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In vitro antibacterial, alpha-amylase inhibition potential of three nudibranchs extracts from South East coast of India

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PEER REVIEW

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Comments

This is a good study, in which the authors had evaluated the antibacterial and alpha amylase inhibitory properties of solvent extracts of nudibranchs. They made preliminary analysis on the activities of the extracts and chemically fingerprinted the presence of various pharmacological targets for drug development.
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ABSTRACT

Objective: To study the antibacterial and antiamylase properties of methanol and acetone extracts of nudibranchs including *Bursatella leachii* (*B. leachii*), *Kalinga ornata* (*K. ornata*), *Aplysia* sp.

Methods: Crude methanol and acetone extracts of sea slugs were tested for inhibition of fish bacterial pathogens' growth through disc diffusion method. The activity was measured based on the formation of inhibition zone around the disc impregnated with crude extracts. The α-amylase inhibitory effect was also measured calorimetrically. The chemical fingerprinting of the extract was recorded with HPTLC and GC-MS.

Results: The solvent extracts of all the three sea slugs showed antibacterial property. The maximum zone of inhibition (>15-20 mm) was recorded for methanol and acetone extracts of *K. ornata*. The methanol extract of *Aplysia* sp. exhibited 93% inhibition against α-amylase, following by *B. leachii* (methanol) 70.6% and *K. ornata* (methanol) 49.03% inhibition respectively. The acetone extracts didn't show any notable inhibition. The presence of free amino acids like lysine, aspartic acid, glutamic acid, arginine *etc.*, terpenoids and pigents were confirmed through HPTLC analysis. The presence of siloxanes and propanoic acid were also revealed through GC-MS.

Conclusions: This study suggests that further scrutinisation of the *B. leachii*, *K. ornata* and *Aplysia* sp. will pave the way for development of antibacterial and α-amylase inhibitory agent for therapeutic application.

KEYWORDS

Antibacterial, α-amylase inhibitory effect, Pigents and terpenoids

1. Introduction

New trends in drug discovery from natural sources emphasize on investigation of the marine ecosystem to explore numerous complex and novel chemical entities. These entities are the source of new lead for treatment of many diseases such as cancer, AIDS, inflammatory condition, arthritis, malaria and large variety of viral, bacterial, fungal diseases[1,2]. Study of marine organisms for their bioactive potential, being an important part of marine ecosystem, has picked up the rhythm in recent years with the growing recognition of their importance in human life[3]. Molluscs, which are widely distributed throughout the world, have many representatives in the marine and estuarine ecosystem[4]. Molluscs also feature prominently in a broad range of traditional natural medicines though the active ingredients in the taxa involved are typically unknown. Some marine gastropods and bivalves have been of great interest to natural products chemists, yielding a diversity of chemical classes and several drug leads currently in clinical

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trials[5]. The rich diversity of marine organisms presumes a great opportunity for the discovery of new bioactive substances. Many studies on bioactivity molecules with wide range of activities like antitumor, antiviral, antimicrobial, anti-inflammatory were reported from Mollusca group. The majority of molluscan natural products research is focused within one of the major groups of gastropods, the opisthobranchs (a subgroup of Heterobranchia), which are primarily comprised of soft-bodied marine molluscs. Molluscs have an impressive array of defenses to protect themselves from a broad array of predators from diverse taxa, including sea anemones, sea stars, crustaceans, fishes and humans[6]. Opisthobranchs are naked molluscs, apparently unprotected by the physical constrain of a shell. Sea hares, belonging to the order of opisthobranchia, subclass Gastropoda, are molluscs having many defense mechanisms against their predators. The chemical defenses of these sessile organisms are built through the secretion of strongly acidic substances, glandular secretions or inbuilt bioactive compounds of their metabolism. As this marine mollusc represents a very interesting source of marine bioactive molecules, the present study was carried out to prospect the bioactive potential of three nudibranchs available in South Indian waters.

