ANTI-INFLAMMATORY PROFILING OF SEED AND RIND EXTRACTS OF *AMOMUM SUBULATUM*

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ABSTRACT

Objective: The study aimed to evaluate the anti-inflammatory activity of *Amomum subulatum* (greater cardamom) seed and rind extracts in Wistar rats.

Methods: The seed and rind of *A. subulatum* were air-dried in the shade, powdered, and subjected to 80% hydroalcoholic extraction in the Soxhlet apparatus. The anti-inflammatory activity of the seed and rind extracts of *A. subulatum* was evaluated by *in vivo* and *in vitro* methods.

Results and Discussion: *In vivo* studies, namely, carrageenan-induced rat paw edema, cotton pellet granuloma, and formaldehyde-induced arthritis model confirmed the anti-inflammatory potential of seed and rind extracts of *A. subulatum*. It was found that rind extract exhibited better inhibition of inflammation as compared to seed extract. *A. subulatum* rind extract at the dose of 500 μg/ml exhibited best results for *in vitro* studies, namely, inhibition of albumin denaturation (73% inhibition), antiproteinase action (58% inhibition), membrane stabilization, heat-induced hemolysis, hypotonicity-induced hemolysis (54% inhibition), anti-lipoxygenase activity.

Conclusions: The results of the study showed that the rind extract of A. subulatum (greater cardamom) possesses significant anti-inflammatory potential in various in vivo and in vitro anti-inflammatory models in the experimental animals.

Keywords: A*momum subulatum*, Carrageenan, *In vitro* anti-inflammatory, Anti-lipoxygenase.

INTRODUCTION

Amomum subulatum Roxb., commonly known as greater cardamom belonging to the family Zingiberaceae, is an evergreen herb native to Eastern Himalayas, near mountain streams in Darjeeling, Sikkim, and Northeast India [1].

The major chemical constituent of the herb is 1,8-Cineole. Other minor constituents include sabinene, γ-terpinene, α-bisabolene, α-terpinyl acetate, α- and β-terpineol, cinnamaldehyde, linalool, cuminaldehyde, terpinene-4-ol, petunidin protocatechualdehyde, protocatechuic acid, 1, 7-bis(3,4-dihydroxyphenyl) hepta-4E, 6E-dien-3-one, and 2,3,7-trihydroxy-5-(3,4-dihydroxy-E-styryl)-6,7,8,9-tetrahydro-5H-benzocycloheptene. Other isolated constituents include cardamonin and alpinetin; the glycosides petunidin 3,5-diglucoside ($C_{28}H_{33}O_{17}$), leucocynidin-3-O-β-D-glucopyranoside ($C_{21}H_{24}O_{12}$); a new aurone glycoside, subulin ($C_{28}H_{32}O_{16}$); chalcone, cardamonin ($C_{16}H_{14}O_4$) and a flavanone, alpinetin. A seed of the plant shows the presence of cardamonin and alpinetin [2-4] Fig. 1.

The herb is useful in congestion of liver; gonorrhea, neuralgia, headache, and stomatitis [5]. It also shows gastric anti-ulcerogenic effect [6], antioxidant activity [7], and antioxidant activity [8].

In the present studies, seeds and rind of the plant are evaluated for their *in vivo* and *in vitro* anti-inflammatory activity.

METHODS

Procurement and authentication of raw material
The fruits of *A. subulatum* were procured from the local market of Sunder Nagar, Dist. Mandi, Himachal Pradesh. The crude drug was authenticated by a senior botanist at National Bureau of Plant Genetic Resources, New Delhi (Voucher number: EP 532).

Preparation of extracts
The rinds and seeds (1000 g + 1500 g resp.) were coarsely powdered and extracted in a Soxhlet apparatus separately with 80% methanol for 72 h. The methanolic extracts were concentrated on a steam bath and dried under reduced pressure to get the brown-colored powder and kept in desiccators.

Assessment of *in vivo* anti-inflammatory activity

Carrageenan-induced rat hind-paw edema method
The effect of oral administration of 100, 250, and 500 mg/kg of the extracts of activity of the rind (ASR) and activity of the seed (ASS), 40 mg/kg diclofenac and vehicle (saline, 10 ml/kg) on the hind-paw edema induced by subplantar injection of 0.1 ml carrageenan (1% w/v) was evaluated according to the method described by Winter *et al.* [9].

