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Gastroprotective effect of Sardine oil (*Sardinella longiceps*) against HCl/ethanol induced ulceration in Wistar rats

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Abstract
Sardine oil is rich in n-3 polyunsaturated fatty acids (PUFAs) like EPA (eicosapentaenoic acid) and DHA (docosahexaenoic acid). The oil showed a significant proportion of n-3 polyunsaturated fatty acids (PUFAs), the percentages of EPA (eicosapentaenoic acid) and DHA (docosahexaenoic acid) being 16 and 14% respectively. Gastroprotective activities of sardine oil (*Sardinella longiceps*) were examined in ulcer induced rats. Sardine oil has significant anti-secretory activity as evidenced by decreased gastric fluid volume, total acidity, and increase in the pH of the gastric fluid in ethanol/HCl induced rats. Our studies also revealed that pretreatment with sardine oil significantly reduced the number of ulcer, ulcer score and ulcer index in ethanol/HCl treated rats. The anti-ulcer activity of sardine oil is further supported by histopathological study, which showed protection of the mucosal layer from ulceration. Furthermore, this oil showed significant reversal of ethanol-diminished activity in antioxidant enzymes, such as SOD, CAT, and GSH. The overall findings conclude that oil of *Sardinella longiceps* has a significant proportion of n-3 polyunsaturated fatty acids and potent anti-ulcer effects. Hence, sardine oil may be a suitable natural source for the prevention and treatment of gastric lesions.

Keywords: Sardine oil, anti-ulcer, n-3 PUFA, gastric mucosa

1. Introduction
Gastric ulcer is very common in present society and it affects 4 million people around the world each year [1]. The mechanism of gastric ulcers is not fully understood; it develops when the imbalance between some factors like stress, nonsteroidal anti-inflammatory (NSAID) drugs, *Helicobacter pylori* infection, etc. and endogenous defense mechanisms like bicarbonate efflux and, increasing of antioxidant levels, prostaglandins, nitric oxide, sulfhydryls [2, 3, 4]. The present treatments for this gastric ulcer includes: prostaglandin analogues, gastroprotective agents, histamine receptor antagonists and protons pump inhibitors (PPI) [5, 6]. Most effective treatment of gastric ulcer is proton pump inhibitors. Proton pump inhibitors (PPIs) significantly reduce the amount of acid produced by the stomach, so we can reduce irritation of the stomach lining and allows an ulcer to heal [7]. But these present therapies can cause serious side effects such as hypersensitivity, arrhythmia and serious hematopoietic disorders [6, 8]. Marine lipids, especially deep sea shark liver oil exerts a potent activity against gastric ulcer and used as an alternative medicine for conventional therapies [9]. Therefore, there is a need to search for new, more effective and safer treatments, with fewer side effects. Fish oils which are a major source of n-3 and n-6 PUFA and other non saponifiable compounds have been reportedly found to be effective against adverse inflammatory response [10]. In India, fish oil is mainly extracted from oil sardine (*Sardinella longiceps*) and is reported as a rich source of n-3 PUFA such as EPA and DHA [11]. The nutritional profile of deep sea fishes like myctophids shows that it can be used as a source for human consumption and formulation of novel foods [12]. Studies show that certain dietary phospholipids and some lipid derivatives have a protective action against gastroduodenal ulceration [13]. In view of all of the above factors, the present study was undertaken to examine the major fatty acids present in sardine oil using GC-MS and to assess its anti-ulcer activity on ethanol/HCl induced ulceration in Wistar rats.

2. Materials and Methods

(DMSO) were purchased from Merck (Darmstadt, Germany). Standards of fatty acid methyl esters purchased from Sigma Aldrich GmbH (Steinheim, Germany). All other chemicals used in this study were of analytical grade.

2.1 Sample collection and oil extraction

Samples (*Sardinella longiceps*) were collected from the local fish market (Cochin, Kerala, India). Oil extraction was achieved by following [14] method, employing a 2:1 mixture of chloroform-methanol to a weighed portion of the liver sample. 20% of water was added to this mixture and the layers were allowed to separate. The aqueous layer was discarded and the solvent chloroform was completely flash evaporated to get the pure oil alone. The oil was stored in amber colored bottles, under nitrogen, at -60˚C. Peroxide value analysis (PV) of fish oil was determined by an iodometric titration as described by Kolanowski [15].

