Evaluating the in vitro activity of depsidones from *Usnea subfloridana* Stirton as key enzymes involved in Inflammation and Gout

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Running Title: Chemical and Biological profile of *U. subfloridana*
Abstract

Background: Traditionally, *Usnea* genus has significant uses in the treatment of swelling and tumors in Africa and Asia. The aim of the present study was to investigate the chemical constituents present in the acetone extract (AE) of *Usnea subfloridana* Stirton and also to evaluate their anti-inflammatory and anti-gout effects.

Methods: Isolation and characterization of secondary metabolites from AE were evaluated by chromatography and spectral studies. Anti-inflammatory activities were assessed through cyclooxygenases (COX1 and COX2) and 5-lipoxygenase (5-LOX) enzyme inhibition assays, while anti-gout effects were evaluated by xanthine oxidase (XO) inhibition assay.

Results: The existence of five known depsidones, identified as galbinic acid (1), conprotocetraric acid (2), constictic acid (3), salazinic acid (4), and lobaric acid (5), were exposed by chemical investigation of AE and confirmed by spectral data. Using *in vitro* enzyme inhibition assays, it was noticed that all the isolates showed dose-dependent activity against all the tested enzymes. Mainly, compounds 2 and 5 showed better inhibition efficiency on COX2 enzyme with the IC$_{50}$ of 7.17±1.07 and 7.01±0.94 nM, respectively, than the reference drug indomethacin (7.3±0.65 nM). Furthermore, all isolates exhibited potent inhibition effects on the XO enzyme.

Conclusion: The results indicated that *U. subfloridana* can be a favorable natural source for the treatment of inflammation and gout. The compounds 2 and 5 were responsible for these biological actions by regulating pro-inflammatory enzymes, namely COXs, 5-LOX, and XO.

Keywords: 5-Lipoxygenase, Anti-inflammation, Cyclooxygenase, Enzyme inhibition, *Usnea subfloridana*, Xanthine oxidase.
Introduction

Inflammation is a normal response from the immune system to a lesion that heals the injured tissues or neutralizes the invaded pathogens\textsuperscript{1}. Though a self-limiting manner, inflammation can turn into chronic, which leads to many deadly diseases\textsuperscript{2,3} like Alzheimer’s\textsuperscript{4}, cancer\textsuperscript{5,6}, atherosclerosis\textsuperscript{7}, rheumatoid arthritis\textsuperscript{8}, and gout\textsuperscript{8}. The molecular and cellular level studies on inflammation identified two major metabolic pathways responsible for chronic inflammation. The cyclooxygenase (COX) pathway controls the production of prostacyclins, prostaglandins (PGs), and thromboxane (TXA\textsubscript{2}) by COX1 and COX2 enzymes. The lipoxygenase (LOX) channel alters the production of leukotrienes and hydroperoxy fatty acids by 5-LOX, 12-LOX, and 15-LOX enzymes\textsuperscript{9}.

Clinically, non-steroidal anti-inflammatory drugs (NSAIDs) were medications of choice to treat inflammation by inhibiting COX enzymes\textsuperscript{10}. Both COX1 and COX2 enzymes are entirely responsible for physiological production and biosynthesis of PGs and TXA\textsubscript{2}, but COX1 shows a fundamental role involved in the modulation of gastrointestinal, renal, and vascular functions. At the same time, COX2 regulates cytokines, endotoxins, and mitogens in inflammation, pain, and fever\textsuperscript{11}. These observations led to the development of selective COX2 inhibitor drugs. As a result, the introduction of highly selective COX2 inhibitors, well known as coxibs (celecoxib, rofecoxib, etc.), came into existence. But, the coxib series were noticed to have adverse lower gastrointestinal side effects. In 2004, FitzGerald, an American scientist, highlighted a cluster of suspected severe cardiovascular effects and increased risk of cardiovascular diseases emanating from the use of coxib drugs\textsuperscript{12}. Furthermore, several studies related to cardiovascular reactions with coxibs are under process\textsuperscript{13}. However, researchers are fascinating to design and develop novel anti-inflammatory agents with low toxicity and adverse effects.

