In vitro Antioxidant and Anticancer Activity of *Pila virens* Extracts on A549 Human Lung Cancer Cell Line

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ABSTRACT

Background: Natural bioactive compounds are unaided or in amalgamation with conventional chemotherapeutic agents, perhaps be used as prospective therapies to fight cancer. In this study to determine the bioactive compounds, antioxidant, and anticancer activities of *Pila virens* methonolic extracts on A-549 human lung cancer cell lines. *Methods:* The *Pila virens* extracts (aqueous, ethanol and methonal in various concentration) antioxidant activities using 2,2-diphenyl-1-picrylhydrazyl (DPPH), scavenging assay and ferrous ions activity in different. *In vitro* anticancer activity on A-549 human lung cancer cell lines was evaluated by (3-(4,5-dimethyl thiazole-2yl)-2, 5-diphenyl tetrazolium bromide) MTT assay. *Results: Pila virens* aqueous, ethanol and methanolic tissue extracts exhibited significant total antioxidant activity TAA, DPPH, Hydroxyl radical scavenging activity, total reducing power, chelating ability on ferrous ions activity which predicted as 9.4±0.34 to 66.69±0.62 µg/ml, 11.73 ± 0.81 to 75.61±0.49 µg/ml, 5.03 ±0.38 to 62.07±0.77 µg/ml and 14.16±0.71 to 60.63±0.48 µg/ml respectively. Methanol and ethanol extracts exhibited higher as compare to aqueous extract. Antioxidant capacities were shown highest in methanol and ethanol extracts based on the test performed. These results concluded that, the tissue extract of *Pila virens* has novel antioxidant potential and it has to further characterize to improve the pharmacological active natural products. These results indicate that *P.virens* could be a source of valuable bioactive materials.

Keywords: Antioxidant activity, Bioactive compounds, *In vitro*, Lung cancer cell, Secondary metabolites

INTRODUCTION

In recent years, there have been rises in infectious disease cases all over the world. Natural products have served as the most productive source of leads for drug development for centuries

(Harvey, 2007). In recent decades, many of the new antibiotics and new antitumor drugs approved by the US Food and Drug Administration (FDA), or comparable entities in other countries, are natural products or derived from natural products (Harvey, 2000; Esmaeelian *et al.*, 2007 and Newman, 2007). Natural products are still the major sources of innovative therapeutic agents for infectious and life-style diseases (Clardy and Walsh, 2004).

Natural products play a relevant role in cancer therapy today with substantial numbers of anticancer agents used in the clinic being either natural or derived from natural products from various sources such as plants, animals and microorganisms. Large-scale anticancer drug discovery and screening programs such as those promoted by the National Cancer Institute (NCI) have played an important role in the development of anticancer natural compounds. Today, more than 60% of the anticancer drugs commercially available are of natural origin (Craigg *et al.*, 1997). The knowledge of the physiological and biochemical features of freshwater organisms might contribute to the identification of natural products of biomedical importance. Many of these natural products have interesting biomedical potential, pharmaceutical relevance and diverse applications and also they provided the significant components of pharmacologically important chemical bioactive substances.

The molecular diversity of chemical compounds found in freshwater animals is the result of the evolution of the organisms and their unique physiological and biochemical adaptations and offers a good chance for the discovery of novel bioactive compounds with a variety of unique structures and diverse biological activities. The secondary metabolites isolated from molluscs fall into a wide range of structural classes, with some compounds predominating in certain taxonomy. Cancer cell proliferation, apoptosis, angiogenesis, invasion and metastasis are regulated by an interconnecting network of cellular signaling pathways involving extracellular ligands, transmembrane receptors, intracellular signaling protein kinases, and transcription factors (Adjei *et al.*, 2005; Arun Baskar et al., 2018).

On a global scale, lung cancer is the most common type of cancer. Lung cancer has consistently increased every year and represents 12.3% of all cancers with a total of 52% of these cases accounting for developed countries. In terms of gender, males are the most affected, accounting for 75% of all cases. Lung cancer histology characterizes the condition in two variants, i.e. the non-small cell lung and the small cell lung cancers (Chen *et al*., 2002; Ferlay *et al*., 2008 and 2014; Brenner *et al*., 2011). The first morphological type originates from bronchial epithelial-cell precursors and comprises large cell carcinoma, adenocarcinoma, squamous cell carcinoma and bronchia alveolar cell carcinoma, all of which account to 85% of lung cancer cases.

Molluscs are another prime species that have a wide range of uses in pharmacology. The bioactive compounds are thought to be concentrated molluscs that have defensive chemical weapons (secondary metabolites) for their predation and protection. Its metabolites have been most commonly tested for neuromuscular blocking action, anti-predator, antimicrobial, anti-neoplastic and cytotoxicity activity. Cytotoxicity potential of a natural product is generally a reliable molecule to exhibit anticancer activity. *Pila virens* methanolic extract kills cancer cells and thus exerts its antitumour effects, it also possesses of anti-oxidant properties. It is also believed that the snails and mussels are very good source of bioactive compounds considering the importance of the group.

