ORIGINAL ARTICLE

Phytochemical Exploration of *Hypnea musciformis* and its Zinc Oxide Nanoparticle Conjugates: Antioxidant and Anticancer Activities

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Abstract

Background: Seaweed-derived phytochemicals have gained attention for their chemotherapeutic potential. *Hypnea musciformis* (*H. musciformis*), a red seaweed, is a rich dietary source of bioactive compounds with reported anticancer activity. Objective: To synthesize zinc oxide nanoparticles (ZnONPs) using *H. musciformis* extract and evaluate their antioxidant and anticancer potential. **Methods:** Phytochemical profiling of the extract was performed using gas chromatography–mass spectrometry (GC–MS). ZnONPs were synthesized via a green synthesis approach and characterized. Antioxidant activity was assessed using DPPH, hydrogen peroxide scavenging, ferric reducing antioxidant power (FRAP), ABTS, and nitric oxide inhibition assays. Anticancer activity was evaluated against MG63 human osteosarcoma cells. **Results**: GC–MS analysis identified major phytochemicals, including 3-nitro-2-methylpropene (49.5%) and diethyl methyl (18.1%). The synthesized ZnONPs exhibited significant antioxidant activity across all assays and demonstrated notable cytotoxic effects against MG63 cells. **Conclusion:** *H. musciformis*-mediated ZnONPs show enhanced antioxidant and anticancer properties, suggesting their potential as a promising candidate for therapeutic applications.

Key words: Anticancer activity, antioxidant activity, *Hypnea musciformis,* phytochemicals, zinc oxide nanoparticles

INTRODUCTION

Marine ecosystem harbors diverse variety of marine plants and animal life and hold the highest biodiversity as compared to terrestrial ecosystem.[1] It is home to seaweeds that are rich in phytochemicals with potent anti-inflammatory, anti-tumor, and analgesic compounds.[2] These compounds have contributed to the growing seaweed market for human use.[3] Seaweeds are being used as food, fertilizers, and soil conditioners,

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and in cosmetics, medicine, and for the production of hydrocolloids.[4] *Hypnea musciformis*, an emerging red seaweed belonging to carragenophyta is recently being explored for its potential sulfated polysaccharide – carrageenan, due to its gelling property.[5] Methanolic and ethanolic extracts of *H. musciformis* have been reported to prevent diseases induced by oxidative stress by exhibiting an effect on enzymes, thereby effectively scavenging reactive oxygen species.[6]

Chemotherapy is a widely used conventional treatment for cancer. Its major mechanism of action involves killing actively dividing cells.[7] Although the treatment is effective, it exhibits side effects by destroying other normal cells that divide continuously in the human body, leading to hair loss, and gastrointestinal reactions.[8] Although, targeted therapy has gained scope, resistance to drugs and relapse pose new problems.[9]

This has led to the implementation of nanoparticles (NPs) to overcome problems related to conventional therapy.[10] Use of toxic chemicals in NP synthesis has side effects, and its toxicity is widely debated.[11] The past decade has seen a shift in the NP synthetic process to green chemistry, where plant extracts are used. This has minimal effects and an environmentally sustainable process.[12]

In this study, we analyzed the phytochemical constituents of *H. musciformis* and demonstrated green synthesis of zinc oxide NPs (ZnONPs) using *H. musciformis* extract. Alongside, we used on MG-63 human osteosarcoma cells to assess the biological activities of the extract and its NP conjugates.

MATERIALS AND METHODS

Seaweed collection, processing, and phytochemical analysis

H. musciformis samples were gathered from the Mandapam coast, Tamil Nadu, India. Samples were thoroughly washed in tap water and distilled water (d.H_2O) to eliminate debris. Samples were shade-dried for 36 h and ground into fine powder. The powdered sample was stored at 4°C before further analysis. Methanolic extract of *H. musciformis* was prepared by soaking 10 g of *H. musciformis* powder in 200 mL of methanol and placed on a shaker incubator at 400 rpm, 72 h and the filtrate was concentrated in a rotary evaporator. This crude extract was subject to phytochemical screening. The chemical profile of the methanolic extract was also assessed using gas chromatography mass spectrometry (GC-MS) analysis.[13]

