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Exploring the therapeutic properties of a resilient marine gastropod *Planaxis sulcatus* (Born, 1778) of Karwar coast, India

Santosh Kumar M[1*], Shivakumar B Haragi[2]

Department of Studies in Marine Biology, KUPG Centre, Karwar, Karnataka, India

[1*]Research scholar, Department of Studies in Marine Biology, KUPG Centre, Karwar, Karnataka, India
[2]Associate Professor, Department of Studies in Marine Biology, KUPG Centre, Karwar, Karnataka, India

Corresponding Author
Address: Santosh Kumar M
Research scholar
Department of Studies in Marine Biology, KUPG Centre, Karwar, Karnataka, India
Tel: +91 9591655131
Corresponding Author:santoshstn22@gmail.com

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Abstract

The Marine environment is a pivotal resource for bioprospecting therapeutically active natural compounds. Molluscs are known to harbour compounds with antimicrobial, antitumor and antioxidant activities. The aim of current investigation was to evaluate the antimicrobial, anticancer, and antioxidant activities of the crude whole body methanol extract of *Planaxis sulcatus*. FTIR was employed to identify the types of chemical bonds and functional groups present in the extract. Antimicrobial activity was assessed using Agar well diffusion assay, and determination of MIC, MBC and MFC of the extract by micro broth dilution approach. Cytotoxic effect of the tissue extract was assessed using MTT assay. Antioxidant attributes was evaluated by DPPH, Ferrous-Ion Chelating, and Nitric oxide scavenging assays. Outcomes of the FTIR analysis of the tissue extract has indicating the occurrence of diverse functional groups in the analyte. The gastropod extract demonstrated noteworthy antibacterial activity and antifungal activity. The minimum inhibitory concentration and minimum bactericidal and fungicidal concentrations was ranging from 0.03125- 0.156 mg/mL against tested bacteria and for *Candida albicans* it was 1 mg/mL.The IC_{50} value obtained for cytotoxicity of the tissue extract by MTT assay was showed 53.99 µg/mL. Antioxidant assays demonstrated IC_{50} value of 565.80, 770.53 and, 758.7 µg/mL for DPPH assay, Ferrous-Ion Chelating assay, and Nitric oxide scavenging assay

The outcome of the present study confirmed the presence of bioactive compounds. Decisively, the bioactive compounds extracted from *P. sulcatus* have significant potential to be employed as therapeutic agents.

Keywords: *Planaxis sulcatus*, FTIR, Anticancer activity, antioxidant activity, antimicrobial activity

1. Introduction

Molluscs represents second largest phylum in the animal kingdom comprising approximately 7% of living organisms. Presently, there are approximately 52,000 recognized species of marine molluscs, with an estimated diversity spanning between 100,000 to 200,000 species (Pechenik, 2000; Bouchet and Duarte, 2006). Amongst eight distinctive classes of Mollusca, class gastropods represent 90% of molluscan habitat with an estimated 75,000 - 150,000 species (Benkendorff, 2000). Gastropods are extremely diversified soft animals covered with single-coiled calcareous shells varying in size, shape, and colour with a feeding habit of algal, detritivores and deposit feeders. Despite the presence of shell animals must regularly extend themselves beyond the shell for feeding and locomotion, thus making them rendering them susceptible to predators and pathogens (Benkendorff, 2000). Mollusca like all vertebrates does not have an acquired immunological memory (Hooper *et al.,* 2007). This implying that a range of diverse antiparasitic, antibacterial, antifungal, and antiviral secondary metabolites must have evolved among the molluscs under distinct environmental and biological pressure.

Marine bioactive compounds play a crucial role in preventing and managing a wide array of chronic conditions, including rheumatoid arthritis, inflammatory diseases, asthma, inflammatory bowel disease, psoriasis, osteoporosis, obesity, and diabetes mellitus (Yashodhara *et al.,* 2008). They are also beneficial in treating neurological and cardiovascular disorders (Mozaffarian and Wu, 2011), various types of cancer (such as breast, colorectal, and prostate), and other inflammatory diseases. The Bioactive compounds found in molluscans extract exhibits unique pharmacological properties (Imsongand Murali, 2022). Natural compounds and their structural analogues derived from molluscs have significant anticancer attributes in clinical trials (Benkendorff, 2000).

