Research Article

Anti-Inflammatory and Analgesic Activity of Methanolic Extract of Areca Seed Collected From *Areca Catechu* Plant Grown In Assam

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ABSTRACT
In our previous study the methanolic extract of areca seed collected from *Areca catechu* plant grown in Assam, was found to have potent antioxidant activity in comparison to well established antioxidant ascorbic acid. Antioxidant drugs may be beneficial in prevention of inflammation and pain arises from cellular degeneration due to oxidative stress. Our objective was to study anti-inflammatory and analgesic potential of the methanolic extract of the areca seed. Results showed that the extract significantly inhibits carrageenan induced inflammation in rat and acetic acid induced visceral pain in mice in a concentration dependent manner. The extract exhibited 14.49% and 27.75% inhibition of occurrence of inflammation in treated rats and also exhibits 35.77% and 58.81% inhibition of pain in treated mice at doses 500 and 1000mg/kg respectively. However in both the cases the extract at all the doses examined, produced relatively lower inhibition of inflammation and pain than the standard drug diclofenac sodium and indomethacin. Diclofenac sodium inhibited inflammation by 35.75% at 4 hours of administration and indomethacin inhibited visceral pain by 70.30%. The results obtained prove that the mehtanolic extract of areca seed examined in this study has significant anti-inflammatory and analgesic activity which are dependent on concentration. However the analgesic and anti-inflammatory potencies of the extract was found relatively less than that of well established analgesic anti-inflammatory drug diclofenac and indomethacin.

Keywords: *Areca catechu* seed, Methanolic extract, Anti-inflammatory activity, Analgesic activity.

INTRODUCTION
The seed of areca catechu Lin (Family-Arecaceae) commonly known as betel nut is extensively chewed as betel quid by about 10% of world population particularly in tropical countries[1,2]. It is a popular Chinese traditional medicine used to treat various diseases. In most parts of India, Sri Lanka and southern china areca nuts are not only chewed along with betel leaf but also used in the preparation of Ayurvedic and traditional Chinese medicines. Fresh seeds are used to heal foot sores.[3] Powdered nuts are prescribed in diarrhea, abdominal distention (as purgative)[4], to remove intestinal parasites such as tapeworms, pinworms, round worms (as anthelmintic)[5] and urinary disorders[6]. It is also used as anti-periodic and treatment of syphilis.[7] In veterinary medicine, an extract of areca seed is used to treat tapeworms in dogs and cattle, and to treat intestinal problems in horses.[8] Areca nut has been reported to contain about nine closely related alkaloids belongs to pyridine group among them arecoline content in ripe seed is 0.12-0.24% of total alkaloid content (0.45%) and is a potent muscarinic agonist[9-12]. It is rich in polyphenolic compounds like tannins chiefly catechol tannins and average tannins content is about 25%[13,14]. Besides alkaloids and tannins it also contains small amount of carbohydrate, fats, fibre and minerals. The concentration of chief chemical constituents like alkaloid and tannins present in the seed is decreases with its maturity and with geographical origin[15]. Several research reports suggests that the areca seed has diverse pharmacological activities including ant-inflammatory and analgesic activity[16-20]. The areca seed selected for this study was collected from the state of Assam, the eastern part of India, in which there is a traditional custom or ritual to chew areca nut (unripe, ripe and dried) usually with betel leaf along with lime. Use

of tobacco with betel nut is common among young and elderly individuals. In our previous study the selected areca seed extract showed significant antioxidant activity in comparison to well established antioxidant ascorbic acid. Most antioxidant drugs have been recognized for their capability to prevent inflammation and subsequent pain, resulting from oxidative cellular degeneration arises from oxidative stress. Our aim is to investigate the anti-inflammatory and analgesic activity of the methanolic extract of areca seed and to determine their potency in comparison to standard reference drug.

MATERIALS AND METHODS

Chemicals and reagents

Methanol (Merck), Carrageenin or carrageenan (Spectrochem Pvt. Ltd. Mumbai, India), Diclofenac sodium injection (Voveran-Novartis), Acetic acid (Merck), Indomethacin capsule (E.M. Pharmaceuticals Pvt. Ltd) and all other chemicals, reagents used were of analytical grade.

Plant material

Mature Areca catechu fruits were purchased from the owner of the *Areca catechu* plant, residing at Nagaon district of the state of Assam, India. Only healthy looking fruits, without infection or damage, were chosen for the examination. The husks was removed, nuts were sliced in to small pieces and dried under the sun for 30 days. The dried nuts were powdered in a mechanical grinder and utilized for extraction.