Cotton pellet-induced granuloma method
The method of Winter and Porter with minor revision was used to study chronic inflammation [7]. The animals were anesthetized with ether. The axillary skin was shaved and disinfected with 70% ethanol. An incision was made and by a blunt forceps subcutaneous tunnels were formed and a sterilized cotton pellet (50 ± 1 mg) was placed in both axillas. The vehicle, test extracts (ASR and ASS) (100, 250, and 500 mg/kg, p.o.), and standard (diclofenac) were administered for 7 consecutive days starting from the day of cotton implantation. On the 8th day, rats were anesthetized again and the cotton pellet (along with granular tissue formed around) was removed surgically and freed from extraneous tissue. The pellets were weighed immediately for wet weight. Then, pellets were dried in an incubator at 60°C until a constant weight was obtained [10].

Formaldehyde-induced arthritis assay
In formaldehyde-induced arthritis models, animals were divided into groups as per treatment, namely, normal control, arthritic control, standard, ASR extract treated, and ASS extract treated. The test extracts were given for 10 days. Thirty minutes after administration of the vehicle/test extracts, arthritis was induced by subplantar administration of 0.1 ml formaldehyde (2%v/v) into the left hind paw of all the animals on days 1 and 3. An increase in

joint diameter was measured on days 0, 2, 4, 6, 8, and 10 as paw volume [11].

Assessment of *in vitro* anti-inflammatory activity

Inhibition of protein denaturation [12,13]

The *in vivo* anti-inflammatory activity of *A. subulatum* extracts (ASR and ASS) was studied using inhibition of albumin denaturation technique. The reaction mixture contained test extracts (ASR and ASS) and 1% aqueous solution of bovine albumin fraction, the pH of the reaction mixture was adjusted using a small amount of 1 N HCl. The sample extracts were incubated at 37°C for 20 min and then heated to 51°C for 20 min, after cooling the samples, the turbidity was measured at 660 nm (ultraviolet–visible spectrophotometer). The percentage inhibition of protein denaturation was calculated as follows:

$$Percentage\,inhibition = \frac{(Abs\,control - Abs\,sample)}{Abs\,control} \times 100$$

Antiproteinase action [13,14]

In this test, the reaction mixture (2 ml) was containing 0.06 mg trypsin, 1 ml 20 mM Tris-HCl buffer (pH 7.4), and 1 ml test extracts (ASR and ASS) of different concentrations (100–500 µg/ml). The mixture was incubated at 37°C for 5 min and then 1 ml of 0.8% (w/v) casein was added. The mixture was incubated for an additional 20 min. A 2 ml of 70% perchloric acid was added to detain the reaction. Centrifugation of cloudy suspension was carried out and the absorbance of the supernatant was recorded at the wavelength 210 nm against buffer as blank. The experiment was performed in triplicate. The percentage inhibition of proteinase inhibitory activity was calculated.

$$Percentage\,inhibition = \frac{(Abs\,control - Abs\,sample)}{Abs\,control} \times 100$$

Membrane stabilization

For the preparation of red blood cells (RBCs) suspension [13,15], the blood was collected from healthy animals that were not involved in any type of anti-inflammatory studies at least for the last 3 weeks and transferred to the centrifuge tubes. Centrifugation of the tubes was carried out at the speed of 3000 rpm for 10 min and then washing was carried out thrice with an equal volume of normal saline. The volume of blood was measured and reconstituted as a 10% v/v suspension with normal saline.

Heat-induced hemolysis [13,16]

The reaction mixture (2 ml) consisted of 1 ml test extracts (ASR and ASS) of different concentrations (100–500 µg/ml) and 1 ml of 10% RBCs suspension, instead of test sample only saline was added to the control test tube. Aspirin was used as a standard drug. All the centrifuge tubes containing reaction mixture were incubated in a water bath at 56°C for 30 min. At the end of the incubation, the tubes were cooled under running tap water. The reaction mixture was centrifuged at 2500 rpm for 5 min and the absorbance of the supernatants was taken at 560 nm. The experiment was performed in triplicates for all the test samples. The percentage inhibition of hemolysis was calculated as follows:

$$Percentage\,inhibition = \frac{(Abs\,control - Abs\,sample)}{Abs\,control} \times 100$$

Hypotonicity-induced hemolysis [17]

