a fluorescent lamp. The ulcer index was calculated using the formula below [14]:

$$\text{Ulcer index} = \frac{\text{No of ulcers x size of ulcer}}{\text{10 (magnification of lens)}}$$

Preventive index (PI) was calculated using the formula below [15, 16]:

$$\text{PI} = \frac{\text{Ulcer index of negative control} - \text{Ulcer index of the group}}{\text{Ulcer index of negative control}} \text{ x \%100}$$

Ulcers found on the glandular portion of the stomach were counted and each was given a size rating on an arbitrary scale of 1 – 4 based on the diameter of the ulcer as follows:

Size 1 = diameter ≤ 1mm; Size 2 = 2mm ≥ diameter > 1mm; Size 3 = 3mm ≥ diameter > 2mm; and Size 4 = perforation of gastric mucosa

Phytochemical analysis of the extract: Standard procedures were used to carry out phytochemical tests on the methanolic leaf extract of *A. muricata* [17] to identify the following constituents: saponins, tannins, alkaloids, starch, carbohydrates and flavonoids.

Statistical analysis

Data obtained from the ulcer parameters were analyzed using One way Analysis of Variance (ANOVA) followed by Duncan post hoc test and least significant difference (LSD) to determine differences among the mean number of ulcers of the two ulcer models. Significance was accepted at the level of $p<0.05$.

RESULTS

The yield of the plant extraction

The percentage yield of the methanolic leaf extract of *A. muricata* was 8.6%. The extract was dark green in colour and of pasty consistency.

Results of acute toxicity

No mortality was recorded in the mice that received 10 ml/kg body weight (bw) distil water and 175 mg/kg bw of the extract. However, mortalities were recorded in mice that received 313 mg/kg bw and 550 mg/kg bw, of the extract. Some mice in the group that received 313 mg/kg first showed signs of depression before dying about 2 hours later while mice in the group that received 550 mg/kg bw showed excitement before dying within 20 minutes of administration. The LD_{50} was calculated to be 354.8 ± 8 mg/kg bw. Postmortem did not reveal any pathologic lesions in the liver and kidney of both the treated and control groups.

Results of the effect of the extract on absolute ethanol-induced ulcer in mice

The results of the effect of methanolic leaf extract of *A. muricata* on absolute ethanol-induced ulcer model are

as presented in Table 1. The result showed that both cimetidine (100 mg/kg bw) and the extract (50 mg/kg and 100 mg/kg bw) produced a significant decrease in the mean number of ulcers. However, there was no significant difference in the mean number of ulcers produced by cimetidine and 100 mg/kg bw of the extract as well as between 50 mg/kg and 100 mg/kg bw of the extract. In group E (200 mg/kg extract), two mice died after administration of extract while the remaining 8 mice showed signs of toxicity such as depression and weakness, and later died after administration of ethanol; thus their ulcers were not read.

Results of the effect of the extract on aspirin-induced ulcer in mice

The results of the effect of methanolic leaf extract of *A. muricata* on aspirin-induced ulcer model are as presented in Table 2. The results showed that both cimetidine (100 mg/kg bw) and the extract (25 mg/kg, 50 mg/kg and 75 mg/kg bw) produced a significant decrease in the mean number of ulcers. Though, there was no significant difference in the mean number of ulcers produced by cimetidine (100 mg/kg bw); 50 mg/kg and 75 mg/kg bw of the extract, their mean number of ulcers were however significantly different from the mean number of ulcers produced by 25 mg/kg bw of the extract.

Table 1. The effect of *Annona muricata* extract on absolute ethanol-induced ulcer in mice.

GROUPS	MEAN NUMBER OF ULCERS (mm) [SEM]	MEAN ULCER INDEX (mm) [SEM]	PREVENTIVE INDEX (%)
A	6.60 [0.40]*	1.98 [0.05]*	0
B	5.00 [0.32]**	1.50 [0.15]**	24.24
C	3.60 [0.51]***	0.98 [0.18]c***	50.51
D	4.40 [0.51]**	1.14 [0.12]**	42.42

* – Different superscripts down the column indicate significant difference down the groups

Group A – Mice treated with 10ml/kg distilled water and administered absolute ethanol

Group B – Mice treated with 100mg/kg Cimetidine and administered absolute ethanol

Group C – Mice treated with 50mg/kg *A. muricata* and administered absolute ethanol

Group D – Mice treated with 100mg/kg *A. muricata* and administered absolute ethanol

Table 2. The effect of *Annona muricata* extract on aspirin – induced ulcer in mice.

