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GC- MS SCREENING *AND IN-SILICO* PREDICTION OF *PILA GLOBOSA* EXTRACT AGAINST THE BONE DISEASES IN CALCITONIN RECEPTOR

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ABSTRACT
India's flora and fauna has diverse medicinal properties and constitutes the major portion of our traditional medicine system. One among them is snails. Snails have a long history of being used for treating diseases in traditional medicinal systems around the world including India and China. *Pila globosa* is one of the largest land snails, mainly used as food. Here in this study, the gas chromatography and mass spectrometry discovered the presence of thirteen bio active constituents with various medicinal applications. The constituent Stigmasta-4, 22-dien-3-beta-ol had the highest retention time and exhibited a efficient binding with the human protein calcitonin receptor ectodomain. The calcitonin receptor types is expressed widely in various tissues and cells but most importantly in the osteoclates and hence a very important receptor in the treatment for bone disorders. The binding of the Pila globosa snail constituent with the human calcitonin receptor proved that a novel drug can be developed from the snail for the treatment of bone disorders such as osteoporosis, hypercalcemia of malignancy and paget's disease. In addition to that, the snail extract exhibited no cytotoxicity and an efficient antioxidant property against DPPH and H_2O_2 free radicals. The FTIR analysis revealed the snail contained biologically significant functional groups with properties suitable for a drug and prevalent in most of the pharmaceutical drugs. Thus, these results imply the safety and efficacy of the snail *Pila globosa* for a novel drug development.

Keywords: *Pila globosa*, Molecular docking, Antioxidant, Cytotoxicity, Drug discovery.

1. INTRODUCTION
India has a great history of art literature and medicine from prehistoric times. The country has a diverse flora and fauna biodiversity. The medicinal properties of the natural products are the fundamentals for the Indian traditional medicine system. After the development of science and technology, many of those products efficacies in treating human diseases had been proven and the ingredients are being used in modern drug development [1].

Fresh water snails are one of the Indian nature's abundance bio resources with varied applications. Amphineura, gastropods, bivalves, cephalopods, and scaphopods make up the freshwater snails, which are a diverse collection of animals in terms of shape and diversity. Snails have been identified to be a significant source of structurally varied bioactive chemicals with pharmacological and biological applications [2].

Natural goods derived from freshwater snails have been put to the test for a wide range of pharmaceutical activities. The fresh water snail metabolites have been most frequently tested for neuromuscular blocking action, anti-predator, antimicrobial, anti-neoplastic, antioxidant and cytotoxic activity [3]. In recent years, more and more researchers have come to the consciousness that freshwater organisms have an immense potential as a source of novel molecules such as antioxidants [4]. Antioxidants play an important role in inhibiting and scavenging radicals, thus providing protection to humans against infections and degenerative diseases, but more importantly against the life threatening disease, cancer [5].

Pila globosa, designated as apple snail, has the common habitat in ponds, lakes, streams, rice fields and tanks (Table 1 and Table 2). The snail commonly exists in India, Europe and USA. Even though they are fresh water snails, they are also found in low salinity brackish water. They are amphibious in nature. The snail hibernates during long period of drought in mud called as Aestivation or summer sleep. Herbivores and source of diet are plant scrapping and aquatic vegetation (succulent such as Vallisneria and Pistia). Sexes are separate. Sexual dimorphism is not seen in these snails. The soft body of the snail is enclosed in a univalve shell which is spirally coiled round the collumella axis and the outside opening called the mouth or the aperture. Right handed coiling called dextral. The snails have a unique well developed calcareous operculum, the closure of the mouth/aperture. Only three parts constitutes the soft body, the head, the foot and the visceral mass/viscera. The head is located on the upper side of the animal, has two pairs of contractile tentacles and a pair of eyes; the main organs are located in the spirally coiled viscera which is covered by mantle to pallium and the muscular flat foot aids in the locomotion of the snail. The visceral mass is covered by a skin called mantle that protects the head anthe viscera. Respiration through Pulmonary sac and ctenidium (gills), mantle serves as an additional respiratory organ. The animal has a heart with the pericardium and renal organs.

Table 1: Classification of *Pila globosa*

Kingdom	Animalia
Phylum	Mollusca
Class	Gastropoda
Order	Architaenioglosaa
Genus	*Pila*
Species	*globosa*

Table 2: Nomenclature of *Pila globosa*

Binomial Nomenclature	*Pila globosa* (William John Swanson, 1822).
Other names	Apple snail, Golden snail, Mystery snail, Golden-Mystery snail.

Pila globosa is widely consumed snails and are also used as animal feed such as in prawn culture and as poultry feed; a good source of animal protein. In recent years, the world trade of the snail has increased. It has great medicinal properties; High source of vitamins, minerals and proteins. Used in treating blood pressure, asthma, rheumatism, calcium metabolism, giddiness and nervousness, high body temperature, constipation, hypertension, hemorrhoids skin diseases such as urticaria, bone disorders such as osteoporosis [6].

The environmental pollution is assessed using snail species. The contaminants accumulation like metals relies on the assessment of internal concentrations after a specified exposure period and in snails the contaminants accumulation capacity, their bioavailability and the intensity with which the pollutants in environment (food and / or soil) transfer to snail are all detected [7]. Invertebrate species accumulate pollutants as a consequence of absorption-assimilation, storage, transmission and excretion processes [8]. Terrestrial snails have a non-regulation approach when absorbing pollutants with an influence of calcium metabolism [9], for the prevention of water loss in excess amount [10]. Itziou *et al.,* in 2011 reported that land snails may serve like a biomarker because they enabled the early detection of organic pollutants in their environment, after observing significant changes in haemolymph and digestive gland of snails exposed to organic contaminants [11].

In the analysis of the pollution accumulation using snail, viscera and foot are the important parts. The viscera refer to the snail's shell organs which includes the kidney, hepatopancreas, heart and reproductive system (a part) that extends into the foot. The foot essentially comprises the anterior part of the digestive tract and the nervous system. Previous case studies had revealed the significant dose-dependent increase in the concentration of pollutants in organs, hepatopancreas and kidney and accumulation of pollutants is based on the organism's bioavailability and environmental concentrations [12]. Snails have an open cardiovascular system, meaning no distinction between the blood and the lymph, so the circulatory fluid is commonly known as haemolymph, instead of blood. The organic and inorganic composition of the haemolymph is variable, and affected by the changes in external factors like temperature, photoperiod, activity, hibernation, hydration and feeding. Moreover, shell size and age, influences the haemolymph pollutants accumulation [13].

FTIR is the versatile rapid and reproducible infrared spectral technique for the identification of all the types of organic substances as well as many forms of inorganic substances. It facilitates the quantification of different species in a complex mixture, determine their

molecular composition and orientation and differentiate structural isomers from that of geometric ones. This technique can be used on a wide variety of samples such as gas, liquid, bulk and powdered solid samples, thin films, and nano materials, thus making it a well-used quantification technique. The principle of the technique is that it measures the infrared radiation absorption /transmission of a material as a function of wavelength and represents it as the IR spectrum. The absorption bands obtained are the result of the functional vibrations within the complex mixture, hence an IR spectra results in the identification of the functional group's in an unknown molecule. It can be either used a standalone technique or can be combined with gas or liquid chromatography for the separation and identification of the unknown species [14].

Freshwater snail extract have been structurally elucidated by aqueous designated. The combination of two different analytical techniques, Gas Chromatography (GC) and Mass Spectrometry (MS), is used to analyze complex organic and biochemical mixtures. The volatile and non-volatile compounds can be separated by GC with greater resolution while their structure can be determined by MS. As both the techniques have limitation when performed individually; together Gas chromatography and Mass spectrometry are, in many ways, highly compatible techniques [15].