2. Materials and methods

2.1. Collection of samples

The nudibranch species [*Bursatella leachii* (*B. leachii*), *Kalinga ornata* (*K. ornata*), *Aplysia* sp.] were collected from the Mudasal Odai and Rameshwaram landing centre and transferred to laboratory in an ice box. The collected samples were washed thoroughly with tap and distilled water. The samples were dissected aseptically and chopped into small pieces and stored at −40 °C until use.

2.2. Extraction

Tissue sample of 25 g of each was homogenized using methanol (bioactive compounds extraction) and acetone (pigent extraction). The homogenate were kept in 4 °C for 24 h extraction and then centrifuged at 3000 r/min for 15 min. Pellets were re-suspended in respective solvents for re-extraction. The collected supernatants were subjected to rotary vacuum evaporator at 40 °C. Further, the samples were lyophilised and stored until use.

2.3. Antibacterial assay

The agar disc diffusion method was employed to screen the antibacterial activity of three nudibranchs extracts against eight fish pathogenic bacterias as described by Brumfitt *et al*. (1990) with slight modification[7]. A loop of bacterial culture inoculated into nutrient broth and incubated for 24 h at 37 °C. The size was adjusted to 0.5 McFarland standard turbidity, approximately 108 CFU/mL). Cell suspension (100 μL of target strain) was introduced into Muller Hinton agar plates and spread finely on the plates using a glass spreader. The disc (6 mm, Wattmann No.1) was impregnated with 25 μL of sample (mg/mL) and standard amounts of the antibiotics as control (amoxicillin, 10 mg/mL). Then, the discs were placed on inoculated agar plates, which were incubated at 37 °C for 24 h. The degree of inhibition was determined by measuring the diameter of zone of inhibition (in millimeters).

2.4. Alpha-amylase inhibition assay

The amylase inhibitory activity was measured by following Nickavar and Mosazadeh (2009)[8]. The starch solution (0.5% w/v) was obtained by stirring and boiling 0.25 g of soluble potato starch in 50 mL of deionised water for 15 min. The enzyme solution was prepared by mixing 0.001 g of α-amylase in 100 mL of 20 mmol/L sodium phosphate buffer (pH 6.9) containing 6.7 mmol/L sodium chloride. The extracts were dissolved in dimethylsulfoxide to give suitable concentrations for the assay. The colour reagent was a solution containing 96 mmol/L 3,5-dinitrosalicylic acid (20 mL), 5.31 mol/L sodium potassium tartrate in 2 mol/L sodium hydroxide (8 mL) and deionised water (12 mL). About 1 mL of each the extracts and 1 mL of the enzyme solution were mixed in a test tube and incubated at 25 °C for 30 min. About 1 mL of this mixture was added to 1 mL of the starch solution and the tube was further incubated at 25 °C for 3 min. Then, 1 mL of the colour reagent was added and the tube was placed into an 85 °C water bath. After 15 min, the reaction mixture was cooled and diluted with 9 mL distilled water and the absorbance value determined at 540 nm using a SHIMADZU UV-vis Spectrophotometer (Kyoto, Japan). Individual blanks were prepared for correcting the background absorbance. In this case, the colour reagent solution was added before the addition of starch solution and then the tube was placed into the water bath. Then, the method was followed as described above. Controls were conducted in an identical manner, replacing extracts with 1 mL dimethylsulfoxide. Acarbose solution was used as positive control. The inhibition percentage of α-amylase was assessed by the following formula.

I α-amylase% =100×(△A Control−△A Sample)/△A Control

Where, △A Control=A Test−A Blank, △A Sample=A Test−A Blank.

2.5. Characterisation

2.5.1. High performance thin layer chromatography (HPTLC)

Qualitative chromatographic analyses were performed to identify the pigents, terpenoids and amino acids extracted from nudibranchs *B. leachii*, *K. ornata* and *Aplysia* sp. Required (5 mg) amount of sample was dissolved in methanol and ethyl acetate separately and analysed on silica gel G-60 TLC plates (10 cm×10 cm) (Merck, Darmstadt, Germany). About 10 μL samples were applied band wise onto the plates using the Linomat V automated TLC sampler III (Camag,

Switzerland) on the TLC stationary layer. The plates were developed in a vapour equilibrated CAMAG HPTLC twin trough chamber (about 10 min) using *n*-hexane:acetone 3:1 (v/v) as a mobile phase for pigents and *n*-butanol:acetic acid:ethyl acetate:water 1:1:1:1 (v/v) for bioactive compounds. After development, the plates were air dried for 5 min, and sprayed with ninhydrin for visualisation of amino acids and with p-anisaldehyde for terpenoids (brown ring confirmation test was also done). Pigents were visualised using UV lamp in Densitometer. The plates were then scanned using CAMAG TLC scanner 3 winCATS software (version 4X) for the presence of pigents, terpenoids and amino acids using the R_f values and images.

