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Isolation of flavonoids from *Oroxylum indicum* (Vent.) stem bark and their antioxidant activity using DPPH assay

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Abstract

Four flavonoids were isolated from the ethyl acetate extract of stem bark of *Oroxylum indicum* (Vent.) and identified as 3,4,5–trihydroxy– 6-(7'-methoxy-4'-oxo-2'-phenyl-5'-hydroxy-3'-H-chromen-6'-yoloxy) tetra hydro-pyran-2-carboxylic acid methyl ester (Compound I, unknown), with three known compounds: Chrysin, Scutellarein, Baicalein. The increasing order of IC_{50} values reported were Quercetin (standard) < Compound 1 < Trolox (standard) < Baicalein < Scutellarein < Ascorbic acid (standard) < Chrysin in DPPH assay.

Keywords: *Oroxylum indicum*, Flavonoid, Antioxidant, Ester, DPPH

Contents

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1. Introduction

Oroxylum indicum is native to the Himalayan foot-hills in the Indian subcontinent with a part extending to Bhutan, Southern China, Indo-China and the Malaysia ecozone. The ethnobotanical uses of the plant as stringent, carminative, diuretic, stomachic, aphrodisiac have been reported [9]. The powder and infusion of the bark are diaphoretic and useful in treatment of acute rheumatism. The plant is used as a component of Chyawanprash, Mentat and 'Cortex Oroxyli' - a traditional Chinese medicine [1,8,11]. The stem bark is also useful for tanning and dyeing and as a remedy for scorpion-sting [4]. The previous studies on this plant resulted in the isolation of flavonoids [5,17] and some of them showed gestroprotective activity [16]. Flavonoids are important constituents in many plants and have received considerable attention as potentially protective factors against cancer and heart diseases owing to their antioxidant potency [6]. In this study, we reported the isolation and identification of a novel flavonoid (compound I) with three known compounds: Chrysin, Scutellarein, and Baicalein from the ethyl acetate extract of *O. indicum* (Vent.) stem bark.

2. Materials and Methods

General experimental procedures

Melting points were measured in open capillary tubes on a microprocessor based melting point apparatus (Veego, India) and are uncorrected. NMR spectra were obtained on a FT-NMR (NMR: Bruker Avance III 500, Germany) ultra shield spectrometer (300 MHz for ^{1}H, ^{13}C). The mass spectrum of compounds was recorded on mass spectrometer (Waters, ZQ – 4000, USA). Infra red spectra was recorded as KBr pelletes by a Perkin Elemer RX-1 spectrometer (UK), TLC: silica-gel plates: GF254. Column chromatography (CC): silica gel (200–300mesh), Flash Chromatography (Combi Flash RF, Teledyne ISCO, USA), 2, 2-Diphenyl-1-picryl-hydrazyl (DPPH) (Sigma-Aldrich, USA), Quercetin, Trolox, Ascorbic acid (Sigma) analytical grade were used**.**

Plant material

The stem bark of *O. indicum* was collected from the Tezpur cantonment, Sonitpur, Assam, India. An initial quality evaluation of the plant material was carried out as per the guidelines on herbal quality control [21] and a voucher specimen (V10/Phyto/DRL/08) has been deposited in the Phytochemistry Division of the Defence Research Laboratory, Tezpur, Assam for further reference. [2,11].

DPPH free radical scavenging activity

The antioxidant activity was determined using DPPH method described by Brands Williams et al; 1995 [20]. The compound was dissolved in methanol (1µg/ml) followed by the addition of 3.9 ml DPPH (6 x 10^{-5} mol/L) and the absorbance was recorded at 515 nm interval upto 15 min until the absorbance remained constant. The free radical scavenging activity was calculated by the following formula - % Inhibition = [(AB – AA) / AB] x 100, where AB = absorbance of blank DPPH solution, AA = absorbance of tested compound (t =15min) and expressed as IC_{50} as compared to standard antioxidant compound.

Hydrolysis of compound

The compound was dissolved in methanolic KOH and refluxed at ambient temperature until completely dissolved in methanol. The reaction mixture was extracted with water followed by ethyl acetate (two times) and combined of all extraction. Further, the extract was dried over sodium sulphate (Na_2SO_4) and evaporated in rotary evaporator which produces corresponding acid resolved by column chromatography [15].

