2.1. INTRODUCTION

Diseases are born with man and drugs came into existence since a very early period to remove the pain of diseases and to cure them. Thus the story of the history of drugs is as old as mankind. Drugs used in medicine today are either obtained from nature or are of synthetic origin. Natural drugs are obtained from plants or animals. Drugs from microorganisms like antibiotics were not known in the early period. Synthetic drugs or syntheticals like aspirin, sulpha drugs, some vitamins and antibiotics are synthesized in laboratories from simple chemical or chemicals through various chemical reactions.

Natural drugs obtained from plants and animals are called drugs of biological origin and are produced in the living cells of plants or animals. Simple drugs are derived from animal organs or entire animals as cantharides, glandular products like thyroid organ or liver extract. Similarly, fish liver oils, musk, bees wax, certain hormones, enzymes, antitoxins are products obtained from animal sources. These are always collected from the same animal species.

Sometimes crude drugs are adulterated. An adulterant is the drug resembling the original or authentic drug but usually quite different or inferior, less, effective, containing less percentage of active constituents and sometimes containing more extraneous matter than permitted. Nature of adulteration can be determined by study of pharmacognosy by evaluation.
Phytochemicals are natural bioactive compounds found in plants and animal foods that work with nutrients and dietary fibre to protect against diseases. Research suggests that phytochemicals working together with nutrients found in fruits, vegetables and nuts, may help to slow the aging process and reduce the risk of many diseases, including cancer, heart disease, stroke, high blood pressure, osteoporosis and urinary tract infections. Pronounced “fight-o-chemicals,” phytochemicals fight to protect ourselves from diseases. They can have complementary and overlapping mechanism of action in the body, including antioxidant effects, stimulation of the immune system, modulation of the action of enzymes and hormones, antibacterial, antiviral and pharmacological properties. “Phyto” is a Greek word that means plant and phytochemicals are usually related to plant pigments. So, fruits and vegetables that have bright colours -yellow, orange, red, green, blue and purple, generally contain the most phytochemicals and the most nutrients. Recently animals of the class, Ascidiacea which are beautifully coloured marine organisms also have been found to contain natural pigments with a variety of pharmaceutically important chemical constituents. These protective compounds are an emerging area of nutrition and health, with new bio active principles.
2.2. IMPORTANCE OF PHARMACOGNOSY AND PHYTOCHEMISTRY

Pharmacognosy is the study of crude drugs obtained from plants, animals and mineral kingdom and their constituents. Even though the science of pharmacognosy is practised since a very early period, the term pharmacognosy was first used by Seydler, a German Scientist in 1815 in his book *Analecta pharmacognostica*. It is derived from two Latin words *Pharmaka* (a drug) and *gignosco* (to acquire a knowledge of). It means knowledge or science of drugs. Pharmacognosy may be defined as the branch of science which deals with biological, physico-chemical features of the crude drugs of animal, plant and mineral origin. In the study of pharmacognosy, drugs are arranged according to the following systems of classification:-

i) Morphological classification

ii) Biological (Taxonomical) Classification

iii) Chemical Classification and

iv) Pharmacological classification

In morphological classification, drugs are arranged according to their morphological or external characters. This classification is useful in the teaching of practical pharmacognosy. In modern pharmacognosy the importance of this classification is decreasing. In biological classification, drugs are
classified as plants and animals are classified. In the chemical classification, the medicinal action of drugs are classified according to the chemical nature of active constituents. Recently, biochemical classification which is more natural and takes into account the biogenetic relationship of natural orders is followed. Thus in modern pharmacognosy more importance is given to chemical and biochemical classifications. In pharmacological classification as the important aspect of the drug is its activity, the drugs are classified according to their pharmacologic action. Pharmacognostical methods of study have enabled us to know the exact constituent responsible for the therapeutic action of the drug. The first step of pharmacognostic study is the correct identification of the species. Identification is followed by:

- Detection of adulteration
- Evaluation of purity and genuineness

Since the passing of the Dietary Supplement Health and Education Act (DSHEA) in the United States in 1994, a large number of phytochemicals are being sold as dietary supplements. Doctors, nutritionists and health practitioners have long advocated a low-fat diet that includes a variety of fruits, vegetables, legumes and whole grains. People who consume such a diet have shown lower rates of certain cancers and heart diseases. A quantitative analysis of 25 species of ascidians of Tuticorin coast has revealed that they contain a maximum of 66% of protein and they are lean with respect to lipids. It is a widely accepted
fact that a diet rich in phytochemicals reduces the risk of cancer, heart disease and illness. But only recently researchers have begun to analyse and learn the food materials containing such phytochemicals. Relatively low rates of breast and endometrial cancers in some Asian countries can be attributed to dietary habits. These cancers are much more common in the United States, possibly because the typical American diet is higher in fat content. Ascidians are consumed as food in many parts of the world and there are coastal aqua farms in Japan as well as Thailand for the culture of ascidians. Because of the number of phytochemicals and the complexity of the chemical processes that are involved in, researchers face a challenging task in trying to determine the type of phytochemicals in food that may fight cancer and other diseases, which have no effect and those that may even be harmful. Researchers have shown much interest in phytochemical supplements in animal source. Studies in cell cultures and animals have shown that certain phytochemicals have pharmacological activity.

