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Antibiotic activity of different solvent extracts of the marine gastropod *Thais bufo* against *Staphylococcus aureus* and *Streptococcus pyogenes*

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ABSTRACT

Objective: To evaluate the antimicrobial activity of different solvent extracts of the species *Thais bufo* against the *Streptococcus pyogenes* and *Staphylococcus aureus*.

Methods: Different types of solvents were used such as acetone, benzene, butanol, chloroform, ethanol, ethyl acetate, hexane, methanol, toluene and distilled water for extraction, based on the polarity basis for the purpose of minimizing the process and error in identification and characterization of the active compound.

Results: Regarding the percentage of yield, aqueous extract yielded the highest percentage, following that methanolic extract and other extracts in the order of ethanol, hexane, chloroform, ethyl acetate, butanol, benzene, acetone and toluene. In the perception of antibiotic activity, ethyl acetate and methanol showed better activity against both the species. Through the results, it is clear that the methanolic extract have mild antimicrobial activity. The antimicrobial activity in the form of zone of inhibition was below the value of 7.5 mm. Further, the qualitative analysis was carried out only for the methanolic extract, due to its overall activity. Through the qualitative analysis by thin layer chromatography it happened to know that 3 bands were formed and their R_f values were 0.07, 0.17 and 0.44 respectively.

Conclusions: The results showed that the solvent extracts of the species *Thais bufo* have no potential antibiotic activity, hence it cannot be considered for the further research in the field of drug discovery.

1. Introduction

About 80% of the organisms in the world is present in ocean[1], which include food, fragrances, pigments, insecticides and therapeutic drug candidates *etc.* Around 60 marine compounds have been found to have a potency of immune and nervous systems, some anti-diabetic and anti-inflammatory effects have also been reported. Finally, 68 promising molecules extracted from marine sources were found to interact with an array of molecular targets and receptors, which may probably contribute to develop various pharmacologically active classes of drugs[2]. Specifically phylum Mollusc are the possible source of pharmacologically active compound among the marine invertebrates. Bioactive compounds from marine mollusc are reported

to have the antitumor, antileukaemic, antibacterial and antiviral activities specifically from class bivalves[3] and gastropods[4]. These are the common marine organisms distributed across the Indian sea beaches and estuaries. Simply, the molluscs are gaining the importance in driving drugs, addition to that they are considered to be an important factor in the concern of ecology and economics[5].

In view of nature of the compounds, it was reported that some of the pharmacological activities are due to the presence of polysaccharide, specifically the sulphated mucopolysaccharides[6]. Except this sulphated carbohydrate, some of the secondary metabolites was also analyzed and reported, still the overall secondary metabolites investigated from the molluscan species are < 1%. Out of the natural products obtained from marine lives, sponges comprise 33%, 18% from coelenterates (sea whips, sea fans and soft corals), 24% from other invertebrates phyla such as ascidians, opisthobranch of mollusc, echinoderms and bryozoans[7]. While considering the ecological significance of the bioactive compounds (secondary metabolites), the marine organisms produce the bioactive compounds in response

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to the ecological pressure such as competition for space, deterrence of predation and the ability to reproduce successfully. Likewise, the marine molluscs have the purpose to produce secondary metabolites as evolved chemical defense (antimicrobial compound) to protect eggs from the microbial infection[8]. The characteristics of the class bivalves and gastropods specifically the divers of chemical classes and drug leads interaction of the natural product chemists[9]. On the view of historical perspective, since 1950's the attempt was made to screen the antimicrobial compounds from the marine organisms. In continuation to that, marine organisms of many phyla were screened in large numbers for the identification of antimicrobial compounds[10]. As the results of the above program, around 300 bioactive compounds as marine natural products were patented between the time boundaries of 1960's to 1990's[11].

Following that the authors are interested to note the importance of the present research work that a serious problem to be considered nowadays is the development of resistance to antibiotics by human pathogenic microorganisms[12], efficacy of the drugs and side effects. In concert to the side effects, synthetic drugs have more adverse side effects while comparing to the naturally derived drugs[13]. In order to overcome the issues, search for natural products from natural source specifically from the marine source may be beneficial.

