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Evaluation of Diuretic Activity of Hydro-Ethanolic Extract of *Moringa Stenopetala* Leaves in Swiss Albino Mice

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Abstract

Context: *Moringa stenopetala* (Baker f) Cufodontis is an evergreen tree of approximately 5 to 10 m height and widely distributed in the Southern parts of Ethiopia with numerous nutritional and medicinal purposes.

Objective: The aim of the study was to evaluate the effect of hydro-ethanolic extract of *M. stenopetala* leaves using *in-vivo* mice model of diuresis.

Methods: Furosemide (10 mg/kg), normal saline (1 ml/100 g), and extract doses (150, 250, 350, 500 and 1000 mg/kg) of body weight were used as standard, control and test substance respectively. All substances were administered orally. The urine output and electrolyte concentration of Na^+, K^+ and Cl^- were determined.

Results: At all doses the experimental group had a significant urine output at 0.5 hour and 1 hour ($P < 0.001$) compared to standard and control. After 2.5 hours, at 500 mg/kg the extract showed an increased urine output compared to the control as well as the extract doses of 150, 250, 350 and 1000 mg/kg ($P < 0.05$). The excretion of Na^+ and Cl^- was significantly increased at 250 and 350 mg/kg test doses ($P < 0.001$) compared to control. At 500 and 1000 mg/kg, there is a comparable Na+ and Cl- excretion and a significant K^+ excretion ((at 500 ($P < 0.01$) and 1000 mg/kg ($P < 0.01$)) compared to standard and control respectively.

Conclusion: The outcome of the study demonstrated the diuretic activity of the hydro-ethanolic extracts of *M. stenopetala* leaves which is in agreement to the folkloric use of the plant for the management of hypertension and heart failure.

Keywords: Urine output; Electrolyte excretion; *In-vivo*

Introduction

Moringa stenopetala (Baker f.) Cufodontis belongs to the family Moringaceae commonly grown in Southern parts of Ethiopia at an altitude of 1100 to 1600 m above sea level. The cooked leaves of *M. stenopetala* are consumed by the locals as a staple food. The leaves, seeds and roots are used in folk medicines as hypotensive [1] antispasmodic [2], hypoglycemic [3,4], antileishmenial and antifertility [5], antibacterial and antimicrobial [6,7] antitrypanosomal [2], antihyperglycemic [3,4,8-10]. It has been reported that essential oils of *M. stenopetala* leaves showed trypanocidal and antileukaemic effects [11]. The leaves, seeds, and seedpods of *M. stenopetala* have good nutritional value and help to restore normal body weight [12-14]. The antibiotic principle of the seeds and antispasmodic effects of leaves of *M. oleifera* and *M. stenopatala* were also reported previously [15]. There were also prior studies on isolated fractions of the aqueous leaf extract of this plant which demonstrated its hypoglycemic and antidiabetic effect in mice [16].

To further substantiate the claim of the traditional medicinal use of the plant for the management of hypertension, the present study was undertaken on the diuretic activity of the hydro-ethanolic extract of *M. stenopetala* leaves in *in-vivo* experimental mice model.

Materials and Methods

Chemicals and Reagents

Absolute ethanol (WINLAB, UK), Sodium Chloride (Riedel-de Haen Germany), Furosemide (ERIS Pharma, Australia), and 9180 Electrolyte Analyzer Reagent (ROCHE, Germany) were used in the study. All the drugs and reagents used complied with the required standard and were of analytical grade.

Plant Collection and Authentication

The fresh *M. stenopetala* leaves were collected from Southern Ethiopia around Arbaminch, a town 502 km away from Addis Ababa. The plant material was authenticated by a taxonomist in the Directorate of Traditional and Modern Medicine Research, Ethiopian Public Health Institute and a sample with voucher number AL-001 was deposited for future reference.

Preparation of Extract

Fresh *M. stenopetala* leaves were shed dried and crushed manually using mortar and pestle. 1500 g of the crushed leaves were macerated with 70 % ethanol for 72 hrs at room temperature under a rotator shaker (VWR, DS-500 Orbital Shaker, U.S.A) until exhaustion. The extract was first filtered using cotton gauze and later with whatman filter paper No.1. And the extract was concentrated under reduced pressure using rota vapor (Buchi, Rotavapor R-210/215, Swizerland). The filtrate residue obtained was dried on a water bath (DVE-Kottermann, D-3162, Uetze-Hanigsen/W, Germany) at 40°C overnight and then freeze dried with a lyophilizer (Labconco, 12 L Console Freeze Dry 230v-60 (7754040), Freeze Dry System, USA). The total yield of the extract was calculated to be 20 % (w/w). The dried extract was kept in a refrigerator until further use.

