Journal of Coastal Life Medicine 2014; 2(3): 214-221

Journal of Coastal Life Medicine

journal homepage: www.jclmm.com

Document heading doi:10.12980/JCLM.2.2014C300

Antihyperglycemic and subchronic toxicity study of *Moringa stenopetala* leaves in mice

Tesemma Sileshi[1*], Eyasu Makonnen[2], Asfaw Debella[3], Birhanu Tesfaye[3]

[1]*Department of Pharmacy, College of Medicine and Health Science, Ambo University, PO Box 19, Ambo, Ethiopia*

[2]*Addis Ababa University, School of Medicine, PO Box 9080, Addis Ababa, Ethiopia*

[3]*Ethiopian Nutrition and Health Research Institute (ENHRI), PO Box 1242, Addis Ababa, Ethiopia*

PEER REVIEW

Peer reviewer

Girma Belachew Gutema, PhD Candidate, School of Pharmacy, University of Oslo, Norway.
Tel: +4796878879
E-mail: girmabbaacabsaa@gmail.com

Comments

The work is of high scientific and literary standards with appreciable qualitative and quantitative approaches to answer the research question raised. The extensive use of robust instrumental techniques, generation and presentation of relevant data set in line with the objective rendered the study of significant contribution to the body of knowledge in this specific thematic area.
Details on Page 220

ABSTRACT

Objective: To evaluate the antihyperglycemic activity and subchronic toxicity of an extract of *Moringa stenopetala* (*M. stenopetala*) leaves in mice.

Methods: Antihyperglycemic activities of various solvent subfractions and chromatographic fractions were investigated in alloxan induced diabetic mice. All fractions were administered intragastrically using oral gavage at a dose of 500 mg/kg. For the subchronic toxicity investigation of the 70% ethanol extract of *M. stenopetala* leaves, a daily dose of 300 or 600 mg/kg body weight was administered to mice over 96 d. Some hematological and plasma biochemical parameters were measured as indices of organ specific toxicity. Preliminary phytochemical screening and antioxidant activity investigation was done using thin layer chromatography method.

Results: Among the solvent subfractions of the 70% ethanol extract tested only butanol subfraction exhibited significant reduction of blood glucose level ($P<0.05$) at 2 h (53.44%) and 4.5 h (46.34%) in diabetic mice and it was further fractionated chromatographically. This resulted in isolation of three chromatographic fractions (fraction 1, 2, and 3) which exhibited maximal blood glucose reduction ($P<0.01$) at 6 h (77.2%), at 4.5 h (69.1%) and at 4.5 h (71.96%) after administration. Furthermore, these fractions exhibited comparable antioxidant activity, and preliminary phytochemical screening indicated the presence of phenolic compounds which may be phenolic glycoside in all fractions. The subchronic toxicity study of the 70% ethanol extract of *M. stenopetala* leaves revealed that there were no significant differences in body weight, between controls and treated mice. Hematological analysis showed no differences in most parameters examined. Furthermore, it did not significantly affect plasma creatinine, urea, cholesterol, triglycerides and CA125 levels. It also did not significantly affect the plasma T3, T4 and THS level. It, however, caused a significant dose-dependent increases in aspartate aminotransferase and alkaline phosphatase. The plasma alanine aminotransferase increased in a dose dependent manner, though not significant.

Conclusions: The present study revealed that the crude ethanol extract and solvent-solvent fractions as well as chromatographic fractions have antihyperglycemic effect. Furthermore, the crude ethanol extract have some effect on liver of the mice on subchronic administration. Therefore, further study should be done to identify the active principal compound responsible for antihyperglycemic effect and to rule out the safety in other animal model.

KEYWORDS

Moringa stenopetala, Diabetes mellitus, Antihyperglycemic effect, Mice, Subchronic toxicity

1. Introduction

Diabetes mellitus is a group of metabolic diseases of multiple etiologies characterized by chronic hyperglycemia with disturbances of carbohydrate, protein and lipid metabolism resulting from defects in insulin secretion, insulin action, or both[1]. Diabetes is associated with reduced life expectancy, significant morbidity due to specific

*Corresponding author: Tesemma Sileshi, Department of Pharmacy, College of Medicine and Health science, Ambo University, Po Box 19, Ambo, Ethiopia.
Tel +2519 11550975
E-mail: teshawa20@yahoo.com

Foundation Project: Supported by the School of Graduate Studies, Addis Ababa University and Traditional and Modern Drug Research Department of the Ethiopian Health and Nutrition Research Institute (grant number GSR/2842/00).

Article history:
Received 5 Feb 2014
Received in revised form 10 Feb, 2nd revised form 19 Feb, 3rd revised form 22 Feb 2014
Accepted 15 Mar 2014
Available online 28 Mar 2014

diabetes related microvascular complications, increased risk of macrovascular complications such as ischemic heart disease, stroke and peripheral vascular disease, and diminished quality of life[2,3].

