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CHARACTERIZATION AND ANTIULCER ACTIVITY OF ISOLATED COMPOUND FROM THE ETHANOL EXTRACT OF *HALYMENIA CEYLANICA* HARVEY EX KUTZING

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ABSTRACT

The purpose of this research was carried out to characterize the bioactive compounds in the *Halymenia ceylanica* Harvey ex kutzing using GC-MS analysis. The analysis revealed the two bioactive compounds such as Kaempferol (96.076%) and Phenytoin (3.924 %) .The FTIR analysis recognized different spectrum revealed the presence of functional groups. The HPLC profile displays two compounds with two different retention times (2.150 min, 4.480 min). The retention time 2.150 min with the peak area percentage 98.3 is the important bioactive compound. PASS software was used to analyze the biological activity of compound. In Pa>0.7, there are 9 different activities are predicated including antiulcerative (95.2%) followed by anti-eczematic (94.8%) etc. The compounds were isolated by preparative HPLC. It revealed the two peaks elute at retention time of 2.150 min with the peak area of 98.4% and 4.480 min with the area of 1.6%. The compound is separated and their structures were determined using advanced spectroscopy methods including Fourier Transform Infrared Spectroscopy (FTIR) and Nuclear Magnetic Resonance (NMR). FTIR spectrum confirms the functional groups of the isolated compound and the ^{1}H NMR and ^{13}C NMR analysis determine the number of hydrogen atoms and carbon atoms present in a molecule and the structural conformation of the isolated compound. Anti-ulcer activity was assessed for the isolated compound by the experimental wistar albino rats. The effect was evaluated by measuring ulcer index and percentage of ulcer healing. The ethanolic extract of 200mg/kg and 400mg/kg was found significant antiulcer activity as evidenced by the data obtained. The reduction of ulcer regions in the gastric wall was most prominent at a dose of 400 mg/kg extract. The current research suggests that the isolated compound of the ethanol extract of *Halymenia ceylanica* Harvey ex kutzing might be effective in the treatment of peptic ulcers.

INTRODUCTION

The pharmaceutical industry is increasingly active in promoting natural therapeutics. The world health organization also promotes the plant derived medicines due to its therapeutic efficacy and safety [1]. Biologically active natural compounds with various structural types are found in the marine environment that is often not available in terrestrial sources [2]. Acid secretion is usually normal or low in a gastric ulcer. Acid secretion is excessive in half of the individuals with a duodenal ulcer, but normal in the other half. Gastric and duodenal ulcers are prevalent diseases that can be triggered by a number of reasons including stress, smoking, dietary deficits, and noxious agents such as non steroidal anti-inflammatory medicines [3]. Marine algae are primarily used to produce a wide range of bioactive secondary metabolites that have the potential to be used in the pharmaceutical sector. Many unique compounds identified from marine species in the last four decades, and these compounds have fascinating biological activities [4]. Marine macro algae include variety of biologically active chemical compounds with antiviral, antioxidant, anticancer, anthelmintic, antibacterial properties etc. [5]. Based on the pigmentation, marine macro algae are classified into three taxonomic groups: Green algae, red algae and brown algae [6]. The distribution of red algae was reported 41 along the coast of the Gulf of Mannar in Tamil Nadu [7]. Red algae are an important category in terms of human requirements, by a large scale application ranging from nutritional to biotechnology usage in labs, industries and medicines [8]. *Halymenia* is a genus of red algae with more than 60 recognized species, most of them found in tropical regions for the warm climate and some of which have been identified in cold temperate zones [9].

The Red algae are rich in biologically active chemical compounds. However, there are only a few studies on the pharmacological effects and chemical composition of the marine red algae. The selected *Halymenia ceylanica* Harvey ex kutzing have important valuable primary and secondary metabolites with medicinal properties.

Hence, the current research intends to achieve the following objectives,

i. GCMS analysis and HPLC analysis of the ethanol extract of *Halymenia ceylanica* Harvey ex kutzing to determine the chemical components
ii. Prediction of the biological activity of the selected compound by the PASS software product
iii. Characterization and isolation of the compound from the crude extract by the preparative HPLC.
iv. The molecular structures were assigned by Fourier Transform Infrared Spectroscopy (FTIR) and Nuclear Magnetic Resonance (NMR).
v. Evaluate the antiulcer property of the ethanolic extract of *Halymenia ceylanica* Harvey ex kutzing

MATERIALS AND METHODS

Algal sample:

The fresh red algal sample of *Halymenia ceylanica* Harvey ex kutzing was collected from Mandapam coastal line, Ramanathapuram district in the south east coast of Tamil Nadu, India with the Latitude 9° 16’ 48.00” N and Longitude 79° 07’ 12.00” E (Figure 1). *Halymenia ceylanica* Harvey ex kutzing was authenticated and deposited in Xavier’s College Herbarium, Centre for Biodiversity and Biotechnology, St. Xavier’s College (Autonomous), Palayamkottai-627002 and the Voucher number was given as XCH20504. The collections were made during the low tidal and sub tidal regions (up to 1m depth) by hand picking. The collected materials were extensively rinsed with marine water in the field to exclude epiphytes and silt particles. The samples were packaged separately in polythene bags and taken to the laboratory and then they were properly washed in tap water followed

by distilled water to remove the salt on the thalli's surface. They were preserved in 5% of formalin solution. Washed specimens were spread out at room temperature in the shade on blotting paper to dry. Using a tissue blender, the shade dried samples were grounded to a fine powder [10].

Figure 1: Natural habit of *Halymenia ceylanica* Harvey ex kutzing

Preparation of extracts:

Halymenia ceylanica Harvey ex kutzing was extracted from 30g of fine powder with ethanol for 8h separately. The sample was shaken periodically for 72 hours in the dark. The solution was filtered via filter paper after incubation and the filtrate was collected as crude extracts [11].

