

Isolation and Evaluation of Cytotoxic, Anti-Inflammatory, Anti-Ulcer Activity of Methanolic Extract of *Ceriops decandra* leaves

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The current study was undertaken to provide scientific validation for the traditional medicinal applications of *Ceriops decandra* leaves in treating gastrointestinal disorders and inflammation. Additionally, the study aimed to isolate a pure component from the extracted leaves for further analysis. Lupeol was extracted from the crude methanolic extract of *Ceriops decandra* leaves by column chromatography as part of a phytochemical inquiry. Its structure was determined using ¹H and ¹³C NMR spectroscopy. In order to assess the cytotoxicity, the unrefined methanolic extract was divided into two fractions: a petroleum fraction and an aqueous fraction, employing the modified Kupchan method. The brine shrimp lethality test revealed that both the aqueous and petroleum ether fractions had significant cytotoxic activity, with LC₅₀ values of 1.93 µg/l and 2.04 µg/l, respectively. These values were compared to the LC₅₀ value of the standard Vincristine Sulphate, which was found to be 0.02 µg/l. The results of the anti-inflammatory trial demonstrated that the administration of the extract at doses of 250mg/kg and 500mg/kg resulted in protection rates of 62.5% and 87.5%, respectively, as compared to the carrageenan control group after 3 hours post-injection. It is worth noting that Ibuprofen exhibited a higher level of protection, with a rate of 91.7%. In the context of ethanol-induced stomach ulcer, the administration of extracts at doses of 250 mg/kg and 500 mg/kg resulted in 45.5% and 59.1% protection against gastric ulcer, respectively. These findings were compared to the protective effect of Omeprazole, which demonstrated 63.6% protection and served as the standard reference. The findings suggest that the methanolic leaf extract of *Ceriops decandra* possesses robust cytotoxic and potent anti-inflammatory and anti-ulcer properties. These results provide support for the traditional application of this extract in the management of gastrointestinal diseases, inflammation, and cancer.

Keywords: Anti-Inflammatory; Anti-Ulcer; Cytotoxicity; Isolation; LC₅₀; NMR.

Only 124 nations and regions have at least one natural mangrove species, with Asia having the most mangrove acreage. Mangrove is essential to the ecosystem by protecting the seashore and

providing a habitat for marine and terrestrial organisms. Mangroves grow in the intertidal zone of extreme environmental conditions like inundation, anaerobic soil, strong wind, and high

salinity¹. Because of their extreme morphological and physiological adaptations, mangroves have a higher chance of producing enhanced bioactive compounds. Traditionally, it is used to treat gastrointestinal disorders, inflammation, cancer and infection².

Ceriops decandra is a mangrove plant of the family Rhizophoraceae. It is found throughout tropical Asian countries like Bangladesh and Eastern Africa, Queensland and Madagascar. It is rich in phytochemicals such as protein, coumarin, phenols (catechin, procyanidins), triterpenoids (lupeol, α -amyrin, oleanolic acid, ursolic acid), flavonoids, saponins, glycosides, alkaloids diterpenoids (ceriopsin A-G), and tannins³.

Despite severe cardiovascular and gastrointestinal side effects, NSAIDs are widely used as analgesic and anti-inflammatory agents. COX 2 selective analgesics such as Celecoxib are safe for Gastro intestinal tract than a nonselective agent. The search for newer and safer analgesic and anti-inflammatory agents continues for this⁴. The lack of knowledge regarding *Ceriops decandra* methanolic extracts cytotoxic, anti-inflammatory, and anti-ulcer properties in relation to Bangladeshi species inspired this work. These findings were accompanied by histological analysis of the anti-inflammatory and anti-ulcer actions as well as chemical component separation.

MATERIALS AND METHOD

Plant materials

The fresh leaves of *Ceriops Decandra* were collected from the Sundarbans mangrove forest in June-July. The plant's identity was confirmed by the Bangladesh National Herbarium, where a voucher specimen was kept (Voucher no- DACB-85929).

Chemicals and drugs

Eskayef Bangladesh Limited provided the omeprazole and ibuprofen that were collected. Carrageenan was acquired from Sigma Aldrich compounds in Germany, and analytical-grade Petroleum ether, ethyl acetate, and other compounds were purchased from Merck in Germany.

