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Extraction, identification of bioactive compounds and *in vitro* antioxidant activity potential in freshwater ampullariidae snail *Pila virens*

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Abstract

Objective: Molluscs are considered as a significant natural source to derive many narrative biological active compounds. Animal extract are usually complex mixtures of bioactive molecules, including proteins and peptides. Freshwater gastropods are found to be a vital source of valuable bioactive substances. These bioactive compounds are involved in various biological functions such as communication, infection, reproduction and self- defense. Discovery of freshwater gastropods bioactive potential and their curious roles are still limited.

Experimental approach: Consequently, the present study was evaluated on the bioactive compound extraction; identification and *In-vitro* antioxidant activity were gritty by total antioxidant activity, DPPH (diphenylpicrylhydrazyl) assay, hydroxyl radical scavenging activity, superoxide radical scavenging, reducing power and ferrous ion chelating from active fraction of *Pila virens* tissue methanolic extract at various concentrations (20-250µg/ml).

Findings: The results of freshwater gastropod methanol tissue extracts exhibited significant total antioxidant activity, DPPH (diphenylpicrylhydrazyl) assay, hydroxyl scavenging and superoxide scavenging activity which predicted as 67.09%, 74.83%, 60.21% and 59.89%.

Conclusion: These results concluded that, the tissue extract of *Pila virens* has novel bioactive compound, antioxidant potential and it has to further characterize to improve the pharmacological active freshwater natural products.

Keywords: freshwater molluscs, gastropods, methanolic extract, bioactive compound and antioxidant activity

Introduction

India is endowed with rich and diverse bio-resources and the molluscs are not an exception. Molluscs are a heterogeneous group of animals both in shape and diversity and are represented by amphineura, gastropods, bivalves, cephalopods and scaphopods. Molluscs have also proven to be rich source of structurally diverse bioactive compounds with the valuable pharmaceutical and biomedical application [1]. Natural products have served as an important source of drugs since ancient times and about half of the useful drugs today are derived from the natural sources. Natural products isolated from molluscan have been tested for an extensive range of natural activities.

Molluscs are said to be pharmacologically significant out let. There are more than thousands of bioactive compounds discovered in freshwater molluscs. They are peptide, sterols, sesquiterpenes, terpenes, polypropionate, nitrogenous compounds, macrolides, prostaglandins miscellaneous compounds and alkaloids [2]. The antioxidant activity of putative antioxidants have been attributed to various mechanisms, among which are prevention of chain initiation, binding of transition metal-ion catalysts, decomposition of peroxides, prevention of continued hydrogen abstraction, reductive capacity and radical scavenging [3, 4].

Molluscan metabolites have been most frequently tested for neuromuscular blocking action, anti-predator, antimicrobial, anti-neoplastic and cytotoxic activity. The use of crude or more sophisticated products from nature in order to acquire health benefits is an ancient human habit; nevertheless, the expenditure of many of these products has been scientifically proven to offer chemoprevention/protection for several human diseases. In recent years, more and more researchers have come to the consciousness that freshwater organisms hold immense potential as a source of novel molecules and new anticancer agents. Antioxidants play an important role in inhibiting and scavenging radicals, thus providing protection to humans against infections and degenerative diseases.

The freshwater gastropod, *P.virens* is known as snail under the family Ampullariidae. They are large freshwater gastropod molluscs having a strong thick calcareous operculum. Among the invertebrates the molluscs are very good source of biomedically important products [5] and have developed very effective mechanisms that are part of their innate immunity [6]. They are considered as one of the important source to derive bioactive compounds that exhibit antitumour, antimicrobial, anti-inflammatory and antioxidant properties [7]. Hence the present study was designed to identify the bioactive compounds present in the flesh extract of freshwater gastropod, *P.virens*.

If a pure compound shows really interesting activity, further pharmacological assays (*in vitro*, *in vivo*, tolerated dose, and so on) and chemical work (structure elucidation, structure modification, preparation of analog, structure activity relationship, total synthesis, cultivation, *etc*.) should be carried out in order to enter the development step [8]. Usually for bio-prospecting, freeze-dried samples of marine organisms are solvent extracted, and the extract is partitioned by various chromatographic techniques including thin layer

chromatography, vacuum liquid chromatography, column chromatography and high-performance reversed-phase liquid chromatography [9].

Freshwater molluscs bioactive compounds have been structurally elucidated and chemically designated. Structural elucidation of such isolates is greatly facilitated also by technologies in mass spectrometry, NMR and other spectroscopic techniques including FT-IR [10]. GC/MS-a combination of two different analytical techniques, Gas Chromatography (GC) and Mass Spectrometry (MS), is used to analyze complex organic and biochemical mixtures. GC can separate volatile and semi-volatile compounds with great resolution, but it cannot identify them. MS can provide detailed structural information on most compounds such that they can be exactly identified and quantified, but it cannot readily separate them. Gas chromatography and mass spectrometry are, in many ways, highly compatible techniques.