Several approaches are presently available to reduce the hyperglycemia in diabetes mellitus. Among these people in Ethiopia use different plants traditionally for the treatment of diabetes mellitus. *Moringa stenopetala* (*M. stenopetala*) is one of these medicinal plants which is widely used for antidiabetic purpose. *M. stenopetala* belongs to the family Moringacae represented only by a single genus *Moringa*. This plant is known by different vernacular names like shiferaw in Amharic. Previous study made on the crude aqueous extract of the leaves of *M. stenopetala* on rabbit[4] and mice[5] indicated the hypoglycemic activity of the plant. Moreover, the fractions isolated from the aqueous extract of the plants were also shown to have both hypoglycemic and antihyperglycemic effects[5]. Furthermore the crude ethanol extracts and solvent fractions of the ethanol extract of leaves of the plant were reported to have both hypoglycemic and antihyperglycemic effect[6]. The administration of butanol fraction of solvent fractions over 28 d also found to reduce hyperglycemia in alloxan induced diabetic mice[7].

Although there are sufficient data about the diverse biological activity of the different parts of plants belonging to the family Moringaceae, there are conflicting reports as to their safety. Mekonnen and coworkers[8] showed that ethanol extract of the leaves and seeds of *M. stenopetala* contain toxic substances that are extractable with organic solvents or are formed during the process of extraction with these solvents while the aqueous extract was found to be safe in the *in-vitro* cytotoxic test.

The antihyperglycemic effects upon further fractionation and Phytochemical screening for the possibly class of compound, and safety in animal model however, were not investigated. The objective of the present study was therefore; to evaluate the antihyperglycemic activity of solvent fractions of ethanol extract of *M. stenopetala* leaves as well as chromatographic fractionation of a butanol fraction, phytochemical screening of the active fractions and safety of the crude extract.

2. Materials and methods

2.1. Collections and preparation of plant materials

The leaves of *M. stenopetala* used in this study were collected from Arbaminch town 502 km south of Addis Ababa in March 2010. The plant material was identified, authenticated and voucher specimen (No. 2147) was deposited in the herbarium of traditional and modern drug Research Department of Ethiopian Health and Nutrition Research Institute (EHNRI). It was then dried under shade and crushed to powder for extraction.

2.2. Chemicals and instruments

Alloxan (Sigma, Alderich, Germany), ethanol (Winlab, England), petroleum ether 60-80 °C (fluka, Germany), sulphuric acid (Farm Italia Carrloerba, Italy), *n*-hexane (Wagtech International Ltd, England), lead acetate (BDH Ltd, England), methanol HPLC grade (Techno PharMCHem, Bahadurgarm, India), ethyl acetate (Fluka, Germany) and Glibenclamide (Zydus Cadila, India) Butanol(Riedel -dethan Germany), dichloromethane (Alderich, Germany), 1,1-diphenyl 2-picryl-hydrazyl (DPPH) (Sigma, USA), fast blue B (Riedel-dethaen, Germany), silica gel (BDH chemical LTD poole, England), vanillin (Merk, Germany), sodium hydroxide (Riedel-dethaen, Germany), rota vapor (buchi rota vapor vac R-500, Switzerland), lyophilizer (Labconco, USA), Accua check active glucometer (Ireland) and Accua check active glucose test strip (Roche diagnostic GMBH, Germany) XT-1800i automated hematological analyzer (Japan), Elecsys 2010 roche diagnostic (GMBH, Germany), Humaster 80 automated clinical chemistry analyzer (Germany) were used in this study.

2.3. Preparation of plant material extract

The powdered leaves of *M. stenopetala* (1.2 kg) were soaked with 70% ethanol (v/v) for 72 h successively. This was repeated three times and the extract was then filtered with Whatman No. 1 filter paper. The filtrates were concentrated using rota vapor (buchi rota vapor vac R-500, Switzerland). To evaporate the remaining solvent, the extract was kept on the water bath, and the dried extract (73 g) was stored in a refrigerator at 80 °C for subsequent experiments.

2.4. Solvent-solvent fractionations of the total ethanol extract

The procedure for solvent-solvent separation was adopted from Hideaki[9] with some modification. A total of 10 g of 70% ethanol extract of plant material was dissolved in 100 mL distilled water. The dissolved ethanol extract was then transferred to separatory funnel and extracted with *n*-hexane (150 mL), dichloromethane (150 mL), and *n*-butanol (225 mL) successively until the extracting solvent became colorless in each case. After completing the separation process, solvents were recovered by rota vapor, and the aqueous residue was lyophilized using a lyophilizer (Labconco, USA). Dried fractions were stored in a refrigerator at 8 °C for subsequent experiments.

2.5. Chromatographic fractionation

A total of 8 g of the butanol fraction was dissolved in small

volume of methanol and adsorbed in 10 g silica gel (mesh size 60). The solvent was then removed under reduced pressure and the silica that adsorbed sample was dried and collected. Silica gel (100 g) slurry made using petroleum ether and packed into a column pre-plugged with a small piece of cotton at bottom and fixed in a clamp. Silica adsorbed sample was then transferred to the column. The column was then filled with eluting solvent and allowed to run at the rate of 40 drops per minute.

