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Evaluation of Diuretic Activity of Methanol Crude Extract of *Thymus serrulatus* Leaves and its Solvent Fraction in Mice

Amelework Eshetu Melka[1], Eyasu Makonnen[1], Asfaw Debella[2], Netsanet Fekadu[2] and Bekesho Geleta[2*]

[1]*Department of Pharmacology, School of Medicine, College of Health Sciences, Addis Ababa University, Addis Ababa, Ethiopia*

[2]*Directorate of Traditional and Modern Medicine Research, Ethiopian Public Health Institute, Addis Ababa, Ethiopia*

[*]**Corresponding author:** Bekesho Geleta, Directorate of Traditional and Modern Medicine Research, Ethiopian Public Health Institute, Addis Ababa, Ethiopia, Tel: +251911091969; E-mail: bekeshog@gmail.com

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Abstract

Background: *Thymus serrulatus* is an indigenous plant to Ethiopia and used widely in the treatment of many illnesses including renal diseases and hypertension. The present study was undertaken to investigate the diuretic activity of the 80% methanol crude extract and solvent fractions of *T. serrulatus* leaves in saline-loaded mice.

Methods: Swiss albino mice of either sex were divided into six groups (five animals in each group). The control group received normal saline (25 ml/kg) in 2% Tween 80, the reference group received hydrochlorothiazide (10 mg/kg). Group-III to Group-XIV received the test substances at dose levels of 125, 250, 500, and 1000 mg/kg orally. At the end of the 5th hour, urine was collected and the total volume of urine excreted by each animal was recorded. Concentration of urinary Na^+, K^+, and Na^+/K^+ ratio were also determined. The acute toxicity test was conducted for the most active fraction of the crude extract.

Results: The findings demonstrated that the crude methanol extract of *T. serrulatus* leaves and its n-butanol fraction have significant ($P<0.01$) diuretic activity. The n-butanol fraction, at the dose of 1000 mg/kg, displayed a pronounced diuretic activity (104.0%) which was greater than the reference drug. It also showed a good natriuretic activity (1.34). However, the chloroform fraction was observed to lack significant diuretic activity and effect on electrolyte excretion except at the highest test dose.

Conclusion: The present findings indicate that the crude methanol extract of the leaves of *T. serrulatus* as well as its n-butanol fraction has significant diuretic effect with increased concentration of urinary electrolytes in mice. Further studies, however, should be carried out to come up with possible mechanism/s of action and look for the active component responsible for the diuretic effect.

Keywords: Diuretic activity; Electrolyte excretion; *Thymus serrulatus*; Extract

Introduction

Diuretics, also commonly referred to as "water pills" are drugs that act on the kidney to aid the elimination of water and sodium from the body. When the kidneys excrete sodium ion, they excrete water along with it which results in a decreased blood volume and pressure on the walls of the arteries. These groups of drugs are used to treat several conditions, such as high blood pressure, heart failure, liver disease and certain types of kidney disease [1]. The mechanism by which diuretics block the reabsorption of ions and the site of action varies; they may act at the proximal tubule (carbonic anhydrase inhibitors), loop of Henle (loop diuretics), distal tubule (thiazide diuretics), collecting tubule (potassium sparing diuretics), or combination of these sites [2].

Thymus serrulatus also locally known as "tossign" is a much-branched perennial shrub that grows in the highlands of Semien Shoa, Tigray and Wollo. It is one of the endemic species of thyme in Ethiopia [3,4]. The leaves of the plant are used as condiments and medicines in the Ethiopian folklore medicine [5]. Tea made from the fresh leaves of *T. serrulatus* is consumed for the management of renal disorders and hypertension [6].

Thyme species are widely utilized around the world for their diuretic activity [7]. The leaf of *T. schimperi,* is also traditionally used for urinary retention and hypertension, and is reported to show a diuretic activity with increased ionic content of urine in rats [8]. Moreover, there is also scientific evidence that aqueous leaf extract of *T. serrulatus* possesses an *in vitro* vasodilatory activity on thoracic aorta of Guniea pigs [9]. Hitherto, no prior scientific reports exist concerning its diuretic activity; therefore the present study attempts to evaluate the diuretic potential of the crude methanol extract of *T. serrulatus* and its solvent fractions in mice.