On the other hand, the usage of natural sources like lichens and their extracts as anti-inflammatory agents is well known for ages. Based on the data of the folklore and documented books, researchers tested lichens and their products for anti-inflammatory properties. They identified many therapeutic agents used to diagnosis acute and chronic inflammation with lesser side effects.

*Usnea* genus belongs to family Parmeliaceae, well recorded in the flora of Asia, and Africa\textsuperscript{14}. This genus consists of around 1000 species worldwide, many of them recorded in countries of Asia\textsuperscript{15,16}. Amongst these, *Usnea subfloridana* Stirton is a fruticose lichen, usually called “Beard lichen,” and “Old man’s beard” in Tamil Nadu, India. In the folklore of many Asian and
African countries, *Usnea* species has been used in the treatment of a fever, skin diseases, swelling, sore throat, and tumors. Mainly, the Asian and African tribes used the species of *Usnea* in the treatment of chronic inflammation and arthritis\(^{17,18}\). Biologically, *U. subfloridana* has been reported to have antibacterial and antifungal activities\(^{19}\). Besides, no chemical examination has been performed on lichen *U. subfloridana*.

Earlier, we have identified and reported the anti-inflammatory capabilities of secondary metabolites and extracts of some lichens\(^{20-24}\). Thus, based on the reports of the folklore and publications on *Usnea* genus as good source for anti-inflammatory agents, the aim of present study is to examine the phytoconstituents present in the acetone extract of under-investigated lichen *U. subfloridana* (AE) employing chromatography and to monitor anti-inflammatory and anti-gout effects of identified secondary metabolites.

**Methods**

**Lichen material**

The lichen *Usnea subfloridana* Stirton (Family: Parmeliaceae) collected on the barks of alpine trees in Wenlock Downs 9th Mile Shooting Point (11°12’18” N and 76°59’92” E), Coimbatore-Ooty-Gundlupet Hwy, Tamil Nadu, India, at 7300 ft. elevation, on 12 December 2018. The sample was authenticated by Dr. D. K. Upreti, Chief Scientist, CSIR-NBRI (National Botanical Research Institute), Lucknow, India, and a voucher specimen (18-035446) was deposited at Lichen herbarium, CSIR-NBRI, India.

**Chemicals and Reagent**

5-lipoxygenase (No. 437996), diclofenac, and xanthine oxidase (No. MAK078) purchased from Sigma Aldrich (USA). COX1 and COX2 (Cat. No.: 560131) purchased from Cayman (USA).

**Extraction and Isolation of compounds**

About 100 g of *U. subfloridana* was shade dried and extracted with acetone at room temperature (3 times). All combined and evaporated under low pressure to obtain an acetone extract of *U. subfloridana* (AE, 5 g, 5%w/w). By using column chromatography (CC) of mesh size 100-200, AE extract (5 g) was fractionated using a hexane/ethyl acetate solvent system (step gradient flow from 100:0, 95:5, 90:10, …, 5:95, 0:100), which yielded five main fractions,
nearly F1-5. Similarly, F1 (200 mg) subjected to CC using the above parameters yielded 1
(110 mg, 0.11%w/w) as colorless sharp needles. By using step gradient flow
dichloromethane/ethyl acetate solvent system (from 100:0, 95:5, 90:10, …, 5:95, 0:100), F2
(250 mg) gave 2 (120 mg, 0.12%w/w) as a pale yellow solid. Similarly, with
dichloromethane/ethyl acetate solvent system, F3 (200 mg) yielded 3 (90 mg, 0.09%w/w) as
colorless needles, F4 (800 g) yielded 4 (500 mg, 0.5%w/w) as pale yellowish needles, F5 (600
g) yielded 5 (400 mg, 0.4%w/w) as a faint yellow solid. Initially, all the compounds obtained
in solid form, they are purified by subjected to re-crystallization using hexane and acetone
(9:1). NMR [Bruker Avance 400 Spectrometer (400 MHz for 1H-NMR and 13C-NMR) and
CNHS (2400 CHNS Organic Elemental Analyzer) analyses were applied for structure
elucidation of isolated compounds.