2.2.1. The need for standardization

Evaluation of the drug means determining their identity, purity and quality or activity. According to Claus, evaluation can be expressed through

- Organoleptic and morphological evaluation
- Microscopical evaluation
- Biological evaluation
• Physical evaluation

• Chemical evaluation

In organoleptic evaluation macroscopic and sensory characters are mentioned. In microscopic evaluation, microscopic characters of drugs are described. In biological, physical and chemical evaluation quality or activity of the drug is determined. The evaluation or standardization prevents misidentification and adulteration of the drug. It is also helpful in finding the quality and purity of the drug. A high grade of quality in a drug is of primary importance, and efforts should be made to obtain and maintain this high quality.

Pharmacognosy is an applied science\textsuperscript{3,4} which deals with botanical, physico-chemical and economical features of crude drugs. Phytochemistry is concerned with the enormous variety of organic substances that are accumulated by plants, deals with the chemical structures of these substances, synthesis, turnover metabolism, their contribution and biological function\textsuperscript{5}. Since ascidians are marine sedentary organisms the phytochemistry of these animals may also have a similar profile compared to that of plants. Qualitative and quantitative estimation of the phytochemical constituents will be very much useful in the standardisation of ascidians. Pharmacologic activity of certain drugs can also be applied for the evaluation and standardisation of crude drugs. Pharmacological screening consists of a specified set of procedures to which a series of compounds or crude drugs can be subjected\textsuperscript{6}.
2.3. PAST WORK ON PHARMACOGNOSTICAL AND PHYTOCHEMICAL STUDIES OF SOME ASCIDIANS

Though pharmacognostical studies involve both morphological and physico-chemical characters, only morphological studies have been carried out in ascidians. A few ascidians which have been subjected to systematic morphological studies have been presented here.

The first comprehensive work on the ascidians of the Indian waters was a report upon the Tunicates in the collection of the Indian museum, presented by Oka\textsuperscript{7}. He described three species - \textit{Polycarpa cryptocarpa}, \textit{Microcosmus manaarensis} and \textit{Polycarpa anandalei}. Gravely reported\textsuperscript{8} ascidians of the littoral fauna of Krusadai Island in the (Gulf of Mannar).

The ascidian fauna of the sea adjoining Tuticorin, Ennur and Bombay was surveyed by Das. He reported the monoascidians of the Indian seas\textsuperscript{9}, the structure and function of the ascidian test\textsuperscript{10}, new ascidian, \textit{Ecteinascidia bombayensis} from Bombay\textsuperscript{11}, new monoascidian \textit{Herdmania ennurensis} from Madras\textsuperscript{12}, a collection of monoascidians from Madras\textsuperscript{13}.

Sebastian studied\textsuperscript{14} the anatomy and larval organisation of \textit{Polyclinum} sp. He also reported the occurrence of new species of synascidian\textsuperscript{15}, \textit{Polyclinum indicum}\textsuperscript{16}, \textit{Perophora listeri indica} var. \textit{nova}\textsuperscript{17} from the Madras coast of India. \textit{Symplegma viride} and \textit{Symplegma viride stolonica}\textsuperscript{18}, two unrecorded fouling organisms from Indian seas and the occurrence of \textit{Herdmania ennurensis}\textsuperscript{19}, a
fouling ascidian from the Kerala coast of India was also reported by Sebastian. Sebastian and Kurian described\textsuperscript{20} the taxonomical aspects of Indian ascidian.

Renganathan carried out a sincere and deeper survey of the distribution of ascidians along the Tuticorin coast during the period from 1981 to 1986. He reported the occurrence of various colonial ascidians like *Didemnum psammathodes*\textsuperscript{21}, *Lissoclinum fraglie*\textsuperscript{22}, new genus of colonial ascidian\textsuperscript{23}, new genera of colonial ascidians from India\textsuperscript{24}, *Eudistoma virdie*\textsuperscript{25}, *Ecteinascidia garstangi*\textsuperscript{26}, *Botrylloides chevalense*\textsuperscript{27}, *Symplegma brakenhielmi*\textsuperscript{28} and *Eudistoma lakshmiani* n.sp, a new colonial ascidian from Tuticorin coast of India\textsuperscript{29}. He also reported the occurrence of simple ascidians like *Styela bicolor*\textsuperscript{30}, *Molgula martensii*\textsuperscript{31}, *Microcosmus curvus*\textsuperscript{32}, *Pyura*\textsuperscript{33}, *Perophora formosona*\textsuperscript{34}, *Aplidium multiplicatum*\textsuperscript{35}, *Pyura lanka*\textsuperscript{36} from Tuticorin coast.

Renganathan and Monniot reported\textsuperscript{37} a colonial ascidian *Sydnium indicum* from Tuticorin coast of India whereas Renganathan and Krishnaswamy reported\textsuperscript{38} two new species of ascidians, *Ecteinascidia krishnani* and *Polyandrocarpa chendurensis* from Indian waters. Two genera of ascidians were reported from Indian waters by Renganathan and Nelson\textsuperscript{39}. Krishnan \textit{et al.}, reported\textsuperscript{40} the occurrence of four species of ascidians which are new to Indian waters.

