



## DIURETIC ACTIVITY OF FLAVONOID COMPOUND ISOLATED FROM *GMELENA ARBOREA* FRUITS EXTRACT

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Article Received on 16/12/2016

Article Revised on 05/01/2017

Article Accepted on 26/01/2017

### ABSTRACT

**Background:** The plant *Gmelina arborea* has been traditionally used in India for several medicinal purposes like anthelmintic, diuretic, antibacterial, hepatoprotective, anti-inflammatory, antioxidant and antidiabetic. It contains phytoconstituents like alkaloids, carbohydrates, anthraquinone glycosides, gums, mucilages, tannins, phenolic compounds and flavonoids. **Aims:** The objective of present study is to isolate a compound from ethanolic extract of *G. arborea* and to explore the diuretic activity of isolated compound. **Material and methods:** The isolation of compound was done by column and thin layer chromatographic methods. The isolated compound was characterized to elucidate its structure by spectroscopic methods like ultraviolet, infrared, nuclear magnetic resonance and mass spectroscopy. The diuretic activity of isolated compound was evaluated by Lipschitz test methods using Wistar rats as animal model. **Statistical analysis used:** All data are verified for statistically significant by using one way ANOVA at 5 % level of significance ( $p < 0.05$ ). **Results:** A flavonoidal compound was isolated as yellow color crystal with melting point  $177 \pm 1$  °C with molecular formula  $C_{16}H_{15}O_5$  and IUPAC name 5,7-dihydroxy-4-methoxy flavone. Urine volume was significantly increased in comparison to normal and standard control groups. The excretion of sodium was also increased by the compound. The diuretic effect of the compound was comparable to that of the reference standard (Furosemide). **Conclusion:** It could be concluded that the isolated compound is a flavonoid and it possess diuretic activity.

**KEY WORDS** *Gmelina arborea*, Flavonoid, Diuretic, Lipschitz.

### INTRODUCTION

*Gmelina arborea* Roxb (Family *Verbenaceae*) fruits are oval in shape,  $\frac{3}{4}$  inches in length and are yellow in color. The fruits are sweet in taste and sometimes astringent.<sup>[1,2]</sup> The plant, *G. arborea* was reported to have several medicinal properties such as aphrodisiac, astringent, analgesic, antipyretic, antidiabetic, diuretic, anti-inflammatory and tonic characteristics.<sup>[3]</sup>

The literature survey reveals that fruits of *G. arborea* contain cardiac glycosides, flavonoids and steroids. The ethanol extract contains alkaloids, carbohydrates, anthraquinone glycosides, gums, mucilages, tannins, phenolic compounds and flavonoids.<sup>[4]</sup> Inflammation is a local response of living mammalian tissues to the injury, which is a body defense reaction in order to eliminate or limit the spread of injurious agents. However, inflammation that is unchecked leads to chronic inflammatory disorders. Oedema formation, leukocyte infiltration and granuloma formation represent components of inflammation. The present study was

aimed to isolate the chemical compound and to evaluate anti-inflammatory activity of isolated compound.

### MATERIALS AND METHODS

#### Chemicals used

The solvents, ethanol AR and ethyl acetate AR 60-80°C (Emsure® ACS) were procured from Merck Pvt. Ltd., Navi Mumbai, Maharashtra, India. Other solvents, ethyl acetate, chloroform, methanol and petroleum ether AR 40-60°C were procured from Loba Chemie Pvt. Ltd., Mumbai, India. Silica gel G (Qualigen) was procured from Fisher Scientific, Mumbai, India. Furosemide was obtained as gift sample from Ranbaxy Pvt. Ltd., Mumbai, India.

#### Instruments used

The ultraviolet spectrophotometer (UV-1700 Shimadzu, Japan), infrared (8400S Shimadzu IR spectrophotometer, Japan), nuclear magnetic resonance (Bruker AM-400 NMR) and mass spectrometer (Bruker APEXIII, APEX Technologies Inc.) were procured from

Shimazu Pvt. Ltd., Japan.

### Collection of plant materials, identification and size reduction

The fruits of *G. arborea* were collected from local area of Koraput district (India) and was authenticated by the Biju Patnaik Medicinal Plants Garden and Research Centre, Dr. M.S. Swami Nathan Research Foundation, Jeypore, Koraput (District), Odisha (Letter no. MJ/DBT (13)/1067, dated 12.04.2013). The fruits were shade dried and were pulverized to form coarse powder by using electrical grinder and stored in a closed air tight container for further use.