Extraction of plant materials

2.5 kg of fresh *C. decandra* leaves were separated, shade-dried, and ground into a coarse powder. The coarse powder was then extracted

with methanol using a Soxhlet apparatus at Jahangirnagar University and vacuum-dried.

Extraction and isolation from *Ceriops decandra*

For approximately six months, 40 g of crude methanolic extract were stored in a refrigerator at 3-5 °C. The substance underwent a natural separation process, resulting in the formation of two distinct layers: a lower layer characterized by its sticky and resinous properties, and an upper layer consisting of a solid component. In this study, the solid component was utilized to extract and separate Lupeol. The solid component was separated into a fraction that is soluble in petroleum ether using a separating funnel. The organic fraction, which is soluble in petroleum ether, was subsequently dried at room temperature, resulting in a yield of 9 grams.

The petroleum ether fraction was subjected to fractionation using silica column chromatography (CC) utilizing a gradient elution system consisting of petroleum ether, ethyl acetate, and methanol in varying proportions (ranging from 99:1 to 20:80, v/v). Therefore, the entirety of the dissolved fraction of petroleum ether (9 g) was partitioned into 75 fractions, each containing a solvent system of 20 ml.

The impure, colorless crystalline substance was formed through the process of evaporating the solvents (85:15-60:40, v/v) of fractions 22 to 31, which consisted of Petroleum ether and ethyl acetate. The production of compound 1 (30 mg, Rf=0.55) involved the repeated washing of crystal bodies with n-hexane (100:0, v/v) and subsequent recrystallization using a mixture of n-hexane and ethyl acetate (50:50, v/v). The resulting crystals were pure.

Modified Kupchan Partitioning Method

Crude methanolic extract (5gm) was fractionated using petroleum ether (100ml, 3 times). Again, the aqueous fraction from this extraction was fractionated using ethyl acetate. The ethyl acetate soluble fraction (4.8 gm) and aqueous fraction (1.75 gm) were then used for the brine shrimp cytotoxicity assay.

Brine shrimp lethality bioassay

The lethality test on brine shrimp was used to determine the plant extract's cytotoxic activity. The concentrations of the dried extract preparations were serially diluted to range from 5 g/ml to 320 g/ml. Positive control was provided by the

conventional medication vincristine sulphate (VS), whose concentrations ranged from 0.312 g/ml to 5 g/ml. The brine shrimp's (*Artemia salina*) eggs were obtained from a neighborhood aquarium store in Dhaka, Bangladesh, and they were incubated in brine for 24 hours with a continual supply of oxygen. After 24 hours, the number of alive nauplii out of 10 were counted. These experiments were conducted on triplets. The lethal concentration (LC50) required to eliminate 50% of the nauplii was calculated using the application "Microsoft Excels 2007"⁹

Anti-inflammatory Activity Experimental Animal

This experiment was designed following the previously described method discussed by Begum *et al.*^{5,6}. Male Albino wistar rats weighing 120 and 150 g were collected from Jahangirnagar University's pharmacy department. They were kept in groups of six rats in polypropylene cages,

with a room temperature of 25 ± 2 °C and a 12-hour light/dark cycle. The rats were given a week of acclimation before the studies, during which they had unrestricted access to free standard laboratory animal food and water. They went without eating the night before the test. All procedures used the anesthetic isoflurane (5% in 100% oxygen) to reduce preventable harm. The principles and guidelines outlined in the Guide for the Care and Use of Laboratory Animals served as the cornerstone for the treatment of animals and research procedures (NIH document no. 85-23; updated in 1985).

Induction of Inflammation in experimental animals

The reference standard utilized was ibuprofen (20 mg/kg), and the anti-inflammatory action was carried out as advised by Winter *et al.* by injecting carrageenan as an edematogenic agent on adult albino rats.

The following five groups, each with six rats, were formed:

Group	Description
Group I	Oral administration of 0.5ml distilled water
Group II	Oral administration of 0.5ml distilled water and 0.1ml of Carageenan injection (Carageenan control, CC)
Group III	Oral administration of Ibuprofen (20 mg/kg) and 0.1ml of Carageenan injection (IP 20)
Group IV	250 mg/kg methanolic extract of <i>C.decendra</i> and 0.1ml of Carageenan injection (MECD-250)
Group V	500 mg/kg methanolic extract of <i>C.decendra</i> and 0.1ml of Carageenan injection (MECD-500)

The extracts were given orally to the treatment group as a suspension containing 2 to 3 drops of tween 80 at 250 and 500 mg/kg doses, respectively. Thirty minutes before injecting carrageenan (0.1 ml of 1% w/v solution of distilled water) (0.5 ml), all test samples were administered orally. Carrageenan was injected into the sub-planter area of the left paw of each rat. However, the normal control group received no carrageenan. Slide callipers were used to gauge and record the paw swelling every hour. The percentage inhibition of paw edema was calculated after three hours.