Materials and Methods

Experimental animals

Swiss albino mice of either sex, age of 4-6 weeks and a weight range of 20-25 g were used for the study. All animals used for this study were bred in the animal breeding facility of the Ethiopian Public Institute (EPHI). An acclimatization period of a week was given for all animals before the experiments were carried out. The laboratory conditions

were maintained on a 12 hours light/dark cycle, with an ambient temperature of 25°C. All animals had free access to standard pellet diet and water and were treated humanely throughout the study period.

Grouping and dosing

The animals were divided into six groups, each group comprised of five mice. Group I received 2% Tween-80 in normal saline (NS) (25 ml/kg) and served as a negative control, Group-II received the standard diuretic drug, hydrochlorothiazide (HCTZ) (10 mg/kg). Groups III to VI received the test substance, methanol crude extract at test doses of 125, 250, 500, and 1000 mg/kg; group VII to X received the test substance, chloroform fraction of methanol crude extract; group XI to XIV received the test substance, n-butanol fraction of methanol crude extract orally using gavage.

Chemicals

Hydrochlorothiazide Tablets (Esidrex 25 mg, Novartis), Absolute methanol (TechnoPharmChem, India), n-butanol (Merck, Germany), Chloroform (Chromasolr, England) were used. All the chemicals were purchased from reliable sources and were of analytical grade.

Plant material

The leaves of *T. serrulatus* were collected from Debresina, a town located 190 km North of Addis Ababa. The plant was identified by a taxonomist, Dr. Dawit Abebe, at EPHI and a voucher specimen (No. TS-2103) was deposited at the herbarium of Traditional and Modern Medicine Research Directorate (TMMRD) of EPHI.

Extraction

Crude extract: Powdered leaves of *T. serrulatus* (600 g) were macerated with 750 ml of 80% methanol for 72 hours at room temperature. The extract was then filtered through Whatmann filter paper No.1, and the marc was re-macerated twice using the same volume of solvent to exhaustively extract the plant material. The solvent was removed under reduced pressure using Rotavapor (Büchi, U.S.A) at 40°C to give a greenish-black colored sticky extract which was kept in a refrigerator until further use. The percentage yield was 8.37%.

Fractionation

The crude extract (42 g) was allowed to suspend in 50 ml of warm water. The suspension was shaken in a separatory funnel with 50 ml of chloroform. The mixture was allowed to settle and form layers from which the bottom layer (chloroform fraction) was collected in a vial. This was repeated three times where the hydroalcoholic residue was then shaken using the same volume of chloroform. The residue was then shaken with 50 ml of n-butanol in the same manner to obtain the n-butanol fraction (upper layer).The resulting fractions were oven dried at a temperature of 40°C.

Screening for diuretic activity

All extracts were screened for their diuretic activity using the Kau method with some modification [10]. The animals were placed in a standard metabolic cage. Food and water were withdrawn 18 hours prior to the experiment. All extracts were dissolved in physiological saline solution to make the required concentrations and were administered orally.

The cumulative urine excreted was measured at the end of the 5th hour in all groups. The parameters taken were total urine volume, urinary concentration of Na^+ and K^+. The volume of the urine excreted in 5th hour of the study by each group is expressed as the percent of the liquid (normal saline) administered giving rise to a measure of "urinary excretion" independent of group weight. The ratio of urinary excretion in the test group to urinary excretion in the control group is used as a measure of the diuretic index for the given dose of a drug [11]. As the diuretic index is prone to variability, a parameter known as Lipschitz value was calculated. To obtain Lipschitz value, the diuretic index of the test substance is compared to that of the standard drug in the test group.

Urinary excretion=$(V_o/V_i) \times 100$

Where, V_o is Total urinary output and V_i is Total volume of fluid administered.

Diuretic index=V_t/V_c

Where, V_t is mean urine volume of test group and V_c is mean urine volume of control group.

Lipschitz value=V_t/V_r

Where, V_t is mean urine volume of test group and V_r is mean urine volume of reference group.

Na^+/K^+ ratio=C_n/C_k

Where, C_n is the concentration of Na^+ in urine of a group and C_k is the concentration of K^+ in urine of same group.

