Progress in Nutrition 2016; Vol. 18, N. 4: 436-442

Original article

Antinociceptive activity investigation of the methanolic crude extract of *Costus speciosus* in Mice

Shofiul Azam[1,2], Prawej Ansari[1,2], Shahnaz Jalil[1], Abdunasir Hussain Ibrahim[1], Nasrin Sultana[1], Mohammad Mosharraf Hossain[3], Jaysee Muhammad Naveed[4], Mohammed Forhad Hossain[2]

[1]Department of Pharmaceutical Sciences, North South University, Block-B, Bashundhara R/A, Dhaka-1229, Bangladesh - E-mail: shofiul_azam@hotmail.com; [2]Department of Pharmacy, International Islamic University Chittagong, 154/A, college road, Chittagong-4203, Bangladesh; [3]Department of Pharmacy, University of Science and Technology Chittagong (USTC), Bangladesh; [4]Department of Pharmacy, Southeast University, 24 Kamal Ataturk Avenue, Dhaka-1213, Bangladesh.

Summary. *Goal:* This study was designed to investigate the anti-inflammatory and analgesic activity of one of common Bangladeshi medicinal plant, *Costus speciosus*. *Methods & materials:* We have included the phytochemical investigation before proceeding to our major objective. The seeds of *Costus speciosus* (MECS) were extracted using methanol. MECS have been used in in vitro and in vivo investigation for possible analgesic and anti-inflammatory effect. In this pre-designed study Formalin-induced writhing test and Tail immersion test was included. *Results:* The methanol extract of *C. speciosus* (at 500 mg/kg) showed significant (P<0.01) anti-inflammatory activity (51.09±1.67% and 75.93±2.58% shown by standard) on red blood cell membrane stabilization study, and the crude extract have included 75.15±2.33% (p<0.05) of protection against protein denaturation. In tail immersion study the extract at the dose of 500 mg/kg increases the response time up to 5.97±0.11 second, which was close enough to reference group (8.51±0.21 second) at 90 min of drug administration. *C. speciosus* relapse the writhing, up to 53.97% during first phase of inflammation induced by formalin, it also reduced pain substantially (59.89%; p<0.05) at second phase of study, where standard group showed 73.78% pain reduction. *Conclusion:* This study presents a very good potentiality in inflammatory mediators inhibition. From the result of our study, it is clear that it possess analgesic and anti-inflammatory activity. Further study is suggested for active compound isolation and adjustment.

Key words: analgesic, anti-inflammatory, *C. speciosus*, HRBC, inflammation, protein denaturation

Introduction

C. speciosus is a quite familiar in Bangladesh as a medicinal herb and we examine this bulb to seek its anti-inflammatory and analgesic property. Apart from its bounded and proper use, it has been advised for anti-inflammatory activity (1, 2) in recent years. The pain response by way of organic arrangement requires a complex array of enzyme activation, mediator liberation, aqueous extravasations, cell migration, and tissue breakdown and all of these aimed toward host defense, and overall activation may cause a lot of suffering altitude (3) including heart attack and Alzheimer's disease (4-6) and cancer (7). This activation shows up with hostile antibody, stimuli, or irritants, characterized through arise of pain, swelling, redness, temperature rising (8, 9). Several phenomena adapt the antigenicity of autogenous proteins, together with protein denaturation and glycosylation. Protein denaturation may happen during enduring analgesic phenomena *in vivo* and albumin denaturation model resembles those patients with arthritic diseases and in rats with chronic inflamed lesions (10). Lysosomal enzymes causes deepening of inflammation as well as enhance the duration of the pain. Likewise, all other NSAIDs, Diclofenac act by inhibiting these lysosomal enzymes or by way of stabilizing the lysosomal membrane. Considering that the membrane of RBC is structurally similar to the lysosomal membrane, the

capability of any agents on stabilization of RBC membrane could also stabilize the lysosomal membrane (11, 12). It has been showing up, those corpuscle proteinases play a primary function within the progress of tissue accident in the course of pain reactions and proteinase inhibitors furnished a solid defense. As a result, inhibition of albumin denaturation, RBC membrane stabilization, and protease inhibition permit protection against chronic inflammatory altitude (13).