Meenakshi and Renganathan described the taxonomical characters and reported the occurrence of rare simple ascidian *Rhodosoma turcicum*\textsuperscript{41}, *Ascidia
sydneiensis\textsuperscript{42}, and Ascidia dorsata\textsuperscript{43} (Asciidiidae) from Tuticorin coast. New ascidian species- Distaplia nathensis sp. nov., two species - Eusynstyela tincta, Phallusia nigra\textsuperscript{44}, three species of Polyclinid ascidians\textsuperscript{45} and Phallusia polytrema\textsuperscript{46}. Ecteinascidia venui a colonial ascidian (Perophoridae) from Tuticorin southeast coast of India\textsuperscript{47} and Trididemnum, a colonial ascidian were reported as new to Indian waters by Meenakshi\textsuperscript{48}.

Meenakshi and Venugopal recorded\textsuperscript{49} a colonial ascidian, Ecteinascidia sluteri as new record to Indian water. The occurrence of new species of colonial ascidian Eudistoma kaverium sp. nov., and four new records of Eudistoma to Indian coastal waters were reported by Meenakshi\textsuperscript{50}.

The complete review of phytochemistry of some ascidians has already been discussed in Chapter 1.
2.4. SCOPE OF THE PRESENT WORK

Ascidians are of great medical importance. The medicinal value of these animals lies in some chemical substances that produce a definite physiological action on the human body. Phytochemicals are the substances responsible for the mechanism of action and the isolation and characterization has been done on ascidians mainly in other parts of the world. In India only meagre work has been carried out. A review of literature reveals that only morphological and anatomical characters have been studied in ascidians. No evidence for the study of physico-chemical characters of ascidians is available. Hence an attempt has been made to perform a complete pharmacognostical and phytochemical study of Phallusia nigra Sav. Pharmacognostical methods of study is a tool which enables us to know the exact constituents responsible for the therapeutic action of the drug. In order to avoid confusion of misidentification and adulteration, it is essential to give details of morphology, anatomy of the different parts used in the official preparations, their chemical composition and also fixing the identity of the animal source. In the absence of these studies it is not possible to differentiate any false specimen from the genuine one. A preliminary survey of literature reveals that no such pharmacognostic studies have been performed in Ascidians especially Phallusia nigra Sav. Hence in the present investigation a systematic pharmacognostical and phytochemical studies have been performed on the Phallusia nigra for the first time.
2.5. RESULTS

2.5.1. Macroscopic characters

2.5.1.1. Systematic position of *Phallusia nigra*:

- **Phylum**: Chordata
- **Sub Phylum**: Urochordata
- **Class**: Ascidiacea
- **Order**: Enterogona
- **Sub order**: Phlebobranchia
- **Family**: Ascidiidae
- **Genus**: *Phallusia*
- **Species**: *Phallusia nigra*
- **Local name**: Oothi
- **Occurrence**: Tuticorin (Lat 8°48’N: Long 78°11’ E).

The following are the synonyms of *Phallusia nigra*:

- *Phallusia nigra* Savigny\(^5\), 1816
- *Ascidia atra* Lesueur\(^5\), 1823
- *Ascidia nigra* Heller\(^5\), 1878
- *Ascidia nigra* Herdman\(^5\), 1880
Ascidia nigra Herdman\textsuperscript{55}, 1882

Phallusia atra Traustedt\textsuperscript{56}, 1882

Ascidia nigra Drasche\textsuperscript{57}, 1884

Ascidia atra Metcalf\textsuperscript{58}, 1897

Ascidia atra Van Name\textsuperscript{59}, 1902

Phallusia atra Hartmeyer\textsuperscript{60}, 1908

Phallusiopsis nigra Hartmeyer\textsuperscript{61}, 1909

Ascidia atra Hecht\textsuperscript{62}, 1916

Ascidia atra Pratt\textsuperscript{63}, 1916

Phallusia atra Hartmeyer\textsuperscript{64}, 1916

Phallusia atra Michaelsen\textsuperscript{65}, 1918

Phallusia atra Van Name\textsuperscript{66}, 1921

Ascidia nigra Van Name\textsuperscript{67}, 1930

Ascidia nigra Pratt\textsuperscript{68}, 1935

Ascidia nigra Plough and Jones\textsuperscript{69}, 1937

Ascidia nigra Van Name\textsuperscript{70}, 1945

Phallusia nigra Van Name\textsuperscript{71}, 1952

Phallusia nigra Peres\textsuperscript{72}, 1958
*Phallusia nigra* Monniot\textsuperscript{73}, 1972

*Phallusia nigra* Millar\textsuperscript{74}, 1975

*Phallusia nigra* Nishikawa\textsuperscript{75}, 1984

2.5.1.2. **External appearance**

Individuals are oval or elongated laterally compressed with the free edges thick and rounded. The size varies from 1.5 cm to 9.5 cm. Attachment is by the posterior end or by one-third of the posterior left side. In a few specimens the posterior basal part had a long flat creeping process for attachment. The anterior end narrows to a terminal branchial siphon. Atrial siphon is one – third from the anterior end on the dorsal surface directed anteriorly. There are 8-10 branchial and 6-8 atrial lobes with ocelli in between them. The lobes are round without any tentacular fringes. The whole anterior part of the body is curved dorsally which is characteristic of the species so that the two apertures are quite close together. Test is firm, smooth, shiny and jet black in colour. Main test vessel leaves the body two-third distance from the anterior end and branches profusely.