Molluscs represent good candidates for anti-cancer natural products research considering their evolutionary and ecological significance. Currently, natural products isolated from molluscs and their structural analogues are particularly well represented in the anticancer compounds in clinical trials (Simmons *et al.,* 2005). Natural antioxidant compounds exhibit their antioxidant activity through various mechanisms; including chain breaking (by donation of hydrogen atoms or electrons that convert free radicals into more stable species) and decomposing lipid peroxides into stable final products (Hussain *et al.,* 2008).

Molluscs are considered as one of the important sources to derive bioactive compounds that exhibit antitumor, antimicrobial, anti-inflammatory, and antioxidant activities (Benkendorff *et al.,* 2011). It is also containing rich nutrients that are beneficial to people in all ages (Anand *et al.,* 2010; Andreo, and Jorge, 2006). In our body, oxidation process leads to cell damage, cancer and degenerative diseases; antioxidant molecules present in different molluscs has prevent cell damage from oxidation reaction (Nagash *et al.,* 2010). It is also believed that the snails are very good source of bioactive compounds considering the importance of the group.

Bioactive compounds such as antioxidants are frequently added to food supplies to provide health benefits outside the basic nutritional values. Food industries have tried to improve the quality and nutrition value of their products, defending the association of ordinary diet supplies with natural compounds contributes to a lower risk in the development of chronic diseases, including cancer and cardiovascular disorders (Biesalski, *et al,* 2009). Most of the pathogens are increasingly resistant to the major classes of the routinely used antibiotic. It is a metabolite, which can or not be synthesized by the organism, such as vitamins, proteins or polyphenols. Those are essential compounds presenting a range of different bioactivities including antitumor, anticoagulant, antifungal, antiviral, anti-flammatory, antimicrobial and antioxidant activities (Bhatnagar & Kim, 2010).

A549 is a malignant cell line which was derived from human lung carcinoma. A549 cells resemble type II cells in a number of important features, because they are readily cultured and derived from a human source, and they are widely used as a model of type II cells (Umimo *et al.*, 2000). For this reason A549 cell line was chosen for this study. The earliest morphological changes in apoptotic cells include cytoplasmic shrinking, loss of cell–cell contacts and active membrane blabbing (Sutherland *et al.*, 2001). Apoptosis is a physiological process leading to cell death (Chen *et al.*, 2002). It plays an important role as a protective mechanism in the organism by removing damaged cells or over proliferating cells due to improper mitotic stimulus (Lee *et al.*, 2010). The induction of apoptosis has become a target strategy for antitumor drug discovery in recent years, and an apoptosis-inducing agent specific for tumor cells may be an ideal antitumor drug (Chen *et al.*, 2002). In this study, we report that the carvacrol was cytotoxic towards a human non-small cell lung cancer (NSCLC) cell line (A549) and caused the cells to undergo apoptotic cell death.

Discovering the natural products against the human diseases is a current focusing of research. Unlike synthetic chemicals natural products have low toxicity, complete biodegradability and eco-friendly. Many anti-oxidant and anticancer agents are used with varying degrees of success, they all have some distinct disadvantages such as treatments may affect normal cells as well and cause serious and potentially life threatening side effects. Therefore, new anticancer treatments are continuously being sought which will prove to be more selective in inhibiting cancer cells, while being less toxic to the normal cells. Hence, in the present study the bioactive compounds / secondary metabolites were isolated from the freshwater gastropod *P.virens* and its *in vitro* anti-oxidant and anti-cancer efficacy was evaluated.

MATERIALS AND METHODS

Collection of samples and extraction

The freshwater gastropods *P.virens* were collected from the 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 (Subha Rao., 2003). The shell removed fresh tissue samples were washed with sterile distilled water. The extraction method was followed by (Vinodhini et al., 2017).

The freshly collected mollusk tissues each 25g in wet weight were soaked in aqueous, ethanol and methanol separately and maintained for 3 days. The extracts were filtered through Whatman No.1 filter paper and the solvents were concentrated by rotary evaporator (VC100A Lark

Rotavapor at 30°C) and freeze dried to give light yellow gummy mass which stored at 4˚C for further analysis.

Sample analysis in GC-MS

The purified gastropod fractions were individually examined using GC SHIMADZU QP2010 Ultra system and gas chromatograph interfaced to a mass spectrometer equipped with Elite-1 fused silica capillary column. For GC-MS 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 250ºC: ion source temperature 200ºC). The Column temperature was programmed from 80^0C (holding time 1 min) – further increase (5°C/min) – upto 300°C (2min) 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.

