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Anti-inflammatory Activity of a New flavone glycosides from Ficus krishnae L.

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ABSTRACT

The stem of Ficus krishnaeisolated a new allelochemicals flavones glycosides3,7,4',5' tetrahydroxy 8,3 ' dimethoxy flavone 7-O-β-D rhamnopyranosyl (1→3) O-β-D galactopyranoside along with one well-known compound Kaempferol from methanolic extract. The present study deals with identification of a new bioactive compounds and characterized with the help of various chemical reactions, Fourier transform infrared spectroscopy (FT-IR), Nuclear magnetic resonance spectroscopy (^{1}HNMR), Mass spectrometry (ESI-MS) and chemical degradations. In vitro anti-inflammatory tests revealed significant anti-inflammatory effects besides the degradation of egg albumin. Compounds FKS illustrated the more percentage inhibitory action of protein denaturation at 800 µg/ml, and Diclofenac sodium used as a standard drug. As a result, Compound FKS inhibits protein denaturation more effectively than Diclofenac sodium at the same concentrations. Therefore, compound FKS may utilize as an anti-inflammatory source.

Keywords: Ficus Krishnae, Moraceae, Allelochemicals, Anti-inflammatory activity, Diclofenac sodium.

INTRODUCTION

Natural have been widely used as a significant sources of drugs and leads for treating various diseases. Their leading role is evident in the approximately 75% of drugs for infectious diseases that are either natural products or natural product derivatives[1]. In some diseases show the several effects on body, which involves the raise of vascular permeability, raise of protein denaturation, cell alteration and defensive response that is results in reddishness, ache, warmth, inflammation and defeat of function that occurs depends on the location and extent of injury[2]. It is a genuine problem of the rural areas people they used many anti inflammation drugs or substances produced by medicinal plants[3]. Anti-inflammatory agents block certain substances in the body that cause inflammation. They are used to treat many different conditions. Some anti-inflammatory agents are being studied in the prevention and treatment of cancer[4].

Ficus krishnae L. (Moraceae) is also called Makhan Katori in Hindi and Krishna's butter cup in English which is native plant to India and distributed in Sri Lanka and Africa as well[5].As a unique characteristic of the *Ficus krishnae* L. tree, the leaves have a pocket-like fold at the base and the leaf to have medicinal values to cure the disease like ulcers, vomiting, fever, inflammations, leprosy, syphilis, biliousness, dysentery, diabetes and inflammation of liverect[6]. There are bioactive compounds found in the stem bark of the plant, including phenols, flavonoids[7], and phytochemicals[8] that have anti-diabetic[9] and antihyperlipidemic properties[10]. In the present paper we report the isolated of a new biologically active flavone glycosides characterized as 3,7,4',5' tetrahydroxy 8,3' dimethoxy flavone 7-O-β-D rhamnopyranosyl (1→3) O-β-D galactopyranoside along with one well-known compound 3,4,5,7 tetrahydroxy flavone (Kaempferol) from methanolic extract of the stems of *Ficus krishnae* L. plant.

MATERIALS AND METHODS

General Experimental Procedure

The Thiele's tube apparatus (Borosil) was used for analysis of melting point and are uncorrected. FTIR studies were made using Bruker Alpha II ECO-ATR Spectrophotometer by KBr disk method. ^{1}H-NMR spectra and ^{13}C-NMR studies was made on a Bruker DRX at 300MHz and 90MHz, respectively using solvent DMSO. The Mass spectra were recorded on a Jeol SX-12 (ESI-MS) spectrometer. The UV spectra were recorded by Labindia UV/Vis, Spectrophotometer using spectroscopic MeOH.

Plant collection

The stem of the *Ficus krishnae* L. plant were collected locally from Department of Botany, Dr. H. S. Gour Vishwavidyalaya, Sagar, (M.P.) in the month December. The plant was authenticated by Taxonomist, Department of Botany, Dr. Hari Singh Gour Vishwavidyalaya, Sagar (M.P.) India. Herbarium voucher specimen (No.BOT./H/08/21/02/05) has been deposited in the Department of Chemistry.

ISOLATION OF THE COMPOUNDS

Dried and powdered stems of this plant (5.0 kg) were extracted with 95 % methanol in Soxhlet apparatus for 76 hrs. The methanolic extract of the stems of the plant was concentrated under reduced pressure to give viscous mass. The methanol soluble fraction was concentrated using a rotatory evaporator under reduced pressure to give brown coloured compound (3.82 gm). It gave two spots on TLC examination indicating it to be mixture of two compounds. These were separated by TLC and purified by column chromatography over silica gel using $CHCl_3$: MeOH as solvent in different proportions (7:3, 5:5, 3:7).On removal of the solvent, it gave crystalline Compound **FKS** (1.85 gm) which was found to be homogeneous on TLC examination.