Gradient mobile phases were used to facilitate the separation of components of butanol fraction that was loaded. The mobile phases used for the column were based on the principle of increasing polarity. Initially the column was run with petroleum ether and then petroleum ether/ ethyl acetate (1:1), ethyl acetate/methanol (10:0.5), ethyl acetate/ methanol (10:1), ethyl acetate/methanol (10:2), ethyl acetate/ methanol (10:4), ethyl acetate/methanol (10:6), ethyl acetate/ methanol (10:8), ethyl acetate/methanol (10:12) respectively and finally methanol. A total of eighteen fractions were collected sequentially in a labeled flask based on their thin layer chromatography (TLC) profile. The solvent was then removed under reduced pressure and chromatographic fractions of each elute were further analyzed on TLC plates and combination of fractions were done using their similar TLC profile. The four combined fractions were finally used to analyze antihyperglycemic activity, phytochemical and antioxidant activity as described below.

2.6. Pharmacological and toxicological evaluation

2.6.1. Experimental animals

Swiss albino mice weighing 20-32 g of both sexes were obtained from Ethiopian Health and Nutrition Research Institute (EHNRI). Animals were allowed free access to water and food. All animals (mice) were maintained on a 12 h light/ dark cycle, at a constant temperature and humidity during the study period. Animals were acclimatized to the test environment prior to the experiments.

2.6.2. Induction of diabetes

Male Swiss albino mice were fasted overnight and administered 180 mg/kg alloxan monohydrate dissolved in distilled water interapertoneal (*i.p.*). Food and water were given to the animals 30 min after alloxan administration. Seven days after the injection, the blood glucose levels were measured. A mouse with a blood glucose level above 200 mg/dL was considered to be diabetic and used in the experiments.

2.6.3. Evaluation of antihyperglycemic activity of solvent-solvent fractions

Diabetic male mice, deprived of food overnight, were divided into seven groups of five animals each. One group served as a control and received a normal saline solution (10 mg/kg body weight). The second group received glibenclamide (a single dose of 0.66 mg/kg body weight) as a reference drug. The other groups received 500 mg/kg of crude 70% ethanol extract, 500 mg/kg of butanol fraction, 500 mg/kg of dichloromethane fraction, 500 mg/kg of hexane fraction and 500 mg/kg of the aqueous residue of solvent-solvent fraction of *M. stenopetala* leaves extract. All treatments were oral. Blood samples were obtained by amputation of the tail tip at 0, 2, 4 and 6 h after treatment. The blood glucose level was determined using aqua check active glucose analyzer.

2.6.4. Evaluation of antihyperglycemic activity of chromatographic fractions

Alloxan induced male diabetic mice, deprived of food overnight, and were divided into six groups of five animals. One group served as a control and received a normal saline solution (10 mg/kg body weight). The second group received glibenclamide (a single dose of 0.66 mg/kg body weight) as a reference drug. The other groups received 500 mg/kg of fractions 1 to 4 of column chromatographic elutes. The solutions and fractions were administered orally. Blood samples were obtained by amputation of the tail tip at 0, 1.5, 3.0, 4.5 and 6.0 h after treatment. The blood glucose level was determined using aqua check active glucose analyzer.

2.6.5. Subchronic toxicity study

The experimental animals used in this study were Swiss albino mice of either sexes each weighing 23-33 g and aged 8-10 weeks. The mice were randomly distributed into three groups of 10 mice per dose with free access to water and food. All mice were maintained on a 12 h light/dark cycle, at a constant temperature and humidity during the study period. The first group was given vehicle and served as the control. The second group received 300 mg/kg of ethanol extract of *M. stenopetala* leaves and the third group received 600 mg/kg of the same extract by oral route. The general state of the mice including the dynamics of body weight changes, appetite, motor activity, and behavior were then recorded.

On the 96th day of treatment blood samples for hematological and biochemical analyses were collected with and without EDTA respectively from the heart. Hematological parameters such as erythrocyte and platelet counts; hemoglobin concentration; mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), and mean corpuscular hemoglobin concentration (MCHC); and white blood cell counts were determined by automated hematological analyzer XT-i1800i (Japan). The biochemical parameters were determined in serum obtained after centrifugation of the whole blood without anticoagulant at 1 500 r/min for 15 min. The level of serum alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP), urea, creatinine, cholesterol and triglycerides were determined

by the automated clinical chemistry analyzer (humostar 80, Germany). Serum concentrations of T3, T4, TSH and tumor marker CA125 were determined by Roche diagnostics, GmbH (Elecsys2010 Roche Hitachi, Germany).

2.7. Phytochemical screening of butanol fraction and chromatographic fractions

Sample of butanol fraction of solvent-solvent separate and its column chromatography elutes were separately dissolved in methanol and spotted onto a TLC plates (silica gel F 254) using micropipette. The plates were then placed in presaturated glass tanks containing mobile phase ethyl acetate/methanol (10:2). After complete elution, the plates were air dried and examined under UV light of wavelength (λ254 and 366). Finally, the plates were sprayed with freshly prepared reagents such as 0.5% fast blue B+0.1N NaOH, 4% sulfuric acid in ethanol, 1% vanillin in sulfuric acid and 2% lead acetate solution and development of different color was recorded and used as an indicator for the presence or absence of secondary plant metabolite[10].

2.8. In vitro antioxidant assay of butanol fraction and chromatographic fractions

TLC screening for antioxidant activity of butanol fraction and column chromatographic elutes was carried out by spotting a concentrated methanolic solution of fractions on silica gel plates. The plates were developed as described above afterwards air-dried and sprayed with 0.2% w/v DPPH spray in methanol. The plates were visualized for the presence of light yellow spots against pink (purple) background.