2.5.1.3. **Internal structure**

The body wall is delicate, semi transparent and black. It is thicker in the anterior region and becomes thin gradually towards the posterior region. Longitudinal muscles radiate from the siphons to the right side of the body, branch and are crossed by many transverse and oblique bands to form an irregular mesh. On the left side, muscles are present only anterior to the gut
loop. Circular muscles are present in the siphons. There are about 50 tentacles of two orders arranged alternately. Prebranchial area in narrow there is a v-shaped peritubercular area. Dorsal tubercle is simple with a horse-shoe shaped orifice with both the horns tuned in. The dorsal ganglion and the neural gland are situated away from the dorsal tubercle at the base of the atrial siphon. Numerous minute accessory openings are present situated along the very long neural duct. Dorsal lamina is double anteriorly with transverse ribs on its left side and a serrated free margin. The branchial sac is with plications. The number of internal longitudinal vessels varies with the size of the animal. Triangular branchial papillae are present at the junction of internal longitudinal vessels with the transverse vessels. No intermediate branchial papillae. 4-5 stigmata are present in a mesh. The gut loop is large and occupies more than half of the left side and is deeply curved. Oesophagus is two-third distance from the anterior end on the dorsal border. Rectum and descending limb of the intestine are distended with mud. Anus is smooth, lies on the dorsal border, a little in front of the oesophageal opening. Gonads are in the gut loop. The ovary is branched and present in the primary gut loop. Testis follicles are numerous spread over the visible parts of the ovary and portions of the intestine adjacent to the ovary. These macroscopic characters are presented in Fig. 2.1a to Fig. 2.1c. The specimen has been deposited in the National collections of ascidians in the Museum of Department of Zoology, A.P.C.Mahalaxmi College for women, Tuticorin, India (Voucher Specimen Number: AS-2083).
2.5.2. Microscopic characters

The test is a living tissue comparable to the connective tissue of the vertebrates. It covers the animal on the outside and is thick and slightly cartilagenous. It consists mainly of protein and polysaccharides with embedded microfibrils of polysaccharides complex, resembling cellulose in structure. Nerve fibrils are present in the test serves a protective and sensory function. Mantle is the body situated inside the test and forms a covering to the inner organs. It is formed of a layer of epidermis. The muscle fibres run in longitudinal and transverse directions crossing each other in several places. Tentacles are endodermal structures, slender, filamentous present at the base of the oral siphon arranged on a ring muscle. Tentacles are unbranched, numerous and act as a strainer for the passage of food.

Dorsal Tubercle is a small, flat cushion, protruding into the pharynx in the dorsal midline onto which the duct of the neural gland opens. The opening is narrow, ciliated and “U” shaped slit. Endostyle is a groove extending from end to end of the pharyngeal sac on the ventral side. It has seven zones; four of them secretory, one flagellated and two ciliated. At the base there is a zone carrying elongated flagellae.

Dorsal Lamina is situated on the dorsal margin in the middle line of the branchial sac, opposite to the endostyle. It extends from the anterior to the posterior end terminating at the opening of the oesophagus. It is membraneous
and provided with short lauguets. The branchial sac is perforated by long narrow apertures. They help in respiration. The number varies with the size of the individual. Internal Longitudinal vessels are longitudinal blood vessels extending down the inner surface of the branchial wall; crossing the transverse vessels. Branchial Papillae are those lie vertical to the plane of the branchial wall that support the internal longitudinal vessels at their junctions with transverse vessels. They are triangular in shape. Neural Gland is a glandular tissue lying ventral to the neural ganglion. The gland is considered to secrete oxytocin, vasopressin and melanocyte stimulating hormone. Accessory openings of neural gland are additional secondary ciliated openings from a long neural duct into the atrial cavity.

Pyloric Glands are the digestive glands. It arises from the stomach and the branches ramify all over the intestinal wall. The gland performs digestive, excretory function and ionic regulation of the blood and the pH of alimentary canal. The secretion helps to soften the food cord. Ovary is numerous and tubular closely associated with the male components. Testis are small, branched and very numerous. The male follicles spread out in the body wall over the gut loop. These microscopic characters are presented in Fig. 2.1d to Fig.2.1g.
2.5.3. Fluorescence Analysis

2.5.3.1. Extraction of the animal material

Epibionts adhering to the surface of the test of *Phallusia nigra* were carefully removed. The specimen was washed several times with sterile sea water. It was dried under shade. The dry weight was taken after 48 hours and drying was continued till a constant weight was achieved. This ensures the complete removal of water from the samples. The dried animals were homogenized to get a coarse powder. The powder was stored in an air-tight container and used for all further investigations.

Many drugs give fluorescence when the cut surface or the powder is exposed to UV radiation. *Phallusia nigra* was shade-dried until constant weight was obtained. Then the animals were powdered and the powder was used for fluorescence analysis. The powder was treated with various chemical reagents and the changes in colour was recorded. The fluorescence characters were determined according to the methods of Chase and Pratt\(^7\) and the results are presented in Table 2.1. A characteristic yellowish-brown fluorescence was noticed in petroleum ether (40\(^0\)-60\(^0\)C), benzene and chloroform extracts and a dark brown fluorescence was observed in methanol and aqueous extracts under UV light (365 nm). This characteristic fluorescence can be used as a diagnostic tool for the correct identification of *Phallusia nigra* and to test adulteration (if any) in the species.
2.5.4. Physico-chemical characters