GROUPS	MEAN NUMBER OF ULCERS (mm) [SEM]	MEAN ULCER INDEX (mm) [SEM]	PREVENTIVE INDEX (%)
A	6.6 [0.40] *	0.84 [0.04] *	0
B	2.20 [0.37] ***	0.32 [0.03] ***	61.9
C	4.40 [0.51] **	0.50 [0.03] b**	40.48
D	2.20 [0.37] ***	0.22 [0.04] ***	73.81
E	1.6 [0.27] ***	0.20 [0.02] ***	76.19

* – Different superscripts down the column indicate significant difference down the groups

Group A – Mice treated with 10ml/kg distilled water and administered aspirin

Group B – Mice treated with 100mg/kg Cimetidine and administered aspirin

Group C – Mice treated with 25mg/kg *A. muricata* and administered aspirin

Group D – Mice treated with 50mg/kg *A. muricata* and administered aspirin

Group E – Mice treated with 75mg/kg *A. muricata* and administered aspirin

Table 3. Results of the Phytochemical Analysis of methanolic *Annona muricata* leaf extract (MAMLE).

MAMLE	Saponins	Tannins	Alkaloids	Flavonoids	Starch	Carbohydrates
±	+	+	+	_	_	_

-, Absent

+, Present.

Results of the phytochemical analysis of extract

The result of the phytochemical analysis is as shown in Table 3. Foams were observed in the test for saponins while no colour changes were observed in the tests for starch, carbohydrates and flavonoids. However, of the two tests carried out for the presence of tannins, the test involving 5% ferric chloride showed no colour change while the other involving lead acetate showed light green precipitate. Light green precipitates were also observed in the tests for the presence of alkaloids using Meyer's reagent and Wagner's reagent while white ring was observed in the test using Dragendorff's reagent.

DISCUSSION

The methanolic leaf extract of *A. muricata* had a yield of 8.6% w/w. This increase in the yield compared to that of Azadirachta indica which was 7.62% [18] could be attributed to the quality of the constituents of the plant and the quantity of methanol used in the extraction. The results of the acute toxicity revealed that the methanolic leaf extract of *A. muricata* has a median lethal dose (LD_{50}) of 354.8± 8 mg/kg bw. Using acute toxicity rating, the extract could be classified as being moderately hazardous [19]. This toxic nature of the extract was further confirmed by the 20 % death of the group that received 200 mg/kg bw of the extract.

In the ethanol-induced ulcer model, both cimetidine and the extract at all doses significantly decreased ($p<0.05$) the mean number of ulcers with 50 mg/kg of the extract producing the least mean number of ulcers (3.60 ± 0.51), least mean ulcer index (0.9 8± 0.05) and highest preventive index (50.51%). This is in agreement with other workers [20], who stated that the ethanolic leaf extract of *A. muricata* significantly reduced the total area of gastric lesions in ethanol-induced ulcer. These therefore suggest that the extract possesses antiulcer activity.

Absolute ethanol induces gastric damage ranging from endothelial microvascular damage to development of macroscopic gastric mucosal lesions, which is attributed mainly to the inhibition of biosynthesis of cytoprotective prostaglandins resulting in overproduction of leukotrienes and other products of the 5-lipoxygenase pathway [21]. These agents break the mucosal barrier and provoke an increase in gastric mucosal permeability and the amount of acid diffusing back into the mucosa. Mast cells in the sub mucosa and lamina propria degranulate upon contact with acid, releasing histamine which stimulates parietal cell secretion of hydrochloric acid and promotes cellular injury. It is this back diffusion of hydrochloric acid that is the principal factor eliciting mucosal erosion and ulceration. Cimetidine, being a H_2-receptor antagonist was able to significantly reduce the mean number of ulcer lesions by inhibiting the secretion of gastric acid and reducing pepsin output.