2.9. Statistical analysis

Data were expressed as mean±SEM. Statistical evaluations were performed using One-way analysis of variance (ANOVA) followed by Tukey test to compare the means of each dose group with the control group. The level of statistical significance was set at $P<0.05$. Statistical analysis was done using graph pad Instat® software.

3. Results

3.1. Antihyperglycemic activity of 70% ethanol extract of M. stenopetala leaves and its solvent fractions

As shown in Table 1 single dose of the crude ethanol extract resulted in significant reduction of blood glucose level at 2 h ($P<0.01$) after administration. The percent blood glucose level reduction were very significant with hexane fraction at 2 and 4 h after administration ($P<0.01$); dichloromethane fraction at 2, 4, and 6 h after administration ($P<0.01$). The percent reduction with butanol fraction was extremely significant at 2 and 4 h after administration ($P<0.001$), while the reduction with aqueous residue was very significant ($P<0.01$) at 2 h and significant ($P<0.05$) at 4 h after administration.

Table 1
Percent blood glucose reduction after administration of solvent fraction of *M. stenopetala* leaves in alloxan induced diabetic mice.

Treatment group	% Blood glucose level reduction at t (h)			
	0 h	2 h	4 h	6 h
Diabetic control	0	−15.66±5.7	−2.88±3.9	7.24±5.8
Standard (0.066 mg/kg)	0	8.44±2.4	53.28±6.6***	56.30±6.80***
Ethanol extract (500 mg/kg)	0	20.10±7.2**	10.90±3.2	21.53±3.25
Hexane fraction (500 mg/kg)	0	29.10±8.0**	39.86±4.0**	32.74±2.50
Dichloromethane fraction (500 mg/kg)	0	27.73±4.9**	27.70±5.5**	43.80±5.30**
Butanol fraction (500 mg/kg)	0	53.44±2.4***	46.40±7.8***	30.00±10.70
Aqueous residue (500 mg/kg)	0	19.44±4.3**	26.66±5.5*	31.00 ±5.24

*: $P<0.05$, **: $P<0.01$, ***: $P<0.001$.

3.2. Antihyperglycemic activity of chromatographic fractions

The results of antihyperglycemic activity of a single dose of chromatographic fractions obtained are shown in Table 2. There were no significant differences in the blood glucose levels with the chromatographic fractions as compared to control at 1.5 h after administration. But 3 h after administrations fraction 2 and 3 showed a very significant reduction of blood glucose level ($P<0.01$). The two fractions showed the same result at 4.5 h and 6 h after treatment. Fraction 1 reduced blood glucose level significantly ($P<0.05$) at 6 h after administration. Similarly the percent blood glucose reduction of all fractions was not significant at 1.5 h after administration. However, fractions 1, 2 and 3 showed extremely significant percent blood glucose level reduction at 3, 4.5 and 6 h after treatment which was higher than that observed by the standard drug ($P<0.001$). On the other hand fraction 4 showed very significant reduction of percent blood glucose level ($P<0.01$) at 3 h and significant ($P<0.05$) at 4.5 and 6 h after treatment.

Table 2
Percent blood glucose reduction after administration of chromatographic fractions of *M. stenopetala* leaves in alloxan induced diabetic mice.

Treatment group	% Blood glucose level reduction at t (h)				
	0 h	1.5 h	3 h	4.5 h	6 h
Diabetic control	0	−11.76±6.13	−15.00±11.5	5.42±6.40	6.48±6.57
Standard (0.066 mg/kg)	0	2.24±1.30	34.50±3.3**	54.00±7.60**	55.80±7.00***
Fraction 1 (500 mg/kg)	0	2.66±12.00	46.66±11.5***	72.66±7.55***	77.20±6.20***
Fraction 2 (500 mg/kg)	0	24.54±10.00	53.80±4.5***	69.10±4.45***	62.94±6.70***
Fraction 3 (500 mg/kg)	0	25.14±9.30	59.32±7.0***	71.96±5.70***	67.60±7.70***
Fraction 4 (500 mg/kg)	0	−7.84±14.00	32.70±6.3**	44.68±11.00*	44.80±9.40*

*: $P<0.05$, **: $P<0.01$, ***: $P<0.001$.

3.3. Phytochemical screening of the butanol fraction and chromatographic fractions

Preliminary phytochemical screening was undertaken for

butanol fraction of solvent-solvent separate and column chromatographic fractions using TLC.

Chemical test and UV detection were performed for butanol fraction of solvent-solvent separate and column chromatographic elutes. TLC was developed using ethyl acetate/methanol (10:2) mobile phase, which was found to be an optimal solvent system to separate components. Detection under UV (λ254 and 366) the fractions showed no clear spot. However, single dark and faded spot were observed in UV detection at λ366.

Spraying of the developed TLC plates with 4% sulfuric acid in ethanol, and 0.5% fast blue+0.1% NaOH, both butanol fraction and column chromatographic elute fraction 1, 2 and 3 produced yellowish spot and violet red spot respectively. However, the intensity of color produced by the fraction 4 of chromatographic elute was very weak. The chromatogram of butanol fraction produced no colored spot when sprayed with 1% vanillin in sulfuric acid.