Animals

The experiment was performed on adult, healthy male Swiss albino mice with a weight range of 25-30 g. The experimental animals were bred and obtained from the Ethiopian Public Health Institute. All the animals used for this study were kept in standard cages and maintained under controlled laboratory conditions of temperature (22 ± 3°C), humidity and 12 hour day-12 hour night and had free access to food (standard pellet diet) and water ad libitum. The animals were treated humanely throughout the study period adhering to the guideline for use and care of animals in declaration of Helsinki (National Research Council, 2011).

Acute Toxicity Testing

The hydro-ethanolic extract of *M. stenopetala* leaves were evaluated for its toxicity in female Swiss albino mice at the age of 6-8 weeks, at a dose of 5000 mg/kg body weight according to OECD guidelines No 425 [17]. The animals were deprived of food for 18 hours with free access to water. Immediately after administration of the extract, the animals were carefully observed continuously for the first 4 hr for any overt signs of toxicity and death and then for the next 24 hour. Thereafter, they were kept under close observation up to 14 days to monitor the presence of any signs of morbidity or mortality. The weight of each animal was recorded at the 1st, 7th, and 14th day of administration to verify any weight change that might have occurred. Finally, after cervical dislocation, the mice were dissected at the 14th day to observe gross pathology of the vital organs such as liver and kidney.

Screening for Diuretic Activity

The hydro-ethanolic extract of *M. stenopetala* leaves was evaluated for its diuretic activity with slight modifications of [18] and other methods [19-23]. Swiss albino mice weighing between 25-30 g were divided into seven groups, (n=8) and were placed in standard metabolic cages. Food/pellet and water was withdrawn 18 hours prior to the experiment session. The different doses of the extract, normal saline and standard drug were administered on the basis of the weight of the animals. All doses of the extract were dissolved in normal saline solution to make the required concentrations and administered orally.

Group-1 received normal saline solution (0.9% NaCl), (1 ml/100 g of body weight) and served as control. Group-2 received the standard diuretic drug Furosemide (10 mg/kg body weight) and served as standard. Group-3 to Group-7 received test substance/extract at dose levels of 150, 250, 350, 500, and 1000 mg/kg, respectively. The cumulative urine excreted was measured every 30 minutes for 5 hours in all groups and at the end of 5th hour the collected urine stored at -20 0C until further analysis. The parameters taken were total urine volume, urinary electrolyte concentration of Na^+, K^+ and Cl^-.

Diuretic action, diuretic index, Lipschitz value, diuretic activity, urinary excretion, saluretic activity, natriuretic activity, carbonic anhydrase inhibition, Na^+, K^+, and Cl^- excretion were the parameters determined in order to compare the effects of the test doses of the extract with Furosemide on diuresis [18]. The urinary excretion was calculated as the total urinary output divided by total liquid administered (Formula-1). The ratio of urinary excretion in test group to urinary excretion in the control group was used as a measure of diuretic action of the diuretics (Formula-2). The diuretic activity was also calculated as the ratio of diuretic action of the test substances to that of the standard drug (Formula-3)[24].

$$\text{Urinary Excretion (EU)} = \frac{\text{Total Urinary output (VO)}}{\text{Total liquid administered (VI)}} \times 100\% \quad \text{(Formula -1)}$$

$$\text{Diuretic action} = \frac{\text{Urinary Excretion in test group (UEt)}}{\text{Urinary Excretion in control group (UEc)}} \quad \text{(Formula -2)}$$

$$\text{Diuretic activity} = \frac{\text{Diuretic action of test group (DAt)}}{\text{Diuretic action of standard drug (DAF)}} \quad \text{(Formula-3)}$$

Determination of Urine Concentration of Ions Excreted

Urinary Na^+, K^+ and Cl^- concentrations of the experimental group (hydro-ethanolic extract), control and standard group were determined using Ion Selective Electrode (ISE) analysis (AVL 9180 Electrolyte Analyzer, Roche, Germany). Dilutions of the urine samples were made as required to bring electrolyte content in the range that can be determined by the electrolyte analyser [25].