### Preparation of solvent extracts

The coarse powder form of dried fruits was extracted by Soxhlation method by using ethanol as solvent. A total amount of 1500 g coarse powdered fruits was extracted with 1200 ml of each solvent. The crude extract was evaporated to dryness in a rotary flash evaporator, with the percentage yield being 2.2 %. Crude extract was kept in closed air tight containers under cool and dark place for further study.

### Isolation of phytoconstituent.<sup>[5-7]</sup>

About 25 g of sample (Ethanol extract) was weighed and dissolved in 100 ml of chloroform for 3 h by continuous stirring. The suspension was filtered by using Whatmann filter paper no. 1 and the supernatant liquid thus collected in the beaker was concentrated by evaporating the solvent by heating at 60 °C for 10 min. The concentrated extract was again redissolved in chloroform, loaded at the top of the column and kept for 3 h. Mobile phases used were: Ethyl acetate - n-hexane (25:75), ethyl acetate - n-hexane (50:50), ethyl acetate - n-hexane (25:75), ethyl acetate (100 %), methanol - ethyl acetate (5:95), methanol - ethyl acetate (10:90), methanol - ethyl acetate (20:80), methanol - ethyl acetate (50:50) and methanol (100 %). About 25 ml fraction was collected at each time. The elutes were collected in test tubes and each fraction was subjected to TLC study.

### Thin Layer Chromatography study

The slurry of adsorbent that is Silica gel-G (Gypsum i.e. Calcium Sulphate) as stationary phase was transferred to various TLC plates by spreading method. The prepared TLC plates were air dried at room temperature for 30 min. The prepared TLC plates were activated by heating the plates at 105°C for 1 h in a hot air oven (ACM-22066-1, ACMAS Technocracy (Pvt.) Ltd., New Delhi). The solvent systems used for development of chromatogram in TLC plates were chloroform and ethyl acetate in the ratio of 5:95. The chromatograms were detected by spraying the above reagents. The resultant bands were also visualized by charring with a reagent containing methanol and concentrated sulphuric acid (85:15). The  $R_f$  values were recorded. The column with the solvent system methanol and ethyl acetate (5:95) gave least number of fractions. The larger band was collected as eluent and subjected to for column

chromatography once again, using chloroform and ethyl acetate in ratio of 5: 95. The column and thin layer chromatography studies were continued until the purification of compounds achieved, which was ensured by obtaining single spot on TLC plate.

### Identification of isolated phytoconstituent

#### Physical and chemical evaluations

The properties like appearance, color, taste, odor, solubility and melting point of the isolated constituents was determined. The isolated constituent was dissolved in ethanol and evaluated chemically for detection of flavonoid by using Shinoda, zinc hydrochloride reduction and alkaline reagent test.<sup>[6,7]</sup>

#### Structural elucidation of isolated phytoconstituent

The compound was dissolved in methanol and ultra violet absorption spectrum was determined by using UV spectrophotometer by scanning in the range of 200 to 800 nm using methanol as blank. The  $\lambda_{max}$  was determined from UV spectral analysis by considering that the corresponding wavelength at which maximum absorbance took place.<sup>[8,9]</sup> The FT-IR was used for IR analysis in the frequency range between 4000 and 600  $cm^{-1}$  and at 1  $cm^{-1}$  resolution. The sample of pure isolated compound was prepared by palletization technique in KBr using IR press. The IR peaks of the sample were analyzed and interpreted to elucidate the structure of isolated compound.<sup>[10]</sup> Nuclear magnetic resonance spectra ( $H^1$  and  $C^{13}$ ) were recorded at 400 MHz for  $H^1$ , 100 MHz for  $C^{13}$  – nuclei respectively.<sup>[11,12]</sup> Electron impact (EI) mass spectra were recorded coupled with PDP 11/34 computer system. High resolution spectrometry (HR-MS) and field desorption mass spectrometry (FD-MS) were also performed on ApexIII mass spectrometer.<sup>[13,14]</sup>

#### Evaluation of diuretic activity (Lipschitz test)

Approval for the research work and ethical clearance was obtained from the Gayatri College of Pharmacy, Gayatri Vihar, Jamadaripali, Sambalpur, Odisha (Ethical committee no. 1339/ac/10/CPCSEA).

