Contents lists available at ScienceDirect

Asian Pacific Journal of Tropical Medicine

journal homepage:www.elsevier.com/locate/apjtm

Document heading doi:

Preliminary studies for a new antibiotic from the marine mollusk *Melo melo* (Lightfoot, 1786)

Kanagasabapathy Sivasubramanian*, Samuthirapandian Ravichandran, Kumaresan M

Centre of Advanced Study in Marine Biology, Annamalai University, Parangipettai 608502, India

ARTICLE INFO

Article history:
Received 20 December 2010
Received in revised form 27 January 2011
Accepted 15 February 2011
Available online 20 April 2011

Keywords:
Antibacterial
Antifungal
TLC
SDS-PAGE
Melo melo

ABSTRACT

Objective: To search for new bioactive compounds from marine mollusks *Melo melo (M. melo)*. **Methods:** Preliminary work for bioactive compound was identified by using disc diffusion methods against human pathogens. Further analyses of compound were done by using TLC, SDS-PAGE. And also estimate the amount of protein in the samples by following Biuret method. **Results:** In antibacterial activity the maximum diameter of 24 mm zone of inhibition was recorded against *Klebsiella pneumoniae (K. pneumoniae)* strain of the mucus extract and minimum zone of inhibition of 11 mm was observed in *Salmonella typhi (S. typhi)* strain of body tissue extract. The antifungal activity of the extraction shows maximum activity against *Trichophyton mentagarophytes (T. mentagarophytes)* (14 mm) and minimum activity was recorded in *Aspergillus flavus (A. flavus)* (11 mm). The extract of mucus, nerve tissue, body tissue and kidney that showed antimicrobial activity was subjected to TLC to determine the presence of the peptides and amide groups, and also subjected to SDS-PAGE to estimate the molecular weight of proteins in a clear band were detected in the gel that represented kidney, body tissue, brain and mucus represent 14, 17, 22, 45 kDa. **Conclusions:** The extracts from marine mollusks *M. melo* is the potential source of producing bioactive compounds against human pathogens and can be used for synthesis of new drugs.

1. Introduction

Marine organisms have been used for medical purposes in India, China, the nearest and Europe, since ancient time. In the recent past, several pharmacological substances of marine origin have been developed. The discovery of the antibiotic activity of *Penicillium notatum (P. notatum)* by Fleming in 1929 has revolutionized medical science loading to the invention of the wonder drug "Penicillin". The "cepholothin" (marketed as 'Keflin' by Lilly pharmaceutical co. USA; Osol *et al* 1967) is an antibiotic, active against number of penicillin resistant bacteria. The term drug need not be limited to its strictly medical usage, but should be synonymous with the term "bioactive compounds" as it also denotes any chemical substance that a specific physiological function.

The wealth of antibiotics that have been isolated from terrestrial organisms is in extreme contrast to the poverty of drugs that have been obtained from marine source. Bioactive substances from marine mollusks gained prominence in recent years. The mollusks constitute the largest group of biotoxic marine invertebrates, widely distributed throughout the world. In gastropods, several species viz, *Haliospecies rufescens (H. rufescens)*, Australian dorid nudibranches, have shown to have high antimicrobial activity. Among mollusks, the cephalopods have largely been neglected for their biomedical research. Some of the mollusks are believed to cure certain diseases like asthma and rickets. Some marine mollusks have shown pronounced activities, useful in the biomedical arena. The potential of marine mollusks as a source of biologically active products is largely unexplored in India[1]. Hence, a broad based screening of marine mollusks for bioactive compounds is necessary.

*Corresponding author: Mr. Sivasubramanian K, Ph.D, Research Scholar, Centre of Advanced Study in Marine Biology, Annamalai University, Parangipettai- 608 502, India.
Tel: +91 9159781715
Fax: 04144 243555
E-mail: siva4516@yahoo.co.in

2. Materials and methods

2.1. Collection and identification

Mollusks was collected from the Pazhayaar landing center (Lat 11°21′32.27″ N; long 79°49′24.92″ E) (Figure 1). *Melo melo (M. melo)* were identified based on the taxonomical features (Lightfoot, 1786). The sample *M. melo* was washed extensively with water upon collection as were dissected the different target organ (*i.e.*, nerves tissue, mucus, body tissue and kidney). The samples were transfer to 100% methanol. Collected organs were stored at −20 ℃ before extraction.

Figure 1. Location of the sampling site.

2.2. Extraction

The collected specimens′ organs were homogenized by crushing the tissue in mortar and pestle. The samples were extracted with methanol and than partitioned with chloroform.