Statistical Analysis

Results are expressed as mean (X) ± standard error of mean (S.E.M) and were subjected to biostatistical interpretation by SPSS windows version 20 statistical packages using one way ANOVA followed by Tukey's post- hoc test for multiple comparisons. The level of significance was set at $P < 0.05$.

Results

Acute Oral Toxicity

Acute oral toxicity of hydro-ethanolic extract of *M. stenopetala* leaves was evaluated in female Swiss albino mice aged of 6-8 weeks according to OECD guideline No 425 [17]. On the basis of oral reports of high consumption of the leaves for diet mixed with cultural foods, the chosen highest dose was 5000 mg/kg extract. The acute toxicity study indicated that the extract did not cause mortality within 24 hrs at 5000 mg/kg body weight dose. After 14 days of observation of the experimental mice, no body weight reduction was observed. Gross physical and behavioural observation also revealed no visible signs of toxicity. Additionally, there was no gross pathological alteration (color, size and texture) of the vital organs.

Effect on Urine Output

The observation of the urine output of all extract doses of hydro-ethanolic extract of M. stenopetala leaves failed to show a significant ($p < 0.001$) urine output for the first 0.5 and 1 hour period compared to standard group. After 2.5 hours, at 500 mg/kg the extract showed an increased urine output compared to the control as well as the extract doses of 250, 350 and 1000 mg/kg ($P < 0.05$). At the end of the 5th hour, there was a significant difference in the urine output between the control group and the groups that received 500 and 1000 mg/kg of the extract ($P < 0.01$ and $P < 0.05$ respectively).This effect was comparable to the urinary output observed in the groups that received the standard drug Furosemide. The highest cumulative urinary output of the extract was observed at 500 mg/kg among all the other experimental and the standard group. This cumulative urinary output is significantly higher ($P < 0.05$) in comparison with the test groups that received 150, 250 and 350 mg/kg of the extract.

Effect on Urinary Excretion and Related Parameters

The highest percent urinary excretion of 7.78 % was observed at 500 mg/kg of the hydro-ethanolic extract of the *M. stenopetala* leaves, and this dose also earned the highest diuretic action and diuretic activity (1.61 and 0.89) among the other test doses of as compared to control and standard groups respectively. The second and third highest diuretic activity in relation to the standard, were observed at 1000 and 350 mg/kg (0.88 and 0.82 respectively) which considerably exceeded the effects observed at the other test doses.

Effect on Electrolyte Concentration Excretion

At 1000 mg/kg of the extract of hydro-ethanolic extract of M. stenopetala leaves there was a significant effect ($P < 0.01$) on K^+ excretion as compared to the control and was comparable to the standard drug. Although exceeded by the highest test dose, the extract at 500 mg/kg had also exhibited a significant ($P < 0.05$) increase in K^+ excretion to standard group. The comparison among extract doses 350 mg/kg ($P < 0.05$), 500 mg/kg ($P < 0.001$), were also observed to have considerable difference compared to 1000 mg/kg dose. The maximum natriuretic activity was observed at 500 mg/kg extract dose whereas saluretic activity, carbonic anhydrase activity and Lipschitz value was that 250 mg/kg extract dose in all cases.

Discussion

Diuretics are drugs that are used to reduce the abnormal accumulation of excess fluid in the body. They achieve this by bringing about an overall increase in urinary volume as well as in the electrolyte output. They decrease cardiac work load by reducing plasma volume and subsequently venous return to the heart .This class of drugs have a wide application in the management of pathological conditions such as congestive heart failure, certain renal diseases and hypertension [26]. Diuretics also relieve pulmonary congestion and peripheral edema and are useful in reducing the syndromes of volume overload, including orthopnea and paroxysmal nocturnal dyspnoea.

