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A new biopotentially active isoflavone glycoside from the stem of *Ficus arnottiana* Miq.

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Abstract: A new bioactive isoflavone glycoside with a molecular formula $C_{34}H_{42}O_{20}$, melting point 261°C-263°C and $[M]^+$ 770 was isolated from the methanol soluble fraction of 90% methanolic extract of the stem of *Ficus arnottiana* Miq. The compound was illustrated as a new bioactive isoflavone glycoside 5,6,5ˈ-trihydroxy-8,3ˈ-dimethoxy-isoflavone 5ˈ-O-α-L-rhamnopyranosyl (1→3) O-α-L xylopyranosyl 5-O-β-D-glucopyranoside were characterized with the help of various chemical reactions, Fourier transforms infrared spectroscopy (FT-IR), Nuclear magnetic resonance spectroscopy (1HNMR), Mass spectrometry (LC-MS) and chemical degradations. Isolated compounds were also used for the antimicrobial activity of micro-organisms worked successfully tested against all strains: *Escherichia coli, Bacillus cereus, and Staphylococcus aureus,* while the least activity by *Pseudomonas aeruginosa*.

Keywords: *Ficus arnottiana* Miq.; Moraceae; Isoflavone glycoside; Antimicrobial activity; Micro-organisms.

1. Introduction

Medicinal plants have been used since the Vedic era [1]. For thousands of years, humans have been infected by bacteria, fungi, viruses, parasites [2], inflammation [3], cold [4], digestive problems [5], pain, and many other health disorders and diseases [6]. Treating infections and health disorders with herbal medicines is usually effective as it involves active natural products of low molecular weight with great structural diversity (so-called secondary metabolites), typical for all plants [7]. These secondary metabolites protect the cells from the damage caused by unstable molecules known as free radicals [8]. There is growing interested in using natural antimicrobial compounds especially extracted from plants, for the preservation of foods. Therefore, need to search for plants of medicinal value. Some are *O*-glycosides [9], Rutin [10], Kaempferol, Quercetin [11], Apigenin [12], etc., important flavonoids are known for their anti-inflammatory [13], anti-allergic [14], hepatoprotective [15], antibacterial [16] and anticancer properties [17].

The *Ficus arnottiana* Miq. belongs to the Moraceae family known as Paraspipal [18]. It is a native plant of India and Shri Lanka [19]. The leaves and bark of plants are used to treat infertility, inflammation, diarrhea, diabetes, burning, ulcers, scabies, wounds, and skin problem [20-23]. The present study deals with the separation, identification, and structural determination of a bioactive isoflavone glycoside 5,6,5ˈ-trihydroxy 8,3ˈ-dimethoxy isoflavone 5ˈ-O-α-L- rhamnopyranosyl (1→3) O-α-L xylopyranosyl 5-O-β-D-glucopyranoside with the help of various chemical reactions, spectroscopic analysis, and chemical degradations. A first-time isolation of these compounds was identified from *Ficus arnottiana* Miq stems.

2. Materials and Methods

2.1. General Experimental Procedure

The Thieleˈs tube apparatus (Borosil) was used to analyze melting point and are uncorrected. FTIR studies were made using Bruker Alpha II ECO-ATR Spectrophotometer by KBr disk method. 1HNMR spectra and 13CNMR studies were made on a Bruker DRX at 300MHz and 90MHz, respectively, using solvent DMSO, whereas, The Mass spectra were recorded on a Jeol SX-12 (EI) spectrometer. The UV spectra were recorded by Labindia UV/Vis, Spectrophotometer (UV 392).

2.2. Collection of Plant Material

The stem of *Ficus arnottiana* Miq. were collected from the Region of Shastra Dhara valley (3 17' 19.356" N and 78 3' 33.94" E), Dehradun (Uttrakhand, India), during the month of April and May (2019). The plants were authenticated by taxonomist Dr. Pradeep Tiwari, Department of Botany, Dr. H. S. Gour University,

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Sagar. Herbarium voucher specimen number (BOT. /H/8/21/1/2) has been deposited in the departmental herbarium.

