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# Bioactive phenolic compounds from a medicinal lichen, *Usnea longissima*

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**Abstract**

Natural products, longissiminone A (**1**) and longissiminone B (**2**), were isolated along with a known compound, glutinol (**3**), from a medicinal lichen, *Usnea longissima*. The structures of compounds **1** and **2** were determined with the help of spectroscopic studies. Compound **1** was found to possess potent anti-inflammatory activity in a cell-based contemporary assay. Cytotoxicity activity measured by cell viability assay showed 100% viability in the presence of 200 μg/mL conc. of these compounds.


*Keywords:* *Usnea longissima*; Lichen; Usneaceae; Glutinol; Longissiminone; Anti-inflammatory; Cytotoxicity

## 1. Introduction

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 Venezuelan Andes (Marcano et al., 1999), lichens have attracted attention as a source of new antibiotics (Hobbs, 1986). *Usnea* species have also been used traditionally for pain relief and fever control (Okuyama et al., 1995). They are also effective in TB, as well as other lower respiratory infections (Hobbs, 1986).

Previous phytochemical studies on *Usnea longissima* Linn., also known as Old Man's Beard, resulted in the isolation of several lichen acids, with anti-inflammatory, analgesic, antipyretic, anti-tumor, anti-cholesterol and nematocidal properties (Yamamoto et al., 1995; Nishitoba et al., 1987). We report here the isolation of two new phenolic compounds, longissiminone A (**1**) and longissiminone B (**2**). Glutinol (**3**) was isolated for the first time from this lichen and which was previously reported from various plants to possess antifungal (Madureira et al., 2003) and analgesic activities (Gaertner et al., 1996). The structures of these compounds were deduced by spectroscopic techniques. Anti-inflammatory and cytotoxic properties of compounds **1**–**3** were also studied.

## 2. Results and discussion

Longissiminone A (**1**) was obtained as a colorless powder. The HREI MS displayed the $M^+$ at *m/z* 210.0501 ($C_{10}H_{10}O_5$, Calcd. 210.0528). The mass fragmentation pattern [*m/z* (rel. int. %) 210 (65), 178 (52), 150 (100), 122 (48) and 94 (43)] was in agreement with the proposed structure **1**. The UV absorptions at 203, 241 and 260 nm indicated the presence of a highly substituted benzene ring (Hatjimanoli et al., 1988), while the IR spectrum showed absorption bands at 3466 (OH), 2854 (CHO), 1701 (C=O) and 1576, 1429 (aromatic C=C) $cm^{-1}$ (Ding and Jia, 1992). The $^1H$ NMR

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spectrum of compound **1** displayed signals for a methoxy group at $\delta$ 3.94 (*s*, 3-$OCH_3$), CHO group at $\delta$ 10.33 (*s*, 1-CHO), methyl group at $\delta$ 2.51 (3H, *s*), aromatic methine proton at $\delta$ 6.28 (*s*, 5-H), and two chelated hydroxyl protons at $\delta$ 12.84 (*s*, 2-OH) and 12.39 (*s*, 6-OH). The $^{13}C$ NMR spectra showed the resonances of 10 carbons including two methine, two methyl and six quaternary carbons. A downfield methine and a quaternary signal at $\delta$ 193.9 and 182.3 were assigned to the aldehydic and ketonic functionalities, respectively (Ding and Jia, 1992). Two quaternary carbon signals at $\delta$ 108.4 (C-1), and 103.8 (C-4) and a methine carbon signal at $\delta$ 112.1 (C-5) were also observed. The $^{13}C$ NMR spectra also showed the presence of a methoxy ($\delta$ 52.3) and an acetyl methyl ($\delta$ 25.2) carbons. In the HMBC spectrum, aldehydic proton ($\delta$ 10.33) showed $^2J$ and $^3J$ correlations with C-1 ($\delta$ 108.4) and C-6 ($\delta$ 166.6), respectively. While, the acetyl methyl protons ($\delta$ 2.51) showed $^2J$, $^3J$ and $^4J$ correlations with carbonyl carbon ($\delta$ 182.3), C-4 ($\delta$ 103.8) and C-5 ($\delta$ 112.1), respectively. Two hydroxyl protons ($\delta$ 12.84 and 12.39) were placed at C-2 and C-6, based on their HMBC correlations with carbon signals at $\delta$ 168.2 and 166.6, respectively. Similarly, the C-5 aromatic methine proton ($\delta$ 6.28) showed HMBC correlations with C-4 ($\delta$ 103.8) and C-6 ($\delta$ 166.6). The methoxy group ($\delta$ 3.94) was substituted at C-3 on the basis of its HMBC correlation with carbon signal at $\delta$ 172.0. The acetyl group at C-4 ($\delta_H$ 2.51) was deduced from the NOE difference studies. These spectroscopic studies led to the structure **1** for this new phenolic derivative.