Antioxidants are defined as any substance with the ability to delay or inhibit oxidation, being widely used in food industries [16]. Recently, the need for natural antioxidants has increased owing to the health hazards from synthetic antioxidants. Several published studies have discovered the cause of skin allergies, gastrointestinal tract problems owing to the long term consumption of synthetic antioxidants and in some cases as extreme as DNA damage, premature senescence and cancer [17-19]. The new integrated approach consisting of 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 favour the extraction and purification of bioactive compounds.

Molecular docking is the common method for structure based drug design since the 1980s [20]. The wide application of the molecular docking is because the technique can be used to model the interaction between a protein target and a drug candidate usually a small molecule at an atomic level and the fundamental biochemical processes can be elucidated by characterizing the behaviour of the molecule of interest at the binding site of target protein [21]. Thus, with the aid of molecular docking various molecules from natural and animal origin are being discovered for their role in drug discovery and the mechanism of action are deciphered. One such example can be seen in a study conducted by Liu *et al.,* in 2020, where the therapeutic effect of the herbal decoction Huangqi Guizhi Wuwu Decoction, used in Chinese traditional medicine for 1000 years for treating rheumatoid arthiritis, was confirmed through molecular docking that identified the presence of certain important active compounds such as quercetin, kaempferol, and beta-sitosterol [22].

Since the limited studies reported on the biological activity of aqueous extraction using the from fresh water snail, this study was carried out to analyse the bioactive compounds through GCMS, FTIR, *in vitro* cytotoxicity, antioxidant and molecular docking activities of aqueous extract from fresh water snail *Pila globosa*.

2. MATERIAL AND METHODS

2.1. Collection and Preparation of Fresh Water Snail

Fresh water Snails *Pila globosa* were collected from Tiruvallur district, Tamil Nadu, India, identified and transported to the laboratory under freeze condition.

2.2. Preparation of Snail powder

The aqueous extract of flesh was prepared through the reported method [23]. The snails were processed to break shells and the removed soft bodies (flesh) were collected in sterile glass containers. The flesh sample was dried in hot air oven at 55°C and then finely powdered. The shell sample was washed, dried at 55°C and blended into fine powder [24].

2.3. Soxhlet extraction

The preparation of extract was performed by modified method [25]. A quantity of 25g powder of formulated Snail sample was extracted in 100ml of aqueous ethanol. In a Seitz-filter with Whatman filter paper, the extract was filtered in sterile condition and using a lyophilizer (-80°C), the powder was obtained. The powdered extract was stored at 4°C until further analysis.

2.4. Fourier transform infra-red (FTIR) spectroscopy

A 2g of the *Pila globosa* snail extract was prepared with KBr and processed using 500 kg/m^3 pressure. The

sample was put into the sample holder and FTIR spectra were recorded ranging 3800-800 cm^{-1} using FTIR spectrometer [Model No.- IRAffinity- 1(SHIMADZU)] [45].

2.5. Gas chromatography-Mass spectrometry (GC-MS) analysis

The bioactive compounds present aqueous extract of formulated Snail sample were deducted using GC-MS (SHIMADZU QP2010). The GC specifications were as follows: column oven temperature was 80°C, injector temperature 250°C at a pressure of 108.0 kpa, injection mode- Split, Split Ratio- 40, Flow control mode was Linear velocity, Column flow was 1.58mL/min, Carrier Gas- Helium 99.99% purity. The MS specifications were as follows: Ion source temperature was 200°C, interface temperature was 250°C, scan range was 40-1000 m/z, event time- 0.5s, solvent cut time was 5 min, start time was 4 min, end time was 35 min, and ionization Washington EI (-70 ev) [26]. The compounds were identified by their identical GC retention times. The identified compound name, chemical formula, chemical nature and molecular mass were ascertained and represented. The relative percentage amount of all the components was calculated and represented as peak area percentage.

2.6. *In vitro* Cytotoxicity Assay

Fibroblast cell line (L929) was used for the cytotoxicity assay. The cell cultures were procured from National Centre for Cell sciences (NCCS), Pune, India. Stock cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% inactivated Fetal Bovine Serum (FBS), penicillin (100 IU/ml), streptomycin (100 µg/ml) and amphotericin B (5 µg/ml) and stored in an humidified atmosphere of 5% CO_2 at 37°C until confluent. TPVG solution (containing 0.2% trypsin, 0.02% EDTA, 0.05% glucose in PBS) was used for the cell dissociation. The stock cultures were grown in 25 cm^2 culture flasks and the experiment performed in 96 well microtitre plates.

2.6.1. Cytotoxicity of snail extract by MTT assay

The fibroblast cell line (L929) were plated separately using 96 well plates with the concentration of 1×10^4 cells/well in DMEM media with 1X Antibiotic Antimycotic Solution and 10% fetal bovine serum (Himedia, India) in CO_2 incubator at 37°C with 5% CO_2. The cells were washed with 200µL of 1X PBS, and then the cells were treated with various test concentration of compound in serum free media and incubated for 24 hour. At the end of the incubation period, the medium was aspirated from the cells and 0.5mg/mL MTT prepared in 1X PBS was added and incubated at 37°C for 4 h using CO_2 incubator. The MYT medium was then discarded followed by washing the cells with 200 µL of PBS. Using 100 µL of DMSO, the formed crystals were dissolved and mixed thoroughly. The solution turned to blue colour. The absorbance of the cells was measured at 570 nm in a microplate reader [27].

2.7. Antioxidant activities

2.7.1. (2, 2-Diphenyl-1-picrylhydrazyl)-DPPH scavenging assay

The free radical scavenging activity of snail extract was estimated using DPPH radical scavenging [28] with slight modification. One millilitre of various concentrations of snail extract (12.5-100µg/mL) was dissolved in 100µL of methanol which was mixed with 1.0 mL of 0.1 mmol DPPH solution. Ascorbic acid was used as the control and DPPH solution was used as the blank without any addition of extracts. The reaction mixture was shaken vigorously and incubated for 30 minutes in dark. The absorbance of the solution against blank measured at the wavelength of 517nm. Percentage of DPPH scavenging activity of snail extract was calculated using following formula.

DPPH scavenging capacity (%) = $[(A_{sample} - A_{blank})/A_{control}] \times 100$

2.7.2. Hydrogen peroxide (H_2O_2) scavenging activity

H_2O_2 scavenging assay was estimated according to the method [29] with some modification. The snail extract at different concentration 1mg/mL (12.5-100µg/mL) prepared in ethanol were added to 0.6 mL of H_2O_2 solution. The incubation period was 14 minutes. At 230nm, the absorbance of the reaction mixture was read. Blank solution includes H_2O_2 solution without any extract and compound substances. H_2O_2 scavenging activity of snail extract was calculated as:

Hydrogen peroxide scavenging (%) = $(A_{control} - A_{sample})/A_{control} \times 100$

2.8. *In-silico* Molecular Docking

Molecular docking of the bioactive constituent in *Pila globosa* against the human calcitonin receptor ectodomain was carried out with the aid of Auto dock

tools (ADT) v1.5.4 and Autodock v4.2 programs [30]. Chemical structure of ligands was retrieved from Pubchem database. Three dimensional structures of target proteins namely human calcitonin receptor ectodomain (PDB ID: 5II0); human Cathepsin K (PDB ID: 1YK8); human MMP-9 (PDB ID: 4XCT) and human purple acid phosphatase (PDB ID: 2BQ8), were retrieved from protein database [31]. The ligand was docked to target protein complexes with the molecule considered as a rigid body and the ligand being flexible. As blind docking, the whole receptor protein was searched. Lamarckian Genetic Algorithm was used for the search; 150 individuals in a population with 0.02 mutation rate evolved for 5 generations. Once the result obtained, the clusters sorted based on their binding energy for evaluation. To determine the trustable solution, a cluster analysis was performed that used root mean square deviation values, with reference to the starting geometry, and identified the lowest energy conformation of the more populated cluster. Docked ligand-receptor interactions were visualized and analyzed using Discovery Studio 2021 version.