The formula for the percentage of inhibition in control and the increase in paw thickness is as follows:

Increase of paw thickness in control or treatment group = PC or PT = Pt – Po
Percentage of inhibition in paw thickness in the treatment group = $[(PC - PT) \times 100] / PC$

Where, Pt = paw thickness at time t,

Po = initial paw thickness,

PC = Increase in thickness of paw of the control group and

PT = Increase in thickness of paw of the treatment group.

Anti-ulcer activity

Induction of Ulcer

This experiment was performed following Begum *et al.*^{6,7}. Five groups of animals were not allowed to access food for a day, other than water, for two hours before the test. The rats were kept apart in their cages to avoid coprophagy throughout the fasting phase. Gastric ulcers were induced in the test sample (MECD at doses of 250 and 500 mg/kg p.o.) and standard (omeprazole at doses of 20 mg/kg, p.o.) using ethanol-acid after 30 minutes of pretreatment. Under isoflurane, 5% in 100% oxygen anesthesia, these rats were euthanized 90

minutes later, and their stomachs were immediately removed. The stomachs were sliced open along the larger curvature, removed, cleaned with distilled water, and stored in 10% buffered formalin. The gastrointestinal mucosa was examined under a microscope for ulcers, and the ulcers were graded as follows:

Tissue architecture	Ulcer score
Normal stomach	0
Red coloration	0.5
Spot ulcer	1
Hemorrhagic streak	1.5
Ulcers	2.0
Perforation	3.0

The average ulcer score for each animal is represented by the ulcer index. The percentage of ulcer prevention was calculated using the formula below: -

$$\% \text{ Protective} = \left[\frac{\text{Control mean ulcer index} - \text{Test mean ulcer index}}{\text{Control mean ulcer index}} \right] \times 100$$

The animals were grouped into five groups, each with six rats:

Histological investigation

Carrageenan-induced paw tissues were collected for this study 3 hours after the induction and kept in freshly made 10% neutral buffered formalin for at least 24 hours. The specimens for the anti-ulcer study were kept in a 10% natural

The animals were grouped into five groups, each with six rats:

Group	Description
Group I	Oral administration of 5 ml/kg/day distilled water (Normal Control)
Group II	Oral administration of 5 ml/kg/day distilled water and 25 ml per kg of 0.3M HCl in 60 % ethanol (Negative control)
Group III	Oral administration of 20 mg/kg/day omeprazole and 25 ml per kg of 0.3M HCl in 60 % ethanol
Group IV	Oral administration of 250 mg/kg methanolic extract of <i>C.decendra</i> 25 ml per kg of 0.3M HCL in 60 % ethanol
Group V	Oral administration of 500 mg/kg methanolic extract of <i>C.decendra</i> 25 ml per kg of 0.3M HCL in 60 % ethanol

buffer. Following that, tissue slices were paraffin-embedded. Using a microtome, the samples were cut into cross-sections stained with hematoxylin and eosin (H and E) and mounted on Canada balsam. Each segment was inspected under a light microscope, and any histopathological alterations, such as edema, erosion, infiltration, and inflammation, were photographed using an Olympus photomicroscope.

Statistical Analysis

The values are tabulated as mean \pm S.E.M, and statistical significance between treated and control groups was analyzed using One-way ANOVA, where $P < 0.05$ was considered statistically significant.

RESULTS

Characterization of isolated compounds

Compound **1** is a pentacyclic triterpene. It

was a white powder. Seven tertiary methyl singlets and one secondary hydroxyl group were seen in the ^1H NMR spectra. Additionally, it displayed olefinic protons at 4.71 and 4.59. The compound's ^1H NMR spectra showed signals at δ 4.71 and δ 4.59, both of which were focused on the furan exomethylene group, as one proton doublets with 2.4 Hz coupling constants and one proton double quartets with 1.2 Hz and 2.4 Hz coupling constants, respectively. A secondary carbinol group may be given to the signals at δ 3.21 that appeared as a doublet of doublets with coupling constants of 11.2 Hz and 5.2 Hz. A wide doublet at 1.66 with a coupling constant of 1.2 Hz was also visible in the spectrum and was attributed to a methine group. Six tertiary methyl singlets could be seen in the resonance signals at 0.79, 0.81, 0.85, 0.97, 0.99, and 1.05. These results closely matched those that had previously been published for a common pentacyclic triterpenoid called lupeol⁷.

