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Phytochemical profiling and *In vitro* Biological activities of *Haloplegma duperreyi* Mont.-a Red Seaweed from Gulf of Mannar

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Abstract

The exploration of marine resources, particularly seaweeds, for new bioactive compounds is essential in the fight against life-threatening diseases worldwide. Red seaweeds, in particular, have garnered significant attention from the pharmaceutical industry due to their potential to yield novel compounds that can be developed into effective treatments for cancer and microbial infections. This study presents, for the first time, a comprehensive phytochemical analysis and evaluation of the biological activities of the methanolic extract of *Haloplegma duperreyi Mont.*, a red seaweed from the Gulf of Mannar. GC-MS analysis of the extract identified a total of twelve phytocompounds in varying ratios. The findings revealed that the methanolic extract of the seaweed contains three major bioactive compounds such as flavones, 9-Octadecenoic acid methyl ester, and Methyl eicosa-5,8,11,14,17-pentaenoate which are known to be the leading phytocompounds that play a significant role in antimicrobial and anticancer activities. The antibacterial activity assay with the extract confirmed the remarkable growth-inhibitory potential of *Haloplegma duperreyi* against human infectious pathogens. Additionally, the DPPH radical scavenging and phosphomolybdenum reduction assays suggested that the methanolic extract has significant antioxidant activity within a short duration. In summary, the in vitro experiments highlight *Haloplegma duperreyi* as a highly promising marine resource with significant potential for the development of effective drugs and innovative biomedical applications.

Key words : Antibacterial, Antioxidant, Cytotoxicity, Seaweeds, *Haloplegma duperreyi.*

Red seaweeds are a valuable marine resource for the pharmaceutical, food, and cosmetic industries. They have garnered significant interest among biomedical scientists searching for effective drugs to combat life-threatening diseases[1]. The bioactive compounds from seaweed present promising opportunities for developing new medicines due to their unique biomolecules, biosafety, and easy availability from natural sources[2-4]. The increasing demand for effective medications has resulted in the production of many synthetic drugs, which often come with unwanted side effects for both humans and the environment. These adverse effects pose significant challenges to public health[5]. In this context, marine-derived drugs offer several advantages, including biocompatibility, sustainability, and cost-effectiveness, carving out a unique niche in the pharmaceutical industry[6]. In various experimental settings, bioactive compounds found in red seaweed extracts demonstrate potent antibacterial, anticancer, and immuno-modulatory effects[7]. Some studies have shown that crude sulfated polysaccharides from red seaweeds possess strong antifungal, antioxidant, and wound-healing properties[8-11]. Over the past two decades, seaweed-derived polysaccharides such as laminarin, alginate, fucoidan, and carrageenan have gained significant attention for their pharmacological applications. Marine polysaccharides and bioactive compounds can also form hydrogels under specific pH conditions[12-16]. Seaweeds provide a diverse array of biofunctional compounds with commercial uses, particularly in functionalized foods and tissue engineering. Seaweed-derived sulfated polysaccharides exhibit remarkable antiviral activity. The effectiveness of this antiviral mechanism relies on the presence of bioactive compounds within the seaweed[17]. Numerous experimental studies have demonstrated that sulfated polysaccharides can block the binding of viruses to host cells and prevent viral replication. They achieve this by altering the immune responses of host cells, thereby hindering the transmission of the virus and its transcription. Recent studies explored the efficacy of sulfated polysaccharides as effective treatments for COVID-19[18]. Among the various red seaweed species in the Gulf of Mannar region, *Haloplegma duperreyi Mont.* is readily available throughout the year[19-20]. However, there have been no reported experimental studies on the biological activity of *Haloplegma duperreyi*. The current study aims to evaluate the antibacterial, antioxidant, and anticancer properties of Haloplegma duperreyi against human pathogens and HT-29 colon cancer cells. The identification of active compounds was conducted using GC-MS analysis. The antioxidant activity of the *H. duperreyi* was assessed through DPPH, phosphomolybdenum reduction, and Fe^{3+} reduction assays. The anticancer potential was evaluated using the MTT cytotoxicity bioassay against HT-29 cells. Upon completion of these experimental studies, *Haloplegma duperreyi* may be proven to be an effective and low-cost marine resource for various antimicrobial and anticancer applications.