The method is based on water and sodium excretion in test animals as compared to rats treated with high dose of Furosemide. The method of Lipschitz *et al.* was employed for the assessment of diuretic activity.<sup>[15-17]</sup> Wistar rats were divided into four groups (3 each). The animals of group (I) served as normal control (Vehicle) which received normal saline water (2 ml/Kg b.w., orally) only. The animals of group (II) served as standard control which received Furosemide (25 mg/Kg b.w., orally). Groups (III) and (IV) received flavonoid (named as acacetin) at doses of 25 and 50 mg/Kg b.w., orally. Male Wistar rats weighing 150 to 200 g were used. They were placed in metabolic cages provided with a wire mesh bottom and a funnel for collecting the urine. Stainless steel sieve was placed in the funnel to retain the feces and it also allows only urine to flow down for collection and measurement. The food and water were

withdrawn at 15 h prior to the test. Three animals were placed in one metabolic cage. The rats of each group were treated with drugs as per the detail mentioned above. Additionally 5 ml of normal saline solution per 100 g was administered to all rats orally. Urine excretion was recorded after 5 h.

The sodium and potassium content of the collected urine was estimated by Flame Photometer (Toshniwal group model TCM-35). The instrument was calibrated with standard solutions containing different concentrations of Na<sup>+</sup> and K<sup>+</sup>. pH was measured with a digital pH meter (MK-VI, Unique instruments & machineries, Calcutta) on fresh urine sample. For the calculation and presentation of results, urine volume excreted per 100 g body weight was worked out. The result are expressed as "Lipschitz value" that is the ratio T/U, where T represents the response of the test compound and U that of urea treatment. The Lipschitz value was calculated for both urine and sodium excretion. The indices of 1.0 or more indicate a positive diuretic effect of test compounds. All observations were done three times and data are represented as mean, standard deviation and standard error mean. All data were analyzed for statistical significance by using one way ANOVA at 5% level of significance ( $p < 0.05$ ). The significance between two proportions was also tested by z-Test in comparison to standard drug, Furosemide.

## RESULTS AND DISCUSSIONS

All phytochemical tests for flavonoid such as shinoda (Magnesium turning), ferric chloride, lead acetate, zinc hydrochloric acid reduction, sodium hydroxide and sulphuric acid were found to be positive. The appearance of the compound was crystalline powder and yield was 2.4%. The color of the compound was yellowish white. It is soluble in methanol, chloroform and n-hexane. The melting point of compound was 176 to 178 °C and optical rotation was  $^{30}[\alpha]_D - 32.5^\circ$  (C = 0.84 in methanol).<sup>[16,7]</sup>

The infrared spectroscopy study (Fig 1) of isolated compound reveals that the major peaks were obtained at wave numbers ( $\nu$ ) of 3405.57, 3211.76, 2954.96, 2834.58, 2722.24, 1594.95, 1497.82, 1348.62, 1302.16, 1252.69, 1168.52, 1090, 1020.49, 831.94, 721.76 and 559.15  $\text{cm}^{-1}$  respectively.<sup>[15,16]</sup> It signifies that compound has hydroxy (OH), methoxy (OCH<sub>3</sub>), keto groups etc. The compound presented the characteristic intensities of C=O absorption band at  $\nu$  of 1594.95  $\text{cm}^{-1}$  and the OH stretching band at  $\nu$  of 3405.57  $\text{cm}^{-1}$ . The  $\nu$  of 1497.82 and 1348.62  $\text{cm}^{-1}$  shows the absorption band of C=C and C-OH. The wave number of frequency 1252.69  $\text{cm}^{-1}$  shows the stretching band for C-O-C. The other wave number frequencies show characteristic bands for C-C and C=C.<sup>[18,19]</sup>

<sup>1</sup>H NMR and <sup>13</sup>C NMR (DMSO, 400 Hz) is shown in Fig 2 and 3. <sup>1</sup>H NMR (DMSO, 400 Hz):  $\delta$  2.514 (1H,ddJ=12.4,2.4Hz, H-2), 2.676 (1H,ddJ=16.8,2.4Hz,