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 Assay

Antioxidant activity of the methanolic extract of *P.virens* were estimated in terms of total antioxidant activity, DPPH, Hydroxyl radical scavenging activity , total reducing power, chelating ability on Ferrous ions method. The two most commonly used synthetic antioxidants; Butylated Hydroxyl Anisole (BHA) and Butylated Hydroxyl Toluene (BHT) are restricted because of their toxicity and DNA damage induction followed by Arjun *et al.*, (2017).

Assay of Total Antioxidant Activity (TAA)

The free radicals oxidize the highly unsaturated beta carotene. Consequently, the orange colored chromophore of beta carotene would be degraded and the results can be monitored spectrophotometrically. The antioxidant activity is determined by the conjugated. The total antioxidant activity was carried out according to the Diene method described by Moovendhan (2016). Briefly, 2.0 ml of sample at various concentrations (50-250 µg/ml) was mixed with 1.0 ml reagent solution (0.6 M sulfuric acid, 28mM sodium phosphate and 4mM ammonium molybdate). The reaction mixture was incubated at 95°C for 90 min in water bath. After the mixture had been cooled to room temperature, the absorbance of each solution was measured at

695nm against a blank. The L-ascorbic acid and BHA were used as standards and the total antioxidant capacity was expressed as ascorbic acid equivalent.

$$\text{Antioxidant activity (\%)} = (A0 - A1 / A0) \text{ X } 100$$

Where; A0 is the absorbance of control and A1 is the absorbance of test. Ascorbic acid and Butylated Hydroxyanisole (BHA) (Lingnert *et al.,* 1979) can be used as a positive control.

Assay of Scavenging ability on 1,1 dipheny l-2 picrylhydrazy radicals(DPPH)

The Scavenging ability on DPPH radicals was determined according to the method Lee *et al.*, (2007). Each sample (50-250 µg/ml) in 2g/l acetic acid solution (4 ml) was mixed with 1ml of aqueous, ethanol and methanol solution containing DPPH radicals, which result a final concentration of 10mmol/l DPPH. Briefly, the concentrations (50-250 µg/ml) of extracts were prepared in methanol. DPPH solution (0.004%) was prepared in ethanol and 1 ml of this solution was mixed with the same volume of aqueous, ethanol and methanol gastropod extracts and standard ascorbic acid, BHA solution separately.

The mixture was shaken vigorously and left to stand for 30 minutes in the dark and the absorbance was then measured at 517nm against a blank. Ascorbic acid and BHA were used for comparison. The scavenging ability was calculated by

$$\text{DPPH Scavenging ability (\%)} = \frac{(A^{\text{control}} - A^{\text{sample}})}{A^{\text{control}}} \times 100.$$

Where A^{control} the absorbance of the control reaction (1 ml of ethanol with 1 ml of DPPH solution), and A^{Sample} the absorbance of the test sample. The results were analyzed in triplicate.

Assay of Hydroxyl Radical Scavenging

The reaction mixture containing sample (50-250µg/ml) was incubated with deoxyribose (3.75 mM), H_2O_2 (1 mM), $FeCl_3$ (100 mM), EDTA(100 mM) and ascorbic acid (100 mM) in potassium phosphate buffer (20 mM, pH 7.4) for 60 minutes at 37ºC. The reaction was terminated by adding 1ml of TBA (1%, w/v) and 1ml of TCA (2%, w/v) and then the tubes were heated in a boiling water bath for 15 minutes. The contents were cooled and the absorbance of the mixture was measured at 535 nm against reagent blank. Decreased absorbance of the reaction mixture indicated decreased oxidation of deoxyribose.

$$\text{Inhibition (\%)} = (A0\text{-}A1) / A0 \text{ x } 100)$$

Where A0 represents control absorbance (a blank without peptides) and A1 represents the sample's absorbance. Final assay values are presented as means taken from separate assays of samples for concentration.

Assay of Reducing Power

Briefly, 1 ml of *P.virens* methanolic extract (50-250µg/ml) in phosphate buffer (0.2 M pH 6.6) was mixed with 1 ml of potassium ferricyanide (1%, w/v) and incubated at 50ºC for 20 min. Afterwards 2.0 ml of TCA (10% w/v) was added to the mixture to terminate the reaction. The solution was mixed with 1.25 ml of ferric chloride (0.1% w/v) and the absorbance was measured at 700nm. The L-ascorbic acid and BHA were used as standards.

Assay of Chelating Ability on Ferrous Ions

Chelating ability was determined according to the method of Dinis *et al.*, (1994). Each sample (50-250µg/ml) in 2 g/l acetic acid solution (1 ml) was mixed with 3.7 ml of methanol and 0.1 ml of 2 mmol/l ferrous chloride. The reaction was initiated by the addition of 0.2 ml of 5 mmol/l ferrozine. After 10 minutes at room temperature, the absorbance of the mixture was determined at 562nm against a blank and Ethylene diamine tetra acetic acid (EDTA) was used as a standard. A lower absorbance indicates a higher chelating power.

$$\text{Metal chelating effect (\%)} = ((Abs1-Abs2)/Abs1) \times 100$$

Where Abs1 was the absorbance of the control and Abs2 was the observed absorbance for the sampled *P.virens* methanolic extract concentration.

