In vitro anti-oxidant potential of new metabolites from *Hypericum oblongifolium* (Guttiferae)

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Phytochemical investigations on *Hypericum oblongifolium* led to the isolation of a flavone named folicitin (1) and a bicyclic conjugated lactone, folenolide (2) from the ethyl acetate fraction of methanolic extract. Both metabolites were characterised as new compounds based on detailed spectroscopic analyses. *In vitro* anti-oxidant potential of both the compounds was evaluated by the DPPH radical scavenging assay. Compound 1 exhibited significant antioxidant activity while compound 2 was found inactive.

**Keywords:** Hypericum oblongifolium; Guttiferae; folicitin; folenolide; flavone; antioxidant

1. Introduction

*Hypericum*, a large genus of herbs and shrubs, grows widely in the temperate regions throughout the globe. *Hypericum oblongifolium* belongs to the family Guttiferae which comprises of 50 genera and 1200 species (Cakir et al. 2003). There are about 400 species of genus *Hypericum* and they are used as traditional medicinal plants in various parts of the world. These plants are the richest sources of flavones and xanthones (Cakir et al. 2003). The genus is represented by nine species in Pakistan. Useful biologically active compounds, dyes, pigments, timbers, gums and resins have been isolated from members of this family (Mabberly 1987).

*H. oblongifolium* Wall., is an evergreen shrub, found at an altitude of 5000–6000 ft, and is common on Khasia Hill in China and in the Himalayas. It is used for the treatment of hepatitis,
nasal haemorrhage, bacterial diseases and as a remedy for the sting of bees and dog bites (Dulger & Gonuz 2005). Most of these species have been used as a treatment of gastric ulcer, external wounds, sedative, antispasmodic and also as antiseptic in folk medicine (Ferheen et al. 2006). The crude extract of *H. oblongifolium* has been found to possess respiratory, gastrointestinal and cardiovascular inhibitory effects (Khan et al. 2010). The aqueous and n-butanol fractions of *H. oblongifolium* possess significant *in vitro* antiglycation, antioxidant and anti-lipid peroxidation activities with no toxic effects (Abbas et al. 2013). The hexane fraction is reported to possess relatively potent anti-proliferative activity (Ali et al. 2011b). Two new anti-inflammatory xanthones, hypericorin A and hypericorin B from the twigs of *H. oblongifolium* were isolated (Ferheen et al. 2005; Ali et al. 2011a). Bioassay guided fractionation of the plant has also led to the isolation of three potent urease inhibitors (Ferheen et al. 2005; Arfan et al. 2010).

Free radicals are produced due to oxidation inside the body and lead to the initiation of chain reactions. These reactions can be harmful for cells and can cause death of cells leading to various human health disorders, such as atherosclerosis, cardiovascular and neurodegenerative diseases (Witztum & Steinberg 1991; Esterbauer et al. 1992). These chain reactions can be stopped by the radical scavenging potential of antioxidants (Sies 1997). Antioxidants are commonly found in fruits, vegetables, tea, coffee and cacao (Gülçin 2012). *Hypericum* species contain various pharmacologically active compounds such as flavonoids, naphthodianthrones, phloroglucinols and xanthones (Robson 1990). Flavonoids are the most common class of plant phytochemicals presenting a wide range of biochemical properties, including anti-inflammatory (Owoyele et al. 2008), antibacterial (Hnatuszyn et al. 2003), antifungal (Li et al. 2005), antioxidant (Bernardi et al. 2007) and anti-cancer activities (Seelinger et al. 2008). Flavonoids have been recorded to act as scavengers of several oxidising species (Tournaire et al. 1993). The aim of the present study was the phytochemical investigation of *H. oblongifolium* and the evaluation of the antioxidant potential of the isolated metabolites.

### 2. Results and discussion

Phytochemical investigation of *H. oblongifolium* led to the isolation of two new metabolites.