Extraction and isolation

The extraction and isolation processes followed here are those of Kamiya et al., 2008, Liu et al., 2009;[10,12] with slight modification whenever necessary. The shade-dried stem bark (5 kg) of *O. indicum* were powdered and extracted with methanol three times under reflux. The combined methanol extract was evaporated in rotary evaporator to afford a gummy residue (710 g). This residue was partitioned and extracted at room temperature with Petroleum ether, Hexane, Chloroform, and Ethyl acetate (EtOAC) successively. The EtOAC extract (80 g) was subjected to Si-gel CC eluting with Dichloromethane (DCM) and EtOAC. The fraction eluted with DCM- EtOAC 15:1 and 25:1 gave a mixture of crude flavonoids that was subjected to Si-gel CC again, Frs of (Fr1, 200 ml) and (Fr2, 250 ml), respectively, were collected. Later Sephadex LH-20 using Ethyl acetate as the eluent and further submitted to Flash chromatography (Telydene Isco, USA, coloumn) afford 1(18 mg), 2(32 mg), 3(60 mg), 4(13 mg).

Compound(I):

3,4,5–trihydroxy–6-(7'-methoxy-4'-oxo-2'-phenyl-5'-hydroxy-3'-H-chromen-6'-yoloxy)tetrahydropyran-2-carboxylic acid methyl ester; yellow, amorphous solid; mp-232°C, UV (λ_{max}) (EtOAc): 344 nm and 245 nm; IR (KBr) v_{max}(cm^{-1}): 3444 (OH stretching), 2920 (Alkyl C-H stretching), 1734 (ester C=O), 1607 (-C=O stretching), 1503 (aromatic ring stretching), 1357 (OH bending), 1289 (asymmetric C-O-C stretching), 1166 (C-O-H), 1096 (symmetric C-O-C stretching) ; ^{13}C (75 MHz, $CDCl_3$)(δ ppm): 182.01 (C-4', carbonyl), 163.10 (carbonyl of ester at C-2), 155.80 (C-7'), 154.17 (C-5'), 151.07 (C-9), 131.20 (C-6),130.86 (C-T), 129.08 (C-3', 5'), 127.82 (C-2',6'), 109.53 (C-10), 104.23 (C-3), 92.44 (C- 8), 59.88 (Ar-OMe); ^{1}H (300 MHz, $CDCl_3$) (δ ppm): 12.93 (1 H, s, OH-5) 7.81-7.83 (2H, m, H-2", 6"), 7.44-7.50 (3H, m, H-3", 4", 5"), 6.60 (1 H, s, H-8), 6.55 (1 H, s, H-3), 3.4-4.50 (m, sugar protons), 3.98 (3H, s, OMe), 3.85 (3H, s, Ar-OMe); Element Analysis: C - 57.3 %, H - 2.98 %, O -39.72%; Calc. C - 58.2 %, H-4.64 %, O - 37.13 %; EIMS m/z: 475.4 (M^{+}+1, 5%), calculated mass for $C_{23}H_{22}O_{11}$: 474.176.

***Compound (II)*:**

Oroxoloside methyl ester (3,4,5–trihydroxy–6-(6-methoxy-4-oxo-2-phenyl-4-H-chromen-7-yoloxy) tetrahydro pyran-2-carboxylic acid methyl ester); Reference compound; mp 201°C; UV (λ_{max}) (MeOH): 345, 285nm; IR (KBr) v_{max} (cm^{-1}): 3395(OH stretching), 2924 (Alkyl C-H stretching), 1735(ester-C=O), 1618(-C=O), 1359(OH bending), 1076(symmetric C-O-C stretching); ^{13}C (300 MHz, DMSO-d_6)(δ ppm): 163.72 (C-2), 104.95(C-3),182.37(C-4), 152.52(C-5),132.04(C-6), 156.08(C-7), 94.07 (C-8),152.17 (C-9), 106.12(C-10), 130.59(C-1′), 126.35(C-2′, 6′), 129.03(C-3′, 5′), 132.06(C-4′), 99.49(C-1″), 75.60(C-2″), 75.25(C-3″), 72.77(C-4″), 71.18(C-5″), 168.96(C-6″), 60.21(Ar-OMe), 51.81(OMe); ^{1}H (200 MHz, DMSO-d_6)(δ ppm) 12.78(1H,s,OH-5), 7.90-8.0(2H, m, H-2′, 6′), 7.48-7.60(1H, m, H-3′, 4′, 5′), 6.84 (1 H, s, H-8), 6.80 (1H,s,H-3), 3.4-5.50(m, sugar portion), 3.78 (3H,s,OMe), 3.82(3H,s,Ar-OMe)**;** EIMS m/z: 475(M^{+} + 1, 100); calculated mass for $C_{23}H_{22}O_{11}$: 474.176.