Determination of urinary Na^+ and K^+

The urinary sodium and potassium concentration was determined with flame photometer using Instrumentation Laboratory model 243.

Acute toxicity study

Swiss albino mice of either sex weighing between 20-25 g were divided into two groups having six animals each. Before the day of the experiment, the animals were deprived of food for 18 hours, and water was given *ad libitum*. The control group received NS while the other group received a limit dose of 5000 mg/kg of the n-butanol fraction of the 80% methanol crude extract orally. Immediately after dosing, the animals were observed continuously for 24 hours giving special attention to the first four hours for overt signs of morbidity and mortality. The animals were kept under observation for up to 14 days thereafter [12].

Phytochemical screening

All extracts used for the *in vivo* study were subjected to phytochemical screening following methods described by Trease and Evans [13].

The extracts along with negative controls were tested for the presence of alkaloids, saponins, polyphenols, flavonoids, terpenoids, anthraquinones, tannins, phytosterols, and glycosides as follows:

Alkaloids: One and half milliliter of 10% HCl was added to 0.5 mg of the extracts in a test tube. The mixture was heated for 20 minutes. It was then cooled and filtered. To 1 ml of the filtrate 5 drops Mayers and

Dragendorff's reagents each were added. Formation of cream and orange colored precipitates respectively indicates the presence of alkaloids in the extracts.

Saponins: Froth test: An aqueous solution of 0.5 mg of the extract in a test tube was vigorously shaken for 2 minutes. Foam which persisted for 30 minutes and doesn't disappear upon warming was taken as an indication of the presence of saponin in the extract.

Polyphenols (Phenolic compounds): Three drops of a mixture of 1 ml 1% $FeCl_3$ and 1% $K_3Fe(CN)_6$ each were added to 2 ml of extracts. Formation of green or blue color was taken as an indication of the presence of polyphenols.

Flavonoids: To 2 ml of aqueous solution of the extract 4 drops of 2% lead acetate solution was added. Development of yellow or orange color confirms the presence of flavonoids.

Terpenoids (Ketonic): One milliliter of 2, 4-dinitrophenylhydrazine solutions (0.5 g dissolved in 100 ml of 2M HCl) was added to 2 ml aqueous solution of the extract. Formation of yellow-orange coloration indicates the presence of a ketonic terpenoids.

Anthraquinones: Borntrager's test: Five milliletr of the extract was dried and shaken with 3ml petroleum ether. The filtrate was added to 2 ml of a 25% ammonia solution. The mixture was shaken and formation of a red coloration was taken as an indication of the presence of free anthraquinones.

Tannins: Three drops of 5% ferric chloride solution was added to 1ml of the extract solution in water. A greenish or blue coloration or precipitation was taken as indication of the presence of tannins.

Phytosterols and Withanoids: Five drops of 3% vanillin in conc. H_2SO_4 was added to a concentrated chloroform solution of extracts. Formation of a rose or reddish brown color indicates the presence of anoids or phytosterols.

Test for glycosides (Keller-Killiani Test)

To 0.5 g of each extract suspended in 5 ml water, 2 ml of glacial acetic acid containing one drop of ferric chloride hexahydrate ($FeCl_3.6H_2O$) solution was added. This was mixed with 1 ml of concentrated sulfuric acid and observed for a brown ring at the interface or a violet ring below the brown ring; alternatively acetic acid was added and observed for a greenish ring above the brown ring which gradually spread throughout this layer.

Statistical Analysis

Results are expressed as mean ± standard error of mean. Statistical analysis was performed using the unpaired student's t- test and $P<0.05$ was considered significant.

Results

Urine output

As shown in Table 1, the 80% methanol crude extract of *T. serrulatus* increased urine volume significantly at all test doses. The lowest and the highest urinary excretion were observed at a dose of 125 mg/kg and 500 mg/kg with a diuretic index of 1.13 and 1.60, respectively. The extract has a diuretic activity of about 88% at 500 mg/kg.