Folecitin (I) was obtained as yellow crystals. ESI-MS showed the M + H ion peak at m/z 449.11 (Calcd. 449.1083 amu) corresponding to the molecular formula C_{21}H_{20}O_{11}. The IR spectrum of I showed absorption bands at 3275 cm\(^{-1}\) (OH), 1655 cm\(^{-1}\) (ketone) and 1603 cm\(^{-1}\) (olefin). The\(^{13}\)C NMR spectrum of I showed a C\(_{\text{v}}\)O group at \(\delta\) 179.7 (C-4), and quaternary alcohol groups at \(\delta\) 136.2 (C-3), 163.3 (C-5), 166.0 (C-7) and 145.6 (C-3\(_{\text{a}}\)). Methine signals at \(\delta\) 71.9 (C-3\(_{\text{o}}\)), 73.2 (C-4\(_{\text{o}}\)), 72.0 (C-5\(_{\text{o}}\)) showed the presence of Z\(\text{OH}\) groups attached to these carbons. A signal at \(\delta\) 17.7 showed the presence of a methyl group in the molecule.

The \(^1\)H NMR spectrum of I showed aromatic proton signals at \(\delta\) 6.19 (H-6, 1H, d, \(J = 2.0\) Hz), 6.37 (H-8, 1H, d, \(J = 2.0\) Hz), 7.30 (H-6\(_{\text{a}}\), 1H, dd, \(J = 8.0, 2.0\) Hz), 6.90 (H-5\(_{\text{a}}\), 1H, d, \(J = 8.0\) Hz) and 7.33 (H-2\(_{\text{a}}\), 1H, d, \(J = 2.0\) Hz), which are characteristic of a flavone skeleton. Carbinylic protons appeared at \(\delta\) 4.21 (H-3\(_{\text{a}}\), 1H, dd, \(J = 3.5, 1.5\) Hz), 3.39 (H-4\(_{\text{a}}\), 1H, m) and 3.73 (H-5\(_{\text{a}}\), 1H, dd, \(J = 9.5, 3.5\) Hz).The methyl signal appeared at \(\delta\) 0.93 (H-7, 3H, d, \(J = 6.0\) Hz). The \(^{13}\)C and \(^1\)H NMR suggested a flavonoid skeleton of I.

The COSY spectrum showed correlations between H-5\(_{\text{a}}\) and H-6\(_{\text{a}}\), thus meaning that these are attached to adjacent carbon atoms. C–H protons at \(\delta\) 4.21 (H-3\(_{\text{a}}\)) showed COSY cross peaks with the proton at \(\delta\) 3.39 (H-4\(_{\text{a}}\)) which in turn showed COSY cross peaks with H-5\(_{\text{a}}\) (\(\delta\) 3.73). H-5\(_{\text{a}}\) also showed COSY interactions with H-6\(_{\text{a}}\) (\(\delta\) 3.42) which in turn showed interaction with the methyl protons (H-7\(_{\text{a}}\)). A relay of the COSY cross peaks between these methine and methyl protons suggested that all of them are in the same cycle and that the methyl group is also in the same ring.
HMBC spectrum of compound 1 showed correlations of H-8 (δ 6.37) with C-6 (δ 99.8) and C-10 (δ 105.9), while proton H-6 in turn showed HMBC cross peaks with C-8 (δ 94.7) and C-10 (δ 105.9). H-6' (δ 7.30) showed HMBC correlations with C-2 (158.6), C-4' (δ 149.8) and C-2' (δ 116.9). H-5' was HMBC correlated with C-1' (δ 122.9) and C-3' (δ 145.6). The methyl protons (H-7''', δ 0.93) showed HMBC cross peaks with C-5'' (δ 72.0).

Based on the above interactions and other spectral analyses, the structure of compound 1 was elucidated as 3,5,7-trihydroxy-2-[3-hydroxy-4-(3,4,5-trihydroxy-6-methyltetrahydro-2H-pyran-2-yloxy)phenyl]-4H-chromen-4-one (Figure 1). Extensive literature survey proved that this compound has not been reported earlier and is thus a new compound.

Folenolid (2) was obtained as white crystals. HR-EIMS showed the molecular formula C_{7}H_{8}O_{4} (M = 156.0432), while M\(^{+}\) appearing at m/z 156.00 (calc. 156.0423). The IR spectrum of 2 showed absorption bands at 3275 cm\(^{-1}\) (OH), 1656 cm\(^{-1}\) (ester) and 1603 cm\(^{-1}\) (olefin). The \(^{13}\)C NMR spectrum of 2 showed a C\(-\)O group at δ 173.14 (C-7) and methine signals at δ 73.6 (C-3), 68.4 (C-4) showed the presence of \(\text{\textasciitilde OH}\) groups attached to these carbons.

