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Anti-inflammatory properties of *Dirinaria consimilis* extracts in albino rats

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ABSTRACT

Background

Earlier, the lichens are used in traditional medicines by different cultures across the world. As the *Dirinaria* genus has been shown to be biologically active against inflammation in folklore, we assessed the *in vitro* and *in vivo* anti-inflammatory profile of *Dirinaria consimilis*.

Material and methods

Initially, the hydroalcoholic extract of lichen, *D. consimilis* **(Dc-HE)** was prepared and re-extracted with *n*-hexane, chloroform, ethyl acetate, acetone and methanol. The resultant extracts were evaluated for their *in vitro* (protein denaturation method), acute toxicity and *in vivo* (formalin-induced rat paw oedema assay) anti-inflammatory studies.

Results

Among all the tested extracts, the acetone and chloroform extract of *D. consimilis* depicted prominent anti-inflammatory activity in both the bioassays. The acetone extract inhibited protein denaturation with IC_{50} value of about 468 µg/mL while the standard (Indomethacin) with 120 µg/mL. Moreover, the **Dc-HE** was screened for acute toxicity studies in male albino rats up to 2000 mg/Kg b.w dosage. The *in vivo* anti-inflammatory analysis of acetone extract (200 mg/mL) showed potent reduction of rat paw oedema nearer to that of the standard, whereas chloroform extract depicted moderate depletion and the other extracts revealed mild inhibitory profile against inflammation.

Conclusion

This study reveals that the lichen, *D. consimilis* might be a good source of anti-inflammatory agents.

Keywords

acute toxicity, *Dirinaria consimilis* (Stirton) D.D. Awasthi, paw oedema, protein denaturation method

Introduction

Inflammation is a usual stimuli to injury and includes the response of the immune system in neutralizing the invading microorganisms, repair of the damaged tissues thereby promoting wound healing. [1] Inflammation process though a self-limiting process, it can become chronic and can further lead to several other serious inflammatory diseases. [2, 3] The chronic inflammations may lead to various diseases such as cancer [4], atherosclerosis [5], Alzheimer's [6] and rheumatoid arthritis. [7] Historically, Willow bark extracts were used to treat the fever, inflammation and pain, thereafter the non-steroidal anti-inflammatory drugs (NSAIDs) which include organic acids and non-acidic compounds and coxibs (celecoxib, rofecoxib, etc) were drugs of choice to treat inflammation. [7, 8] In contrast, these synthetic drugs were noticed to have adverse effects on cardiac, gastrointestinal, renal, and vascular functions. [8] Therefore, keeping in mind of the aforementioned drawbacks, the evolution of novel anti-inflammatory agents having lesser adverse effects are highly desirable. In this context, the screening of anti-inflammatory activity of natural product extracts (or) natural product like is an upcoming topic of research owing to lesser side effects. [9, 10]

With this in mind, our attention was drawn towards "Lichens - mutualistic existence of algae and fungi". Lichen and their secondary metabolites exert a diverse range of pharmacological actions including analgesic, antibiotic, anti-inflammatory, antimycotic, antipyretic, antiviral and cytotoxic effects. [11] *Dirinaria consimilis* (Stirton) D.D. Awasthi is a foliose lichen belongs to the *Dirinaria* genus which possesses anticaries, antioxidant, antimicrobial, cytotoxic and larvicidal activities. [12-15] However, there are no pharmacological study reports revealing anti-inflammatory potentiality of *D. consimilis* and also keeping in mind of the aforementioned the biological profile of *Dirinaria* genus and the drawbacks of anti-inflammatory therapeutics, we anticipated *Dirinaria consimilis* might be active good source for anti-inflammatory activity.

Material and methods

The present study was undertaken in the Pharmaceutical Chemistry Department, AU College of Pharmaceutical Sciences, Andhra University, Visakhapatnam, India, during the period of 2016-2017.

Collection

The specimens of mangrove lichen, *Dirinaria consimilis* (Stirton) D. D. Awasthi was collected on the bark of mangrove plant, *Excoecaria agallocha* from Vainateya Island, Godavari estuary, Andhra Pradesh, India (16°44'48"N and 81°98'19"E with 0 m elevation) in February, 2015. This species were authenticated by Dr. D. K. Upreti, CSIR-National Botanical Research Institute (NBRI), Lucknow and deposited at Lichen herbarium, CSIR-NBRI, Lucknow, India with accession numbers 15-027173.

