ISOLATION AND CHARACTERIZATION OF BIOACTIVE COMPOUNDS, MOLECULAR DOCKING AND ANTICANCEROUS PROPERTY OF *FILIFUSUS FILAMENTOSUS* (RODING, 1798)

A dissertation

submitted to

ST.MARY'S COLLEGE (Autonomous), THOOTHUKUDI

affiliated to

MANONMANIAM SUNDARANAR UNIVERSITY, TIRUNELVELI

in partial fulfillment for the award of the degree of

MASTER OF PHILOSOPHY IN ZOOLOGY

By

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JUNE - 2019

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BONAFIDE CERTIFICATE

Certified that this dissertation entitled "**ISOLATION AND CHARACTERIZATION OF BIOACTIVE COMPOUNDS, MOLECULAR DOCKING AND ANTICANCEROUS PROPERTY OF *FILIFUSUS FILAMENTOSUS* (RODING, 1798)**" is the bonafide work of **JEBA NESAM.B** Reg. No.18MLZO01 who carried out the work under my supervision. Certified further that to the best of my knowledge the work reported herein does not form part of any other thesis or dissertation on the basis of which a degree or award was conferred on an earlier occasion on this or any other candidate.

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DECLARATION

I do hereby declare that this dissertation entitled, **ISOLATIONAND CHARACTERIZATION OF BIOACTIVE COMPOUNDS, MOLECULAR DOCKING AND ANTICANCEROUS PROPERTY OF *FILIFUSUS FILAMENTOSUS* (RODING, 1798),** submitted by me in partial fulfillment for the award of the degree of Master of Philosophy in Zoology, is the result of my original and independent research work carried out under the guidance of **Dr. P. Subavathy M.Sc., M.Phil., SET., Ph.D.,** Assistant Professor, PG and Research Department of Zoology, St.Mary's College (Autonomous), Thoothukudi and it has not been submitted elsewhere for the award of any other degree.

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ACKNOWLEDGEMENT

I bow and surrender before thy **Lord** for his choicest blessing and immense grace for carrying me all the way to complete my dissertation successfully.

I would like to express my profound gratitude to **Rev. Dr. Sr. A.S.J. Lucia Rose M.Sc.,PGDCA., M.Phil., Ph.D.,** Principal, St. Mary's College (Autonomous), Thoothukudi for her constant support and encouragement which enabled me to complete the work successfully.

I am indeed debated to my guide **Dr.P.Subavathy M.Sc., M.Phil., SET., Ph.D.,** Assistant Professor, PG and Research Department of Zoology, St.Mary's College (Autonomous), Thoothukudi for her continuous encouragement, support and critical comments made this interesting work possible.

I extend my profound thanks to **Dr. Hermin Pasangha., M.Sc.,B.Ed.,Ph.D.,** Head, PG and Research Department of Zoology, St.Mary's College (Autonomous), Thoothukudi for providing me all facilities during my study period.

I am extremely grateful to **Dr. R.D. Thilaga M.Sc.,M.Phil.,M.Ed., Ph.D.,** former Head, PG and Research Department of Zoology, St.Mary's College (Autonomous), Thoothukudi. Her guidance and encouragement will always be remembered.

I extent my heartfelt thanks to Dr. Savithiri Shivakumar, Aaranya Biosciences, Chennai for her timely help in getting the laboratory reports.

Special word of thanks to Miss. K. Jesima for her timely suggestion and encouragement throughout the period of my work.

I express my deep sense of gratitude to the faculty members of Department of Zoology, St. Mary's College (Autonomous), Thoothukudi for their encouragements, help and support for carrying out my work.

Special gratitude to the Laboratory Assistants, St. Mary's College (Autonomous), Thoothukudi for their support.

Words become endless to express the motivation and encouragement rendered by my husband **Mr. M. Sathish Pandian**. Heartfelt joyous thanks to my dear son **Master. S. Mofrin** whose smile facilitated me to reach the set goals.

CONTENTS

LIST OF TABLES

LIST OF PLATES

LIST OF FIGURES

1. INTRODUCTION

Marine species comprise half of the world's total biodiversity. The phylum Mollusca has about 1,00,000 described species (Strong *et al.,* 2008). It is divided into seven classes (Ruppert *et al.,* 2004) and is present on nearly every continent. Molluscs are the second largest animal phylum on earth, constituting approximately 7% of living animals. There are currently around 52,000 named species of marine molluscs (Bouchet, 2006) and an estimated diversity of 100,000 - 200,000 species (Pechenik, 2000). Molluscs are diverse not only in terms of their species richness, but also encompass a wide range of morphologies and ecological niches.

Throughout history, molluscs have provided a wide range of human resources, including food, shells, dyes and medicine. In many cultures shelled gastropods and bivalves are regarded as a delicacy or healthy food and they also feature in a range of traditional natural remedies (Herbert *et al.,* 2003; Prabhakar and Roy, 2009). In most cases there has been no scientific research undertaken to substantiate the health benefits of molluscs. However, there is increasing interest in the bioactivity of mollusc extracts and secondary metabolites (Cimino and Gavagnin, 2006). 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). Nevertheless, it is presently unclear whether the production of bioactive secondary metabolites is ubiquitous within the phylum mollusca.

Consequently, there should be much scope for future drug discovery within this phylum. The continual discovery of novel drug leads from the enormous pool of

available species requires a strategic approach, such as the investigation of traditional medicines and previously unstudied sources that are likely to have independently evolved novel pathways for secondary metabolism. Consequently, it could be predicted that distinct chemical structures will occur within molluscan groups that have evolved under different environmental and biological pressures.

In recent years, the marine environment has been recognized as a resource with high biodiversity but still unexplored (Crue Ramirez *et al.,* 2015). The bioactive compounds isolated from molluscs exhibit anti-inflammatory and antitumor activities (Zhukova, 2014) which prevent free radical oxidation process that cause cell damage, cancer and degenerative diseases (Ravi *et al.,* 2012). These researches prove that these marine invertebrate can become an important source of new compounds with potential application in biomedicine. In the last few years, pharmaceutical industry encourages lead compounds from marine organisms thus paved the way for more collaborative efforts between academia and pharmaceutical industry to translate the natural products into clinical trials (Glaser, 2009).

Gas Chromatography - Mass Spectrometry (GC-MS) is a sensitive analytical technique that is used in a wide range of applications such as environment monitoring, flavor and fragrance analysis (Paranthaman *et al.,* 2012), pesticide analysis, metabolite analysis (Reade *et al*., 2014) forensic and criminal cases etc (Chauhan *et al.,* 2014). It is considered as the method of choice for detection of volatile compounds due to its high sensitivity over other analytical techniques like Liquid Chromatography - Mass Spectrometry (LC-MS) (Agilent Technologies, 2007). With the selection of suitable column, a wide range of compounds such as eicoscanoids, essential oils, FAs, wax, esters, perfumes, terpenes can be analyzed in

GC-MS (Agilent Technologies, 2007). GC-MS combines the separation capability of GC with fragments identification capability of MS. Thus the results obtained in GC-MS are much more confirmatory compared to GC.

Apart from the food, the marine environment is an exceptional reservoir of bioactive natural products, many of which exhibit structural and chemical features not found in terrestrial natural products (Ireland *et al.,* 1988). Many of these organisms are known to possess bioactive compounds as a common means of defense (Indap and Pithare, 1998) and to maintain homeostasis in the environment. Isolation of natural products from marine organisms increases rapidly and hundreds of new compounds being discovered every year (Faulkner, 2002; Proksch and Muller, 2006).

So far approximately 6500 bioactive compounds were isolated from marine organisms (Kamboj, 1999). These compounds belongs to different structural types such as diterpenoids (37%), steroids or sterols, glycosides (18%), sesquiterpenoids (17%) and the remaining were alkaloids, amino acids, fatty alcohol esters and glycosides (Kamboj, 1999). They are considered as one of the important source to derive bioactive compounds that exhibit antitumour, antimicrobial, antiinflammatory and antioxidant properties (Nagash *et al.*, 2010).

The study of marine organisms for their bioactive potential and importance in the marine ecosystem has accelerated in recent years along with the growing recognition of their importance in human life (Nazar *et al.*, 2009). In the last several decades, research has expanded from land to ocean in order to find new drug and

this diversity has provided a unique source of chemical compounds with potential bioactivities that could lead to potential new drugs candidates.

The study of the molecular interactions between biologically active natural products and the corresponding cellular receptors is of great importance from a biological as well as medicinal point of view (Gautam, 2007). Docking is a method which predicts the preferred orientation of one molecule to a second when bound to each other to form a stable complex. This is called as induced fit. Molecular docking is an internet service that calculates the sites, the energy of small molecules and geometry of interacting protein (Schames, 2004). After docking stimulation, well - docked protein - ligand complexes are produced in experimental laboratories for testing.

Computation methods such as molecular docking is very useful and reasonably reliable for prediction of putative binding modes and affinities of ligands for macromolecules. Such methods are gaining popularity because the experimental determination of complex structure is rather difficult and expensive. Over the year, the speed and accuracy of computational docking methods has improved, and these methods now play a significant role in structure-based drug design (Rita Borik *et al.*, 2018).

Docking of the molecules into their respective 3D macromolecular target is a widely used method for lead optimization (Kontoyianni *et al.*, 2004). Docking programs find their most important application in visual database screening approaches in which thousands of molecules are docked into the binding pocket to identify plausible binders (Wang *et al.*, 2003). It was reported that docking programs

are able to predict experimental poses with deviation average from 1.5 to 2.0 Å root mean square deviation (RMSD) (Bissantz *et al.,* 2000).

One of the most well-known docking program is a AutoDock (Morris *et al.*, 2009). AutoDock is an automatic docking tool. It is designed to predict how small molecules, such as substrates, bind to a receptor of known 3D structures. A graphical user interface called AutoDock tool or ADT was utilized to generate grids, calculate the docking score and evaluate the conformers (Nurfina, 1997). Docking is most commonly used in the field of drug design, may be applied to hit identification, lead optimizations and bio-remediation. Structure - based drug design methods utilize knowledge of the three - dimensional structure of a receptor complex with a lead molecule is an attempt to optimize the bound ligand or a series of congeneric molecules. Using a model with a given structure, a medical chemist can compute an activity of a molecule (Lewis, 2005).

Cancer is the leading cause of mortality worldwide and the number of cases is increasing every year (World Health Organisation, 2010). Complete removal of the cancer without damage to the rest of the body is the goal of treatment, but the property of cancers invade adjacent tissue or any other organs by microscopic metastasis often limits its effectiveness (Jemma Hermelin Jesy Diaz *et al.,* 2014). A successful anticancer drug must target or inhibit tumor cells while causing minimal damage to healthy cells.

Cancer develops through multistep carcinogenesis process that encompass various cellular physiological system such as cell signaling apoptosis, thus making it a very complex disease. The majority of currently used anticancer drugs, which have

been obtained by synthesis of novel compound or from natural sources are toxic to normal cells in addition to cancer cells and thus have first substantial harmful side effects. There is therefore a continuous search for innovation chemotherapeutic drugs that act as “Magic Bullets” specifically targeting cancer cells with minimal damage to normal cells (Saleem *et al.,* 2019).

Breast cancer is cancer that starts in the breast, usually in the inner lining of the milk ducts or lobules. The various factors which influence the breast cancer are as follows age, race, alcohol intake, obesity, radiation, physical activity and adult height, reproductive, hereditary, hormonal, environmental and lifestyle factors (Rudden, 2007). Symptoms of breast cancer are the presence of lumps or thickening in the breast, swelling, dimpling, redness and soreness of skin, change in shape of nipple and nipple discharge. Detection and diagnosis of breast cancer can be by breast examination, mammography, ultrasound, breast MRI, fine needle aspiration, core needle biopsy, breast tumor pathology and lymph node biopsy. Treatment of breast cancer includes surgery, radiation, chemotherapy, hormonal therapy and alternative medicines.

Various compelling experimental and epidemiological studies concluded estrogen hormone as the main etiology for about 80% of the breast cancer and approximately 5% of cases are due to hereditary syndromes. All the environmental and reproduction risk factors finally increase the level of this hormone (Reynolds and Schecker, 1995). This type of cancer is also known as the hormone sensitive cancers. Types of breast cancer are invasive and non-invasive carcinoma, whose subtypes include ductal *in situ* (DCIS) and lobular carcinoma *in situ* (LCIS).

Another type of breast cancer is that arises from the muscle, fat or connective tissue of the breast and is known as sarcomas.

Chemoprevention is a long-term strategy with the best cost-effectiveness ratio for cancer control, which consists of certain chemicals, natural or synthetic, with the aim of preventing carcinogenesis or reversing the development of invasive malignancy (Martinez, 2006). Conventional anticancer treatment such as ionizing radiation, hyperthermia, alkylating agent, DNA topoisomerase inhibitors and platinum compounds induce DNA damage indiscriminately, thus killing the normal cell and the rapidly proliferating cancer cell. Since these drug are not specifically selective for cancer cells, cancer patient suffer from adverse side effects (Kawabe, 2004). These cytotoxic anticancer treatments are being supplemented by targeted therapies that recognize specific target for cancer cell to improve efficacy of treatment and reduce side effects.

Targeted therapies include apoptosis-inducers, angiogenesis inhibitors, signal-transduction inhibitors, monoclonal antibodies and gene therapy (Maione *et al.*, 2004). Apoptosis - inducers in particular can be used to induce cell death through the genes and proteins that control a apoptosis, since tumor-specific alternations in apoptotic programs provide opportunities to target cell death in a selective manner (Rossi and Gaidano, 2003). Using these genes and proteins as potential drug targets, the apoptosis-inducers that directly induce apoptosis may provide less opportunity for acquired drug resistance, decrease mutagenesis and reduce toxicity. It is for these reasons that we have a general interest in identifying compounds that possess apoptosis-inducing capabilities.

Complementary treatment approaches are increasingly requested by cancer patients and survivors to reduce side effects of conventional cancer treatment and enhance health related quality of life (Middha *et al.,* 2013; Cox and Balick, 1994). Nearly 60% of the currently used anti-cancer drugs are derived from natural agents including plants, micro and marine organism and many are approved by Food and Drug Administration (FDA).

Programmed cell death leading to apoptosis is essential for normal development and homeostasis in plants and throughout the animal kingdom. Although there are differences in apoptosis mechanism between lower animals and vertebrates, crucial biochemical compounds of the programmed cell death pathways remained remarkably conserved throughout evolution (Tibor Kiss, 2010). Programmed Cell Death (PCD) is a genetically determined capacity in virtually all cells of eukaryotes. It is important for the maintenance of normal organismal functioning including embryonic development, tissue homeostasis and pathological process.

Abnormalities in the PCD machinery cause a number of malfunctions, including cancer, neurodegenerative and other type of diseases (Green and Kroemer, 2004; Pollard and Earnshaw, 2002). Apoptosis is a highly orchestrated suicide program by which cells are physiologically eliminated in metazoans without inducing inflammation around the dying cell (Edinger and Thompson, 2004). Cell death by apoptosis is also a highly conserved evolutionary process regulated tightly at both transcriptional and post - translational levels. Despite decades of studies on the neurobiology and development of molluscs, comparatively little is known about the mechanisms of apoptosis in this phylum.

This ideal situation may be achievable by the induction of apoptosis in cancer cells. Thus, apoptosis modulation may be a key factor in the prevention and treatment of cancer. Recently, apoptosis induction in cancer cells has been the focus for an innovative mechanism to base drug discovery (Jemal *et al.*, 2011). Apoptosis is a mechanism of programmed cell death that is characterized by highly organized biochemical processes that eradicate injured or abnormal cells.

Apoptosis serves a key function as a protective mechanism against cancer, by removing genetically damaged cells, or cells that have become cancerous. When apoptosis is triggered in response to certain physiological signals, a proteolytic cascade involving different caspases is initiated in the suicidal cells. This cascade leads to activation of nucleases that initiate the degradation of chromosomal DNA. This type of DNA fragmentation is considered hallmark of the apoptosis process (Gibbs, 2000; Debatin, 2004). The study of apoptosis is an important field of biological inquiry since a deficiency or an excess of apoptosis is one of the causes for cancers, autoimmune disorders, diabetes, Alzheimer's, organ and bone marrow transplant rejection and many others diseases (Deborah Ribble *et al.*, 2005).

Based on the above approach, the present study aims to design a new series of bioactive compounds structurally containing 13 components and derivatives to develop a good target for drug discovery. For this target, docking of the newly synthesized compounds was done using AutoDock 4.2. The cytotoxic effect, apoptotic property, nuclear cell morphology and DNA fragmentation of the marine gastropod *Filifusus filamentosus* was investigated.

2. REVIEW OF LITERATURE

The ocean harbor a variety of life forms ranging from microorganisms to vertebrates, which in turn provide mankind with several benefits biologically and medicinally. This feature of wide diversity in marine life forms has been identified as chief source for unique biologically active compounds that exhibit tremendous potential for pharmaceutical applications (Jain, 2009). Apart from the food that is derived from the marine environment, a wide variety of bioactive substances is being isolated and characterized several with great promise for the treatment of human disease.

A number of biologically active compounds with varying degrees of action such as antimicrobial, antitumour, anticancer, antileukemic, antibacterial, antiviral, antiproliferative, cytotoxic, photoprotective, as well as antibiotic and antifouling properties, have so far been isolated from marine source (Benkendorff *et al.,* 2001; Villa, 2010; Mayer *et al.,* 2001; Blunt *et al.,* 2011; Rajaganapathi *et al.,* 2002; Prem and Patterson, 2002; Haug *et al.,* 2003).

However, the biologically active products from marine molluscs are largely explored in India (Sarumathi *et al.,* 2012). The marine gastropod choosen for the present study *Filifusus filamentosus* is abundant in Gulf of Mannar coastal region and consumed for its delicacy and nutritive valve. Biomedical screening of solvent extract of marine molluscs would provide valuable base for new leads and ultimately used for treatment of chronic disease (Mayer *et al.,* 2013). Hence, the present study has been planned to investigate the broad spectrum biological activities such as

GC-MS analysis, molecular fragmentation or cell morphology and DNA fragmentation assay in the marine neogastropod *Filifusus filamentosus.*

Indole derivatives from the egg masses of muricid molluscs were isolated by Benkendroff *et al.* (2001). Aneiros and Garateix (2004) studied bioactive peptides from marine source. Rajee Kumar and Xu Zi-Rong (2004) investigated the presence of bioactive compounds in marine organisms. Anbuselvi *et al.* (2009) observed the bioactive potential of coral associated gastropod *Trochus tentorium* of Gulf of Mannar, Southeastern India.

Furunashi *et al.* (2009) determined the GC-MS and IR spectroscopy in chitin analysis of molluscan shells. Bioactive compounds from cone snails, terebrids and turrids were isolated by Puillandre and Holford (2010). Manjusha *et al.* (2011) evaluated a antimicrobial screening and GC-MS analysis of marine sponge *Crambe crambe.* Priya *et al.* (2012) analyzed the antimicrobial and GC-MS activity of bioactive compounds in *Pleurotus ostreatus.* Edwards (2012) reported the bioactive compounds from the marine mollusc *Dicathais orbita* on human reproductive cells and human reproductive cancer cells.

Kaviarasan *et al.* (2012) isolated the compounds Kabiramid C, Aplysianin E, Aplysianin A, Thisaplysianin E and Tyrian purple from the egg capsule of gastropods. GC-MS analysis of fatty acid of prosobranch gastropod species *Thais carinifera* from Pakistan coast (North Arabian sea) was assessed by Nuzhat Afsar *et al.* (2012). Maushmi Kumar and Asim Pal (2013) carried out GC/MS-MS analysis of some bioactive constituents from marine sponge, *Spongosorites halichondriodes* (Dendy, 1905).

The bioactive compounds in the mucus of *Dendropoma maxima* were detected by Kloppel *et al.* (2013). Kiran *et al.* (2014) screened the bioactive compounds with antimicrobial peptides from some species of molluscs and crustaceans. The bioactive compounds from marine prosobranch gastropods from Gulf of Mannar were observed by Santhi (2014). Ashish Chauban *et al.* (2014) studied the GC-MS technique and its analytical application in science and technology. Smiline Girija *et al.* (2014) characterized the bioactive constituents with antimicrobial potential from the pigmentation ink of *Loligo duvauceli.*

Pankaj Gupta *et al.* (2014) showed the screening of antiangiogenic potential of twenty two marine invertebrate extracts of phylum Mollusca from Southeast coast of India. Maushmi Kumar (2015) isolated the bioactive compounds and pharmacological properties of marine sponge from Mumbai coastal region. Chairman *et al.* (2015) isolated and structurally characterized the bioactive compounds in *Aurora globostellata*. Mahanty *et al.* (2015) carried out the GC-MS fingerprinting of fatty acids of freshwater mollusc *Lamellidens marginalis* using different colmns, TR-waxms and TR-FAME.

Jemma Hermelin Jesy Diaz and Thilaga (2015) studied the GC-MS analysis of methanolic extract from the internal shell of cephalopods. Santhi *et al.* (2016) found out the bioactive compounds from marine prosobranch *Purpura persica* from Tuticorin coast. Subavathy and Thilaga (2016) have carried out the GC-MS analysis of bioactive compounds from whole body tissue methanolic extract of *Cypraea arabica.* Gayathri *et al.* (2017) extracted and identified the bioactive compounds and *in vitro* antioxidant potential in freshwater ampullariidae snail *Pila virens.* Divya (2018) observed FT-IR and GC-MS analysis on the ethanol extract of *Aplidium*

multiplicatum from Vizhinjam, Kerala. Matthew and Susan (2018) assessed the bioactive compounds from marine organism: potential from bone growth and healing. Ashlin *et al.* (2018) investigated the bioactive compounds from neglected predatory marine gastropods.

Synthesis, antitumor activity and molecular docking study of novel benzofuran-2-yl pyrazole pyrimidine derivatives was assessed by Magdy *et al.* (2011). Hamed *et al.* (2011) studied structure-based drug design and AutoDocking study of potential protein tyrosine kinase inhibitors. Chella Perumal *et al.* (2014) identified novel PPARγ agonist from GC-MS analysis of ethanolic extract of *Cayratia trifolia* (L.) a computational molecular simulation studies.

Vijayalakshmi *et al.* (2014) described *in silico* docking analysis of secondary metabolites of *Bauhinia variegata* and *Garcinia cambogia* with retinol binding protein 4 as target for obesity. Talambedu Usha *et al.* (2014) to analysed the molecular docking studies of anti-cancerous candidates in *Hippophae rhamnoides* and *Hippophae salicifolia.* Karpakavalli *et al.* (2016) have carried out docking, studies synthesis, characterization and anticancer activity of 4-(4'-hydroxy, 3'-methoxy) phenyl, but-2-one-3-ene, a curcumin analogue precursor.

Kiran and Shwetha (2016) observed acetyl cholinesterase activity of plant extract using GC-MS and autodock analysis. Rina and Gunawan (2017) evaluated the molecular docking analysis: Interaction studies of natural compounds to anti-inflammatory targets. Rita *et al.* (2018) assessed the design, synthesis, anticancer evaluation and docking studies of novel heterocyclic derivatives obtained via reactions involving curcumin. GC-MS analysis for identification of active

compounds in propolis and molecular docking studies of selected compounds against chronic hepatitis B protein (Large Envelop Protein) was studied by Flora *et al.* (2018).

Antibacterial and antineoplastic protein secretion of a seahare *Aplysia juliana* was purified by Kamiya *et al.* (1989). Jimeno (2002) identified marine derived anticancer compounds. Kang *et al.* (2004) screened the controlled release of paclitaxel from microemultion containing PLGA and evaluation of anti-tumor activity *in vitro* and *in vivo*. The marine natural products as anticancer drugs were described by Simmons *et al.* (2005). Faircloth and Cuevas (2006) evaluated Kahalalide F and ES285: potent anticancer agents from marine molluscs. Magesh (2010) reported molecular basis of anticancer activity of *Indigofera tinctoria* (Lin).

Magbubah Essack *et al.* (2011) determined the recently confirmed apoptosis – inducing lead compounds from marine sponge of potential relevance in cancer treatment. Benkendorff *et al.* (2011) described bioactive potential of the *Murex* and Australian muricid mollusc extracts against human cancer cells. Candida Nastrucci *et al.* (2012) discovered anticancer drug from the marine environment. Sarfaraj Hussain *et al.* (2012) found out marine natural products: a lead for anti-cancer. Krishnamoorthi and Yogamoorthi (2013) have carried out MTT assay in crude solvent extract of gastropod *Cellana*. Priya Senan *et al.* (2013) carried out anticancer property of purified fraction of cuttle fish (*Sepia pharaonis*) ink on cervical cancer. Westley *et al.* (2013) assessed gastrointestinal and hepatotoxic activity of an anticancer extract from muricid molluscs. Jemma Hermelin Jesy Diaz *et al.* (2014) analyzed anticancer activity of marine cephalopod (*S. pharaonis*) from Gulf of

Mannar. Braga (2014) reported antioxidant and antitumor activity of marine invertebrate extracts.

Sugesh *et al.* (2014) assessed the cytotoxic effects of two edible bivalves *Meretrix meretrix* and *Meretrix casta.* Vera Gesheva *et al.* (2014) investigated anticancer properties of gastropodan hemocyanins in murine model of colon carcinoma. Praveena and Kaneez Fathima (2015) studied anticancer activity of protein extract from *Perna viridis* (Green Mussel) and *Meretrix meretrix* (Great clam). Newman (2015) evaluated anticancer properties of *Lamellarins.* Premalata Pati *et al.* (2015) showed marine molluscs as a potential drug cabinet : an overview. Susana-Gabriela *et al.* (2015) determined the antimutagenic, antiproliferative and antioxidant effect of extract obtained from *Paraoctopus limaculatus.* Subavathy *et al.* (2015) assessed *in vitro* anticancer activity of the marine gastropod *Cypraea arabica* (L.1758). Maria Letizia Ciavatta *et al.* (2017) reported marine mollusc-derived agents with antiproliferative activity as promising anticancer agents to overcome chemotherapy resistance.

Kahanda *et al.* (2016) studied tracked anticancer drug activity using DNA devices. The cytotoxic effect of *Turbo coronatus* extract on cancer cell lines was observed by Majid Honari *et al.* (2017). Ashutosh Bahuguna *et al.* (2017) evaluated the cytotoxic potential of drug. Veronica Ruiz *et al.* (2017) reviewed marine anticancer compounds : the use of virtual screening for the discovery of small-molecule cancer drug. Sithranga Boopathy and Kathiresan (2017) showed anticancer drugs from marine flora : A overview. Amir Shankouri *et al.* (2018) reported anticancer activity of Liposomal medical leech saliva extract.

Priya *et al.* (2006) studied induction of apoptosis in a human cervical cancer cell line by peptidoglycan from cuttle fish ink. Gifondorwa and Leisa (2006) observed programmed cell death in the apical ganglion during larval metamorphosis of the marine mollusc *Llyanassa obsolete.* Oberst *et al.* (2008) studied the living with death: the evolution of the mitochondrial pathway of apoptosis in animal. Terahara and Takahashi (2008) tested the mechanisms and immunological roles of apoptosis in molluscs. Trevor Williams *et al.* (2009) analysed the invertebrate iridovirus modulation of apoptosis.

Alejandro Romero *et al.* (2011) gave a new insight into the apoptotic process in molluscs: characterization of caspase gene in *Mytilus galloprovincialis.* Babak Esmaeelian *et al.* (2014) investigated 6-Bromoisatin found in muricid mollusc extracts inhibits colon cancer cell proliferation and induces apoptosis, preventing early stage tumor formatting in a colorectal cancer. Joseph and Kavimani (2014) evaluated apoptosis regulating efficacy of chosen marine sponge extracts. Pazhanimuthu Annamalai *et al.* (2015) determined the ethyl acetate extract of marine sponge *Hyattella cribriformis* inhibit potent anticancer activity by promoting tubulin polymerization as evidenced miotic arrest and induction of apoptosis.

Yi-Peng Gu *et al.* (2017) studied chemotherapy - induced apoptosis of testicular cell by squid ink polysaccharide. Zhi Zhang *et al.* (2017) observed *Sepia* ink oligopeptide induces apoptosis and growth inhibition in human lung cancer cells. Sweta Agarwal *et al.* (2017) reported methanolic extract of *Euchelus asper* exhibits *in vovo* antiangiogenic and *in vitro* anti-proliferative activities. Aldairi *et al.* (2018) assessed antiproliferative activity of glycosaminoglycan like polysaccharides

derived from marine molluscs. Andrey Victorovich Boroda *et al.* (2018) have carried out the chemical modulation of apoptosis in molluscan cell cultures.

Cytotoxic and antibacterial properties of *Mytilus galloprovincialis*, *Ostrea edulis* and *Crassostrea gigas* haemolymph was analysed by Hubert *et al.* (1996). Jemina Barsiene *et al.* (2008) evaluated genotoxic and cytotoxic effects in the molluscs *Macoma balthica* and *Mytilus edulis* from the Baltic sea. Jorge Pereira *et al.* (2011) described an efficient method for genomic DNA extraction from different molluscs species of different orders.

Yalda Rahba Saadat *et al.* (2015) studied an update to DNA ladder assay for apoptotis detection. Kjelland *et al.* (2016) investigated DNA fragmentation in blue mussel (*Mytilus edulis*) sperm: aquaculture and fisheries implication. Jacky Bhagat *et al.* (2016) showed DNA damage and oxidative stress in marine gastropod *Morula granulate* exposed to phenanthrene. Daoud Ali *et al.* (2017) noted genotoxicity in the fresh water gastropod *Lymnaea luteola* (L): assessment of cell type sensitivities to lead nitrate. Vasanth *et al.* (2017) have carried out impairment of micronucleus and DNA in the 2-chloro-4-ethylamino-6-isopropylamino -1,3,5-triazine exposed *Poecilia sphenops.*

The above literature review highlighted the importance of bioactive compounds, molecular docking, antiproliferative effect, apoptosis, cell nuclear morphologhy and DNA fragmentation analysis from marine molluscs. Based on the paucity of information available in marine gastropods the present study has been undertaken to assess the above stated biological activities from the marine gastropod *Filifusus filamentosus.*

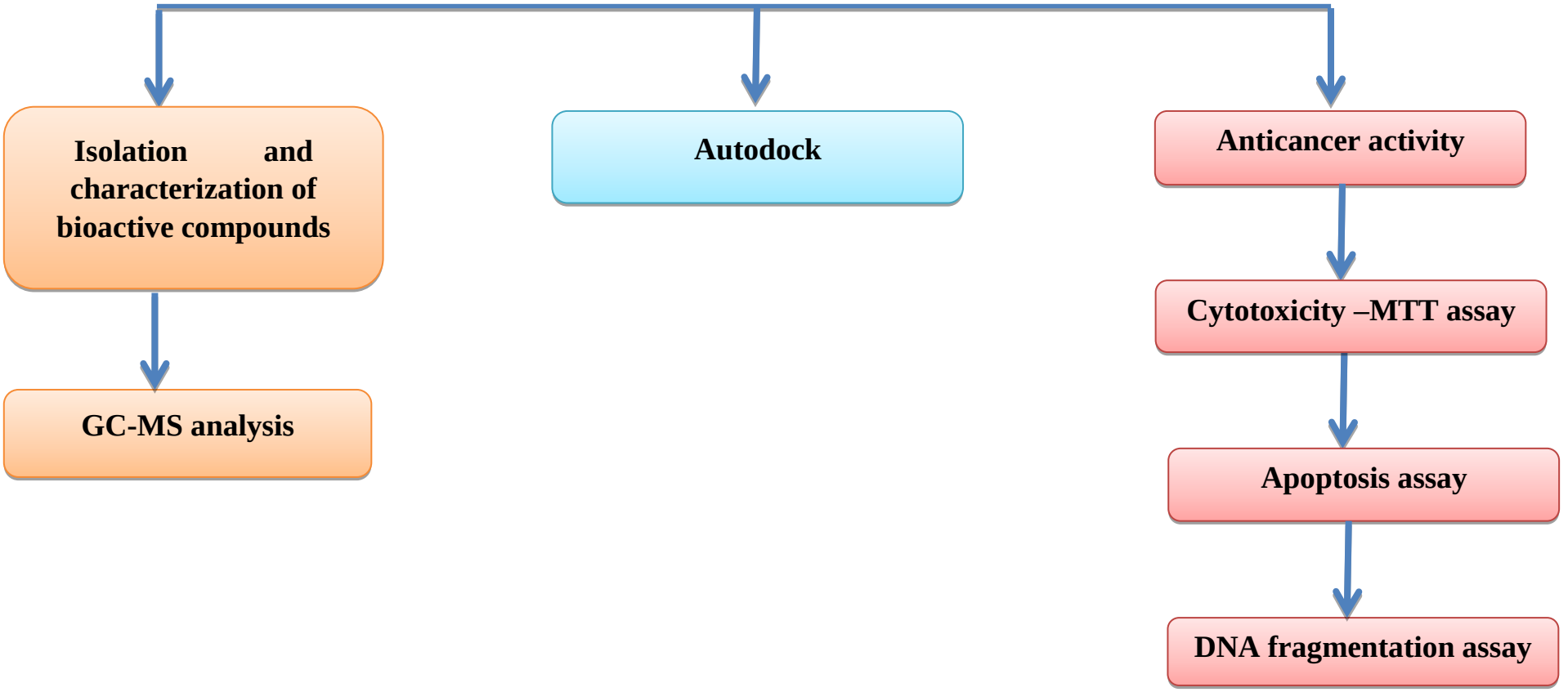
3. OBJECTIVES

Ocean is a treasure house of many living and nonliving resources, with about 26 phyla of marine organisms found therein. The marine environment differs from land-based ecosystem and offers a great chemical diversity and high biochemical specificity. Throughout the ages, marine natural products play an important role in modern medicine. Almost half of the drugs approved nowadays are based on marine natural products. In the past 30 years, interest towards marine bioprospecting has increased among researchers in the whole world. Marine organism are therefore considered as treasures that remain a relatively unexplored source for novel bioactive compounds that could eventually be developed into therapeutics. So, the present study has been carried out with the following objectives:

- To isolate, purify and characterize the bioactive compounds from *Filifusus filamentosus* by GC-MS analysis.
- To discover molecular docking simulation of experimental organism *F. filamentosus* by analysing the potential binding interaction.
- To investigate the cytotoxic and apoptotic properties of methanolic extract of *F. filamentosus* on MCF-7 cell line.
- To analyse the cell nuclear morphology of methanolic extract of *F. filamentosus.*
- To determine the molecular mechanism of DNA fragmentation in *F. filamentosus.*

4. EXPERIMENTAL DESIGN

Collection of experimental organism *Filifusus filamentosus* from Gulf of mannar coastal region of Thoothukudi



5. MATERIALS AND METHODS

5.1 Description of the study area

The Gulf of Mannar is located between India and Srilanka, stretches from the longitude 78^{o}08' to 79^{o}30' E and along the latitude from 8^{o}35' to 9^{o}25' N. It is a part of the southward extension of the Bay of Bengal and meets in the Indian Ocean. This geographical area runs from Pamban Island including Rameshwaram to Cape Comorin along the Southeast coast of India to a distance of about 170 nautical miles. The Gulf of Mannar biosphere reserve has an area of about 10,500 km^{2} and is considered as 'Biologist's Paradise' for, it has 3600 species of flora and fauna. This coast maintains a rich biological diversity perspective of flora and fauna largely due to diversified microhabitats such as mangroves, corals, seaweed beds, sea grasses, sandy, rocky and muddy shore etc. The faunal diversity is also well pronounced with reference to different molluscan groups (Fig. 1).