Group	Cumulative volume of urine (ml)	Urinary excretion (V_o/V_i) x 100	Diuretic index (V_t/V_c)	Lipschitz Value V_t/V_r
Control (2% Tween-80 in NS)	3.75 ± 0.41	75	-	-
Hydrochlorothiazide (10 mg/kg)	6.75 ± 0.91***	135	1.8	-
Ts-125 mg/kg	4.25 ± 0.37*	85	1.13	0.62
Ts-250 mg/kg	4.75 ± 0.56*	95	1.27	0.7
Ts-500 mg/kg	6.0 ± 0.82**	120	1.6	0.88
Ts-1000 mg/kg	5.5 ± 0.69**	110	1.47	0.81
Note: Cumulative values are expressed as Mean ± S.E.M (n=5); *P<0.05; **P<0.01; ***P<0.001; Ts-*T. serrulatus;* NS-Normal Saline				

Table 1: Effects of the 80% methanol crude extract of *T. serrulatus* on urine volume of normal mice.

The chloroform fraction of the 80% methanol crude extract as shown in Table 2 failed to show increased urinary output at 125, 250 and 500 mg/kg. However, significant increase in urine volume occurred at 1000 mg/kg ($P<0.01$) indicating a 92% diuretic activity as compared to the standard drug.

Group	Cumulative volume of urine (ml)	Urinary excretion (V_o/V_i) x 100	Diuretic index (V_t/V_c)	Lipschitz Value V_t/V_r
Control (2% Tween-80 in NS)	4.75 ± 0.67	95	-	-
Hydrochlorothiazide (10 mg/kg)	7.0 ± 1.01*	140	1.47	-
Ts-125 mg/kg	5.0 ± 0.31	100	1.05	0.71
Ts-250 mg/kg	4.75 ± 0.77	110	1.16	0.67
Ts-500 mg/kg	4.25 ± 0.27	85	0.89	0.6
Ts-1000 mg/Kg	6.50 ± 1.27*	130	1.37	0.92
Note: Cumulative values are expressed as Mean ± S.E.M for n=5; *P<0.05; **P<0.01; ***P<0.001; Ts- *T. serrulatus;* NS-Normal Saline				

Table 2: Effect of the chloroform fraction of the 80% methanol crude extract of *T. serrulatus* on urine volume in mice.

Unlike the chloroform fraction, the n-butanol fraction of the 80% methanol crude extract of *T. serrulatus* increased urine volume significantly at all doses (Table 3). The highest diuretic index ($P<0.01$) was evident at 1000 mg/kg which was even greater than that of the HCTZ. The fraction at 500 mg/kg also displayed a comparable diuretic activity with the standard drug though less than the one observed with the highest dose. The least urinary excretion observed for this fraction was at 250 mg/kg.

Group	Cumulative volume of urine (ml)	Urinary excretion (V_o/V_i) x100	Diuretic index (V_t/V_c)	Lipschitz Value V_t/V_r
Control (NS)	3.5 ± 0.12	70	-	-
Hydrochlorothiazide (10 mg/kg)	5.75 ± 0.33**	115	1.64	-
Ts-125 mg/kg	5.2 ± 0.2*	104	1.49	0.9
Ts-250 mg/kg	4.0 ± 0.74*	80	1.14	0.69
Ts-500 mg/kg	5.5 ± 0.85**	110	1.57	0.95
Ts-1000 mg/kg	6.0 ± 1.2**	120	1.71	1.04
Note: Cumulative values are expressed as Mean ± S.E.M for n=5; **P<0.05; **P<0.01; ***P<0.001; Ts- *T. serrulatus*; NS-Normal Saline				

Table 3: Effect of the n-butanol fraction of the 80% methanol crude extract of *T. serrulatus* on urine volume in mice.

Electrolyte excretion

Table 4 shows that the 80% methanol crude extract of *T. serrulatus* induced significant increase in urinary excretion of both Na^+ and K^+. The extract showed the highest natriuresis at 500 mg/kg (P<0.001), and the least was observed at 125 mg/kg. Significant kaliuresis was observed at 250 mg/kg (P<0.01), 500 mg/kg (P<0.05) and 1000 mg/kg (P<0.01).

