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BIOACTIVITY, PHYLOGENETIC AND CHEMICAL ANALYSIS OF BIVALVE *MERETRIX CASTA*

Chandra B. Maurya[1]* and Sarika Chhabria Talreja[2]

[1]Department of Chemistry, G. N. Khalsa College, Matunga, Mumbai, India.

[2]Department of Chemistry, Smt. C.H.M College, Ulhasnagar, Maharashtra, India.

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***Corresponding Author**
Dr. Chandra B. Maurya
Department of Chemistry,
G. N. Khalsa College,
Matunga, Mumbai, India.

ABSTRACT

Meretrix casta is a marine clam found along both the coasts of India. Earlier commonly distributed, it has recently received a rare status by International Union of Conservation highlighting the need for more scientific studies on it using modern molecular tools. The present study reports antimicrobial activity of the extract prepared from this bivalve mussel using petroleum ether. Phylogenetic analysis of the mussel was carried out using the sequence of the amplified 18S rRNA gene and comparing it with the nucleotide database. Thin layer chromatography of the bioactive extract revealed the presence of steroids.

KEYWORDS: *Meretrix casta*, 18S rRNA, bivalve mussel, phylogenetic, bioactivity.

INTRODUCTION

Fish and shellfish are important sources of dietary proteins. Shellfish fishery is an important sector of fishing industries throughout the world. India has a rich diversity of clams and oysters. *Meretrix casta* (Chemnitz) is a mollusc (Bivalvia: Family Veneridae) of considerable commercial importance and occurs in estuaries and backwaters of both the east and west coasts of India. It is a common edible species of clam along the Konkan region of India.

Better known as the backwater clam, *M. casta* has received considerable scientific attention and there are many research papers on it. However, majority of the studies are focused on studying it's general biology [1,2], growth aspects [3,4], dimensional relationships [5,6], Geo-biochemical aspects [7,8], toxicity studies [9,10] and population dynamics [11] and there are hardly any reports available on its phylogenetic analysis and potential bioactivity.

Considering this, the present investigation was undertaken on *M. casta* collected from Malvan coastal region of Maharashtra State, India.

MATERIALS AND METHODS

Collection of Mussels

Meretrix casta was collected from coastal habitat of Malvan, Sindhudurg district (Maharashtra). After collection, fifty bivalve specimens were cleaned with freshwater and de-shelled.

Preparation of mussel extract

The meat (250 g) and mantle fluid (approx. 40 ml) was collected in a beaker. The meat of the bivalve was finely chopped and extracted with 500 ml of petroleum ether (60:80). The mixture was sonicated for 30 mins and the extract was filtered and collected separately. The extraction procedure was repeated two more times, every time by adding fresh petroleum ether. The extract obtained from three repetitions was pooled together and solvent was evaporated by using rotary evaporator. The dried extract (18 g) was kept in refrigerator till further use.

Antimicrobial activity of bivalve extract

Antibacterial activity was tested in triplicate using the standard paper disc diffusion method. Stock solution of bivalve petroleum ether extract was prepared by dissolving in 50 mg/ml of petroleum ether. The test concentrations of extract were 100 µg, 500 µg and 1 mg per disc. The extract from stock solution was applied to sterile paper discs (6 mm in diameter). The discs were dried before they were placed onto agar plates that had been seeded with reference microbial strains. The diameters of the inhibition zones (diameter of inhibition zone minus diameter of disc) were measured in millimeters after incubation at 30°C for 24 hours. Solvent (pet. ether) control discs without extract prepared in the same manner were never observed to inhibit bacterial growth.

Phylogenetic analysis of *M. casta*

Extraction and quantification of shellfish DNA

DNA Extraction from bivalve sample (*M. casta*) was carried out using G1N10 GenElute Genomic DNA extraction kit of Sigma–Aldrich. The frozen tissue (20 mg) was treated with lysis solution, Proteinase K and RNase A solution. The extracted DNA was stored at 4°C till further use. Agarose gel electrophoresis was performed to check the presence of DNA using

0.8% Agarose. DNA concentration was determined using Quant-iT^{TM} dsDNA BR Assay Kit of in vitrogen. The concentrated DNA samples were then stored at -20°C till further use.