Synthesis and characterization of ZnO NPs

ZnONPs were synthesized using *H. musciformis* extract as a reducing and stabilizing agent. Briefly, 5 g of *H. musciformis* crude extract was dissolved in 40 mL of d.H_2O by heating for 30 min at 80°C. 25 mM zinc nitrate solution was prepared in d.H_2O, and added to the boiling solution and stirred for 3 h. The NP formation can be seen as white precipitate settling at the bottom. The supernatant was discarded, washed thrice with d.H_2O by centrifuging at 5,000 rpm for 10 min.[14]

The synthesized ZnONPs were characterized using a ultraviolet (UV)-visible spectrophotometer to determine their absorption spectrum, and X-ray diffraction (XRD) analysis to investigate phase and crystalline structure. The functional groups were identified by Fourier transform infrared spectroscopy (FTIR) (Spectrum Two, Perkin Elmer) with 64 scans per sample in the 4,000–400 cm^{-1} wavelength spectrum and resolution of 4/cm.[15]

Evaluation of antioxidant activity

2, 2-diphenyl-1-picrylhydrazyl (DPPH) antioxidant assay

Scavenging of free radicals by ZnONPs was determined by the Abdelbaky method with simple modifications.[16] 2 mL of freshly prepared DPPH solution (100 mM in methanol) was added to 100 µL of different concentrations of ZnONPs (10–50 µg mL^{-1}) and mixed well. The reaction mixture was incubated in the dark for 30 min at room temperature. Absorbance was recorded at 517 nm. Each measurement was taken in 3 repetitions. Free radical scavenging activity is represented as percent inhibition of free radicals and calculated by equation (1). IC_{50} values are determined from a relationship from % Inhibition versus concentration of the sample curve.

$$\text{Inhibition (\%)} = \{(A_{control} - A_{sample})/A_{control}\}*100 \quad (1)$$

Where A – absorbance.

Hydrogen peroxide (H_2O_2) scavenging assay

H_2O_2 scavenging activity of ZnONPs was estimated by following the method of Chandramohan *et al*., with simple modifications.[17] 600 µL of 43 mM H_2O_2 was added to 500 µL of ZnONPs at concentrations ranging from 10 to 50 µg/mL. Then phosphate buffer (0.1M) was aliquoted to the reaction mixture for a 4.5 mL make-up and 10 min of dark incubation at room temperature before recording absorbance at 230 nm, and percent scavenging was calculated by equation (1).

Ferric reducing antioxidant potential assay

Ferric reducing antioxidant power (FRAP) reagent was made in a 1:1:10 (v/v/v) ratio of 10 mM 2, 4, 6 – Tris (2-pyridyl)-s-triazine solution in 40 mM HCl, 20 mM $FeCl_3 \cdot 6H_2O$ solution, and 0.3 M acetate buffer (pH 3.5). 500 µL of acetate solution of ZnONPs at different concentrations (10–50 µg mL^{-1}) was aliquoted into 2 mL of FRAP reagent, vortexing followed by 30 min darkincubation. Absorbance was measured at 593 nm. FRAP and acetate buffer served as a control.[18]

2, 2-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) assay

A 1:1 (v/v) ratio of 7 mM ABTS and 2.45 mM potassium persulfate was prepared and pre-incubated overnight in the dark before use. Methanol was used to dilute the stock solution. 500 µL of aqueous ZnONPs at several concentrations (10–50 µg mL^{-1}) were added to 2 mL of ABTS solution, followed by room temperature incubation in the dark for 15 min, and absorbance was recorded at 734 nm. Solution of ABTS in d.H_2O serves as control.[19]

NO inhibition assay

10 mM sodium nitroprusside solution in phosphate-buffered saline (pH 7.4) is prepared, and 2 mL is added to 500 µL of different concentrations of ZnONPs (10–50 µg/mL) and incubated at ambient temperature for 150 min. Thereafter, 500 µL of the incubated solution was combined with 1 mL of sulfanilic acid (33% sulfanilic acid in 2% acetic acid) and incubated for 5 min. 1 mL of 0.1% naphthyl ethylenediamine dihydrochloride was added to the mixture and re-incubated for 30 min. Absorbance was recorded at 540 nm. Scavenging activity was calculated by equation 1.[20]

