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Original Research

Protective effects of bitter almond kernel oil on some biochemical parameters in brain tissue of diabetic rats

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Abstract
Aim: In this study, we aimed to determine possible protective effects of the bitter almond kernel oil extract on the lipid-soluble vitamins, cholesterol, GSH, total protein, MDA and fatty acid levels of brain tissue in the streptozotocin-induced diabetic Wistar rats.
Method: The lipid-soluble vitamins, cholesterol and other sterol levels were measured by HPLC, the fatty acid levels were measured by GC, MDA, GSH and total protein levels were measured by UV-Vis spectrophotometer.
Results: Whereas the α-tocopherol and GSH levels were decreased in the diabetes (D) group, these parameters were protected in the diabetes + almond oil (D+AO) group when compared to control (C) group. The total protein and MDA levels were increased in the D group, but their levels were not changed in the D+AO group. The palmitic acid and stearic acid levels were increased in the D and D+AO groups. The arachidonic acid and docosahexaenoic acid levels were increased in the D group, but these fatty acid levels were not changed in the D+AO group when compared to the C group. In conclusion, according to our results, bitter almond oil was shown that some positive effects on the biochemical parameters of brain tissue in the diabetic rats.
Conclusion: This oil was protected or prevented the GSH and MDA levels in bitter almond oil extract given group, and these values were closed to control group values.

INTRODUCTION

Diabetes mellitus is a significant endocrine system disease and it is usually characterized by low level of blood insulin or the insulin insensitivity in target tissues [1]. Chronic hyperglycemia, which occurred in diabetes, affected the central nervous system and can contribute to the formation of various complications [2]. The relationship between diabetes and central nervous system has been interesting subject in recent times. Diabetes-induced neurological complications can be caused the changes of morphological, neurochemical and peripheral neuropathies [3]. Hyperglycemia can lead to irreversible damages -such as stroke- due to effects of neurochemical on the central nervous system [4,5]. Also, it has been shown the hyperglycemia cause significant changes in neuronal and glial cells [6]. The polyunsaturated fatty acids (PUFA) are abundant in the brain structure and these are particularly sensitive to oxidative stress due to can easily oxidize [7]. The increasing of oxidative stress with decreasing of the antioxidant defense system activity of the brain, make clear brain to pathological changes in diabetes [8]. The previous studies have shown that experimental hyperglycemia induces oxidative damage and increases lipid peroxidation of the brain tissue in the diabetic rats [9-12].

Epidemiological studies have indicated that more than 1200 plants used for the hypoglycemic activity in traditional folk medicine [13-15]. In literature, it was specified these plants have hypoglycemic and hypolipidemic activity via they contain some important

chemical compounds, such as flavonoids, phenolics, sterols, vitamins etc. Since ancient times, diabetes mellitus attempted to treat with herbal medicines [16]. Almond (*Prunus dulcis*) is a plant belonging to the Roseaceae family, and it is cultivated in many regions of the world [17]. It was reported that the almond an important source of mono and poly unsaturated fatty acids, phenolic acids, flavonoids and tocopherols. There is an important relationship between almond consumption and reducing of the chronic disease risks. It was indicated that mentioned compounds reduce blood pressure, total cholesterol and these substances protect the body from oxidative stress. In previous studies, it was expressed that the almond oil has free radical scavenging activity and it used for the reduction of blood glucose level in diabetes patients [18-20].

The aim of this study was determined the possible protective effects of bitter almond kernel oil on the fatty acid composition, lipid soluble vitamin contents, GSH, total protein, sterols and MDA levels of brain tissue of streptozotocin-induced diabetic rats.

MATERIALS AND METHODS

Chemicals

All the chemicals and reagents were used of analytical grade and these chemicals and reagents were obtained from Sigma Chemical Co. (Germany).

Animals

All the experimental protocols were approved by the Ethical Committee of Firat University (Elazig, Turkey; Ethical Decision Number: 05.05.2011/81). Thirty healthy male Wistar albino rats, aged 8-10 weeks and weight in the range of 225-250 g, were obtained from Firat University Experimental Research Centre (Elazig, Turkey). The animals were housed in polycarbonate cages in a room with a 12 h day-night cycle, at the temperature of 24 ± 3 °C and humidity of 45–65%. During the whole experimental period, animals were fed with a balanced commercial diet and water ad libitum.

Experimental Design

The thirty rats were randomly divided into three groups each containing ten rats.

Group C (Control): to the rats injected 0.5 mL DMSO twice a week, the rats received tab water and fed with standard pellet diet as ad libitum.

Group D (Diabetes): to the rats injected intraperitoneally streptozotocin 40 mg/kg a single dose, 0.5 mL DMSO twice a week and fed with standard pellet diet as ad libitum.

Group D+A (Diabetes+Almond Oil): to the rats injected intraperitoneally streptozotocin 40 mg/kg a single dose, and rats received 1 mg/kg almond bitter oil extract every day and fed with standard pellet diet as ad libitum.

All treatments were continued for 60 days. Each experimental rat was decapitating after one week the last injection DMSO and the last intake bitter almond extract. At the end of the experimental period, animals were fasted overnight and sacrificed by cervical decapitation. Brain tissue was dissected out washed in ice-cold saline to remove blood. The tissue was homogenized in Tris-HCl and EDTA buffer at pH = 7.4 and then centrifuged at 9000 rpm for 20 min. The supernatants were used for of lipid peroxidation, total protein and pellet were used for fatty acids, vitamin and sterol analysis.

Induction of Diabetes

Overnight fasting animals were made diabetic by a single intraperitoneally injection of freshly prepared streptozotocin (STZ) (40 mg/kg) dissolved in 0.1 M citrate buffer (pH=4.5) [21]. Control rats were injected with the same volume of isotonic saline. Plasma glucose was determined at the end of 72 h, and those rats with fasting glucose levels in the range of 140-200 mg/dL were used in the present study [22]. The blood glucose levels were measured by using reagent strips (Contour, Bayer, Leverkusen, Germany).

Determination of Reduced Glutathione (GSH)

Reduced glutathione (GSH) was determined by the method of Ellman [23]. Briefly 1 mL of tissue homogenate was treated with 1 mL of 10% trichloroacetic acid (TCA), the mixtures were centrifuged in 5000 rpm and the supernatants were taken. After deproteinization, the supernatants were allowed to react with 1 mL of Ellman's reagent (30 mM, 5,5'-dithiobisnitro benzoic acid in 100 mL of 0.1% sodium citrate). The absorbance of the yellow product was read at 412 nm by a spectrophotometer. Pure GSH was used as standard for establishing the calibration curve.

Determination of Total Protein

Total protein was estimated by the method of Lowry et al. [24]. To 10 µL of tissue homogenate and 4.0 mL of alkaline copper reagent were added and kept at room temperature for 10 min. Then 0.5 mL of Folin's-Ciocalteau reagent was added and the colour developed was read after 30 min at 750 nm. A standard curve was obtained using bovine serum albumin. The level of protein was expressed as mg albumin/g of tissue.

Determination of MDA Level

Lipid peroxides (MDA) in tissue homogenate were estimated using thiobarbituric acid reactive substances by the method of Ohkawa et al. [25]. To 1.0 mL tissue homogenate, 0.5 mL of 8.1% sodium dodecyl sulfate (SDS), 1.0 mL of (20% acetic acid / NaOH, pH = 3.5), 1.0 mL of 10% TCA, 50 µL of 2% BHT and 1.0 mL of 0.8% TBA were added. The mixture was heated in a water bath at 95 °C for 60 min. After cooling, 4 mL of n-butanol/pyridine mixture were added and shaken vigorously. After centrifugation at 4250 rpm for 15 min, the organic layer was taken and its absorbance at 532 nm was measured. 1,1,3,3-tetramethoxypropane was used as standard. Results were calculated as nmol MDA/g tissue.

Fatty Acid Analysis

Lipids of tissue samples were extracted with hexane–isopropanol (3:2, v/v) according to the method of Hara and Radin [26]. 1 g tissue sample was homogenized with 10 mL hexane-isopropanol mixture. Fatty acids in the lipid extracts were converted into methyl esters including 2% sulfuric acid (v/v) in methanol [27]. The fatty acid methyl esters were extracted with 5 mL n-hexane. Analysis of fatty acid methyl ester was performed in Shimadzu GC-17A instrument gas chromatography equipped with a flame ionization detector (FID) and a 25 m, 0.25 mm Permabond fused-silica capillary column (Machery–Nagel, Germany). The oven temperature was programmed between 145 and 215 °C, 4 °C/min. Injector and FID temperatures were 240 and 280 °C, respectively. The nitrogen carrier gas flow was 1 mL/min. The methyl esters of fatty acids were identified by comparison with authentic external standard mixtures analyzed under the same conditions. Class GC 10 software version 2.01 was used to process the data. The results were expressed as percent amount/tissue.

Lipid-Soluble Vitamin and Phytosterol Analysis

Lipid-soluble vitamins and phytosterols were extracted from the lipid fraction according to the method of Sanchez-Machado et al. [28] with minor modifications. 5 mL of n-hexane/isopropyl alcohol mixture treated with 5 mL of KOH solution (0.5 M in methanol) were added and immediately vortexed for 20 s. The tubes were placed in a water bath at 80 °C for 15 min. Then after cooling in iced water, 1 mL of distilled water and 5 mL of hexane was added, and the mixture was rapidly vortexed for 1 min, then centrifuged for 5 min at 5000 rpm. The supernatant phases were transferred to another test tube and dried under nitrogen. The residue was redissolved in 1 mL of the HPLC mobile phase (68:28:4, v/v/v, methanol/acetonitrile/water). Finally, an aliquot of 20 µL was injected into the HPLC column. Before injection, the extracts were maintained at −20 °C away from light.

Chromatographic Conditions

Chromatographic analysis was performed using an analytical scale (15 cm×0.45 cm) Supelco LC 18 DB column with a particle size 5 µm (Sigma, USA). HPLC conditions were as follows: mobile phase 60:38:2 (v/v/v): acetonitrile/methanol/water; a flow rate of 1 mL/min; column temperature 30 °C. The detection was operated using two channels of a diode-array spectrophotometer, and 202 nm for α-tocopherol and cholesterol. α-tocopherol and cholesterol were identified by retention and spectral data [29,30].

Statistical analysis

All the data were statistically evaluated with SPSS 15.0 software. The statistical significance of the data is determined using one-way analysis of variance (ANOVA), and the group means have been compared by Duncan's multiple range test (DMRT). P values of less than 0.05 were considered to indicate statistical significance.

RESULTS

The lipid-soluble vitamins, sterols, GSH, MDA and total protein levels in the brain of diabetic rats are shown in the Table 1. While the MDA level was significantly increased in the D group ($p<0.001$), the GSH level was significantly decreased in the same group ($p<0.001$) when compared to the C group. In the D+AO group, the MDA level was signficanlty decreased ($p<0.001$) and its level was closely to control group values, in addition the GSH level was significantly increased ($p<0.001$) when compared to the D group. When compared to the C group, vitamin K_2, retinol and vitamin K_1 levels were significantly increased ($p<0.001$), α-tocopherol, cholesterol and stigmasterol levels were significantly decreased ($p<0.001$) in the D group. When compared to D group, vitamin K_2, δ-tocopherol, vitamin K_1, α-tocopherol, cholesterol and stigmasterol were significantly decreased in the D+AO group ($p<0.001$).

The important fatty acid levels in the brain of diabetic rats are shown in the Table 2. It was determined that while the palmitic acid (16:0), palmitoelic acid (16:1), stearic acid (18:0), linoleic acid (18:2), arachidonic acid (20:4) and docosahexaenoic acid (22:6) levels were significantly increased in the D group ($p<0.001$), the oleic acid (18:1) level was significantly decreased in the same group ($p<0.001$) when compared to the C group. In the D+AO group, while the palmitic acid (16:0), stearic acid (18:0), arachidonic acid (20:4) and docosahexaenoic acid (22:6) levels were significantly decreased ($p<0.001$), the oleic acid (18:1) and linoleic acid (18:2) levels were significantly increased ($p<0.001$) when compared to the D group.

Table 1. The biochemical parameters in brain of Wistar rats

Biochemical Parameters	Control	Diabetes	Diabetes + Almond Oil
Retinol (µmol/g)	0.22±0.02[b]	1.30±0.02[a]	1.27±0.02[a]
α-tocopherol (µg/g)	17.63±0.26[a]	14.21±0.15[c]	15.25±0.14[b]
δ-tocopherol (µg/g)	0.42±0.02[b]	0.40±0.02[b]	0.48±0.01[a]
Vitamin D_3 (µg/g)	1.05±0.02[c]	1.66±0.67[a]	1.31±0.01[b]
Vitamin K_1 (µg/g)	6.48±0.22[c]	6.98±0.14[b]	8.84±0.10[a]
Vitamin K_2 (µg/g)	8.90±0.10[c]	10.30±0.40[b]	14.68±0.18[a]
Cholesterol (µmol/g)	8.59±0.14[a]	6.98±0.10[b]	8.63±0.09[a]
β-Sitosterol (µg/g)	0.96±0.02[c]	1.29±0.02[b]	1.66±0.90[a]
Stigmasterol (µg/g)	24.68±0.39[b]	23.08±0.33[c]	34.55±0.24[a]
GSH (µmol/g)	0.74±0.01[a]	0.44±0.01[c]	0.56±0.01[b]
Total Protein (µg/g)	45.48±0.26[b]	47.52±0.32[a]	44.44±0.19[c]
MDA (nmol/g)	9.17±0.10[c]	10.38±0.04[a]	9.67±0.03[b]

Data are mean ± SD values for ten rats in each group. Values not sharing a common superscript horizontal differ significantly at $p<0.001$ (DMRT). There is no statistically significant difference among the same letter groups. All the groups were compared with each other.

Table 2. The fatty acid composition in brain of Wistar rats (%)

Fatty Acids	Control	Diabetes	Diabetes + Almond Oil
16:0	17.46±0.05[c]	19.76±0.13[a]	18.58±0.08[b]
18:0	16.96±0.03[c]	18.26±0.03[a]	17.84±0.13[b]
18:1	25.49±0.02[a]	23.63±0.17[c]	24.69±0.19[b]
18:2	0.61±0.02[c]	0.71±0.02[b]	0.83±0.01[a]
20:4	10.67±0.16[b]	11.85±0.12[a]	10.69±0.12[b]
22:6	10.60±0.15[c]	12.20±0.14[a]	10.94±0.12[b]
Others	18.21±0.41	13.59±0.48	16.43±0.77
∑SFA	34.42±0.42[b]	38.02±0.47[a]	36.42±0.36[a]
∑MUFA	25.49±0.02[c]	23.63±0.17[c]	24.69±0.19[b]
∑PUFA	21.88±0.17[a]	24.76±0.11[b]	22.46±0.22[b]
∑USFA	47.37±0.29[a]	48.39±0.44[b]	47.15±0.18[b]

MUFA: monounsaturated fatty acids; PUFA: polyunsaturated fatty acids; SFA: saturated fatty acids; USFA: unsaturated fatty acids. Data are mean ± SD values for ten rats in each group. There is no statistically significant difference among the same letter groups. Values not sharing a common superscript horizontal differ significantly at $p<0.001$ (DMRT). All the groups were compared with each other.

DISCUSSION

Streptozotocin (STZ) is widely used diabetogenic agent in experimental animals for forming the diabetes. It leads to impairment the activity of insulin producing pancreatic beta cells via damaging of these cells. Many studies were expressed that STZ effectively forms the diabetes with its intraperitoneal administration in rats [31,32].

The appearing complications in the diabetes patients were negatively affected to patient's life quality. Hyperglycemia caused to increasing of reactive oxygen species (ROS) and to destruction of between oxidants and antioxidants, thus it accelerates forming of the diabetes-related complications [33]. Many studies were revealed the relationship between formation of diabetes-specific complications and the lipid peroxidation (LPO) level [34]. In this respect, it is more clearly understood the importance of controlling of LPO level in diabetes mellitus. In the present study, while the brain tissue MDA level was significantly increased in the D group ($p<0.001$), but its level was

significantly decreased and this level closed to the C group values in the bitter almond oil given D+AO group ($p<0.001$). The various antioxidant compounds found in the bitter almond oil may be supported the antioxidant defense system of brain, and this extract prevented some reactions inducing by the STZ. Therefore we think that the bitter almond oil extract suppressed the formation of LPO and it reduced the MDA level in the brain. Many researchers were reported that the almond oil was rich as tocopherols, fatty acids, phenolic compounds, which have effective antioxidant activity [18,35]. Additionally, some studies were indicated that the almond reduced the LPO and oxidized LDL-C levels in serum, and it decreased the isoprostan level in urine in the hyperlipidemic peoples [36,37].

Glutathione (GSH) is found in many mammalian cells and it is a most important non-enzymatic antioxidant molecule against the oxidative stress. This molecule scavenges the free radicals and it repairs the biological damages caused by these radicals. Prabakaran and Ashokkumar [38] have shown that the GSH level was decreased in the STZ-induced diabetic rats when compared to control group. In our study, while the GSH level was significantly decreased in the diabetic group when compared to control group, its level was significantly increased in the bitter almond oil given group when compared to the diabetic group. Decreasing of the GSH levels in tissues is one of the most important factors predisposing to an increase of LPO level. Because fall below the normal levels of GSH stimulate the production of ROS with oxidative stress, and this situation will lead to start of some chain reactions, which affect the functions of the organelle membranes and the structural integrity of the cells [39]. In this study, decreasing of the MDA level and increasing of the GSH level have shown that the bitter almond oil extract protected from oxidative damage caused by the diabetes in the brain tissue of diabetic rats. From this standpoint, the obtained results are very important for the diabetes researches and the diabetic patients. Oxidative stress is a common feature of chronic degenerative diseases such as diabetes, the consumption of high antioxidant potential foods such as almond may prevent its occurrence [40].