The determination of ash value is useful for detecting low grade products, exhausted drugs and excess of sandy or earthy matter, it is more applicable to powder drugs. The percentage of total ash, acid-insoluble ash, water-soluble ash, residue on ignition and extractive values are presented in Table 2.2. The extractive value was found to be minimum in petroleum ether (0.64%) and maximum in methanol (12.62%) and water (10.14%).
2.5.5. PHYTOCHEMICAL STUDY

2.5.5.1. Preliminary phytochemical screening

100 g of the powdered animal material was extracted successively with solvents like petroleum ether (40°C - 60°C) benzene, chloroform, methanol and water using Soxhlet apparatus. The extract was cooled to room temperature. It was then evaporated in a vacuum evaporator under reduced pressure to get a brownish black sticky residue. Preliminary phytochemical screening for the presence of alkaloids, terpenoids, steroids, coumarins, tannins, saponins, flavonoids, quinones, anthraquinones, phenols, aromatic acids, catechins, proteins, xanthoprotein, amino acids, carbohydrate, starch and lipids were tested qualitatively using the crude extracts of petroleum ether (40°C - 60°C), benzene, chloroform, methanol and water. The results are presented in Table 2.3. Petroleum ether (40°C - 60°C) and benzene extracts showed the presence of alkaloids, terpenoids, steroids, saponins, flavonoids, quinones, anthraquinones, proteins, carbohydrate and lipids. Chloroform extract showed the presence of alkaloids, terpenoids, steroids, saponins, flavonoids, quinones, anthraquinones and carbohydrates. The methanol extract showed the presence of alkaloids, terpenoids, steroids, flavonoids, quinones, phenols, protein, aminoacids, carbohydrate, sugar and lipids. Water extract showed the presence of alkaloids, terpenoids, steroids, saponins, flavonoids, quinones, anthraquinones, phenols, protein, aminoacids, carbohydrate, sugar and lipids.
2.5.6. Chromatographic studies

2.5.6.1. Thin layer chromatography:

Crude extracts of petroleum ether ($40^\circ - 60^\circ$C), benzene, chloroform and methanol of *Phallusia nigra* have been subjected to thin layer chromatographic study using pre-coated plates of silica gel G (E-Merck, Germany). The solvent systems were chosen after trial and error of the various solvents. No common solvent could be identified for all the extracts. Different solvent systems are found to be effective to get the maximum number of spots for the various extracts.

The developed TLC plates have been first viewed through ultraviolet fluorescence viewing cabinet (365 nm) before keeping in an Iodine chamber and the $R_f$ values of the fluorescing spots and the spots appeared after keeping in Iodine chamber were measured. The results are presented in Table 2.4.

2.5.6.2. Paper chromatography

Paper chromatographic study has been performed for the aqueous extract of *Phallusia nigra*. The solvent system used is a mixture of n-butanol: acetic acid: water (8.5:0.5:1.0). The $R_f$ values of the spots are calculated and presented in the Table 2.4.
2.5.7. GC-MS Analysis

GC-MS chromatogram of the methanolic extract of *Phallusia nigra* (Fig. 2.2) showed peaks indicating the presence of 11 chemical constituents. On comparison of the mass spectra of the constituents with the NIST library the 11 constituents were characterized and identified (Table 2.5). The various chemical constituents which contributed the medicinal activity to the animal is presented in Table 2.5. These eleven chemical constituents have not been reported elsewhere from ascidians, especially from *Phallusia nigra*. GC-MS chromatogram of the 11 chemical constituents are presented in Fig. 2.3 to Fig. 2.13.
2.5.8. HPTLC Studies

HPTLC studies have been performed for the methanol extract of *Phallusia nigra*. Fig. 2.14 shows the peak area of various phytochemical constituents identified and their concentrations. R<sub>f</sub> values of the different spots were measured and are presented in Table 2.6.
2.6. DISCUSSION

Many phytocompounds exhibit fluorescence when suitably illuminated. This is specific for each compound. A non fluorescent compound may fluoresce if mixed with impurities that are fluorescent. The fluorescent method is adequately sensitive and enable the precise and accurate determination of the analyte over a satisfactory concentration range without several time consuming dilution steps prior to analysis of pharmaceutical sample. A characteristic yellowish – brown fluorescence was noticed in petroleum ether (40°C-60°C), benzene and chloroform extracts and a dark brown fluorescence was observed in methanol and aqueous extracts under UV light (365 nm).

The physical constant evaluation of *Phallusia nigra* is one of the important parameters in detecting adulteration or improper handling of drugs. Equally important in the evaluation of crude drugs is ash value and acid insoluble ash value determination. The total ash is particularly important in the evaluation of purity of drugs i.e the presence or absence of foreign organic matter such as metallic salts and or silica. The ash values of *Phallusia nigra* are

i) Total ash – 12.19%

ii) Acid insoluble ash – 0.98%

iii) Water soluble ash – 4.42%

These ash values are indicative of the impurities present in the drug. Since the ash values are constant for a given drug these values are also one the
diagnostic parameters of the drug. Water soluble ash of *Phallusia nigra* is more than that of the acid insoluble ash.

A knowledge of the phytochemical screening is desirable, not only for the discovery of therapeutic agents, but also because such information may be of value disclosing new sources of such economic materials as tannins, oils, gums, precursors for the synthesis of complex chemical substances etc. Major phytochemical constituents such as alkaloids, steroids, flavonoids, quinones, saponins and anthraquinones are present in almost all the extracts.