Chemicals used :

Ammonium molybdate, Methanol, Bradford reagent, DPPH, ethylenediaminetetraacetic acid (EDTA), MTT, n-hexane, ferrozine, Muller Hinkton agar, Phosphomolybdenum, and Nutrient broth, were purchased from Merck. Colon cancer cell line (HT-29),

was purchased from the NCCS, Pune, India.

Collection of seaweed :

The seaweed was collected from the Gulf of Mannar, Rameswaram coastal area, Tamil Nadu, India (9° 1656.35°N 79° 124.71°E). It was identified as *Haloplegma duperreyi* in the Botanical Survey of India, Coimbatore. The Collected seaweed was washed thrice with double-distilled water to remove unwanted debris. Then shadow-dried for one week, and the fine powder was ground. The powder was stored at 4°C in an airtight container.

Methanolic extraction :

The dried seaweed powder (100 g) was extracted thrice with Methanol at 60 °C for 4 h and filtered by a filter paper (Whatman No. 1). The prepared extract was concentrated at 50 °C in a vacuum evaporator to 1:3 volume, and partitioned to furnish the fraction of n-hexane, DCM, and EtOAc.

Phytochemical analysis :

The Preliminary Qualitative phyto-chemicals of *H. duperreyi* seaweed were examined by Sobuj *et.al.,* method[3]. Alkaloids were identified by Dragendorff's test, tannins using ferric chloride, and flavonoids using the Alkaline Reagent test. The saponins test was done with the Foam test, and the Salkowski test confirmed the terpenoid. Other phytochemicals such as phenols, steroids and cardiac glycoside are also tested with standard procedures[8]. Quantification of Phytochemical compounds such as phenol and flavanoid in the *H. duperreyi* seaweed were examined according to the previous method with slight modification[21].

Gas chromatography–Mass Spectrometry (GC–MS) :

The methanolic extract of *H. duperreyi* was analyzed using gas chromatography-mass spectrometry (GC-MS) at the Vellore Institute of Technology, India. The analysis was performed with a Clarus 680 model instrument (Perkin-Elmer) using Clarus 600 electron ionization. The mass detector was maintained at a transfer line temperature of 250 °C and operated at an electron ionization energy of 70 eV. The instrument was equipped with fused silica column packs of Elite-5MS. Bioactive components of the extract were separated using helium as an inert carrier gas at a flow rate of 1 mL/min. The chromatogram revealed three prominent compound peaks, with details of the predicted compounds provided in the table below. The GC-MS spectra were examined using Turbo Massver 5.4.2 software and compared with the NIST-2008 standard library database[6].

DPPH *radical scavenging activity :*

The antioxidant activity of *Haloplegma duperreyi* extracts was assessed using the standard DPPH radical scavenging assay[8]. Stock solutions of the extracts were prepared using methanol. Samples were diluted to concentrations ranging from 100 to 600 µg/mL. Diluted extract solutions (1 mL) were mixed with 2 mL of DPPH· solution in methanol. The reaction mixtures were shaken and incubated at 30 ºC for 30 minutes in the dark. The absorbance of the samples was measured at 517 nm using a UV-Vis spectro-photometer. A control group was established

using 2 mL of DPPH· solution mixed with methanol (2 mL). The DPPH assay was performed three times. The percentage of free radical scavenging was calculated using the following equation:

$$\%\ of\ inhibition = \frac{Absc - Abst}{Absc} X\ 100$$

where Absc- Absorbance of the control, and Abst- Absorbance of the treated sample.