3.4. Antioxidant activity of the butanol fraction and chromatographic fractions

The TLC of butanol fraction and chromatographic elutes were developed as described above and sprayed with 0.2% DPPH in methanol to check for the presence of antioxidant component. Butanol fraction and fraction 1, 2 and 3 of the chromatographic elutes produced comparable white color while fraction 4 produced very weak white color against the pink background.

3.5. Subchronic toxicity

No clinical signs or deaths related to extract administration were observed throughout the experimental period. Clinical observations noted during the study included, general state of the animals including the dynamics of body weight changes, skin abrasions, minor hair loss, appetite, motor activity, and behavior of the animals. The incidences of these signs were similar in control and 300 mg/kg and 600 mg/kg extract administered mice. Furthermore, there was no significant treatment-related effect on food consumption.

The results of the hematological studies are presented in Table 3. The data show that RBC, WBC, HGB, HCT, MCV, MCH, and MCHC, levels in both 300 mg/kg and 600 mg/kg of 70% ethanol extract of *M. stenopetala* leaves treated mice were not significantly different from those of the control group (P>0.14 in all cases). However, platelet counts were significantly higher (P=0.0032) in 300 mg/kg treatment group as compared to the control.

Plasma biochemical and hormonal data at the end of the study are presented in Table 4. No significant changes were observed in the clinical chemistry parameters (urea, creatinine, CA125, TSH, FT3, ALT, TGs, and cholesterol) in extract treatment groups and control. However, FT4 was significantly reduced in the 300 mg/kg received group (P=0.007 2) as compared in 600 mg/kg received group, and the levels of AST (P=0.009 8) and ALP (P=0.046 2) were significantly higher in the mice received 600 mg/kg of the extract in a dose dependent manner.

Table 3

Hematological data at the end of 70% ethanol extract of *M. stenopetala* leaves administration for 96 d.

Treatment group	WBC (10^3/UL)	RBC (10^6 mm^{-3})	HGB (g/dL)	MCV (fL)	MCH (pg)	MCHC (g/dL)	Platelet (10^3/UL)
Control (n=5)	2.597 0±0.784 0	11.140±0.111	15.800±0.125	50.567±1.271	14.167 0±0.233 0	28.067 0±0.233 0	791.000±54.720
300 mg/kg (n=4)	1.460 0±0.220 0	11.615±0.145	16.120±0.250	49.150±0.050	13.900 0±0.060 0	28.250 0±0.050 0	1 415.000±24.000**
600 mg/kg (n=5)	3.275 0±0.577 4	11.512±0.263	16.075±0.075	50.600±1.115	13.975 0±0.370 5	27.625 0±0.193 1	859.000±81.540

Results are mean±SEM, **: P< 0.01 as compared to control.

Table 4

Biochemical and hormonal data at the end of 70% ethanol extract of *M. stenopetala* leaves administration for 96 d.

Treatment group	TSH (U/L)	FT4 (μg/dL)	FT3 (ng/dL)	AST (U/L)	ALT (U/L)	ALP (U/L)	TG (mg/dL)	Cholesterol (mg/dL)	Urea (mg/dL)	Creatinine (mg/dL)	CA125 (IU/mL)
Control (n=5)	0 .015 80±0.001 020	3.726±0.348 9	3.754 0±0.463 0	93.80±2.354	68.8±3.810	66.2±4.04	138.6±10.890	122.00±17.590	54.0±4.041	0.650 00±0.052 0	<0.6
300 mg/kg (n=4)	0.017 75±0.007 973	2.630±0.312 6	2.662 5±0.193 8	110.50±6.380	72.0±5.292	200.0±63.49	176.6±32.100	155.75±3.680	57.0±7.024	0.477 00±0.158 0	<0. 6
600 mg/kg (n=5)	0.012 40±0.002 502	4.242±0.149 7	3.402 0±0.203 6	122.25±7.146**	77.0±10.136	210.6±42.07*	141.4±14.445	133.40±10.703	62.6±10.088	0.526 67±0.062 4	<0.6

Results are mean±SEM, *: P< 0.05, **: P<0.01.

4. Discussion

In this work the antihyperglycemic effect of the solvent fraction and chromatographic elutes was assessed in alloxan induced diabetic mice. Alloxan, a well-known diabetogenic agent is widely used to induce type 2 diabetes in animals[11]. Alloxan induces diabetes through the generation of a redox cycle with the formation of superoxide radicals which undergo dismutation to hydrogen peroxide. These radicals accumulate in the cytosol simultaneously with calcium and cause rapid destruction of the pancreatic β-cells[12]. The dose of alloxan required to induce diabetes depends on the animal species, route of administration and nutritional status[13]. The dose proposed to cause type 2 diabetes in mice is in the range of 100-200 mg/kg. Therefore, 180 mg/kg of alloxan was administered to induce diabetes in this study.