2.2 Fatty acid analysis by Gas Chromatography

Fatty acid profile of extracted oil was done by Folch & Stanley method [14]. A fraction of the lipid extract was saponified with methanolic NaOH followed by methylation in 14% boron trifluoride in methanol (BF3 /MeOH). Methyl esters of the fatty acids thus obtained were separated by gas chromatography (Thermo Trace GC Ultra) equipped with a Perkin Elmer Elite 225® capillary column (30 ×0.25 mm × 0.25 μ) and a flame ionization detector. Identification and quantification were done with the help of FAME external standard mixture.

2.3 Animals

Wistar strain male albino rats (160-200 g) were used for this experiment. They were housed individually in polyurethane cages under hygienic conditions, maintained at room temperature (28±2 °C) and provided food and water ad libitum. Animal experiments were performed as per the guidelines of the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), New Delhi, India and approved by the Institutional Animal Ethics Committee (IAEC), Central institute of fisheries technology (ICAR-CIFT), Cochin, Kerala, India.

2.4 Anti-ulcer activity

Anti-ulcer activity was determined, by the method of Hara & Okabe [16]. Male and female wistar rats kept in standard laboratory conditions were randomly assigned to four groups consisting of 6 animals each. Group I (Positive control) received hydrochloric acid-ethanol (0.6%v/v) to induce ulcer while group II acted as negative control and was fed with regular diet only. Group III was pre-treated with sardine oil along with the vehicle (Oil: DMSO = 4:1) at 0.5 g kg-1 body weight prior to induction of ulcer. Groups IV was administrated DMSO alone. All the animals were fasted overnight before the induction of ulcer. After 4 h of induction of ulcer, all animal groups underwent surgery as per the procedure of Takeuchi [17]. The stomach was inflated with normal saline and then incised and taken to counting the number of lesions and histopathology. Gastric juice was taken for determining the p^H. The ulcer index was calculated by adding the total number of ulcers per stomach and the total severity of ulcers per stomach [18].

The number and severity recorded for ulcer scores are as follows:

0 = normal stomach; 0:5 = red coloration;
1:0 = spot ulcers; 1:5 = hemorrhagic streaks;
2:0 = > 3 but < 5 ulcers; 3:0 = > 5 ulcers

The mean ulcer score for each animal is expressed as the ulcer index.

The percentage protection is expressed as:

Percentage protection = 100 − Ut/Uc*100

Where

Ut = the ulcer index of the treated group; and
Uc = the ulcer index of the control group:

2.5 Preparation of subcellular fractions of stomachs

Tissue samples from the stomach exposed to HCl and ethanol were taken for the biochemical assays. The lesion part were weighted and homogenized with 200 mM potassium phosphate buffer (pH 6.5). The homogenate was used to measure the reduced glutathione (GSH) levels and then centrifuged at 11,000 rpm for 20 min at 4 °C. The supernatant was used to establish superoxide dismutase (SOD) and catalase (CAT) activity.

2.6 Determination of reduced glutathione (GSH) levels

GSH levels in gastric mucosa were determined as described earlier [19]. Tissue homogenate previously prepared were mixed with 12.5% trichloroacetic acid and centrifuged for 4000 rpm, 15 min at 4 °C. The absorbance of supernatant plus TRIS buffer (0.4 M, pH 8.9) and 5,5′-dithiobis 2-nitrobenzoic acid (DTNB, 0.01 M) absorbance of the supernatant was measured at 420 nm and expressed as mg GSH/g of tissue.

2.7 Determination of superoxide dismutase (SOD) activity

The activity of SOD was determined as described previously by Marklund and Marklund [20]. Aliquots of tissue homogenate were mixed with Pyrogallol (1 mM) and buffer solution (Tris HCl 1 mM – EDTA 5 mM, pH 8.5). The reaction was incubated for 20 min, stopped with the addition of 1 N HCl and then centrifuged for 4 min at 14,000 rpm. The absorbance of the supernatant was measured at 405 nm. The amount of SOD that inhibited the oxidation of pyrogallol by 50%, relative to the control, was defined as one unit of SOD activity. The enzymatic activity was expressed as U/mg of protein.

2.8 Determination of catalase (CAT) activity

Catalase activity was measured as described earlier [21]. Samples aliquots of supernatant were mixed with a solution containing 30% H2O2, milli-Q water and buffer 5 mM Tris EDTA, pH 8.0. The absorbance was determined by spectrophotometry at 240 nm for 60 s. The enzymatic activity was expressed as mmol/ min/mg of protein

2.9 Statistical analysis

Statistical analysis for triplicate values has been done and the results are expressed as mean ± S.D.

