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Characterization and bioactivities of a novel copper–zinc superoxide dismutase from the marine snail *Rapana venosa*: antioxidant, antimicrobial, and anticancer effects

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Abstract

Background Marine organisms, particularly snails, are rich sources of bioactive compounds with potential antioxidant, antimicrobial, and anticancer properties, offering promising avenues for drug discovery.

Methods In this study, *Rapana venosa* extracts were evaluated for antioxidant enzyme activities and tested for antimicrobial and anticancer effects. Furthermore, a superoxide dismutase (SOD) isoenzyme, designated *Rapana venosa* superoxide dismutase 1 (RvSOD1), was purified from *R. venosa* using chromatographic techniques, characterized, and evaluated for its antimicrobial and anticancer activities.

Results The crude *R. venosa* extract demonstrated substantial antioxidant activity, with measurable catalase (CAT), superoxide dismutase (SOD), glutathione S-transferase (GST), and glutathione peroxidase (GPx) activities. It exhibited antimicrobial effects, inhibiting *Escherichia coli* by 71.2% and *Candida albicans* by 90.8%. The extract also showed cytotoxicity against human cancer cell lines MCF-7 (breast cancer), MDA-MB-231 (triple-negative breast cancer), and A549 (lung adenocarcinoma). The purified RvSOD1 displayed a specific activity of 600.7 U/mg (7.5-fold purification; 30.4% yield). Characterization indicated that RvSOD1 is a copper–zinc SOD, sensitive to potassium cyanide and activated by Zn^{2+} and Cu^{2+} , with an optimum pH of 8.0 and an apparent molecular mass of ~90 kDa. Functionally, RvSOD1 showed antifungal activity against *C. albicans* (79.9% inhibition) and antibacterial activity against *E. coli*, *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, and *Staphylococcus aureus*. In anticancer assays, RvSOD1 was active against MCF-7 and MDA-MB-231 cells but was less effective against A549 cells. Notably, the crude extract was more effective than purified RvSOD1 against all three cell lines, suggesting synergistic effects or additional bioactive compounds.

Conclusion Both the *R. venosa* extract and the isolated RvSOD1 possess significant antioxidant, antimicrobial, and anticancer properties. These findings highlight their potential as promising multifunctional therapeutic candidates for future pharmaceutical and biomedical applications.

Keywords *Rapana venosa*, Antioxidant, Antimicrobial, Antitumor, Superoxide dismutase, Purification, Characterization

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1 Background

The marine environment represents an unparalleled reservoir of novel bioactive molecules, attributed to its vast biodiversity and the unique adaptive biochemical strategies evolved by marine organisms [1, 2]. Marine invertebrates, in particular, thrive under extreme and dynamic conditions, such as fluctuating salinity, high pressure, and low light availability. These environmental pressures have driven the evolution of distinct molecular structures and potent bioactivities not commonly found in terrestrial sources [3]. A critical factor for the survival of marine organisms in these challenging environments is their robust ability to counteract oxidative stress. Oxidative stress, a pathological state characterized by an imbalance between the production of reactive oxygen species (ROS) and the body's antioxidant defense mechanisms, can lead to significant damage to molecular structures and cellular integrity [4, 5]. While physiological concentrations of ROS are essential for cellular signaling, excessive ROS can induce oxidative modifications in vital biomolecules, including lipids, proteins, and nucleic acids, thereby disrupting cellular homeostasis and contributing to the progression of numerous diseases [6].

Oxidative stress is increasingly recognized as a fundamental contributor to the pathogenesis of various chronic diseases, including cancer, cardiovascular disorders, neurodegenerative conditions, and inflammatory diseases [7, 8]. The global health community has consequently placed a growing emphasis on integrating oxidative stress mitigation into disease prevention strategies, acknowledging its role as a modifiable risk factor underlying degenerative diseases such as cancer [9]. This recognition has spurred an accelerated search for bioactive compounds possessing both antioxidant and anticancer properties, aiming to simultaneously address the critical public health challenges of reducing oxidative damage and curbing cancer incidence. Cellular systems employ a sophisticated hierarchy of antioxidants, broadly classified into enzymatic and non-enzymatic (small-molecule) categories, to neutralize ROS. The primary enzymatic defense line against ROS includes key enzymes such as superoxide dismutase (SOD), catalase (CAT), glutathione S-transferase (GST), and glutathione peroxidase (GPx), which directly neutralize reactive species [10–12].

Marine invertebrates, encompassing a diverse range of organisms like sponges, corals, and mollusks, have proven to be prolific sources of secondary metabolites and proteins exhibiting potent pharmacological activities through various mechanisms [13, 14]. Over recent decades, a wide array of marine-derived compounds with diverse therapeutic effects, including antioxidant, antimicrobial, antitumor, antiviral, cardioprotective, and immune-modulatory properties, have been identified

[15–17]. Marine mollusks, in particular, are well-established as rich sources of nutraceuticals and natural medicinal compounds. Their tissues contain a variety of bioactive constituents, such as proteins, peptides, polysaccharides, lipids, and pigments, which contribute to their defense and survival in the marine environment [18, 19].

Among these, the veined rapa whelk, *Rapana venosa*, has garnered increasing scientific interest. The hemolymph of *R. venosa* contains a glycoprotein, common in mollusks, that has demonstrated immune-stimulatory and anticancer activities. Biologically active compounds isolated from *R. venosa* have been shown to significantly decrease cancer cell viability, induce morphological changes in cancer cells, exhibit synergistic effects with standard chemotherapeutics [20, 21], and exert antioxidant and antiproliferative effects by inhibiting tumor cell proliferation and reducing cell migration through interactions with ROS and extracellular matrix components [22]. These collective findings underscore the versatility and therapeutic promise of *R. venosa*-derived compounds in drug development, particularly as novel agents with enhanced specificity and reduced toxicity. Despite this potential, *R. venosa* remains an under-investigated source of enzymatically accessible proteins, and its antioxidant enzyme repertoire (CAT, SOD, GST, and GPx) and putative cytotoxic properties are largely unexplored.

Therefore, the present study aims to comprehensively investigate the bioactive potential of *R. venosa*. Specifically, this research endeavors to: (i) extract proteins from *R. venosa*; (ii) characterize their antioxidant enzyme activities and isoenzyme patterns; (iii) assess their antimicrobial effects against various microbial strains; and (iv) evaluate their in vitro antiproliferative effects against human cancer cell lines (A549, MCF-7, and MDA-MB-231) as well as the normal lung cell line WI-38 (ATCC® CCL-75) in comparison with doxorubicin used as a reference drug. Furthermore, given the prominent activity observed for superoxide dismutase, a significant portion of this study focuses on the purification and detailed characterization of a novel SOD isoenzyme from *R. venosa*. The antimicrobial and anticancer characteristics of this purified *R. venosa* SOD will be thoroughly investigated, with the ultimate goal of contributing to the development of new therapeutic agents for infectious diseases and various cancers.

2 Materials and methods

2.1 Sample collection

R. venosa snails were manually collected from the intertidal and shallow subtidal zones of the Mediterranean Sea at a depth of 1–3 m. Snails were washed with seawater to remove attached sand, pebbles, and other impurities.