It is known to change the fatty acid composition in both experimental and clinical diabetes. In the experimental diabetes studies, the administration of herbal treatment was prevented to changes in the fatty acid composition be based on the diabetes [41]. In the present study, whereas the fatty acid composition was changed in the diabetic group when compared to the control group, these changes were prevented by the bitter almond oil extract in the brain tissue of the D+AO group. The saturated fatty acids palmitic and stearic acid levels were significantly increased in the diabetes group, but this increasing was significantly reduced in the bitter almond oil given group and these values were closed to the control group values. These findings are similar to previous study results [42]. In the brain tissue, while the oleic acid level was significantly decreased in the diabetes group when compared to the control group, its level was significantly increased and was closed to the control group values in the bitter almond oil given group. Linoleic, arachidonic and docosahexaenoic acids are polyunsaturated fatty acids found in the brain tissue, and these fatty acids are very important for maintaining a healthy way the operation and functions of the brain. These fatty acids are particularly sensitive to oxidative stress due to the double bands in the bodies [43]. It was expressed that the brain tissue fatty acid composition changed due to the decrease in the level of insulin in the diabetic rats [42]. Also it has been suggested that the activity of some enzymes, which are involved in the fatty acid biosynthesis, may change depending upon feeding [44-46]. The almond is an important nutrient in point of the unsaturated fatty acids [47]. Some researchers have been proposed that diet rich unsaturated fatty acids may alter the fatty acid composition in tissues [48-50]. The unsaturated fatty acids found in the bitter almond oil may be affected to the activities of some enzymes on duty fatty acid biosynthesis. And this situation may be caused the changes in the fatty acid composition of the brain tissue.

Shin et al. [51] have shown that the linoleic acid level was increased in the diabetic rat tissues. This result is parallel with our study results. This fatty acid level was significantly increased in the brain tissue of the diabetes group. In our study, this fatty acid level was increased in the bitter almond oil given group. This situation may be caused by the high linoleic acid level found in the almond oil [52]. Besides it has been indicated that the increasing of the arachidonic acid and docosahexaenoic acid level is in unison with the results of previous studies [53,54].

The brain is considered the richest an organ of the body in cholesterol. It was suggested that a number of neurological and chemical changes occur in the brain tissue due to the decrease of cholesterol synthesis in the diabetes [55]. In our study, the cholesterol level was decreased in the brain tissue of the diabetic rats. In contrast, its level was increased and it was closed to the control group values as a result of the bitter almond oil administration in the D+AO group. The resulting increase in the level of the cholesterol can be caused by the protective effects of the bitter almond oil on the neurological and metabolic changes in the brain tissue of diabetic rats.

As known the potential structural and functional

changes in the diabetes can be prevented by means of antioxidants. Vitamin E is an antioxidant, which feature chain breaking. It was indicated that this vitamin has medicinal functions on some biochemical and metabolic parameters in the STZ-induced diabetic rats [56]. In the present study, whereas α-tocopherol and δ-tocopherol levels were decreased in the diabetes group, in contrast, these levels were increased in the bitter almond oil given group when compared to the control group. According to this result, it can be stated that the bitter almond oil was contributed to the protection of brain tissue from oxidative stress causing by the diabetes via increasing of α-tocopherol and δ-tocopherol levels. Lipophilic vitamins are necessary to maintain the physiological functions of the organisms and promoting of growth activity, and these play an important role in the digestion of nutrients. Some studies have shown that the vitamin deficiency increases the tendency of some diseases [57]. In this study, retinol and vitamin D_3 levels were significantly increased in the diabetic rats. These increases may be caused by the decrease of the activities of retinol and vitamin D carrier proteins [58,59]. These vitamin levels were decreased in the bitter almond oil given group when compared to the control group. This situation can be explained that the bitter almond oil was positively affected to the activities of carrier proteins of these vitamins.

Vitamin K is plenty present in the brain tissue and it is essential for brain functions [60]. In the present study, while the levels of vitamin K were increased in the diabetic rats, these levels were more increased in the bitter almond oil given group when compared to the control group. The diabetes increases to the sensitivity of tissues against to the oxidative stress and brain is one of these tissues. The increasing of the vitamin K levels can be caused by the relationship between balancing of oxidative stress and reducing of negative effects of its on the brain tissue in the diabetes. Previous studies have been reported that the vitamin K is necessary for brain structure and functions due to the inhibition ability of oxidative stress [60,61].

CONCLUSION

In conclusion, while the MDA level was significantly increased in the diabetes group, but its level was not changed in the bitter almond oil given group. Also, whereas the GSH level was significantly decreased in the diabetes group, its level was protected in the bitter almond oil given group. The fatty acid composition, lipid-soluble vitamins and cholesterol levels were protected or prevented by the bitter almond oil extract in the brain tissue of diabetic rats. The observed these positive results can be attributed to bitter almond oil, because it contains phytochemical compounds for used antioxidative and medicinal purposes, such as phenolics, flavonoids, sterols, vitamins.

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Original Research

In vitro erythrocyte oxidative damage of *Morinda citrifolia* L. (noni) leaves extract.

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Keywords: Red blood cell toxicity, water extract, fractions, methemoglobin, lipid peroxidation

Abstract

Slight decrease of hemoglobin and erythrocyte count was observed previously after subchronic oral dosing of Morinda citrifolia L leaves extract in rats. Induction of erythrocyte membrane damage could be the cause for these effects.

Aims: The objective of this investigation was to assess the in vitro cytotoxicity of Morinda citrifolia L leaves extract and fractions on rat erythrocytes.

Methods: Hemolytic damage was assayed in rat erythrocytes. Oxidative stress was assessed by measuring methemoglobin formation, thiobarbituric acid reactive substances (TBARS) and enzyme antioxidant activities, superoxide dismutase (SOD) and catalase (CAT).

Results: Morinda citrifolia L extract caused no hemolysis and induced oxidative damage to red cells in vitro. Methemoglobin increase was observed at concentration between 2 and 8 mg/ml of the extract. Lipid peroxidation was increased and CAT and SOD activities were depleted indicating a possible increase of hydrogen peroxide and superoxide radicals in erythrocytes. Ethyl acetate, dichloromethane and butanol fraction did not cause methemoglobin formation while water fraction increased methemoglobin level at doses up to 6 mg/ml.

Conclusions: We concluded that high doses of Morinda citrifolia L extract promote erythrocyte oxidative damage due to metabolites present in water fraction. These could be the cause of decreased erythrocyte and hemoglobin levels observed.

INTRODUCTION

Morinda citrifolia Linn, popularly known as ‘‘noni,’’ has been used in traditional Polynesian medicine for over 2,000 years. *M. citrifolia* (Rubiaceae) is native from southeast Asia to Australia and is cultivated in Polynesia, India, the Caribbean region, and central and northern South America [1, 2].Cultures native to these regions favored using *M. citrifolia* for treating major diseases and used it for nourishment in times of famine. Noni fruit has been recognized by cultures as an excellent source of nutrition [3]. Noni leaves have been consumed as a vegetable by many cultural groups. For this reason, it is included in the World Health Organization’s and Food and Agriculture Organization’s food composition tables for East Asia and the Islands of the Pacific [4].

Whereas noni juice and fruit have been well characterized chemically and pharmacologically [1-3], few data are available regarding the properties of *M. citrifolia* leaves. Despite the lack of experimental data on potential therapeutic properties, the use of noni for different purposes is still widespread in many parts of

the world. However, infusions prepared with the aerial parts (stems and leaves) of noni are used in folk medicine to treat painful conditions and as a sedative.

Previous evaluation of oral toxicity of *M. citrifolia* leaves extract in rats show slight variations in few hematological parameters after subchronic oral dosing. Hemoglobin and differential leukocyte count were significantly affected and erythrocyte count was marginally affected after *M. citrifolia* subchronic exposure. Hematological varations were within or close to normal range and reversibly. The extract was non-toxic and non-genotoxic according to the results of sub-acute and genotoxicity assays [5]. A possible explanation for the erythrocyte and hemoglobin reduction in treated animals could be the induction of oxidative erythrocyte membrane damage. The objective of this investigation was to assess the in vitro cytotoxicity of *Morinda citrifolia* L leaves aqueous extract and fractions on rat erythrocytes.

MATERIALS AND METHODS

Test substances

Leaves of *M. citrifolia* were collected in April in the Medicinal Plant Experimental Station "Dr. Juan Tomás Roig" (Güira de Melena, Artemisa, Cuba). Voucher specimen (Nº 4741) was deposited at the "Dr. Juan Tomás Roig" herbarium in the cited Experimental Station. The leaves were dried in a recycled air stove at 45ºC for two days. Dried *M. citrifolia* leaves were extracted with demineralized water at 100ºC for one hour with agitation. The extract obtained was dried with spray drier equipment as described previously [6]. Total dry powder obtained was used for the studies and was fractionated by solvent-solvent extraction procedure into dichloromethane, ethyl-acetate, *n*-butanol, and aqueous fractions for two successive 24 hr periods respectively.

To detect the presence of various chemical constituents in *M. citrifolia* extract, phytochemical screening was performed according to the method described by García [7]. The extract was qualitatively analyzed for the presence of essential oils, terpenoids, flavonoids, glycosides, amines, amino acids, oligosaccharides, alkaloids, anthraquinone compounds, and coumarins. The phytochemical screening of the extract showed the presence of terpenoids, flavonoids, amines, amino acids, and anthraquinone compounds.

The extract was standardized in accordance with the content of anthraquinone compounds and total anthracen-derived. Anthraquinone compounds were performed by quantification of colored phenols obtained by chemical reaction of alkali and anthracen-derived. Total anthracen-derived content was determined by quantification of colored phenols obtained by anthracen-derived phenols oxidation with ferric chloride in acid medium. Quantification was performed by using a spectrophotometer at 525 nm. Reference substance used was cobalt chloride 1% in ammonium alkaline solution equivalent to 0.43 mg of oxianthraquinone. Results were expressed as % w/v from calibration curve (r^2=0.999)[8]. *M. citrifolia* total extract with 2.09% of anthraquinone compounds and 11.21% of total anthracen-derived was used in the studies [6].

Animals

Animal care was performed in conformity with Canadian Council for Animal Care guidelines [9]. Healthy male Wistar (Cenp:Wistar) rats, 200-250 g of body weight, were used for obtaing blood. Animals were obtained from the Laboratory Animal National Centre (CENPALAB), Havana, Cuba and were housed together in polycarbonate cages in a light- and humidity-controlled biohazard suite (24 ± 2 °C; 55 ± 5% relative humidity), with a 12-hour light-dark cycle, and free access to drinking water and a standard laboratory diet CMO1000 (CENPALAB).

Evaluation with M. citrifolia total extract

Hemolysis test: Rat blood, containing heparin as anticoagulant, was centrifuged and the plasma and buffy coat discarded. The cells were washed four times with isotonic phosphate buffer (pH 7.4) and adjusted via oxyhemoglobin concentration (0.125 mmol/l) corresponding to about $8x10^9$ cells/ml for hemolysis test. Hemolysis assay was performed as described previously [10]. Five equidistantly increasing concentrations of the test sample and sodium dodecyl sulfate (SDS) as positive control were assayed. Percentage of hemolysis was determined by comparing the absorbance (560nm) of the supernatants with that of hemolyzed control samples.

For erythrocyte oxidative damage measure, the red blood cell (RBC) suspension was resuspended in isotonic phosphate buffer to a hematocrit of 5%. Test materials were added as solutions in purified water at concentration between 0 and 8 mg/ml; control suspensions received purified water alone. Additionally, a control vial with high dose of the test solution without RBC suspension was tested to discard color interference. The cell suspensions were incubated with test solutions at 37ºC in a shaking water-bath. All experiments were performed in triplicate.

Methemoglobin formation: Methemoglobin level in erythrocytes was determined by the method of Evelyn and Malloy [11] in which the conversion of methemoglobin into cyan-methemoglobin is monitored spectrophotometrically at 635nm following the

addition of sodium cyanide.

Measurement of lipid peroxidation: Oxidative damage in erythrocytes was assessed by measuring the rate of lipid thiobarbituric acid reactive substances (TBARS) which were determined by a method based on the reaction of thiobarbituric acid with malondialdehyde (MDA) or MDA-like substances to produce a pink pigment with an absorbance maximum at 532nm [12].

Measurement of antioxidant enzyme activities: Catalase (CAT) activity was determined by the kinetic assay following the method of Beers and Sizerin [13] in which the disappearance of peroxide is monitored spectrophotometrically at 240nm. Catalase activity was expressed as unit of CAT / mg of protein.

Superoxide dismutase (SOD) activity was determined indirectly by a method based on inhibition of pyrogallol autoxidation [14]. Autoxidation of pyrogallol was recordered spectrophotometrically at 420 nm during 1 min after addition of pyrogallol (0.1 mM final concentration) in medium containing 50 mM TRIS buffer (pH 8.2). The linear slope (with r^2>0.99) was calculated, and the increase in absorbance after this time was taken as the initial rate of autoxidation. Linear slope inhibition was indicative of SOD activity.

Evaluation with M. citrifolia fractions

Dichloromethane, ethyl-acetate, *n*-butanol, and aqueous fractions were tested for methemoglobin formation as described above to identify the fractions with erythrocyte toxicity *in vitro*. Dichloromethane, ethyl-acetate, and *n*-butanol fractions were re-suspended in dimethyl sulfoxide, and dissolved in purified water up to concentration of 10 mg/mL. Fractions were tested at concentrations between 0 and 0.8 mg/mL. Upper concentrations were not tested for solubility. Water fraction was tested in purified water at concentration between 0 and 14 mg/ml.

Statistics

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

RESULTS

Evaluation with M. citrifolia total extract

No hemolysis was observed when *M. citrifolia* extract was incubated with rat erythrocytes (Fig. 1). *M. citrifolia* extract induced a significant and dose dependent increase in methemoglobin levels at concentrations between 2 and 8 mg/ml (Fig. 2).

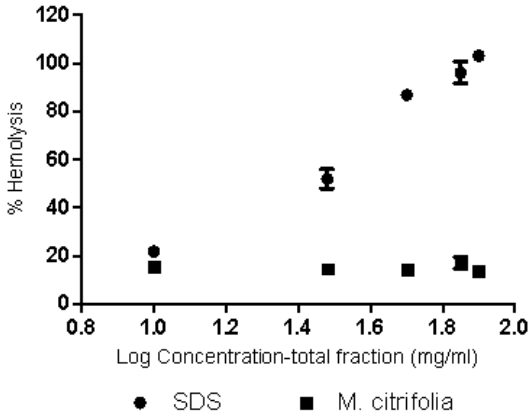

Figure 1. Relationship between logarithm of test samples concentration and % of hemolysis. The data are expressed as mean ± SEM of 3 determinations.

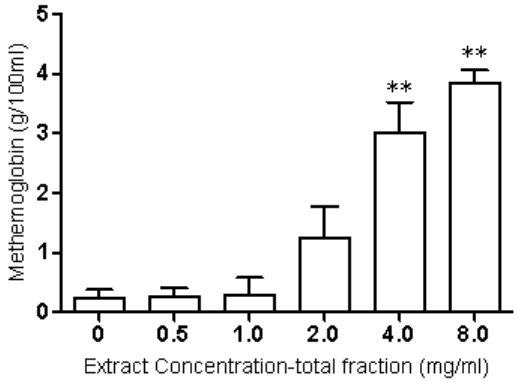

Figure 2. Effect of *M. citrifolia* extract on rat erythrocyte methemoglobin (g/100ml) level. The data are expressed as mean ± SEM of 3 determinations. ***p* < 0.01 (significantly different from control).

M. citrifolia extract induced an increase in TBARS levels of rat erythrocytes at doses between 2.0 and 8.0 mg/ml, which was significantly different at doses of 8.0 mg/ml (Fig. 3). Catalase activity was significantly reduced after incubated *M. citrifolia* extract with rat erythrocytes. Inhibition of spontaneous oxidation of pyrogallol was observed similarly in untreated and treated erythrocytes at 0.5-2.0 mg/ml of *M. citrifolia* extract (Fig. 4). The doses of 4.0 and 8.0 mg/ml of *M. citrifolia* extract affected the spontaneous oxidation of pyrogallol. This effect was statistically significant at dose of 8.0 mg/ml (p < 0.05).

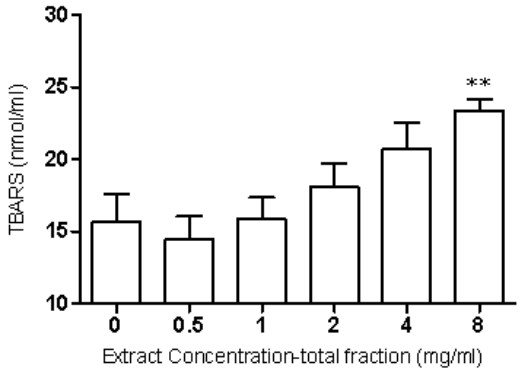

Figure 3. Effect of *M. citrifolia* extract on erythrocyte TBARS (nmol/ml) level in rats. The data are expressed as mean ± SEM of 3 determinations. $^{**}p < 0.01$ (significantly different from control).

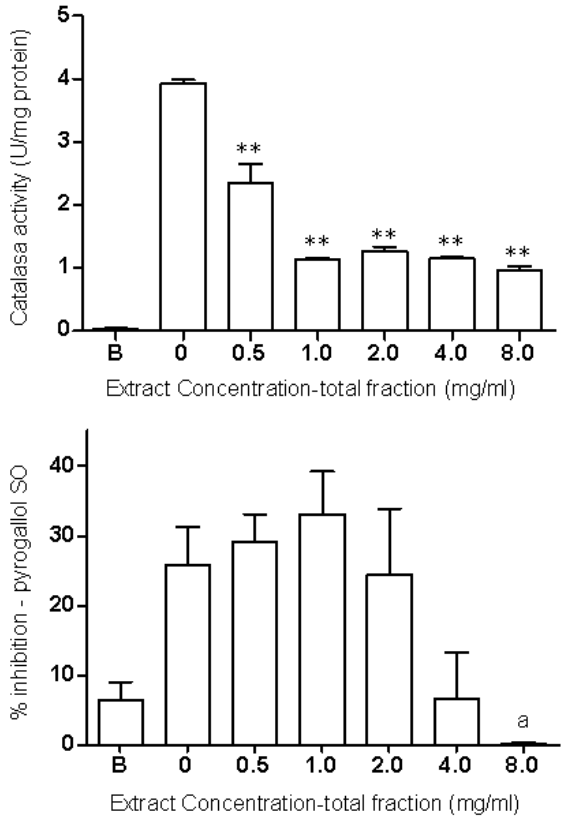

Figure 4. Effect of *M. citrifolia* extract on rat erythrocyte CAT and SOD activities. The data are expressed as mean ± SEM of 3 determinations. $^{**}p < 0.01$ (significantly different from control). SO: spontaneous oxidation, a: significantly different from control, 0.5, 1.0 and 2.0 mg/ml of *M. citrifolia* extract, ($p < 0.05$).

