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**Evaluation of Analgesic, Anti-inflammatory and Anti-cancer Activities of Cartilage Extract of *Aetobatus narinari***

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**ABSTRACT**

To evaluate the analgesic, anti-inflammatory and anti-cancer activities of the ethanol extract of cartilage of *Aetobatus narinari* in different experimental models in mice and rats. The dried cartilage was reduced to fine powder and refluxed with ethanol. Analgesic activity of cartilage extract of *Aetobatus narinari* was assessed in tail flick model in rats (n = 5), and acetic acid induced writhing in mice (n = 5), Anti-inflammatory activity was evaluated using carrageenan induced rat paw edema and the anti-cancer activity (In-vitro dye exclusion method) was assessed in DAL cells bearing mice. The cartilage extract exhibited  $38.2 \pm 4.33$  and  $22.6 \pm 1.17$  writhing and shown the protection of 50.52% and 70.73% at 200 and 400 mg·kg<sup>-1</sup>, respectively. The tail flick method revealed an increase in basal reaction time from  $2.4 \pm 0.25$  and  $2.6 \pm 0.40$  sec at 0 min to  $4.6 \pm 0.51$  and  $6.4 \pm 0.40$  sec at 90 min at a dose of 200 and 400 mg·kg<sup>-1</sup>, respectively. The extract at 200 mg·kg<sup>-1</sup> inhibited the edema volume by 70% with a mean edema volume of  $0.140 \pm 0.006$  at 120 min, whereas the crude ethanol cartilage extract at 400 mg·kg<sup>-1</sup> concentration produced 80% inhibition of edema volume with a mean  $0.120 \pm 0.009$  at 120 min. The ethanol extract of *Aetobatus narinari* showed a significant cytotoxic activity to the tune of 88% at a concentration of 200 µg in Dalton's Ascites Lymphoma cells in vitro. The present investigation confirmed the analgesic, anti-inflammatory and anticancer properties of *Aetobatus narinari*, and further investigations are required to augment such potentials.

**Keywords:** *Aetobatus narinari*, Cartilage, Analgesic, Anti-inflammatory, Anticancer.

**INTRODUCTION**

Cartilages of the Elasmobranchii have been of great interest due to their potential medicinal properties. Cartilage is an acidic mucopolysaccharides, also contains collagen and glycosaminoglycans including chondroitin sulfates. Chondroitin sulfate isolated from shark cartilage is used both as an angiogenesis inhibitor in the treatment of cancer and as a joint lubricant in arthritis<sup>1,2</sup>. Water soluble fraction of shark cartilage has proved to contain antinociceptive and anti-inflammatory properties<sup>3</sup>. *Aetobatus narinari*, commonly known as spotted eagle ray, belongs to the Elasmobranchii, and is traditionally used to treat inflammatory diseases and arthritis. However, this practice had not been justified with scientific experiments. Therefore the present study was aimed to investigate the analgesic, anti-inflammatory, and anti-cancer activity of the ethanol cartilage extract of *Aetobatus narinari* in animal models.

**MATERIALS AND METHODS**

**Collection of fish material**

Fresh fish were collected directly from fishing vessels of Puducherry coastal waters (11°46' and 12°03' N; 79°36 and 79°53' E). Fish were identified using the keys given by Ramaiyan and Sivakumar<sup>4</sup>.

### Preparation of extract

Freshly collected fish was cut into pieces and dehydrated for 2-3 days. The dried pieces were re-hydrated with hot water in order to separate the cartilage from the flesh and dried for a day. The dried cartilage was reduced to fine powder (75g) and refluxed three times with ethanol for 4-6 h and left to cool overnight<sup>5</sup>. Then the extract was concentrated in vacuum to yield a concentrated crude extract (7.5g). The crude extract dissolved in water and used for further studies.

### Animals

Swiss mice (25-30 g) and Wistar albino rats (200-250 g) of each sex were procured from the College of Pharmacy, Mother Theresa Institute of Health Sciences, Puducherry. The animals were kept at room temperature and maintained in a 12 h light/ dark cycle, and fed *ad libitum* with standard food and water. They were fasted for 24 h before the experiment. All the test doses were administered by the intraperitoneal route and were 10 times lower than the LD<sub>50</sub> dose. All experimental procedures followed the guidelines on ethical standards for investigations and were carried out according to a protocol approved by the local Animal Ethics Committee in compliance with National and International standards on the care and use of laboratory animals<sup>6</sup>.

