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NOVEL BIOACTIVE FLAVONOID FROM FLOWERS OF *CALOTROPIS GIGANTEA* LINN.

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ABSTRACT

Calotropis gigantea Linn. is commonly known as Ak in Hindi. It is a shrub or small tree, eight to ten feet in height, bearing unscented white flowers with five pointed petals and a small elegant crown rising from the centre which holds the stamens. The plant has oval, light green leaves and milky stem. The milky exudation from the plant is corrosive in nature, having mercury like effect on human body and is therefore sometime referred to as vegetable mercury. The plant mainly contains cardiac glycosides. Earlier workers have isolated various constituents from this plant, the present paper deals with isolation and structural elucidation of a new compound A, identified as 3,5,4'-trihydroxy-6,7dimethoxyflavone-3-O-α-L--arabinopyranosyl-5-O-β-D-galactopyranosyl-(1→4)-O-β-D xylopyranoside, along with a known compound B, identified as Myricetin from the methanol soluble ethanolic extract of the flowers of the plant. The isolated compounds were identified and characterized by various colour reactions, chemical degradation and spectral analysis. Flower extract of the plant was utilized For Behaviour Despair Study in animal model, by employing Forced Swim Test and Tail Suspension Test. These tests indicated the potential antidepressant effect of the plant extract.

KEYWORDS: *Calotropis gigantea* Linn., Ascelpiadaceae, Flowers, Myricetin, Flavonoidal Glycosides.

INTRODUCTION

Calotropis gigantea Linn.[1-3] belongs to Ascelpiadaceae family. It is known as 'Ak' in Hindi and 'Madar' in Sanskrit. It is commonly found throughout India. It is a shrub or small tree eight to ten feet in height, bearing unscented white flowers. Its root, bark and juice are used in

medicine for their emetic, diaphoretic, alterative and purgative properties. Its flowers are considered digestive, stomachic, tonic, useful in asthma, catarrh and in loss of appetite. Its leaves used in tinctured form act as an antiperiodic in case of intermittent fevers. The milky exudation from the plant is corrosive in nature having mercury like effect on the human body and is therefore sometime referred to as vegetable mercury.

The plant mainly contains cardiac glycosides. Earlier workers [4-8] have isolated and reported various constituents from this plant. The present work deals with the isolation and structural elucidation of a new compound A, identified as 3, 5, 4'-trihydroxy-6, 7 dimethoxy flavone-3-O-α-L-arabinopyranosyl-5-O-β-D-galactopyranosyl- (1→4)-O-β-D xylopyranoside along with a known compound B, identified as Myricetin from the methanol soluble ethanolic extract of the flowers of the plant. Flower extract of the plant was also utilized for Behaviour Despair Study in animal model by employing Forced Swim Test and Tail Suspension Test. These tests reported antidepressant
activity of the plant.

EXPERIMENTAL SECTION

General Experimental Procedure

All the melting points were determined on a thermoelectrical melting point apparatus and are uncorrected. The IR spectrum was recorded in KBr disc on Perkin Elmer Spectrum RXI (4000-4500 cm^{-1}). ^{1}H NMR and ^{13}C NMR spectra were recorded at 300 MHz on Bruker DRX NMR Spectrometers using TMS as internal standard and $CDCl_3$ as solvent. Mass spectra was run on Jeol SX-102 FAB Mass Spectrometer.

The antidepressant activity of flower extract of the plant *Calotropis gigantea* on mice was evaluated by employing animal models and tools like Forced swim test and Tail suspension test. The animal handling was performed according to the Good Laboratory Practice (GLP) guidelines. Institutional animal ethics committee constituted as per the direction of the Committee for the Purpose of Control and Supervision of Experiment on Animals approved the protocol.

PLANT MATERIAL

Flowers of *Calotropis gigantea* Linn. were procured from Sagar region and were taxonomically authenticated by Taxonomist, Department of Botany, Dr. H.S. Gour Central

University Sagar. A voucher specimen (AY-27CG) has been deposited in the Natural Products Laboratory, Department of Chemistry, Dr. H.S. Gour University, Sagar (M.P.) India.