3. Results

3.1 Fatty acid analysis

Around 500ml of oil has been obtained from 5 Kg of sardine. Primary oxidation of oil and the measurement of hydroperoxides are determined by peroxide value analysis. The peroxide value of oil sample extracted from *Sardinella longiceps* is 1.65 meq/kg. The Mass spectrometry analysis of

Sardine (*Sardinella longiceps*) fish oil methyl ester was carried out according to AOAC official method highlighting 23 compounds. Fatty acid profile of sardine oil extracted from *Sardinella longiceps* contains major nutritionally significant n-3 PUFA (like EPA and DHA) and they contribute to the major bioactivity of the oil (Fig.1). The major fatty acids observed were Palmitic acid (21.29%), 8, 11, 14-eicosatrienoic acid (16.02%), Docosahexenoic acid (14.00%), Palmitoleic acid (10.57%). Myristoleic acid is present in very small amount (0.05%) compared to other compounds present in the sardine oil. Fig. 2 and Fig.3 shows saturated and monounsaturated fatty acids. In our study, we can understand that the fish oil contains different types of polyunsaturated fatty acid. In which Eicosapentaenoic acid and Docosahexenoic acid are present in high amount. EPA and DHA are known to reduce inflammation, improve heart health, memory and have various other health benefits [22]. Fig.4 depicts gas chromatography–single quadrupole mass spectrometry chromatogram (TIC) of sardine oil.

3.2 Effect of sardine oil on ulcer models

The anti-ulcer effect of sardine oil was evaluated by comparing the degree of gastric ulceration in treated versus control animals by, histopathology. Ethanol/HCl caused histopathological lesions including degeneration, hemorrhage, and edematous appearance of the gastric tissue.

Pretreatment with sardine oil (0.5 g/kg) and offered significant protection against all such damage to the mucosa. (Fig.4). Oxidative stress is one of the main factors and plays an important role in the pathogenesis of gastric mucosal injury. HCl/ethanol induced gastric lesions are thought to arise as a result of direct damage of gastric mucosal cells, resulting in the development of free radicals and hyper oxidation of lipid [23]. The gastric lesions in the stomachs of ethanol-treated rats were significantly higher when compared to the normal control. Reduction of ulcer lesions (Fig. 5) was observed in sardine oil-treated groups. Severe blood vessel damage and tissue damage with 11-17 lesions were observed for positive control. Whereas reduction of tissue and blood vessel damage was noticed for oil-treated sample. The number of lesions has also been reduced to 2-3. Oils of different origin were proven as effective anti-ulcer agents. Shark liver oils are reportedly known for their anti-ulcer properties [9]. Bioactive compounds present in oils could be the reason behind their anti-ulcer properties [24]. The anti-ulcer effect may be due to the presence of bioactive fatty acids and other potent compounds in the fish oil [25]. Sardine oil (0.5g/kg) treatment showed significant protection from ulcerative lesions caused by ethanol and HCl. In the ethanol-induced ulceration study, pretreatment with sardine oil produced a significant decrease in number of ulcer, ulcer score and ulcer index (Table 1). The ulcer inhibition was found to be 80% at a dose of 0.5g/kg (Table.1). The result shows that the SOD, CAT, and GSH levels in the ethanol treated groups were significantly lower than that of the control group. Sardine oil increased the activity of antioxidant enzymes (Table. 2), which in turn demonstrates that the oil is more efficient in reducing oxidative damage, and therefore, has a greater potential to promote higher quality gastric healing. Increase in gastric volume, low pH value and acidity are the major parameters in assessing severity of ulcer. Increase in pH and reduced gastric volume is highly correlated with effective anti-ulcer activity. In our results we observed and elevation of pH of 6.8 and decreased acidity, (Table.3) which could be an effective indication of protection of sardine oil against gastric ulcer.

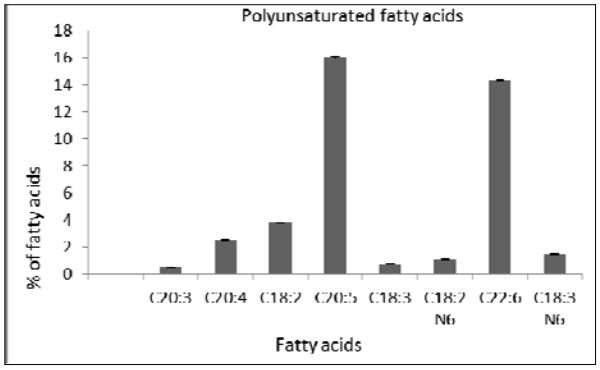

Fig 1: The main polyunsaturated fatty acids from oil of *Sardinella longiceps*. Data expressed as a percentage of wet weight. The value represents the mean ± SD.