H-3a), 3.247 (1H,ddJ =17.0,4.4Hz, H-3b), 5.895 (1H,dJ=8.2Hz, H-6), 5.895 (1H,dJ =2.8Hz, H-8), 7.445 (1H,dJ =8.8Hz, H-2'), 6.981 (1H,dJ =8.4Hz, H-3'), 6.981 (1H,dJ =8.4Hz, H-5'), 7.445 (1H,dJ =8.8Hz, H-6'), 12.141(1H,brs, OH-5), 10.801 (1H,brs,OH-7) and 3.776 (3H,s, OMe-4'). <sup>1</sup>H NMR spectra showed the presence of three protons attached to C-4' bearing methoxy group appeared at  $\delta$  55.16. The proton attached to C-5 and C-7 carbon bearing hydroxyl groups appeared at  $\delta$  12.141 and 10.801 as multiplet. The <sup>1</sup>H NMR signals at 12.141 ppm should be attributed to hydroxyl protons of isolated constituent, which participated in a strong intramolecular hydrogen bond, between the hydrogen atom of the hydroxyl group OH (C-5) and the oxygen atom of the carbonyl group CO (C-4).<sup>[18,19]</sup>

<sup>13</sup>C NMR (DMSO, 400 Hz):  $\delta$  78.14 (CH-2), 41.99 (CH<sub>2</sub>-3a), 196.10 (C-4), 163.47 (C-5), 95.85 (CH-6), 166.66 (C-7), 95.00 (CH-8), 130.62 (C-1'), 128.15 (CH-2'), 113.88 (CH-3'), 159.45 (C-4'), 113.88 (CH-5'), 128.15 (CH-6'), 101.78 (C-4a), 162.80 (C-8a), 55.16 (CH<sub>3</sub>-OMe-4'). <sup>13</sup>C NMR showed the presence of one quartet, one triplet, seven doublets and seven singlets corresponds to one methyl, one methylene, seven methine groups and seven tertiary carbon atoms. The spectrum of isolated constituent showed product on aromatic groups ranging from 6 to 8 ppm, and a strong intramolecular hydrogen bonding at 12.141 ppm.<sup>[18,19]</sup>

From the mass spectroscopy study (Fig 4), the result showed a peak at 286 (molecular peak ion - m/z value) which corresponds to molecular weight of isolated compound was found to be 286 (M<sup>+</sup>) with molecular formula of C<sub>16</sub>H<sub>15</sub>O<sub>5</sub>.<sup>[18,19]</sup>

From the analytical studies such as ultraviolet, infrared, nuclear magnetic resonance and mass spectroscopy study, it can be concluded from their interpretation of their spectral data, the isolated compound is a derivative of flavonoid with a chemical structure "5, 7-dihydroxy-4 methoxy flavone" named as acacetin and the chemical structure is depicted in Fig 5.

Acacetin, at two doses (25 and 35 mg/Kg b.w.) was screened for diuretic activity by administering orally. The sodium and potassium ions concentration, volume and pH of urine were recorded. The ratio of the concentration of Na<sup>+</sup>/K<sup>+</sup> and diuretic index were calculated at the end of 5 h to assess the diuretic activity of the extracts. The results are shown in Table 1 and 2. In the control and standard groups, the excretion of sodium in urine after 5 h was found to be 109.6±1.25 and 121.34±1.08 mmol/l respectively. The excretion of sodium in urine after 5 h was 131.82±1.08 and 144.32±1.17 mmol/l for acacetin at 25 and 35 mg/Kg b.w., respectively. In the control and standard groups, the excretion of potassium was found to be 67.82±1.17 and 77.61±1.05 mmol/l respectively while the excretion of potassium was 84.43±1.13 and 93.52±1.19 mmol/l for acacetin at 25 and 35 mg/Kg b.w., respectively. The test

material produced a significant increase in Na<sup>+</sup> and K<sup>+</sup> ions excretion, when compared with the control group. The normal value of Na<sup>+</sup>/K<sup>+</sup> ratio is reported to be 1.551 to 1.616. The concentration of aldosterone is found to be dependent on Na<sup>+</sup>/K<sup>+</sup> ratio. If the Na<sup>+</sup>/K<sup>+</sup> ratio falls below the normal level (in plasma), the aldosterone secretion will be decreased and if the ratio rises above the normal value the aldosterone secretion will be increased. Significant increase in Na<sup>+</sup>, and K<sup>+</sup> excretion was observed for both the doses of acacetin and it was more than the standard control (Furosemide).