Previous studies done on the crude aqueous extract and its solvent fractions[5], and crude ethanol extract and its

solvent fraction revealed the antihyperglycemic effect of *M. stenopetala*[6]. Among the solvent fractions of the crude aqueous extract and crude ethanol extract of these studies, butanol fraction showed highest antihyperglycemic effect. In our study, the antihyperglycemic effect of the ethanol extract and its fractions observed are in agreement with the previous study. Therefore, the butanol fraction was further fractionated into four fractions using column chromatography. Of the four fractions obtained, fraction 4 was observed to be the only fraction with a weak antihyperglycemic activity which was less than the butanol fraction. Fraction 1, 2 and 3 showed highly significant antihyperglycemic activity as it resulted in about 70% reduction of blood glucose level at 4.5 h which was higher than with the standard drug. When the antihyperglycemic effect of the three active fractions were compared among themselves and also with that of glibenclamide, fraction 2 and fraction 3 were found to be significant. However, fraction 1 also exhibited optimum antihyperglycemic activity and its activity increased with time. Therefore, all the three fractions might have active compound(s) which was/were responsible for the observed antihyperglycemic activity.

A wealth of evidence largely suggests increased oxidative stress in diabetes. Decreased antioxidant capacity, increased production of ROS, and elevated oxidation products of lipids, and proteins have been reported in plasma, urine, and various tissues, suggestive of systemic and organ specific oxidative stress. Hyperglycaemia results in the generation of free radicals which can exhaust antioxidant defenses thus leading to the disruption of cellular functions, oxidative damage to membranes and enhanced susceptibility to lipid peroxidation[14,15]. The fact that the role of antioxidant compounds in both protection and therapy of diabetes mellitus was also emphasized in previous scientific researches[16], in parallel with their antihyperglycemic activity evaluation, antioxidant activity evaluation of the fractions which were found to have an antihyperglycemic effect was carried out in this experiment.

The DPPH assay has been largely used as a quick, reliable and reproducible parameter to search the *in vitro* general antioxidant activity of pure compounds as well as plant extracts[17,18]. Because of its odd electron, 2, 2-diphenyl-picryl-hydrazyl radical (DPPH) gives a strong absorption band at 517 nm in visible spectroscopy (deep violet colour). As the electron becomes paired off in the presence of a free radical scavenger, the absorption vanishes, thus the resulting decolorization is stoictiometric with respect to the number of electrons taken up and the white color against the pink (purple) background indicates the antioxidant activity.

The result of antioxidant activity assay in this study revealed that butanol fraction, fraction 1, 2 and 3 of column chromatographic elutes which manifested in the rapid discolouration of the purple DPPH; suggest that they have strong antioxidant activity. But fraction 4 obtained from column chromatography exhibited weak activity in the discolouration of the purple DPPH. Interestingly this fraction has also shown weak antihyperglycemic activity suggesting overlapping of activity which was considered to be a benefit in diabetes therapy.

Preliminary phytochemical screening performed using TLC technique for butanol fraction and its column chromatographic fractions indicated the presence of flavonoids, phenolic compounds and phenolic glycosides. However, fraction 4 of chromatographic elutes showed very weak color change, which was used as indicator of these classes of compounds, in the entire test performed indicating scarcity of this class of compound in this fraction. Furthermore, this fraction exhibited weak activity in their antihyperglycemic and antioxidant activity indicating the possibility that these classes of compounds may be responsible for antihyperglycemic and antioxidant activity observed.

In the 13-week subchronic oral toxicity study, mice were administrated 300 mg/kg and 600 mg/kg of crude 70% ethanol extract daily. The results presented in this study did not reveal any overt toxic reactions of ethanol extract of *M. stenopetala* leaves as reflected in the body weight change, hematological, biochemical and hormonal data at these dose levels in the study period. Animals in the three study groups gained weight during the 96-day period, although the mice group treated with 300 mg/kg slightly decreased in weight in the first 10 d of treatment. The observed weight reduction was not in a dose and time dependent manner and it might have resulted from physiological variation in mice such as food intake, and metabolism.

Certain medicinal plant preparations adversely affect various blood components[19]. The level of assessed hematological parameters such as erythrocyte, and platelet counts; hemoglobin concentration; MCV, MCH, and MCHC; and white blood cell counts were not significantly affected following repeated administration of 300 mg/kg and 600 mg/kg of 70% ethanol extract of *M. stenopetala* leaves during the period of study. However, PLT counts, significantly increased in 300 mg/kg treatment group as compared to control. PLT alone, however, cannot be taken as indicative of hematotoxicity. Furthermore, the increase in PLT was not in a dose dependent manner.

Some plant medicines have hepato and nephrotoxic effects. Damage to these organs often results in elevation of the clinical chemistry parameters such as serum enzymes like AST and ALT and analytes like, urea and creatinine[20-22]. Testing of this serum/plasma clinical chemistry is therefore, used to determine potential target organs of toxicity and associated time courses of that damage without the need of biopsy or necropsy samples.