Culturing of cell lines

The Vero and human lung cancer cells (A549) were obtained from Kings Institute of Preventive Medicine and Research, Guindy, Chennai. The cells were grown in 96-well tissue culture (TC) plate in Dulbecco's Minimum Essential Medium (MEM) with Trypsin-phosphate-versene-glucose (TPVG) solution, 10% New Born Calf Serum (NBCS) (Gibco-Invitrogen), 100 U/mL of penicillin (Gibco-Invitrogen) and 100 μg/mL of streptomycin (Gibco-Invitrogen). The cells were incubated in CO_2incubator (Haier Electric Co., Ltd.,) at 37^{o}C in 95% humidified atmosphere enriched by 5% CO_2 and sub-cultured every 3-4 days once.

MTT cell viability assays

The MTT assay was done using the methodology of Siddiqui *et al* (2010). The monolayer of cell culture was trypsinized and the cell count was adjusted to $1.0x10^5$ cells/ml using growth medium. To each well of the 96 well microtitre plate, 0.1ml of the diluted cell suspension (approximately10, 000 cells /well) was added. After 24 hours, when a partial monolayer was formed, the supernatant was flicked off, the monolayer was washed once and 100µl of *Pila virens* methanolic extract with different concentrations (100, 200, 250,500 and 1000µg/ml) was added to each well. The plates were then incubated at 37^{o}C for 3 days in 5% CO_2 atmosphere, and microscopically examined at the end of 6, 12, 24 and 36 hours for recording the result. After

72 hours, the test solutions in the wells were discarded and 50µl of MTT in HBSS-PR was added to each well. The plates were gently shaken and incubated for 3 hours at 37°C in 5% CO_2 atmosphere. The supernatant was removed and 50µl of propanol was added and the plates were gently shaken to solubilize the formed formazan. The absorbance was measured using a microplate reader at the wavelength of 540nm. The percentage growth inhibition was calculated using the formula given below:

$$\% \text{ Cytotoxicity} = (1\text{-Abs test /Abs Control}) \times 100$$

Statistical analysis

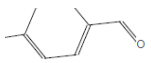
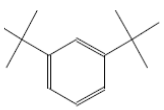
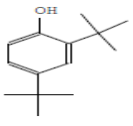
The results were expressed as mean ± standard deviation. Descriptive statistics was used to analyze the mean, standard deviation, variation, and level of statistical significance between groups. When $p < 0.05$ and $p < 0.01$, it was considered statistically significant for analysis of percent inhibition of cell growth.

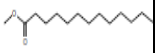
RESULTS AND DISCUSSION

Extraction and chemical characterization of secondary metabolites

Isolation and purification of bioactive compounds extracts of the snail for its biological activity. The active principles with their retention time (RT), molecular formula, molecular weight (MW), and concentration (Peak area %) are recorded. *Pila virens* whole animal extract by GC-MS analysis clearly showed the presence of four compounds they are 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).

Table 1. Activity of components identified in the whole body muscle extract of *P.virens*

Peak	Retention Time	Peak Area %	Molecular Formula	Molecular Weight	Name	Activity	Compound Structure
1	9.844	40.29	C_8H_8O	120	Benzaldehyde, 4-Methyl	Neurotoxicity , Antioxidant	
2	14.573	17.83	$C_{14}H_{22}$	190	Benzene, 1,3-Bis(1,1-Dimethylethyl)	Antibacterial activity, Antioxidant, Antiseptic	
3	21.337	7.21	$C_{14}H_{22}O$	206	Phenol, 2,4-bis(1,1-Dimethylethyl)	Analgesic, Anesthetic, Antioxidant, Antiseptic,	

						Antibacterial, Antiviral, Cancer preventive	
4	30.613	4.68	$C_{17}H_{34}O_2$	270	Hexadecanoic Acid, Methyl Ester	Antioxidant, Antandrogenic, Hyporcholesterolemic	