The most used drugs in the reduction of inflammatory belongs to NSAID class. However, they have many drawbacks. All NSAIDs works on an identical pathway, they inhibit both COX-1 and COX-2 or some chosen medicines simplest blocks COX-2 enzyme. Equally, they are interrupting enzyme activity; long run utilization of these medicines causes critically opposed activities like gastric or peptic ulcer (14, 15), cardiovascular disorders, and renal failure (16). For this reason, researchers are looking for novel medicines to be much less or no adversarial toward body mechanism and more potent (17). Plant centered remedy has gained good attention in different critical cases, in view that they give much less or no facet outcomes, availability, and low cost (18).

The medicinal use of our present sample in locality grows interest so much; this the plant has been stated as medication of lots of sicknesses like diarrhea, arthritis, jaundice, anti-vermin, abortifacient, and anti-emetic and so forth (19-26). The phytochemical study experiences the presence of following materials in methanolic extract of *C. speciosus*: alkaloids, steroids, tannins, flavonoids, glycosides, phenols, and saponin. The bearing of the flavonoids, phenols, etc. encourages us extra to investigate this plant, because these contents are liable for an extensive scope of anti-oxidant property (27), that suggests they aid security against ROS and lower the impairment of cells and so they can be equipped to guard towards the discharge of inflammatory mediators. Based on such phytoconstituents presence, we have attempted to investigate *in vitro* and *in vivo* anti-inflammatory activity.

Experimental procedure

Collection and Extraction

The plant was collected from Chittagong Hill tract nursery, in June-July 2013 and was identified by National Herbarium Chittagong, Bangladesh. The plant was washed with water, and air-dried in the shade. The plant part (leaves) were pulverized in a mechanical grinder and powdered that was used for extraction by using soxhlet method with 50% methanol. The extract was kept in a water bath for evaporating the remaining methanol and the deep brown residue obtained was kept in a desiccator for further experiments. The percentage yield was 4.9%.

Anti-inflammatory testing

Animal selection

Healthy male rats were selected weighing between 120-180g. They were retained at room temperature with 12-h light and dark cycle. Animals in this study were selected from the animal house of International Islamic University Chittagong (IIUC) and the ethical committee of IIUC approved their utilization in this protocol (Ref.Pharm-P&D-44/07'13-07).

Determination of anti-inflammatory activity In vitro
Membrane stability testing
Preparation of erythrocyte suspension

New complete blood specimen was collected from 10 healthy volunteers, have no drug history in past 4 weeks and non-smoker, using a hypodermic syringe (containing anticoagulant 3.1% sodium citrate). The collected blood was washed three times with isotonic solution (154 mM NaCl) in 10 mM sodium phosphate buffer (pH 7.4) via centrifugation. Blood specimens were centrifuged for 10 minutes each time at 3000 rpm.

Hypotonicity-impelled HRBC hemolysis

The impacts of the *C. speciosus* on hemolysis of HRBC impelled through hypotonic association, that have been assessed following the method designed by Shinde *et al* (28) with a few changes. The test specimen made out of stock erythrocyte (RBC) suspension (0.50 ml) mixed with 5 ml of hypotonic solution (50 mM NaCl) in 10 mM sodium phosphate buffered saline (pH 7.4) containing the *C. speciosus* (62.5-500 µg/ml) or hydrocortisone (500 µg/ml). The control made from 0.5 ml of RBC blended with hypotonic saline completely. The mixture was incubated for 10 min at

room temperature and centrifuged for more 10 min at 3000 rpm and the absorbance of the supernatant was measured at 540 nm. The restraint of hemolysis or membrane stability was calculated as:

- % Inhibition of hemolysis = [(OD1 - OD2)/OD1] × 100;
- Where, OD1 = Optical thickness of hypotonic-cradled saline, and
- OD2 = Optical thickness of test example in hypotonic solution

Protein denaturation inhibition

The claim of anti-inflammatory activity of *C. speciosus* taken into consideration by using bovine protein albumin denaturation, as indicated by Mizushima *et al* (29) and Sakat *et al* (30) with minor alterations. The reaction mixture was involved with test concentrates and 1% fluid solution of bovine albumin and pH of the mixture turned into balance using 1N HCl. The extract was heated at 37˚C for 20 minutes and after that warmed again to 51˚C for 20 mins, following that specimens were cooled, the turbid solution was measured at 660 nm. The percent prevention of protein denaturation was figured out from the formula below: Percentage restraint of protein = (Abs control − Abs sample) × 100/Abs control

Determination of anti-inflammatory activity In vivo
Formalin induced writhing test

The approach used was defined formerly (31, 32), with a few necessary modifications. The mice were divided into four groups every containing 5 mice and each group received their treatment as prescribed; for example, distilled water (1 ml/kg, i.p.), methanolic extract of *C. speciosus* (250 and 500 mg/kg, i.p), Diclofenac sodium (10 mg/kg, s.c.), respectively. Thirty mins after this treatment; 50 µl of a freshly organized 0.6% solution of formalin was injected subcutaneously underneath the plantar area of the left hind paw of each mouse. The mice had been located in a surveillance chamber and monitored for one hour. The time (S) spent in licking and biting responses of the injected paw was taken as an indication of pain response. Anti-nociceptive effect was concluded in two levels. The early phase (segment 1) was recorded at some stage within the first minutes, at the same time as the late phase (segment 2) was recorded after 20–30 minute, after formalin injection.

