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Antifungal activity of separated fractions from the hemolymph of marine snail *Rapana venosa*

E. Krumova[1], P. Dolashka[2], R. Abrashev[1], L. Velkova[2], A. Dolashki[2], A. Daskalova[2], V. Dishliyska[1], V. Atanasov[2], D. Kaynarov[2], M. Angelova[1]*

[1] *The Stephan Angeloff Institute of Microbiology, Bulgarian Academy of Sciences, Sofia, Bulgaria*
[2] *Institute of Organic Chemistry with Centre of Phytochemistry, Bulgarian Academy of Sciences, Acad. G. Bonchev Str. Bl. 9, 1113 Sofia, Bulgaria*

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For the first time the antifungal activities of the hemolymph isolated from mollusks marine snail *Rapana venosa* have been tested. Three protein fractions containing compounds with Mw<10 kDa (Rv/10), Mw between 10-50 kDa (Rv/10-50), and Mw between 30-100 kDa (Rv/30-100), were obtained by ultrafiltration of hemolymph with Mw bellow 100 kDa. Their effect against strains belonging to the species *Fusarium oxysporum, Penicillium griseofulvum, Alternaria solani, Mucor hiemalis, Aspergillus niger, Botrytis cinerea, and Candida albicans* was determined. Fungal growth inhibition and Minimum Inhibitory Concentration (MIC) were assayed by agar well diffusion (AWD) and broth micro-dilution (BMD) methods. The fraction Rv/30-100 was found to be the most effective against all tested strains. Other fractions, Rv/10-50 and Rv/10, displayed growth inhibitory activity against *F. oxysporum, P. griseofulvum, B. cinerea,* and *F. oxysporum*, respectively.

Key words: *Rapana venosa*; hemolymph; protein fraction; antifungal activity; fungi; MIC

INTRODUCTION

Fungal diseases are a major medical problem worldwide. These diseases are now more important and troublesome than ever before [1, 2]. Today almost a billion people suffer from skin, nail, and hair fungal infections. According to published data, many 10's of millions mucosal candidiasis and more than 150 million people have serious fungal diseases [3, 4]. The therapy for most invasive fungal diseases remains unsatisfactory given their high morbidityand mortality despite the available antifungal treatment. For example, fungal diseases such as aspergillosis have high mortality even when treated with appropriate therapy and are often incurable in hosts with impaired immunity [5]. In the near horizon, the prevalence of fungal diseases is likely to increase, as there will be more hosts with impaired immunity and drug resistance will inevitably increase after selection by antifungal drug use. Different species, belonging to genera *Aspergillus, Mucor, Penicillium, Cladosporium*, etc. have been reported as etiological agents of well-characterized respiratory disorders [6]. *Fusarium*, conventionally regarded as agents of onychomycosis, is now well known to cause fatal respiratory mycosis. *Aspergillus* spp. is the major culprit of severe asthma with fungal sensitization (SAFS), although a range of other fungi, such as *Alternaria* and *Cladosporium* spp., are also involved [3]. In line with Fungal Infection Trust (7) in 2021, nearly 20 million people are living with aspergillosis, and over a million die each year. Moreover, new pathophysiological associations hitherto unknown, such as fungal sensitization and allergic bronchopulmonary mycosis in patients with chronic obstructive pulmonary disease, are unfolding [8].

The treatments with currently used antifungal agents require long term administration protocols capable of causing toxic. The standard antifungal therapies can be also limited because of low efficacy rates and drug resistance [9, 10]. Multidrug resistance (MDR) is a serious complication during treatment of the opportunistic fungal infections that frequently afflict immunosuppressed patients [2, 11]. These patients require antifungal therapy as part of their supportive care. Despite improvement of antifungal therapies over the last 30 years, the phenomenon of antifungal resistance is still of major concern in clinical practice [12, 13].