### Analgesic effect

The writhing model represents a chemical nociceptive test based on the induction of peritonitis like condition in animals by injecting irritant substances<sup>7</sup>. The mice were divided into four experimental groups of five animals each. Group 1 served as control and received acetic acid (10 mL/kg), Group 2 treated with standard drug (reference) aspirin (100 mg/kg). Groups 3 and 4 were administered the crude ethanol cartilage extract of 200 mg/kg and 400 mg/kg respectively. After 30 minutes of test extracts administration, 0.1 ml of 1% acetic acid solution was injected. Mice were placed in an individual box (25×25 cm) and five minutes were allowed to elapse. They were then observed for a period of 15 minutes and the numbers of writhes were recorded in each animal. For scoring purpose, a writhe is indicated by stretching of the abdomen with simultaneous stretching of at least one hind limb. The percentage of inhibition was calculated for each animal group using the following formula.

$$\% \text{ inhibition} = [(Wc - Wt) / Wc]$$

Where, Wc = Number of writhes in control groups, Wt = Number of writhes in test groups.

### Analgesic effect

Analgesia was measured by tail flick (thermal) method using an analgesiometer<sup>8</sup>. The rats were divided into four experimental groups of five animals each. Group 1 served as control and received normal saline (2 mL/Kg), Group 2 treated with standard drug (reference) pentazocine (5 mg/kg). Groups 3 and 4 were administered test extracts as in writhing method. Reaction time in seconds was used as the unit for measurement of pain and an increase in reaction time was indicative of analgesia. Time between placing the tail of the rat on the radiant heat source (5.26 Amps) and sharp withdrawal of the tail was recorded as “reaction time”. Cut off time of 15s was imposed in all sets of experiments taken as maximum latency so as to rule out thermal injury while noting down the reaction time. Animals that showed a mean reaction time outside the range of 5-6 s were discarded. In all the groups, tail-flick test was performed before (0) and at 15, 30, 60, 90 and 120 min after drug administration and the increase in reaction time against control was recorded.

### Anti-inflammatory activity

The carrageenan-induced paw edema assay was carried out in Wister albino rat (200-250 g) described by Winter *et al.*<sup>9</sup>. Edema was induced by sub-plantar injection of 0.1 mL of freshly prepared 1% carrageenan (W/V) into the right hind paw of the rats of four groups of five animals each. Group 1 served as carrageenan control (0.1 mg/kg), Group 2 treated with standard drug (reference) diclofenac sodium (20 mg/kg). Groups 3 and 4 were administered test extracts as in writhing method. The volume of pedal

edema was measured at 0 and 15, 30, 60, 90 and 120 min after injection of carrageenan using a plethysmometer (Ugo Basile). All of the treatments were given 30 min prior to the injection of carrageenan. The percentage of edema inhibition was calculated for each animal group using the following formula.

$$\% \text{ inhibition} = 1 - T / C \times 100$$

Where, T = Increase in paw volume in groups treated with test extract, C = Increase in paw volume in control group.

### Anti-cancer activity

Dalton's Ascites Lymphoma (DAL) cells bearing mice were obtained from Amala Cancer Research Centre, Thrissur, Kerala, India. The cells were aspirated from the peritoneal cavity of cancer bearing mice. The cells were washed three times with phosphate buffered saline. The viability of the cells was checked using trypan blue and different dilutions of  $10^{-1}$ ,  $10^{-2}$  and  $10^{-3}$  were made. The number of cells in the  $10^{-3}$  dilution was counted using haemocytometer and the number of cells were adjusted to  $1 \times 10^6$  cells/ml. The experiment was done by incubating different concentrations of ethanol cartilage extract (10, 20, 50, 100 and 200  $\mu\text{g/ml}$ ) of *Aetobatus narinari* with  $1 \times 10^6$  cells/ml. The final volume of the assay mixture was made up to 1 ml using phosphate buffered saline and incubated at  $37^\circ \text{C}$  for 3 h. After the incubation, 0.1 ml of trypan blue was added and the number of dead cells was counted by hemocytometer<sup>10</sup>. Counting of non-viable cells for every 100 cells is an indicative of anti-tumor activity and the percentage cytotoxicity was calculated for different concentrations using the following formula.

$$\% \text{ cell viability} = \frac{\text{Number of dead cells}}{\text{Number of viable cells} + \text{Number of dead cells}} \times 100$$

### Statistical analysis

The data were expressed as mean  $\pm$  SEM, students 't' test were used for statistical analysis. A probability value ( $P < 0.001$ ) was considered significant.