Previous chemical profile investigations on *P. sulcatus* have been demonstrated the occurrence of cembronoids, and steroid derivatives (Alam *et al.,* 1993). Recent investigations implies that this species exhibits promising immunomodulatory and potential cytotoxic activities (Alam *et al.,* 1993; Khan *et al.,* 2022). Most predominantly the marine molluscs were serves as a reservoir of biologically active components, their exploration and research in India have been limited (Anbuselvi*et al.,* 2009; Chakraborty and Joy, 2020). Therefore, it is necessary to conduct thorough screenings of marine molluscs for bioactive compounds. The current investigation aimed to evaluate the antimicrobial, anticancer, and antioxidant activities of the bioactive components present in the whole-body tissue extracts of the gastropod *P. sulcatus* (Born, 1778).

This is a marine prosobranch gastropod of the family Planaxidae. Members of this family of gastropods are widespread across the Indo-Pacific region and abundant along the rocky intertidal zones of west coast of India (Houbrick, 1987).

2. Materials and Methods

2.1. Materials

Ascorbic acid, MTT were purchased from Sigma-Aldrich, Germany. 2, 2-diphenyl 1-picrylhydrazyl (DPPH), ferrozine, ferric chloride, and Agar were purchased from Hi-media laboratories, India. The other analytical grade chemicals were procured from local distributers.

2.2. Studied specimen

During this investigation, live marine gastropod *P. Sulcatus* (Born, 1778) (Family: Planaxidae) were collected in a random way during the daytime low tide period by hand picking from the intertidal rocky shore, Majali, Karwar, Uttar Kannada district of Karnataka, situated on the West coast of India (14.88°N, 74.11°E). The live specimens were transported to the laboratory in zip lock bag and verified up to species, by utilizing the Sea Shells of India (Apte, 2014) and systematic classification was authenticated from the WRoM (World Registrar of Marine Species) (https://www.marinespecies.org). The gastropod samples were validated by the Marine Biology Regional Centre, ZSI (Zoological Survey of India), Chennai, India.

2.3. Extraction of sample

100g of dried whole-body sample of *P. sulcatus* was taken in a conical flask and 200mL of methanol was added to the sample. The flask was kept in a shaker at 45° C for 72 Hrs with 120 rpm, sample was filtered following the incubation period and placed at 65° C for complete evaporation. The residue, called as “the extract” hereafter was collected in air-tight containers and stored in a cool place for further use. (Chellaram *et al.,* 2004).

2.4. Fourier Transform Infra-Red (FTIR) Spectroscopic Analysis

FT-IR-ATR spectroscope (Aligent FT-IR-7600), fitted with a single bounce diamond crystal and a deuterated triglycinesulfate detector, was used to identify the functional groups present in the extract. The resolution applied for the analysis was $4cm^{-1}$ and $4000\text{-}600cm^{-1}$ infrared (IR) range. 32 transmittance mode scans were used to gather each spectrum. The measurements were done

in triplicates, and the average values were applied. The FTIR spectra present transmittance values in y-axis as a function of wave number in x-axis.

2.5. Antimicrobial activity

2.5.1. Determination of the Minimum Inhibitory Concentration (MIC)

The Clinical and Laboratory Standard Institute (CLSI) protocol was followed for determining the Minimum Inhibitory Concentration (MIC). Distilled water (DW) was used to create the *Planaxis sulcatus* extract stock solution. Extract from *P. sulcatus* was serially diluted twice in Brain Heart Infusion (BHI) broth, with concentrations ranging from 1 mg/mL to 0.0019 mg/mL (20 mg/mL). Each micro test tube received one hundred microliters of previously prepared cultures of *Streptococcus mutans*, *Staphylococcus. aureus*, *Bacillus subtilis*, *Fusobacterium nucleatum,* and *Actinobacillus actinomycetemcomitans*. Anaerobic conditions were maintained in the tubes for 48 hours at 37 °C, while aerobic conditions were maintained for 24 hours with *Bacillus subtilis*. The bacterial growth was observed visually following the incubation period. Once 100 µL of the broth was incubated, 30 µL of Resazurin dye was added and incubated for 4 hours at 37° C. The color change of the wells indicated the presence of bacterial growth. The minimum inhibitory concentration (MIC) was defined as the concentration at which no bacterial growth was observed. The MIC of the extract against *Candida albicans* was also examined for 72 hours at 25° C.