Antifungal activity of bioactive compounds is a new direction of scientific searching. Mollusks are a huge source to discover bioactive natural products [14]. In most cases these are substances with antibacterial effect. For example, methanol extracts of sea invertebrates demonstrated activity against

* To whom all correspondence should be sent:
E-mail: *mariange_bg@yahoo.com*

Pseudomonas aeruginosa [15]. The hemolymph of several molluscan species such as sea hares, sea slung, oysters, and mussels possess antibacterial and antiviral activities [16-18]. On the other hand, different snail proteins have been studied for their antimicrobial effect. But the compounds exhibiting antifungal effect are very rare found [10, 19, 20]. For instance, crude proteins extracted from the snail *Cryptozona bistrialis* demonstrated antifungal effect against *Candida albicans*, *Penicillium chrysogenum*, *Aspergillus fumigatus*, and *Mucor racemosus* [18]. The mucus secreted by *Achatina fulica* (*Mollusca*) has potential antifungal properties [21]. Suresh *et al.* [22] determined significant growth inhibition against *Babylonia zeylanica* and *Harpa conoidalis* (Molluscas) by *Candida albicans* and *Aspergillus niger*. Even less is known about the antifungal activity of peptide fractions (antifungal peptides, AFPs) isolated from mollusks and arthropods [20]. A cysteine-rich peptide, named mytimycin, isolated from mussels *Mytilus edulis* inhibited growth of *Neurospora crassa* and *Fusarium culmorum* [23]. Such strictly antifungal peptide from *M. edulis* was reported by Charlet *et al.* [24].

In our previous studies we investigated a broad range of peptide fractions isolated from marine and terrestrial molluscs for their antifungal properties. The present study was designed to determine the effect of fractions isolated from the hemolymph of marine snail *R. venosa* against several fungal strains belonging to the genera *Aspergillus, Penicillium, Fusariun, Alternaria, Mucor, Botrytis*, and *Candida*.

EXPERIMENTAL

Isolation of bioactive fractions from the hemolymph of marine snail R. venosa

The hemolymph was collected, which lives freely in the Black Sea. It is known that hemolymph of *R. venosa* contained above 90% hemocyanin as a major protein. After several purification steps, including filtration, centrifugation at 5000xg at 4 °C for 20 minutes to remove coarse particles and haemocytes and anew filtration, a crude hemolymph extract was obtained [25]. This extract was subjected to ultrafiltration under pressure (4 bar) on a 100 kDa membrane (Millipore Ultrafiltration Membrane Filters) of Amicon® Stirred Cell, which resulted in two fractions - one fraction with molecular masses (Mw) above 100 kDa to obtain hemocyanin, and another fraction with Mw bellow 100 kDa. Hemolymph with Mw bellow 100 kDa was separated by ultrafiltration in three protein fractions containing compounds with Mw<10 kDa (Rv/10), Mw between 10-50 kDa (Rv/10-50), and Mw between 30-100 kDa (Rv/30-100).

Disc membranes from ultracel regenerated cellulose from 10 NMW, 30 NMW 50 NMW and 100 NMW (Millipore™ Corporation, Billerica, U.S.A) were used for processes of ultrafiltration.

SDS-PAGE Electrophoresis

Protein fractions were analyzed by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE), according to Laemmli method with modifications [26]. Equal volumes containing approximately 20 μg/lane of the samples dissolved in Laemmli sample buffer and protein standard mixture (Precision Plus Protein., All Blue, Bio-Rad, Feldkirchen, Germany) were separated by 12.5% SDS-PAGE and visualized by staining with Coomassie Brilliant Blue G-250.

Microorganisms and culture conditions

Fungal strains *Fusarium oxysporum* NBIMCC 124, *Penicillium griseofulvum* P29, *Alternaria solani* CBS 106.21, *Mucor hiemalis* M2, *Aspergillus niger* 26, *Botrytis cinerea* NBIMCC 120, and *Candida albicans* 14 were used in this study. They were chosen as representatives of fungal infection. All of them belong to the Mycological collection of the Stephan Angeloff Institute of Microbiology, Sofia. Long-term preservation of these fungi was carried out in the Microbank system (Prolab Diagnostics, Richmond Hill, Canada) consists of sterile vials that contain 25 porous, colored beads and a cryopreservative fluid at -80°C. Before use, the conidiospores were grown on Beer agar medium [27] at 28 °C for 7 days.

Preparation of standardized spore suspension

Fungal strains were freshly subcultured on sterile on potato-dextrose agar (PDA) and incubated at 28 °C for 7 days. The resultant spores were washed into sterile solution of Triton X-100 and adjusted to a concentration of $2x10^8$ spores/mL.