Evaluation with Morinda citrifolia fractions

Cytotoxicity was not observed on erythrocytes with all fractions at doses between 0.1 and 0.8 mg/ml. Dichloromethane, *n*-butanol and ethyl acetate fractions did not increase methemoglobin formation. Increase of methemoglobin formation was observed with water fraction at doses up to 6 mg/ml (Fig. 5). These results were similar to the cytotoxicity observed with total extract.

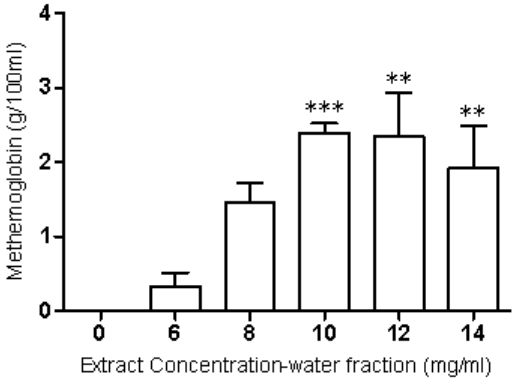

Figure 5. Effect of *M. citrifolia* water fraction on rat erythrocyte methemoglobin (g/100ml) level. The data are expressed as mean ± SEM of 3 determinations. $^{**}p < 0.01$, $^{***}p < 0.001$ (significantly different from control).

DISCUSSION

Some changes were observed in the previous study [5] carried out to evaluate the subchronic 13-week repeated oral dose toxicity of *M. citrifolia* extract. Hemoglobin and differential leukocyte count were significantly affected and erythrocyte count was marginally affected after *M. citrifolia* subchronic exposure. Hematological variations were within or close to normal range and reversibly [5]. A possible explanation for the erythrocyte and hemoglobin reduction in treated animals could be the induction of erythrocyte membrane damage. In our study we evaluated the *in vitro* cytotoxicity of *M. citrifolia* leaves aqueous extract and fractions on rat erythrocytes. Membrane rupture was not observed after incubation of *M. citrifolia* extract with rat erythrocytes.

Dose dependent increase of methemoglobin level at concentration between 2 and 8 mg/ml of the extract was observed. This result indicates the hemoglobin oxidation in erythrocytes after exposure with the extract probably due to oxidative damage of RBC. Lipid peroxidation was increased and CAT and SOD activity were depleted at dose dependent manner indicating a possible increase of hydrogen peroxide and superoxide radicals in erythrocytes.

The reactive oxygen species (ROS) generation in tissues is efficiently scavenged by the enzymatic and nonenzymatic antioxidants. The decrease in the activities of antioxidant enzymes is in close

relationship with the induction of lipid peroxidation [15]. The components of the defense system, which have evolved to reduce and contain the injury from free-radical attack, include several enzymes and a few free-radical scavenger molecules [16]. SOD plays an important role in protecting the cells from oxidative damage by converting superoxide radicals into hydrogen peroxide, which is further metabolized by CAT to molecular oxygen and water. SOD is the first enzyme of the scavenger enzyme series to ameliorate the damage caused to cells by free radicals [17], while CAT is one of the several cellular antioxidant enzymes that provide a defense system for the scavenging of reactive oxygen metabolites.

The inhibition of pyrogallol autoxidation brought about by superoxide dismutase can be employed in a rapid and convenient method for the determination of the enzyme. Results suggested that SOD activity could be depleted at 4.0 and 8.0 mg/ml *M. citrifolia* extract treated erythrocytes probably due to superoxide radical generation. Dimitrova *et al*. [18] suggested that superoxide radicals and/or their transformation to hydrogen peroxide cause a cysteine oxidation in the enzyme and decrease SOD activity.

M. citrifolia leaves extract has been reported to possess antioxidant activity. Serafini *et al*. [19] reported the significant antioxidant effect of aqueous extracts from *M. citrifolia* leaves by protection against lipid peroxidation, hydroxyl radical–scavenging capacity and nitric oxide–scavenging activity at doses between 1 µg/ml and 1mg/ml. However, the authors suggested that the dosage used could be high, and recommended additional studies to evaluate the potential toxicity of the extract.

In the present study we evaluated the toxicity of *M. citrifolia* leaves extract. Doses over 2 mg/ml of *M. citrifolia* leaf extract produced *in vitro* hemoglobin oxidation with increase of H_2O_2 and $O_2\bullet-$ generation. Antioxidative activity of the methanol crude extract and ethyl acetate extract of leaves, fruit and roots of *M. citrifolia* was report by Zin *et al*. [20]. The antioxidative activities were measured using ferric thiocyanate (FTC) and thiobarbituric acid (TBA). The FTC method was used to measure the peroxide level during the initial stage of lipid oxidation. In this method, low absorbance values would indicate high levels of antioxidative activity. Results showed that the methanol extracts of both fruit and leaf of *M. citrifolia* had negligible antioxidative activities, and were not significantly different from the control. However, the results showed significant increase in absorbance values for leaves and fruit extract. Measurement of malonaldehyde by the TBA method characterize the gradual decomposition of peroxides during the oxidation process. In this report, significant increase in absorbance values for leaf and fruit extract was observed too. The authors did not discuss this finding but increased peroxide generation could be occurring with leaves and fruit extracts.

Findings of this study showed that high concentrations of *M. citrifolia* extract could induce erythrocytes toward oxidative damage. Oxidative stress has been suspected in several pathologies including intoxication, genotoxicity and cancer development [21, 22]. Reductions in the erythrocyte count of treated rats observed previously [5] could be a consequence of oxidative stress complication which is incriminated to induce hemolysis by shortening RBC survival and increasing their fragilities. Results of fractions evaluation on hemoglobin oxidation of rat erythrocytes suggest that resposible metabolite is content in water fraction.

CONCLUSIONS

M. citrifolia leaves extract at high dose could be provoque hemoglobin oxidation in rat erythrocytes *in vitro* with lipid peroxidation and increase of H_2O_2 and $O_2\bullet-$ generation. Components responssible for this effect could be found in water fraction of the extract. These effects could be the cause of erythrocyte and hemoglobin decrease observed previously in rats.

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Original Research

Evaluation of the anti-ulcer properties of the aqueous methanolic leaf extract of *Palisota hirsuta* and its fractions in mice.

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Abstract

Objective: This study evaluated the effects of aqueous methanolic leaf extract of Palisota hirsuta (MLEPH), against experimentally induced gastric ulceration in mice.

Materials and methods: The plant material was extracted with 70% methanol for 48h and concentrated in vacuo with rotary evaporator, yielding 8.77%w/w MLEPH. MLEPH (50, 100, 150 mg/kg) and MLEPH fractions (MLEPHfr) (50 mg/kg) were studied in various ulcer models: acetyl salicylic acid (ASA), HCl-ethanol and cold restraint stress-induced ulcer models.

Results: MLEPH at all doses used significantly reduced ($P<0.05$), the mean number of ulcers (MNU), while mean ulcer index (MUI) was significantly reduced by 50 and 100 mg/kg in ASA-induced ulcer compared with 10 ml/kg distilled water. The highest ulcer preventive index (UPI) of MLEPH (50 mg/kg) was 77% compared with 100 mg/kg cimetidine (83%). In the HCl-ethanol-induced gastric ulceration, MLEPH at 50 mg/kg reduced the MUI though not significantly ($P>0.05$) and gave a UPI of 33%. In cold restraint stress, MLEPH (50 and 100 mg/kg) decreased both MNU and MUI. However this was not significant ($P>0.05$) compared with the control. Both doses produced UPI of 57% compared with cimetidine (95%). The MLEPHfr7 (50 mg/kg), significantly decreased ($P<0.05$) MNU compared with both distilled water and cimetidine and MUI compared with cimetidine in HCl-ethanol-induced gastric ulceration. The UPI for MLEPHfr7 was 58% compared with cimetidine. The phytochemical constituents of MLEPHfr7 include tannins and reducing sugars.

Conclusion: Our study shows that MLEPH and MLEPHfr exhibited promising antiulcer properties, mediated by tannins and reducing sugars through cytoprotective mechanisms.

INTRODUCTION

Peptic ulcer is a common clinical problem in humans with a lifetime risk of at least 10% [1]. Millions of people are affected each year, imposing a major economic burden on the health care system. Increasing age of the population at risk, changes in smoking prevalence, and increasing use of non-steroidal anti-inflammatory drugs for pain and arthritis, have impacted negatively on the epidemiology of peptic ulcer [2, 3]. The current therapeutic agents used in the management of peptic ulcers are not free of serious side effects [4]. Medicinal plants could be a source of alternative therapy for the treatment and management of peptic ulcer. But surprisingly most of the plants used for this purpose have not been scientifically tested for efficacy and safety. Therefore the need for accurate and up to date information on the properties, uses, efficacy, safety and quality of plant preparations has arisen. Thorough search through literature has not shown any scientific report to support the use of *P.hirsuta* in the treatment of peptic ulcer. *Palisota. hirsuta* K. schum. (*Commelinaceae*) is a robust herb of forest regions,

about 2 – 4 cm high reproducing from seeds. The leaves are arranged in rosettes, mostly at the terminal of the stem and are obvate to oblanceolate about 15 – 30 (40) cm long and 4 – 11.5 cm broad. It is found in farm lands of forest zones of eastern Nigeria, Northern Cameroun, Guinea and Ghana [5]. It is called "Thumb" in English, "*Ikpere anukwu*" (knee of a great animal) in the Igbo tribe of South-Eastern Nigeria. Various parts are used traditionally for different purposes. Roots are used to treat dysentery, anemia and rheumatism. Whole plant for analgesic and antiseptic while a leaf decoction is used for colic, peptic ulcer, diarrhea, boil and cough [6, 7, 8]. The ethanolic leaf extract of *P. hirsuta* has been reported to have anxiolytic and anti-depressant effects in mice [9]. Total flavonoids extracted from the leaves showed significant sexual stimulation in rats [10]. The anti-inflammatory and anti-pyretic effects of an ethanolic root extract of *P. hirsuta* in chicks and rats have been reported [11]. Anti-nociceptive, local analgesic and anti-bacterial effects of the methanolic leaf extract have also been demonstrated [12].

In this study we evaluated the effects of the aqueous methanolic leaf extract of *P. hirsuta* and its fractions against acetyl salicylic acid, HCl-ethanol and cold restraint stress-induced gastric ulcerations.

MATERIALS AND METHODS

Plant Extraction

Fresh green leaves of *Palisota hirsuta*, were collected from Obukpa in Nsukka Local Government Area of Enugu state, South Eastern Nigeria. They were authenticated as *P. hirsuta* at the Department of botany University Nigeria Nsukka. Voucher specimen was also kept in the Department of Botany herbarium. The leaves were dried under laboratory conditions at the temperature range of 25-27°c for about 10 days and pulverized to a coarse powder (mesh size1.00 mm) using hammer mill. About 382 g of the plant material was extracted by cold maceration using 70% methanol with intermittent shaking for 48 h. The extract (MLEPH) was filtered with whatmann No.1 filter paper and concentrated *in vacuo* to dryness using rotary evaporator ((Buchi Labotechnik, Switzerland). The MLEPH was separated into fractions in accordance with the method described by Harbourne [13]. Briefly Silica gel 60 G for column chromatography was used as the stationary phase, while petroleum-ether-chloroform-ethyl acetate-methanol was the mobile phase (table 1). The eluents were spotted on pre-coated silica gel F254 aluminum plate and eluted with chloroform-methanol-ethyl acetate (1:3:1) in a small chromatographic tank to separate the various fractions based on their relative mobility and color reactions with UV light ((Buchi Labotechnik, Switzerland). The fractions were concentrated to dryness using rotary evaporator at 200 milibar and 40°c. The percentage yield (w/w) of the extract and fractions were calculated using the formula below:

$$\frac{\text{Weight of extract}}{\text{Weight of starting material}} \times 100 = X\ \%w/w$$

Table 1. Solvent System for Chromatographic Separation of MLEPH

Pet ether %	Chloroform %	Ethyl acetate %	Methanol %
100			
70	30		
50	50		
20	70	10	
	80	20	
	60	40	
	40	50	10
	20	60	20
		70	30
		50	50
		30	70
		10	90
			100

Experimental animals

Mature albino mice of both sexes (20 – 30g) were procured from the laboratory animal unit of Faculty of Veterinary Medicine University of Nigeria Nsukka. They were provided feed and water *ad libitum.* All animal experiments were in accordance with the guide line stipulated by National institute of Health for care and use of laboratory animals (Pub. No. 85: 23 revised 1985).

Drugs: Acetyl salicylic acid tablets (Sigma, Spain), cimetidine (Zim laboratories Ltd India), ethanol (Reidel De Haen, Germany), HCL (B.D.H. England). All solvents for chromatography were of analytical grade from Sigma Aldrich.

Chromatographic materials: Silica gel (60G) (Vicker' West York England), silica gel TLC plate (GF254) (Merck, Germany).

Acetyl salicylic acid (ASA)-induced Gastric Ulceration

Mice fasted for 16 h prior to experiment, but have access to clean water were randomly divided into five groups of five. Gastric ulceration was induced as described by Williamson *et al* [14]. They were treated with 50, 100 and 150 mg/kg b.w. MLEPH respectively; while the control groups received either 10ml/kg b.w. distilled water or 100 mg/kg b.w.

cimetidine. Ulcer was induced 1 h later with 200 mg/kg b.w. ASA. All the animals were sacrificed 4 h post administration of ASA under light ether anesthesia and their stomachs isolated, incised along the greater curvature, and then rinsed in normal saline. The stomachs were pinned on a cork-board and observed with a hand lens by an independent observer who did not know about the treatment protocol to avoid bias. The number and size of ulcers were determined and the mean number of ulcer (MNU), mean ulcer index (MUI) and preventive index (UPI) for each group were determined according to the method described by Obi *et al* [15]as follows: 0 = no ulcer, 1 = superficial ulcer, 2 = deep ulcer, 3 = perforation.

$$\textbf{UI} = \frac{\text{no. of ulcer} \times \text{ulcer size}}{10}$$

(10 = magnificationof hand lens)

$$\textbf{UPI} = \frac{(\text{UIc} - \text{UIt})}{\text{UIc}} \times 100$$

(UIc = ulcer index of negative control, UIt = ulcer index of any treated group).

HCl-ethanol-induced Gastric Ulceration

Gastric mucosal damage was induced according to the method of Malairajan *et al* [16]. Briefly, twenty five mice were randomly divided into five groups of five per group. They were fasted for 16 h before the experiment, but have free access to clean water. Group1 received 10 ml/kg b.w. distilled water, group 2 received 100 mg/kg b.w. cimetidine, while groups 3 - 5 received 50, 100 and 150 mg/kg b.w. MLEPH respectively. For the MLEPH fractions, 45 mice grouped into 9 groups of 5 each were treated with 50 mg/kg b.w. of fractions 1 – 7, while the controls either received 10 ml/kg b.w. distilled water or 100 mg/kg b.w. cimetidine. Ulcer was induced with HCl-ethanol (0.1N HCl and 80% ethanol), 1 h post treatment. The animals were sacrificed 1 h later under light ether anesthesia and stomachs isolated, incised along the greater curvature and rinsed in normal saline. The stomachs were pinned on a cork board and observed by an independent observer, who did not know anything about treatment protocol to avoid bias. The number of ulcers was determined and each ulcer given a severity score on a scale of 1 - 4 as follows: ≤1mm = 1, ≤ 2mm = 2, ≤ 3mm = 3 or perforations = 4 [17, 18]. MUI and UPI were determined as earlier described.

Cold Restraint Stress-induced gastric Ulceration

Gastric mucosal damage was induced according to the method File and Pearce [19], Al-rehaily *et al* [20] with modifications. Twenty five albino mice fasted for 16 h, but have free access to clean water, were randomly divided into five groups of five each and were treated with 50, 100 and 150 mg/kg b.w. MLEPH, while the controls received either 10 ml/kg b.w. distilled water or 100 mg/kg b.w. cimetidine. The mice were immobilized in a restraint cage and placed in a ventilated refrigerator maintained at 4°c and 2 h later they were removed and sacrificed under light ether anesthesia and stomachs isolated, incised along the greater curvature and rinsed in normal saline. The stomachs were pinned on a cork board and observed by an independent observer, who did not know anything about the treatment protocol to avoid bias. The number of ulcers, size of ulcers, MUI and UPI were determined as already described [15].

Data obtained for number of ulcers and ulcer index were analyzed using one way analysis of variance and results expressed as means ± standard error of mean. Variant means were separated post-hoc using the least significant difference (LSD). Significance was accepted at the probability level $P<0.05$. Preventive index were presented as percentages.

RESULTS

Plant Extraction and phytochemical Constituents of Fractions

The methanolic leaf extract of *P.hirsuta* (MLEPH) yielded 8.77 %w/w material which was dark green in color, pasty in consistency and has a sharp pungent smell. Column chromatographic separation of MLEPH yielded seven fractions. The phytochemical constituents of MLEPHfr7 (most active fraction) include tannins and reducing sugars.