***Compound (III)*:**

Chrysin (5,6-Trihydroxy-2-phenyl-4H-1-bezopyran-4-one), amorphous solid, Yellow in colour, m.p. 271 ± 2 °C UV λ_{max} (MeOH) :363 nm; IR (KBr) v_{max} (cm^{-1}): 3414 (OH stretching), 2924.09 (Alkyl -CH stretching), 1654.3 (-

C=O), 1608.63(-C=C) , 1500.62 (aromatic ring stretching), 1355.96(OH bending), 1290 (asymmetric C-O-C stretching), 1166.93(C-O-H), 1095.57(symmetric C-O-C stretching); ^{13}C (400 MHz, DMSO-d_6) (δ ppm):163.51 (s,C-2), 105.56 (s,C-3), 182.25 (s,C-4), 157.84 (C-9), 161.89 (s, C-5), 99.44 (C- 6), 164.84 (C-7), 94.52 (C-8), 104.39 (C-10), 131.12 (s, C-1'), 126.77 (C-2',6'), 129.49 (C-3',5'), 132.36 (C-4'). ^{1}H (400 MHz, DMSO-d_6) (δ ppm): 12.79 (1 H, s, OH-5) 10.89 (1H, s, OH-7) 8.00 (2H, d, J = 8.0 Hz, H-2', 6') 7.51-7.56 (3H, m, H-3', 4', 5'),6.89 (1 H, s, H-3), 6.47 (1H, s, H-8),6.18 (1H, s, H-6) ; Elemental analysis: C-71.42%, H-2.66 %, O - 25. 92 %, calculated – C-70.86 %, H- 3.93%, O - 25. 1 %; ESI-MS m/z: 255 (M^+, 100 %); calculated mass for $C_{15}H_{10}O_4$: 254.23

Compound (IV):

Scutellarein (5,6,7,4'-Tetrahydroxy-2-phenyl-4H-1-bezopyran-4-one) Yellow green, amorphous, mp-297 °C; UV λ_{max} (MeOH): 285 and 335 nm; IR (KBr) v_{max}(cm^{-1}): 3442(OH stretching), 1660(-C=O), 1617(-C=C), 1496(aromatic ring stretching), 1365(OH bending), 1275(asymmetric C-O-C stretching) **;** ^{13}C (400 MHz, DMSO-d_6) (δ ppm): 163.49 (s, C-2), 102.24(s,C-3), 182.0 (s, C-4), 147.02(s, C-5), 129.12 (s, C-6), 149.64(s, C-7), 93. 83 (C-8), 153.29 (s, C-9), 103.95(s, C-10), 121.44 (s, C-1'), 128.33 (s, C-2' , 6'), 115.92 (d, C-3', 5'), 160.95 (s, C-4'); ^{1}H (400 MHz, DMSO-d_6) (δ ppm): 12.78 (H-1,s,HO-5), 6.72 (1H, s, H-3), 8.73 (1H, s, HO-6), 10.30 (2H,s,HO-7, 4'), 6.56(1H, s, H-8), 7.88 (2H, d, *J*=8.0, H-3, 5); Elemental analysis: C-61.80%, H-2.97%; O-35.33%; Calculated: C-62.94%, H-3.52%, O-33.54%; ESI-MS m/z: 286 (M^+, 100 %) ; calculated mass for $C_{15}H_{10}O_6$: 286.23.

