Evaluation and characterization of herbal extract of *Halodule uninervis* and its anti diabetic activity

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Submitted By

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CERTIFICATE

This is to certify that the thesis entitled **"Evaluation and characterization of herbal extract of *Halodule uninervis* and its anti diabetic activity"** submitted to the Tamil Nadu Dr. M.G.R. Medical University, Chennai for the award of the degree of **Doctor of Philosophy** (Faculty of Pharmacy)**,** is a record of research work done by **Mr. R.KARTHIKEYAN,** Department of Pharmaceutical Chemistry during the period 2014 to 2018 at the Institute of K.M. College of Pharmacy, Madurai under my guidance. The thesis has not previously formed the basis for the award of any other degree, diploma, associate ship, fellowship or other similar title to the candidate and the thesis represents independent work on the part of the candidate.

(Dr. M.Sundarapandian)

Guide

DECLARATION

I declare that the thesis entitled **"Evaluation and characterization of herbal extract of *Halodule uninervis* and its anti diabetic activity"** submitted to the Tamil Nadu Dr. M.G.R. Medical University, Chennai for the degree of **Doctor of Philosophy** (Faculty of Pharmacy), is a record of research work carried out by me during the period 2014 to 2018 under the guidance of **Dr.(Mr). M.SUNDARAPANDIAN, M. Pharm., Ph.D.,** Professor, Principal, K. M. College of pharmacy, Madurai**.** The thesis has not previously formed the basis for the award of any degree, diploma, associate ship, fellowship title in this or any other university or similar institute of higher learning.

(R.KARTHIKEYAN)

Dedication


I thank
my beloved Parents
Mrs. V. Suseela &
Mr. K. RAMAKRISHNAN
My Wife Mrs. C.Shobia
and my Daughters
Deepthika.K & Kaashika Sri. K
for their unwavering Support and help.

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I humbly submit this thesis with gratitude to The Omnipresent The Almighty.

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At the outset, I am privileged to take this opportunity with immense pride in expressing my deep sense of gratitude and agathodaimon to my guide **Dr. M.Sundarapandian M.Pharm, Ph.D,** Principal, Department of Pharmaceutical chemistry. Thanks for his boundless enthusiasm, motivation, constant encouragement, guidance and valuable advice which greatly affiliated this work.

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Chapter I
Introduction

INTRODUCTION

Natural products play the most significant role in the drug discovery and drug development process. The medicinal plants are best source of treatment of illness and it can be traced back by the written documents evidence of the early civilizations for long period of time in India. Studies on natural therapies provide that to maintain human health plants have been a valuable source of natural products, in the last decade, with more effective manner.

The kingdom of plants are the reservoir of a largely unexplored biologically active compounds not only as drugs but also the unique components that could serve as a starting material an intermediates for synthetic compounds. Plants kingdom also serve to understanding of biological processes leads to invent the better synthetic routs to produce pharmaceutical active components with cost effective manner. In India the use of plant materials and compounds for medicinal and manufacturing pharmaceutical actives has measurably increased. World Health Organization accepted that plants would be the best source to obtain many of pharmaceutically active compounds. Usage of traditional medicine and compounds derived from medicinal plants by the individuals increasing widely in developed countries.

Plants have been demonstrated to be a very viable source of clinically relevant antidiabetic compounds. However, ethnopharmacologic information has been poorly utilized in the past in the search for new principles against diabetic. In many ethnomedical systems, reports of specific diabetic type uses of plants are rarely found, mainly because diabetic is a disorder that involves a complex set of signs and

symptoms. Since, many of the plant products and their derivatives are approved for diabetic control; the development of new drugs to play an important role in diabetic control is greatly desired. Plants contain and produce a variety of chemical substances that act upon the body and give most successive results. Different parts of plants like leaves, flowers, stems, berries and roots used to prevent, relieve, and treat illnesses. A number of herbal plants and their compounds have been used, and have served as models for modern medicine. Many conventional medications and listed drugs were originally derived from plants [1].

Among them marine products possess quite novel structures which lead to pronounced biological activities and novel pharmacology. The study of such compounds lead to most successive results for the development of new pharmacological classes with cost effective manner. Indian costal regains hold treasures in terms of natural products which have many pharmaceutical potential values as like the natural products in the land. It is infinite and will remain undiscovered unless research into drug development establishes the medicinal value of the chemical compounds they hold. Marine herbal medicine sometimes referred as herbalism or botanical medicine because for their medicinal or therapeutic value and has been used throughout history by all cultures [2].

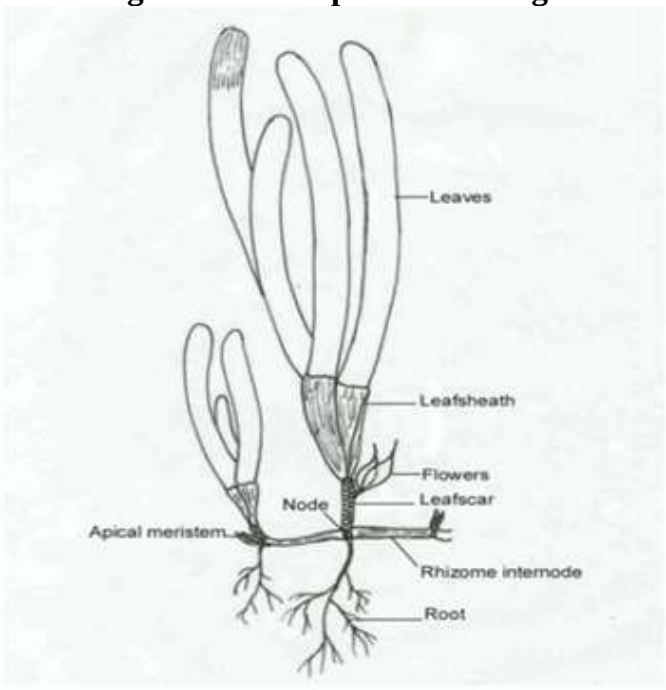
1.1. SEAGRASSES:

Seagrasses are marine flowering plants. They are capable of completing their life cycle when they are submerged in sea water. Ecosystem of Seagrass is one of the most widespread coastal vegetation types as compared to the ecosystems of coral and

mangrove [3]. Seaweeds (Algae), the plants that also colonise the sea are often confused with seagrasses; however, they are more primitive than seagrasses. In contrast to seagrasses, algae do not have a true root system (they have holdfasts) and do not have veins that carry molecules around the plant. Algae have spores and do not flower or produce fruit, while seagrasses have seeds and fruit. Seagrasses represent one of the highly productive coastal ecosystems of the world. The scientific communities and environmentalists in the tropics and particularly in India have given low priority in research on seagrass ecosystem. But, in the late nineties and early 2000's, the scenario has changed slowly and research activities started gaining momentum. Indian seagrass habitats are mainly limited to mud flats and sandy regions. It extend from the lower inter tidal zone to a depth of 10-15 m along the open shores and in the lagoons around the islands. Fresh seagrasses are the direct food source to sea urchins, fishes, seacows, geese and ducks and when they get decomposed, innumerable insect larvae and amphipods feed on them[4]. The major role of these plants is in protecting our planet from the increasing build-up of carbon dioxide and is responsible for about 15% of total carbon storage in the ocean. They can act as carbon sink, sequestering carbon dioxide from the atmosphere. Seagrasses are assigned mainly to six families encompassing 14 genera. Seagrass flora of India is represented by 6 genera and 13 species, out of which the Gulf of Mannar and Palk Bay harbour the maximum number of species followed by Andaman and Nicobar and Lakshadweep islands.**Fig.1 and Fig.2** shown the Seagrass and the typical plant parts of seagrass[5].

Fig.1: Seagrass beds in deep sea

Fig.2: Basic components of seagrass



Basic components of seagrass architecture

1.2. PHYTOCHEMICAL CONSTITUENT OF SEA GRASSES:

Phytochemical study on sea grasses has clearly shown that seagrasses have pharmaceutically active secondary metabolites. Different studies on these seagrass

metabolites proven that the extracted active components from saegrasses have alkaloids, glycosides, galactomannan gum, polysaccharides, peptidoglycan, hypoglycans, guanidine, steroids, carbohydrates, glycopeptides, terpenoids, amino acids and inorganic ions which directly or indirectly have a effective action against infection, pharmaceutically active hypolipemic agents, reduce blood pressure, and blood cholesterol levels. Phenolic compounds are effective secondary metabolites of seagresses which play a main role in various bio relevant activities in human body maintenance[6]. These phenolic compounds, which include phenol, tannin, and flavonoids.. The presence of phytoconstituents, such as flavonoids, phenols, and tannin, in seaweeds and seagrasses indicates a possibility that extracts may have anti-oxidant activity and anti diabetic activity. Roughage which is the crude fiber of seagrass constituents, consists of hemicelluloses and cellulose and a heterogeneous group. Pentagons from this heterogeneous group are generally predominant over pectin, cutin and lignin substances. This shows seeagrasses are the rich fiber foods as per the current dietary recommendations. These fibers have a many of physiological effects and provide many health benefits to human body. Ascorbic acid is an essential nutrient for humans, but an external dietary source is required because it is not synthesized by human metabolism. Ascorbic acid is involved in collagen synthesis, and bone and tooth calcification[7]. It is an oxidant and protects hydrogen/electron carriers within the cell and maintains suitable redox levels for the enzyme system. Ascorbate is one of the active metabolite components of seagrass which involved in the hormones and DNA biosynthesis. Essential vitamin Vitamin B3 (nicotinic acid) and Niacin is a true anti-pellagra agent is enriched in seagrasses. Detailed study on seagrasses will give effective

output to identify and invent pharmaceutically active compounds with cost effective manner to serve the society[8].

Seagrasses contain a combination of multiple compounds which need to be isolated and purified to achieve required therapeutic activity. This includes isolation of pure chemical substance, chemical structure elucidation, degradation chemistry, biological testing and analytical tests of the substance where the focus is on identification and quantification of the substance[9].

Chemical and physical method involves in the structural determination of isolated components which includes melting point, boiling point, determination of molecular weight, identification of active constituents by qualitative chemical test, UV Spectroscopy, mass spectrometry, nuclear magnetic resonance methods and molecule crystallography[10].

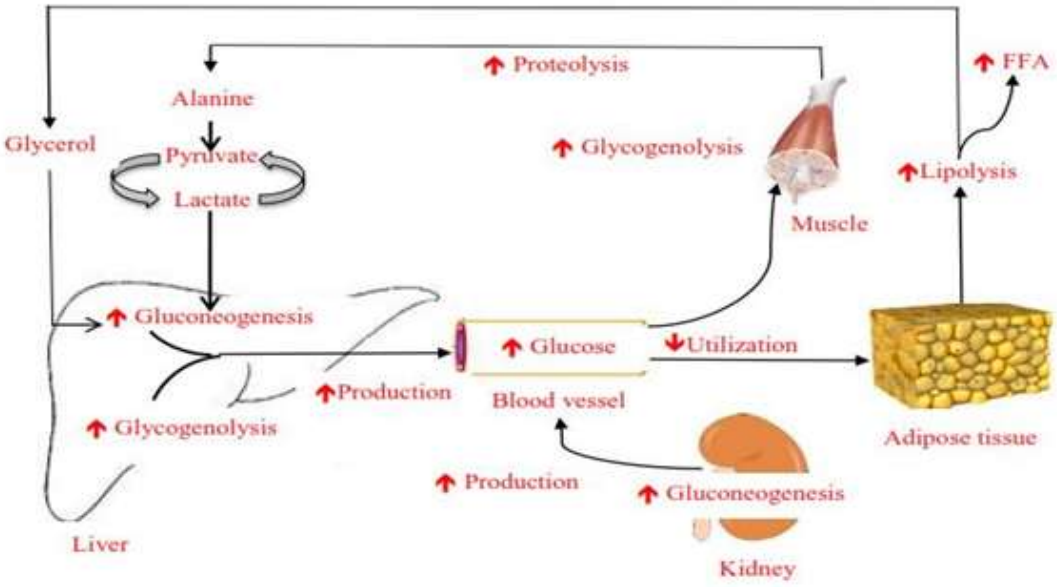
HPLC and High performance thin layer chromatography are the most familiar unique separation techniques used for isolation and determining purity, assay values of components.

1.3. DIABETES MELLITUS

The current research focuses on identification of a effective active component from the marine source for the treatment diabetes mellitus, Insulin insufficiency or insulin dysfunction cause the complex metabolic disorder which is knows as Diabetes mellitus, a major disorder in the world leading to massive economic losses[11].

As per the studies Diabetes is a chronic disorder of carbohydrate, fat and protein metabolism characterized by increased fasting and fed blood sugar levels in the human body. According to survey the world predominance of diabetes is measured 4% in 1995 and increase to 5.4% by the year of 2025. World health organization predicted that the developing countries will face major burden due to Diabetes. Studies conducted in India, highlighted that the prevalence of diabetes is high and also the rapid increase in the urban population. In India a study estimated that about 33 million adults affect with diabetes in the year 2025. The expected number of Diabetes patient will be around 57.2 million12.

Fig.3: Common metabolic disorders in diabetes mellitus patients. (↑) indicates increase or activation level (↓) decrease level or suppression.



