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ACUTE DIURETIC ACTIVITY OF A NEW FLAVONE GLYCOSIDE FROM THE STEMS OF TEPHROSIA PURPUREA LINN IN NORMAL RATS

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ABSTRACT

Abstract: The aim of the present study is to isolate and structurally elucidate a new flavones glycoside 5,7,3′,4′-tetrahydroxy-8-methoxyflavone-5-O-α-L-rhamnopyranosyl(1→6)O-β-D-glucopyranosyl(1→3)O-β-D-xylopyranoside together with two known compounds Apigenin and Kaempferol from the methanolic extract of the stems of *Tephrosia purpurea* Linn. and also evaluate the diuretic potential and effect on urinary electrolytes in normal rats. The structure was elucidated on the basis of various chemical degradations, color reactions and spectroscopic analysis.

KEYWORDS: *Tephrosia purpurea* Linn., Leguminosae, flavone glycoside, Diuretic activity, Furosemide.

1. INTRODUCTION

Tephrosia purpurea Linn [1]. belongs to family Leguminosae and commonly known as Sarphonka. It is found throughout in India. Its plant tonic, used as antihelminthic for children and used internally as a purifier of the blood and considered as a cordial. Its root are bitter and given in timpanists, dyspepsia and used as a fish poison. Its fresh root bark are ground and made into a pill with a little black pepper and used in case of obstinate colic.

Plant medicine was commonly used for traditional treatment of renal diseases and lot of plants have been reported to show significant diuretic activity[2-3]. This study was aimed to evaluate the diuretic potential and effect on urinary electrolytes of aqueous extracts of *Tephrosia purpurea* Linn. in normal rats.

Earlier workers have been reported [4-9] various constituents from this plant. In the present study, we report the isolation and structure elucidation of a new compound A from the methanolic extract of the stems of this plant. The structure of this new compound has been characterized as 5, 7, 3′, 4′-tetrahydroxy-8-methoxyflavone-5-O-α-L-rhamnopyranosyl-(1→6)O-β-D-glucopyranosyl(1→3)O-β-D-xylopyranoside .

2. MATERIALS AND METHODS

2.1 General experimental procedures

All the melting points were determined on a thermoelectrically melting point apparatus and are uncorrected. UV Spectra were determined on Shimadzu-120 double beam spectrometer in MeOH. The IR spectra were recorded on Shimadzu FT-IR 8400S in KBr disc. ^{1}H-NMR Spectra were recorded on Bruker DRX 300 MHz spectrometer in DMSO-d_6 using TMS as internal standard. ^{13}C-NMR Spectra were recorded on Bruker DRX 75 MHz spectrometer using DMSO-d_6. The chemical shift values are reported in ppm (δ) units and coupling constant (J) in Hz. The FAB mass spectra were recorded on a Jeol-SX (102) Mass spectrometer.

2.2 Plant material

The stems of *Tephrosia purpurea* Linn. were procured from Sagar region in the month of January 2012 and were taxonomically authenticated by the Department of Botany, Dr. H. S. Gour University Sagar. A voucher specimen has been deposited in the Natural Products Laboratory, Department of Chemistry, Dr. H. S. Gour University, Sagar (M.P.) INDIA..

2.3 Extraction and Isolation

Air dried and powdered stems (6.0 kg) of the plant were extracted with petroleum ether (40-60°C) in Soxhlet apparatus for 6 days. The stems of the plant were successively extracted with methanol for four days. The MeOH soluble fraction of the plant was concentrated under reduced pressure, to yield a light brown viscous mass (3.85 g) which was subjected to TLC examination over silica gel-G using n BAW (4:1:5) as solvent and I_2 vapors as visualizing agent, showed three spots, indicating it to be a mixture of three compounds A, B and C. These compounds were separated and purified by column chromatography over silica gel using $CHCl_3$: MeOH in various proportions (2 : 6, 3 : 5, 4 : 4). After removal of the solvent and crystallization from ether, above eluates yielded compound A (1.75 g), compound B (1.02 g) and Compound C (0.98 g).