GCMS analysis

A Perkin Elmer Turbo Mass Spectrophotometer (Norwalk, CTO6859, and USA) was used to perform GC-MS analysis using a Perkin Elmer Auto sampler XLGC. The column utilised was a Perkin Elmer Elite - 5 capillary column measuring 30 m × 0.25 mm with a film thickness of 0.25 mm composed of 95% Dimethyl polysiloxane. At a flow rate of 1.0 ml/min, helium was employed as a carrier gas. A sample injection volume of 1 µl was used. The equipment was set to an initial temperature of 60°C and kept at that temperature for 2 minutes, after which the oven temperature was increased to 300°C at a rate of 10°C/min for 6 minutes. The temperature of the injection port was maintained at 250°C. Total run time was 90 min. The data was examined using total ion count to identify the molecule and quantification. Mass spectra were assessed using electron impact ionisation at 70 eV with an ion source temperature of 240°C and an interface temperature of 240°C. The mass units scanned ranged from 50 to 600. The chemical constituents were identified by GC-MS. The National Institute of Standards and Technology (NIST) database, which contains over 62,000 patterns, was used to interpret the mass spectrum of the GC-MS. The component's spectrum was compared to a database of known component's spectra maintained in the NIST library. Turbo-Mass OCPTVS-Demo SPL software was used to measure and analyze data from peak locations [12].

Fourier Transform Infrared (FTIR) spectral analysis

The functional groups were analyzed using a Shimadzu 8400S FTIR spectrophotometer. About 1mg of sample extract was separately made in to thin discs with 10 to 100mg of potassium bromide using a mold and press under anhydrous conditions. The measurements in the range of 400 to 3900cm^{-1} were taken in an automated recording FTIR spectrophotometer. The FTIR peak points were recorded and compared to the standard peak value [13].

High Pressure Liquid Chromatography (HPLC) analysis

The phytoconstituents identified in the various crude extracts were detected using High Pressure Liquid Chromatography. A Shimadzu LC-10AT VP HPLC system was used to analyze the crude extracts. The instrument includes with two LC-10AT VP pump, a UV-Visible detector SPD-10AT, a CTO-10AS VP column, a SCL 10A VP system controller, a Rheodyne injector with a 20 µl loop, and a SIL-10AT auto injector, a Hypersil BDS C-18 column (4.6250mm, 5m size), along with a C-18 guard column. At ambient temperature (25-28°C), the elution was carried out using gradient solvent systems with a flow rate of 1ml/min. The mobile

phase consists of two solvents. Solvent a methanol and the solvent B is water with the ratio of 45:55 were filtered through a 0.2 µ membrane filter before use and pumped from the solvent reservoir at a flow rate of 1ml/min, resulting in 260-270kgf/cm^2 column backup pressure. The mobile phase was prepared daily [14].

Prediction of Biological Activity

The concept of biological activity spectrum served as a basis for developing prediction of activity spectra for substances (PASS) software product (version 9.1, http://195.178.207.233/PASS). Over 4000 types of biological activity are predicted by PASS software online, including pharmacological effects, modes of action, interactions with metabolic enzymes, influence on gene expression, toxic and unfavorable consequences. A precise description of the PASS methodology, as well as some examples of its applications, can be found, and the quality of the prediction may be evaluated by submitting material structures with known functions [15]. Biological activities based on structural formula of a chemical compound evaluates the possibility that the predicted compound belongs to the category of active compounds represent as Pa and evaluates the possibility that the predicted compound belongs to the category of inactive compounds represent as Pi. Being probabilities, the Pa and Pi esteems fluctuate from 0.000 to 1.000 and their sum is not equal to zero i.e. $Pa+Pi\neq1$, since these probabilities are determined independently. (i) only functions with Pa>Pi can work for a particular compound; (ii) if Pa>0.7, the function is more likely to be detected experimentally; (iii) if 0.5<Pa<0.7, the chance to discover the action tentatively is less, but the compound may not be as well known pharmacologists; (iv) if Pa<0.5, the chance to find the activity experimentally is less, but the possibilities of structurally new compound detection that is, new chemical entries is higher [16]

Preparative HPLC

A Preparative HPLC system associated with large columns and high flow rate. The objectives of the preparative HPLC are isolation and purification of the crude extracts. The phytochemical was purified using a Shimadzu HPLC fitted with a fraction collector (FRC-10A). Preparative reversed-phase HPLC was performed Shimpack PRC-ODS (250X20mm id, 5µm, Shimadzu). The instrument is consisted of an auto sampler, two pumps (main pump and make up pump) and a photodiode array detector connected with the fraction collector. The column configuration consists of C18 reversed-phase column with 100 mm. The temperature of the auto sampler tray was maintained at 35°C and the column was kept at room temperature. HPLC grade methanol as 80 % v/v and 20% of water made up the mobile phase. With a total flow rate of 5.0 mL min^{-1}, the detection wavelength was set to 190-370 nm. Injection volumes were 5.0ml. The fraction collector was used to isolate and collect fractions. A photodiode array detector was used to detect phyto-constituents at a wavelength of 254nm and an individual peak was collected and the solvent was removed using rotary evaporation [17].

NMR spectroscopy

The NMR spectrometer is made up of three main parts: (1) The probe, which holds the sample to be tested, is attached to the superconducting magnet; (2) The console, which includes all of the electronics required for transmitting and receiving radio frequency (rf) pulses to the probe through the preamplifier and (3) The computer on which the experiment is executed and the collected NMR data is processed. The Bruker model AVANCE III HD (Switzerland) used to analyze the 1H NMR and the DRX-300 Mega Hz Bruker (Switzerland) used to analyze the 13C NMR. All NMR measurements were acquired at 298K (25°C). Topspin 3.1 software was used to examine the data.

Chemical shifts (δ) were calculated using residual solvent signals can evolve only in the state of magnetization and it expressed in ppm. For the recording of 1H and 13C NMR spectra, the following conditions were used: 30° pulse experiment; acquisition time 4.1 seconds; relaxation time 1.0 second; sweep width 15.1ppm (8012Hz); data points 65536 and dummy scan 2. Line broadening 0.1Hz was used to process the data. A total of 16 scans were acquired for each sample. For improved resolution, free induction decays were Fourier processed with a line widening factor of 0.1Hz. Manual phase adjustment was performed on the spectra. For quantitative analysis, peak integration was performed. At least triplicate measurements were taken in each experiment [18].

Antiulcer activity

Experiment animals

Wistar albino mice (160-200g) of either gender were obtained from Venkateswara Enterprises, Bangalore, Karnataka, India. The animals were acclimated for 7 days under typical husbandry circumstances, which included ambient temperature of 35±1°C, relative humidity of 45-55%, and light/dark cycle 12/12h. The experiment animanls were fed a conventional rodent pellet diet and had unlimited access to water. The composition of diet is 10% protein, 4% Arachis oil, 1% fibers, 1% calcium, 1000IU/g vitamin A and 500IU/g vitamin D. All the animals were acclimatized to the laboratory conditions prior to experimentation. All of the studies were carried out between the hours of 10.00 and 17.00 in compliance with the ethical criteria of International Association for the Study of Pain [19]. All the experiments followed the rules for the care and use of experimental animals, which were authorized by the Committee for the Purpose of Control and Supervision of Animal Experiments (CPCSEA).