In the aspirin-induced ulcer model, both cimetidine and the extract at all doses significantly decreased ($p<0.05$) the mean number of ulcers with 75 mg/kg of the extract producing the least mean number of ulcers (1.60±0.27), least mean ulcer index (0.20 ± 0.02) and highest preventive index (76.19%). This strongly proved the efficacy of the extract in the treatment of ulcer. Aspirin directly injures gastric epithelial cells and impairs prostaglandin E production by the inhibition of cyclo-oxygenase (COX) enzyme(s) [22]. This inhibition of prostaglandin E production ultimately results in the same cascade of reactions seen in ethanol-induced ulcers. Cimetidine, therefore was able to significantly reduce the mean number of ulcer lesions by inhibiting the secretion of gastric acid and reducing pepsin output. It is however worth noting that cimetidine produced less mean number of ulcers, less mean ulcer index and higher preventive index in aspirin-induced ulcer (2.20 ± 0.37, 0.32± 0.03 and 61.90% respectively) than in ethanol-induced ulcer (5.00 ± 0.32, 1.50 ± 0.15 and 24.24%) respectively), indicating that cimetidine is more efficacious in preventing aspirin-induced ulcer than ethanol-induced ulcer.

Preliminary phytochemical analysis of the extract showed the presence of saponins, alkaloids and traces of tannins, all of which have antiulcer properties [23, 24, 25]. The pharmacological activities of saponins in plants, such as their anti-inflammatory, antitumour, antiexudative, antiulcer, analgesic, antipyretic and immunostimulant effects, have been known for many years, while new activities are continually being discovered [26]. Several plants containing high amounts of saponins have been shown to possess anti-

ulcer activity in several experimental ulcer models [22]. The protective activities of all these active saponins are not due to inhibition of gastric acid secretion but probably due to activation of mucous membrane protective factors [27]. The antiulcer activity of the extract could thus be attributed to the presence of saponins. Alkaloids are secondary metabolites found in about 20% of plant species. A wide range of biological activities of alkaloids have been reported and include emetic, anti-cholinergic, antitumor, diuretic, sympathomimetic, antiviral, antihypertensive, hypnoanalgesic, antidepressant, miorelaxant, antitussigen, antimicrobial and anti-inflammatory properties [28]. The mechanism of action of the antiulcer activity of alkaloids depends on the particular alkaloid involved. Anisodamine and anisodine, both of which are analogs of atropine, have been shown to produce antiulcer effects against indomethacin, reserpine, stress, pylorus ligature, acetic acid or absolute ethanol induced ulcers by altering the gastric acid secretion through increase of luminal gastric output of basal bicarbonate and pH [29]. A well known pyridine alkaloid, nicotine, has been shown to protect the stomach from damage induced by aspirin by decreasing hemorrhages and increasing the pH gradient/gastric fluid volume [30]. Nigakinone and methylnigakinone which are indole alkaloids have been shown to possess antiulcer effects associated with decreases in gastric acid/pepsin secretions and protection of the mucous membrane [31, 32]. The anti-inflammatory as well as the antiulcer activity of alkaloids may thus be equally responsible for the antiulcer activity of the plant extract. Tannins are water soluble poly phenols present in plants. They are used in medicine primarily because of their astringent properties. Tannins precipitate micro proteins at the site of the peptic ulcer, forming an impervious layer over the lining that hinders gut secretions and protects the underlying mucosa from toxins and other irritants [33, 34] and promote resistance to the action of proteolytic enzymes, an associated activity against *Helicobacter pylori* [35]. They have also been shown to inhibit gastric acid secretion. However, because of the little presence of tannins, this antiulcer activity can be said to be mainly due to alkaloids.

CONCLUSION

The methanolic leaf extract of *A. muricata* can be concluded to be moderately toxic since it has an LD_{50} of 354.8 ± 8mg/kg using the WHO [19] classification. The antiulcer activity of the extract can be linked to the presence of saponins, alkaloids and tannins. However, more research work is needed to find out the active principle in this plant extract so that it can be purified, characterized and commercialized for the treatment of gastric ulcer.

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Monograph

Vangueria madagascariensis J.F. Gmelin (Rubiaceae) - an under-utilised African traditional medicinal food plant with potential applications

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Abstract
Vangueria madagascariensis J.F. Gmelin (Rubiaceae) is one of the most common species of the genus *Vangueria* which have received scientific attention for its extensive ethnomedicinal applications worldwide. Generally cultivated for its sweet-sour fruits, this plant has also brought significant contribution in the African *materia medica* for its antimicrobial and anthelmintic properties since time immemorial. *In vitro* data revealed the presence of a number of bio-constituents with pluripotential mechanism of action(s) which might be responsible for its medicinal virtues. Recent findings also support its promising potential for use against inflammatory diseases and as a functional food/dietary adjunct for the management of diabetes mellitus and related complications. The present monograph endeavours to highlight the botanical description, ethnopharmacological uses, and main therapeutic benefits of *Vangueria madagascariensis*. Special emphasis has been geared towards recent *in vitro* data which tend to support its ethnopharmacological use in the traditional medicine of many countries