Effects of MLEPH on ASA-Induced Gastric Ulceration

The effect of MLEPH on ASA-induced gastric ulceration is presented in table 2. The extract (50, 100 and 150 mg/kg b.w.) caused significant ($p<0.05$) reduction in the mean number of ulcers when compared with 10 ml/kg distilled water. Mean ulcer index was also significantly ($p<0.05$) decreased by 50 and 100 mg/kg b.w. MLEPH when compared with 10 ml/kg distilled water. There was no significant ($p>0.05$) difference between the MLEPH treated groups and cimetidine (100 mg/kg). The highest ulcer preventive index of MLEPH (50 mg/kg) was 77 %, compared with 100 mg/kg cimetidine (83 %).

Table 2. Effects of MLEPH on ASA-induced Gastric Ulceration

Groups	Mean no. of ulcers	Mean UI	PI (%)
A	6.6±0.68	1.30 ± 0.20	0
B	1.4±0.51*	0.22 ± 0.09*	83
C	2.2±0.66*	0.30 ± 0.11*	77
D	2.4±0.51*	0.44 ± 0.20*	66
E	2.4±0.51*	0.68 ± 0.39	48

Mean ±standard error of mean* is significant (P<0.05) compared with A. A: 10 ml/kg distilled water treated mice; B: 100 mg/kg cimetidine treated mice; C-E (50, 100 and 150 mg/kg) MLEPH treated mice respectively.

Effects of MLEPH on HCl-ethanol-induced Gastric Ulceration

MLEPH (50 and 150 mg/kg) decreased the ulcer index, though not significantly (p>0.05) when compared with the control as shown in table 3. There ulcer preventive indexes were 33 and 23% respectively and these effects were better than that of cimetidine (100 mg/kg).

Table 3. The effects of MLEPH on HCl-ethanol-induced gastric ulceration

Groups	Mean No. of ulcers	Mean UI	PI (%)
A	15.2 ± 1.16	4.32± 0.54	0
B	14.6 ± 1.89	4.38 ± 0.57	0
C	13.2 ± 1.66	2.88 ± 0.91	33
D	16.4 ± 1.63	4.44 ± 0.89	0
E	14.0 ± 2.43	3.30 ± 1.09	23

Mean ± standard error of mean. A: 10 ml/kg distilled water treated mice; B: 100 mg/kg cimetidine treated mice; C-E (50, 100 and 150 mg/kg) MLEPH treated mice respectively.

The Effects of MLEPH on Cold Restraint Stress-induced Gastric Ulceration

MLEPH (50 and 100 mg/kg) decreased both mean number of ulcers and mean ulcer index, though the decrease was not significant (p>0.05) when compared with the control. Both doses of MLEPH also produced ulcer preventive index of 57% compared with cimetidine (95 %) (table 4)

Table 4. Effects of MLEPH on cold restraint stress-induced gastric ulceration

Groups	Mean No. of ulcers	Mean UI	PI (%)
A	7.2 ± 2.92	0.88 ± 0.04	0
B	0.4 ± 0.24*	0.04 ± 0.02*	95
C	2.8 ± 1.50	0.38 ± 0.19	57
D	2.4 ± 0.81	0.38 ± 0.12	57
E	7.0 ± 1.61	0.94 ± 0.27	0

Mean ± standard error of mean*is significant (P<0.05) compared with A. A: 10 ml/kg distilled water treated mice; B: 100 mg/kg cimetidine treated mice; C-E (50, 100 and 150 mg/kg) MLEPH treated mice respectively.

The Effects of MLEPHfr on HCl-ethanol-induced Gastric Ulceration

Fraction7 caused significant (p<0.05) decrease in mean number of ulcers when compared with both 10 ml/kg distilled water and 100 mg/kg cimetidine, while fractions 5, 6 and 7 significantly (p<0.05) decreased the mean ulcer index when compared with cimetidine (100 mg/kg). The MLEPHfr7 produced the highest ulcer preventive index (58%), followed by MLEPHfr5 and 6 (52%) (table 5). Thus MLEPHfr7 was considered the most active fraction.

Table 5. Effects of MLEPH fractions on HCl-ethanol-induced gastric ulceration

Groups	Mean no. of ulcers	Mean UI	PI (%)
A	15.0±3.51	3.77 ± 0.81	0
B	16.0±4.04	4.60 ± 0.91	0
C	8.33±3.28	2.03 ± 0.93	46
D	8.67±2.03	2.63 ± 1.1	30
E	8.67±3.38	2.67 ± 1.3	29
F	10.33±0.88	2.00±0.15	45
G	10.67±3.18	1.80±0.90*	52
H	8.00±2.08	1.80±0.55*	52
I	5.67±2.03*	1.60±0.78*	58

Mean ± standard error of mean* is significant (P<0.05). Mean ± sem** compared with B. A: 10 ml/kg distilled water treated mice; B: 100 mg/kg cimetidine treated mice; C-I (50 mg/kg) MLEPHfr1-7 treated mice respectively.

DISCUSSION

The outcome of this study showed that the leaf extract of *P. hirsuta* and its fractions exhibited antiulcer activity by providing different degrees of protection against ASA, HCl-ethanol and cold restraint stress-induced gastric ulcerations in treated mice. Ulcers are as a result of imbalance between protective (mucous and bicarbonate) and aggressive (acid and pepsin) factors in the stomach [21]. Acetyl salicylic acid-induced gastric damage is due to direct irritant effect of ASA on gastric mucosa, increased acid secretion and decreased mucin secretion due to inhibition of prostaglandins (PG) synthesis [22, 4]. These effects ultimately break down gastric mucosal barriers. Pretreatment of mice with MLEPH and subsequent protection from gastric mucosal damage induced by ASA reflected a clear tendency to enhance gastric mucosal protective mechanisms in spite of ASA-induced depletion. Gastric damage induced by HCl-ethanol is possibly through direct corrosive effect on gastric epithelium, leading to mucosal damage on the

glandular part of the stomach [16]. Biochemical changes include disturbances of mast cells and release of histamine [23]. Since cytoprotective agents are known to inhibit HCl-ethanol-induced gastric damage [24], it is most likely that MLEPH and MLEPHfr7 has cytoprotective properties. HCl-ethanol-induced ulcer model was chosen for the evaluation of the MLEPH fractions because it produces stronger gastric irritation and mucosal damage than the other models, therefore it will make for observable differences in the antiulcer effects of the fractions. MLEPHfr7 exhibited stronger ulcer protective effect than MLEPH, suggesting that purification of MLEPH to concentrate the active principles is necessary for maximal effect. Cold restraint is one of the best models of stress in mice as it provides both psychological and physiological stress to the animal [25]. Pathogenesis of cold restraint stress-induced ulcer is related to hyper-secretion of gastric acid, decrease in gastrointestinal mucosal blood flow and hyper-contractility [25, 26, 27, 28]. The gastric mucosal protection produced by MLEPH against cold restraint stress suggests anti-acid secretory activity and modulation of mucosal microcirculation and contractility. The phytochemical constituents of MLEPHfr7 which are tannins and reducing sugars seem to mediate the antiulcer effects of MLEPH as they are known to have cytoprotective effects in other medicinal plants. Plant tannins are astringent in nature and confer on them the ability to form complexes with proteins, starches and other macromolecules, thus forming impervious layer over the mucosal linings and protecting them from irritants [29]. Reducing sugars are known to be antioxidants, therefore could have protected the gastric mucosa from the oxidative effects of HCl-ethanol [30]. Generally in all the ulcer models used in this work the lowest dose of the extract (50 mg/kg, b.w.) produced the highest ulcer preventive index showing that higher doses of the extract could be irritating to the gastric mucosa. In conclusion, the methanolic leaf extract of *P. hirsuta* exhibited different degrees of antiulcer properties in different ulcer models through multiple mechanisms possibly due the different phytochemical constituents. Fraction 7 exhibited the strongest cytoprotective effect mediated by tannins and reducing sugars, justifying the folkloric use of *P. hirsuta* in the treatment of peptic ulcers in Nigeria.

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Original Research

Effects of long-term use of polyphenols on the absorption and tissue distribution of orally administered metformin and atenolol in rats

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Keywords: Polyphenols, metformin, atenolol, absorption, tissue distribution

Abstract

Aim: To evaluate the effect of long-term use of silibinin, epigallocatechin (ECGC), quercetin and rutin on the absorption and tissue distribution of metformin and atenolol. Materials and

Methods: Thirty male rats were used, allocated into 5 groups and treated as follow: 1st group treated with olive oil and served as control; the other 4 groups were treated with either silibinin, EPGC, quercetin or rutin, administered orally as oily solutions for 30 days. At day 30, a 300mg/kg metformin and 50mg/kg atenolol were administered orally; 3.0 hrs later, the animals were sacrificed and blood samples, tissues of brain, kidney and liver were obtained for evaluation of the drugs level.

Results: The polyphenols increased both serum and tissue levels of metformin compared with controls. This effect was relatively varied according to the structural differences among flavonoids.

Conclusion: Long-term administration of silibinin, EGCG, quercetin or rutin increase oral absorption and tissue distribution of metformin, while atenolol was not affected; the effects of the studied polyphenols varied in accordance with the variations in their structural formulas.

INTRODUCTION

With the increasing interest in complementary therapy, plant-derived products are consumed by at least 10% of the general population and 30-70% of individuals with specific pathological conditions [1,2]. However, dietary supplements are not classified as drugs and do not require Food and Drug Administration (FDA) approval to be marketed. Co-administration of flavonoids with certain clinically used drugs may cause flavonoid-drug interactions by modulating the pharmacokinetics of certain drugs, which results in an increase in their toxicity or a decline in their therapeutic effect, depending on the structure of the ingested polyphenols [3]. This subject is of particular importance in assessing the safety of concentrated flavonoid food supplements or plant-derived products, particularly if their plasma concentrations stay high after ingestion [4,2]. Concerning the safety threatening aspect, the risks of adverse effects due to pharmacological interactions between herbal medicinal products and conventional therapies are highly considered [5]. These are often underestimated for two main reasons: consumers generally consider herbal medicinal products “safe” because of their natural origin, and as self-care products they are often taken without consulting a physician [6]. The efficacy of drug therapy depends on many factors related to a drug's pharmacokinetic and pharmacodynamic properties, which can be modified by differences in genetic polymorphisms, age, gender, circadian rhythms, intestinal bacteria,

pathophysiological conditions, pharmaceutical dosage form and xenobiotics [7-11]. One particular case is the co-administration of traditional drugs and herbal medicinal products (*i.e.* dietary supplements containing medicinal herbs or the herbal medicines traditionally used in phytotherapy for treating or preventing diseases), which may cause unexpected interactions [12]. Most interactions affecting absorption usually result in a reduction of the absorption of the drug, although increases in absorption can occur. Herbal drugs are more likely to inhibit absorption by forming complexes, for example with metal cations such as calcium, tannins and polyphenols in water extracts. Moreover, drug displacement from protein-bound forms, by concurrent drug administration, causes an increase in serum drug levels and therefore an increase in the therapeutic effect. It has been realized that this mechanism of interaction was grossly over-emphasized, because *in vitro* studies are not necessarily reflected by what happens *in vivo* [13]. The present study was designed to evaluate effect of long-term use of the flavonoids, silibinin, EGCG, quercetin and rutin, on the intestinal absorption and distribution of orally administered single doses of metformin and atenolol in rats.

MATERIALS AND METHODS

Chemicals and reagents

Silibinin dihemisuccinate (SDH) (98% purity) was obtained from Tolbiac SRL, Argentina; Quercetin dihydrate (98% pure standardized extract) was purchased from Xian Co, China; Epigallocatechin gallate (EGCG) was a gift from Al-Razi Pharm Ind, Syria; Rutin was obtained from Merck Laboratories, Germany; atenolol and metformin were obtained as a standardized powder from SDI, Iraq.

Animals and study design

Thirty male adult Sprague Dawly rats of body weight 200-250g were obtained from the Animal House, Department of Pharmacology and Toxicology, College of Pharmacy, Baghdad University, and the experiments were carried out in the Department of Pharmacology, College of Pharmacy, Al-Basra University, Iraq. The rats were housed under controlled conditions (22-25°C) on a 12 h light/12 h dark cycle, and received the standard pellet diet (National Center for Drug Research and Quality Control, Baghdad) and water *ad libitum*. The study protocol was approved by the Institutional Animal Ethical Committee (IAEC), College of Pharmacy, University of Baghdad. After acclimatization for a period of one week, the animals were allocated into five groups consisting of 6 rats each; first group was treated with vehicle (olive oil) as control group; the other four groups are treated with one of the flavonoids: SDH (100 mg/kg), EGCG (25mg/kg); Quercetin (50mg/kg) and Rutin (500mg/kg). All flavonoids are prepared as oily solutions dissolved in olive oil and introduced as single daily doses administered orally using gavage tube for 30 consecutive days; the control group receives 0.2 ml/day of olive oil in the same way. At day 30, all groups of rats received orally single doses of metformin (300mg/kg) and atenolol (50 mg/kg) by gavage needle administered 2.0 hrs after the last doses of the flavonoids and the vehicle.

Sample preparation

After 3.0 hrs of drugs administration, all animals were sacrificed after short duration anesthesia with anesthetic ether; blood samples were drawn and collected in polyethylene tube, centrifuged at 10000 rpm for 20 min and the resulted serum was kept frozen at -20°C until analysis. The liver and both kidneys were quickly removed, and perfused with ice-cooled saline; the brain was carefully excised, rinsed with ice-cooled saline and the arachnoid membrane was carefully removed. Ten milligrams tissue from each organ was homogenized in ice-cooled phosphate buffer saline, and utilized for analysis of atenolol and metformin tissue levels.

Analysis of atenolol and metformin in serum and tissues

A 50 µL aliquot of serum or tissue homogenate sample was deproteinized with a 100 µL aliquot of acetonitrile. After vortex-mixing and centrifugation (16,000 g, 10 min), a 30 µL aliquot of the supernatant was injected directly onto a reversed-phase (C18) HPLC (Knauer, Germany). The mobile phase (25% acetonitrile and 75% pH 7.0, 0.03 M $(NH_4)_2$ HPO_4) was pumped at a flow-rate of 1.0 ml/min. The prepared mobile phase was filtered through a 0.45-mm Millipore filter and degassed ultrasonically before used. The UV-detector wavelength was set at 240 nm [14,15].

Statistical Analysis

Values were expressed as mean±S.D; the values were statistically evaluated using unpaired Student's t-test and one way analysis of variance (ANOVA), supported by Bonferroni's *post hoc* analysis. Values with $P<0.05$ were considered significantly different. Analysis was performed using GraphPad Prism software for Windows (version 5.0, GraphPad Software, Inc., San Diego, CA).

RESULTS

After administration of the four polyphenols (Silibinin, EGCG, Quercetin and Rutin) for 30 days, serum levels of metformin and atenolol were measured to explain drugs-polyphenols interactions. Figure 1 showed that all polyphenols produced significant increase (P<0.05) in serum levels of metformin compared with control group; the serum levels of metformin in all groups were found comparable. In other hand, serum atenolol levels revealed no significant differences compared with controls, except for silibinin (Figure 2), where significant increase was reported. No significant differences in serum atenolol levels were reported among all groups. In table 1, long-term administration of silibinin significantly increases tissue levels of metformin in brain and kidney (brain>kidney) compared to control group, while no such effect reported in liver tissue. Concerning the effect of EGCG in this respect, long-term use produced significant increase in metformin contents in the three tested organs (bran, kidney and liver) compared with control group; inter-groups analysis showed that highest level was reported in the kidney, which was significantly different with respect to that reported in brain and liver, where the latter two found comparable. In the quercetin-treated group, long-term use increases tissue levels of metformin in all the targeted organs (Table 1) compared to control group, with highest level reported in kidney tissues. Concerning the effect of rutin, long-term use significantly increases metformin levels in kidney tissue only compared with that reported in control group (Table 1). As shown in table 2, atenolol was not detected (within the limits of analysis method) in all targeted brain tissues and in all animal groups including the controls. Moreover, the detected levels of atenolol in kidney and liver tissues are not found significantly different after administration of polyphenols after long-term use, these levels are also found comparable to those reported in control group (Table 2). In the present study, the relative tissue availability in brain, kidney, and liver with respect to serum levels of metformin and atenolol were measured as an indicator of for the ability of polyphenols to modulate drug distribution in those organs, and give an idea about the ability of drugs to cross tissue specific barriers like blood brain barrier. Figure 3 demonstrates that in brain tissue, only silibinin, EGCG and quercetin increased the relative tissue availability of metformin in this organ; while rutin fails to show similar effect. The maximum organ delivery was attributed to the effect of quercetin compared with others. Meanwhile, in kidney tissue, all the four polyphenols significantly increased relative tissue availability of metformin in the kidney compared with control group (Figure 3), and their effects in this respect are found comparable and not significantly different. In the liver, only quercetin produced significant increase in the relative tissue availability of metformin compared with control; however, both silibinin and rutin significantly decreased such tissue availability compared with control. Meanwhile, EGCG do not show significant changes in this respect. In figure 4, long-term administration of the four polyphenols did not produce significant changes in relative tissue availability of atenolol in all targeted organs (brain, kidney and liver); as mentioned previously, atenolol was not detected in brain tissues in all circumstances.

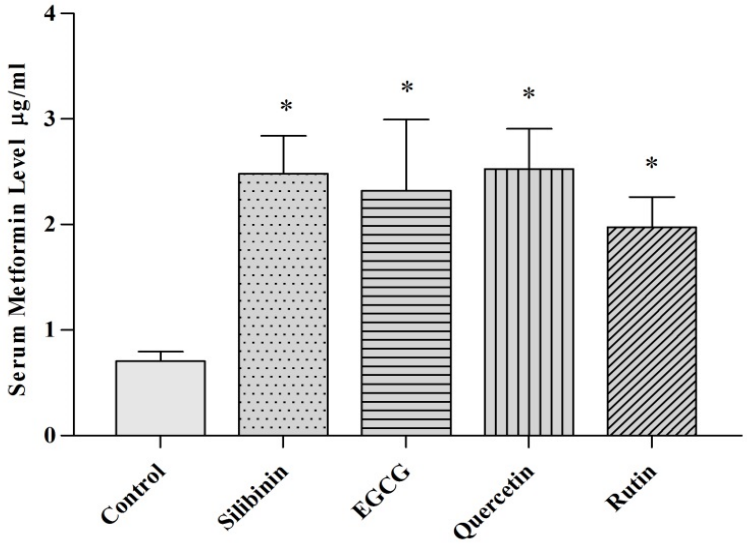

Figure 1. Effects of long-term use of Silibinin, EGCG, Quercetin and Rutin on serum levels of orally administered single dose of Metformin; values are presented as mean±S.D; * significantly different compared to control (P<0.05); no significant differences among treated groups (P>0.05).

