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RADICAL SCAVENGING AND LIPOXYGENASE INHIBITION STUDIES OF THE COMPOUNDS ISOLATED FROM A MEDICINAL LICHEN, *Usnea longissima*

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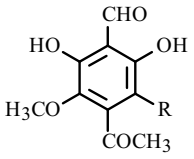
The genus *Usnea* (Usneaceae) is a large, hanging hair lichen (part fungus, part algae) that grows throughout the northern temperate zones, especially the sub-arctic and coastal rainforests of Europe, Asia, and North America. Because of a long tradition of their use as antimicrobial agents by indigenous communities such as the Venezuelan Andes [1], lichens have attracted attention as a source of new antibiotics [2]. *Usnea* species have also been used traditionally for pain relief and fever control [3]. They are also effective in treating respiratory infections [2].

Antioxidants can inhibit the oxidation of oxidizable materials in living cells. Free radicals are generated as a result of oxidation [4] and may be involved in the pathogenesis of various disease such as cancers. They may also cause many other diseases like heart disease, diabetes, arteriosclerosis, and arthritis [5]. Antioxidants retard such processes as premature aging [6], cancers [7], and heart disease [8] by inhibiting of certain enzyme and quenching free radicals that may result in reactive oxygen species (ROS) [4] during their catalytic reactions.

Lipoxygenases constitute a family of non-heme iron containing dioxygenases that are widely found in animals and plants. In mammalian cells, these are the key enzymes in the biosynthesis of a variety of bioregulatory compounds such as hydroxyeicosatetraenoic acids (HETEs), leukotrienes, lipoxins, and hepoxylins [9]. Lipoxygenase products play an important role in a variety of disorders such as bronchial asthma, inflammation [10], autoimmune diseases, and tumor angiogenesis [11]. Lipoxygenases are therefore potential targets for rational drug design and discovery of mechanism-based inhibitors for the treatment of these diseases.

Our previous phytochemical studies on *Usnea longissima* Linn., also known as Old Man′s Beard, resulted in the isolation of two new phenolic compounds, longissiminone A (**1**) and longissiminone B (**2**), and a new source, glutinol (**3**) [12]. The structures of these compounds were deduced by spectroscopic techniques. Anti-inflammatory and cytotoxic properties of compounds **1–3** were also studied [12]. In continuation of our studies, we report here the DPPH radical scavenging and lipoxygenase inhibition activities of the isolated compounds.



1, 2

1: R = H
2: R = Cl

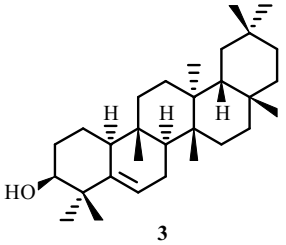


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TABLE 1. *In vitro* DPPH Radical Scavenging Activities of Compounds **1–3** Isolated from Methanolic Extract of *Usnea longissima*

Compounds	DPPH scavenging activity (% inhibition) at 1 mM conc.	Compounds	DPPH Scavenging Activity (% Inhibition) at 1 mM conc.
1	–	Propyl gallate*	92.0
2	24.0	3-*t*-Butyl-4-hydroxyanisole*	91.2
3	–		

*Standard radical scavengers.

TABLE 2. *In vitro* Quantitative Inhibition of Lipoxygenase by Compounds **1–3** Isolated from Methanolic Extract of *Usnea longissima*

Compounds	IC_{50} (μM) ± S. E. M. against lipoxygenase	Compounds	IC_{50} (μM) ± S. E. M. against lipoxygenase
1	> 400	**3**	> 400
2	> 400	Baicalein*	22.7 ± 0.5

IC_{50} – Concentration of a test compound; S. E. M. – standard error of the mean of five assays; *positive control used for the inhibition of lipoxygenase.

