In vitro potential of *Hormophysa cuneiformis* methanolic extract as antioxidant, anti-inflammatory, anticoagulant, and anticancer

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ABSTRACT: Recently, research on bioactive natural compounds used in diverse industries, including biomedicine, and food, has been increasing rapidly. *Hormophysa cuneiformis* methanolic extract (*H. cuneiformis* ME) was evaluated for its antioxidant, anti-inflammatory, anticoagulant, and anticancer activities by assessing its cytotoxicity and selectivity index. The phytoconstituents, total phenolics, flavonoids, and tannins content, and their fractions were estimated. The antioxidant activity methods included DPPH scavenging activity (DPPH SA) and ferric-reducing antioxidant power (FRAP). The anti-inflammatory, anticoagulation, and anticancer properties were assessed by determining the inhibition of COX-2 enzyme, prolongation of clotting time (APPT and PT), and cytotoxicity against PC-3, HepG-2, and VERO cell lines, respectively. The algal extract showed an increase of DPPH scavenging and FRAP % proportionately with increasing its concentration, with maximum values of 81.14% with IC50=175.31µg/ml and 70.73% with IC_{50}= 197.42 µg/ml, respectively. The algal extract has a concentration-dependent COX-2 inhibitory % (74.92% with IC50= 172.81 µg/mL) and clotting time prolongation (APTT (65 sec) and PT (54.8 sec). *In vitro* antitumor activity of *H. cuneiformis* methanolic extract revealed an inhibitory effect against PC-3, and HepG-2 (prostate and liver carcinoma) cell lines with IC_{50} of 53.31 and 40.80 µg/ml, and selectivity index 9.10 and 11.89, respectively. High-performance liquid chromatography (HPLC) revealed that the algal extract is rich in various bioactive phenolics, flavonoids, and tannin compounds. *H. cuneiformis* ME exhibited effective antioxidants, anti-inflammatory, anticoagulation, and anticancer activities with a high selectivity index, which recommends its further prospective use in pharmaceutical and medicinal domains.

Keywords: Bioactive compound, anticoagulation, cytotoxicity, selectivity index, *Hormophysa cuneiformis*, brown algae

INTRODUCTION

Due to their characteristics, molecule diversity, and novel chemical structures that are complicated and challenging to synthesize chemically, marine macroalgae, commonly referred to as seaweeds, have drawn the interest of numerous researchers as a provenance of promising biomolecules (Ruiz-Medina et al., 2022). It was established that seaweed is a significant, quintessential naturally occurring source of highly valuable, structurally diversified bioactive chemicals that satisfy demands in the pharmacological, therapeutic, and nutritional domains (Mohy El-Din and Alagawany, 2019). They are a valuable source of dietary substances like carbohydrates, proteins, minerals, and vitamins (Gomez-Zavaglia et al., 2019). Otherwise, they produce many secondary metabolites, including polyphenols, alkaloids, steroids, terpenoids, glycoproteins, phytosterols, and more (Park et al., 2023). Numerous biological effects of these compounds have been demonstrated, including antioxidant, antiangiogenic, anticoagulant, anti-inflammatory, antifungal, antiviral, antihypertensive, antimutagenic, antiproliferative, anticancer properties, and more (Patra et al., 2016; Ismail et al., 2021). Accordingly, seaweed can be utilized confidently in the search for medications with fewer adverse effects meant to be employed as a therapy for diabetes, cancer, obesity, hypertension, and cardiac illnesses (Shannon and Abu-Ghannam, 2019). In addition, macroalgae offer advantages over plants in that they have a rapid cycle of cultivation and collecting, as cultivation could be carried out even in sewage and enhanced medication (Ercolano et al., 2019).

Algal metabolites' antioxidant power protects against challenging conditions (Haq et al., 2019). Numerous studies have demonstrated the elevated phenolic content and high antioxidant capacity of various algal species (Premkumar et al., 2019; Ismail et al., 2023). Apart from their antioxidant properties, phenols have demonstrated their potential as antibacterial, antidiabetic, anti-inflammatory, anticancer, anticoagulant, antihypertensive, and antiallergic agents, rendering them highly valuable in the dietary and pharmaceutical sectors (Ruiz-Medina et al., 2022). Flavonoids have potent antioxidant activity, antimicrobial, anticarcinogenic, anti-obesity, and antidiabetic properties (Alghazeer et al., 2017; Yan et al., 2019). Because of their widely recognized pharmacological effects, flavonoids are gaining more and more attention. As a result, preventive effects have been reported in conditions including heart disease, viral infections, stomach and duodenal ulcers, cancer, and inflammations (Albuquerque et al., 2021). Numerous studies scouted various forms of flavonoids found in multiple algae (Bulut et al., 2019; Fernando et al., 2022). Tannins, a type of flavonoid, are well-known for their therapeutic benefits in managing allergies, malignancies, heart conditions,

and platelet aggregation (de Melo et al., 2023). The diverse range of bioactivities phenolic compounds generated by seaweed makes them attractive possibilities for product development or as components in the food, health, and cosmetics industries (Aydin, 2022).