## RESULTS

The results (mean  $\pm$  SEM) of the control group showed  $77.2 \pm 4.21$  writhing, whereas the groups treated with ethanol cartilage extract exhibited  $38.2 \pm 4.33$  and  $22.6 \pm 1.17$  writhing and shown the protection of 50.52% and 70.73% at 200 and 400 mg/kg, respectively. Aspirin treated group showed  $12.4 \pm 0.93$  writhing with a percentage inhibition of 83.94% (Fig. 1). The tail flick method revealed that the cartilage extract exhibited an increase in basal reaction time from  $2.4 \pm 0.25$  and  $2.6 \pm 0.40$  sec at 0 min to  $4.6 \pm 0.51$  and  $6.4 \pm 0.40$  sec at 90 min at a dose of 200 and 400 mg/kg, respectively (Fig. 2). The basal reaction time of control group were  $2.4 \pm 0.25$ , whereas the standard drug (reference) pentazocine showed maximum reaction time of  $8.2 \pm 0.58$  at 120 min.

Table 1 illustrates the inhibitory effect of cartilage extract of *Aetobatus narinari* on carrageenan-induced rat paw edema in all the groups. The extract at 200 mg/kg inhibited the edema volume by 70% with a mean edema volume of  $0.140 \pm 0.006$  at 120 min, whereas the crude ethanol cartilage extract at 400 mg/kg concentration produced 80% inhibition of edema volume with a mean  $0.120 \pm 0.009$  at 120 min. The carrageenan control induced inflammation with a mean edema volume from  $1.130 \pm 0.012$  at 0 min to  $0.230 \pm 0.012$  at 120 min. Percentage inhibition (90%) of acute inflammation was greater in diclofenac sodium treated group at all time intervals. The results of the anti-cancer activity of ethanol cartilage extract of *Aetobatus narinari* on DAL cells are shown in Fig. 3. The percentage of cytotoxicity effect was found to be 88%, 24%, 16%, 12% and 10% at 200, 100, 50, 20 and  $10 \mu\text{g}$  respectively.

**Table 1 Anti-inflammatory activity of ethanol cartilage extract of *Aetobatus narinari***

Group	Treatment	Dose (mg/kg)	Paw edema volume after administration (Mean $\pm$ SE, n = 5)						Inhibition %
			0 min	15 min	30 min	60 min	90 min	120min	
1	Carrageenan (control)	0.1	0.110 $\pm$ 0.012	0.140 $\pm$ 0.010	0.204 $\pm$ 0.003	0.220 $\pm$ 0.012	0.230 $\pm$ 0.012	0.230 $\pm$ 0.012	-
2	Diclofenac	20	0.110 $\pm$ 0.010	0.180 $\pm$ 0.030	0.160 $\pm$ 0.010	0.130 $\pm$ 0.012	0.120 $\pm$ 0.020	0.120 $\pm$ 0.012	90
3	Test extract	200	0.110 $\pm$ 0.010	0.210 $\pm$ 0.019	0.190 $\pm$ 0.013	0.166 $\pm$ 0.014*	0.146 $\pm$ 0.004**	0.140 $\pm$ 0.006**	70
4	Test extract	400	0.110 $\pm$ 0.010	0.220 $\pm$ 0.012	0.196 $\pm$ 0.016	0.170 $\pm$ 0.012*	0.132 $\pm$ 0.008**	0.120 $\pm$ 0.009**	80

