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Research Article

STUDY OF *IN VITRO* ANTI-INFLAMMATORY ACTIVITY OF ETHNOMEDICINAL PLANTS OF SIKKIM *VISCUM ARTICULATUM* AND *ACORUS CALAMUS*

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ABSTRACT

Objective: The present study was carried out to evaluate the anti-inflammatory property of ethnomedicinal plants *Viscum articulatum* and *Acorus calamus.*

Methods: Human red blood cells membrane stabilization method was applied to assess the anti-inflammatory property of both the plants.

Results: It was observed that both plant extracts have the anti-inflammatory potential comparable with that of the drug indomethacin. However, *A. calamus* was found to provide slightly more inhibition of hemolysis (76.8%) than that of standard drug (indomethacin) (72.8%), whereas *V. articulatum* provided slightly lesser inhibition (68%) in comparison to the drug. There was the tendency of increase protection along with the increase in the concentration of the extract, and maximum protection was achieved at the maximum concentration of 5000 µg/ml.

Conclusion: The study proves the anti-inflammatory efficacy of both the plants and they hold a good prospect for drug development against inflammation.

Keywords: Anti-inflammatory, *Viscum articulatum*, *Acorus calamus*, Sikkim.

INTRODUCTION

Sikkim is part of the Himalayan region and with its exquisite ecosystem hosts varieties of medicinal plants. Over 400 plants possessing therapeutic properties have been recorded from this region [1]. The tremendous medicinal plant wealth of Sikkim Himalayas has been part of the traditional system of medicine (Ayurveda, Homeopathy, Naturopathy, and Unani and Siddha system of medicine) [2]. Ancient medicinal systems abound in Sikkim are popularly nurtured by Buddhist groups for their traditional Tibetan pharmacopeia [3]. In traditional medicine, many of the plants are used in combination as well as individually to produce the desired effect.

Inflammation has been associated with many diseases which bring about the hazardous effect on the patients sometimes causing a threat to his/her life. This has attracted the attention of researchers to this field. There are many anti-inflammatory drugs to treat the consequences of inflammation belonging to steroidal or non-steroidal anti-inflammatory drugs (NSAIDS). However, studies suggest that these drugs are not free from adverse effects, as they are responsible for gastrointestinal complications such as mucosal damage and bleeding [4]. Moreover, NSAIDS can also cause acute renal failure [5]. For these reasons, many researchers have shifted their focus on finding the medicinal plants, which have gotten the anti-inflammatory property and can serve as a potential ingredient for future drug development [6].

Many studies had been conducted on plants to investigate its anti-inflammatory potential, and also these plants have been screened for phytochemicals, which are actively associated with its anti-inflammatory property. Over the years, one of the most popular methods to study *in vitro* anti-inflammatory property of plant extract has been membrane stabilization method of red blood cells (RBC). In one such study, anti-inflammatory activity was evaluated on extracts of fresh leaves of *Clerodendrum paniculatum* by *in vitro* (human red blood cells [HRBC] membrane stabilization method) and *in vivo* methods (0.1 ml of 1% w/v carrageenan-induced rat paw edema model). Petroleum ether, chloroform, ethyl acetate, alcohol, and aqueous extracts of *C. paniculatum* were screened for *in vitro* anti-inflammatory activity. Among them, petroleum ether and chloroform extracts showed the best *in vitro* anti-inflammatory activity at the dose level of 200 and 400 mg/kg. The study demonstrated that petroleum ether and chloroform extracts possess statistically significant ($p<0.001$) anti-inflammatory potential which provides a scientific basis for the traditional claims of *C. paniculatum* leaves as an anti-inflammatory drug [7]. In another study evaluation of the anti-inflammatory property of the leaf exacts of *Gendarussa vulgaris* was carried out by Saleem *et al.* [8], the aqueous and alcoholic extracts of the leaves were used in both *in vitro* and *in vivo* methods. *In vitro* anti-inflammatory property was estimated by HRBC membrane stabilization method. The results concluded that the alcoholic extract at a concentration of 300 mg/ml showed potent activity on comparing with the standard drug diclofenac sodium. Another study was carried out to evaluate the *in vitro* anti-inflammatory activity of *Centella asiatica* by HRBC membrane stabilization. The maximum membrane stabilization of *C. asiatica* extracts was found to be 94.97% at a dose of 2000 µg/ml. The study revealed the potency of active constituents from *C. asiatica* in treating inflammations [9]. Further, phytochemical analysis of *C. asiatica* plant extracts revealed the presence of biochemical compounds such as triterpenoids and flavonoids, which have been observed to have remarkable anti-inflammatory activity. Recently, a study was conducted to evaluate the membrane stabilization activity of *Spilanthes paniculata* leaves. The results showed that at the concentration of 1 mg/ml, ethanol extract, and n-hexane and ethyl acetate soluble fractions significantly inhibited hypotonic solution-induced lysis of the HRBC (27.406±3.57, 46.034±3.251, and 30.72±5.679%, respectively), whereas standard drug acetylsalicylic acid (concentration 0.1 mg/ml) showed 77.276±0.321% inhibition. In the case of heat-induced HRBC hemolysis, the plant extracts also showed significant activity (34.21±4.72%, 21.81±3.08%, and 27.62±8.79% inhibition, respectively). The study showed n-hexane extract has better effects than the other extracts. It was concluded that the leaves of *S. paniculata* possess remarkable pharmacological effects and has the potential to act as an anti-inflammatory agent [6]. Further, Somlata (*Sarcostemma acidum*) highly used by the rural and tribal