Different concentrations of test extract (ASR and ASS) (100–500 µg/ml), reference sample, and control were separately mixed with 1 ml of phosphate buffer, 2 ml of hyposaline, and 0.5 ml of RBC suspension. For comparison with standard, diclofenac sodium (100 µg/ml) was used. All the assay mixtures were incubated at 37°C for 30 min and centrifuged at 3000 rpm. Hemoglobin was estimated by decanting the supernatant liquid and recording absorbance at 560 nm. The percentage of hemolysis was estimated by assuming the hemolysis produced in the control as 100%.

$$Percentage\,protection = 100 - (OD\,sample / OD\,control) \times 100$$

Anti-lipoxygenase activity [16]

Anti-lipoxygenase activity was studied using linoleic acid as a substrate and lipoxidase as an enzyme. Test extracts (ASR and ASS) at different dose levels (100–500 µg/ml) were dissolved in 0.25 ml of 2M borate buffer pH 9.0 and added 0.25 ml of lipoxidase enzyme solution (20,000 U/ml) and incubated for 5 min at 25°C. Later, 1 ml of linoleic acid solution (0.6 mM) was added, mixed well and absorbance was measured at 234 nm. Indomethacin was used as a reference standard. The percent inhibition was calculated from the following equation,

$$\%inhibition = [\{Abs\,control - Abs\,sample\}/Abs\,control] \times 100$$

A dose–response curve was plotted to determine the IC_{50} values. IC_{50} is the concentration sufficient to obtain 50% of a maximum scavenging capacity. All tests and analyses were run in triplicate and averaged.

Statistical analysis

Results are expressed as Mean±SD. The difference between experimental groups was compared by one-way ANOVA followed by Dunnett's multiple comparison test (control vs. test) using the software GraphPad Instat.

RESULTS AND DISCUSSION

Effect of *A. subulatum* extracts on carrageenan-induced rat paw edema

About 80% methanolic extracts of rind and fruits of *A. subulatum* were studied for their acute anti-inflammatory activity on three dose levels, namely, low (100 mg/kg), medium (250 mg/kg), and high (500 mg/kg). Both the extracts were found to have anti-inflammatory activity. The % inhibition was seen maximum in case of rind extract (500 mg/kg) treated animal group.

Effect of *A. subulatum* extracts on cotton pellet granuloma in rats

The cotton pellet granuloma model has been studied for testing the proliferative phase (granuloma formation). The extracts of seed and rind of *A. subulatum* showed significant % inhibition of 46.08 and 51.32, respectively. No signs of ulcerations were seen in either of the cases.

Effect of *A. subulatum* extracts on formaldehyde-induced arthritis in rats

The results of this experiment showed that *A. subulatum* rind extract on the 10th day at 500 mg/kg dose level showed a significant reduction in paw edema, i.e., 75.86% as compared to the arthritic control group and these results were almost comparable with the standard drug (77.45%). However, *A. subulatum* seed extract at 500 mg/kg dose level exhibited 71.12% reduction in paw diameter.

***In vitro* parameters**

Effect of A. subulatum extracts on inhibition of albumin denaturation

In protein denaturation, proteins lose their tertiary structure and secondary structure by application of external stress or compound, such as strong acid or base, a concentrated inorganic salt, an organic solvent or heat. When studied, it was found effective in inhibiting heat-induced albumin denaturation. Maximum inhibition of 73% was observed at 500 µg/ml in *A. subulatum* rind extract. The standard anti-inflammation drug showed the maximum inhibition of 75% at the concentration of 100 µg/ml compared with control.
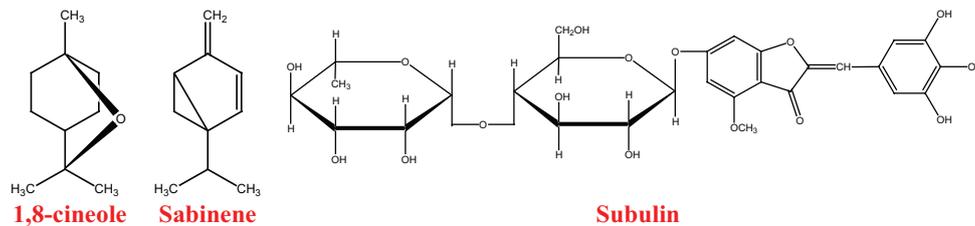
1,8-cineole   **Sabinene**   **Subulin**

