Isolation and Antimicrobial Studies of the Compounds Isolated from the Stem Bark of *Ficus hispida* Linn.

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Three new glycosides isolated from chloroform and methanol extracts of the stem bark of *F. hispida* Linn which is widely used in Manipur, India for controlling *Diabetes mellitus*, as 3',4',5',5,7-pentamethoxy-4-acetyldelphinidin-3-O-α-L-rhamnoside (**2**) 4',5,7-trimethoxy pelargonidin-6-C-glucopyranosyl-3-O-α-L-rhamnoside (**3**) and 3',4',5',5,7-pentamethoxy delphinidin-3-O-α-L-rhamnoside (**4**) along with known compounds have been reported. A new ketoester, 24-ketopentacosyl-γ-hydroxypentanoate (**1**) has also been isolated from the petroleum extract. Compound **1** showed high inhibitory effect on the growth of coliform organism and compound **2** had antibiotic property in penicillium species.

Key Words: *Ficus hispida* Linn., *Diabetes mellitus*, Pelargonidin, Delphinidin, Hypoglycemic, Coliform.

INTRODUCTION

Ficus hispida Linn (Moraceae)[1] is a genus of about 800 species are found in most parts of India. The stem bark of the plant is used traditionally by the people of Manipur, India for treatment of *Diabetes mellitus*[2]; when the decoction of the bark is used, the person is relieved from tiredness and repeated urination and it controls the increase in blood glucose level. All parts of the plant are astringent to the bowels and useful in biliousness, *psoriasis anaemia*, pile and jaundice[3]. The chemical constituents of the plant have not been investigated, although hypoglycemic effect of its stem bark and the antidiarrhoeal activity of leaf extract were already observed[4,5].

EXPERIMENTAL

Melting points were determined in open capillary tubes and are uncorrected. UV-spectra were recorded on a Shimadzu 1201. IR-spectra were recorded on an ATF Mattson Genesis FTIR spectrometer; low resolution CI and EIMS were recorded on a Fisons TRIO 2000 quadrupole mass spectrometer. FAB-MS were recorded on a VG-Autospec 3000 mass spectrometer; ^{1}H NMR spectra on Varian Unity (500MHz), Bruker AC-300 and Varian XL (300 MHz) spectrometer and ^{13}C NMR spectra on Bruker AC-300 and Varian XL (75 MHz) spectrometers.

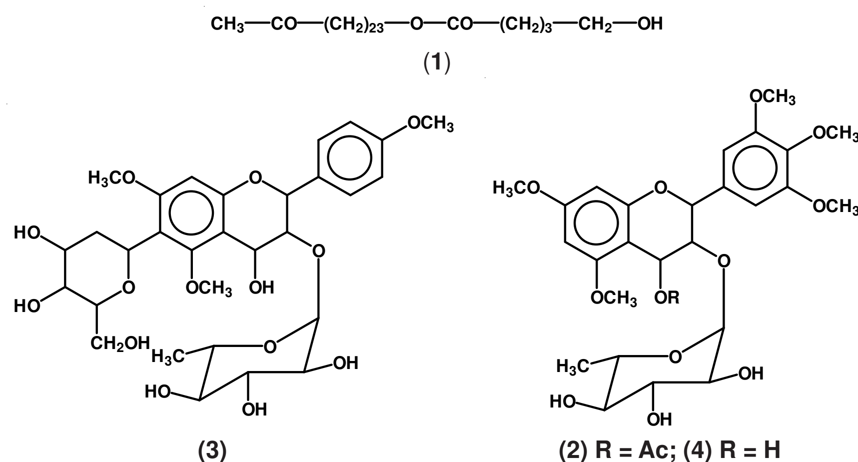
Isolation of compounds from crude extract

The collection of the barks of *Ficus hispida* was done during July-November 2006 from various parts of Manipur. The barks were cut into small pieces and air dried. The air-dried barks (2.25 kg) were extracted first with light petroleum ether using Soxhlet apparatus for 20 h and the solvent was evaporated using rotary vacuum evaporator at a temperature of 30-40 ºC to give a crude mass (0.54 kg). The crude mass was fractionated and purified by silica gel (60-120 mesh) column chromatography using petroleum ether, EtOAc and MeOH. Altogether eighteen fractions were collected and most of the compounds were found to be hydrocarbons, fats and oils which are known. Lupenyl acetate, 10-ketotetracosyl arachidate, 24-ketopentacosan-1-ol and β-sitosterol were also isolated from the petroleum ether extract and compound, 24-ketopentacosyl-γ-hydroxypentanoate (**1**) was isolated with petroleum ether- ethyl acetate (1:4 v/v).