Tail immersion method

The tail immersion assay was made according to the description by way of Luiz *et al* (33). Mice were divided into four groups, of 5 each. The rear end of the animal of each group, as much as 5 cm, was dipped into hot water, temperature maintained at 55 ± 0.1˚C. The time was taken from mice in reaction, which means withdrawal of the tail, was recorded in seconds (unit). Reaction time was counted before and 15 min after oral gavaging (p.o.) of control stock, the MECS (methanolic extract of *C. speciosus*) at the dose of 250 and 500 mg/kg, (p.o.) and intra peritoneal (i.p.) injection of morphine (standard) at 5 mg/kg. The reaction time was evaluated every 15-minute interval over a 90-minute long study.

Phytochemical screening

Different phytochemical tests were done to discover the presence of alkaloids, steroids, triterpenoids, flavonoids, saponins, tannins, glycosides and reducing sugars in extracts (34) (Table-1).

Table 1. Anti-inflammatory activity of *C. speciosus* seeds

Group	0 min	15 min	30 min	45 min	60 min	90 min
Control	1.38 ±0.05	1.63± 0.08	1.91 ± 0.10	2.13 ± 0.11	1.95 ± 0.07	2.01 ± 0.08
Standard (5 mg/kg)	1.09 ± 0.01	3.96 ± 0.04	6.25 ± 0.14	7.85 ± 0.21	8.25 ± 0.13	8.51 ± 0.21
Extract_250 mg/kg	1.11± 0.02	2.17 ± 0.06	3.04 ± 0.08	4.19 ± 0.10	4.39 ± 0.11	4.61 ± 0.15
Extract_500 mg/kg	1.13± 0.04	3.23 ± 0.10	4.01 ± 0.04	5.55 ± 0.23	5.17 ± 0.08	5.97 ± 0.11

Data represented as mean ± SEM, from one-way ANOVA analysis followed by Dunnett's test P <0.001, each findings were compared with control and standard for the evaluation of efficacy.

Statistical analysis of data

Values for analgesic and anti-inflammatory activity were expressed as mean ± SEM. The significance of divergence between the means was analyzed by one-way ANOVA followed by Dunnett's multiple comparison tests. *In vitro* models were diagnosed by student 't' test for statistical significance. The difference was considered significant when P <0.05. All statistical analysis was carried out through GraphPad Prism-6 software.

Result and Discussion

Result and discussion of in vitro anti-inflammatory effect

C. speciosus seed extracts exhibited membrane stabilization activity by inhibiting hypotonic lysis of the erythrocyte membrane. The erythrocyte membrane is similar to the lysosomal membrane (35) and its stabilization implies that the extract may as good to stabilize lysosomal membranes. It is important to stabilize the lysosomal membrane to restrict the inflammatory response by stopping the discharge of lysosomal materials of an activated neutrophil causes additional tissue infection and harm upon extracellular release (36). Nonetheless, the specific mechanism of membrane stabilization via the extract yet to be confirmed, but hypotonic hemolysis may just come up from burst out of the cells because of the osmotic pressure and results in intracellular electrolyte and fluid contents release. The process could stimulate or enhance the efflux of these intracellular accessories, which can be averted through the extract (37). The methanol extract of *C. speciosus* showed significant (P <0.01) anti-inflammatory activity (51.09 ± 1.67% and 75.93 ± 2.58 % shown by standard) on the conc. of 500 µg/ml (Figure 1).