Brine Shrimp Cytotoxicity

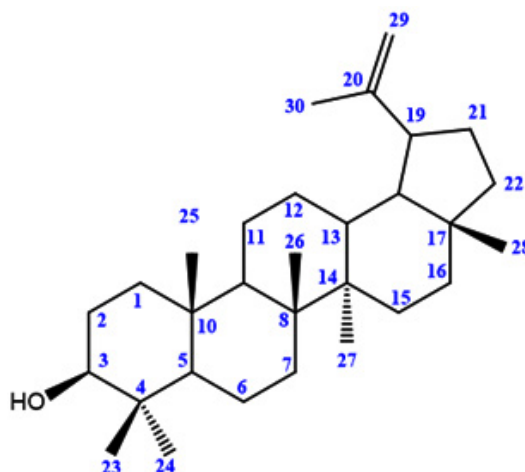
The effect of methanolic extract of *Cerriops decandra* as well as Vincristine sulphate on brine shrimp nauplii has been tabulated and graphically represented.

Anti-inflammatory activity of *Cerriops decandra*

In this study, animals given methanolic extract (250 and 500 mg/kg, p.o.) and ibuprofen (20 mg/kg, p.o.) for 1 to 3 hours showed considerably reduced paw thickness and redness than the carrageenan control group. After the investigation, it was found that when compared to the carrageenan control group at 3 hours after carrageenan injection, oral administration of methanolic extract of 250mg/kg and 500mg/kg reduced paw edema by 62.5% and 87.5%, respectively.

Effect of methanolic extract of *Cerriops decandra* on the carrageenan induced paw edema

In the healthy control (2a) there was no inflammation or erythema observe. However, in Carrageenan control (2b) significant erythema and edema were observed. In case of Ibuprofen 20 mg/kg (2c), minor erythema and edema is seen. The paw of *C. decandra* 250 mg/kg (2d) methanolic extract treated rat shows mild erythema and edema



¹³C NMR value: (δ 178.2, C-28), (δ 151.6, C-20), (δ 126.0, C-29), (δ 178.2, C-28), (δ 79.1, C-3), (δ 55.8, C-17), (δ 56.1, C-5), (δ 51.1, C-9), (δ 50.0, C-19), (δ 46.8, C-18), (δ 41.8, C-14), (δ 41.0, C-24), (δ 40.0, C-4), (δ 40.1, C-1), (δ 37.9, C-13), (δ 38.0, C-10), (δ 37.5, C-22), (δ 178.2, C-28), (δ 34.8, C-7), (δ 32.5, C-16), (δ 31.0, C-15), (δ 29.9, C-21), (δ 28.1, C-23), (δ 28.4, C-2), (δ 26.0, C-12), (δ 21.0, C-11), (δ 19.0, C-30), (δ 18.7, C-6), (δ 16.0, C-25), (δ 15.2, C-27)⁸.

Fig. 1. Lupeol

Table 1. Effect of aqueous and petroleum ether fraction of *Cerriops decandra* as well as Vincristine sulphate on brine shrimp nauplii

<i>Cerriops decandra</i> leaf extract			Vincristine Sulphate			
Concentration, C (µg/ml)	Log C	% mortality (aqueous fraction)	% mortality (Petroleum ether fraction)	Concentration, C _v (µg/ml)	Log C _v	% mortality
5	0.693	25	5	0.312	-0.505	10
10	1	30	15	0.625	-0.204	30
20	1.301	35	20	1.25	0.096	60
40	1.602	40	30	2.50	0.397	80
80	1.903	50	45	5.0	0.698	100
160	2.204	55	55			
320	2.505	65	70			

Table 2. LD50 Value of extracts *Cerriops decandra* aqueous and petroleum ether part as well as vincristine sulphate

Sample	LC ₅₀ (ig/ml)	Regression line equation	R2
<i>Cerriops decandra</i> (Aqueous fraction)	1.93	y = 21.899x + 7.7936	0.9831
<i>Cerriops decandra</i> (petroleum ether fraction)	2.04	y = 35.513x - 22.575	0.9822
Vincristine sulphate	0.02	y = 76.485x + 48.627	0.9934