1.4. CLASSIFICATION OF DIABETES MELLITUS

Classification of Diabetes mellitus is based on the pathogenic process which leads to hyperglycemia. Diabetes mellitus classified as: Type I Diabetes, type II Diabetes, gestational and other specific types of diabetes mellitus[13]

1.4.1. Type I -Diabetes mellitus:

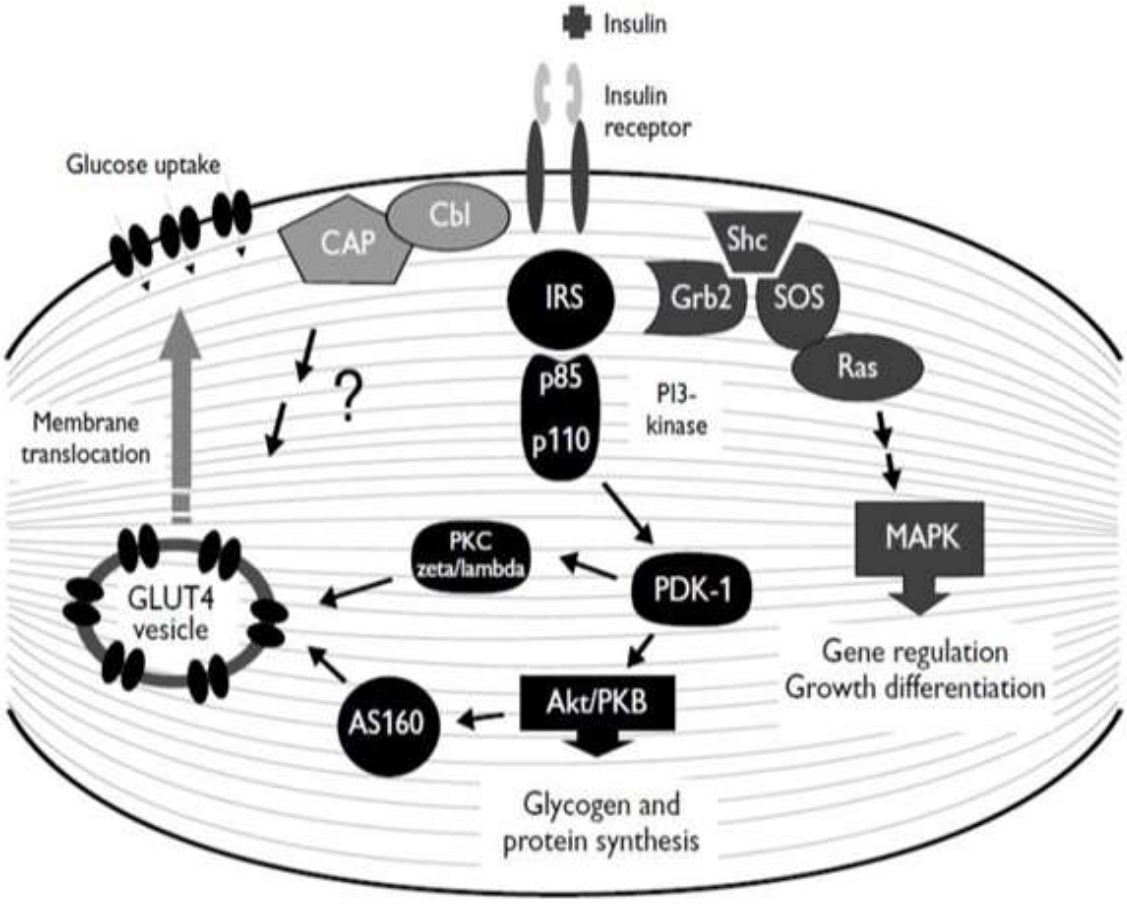
Diabetes mellitus type-I also called as insulin dependent diabetes mellitus or IDDM. Studies show that Diabetic patients about 10% have type I diabetes. Pancreatic β-cells autoimmune destruction leads to decrease plasma insulin concentration which increases the concentration plasma glucose. Also lack of insulin leads liver to produce excessive production of glucose and ketone and reduced the glucose utilization of the peripheral tissues. Then the lipolysis and proteolysis extended and the body enters into catabolic state. When the renal glucose re-absorption rate is maximum, the rate of filtration of glucose exceeds at kidney ,this condition leads to dehydration if this is untreated. Ketone formation causes diabetic ketoacidosis which is the complication of type I diabetes. This diabetic ketoacidosis which is potentially causes metabolic acidosis[14].

1.4.2. Type II -Diabetes mellitus

Type II diabetes mellitus also called as non-insulin dependent diabetes mellitus or NIDDM. Studies show that Diabetic patients about 90% have type II diabetes. However, Type II diabetes is typically a progressive disease but for significant percentage of patients Type II diabetes remains undiagnosed for several years. Obesity and lack of exercise leads to Type II diabetes. Worldwide Proportions of this type II

diabetes is increasing in large proportion. Multiple causes like life style and food habits lead to the development of Type II diabetes. This causes defects of target organs which respond to insulin (insulin resistance), with deficiency of β-cells shows how diabetes mellitus type II patients affected the signaling pathways of insulin- in the insulin receptor downstream of skeletal muscle[15].

Fig.4: Overview of insulin-signaling pathways in skeletal muscle (Glund and Zierath, 2005).



1.4.3. Gestational diabetes mellitus

This type of diabetes mellitus arises during second or third trimester of pregnancy. The women about 20-50% are affected with this type. Insufficient insulin production during pregnancy to meet the extra needs leads to Gestational diabetes[16].

1.4.4. Other specific types of diabetes mellitus

Maturity onset diabetes of the young (MODY) is a non-insulin dependent diabetes. Studies show that 1% to 2% of diabetes cases Diabetic patients about have this diabetic type[17].

1.5. DIABETES MELLITUS AND DYSLIPIDEMIA

Hyperlipidemia is a known cause in diabetic patients. Study says about 40% of diabetic patients affected through various metabolic derangements due to Hyperlipidemia. This leads to moderate increase in triglyceride (TG) levels, lower the high density lipoprotein cholesterol (HDL-C) levels. In type-II diabetic patients after eating TG-rich lipoproteins remnant lipoproteins, apolipoprotein B 100 (ApoB) are increased. Also, LDL-C particles are small in size and crowded, each particles are carrying less cholesterol so there are many LDL particles present in an type II diabetes individuals[18], which explained in the below

Increases the level of small dense LDLl. If the VLDL transported triglyceride is in high concentration then CETP promotes the LDL cholesterylester transfer or triglyceride are in exchange with HDL cholesterylester. Triglyceride rich HDL-C or

LDL-C can undergo hydrolysis by hepatic lipase or lipoprotein hydrolysis the Triglyceride rich HDL-C or LDL-C.

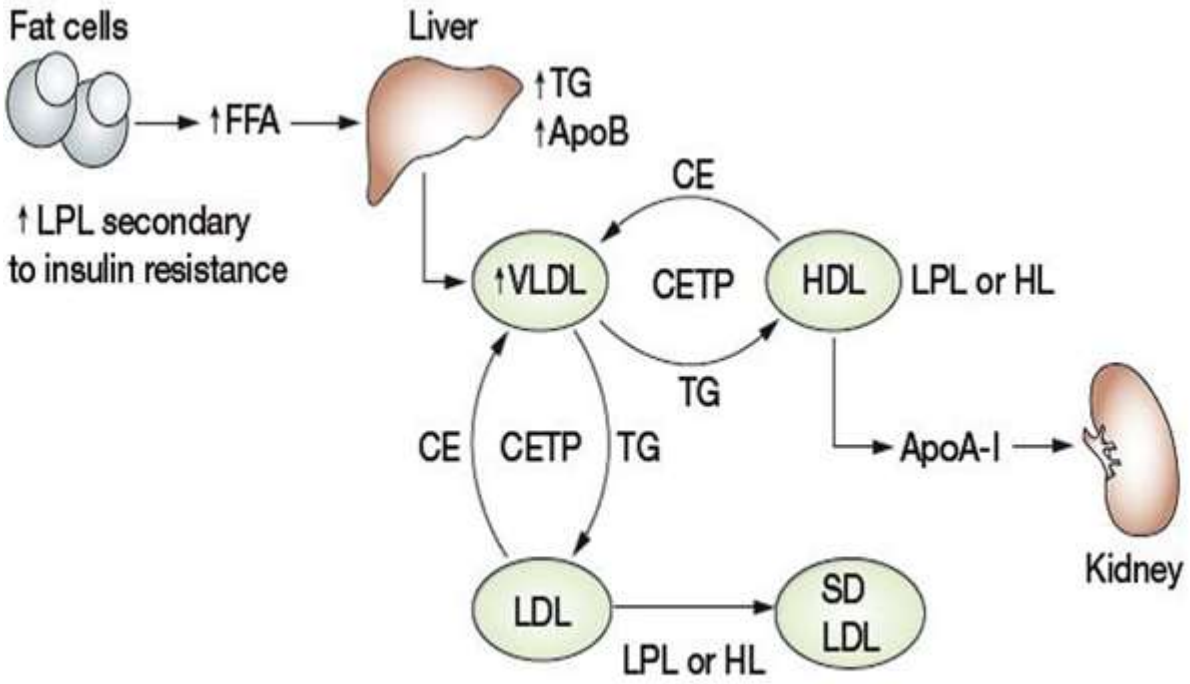


Fig.5: Insulin resistance role in diabetic dyslipidemia resistance of Insulin initiates the high triglyceride level and lower the level of HDL-C.

Abbreviations: increased level (↑);apolipoprotein A-1(ApoA-1,); , apolipoprotein B(ApoB);, cholesteryl ester (CE);, cholesterylester transfer protein (CETP);, free fatty acid (FFA); hepatic lipase (HL); lipoprotein lipase (LPL); small dense LDL cholesterol (SD LDL); triglyceride (TG)[19]

1.6. DIAGNOSIS OF DIABETES MELLITUS

Healthy human blood glucose level is about 100 mg/dL and up to 160 mg/dL on fasting state. 1.5.1. Fasting blood glucose and insulin levels

Above 126 mg/dL or 7.0 mmol/L Fasting blood glucose level indicates diabetes mellitus. Plasma insulin level is very low or undetectable during fasting and even after a meal indicates type I diabetes mellitus. A standard glucose load during glucose tolerance test increases plasma insulin concentration may be several times higher than the normal level [20]

1.6.1. Oral glucose tolerance test

When blood glucose concentration is in the range of 5.5-11.1 mmol/ L then oral glucose tolerance test recommended by WHO (World health organization). Above 200 mg/dL or 11.1 mmol/L Plasma glucose level after two hours of 75 g oral anhydrous glucose in water indicates diabetes mellitus[21].

1.6.2. Glycated hemoglobin (HbA1c)

Long term diabetes and its complications can be diagnosed by Glycated hemoglobin level this is a reliable measure of chronic glycemic control. Fasting or timed sample, may not required for this diagnosis. This HbA1c diagnosis method is a good screening tool for type II diabetes[22] .

1.7. TREATMENT OF DIABETES MELLITUS:

1.7.1 Type I diabetes mellitus treatment:

Metabolism of normal carbohydrates, proteins, and fats required Insulin

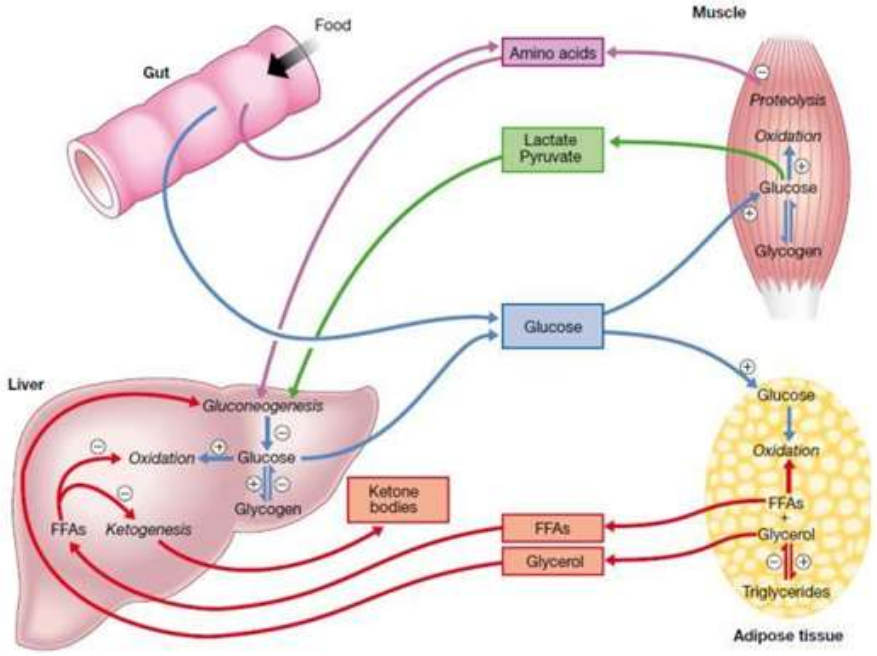


Fig.6: The actions of insulin on major metabolic pathways of fuel metabolism (+ve) indicates stimulation by insulin and (-ve) suppression by insulin.

Enough Insulin not produces for the patients with Type I diabetes mellitus therefore on exogenous insulin required for their survival. Preventing the micro and macro vascular complications and maintaining near normal glycemic control in the diabetic patients thereby they are live out their natural life span is the primary goal of diabetes mellitus treatment[23].

1.7.2 Type II diabetes mellitus treatment

Administration of oral hypoglycemic agents along with diet and exercise are encouraged for type II diabetes mellitus. Oral diabetic treatments subdivided into the hypoglycemic drugs and anti-hyperglycemic drugs, hypoglycemic drugs are sulfonylureas and benzoic acid derivatives and anti-hyperglycemic drugs are

biguanides, α-glucosidase inhibitors, and thiazolidinediones[24]. The action of these drugs shown in **Fig.7**

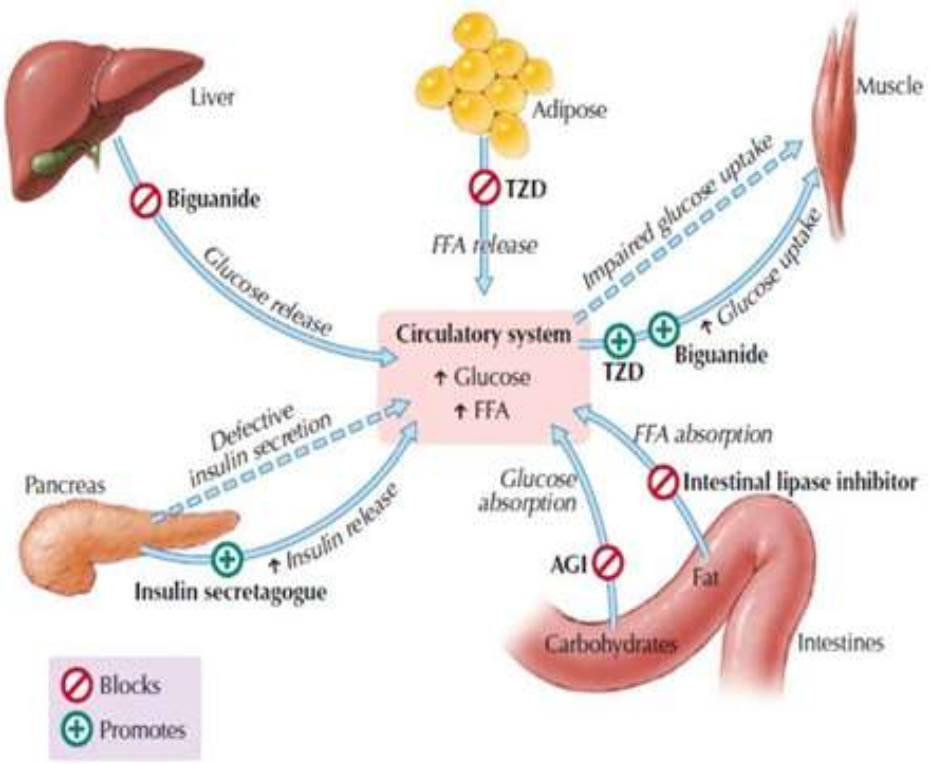


Fig.7: Major target organs and actions of oral hypoglycemic and antihyperglycemic agents in type-II diabetes. Thiazolidinedione (TZD) ; Free fatty acid(FFA); α-glucosidase inhibitor(AGI).