Antioxidants widely used in food industries are defined as any substance with the ability to delay or inhibit oxidation [11]. Some synthetic antioxidants such as butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT) and propylgallate are commonly added to prevent lipid oxidation [12]. Recently, the search for natural antioxidants is growing due to health hazards from synthetic antioxidants. The new integrated approach consisting of a screening of extraction conditions and an *in vitro* measuring of functional activities together with an exhaustive chemical characterization will provide us with a new tool to discover new bioactive compounds. Several examples can be found in the literature about integrated processes that may favor the extraction and purification of bioactive compounds.

In this study, bioactive compounds of *P.virens* have been evaluated using GC-MS techniques. The experimental approach consists of a systematic investigation about the action of crude and purified extract on physiological preparations of whole animals to outline the profile of their biological activities and mechanism of action still incomplete, the aim of this work focuses on the isolation and purification of bioactive compounds from the *P.virens* snail tissue extracted with methanol and its antioxidant activity at various concentrations. The freshwater snail bioactive compound and antioxidant study of *P.virens* is very meager so consequently the present study on *P.virens* is the first work on Indian Ampullariidae. Literature survey revealed the urgent need to explore freshwater gastropods and not much work has been carried out in *P.virens*. Hence, the *P.virens* was chosen for the present study with well defined executable objectives.

Consequently, our objectives for the current effort are to (i) Determine the chemical composition of freshwater apple snail *P.virens* (ii) The *P.virens* methanol extract antioxidant activity and (iii) To compare the antioxidant activity of the optimized *P.virens* methanol extract to L-ascorbic acid and BHA were used as standards and the total antioxidant capacity was expressed as ascorbic acid equivalent.

Materials and methods

Sample collection and extraction

Live specimens of freshwater gastropod *P.virens* was randomly collected by hand picking from in the coleroon river, Lower Anaicut Reservoir, Thanjavur district, Tamilnadu. The collected fresh molluscs were preserved with ice and transported to the laboratory and identified by the standard literature of [13]. The methanolic extract of flesh was prepared by the method of Chellaram [14]. The specimen was brought to the laboratory and their soft bodies were removed by breaking the shell. The flesh sample was dried using hot air oven at 60^0C and powdered. 25grams sample was soaked in methanol and maintained for 3 days. The extract was filtered through Whatman No.1 filter paper. The resultant extract was concentrated by using rotary vaccum evaporator with reduced pressure. The resultant extract were then kept in airtight container and stored at 4^0C for further analysis.

Thin layer chromatography (TLC)

Partial purification of bioactive compounds was carried out using read made silica gel (60-F 254mm]. Crude extract of *P.virens* were spotted at the bottom of the TLC sheet using capillary tube and placed in TLC chamber. n- Butanol, acetic acid and water (6:2:2) mixture was served as the mobile phase. After running the chromatogram, the TLC plate was air dried and placed in closed iodine chamber to clearly visualize the separated compounds as spots. The spots were labeled and their distance from the baseline was measured. The distance between the baseline and the solvent front was also measured. The retention factors of the samples were then determined. The retention factor, Rf is defined as the distance travelled by the compound divided by the distance travelled by the solvent.

$$RF = \frac{\text{Distance travelled by the compound}}{\text{Distance travelled by the solvent}}$$

Confirmation of some of the characterized compounds by HPLC

In order to prepare the correct solvent system for the most potent fraction of test animal the silica gel column purified extracts of test animals were repurified by alumina column and TLC for HPLC analysis. High Performance Liquid Chromatography fitted with an Auto sampler and a UV Detector (HPLC, Shimadzu, LC 2010A, Japan), was used for the analysis. The column used was Phenomenex C18 measuring 250 mm × 4.6 mm, 5"m (California, USA). The flow rate was adjusted to 1.0 ml/min. Injection volume was adjusted to 20"l and detection was made at 366 nm [15]. Standards were run to confirm the presence of the same in the isolated compounds of the *P.virens*.

Fourier Transform Infra-Red spectrum analysis

FT-IR spectroscopy is one of the most powerful analytical techniques used for structural elucidation and identification compound. It has been used to examine and provide important data on a wide variety of biological molecules. The functional group present in the extract isolated from *P.virens* was determined using FT-IR spectroscopy (Bio – read FTIR – 40 models, USA). Briefly 10 mg of sample was mixed with 100 mg of dried potassium bromide (KBr) and compressed to prepare as a salt disc (10 mm diameter) for reading the spectrum. The absorption was read between 500 and 4000 cm-1.

Identification of bioactive compounds by GCMS

The purified gastropod fractions were separately examined using GC SHIMADZU QP2010 Ultra system and gas chromatograph interfaced to a mass spectrometer equipped

with Elite-1 fused silica capillary column. For GCMS detection, an electron ionization energy system with ionization energy of 70eV was used. Helium was used as carrier gas at constant flow rate 4 ml/min and an injection volume of 2µl was employed (injector temperature 250^0C: ion source temperature 200^0C). The Colomn temperature was programmed from 80^0C (holding time 1 min) – further increase (5°C /min) – upto 300°C (2 min) with column flow rate of 1 µl /min. The sample was run for 47 min with solvent out time of 3.5 min. Mass spectra were taken with scan interval of 10 min. Explanation on mass spectrum was achieved by using data base of WILEY8.LIB and NIST11S LIB for different bioactive compounds.