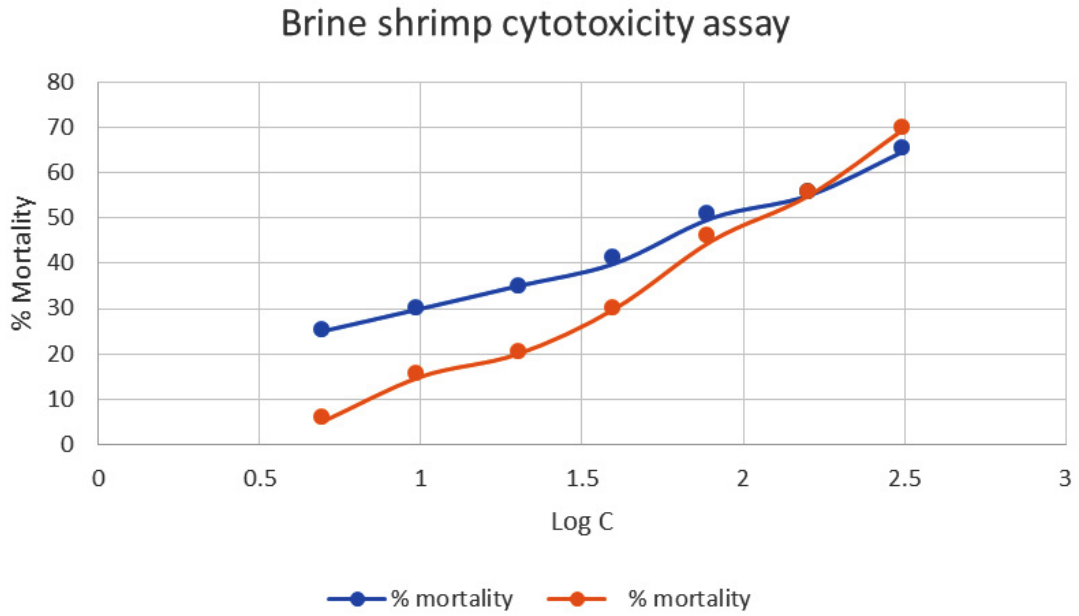


Fig. 1. Plot of log C % mortality of *Ceriops decandra* petroleum ether and aqueous fraction



Fig. 2. Effect of methanolic extract of *Ceriops decandra* on carrageenan induced paw edema after 3 h

and 500 mg/kg methanolic extract of *C. decandra* (2e) treated rat shows comparatively less edema and erythema.

Histopathological Evaluations of Paw Tissue

When paw tissue was examined histopathologically, it can be deduced that tissues from normal controls (3a) have few leukocytes. In contrast, tissues from carrageenan-induced rat paws (3b) have a large number of inflammatory cells. On the other hand, tissues from rat paws treated with the standard drug Ibuprofen (3c) and *C. decandra* methanolic extract at dosages of 250 (3d) and 500 mg/kg (3e) exhibit only a moderate to low level of inflammatory cell infiltration.

Ethanol-Induced Gastric Ulcer

After dissecting the stomachs in the greater curvature, the ethanol-treated rat tissues (4b) have thick, dark red and black lesions in the glandular portion. As compared to normal control (4a), Omeprazole (20mg/kg) (4c), Methanolic extract at 250mg/kg (4d) and 500 mg/kg (4e) treated rat tissues have much better ulcer protection with a significant protection index of 63.6 %, 45.5% and 59.1% respectively.

Histological Evaluation of Gastric Lesions

Figure 5a shows a section of normal control which only received distilled water. On the contrary, the ethanol control group 5b shows ulceration with