Patients with type II diabetes are not dependent on insulin treatment for survival as like type I diabetes mellitus patients. However, many of these patients will show decreased insulin production over a period of time during stress or illness or when oral antihyperglycemic and hypoglycemic drugs fail to lower blood glucose[25].

The below **Table-1** shows the detail of some anti-diabetes mellitus drugs and their molecular target, site of action and adverse reactions.

Table-1: Anti-diabetes mellitus drugs and their molecular target, site of action and adverse reactions:

Antidiabetic Drugs	**Molecular target**	**Site of action**	**Limitations/Side Effects**
Sulfonylureas	SU receptor, K+ ATP Channel	Pancreatic β-cells	Hypoglycemia, weight gain
Biguanides	Not clear	Liver and muscles	Gastrointestinal disturbances, lactic acidosis
Alpha-glucosidase inhibitors	α-glucosidase	Intestine	Gastrointestinal disturbances
Thiazolidinediones	PPAR-γ	Fat , liver and muscles cells	Liver toxicity, weight gain, high LDL-C, high cost
Exenatide	GLP-1 receptor	Pancreatic α-cells, intestinal mucosal cells	Nausea, hypoglycemia, diarrhea
Sitaglipitin	DPP-4	Intestine	Headache, nausea
Insulin	Insulin receptor	Liver, Muscle and fat cells	Hypoglycemia, weight gain

1.8. Natural products and diabetes mellitus

Prolonged medications required for the treatment of diabetes mellitus, considering the limitations and side effect in the synthetic drugs, Natural products derived from Medicinal plants and marine products are being looked for the treatment of diabetes. However components of medicinal plants and marine products are the base of many conventional drugs. One of the majorly using oral glucose lowering agent Metformin development was based on the diabetes treatment using the plant Galega officinalis. This plant is containing hypoglycemic component guanidine. Clinically Guanidine proved as toxic compound, Guanidine derivatives alkyl biguanides synthalin

A and alkyl biguanides synthalin B were introduced as oral antidiabetic agents in the year 1920 in Europ. But after insulin became more widely available, guanidine derivatives were discontinued[26].

Experience of guanidine and biguanides over the treatment of diabetes leads to the development of anti diabetic drug metformin.

Studies indicates over 1200 plants and marine products are traditionally used for the treatment of diabetes mellitus among them more than 200 pure compounds have showed lowering blood glucose activities. considering low toxic, ready availability and cost effective, usage of natural products for the treatment of diabetes mellitus has recommended and encouraged by WHO (World Health Organization) worldwide[27].

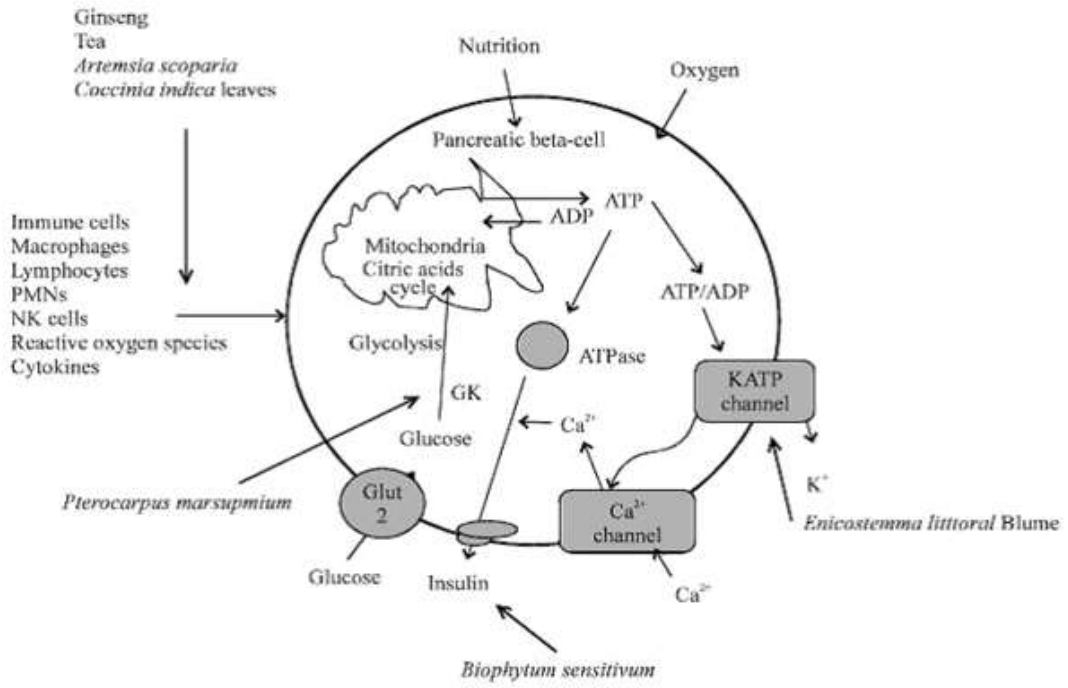
Among the marine products, seagrasses place an important role in the diabetic treatment.

The main active constituents derived from seagrasses which have antidiabetic activity include alkaloids, glycosides, galactomannan gum, polysaccharides, peptidoglycan, hypoglycans, guanidine, steroids, carbohydrates, glycopeptides, terpenoids, amino acids and inorganic ions which directly or indirectly affect the level of blood glucose in the human body.

The antidiabetic activity of seagrasses depends upon variety of mechanisms. like adrenomimeticism, pancreatic beta cell potassium channel blocking, cAMP (secondary messenger) stimulation; providing certain necessary elements like calcium, zinc, magnesium, manganese and copper for the beta-cells. These active constituents of

natural sources reduce the blood glucose level by activate or regenerate the βcells which leads to enhance the insulin release. Also inhibit β-galactocidase and α-glucocidase which prvent the oxidative stress. This avoid the pancreatic β-cell dysfunction which is a well known cause of diabetes. The active constituent also Stimulate the glycogenesis, glycolysis and citric acid cycle which activily involve the glucose metabolism via reduce the blood glucose level[28].

The mechanism of action of some hypoglycemia herbal drugs and action sites are explained by the **Fig.8**



Administration of hypoglycemia herbal drugs enhances the insulin secretion by closing the potassium channels (KATP) by increasing the ratio of ATP and ADP in cytoplasm resulting from glycolysis and Krebs cycle. Closing of ATP- sensitive potassium channels (KATP) leads to opening voltage-gated Ca2+ channels which increase free Ca2+ concentration in the cytoplasm and finally insulin secretion triggers.

The herbal medicines from plant and marine sources have equal efficient like synthetic drugs in the diabetic treatment. But considering low toxic, ready availability and cost effective, usage of natural products for the treatment of diabetes mellitus mostly recommended WHO (World Health Organization) worldwide and WHO encourages the further investigation on treatment of diabetes mellitus using herbal drugs[29].

Chapter II
Aims And Objectives

AIM AND OBJECTIVE OF THE STUDY

This study is to evaluate the medicinal values and the application of seagrass *Halodule uninervis* (Forsk.) Asch. belongs to the family Cymodoceaceae. This seagrass posses medicinal activities and used for traditional treatment. However, there are few scientific studies carried out to explain the medicinal activities of this sea plant. The current study on *Halodule uninervis* to target a detailed evaluation of its constituents and the important pharmacological activity of those particular constituents[30]. The study details are as follows

- Preparation of a monograph of *Halodule uninervis*.
- Find out the ash content, acid insoluble ash, acid soluble ash and fluorescence analysis for powdered plant.
- Extractions of active constituents from the plant as crude.
- Preliminary phytochemical screening by TLC and qualitative chemical analysis
- Find out the adulteration due to any metallic material by atomic absorption spectroscopy.
- To explore the main pharmacological activity of Methanolic extract of *Halodule uninervis by* in *vivo* and in *vitro* studies.
- Isolation and Purification of selective and active phyto constituents by column chromatography.
- Determination of purity and Elucidation of structures for the isolated components by various spectroscopic methods.

Chapter III
Review of Literature

REVIEW OF LITERATURE

Akinmoladun *et al* 2007 reported that medicinal plants are now being paid more consideration than ever since they have potential of numerous benefits to the world or indeed to all especially in the line of medicinal and pharmacology. The bioactive of phytochemical constituents in the plants are responsible for the medicinal value of these plants this produce specific pharmacological activity in human body[31].

The WHO (World Health Organization) has predicted that for about 3.4 billion people from the developing country may use the plants as primary source of medicines which means about 88% of the population in the world depends on conventional herbal medicines for their key health care (Famsworth, 1985; Farnsworth, 1988)[32].

Butler *et al, 2004* studied all globally prescribed drugs were derived from natural products between 1983 and 1994 were about 41%. In the year of 2000 the percentage usage drugs that derived from natural products were 40% which has approximately remained constant after the year 2001[33].

Farnsworth *et al*, 1988 reported that the traditional medicines tender assured results to look the global increasing requirements for new pharmacological active agents. Inadequate evident for most plants to assure their safety, efficacy and quality. Plants and their parts include number of constituents and few of them are more toxic eg. most anti-cancer drugs that derived from plant, pyrrolizidine alkaloids and digitalis[34].

Brown, *et al* 1992 reported phytotherapeutic agent's adverse effects are less frequent when compared against drugs that synthesized from chemicals; Well-explained clinical trials now established that such pharmacological activity effects really exist[35] .

Rajendran *et al, 2003* reported the marine products possess quite novel structures which lead to pronounced biological activities and novel pharmacology. The study of such compounds lead to most successive results for the development of new pharmacological classes with cost effective manner[36].

Thakur Narsinh L *et al*, 2005, evaluated the Indian costal regains hold treasures in terms of natural products which have many pharmaceutical potential values as like the natural products in the land. It is infinite and will remain undiscovered unless research into drug development establishes the medicinal value of the chemical compounds they hold. Marine herbal medicine sometimes referred as herbalism or botanical medicine because for their medicinal or therapeutic value and has been used throughout history by all cultures[37].

Kannan, L. and Veluswamy, K. (1989). studied and reported that seagrasses are the plants similar to grass which grow in or around the aquatic marine ecosystems. The seagrass marine flowering plants and produce fruits and seeds which differentiate this from marine algae [38].

Prof. L. Kannan* and Dr. T. Thangaradjou studied about the major taxonomic relation between Seagrasses and grass family (Poaceae). Seagrasses are the group of angiosperms, they are specifically adapted to grow in marine ecosystems. Seagrass includes 13 genera and approximately 72 species that belong to the families

Zosteraceae, Potamogetonaceae, Posidoniaceae, Cymodoceaceae, Hydrocharitaceae and Ruppiaceae. They adapted to grow in in both tropical and temperate regions of coastal marine environments on almost every part of the world [39].

McRoy, C.P, and McMillan, C. (1977) explained about the production ecology and physiology of seagrasses. In tropical seas, genera such as Halodule, Cymodocea, Halophila, Enhalus, Syringodium, Thalassodendron and Thalassia are represented. However, in moderate regions some species of these genera are also found. Species of Amphibolis, Phyllospadix, Heterozostera, Posidonia, Zostera and Pseudalthenia are usually seen only to moderate seas. Seagrasses growing vertical from rhizomes rooted in the sediment and remains on the ocean floor, they are the only angiosperms that are able to succeed underwater in marine environments [40].

AF Newmaster *et al,* 2011 explained about seagrasses population world wide , seas, bays, backwaters, estuaries and lagoons of shallow and sheltered localities of tidal and subtidal zones. Seagrasses are usually prefer sandy, muddy, coral rubble substrate, and clayey, they also grow in crevices and on rocks. Seagrasses grow either homogenously or heterogeneously and forming dense, thick meadows which produce large biomass that provide tremendous environment in the marine system [41].

Ramamurthy et al, 1992, evaluated and reported that the Seagrasses produce world's most productive and biodiverse marine ecosystems. Seafloor residue stabilization and nutrients recycling in the sea are some valuable ecosystem services of the seagrasses. The erosion during the storms prevented by these services of the seagrasses and provides significant environment for many infantile marine invertebrates and spawning

fish. Seagrasses are also primary rich food sources for dugongs, green sea turtles and manatees, and they serves as a hidden areas for small fishes and sea animals form big fish and predators like some large mammals and birds to save their lives themselves[42].

Vasanthi et al, 2006 eveluated different studies on these seagrass metabolites proven that the extracted active components from saegrasses have alkaloids, glycosides, galactomannan gum, polysaccharides, peptidoglycan, hypoglycans, guanidine, steroids, carbohydrates, glycopeptides, terpenoids, amino acids and inorganic ions which directly or indirectly have a effective action aganst infection, pharmaceutically active hypolipemic agents, reduce blood pressure, and blood cholesterol levels[43].

Achamlale S *et al,* 2009 evaluated the presence of phenolic compounds are effective secondary metabolites of seagresses which play a main role in various bio relevant activities in human body maintenance. These phenolic compounds, which include phenol, tannin, and flavonoids[44].

Kannan, L et al, 2005 evaluated the presence of phytoconstituents, such as flavonoids, phenols, and tannin, in seaweeds and seagrasses indicates a possibility that extracts may have anti-oxidant activity and anti diabetic activity. Roughage which is the crude fiber of seagrass constituents, consists of hemicelluloses and cellulose and a heterogeneous group[45].

Krishnamurthy, V. 2005, evaluate the presence of pentasons from this heterogeneous group are generally predominant over pectin, cutin and lignin substances. This shows seeagrasses are the rich fiber foods as per the current dietary recommendations. These

fibers have a many of physiological effects and provide many health benefits to human body[46].