The plant residue of the petroleum ether extract was extracted with $CHCl_3$ using Soxhlet apparatus (22 h) and after evaporation in rotary vacuum evaporator produced 0.58 kg crude mass. The crude mass was then separated and purified by passing through a column packed with silica gel and eluted with petroleum ether, EtOAc, $CHCl_3$ and MeOH mixture in increasing order of polarity. 32 Fractions were isolated. Finally, fractionation of the column on silica gel using petroleum ether-EtOAc (75:25 v/v) produced the compound, 3',4',5',5,7-pentamethoxy-4-acetyldelphinidin-3-O-α-L-rhamnoside (**2**).

Finally, the residue of $CHCl_3$-extract was extracted with MeOH and concentrated under vacuum to give a crude mass (0.70 kg). The crude mass was partitioned between H_2O and *n*-BuOH to remove water soluble inorganic salts and free sugars. The *n*-BuOH fraction (0.13 kg) on repeated chromatographic purification over a column of silica gel led to the isolation of two compounds, 4',5,7-trimethoxy pelargonidin-6-C-gluco-pyranosyl-3-O-α-L-rhamnoside (**3**) and 3',4',5',5,7-pentamethoxy delphinidin-3-O-α-L-rhamnoside (**4**) (**Scheme-I**). The *n*-BuOH extract was first extracted with petroleum ether, then with a gradient of petroleum ether-EtOAc with increasing EtOAc. A fraction collected from EtOAc-petroleum ether (25:75 v/v) elution yielded compound **3**. Compound **4** was obtained by elution with MeOH-$CHCl_3$ (5:95 v/v) as eluent.

Acid hydrolysis of compounds **2** and **4** were performed as described in the literature[8], each compound was treated with 2 N HCl (1 h of reaction time at 100 ºC) and after cooling, the reaction mixture was extracted with ether. Sugars in the neutralized acidic phase were analyzed on TLC and compared with authentic samples. The aglycone residues were identified by comparison with authentic samples.



Scheme-I

Antimicrobial activities fo the unknown compounds

The biological activities in terms of the antifungal and antibacterial properties of the crude as well as the compounds isolated from the plant *Ficus hispida* were determined by the standard disc diffusion method[9]. The bacteria and fungi were grown in nutrient agar and potato dextrose agar (PDA) plates, respectively. The compounds to be tested were dissolved in DMSO to a final construction of 1 % and soaked in filter paper discs of 10 mm diameter and 0.5 mm thickness. The filter papers (Whatmann filter paper no. 42) were found to hold a quantity of 0.05 mL solution. These discs were placed on the already seeded plates (of test organisms with an appropriate density of 1×10^6 CFU/mL) and incubated at 27 ± 0.2 ºC for a period of 24 h. A clearing around the disc indicated the inhibitory activity of the compound on the organism.

RESULTS AND DISCUSSION

The concentrated petroleum extract of the bark of *Ficus hispida* was fractionated by successive elution with petroleum ether and ethyl acetate. Compound **1** was isolated with petroleum ether-ethyl acetate (1:4 v/v) and purified. The compound **1** was identified as 24-ketopentacosyl-hydroxy-pentanoate.

Compound **1** ($C_{30}H_{58}O_4$) showed a parent ion at m/e 482, base peak 409. Its IR-spectrum showed strong absorption of hydroxyl groups at 3451, 1735 and 1639 cm^{-1}. ^{1}H NMR spectrum showed triplets at 4.05 (2H, CH_2-O-), at 2.38 (2H, CH_2-CO-) and at 2.29 (2H, CH_2-CO-) and its ^{13}C NMR spectrum exhibited peaks at 173.6 and 170.9 due to carbonyl carbon atoms. These facts indicated that compound **1** was 24-ketopentacosyl-γ-hydroxy pentanoate, confirmed by alkali hydrolysis and by comparing with the authentic compound.