Anticancer activity

The biological activities of the synthesized *H. musciformis* ZnONPs were assessed through cytotoxicity. Cytotoxicity was evaluated using the MTT assay on human cancer cells. Briefly, MG63 human osteosarcoma cells were initially treated with ZnONP concentrations 2.5–60 µg/mL and allowed to grow until 80% confluency was observed, then 200 µL of MTT (3 mg/mL) was aliquoted, incubated for about 4 h. Then, the MTT dissolving solution (dimethyl sulfoxide) was combined and incubated on a shaker for 15 min, followed by optical density measurement at 570 nm. IC_{50} values were determined.[21]

Statistical analysis

Each experiment was replicated thrice, and the results were represented as the mean ± standard deviation. Statistical analysis was performed by analysis of variance and *post hoc* test. A significant value, $P < 0.05$, was considered to evaluate differences.

RESULTS

Phytochemical composition and GC-MS analysis of *H. musciformis* extract

The qualitative phytochemical analysis of *H. musciformis* methanolic extract revealed a diverse profile of bioactive compounds [Figure 1]. Alkaloids were detected through the formation of a reddish-brown precipitate in the Mayer's test, phenols by bulky white precipitate in the lead acetate test, cardiac glycosides through brown ring formation in the Keller–Kiliani test, steroids through a positive Lieberman–Burchard reaction, and terpenoids by reddish brown coloration at the interface in the Salkowski test. A high-performance liquid chromatography analysis study by Paul and Raja[22] on *Hypnea* species collected from the Manapad region of Tamil Nadu, India, revealed the separation of 6 compounds at different retention times, and FTIR peaks of these compounds correspond to amides, phosphorous, and halogen compounds, alcohols, and phenols. These results are consistent with our results, suggesting a similar phytochemical composition.

GC-MS analysis of the methanolic extract of *H. musciformis* revealed several volatile compounds, as listed in Table 1. The major components are 3-nitro-2-methyl propene (49.5%), borane, diethyl methyl (18.1%), and ethenamine, n-methylene (3.98%). A similar study on *H. musciformis* collected from the Gulf of Mannar reported more diverse phytochemical composition with triterpenoids, carotenoids, steroids, glycosides, flavonoids, and prostaglandins, such as PGF2 and PGF2α.[23] The difference in chemical profile in our extract can be attributed to change in geographical location

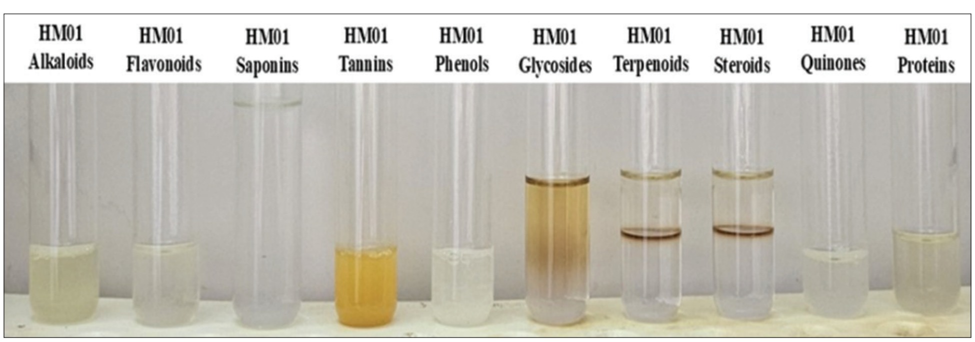

Figure 1: Phytochemical screening of *Hypnea musciformis* methanolic extract. Test tubes show results of specific assays for alkaloids, flavonoids, saponins, tannins, phenols, glycosides, terpenoids, steroids, quinones, and proteins. Characteristic colour changes or precipitates indicate the presence of each phytochemical

and climatic conditions.[24] Nevertheless, *H. musciformis* from both locations has higher bioactive compositions that are transferable to therapeutic formulations.

