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Purification, Characterization and Cytotoxic Activity of Two Flavonoids from *Oroxylum indicum* Vent. (Bignoniaceae)

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Abstract: Phytochemical examination of the ethyl acetate extract of root parts of *Oroxylum indicum* Vent. has led to the isolation of two flavonoid: 2,5-dihydroxy-6,7-dimethoxy flavone and 3,7,3′,5′-tetramethoxy-4-hydroxy flavone by a combination of column and preparative thin layer Chromatography. The structure of these compounds were determined by spectroscopic analysis as well as by comparison of their spectral data with previously reported values. The cytotoxicity of these two compounds and crude petroleum ether, ethyl acetate and methanol extracts was done by brine shrimp lethality bioassay. The LC_{50} values of compound 1 and 2 and petroleum ether, ethyl acetate and methanol extracts were found to be 14.12, 3.80, 3.97, 6.02 and 8.70 μg/ml, respectively.

Key words: *Oroxylum indicum*, characterization, 2,5-dihydroxy-6,7-dimethoxy flavone, 3,7,3′,5′-tetramethoxy-4-hydroxy flavone, cytotoxic activity

Introduction

Extracts or compounds isolated from extracts have been playing an important role in the development of medicinal agents that afford many opportunities for the design of new therapeutics to treat diseases with fewer side effects or to combat the growing and serious problems of drug resistance (Alfred, 1960; Sofora, 1982; Wallis, 1985). Aspects of these opportunities we selected a medicinal plant *Oroxylum indicum* Vent. (Family: Bignoniaceae). The plant is an important herbal medicine in many Asian countries and is used in folk medicine as a cure of various diseases (Biswas and Ghosh, 1994). The root bark is used in fever, bronchitis, intestinal worms, leucoderma, asthma, inflammation, anal troubles etc. The fruit and seeds are used as expectorant, purgative and bitter tonic (Kirtikar and Basu, 1996). In hindu medicine the root, bark, stem and leaf are prescribed for snake bite in diarrhea and dysentery (Ghani, 1998).

Keeping in mind the folkloric reputation, the plant was chosen for phytochemical and biological investigation to isolate more potent bioactive compounds for better health cure system of common people of third world countries.

We, herein, report the isolation, purification and structure elucidation of two flavonoids from the root bark of the plant *Oroxylum indicum* and their cytotoxic activity.

Materials and Methods

Collection of plant materials: The root barks of the plant *Oroxylum indicum* Vent. was collected from Gobindoganj, Gaibandha, Bangladesh during July-August,2000. The plant was taxonomically identified by Professor A.T.M. Nadiruzzaman, Department of Botany, Rajshahi University, Bangladesh.

Extraction, isolation and characterization: The barks were dried in an oven at 45°C, crushed and then extracted (850 gm) in soxhlet apparatus using petroleum ether (to remove fatty materials) in "Phytochemistry Research Laboratory, Department of Pharmacy, Rajshahi University, Bangladesh". The residue was further extracted gradually with ethyl acetate and methanol and the amounts of each extract obtained were 32.50 gm and 20.50 gm respectively. A portion of ethyl acetate extract was subjected to column chromatography on silica gel of 60-120-mesh size (Beckett and Stenlake, 1986). The column was first eluted with n-hexane and then n-hexane with increasing portions of ethyl acetate, then with ethyl acetate and finally with methanol which gave 29 fractions.

Compound 1 was obtained from fraction 8-10 when subjected to PTLC (pet ether: ethyl acetate 3:1), while compound 2 was obtained from the fraction 11-16 using same solvent system.

Compound 1 (as 2,5-dihydroxy-6,7-dimethoxy flavone): Fine crystals. R_f value: 0.621 (pet ether : ethyl acetate 3:1).mp. 195-198°C. ^{1}H-NMR (500 MHZ, $CDCl_3$): δ_H 2.498

(1H, d, J=17 Hz, H-3), 3.312 (1H, dd, J=17.2 Hz, H-3), 3.673 (3H, s, -OCH_3), 3.861 (3H, s, -OCH_3), 6.291 (H-8, s), 7.393 (1H, m, H-4′), 7.481 (2H, m, H-3′/5′), 7.619 (2H, m, H-2′/6′), 7.745 (1H, d, J=2.0 Hz, -OH at C-2), 11.802 (1H, s, -OH at C-5); ^{13}C-NMR (125 MHZ, $CDCl_3$): δ_C: 102.113 (C-2), 48.392 (C-3), 196.014 (C-4), 153.682 (C-5), 128.611 (C-6), 160.355 (C-7), 92.873 (C-8), 155.712 (C-9), 101.934 (C-10), 142.195 (C-1′), 125.453 (C-2′, 6′), 128.162 (C-3′, 5′), 128.235 (C-4′), 60.122 (-OMe), 56.271 (-OMe).