people of India for various disorders such as asthma, swelling, fever and cold, dyspepsia, inflammatory infection, and gastric problem was investigated for *in vitro* anti-inflammatory activity by HRBC membrane stabilization method. Four different concentrations of the extract were used for HRBC membrane stabilization method among which concentration of 1 mg/ml showed 30% protection, concentration of 2 mg/ml showed 42.8% protection, concentration of 4 mg/ml showed 54.0% protection, and concentration of 6 mg/ml showed 67.6% protection of HRBC in hypotonic solution. The study revealed that the ethyl acetate extract has significant membrane stabilizing action on HRBC when compared to standard drug indomethacin which showed 69.6% protection of HRBC in hypotonic solution [10].

In the present study, two medicinal plants of Sikkim *Viscum articulatum* and *Acorus calamus* were investigated for their anti-inflammatory property. The authors were stimulated for the investigation as both these plants are traditionally used by local healers and ethnic communities of Sikkim to treat the inflammation associated diseases [11]. The anti-inflammatory property was studied by taking the human RBC as a model of the lysosome. Human RBC is used for this purpose because the membrane of RBC is considered analogous to the membrane of the lysosome. The lysosome contains potent inflammatory mediators, thus structural well-being of this organelle is of significance in the process of inflammation. Membrane disruption of the lysosomes by any means leads to release of the inflammatory mediators including hydrolytic enzymes which contribute to the process of inflammation. Hence, stabilization of membrane can be seen as a way to avoid the release of inflammatory mediators and lessen the effect of inflammation. Thus, the protection provided by plant extract against membrane rupture of RBC can be considered as a potential tool for the assessment of anti-inflammatory activity. Hence, the present preliminary study has been undertaken to investigate: (i) *In vitro* anti-inflammatory activity of methanolic extract of *V. articulatum* and *A. calamus* by HRBC stabilization method (ii) to compare the anti-inflammatory activity of both the plants to that of the standard anti-inflammatory drug indomethacin, (iii) to qualitatively assess the presence of some of the anti-inflammatory phytochemicals present in the plants. The study may help to throw light on the anti-inflammatory property of the plants (if any) which may further unfold its potential for future drug development.

METHODS

Collection and identification of the plant material

V. articulatum, which was found to be parasitic on *Quercus semicarpifolia* was collected from the forest area approximately 5 km from Namrang village, Tumin, East Sikkim. *A. calamus* was collected from the same village area, where villagers cultivate them in patches/batches in around semi-aquatic habitats for medicinal purposes. Both the plants were collected in the month of February 2015 and brought to the laboratory of Department of Zoology, Sikkim University in the same month.