Phosphomolybdenum *reduction activity :*

The antioxidant potential of the seaweed extract was assessed using the phosphomolybdenum reduction assay[21]. The reaction mixture included sodium phosphate (25 mM), ammonium molybdate (3 mM), and H_2SO_4 (0.5 M). The seaweed extract was uniformly diluted in dichloromethane within a concentration range of 100 to 600 µg/mL. The treated samples were incubated at 90 °C for 60 minutes. Visual color changes were observed, and the absorbance of the phosphomolybdenum complex was measured at a wavelength of 695 nm using a UV-Vis spectrophotometer. Ascorbic acid (2 mM) was used as a reference sample. The phosphomolybdenum reduction potential of the seaweed extract was quantified in terms of Ascorbic acid equivalents (mg of ascorbic acid per gram of extract).

Fe^{3+} *reducing power activity :*

The efficacy of ferrous ion reduction by *Haloplegma duperreyi* was examined[21]. A mixture was prepared by combining 1 mL of ferric sulfate (0.5 mM), 1 mL of ferrozine (0.5 mM), and 1 mL of seaweed extract at various doses from 100 to 600 µg/mL. The reaction solution was maintained without disturbances to equilibrate at room temperature for 15 minutes. After observing a color change in the solution, the reduction of the iron (II)-ferrozine was quantified at an absorbance of 562 nm. The total percentage of ferrous ion reduction was calculated using the following equation:

$$Chelating\ ability\ \% = \frac{Absc - Abst}{Absc} X\ 100$$

Absc-absorbance of control, and Abst-Absorbance of the treated sample

Antibacterial activity :

The antibacterial effectiveness of *Haloplegma duperreyi* was assessed against five human pathogens *Micrococcus luteus* MTCC 1538, *Bacillus subtilis* MTCC 441, *Staphylococcus aureus* MTCC 3166, *Escherichia coli* MTCC 1302, and *Pseudomonas aeruginosa* MTCC 1688, using a well-diffusion assay[19]. The 12-hour bacterial culture (100 µL) was swabbed onto the surface of nutrient agar. Different concentrations of seaweed extract (250, 500, and 750 µg/mL) were added to the wells, with the commercial antibiotic Tetracycline included as a positive control for comparison. The agar plates treated with the extract and containing the bacteria were incubated at 37 °C for 12 hours. The antibacterial efficacy was examined from the zones of inhibition on the agar plates. The growth inhibition diameters were assessed using a microbiology zone scale.

Anticancer activity :

The anticancer potential of *Haloplegma*

duperreyi extract was evaluated against HT-29 colon cancer cells using the MTT cytotoxicity assay, following the methodology outlined[19]. The HT-29 cells were cultured overnight at 37 ºC in a continuous flow of CO_2. The cells were then exposed to different doses of the seaweed extract (ranging from 50 to 250 µg/mL) and incubated for 24 hours. After this incubation period, the cells were treated with 100 µL of MTT solution (1 mM) and incubated at 37 ºC for an additional 4 hours. Finally, the absorbance of the cell medium was calibrated at 570 nm using a Spectramax M3 multi-plate reader with Softmax Pro V5 5.4.1 software. Cytotoxic percentage was quantified using a specific formula.

$$Cell\ Viability\ \% = \frac{Absc - Abst}{Absc} X\ 100$$

Absc-absorbance of control, and Abst-Absorbance of the treated sample

Statistical analysis :

The IC_{50} and LD_{90} values were calculated by using a one-way analysis of ANOVA software and Prism8pro software. Statistical significance of results considered at the level of p**<0.05. All test results were expressed as Mean ± SD of triplicates.

Phytochemical screening of the extracts :

The external appearance of seaweed (Fig. 1A) has been evaluated and species was identified as *Haloplegma duperreyi* belongs to the family Wrangeliaceae by BSI, Ciombatore (Ref. No.BSI/SRC/5/23/2021/Tech). The presence of phytochemicals such as tannins, alkaloids, flavonoids, and phenolic compounds was confirmed by standard tests (Fig. 1B). The total phenolic compound of the methanolic extract is 42.84 µg/mg and the flavonoids are 5.89µg/mg respectively (Fig. 1C). The list of phytochemicals found in the methanolic extract is presented in Table-1.