Omaye ST et al, 1979 evaluated the essential of Ascorbic acid nutrient for humans, but an external dietary source is required because it is not synthesized by human metabolism. Ascorbic acid is involved in collagen synthesis, and bone and tooth calcification. It is an oxidant and protects hydrogen/electron carriers within the cell and maintains suitable redox levels for the enzyme system. Ascorbate is one of the active metobolitic components of seagrass which nvolved in the hormones and DNA biosynthesis[47].

Poppy Mary Vimalabai et al 2003, evaluated the Essential vitamin Vitamin B3 (nicotinic acid) and Niacin is a true anti-pellagra agent is enriched in seagrasses. Detailed study on seagrasses will give effective output to identify and invent pharmaceutically active compounds with cost effective manner to serve the society[48].

WHO,2009 studied among the world population, 246 million was estimated as global diabetes mellitus burden in 2007, As per the IDF (International Diabetes Federation) by the year 2025 this figure may rise to 380 million and in women likely 55% deaths occur[49]

WHO,2013, Estimated around 347 million diabetic people among the world population and by the year 2030 this may rise to 438 million, if proper precautions and execution strategies are not kept in place. In India the conducted studies highlighted that the occurrence of diabetes is high and also the rapid increase in the urban population mostly due to environmental factors such as nutrition, food habits and toxins of

chemicals. In India by the year 2025, a study estimated that about 33 million adults expected to affect with diabetes. The expected number of Diabetes patient will be around 57.2 million in overall population[50].

Wild *et al*., 2004 and WHO 2002 estimated the occurrence of diabetes for all age-groups worldwide was expected to be 2.8% in 2000, and is likely to rise to 4.4% in 2030. The people between the age group 35 and 64 years in developing countries affected most frequently by diabetes mellitus, most probably type II diabetes[51].

WHO 2002, expected that worldwide high blood pressure may attribute 7.1 million deaths, high cholesterol 4.4 million and excessive body weight may attribute 2.6 million deaths globally. This may directly or indirectly relate to diabetes mellitus[52].

WHO,1985 reveled the major known reasons for diabetes are identified as genetic predisposition (inheritance) and environmental factors such as nutrition, food habits and toxins of chemicals[53].

The Expert Committee on the Diagnosis and classification of Diabetes, 1997 studied abput The chronic hyperglycemic nature of diabetes is linked with extended damage, failure, dysfunction of multiple organs, mainly the eyes, heart, kidneys, blood vessels and nerves. A number of pathophysiological processes are mixed up in the progress of diabetes mellitus. These range from autoimmune damage of the β-cells in pancreatic followed by abnormal insulin deficiency leads to insulin action resistant. Abnormal fats , proteins and carbohydrates, metabolism observed due to lack and inadequate action of insulin on tissues target that lead to abnormalities and failure of multiple organ. Growth destruction and possibilities of certain infections may also

connect with chronic hyperglycemia. Retinol dysfunction associated with loss of vision; renal failure due to nephropathy; foot ulcers resulting from peripheral neuropathy, amputations, cardiovascular symptoms charcot joints, sexual dysfunction and autonomic neuropathy causing gastrointestinal, genitourinary are the prolonged complications of diabetes[54].

Davis et al, 1996 evaluated the symptoms of hyperglycemia consist of; Frequent urination (polyuria), Frequent hungry (polydipsia), body weight loss, sometimes with polyphagia, and vision deficiency. Non-ketotic hyperosmolar syndrome and hyperglycemia with ketoacidosis are the delicate life threatening consequences of uncontrolled diabetes[55] .

Riturparna and Neeraj, 2007 reported the oxidative stress is a causative factor in the diabetes mellitus[56].

Brownlee *et al*, 1995 evaluated the tissue damaging reactive oxygen species increases due to diabetes this increases glucose production by non-enzymatic protein glycosylation and glucose auto oxidation[57].

Wild *et al*., 2004 reporetd the oxidative complication of diabetes takes place in the major site known as vascular endothelium. Increase in Hydrogen peroxide (H_2O_2) production and superoxide anion (O_2^-) production, decrease the bio availability of nitrogen oxide (NO) in in vascular tissue and the cultured endothelial cells due to hyperglycemia (Riturparna and Neeraj, 2007),. Endothelial dysfunction is a well-known characteristic occurrence in diabetes mellitus[58] .

Riturparna and Neeraj, 2007 were evaluated there is a constant increase in the diabetes mellitus patients world wide due to: lifestyles, population growth, aging, low birth weight, less physical activity, urbanization, excess diets, psychological stress, smoking, and increase in occurrence of obesity[59].

Siddharth, 2001 reported In 21st century Diabetes mellitus is the major challenging metabolic disorder because it increases the risk of renal and cardiac disorders and affects every cell's essential biochemical activities in the body. The global survey stated that nearly 10% of the world population affected by Diabetes mellitus[60].

Davis and Granner, 1996 reported diabetes mellitus is characterized by excessive blood glucose in the due to insufficient insulin production by the pancreas or insulin ineffectiveness to control blood sugar level[61].

Sharma, 1993 was evaluated this disorder affects the metabolism of protein, fat and carbohydrate, eyes, kidneys and nerves affected possible secondary complications due to glycation that caused by chronic hyperglycemia[62].

Greenspan and Gardner, 2001 explained about the type of diabetes mellitus. diabetes mellitus classified into Type I or insulin-dependent diabetes mellitus (IDDM) Type-II or non-insulin-dependent diabetes mellitus (NIDDM). In these Type I Diabetes patients the pancreatic β-cells no longer produce insulin due to the destruction of β-cells by immune system has destroyed them. Treatment for IDDM usually involves Taking insulin injections or induce insulin through insulin pump, controlled diets, routine physical exercise, cholesterol and blood pressure controlling are the right choice to control the I or insulin-dependent diabetes mellitus [63].

The Expert Committee on the Diagnosis and classification of Diabetes, 1997 reported, Worldwide 5-10% of patients with diabetes Type I diabetes mellitus.

Davis, 1996 reported, the β-cell destruction rate is varying age group wise. Infants, destruction of β-cell quit fast and slow in children and adults. Keto-acidosis is the first symptom in children and adult may present with the diabetes mellitus and leads to hyperglycemia [64].

Kaplan *et al.,* 1989 reported about 90-95% among the diabetic population affected by type II diabetes. This type of type II diabetes mostly occurs after 40 years of age. It is also known as adult's onset diabetes.[65]

Abel *et al*., 2001 evaluated the patients with insulin resistance and have relative insulin deficiency affected by Type II diabetes. In type II diabetes Insulin resistance due to insufficient reaction to circulate insulin by insulin targeted tissues like skeletal muscle, adipose, and liver usually leads to the characteristic hyperglycemia [66].

Schenk *et al*., 2008 reported the obesity in type II diabetes patients leads to insulin resistant . Hyperglycemia gradually develops several years and in earlier stages it was not harsh enough to identify due to identify any diabetes symptoms. Even though patients with this stage of diabetes may have normal or elevated insulin levels, blood glucose larger levels would be anticipated to higher insulin values result and even function of β-cells also normal. Slow development of insulin resistance due to is defective in insulin secretion in these leads to the risk of developing this type-II diabetes and increases with age, lack of physical activity and obesity[67].

WHO, 1985 explain the gestational diabetes and brittle diabetes are other forms of diabetes mellitus. Monogenic defect in β-cell activity are connected with hereditary in an autosomal governing pattern, and are often characterized by onset of hyperglycemia at an early age, usually before the age of 22 years and called as maturity-onset diabetes (MOD) and are diagnosed by lack of insulin secretion with low or no defects in the action insulin (WHO, 1985)[68].

Huang and Czech et al,. 2007 explained about the Insulin physiological effects in the human body are far reaching. More or Less insulin in body circulation directly related to the defects observes in the human body. The major effect of this hormone play main role in the storage, and consumption of carbohydrates, proteins and fats. Major insulin effect observed in the skeletal muscle, adipose tissue, smooth muscle, cardiac muscle, pancreas and liver. Normal glucose levels in the human body maintained by Adipose tissue. Its main role is the energy storage in triglycerides form of glucose disposal is the key role for the skeletal muscle. GLUT 4, GLUT 8 and GLUT 12 are the glucose transporters in Adipose tissue that are responsible for the transport of glucose into the cells. The major hexose transporter in adipose tissue is GLUT[69].

Duncan *et al*., 2007 et al., reported Adipose tissues have visceral fat storage in very elevated lipolytic rates, this resulting in the release of large amounts of fatty acids into the system. Insulin normally suppresses the lipase and adipocytes triglyceride lipase enzyme that hydrolyses intracellular triglyceride but in the insulin resistant state, the activity of this enzyme is enhanced resulting in a free fatty acid flux[70].

Ruan and Lodish, 2002 explained about the large amounts of a protein released by Adipose tissue which known as tumour necrosis factor (TNF-α) that plays a major role in the repression of many genes in the body which are responsible for the uptake and storage of glucose as well as fatty acids. TNF-α also mediates the inflammatory process which is associated with obesity and type II diabetes[71].

Huang and Czech, 2007 reported the glucose stored as glycogen in skeletal muscle which oxidizes when required producing energy[72].

Perriot *et al*., 2001 reported that the deficiency of glucose-6-phosphatase enzyme in this tissue, accounts for around 75% of the whole insulin in human body stimulated glucose uptake. In a normal 70 kg man muscle tissues stored around 500 to 600 gram of glycogen, but because of the deficiency of glucose-6-phosphatase enzyme in this tissue[73].

Perriot *et al*., 2001 reported that the GLUT 4 is a specialized transmembrane sugar transporter of glucose. This transport the glucose into the cells and catalyses transport of glucose through the plasma membrane. This GLUT 4 transporter works in a cycle with other transporter like GLUT 1, 5 and 12 to improve the glucose transfer via facilitative diffusion. Insulin has many effects on the muscle, with the most important ones being the increased entry of glucose. When insulin binds to its receptors, tyrosine phosphorylation of protein substrates occurs and this activates the P13 kinase pathway[74].

Huang and Czech, 2007 studied about GLUT 4 acivate the subsequent signaling pathways with eventually moving from its intracellular stores to the plasma membrane.

This transporter has a unique characteristic such that its N and COOH terminals direct both endocytic and exocytic processes[75].

Abel *et al*. (2001) demonstrated that sensitivity to insulin was markedly higher as was the response of uptake of glucose in transgenic mice which had higher expression levels of GLUT 4. In type II diabetic patients there is as much as a 90% reduction in levels of GLUT 4 that are responsive to insulin[76].

Huang and Czech, 2007 explained about translocation of transporters interfered with subsequent signaling pathways. This is one way that results in the characteristic insulin resistant (IR) state found in type II diabetics. There is also an increased GLUT 4 expression in muscle in response to exercise[77].

Lewis *et al*., 2002 reported the Insulin first reaches liver through bloodstream. Insulin appaly effects on the liver by either promoting anabolism or inhibiting catabolism. The liver facilitates the body to keep normal glucose concentrations in the blood in fasting and postprandial states. When insulin levels are low, results glycogenolysis and increase the production of hepatic glucose[78] .

Luca and Olefsky, 2007 reported 100 to 110 g of glycogen or approximately 440 kilo calories of energy can able to store in the liver which is the maximum storage capacity. Glycogen synthesis promoted by Insulin and store as well as prevent glycogen breakdown to glucose. These effects are mediated by changes in the activity of enzymes in the glycogen synthesis pathway. Insulin inhibits the expression of key gluconeogenic enzymes such as G-6-phospatase leading to elevated levels of glucose production in the liver[79] .

Greenspan and Gardner, 2001 studied the Insulin increases both protein and triglyceride synthesis and very low density lipoproteins (VLDL) formation by the liver. Individuals with type II diabetes have a higher incidence of liver function transferases (LFTs) abnormalities than individuals without diabetes[80].

Harris, 2005 reported that the alanine aminotransferase (ALT) most common abnormality. ALT levels generally decreases due to antidiabetic agents which reduce blood glucose level in the body. The human pancreas is build up by two verities of tissues, called exocrine and endocrine. The exocrine tissue (acini) produces digestive enzymes. This digestive enzymes help to breakdown proteins, carbohydrates, fats and acids in the duodenum where as the endocrine pancreas (islets of langerhans) has a hormonal activity. It produces insulin, somatostatin, gastrin and glucagon. These hormones play important roles in maintaining salt homeostasis and glucose level in the body[81].

Riturparna and Neeraj, 2007 stated that in the developing nations the continued increase in prevalence of diabetes can be largely recognized to economic development, urbanization and westernization. Risk factors which are majorly contributing are population ageing, obesity, sedentary lifestyles, over-processed diets, smoking, psychological stress and low birth weight[82].

Qatanani and Lazar, 2008 reported, in obesity associated type II diabetes mellitus, there is an increased buildup of visceral fat which made up of pro-inflammatory molecules such as α-tumour necrosis factor (TNF-α), which involved in the insulin sensitivity regulation in the human body[83].

Qatanani and Lazar, 2008 studied and reported the other molecules such as adiponectin whose levels are low in obesity improves insulin sensitivity, reduce output of glucose and oxidation of fatty acid in the liver[84] .

Lichtenstein and Schwab, 2000 reported the the contributing factor to obesity is poor dietary choice this poor diet associated with disorders like type II diabetes mellitus. Epidemiological evidence has demonstrated that saturated fatty acid intake is associated with increased risk of insulin resistance, diabetes and impaired glucose tolerance. The inclusion of foods rich in *trans*-fatty acids and high ratios of saturated to unsaturated fats results in weight gain and predisposition to diabetes. Foods such as red meats, refined grains, sweets and high fat dairy products have been linked to risks of type II diabetes. In contrast, weight loss is characterized by reduction in fat cell mass especially visceral fat which contain inflammatory markers associated with insulin resistance and decreased insulin sensitivity[85].

Mlinar *et al*., 2006 reported that reduced visceral fat due to weight loss is accompanied by decreased adipose TNF-α release resulting to improved insulin sensitivity[86].

WHO, 2002 stated the diagnosis of diabetes mellitus criteria accepted by world health organization (WHO) based on a venous plasma glucose concentration of more than 11.1 mM, after two hours of a 75 g glucose tolerance test[87].