5.2 Collection of experimental organism

In the present study the gastropod *Filifusus filamentosus* was collected from the Gulf of Mannar coastal region (Plate 1). The mesogastropod *F. filamentosus* was collected from the landed by-catch from fishing trawlers operated for crabs and prawns along the Thoothukudi coastal region. The gastropod was collected during the month of November - December 2018. The freshly collected samples were brought to the laboratory, cleaned and washed with fresh sea water to remove all impurities. The shells were broken, tissues were removed and then dried in hot air oven at 56^{o}C for 48 hours and used for further studies.

5.3 Description of experimental organism

5.3.1 Systematic position of *Filifusus filamentosus* (Roding, 1798)

Phylum	**:**	**Mollusca**
Class	**:**	**Gastropoda**
Subclass	**:**	**Caenogastropoda**
Order	**:**	**Neogastropoda**
Super family	**:**	**Buccinoidea**
Family	**:**	**Fasciolariidae**
Genus	**:**	***Filifusus***
Species	**:**	***filamentosus***

Filifusus filamentous is a marine gastropod in the family Fasciolariidae. It is also known as the spindle snails. Shell is large upto 115 mm in height, solid, spindle shaped, suture adpressed, shoulders round. The spire is elongated. The siphonal canal is well developed and is long to moderately long. Aperture elongate with close-set yellowish spiral ridges within, outer lip with dark brown denticles, columella with callus anteriorly glazed, arched and with three to four plaits anteriorly, anterior canal elongate and slightly twisted. Surface sculptured with prominent spiral cords crossed by faint axial striae, a row of axial nodules on each whole. Colour bluish - white, cords reddish brown. The radula is characteristic with narrow central teeth with three cusps. The wide lateral teeth show numerous ctenoid cusps. They are found mainly in shallow water. The embryos develop into planktonic trocophore larvae and later into juvenile veligers before becoming fully grown adults. They are mainly carnivorous. They feed on other gastropods and on bivalve. And also prey on worms and barnacles.

5.4 Preparation of extract

Methanol extract of the whole body tissues were prepared following the slightly modified technique given by Thilaga (2005). Dried tissues were soaked in 100% A.R grade methanol for 10 days at room temperature. After filtration with Whatman No.1 paper, the methanol extract was reduced by vacuum evaporation. The extract residue was resuspended in 20 ml of 100% A.R grade methanol. The methanol soluble extracts were dried and solubilized in deionized water. Different concentrations of extracts were prepared and stored at 0ºC for further use.

5.5 GC-MS analysis

GC-MS analysis was carried out on a GC Clarus 500 Perkin Elmer System comprising a AOC 20i auto sampler and gas chromatography interfaced to a mass spectrophotometer (GC-MS) instrument employing the following conditions such as Column elite – 5MS fused silica capillary column (30 × 0.25mm ID × 0.25µm df, composed of 5% Diphenyl/95% Diphenyl Poly Siloxane), operating in electron impact mode at 70 eV: Helium (99.999%) was used as a carrier gas at constant flow of 1 ml/min and an injection volume of 3 µl (split ratio of 10:1) injector temperature of 250ºC. The oven temperature was programmed from 110ºC (isothermal for 2 min), with an increase of 10°C/min to 200°C, then 5ºC/min to 280ºC. Mass spectra were taken at 70 eV; a scan interval of 0.5 s and fragments from 45 to 450 $\mathcal{D}$a.

5.5.1 Identification of compounds

Interpretation on mass spectrum was conducted using the database of National Institute of Standard Technology (NIST Ver.21), WILEY 8 and FAME

having more than 62,000 patterns. The unknown components found in the body tissues of *Filifusus filamentosus* were matched with the spectrum of the known components stored in NIST, WILEY and FAME, the MS library and predicted from Duke's Ethno Botanical Database.

5.6 AutoDock protocol

Computer-Aided Docking is an important tool for gaining understanding of the binding interactions between a ligand and its target receptor. Docking is a computational technique to predict whether a molecule (ligand) can bind to the receptor (Protein). The affinity is measured based on the energy stability of the system and usually most stable interaction has low or negative free energy. Docking is virtual screening of a database of compounds and predicts the strongest binders based on various scoring functions. AutoDock is a suite of automated docking tools. It is designed to predict how small molecules such as substrates or drug candidates, bind to a receptor of known 3D structure. In the present study AutoDock 4.2 was used for docking simulation studies.

The native crystal structure of a chain of 5p21 protein was retrieved from Research Collaboratory for Structural Bioinformatics (RCSB) Protein Data Bank (PDB, ID: 5P21) (http://www.rcsb.org/pdb/home/home.do). RCSB is the single, global archive for information about the 3D structure of macromolecules (proteins and DNA) and their complexes, as determined by X-ray crystallography, NMR spectroscopy and cryoelectron microscopy. The PDB file was cleaned and the hetero atoms of the receptor were removed manually, since these are non-standard residues of protein.

5.6.1 Preparation of ligand

Based on the GCMS analysis, the test sample contains the compound 2,4-Di-tert-butyl phenol with maximum percentage peak area. 2,4-Di-tert-butyl phenol was choosed as the ligand for the present docking study. Also salirasib, which is one of the most potent inhibitor of 5p21 protein is considered as the standard drug.

5.6.2 Preparation of standard drug and ligand structure

The structure of salirasib and ligand 2,4-Di-tert-butyl phenol were obtained from the PUBCHEM database. Before docking, the ligand and standard drug were prepared using the Avogadro software to minimize the energy of the ligands. AutoDock identifies the conformational space of the ligand by using Genetic algorithm 4.2.

The program Pymol was used to transform the receptor PDB file into the PDBQT format file containing the receptor atom coordinates, partial charges and solvation parameters. The grid calculations and maps were calculated with the AutoGrid program. The grid maps were centered on the ligand's binding site obtained from CASTp. Following this, the proteins and ligands were docked. The AutoDock scores and binding energies of the docked ligands were estimated. The result were then compared and interaction were visualized using Chimera software.

5.7 Anticancer activity (Mosmann, 1983)

5.7.1 *In vitro* cytotoxicity assay (MTT assay)

To determine the cytotoxic effects of the methanol extract of *Filifusus filamentosus*, MTT 3- (4, 5- dimethyl thiazol - 2- yl) - 2, 5 - diphenyl tetrazolium

bromide assay was performed using MCF-7 (Breast carcinoma) cells. MCF-7 (Breast carcinoma) cell line was procured from National Centre for Cell Sciences (NCCS), Pune, India. Stock cells were cultured in DMEM supplemented with 10% inactivated Fetal Bovine Serum (FBS), Penicillin (100 U/ml), Streptomycin (100 µg/ml) and amphotericin B (5 µg/ml), in a humidified atmosphere of 5% CO_2 at 37^{o}C until confluent. The cells were dissociated with TPVG solution (0.2% trypsin, 0.02% EDTA, 0.05% glucose in PBS). The stock cultures were grown in 25 cm^2 culture flasks and all experiments were carried out in 96 microtitre plates (Tarsons India Pvt Ltd., Kolkata, India).

The ability of the cells to survive a toxic insult has been the basis of most cytotoxicity assays. This assay is based on the assumption that dead cells or their products do not reduce tetrazolium. The assay depends both on the number of cells present and on the mitochondrial activity per cell. The principle involved is the cleavage of tetrazolium salt 3-(4,5 dimethyl thiazole -2-yl) -2-5-diphenyl tetrazolium bromide (MTT) into a blue coloured product (formazan) by mitochondrial enzyme succinate dehydrogenase. The number of cells was found to be proportional to the extent of formazan production by the cells.

The monolayer cell culture was trypsinized and the cell count was adjusted to 1.0×10^5 cells/ml using DMEM containing 10% FBS. To each well of the 96 well microtitre plate, 0.1 ml of the diluted cell suspension (approximately 10,000 cells) was added. After 24 hr, when a partial monolayer was formed, the supernatant was flicked off, washed once and different test concentrations of test drugs were added on to the partial monolayer in microtitre plates to obtain final concentrations of 100,

200, 300, 400 µg/ml. The plates were then incubated at 37^{o}C for 3 days in 5% CO_2 atmosphere and microscopic examination was carried out and observations were noted every 24 h interval. After 72 h, the drug solutions in the wells were discarded and 50 µl of MTT in PBS was added to each well. The plates were gently shaken and incubated for 3 h at 37^{o}C in 5% CO_2 atmosphere. The supernatant was removed and 100 µl of propanol was added and the plates were gently shaken to solubilize the formed formazan. The absorbance was measured using a microplate reader at a wavelength of 570 nm. The untreated cells were used as the control. The effect of the samples on the proliferation of MCF-7 breast carcinoma cells was expressed as the percentage cell viability using the following formula:

$$\% \text{ of viability} = \frac{\text{Absorbance of the sample}}{\text{Absorbance of the control}} \times 100$$

$$\% \text{ of toxicity} = 100 - \% \text{ of viability}$$

5.8 Acridine orange and ethidium bromide staining

Acridine orange and ethidium bromide (AO/EB) staining was carried out to detect morphological evidence of apoptosis. MCF-7 cells were treated with the methanolic extract *Filifusus filamentosus* at different concentration of 6.25 µg/mL and 12.5 µg/mL respectively. The cells were washed with PBS (pH 7.4) and 10 µL of acridine orange and ethidium bromide solution (60 µg/mL of acridine orange and 100 µg/mL of ethidium bromide in PBS) and made up to 100 µL using PBS and incubated for 5 min. The cells were then washed with PBS and examined under Nikon Eclipse T*i* fluorescence microscope (Nikon Instruments Inc., NY, USA).

5.9 DAPI staining

Cell nuclear morphology was evaluated by fluorescence microscopy following DAPI staining. MCF-7 cells were treated with the methanolic extract of *Filifusus filamentosus* at different concentration of 6.25 µg/mL and 12.5 µg/mL respectively 24 hrs. The cells were washed with PBS (pH 7.4), fixed with ice cold 70% ethanol and resuspended in DAPI, and incubated for 15min at 37^{o}C wrapped in aluminium foil. The cells were then washed with PBS and examined under Nikon Eclipse T*i* fluorescence microscope (Nikon Instruments Inc., NY, USA).

5.10 DNA fragmentation analysis

To confirm the apoptotic mode of cell death, DNA fragmentation assay was performed. The DNA fragmentation assay was carried out by following the methods of Tyagi *et al.*, 2014. The MCF-7 cell lines and methanolic extract of *Filifusus filamentosus* were incubated for 24 hrs in 5% of CO_2 incubator. After incubation the trypsin phosphate versene glucose reagent (TPVG) was added and then it was centrifuged at 15,000 rpm for 10 minutes. The pellet was collected and washed with proteinase K and incubated at 55^{o}C for 3 hrs. Then phenol, chloroform and isoamyl alcohol (in the ratio of 25:24:1 v/v) was added and vortexed vigorously and incubated on ice for 5 min. Then it was centrifuged at 10,000 rpm for 10 minutes and the aqueous layer was transferred to a new eppendrof tube and phenol, chloroform and isoamyl alcohol extraction was repeated. The aqueous layer was combined with 50 ml of 3 M sodium acetate, 2.5 ml of 100% cold ethanol and stored at -20^{o}C overnight. Then, it was centrifuged at 15,000 rpm for 5 min at 4^{o}C. The pellet was air dried for 5-10 min. Next, the dried powder was resuspended in 100 µl

buffer (10 mM Tris /1 mM EDTA) and subjected to 1% agarose gel. The gel was stained with 1 μg/ml ethidium bromide. The clear bands were visualized and photographed.

6. RESULTS

6.1 GC-MS analysis

The methanolic extract of *Filifusus filamentosus* of was subjected to GC-MS analysis. GC-MS analysis from experimental organism *Filifusus filamentosus* revealed 13 compounds that could be identified as Cyclotetrasiloxane, octamethyl, Cyclododecane, 2,4-Di-tert-butyl phenol, E-14-Hexadecanol, Cholestrol, 9-Octadecenamide, (Z) - [Oleic acid amine], Undecanal, 2-methyl-, Cyclohexanol,2-amino-,trans-, Butanal, O-methyloxime, E-2-Tetradecen-1-ol, 1,1-Cyclopropane dicarbonitrile, 2,2-dimethyl-, Cyclopentanol, 2-(aminomethyl)-, cis, Cholan-24-oic acid, 3-oxo-, methyl ester, (5á). The bioactive compound with their retention time (RT), molecular formula, molecular weight (MW) and concentration (area) are present in Table 1. The mass spectrum and structure of the compounds indentified were present in the crude methanolic extract of *Filifusus filamentosus* were depicted in Figure 2-14 respectively, which could be responsible for anticancer, antioxidant, antimicrobial, analgesic, anti-inflammatory, antipyretic, hepatoprotective, diuretic, antiviral activities etc.

6.2 AutoDocking

In the present study the AutoDock binding affinities of various structure for their potential anticancer activity was investigated. In the present study, AutoDock software were used to perform the docking simulation between the ligand 2,4-Di-tert-butyl phenol and standard drug salirasib with the protein 5p21. The results of the docking simulations are presented in Table 2. The results showed that the ligand 2,4-Di-tert-butyl phenol has a lower binding energy (- 4.67 kcal/mol) that indicated

higher affinity with the protein. Also ligand has highest root mean square deviation (RMSD) score (40.37) and lowest inhibition constant (375.78 µm) when compared to the standard drug. Moreover the stability of the ligand is also high than that of standard drug and has succeeded in making strong hydrogen bond with the target protein. The distance and site of hydrogen bond is shown in the (Figures 15 and 16) and (Plate 2).

6.3 Anticancer activity of methanolic extract of *Filifusus filamentosus*

Anticancer activity of the methanolic extract of *F. filamentosus* was carried out against MCF-7 human breast cancer cell line. The results of anticancer activity of *F. filamentosus* extract on MCF-7 human breast cancer cell line were shown in (Plates 4) and (Figure 17) respectively. The results of the anticancer activity revealed that *F. filamentosus* showed good anticancer potential which has the IC_{50} of 231.50 µg/ml against MCF-7 breast cancer cells. In the present study, the cell viability was found to be increased with increase in concentration. The methanolic extract of *F. filamentosus* showed 72.92% viability at 100 µg/ml followed by 78.03%, 88.55% and 92.20% at 200, 300 and 400 µg/ml concentrations respectively. The percentage toxicity was found to be 27.08% at 100 µg/ml, 21.97% at 200 µg/ml, 11.48% at 300 µg/ml and 7.8% 400 µg/ml at different concentrations respectively.

6.4 Apoptotic effect of *F. filamentosus* extract in MCF-7 cells

Acridine orange and ethidium bromide staining as performed to evaluate the cellular morphological changes in MCF-7 cells treated with the methanolic extract of *Filifusus filamentosus*. Usually acridine orange will enter the nucleus and stain live cells as green colour and ethidium bromide will penetrate the nucleus of dead

cell due to loss of membrane integrity and stain as red colour. In the present study, viable cells appeared as green fluorescence with highly organised nuclei. Apoptotic cells appeared as green-orange colour nuclei with condensed or fragmented chromatin at the concentration of 6.25 µg/ml respectively. Apoptotic cells appeared as orange to red colour with highly condensed or fragmented chromatin and apoptotic bodies at the concentration of 12.5 µg/ml respectively. The methanolic extract of *F. filamentosus* treated cell showed typical apoptotic morphological features such as condensed nuclei, membrane blebbing and formation of apoptotic bodies in a concentration-dependent manner, which were clearly observed under the fluorescence microscope and quantitated (Plate 5) and (Figure 18).

6.5 Apoptotic cell nuclear morphological changes of *Filifusus filamentosus* in MCF-7 cells

Apoptosis can be differentiated from necrosis by their characteristic nuclear changes. DAPI is a nuclear stain which is observed as blue fluorescence when excited under fluorescence microscope. In the present study, DAPI staining revealed the changes associated with apoptosis in MCF-7 cells treated with the methanolic extract of *F. filamentosus.*

The methanolic extract of *F. filamentosus* treated MCF-7 cells appeared as bright blue with apoptotic nuclear morphological changes. The morphological changes include chromatin condensation, nuclear fragmentation and marginalization, DNA condensation and fragmentation and formation of apoptotic bodies in MCF-7 treated cells at the concentration of 6.25 µg/ml and 12.5 µg/ml

respectively. In untreated cells, there were no morphological changes, nuclei fluoresced as faint blue which was homogenous (Plate 6) and (Figure 19).

6.6 Apoptosis confirmation by DNA fragmentation assay

To gain further insights into the mode of cell death caused by methanolic extract of *F. filamentosus* DNA fragmentation assay, a widely used technique for the detection of apoptosis was adopted. The treatment resulted in a dose-dependent increase in the DNA fragmentation levels in MCF-7 cells. DNA fragmentation was clearly visible in the groups with the dose-dependent treatment of the methanolic extract (Lanes 3, 4) compared to the control group (Plate 7)

DNA laddering assay was performed on agarose gel electrophoresis, to determine methanolic extract of *F. filamentosus* cell death of MCF-7 breast cancer cell line via apoptotic. A clear fragmented DNA ladders were observed in MCF-7 breast cancer cell lines treated with methanolic extract of *F. filamentosus* and control don’t show any DNA fragmentation (Plate 7).

Figure 1 : Map showing the study area Thoothukudi coastal region

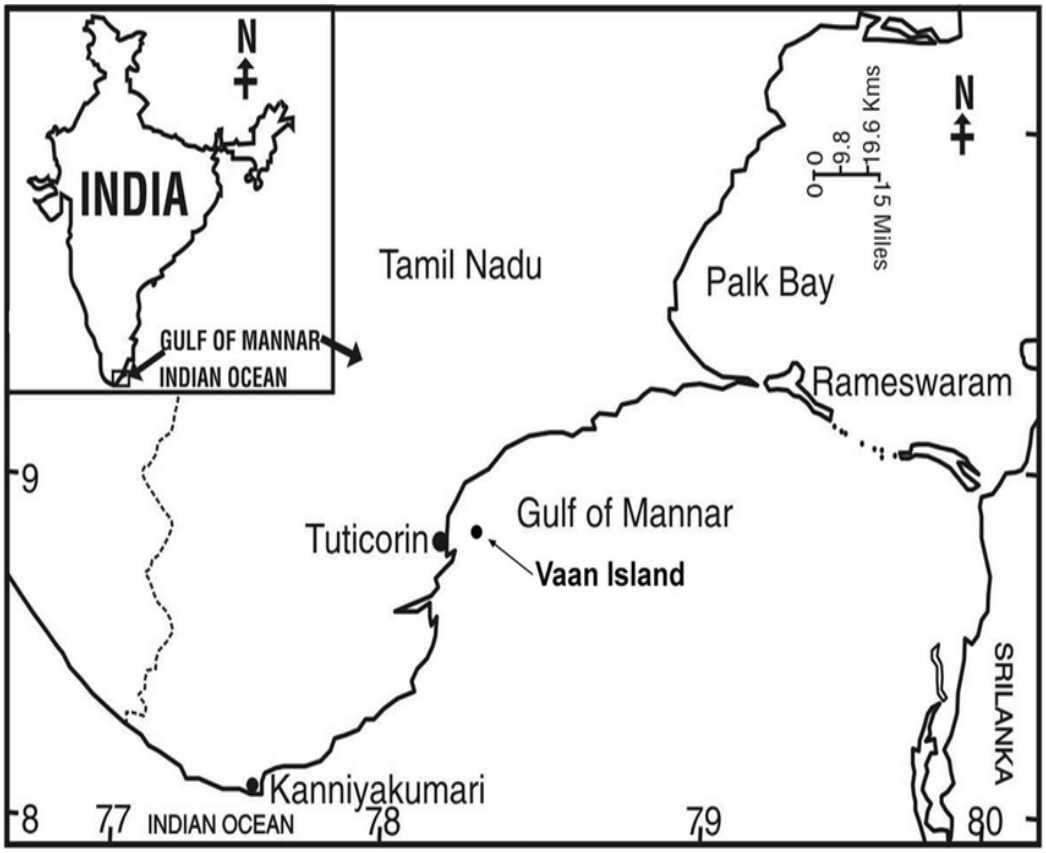


Plate 1 : Experimental animal

Dorsal view of *Filifusus filamentosus*

Ventral view of *Filifusus filamentosus*

Table 1 : Activity of components identified in the methanol extract of *Filifusus filamentosus* by GC - MS study

Sl. No.	RT	Name of the Compound	Molecular Formula	Molecular Weight (g/mol)	Peak area %	**Activity
1	3.52	Cyclotetrasiloxane, octamethyl	$C_8H_{24}O_4Si_4$	296.616	92.61	Wound healing, Anti-ulcer, Antisoriatics, Antiseborrheics, Antioxidant, Antiarthritics, Analgesic, Antipyretic, Antiinflammatory, Antiviral, Antineoplastic, Immunomodulators
2	6.89	Cyclododecane	$C_{12}H_{24}$	168.324	84.67	Antiadthmatics, Bronchodilators, Anti-spasmodics, Anti-acne, Antioxidants, Antigout, Anaesthetics, Anticonveulsants, Antibacterial.
3	8.41	2,4-Di-tert-butyl phenol	$C_{14}H_{22}O$	206.329	95.61	Antioxident, Anticancer, Antiinflammatory, Analgesic, Antipyretics
4	9.51	E-14-Hexadecanol	$C_{22}H_{38}$	302.546	79.16	Antioxident, Anticancer, Antiviral, Antifungal
5	36.39	Cholestrol	$C_{27}H_{46}O$	386.654	89.46	Antioxident, Anticancer, Hepatopotective, Antiinflammatory, Antimicrobial, Analgesic, Antipyretic

6	16.32	9 - Octadecenamide, (Z)- [Oleic acid amine]	$C_{18}H_{35}NO$	339.564	8.80	Antiinflammatory, Antiandrogenic, Cancer preventive Dermatitigenic, Hypocholesterolemic, 5-Alpha reducatase inhibitor, Anemiagenic Insectifuge, Flavor
7	3.01	Undecanal, 2-methyl-	$C_{12}H_{24}O$	184.323	2.85	Antimicrobial
8	10.85	Cyclohexanol,2-amino-,trans-	$C_6H_{13}NO$	172.268	1.73	Antiinflammatory
9	12.59	Butanal, O-methyloxime	$C_5H_{11}NO$	101.149	7.29	Antimicrobial
10	14.76	E-2-Tetradecen-1-ol	$C_{14}H_{28}O$	210.361	1.77	Antimicrobial
11	16.89	1,1-Cyclopropane dicarbonitrile, 2,2-dimethyl-	$C_7H_8N_2$	68.119	1.18	Antimicrobial
12	17.46	Cyclopentanol, 2-(aminomethyl)-, cis-	$C_6H_{13}NO$	100.161	0.41	Antimicrobial
13	28.85	Cholan-24-oic acid, 3-oxo-, methyl ester, (5á)-	$C_{25}H_{40}O_3$	388.592	50.06	Antimicrobial Antiinflammatory, Anticancer, Antiasthma, Diuretic, Antiarthritic

Chromatogram of methanolic extract of *Filifusus filamentosus*

Figure : 2

Name : Cyclotetrasiloxane, octamethyl

Formula : $C_8H_{24}O_4Si_4$

Molecular Weight : 296.616 g/mol

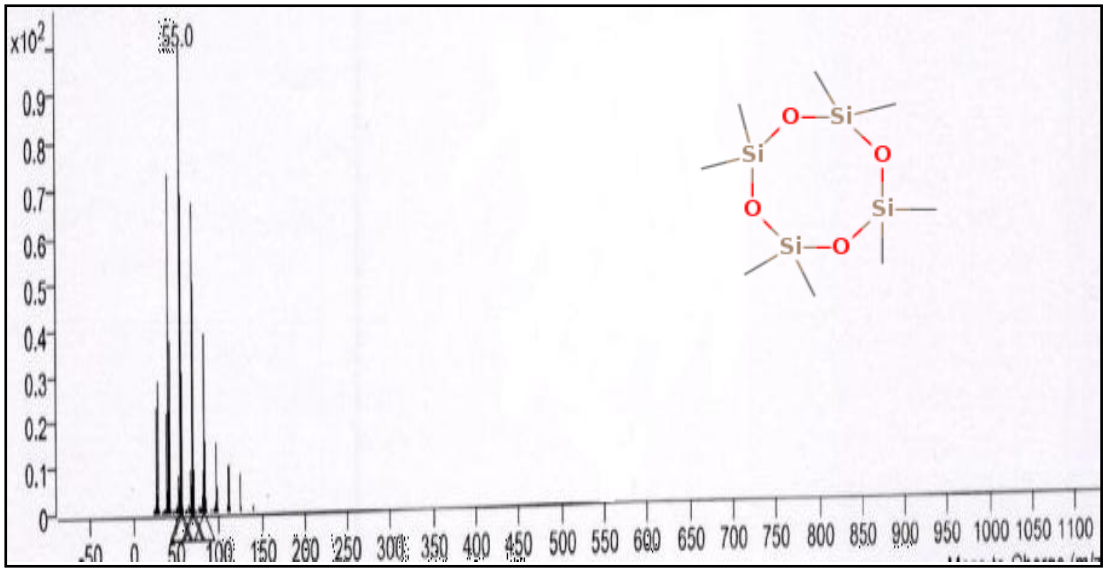


Figure : 3

Name : Cyclododecane

Formula : $C_{12}H_{24}$

Molecular Weight : 168.324 g/mol

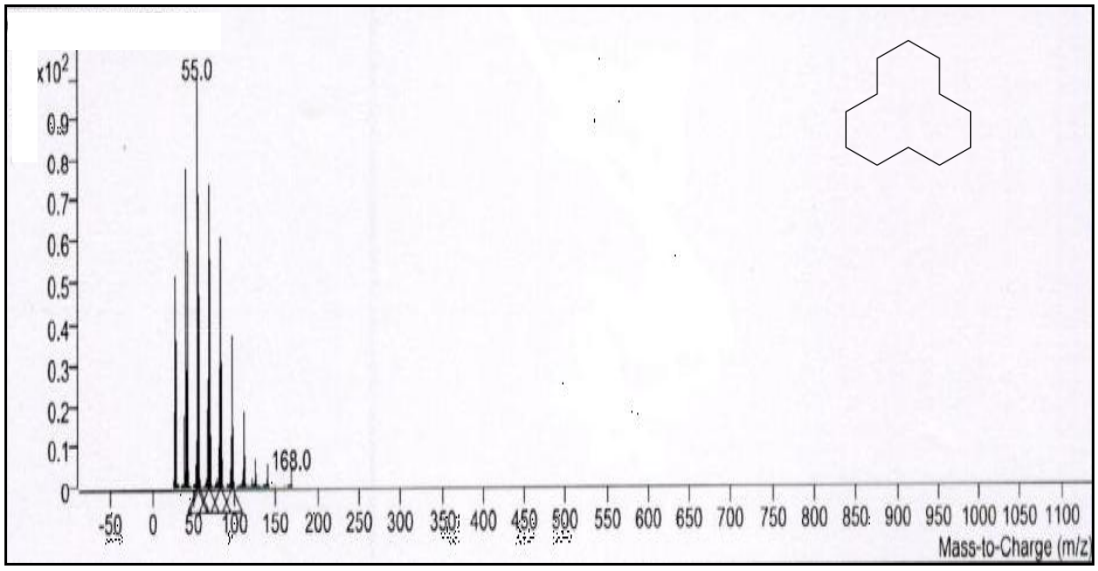


Figure : 4

Name : 2, 4-Di-tert-buty1 phenol

Formula : $C_{14}H_{22}O$

Molecular Weight : 206.329g/mol

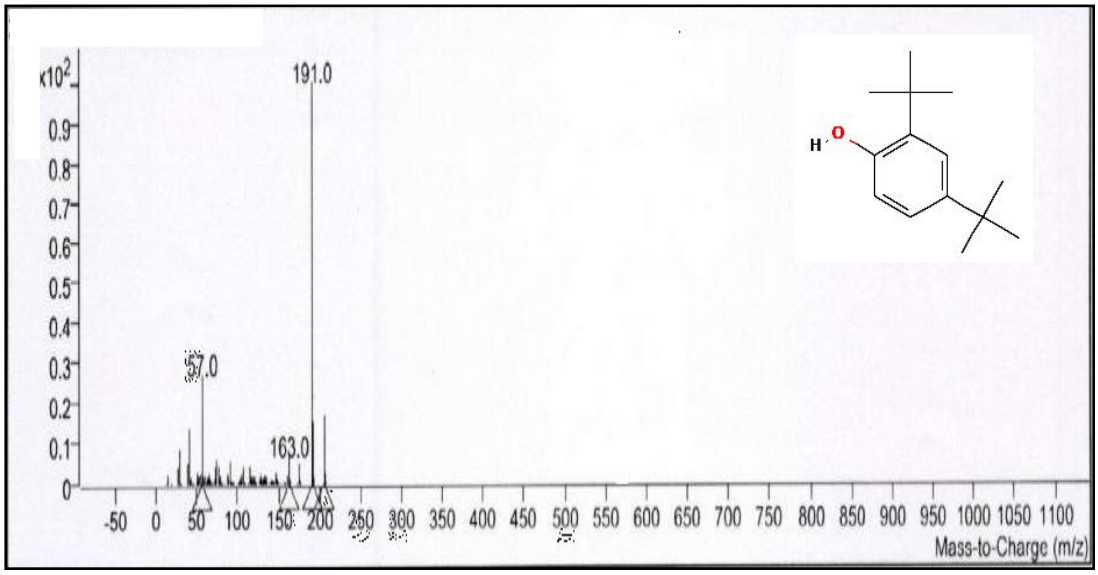


Figure : 5

Name : E-14-Hexadecanol

Formula : $\underline{C_{16}H_{34}O}$

Molecular Weight : 242.447 g/mol

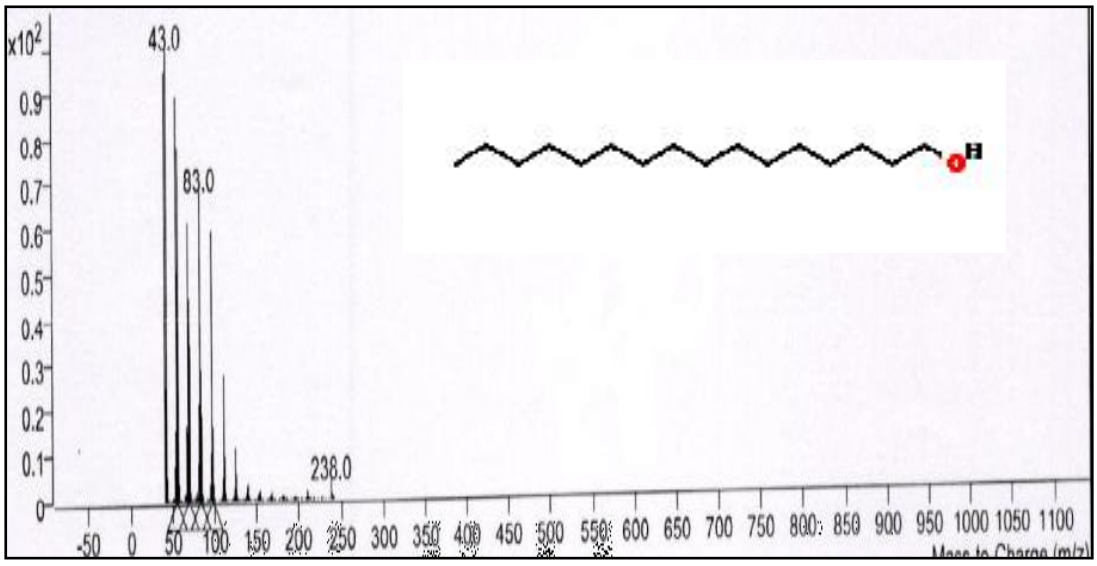


Figure : 6

Name : Cholestrol

Formula : $C_{27}H_{46}O$

Molecular Weight : 386.664 g/mol

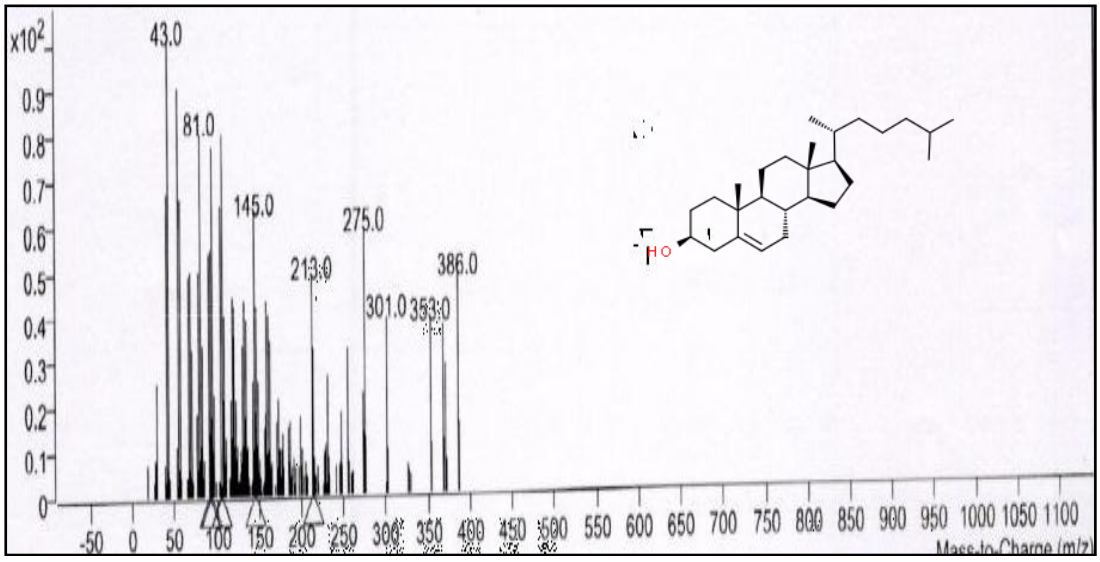


Figure : 7

Name : 9 - Octadecenamide, (Z) - [Oleic acid amine]