3. RESULTS AND DISCUSSION

3.1. Fourier transform infra-red (FTIR) spectroscopy

The *Pila globosa* snail powder was analyzed by FTIR spectroscopy in order to identify diverse functional groups present in the bioactive components based on its peak ratio as well as electron transition of compounds Table 3 and Fig. 1. The peaks were observed between 3452-1573 $cm^{-1.}$ The functional groups present in *Pila globosa* were Amide (N-H), Alkyl (C-H), Aldehyde (CHO), Alkyne (C triple bond C), Carboxylic acid (O-H), ketone (C=O), Amide (C=O) and Benzene ring (C=C).

Table 3: Functional groups of *Pila globosa* at different wavelength through FTIR spectroscopy

Nanometer (nm)	Functional group	Peak strength
3452	Amide (N-H)	Medium and broad
2932	Alkyl (C-H)	variable
2879	Alkyl (C-H)	variable
2724	Aldehyde (CHO)	Medium; two peaks
2482	Carboxylic acid (O-H)	Strong
1885	Alkyne (C triple bond C)	variable and sharp
1745	Ketone (C=O)	Medium
1654	Amide (C=O)	Medium
1573	Benzene ring (C=C)	Medium

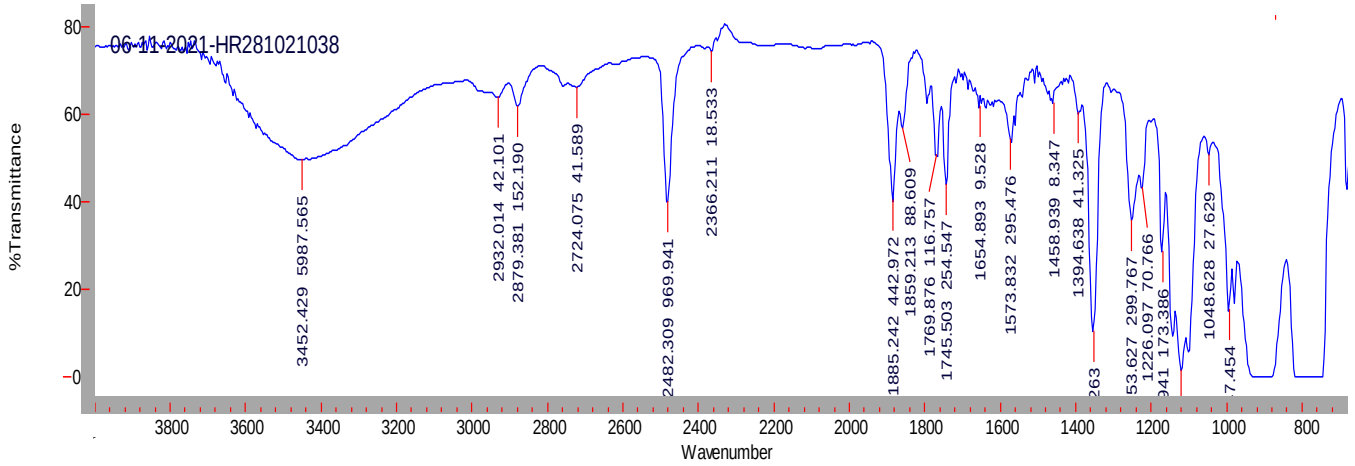


Fig. 1: FTIR spectroscopy of *Pila globosa*

3.2. Gas chromatography-mass spectrometry (GC-MS) analysis

The *Pila globosa* GC-MS chromatogram exhibited thirteen peaks i.e. thirteen compounds. The first compound eluted with the least retention time of 12.854 minutes was tetradecanoic acid and the compound with the longest retention time was Stigmast-7-en-3-ol, (3-beta,5-alpha) with a retention

time of 30.834 minutes. The compounds eluted from the GC was analysed in a mass spectrometer. The mass spectra of the different unknown components at the different retention time were compared with spectra of the known components in the National Institute of Standard and Technology (NIST) library and the best three hits were given in Table 4 and Figs. 2 & 3. The biological significance of the identified constituents was discussed.

Table 4: Bio-active components identified in the *Pila globosa* snail by GC-MS analysis

Peak No.	Retention Time (min)	Hit No.	Compound Name	Compound Nature	Molecular Formula	Molecular Weight	Peak Area (%)
1	12.854	1	Tetradecanoic acid	Fatty acid	$C_{14}H_{28}O_2$	228.4	1.49
		2	Tetradecanoic acid	Fatty acid	$C_{14}H_{28}O$	228.4	
		3	Tetradecanoic acid	Fatty acid	$C_{14}H_{28}O$	228.4	
2	15.878	1	n-Hexadecanoic acid	Fatty acid	$C_{16}H_{32}O_2$	256.4	4.50
		2	Tridecanoic acid	Fatty acid	$C_{13}H_{26}O_2$	214.3	
		3	n-Hexadecanoic acid	Fatty acid	$C_{16}H_{32}O_2$	256.4	
3	18.252	1	9,12-Octadecadienoic acid (Z,Z)	Fatty acid	$C_{18}H_{32}O_2$	280.4	1.57
		2	9,12-Octadecadienoic acid (Z,Z)	Fatty acid	$C_{18}H_{32}O_2$	280.4	
		3	9,12-Octadecadienoic acid (Z,Z)	Fatty acid	$C_{18}H_{32}O_2$	280.4	
4	18.338	1	6-Octadecenoic acid	Fatty acid	$C_{18}H_{34}O_2$	282.5	4.97
		2	9-Octadecenoic acid, (E)	Fatty acid	$C_{18}H_{34}O_2$	282.5	
		3	6-Octadecenoic acid, (Z)	Fatty acid	$C_{18}H_{34}O_2$	282.5	
5	18.654	1	Octadecanoic acid	Fatty acid	$C_{18}H_{36}O_2$	284.5	1.55
		2	Octadecanoic acid	Fatty acid	$C_{18}H_{36}O_2$	284.5	
		3	Octadecanoic acid	Fatty acid	$C_{18}H_{36}O_2$	284.5	
6	20.412	1	5,8,11-Heptadecatrien-1-ol	Fatty acid	$C_{17}H_{30}O$	250.4	1.52
		2	Arachidonic acid	Fatty acid	$C_{20}H_{32}O_2$	304.5	
		3	1,2-Dihydropyridine, 1-(1-oxobutyl)	Ester	$C_9H_{13}NO$	151.2	
7	21.621	1	Eicosane	Alkane	$C_{20}H_{42}$	282.5	4.24
		2	Sulfurous acid, butyl heptadecyl ester	Ester	$C_{21}H_{44}O_3S$	376.6	
		3	Pentadecane, 2-methyl	Alkane	$C_{16}H_{34}$	226.4	
8	28.672	1	27-Norergosta-5,22-dien-3-ol, (3.beta.,22Z)	Steroid	$C_{27}H_{44}O$	384.6	0.86
		2	6-Chloro-3-nitro-4-phenyl-quinolin-2-ol	Steroid	$C_{15}H_9ClN_2O_3$	300.7	
		3	3-methoxy-17-methylestra-1,3,5(10)-trien-17-ol	Steroid	$C_{20}H_{28}O_2$	300.4	
9	29.068	1	Cholesterol	Sterol	$C_{27}H_{46}O$	386.7	62.49
		2	Cholest-8-en-3-ol, (3.beta.)	Sterol	$C_{27}H_{46}O$	386.7	
		3	Cholestane, 5,6-epoxy-, (5.alpha…	Steroid	$C_{27}H_{46}O$	386.7	
10	29.434	1	Cholecalciferol	Steroid	$C_{27}H_{44}O$	384.6	3.84
		2	Pyrrol-2-one, 4-acetyl-1-(4-fluo…	-	-	-	
		3	Cholesta-5,7-dien-3-ol, (3.beta.)	Steroid	$C_{27}H_{44}O$	384.6	
11	30.022	1	.gamma.-Ergostenol	Steroid	$C_{28}H_{48}O$	400.7	7.74
		2	Cholesterol, 7-oxo	Sterol	$C_{27}H_{44}O_2$	400.6	
		3	Cyclopropa[5,6] cholestan-3-ol, 3…	Sterol	$C_{27}H_{48}O$	388.7	
12	30.255	1	Stigmasta-4,22-dien-3.beta.-ol	Steroid	$C_{29}H_{46}O$	410.7	2.55
		2	Cholest-7-en-3-one, 4,4-dimethyl	Steroid	$C_{29}H_{48}O$	412.7	
		3	22,23-Dihydrospinasterone	Sterol	$C_{29}H_{48}O$	412.7	
13	30.834	1	Stigmast-7-en-3-ol, (3.beta.,5 alpha)	Sterol	$C_{29}H_{50}O$	414.7	2.68
		2	Stigmast-8(14)-en-3.beta.-ol	Sterol	$C_{29}H_{50}O$	414.7	
		3	.beta.-Peltatin	Sterol	$C_{22}H_{22}O_8$	414.4	