3. RESULTS AND DISCUSSION

3.1 Study of Compound A

Compound A has m.p. 256-257 ºC, m.f. $C_{33}H_{40}O_{20}$ $[M]^+$ 756 (FABMS). It gave Molisch and Shinoda tests [10-11] showing its flavonoidal glycosidic nature. The UV spectrum showed λ_{max} at 320 nm (Band I) given strong indication of the presence of flavone skeleton and also exhibited λmax at 257 nm plus a shoulder at 275 nm indicated di-O-substituted B ring.[12-14] Its IR spectrum showed absorption bands at 3450 (-OH), 1652(α-β unsaturated C=O), 1610, 1540, 1506 (aromatic ring system). In ^{1}H-NMR spectrum of compound A, two singlets at δ 6.85 (1H, s) and 6.97 (1H, s) were assigned for H-3 and H-6 position in ring C and ring A respectively. Doublets at δ 7.88 (1H, d, J 2.4 Hz) and δ 7.14 (1H, d, J 8.9 Hz) were assigned for H-2′ and H-5′ in ring B. A double doublet at δ 7.49 (1H, dd, J 8.9, 2.4 Hz) was assigned for H-6′. In ^{1}H-NMR two singlets at δ 12.53 and 9.90 were assigned for -OH groups at C-5 and C-7 position. Another singlet at δ 3.85 confirmed the presence of OCH_3 group at C-8 position. The anomeric proton signals at δ 4.50 (1H, d, J 7.4 Hz), δ 5.21 (1H, d, J 7.1 Hz) and δ 4.57 (1H, br, d,J 1.6 Hz) were assigned to H-1′′′′ of D-xylose, H-1′′′ of D-glucose and H-1′′ of L-rhamnose respectively. A coupling constant at (J 1.6 Hz) of H-1′′ confirmed the α-configuration for the L-rhamnose. Two coupling constants at (J 7.1 Hz) and (J 7.4 Hz) confirmed the β configuration for the D-glucose and D-xylose at H-1′′′and H-1′′′′ respectively. [15-16]

In the mass spectrum of the compound A, characteristic ion peaks at m/z 756 $[M]^+$, 610 [M^+-L-rhamnose], 448 [M^+-D-glucose] and 316 [M^+-D-xylose, aglycone] were found by subsequent losses from the molecular ion of each molecule of L-rhamnose, D-glucose and D-xylose revealing L-rhamnose, D-glucose and D-xylose were attached to aglycone at C-5 position.

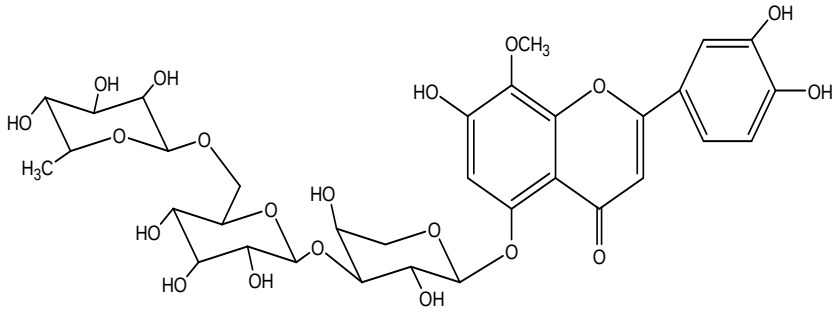
Acid hydrolysis of compound A with 10% ethanolic H_2SO_4 gave aglycone A-1, m.p. 216-217 ºC, m.f $C_{16}H_{12}H_7$ $[M]^+$ 316 (FABMS) which was identified as 5, 7, 3′ 4′- tetrahydroxy-8-methoxy flavone (see in experimental section). The aqueous hydrolysate obtained was neutralized with $BaCO_3$ and the $BaSO_4$ was filtered off. The filtrate was concentrated and subjected to Paper chromatography examination on Whatman filter Paper No. 1 and showed the presence of L-rhamnose (R_f 0.37), D-xylose (R_f 0.28) and D-glucose (R_f 0.16). Quantitative estimation of sugars revealed that all the three sugars were present in equimolar

ratio 1:1:1. [17] Periodate oxidation of compound A confirmed that all sugars were present in the pyranose form.[18]

The position of the sugar moieties in compound A were determined by permethylation followed by acid hydrolysis, yielded methylated aglycone and methylated sugars. The methylated aglycone was identified as 5- hydroxy-7, 8, 3′, 4′- tetramethoxy flavone which confirmed that glycosidation was involved at C-5-OH of aglycone. The methylated sugars were identified as 2, 3, 4-tri-O-methyl-L-rhamnose (R_G 1.01), 2, 4-di-O-methyl-D-xylose (R_G 0.66) and 2, 3, 4-tri-O-methyl-D-glucose (R_G 0.64) by paper chromatography with authentic samples.