Formula : $C_{18}H_{35}NO$

Molecular Weight : 339.564 g/mol

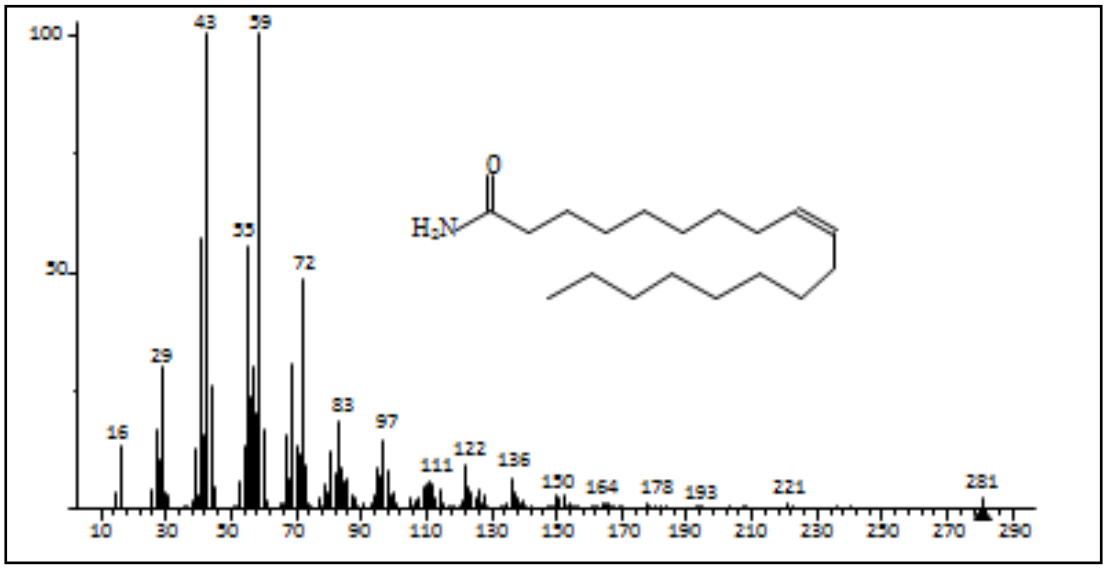


Figure : 8

Name : Undecanal, 2-methyl-

Formula : $\underline{C_{12}H_{24}O}$

Molecular Weight : 184.323 g/mol

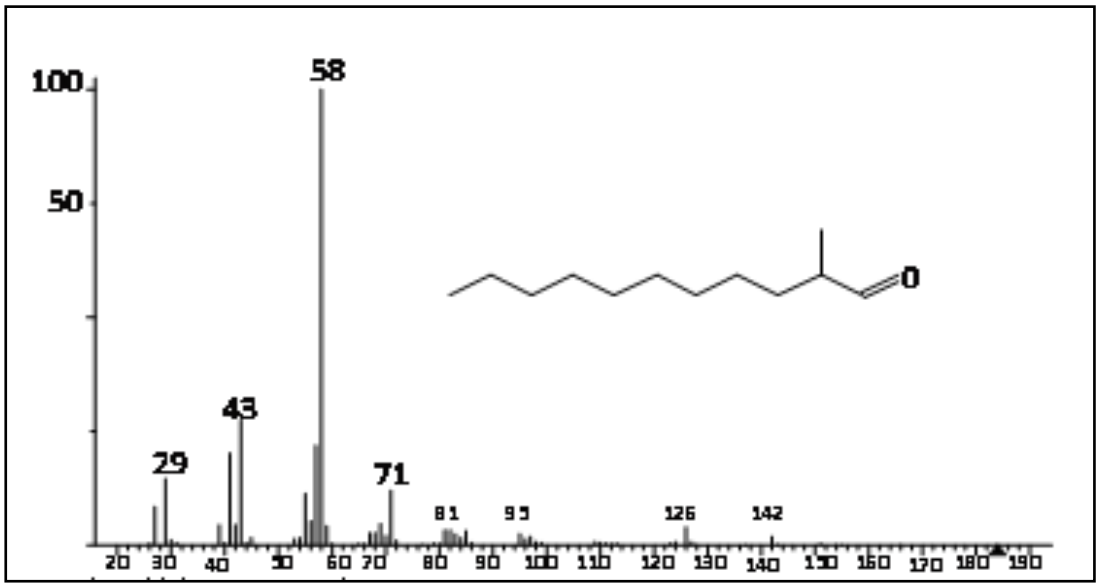


Figure : 9

Name : Cyclohexanol, 2-amino-,trans-

Formula : $C_6H_{13}NO$

Molecular Weight : 115.176 g/mol

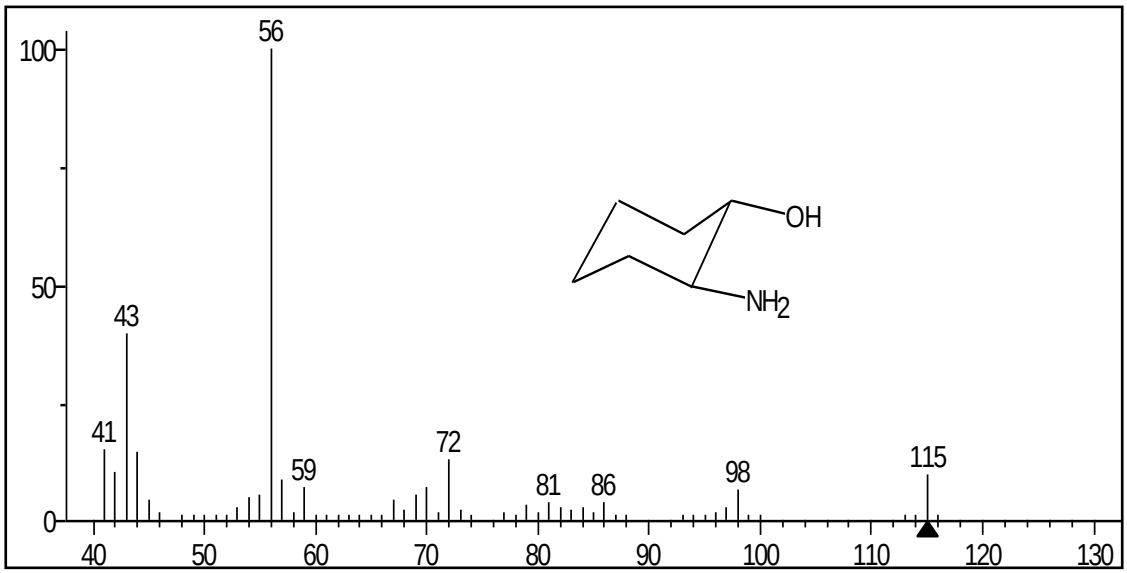


Figure : 10

Name : Butanal, O-methyloxime

Formula : $C_5H_{11}NO$

Molecular Weight : 101.149 g/mol

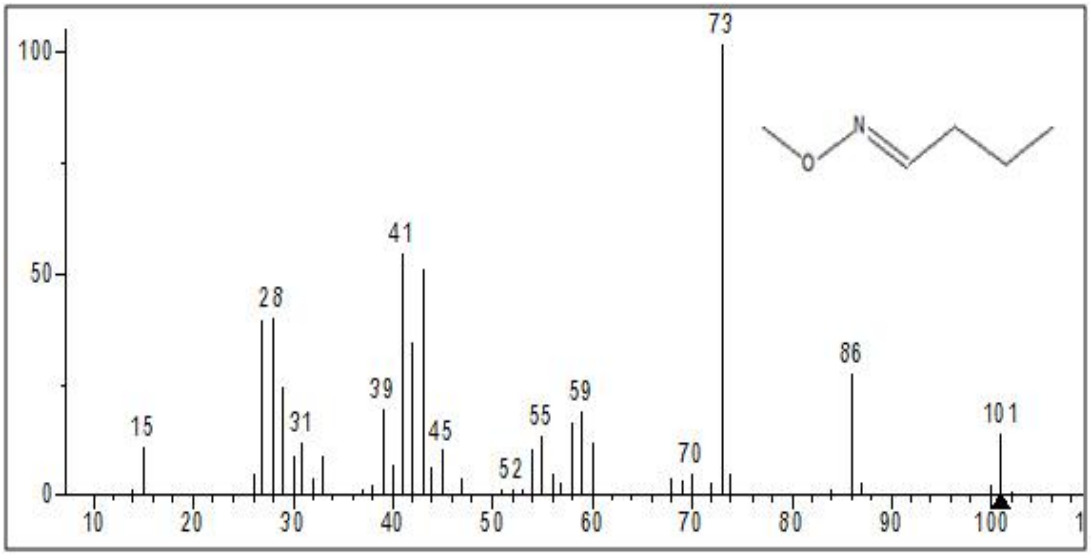


Figure : 11

Name : E-2-Tetradecen-1-ol

Formula : $C_{14}H_{28}O$

Molecular Weight : 212.377 g/mol

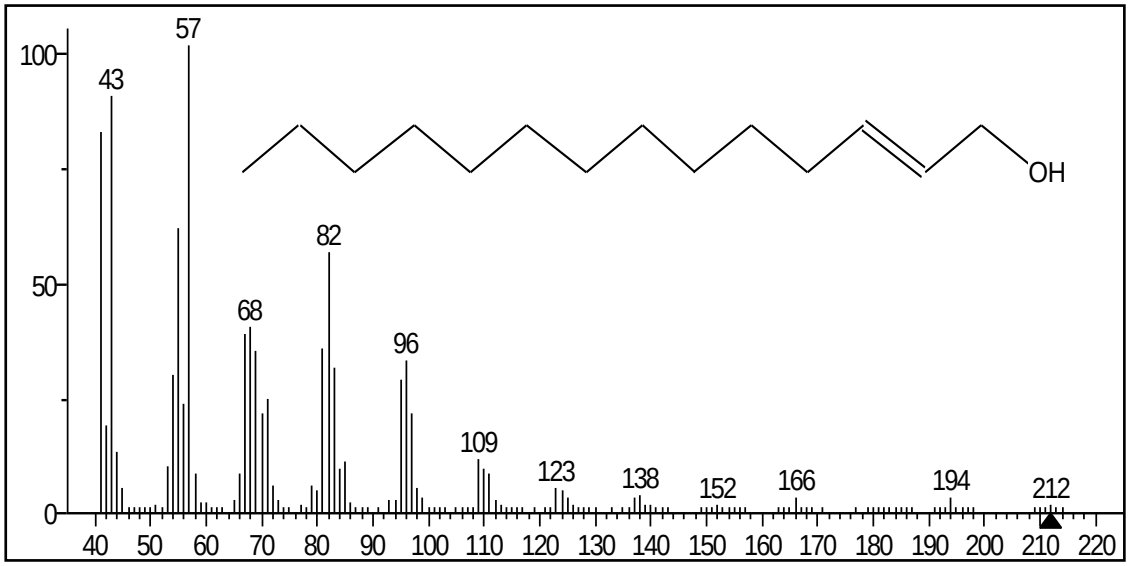


Figure :12

Name : 1,1-Cyclopropane dicarbonitrile, 2,2-dimethyl-

Formula : $C_7H_8N_2$

Molecular Weight : 68.119 g/mol

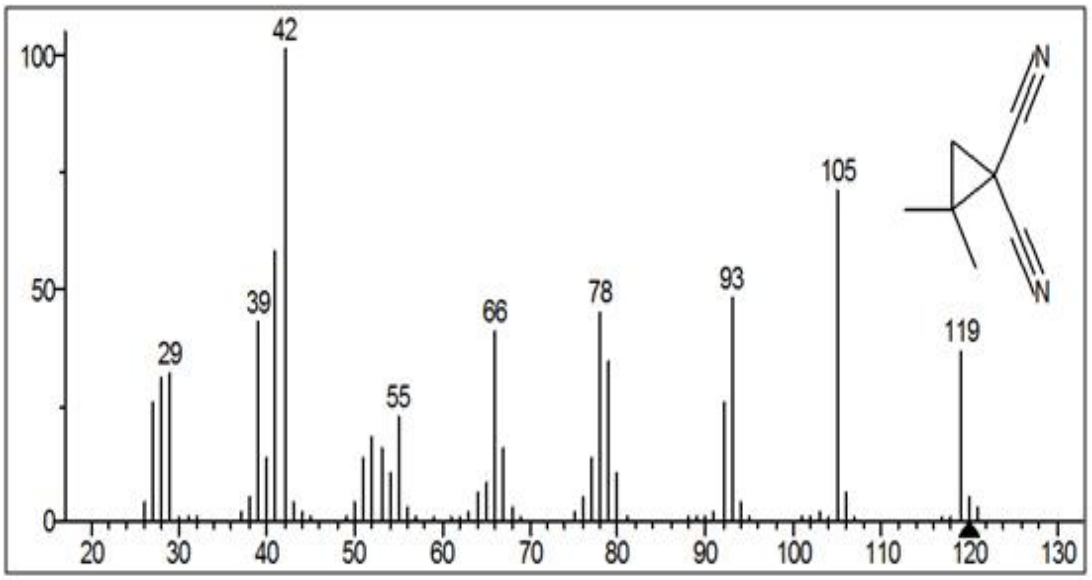


Figure : 13

Name : Cyclopentanol, 2-(aminomethyl)-, cis-

Formula : $C_6H_{13}NO$

Molecular Weight : 115.176 g/mol

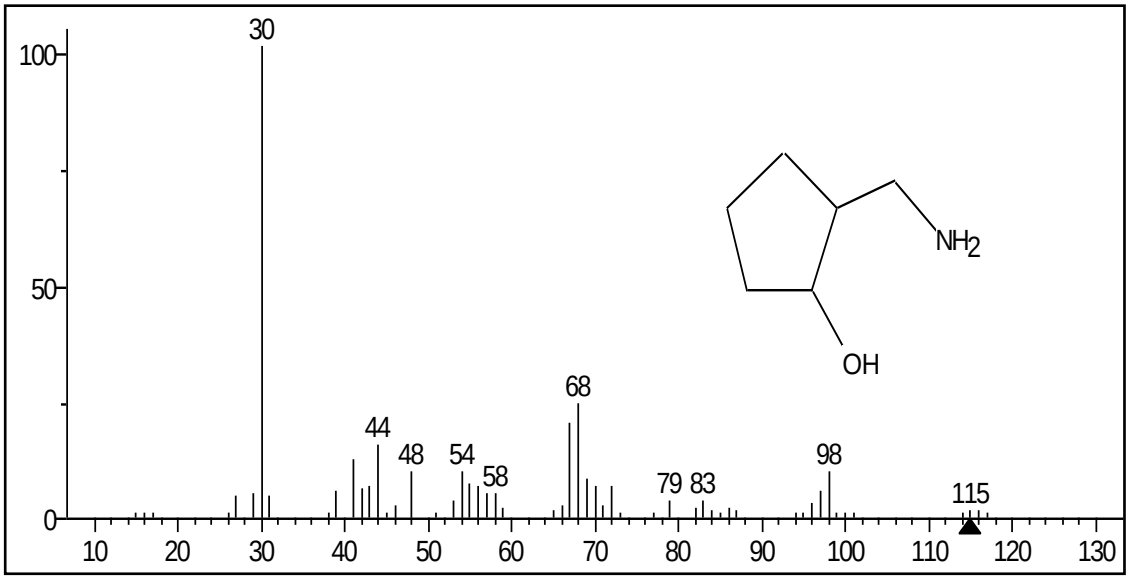


Figure : 14

Name	:	Cholan-24-oic acid, 3-oxo-, methyl ester, (5á)-
Formula	:	$C_{25}H_{40}O_3$
Molecular Weight	:	388.592 g/mol

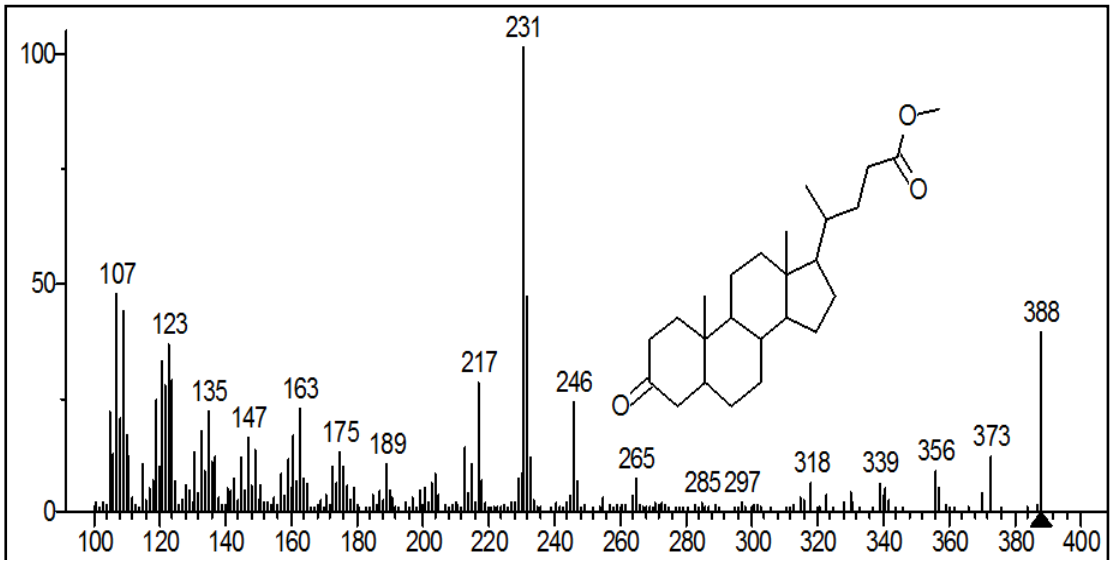


Table 2 : Docking result of protein 5p21

Ligand	2,4-Di-tert-butyl phenol	Salirasib
Binding energy	-4.67 kcal/mol	-4.49 kcal/mol
Ligand efficiency	-0.31	-0.18
Inhib_constant	375.78 µM	515.44 µM
H acceptor	UNL1:H	UNL1:O
H donor	A:GLU31:O	A:LYS16:NZ
Distance	1.926 Å	2.892 Å
RMSD Score	40.37	40.23

Figure 15 : Docking simulation between 5p21 and Standard Salirasib

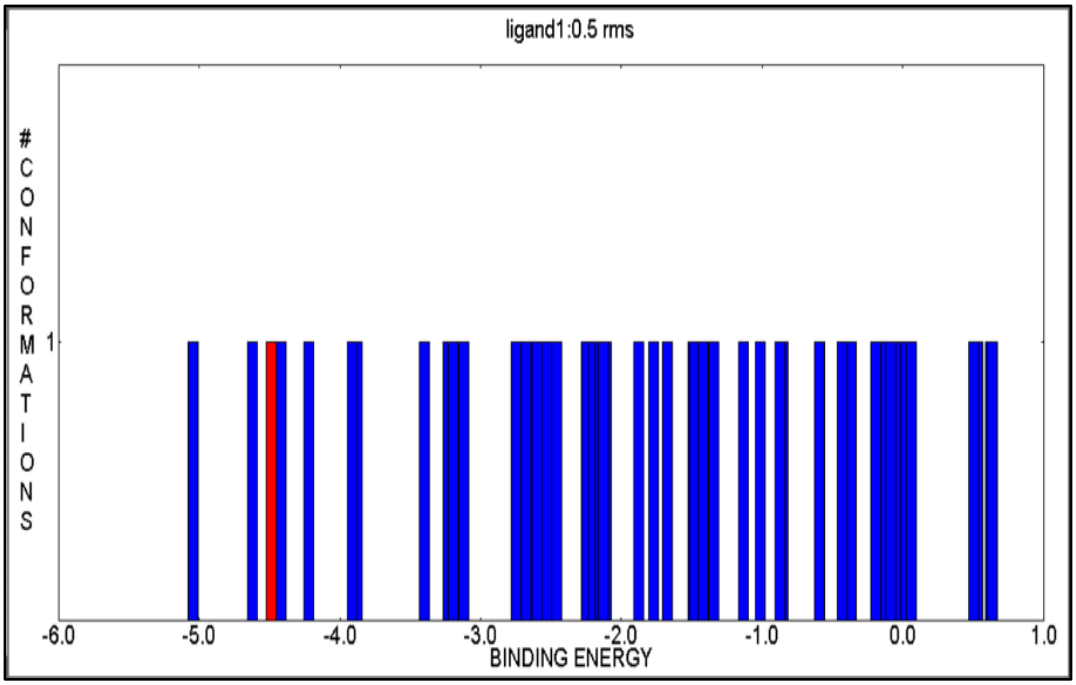


Figure 16 : Docking simulation between 5p21 and 2,4-Di-tert-butyl phenol

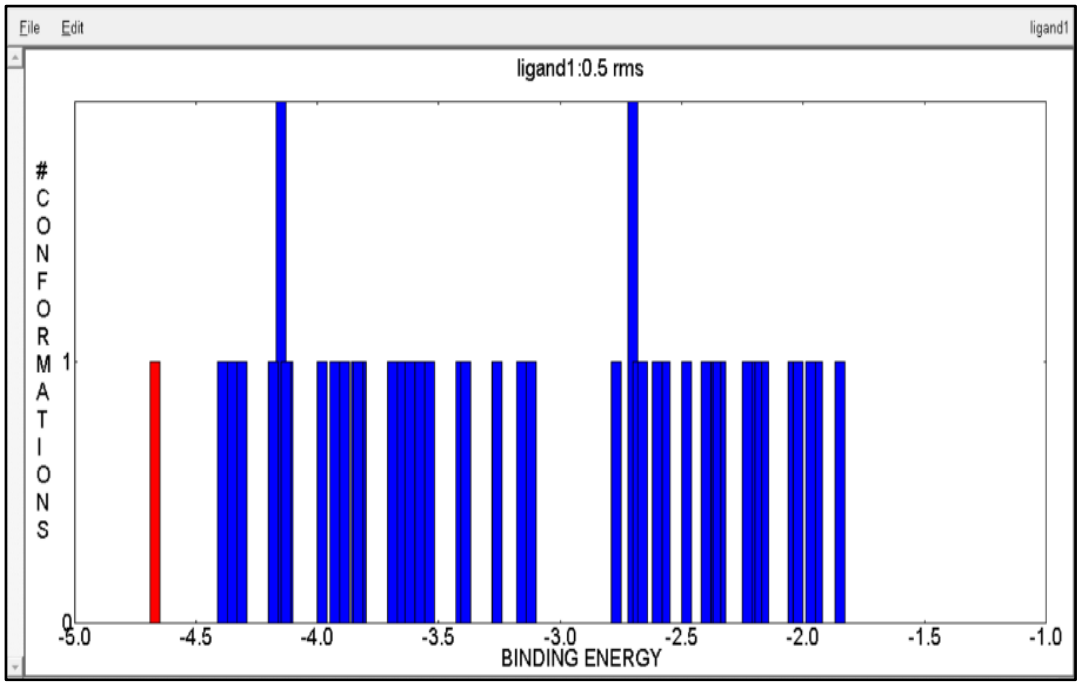
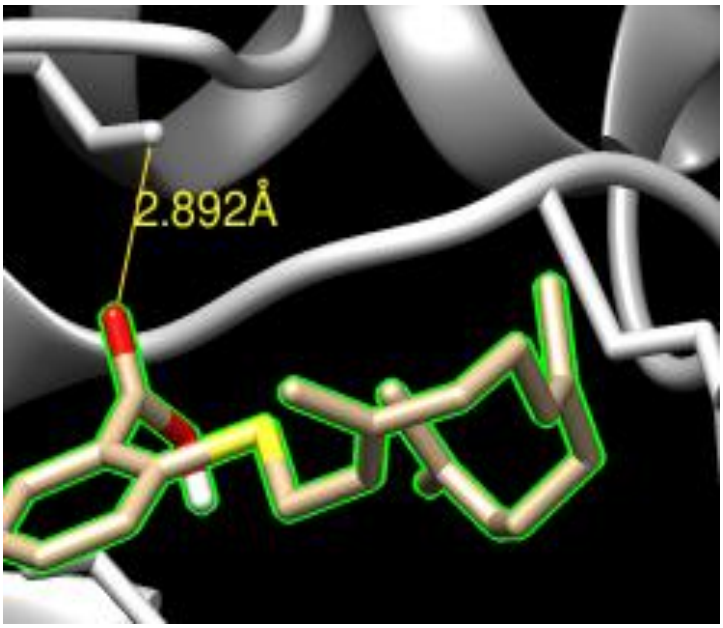
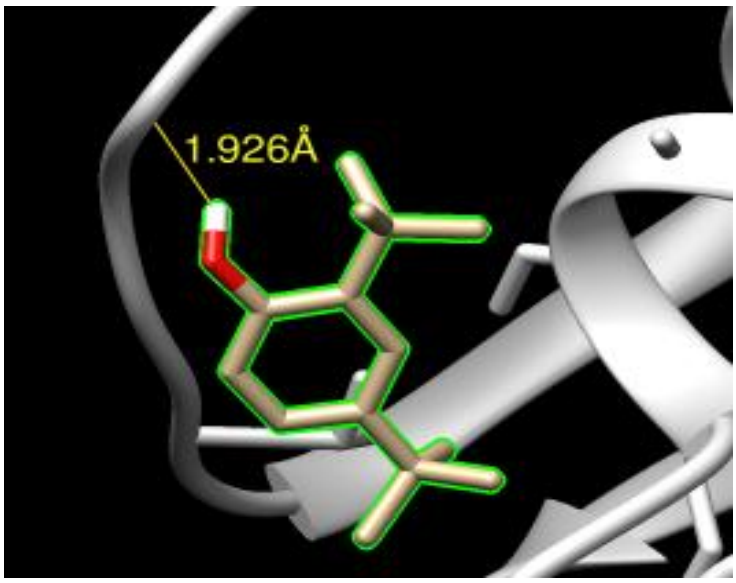


Plate 2 : Visualization of interactions of ligand and standard drug with the target protein 5p21.

a. Interaction of Salirasib with 5p21



b. Interaction of 2,4-Di-tert-butyl phenol with 5p21



Green chains represents the standard drug and ligand

Figure 17 : Anticancer activity of methanolic extract of *Filifusus filamentosus* in MCF-7 cell line

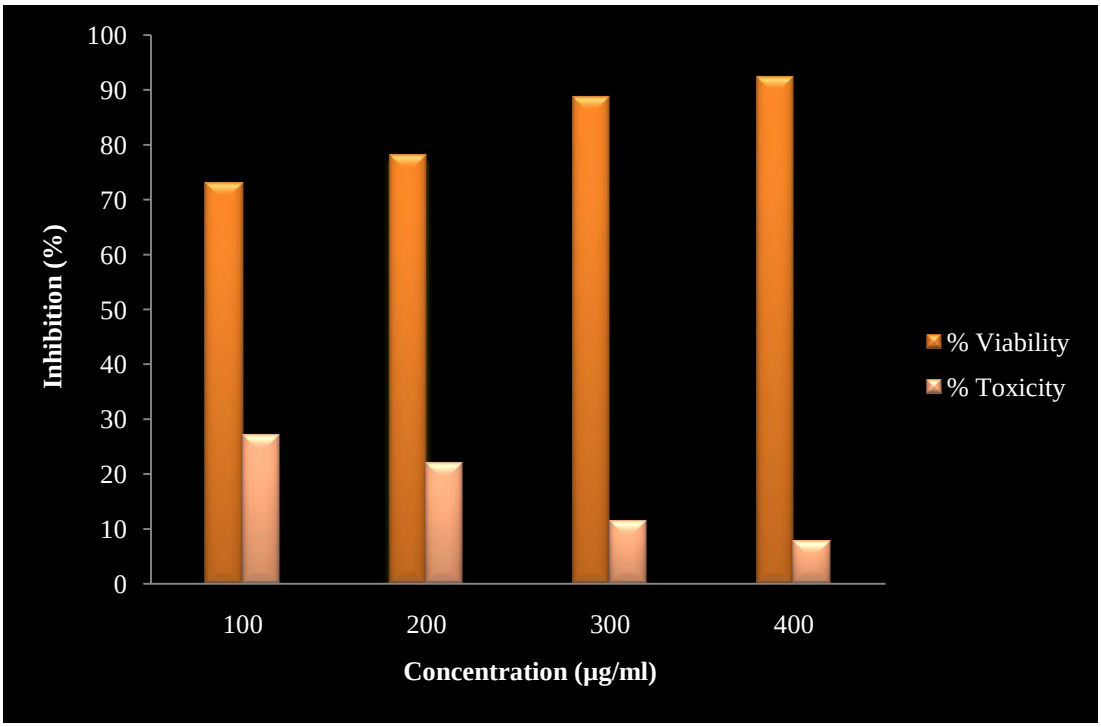
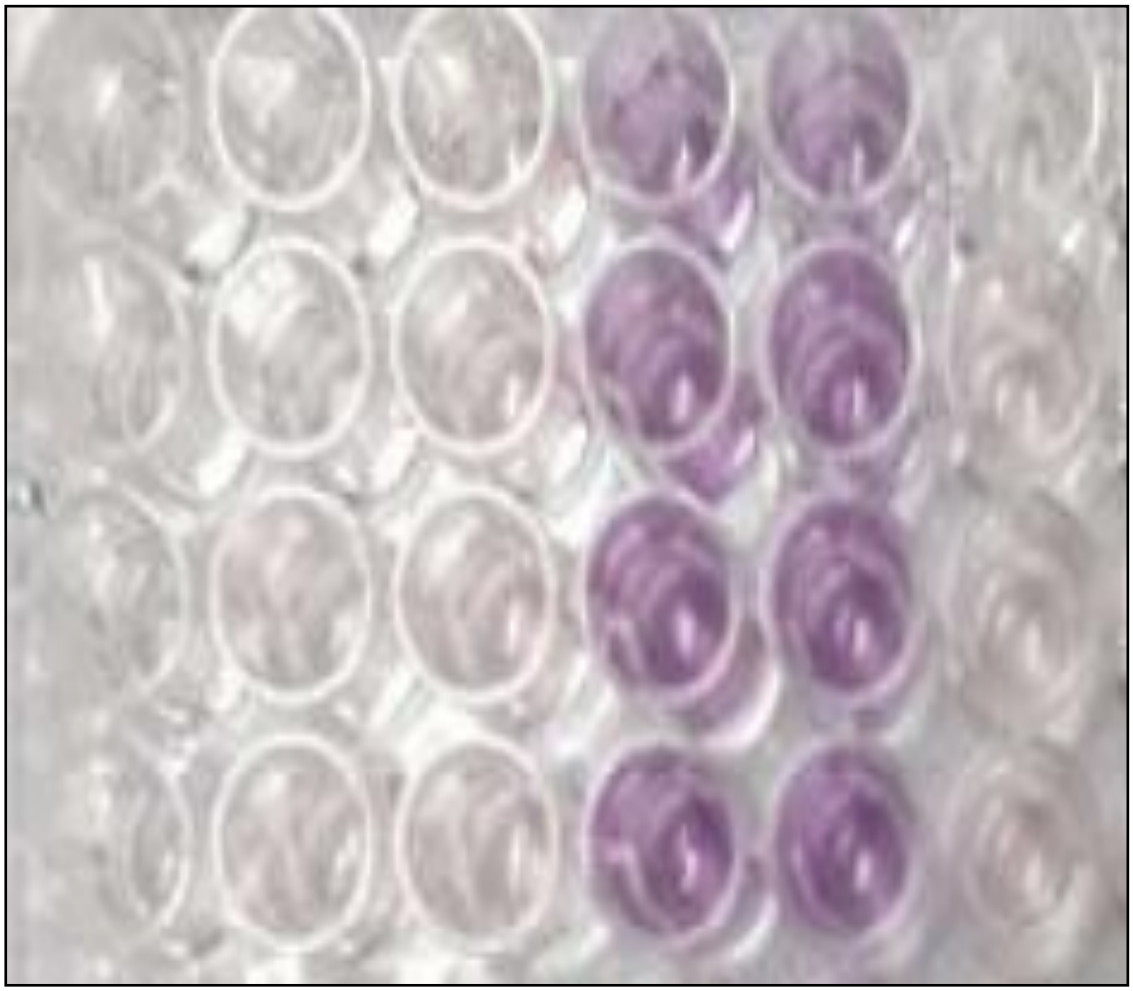


