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ANTIOXIDANT ACTIVITY OF NEW POTENTIAL ALLELOCHEMICAL FROM STEMS OF *CASSIA SIAMEA* LAM.

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Keywords:

Cassia Siamea Lam., Leguminosae, Allelochemical, Antioxidant activity.

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ABSTRACT: A new potential allelochemical (A) m.f. $C_{34}H_{42}O_{19,}$ m.p 168-170 $^{\circ}$C, $[M]^{+}$ 754 (FABMS) along with two known compounds Scutellarin 7-O-β-D-glucopyranoside (B), Luteolin (C) were isolated from methanolic extract of the stems of *Cassia Siamea* Lam. The structure of a new allelochemical (A) was characterized as 3,7-dihydroxy-5,3',4'-trimethoxyflavone-3-O-β-D-xylopyranosyl-7-O-α-L-rhamnopyranosyl(1→4)-O-α-L-arabinopyranoside, on the basis of extensive spectroscopic analysis, colour reactions and various chemical degradations. Compound A exhibited higher radical scavenging activity in the (DPPH) (1,1-diphenyl-2-picrylhydrazyl) assay system.

INTRODUCTION: *Cassia Siamea* Lam.[1-6] belongs to family Leguminosae and commonly known as Kassod in Hindi. It is a moderate-size tree and cultivated throughout India and Burma. Its leaf extract is useful in controlling blood glucose level as well as improving lipid metabolism[7].

Its stem bark is traditionally use against constipation, malaria and associated diseases such as fever and jaundice, A decoction of bark is given to diabetic patients while a paste is used as antipyretic and leaves for constipation, hypertension, Insomnia and asthma. Its flowers show antioxidant and antihypertensive activity [8-10]. It also shows laxative activity, antimalarial, antiplasmodial activity and sedative activity [11-15].

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Earlier workers have reported [16-19] the presence of various active constituents from this plant. In the present paper we report the isolation and structural elucidation of a new compound (A) 3,7-dihydroxy-5,3',4'-trimethoxyflavone-3-O-β-D-xylopyranosyl-7-O-α-L - rhamnopyranosyl (1→4) – O - α-L-arabinopyranoside alongwith two known compounds Scutellarin-7-O-β-D-glucopyranoside (B), Luteolin(C), from methanolic extract of stems of this plant.

General Experimental Procedure:

The plant extract was concentrated under reduced pressure by rotary vaccum evaporator (R/178). All of the melting points were determined by Thermoelectrically melting point apparatus and are uncorrected. The IR spectra were recorded on Simadzu 84005 FTIR spectrophotometer in KBr pellets, UV spectra were recorded on Systronics-2201 UV/Vis Double Beam spectrophotometer in MeOH. ^{1}H NMR spectra were recorded on Bruker DRX-300 Spectrometer operating at 300 MHz using $CDCl_3$ as solvent and TMS as internal standard, ^{13}C NMR spectra

were recorded on Bruker DRX-75 MH_Z spectrometer using $CDCl_3$. The chemical shift values are reported in ppm(δ) units and coupling constant (*J*) in Hz.The mass spectra were recorded on Jeol-SX (102) mass spectrometer.

Plant material:

The stems of the plant were collected locally around Sagar region and were taxonomically authenticated by Taxonomist, Department of Botany, Dr. H. S. Gour Central University, Sagar (M.P.) India. A voucher specimen has been deposited in the Natural Products Laboratory, Department of Chemistry of this university.

Extraction and isolation:

Air dried and powdered stems (4.5Kg) of the plant were extracted with ethanol in a Soxhlet apparatus for 4 days. The ethanolic extract of stems of the plant were further successively partitioned with chloroform, ethyl acetate, acetone, and methanol. The methanol soluble fraction was concentrated under reduced pressure by rotary vaccum evporator to give brown viscous mass (4.12g), which was subjected to TLC examination using silica gel-G and nBAW (4:1:5) as solvent and I_2 vapours as visualizing agent. It showed three spots indicating it to be mixture of three compounds A, B and C. These compounds were separated by column chromatography over silica gel using $CHCl_3$: MeOH (4:8) as eluent and studied separately.