Extraction

The lichen specimens were gently collected from the barks of mangrove plant and shade dried. The dried lichen materials (150 g) were powdered, suspended in ethanol-water (1:1) for a week and evaporated under reduced pressure to obtain hydroalcoholic extracts from *D. consimilis* (**Dc-HA**, 10.91 g). The **Dc-HA** were re-extracted with solvents of increasing polarity, concentrated to obtain dry extracts of acetone for three times, dried over anhydrous sodium sulphate and concentrated to obtain dry of *D. consimilis* i.e., *n*-hexane (**DH**, 500 mg), chloroform (**DC**, 540 mg), ethyl acetate (**DE**, 602 mg), acetone (**DA**, 444 mg) and methanol (**DM**, 780 mg), which were preserved at 4°C earlier to use.

In vitro anti-inflammatory assay

Protein denaturation method [16] was employed for the evaluation of *in vitro* anti-inflammatory activity for *D. consimilis* extracts in three sets and the Mean±SD values are reported. Bovine serum albumin protein was used in this study. Study Design - The protein was solubilized to 1% concentration using sodium phosphate buffer (50 mM, pH 6.4). To 0.2 mL of prepared protein, 0.1 mL of extract dissolved in DMSO (Sample size - 0.1, 0.2, 0.4, 0.6, 0.8 and 1 mg/mL for sample) was added and final volume adjusted to 5 mL with buffer. Then the sample tubes are incubated at 37°C for 20 min. Exclusion criteria - Thereafter, the tubes are heated in steam bath at 95°C for 20 min and then cooled to room temperature. Finally, the turbidity in the cooled tubes are measured at 660 nm by UV–Visible Spectrophotometer (Model SL 210, Elico India Ltd.). The percentage inhibition of serum albumin protein denaturation was determined as follows and IC_{50} values were calculated by plotting concentration vs percentage inhibition.

Percentage inhibition = $[(C-S)/C] \times 100$
where C is absorbance of Control
S is absorbance of Sample

Animals

Healthy albino rats of both sex weighing 180-200 g, matured between 2-3 months are fed with water (*ad libitum*) and standard pellet diet are used for the contemporary study. Selected animals were adapted to the standard conditions for at least seven days before the exploration. The experimental protocols are maintained as per the Institutional Animals Ethical Committee and Control and Supervision of Experiments on Animals (CPCSEA) guidelines for experimental clearance (516/PO/c/01/CPCSEA).

Acute Toxicity studies

The acute toxicity studies of the **Dc-HA** were performed by Oral Acute Toxic Class method [17] and as per OECD

Table 1: *In vitro* anti-inflammatory effect of *D. consimilis* extracts against serum albumin protein (n=3)

Sample	Percentage inhibition at different concentration (%)					
	0.1 mg/mL	0.2 mg/mL	0.4 mg/mL	0.6 mg/mL	0.8 mg/mL	1 mg/mL
DH	3.15±0.69	4.10±0.09	7.66±0.65	9.29±0.45	15.30±0.89	21.04±0.73
DC	13.40±1.15	17.45±2.96	25.95±0.82	41.01±1.62	57.92±0.81	71.04±1.77
DE	0.82±0.018	2.72±0.90	4.36±0.87	8.47±1.18	15.84±0.97	23.50±0.99
DA	20.22±0.09	34.96±1.16	45.64±1.11	58.44±2.09	71.08±2.72	80.08±2.61
DM	0.27±0.47	1.36±0.45	2.18±0.43	3.82±0.89	10.94±0.72	14.22±0.79
Indo	48.63±0.62	62.57±2.21	70.46±2.27	78.70±1.97	85.79±3.28	93.71±1.29

guidelines for their LD_{50} in male rats. In this evaluation, two groups of male albino rats (n=6) administered intraperitoneally with at 1000 and 2000 mg/Kg body weight (b.w) and all the animals were retained under examination for 24 h. During this duration biological and physiological changes like aggressiveness, respiratory movements, mortality, skin changes etc., were noted.