Proteins, lipids, and DNA inside the structure of an organism can all be harmed by free radicals produced during usual metabolic pathways. The body's natural antioxidant defense systems scavenge these free radicals, preserving the equilibrium between oxidation and antioxidants. However, this equilibrium is upset by external oxidative agents that enter the body, such as pollution, ozone, radiation, smoking, and industrial chemicals, which lead to oxidative damage in cells (Haq et al., 2019). Numerous health issues, including aging, heart, muscle, lung, and liver disorders, arteriosclerosis, diabetes, cancer, inflammation, and Alzheimer's disease, can be brought on by oxidative damage (Ruiz-Medina et al., 2022; Aydin 2022). Exterior antioxidants must be administered to regain this equilibrium, so natural antioxidants derived from natural resources must be considered for their safety (Aydin, 2022).

Inflammatory illnesses are now one of the leading global causes of health problems and have a significant impact on medical expenses. Where the consequences of inflammation, particularly in a chronic state, may lead to injurious health problems, such as insulin resistance, irritable bowel syndrome, multiple sclerosis, atherosclerosis, inflammatory arthritis, dermatitis, and a variety of other diseases (Ghallab et al., 2024). Thus, anti-inflammatory substances are essential for treating inflammatory illnesses. Nowadays, there are numerous anti-inflammatory treatment medications available, but using them predominantly results in problems and significant side effects that affect organs and systems. Furthermore, there are several contraindications to their use. In this regard, the hunt for naturally occurring anti-inflammatory compounds, including those found in marine organisms, especially polyphenols, is currently receiving much attention (Besednova et al., 2022). Marine algae have been pointed out as a well-undiscovered source of unparalleled anti-inflammatory substances. These comprise polyphenols, terpenes, proteins, sulfated polysaccharides, fatty acids, and other bioactive phytoconstituents. Thus, consuming these marine algae may help prevent and counter the pathophysiology of many chronic inflammatory disorders. With more research, algal anti-inflammatory phytochemicals may be employed as medicines or to create structural analogs with potent anti-inflammatory properties and fewer side effects (Ghallab et al., 2024).

Thrombotic illnesses are death-causative agents globally. Showing the rising prevalence of thrombotic disorders, effective medications are desperately needed. Heparin, an anticoagulant medication, is used as a standard drug. However, it has some adverse effects (Qin et al., 2023). Therefore, looking for natural substitute sources of anticoagulants is necessary. Sulfated polysaccharides in Seaweed have an effective anticoagulant action. Thus, its importance has been rapidly increasing recently. According to previous studies, the most reported potent natural anticoagulants from marine algae are sulfated polysaccharides (SPs) and phlorotannins; nevertheless, many phenolic, flavonoids, sterols, alkaloids, and terpenoid compounds exhibit anticoagulation properties (Kim and Wijesekara, 2011; Sahagun et al., 2021; Abd El Monsef et al. 2023 a,b).

When normal cells undergo transformations or deformations, this leads to cancer. Cancer can be caused by uncontrolled cell proliferation and metastasis in distant body regions. As a result, any tissue in the body has the potential to become cancer (Labidi et al., 2016). Owing to the significance of prostate diseases, particularly cancer, laboratory research aims highly to discover a medication or novel approach for treating endocrine disorders. On the other hand, PC chemical therapies are linked to a range of restrictions, so we should direct them to other natural therapies (Mirzaei et al., 2019). The prevalence of liver illnesses is higher in developing countries (Anwanwan et al., 2020). The liver has a critical function in metabolism. It plays a significant part in the metabolism of external substances. Therefore, malignant growth in the liver can change the body's internal environment. Although certain liver chemotherapeutic drugs, such as doxorubicin, are successful in causing apoptosis, their adverse effects prevent many people from using them (Zhao et al., 2023). Thus, finding novel natural substances with anticancer properties is crucial due to their high safety and low cost (Van Weelden et al., 2019). A quarter or so of the existing anti-cancer medications are of natural origin, and an additional quarter are chemically modified versions of natural substances (Foo et al., 2018). The advantageous properties of macroalgae-derived substances on cancer have been increasingly discovered, offering them a primary

substitute for developing novel medications and/or adjuvants for first-line drugs reducing their truculence (Agena et al., 2023).

Hormophysa cuneiformis belongs to Phaeophyceae, the family Sargassaceae. To the best of our knowledge and the available literature, few reports have been conducted on the anti-inflammatory activity and anticancer ability of *H. cuneiformis*, and they have done so without the assessment of selectivity index (SI). In addition, all previous research on the anticoagulation activity of algae has been conducted on algal polysaccharides. Therefore, this work aims to evaluate the *in vitro Hormophysa cuneiformis* methanolic extract's anti-inflammatory, anticoagulation activities, cytotoxicity and selectivity index against PC-3, HepG-2, and VERO cell lines with the assessment of its antioxidant activity.

MATERIALS AND METHODS

Hormophysa cuneiformis sampling

The brown seaweed *Hormophysa cuneiformis* (J.F.Gmelin) P.C.Silva 1987, belongs to the family *Sargassaceae*, was collected from the Red Sea shore at Gulf of Suez, Ras Sudr between latitude 29°71'60.34''N and longitude 32°69'69.93''E, Egypt. Based on the descriptions provided in taxonomic references by Aleem (1978) and Lipkin and Silva (2002), the seaweed species was identified under a microscope using its morphological features. This identification was validated by the Algae Base website (Guiry 2020). Thalli were washed following harvesting by seawater to remove any epiphytes and sand particles. To avoid microbial contagion, rhizoidal parts were also removed. Algal samples were preserved in sterile, spotless plastic containers and brought to the laboratory. Algal samples were thoroughly washed once more in the laboratory using tap water and then ionized water. Drying took place in shaded air for 2–3 days, then in an oven at 60 °C for 3 h, after which ground occurred with an electric blender and preserved in sterile, spotless plastic bags at room temperature.