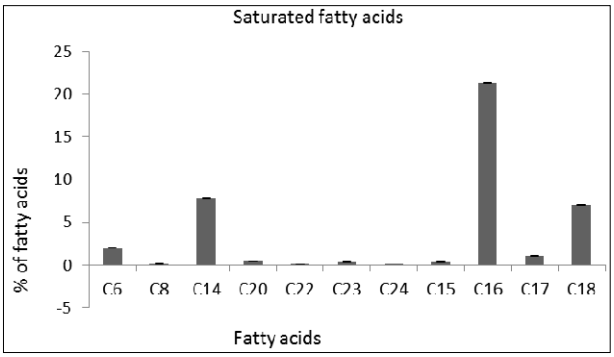

Fig 2: Saturated fatty acids from the oil of *Sardinella longiceps*. Data expressed as a percentage of wet weight. The value represents the mean ± SD

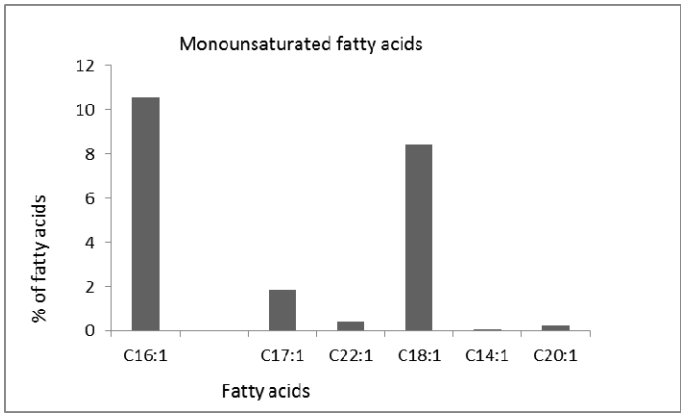

Fig 3: Mono unsaturated fatty acids from oil of *Sardinella longiceps*. Data expressed as a percentage of wet weight. The value represents the mean ± SD

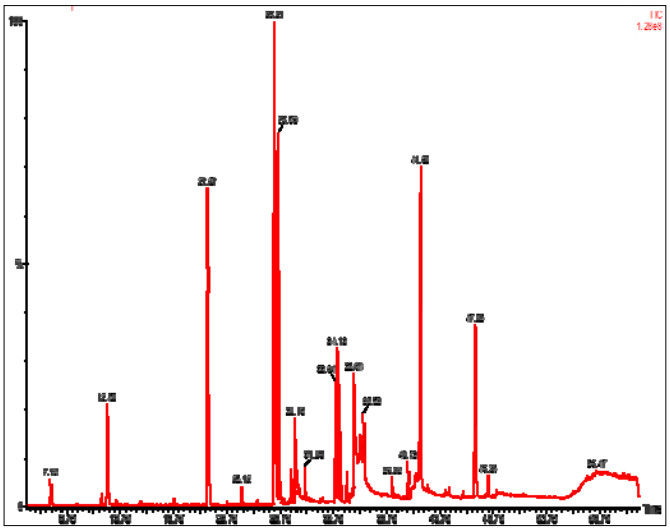

Fig. 4. Gas chromatography–single quadrupole mass spectrometry shows total ion chromatogram (TIC) of sardine oil.