Acute toxicity test

Acute oral toxicity analysis was carried out in accordance with OECD-423 criteria [20]. For the acute toxicity assessment, Wistar albino mice (n=6) of either sex were selected using a random sample method. The animals were fasted for one night and provided only water after which the extract (50% ethanolic extract) was administered orally at the dose level of 5mg/kg body weight via gastric incubation and being monitored for 14 days. If mortality is observed in 2 out of 3 animals, then the dose administered would be assigned as hazardous. If one animal dies, the same dose is given twice to confirm the toxic dose. If no mortality occurs, the process will be repeated with increased doses of 500, 1000, 1500, and 2000 mg/kg body weight. The doses for the trials were set based on the results of the acute toxicity test.

Aspirin induced gastric ulceration and experimental design [21]

Wistar albino rats of either sex were separated into four groups, each with six animals. Before the research, the animals were fasted for 24 hours but had unrestricted access to water. Only distilled water was given to the animals in the control group. The animals in the treatment group were administered ethanol extracts of *Halymenia ceylanica* Harvey ex kutzing. Ranitidine (10mg/kg) was used as a standard. They were anaesthetized with anaesthetic ether and the abdomen was opened by a minor midline incision after 1 hour of medication therapy. The pyloric part of the stomach was slightly lifted out and ligated according to method of Shay et al. [22] avoiding traction to the pylorus or damage to its blood supply. The stomach was replaced carefully and the abdominal wall was closed by interrupted sutures. Rats were sacrificed by an over dose of anesthetic ether after four hours of pyloric ligation. The abdomen was opened, cardiac end of the stomach was dissected out and the contents were drained into a glass tube. The volume of the gastric juice was measured and centrifuged at 2000rpm for 10min. The pH, total, and free acidity of the supernatant were determined using aliquots (1ml each). The fore stomach section of

each stomach was evaluated for lesions and indexed according to severity.

Macroscopic evaluation of stomach

The stomachs were opened along the larger curvature, cleaned with saline to eliminate gastric contents and blood clots, and viewed with a 10X magnifying lens to determine whether ulcers had formed. The numbers of ulcers were counted.

Scoring of ulcer will be made as follows:

Normal colored stomach..... (0)
Red coloration..... (0.5)
Spot ulcer.....…..... (1.0)
Hemorrhagic streak..... (1.5)
Deep Ulcers.....… (2.0)
Perforation.....…..... (3.0)

Mean ulcer score for each animal will be expressed as ulcer index. The percentage of ulcer protection was determined as follows:

Ulcer index (UI) was measured by using following formula:

$$UI = UN + US + UP \times 10{-}1$$

Where,

UI= Ulcer Index; UN = Average number of ulcers per animal; US = Average number of severity score; UP = Percentage of animals with ulcers

Percentage of inhibition of ulceration was calculated as below

% Inhibition of Ulceration= Ulcer index (control)-Ulcer index (test)/Ulcer index (control) × 100

RESULT AND DISCUSSION

GC-MS Analysis

The GC-MS analysis of the ethanol extract of *Halymenia ceylanica* Harvey ex kutzing revealed two peaks which indicate the presence of two compounds. Kaempferol (96.076 %) and Phenytoin (3.924 %) were the compounds which are present in the ethanol extract of *Halymenia ceylanica* Harvey ex kutzing. The presence of two components with Retention Time (RT) of 22.376 and 24.867 was verified by the GC-MS spectrum profile (Figure 2). The identified compounds with the Retention Time (RT), Percentage of Peak Area (PA%), Molecular Weight (MW), Molecular Formula (MF) and structure are listed in the Table 1.

Prediction of Biological activity spectra and effects

The identified compounds in the ethanol extract of *Halymenia ceylanica* Harvey ex kutzing by GC-MS analysis were Kaempferol (96.076 %) and Phenytoin (3.924 %). Kaempferol occupies 96.076 percentage of area, were analyzed for the biological activity by PASS software. There are 98 different activities predicted in Pa>0.7. All the 98 activities are fall in the four categories are mechanism of action, metabolic terms, pharmacotherapeutic effects, and adverse and toxic effects (Table 4). There are 71 different types of mechanism of action including Chlordecone reductase inhibitor, Membrane permeability inhibitor, ATPase inhibitor etc; 18 types of metabolic terms including CYP1A1 substrate, CYP2C12 substrate, UGT1A3 substrate, UGT1A1 substrate etc and there are 9 types of pharmacotherapeutic effects including Antiulcerative (95.2%), Antieczematic (94.8%), Antihemorrhagic, Antioxidant etc (Table 5).

Fourier Transform Infrared Spectroscopy (FTIR) analysis

The FTIR spectrum was used to identify the functional groups of the active components based on the peak value in the region of infra red radiation. The ethanol extract of *Halymenia ceylanica* Harvey ex kutzing was passed into the FTIR spectroscopy and the functional groups of the components were predicted based on its peak ratio. The isolated compound shows the following frequencies of intense peak in spectral data 620.07, 652.86, 677.93, 765.69, 854.41, 990.38, 1038.60, 1073.31, 1117.67, 1174.57, 1217.00, 1383.83, 1442.66, 1475.44, 1511.12, 1605.63, 1718.46, 2307.67, 2827.45, 2885.31, 2975.96 and 3433.06 (Figure 3). These FTIR spectrums reveals the possible compounds are alkenes, alkanes, aromatics, aliphatic amines, alkyl halides, 1° amines,

esters, carboxylic acids, amino acids and phenols (Table 2).

High Pressure Liquid Chromatography (HPLC) analysis

For standardization of ethanol extract of *Halymenia ceylanica* Harvey ex kutzing, HPLC is a sensitive and precise technology that is frequently used for the quality evaluation. The qualitative HPLC fingerprint profile of the ethanol extract of *Halymenia ceylanica* Harvey ex kutzing as selected at a wavelength of 660nm due to the clarity of the peaks and adequate baseline. The ethanol extract prepared by hot extraction was subjected to HPLC for the separation and identification of constituents present in the *Halymenia ceylanica* Harvey ex kutzing. The chromatogram shows the two constituents with two different retention times are 2.150 min and 4.480 min (Figure 4 and Table 3). In which the compound having the retention time 2.150 min with the peak area percentage 98.3 is the main constituents in ethanol extract of *Halymenia ceylanica* Harvey ex kutzing.