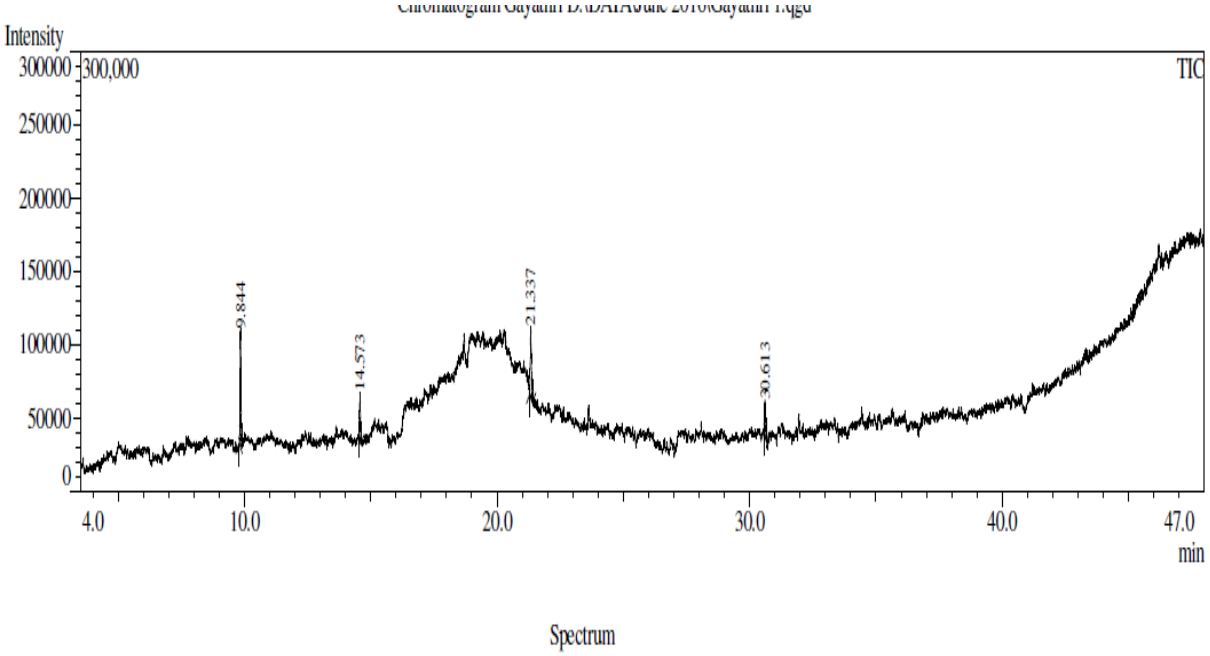


Fig. 1. GC-MS analysis of bioactive compound of *P.virens*

Total Antioxidant Activity

The *Pila virens* methanolic extract showed the total antioxidant activity in the range of 9.4±0.34 to 18.86±0.44 µg/ml, 12.17±0.45 to 28.16±0.42 µg/ml and 20.59±0.49 to 66.69±0.62 µg/ml at different concentrations 50 - 250 µg/ml. The maximum of 66.69±0.62 inhibition was observed at the concentration of 250 µg/ml of *Pila virens* methanolic extract to compared aqueous and ethanolic 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.

DPPH Radical Scavenging Activity

The effect of *Pila virens* methanolic extract on oxidative damage induced by hydroxyl radical at different concentrations (50 - 250 µg/ml) was found between 11.73 ± 0.81 to 18.53 ±0.52 µg/ml, 15.72 ± 0.58 to 53.22 ±0.28 µg/ml, and 22.02 ±0.80 to 75.61±0.49 µg/ml of *Pila virens* methanolic extract to compared aqueous and ethanolic extract. The maximum of 75.61%

inhibition was observed at the highest concentration of 250 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 250 µg/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.

Hydroxyl Radical Scavenging Activity

The effect of *Pila virens* methanolic extract on oxidative damage induced by hydroxyl radical at different concentrations (50 - 250 µg/ml) was found between 5.03 ±0.38 to 12.4±0.58 µg/ml, 7.26±0.26 to 33.58±0.50µg/ml, and 10.02±0.39 to 62.07±0.77µg/ml of *Pila virens* methanolic extract to compared aqueous and ethanolic extract. The maximum of 62.07 % inhibition was observed at the highest concentration of 250 µg/ml of *Pila virens* methanolic extract. The hydroxyl radical scavenging activity of L-ascorbic acid and BHA was 80.34 and 82.56% at 250 µg/ml.

Superoxide Radical Scavenging Activity

The effect of *Pila virens* methanolic extract on oxidative damage induced by superoxide radical at different concentrations (50 - 250 µg/ml) was found between 14.16±0.71 to 24.86±1.14 µg/ml, 22.48±0.94 to 53.91±0.46 µg/ml, and 26.19±0.29 to 60.63±0.48 µg/ml of *Pila virens* methanolic extract to compared aqueous and ethanolic extract. The maximum of 60.63 % inhibition was observed at the highest concentration of 250 µg/ml of *Pila virens* methanolic extract. The hydroxyl radical scavenging activity of L-ascorbic acid and BHA was 82.45 and 85.29% at 250 µg/ml.

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 *Pila 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 (Fe^{2+}) 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 (Stohs, 1995). The complex formation of the ferrous ion is disrupted when chelating agents are present, resulting in decreased of color (Thiansilakul *et al*., 2007). As for reducing power, the presence of antioxidants in causes the *Pila virens* methanolic extract reduction of the ferricyanide complex to its ferrous form.