Compound 2 (as 3,7, 3′, 5′-tetramethoxy-4-hydroxy flavone): Needle shape crystals, R_f value: 0.721 (pet ether: ethyl acetate, 3:1). Mp. 210-211°C. ^{1}H-NMR (500 MHZ, $CDCl_3$): δ_H 7.572 (1H, d, J=8.0 Hz, H-5), 6.905 (1H, dd, J=8.2 Hz, H-6), 7.235 (1H, d, J=2.0 Hz, H-8), 7.291 (1H, d, J=2.0 Hz, H-2′), 7.293 (1H, d, J=2.0 Hz, H-6′), 3.842 (3H, s, -OCH_3 at C-3), 3.925 (3H, s, -OCH_3 at C-7), 3.944 (3H, s, -OCH_3 at C-3′), 3.946 (3H, s, -OCH_3 at C-5′); ^{13}C-NMR (125 MHZ, $CDCl_3$): δ_C: 158.132 (C-2), 140.001(C-3), 180.401 (C-4), 125.023 (C-5), 111.722 (C-6), 163.715 (C-7), 98.799 (C-8), 159.263 (C-9), 116.122 (C-10), 121.061 (C-1′), 105.203(C-2′), 146.91 (C-3′), 136.133 (C-4′), 146.921 (C-5′), 105.192 (C-6′), 58.124 (OMe at C-3), 57.025 (OMe at C-7), 56.801 (OMe at C-3′), 56.802 (OMe at C-5′).

Determination of cytotoxic activity: The cytotoxic activity of petroleum ether, ethyl acetate and methanol extracts and isolated compounds (1 and 2) were determined by brine shrimp lethality bioassay (Mayer *et al.*, 1982; Persoone *et al.*, 1980).

Preparation of seawater: 38 g of sea salt was weighted, dissolved in one liter of distilled water and filtered off.

Hatching of shrimps: Seawater was kept in a small tank and *Artemia salina* Leach (brine shrimp eggs) was added to the divided tank. Constant oxygen supply was provided and constant temperature (37±1°C) was maintained for 48 hrs to hatch and mature the shrimp as nauplii (larvae).

Preparation of sample: 1 mg of each sample (petroleum ether, ethyl acetate and methanol extract and compound 1 and 2) were initially dissolved in 200 µl of dimethyl sulfoxide (DMSO) to get a concentration of 5 µg/µl. Forty clean vials were taken for the 4 samples in five concentrations (two vials for each concentration) and two vials also taken for control test for each sample. 5 ml seawater containing 10 brine shrimp nauplii was given to each of the 5 vials and specific volume of samples were transferred from the stock solutions to the vials to get final concentration of 5, 10, 20, 40 and 80 µg ml^{-1}. Control vials contain 5 ml of seawater and same volume of DMSO as in the sample vials. After 24 hrs the number of survivals in each vial was counted. The percentage of mortality of the brine shrimp was calculated for each concentration and the median lethal concentration (LC_{50}) values were determined.

Results and Discussion

The ethyl acetate extract of the root barks of the plant *Oroxylum indicum* yielded two pure compounds 1 and 2 by column and preparative thin layer chromatography. The isolated compounds were identified by spectroscopic method of analysis as well as by comparison of their spectral data with previously reported values.

The ^{13}C-NMR spectrum of compound 1 displayed seventeen carbon peaks including one intra molecularly hydrogen bonded carbonyl carbon (δ 196.014), eleven aromatic carbon (δ 142.195, 125.453 (2 C), 128.162 (2 C), 128.235, 153.682, 128.611, 160.355, 92.873, 155.712 and 101.934), two methoxyl carbon at δ 60.122 and 56.271 and two methylene carbon at δ 48.392 and 102.113 (attached with a transdiaxial coupled hydroxyl proton).

The ^{1}H-NMR spectrum showed signals for two methoxyl group at δ 3.673 and 3.86 (each 3H, s) attached with C-6 and C-7, one aromatic singlet at δ 6.291 for H-8 and five aromatic protons at δ 7.39 (1H), 7.481 (2H) and 7.619 (2H) assigned to H-4, H-3′/5′ and H-2′/6′ respectively. The spectrum included a broad singlet at d 11.802 attributed to the aromatic hydroxyl proton intra molecularly hydrogen bonded with carbonyl group in ortho position (Sharma, 1997) and a singlet at δ 7.745 due to the presence of a hydroxyl proton which showed a small transdiaxial coupling with δ 3.3 (dd, J=17.2 Hz, H-3).