2.5.2. Gas chromatography-mass spectrometer (GC-MS) analysis

The sample was injected in GC column (Accu-TOF GCV-JMS-T100GCV, JEOL Asia, Japan). The column temperature was maintained at 80-280 ° C, the split ratio was 20 and the helium flow rate was maintained at 1 mL/min. Then the possible groups were identified for the sample peaks using MS library.

3. Results

3.1. Extraction of bioactive compounds

The wet weight and net yield of the methanol and acetone extracts of nudibranchs were tabulated (Table 1).

Table 1
The yield of all the extracts from the fresh animals were tabulated.

Organism	Source	Solvent	Wet Weight (g)	Net yield (g)
B. leachii	Body	Acetone	7.47	0.128 1
	Body	Methanol	63.25	0.664 0
K. ornata	Body	Acetone	36.04	0.325 7
	Body	Methanol	5.00	0.098 9
Aplysia sp.	Body	Acetone	108.07	3.094 3
	Body	Methanol	5.00	0.101 2

3.2. Antibacterial assay

The antibacterial activity was tested using agar diffusion method against seven fish pathogens (*Bacillus firmus*, *Bacillus flexus* (*B. flexus*), *Bacillus vietnamensis* (*B. vietnamensis*), *Bacillus cereus*, *Halomonas* sp., *Staphylococcus* sp., *Bacillus* sp. The maximum area of zone of inhibition was observed for the crude acetone and methanol extracts of *K. ornata* against *Staphylococcus* sp, *Bacillus* sp and *B. vietnamensis* whereas the minimum activity was observed for the crude extract of *B. leachii* (methanol) against *B. flexus*, *Halomonas* sp. and *Aplysia* sp. exhibited limited activity (acetone and methanol) against *Halomonas* sp. The results were tabulated (Table 2).

3.3. Alpha-amylase inhibition assay

The α-amylase inhibitory activity was measured by using reducing sugar method. The crude methanol extract of *Aplysia* sp. exhibited the maximum activity of 93%, whereas *B. leachii* (methanol) arrested α-amylase activity by 70.6%. The methanol extract of Kalinga inhibited moderately around 49.03% respectively. Thus methanol extracts from nudibranch under investigation showed alpha amylase inhibition activity whereas acetone extracts didn't show any inhibitory effect (Figure 1).

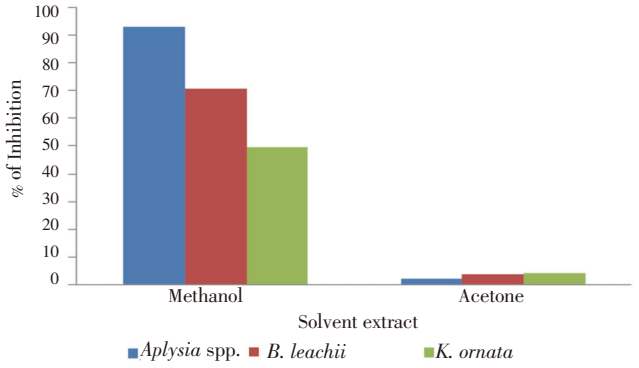

Figure 1. The α-Amylase inhibitory activity of methanol and acetone extracts of nudibranchs.

3.4. HPTLC characterisation of extracts

3.4.1. Pigent analysis

The presence of pigents was identified at 280 nm. Acetone extracts showed significant intense peak at 280 nm while methanol extracts showed slight absorbance when scanned using CAMAG scanner 3. Nevertheless, the magnitude of pigents distribution varied depending upon the species. The absorbance spectrum of samples is shown in Figure 2.