The dried lichen, *Usnea longissima*, was extracted with methanol. The methanolic extract was concentrated to a residue (260.2 g), which was dissolved in distilled H_2O (2 L) and defatted with hexane (6 L). The defatted aqueous extract was further fractionated with $CHCl_3$, acetone, and butanol. The resulting $CHCl_3$ extract (97.8 g) was loaded on a silica gel (1.3 kg) column and eluted with a gradient of hexane, $CHCl_3$, and acetone (hexane–$CHCl_3$ to $CHCl_3$: acetone mixtures from 9.0:1.0 to 7.0:3.0, respectively, each 2 L) to afford three important fractions: fraction A ($CHCl_3$–hexane, 5.8:4.2, 500 mL) containing mainly compound **1**, fraction B ($CHCl_3$–hexane, 5.0:5.0, 800 mL) containing mainly compound **2**, and fraction C ($CHCl_3$–hexane, 3.4:6.6, 250 mL) found to contain compound **3**. Fraction A was purified by column chromatography (silica gel 3.0 g) to yield compound **1** (24.2 mg, $CHCl_3$–hexane, 5.5:4.5). Fraction B was also subjected to column chromatography (silica gel 2.5 g) to obtain compound **2** (20.6 mg, $CHCl_3$–hexane, 4.7:5.3). Fraction C was also purified on column chromatography (silica gel 7.0 g) to obtain compound **3** (62.8 mg, $CHCl_3$–hexane, 3.0:7.0).

Longissiminone A (1), white amorphous solid, mp 132°C. UV (MeOH, λ_{max}, nm) (log ε): 260 (3.90), 241 (4.03), 203 (3.79). IR ($CHCl_3$, ν_{max}, cm^{-1}): 3466 (broad OH), 2854 (CHO), 1701 (C=O), 1576, 1429, (aromatic C=C), 1372 (C-O). EI-MS *m/z* (rel. int. %): 210 $[M]^+$ (65), 178 (52), 150 (100), 122 (48), 94 (43). HR-EI-MS *m/z* 210.0501 ($C_{10}H_{10}O_5$, calcd 210.0528). ^1H NMR (400 MHz, $CDCl_3$, δ, ppm): 2.51 (3H, s, CH_3), 3.94 (3H, s, 3-OCH_3), 6.28 (1H, s, 5-H), 10.33 (1H, s, CHO), 12.39 (1H, s, 6-OH), 12.84 (1H, s, 2-OH). ^{13}C NMR (125 MHz, $CDCl_3$, δ, ppm): 25.2 (CO<u>C</u>H_3), 52.3 (OCH_3), 103.8 (C-4), 108.4 (C-1), 112.1 (C-5), 166.6 (C-6), 168.2 (C-2), 172.0 (C-3), 182.3 (<u>C</u>OCH_3), 193.9 (CHO).

Longissiminone B (2), white amorphous material, mp 113°C. UV (MeOH, λ_{max}, nm) (log ε): 206 (4.41), 237 (4.38), 275 (4.34). IR ($CHCl_3$, ν_{max}, cm^{-1}): 3578 (broad OH), 2846 (CHO), 1706 (C=O), 1606, 1446 (aromatic C=C), 1322 (C-O). EI-MS *m/z* (rel. int. %): 246 $[M\ (^{37}Cl)]^+$ (9), 244 $[M\ (^{35}Cl)]^+$ (26), 212 (20), 184 (100), 156 (18), 128 (10), 93 (13). HR-EI-MS *m/z* 244.0072 ($C_{10}H_9O_5Cl$, calcd 244.0098). ^1H NMR (400 MHz, $CDCl_3$, δ, ppm): 2.68 (3H, s, CH_3), 3.97 (3H, s, 3-OCH_3), 10.33 (1H, s, 1-CHO), 12.62 (1H, s, 2-OH), 13.10 (1H, s, 6-OH). ^{13}C NMR (125 MHz, $CDCl_3$, δ, ppm): 20.7 (CO<u>C</u>H_3), 52.7 (OCH_3), 105.2 (C-4), 108.6 (C-1), 115.0 (C-5), 162.6 (C-6), 165.5 (C-2), 171.5 (C-3), 188.9 (<u>C</u>OCH_3), 193.7 (CHO).