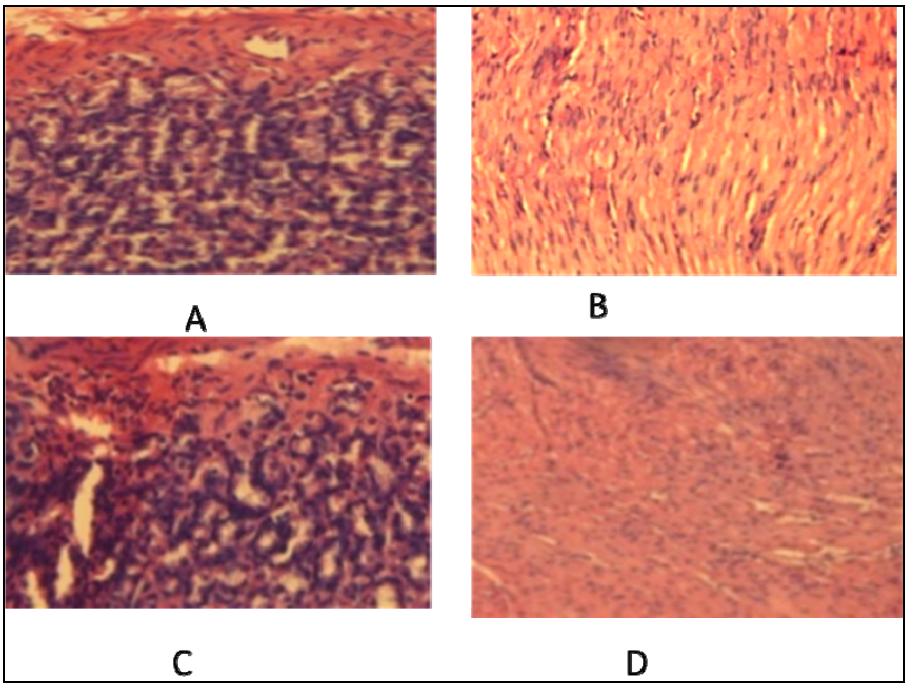

Fig 4: The anti - ulcer activity of oil extracted from *Sardinella longiceps* on gastric mucosa: Histopathological section. A: Positive control (HCl/ethanol induced group), B: Negative control (Normal diet received group), C: DMSO treated group (Vehicle), D: Sardine oil treated group.

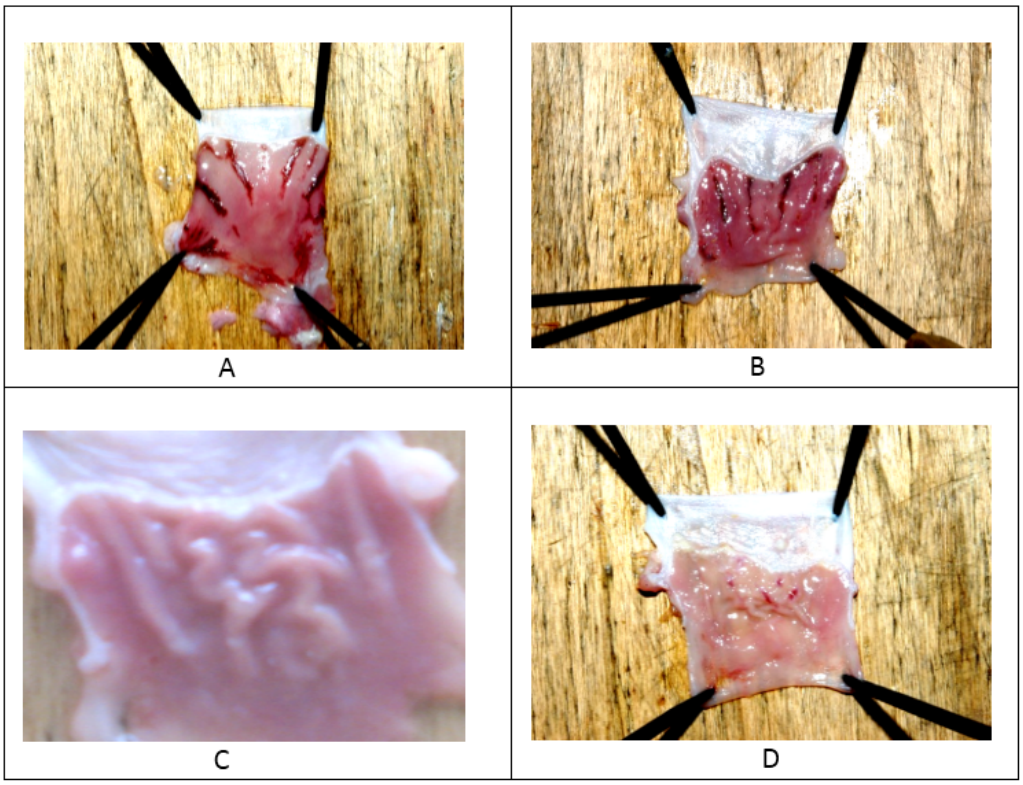

Fig 5: Antiulcer activity of sardine oil (*Sardinella longiceps*) on gastric mucosa. A: Positive control (HCl/ethanol induced group), B: DMSO treated group (Vehicle), C: Negative control (Normal diet received group), D: Sardine oil treated group