Validation Tests Validation trials were run to determine the actual yield and antioxidant activity under the stated optimized conditions. Experimental results for optimized hydrolysis produced a 9.08% yield with an averaged antioxidant activity of 60.21%. Both values were less than predicted values which anticipated a yield of 19.78% and antioxidant activity of 67.09%. These lower results were likely due to losses occurring during the process of freeze drying as a consequence of small batch drying (Abdul Hamid and Bee, 2002).

Table 2. Antioxidant activity of *P.virens* different extracts (Water, Ethanol and Methanol)

Concentration	50 µg/ml			100 µg/ml			150 µg/ml			200 µg/ml			250 µg/ml		
Extracts	**A**	**E**	**M**	**A**	**E**	**M**	**A**	**E**	**M**	**A**	**E**	**M**	**A**	**E**	**M**
Total antioxidant	9.4± 0.34	12.17 ±0.45	20.59 ±0.49	11.23 ±0.27	19.83 ±0.77	30.01 ±0.81	13.5± 0.54	24.03 ±0.79	41.88 ±0.48	16.93 ±1.11	25.2± 0.43	50.9± .0.41	18.86 ±0.44	28.16 ±0.42	66.69 ±0.62
DPPH	11.73 ± 0.81	15.72 ± 0.58	22.02 ±0.80	13.7 ±0.60	23.80 ± 0.53	33.7± 0.56	14.83 ±0.51	36.53 ±0.51	48.88 ±0.41	15.4 ±0.21	44.47 ±0.20	61.02 ±0.40	18.53 ±0.52	53.22 ±0.28	75.61 ±0.49
Hydroxyl	5.03 ±0.38	7.26± 0.26	10.02 ±0.39	7.2 ±0.69	13.16 ±0.31	19.07 ±0.36	8.06± 0.36	16.25 ±0.27	30.48 ±0.54	10.5± 0.54	25.53 ±0.20	49.24 ±0.27	12.4± 0.58	33.58 ±0.50	62.07 ±0.77
Superoxide	14.16 ±0.71	22.48 ±0.94	26.19 ±0.29	13.7± 0.21	27.59 ±0.49	39.07 ±0.36	16.46 ±0.55	37.8± 0.78	44.12 ±0.74	20.6± 0.49	48.26 ±0.66	54.39 ±0.21	24.86 ±1.14	53.91 ±0.46	60.63 ±0.48

Cytotoxicity activity of P.virens

The *Pila virens* methanolic extract was initially evaluated for their cytotoxic effects on vero (Normal) cell lines. The maximum cytotoxicity (20%) of methanolic extract was observed at 1000 µg/ml concentration.

Anticancer Activity on A549 Cell Lines

The *Pila virens* methanolic extract showed the anticancer activity against A549 cell line in a dose dependant manner. A549 cells treated with *Pila virens* methanolic extract in different hours (6, 12, 24 and 36 hours) after the 36 hours the cells growth are controlled. The *Pila virens* methanolic extract showed a maximum inhibition of 68% at 500µg/ml against A549 lung cancer cells. The present investigation the minimum inhibition of 12% was recorded at 100µg/ml concentration. The *P.virens* methanolic extract showed the anticancer activity against A549 cell line in a dose dependant manner.A549 cells treated with *P.virens* methanolic extract in different concentration level (100, 200, 250, 500 and 1000 µg/ml) after the 36 hours the cells growth are changes occurred. A549 cell normally has a polygonal shape and sheet like pattern in normal monolayer culture, which is compatible with its epithelial origin. The *Pila virens* methanolic

extract showed a maximum inhibition of 68% at 500µg/ml against A549 lung cancer cells. The present investigation the minimum inhibition of 12% was recorded at 50µg/ml concentration.