The volumes of urine collected from the treated animals at the end of 5 h were found to be 3.43±1.24, 4.61±1.16, 6.21±1.04 and 7.87±1.13 ml for control, standard, acacetin at 25 and 35 mg/Kg b.w., respectively. The pH of urine collected from treated animal was found to be 7.02±0.71 and 6.85±0.69 for acacetin at 25 and 35 mg/Kg b.w., respectively. No change of pH in urine was observed for standard drug as well as with both doses of acacetin when compared to normal.

Data are found to be significant (*F value* < *F crit*) by testing through one way ANOVA at 5 % level of significance (*p* < 0.05 that is *p* = 0.01993) followed by *z*-

test. The results of the present study revealed that both the doses of acacetin present in fruits of *G. arborea* showed significant diuretic activity and is well comparable with the standard drug (Furosemide). The acacetin showed an increased diuretic activity with increase in dose. The acacetin exhibited better diuretic activity in comparison with standard drug, Furosemide. A complex set of interrelationships exists among the cardiovascular system, the kidneys, the central nervous system (Na<sup>+</sup>, appetite and thirst regulation) and the tissue capillary beds (distribution of extra cellular fluid volume), so that perturbation at one of these sites can affect all the remaining sites. The effect may be produced by stimulation of regional blood flow or initial vasodilation<sup>20</sup>, or by producing inhibition of tubular reabsorption of water and anions<sup>21</sup>, the result in both cases being diuresis. The increased sodium and water excretion activity also provides strong basis for its proved anti-hypertensive action<sup>22</sup>. The test compound acacetin exhibited confirmed diuretic effect and the present study supports its ethno pharmacological use as diuretic. This effect may be explored in the use of the plant in the management of some cardiovascular diseases.

**Table No.1: Summary of different parameters of diuretic activities of acacetin.**

Groups	Dose (mg/Kg)	Na <sup>+</sup> (mmol/l) (X±SD)	K <sup>+</sup> (mmol/l) (X±SD)	Lipschitz value (Na <sup>+</sup> ) (T/U)	Na <sup>+</sup> /K <sup>+</sup> ratio
I (C)	2 ml/Kg	109.6±1.25	67.82±1.17	--	1.616
II (U)	25	121.34±1.08*	77.61±1.05*	--	1.563
III (T)	25	131.82±1.08*	84.43±1.13*	1.086	1.561
IV(T)	35	144.32±1.17*	93.52±1.19*	1.189	1.543

Data are presented in mean ± standard deviation, n = 3. Standard error of mean < 0.721. Group I – Control (Normal saline water), group II - Standard (Furosemide - 25 mg/Kg b.w.), groups III and IV – Acacetin (At 25 and

35 mg/Kg b.w.) respectively. ns = Non-significant. \**P*<0.05 and \*\**P*<0.01 (Test of significance between two proportions by *z*-Test) in comparison to standard compared to control.

**Table No.2 Diuretic effect of acacetin of *G. arborea* fruits extracts in Wistar rat.**

Drugs	Dose (mg/Kg)	Urine volume (ml/100g/5h) (X±SD)	Diuretic index (T/C)	Lipschitz value (Urine) (T/U)	Urine pH (X±SD)	
NSW (C)	2 ml/kg	3.43±1.24	-	--	7.09±0.78	
Furosamide (U)	25	4.61±1.16*	1.344	--	6.75±0.81	
Acacetin	25	6.21±1.04*	1.811	1.347	7.02±0.71	
Acacetin	35	7.87±1.13**	2.294	1.707	6.85±0.69	
ANOVA						
Source of Variation	SS	df	MS	F	P-value	F crit
Between Groups	3.906012	1	3.90601	2.08009	0.01993	5.98738
Within Groups	11.266875	6	1.87781			
Total	15.172888	7				

Data are presented in mean ± standard deviation, n = 3. Standard error of mean < 0.716. ns = Non-significant. \**P*<0.05 and \*\**P*<0.01 compared to control (ANOVA followed by Dunnett's test). Data are found to be significant (*F value* < *F crit*) by testing through one way ANOVA at 5 % level of significance (*p* < 0.05 that is *p* = 0.01993).