Compound (V):

Baicalein (5,6,7-Trihydroxy-2-phenyl-4H-1-bezopyran-4-one**)**; light brown; amorphous solid; mp-265 °C; UV (λ_{max}) (MeOH): 273nm, 324nm; IR (KBr) v_{max}(cm^{-1}): 3413(OH stretching), 1657(-C=O); ^{13}C (400 MHz, DMSO-d_6) (δ ppm): 163.01 (s, C-2), 104.46(s, C-3), 180.16 (s, C-4), 153.63(s,C-5), 129.34(s, C-6), 146.90(s, C-7), 94.01(s, C-8), 149.83(s,C-9), 104.29(s,C-10), 130.96(s,C-1'), 126.29(d,C-2', 6'), 129.12 (d, C-3',5'), 131.77(d, C-4'); ^{1}H (400 MHz, DMSO-d_6) (δ ppm): 6.93(1H, s, H-3), 12.60 (1H, s, HO-5), 8.82(1H, s, HO-6),10.59(1H, s, HO-7), 6.63 (1H, s, H-8), 8.02 (2H, dd, J=8.0 Hz, H-2' 6'), 7.53(d, J=8.0Hz, H-3',5'), 7.57(1H, m, H-4'); Elemental analysis: C-65.02%, H-2.89%, O- 32.09%; calculated for $C_{15}H_{10}O_5$: C-66.72%, H-3.73%, O-29.55%, ESI-MS m/z: 270 (M^+, 100 %),calculated mass for $C_{15}H_{10}O_5$:270.23.

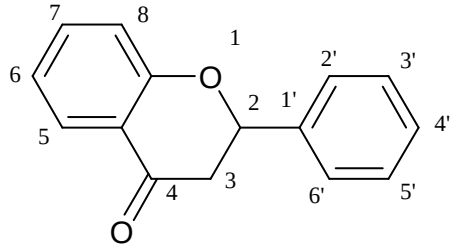

Figure 1

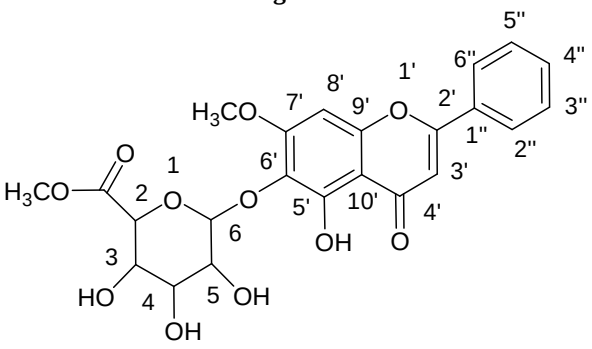

Figure 2

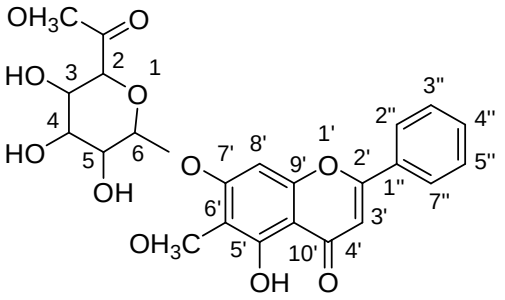

Figure 3

Table 1. IC_{50} Values (µg/ml) of standards and isolated compounds from ethyl acetate extracts of *O. indicum* stem bark in DPPH assay

Entry	Compounds	IC_{50}(µg/ml)
01	I	3.99 ± 1.03
02	Chrysin	8.27 ± 1.34
03	Scutellarein	5.77 ± 0.53
04	Baicalein	4.85 ± 0.24
05	Ascorbic acid (standard)	6.70 ± 0.98
06	Quercetin (standard)	3.37 ± 0.67
07	Trolox (standard)	4.09 ± 0.95

Table 2. Different groups are attached with different position in figure 1

Entry	Compounds	5	6	7	4'
01	Chrysin (III)	-OH	-	-OH	-
02	Scutellarein (IV)	-OH	-OH	-OH	-OH
03	Baicalein (V)	-OH	-OH	-OH	-