Preparation of methanolic algal extract (ME)

An algal sample weighing twenty grams was mixed with thirty times its volume of absolute methanol in a sealed Perkin Elmer flask. The mixture was retained in a shaker at room temperature for three days. Then centrifugation occurs for 20 min at 7200 rpm. A rotary evaporator was used to evaporate the solvent and reduce the extract volume at 50°C and reduced pressure of 72 mbar, and then a lyophilizer was used to dry it well. The resulting powder was dissolved in dimethyl sulfoxide (DMSO) (5 mg/ml) and retained at -20°C until needed, with the evaluation of extraction yield percentage using the formula of Maisuthisakul and his colleague (Maisuthisakul et al., 2007).

$$\text{Extraction yield}\% = (\text{Weight1}/\text{Weight2}) \times 100$$

Weight1 is *H. cuneiformis* dry methanolic extract weight, and Weight2 is *H. cuneiformis* powder weight (20 gm).

Total Phenolics determination

The Folin-Ciocalteau method was applied for phenolics estimation, according to Taga et al (1984). Concisely, 50 µl of *H. cuneiformis* ME was mixed with 1 ml of 2% sodium carbonate at dark 25 °C conditions for approximately 5 minutes and then mingled with 50 µl of 50% Folin-Ciocalteau phenol reagent, and mixture incubation occurs at 25 °C in the dark for arround 30 min. Absorbance was measured at 720 nm. Distilled water was used as a blank and for control. A calibration curve of gallic acid was prepared, and phenolic contents were determined from the linear regression equation of this curve. The results are reported as mg gallic acid equivalents per gm extract (GAE/gm).

Flavonoids estimation

According to Chang et al. (2002) 1 ml of *H. cuneiformis* ME was mingled with 1 ml of $AlCl_3$ (10%), 1mL of Potassium acetate ($C_2H_3KO_2$) (1 M), and 28 ml of methyl alcohol, retained for 30 minutes at 25 °C, absorbance was performed at 415 nm. Distilled water was used as a blank and control. A calibration curve of quercetin was prepared, and flavonoid contents were determined from the linear regression equation of the calibration curve. The content of flavonoid was expressed in mg quercetin equivalents per gm extract (QE/gm).

Tannins estimation

Based on Julkunen-Titto (1985) 100 µl of *H. cuneiformis* ME were added to 3 ml of 40% vanillin, the mixture was mingled with 1.5 ml of HCl, agitated strenuously, and left in the dark at 25°C for approximately 30 min. The absorbance was carried out at 500 nm. A calibration curve of tannic acid was prepared, and total tannins content was expressed in mg tannic acid equivalent per gm extract (TAE/gm).

HPLC analysis

This analysis used a Kromasil column (150 mm × 4.6 mm) using an HPLC device (GBC, Australia) with a

UV/Vis detector and LC 1110 Pump. The ratios of methanol to water to tetrahydrofuran to acetic acid (23:75:1:1 v/v/v/v) as a mobile phase with 1 ml/min as flow rate and chromatograms were registered at UV 280 nm in the case of phenols. For flavonoids, acetonitrile to water to formic acid (85:14:1 v/v/v) was used as a mobile phase with a flow rate was 0.8 ml/min, and chromatograms were registered at UV 356 nm. For tannins, methanol to water (50: 50 v/v, isocratic mode) was used as a mobile phase and the flow rate was 1 ml/min; the detector was set at 280 nm with the mobile phase (Gupta and Garg, 2014).

DPPH scavenging activity (SA)

DPPH methanolic solution (0.004%w/v) was freshly performed and kept in the dark at 10 °C. *H. cuneiformis* ME (0.5-1000 µg/ml) was prepared with 3 ml of DPPH solution. Absorbance values were promptly recorded using a UV-visible spectrophotometer (Milton Roy, Spectronic 1201). DPPH radical absorbance without antioxidant (control) and antioxidant natural standard (ascorbic acid) was also determined. All measurements were carried out three times at 517 nm. The DPPH SA (PI) was estimated using the following formula:

$$PI = [\{(AC - AT)/ AC\} \times 100]$$

Where *A*C = absorbance of the control at t = 0 min and *A*T = absorbance of the sample + DPPH at t = 16 min (Barroso et al., 2016).

The fifty-percentage inhibitory concentration (IC_{50}) required to achieve 50% DPPH SA was determined from graphic plots of the concentration-response curve using GraphPad Prism software (San Diego, CA. USA).