In vitro assays of anti-inflammatory activity

Cyclooxygenase (COX1/2) inhibitory assay: The abilities of depsidones (1-5) to inhibit
isoenzymes COX-1/2 were performed using COX (ovine/human) inhibitor assay kit (Cayman,
No.: 560131)25. To 10 µl of either COX1 or COX2 added 0.1 M Tris-HCl buffer (960 µl) and
different concentrations of test samples and incubated at 37 °C for 10 min. Later 10 µl of 100
µM arachidonic acid, after 2 min 1 M HCl of 50 µl and Ellman’s reagent, were added. The
absorbance was noted spectrophotometrically at 410 nm against the blank. The % inhibition
was deliberated with the absorbance values by which IC50 values were calculated by linear
regression.

5-lipoxygenase (5-LOX) inhibitory assay: The depsidones (1-5) were tested against 5-LOX
(human recombinant) using 5-LOX assay kit (No. 437996, Sigma Aldrich)26. To 90 µl of 5-
LOX enzyme solution added different test sample concentrations, 100 µl of de chromogen, and
finally added 10 µl of the substrate (arachidonic acid) and gently shake or 10 min and
absorbance was recorded at 490 nm against the blank. The % inhibition was deliberated with
the absorbance values by which IC50 values were calculated by linear regression.

Xanthine oxidase (XO) inhibitory assay: All the isolated depsidones (1-5) were subject to XO
inhibitory assay27 using assay Sigma Aldrich assay kit. To 10 µl of the substrate (xanthine, 5
mM), added of sodium phosphate buffer (470 µl), different test sample concentrations, and 10
µl of XO enzyme and incubated for 5 min at 25 ºC and absorbance was recorded at 295 nm
against the blank. The % inhibition was deliberated with the absorbance values by which IC\textsubscript{50} values were calculated by linear regression.

**Statistical analysis**

The percentage of inhibition outcomes of the current study were denoted as mean±SD, employing one-way ANOVA followed by a t-test, where \( p < 0.05 \) was statistical significance.

**Results**

**Chemical constituents**

Five known depsidones (1-5) were successfully isolated and identified from the acetone extract of *U. subfloridana* (AE) for the first time by utilizing chromatographic and re-crystallization methods and analyses of their spectral NMR data and elemental composition. The obtained data were interrelated with those reported in the previous literature (Figure 1).

**Spectral data for isolated depsidones**

**Galbinic acid (1)** - Colourless sharp needles (110 mg, 0.11%w/w); m.p. 262-263 °C; \textsuperscript{1}H NMR (400 MHz, DMSO-\textit{d}\textsubscript{6}): 1.98 (3H, s, 18-CH\textsubscript{3}), 2.28 (3H, s, 20-CH\textsubscript{3}), 5.27 (2H, s, 16-CH\textsubscript{2}), 6.60 (1H, s, 5-Ar-H), 8.08 (1H, s, 11-Ar-H), 8.32 (1H, s, 14-OH), 8.52 (1H, s, 11-OH), 9.03 (1H, s, 4-OH), 9.52 (1H, s, 11-CHO); \textsuperscript{13}C NMR (400 MHz, DMSO-\textit{d}\textsubscript{6}): 22.14 (C-18), 22.21 (C-20), 60.90 (C-16), 97.59 (C-11), 113.70 (C-1), 114.46 (C-3), 116.57 (C-13), 117.49 (C-5), 127.42 (C-15), 137.09 (C-10), 144.02 (C-9), 152.53 (C-8), 152.94 (C-6), 158.93 (C-14), 163.16 (C-7), 165.75 (C-2), 167.57 (C-4), 167.72 (C-12), 172.77 (C-17), 188.38 (C-19); CHNS analysis for galbinic acid [C\textsubscript{20}H\textsubscript{14}O\textsubscript{11}]: C-55.98, H-3.29(%), calcd. C-55.82, H-3.28(%).