2.5.2. Determination of Minimal Bactericidal Concentration MBC

Following the standards set out by the Clinical and Laboratory Standard Institute (CLSI) for minimal bactericidal concentration (MBC), samples were streaked onto agar plates utilizing MIC test tubes, and the bacterial and fungal growth was observed over the course of 48 hours at 37°C and 25°C, respectively for fungal and bacterial inoculants (Mishra *et al.,* 2017).

2.5.3. Agar well diffusion method

Wells were prepared in BHI agar plates after being inoculated with the test organisms (*Staphylococcus aureus, Fusobacterium nucleatum, Actinobacillus actinomycetemcomitans, Streptococcus mutans, Bacillus subtilis,* and *Candida albicans*) and *P. sulcatus* extract sample.

Every plate was maintained for 24 hours at 37°C. Following incubation, a scale was used to measure the widths of any clear zones surrounding the wells or containing antimicrobials.

2.6. Anticancer assay (3-[4,5-dimethylthiazo-2yl]-2,5 diphenyl tetrazolium bromide (MTT) assay)

The anticancer efficiency of the methanolic extract of *P.sulcatus* on Human breast cancer was evaluated by MTT assay utilizing MCF-7 cell line. The cells were introduced in a 96-well microplate and were kept for overnight at 37ºC, 95% humidity, and 5% CO_2. Varying sample concentrations (100, 50, 25, 12.5, 6.25, 3.125 µg/ml) were subjected to treatment. An additional 48 hours were kept for incubating the cells. After two PBS washes. To each well, 20 µL of MTT staining solution was applied, and the plate was incubated at 37ºC. Following four hours, 100 µL of DMSO was introduced to dissolve the formazan crystals to each well, and a microplate reader (Bio-Rad Microplate Reader 550) was employed to record the absorbance at 570 nm (Kumbar *et al.,* 2021).

2.7. Anti-oxidant assay

2.7.1. 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay

Samples (0 to 500 µg/mL) varying concentration were added, along with 2 mL of DPPH (100 µM) and 3 mL of methanol. The assay mixture was maintained in the dark at ambient temperature for 45 minutes. Employing a spectrophotometer (Shimadzu UV-1800, Kyoto, Japan) intensity of the incubated sample was measured at 517 nm, against the blank (no sample/standard). The free radical scavenging efficacy of the extract is reported by way of IC_{50} values (Blois, 1958).

2.7.2. Ferrous-Ion Chelating Assay

0.05ml of 2Mm $FeCl_2$solution was mixed separately with varying concentrations (0 to 500µg/mL) of appropriately diluted samples of the extract, initiated the reaction by adding 5mM Ferro-zine (0.1 mL), and the samples were maintained at room temperature for 10 minutes. Utilizing a spectrophotometer, absorbance of the chromogen was monitored at 562nm against a blank (without sample or standard), the % inhibition was computed and reported by the way of IC_{50} values (Dinis *et al.,* 1994).

2.7.3. Nitric oxide scavenging assay

Varying concentrations of sample (0-500µg/mL) were combined with sodium nitroprusside (10mM) in phosphate-buffered saline (PBS, pH 7.4) and incubated for 150 minutes at room temperature. The control was the same reaction mixture without the sample or standard. Followed by the addition of 0.5mL of Griess reagent (1% N-(1-naphthyl) ethylenediamine HCl 1% Sulfanilamide, and 2% H_3PO_4), the absorbance of incubated samples was recorded at 546 nm (Sreejayan and Rao, 1997). By comparing the absorbance values of the test and control (L-ascorbic acid) preparations, the percent nitric oxide scavenging activity was ascertained. The results were represented in terms of IC_{50} values.