Preparation of extract

A weighed amount of powdered areca seed was macerated in petroleum ether for 7 days and filtered using Whatman No. 1 filter paper made in England. The residue was washed thrice with petroleum ether, filtered to remove fatty materials. The remaining residue was macerated again in methanol for 7 days and filtered. The residue was washed thrice, filtered and the filtrates were mixed. The solvent of the extract was evaporated in a rotary evaporator (HAHNVAPOR, Model No. HS-2005V, Made in Korea) to form concentrated thick mass and then dried using Freeze drier. The dried extract was stored at 4^0c until use.

Experimental Animals

Wister rates of average weight 120 gm and Swiss albino mice weighing 14 to 28.2 gram of either sex were used for the study anti-inflammatory and analgesic activity respectively. The animals were grouped and housed in poly acrylic cage of dimension 38x23x10 cm, with stainless steel coverlid, not more than five per cage and maintained under standard laboratory conditions: temperature 25±2°C, 12:12 hours light and dark cycles. They were fed with standard dry pellet food (Hindustan Unilever, Kolkata, India) and water ad libitum. The animals were randomly selected and marked to permit individual identification and acclimatized to laboratory conditions for a period of one week. Animals were fasted, but allowed water for 12 hours duration prior to commencement of the experiment. The ethical clearance was obtained from Jadavpur University Ethical Committee for using animals in the present study.

Methods

Anti-inflammatory activity

Anti-inflammatory activity was evaluated by Carrageenan induced rat paw oedema method described by Winter et al. (1962) [21,22,23,24]. Wister strain of Albino rats of either sex weighing 120 g in average, were divided in 4 (four) groups, each containing 6 (six) animals (N=6). Group-I was marked as control, received normal saline in a dose of 10 ml/kg/b. wt. , Group-II and III received methanolic extract (ME) of areca seed, in a dose of 500 and 1000 mg/kg/b. wt. orally, respectively. Group- IV received standard anti-inflammatory drug “Diclofenac sodium” in a dose of 10 mg/kg/b. wt. subcutaneously. One hour after treatment, 0.1 ml of 1% suspension of carrageenan in normal saline was injected into the sub-planter region of left hind paw to induce oedema. Treatment schedule of different groups are summarized in table 1.

The paw volume up to tibiotarsal articulation was measured using Plethysmometer, initially at 0 hour and at

1, 2, 3, 4, 5 hours after administration of carrageenan injection. The difference between the initial and subsequent values gave the actual oedema volume. The inhibition of oedema development at the site of injection of the animals treated with test materials 1 hour before carrageenan administration was considered the indication of anti-inflammatory activity. The percentage inhibition of oedema in comparison to that of control was taken as a measure of anti-inflammatory ability for the test materials. The efficacy or potency of anti-inflammatory activity of the test substance was than determined by comparing with that of standard drug.

The percentage inhibition of the inflammatory reaction was determined for each animal by comparing with that of control and calculated using the following formula:

$$\textbf{\% inhibition (\% I)} = \frac{(V_C - V_T)}{V_C} \times 100$$

Where, V_C and V_T represent mean paw oedema volume of control and treated groups.
$(V_C - V_T)$ = Mean paw oedema volume inhibited.

Table 1: Treatment schedule of anti-inflammatory activity study

Sl. No.	Groups	Treatment & doses	
1	I	Vehicle: Normal saline 10ml/kg b. wt.	0.1 ml Carrageenan 1% after 1 hour of treatment with vehicle and drugs.
2	II	ME: 500 mg/kg b. wt.	
3	III	ME: 1000 mg/kg b. wt.	
4	IV	Standard: Diclofenac sodium- 10 mg/kg b. wt.	

Analgesic activity

The analgesic activity of the extract was evaluated by acetic acid induced writhing test method.[25,26,27,28] In this method 0.6% v/v acetic acid was used to induce abdominal pain in experimental animals (mice). This pain response was recognized by its characteristic wave of contractions of the abdominal musculature followed by extension of the hind limbs (Writhing). Indomethacin was used as standard in a dose of 10mg/kg b. wt. to compare the analgesic activity of the extract. The animals were divided randomly in to four groups of six animals each. All animals were weighed for calculating accurate dose before administration. Acetic acid was administered at a dose of 10ml/kg b. wt. intraperitoneally to all the animals after one hour of oral administration of vehicle, extract and indomethacin. Immediately after administration of acetic acid the animals were placed in clean poly-acrylic cages and the number of writhing movement made by the mice was counted individually for a period of 15 minutes. The number of writhing movement recorded for the groups, treated with extract and standard was then compared with that of control. Percentage inhibition of both extract and standard groups were calculated using the equation given below and compared to evaluate analgesic ability of the extract at the doses tested.

$$\textbf{\% inhibition} = \frac{(C - T)}{C} \times 100$$

Where, C and T represents mean writhing numbers of control and treated groups respectively.
(C-T) = Mean writhing numbers inhibited by groups treated with extract or standard

Table 2: Treatment schedule of analgesic activity study

Groups		Treatment Drug & dose	Rout	Writhing inducer
I	Control	Distilled water	IP	0.6% Acetic acid in a dose of 10 ml/kg i. p. one hour after drug & vehicle administration
II	Treated	ME 500 mg/kg	Oral	
III		ME 1000 mg/kg		
IV	Standard	Indomethacin 10mg/kg		

Statistical analysis

Data obtained were analyzed by one way analysis of variance (ANOVA) followed by Dunnett's multiple comparison test using software GraphPad Prism5 and expressed as mean ± standard error mean. All data were considered significant in comparison to control when $p < 0.05$.