**Conprotocetraric acid (2)** - Pale yellow solid (120 mg, 0.12%w/w); m.p. 234-235 °C; \textsuperscript{1}H NMR (400 MHz, DMSO-\textit{d}\textsubscript{6}): 2.29 (3H, s, 14-CH\textsubscript{3}), 2.85 (3H, s, 15-CH\textsubscript{3}), 5.03 (2H, s, 17-CH\textsubscript{2}), 5.31 (2H, s, 18-CH\textsubscript{2}), 6.50 (1H, s, 12-Ar-H), 7.30 (1H, s, 11-OH), 8.03 (1H, s, 18-OH), 8.65 (1H, s, 5-OH), 8.97 (1H, s, 17-OH), 9.17 (1H, s, 16-COOH); \textsuperscript{13}C NMR (400 MHz, DMSO-\textit{d}\textsubscript{6}): 14.02 (C-15), 22.21 (C-14), 58.06 (C-18), 58.41 (C-17), 114.67 (C-8), 117.30 (C-12), 117.39 (C-10), 120.27 (C-4), 126.29 (C-6), 142.02 (C-3), 146.12 (C-2), 147.96 (C-1), 149.36 (C-13), 160.25 (C-5), 163.16 (C-7), 164.24 (C-9), 164.72 (C-11), 174.00 (C-16); CHNS analysis for conprotocetraric acid [C\textsubscript{18}H\textsubscript{16}O\textsubscript{5}]: C-57.74, H-4.14(%), calcd. C-57.45, H-4.29(%).
Constictic acid (3) - Colourless needles (90 mg, 0.09%w/w); m.p. 204-205 °C; ¹H NMR (400 MHz, DMSO-δ6): 2.31 (3H, s, 19-CH₃), 3.75 (3H, s, 18-OCH₃), 4.94 (2H, s, 16-CH₂), 6.69 (1H, s, 5-Ar-H), 8.13 (1H, s, 11-Ar-H), 8.43 (1H, s, 14-OH), 8.47 (1H, s, 11-OH), 9.14 (1H, s, 16-OH), 9.31 (1H, s, 17-CHO); ¹³C NMR (400 MHz, DMSO-δ6): 22.00 (C-19), 58.25 (C-18), 58.39 (C-16), 97.58 (C-11), 114.81 (C-13), 114.97 (C-5), 115.07 (C-1), 118.53 (C-3), 130.00 (C-15), 136.06 (C-10), 142.80 (C-9), 150.11 (C-6), 154.56 (C-8), 161.01 (C-4/14), 163.15 (C-7), 166.07 (C-2), 167.71 (C-12), 186.78 (C-17); CHNS analysis for constictic acid [C₁₉H₁₄O₁₀]: C-56.72, H-3.41(%), calcd. C-56.72, H-3.51(%)²⁸.

Salazinic acid (4) - Pale yellowish needles (500 mg, 0.5%w/w); m.p. 274-275 °C; ¹H NMR (400 MHz, DMSO-δ6): 2.25 (3H, s, 18-CH₃), 4.93 (2H, s, 16-CH₂), 6.57 (1H, s, 5-Ar-H), 7.28 (1H, s, 16-Ar-H), 7.62 (1H, s, 11-OH), 8.08 (1H, s, 11-Ar-H), 8.46 (1H, s, 14-OH), 8.92 (1H, s, 4-OH), 9.46 (1H, s, 17-CHO); ¹³C NMR (400 MHz, DMSO-δ6): 22.21 (C-18), 58.40 (C-16), 97.59 (C-11), 113.70 (C-1), 114.46 (C-3), 114.82 (C-13), 117.49 (C-5), 130.01 (C-15), 136.07 (C-10), 142.81 (C-9), 152.94 (C-6), 154.57 (C-8), 161.01 (C-14), 163.16 (C-7), 165.75 (C-2), 167.57 (C-4), 167.72 (C-12), 188.37 (C-17); CHNS analysis for salazinic acid [C₁₈H₁₂O₁₀]: C-55.57, H-3.20(%), calcd. C-55.68, H-3.12(%)²⁸.