Therefore it was concluded that C-1′′-OH of L-rhamnose was linked with C-6′′′ of D-glucose, C-1′′′-OH of D-glucose was attached with C-3′′′′ position of D-xylose, and C-1′′′′ of D-xylose was attached with C-5 position of aglycone. The interglycosidic linkages (1→6) and (1→3) were found between L-rhamnose and D-glucose as well as D-glucose and D-xylose.

Enzymatic hydrolysis [19] of compound A with takadiastase enzyme liberated L-rhamnose indicating the presence of α linkage between L-rhamnose and 5, 7, 3′, 4′-tetrahydroxy-8-methoxyflavone-5-O-β-D-glucopyranosyl-(1→3)O-β-D-xylopyranoside as proaglycone. Proaglycone on further hydrolysis with almond emulsin enzyme liberated D-glucose first followed by D-xylose and aglycone. Thus compound A was identified as 5, 7, 3′, 4′ tetrahydroxy-8-methoxyflavone-5-O-α-L-rhamnopyranosyl (1→6)O-β-glucopyranosyl (1→3)O-β-D-xylopyranoside.



Compound A

Figure 1

3.3 Spectral Data of compound A

It was analyzed for m.f. $C_{33}H_{40}O_{20}$, m.p. 256-257 ºC $[M]^+$ 756 found (%) C 51.90 , H 5.24, O 42.54, Calcd for m.f. $C_{33}H_{40}O_{20}$ found (%) C 52.3 , H 5.29, O 42.3, UV (MeOH) λ_{max} nm

320, 275 and 257. IR (kBr) ν_{max} (cm-1); 3450, 1652, 1610, 1540, 1506. ^{1}HNMR (300 MHz, DMSO-d_6); δ 12.52 (1H, s, 5-OH)), 9.90 (1H, s, 7-OH), 3.85 (3H, s, OMe), 6.85 (1H, s, H-3), 6.97 (1H, d, J 2.7 Hz, H-6), 7.88 (1H, d, J 2.4 Hz, H-2′), 7.14 (1H, d, J 8.9 Hz, H-5′), 7.49 (1H, dd, J 8.9, 2.4 Hz, H-6′), 4.57 (1H, br, d, J 1.6 Hz, H- 1′′), 3.69 (1H, m, H-2′′), 3.48 (1H, dd, J 9.5, 3.4 Hz, H-3′′), 3.19 (1H, m, H-4′′), 3.43 (1H, dd, J 9.5, 6.3 Hz, H-5′′), 5.21 (1H, d, J 7.1 Hz, H-1′′′), 3.53 (1H, m, H-2′′′), 3.55 (1H, m, H-3′′′), 3.22 (1H, t, J 9.3, H-4′′′), 3.66 (1H, m, H-5′′′), 3.88 (1H, dd, J 11.4, 1.6 Hz, H-6a′′′), 3.46 (1H, dd, J 11.4, 6.3 Hz, H-6b′′′), 4.50 (1H, d, J 7.4 Hz, H-1′′′′), 3.03 (1H, dd, J 8.5, 7.4 Hz, H-2′′′′), 3.19 (1H, m, H-3′′′′), 3.29 (1H, m, H-4′′′′), 3.68 (1H, dd, J 11.4 5.3 Hz, H-5′′′′a), 3.13 (1H, dd, J 11.4 9.9 Hz, H-5′′′′b), ^{13}C NMR (75 MHz, DMSO-d_6); δ 166.0 (C-2), 106.0 (C-3), 184.0 (C-4), 164.0 (C-5), 100.0 (C-6), 165.80 (C-7), 95.80 (C-8), 157.90 (C-9), 106.90 (C-10), 122.80 (C-1′), 114.80 (C-2′), 149.20 (C-3′), 152.40 (C-4′), 117.0 (C-5′), 114.90 (C-6′), 104.70 (C-1′′), 74.20 (C-2′′), 75.85 (C-3′′), 69.40 (C-4′′), 65.70 (C-5′′), 98.38 (C-1′′′), 81.95 (C-2′′′), 76.86 (C-3′′′), 69.15 (C-4′′′), 75.45 (C-5′′′), 65.90 (C-6′′′), 100.45 (C-1′′′′), 70.20 (C-2′′′′), 70.60 (C-3′′′′), 70.00 (C-4′′′′), 77.25 (C-5′′′′).