Synthesis and characterization of ZnONPs

The successful green synthesis of ZnONPs using *H. musciformis* extract was confirmed by UV-Vis spectroscopy [Figure 2a]. UV-visible spectroscopy revealed a characteristic absorption peak at 345 nm, attributed to the surface plasmon resonance of ZnONPs. Kokabi *et al*. (2017) synthesized ZnONPs from aqueous extracts of *H. musciformis* and found absorption maxima at 375 nm, and the particle size was determined to be 16.5 nm. The low absorption of ZnONPs indicates a smaller size than previously reported for algae-mediated ZnONPs.

FTIR spectroscopy [Figure 2b] identified key functional groups involved in the biosynthesis and stabilization of ZnONPs, with characteristic peaks at 3340.81 cm^{-1} (O-H group), 2343.66 cm^{-1} (C-H stretch), 1616.36 cm^{-1} (C=N group), and in the region between 1,000 and 1,300 cm^{-1} (C-O group). These spectral features indicate the involvement of hydroxyl-containing compounds, proteins, and polysaccharides from the *H. musciformis* extract in NP formation. Similar functional group involvement was documented by Abotaleb[25] in their study of seaweed-mediated NP synthesis, though our results show stronger involvement of C=N-containing compounds, likely originating from the protein-rich composition of *H. musciformis*.

XRD analysis of the ZnONPs synthesized using *H. musciformis* extract confirmed the crystalline nature. Sharp diffraction peaks were observed at 2θ values of 19.76°, 25.72°, 29.11°, and 34.24°, corresponding to the (220), (311), (400), and (440) planes, respectively [Figure 2c]. The crystallinity index of the ZnONPs was calculated to be 32.6%, indicating a significant amorphous content. This ratio differs from that reported by the Fouda group,[26] who observed a higher 65% crystallinity in ZnONPs, synthesized using *Ulva fasciata.* This suggests that the algal phytochemical composition influences the crystallization process of the synthesized NPs.

Table 1: Compounds identified in the methanolic extract of *Hypnea musciformis* by GC-MS

Peak No.	Compound name	Molecular formula	Percentage peak area
1	3-Nitro-2-methylpropene	$C_4H_7NO_2$	49.5
2	Diethylmethylborane	$C_5H_{13}B$	18.1
3	N-methyleneethenamine	C_3H_5N	3.98
4	Propargyl alcohol	C_3H_4O	3.98
5	But-3-enyl ethyl carbonate	$C_7H_{12}O_3$	3.67
6	2-methylbutanenitrile	C_5H_9N	3.24
7	Allyl acrylate	$C_6H_8O_2$	2.09

GC-MS: Gas chromatography mass spectrometry

Antioxidant activity

H. musciformis conjugated ZnONPs demonstrated remarkable scavenging of DPPH radical, with 88.31% inhibition at 50 µg/mL, comparable to the standard ascorbic acid [Figure 3a]. Furthermore, ZnONPs demonstrated significant scavenging activity in a dose-dependent manner, highest at 50 µg/mL, against H_2O_2, FRAP, ABTS, and nitric oxide (NO), with inhibition percent of 86.9%, 88.63%, 88.45% and 91.38%, respectively [Figure 3b-e]. A study on red seaweeds *Ulva lactuca* and *Sargassum muticum* showed a DPPH scavenging of 86.47% and 84.30%, respectively. This efficacy is comparable to our results. Another study on ZnONPs synthesized by Sea Lavender *Limonium pruinosum* extract reported 75.2% DPPH scavenging at a very concentration of 1,000 µg/mL.[27] Uvarajan synthesized ZnONPs from *Sargassum polycystum,* a brown algae, and reported that H_2O_2 was dose dependent and scavenged by 61.59% and NO by 66.69% at the highest concentration of 250 µg/mL.[28] The high antioxidant activity of *H. musciformis* conjugated ZnONPs is due to the presence of secondary metabolites, as confirmed by GC-MS and FTIR. The terpenoids and phenolics in the *Hypnea* extract are potent antioxidants. These phytochemicals act as capping agents, rendering unique surface properties and possibly related to their antioxidant activity.