Ferric chloride antioxidant power (FRAP)

Barroso et al.'s (2016) method measures the FRAP of *H. cuneiformis* methanolic extract. Samples in 1ml of methanol were mingled with equal amounts (2.5 ml) of both sodium phosphate buffer (0.2 M, pH 6.6) and potassium ferricyanide [$K_3Fe(CN)_6$] (1%, w/v), then stand for incubation for 20 min at 50 °C, then, addition of 2.5 mL of trichloroacetic acid (10%, w/v) was done followed by 10 min centrifugation at 1000 rpm. Equal amounts of supernatant and deionized water were mixed with freshly made ferric chloride (0.1%, w/v); absorbance was determined at 700 nm compared to a blank. Ascorbic acid was utilized as a reference natural antioxidant standard. As reported by Canabady-Rochelle et al. (2015), the percentage is computed as follows:

$$\text{Reducing capability } (\%) = 100 - \left[\frac{Ao - As}{Ao} x100\right]$$

Where, A_0: absorbance of the control solution. As: sample absorbance.

In vitro cyclooxygenase (COX-2) inhibition assay

Herein, Leuco-2,7-dichlorofluorescein diacetate (5 mg) was hydrolyzed in 1 M sodium hydroxide (50 µL) for ten minutes. 1-DCF and COX-2 were diluted using 0.1 M Tris-buffer, pH 8. Incubation of algal extract or standard (20 µL) with COX-2 was done for five minutes with hematin, with the addition of 1-DCF and arachidonic. Absorbance was measured at 502 nm with consideration blank and IC_{50} calculation (Elaasser et al., 2020).

Anticoagulation assay

According to Mohy El-Din and Alagawany (2019), this assay was done using blood of healthy mice with normal plasma, then 9 units of blood mixed with one unit of Tri sodium citrate buffer (3.8%), centrifugation at 10000 rpm for 10 min, the supernatant fluid was plasma. Active partial thromboplastin time (APTT) was estimated by mixing 50 µl of *H. cuneiformis* ME with 100 µl plasma and standing for a minute, then adding, 100 µl APTT and thrombin reagent to withstand for 5 min. The coagulation will occur by adding 100 µl $CaCl_2$ (0.025 M) and the time was recorded (prothrombin time PT).

Evaluation of cytotoxic effects
Mammalian cell lines

HepG-2, PC-3, and VERO (African green monkey kidney cells) were obtained from the American Type Culture Collection (ATCC, Rockville, MD). Dimethyl sulfoxide (DMSO), Fetal Bovine serum, MTT, and trypan blue dye were purchased from Sigma (St. Louis, Mo., USA). RPMI-1640**,** HEPES buffer solution, L-glutamine, gentamycin, and 0.25% trypsin-EDTA were purchased from Lonza (Belgium).

Cytotoxicity

Cytotoxicity assay was done by suspending $5x10^4$ cell/well of 96-well tissue culture plates (96WTCP) for a day, after that, *H. cuneiformis* extract was added and 0.5 % DMSO was used, via using MTT assay. The viable cells were determined according to Hamza et al. (2022), as the media used in 96WTCP was replaced with 100 µl of fresh ones, without phenol red, then 10 µl of MTT solution were added and then incubated for 4 hrs at 37 °C and 5% carbon dioxide, addition and mixing of 50 µl of DMSO were done and incubated for

10 min. At 590 nm, the optical density (OD) was determined using a microplate reader (Sunrise, TECAN, Inc, USA), and then viability% (V%) was defined as follows:

$$V\% = [(ODt/ODc)]x100\%$$

Where ODt is the mean OD of wells treated with the *H. cuneiformis* extract and ODc is the mean OD of untreated cells. With the determination of IC_{50}, the selectivity Index (SI) was determined using the Indrayanto et al. (2021) formula as follows:

SI = IC_{50} for normal cells/IC_{50} for cancer cells

RESULT AND DISCUSSION

Extraction yield

The crude extract was obtained using methanol; the methanol's polarity index was 5.1. Methanol was chosen as a solvent because of its polar nature. There was a tendency in both brown and red algae to exhibit higher yields from polar solvents compared to non-polar ones with the continuity of brown algae as the superior yielder (Agregán et al., 2018; El-Sheekh et al., 2020). In agreement with this Truong et al. (2019), reported that in *Severinia buxifolia*, the extraction efficiency is achieved by the more polar solvents. El-Sheekh et al. (2020) examined the antioxidant capacity of the extract's bioactive components to understand how the type of solvent influences the extraction yield. The considerable differences in extraction yield found throughout various seaweed species may be attributed to the polarity of different components found in these different species. The H. cuneiformis methanol extract (H. cuneiformis ME) obtained was of yield 10%.

Phytochemical analysis

H. cuneiformis ME total phenolics, flavonoids, and tannins were determined (Table 1)*, H. cuneiformis* contains about 26.93, 6.25, and 13.59 mg/g of phenols, flavonoids, and tannins respectively. The data demonstrated that the algal extract is rich in various phytochemicals (Table 2 and Figure 1). Six different types of phenolic compounds were identified: syringenic, p-coumaric acid, caffeic acid, pyrogallol, gallic acid, and ferulic acid, all of them occur in a considerable concentration except pyrogallol which has low concentration (0.54 µg/gm). A maximum concentration was recorded for gallic acid (22.17 µg/gm). Six types of flavonoids were also detected: naringenin, rutin, quercetin, chlorogenic acid, luteolin, and apegenin. A maximum value was recorded for naringenin (14.56 µg/gm) and the lowest value for chlorogenic acid (5.10 µg/gm). Four types of tannins were identified: eckol, diphlorethohydroxycarmalol, phlorofucofuroeckol, and fucodiphloroethol. All of them belong to phlorotannins and were found in a considerable concentration, which proves that brown algal species possess distinctive secondary metabolites known as polyphenols, which include phenolics, flavonoids, tannins, and other derivatives (Blunt et al., 2009; Hakim and Patel, 2020; Bié et al., 2023). Due to the polyphenols' wide range of biological activities, brown algae can be utilized as main ingredients in pharmaceutical, cosmetic, nutraceutical, and therapeutic products. This allows them to operate as natural alternatives to various industrial formulations without hurting humans. Jimenez-Lopez et al. (2021) revealed that algal phenols, flavonoids, and tannins have various health-boosting properties in humans.