EXPERIMENTAL ANALYSIS

STUDY OF COMPOUND-FKS

Compound-FKS was obtained as a light brown crystalline solid having molecular formula $C_{29}H_{34}O_{17}$ yielded 1.85 gm, melting point 228-230^{0}C, [M^+] 654 (EI-MS); found (calculated) (%): C 53.46(53.21), H 5.18(5.24), O 41.55(40.65).

UV : λ_{max} MeOH 265, 330 nm.

FT-IR (KBr) : ν_{max} 3350 (OH), 2780, 1725 (α, β-unsaturated >C=O group), 1615(Aromatic ring system), 1234, 1036, 959, 895 cm^{-1}

^{1}HNMR (300MHz, DMSO-d6) δ (ppm);

10.68 (1H, s, 3-OH), 9.38(1H, s, 4'-OH,5'-OH), 3.71 (3H,s, 8-OCH_3), 3.83 (3H,s, 3'-OCH_3),7.71(1H,d , *J* =8.0 Hz, 5-H,),6.73(1H,d , *J* =7.8 Hz, 6-H),), 6.23 (1H, d, *J*=7.2 Hz, 2'-H),6.20(1H, d, *J*=6. 1 Hz, 6'-H) 5.80(1H, dd, *J*=5.8 Hz, H-1''),3.19 (1H, d, H-2''),3.16-4.08 (3H, m, H-3'', H-4'', H-5''), 1.96(3H, s, *J* =6.4 Hz, CH_3-6''),5.40 (1H, dd, *J* =1.25Hz, H- 1'''), 3.55-4.78 (3H, m, H-2''', H-3''', H-4'''), 4.85 (1H,d , *J* =8.7 Hz, H-5'''),

^{13}CNMR (90 MHz, DMSO-d6), δ(ppm);

161.3 (C-2), 150.2(C-3), 182.6 (C- 4), 113.6(C-5), 101.1 (C-6), 164.3 (C-7) , 127.8 (C-8), 61.9 (8-OCH_3), 144.8 (C-9), 112.9 (C-10),118.5 (C-1'), 100.2(C-2'), 152.9(C-3'), 123.9(C-4'), 146.2 (C-5'), 102.6 (C-6'), 56.1 (3'-OCH_3), 82.1 (C-1''), 70.3 (C-2''), 76.9(C-3''), 69.1(C-4''), 79.7 (C-5''), 66.8(C-6''),99.3 (C-1'''), 71.4(C-2'''), 70.5(C-3'''), 72.7(C-4'''), 73.8(C-5'''), 17.0(C-6''').

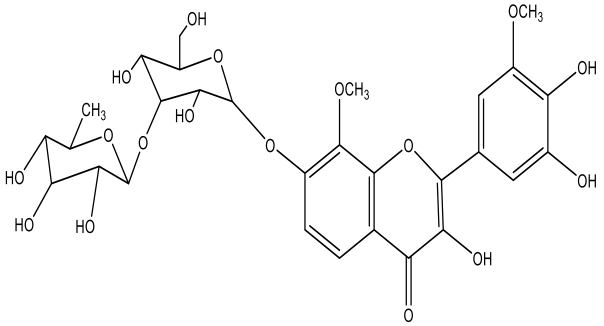


Fig. 1 Structure of Compound FKS

Acid Hydrolysis of Compound FKS

The compound **FKS** was refluxed with 25 ml of ethanol and 10% H_2SO_4in 150 ml round bottomed flask for 6 hrs. The contents were concentrated and allowed to cool and filtered. The residue was recrystallized from chloroform to give light red amorphous powder (228 mg) as aglycone identified as a 3,7,4', 5' tetrahydroxy- 8, 3', dimethoxy flavone. Concentrated filtrate was subjected to paper chromatography nBAW (6:1.5:7.5) as developer and Ninhydrin as visualizing reagent, showed the presence of D-rhamnose (R_f 0.73), D - galactose (R_f 0.39).