Arthritis is a form of a joint disease that involves irritation of one or more joints, liable for painful swelling, stiffness, loss of function within the joint. Denaturation of protein is one of the causes of arthritis as documented. Generation of auto antigenic arthritis is one of impact of denaturation of the protein. The mechanism of denaturation, in general, involves alteration electrostatic hydrogen, hydrophobic and disulphide bonding (38). Right here, the methanol extract have shown enormous (P < 0.01) activity at various concentrations and the results were when put next to

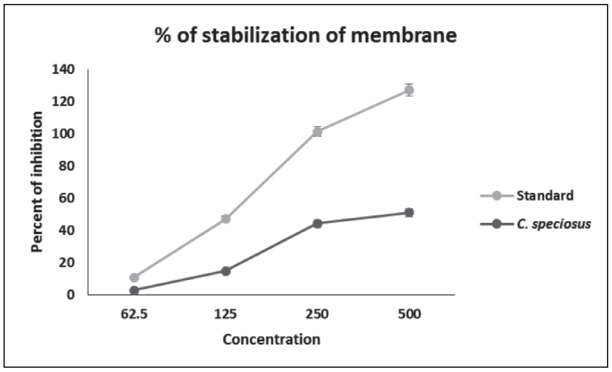

Figure 1. Data represented here are percent of protection given by methanolic crude extract from heat induced membrane lysis. It is observable that extract shows dose dependent activity likewise standard, and it was significant with P <0.01, from student 't' test analysis.tween BMI and FFMI in men for total subjects; r1= the correlation coefficient of study group; r2=the correlation coefficient of control group.

the standard, diclofenac sodium, at maximum dose, 500 µg/ml, studied crude extract have included 75.15 ± 2.33 % from protein denaturation (Figure 2). Consistent with the outcome, the *C. speciosus* extract has the capacity of controlling the production of autoantigen to inhibit the denaturation of protein, enough to call as an anti-inflammatory agent.

Result and discussion of in vivo anti-inflammatory activity

The anti-inflammatory activity of methanolic extract of *C. speciosus* on mice was found effective against inflammatory mediators. Extract at the dose of 500 mg/kg increases the response time up to 5.97 ± 0.11, which

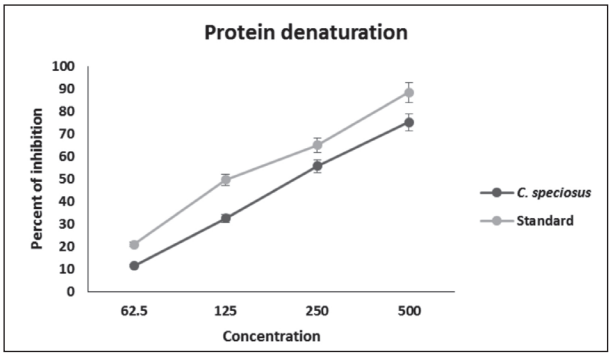

Figure 2. Percentage of protection against protein denaturation in comparison with standard. Extract is showing dose dependent potency as it is increasing in a linear manner. After performing student 't' test for statistical interception, it is found significant, P <0.01, compared to control.

was close enough to reference group (8.51 ± 0.21) at 90 min of drug administration (Table 1 and Figure 3). The effect of the extract of *C. speciosus* on formalin-induced pain in mice is shown in Table 2. The extract significantly inhibited the licking response in both the early phase and the late phase of the formalin test, which were comparable to those of the standard drug (Table 3, and Figures 4 and 5). Both these inhibitions were dose dependent.

The formalin experiment is a model of carrying on with pain including peripheral irritation and central sensitization. The process displays a biphasic reaction comprising of an early (neurogenic) and a late (inflammatory) segment reaction. The neurogenic infection followed through participation of kinins and leukocytes with their pre-inflammatory mediators including prostaglandins (39). The acute inflammation resulting from formalin induction transform into cell damage, which plays a major role in the production of endogenous mediators (40). Results of this study shows that the plant extract produced antinociception in opposition to both neurogenic and inflammatory segment of formalin induction. The fact that the extract on the prescribed doses produced analgesia in all nociceptive models is indicative that it possesses both central and peripheral antinociceptive effects and the mechanism of the extract may partially be involving lipooxygenase and/or cyclooxygenase of the arachidonic acid cascade and/or opioid receptors. From the phytochemical study (see Table 4) the leaves of *C. speciosus* contains flavonoids, steroids and some other anti-oxidant content, the anti-inflammatory effect we observed in this study may be due to their presence.

It is well known that tail immersion model is a

Figure 3. Acute anti-inflammatory activity of methanolic plant extract through tail immersion methods. The response time means the time taken by animals to respond to the heat that induced pain in animal.

