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Dibenzofurans from *Cladonia corniculata* Ahti and Kashiw inhibit key enzymes involved in inflammation and gout: An *in vitro* approach

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The usage of natural sources like lichen extracts and their metabolites 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 and they identified many therapeutic agents used to diagnosis acute and chronic inflammation with lesser side effects. In this study, we aimed to examine the acetone extract of *Cladonia corniculata***(AE)**and its metabolites for *in vitro* anti-inflammatory and anti-gout effects. Through chemical investigation, we have successfully isolated and identified three known dibenzofurans, namely alectosarmentin **1**, porphyrilic acid **2**, and strepsilin **3** from the **AE**. All isolated dibenzofurans**1-3** showed prominent inhibition of cyclooxygenase enzymes, whereas compounds **1** and **2** exhibited noticeable inhibition of 5-lipoxygenase enzyme. Only compound **2** showed significant inhibition of xanthine oxidase enzyme with an IC_{50} value of 80.17±0.66 µg/mL, while standard drug allopurinol with 9.10±0.64 µg/mL. The results indicate that *C. corniculata*can be a favourable natural source for the treatment of inflammation and gout and these actions are linked to the natural active dibenzofurans**1-3**.

Keywords: 5-Lipoxygenase, *Cladonia corniculata*, cyclooxygenase, lichen, xanthine oxidase

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[1]. It has been reasoning that lichens produced unique substances that support their survival and growth in extreme conditions[2]. Different groups have applied chromatography techniques to analyse 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*.[3,4] 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[4,5].

Cladonia genus belongs to family Cladoniaceae comprising about 400 species around the world, well recorded in the flora of Bhutan, India, Nepal, Himalayas, Thailand, and Vietnam[6]. *Cladonia corniculata* Ahti & Kashiwis a fruticose lichen, usually called as "Cup Lichen".[7] In the folklore, *Cladonia* species has wide applications in the treatment of microbial infections, inflammation, and tumours. Mainly, the Asian tribes used *C. corniculata* in the treatment of microbial infections and chronic inflammation. Biologically, *Cladonia* reported for anticancer[8,9], antifungal[10,11], antioxidant[9], anti-microbial[9], anti-inflammatory[12], allelopathic[13] and bioherbicidal[13] potentialities. To date, no proper chemical investigation has attempted to evaluate the chemical constituents of whole lichen *C. corniculata*. Thus, based on the reports of the folklore and publications on *Cladonia* genus as good source for anti-inflammatory agents, the present study aims to examine the phyto-constituents present in the acetone extractof under-investigated lichen *C. corniculata* **(AE)** employing chromatography and to monitor anti-inflammatory and anti-gout effects of identified secondary metabolites.

Results and Discussion

Chemistry

Three known dibenzofurans **(1-3)** were successfully identified from the **AE** by utilizing chromatographic and analyses of their spectral NMR data and elemental

composition. The obtained data were interrelated with those reported in the previous literature.

Compound **1** [Alectosarmentin][14](Figure 1): m.p.290-291°C. R_f: 0.6 (hexane:ethyl acetate, 1:1); ^{1}H NMR (400 MHz, DMSO-d_6): δ2.27 (s, 1H, OH), 2.98 (s, 1H, OH), 3.93 (s, 1H, OH), 5.03 (s, 1H, OH), 6.35 (s, 1H, Ar-H), 6.45 (s, 1H, Ar-H), 7.12-7.13 (d, 2H,*J*= 4 Hz, Ar-H), 7.61-7.63 (d, 2H,*J*= 8 Hz, Ar-H) (Fig. S1);^{13}C NMR (400 MHz, DMSO-d_6): δ94.96 (C-8), 100.15 (C-6), 104.82 (C-4), 116.49 (C-12/14), 122.48 (C-10), 131.48 (C-11/15), 136.89 (C-2), 146.42 (C-1), 158.13 (C-9), 160.36 (C-13), 160.49 (C-5), 164.68 (C-7), 175.78 (C-3) (Fig. S2). Anal.Calcd for: C,62.94; H,3.52. Found: C,62.96; H,3.54%. ESI-MS: Calcd *m/z* for $C_{15}H_{10}O_6$: 286.23 [M].Found: 285.14 [M + H^+], 287.24 [M - H^+] (Fig. S3).