\* $P < 0.001$  vs the control group; \*\* $P < 0.001$  vs the diclofenac group

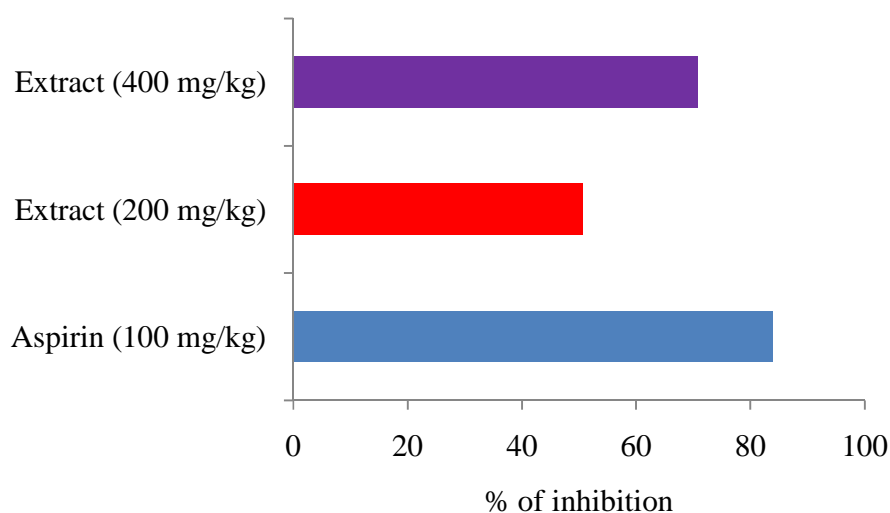
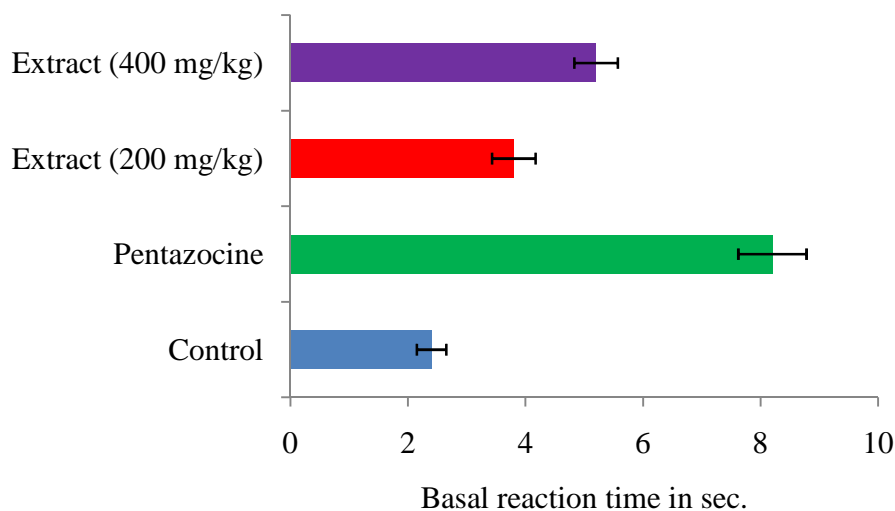
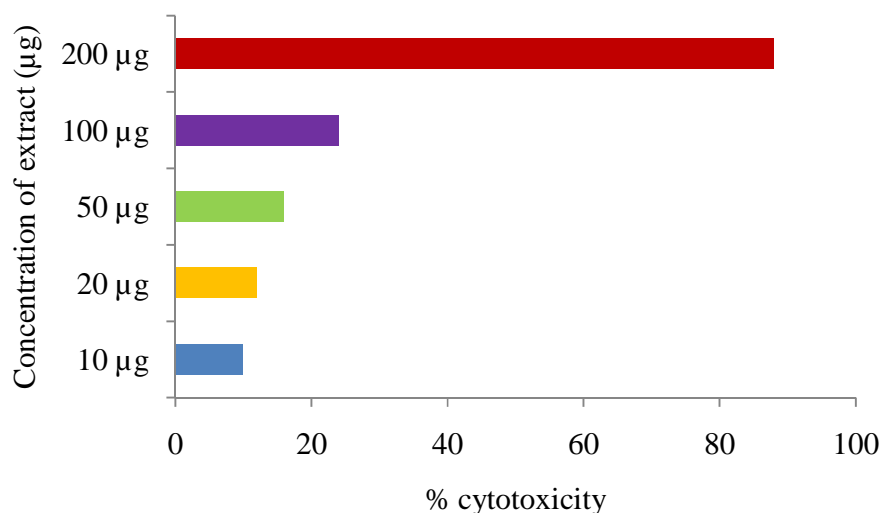
**Fig. 1. Analgesic activity of ethanol extract of cartilage of *Aetobatus narinari* – Acetic acid induced writhing (Mean  $\pm$  SE, n = 5)****Fig. 2. Analgesic activity of ethanol extract of cartilage of *Aetobatus narinari* – Tail flick method (Mean  $\pm$  SE, n = 5)**

Fig. 3. Cytotoxic effect of ethanol extract of cartilage of *Aetobatus narinari* on DAL cells