Carpenter and Coustan (1982) reported the criteria for unusual glucose tolerance in pregnancy[88].

Proposal from the Fourth International Conference of American Diabetes Association's on Gestational Diabetes Mellitus in March, 1997 recommended the use of Carpenter and Coustan diagnostic criteria as well as the other use of a diagnostic 75g, two hour oral glucose tolerance test (OGTT) (EC, 1997)[89].

As per National Diabetes Data Group, 1979, the new diagnostic criteria for diabetes mellitus have been very much simplified. The OGTT which was earlier recommended by the National Diabetes Data Group in 1979, has been substituted with the proposal that diagnosis based on two fasting plasma glucose levels of 2500 mg/L (13.8 mM) or more (WHO, 2002; WHO, 2009)[90].

WHO, 1985 and Gutteridge, 1999 reported fasting glucose plasma level determination is the a perfect diagnostic test, but grouping of any two abnormal test results can be used. of Fasting Glucose plasma determination is considered as the primary diagnostic test; as this measure the adverse outcomes like retinopathy[91] .

WHO, 2002 stated diet and physical activity are the non-pharmacological treatment of diabetes (). Hydrotherapy, Acupuncture and conventional drugs includes; oral hypoglycemic agents, exogenous insulin, transplantation and mineral supplementation (WHO, 2002). Are other methods of treatment of diabetic? About five types of oral glucose reducing drugs that approved by the management of diabetes mellitus. Oral therapy is specifying in any patient in whom fail in exercise and diet to attain suitable glycemic control. Oral hypoglycemic drugs may lose their action though the earlier response may be good, in a major percentage of patients.

The glucose lowering drug types include; biguanide, sulfonylurea, α-glucosidase inhibitor, meglitinide and thiazolidinedione[100].

Pandey *et al*., 2011 reported various side effects resulting the administration of these drugs which includes; weight gain caused by of sulfonylurea due to hyperinsulinemia, biguanide administration leads to body weakness, tiredness, lactic acidosis and thiazolidinediones-a alpha glucosidase inhibitor may increase LDL-cholesterol level leads to diarrhea[101].

Ribes *et al*., 1986 reported when glucose control is sub-optimal at maximum dose of oral treatment then Insulin is usually given as an oral agent. Common side effects of insulin are weight gain and hypoglycemia. Increase in atherogenesis may also a side effect of vigorous insulin treatment[102].

Kelly *et al*., 1995 reported insulin secretion enhanced by stimulating the pancreatic β cells when treating with oral glucose-lowering agent like sulfonylurea, tolbutamide and glyburide. These act on liver cells enhancing glucose breakdown in glycolytic pathway and preventing glucose generation[103].

Foye et al., 1995 reported Sulphonylureas acts by preventing KATP channels in pancreatic β cells plasma membranes. The prevention works to enhance the insulin secretion which is comparable to that formed by glucose in the body but is of a different mechanism. Particularly in patients who cannot tolerate metformin, then these drugs may be used as first-line drugs in a case where oral hypoglycemic medication is necessary[104].

Foye et al., 1995 stated recent drugs in this group such as glipizide and glimipramide appear to afford equal effectiveness than earlier drugs such as gliclazide[105].

Malender *et al.,* 2004 reported Sulphonic acid-urea nucleus present in all sulphonylureas drugs and to make different drugs a different chemical moieties are added at various positions. The mechanism of action of the derived drugs may have the preferred effect, however, the influence and efficiency may differ considerably[106].

Zhou *et al*., 2001 studied and reported Metformin a biguanides derivative act in Presence of endogenous insulin and increasing glucose transport across the skeletal muscle cell membrane. Insulin resistance patients Metformin is widely used for treatment of diabetic because safely it can be used as an addition to diet treatment in obese patients to reduce their high glucose levels particularly those who are not approachable to other treatments. The right mode of action of metformin is uncertain. activates adenosine monophosphate protein kinase (AMPK) in liver cells leads to increase fatty acid oxidation and glucose uptake by cells. A general reduction in lipogenesis and hepatic glucose generation is usually observed. Metformin has antioxidant property which is useful in treatment of cardiovascular disease and diabetes treatment[**107**].

Rahimi *et al*., 2005, explained to inhibit xanthine oxidase and phosphodiesterase, advanced glycation end product formation and decreased production of tumour necrosis factor[108] .

Qatanani and Lazar, 2007 reported Thiazolidinediones act by raising the sensitivity of peripheral tissues against insulin by disturbing the expression of specific genes.

They attain this by binding and activate the γ peroxisome proliferator receptor (PPAR-γ) which is a nuclear receptor [109].

Greenspan and Gardner, 2001 ststed the effects of this gene response include, the increase in the expression of the glucose transporters will increase the differentiation of pre-adipocytes to adipocytes and decrease the hepartic glucose output[110].

Sharma and Staels, 2007 reported the high attraction of this drug to PPAR-γ is significant in the management of insulin resistant because large adipocytes that distinguish from smaller one to produce TNF-α which raise insulin resistant. Thiazolidinediones therefore suppress the activity of these adipokines which involved in insulin resistant[111].

Greenspan and Gardner, 2001 studied thet Alpha-glucosidase acts by reducing the alpha glucosidase enzyme in the brush border of the small intestine. This delays the glucose absorption by reducing the breakdown of carbohydrate complex by enteric digestive enzymes. Some of the most usually used α-glucosidase inhibitors like acarbose lead to severe gastrointestinal side effects such as flatulence, diarrhoea, and pain in abdomen. This leads to the need for alternate sources of these inhibitors that have less or no side effects . The most apparent choice for these substitutes would be drugs which obtained from plants with ethnomedical uses in treatment of diabetes[112].

Rai and Carpinella, 2006 reported long-standing medications required for the treatment of diabetes mellitus, considering the limitations and side effect in the synthetic drugs, Natural products derived from Medicinal plants and marine products are being looked for the treatment of diabetes. However components of medicinal

plants and marine products are the base of many conventional drugs. One of the majorly using oral glucose lowering agent Metformin development was based on the diabetes treatment using the plant Galega officinalis. This plant is containing hypoglycemic component guanidine. Clinically Guanidine proved as toxic compound, Guanidine derivatives alkyl biguanides synthalin A and alkyl biguanides synthalin B were introduced as oral antidiabetic agents in the year 1920 in Europ. But after insulin became more widely available, guanidine derivatives were discontinued[113].

Studies indicates over 1200 plants and marine products are traditionally used for the treatment of diabetes mellitus among them more than 200 pure compounds have showed lowering blood glucose activities. considering low toxic, ready availability and cost effective, usage of natural products for the treatment of diabetes mellitus has recommended and encouraged by WHO (World Health Organization) worldwide[114].

Among the marine products, seagrasses place an important role in the diabetic treatment.

The antidiabetic activity of seagrasses depends upon variety of mechanisms. like adrenomimeticism, pancreatic beta cell potassium channel blocking, cAMP (secondary messenger) stimulation; providing certain necessary elements like calcium, zinc, magnesium, manganese and copper for the beta-cells[114].

These active constituents of natural sources reduce the blood glucose level by activate or regenerate the βcells which leads to enhance the insulin release. Also inhibit β-galactocidase and α-glucocidase which prvent the oxidative stress. This avoids the pancreatic β-cell dysfunction which is a well known cause of diabetes. The active

constituent also Stimulate the glycogenesis, glycolysis and citric acid cycle which activily involve the glucose metabolism via reduce the blood glucose level[115].

Szkudelski, 2001 reported that the Streptozotocin -induced diabetic male BALB/c mice is one of the most useful tools existing for estimation of the value and safety of anti diabetic activity of medicinal plants[115] .

Ghatta and Ramarao, 2004 stated mice are majorly used in scientific research because they are very ease to handle and having more degree of relation they share with the human beings. For type I and II diabetes mellitus, obesity, and insulin resistance study selective breeding has many models and strains. Many new animal Mice models are available to test routes of delivery , dosage , toxicity, and efficacy of a newly invented drugs . high fat diet feeding mice model is representing as a working model for investigating the effects of excessive fat diet intake in human, and this model of mice be a symbol of non-insulin dependent diabetes mellitus (NIDDM). This excessive fat diet fed mice model extensively used for elucidating prevention and/or reversal of insulin resistance mechanisms[116].

H. Bagg, 1913, the BALB/c mice obtained from the albino stock model and so named as BALB or Bagg albino. They were inbred by MacDowell, Cold Spring Harbor, NY, USA in 1923 and scientist Snell, who included the ‘c’ in 1936 for the albino to differentiate from other models and hence getting the name BALB/c[118].

Abbort *et al*., 1997 reported biochemical markers are majorly applied to identify, analyze and observe the treatment-related outcome following of exposure to xenobiotics. There are three main roles they play in toxicology, to verify deleterious

agent exposure, to provide a system to monitor individual susceptibility to a toxicant and to quantities assess harmful effects of a toxicant to an organism or individual (Amacher, 2002). Because the liver is a common target for adverse effects of all drugs and other chemicals. Improper biomarkers hepatic responses to xenobiotics are of specific interest to the pharmaceutical toxicologist[119].

3.1 PLANT PROFILE

Botanical Name : *Halodule uninervis* (Forsk.) Asch[120].

English Name : Narrowleaf seagrass[121]

Family : Cymodoceaceae[122]

Morphology:

Halodule uninervis is a sublittoral seagrass found from the mid-intertidal to a depth of 20 m. Its characteristic features include: shoots up to 30 cm long and erect, having 2-4 leaves in each branch, leaf linear, narrowed at base with sheath, margin entire, nerves 3, midrib conspicuous and lateral ribs inconspicuous, ending in well developed lateral teeth at leaf apex, teeth tridentate. It can grow in a range of different habitats and very common between 0-3 m in sub-littoral lagoons and in front of reefs. It is very fast growing colonizes rapidly and can flower prolifically. It can form dense meadows at some sites or is patchy and intermixed with other seagrass species, and frequently observed on back reefs in association with larger algae[122].

Vernacular names[123]:

English Name : Narrowleaf seagrass

Tamil : Sinna eekku passi

Malayalam Poduvu koraai

Arabic A'shab bahriya

Ethnomedical properties and uses:

The leaves, rhizomes and roots of Sinna eekku passi have great medicinal value. The plant is used both *in-vivo* and *in-vitro.* The leaf paste used for the treatment of dandruff and skin disease like burns and boils. Even the leaf extract reported to be

effective in the early stages of Leprosy. The extract showed antifungal and anti microbial activities. Dried root used as medicine for fever, malaria and in pneumoniae. Powder of dried rhizome used for the treatment of mental illnesses. Dried powder of whole plant used for the treatment of cough and used as a tranquillizer for babies. Leaves mixed with sesame oil and consumed with daily meals to cure the iron deficiencies. Seagrasses are known to produce secondary metabolites as defence mechanism under stress conditions and these compounds are found to be anti-oxidative in nature. Biochemical analysis of *H.uninervis* estimated carbohydrate protein, lipid, tannin and phenol, Cardiac alkaloids, flavone glucosides, saponins, palmitic acid, linoleic acid, phenylethane derivative, (S)-methoxy-(3-,5-dimethoxy-4-hydroxyphenyl) ethanediol, 3,4,5-trihydroxy benzoic acid, (E)-3-(4-methoxyphenyl)-2-propenoic acid, syringin (7), 5-hydroxy-3-4 7-trimethoxyflavone, and 4'-hydroxy-3',5,7-trimethoxy flavones [124].

3.2. MORPHOLOGICAL CHARACTERS OF *Halodule uninervis*

3.2.1.Description:

Halodule uninervis is a sublittoral seagrass which is a flowring plant which found from the mid-intertidal to a 20 meter depth in the sea. This has leaves, root, stem raizomes. *Halodule uninervis* can raise in a range of multi habitats and vary in sub-littoral lagoons common between 0-3 m and in front of reefs. It grow very fast as colonizes and can flower generously, can form dense meadows or at some areas patchy or sometimes mixed with other seagrass species . It can regularly observe in association with larger algae. It is a favoured foods of the Dugong and fishes and many sea animals[125].

3.2.2.Pharmacognosy:

Halodule uninervis has long narrow stem tiny roots. whole plant is soft to the touch, and tiny. Shoots up to 30 cm long and vertical, Rhizomes creeping, white to pale brown, having flat narrow, short leaves. each branch having 2-4 leaves, leaves are linear, pointed at base with entire cover margin, 3 nerves , conspicuous midrib and inconspicuous lateral ribs, well developed lateral teeth ending in leaf apex and tridentate teeth. Root bark is 0.5 mm to 1.5 mm thick, outer surface brown, longitudinally wrinkled. Fracture is tough, fibrous, taste slightly bitter. Microscopically the root shows tetrarch stele. Transverse section of a mature root shows periderm consisting of 6-9 layers of cork, 1-4 layered phellogen, 5-7 layers of phelloderm and a wide zone of phloem and the central wood. The phloem consists of sieve tubes, companion cells, phloem parenchyma, a few stone cells traversed by phloem rays[126].

Stem Bark: The bark stem crop up in pieces shoots up vertical, 30 cm long and 3-6 mm thick. Outer surface is green to brownish green colored and rough due to the presence of lenticels. Having smoothie greenish white Inner surface.

Leaves: The leaves are long narrow, thin, pointed at base with entire cover margin, 3 nerves , conspicuous midrib and inconspicuous lateral ribs, well developed lateral teeth ending in leaf apex and tridentate teeth[127].

3.2.3 Distribution:

The global distribution of this species is recorded in costal area of India, Indonesia, South africa and SriLanka. In India, it occurs in Lakshadweep islands, Gulf of Mannar, Palk Bay, Tamilnadu Anthra Pradesh coast, Andaman and Nicobar islands[128].

3.2.4 Cultivation:

It can be propagated through sea weeds and raizomes[129].

Halodule uninervis

Chapter IV
Scope and plan of work

SCOPE AND PLAN OF WORK

From the extensive review of literature done, it was found that the whole plant of *Halodule uninervis* have not been subjected to any scientific studies for their antidiabetic potential so far or very little work has been carried out. Hence these plant will be explored for characterization and evaluation of antidiabetic activity by subjecting them to a series of tests.