Permethylation of compound FKS

In permethylation a mixture of compound FKS (8mg), MeI (10 mL) and Ag_2O (60 mg) was kept refluxing in presence of DMF (20 ml) for 2-3 days and then pass through a filter. The filtrate was dried in vacuum and hydrolyzed with 7% methanolic H_2SO_4 for 6-8 h. The methylated aglycone identified a 7-hydroxy- 3,8, 3', 4', 5' pentamethoxy flavone and methylated sugar, , which confirmed that glycocylation was involved at C-7 -OH position of the aglycone and methylated sugars identified as 2,4,6-tri-O-methyl-D-galactose (R_G 0.62) and 2,3,4,- tri-O-methyl D-rhamnose (R_G1.02),(by Co-PC).

Enzymatic Hydrolysis of Compound FKS

Compound FKS was treated with 20 mL MeOH and then hydrolyzed the same quantity of Takadiastase enzyme at 37^0C for 48 hrs and no reaction, which showed the absence of sugar in L-position. The proaglycone was treated with MeOH (20mL) and hydrolyzed with same quantity of almond emulsion yielded D-galactose(R_f 0.39), D-rhamnose (R_f 0.73) and aglcone.

Therefore, it was confirmed that C-1'''-OH of D-rhamnose was linked with C-3''-OH of D-galactose and C-1'' -OH D-galactose of attached with C-7-OH of the aglycone.

STUDY OF THE AGLYCONE (FKS-1)

It was analyzed for molecular formula $C_{17}H_{14}O_8$, melting point 198-200^0C, $[M^+]$ 346 (EIMS).It was crystallized from acetone to give lemon yellow amorphous powder (228 mg). It was found to be homogeneous on TLC examination. It responded all the characteristic color reactions of flavonoids. found (calculated) (%): C 53.46(53.21), H 5.18(5.24), O 41.55(40.65). UV: λ_{max} MeOH 260, 345

FT-IR (KBr) : ν_{max} 3348 (OH), 2775, 1720 (α, β-unsaturated >C=O group), 1608(Aromatic ring system), 1230, 1040, 943, 885 cm^{-1}

^{1}HNMR (300MHz, DMSO-d6) δ (ppm);

10.46 (1H, s, 3-OH), 9.48(1H, s, 7-OH), 3.71 (3H,s, 8-OCH_3), 7.54(1H, s, 5-H,), 6.79(1H,s, 6-H),), 6.23 (1H, d, *J*=2.0 Hz, 2'-H), 6.20(1H, d, *J*=8.2 Hz, 6'-H) 8.73(1H, d, *J*=8.1 Hz, 4'-OH), 9.48 (1H, s, 5' -OH), 3.86 (3H,s, 3' -OCH_3)), 6.02 (1H, d, *J*=2.0 Hz, 1''-H)3.16-4.08 (3H, m, H-2'', H-3'', H-4'', H-5''), 1.96(3H, s, *J* =6.4 Hz, CH_3-6''),5.40 (1H, dd, *J* =1.25Hz, H- 1'''), 3.55-4.78 (3H, m, H-2''', H-3''', H-4'''), 4.85 (1H,d , *J* =8.7 Hz, H-5''').

^{13}CNMR (90 MHz, DMSO-d6), δ(ppm);

146.9 (C-2), 136.5(C-3), 172.6 (C- 4), 120.5(C-5), 115.1 (C-6), 156.1(C-7) , 134.6 (C-8), 60.8 (8-OCH_3), 150.5(C-9), 114.6 (C-10),126.7 (C-1'), 104.5(C-2'), 148.4(C-3'), 134.7(C-4'), 145.7 (C-5'), 107.6 (C-6'), 56.1 (3'-OCH_3), 82.1 (C-1''), 70.3 (C-2''), 76.9(C-3''), 69.1(C-4''), 79.7 (C-5''), 66.8(C-6''),99.3 (C-1'''), 71.4(C-2'''), 70.5(C-3'''), 72.7(C-4'''), 73.8(C-5'''), 17.0(C-6''').