3.4 Acid hydrolysis of compound A

Compound A (70 mg) was dissolved in ethanol (15 ml) and refluxed with 10% H_2SO_4 on water bath for 6 h. The reaction mixture was concentrated and allowed to cool and residue was extracted with diethyl ether. The ether layer was washed with water and the residue was chromatographed over silica gel using $CHCl_3$: MeOH as solvent to give aglycone A-1, which was identified as 5,7,3′,4′-tetrahydroxy-8-methoxyflavone. The aqueous hydrolysate was neutralized with $BaCO_3$ and the $BaSO_4$ was filtered off. The filtrate was concentrated and subjected to paper chromatography examination using nBAW (4:1:5) solvent system and sugars were identified as L-rhamnose (R_f 0.37), D-xylose (R_f 0.28) and D-glucose (R_f 0.16) (Co-PC and Co-TLC).

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3.5 Permethylation of compound A

Compound A (40 mg) was dissolved in DMF (35 ml) and treated with MeI (5 ml) and Ag_2O (20 mg) in a 150 ml round bottomed flask fitted with air condenser and refluxed for 2 days. The reaction mixture was filtered and washed with DMF. The filtrate was concentrated under reduced pressure and hydrolysed with 10% H_2SO_4 to give methylated aglycone 5-hydroxy-7, 8, 3′, 4′-tetramethoxy flavone. The aqueous hydrolysate obtained after the removal of

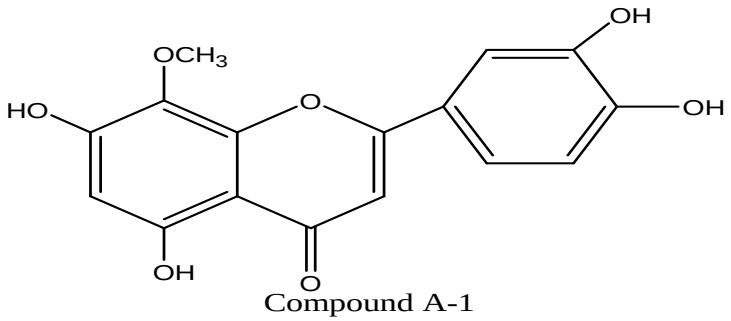
aglycone was neutralized with $BaCO_3$ and the $BaSO_4$ was filtered off. The filtrate was concentrated under reduced pressure and subjected to Paper chromatography examination using nBAW (4:1:5) as solvent system and aniline hydrogen phthalate as spraying agent. The methylated sugars were identified as 2,3,4-tri-O-methyl-L-rhamnose (R_G 1.01), 2,3,4-tri-O-methyl-D-glucose (R_G 0.64) and 2,4-di-O-methyl-D-xylose (R_G 0.66) (by m.m.p and Co-PC).

3.6 Enzymatic hydrolysis of compound A:

Compound A (30 mg) was dissolved in MeOH (20 ml) and hydrolyzed with equal volume of takadiastase enzyme. The contents were allowed to stay at room temperature for 3 days and filtered. The hydrolysate was concentrated and subjected to paper chromatography examination using nBAW (4:1:5) as solvent system and aniline hydrogen phthalate as a spraying reagent which showed the presence of L-rhamnose (R_f 0.37) The proaglycone was dissolved in MeOH (20 ml) and further hydrolyzed with equal volume of almond emulsin enzyme at room temperature as usual procedure yielded aglycone identified as 5, 7, 3′, 4′-tetrahydroxy-8-methoxy flavone and sugars were identified as D-glucose (R_f 0.16) and D-xylose (R_f 0.28).