EXTRACTION AND ISOLATION

Air-dried and powdered flowers (4.5 kg) of the plant were extracted with 95% Ethanol (60-80^{o}C) in a Soxhlet apparatus for seven days. The total ethanolic extract was concentrated under reduced pressure and successively partitioned with $CHCl_3$, $CH_3COOC_2H_5$, CH_3COCH_3 and CH_3OH. The methanol soluble fraction of the plant was concentrated under reduced pressure to give a dark brown viscous mass (3.40 gm). It yielded two spots on TLC examination, using chloroform : methanol : water (6:4:2) as solvent system and I_2 vapours as visualizing agent, indicating it to be a mixture of two compounds, A and B. These were separated by column chromatography over a silica gel column using $CHCl_3$: MeOH (3:7, 4:6, 5:5) as eluent and purified by preparative TLC, yielding compounds A and B.

STUDY OF COMPOUND A

It was crystallized from methanol to give light yellow needle compound, yield 1.95 gm; m.p. 224-226^{o}C; Molecular formula $C_{33}H_{40}O_{20}$, [M^+] m/z 756 (FABMS). Found: C, 52.43%; H, 5.36%; Calculated for Molecular Formula $C_{33}H_{40}O_{20}$: C, 52.38%; H, 5.29%; UV(MeOH) λ_{max} nm; 258, 343; IR (KBr) υ_{max} 3372, 2930, 2835,1665,1242,1064,862,840 cm^{-1}; ^{1}H NMR (300 MHz, $CDCl_3$): δ 3.78 (3H, s, 7-OCH_3), 3.61 (3H, m, 6-OCH_3), 6.54 (1H, s, H-8), 7.31 (1H, d, J 7.2 Hz, H-2'), 7.41 (1H, d, J 6.9 Hz, H-3'), 8.28 (1H, s, 4'-OH), 7.41 (1H, d, J 6.9 Hz, H-5'), 7.31 (1H, d, J 7.2 Hz, H-6'); 5.13 (1H, d, J 6.7 Hz, H-1''), 4.32 (1H, dd, J 3.1, 10.4 Hz, H-2''), 4.11 (1H, dd, J 2.9, 10.4 Hz, H-3''), 4.33 (1H, m, H-4''), 4.41 (2H, d, J 6.1 Hz, H-5''); 5.38 (1H, d, J 7.3 Hz, H-1'''), 3.77 (1H, dd, J 7.4, 8.1 Hz, H-2'''), 4.19 (1H, d, J 8.3 Hz, H-3'''), 4.11 (1H, s, H-4'''), 3.67 (2H, d, J 8.8 Hz, H-5'''); 5.69 (1H, d, J 7.8 Hz, H-1''''), 3.78 (1H, dd, J 6.9, 8.6 Hz, H-2''''), 3.65 (1H, dd, J 7.2, 3.8 Hz, H-3''''), 4.31 (1H, dd, J. 8.2, 2.8 Hz, H-4''''), 3.26 (1H, d, J 6.1 Hz, H-5''''), 4.52 (2H, dd, J 9.8, 4.3Hz, H-6''''); ^{13}C NMR (300 MHz, $CDCl_3$): δ 162.77 (C-2), 146.98 (C-3), 180.91 (C-4), 155.89 (C-5), 59.89 (C-6, OCH_3), 57.68 (C-7, OCH_3), 94.22 (C-8), 149.42 (C-9), 111.51 (C-10), 118.11 (C-1'), 131.83 (C-2'), 117.54 (C-3'), 164.19 (C-4'), 117.54 (C-5'), 131.83 (C-6'), 104.7 (C-1''), 73.8 (C-2''), 73.2 (C-3''), 71.7 (C-4''), 68.2 (C-5''); 102.8 (C-1'''), 72.6 (C-2'''), 80.4 (C-3'''), 71.3 (C-4'''), 69.1 (C-5'''); 103.7 (C-1''''), 76.3 (C-2''''), 74.3 (C-3''''), 69.2 (C-4''''), 76.4 (C-5''''), 62.6 (C-6'''').