All the proton peaks in the ^{1}H-NMR data are in good agreement with the compound 2-Hydroxy flavanone isolated from *Collinsonia canadensis* (Jan *et al.*, 1999). The ^{13}C-NMR shifts are also in accord with the structure assigned.

On the basis of ^{1}H-NMR and ^{13}C-NMR spectral data and comparison with aurhontic flavonoidal compound (Hase *et al.*, 1995; Barua, 1989; Jan *et al.*, 1999, Table 1) the structure of Compound was determined as 2,5-dihydroxy-6,7-dimethoxy flavone (Fig. 1). Although it is a known natural product but this is the first report from this plant.

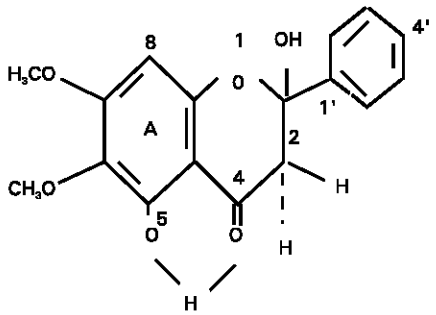

Fig. 1: 2, 5-dihydroxy-6, 7-dimethoxy flavone (1)

Table 1: ^{1}H and ^{13}C-NMR spectral data of compound 1 and comparison with authentic flavanone

^{13}C-NMR data Position of C	^{1}H-NMR data of compound 1	^{13}C-NMR data of compound 1	of flavanone (Jan*et al.*, 1999)
2	-	102.113	102.2
3	2.498 (1H, d, J=17 Hz), 3.312 (1H, dd, J=17.2 Hz)	48.392	48.5
4	-	196.014	196
5	-	153.682	153.7
6	-	128.611	128.6
7	-	160.355	160.5
8	6.291 (1H, s)	92.873	92.9
9	-	155.712	155.7
10	-	101.934	101.9
1′	-	142.195	142.2
2′, 6′	7.619 (2H, m)	125.453	125.4
3′, 5′	7.481 (2H, m)	128.162	128.1
4′	7.393 (1H, m)	128.235	128.2
OH-2	7.745 (1H, d, J=2.0 Hz)	-	-
OH-5	11.802 (1H, s)	-	-
OMe-6	3.861 (3H, s)	60.122	60.1
OMe-7	3.673 (3H, s)	56.271	56.2

Table 2: ^{13}C-NMR and ^{1}H-NMR spectral data of compound 2

Position of C	^{13}C-NMR data	^{1}H-NMR data
2	158.132	-
3	140.001	-
4	180.401	-
5	125.023	7.572 (d, J=8.0 Hz)
6	111.722	6.905 (dd, J=8, 2 Hz)
7	163.715	-
8	98.799	7.235 (d, J=2.0 Hz)
9	159.263	-
10	116.122	-
1′	121.061	-
2′	105.203	7.291 (d, J=2.0 Hz)
3′	146.91	-
4′	136.133	-
5′	146.921	-
6′	105.192	7.293 (d, J=2.0 Hz)
OMe at C-3	58.124	3.842 (s)
OMe at C-7	57.025	3.925 (s)
OMe at C-3′	56.801	3.944 (s)
OMe at C-5′	56.802	3.946 (s)

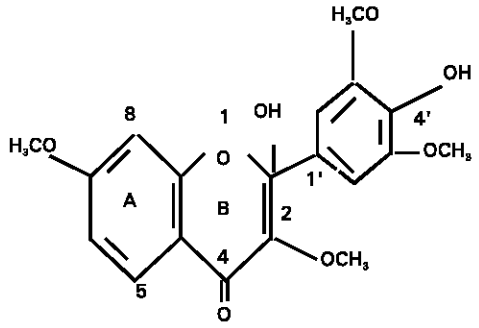

Fig. 2: 3, 7, 3', 5'-tetra methoxy-4 hydroxy flavone (2)

The ^{13}C-NMR spectrum of the compound 2 showed nineteen aromatic carbons (δ 158.132, 140.001, 125.023, 111.722, 163.715, 98.799, 159.263, 116.122, 121.061, 105.203, 146.91, 136.133, 146.921, 105.192) and four methoxyl carbon (δ 58.124, 57.025, 56.801, 56.802).