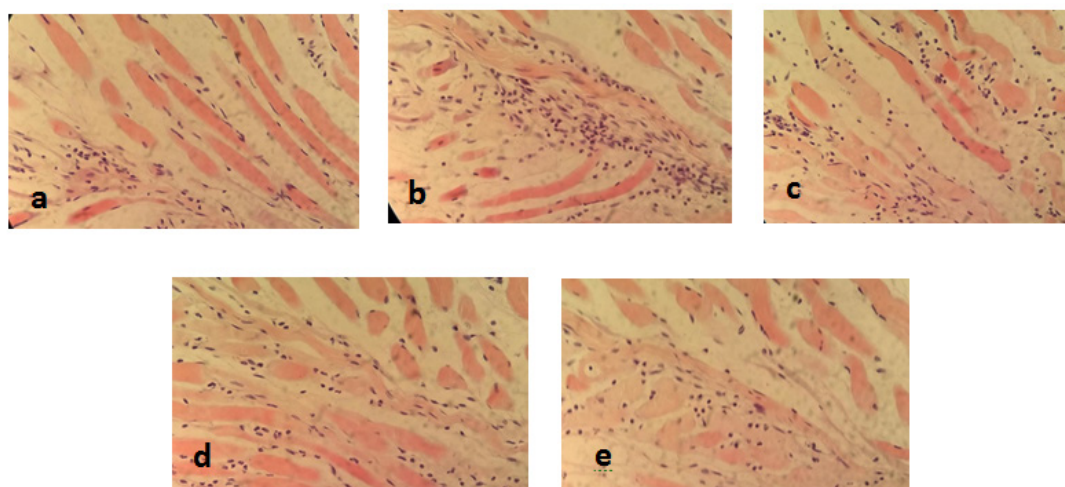


Fig. 3. Histopathological Evaluations of Paw Tissue of a) Normal control b) Carrageenan control c) 20mg/kg Ibuprofen d) Methanolic extract of *Ceriops decandra* leaves at dose 250mg/kg e) Methanolic extract of *Ceriops decandra* leaves at dose 500mg/kg. Each group was viewed with 400x magnification, scale bar: 20 μ m.

Table 3. Anti-inflammatory effect of *Ceriops decandra* methanolic extract on the carrageenan induced changes in paw thickness in experimental rats

Groups Dose mg/kg	NC	CC	IP-20 (20mg/kg Ibuprofen)	MECD-250 (250mg/kg extract)	MECD-500 (500mg/kg extract)
Initial paw thickness (cm)	0.48 \pm 0.004	0.49 \pm 0.005 ^c	0.46 \pm 0.003 ^{b**}	0.49 \pm 0.003 ^b	0.48 \pm 0.004 ^c
Paw thickness (cm) after 1h	0.48 \pm 0.004	0.67 \pm 0.004 ^{a**}	0.57 \pm 0.006 ^{b**}	0.64 \pm 0.004 ^b	0.53 \pm 0.007 ^{b**}
Paw thickness (cm) after 2h	0.48 \pm 0.002	0.70 \pm 0.007 ^{a**}	0.54 \pm 0.003 ^{b**}	0.61 \pm 0.007 ^{b*}	0.52 \pm 0.004 ^{b**}
Paw thickness (cm) after 3h	0.48 \pm 0.003	0.73 \pm 0.004 ^{a**}	0.48 \pm 0.003 ^{b**}	0.58 \pm 0.003 ^{b**}	0.51 \pm 0.004 ^{b**}

Each value is presented as Mean SEM (n=6). NC stands for "Healthy Control," CC stands for "Carrageenan Control," IP-20 stands for "Ibuprofen 20 mg/kg/day," MECD-250 stands for "Methanolic Extract of *C. Decandra* 250 mg/kg," and MECD-500 stands for "Methanolic Extract of *C. Decandra* 500 mg/kg." Dunnett's multiple comparison test (*p 0.05 and **p0.01) is used after one way analysis of variance to show statistically significant differences from the respective group. uses one-way analysis of variance to demonstrate that there is no statistically significant difference from the corresponding group, followed by Dunnett's multiple comparison test (p > 0.05). when contrasted with the control (carrageenan) and the control (normal).

broken mucosal barrier and migration of numerous inflammatory cells. However, omeprazole (20 mg/kg) (5c) administration changed histopathology significantly, with minimal hemorrhage presence and intact mucosal lining. Methanolic extract of *C. decandra* at 250 mg/kg (5d) showed moderate regeneration of gastric mucosa, while 500 mg/kg (5e) showed significant regeneration.

DISCUSSION

Based on the ^1H and ^{13}C NMR values for each proton and carbon that were determined and provided in the results and discussions lupeol skeleton for compound **1**, the lack of a hydroxyl group, and the appearance of a carbonyl group in the ^{13}C NMR spectrum data of **1**. As a result, structure **1** was determined to be that of the well-known chemical lupeol, which was supported

by the physical and spectral information in the literature⁹.