1.1. Rheumatic heart disease

Though there are many kinds of human pathogenic microorganism, the main purpose of choosing *Staphylococcus aureus* (*S. aureus*) and *Streptococcus pyogenes* (*S. pyogenes*) is because these are reported to responsible for the illness of rheumatic fever and rheumatic heart disease. Among these two species, *S. pyogenes* is the main causative pathogen causing infection on children and adolescents. Further, it associates with a wide spectrum of infections and diseases[14], among that the rheumatic fever and rheumatic heart disease are the prominent conditions to be considered, hence few of the properties alone is given that *S. pyogenes* is the only species in group A *Streptococcus*, and it is a Gram-positive coccoid-shaped bacterium. On media, it forms white to grey colonies with a clear zone of β -hemolysis on blood agar. As mentioned earlier, *S. pyogenes* is responsible for the group A streptococcal infection, which leads to the acute rheumatic fever, an inflammatory disorder and a serious problem of rheumatic heart disease. In many countries, rheumatic heart disease is the most common acquired disease, most specifically in developing countries, and mostly affecting the children. It can be prevented and treated by the antibiotics. The infection mainly affects heart, joints and central nervous system. Further the rheumatic fever cause the fibrosis of heart valves, it leads to crippling valvular heart disease, heart failure and death[15]. Following the illness named rheumatic fever, an additional complication is associated called carditis, is the condition. It occurs in 50% to 60%

of the acute rheumatic fever cases, finally it leads to morbidity and mortality[14]. Therefore, the present study was focused to evaluate the antimicrobial activity of different solvent extracts of the species *Thais bufo* against the given human pathogenic microorganisms, most specifically against the *S. pyogenes* and *S. aureus*.

The main objective of the present research work was to screen the metabolite to check whether the compounds having antibiotic activity (antimicrobial activity), hence it not only intended to focus only on the antimicrobial activity, but also to concentrate on the properties and importance of solvents for the efficient metabolites extraction and safety purposes. In this current research work, different types of solvents were used such as acetone, benzene, butanol, chloroform, ethanol, ethyl acetate, hexane, methanol, toluene and distilled water, based on the polarity basis for the purpose of minimizing the process and error in identification and characterization of the active compound, in case of the most promising and considerable activities. In the aspect of efficient extraction, the polarity index is the important factor to be considered, but bases on the hazardous nature solvents are classified as 3 types, the solvents selected for the present research work coming under all the 3 categories. Those classes of solvents and alternative way of replacing the solvents with best solvating property but having hazardous property are discussed in the section of discussion.

2. Materials and methods

2.1. Sample collection and identification

Live species of *Thais bufo* was collected from the coastal area of Kovalam, Southeast coast of Tamil Nadu, India. The collected animals were washed with the sea water roughly, packed in icebox and transported to the laboratory, where the animals were washed with fresh water and then with distilled water. The collected samples were identified by the standard literature[16]. Later the shells were broken with small hammer to collect the tissue.

2.2. Extraction

Around 100 g of the tissue was obtained from the *Thais bufo*, and extracted with distilled water and other solvents. The 100 g of the tissue was kept in a cleaned mortar and pestle and homogenized well. Then the homogenized tissue was divided into several portions to get the extracts of solvents such as acetone, benzene, butanol, chloroform, ethanol, ethyl acetate, hexane, methanol, toluene and distilled water. The exact quantity of the tissue extract is divided into 10 g each, following that the respective solvents were added to the flasks and mixed well with the help of vortex for 10 min. Then the mixture was centrifuged at 15000 r/min for 30 min for the aqueous sample, for the solvent mixture filtration was preferred to separate the tissue debris

and the organic phase, was collected in different flasks.

2.3. Extraction yield

Quantity of the extracts obtained from each 10 g of the tissue homogenate was determined. The clean and dry flasks were marked and preweighed, then the organic extracts were collected in the separate preweighed flasks. Then the solvent extracts alone condensed in a water bath at 100 °C. Finally the condensed extracts were dried in a hot air oven at 75 °C and cooled in a desiccator then weighed to calculate the extraction yield. While considering the aqueous extract, rotary vacuum evaporator was used to condense the extract and followed the same procedure to determine the extraction yield.