PLAN OF WORK

- Collection, identification and authentication of plant materials.
- Successive Soxhlet extraction of the powdered seeds of *Lepidium sativum* (n-hexane, chloroform, ethyl acetate and methanol) and powdered whole plants of *Halodule uninervis* (petroleum ether, chloroform, ethyl acetate and methanol)
- Phytochemical screening of *Halodule uninervis* plant extracts.
- Evaluation of antioxidant activity of *Halodule uninervis* plant extracts by
    * DPPH Photometric assay
    * Hydroxyl radical inhibition activity
    * Superoxide anion scavenging activity
- Estimation of flavonoid content in *Halodule uninervis* plant extracts.
- Evaluation of *in-vio* antidiabetic activity of methanolic extracts of *Halodule uninervis* in streptozotocin induced rats.
    - Acute toxicity study by OECD guidelines 423
    - Study of Glucose tolerance

- Preparation study protocol
- Study of anti diabetic activity in streptozotocin induced rats.
- Study of Liver and kidney functions
- Antioxidant status
- Estimation of Biochemical parameter
- Histopathological Investigation
- Statistical Analysis

➢ Isolation of the active ingredients from methanolic extract of *Halodule uninervis* plants by column chromatographic method.

➢ Characterization and identification of the isolated active principles by FT-IR, ^{13}CNMR, ^{1}HNMR and GC-MS.

➢ Assessments of in-vitro pharmacological activities of methnolic extract and isolated compounds.

- Evaluation of anti oxidant activity of Methanolic extract of *Halodule uninervis and isolated compounds* include DPPH free radical scavenging activity, Xanthine oxidase inhibition assay and Hydrogen peroxide scavenging assay.
- Anti microbial activity of methanolic extract and isolated active constituents from *Halodule uninervis*
- Evaluation of the angiogenesis activity of *Halodule uninervis* extract using the chorio allantoic membrane assay in chick embryos.

Chapter V
Materials & Methods

MATERIALS AND METHODS

5.1 COLLECTION OF PLANT MATERIAL:

Whole plant of *Halodule uninervis* were collected in and around local costal area of Ramanathapuram, Tamilnadu during December and authenticated by the Botanist Prof.Chelladurai, Department of Botany, Govt. Siddha Medical College, Tirunelveli. A voucher herbarium specimen number KMCP/HU/02/2015 was also preserved in the K.M.College of Pharmacy, Madurai. All the collected samples were washed immediately using native sea water to remove the adhering salts and sands, several times with distilled water. The whole plants were dried in shade for 3weeks. Then about 1 kg of the shade dried plant was made in to segregated, pulverized by a mechanical grinder and passed through a 40 mesh sieve. The powdered plant materials were stored in an airtight container, and used for further studies and investigation[130].

5.2 PHYSICOCHEMICAL ANALYSIS:

The Physicochemical parameters are Loss on drying, total ash, acid insoluble ash, water soluble ash and fluorescence analysis were performed[131]to find out the contamination of any mineral content as per the procedure below and The results obtained are given in **Table -2 to 3.**

5.2.1 Determination of Total ash

Place about 3 g of the ground material, accurately weighed, or the quantity specified in the monograph, in a suitable tared dish (for example, of silica or platinum), previously ignited, cooled and weighed. Incinerate the material by gradually increasing

the heat, not exceeding 450 °C, until free from carbon; cool, and weigh. If a carbon-free ash cannot be obtained in this way, exhaust the charred mass with hot water, collect the residue on an ashless filter-paper, incinerate the residue and filter-paper, add the filtrate, evaporate to dryness, and ignite at a temperature not exceeding 450 °C. Calculate the content in mg of ash per g of air-dried material[132].

5.2.2 Determination of acid-insoluble ash

Boil the ash for 5 minutes with about 25 mL of hydrochloric acid (~70 g/l) TS; collect the insoluble matters in a sintered silica crucible, wash with hot water and filtered with ashless filter-paper, The residue was ignite in a sintered silica crucible at about 600 to 650 °C to constant weight. Calculate the content in mg of acid-insoluble ash per g of air-dried material[133].

5.2.3 Extractive values

The extractive values were recorded in alcohol and water with a view to study the distribution of various constituents of plant powder of *Halodule uninervis*. Accurately weighed about 4.0 g of coarsely powdered air-dried material was placed in a glass-stoppered conical flask and macerated with 100 ml of the solvent for 6 h, shaking frequently, and then allowed to stand for 18 h. The mixture was filtered rapidly taking care not to lose any solvent. Twenty-five millilitres of the filtrate was transferred to a tared flat-bottomed dish and evaporated to dryness on a water bath. The residue was dried at 105°C for 6 h, cooled in a desiccator for 30 min, and weighed without delay[134].

5.2.4 Determination of water soluble Ash

The ash was boiled for about 5 minutes with 25ml of distilled water. Insoluble matter collected in crucible or on an ash less filter paper and washed with hot water, ignited at about 600°C and weight. Percentage of water insoluble ash was calculated with reference to the air dried drug[135].

5.2.5 Extractive values

The extractive values were recorded in petroleum ether and chloroform with a view to study the distribution of various constituents of plant powder of *Halodule uninervis*. Accurately weighed 4.0 g of coarsely powdered air-dried material was placed in a glass-stoppered conical flask and macerated with 100 ml of the solvent for 6 h, shaking frequently, and then allowed to stand for 18 h. The mixture was filtered rapidly taking care not to lose any solvent. Twenty-five millilitres of the filtrate was transferred to a tared flat-bottomed dish and evaporated to dryness on a water bath. The residue was dried at 105°C for 6 h, cooled in a desiccator for 30 min, and weighed without delay[136].

5.2.6 Fluorescence Analysis

The plant powder was subjected to fluorescence analysis after being separately treated with water, NaOH, H_2SO_4, HCl, HNO_3, nitrocellulose. Since many herbs fluorescence when powder is exposed to UV light and this can help in their identification method. The fluorescence character of the plant powder was studied both in day light and UV light (254 and 366 nm)[137].

5.3 PHYTOCHEMICAL INVESTIGATION OF THE VARIOUS EXTRACTS OF *Halodule uninervis*

5.3.1 Extractions of active constituents from the plant *Halodule uninervis*

The powdered plant materials of *Halodule uninervis* was subjected to preliminary Phytochemical screening for the detection of various plant constituent on the following lines[138].

5.3.1.1 Plant Parts used:

Shade dried powdered plant materials passed through 40 mesh sieve of *Halodule uninervis.*

5.3.1.2 Method:

Hot Percolation

5.3.1.3 Materials used:

Materials used for extraction with various solvents

1. Soxlet apparatus
2. R.B. flask
3. Condenser
4. Heating Mantle
5. Rotary vacuum evaporator

5.3.1.4 Chemicals Used:

Petroleum Ether

Chloroform.

Ethyl acetate

Methanol

Previously soaked about 1000 gm of dried coarsely powdered plant material was extracted in soxhlet assembly with petroleum ether at 40◦ – 60◦ C by continuous hot percolation method. The extraction was continued for 72 hours. The petroleum ether extract was filtered and concentrated by using rotary vacuum evaporator to a dry mass (9.7 gm). A Dark green residue was obtained. The marc left, after petroleum ether extraction was taken for further 72 hours extraction with chloroform. The chloroform extract was concentrated to the dry mass. A greenish brown residue was obtained (12.3 gms).The marc left, after chloroform extraction was taken and further extracted with Ethyl acetate for 72 hour. The concentrated ethyl acetate extract was in Yellowish Brown residue (16.8 gms).

The further extraction of marc from ethyl acetate was taken and extracted with Methanol for about 72hrs obtained Brownish green residue (65.3gms) [139].

5.3.1.5 Calculation of percentage yield:

The percentage yield was calculated for the extracts and major compounds with reference to the crude material taken using the formula given below.

$$\% \text{ yield of extract} = \frac{\text{Weight in grams of extracts obtained}}{\text{Weight in grams of plant material taken}} \times 100$$

5.3.1.6 Results:

The percentage yield of successive solvent extractive values of *Halodule uninervis* is tabulated in **Table-4**.

The extracts above obtained were stored in air-tight amber- coloured glass containers at 4°C - 6°C and the storage containers labelled as

- **Petroleum ether extract - *Halodule uninervis* [PE-HU]**
- **Chloroform extract - *Halodule uninervis* [CE-HU]**
- **Ethyl acetate extract- *Halodule uninervis* [EAE-HU]**
- **Methanol extract - *Halodule uninervis* [ME-HU]**

5.3.2 Thin Layer Chromatography:

To identify the major components present in the Extract a different number of solvent systems were tried and the solvents n-Hexane: Ethyl acetate: Formic acid was identified. Thin layer chromatographic studies of the hexane extract of Halodule uninervis.

5.3.2.1 Test solution: Reflux 5 gm of crude extract with 25 ml of methanol for 1 hour. Filter and evaporate to dryness. The residue Dissolved in 2 ml Methanol.

Solvent system I: n-Hexane: Formic acid (8.5:1.5)

Solvent system II: n-Hexane: Ethyl acetate: Formic acid (6:2:2),

Solvent system III: n-Hexane: Ethyl acetate: Formic acid (5:4:1),

Solvent system: IV: n-Hexane: Ethyl acetate: Formic acid (3.5:5:1.5),

Solvent system V: n-Hexane: Ethyl acetate: Formic acid (3:5:2),

5.3.2.2 Detection: Visual, UV light and fluorescent light **Fig. 9**. Rf values of different solvent systems tabulated in **Table-5**

5.4 QUALITATIVE CHEMICAL EVALUATION OF EXTRACTS:

The whole plant extracts like petroleum ether, chloroform, ethyl acetate, Methanol extracts and the powder were subjected to qualitative chemical analysis as per the following procedure [140]

5.4.1 Tests for alkaloids:

5.4.1.1 Dragondroff's Test

To 1 ml of the extract, 1 ml of Dragondroff's reagent was added; formation of orange red precipitate indicated the presence of alkaloids.

5.4.1.2 Wagner's Test

To 1 ml of the extract, 2 ml of Wagner's reagent was added; the formation of a reddish brown precipitate indicated the presence of alkaloids.

5.4.1.3 Mayer's Test

To 1ml of the extract, 3 ml of Mayer's reagent was added; the formation of full white precipitate confirmed the presence of alkaloids.

5.3.1.4 Hager's Test

To1ml of the extract, 3 ml of Hager's reagent was added; the formation of yellow precipitate confirmed the presence of alkaloids.

5.4.2. Test for carbohydrates:

5.4.2.1 Molisch Test

To 2ml of the extract, 1 ml of α-naphthol solution was added, and concentrated sulphuric acid was added through the sides of test tube. Purple or reddish violet colour at the junction of the two liquids revealed the presence of carbohydrates.

5.4.2.2 Fehling's Test

To 1ml of the extract, equal quantities of Fehling's solution A and B were added, upon heating formation of a brick red precipitate indicated the presence of carbohydrates.

5.4.2.3 Benedict's test

To 5ml of Benedict's reagent, I ml of extract solution was added and boiled for 2 minutes and cooled. Formation of a red precipitate showed the presence of carbohydrates.

5.4.3 Tests for proteins and amino acids:

5.4.3.1 Biuret Test

To 1 ml of the extract add 1 ml of 40% sodium hydroxide solution was added followed by 2 drops of 1% copper sulphate solution. Formation of a violet colour showed the presence of proteins.

5.4.3.2 Xanthoprotein Test

To 1 ml of the extract 1 ml of concentrated nitric acid was added. A white precipitate was formed, it was boiled and cooled. 20% of sodium hydroxide or ammonia was subsequently added, and orange colour produced was indicated the presence of aromatic amino acids.

5.4.3.3 Lead Acetate Test

To the extract, 1 ml of lead acetate solution was added. Formation of a white precipitate indicated the presence of proteins.

5.4.3.4 Ninhydrin Test

Two drops of freshly prepared 0.2% ninhydrin reagent was added to the extract solution and heated. Blue colour was developed and it was revealed the presence of proteins, peptides or amino acids.

5.4.4 Tests for phytosterol:

5.4.4.1 Libermann Burchard Test

The extract was dissolved in 2 ml of chloroform in a dry test tube. 10 drops of acetic anhydride and 2 drops of concentrated sulphuric acid were added. The solution became red, then blue and finally bluish green, indicated the presence of steroids.

5.4.4.2 Salkowski Test

The extract was dissolved in chloroform and equal volume of concentrate sulphuric acid was added. Formation of bluish red to cherry red colour in chloroform

layer and green fluorescence in the acid layer represented the steroid components in the tested extract.

5.4.5 Tests of glycosides:

5.4.5.1 Legal Test

The extract was dissolved in pyridine and sodium nitro prusside solution was added to make it alkaline. The formation of pink red to red colour showed the presence of glycosides.

5.4.5.2 Baljet Test

To 1 ml of the test extract 1 ml sodium picrate solution was added and the yellow to orange colour revealed the presence of glycosides.

5.4.5.3 Borntrager's Test

A few ml of dil HCl was added to 1 ml of the extract solution. It was then boiled, filtered and the filtrate was extracted with chloroform. The chloroform layer was then treated with 1 ml of ammonia. The formation of red colour showed the presence of anthraquinone glycosides.

5.4.5.4 Keller Killiani Test

The extract was dissolved in acetic acid containing traces of ferric chloride and it was then transferred to a test tube containing sulphuric acid. At the junction, formation of a reddish brown colour, which gradually became blue, confirmed the presence of glycosides.

5.4.6 Test for saponins:

Foam test 1ml solution of extract was diluted with distilled water to 20 ml and shaken in a graduated cylinder for 15 minutes. Development of stable foam suggests the presence of saponins.

1ml extract was treated with 1% lead acetate solution. Formation of white precipitates indicates the presence of saponins.

5.4.7 Test for flavonoids:

5.4.7.1 Shinoda Test

To 1 ml of the extract, magnesium turnings were added followed by the addition of 1-2 drops of concentrated hydrochloric acid. Formation of red colour showed the presence of flavanoids.

5.4.8 Test for tannins and phenolic compounds:

To 1 ml of the extract, ferric chloride was added, formation of a dark blue or greenish black colour product showed the presence of tannins.

To the extract, potassium dichromate solution was added, formation of a precipitate showed the presence of tannins and phenolic compounds.

5.4.9 Test for triterpenoids:

Two or three granules of tin metal in 2 ml thionyl chloride solution was dissolved and 1 ml of the extract was added into the test tube. The formation of a pink colour indicated the presence of triterpenoids.

5.4.10Test for fixed oils:

5.4.10.1 Spot Test

A small quantity of extract was pressed between two filter papers. Oil stains on paper indicated the presence of fixed oils.