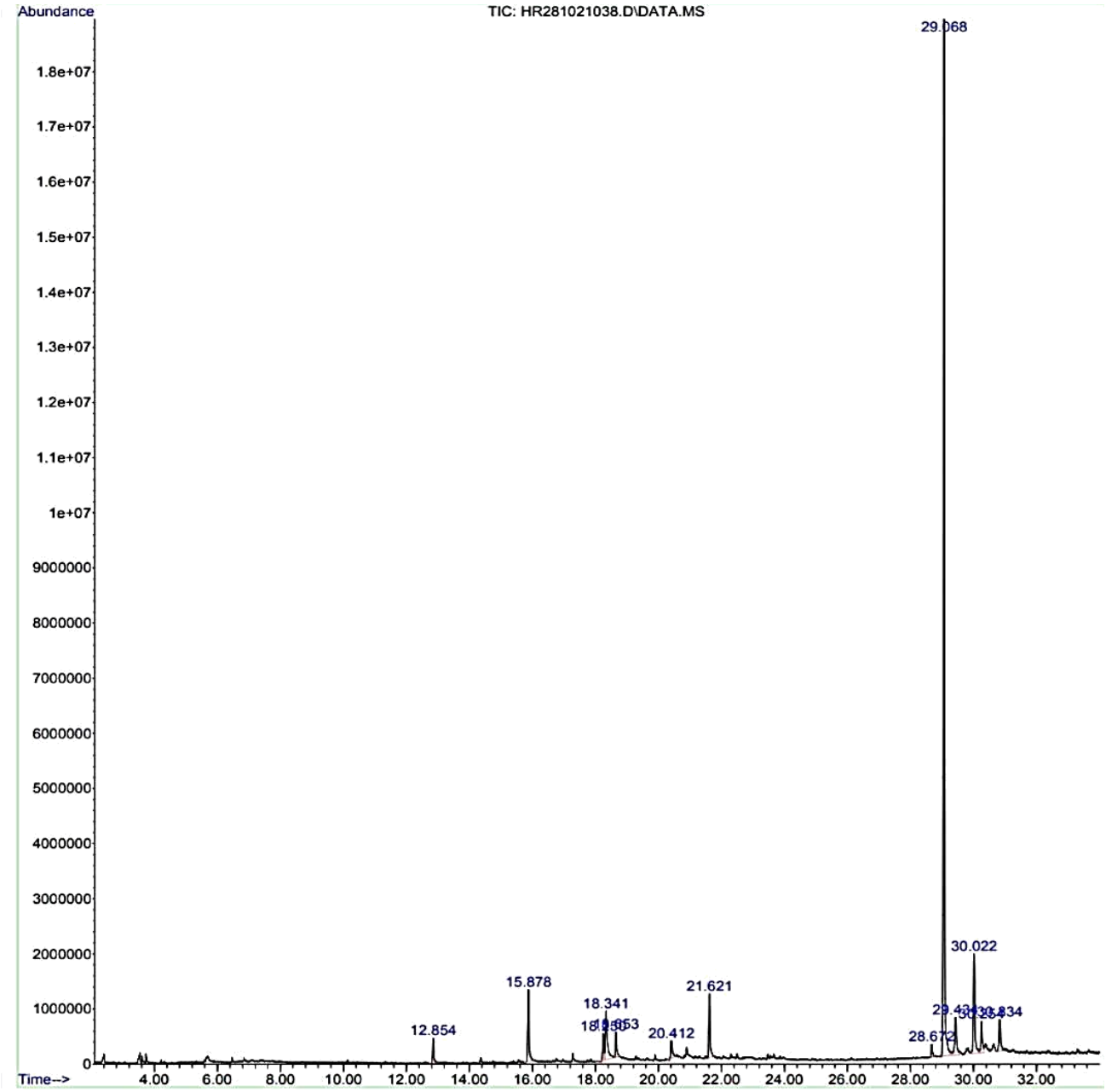
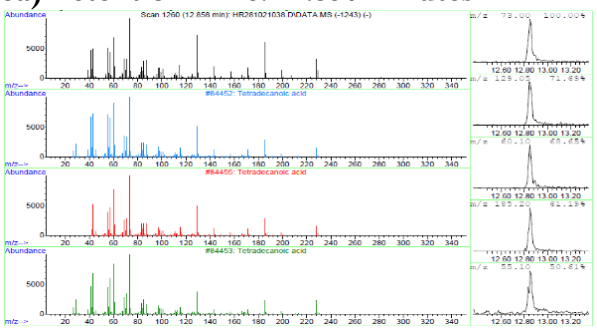
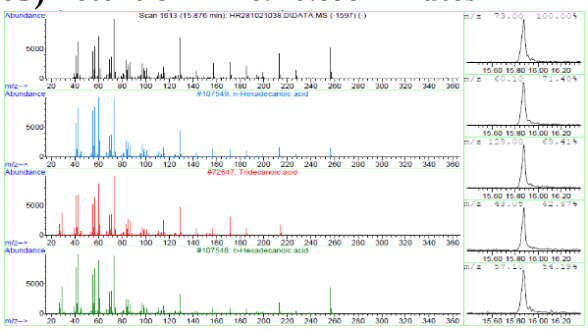