Preparation of extracts

The plants were washed and dried completely in the sunlight. After few days, when the plant material was completely desiccated, the whole plant of *V. articulatum* and rhizome of *A. calamus* were grounded into powdery fine particles. 10 g of powdery *V. articulatum* and *A. calamus* were added to 80 ml of 70% ethanol and mixed. The solution was kept for 3 days with occasional stirring and then it was filtered through Whatman filter paper No.1. The filtered content was kept in an incubator at 37°C for drying. A thick, sticky plant extract was obtained, and it was collected and quantified at appropriate proportion to make a stock solution.

Preparation of stock solutions

The stock solution of both the plants was prepared by adding 1 ml of water to 50 mg of plant extract, constituting the solution to be of 50 mg/ml. The solution was further diluted to make 10 mg/ml solution. The stock solution of standard drug was prepared by adding 1 ml of water to 75 mg of indomethacin, constituting the solution to be of 75 mg/ml. This solution was further diluted by adding distilled water to make 10 mg/ml solution. The 10 mg/ml solution of both the plants and the extract was used to make appropriate dose with specific dilution.

Selection of doses

For assessment of the anti-inflammatory activity of *V. articulatum* and *A. calamus* extract, six dose levels were chosen in increasing concentration (0.5-5 mg/ml). The similar dose level, in increasing concentration (0.5-5 mg/ml) was also chosen for the drug (indomethacin).

Screening method for anti-inflammatory activity

Preparation of human RBC suspension

Fresh 5 ml blood was collected from the investigators themselves. Administration of NSAIDS for 2 weeks before taking the blood was avoided. Collected blood was mixed with equal volume of sterilized Alsever's solution (2% dextrose, 0.8% sodium citrate, 0.5% citric acid, and 0.42% sodium chloride). The blood was centrifuged at 3000 revolutions per minute (rpm) for 10 minutes, and packed cells were washed three times with isosaline (0.85% w/v NaCl). The volume of the blood was measured and reconstituted as 10% v/v suspension with isosaline.

Hypotonicity-induced hemolysis

Various concentrations of the extract were prepared in a test tube (500, 1000, 2000, 3000, 4000, and 5000 µg/ml) using distilled water and to each concentration 1 ml phosphate buffer, 2 ml hyposaline, and 0.5 ml HRBC suspension were added. Indomethacin at different concentrations (500, 1000, 2000, 3000, 4000, and 5000 µg/ml) was used as the standard drug and compared with respective concentrations of plant extract. Standard and control were prepared omitting the extracts. These were incubated at 37°C for 30 minutes and centrifuged at 3000 rpm for 20 minutes. The hemoglobin content in the supernatant solution was estimated spectrophotometrically at 560 nm using an ultraviolet spectrophotometer (COSLAB).

The percentage of hemolysis of HRBC membrane was calculated as follows:

% Hemolysis=(O.D of test sample÷O.D of control)×100

The percentage of HRBC membrane stabilization was calculated as follows:

% Protection=100−(O.D of test sample÷O.D of control)×100]

Statistical analysis

Statistical analysis was done by SPSS 16 software. Analysis of variance was performed, followed by pairwise Pearson correlation test and spearman's correlation test. The $p \leq 0.05$ was considered significant.

RESULTS

The results of the assay are presented in Table 1. It was observed that both plant extracts have the anti-inflammatory property comparable with that of the standard drug. However, there was the tendency of increase protection along with the increase in the concentration of the extract with the maximum protection achieved at the maximum concentration of 5000 µg/ml. The Spearman's correlation analysis was done to test this trend (Table 2). The test indicated that protection provided by drug and *V. articulatum* is dependent on concentration, i.e., with an increase in concentration, the protection also increases. However, *A. calamus* did not follow this trend suggesting that both minimal and maximal concentrations of the plant are equally efficient in providing protection. Further, one-way analysis between plants extracts and the drug showed no statistically significant difference in protection to hemolysis. However, *A. calamus* was found to provide

Table 1: Effect of *V. articulatum, A. calamus* and indomethacin on HRBC membrane hemolysis and protection