5.4.10.2 Saponification Test

To 1 ml of the extract few drops of 0.5N alcoholic potassium hydroxide was added along with a drop of phenolphthalein. The mixture was heated on a water bath for 1-2 hours. The formation of soap or partial neutralization indicated the presence of fixed oils.

The obtained results were given in the **table-6**

5.5 DETERMINATION OF CONTAMINATION LEVELS OF HEAVY METALS IN *Halodule uninervis* PLANT BY ATOMIC ABSORPTION SPECTROPHOTOMETRIC ANALYSIS

Medicinal plants play an important role in traditional medicine and are widely consumed as home remedies. The past decade has seen a significant increase in the use of herbal medicine due to their minimal side effects, availability and acceptability to the majority of the population of under developing countries and wild fruits to overcome the malnutrition(192). Since times immemorial, plant based drugs have been in use in the amelioration of various ailments ranging from common cold to cancer. Relatively high levels of essential elements, such as Fe, Mn, Zn, and Ca, have been demonstrated to influence the retention of toxic elements in animals and human beings[141]. The quantitative estimation of various trace element concentrations is

Important for determining the effectiveness of the medicinal plants in treating various diseases and also to understand their pharmacological action142. The continuous intake of diets that are excessively high in a particular trace element can influence changes in the functioning, forms, activities of some organs or concentrations of such element in the body tissue and fluids can rise above the permissible limit143. Heavy metals (HMs) pollution is a result of increasing industrialization throughout the world, which has penetrated into all sectors of the food industry [144].

In 1991 Codex Alimentarius Commission give safe and maximum allowable limits of elements in fruits and vegetables for Cd (0.2mg/kg dry weight), As (0.2mg/kg dry weight), Hg (10mg/kg dry weight), Cu (40mg/kg dry weight), and Zn (60mg/kg dry weight) (Codex Alimentarius Commission, 1991)200. WHO-2003 set the provisional tolerable weekly intake (PTWI) for As (0.015mg/kg body weight), WHO-1995 set

intake for Pb (0.025mg/kg body weight) whilst WHO- 1991 set the total intake of mercury (0.005mg/kg body weight)[145].

5.5.1 Standard preparation

The selected heavy metals were cadmium, lead, arsenic, and mercury. For each of the selected metals a standard linear calibration curve of various concentrations ranging from 0.5000 ppm, 1.0000 ppm and 1.5000 ppm (three points) were analyzed by AAS and storing as stock solutions in a quartz flask.

Heavy metal analysis performed in selected plant extract by Varian model AA 240 FS atomic absorption spectrophotometer (AAS). Measurements were made for cadmium, lead, arsenic, and mercury at wavelengths of 228.80 nm, 283.31 nm, 193.70 nm and 253.7 nm respectively using a hollow electron discharge lamp (EDL) with the 0.5nm slit width. Analysis was performed by testing samples at three different concentrations 0.5000 ppm, 1.0000 ppm and 1.5000 ppm to ensure that the method has wide adaptability and good accuracy. Parameters used for Atomic absorption spectroscopy is tabulated in **table-7**

Table-7: Parameters used for Atomic absorption spectroscopy:

Parameters	**Cadmium**	**Lead**	**Arsenic**	**Mercury**
Lamp	Cadmium Electron Discharge Lamp	Lead Electron Discharge Lamp	Arsenic Electron Discharge Lamp	Mercury Electron Discharge Lamp
Wavelength	228.80nm	283.31nm	193.70nm	253.65nm
Fuel gas	2.5L/min (Acetylene)	2.5L/min (Acetylene)	5.5L/min (Argon)	5.5L/min (Argon)
Support gas	15.0 L/min (Air)	15.0 L/min (Air)	15.0 L/min (Air)	15.0 L/min (Air)

Contamination levels of heavy metals in *Halodule uninervis* Plant and Results Obtain from atomic absorption spectrophotometer (AAS) given in **Table-8.**

5.6 ESTIMATION OF TOTAL FLAVONOID CONTENT IN VARIOUS EXTRACTS OF *HALODULE UNINERVIS* ESTIMATION OF TOTAL FLAVONOIDS [134]

Instrument: Shimadzu UV Visible spectrophotometer, Model 1800

Reagents: 1% Vanillin in 70% conc. H_2SO_4.

Procedure:

To different concentrations of the extract, 4ml of the vanillin reagent was added and kept in a boiling water bath for 15min and the absorbance was read at 360 nm. Catechol was used as a standard. The amount of flavonoids present was determined by linear regression analysis and expressed as mg catechol equivalents /g of extract. The results obtained are illustrated in **Table 9**.

5.7 EVALUATION OF *IN-VIVO* PHARMACOLOGICAL ACTIVITY STUDIES OF METHANOLIC EXTRACT OF *HALODULE UNINERVIS*.

The Methanol extract obtained from the successive extraction of *Halodule uninervis* was shown a well response for most of the qualitative chemical tests which conforms that the effective extraction of maximum constituents from the plant material. As the more constituents present in the Methanolic extract, Evaluation of the pharmacological activity further proceed[149].

Antidiabetic activity study against the streptozotocin induced diabetic in mice

Experimental animal Male Swiss albino mice (2-3 months old) mice, weighing about 25 to 30g, was kept under standard conditions, about 12 hours in both light and dark and fed with standard diet pellet, water, ad libitum. Animals were adapted to laboratory condition for a period of one day before conducting the experiments. All animal experiments were conducted according to the rules and regulations of institutional animal ethical committee clearance (R.Karthikeyan/ PhD/ IAEC/ KMCP/ 105/2013-2017).

5.7.1 Acute Toxicity Studies:

Acute toxicity study was carried out according to OECD guideline. Methanolic extract of *Halodule uninervis* a dose range of 50 mg to 450 mg / kg was administered intraperitoneally to different group of animals (six in each group). The animals kept under observation for 2h, to identify any symptoms of toxicity (behaviors like pattern, tremors, sleep, coma) and or death. The observation was continued for further 2 weeks of time [150].

5.7.2 Glucose tolerance:

Overnight fasted animals were divided to group of three (n=6). 1 mL of Normal saline given orally to Group I. Concentrations of 50, 450 mg / kg *of Halodule uninervis* extract was administered to Groups II and III, respectively. Glucose (2 g/kg body weight.) was administered and blood samples were taken at 0.5 hr, 1hr, 2hr and 4hr time from the vein in glucose administration. Blood Glucose was estimated and the Data was used as a hypothetical reference to determine the extract dose level. This data used in evaluation effects of *Halodule uninervis* extract on diabetic rats.

5.7.3 Treatment Protocol:

Animals were divided in to five groups; six in each group received the treatment schedule as tabulated below.

Table-10: Treatment schedule

Group	Study
I	Normal control (saline).
II	Streptozotocin treated control (150 mg/kg.ip)
III	Streptozotocin (150 mg/kg.ip) + *Halodule uninervis* extract (50mg/kg.ip)
IV	Streptozotocin (150 mg/kg.ip) + *Halodule uninervis* extract (150 mg/kg.ip)
V	Streptozotocin (150 mg/kg.ip) + *Halodule uninervis* extract (250 mg/kg.ip)

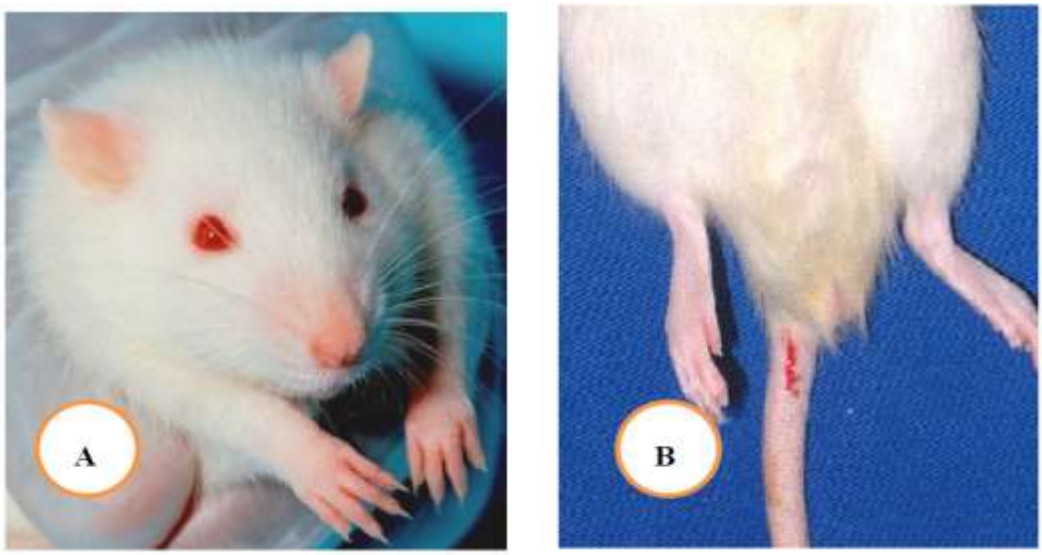
5.7.4 Work Design:

Animals were divided in to five groups as shown in table. Group-I is the control for which no drug administered. Group –II is diabetic control for which 150mg/kg streptozotocin was injected to induce diabetic. Each Group III, IV and group-V received 150mg of Streptozotocin along with 50, 150 and 250mg of methanolic extract of Halodule *uninervis.*

Experimental animal Male Swiss albino mice (5-9 weeks old) mice, weighing about 20 to 30g, was kept under standard conditions, about 12 hours in both light and dark and fed with standard diet pellet, water. Animals were adapted to laboratory condition for a period of one day before conducting the experiments. All animal experiments were conducted according to the rules and regulations of institutional animal ethical committee clearance.

Overnight fasted animals were divided to group of three (n=6). 1 mL of Normal saline given orally to Group I. Concentrations of of 50, 450 mg / kg of *Halodule uninervis* extract was administered to Groups II and III, respectively. Glucose (2 g/kg body weight.). blood samples were taken at 0.5 hr, 1hr, 2hr and 4hr time from the vein in glucose administration. Blood glucose concentrations (mg/100 ml) were estimated using a Glucometer- by glucose oxidase determination method. From the tip of tail, blood samples were collected at the mentioned time patterns and the hypothetical data were used to determine the extract dose level. This data used in evaluation effects of *Halodule uninervis* extract on diabetic rats[152].

Male Swiss albino mice (A-**Fig.9**) and incision of tail vein for blood reading (B-**Fig.10**)



5.7.5 Anti diabetic effects:

Treatment was given as per the protocol. Blood samples were collected at 2, 4, 6 and 8 hours intervals from the fasted rat's tail vein prior to administration of the extract. Glucose levels were measured from the separated Serum According to the protocol the non-fasted animals daily treated with Methanolic extract of *Halodule uninervis* for 18 days. Blood samples were collected on 6^{th} day, 9^{th} day, 12^{th}, 15^{th} and 18^{th} day after Streptozotocin and glucose levels in blood serum were measured. The graphical representation given in **Fig.12 and Fig.13**.

5.7.6 Liver and kidney functions:

ALP (Alkaline phosphatase), creatinine, GPT (glutamate pyruvate transaminase) and BUN (Blood urea nitrogen) and creatinine were measured (**Table-13)**. Total WBC count was determined by using a hemocytometer and the body weight of treatment rats were checked. The results tabulated in **Table-14**. The graphical representation given in **Fig.14 and Fig.15**.

5.7.7 Antioxidant status:

GSH (reduced gluta-thione), GPx (glutathione peroxidase), SOD (superoxide dismutase), catalase, and MDA (malondialdehyde) levels were determined in In liver homogenates by spectrophotometrically using assay kits available commercially. Total nitrites present in aortic tissues were colorimetrically estimated by using Griess reagent. Concentration of nitrite in the sample was calculated using sodium nitrite standard and normalized to the aorta protein content[153]. the results given in **Table-15**.

5.7.8 Collection of blood, pancreas and kidney:

At the end of the experimental period, all rats were fasted overnight and anesthetized with ether for blood collection. Blood was collected from the retroorbital plexus from each rat by capillary tube and introduced into clean dry bottles (EDTA

bottles) for hematological parameters while heparinized tubes were used to collect blood for biochemical estimation. The heparinized blood was centrifuged within 5 min of collection at 2500 rpm for 10 minutes. Serum was collected into a clean dry sample container[154]. The results were tabulated in **Table-14.**

5.7.9 Estimation of Biochemical parameter:

Serum glucose , plasma insulin (estimated by ELISA method using Boehinger Mannheim Gmbh kit, werk Penzberg, Germany), serum lipid profile were determined standard procedures in an auto analyzer using Ecoline kits (E.Merck, Mumbai, India).

5.7.10 Histopathological Investigation:

The tissues were processed by standard histopathological technique (i.e dehydration through graded isopropyl alcohol, cleaning through xylene and impregnated in paraffin wax for 2 hr). Wax blocks were made, sections were used for cutting the microtome and stained by haematoxylin eosin method and photographed (**Fig 16, Fig-17**).

5.7.11 Statistical Analysis:

Statistical evaluation for all the grouped data performed by ANOVA. Values were expressed as mean ± SEM (Standard Error of Mean) for six animals in each group. Unpaired student t-test is used for statistical comparison between the four different groups. Changes were considered to be statistically significant if the P-value was < 0.05.

$+p < 0.05$, $++p < 0.01$, $+++ p<0.001$ and $*p<0.0001$ was considered statistically significant[151].

5.8 ISOLATION OF ACTIVE CONSTITUENTS FROM METHANOLIC EXTRACT OF *Halodule uninervis*.

5.8.1 Isolation of active constituents by column chromatography from methanol extract:

The Methanol extract obtained from the successive extraction of *Halodule uninervis was* shown a well response for most of the qualitative chemical tests which conforms that the effective extraction of maximum constituents from the plant material and shows effective in-vivo and in-vitro pharmacological activity. The Brownish green Methanol extract was (% yield about 6.53) taken for the isolation using silica gel (100 - 120 mesh) packed column by using the solvents with increasing polarities. The elute was collected and concentrated. These fractions were tested for the presence of various constituents and nature of the compounds.