Glutinol (3), white amorphous material, mp 212°C. UV (MeOH, λ_{max}, nm) (log ε): 212 (4.36). IR ($CHCl_3$, ν_{max}, cm^{-1}): 3436 (OH), 1622 (C=C). EI-MS *m/z* (rel. int. %): 426 (55), 408 (16), 274 (100), 259 (83), 205 (42), 152 (27) and 134 (56). HR-EI-MS *m/z* 426.3818 ($C_{30}H_{50}O$, calcd 426.3812). ^1H NMR (400 MHz, $CDCl_3$, δ, ppm, J/Hz): 0.83 (3H, s, CH_3, H-25) 0.93 (3H, s, CH_3, H-29), 0.97 (3H, s, CH_3, H-28), 0.98 (3H, s, CH_3, H-26), 1.02 (3H, s, CH_3, H-24), 1.07 (3H, s, CH_3, H-27), 1.12 (3H, s, CH_3, H-23), 1.14 (3H, s, CH_3, H-30), 3.45 (1H, t, $J_{3,2}$ = 3.0, H-3α), 5.61 (1H, m, $W_{1/2}$ = 4.0, H-6).

Compounds **1**–**3** from the methanolic extract of the lichen *Usnea longissima* were previously reported [12], and their anti-inflammatory activities were also examined, where compound **1** showed the highest activity while compounds **2** and **3** did not show any significant activity at 400 μg/mL concentration. It was also previously reported that compound **3** possesses antifungal [13] and analgesic activities [14].

Now we further studied their antioxidant activities relative to their medicinal importance. The antioxidant activities of these compounds were evaluated by using a DPPH radical scavenging assay. Propyl gallate and 3-*t*-butyl-4-hydroxyanisole were used as positive controls. No compound exhibited significant activity against DPPH radicals. Compound **2** was found to be a weak inhibitor, whereas compounds **1** and **3** did not show any activity. The results of the antioxidant activity are summarized in Table 1.

Compounds **1**–**3** were also tested for lipoxygenase inhibitory activity. Baicalein was used as a positive control. The results of the enzyme inhibition study are presented in Table 2. No compound exhibited any activity against the enzyme.

Plant Material. The preserved dried lichen, *Usnea longissima* Linn. (11 kg), was purchased from the shop "Amjad Yunani Medicine" in the herbal market of Liaquatabad, Karachi, Pakistan.

DPPH (1,1-Diphenyl-2-picryl hydrazyl) Free Radical Scavenging Antioxidant Assay. Antioxidant activity was assayed by using the nonphysiological DPPH free radical scavenging assay. Different concentrations of the test compounds were taken in the reaction mixture, ranging from 1000–10 μM, while the concentration of DPPH was kept constant at 300 μM. The reaction mixture containing 5 μL of test compound in DMSO (1 mM) and 95 μL of DPPH in ethanol (300 μM) was taken in 96-well microtiter plates (Molecular Devices, SpectraMax 340, USA) and incubated at 37°C for 30 min. The absorbance was measured at 515 nm. The percent radical scavenging activity of the test compounds was determined by comparing with the DMSO-treated control group. The IC_{50} values represent the concentration of compounds required to scavenge 50% of DPPH free radicals, which were calculated by using the EZ-Fit Enzyme Kinetics program (Perrella Scientific Inc., Amherst, USA). Propyl gallate (PG) and 3-*t*-butyl-4-hydroxyanisole (BHA) were used as positive controls [15, 16].

Lipoxygenase Inhibition Assay. Lipoxygenase (EC. 1.13.11.12) inhibiting activity was conveniently measured by modifying the spectrophotometric method developed by A. L. Tappel [17]. The reaction mixture, containing 165 μL of 100 mM sodium phosphate buffer (pH 8.0), 10 μL of test compound solution, and 20 μL of lipoxygenase solution, was incubated for 10 min at 25°C. The reaction was then initiated by the addition of 10 μL linoleic acid (substrate) solution. After the formation of (9*Z*,11*E*)-(13*S*)-13-hydroperoxyoctadeca-9,11-dienoate, the changes in absorbance at 234 nm were measured for 6 min. The test compounds and control were dissolved in methanol. All the reactions were performed in triplicate in 96-well microplates in SpectraMax 384 Plus (Molecular Devices, USA). The IC_{50} values were then calculated using the EZ-Fit Enzyme Kinetics program (Perrella Scientific Inc., Amherst, USA). The percentage (%) inhibition was calculated as follows:

$$\% \text{ Inhibition} = (E - S)/E \times 100,$$

where E is the activity of the enzyme without test compound and S is the activity of the enzyme with test compound.

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