2.3. Extraction and Isolation

The shade-dried and crushed stem of the *Ficus arnottiana* Miq. extracted by Soxhlet apparatus with different solvents according to their polarity, i.e., Petroleum ether (50°-60°C), C_6H_6, $CHCl_3$, EtOAc, and MeOH for 12 days. The methanolic extract was condensed using a rotatory evaporator under reduced pressure to yield a dark brown-colored sticky compound. The TLC examination reported two spots on the TLC plate with the help of a solvent system of n-BuOH: CH_3COOH: H_2O (8:2:10) and I_2vapours as visualizing reagent. Two spots from the TLC analysis indicated a mixture of compounds A and B. These compounds were purified and separated by silica-gel G using column chromatography and eluted with $CHCl_3$: MeOH in different ratios (60:40, 50:50, 40:60). Compound B was obtained in a very small amount (0.002), so it was not sufficient for further characterization. Crystallization of compound A using methanol resulted in 2.8 gm needles shaped copper brown colored crystal.

2.4. Study of compound A

Compound A was obtained as a copper brown crystalline solid having molecular formula $C_{34}H_{42}O_{20}$, melting point 261°–263°C, HRESI-MS [M-H]-769.221 Negative Mode, soluble in methanol.

UV-Visible band (λ_{max}) at 245 nm to 270 nm for isoflavone.

IR (KBr) υ_{max} observed at 3278–3520 cm^{-1} (-OH), 2920 cm^{-1} (C–H), 1612- 1710 cm^{-1} (C=O), 1513 cm^{-1} (ring compound), 1450-1012 cm^{-1} (O-glycosidic linkage), 913 and 838 cm^{-1} (aromatic compound).

1HNMR (300MHz-DMSO-d_6)

11.83 (1H, s, C-6 OH), 10.68 (1H, s, C-5 -OH), 9.08 (1H, s, C-5' OH), 3.94 (3H, s C-8 OMe), 3.83 (3H, s, C-3'OMe), 8.43 (1H, s, J.2:0 Hz; H-2), 7.36 (1H, s, J. 6:7Hz; H-7), 7.17 (1H, d, H-2'), 7. '6 (1H, d, H-4'), 7.28 (1H, d H-6'), 5.76 (2H, dd, H-1''), 5.28 (1H, dd, J . 8.2Hz; H-2'') 4.87 (1H, dd, J. 8.2; 10.9Hz; H-3''), 3.66 (1H, m J. 3.6; 9.8Hz; H-4''), 3.98 (1H, m, J 3.6Hz; H-5''), 3.86 (1H, dd, J. 7.0Hz; H-6''), 5.46 (2H, dd, J. 7.6 11.4Hz; H-1''') 5.97 (1H, d H-2'''), 5.02 (1H, dd, J. 7.6Hz; H-3''') 4.'8(1H, m, J .7.9, 8.6Hz; H-4'''), 3.63 (1H, m J 7.4Hz; H-5'''), 5.46 (1H, d, J .1.6Hz; H-1''''), 4.73 (1H, dd, H-2''''), 4.28 (2H, dd, J.5.8; Hz; H-3''''), 3.4' (1H, dd J 7.8Hz; H-4''''), 3.91 (1H, m, H-5''''), 3.23 (2H, dd, H-6'''').

13CNMR (90 MHz, DMSO-d_6)

152.5 (C-2), 135.7 (C-3), 168.7 (C-4), 144.9 (C-5), 11'.6 (C-6), 166.8 (C-7), 98.6 (C-8), 154.5 (C-9), 1'8.6 (C-1'), 124.5 (C-1'), 1'6.4 (C-2'), 142.6 (C-3'),132.4 (C-4'), 156.6 (C-5'), 11'.2 (C-6') 1'5.5 (C-1''), 69.9 (C-2''), 75.5 (C-3''), 7'.4 (C-4''),68.5 (C-5''),58.4 (C-6''),11'.6 (C-1'''), 75.6 (C-2'''), 69.8 (C-3'''), 66.7 (C-4''')72.7 (C-5'''), 1'4.7 (C-1'''') 88.6 (C-2''), 71.9 (C-3''), 68.7 (C-4),67.6 (C-5), 23.6(C-6'''').