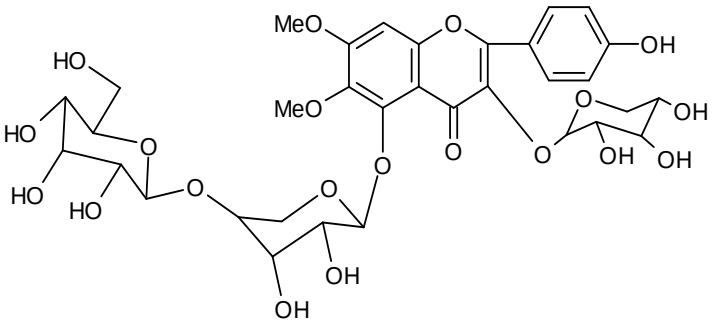

Fig: 1 Compound A

ACID HYDROLYSIS OF COMPOUND A

Compound A (600 mg) was dissolved in ethanol (20 ml) and refluxed with 10% H_2SO_4 (50 ml) in 150 ml round bottomed flask on water bath for 6 hrs. The contents were 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-I which was identified as 3, 5, 4'-trihydroxy-6, 7-dimethoxy flavone by comparison of its spectral data with reported literature values. 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 *n*-Butanol: Acetic Acid: Water (4:1:5) as solvent system and the sugars so obtained were identified as L-arabinose (R_f 0.20), D-xylose (R_f 0.26), and D-galactose (R_f 0.18) by (Co-PC and Co-TLC).

STUDY OF COMPOUND A-I

It was obtained as light brown compound, m.p. 265-267^oC; Molecular formula $C_{17}H_{14}O_7$, [M^+] m/z 330 (FABMS); Found: C, 61.74%; H, 4.19%; Calculated for Molecular Formula $C_{17}H_{14}O_7$: C, 61.81%; H, 4.24 %; UV (MeOH) λ_{max} nm; 262, 346; IR (KBr) υ_{max} 3368, 2932, 2830, 1673, 1666, 1244, 1137, 867, 842 cm^{-1}; ^1H NMR (300 MHz, $CDCl_3$): δ 3.46 (3H, s, 6-OCH_3), 3.53 (3H, s, 7-OCH_3), 6.61 (1H, s, H-8), 9.25 (1H, br, s, 3-OH), 8.13 (1H, s, 4'-OH), 10.12 (1H, br, s, 5-OH), 7.68 (1H, d, J 7.8 Hz, H-2'), 7.24 (1H, d, J 7.9 Hz, H-3'), 7.24 (1H, d, J 7.9 Hz H-5'), 7.68 (1H, d, J 7.8 Hz, H-6'); ^{13}C NMR (300 MHz, $CDCl_3$): δ 161.74 (C-2), 128.77 (C-3), 181.91 (C-4), 155.69 (C-5), 124.27 (C-6), 158.86 (C-7), 92.12 (C-8), 148.42 (C-9), 110.21 (C-10), 56.13 (C-7, OCH_3), 60.73 (C-6, OCH_3), 118.16 (C-1'), 132.13 (C-2'), 118.46 (C-3'), 163.39 (C-4'), 117.46 (C-5'), 132.13 (C-6').

STUDY OF COMPOUND B

It was crystallized from methanol to give pale yellow powder, yield 0.310 gm; m.p. 343-344^{o}C; Molecular formula $C_{15}H_{10}O_8$, [M^+] m/z 318 (FABMS). Found: C, 56.63%; H, 3.17%; Calculated for Molecular Formula $C_{15}H_{10}O_8$: C, 56.60%; H, 3.14%; UV(MeOH) λ_{max} nm; 287, 328; IR (KBr) υ_{max} 3378, 2924, 1635, 1460, 1366 cm^{-1}; ^{1}H NMR (300 MHz, $CDCl_3$):δ 6.41(2H, s, H-2'), 9.27-8.48 (1H, br, s, OH, 3', 4', 5'), 6.41 (2H, s, H-6'), 12.29 (1H, s, OH-5), 6.24 (1H, d, J 1.8 Hz, H-6), 10.31 (1H, br, s, OH-7), 6.39 (1H, d, J 9.1 Hz, H-8); ^{13}C NMR (300 MHz, $CDCl_3$) δ 147.13 (C-2), 137.61 (C-3), 174.42 (C-4), 161.81 (C-5), 99.52 (C-6), 163.72 (C-7), 94.86 (C-8), 156.83 (C-9), 102.34 (C-10); 120.91 (C-1'), 106.84 (C-2'), 145.21 (C-3'), 136.34 (C-4'), 145.21 (C-5'), 106.84 (C-6').