Live snails were transported in seawater-filled plastic aquaria to the Medical Malacology Department, Theodor Bilharz Research Institute, Giza, Egypt. For taxonomic identification, specimens were quickly rinsed with sterile water, fixed in 2% buffered formaldehyde, and transferred to absolute ethanol [43]. Species-level identification was performed based on published taxonomic keys [23] and verified using the World Register of Marine Species [24].

2.2 Chemicals

Bovine serum albumin (BSA), cytochrome c, 1-chloro-2,4-dinitrobenzene (CDNB), nitroblue tetrazolium (NBT), reduced glutathione (GSH), phenazine methosulfate (PMS), glutathione reductase (GR), xanthine oxidase, xanthine sodium salt, fetal bovine serum (FBS), RPMI-1640 medium, 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) salt, nicotinamide adenine dinucleotide phosphate (NADPH), doxorubicin hydrochloride, and dimethyl sulfoxide (DMSO) were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA), unless otherwise stated. All other chemicals used were of analytical grade.

2.3 Extraction of *R. venosa* snail proteins

All extraction steps were conducted at 4 °C to maintain enzyme stability. Approximately 20 g of *R. venosa* tissue was homogenized in 20 mmol/L potassium phosphate buffer (pH 7.5) using a mixer homogenizer (Sourval Instruments, New Delhi, India). The homogenate was centrifuged at 10,000 × g for 30 min at 4 °C using a refrigerated centrifuge (Sigma Laborzentrifugen GmbH, Osterode am Harz, Germany). The supernatant (crude extract) was collected for subsequent analyses [25].

2.4 Protein assessments

Protein concentration was measured at all stages using the Bradford method with bovine serum albumin (BSA) as a standard [26] using a Shimadzu UV-2401 spectrophotometer (Shimadzu Corporation, Kyoto, Japan).

2.5 Enzyme assays

2.5.1 Catalase (CAT) activity

CAT activity was measured in a 3 mL reaction mixture containing 0.05 M potassium phosphate buffer (pH 7.0) and 0.02 M hydrogen peroxide (H_2O_2). The reaction was initiated by adding the enzyme solution, and H_2O_2 decomposition was monitored at 240 nm for 2 min as the decrease in absorbance. One unit of CAT activity was defined as the amount of enzyme that decomposes 1 μ mol H_2O_2 per min, using an extinction coefficient of 43.6 $M^{-1} cm^{-1}$ [27].

2.5.2 Superoxide dismutase (SOD) activity

SOD activity was assayed in a 1 mL reaction mixture containing 50 mmol/L potassium phosphate buffer (pH 7.8), 10 mmol/L cytochrome c, 100 mmol/L EDTA (ethylenediaminetetraacetic acid), and 50 mmol/L sodium xanthine. The reaction was initiated by adding 21 mU xanthine oxidase. One unit of SOD activity was defined as the amount of enzyme that inhibits cytochrome c reduction by 50% at 550 nm [28].

2.5.3 Glutathione S-transferase (GST) activity

GST activity was assayed in a 1 mL reaction mixture containing 100 mmol/L potassium phosphate buffer (pH 6.5), 1 mmol/L 1-chloro-2,4-dinitrobenzene (CDNB), and 1 mmol/L reduced glutathione (GSH). The reaction was initiated by adding the enzyme solution, and the increase in absorbance at 340 nm was recorded for 3 min against a blank prepared without enzyme. One unit of GST activity was defined as the amount of enzyme that conjugates 1 μ mol of CDNB per min, using an extinction coefficient of 9.6 $mM^{-1} cm^{-1}$ [29].

2.5.4 Glutathione peroxidase (GPx) activity

GPx activity was assayed in a 1 mL reaction mixture containing 0.05 M potassium phosphate buffer (pH 7.0), 5 mmol/L EDTA, 75 mmol/L H_2O_2 , 5 mmol/L GSH, 280 mmol/L NADPH, 1 unit of glutathione reductase (GR), and the enzyme solution. The decrease in absorbance at 340 nm was monitored for 3 min against a blank prepared without enzyme. One unit of GPx activity was defined as the amount of enzyme that oxidizes 1 μ mol of NADPH per min, using an extinction coefficient of 6.22 $mM^{-1} cm^{-1}$ [30].

2.5.5 Isoenzyme profiling

Crude *R. venosa* extract was separated by 7% native polyacrylamide gel electrophoresis (native-PAGE) using a vertical electrophoresis system (GE Healthcare Life Sciences, Uppsala, Sweden), followed by activity staining for CAT, SOD, GST, and GPx isoenzymes.

2.5.6 CAT isoenzyme pattern

For CAT, after electrophoresis, the gel was immersed in 3% H_2O_2 for 15 min and then rinsed thoroughly with distilled water. The gel was subsequently incubated in a freshly prepared 1:1 mixture of 2% potassium ferricyanide ($K_3[Fe(CN)_6]$) and 2% ferric chloride ($FeCl_3$) with gentle agitation until yellow bands appeared on a blue-green background, indicating CAT activity [31].

2.5.7 SOD isoenzyme pattern

For SOD, after electrophoresis, the gel was incubated in 50 mL of 100 mmol/L Tris–HCl buffer (pH 8.6) containing 20 mg nitroblue tetrazolium (NBT) and traces of phenazine methosulfate (PMS), then exposed to daylight until achromatic bands appeared on a blue background, indicating SOD activity [28].

2.5.8 GST isoenzyme pattern

For GST, after electrophoresis, the gel was incubated in 50 mL of 100 mmol/L potassium phosphate buffer (pH 6.5) containing 1 mmol/L CDNB, 4.5 mmol/L GSH, and 1 mmol/L NBT at 37 °C for 10 min with gentle agitation. The gel was then washed twice with distilled water and incubated at room temperature in 50 mL of 100 mmol/L Tris–HCl buffer (pH 9.6) containing 3 mmol/L PMS until blue formazan covered the gel surface except for clear GST activity zones [32].

2.5.9 GPx isoenzyme pattern

For GPx, after electrophoresis, the gel was incubated in 50 mmol/L Tris–HCl buffer (pH 7.9) containing 13 mmol/L GSH and 0.004% H₂O₂ with gentle agitation for 20 min. The gel was then washed thoroughly with distilled water and incubated in 50 mmol/L potassium phosphate buffer (pH 7.0) containing 1.2 mmol/L MTT and 1.6 mmol/L PMS until clear zones appeared against a brown background, indicating GPx activity [33].

2.6 Purification of *R. Venosa* SOD isoenzymes

2.6.1 Diethylaminoethyl (DEAE)-cellulose chromatography

R. venosa extract was loaded onto a diethylaminoethyl (DEAE)-cellulose anion-exchange column (12×2.5 cm) packed with DEAE-cellulose resin (Sigma-Aldrich Chemical Co., St. Louis, MO, USA) and pre-equilibrated with potassium phosphate buffer (pH 7.5). Bound proteins were eluted using a linear 0–1.0 M NaCl gradient prepared in the same buffer at a flow rate of 60 mL/h. Fractions were collected and assayed for SOD activity; active fractions were pooled and concentrated for further purification.