The \(^{1}\)H NMR spectrum of 2 showed a downfield proton signal at δ 6.75 (H-5, 1H, t, J = 2.0 Hz), corresponds to olefinic proton. Carbinyl protons attached to hydroxyl groups showed signals for their corresponding protons at δ 3.73 (H-3, 1H, q, J = 4.5, 12.5 Hz) and 4.40 (H-4, 1H, t, J = 4.0, 4.5 Hz). The spectrum also displayed signals for methylene protons at δ 2.18 (H-8, ddt, J = 1.5, 6.5, 17.5 Hz) and 2.70 (H-8, ddt, J = 1.5, 5.5, 18 Hz).

The COSY spectrum showed correlations between H-2 and H-8, thus meaning that these are attached to adjacent carbon atoms. Methine proton at δ 3.98 (H-2) showed COSY cross peaks with the proton at δ 3.73 (H-3) which in turn showed COSY cross peaks with H-4 (δ 4.40). H-4 also showed COSY interactions with H-5 (δ 6.75).

HMBC spectrum of compound 2 showed correlations of H-3 (δ 3.73) with C-5 (δ 139.3) and C-8 (δ 33.0), while proton H-4 showed HMBC cross peaks with C-5 (δ 33.0). H_{2}-8 (δ 2.18 and 2.70) showed HMBC correlations with C-2 (δ 69.0), C-3 (δ 73.6), C-5 (δ 139.3), C-6 (δ 133.0) and C-7 (δ 173.0).

Based on the above interactions and other spectral analyses, the structure of compound 2 was elucidated as 3,4-dihydroxy-6-oxabicyclo[3.2.1]oct-1-en-7-one (Figure 1). To the best of our knowledge this compound has not been reported earlier and is thus regarded as a new compound.

Compounds 1 and 2 were evaluated for their anti-oxidant potential using the DPPH radical scavenging assay (Table 1).

### 3. Experimental
#### 3.1. Plant material
The whole plant (12 kg) was collected in the month of April and May, 2011, from Bara Gali, District Abbottabad, Khyber Pakhtunkhwa, Pakistan. The plant was identified by Dr. Muhammad Ibrar, Department of Botany, University of Peshawar, and a voucher specimen (08823) was deposited in the herbarium of the same department.

| Compound | IC_{50} (μg/10 μL) | SEM\(^{a}\) |
|----------|-------------------|-------------|
| 1        | 25.2 ± 0.26       |             |
| 2        | NA\(^{b}\)        |             |
| BHA (butylated hydroxyanisole)\(^{c}\) | 44.2 ± 0.87 |             |

\(^{a}\) Standard error mean.

\(^{b}\) Not active.

\(^{c}\) Standard used.
3.2. General experimental conditions
Thin layer chromatography (TLC) was carried out on pre-coated silica gel plates (G-60, F254,
Merck, Darmstadt, Germany). Column chromatography (CC) was performed on silica gel (G-60,
70–230 mesh). 1H and 13C NMR spectra were recorded in deuterated methanol on Bruker
Avance-NMR spectrometers (MA, USA) with tetramethylsilane as an internal standard. The
chemical shifts (δ values) are given in parts per million (ppm), and the coupling constants (J
values) in Hertz. JEOL JMS-600H Mass spectrometer (Tokyo, Japan) was used for recording EI-
MS and HR EI-MS; in m/z (rel. %).
Visualisation of chromatograms was achieved under UV (254 and 365 nm) before using
spraying reagents (exposure to ammonia vapour or aluminium chloride spray).

3.3. Extraction and isolation
The powdered plant material was macerated in 20 % methanol for extraction. The crude extract
(2.5 kg) obtained after maceration, was fractionated in n-hexane, ethyl acetate, n-butanol and
water, respectively. The ethyl acetate fraction (490 g) was subjected to column chromatography
over silica gel. This fraction was eluted with gradient n-hexane-ethyl acetate and ethyl acetate-
methanol solvent systems. The column provided 300 fractions were made and pooled based on
TLC analysis, resulting in 16 major fractions. Fraction 14 (2 g) was further subjected to column
chromatography over flash silica gel (ethyl acetate–chloroform, 1:1) leading to the isolation of
compound 1 (40 mg). The fraction 16 (ethyl acetate–n-hexane, 1:3) was re-chromatographed
and eluted with acetone–chloroform (2:3) solvent system to get compound 2 (30 mg) as a white
powder.