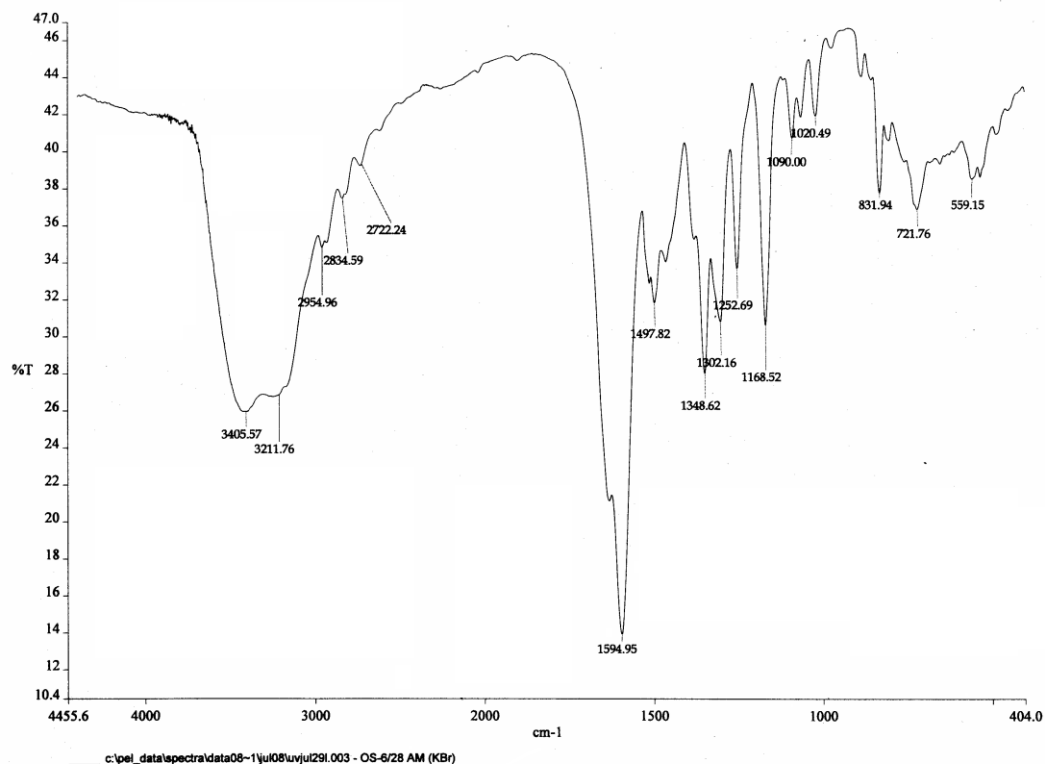


Fig 1. Infrared spectral data of isolated compound in the frequency range between 4000 and 600  $\text{cm}^{-1}$ .