Antioxidant activity (AA)

Figures 2 and 3 showed the potent antioxidant activity of *H. cuneiformis* ME, as it activated DPPH scavenging and FRAP in a concentration-dependent manner, without exceeding ascorbic acid (natural reference standard). The maximum DPPH scavenging activity (%) and FRAP (%) of 81.14 with IC_{50}=175.31 ±10.78 µg/ml and 70.73% with IC_{50}= 197.42 ±11.22 µg/ml, respectively occurred at 1000 µg/ml concentration. The results are in harmony with those of Gunathilaka et al. (2020) who found that ME of brown seaweed *Chnoospora minima* possessed high AA, which increased linearly with increasing extract concentration.

Research on screening natural items for antioxidant activity is expanding quickly. Over the past few years, numerous researchers have attempted to identify more potent oxidation inhibitors that may be employed as antioxidants in food or medication formulations without causing adverse effects. Duh (1998) proved that reducing properties are mainly due to reductants being present, which react with peroxide precursors and stop the peroxide production (Makhlof et al., 2024). *H. cuneiformis* extract's reducing power is primarily due to the presence of different types of phenolics, flavonoids, and tannin compounds (Tables 1 and 2). This is substantiated by several earlier studies that proved the potent antioxidant capabilities of p-coumaric acid, caffeic acid, gallic acid, ferulic acid (Skroza et al., 2022), naringenin (Stabrauskiene et al., 2022), rutin (Choi et al., 2021), chlorogenic acid (Chiang et al., 2015), quercetin, luteolin, apigenin (Tian et al., 2021),

Table 1. Total phenolics, flavonoids and tannins content of *H. cuneiformis* methanolic extract.

Compounds	
Total Phenolics (mg/gm)	26.93 ± 1.09
Total Flavonoids (mg/gm)	6.25 ± 0.46
Total Tannins (mg/gm)	13.59 ± 0.61

Table 2. HPLC fractions of phenolics, flavonoids and tannins content of *H. cuneiformis* methanolic extract

	Compounds	Retention time (Min)	Concentration µg/gm
Phenolics	Syringenic acid	4.9	11.24
	p-Coumaric acid	6.0	5.36
	Caffeic acid	8.0	7.40
	Pyrogallol	9.0	0.54
	Gallic acid	9.8	22.17
	Ferulic acid	10.8	3.14
Flavonoids	Naringenin	4.4	14.56
	Rutin	5.4	7.41
	Quercetin	7.0	6.23
	Chlorogenic acid	8.0	5.10
	Luteolin	9.0	10.69
	Apigenin	10.0	9.74
Tannins	Eckol	4.9	8.95
	Diphlorethohydroxycarmalol	7.0	14.4
	Phlorofucofuroeckol	9.0	12.32
	Fucodiphloroethol	12.8	10.78

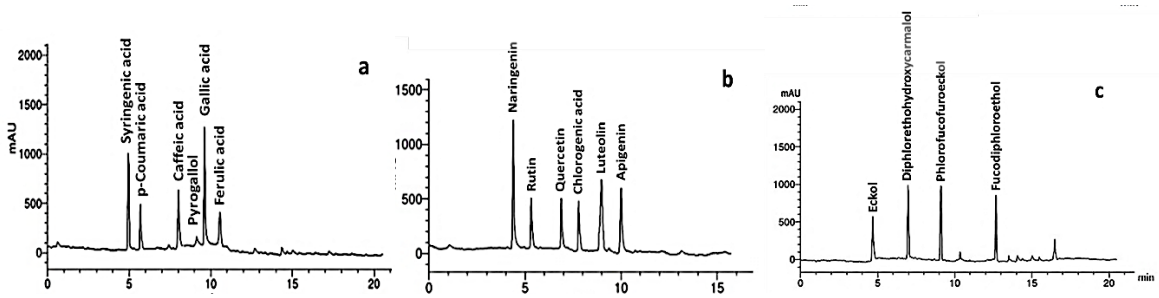

Figure 1. HPLC chromatogram of *H. cuneiformis* phenolics (a), flavonoids (b) and tannins (c).