A total of twelve bioactive compounds were confirmed through the GC-MS technique of methanolic extracts from *H. duperreyi* (Table-2). Among the twelve bioactive compounds, three primary compounds were highlighted based on their peak areas and retention times, as depicted in the chromatogram (Fig. 2A). The GC-MS spectra of the extract were compared with the NIST-2008 standard library database. Table-2 provides details on the bioactive compounds in the methanolic extract, including their molecular weights and molecular formulas identified during the GC-MS analysis. The secondary metabolites from seaweeds are crucial for drug discovery and the cosmetics industry. The extract of *H. duperreyi* showed a range of promising compounds, including flavones (Fig. 2B), dodecanoic acid (10-methyl-, methyl ester), 9-octadecenoic acid (z)-, methyl ester, caryophyllene, and 6-dihydroequilenin, among others. Flavones and 9-octadecenoic acid (z)-, methyl ester have been documented for their antimicrobial, antioxidant, and anticancer properties. The leading compound identified in the methanolic extract of *H. duperreyi* is 9-Octadecenoic acid methyl ester (Fig. 2C), which has shown significant tumor-inhibiting activities against various cancer cell lines. Additionally, the extract contains 6-dihydroequilenin, a prominent component used in estrogen hormone replacement therapies. Most of the phytocompounds listed in the GC-MS analysis of the

Table-1. Qualitative analysis of phytochemicals in the methanolic extract of *H. duperreyi*

Phytochemicals	Test	Inference	Result
Alkaloids	Dragendorff's test	Brown precipitate appears	+
	Hager's test	Yellow precipitate appears	+
Terpenoids	Salkowski test	A red ring appears.	+
Steroids	Libermann-Burchard's test	Dark violet color appears	+
Phenolic compounds	Ferric chloride test	Green color appears	+
Flavonoids	Alkaline Reagent test	Yellow colour appears	+
Tannins	Lead acetate test	White precipitate appears	+
Glycosides	Legal's test	Blood red color appears.	+
Carbohydrates	Molisch test	Violet color appears	+
Saponins	Foam test	Foam appears	+

Table-2. GC-MS list of Phytochemical in the methanolic extract of *H. duperreyi*

Name of the compound	Molecular weight(g/mol)	Molecular Formula
5-Methyl-2,4-disoprophylphenol	178.27	$C_{12}H_{18}O$
Caryophyllene	204.35	$C_{15}H_{24}$
Phenol,2,4-bis[1,1-dimethylethyl]-	532.7	$C_{35}H_{49}O_2P$
Flavone	222.24	$C_{15}H_{10}O_2$
Dodecanoic acid, 10-methyl-, methyl ester	200.32	$C_{12}H_{24}O_2$
6-Dihydroequilenin	268.3	$C_{18}H_{20}O_2$
9-Octadecenoic acid[z]-, methyl ester	282.5	$C_{18}H_{34}O_2$
Methyl eicosa-5,8,11,14,17-pentaenoate	316.5	$C_{21}H_{32}O_2$
4-Androsten-6a-ol-3,17-dione	302.4	$C_{19}H_{26}O_3$
z,z-3,11-octadecadien-1-ol-acetate	308.5	$C_{20}H_{36}O_2$
1-tetradecane, 2-decyl-	230.39	$C_{14}H_{30}O_2$
Phenol, 2,6-bis[1,1-dimethylethyl]-4-[4-hydroxy-3, 5-dimethylphenyl]methyl]-	340.5	$C_{23}H_{32}O_2$

extract exhibit potential antimicrobial activity against various human infectious bacteria and fungal species. Some reports indicate that esters based on methyl eicosa-5,8,11,14,17-pentaenoate (Fig. 2D) have significant antibacterial effects against *Bacillus, Streptococcus*, and *Candida* species. The GC-MS analysis confirms that *H. duperreyi* seaweed contains effective bioactive compounds, making it a promising marine resource for new drug discovery. The 9-octadecenoic acid methyl ester also induces cell death by generating ROS and free radicals, leading to mitochondrial damage in bacteria[21]. Furthermore, the presence of flavonoids enhances the anticancer properties against HT-29 colon cancer cells.