Table 3: Results of the brine shrimp lethality bioassay of extracts and isolated compounds

Test sample	Concentration (µg/ml)	Log Concentration	% mortality	LC_{50} (µg/ml)
Petroleum ether	5	0.69	50.33	3.97
	10	1.00	66.66	
	20	1.30	73.33	
	40	1.60	86.66	
	80	1.90	96.66	
Ethyl acetate	5	0.69	46.66	6.02
	10	1.00	56.66	
	20	1.30	72.33	
	40	1.60	83.33	
	80	1.90	86.66	
Methanol	5	0.69	41.66	8.70
	10	1.00	53.33	
	20	1.30	66.66	
	40	1.60	77.66	
	80	1.90	80.00	
Compound 1	5	0.69	40.00	14.12
	10	1.00	46.66	
	20	1.30	56.66	
	40	1.60	60.00	
	80	1.90	66.66	
Compound 2	5	0.69	53.33	3.80
	10	1.00	60.00	
	20	1.30	66.66	
	40	1.60	80.00	
	80	1.90	83.33	

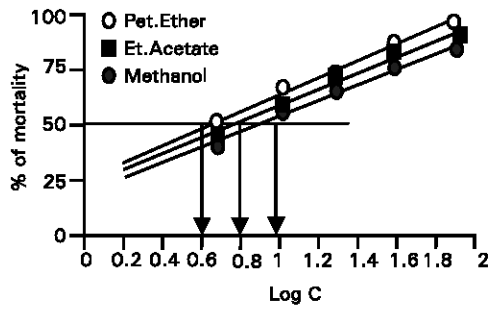

Fig. 3: Determination of LC_{50} of petroleum ethyl acetate and methanol extract

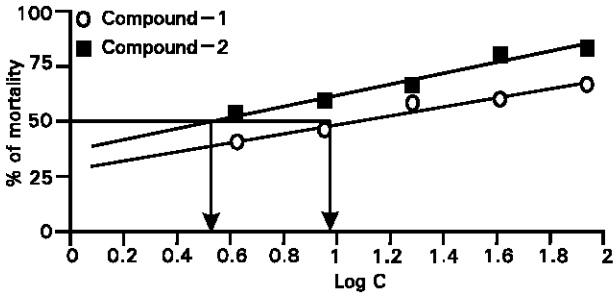

Fig. 4: Determination of LC_{50} of compound 1 and compound 2

The ^{1}H-NMR spectrum of compound 2 displayed signals for four methoxy group at δ 3.842, 3.925, 3.944 and 3.946 assignable to C-3, C-7, C-3′ and C-5′ respectively (Table 2). The spectrum included one set of a ABX

mutually coupled proton system at δ 7.572 (1H,d, J=8 Hz), 6.905 (1H, dd, J=8 Hz, 2Hz) and 7.235 (1H, d, J=2 Hz) assigned to H-5, H-6 and H-8 respectively and two meta coupled protons at δ 7.291 (1H, J=2 Hz) for H-2′ and δ 7.293 (1H, J=2Hz) for H-6′. The ^{1}H-NMR data was found to be identical to that reported for 3,7, 3′, 5′-tetramethoxy-4-hydroxy flavone by Agarawal (1989). The ^{13}C-NMR shifts are also in accord with the structure assigned.

Thus the compound 2 was assigned to be 3,7, 3′, 5′-tetramethoxy-4-hydroxy flavone which is the first report from this plant (Fig. 2).

The result of the brine shrimp lethality bioassay is shown in Table 3. The median lethal concentration (LC_{50}) was determined by extrapolation from the graph (Fig. 3 and 4) and the values were found to be 3.97, 6.02 and 8.70 μg ml^{-1} for petroleum ether, ethyl acetate and methanol extract respectively. For the compound 1 and 2 the LC_{50} values were 14.12 and 3.80 μg ml^{-1}. respectively.

In this study, all the extracts and compound 1 and 2 showed strong positive results, indicating that the extracts and the compounds are highly cytotoxic as well as biologically active. Each of the test samples showed different mortality rate at different concentrations and was found to be increased with increasing concentration of the sample. Logarithm of concentration versus percentage mortality (Goldstein *et al.*, 1974) was plotted on the graph paper that showed an approximate linear correlation. There was no mortality in the control group. So it is evident that all the test materials were highly lethal to brine shrimp nauplii. However compound 2 and petroleum ether extract were more active with minimum LC_{50} value and the compound 1 was comparatively less active with maximum LC_{50} value.

In conclusion, we have successfully isolated, purified and characterized two flavonoids: 2,5-dihydroxy-6,7-dimethoxy flavone and 3,7,3′,5′-tetramethoxy-4-hydroxy flavone from the ethyl acetate extract of root parts of *Oroxylum indicum* Vent. Both the compounds possess cytotoxic activity. However, a detailed investigation is now under progress for evaluation of it's therapeutic use.

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