***In vivo* assay**

The *in vivo* anti-inflammatory activity was performed by formalin-induced hind rat paw oedema assay by using plethysmographic measurement of rat paw oedema caused by sub-plantar administration of 1%w/v formalin in the hind rat paw [18, 19]. The healthy albino rats of both sex were categorized into batches containing six albino rats (n=6) each. First batch served as control (administered only with 0.5% carboxymethyl cellulose), second for standard drug, indomethacin (100 mg/Kg b.w) and the remaining batches served for test extracts. In order to attain accurate measurement of the paw oedema, spotting was done at just behind the tibiotorsal junction on both the hind rat paws, so that to ensure constant paw volume the paw was inserted in mercury column up to fixed mark. Inclusion criteria - All the test samples (100, 200 mg/Kg b.w) were deliquesced in 0.5% carboxymethyl cellulose and administered intraperitoneally. Immediately 30 min after sample dosage, 0.1 mL of 1% w/v formalin was administered in the plantar region of the rat left paw. The non-inflammated right paw is assisted as a reference standard. Exclusion criteria - The rat paw volume of all tested animals were measured at 2, 4 and 6 h after the sample dosage. Hence, the percentage of variation in rat paw oedema was measured and compared with that of the standard tested drugs at respective intervals of time i.e., 2, 4 and 6 h. The percentage reduction of rat paw oedema at mentioned intervals of time in the treated rats were measured by using below equation.

Percentage inhibition = (C – T)/C x 100

C = volume of paw rat oedema in control animals

T = volume of paw rat oedema in treated animals

Statistical analysis

The results are mentioned as Mean±SEM values of six independent experiments. Statistical significance (AP<0.05) value between the batches was measured by one-way analysis of variance (ANOVA) followed by Dunnett's test.

Results

***In vitro* anti-inflammatory studies**

The denaturation of biological proteins causes inflammation. The denaturation pathways can be by acidic (or) alkaline reactions, heat treatment, radiation reactions, etc. Proteins lose their complex tertiary structure because of the externally induced stress under the above mentioned conditions thus leading to denaturation. The *in vitro* anti-inflammatory capability of *D. consimilis* extracts (**DH, DC, DE, DA** and **DM**) were initially subjected to protein denaturation method [16] using different concentration (0.1-1 mg/mL) for extracts as well as standard (indomethacin) and data is tabulated in Table 1.

The data evident that the **DC** and **DA** revealed prominent anti-inflammatory profile, besides, **DH, DE** and **DM** depicted moderate anti-inflammatory activity (Table 1). The **DA** and **DC** (1 mg/mL) showed good inhibitory activity against protein denaturation with 80.08±2.61 and 71.04±1.77%, respectively, while standard drug (Indo, 1 mg/mL) with 93.71±1.29% (Table 1). Similarly, the percentage inhibition of bovine albumin protein denaturation for **DH, DE** and **DM** were noticed to be 21.04±0.73, 23.50±0.99 and 14.22±0.79%, respectively (Table 1). As shown in illustrated in Figure 1, the IC_{50} values for **DC** and **DA** were 0.706 and 0.468 mg/mL, respectively, with respect to standard (Indo, 0.120 mg/mL), whereas **DH, DE** and **DM** did not show better inhibition of albumin protein up to 1 mg/mL concentration.

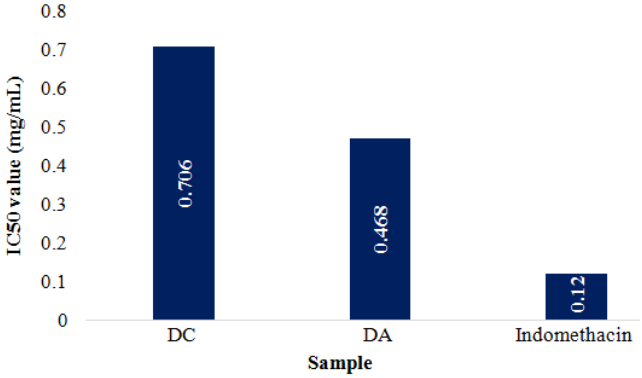

Figure 1: IC_{50} values of **DC** and **DA** extracts in protein denaturation method