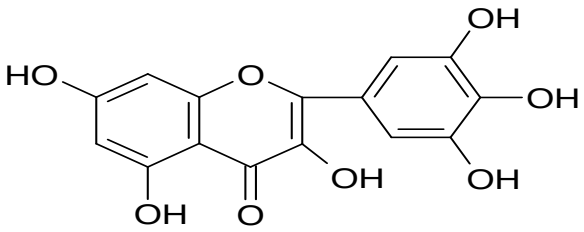

Fig: 2 Compound B

PERMETHYLATION OF COMPOUND A

Compound A (25 mg) was dissolved in DMF (50 ml) and treated with MeI (5 ml) and Ag_2O (20 mg) in 150 ml round bottomed flask fitted with condenser and refluxed for 2 days. The contents were filtered and washed with DMF. The filtrate was concentrated under reduced pressure and treated with $CHCl_3$ (25 ml) and washed with water. After removal of solvent a syrupy mass was found which was hydrolysed with 10% ethanolic H_2SO_4 (5 ml) to give methylated aglycone, identified as 3, 5-dihydroxy- 6, 7, 4'-tri-methoxy flavone and methylated sugars.The aqueous hydrolysate obtained after 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 on Whatmann filter paper number 1 using *n*-Butanol: Acetic acid : Water (5:1:4) as solvent system and aniline hydrogen phthalate as spraying agent. The methylated sugars were identified as 2, 3, 4 tri-O-methyl L- arabinose (R_G0.96); 2, 3, di-O-methyl D-xylose (R_G0.73) and 2, 3, 4, 6-tetra-O-methyl D-galactose (R_G1.01), (Co-PC).

ENZYMATIC HYDROLYSIS OF COMPOUND A

Enzymatic hydrolysis of the glycoside (A) with takadiastase enzyme liberated L-arabinose (R_f 0.20) and 3, 5, 4'-trihydroxy-6, 7-dimethoxy flavone-5-O-β-D-galactopyranosyl-(1→4)-O-β-D-xylopyranoside as proaglycone, revealing the presence of α-linkage between L-arabinose and proaglycone. The proaglycone on further hydrolysis with almond emulsin enzyme liberated D-galactose and proaglycone 3, 5, 4'-trihydroxy-6, 7-dimethoxy flavone-5-O-β-D-xylopyranoside which on further hydrolysis liberated D-xylose and aglycone, confirming the presence of β-linkage between D-galactose and D-xylose as well as between D-xylose and aglycone.

RESULT AND DISCUSSION

Compound A was obtained as light yellow needle compound, m.p. 224-226^{o}C, Molecular Formula $C_{33}H_{40}O_{20}$ [M^{+}] 756 (FABMS). It gave positive Molisch[9] and Shinoda[10] test showing its flavonoidal glycosidic nature. Absorption bands at 258 and 343 nm in UV suggested its flavone structure. Its IR spectra showed absorption at 3372, 2930, 2835, 1665, 1242, 1064, 862 and 840 cm^{-1}. In ^{1}H NMR three singlet at δ 3.78 (3H, s, 7-OCH_3) and multiplet at δ 3.61(3H, m, 6-OCH_3) were assigned to methoxy group at H-6, and H-7 respectively. Characteristic singlet peak was observed at δ 8.28 (1H, s, 4'-OH) for hydroxyl group at H-4'. Doublets at 7.31 (1H, d, J 7.2 Hz) for H-2', H-6' and at 7.24 (1H, d, J 6.9 Hz) were present for H-3', H-5' respectively.The anomeric proton signals at δ 5.13 (1H, d, *J* 6.7 Hz), δ 5.38 (1H, d, *J* 7.3 Hz) and at δ 5.69 (1H, d, *J* 7.8 Hz) were assigned to H-1" of L-arabinose, H-1'" of D-xylose and H-1'"" of D-galactose respectively. The ^{13}C NMR spectrum of compound A exhibited signals at δ 59.89 and δ 57.68 for methoxy group at C-6 and C-7 respectively. A signal at δ 164.19 suggested the presence of hydroxyl group at C-4'. Thus the ^{13}C NMR spectrum of compound A exhibited 33 signals, 17 of which could be assigned to the aglycone and 16 signals to the sugar moieties revealing the nature of compound A as bidesmosidic.

Acid hydrolysis of compound A with 10% ethanolic H_2SO_4 gave aglycone A-I, m.p. 265-267^{o}C, Molecular Formula, $C_{17}H_{14}O_7$ [M^{+}] 330 (FABMS). It was identified as 3, 5, 4'-trihydroxy-6, 7-dimethoxy flavone by comparison of its spectral data with reported literature values.[11-13] The aqueous hydrolysate so obtained from acid hydrolysis process was neutralized with $BaCO_3$ and the $BaSO_4$ was filtered off. The filtrate was concentrated and subjected to paper chromatography[14] which indicated the presence of L-arabinose, D-xylose and D-galactose.