Longissiminone B (**2**) was obtained as a colorless powder. The HREI MS displayed the $M^+$ at *m*/*z* 244.0072, corresponding to the formula $C_{10}H_{10}O_5Cl$ (Calcd. 244.0098). The mass fragmentation pattern [*m*/*z* (rel. int. %) 246 (9) {$M^+(^{37}Cl)$}, 244 (26) $M^+(^{35}Cl)$, 212 (20), 184 (100) and 156 (18)] supported the proposed structure **2**. The spectral data of compounds **1** and **2** were distinctly similar, with the $^1H$ NMR spectrum of **2** showing no aromatic proton signal. The $^{13}C$ NMR of **2** showed an aromatic carbon at $\delta$ 115.0, where –Cl was thus placed. These spectroscopic studies led to the deduction of structure **2** for this new phenolic derivative.

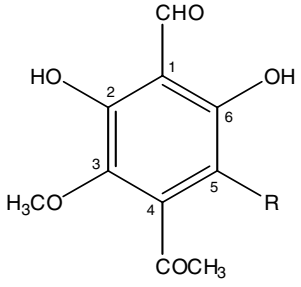
Compound **3** was isolated as a white amorphous material with the HREI MS displaying the $M^+$ at *m*/*z* 426.3818, corresponding to the formula $C_{30}H_{50}O$ (Calcd. 426.3812). The spectral data ($^1H$, $^{13}C$ NMR, MS, UV and IR) of compound **3** was found to be identical to the reported compound, glutinol (Gonzalez et al., 1987). This compound was previously isolated from *Salvia glutinosa* and *Scoparia dulcis* (L.) and this is the first report of its isolation from *U. longissima*.

A cell-based in vitro bioassay was used to examine the anti-inflammatory activity of compounds **1**–**3** (Tan and Berridge, 2000). The concentration of compounds and their $IC_{50}$ are shown in Table 1. Indomethacine and aspirin, clinically used anti-inflammatory drugs, were used as positive control. Compound **1** showed the highest activity comparable to the standards, while compounds **2** and **3** did not show any significant activity at 400 μg/mL conc. Table 1 summarizes the $IC_{50}$ values, i.e. the concentration of compound at which the superoxide production inhibited upto 50% and percentage inhibition of reduction of WST-1 compared to positive control. Cytotoxic evaluation of compounds **1**–**3**, using freshly isolated human neutrophils, showed that they were non-toxic at 200 μg/mL conc. in this assay. The cell viability was compared with 0.1% Triton X-100, as a negative control, which showed 98% cytotoxicity.

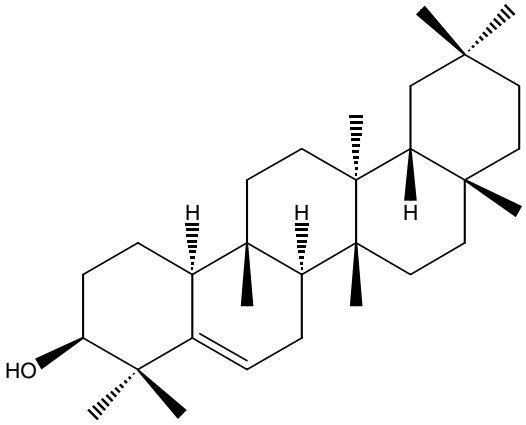
Table 1
$IC_{50}$ (μg/mL) values of compounds **1**–**3** and positive controls at 400-μg/mL concentration

| Compounds | % Inhibition at 400 g/mL | $IC_{50} \pm SEM$[a] (μg/mL) |
|---|---|---|
| Longissiminone A (**1**) | 72.13 | $165.07 \pm 0.848$ |
| Longissiminone B (**2**) | 34.34 | – |
| Glutinol (**3**) | 11.44 | – |
| Indomethacine[b] | 58.82 | $271.212 \pm 5.90$ |
| Aspirin[c] | 70.45 | $50.30 \pm 4.42$ |

[a] SEM was standard error mean of five assays.
[b] Positive control used in the assay.
[c] Positive control used in the assay.