Fig. 1: Structures of major phytoconstituents of *A. subulatum*

Effect of *A. subulatum* extracts on Carrageenan on rat paw edema

Effect of *A. subulatum* extracts on cotton pellet granuloma in rats

Effect of *A. subulatum* extracts on formaldehyde induced arthritis in rats

Effect of *A. subulatum* extracts on heat-induced protein denaturation

Effect of A. subulatum extracts on Antiproteinase action

Effect of *A. subulatum* Heat-induced hemolysis

Effect of *A. subulatum* extracts on hypotonicity induced hemolysis of erythrocyte

Effect of *A. subulatum* extracts on Anti-lipoxygenase activity
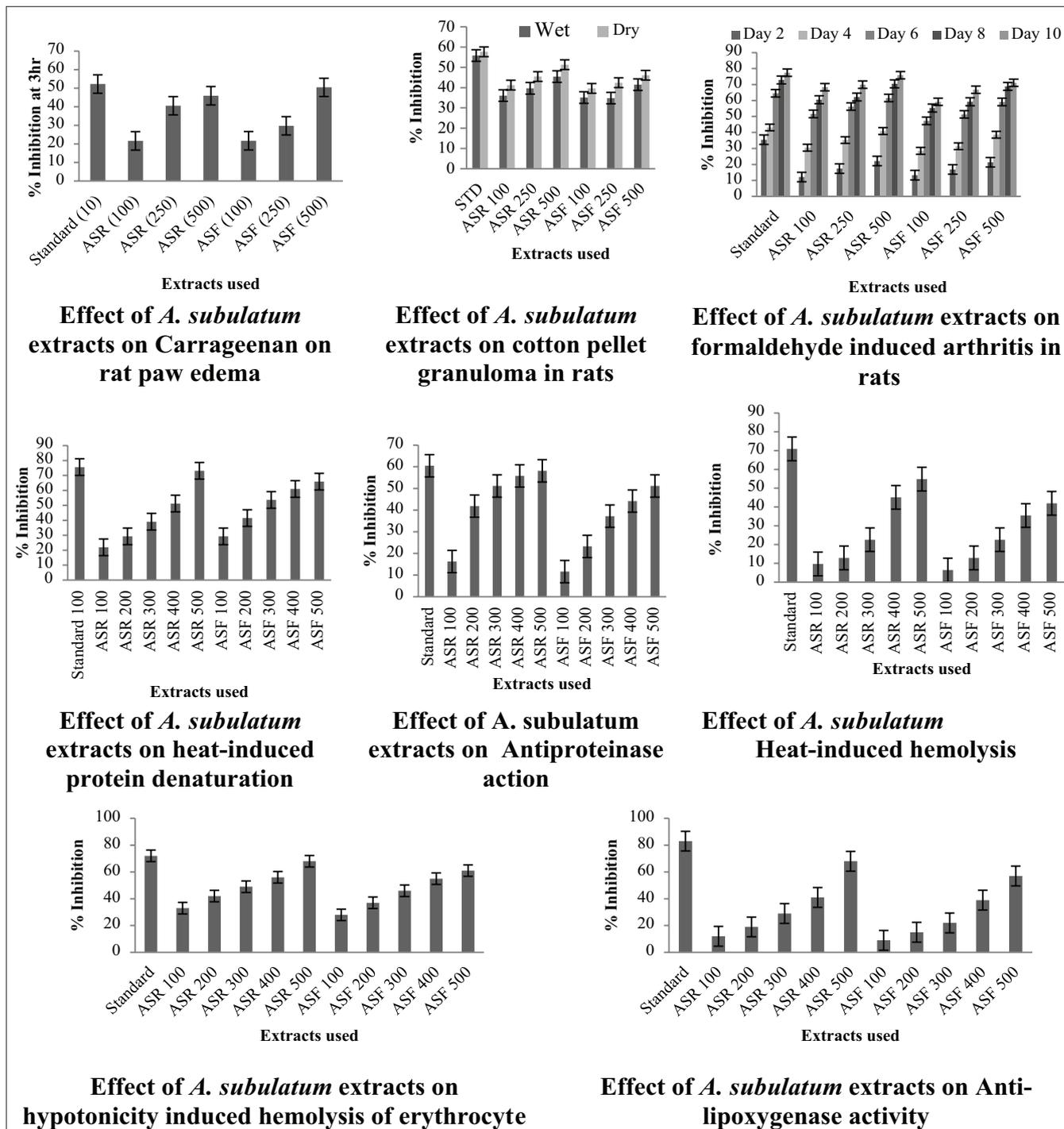
Fig. 2: Graphical presentation of various *in vivo* and *in vitro* parameters evaluated to check the anti-inflammatory activity of rind and fruit extracts of *Amomum subulatum*