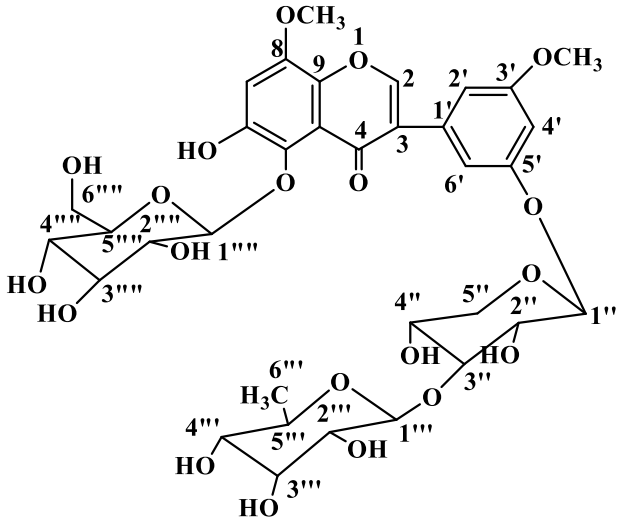

Figure 1. Structure of compound A (5,6,5' trihydroxy 8,3'-dimethoxy isoflavone-5'-O-α-L- rhamnopyranosyl (1→3) O-α-L xylopyranosyl 5-O-β-D-glucopyranoside)

2.5. Acid hydrolysis of compound A

In acid hydrolysis, 50mL of compound A dissolved in 20 mL MeOH hydrolyzed with 7.5 % of H_2SO_4 by refluxing for 4 hrs on a water bath and then concentrating the acidic solution by cooling. The residue was separated with Et_2O and chromatographed over silica gel G using solvent $CHCI_3$: MeOH (7:3) to give aglycone (Compound A-1). The aqueous hydrolysate was neutralized with $BaCO_3$, and the resulting $BaSO_4$ was removed. The sugar from the

aqueous mother liquor was recognized as L-xylose (R_f 0.66), D-glucose (R_f 0.43), and L-rhamnose (R_f 0.73) by paper chromatography [24]. Aglycon A-1 was identified as 5,6,5′-trihydroxy 8,3′-dimethoxy isoflavone.

2.5.1. Study of Aglycon A-1

Molecular formula $C_{17}H_{14}O_7$ was analyzed by m.p. 215°–218°C, (M^+) 330 (EIMS). Elemental analysis found (%) C, 61.81 %, H 4.27 %, O 33.91 %, Calculated found(%), 61.78 %, H 4.30 %, O 32.06 %.UV (MeOH) λ max (nm) 268, 308, IR (KBr) spectra showed absorption band 3448-3200 cm^{-1}, 1664 cm^{-1}, 1560 cm^{-1}, 1515 cm^{-1}.

1HNMR (300MHz-DMSO-d_6)

9.48 (1H, s, C-6 OH), 9.20 (1H, s, C-5 -OH), 9.07 (1H, s, C-5′ OH), 3.83 (3H, s C-8 OMe), 3.77 (3H, s, C-3′OMe), 6.63(1H, s, J.2:0 Hz; H-2), 3.22 (1H, s, J. 6:7Hz; H-4), 6.08 (1H, s, J. 6:7Hz; H-7), 6.40 (1H, d, H-2′), 6.30 (1H, d, H-4′), 6.38 (1H, d H-6′).

13CNMR (90 MHz, DMSO-d_6)

153.5 (C-2), 133.7 (C-3), 180.7 (C-4), 145.9 (C-5), 143.6 (C-6), 106.8 (C-7), 141.6 (C-8), 142.5 (C-9), 115.6 (C-10), 139.5 (C-1′), 121.4 (C-2′), 130.6 (C-3′),115.4 (C-4′), 158.6 (C-5′), 112.2 (C-6′).

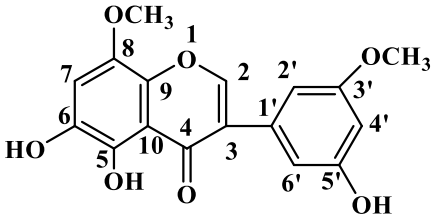

Figure 2. Structure of Compound A-1 (5, 6, 5′-trihydroxy 8,3′-dimethoxy isoflavone)

2.6. Permethylation of Compound A

A solution of compound A (8mg), MeI (10mL), and Ag_2O (34mg) was prepared in the presence of DMF (5 mL). The solution was refluxed at 37°C for 24 hrs and then filtered. The filtrate was concentrated under reduced pressure and hydrolyzed by 7% alcoholic H_2SO_4 for 6 hrs which filtrate methylated aglycone recognized as 5,5′-dihydroxy 6,8,3′-tri methoxy isoflavone and methylated sugars were recognized as 2,3,4-trimethoxy-L-rahmnose (R_G1.02) 2,4-dimethoxy-L-xylose (R_G 0.83) and 2,3,4,6 tetramethoxy-D-glucose (R_G 0.63) by paper chromatography.