Identification of components

Interpretation of mass spectrum GC-MS was conducted using the database of National Institute of Standard and Techniques (NIST11s) and WILEY8 having more patterns. The spectrum of the unknown component was compared with the spectrum of the known components stored in the NIST11s and WILEY8 library. The name, molecular weight, molecular formula and structure of the component of the test material were identified.

Antioxidant activity reagents

Ammonium molybdate, Phosphate buffer, Ascorbic acid, Hydrogen peroxide solution, 1, 1-diphenyl-2-picrylhydrazyl (DPPH), Sodium nitroprusside, Sulfanilic acid, Naphthyl ethylenediamine dihydrochloride, Sulfuricacid, Butylated Hydroxyltoluene, Potassium ferricyanide, were purchased from Sigma Chemical Co. (St. Louis, MO). TCA, $FeCl_3$ and H_2O_2 were obtained from Merck Co. (Darmstadt, Germany). Other chemicals used were of analytical grade.

Antioxidant Assays

The antioxidant activity of the methanolic extract of *Pila virens* were estimated in terms of total antioxidant activity [16], DPPH assay [17], Hydroxyl radical scavenging activity [18], total reducing power [19], chelating ability on ferrous ions assay [20] followed by the method with slight modification.

Results and discussion

Molluscan extracts are usually complex mixtures of bioactive molecules mainly proteins, peptides and sterols. The methanol extraction of the *P.virens* showed significant antioxidant activity. Hence this fraction was subjected to chromatography which is used for the preliminary compound separation and then to GC-MS analysis to characterize the compound responsible for antioxidant activities.

FT-IR- spectra represented to crude methanolic extract of *P. virens*

Fourier Transform- Infra Red spectroscopic analysis led to the identification of functional groups. KBr was used as the standard. In the present study scrutiny of *P.virens*, from the graph plotted with wave number against intensity 7 peaks with frequencies ranging from 403.12 to 3994.58^{cm-1}. Here, ten functional groups iodide (C-I), Bromide (C-Br), Chloride (C-Cl), phosphates, nitrates, ketones (C=O), arenes (C=C), ketones (C=O), cyanide (C N), and alcohol (O-H) were found. The wave numbers 3840, 3421,3196,2584,2499 and 2310 distinctive of asymmetrical stretching of CH2 and 1992, 1627, 1271 and 1016 and 682 – 403 positions of the spectrums are the characteristic C=O stretching, COH, CH, C-O and Skeletal stretch respectively. (Fig.1)

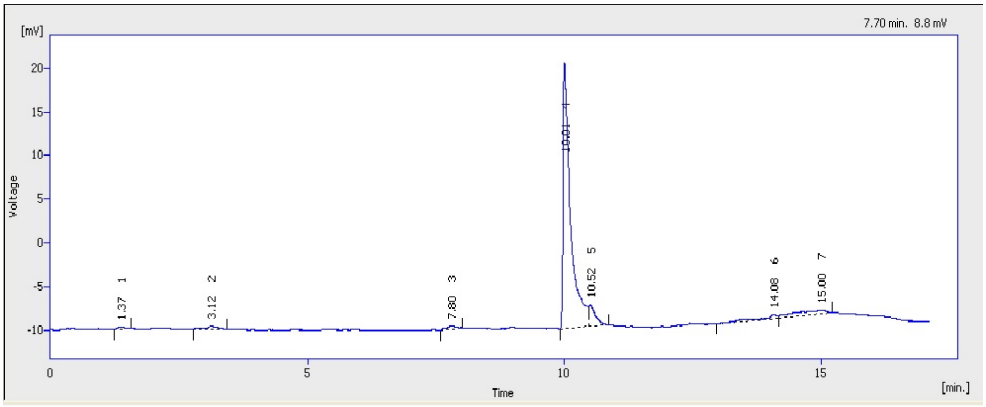


Fig 1: The FTIR spectrum of crude sample in *Pila virens*

The studies on the active principles in the *P.virens* whole animal methanol extract by GC-MS analysis clearly showed the presence of four compounds (Fig 2) (Table 1). The active principles with their retention time (RT), molecular formula, molecular weight (MW), and concentration (Peak area %) are presented in Table. The GC-MS chromatogram of the four peaks of the compounds detected was shown in figure. The mass spectrum and structure of the compounds identified were presented and the total number of compounds identified in methanol extracts. The results revealed that Benzaldehyde, 4-Methyl (9.844), Benzene, 1,3-Bis(1,1-Dimethylethyl)- (14.573), Phenol, 2,4-Bis(1,1-Dimethylethyl)- (21.337) Hexadecanoic Acid, Methyl Ester (30.613).