2.4. Microbial cultures

Human pathogenic species such as *S. aureus* and *S. pyogenes* were used for the antibiotic activity and these were obtained from Academy of Maritime Education and Training University, Katangulathur, Tamilnadu.

2.5. Inoculum preparation

Himedia nutrient broth was prepared and sterilized in the autoclave at the temperature of about 121 °C and 6.8 kg pressure for 15 min. Then *S. aureus* was inoculated in the sterilized nutrient broth and incubated at 37 °C for 24 h. To carry out the disc diffusion method of antibiotic activity, Mueller-Hinton agar was prepared, sterilized in the autoclave at the same conditions done for broth sterilization. After the sterilization the media was poured into the sterilized Petri plates in the sterile laminar blower and allowed the media to solidify. Then the 24 h old bacterial broth culture was inoculated using the sterile cotton swabs on the solidified Mueller-Hinton agar in the Petri plate[17]. But for *S. pyogenes*, 5% blood Mueller-Hinton agar plates were used. After placing the sample discs, plates were incubated with 5% CO₂ at 37 °C for 20 h, the same conditions was used for *S. pyogenes* inoculation broth preparation[4].

2.6. Antibacterial activity

Twenty-four hours old bacterial strains were used in the antibacterial activities. The antimicrobial activity of the tissue extracts were followed by standard disc diffusion method by Kelman *et al.*[17]. In this method, the microbial strains were swabbed on the respective agar plates and discs (Whatman No.1 filter paper with 9 mm diameter) were impregnated with the gastropod tissue extracts. Then the Petri plates were incubated with the same condition mentioned in the inoculum preparation. The susceptibility of the test organisms were determined

by radius of the inhibition zone formed around the disc. The sterile discs impregnated with the corresponding solvents were used as the negative controls and the erythromycin 15 µg and penicillin G 1 µg were used as positive controls.

2.7. Qualitative analysis of the extracts by thin layer chromatography (TLC)

Aqueous and the solvent extracts were included for the simple qualitative analysis. Merck, TLC silica gel 60 F254 (20 cm × 20 cm) were used. The TLC was carried out only for the methanolic extract to check the metabolites extracted. While considering the mobile phase, different combinations of solvents as mobile phase was used with different polarities for the purpose of better separation of the metabolites. The TLC plates were exposed to the universal detection agent “iodine vapor” and all the plates were visualized directly after drying and observed under UV light at two different wave lengths of about 254 and 366 nm in UV TLC plate viewer. The partition of the analytes on the TLC plate were measured and expressed as R_f .

3. Results

3.1. Percentage yield of tissue extracts

While considering the percentage of yield, aqueous extract yield the highest percentage, following that methanolic extract and other extracts in the order of ethanol, hexane, chloroform, ethyl acetate, butanol, benzene, acetone and toluene, as shown in Table 1 and Figure 1. In the perception of antibiotic activity, ethyl acetate and methanol showed better activity against both the species. Through the results it is clear that the methanolic extract have mild antimicrobial activity, those were denoted in Figure 2. The antimicrobial activity in the form of zone of inhibition was below the value of 7.5 mm. The main reason for qualitative analysis of methanolic extract alone is due to its overall activity. Through the simple qualitative analysis by TLC it happened to know that 3 bands were formed, R_f value for those were 0.07, 0.17 and 0.44 respectively, as shown in Figure 3.

Table 1
Percentage of yield.

Solvents used	Percentage of yield
Acetone	1.5
Benzene	2.0
Butanol	3.2
Chloroform	4.0
Ethanol	10.0
Ethyl acetate	3.8
Hexane	8.0
Methanol	25.0
Toluene	1.2
Distilled water	28.0

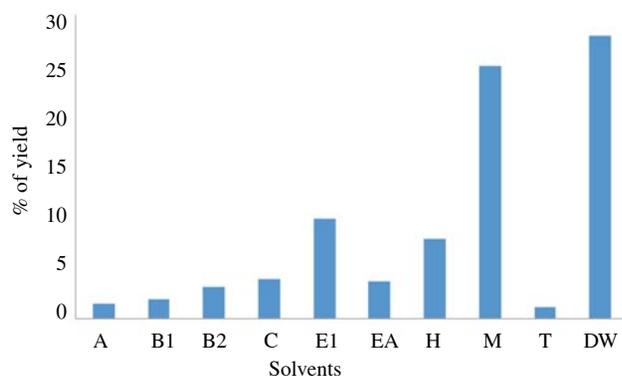


Figure 1. Percentage yield.