Table 2. Results of the Formalin induced writhing test

Groups	Phase 1 (0-5 min)	Phase 2 (20-30 min)
Control	31.8 ± 3.45	19.1 ± 1.71
Standard (10 mg/kg)	7.9 ± 0.79	3.2 ± 0.47
CS_250 mg/kg	17.3 ± 2.39	7.8 ± 0.75
CS_500 mg/kg	13.7 ± 2.17	6.4 ± 0.29

Values shown here is mean ± SEM, with significantly (P <0.001) different, each value was compared to control and finally intercept through one-way ANOVA followed by Dunnett's test.

Table 3. Results of the Percent inhibited by extract

Group	1st phase	2nd phase
Standard (10 mg/kg)	68. 93%	73.78%
CS_250 mg/kg	43.39%	55.91%
CS_500 mg/kg	53.59%	59.89%

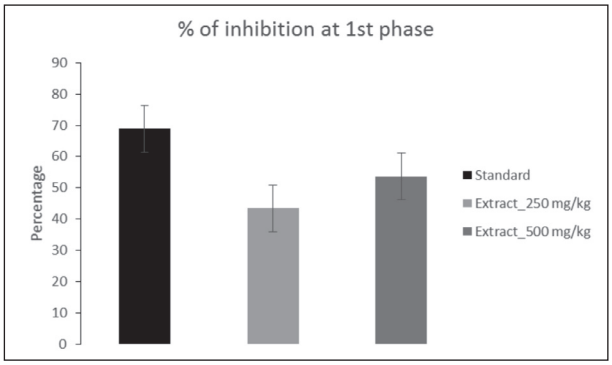

Figure 4. Percent inhibited by different doses of extract in 1st segment of inflammation development

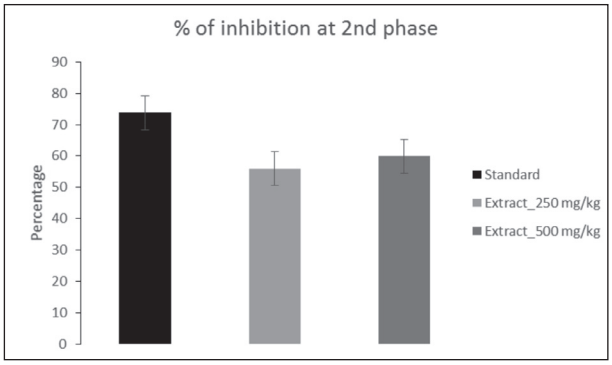

Figure 5. Percent inhibited by different doses of extract in 2nd segment of inflammation development

Table 4. Presence or absence of phytochemical compounds in extract of *C. specious*

Phytochemical compounds	Presence/absence
Carbohydrate	+
Cholesterol	+
Steroid	-
Alkaloid	+
Tannin	+
Flavonoid	+
Resin	-
Saponin	+
Phenol	+

method for measuring the central analgesic effects of drugs that acts on opioid receptor (41). Our present study demonstrated that methanolic extract of *C. speciosus* was effective against this entire model at 500 mg/kg dose that was comparable with the standard drug (Table 1, Figure 3). Narcotic analgesics are active against both peripheral and central ache, while nonsteroidal anti-inflammatory drugs inhibit only peripheral pain (42, 43). Our present used method and its impact from the extract, we worked with, indicates clearly that this plant is acting like a narcotic analgesic drugs.

Both models, increase in pain reaction time or latency period indicates the level of analgesia of drug or extract and so it is done by the extract in both cases.

Conclusion

The outcomes from our present study allows us to conclude saying that the methanolic extract of *C. speciosus* has good analgesic and anti-inflammatory property. This property offers in general due to the presence of the antioxidant element like the flavonoid and the steroidal content material in it. The mechanism of action is just not corroborated, however from the point of view of our applied methods, the mechanism of action is somehow predictable. The anti-inflammatory activity shown by the methanolic extract is possibly due to inhibition of COX enzyme followed by the inhibition of synthesis of prostaglandin and the large analgesic effect is a result of its action over nociceptors in CNS. For that reason, these findings of our present study demonstrates that the methanolic extract of *C. speciosus* is a potent anti-inflammatory and analgesic herbal extract and its use in normal treatment to deal with inflammatory and painful stipulations is justified. The outcome also provides an indication that the obtained results of this plant may be because of its free radical scavenging ability, as well.

Acknowledgement

We would like to thank International Islamic University Chittagong for permitting us to use their lab, without which we would not be able to complete this project.

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Correspondence:
Shofiul Azam,
Department of Pharmaceutical Sciences
North South University, Dhaka-1229, Bangladesh
E-mail: shofiul_azam@hotmail.com