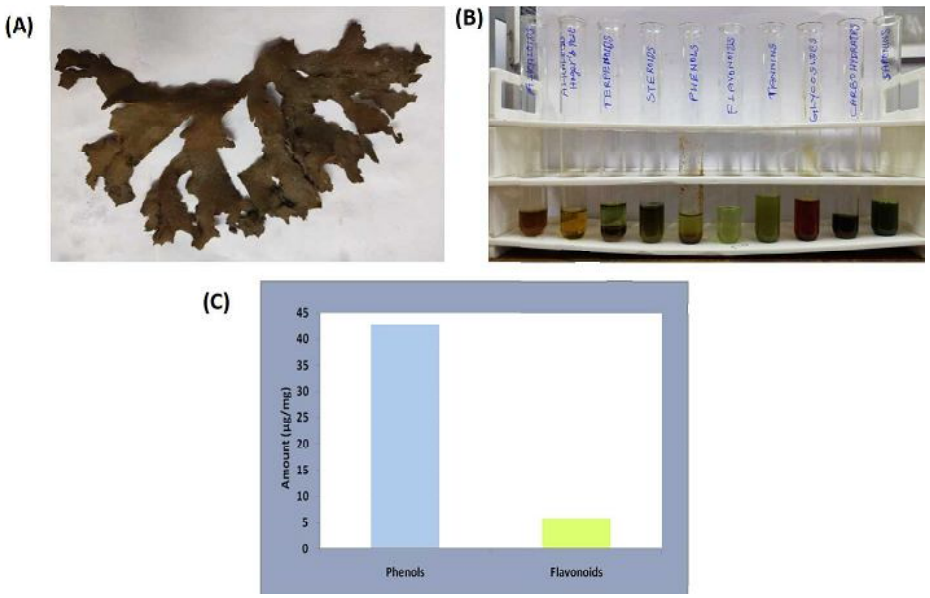

Fig. 1. (A) Photographic images of fresh *Haloplegma duperreyi* Mont. red seaweed, (B) Phytochemical analysis, (C) Quantitative analysis of Flavonoid and Phenol in methanolic extract of *H. duperreyi*