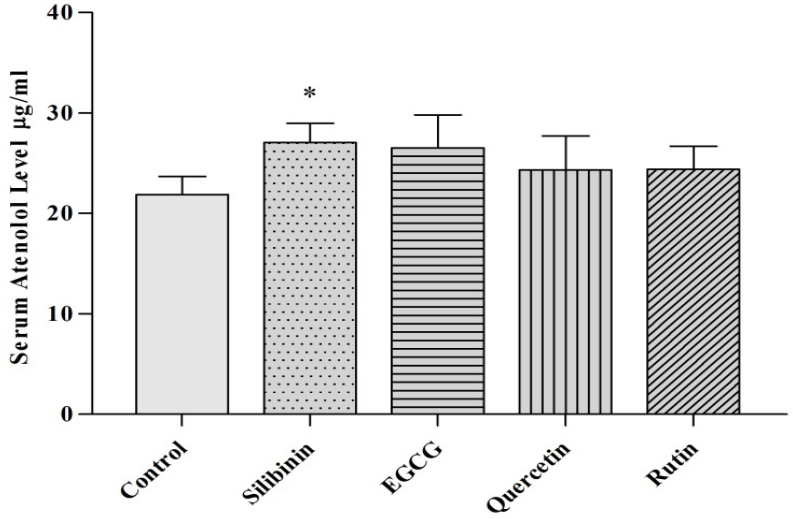

Figure 2. Effects of long-term use of Silibinin, EGCG, Quercetin and Rutin on serum levels of orally administered single dose of Atenolol; values are presented as mean±S.D; * significantly different compared to control (P<0.05); no significant differences among treated groups (P>0.05).

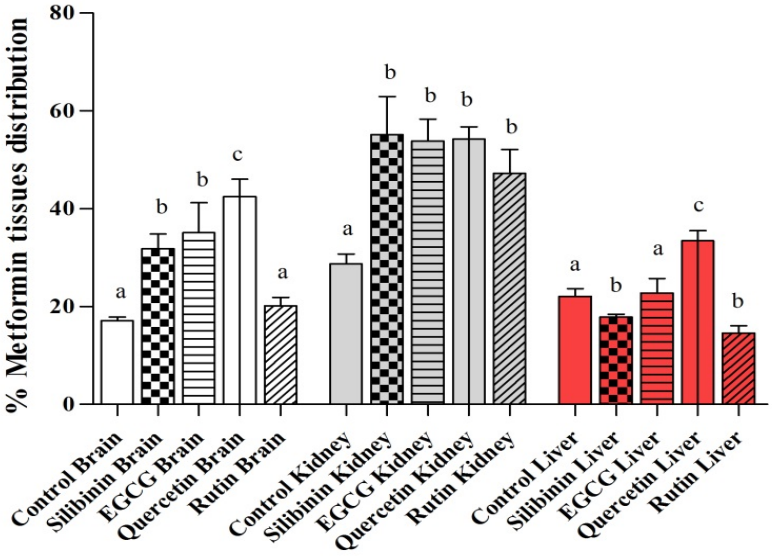

Figure 3. Effects of long-term use of silibinin, EGCG, quercetin and rutin on % metformin distribution in brain, kidney and liver after single oral dose; values are presented as mean±S.D; values with non-identical letters (a,b,c) represent significant differences among groups within the same organ.

Table 1. Effects of long-term administration of silibinin, EGCG, quercetin or rutin on the distribution of orally administered single dose (300) metformin in brain, kidney and liver tissues of rats.

Type of flavonoid	Metformin level µg/g tissue			*P* value
	Brain	Kidney	Liver	
Control	0.16±0.02	0.2±0.03	0.17±0.02	n.s
Silibinin	0.78±0.08*[a]	1.37±0.23*[a]	0.45±0.07[a]	0.01
EGCG	0.83±0.28*[a]	1.24±0.31*[a]	0.52±0.11*[a]	0.01
Quercetin	1.08±0.23*[a]	1.38±0.24*[a]	0.84±0.11*[b]	0.01 Kidney *vs*. Liver
Rutin	0.4±0.09[b]	0.93±0.18*[b]	0.3±0.05[a]	n.s Brain *vs.* Liver

Values were expressed as mean±SD; number of animals=6 in each group; * significantly different compared to the control within the same tissue (P<0.05); values with non-identical superscripts (a,b) within the same tissue were considered significantly different (P<0.05); P=0.01: significant differences between tissues within the same group.

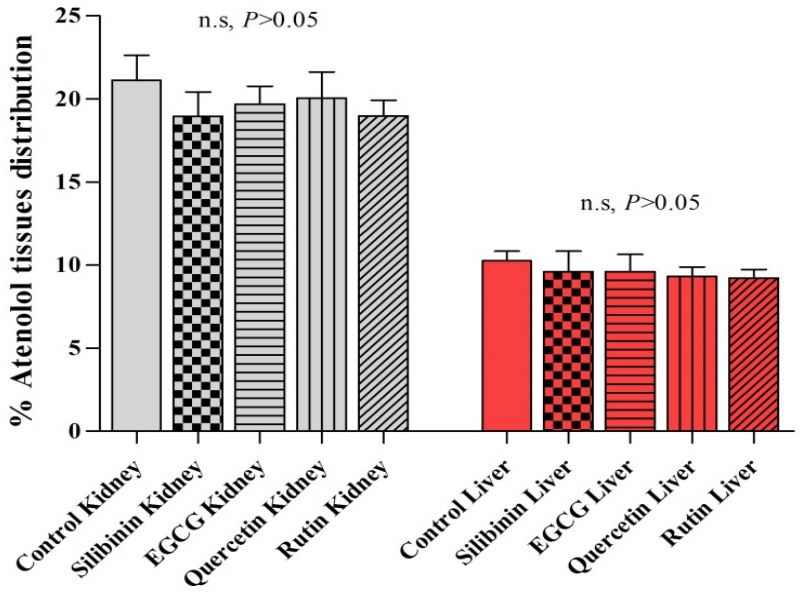

Figure 4. Effects of long-term use of silibinin, EGCG, quercetin and rutin on % atenolol distribution in kidney and liver after single oral dose; values are presented as mean±S.D; n.s=non-significantly different among groups within the same organ.

Table 2. Effects of long-term administration of silibinin, EGCG, quercetin or rutin on the distribution of orally administered single dose (50mg/kg) atenolol in brain, kidney and liver tissues of rats.

Type of flavonoid	Atenolol level µg/g tissue			*P* value
	Brain	Kidney	Liver	
Control	N. D	4.8±0.25	2.49±0.24	0.01
Silibinin	N. D	5.12±0.39	2.61±0.4	0.01
EGCG	N. D	5.2±0.7	2.56±0.5	0.01
Quercetin	N. D	4.86±0.64	2.28±0.39	0.01
Rutin	N. D	4.64±0.62	2.26±0.26	0.01

Values were expressed as mean±SD; number of animals=6 in each group; *P*=0.01: significant differences between tissues within the same group; N.D: not detected; no significant differences compared to control within the same tissue for each flavonoid (*P*>0.05).

DISCUSSION

Researches on polyphenols-drug interactions have illustrated the ability of some polyphenols to affect the bioavailability and activity of drugs, usually resulting in adverse effects. However, it seems that these interactions can be utilized to design specific synergies between polyphenols to potently increase their bioactivity, and hence, their beneficial health-promoting effects [16]. Information on the bioavailability and organ distribution of drugs after long term administration of polyphenols is important for understanding whether flavonoids inhibit or enhance absorption and organ distribution of several compounds. To our knowledge, this is the first project that studies the effect of silibinin, EGCG, quercetin or rutin (long-term use) on absorption and distribution of metformin and atenolol in rats. Interactions of nutritional components in food with these drug transporters and metabolizing enzymes give insight into several important issues. Thus, polyphenols may dramatically affect the blood concentrations of clinically used drugs, resulting in overdose or loss of therapeutic effect. Some polyphenols are high affinity inhibitors, whereas others may be substrates themselves [17]. In the present study, orally administered polyphenols significantly increased both serum levels and tissue availability of metformin; specific efflux transporters, which thought to be inhibited by

polyphenols, controlled metformin absorption and membrane transport. This inhibition largely depends on the polyphenolic structure and hydrophobicity, especially for their interaction with the hydrophobic regions of such transporters [18]. These transporters are widely expressed in the blood-brain barrier, intestine, kidney and liver, and have remarkable influence on the absorption and distribution of many drugs [19,20]. Accordingly, polyphenols can be considered as good candidate molecules to modulate the effects of these regulatory proteins, resulting in either enhancement or inhibition of their activities. This condition was highly expected in the finding of the present study, especially metformin. Metformin is a well-known substrate for influx transporters, including plasma monoamine transporter in the intestine, as well as efflux transporters including MDR and P-gp; since metformin is also a substrate for the efflux pump P-gp [21], the oral bioavailability of metformin might be affected by transporter inhibitors like polyphenols. Polyphenols are abundantly found in our daily foods; in addition to their antioxidant properties, they are capable to inhibit drug efflux by MDR [22]. The present study provides *in vivo* evidence that all studied polyphenols might increase absorption of metformin through the inhibition of P-gp-mediated efflux during the absorption phase in the intestine; this effect varies with variations in structural features of the flavonoids. In the present study, the increase in serum and tissue levels of metformin after long-term oral administration of polyphenols came in tune with many previously reported data. Shin and Choi (2009) reported that oral administration of quercetin, morin and EGCG significantly increased the C-max and the AUC of tamoxifen in rats [23]; while Rajnarayana *et al* (2004) demonstrated that pretreatment with silymarin led to significant increase in the disposition of metronidazole and its active metabolite, hydroxyl-metronidazole [24]. Moreover, Tamaki *et al* (2010) demonstrated that some herbal and dietary supplements and isoflavonoids increase the systemic availability of many BCRP substrates when concomitantly given orally [25]. The clinical outcome of such types of interactions may be of serious consequences during treatment with drugs that have low margin of safety and narrow therapeutic index. In this respect, quercetin increases oral bioavailability of digoxin in pigs and resulted in serious toxicity [22]. After long-term administration, polyphenols significantly increase tissue availability of metformin compared with control; the order of increase can be ranked as follow: kidney>brain>liver; this may be attributed to the highest concentration of polyphenols achieved in intestine and kidney compared to liver, which makes modulation of transporters in those organs more prominent. This result was in agreement with previous observation by Chen *et al* (1997), who reported that highest EGCG, epigallocatechin and epicatechin AUC levels were detected in the intestine and kidney and excreted through both the urine and bile [26]. The reported differences between the effects of the studied polyphenols can be related to the variation in certain structural properties, including the number and distribution of hydroxyl groups at specific parts of the structural formula; this will consequently affect the physicochemical properties of these flavonoids, especially lipid solubility and interactions with biological targets. Kitagawa (2006) reported that EGCG, as inhibitor of P-gp, was more effective than verapamil and quercetin; so, there is a possibility many polyphenols have such inhibitory activities on P-gp function, and the large hydrophobic region in addition to the phenolic hydroxyl groups seems to be commonly necessary for this activity [18]. The present study demonstrates also that metformin can pass the BBB, and the polyphenols can affect the transport function of this barrier, with consequent increase in relative availability of metformin in brain tissue; this finding was in tune with previous data in this respect [27]. In the present study, we also evaluate the effects of long-term administration of polyphenols on GIT absorption and tissue availability of atenolol, not P-gp substrate, to enable precise estimation of polyphenols effects on membrane transporters. Orally administered doses of the four polyphenols (multiple doses) had no effects on either serum levels or tissue availability of atenolol compared to control group. This finding can be explained according to that atenolol is not a well-known substrate for P-gp; so, its absorption and distribution are not influenced by the polyphenols as in case of metformin. This finding was in tune with the report of Yang et al (2000), who indicate that atenolol, metoprolol and alprenolol are not substrates for P-gp (in contrast to propranolol) and not affected by modulating its activity *in vitro* [28]. Such finding was also supported by the idea that atenolol was absorbed and distributed mainly through paracellular absorption pathway [29,30]. Atenolol follows the passive diffusion pathway with minimal first pass metabolism effect, implying no effect of P-gp on its transport or movement in apical basolateral direction [31]. In tune with this finding, the present study showed that there is no effect for the evaluated polyphenols, as p-gp modulators, on absorption or tissue distribution of atenolol. In the present study, atenolol was not detected (within the method limitations) in brain tissues of all animal groups including the controls. This result was consistent with many other studies; Wang *et al* (2005) considered atenolol as CNS negative compound, and a P-gp substrate [32], while others considered passage of atenolol across BBB into the brain as very limited [33].

CONCLUSION

Long-term administration of silibinin, EGCG, quercetin or rutin increase oral absorption and tissue distribution of metformin, while atenolol was not affected; the effects of the studied polyphenols varied in accordance with the variations in their structural formulas.

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Original Research

Ethnobotanical uses of *Lantana trifolia L.* and *Sida cuneifolia Roxb.* in Mukungwe and Wabinyonyi sub-counties of central Uganda

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Keywords: Conservation, culture, ethnomedicine, ethnoveterinary, plants

Abstract
Aim: This was an ethnobotanical study that was carried out to establish the traditional uses of *Lantana trifolia* L. and *Sida cuneifolia* Roxb. plants in selected parts of Central Uganda.
Methods: The ethnobotanical study was done in August and September, 2012 in Mukungwe and Wabinyonyi sub-counties in Masaka and Nakasongola Districts respectively located in Central Uganda. Study sites and respondents were purposefully selected and information was obtained through semi-structured interview guides, key informant interview guides as well as observations. Eighty respondents were considered for semi-structured interviews and 15 for key informant interviews.
Results: Seven ethnobotanical uses for *Lantana trifolia* were cited by respondents and majority (46.25%) of them used it as a herbal remedy. As a herbal remedy, *Lantana trifolia* managed 13 human disease conditions and mainly used in the management of cough and common colds by 22.5% of the respondents. Four ethnobotanical uses were cited for *Sida cuneifolia* and majority of the respondents (62.5%) used it as a herbal remedy as well as sweeping brooms. As a herbal remedy, *Sida cuneifolia* was reported to be useful in management of 12 disease conditions, fractures and sprains (bone setting) being mentioned by the majority of the of respondents (36.25 %).
Conclusion: In conclusion, *Lantana trifolia* and *Sida cuneifolia* were culturally important ethnomedicines. Scientific validation of traditional claims as well as conservation of these plants should be encouraged in order to preserve and promote their use.

INTRODUCTION

Uganda is endowed with a wide diversity of plant resources that people have interacted with for years, which form part of their traditional knowledge (TK) systems to improve their livelihoods. This knowledge can contribute to local economic and political empowerment, preserves local cultures through instilling pride and represents an important component of global knowledge on development issues [1]. However, TK faces an ever-growing threat of extinction through ethnocide of indigenous cultures and languages, ecocide of indigenous habitats and genocide of indigenous peoples [2]. Urgent action is required to safeguard traditional knowledge as this will contribute to the conservation and sustainable use of biological diversity [3]. Integrating cultural context in all medicinal plant research is also important in shaping the health and well being of traditional knowledge custodians in addition to the scientific world [4]. *Lantana trifolia* L. (Verbenaceae) and *Sida cuneifolia*

Roxb. (Malvaceae) are some of the culturally important plants in Central Uganda but the information about their cultural significance has not been adequately documented. This study therefore highlighted and documented the traditional knowledge systems associated with *Lantana trifolia* and *Sida cuneifolia* in two selected areas of Central Uganda.

MATERIALS AND METHODS

Study area description

The study was conducted between August and September 2012 in two areas of Central Uganda, Mukungwe and Wabinyonyi sub-counties of Masaka and Nakasongola districts respectively, on traditional usage of *Lantana trifolia* and *Sida cuneifolia*.

Masaka is located in the Lake Victoria basin dominated by tropical grassland vegetation. According to Fungo, Grunwalds (5), the climate regime is characterized by bimodal rainy seasons. The altitude ranges from 1,200-1,260 meters above mean sea level, slopes are gentle to moderate and soils are classified as ferralsols. It is located on the East African plateaus and characterized by hills and ridges that are highly dissected by streams and drainage ways. Agro-economically the area is part of the banana-coffee system and farmers rely on rain fall for cultivation which forms the base of agricultural production. The main inhabitants are Baganda and Luganda is the most widely spoken language. Nakasongola District is located south of Lake Kyoga and forms part of the cattle corridor dominated by semi-arid rangelands. According to Kisamba-Mugerwa (6), this zone covers natural grassland, bush land and wood land. The area is characterized by low and erratic rain fall regimes leading to frequent and severe droughts and fragile soils. Agro-economically, pastoralism is the main activity. The most widely spoken languages in this area are Luruli and Luganda.

Data collection methods

This was a qualitative cross-sectional ethno-botanical study. Methods used included semi-structured interviews, key informant interviews and observations (7, 8). Pre-tested Interview guides were used during the interview sessions and interviews were conducted in Luganda the language used by the locals. Purposeful sampling and snow-ball sampling (9) were used to select the study sites and population. Eighty purposefully selected respondents, 40 per sub-county rich in general ethno-botanical knowledge were selected for semi-structured interviews. The selection criteria also considered study sites with prominent poultry production since use of these plants in poultry farming was important to this study. Eleven key informants were interviewed for classified ethnobotanical knowledge about the two plants which included herbalists, bone-setters and traditional birth attendants.