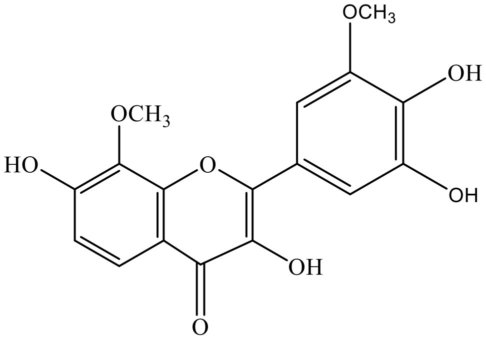


Fig. 2 Structure of Aglycone

STUDY OF KNOWN COMPOUND

The 0.30 gm of compound was crystalline in acetone. It has molecular formula $C_{15}H_{10}O_6$, melting point $203-210^0C$ $[M^+]$ 286 (EMIS) found (calculated) %: C 63.84(63.45), H 3.12(3.10), O 33.06(34.40). UV λmax (nm) +:(MeOH) 264, 338(+NaOCH3) 261, 385 (+$AlCl_3$) 274, 312, 356, 402. IR (KBr) υ_{max} 3454, 1638, 1608, 1105, 842. ^{1}HNMR (300MHz, $CDCl_3$) δ (ppm); 5.24 (1H, d, *J*=2.0Hz, 6-H), 5.40(1H,dS , *J*=1.2Hz, 5-H), 12.8(1H, s, 3-OH), 5.50(1H, s, 5-OH),5.35(1H, s, 7-OH), 7.62(2H, d, *J* 9.0 Hz, 2'-Hand 6'-H), 6.60(1H, d, *J* 8.2 Hz, 3'-H and5'-H), 5.64(1H, s, 3'-OH).^{13}CNMR (90MHz, DMSO-d6), δ(ppm), : 146.9 (C-2), 136.5(C-3), 176.1(C-4), 161.8(C-5), 98.3 (C-6), 166.4(C-7),94.0 (C-8), 158.8(C-9), 104.5(C-10), 122.9 (C-1'), 129.2(C-2' and C-6') , 115.8(C-3' and C-5'), 157.7(C-4'). Then one well-known compound 3,4,5,7 tetrahydroxy flavones Compound B (Kaempferol) was identified from methanolic extract and similarity of its spectral data with reported literature [15-31].

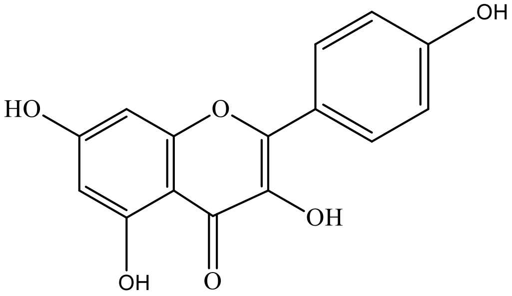


Fig. 3 Kaempferol

DETERMINATION OF ANTI-INFLAMMATORY ACTIVITY OF COMPOUNDS FKS

The anti-inflammatory activity of Compound FKS was studied by egg albumin denaturation technique[11]followed with slight conversion. 1.05 ml of egg albumin 2.25 ml of phosphate buffered saline (PBS, pH 6.5) and 1.70 ml of standard drug Diclofenac sodium mix together for prepared 5ml of reaction mixture. After that different concentrations of Compound FKS to assemble the ultimate concentration of (50, 100, 200, 400, 800μg/mL) respectively. Diclofenac sodium used as a standard drug and same quantity of mineral water has been used as control. Whole the reaction mixture were incubated at room temperature for 15 min. and then warm up at 60^0C for 10 min. After cooling, their absorbance of the turbidity is measured at 660nm by Systronic-2201 UV-Visible Double Beam Spectrophotometer. The test analysis was carried out in triplicate. The % inhibition of protein denaturation was calculated using following formula[12]:

$$\%\ Inhibition = \frac{(At - Ac)}{Ac} * 100$$

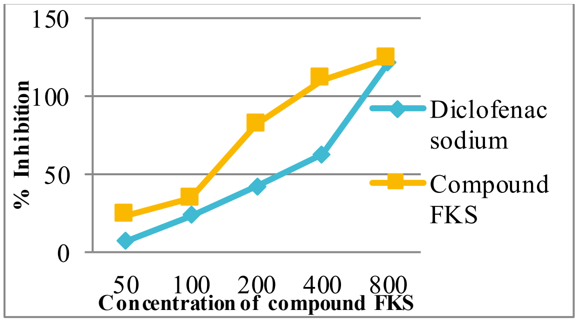
Where Ac = Absorbance of control, At = Absorbance of test sample. Absorbance of control=0.322

ANTI INFLAMMATORY ACTIVITY OF COMPOUND III (FKS)

Conc.	Diclofenac sodium		Compound FKS	
	Abs.	% Inhibition	Abs.	% Inhibition
50	0.344	6.83	0.395	20.45
100	0.398	23.60	0.432	34.16
200	0.456	41.61	0.586	81.98
400	0.524	62.73	0.679	110.86
800	0.712	121.11	0.722	124.22