The peaks from a to e show the presence of pigents in

Table 2
Antibacterial activity of nudibranch extracts against bacterial fish pathogens.

Samples Tested	Pathogen name						
	Bacillus firmus	*B. flexus*	*B. vietnamensis*	*Bacillus cereus*	*Halomonas* sp.	*Staphylococcus* sp.	*Bacillus* sp.
	Zone of inhibition in mm						
B. leachii Methanol	16	12	14	12	12	17	15
B. leachii Acetone	12	10	15	11	09	14	13
K. ornata Methanol	17	19	16	18	14	23	20
K. ornata Acetone	19	17	20	17	16	21	18
Aplysia spp. Methanol	14	12	13	09	10	16	13
Aplysia spp. Acetone	12	11	13	11	10	12	12
Control	24	15	25	20	19	16	17

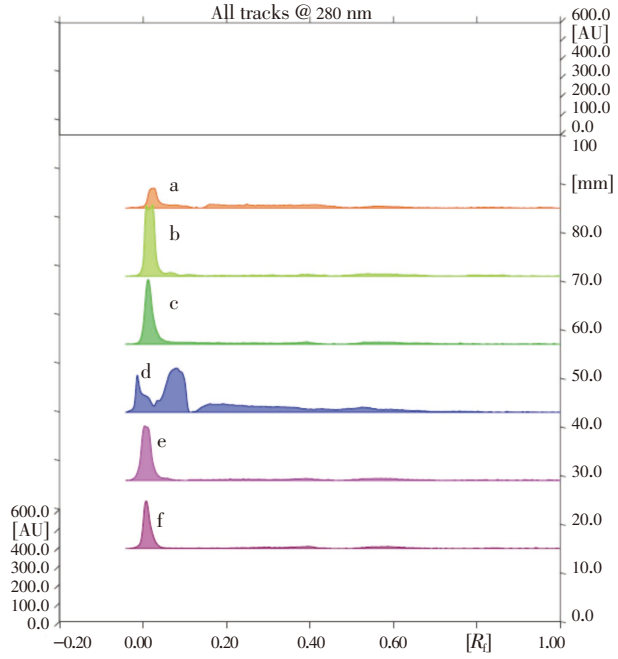

Figure 2. Spectrum shown by the pigents at 280 nm when scanned with Camag scanner 3.

methanol and acetone extracts of *B. leachii*, *K. ornata*, *Aplysia* spp.

3.4.2. Terpenoids analysis

The presence of terpenoids was confirmed at two levels. The preliminary qualitative analysis of terpenoids was carried out using brown ring formation test (Salkowski test) and further substantiated by HPTLC analysis. Terpenoids present in the sample showed purple spots when sprayed with anisaldehyde reagent. Terpenoids (R_f: 0.94) were detected only in the methanol extracts of nudibranch (Figure 3). The difference in their intensity supports their diverse presence in the extract.

3.4.3. Free amino acids analysis

The presence of free amino acids in the samples was confirmed through HPTLC in comparison with standard amino acids. Among the 21 amino acids, few of them were detected in the nudibranchs extract which was confirmed by their corresponding R_f values of standards and samples (Figure 4) whereas the other components remain unknown which may be due to the presence of impurities and other non standard amino acids. The list of amino acids present in the sample were tabulated (Table 3).

Table 3

Shows the free amino acid analyses in the nudibranchs extract.

Sample R_f peaks	Possibile amino acids in sample by comparing with standard R_f value	1	2	3
0.22	Arginine, histidine, lysine	*	*	*
0.29	Cysteine	*	*	–
0.45	Glycine, aspartic acid, glutamine, proline, aspargine, serine, hydroxyproline	*	*	*
0.62	Glutamic acid, threonine	–	*	*
0.75	Valine, methionine	*	*	*
0.92	Tyrosine, tryptophan, phenyl-alanine, leucine	*	*	*