2.6.2 Size-exclusion chromatography

The DEAE-cellulose concentrate containing SOD activity was further purified on a size-exclusion column (141×1.8 cm) packed with Sephacryl S-300 (Cytiva, Uppsala, Sweden) and equilibrated with potassium phosphate buffer (pH 7.5). Proteins were eluted with the same buffer at a flow rate of 30 mL/h, and fractions were assayed for SOD activity. The column was calibrated with molecular weight standards (ferritin, 440 kDa; catalase, 240 kDa; alcohol dehydrogenase, 150 kDa; albumin, 67 kDa; myoglobin, 17 kDa; Sigma-Aldrich Chemical Co.,

St. Louis, MO, USA) to estimate the molecular mass of the *R. venosa* SOD enzyme.

2.6.3 Polyacrylamide gel electrophoresis

Fractions obtained during purification were analyzed by 7% native polyacrylamide gel electrophoresis (native-PAGE) [34] or 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) [35, 36]. Protein bands were visualized by staining with 0.25% Coomassie Brilliant Blue R-250 (Sigma-Aldrich Chemical Co., St. Louis, MO, USA).

2.6.4 Determination of optimal pH

The optimal pH for purified RvSOD1 activity was determined using potassium phosphate and Tris–HCl buffers over a pH range of 6.0–9.0.

2.6.5 Effect of divalent cations and inhibitors

The type of the isolated SOD isoform was determined by assessing the effects of divalent cations and specific inhibitors on SOD activity relative to a control assay without additives [37].

2.6.6 Antimicrobial activity

Antimicrobial activity was assessed using the microtiter plate (MTP) assay [38]. The tested microorganisms included *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa* (*P. aeruginosa*), *Escherichia coli* (*E. coli*), and *Candida albicans*. Cultures were grown in sterile media and adjusted to a standardized concentration using phosphate-buffered saline (PBS). Ciprofloxacin and nystatin (Sigma-Aldrich Chemical Co., St. Louis, MO, USA) were used as positive controls. In a 96-well flat-bottom polystyrene plate, 10 µL of sample and 80 µL of lysogeny broth were mixed with 10 µL of the microbial culture (log phase) and incubated overnight at 37 °C. Growth inhibition was assessed by measuring absorbance at 600 nm using a microplate reader (SPECTROstar Nano, BMG LABTECH, Ortenberg, Germany).

2.6.7 Cytotoxicity evaluation

The cytotoxicity of the *R. venosa* crude extract and purified RvSOD1 was evaluated using the MTT assay [39]. Human breast cancer cell lines MCF-7 and MDA-MB-231, the lung cancer cell line A549 (ATCC® CRM-CCL-185™), and the normal lung cell line WI-38 (ATCC® CCL-75™) were obtained from the Medical Experimental Research Center (MERC), Faculty of Medicine, Mansoura University, Mansoura, Egypt. Cells were seeded in 96-well flat-bottom plates at a density of 5×10³ cells/well in 200 µL of complete culture medium and allowed to adhere for 24 h at 37 °C in a humidified 5% CO₂ incubator. Cells were treated with various concentrations of the

crude extract, purified RvSOD1, or doxorubicin (DOXO) as a reference drug. After 72 h, cell viability was assessed using the MTT assay. Formazan crystals were dissolved in DMSO, and absorbance was measured at 570 nm using a microplate reader (BioTek Instruments, Winooski, VT, USA). Half-maximal inhibitory concentration (IC_{50}) values were determined and used for subsequent experiments. The effect of different concentrations on cell proliferation was expressed as percentage cell viability relative to untreated control cells, as follows:

$$\% \text{ Cell viability} = (A_{570} \text{ of treated cells} / A_{570} \text{ of control cells}) \times 100.$$

2.6.8 Statistical analysis

All assays were performed in at least three independent replicates, and data are presented as mean $\pm$ standard deviation (SD). Data from enzyme activity tests as well as MTP and MTT assays were analyzed using GraphPad Prism software (version 8.1.1; GraphPad Software, San Diego, CA, USA) [40].

3 Results

3.1 Electrophoretic analyses and antioxidant activity of *R. venosa* snail

Analysis of the *R. venosa* crude extract by 12% SDS-PAGE revealed a complex protein profile, with more than ten distinct protein bands ranging from approximately 10 kDa to over 180 kDa (Fig. 1a). The specific activities of catalase (CAT), superoxide dismutase (SOD), glutathione S-transferase (GST), and glutathione peroxidase (GPx) were 12.4 ± 2.08 , 86.3 ± 12.82 , 2.7 ± 0.28 , and 0.66 ± 0.038 U/mg protein, respectively (Table 1). Consistent with these measurements, 7% native-PAGE showed one isoenzyme band each for CAT and GPx, two isoenzyme bands for GST, and four isoenzyme bands for SOD, indicating multiple SOD isoforms in the *R. venosa* crude extract (Fig. 1b).

3.2 Antimicrobial activity of *R. venosa* extract

The antimicrobial potential of the *R. venosa* crude extract was evaluated against a panel of bacterial and fungal microorganisms. The extract exhibited weak inhibitory effects against *S. aureus* (5.2% inhibition) and *K. pneumoniae* (21.2% inhibition), moderate inhibition against *P. aeruginosa* (51.1% inhibition), and strong inhibition against *E. coli* (71.2% inhibition). Notably, the crude extract showed pronounced antifungal activity, achieving 90.8% inhibition against *C. albicans* (Fig. 2).

3.3 Cytotoxic effects of *R. venosa* extract

The cytotoxic effects of the *R. venosa* crude extract were assessed on human breast cancer cell lines (MCF-7 and MDA-MB-231), the human lung cancer cell line A549,