Fig. 2: GC-MS chromatogram of *Pila globosa*

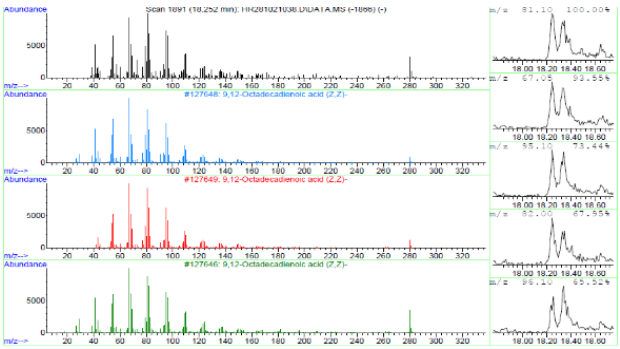
3a) Retention Time: 12.854 minutes



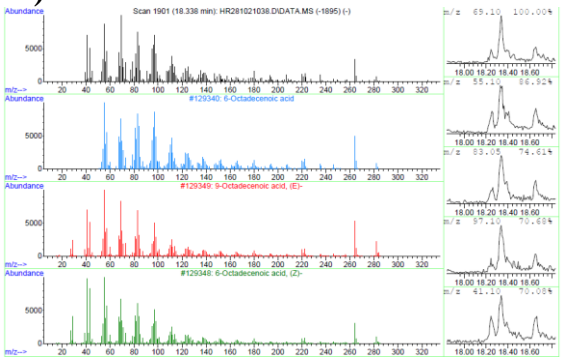
3b) Retention Time: 15.878 minutes



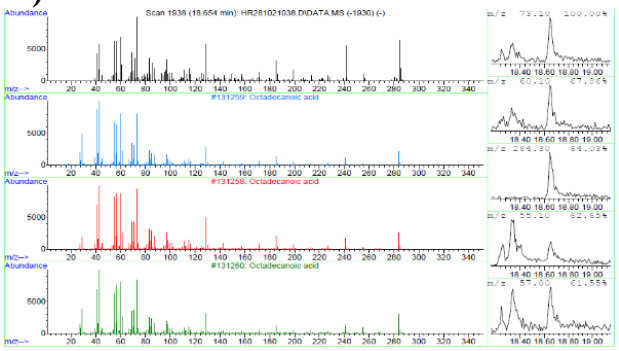
3c) Retention Time: 18.252 minutes



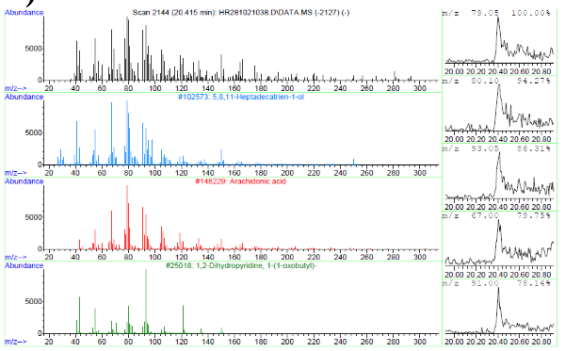
3d) Retention Time: 18.338 minutes



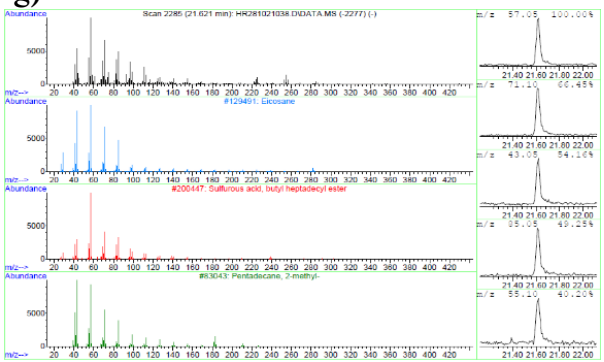
3e) Retention Time: 18.654 minutes



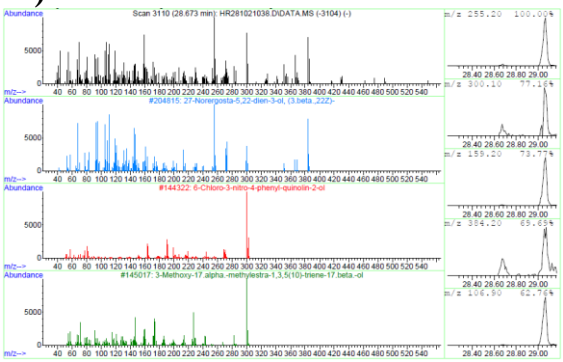
3f) Retention Time: 20.412 minutes



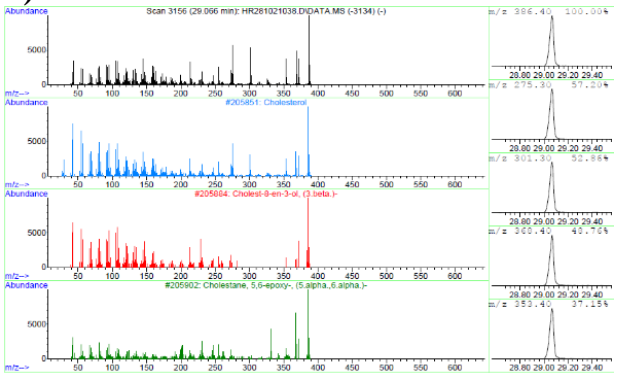
3g) Retention Time: 21.621 minutes



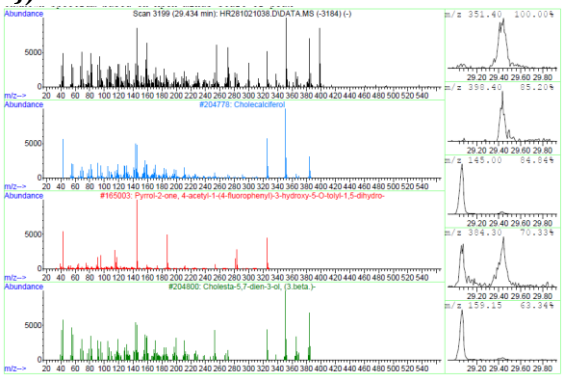
3h) Retention Time: 28.672 minutes



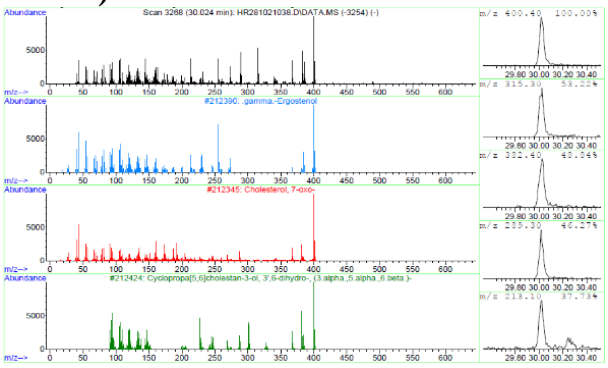
3i) Retention Time: 29.068 minutes



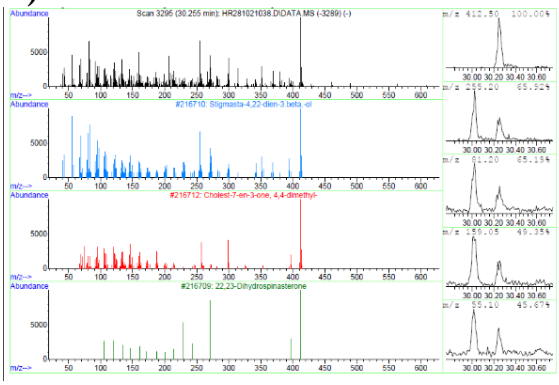
3j) Retention Time: 29.434 minutes



3k) Retention Time: 30.022 minutes



3l) Retention Time: 30.255 minutes



3m) Retention Time: 30.834 minutes

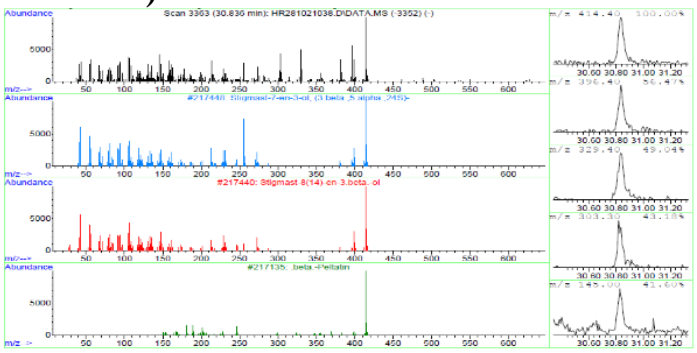


Fig. 3: GC-MS chromatogram of three hits of different constituents of *Pila globosa* represented in terms of retention time

3.3. *In vitro* cytotoxicity assay

The results of the absorbance of different concentrations of snail extract in fibroblast cell line measured at 570nm in the microplate reader were tabulated in Table 5 and based on the optical density of control and samples, % cell viability of each concentration were measured and tabulated in Table 6 and Fig. 4. The microscopic images of the control and different concentrations of snail in MTT assay was represented in Fig. 5. The concentrations were analysed in triplicates at 570 nm. The control wells exhibited an optical density of 0.295, 0.303 and 0.312 with 100% cell viability. The % cell viability of the snail extract was found to be inversely proportional to the test concentrations. At 100 µg/ml, % cell viability were 79.54%, 81.85% and 79.87% and exhibited an increasing viability with decreasing concentration, the highest viability seen at 12.5 µg/ml as 99.34%, 98.35% and 99.01% and similar to the controls.