3.4.4. GC-MS

Interpretation on mass spectrum of GC-MS was conducted using the database of National Institute of Standard and Technology (2005) having more than 62 000 patterns. The spectrum of the separated components was compared with the spectrum of National Institute of Standard and Technology library database. The identity of the spectra should be above 95% for the identification of components. The methanol extracts were analysed with GC-MS to identify the possible bioactive molecules. Extracts showed so many junk peaks along with our compounds of interest, since it was a crude sample. Siloxane was the major group commonly found in all the methanol extracts next to that propanoic acid which was commonly present in all the samples. Besides this, some of the major peaks contributed to the presence of, 2-(methyl thio), phenol, 2, 4-bis (1, 1-dimethyl ethyl) in sample *B. leachii*. Whereas in *Aplysia* sp. peaks responsible for the presence of (1, 3) thiazinane-4-carboxylic acid, 3-methyl and 2, 2, 5-trimethyl -piperidin-3-ol were recorded. *K. ornata* contains glycine, N-(methoxyoxoacetyl)-, methyl ester, (1, 3) thiazinane-4-carboxylic acid, 3-methyl methyl tetradecanoate. The GC-MS of the methanol extracts of nudibranchs were shown in Figure 5.

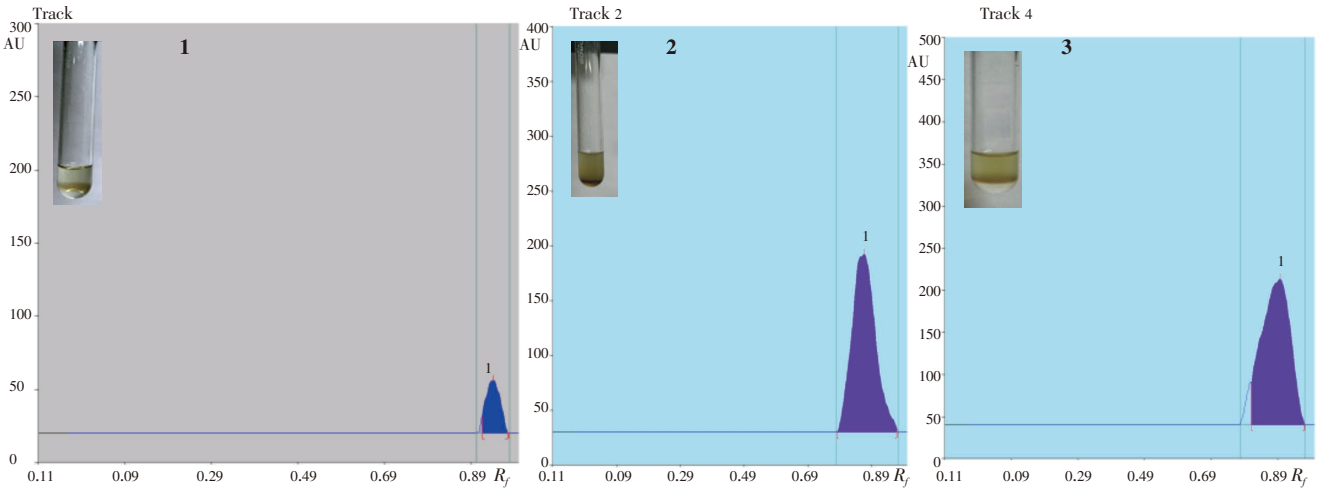

Figure 3. Shows the peak responsible for terpenoids when scanned at 463 nm. 1. *B. leachii* methanol, 2. *K. ornata* methanol, 3. *Aplysia sp*. methanol. (Photos on the left side depicts brown ring formation responsible for terpenoids in the sample).