2.3. Anti-microbial activity

2.3.1. Microbial strain used

Antibacterial activity of mucus, nerves tissue, body tissue and kidney extract of Mollusks was screened against 10 different bacterial strains *Staphylococcus aureus (S. aureus)*, *Salmonella typhi (Salmonella typhi)*, *Salmonella paratyphi (S. paratyphi)*, *Klebsiella oxytoca (K. oxytoca)*, *Pseudomonas aeruginosa (P. aeruginosa)*, *Escherichia coli (E. coli)*, *Proteus mirabilis (P. mirabilis)*, *Vibrio cholera (V. cholera)*, *V. parahemolyticus*, *Klebsilla pneumonia (K. pneumonia)* and ten fungal strains *Aspergillus niger (A. niger)*, *Aspergillus flavus (A. flavus)*, *Alternaria alternaria (A. alternaria)*, *Candida albicans (C. albicans)*, *Epidermophyton floccossum (E. floccossum)*, *Trichophyton mentagrophytes (T. mentagrophytes)*, *Trichophyton rubrum (T. rubrum)*, *Pencillium* sp, *Rhizopus* sp, *Mucor* sp.

These pathogen strains were obtained from the Department of Medical Microbiology (Raja Muthiah Medical College hospital) Annamalai University, Annamalai Nagar.

2.3.2 Anti-microbial assay of crude extract

In vitro anti-microbial assay was carried out by disc diffusion method.

2.4. Thin-layer chromatography (TLC)

As the soluble fractions showed ninhydrin positive spots they were subjected to purification by gel permeation chromatography sephadex LH 20 using methanol as eluent and monitored by TLC. Diagnostic thin layer chromatography was performed on methanol and chloroform extracts. They were also spotted and plates developed in varying proportions. Detection was done with specific color reagent ninhydrin, for detecting the compounds.

2.5. Estimation of protein

Mucus, nerve tissue, body tissue and kidney were collected from *M. melo* with a fine sterile scissor. The amount of protein was measured by spectrometry according to the method of following using a calibration curve prepared with different concentration of (0.1-0.5 mg/mL) of bovine serum albumin (BSA) as standard. Biuret reagent was used as a color reactant and concentration was calculated in response to absorbance at 540 nm in spectrophotometer (spectro UK-VIS RS).

2.6. Determination of molecular weight by using SDS-PAGE

SDS-PAGE was performed in 12% separating gels, according to the method described by Laemmli (1970). The reference proteins for molecular weight estimation were different molecular weight marker proteins, which were used in alpha actinin (97 kDa), alpha internexin (66 kDa), podoplanin (43 kDa), calretinin (29 kDa), CDK6 inhibiting protein (18 kDa), ubiquitin conjugating enzyme (E2) (14 kDa). The electrophoresis was carried out at constant 100 V for 3 h. Following electrophoresis, the protein bands were visualized by staining with Coomassie Brilliant-250.

3. Results

3.1. Antimicrobial assay

Antibacterial activity of mucus, nerves tissue, and body tissue and kidney extract of *M. melo* was evaluated comparing to the positive control tetracycline (C). The zone of inhibition in different bacterial strains against

M. melo organs is shown in Figure 2. Among the various strains maximum diameter of (24 mm) zone of inhibition was recorded in *K. pneumoniae* against mucus sample and lowest zone of inhibition of (11 mm) was observed in *V. parahemolyticus* and *S.typhi* strain against nerve tissue and body tissue samples respectively. Among the tested ten pathogenic strains *V. cholerae* , *P. aeruginosa*, *E. coli* and *K. oxytoca* shows negative activity and rest of them shown positive activity.

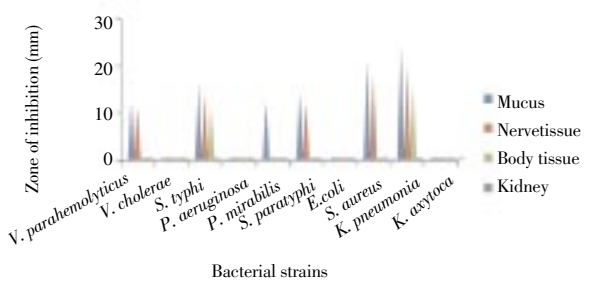

Figure 2. Antibacterial activity of *M. melo* against bacterial strain.

The antibacterial agent of tetracycline showed activity against all the tested bacterial strains. Maximum activity against *P. aeruginosa* was 25 mm and the minimum activity was observed against *P. mirablis* (16 mm).