3. Results and Discussion

Compound 1, (Figure 2), obtained as a yellow, amorphous solid and showed a positive HCl–Mg reaction, indicating a flavonoid. The molecular formula was established as $C_{23}H_{22}O_{11}$ based on EIMS m/z: 475.4 (M^++1, 5%). calculated mass for $C_{23}H_{22}O_{11}$: 474.176. The IR spectrum showed the presence of absorptions are 3444 (O-H stretching), 2920 (C-H stretching), 1734 (ester C=O stretching), 1607 (C=C, stretching), 1503 (aromatic ring stretching), 1357 (O-H stretching), 1289 (asymmetric, C-O-C stretching), 1166 (C-O stretching) and 1096 (symmetric, C-O-C stretching) cm^{-1}. The IR, ^1H NMR and ^{13}C NMR of compound 1 resembled closely those of Oroxoloside methyl ester (II). A significant difference was observed in the chemical shifts of C-ring protons and carbons. NMR spectra of the compound are similar to those of compound II (Reference compound), (Figure 3) [17] with only minor differences to chemical shift values. In proton NMR a singlet at 12.93 represents a phenolic proton similar to one at 12.78 of compound II. The singlet signal at 3.93 of the ester functionality ($-CO_2CH_3$) corresponding to singlet signal at 3.78 of compound II. Singlet signals at 6.60 and 6.55 correspond to signals at 6.84 and 6.80 respectively. These two signals represent the olefinic proton at position 8 or 3 respectively in compound II. Other proton signals are (Bracketed values are corresponding values for compound II): 7.81-7.83 (7.90-8.0, 2H, m, H-2', 6'), 7.43-7.50 (7.48-7.60) (3H, m, H-3', 4', 5'), 3.80-4.30 (3.4-5.50, m, sugar proton) and 3.85 (3.82, 3H, s, Ar-OMe). It is therefore clear that the compound 1 has a different set of ^1H NMR spectral data but the values resembles closely to those of oroxoloside methyl ester. The m.p. of the compound is 232 °C which is different from that of oroxoloside methyl ester (201 °C).

These observations led us to believe that the new compound has a different structure but it should closely resemble to that of compound II. Taking this into consideration, we strongly suggested that structure I is possibly the correct structure. More spectral and other analytical evidences are presently being collected to confirm this. In order to get into the structure of the compound I, it was subjected to different analytical tools such as UV-Visible, IR, Mass and NMR. The elemental analysis showed that the compound contain C-56.3 %, H-2.98 %. Mass spectrum shows molecular ion peak at m/z -475.4 and this indicates that this compound may be oroxoloside methyl ester (II) already reported or one of its isomers. The compound I on alkaline hydrolysis, gave an acid derivatives whose ^1H NMR showed complete disappearance of ester absorption at δ 3.78, which supported establishment of the structure. Therefore, the structure was characterized as 3,4,5–trihydroxy– 6-(7'-methoxy-4'-oxo-2'-phenyl-5'-hydroxy-3'-H-chromen-6'-yloxy) tetrahydropyran-2-carboxylic acid methyl ester (Figure 2).

DPPH free radical scavenging activity

The increasing order of IC_{50} values recorded were Quercetin (standard) < Compound 1 < Trolox (standard) < Baicalein < Scutellarein < Ascorbic acid (standard) < Chrysin (Table 1). The compound 1 having better free radical scavenging activity showed the lowest IC_{50} value among tested compounds and standards. When the flavonol quercetin (3,5,7,3',4'-pentahydroxyflavone) reacts with a free radical, it donates a proton and becomes a radical itself, but the resulting unpaired electron is delocalized by resonance, making the quercetin radical too low in energy to be reactive [14]. Three structural groups aid in quercetin's ability to maintain its stability and act as an antioxidant when reacting with free radicals: the B ring *o*-dihydroxyl groups, the 4-oxo group in conjugation with the 2,3-alkene, and the 3- and 5-hydroxyl groups [7]. The functional groups can donate electrons to the rings, which increase the number of resonance forms available in addition to those created by the benzene structure [14]. similarly compound 1 have basic flavonoid structure with 4'-oxo group in conjugation with the 2,3-alkene, and the 3-,4-,5- and 5'- hydroxyl groups and better free radical scavenger similar to quercetin according to IC_{50} value. The

hydrogen–donor capacities of polyphenols for DPPH radical were found proportional to the number of hydroxyl groups [13] and the amount of inactivated DPPH radical was found proportional to the concentration of added flavonoids.

4. Conclusion

The present investigation showed that the four isolated compounds where unknown compound I (Figure 2) was 3,4,5–trihydroxy–6-(7'-methoxy-4'-oxo-2'-phenyl-5'-hydroxy-3'-H-chromen-6'-yoloxy)tetrahydropyran-2-carboxylic acid methyl ester and other three known compounds were Chrysin, Scutellarein and Baicalein. Compound 1 was showed highest antioxidant activity (lowest IC_{50} value value) in comparison to other isolated compounds with standards. The isolated compounds and mixture of ethyl acetate extract of *O. indicum* stem bark may be use in new drug candidates in current medicine system.

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