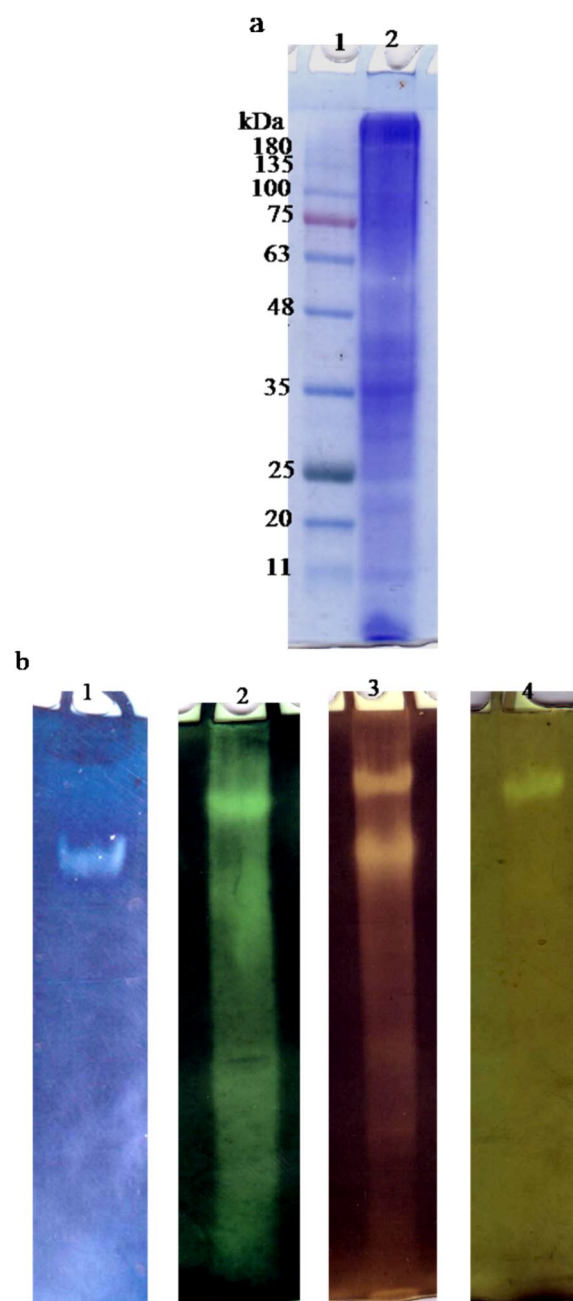


Fig. 1 **a** Protein profile of *R. venosa* crude extract analyzed by 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Lane 1: molecular weight markers; lane 2: *R. venosa* crude extract. **b** Antioxidant isoenzyme patterns of *R. venosa* crude extract resolved by 7% native polyacrylamide gel electrophoresis (native-PAGE). Lane 1: catalase (CAT) isoenzyme; lane 2: superoxide dismutase (SOD) isoenzymes; lane 3: glutathione S-transferase (GST) isoenzymes; lane 4: glutathione peroxidase (GPx) isoenzyme

and the normal human lung cell line WI-38 after 72 h of exposure using the MTT assay. Doxorubicin (DOXO) served as a positive control. The *R. venosa* extract

Table 1 Specific activities of antioxidant enzymes in *R. venosa* snail tissue

Enzyme	Specific activity
Catalase (CAT)	12.4 ± 2.08
Superoxide dismutase (SOD)	86.3 ± 12.82
Glutathione S-transferase (GST)	2.7 ± 0.28
Glutathione peroxidase (GPx)	0.66 ± 0.038

Values represent the mean ± standard deviation (SD) of three independent experiments. Enzyme activities were expressed as units per milligram of protein (U/mg protein)

exhibited potent cytotoxic effects across the tested cancer cell lines, with IC_{50} values of 1.102 μ g/mL for MCF-7, 1.513 μ g/mL for MDA-MB-231, and 1.668 μ g/mL for A549 (Fig. 3a). These values compared favorably with those of DOXO (IC_{50} = 1.844, 1.238, and 6.89 μ g/mL for MCF-7, MDA-MB-231, and A549, respectively) (Fig. 3c). In contrast, the crude extract caused only a limited reduction in WI-38 cell viability (~ 75% viability

at the highest dose), indicating low cytotoxicity toward normal cells (Fig. 3d).

3.4 Purification of *R. venosa* superoxide dismutase isoenzymes

The purification of superoxide dismutase isoenzymes from *R. venosa* was achieved through a two-step chromatographic procedure, resulting in substantial increases in SOD specific activity and purification fold. The initial specific activity of SOD in the *R. venosa* crude extract was 79.6 U/mg protein. During DEAE-cellulose anion-exchange chromatography, two distinct peaks exhibiting SOD activity were resolved (Fig. 4a). The major isoenzyme, designated *R. venosa* superoxide dismutase 1 (RvSOD1), eluted with 0.0 M NaCl, while a minor isoenzyme (RvSOD2) eluted with 0.2 M NaCl. The specific activities of RvSOD1 and RvSOD2 increased by 1.8-fold and 1.7-fold, respectively, compared with the crude extract, with corresponding recoveries of 53.4% and 27.2%. The fractions containing RvSOD1 were pooled, concentrated, and subjected to further purification using Sephacryl S-300 size-exclusion chromatography.

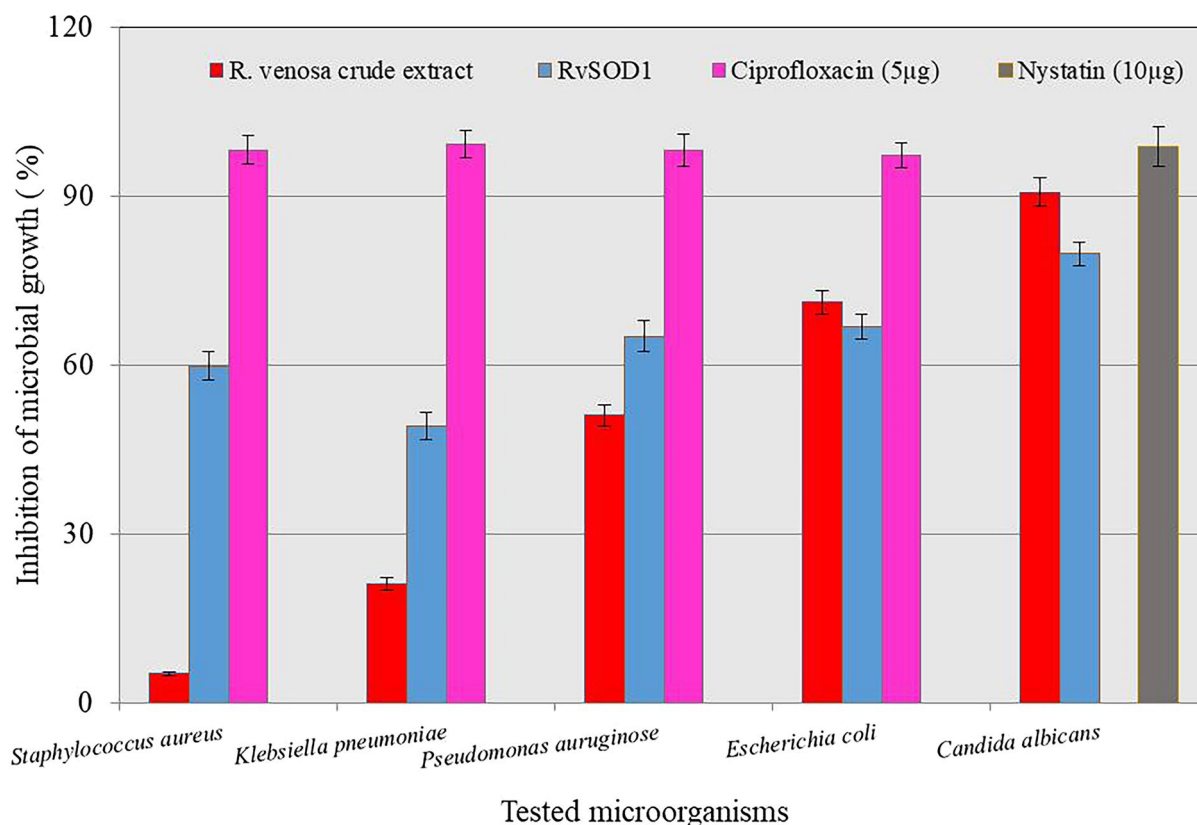
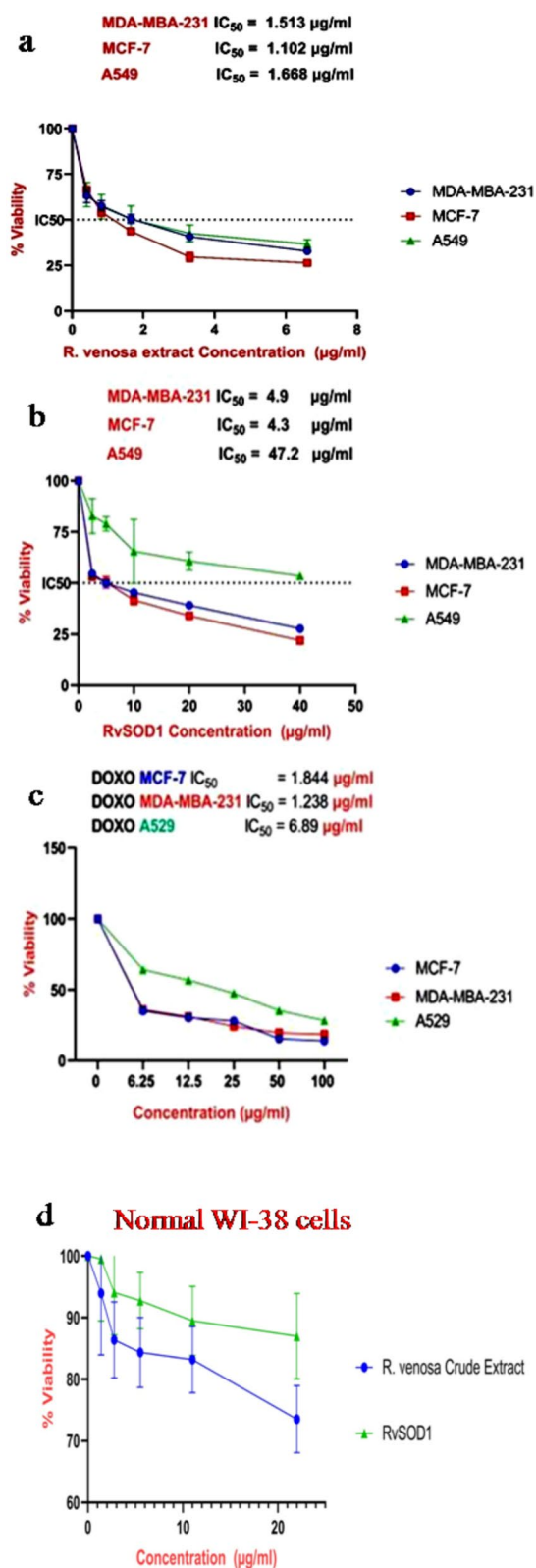