The liver is a major organ of early screening efforts in

the pharmaceutical industry and a major target organ in the repeated-dose nonclinical safety studies used to support clinical trials. There are a variety of clinical chemistry tests for evaluation of liver health and function appropriate for use with laboratory animals. Among the commonly used clinical chemistry tests, AST and ALT are used for hepatocellular evaluation while ALP are used for hepatobiliary evaluation[23,24]. In the current study the level of AST increased in a dose dependent manner significantly. However, ALT which is a gold standard in hepatocellular evaluation increased non-significantly. On the other hand, the level of ALP increased significantly again in a dose dependent manner. Furthermore, there was no significant change in the level of cholesterol in the experimental groups. Excessive serum cholesterol provides supportive evidence for hepatic disease. In obstructive biliary disease, hypercholesterolemia may be due to retrograde flow through the biliary system and/or bile salt retention. As a result, cholesterol remains in a soluble state and there is a reduction in tissue uptake. In severe hepatocellular disease with loss of hepatic synthetic capacity, a progressive decrease in total cholesterol occurs[25]. From the present work, it is difficult to conclude that the extract has toxic effect on liver cell and function but the results are in line with the work of Makonen and coworkers[8] and warrants further studies especially histological assessment of this organ.

Toxic agents may affect kidneys and impair their physiological functions. These can be detected by cross checking the normally expected function of the kidneys, for example in excreting waste product like blood urea nitrogen BUN and creatinine[26]. In this work renal toxicity was assessed by measuring BUN and creatinine. And the results indicated non toxic effect of the extract on the kidney.

Previous study of the chemical composition of *M. stenopetala* revealed the presence of rutin, 4-(4′-O-acetyl-L-rhamnosyloxy)-benzylisothiocyanate and O-(rhamnopyranosloxy) benzyl glucosinolates[27]. Glucosinolates are known to impair the thyroid function[28] with deleterious consequences on growth. Glucosinolates toxicity is caused by their derivative products, such as thiocyanate anions, and isothiocyanates. This breakdown is mainly caused by myrosinase, which is a specific plant hydrolytic enzyme, but also by high temperatures or the activity of the intestinal microflora[29]. With this conjecture the effect of ethanol extract of *M. stenopetala* on thyroid function was assessed by measuring serum T3, T4 and TSH. The results of these studies showed no significant dose dependant difference between extract administered and control group.

Conflict of interest statement

We declare that we have no conflict of interest.

Acknowledgements

This study was supported by the School of Graduate Studies, Addis Ababa University and Traditional and Modern Drug Research Department of the Ethiopian Health and Nutrition Research Institute (Grant number GSR/2842/00). We appreciate the assistance of Ato Tesfaye Asaye, Mesay Getachaw and Daniel Melaku for their technical support in laboratory work.

Comments

Background

The research theme in general and the topic taken up in particular is very relevant and interesting to the science community. The methodological approach the investigators took is scientifically sound. The findings clearly address the objective set in the study.

Research frontiers

As part of the move to capitalize on what have been done on the selected medicinal plant, the leave extracts of *M. stenopetala*, this research showed the possible use of its extracts as an antihyperglycemic agent, which substantially augments the accumulating evidences on safety and efficacy of the said medicinal agent.

Related reports

The work is done in line with the standard methodological approaches followed in such scientific researches and it was done as a continuation of some preliminary works on the same plant with crude and aq. extracts. The fact that the investigators went on further fractionation techniques with standard solvents to check the biological activities and subchronic toxicity profiles of the extracts made the work very valuable.

Innovations and breakthroughs

The fractionation of the extracts and investigations on their effects on the liver and their antioxidant effects can be considered as new reports and novel findings in the study.

Applications

The research generally is a good input for the ongoing global effort to rule out the safety and efficacy profiles of medicinal plants with certain useful claim in the traditional medicines of African communities, thereby augmenting formulation and the marketing attempts underway in most countries including Ethiopia.

Peer review

The work is of high scientist and literary standards with appreciable qualitative and quantitative approaches to

answer the research question raised. The extensive use of robust instrumental techniques, generation and presentation of relevant data set in line with the objective rendered the study of significant contribution to the body of knowledge in this specific thematic area.

References

[1] American Diabetes Association. Diagnosis and classification of diabetes mellitus. *Diabetes Care* 2012; **35**(Suppl 1): S64-S71.

[2] Rao MU, Sreenivasulu M, Chengaiah B, Reddy KJ, Chetty CM. Herbal medicine for diabetes mellitus: a review. *Int J PharmTech Res* 2010; **2**(3): 1883-1892.

[3] American Diabetes Association. Standards of medical care in diabetes-2011. *Diabetes Care* 2011; **34**(1): S11-S61.

[4] Makonnen E, Hunde A, Damecha G. Hypoglycemic effect of *Moringa stenopetala* aqueous extracts in rabbits. *Phytother Res* 1997; **11**: 147-148.

[5] Mussa A, Makonnen E, Urga K. Effect of the crude aqueous extract and isolated fractions of *Moringa stenopetala* leaves in normal and diabetic mice. *Pharmacologyonline* 2008; **3**: 1049-1055.

[6] Nardos A, Makonnen Y, Debella A. Effects of crude extracts and fractions of *Moringa stenopetala* (Baker f) Cufodontis leaves in normoglycemic and alloxan-induced diabetic mice. *Afr J Pharm Pharmacol* 2011; **5**(20): 2220-2225.

[7] Toma A, Makonnen E, Debella A, Tesfaye B. Antihyperglycemic effect on chronic administration of butanol fraction of ethanol extract of *Moringa stenopetala* leaves in alloxan induced diabetic mice. *Asian Pac J Trop Biomed* 2012; **2**(Suppl 3): S1606-S1610.