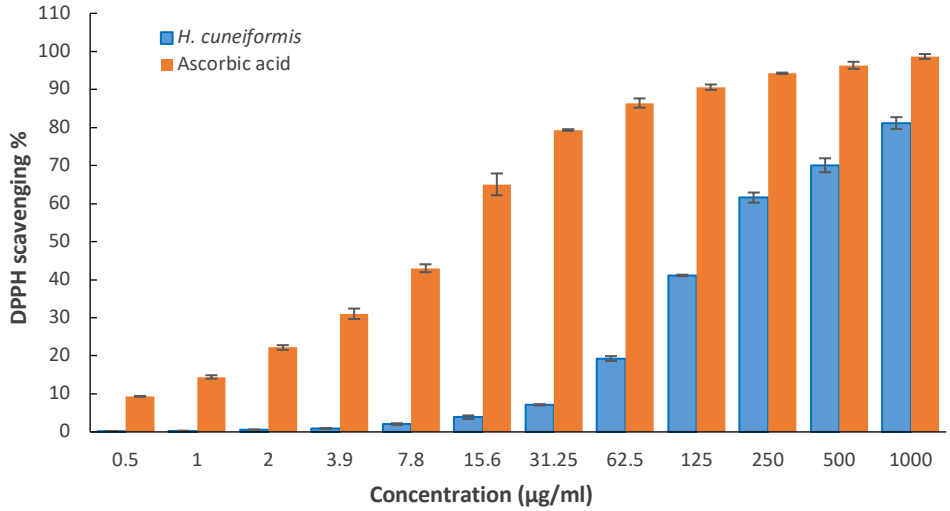

Figure 2. DPPH scavenging activity (%) of different concentrations of *H. cuneiformis* methanolic extract and ascorbic acid (natural antioxidant standard).

eckol and diphlorethohydroxycarmalol (Senevirathne and Kim 2013). All the above-mentioned compounds were detected in the studied seaweed extract (Table 2).

Anti-inflammatory test

The anti-inflammatory activity of *H. cuneiformis* was evaluated by studying the inhibitory effect of the algal methanolic extract on cyclooxygenase-2 (COX-2), a proinflammatory enzyme, using celecoxib as a standard reference. Figure 4 showed COX-2 inhibition by *H. cuneiformis* extract in concentration dependent manner and attained a maximum inhibition (%) of 74.92% at 1000 µg/ml concentration with IC_{50} = 172.81 ±4.27 µg/ml but did not exceed celecoxib which fulfilled 92.43% inhibition at 1000 µg/ml concentration with IC_{50} = 13.12 ±0.54 µg/ml. Han et al. (2015) demonstrated the potential of brown alga *Hizikia fusiformis* as a therapeutic drug for inflammation and oxidative stress-related diseases which agrees with our findings.

There are a lot of negative side effects associated with current available anti-inflammatory medications, such as bleeding, perforation, and gastrointestinal ulceration. COX-2 selective inhibitors have been shown to provide anti-inflammatory effects attributes with significantly lower gastrointestinal toxicity when compared to conventional non-steroidal anti-inflammatory drugs (NSAIDs) (Besednova et al., 2022). Finding natural inhibitors that specifically block COX-2 while causing no adverse effects is essential to creating innovative anti-inflammatory medications.

Several investigations have found that phenolics and flavonoids are the primary contributors to anti-inflammatory activity (Pradhan et al., 2022). Zielińska et al. (2021) and Bai et al. (2021) revealed that caffeic acid and gallic acid are distinctive with anti-inflammatory action by inhibiting COX-2, prostaglandin E2, and chemokine production, as ferulic acid and naringenin are widely utilized in cosmetics and is even used in clinics to treat numerous diseases (Shukla et al., 2019; Shi et al., 2021). Muvhulawa et al. (2022) demonstrated, rutin's strong antioxidant qualities allow it to reduce inflammation efficiently. Quercetin has been identified as a long-lasting potent anti-inflammatory agent (Li et al., 2016). Luteolin has powerful anti-inflammatory properties both in vitro and in vivo and is frequently present in medicinal plants (Aziz et al., 2018).

Anticoagulation activity

The anticoagulation activity of *H. cuneiformis* ME was evaluated using heparin as a standard reference. Table 3 shows that *H. cuneiformis* ME has anticoagulation properties where it significantly prolonged APTT and PT proportionately by increasing its concentration, but its effectiveness does not exceed heparin (reference). APTT and PT of *H. cuneiformis* ME increased by 2.48 and 2.43 folds more than the control, respectively (65 and 54.8 sec, respectively) at concentration 0.8 mg/ml.

Blood coagulation evolved from hemostasis failure, which gives rise to vascular occlusion and, on occasion, death. Certain higher plants and algae impede blood coagulation, which can be deemed safe and innocent in comparison to other synthetic substances (Mohy El-Din and Alagawany, 2019). Phenolics and flavonoids are powerful antioxidants and possess other therapeutic properties including anti-inflammatory, antiplatelet, and anticoagulant. Because of the above qualities, plant extracts rich in polyphenols and flavonoids could effectively prevent and treat thromboembolic issues (Lamponi, 2021). Furthermore, algal phlorotannins have been demonstrated to have strong anticoagulant properties. Therefore, the anticoagulation property of *H. cuneiformis* ME was predicted due to the existence of various compounds of phenolic, flavonoids, and tannins. Based on earlier research, chlorogenic acid inhibited protease enzymatic activity and broke down blood clots, delaying the prothrombin, thrombin and APTT (Choi and Kim, 2016). Ferulic acid exhibited anticoagulant effects (Choi et al., 2017). Eckol demonstrated anticoagulant action *in vivo* (Kim et al., 2012). The suppression of coagulation factors demonstrated that the algal extract contains numerous compounds capable of inhibiting the clotting process, which can be utilized in blood clots and stroke treatment. Thus, the algal extract can be presented as a novel therapeutic option for blood coagulation ailments.