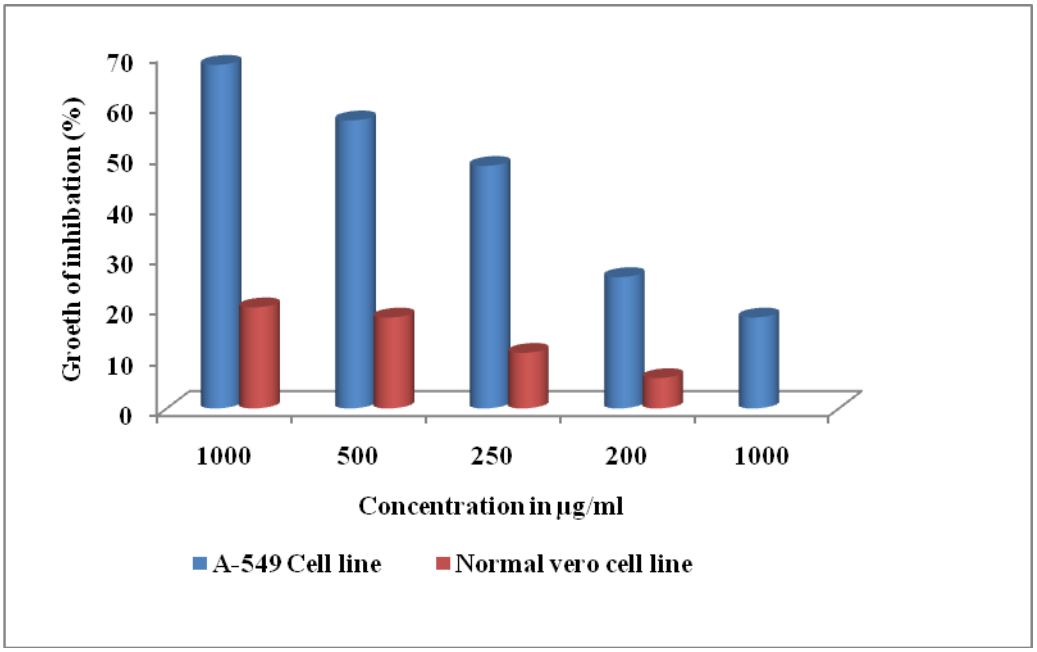


Fig. 2. Compared anticancer activity on normal Vero cell line to A549 cell lines in different concentration

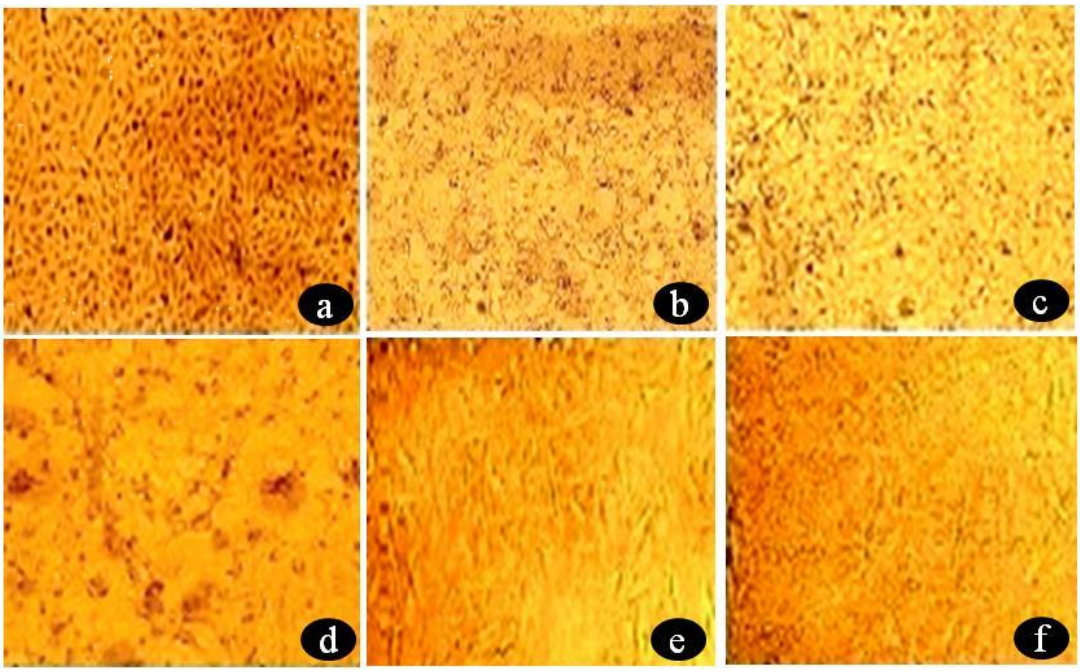


Fig. 3. A 549 Lung cancer cell culture in different concentration

a. Control, b. 100 µg/ml, c. 200 µg/ml, d. 250 µg/ml, e. 500 µg/ml, f. 1000 µg/ml.

Discussion

The secondary metabolites derived from number of molluscs possess antibiotic, anti-parasitic, antiviral and anti-cancer activities. Protein is a major biochemical constituent in all invertebrate and received highly attention due to their potential bioactive and functional properties. Many molluscs have evolved chemical defense mechanism for their *P.virens* methanolic extract and thus producing secondary metabolites which possess antioxidant activities.

Diphenylpicrylhydrazyl (DPPH) is stable nitrogen centered free radical which can be effectively scavenged by antioxidants (Vilano *et al.*, 2007) and also considered as a good kinetic model for peroxylradicals (Rackova *et al.*, 2007). 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 substance that can donate a hydrogen atom then this give rise to the reduced form (Diphenylpicrylhydrazine) which indicates the loss of violet color (Molyneux, 2004).

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 (Zou *et al.*, 2004). Hydrogen peroxide is a weak oxidizing agent (Pandimadevi *et al.*, 2008) and once inside the cell it can probably react with Fe^{2+} and possibly Cu^{2+} to form hydroxyl radicals and this may be the origin of toxic effects (Kulshreshthal *et al.*, 2011).