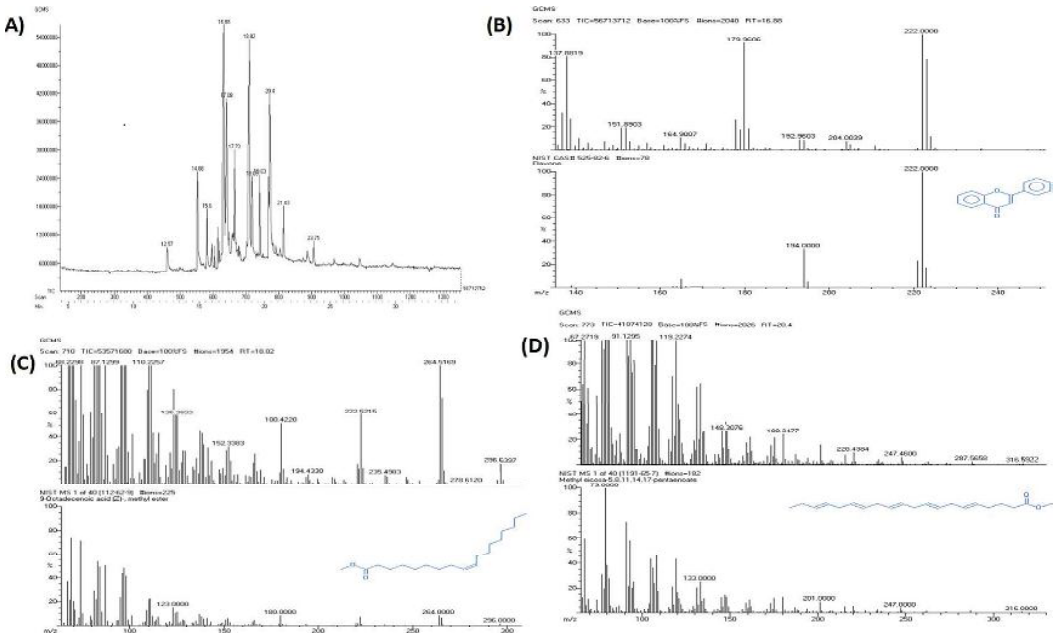

Fig. 2. (A) GC-MS analysis of a methanolic extract of *H. duperreyi*, (B) GC-MS spectra of Flavone, (C) GC-MS spectra of 9-octadecenoic acid (z)-, methyl ester, (D) GC-MS spectra of methyl eicosa-5,8,11,14,17-pentanoate

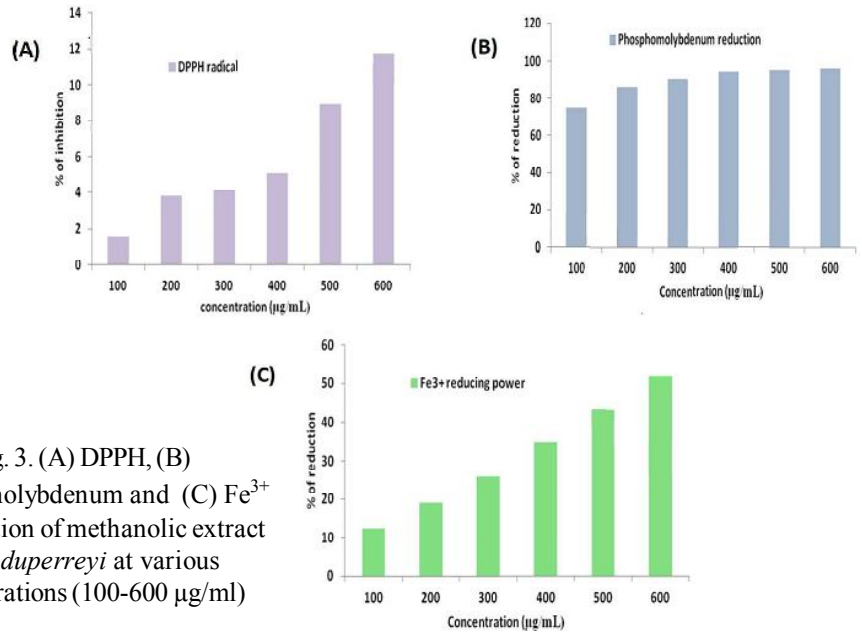

Fig. 3. (A) DPPH, (B) Phosphomolybdenum and (C) Fe^{3+} ion reduction of methanolic extract of *H. duperreyi* at various concentrations (100-600 µg/ml)

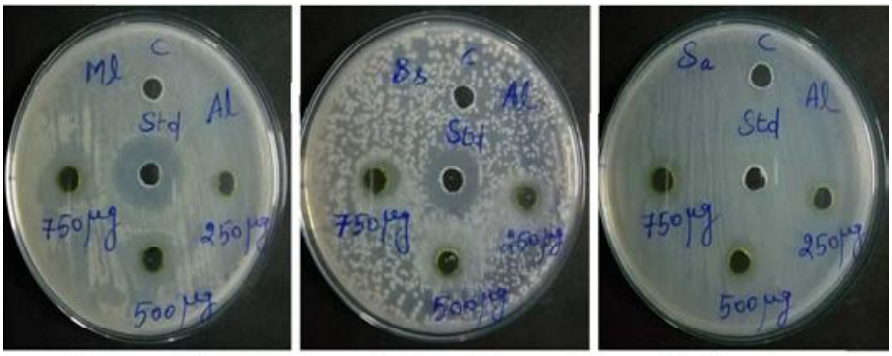

Fig. 4. Antibacterial activity of methanolic extract of *H. duperreyi* against infectious pathogens at various concentrations (250-750 µg/ml)