RESULTS

Anti-inflammatory activity

The carrageenan induced paw oedema volume of all individual rats belongs to both control and treated groups were measured at every one hour intervals from 0-5 hours. These raw data were statistically analyzed to determine mean and standard error mean which are shown in table 3. The paw oedema volume found increased with time and attained maximum at 3 hour which was then decreased.

Table 3: Rats paw oedema volume of control and treated groups recorded in one hour interval from 0-5 hours

Groups	Paw oedema volume in ml					
	0 hour	1 hour	2 hour	3 hour	4 hour	5 hour
Control	0.4±0.007	0.49±0.008	0.61±0.019	0.72±0.011	0.69±0.034	0.66±0.026
ME 500	0.44±0.023	0.48±0.021	0.54±0.022	0.61±0.016	0.59±0.011	0.58±0.018
ME 1000	0.43±0.015	0.45±0.017	0.49±0.024	0.51±0.011	0.49±0.017	0.49±0.010
Diclofenac	0.41±0.011	0.42±0.006	0.44±0.005	0.44±0.004	0.43±0.006	0.42±0.008

Data expressed as mean paw volume in ml ± SEM, N=6. Raw data analyzed by ANOVA.

The percentage inhibitions of carrageenan induced paw oedema volume was then calculated for all the groups treated with methanolic extract and standard drug Diclofenac sodium which are shown in table-4 and figure-1. These data were analyzed by ANOVA followed by Dunnet's Multiple Comparison test and found significant. Maximum inhibition was observed at 3 hour with methanolic extract and standard. Methanolic extract of areca seed at a dose of 500mg/kg and 1000mg/kg inhibits carrageenan induced paw oedema volume by 15.27% and 28.39% respectively. Diclofenac sodium was used as standard to compare inhibitory activity of methanolic extract of areca seed which produced highest inhibition (38.69%) at 3 hour from administration than at all the doses of extracts examined.

Table 4: Percentage inhibition of paw oedema volume of rats treated with methanolic extract and diclofenac sodium

Groups	% Inhibition of carrageenan induced Paw oedema volume				
Treated with	1 hour	2 hour	3 hour	4 hour	5 hour
ME 500	2.04	10.47	15.27	14.49	12.12
ME 1000	8.16	19.67	28.39	27.75	25.63
Diclofenac	13.24	26.95	38.69	37.46	35.75

Data analyzed by ANOVA followed by Dunnett's Multiple Comparison Test. $P < 0.05$.

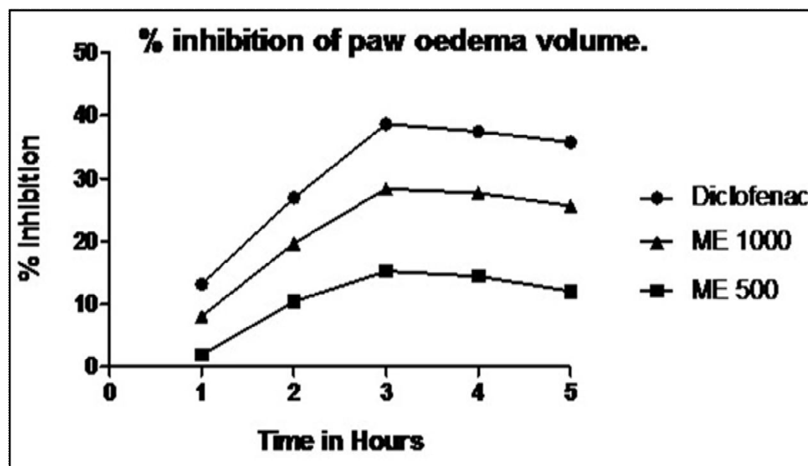

Fig. 1: Percentage inhibition of carrageenan induced paw oedema volume of rats treated with standard drug diclofenac sodium and methanolic extract of areca seed

Analgesic activity

The occurrence of writhing movements as a response to pain induced by acetic acid were found reduced by methanolic extract of areca seed at all the doses examined and were found statistically significant in comparison to control which are summarized in Table 5. Indomethacin was used as standard to compare the analgesic ability of the extract, exhibited significant reduction of the number of writhing, recorded in 15 minutes. Methanolic extract of areca seed inhibited the acetic acid induced writhing in a dose dependent manner. At doses of 500 and 1000mg/kg the extract exhibited 35.77 and 58.81% inhibition respectively. However at both the doses the analgesic activity of the extract was found less than that of standard analgesic indomethacin which produced 70.3% inhibition.