Compound **2** [Porphyrilic acid][15,16](Figure 1): m.p.301-302°C. R_f: 0.5 (hexane:ethyl acetate, 1:1); ^{1}H NMR (400 MHz, DMSO-d_6): δ2.97 (s, 1H, OH), 3.55 (s, 1H, OH), 3.85 (s, 3H, OCH_3), 3.87 (s, 3H, OCH_3), 5.03 (s, 1H, OH), 6.23 (d, 1H,*J*= 1 Hz, Ar-H), 6.24-6.25 (d, 1H, *J*= 4 Hz, Ar-H), 6.85-6.87 (d, 1H, *J*= 8 Hz, Ar-H), 7.02-7.06 (m, 2H, Ar-H) (Fig. S4);^{13}C NMR (400 MHz, DMSO-d_6): δ57.07 (C-10), 57.81 (C-17), 93.54 (C-8), 98.70 (C-6), 106.03 (C-4),113.24 (C-15), 116.39 (C-12), 121.00 (C-16), 124.86 (C-11), 138.13 (C-2), 146.92 (C-13), 147.87 (C-1), 150.82 (C-14), 159.09 (C-9), 161.63 (C-5), 166.42 (C-7), 176.27 (C-3) (Fig. S5). Anal. Calcd for $C_{16}H_{10}O_7$: C,61.15; H,3.21. Found: C,61.16; H, 3.24%. ESI-MS: Calcd *m/z* for $C_{16}H_{10}O_7$: 314.24 [M].Found: 331.63 [M + H^+], 329.20 [M - H^+] (Fig. S6).

Compound **3** [Strepsilin][14](Figure 1): m.p.324-325°C. R_f: 0.4 (hexane:ethyl acetate, 1:1); ^{1}H NMR (400 MHz, DMSO-d_6): δ2.77 (s, 1H, OH), 3.01 (s, 1H, OH), 3.50 (s, 1H, OH), 3.87 (s, 1H, OH), 5.01 (s, 1H, OH), 6.19 (d, 1H,*J*= 1 Hz, Ar-H), 6.19-6.20 (d, 1H, *J*= 4 Hz, Ar-H), 6.77-6.78 (d, 1H,*J*= 4 Hz, Ar-H), 6.98-7.01 (m, 2H, Ar-H) (Fig. S7);^{13}C NMR (400 MHz, DMSO-d_6): δ94.81 (C-8), 99.99 (C-6), 104.66 (C-4), 116.36 (C-11), 116.67 (C-14), 122.01 (C-15), 122.19 (C-10), 137.48 (C-2), 145.68 (C-12), 147.21 (C-1), 148.73 (C-13), 157.97 (C-9), 160.34 (C-5), 164.52 (C-7), 175.62 (C-3) (Fig. S8). Anal. Calcd for: $C_{15}H_{10}O_5$: C,66.67; H,3.73. Found: C,66.62; H,3.74%. ESI-MS: Calcd *m/z* for $C_{15}H_{10}O_5$: 270.23 [M]. Found 303.66 [M + H^+], 301.25 [M - H^+] (Fig. S9).

Anti-inflammatory anti-gout activity

The *in vitro* anti-inflammatory and anti-gout effects of isolated compounds **(1-3)** were performed using cyclooxygenase (COX1/2), 5-lipoxygenase (5-LOX), and xanthine oxidase(XO) enzymes, and the results were reported in IC_{50} values (Table I). The concentration required for 50% inhibition of COX1 enzyme for compounds **1**,**2**,**3** and **AE** were found to be 82.17±2.24, 56.75±0.42, 89.58±0.21 and 42.11±1.13 µg/mL, respectively, whereas reference drug, indomethacin with 5.74±0.68 µg/mL (Table I). From