PCR amplification of 18S rRNA gene

PCR amplification was carried out following the method of Williams and Ozawa [12]. The PCR reaction mix contained 2.5µl of 10X buffer, 1µl of each primer (18S5 forward and 18S1100 reverse), 2.5 µl of 2.5mM of each dNTP, 2.5 Units of Taq DNA polymerase and 1µl Template DNA and 8.5µl nuclease free water. The PCR amplification cycle consists of, a cycle of 5 min at 94°C; 35 cycles of 1 min at 94°C, 1 min at respective annealing temperature, 2 min at 72°C; and additionally 1 cycle of 7min at 72°C. The reagents used are procured from Bangalore GeNei. Agarose Gel electrophoresis of PCR product was carried out and molecular weights were compared with High range DNA ruler 100bp to 10kb of GeNei (61265297100A). Gel picture was taken through UV transilluminator and the size of PCR product was determined.

Sequencing of PCR products and Blast analysis

The bivalve 18S rRNA gene PCR product was purified using PCR product purification kit and was sequenced at MWG Biotech, Bangalore, India. The chromatograms and sequences obtained were analysed by using Basic Local Alignment Search Tool (BLAST). Nucleotide – nucleotide BLAST was carried with facility of National Center for Biotechnology Information (NCBI). A phylogenetic tree was constructed and the evolutionary history was inferred using the Neighbour-Joining method. Evolutionary analyses were conducted in MEGA5.

Thin layer chromatography of bivalve crude extract

TLC of crude petroleum ether extract obtained from bivalve was performed on a silica gel 60 plate (Merck). The solvent system petroleum ether: ethyl acetate (80:20, v/v) was used for separation of organic compounds on plate. TLC plate was checked by spraying with 10% ethanolic solution of sulphuric acid followed by heating at 100° C for 2 to 4 mins. The plate was observed for the presence of compounds.

Qualitative test for presence of steroids in crude extract

In order to check steroids in crude petroleum ether extract of bivalve a qualitative test was carried out. Crude extract 0.05 g was dissolved in 2 ml chloroform. 2 ml each of concentrated

Sulphuric acid and acetic acid was added in the extract. After addition, the tube was checked greenish colour development [13].

RESULTS

Antimicrobial activity of bivalve extract

The crude petroleum ether extract of bivalve *M. casta* showed a promising antimicrobial activity against a few test organisms. Highest inhibition was seen that of *Salmonella typhi* with a zone of inhibition of 11 mm and 10 mm against *Aspergillus* sp. No activity was observed against *Staphylococcus aureus* at any concentration. The extract could not show any activity against *E. coli* and *Vibrio cholera* at 50 µg/disc concentration but showed the activity of 3 and 8 mm at 100 µg/disc concentration (Table 1).

Table 1: Antimicrobial activity of crude extract obtained from bivalve.

Test bacterial strains	50µg/disc	100µg/disc
Aspergillus sp.	6±1	10±1
Escherichia coli	-	3±1
Pseudomonas aeroginosa	4±1	7±1
Staphylococcus aureus	-	-
Vibrio cholera	-	8±1
Salmonella typhi	4±1	11±1

Phylogenetic analysis of *M. casta*

Gel electrophoresis indicated that the PCR product size obtained was approximately 1100 bp for the amplified 18S rRNA gene of the clam *M. casta*. This gene when sequences yielded a product of 675 base pairs as shown in the fig. 1.

>18S rRNA *M. casta* (Sequence length: 675bp)

TGACGAAAAATAACAATGCGGGACTCTTTCGAGGCCTCGTAATTGAAATGAGTA
CACTCTAAATCCTTGACACGAGGATCCATTGGAGGGCAAGTCTGGTGCCAGCAG
CCGCGGTAATTCCAGCTCCAATAGCGTATACACGGGTTGCTGCAGTTAAAAAGCT
CGTAGTTGGATCTCGGGCGCGGGCTCGCGGTGTGCTCGTCGAGGCGACCATCGCT
TGTCCAAGCCTCCCAGCCGGACATTGAAAGTCCCTGGTGCTCTTCATCGAGCGTC
TTGGACGGCCGGCGTGTTTACTTTGAAGAAATTAGAGTGCTCAAAGCAGGTCTAG
CCAGCCTGAATACTGTTGCATGGAATAATGGAATAGGACCTCGGTTCTATTTTGT
TGGTTTTGAGAGCACGAGGTAATGATTAAGGAAGCTGACGGGGGCTTACGTACT
GCGGCGCGAGAGGTGAAATTCTGTGACCGTCGCAAGACGAACGAGAGCGAAAG
CATTTGCCAAGCATGTTTTCGTTAATCAAGAACGAAAGTCAGAGGTTCGAAGAC
GATCAGATACCGTCGTAGTTCTGACTGTAAACTATGCCAACTGTCGATCCGCCGT
GGTTACTTCAATGACCCGGTGTGCAGCCTCCGGGAAACCAAAGTCTTTGGGTTCT
GGGGGGGAGTATGGTTGCAAA