2.8. Statistical analysis

Each trial was evaluated in thrice; data were tabulated as mean ± SD (standard deviation). IC_{50} (The IC_{50} value is the 50% of inhibition of its activity under the assay conditions) values, from the *in vitro* data, were determined by regression analysis utilizing Graph pad prism software.

3. Results

3.1. Fourier Transform Infra-Red (FTIR) Spectroscopic Analysis

The Fourier transform infrared spectroscopic analysis of the methanolic extract shows each functional group in the form of a peak, the transmittance displayed on the y-axis, while the wave number (cm^{-1}) represented on the x-axis. Outcomes of analysis of the body tissue of *P. sulcatus* has yielded 35 major peaks with varying wave intensities forecasting compounds with multiple functional groups. Major components detected in the sample includes alkanes, alkyne, alene, alcohol, carboxylic acids, ketones, halo compounds, nitro compounds, sulfone and sulfoxide as shown in the in Fig. 1, and Table 1.

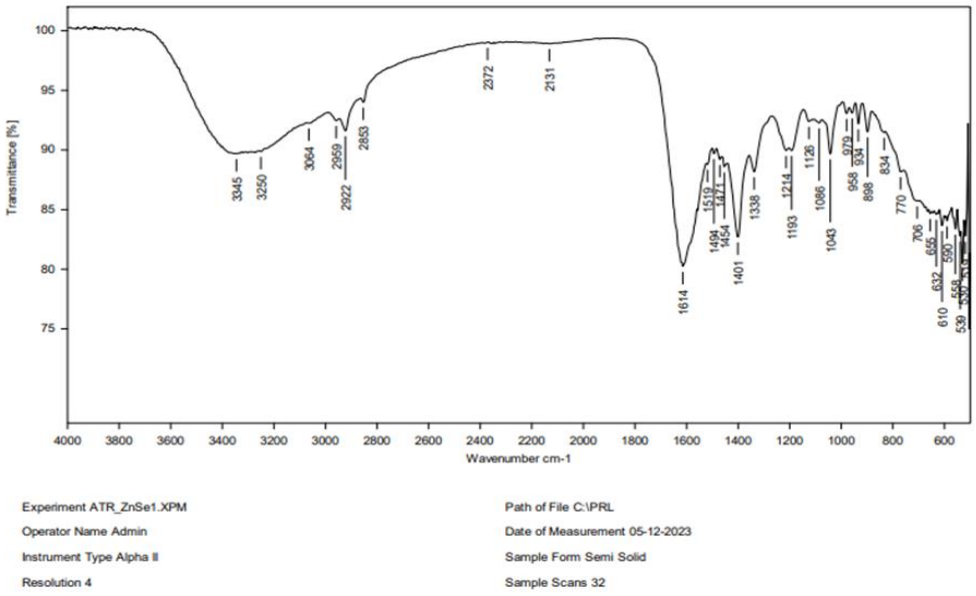

Figure 1: FTIR spectrum of methanolic extract of *Planaxis sulcatus* (Born, 1778)