Antifungal activity assay by agar well diffusion (AWD) method

The antifungal activity was assayed trough a diffusion technique on PDA growth medium. Spore suspension of each fungal strain (200 μL) was spread onto the surface of the Petri dishes. Then, 10-mm-diameter holes were punched and filled with 100 μL of the previously prepared sampling fractions in decreasing dilutions (0.65, 0.32, and 0.17 μg/mL). The used concentrations were selected based on our preliminary experiments. As control samples for each variant, Triton X-100 (negative control) and the fungicide nystatin (0.1%, positive control) were used. Subsequently, the plates were incubated at 28 °C. Each extract form was evaluated with 3 repetitions, and the assessment was conducted after 24, 48, 72, and 168 h by measuring the diameter of the inhibition of the fungi mycelial growth (clear zone of inhibition formed around were considered indicative of antifungal activity).

Antifungal activity assay by broth micro-dilution (BMD) method

Antifungal activity was tested by determining the minimum inhibitory concentration (MIC) using the microdilution method with resazurin, an indicator of microbial growth [23]. In general, the 96-well plates were prepared by dispensing into each well 50 μL of potato dextrose broth ammended with 50 μL tested fraction at 0.32, 0.17, 0.08, and 0.04 μg/mL. Then, 10 μL spore suspension and 30 μL of 0.02% resazurin were added and plates were incubated at 28 °C. Effect of tested fraction on fungal growth was evaluated after 24, 48, 72, and 168 h by visual inspection. Control samples contained 50 μL nystatin in concentration 1 mg/mL instead of tested fraction.

The MIC was determined as the lowest concentrations that caused complete growth inhibition (100%) compared with control probes without antifungal compounds.

RESULTS AND DISCUSSION

The hemolymph of *R. venosa* is rich in different bioactive components. Up to now some extracellular proteins are identified, such as actin and several FUs of *R. venosa* hemocyanin, the remaining proteins are unknown. Therefore, the aim of our study was to evaluate the antifungal effect of three fractions isolated from *R. venosa* hemolymph with Mw <100, containing different natural compounds. The main protein in the hemolymph of the sea snail (over 90%) is *R. venosa* hemocyanin (RvH) characterized previously [25, 29]. Moreover, antimicrobial proline-rich peptides with molecular masses below 10 kDa isolated from the hemolymph of marine snail *R. venosa* have been studied [30]. They have demonstrated antibacterial activity against Gram-positive (*S. aureus*) and Gram-negative (*K. pneumoniae*) bacteria [30].

The distribution of the proteins and peptides in three fractions of *R. venosa* hemolymph - Rv/10, Rv/10-50 and Rv/30-100 is represented in the 12.5% SDS-PAGE (Fig. 1). It was detected that the fraction below 10 kDa contained peptides with molecular masses between 3000 and 9500 Da, determined by mass spectrometric analysis in the study [25]. The presence of proteins in the region between 35-45 kDa was observed in both fractions. The proteins with Mw at ~ 16 kDa ~ 20 kDa and ~ 25 kDa are specific for fraction Rv/10-50. The protein band at ~26 kDa and ~30 kDa are more extensively expressed in fraction Rv/10-50 in comparison to the other fraction. The proteins with Mw at ~ 52 kDa, ~ 65 kDa and ~ 100 kDa are specific for fraction Rv/30-100. Moreover, on this lane, traces from protein bands with Mw above 100 kDa were observed (Fig. 1).

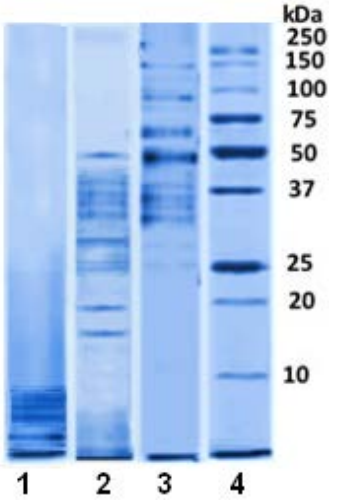

Fig. 1. 12.5% SDS-PAGE analysis, visualized by staining with Coomassie G-250, position: 1) peptide fraction with Mw<10 kDa; 2) Fraction from *R. venosa* hemolymph with Mw 10-50 kDa; 3) Fraction from *R. venosa* hemolymph with Mw 30-100 kDa; 4) Molecular weights of standard proteins from Bio-Rad.

In this study, three fractions (Rv/10, Rv/10-50 and Rv/30-100) of *R. venosa* were tested against seven potentially pathogenic fungal strains. Table 1 shows the antifungal activity determined by AWD method. Among these, the fraction Rv/30-100

Table 1. Antifungal inhibitory activity of the extracts using AWD method.