3.4. Physical and spectral data

**Folecitin (1)**
Yellowish brown crystalline solid (40 mg). M.P. 187–189°C, UV: λmax = 350 (ε0 = 6.6),
[α]D = −377, IR (cm⁻¹): C=O (1655), −OH (3275), C==C (1603), 1H NMR (δ in ppm, J in
Hz, CD3OD, 500 MHz): H-6 (6.19, d, J = 2.0 Hz), H-8 (6.37, d, J = 2.0 Hz), H-2' (7.33, d,
J = 2.0 Hz), H-5' (6.90 d, J = 8.0 Hz), H-6' (7.30, dd, J = 8.0, 2.0 Hz), H-2'' (5.34, d,
J = 1.5 Hz), H-3'' (4.21, dd, J = 3.5, 1.5 Hz), H-4'' (3.39, m), H-5'' (3.73, dd, J = 9.5, 3.5 Hz),
H-6'' (3.42, m), H-7'' (0.93, d, J = 6.0 Hz). 13C NMR (δ in ppm, CD3OD, 150 MHz): C-2
(158.6), C-3 (136.2), C-4 (179.7), C-5 (163.3), C-6 (99.8), C-7 (145.6), C-8 (94.7), C-10 (105.9),
C-1' (122.9), C-2' (116.9), C-3' (145.6), C-4' (149.8), C-5' (116.4), C-6' (122.8), C-2'' (103.5),

![Figure 1. Folecitin (1) and folenolide (2).](image-url)
C-3'' (71.9), C-4'' (73.2), C-5'' (72.0), C-6'' (72.1), C-7'' (17.7). ESI-MS: (M + H)^+ (449.1083, C_{21}H_{21}O_{11}) EI-MS: m/z (rel. int. %) 302 (100), 285 (3.5), 275 (2.2), 257 (3.4), 245 (4.7), 229 (8.1), 217 (2.3), 204 (1.1).

**Folenolide (2)**
White crystalline solid (30 mg). M.P. 175–176°C, UV: \(\lambda_{\text{max}} = 349 (\varepsilon_0 = 5.36), [\alpha]_D = -322,\) IR (cm\(^{-1}\)): C=O (1656), –OH (3275), C==C (1603), ^1H NMR (δ in ppm, J in Hz, D_2O, 500 MHz): H-2 (3.98, m), H-3 (3.73, q, J = 4.0 Hz), H-4 (4.40, t, J = 4.5 Hz), H-5 (6.75, d, J = 2.0 Hz), H-8 (2.18, ddt, J = 17.5, 6.5, 1.5 Hz; 2.70, ddt, J = 18, 5.5, 1.5 Hz). ^13C NMR (δ in ppm, D_2O, 125 MHz): C-2 (69.0), C-3 (73.6), C-4 (68.4), C-5 (139.3), C-6 (133.0), C-7 (173.0), C-8 (33.0). FAB^+: m/z (MH^+157.05, C_7H_9O_4), EI-MS: m/z (rel. int. %) 156 (13.9), 138 (32.1), 127 (10.7), 115 (27.9), 110 (31.9), 102 (1.6), 96 (24.6), 82 (0.9).

### 3.5. DPPH radial scavenging assay
The free radical scavenging activity was determined by 1,1-diphenyl-2-picryl-hydrazyl (DPPH) using the method described by Gülçin et al. (2005). The solution of DPPH of 0.3 mM was prepared in ethanol. Five μL of each sample of different concentrations (62.5–500 μg) was mixed with 95 μL of DPPH solution in ethanol. The mixture was dispersed in 96-well plates and incubated at 37°C for 30 min. The absorbance at 515 nm was measured by microtitre plate reader (Spectramax plus 384 Molecular Device, Union City, CA, USA) and percent radical scavenging activity was determined in comparison with the dimethyl sulfoxide (DMSO) treated control. Butyl hydroxyl anisole was used as a standard.

\[
\text{DPPH scavenging effect (\%) = } \frac{\text{Ac} - \text{As}}{\text{Ac} \times 100},
\]

where, Ac = Absorbance of control (DMSO treated). As = Absorbance of sample.

### 4. Conclusion
In conclusion, the phytochemical investigation of *H. oblongifolium* was carried out which yielded two new metabolites, a flavone, folicitin (1) and a bicyclic conjugated lactone, folenolide (2). DPPH radical scavenging assay proved that compound 1 is a potential anti-oxidant compound.

### Supplementary material
Supplementary material relating to this paper is available online, alongside Figures S1–S4 and Tables S1–S2.

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