The data obtained through semi-structured interviews included: general uses, the human ailments treated, use in livestock management especially poultry. The data obtained through key informant interviews included: medicinal preparations and administration, cultural beliefs/values and any conservation efforts. Observations were made on cultivation, availability and the practical use of these plants. The two plants were collected and identified and voucher specimen prepared and preserved as *Lantana trifolia* L. (RN 01) and *Sida cuneifolia* Roxb. (RN 02) at the Botany unit herbarium of Makerere University, Kampala. The names and families of plants were according to Brummitt and Powell (10). Ethical clearance was obtained from the Ethical Review Board of the College of Veterinary Medicine, Animal Resources and Biosecurity, Makerere University, reference number, VAB/REC/13/05. Permission was sought from the authorities in the study areas. Oral informed consent was also obtained from the respondents.

Data analysis

Data was analysed qualitatively and the methods used for analysis of written records from the interviews were content analysis and thematic analysis (11). Descriptive statistics (frequencies and percentages) were used to express the results.

Quantitative value indices were also calculated for the general uses of these plants. Relative frequency of citation (RFC) was calculated by dividing the frequency of citation (FC) by the total number of informants in the survey: RFC=FC/N (12).

The percentage of respondents who had knowledge (PRK) regarding the use of a species (percent use value) in the treatment of diseases was estimated using the formula: (number of people interviewed citing species/ the total number of people interviewed) × 100 (13).

RESULTS

Gender distribution of respondents

Among respondents for semi-structured interviews, the gender distribution in Mukungwe sub-county showed that 50% of the respondents were males and 50% were females. In Wabinyonyi sub-county, 52.5% were males and 47.5% were females. For the key informant interviews, only one respondent from Mukungwe sub-county was a male while the rest of the fourteen respondents were females.

General uses of *Lantana trifolia* and *Sida cuneifolia* plants

Considering the total frequency citations on plant usage, the knowledge was more distributed amongst females (99 for Mukungwe and 49 for Wabinyonyi) compared to males (20 for Mukungwe and 30 for Wabinyonyi). Respondents in Mukungwe cited uses of these plants more frequently (20 for males and 99 for females) compared those in Wabinyonyi (30 for males and 49 for females). *Lantana trifolia* was most commonly used as herbal remedies with RFC of 0.4625 giving a percentage of 46.25% respondents. *Sida cuneifolia* was commonly used as sweeping brooms and for human medicine with RFC of 0.625, giving a percentage of 62.5% respondents. (Table 1).

Human diseases/conditions that are managed, preparation and administration of the remedies

Cough and common colds were the most commonly cited conditions treated by *Lantana trifolia* with a percentage respondents' knowledge (PRK) of 22.5. *Sida cuneflolia* was most commonly cited for treatment of fractures and sprains (bone setting) with PRK of 36.25 (Table 2).

Uses of *Lantana trifolia* and *Sida cuneifolia* plants in livestock production and management

Lantana trifolia and *Sida cuneifolia* were used in preparation of ethnoveterinary remedies and as an animal fodder. *Lantana trifolia* was commonly used for management of respiratory symptoms and diarrhea in poultry with PRK of 7.5 while *Sida cuneifolia* was commonly used for induction of labour in ruminants with PRK of 6.25 (Table 3).

Cultural beliefs associated with these plants

Locally, *Sida cuneifolia* was called "akakumilizi" or "akeeyeyo" or "akabamba maliba". The word "akakumilizi" comes from a Luganda word, "okukumilira" which means to collect or to gather. This plant was traditionally used to gather and accumulate wealth with the assumption that it brings good luck, hence the name "akakumilizi". The word "akeeyeyo" comes from the word "okweera" literally meaning to sweep, this plant is locally used to sweep compounds. The aerial part of *Sida cuneifolia* was put in the brooms which swept the houses, shops and market stalls for cleansing intentions.

Table 1. General uses of *Lantana trifolia* and *Sida cuneifolia* in central Uganda

Plant uses	Mukungwe FC*		Wabinyonyi, FC		Total FC per plant use	RFC**	Percentage citation
	Males	Females	Males	Females			
Lantana trifolia							
Human medicinal remedies	6	20	4	7	37	0.4625	46.25
Tooth brushes/oral hygiene	4	16	2	5	27	0.3375	33.75
Food (fruits are eaten)	2	5	8	3	18	0.225	22.5
Animal production and management		5		1	6	0.075	7.5
Herbal bath (ekyogero)		4			4	0.05	5
Female genital modification		2			2	0.025	2.5
Cultural rituals		3		3	6	0.075	7.5
Sida cuneifolia							
Human medicinal remedies	5	21	9	15	50	0.625	62.5
Sweeping brooms	3	16	7	14	50	0.625	62.5
Animal production and management		3		1	4	0.05	5
Cultural rituals		4			4	0.05	5
Total frequency citations	20	99	30	49	198		

FC*: Frequency of citation

RFC**: Relative frequency of citation = frequency of citation (FC) divided by the number of informants who were interviewed (n=80).

Table 2. Frequency citations of *Sida cuneifolia* and *Lantana trifolia* in management of human diseases/conditions

Parts used per plant species	Body organ/system treated	Disease/condition	Mode of preparation and administration	Use reports n=80	PRK*
Sida cuneifolia					
Leaves	Respiratory system	Cough and common colds	Squeeze leaves in clean water, wash the face, apply 2-3 drops in the nose	1	1.25
Leaves	Reproductive system	Induction of labour during child birth	Squeeze leaves in cool boiled water, drink 500mls thrice daily	3	3.75
Leaves	Reproductive system	Menstrual pains	Squeeze leaves in cool boiled water, drink 500mls thrice daily	3	3.75
Leaves	Reproductive system	Manhood enhancement	Chew leaf	1	1.25
Leaves	Nervous system	Epilepsy and infant cerebral malaria	Squeeze leaves in clean water, pour on head	2	2.5
Leaves	Nervous system	Dizziness	Squeeze leaves in clean water, pour on head	3	3.75
Leaves	Head	Headache	Squeeze leaves in clean water, pour on head	5	6.25
Leaves	Several organs and systems	Fever	Squeeze leaves in clean water, bath	2	2.5
Leaves, stem	Teeth	Toothache	Chew leaves or stem back	2	2.5
Leaves	Skeletal system	Bone setting	Pound leaves, mix with local cow ghee, align the bones, apply preparation, topically	29	36.25
Leaves	Integumentary system	In rashes due to measles and other skin rashes	Powder mixed with jelly and smeared topically on skin	3	3.75
Leaves	Immune system	Prevention of allergies due to meat consumption	Use powder in to make tea and drink or add in food	2	2.5
Lantana trifolia					
Leaves	Respiratory system	Cough and flue	Boil leaves in water or local banana juice drink or Squeeze leaves to produce juice apply 2-3 drops in the nose Burn leaves to ashes and leak	18	22.5
Roots and Leaves	Respiratory system	Asthma and sinusitis	Burn leaves to ashes and leak or dry roots and pounded to make a fine powder, add 2 tea spoons to 500mls of boiled water, drink	4	5
Leaves	Respiratory system	Chronic rhinitis	Squeeze leaves to produce juice apply 2-3 drops in the nose	2	2.5
Leaves	Reproductive system	Menstrual pains	Boil leaves in water, drink 500mls thrice daily	1	1.25
Leaves	Nervous system	Epilepsy and Infant cerebral malaria	Boil leaves in water, drink 500mls thrice daily	1	1.25
Leaves	Nervous system	Madness	Squeeze leaves to produce juice, apply 2-3 drops in the nose thrice daily	1	1.25
Leaves	Eye	Eye infections (trachoma, conjunctivitis)	Squeeze leaves to produce juice, apply 2-3 drops topically on the eyes thrice daily	3	3.75
Leaves	Ear	Otitis	Squeeze leaves to produce juice, apply 2-3 drops topically on the eyes thrice daily	2	2.5
Leaves	General body systems	Fever	Boil leaves in water, drink 500mls thrice daily	2	2.5
Leaves, Stem	Teeth	Toothache	Steam leaves to soften them, squeeze to produce juice, apply on the affected tooth Or chew clean leaves Or dry leaves and flowers, pound to powder and add salt, apply on a tooth brush and brush the affected teeth Or Brush teeth with the stem	5	6.25
Leaves	Blood	Sickle cell anaemia	Boil leaves in water, drink 500mls thrice daily	1	1.25
Leaves	Stomach	Stomachache	Boil leaves or root in water, drink 500mls thrice daily	2	2.5
Leaves	Integumentary system	Skin rashes due to measles and other skin rashes	Squeeze or boil leaves in water, orally administer 250mls thrice daily Or squeeze leaves, apply topically all over the skin Or powder mixed with jelly and smeared topically on skin	2	2.5

PRK*: Percentage respondents' Knowledge

Table 3. Uses of *Lantana trifolia* and *Sida cuneifolia* in animal production and management

Parts used per plant species	Livestock	Uses	Preparation and administration	Use reports n=80	PRK*
Sida cuneifolia					
Leaves, Roots	Poultry	Management of diarrhea	Squeeze or boil leaves in water, mix in drinking water given to poultry Or pound roots, add to boiled water, mix with drinking water for poultry.	3	3.75
Leaves	Ruminants	Induction of labour during calving	Squeeze leaves in water, administer orally	5	6.25
Leaves	Poultry	Animal feed	Suspend in poultry house with other vegetables	3	3.75
Leaves	Swine		Mix fresh leaves with other feeds	2	2.5
Lantana trifolia					
Leaves, roots	Poultry	Management of respiratory symptoms and diarrhea	Squeeze or boil leaves in water, mix in drinking water given to poultry Or pound roots, add to boiled in water, mix with drinking water for poultry	6	7.5
Leaves	Poultry	Improving egg size, yolk colour and strengthening of the uterus	Dry leaves, mix with other feeds	3	3.75
Leaves	Swine	Animal feed	Mix fresh leaves with other feeds	1	1.25
Leaves	Goats	Animal feed	Goats browse this shrub	4	5

PRK*: Percentage respondents' Knowledge

For prevention of fatal bad omens on babies whose parents committed adultery, *Sida cuneifolia* leaves were squeezed and the parent passed it in his or her hands and topically all over the child's body. This would prevent the child from acquiring bad omens from their parents. The root of *Sida cuneifolia* was also tied around the baby's waist in form of a belt to alleviate conditions like weakness and emaciation which affected infants due to poor birth spacing. When using *Sida cuneifolia* to make any concoction, the practitioner was advised to make the preparations from the leaves on a standing live plant. This was done in an effort to conserve this plant.

Locally, *Lantana trifolia* was called "akayukiyuki akasekera nyonyi" comes from a Luganda word meaning a plant that makes birds happy. The fruits of this plant are so attractive to birds and they eat them especially for deworming purposes. *Lantana trifolia* was also called "akasekera" because the gods are always very happy with it when it is used in most of the cultural rituals. *Lantana trifolia* was involved in most cultural rituals to appease gods. The leaves of *Lantana trifolia* were also put in the "herbal bath" for babies to acquire blessings. People also brushed their teeth using *Lantana trifolia* stem in the morning to acquire good luck and fortune during the day. The stem of *Lantana trifolia* was put on the roof on the house by parents who had stubborn children to prevent them from committing repeated crimes. *Lantana trifolia* was also used to curtail criminal investigations for those who were being accused for crimes.

Plant propagation and distribution

There was limited domestication of these two plants in Wabinyonyi sub-county compared to Mukungwe sub-county. *Sida cuneifolia* was observed frequently on cultivated land and in compounds. In Mukungwe and Wabinyonyi, seventeen and five respondents respectively had this plant in their compounds. It was propagated mainly by seeds through the habit of sweeping the compound hence encouraging seed dispersion. However, it could also be propagated by root stalks. *Lantana trifolia*, was very common in Mukungwe, where it grew mainly in the wild and was rarely cultivated. However, two respondents had *Lantana trifolia* in their gardens. It was mainly dispersed by the birds that eat the fruits but it could also be planted by people using the seeds and the root stalks. In Wabinyonyi, *Lantana trifolia* was very rare and if found, it was only in the wild and never domesticated. Respondents also reported that other *Lantana species* which were more invasive competed with *Lantana trifolia* making its conservation difficult.

Table 4. Previous research findings on the two plants

Species	Reference notes
Sida cuneifolia	
Antimicrobial activity	Showed good antimicrobial activity against *Bacillus cereus*, *Staphylococcus aureus*, *Escherichia coli*, *Candida albicans* [40]
	Showed good antibacterial activity against *Streptococcus faecalis*, *Staphyloccus aureus*, *Escherichia coli*, *Salmonella typhimurium* [25]
Some perceived traditional claims	Used in Western Uganda for treatment of chest and muscle pains [41].
	Used in Uganda for treatment of HIV and related conditions [42].
	Used in Uganda for fixing of dislocated bones [43]
	Used in Western Uganda by pregnant women for induction of labour [34].
	Used in Southern Uganda for treatment of athlete's foot, broken bones, prevention of miscarriage, eaten by pregnant women to boost foetal health [44].
	Used in Western Kenya for treatment of stomachache [45].
Lantana trifolia	
Bioactivity	The ethanolic and ethyl acetate extracts contain flavone glycosides which exhibit intense sedative effect [23].
	The plant extracts demonstrated good anti-inflammatory in rats in a study in Venezuela [26].
	The leaves contain Umuhengerin, a flavonoid exhibiting good anti Staphylococcal activity [18].
	The aqueous extracts of the chewing sticks from this plant showed good anti-streptococcal activity [27].
	The essential oils from this plant contain sesquiterpenes with good antimycobacterial activity [46].
	This plant showed good antibacterial activity [22].
	Used by the Amazonians for treatment of Central Nervous System disorders and exhibited good psychoactive activity on experimental animals [28].
	The plant was effective in treatment of cutaneous Leishmaniasis [47].
	The plant extracts exhibited good antimicrobial and antiviral activities [20].
	Showed good antibacterial activity against *Streptococcus faecalis*, *Staphyloccus aureus*, *Escherichia coli*, *Salmonella typhimurium* [25].
	The hexane and dichloromethane leaf extracts are active on *Mycobacterium tuberculosis* [21].
	The leaf extracts are active on *Mycobacterium fortuitum* [19]
	The methanol leaf extracts are active on *Staphylococcus aureus* and *Pseudomonus aeruginosa* [24].
Toxicity studies	The ethanol extracts showed mild toxicity on Brine shrimp [48].
Some perceived traditional claims	Used in Bulamogi, Uganda for treatment of cough [49].
	Used in Southern Uganda for oral hygiene [44].
	Used in Rwanda for treatment of gonorrhoea and heart failure [19].
	Used in Rwanda for treatment of angina, gonorrhea and hepatitis [20].
	Used in Uganda for treatment of tuberculosis and related ailments [50].
	Used in Kibale, Western Uganda for treatment of malaria, yellow fever diarrhea and cough [41].
	Used in Nyakayogo sub-county, Western Uganda for treatment of malaria [51].
	An ethnomedicinal plant in Ethiopia [52].
	Used by the people of Kagera region Tanzania for treatment of Malaria [48]..
	Used in Loita, Kenya by Masai people for cultural rituals involving livestock mainly for cleansing and blessing [31].
	Used in Bagamoyo District, Tanzania as an ingredient in ritual bath water and in preparation of medicine which helps to give birth to male children [30].
	The fruits are consumed in Konso, Ethiopia as a wild food plant [29].

DISCUSSION

Studies have been done in some parts of Uganda and the world on *Lantana trifolia* and *Sida cuneifolia* which listed some ethnomedicinal uses with some experimental evaluations of their medicinal values (Table 4). However, the uses of *Sida cuneifolia* in the treatment of epilepsy, infant cerebral malaria, menstrual pains, skin conditions, allergies and manhood enhancement have been documented for the first time. The uses of *Lantana trifolia* in the treatment of asthma, sinusitis, menstrual pains, epilepsy, infant cerebral malaria, madness, eye infections, sickle cell anaemia, stomachache and skin conditions have also been documented for the first time in Uganda. The uses of these two plants to carry out cultural rituals and as livestock feeds have also been documented for the first time in Uganda.

The usage of *Lantana trifolia* and *Sida cuneifolia* plants varied among respondents. According to gender, women were more knowledgeable compared to men. In many human societies, women are in charge of the domestic arena [14] explaining why women are the main upholders of traditions linked to these plants [15]. *Lantana trifolia* and *Sida cuneifolia* had common uses in these two study areas. Consistent pattern of ethnomedicinal usage of plants within or between cultural groups is attributable to probable scientifically reliable biological effectiveness [16]. The common usage patterns could also indicate common ancient culture inherited vertically to these two communities [17].

Lantana trifolia was reportedly used in management of various conditions, which is probably attributed to its biological effects as demonstrated by previous researchers [Table 4]. Management of various infections in humans and livestock could be as a result of its antimicrobial properties as demonstrated by [18-25]. Silva, Martins [26] reported anti-inflammatory activity of *Lantana trifolia*, which probably explains the basis of its use in management of various inflammatory conditions and pains. The use of *Lantana trifolia* for oral hygiene has been reported by earlier studies carried out in Uganda and has been validated for anti-streptococcal activity [27]. The plant also has sedative effects [23] and psychoactive activity [28] hence its use for treatment of nervous conditions. Consumption of *Lantana trifolia* fruits has also been reported in Konso, Ethiopia [29] and this indicates that its use as food is common in the two counties.

The use of *Lantana trifolia* as an ingredient of the herbal bath corresponds with findings by Hurskainen [30] who documented its use in ritual bath water in Bagamoyo District of Tanzania. The use of *Lantana trifolia* for cultural rituals is comparable to that of the Masai in Kenya who use it to cleanse and bless their livestock as reported by Maundu, Berger [31]. Uganda, Kenya and Tanzania are neighboring countries and this may explain the similarities in some of the rituals about *Lantana trifolia*. Previous studies have indicated that plants play an important role in cultural practices, have associated taboos and such lifestyles link people to nature and are important elements of conservation and sustainable use in many cultures [32]. However, this mysterious form of traditional medicine cannot easily be investigated, rationalized or explained scientifically and its explanation is beyond the ordinary scientific human intelligence or intellectual comprehension [33].