2.7. Enzymatic hydrolysis of compound A

An excess ethereal method is used to hydrolyze 50mL of Compound A, with Takadiastase enzyme, in a conical flask at 37°C for 48 hrs. Examination of hydrolysate by paper chromatography using nBuOH: CH_3COOH: H_2O (8:2:10) as a developer and Ninhydrine as visualizing reagent, which showed the presence of L-rhamnose (R_f 0.72), L-xylose (R_f 0.66) showing α linkage with proaglycone [25]. The pro aglycone (50 mg) in MeOH was treated with an equal amount of almond emulsion solution, yielded D-glucose (R_f 0.43), showing β linkage between D-glucose and proaglycone [26].

2.8. Antimicrobial activity of compound A

2.8.1. Agar diffusion Method

The antimicrobial activity of compound A was determined by the agar diffusion method. Compound A showed an inhibitory effect on gram-positive and gram-negative bacterial strains. The stock solution of compound A has been prepared with methanol and further diluted with methanol to prepare the desired concentrations (25, 50, 75, and 100 µg/ml). Ciprofloxacin was used as a standard drug [27]. The bacterial strains were subcultured in Muller-Hinton agar and developed at 37°C for 22 hrs. Turbidity of the bacterial suspension has been used to the McFarland standard (0.5), and disinfected cotton swabs scrubbed in broth and then streaked on an MHA plate in a zigzag manner [28]. All experimental samples (25 µg/ml) were introduced on the disc (5 mm) and applied the disc on MHA plates, while the whole experiment was performed at 37°C for 18 hrs.

The antimicrobial activity was taken on the basis of the diameter of the zone of inhibition (mm). Experiments were conducted in triplicates. The percentage activity of the methanolic extract [Eq. 1] shows the total antimicrobial strength of the extract in particular.

$$\text{Activity } \% = \frac{(100 * \text{Num.})\ (\text{Total number of strains tested})}{\text{Number of susceptible bacterial strains extract}} \quad 1$$

The Index bacterial susceptibility (IBS) [Eq. 2] shows that the number of microbes liable to extract evaluated ranges from zero (conflict extract every sample test) to hundred (liable to total extract) [27].

$$\text{IBS} = \frac{100 * \text{Number of effective extracts for each bacterial strain}}{\text{Number of strain}} \quad 2$$

Table 1. Antimicrobial Activity of Compound A against Bacterial strains.

Microorganisms	Concentration of Compound A				Ciprofloxacin (Stand. drug)
	25 µg/ mL	50 µg/ mL	75 µg/ mL	100 µg/ mL	(10 µg/mL)
	Zone of inhibition (mm)				
E. coli	11.9 ± 0.03	13.5 ± 0.10	16.2 ± 0.06	20.8 ± 0.40	22.8 ± 0.15
Bacillus cereus	10.5 ± 0.05	14.8 ± 0.14	15.1 ± 0.05	18.1 ± 0.32	24.2 ± 0.12
S. aureus	8.3 ±0.05	9.6 ± 0.06	12.3 ± 0.13	16.8 ± 0.23	27.3 ± 0.91
P. Aeruginosa	9.2 ± 0.21	10.8 ± 0.08	14.01 ± 0.7	15.2 ± 0.17	23.1 ± 0.13

Notes: Data are expressed as Mean ± SEM. All values were taken in triplicates.

2.8.2. MIC Study

MICs of compound A were determined using the broth microdilution method. This method evaluates multiple dilutions twice in a disposable petri dish of 90 mm in diameter. In brief, desired bacterial cultures were activated by transferring a loopful of strains from stock cultures and inoculating them with Nutrient-broth (NB) medium for 24 hrs [29]. Bacterial cultures were diluted in fresh NB medium to a 100 g/ml concentration and then serially diluted to 50, 25, 12.5, 6.25, 3.12, and 0.78 g/ml. After that, 20µL of microbial suspension of each tube was inoculated in a Petri dish and incubated at 37°C for 20 hrs [30]. The results are recorded in Figure 3.