In this work, a methanolic extract of *Ceriops decandra* showed a dose-dependent anti-inflammatory activity in paw edema caused by carrageenan. The outcome is nearly as significant as the standard drug Ibuprofen. The suppression of mediators such as prostaglandins, leukotrienes, histamine, serotonin, and bradykinins, which cause pain and fever, may cause anti-inflammatory action¹⁰.

The histological analysis of the hind paw tissue in our study showed that the methanolic extract of *Ceriops decandra* reduced the massive influx and buildup of inflammatory cells in the paw tissue after carrageenan induction in a dose-dependent manner. TNF- α is a crucial element of inflammation. Among other things, it can trigger the release of pro-inflammatory cytokines and the activation and chemotaxis of leukocytes.

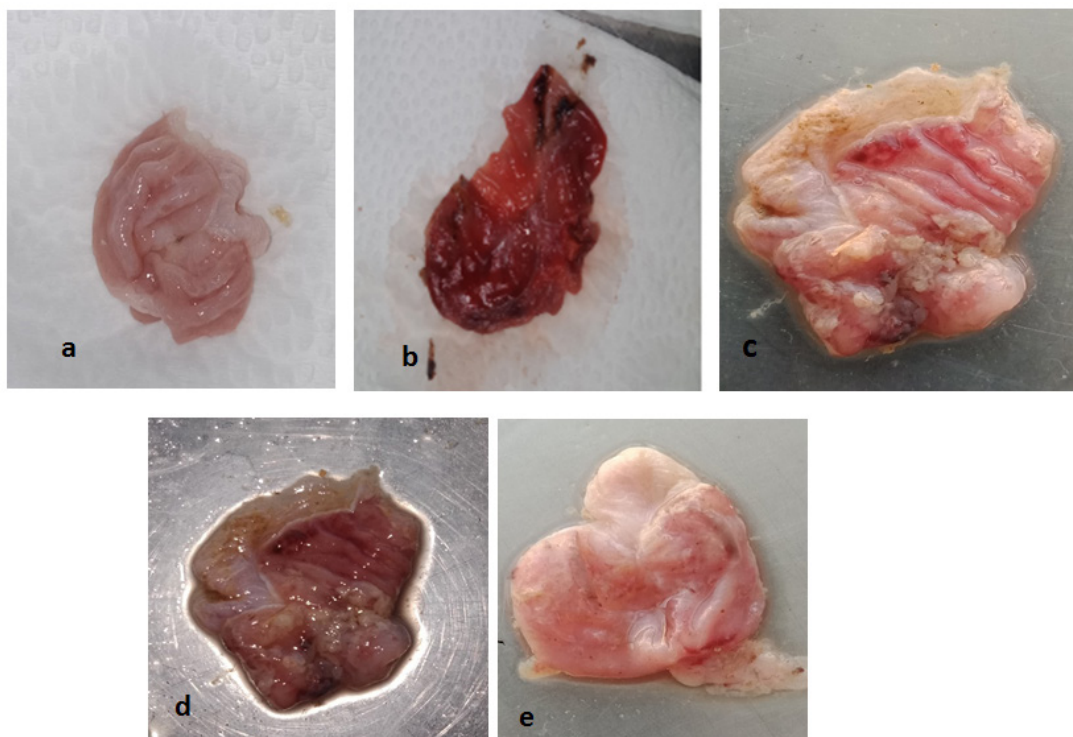


Fig. 4. Gross appearance of the gastric mucosa: a) Normal control (healthy stomach) b) Ethanol control (developed significant ulceration with mucosal injury) c) Omeprazole 20 mg/kg (less area of gastric ulceration) d) Methanolic extract of *C. decandra* 250 mg/kg (reduced area of gastric ulceration, with dark red, black lesion compared to ethanol control) e) Extract of *C. decandra* 500 mg/kg (reduced area of gastric ulceration and flattening of gastric mucosal folds)