Study of Compound A:

It was crystallized from acetone to yield 1.72 gm. It had m.f. $C_{34}H_{42}O_{19,}$ m.p 168- 170°C, $[M]^+$ 754 (FABMS); found (%):C 54.08,H 5.66, O 40.24,calcd.(%) for m.f. $C_{34}H_{42}O_{19}$: C 54.11, H 5.61, O 40.28 ; UV λ_{max} MeOH (nm) 348, 254,272 ;(+$AlCl_3$) 408; (+NaOMe) 398; (+NaOAc) 260. IR (KBr) $\nu_{max}(cm^{-1})$ 3420, 1685, 1610, 1432, 1068. ^{1}HNMR (300MH_Z, $CDCl_3$), δ(ppm); 3.89 (3H, s, 5-OCH_3), 6.34 (1H, d, J 2.2H_Z, H-6), 6.45 (1H, d, J 2.1H_Z, H-8), 7.92 (1H, d, J 2.0H_Z, H-2′), 3.73(3H, s, 3′-OCH_3), 3.84(3H, s, 4′-OCH_3), 6.07 (1H, d, J 8.8H_Z, H-5′) , 7.79(1H, dd, J8.7,2.1 H_Z,H-6′), 5.53(1H, d, J 7.1H_Z, H-1″), 4.75(1H, d,J 2.1 H_Z ,H-2″), 3.49 (1H, m, H-3″), 3.53(1H, m, H-4″), 3.72(1H, m, H-5″), 5.81(1H, d,J 1.8H_Z, H-1‴), 3.54(1H, m, H-2‴), 3.42(1H, m, H-3‴), 3.62(1H, m, H-4‴), 3.74(1H, m, H-5_a‴), 3.35(1H, m, H-5_b‴), 5.45(1H, d, J-1.6 H_Z, H-1⁗), 4.22(1H, br , H-2⁗), 3.95(1H, m, H-3⁗), 3.43(1H, m, H-4⁗), 3.66(1H, m , H-5⁗), 1.12(3H, d, J 6.2 H_Z, H-6⁗). ^{13}C NMR (75 MH_Z,$CDCl_3$), δ(ppm): 143.7(C-2), 132.6(C-3) ,171.9(C-4), 163.5(C-5), 95.2(C-6), 162.3(C-7), 94.5(C-8), 158.7(C-9), 107.3(C-10), 128.6(C-1′), 120.2(2′), 137.3(C-3′), 149.8(C-4′), 112.0(C-5′), 126.5(C-6′), 56.4(5-OCH_3), 56.2(3′-OCH_3), 56.0(4′-OCH_3), 98.5(C-1″), 68.3(C-2″), 72.4(C-3″), 65.1(C-4″), 73.6(C-5″), 103.4(C-1‴), 64.2(C-2‴), 72.3(C-3‴), 65.7(C-4‴), 71.5(C-5‴), 92.6(C-1⁗), 60.8(C-2⁗), 65.7(C-3⁗), 72.4(C-4⁗), 75.2(C-5⁗), 12.5(C-6⁗).