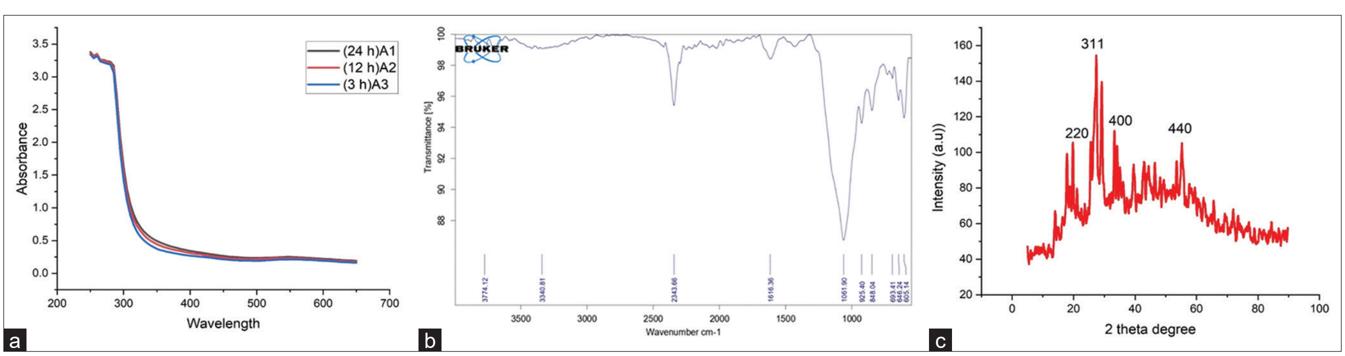

Figure 2: (a) Ultraviolet-Vis absorption spectrum, (b) Fourier transform infrared spectroscopy spectrum, and (c) X-ray diffraction pattern spectrum of Zinc oxide nanoparticles synthesized using *Hypnea musciformis* extract

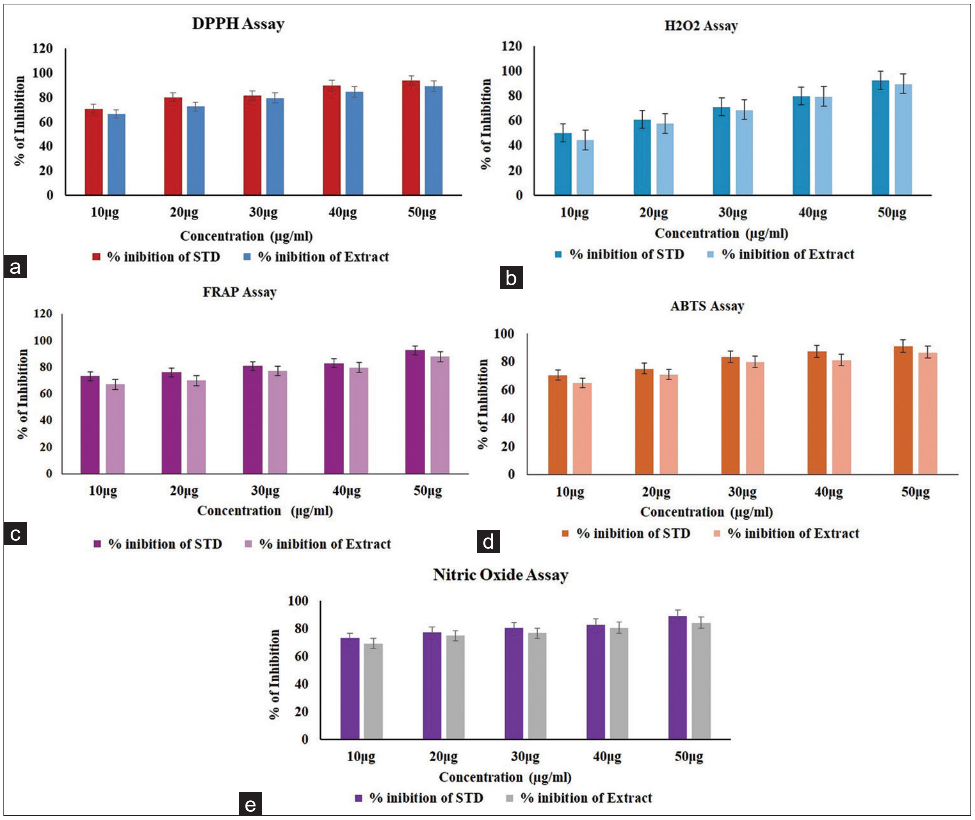

Figure 3: Antioxidant potential of *Hypnea musciformis* Zinc oxide nanoparticles on (a) 2, 2-diphenyl-1-picrylhydrazyl, (b) H_2O_2, (c) ferric reducing antioxidant power, (d) 2, 2-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid), and (e) nitric oxide