Table 2: *In vivo* anti-inflammatory effect of *Dirinaria consimilis* extracts in formalin hind rat paw edema assay

Sample	Dosage (mg/Kg)	2 h		4 h		6 h	
		Raise in Paw edema volume[#]	% inhibition[#]	Raise in Paw edema volume[#]	% inhibition[#]	Raise in Paw edema volume[#]	% inhibition[#]
DH	100	-	-	-	-	-	-
	200	1.71±0.11	5.73±0.56	2.02±0.08	4.88±1.00	2.19±0.06	1.57±0.27
DC	100	1.53±0.08	15.26±0.80	1.87±0.07	12.17±0.61	2.00±0.05	10.02±0.46
	200	1.34±0.08	26.09±0.75	1.29±0.04	39.14±1.70	1.71±0.03	23.16±1.57
DE	100	-	-	-	-	-	-
	200	1.69±0.10	6.83±0.53	1.95±0.07	8.40±0.27	2.16±0.06	3.07±0.80
DA	100	1.26±0.07	30.30±0.88	1.41±0.06	33.83±1.16	1.51±0.05	32.09±0.90
	200	1.08±0.08	40.60±1.21	0.90±0.05	56.43±0.75	1.22±0.003	45.10±0.82
DM	100	-	-	-	-	-	-
	200	1.80±0.11	0.56±0.03	2.10±0.07	1.10±0.23	2.22±0.06	0.52±0.18
Indo	100	0.98±0.06	45.81±1.00	0.83±0.03	61.14±0.70	0.73±0.02	67.31±0.21

Acute toxicity studies

By following the Oral Acute Toxic Class method, **Dc-HA** were evaluated for median lethal dose (LD_{50}) value in male albino rats using standard protocol. The **Dc-HA** showed no clinical signs in the albino rats as evidenced by the LD_{50} value of above 2 g/Kg b.w. From the observations, the dosage was deliberated by Smith method and found to be 100 and 200 mg/Kg b.w.

***In vivo* assay**

The extracts of *Dirinaria consimilis* are active against *in vitro* denaturation of protein, we extended the study to evaluate them for *in vivo* anti-inflammatory activity by using formalin-induced rat paw oedema assay by plethesmography. [18, 19] The results are represented as the percentage reduction of oedema measured to basal paw volume.

Considering the *in vitro* anti-inflammatory analysis and acute toxicity studies, the extracts at 100 and 200 mg/Kg b.w were subjected to standard *in vivo* protocol (formalin-induced rat paw oedema assay by plethesmography) of anti-inflammatory activity against standard drug (Indo, 100 mg/Kg b.w). It is evident for the data that the all the extracts depicted dose dependent reduction of paw oedema in formalin-induced rats. The animals administered with lower dose of **DA** (100 mg/Kg b.w) depicted 30.30±0.88, 33.83±1.16 and 32.09±0.90% reduction of their paw oedema at 2, 4 and 6 h, respectively, while more pronounced effect of 40.60±1.21, 56.43±0.75 and 45.10±0.82%, respectively, depletion of paw oedema was observed in rats treated with higher dose of **DA** (200 mg/Kg b.w), which was almost nearer to that of the standard drug (Indo, 100 mg/Kg b.w) (Table 2). The **DC** showed moderate percentage reduction of paw oedema at 2, 4 and 6 h, whereas the **DH, DE** and **DM** revealed mild anti-inflammatory activity at tested doses during the noted hours (Table 2).

At higher doses of **DC** (200 mg/Kg b.w) exhibited better depletion of rat paw oedema than the lower doses. The rats treated with **DC** at 100 mg/Kg b.w revealed 15.26±0.80, 12.17±0.61 and 10.02±0.46% reduction in their paw oedema at 2, 4 and 6 h respectively, while **DC** at 200 mg/Kg b.w showed 26.09±0.75, 39.14±1.70 and 23.16±1.57% at 2, 4 and 6 h, respectively (Table 2). From the outcomes it can be deduce that the acetone and chloroform extracts of *Dirinaria consimilis* are highly effective in reducing paw oedema in rats as that of the standard drugs.