Fig. 2 Antimicrobial activity of *R. venosa* crude extract and purified *Rapana venosa* superoxide dismutase 1 (RvSOD1) against various bacterial and fungal microorganisms, expressed as percent inhibition (%). Values represent the mean ± standard deviation (SD) of three independent experiments



◀ **Fig. 3** Cytotoxic effects were evaluated after 72 h of treatment using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. **a** Effect of *R. venosa* crude extract on the viability of MCF-7, MDA-MB-231, and A549 cancer cell lines. **b** Effect of purified *Rapana venosa* superoxide dismutase 1 (RvSOD1) on the viability of MCF-7, MDA-MB-231, and A549 cancer cell lines. **c** Effect of doxorubicin (DOXO) on the viability of MCF-7, MDA-MB-231, and A549 cancer cell lines. **d** Cytotoxic effects of *R. venosa* crude extract and purified RvSOD1 on the normal human lung cell line WI-38. Values represent the mean $\pm$ standard deviation (SD) of three independent experiments

RvSOD1 eluted as a single prominent peak on Sephacryl S-300 (Fig. 4b). This step enhanced the specific activity of RvSOD1 to 600.7 U/mg, representing a 7.5-fold purification and a 30.4% yield (Table 2). The molecular mass of purified RvSOD1 was estimated to be approximately 90 kDa based on its elution volume from the calibrated Sephacryl S-300 column.

3.5 Electrophoresis analysis of RvSOD1

To monitor the purification progress of RvSOD1, aliquots from different stages (crude snail extract, DEAE-cellulose concentrate, and Sephacryl S-300 fraction) were analyzed by 7% native-PAGE. The native-PAGE analysis revealed a single protein band for RvSOD1 that precisely comigrated with the SOD activity band, confirming the successful isolation and homogeneity of the enzyme (Fig. 5a, b). Furthermore, electrophoretic separation of the purified RvSOD1 on 12% SDS-PAGE, in comparison with protein molecular weight markers, showed a single band at approximately 90 kDa (Fig. 5c), consistent with the size estimated by size-exclusion chromatography.

3.6 Effects of pH, metal ions, and inhibitors on purified RvSOD1

The optimal pH for RvSOD1 activity was investigated using potassium phosphate and Tris-HCl buffers across a pH range of 6.0–9.0. RvSOD1 exhibited maximal activity at pH 8.0 (Fig. 5d). The effects of various ions and specific inhibitors on RvSOD1 activity were assessed relative to a control (100% activity). CuCl_2 , ZnCl_2 , and CoCl_2 enhanced activity, whereas CaCl_2 , FeCl_2 , MgCl_2 , MnCl_2 , and NiCl_2 reduced it (Table 3). Potassium cyanide, hydrogen peroxide, potassium dichromate, and DL-dithiothreitol inhibited RvSOD1, while the remaining tested inhibitors had no noticeable effect (Table 4).

3.7 Antimicrobial activity of purified RvSOD1

The antimicrobial effects of purified RvSOD1 were evaluated against one fungal and four bacterial strains, with nystatin (10 μg) and ciprofloxacin (5 μg) serving as standard control drugs. RvSOD1 demonstrated antibacterial activity with inhibition rates of 49.2% against

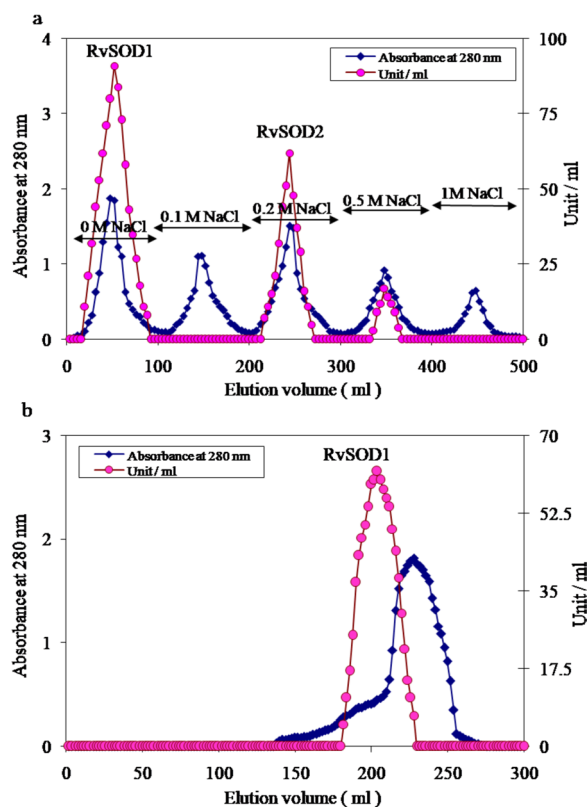


Fig. 4 **a** Chromatographic profile of *R. venosa* crude extract on a diethylaminoethyl (DEAE)-cellulose anion-exchange column (10×2.8 cm) equilibrated with 0.02 M potassium phosphate buffer (pH 7.5). **b** Chromatographic profile of the DEAE-cellulose *Rapana venosa* superoxide dismutase 1 (RvSOD1) fraction on a Sephacryl S-300 size-exclusion column (142×2.4 cm) equilibrated with 0.02 M potassium phosphate buffer (pH 7.5)

K. pneumoniae, 59.9% against *S. aureus*, 65.2% against *P. aeruginosa*, and 66.9% against *E. coli*. Although these effects were notable, ciprofloxacin showed higher activity against all tested bacterial pathogens. Importantly, RvSOD1 displayed potent antifungal activity, achieving

79.9% inhibition against *C. albicans*, compared with 98.9% for nystatin (Fig. 2).