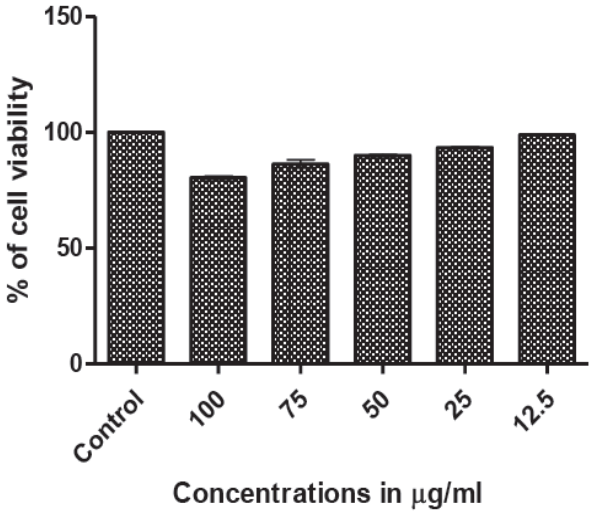


Fig. 4: Graphical representation of % cell viability against different concentrations of snail extract

Table 5: Absorbance of different concentrations of snail extract in MTT assay

Pila globosa powder concentration(µg/ml)	OD at 570nm (Triplicate values)		
100	0.241	0.248	0.242
75	0.251	0.265	0.269
50	0.275	0.27	0.274
25	0.285	0.281	0.283
12.5	0.301	0.298	0.3
Control	**0.295**	**0.303**	**0.312**

Table 6: % cell viability of different concentrations of snail extract in MTT assay

Pila globosa powder concentration(µg/ml)	% Cell viability (Triplicate values)		
100	79.54	81.85	79.87
75	82.84	87.46	88.78
50	90.76	89.11	90.43
25	94.06	92.74	93.40
12.5	99.34	98.35	99.01
Control	100	100	100

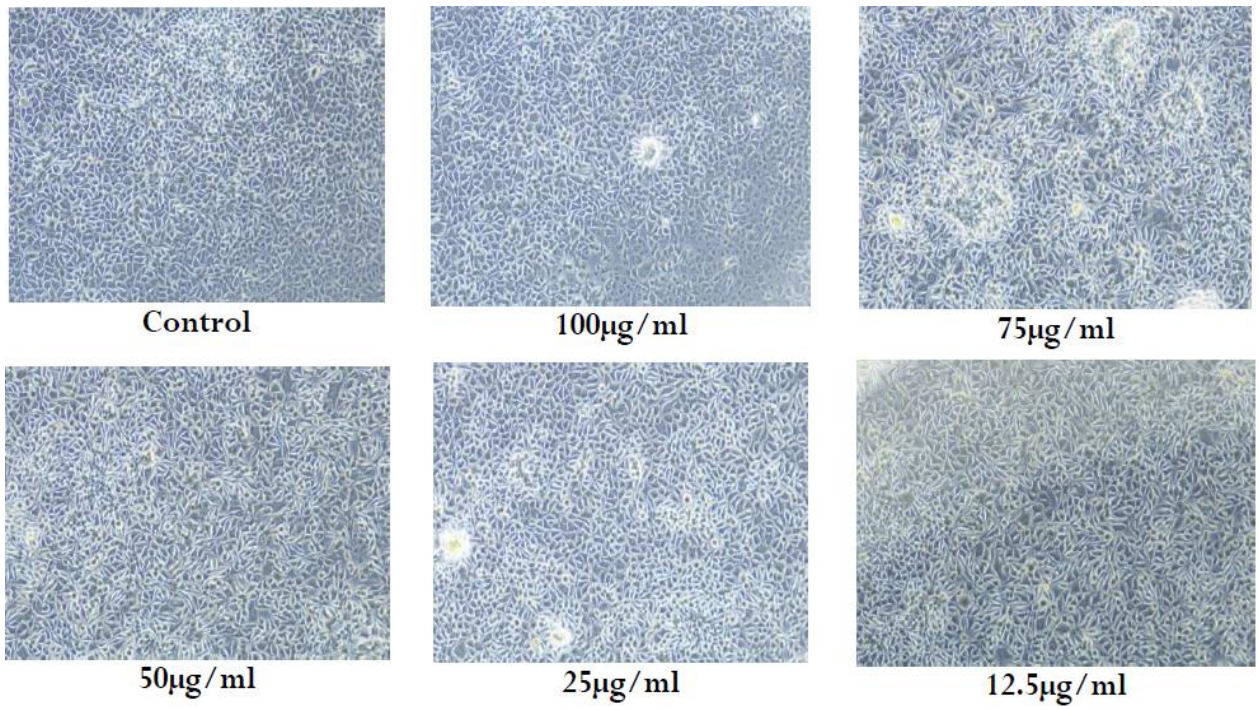


Fig. 5: Microscopic images of MTT assay of control and concentrations of snail extract

3.4. Antioxidant Activity

3.4.1. 2,2-Diphenyl-1-picrylhydrazyl (DPPH) scavenging assay and Hydrogen peroxide (H2O2) scavenging activity

The results of the *Pila globosa* snail powder extract scavenging activity against DPPH radical and H_2O_2 radical against the standard antioxidant (ascorbic acid) were presented as bar diagrams in Figs. 6 and 7 respectively. The snail powder exhibited an increasing percentage of inhibition of both DPPH and H_2O_2 radical with increasing concentration; however, when compared with the ascorbic acid, the inhibition percentage shown by the snail powder was comparable to that of the standard antioxidant at the highest concentration.

3.5. *Insilico* Molecular Docking

The constituent Stigmasta-4,22-dien-3.beta.-ol was selected for molecular docking with human proteins calcitonin receptor ectodomain, cathepsin K, human MMP-9 and purple acid phosphatase. Out of the four proteins, the ligand bound only with human calcitonin receptor ectodomain. Among the different conformations, the protein ligand cluster with the lowest binding energy (-3.87 kcal/mol) was determined as the best binding confirmation as already stated in a previous study that the lower the protein ligand binding energy score, the better the binding energy [32]. The binding energy of the protein and ligand was given in Table 7.