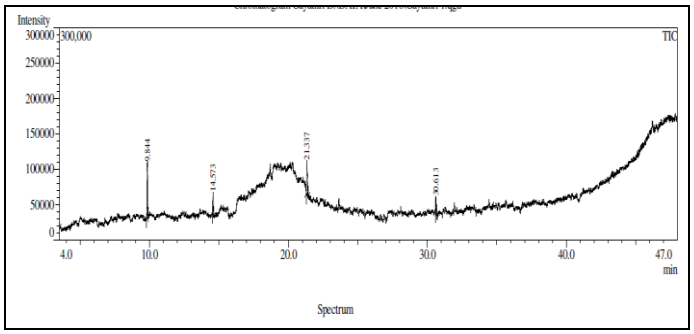
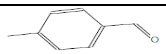
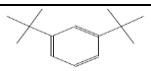
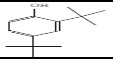
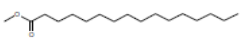


Fig 2: GC-MS chromatogram of *P.virens* methanolic extract

Table 1: Bioactive compounds identified in the *Pila virens* Total Antioxidant Activity (TAA)

Peak	Retention Time	Peak Area %	Molecular Formula	Molecular Weight	Name	Compound Structure
1	9.844	40.29	C_8H_8O	120	Benzaldehyde, 4-Methyl	
2	14.573	17.83	$C_{14}H_{22}$	190	Benzene, 1,3-Bis [1,1-Dimethylethyl]	
3	21.337	27.21	$C_{14}H_{22}O$	206	Phenol, 2,4-bis [1,1-Dimethylethyl]	
4	30.613	14.68	$C_{17}H_{34}O_2$	270	Hexadecanoic Acid, Methyl Ester	

The *Pila virens* methanolic extract showed the total antioxidant activity in the range of 19.78 to 67.09% at different concentrations 50 - 250 µg/ml. The maximum of 67.09% inhibition was observed at the concentration of 250 µg/ml of *Pila virens* methanolic extract. It was observed that the total antioxidant activity was found increasing with increasing concentration. On comparison the standards L-ascorbic acid and BHA reported 80.12% and 88.05% of total antioxidant activity at the highest concentration of 250µg/ml respectively (Fig 2).

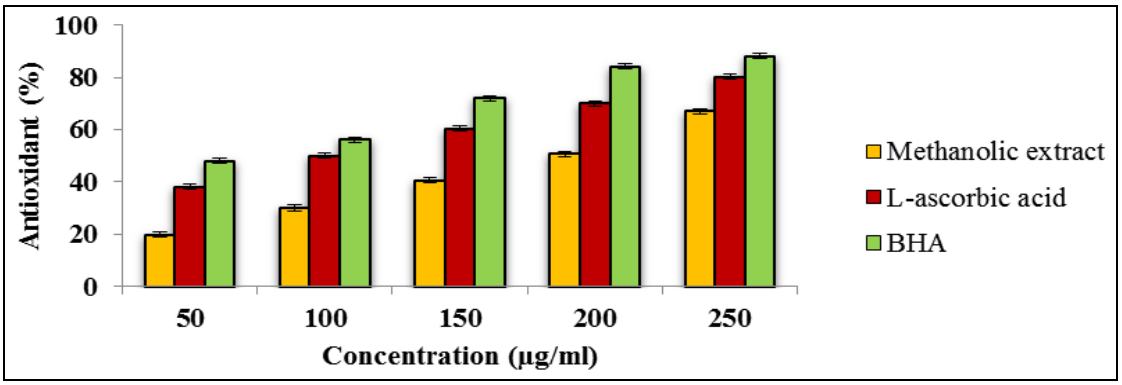


Fig 2: TAA of *P.virens* methanolic extract (50-250 µg/ml), compared with standard L- Ascorbic acid and BHA

DPPH Radical Scavenging Activity

The effect of *Pila virens* methanolic extract on oxidative damage induced by hydroxyl radical at different concentrations (0.1 – 2.0 µg/ml) was found between 20.06% and 74.83%. The maximum of 74.83% inhibition was observed at the highest concentration of 2.0 µg/ml of *Pila virens* methanolic extract. The hydroxyl radical scavenging activity of L-ascorbic acid and BHA was 79.8 and 80.2% at 2.0µg/ml. The decrease in absorbance of DPPH radical is caused by antioxidants, because of the reaction between antioxidant molecules and radicals, progresses, which results in the scavenging of the radical by hydroxyl donation (Fig 3).