3.6.Study of Compound A-1

It was analyzed for m.f. $C_{16}H_{12}O_7$, m.p. 216-217 ºC, $[M]^+$ 316 found (%) C 60.74, H 3.75, O 35.47, Calcd for m.f. $C_{16}H_{12}O_7$ found (%) C 60.78, H 3.80 O 35.44, UV(MeOH) λ_{max} nm 320, 275 and 257. IR (kBr) ν_{max} (cm-1); 3450, 1652, 1610, 1540, 1509. ^{1}HNMR (300 MHz, DMSO-d_6); δ 12.52 (1H, s, 5-OH)), 9.90 (1H, s, 7-OH), 3.85 (3H, s, OMe), 6.85 (1H, s, H-3), 6.97 (1H, d, J 2.7 Hz, H-6), 7.88 (1H, d, J 2.4 Hz, H-2′), 7.14 (1H, d, J 8.9 Hz, H-5′), 7.49 (1H, dd, J 8.9, 2.4 Hz, H-6′),^{13}C NMR (75 MHz, DMSO-d_6); δ 166.0 (C-2), 106.0 (C-3), 184.0 (C-4), 164.80 (C-5),100.0 (C-6), 165.0 (C-7), 95.80 (C-8), 157.90 (C-9), 106.90 (C-10), 122.80 (C-1′), 114.80 (C-2′), 149.20 (C-3′), 152.40 (C-4′), 117.0 (C-5′), 114.90 (C-6′).

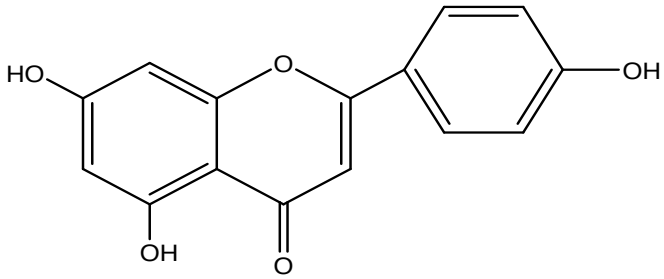


Compound A-1

Figure-2

3.7. Study of Compound B (Apigenin) :

It was analyzed for m.f. $C_{15}H_{10}O_5$, $[M]^+$ 270 found (%) C 66.7, H 3.75, O 29.64, Calcd for m.f. $C_{15}H_{10}O_5$ found (%) C 66.6, H 3.70, O 29.62, UV(MeOH) λ_{max} nm 268, 300 and 335. IR (kBr) ν_{max} (cm-1); 3410, 1652, 1609, 1544, 1507. ^{1}HNMR (300 MHz, DMSO-d_6); 6.59 (1H, s, H-3), 6.84 (1H, d, J 2.1 Hz, H-6), 6.72 (1H, d, J 2.1 Hz, H-8), 7.84 (2H, d, J 8.8 Hz, H-2′ and H-6′), 6.92 (2H, d, J 8.8 Hz, H- 3′ and 5′). ^{13}C NMR (75 MHz, DMSO-d_6); δ 164.42 (C-2), 106.58 (C-3), 180.45 (C-4), 164.95 (C-5),104.85 (C-6), 160.16 (C-7), 99.34 (C-8), 160.73 (C-9), 109.40 (C-10), 123.24 (C-1′), 129.08 (C-2′), 117.07 (C-3′), 161.40 (C-4′), 117.07 (C-5′), 129.08 (C-6′).

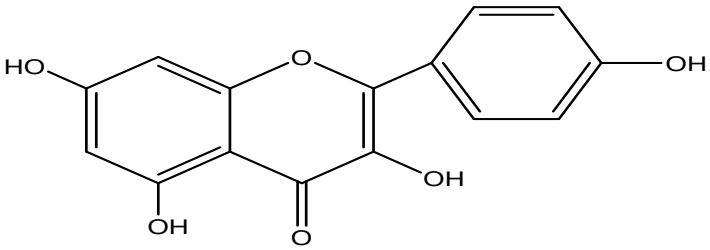


Compound B

Figure-3

3.8 Study of Compound C (Kaempferol)

It was analyzed for m.f. $C_{15}H_{10}O_6$, $[M]^+$ 285 found (%) C 63.12, H 3.14, O 33.69, Calcd for m.f. $C_{15}H_{10}O_6$ found (%) C 63.15, H 3.15 O 33.68, UV(MeOH) λ_{max} nm 206, 266 and 350. IR (kBr) ν_{max} (cm-1); 3400, 1650, 1608, 1545, 1508. ^{1}HNMR (300 MHz, DMSO-d_6); 6.08 (1H, d, J 2.0 Hz, H-6), 8.00 (1H, d, J 9.0 Hz, H-2′ and H-6′), 6.90 (1H, d, J 9.0 Hz, H- 3′ and 5′). ^{13}C NMR (75 MHz, DMSO-d_6); δ 158.48 (C-2), 133.87 (C-3), 178.74 (C-4), 162.89 (C-5),100.0 (C-6), 170.49 (C-7), 95.88 (C-8), 157.90 (C-9), 104.62 (C-10), 123.24 (C-1′), 132.08 (C-2′), 116.24 (C-3′), 161.40 (C-4′), 116.24 (C-5′), 132.08 (C-6′).