[8] Mekonnen N, Houghto NP, Timbrell J. Toxicity of extracts of plant parts of *Moringa stenopetala* in HEPG2 cells *in vitro*. *Phytother Res* 2005; **19**: 870-875.

[9] Hideaki otsuka. Purification by solvent extracting using partition coefficient. In: Sarker SD, Latif Z, Gray AI, editors. *Methods in biotechnology, Vol 20, Natural product isolation*. 2nd ed. Totowa, New Jersey: Humana Press Inc.; 2006, p. 269-273.

[10] Debella A. *Manual for phytochemical screening of medicinal plants*. Addis Ababa Ethiopia: Department of Drug Research, Ethiopian Health and Nutrition Research Institute; 2002, p. 1-84.

[11] Suresh K, Rajeshwar S, Neeru V, Sunil S. Acute and chronic animal models for the evaluation of anti-diabetic agents. *Cardiovasc Diabetol* 2012; **11**: 9.

[12] Lenzen S. The mechanisms of alloxan and streptozotocin-induced diabetes. *Diabetologia* 2008; **51**: 216-226.

[13] Frode TS, Medeiros YS. Animal models to test drugs with potential antidiabetic activity. *J Ethnopharmacol* 2008; **115**: 173-183.

[14] Matough FA, Budin SB, Hamid ZA, Alwahaibi N, Mohamed J. The role of oxidative stress and antioxidants in diabetic complications. *Sultan Qaboos Univ Med J* 2012; **12**(1): 5-18.

[15] de M Bandeira S, da Fonseca LJS, da S Guedes G, Rabelo LA, Goulart MOF, Vasconcelos SML. Oxidative stress as an underlying contributor in the development of chronic complications in diabetes mellitus. *Int J Mol Sci* 2013; **14**: 3265-3284.

[16] Giacco F, Brownlee M. Oxidative stress and diabetic complications. *Circ Res* 2010; **107**(9): 1058-1070.

[17] Zhang CY, Wu WH, Wang J, Lan MB. Antioxidant properties of polysaccharide from the brown seaweed *Sargassum graminifolium* (Turn.), and its effects on calcium oxalate crystallization. *Mar Drugs* 2012; **10**: 119-130.

[18] Gupta D, Bhardwaj R, Gupta RK. *In vitro* antioxidant activity of extracts from the leaves of abies pindrow royle. *Afr J Tradit Complement Altern Med* 2011; **8**(4): 391-397.

[19] Ofem OE, Ani EJ, Eno AE. Effect of aqueous leaves extract of *Ocimum gratissimum* on hematological parameters in rats. *Int J Appl Basic Med Res* 2012; **2**(1): 38-42.

[20] Hor SY, Ahmad M, Farsi E, Yam MF, Hashim MA, Lim CP, et al. Safety assessment of methanol extract of red dragon fruit (*Hylocereus polyrhizus*): acute and subchronic toxicity studies. *Regul Toxicol Pharmacol* 2012; **63**(1): 106-114.

[21] Ashafa AO, Sunmonu TO, Afolayan AJ. Toxicological evaluation of aqueous leafand berry extracts of *Phytolacca dioica* L. in male Wistar rats. *Food Chem Toxicol* 2010; **48**: 1886-1889.

[22] Ashafa AO, Orekoya LO, Yakubu MT. Toxicity profile of ethanolic extract of *Azadirachta indica* stem bark in male Wistar rats. *Asian Pac J Trop Biomed* 2012; **2**(10): 811-817.

[23] Coolborn AF, Bolatito B, Clement AF. Study of acute and subchronic toxicity of *Spathodea campanulata* P Beav leaf. *Int Proc Chem Biol Environ Eng* 2012; **41**: 76-80.

[24] Oduola T, Bello I, Adeosun G, Ademosun AW, Raheem G, Avwioro G. Hepatotoxicity and nephrotoxicity evaluation in Wistar albino rats exposed to *Morinda lucida* leaf extract. *N Am J Med Sci* 2010; **2**(5): 230-233.

[25] Hall RL. Principles of clinical pathology for toxicology studies. In: Wallace Hayes A, editor. *Principles and methods of toxicology*. 4th ed. Philadelphia: Tyler and Francis; 2001, p. 1001-1034.

[26] Nafiu M, Akanji M, Yakubu M. Effect of aqueous extract of *Cochlospermum planchonii* rhizome on some kidney and liver functional indicies of albino rats. *Afr J Tradit Complement Altern Med* 2011; **8**(1): 22-26.

[27] Mekonnen Y, Dräger B. Glucosinolates in *Moringa stenopetala*. *Planta Med* 2003; **69**: 380-382.

[28] Dolan LC, Matulka RA, Burdock GA. Naturally occurring food toxins. *Toxins* 2010; **2**: 2289-2332.

[29] Burel C, Boujard T, Kaushik SJ, Boeuf G, Mol KA, Van der Geyten S, et al. Effects of rapeseed meal-glucosinolates on thyroid metabolism and feed utilization in rainbow trout. *Gen Comp Endocrinol* 2001; **124**(3): 343-358.