Lobaric acid (5) - Faint yellow solid (400 mg, 0.4%w/w); m.p. 196-197 °C; ¹H NMR (400 MHz, DMSO-δ6): 0.85 (3H, s, 24-CH₃), 0.86 (3H, s, 19-CH₃), 1.20-1.26 (6H, m, 18,22,23-CH₂), 1.39-1.42 (2H, m, 17-CH₂), 1.47-1.52 (2H, m, 21-CH₂), 2.48-2.51 (2H, t, J = 4, 8 Hz, 16-CH₂), 3.11-3.14 (2H, t, J = 4, 8 Hz, 20-CH₂), 3.68 (3H, s, 14-OCH₃), 6.72 (1H, s, 13-Ar-H), 6.82 (1H, s, 3-Ar-H), 6.86 (1H, s, 5-Ar-H), 7.68 (1H, s, 12-OH), 8.56 (1H, s, 25-COOH); ¹³C NMR (400 MHz, DMSO-δ6): 12.12 (C-19/24), 20.28 (C-18), 21.04 (C-23), 26.76 (C-17), 28.16 (C-21), 28.74 (C-22), 29.45 (C-20), 38.84 (C-16), 54.14 (C-14), 104.19 (C-13), 106.48 (C-3), 108.63 (C-5), 108.74 (C-11), 112.94 (C-1), 136.02 (C-10), 139.31 (C-9), 142.27 (C-6), 146.43 (C-8), 157.57 (C-2), 157.78 (C-12), 160.21 (C-7), 162.02 (C-4), 169.95 (C-25), 200.51 (C-15); CHNS analysis for lobaric acid [C₂₅H₂₈O₈]: C-65.40, H-6.10(%), calcd. C-65.78, H-6.18(%)²⁸.

Anti-inflammatory activity

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The in vitro anti-inflammatory (Table S1-S2) and anti-gout (Table S3) effects of isolated depsidones (1-5) were performed using COX-1/2, 5-LOX, and XO enzymes and the results were reported in IC$_{50}$ values (Table 1).

The concentration required for 50% inhibition of COX1 enzyme for compounds 1, 2, 3, 4 and 5 found to be 18.9±1.20, 11.9±1.12, 21.5±2.73, 15.7±1.03 and 17.1±1.24 nM, respectively, whereas reference drug, indomethacin with 7.3±0.44 nM (Table 1). From the results of COX2 enzyme inhibitory assay, it is noticed that the compounds 2 and 5 showed potent inhibition efficiency on COX2 enzyme with the IC$_{50}$ of 7.17±1.07 and 7.01±0.94 nM, respectively, compared to indomethacin with 7.3±0.65 nM (Table 1). Moreover, the IC$_{50}$ values of compound 1, 3, and 4 on COX2 found to be 11.9±1.30, 21.8±1.75, and 18.5±1.21 nM, respectively, while indomethacin with 7.30±0.65 nM (Table 1).

The concentration of 1, 2, 3, 4 and 5 needed to inhibit 5-LOX activity at 50% was found to be 21.8±1.35, 11.4±1.59, 19.3±1.55, 22.4±2.03 and 16.6±1.30 nM, respectively, while that of diclofenac was 9.4±0.52 nM, respectively (Table 1). Except compound 3, all the isolated depsidones 1, 2, 4 and 5 showed noteworthy inhibition of XO enzyme with IC$_{50}$ values of 13.0±1.40, 14.6±1.02, 19.3±1.68 and 14.6±1.35 nM, respectively, whereas allopurinol with 2.25±0.15 nM (Table 1). The concentration of 3 required for 50% reticence of the XO enzyme was found to be 24.6±2.17 nM (Table 1).

Discussion

Lichens are recognized as an integral part of all ecosystems that can colonize and grow on bare rock surfaces, soil, trees, or even in intertidal zones and freshwater streams$^{14,16}$. It has been reasoning that lichens produced unique substances that support their survival and growth in extreme conditions. Different groups have applied chromatography techniques to analyze lichen extracts and elucidated approximately 1050 unique phytoconstituents, to date, which falls in the classes of carbohydrates, amino-acid derivatives, chromones, xanthones, anthraquinones and naphthoquinones, depsides, tridepsides, depsidones, steroids, etc.$^{18}$

Among all classes of lichen constituents, depsidones are the most remarkable secondary metabolites, comprising of two 2,4-dihydroxybenzoic acid rings connected by both ester and ether bonds. Also, they are well-acknowledged to have antibacterial, antifungal, antioxidant, anti-inflammatory, and cytotoxic properties$^{29}$. On the other hand, the Usnea genus is the biggest of the fruticose lichens comprising about 1000 species around the world. The studies on species of Usnea are well-reported to have a significant content of depsidones such as usnic.
acid, galbinic acid, salazinic acid, conprotocetraric acid, and constictic acid, etc. Previously, these compounds are screened for their antimicrobial and antioxidant activities\textsuperscript{30}.