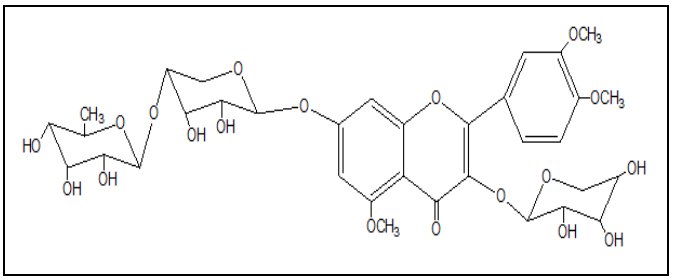

FIG. 1: Compound A

Acid Hydrolysis of Compound A:

460 mg of compound A was dissolve in ethanol (40ml) and refluxed with 25 ml of 10% H_2SO_4 on water bath for 6 h. The contents were concentrated and allowed to cool and residue was extracted with diethyl ether (Et_2O). The ether layer was washed with water and evaporated to dryness. The residue was subjected to column chromatography over silica gel using $CHCl_3$:MeOH (3:8) to give compound A-1, which was identified as 3,7-dihydroxy-5,3',4'-trimethoxy flavone. 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) as a solvent and aniline hydrogen phthalate as detecting reagent. The sugars were identified as D-xylose (R_f 0.28) and L-arabinose (R_f 0.21), L-rhamnose (R_f 0.37) (Co-PC).

Study of Compound A-1:

It was crystallized from acetone to yield 295 mg. It had m.f. $C_{18}H_{16}O_{7,}$ m.p 158-161 °C, $[M]^+$ 754 (FABMS); found (%):C 62.70, H 4.71, O 32.55, calcd.(%) for m.f. $C_{18}H_{16}O_7$: C 62.79, H 4.68, O 32.53 ; UV λ_{max} MeOH (nm) 348, 254,272 ;(+$AlCl_3$) 408; (+NaOMe) 398; (+NaOAc) 260. IR

(KBr) ν_{max}(cm^{-1}) 3420, 1685,1610,1432,1068. ^{1}HNMR (300MH_Z, $CDCl_3$), δ(ppm); 3.99 (3H, s, 5- OCH_3), 6.37 (1H, d, J 2.2H_Z, H-6), 6.55 (1H, d, J 2.1H_Z, H-8), 7.94 (1H, d, J 2.1H_Z, H-2′), 3.91(3H, s, 3′-OCH_3), 3.83(3H, s, 4′-OCH_3), 6.09 (1H, d, J 8.7H_Z, H-5′) , 7.71(1H, dd, J8.7,2.0 H_Z,H-6′),^{13}C NMR (75 MH_Z,$CDCl_3$), δ(ppm): 142.5(C-2), 131.5(C-3) ,170.2(C-4), 162.5(C-5), 94.2(C-6), 160.4(C-7), 93.5(C-8), 157.5(C-9), 106.3(C-10), 127.6(C-1′), 119.1(2′), 136.3(C-3′), 148.2(C-4′), 111.2(C-5′), 125.4(C-6′), 55.4(5-OCH_3), 55.2(3′-OCH_3), 56.1(4′-OCH_3).

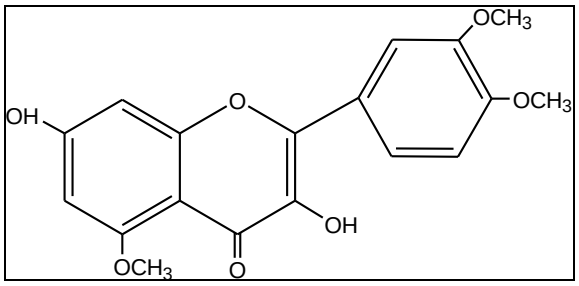

FIG. 2: COMPOUND A-1

Permethylation of Compound A:

Compound A (45mg) was dissolved in 20mg DMF and treated with MeI (10 ml) and Ag_2O (20 ml) in round bottomed flask fitted with air condenser and refluxed for one day and the reaction mixture was filtered and washed with DMF. The filtrate was concentrated under reduced pressure and hydrolysed with 10% ethanolic H_2SO_4 for 6h to give methylated aglycone identified as 3,7-dihydroxy-5,3',4'-trimethoxy flavone and the aqueous hydrolysate obtained after the removal of aglycone was neutralized with $BaCO_3$ and the $BaSO_4$ filtered off. The filtrate was concentrated and subjected to paper chromatography examination using nBAW(4:1:5) as a solvent and aniline hydrogen phthalate as detecting reagent. The methylated sugars were identified as 2,3,4-tri-O-methyl-D-xylose [R_G 0.94], 2,3-di- O-methyl-L-arabinose [R_G 0.64], 2,3,4-tri-O- methyl-L-rhamnose [R_G 1.01].