Concentration (µg/ml)	Hemolysis (in %)			Protection (in %)		
	V. ariculatum	*A. calamus*	Indomethacin	*V. articulatum*	*A. calamus*	Indomethacin
500	41.6±0.50	35.0±0.46	40.0±0.43	58.4±0.48	65.0±0.49	60.0±0.47
1000	41.6±0.48	32.0±0.48	38.4±0.46	58.4±0.45	68.3±0.45	61.6±0.49
2000	38.4±0.45	33.6±0.47	36.9±0.45	61.6±0.46	66.4±0.46	63.2±0.43
3000	38.4±0.52	32.0±0.49	33.6±0.48	61.6±0.5	68.0±0.41	66.4±0.44
4000	35.0±0.48	32.8±0.50	33.6±0.45	65.0±0.52	67.2±0.48	66.4±0.48
5000	32.0±0.47	23.2±0.49	27.2±0.51	68.0±0.4	76.8±0.50	72.8±0.49

Values are mean±SD; n=3 in each concentration. SD: Standard deviation, *V. articulatum: Viscum articulatum, A. calamus: Acorus calamus*, HRBC: Human red blood cells

Table 2: Spearman's rank correlation coefficient (r) of dose-dependent pairwise correlation between drug, *Viscum articulatum* and *Acorus calamus* with inhibition of hemolysis

Treatment	r	p
Indomethacin (drug) versus concentration	r=0.986	p≤0.01
Viscum articulatum versus concentration	r=0.971	p≤0.01
Acorus calamus versus concentration	r=0.667	p=0.148

Table 3: Phytochemical screening of methanolic extract of *V. articulatum* and *A. calamus*

Phytochemicals	Present (+)/absent (-)	
	V. articulatum	*A. calamus*
Flavonoids	+	-
Triterpenoids	-	-
Saponins	+	-
Steroids	+	+
Alkaloids	+	+

V. articulatum: Viscum articulatum, A. calamus: Acorus calamus

slightly more inhibition of hemolysis than that of the drug, whereas *V. articulatum* provided slightly lesser inhibition in contrast to the drug. The preliminary qualitative phytochemical assessment of the plants showed the presence of flavonoids, saponins, steroids, alkaloids in *V. articulatum* and steroids, alkaloids in *A. calamus* (Table 3).

DISCUSSION

The present investigation is the first time approach to assessing the anti-inflammatory activity of two ethnomedicinal plants *V. articulatum* and *A. calamus* of Sikkim Himalayas by HRBC membrane stabilizing method. In the traditional medicinal system, both the plants have been used by different ethnic communities of Sikkim and other tribes of India in the treatment of inflammation associated ailments [11,12]. However, as far as a review of the literature is concerned only a few laboratory studies have been conducted to investigate the anti-inflammatory activity of both these plants.

In this study, RBC membrane stabilization of method was employed to study anti-inflammatory property *V. articulatum* and *A. calamus* because RBC resembles lysosomes in terms of the membrane similarity which was first noted by Chou in 1997 [13]. Owing to the intricate association of burst of the lysosomes with consequences of inflammation [14]. The study aimed only to find out the membrane stabilizing potential of plant extract for RBC that would be more or less same for the lysosomes.

The results of the present investigation showed the anti-inflammatory of the property of *V. articulatum* and *A. calamus*, which is comparable to some of the studies and which was conducted on the other plant species [6-10]. In the line of these studies, Vijayasarthi *et al*. (1981) [15] also observed the anti-inflammatory activity of *A. calamus*, which also corroborates with our study. However, the present study was undertaken *in vitro* condition, whereas Vijayasarthi *et al.* (1981) used *in vivo* method for the study, which was the major difference between the two studies.

Further studies on the mechanism of action of *A. calamus* have shown that it can inhibit the expression of interleukin-8 (IL-8) and IL-6 ribonucleic acid (RNA) and protein levels and attenuated the activation of Nuclear Factor Kappa B and interferon regulatory factor 3. Thus, *A. calamus* inhibits the production of pro-inflammatory cytokines through multiple mechanisms Kim *et al.* (2009) [16]. The major difference between the study of Kim *et al.* (2009) and the present study is the choice of model for anti-inflammatory study, which was human keratinocyte HaCaT cells. Moreover, Kim *et al*. (2009) used leaf extract for the study. As the mode of action of the plant, extract may vary depending on experimental designs. The mode of action of the *A. calamus* extract in the present study may not be similar as shown by Kim *et al.* (2009). In a recent study by Shi *et al.* (2014), the anti-inflammatory activity of fresh roots and rhizomes of *A. calamus* was evaluated by the real-time reverse transcription polymerase chain reaction techniques in the lipopolysaccharide-induced RAW 264.7 cells test. Aqueous extracts used in the study were found to effectively inhibit the mRNA expressions of inflammatory mediators induced by lipopolysaccharide in RAW 264.7 cells [17].