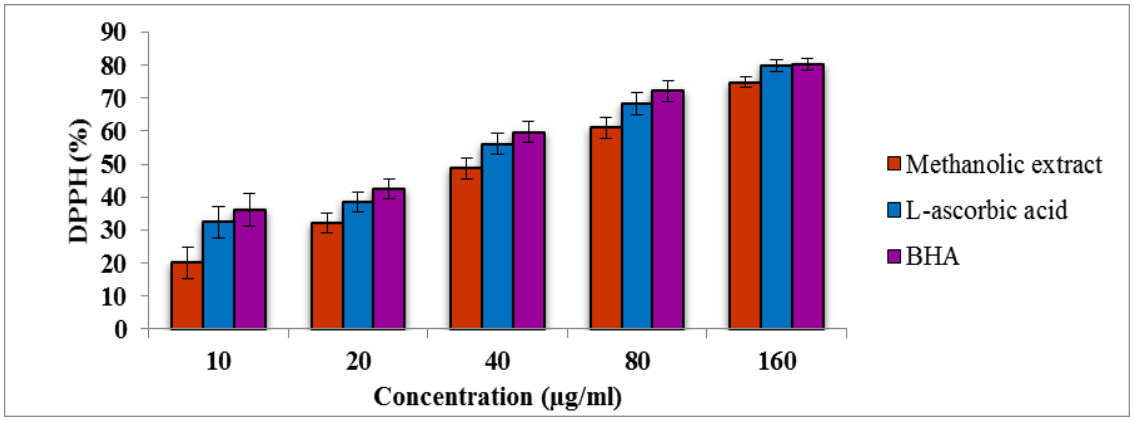


Fig 3: DPPH of *P.virens* methanolic extract (10-160µg/ml), compared with standard L- Ascorbic acid and BHA

Hydroxyl Radical Scavenging Activity

The effect of *P.virens* methanolic extract on oxidative damage induced by hydroxyl radical at different concentrations (0.1 – 2.0 µg/ml) was found between 9.08% and 60.21%. The maximum of 60.21% inhibition was observed at the highest concentration of 2.0 µg/ml of *P.virens* methanolic extract. The hydroxyl radical scavenging activity of L-ascorbic acid and BHA was 80.34 and 82.56% at 2.0µg/ml. (Fig 4)

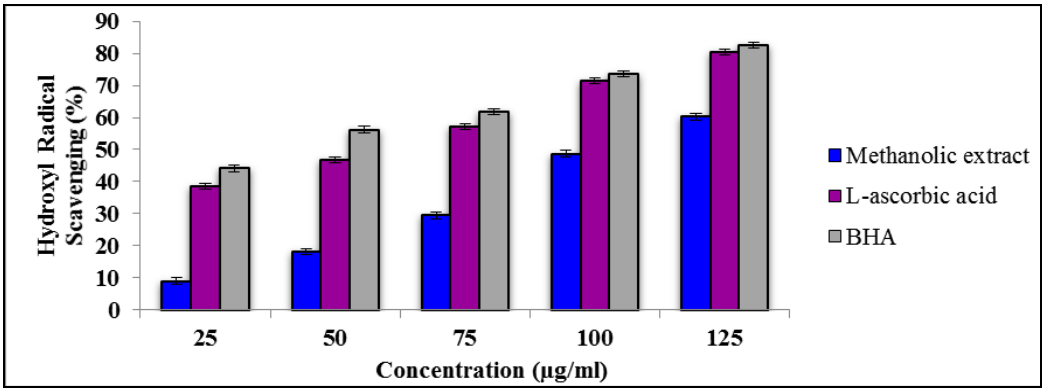


Fig 4: Hydroxyl radical scavenging activity of *P.virens* methanolic extract (25-125 µg/ml), compared with standard L- ascorbic acid and BHA

Superoxide Radical Scavenging Activity

The effect of *P.virens* methanolic extract on oxidative damage induced by superoxide radical at different concentrations (0.1 – 2.0 µg/ml) was found between 25.59% and 59.89%. The maximum of 59.89% inhibition was observed at the highest concentration of 2.0 µg/ml of *P.virens* methanolic extract. The hydroxyl radical scavenging activity of L-ascorbic acid and BHA was 82.45 and 85.29% at 2.0µg/ml. (Fig 5).

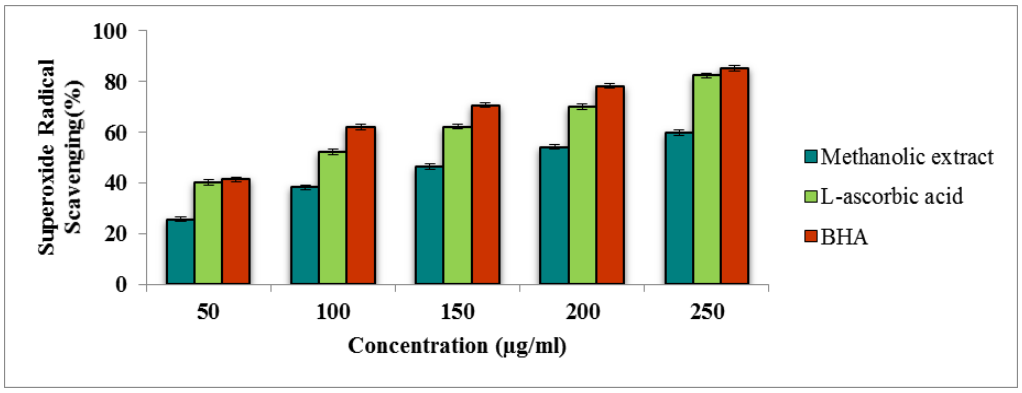


Fig 5: Superoxide radical scavenging activity of *P.virens* methanolic extract (50-250 µg/ml), compared with standard L- ascorbic acid and BHA

Antioxidant Activity

Antioxidants play an important role in food industries for purposes of nutritional preservation and prevention of color and flavor deterioration. Analysis of antioxidant activity on *P.virens* methanolic extract showed higher ferrous chelating (67.09%) and hydroxyl radical scavenging activities (60.21%) compared to BHA (80.34 % and 82.56%).