Antifungal activity of the different organs of *M. melo* was used for the present study. The positive control agents fluconazole (C) showed activity against all the fungal strains tested. The maximum activity against *A. niger* was 17 mm and the minimum activity was observed against *C. albicans* (14 mm). Concerning the antifungal activity of the different organs of *M. melo* the maximum zone was observed against *T. mentagarophytes* strain against mucus sample and the minimum inhibition zone was observed in *A. flavus* strain against body tissue sample. Among the ten pathogenic strains *C. albicans*, *Mucor* sp, *Rhizopus*, *E. floccosum*, *T. rubrum* shows negative activity Figure 3.

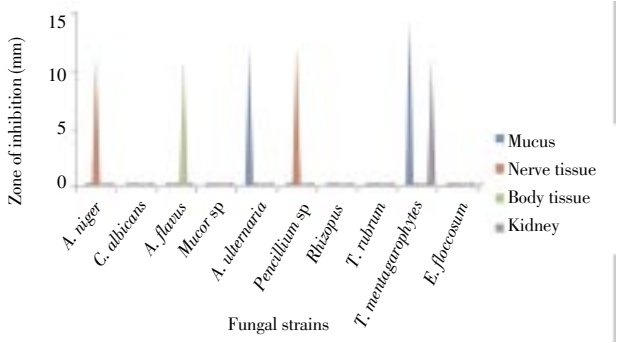

Figure 3. Antifungal activity of *M. melo* against fungal strains.

3.2. Thin layer chromatography

TLC profiling was done for the samples of nerves tissue and mucus extract in solvent system of butanol, acetic acid and water (B:A:W) in proportions of 5:1:4. The plates when developed in the solvent systems showed light pink spots in the case of nerves tissue and mucus extract, when the TLC plate was sprayed with ninhydrin. The plate with fractions developed in BAW as the solvent system and sprayed with ninhydrin, showing pink spots indicating the presence of amino acids and peptides is shown in Figure 4.

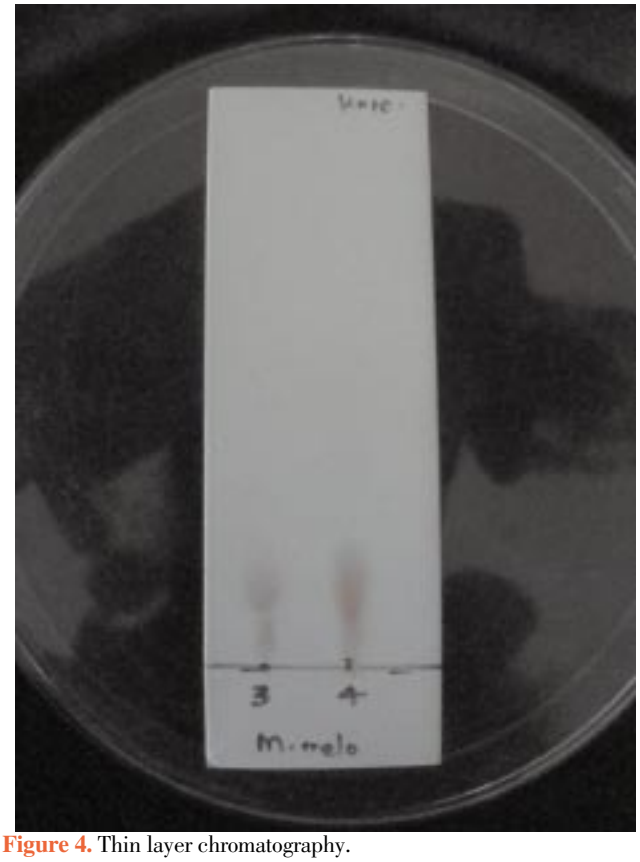

Figure 4. Thin layer chromatography.

3.3. Estimation of protein

Extracted samples were centrifuged and a supernatant part is aspirated through micropipette. 0.5 mL separated supernatant part is subjected to protein quantification by Biuret method. The amount of protein present in the mucus extract is 11.6%, body tissue 15.8%, kidney 0.5% and the nerves tissues extract contains 12.3%.

3.4. Determination of molecular weight by using SDS-PAGE

The *M. melo* samples showed antibacterial activity was subjected to SDS-PAGE to estimate the molecular weight of proteins present in it. Different standard were used to determine the molecular weight of sample proteins. The stained gel revealed that the sample contained a simple population of proteins. They are different molecular weight marker proteins used in alpha actinin (97 kDa), alpha internexin (66 kDa), podoplanin (43 kDa), calretinin (29 kDa), CDK6 inhibiting protein (18 kDa), ubiquitin conjugating enzyme (E2) (14 kDa). The clear bands are detected in the gel of kidney, body tissue, nerve tissue and

mucus represent 14, 17, 22, 45 kDa is shown in Figure 5.