Longissiminone A (**1**) R = H
Longissiminone B (**2**) R = Cl




Glutinol (**3**)

## 3. Experimental

### 3.1. General experimental procedures

The $^1$H NMR spectra were recorded in $CDCl_3$ at 400 MHz on Bruker AM-400 NMR spectrometers with TMS as internal standard using XWIN-NMR on UNIX operating system. The $^{13}$C NMR spectra were recorded in $CDCl_3$ at 125 MHz on Bruker AMX-500 NMR spectrometer. The HREI MS were recorded on Jeol JMS 600 and HX 110 mass spectrometers with the data system DA 5000. The IR spectra were recorded on a Jasco A-302 spectrometer. The UV spectra were recorded on a Hitachi U-3200 spectrophotometer. The optical rotations were measured on JASCO DIP-360 digital polarimeter. The melting point was determined on a Buchi 510 apparatus. Column chromatography (CC) was carried on silica gel column (70–230 mesh). Purity of the samples was checked by TLC on pre-coated silica gel GF-254 preparative plates (20 × 20 cm, 0.25 mm thick, Merck) and were detected under UV light (254 and 366 nm), while ceric sulphate was used as spraying reagent. WST-1 was purchased from Dojindo Laboratories (Kumamoto, Japan), Zymosan A and Triton X-100 were purchased from Sigma Chemicals (St. Louis, MO, USA), while Ficoll paque was purchased from the Pharmacia Biotech Amersham (Uppsala, Sweden). All reagents were of analytical grades. Deionized water was used in all experimental procedures. Absorbance were measured on SpectraMAX 340 microplate reader (Molecular Devices).

### 3.2. Plant material, extraction and isolation of compounds

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

The dried lichen, *U. 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 the gradient of hexane, $CHCl_3$ and acetone (hexane:$CHCl_3$ to $CHCl_3$:acetone mixtures from 9.0:1.0–7.0:3.0, 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).

#### 3.2.1. Longissiminone A (*1*)

White amorphous solid; (24.2 mg, $2.18 \times 10^{-4}$%); m.p. 132 °C; UV (MeOH) $\lambda_{max}$ nm (log $\varepsilon$): 260 (3.90), 241 (4.03), 203 (3.79); IR ($CHCl_3$) $\nu_{max}$ cm$^{-1}$: 3466 (broad OH), 2854 (CHO), 1701 (C=O), 1576, 1429, (aromatic C=C), 1372 (C–O). EIMS *m*/*z* (rel. int. %): 210 $[M]^+$ (65), 178 (52), 150 (100), 122 (48), 94 (43); HREIMS *m*/*z*: 210.0501 ($C_{10}H_{10}O_5$, calcd. 210.0528); $^1$H NMR (400 MHz, $CDCl_3$) $\delta$: 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, $CDCl_3$) $\delta$: 25.2 ($CO\mathit{C}H_3$), 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 ($\mathit{C}OCH_3$), 193.9 (CHO).

#### 3.2.2. Longissiminone B (*2*)

White amorphous material; (20.6 mg, $1.82 \times 10^{-4}$%); m.p. 113 °C; UV (MeOH) $\lambda_{max}$ nm (log $\varepsilon$): 206 (4.41), 237 (4.38), 275 (4.34); IR ($CHCl_3$): $\nu_{max}$ cm$^{-1}$: 3578 (broad OH), 2846 (CHO), 1706 (C=O), 1606, 1446 (aromatic C=C), 1322 (C–O). EIMS *m*/*z* (rel. int. %): 246 [M ($^{37}$Cl)]$^+$ (9), 244 [M ($^{35}$Cl)]$^+$ (26), 212 (20), 184 (100), 156 (18), 128 (10), 93 (13); HREIMS *m*/*z*: 244.0072 ($C_{10}H_{10}O_5Cl$, calcd. 244.0098); $^1$H NMR (400 MHz, $CDCl_3$): 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, $CDCl_3$) $\delta$: 20.7 ($CO\mathit{C}H_3$), 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 ($\mathit{C}OCH_3$), 193.7 (CHO).