4. Discussion

Sardine oil has been used as medicinal purpose for more than 50 years. It was observed that sardine oil can be used as immunobooster against infectious diseases [26, 10]. Sardine oil contains comparatively high amount of PUFA and n-3 PUFA dominant among fatty acids [27]. The gas chromatographic analysis of Sardine (*Sardinella longiceps*) fish oil highlighting nutritionally significant n-3 PUFA (like EPA and DHA) and they contribute to the major bioactivity of the oil. In our studies, we went a step ahead and have studied gastric antisecretory and antiulcer activities of sardine oil in ethanol induced ulcer model. Sardine oil showed a significant protective effect in ethanol/ HCl induced ulceration in experimental rats. Ethanol and HCl are known to induce peptic ulceration by causing mucosal damage. The mode of action of Ethanol/HCl is by increasing the acid secretion and back diffusion of H+ ions also it breaks the superficial mucosal barrier. Alcohol stimulates mucosal mast cells to release histamine, platelet aggregating factor (PAF), and leukotrienes [28]. In the present study, sardine oil administration decreased the gastric juice volume, total acid output and p^H induced by ethanol and HCl. This may possibly be due to the anti-secretory property of sardine oil. In ethanol/ HCl induced gastric ulcers at 0.5g/kg dose of sardine oil, significant reductions in the ulcer index, total acidity was observed relative to the control. The gastric lesions in the stomachs of ethanol-treated rats were significantly higher when compared to the normal control. There was a significant reduction in the ulcer index in the sardine oil treated group compared to the ethanol group. Reactive oxygen species (ROS), histamines and PAF (Platelet aggregating factor) are the major mediators responsible for gastric mucosal injury associated with ethanol consumption [29]. There is a need to evaluate the antioxidant mechanism with regard to the ulceration process in all stomach tissues; the antioxidant levels (SOD, CAT, GSH and LPO) were evaluated. The result shows that the SOD, CAT, and GSH levels in the ethanol treated groups were significantly lower than that of the control group. Sardine oil increased the activity of antioxidant enzymes, which in turn demonstrates that the oil is more efficient in reducing oxidative damage, and therefore, has a greater potential to promote higher quality gastric healing. The gastric mucosa of rats treated with ethanol showed some morphological changes like hemorrhage, congestion and eroding. Pretreatment with sardine oil (0.5g/kg) offered 80% protection against the ethanol induced ulceration in the gastric mucosa. The results reinforce that the gastroprotective effects of sardine oil are dependent on a group of fatty acids and some other non saponifiable compounds. Therefore, the oil from this species (*Sardinella longiceps*) may be used for the development of a medicine against gastric ulcer.

Table 1: Effect of sadine oil on ulcer score, ulcer index and ulcer inhibition in ulcer induced rats. Values are expressed as mean±S.E.M. for six animals in each group.

Treatment	No. of ulcer	Ulcer score	Ulcer index	Ulcer inhibition (%)
Normal Control	0.0±0.0	0.0±0.0	-	-
Ethanol/HCl induced	17±0.5	3±0.8	20±0.8	-
DMSO treated	13±0.3	3±0.6	16±0.7	20
Sardine oil treated	3±0.4	1±0.7	4±0.5	80

Table 2: Effect of sardine oil treatment on superoxide dismutase (SOD), catalase (CAT) and non-protein sulfhydryl groups (GSH) at ulcer site induced by ethanol and HCl in rats. Results as mean ± S.E.M for six rat of each group.

Group	SOD (U/mg of protein	CAT(mmol/min/mg of protein)	GSH (µg/g of tissue)
Normal Control	1684±104	3018±28	481±34
Ethanol/HCl induced	285±98	265±82	289±48
DMSO treated	435±79	486±47	317±38
Sardine oil treated	1348±211	2897±77	514±28

Table 3: Effect of sardine oil on gastric secretion, pH of gastric content and total acidicity. Results as mean ± S.E.M

Treatment	Gastric content (ml)	pH of gastric content	Total acidicity(mEq/L)
Normal Control	2.12±0.2	4.02±0.1	44.32±0.2
Ethanol/HCl induced	3.59±0.2	2.26±0.7	74.23±0.3
DMSO treated	2.92±0.7	2.87±0.3	60.33±0.2
Sardine oil treated	1.58±0.5	6.08±0.2	41.38±0.1

5. Conclusion

The present study clearly depicts the fatty acid analysis and anti-ulcer activities of oil extracted from sardines (*Sardinella longiceps*). Which are supported by biochemical and histopathological studies of the stomach wall tissues of differently treated groups of rats.

6. Conflict of interest statement

The authors declare that there are no conflicts of interest.

7. Acknowledgements

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