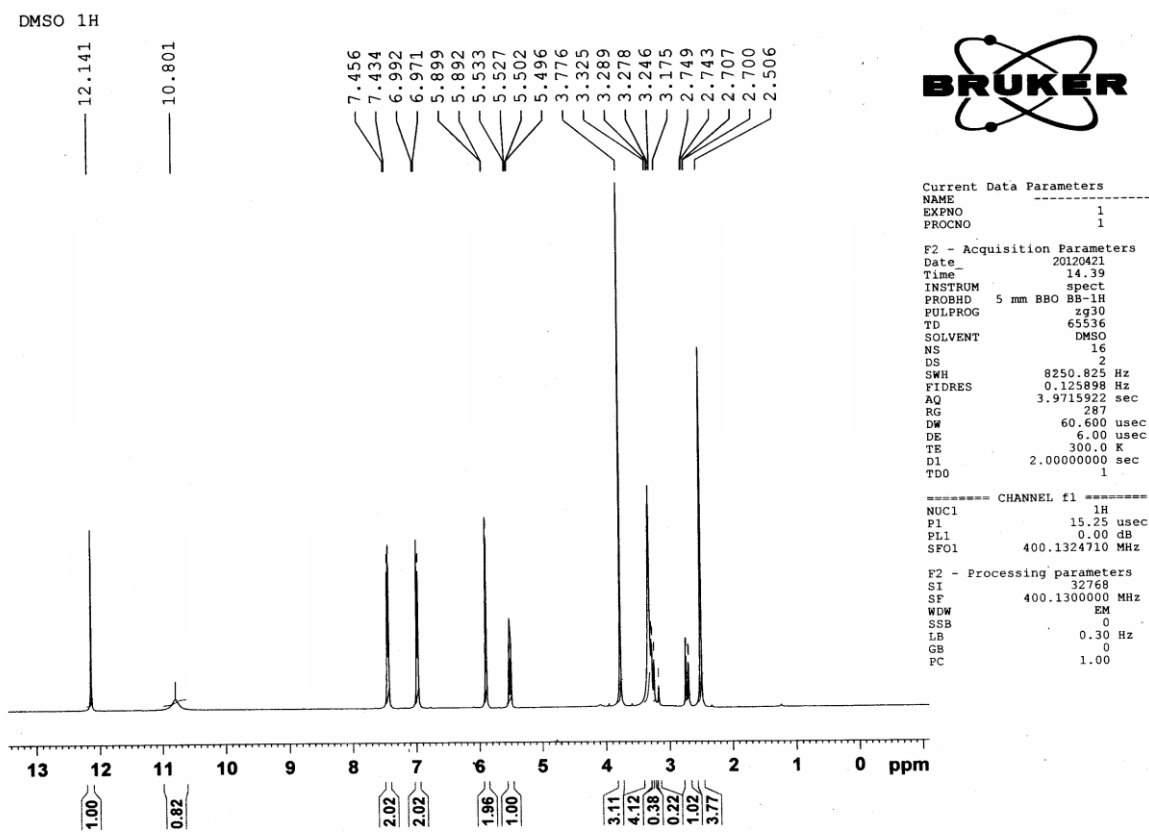


Fig 2.  $\text{H}^1$  (Proton) NMR data of isolated compound at 400 Hz.

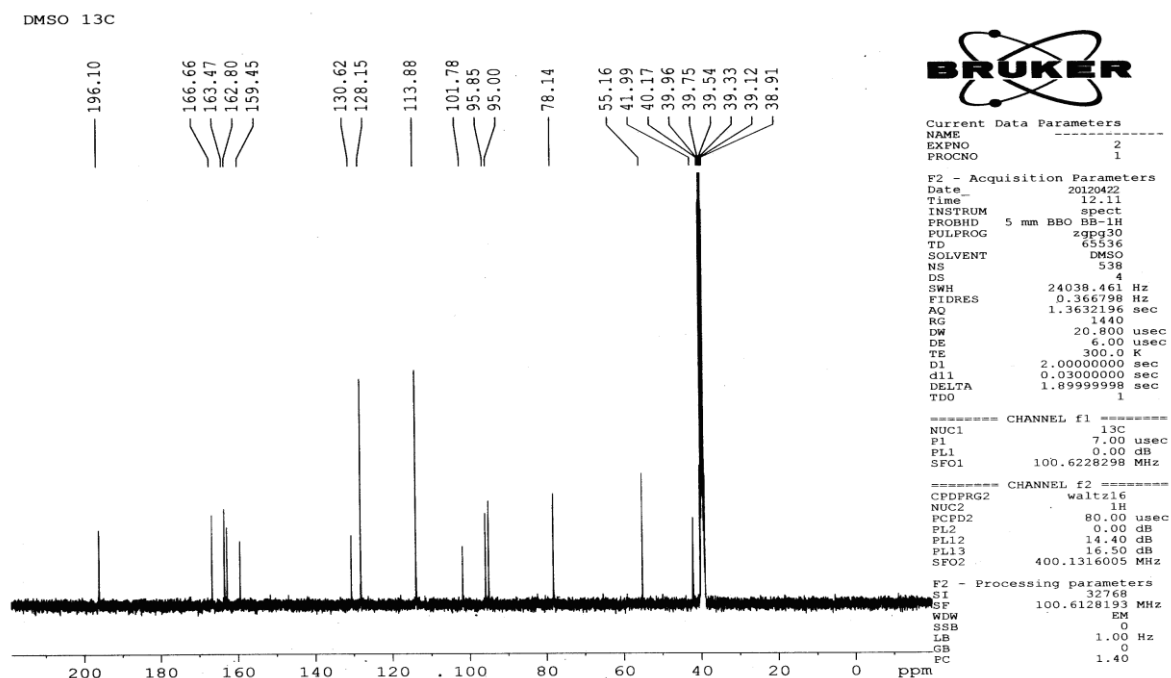
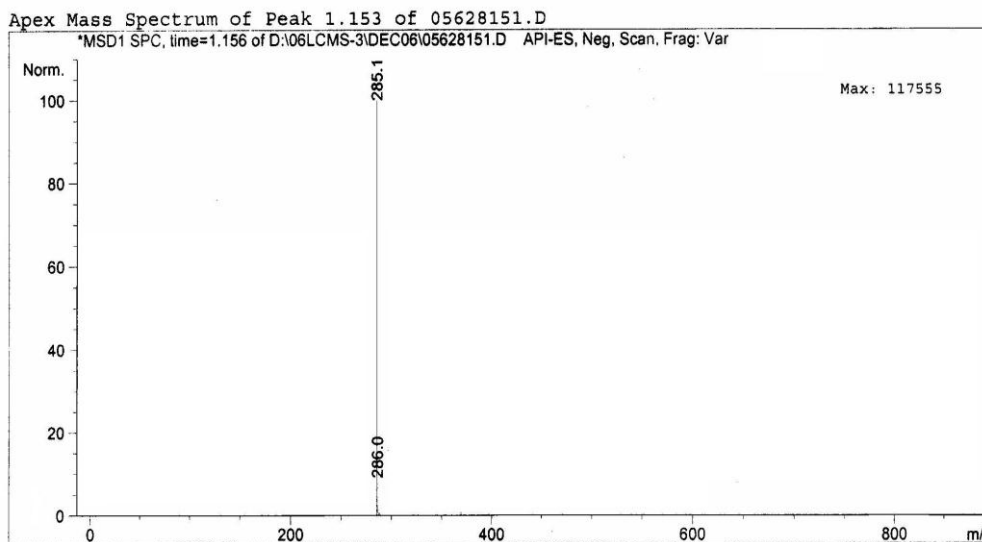
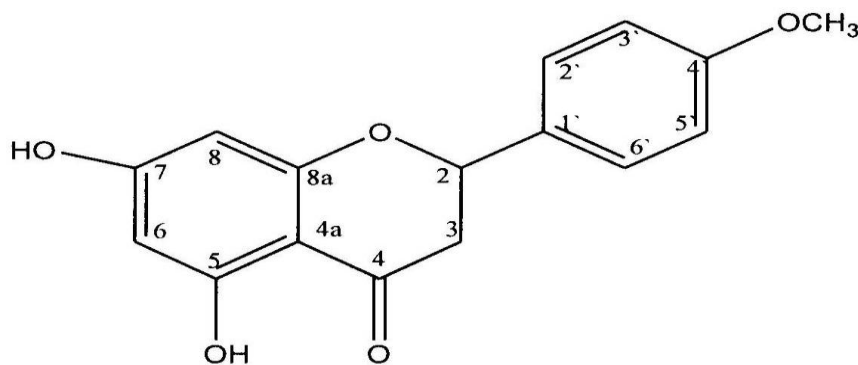
Fig 3.  $C^{13}$  NMR data of isolated compound at 400 Hz.