Enzymatic Hydrolysis of Compound A:

Compound A (37mg) was dissolved in MeOH (25ml) and hydrolysed with an equal volume of almond emulsin enzyme. The reaction mixture was allowed to stay at room temperature for two days and filtered. The hydrolysate was concentrated and subjected to paper chromatography examination using nBAW (4:1:5) as a solvent and aniline hydrogen phthalate as a spraying reagent which showed the presence of D-xylose (R_f 0.28). The proaglycone was dissolved in MeOH (20ml) and further hydrolysed with equal volume of takadiastase enzyme at room temperature as usual procedure yielded aglycone identified as 3,7-dihydroxy-5,3',4'-trimethoxy flavone and sugar were identified as L - rhamnose (R_f 0.37) , L-arabinose (R_f 0.21) (Co-PC).

Study of Compound B:

It was analyzed for m.f. $C_{21}H_{20}O_{11}$,m.p. 183 - 185 ^{0}C, $[M]^+$ 448 (FABMS); found (%):C 56.15, H 4.48, O 39.50, calcd.(%) for m.f. $C_{21}H_{20}O_{11}$: C 56.25, H 4.50, O 39.25, UV λ_{max} MeOH (nm) 348, 271, (+NaOAc) 276, 287, IR (KBr): ν_{max} cm^{-1} 3250 and 1648,

^{1}H-NMR (300 MHz, $CDCl_3$): δ 7.54 (2H, d, J 9.1 Hz, H-3′ and H-5′), 7.10 (2H, d, J 9.3 Hz, H-2′ and H-6′), 6.50 (1H, d, J 3.0 Hz, H-8), 6.50 (1H, s, H-3), 5.12 (1H, d, J 7.4, H-1′′), 4.42 (m, H-4′′), 3.82 (1H, dd, J 2.1 Hz, 11.8 Hz, Hβ-6′′), 3.66 (1H, m, H-2′′), 3.75 (1H, dd, J 5.6 Hz, 11.7 Hz, Hα-6′′), 3.69 (m, H-3′′), 3.36 (m, H-5′′), ^{13}C-NMR (75 MHz, $CDCl_3$): δ 163.8 (C-2), 102.5 (C- 3), 186.2 (C-4), 148.5 (C-5), 134.7 (C-6), 153.8 (C-7), 98.6 (C-8), 150.6 (C-9), 107.2 (C-10), 124.4 (C-1′), 127.44 × 2 (C - 2′and C - 6′), 119.0 × 2 (C - 3′ and C - 5′), 165.36 (C - 4′), 101.3 (C-1′′), 76.1 (C-2′′), 70.3(C-3′′), 68.6 (C- 4′′), 74.2 (C- 5′′), 59.6 (C-6′′).Thus it was identified as Scutellarin 7-O-β-D-glucopyranoside by comparison of its spectral data with reported literature values[20].

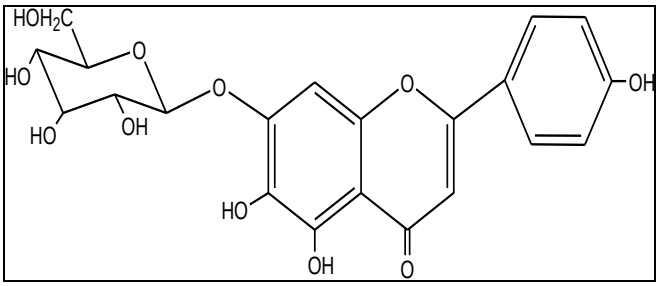

FIG. 3: COMPOUND B

Study of Compound C:

It was analyzed for m.f. $C_{15}H_{10}O_6$, m.p 232-233 °C, $[M]^+$ 286 found (%) C 62.98, H 3.53, O 33.59, Calcd (%) for m.f. $C_{15}H_{10}O_6$,C 62.94, H 3.52, O 33.54. UV (MeOH) λ_{max} nm 274, 321 and 375. IR

(kBr) υ_{max} (cm^{-1}); 3461, 1650, 1600, 1560, 1507. ^{1}HNMR (300 MHz, $CDCl_3$); δ 6.72 (1H, s, H-3), 6.48 (^{1}H, br, d, J 2.0 Hz, H-6), 7.07 (1H, br, s, H-8), 7.76 (1H, br, s, H-2'), 6.88 (1H, d, J 8.4 Hz, H-5'), 7.42 (1H, dd, J 8.4-2.3 Hz H-6'). ^{13}C NMR (75 MHz, $CDCl_3$); δ 164.42 (C-2), 102.40 (C-3), 182.80 (C-4), 163.80 (C-5), 102.10 (C-6), 162.54 (C-7), 97.50 (C-8), 160.40 (C-9), 105.52 (C-10), 121.12 (C-1'), 113.10 (C-2'), 145.89 (C-3'), 149.70 (C-4'), 115.68 (C-5'), 118.36 (C-6'). It was identified as Luteolin by comparison with reported literature values [21].

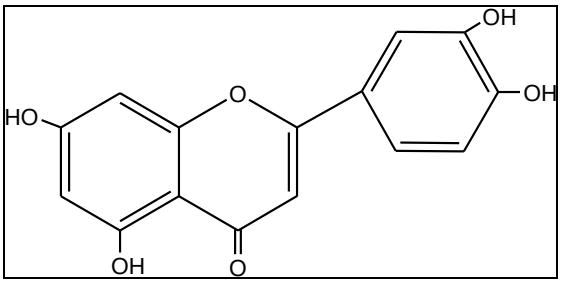

FIG. 4: COMPOUND C

Evaluation of antioxidant activity by DPPH radical scavenging method:

For the determination of scavenging activity of DPPH free radical by the compound A, solution of 0.002% DPPH in methanol was prepared and ascorbic acid was taken as the reference standard. Different concentration of compound A and standard drug [5,10,25,50,100µg/ml] were prepared using methanol 2.0ml of 0.002% DPPH solution was mixed with 2.0ml of all the concentration of compound A and standard separately. The reaction mixture was vortexed thoroughly and left in the dark at room temperature for 30 min. The absorbance of the mixture was measured spectrophotometrically at 517 nm.

The ability of plant extract to scavenge DPPH radical was calculated by the following equation:

$$\text{DPPH radical scavenging activity} = (A_{control} - A_{sample} / A_{control}) X\ 100$$

Absorbance of 0.002% DPPH (control) =0.733

TABLE 1: ANTIOXIDANT ACTIVITY OF COMPOUND A

Conc. (µg/mL)	Absorbance of Ascorbic acid (Mean value)	% inhibition of (Ascorbic acid)	IC_{50} (µg/mL) of (Ascorbic acid)	Absorbance of Compound A (Mean value)	% Inhibition of Compound A	IC_{50} (µg/mL) of Compound A
5	0.534	27.1487		0.553	24.5566	
10	0.498	32.0600		0.512	30.1500	
25	0.456	37.7899		0.413	43.6562	
50	0.398	45.7025	52.90	0.352	51.9781	40.08
100	0.198	72.9877		0.055	92.4965	