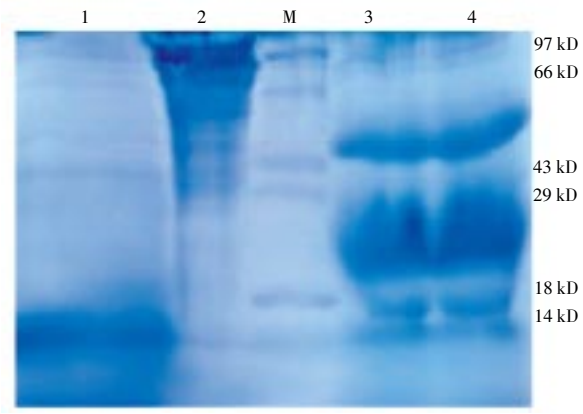

Figure 5. SDS PAGE Lane.
M: Molecular weight marker proteins.
Alpha actinin (97 kDa), alpha internexin (66 kDa), podoplanin (43 kDa), calretinin (29 kDa), CDK6 inhibiting protein (18 kDa), ubiquitin conjugating enzyme (E2) (14 kDa).

4. Discussion

In the present investigation a prominent antimicrobial activity has been observed against some bacterial strains. The methanol extraction of *M. melo* shows activity against both bacterial and fungal strains. In antibacterial activity the maximum diameter of zone of inhibition was recorded in *K. pneumoniae* strain and minimum zone of inhibition was observed in *S. typhi* strain. The antifungal activity of the *M. melo* against the fungal strains the maximum activity was showed against *T. mentagarophytes* and minimum activity showed against *A. niger*. The study of bioactive compound of coral associated gastropod, *Trochus tentorium* against the human pathogens has shown the similar result[1]. Dolastatins are a group of cyclic and linear peptides isolated from the marine mollusk *Dolabella auricularia*, with prominent cell growth suppressing activity. From mollusks it has been also isolated another prominent family of peptides, in this case highly compact and stable linear peptides, known as conotoxins but with specific actions on ion channels and membrane receptors of excitable cells[2]. The antimicrobial activity of gill extraction of *Perna viridis* was observed maximum against *S. aureus*, *P. mirabilis*, *K. pneumonia* and *K. oxytoca*[3]. This study corroborates the results of present investigation. Very similar to this pronounced inhibition was conferred by the acetone extracts of *D. margariticola* against dreadful human bacterial pathogens[4].

The presence of antibacterial compound in the oyster *Pteria chinensis*, crab *Portunus sanguinolentus* and bivalve *Perna viridis* has been reported using the various solvent extracts[5, 10], the recombinant abalone lysozyme of *Haliotis discus hannai* was determined by using turbidimetric assays[6]. Bursatellanin-P, a 60 kDa protein was purified from the purple ink of the sea hare *Bursatella leachii*. The protein exhibited anti-HIV activity. The first total syntheses of aplyolides B-E, ichthyotoxic macrolides isolated from the skin of sea hare *Aplysia depilans*, have been reported confirming the absolute stereochemistry reported for the metabolites[7]. A saccharothrixmicine were isolated from the actinomycete *Saccharothrix espanaensis* An 113 associated with the marine mollusk *Anadara broughtoni* containing fraction exhibited activity towards *C. albicans* and *Xanthomonas* sp. *pv. badrii* where as the diketopiperazines showed antibiotic activities against *V. alginolyticus* and *V. parahaemolyticus*[8].Marine mollusk antimicrobial peptides, specifically those isolated from mussels, scallops, oysters, venerid clams and abalone, which mainly include MGD, mytilin, myticin, mytimycin, big defensin, and RPD-1. Their structural characteristics, antibacterial activity, and expression pattern as well as peptide distribution and their release following microbial challenge[9].

It was found, that mollusk specimens are colonized by a community of diverse cultivable microorganisms, which may supply their host bivalve with bioactive metabolites providing vital functions or chemical protection from colonization by opportunistic microorganisms. The study of marine mollusc-associated bacteria is of importance for our understanding of their ecological role in the interaction with animals and between themselves, and also for their biotechnological application as producers of bioactive compounds[11]. The polysaccharides have been extracted from the cuttlebone of *S. aculeata* and *S. brevimana* showed promising antibacterial and antifungal activity against the human pathogenic strains[12,13]. Two antimicrobial peptides, defensins A and B, Mytilins A and B, Mytimycin Myticin A and B display antibacterial activity against gram-positive bacteria, and myticin B is active against the fungus *Fusarium oxysporum* and gram-negative bacteria *E. coli* D31 from *Mytilus edulis* and *Mytilus galloprovincialis*. In addition this, antibacterial activity has been measured in unfractionated plasma from the mussel *Geukensia demissa* and from the oyster *Crassostrea virginica*[14]. In the present study methanol extract of mucus, nerve tissue, body tissue and kidney that showed antimicrobial activity was subjected to TLC to determine the presence of the peptides and amide groups and also subjected to SDS-PAGE to estimate the number and molecular weight of proteins present. After electrophoresis the clear band were detected in the gel which represented proteins of molecular weight of kidney, body tissue, brain and mucus represent 14, 17, 22, 45 kDa. From the present study the marine mollusks *M. melo* have the potential of antimicrobial substance and also used for synthesis of the new drugs formulation.