24-Ketopentacosyl-γ-hydroxy pentanoate (1): White solid, m.p. 210-212 ºC; IR (KBr, ν_{max}, cm^{-1}): 3451, 1735, 1639; Mass (m/z, %): 482 (M^+, 5), 468 (50), 409 (100), 365 (2), 351 (2); ^{1}H NMR ($CDCl_3$): δ 5.53 (2H, t, *J* = 8.4 Hz, CH_2-O-CO-), 4.52 (2H, t, *J* = 7.6 Hz, CH_2OH), 4.05 (2H, t, *J* = 8.0 Hz, CH_2CO-O-), 2.37 (2H, t, *J* = 7.9 Hz, CH_2CO), 2.31 (2H, t, *J* = 8.1 Hz, CH_2CO), 0.90 (3H, t, *J* = 4.8 Hz, CH_3); ^{13}C NMR ($CDCl_3$): δ 170.9, 139.6, 124.3, 80.9, 59.0, 55.4, 53.9, 50.0, 47.6, 42.0, 41.5, 40.7, 38.4, 37.7, 35.0, 32.8, 31.2, 29.7, 28.2, 27.3, 26.6, 23.6, 23.2 21.3, 20.7, 18.1, 17.5, 16.3, 15.7, 13.7.

The crude mass of chloroform extract of the bark was fractionated by successive elution with petroleum ether, ethyl acetate, chloroform and methanol. Compound **2** was obtained by elution with chloroform-methanol (4:1 v/v) mixture as brown powder. Compound **2** ($C_{28}H_{36}O_{13}$) gave positive test for reducing sugars and identified as L-(–)-rhamnose and the aglycone as delphinidin after demethylation by conventional methods and showed methoxyl groups at positions 5 and 7 of ring. Its mass spectrum showed peak at m/e 580 (M^+), 538 (M-acetyl) and 433 (M-sugar). IR spectrum showed absorption at 3451, 3454, 3457, 2918, 1713 and 1262 cm^{-1}. The ^{1}H NMR and ^{13}C NMR spectral data were similar to those of delphinidin-3-O-α-L-rhamnoside isolated from *Ficus bengalensis*[3], except for lacking the signals due to the acetyl group and two extra methoxyl groups. Peaks at 0.98 and 1.06 were due to methyl groups of acetyl and rhamnopyranosyl, respectively. Its ^{13}C NMR spectrum exhibited peaks at 170.9 due to carbonyl C-atom and 13.2 and 15.7 due to methyl groups.

Compound **2** consumed two moles of periodate and produced one mole of HCOOH per mole of glucoside showing that rhamnose was present in the pyranose form. The glycoside was not hydrolyzed by enzyme emulsion showing that an α-linkage was present. Thus, from the spectral data and chemical studies, it could be concluded that compound **2** was 3',4',5',5,7-pentamethoxy-4-acetyl-delphinidin-3-O-α-L-rhamnoside which is an unknown compound.

3',4',5',5,7-Pentamethoxy-4-acetyl-delphinidin-3-O-α-L-rhamnoside (2): Reddish-brown powder, m.p. 120-125 ºC; IR (KBr, ν_{max}, cm^{-1}): 3457, 3454, 3451, 2918, 1713, 1467, 1262, 826, 730, 721; Mass (m/z, %): 581 (M+1, 40), 580 (M^+, 25), 538 (M-acetyl, 10), 433 (M-sugar, 15), 419 (20), 405 (5), 391 (55), 307 (65); negative FAB-MS m/z: 579.0950 $[M-H]^-$ (Calcd. for $C_{28}H_{36}O_{13}$: 579.0970); ^{1}H NMR ($CDCl_3$): δ 6.77-6.82 (2H, m, 2′-H, 6′-H), 6.17-6.22 (2H, m, 6-H, 8-H), 5.55 (1H, m, 4-H), 4.88 (1H, m, 3-H), 4.12 (1H, d, *J* = 6.7 Hz, 2-H), 3.43-3.78 (15H, m, 5xOCH_3), 3.12-3.24 (5H, br s, sugar protons), 1.06 (3H, s, CH_3), 0.98 (3H, s, CH_3); ^{13}C NMR ($CDCl_3$) δ: 170.9, 129.8, 121.3, 75.2, 71.5, 56.1, 51.4, 49.5, 45.2, 42.8, 41.7, 40.3, 39.2, 38.2, 36.7, 34.1, 32.1, 31.3, 29.1, 28.7, 27.6, 25.4, 24.7, 23.8, 22.6, 18.8, 15.2, 13.2.

The crude mass of methanolic extract was fractionated with *n*-BuOH treatment. The *n*-BuOH extract was subjected to column chromatography on silica gel to yield compounds **3** and **4**. Compound **3** was identified as 4',5,7-trimethoxy-pelargonidin-6-C-glucopyranosyl-3-O-L-rhamnoside on the basis of acid hydrolysis, spectral data (UV, ^{1}H NMR, ^{13}C NMR and FAB-MS) and comparison with published data.