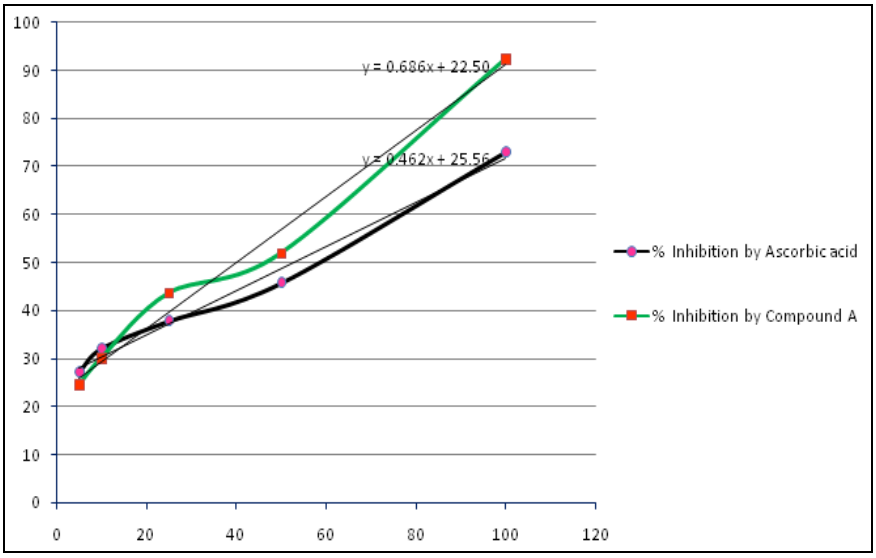

FIG. 5: GRAPHICAL REPRESENTATION OF DPPH RADICAL SCAVENGING ACTIVITY OF COMPOUND A.

RESULTS AND DISCUSSION: Compound A has molecular formula $C_{34}H_{42}O_{19}$, m.p.168- 170°C, $[M]^+$ 754 (FABMS), It gave positive Molisch and Shinoda tests [22-25] showing its flavonoidal glycosidic nature. Its IR spectrum showed absorption bands at 3420cm^{-1} (-OH), 1685cm^{-1}

(>C=O α-β unsaturated), 1610 cm^{-1} (aromatic ring system), 1432 cm^{-1} (CH_3 bending), 1068 (glycosidic linkage). Its UV spectrum showed absorption bands at 352nm showed its flavonoidal skeleton and bands at 256 with its shoulder at 271 suggested that it has di-O-substituted-B-ring, absorption bands at 412 and 402 nm with $AlCl_3$ and NaOMe showed the presence of –OH groups at C-3, C-7 respectively [26].

In ^{1}H NMR spectrum of compound showed a singlet at δ 3.89, 3.73, 3.84 confirmed the presence of –OMe group at C-5, C-3′, C-4′ position. A doublet at 6.34 (1H, d, J 2.2H_Z) assingned to H-6, a doublet at 6.45(1H, d, J 2.2 H_Z) assingned to H-8 of ring C. Two doublets at 7.92(1H, d, J 2.1 H_Z), 6.07 (1H, d, J 8.8 H_Z) assingned for H-2′, H-5′ respectively and double doublet at 7.79(1H, dd, 8.7, 2.1 H_Z) assingned for H-6′ of ring B. The anomeric proton signals at δ 5.53(1H, d, J 7.1H_Z,), δ 5.81(1H, d, J 1.8H_Z,), δ 5.45(1H, d, J 1.6 H_Z,) were assigned for H-1″, H-1‴, H-1⁗of D-xylose, L-arabinose and L-rhamnose respectively. In ^{1}HNMR spectrum coupling constants at J 7.1 H_Z of H-1″confirmed the β-anomeric configuration for the D-xylose. Two coupling constant at J 1.8H_Z and J 1.6H_Z for the anomeric protons of L-arabinose and L-rhamnose respectively, confirmed the α-configuratios of L-arabinose and L-rhamnose [27].