#### 3.2.3. Glutinol (*3*)

White amorphous material, (62.8 mg, $5.6 \times 10^{-4}$%). m.p. 212 °C; $[\alpha]_D^{25}$ : 63.3 ($CHCl_3$, *c* 0.71); UV (MeOH) $\lambda_{max}$ nm (log $\varepsilon$): 212 (4.36); IR ($CHCl_3$) $\nu_{max}$ cm$^{-1}$: 3436 (OH), 1622 (C=C). EIMS *m*/*z* (rel. int. %): 426 (55), 408 (16), 274 (100), 259 (83), 205 (42), 152 (27) and 134 (56); HREIMS *m*/*z*: 426.3818 ($C_{30}H_{50}O$, calcd. 426.3812). See $^1$H and $^{13}$C NMR data in the literature (Gonzalez et al., 1987).

### 3.3. Assay procedures

#### 3.3.1. Isolation of human neutrophils

Heparinized fresh venous blood was drawn from healthy volunteers in a local blood bank and neutrophils were isolated by the method of Siddiqui et al. (1995). Whole blood was mixed with Ficoll paque and left for the sedimentation of unwanted red blood cells. After 30 min, the buffy coat was layered on the Ficoll paque in a centrifuge tube. It was centrifuged for 30 min at

1500 rpm. After discarding the supernatant, RBCs were lysed by mixing it with hypotonic ammonium chloride solution (0.83%). It was centrifuged again and the neutrophils were washed with MHS (modified Hank's solution, pH 7.4) and resuspended at conc. of $1 \times 10^7$ cells/mL.

### 3.3.2. Anti-inflammatory assay

Anti-inflammatory activity of test compounds was determined by using modified assay of Tan and Berridge (2000). This in vitro assay is based on the reduction of highly water-soluble tetrazolium salt (WST-1) in the presence of activated neutrophils. Anti-inflammatory activity was determined in a total volume of 250 μL MHS (pH 7.4) containing $1.0 \times 10^4$ neutrophils/mL, 500 μM WST-1 and various concentrations of test compounds. The control contained buffer, neutrophils and WST-1. All compounds were equilibrated at 37 °C and the reaction was initiated by adding opsonized Zymosan A (15 mg/mL), which was prepared by mixing it with human pooled serum, followed by centrifugation at 3000 rpm and pallet was resuspended in PBS buffer. Absorbance was measured at 450 nm. Aspirin and indomethacine were used as positive controls which are widely used as non-steroidal anti-inflammatory drugs (NSAIDs) for the treatment of several inflammatory diseases (MacDonald et al., 2002; Martin et al., 2002). $IC_{50}$ Values were calculated by comparing to the DMSO used as blank and expressed as % inhibition of superoxide anions produced.

### 3.3.3. Cell viability assay

Living (metabolically active) cells reduce tetrazolium salts into colored formazan compounds. Thus, tetrazolium salt-based colorimetric assays detect viable cells exclusively. These sensitive assays can readily be performed in a microtiter plate (MTP) with relatively few cells by using modified method of Berridge et al. (1996). During this study, the human isolated neutrophils ($1 \times 10^7$ cells/mL) were incubated with test compounds for 30 min then WST-1 (250 μM) was added and incubated in shaking water bath at 37 °C for 3 h. The absorbance was measured at 450 nm. Triton X-100 (0.1%) was used as negative control showed 0.00% cell viability. The OD is the mean of the five experimental replicates. % Cell viability was calculated by using the following formula and results are shown in Table 2:

$$\%\ \text{Viability of cells} = (\text{OD test compound}/\text{OD control}) \times 100$$

Table 2
% Cell viability of compounds **1–3** at different concentrations

| Concentration (μg/mL) | % Cell viability of compounds | | |
|---|---|---|---|
| | Longissiminone A (**1**) | Longissiminone B (**2**) | Glutinol (**3**) |
| 200 | 52.26 | 68.85 | 72.29 |
| 100 | 52.91 | 80.98 | 100 |
| 50 | 90.05 | 100 | 100 |
| 25 | 77.18 | 91.75 | 100 |
| 12.5 | 89.68 | 78.81 | 100 |

*Note*. Triton X-100 (0.1%) was used as negative control, showed 0.00% cell viability.

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