	Fractions	Antifungal effect						
		F. ox.	***P. gr.***	***A. sl.***	***M. mh.***	***A. ng.***	***B. cn.***	***C. al.***
1	Rv/10-50	FC/48***	N/I	N/I	N/I	N/I	FC/48***	N/I
2	Rv/10	N/I	N/I	N/I	N/I	N/I	N/I	N/I
3	Rv/30-100	FC/48** RG/72***	FC/48** RG/168***	FC/168*	FC/168*	FC/48**	FC/168*	FC/168*

Note: Fc – fungicidal activity; Fs – fungistatic activity; RG –retarded growth; N/I = no inhibition; 24-168 – number of hours; Minimal concentration dose * - 0.17; ** - 0.32; *** - 0.65 µg/mL. F. ox – *F. oxysporum*; P. gr. – *P. griseofulvum*; A. sl. – *A. solani*; M. mh. - *M. hiemalis*; A. ng. - *A. niger*; B. cn. - *B. cinerea*; C. al. - *C. albicans*

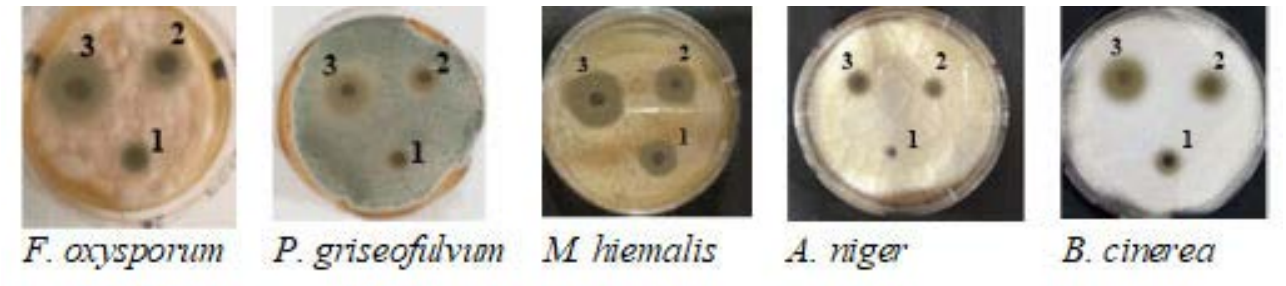

Fig. 2. Fungal growth inhibitory activity of the fraction Rv/30-100 in a concentration: 1 - 0.17 µg/mL; 2 - 0.32 µg/mL; 3 - 0.65 µg/mL.

Table 2. Antifungal inhibitory activity of the plant extracts using broth microdilution method.

№	Fractions	Antifungal effect						
		F. ox.	***P. gr.***	***A. sl.***	***M. mh.***	***A. ng.***	***B. cn.***	***C. al.***
1	Rv/10-50	IG/24****	FS/48****	N/I	N/I	N/I	FC/48***	N/I
2	Rv/10	IG/24****/	N/I	N/I	N/I	N/I	N/I	N/I
3	Rv/30-100	IG/48*** RG/72***	IG/24*** RG/72****	IG/168***	IG/168***	IG/48***	IG/168***	IG/72****

Note: IG – inhibition effect on mycelium growth; N/I = no inhibition effect; 24-168 – number of hours; Minimal concentration * - 0.04, ** 0.08, *** 0.17, **** 0.32 µg/mL; F. ox – *F. oxysporum*; P. gr. – *P. griseofulvum*; A. sl. – *A. solani*; M. mh. - *M. hiemalis*; A. ng. - *A. niger*; B. cn. - *B. cinerea*; C. al. - *C. albicans*

exhibited clear fungicidal effect towards all the seven tested fungi. These results are also demonstrated in Fig. 2.

At the same time, the fraction Rv/10-50 displayed growth inhibitory activity against *F. oxysporum* and *B. cinerea*. In contrast, fraction Rv/10 showed negative activity against all tested fungal strains. The most sensitive fungal strains were *F. oxysporum* and *B. cinerea* that demonstrated fungicidal effect towards two fractions (Rv/10-50 and Rv/30-100), followed by *P. griseofulvum, A. solani*, *M. hiemalis*, *A. niger*, and *C. albicans* whose growth was inhibited by Rv/30-100 only. It should be emphasized that the fraction Rv/30-100 revealed the most significant antifungal activity compared to other two fractions.