The result 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. Nevertheless, 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 Fe^{2+} were determined by the formation of ferrozine -Fe^{2+} complexes.

Results also showed that the antioxidant activity of *P.virens* methanol extract has potential as a radical scavenger due to its high chelation of the ferrous ion as well as hydroxyl radical scavenging activity. In addition, although lower than that of BHA, *P.virens* methanolic extract protein hydrolysate has reducing power activity. In the present investigation, the *P.virens* antioxidant activity correlated with 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 65ºC with a 2% enzyme concentration, a pH of 10, and hydrolysis time of 60 minutes. Kaensombath, (2005) and Yahya *et al.*, (2006) reported that the *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}). The involvement of free radicals, especially their increased production, appears to be a feature of most, if not all human diseases, including cardiovascular disease and cancer (Deighton *et al.*, 2000; Hariprasath et al., 2017).

Sivakumar and Rajagopal, (2011) have been reported that the highest antioxidant activity was observed in methanol extract from eight green algal species. Nevertheless, Uma *et al.*, (2011) observed that the methanolic extracts displayed greater potential in all antioxidant assays when compared to ethanolic and acetone extracts of green microalgae *Desmococcus olivaceous* and *Chlorococcum humicola.* Similarly, Lee *et al.*, (2010) reported that 80 % of methanol extract and organic solvent fractions (n-hexane, chloroform, and ethyl acetate of *Halochlorococcum porphyrae* and *Oltamannsiellopsis unicellularis* showed notable activities indicating the higher efficacy for scavenging of free radicals. These implications are important as radical scavengers may protect cell tissues from free radicals, thereby preventing diseases such as cancer. Free radicals such as superoxide radical (O_2), hydroxyl radical (OH) and other reactive oxygen species are associated with multistage carcinogenesis and mutagenesis. 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.

The present investigation was to purify and characterize methanolic extracted from the *P.virens* snails. Moreover the antioxidant property is derived from in the marine ecosystem only although present in the freshwater ecosystem. Nevertheless antitumor pharmacological studies were conducted with potentially promising *in-vitro* cytotoxicity data generated with freshwater snail

and human tumor cell lines were reported extremely scanty. In the scientific research, freshwater snails are used as model animals especially in molecular biology and immunology.

Cancer is a growing health problem around the world. Cancer continues to be the largest cause of mortality in the world and claims over 6 million lives every year (Hemminkin and Mutanen, 2001). Natural products have long been used to prevent and treat many diseases, including cancer, and thus they are good candidates for the development of anticancer drugs (Smith Warner *et al.*, 2000). Molluscs also contain highly rich nutrients, which are beneficial to humans. Many of these natural products have interesting biomedical potential, pharmaceutical relevance and diverse applications (Anand *et al.,* 2010). In the present investigation, the lung cancer cells are treated with *P.virens* methanolic extract. Naturally some bioactive compounds are present in the freshwater species still down with explained.

Cytotoxicity is one of the chemotherapeutic hallmarks of anti-tumor activity (Benkendorff , 2010). Cancer Resource is a database that integrates cancer-relevant relationships of compounds and targets complemented with experimental and supporting information on genes and cellular effects (Ahmed *et al.,* 2011). Cancer-relevant genes have been intensively studied and the fundamental hallmarks of cancer were established by Hanahan and Weinberg (2011) and the applicability of these concepts will increase the development of new avenues to treat human cancer. Nearly all cancers are caused by abnormalities in the genetic material of the transformed cells. The biomolecule also appears to inhibit the expression of certain specific genes that are involved in DNA replication and cell proliferation, thereby inhibiting tumor spreading and growth. Once a cancer diagnosis is confirmed, it is determined how to treat it. Conventionally, there are three ways to treat cancer; these are chemotherapy, radiation, and surgery hormone therapy. Any of these or combination of these is used to treat cancer.

CONCLUSION

In India the molluscan species occur in a wide range of habitats in the freshwater environment. The species abundance and economic resources pertaining to this group are vast. The conservation of molluscs is very important because of these great economic values, although the conservation is impossible without proper understanding of mollusc biology, habit and habitat and their physiological activities. To conserve the molluscs, understanding of defense mechanism through research study is essential. Understanding the immune defenses of these creatures is very important for improving disease resistance and increasing the survival of ecologically and economically important molluscs.

Natural products in simplest term are the chemical compounds, produced by living organisms. The cells of living organisms can be considered as micro chemical reactors producing large number of chemical compounds through metabolic reactions. Natural products unlike synthetic

chemicals have low toxicity, complete biodegradability, availability from renewable sources and some cases low cost. It is because of these reasons that health care products and environmentally acceptable agricultural compounds are mainly natural products or are derived by modification of natural product leads.

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