Table 1: FT-IR analysis of methanol extract of *Planaxis sulcatus*

Sl. No.	Wavenumber (cm^{-1})	Band Assignments	Band interaction	Possible compounds
1	519	C-Br	Stretching	Halocompounds
2	530	C-I	Stretching	halo compound
3	539	C-Br	Stretching	Halocompounds
4	558	C-Cl	Stretching	halo compound
5	590	C-I	Stretching	halo compound
6	610	C-Br	Stretching	halo compound
7	632	C-Br	Stretching	halo compound
8	655	C-Br	Stretching	halo compound
9	706	C=C	Bending	alkene
10	770	C-H	Bending	monosubstituted
11	834	C-H	Bending	1,2,3,4-tetrasubstituted
12	898	C-H	Bending	1,3-disubstituted
13	934	--	--	---
14	958	C=C	Bending	alkene
15	979	C=C	Bending	alene
16	1043	CO-O-CO	Stretching	anhydride
17	1086	S=O	Stretching	sulfoxide
18	1126	C-O	Stretching	tertiary alcohol
19	1193	C-O	Stretching	secondary alcohol
20	1214	C-O	Stretching	vinyl ether
21	1338	S=O	Stretching	sulfone
22	1401	O-H	Bending	carboxylic acid
23	1454	C-H	Bending	alkane
24	1471	C-H	Bending	alkane
25	1494	N-O	Stretching	nitro compound
26	1519	C=C	Stretching	cyclic alkene
27	1614	C=C	Stretching	α, β-unsaturated ketone
28	2131	C≡C	Stretching	alkyne
29	2372	O=C=O	Stretching	carbon dioxide
30	2853	C-H	Stretching	alkane
31	2922	O-H	Stretching	alcohol
32	2959	C-H	Stretching	alkane
33	3064	C-H	Stretching	alkene
34	3250	O-H	Stretching	alcohol
35	3345	O-H	Stretching	alcohol

Antimicrobial activity

3.1.1. Agar well diffusion method

Inhibition zone of *P.sulcatus* methanol extract against *S. aureus* and *F. nucleatum* was found to be 10mm whereas *B. subtillis* demonstrated 5mm and standard showed 44mm. Further *A. actinomycetemcomitans* was found to be 8mm and *S. mutans* have showed inhibition zone of 15mm radius and standard showed 44mm and 22mm respectively. *Candida albicans* was found to be 8mm and standard displayed 36mm of inhibition (Fig. 2).

3.1.2. Determination of the Minimum Inhibitory Concentration (MIC)

The MIC values of the methanol extract of *P. sulcatus* against 6 microorganisms comprising 5 bacteria and one fungal species is depicted in Table 2. Among the 6 organisms tested, *S. aureus* and *B. subtillis* demonstrated greater susceptibility to the test sample. *C. albicans* displayed the least susceptibility.

3.1.3. Determination of the Minimum Bactericidal Concentration (MBC) and Minimum fungicidal Concentration (MFC)

The MBC of methanol extract of *P. sulcatus* against the test organisms, displayed similar value of 0.03125 mg/mL for *S. aureus*, *B. subtillis*, *F. nucleatum*, and *S. mutans***.** MBC for *A. actinomycetemcomitans* was recorded as 0.125 mg/mL and that for *C. albicans* as 1mg/mL. Highest MBC of 1mg/mL was recorded for *C. albicans* (Table 2).

Table 2: Antimicrobial activity of methanol extract of *P.sulcatus*

Microorganisms	**MIC (mg/mL)**	**MBC/MFC (mg/mL)**
Staphylococcus aureus	0.0156	0.03125
Fusobacterium nucleatum	0.03125	0.03125
Actinobacillus actinomycetemcomitans	0.125	0.125
Streptococcus mutans	0.03125	0.03125
Bacillus subtilis	0.0156	0.03125
Candida albicans	1.0	1.0

