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Pharmacological investigation and spectral characterization of bioactive compounds from crude extracts of sting ray, *Dasyatis jenkinsii* (Annandale, 1909)

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[ABSTRACT] AIM: *Dasyatis jenkinsii* is used traditionally to treat inflammatory complaints and arthritis by the fisherman community and local population. The present study was designed to scientifically investigate the traditional practice through the analgesic, anti-inflammatory, and organ toxicity studies and characterization of bioactive compounds of crude extracts of *D. jenkinsii*. **METHOD:** Solvent extract of homogenized fresh fish was prepared using petroleum ether and diethyl ether. The chemical and spectral analyses of extracts were carried out using FT-IR and GC-MS. Analgesic and anti-inflammatory activities were assessed by hot plate, tail clip, and carrageenan induced rat paw edema methods. The organ toxicity of each extract was assessed on brain, liver, and kidney of mice. **RESULTS:** The IR spectrum indicated the presence of aromatic and aliphatic compounds. GC-MS analysis revealed the presence of 1-(4-carboxy)phenylnona-2, 5-diene and 3-hydroxymono-glyceryl hydrogen phthalate in the petroleum ether extract and carboxyl serine, dihydrotryptophan, and indolyl carboxylic acid in the diethyl ether extract. Both extracts showed significant analgesic and anti-inflammatory effects in all the methods tested. The vital organs of the test animals were not affected by the crude extracts significantly. **CONCLUSIONS:** The presence of biologically active compounds in the crude extracts with analgesic and anti-inflammatory properties justifies the traditional knowledge and paves the way for isolation of these compounds for further experimentation.

[KEY WORDS] Elasmobranch; *Dasyatis jenkinsii*; Analgesic; Hot plate; Anti-inflammatory; Sharp nose stingray

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1 Introduction

The traditional knowledge regarding the medicinal value of fish is prevalent among the local communities from time immemorial. This knowledge has become a tool to identify and explore the invisible marvels of bioactive compounds that are contained in these fish. Members of the Elasmobranchii have been of great interest due to their potential medicinal properties. Shark cartilage is used both as an angiogenesis inhibitor in the treatment of cancer and as a joint lubricant in arthritis [1]. Chondroitin sulfate isolated from shark cartilage appears to improve osteoarthritis symptoms [2]. The petroleum ether and diethyl ether fraction of *Dasyatis zugei* showed significant analgesic and anti-inflammatory properties in animal models [3]. *Dasyatis jenkinsii*, commonly known as sharp nose stingray, belongs to the Elasmobranchii, and is traditionally used to treat inflammatory diseases and arthritis. A pudding of fish flesh is given to a person suffering from arthritis. An ethnopharmacological survey based on the Foundation for Revitalization of Local Health Traditions (FRLHT), Bangalore, India among the traditional practitioners of the fisherman community confirms these uses. However, no scientific data have been published to support the ethno pharmacological use. The present study was aimed to investigate the analgesic, anti-inflammatory, and organ toxicity of the petroleum ether and diethyl ether fractions of the fish extract in animal models. The bioactive compounds responsible for pharmacological activities were also studied through FT-IR and GC-MS.

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These authors have no conflict of interest to declare.

2 Methodology

2.1 *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 [4].

2.2 *Preparation of extract*

Fresh fish (500 g) was collected and homogenized at room temperature. The homogenized flesh was refluxed three times with petroleum ether and diethyl ether separately for 4−6 h and left to cool overnight [5]. Then the extracts were concentrated in vacuum to yield a thick, viscous, dark reddish brown mass (12 g). The crude extract obtained in each case was solubilized in DMSO before being administered to the mice and rats.

2.3 *Animals*

Male albino rats (150−175 g) and male albino Swiss mice (25−30 g) were procured from the College of Pharmacy, Mother Theresa Institute of Health Sciences, Puducherry for use in the experiment. 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_{50} 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 [6].