Effect of A. subulatum extracts on antiproteinase action

Neutrophils are known to be a rich source of serine proteinase and are localized at lysosomes. It was previously reported that leukocytes proteinase plays an important role in the development of tissue damage during inflammatory reactions and a significant level of protection was provided by proteinase inhibitors [18]. *A. subulatum* extracts exhibited significant antiproteinase activity at different concentrations. The rind extract showed maximum inhibition of 58% at 500 μg/ml. The standard drug showed maximum inhibition of 60% at 100 μg/ml.

Effect of A. subulatum extracts on membrane stabilization

The human RBC membrane stabilization has been used as a method to study the *in vitro* anti-inflammatory activity because the erythrocyte membrane is analogous to the lysosomal membrane [19,20] and its stabilization implies that the extract may well stabilize lysosomal membranes. Stabilization of the lysosomal membrane is important in limiting the inflammatory response by preventing the release of lysosomal constituents of activated neutrophil, such as bacterial enzymes and proteases, which causes further tissue inflammation and damage on extracellular release. The lysosomal enzymes released during inflammation produce various disorders. The extracellular activity of these enzymes is said to be related to acute or chronic inflammation. The nonsteroidal drugs act either by inhibiting these lysosomal enzymes or by stabilizing the lysosomal membrane [21].

Effect of A. subulatum extracts on heat-induced hemolysis

The extracts were effective in inhibiting the heat-induced hemolysis at different concentrations. The results showed that the rind extract of *A. subulatum* at concentration of 400 and 500 μg/ml protects significantly the erythrocyte membrane against lysis induced by heat. Standard drug (100 μg/ml) offered significant protection against the damaging effect of heat solution.

Effect of A. subulatum extracts on hypotonicity-induced hemolysis

The results showed that the rind extract of *A. subulatum* at a concentration range of 200–500 μg/ml protects significantly the erythrocyte membrane against lysis induced by hypotonic solution. The standard drug (100 μg/ml) offered significant protection against the damaging effect of hypotonic solution. At the concentration of 500 μg/ml, it showed a maximum of 54% protection, whereas the standard drug (100 μg/ml) showed 70% inhibition of RBC hemolysis when compared with control.

Effect of A. subulatum extracts on anti-lipoxygenase activity

LOXs are sensitive to antioxidants and most of their action may consist of inhibition of lipid hydroperoxide formation due to scavenging of lipidoxy or lipid peroxy radicals formed in course of enzyme peroxidation. Extracts of *A. subulatum* seed and rind were tested for their potential against lipoxygenase activity at various dose levels ranging from 100 to 500 μg/ml. From the result, the strongest inhibition was obtained by rind extract at a concentration of 500 μg/ml. The standard drug showed 83% inhibition at a concentration of 100 μg/ml.

The results obtained from our studies Fig. 2 on seed and rind extracts of *A. subulatum* have shown a potential anti-inflammatory activity. The extracts inhibited the lipoxygenase enzyme activity. This indicates that *A. subulatum* is more useful in studies of inflammation and can be explored in other related physiological studies, aging, and diseases such as cancer and neurological disorder.

CONCLUSIONS

In the present study, results indicate that the methanolic extracts of seed and rind of *Amomum subulatum* possess anti-inflammatory properties. These activities may be due to the strong occurrence of polyphenolic compounds such as flavonoids, tannins, terpenoids,

steroids, and phenols. The extract fractions serve as free radical inhibitors or scavenger or acting possibly as primary oxidants and inhibited the heat-induced albumin denaturation, proteinase activity, and stabilized the RBCs membrane. The extracts also reduced the activity of lipoxygenase. The rind extract was found more effective as compared to seed extract. Purification of each bioactive compound is necessary and this purified form of the compound can be used which may show increased activity.

AUTHORS' CONTRIBUTIONS

The author herself has carried out the present study.

CONFLICTS OF INTEREST

There are no conflicts of interest concerning the present study.

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