In the mass spectrum of the compound A, characteristic ion peaks at *m/z* 754 [M^+], 622 [M^+-D- xylose], 476 [M^+-L-rhamnose], 344 [M^+-L-arabinose], were found by subsequent losses from the molecular ion of each molecule of D- xylose , L-rhamnose, and L-arabinose, revealing D- xylose at C-3 position and L-rhamnose attached with L-arabinose were linked to aglycone at C-7 position. Acid hydrolysis of compound A with 10% ethanolic H_2SO_4 gave aglycone A-1 m.f. $C_{18}H_{16}O_7$, m.p 158-161 °C, $[M]^+$ 754 (FABMS), and sugar moieties. These were separated and studied separately. The aglycone A-1 was identified as 3,7-dihydroxy-5,3',4'-trimethoxy flavones (See in Experimental section).

The aqueous hydrolysate was neutralized with $BaCO_3$ and $BaSO_4$ filtered off. The filtrate was concentrated and subjected to paper chromatographic examination (using Whatman filterpaper No.1) and sugars were identified as D-xylose (R_f 0.28) and L-arabinose (R_f 0.21), L-rhamnose (R_f 0.37) (Co-PC).[28]

Quantitative estimation[29] of sugars revealed that all the three sugars were present in equimolar ratio 1:1:1. Periodate Oxidation [30] of compound a confirmed that all the sugars were present in the pyranose form. The position of sugars moieties in compound A were determined by permethylation[31] followed by acid hydrolysis yielded methylated aglycone identified as 3,7-dihydroxy-5,3',4'-trimethoxy flavone showed that glycosydation was involved at C-3 and C-7 positions of aglycone. The methylated sugars were identified as 2,3,4-tri-O-methyl-D-xylose [R_G 0.94], 2,3-di-O-methyl-L-arabinose [R_G0.64], 2,3,4-tri-O-methyl-L-rhamnose [R_G 1.01]. indicating that C-1″ of D-xylose was attached with -OH group at C-3 position of the aglycone and C-1⁗-OH of L- rhamnose was linked with C-4‴ -OH of L-arabinose, C-1‴ of L-arabinose was attached with -OH group at C-7 position of aglycone the inter glycosidic linkage (1→4) was found between L- rhamnose and L-arabinose.

Enzymatic hydrolysis [32] of compound A with almond emulsin enzyme liberated D-xylose indicating the presence of β-linkage between D-xylose and proaglycone. Proaglycone on further hydrolysis with takadiastase enzyme liberated L-rhamnose first followed by L-arabinose showed the presence of α-linkage between L- rhamnose and L-arabinose. Thus the compound A was identified as 3,7-dihydroxy-5,3',4'-trimethoxy flavone-3-O - β - D - xylopyranosyl- 7 - O – α - L-rhamnopyranosyl(1→4)-O-α-L-arabinopyranoside.

On the basis of above evidences, the structure of compound A was established as 3,7-dihydroxy-5,3',4'-trimethoxyflavone-3-O-β-D-xylopyranosyl-7- O - α - L - rhamnopyranosyl(1→4) – O – α - L-arabinopyranoside. The IC_{50} value of the compound A was 40.08μg/ml, as opposed to that of IC_{50} value of ascorbic acid was 52.90 μg/mL. Total antioxidant activity was also found to increase with increasing concentration, compound A exhibited higher radical scavenging activity in the 1,1-diphenyl-2-picryl-hydrazyl (DPPH) assay system.

CONCLUSION: The above results and evidences showed the presence of a new allelochemical (A) 3,7-dihydroxy-5,3',4'-trimethoxyflavone-3-O-β - D-xylopyranosyl-7-O-α-L-rhamnopyranosyl (1→4)-O-α-L-arabinopyranoside. along with two known compounds Scutellarin 7-O-β-D-glucopyranoside (B), Luteolin (C) have been isolated from the methanolic extract of the stems of *Cassia Siamea* Lam. Compound A showed good antioxidant activity in DPPH (1,1-diphenyl-2-picrylhydrazyl) assay system.

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