2.4 *Chemical and spectral analysis*

The chemical analysis of the petroleum ether and diethyl ether extracts was carried out to ascertain the aliphatic or aromatic, saturated, or unsaturated nature of the constituents. FT-IR spectrum was recorded between 4 000 and 600 cm^{-1} for all the extracts using a Bruker IFS 85 FT-IR, Germany. The elucidation and molecular mass of the compounds from the crude extracts were performed on a GC-MS (Shimadzu QP 5 000, Japan). The methanol-dissolved sample (2 mL) was injected into a CBP-1 packed column (25 mm × 0.25 mm dia), the temperature was increased linearly from 50 to 320 °C and the carrier gas pressure was fixed at 79.80 Kpa for all the samples. Electron impact mass spectra were recorded for each compound separated in succession by GC, the relative intensities corresponding to their R_t of the molecular ion peak and the fragmented ion peaks were normalized with respect to the base peak.

2.5 *Analgesic effect – hot plate method*

The analgesic activity was assessed by the hot plate (thermal) method as described by Eddy and Leimbach [7]. The mice were divided into six experimental groups of six animals each. Group 1 served as control and received normal saline (2 $mL \cdot kg^{-1}$). Groups 2, 3, and 4 were treated with standard drugs (reference) buprenorphine (5 $mg \cdot kg^{-1}$), pethidine (30 $mg \cdot kg^{-1}$), and pentazocine (5 $mg \cdot kg^{-1}$), respectively. Groups 5 and 6 were administered the crude petroleum ether extract (80 $mg \cdot kg^{-1}$) and diethyl ether extracts (95 $mg \cdot kg^{-1}$). The animals were placed on a hot plate (Analgesiometer, Techno) maintained at a temperature of (55 ± 0.5) °C. The basal reaction time, when the animals licked their paw or jumping occurred was recorded by a stop watch before (0) and at 15, 30, 60, 90, and 120 min after administration of the crude extracts. A cut off time of 15 s was used. The increase in reaction time against control was calculated.

2.6 *Analgesic effect – Haffner's tail clip method*

Haffner's tail clip method was performed as described by Bartoszyk and Wild [8]. Groups 1 to 6 received the control, standard drugs (reference) and test extracts as in the hot plate method. An artery clip with thin rubber sleeves was applied at the base of the animal tail. The time taken by the animal to make an effort to dislodge the clip was recorded before (0) and at 15, 30, 60, 90 and 120 min after administration of crude extracts. The increase in reaction time against control was recorded.

2.7 *Anti–inflammatory activity – Carrageenan-induced rat paw edema method*

The carrageenan-induced paw edema assay was carried out in male albino rat (150−170 g) described by Winter *et al* [9]. 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 six groups of six animals each. Group 1 served as carrageenan control (0.1 $mg \cdot kg^{-1}$g), Groups 2, 3, and 4 were treated with standard drugs (reference) diclofenac sodium (10 $mg \cdot kg^{-1}$), ketorolac (10 $mg \cdot kg^{-1}$), and phenylbutazone (200 $mg \cdot kg^{-1}$), respectively. Groups 5 and 6 were given the crude petroleum ether (80 $mg \cdot kg^{-1}$) and diethyl ether extracts (95 $mg \cdot kg^{-1}$) of *D. jenkinsii*, respectively. The volume of pedal edema was measured at 0 and 1/2, 1, 2, 3, and 4 h 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.

2.8 *Histopathological studies*

The organ toxicity of each extract was assessed on the brain, liver, and kidney in mice. Five animals from each control (2 $mL \cdot kg^{-1}$), petroleum ether (80 $mg \cdot kg^{-1}$), and diethyl ether (95 $mg \cdot kg^{-1}$) treated groups were sacrificed by cervical dislocation at the end of 24 h. The vital organs of each group were dissected out, rinsed in distilled water, and were fixed in 10% buffered neutral formalin. Paraffin blocks were prepared and 5 mm thick sections were cut on a rotary microtome and stained with haematoxylin and eosin. Prepared slides were observed under light microscope and gross pathological changes were observed.

2.9 *Statistical analysis*

The analyses of variance, ANOVA, followed by the Fisher test were used for statistical analysis. Data were expressed as mean ± SE. A probability value ($P < 0.05$) was considered significant.

3 Results and Discussion

The results of the elemental analysis indicated the pres-

ence of carbon, hydrogen, oxygen and the absence of nitrogen and sulfur. The functional group analysis gave positive tests for the presence of carboxylic acid and ester groups. The aromatic nature of the compounds were derived from the sooty flame produced by the concentrate of the extract.