The antifungal activity of the *R. venosa* fractions was tested also using BMD method with resazurin (7-Hydroxy-3H-phenoxazin-3-one 10-oxide). The resazurin is a blue dye which can be irreversibly reduced to a pink by oxidoreductase within viable cells. The results concerning growth inhibition effect on used fungal strains are shown in Table 2.

Visual inspection was sufficient to easily identify strains that are more sensitive to *R. venosa* fractions, i.e., their wells show more purple or less pink color than the control after a set time of incubation. The fraction Rv/30-100 showed significant activity against all strains tested (Fig. 3). This fraction completely inhibited the growth after 24 h of incubation for *P. griseofulvum*, 48 h for *F. oxysporum* and *A. niger*, 72 h for *C. albicans*, and 168 h for *A. solani*, *M. hiemalis*, and *B. cinerea*. The fraction Rv/10-50 proved less effective then Rv/30-100. Among the 7 tested species, 3 species (*F. oxysporum*, *P. griseofulvum*, and *B. cinerea*) showed positive results for antifungal activity for 24 or 48 h. Surprisingly, the fraction Rv/10

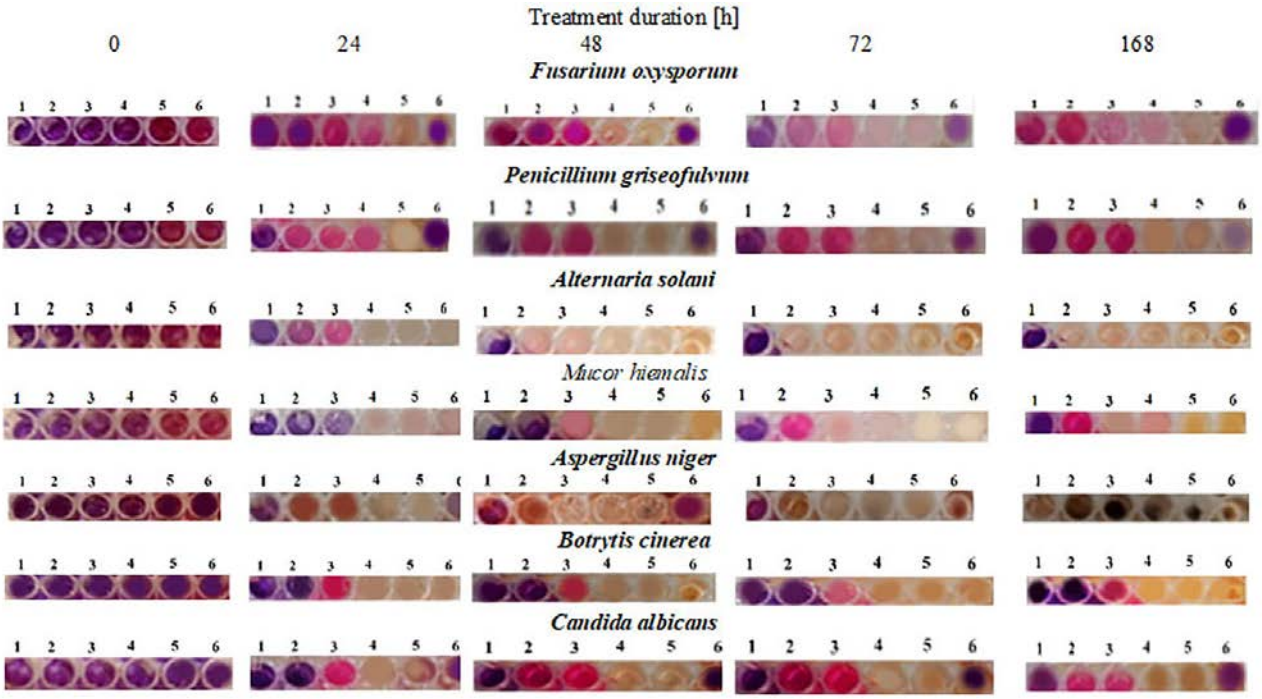

Fig. 3. Fungal growth inhibition by protein fraction Rv/30-100. Note: 24-168 – Number of hours; Minimal concentration: 1 - 0.32; 2- 0.17; 3- 0.08; 4 - 0.04 µg/mL; 5 – Negative control (non-treated culture); 7 – Positive control (culture treated with nystatin).

Table 3. The MIC values of *R. venosa* fractions against tested fungal strains assayed by AWD and BMD methods.