Figure 1: 18S rRNA gene sequence of *Meretrix casta.*

This nucleotide sequence when analyzed with BLAST tool indicated that the 18S rRNA gene sequence of *M. casta* was 99% identical to the existing gene sequence of 18S ribosomal RNA of the other marine clams belonging to the genus *Meretrix* such as *M. lyrata*, *M. lusoria* and *M. meretrix*. The gene sequence was also found to be 90% similar to the gene sequence of marine clams such as *Callista brevisiphonata, Callista disrupta, Saxidomus purpuratus* and *Venus verrucosa* indicating an existence of a connecting link that is still present between different marine mussels during the course of evolution (Table 2). The phylogenetic tree drawn also showed the evolutionary relationship between the *M. casta* and its neighbours (Fig. 2).

Table 2: BLAST results of *M. casta* 18S rRNA gene sequences.

Description	Max score	Identity	Accession
Meretrix lyrata isolate MeMly 18S ribosomal RNA gene, partial sequence	1531	99%	JN996715.1
Meretrix lusoria isolate MeMlu 18S ribosomal RNA gene, partial sequence	1526	99%	JN996714.1
Meretrix meretrix 18S ribosomal RNA gene, complete sequence	1526	99%	EF426291.1
Callista brevisiphonata isolate CaCbr 18S ribosomal RNA gene, partial sequence	1062	90%	JN996709.1
Callista disrupta partial 18S rRNA gene, specimen voucher BMNH 20070276 (Natural History Museum, London)	1062	90%	AM774567.1
Saxidomus purpuratus 18S ribosomal RNA gene, complete sequence	1062	90%	EF426294.1
Venus verrucosa voucher BivAToL-176 18S ribosomal RNA gene, partial sequence	1057	90%	KC429391.1
Chione elevata voucher BivAToL-4 18S ribosomal RNA gene, partial sequence	1057	90%	KC429387.1
Irus mitis gene for 18S rRNA, partial sequence, specimen_voucher: personal:Goto R.:GOTO_41	1057	90%	AB714785.1
Ruditapes variegatus isolate GN8 18S ribosomal RNA gene, partial sequence	1057	90%	JN807348.1