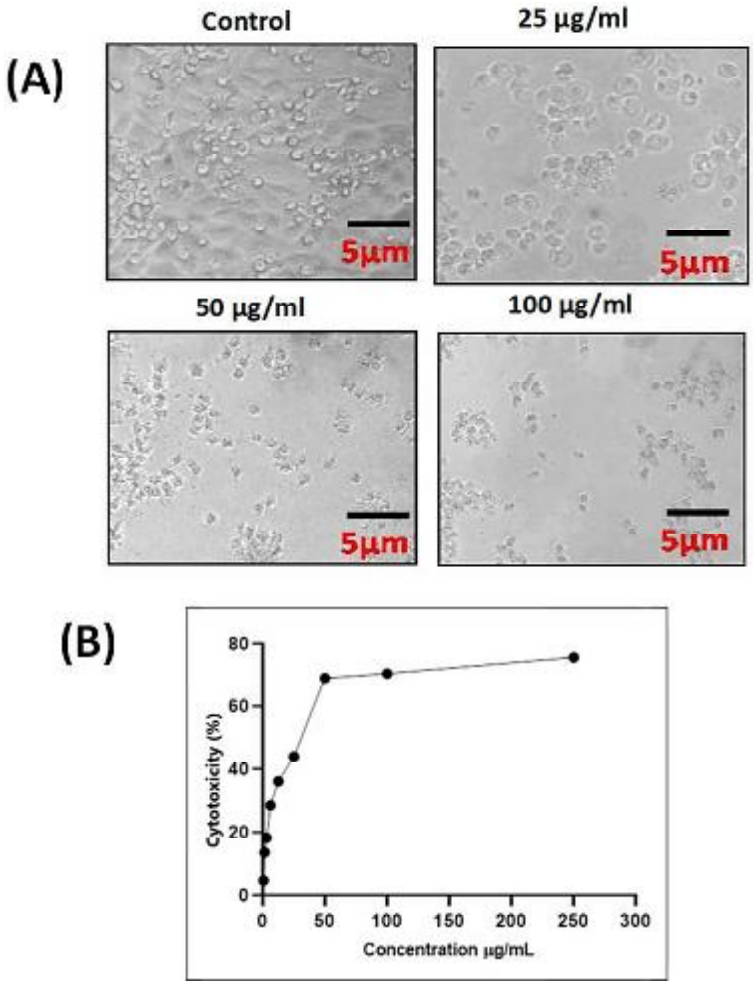

Fig. 5. Cytotoxicity of methanolic extract of *H. duperreyi* against HT-29 colon cancer cells (A) Inverted phase contrast microscopic images HT-29 cells after treatment, (B) cytotoxic percentage of methanolic extract at various doses.

Antioxidant activity of H. duperreyi extract:

Seaweed extracts have demonstrated potential in free radical scavenging, thereby preventing the generation of ROS and protecting cells from damage. The methanolic extract of *Haloplegma duperreyi* shows a dose-dependent efficacy in free radical scavenging. Notably, the scavenging activity against the DPPH free radical significantly increased at higher concentrations, with the highest concentration of 600 µg/mL exhibiting an antioxidant potential of 11.75% within 30 minutes (Fig. 3A). The results from the phosphomolybdenum reduction assay indicate a remarkable 96.14% reduction at the same concentration of 600 µg/