However, BHA showed higher reducing power (2.52%). The ferrous ion (Fe2+) is a pro-oxidant that interacts with hydrogen peroxide (Fenton reaction) to produce reactive oxygen species (ROS) and the hydroxyl (OH) free radical, which may initiate and/or accelerate lipid oxidation [21]. The complex formation of the ferrous ion is disrupted when chelating agents are present, resulting in decreased of color [22]. As for reducing power, the presence of antioxidants in causes the *Pila virens* methanolic extract reduction of the ferric cyanide complex to its ferrous form.

In the present study discuss and present some selected applications of the extraction of target compounds with, among others, antioxidant, antimicrobial, and antiproliferative activities from different sustainable molluscs sources. The FT-IR spectrum of polysaccharides showing antioxidant activity respectively *P.virens* 3840, 3421, 3196, 2584, 2499 and 2310 distinctive of asymmetrical stretching of CH2 and 1992, 1627, 1271 and 1016 and 682 – 403 positions of the spectrums are the characteristic C=O stretching, COH, CH, C-O and Skeletal stretch A broad peak at 3840 cm-1 is reported to be indicative of the hydroxyl stretching vibration and the sharp peak at 2929.87 cm-1 represents the characteristic -CH- stretching vibrations.

An attempt has been made to outline the most important aspects of the empirical approach to find new lead compounds from natural resources such as animals. In the present study sixteen chemical constituents have been identified from methanol extract of the whole animal of *P.virens* by gas chromatogram mass spectrometry (GC-MS) analysis. The presence of various bioactive compounds justifies the use of whole animal for various ailments by traditional practitioners. More than 2600 scientific studies have been carried out over the last 20 years testify to the important contribution of toxins extracted from gastropod snails to medicine and cellular biology [23]. Chemical drugs may lead to adverse effects and recent researchers have focused on pharmacologically active compounds from the natural sources. GC-MS is used to identify the constituents of volatile matter, long chain and branched chain hydrocarbons, alcohols, acids and esters.

Results also showed that the antioxidant activity of *P.virens* methanol extract has potential as a radical scavenger due to its high chelating of the ferrous ion as well as hydroxyl radical scavenging activity. In addition, although lower than that of BHA, *P.virens* methanolic extract has reducing power activity. In the present investigation of *P.virens* antioxidant activity compared to *Pomacea canaliculata* respectively antioxidant scavenging activity on DPPH ranged from 11.8% to 92.60%

with the highest activity (92.60%) was recorded at 65˚C with a 2% enzyme concentration, a pH of 10, and hydrolysis time of 60 minutes. [24] and [25] According to previous analysis that *Pomacea canaliculata* their chemical content includes 65.0% crude protein (flesh, excluding shell) and 13.5% dry matter with high mineral and vitamin content and appears to be a good mineral source as indicated by their calcium (35% in the shell) and phosphorus content (1.1%), and they are also a good source of energy (12.55 MJ kg-1).

Diphenylpicrylhydrazyl (DPPH) is stable nitrogen centered free radical which can be effectively scavenged by antioxidants [26]. DPPH is also considered as a good kinetic model for peroxylradicals [27]. The ability of protein to scavenge DPPH radical was determined by the decrease in it is absorbance in spectrophotometer. When, the solution of diphenylpicrylhydrazyl was mixed with that of a substance that can donate a hydrogen atom then this give rise to the reduced form (Diphenylpicrylhydrazine) which indicates the loss of this violet color [28]. The present, investigation shown that the partial purified from *P.virens* methanolic crude extract exhibited DPPH scavenging activity. Since the effect of antioxidants on DPPH radical scavenging is thought to be due to their hydrogen donating ability. The reducing power ability of partial purified protein of *P.virens* methanolic extract greatly depends on the presence of reductions, which have exhibit antioxidant potential by breaking the free radical chain by donating a hydrogen atom [29]. Hydrogen peroxide is a weak oxidizing agent [30] and once inside the cell it can probably react with Fe2+ and possibly Cu2+ to form hydroxyl radicals and this may be the origin of toxic effects [31]. The result of present study reveals that there is a strongest H_2O_2 scavenging activity was observed for protein at various concentrations when compared to be good scavenger of hydrogen peroxide. But the maximum activity has observed in *P.virens* methanolic extract can be a good antioxidant for removing hydrogen peroxide free radicals.