Plate 3 : MTT assay

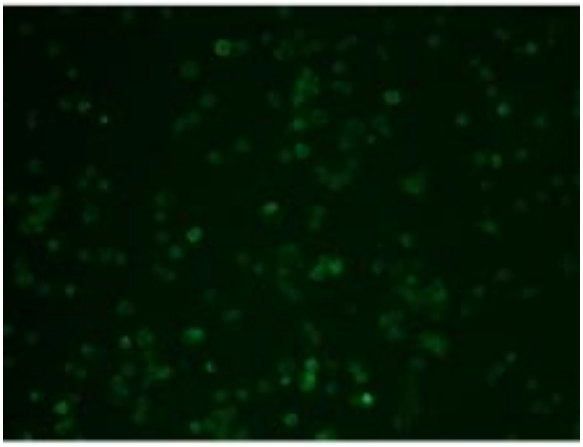
S C



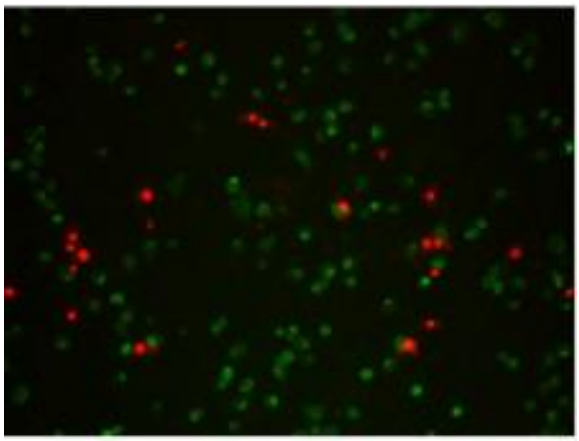
Sample	100 µg/ml	200 µg/ml	300 µg/ml	400 µg/ml
Control	100 µg/ml	200 µg/ml	300 µg/ml	400 µg/ml

Plate 5 : Acridine orange and ethidium bromide staining assay of *Filifusus filamentosus* in MCF-7 cells

Control



6.25 µg/ml



12.5 µg/ml

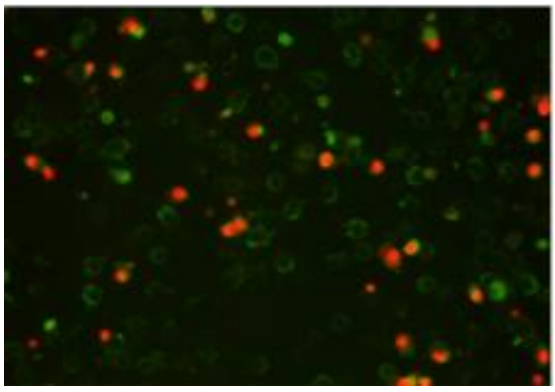


Figure 18 : Acridine orange and ethidium bromide staining assay of *Filifusus filamentosus*

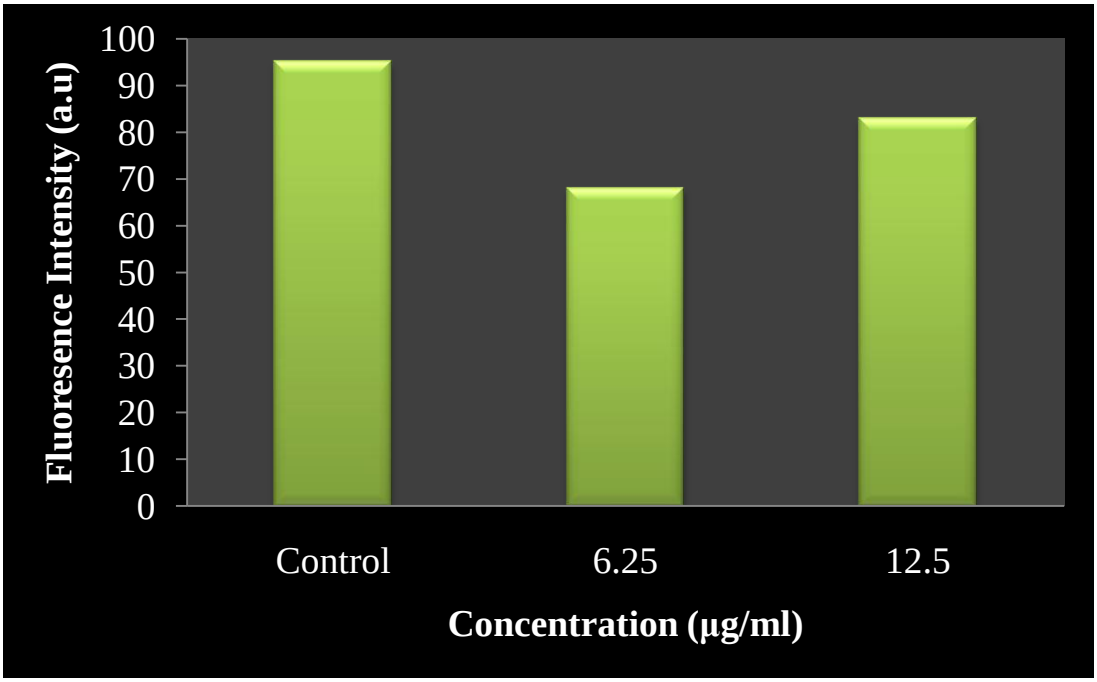
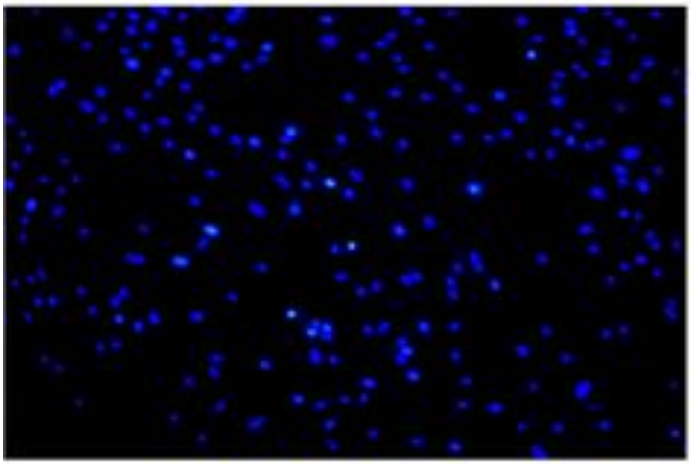
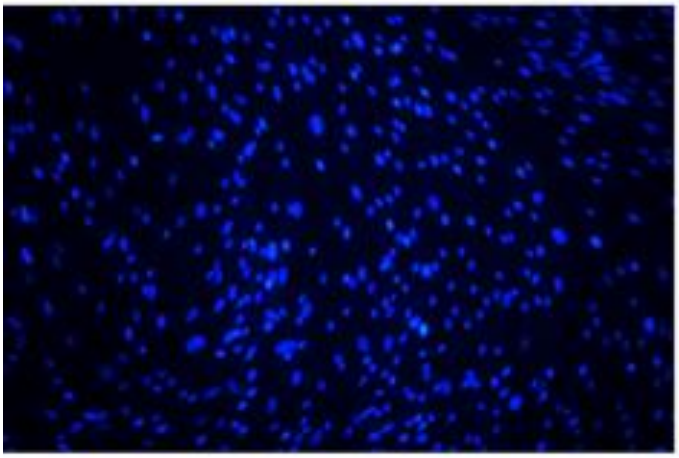


Plate 6 : DAPI Staining assay of *Flifusus filamentosus*

Control



6.25 µg/ml



12.5 µg/ml

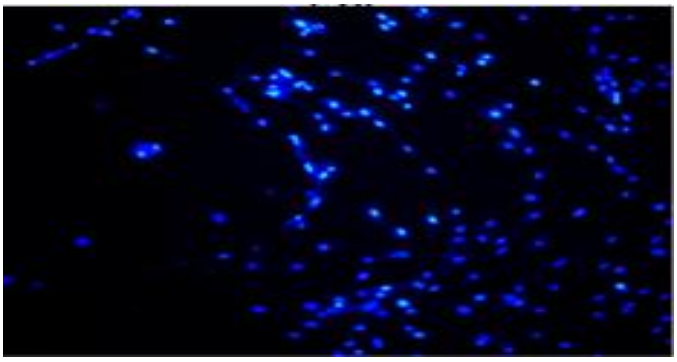


Figure 19 : DAPI staining assay of *Filifusus filamentosus*

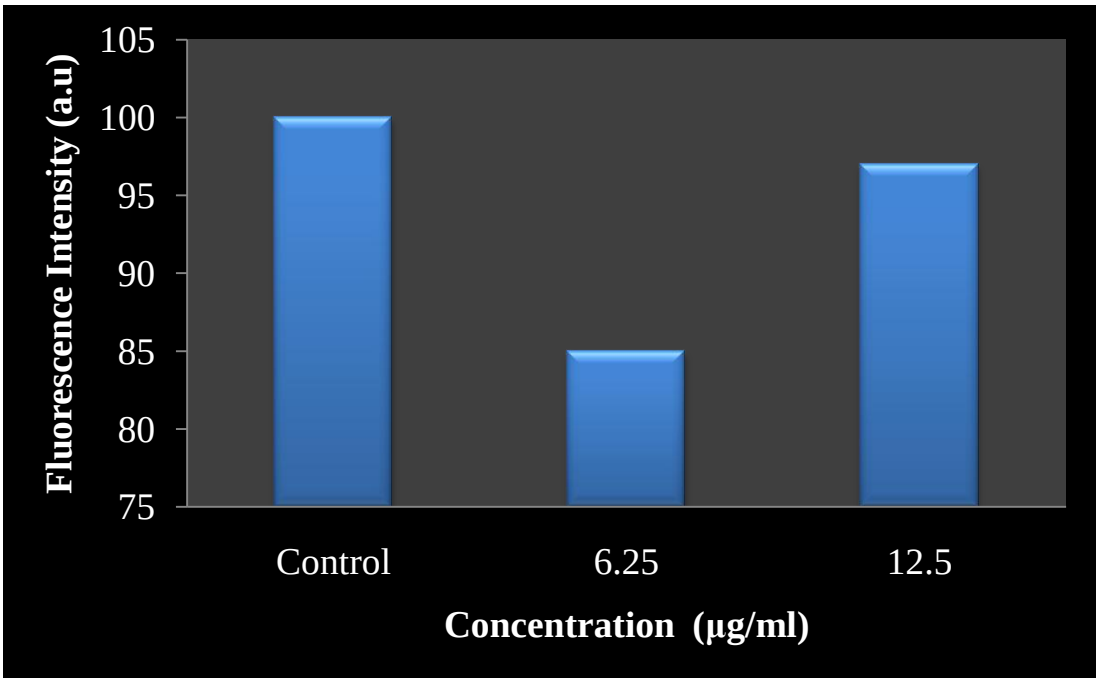
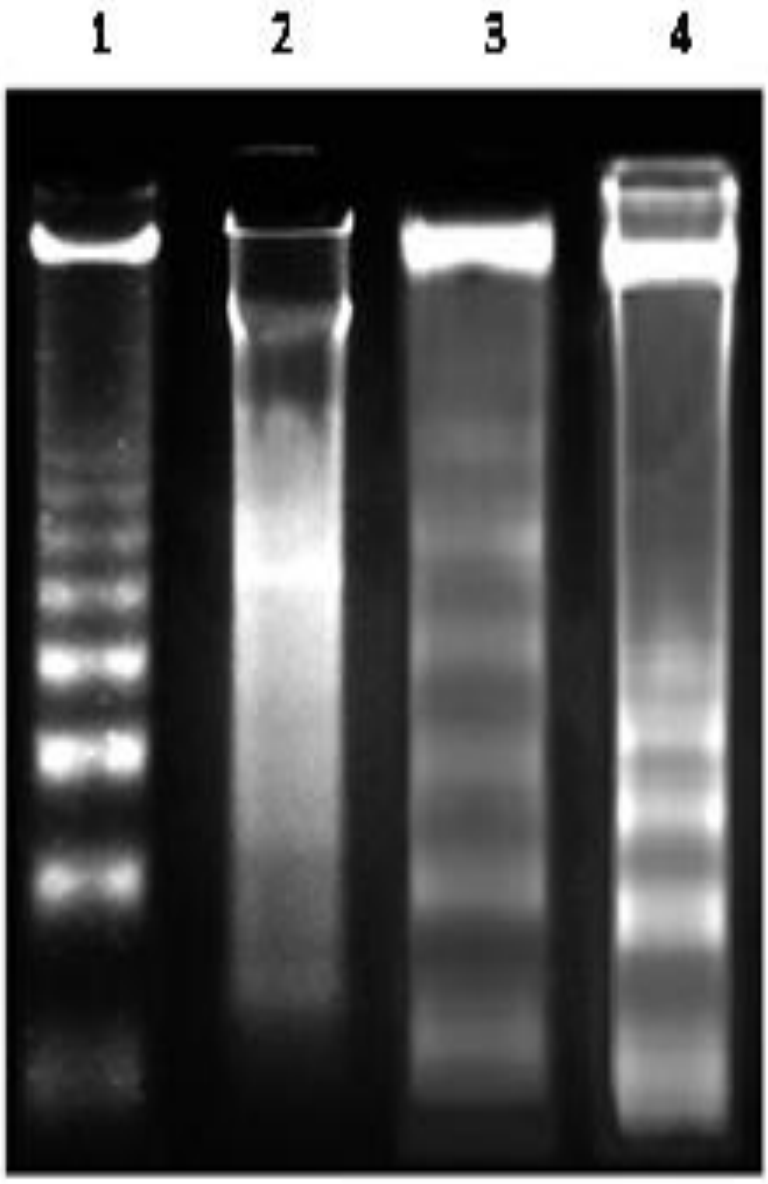


Plate 7 : DNA fragmentation assay in *Filifusus filamentosus*



Lane 1 – 1kb DNA Ladder

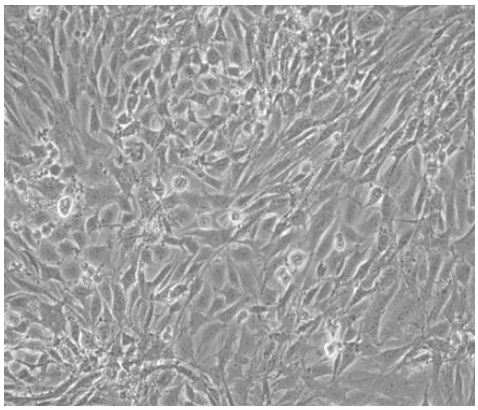
Lane 2 – Control

Lane 3 – 6.25 µg/ml

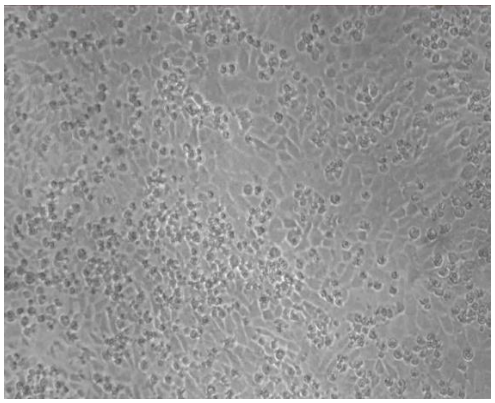
Lane 4 – 12.5 µg/m

Plate 4 : Anticancer activity of methanolic extract of *Filifusus filamentosus*

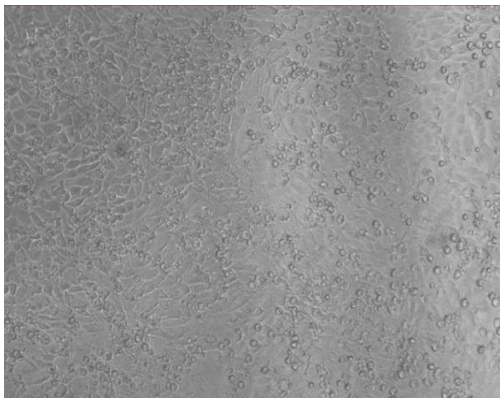
Control



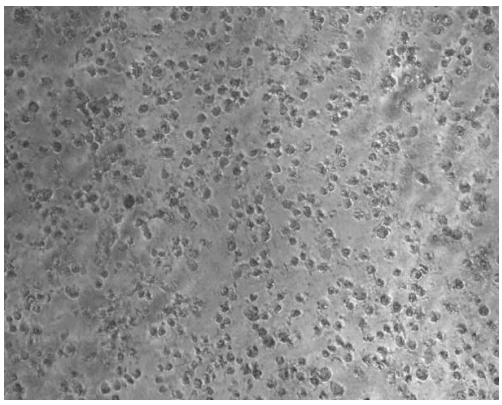
100 µg/ml



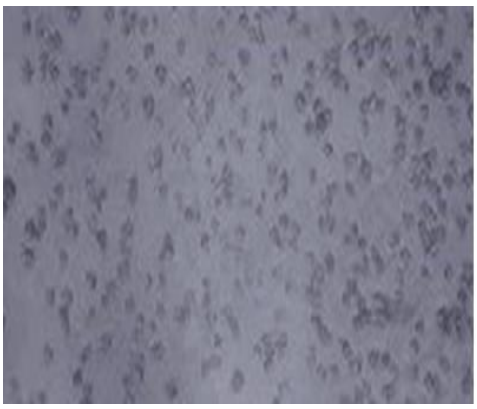
200µg/ml



300 µg/ml



400 µg/ml



7. DISCUSSION

Among the invertebrates, the molluscs are very good source for biomedical products (Balcazar *et al.,* 2006). Many classes of molluscs with bioactive compounds like antitumour, antileukemic, antibacterial, cytotoxic, antiinflammatory and antiviral properties have been reported by Blunt *et al.* (2006) and Santhana Ramasamy and Murugan (2005). These reports suggest that molluscs are the rich source for discovering novel lead compounds for the possible development of new types of drugs for pharmaceutical use. Keeping the importance of gastropods in terms of bioactive compounds with anticancer property, the present study has been undertaken to determine the GC-MS analysis, docking studies, anticancer activity, apoptosis effect, cell nuclear morphology and DNA fragmentation of methanolic extract of *F. filamentosus*.

GC-MS is used to identify the constituents of volatile matter, long chain and branched chain hydrocarbons, alcohols, acids and esters. GC-MS is a powerful tool that is being increasingly used in biomarker discovery. It can be used for analysis of wide range of biological compounds including FAs, essential oils, eicoscanoids, wax, esters by the selection of suitable columns. As many of the FAs are non-volatile compounds, these needs to be derivatized into their methyl ester forms for analysis in GC-MS.

GC-MS chromatogram of the methanolic extract of *Cypraea arabica* showed 23 peaks indicating the presence of 23 compounds. The steroid compound exhibits antiarthritic, hepatoprotective, antiasthma, anti-inflammatory, diuretic and anticancer activities. On the other hand alcoholic, fluro and aldehyde compounds are

responsible for antimicrobial activity. Sesquiterpene oxide has anti-tumour, analgesic, antibacterial, anti-inflammatory and fungicide effect. Fatty acid is responsible for nematicide and pesticide activities and can also be used as flavouring agent. Myristic acid shows antioxidant, cancer preventive, nematicide hypercholesterolemic and also acts as lubricant. In addition to antioxidant and pesticide activity, palmitic compound act as antiandrogenic, nematicide, lubricant and 5 alpha reductase inhibitors. Monounsaturated fatty acid shows anti-inflammatory, antiandrogenic, cancer preventive, insecticide, perfumery, 5 alpha reductase inhibitor and dermatigenic activities (Subavathy and Thilaga, 2016).

Marine molluscan extracts are usually complex mixtures of bioactive molecules containing mainly proteins, peptides and sterols. Jemma Hermelin Jesy Diaz and Thilaga, (2015) characterized the bioactive substance present in *Loligo duvauceli* (squid) and *Sepia pharaonis* (cuttle fish) internal shell by GC-MS analysis. GC-MS analysis revealed the presence of ten active compounds in the squid. Among the identified compound an amide compound 9 - octadecenamide was found to be in the maximum percentage (15.08). Similarly 'Pachydictyol-A' extracted from digestive gland of *Aplysia depilans* showed mild antibiotic activity against *Staphylococcus aureus* (Minale and Riccio, 1976). Kubota *et al*. (1985) purified and characterized an antibacterial factor from snail mucus. Chromodorolide - A isolated from *Chromocloris cavae* exhibits *in vitro* antimicrobial and cytotoxic activities (Morris *et al.,* 1990). High concentrations of macrolides, extracted from egg of Spanish dancer nudibranch, *Hexabranchus sangiuneus* prevented the growth of pathogenic micro organism (Pawlik, 1992).

Similar findings were reported by Charlet *et al*. (1996) isolated Mytilins A and B cysteine rich antimicrobial peptides from *Mytilus edulis*. A new class of water - soluble broad - spectrum antibiotics, Squalamine amino-steroid isolated from the stomach extract of shark (*Squalus acanthisa*) also inhibit the angiogenesis of tumours (Moore *et al.,* 1993). Lectin isolated from noise mussel *Modiolus modiolus* exhibited strong antibacterial effect against *Vibrio anguillarum* and *V. salmonicida* (Tunkijanukij and Olafsen, 1998). The mytilin isoforms C, D and G1 were isolated from *Mytilus galloprovincialis* exhibited complementary antimicrobial properties (Mitta *et al.,* 2000).

Similar finding was reported by Wayan Mudianta *et al.* (2010) in an Indonesian sponge *Halichondria* sp. has provided 3-alkyl piperidine alkaloids tetradehydrohaliclonacyclamine A, the mono-N-oxide and a C-2 epimer. Brominated indoles 6 - bromo 2 - methylthioindolin-3-one extracted from Australian muricid *Dicathais orbita* has been identified as anticancer drug indole derivatives of 6, 6´ -dibromoindigo (Benkendorff *et al.,* 2001).

A rich source of nitrogen compounds with a wide range of biological activities viz., Didemnins, Cyclodepsipeptides isolated from the Caribbean ascidian *Tridemnum solidum* displayed potent cytotoxic, immunosuppressant and antibiotic activities (Britton *et al.,* 2001). Ulapualide-A, a sponge derived macrolide isolated from the nudibranch *Hexabranchus sanguineus* exhibits cytotoxic activity against L1210 murine leukemia cells and antifungal activity (Rorsener and Scheuer, 1986).

Emiliano Manzo *et al*., 2007 reported that two novel triterpenoids, aplysoils A and B, β Etzionin a tyrosin derived compound exhibited antibacterial activity

against *Bacillus subtilis.* An antimicrobial peptide from the seminal plasma of the mud crab *Scylla serrata* was isolated by Wang *et al*. (2007). A polyproline type AMP isolated from the Chilean scallop *Argopecten purpuratus* showed antifungal activity against *Fusarium oxisporum* and *Saprolegnia parasitica* (Arenas *et al.*, 2009).

The result are in agreement with Tamil Muthu and Selvaraj (2015) that *Turbo brunneus* flesh extract revealed eight compound with various biological activities like herbicidal, anti-inflammatory, acaricidal, antiparasitic, insecticidal, antioxidant, analgesic and antimicrobial properties. Santhi *et al.* (2016) isolated bioactive compound frommarine prosobranch *Purpura persica* from Tuticorin coast. Janaki *et al.* (2015) revealed the presence of eleven bioactive compounds in *Fusinus nicobaricus* from Gulf of Mannar.

GC-MS analysis of the present study revealed the presence of 13 compounds with antimicrobial, antioxidant, anticancer, anti-inflammatory, diuretic, hepatopotective, antiviral, analgesic, antipyretic activities etc (Table 1) (Figure 2-14). The present study agree well with the above findings. Further investigations are intended to purify these active compounds present in the methanolic extract *F. filamentosus* shall pave the way for the development of either the base or a new drug itself in future and the application of the extract as drug for human administration, need more research.

Molecular docking discovers the binding geometry of two interacting molecules with known structures. It predicts the preferred orientation of receptor and ligand to each other to form a stable complex (Usha *et al.,* 2013; Brooks *et al.,* 2009;

Madeswaran *et al.,* 2012). Currently, the use of computers to determine the binding of datasets of small molecules to known receptors is a major component of drug discovery.

Singh and Konwar (2012) reported molecular docking studies of quercetin and its analogues as anticancerous agents. Though all the compounds occupied the similar active site of H-Ras, the evident difference in binding energies may be due to wide structural variations and based on specificity of the natural compounds (Middha *et al.,* 2013).

The partial plasma binding ability of the compounds temporarily influences the efficacy of the drug, since the bound fraction shielded from metabolism and only the unbound fraction exhibits pharmacological effect (Susnow and Dixon, 2003). Toxicity of the compounds may also be due to poor intestinal absorption levels. Intestinal absorption is defined as a percentage absorbed rather than as the ratio of concentrations (Dixon and Merz, 2001; Egan *et al.,* 2000).

Autodock a molecular modeling was effective for protein – ligand docking. In the present study docking simulation was performed between the ligand 2,4-Di-tert-butyl phenol and standard drug Salirasib with the protein 5p21. The results revealed that the ligand 2,4-Di-tert-butyl phenol has a lower binding energy (-4.67 Kcal/mol) that indicated higher affinity with the protein (Plate 2). Affinity is defined as the change in energy (in K cal/mole) of a neutral atom (in the gaseous phase) when an electron is added to the atom to from a negative ion. The electron affinity is a measure of the attraction between the incoming electron and the nucleus

i.e, the ligand and the protein respectively. The strong the attraction, the more energy is released. The negative sign for the affinity shows that energy is released.

Currently most anti-cancer drugs being developed are derived from natural sources. This eliminates the risk of side effects, to an extent, when compared to synthetic drugs. *H. salicifolia* and *H. rhamnoides* offer immense potential in the development of a new cancer drug that targets the Ras pathway. Attempts to develop drugs that target oncogenic Ras proteins have been unsuccessful (Neidle and Thurston, 2005). Hence, tumors having these mutations remain the most difficult to treat. These two plant species contain a few compounds that are capable of binding to and inhibiting the Ras protein and thereby preventing cell proliferation in an uncontrolled manner.

As determined by Autodock 4.0, zeaxanthin, translutein, quercetin 3-glucoside 7-rhamnoside and isorhamnetin 7-rhamnoside showed promising association to the binding site of the Ras protein. From the ADMET studies, found that quercetin 3-glucoside 7-rhamnoside is a better drug candidate and shows the least adverse effects. Hence, quercetin 3-glucoside 7-rhamnoside could prove to be a probable anti-cancer drug. However, this molecular docking study is only one way of predicting the activity of the molecules involved. Therefore, *in vitro* and *in vivo* studies need to be performed on animal models to confirm the anti-cancerous activity of the compounds. The role of some important amino acids involved in the appropriate binding of inhibitors with the active site of H-Ras can be helpful for designing better drugs to combat cancer.

Flora *et al.* (2018) reported *in silico* molecular docking studies of compound present in propolis methanolic extract against large envelop protein. *In silico* molecular docking study revealed that the intraction of Desmethyldeprenyl with large envelop protein showed higher docking score (-1.83 K cal/Mol) than the 1,2 - Benzenedicarboxylic acid with the docking score (-1.63 K cal/Mol). Similar type of studies were performed with fucoidan compound against $HepG_2$ cell line protein by Ashok and Sivakumari (2015), Kappa - Carragenan present in *Kappaphycus alvarezi* against InhA enzyme by Mayakrihanan *et al.* (2015), quercetin compound against HaLa cell line proteins by Muthukala *et al.* (2015), resveratrol compound against Kβ cell line proteins by Manimaran *et al.* (2015), stearic acid against transferring and plasminogen protein present on $HepG_2$ cell present in *Cardiospermum halcacabum* by Rajesh *et al.* (2016).

In the present study, the result clearly indicates that there is binding site between the protein and ligands. This is the first report of molecular docking studies of compound present in methanolic extract of *Filifusus filamentosus* against 5p21 protein has not carried out and in future these compounds can be used as a potential and natural therapeutic agents to treat breast cancer. The present study corroborate well with the above findings.

Cancer is the third leading cause of death worldwide, preceded by cardiovascular and infectious diseases. There are standard chemotherapeutic drugs for treatment of cancers. Several studies have shown that chemotherapeutic drugs have harmful effects on health and can lead to the development of drug resistance in tumor cells, which limit the clinical success of cancer chemotherapy (Von *et al.*, 1979; Raghavan *et al.*, 1997). Recent reports show that chemotherapeutic drugs and

natural compounds with known anticancer activity could be used in combination therapy to reduce the systemic toxicity of chemotherapeutic agents (Hortobagyi, 2001; Tyagi *et al.,* 2002). So called natural compounds with anticancer activity often modify many intracellular signaling pathways simultaneously.

Cancer is a multi-factorial disease that commonly presents numerous complications and requires a holistic approach to treatment, control and prevention. Cancer develops via a multistep carcinogenesis progression involving a number of cellular physiological systems, such as cell signaling and apoptosis, making it a very complex disease (Reichert and Wenger, 2008). According to global cancer statistics from 2011, cancer rates are increasing at an alarming rate (Jemal *et al.,* 2011).

Sulfated ink polysaccharide isolated from cuttle fish *Sepiella maindroni* are known for their inhibition potential of MMPs (Wenjie *et al.,* 2017) and especially the metastasis in cancer is strong inhibition by the O-sulfated polysaccharide (Borgenstrom *et al.,* 2007). But in contrast Priya *et al.* (2006) reported that an uronic acid rich peptideoglycan isolated from the ink of the cuttlefish *Sepia pharaonis* showed cytotoxity against human cervical cancer Hela cells (CC50 = 135 g/ml). Guo-Fang Ding *et al.* (2011) investigated the anticancer activity of peptides isolated from hydrolysates of Sepia ink. A previous study reported that crude extract of *Meretrix meretrix* and *Meretrix casta* exhibited potential anticancer activity against hepatoma cell line HepG2 (Sugesh *et al.,* 2014)

Sreejamole and Radhakrishnan (2013) reported significant cytotoxicity in green mussel *P. viridis* against two human cancerous cell lines MCF-7 and HCT-116. Similar finding was observed by Priya Senan *et al.* (2013) in ink extracts

of cuttle fish and squid. Ramesh *et al.* (2014) demonstrated that venom extract of *C. amadis* significantly suppress the proliferation of liver cancer cells (Hep G_2) *in vivo.* The present study also demonstrated the antiproliferative effect of bioactive compounds of *F. filamentosus* as anticancer agents. In the present study, the cell viability was found to be increased with increase in concentration. The results of the anticancer activity of *F. filamentosus* showed good anticancer property with IC_{50} of 231.50 µg/ml respectively (Figure 17). The present study ratify well with the above findings. Further studies are required to fully evaluate the underlying mechanisms of action of the compounds, prior to further development as potent anticancer agents.

Natural bioactive substances can modify redox status and interfere with basic cellular functions (cell cycle, apoptosis, inflammation, angiogenesis, invasion and metastasis) (Kampa *et al.,* 2007). Apoptosis is important in embryological development, cell proliferation, cell differentiation, elimination of seriously damaged cells or tumor cells by chemopreventive or chemotherapeutic agents and many other physiological processes (Galati *et al.*, 2000). Apoptotic cells and bodies are rapidly recognized by macrophages before cell lysis, and then can be removed without inducing inflammation. Therefore, the induction of apoptosis is an important mechanism of chemoprevention and chemotherapy of cancer.