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GENERAL INFORMATION

Vangueria madagascariensis (VM) J.F. Gmelin, commonly known as Tamarind of the Indies or wild medlar is a perennial plant of the Rubiaceae family which is native to tropical Africa and Madagascar [1]. It grows naturally in riverine-lowland forests and volcanic ash soils throughout Africa and Asia. The plant can also be found in the Republic of Mauritius (including Rodrigues Island), Seychelles, India, Northern Australia, Singapore and Trinidad [2]. The generic name '*Vangueria*' is derived from the Madagascan vernacular name 'voa-vanguier'. Other common vernacular names include Voavanga and Vavandrika in Madagascar; Vavang and Vavangue in Mauritius, Madagascar and Seychelles; and mviru or muiru in Swahili [3-5]. The synonyms for this plant are *Vangueria acutiloba* Robyns., *Vangueria edulis* Vahl. and *Vangueria venosa* Hochst. Ex A. Rich. [6].

BOTANICAL DESCRIPTION

VM is a profusely branched tree of 1.5-15 m tall, with smooth grey bark. Leaves, with stalks 5-10 mm long, are opposite, elliptic-ovate or rotundate, dark green above and paler beneath with prominent venation. The plant bears small flowers in clusters which are greenish to yellowish in colour with calyx 1.2-3 mm in length. Fruits are more or less globular, smooth, and shiny with diameter 2.5-5 cm and contain 4-5 pyrenes, varying from green to brownish red. They appear greenish when immature, changing to brownish-red when ripe, and contain 4-5 woody seeds up to 1.6 cm long [7, 8].

Figure1. *Vangueria madagascariensis,* A) leaves, B) unripe fruits, C) seeds and D, E and F) ripe fruits

ETHNOPHARMACOLOGICAL USES

Some species of genus *Vangueria* are widely studied and used in traditional medicine in various countries. For instance, in Tanzania different parts of the species *Vangueria infausta* have traditionally been used for the treatment of malaria, wounds, menstrual and uterine problems [9]. With respect to VM, available folk data suggest its use as an athelmintic against roundworms, as antiplamodial, as astringent against cholagogue and as expectorant, for the treatment of smallpox and sores, herpes labialis, and ophtalmia [10]. Roots are also reported to be macerated and administered orally for the treatment of diabetes mellitus in South-eastern Sudan [11]. In Mauritius, an infusion of the leaves of VM, ingested once a week, has also been reported for the same purpose whilst a bark decoction is used against dysentery, cardiac palpitations or nausea [12]. In Rodrigues, a decoction made from VM, *Jatropha curcas*, *Toddalia asiatica* and *Sporobouls capensis* is used as mouthwash for teething children. Additionally, a decoction of leaves of VM, *Jatropha curcas*, *Azadirachta indica* and pieces of *Ipomoeaa-pes-caprrae* (liana) with a pinch of salt is prepared for bath for treatment of abscesses, scurf, and carbuncle [13].

NUTRITIONAL VALUE

The proximate nutritional composition of the wild medlar per 100g (Table 1) was established based on the pulp to fruit ratio of 47.5% [14].

Table 1. The proximate nutritional composition of the wild medlar per 100g

Nutrient	Perecntage
Energy (kJ)	498
Protein	1.4
Lipid	0.1
Carbohydrate	28
Fibre	4.7
Vitamins	mg
Ascorbic acid	4.7
Thiamine	0.04
Riboflavin	0.03
Niacin	0.61
Minerals	mg
Calcium	25
Iron	1.1
Magnesium	39
Phosphorus	36.6
Potassium	521
Sodium	28

SELECTED CONSTITUENTS

Preliminary phytochemical screening of the leaves and stems indicate the presence of alkaloids, terpenes, cyonogenetic heterosides as well as phenols, tannins, and saponosides which may likely be responsible for its documented antimicrobial effects [12]. Interestingly, fractionation and purification of the alcoholic extract of the leaves and stem of VM cultivated in Egypt yielded thirteen compounds identified as: β-sitosterol acetate, stigmasterol, palmitic acid, scopoletin, *p*-coumaric acid, protocatechuic acid, esculetin, ethyl-1-O-glucosyl-4-O-(E) caffeoyl quinate, kaempferol-3-O-rhamnoside,7-O-rutinoside, β-sitosterol, ceryl alcohol, vanillic acid and β-sitosterol-3-O-β-D-glucopyranoside [15].