In the present study, *P.virens* methanolic extract at the concentration from 20 to 250µg/ml exhibited 67.09%, 74.83%, 60.21% and 59.89% respectively. The present result suggests that the *P.virens* methanolic crude extract might be potent agent for scavenging assay. In the present study of *P.virens* extract at the various concentrations showed higher absorbance to indicating the tissue extract is the best source of antioxidant compounds. The chelating effects of various extracts on Fe2+ were determined by the formation of ferrozine -Fe2+ complexes. Chelating agents are able to capture ferrous ion before ferrozine, thus hindering the formation of ferrozine -Fe2+. Numerous antioxidant methods and modifications have been proposed to evaluate antioxidant activity and to explain how antioxidants function. Of these, total antioxidant activity, reducing power, DPPH assay, metal chelating, active oxygen species such as H_2O_2, O_2•− and OH• quenching assays are most commonly used for the evaluation of antioxidant activities of extracts [32-34]. In this study methanolic extract of *P.virens* and standard compounds have potential of antioxidant activity. Ferrozine can quantitatively form complexes with Fe^{2+}. In the presence of chelating agents, the complex formation is disrupted, resulting in a decrease in the red colour of the complex. Measurement of colour reduction therefore allows estimating the metal chelating activity of the coexisting chelator. In this assay methanolic extracts of *Pila virens* and standard compounds are interfered with the formation of ferrous and ferrozine complex, demonstrating that they have chelating activity and are able to capture ferrous ion before ferrozine.

Spectrophotometer assessment of ferrozine-Fe2+ absorbance can accordingly be used to calculate ferrous ion chelating activity. The metal chelating capacity is important since it reduces the concentration of transition metals that may act as catalysts to generate the first few radicals to initiate the radical-mediated oxidative chain reactions in biological and food systems. Ion chelating agents also may inhibit the Fenton reaction and hydro peroxide decomposition [35]. Through the formation of ferrozine complex indicate the ferrous ion chelating ability of peptides from *P.virens* methanolic crude extract.

In present study, antioxidant activity of purified crude methanolic extract of *P.virens* was investigated. Although radical scavenging and antioxidant activities, as determined by scavenging effect on the total antioxidant activity, DPPH, chelating effect on ferrous ions, Hydrogen peroxide scavenging activity, superoxide scavenging activity and reducing power. Hence the present study concluded that *P.virens* methanolic extract possessed the potential bioactive compound and antioxidant activity it could be used as natural accessible source for treating human diseases.

Conclusion

The methanolic extract of *Pila virens* proved to be a reservoir of bioactive constituents, which could be used in various diseases in future. However, isolation of individual compounds and their biological activities needs to be uncovered further to enhance its pharmacological importance and open new avenues in research. It could be concluded that, freshwater snails *P.virens* contains various bioactive compounds and may be recommended as a freshwater gastropods of pharmaceutical importance.

References

1. Shanmugam M, Mody KH. Heparinoid-active sulphated polysaccharides from marine algae as potential blood anticoagulant agents. Curr Sci. 2000; 79:1672-1683.
2. Blunt J, Copp B, Munro M, Northcote P, Prinsep M. Marine natural products. 2006.
3. Diplock AT. Will the good fairies please proves to us that vitamin E lessens human degenerative of disease? Free Radical Research. 1997; 27:511-532.
4. Oktay M, Gulcin I, Kufrevioglu OI. Determination of *in vitro* antioxidant activity of fennel (Foeniculum vulgare) seed extracts, Leb Wissen Technol. 2003; 36:263-271.
5. Shenoy AS. Octopus a delicacy in Japan. Sea Food Exp. 1988; J, 20:21-25.
6. Tincu JA, Taylor SW. Antimicrobial peptides from marine invertebrates. Antimicrobial Agents Chemother. 2004; 48:3645-3654.
7. Nagash YS, Nazeer RA, Sampath Kumar NS. *In vitro* antioxidant activity of solvent extracts of molluscs (Loligo duvauceli and Donax strateus) from India. World J Fish Mar Sci. 2010; 2:240-245.
8. Riguera R. Isolating bioactive compounds from marine organisms. J. mar.biotechnol. 1997; 5:187-193.
9. Ebada SS, Edrada RA, Lin W, Proksch P. Methods for isolation, purification and structural elucidation of