Preparative HPLC

Preparative high pressure Liquid chromatography (PHPLC) is a well-known and very effective purification process that was created for the isolation of impurities, for chromatographic purifications or as a part of a scale up process and identification of important products in the chemical and pharmaceutical industries. The success of preparative HPLC on a production scale has been made possible because of significant improvements made in several areas like i) column technology - compressed columns are used ii) packing materials - pressure stable spherical particles with high homogeneity either nonchiral or chiral iii) the understanding of the nonlinear processor in preprative HPLC. Preparative HPLC is a highly expensive technique for distillation, crystallization, or extraction when compared to other classic methods. Purification can only be utilized for rare or expensive items that have been obtained extremely fast and efficiently through the invention of preparative liquid chromatography, which can be used to manufacture pure chemicals. The ethanol extract of *Halymenia ceylanica* Harvey ex kutzing was subjected to Preparative HPLC for the purification of impurities. It revealed the one peak elute at retention time of 2.150 min (Figure 5). The peak area of one fraction is 98.4% (Table 6). Fractions of the peaks in the preparative HPLC chromatogram peaks were collected and analyzed further using Fourier Transform Infrared Spectroscopy (FTIR) and Nuclear Magnetic Resonance (NMR) to determine the structure of the isolated purified compound.

The Spectroscopic Techniques

A combination of various significant spectroscopic methods includes Fourier Transform Infrared Spectroscopy (FTIR), ^{1}H NMR and ^{13}C NMR is frequently used to identify an isolated compound. The target isolated compound can be identified by comparing its characteristic features with literature values.

Fourier Transform Infrared Spectroscopy (FTIR) analysis

The absorption of Infrared radiations by a molecule is the basis of Infrared absorption spectroscopy. One of the most extensively used techniques for identifying organic compounds is infrared spectroscopy. Infrared spectra are particularly dependable in elucidating the structure of many organic molecules, notably for the presence of functional groups. Infrared spectroscopy relies on the vibration and rotation of atoms. The compound with high peak area percentage is isolated and purified. The isolated compound was subjected to Infrared spectroscopy. The isolated compound of the ethanol extract of *Halymenia ceylanica* Harvey ex kutzing was subjected to FTIR for the identification of functional groups. The isolated compound shows the following frequencies of intense peak in spectral data 652.86 cm^{-1}, 678.90 cm^{-1}, 1383.83 cm^{-1}, 1459.05 cm^{-1}, 1740.64 cm^{-1} , 2853.49 cm^{-1}

and 2924.85 cm^{-1} (Figure 6). In FTIR spectrum, the each different peak indicates the presence of alkenes, aromatics and carboxylic acids. (Table 7). It is likely that the component of fraction from ethanol extract of *Halymenia ceylanica* Harvey ex kutzing is flavonoid based on these patterns.

Nuclear Magnetic Resonance (NMR) Spectroscopy

Edward Purcell and Felix Bloch discovered Nuclear Magnetic Resonance (NMR) in condensed matter in 1946 using separate apparatus and procedures. NMR spectroscopy is used to identify and analyze the structure of chemical molecules, proteins, and other complex substances. This method provides useful information on each proton and carbon atom present in the compound. NMR spectroscopy has been used to investigate a vast variety of natural compounds, revealing the structure of the compound

^{1}H NMR Analysis

The isolated compound of the ethanol extract of *Halymenia ceylanica* Harvey ex kutzing was subjected to ^{1}H NMR analysis for determine the types and number of hydrogen atoms present in a molecule. The isolated compound shows the chemical shift values (δ) at the following ppm 6.81, 7.26, 7.63, 7.26 and 6.81. (Figure 7 and Table 8). Among these chemical shifts, 6.81 ppm, 7.26 ppm, 7.63 ppm and 7.26 ppm indicated the presence of Aromatic - Benzene Ring (Ar - H) and 10.88 ppm revealed the presence of Carboxyl group (R – COOH) (Table 9). The ^{1}H NMR spectra of the isolated compound of the ethanol extract of *Halymenia ceylanica* Harvey ex kutzing revealed the existence of proton signals.

^{13}C NMR Analysis

The isolated compound of the ethanol extract of *Halymenia ceylanica* Harvey ex kutzing was subjected to ^{13}C NMR analysis for determine the types and number of carbon atoms present in a molecule. The isolated compound shows the chemical shift values (δ) at the following ppm 76.71, 77.03, 77.34, 118.05, 124.13, 132.01, 143.38 and 171.32. (Figure 8 and Table 10). Among the nine different chemical shifts 76.71 ppm, 77.03 ppm and 77.34 ppm represent a chloroform solvent. 118.05 ppm, 124.13 ppm, 132.01ppm and 143.38 ppm indicated the presence of aromatic (Phenyl Ring C) and 171.32 ppm indicated the presence of carboxyl group (R – COOH) (Table 11). The ^{13}C NMR spectra of the isolated compound of the ethanol extract of *Halymenia ceylanica* Harvey ex kutzing revealed the existence of carbon signals

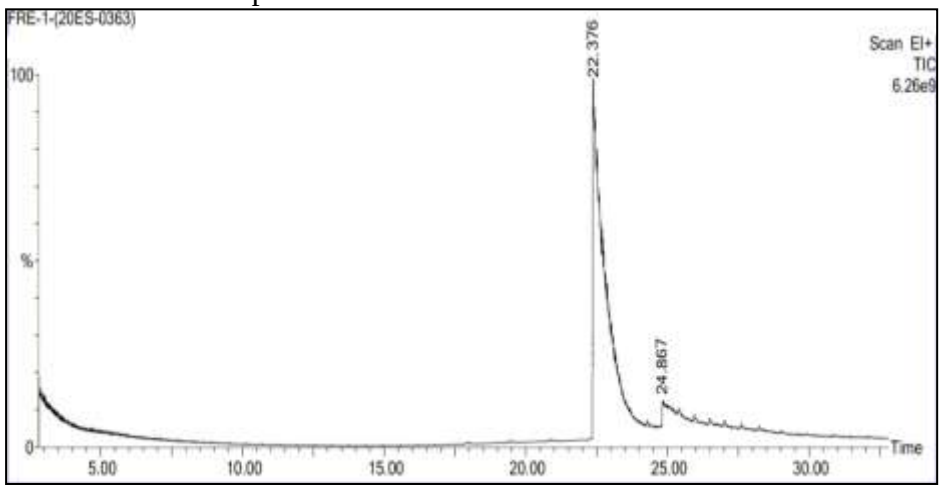
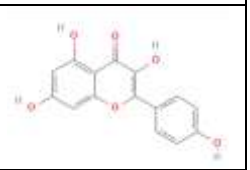
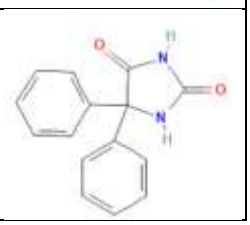