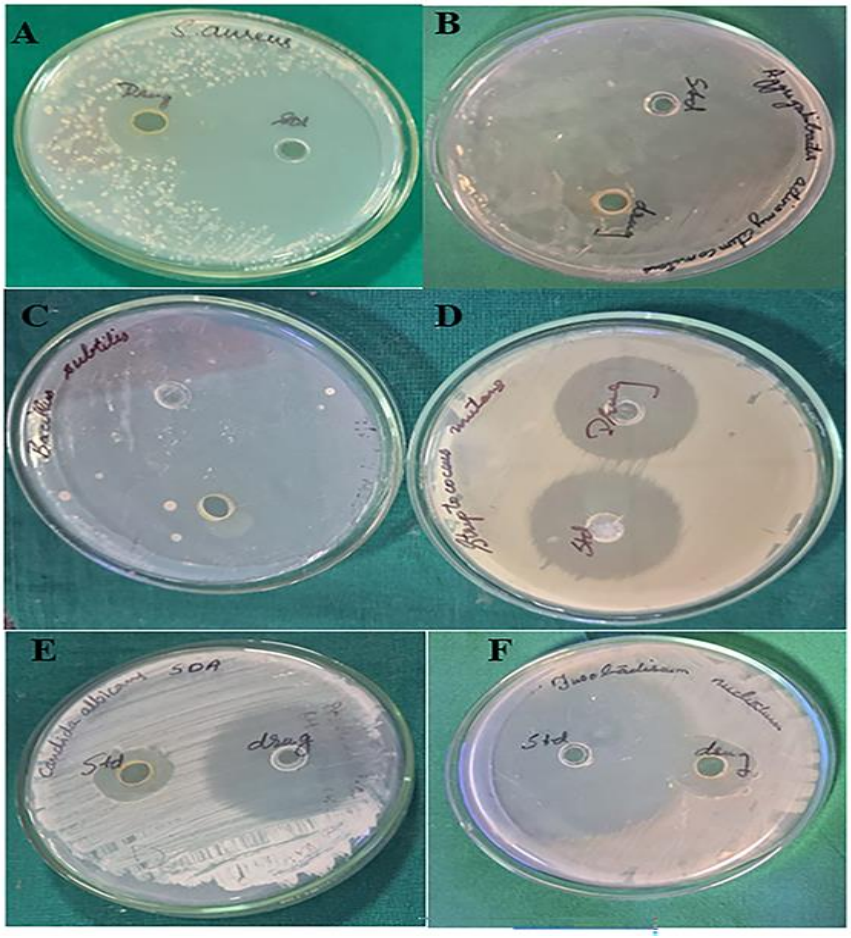

Figure 2: Antimicrobial activity of methanol extract of *P.sulcatus.* A-*Staphylococcus aureus*; B-*Actinobacillus actinomycetemcomitans*; C-*Bacillus subtilis*; D-*Streptococcus mutans*; E-*Candida albicans*; F-*Fusobacterium nucleatum.*

3.2. Anticancer activity (MTT assay)

The cytotoxicity efficacy of tissue sample was ascertained utilizing MCF-7 cells and Doxorubicin in terms of % viability is represented. A dose-dependent reduction in cell viability was observed in cultured MCF-7 cells upon treatment with the tissue extract. The tissue extract, at a concentration of 6.25 µg /mL demonstrated significant reduced cell viability (84.06%) with IC_{50}value of 53.99 µg /mL (Fig 3).

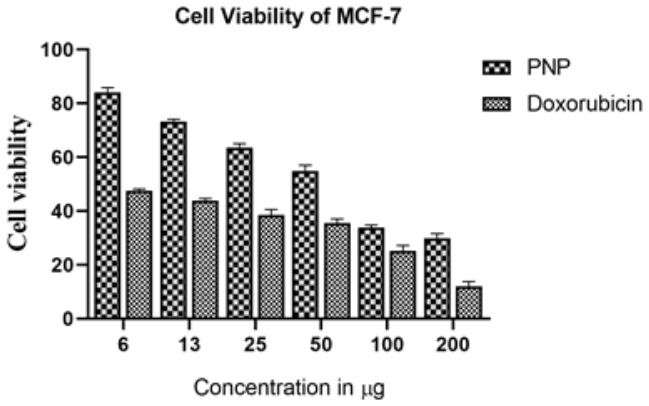

Figure 3: I*n vitro* Cytotoxicity assay against MCF cells

3.3. Antioxidant assay

3.3.1. DPPH (2,2-diphenyl-1-picrylhydrazyl) assay

The DPPH assay was used to determine the antioxidant properties of the methanolic extract of *P. sulcatus*. Ascorbic acid served as conventional standard, which demonstrated an IC_{50} value of 1.54µg/ml, whereas the extract exhibited with IC_{50} value of 565.80 µg/ml (Fig.4).