A: Acetone; B1: Benzene; B2: Butanol; C: Chloroform; E: Ethanol; EA: Ethyl acetate; H: Hexane; M: Methanol; T: Toluene; DW: Distilled water.

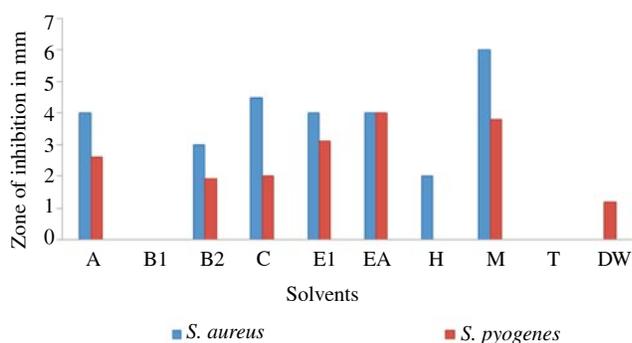


Figure 2. Antimicrobial activity.

A: Acetone; B1: Benzene; B2: Butanol; C: Chloroform; E: Ethanol; EA: Ethyl acetate; H: Hexane; M: Methanol; T: Toluene; DW: Distilled water.



Figure 3. Qualitative analysis of methanol extract by TLC.

4. Discussion

The reason of variation in the antimicrobial activity of the gastropod extracts may be depend on extraction capacity of the solvents and the compounds extracted[18]. From results of the present research, it could be seen the fact that the aqueous and methanolic extracts give the highest percentage of yield but the aqueous extract didn't show considerable result. Through extracts of many organisms showing antimicrobial activity, actually they have not been active enough to complete with classical antimicrobial activity against the pathogenic microorganisms[19]. In the present research work, even the methanolic extracts shows antimicrobial activity, the overall antimicrobial activity is less than 7.5 mm in diameter. It is not the considerable zone of inhibition, further, it cannot be recommended as a source of good antimicrobial compound. Hence considering and recommending the species *Thais bufo* as the source of good antimicrobial compound is worthless.

Further, the antimicrobial activity of some of the marine mollusk are discussed here that the methanolic extracts of *Hemifusus pugilinus* and *Anadara granosa* was reported to highly active against *E. coli* and less effective against *Klebsiella oxytoca* and *Salmonella typhi* respectively[20]. Likewise the acetone extract of the species *Trochus tentorium* was exhibited to have activity against the human pathogen *Streptococcus pneumonia*[21]. Except the scientifically proven biologically active species, some of the marine mollusc are traditionally used as folk medicine, for example the bivalve *Meretrix meretrix*, it was used to treat many liver diseases like jaundice, hepatitis A and B in India and China[22].

As mentioned in the introduction part, solvent extraction is not the major objective of the present research work, some important facts to be discussed here regarding the solvents, choice of usage, daily exposure limit of the solvents and their classes. Based on the effect of solvent on humans and the environment solvents are classified as 3 types such as Class 1, Class 2, and Class 3 solvents. In addition to a general regarded as safe solvents[9]. These classes represents the important hints that solvents to be avoided, solvents to be limited and solvents with low toxic potentials respectively, of which Class 1 symbolized as human carcinogens, environmental hazards, Class 2 solvents are non genotoxic animal carcinogens and Class 3 solvents are consider to be low toxic potential to man and this type of solvents have the permitted daily exposure value of 50 mg or more per day or permitted daily exposure can be in terms of mg/day.