Figure 2: Gas Chromatography Mass spectroscopy chromatogram Ethanol extract of *Halymenia ceylanica* Harvey Ex Kutzing

Table 1: Active Compounds identified in the Ethanol extract of *Halymenia ceylanica* Harvey Ex Kutzing

S.No.	Name of the compound	RT	PA %	MW	MF	Structure
1	Kaempferol	22.376	96.076	286	$C_{15}H_{10}O_6$	
2	Phenytoin	24.867	3.924	252	$C_{15}H_{12}O_2N_2$	

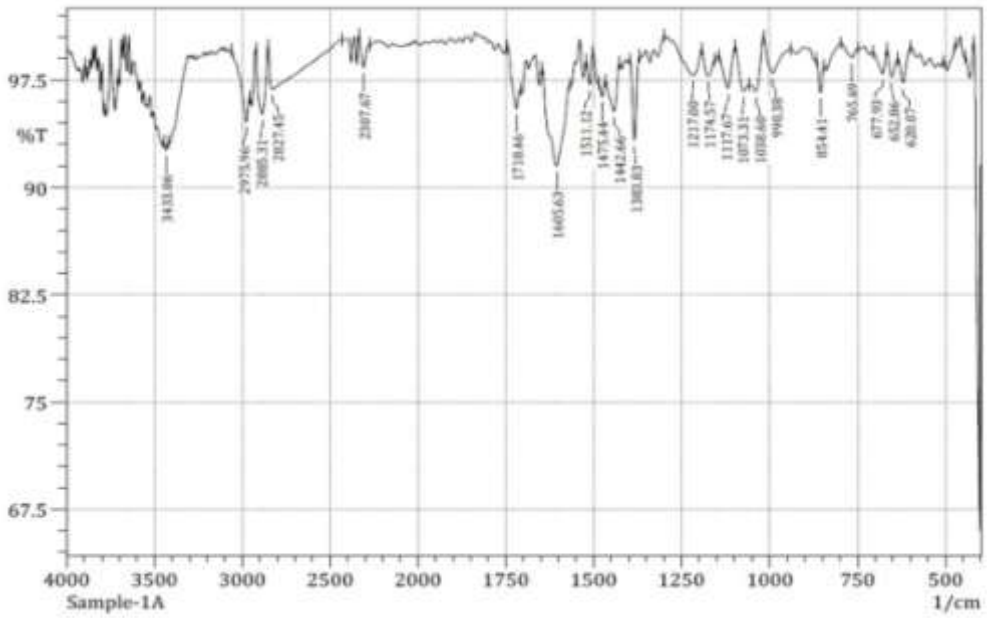


Figure3: FTIR spectrum of ethanol extract of *Halymenia ceylanica* Harvey ex Kutzing

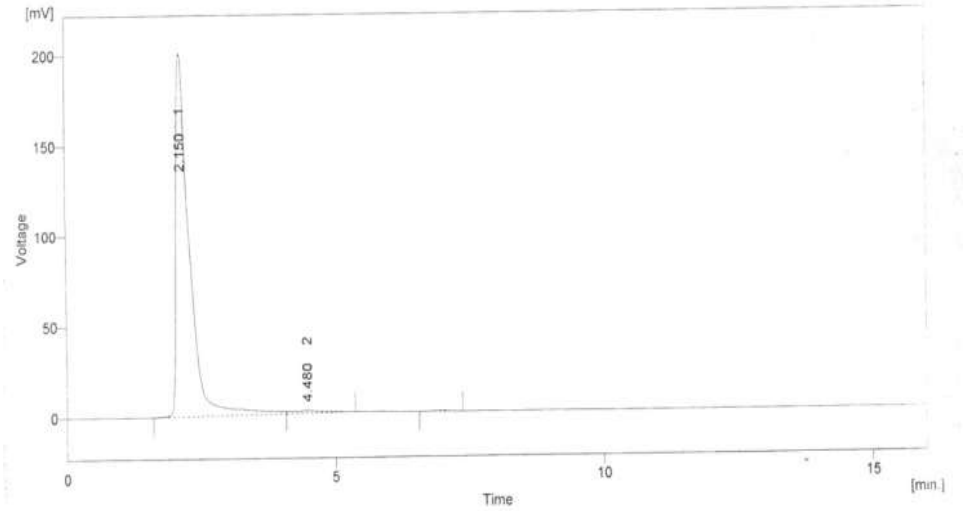


Figure 4: High Pressure Liquid Chromatography Spectrum of Ethanol extract of *Halymenia ceylanica* Harvey Ex Kutzing

Table 3: High Pressure Liquid Chromatography profile of ethanol extract of *Halymenia ceylanica*

S.No	Reten. Time (min)	Area (mV.s)	Height (mV)	Area (%)	Height (%)	W05 (min)
1	2.150	3502.156	201.449	98.3	99.3	0.24
2	4.480	59.363	1.475	1.7	0.7	0.81
	Total	3561.519	202.924	100.0	100.0	

Harvey ex Kutzing

Table 2: FTIR spectrum of ethanol extract of *Halymenia ceylanica* Harvey ex Kutzing