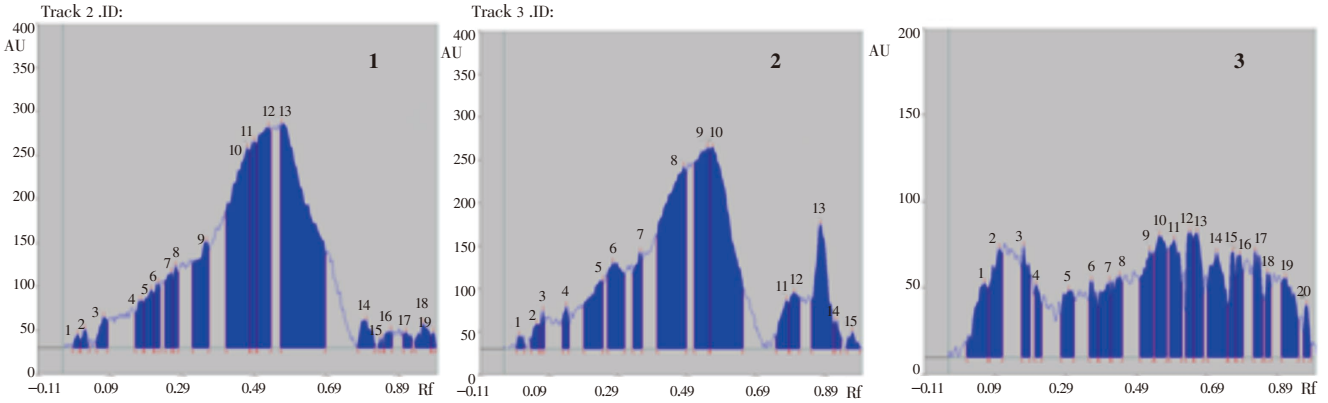

Figure 4. HPTLC analysis of free amino acids of Nudi extracts. 1) *B. leachii* methanol, 2) *K. ornata* methanol, 3) *Aplysia Sps* methanol

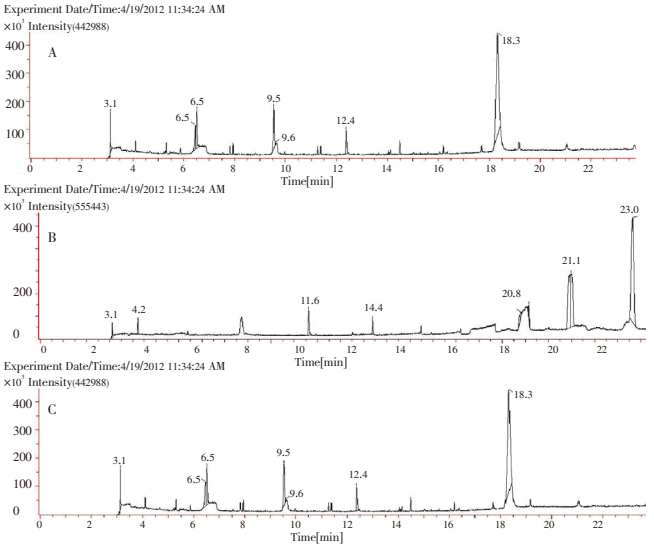

Figure 5. A) GC-MS spectrum of *B. leachii* methanol extract; B) GC-MS spectrum of *K. ornata* methanol extract; C) GC-MS spectrum of *Aplysia* sp. methanol extract.

4. Discussion

In the last three decades, the study of marine chemical ecology has experienced great progress due to the new technological advances for collecting and studying marine samples[9,10]. The largely unexplored marine world that presumably harbors the most biodiversity may be the vastest resource to discover novel 'validated' structures with novel modes of action that cover biologically relevant chemical space[11]. Thus, knowing the importance of sea hare in the development of new bioactive peptides, we have attempted a study to reveal the potency of acetone (pigents) and methanol (bioactive molecule) extracts of three different sea hares *B. leachii*, *K. ornata* and *Aplysia* sp.

The present study proved that both methanol and acetone extracts were potent antibacterial agents against fish pathogens investigated. The maximum area of inhibition zone was observed for the crude acetone and methanol extracts of *K. ornata* against *Staphylococcus* sp., *Bacillus* sp. and *B. vietnamensis*, *Staphylococcus* sp. respectively. Furthermore, the antibacterial activity of purple fluid of sea hare *Dolabella auricularia* (*D. auricularia*) against human pathogens was reported by Abirami *et al*[12]. Earlier, Gunthorpe and Cameron screened bioactive properties of 21 Australian dorid nudibranchs extracts and reported that antimicrobial activity was present 80% of the extracts[13]. Also the protein isolated from the ink of *Aplysia californica* inhibited the growth of gram positive and gram negative bacteria including marine bacteria, yeast and fungi[14]. Similarly in our study, all the nudi samples showed considerable inhibition against all the fish pathogens tested. In which, *K. ornata* sample showed significant inhibition potency. Thus, the current result proved that our antibacterial agents possess a broad spectrum of activity in nature.