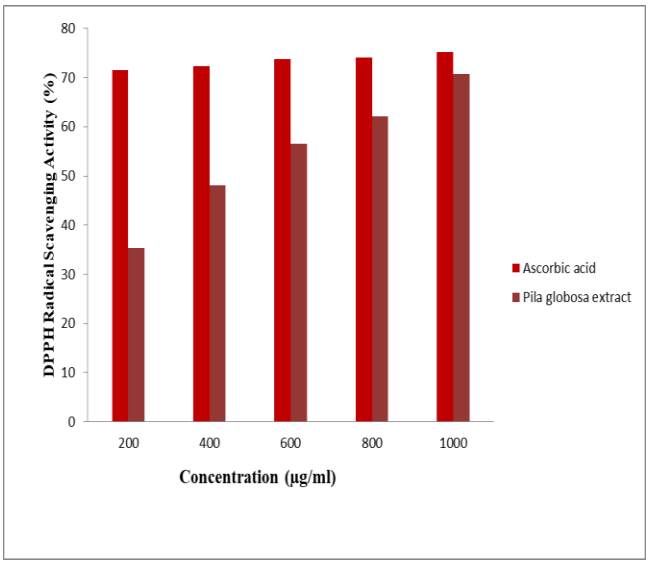


Fig. 6: DPPH radical scavenging activities of *Pila globosa* powder extract using ascorbic acid as a standard

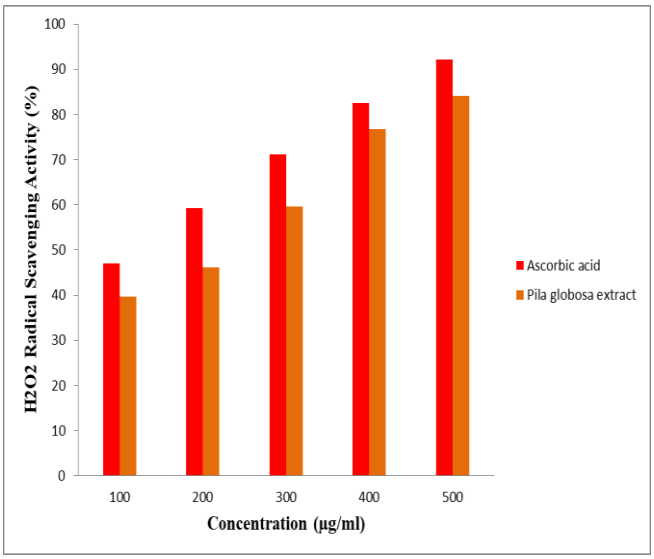


Fig. 7: H_2O_2 radical scavenging activities of *Pila globosa* powder extract using ascorbic acid as a standard

Table 7: Binding energy of human protein and ligand

S. No.	Protein	Ligand	Pub-chem ID	Binding energy	Ligand Efficiency	Inter-mole energy	Ligand atoms (ring)	Docked amino acid residue (bond length)
1	calcitonin receptor ectodomain	Stigmasta-4,22-dien-3.beta.-ol	15215516	-3.87	-0.13	-4.67	Conventional Hydrogen Bond: C3-OH C3-O Pi-Alkyl Hydrophopic interaction: C O Alkyl Hydrophopic interaction: C O C	Chain A: GLN`93'OE1'(2.72 Å) Chain B: LYS`46'HZ1'(2.20 Å) Chain A: PHE`94'(5.39 Å) Chain A: PHE`94'(5.28 Å) Chain B: LYS`46'(4.60 Å) Chain B: LYS`46'(5.02 Å) Chain B: LYS`47'(5.39 Å)
2	Cathepsin K	Stigmasta-4,22-dien-3.beta.-ol	15215516	No interaction	-	-	-	-
3	MMP-9	Stigmasta-4,22-dien-3.beta.-ol	15215516	No interaction	-	-	-	-
4	purple acid phosphatase	Stigmasta-4,22-dien-3.beta.-ol	15215516	No interaction	-	-	-	-

4. DISCUSSION

The FTIR analysis of the snail whole viscera powder determined the presence of functional groups of significant biological activities such as Amides, Alkyl, Aldehyde, Benzene ring, ketone, Alkyne and Carboxylic acid. Functional groups are the various compounds present in a drug molecule that provides the drug with certain specific properties so that the drug molecules can exert their pharmacokinetic and pharmacodynamic effects. The significant role of any functional group for a drug molecule may include but not limited to their mechanism of action, route of administration, water/lipid solubility, metabolism and elimination, action duration, therapeutic situation and drug interactions or adverse effects. A drug molecule will contain many functional groups with different properties. In general, functional groups are the backbone of a drug molecule [33].

Amides, the most abundant form of bond in any organic molecules, have high stability and high polarity; hence most prevalent in many medicinal drugs, as amide containing drugs can interact with biological receptors and enzymes [34, 35]. Alkyls are the substituent of alkanes without one hydrogen atom. The alkyl chains incorporated compounds have exhibited increased lipophilicity which in turn increases the antimicrobial activity of chalcones and flavanones [36]. Alkynes contain a carbon triple bond carbon in its structure which is ubiquitous in environment [37] and highly bioactive. It is present in pharmaceutical drugs like contraceptive noretynodrel, antiretroviral Efavirenz, antifungal Terbinafine [38, 39]. Aldehydes are also ubiquitous in the nature. Naturally derived Aldehydes such as cinnamaldehyde, citraldehyde and anisealdehyde acts as flavouring agent to the foods and exerts medicinal properties like cinnamaldehyde exhibiting anti-inflammatory activities [40]. Benzene are used in the treatment of polythemia and malignant lymphoma [41]. Many of the pharmaceutical drugs contains benzene derivatives such as Mesalazine, to treat ulcerative colitis [42], Tolcapone, a catechol-O-methyltransferase (COMT) inhibitor for Parkinson's disease [43], Zucapsaicin, topical analgesic for osteoarthritis [44]. As evident from numerous studies, Ketone supplement administration proven to exert potential therapeutic effects in central nervous system associated psychiatric diseases by positively affecting the mitochondria, glycolysis, neurotransmitter levels and histone deacetylase [45]. Carboxylic acids occur in abundance in nature and found in many of the life cycles such as fermentation and kerb's cycle. They play an important role in food properties (as additives and flavourings), food stability and as antimicrobials [46]. FTIR had played a major role in the discovery of novel functional groups in various snails' species. The FTIR analysis of the shell powder of the garden snail *Helix aspersa* determined the presence hydroxyapatite (HAP) in an attempt to produce the compound from biological waste for biomedical applications [47]. In the horn snail *Telescopium* sp shell, the presence of carbonate functional group identified through FTIR for the use of toxic metal removal from aquatic environments [48].

In this study, the Gas chromatography and Mass spectrometry of the *Pila globosa* whole snail tissue revealed the presence of thirteen bioactive constituents of significant importance. Gas chromatography coupled with mass spectrometry have significant application in the scientific field as it provides more precise information in both the qualitative analysis and quantitative determination experiments [49]. Identification of the bioactive constituents were based on their retention time. In a chromatogram, the peaks represent the various compounds eluted as a function of the retention time. The height of the peaks in the chromatogram is directly relative to the concentration of the respective compound. The results indicated that the major compound present in the *Pila globosa* snail was cholesterol with an area of 62% followed by gamma.-Ergostenol (7.74%), Eicosane (4.24%), n-Hexadecanoic acid (4.50%), 6-Octadecenoic acid (4.97%) and Cholecalciferol (3.84%). All of these compounds possess many significant medicinal properties. Cholesterol is the common steroid found in any fresh water molluscs. Alicia and Edurado *et al.,* (1984) reported the presence of a certain form of cholesterol, 7-oxo-cholesterol in snail *Biomphalaria tenagophila,* but not in the bivalves found in the same fresh water source indicating the physiological importance of snails among other fresh water organisms [50]. According to a patent, Ergostenol is a pharmaceutically accepted component for preventing or treating dermatitis and also can be used in cosmetics to alleviate atopy condition [51]. Eicosane had shown to have anti-inflammatory, analgesic and anti-pyretic activities [52]. Hexadecanoic acid, also known as palmic acid, has antioxidant, nematicide and hypocholesterolemic activities [53]. Octadecenoic acid has anti-inflammatory activity [54]. Cholecalciferol or otherwise known as Vitamin D3 is an essential vitamin in a dietary supplement to treat vitamin D deficiency. It was also known to improve heart function and reduce the risk of heart attack [55]. Diet containing functional lipids is emerging to be a great source for the prevention of many diseases, improving human health and exerting beneficial effects [56]. Several studies have revealed the presence of various naturally occurring products with medicinal values in snails. Bashir Lawal *et al.,* in 2015 had discovered the presence of 26 constituents in giant African Snail (*Archachatina maginata*) haemolymph including fatty acids, alkane, ester, alcohol and phthalate with antioxidant, anti-inflammatory, anti-alopecic and anti-mutagenic properties [24]. In the three land snails' species (*Eobania vermiculata*, *Theba pisana* and *Monacha obstructa),* oxime, methoxy phenyl, cyclotrisiloxane and hexamethyl were found to be the major components with species specific diversity in concentrations [57]. Abdel Hamid *et al.,* (2005), analyzed the compositions of different sterols in the tissue, hemolymph or

hemocyanin extracts of the three snail *Biomphalaria alexandrina, Lymnaea truncatula* and *Physa acuta* and reported the presence of sterols with carbon [58].