S.No	Wave Number cm^{-1}	Wave Number cm^{-1} (Reference article)	Intensity Estimation	Functional Group	Type of vibration	Possible compounds
1	620.07	650 - 1000	weak	=C-H	bend	Alkenes
2	652.86	650 - 1000	weak	=C-H	bend	Alkenes
3	677.93	650 - 1000	weak	=C-H	bend	Alkenes
4	765.69	650 - 1000	weak	C-H	rock	Alkanes
5	854.41	675 - 900	medium	C-H	oop bend	Aromatics
6	990.38	650 - 1000	weak	=C-H	bend	Alkenes
7	1038.60	1020 - 1250	medium	C-N	stretch	Aliphatic amines
8	1073.31	1020 - 1250	medium	C-N	stretch	Aliphatic amines
9	1117.67	1020 - 1250	medium	C-N	stretch	Aliphatic amines
10	1174.57	1020 - 1250	weak	C-H	wag	Alkyl halides
11	1217.00	1020 - 1250	weak	C-H	wag	Alkyl halides
12	1383.83	1350 -1390	medium	C-H	rock	Alkanes
13	1442.66	1400 - 1520	medium	C-C	Stretch	Aromatics
14	1475.44	1400 - 1520	weak	C-C	stretch	Aromatics
15	1511.12	1400 - 1520	weak	C-C	stretch	Aromatics
16	1605.63	1580 -1650	strong	N-H	Bend	1° amines
17	1718.46	1690 - 1760	weak	C=O	stretch	Esters, Carboxylic acids, Carbonyls
18	2307.67	2500 - 3330	weak	S–H	Stretch	Aminoacids
19	2827.45	2500 - 3330	weak	O–H	stretch	Carboxylic acids
20	2885.31	2850 - 3000	medium	C–H	Stretch	Alkanes
21	2975.96	2850 - 3000	medium	C–H	Stretch	Alkanes
22	3433.06	3200 - 3500	strong	O-H	stretch	Phenols

Table 4: The number of biological activities of Kaempferol predicted by the PASS in the ethanol extract of *Halymenia ceylanica* Harvey ex kutzing

S.No	Biological Spectra (Categories)	Number of Activities	Examples
1	Mechanism of Action	71	Chlordecone reductase inhibitor, Membrane permeability inhibitor, ATPase inhibitor etc
2	Metabolic terms	18	CYP1A1 substrate, CYP2C12 substrate, UGT1A3 substrate, UGT1A1 substrate etc
3	Pharmacotherapeutic effects	9	Antiulcerative, antioxidant, Antieczematic, Antihemorrhagic, etc
4	Adverse and toxic effects	-	-

Table 5: Prediction activity spectra in pharmacotherapeutic effects for Kaempferol by PASS system

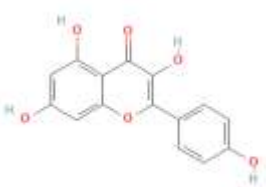
Compound Name and Structure	Active, P_a	Inactive P_i	Activity Spectra
Kaempferol 	0,952	0,001	Antiulcerative
	0,948	0,001	Antieczematic
	0,894	0,002	Antihemorrhagic
	0,856	0,003	Antioxidant
	0,852	0,010	Antiseborrheic
	0,814	0,003	Cardioprotectant
	0,796	0,002	Hemostatic
	0,791	0,013	Antineoplastic
	0,715	0,008	Anticarcinogenic

Figure 5: Preparative High Pressure Liquid Chromatography Spectrum of Ethanol extract of *Halymenia ceylanica* Harvey Ex Kutzing

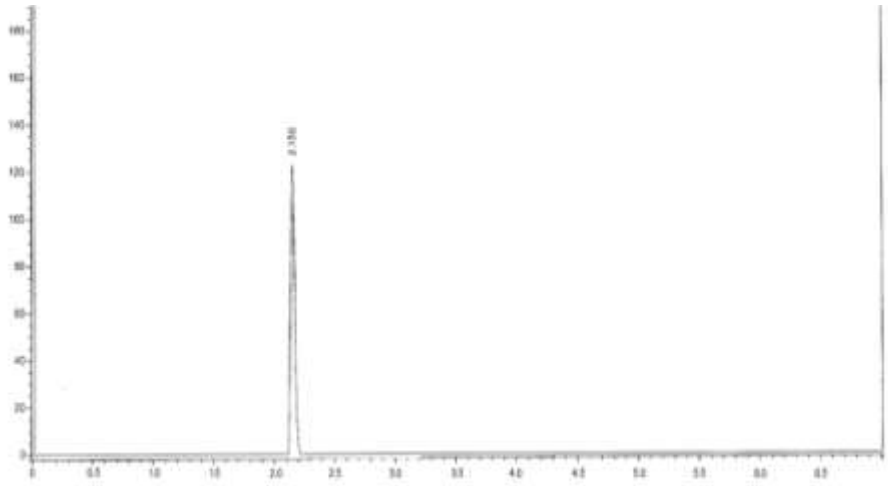


Table 6: Spectrum details of Preparative High Pressure Liquid Chromatography in the Ethanol extract of *Halymenia ceylanica* Harvey Ex Kutzing

Name	Retention Time (min)	Peak Area (mV.s)	Peak Height (mV)	Peak Area %
Peak 1	2.150	3492.486	124.030	98.4

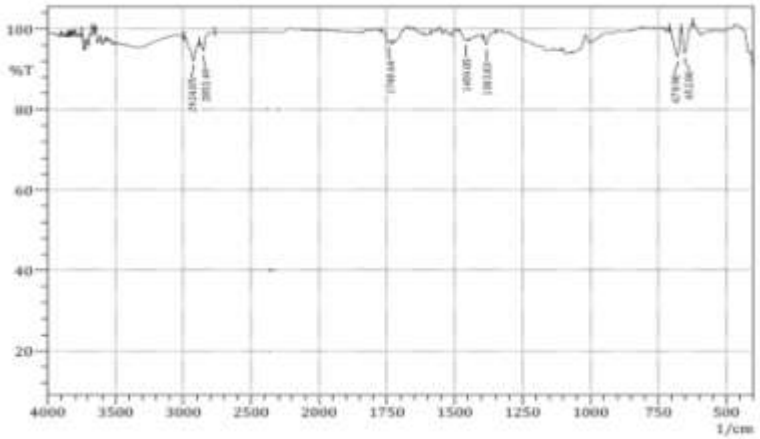


Figure 6: Fourier Transform Infrared Spectroscopy (FTIR) Spectrum of isolated compound of Ethanol extract of *Halymenia ceylanica* Harvey Ex Kutzing

Table 7: Fourier Transform Infrared Spectroscopy (FTIR) peak value of Ethanol extract of *Halymenia ceylanica* Harvey Ex Kutzing

S.No	Wave Number cm^{-1}	Wave Number cm^{-1} (Reference article)	Intensity Estimation	Functional Group	Type of vibration	Possible compounds
1	652.86	650 - 1000	weak	=C-H	bend	Alkenes
2	678.90	650 - 1000	weak	=C-H	bend	Alkenes
3	1383.83	1350 -1390	medium	C-H	rock	Alkanes
4	1459.05	1400 - 1520	weak	C-C	stretch	Aromatics
5	1740.64	1690 - 1760	weak	C=O	stretch	Carboxylic acids
6	2853.49	2500 - 3330	weak	O–H	stretch	Carboxylic acids
7	2924.85	2500 - 3330	weak	O–H	stretch	Carboxylic acids