To determine whether the inhibition of cell proliferation by methanolic extracts of *F. filamentosus* was due to the induction of apoptosis, we assessed the latter with the acridine orange and ethidium bromide method. Proapoptotic activity of methanolic extracts of *F. filamentosus* was investigated with respect to the morphological shape of cells by fluorescence microscopy. Fluorescence microscopy images clearly showed morphological changes such as condensed nuclei, membrane

blebbing, chromatin condensation, nuclear fragmentation and formation of apoptotic bodies of treated cells (Plate 5).

Apoptosis maintains the balance between cell death and renewal thereby it eliminates excess, damaged and abnormal cells from the body. Therefore, activation of apoptosis in cancer cells could be an effective strategy for cancer treatment. Various studies have reported that many plant extracts and plant-derived compounds induce cancer cell death through apoptosis without a significant amount of side effects (Desai *et al.,* 2008; Li *et al.,* 2009). The presence of apoptotic U937 cells under these conditions was confirmed by two morphologic criteria: green or yellow to red or orange shifts using acridine orange and ethidium bromide staining techniques and cell surface annexin V binding indicative of PS appearance on the external plasma membrane.

Apoptotic is a form of cell death that allows for the elimination of damaged or unwanted cells without damaging the organism. The most obvious characteristics of this form of cell death are cytoplasmic and nuclear condensation, followed by internucleosomal DNA cleavage, membrane blebbling and finally cell fragmentation (Fritzer-Szekeres *et al.,* 2002).

Nuclear changes such as chromatin condensation around the nuclear membrane was noticed by acridine orange and ethidium bromide staining (Plate 5). Acridine orange stain is membrane permeable and marks the nuclei green and EtBr, which binds to DNA, is mainly taken up by cells when membrane integrity is lost and stains the nuclei red. Since AO intercalates in the DNA but only interacts with

the RNA, viable cells do not uptake EtBr and these cells exhibit green nuclei. However, EtBr is taken up by dying cells, which turn red (Giral *et al.,* 2007).

Treatment with a combination of AO and EtBr has been used as a reliable index of cellular degeneration (Campos-da-Paz *et al.,* 2008). Sanguinarine, a benzophenanthridine alkaloid derived from the root of *Sanguinaria canadensis*, induced apoptosis in human cancer cells, which was assessed by AO and EtBr staining (Han *et al.,* 2008).

The acridine orange and ethidium bromide staining showed that the methanolic extract of *F. filamentosus* induced apoptosis in MCF-7 cancer cells (Figure 18). Kabeer *et al.* (2012) stated that acridine orange and ethidium bromide staining showed isodeoxyelephantopin could inhibit the proliferation of breast cancer cells and lung cancer cells and induce apoptosis in treated cells. Similarly AO/EB assay and FACS analysis clearly demonstrated that 5-Fluorouracil (5-FU) nanoparticles induce apoptosis in glioma (U87MG) and breast cancer (MCF-7) cells compared with free drug (Nirmaladevi *et al.,* 2012).

Zerumbone treated PANC-1 cells exhibited obvious apoptotic morphological changes in the nuclear chromatin, such as cell shrinkage, chromatin condensation and cell nuclear fragmentation (Zhang *et al.,* 2012). The methanolic extract treated *F. filamentosus* exhibited morphological changes typical of apoptosis, including cell shrinkage, plasma membrane blebbing, chromatin condensation and nuclear fragmentation compared with the control cells with prominent rounded nuclei and defined plasma membrane contours.

DAPI staining is used to observed the nuclear contents in shrunken cells. DAPI has been used to study pamidronate, anti-proliferative, apoptotic and anti-migratory effects in hepatocellular carcinoma cells (Wada *et al.,* 2006). The cell with altered nuclear changes were found to be greatest at 12.5 µg/ml concentration of methanolic extract of *F. filamentosus.*

The extract of *Zea mays* leaves were also highly efficient in induced apoptosis in cancer cells (Kimthika and Padma, 2013). Human breast cancer cells treated with the extract of *Astrodaucus persicus* also showed potential decreased in the cell proliferation by staining with DAPI (Abdolmohammadi *et al.,* 2008). In the present study results are in agreement with these supporting literatures that the methanolic extract of *F. filamentosus* induce apoptotic cell death.

Chemicals with the potential to damage DNA are increasingly present in the terrestrial and aquatic environment. Recently compelling evidence has accumulated that supports the notion that apoptosis induced by DNA damage might have an important role in regulation of the population dynamics (Sokolova *et al.,* 2004; Micic *et al.,* 2001). The possible role of apoptosis in manipulating the population was demonstrated in a series of studies on the intertidal mud snail, *Ilyanassa obsoleta*, where the imposex condition was linked to pollution by the chemical TBT. Imposex is the occurrence of pollutant-induced male sex characteristics (for example the penis and the vas deferens) superimposed on normal female snails. Gastropods bioaccumulate TBT and its endocrine disruptive effects then result in elevated testosterone levels giving rise to imposex. The pathological condition induced by TBT affected females of more than 200 species of marine gastropods. In organotin-polluted areas marine mollucks have shown reproductive failure due to oviduct

blockage by vas deferens formation resulting in population decline or mass extinction (Oehlmann *et al.*, 1991; Nishikawa, 2006).

The morphological feature of cells demonstrated that suppression of cell growth may be caused by apoptosis rather than the inhibition of cell proliferation. The extrinsic apoptosis has been induced by specific trans-membrane receptors such as FAS/CD95 ligand, TNF α and TNFSF10 (Abu *et al.,* 2013). The intrinsic apoptosis could be caused by intracellular DNA damage, oxidative stress, cytosolic Ca2+ overload and accumulation of unfolded proteins in the endoplasmic reticulum. The process was consequently initiated by cascade signaling of caspases and leads to apoptosis (Weerapreeyaku *et al.*, 2016). Sugesh *et al.* (2014) revealed that mollusc extract exerted anticancer activity through lactate dehydrogenase (LDH) leakage, depletion of glutathione (GSH), DNA damage and increased the expression of apoptosis stimulating factor such as caspases.

Kjell and *et al.* (2016) showed DNA fragmentation in *Mylilus edulis* and Jacky Bhagat *et al.* (2016) reported DNA damage and oxidative stress in *Morula granulate.* In the present study DNA showed a clear ladder pattern at two different concentration of 6.25 and 12.5 µg/ml respectively. The present study corroborate well with the above finding.

So the current regimen of chemotherapy has two major disadvantages, one is, it utilizes drugs which inhibit DNA synthesis, and another serious problem is the resistance of the drugs in use. Therefore there is an urgent requirement for development of new cancer drugs which overcome these disadvantages. Hence, nowadays research works carried out with synthetic compounds were concerned on

alternative approach other than inhibition of DNA synthesis, one of the alternative approach used nowadays involves the evaluation of compounds involved in the inhibition of signal transduction, by substituting one or more substituents to the parent nucleus.

Marine organism have many compound with various bioactive, including antioxidant, anti-inflammatory and anticancer activities. Therefore money molluscs have been examined to indentify new and effective anticancer compounds, as well as to elucidate the mechanisms of cancer prevention and apoptosis. The aim of anticancer agents is to trigger the apoptosis signaling system in the cancer cells while disturbing their proliferation. So the present findings suggest that the methanolic extract of *F. filamentosus* most likely have anticancer properties. The presence of bioactive compounds in *F. filamentosus* may contribute to its medicinal properties. The analytical chemistry and molecular target identification methods are needed to transform the bioactive compounds into clinically valuable drugs or drug scaffolds.

8. SUMMARY

The present study was carried out in the marine gastropod *Filifusus filamentosus* collected from Thoothukudi coastal region. In the present analysis GC-MS, AutoDocking, Anticancer activity, apoptotic effect and DNA fragmentation assay were studied and the following results were obtained.

- GC-MS analysis from experimental organism *Filifusus filamentosus* revealed 13 compounds that could be identified as Cyclotetrasiloxane, octamethyl, Cyclododecane, 2,4-Di-tert-butyl phenol, E-14-Hexadecanol, Cholestrol, 9-Octadecenamide, (Z) - [Oleic acid amine], Undecanal, 2-methyl-, Cyclohexanol,2-amino-,trans-, Butanal, O-methyloxime, E-2-Tetradecen-1-ol, 1,1-Cyclopropane dicarbonitrile, 2,2-dimethyl-, Cyclopentanol, 2-(aminomethyl)-, cis, Cholan-24-oic acid, 3-oxo-, methyl ester, (5á).

- The mass spectrum and structure of the compounds identified were present in the crude methanolic extract of *Filifusus filamentosus* which could be responsible for anticancer, antioxidant, antimicrobial, analgesic, anti-inflammatory, antipyretic, hepatoprotective and diuretic, antiviral activities etc.

- In the present study, AutoDock software were used to perform the docking simulation between the ligand 2,4-Di-tert-butyl phenol and standard drug salirasib with the protein 5p21.

- The ligand 2,4-Di-tert-butyl phenol has a lower binding energy (-4.67 kcal/mol) that indicated higher affinity with the protein. Also ligand has highest root mean square deviation (RMSD) score (40.37) and lowest inhibition constant (375.78 µm) when compared to the standard drug.

- The anticancer activity revealed that *F. filamentosus* showed good anticancer potential which has the IC_{50} of 231.50 µg/ml against MCF-7 breast cancer cells.

- The methanolic extract of *F. filamentosus* showed 72.92% viability at 100 µg/ml followed by 78.03%, 88.55% and 92.20% at 200, 300 and 400 µg/ml concentrations respectively.

- Apoptotic cells appeared as green-orange colour nuclei with condensed or fragmented chromatin at the concentration of 6.25 µg/ml respectively. Apoptotic cells appeared as orange to red colour with highly condensed or fragmented chromatin and apoptotic bodies at the concentration of 12.5 µg/ml respectively.

- DAPI staining revealed the changes associated with apoptosis in MCF-7 cells treated with the methanolic extract of *F. filamentosus*

- The methanolic extract of *F. filamentosus* treated MCF-7 cells appeared as bright blue with apoptotic nuclear morphological changes. The morphological changes include chromatin condensation, nuclear fragmentation and marginalization, DNA condensation and

fragmentation and formation of apoptotic bodies in MCF-7 treated cells at the concentration of 6.25 µg/ml and 12.5 µg/ml respectively.

- A clear fragmented DNA ladders were observed in MCF-7 breast cancer cell lines treated with methanolic extract of *F. filamentosus* and control don't show any DNA fragmentation.

9. CONCLUSION AND SUGGESTION

The marine environment is a huge source for discovering many novel drugs. Apart from the food that is derived from the marine environment, wide varieties of drugs are being isolated and characterized with great promise for the treatment of human diseases. Studies on biomedical screening give new insights into the extraction of bioactive compounds from marine molluscs. The presence of various bioactive compounds justifies the use of *Filifusus filamentosus* for various ailment.

In the present study, autodocking was carried out with AutoDock 4.2. This study showed that this gastropod contain a few compounds that are capable of binding to and inhibit the 5p21 protein and thereby preventing cell proliferation in an uncontrolled manner. As determined by AutoDock 4.2, 2-4-Di-tert-butyl-phenol show promising association to the binding site of the 5p21 protein. The cytotoxic effect of *Filifusus filamentosus* was studied against MCF-7 cell line. The search for chemopreventive compounds in marine organism has been extensively reported, however the presence of these compounds in *F. filamentosus* has been incipiently un explored.

In this present study apoptotic potential of methanolic extract of *F. filamentosus* was investigated. The results from this research suggested that methanolic extract of *F. filamentosus* contained compounds with chemopreventive and anticancer properties; however, further research is required for their full characterization. Likewise, its moderate cytotoxic effect is due to its ability to induce apoptosis in MCF-7 cells. The mode of cell death of cancer cells by

F. filamentosus predominantly followed apoptosis. Thus, the results of this study have led to a new source of mollusc exerting potent apoptotic effect.

The present study also suggests the possibility of a life saving drug combating different types of cancers especially breast cancer. Regarding the role and regulatory mechanisms of apoptosis in molluscs, it was believed that molluscan models will faster the emergence of new concepts in the future and contribute to the unravelling of the molecular organization of living cells in general. Further studies are required to assess molecular mechanism and other pathway for apoptosis. These results provide a strong scientific foundation for the development of novel anticancer agents from the bioactive ingredients in the most effective fraction.

That it can be concluded from the above study that *F. filamentosus* extract act as therapeutic agent which is of great interest in pharmaceutical industry. This exploration creates the new standard where the extract can be a powerful weapon against cancer. This encouraging results provides useful information for designing a much better anticancer compound using *F. filamentosus* extract with minimal side effects. Hence *F. filamentosus* could prove to be a probable anticancer drug.

10. BIBLIOGRAPHY

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SURVEY OF BY- CATCH OF CRAB FISHERY FROM THOOTHUKUDI COAST

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ABSTRACT

Trawling though one of the most efficient methods of fish capture is also found to be the most important human caused physical disturbance on the world's continental shelves and hence the physical destruction of ecosystems. The main objective of the present work is to know the species composition of by-catch from crab fishery landed out Therespuram landing centre, Thoothukudi coast. Crab fishery in Thoothukudi coast is constituted by five commercially important species namely *Scylla serrate, Portunus pelagicus, Portunus sanguinolentus, Charybdis natator* and *Charybdis feriatus.* The edible species of organisms recorded from the discard during the study period were gastropod mollusk *Turbinella pyrum* and the finfishes *Lutjanus eherenbergii* and *Nibea Maculata.* Out of the 45 species collected 11 species were of commercially important molluscs. In the present study about 15 species of molluscs were identified from the by-catch. In the present study 43 species of organisms were identified with unidentified corals and sponges. In the present study, the organisms recorded from by-catch are 10 species of gastropod molluscs, 5 species of bivalve molluscs , 3 species of star fishes, 6 species of inedible crabs, 5 species of finfishes, corals, ascidians, sea cucumber, sea urchins and sea horse.

KEYWORDS: By-catch, bottom set gill net, crab net, star fish, finfishes, molluscs.

ANTIMITOTIC ACTIVITY OF *FUSINUS NICOBARICUS* EXTRACT ON *ALLIUM CEPA* ROOT TIP

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ABSTRACT

Molluscs are a rich source for discovering novel compounds for the possible development of new types of antibiotics for pharmaceutical use. Cancer is a class of disease characterized by out-of-controlled cell growth. The studies on antimitotic property with the help of onion root tips also pointed out the evidence of the presence of antitumour agents in molluscs. Methylene chloride extract of *Fusinus nicobaricus* showed maximum inhibition with 36.99% followed by hexane extract with 20.78%, methanol extract with 17.62% and benzene extract with 10.31%. It is evident that all extracts reduced the mitotic index significantly. The reduction in number of dividing cells in the root meristem showed the antimitotic effects of the substances that found in gastropod *Fusinus nicobaricus* extracts. *Fusinus nicobaricus* contains antimitotic constituents that can stop the mitosis in anywhere of the cell cycle. They also affect the cytoskeleton or tubulin polymerization or degradation. A new generation of antimitotic drugs is being developed to understand how cancer cells respond to them. So, the present study has been carried out with a view to investigate antimitotic activity of *Fusinus nicobaricus*.

KEYWORDS: *Fusinus nicobaricus*, Antimitotic activity, *Allium cepa*, Methylene chloride, Hexane

EVALUATION OF BIOACTIVE PEPTIDES AND BIOMEDICAL POTENTIAL OF *LAMBIS LAMBIS* (LINNAEUS, 1758)

A dissertation

submitted to

ST. MARY'S COLLEGE (Autonomous), THOOTHUKUDI

affiliated to

MANONMANIAM SUNDARANAR UNIVERSITY, TIRUNELVELI

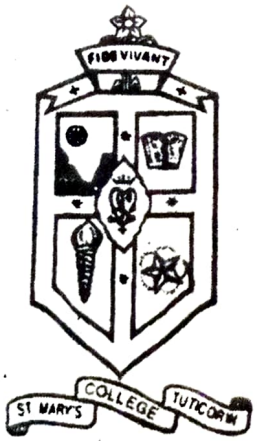
in partial fulfillment for the award of the degree of

MASTER OF PHILOSOPHY IN ZOOLOGY

by

JESIMA.K

Reg. No. 18MLZO02



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JUNE - 2019

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BONAFIDE CERTIFICATE

Certified that this dissertation entitled **"EVALUATION OF BIOACTIVE PEPTIDES AND BIOMEDICAL POTENTIAL OF *LAMBIS LAMBIS* (LINNAEUS, 1758)"** is the bonafide work of **JESIMA.K Reg.No. 18MLZO02** who carried out the work under my supervision. Certified further that to the best of my knowledge the work reported here in does not form part of any other thesis or dissertation on the basis of which a degree or award was conferred on an earlier occasion on this or any other candidate.

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DECLARATION

I do hereby declare that the dissertation entitled, **"EVALUATION OF BIOACTIVE PEPTIDES AND BIOMEDICAL POTENTIAL OF *LAMBIS LAMBIS* (LINNAEUS, 1758)"** submitted by me in partial fulfillment for the award of the degree of Master of Philosophy in Zoology, is the result of my original and independent work carried out under the guidanc of **Dr.P.Subavathy M.Sc.,M.Phil.,SET.,Ph.D.,** Assistant Professor of the PG and Research Department of Zoology, St. Mary's College (Autonomous), Thoothukudi and it has not been submitted elsewhere for the award of any other degree.

STATION: THOOTHUKUDI

DATE: 26.06.2019

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ACKNOWLEDGEMENT

First and foremost, I express my sincere thanks to God Almighty, for blessing me with good health and soaring spirits in fulfilling the task of writing this dissertation.

I express my deep sense of gratitude to **Rev.Dr.Sr. A.S.J. Lucia Rose M.Sc., PGDCA., M.Phil., Ph.D.,** Principal, St. Mary's College (Autonomous), Thoothukudi for her constant support and encouragement which enabled me to complete the work successfully.

With profound sense of gratitude, I owe my indebtedness to my guide **Dr.P.Subavathy M.Sc., M.Phil., SET., Ph.D.,** Assistant Professor, PG and Research Department of Zoology, St. Mary's College (Autonomous), Thoothukudi for her efficient and able guidance for the completion of my work and I also record my sincere thanks for her constant encouragement throughout the period of my study.

My sincere thanks are due to **Dr.HerminPasangha M.Sc., B.Ed., Ph.D.,** Head and Associate Professor, PG and Research Department of Zoology, St.Mary's College (Autonomous), Thoothukudi for providing me all facilities during my study period.

It is a great pleasure to thank **Dr.R.D.Thilaga M.Sc., M.Phil., M.Ed., Ph.D.,** former Head, PG and Research Department of Zoology, St.Mary's College (Autonomous), Thoothukudi for her motivation, encouragement and for providing all facilities to carry out my work.

I thank Dr. SavithiriShivakumar, Aaranya Biosciences, Chennai for her timely help in getting the laboratory reports.

I am grateful to Mrs. JebaNesam for her constant support, encouragement and timely help throughout the period of my work.

My special thanks are due to the faculty members of PG and Research Department of Zoology, St.Mary's College (Autonomous), Thoothukudi for their help and encouragement.

I am indeed grateful to the Laboratory Assistants, St. Mary's College (Autonomous), Thoothukudi for their timely help and support.

I am extremely indebted to all my family members for their support, help, encouragement and prayers throughout my study period.

CONTENTS

LIST OF TABLES

LIST OF PLATES

LIST OF FIGURE

1. INTRODUCTION

The ocean turned out to be an attractive field. Since then, giant efforts have been accomplished worldwide aiming the isolation of new metabolites from marine organisms. The search for new biomedicals from marine organisms resulted in the isolation of more or less 10,000 metabolites (Fusetani, 2000), many of which endowed of pharmacodynamics properties. A broad spectrum of biological activities such as antibiotic, antifungal, cytotoxic, neurotoxic, antimitotic, antiviral, antineoplastic and cardiovascular activities has been detected.

Within, marine environment invertebrates represent a great majority of all macroscopic life and inhabit all ecosystem, from estuaries to the deep sea (Ludovic Donaghy *et al.,* 2015). As many as 34 phyla, of the total 36 known animal phyla were reported from marine biosphere against 17 phyla from land (Mohapatra *et al.,* 2013). Marine organisms are used as nutritious foods, animal feed, ornamental and recreational items and also as potential source of marine natural products in health care since ancient times. Molluscs which are widely distributed throughout the world, have many representatives in the marine and estuarine ecosystem.

Phylum Mollusca is the second largest phylum of all invertebrates. Gastropods, are a large taxonomic class within the phylum Mollusca, commonly known as snails and slugs. The classgastropoda has an extraordinary diversification of habitats. They have greatest numbers of named molluscs species (Kohan *et al.,* 2012). Gastropod have a worldwide distribution from the near arctic and antarctic zones to the tropics (Lindberg *et al.,* 2004). The molluscs have received a considerable amount of research effort, reflecting both their ecological and economic importance and now gaining importance in deriving drugs (Huges, 1986).

Synthesis of secondary metabolites is their unique ability that helps them to protect against the ill effects of environmental hazards and microbial infections and many of these are found as promising source to meet the human health care demand (Sokolova, 2009). Marine organisms contain much undiscovered bioactive compounds, the number of new active compounds isolated from marine organisms are estimated to be 10,000 (Kelecom, 2002). Molluscs are considered as one of the important sources to derive bioactive compounds that exhibit antitumor, antimicrobial, anti-inflammatory and antioxidant activities (Anbuselvi*et al.,* 2009; Chellaram and Edward, 2009b and Benkendroff*et al.,* 2011). Molluscs also contain rich nutrients that are beneficial to people of all ages (Anand *et al.,* 2010). Some marine molluscs have shown pronounced activities, useful in the biomedical arena. The potential of marine molluscs as a source of biologically active products is largely unexplored in India (Anbuselvi*et al.,* 2009).

Marine molluscs have become the focus of many chemical studies aimed at isolating and identifying novel natural products. The secondary metabolites isolated from molluscs fall into a wide range of structural classes, with some compounds predominating in certain taxa. In the gastropoda, terpenes dominate, whereas fatty acid derivatives are relatively uncommon. Molluscs used directly as a food source may also contribute to the prevention of disease by providing essential nutrients, as well as immune – stimulatory compounds and other secondary metabolites with direct biological activities (Benkendroff, 2010).

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 (Riguera, 1997). Usually for bioprospecting, 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 (Ebada*et al.,* 2008).

The molecular diversity of chemical compound found in marine 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 peptides with diverse biological activities (Evans- Illidge*et al.,* 2013). Secondary metabolites, also known as natural products, are compounds that are not directly required for an organism's primary metabolism. These secondary metabolites are usually unique to specific organisms and are not present during all environmental conditions. Although most metabolites can be categorized as primary or secondary there is some overlap between the two. Some are essential for primary metabolism but are only synthesized by specific species and are therefore called as secondary metabolites.

A preliminary thin layer chromatograpphy was used to determine the qualitative status of free amino acids (Preet and Gupta, 2009). The protein contained nutritionallly useful quantities of most of the essential amino acids, including sulphur containing amino acids (Adeyeye and Afolabi, 2004). SDS- PAGE is the most widely used method for analyzing protein mixture qualitatively. It is particularly useful for monitoring protein purification and this method is based on separation of proteins according to their size, it can also be used to determine the relative molecular mass of proteins (Wilson and Walker, 2011).

Screening of organic extracts from marine organisms is a common approach to identify compounds of biomedical importance. Diabetes mellitus (DM) is a group of metabolic diseases characterized by hyperglycemia resulting from defects of insulin (American Diabetes Association, 1998) . Diabetes mellitus is ranked seventh among the leading causes of death and is considered third when its fatal complications are taken into account. In people with diabetes, either not enough insulin is produced or it is not working properly and therefore, glucose builds up in the blood (Trivedi *et al.,* 2004). Statistical projections about India suggest that Indians are genetically more susceptible to diabetes, and the World Health Organization predicts the number of diabetic person in India would go upto 40 million by 2010 and to 74 million by 2025 (Pillai, 2006).

The deficiency of insulin leads to diabetes mellitus (DM). The World Health Organization reported that 5.0 % of global population has been affected by diabetes mellitus and at present, these is no drug to give permanent remedy to diabetes mellitus (Geethalakshmi*et al.,* 2010). Alpha amylase and glucosidase are the key hydrolysing enzymes involved in the digestion of carbohydrates (Ali *et al.,* 2006). Inhibition of these enzymes resulted in decreasing the rate of digestion of carbohydrate, prolonged the time of the carbohydrate digestion and thereby reduced the amount of glucose level in blood. The antidiabetic agent which was isolated from molluscs and studied in rat models showed significant hypoglycemic effect (Tiwari *et al.,* 2008).

Diabetes treatment comprises one or more of the following modalities viz., medical nutrition therapy, exercise, insulin and non - insulin agents, including oral medications and the non-insulin injectable drug exenatide (Silverman, 1986). The modern oral hypoglycemic agents produce undesirable side effects, and so the

management of diabetes is a global problem until now, and successful treatment is not yet discovered. Thus, alternative therapy is required (Li *et al.,* 2004). In contrast, marine bacteria and fungi are poorly investigated for antidiabetic activity but may be of great promise in the search for new antidiabetic drugs for the future (Seo*et al.,* 2009). Almost 80% of diabetes deaths occur in low and middle - income countries (Shrivastuva*et al.,* 2013). Many studies revealed that molluscs has antitumour, antileukemic and antiviral activites (Anand *et al.,* 2002). This rich diversity to marine organisms assumes a greater opportunity for the discovery of new bioactive compounds.

Diabetes is one of the major causes of premature death worldwide. Diabetes affects mainly the developing countries like India. Indeed, India presently has the largest number of diabetic patient in the world and has been infamously dubbed as the 'diabetic capital of the world' (Abate and Chandalia, 2007). Diabetes mellitus is epidemic in India as a result of societal influence and changing lifestyles. Diabetes has been known in India for centuries as 'a disease of richman' but now spread among all masses (Gupta and Misra, 2007). Inhibition of amylase and glucosidase enzymes can be an important strategy in management of post prandial blood glucose level in type 2 diabetes patient (Ali *et al.,* 2006).

Diabetes mellitus leads to a series of chronic diabetic complications such as heart disease, kidney failure, retinopathy, neuropathy and stroke (Davoudi and Sobrin, 2015). One of the main causes for these complications was persistent hyperglycemia. In the diabetic patient, sustained hyperglycemia results in protein glycosylation and glucose antioxidation, which could cause excessive production of reactive oxygen species (ROS) (Dakhale*et al.,* 2011). Some literatures also reported that the pancreatic

beta - cell mass was reduced by oxidative stress in type 1 and type 2 diabetes (Rosenberg, 1995 and Donath *et al.,* 2011).

ROS could activate nuclear factor – KB (NF-KB) signaling pathway associated with inflammatory response. Inflammation plays a very important role in diabetes, insulin resistance and complication (Gratas – Delamarche*et al.,* 2014 and Mahmoud *et al.,* 2013). Therefore, the drugs which have antihyperglycemic and antioxidant activities may be useful for the treatment of diabetes mellitus. A number of natural products were reported to have antidiabetic effects and some have been used in clinical trials for a long time (Hung *et al.,* 2012). Although different classes of drugs are available to control type 2 diabetes, still it is a challenging task to bring a better molecule which is devoid of undesirable adverse effects than existing drugs (Subramanian Ramachandran *et al.,* 2013).

Reactive oxygen species (ROS) are small molecules considered in vertebrates as they are involved in internal defense through the elimination of internalized non – self particles such as pathogens (Lam *et al.,* 2010). ROS can however, interact with host cells proteins, lipids, carbohydrates and nucleic acid, irreversibly altering the spatial conformation and function of the impacted molecule (Nahorevanian, 2009). Consequently, the concept of ROS as agents of cellular damage has been widely accepted for a long time.

Indeed, a plethora of biological activities have been ascertained by the use of marine collagen-derived peptides such as antimicrobial, antihypertensive, antidiabetic, opioid, calciotropic, secretagogue, joint and bone-regenerative, antioxidant, wound-healing, UV-protective and antityrosinase activities, both *in vitro* and *in vivo*, alternatively administered orally or systemically or even in topical concoctions

(Venkatesan and Goh *et al.,* 2017). In the recent years, particular attention has been given to the antioxidant properties of these peptides, since excess of intercellular reactive oxygen species (ROS) has been linked to the development and chronicization of many pathological conditions sush as cardiovascular, neurodegenerative, inflammatory, cancer and age-related illnesses (Correa *et al.,* 2005). Thus, the search for new molecules with antioxidant activity, especially from natural sources, is continuously pursued as potential drugs for many pathological conditions.

Reactions of free radicals such as superoxide radical, hydroxyl radical, peroxyl radical and other reactive oxygen and nitrogen are associated with diseases such as atherosclerosis, dementia and cancer. Antioxidants delay or prevent oxidative damage and thus they may be useful as therapeutics or food additives. In our body, oxidation process leads to cell damage, cancer and degenerative diseases, antioxidant molecules present in different molluscs prevent cell damage from oxidation reaction (Nagash*et al.,* 2010). Recently, the search for natural antioxidants is growing due to health hazards from synthetic antioxidants.

Oxidative damage to DNA can result in a variety of lesions, including base damage, inter and intrastrand crosslinks, DNA-protein crosslinks, single-strand breaks and doublestrand breaks (Gracy*et al.,* 1999 and Dahm - Daphi*et al.,* 2000). Fixation of such damage has been implemented in numerous diseases such as diabetes, heart failure, neurodegeneration, aging and cancer (Flora, 2007). Likewise, scavenging of ROS has become an important target for radioprotectors (Grdina*et al.,* 2005) and anticancer. Many methods have been reported for quantifying intracellular ROS generated either endogenously or from radiation exposure. A plethora of fluorescent probes have been described, each characteristically able to efficiently detect specific radical species (Gomes *et al.,* 2005).

2`7`- Dichlorofluorescin diacetate (DCFH-DA) has been used previously to measure radiation - induced ROS. DCFH-DA is permeable to the cellular membrane and once inside the cell is rapidly hydrolyzed by cellular esterases to non-fluorescent DCFH (LeBel, 1992). Oxidation of DCFH by hydrogen peroxide or other ROS produces the fluorescent indicator DCF (Gomes *et al.,* 2005). Measurement methods using DCFH-DA to quantify radiation-induced ROS have been reported using fluorescence microscopy (Yang *et al.,* 2005), video microscopy (Leach *et al.,* 2005), flow cytometry (Takada *et al.,* 2005) and microtiter plate analysis (Wan *et al.,* 2003).

Antioxidant from natural sources play a paramount role in helping endogenous antioxidants to neutralize oxidative stress (Sasikumar *et al.,* 2009). The generation of reactive oxygen species (ROS) is an unavoidable consequence of life in an aerobic environment. In which, the production of ROS is essential to many organisms for the production of energy to fuel biological process (Yong-Xu *et al.,* 2010). On the other hand, ROS such as hydroxyl, superoxide and peroxyl radicals are formed in human cells by endogenous factors and exogenously result in extensive oxidative damage (Aruoma*et al.,* 1999). This uncontrolled generation of free radicals is associated with lipid and protein peroxidation, resulting in cell structural damage, tissue injury or gene mutation and ultimately lead to the development of various health disorders such as Alzheimer's disease, cancer, atherosclerosis, diabetes mellitus, hypertension and ageing (Mantle *et al.,* 2000). These free radicals are naturally scavenged by antioxidant mechanisms in mammal.

Thus the free radicals and ROS are considered important because the human body constantly quenches excessive oxidants through various scavenging mechanisms such as use of antioxidant enzymes and molecules. These antioxidants refer to any

substance that hinders the reaction of a substance that inhibits free radical reaction (Abdel – Satter*et al.,* 2007). In certain circumstances, this *in-situ* capacity becomes inefficient, which makes mandatory dietary intake of antioxidant compounds as an alternative, suggesting that there is an inverse relationship between dietary intake of antioxidants and the incidence of disease caused by the deficiency of these substances (Sharma *et al.,* 2010). Hence, antioxidants are called as free radical scavengers.

Generally, synthetic antioxidants such as butylated hydroxyanisole (BHA), butylated hydroxytoleune (BHT), tert-butyl-hydroquinone (TBHQ) and propyl gallate (PG) have been in use to reduce the deleterious effect of oxidative-induced reaction in food and biological system (Becker and Grice, 1993). However the potential toxicity of these synthetic antioxidants has aroused an increased interest among scientists to focus on isolation and characterization of natural antioxidants from natural sources (Wang and Linn, 2000).

The antioxidant activity of putative antioxidants have been attributed to various mechanisms, among which are prevention of chain inititation, binding of transition metal-ion catalyst, decomposition of peroxides, prevention of continued hydrogen abstraction, reductive capacity and radical scavenging (Diplock, 1997 and Oktay*et al.,* 2003). 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 or protection for several human diseases.

By keeping in view the above importance of phylum mollusca the present study has been carried out to investigate the biomedical efficacies such as protein isolation through TLC, SDS – PAGE, antidiabetic, ROS and antioxidant activities of the marine gastropod *Lambislambis*.