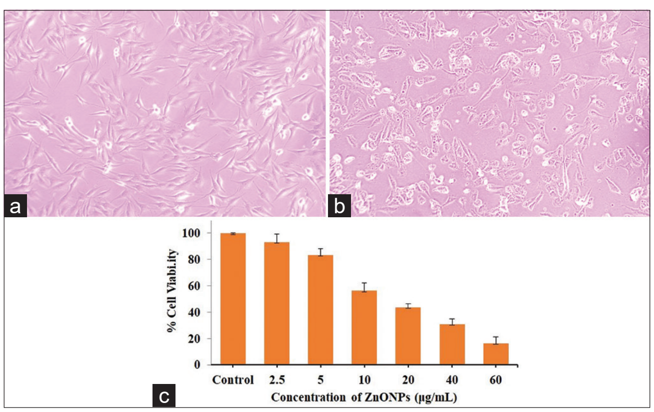

Figure 4: Antiproliferative activity of *Hypnea musciformis*- mediated Zinc oxide nanoparticles (ZnONPs) against MG63 human osteosarcoma cells. (a) Untreated control cells exhibiting typical spindle-shaped morphology and intact cell membranes. (b) Treated cells showing marked morphological alterations, such as cell shrinkage, rounding, and membrane blebbing, indicative of apoptosis. (c) Dose-dependent cytotoxicity of ZnONPs assessed by MTT assay

Anticancer potential

The cytotoxic effect of *H. musciformis* ZnONPs was evaluated on MG63 human osteosarcoma cells. After 24 h of treatment, the MTT assay showed a dose-dependent cytotoxicity with 17% cell viability at 60 µg/mL ZnONPs concentration, and IC_{50} was calculated to be 15.32 ± 1.36 µg/mL (Figure 4). Alongside, significant morphological changes were noted, including membrane blebbing and cell shrinkage. A study on *S. muticum* ZnONPs on different murine cancer cell lines showed potent anticancer activity after 72 h of treatment, and IC_{50} was determined to be 21.7, 17.45, 11.75, and 5.6 µg/mL for 4T1, CRL-1451, CT-26, and WEH1-3 cells, respectively.[29] Another study involving *S. muticum* ZnONPs on human liver cancer cells HepG2 reported that the cell viability reduced to 44% after 48 h of treatment with 175 µg/mL and decreased further in a dose-dependent manner, reaching a maximum of 95.5% at 2800 µg/mL.[30] These reports are consistent with our results, and it is important to note that we have achieved greater cytotoxicity against MG63 cells at a lower dosage.

CONCLUSION

This comprehensive study on *H. musciformis* and its ZnONPs conjugates reveals the phytochemical constituents. The terpenoids and phenols in the *H. musciformis* have increased the anti-oxidant and anti-proliferative potential of ZnONPs. These findings highlight the importance of the application of seaweeds in therapeutic development. To comprehend the underlying mechanism of action and optimize for *in vitro* and *in vivo* applications, more research is required.

AUTHOR CONTRIBUTION

Concept and design: Remya Rajan Renuka, Data Acquisition: Kolli Eswar Teja, Data analysis: Veena Markandan, Drafting Manuscript: Naven Kumar Rajagopal Kumaran, Syed Shahid Afridi, Supervision: Mukesh Kumar Dharmalingam Jothinathan.

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HUMAN AND ANIMAL RIGHTS DECLARATION

All authors hereby declare that there are no ethical concerns related to human or animal rights in this study.

DATA AVAILABILITY

The data sets used and analyzed during the present study are available from the corresponding author upon reasonable request.

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