Phenolic compounds, saponins and flavonoids may be linked or suggested to be involved with antibacterial, antiviral and antidiarrhoeal activity as suggested by Majaw and Moirangthem\textsuperscript{80}, in plants. Investigations on the mode of action by Enzo\textsuperscript{81} in plants indicate that flavonoids increase colonic water, electrolyte reabsorption and other chemicals act by inhibiting intestinal mobility while some components have been shown to inhibit particular entero pathogens. Alkaloids are reported to have cardiovascular effects by Juge *et al*\textsuperscript{82}. As ascidians are sedentary animals, the same role may be suggested in these animals as defence mechanism.

Thin layer chromatographic studies of the various extracts of *Phallusia nigra* was carried out using silica gel G plate and the $R_f$ values of the spots are presented in Table 2.4. In TLC studies, petroleum ether (40\textdegree-60\textdegree C) and
benzene extracts show two spots whereas in chloroform and methanol extracts a maximum of four spots were observed.

Separation of amino acid was carried out for the water extract of Phallusia nigra using paper chromatography. On comparison with the standard amino acids with the water extract, the amino acids present in the aqueous extract was determined. Serine, norvaline, diiodotyrosine, α – alanine, lysine, aspartic acid and arginine were found to be present in Phallusia nigra in the water extract. Chloroform and ethanol extracts showed a thick spot with Rf value 0.72 which showed an yellowish brown fluorescence.

The GC-MS study clearly indicates that the methanolic extract of Phallusia nigra was rich in many phytochemicals like Methyl 3-bromo-1-adamantaneacetate, n-Hexadecanoic acid, (Z)-11-Hexadecen-1-ol, 2,6-Dimethyl-6-trifluoroacetoxyoctane which contributes to the activities like antimicrobial, antioxidants, hypocholesterolemic, nematicide, pesticide, antiandrogenic, hemolytic and antifouling activity.

In HPTLC studies, six peaks were noticed in the chromatogram. Gallic acid, ferulic acid, caffeic acid and flavonoids such as rutin, isoquercitrin and quercetin were found to be present. A maximum peak area (27773.44) corresponding to quercetin was observed with 221.89 μg/g concentration.
2.7. EXPERIMENTAL

2.7.1. Collection of animals

Small plastic containers or buckets with sufficient sea water to cover the collected specimens were used as containers during field collections. Specimens were carefully dislodged from the substratum. Any larger coral or rock fragments that can damage the specimen were removed. Before removing the animal its colour, appearance its habitat were noted. Colour Photographs were taken. A label indicating date of collection, location, depth and colour of the specimen was pasted.

2.7.2. Sectioning

Tissue Processing

Micro technique:

Tissues of *Phallusia nigra* like test, mantle, stigmata, endostyle and gonads were fixed in 5% sea water-formalin for 15 hours. They were washed in running tap water overnight and then dehydrated in ascending grades of isopropyl alcohol. Then the tissues were completely dehydrated through two changes of 100% alcohol for 20 to 30 minutes each. The dehydrated tissues were dealcoholised by two changes in xylol, 20 to 30 minutes in each change. This was followed by infiltration in xylol saturated with paraffin wax at a temperature of 58° C to 60° C. The tissues were left in molten wax for at least
30 minutes in the embedded bath. In order to remove all the clearing agent (xylol) three changes in the paraffin were made for thorough infiltration.

The tissues were embedded in labelled paper “boats” after proper orientation of the processed tissues. The whole process of embedding was carried out after. The embedded tissues were sectioned at 8µm (microns) thickness in a rotary microtome. The ribbon of section was cut into strips and floated on warm water which was poured over acid cleaned glass slides. The ribbons were then pasted with a thin film of egg albumin as affixative.

Flattening and spreading of the sections was achieved by floating the sections on water medium. When the sections become flat and well spread the water was drained. After complete drying for a few hours, the slides were left over night on the slide warmer whose temperature was maintainer at 10 to 15º C below melting point of the paraffin wax.

The sections were deparaffined in xylene and rehydrated in desending grades of isopropyl alcohol.

Staining technique:

Ehrlich’s hematoxylin and Eosin:

Aqueous 0.2% Eosin was used as the cytoplasmic stain and Ehrlich’s hematoxylin as the nuclear stain. Slides were regressively stained in Ehrlich’s hematoxylin. They were rinsed in distilled water and left in the stain for 15 minutes to over stain. After rinsing in tap water and making the sections blue,
they were observed in wet condition under the microscope. The over stained section were then differentiated in 1% hydrochloric acid in water. After a brief rinse in the acid, the sections were washed in tap water and again observed under the microscope. This process was repeated again till the nuclei was properly differentiated and the cytoplasmic back ground colourless. The sections were counter stained with 0.2% aqueous Eosin for 10 to 15 seconds. In order to remove the excess Eosin, the slides were rinsed in distilled water and quickly run through two changes of 95% alcohol. They were finally placed in 100% alcohol I and II for 15 minutes in each solution and passed through xylol I and II. Sections were then mounted on DPX.