The crude petroleum ether extract of *D. jenkinsii* (PEEDJ) exhibited strong IR signals appearing at 3 010, 2 922, and 2 852 cm^{-1} corresponded to the aromatic and aliphatic C-H stretching, and strong absorption at 1 709 cm^{-1} was due to the C=O stretching frequency (Fig. 1). The GC-MS analysis suggested the presence of three peaks with the retention times ranging from 17.942 to 20.225 min (Fig. 2A). The positive EIMS and their fragmentation pattern were recorded for three compounds separated by GC. Compound 1 corresponding to the first peak gave a molecular ion peak at *m/z* 244 inferred from the EIMS was in accordance with $C_{16}H_{20}O_2$ as 1-(4-carboxy)phenylnona-2, 5- diene (Fig. 2B). The EIMS pattern of compound 2, with a molecular ion peak at *m/z* 256, suggested 3-hydroxymono- glyceryl hydrogen phthalate with the molecular formula $C_{11}H_{12}O_7$ (Fig. 2C). The molecular ion peak for compound 3 appeared at *m/z* 149 in its positive EIMS due to anhydride ion, suggesting protonated phthalic anhydride with the formula $C_8H_5O_3$ (Fig. 2D).

The elemental analysis of the crude diethyl ether extract of *D. jenkinsii* (DEEDJ) disclosed the presence of carbon, hydrogen, oxygen, and nitrogen, and the functional group tests revealed the presence of acid and amine groups in an aromatic system.

The broad band at 3 344 cm^{-1} of FT-1R spectrum could be assigned to NH and O-H stretching, while 1 623 cm^{-1} was C=O stretching. The band at 1 042 cm^{-1} was assigned to C-N stretching frequency (Fig. 3). GC-MS analysis displayed three peaks with the retention times ranging from 6.201 to

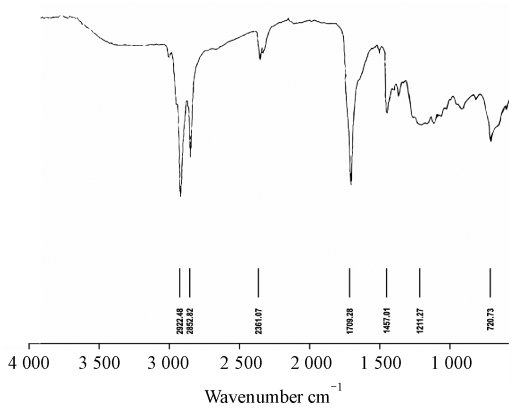

Fig. 1 FT-IR spectrum of crude petroleum ether extract of *D. jenkinsii*

24.026 min (Fig. 4A). The fragmentation pattern that resulted from the EIMS of compound 1 contained the molecular ion peak at *m/z* 149 corresponding to the molecular formula $C_4H_7O_5N$ and the fragment ion peaks at *m/z* 105 suggested the compound to be 3-carboxyl serine, an acidic amino acid (Fig. 4B). EIMS of compound 2 exhibited a molecular ion peak at *m/z* 206 with the molecular formula $C_{11}H_{14}O_2$. The fragmentation pattern revealed the compound was 2, 3- dihydrotryptophan (Fig. 4C). The molecular ion peak at *m/z* 161 observed in the positive EIMS of compound 3 was compatible with the molecular formula $C_9H_7NO_2$. Based on the fragmentation pattern the compound was characterized as indolyl-3-carboxylic acid (Fig. 4D). All the compounds characterized in the present study were compared with those compounds already reported in herring, mackerel, cod liver oil, and shark liver oil [10].