Fig 4. Mass spectral data of isolated compound of ethanol fraction.



5,7-dihydroxy-4'-methoxyflavone

Fig 5. Chemical structure of the isolated flavonoid (Derivative) compound.

**TABLE LEGENDS**

**Table No.1 Summary of different parameters of diuretic activities of acacetin.**

**Table No.2 Diuretic effect of acacetin of *G. arborea* fruits extracts in Wistar rat.**

**FIGURE LEGENDS**

**Fig. 1 Infrared spectral data of isolated compound in the frequency range between 4000 and 600 cm<sup>-1</sup>.**

**Fig. 2 H<sup>1</sup> (Proton) NMR data of isolated compound at 400 Hz.**

**Fig. 3 C<sup>13</sup> NMR data of isolated compound at 400 Hz.**

**Fig. 4 Mass spectral data of isolated compound of ethanol fraction.**

**Fig. 5 Chemical structure of the isolated flavonoid (Derivative) compound.**

**CONCLUSION**

It could be concluded that the isolated compound was a derivative of flavonoid with a chemical structure "5, 7-dihydroxy-4 methoxy flavone" named as acacetin. The flavonoid compound exhibited significant diuretic effect and the present study supports its ethno pharmacological use as diuretic. This effect may be explored in the use of the plant in the management of some cardiovascular diseases.

**ACKNOWLEDGEMENT**

Author wish to thanks Director of M.S. Swaminathan Research Foundation for identification and authentication of plant product. Author also wishes to thanks Ranbaxy Pvt. Ltd. For supplying Furosemide as gift sample.

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