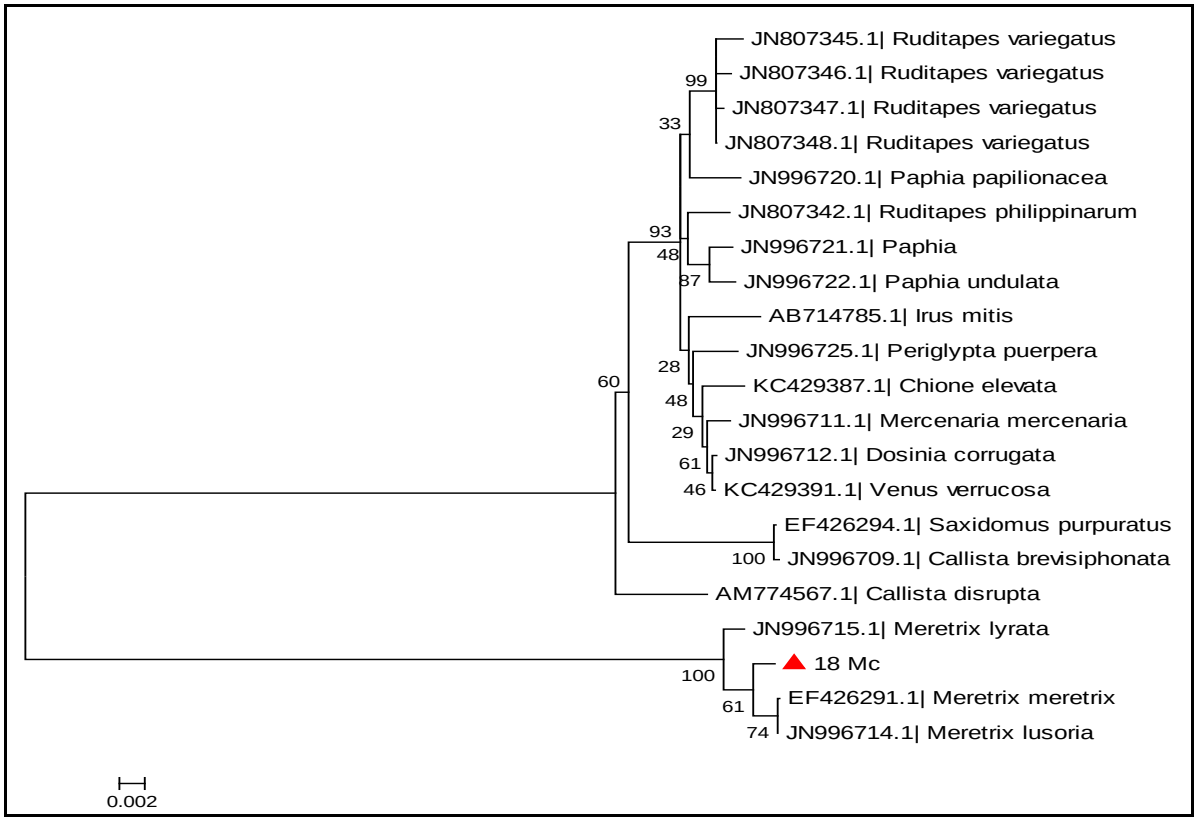

Figure 2: Phylogenetic tree showing evolutionary relationships of *M. casta*

Thin layer chromatography of the bivalve extract

The developed TLC plate is shown in Figure 3. After using very specific spray reagents, TLC of the crude extract showed presence of steroids. TLC of the crude extract showed more than one compound. The RF value was not possible to calculate but presence of steroids was detected.

Figure 3: TLC of the crude bioactive petroleum ether extract of bivalve.

Biochemical analysis of the bivalve extract

When sulphuric acid and acetic acid were added to the crude petroleum ether extract of the bivalve, formation of a dark green colouration was observed which indicated the presence of steroids (Fig. 4).

Figure 4: Detection of steroid in crude bioactive petroleum ether extract of bivalve.

DISCUSSION

Crude petroleum extract of *M. casta* showed promising inhibition of both fungi and bacteria indicating its broad antimicrobial spectrum. Results of the present study and those by Mariappan *et al*., [14] who reported encouraging bioactivity of extracts prepared from mussels *M. casta* and *Tridacna maxima* against human pathogens highlight that bivalve extracts could have potential bioactivity. Mariappan *et al*., (2010) used ethanol, methanol, hexane, acetone, butanol and water to prepare bivalve extracts. However, they did not use petroleum ether to make extracts. Therefore, this is a maiden report and more studies are ongoing to characterize the bioactive fraction. As indicated by TLC and chemical analysis, the bioactivity of the extract could be attributed to the steroids which were detected in the extract.

Reliable species identification is prerequisite to undertake any scientific, ecological, genetic and conservation studies. Mussel species cannot be easily distinguished based on morphology as many shell characters tend to overlap. Hence, any research could be biased when only shell morphology or morphometry is used for species identification. Therefore, it is strongly recommended that sophisticated molecular marker such as 18S rRNA should be employed as a diagnostic tool.

The results of the present study clearly indicate usefulness of 18S rRNA gene sequencing to confirm the phylogenetic position of the marine clam *Meretrix casta* and suggest that such markers can effectively identify a given bivalve mussel up to its generic level.

So observations made in the present study vindicate the earlier reports by Rice *et al*., [15], Adamkewicz *et al.*, [16], Maruyama *et al*., [17], Canapa *et al*., [18], Espiñeira *et al*., [19], Upadhye *et al*., [20] and Magare *et al*., [21] who have already suggested that the 18S rRNA comparative studies of various molluscan taxa can be reliable in elucidating the phyletic relationships below the class level.

CONCLUSION

The crude extract of bivalve *M. casta* obtained in non polar petroleum ether showed very good antimicrobial activity against *Aspergillus* sp. and *S. typhi*. The bioactivity of the crude extract was found to be due to the presence of steroid. The bioactive producing metabolites bivalve was identified as *Meretrix casta* with the help of shell morphology and upto genus level as *Meretrix* sp. using 18S rRNA gene sequences.

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