Conflict of interest statement

We declare that we have no conflict of interest.

Acknowledgements

Authors are thankful to the Dean, Faculty of Marine science ,Centre of Advanced Study in Marine Biology Annamalai University, Parangipettai for their encouragement and provided facilities.

References

[1] Anbuselvi S, Chellaram C, Sreenivasan RS, Jonesh S, Jayanthi L, Edward JKP. Bioactive potential of coral associated gastropod, *Trochus tentorium* of Gulf of Mannar, Southeastern India. *J Med Sci* 2009; **9**(5): 240-244.

[2] Aneiros A, Garateix A. Bioactive peptides from marine sources: pharmacological properties and isolation procedures. *J Chromatogr B* 2004; **803**: 41-53.

[3] Chandran B, Rameshkumar G, Ravichandran S. Antimicrobial activity from the gill extraction of *Perna viridis* (Linnaeus; 1758). *Global J Biotechnol Biochem* 2009; **4**(2): 88-92.

[4] Chellaram C, Sreenivasan RS, Jonesh S, Anand TP, Edward JKP. *In vitro* antibiotic bustle of coral reef associated gastropod, *Drupa margariticola* (Broderip, 1832) of tuticorin coastal waters, *Southeastern India. Biotechnol* 2009; **8**: 456-461.

[5] Ding J, Li J, Bao Y, Li L, Wu F, Zhang G. Molecular characterization of a mollusk chicken-type lysozyme gene from *haliotis discus hannai* ino, and the antimicrobial activity of its recombinant protein. *Fish Shellfish Immunol* 2011: **30**(1): 163-172.

[6] Chellaram C, Gnanambal KM, Edward JKP. Antibacterial activity of the winged oyster, *Pteria chinensis* (Pterioida: Pteridae). *Indian J Mar Sci* 2004; **33**: 369 -372.

[7] Jha RK, Xu Zi-rong. Biomedical compounds from marine organisms. *Mar Drugs* 2004; **2**: 123-146.

[8] Kalinovskaya NI, Kalinovsky AI, Romanenko LA, Dmitrenok PS, Kuznetsova TA. New angucyclines and antimicrobial diketopiperazines from the marine mollusk-derived actinomycete Saccharothrix espanaensis An 113. *Nat Prod Commun* 2010; **5**(4): 597-602.

[9] Li C, Song L, Zhao J. A review of advances in research on marine molluscan antimicrobial peptides and their potential application in aquaculture. *Molluscan Res* 2009; **29**: 17-26.

[10] Maripandi A, Prakash Ali L, Al-Salamah A. HPTLC Separation of antibacterial compounds from *Perna viridis* and *Portunus sanguinolentus* and its activity tested against common bacterial pathogens. *Adv Biotech* 2010; **9**(9): 24-28.

[11] Romanenko LA, Uchino M, Kalinovskaya NI, Mikhailov VV. Isolation, phylogenetic analysis and screening of marine mollusc-associated bacteria for antimicrobial, hemolytic and surface activities. *Microbiol Res* 2008; **163**(6): 633-644.

[12] Santhana Ramasamy M, Murugan A. Potential antimicrobial activity of marine molluscs from Tuticorin, Southeast coast of India against 40 biofilm bacteria. *J Shellfish Res* 2005; **24**(1): 243-251.

[13] Shanmugam A, Mahalakshmi TS, Vino AB. Antimicrobial activity of polysaccharide isolated from the cuttlebone of *Sepia aculeate* (Orbingy, 1848) and *Sepia brevimana* (Steenstrup, 1875): an approach to selected antimicrobial activity for human pathogenic microorganisms. *J Fish Aquatyic Sci* 2008; **3**: 268-274.

[14] Tincu JA, Taylor SW. Antimicrobial peptides from marine invertebrates. *Antimicrobial Agents Chemother* 2004; **48**: 3645-3654.