3.8 Cytotoxic effects of purified RvSOD1

The cytotoxic effects of purified RvSOD1 were assessed on the cancer cell lines MCF-7, MDA-MB-231, and A549, as well as the normal WI-38 cell line, after 72 h using the MTT assay, with DOXO as a positive control. RvSOD1 exhibited cytotoxicity against MCF-7 and MDA-MB-231 cells, with IC_{50} values of 4.3 μ g/mL and 4.9 μ g/mL, respectively. However, its efficacy was considerably lower against A549 cells (IC_{50} = 47.2 μ g/mL) (Fig. 3b). For comparison, the IC_{50} values of DOXO were 1.844 μ g/mL for MCF-7, 1.238 μ g/mL for MDA-MB-231, and 6.89 μ g/mL for A549 (Fig. 3c). In WI-38 cells, RvSOD1 maintained viability above 85% even at the highest tested concentration, indicating low cytotoxicity toward normal cells (Fig. 3d).

4 Discussion

Marine organisms are increasingly recognized as valuable sources of biologically active compounds with therapeutic potential. Marine natural products exhibit a wide spectrum of bioactivities, including antibacterial, antiviral, anticancer, and anti-inflammatory properties [41]. Mollusks, in particular, have been reported to possess notable antioxidant, antibacterial, antifungal, and anticancer capacities [11, 25, 37, 41, 42]. Building on this context, the present study comprehensively evaluated the antioxidant, antimicrobial, and anticancer activities of the crude extract derived from *R. venosa*.

Bioactive protein extraction from *R. venosa* was achieved through precise specimen collection, taxonomic verification, and cold-chain processing. Specimens were collected from shallow Mediterranean waters (1–3 m), consistent with known habitats [43], and identified using morphological keys [23] and the World Register of Marine Species [24]. Protein extraction at 4 °C in potassium phosphate buffer (pH 7.5) preserved enzyme

Table 2 Purification summary of superoxide dismutase (SOD) isoenzymes from *R. venosa* snail tissue

Purification step	Total mg proteins	Total units	Recovery (%)	Specific activity	Fold purification
<i>R. venosa</i> crude extract	72	5731	100.0	79.6	1.0
DEAE-cellulose fractions					
0.0 M NaCl (RvSOD1)	22	3088	53.4	140.4	1.8
0.2 M NaCl (RvSOD2)	11.4	1556	27.2	136.5	1.7
Sephacryl S-300 fractions					
RvSOD1	2.9	1742	30.4	600.7	7.5

The enzyme was purified sequentially using diethylaminoethyl (DEAE)-cellulose and Sephacryl S-300 chromatography. Values represent total protein content, total enzyme units, recovery percentage, specific activity, and purification fold at each purification step

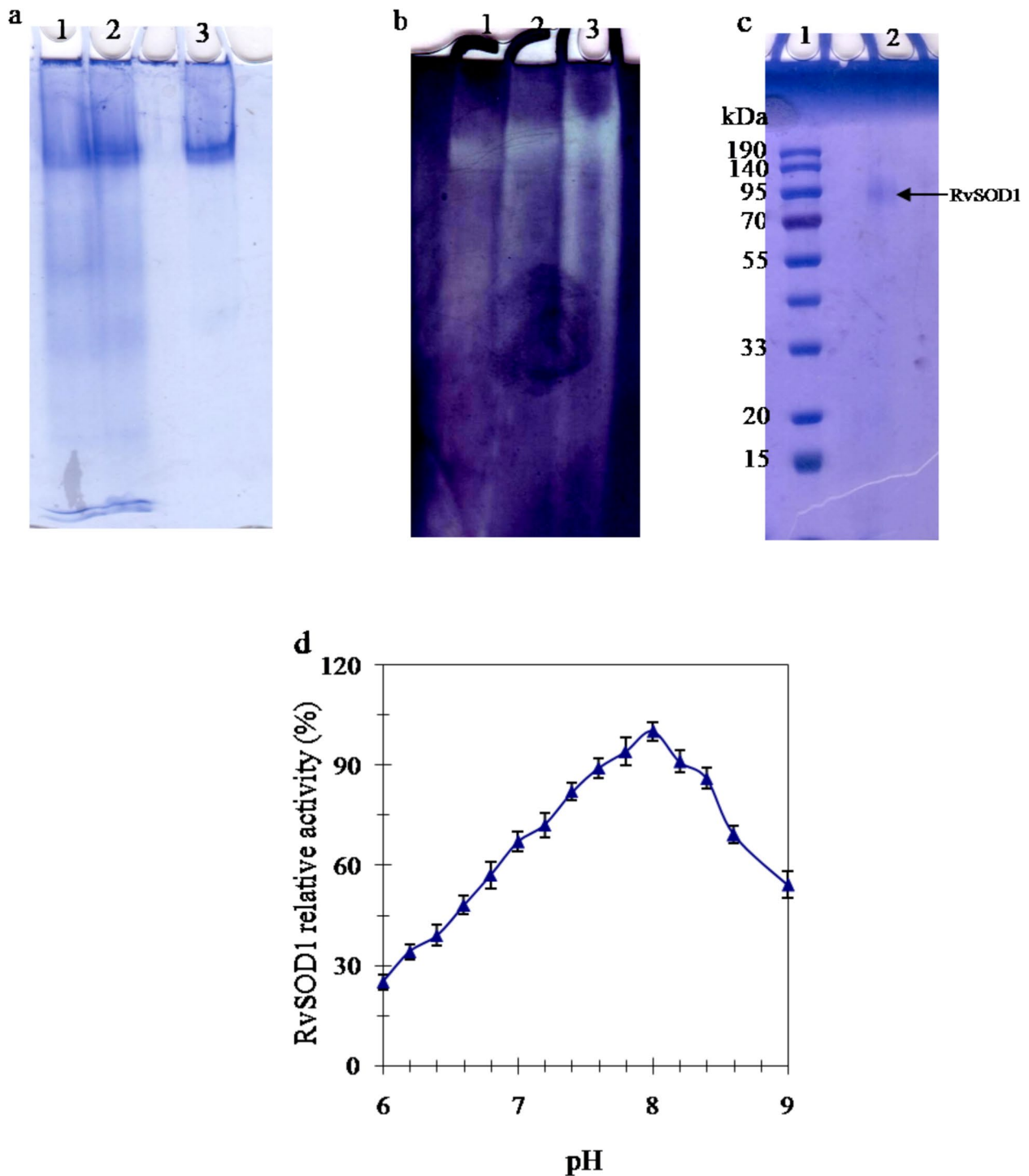


Fig. 5 **a** Protein pattern of *Rapana venosa* superoxide dismutase 1 (RvSOD1) purification steps on 7% native polyacrylamide gel electrophoresis (native-PAGE). Lane 1: *R. venosa* crude extract; lane 2: diethylaminoethyl (DEAE)-cellulose RvSOD1 fraction; lane 3: Sephacryl S-300 RvSOD1 fraction. **b** Corresponding superoxide dismutase (SOD) isoenzyme pattern of RvSOD1 purification steps on 7% native-PAGE. **c** Subunit molecular weight determination of purified RvSOD1 by 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Lane 1: molecular weight marker proteins; lane 2: purified RvSOD1. **d** Effect of pH on the activity of purified RvSOD1 from *R. venosa* snail tissue. Enzyme activity was measured in potassium phosphate buffer (pH 5.7–8.0) and Tris–HCl buffer (pH 8.0–9.2). Values represent the mean $\pm$ standard deviation (SD) of three independent experiments