BIOLOGICAL ACTIVITIES

The *n*-hexane and *n*-butanol fractions of the leaves and chloroform, *n*-hexane fractions of the stem-bark (400 mg/kg) showed potent anticonvulsant activity against pentylene tetrazole induced convulsion in rats compared with carbamazepine. Different fractions of the leaves and bark (*n*-hexane, chloroform, ethyl acetate) had significant inhibiting effects on Gram-negative bacteria *Escherichia coli, Klebsiella pneumonia, Pseudomonas aeruginosa* and the fungus *Candida albicans* when compared with positive control Gentamicin (bacteria) and Clotrimazole (fungus) [15]. Potent anti-inflammatory activity compared with indomethacin was observed in rats administered with chloroform and ethyl acetate fractions of the leaves, with maximum effect being obtained after 2 hrs. The activity of the chloroform and ethyl acetate fractions may be attributed to scopoletin which has anti-inflammatory activity. Indeed, scopoletin is a specific inhibitor of the production of inflammatory cytokines in mast cells and may be beneficial in the treatment of chronic inflammatory diseases [16].

RECENT FINDINGS

We recently showed that only leaf decoction (IC_{50}=1.13±0.24 mg/ml), leaf methanol (IC_{50}=1.46±0.45 mg/ml), and unripe fruit methanol (IC_{50}=1.38±0.06 mg/ml) extracts of VM significantly (p<0.05) decreased α-amylase activity *in vitro* [17]. Enzyme kinetic studies revealed that VM extracts decreased both the maximal velocity and Michaelis-Menten constant, indicating a mixed non-competitive type of inhibition. Active extracts which were found to inhibit α-glucosidase were unripe fruit methanol (IC_{50}=0.36±0.07 mg/ml), unripe fruit decoction (IC_{50}=0.50±6.0 mg/ml), leaf decoction (IC_{50}=0.61±0.21mg/ml) and ripe fruit methanol (IC_{50}=3.28±0.45 mg/ml), where values were significantly lower than the positive control acarbose (IC_{50}=5.03±0.14 mg/ml). However, methanol and decoction extracts of the leaves, ripe and unripe fruits and seeds did not significantly retard glucose movement across dialysis tube. Antimicrobial activity was noted for *Escherichia coli* using unripe fruit decoction extract (12.67±0.58 mm) whereas for *Staphylococcus aureus,* leaf methanol extract produced highest inhibition (11.67±1.53 mm). However, the inhibitory zones were significantly lower (p<0.05) than the mean standard antibiotic, ampicilin. Decoction and methanol extracts of the leaves, ripe and unripe fruits and seeds did not show antifungal properties. Total phenolic content showed a strong negative correlation with 2,2-diphenyl-1-picrylhidrazyl (DPPH) radical scavenging activity (r=-0.77, p<0.05) whereas for ferric reducing antioxidant power (FRAP), strong positive relationship was found (r=0.88, p<0.05). Methanol leaf extract with an IC_{50}=43.22±0.59 μg/ml, demonstrated efficient nitric oxide scavenging potential and was significantly (p<0.05) lower than the control ascorbic acid (IC_{50}=546.54±9.79 μg/ml). Concerning hydroxyl radical scavenging assay, only methanol extracts of leaf (IC_{50} =0.09±0.04 μg/ml), unripe fruit (IC_{50}=0.29±0.08 μg/ml) and ripe fruit (IC_{50}=0.26±0.02 μg/ml) had IC_{50} values significantly lower (p<0.05) than α-tocopherol (IC_{50}=0.50±0.11 μg/ml) [17].

CONCLUSIONS

This paper has tried to highlight some of the medicinal values of VM validated through different *in vitro* assays. Recent findings showed the promising antioxidant, antimicrobial and anti-diabetic activity of the fruits, seeds and leaves of VM. However, this paper provides essential information for literature only. Further studies are needed to ensure that *in vitro* effects can be translated into a safe and effective use of VM as a functional food and thus validate its folkloric use.

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