Quantitative estimation [15] of sugars revealed that all the three sugars were present in equi-molecular ratio (1:1:1). Periodate oxidation [16] of compound A, further confirmed that all the three sugars were present in pyranose form.[17]

Permethylation [18] followed by acid hydrolysis was carried out to determine the position of sugar moieties in compound A. The permethylation process yielded methylated aglycone and methylated sugars, confirming that hydroxyl groups at C-3 and C-5 position of aglycone were involved in glycosidation. The methylated aglycone was identified as 3, 5-dihydroxy- 6, 7, 4'-tri-methoxy flavone and the methylated sugars were identified as 2, 3, 4, tri-O-methyl L-arabinose (R_G 0.96); 2, 3, di-O-methyl D-xylose (R_G 0.73); 2, 3, 4, 6-tetra-O-methyl D-galactose (R_G 1.01), (Co-PC). [19] Therefore it was concluded that C-1" of L-arabinose was linked with C-3 position of aglycone likewise C-4"' of D-xylose is linked to C-1"" of D-galactose. The inter glycosidic linkage (1→4) was found between D-xylose and D-galactose.[20]

Enzymatic hydrolysis [21] of the glycoside (A) with takadiastase enzyme liberated L-arabinose (R_f 0.20) and 3, 5, 4'-trihydroxy-6, 7-dimethoxy flavone-5-O-β-D-galactopyranosyl-(1→4)-O-β-D-xylopyranoside as proaglycone, revealing the presence of α-linkage between L-arabinose and proaglycone. The proaglycone on further hydrolysis with almond emulsin enzyme liberated D-galactose and proaglycone 3, 5, 4'-trihydroxy-6, 7-dimethoxy flavone-5-O-β-D-xylopyranoside which on further hydrolysis liberated D-xylose and aglycone, confirming the presence of β-linkage between D-galactose and D-xylose as well as between D-xylose and aglycone. Thus on the basis of above evidences the structure of compound A was assigned as 3, 5, 4'-trihydroxy-6, 7 dimethoxy flavone-3-O-α-L-arabinopyranosyl-5-O-β-D-galactopyranosyl-(1→4)-O-β-D xylopyranoside.

Compound B was analyzed for Molecular Formula, $C_{15}H_{10}O_8$, m.p. 343-344^oC [M^+] m/z 330 (FABMS) and was identified as Myricetin by comparison of its spectral data with reported literature values. [22]

ANTIDEPRESSANT ACTIVITY

To investigate the ability of herbal drug in elevation of suppressed mood, behaviour despair study was performed by making use of flower extract of *Calotropis gigantea*. For the purpose two animal models, Forced Swim Test [23-26] developed by Porsolt et al., (1981); [27] and Tail Suspension Test [28-31] developed by Steru et al., (1985); [32] were employed. The immobility

displayed by rodents when subjected to unavoidable stress such as forced swimming or tail suspension test is thought to reflect a state of despair or lowered mood, which are also thought to reflect depressive disorders in humans. [33-34] *Calotropis gigantea* is rich in phytoconstituents like alkaloid, tannins, proteins, flavonoids etc. Drug dosages of flower extract of *Calotropis gigantean* showed antidepressant action on the test species of the experimental process. It has also been found that flavonoids isolated from plant species such as *Hypericum perforatum* showed antidepressant activity. [35] Thus it can be attributed that the possible antidepressant action of *Calotropis gigantea* can be due to the presence of flavonoid components along with other phytoconstituents. [36-39]

CONCLUSION

Thus above evidences establish the presence of a novel bioactive flavonoid compound A, identified as- 3,5,4'-trihydroxy-6,7dimethoxyflavone-3-O-α-L--arabinopyranosyl-5-O-β-D-galactopyranosyl-(1→4)-O-β-D xylopyranoside, along with a known compound B, identified as Myricetin, from the methanol soluble ethanolic extract of the plant, *Calotropis gigantea* Linn., The plant extract when examined for antidepressant activity in animal model by employing Forced Swim Test and Tail Suspension Test clearly indicated the potential for the use of these herbal drugs as an adjuvant in the treatment of depression. Further research however is required to gain closer insights into exact mechanism of their action.

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