Table 3 Effect of different metal ions (5 mM) on the activity of purified *Rapana venosa* superoxide dismutase 1 (RvSOD1) from *R. venosa* snail tissue

Reagent (5 mM)	Residual activity
Control	100.0
CaCl ₂	25.8 ± 3.62
CoCl ₂	106.8 ± 2.77
CuCl ₂	113.2 ± 4.29
FeCl ₂	43.1 ± 3.11
MgCl ₂	36.5 ± 2.43
MnCl ₂	56.2 ± 1.97
NiCl ₂	81.2 ± 5.03
ZnCl ₂	117.4 ± 4.32

Residual enzyme activity was expressed as a percentage relative to the control (100%). Values represent the mean ± standard deviation (SD) of three independent experiments

Table 4 Effect of different inhibitors on the activity of purified *Rapana venosa* superoxide dismutase 1 (RvSOD1) from *R. venosa* snail tissue

Inhibitor (5 mM)	Inhibition %
Control	0.0
Potassium cyanide (KCN)	43.1 ± 2.86
Hydrogen peroxide (H ₂ O ₂)	49.7 ± 3.13
Sodium Azide (NaN ₃)	6.2 ± 1.46
Sodium dodecyl sulfate (SDS)	2.1 ± 0.3
Ethylenediaminetetraacetic acid (EDTA)	3.5 ± 0.86
DL-Dithiothreitol (DTT)	56.1 ± 4.21
β-Mercaptoethanol	4.8 ± 1.17
Potassium dichromate	14.9 ± 2.04
1,10-Phenanthroline	0.8 ± 0.18

Enzyme inhibition was expressed as a percentage relative to the control (0.0%). Values represent the mean ± standard deviation (SD) of three independent experiments

activity, yielding extracts with notable antioxidant properties. This approach ensured biochemical integrity and enabled functional validation, highlighting *R. venosa* as a potential source of therapeutic bioactives. The antioxidant defense system in biological organisms relies heavily on enzymatic activities to mitigate oxidative stress caused by ROS. In *R. venosa* snail extract, four key enzymes were evaluated: CAT: 12.4 ± 2.08 U/mg, SOD: 86.3 ± 12.82 U/mg, GST: 2.7 ± 0.28 U/mg, and GPx: 0.66 ± 0.038 U/mg. Notably, SOD exhibited the highest specific activity, while GPx showed the lowest. These quantitative findings were further supported by electrophoretic analysis, which provided visual confirmation of enzyme activities and isoenzyme patterns on polyacrylamide gels. The isoenzyme analysis revealed a robust SOD activity, characterized by the presence of four distinct SOD isoenzyme bands; alongside two GST bands and one isoenzyme

band each for CAT and GPx. These observations are consistent with previous reports detecting significant levels of antioxidant enzymes such as CAT, SOD, GPx, and GR in various marine species [44, 45]. They are also concordant with prior enzyme-focused studies in mollusks—e.g., GST purified from *Turbo radiatus* and copper–zinc superoxide dismutase (Cu/Zn-SOD) purified from *Cel-lana rota*—where high antioxidant capacities paralleled downstream bioactivities [11, 25]. Collectively, the high antioxidant capacity of *R. venosa* supports a plausible role in mitigating oxidative stress-related pathologies, in line with earlier findings on molluscan extracts [42].

The antimicrobial activity of the *R. venosa* crude extract demonstrated a species-selective inhibitory profile. While it showed relatively weak inhibition against *S. aureus* (5.24%) and *K. pneumoniae* (21.2%), it exhibited significant effectiveness against *E. coli* (71.2%) and, most notably, a potent antifungal effect against *C. albicans* (90.8%). This strong antifungal efficacy suggests the presence of potent small-molecule compounds or thermostable components within the extract with broad-spectrum action. When benchmarked against ciprofloxacin, a synthetic antibiotic with universal efficiency (typically exceeding 97% across bacteria), the snail fractions showed limited comparative efficacy. However, their varied inhibitory patterns suggest unique, potentially synergistic mechanisms of action that warrant further investigation. These findings align with previous studies reporting antimicrobial activities in tissue extracts from other snail species, including *Lambis* [46], *Babylonia spirata* [47], *Tectus conus*, *Maninella alounia*, and *Trochus maculatus* [48], and they are congruent with recent demonstration of selective antibacterial effects by a purified GST from *T. radiatus* [11], SOD from *Tectus dentatus* [37], and broader antimicrobial activities observed in molluscan studies [25, 42].

In terms of cytotoxic effects, the *R. venosa* crude extract demonstrated potent activity against human cancer cell lines MCF-7, MDA-MB-231, and A549, with IC₅₀ values of 1.102, 1.513, and 1.668 µg/mL, respectively. These values were comparable to—and in some cases superior to—those of the positive control doxorubicin (DOXO) (IC₅₀ = 1.844, 1.238, and 6.89 µg/mL for MCF-7, MDA-MB-231, and A549, respectively). The minimal impact on WI-38 viability further supports the extract's safety profile and therapeutic selectivity, underscoring its promise as a source of marine-derived anticancer agents. Similar anticancer activities have been reported for extracts from marine macroalgae against MCF-7, HeLa, and Vero cell lines [49], and for different fractions of *Holothuria leucospilota* and *Echinometra mathaei* against HeLa cancer cells [50]. Importantly, our recent work on *R. venosa* peptides showed selective cytotoxicity toward breast cancer

cells (MCF-7 and MDA-MB-231) and synergy with doxorubicin, lending mechanistic plausibility to the strong effects observed here [21].

Given the remarkable and diverse SOD activity observed in the crude extract—evidenced by its high specific activity and the presence of four distinct isoenzyme bands on native gels—we proceeded to purify and characterize the most active isoenzyme, designated RvSOD1, and to evaluate its antimicrobial and anticancer activities. Marine SODs have been widely reported to contribute to antioxidant defense and to exhibit antimicrobial and anticancer effects [25, 51]. Our chromatographic workflow enriched RvSOD1 to 600.7 U/mg (7.5-fold), yielding a homogeneous enzyme on native-PAGE and an apparent ~90 kDa band on SDS-PAGE, consistent with previous Cu/Zn-SOD purification from *C. rota* and *T. dentatus* and with molluscan antioxidant enzymology [11, 25, 37].