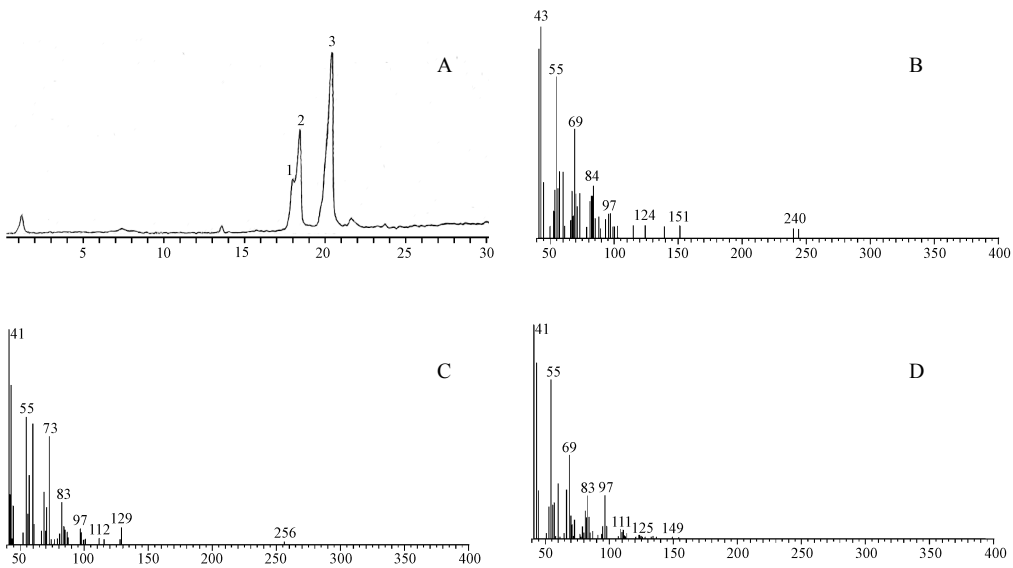

Fig. 2 A. GC-MS of the crude petroleum ether extract of *D. jenkinsii*; B. EIMS of compound 1 from the crude petroleum ether extract of *D. jenkinsii*; C. EIMS of compound 2 from the crude petroleum ether extract of *D. jenkinsii*; D. EIMS of compound 3 from the crude petroleum ether extract of *D. jenkinsii*

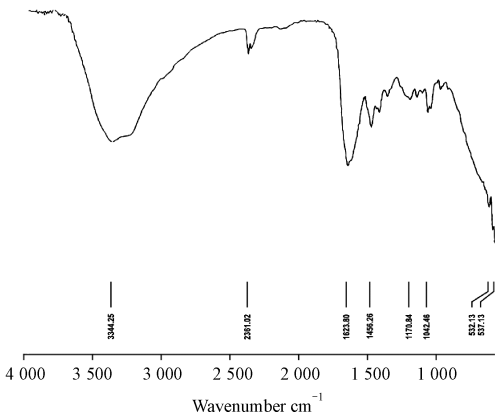

Fig. 3 FT-IR spectrum of the crude diethyl ether extract of *D. jenkinsii*

The results (mean ± SE) of the hot plate method showed that the crude petroleum ether and diethyl ether extracts exhibited an increase in basal reaction time from 2.217 ± 0.070 and 2.117 ± 0.048 at 0 min to 7.100 ± 0.037 and 9.783 ± 0.031 at 120 min, respectively (Fig. 5). The tail clip method revealed a marked increase in basal reaction time of 7.817 ± 0.031 in petroleum ether and 9.850 ± 0.043 in diethyl ether extract at 120 min (Fig. 6). The basal reaction time of control groups were 2.233 ± 0.061 and 2.667 ± 0.033 in the hot plate and tail clip methods, respectively, whereas the standard drugs (reference) buprenorphine, pethidine, and pentazocine showed maximum reaction time of 15.000 ± 0.000 in both the models at 120 min.

The results of the inhibitory effect of fish extracts on carrageenan-induced rat paw edema are shown in Table 1. The crude petroleum ether inhibited the edema volume by