2. REVIEW OF LITERATURE

Diversity of living marine resources provide new hope for drug discovery from natural sources. Marine organisms living continuously in the marine environment experience a variety of pressures causing organisms to produce bioactive compounds for self – defense in the environment and predation. Research on bioactive substances from marine organisms has now been growing rapidly and producing a variety of compounds for potential new drug substance. Molluscs are one of the marine organisms, with potential as natural sources produce many bioactive compounds such as antioxidant (Anand *et al.,* 2010), antidiabetic (Sadhasivam*et al.,* 2013 and Ravi *et al.,* 2012), anti-inflammatory (Ravi *et al.,* 2012) and antibacterial activities (Ravi *et al.,* 2012 and Ramasamy *et al.,* 2013).

Molluscs are the largest marine phylum comprising about 23% of all the named marine organisms. Molluscs have more varied forms than any other animal phylum. They include snails, slugs, other gastropods, clams and other bivalves. Molluscs are species that have a wide range of uses in pharmacology. They are considered as an important natural source to derive many novel biological active compounds. Many of these natural products have interesting biomedical potential, pharmaceutical relevance and diverse application.

Lijima*et al.* (2003) identified a novel antimicrobial peptide from the sea hare *Dolabellaauricularia*. Aneiors andGarateix (2004) studied bioactive peptides from marine sources. Rajeev Kumar and Xuzirong (2004) investigated the presence of bioactive compounds in marine organisms. Secondary metabolites from marine molluscs are analysed by Fontana (2006). Balamurgan *et al.* (2009) isolated and identified heparin like substances from the body tissue of *Conus musicus*. Benkendroff (2010)

isolated secondary metabolites from marine molluscs. Bioactive compounds from cone snails, terebrids and turrids were isolated by Puillandre and Holyord (2010).

Preet and Gupta (2009) studied the thin layer chromatographic analysis of free amino acids in the Haemolymph and DGG of *Indoplanorbisexustus* (Mollusca : Gastropoda) naturally injected with digenetic trematodes. Chandran *et al.* (2009) investigated antimicrobial activity from the gill extraction of *Pernaviridis* (Linnaeus, 1758). Sugesh (2010) analyzed antimicrobial activities of bivalve Mollusca *Meretrix meretrix*(Linnaeus, 1758) and *Meretrix casta* (Gemlin, 1791). Vennila*et al.* (2011) investigated antimicrobial and plasma coagulation property of some molluscan ink extracts: Gastropods and Cephalopods. The electrophoretic profile of the ink samples showed the presence of small to high molecular weight protein.

Screening on TLC, FT-IR and antimicrobial activity of marine gastropods *Babylonia zeylanica* (Brugiere, 1789) and *Harpaconoidalis* (Lamarck, 1822) from Mudasalodai, Southeast coast of India was reported by Suresh *et al.* (2012). Sarumathi*et al.*, (2012) studied the bioprospecting potential of a gastropod mollusc*Cantharus tranquebaricus* (Gemlin, 1791). Molecular weight of the crude extract of gastropod was determined by using SDS – PAGE. Periyasamy*et al.* (2012) have carried out antimicrobial activities of the tissue extracts of *Babylonaspirata* (Linnaeus, 1758) (Mollusca : Gastropoda) from Thazhanguda Southeast coast of India. Priya Senan*et al.* (2013) observed the cytotoxic effect of ink extracts of cuttlefish and squid on chick embryo fibroblasts. Sugesh and Mayavu (2013) analyzed the TLC profile and SDS – PAGE for the estimation of the molecular weight of proteins in two edible bivalves *M.meretrix* and *M.casta*.

Monolisha*et al.* (2013) determined molecular characterization of unknown protein by SDS – PAGE of *Octopus aegina*and *Octopus dolfusii* in Gulf of Mannar coast. Ramya *et al.* (2014) characterized antimicrobial compound by TLC, SDS – PAGE and FITR from the sea slug *Armina babai*. Mohanraj*et al.* (2014) screened the biomedical properties of whole body and ink fluid extracts of marine sea slug from Southeast coast of India. The antibacterial, cytotoxic, thin layer chromatography and functional characterization of the whole body and ink fluid crude extracts from *Kalinga ornata* and *Bursatellaleachii* was investigated.

Packialekshmi*et al.* (2015) screened antibacterial activity of fresh water crab and snail and isolated of antibacterial peptides from haemolymph by SDS – PAGE. Tamil muthu and Selvaraj (2015) analysed the bioactive constituents from the flesh of *Turbo brunneus* (Roding, 1798) by GC – MS. Shruti shukla (2016) evaluated therapeutic importance of peptides from marine source : a mini review. Abdolghani Ameri*et al.* (2017) have carried out antibacterial evaluation and biochemical characterization of *Thais savignyi* gastropod extracts from the Persian Gulf.

Punitha*et al.* (2006) analyzed antidiabetic activity of benzyl tetra isoquinoline alkaloid berberine in streptozoin - nicotinamide induced type -2 diabetic rats. Anbuselvi*et al.* (2009) analyzed the bioactive potential of coral associated gastropod *Trochus tentorium* of Gulf of mannar South eastern India. Sadhasivam et al. (2013) assessed *in vitro* antibacterial, alpha – amylase inhibition potential of three nudibranchs extract from South east coast of India. Suganya*et al.* (2014) analyzed *in vitro* activity of antidiabetic, antioxidant, antiinflammatory activity of *Clitoriternatea L*. Reni TriCahyani*et al.* (2015) have carried out antidiabetic potential and screened secondary metabolites from mangrove gastropod *Cerithideaobtusa*. Makkarchakraborty (2016)

analyzed antidiabetic and antiinflammatory potential of sulphated polygalactons from red seaweads*Kappaphycus, Alvareezul*and *Gracilaria opuntia.* Harikrishna Jana*et al.* (2017) compared the antimicrobial activity, antioxidant, antidiabetic and antiinflammatory property of freshwater molluscs and marine molluscs. Kajal Chakraborty and Minjujoy (2017) have carried out the antidiabetic and antiinflammatory activities of commonly available cephalopods. Fang Wang*et al.* (2018) observed antidiabetic activity and chemical composition of *Sanbai* melon seed oil.

Chen *et al.* (2007) described free radical scavenging activities of melanin from Squid ink. Gastropod have not been utilized in identyfing and extending the natural antioxidant present in them and screened for their antioxidant potential (Subhapradha*et al.,* 2013). Reactive oxygen species and free radicals are formed in the body as a consequence of normal metabolic reactions, exposure to ionizing radiation and by the influence of many xenobiotics, reactive oxygen species are an important part of the defense mechanisms against infection, but excessive generation of free oxygen radicals damage tissues (Prem Anand *et al.,* 2014).

Ludovic Donaghy *et al.,* (2012) evaluated reactive oxygen species in unstimulated hemocytes of the pacific oyster *Crassostrea gigas.*PatcharawanSittisart and BenjamartChitsomboon (2014) analysed intracellular ROS scavenging activity and down regulation of inflammatory mediators in RAW 264.7 macrophage by fresh leaf extracts of *Pseuderanthemumpalatiferum.*Ludovic Donaghy*et al.* (2014) investigated the known and unknown sources of reactive oxygen and nitrogen species in haemocytes of marine bivalve molluscs. Kamalini Ghosh*et al.* (2016) assessed withaferin A induced ROS - mediated paraptosis in human breast cancer cell lines MCF-7 and MDA-MB-231. Marina Pozzolini*et al.* (2018) studied ROS scavenging activity,

photoprotective and wound healing properties of collagen derived peptides from the marine sponge *Chondrosiareniformis*.

Nagesh *et al.* (2010) found out *in vitro* antioxidant activity of solvent extracts of molluscs (*Loligoduvauceli* and *Donaxstrateus*) from India. Nazeer and Nagash (2011) analyzed *in vitro* antioxidant activity of two molluscs, *Loligoduvauceli* and *Donax cuneatus* by solvent extraction methods. Shenai – Triodkar*et al.* (2012) compared *in vitro* study on free radical scavenging potential of selected bivalve species. Shanmugam *et al.* (2012) characterized biopolymer chitosan from the shell of Donacid clam *Donaxscortum* (Linneaus, 1758) and its antioxidant activity. Purwaningish (2012) described antioxidant activity and nutrient composition of Matah Merah snail *(Cerithideaobtusa).* Ravi *et al.* (2012) carried out isolation and biomedical screening of the tissue extract of two marine gastropod *Hemifususpugilinus* and *Natica didyma*. Fahmy and Soliman (2013) reported *in vitro* antioxidant, analgesic and cytotoxic activities of *Sepia officinalis* ink and *Coelaturaaegyptiaca* extracts. Sreejamole and Radhakrishnan (2013) studied the antioxidant and cytotoxic activities of ethyl acetate extract of the Indian green mussel *Pernaviridis*. Subhapradha*et al.* (2013) investigated antioxidant potential of crude methanolic extract from whole body tissue of *Bursa spinosa* (Schumacher, 1817).

Liu *et al.* (2013) studied isolation and structural characterization of novel antioxidant mannoglucan from a marine bubble snail, *Bullactaexarata* (Philippi). Rajalakshmi *et al.* (2013) described antioxidant activity of the Chitosan extracted from shrimp exoskeleton. Sivaperumal*et al.* (2013) showed antimicrobial peptide from crab haemolymph of *Ocypodamacrocera* (Milne Edwards, 1852) with reference to antioxidant : A case study.

Madhu *et al.* (2014) analyzed antibacterial and antioxitant activities of the tissue extract of *Pernaviridis* (Mollusca : Bivalvia) from Versova coast, Mumbai. Pawan kumar*et al.* (2014) observed the pharmacological studies on the venom of the marine snail *Conus lentiginosus* (Reeve, 1844). SadeeshkumarVelayutham*et al.* (2014) carried out *in vitro* antioxidant activity on methanolic extract of *Babylonia zeylanica* (Bruguiere, 1789) from Mudasalodai, Southeast coast of India. Ramesh *et al.* (2014) have shown the effect of drug against antioxidant and cytotoxic (HepG 2 cell line) activity *Conus amadis* venom (Gemlin, 1791). *In vitro* antioxidant activity of different gastropods, bivalves and echinoderm by solvent extraction method was carried out by Pachaiyappan*et al.* (2014).

Umayaparvathi*et al.* (2014)have carried out antioxidant activity and anticancer effect of bioactive peptides from enzymatic hydrolysate of oyster *(Saccostrea cucullata).* Chakraborty *et al.* (2014) assessed antioxidant activities and phenolic contents of three red seaweads harvested from the gulf of mannar of peninsular India. Gayatheri*et al.* (2017) extracted and identifiedbioactive compound and *in vitro* antioxidant activity potential in freshwater ampullaridae snail *Pila virens*.

From the perusual of literature it is clearly understood that there is paucity of information on *Lambislambis* along Gulf of Mannar coastal region of Thoothukudi. Hence, the present study has been carried out with a view to assess the biomedical importance of the marine gastropod *Lambislambis.*

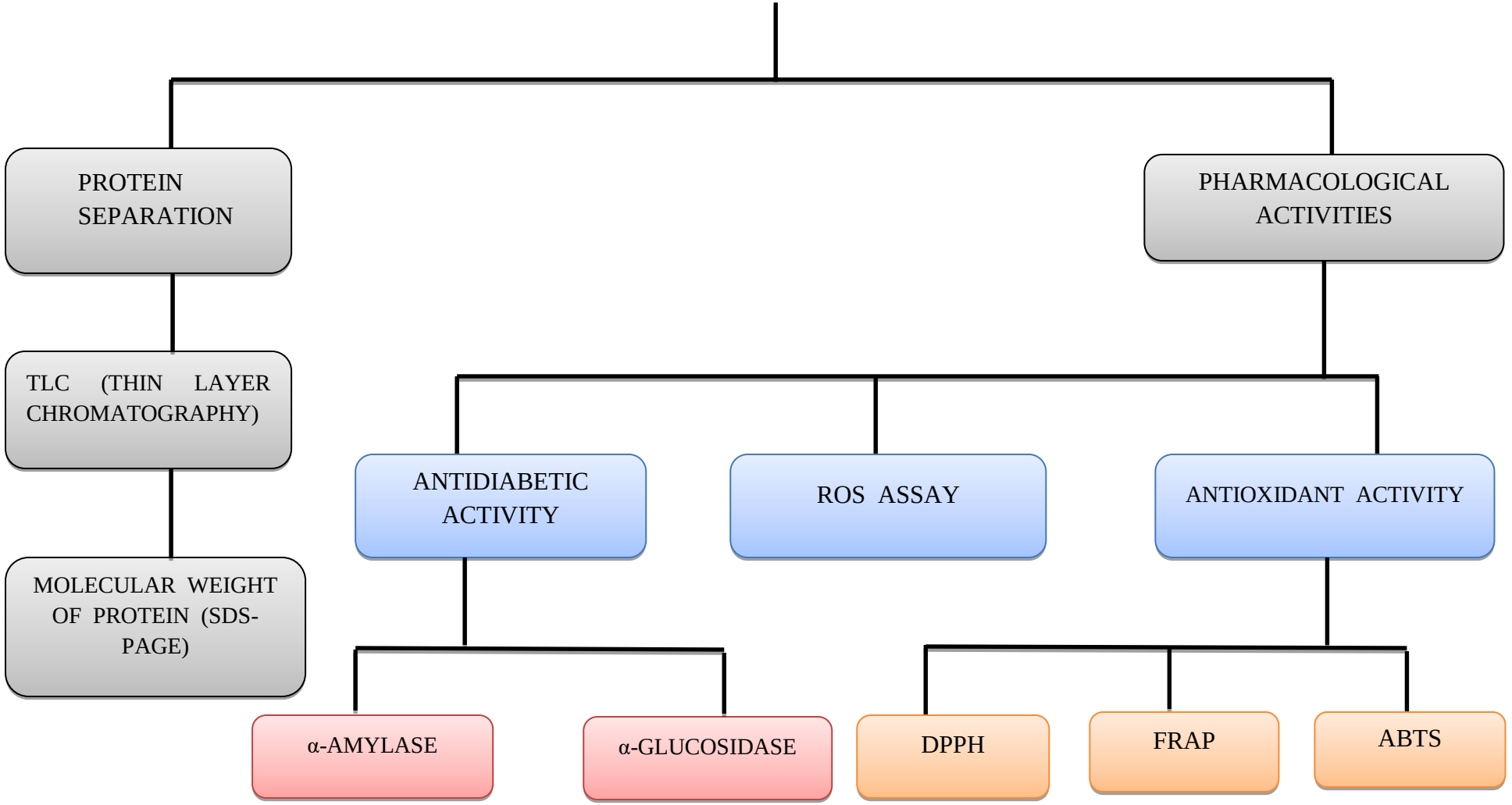
3. OBJECTIVES OF THE PRESENT STUDY

Over 7000 natural products have been isolated from marine organisms so far and this number is increasing with addition of new products every day. The recent research findings have further shown that many more species of marine organisms, particularly the molluscs stand as a prospective source of valuable bioactive compounds with great potential for new leads. It is hoped that it would provide basic insight in the yield of pharmaceutical discoveries from the molluscs from the sea in order to battle with the growing health issues in new millennium.

- To identify the proteins from experimental organism *Lambislambis*for its therapeutic importance.
- To evaluate antidiabetic effect of *Lambislambis* through α – amylase and α – glucosidase activity.
- To investigate *in vitro* ROS activity in MCF – 7 cell line.
- To assess antioxidant potential of *Lambislambis*through DPPH, FRAP and ABTS activities.

4. EXPERIMENTAL DESIGN

Collection of marine gastropod *Lambis lambis* from Thoothukudi coastal region



5. MATERIALS AND METHODS

5.1 Description of the study area

The Gulf of Mannar is located between India and Srilanka, stretches from the longitude 78°08′ to 79°30′ E and along the latitude from 8°35′ to 9°25′ N. It is a part of

the southward extension of the Bay of Bengal and meets in the Indian Ocean. This geographical area runs from Pamban Island including Rameshwaram to Cape Comorin along the Southeast coast of India to a distance of about 170 nautical miles. The Gulf of Mannar biosphere reserve has an area of about 10,500 km^2 and is considered as 'Biologist's Paradise' for, it has 3600 species of flora and fauna. This coast maintains a rich biological diversity perspective of flora and fauna largely due to diversified microhabitats such as mangroves, corals, seaweed beds, sea grasses, sandy, rocky and muddy shore etc. The faunal diversity is also well pronounced with reference to different molluscan groups (Fig.1).

5.2 Collection of experimental organisms

In the present study the mesogastropod *Lambis lambis* was collected from the Thoothukudi coastal region (Plate 1). The mesogastropod *L.lambis*was collected from the landed by-catch from fishing trawlers operated for crabs and prawns along the Therespuram coastal region. These gastropods were collected during the month of November - December 2018. The freshly collected samples were brought to the laboratory, cleaned and washed with fresh sea water to remove all impurities. The shells were broken, tissues were removed and then dried in hot air oven at 56^oC for 48 hours and used for further studies.

5.3 Description of experimental organism

5.3.1 Systematic position of *Lambis lambis* (Linnaeus, 1758)

Phylum : Mollusca

Class : Gastropoda

Subclass	**:**	**Caenogastropoda**
Order	**:**	**Mesogastropoda**
Super family	**:**	**Stromboidea**
Family	**:**	**Strombidae**
Genus	**:**	***Lambis***
Species	**:**	***lambis***

The maximum shell length for this species is upto 29 cm and average length stands for 18 cm. *Lambislambis* has a very large, robust and heavy shell. One of its most striking characteristics is its flared outer lip, ornamented by six hallow marginal digitations. These digitations present subtle differences in shape between genders in this species, as the three anterior most digitations are short and posteriorly bent in male individuals and longer and dorsally recurved in females. The color of the shell is highly variable, being white or cream externally and often presenting brown purplish or bluish black patches. The interior is glazed and may be pink, orange or purple.

5.4 Preparation of extract

One gm of the sample was dissolved in 20ml of the methanol solvent and mixed well in conical flask, after mixing the sample was incubated at 40°C, 60-70rpm in orbital shaker for 24 hrs. The extract was filtered through Whatman No. 1 filter paper and the extract residue was resuspended in 20 ml of 100% A.R grade methanol. The methanol soluble extracts were dried and solubilized in deionized water. Different concentrations of extracts were prepared and stored at 0°C for further use.

5.5 Thin layer chromatography (TLC) analysis

Thin layer plates of silica gel were used for qualitative (chromatographic) analysis of secondary metabolites (i.e for separation of proteins, peptides and amino acids). Thin plates of uniform thickness were prepared using silica gel. The plates were activated in an oven at 100^0 C for 1hr and stored in dry place and used.

The methanolic extract of gastropod were loaded on TLC plate with the help of micropipette for the separation of peptides and amino acids. The solvent employed was butanol, acetic acid and distilled water in the proportion of 5:1:4. When the TLC plate was sufficiently developed it was removed from the glass jar, air dried and sprayed with Ninhydrin reagent (0.5% solution of Ninhydrin in 100 ml 80% acetone). The pink spots were developed which indicated the presence of proteins, peptides and amino acids.

$$R_f = \frac{\text{Distance travelled by the solute}}{\text{Distance travelled by the solvent}}$$

5.6 Molecular characterization of protein (SDS - PAGE)

SDS - PAGE was performed following the method described by Laemmli (1970). SDS-PAGE is the most widely used method for analyzing protein mixture

qualitatively. Polyacrylamide gel electrophoresis in the presence of SDS separates the polypeptide chains according to their molecular weight. Thus the molecular weight of the polypeptide chains of a given protein can be determined by comparing their electrophoretic mobility on SDS gels to the mobility of marker proteins with polypeptide chains of known molecular weight.

Proteins are reacted with anionic detergent SDS to form negatively charged complexes. Initial heating of the protein sample at 95^0C in the presence of excess SDS and thiol reagent (2-β-mercaptoethanol) denatures the protein mixture and disrupts the disulphide bonds. Under these conditions, all reduced polypeptides bind the same amount of SDS on a weight basis (1.4 g of SDS per gram of polypeptide) independent of amino acid composition and sequence. The resolving power of SDS-PAGE is greatly enhanced by preceding the protein separation phase with a "Stacking gel" which employs the principle of isotachophoresis to concentrate samples from relatively large volumes into very small bands. In the separating gel, the negatively charged SDS-protein complexes migrate through the sieve like polyacrylamide matrix and are separated solely on molecular weight differences. The glass plates, spacers and comb were washed with detergent, rinse with water. The glass plates were wiped with ethanol and were assembled. The separating and stacking gel were prepared as follows:

Table 1 : 10% SDS composition

Reagents	Separating gel (10ml)	Stacking gel (5ml)
Distilled water	4 ml	3.4 ml
30% acrylamide	3 ml	0.83 ml
1.5M Tris (pH8.8)	2.5 ml	-
1M Tris (pH6.8)	-	0.63 ml
10% SDS	0.1 ml	0.05 ml
10% APS	0.1 ml	0.05 ml
TEMED	0.008 ml	0.006 ml

The test samples were prepared by diluting (1:1) in the sample solubilizing buffer (100^0C), which were placed for 10 min in a boiling bath. After cooling to room temperature, the sample was spun for a minute. The separating gel solution was gently mixed and poured into the glass plate using a pipette upto a level of about 3 cm from the top. The gel was allowed to stand for 15 to 20 minutes to polymerize. The stacking gel solution was gently mixed and poured the solution into the glass plate using a pipette then the comb was gently inserted between gel plates without air bubbles. The gel was allowed to stand for 10 minutes to polymerize. The test sample was mixed with sample buffer and was heated for 3 min at 95^0C. The glass plate

was fixed to the gel tank tightly with clips on both sides. The upper and lower chamber was filled with running buffer until marked buffer level and the comb were gently removed from the gel. The prepared protein sample was loaded into each well using micropipette. The gel runs at 100 V until the tracking dye reached approximately 0.5 cm from the bottom of the gel. After electrophoresis, the gel was stained with 0.25% Coomassie Brilliant Blue for overnight. The stained gel was treated with a destaining solution (methanol: acetic acid: distilled water - 4.5:1:4.5) for two times with 15 min interval. The stained gels were placed on White Transilluminator and the bands were observed and molecular weight of the protein was determined.

5.7 *In vitro* antidiabetic activity

5.7.1 Inhibition of alpha amylase activity

A total of 0.5 ml of different concentrations of sample i.e 100 µg/ml, 200 µg/ml, 300 µg/ml, 400 µg/ml, 500 µg/ml were taken from test samples. To that 500 µl of 0.20 mM phosphate buffer (pH 6.9) containing α - amylase (0.5mg/ml) solution was added and incubated at 25°C for 10 min. After these, 500 µl of a 1% starch solution in 0.02 M sodium phosphate buffer (pH 6.9) was added to each tube. The reaction mixtures were then incubated at 25°C for 10 min. The reaction was stopped with 1.0 ml of 3, 5 dinitrosalicylic acid colour reagent. The test tubes were then incubated in a boiling water bath for 5 min, cooled to room temperature. The reaction mixture was then diluted after adding 10 ml distilled water and absorbance was measured at 540 nm. Control represent 100% enzyme activity and were conducted in similar way without adding the sample extract (Thalapaneni et al., 2008 and Heidari et al., 2005).

5.7.2 Inhibition of alpha glucosidase activity

The inhibitory activity was determined by incubating a solution of starch substrate (2 % w/v maltose or sucrose) 1ml with 0.2 M Tris buffer pH 8.0 and

different concentrations of sample extract i.e 100 to 500 µg/ml for 5 min at 37°C. The reaction was initiated by adding 1ml of α-glucosidase enzyme (1U/ml) to it followed by incubation for 10 min at 37°C. Then, the reaction mixture was heated for 2 min in boiling water bath to stop the reaction. The amount of liberated glucose is measured by glucose oxidase peroxidase method(Andrade – Cetto et al., 2008).

The concentration of the gastropod extracts required to scavenge 50% of the radicals (IC_{50}) was calculated by using the percentage scavenging activities at five different concentrations of the extract. Percentage inhibition (I %) was calculated by

$$\text{Percentage inhibtion} = \frac{\text{Absorbance of Control} - \text{Absorbance of Test}}{\text{Absorbance of Control}} \times 100$$

5.8 Intracellular ROS scavenging activity

Intracellular oxidative stress was detected using DCFH-DA as described by Kim *et al.,* (2011) with slight modification. Briefly, MCF-7 cells (4 × 104 cells/well) were plated in 96-well clear bottom plate and incubated for 16–18 hr at 37°C and 5% CO_2. After incubation, the cells were washed with PBS twice. To assess antioxidant activity, the cells were pre exposed to different concentrations of test sample (100, 200, 300, 400 μg/ml) for 24 hr. After washing twice with PBS, the cells were exposed to 20 μM DCFH-DA in HBSS and further incubated in the dark for another 30 min. The DCFH-DA was removed by washing the cells with PBS two times. Then, 500μM tBuOOH was added. The unstimulated DCFH-DA (no tBuOOH) in the unexposed MCF 7 cells served as the naive control (NA). The intensity of the fluorescence signal was detected time dependently with an excitation wavelength of 485 nm and an emission wavelength of 535 nmusing a Gemini EM fluorescence microplate reader (Molecular Devices, Sunnyvale, CA, USA).

5.9 Antioxidant activity

5.9.1 DPPH radical scavenging activity (Harborne and Baxter, 1995)

The antioxidant activity of the methanol extract of *L.lambis*was determined using the DPPH radical scavenging activity. DPPH solution (0.004% w/v) was prepared in 95% ethanol. A stock solution of methanol extract was prepared in the concentration of 10 mg/100 ml. From stock solution 100 µg/ml to 500 µg/ml concentration of this solution were taken in five test tubes respectively. 2 ml of freshly prepared DPPH solution (0.004% W/V) was added in each of these test tubes. The reaction mixture was incubated in the dark for 15 min and thereafter the optical density was recorded at 517 nm using spectrophotometer against the blank. For the control, 2 ml of DPPH solution in ethanol was mixed with 10 ml of ethanol and the optical density of the solution was recorded after 30 min. The assay was carried out in triplicate. The decrease in optical density of DPPH on addition of test samples in relation to the control was used to calculate the antioxidant activity, as percentage inhibition (%IP) of DPPH radical. The scavenging ability of DPPH radical was calculated using the following equation.

$$\text{Scavenging ability (\%)} = \frac{\text{Control (ac)} - \text{Test (at)}}{\text{Control (ac)}} \times 100$$

where ac = absorbance of control

at = absorbance of test samples

5.9.2 Ferric reducing antioxidant power

Ferric reducing antioxidant power (FRAP) was assayed according to the method of Benzie and Strain (1996). Stock solution included 300 mM acetate buffer (pH 3.6),

10 mM TPTZ solution in 40 mM HCl and 20 mM $FeCl_3$.$6H_2O$ solution. A working solution was freshly prepared by mixing 25 ml of acetate buffer, 2.5 ml of TPTZ solution and 2.5 ml of $Fecl_3$. $6H_2O$ solution. The mixed solution was incubated at 37ºC for 30 min and was referred to as FRAP solution. Differrent concentration of test sample (100 t0 500 µg/ml) was mixed with 2,850 µl of FRAP solution and was kept for 30 min in the dark. The ferrous tripyridyltriazine complex (colored product) was measured by reading the absorbance at 593 nm. The control was prepared in the same manner except that deionized water was used instead of the sample. The percentage inhibition was calculated by using the following formula

$$\text{Percentage inhibtion} = \frac{\text{Absorbance of Control} - \text{Absorbance of Test}}{\text{Absorbance of Control}} \times 100$$

5.9.3 ABTS Radical Scavenging Activity

ABTS radical scavenging activity was determined according to the method of Binsan *et al.,* (2008). The stock solutions included 7.4 mM ABTS solution and 2.6 mM potassium persulphate solution. The working solution was prepared by mixing the two stock solutions in equal quantities and allowing them to react for 12 hr at room temperature in the dark. The solution was then diluted by mixing 1 ml of ABTS solution with 50 ml of methanol in order to obtain an absorbance of 1.1 ± 0.02 units at 734 nm using a spectrophotometer. Different concentrations test sample 100 to 500 µg/ml was mixed with 2,850 µl of ABTS solution and the mixture was left at room temperature for 2 hr in the dark. The absorbance was then measured at 734 nm. The scavenging activity of ABTS was calculating using the following formula.

$$\text{Scavenging activity} = 1 - \left(\frac{\text{Absorbance of Sample}}{\text{Absorbance of Control}} \times 100 \right)$$

6. RESULTS

6.1 Thin layer chromatography

TLC profiling was done for the methanolic extract of *Lambislambis* in solvent system of n- butanol: acetic acid: water (B:A:W) in proportions of 6:2:2. The plates were developed in the solvent systems and showed the development of pinkish colored spot in tissue extract, when the TLC plate was sprayed with ninhydrin. The plate with fractions when developed in BAW as the solvent system and sprayed with ninhydrin, showed pink spots indicating the presence of amino acids and peptides. The R_f value was calculated as 0.20, 0.29, 0.39, 0.42 and 0.73. The R_f value corresponds to five different amino acids visualizing arginine, histidine, threonine, cystine, phenyl alanine (Plate 2).

6.2 SDS – PAGE

The test sample was subjected to SDS - PAGE to estimate the molecular weight of active proteins present in it. The stained gel revealed that the sample contained a simple population of proteins. The clear bands detected in the gel represent the moleculalr weight of 60 kDa (Plate 3).

6.3 Antidiabetic activity

6.3.1 *In vitro* α- amylase activity

The methanol extract of *L.lambis* revealed a significant inhibitory action on α-amylase enzyme. The percentage inhibition at 100 – 500 µg/ml concentrations of *L.lambis* extract showed a concentration dependent increase in percentage inhibition. The percentage inhibition varied from 26.03% to 76.71% for various concentrations of 100 to 500 µg/ml. The percentage inhibition was found to be maximum of

76.71% at 500 µg/ml of concentration followed by 62.32% at 400, 54.11% at 300, 34.93% at 200 and minimum of 26.03% at 100 µg/ml of concentration respectively. The 50% inhibition (IC_{50}) was found to be 280 µg/ml respectively (Fig. 1).

6.3.2 *In vitro* α - glucosidase activity

There was a dose - dependent increase in percentage inhibitory activity against α- glucosidase enzyme. *L.lambis*showed 32.78% at 100 µg/ml followed by 39.67% at 200 µg/ml, 58.08% at 300 µg/ml, 65.02 % at 400 µg/ml and 86.71% at 500 µg/ml concentration respectively. The IC_{50} value was found to be 251.58 µg/ml (Fig.2).

6.3 Intracellular reactive oxygen species (ROS) levels in MCF-7 cell line

Ability of methanolic extract of *L.lambis* to induce intracellular ROS production is demonstrated in Fig. 3 . There was a strong decrease in intracellular ROS production in MCF – 7 cell line. The intracellular ROS production decreased progressively in concentration independent manner. The fluorescence intensity was found to be higher at 100 µg/ml of 7797.7 au followed by 6610.3 au at 200 µg/ml, 3601.7 au at 300 µg/ml and lower at 400 µg/ml of 2429 au respectively.

6.4 Antioxidant activity

The principle of antioxidant activity is the availability of electrons to neutralize any so called free radicals. Since, antioxidant mechanisms are diverse, a variety of *in vitro* techniques has been developed. It is better to use different assays based on different mechanisms to evaluate the antioxidant capacity. In this study, the antioxidant activity of methanolic extract of *L.lambis* was evaluated using DPPH scavenging, FRAP and ABTS assays.

6.4.1 DPPH scavenging assay

The antioxidant activity of methanol extract of *L.lambis* was found to possess good scavenging ability. The DPPH assay was performed at different concentrations of 100 to 500 µg/ml concentrations. The percentage inhibition of 65% was observed at 100 µg/ml followed by 69.31% at 200 µg/ml, 73.84% at 300 µg/ml, 79.26% at 400 µg/ml and 83.32% at 500 µg/ml concentration respectively and the IC_{50} value was found to be 237.30 µg/ml.

6.4.2 FRAP assay

In the present study, antioxidant activity of methanolic extract of *L.lambis* against FRAP assay was observed as 58.21% at 100 µg/ml, 65.83% at 200 µg/ml, 71.43% at 300 µg/ml, 73.58% at 400 µg/ml and 81.76% at 500 µg/ml concentration respectively. The methanolic extract of gastropod *L.lambis* was found to exhibit a good scavenger of FRAP radical with an IC_{50} value of 248.38 µg/ml respectively.

6.4.3 ABTS assay

The antioxidant activity of methanolic extract of *L.lambis* was measured by ABTS assay. The extract exhibits different levels of antioxidant activity in different concentration viz., 100 to 500 µg/ml. The result shows that the mesogastropod had significant ABTS scavenging activity of 92% at 500 µg/ml concentration respectively. The IC_{50} value was found to be 215.51 µg/ml.