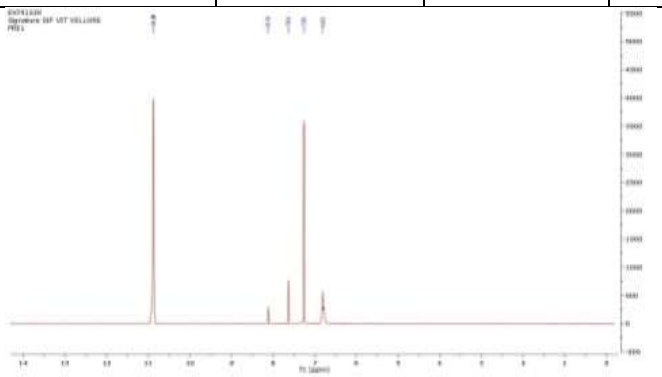
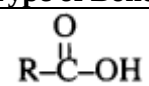
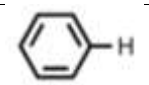
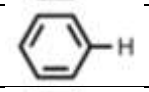
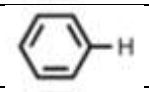
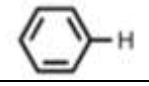


Figure -7 ^{1}H NMR Analysis of isolated compound of Ethanol extract of *Halymenia ceylanica* Harvey Ex Kutzing

Table - 8 ^{1}H NMR Analysis of isolated compound of Ethanol extract of *Halymenia ceylanica* Harvey Ex Kutzing

S.No	Assign.	Shift (ppm)	Range (ppm)	H's	Integral	Class
1	A	10.88	10.64 – 11.04	1	1.298	Singlet
2	B	8.13	8.07 – 8.19	2	2.307	Singlet
3	C	7.63	7.51 – 7.75	2	1.925	Singlet
4	D	7.26	7.17 – 7.35	4	3.614	Singlet
5	E	6.81	6.64 – 6.98	1	1.473	Singlet

Table - 9 Compounds retrieved from ^{1}H NMR Analysis peak of isolated compound of Ethanol extract of *Halymenia ceylanica* Harvey Ex Kutzing

S.No	Shift (ppm)	Type of Bond	Description
1	10.88		Carboxylic
2	8.13		Aromatic (Benzene Ring)
3	7.63		Aromatic (Benzene Ring)
4	7.26		Aromatic (Benzene Ring)
5	6.81		Aromatic (Benzene Ring)

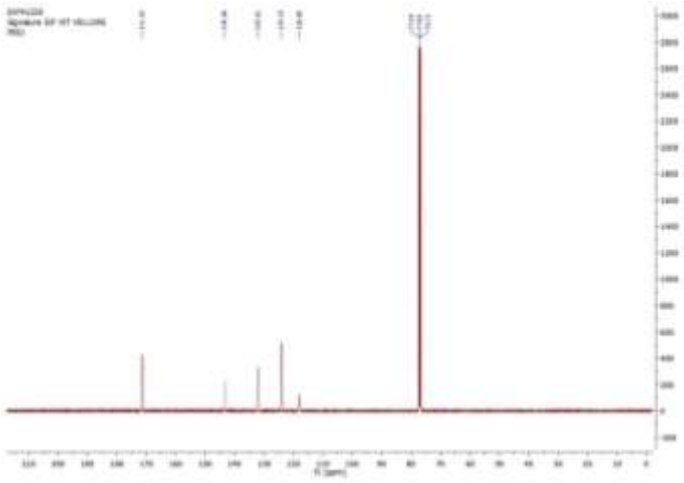
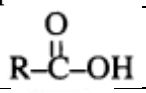


Figure -8 ^{13}C NMR Analysis of isolated compound of Ethanol extract of *Halymenia ceylanica* Harvey Ex Kutzing

Table - 10 ^{13}C NMR Analysis of isolated compound of Ethanol extract of *Halymenia ceylanica* Harvey Ex Kutzing

S.No	Shift (ppm)	Intensity	Width	Type
1	171.32	412.62	1.47	Compound
2	143.38	227.37	1.94	Compound
3	132.01	89.11	1.62	Compound
4	124.13	334.83	1.86	Compound
5	118.05	118.29	1.76	Compound
6	77.34	2742.27	2.04	Solvent
7	77.03	2731.36	1.92	Solvent
8	76.71	2736.98	1.81	Solvent

Table - 11 Compounds retrieved from ^{13}C NMR Analysis peak of isolated compound of Ethanol extract of *Halymenia ceylanica* Harvey Ex Kutzing

S.No	Shift (ppm)	Type of Carbon	Description
1	171.32		Carboxyl
2	143.38		Aromatic (phenyl Ring C)
3	132.01		Aromatic (phenyl Ring C)
4	124.13		Aromatic (phenyl Ring C)
5	118.05		Aromatic (phenyl Ring C)

Anti Ulcer Activity Analysis

Acute toxicity study was performed in which the animals were treated with the ethanol extract of *Halymenia ceylanica* Harvey ex kutzing at a dose of 5mg/kg body weight via gastric incubation and being monitored for 14 days. At this dosage, all the tested animals were alive and did not exhibit significant visible signs of toxicity. During the monitoring period, there were no aberrant symptoms, behavioral changes, body weight changes, or morphological findings. Hematology and serum biochemistry indices such as triglycerides, creatinine, urea, haemoglobin, AST, ALT, and ALP of the treated rats did not differ significantly from those of the control normal rats. These findings suggest that the extract is highly secure even at these heigher dosages, with no acute toxicity and an oral lethal dose (LD50) of greater than 5 g/kg body weight for both male and female rats.