The use of *Sida cuneifolia* for inducing child birth seems to be widely practiced since it was also reported in Western Uganda by Kamatenesi-Mugisha and Oryem-Origa [34]. *Sida cuneifolia* contains and therefore provides nutrients such as ascorbic acid, potassium, magnesium and vitamin K that are thought to be associated with improved bone health. In addition it produces alkaline metabolites that might improve bone health by reducing calcium excretion [35]. Plants also contain flavonoids which improve femoral bone mineral density and markers of bone turnover as demonstrated by Hyson [36]. These attributes could be the reasons for their use in bone setting. The use of *Sida cuneifolia* for treatment of diarrhea in poultry has also been cited in earlier studies and this could be attributed to the antimicrobial activity against some disease-causing agents which manifest with diarrhea as a symptom [25, 37].

These plants were more distributed in Mukungwe compared to Wabinyonyi. This is probably because people in Mukungwe made use of these plants more frequently than those in Wabinyonyi. Local communities where traditional cultures persist have been appreciated as repositories not only of knowledge but also of biological diversity [38]. Abandoning these beliefs reduces traditional influences hence potentially leading to destruction of formerly protected natural resources [32]. *Lantana trifolia* was mainly distributed in the wild contrally to *Sida cuneifolia* which was frequently domesticated. There have been concerns about collection of most medicinal plants from the wild and rare domestication, yet wild medicinal plant resources are increasingly under threat from habitat destruction caused by encroachment [39].

CONCLUSION AND RECOMMENDATIONS

The study has revealed that *Lantana trifolia* and *Sida cuneifolia* are useful plants in the traditional knowledge system of central Uganda and therefore conservation and domestication of these plants should be

encouraged. Scientific validation of the therapeutic claims of *Lantana trifolia* and *Sida cuneifolia* should be carried out especially their efficacy and safety since this information is scanty. This will benefit both the scientific world and the consumers of this medicine. There is need to follow up this preliminary study by focusing more on quantitative aspects to establish the general representation of the community. Since this work was carried out in only two sub-counties, the findings do not necessarily represent the entire central Uganda. Similar studies should be done in other areas of this region for comparison purposes.

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Original Research

Antiobesity, antioxidant and antidiabetic activities of red Ginseng plant extract in obese diabetic rats

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Abstract

Aim: This study aimed to investigate the effects of red ginseng extract (RGE) on adiposity index, some serum biochemical parameters and tissue antioxidant activity in obese diabetic rats.

Methods: Five groups of male Sprague-Dawley rats were used. Group (1) was negative control and the other 4 groups were fed on high fat-diet for 6 weeks to induce obesity. The obese rats were then rendered diabetic by intraperitoneal injection of alloxan for 5 days. Group (2) was kept obese diabetic (positive control) and the other 3 groups were orally given RGE at 100, 200 and 400 mg /kg /day, respectively, for 4 weeks. Blood samples were collected for biochemical analyses and kidneys were taken to assay of activities of antioxidant enzymes.

Results: oral dosage of RGE to obese diabetic rats significantly ($P < 0.05$) reduced adiposity index; decreased serum levels of aspartate aminotransferase (AST), alanine aminotransferase (ALT), gamma- glutamyl transpeptidase (GGT) enzymes, total cholesterol (TC), triglycerides (TG), and low density lipoproteins (LDL-c) and improved atherogenic index. Blood glucose and leptin hormone decreased, but insulin increased by administration of RGE. it increased activities of superoxide dismutase (SOD), glutathione peroxidase (GPx) and catalase (CAT) antioxidant enzymes in kidneys tissues.

Conclusion: Red ginseng extract produces antiobesity, antioxidant, and antidiabetic activities in obese diabetic rats. The study suggests that red ginseng plant may be beneficial for the treatment of patients who suffer from obesity associated with diabetes.

INTRODUCTION

Obesity is an excessive fat accumulation in the body that results from an imbalance between energy intake and energy expenditure associated with genetic, metabolic, and behavioral components. Despite of a major contribution of genetic susceptibility, the rapid development of obesity might reflect substantial changes of other factors such as dietary habits [1]. The prevalence of obesity is rising dramatically among all ages with the changes of lifestyles and dietary fat intake [2]. Obesity represents a serious health problem that increased the risk for many diseases such as hypertension and diabetes mellitus [3]. Obesity and insulin resistance are strongly associated with the infiltration of adipose tissue by inflammatory cells [4].

Diabetes mellitus is a chronic and progressive metabolic disease characterized by hyperglycaemia due to insulin deficiency, or resistance, or both. Hyperglycemia occurs when the cells become unable to utilize glucose and/or the liver and skeletal muscles cannot store glycogen [5, 6]. The increased extracellular and intracellular glucose concentrations result in oxidative stress due to increased production of reactive oxygen species (ROS) and sharp decrease in antioxidant body defenses [7]. Oxidative stress plays a key role in the onset and development of diabetes complications, notably diabetic nephropathy [8]. Insulin resistance, a common accompaniment of obesity, is a major risk factor for diabetes mellitus [9]. Because synthetic chemical drugs prescribed for

treating obesity and diabetes had many adverse side effects, therefore there is a great need to search for new and safe alternative drugs from medicinal plants.

Red ginseng (Family *Araliaceae*) is one of medicinal plants with fleshy roots. The Roots of red ginseng are rich in glycosylated saponins (ginsenosides) which have been reported to possess various biological properties. The crude extract of red ginseng roots and the isolated ginsenosides were found to produce hypoglycemic and antidiabetic activities [10, 11, 12, 13]; anticarcinogenic effect [14]; hepatoprotective action [15] and hypocholesterolemic and antihyperlipidemic effects [16] in humans and experimental animals. The crude saponins of Korean red ginseng roots were reported to possess anti-obesity effect in rats fed on high fat-diet [17]. .

MATERIALS AND METHODS

Plant

Dried roots of red ginseng (Family *Araliaceae*) were purchased from a local market of Agricultural Herbs, Spices and Medicinal plants, Cairo, Egypt. The roots were grinded using an electric mixer into a fine powder and thereafter subjected to the alcohol extraction.

Alloxan and biochemical kits

Alloxan was purchased from El-Gomhoryia Company for Chemicals; Cairo, Egypt. It is dispensed in the form of white powder packed in tightly closed brown bottles each containing 25 gram alloxan monohydrate. Kits for biochemical determinations of blood glucose, leptin hormone (radioimmunoassay) and insulin hormone (enzyme-linked immunoassay) were purchased from Gamma Trade Company, Egypt. The other biochemical kits were obtained from Biodiagnostic Company, Dokki, Egypt.

Rats and feeding

Forty five mature male Sprague Dawley rats weighing 185 -200 g body weight and 10-12 weeks old were used in this study. Animals were obtained from the Laboratory Animal Colony, Agricultural Research Center, Egypt. Rats were housed in a well ventilated animal room under standard conditions of 24 ℃ temperature, 50% relative humidity and 12 hr light/12 hr dark cycle. Basal diet was prepared according to report of American Institute of Nutrition (AIN) [18] and water was provided *ad libitum*.

Preparation of plant extract

The crude extract of dry red ginseng roots was prepared according to the method described by Shalaby and Hamowieh [19]. Two hundred grams of powdered red ginseng roots were soaked in 1 liter of 90% ethyl alcohol and kept in a refrigerator with daily shaking for 5 days. Ethanol was thereafter evaporated using a rotatory evaporator connected to vacuum pump. Twenty grams of the obtained semisolid extract were mixed with 2 ml of Tween 80 (suspending agent) and distilled water (98 ml) was gradually added to obtain 20% liquid extract.

Induction of obesity and diabetes

Obesity and acute hyperlipidemia was induced by feeding rats on high fat-diet (HFD) which supplies 45 % calories from fat (lard) for 6 weeks according to Bhatt *et al.* [20], while the basal diet supplies 11% calories from fat (corn oil). This model of obesity closely resembles the reality of obesity in humans. The obese rats were thereafter rendered diabetic by intraperitoneal injection of alloxan in a dose of 120 mg/kg/day for 5 days as described by Ashok *et al.* [21].

Experiment and groups of rats

The experiment was carried out on forty five mature Sprague Dawley male rats randomly distributed into 5 equal groups. Group (1) was fed on basal diet and kept negative control, while the other 4 groups were fed on HFD for 6 weeks to induce obesity. The obese rats were then rendered diabetic by intraperitoneal injection of alloxan (120 mg/kg/day) for 5 days. After induction of diabetes, the group (2) was kept obese diabetic (positive control), while groups (3), (4) and (5) were orally given red ginseng extract in doses 100, 200 and 400 mg/kg, respectively once daily for 4 weeks. At the end of experimental period, final body weights of rats were recorded and the adiposity index (Ad I) was calculated by dividing the total weight of mesenteric, visceral, epididymal and retroperitoneal adipose tissue by the body weight and multiplied by 100 i.e. Ad I = fat weight/body weight x 100 according to Pichon *et al.* [22]. Rats were then euthanized and blood samples were collected from retro-orbital plexuses of veins of eye using capillary tubes. Blood was left to clot and centrifuged at 3000 rpm for 15 min. at 4 °C for separating the serum which was frozen and stored at −18°C until biochemical analyses. Kidneys were taken to assay the activities of tissue antioxidant enzymes.

Biochemical analysis

Serum aspartate aminotransferase (AST) and alanine aminotransferase (ALT) [23]; gamma- glutamyl transpeptidase (GGT) [24]; total cholesterol (TC) and high density lipoprotein (HDL-c) cholesterol [25] and triglycerides (TG) [26] were chemically determined. Low density lipoprotein (LDL-c) cholesterol was calculated using this formula: LDL-c = TC – (TG/5) – HDL-c). Blood glucose was determined using glucose enzymatic kit according to Siest *et al.* [27]. Insulin was

estimated using antibody radioimmunoassay (RIA) assay [28] and leptin hormone was determined using enzyme-linked immunosorbent (ELISA) assay [29].

Antioxidant activity

One gram of kidney tissue was washed in ice-cooled 0.9% NaCl solution and homogenized in ice-cooled 1.15% solution of potassium chloride and 50 mMol potassium phosphate buffer solution (pH 7.4) to yield 10% (w/v) homogenate. Homogenization was performed using Sonicator, 4710 Ultrasonic Homogenizer. Kidney homogenates were centrifuged at 4000×g for 10 min. at 4°C and the supernatants were used to assay activities of antioxidant enzymes superoxide dismutase, glutathione peroxidase and catalase according to Nishikimi *et al.* [30], Paglia and Valentine [31] and Sinha [32], respectively.

Statistical analysis

Statistical analysis was carried out using one-way analysis of variance (ANOVA) followed by Duncan's multiple range test with SPSS computer program [33]. Differences between the controls and treated groups were considered significant at $P < 0.05$ level.

RESULTS

Feeding of male rats on high fat-diet (HFD) for 6 weeks significantly ($P < 0.05$) increased the final body weight, fat weight and adiposity index as compared to negative control rats fed on basal diet. Oral administration of red ginseng extract at doses 100, 200 , 400 mg/kg to obese diabetic rats for 4 weeks caused significant ($P < 0.05$) decreases in the final body weight, fat weight and adiposity index as compared to positive (obese diabetic) control rats, in a dose dependent manner, as shown in Table (1).

The results showed that male rats fed on high fat-diet (HFD) for 6 weeks had significant ($P < 0.05$) increases in serum levels of liver enzymes aspartate aminotransferase (AST), alanine aminotransferase (ALT) and gamma-glutamyl transpeptidase (GGT) when compared with negative control rats fed on basal diet. Oral administration of red ginseng extract at doses 100, 200 and 400 mg/ kg to obese diabetic rats for 4 weeks induced significant ($P < 0.05$) reductions of the elevated serum levels of AST, ALT and GGT enzymes when compared to the positive control group, in a dose dependent fashion, as recorded in Table (2).

Table 1. Effect of red ginseng extract (RGE) on body weight (B.wt.), fat weight (F.wt.) and adiposity index (Ad I) in rats.

Parameters Groups	B.wt. (g)	F.wt. (g)	Ad I (%)
Group (1) **Negative control**	261 ± 13.0^{c}	7.46 ± 0.12^{c}	2.86 ± 0.15^{a}
Group (2) **Obese diabetic control**	315 ± 19.0^{a}	16.61 ± 0.22^{a}	5.27 ± 0.17^{c}
Group (3) **REG (100mg/kg)**	295 ± 10.0^{b}	14.12 ± 0.25^{b}	4.79 ± 0.24^{b}
Group (4) **REG(200 mg/kg)**	283 ± 13.0^{b}	11.20 ± 0.17^{b}	3.96 ± 0.16^{b}
Group (5) **REG(400mg/kg)**	275 ± 12.0^{b}	9.45 ± 0.19^{b}	3.44 ± 0.19^{b}

Means ± SE with different letters superscripts in the same column are significant at $P < 0.05$ using one way ANOVA test.
n= 9 rats/group.

Table 2. Effect of red ginseng extract (RGE) on levels of aspartate aminotransferase (AST), alanine aminotransferase (ALT) and gamma- glutamyl transpeptidase (GGT) liver enzymes in rats.

Parameters Groups	AST (U/L)	ALT (U/L)	GGT (U/L)
Group (1) **Negative control**	44.0 ± 2.11^{d}	36.0 ± 2.12^{d}	23.5 ± 1.15^{d}
Group (2) **Obese diabetic control**	82.0 ± 6.12^{a}	64.0 ± 5.41^{a}	44.0 ± 3.17^{a}
Group (3) **REG (100mg/kg)**	74.0 ± 5.14^{b}	55.0 ± 4.25^{b}	38.0 ± 2.24^{b}
Group (4) **REG(200 mg/kg)**	60.0 ± 5.34^{b}	47.0 ± 3.17^{c}	36.0 ± 2.16^{b}
Group (5) **REG(400mg/kg)**	49.0 ± 3.13^{c}	33.0 ± 2.19^{c}	26.0 ± 2.19^{c}

Means ± SE with different letters superscripts in the same column are significant at $P < 0.05$ using one way ANOVA test.
n= 9 rats/group.

As demonstrated in Table (3), feeding of male rats on high fat-diet (HFD) for 6 weeks significantly (P <0.05) increased serum levels of total cholesterol (TC) and triglycerides (TG) when compared to those fed on basal diet. Oral administration of red ginseng extract at doses 100, 200 and 400 mg /kg to obese diabetic rats for 4 weeks significantly (P < 0.05) decreased the elevated serum levels of TC and TG when compared with obese diabetic control rats.

Serum analysis revealed that male rats fed on high fat-diet (HFD) for 6 weeks had a significant decrease in high density lipoprotein (HDL-c), increase in low density lipoprotein (LDL-c), and high atherogenic index (AI) when compared with the negative control group. Oral administration of red ginseng extract to obese diabetic rats for 4 weeks increased serum HDL-c, decreased LDL-c and improved AI as compared with the positive control group (Table 4).

Data in Table (5) showed that male rats when fed on high fat-diet (HFD) for 6 weeks had significant (P < 0.05) increases in serum glucose and leptin hormone and decrease in insulin hormone levels when compared to those fed on basal diet (negative control group). Red ginseng extract when orally given at doses 100, 200 and 400 mg kg to obese diabetic rats for 4 weeks significantly (P < 0.05) decreased serum glucose and leptin hormone, but increased insulin levels when compared with positive control rats, in a dose dependent manner.

Feeding a high fat-diet (HFD) to male rats for 6 weeks caused significant (P < 0.05) decreases in renal tissue levels of superoxide dismutase (SOD), glutathione peroxidase (GPx) and catalase (CAT) antioxidant enzymes when compared to those fed on basal diet. Oral administration of red ginseng extract at doses 100, 200 and 400 mg/kg to obese diabetic rats for 4 weeks significantly (P < 0.05) increased tissue levels of SOD, GPx and CAT enzymes when compared with the positive control group, in a dose dependent manner(Table 6).

Table 3. Effect of red ginseng extract (RGE) on serum total cholesterol (TC) and triglycerides (TG) in rats.

Parameters Groups	TC (mg/dL)	TG (mg/dL)
Group (1) **Negative control**	95.29 ± 2.03^{d}	63.94 ± 2.19^{d}
Group (2) **Obese diabetic control**	152.70 ± 3.56^{a}	172.60 ± 6.73^{a}
Group (3) **REG (100mg/kg)**	122.65 ± 7.34^{b}	144.12 ± 6.25^{b}
Group (4) **REG(200 mg/kg)**	118.50 ± 6.42^{b}	138.20 ± 7.17^{b}
Group (5) **REG(400mg/kg)**	105.60 ± 4.32^{c}	120.82 ± 6.19^{c}

Means ± SE with different letters superscripts in the same column are significant at P < 0.05 using one way ANOVA test.
n= 9 rats/group.

Table 4. Effect of red ginseng extract (RGE) on levels of high density lipoprotein (HDL-c), low density lipoprotein (LDL-c) cholesterol and atherogenic index (AI) in rats.

Parameters Groups	HDL-c (mg/dL)	LDL-c (mg/dL)	AI LDL-c / HDL-c
Group (1) **Negative control**	70.97 ± 1.89^{a}	12.48 ± 3.11^{d}	0.176
Group (2) **Obese diabetic control**	53.34 ± 2.52^{c}	67.06 ± 5.65^{a}	1.257
Group (3) **REG (100mg/kg)**	59.66 ± 3.22^{b}	44.83 ± 2.25^{b}	0.751
Group (4) **REG(200 mg/kg)**	61.45 ± 4.12^{b}	33.20 ± 2.17^{c}	0.540
Group (5) **REG(400mg/kg)**	65.50 ± 5.16^{b}	31.45 ± 3.19^{c}	0.480

Means ± SE with different letters superscripts in the same column are significant at P < 0.05 using one way ANOVA test.
n= 9 rats/group.

Table 5. Effect of red ginseng extract (RGE) on blood glucose (BG), leptin and insulin hormones levels in rats.