4',5,7-Trimethoxy-pelargonidin-6-C-glucopyranosyl-3-O-α-L-rhamnoside (3): Reddish brown solid, m.p. 215-218 ºC; IR (KBr, ν_{max}, cm^{-1}): 2912, 1467, 1380, 1074, 1023, 826, 730, 721; Mass (m/z, %): 661 (M+1, 5), 660 (M^{+}, 15), 391 (65), 307 (45); negative FAB-MS m/z: 659.0716 $[M-H]^{-}$ (Calcd. for $C_{28}H_{36}O_{13}$: 659.0712); ^{1}H NMR ($CDCl_3$) δ: 6.77, 6.82 (2H, m, 2′-H, 6′-H), 6.17-6.22 (1H, m, 8-H), 5.55 (1H, m, 4-H), 4.88 (1H, m, 3-H), 4.12 (1H, d, *J* = 7.7 Hz, 2-H), 3.43-3.78 (15H, m, 9xOCH_3), 3.12-3.24 (5H, br s, sugar protons), 0.84 (3H, s, CH_3); ^{13}C NMR ($CDCl_3$): δ 129.5, 80.9, 55.7, 44.4, 40.0, 39.8, 39.5, 39.2, 38.0, 34.4, 31.9, 31.1, 30.8, 29.7, 29.3, 28.7, 28.0, 27.2, 25.9, 25.2, 23.6, 23.3, 22.4, 22.0, 18.5, 18.0, 16.8, 16.1, 15.4, 13.8.

Compound **4** was obtained as dark brown amorphous compound by elution with MeOH-$CHCl_3$ (5:95 v/v). The ^{1}H NMR and ^{13}C NMR spectral data were similar to those of compound **2**, except for the absence of the acetyl group. The structure of compound **4** was established as 3',4',5',5,7-pentamethoxy-delphinidin-3-O-α-L-rhamnoside.

3',4',5',5,7-Pentamethylethoxy-delphinidin-3-O-α-L-rhamnoside, (4): Dark-brown solid, m.p. 201-205 ºC; IR (KBr, ν_{max}, cm^{-1}): 3452, 3448, 3442, 3435, 2851, 1469, 1374, 1178, 803, 772, 722; Mass (m/z, %): 539 (M+1, 10), 538 (M^{+}, 20), 391 (50), 307 (75); negative HR FAB-MS m/z: 537.0603 $[M-H]^{-}$ (Calcd. for $C_{26}H_{34}O_{12}$: 537.0607); ^{1}H NMR ($CDCl_3$): δ 6.47-6.50 (2H, m, 2′-H, 6′-H), 5.88-5.94 (2H, m, 6-H, 8-H), 5.34 (1H, m, 4-H), 4.42 (1H, m, 3-H), 4.12 (1H, d, *J* = 6.9 Hz, 2-H), 3.29-3.73 (15H, m, 5xOCH_3), 2.39-2.91 (5H, br s, sugar protons), 0.84 (3H, s, CH_3); ^{13}C NMR ($CDCl_3$): δ 124.3, 70.3, 59.0, 55.2, 53.9, 47.6, 42.0, 41.5, 40.7, 39.6, 39.2, 38.4, 37.7, 35.0, 32.8, 31.2, 29.7, 28.2, 27.8, 26.6, 23.7, 21.4, 20.7, 18.2, 17.5, 14.11.

The results of antibacterial and antifungal studies are given in the Table-1. Compound **1** was found to have the highest activity against coli form organisms whereas other compounds were moderated and have less activity. The high inhibitory effect against the growth of coli form organisms is the important cause of anti-dysenteric effect of *Ficus hispida*. The studied compounds were found to be inactive against some species of fungi, except Compound **2** was found to have antibiotic property in penicillium species.

TABLE-1
ANTIBACTERIAL AND ANTIFUNGAL ACTIVITIES DATA OF COMPOUNDS (**1-4**) ISOLATED FROM THE STEM BARK OF *Ficus hispida* Linn

Test organism/compd.	DMSO	**1**	**2**	**3**	**4**	Crude
Fungal culture (zone in mm)						
Penicillium sp	11	11	12	11	11	11
Trichoderma sp	11	11	11	11	11	11
Fusarium sp	11	11	11	11	11	11
Aspergillus sp	11	11	11	11	11	11
Mucor SP	11	11	11	11	11	11
Bacterial culture (zone in mm)						
E. coli	11	15	14	12	12	13
Bacillus species	11	11	11	11	12	11

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