While considering the terms of representation of the exposure limits of toxic chemicals, different terms are using such as tolerance daily intake and acceptable daily intake which is used by the international program on chemical safety and the World Health Organization and other national and international health authorities

and institutes. Except that a new term is defined that is permitted daily exposure which is defined as a pharmaceutically acceptable intake of residual solvents to avoid confusion of differing values for acceptable daily intake's of the same substance[23]. In connection to the above context, the main purpose of representing the terms and exposure limits are to minimize the hazardous nature of such solvents by limiting the exposure time. For example, the aim of permitted daily exposure is to ensure human safety[24]. But the solvents used in the preparation of crude extract were not based on this classification and even it was not considered because the exposure period was within the given time limit. As already mentioned, totally 9 types of solvents were used in the extraction such as acetone, benzene, butanol, chloroform, ethanol, ethyl acetate, hexane, methanol, toluene and water. Further, the solvents having the polarity index about 5.1, 2.7, 4.0, 4.1, 5.2, 4.4, 0.0, 5.1, 2.4 and 9.0 respectively.

In the present research work, all the 3 types of the solvents were used. Out of the solvents used, benzene alone is the Class 1 solvent, and others are Class 2 and Class 3 solvents. Except that four of the solvents are Class 2 solvents and 3 are Class 3 solvents. They are chloroform, hexane, methanol and toluene, then butanol, ethanol and ethyl acetates respectively. While considering the polarity index, water has the highest polarity index value of 9.0, following this ethanol has 5.2 and methanol, acetone have the same polarity index about 5.1. Because of the higher polarity index value, these solvents may extract the compounds efficiently than the other solvents. An important point to be registered here is that the quantity of the extract may not be propositional to the antimicrobial activity. For example water and ethanol have the highest polarity index so the solvents may extract the compounds in high quantity but it is not compulsion that those two extracts should contain effective activity against the human pathogens, addition to that hexane has the value of 0.0 but it does not mean that it cannot extract any of the compounds but it may extract fat and fat soluble compounds. In addition to these all, one of the solvents used in this experiment is Class 1 solvent named benzene which has the solvating value of 2.7. It is a well known hazardous solvent. If such solvent extract shows the promising antimicrobial or any of the activities than it may be the compulsion of using the same solvent for mass scale preparation.

In the safety aspect, long time exposure of the solvent can leads to hazardous effect in human. In the vision of avoiding this, a concept was explained named Hildebrand solubility parameter. It is the total Van der Waals force, which is reflected in the Hildebrand solubility parameter (the simplest solubility parameter). The relative solvency behavior of a specific solvent was specified by numerical value of the solubility parameter, further, it can be derived from solvent's cohesive energy density, which in turn

is derived from the heat of vaporization[25,26]. By averaging the Hildebrand values of the individual solvents by volume, Hildebrand value of a solvent mixture can be determined. For example it was given that two parts toluene and one part acetone mixture having the Hildebrand value of 18.7 ($18.3 \times 2/3 + 19.7 \times 1/3$), about the same as chloroform. That means 2:1 toluene/acetone mixture should have solubility behavior similar to chloroform[26]. So, it may be possible to formulate the solvent system with the same solvating property to that of the Class 1 solvents, through this, Class 1 solvents can be avoided.

However the main objectives of the present research work is to evaluate the antimicrobial activity against the human pathogens, type of solvent used for the extraction, class of the solvent, polarity and some of the physical properties also important and the above facts should be considered for safe and better extraction. Otherwise, some of the factors already exist to choose the green solvent those can be considered. For example, Rowan solvent selection table and GlaxoSmith's pharmaceutical solvent selection table.

4.1. Way of searching new antibiotics

The extensive biochemical capacity of the microorganism can produce new compounds. To obtain this, five different approaches were listed. They are soil screening, semisynthetic modifications, bio transformations, protoplast fusion and gene cloning[27], through the approaches we can produce effective antibiotic compounds.

The results showed that the solvent extracts of the species *Thais bufo* have no potential antibiotic activity, hence it cannot be considered for the further research in the field of drug discovery, and the new concept of Hildebrand solubility parameter and Rowan solvent selection table and GlaxoSmith's pharmaceutical solvent selection table can be considered for the safe and eco-friendly extraction.

Conflict of interest statement

We declare that we have no conflict of interest.

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