Methylene Blue:

Methylene blue chloride stain was also employed in certain sections, without counter staining, for the following reason. In the aged, Methylene blue solution which is generally recommended, some of the dye is apparently converted to the azuves which are constituents of Giemsa’s. The presence of several oxidation products gives the stain a plouchrome effect so that it stains in various states of blue, purple and lilac. Similarly Crystal violet was used in the place of Methylene blue in certain section.
2.7.3. Photomicrographs

The slides observed under low and high power in OLYMPUS Trinocular microscope fitted with NIKON F 310 SLR Camera for photomicrography. The micrographs were taken under low, medium and high magnifications and the high magnification were taken in oil immersion using blue sub stage filter.

2.7.4. Fluorescence analysis

The animal powder and their extracts in various solvents were examined under ordinary light and UV light (365 nm). These powders was also treated with 1N NaOH (aqueous), 1N NaOH (ethanolic), 1N HCl, 1:1 H₂SO₄ and 1:1 HNO₃ and changes in colour were recorded and are presented in Table 2.1. The fluorescence characters were determined according to the methods of Chase and Pratt⁷⁶.

2.7.5. Quantitative determination

The percentage of loss of weight on drying, total ash, acid-insoluble ash, water-soluble ash and residue on ignition were obtained by employing standard method of analysis described in Pharmacopoeia of India⁸³.

2.7.5.1. Determination of loss of weight on drying

A known quantity of fresh animals studied in the present investigation were weighed separately and allowed to dry under shade till a constant weight
was obtained. The loss of weight on drying was calculated. The percentage of loss of weight on drying is presented in Table 2.2.

2.7.5.2. Determination of total ash

5g of air dried powdered sample was taken in a previously weighed Nickel crucible and ignited carefully, not exceeding dull red heat until the ash was free from carbon. The crucible was cooled and weighed. The percentage of ash with reference to the air-dried sample was calculated. The percentage of total ash is presented in Table 2.2.

2.7.5.3. Determination of acid-insoluble ash

A known weight of ash (200mg) was boiled with 25ml of 4N hydrochloric acid. The insoluble matter was collected in a previously weighed sintered crucible, washed with hot water, dried to constant weight and weighed. The percentage of acid-insoluble ash with reference to the air-dried sample was calculated and is presented in Table 2.2.

2.7.5.4. Determination of water-soluble ash

A known weight of ash (200mg) was boiled with 25ml of distilled water. The insoluble matter was collected in a previously weighed sintered crucible washed with hot water, dried to constant weight and weighed. The percentage of water-soluble ash with reference to the air-dried sample was calculated and presented in Table 2.2.
2.7.5.5. Determination of residue on ignition

3g of air-dried, powdered sample was taken in a previously weighed Nickel crucible and ignited carefully, not exceeding dull red heat until the ash was free from carbon. Then the ash was strongly ignited and weighed. The percentage of ignited ash with reference to the air-dried sample was calculated. The percentage of residue on ignition of the sample is presented in Table 2.2.

2.7.5.6. Determination of extractive values

The extractive value of the animal powder in petroleum ether (40\(^0\)-60\(^0\)C), benzene, chloroform, methanol and water were determined by employing the methods of analysis described in Pharmacopoeia of India\(^83\).

About 5g of air-dried sample was taken in a stoppered flask. 100ml of the solvent was added, shaken well and allowed to stand for 24 hours with occasional shaking. Then the content was filtered. 50ml of the filtrate was pipetted out into a clean, previously weighed china dish and evaporated on a water bath. Finally it was dried at 105\(^0\)C, cooled and weighed. The percentage of solvent soluble extractive with reference to the air-dried sample was calculated. The percentage of extractive value in various solvents is presented in Table 2.2.
2.7.6. Preliminary phytochemical screening

Standard procedures as suggested by Brindha\textsuperscript{84} \textit{et al.}, Trease and Evans\textsuperscript{85} and Harborne\textsuperscript{86} were followed.

**Test for alkaloids: Dragendorff's test:** One ml of extract was taken and few drops of Dragendorff's reagent was added. Appearance of orange colour indicates the presence of alkaloids.

**Mayer's test:** One ml of extract was taken in a test tube. 2 ml of Mayer’s reagent was added. Appearance of dull white precipitate indicates the presence of alkaloids.

**Test for terpenoids:** One ml of extract was warmed with a piece of tin and one ml of thionyl chloride. Appearance of pink colour indicates the presence of terpenoids.

**Test for steroids:** One ml of extract was dissolved in chloroform and equal volume of concentrated sulphuric acid was added. Bluish red to cherry red colour in chloroform layer and green fluorescence in the acid layer shows the presence of steroids.

**Test for coumarins:** Sodium hydroxide was added to one ml of extract. Appearance of yellow colour indicates the presence of coumarin.

**Test for tannins:** One ml of extract was mixed with basic lead acetate solution. Presence of white precipitate indicates tannins.
**Test for saponins:** To 5 ml of the extract, few drops of sodium bicarbonate was added and shaken vigorously for few minutes and kept aside. The formation of honey comb like froth shows the presence of saponins.

**Test for flavonoids:** A piece of magnesium turning and one to two drops of concentrated hydrochloric acid was added and boiled for 5 minutes with few ml of extract. Appearance of red colour represents flavonoids.

**Test for quinones:** One ml of extract was taken and one ml of concentrated sulphuric acid was added. Appearance of red colour indicates quinones.

**Test for anthraquinones:** Extract was macerated with ether and after filtration, aqueous ammonia was added. Appearance of pink red or violet colour in the aqueous layer after shaking indicates the presence of anthraquinones.