Group	Electrolyte concentration (MEq/l)		Na^+/K^+
	Na^+	K^+	
Control (2% Tween-80 in NS)	74.2 ± 1.12	47.5 ± 1.11	1.56
Hydrochlorothiazide (10 mg/kg)	112.7 ± 1.8***	73.9 ± 1.38*	1.52
Ts-125 mg/kg	85.3 ± 1.32	50.4 ± 1.09	1.69
Ts-250 mg/kg	101.2 ± 1.37**	69.1 ± 1.46**	1.46
Ts-500 mg/kg	110.7 ± 1.53***	57.2 ± 1.16*	1.93
Ts-1000 mg/kg	91.2 ± 1.02**	63.1 ± 1.25**	1.44
Cumulative values are expressed as Mean ± S.E.M (n=5); *P<0.05; **P<0.01; ***P<0.001; Ts- *T. serrulatus;* NS-Normal Saline			

Table 4: Effect of the 80% methanol crude extract of *T. serrulatus* on electrolyte excretion in mice.

The chloroform fraction of the crude extract didn't cause significant urinary excretion of both sodium and potassium ion as shown in Table 5. However, the extract showed significant kaliuresis only at 1000 mg/kg (P<0.05).

As it shown in Table 6, the n-butanol fraction of the 80% methanol extract of *T. serrulatus* showed a dose- dependent increase in the urinary excretion of Na^+ and K^+. The highest natriuresis and kaliuresis were observed at the dose of 1000 mg/kg (P<0.001 and P<0.01, respectively).

Group	Electrolyte concentration (MEq/l)		Na^+/K^+
	Na^+	K^+	
Control (2% Tween-80 in NS)	81.2 ± 0. 97	53.71 ± 1.21	1.51
Hydrochlorothiazide (10 mg/kg)	117.7 ± 1.83**	60.5 ± 0.87*	1.94
Ts-125 mg/kg	82.6 ± 1.09	51.2 ± 0.62	1.61
Ts-250 mg/kg	83.2 ± 0.89	56.7 ± 0.67	1.46
Ts-500 mg/kg	72.3 ± 0.52	49.2 ± 0.38	1.46
Ts-1000 mg/kg	81.9 ± 1.45	54.8 ± 1.81*	1.49
Note: Cumulative values are expressed as Mean ± S.E.M for n=5; *P<0.05; **P<0.01; ***P<0.001; Ts-*T. serrulatus;* NS-Normal Saline			

Table 5: Effect of the chloroform fraction of 80% methanol extract of *T. serrulatus* on electrolyte excretion in mice.

Group	Dose (mg/kg)	Electrolyte concentration (MEq/l)		Na^+/K^+
		Na^+	K^+	
Control (2% Tween-80 in NS)	-	65.17 ± 0.89	58.1 ± 0.67	1.12
Hydrochlorothiazide (10 mg/kg)	10	120.5 ± 1.71***	66.3 ± 1.12*	1.81
Ts-125 mg/kg	125	72.2 ± 1.25*	69.2 ± 1.08*	1.04
Ts-250 mg/kg	250	80.6 ± 1.18*	67.9 ± 0.91*	1.19
Ts-500 mg/kg	500	98.4 ± 1.86**	81.7 ± 1.64**	1.2
Ts-1000 mg/kg	1000	115.9 ± 2.18***	86.2 ± 1.78**	1.34
Note: Cumulative values are expressed as Mean ± S.E.M for n=5; *P<0.05; **P<0.01; ***P<0.001; Ts- *T. serrulatus;* NS-Normal Saline				

Table 6: Effect of the n-butanol fraction of 80% methanol extract of *T. serrulatus* on electrolyte excretion in mice.

Acute toxicity test

After administration of the oral limit dose of 5 g/Kg of n-butanol fractions of the crude methanol leaf extract of *T. serrulatus* all animals didn't show any overt sign of behavioral abnormality as well as morbidity during the observation period. Additionally, no mortality was observed during the same period which indicates that the LD_{50} of the fraction is greater than the limit dose employed here.

Phytochemical screening

Basic investigations of the extracts for their major phytocompounds is vital as the active principles of many drugs are these secondary metabolites found in plants. The various phytochemical screening tests performed on the crude extracts and solvent fractions *Thymus serrulatus* leaves revealed the presence of different secondary metabolites (Table 7).