### DISCUSSION

In the present study, the effects of ethanol extract of cartilage of *Aetobatus narinari* against selected animal models to establish the analgesic, anti-inflammatory and anti-cancer properties. The experimental models used for screening the analgesic effect are belonging to both chemical and thermal methods. Acetic acid induced writhing in rats attributed visceral pain finds much attention of screening analgesic drugs<sup>11</sup>. Pain sensation in acetic acid induced writhing method is elicited by triggering localized inflammatory response resulting release of free arachidonic acid from tissue phospholipid via cyclooxygenase (COX), and prostaglandin biosynthesis<sup>12</sup>. The two different doses (200 and 400 mg/kg) of cartilage extract of *Aetobatus narinari* showed significant analgesic action but at 400 mg/kg was found to exhibit higher analgesic activity of 70.73% against acetic acid induced pain in rats. Maximum analgesic activity of aspirin was observed in this model. The significant pain reduction of the cartilage extract might be due to the presence of analgesic principles acting with the prostaglandin pathways. Therefore, the result of the acetic acid-induced writhing strongly suggests that the mechanism of this action may be linked to the inhibition of cyclooxygenase in the peripheral tissues, thereby reducing prostaglandin synthesis and interfering with the mechanism of transduction in primary afferent nociceptors<sup>13</sup>.

The tail flick (thermal) method of analgesia is effective in estimating the efficacy and potency of centrally acting analgesics based on the enhancement of pain threshold by the compounds present in the test extract<sup>14</sup>. This was evident in the present study wherein the pain threshold increased significantly during the period of observation in the groups treated with test extract with maximum effect observed at 400 mg/kg suggesting dose-dependent increase in pain threshold. The standard drug, pentazocine (a  $\kappa$ -receptor agonist) was used in the present study exerted a significant analgesic effect by preventing the sensitization of the opioid receptors suggesting that the bioactive compounds present in the ethanol extract of cartilage of *Aetobatus narinari* are capable of relieving pain by preventing the sensitization of the receptors through chemical stimulation by enhancing the pain threshold<sup>15</sup>.

Carrageenan-induced hind paw edema is the standard experimental model for acute inflammation. Carrageenan is the phlogistic agent of choice for testing anti-inflammatory drugs as it is not known to be antigenic and is devoid of apparent systemic effects<sup>16</sup>. The ethanol cartilage extract of *Aetobatus narinari* showed considerable biological potential to inhibit the inflammation significantly. The extract at 400 mg/kg caused a significant inhibition, with an inhibition percentage of 80% almost close to standard drug diclofenac sodium (90%), whereas at 200 mg/kg concentration the extract showed 70% reduction in edema volume. The development of edema has been described in three distinct phases. The initial phase is

attributed to the release of histamine and serotonin. A second phase is mediated by kinins and a more pronounced third phase is related to the release of the most important mediator prostaglandins<sup>17</sup>. The standard drug (reference) used in the present study is the most widely used drug for the treatment of inflammatory conditions and are non-selective direct inhibitors of cyclooxygenase enzyme. The present result shows that the extract at both concentrations caused a significant inhibition in rat paw edema only during the 3<sup>rd</sup> and 4<sup>th</sup> h, where as the inhibition caused at the end of the 1<sup>st</sup> and 2<sup>nd</sup> h was not significant, suggesting the probable mechanism of anti-inflammatory action may be due to the inhibition of prostaglandin biosynthesis by interfering with the cyclooxygenase pathway by the effect of bioactive compounds present in the cartilage extract<sup>18</sup>.

The ethanol extract of cartilage of *Aetobatus narinari* showed a significant cytotoxic activity to the tune of 88% at a concentration of 200 µg in Dalton's Ascites Lymphoma cells *in vitro*. Previous study<sup>19</sup> revealed that bioactive compounds isolated from cartilage of elasmobranchii is known anti-angiogenesis and able to block growth factors signal pathway. Since only *in vitro studies* were carried out on ethanol extract of cartilage of *Aetobatus narinari* it is difficult to establish its exact mechanism of action, but the possible mechanism of cytotoxic potential of the extract may be due to blockade of angiogenesis<sup>20</sup>.

In conclusion, the present investigation confirmed the capability of the ethanol extract of cartilage of *Aetobatus narinari* to suppress abdominal writhes, increase pain threshold latency, inhibition of the phases of carrageenan-induced inflammation and cytotoxicity confirms the analgesic, anti-inflammatory and anti-cancer properties. These findings justify conventional use of this ray fish in the treatment of pain and other inflammatory conditions and validate its claim of being used for the said purpose in traditional practice.

#### CONFLICT OF INTEREST STATEMENT

We declare that we have no conflict of interest.

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