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

Fig. 4:Anti inflammatory activity by egg albumin denaturation



RESULT AND DISCUSSION:

It was analysed for molecular formula $C_{29}H_{34}O_{17}$ yielded 1.85 gm, melting point 228-230^{0}C, $[M^+]$ 654 (EIMS). It responded to positive Molisch and Shinoda tests indicating glycosidic nature as well as various characteristics colour reaction for flavonoids. In UV spectrum band at 265 and 330 nm confirmed for flavonoid glycoside. In IR spectrum peak at ν_{max}^{KBr} 3350cm^{-1} , 2780 cm^{-1} showed the presence of hydroxyl group(s) and methoxy group(s) respectively.1725 cm^{-1} showed the presence of α, β-unsaturated >C=O group. ^{1}H NMR spectrum showed three singlets at δ 10.68, 9.38 and 9.08 for OH groups at C-3, C-4' and C-5' position. Two singlets at δ 3.71 and 3.83 were assigned for -OMe group at C-8 and C-3' position. In ^{1}HNMR spectrum of the compound FKS given four doublet at δ 7.71 , δ 6.73, δ 6.23 and δ 6.20 was assigned to H-5 , H-6, H-2' and H-6' in ring A and B. The anomeric proton at δ 5.80 (d, *J =5.8* Hz, 1H) and δ 5.40 (d, *J*= 1.25 Hz, 1H) were assigned for H-1", H-1'"of D- galactose and D- rhamnose respectively. In the MS of compound FKS, characteristic ion peaks at *m/z* 654 $[M^+]$, 508 $[M^+]$ and 346$[M^+]$ were shown by consequent losses from the ion of D- rhamnose, and D- galactose was linked to aglycone at C-7 position.

In permethylation a mixture of compound FKS separated methylated aglycone and sugar. Methylated aglycone identified as a 7-hydroxy-3,8, 3', 4', 5' pentamethoxy flavone and and methylated sugars identified as a 2,4,6-tri-O-methyl-D-galactose (R_G 0.62) and 2,3,4,- tri-O-methyl D-rhamnose (R_G1.02),(by Co-PC). Therefore, it was concluded that C-1'"- OH of the D-rhamnose was linked with C-3"-OH of D-galactose and C-1"-OH of D-galactose was attached with C-7-OH of aglycone, which confirmed that interlinkage(1→3) was present between D-galactose and D-rhamnose ,and also showed that both the sugars were present in pyranose form[39].

Enzymatic hydrolysis of compound FKS by almond emulsion enzyme , confirming the presence of β-linkage between D-galactose (Rf 0.39) and D-rhamnose(Rf 0.73) as well as between D-galactose and aglycone. Therefore, it was confirmed that C-1'''-OH of D-rhamnose was linked with C-3''-OH of D-galactose and C-1'' -OH D-galactose of attached with C-7-OH of the aglycone.

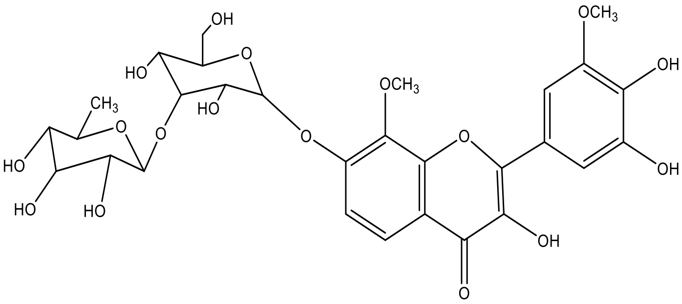


Fig. 5 Structure of Compound FKS

In vitro anti-inflammatory tests revealed significant anti-inflammatory effects besides the degradation of egg albumin. Compounds FKS illustrated the high percentage inhibitory action of protein denaturation at 800 µg/ml, and Diclofenac sodium also demonstrated inhibitory effect at same concentration as a standered. As a result, Compound FKS inhibits protein denaturation more effectively than Diclofenac sodium. Therefore, compound FKS may utilize as an good anti-inflammatory source.

CONCLUSION

Therefore, on the basis of all above facts, proved that compound FKS 3,7,4',5' tetrahydroxy 8,3' dimethoxy flavone 7-O-β-D rhamnopyranosyl (1→3) O-β-D galactopyranoside along with one well-known compound 3,4,5,7 tetrahydroxy flavone (Kaempferol) from methanolic extract of *Ficus krishnae* L. Compound FKS which have been shown to be bio potentially effective as can be used as natural alternative preventives to anti inflammatory activity.

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