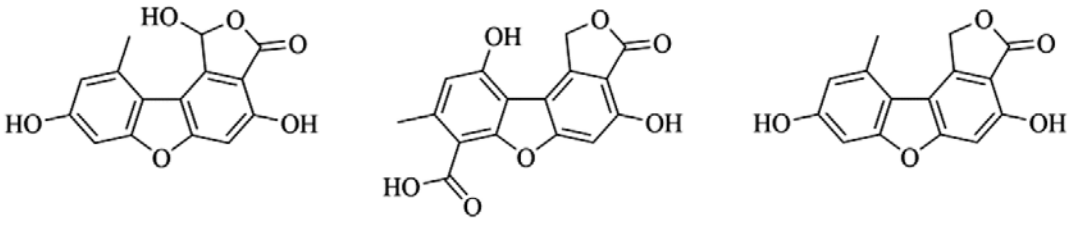

Figure 1 — Chemical representation of isolated dibenzofurans **1-3** from *Cladonia corniculata*

Table I — Effects of **1-3** and **AE** on cyclooxygenase (COX1/2), 5-lipoxygenase (5-LOX), and xanthine oxidase (XO) enzymes

Sample	IC_{50} values (µg/mL)*			
	COX1	COX2	5-LOX	XO
1	82.17±2.24	42.18±2.96	71.14±2.17	>100
2	56.75±0.42	65.18±0.97	49.38±0.28	80.17±0.66
3	89.58±0.21	85.25±0.54	>100	>100
AE	42.11±1.13	50.15±1.15	69.18±1.87	82.16±2.18
Standard	5.74±0.68	6.17±0.71	7.00±0.74	9.10±0.64

*mean±SD values (*n*=3)

the results of COX2 enzyme inhibitory assay, it was noticed that the compounds **1**,**2**,**3** and **AE** showed potent inhibition efficiency on COX2 enzyme with the IC_{50} of 42.18±2.96, 65.18±0.97, 85.25±0.54 and 50.15±1.15 µg/mL, respectively, compared to indomethacin with 6.17±0.71 µg/mL (Table I). The concentration of **1**,**2** and **AE** needed to inhibit 5-LOX activity at 50% was found to be 71.14±2.17, 49.38±0.28 and 69.18±1.87 µg/mL, respectively, while that of diclofenac was 7.00±0.74 µg/mL (Table I).

Inflammation is regulated by higher levels of eicosanoids, namely prostaglandins, thromboxanes, and leukotrienes in the human body[17,18]. COXs and 5-LOX are key enzymes that catalyse the production of prostaglandins, thromboxanes and leukotrienes, and hydroperoxy fatty acids from arachidonic acid[19,20]. Particularly, inhibition of any one of the eicosanoids will activate the other pathway and prolongs inflammation[21]. 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[22]. Therefore, COXs and 5-LOX (dual inhibitors) drugs inhibit the production of eicosanoids (prostaglandins, thromboxanes, and leukotrienes) and entirely prevent inflammation by lesser adverse effects[21–23]. Generally, NSAIDs are the drugs of choice to control the production of eicosanoids, and eventually relief from inflammation[24]. The outcomes of our study exhibited that isolated compounds **1**,**2** and **3** act as both COXs and 5-LOX (dual) inhibitors, whereas compound **3** prominently inhibit COX1/2 enzymes (Table I).

The isolated compound **2** and **AE** exhibited significant inhibition of XO enzyme with IC_{50} values of 80.17±0.66 and 82.16±2.18 µg/mL, respectively, whereas allopurinol with 9.10±0.64 µg/mL (Table I). The concentration of compounds **1** and **3** required for 50% reticence of the XO enzyme was found to be above 100 µg/mL (Table I).