Compound C

Figure 4

4. DIURETIC ACTIVITY

4.1.Preparation of aqueous extract- Air dried and powdered stems (2.5 kg) of the plant *Tephrosia purpurea* Linn. were extracted with distilled water in Soxhlet apparatus for 6 days.

4.2.Reference drug - Furosemide, a high- ceiling loop diuretic was used as the reference drug, administered 10 mg/kg intraperitoneally.

4.3.Animal and Treatment - Male wistar rats weighing 200±20g were provided by the animal house of TIT Pharmacy, Bhopal. All animals were maintained in standard propylene cages and with free access to standard diet and continues water supply. The animals were housed at room temperature (20±2˚C) on a normal day-night cycle (0600 h to 1800 h). All animal experimentation was carried out after approval of the protocol by the Institutional Animal Ethical Committee according to the guidelines of CPCSEA, India.

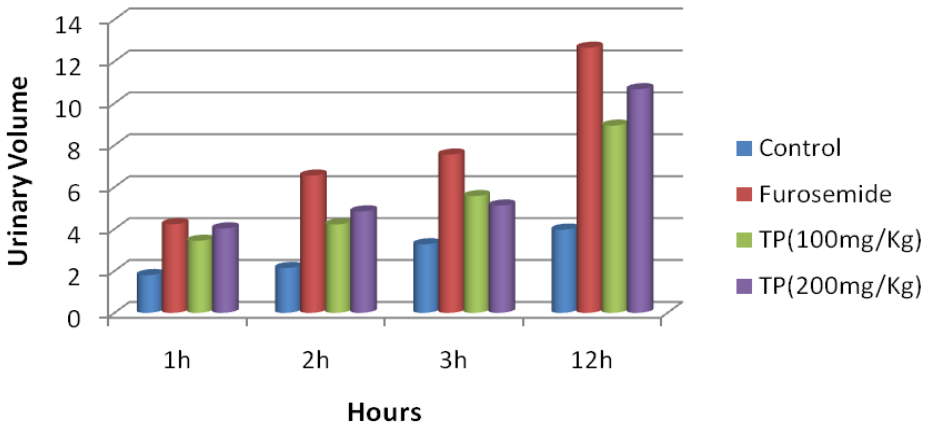
4.4.Determination of acute toxicity - To determine acute oral toxicity studies were carried out on normal healthy rats, the method of acute oral toxicity at fixed doses was used [20]. The TP (50,100,150,200,300 mg/kg/day) was administered orally for 4 days of six groups of rats (n=6).

5. Evaluation of diuretic activity

The animals were kept under observation for mortality as well as any behavioral changes for evaluation of a possible diuretic effect Animals were maintained under standard condition of temperature and humidity and underwent for an adaptation period of three days. The animals were divided into four groups (n= 6). **Group 1,** as control, received normal saline solution (25 ml/kg oral administration). **Group 2,** received the reference diuretic, furosemide at 10 mg/kg administered intraperitoneally; **Groups 3 and 4** received TP 100 mg/kg and 200 mg/kg respectively, in normal saline solution (25 ml/kg) [21]. The animals were placed in metabolic cages (1 per cage), especially designed to separate urine and feces, and kept at a controlled temperature of 22-25 ºC. At the end of 1, 2, 4 and 12 h, the volume of urine collected was measured. During this period, no food and water was available to the animals. During the experimental period, total urine volume, urinary pH and concentration of Na^+ and K^+ in the urine were determined by an ion sensitive electrode automatic analyzer.