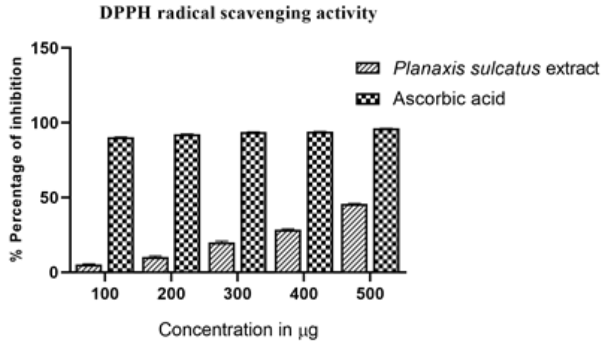

Figure4: DPPH radical scavenging activity of methanolic extract of *Planaxis sulcatus*

3.4.2. Ferrous-Ion Chelating Assay

In the present investigation, as concentration increased(100–500µg/mL), the chelating activity improved. At 500µg/mL concentration of ascorbic acid and methanolic extract have the maximum chelating activity with percent inhibition of 92.69% and 29.16%, demonstrated with the IC_{50} values of 0.71µg/ml and 770.53 µg/ml respectively (Fig. 5). The ascorbic acid IC_{50} was lower than that of the methanolic extract. This indicates that, contrary to ascorbic acid, the methanolic extract is a mild metal chelating agent.

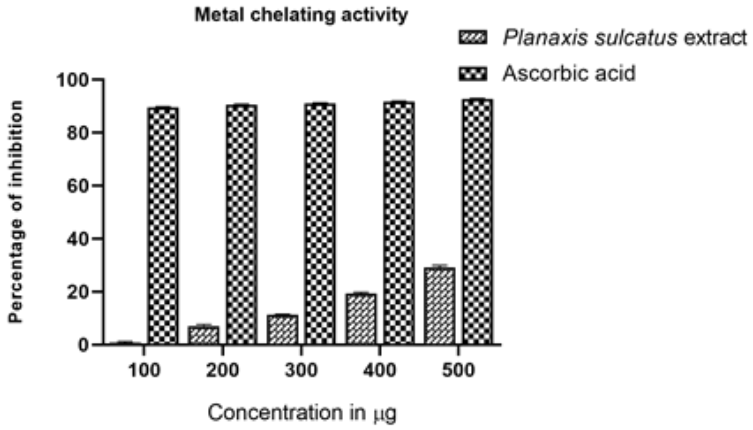

Figure 5: Metal chelating activity of methanolic extract of *Planaxis sulcatus*

3.4.3. Nitric oxide scavenging assay

The Nitric oxide scavenging assay was used to determine the antioxidant properties of the methanolic extract of *P. sulcatus* (Fig. 6). Utilizing ascorbic acid as reference compound, which has an IC_{50} value of 8.6µg/ml, the extract exhibited the ability to scavenge nitric oxide, with an IC_{50} value of 758.7µg/ml (Table 3).

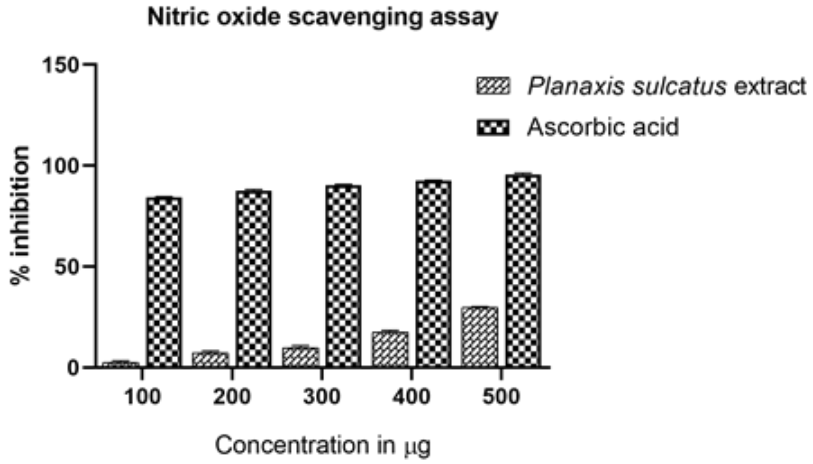

Figure 6: Nitric oxide scavenging activity of methanolic extract of *Planaxis sulcatus*