The qualitative phytochemical screening performed in this study for *A. calamus* showed the absence of flavonoids and triterpenoids, both known to have remarkable anti-inflammatory activity [9]. Instead of the absence of this phytochemical membrane protection was achieved, which hints that there may be other phytochemical present in the plant extract that may have given protection to the RBC membrane. Besides, it has been observed that other phytochemicals such as glycosides or steroids have the anti-inflammatory role [18]. These phytochemicals act by the inhibition of mediators which probably play a key role in preventing inflammation, as because inflammatory cytokines induces cyclooxygenase-2 (COX-2) and prostaglandin E2 synthesis, which have a critical role in the pathogenesis of inflammatory diseases [19]. Apart from this, phytosterols have been reported to lower some of the pro-inflammatory cytokines including C-reactive protein [20] and alkaloids have been found to have pain-killing activity [21] suggesting their role against inflammation.

As with the *A. calamus*, *V. articulatum* also provided significant anti-inflammatory activity but was found to have a lesser potential to stabilize the RBC membrane with respect to *A. calamus* and indomethacin (standard drug). As far as literature review is concerned very few studies were conducted to investigate the anti-inflammatory property of *Viscum* sp. More specifically no literature is available to study anti-inflammatory property of *V. articulatum* with the help of HRBC stabilization method in this respect. However, another variety of mistletoe species, *Viscum album* has been found to have anti-inflammatory potential [19]. The studies on *V. album* postulated that destabilization of COX-2 mRNA caused by plant preparations as a possible mechanism for COX-2 inhibition, implicating the role of the plant in anti-inflammation. In the context of, *V. articulatum* the study by Saha *et al*. (2015) hints on the *in vitro* membrane stabilizing potential, but the idea of COX-2 inhibition by *V. articulatum* in the similar way of *V. album* would be very illogical as this two mistletoes are of a different variety and their habitat also differs. Apart from this,

V. articulatum being a hyper-parasitic plant further opens up challenges as the phytoconstituents of the plant can vary depending on the host it parasitizes. However, the mechanism of action of *V. articulatum* can be extended by contemplating on the results of our phytochemical screening, which showed the presence of flavonoids and triterpenoids which are known to be remarkable anti-inflammatory phytochemicals. With the presence of flavonoids in *V. articulatum,* it can be considered that it acts by directly inhibiting the COX enzyme. In addition to it, a study carried out by Leu *et al.* (2004) implies the role of Oleanolic acid in inhibition of superoxide anion generation by human neutrophils in response to Formyl-L-Methionyl-L-Leucyl-L-Phenyl-alanine but not to phorbol myristate acetate. This study suggests the other way by which *V. articulatum* can be the potent anti-inflammatory agent [22].

As the present study is a preliminary one to assess the anti-inflammatory potential of *V. articulatum* and *A. calamus,* the potential mechanism of action of these plants were not studied. Moreover, only a few qualitative phytochemical analysis was performed which is the limitation of the present study. Considering these limitations present study provides evidence for the anti-inflammatory property of *V. articulatum* and *A. calamus.* In the present investigation, *A. calamus* was found to provide even more protection to HRBC than the standard drug which is the new finding of the study. Moreover, extensive studies are required to confirm the efficacy of these plants using *in vivo* anti-inflammatory study models. Thus, the present study opens up the quest to isolate, identify, and investigate the possible mechanism of action of phytochemicals present in these plants.