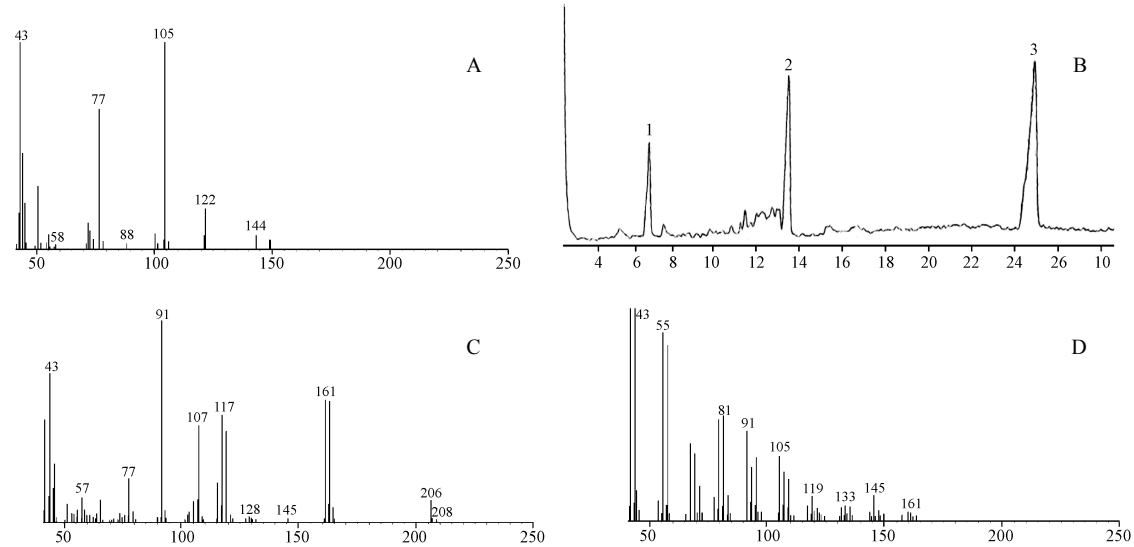

Fig. 4 A. GC-MS of the crude diethyl ether extract of *D. jenkinsii*; B. EIMS of compound 1 of the crude diethyl ether extract of *D. jenkinsii*; C. EIMS of compound 2 of the crude diethyl ether extract of *D. jenkinsii*; D. EIMS of compound 3 of the crude diethyl ether extract of *D. jenkinsii*

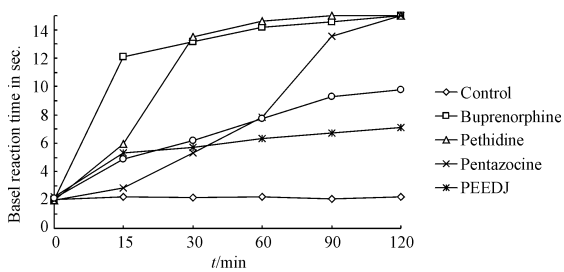

Fig. 5 Analgesic effect of the crude petroleum ether and diethyl ether extracts of *D. jenkinsii* (hot plate method) (Mean ± SE, *n* = 3)

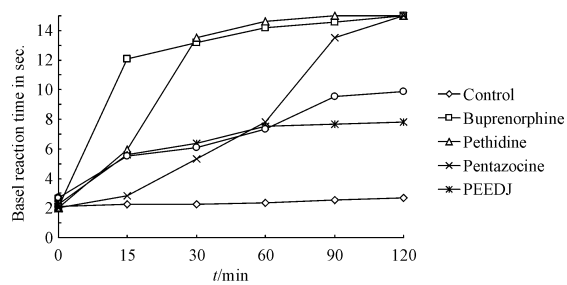

Fig. 6 Analgesic effect of the crude petroleum ether and diethyl ether extracts of *D. jenkinsii* (tail clip method) (Mean ± SE, *n* = 3)

Table 1 Anti-inflammatory activity of crude petroleum ether extract (PEEDJ) and diethyl ether extract (DEEDJ) of *D. jenkinsii*

Group	Treatment	Dose/ (mg·kg^{-1} bw)	Paw edema volume after administration (Mean ± SE, n = 3)						Inhibition /%
			0 h	½ h	1 h	2 h	3 h	4 h	
1	Carrageenan (control)	0.1	2.500 ± 0.026	2.983 ± 0.031	3.517 ± 0.031	3.800 ± 0.037	3.967 ± 0.021	4.000 ± 0.026	–
2	Diclofenac	10	2.500 ± 0.026	2.967 ± 0.033	3.467 ± 0.021	3.033 ± 0.0321	2.850 ± 0.034	2.750 ± 0.022	84
3	Ketorolac	10	2.500 ± 0.000	2.917 ± 0.031	3.467 ± 0.021	3.200 ± 0.022	3.033 ± 0.021	2.983 ± 0.017	68
4	Phenylbuta-zone	200	2.500 ± 0.000	2.917 ± 0.031	3.467 ± 0.021	3.200 ± 0.022	3.183 ± 0.021	3.133 ± 0.017	60
5	PEEDJ	80	2.550 ± 0.034	3.067 ± 0.033^{abcd}	3.533 ± 0.021^{abcd}	3.500 ± 0.026^{abcd}	3.433 ± 0.021^{abcd}	3.350 ± 0.022^{abcd}	53
6	DEEDJ	95	2.550 ± 0.026	3.033 ± 0.021^{cd}	3.600 ± 0.026^{abcd}	3.767 ± 0.021^{bcd}	3.417 ± 0.017^{abcd}	3.017 ± 0.017^{ab}	65