Parameters Groups	BG (mg/dL)	Leptin (ng/ml)	Insulin (ng/ml)
Group (1) **Negative control**	220 ± 12.0[d]	2.50 ± 0.15[d]	2.95 ± 0.15[a]
Group (2) **Obese diabetic control**	285 ± 10.0[a]	4.90 ± 0.11[a]	0.89 ± 0.13[d]
Group (3) **REG (100mg/kg)**	266 ± 13.0[b]	4.10 ± 0.18[b]	1.82 ± 0.24[b]
Group (4) **REG(200 mg/kg)**	245 ± 11.0[b]	3.35 ± 0.17[b]	2.43 ± 0.12[b]
Group (5) **REG(400mg/kg)**	237 ± 10.0[c]	2.75 ± 0.19[c]	2.52 ± 0.14[c]

Means ± SE with different letters superscripts in the same column are significant at
P < 0.05 using one way ANOVA test.
n= 9 rats/group.

Table 6. Effect of red ginseng extract (RGE) on activities of tissue superoxide dismutase (SOD), glutathione peroxidase (GPx) and catalase (CAT) antioxidant enzymes in rats.

Parameters Groups	SOD (U/mg protein)	GPx (nmol/min/mg protein)	CAT (nmol/min/mg protein)
Group (1) **Negative control**	58.70 ± 2.24[a]	0.69 ± 0.01[a]	0.185 ± 0.001[a]
Group (2) **Obese diabetic control**	38.50 ± 2.88[d]	0.18 ± 0.04[d]	0.138 ± 0.002[d]
Group (3) **REG (100mg/kg)**	44.74 ± 3.46[c]	0.22 ± 0.03[b]	0.145 ± 0.001[b]
Group (4) **REG(200 mg/kg)**	48.95 ± 2.58[c]	0.24 ± 0.01[b]	0.158 ± 0.001[b]
Group (5) **REG(400mg/kg)**	55.25 ± 2.73[b]	0.49 ± 0.01[c]	0.175 ± 0.002[c]

Means ± SE with different letters superscripts in the same column are significant at
P < 0.05 using one way ANOVA test.
Unit of GPx= nmol of GSH utilized/min/mg protein.
Unit of CAT= nmol of H_2O_2 utilized/min/mg protein.
n= 9 rats/group.

DISCUSSION

This study aimed to investigate the effects of red ginseng extract on adiposity index, serum liver enzyme, lipid profile, blood glucose, leptin and insulin hormones levels as well as the activities of renal antioxidant enzymes in obese diabetic rats.

In the present era, medicinal plants and culinary herbs with antihyperlipidemic and antidiabetic activities have gained much attention, especially those with little toxicity properties. It has been widely accepted that the biological value of plants depends on their bioactive constituents such as flavonoids, anthocyanins, saponins, diterpenes and other phytochemicals [34, 35].

In the current study, obesity was experimentally induced by feeding rats on high fat-diet for 6 weeks according to the method described by Bhatt *et al.* [20]. This model of obesity in rats closely resembles the reality of obesity in humans. However, the experimental obesity could be also induced in rats and mice by other methods such as feeding on high carbohydrate diet, damage in anterior hypothalamus and genetically induced obesity [36]. In this study, the rat model used was obese diabetic rats where the obese rats were rendered diabetic by intraperiotoneal injection of alloxan for 5 days.

The results of the present study showed that the extract of red ginseng (RGE) when given orally to obese diabetic rats for 6 weeks caused marked decreases in the body weight, fat weight and adiposity index. The anti-obesity effect of RGE that reported in this study was similar to the previously reported [17, 37, 38]. The previous authors reported that the crude saponins of Korean red ginseng induced an antiobesity effect in rats fed on high fat-diet. Feeding rats on high fat-diet was previously reported to increase the final body weight, fat weight and concentrations of serum triglycerides (TG), total cholesterol (TC), and low density lipoprotein (LDL-c) cholesterol when compared to rats fed on the basal diet [39].

The mechanism(s) underlying the antiobesity effect of red ginseng extract could (RGE) be possibly explained by its hyperinsulinemic effect that was evident in the present study. It is evident that hyperinsulinemia and insulin resistance are common features of obesity in humans [40] and rats [39]. In addition, the antiobesity activity of RGE could also be attributed to the high level of leptin hormone caused by RGE that reported in the current study. It is known that leptin is a peptide hormone secreted by adipose tissue in proportion to its mass and when leptin circulates in blood and acts on the brain to regulate food intake (appetite) and energy expenditure When body fat mass decreases, the plasma leptin levels decreases so stimulating appetite and suppressing energy expenditure till fat mass is restored [29]. On this basis, the reduced adiposity index following administration of RGE to obese diabetic rats could be attributed to the low serum leptin level in the treated rats.

The hepatoprotective effect of red ginseng extract (RGE) reported in this study was evident by the significant decreases of the elevated serum levels of liver enzymes (AST, ALT and GGT) in the treated rats. The reported hepatoprotective effect RGE agreed with that demonstrated by previous authors [41, 42]. The authors concluded that the isolated saponins of Korean red ginseng caused hepatoprotective effect and induced restoration of hepatic enzymes in CCl_4-intoxicated rats. In addition, Korean red ginseng extract inhibited the high levels of AST and ALT enzymes and ameliorated liver injury after 70 % hepatectomy in rats [43]. The mechanism of hepatoprotection of red ginseng saponins was assumed to be through an inhibition of the activity of cytochrome P450 enzymes in the rat liver microsomes [44].

The decrease in serum levels of total cholesterol, triglyceride and LDL-c caused by RGE, in this study, was similar to that previously reported [13, 16, 37]. The authors concluded that RGE and its saponins fraction lowers the levels of total cholesterol, triglycerides and LDLc in man and rats. The hypolipidemic effect of RGE was attributed to its content of saponins which inhibited the intestinal absorption of cholesterol and reduced serum cholesterol levels in rats. In man and rabbits, red ginseng extract reduced serum total cholesterol, LDL-c, and triacylglycerol and so improved serum lipid profile [16]. .

Rats fed on high fat-diet for 6 weeks had significantly lower serum insulin levels than those fed on basal diet. This effect agreed with that the pervious finding that feeding high-fat diet to rats resulted in impaired pancreatic function and decreased insulin secretion [45]. Red ginseng extract when orally given to obese diabetic rats at doses 100, 200, and 400 mg/kg caused hyperinsulinemia, in a dose dependant manner. The hyperinsulinemic and hypoglycemic effects of RGE were similar to that those reported in diabetic mice [10] and in obese rats [11]. Some previous studies revealed that hyperinsulinemia and insulin resistance are common features of obesity in humans [40] and in rats [39]. The mechanism(s) of antidiabetic and antiobesity effects of Korean red ginseng (KRG) extract (200mg/kg, oral) was examined in obese insulin resistant rat model. The results showed that KRG led to a significant reduction in body weight, fat mass reduction associated with increased insulin sensitivity. The authors concluded that KRG may have antidiabetic and antiobesity effects due to partly increased insulin sensitivity by increased adipokines (cytokines secreted by adipose tissue) and partly due to enhanced insulin signaling [12]. .

The present results showed that rats fed on high fat-diet (HFD) had high serum leptin hormone level when compared with those fed on basal diet. This finding agreed with that reported by Huang *et al*. [45] who found that HFD elevated serum leptin level in rats. Leptin plays a key role in regulating energy intake and energy expenditure. Leptin is primarily manufactured in the adipocytes of white adipose tissue, and the level of circulating leptin is proportional to the total amount of fats in the body. RGE significantly decreased serum leptin levels in obese diabetic rats. This result agreed with that previously reported that saponins of red ginseng reduced body weight, decreased serum leptin level and depressed appetite in obese rats [45].

The activity of antioxidant enzymes (SOD, GPx and CAT) decreased in renal tissues in obese diabetic rats fed on HFD, This finding can be explained by hyperglycemia due to alloxan injection that causes renal oxidative stress . It is known that oxidative stress plays a key role in the onset and development of diabetes complications, notably diabetic nephropathy [8]. Red ginseng extract (RGE) when given to obese diabetic rats induced an antioxidant effect that evident by increased activities of renal tissue SOD, GPx and CAT antioxidant enzymes. The antioxidant effect of RGE could be attributed to its hypoglycemic activity that reported in this study. This activity of RGE was similar to that previously reported [11, 12, 13].

In conclusion, oral administration of red ginseng extract to obese diabetic rats exhibited antiobesity, antidiabetic, hepatoprotective, antihyperlipidemic and antioxidant activities. These results suggest the possibility of use of red ginseng plant for treating obese patients who suffer from diabetes mellitus due to its good antiobesity, antioxidant and antidiabetic effects.

CONFLICT OF INTEREST

None.

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Original Research

The role of diltiazem enriched with zinc sulphate in anal fissure

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Abstract

Aim: Chronic anal fissure has traditionally been treated surgically. Initial enthusiasm for chemical sphincterotomy has waned because of poor outcomes with glyceryltrinitrate ointment. In this study the use of topical 2% diltiazem enriched with 2% Zinc sulphate ointment has been investigated as an alternative method of chemical sphincterotomy. To evaluate the effectiveness and safety of combined ointment of 2% diltiazem enriched with Zinc sulphate in the treatment of anal fissure, and to analyze the relationship between healing duration of the treatment, and the number of applications.

Method: A prospective observational study of 55 patients diagnosed with anal fissure that began treatment with three groups; control group A; using Vaseline ointment (15 cases), group B; (15 cases) topical diltiazem ointment 2%, and group C; (20 cases) using combined ointment diltiazem 2% enriched with Zinc sulphate 2%, between August and December 2012. Diltiazem and zinc sulphate ointment was prepared in the Pharmacy Service.

Results: Effectiveness and safety were assessed by regular visits as an outpatient management and a telephone survey conducted with each patient within 8 weeks of treatment, adding it to the patient's clinical records. Variables were analyzed including healing, adverse effects, duration of treatment and number of applications, among others. Follow-up was carried out for up to 3 months until complete recovery of the fissure. A total of 55 patients were included in the study, 50% of anal fissure cases were healed especially group C. Recovery occurred in 15% of patients with anal fissure in group B and in 3% of patients with anal group A. 50 % of patients fissure were cured who underwent treatment for a month or more.

Conclusion: Treatment of chronic anal fissures with 2% diltiazem ointment enriched with Zinc sulphate 2% for two months has avoided surgery in nearly 50 % of patients, with few adverse effects.

INTRODUCTION

Anal fissure is a linear or rocket-shaped ulcer in the distal anal canal, which most often occurs in the posterior midline (in about 90% of all cases). Generally patients with anal fissure have anal pain after defecation (which may last up to several hours).The anal pain may be associated with blood appearing on the stool or toilet paper [1,2]. The most consistent finding in typical fissures is the spasm of the internal anal sphincter [1,3]. It is a widely held view that the anal pain is caused by spasm-induced ischemia of the sphincter. The evidence for this is the association of spasm relief with the relief of pain and fissure healing. However, it is not clear if anal sphincteric hypertonia and ischemia are the cause of anal fissure. After a period of 2-3 months, the fissure acquires chronic features comprising induration at the edges, a sentinel tag of skin or/and hypertrophied anal papillae [4]. Post mortem morphometric studies revealed that the blood supply of the anoderm at the posterior midline is significantly lower than that of the other sides of the anal canal [3]. Using a laser Doppler flow meter [5],

demonstrated that an anodermal blood flow is negatively correlated with the resting anal pressure. These findings form the basis for the understanding of fissure predilection in the posterior midline of the anal canal and the rationale for the use of drugs that reduce sphincter spasm to treat chronic anal fissure [6,7]. Voltage-dependent calcium channels are ion pores (multi-subunit complexes) in the plasma membrane of electrically excitable cells that regulate the entry of extracellular calcium into electrically excitable cells and serve as signal transduction centers. Contractions of smooth muscles are regulated by the cytosolic Ca2+ level. Calcium blockers (diltiazem) act by inhibiting the flow of Ca2+ into the sarcoplasm, with consequent saving of oxygen and decrease in the mechanical contraction of the muscle fibers [8,9].

Calcium channel blockers are as effective as topical Glyceryl trinitrates in reducing persistence of fissure at 30 days to 6 months [8,16]. Chronic anal fissure (acute fissure lasting for more than 2-3 months) has traditionally been treated surgically. Initial enthusiasm for chemical sphincterotomy has waned because of poor outcomes with glyceryltrinitrate ointment. In this study the use of topical 2 % diltiazem enriches with zinc sulphate ointment has been investigated as an alternative method of chemical sphincterotomy. Diltiazem hydrochloride works by relaxing the muscle around the anus (the anal sphincter), thereby reducing the anal pressure and increasing the blood flow to the area to allow healing to occur.

Zinc is an essential trace element for life in humans, it serve as potential antioxidants. It plays a critical role in many biochemical functions including DNA, RNA, and protein synthesis, cell growth, proliferation and regeneration, stebilization of biomembrane structure, wound healing, alcoholic metabolism, intellectual development, reproduction, disease resistance, and immunocompetence [11]. It serves as a catalytic component over 300 enzymes and a structural component of various proteins, hormones and nucleotides [12]. An essential biochemical function of zinc is to retard oxidative processes and it serves as potential antioxidant [13]. Zinc compounds have been used therapeutically in different medical areas because of their healing properities at the same time, it posse's low toxicity and well tolerate [14,15].

MATERIALS AND METHOD

Our study done to find the role of 2% Diltiazem enrich with 2% zinc sulphate in chronic anal fissure cases. It was carried out on 55 patients with age range (35y-50y) over period of 8weeks as an outpatient management. They were allocated into three groups A, B and C.

Group A; Treated with Vaseline, once daily, (control group) – 15 cases.

Group B; Treated with 2% Diltiazem ointment (once daily at night)) - 15 cases.

Group C; Treated with 2% diltiazem enrich with zinc sulphate 2% (once daily at night) - 20 cases.

Follow up done every week by regular visits weekly in UR Day Clinic of Surgery/Baghdad between August to September 2012, for 8 weeks to look for the progress of the anal verge changes by either visual or digital per rectal examination to asses the progress of the anal fissure and to show which is more effective locally. Treatment generally continues until the pain has resolved or for up to a maximum of eight weeks.

The pharmacy department has supplied cream which should last about three weeks. We depend on certain criteria (complication, pain, blood in stool, recurrence and failure of chemical sphinecterotomy - surgery), patient call us once he feel any improvement in symptom, we advise the patients to apply the ointment once time daily after hot bath either when going rest afternoon or at night.

RESULTS

Twenty seven patients (50 %) experienced healing of the fissure after 2-3 months of treatment (11 of group B and 15 of group C). Five of group C escape contact while the other four from group B, who did not heal were treated 2% diltiazem enrich with zinc sulphate 2% for a further 8 weeks and had recovery. Four patients of group C experienced perianal dermatitis and all patients of group B and C experienced headache. No other side-effects were recorded. After a range of 2-3 month follow-up following completion of treatment, 34 patients of 50 available remain symptom free regarding anal fissure and return to normal life after 2-4 months. 19 cases with recurrent fissure were treated successfully by repeat chemical sphincterotomy using (2% Diltiazim enrich with Zinc sulphate ointment), most recurrence cases were from group A. The most common unwanted effects of Diltiazem hydrochloride (which occur in most of the cases that use it) are: Headache. Most headaches will not be severe or long lasting and can be treated with simple Pain killers (e.g. paracetamol).

Light-headedness or dizziness especially when getting up from a sitting or lying down position, if the patients begin to feel dizzy we told them to sit or lie down until the feeling passes. Local burning or itching or rectal bleeding reported also in some patients.

Table 1. Changes in improvement of anal fissure according to the line of management during the first 3 months of illness.

Cases	Parameters	1st month	2nd month	3rd month
Group A	Symptoms & signs	mild pain	Pain + blood in stool after defecation	same
	complication	nil	Anal fissure(acute)	Anal fissure (chronic)
	Recurrence	nil	-	15 cases
	Surgery	-	-	Not Indicated
Group B	Symptoms & signs	Mild pain	No pain, no blood in stool in 11 cases	4 cases still had pain & blood during defecation
	Complication	Headache	same	same
	Recurrence	nil	nil	4 cases treated by diltiazem encircled with zinc
	Surgery	not indicated	Not indicated	Not done
Group C	Symptoms & signs	Mild pain	No pain or blood in stool in 15 cases	Nil
	Complication	Headache, Dermatitis treated by local steroid	nil	nil
	Recurrence	nil	nil	nil
	Surgery	not indicated	not indicated	not indicated

DISCUSSION

The autonomic nervous system and enteric neural stimulation regulate the anal sphincter tone. Certain modulators like Calmodulin, caldesmon, calponin and myosin have an influence on Ca2+ regulation in the cytosol and on muscle contraction but the precise mechanism responsible for prolonged contraction of smooth muscle tone is unclear [16,17,19]. It is widely known that a decrease in cytosolic Ca2+ causes relaxation of the smooth muscle, When the direct influx of extracellular Ca2+ through the membrane of Ca2+ channels is blocked, It is worthwhile noting that the ultimate neural modulation of the anal Sphincter contraction is caused by a different neurotransmitters, such as noradrenaline (NA), acetylcholine (Ach), Prostaglandin E2 (PGE2), Prostaglandin F2 (PGF2), 5-hydroxytryptamine (5- HT), and dopamine. [18,20].

A thorough identification of different pathways and the understanding of this modulation are extremely difficult in part due to the fact that several of the transmitters may be released from the same neurons. Diltiazem one of a strong calcium channel blocker and in low concentration 2% reduce the incidence of complication (headache) in spite of longer time of treatment., meanwhile zinc sulphate play major role as antioxidant to promote healing process and minimizing dermatitis.

CONCLUSION

Topical 2% diltiazem ointment enriched with zinc sulphate ointment 2% used as an agent for chemical sphincterotomy in chronic anal fissure offers significant healing rates up to 50% with a significant low side-effect profile (headache, dermatitis) which may aid in further treatment. Early recurrences are common but usually amenable to further chemical sphincterotomy.

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