XO is an enzyme that catalysed purines xanthine/hypoxanthine to form uric acid. To some extent, the formation of uric acid does not cause any biological effects in human body[25]. Beyond the limits, the higher deposition of uric acid, especially in the joints of the human body, leads to painful inflammation in joint pains, termed as gout[25,26]. Also, XO is an excellent source for free radicals (containing oxygen) that cause inflammatory-related diseases such as atherosclerosis and cancer[26]. Hence, inhibition of XO results in controlling gout, as well as its related conditions. This study suggests that isolated compound **2** possess prominent XO inhibitory effects (Table I) that might be supportive in the treatment of gout and its complications. Also, based on the chemical structures, it is interesting to notice that the carboxylic acid (-COOH) group present in compound **2** plays a crucial role in attaining the biological activity. Taken together, *C. corniculata* scientifically proved as a potential source for the management of inflammation and gout.

Material and Methods

Collection

The whole lichen of *Cladonia corniculata* Ahti & Kashiw was collected at Seshachalam Hills, Tirupati, Andhra Pradesh, India, in February 2019, and a voucher specimen (DB-SVU-2019-3478) has been deposited at Department of Botany, Sri Venkateswara University, Tirupati, Andhra Pradesh, India.

Extraction and Isolation

The whole lichen (250 g) was dried and powdered and extracted three times with acetone (96%) at 25°C. All extracts were combined and concentrated under low pressure to obtain an acetone extract of *C. corniculata* (**AE**,5 g)[27]. By using column chromatography (CC) of mesh size 100-200, **AE** extract (3 g) was fractionated using a hexane/ethyl acetate solvent system (step gradient flow), which yielded three main fractions, namely F1-3. Similarly, F1 (700 mg) subjected to CC using the above parameters yielded **1** (450 mg) as sharp yellow needles. By using step gradient flow dichloromethane/ethyl acetate solvent system, F2 (250 g) gave **2** (150 mg) as colourless needles. Similarly, with dichloromethane/ethyl acetate solvent system, and F3 (550 g) yielded **3** (200 mg) as colourless needles. All the isolated compounds were recrystallized using acetone and hexane (9:1).

Anti-inflammatory assays

Cyclooxygenase (COX1/2) inhibitory assay

The abilities of compounds **(1-3)** and **AE** to inhibit isoenzymes COX-1/2 were performed using COX (ovine/human) inhibitor assay[28] kit (Cayman, No.: 560131). To 10 µL of either COX1 or COX2 added 960 µL of 0.1 M Tris-HCl buffer and different concentrations of test samples and incubated for

10 min at 37°C. 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 percentage of inhibition was calculated with the OD values by which IC_{50} values were determined by linear regression.

5-Lipoxygenase (5-LOX) inhibitory assay

The compounds **(1-3)** and **AE** were tested against 5-LOX (human recombinant) using 5-LOX assay[28] kit (No. 437996, Sigma Aldrich). 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 shaken for 10 min and absorbance was recorded at 490 nm against the blank. The percentage of inhibition was calculated with the OD values by which IC_{50} values were determined by linear regression.

Anti-gout assay

Xanthine oxidase (XO) inhibitory assay

All the isolated compounds **(1-3)** and **AE** were subject to XO inhibitory assay[29] 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 room temperature and absorbance was recorded at 295 nm against the blank. The percentage of inhibition was calculated with the OD values by which IC_{50} values were determined by linear regression.

Conclusion

To conclude, the results of the present study indicated that the dibenzofurans **(1-3)** from acetone extract of *C. corniculata* displayed anti-inflammatory activity by inhibiting COXs and 5-LOX, and anti-gout activity by XO inhibition. The key metabolite responsible for *in vitro* activities is claimed to be compound **2**. The results provide evidence that supports the traditional uses of *C. corniculata*. Also, these findings suggest that the lichen *C. corniculata* can take an account as a good natural source of remedial medicine for inflammation and gout. Hence, the results of the current study remain useful for further research to identify the potential bioactive molecules from *Cladonia* genus. The future scope is to identify the binding affinity of compounds **1, 2** and **3** against COXs, 5-LOX, and XO enzymes using *in silico* studies, which eventually helps in selective derivatization of parent moieties.

Supplementary Information

Supplementary information is available in the website http://nopr.niscair.res.in/handle/123456789/60.

Conflict of interest

There is no conflict of interest between any of the authors.

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