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REFERENCES

1. Srivastava TN, Kapaki BK. Resource survey of plants of potential economic value of Sikkim Himalaya. Bull Medico Ethno Bot Res 1990;12(1-2):1-11.
2. Panda AK, Misra S. Health traditions of Sikkim Himalaya. J Ayurveda Integr Med 2010;1(3):183-9.
3. Das B. Encyclopedia of Tibetan Medicine. Vol. 1-3. Delhi: Sri Satguru Publication; 1994. p. 103-10.
4. Singh G. Recent considerations in nonsteroidal anti-inflammatory drug gastropathy. Am J Med 1998;105(1B):31S-8.
5. Griffin MR, Yared A, Ray WA. Nonsteroidal anti-inflammatory drugs and acute renal failure in elderly persons. Am J Epidemiol 2000;151(5):488-96.
6. Hossain MM, Ahamed SK, Dewan SM, Hassan MM, Istiaq A, Islam MS, *et al. In vivo* antipyretic, antiemetic, *in vitro* membrane stabilization, antimicrobial, and cytotoxic activities of different extracts from *Spilanthes paniculata* leaves. Biol Res 2014;47(1):45.
7. Joseph J, Bindhu AR, Aleykutty NA. *In vitro* and *in vivo* Anti-inflammatory activity of *Clerodendrum paniculatum* Linn. Leaves. Indian J Pharm Sci 2013;75(3):376-9.
8. Saleem TK, Azeem AK, Dilip C, Sankar C, Prasanth NV, Duraisami R. Anti-inflammatory activity of the leaf extacts of *Gendarussa vulgaris* Nees. Asian Pac J Trop Biomed 2011;1(2):147-9.
9. Chippada SC, Volluri SS, Bammidi SR, Vangalapati M. *In vitro* anti-inflammatory activity of methanolic extract of *Centella asiatica* by HRBC membrane stabilisation. Rasayan J Chem 2011;4(2):457-60.
10. Shailesh G, Seema K, Dwivedi S. *In-vitro* anti-inflammatory activity of *Sarcostemma acidum* Wight. and Arn. Indian herb by Human red blood cell membrane stabilization method. Int J Pharm Teach Pract 2011;2(4):184-8.
11. Panda AK. Medicinal plants use and primary health care in Sikkim. Int J Ayurvedic Herb Med 2012;2(2):253-9.
12. Joshi P. Ethnobotany of the Primitive Tribes in Rajasthan. Jaipur, India: Printwell; 1995. p. 89.
13. Chou CT. The anti-inflammatory effect of *Tripterygium wilfordi* Hook on adjuvant induced paw edema in rats and inflammatory mediator's release. Phytother Res 1997;17:152-4.
14. Sadique JA, Al-Rqobah WA, Bugharith ME, El-Gin-dy AR. The bioactivity of certain medicinal plants on the stabilization of RBC membrane system. Fitoterapia 1989;6:525-32.
15. Vijayasarathy V, Sharma LK, Prakash A. Indigenous drug treatment for hemorrhoids. Probe 1981;120:285-7.
16. Kim H, Han T, Lee SG. Anti-inflammatory activity of a water extract of *Acorus calamus* Leaves on keratinocyte HaCa T cells. J Ethnopharmacol 2009;122(1):149-56.
17. Shi GB, Wang B, Wu Q, Wang TC, Wang CL, Sun XH, *et al*. Evaluation of the wound-healing activity and anti-inflammatory activity of aqueous extracts from *Acorus calamus* L. Pak J Pharm Sci 2014;27(1):91-5.
18. Di Rosa M, Giroud JP, Willoughby DA. Studies on the mediators of the acute inflammatory response induced in rats in different sites by carrageenan and turpentine. J Pathol 1971;104(1):15-29.
19. Saha C, Hegde P, Friboulet A, Bayry J, Kaveri SV. *Viscum album* - mediated COX-2 inhibition implicates destabilization of COX-2 mRNA. PLoS One 2015;10(2):e0114965.
20. Othman RA, Moghadasian MH. Beyond cholesterol-lowering effects of plant sterols: Clinical and experimental evidence of anti-inflammatory properties. Int Life Sci Inst 2011;69(7):371-82.
21. Kam PC, Liew S. Traditional Chinese herbal medicine and anaesthesia. Anaesthesia 2002;57(11):1083-9.
22. Leu YL, Kuo SM, Hwang TL, Chiu ST. The inhibition of superoxide anion generation by neutrophils from *Viscum articulactum*. Chem Pharm Bull (Tokyo) 2004;52(7):858-60.