Type of extract	Alkaloids	Saponins	Polyphenols	Flavonoids	Terpenoids	Anthraquinones	Tannins	Phytosterols	Cardiac glycosides
Aqueous crude	+	+	+	-	-	-	+	+	-
Dichloromethane fraction	-	+	-	+	-	-	+	+	-
n-butanol fraction	+	+	+	-	-	-	+	+	-
Negative control (vehicle)	-	-	-	-	-	-	-	-	-
Note: +: present; -: absent									

Table 7: Phytochemical screening of aqueous crude extracts and solvent fractions of *Thymus serrulatus* leaves.

Discussion

Diuretics are substances that promote production of urine and elimination of ions such as sodium from the body. Despite their use in the treatment of edematous conditions they are associated with adverse effects such as hypovolemia, hypokalemia, hyperkalemia, hyponatremia, metabolic alkalosis, metabolic acidosis and hyperuricemia [14]. There is growing attention towards the use of medicines of herbal origins to combat a range of diseases. Numerous studies now exist affirming diuretic effects of traditional medicines [15]. The main advantage of using plant based diuretics is that they exhibit less adverse effects as compared to conventional diuretics available in the market. This property could allow their use as add-on therapy to improve tolerance to more potent drugs [16].

Scientific validation of these plants is a key component in the search for alternative plant based diuretics. In the present study, the diuretic effect of orally administered methanol extract of the leaves of *T. serrulatus* and its solvent fractions were evaluated in saline loaded mice at different test doses. The diuretic activities were compared with that of hydrochlorothiazide, a widely used diuretic in clinical practice. The methanol crude extract and its n- butanol fraction showed a significant urinary output as well as increase in ionic excretion. The diuretic potential of any substance is considered to be good if the diuretic index values are greater than 1.50, moderate if the values are between 1.00 and 1.50, mild if the values lie between 0.72 and 1.00 and nil if the value is <0.72 [17]. Accordingly, the n-butanol fraction has a "good" diuretic potential with a diuretic index of 1.54 and 1.71 at doses of 500 mg/kg and 1000 mg/kg, respectively. The crude methanol extracts at all test doses as well as its chloroform fraction at the highest test dose elicited a "moderate" diuretic activity.

Unlike the crude extract and its n-butanol fraction, the chloroform fraction increased urine output at only the highest dose employed and failed to show appreciable electrolyte excretion. The observed difference in pharmacological response might be due to differential distribution of active phytoconsituents in the extract as well as its fractions. Na^+/K^+ appears to be translatable biomarker of mineralocorticoid receptor (MR) antagonism following administration of single or multiple doses of compounds where in MR blockade causes an increase in urinary Na^+/K^+ in rats [18]. Na^+/K^+ is also an important indicator of natriuresis as sodium is considered an important external factor responsible for primary hypertension as well as water retention [16]. Beside serving as an indicator of a good natriuretic activity, a Na^+/K^+ ratio of >2 also shows the ability of the test substance to excrete more large sodium ion than potassium ion which is a very essential quality for a good diuretic [19]. Whereas values >10 indicate a potassium sparing activity which was not seen in all test substances including the thiazide reference drug in this study.

The essential oil of the leaves of *T. serrulatus* were found to contain p-cymene (13%), gamma-terpinene (13%) and thymol (49%) as major components [20]. Moreover, the phytochemical screening of the powdered dried leaves of *T. serrulatus*, revealed the presence of polyphenols, alkaloids, tannins, saponnins and phytosterols [21]. Phytoconstituents such as theobromine (alkaloid) [22], 2 (Aminomethyl) phenols [23] are known for their appreciable diuretic activity. While it is impossible to predict the phytoconstituents responsible for the observed diuretic activity of *T. serrulatus*, it can be suggested that its diverse content of polar phytoconstituents such as phenolic compounds might be responsible for the perceived response of the n- butanol fraction in mice.

Conclusion

In the light of the outcome of this study, it is reasonable to infer that the 80% methanol crude extract of the leaves of *T. serrulatus* and its n-butanol fraction have a diuretic spectrum similar to that of the standard diuretic, HCTZ, which correlates well with the traditional use of the plant in the treatment of renal disorders and hypertension. However, further studies have to be pursued to come up with the possible mechanism/s of diuretic action and the active component responsible for the diuretic action.

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