Table 5: Writhing, percentage inhibition and statistical significance of analgesic activity of methanolic extract at different doses and standard-indomethacin in comparison to control

Groups		No. of animals	Mean writhing ± SEM recorded in 15 minutes	Significant	% inhibition
I	Control	6	27.51±0.885	-	-
II	ME 500	6	17.67±0.988	Yes*	35.77
III	ME 1000	6	11.33±0.714	Yes**	58.81
IV	Indomethacin	6	8.17±0.477	Yes**	70.30

DISCUSSION

Carrageenan induced paw oedema model is the simplest, standard and most commonly used model to screen the anti-inflammatory activity of new compounds[29] or natural products[30]. Carrageenan causes release of several mediators at the site of injection which activate and sensitize the peripheral nerve endings leading to development of oedema resulting acute inflammation and pain[31, 32]. This phenomenon is believed to be biphasic. The first phase from 0-1 hour of carrageenan injection involves the prompt release of serotonin, histamine and bradykinin. The second phase after 1 hour

involves with the synthesis and release of prostaglandins with the help of cyclooxygenases (COXs) from arachidonic acid and the effect last about 3 to 6 hours.[33,34,35,36,37] This model has been commonly used to evaluate the effect of non-steroidal anti-inflammatory drugs (NSAIDs) that primarily inhibit the COXs, involved in the prostaglandins synthesis.[38] The reduction of paw oedema volume of the rats than the control after one hour of carrageenan injection were taken as a measure of anti-inflammatory activity of the test materials. Table4 shows that the methanolic extract reduces paw oedema volume significantly in a concentration dependent manner which indicates that the extract contains compounds having anti-inflammatory activity. The fashion of anti-inflammatory activity produced by the extract was observed similar to that of Diclofenac sodium which is a well known potent COX-inhibitor. Thus the anti-inflammatory activity of the extract may be related to the inhibition of cyclooxygenases responsible for prostaglandin synthesis. Moreover, figure-1 shows that the extract at both the tested doses exhibited lesser percentage of inhibition than that of diclofenac sodium which indicates that the anti-inflammatory potency of the methanolic extract of areca seed is comparatively less than diclofenac sodium.

The analgesic activity of the extract was evaluated by acetic acid induced writhing method in mice which is a visceral pain model most commonly used for screening analgesic activity of drugs, regardless of the central or peripheral causes[39, 40]. Intraperitoneal administration of acetic acid causes immediate acute inflammation due to release of inflammatory mediators and stimulation of nociceptors at the site of injection. This inflammatory response subsequently enhances the release of arachidonic acid from tissue phospholipid which is then converted in to prostaglandins by cyclooygenases leading to increase prostaglandins level in the peritoneal cavity resulting inflammatory pain by increasing capillary permeability.[41,42,43,44] This acute visceral pain (nociceptive pain) in mice can be identified by a spontaneous twisting or stretching syndrome, occurs within 2 minutes after intraperitoneal injection of acetic acid, characterized by wave of contraction of abdominal musculature followed by extension of the hind limbs (writhing movement). This state of writhing lasts for about a hour.[45] Number of writhing movements inhibited in the test groups of mice in comparison to control was taken as a measure of analgesic activity of the test materials. Acetic acid induced visceral pain is sensitive to both centrally acting opioid antagonists and peripherally acting NSAIDs and thus can be used as positive control. Table-5 shows that the methanolic extract of areca seed exhibited significant analgesic activity in concentration dependent manner. Moreover the extract showed similar type of inhibition of the writhing movements produced by the positive control ‘indomethacin’ which is a well known NSAID and acts by inhibiting cyclooxygenses. This indicates that the extract contains compounds capable of inhibiting cyclooxygenases responsible for prostaglandin synthesis. Although the extract exhibited considerable analgesic activity but it was noticed less than that of indomethacin.

CONCLUSION

The results obtained from the in-vivo animal studies performed suggests that the methanolic extract of areca seed examined in this study has considerable, dose dependant anti-inflammatory and analgesic activity but is less potent than the reference drugs diclofenac and indomethacin respectively. Its analgesic and anti-inflammatory properties may be due to presence of compounds capable of inhibiting cyclooxygenases involving in the synthesis of prostaglandins or other arachidonic acid metabolites responsible for inflammation and nociceptive pain. These experimental findings indicate that the methanolic extract of areca seed may be beneficial in painful inflammatory conditions associated with diseases or injuries. However further study is necessary to identify its active principle responsible for the analgesic and anti-inflammatory properties and to ensure its

safety prior to development it in to herbal drug or food supplement.

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