Enzyme activity is highly dependent on the physiological conditions of the medium, particularly pH. In this study, RvSOD1 exhibited optimal activity at pH 8.0, which is consistent with the pH optima reported for SODs from pearl millet [52] and tea [53], and closely resembles that of tick larvae SOD [28]. The classification of SOD enzymes often relies on their reactivity to various ions and their sensitivity to specific inhibitors like H₂O₂ and KCN. Our results showed that RvSOD1 activity was enhanced by CoCl₂, CuCl₂, and ZnCl₂, while it was significantly inhibited by CaCl₂, FeCl₂, MgCl₂, MnCl₂, and NiCl₂. This strongly suggests that Cu²⁺ and Zn²⁺ ions play a crucial role in RvSOD1 activity, likely serving as essential cofactors. Indeed, copper and zinc are integral components of many Cu/Zn-SODs, as observed in shrimp muscle tissue [54], tick larvae [28], *C. rota* snail [25], and *T. dentatus* snail [37]. The observed inhibition of RvSOD1 by KCN and H₂O₂ further supports its classification as a Cu/Zn-SOD isoenzyme, a characteristic feature of this class known for its sensitivity to both inhibitors. Cyanide ions typically bind to the copper or zinc cofactor at the enzyme's active site, thereby preventing the dismutation of superoxide radicals, while H₂O₂ can induce oxidative damage to the enzyme's metal center. These findings are consistent with previous characterizations of Cu/Zn-SODs [25, 28, 37]. Furthermore, the potent inhibition of RvSOD1 by the reducing agent DL-dithiothreitol (DTT) suggests the presence of critical disulfide linkages within the enzyme structure that are essential for its stability and function, similar to observations in yeast SOD [55]. Overall, the biochemical fingerprint we report for RvSOD1 is highly consistent with Cu/Zn-SODs reported in marine systems [25, 37].

The antimicrobial properties of *R. venosa* snail derivatives, particularly RvSOD1, demonstrate a complex and

specific range of biological effects. RvSOD1 exhibited remarkable antifungal efficacy, achieving 79.8% inhibition against *C. albicans*. This positions RvSOD1 as a promising candidate for fungal management, potentially through oxidative destabilization processes inherent to its enzymatic action. The bacterial response, however, revealed a more nuanced susceptibility. Gram-negative species, including *E. coli*, *P. aeruginosa*, and *K. pneumoniae*, showed moderate-to-high sensitivity (66.9%, 65.2%, and 49.2% inhibition, respectively), suggesting RvSOD1's ability to penetrate or interact with their outer membrane structures. Notably, *S. aureus*, a Gram-positive strain characterized by a robust peptidoglycan matrix, also exhibited significant inhibition (59.9%), indicating a potent affinity or efficient penetration of RvSOD1 into its cellular components. This may imply a selective redox imbalance mechanism that specifically targets oxidative stress sensitive sites within these bacteria. While RvSOD1's overall potency is less than that of ciprofloxacin's almost complete bacterial suppression or nystatin's targeted antifungal effectiveness, it presents a distinctive hybrid profile encompassing both microbiological domains. This dual activity agrees with previous observations of molluscan enzyme-linked antimicrobial effects [25, 37] and selective antibacterial actions of molluscan GST [11], and complements broader bioactivity reported from marine snail extracts [42].

In terms of anticancer activity, RvSOD1 demonstrated cytotoxicity against MCF-7 and MDA-MB-231 cell lines, with IC₅₀ values of 4.3 µg/mL and 4.9 µg/mL, respectively, whereas it was considerably less effective against A549 cells (IC₅₀ = 47.2 µg/mL). Interestingly, the crude *R. venosa* extract exhibited superior efficacy against all three tested cancer cell lines compared with purified RvSOD1, suggesting that additional constituents in the crude extract may contribute to the observed activity. Importantly, RvSOD1 showed minimal cytotoxicity toward WI-38 cells, maintaining cell viability above 85% even at high concentrations, which underscores its biocompatibility and therapeutic selectivity. Similarly, *R. venosa*-derived peptides have been reported to act synergistically with doxorubicin against breast cancer cells [21]. Mechanistically, enzyme-mediated redox regulation and peptide-induced apoptosis or cell-cycle modulation represent promising targets for future validation [21, 25].

5 Conclusion

In conclusion, this study provides compelling evidence that the crude extract of the marine snail *R. venosa* possesses significant antioxidant, antimicrobial, and cytotoxic activities, suggesting the presence of novel bioactive compounds with potential applications in various biomedical fields. Furthermore, this research

represents the first successful isolation and characterization of a copper–zinc superoxide dismutase (Cu/Zn-SOD) isoenzyme, designated RvSOD1, from *R. venosa* using a straightforward purification strategy. The purified RvSOD1 demonstrated potent antimicrobial and selective anticancer properties, particularly against breast cancer cell lines, while exhibiting minimal cytotoxicity toward normal cells. These findings highlight the promising potential of *R. venosa* extract and its isolated RvSOD1 enzyme as candidates for antibacterial, antifungal, and anticancer therapies. Nevertheless, this study has limitations: (i) the bioactivity assessments were performed in vitro and therefore require in vivo validation; (ii) the molecular mechanisms underlying the observed antimicrobial and anticancer effects were not investigated; (iii) additional bioactive constituents in the crude extract were not isolated or chemically identified; and (iv) only a limited panel of microbial strains and cell lines was tested. Future studies should address these points through in vivo evaluations, mechanistic assays, and detailed compound characterization to fully assess therapeutic potential and safety.

Abbreviations

SOD	Superoxide dismutase
RvSOD1	<i>Rapanavenosa</i> Superoxide dismutase 1
ROS	Reactive oxygen species
CAT	Catalase
GST	Glutathione S-transferase
GPx	Glutathione peroxidase
GR	Glutathione reductase
BSA	Bovine serum albumin
ATCC	American type culture collection
DEAE	Diethylaminoethyl
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
IC ₅₀	Half-maximal inhibitory concentration
PAGE	Polyacrylamide gel electrophoresis
CDNB	1-Chloro-2,4-dinitrobenzene
GSH	Reduced glutathione
NBT	Nitroblue tetrazolium chloride
PMS	Phenazine methosulfate
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
PBS	Phosphate-buffered saline
DOXO	Doxorubicin
DMSO	Dimethyl sulfoxide
NADPH	Reduced nicotinamide adenine dinucleotide phosphate

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Author contribution

MFAH, MRH, and HMM designed the study; MRH SRA, and MAS collected the samples; HMM, MRH, and MAS processed samples and extracted proteins; HMM and MAS performed chromatography and enzyme characterization; SRA, MAS, MRH, and HMM analyzed data and wrote the manuscript; SRA, MFAH, MRH, and HMM reviewed the manuscript. All authors read, revised, and approved the final manuscript.

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Data availability

No datasets were generated or analyzed during the current study.

Declarations

Ethics approval and consent to participate

Ethical approval for this research was granted by the Ethics Committee of the Faculty of Science, Mansoura University (Approval No. Sci-Z-ph-2025–156).

Competing interests

The authors declare no competing interests.

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