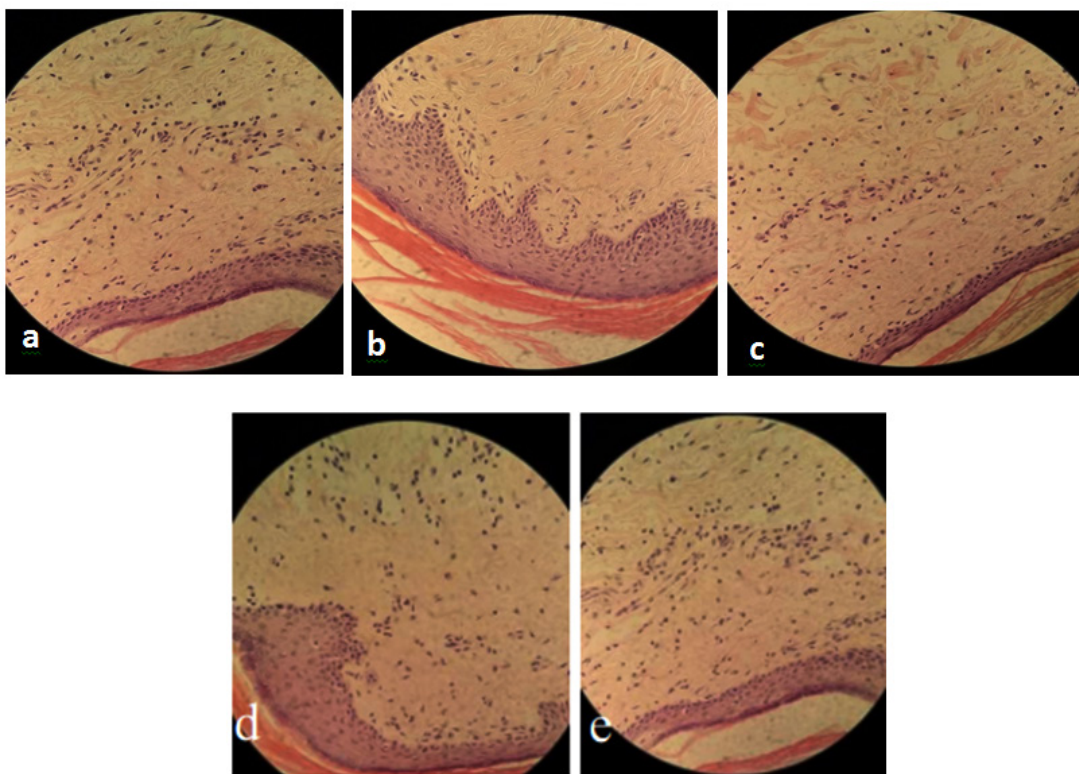


Fig. 5. Histological Evaluation of Gastric Lesions a) Normal control, b) Ethanol control, c) Omeprazole 20 mg/kg, d) Methanolic extract of *C. decandra* (250 mg/kg), e) Extract of *C. decandra* (500 mg/kg)

Leukocyte influx-induced inflammation can be avoided by reducing the release of mediators such as MDA, CRP, NO, MPO, and pro-inflammatory cytokines (TNF- α and IL-1 β)¹¹.

Since ethanol may permeate the stomach mucosa, the mucosal barrier will break down, increasing the permeability of sodium and water and causing calcium ions to accumulate. Additionally, it increases the production of superoxide ions and hydroperoxyl free radicals, which results in stomach mucosal ulcers¹⁰. The stomach cryoprotection through the anti-oxidation mechanism and the decreased release of gastric acid and prostaglandins may all contribute to the anti-ulcer effects of the methanolic extract of *Ceriops decandra*¹¹. The anti-ulcer effect was dose-dependent, which is almost identical to the standard drug omeprazole. The considerable decrease in the intracellular antioxidant Glutathione (NP-SH/GSH) may also be to blame for this result. Lupeol, a compound derived from the leaves of *Ceriops*

decandra, has demonstrated free radical scavenging abilities that are on par with misoprostol¹².

As a functional, living food supply for marine ecology, artemia is regarded as one of the most critical marine ecotoxicity testing organisms¹³. Cytotoxicity and cell death are also brought on by certain polyphenols' nonspecific attachment to the membrane^{14,15,16,17,18,19}.

CONCLUSION

The results of our study revealed that different fractions of the methanolic extract from *Ceriops decandra* possess cytotoxic properties. These findings suggest the need for further investigation utilizing human cell lines, both *in vivo* and *in vitro*. The utilization of this plant for the production of novel anti-cancer medications involves the identification and isolation of its primary cytotoxic compound.

Hence, more investigation is warranted to ascertain the specific phytoconstituents and underlying processes that account for the observed activities as indicated by the findings.

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Conflicts of Interest

There is no conflict of interest.

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