In our phytochemical analysis of the acetone extract of the lichen \textit{U. subfloridana} (AE) yielded five known depsidones (1-5), which affords novel data on the chemical profile of \textit{U. subfloridana}. Previously, galbinic acid (1) was isolated from \textit{Usnea undulata} Stirt., conprotocetraric acid (2) was isolated from \textit{Usnea trichodeoides} Vain., constictic acid (3) was isolated from \textit{Usnea aciculifera} Vain., salazinic acid (4) and lobaric acid (5) were isolated from \textit{Usnea barbata}\textsuperscript{28}. The \textit{in vitro} enzymatic screening of these depsidones (1-5) from \textit{U. subfloridana} proved its aptitude to treat inflammation and gout. It was even justifying that compounds 2 and 5 have potent inhibition of COX2 and XO enzymes.

As said earlier, inflammatory is regulated by higher levels of eicosanoids, namely prostaglandins, thromboxanes, and leukotrienes in the human body. COXs and 5-LOX are key enzymes that catalyzes the production of prostaglandins, thromboxanes and leukotrienes, and hydroperoxy fatty acids from arachidonic acid\textsuperscript{31}. Particularly, inhibition of any one of the eicosanoids will activate the other pathway and prolongs inflammation. For instance, inhibition of only prostaglandins will lead to elevated levels of leukotrienes by activation of the alternative path, i.e., 5-LOX pathway. Thus, routes of COXs and 5-LOX are chosen for the rate-limiting steps to reduce pain, as well as inflammation. Therefore, COX and 5-LOX (dual inhibitors) drugs inhibit the production of eicosanoids (prostaglandins, thromboxanes, and leukotrienes) and entirely prevent inflammation by lesser adverse effects\textsuperscript{33,34}. Generally, NSAIDs are the drugs of choice to control the production of eicosanoids, and eventually relief from inflammation\textsuperscript{32}. The outcomes of our study exhibited that isolated depsidones (1-5) act as a dual (COX and 5-LOX) inhibitor, while compounds 2 and 5 highly constrain towards COX2 enzyme.

On the other hand, XO is an enzyme that catalyzed purines xanthine/hypoxanthine to form uric acid. To some extent, the formation of uric acid does not cause any biological effects in the human body. Beyond the limits, the higher accumulation of uric acid, especially in the joints of the human body, chiefly leads to painful inflammation in joints, termed as gout\textsuperscript{35,36}. Also, XO is an excellent source for free radicals (containing oxygen) that cause inflammatory-related diseases such as atherosclerosis and cancer\textsuperscript{37-40}. Hence, inhibition of XO results in controlling gout, as well as its related conditions. This study suggested that isolated depsidones (1-5) possess prominent XO inhibitory effects (Table 1) that might be supportive in the treatment of
gout and its complications. Taken together, lichen *U. subfloridana* scientifically proved as a potential source for the management of inflammation and gout.

**Conclusion**

To conclude, this is the first report on the chemical and anti-inflammatory examination of *U. subfloridana*. The significant secondary metabolites present in acetone extract from the *U. subfloridana* (AE) were identified as 1-5. The outcomes indicated that these secondary metabolites (1-5) displayed anti-inflammatory and anti-gout effects by regulating enzymes COXs, 5-LOX, and XO, which justifies the traditional uses of *U. subfloridana*. Hence, this study identified a new natural source (*U. subfloridana*) to treat inflammation and gout. However, further studies (docking and in vivo) are required to determine the molecular mechanism of action of these isolated depsidones in the control of inflammation and related diseases.

**References:**


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*<sup>n=3, mean ± SD values</sup>

Table 1. IC<sub>50</sub> values of 1-5 against Cyclooxygenase (COX1 & COX2), 5-lipoxygenase (5-LOX) and xanthine oxidase (XO) enzymes

Figure 1. Known depsidones (1-5) isolated from acetone extract of *Usnea subfloridana* Stirton

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