Aside from being highly revered for its medicinal value, *M. stenoptala* has also served the community as a source of food for centuries. With this prior information of safety, limit test was found to be the appropriate test for assessing the acute toxicity profile as per the OECD guidelines [17]. The acute toxicity study of oral administration of the limit dose of 5000 mg/kg of the hydro-ethanolic extract of *M. stenopetala* leaves indicated that there was no mortality observed in all test animals during the course of the study period. Furthermore, no overt behavioural and physical signs of toxicity were discerned at this dose. Consequently, the facts that there was no sign of morbidtiy as well as mortality at this specific high dose would infact allow making suggestions that the oral medial lethal dose (LD_{50}) of the extract could be greater than 5000 mg/kg. Whereas, the previous study done on sub-chronic administration of hydro-ethanolic extracts of *M. stenopetala* has reported that the level of the aspartate aminotransferase which is used for hepatocellular evaluation increased in a dose dependent manner [9]. This shows that the extract may not cause observable toxicity in single dose but the toxic effect might increase with repeated dose adminsitration.

The hydro-ethanolic extract of *M. stenopetala* leaves had shown a strong diuretic activity based on the urine output measurement (Table 1A) done with 30 minutes time interval for the total duration of 5 hours.

Treatment	30min	1hr	1:30 hr	2 hrs	2 :30hrs	3 hr	3:30hrs	4 hr	4:30hr	5hr
N/S	0.07 ± 0.05 b3	0.32 ± 0.07	0.54 ± 0.10	0.75 ± 0.08	1.00 ±0.12	1.14 ±0.09	1.29 ±0.13	1.43±0.14	1.50 ± 0.15	1.60±0.12 d1c3,b3
Frusemide	0.93 ± 0.07 a3	1.46 ± 0.07 a3	1.89 ± 0.07	2.19 ± 0.09	2.18 ± 0.07a3	2.25 ± 0.09 a3	2.25 ± 0.09	2.297± 0.09	2.361 ± 0.09	2.46 ± 0.10 a3
250 mg/kg	0.036 ±. 036c1,b3	0.54 ± 0.13,b3,d1	0.79 ± 0.18,d1, b3	0.86 ± 0.16.c1,b3	1.04 ± 0.22 ,b3	1.18 ± 0.25 b3	1.39±0.34	1.43 ± 0.36	1.68 ± 0.39	1.75 ±0.43 c1
350 mg/kg	0.16 ± 0.08b3	0.53 ± 0.10 ,b3,d1	0.72 ± 0.14 b3,2	0.81 ± 0.12 e,c1,b3	1.00 ± 0.09 ,b3,c1	1.12 ± 0.13 b3	1.28 ± 0.10	1.47 ± 0.14	1.59 ± 0.16	1.78 ±0.16 c1
500 mg/kg	0.28 ± 0.07b3	0.63 ± 0.08 d3,b3	0.97 ±.09 d2,b3	1.38 ± 0.12d2,b3,a2	1.59 ± 0.13a1,d2,b1	1.69 ± 0.11	1.91 ± 0.21	2.13 ± 0.25 a1	2.19 ± 0.27 a1	2.50 ±0.33 a3
1000 mg/kg	0.00 ± .00b3	0.07 ± 0.07b3	0.21 ± 0.06b3	0.68 ± 0.07b3	0.89 ±. 09b3	1.12 ± 0.17b3	1.21 ± 0.27	1.54 ± 0.34	1.68 ± 0.48	2.00 ±0.65 a1

Table 1A: Effect of hydro-ethanolic extract of *M. stenopetala* leaves on Urine output of mice treated with different doses over a period of 5 hrs.

At the screening dose of 500 mg/kg specifically, a significant urine output as compared to the control was observed starting from the first 2 hour ($P < 0.001$). This effect was maintained throughout the observation period and resulted in a total urinary output which was significantly higher than all the test doses of the extract as well as the control group. The extract only at 500 mg/kg resulted in a slightly higher cumulative urine output compared to the standard although it failed to reach a statistically significant level.

Treatment	Dose (mg/kg)	Total liquid administered (ml)	Cumulative urine volume (ml) ± S.E.M	Diuretic index1	Lipschitz value2	% Urinary excretion	Diuretic action	Diuretic activity
N/S	1 ml/100 g	0.33	1.59 ± 0.27	1	---	4.84	1	---
Frusemide	10	0.28	2.41 ± 0.3	1.52	---	8.76	1.81	1
Hydro-ethanolic extract	150	0.29	1.75 ± 1.14	1.10	0.73	6.09	1.26	0.70
	250	0.37	1.86 ± 0.83	1.17	0.77	5.07	1.05	0.58
	350	0.25	1.78 ± 0.45	1.12	0.74	7.16	1.48	0.82
	500	0.32	2.50 ± 0.94	1.57	1.04	7.78	1.61	0.89
	1000	0.26	2.00 ± 1.58	1.26	0.83	7.69	1.59	0.88

Table 1B: Effects of hydro-ethanolic extract of *M. stenopetala* leaf on cumulative urine volume of mice on different parameters.