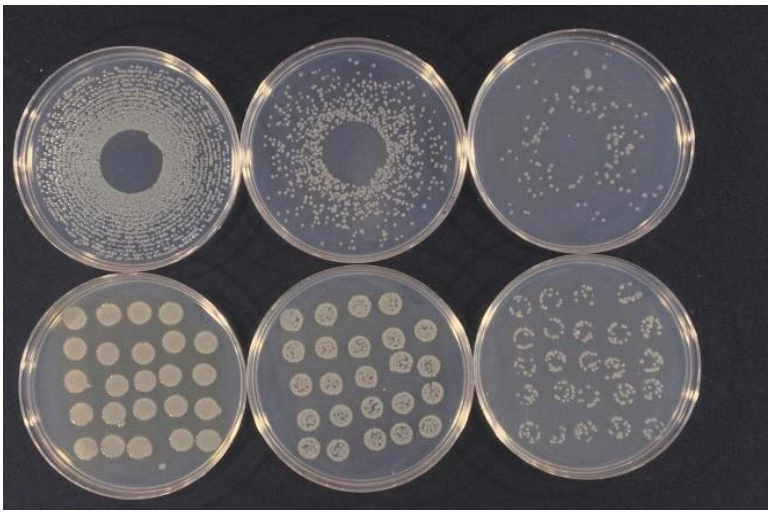

Figure 3.-Growth of E. coli on Nutrient Agar plates depicting MIC study

3. Results and Discussion

A new bioactive compound A, having molecular formula $C_{34}H_{42}O_{20}$, melting point 261–263°C, $[M]^+$ 770 (EIMS), was isolated from 90% methanolic extract stem of Ficus *arnottianan* Miq. Its UV-Vis. The spectrum showed an adsorption band (λ_{max}) at 245 nm to 270 nm for the presence of isoflavone. Its IR spectral data showed absorption band at 3278–3520 cm^{-1} (-OH), 1513 cm^{-1} (ring compound), 1450–1012 cm^{-1} (O-glycosidic linkage) 913 cm^{-1}, and 838 cm^{-1}(aromatic compound). The 1HNMR spectrum showed two singlets at δ 3.94, and 3.83, assigned to two OMe groups at C-8 and C-3' positions. Ananomric protons as one singlet at δ7.36 for the C-7 position in ring A and a doublet at δ 7.17, 7.06, and 7.28 for C-2', C-4', and C-6' position in ring B. One-proton singlets at 8.43 for C-2 in ring B. The anomeric proton signals at δ 5.68(1H, d, J 8.2 Hz), 5.42 (1H, d, J 7.6 Hz), and 5.98 (1H, d, J 2.6 Hz) were assigned to H-1'''H-1''''and H-1'''''of L-rhamnose L-xylose and D-glucose respectively. Coupling constant values at 8.2 and 7.6 Hz represent the β-linkage of L-rhamnose L-xylose, while a coupling constant value at 2.6 represents the α-linkage of D-glucose.

The methanol extract was found to be highly active against *E. coli* (ZI=21 mm), followed by *Bacillus cereus* (ZI=18 mm) and *Staphylococcus aureus (*ZI=17 mm), while least by *Pseudomonas aeruginosa* (ZI=15 mm). MIC study reveals that the highest antimicrobial activity

of methanol extract of *Ficus arnottiana* was observed for *E. coli*. The methanolic extracts were 50% effective for *S. aureus*, 75% for *Bacillus cereus and P. aeruginosa*, and 100% for *E. coli* in broad-spectrum analysis. The activity percentage was 100%, and the Index of bacterial susceptibility (IBS) was 82% in the various methanolic extracts.

4. Conclusion

Therefore, all the above facts and spectral analysis, which is the structure of compound A was identified as 5,6,5'-trihydroxy-8,3'-dimethoxy isoflavone-5'-O-α-L-rahmnopyranosyl (1→3) O-α-L xylopyranosyl 5-O-β-D-glucopyroanoside from the methanolic extract of the stems of the *Ficus arnottiana Miq.* Plant. MIC study reveals the highest antimicrobial activity of methanol extract of *Ficus arnottiana* Miq. observed in *E. coli*. Compound **A** showed significant antibacterial activity, and thus it may be used as a potent antibacterial agent against diseases caused by bacteria.

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