Obesity, and resultant health hazards which include diabetes, cardiovascular disease and metabolic syndrome, are worldwide medical problems. Control of diet and exercise are cornerstones of the management of excess weight[15]. Amylases are a class of enzymes that hydrolyze starch to yield low molecular weight dextrins and sugars and they play important role in the digestion of carbohydrates. Inhibition of α-amylase along with α-glucosidase can significantly reduce the post-prandial increase of blood glucose and can be an important strategy in the control of blood glucose level in the type-2 diabetic patients. Hence, pancreatic a amylase and gastric glucoamylases are the major therapeutic targets for the type-2 diabetes mellitus[16]. About 246 million people suffer from type-II DM worldwide, and its incidence and serious complications continue to grow rapidly. Although there are several classes of antidiabetic drugs, achieving and maintaining long-term glycemia control is often challenging. In addition, many current agents have treatment-limiting side effects[17]. Inhibition of α-amylase, enzyme that plays a role in digestion of starch and glycogen, is considered a strategy for the treatment of disorders in carbohydrate uptake such as diabetes and obesity as well as dental caries and periodontal diseases[18].

Besides this, due to the gastro intestinal side effects exhibited by oral anti-hyperglycaemic agents, searching for new amylase inhibitors became essential in treatment of diabetes. Hence, in the present study, the potency of nudi extracts were tested for α-amylase inhibition activity where methanol extract of *Aplysia* sp. showed strong inhibition (93%), whereas *D. auricularia*, and *K. ornata* showed average inhibition. Similarly, Abirami and her colleagues have reported alpha amylase's inhibitory effect in the purple fluid of *D. auricularia*[12]. Further, Ravi *et al.*

observed alpha amylase inhibitory activity of 72% in acetone extracts of two marine gastropods, *Hemifusus pugilinus* and *Natica didyma*, whereas, here acetone extracts didn't show notable results[19]. Furthermore, Tiwari *et al.* established the anti hyperglycemic activity of the crude extract of bivalve mollusc *Scapharca* inaequivalvisin rat models[20].

Marine animals especially those from tropical waters are often brilliantly coloured, and this is widespread in both sessile and non-sessile invertebrates where distribution and function of pigents seem to vary between invertebrate groups[21]. The crude acetone and methanol extracts of *B. leachii*, *K. ornata* and *Aplysia* sp. were tested for the presence of pigents, terpenoids and amino acid analysis through HPTLC by comparing with those of standards. Accordingly, *K. ornata* showed orange pigented spots whereas *Aplysia* sp. showed dark green spots through out the body. Consequently, the intensity of the peaks recorded for *K. ornata* and *Aplysia* sp.were high when compared to that of *D. auricularia* pigents in the crude extracts of the samples identified based on their spectral absorbance pattern at 280 nm. The presence of amino acids in the crude extracts were confirmed through comparison with standard amino acids. Commonly, glycine, aspartic acid, glutamine proline, aspargenine, serine, hydroxyproline, tyrosine, tryptophan, phenylalanine, leucine were present in all the methanol extracts of samples. Some amino acids were exceptionally present in specific samples. Apart from the 22 standard amino acid, some bands were observed which may be due to the presence of non standard amino acids. Some amino acids have identical migration point when used in particular solvents which are supported by Carol and Prescott[22]. As stated above, some amino acids in the extracts have same migration point and could not be identified distinctly.