The electrolyte excretion potential of *M. stenopetala* on Na^+ and Cl^- was significant at the doses of 250 and 350 mg/kg ($P < 0.001$) in comparison to control group. Although the extract doses showed a magnified excretion of both ions compared to control they did not cause a significant K^+ excretion. On the other hand, excretion of K+ was found to be significant only at 1000 mg/kg ($P < 0.01$) compared to control. This finding with some reservations may indicate the possibility that M. stenopetala at higher doses elicits its diuretic activity possibly by inhibiting $Na^+/K^+/Cl^-$ co-transporter at the thick ascending loop of henle similar to the standard drug Furosemide. This could explain the K+ loss observed at the high extract dose and the significant urine output that is somewhat distinctive to loop diuretics.

The data analysis of the urine volume output measurement, for the duration of 5 hours, confirmed *M. stenopetala* has a slow onset of action as compared to Furosemide which induced a significant diuresis within the first 30 minutes of administration. The Lipschitz value which relates the response of the test compound to the response of the standard in this case was found to be greater than 1.00 for the test dose of 500 mg/kg which is regarded as a positive effect, which gives insight into the substantial diuretic activity the extract, possesses [27]. The saluretic activity calculated as the sum of Na^+ and Cl^- excretion was the highest for 250 mg/kg of the extract. The natriuretic activity given as the ratio of Na^+/K^+ is calculated to determine a potassium-sparing effect of the extract (for values greater than 10.0). None of the test doses reached values greater than 2.0 which is indicative of a favorable natriuretic effect [17].

Treatment		Concentration of excreted ions			Natriuretic activity: [Na+/K+]	Saluretic activity [Na+ + Cl–]	Carbonic anhydrase inhibition: Cl- / (Na+ + K+)]
		Na+ (mMol/L)	K+ (mMol/L)	Cl- (mMol/L)			
N/S		74.83 ± 29.47	47.67. ± 8.68 b1	86.33 ± 31.81	-	-	-
Frusemide		96..25 ± 9.98	168.64 ± 3.79 a1	112.13 ± 4.54	0.57	208.38	0.42
Hydro-alcoholic extract	150 mg/kg	86.60 ± 14.95	99.56 ± 18.37 b1, d1	117.80 ± 34.22	0.74	204.4	0.63
	250 mg/kg	124.67 ± 34.57a3, b1,c2,d2	120.20 ± 23.98a1	162.17 ± 25.22a3	1.04	286.84	0.66
	350 mg/kg	103.13 ± 28.53 a3	90.00 ± 6.89d1, b1, c1	125.13 ± 30.35a3	1.15	228.26	0.65
	500 mg/kg	97.00 ± 30.88	152 .24 ± 5.65 a2	97.13 ± 30.19	0.64	194.13	0.39
	1000 mg/kg	88.00 ± 47.70	168.08 ± 39.39a2	119.00 ± 43.28	0.52	207.00	0.46

Table 2: Effect of the hydro-ethanolic extract of *M.stenopetala* leaves on electrolyte excretion in mice

Slight carbonic anhydrase inhibition of hydro-ethanolic extract of *M. stenopetala* leaves can be also be assumed as a possible mechanism of action of the extract since ratio of $Cl^-/Na^+ + K+$ is calculated to be below 0.8 [17]. The increased Na^+ and Cl^- level in the urine and increased cummulative urinary excretion also reiterate the strong basis for the plants use for the management of hypertension in folkloric medicine.

Conclusion

As evidenced by the outcome of this study, it is safe to infer that the crude hydro-ethanolic extract of M. stenopetala leaves possesses a diuretic activity in mice model of diuresis. Testing the diuretic activity of different fractions of the crude extract also is also crucial to further substantiate this finding and shed light on which phytoconstituents that are responsible for the observed activity. Moreover, in-depth investigations are required in order to isolate and identify the phytoconstituents that are responsible for the plants diuretic activity as no prior studies have been undertaken in this regard.

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