Figure 1: Map showing the study area Gulf of Mannar - Thoothukudi Coastal region

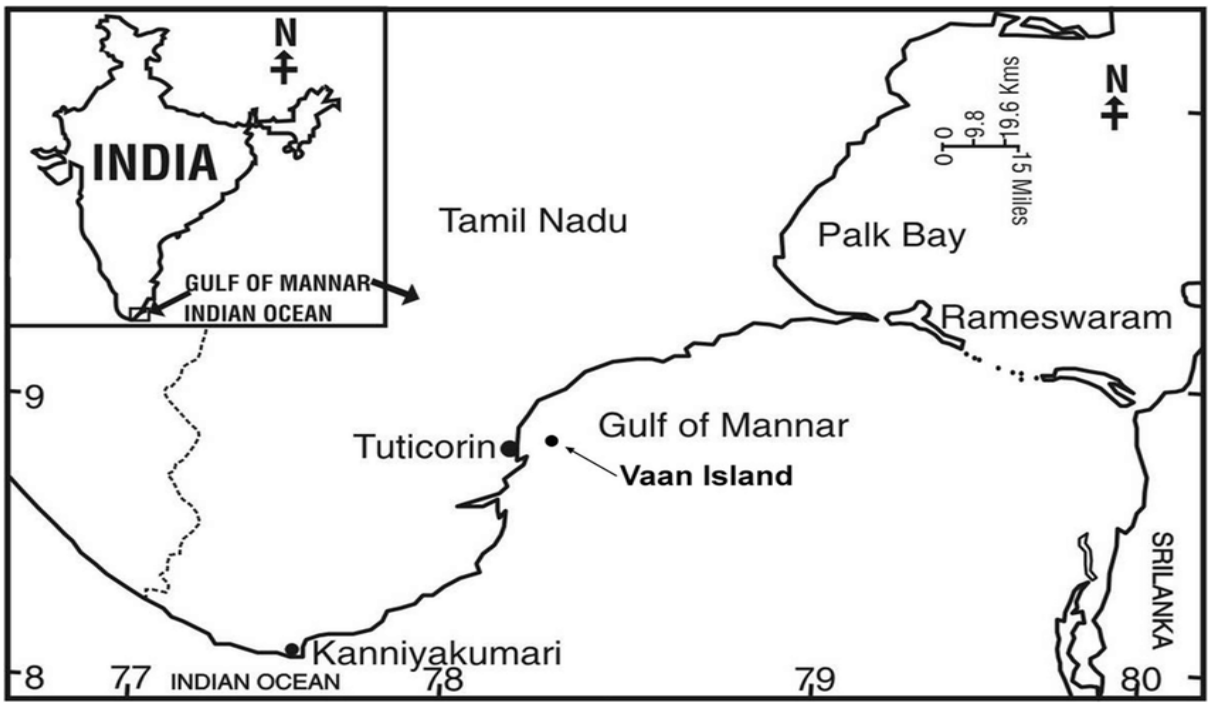
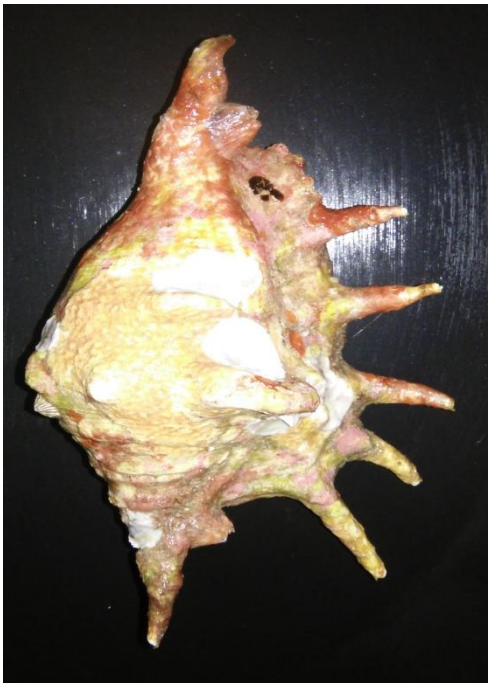


PLATE 1

Dorsal view of the shell – *Lambis lambis*



Ventral view of the shell – *Lambis lambis*

Table 2: Thin layer chromatography

S.No	R_f value	Amino acid
1.	0.20	Arginine
2.	0.29	Histidine
3.	0.39	Threonine
4.	0.42	Cystine
5.	0.73	Phenyl alanine

PLATE - 2

Thin layer chromatography of *Lambis lambis*

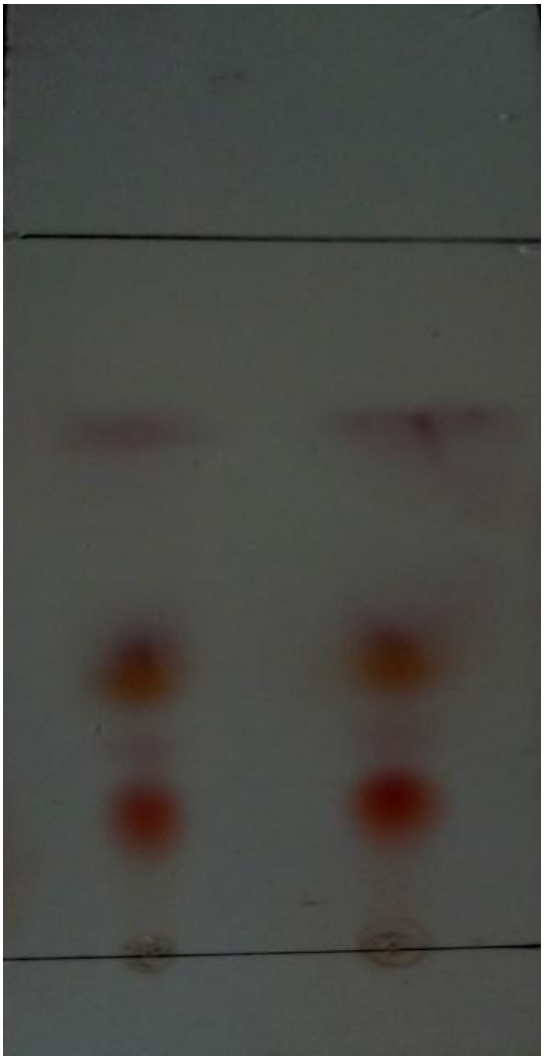
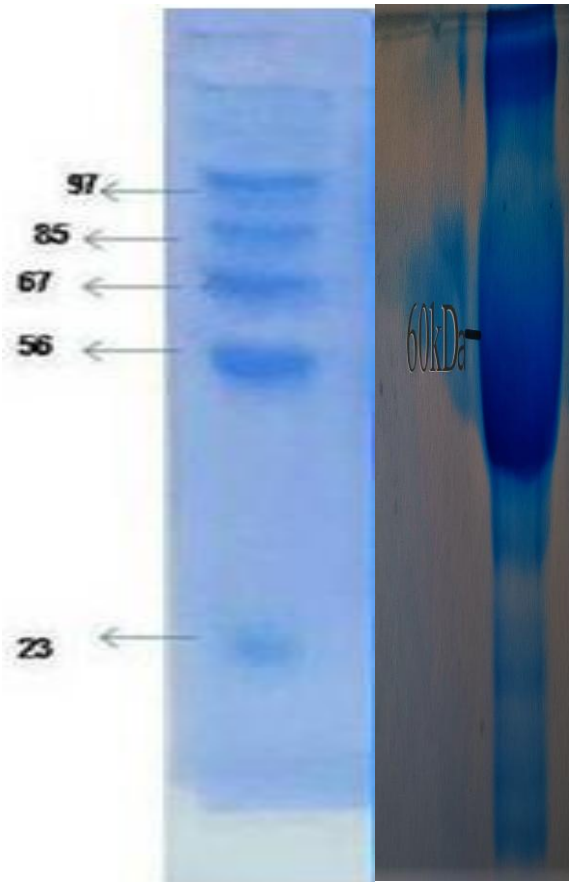


PLATE - 3

Molecular weight determination of protein using SDS - PAGE

Marker **Lane 1**



Lane 1 – *Lambis lambis*

Figure 1: Antidiabetic activity of *Lambis lambis* by α - amylase activity

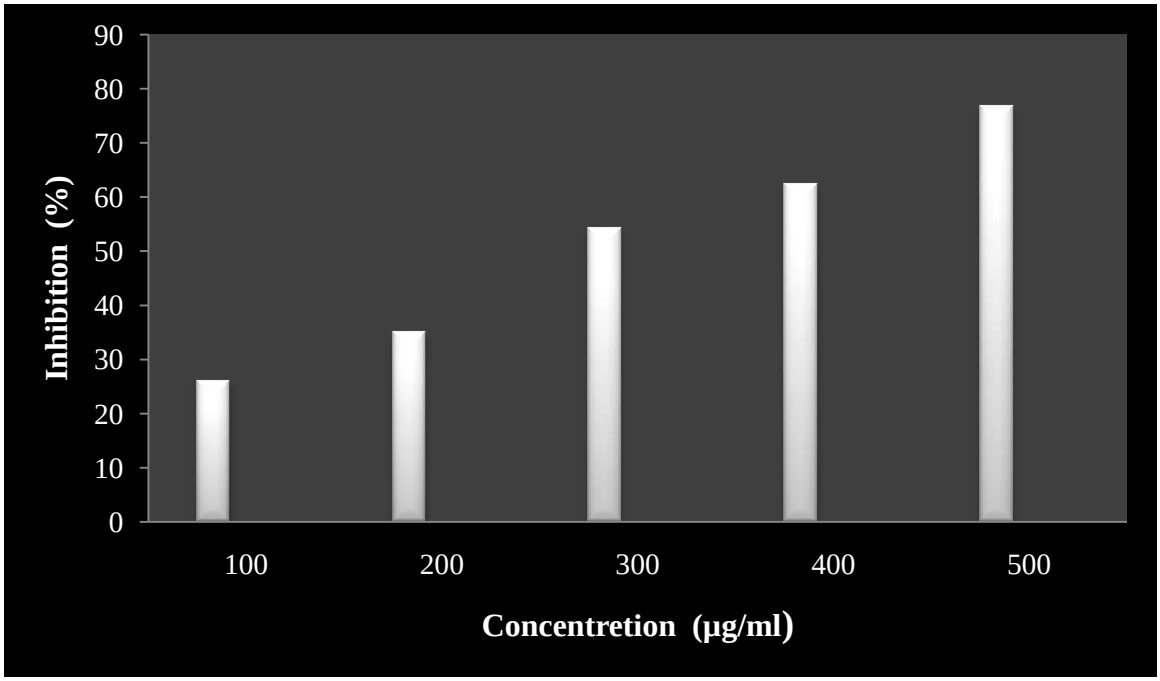


Figure 2 : Antidiabetic activity of *Lambis lambis* by α - glucosidase activity

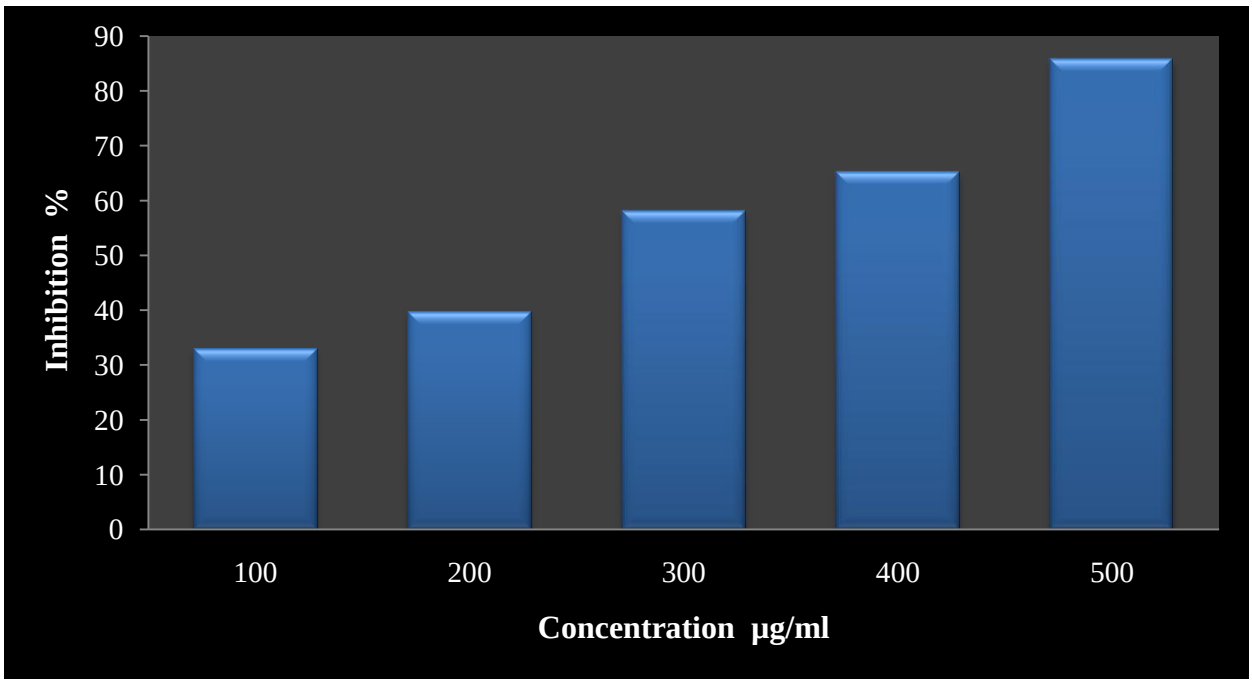


Figure 3 : ROS production of *Lambis lambis* by DCFH-DA assay in MCF-7 cell line

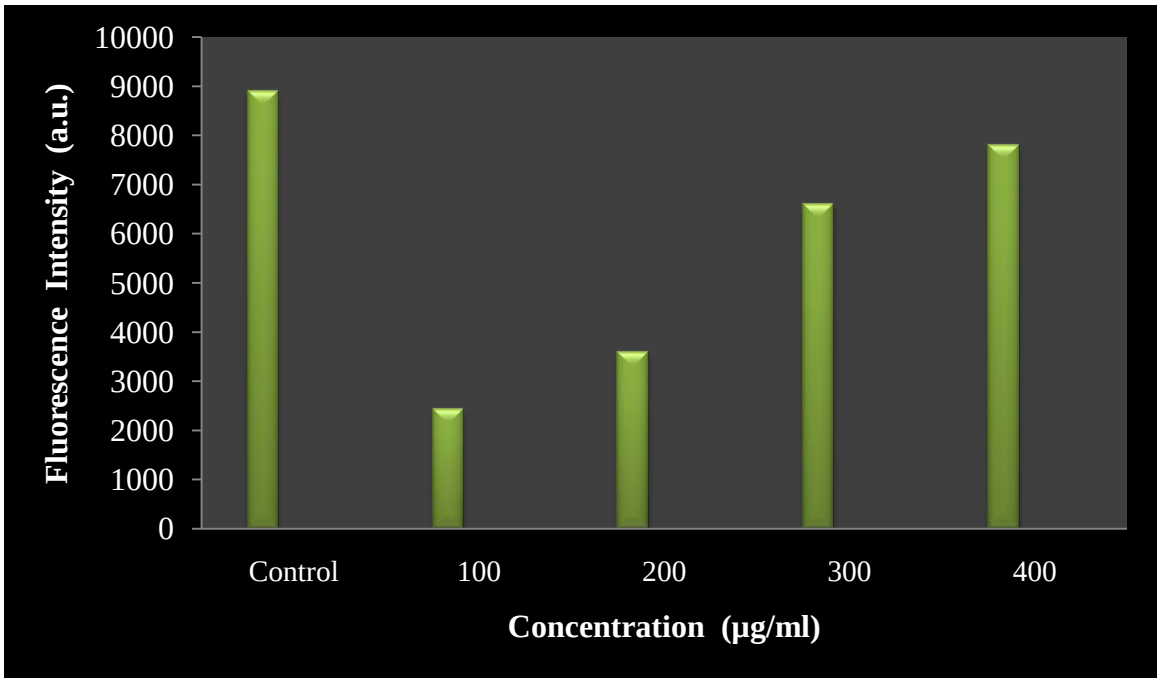
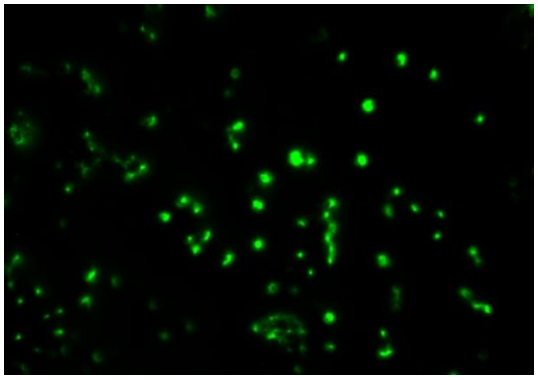
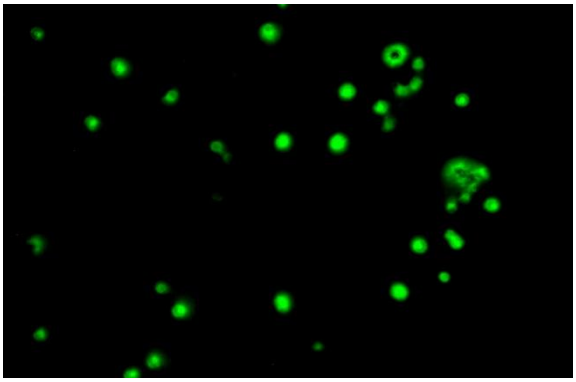


Plate 4 - ROS aSSAY in MCF- 7 CELL lINE

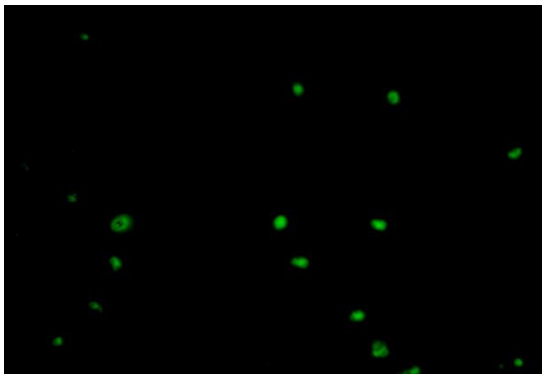
Control



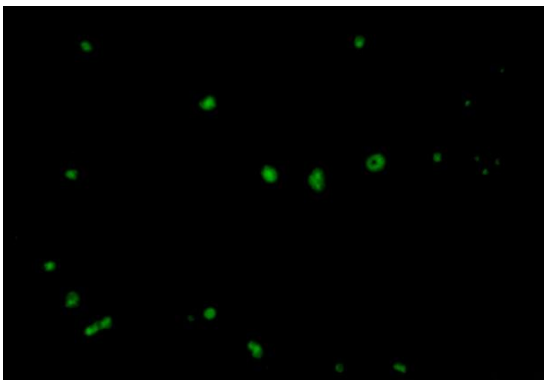
100 µg/ml



200 µg/ml



300 µg/ml



400 µg/ml

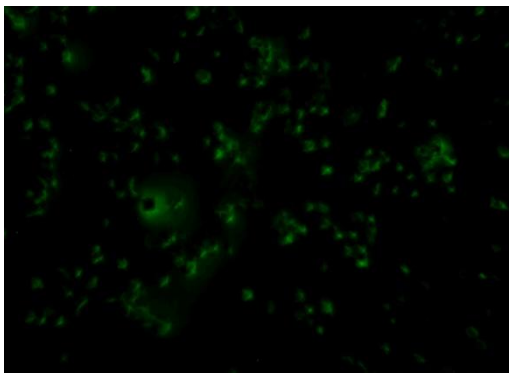


Figure 4 : Antioxidant activity of *Lambis lambis* by DPPH assay

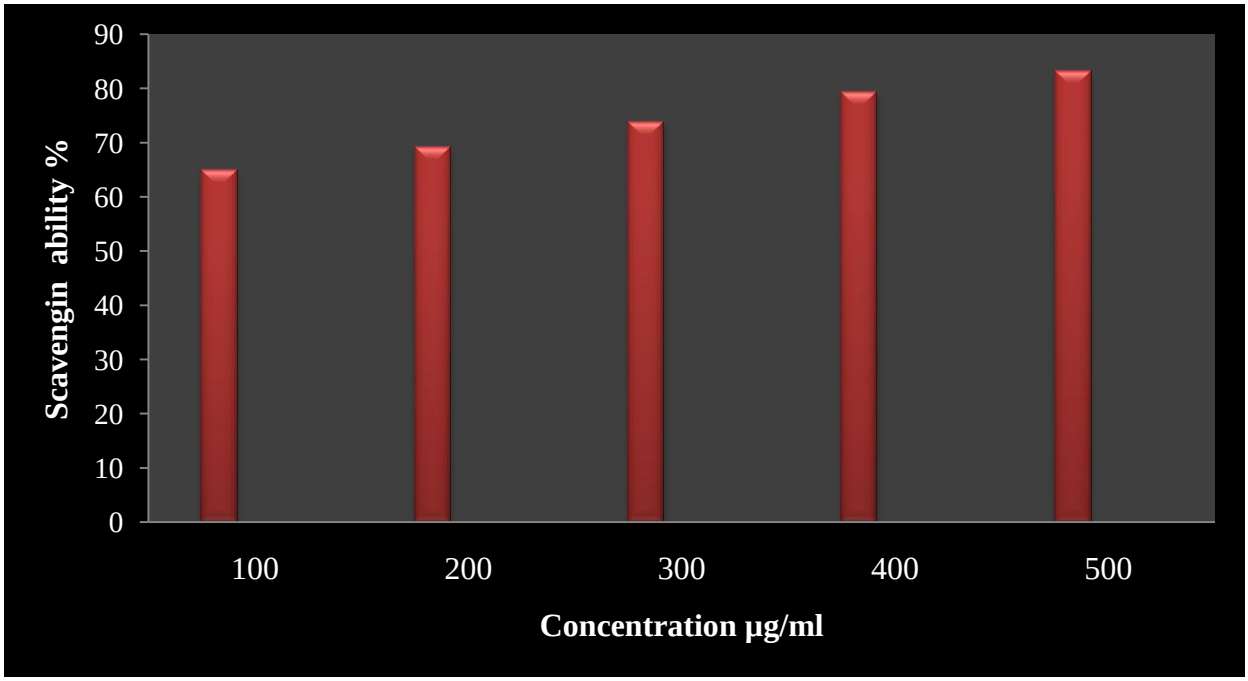


Figure 5: Antioxidant activity of *Lambis lambis* by FRAP assay

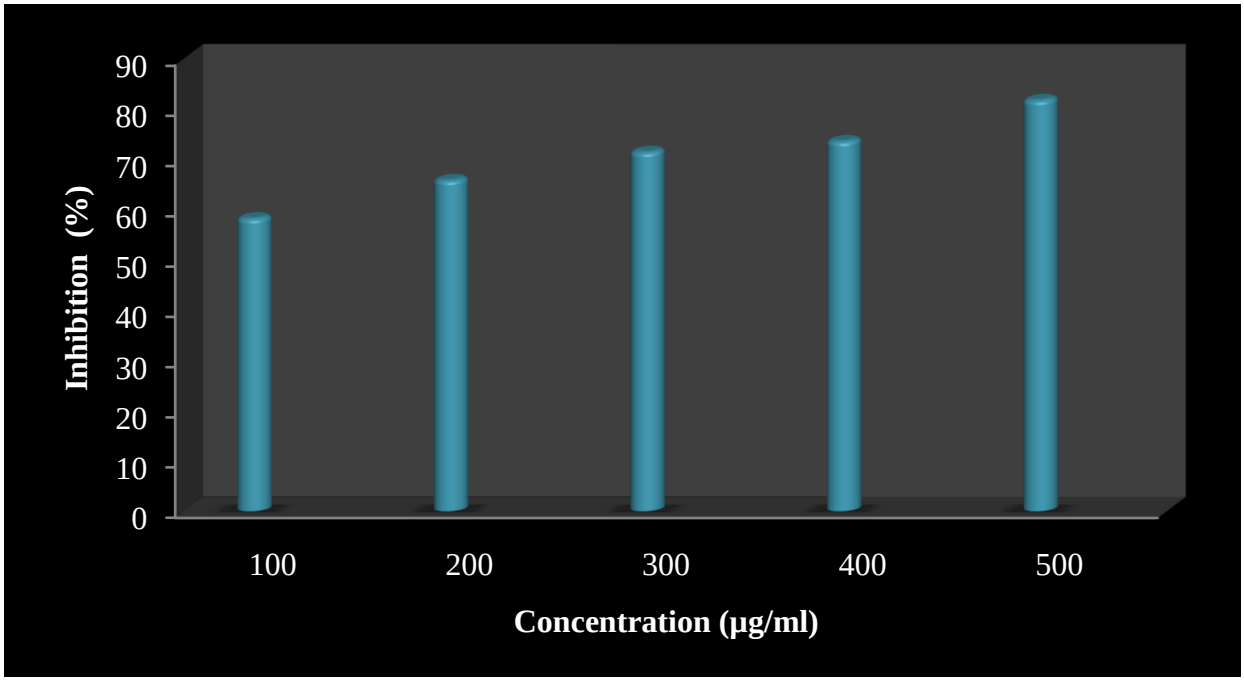
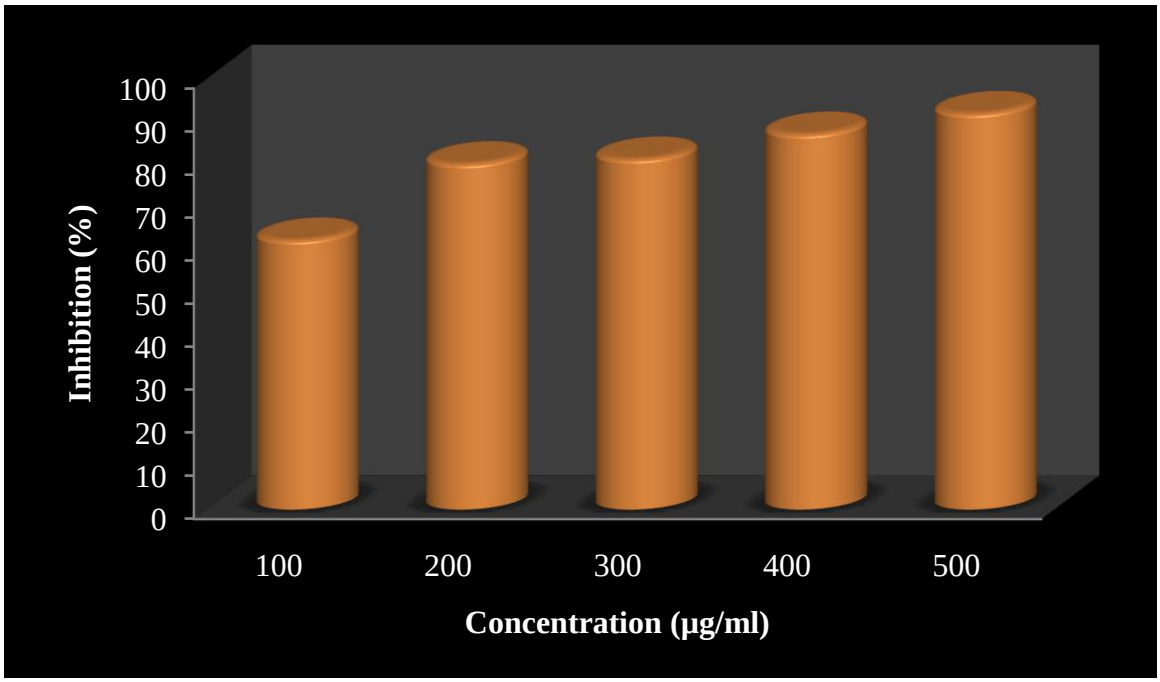


Figure 6: Antioxidant activity of *Lambis lambis* by ABTS assay



7. DISCUSSION

Marine organisms were shown to be rich sources of bioactive compounds, which have a positive influence on human health and may open a new perspective for pharmacological development (Blunt *et al.,* 2005; Makkar and Chakraborty, 2016; Chakraborty *et al.,* 2014 and Chakraborty *et al.,* 2016). Even though many bioactive compounds have been isolated from marine animals, much of the potential compounds still remain unharnessed especially the organisms belonging to the phylum Mollusca. The present study is the first of its kind to report the biomedical properties of the marine gastropod *L.lambis* with regard to its protein isolation, antidiabetic and antioxidant activities by various *in vitro* mechanisms.

Secondary metabolites are compounds that are synthesized by living organisms not to meet their basic needs, but to withstand extreme environmental conditions. Several metabolites, in particular their structures and biological activities have been isolated from marine animals. These metabolite compounds have potential as a drug. Bioactive compounds that are interesting to be studied are generally isolated from marine sponges, jelly fish, coral reefs, molluscs, echinoderms and crustaceans. Bioactive compounds that have been isolated from marine animals are steroids, terpenoids, isoprenoid, non isoprenoid, quinone and nitrogen heterocycles (Bhakuni*et al.,* 2005).

The secondary metabolites derived from number of molluscs possess antibiotic, antiparasitic, antiviral and anticancer activities. Protein is a major biochemical constituent in all invertebrate and received high attention due to their potential bioactive and functional properties (Gayathri *et al.,* 2017). Dolastatins are a group of cyclic and linear peptides isolated from the marine mollusc*Dolabellaauricularia,*with

prominent cell growth suppressing activity. It was found, that mollusc specimens are colonized by a community of diverse cultivable microorganisms, which may supply their host bivalve with bioactive metabolites providing vital functions or chemical protection from colonization by opportunistic microorganisms.

Similar report was studied by Bragadeeswaran *et al.,* (2011) that ascidian extracts of TLC shows the R_f value of 0.2 and 0.3 with ethyl acetate: dichloromethane solvents (9:1 v/v), that the results alkaloids and peptides were clearly detected. Arularasan *et al.,* (2012) studied thin layer chromatography with solvents mixture of n - Butanol: Acetic acid :Water (6:2:2 v/v) clearly showed the R_f value of 0.92 and 0.87 respectively. In the present study, thin layer chromatography was carried out in the methanolic extract of *Lambis lambis*. The result revealed that R_f value of 0.20, 0.29, 0.39, 0.42 and 0.73 corresponds to five different amino acids (Plate 2). The present study agree well with the above findings.

Sugesh and Mayavu (2013) reported SDS-PAGE on 12% gel, the crude proteins of *M.meretrix* and *M.casta*showed 5-6 bands ranged from 45-223 kDa. Sumitha*et al.,* (2009) observed that unclear bands ranging from 14 and 29 kDa in marine bivalves *M.casta*and*P.viridis*. Scotti *et al.,* (2001) observed the similar result from *Perna canaliculus* containing the protein with molecular weight of 35 kDa. Chandran *et al.,* (2009) observed 9.7 kDa proteins in estuarine bivalve *P.viridis.* Very similar to the present study a 60 kDa protein was isolated from the secreted purple fluid of *A.kurodai* and called *Aplysianin P*. *ApylsianinP* displayed cytolytic and antibacterial effects. The present study suggests that methanolic extract of *L.lambis* showed the presence of 60 kDa clear band (Plate 3). Also, the effect of Apylsianin P as a nucleic acid synthesis inhibitor was established (Yamazaki *et al.,* 1989). The present study corroborate well with the above results.

Drugs having an inhibitory action on both of these enzymes (α-amylase and α-glucosidase) possess an ability to control of postprantial blood glucose level specifically in type 2 diabetic patients. Currently, available drugs in this category are acarbose and miglitol, which competitively inhibit above enzymes. But these drugs have common side effects such as flatulence and abdominal bloating (Kuppusamy*et al.,* 2011; Brenner and Stevens, 2006). New drugs or formulations which are devoid of the above side effects will improve the compliance in type 2 diabetic patients.

Anti α - amylase activities of the molluscs were supported by Sadhasivam*et al.,* (2013) explained the α-amylase inhibitory properties of the methanolic extract of three marine molluscs namely *Aplysia sp, Bursatellaleachii* and *Kalinga ornata*(93.0, 70.6 and 50.0% respectively at 0.1mg/ml). Abirami*et al .,* (2011) also observed moderate α-amylase inhibitory activity by the purple fluid of the marine gastropod *Dolabellaauricularia.* An α - amylase inhibition of 72% was observed by Ravi *et al.,* (2012) for the methanolic extract of two marine molluscs*Hemifususpugilinus*and*Natica didyma*. He also observed that the methanol extract of gastropod *Hemifususpugilinus* exhibited greater anti – α - glucosidase activity (IC_{50} 20.27 mg/ml) than the methanol extract of *Natica didyma* (IC_{50} 56.44 mg/ml), although the anti - diabetic properties of this group of molluscs were significantly lesser than the EtOAc-MeOH extracts of the cephalopods *A.marginatus, U.duvauceli, S.pharaonis, S.inernus* and *C. indicus* (IC_{90} 1.69- 5.37 mg/ml; $p<0.05$). The methanol extract of the marine gastropod *Cerithideaobtusa* extract was found to possess moderate anti-α-glucosidase inhibitory activity (IC_{50} 36.40 mg/ml) (Chayani*et al.,* 2015). In the present study, Ic_{50} value of α – glucosidase activity was found to be 251.58 µg/ml.