mL. Seaweed-derived antioxidants as a game changer in neutralization of excessive ROS production by donating electrons, effectively acting as a natural regulatory mechanism for free radicals (Fig. 3B). Additionally, the antioxidant potential of oxymatrine has been assessed through the DPPH assay, revealing excellent reduction of Fe^{3+} ions and scavenging of H_2O_2 free radicals under physiological conditions (Fig. 3C). The presence of flavones in the extract may help reduce lipoprotein oxidation and prevent cardiovascular diseases. Moreover, antioxidants can be utilized in conjunction with chemotherapy for cancer treatment. The antioxidant efficacy of these extracts could heighten ROS stress in cancer cells, influencing transcription factors and activating apoptotic pathways[16,18]. This synergy may be advantageous for potential drug discovery.

Antibacterial potential of H. duperreyi :

The antibacterial potential of the methanolic extract of *Haloplegma duperreyi* was evaluated using the well diffusion technique (Fig. 4). Interestingly, the synthesized extract demonstrated excellent efficacy in inhibiting the growth of human pathogens. The zones of inhibition measured for the extract-treated pathogens were as follows: *Micrococcus luteus* (17 mm), *Bacillus subtilis* (14 mm), *Staphylococcus aureus* (13 mm), *Escherichia coli* (12 mm), and *Pseudomonas aeruginosa* (16 mm). Additionally, the presence of 9-octadecenoic acid (methyl ester) enhanced the antibacterial activity of the extract. Many reports suggest that 9-Octadecenoic acid (methyl ester) is an effective antibacterial and antifungal agent for biomedical applications[4]. Furthermore, the combination of flavones and 9-octadecenoic acid (methyl ester) demonstrated a synergistic biological effect against human pathogenic bacteria[19].

Anticancer efficacy of H. duperreyi :

The anticancer potential of the methanolic extract of *Haloplegma duperreyi* seaweed was evaluated against HT-29 colon cancer cells. The results from the MTT cytotoxicity bioassay demonstrated significant cell toxicity even at the lowest doses. Flavones found in the methanolic extract have shown a wide range of applications in cancer treatment. These seaweed flavones modulate reactive oxygen species (ROS) scavenging activities and induce the G2/M phase cell cycle arrest[13]. The dysfunction of the cell cycle mechanism contributes to apoptosis, autophagy, and the invasiveness of cancer cells[3]. Moreover, flavones can promote cell death through the overexpression of death genes such as caspase-8 and FAS[16]. In contrast, the presence of 9-octadecenoic acid (methyl ester) affects the antioxidant balance, which may lead to the elimination of cancer cells. The IC_{50} value of the extract is 20.22 ± 0.5 µg/ml and the methanolic extract results in an 80% reduction in viability at 250 µg/ml. The cytomorphological changes in extract-treated HT-29 cells were examined using an inverted phase contrast microscope (Fig. 5). Microscopic observations indicated that the extract-treated cells exhibited apoptotic characteristics, including shrinkage, reduced cell density, and condensed chromatin formation. In contrast, control cells did not show any signs of apoptosis. The cytotoxicity assay confirms that the methanolic extract of *Haloplegma duperreyi* is highly promising for anticancer therapy.

This research demonstrates that the methanolic extract of the red seaweed *Haloplegma duperreyi* Mont. exhibits significant antibacterial and anticancer potential against human pathogens and HT-29 colon cancer cells. The antibacterial, antioxidant, and anticancer activities of this methanol extract showed dose-dependent increases when tested against various bacterial strains and HT-29 cells. According to the MTT assay, the methanolic extract can impair mitochondrial function and increase ROS production in HT-29 cells, primarily due to the availability of leading compounds such as flavones and methyl 9-octadecenoate. Additionally, the excellent antioxidant properties of *Haloplegma duperreyi* may help prevent cell damage related to oxidative stress. Overall, this study supports the potential for developing effective antimicrobial and anticancer agents. The presence of phenolic compounds with antioxidant potential suggests that the methanolic extract of *Haloplegma duperreyi* red seaweed could serve as a promising therapeutic agent for cancer and antibiotic treatments.

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