Table 3: Nitric oxide scavenging assay of Methanol extract of *P. sulcatus*

	% Inhibition					
Concentration µg/ml	**Sample 1**			**ASC**		
500	29.253	30.218	29.507	94.972	95.835	96.14
400	18.385	17.674	16.658	92.788	92.534	92.28
300	8.837	10.818	9.751	90.35	90.3	90.808
200	6.501	7.212	8.228	87.456	87.049	88.217
100	2.59	3.352	1.828	84.408	84.053	84.662

4. Discussion

Marine organisms are known to possess abundant bioactive chemicals, many of them beneficial to human health and offer new sources for novel pharmaceuticals. Molluscs exhibit significant diversity in range of compounds with bioactive characteristics. Marine gastropods are abundant in terms of species and population as well. However, they are less explored in terms of bioprospecting. In light of these observations, we have selected the common gastropod species of Karwar coast, *P. sulcatus* for the current investigation on bioprospecting in terms of therapeutic properties (Ghosh *et al.,* 2022).

FTIR analysis of the extract of *P. sulcatus* has yielded 35 major peaks between 519cm^{-1} and 3345cm^{-1}, indicating the presence of multiple compounds with different functional groups. Our earlier report of GC-MS analysis has confirmed the presence of more than 100 bioactive compounds in the tissue extract of this mollusc of which many are reported to be pharmacologically active (Kumar and Haragi, 2023).

Microbial infections pose serious challenges to the health of animals and humans. Overuse of antibiotics since decades in healthcare measures has resulted in the development of drug resistance by many of the pathogenic bacteria. Search for substitute antimicrobials from natural sources is crucial for the development of effective and affordable antibiotics from time to time (Prestinaci *et al.,* 2015). The marine environment is a rich source of bioactive compounds with potential applications in healthcare. Of these, marine molluscs act as a ready source of effective antibacterial agents. Many of them produce these antibacterial components as a first line of defense against pathogenic microbial infection. These extracts may also be a promising source of novel bioactive compounds for use in clinical settings. Our study has confirmed the antimicrobial activity of *P. sulcatus* against multiple bacteria and fungi.

Antitumor and anticancer compounds are highly sought-after bioactive molecules in recent times. Cembronids are a group of bioactive molecules of marine environments with proven antimicrobial and antitumor activities. The presence of cembronids has been reported from *P. sulcatus* along with steroid derivatives by some earlier investigations (Alam *et al.,* 1993). We have reported the presence of Nonanoyl chloride, a known anticancer compound from the tissue extract of this mollusc (Al-Marzoqi, *et al.,* 2016; Kumar and Haragi, 2023). In the current study, crude tissue extract of *P. sulcatus* displayedIC_{50} values at a comparable level to that of the standard drug, doxorubicin. Purification of the extract can yield better results.

Another aspect of the bioactivity of the molluscan extract evaluated in this study is the antioxidant activity. Molluscs are vulnerable to oxidation stress, as their body is very fragile and the marine environment is complex with many oxidizing agents. The polyphenolic components present in them have been reported acting as free radical scavenging agents to protect them from photooxidation. Because of their distinct and heavy hydroxylation pattern, polyphenolics have a high redox potential and function as potent electron donors (Ghareeb *et al.,* 2014). In the current study, *P. sulcatus* extract has demonstrated comparatively lower antioxidant activity in all three assays such as DPPH, Ferrous ion chelating, and Nitric oxide scavenging assays compared to those of the standard (Ascorbic acid). The purified extract will have better performance.

5. Conclusion

The current study has confirmed the presence of multiple bioactive compounds with different functional groups in the methanol extract of the whole-body tissue of *P. sulcatus*. Further, the study has demonstrated the antibacterial, antifungal, anticancer, and antioxidant potentials of the extract through *in vitro* bioassays.

6. Conflict of Interest

The authors declare no conflicts of interest.

7. Author's Contribution

SKM: Performed work, SBH: Manuscript revision and design. We ensure and hereby declared that all authors have read and approved the manuscript.

8. Acknowledgment

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