[a]$P < 0.05$ *vs* the control group; [b]$P < 0.05$ *vs* the diclofenac group; [c]$P < 0.05$ *vs* the ketorolac group; [d]$P < 0.05$ *vs* the phenylbutazone group

53% with a mean edema volume of 3.350 ± 0.022 at 4 h, whereas the crude diethyl ether extract produced 65% inhibition of edema volume with a mean 3.017 ± 0.017 at 4 h. The carrageenan control induced inflammation with a mean edema volume from 2.500 ± 0.026 at 0 h to 4.000 ± 0.026 at 4 h (Table 1). The standard drugs (reference) diclofenac sodium, ketorolac, and phenylbutazone showed inhibition of edema volume of 84%, 68%, and 60%, respectively. The histopathological examinations of the crude petroleum and diethyl ether extract-treated animal groups did not reveal any gross changes in brain, liver, and kidney.

In the present study, the effects of the crude petroleum ether and diethyl ether extracts of *D. jenkinsii* against selected animal models to establish the analgesic and anti-inflammatory effects. The experimental models used for the screening of fish extracts for their analgesic effect are centrally-mediated analgesic activity, and are widely used for analgesic screening based on the enhancement of pain threshold by the compounds present in the crude extracts. In the present study, the petroleum ether and diethyl ether extracts of *D. jenkinsii* were found to be effective as an analgesic, since they significantly ($P < 0.05$) enhanced the basal reaction time. The standard drugs, namely buprenorphine, pethidine (a lipophilic opioid receptor agonist), and pentazocine (a κ-receptor agonist) were used in the present study and exerted a significant analgesic effect in all pain models by preventing the sensitization of the opioid receptors. The crude extracts of *D. jenkinsii* contained compounds including 1-(4-carboxy)phenylnona-2, 5-diene, 3-hydroxymono-glyceryl hydrogen phthalate, protonated phthalic anhydride, and carboxylic acid substituted amino acids. These compounds are capable of relieving pain by preventing the sensitization of the receptors through chemical stimulation by enhancing the pain threshold [11-12].

The petroleum ether and diethyl ether extracts of *D. jenkinsii* showed considerable biological potential to inhibit the inflammation significantly. The diethyl ether extract caused a significant inhibition, with an inhibition percentage (65%) higher than that of the standard drug phenylbutazone (60%), where as the petroleum ether extract showed 53% reduction in edema volume. Carrageenan-induced rat paw edema model is a widely used acute inflammatory model to evaluate the anti-inflammatory activity of any given drug. 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 [13-15].

The twenty-carbon fatty acid arachidonic acid (AA; 20 : 4n-6) derived from the dietary linoleic acid by the action of phospholipase A_2 (PLA_2) then undergoes oxidative transformation via the cyclooxygenase pathway and generates prostaglandin E_2 [16-17]. The standard drugs (reference) used in the present study are the most widely used drugs for the treatment of inflammatory conditions and are non-selective direct inhibitors of cyclooxygenase enzyme [17-19]. The present result shows that both extracts caused a significant inhibition in rat paw edema only during the 3rd and 4th hours, whereas the inhibition caused at the end of the 1st and 2nd 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 combined effect of bioactive compounds, namely carboxyl serine, indolyl-3-carboxylic acid and monoglyceryl hydrogen phthalate present in the crude extracts. Previous studies [20-22] revealed that the carboxyl group substitution in naturally occurring amino acids and peptides is known to produce analgesic and anti-inflammatory activity and the ability to inhibit prostaglandin biosynthesis.

In conclusion, the present ethnopharmacological investigation confirmed the analgesic and anti-inflammatory properties of *D. jenkinsii*. Therefore, this finding validates the traditional practice of using the *D. jenkinsii* in the treatment of inflammatory disease and arthritis among the fisherman community. Further the fish is safe for greater consumption as there were no significant histopathological effects on the vital organs.

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