Cytotoxicity and selectivity index of *H. cuneiformis* methanolic extract

Cancer is a dysregulated cell proliferation that can invade, metastis, and spread to distant regions. According to the growing body of knowledge about cancer biology, new biological therapies targeting diverse aspects of the tumour have been made possible (Zugazagoitia et al., 2018).

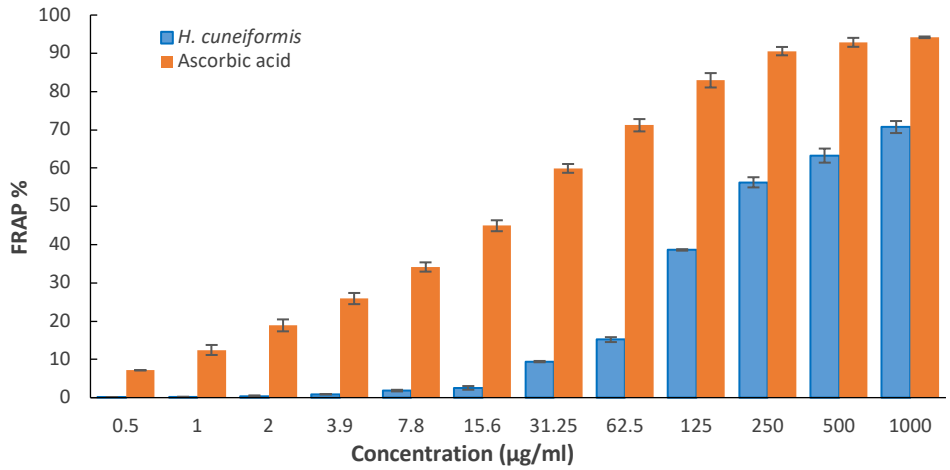

Figure 3. Ferric reducing antioxidant power (FRAP) (%) of different concentrations of *H. cuneiformis* methanolic extract and ascorbic acid (natural antioxidant standard).

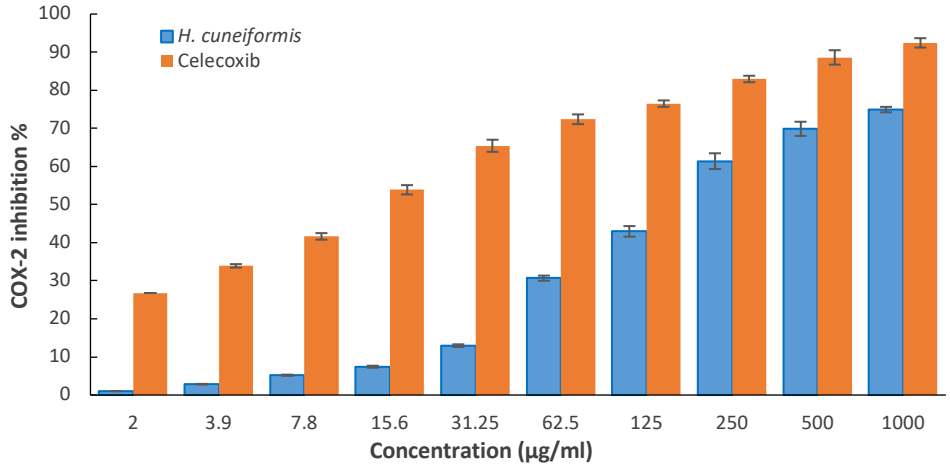

Figure 4. COX-2 inhibition (%) of different concentrations of *H. cuneiformis* methanolic extract and celecoxib (reference standard).

Table 3. Activated partial throboplastin time (APTT) and prothrombine time (PT) of *H. cuneiformis* methanolic extract.

Concentration (mg/ml)	*H. cuneiformis*		Heparin	
	APPT (sec)	PT (sec)	APPT (sec)	PT (sec)
Control	44.0 ± 0. 2	38.4 ± 0.4	43.2 ± 0.4	36.0 ± 0.15
0.05	52.2 ± 0.4	47.8± 0.35	57.0 ± 0.25	53.3 ± 0.3
0.1	67.0 ± 0.6	53.0 ± 0.2	104.6 ± 0.53	88.4 ± 0.6
0.2	79.7 ± 0.3	60.4 ± 0.35	125.4 ± 0.17	104.0 ± 0.92
0.4	88 ± 0.25	72.4 ± 0.72	188.2 ± 0.26	159.0 ± 0.42
0.8	109.0 ± 0.25	93.2 ± 0.53	238.0 ± 0.9	204.4 ± 0.6

Evaluating a sample's anticancer effectiveness on cancer cell lines without determining the selectivity index is a weak indicator for future (clinical) research. Many medicinal plants and isolated biochemical substances are bioactive in countless scientific publications; sadly, most of these reports have not included the SI data. Several researchers stated that the presented publication had very little relevance without including the SI of the reported bioactive sample (Indrayanto et al., 2021). For this reason, *H. cuneiformis* ME was evaluated for cytotoxicity with the determination of its selectivity index. Table 4 illustrates that *H. cuneiformis* ME had an anticancer effect against used cell lines at all its concentrations except concentrations of 1-3.9 µg/ml for PC-3 and 1, 2 µg/ml for HepG-2 where there was no effect, with IC_{50} of 53.31 and 40.80µg/ml in PC-3 and HepG-2 cells, respectively.