Gross evaluation of gastric lesions

Anti-ulcer potential of *Halymenia ceylanica* Harvey ex kutzing showed a dose dependent protection against aspirin (500mg/kg body weight) induced ulcers in rats. There are four different tests for the antiulcer activity includes control, Ranitidine 10 mg/kg, test sample with the dose of 200 mg/kg and test sample with the dose of 400 mg/kg. The area of gastric ulcer formation and the volume of gastric juices are reduced compared to the control. For the test sample, the gastric mucosa becomes no injuries and also showed the flattening of gastric mucosa. All the test doses produced a decrease in ulcer index and total acidity as compared to the control. Gastric pH was also shown to be increased in all drug treated groups as compared to control, with maximum increase being produced by ranitidine as standard drug. The aspirin induction has caused the accumulation of gastric secretions of 6.7ml with pH 6.8 in the control group. The total acidity and free acidity of the gastric secretions were found to be 75.21 and 39.33mEq/l respectively. At dosages of 200 and 400 mg/kg, pre-treatment with the ethanol extract of *Halymenia ceylanica* Harvey ex kutzing substantially ($P<0.05$) reduced the volume of gastric secretions 5.1 and 4.9ml, respectively. Moreover the total acidity and free acidity of 200mg/kg dosage extract is 44.11mEq/l and 26.03mEq/l, as well as the total acidity and free acidity of 400mg/kg dosage extract is 40.17mEq/l and 22.11mEq/l, were considerably decreased ($P<0.05$). The percentage of the inhibition of ulcer for the test sample 200 mg/kg is 62.15% and for the test sample 400 mg/kg is 71.32% (Figure 9 and Table 12). The result of the antiulcer analysis concludes is aspirin induction has caused gastric ulcerations and pre-treatment with the ethanol extract of *Halymenia ceylanica* Harvey ex kutzing has reduced significantly ($P<0.05$) in a dose dependent manner.

Discussion

The world biodiversity is rich in the source of chemicals. Marine algae are potentially abundant sources of highly bioactive secondary metabolites that might lead to the creation of novel medicinal medicines. Phytochemical compounds are the most valuable and having the properties of antiulcer, anticancer, wound healing activities. Marine organisms have the potential to be used as alternate sources for isolating new metabolites with biological and pharmacological capabilities. The extract of the marine algae with the serial dilution from low polarity to high polarity of solvent can separate the phytochemical compounds uniquely.

The present study is screening, characterization and standardization of the phytochemical compound and applied to the specific therapeutic approach. The ethanolic extract of *Halymenia ceylanica* Harvey ex kutzing shows the important phytochemical compounds. FTIR analysis is used to determine the functional group of the compound. By the presence of alkenes, aromatics and carboxylic acids, some of the flavonoid group is present. The HPLC analysis is used to standardize the biological compounds. The GCMS analysis is used to

screening the compounds of the extract. The phytochemical compounds are noted in the GCMS analysis and identified the properties of the phytochemical compounds. Kaempferol is the flavonoid group and having the properties of antiulcer activity. The selected compound is separated by use of preparative HPLC and Fourier Transform Infrared Spectroscopy (FTIR), ^{1}H NMR and ^{13}C NMR is used to identify an isolated compound. FTIR confirms the functional group of the Kaempferol compound. And NMR gives the structure of the compound. Kaempferol compound shows the antiulcer activity with the good results

The flattening of mucosal folds shows that the gastro protective action of Halymenia ceylanica extract is attributable to a reduction in gastric motility. Gastric motility alterations might have a role in the development and prevention of experimental gastric lesions and contraction of the circular muscles of the rat fundus strip. This type of contraction can cause mucosal compression at the site of the greatest mechanical stress, at the crests of mucosal folds which can lead to necrosis and ulceration. The present study shows the ethanol extract of *Halymenia ceylanica* Harvey ex kutzing is the good source for antiulcer activity

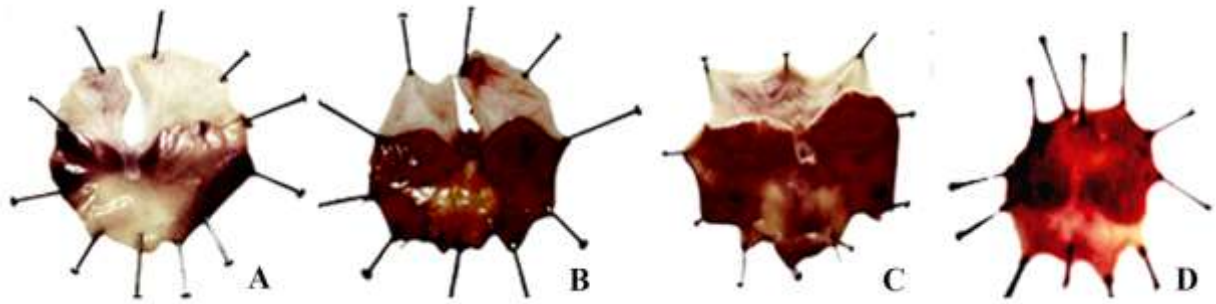


Figure -9: Antiulcer activity of Ethanol extract of *Halymenia ceylanica* Harvey Ex Kutzing

A - Control; Severe injuries in the gastric mucosa
B - Treated with Ranitidine 10 mg/kg. No injuries in the gastric mucosa
C - Treated with the test sample (200 mg/kg). Fewer injuries in the gastric mucosa
D - Treated with the test sample (400 mg/kg). No injury and flattening of gastric mucosa.

Table - 12 Antiulcer activity of Ethanol extract of *Halymenia ceylanica* Harvey Ex Kutzing

S. No	Group	Volume of Gastric juice	pH	Acidity		Ulcer Index	Percentage of Inhibition of ulcer
				Free	Total		
1	Control	6.7±0.23	6.8±0.15	39.33±1.92	75.21±2.21	11.23±0.82	-
2	Ranitidine 10mg/kg	4.9±0.21	4.2±0.11	20.14±1.02	35.11±1.03	3.21±0.21	71.41
3	200mg/kg Ethanol extract	5.1±0.21	5.5±0.41	26.03±1.27	44.11±1.37	4.25±0.41	62.15
4	400mg/kg Ethanol extract	4.9±0.11	4.9±0.12	22.11±1.04	40.17±1.12	3.22±0.27	71.32

Conclusion

The flattening of mucosal folds shows that the gastro protective action of *Halymenia ceylanica* extract is attributable to a reduction in gastric motility. Gastric motility alterations might have a role in the development and prevention of experimental gastric lesions and contraction of the circular muscles of the rat fundus strip. This type of contraction can cause

mucosal compression at the site of the greatest mechanical stress, at the crests of mucosal folds which can lead to necrosis and ulceration. The results of the present investigation recommended the kaempferol compound isolated from the ethanolic extract of *Halymenia ceylanica* possesses the potential anti-ulcer activity in both the doses of 200mg/kg and 400mg/kg. The reduction of ulcer regions in the gastric wall was most prominent at a dose of 400 mg/kg extract. Further studies are required to determine the active components responsible for the mechanism of anti-ulcer of *Halymenia ceylanica* extracts.

Conflict of Interest

The authors declare that they have no conflict of interest.

Data availability

The data sets created and/or analyzed during the current study are available from the corresponding author on reasonable demand.

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