Strain	Fractions/MIC (µg/mL)					
	AWD method			BMD method		
	Rv/10-50	Rv/10	Rv/30-100	Rv/10-50	Rv/10	Rv/30-100
F. oxysporum	0.65	-	0.32	0.32	0.32	0.17
P. griseofulvum	-	-	0.32	0.32	-	0.17
A. solani	-	-	0.32	-	-	0.17
M. hiemalis	-	-	0.17	-	-	0.17
A. niger	-	-	0.32	-	-	0.17
B. cinerea	0.65	-	0.17	0.32	-	0.17
C. albicans	-	-	0.17	-	-	0.32

Note: Not detected (-)

exhibited antifungal activity against *F. oxysporum* (for 24 h). It should be noted that the fraction Rv/30-100 proved to be more suitable for growth inhibition of the strains *A. solani*, *M. hiemalis*, and *Botrytis cinerea* compared to the antifungal drug nystatin (Fig. 3).

The results shown in Table 3 give information about the MIC values determined by both the methods, AWD and BDM. The fraction Rv/30-100 was most effective having MIC value with widest spectrum of antifungal activity compared to the other tested fractions. Its MIC value according AWD method was 0.17 µg/mL against *M. hiemalis*, *B. cinerea*, and *C. albicans* and 0.32 µg/mL against *F. oxysporum*, *P. griseofulvum*, *A. solani* and *A. niger*. The MIC value for Rv/10-50 was 0.65 µg/mL against *F. oxysporum* and *B. cinerea*. The fraction Rv/10 showed non-detectable antifungal activity for the tested strains. As can be seen in Table 3, MIC values assayed by BDM method were 2-fold lower that those obtained by AWD method. Furthermore, the BDM method allowed to determine a MIC dose for Rv/10 against *F. oxysporum* (0.32 µg/mL). The two protein fractions have common proteins as well as specific proteins. Therefore, we hypothesized that differences in the observed antifungal activity against various tested pathogen strains is likely due to the specific proteins of each of both fractions. The similar antifungal activity against *F. oxysporum* and *B. cinerea* is probably due to the proteins common for both fractions, but present in different concentrations.

Taken together, our results revealed that the fraction containing proteins with Mw 30-100 is the most active against the growth of a wide range of fungal strains. This effect can be explained by clearly expressed antifungal activity of these proteins.

In the last three decades marine mollusks become a favorable object for searching biologically active molecules with health benefits, including antibacterial and antifungal activity. Potential antifungal activity of extracts of marine mollusks has been reported by Umayaparvathi *et al.* [31]. The authors suggested that this effect is due to the presence of antifungal peptide. The results of Ulagesan and Kim [22] also demonstrated that proteins extracted from seven different snails act as the bioactive compound against the pathogenic fungi belonging to the genera *Mucor*, *Aspergillus*, *Penicillium*, and *Candida*. Similar results have been described about plasma of the mussel *Mytilus galloprovincialis* [32] and *Perna viridis* [33], molluscs from the family *Muricidae* [34]. In contrast, the efforts of many authors to determined antifungal activity of mollusks and arthropods remain without a positive result [35]. It was proved that peptides from freshwater snail (*Pomacea insularium*) and crab (*Callinectes sapidus*) haemolymph ranged in molecular mass from 9 to 110 kDa and 40 to 100 kDa, respectively, possesses high antimicrobial activity but not antifungal one [36]. Our main finding is that the fractions from *R. venosa* hemolymph have significant inhibition effect on the growth of potentially pathogenic fungi. It should be noted that the amount of MIC was lower compared to the reported results against strains belonging to the genera *Candida*, *Penicillium*, *Aspergillus*, *Mucor*, etc. [18, 19, 37].

CONCLUSION

In the present study, the bioactive fractions from the hemolymph of marine snail *R. venosa* were found to be promising source of highly potent antifungal agents. The used fractions exhibited remarkable activity against seven potentially pathogenic fungal strains. The results clearly demonstrated that the fraction containing proteins with Mw 30-100 used in a concentration 0.17 µg/mL, completely inhibited the fungal growth for a long period (168 h). Moreover, the fractions Rv/10-50 and Rv/10 in a concentration 0.32 µg/mL could be useful against *F. oxysporum*, *P. griseofulvum*, *B. cinerea*, and *F. oxysporum*, respectively.

Dedication: *We dedicate this article to Prof. Wolfgang Voelter from the University of Tuebingen, Germany, who died on January 21, 2021.*

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