Table 4. Cytotoxic activity of *H. cuneiformis* methanolic extract against PC-3, HepG-2 and VERO cell lines.

Algal extract concentration (µg/ml)	PC-3 cell line IC_{50} = 53.31 ± 5.43 µg/ml.		HepG-2 cell line IC50 = 40.80± 5.15 µg/ml		VERO cell line IC50 = 485.14±11.34 µg/ml	
	Viability %	Inhibitory %	Viability %	Inhibitory %	Viability %	Inhibitory %
0	100	0	100	0	100	0
1	100	0	100	0	100	0
2	100	0	100	0	100	0
3.9	100	0	98.73	1.27 ± 0.35	100	0
7.8	98.84	1.16 ± 0.62	94.02	5.98 ± 0.46	100	0
15.6	90.63	9.37 ± 1.05	79.54	20.46 ± 1.22	99.72	0.28 ± 0.14
31.25	67.31	32.69 ± 2.78	54.19	45.81 ± 2.59	98.22	1.78± 2.12
62.5	39.85	60.15 ± 2.81	31.78	68.22 ± 1.84	94.35	5.65 ± 0.63
125	26.86	73.14 ± 1.41	14.31	85.69 ± 1.27	81.47	18.53 ± 3.19
250	17.56	82.44 ± 1.42	9.33	90.67± 0.70	68.57	31.43 ± 2.37
500	7.81	92.19 ± 0.67	5.94	94.06 ± 0.38	48.02	51.98 ± 4.26

PC-3 and HepG-2 cells were affected by *H. cuneiformis* ME in a concentration-dependent manner until it reached 7.81% and 5.94% at 500 µg/mL *H. cuneiformis* ME, and this agrees with Makhlof et al. (2024). Thus, *H. cuneiformis* methanolic extract was more effective against liver cancer than prostate cancer. Also, all *H. cuneiformis* ME concentrations exhibited cytotoxicity in VERO cells except 1–7.8 µg/ml, with IC_{50} of 485.14 µg/ml. The selectivity index of *H. cuneiformis* ME, which is the ratio of its IC_{50} in normal cells (VERO) and carcinoma cells (PC-3, HepG-2 cells), was 9.10 and 11.89 for PC-3 and HepG-2 cell lines, respectively. A SI value greater than or equal 10 was proposed to be considered (Indrayanto et al., 2021). The results demonstrate that *H. cuneiformis* ME is selectively cytotoxic to cancer cells and can be regarded as a potential prospective anticancer that can be further investigated.

There is mounting evidence that bioactive compounds from algae have cytotoxic effects through various methods, including inducing apoptosis in cancerous cells and suppressing cancer progression at all stages (Makhlof et al., 2024). Furthermore, the alga's high antioxidant activity enhances its anticancer potential (El Sheekh et al., 2022). Polyphenols have been involved in various anticancer mechanisms (Niedzwiecki et al., 2016). Caffeic acid, pyrogallol, naringenin, rutin, luteolin, chlorogenic, eckol, phlorofucofuroeckol, diphlorethohydroxycarmalol, and fucodiphloroethol which are found in the extract, have been connected to the prevention and suppression of the progression of several types of cancer by diverse mechanisms (Afshari et al., 2022, Gupta, et al., 2022; Matulja et al., 2022; Arjsri et al., 2023; de Luna et al., 2023).

The study's findings show that the methanolic extract *of Hormophysa cuneiformis* has some enjoyable biological activity in vitro. Still, a few constraints must be resolved before it can be used in clinical trials. One problem is that the results are based on vitro studies, which cannot explain human pharmacology, bioavailability, and systemic effects. Improving the extract's safety and effectiveness in living organisms requires in vivo trials. Second, results may not be reproducible or consistent because environmental and seasonal factors were not considered when determining the extract's composition. In the future, pharmaceutical and commercial uses will require the standardization of extraction techniques and the identification of bioactive markers. Ultimately, the study only tested two cancer cell lines, PC-3 and HepG-2; further testing with other cell lines, including cancer cells, would give a more complete picture of its anticancer potential. Finally, the chemical mechanisms that cause these biological effects have not been studied. These mechanisms should be further understood in future research by using cutting-edge methods like molecular docking or omics approaches. Investigations into the extract's cost-effectiveness, scalability, and possible synergistic effects with current treatments may also lead to its use in clinical and industrial settings. Following these procedures will guarantee that *H. cuneiformis* and its potential as an all-natural medicinal agent are thoroughly evaluated.

CONCLUSION

Hormophysa cuneiformis was found to contain various compounds of phenolics, flavonoids, and tannins which possess a broad spectrum of biological functions, that are responsible for its tested promising in vitro biological activities, therefore, *H.*

cuneiformis may be used in the pharmacological domain for the prevention and therapy of chronic diseases, especially human cancers in near future. However, further in vivo studies in animals and clinical studies are required to ensure their safety and efficacy before its approval for treatment of diseases in human beings. Further investigation is also required for isolation and identification of novel bioactive metabolites with enhanced pharmacological activities that can replace the current generation drug candidates.

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