The α- amylase inhibitory activities of *C.indicus, S.inermis* and *U.duvauceli* were recorded to be significantly greater (IC_{90} ~ 1.7 mg/ml) when compared with

A.marginatus and *S.pharaonis* (IC_{90} 1.9-2.5 mg/ml; $p<0.05$). *A.marginatus* displayed least α-amylase inhibitory activity (IC_{90} 2.5mg/ml). Therefore, the anti-diabetic potential of EtOAc-MeOH extract of cephalopods by α-amylase inhibition demonstrated its effectiveness as an anti - diabetic agent. The solvent extracts derived from the members of the order Octopoda demonstrated fairly good α-amylase inhibitiory activity ($IC_{90} \leq$ 2.5mg/ml) and in which *C.indicus* displayed highest anti- α-amylase property (IC_{90} 1.69 mg/ml). Thus, the use of glucosidase inhibitor, such as α-amylase and α-glucosidase inhibitors, would be a prospective therapeutic agent for the effective management of diabetes (Kajal Chakaraborty and Minju Joy, 2017). The present report corroborates well with the above findings. In the present study the methanol extract of *L.lambis* showed good α - amylase inhibitory activity. The present study unequivocally proved that experimental organism *L.lambis* belonging to Mesogastropoda possess potential bioactivities capable of inhibiting the α-amylase and α-glucosidase enzymes. Therefore, this species can be considered to be the potential candidate species for use against diabetes mellitus.

Antidiabetic activity can be measured *in vitro* by α - glucosidase inhibition method. Alpha - glucosidase is an enzyme that catalyzes the breakdown of 1.4 α - glycoside bond on the non-reducing ends of maltooligosaccharide by releasing β - D - glucose. This enzyme can also hydrolyze 1,6-α-D-glucosidic bond slowly, thus it can continue α- amylase work, which is further hydrolyze α-limit dextrins to glucose (Houlilan, 1979). Inhibition of α- glucosidase work is based on the substrate breakdown to produce a colored product, in which its absorbance is measured during a specific time period. After being hydrolyzed, P-NPG substrate will turn into α-D-glucose and P- nitrophenol which has a yellow color. The resulting yellow color is an indicator of the inhibitor ability to inhibit the reaction. When the ability of

inhibitor to inhibit α - glucosidase work is greater, the resulting yellow color of the solution will be paler compared to the solution without inhibitor (Sugiwati*et al.,* 2009). Inhibition of carbohydrate hydrolysis enzymes such as α- glucosidase become an important strategy in the control of blood glucose levels in the therapeutics of type 2 diabetes.

Sadhasivam*et al.,* (2013) stated that the methanol extract of *Aplysiasp.* showed the highest α - amylase inhibitory activity at 93%, *Bursatellaleachii* at 70.60% and *Kalinga ornata*at 49.03%, while on the acetone extract, α - amylase inhibitory activity showed the percentage below 10%. This was supported with the research by Reni Tri Cahyani*et al.,* (2015) stated that the antidiabetic activity of methanol extracts of *Cerithideaobtusa* was measured with various concentration and generated IC_{50} value of 36.40 mg/ml. In the present study, the 50% inhibition of α – amylase was found to be 280 µg/ml respectively.

Lack of insulin affects the metabolism of carbohydrates, proteins, fat and causes significance disturbance of water and electrolyte homeostasis (Frier and Fisher, 2006). Recent advances in understanding the activity of intestinal enzymes (α - amylase and α - glucosidase both are important in carbohydrate digestion and glucose absorption) have lead to the development of newer pharmacological agents. A high postprandial blood glucose response is associated with micro and macrovascular complications in diabetes and is more strongly associated with the risk for cardiovascular diseases than fasting blood glucose. Hence one of the therapeutic approaches for reducing postprandial (pp) blood glucose levels in patient with diabetes mellitus is to prevent absorption of carbohydrate after food intake.

Inhibition of these enzymes (α-amylase and α- glucosidase) reduced the high postprandial (pp) blood glucose peaks in diabetes (Confort*iet al.,* 2005). The α-amylase inhibitiors act as an antinutrient that obstructs the digestion and absorption of carbohydrate. α - glucosidase enzymes in the intestinal lumen and in the brush border membrane play main roles in carbohydrate digestion to degrade starch and oligosaccharides to monosaccharides before they can be absorbed. It was proposed that suppression of the activity of such digestive enzymes would delay the degradation of starch and oligosaccharides, which would in turn cause decrease in the absorption of glucose and consequently the reduction of postprandial blood glucose level elevation (Pils*et al.,* 1997). Alpha - glucosidase inhibitor retards the digestion of carbohydrates and slows down the absorption.

Synthetic inhibitor causes side effect such as abdominal pain, diarrhea and soft faces in the colon. The reaction mechanisms involved in inhibition of α-amylase enzymes by protein inhibitors are not clearly understood. But these are some suggestion that the protein might cause conformational changes in structure (Kim *et al.,* 2000). The results suggest that methanol extract of *L.lambis*efficiently inhibits α-amylase and glucosidase enzymes *in vitro.* The percentage inhibition at 100-500 µg/ml concentrations of methanolic extract of *Lambislambis* showed a concentration dependent increase in percentage inhibition. The antidiabetic action of *L.lambis* can also be attributed to the intestinal α-glucosidase inhibitory activity.

Reactive oxygen species (ROS) have a crucial role in cell signaling and cellular functions. Mounting evidences suggest that abnormal increase of ROS is often observed in cancer cells and that this biochemical feature can be exploited for selective killing of the malignant cells. Oxidative stress occur when there is an imbalance between generation reactive oxygen species (ROS) and inadequate

antioxidant defense system. Oxidative stress can cause cell damage either directly or through altering signaling pathways. Oxidative stress is a consolidating mechanisms of injury in many types of diseased and pathological conditions (Dryden, 2005).

Reactive oxygen species include a number of molecules that damage DNA and RNA and oxidative proteins and lipids (lipid peroxydation). These reactive molecules contain an oxygen and include H_2O_2 (Hydrogen peroxide), NO (Nitric oxide), O_2^- (Oxide anion), peroxynitrite ($ONOO^-$), hydrochlorous acid (HOCL) and hydroxyl radical (OH^-). Oxidative species are produced not only under pathological situations (cancer, ischemic, reperfusion, neurologic and cardiovascular pathologies, infections diseases, inflammatory diseases, autoimmune disease, etc) but also during physiological (non – pathological) situations such as cellular metabolisms. Indeed, ROS play important roles in many cellular signaling pathways (proliferation, cell activation, migrationetc). ROS can be determined ('It is then referred to as oxidative and nitrosative stress') when produced in high amounts in the intracellular compartments and cells generally respond to ROS by upregulating antioxidants such as superoxide dismutase (SOD) and catalase (CAT), glutathione peroxidase (GPx) and glutathione (GSH) that protects then by converting dangerous free radicals to harmless molecules (i.e water). Vitamins C and E have also been derived as ROS scavengers (antioxidase).

ROS are considered as one of the most important chemically reactive molecules in regulation of cell functions. Moderate increase of ROS an promote cell differentiation and proliferation, whereas excessive ROS accumulation will cause oxidative stress and damage to cells (Trachootham*et al.,* 2009). It is well known that malignant cancer cells have increased ROS level and are more dependent on endogenous antioxidant to maintain a redox balance for cell survival, suggesting that

pharmacological ROS modulation could be exploited to gain targeted therapeutic benefits (*Raj et al.,* 2011).

Abnormal oxidative stress can cause damage to mitochondrial function (Ishikawa *et al.,* 2008). Meanwhile, cell cycle arrest and apoptosis are closely regarded as cellular protection mechanisms under oxidative stress (Shackelford et al., 2000). The oxidation of 2'7'-dichlorofluorescin (DCFH) to fluorescent 2' 7'-dichlorofluorescein (DCF) is commonly used for the detection of radiation-induced ROS. The DCF assay was adapted for efficient, systematic flow cytometry quantification of low-linear energy transfer (LET) γ - radiation - induced ROS *in vitro* in Chinese hamster ovary (CHO) cells. This method is optimized for increased sensitivity to radiation - induced ROS and to discriminate against measurement of extracellular ROS.

Although physiologically low levels of ROS have important role as signaling molecules, the excessive ROS production can contribute to cancer instability and malignancy (Liou and Storz, 2010). Paradoxically, this imbalance in cellular redox homeostasis renders cancer cells more vulnerable to ROS-induced cell death (Jaganjac*et al.*, 2008; Nogueira and Hay, 2013).

In the present study, methanolic extract of *L.lambis* increases intracellular ROS production and consequently ROS-induced cell death. Redox state of the cell also plays a crucial role in regulating apoptosis and mitochondrial electron transport chain is one of the major sites of cellular ROS generation (Trachootham*et al.*, 2008). Furthermore, the intracellular ROS could cause cellular apoptosis via both mitochondria-dependent and independent pathways (Sinha *et al.*, 2013).

So, in the present study there was a strong increase in intracellular ROS production in MCF-7 cell line. The intracellular ROS production increased progressively in concentration dependent manner (Fig. 3). The present study agree well with the above findings.

Diphenylpicrylhydrazyl (DPPH) is stable nitrogen centered free radical which can be effectively scavenged by antioxidants (Vilano*et al.,* 2007). DPPH is also considered as a good kinetic model for peroxyradicals. 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 (Gayathri *et al.,* 2017).

The DPPH radical scavenging assay has been widely used to investigate the scavenging capacity of antioxidant compounds. In DPPH assay, the extract has antioxidative activity and hydrogen - transfer capability, solution's color, because of the addition of extract, changes from purple to yellow. In addition, decrease in absorbance at 515nm indicates the hydrogen transfer and reduction of free radicals by cuttlefish extract (Gangwar*et al.,* 2014; Hmidet, 2011 and Suetsuna, 2000). Aewsiriv*et al.,* (2009) investigated the antioxidant activity with IC_{50} of 249.87 µmol in *Sepia pharaonis*. Hmidet*et al.,* (2011) showed antioxidant activities of cuttlefish *Sepia officinalis* protein hydrolysates, using DPPH assay. Hydrolysate obtained by treatment with A21 proteoses showed a DPPH radical- scavenger with IC_{50} of about 0.52 ±0.01 mg/ml. This IC_{50} is considerably less than the one calculated for cuttlefish's pepsin extract (Hmidet*et al.,* 2011).

Many studies reported antioxidant activities of proteins (Suetsuna, 2000). Peptide fraction obtained from tuna extract has a DPPH scavenging effect of 82.19%. In another study, peptides obtained from enzymatic degradation of *Scomberaustriasicus* have antioxidant effects (Binsan*et al.,* 2008; Jao and Ko, 2002 and Wu *et al.,* 2003). Therefore, DPPH antioxidant activity of *L.lambis* can be because of its peptide and protein compounds.

In the present study antioxidant activity of methanolic extract of *L.lambis* was found to possess good scavenging activity. The percentage inhibition was 83.32% at 500µg/ml concentration and the IC_{50} value was found to be 237.30 µg/ml (Fig. 4).

In FRAP assay, antioxidant activity is to measure the ability of antioxidant presence in the desired extract to reduce the ferric ion Fe^{3+} - TPTZ complex to blue coloured ferrous ion Fe^{2+} - TPTZ by electron donor in acidic medium (Siahposh Abd Souhangir, 2014). The parameter equivalent concentration (EC1) of pepsin extract of *S.pharaonis* was 21.75 mg/ml (Siahposh and Alikhani, 2016). Aewsiri*et al.,* (2009) reported that the FRAP of skin gelatin of *S.pharaonis* was 13.20 µmol/Trolex equivalent/ mg protein. Binsan*et al.,* (2008) investigated the antioxidant activities of soluble fractions of Mungoong produced from the cephalothorax of white shrimp. The water fraction from Mungoong had the highest ferric reducing antioxidant powder (Binsan*et al.,* 2008). The FRAP activity was 13.51 ± 0.26 µmol / Trolox equivalent / gm sample.

In FRAP assay, peptide fractions from squid and tuna skin gelatin indicated antioxidative effects and EC_1 was calculated to be 9.12 ± 0.38 for 10kDa fraction (Jao and Ko, 2002). In a study on catfish hydrolyzed proteins, the fish's extract had a power to reduce iron (Theodore, 2008). So the existence of protein in

*L.lambis*extract can be an effective compound for reducing iron (III) to iron (II) resulting a blue color in FRAP assay. The methanolic extract of gastropod *Lambislambis*was found to exhibit a good scavenger of FRAP radical with an IC_{50} value of 248.38 µg/ml respectively. The present study ratify well with the above reports.

$ABTS^+$ assay is based on the production of $ABTS^+$ radical and it is an excellent tool for determining the antioxidant activity of hydrogen- donating antioxidants (scavengers of aqueous phase radicals) and chain breaking antioxidants (scavenger of lipid peroxyl radicals) so this test is usable in a wide range of pH and in both hydrophilic and lipophilic conditions (Binsan*et al.,* 2008). The IC_{50} of cuttlefish *Sepia pharaonis*extract at 2,4 and 6 min were 129.38, 9.18, 73.21 mg/ml respectively.

Aewsiri*et al.,* (2009) showed that $ABTS^+$ scavenging activity of skin gelatin of *S.pharanis* was 13.20 µmol trolox equivalent/mg protein. Binsan*et al.,* (2008) concluded that $ABTS^+$ activity of soluble fractions of Mungoong produced from the cephalothorax of white shrimp was 75..33 ± 0.12 µ mol Trolox equivalent/gm sample (Binsan*et al.,* 2008). Using different assays, Chalamaiah*et al.,* (2012) measured different fish's antioxidative effects of hydrolyzed proteins. Many peptide sequence isolated from hydrolyzed proteins of *Miisgurnusanguillicaudatu, Lutjanus vitta, Nemipterushexodon* and Merluccius produetus had $ABTS^+$ radical scavenging activity (Chalamaiah*et al.,* 2012). So, the ABTS activity of *L.lambis* can be attributed to its tissue protein. In the present study, results are in agreement with those supporting literatures that the mesogastrood had significant ABTS scavenging activity of 92% at 500µg/ml concentration (Fig. 6).

The methanolic extract of *L.lambis* was subjected to TLC to determine the presence of amino acids and e[tides and also subjected to SDS- PAGE to estimate the molecular weight of protein present in it. From the present study, it was confirmed that the marine molluscs*L.lambis* have good biomedical potential and also it cn be used for synthesis of the new drugs formulation. These findings are useful in designing new strategies for the development of new therapeutic agents.

8. SUMMARY

The present study was made in the mesogastropod*Lambislambis*collected from the gulf of mannar coastal region Various pharmacological studies like separation of peptides and protein, antidiabetic , ROS and antioxidant activities of methanolic extract of *L.lambis* has been assessed and the following results were emerged.

- TLC profiling was done for the methanolic extract of *Lambislambis* in solvent system of n - butanol: acetic acid: water (B:A:W) in proportions of 6:2:2.
- The R_f value was calculated as 0.20, 0.29, 0.39, 0.42 and 0.73. The R_f value corresponds to five different amino acids visualizing arginine, histidine, threonine, cystine, phenyl alanine.
- The test sample was subjected to SDS - PAGE to estimate the molecular weight of active proteins present in it.
- The clear bands detected in the gel represent the moleculalr weight of 60 kDa.
- The methanol extract of *L.lambis* revealed a significant inhibitory action on α - amylase enzyme.
- The percentage inhibition varied from 26.03% to 76.71% for various concentrations of 100 to 500 µg/ml.
- *L.lambis*showed 32.78% at 100 µg/ml followed by 39.67% at 200 µg/ml, 58.08% at 300 µg/ml, 65.02 % at 400 µg/ml and 86.71% at 500 µg/ml concentration respectively in α – glucosidase activity.
- There was a strong increase in intracellular ROS production in MCF – 7 cell line. The intracellular ROS production increased progressively in concentration dependent manner.

- The DPPH assay was performed at different concentrations of 100 to 500 µg/ml concentrations. The percentage inhibition of 65% was observed at 100 µg/ml followed by 69.31% at 200 µg/ml, 73.84% at 300 µg/ml, 79.26% at 400 µg/ml and 83.32% at 500 µg/ml concentration respectively and the IC_{50} value was found to be 237.30 µg/ml.
- In the present study, antioxidant activity of methanolic extract of *L.lambis* against FRAP assay was observed as 58.21% at 100 µg/ml, 65.83% at 200 µg/ml, 71.43% at 300 µg/ml, 73.58% at 400 µg/ml and 81.76% at 500 µg/ml concentration respectively.
- The result shows that the mesogastropod had significant ABTS scavenging activity of 92% at 500 µg/ml concentration respectively. The IC_{50} value was found to be 215.51 µg/ml.

9. CONCLUSION AND SUGGESTIONS

Among different forms of life, molluscs form a dominant group which provides lots of socio – economic benefits, serving as source of food, ornaments and home decorative items and shells as raw materials for lime production. In addition, several species have antimicrobial, antioxidant, anti-inflammatory, antitumour properties offering tremendous opportunity to harness this resource for production of new drugs. Thus marine molluscs can stand as a competent source of secondary metabolites and that could be of great therapeutic use in the new millennium.

The thin layer chromatographic separation revealed that the antidiabetic and antioxidant principle may be of peptide molecules. The results of this study clearly indicates that the methanolic extract of *L.lambis* has potent antidiabetic and antioxidant activities. So the present study concluded that the *Lambislambis* extract showed good biomedical potential. Further analysis of elucidating the structure of bioactive molecules in the marine gastropod *Lambislambis* will pave the road to explore the new drug formulations.

Exploration and exploitation of sea based resources have witnessed a paradigm shift in recent years. Rapid fall in the availability of land based natural resources, the scientists have shifted their research to marine environment which has been found as the hidden ground for a plethora of biomolecules which can be used for discovery of new drugs. The recent research findings have further shown that many more species of marine organisms, particularly the molluscs, stand as a prospective source of valuable bioactive compounds with great potential for new leads.

The level of research in India in context to exploitation of marine molluscan resource to derive some bioactive compounds is mostly limited to screening and

isolation of a new peptides. Marine molluscs could be an important source for development of new generation therapeutic agents to battle with several infectious diseases.

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NATIONAL SEMINAR

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This is to certify that Dr./Mr./Ms ....K. Jesima....II M.Sc Zoology.....

has participated and presented a Research paper on ....The impact of Cowmilk Supplementation

.....on the biological and Cocoon characters of Silkworm Bombyx mori L

in the National Seminar on Research in India - Current State and Future Prospects at St. Mary's College (Autonomous), Thoothukudi, on 11th April 2018.

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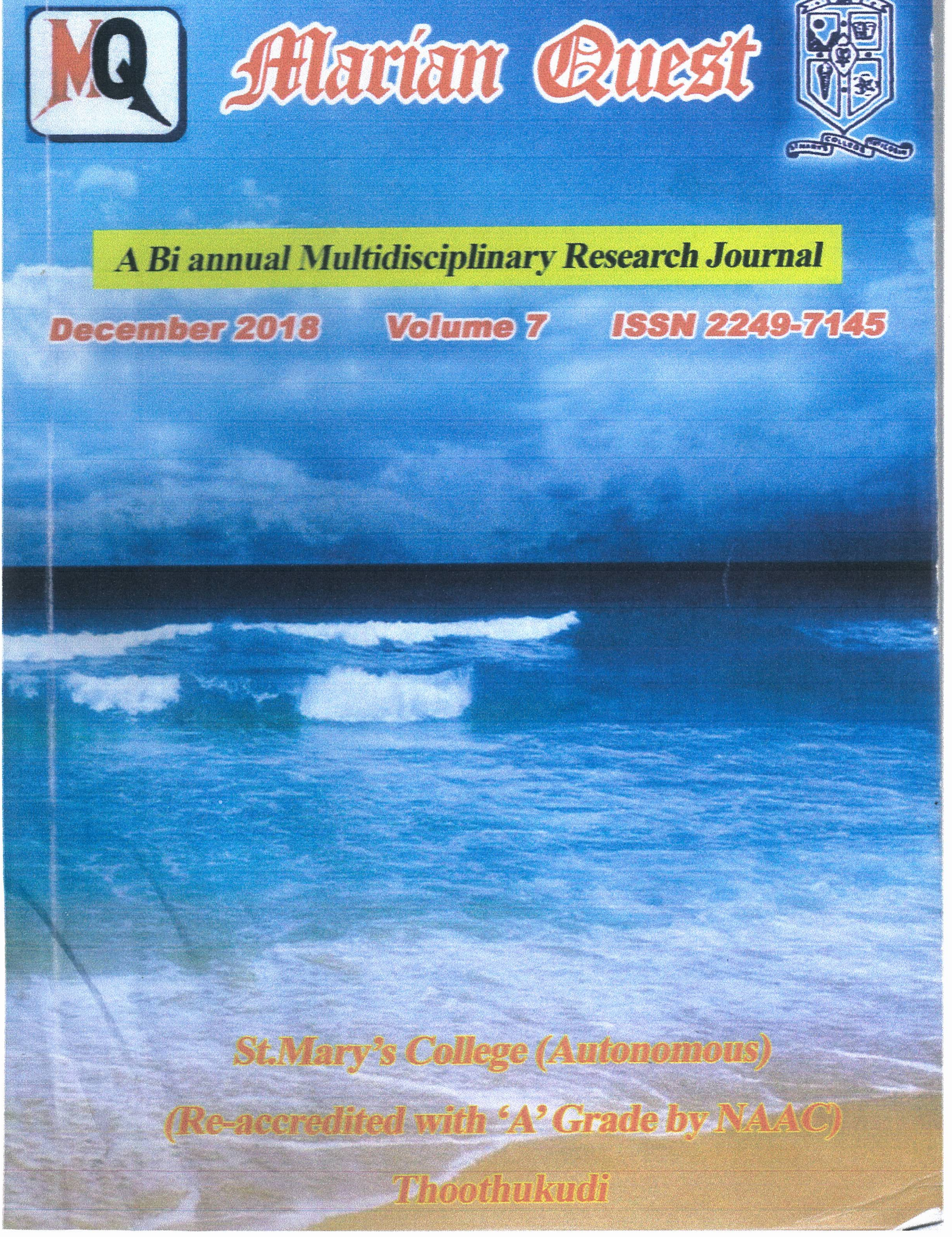
A Bi annual Multidisciplinary Research Journal

December 2018 Volume 7 ISSN 2249-7145



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Marian Quest – A Bi-annual Multidisciplinary Research Journal. Vol.. 7 ISSN 2249 - 7145
December 2018

"IMPACT OF COW MILK ON THE BIOLOGICAL AND COCOON CHARACTERS OF SILKWORM *BOMBYX MORI L.*"

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Abstract- Silkworm is a monophagous insect which has special significance in sericulture industry. *Bombyx mori L.*is a well studied lepidopteran model system because of its morphology, life cycle, and economic importance. Many person have placed importance on enhaccing the economic traits of *B. mori* because it's larvae, silkworms, are vital in the production of silk. In this study the effect of cow milk on *B.mori* growth was tested. Cow milk contain several components that aid in healthy growth. The treatment was given to V instar *B.mori* larvae because the fifth period is when *B.mori* eats and maximum growth of larval stage. The larvae were treated with fresh mulberry leaves , and mulberry leaves dipped in milk from the first day of the V instar. Treatments were given on alternate days and the silkworms were weighed every day to enhancing the weight of the larvae. Cocoon weights were measured as the weight indicates the approximate amount of silk that can be reeled. The result showed that larval gained 100% more weight by end of the fifth instar larval, when fed with mulberry leaves dipped in cow milk. these result suggested that *B.mori* larvae can be fed mulberry leaves treated with cow milk for better growth rate and increased silk production.

Key words: Growth rate , Silkworm *B.mori L.*, Cow milk, Mulberry leaves, economy traits.

INTRODUCTION

Silkworm *Bombyx mori L.*, is a very important economic insect that contributed to the national economy of India. Sericulture has indeed become business oriented and helps in uplifting millions of small and poor sericulturists and others involved in silk industry.

The most important factor influencing the growth of silk worm or for that matter in any organisms is nutrition (kanafi *et al.*, 2007). Mulberry (morus species) leaf is the solo food and source of nutrition for the silkworm, *Bombyx mori L.*, due to the presence of morin (Tribhuwan *et al.*, 1989). The growth and development of larva and subsequent cocoon production are greatly influenced by nutritional quality of mulberry leaves. Supplementations in silk worm nutrition like protein substitute fortified with food stuff are needed for nutritional requirement among several insects(House, 1996). Fortification of mulberry leaves by using supplementary nutrient and feeding the silkworm is an useful modern technique to increase economic value of cocoon (Kamaraj *et al.*, 1972).

Nutritive values of different proteins, amino acids, fatty acids for the silkworm varied largely and it goes without saying that nutrition of the silkworm closely related to the synthesis of silk protein as well as the growth of silk glands.

Phospholipids extracted from the soya bean were also proved to be effective for growth improvement (Russo 2009). Similarly the B vitamin such as biotin, choline, inositol, nicotinic acid, pantothenic acid, pyridoxine, riboflavin and thiamine were also found to be essential for the silkworm.

Konala *et al.*, (2013), suggested that *B.mori* larvae can be fed with bovine milk treated mulberry leaves for increased growth rate and silk production. Cow milk is a source of proteins, carbohydrates, fatty acids, minerals, and other nutrients that facilitate healthy growth and development. Peptides, polyamines, and enzymes also comes from it. Considering the beneficial properties of milk, the importance of protein, carbohydrates, and lipids in the insect diet, and the economic importance of *B.mori*, the present study was undertaken to find out the effect of cow milk on the biological and economic characters of the silk worm in *B.mori*.

MATERIALS AND METHODS:

The eggs of silkworm race L x NB4D² were obtained from the sericulture Demonstration cum Training Centre, Konam, Nagercoil. It is a hybrid variety between the mysore local variety and a Japanese bivoltine variety, introduced by Centrl Sericultural Research and Training Institute (CSRTI), Mysore.

EXPERIMENTAL DESIGN

About 175 freshly moulted IV instar larvae obtained from the same moth were selected and used for the experiment. They were reared in the experimental trays in 5 sets, 4 for experimental purpose and one as control each containing 35 larvae in them. The larvae selected for the experiment were first weighed and reared separately in each tray. The mulberry leaves dipped in different concentration of the raw milk extract were chopped and provided to the each of the respective sets of larvae in required quantities. The leaves provided to the each time of feeding were weighed accurately (20 mgs) and the quantities of leaves given to each set were noted down. The larvae were fed in uniform quantities at each time. The initial weight of the larvae was measured. All weights were made in digital balance. The producer was followed till the larvae reached the spinning stage. The matured larvae were isolated and mounted on separate chandrike. Few cocoons from each treatment were allowed to hatch out. When the moths emerged out they were allowed to mates and then introduced in to the black box and allowed to the eggs.

RESULT AND DISCUSSION

Cow milk which is rich in protein, carbohydrates, fatty acid, minerals, peptides, polyamines and enzyme has profound impact on the growth and development of silkworm *Bombyx mori*. Growth and growth index were enhanced by cow milk in higher concentration. The present result implies the positive and enhancing effect of cow milk on the silk protein of *Bombyx mori* . The present investigation results indicated an enchancing effect on the cocoon parameters. Nutrition plays an important role in improving the growth and development of *Bombyx mori* Kanafi et al., (2007). The supplementation in fortification of mulberry leaves is a recent technique in sericulture research. Murugan et al.,(1998).

Supplementation of cow milk was observed to have an enchanced effect on result silk protein, fibroin, sericin, and total silk protein in the silk gland. The amount of silk proteins in the glands of supplemented larvae increased with the increasing concentration of cow milk. It might be due to the growth promoting and nutritive effect of proteins, carbohydrates fatty acids and minerals present in cow milk. Similar result were obtained by Saravanan et al., (2011) *Vigna unguiculata* aqueous extract supplementation which enchance quality of silk in *Bombyx mori*

TABLE 1

Effect of cow milk on the silk protein of B. mori L

Concentration	Fibroin	Sericin	Total silk protein
Control	0.235	0.23	0.465
30%	0.107	0.016	0.123
70%	0.306	0.127	0.433
100%	0.359	0.46	0.819

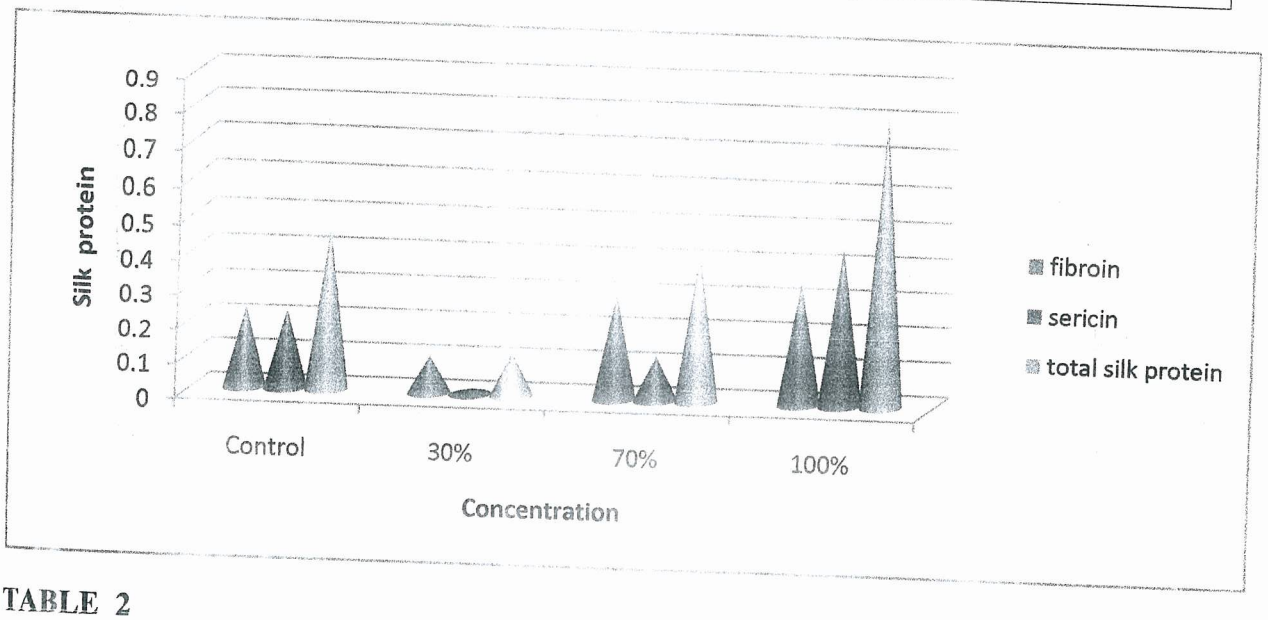
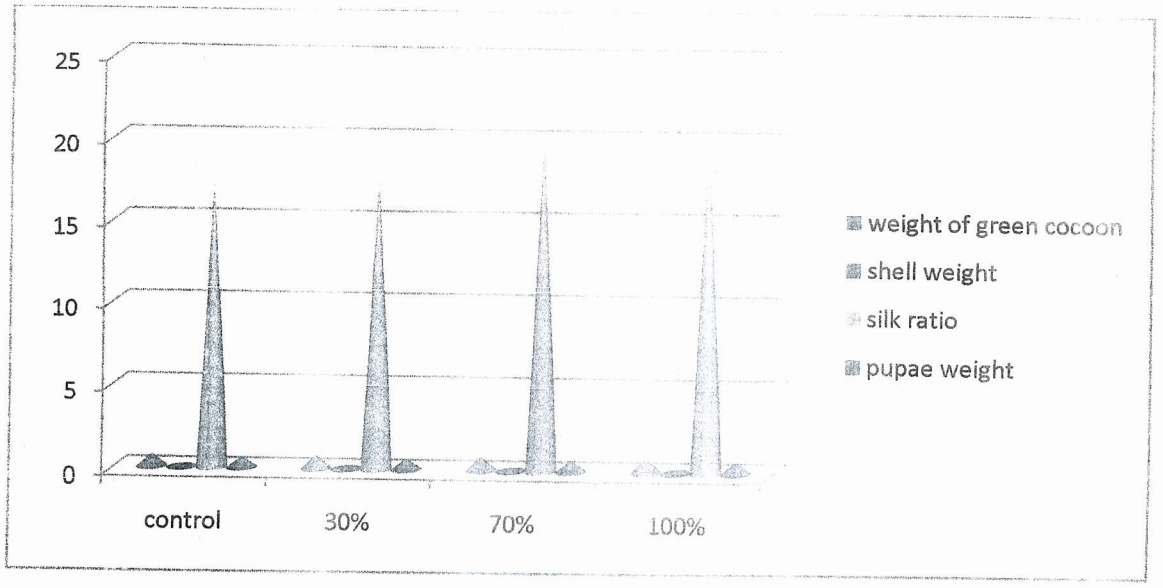


TABLE 2

Cocoon characters of Bombyx mori fed on raw milk supplemented mulberry leaves.

Concentration	Weight of green cocoon (gm)	Shell weight (gm)	Silk Ratio	Pupae weight (gm)
Control	0.759	0.133	17.52	0.626
30%	0.797	0.142	17.81	0.655
70%	0.839	0.170	20.26	0.669
100%	0.859	0.177	20.60	0.682



CONCLUSION

The present study unveilad the nutritive effect of cow milk supplementation on the growth and commercial qualities of *Bombyx mori*. Cow milk which is rich in protein, carbohydrates, folic acid, minerals, peptides, polyamines and enzyme has perfound impact on the growth and development of silkworm *Bombyx mori* . The present works has reveeled that the enrichement of mulberry leaves by cow milk has trastically promoted food consumption economic parameters and silk quality of *Bombyx mori*.The higher concentration had maximum output. Nutritional supplementation would be one of the waste to improve silk production.

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