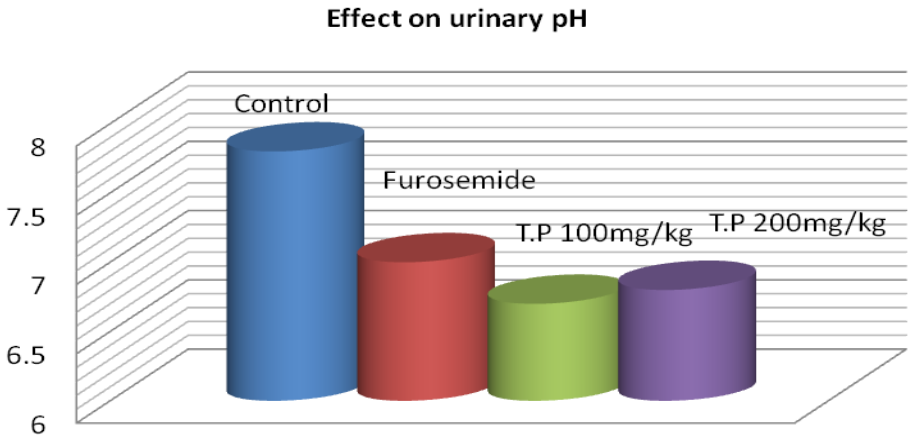
I. Effect on urine volume

Treatment	Dose (mg/kg)	Urine volume (1h)	Urine volume (2h)	Urine volume (4h)	Urine volume (ml/100 g/12 h)
Control	-	1.7 ± 0.10	2.0 ± 0.14	2.6 ± 0.67	3.17 ± 0.80
Furosemide	10	4.1 ± 0.13	6.1 ± 0.45	7.2 ± 0.35	11.25 ± 1.4**
ME	100	3.2 ±0.24	4.0 ± 0.22	5.0 ± 0.57	7.932 ± 1.0*
ME	200	3.7 ± 0.33	4.5 ± 0.34	5.9 ± 0.22	9.67 ± 1.0**



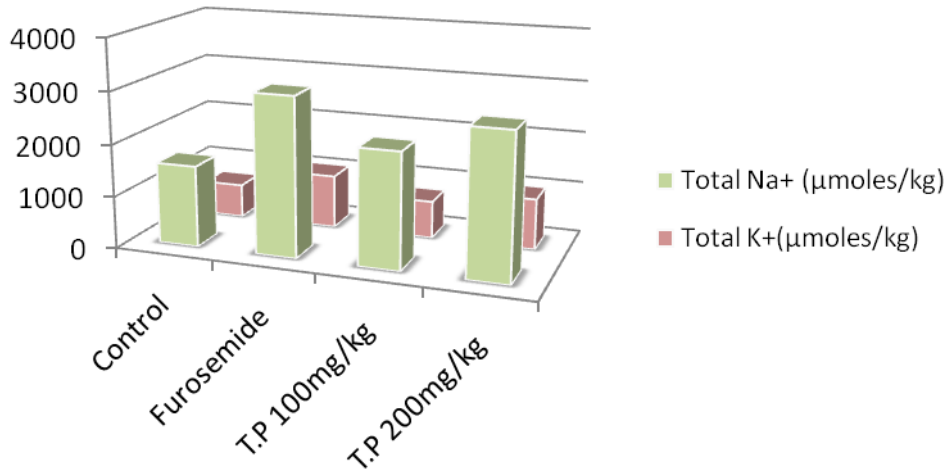
II. Effect on urinary pH

Treatment	Dose (mg/kg)	Urinary pH (ml/100 g/12 h)
Control	-	7.4 ± 0.4
Furosemide	10	6.9 ± 0.1
ME	100	6.4 ± 0.3
ME	200	6.7 ± 0.1



III. Effect on urinary electrolyte excretion

Treatment	Dose (mg/kg)	Total Na^+ (µmoles/kg)	Total K^+ (µmoles/kg)
Control	-	1560 ± 1.0	650.0 ± 1.0
Furosemide	10	3055 ± 1.5***	1029.0 ± 1.9***
ME	100	2215± 1.0**	720.5 ± 1.7**
ME	200	2770 ± 1.7***	976.0 ± 1.8***



Values are expressed as the mean _ SEM; *p < 0.001 compared to the control group, **p < 0.001 compared to Furosemide group (ANOVA followed by Dunnett's test.

6. CONCLUSION

It was concluded that aqueous extract administered, particularly at the dose of 200 mg/kg induced significant effect on urinary output of water and electrolytes and justify their use as diuretic remedy in traditional medicine.

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