The western medicine has always been thriving for novel bioactive molecules for the preparation of novel drug molecules with zero toxicity to humans. With increasing risk of diseases, the new generation, cost effective and target oriented novel medicinal products demand is also increasing. On the other hand, snails have a wide range of applications in the food, cosmetic and pharmaceutical industries. Snails have been traditionally used as medicine since ancient times. Zicotinide (SNXIII), a synthetic peptide in the venom from *Conus magus* was used as a pain killer until the late 19th century, later was approved by US FDA [59]. Snails are enriched with abundant and unique source of bioactive compounds such as glycans, peptides and also other molecules in their body parts. The medicinal properties of different snail species can be evident from the previous studies that exploited their potential ranging from antimicrobial activity [60, 61], anti-inflammatory activity and wound healing [62] and to an extent to cancer treatment [63]. But still more vigorous research is needed. However, it is important to know about the safety and any toxicity to provide scientific validation to support any pharmaceutical application. In this study, the MTT assay demonstrated that the *Pila globosa* snail extract exhibited only slight cytotoxic effect at the highest concentration (100µg/ml) on L929 Mouse fibroblast cells as the cells were cultivated with different concentrations of the snail *Pila globosa*. In a previous study Salimi *et al.,* 2021, reported the crude venom and fractions of Persian Gulf snail (*Conus textile*) did not induce apoptotic effects on normal lymphocytes when compared to Chronic Lymphocytic Leukemia (CLL) B-lymphocytes, and can be a potential drug candidate for CLL [64]. *Helix aspersa muller* is one of the most consumed snail species of African origin, also well used for the snail's mucus medicinal properties in skin from ancient times and worldwide use in cosmetics. Thus the safety of the mucus was evaluated against fibroblasts and neither cytotoxic effect nor morphology changes were observed, furthermore the mucus induced fibroblasts cells proliferation [65]. Similar result has been observed by Gentili *et al.,* in 2020 against human keratinocytes. These results support the safety of the mucus as the potential candidate for skin treatment and new protective technology against pollution induced skin damage [66].

Snail meat contains high nutrition, low fat cholesterol content and good amino acid profile. The high iron content can be useful in treating iron deficiency and anaemia. Thus, snails are in high demand in local and international markets as food and medicine. In the food industry, antioxidants play a major role in nutrition, colour and flavour preservation. In this study, the antioxidant activity was discovered against DPPH and H_2O_2 scavenging radicals. The crude methanol and ethanol extract of apple snail *Pila globosa* exhibited 84% and 92% antioxidant activity for DPPH and hydrogen peroxide radicals respectively at the highest concentration. The % scavenging activity increased with the increasing concentrations. Particularly, the ethanol extract exhibited higher activity than the methanol extract. The antioxidant potential of the snail extracts and compounds derived from snail tissues had been evident from many previous studies. DPPH free radical scavenging activity of fresh water apple snail *Pila maculata* in methanol extract was 83% [67]. Marimuthu Gayathri *et al.,* 2017 reported 74.83% DPPH radical scavenging activity of methanol extract of *Pila virens* [2]. Another fresh water mollusc *Ficus gracilis* when extracted in methanol reported to exhibit 70% activity in scavenging DPPH radical [68]. In another study, the antioxidant activity of the mucus extracted from the garden snail *Cornu aspersum* was analysed. The mucus of the snails has been known for its rich bioactive natural compounds source and being commercially used in the compositions for skin care and ailments. The study found that the mucus extract, specifically the low molecular weight fractions in the extract, exhibit 83% activity against the DPPH radical [69]. *Achatina fulica*, the mollusc used in Chinese medicine and profoundly consumed in large quantities, found to contain a novel glucan that exhibited tremendous DPPH and superoxide anion scavenging [70].

Molecular docking enables the researchers to study an interaction between a small molecules i.e. a drug candidate and a protein at an atomic level and to elucidate the functional biochemical processes. Many computer algorithms have been developed to enable the interaction study. Thus molecular docking has become an important tool in pharmaceutical research since its invention in 1981. They have revolutionized the art of drug research. The constituent Stigmasta-4,22-dien-3.beta.-ol found in the snail *Pila globosa* exhibited an effective binding efficiency with the human protein calcitonin receptor ectodomain with a binding energy of -3.87. The calcitonin receptor, a type of class B1 GPCR

(G protein-coupled receptors), has a broad expression in a variety of tissues and cells that includes brain, kidney, lung, leucocytes, gastrointestinal tract and reproductive organs but most highly expressed in the bones on osteoclates. Thus, calcitonin receptor had been very much exploited and clinically validated to treat bone diseases like osteoporosis, hypercalcemia of malignancy and paget's disease [71]. The binding potential of the snail *Pila globosa* constituent with the calcitonin receptor signifies its potential as a suitable drug candidate for the treatment of bone disorders. Snails had been majorly utilized in numerous historical medicinal structures for the remedy of bone associated issues and diseases. In Indian and Chinese ancient medicine, snail had been mentioned to ail in the prevention of bone disorders and strengthens bone [72]. In South western part of Nigeria exists a medicinal practice to consume *Archanchantina maginata* snail fluid for treatment of rheumatism [73]. In North Bihar of India, the people of Kosi River to treat arthritis do consume the soup made from the foot pad of the fresh water snail *Bellamia bengalensis* [74]. The anti-arthritic effect of *Bellamia bengalensis* was later proved in a research with a rat model. In arthritis induced animals, the snail extract partially restored the cartilage erosion and ruptured synovial membrane in the knee joints [75]. Kieho Sohn and Taehee Kim in 2018 had discovered that the continuous administration of snail extract to female wistar rats over a period of 8 weeks resulted in the significant increase in the Bone mineral density (BMD), serum concentrations of insulin-like growth factor 1 (IGF-1) and insulin like growth factor-binding protein 3 (IGFBP-3) [76]. The study also proved that the *in vitro* treatment of the snail extract on the MG-63 cells i.e. the human osteoblast like cells significantly increased the beta-catenin expression and resulted in the increase in longitudinal growth and growth factor levels proving the snail extract as the potential therapeutic agent for formation of bones. Another Indian fresh water snail *Viviparous bengalensis* flesh extract exhibited potential anti-osteoporosis and anti-osteoarthritis activity in an experimental rat model [77].

5. CONCLUSION

Global outbreaks of diseases with higher mortality rates had become the major concern in the 21st century. The raise of novel diseases demands the need for novel alternate medicines. Alternate medicines from plant and animal sources can be relatively safe without any adverse effects. The *in vitro* and *in silico* assessment of *Pila globosa* disclosed the potential of the snail in the various aspects of the drug discovery. The presence of medicinally significant bioactive constituents and functional groups in the snail makes it a suitable candidate for the development of a novel alternate drug in the future.

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Conflicts of interest

There was no conflict of interest with authors.

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