Terpenoids represent a promising and expanding source for biologically active natural compounds whose potential for research and development of new substances with pharmacologic activity[18]. There have been many applications of terpenes in human societies. Pharmaceutical and food industries have exploited them for their potentials and effectiveness as medicines and flavour enhancers[23]. The presence of terpenoids in the crude extracts was detected at 463 nm. The crude methanol extracts of *B. leachii*, *K. ornata* and *Aplysia* sp. showed peak at R_f value 0.94 which shows the presence of terpenoids. Terpenes represent the major group of secondary metabolites isolated from opisthobranchs. Earlier Souza *et al.* reported the role of terpenoids in antimicrobial activity against cariogenic bacteria[24]. This greatly supports our results on the role of terpenoids in biological activities tested. Most of these compounds show both interesting biological properties and unique chemical structures. In particular, diterpenes include some of the most interesting examples of bioactive molecules; hence, the occurrence of terpenoids was tested in the crude methanol extracts where all the samples showed the presence of terpenoids at notable level. Hence, through HPLTC, the presence of pigents, terpenoids, amino acids were confirmed in the crude extracts of nudibranchs.

Besides this, to identify the other bioactive molecules, the extracts were analysed with GC-MS. GC-MS results revealed the presence of siloxane, a chemical compound which has various applications found in products such as cosmetics, deodorant, water repelling windshield coating, lubricants, molded lenses, food additives, and some soaps. Besides this, peak responsible for the occurrence of propanoic acid, a naturally occurring carboxylic acid was recorded. It is used to make pesticides and preservatives in pharmaceutical industries, as it inhibits the growth of mold and some bacteria. This substantiates that propanoic acid may be one of the contributing factors for the antibacterial activity exhibited by the nudibranch extracts.

Nudibranchs being a slow moving animal secrete bioactive molecules either for defence or prey capture. Thus, it represents as a source of bioactive compounds to be explored. Hence, this present study would gain us knowledge about the bio-potency of crude acetone and methanol extracts of *B. leachii*, *K. ornata* and *Aplysia* sp., which may pave a way for the isolation of specific protein or novel compounds and/or development of new therapeutic strategies complementary to conventional therapy and also imply that further investigation on the bioactive potential of this sea slugs will provide more bioactive molecules of biomedical impact.

Conflict of interest statement

We declare that we have no conflict of interest.

Acknowledgements

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Comments

Background

It is accredited that marine invertebrates produce bioactive natural products that may be useful for developing new drugs. However, mining the marine environment will clear the way for chemical and biological novelties as well. Hence, bioprospecting in marine has potential and promising outcome.

Research frontiers

The reported research work portrayed that the solvent extracts exhibited significant inhibition against fish pathogens and alpha amylase enzyme. Further, the extracts were well characterized and correlated with the bioactivity of the extract investigated.

Related reports

Earlier crozier identified the presence of pigents in

Chronodoris zebra Heilprin, the largest of the nudibralichs found in Bermuda. Rajaganapathi (2002) identified the anti-HIV activity of purple ink of *B. leachii*. *Aplysia dactylomela* ink has shown antimicrobial activities that inhibited the growth of bacteria, as well as being toxic to many larger organisms such as brine shrimp (Melo VMM, 1998).

Innovations and breakthroughs

The marine bioprospecting is the promising field in discovery of natural products. The opisthobranchs of the tropical and temperate Pacific are remarkably diverse, which were reviewed in a series of regional monographs on Hawaii (Gosliner 1980), South Africa (Gosliner and Griffiths 1981), and the Indian Ocean and the South-west Pacific (Miller 1971, 1974, 2001, Rudman 1980, Schrodl 2003). Hence the nudibranch collected from tropical waters of Indian coast will have different metabolites depending on their predation and feeding mechanism. Further, identification of a lead from a source remains as milestone in drug discovery venture. Accordingly in this piece of research work, they have identified the potent inhibitor of pathogens for further development of a drug.

Applications

Nudibranch is a potential sessile organism renowned for its chemical defense and its potential in human therapeutic. This research reports further supports the uses of the chemical molecules isolated from nudibranchs in antibacterial and diabetic control and also suggests that further investigation will pave the way for development of a drug.

Peer review

This is a good study, in which the authors had evaluated the antibacterial and alpha amylase inhibitory properties of solvent extracts of nudibranchs. They made preliminary analysis on the activities of the extracts and chemically fingerprinted the presence of various pharmacological targets for drug development.

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