Discussion

Inflammation is immensely complex and captivating. It is an elementary way in which the living tissue retaliates to injury, infection or irritation by engendering the cardinal signs i.e., calor, dolor, function laesa, rubor and tumor [1]. Basically inflammation is categorized into two forms – acute (rapid stimuli to injury by furnishing plasma proteins and leukocytes to the active site of injury) and chronic inflammation (perpetuated period of inflammation) [5, 6]. Generally, acute inflammation leads to appendicitis, bronchitis, dermatitis, meningitis, sinusitis, sore throat and tonsillitis, while chronic inflammation causes aging, Alzheimer's, asthma, atherosclerosis, cancer, Crohn's disease, hepatitis, peptic ulcer, periodontitis, psoriasis, rheumatoid arthritis, sclerosis, sepsis and tuberculosis diseases [4]. On the other hand, naturally occurring anti-inflammatory extracts or metabolites are preferred due to lesser side effects. In this context, lichen extracts and metabolites are chosen due to their strong pharmacological reports against inflammation [11, 12].

In the present study, preliminary screening for anti-inflammatory activity was performed with protein denaturation method using bovine serum albumin protein.

The tested extracts produced mild to significant inhibitory profile towards protein denaturation. Among all the tested extracts, the **DA** and **DC** depicted prominent *in vitro* anti-inflammatory activity, which were nearer to that of the standard (indomethacin) (Figure 1). Considering the *in vitro* anti-inflammatory profile, we have initially screened for acute toxicity studies using male albino rats and found that the *D. consimilis* was non-toxic up to 2 g/Kg b.w. Based on the *in vitro* assay and oral acute toxic class method, the *in vivo* anti-inflammatory screening was evaluated in albino rats using formalin induced hind rat paw oedema assay at low and high doses. At both the dosage, the **DA** and **DC** showed potent inhibitory profile against rat paw oedema. Moreover, both the bioassays outcomes are almost similar and consistent.

The illustrations of the present experiment propose that the *D. consimilis* extract has prominent anti-inflammatory profile against chronic models like formalin-induced rat hind paw oedema. This *in vivo* model is not only suitable for screening anti-inflammatory activity but also anti-arthritic activity. Hence, the research findings justify that the utilization of *D. consimilis* extracts in traditional medicine assist for the treatment of acute and chronic inflammation, in particularly associated with arthritis. Furthermore, the suppression effect of the extracts of *D. consimilis* may be due to the inhibition of the biosynthesis of prostanoids (PGE_2, $PGF_{2\alpha}$, PGD_2, PGI_2), thromboxane (TXA_2) and Interluekin-8 which are well known COX pathway products or modulation of reactions between mediators and respective receptors or by irreversible blockade of the receptor activity.

Conclusion

This is a preliminary report of pharmacological profile of lichen, *Dirinaria consimilis*. The anti-inflammatory activity was assessed by two experimental models. In protein denaturation method (*in vitro*), the DA depicted prominent protein inhibitory profile with IC_{50} values of 468 µg/mL. In formalin induced hind rat paw oedema assay (*in vivo*), the DA (at both doses) revealed good inhibitory profile against rat paw oedema, demonstrative of both acute and chronic inflammation. Hence, both the bioassays depicted that the lichen, *D. consimilis* has good inhibitory profile against inflammation. Hence, it can be concluded that the *D. consimilis* have an aptitude to reduce rat paw oedema as well as inhibit protein denaturation

Abbreviations

Acetone extract of *Dirinaria consimilis* (DA), Chloroform extract of *Dirinaria consimilis* (DC), Dimethyl sulfoxide (DMO), Ethyl acetate extract of *Dirinaria consimilis* (DE), Hydroalcoholic extract of *Dirinaria consimilis*(Dc-HE), Lethal dose (LD), Methanol extract of *Dirinaria consimilis* (DM), *n*-hexane extract of *Dirinaria consimilis* (DH), Organization for Economic Cooperation and Development (OECD), Standard Error of the Mean (SEM)

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Authors' contribution

Both the authors have equally contributed.

Competing interests

The authors declare no conflicts of interest.

Limitations & future scope of the study

The chemical components which are responsible for anti-inflammatory abilities are currently unclear. Therefore, the chemical and biological examination of the extracts of *D. consimilis* are under progress in our research laboratory in order to isolate and identify the bioactive compounds present in this lichen.

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