**Test for phenols:** One ml of extract was diluted with 2 ml of distilled water. Few drops 10% aqueous ferric chloride was added. Appearance of bluish green colour indicates phenols.

**Test for aromatic acids:** To the test solution, sodium bicarbonate was added. Brisk effervescence indicates the presence of aromatic acids.

**Test for catechins:** To the test solution, few drops of Ehrlich Reagent and concentrated hydrochloric acid was added. Appearance of a pink colour represents catechins.
**Test for proteins: Biuret test:** To few ml of extract, one ml of 40% sodium hydroxide solution and two drops of 1% copper sulphate solution was added. Formation of violet colour indicates the presence of proteins.

**Xanthoprotein test:** To one ml of extract, few drops of concentrated nitric acid was added. White precipitate if formed was boiled and cooled. Then 20% sodium hydroxide was added. Formation of orange colour indicates the presence of proteins.

**Lead acetate test:** One ml of lead acetate was added to one ml of extract. Formation of white precipitate indicates the presence of proteins.

**Millon’s test:** Two drops of freshly prepared Millon’s reagent was added to the extract and boiled. Formation of white precipitate indicates the presence of proteins.

**Ninhydrin test:** Few drops of ninhydrin was added to the extract and boiled for few minutes in a water bath. Formation of violet colour indicates free amino acids.

**Test for carbohydrates: Molisch’s test:** One ml of extract was mixed with one ml of α naphthol solution. Concentrated sulphuric acid was added through the sides of the test tube. Appearance of pink colour at the junction of the two liquids reveals the presence of carbohydrate.

**Fehling’s test:** Equal quantity of Fehling’s A & B was added to one ml of extract and boiled. Formation of brick red precipitate indicates carbohydrate.
**Test for glycogens:** 1% of iodine – potassium iodide was added to one ml of extract. Appearance of blue colour indicates glycogen.

**Test for glycosides:** To one ml of extract, a pinch of anthrone was added followed by a few drops of concentrated sulphuric acid. The mixture was warmed in a water bath. Appearance of dark green colour indicates glycosides.

**Test for lipids: Bragdon test:** Few drops of chloroform was added to one ml of extract followed by one ml of potassium dichromate reagent. Appearance of greenish blue colour indicates presence of lipids.
2.7.7. Chromatographic studies

2.7.7.1. Thin layer chromatography

Thin layer chromatographic studies have been performed for petroleum ether (40° – 60°C), benzene, chloroform and methanol extracts using pre-coated plates of Silica gel for TLC (E-Merck, Germany). The Silica gel-G for TLC was poured as thin layers on glass plates by preparing semi-solid slurry with distilled water. The plates were dried until they are free from moisture and activated in an air-oven at about 110°C for about 3 hours. Different solvent systems were employed for various extracts of the samples. The plates were viewed under UV light (365nm) using a UV visible viewing cabinet. The fluorescence spots were located and \( R_f \) values were measured. The plates were then developed in an iodine chamber and the \( R_f \) values of the spots were calculated. The solvent systems for each extracts were standardized after much trial and error. TLC analysis of the various solvents tried for the thin layer chromatographic techniques, no common solvent could be identified for all the extracts. The solvent system employed and the \( R_f \) values obtained are presented in Table 2.4.

2.7.7.2. Paper chromatography

Paper chromatographic studies has been performed for the water extract of *Phallusia nigra*. The solvent system used was a mixture of \( n-\)butanol : acetic acid : water. The solvent mixture was shaken well in a separating funnel and allowed the phases to separate. The upper organic layer
was used for developing chromatogram. Whatmann No.1 filter paper was used for this purpose. Fluorescence spots were located first and the $R_f$ values were calculated by observing the paper chromatogram in a UV-viewing cabinet (365 nm). The paper chromatogram was then developed in an iodine chamber and $R_f$ values of the spots were calculated and the results are presented in Table 2.4.

2.7.8. 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 chromatograph interfaced to a mass spectrometer (GC-MS) instrument employing the following conditions: column Elite -1 fused silica capillary column ($30 \times 0.25$ mm $1D \times 1EM$ df, composed of 100% Dimethyl poly siloxane), operating in electron impact mode at 70 ev; helium (99.999%) was used as carrier gas at a constant flow of 1ml/min and an injection volume of 0.5 El was employed (split ratio of 10:1) injector temperature 250$^0$C; ion source temperature 280$^0$C. The oven temperature was programmed from 110$^0$C (isothermal for 2 min), with an increase of 10$^0$C/min, to 200$^0$C/min, then 5$^0$C to 280$^0$C/min, ending with a 9 min isothermal at 280$^0$C. Mass spectra were taken at 70 ev; a scan interval of 0.5 s and fragments from 40 to 550 Da.

2.7.9. HPTLC studies

Shimadzu CLASS-VP V6.13 SP2 instrument was used to carry out HPTLC analysis employing the following conditions: Column 4.6 x 75 mm
Zorbax Eclipse XDB-C18,3.5 im; Mobile phase A=water, B=methanol, Gradient at 0 min 90% B, at 20 min 100% B, Column wash at 21 min 90% B; Flow rate-1.0 ml/min; UV detector-variable wave length detector 210 nm, standard cell, Column compartment temperature 200C; Stop time 21 min, Post time 5 min; Injection volume – 5 micro liter.
2.8. REFERENCES