bioactive secondary metabolites from marine invertebrates. Nat. Protocols. 2008; 3:1820-31.
10. Koehn. High impact technologies for natural productus screening. Natural compounds as drugs. Springer. Prog drug res. 2008; 65(175):177-210.
11. Chalamaiah M, Dinesh Kumar B, Hemalatha R, Jyothirmayi T. Fish protein hydrolysates: Proximate composition, amino acid composition, antioxidant activities and applications: A review. Food Chemistry. 2012; 135:3020-2038.
12. Umayaparvathi S, Meenakshi S, Vimalraj V, Arumugam M, Sivagami G, Balasubramanian T. Antioxidant activity and anticancer effect of bioactive peptide from enzymatic hydrolysate of oyster (Saccostrea cucullata). Biomedicine and Preventive Nutrition. 2014; 233:1-11.
13. Subha Rao NV. Indian seashells (Part: 1) Polyplacoptera and Gastropoda. Zoological Survey of India, Occasional. 2003; 192:426.
14. Chellaram C, Edward JK. *In vivo* anti-inflammatory bustle of reef associated mollusc, Trochus tentorium. Adv Biotech. 2004; 8(12):32-34.
15. Guardino X, Obiols J, Rosell MG, Farren A, Serra C. Determination of chlorpyrifos in air leaves and soil from a greenhouse by gas chromatography with nitrogen phosphorus detection, high – performance liquid chromatography and capillary electrophoresis. Journal of chromatography. 1998, 91-96.
16. Ashwini P, Krishnamoorthy M. Antioxidant activity of ethanolic extract of Cassia toral. IJRAP. 2011; 2(1):250-252.
17. Susithra E, Mallikarjuna Rao K, Ramesh KV, Meena S. Evaluation of *in vitro* antioxidant activity of isolated compounds of lichen, Usnea undulate. J pharm res. 2011; 4(2):352-355.
18. Pandimadevi K, Suganthi N, Kesika P, Karuthapandian S. Bioprotective properties of seaweeds: *In vitro* evaluation of antioxidant activity and antimicrobial activity against food borne bacteria in relation to polyphenolic content. BMC Compl Alt Med. 2008; 8(38):1-11.
19. Oloyede GK, Farombi OE. Antioxidant properties of Crinum ornatum bulb extract. World J Chem. 2010; 5(1):132-36.
20. Lim TY, Lim YY, Yule CM. Evaluation of antioxidant, antibacterial and anti-tyrosinase activities of four Macaranga species. Food Chem. 2009; 114:594-599.
21. Stohs SJ. Oxidative stress induced by 2, 3, 7, 8-tetrachlorodibenzo-p-dioxin (TCDD). Free Radical Biological Medicine. 1995; 9:79-90.
22. Thiansilakul Y, Benjakul S, Shahidi F. Compositions, functional properties and antioxidative activity of protein hydrolysates prepared from round scads (Decapterus maruadsi). Food Chemistry. 2007; 103(4):1385-1394.
23. Pickrell J. Wonder Drug Snails face threats, Expers warn. National Geographic News. 2003, 1-2.
24. Yahya H, Wan ZM, Mohd IHS. The golden apple snail (Pomacea sp.) as a potential animal and aquaculture feed. Malaysian Agricultural Research and Development Institute, MARDI, Serdang, Malaysia. 2006.
25. Kaensombath L. Evaluation of the nutritive value of ensiled and fresh Golden Apple snails (pomacea spp) for growing pigs. Research Papers, National University of Laos, Vienetiane, Laos. 2006.
26. Vilano D, Fernandez-Pachona MS, Moyab ML, Troncosoa AM, Garcia-Parrilla MC. Radical scavenging ability of polyphenolic compounds towards DPPH free radical. Talanta. 2007; 71:230-235.
27. Rackova L, Oblozinsky M, Kostalova D, Kettmann V, Bezakova L. Free radical scavenging activity and lipoxygenase inhibition of Mahonia aquifolium extract and isoquinoline alkaloids. J Inflamm. 2007; 4:15-21.
28. Molyneux P. The use of the stable free radical diphenylpicrylhydrazyl (DPPH) for estimating antioxidant. Songklanakarin J Sci Technol. 2004; 26:211-219.
29. Zou YP, Lu YH, Wei DZ. Antioxidant activity of a flavanoid rich extract of Hypericum perforatum L. *in vitro*. J Agric Food Chem. 2004; 52:5032-5039.
30. Pandimadevi K, Suganthi N, Kesika P, Karuthapandian S. Bioprotective properties of seaweeds: *In vitro* evaluation of antioxidant activity and antimicrobial activity against food borne bacteria in relation to polyphenolic content. BMC Compl Alt Med. 2008; 8(38):1-11.
31. Kulshreshthal M, Goswami M, Raol CV, Ashwlayan VD, Yadav S. Estimation of antioxidant potential of aqueous extract of Ficus bengalensis leaf on gastric ulcer. Int. J Phar Sci Rev Res. 2011; 9(1):122-126.
32. Duh PD, Tu YY, Yen GC. Antioxidant activity of water extract of Harug Jyur (Chrysanthemum morifolium Ramat), Lebensmittel- Wissenchaft and Technology. 1999; 32:269-277.
33. Amarowicz R, Naczk M, Shahidi F. Antioxidant activity of crude tannins of canola and rapeseed hulls. J. American Oil Chemist‟s Society. 2000; 77:957-961.
34. Chang LW, Yen WJ, Huang SC, Duh PD. Antioxidant activity of sesame coat, Food Chemistry. 2002; 78:347-354.
35. Liu W, Wang H, Yao W, Gao X, Yu L. Effects of sulfation on the physicochemical and functional properties of a water-insoluble polysaccharide preparation from Ganoderma lucidum. J Agric Food Chem. 2010; 58:3336-3341.