5.8.2 Preparation of column:

The silica gel (100 – 120 mesh) slurry was prepared in petroleum ether and packed in a glass column of 2.4 cm diameter and height of 20 cm in order to establish a column of 600 ml. The column was equilibrated by passing one-column volume of hexane before the fraction was loaded. The solvent was kept 5 cm above the bed and the residue was carefully loaded in the form of hexane and ethyl acetate slurry. The column was then developed with a series of solvent starting with hexane, ethyl acetate and methanol in increasing polarity. The different ratios with succeeding solvents were fixed and were shown in the Table. Fractions of 100ml were collected up to methanol system and thereafter, fraction in smaller volumes were collected and checked with T.L.C. Then the fractions were concentrated and processed further the column chromatography of methanol extract tabulated [155] in **table-16**

Table-16: Isolation of active constituents by column chromatography from methanol extract

S.No	Number of Fractions	% of Solvent	Volume of Solvent (ml)	Compounds
1	1-60	98% n-Hex: 2% Etoac	600	-
2	61-95	95% n-Hex: 5% Etoac	500	-
3	96-130	90% n-Hex :10% Etoac	500	-
4	131-160	85% n-Hex:15% Etoac	600	-
5	161-175	80% n-Hex:20% Etoac	800	Compound 1-ME-HU-C-1
6	176-200	75% n-Hex:25% Etoac	800	-
7	201-230	100% Etoac	400	-
8	231- 240	98% Etoac: 2% MeOH	350	Compound 2- ME-HU-C-2
9	241 -250	90% Etoac: 10% MeOH	250	-

Etoac - Ethyl Acetate**; n-Hex –** n-Hexane**;MeOH**-Methoanol

5.9 IDENTIFICATION AND CHARACTERIZATION OF ISOLATED ACTIVE CONSTITUENTS FROM METHANOLIC EXTRACT OF *HALODULE UNINERVIS.*

5.9.1 Thin Layer Chromatography:

A large number of solvent systems were tried to identify the components present in the Extract. Finally, Solvent system: Ethyl acetate: Formic Acid: Water (8:1:1) gave good resolution between the two components.

5.9.1.1 Test solution:

Reflux 5 gm of powdered drug with 25 ml of methanol for 1 hour. Filter and evaporate to dryness. The residue was dissolved in 2 ml Methanol.

5.9.1.2 Solvent system: Ethyl acetate: Formic Acid: Water (8:1:1).

Rf: Rf of isolated compounds were determined.

5.9.1.3 Visualization of spots:

Sprayed the plate with the reagent consisting of (15ml 3% boric acid solution and 5ml 10% oxalic acid)

5.9.1.4 Detection: Ultra violet light.

TLC of Methanolic extract of *Halodule uninervis* is shown in the **table-17**

5.9.2 HPTLC – Determination of methanolic extract of *Halodule uninervis*

HPTLC method is a simple, precise, sensitive, rapid and reproducible, preliminary determination method. The methanolic extract of *Halodule uninervis was analysed by HPTLC to identify and understand the components present*. The detection

was done densitometrically using UV detector in absorbance mode. The R_f value of the sample proves the good resolution between them. The method is so sensitive to detect the constituents in the nanogram level[156].

5.9.2.1 Materials And Methods

Instrument Used	:	CAMAG
Software	:	CAMAG Upgraded software.
Sample Applicator	:	CAMAG Automatic sample Applicator
Scanning	:	CAMAG TLC plate scanner
Plates	:	Silica gel 60 F 254 HPTLC Plates
Solvent System	:	Ethyl acetate: Formic Acid: Water (8:1:1)
Detection	:	Sprayed the plate with the reagent consisting of (15ml 3% boric acid solution and 5ml 10% oxalic acid) and examined under Visual and UV wavelenght at 254nm
Sample solution	:	ME-HU in methanol

5.9.2.2 Sample preparation

5 gm of Methanolic crude methanolic extract refluxed with 25 ml of methanol for 1 hour. Filter and evaporated under reduced pressure using rotovac evaporator. The residue Dissolved in 2 ml Methanol.. 20 micro liters of the extract was taken in the CAMAG syringe. The extract in the syringe was applied in the Pre coated Silica plate using automatic CAMAG Applicator.

5.9.2.3 Developing solvent system

Based on the TLC method development a satisfactory resolution was obtained in the solvent system n-Hexane: Ethyl acetate: Formic acid (5:4:1).

5.9.2.4 Sample application

Application of bands of extract was carried out using spray technique. Sample was applied in duplicate on Aluminum coated Silica gel 60 F 254 HPTLC Plates with the help of Linomat 5 applicator attached to CAMAG HPTLC system, which was programmed through CAMAG software.

5.9.2.5 Development of chromatogram

Developing solvent mixture n-Hexane: Ethyl acetate: Formic acid (5:4:1) was added in twin trough glass chamber 10x 10 cm and allowed to saturated for about 60 min. After the application of sample on the Silica gel 60 F 254 HPTLC Plates, the plate kep inside the developing chamber and allowed to develop.

5.9.2.6 Detection of spots

The air-dried plates were viewed in ultraviolet radiation at 254nm. **Fig.18**.The chromatograms were scanned by densitometer at 200- 400 nm. The Rf values and finger print data were recorded by CAMAG updated software. The corresponding HPTLC chromatogram was presented in **Fig.19.** The HPTLC profile of the methanolic extract *Halodule uninervis* was presented. Results were tabulated in **Table-18**

5.9.3 FT-IR Analysis of crude extract.

Infra red spectral studies were performed for the crude extracts (methanol and ethyl acetate) in the group frequency region (4000- 1250 cm-1). The IR spectras of

crude extracts given in **Fig.20** and **Fig.21** and the data given in the **table-19** and **table-20.**

5.9.4. Qualitative identification of isolated compounds

5.9.4.1 By chemical test

The isolated compounds were tested to identify the active group and the compounds were initial investigation and the results were represented in **Table-21**

Shinoda Test

To 1 ml of the extract, magnesium turnings were added followed by 1-2 drops of concentrated hydrochloric acid. Formation of red colour represents the presence of flavonoid.

5.9.4.2 Physical characters evaluation:

The isolated compounds physical parameters like description, apparent solubility, melting range were evaluated and the resulted were tabulated in **Table-22**

5.9.5 Characterization of isolated compounds

5.9.5.1 UV spectroscopy:

As most of the isolated components are UV active, photo diode array detectors or ultraviolet (UV) detectors were used in the HPLC systems. Identification of wavelength maximum of the components is very important to detect the components by the detector; the maximum wavelength absorbance of the component can be determined by dissolving the component in the selected diluents and scan between 200nm to 800nm by the UV-Visibile spectrophotometer. **Fig.22 and 23** stating the UV spectrum of isolated compounds.

The absorbance maximum of the components at the particular wavelength considers the working wavelength for that particular component and taken for the further method development.

Isolated Compound dissolved in methanol and scanned in Shimadzu UV-1800 from200 to 800nm and the wave length maximum of compound determined.

5.9.5.2 IR :

IR Spectrum of isolated compounds were measured between 450 to 4500cm^{-1} using KBr- media using Alpha- Bruker IR spectrophotometer. The IR spectrum given in **Fig.24 and 25** and the characteristic peak table given in **Table-23 and 24.**

5. 9.5.3 NMR:

^{1}H NMR and ^{13}C NMR was taken on (Bruker Advance II 400 NMR spectrophotometer), CDCl3 as solvent for both isolated compounds. The ^{1}H NMR and ^{13}C NMR spectra for the isolated compounds were given in the **Fig. 25-29.**

5. 9.5.4 MASS Spectroscopy:

MASS spectrum was taken using neat on (TOF MS ES - 3.26e3 spectrophotometer). The spectrum was given in **Fig. 30 and 31.**

5. 9.5.5 Determination of purity of isolated components by HPLC:

Determination of purity of a pharmaceutically active component plays on important role to produce a toxic free component. It is necessary to produce a pure pharmaceutically active component either by synthetic or through isolation from natural elements. Impure active components may leads to toxic to human and lead to any organ failure or death. Different techniques are involved in the purification.

Filtration, Re-crystallization, multi level purification using different solvents, Isolation through column and preparative HPLC are the major well known purification processes. After purification the purity of component should be quantified and reported. Several analytical techniques were involved in the determination of purity of isolated components among them HPLC method is the universally preferred to quantify the purity of isolated components especially in the compounds isolated from natural products.

Reverse phase HPLC method was developed to quantify the Purity of isolated compound HPLC area normalization method and the below chromatographic conditions derived to quantify the purity of both compounds[158].

Analytical method development to determine the purity of isolated compounds:

5. 9.5.5.1 Solubility determination:

It is very important to select an appropriate solvent to dissolve the component which is required to analyze. Improper solubility of component leads to incompatibility with the mobile phase and the results may not be the correct one. To select proper solvent or diluents the 1-2mg of isolated components were dissolved in 0.5mL to 1mL of methanol, Acetonitrile and Water . In methanol and Acetonitrile a clear solution were observed.

5. 9.5.5.2 Determination of Wavelength maximum:

As most of the isolated components are UV active, photo diode array detectors or ultraviolet (UV) detectors were used in the HPLC systems. Identification of wavelength maximum of the components is very important to detect the components by

the detector, the maximum wavelength absorbance of the component can be determined by dissolving the component in the selected diluents and scan between 200nm to 800nm by the UV-Visibile spectrophotometer.

The absorbance maximum of the components at the particular wavelength considers the working wavelength for that particular component and taken for the further method development.

5. 9.5.5.3 Selection of Stationary phase:

A C18 column with 250mm length and 5µm was taken for initial method development.

5. 9.5.5.4 Selection of Mobile phase:

a) Determination of pH of the components:

pH and pKa of the component majorly play the role in the mobile phase pH selection. Generally the pH of the mobile phase may be ±1 or 2 to the pKa of the components. This leads to proper ionization of the components in the mobile phase and the retention of component in the column is prominent and reproducible.

A small quantity of component (1 to 2mg) dissolved in water and the pH was measured. The resulting pH tabulated below

As the component pH was slight basic, selection of pH towards acidic side may leads to better result.

b) Selection of Mobile phase buffer:

1ml of formic acid in 1000 mL water (0.1% formic acid in water) was taken for the initial method development. Component having better solubility in Acetonitrile and less soluble in water shows the component may less polar. So only the buffer used as mobile phase then the component may retain in the column, by using the organic in the mobile leads to fast elution of component. So a gradient run was performed by using

0.1% formic acid as mobile phase A and Acetonitril as Mobile phase with 1ml/minute flow rate.

Table-25: Gradient Program:

Time	Mobile Phase-A: 0.1% formic acid	Mobile Phase-B: Acetonitrile
0.01	100	0
10	100	0
15	80	20
20	80	20
25	60	40
30	60	40
40	50	50
45	50	50
50	40	60
55	40	60
60	20	80
65	20	80
75	0	100
85	0	100

c) Optimaization of mobile phase composition:

The component was eluted between 15 to 20 minutes. So the mobile phase prepared with the mixture of 1ml of formic acid in 1000mL water and Acetonitrile in the ratio of 800:200.

d) Optimization of peak resolution:

Some closely eluted peaks were observed. To separate the peaks the the below trails were taken.

1) Change in the mobile phase composition: Mobile phase composition altered by decreasing the mobile phase composition may lead to better resolution viz retain the peaks. But there is no any improvement observed.

2) pH of the mobile phase (0.1% formic acid in water) adjusted to pH 3.0 from pH 2.2 with KOH leads to well separation between all unknown peaks and peak of interest.

Optimization of peak symmetry:

Peak shape was not proper in the 0.1% OPA in water pH 3.0 mobile phase. To improve the sharpness of peak the buffer strength increased by adding some salt. The frequently and universally used monobasic potassium hydrogen phosphate 0.01M was added in the mobile phase and the pH was adjusted to pH 3.0 with Orthophosphoric acid and found satisfactory symmetry peak shape.

The final chromatographic condition was tabulated below

Table-26: Chromatographic conditions:

Mobile phase	:	0.01M monobasic potassium phosphate pH 3.0 : Acetonitrile (800:200) with 1ml formic acid
Column	:	C18, 250mm X 4.6, 5µm (Hypersil ODS C18 250mm X4.6mm, 5µ)
Wave length	:	254nm
Injection volume	:	50µl
Diluent		Mobile phase

The HPLC purity of peak was determine for the isolated components which tabulated in **Table- 27**.The HPLC chromatogram were given in **Fig. 32 and 33.**

5.10 EVALUATION OF *IN-VITRO* PHARMACOLOGICAL ACTIVITY STUDIES OF METHANOLIC EXTRACT OF *Halodule uninervis*.

5.10.1 Evaluation of anti oxidant activity of Methanolic extract of *Halodule uninervis and isolated compounds*

An antioxidant activity of a molecule is defined as a molecule which has the ability to inhibit the oxidation of other molecules. Oxidation reactions can produce free radicals which in turn can start chain reactions and they may have the potential to interact with cellular components like DNA and cell membrane and causes cell death. Free radicals can trap low density lipoprotein in artery wall and begins the formation of plaque. All plants produce various secondary metabolites among which phenolics play prominent role in showing good antioxidant activity. The phenolics are generally characterized by the presence of one aromatic ring to which one or more hydroxyl groups are linked. Antioxidants terminate the chain reactions by removing free radical intermediates and inhibit cell death and other oxidation reactions[159].

Majority of the diseases/disorders are mainly linked to oxidative stress due to free radicals. Free radicals are fundamental to any biochemical process and represent an essential part of aerobic life and metabolism. The most common reactive oxygen spices (ROS) include super oxide anion (O2.), hydroxyl radical (OH.), hydrogen peroxide (H2O2) peroxyl radical radicals (ROO.). The nitrogen derived free radicals are nitric oxide (NO.) and peroxynitrite anion (ONOO.). ROS have been implicated in over a hundreds of diseases states which range from arthritis and connective tissue disorders to carcinogenesis, aging, physical injury, infection and cardio vascular malfunction. In treatments of these diseases, antioxidant therapy has gained an immense importance.

Current research is now directed towards finding naturally occurring antioxidants of plant origin. Antioxidants have been reported to prevent oxidative damage by free radical and ROS; any may prevent the occurrence of disease, cancer and aging. It can interfere with the oxidation process by reacting with free radicals, chelating, catalytic metals, and also by acting as oxygen scavengers. Plant and plant products are being used as a source of medicine since long. The medicinal properties of plants have been investigated in the recent scientific developments throughout the world, due to their potent antioxidant activities, no side effects and economic viability[160].

Therefore, the objectives of the present study were to investigate the *in vitro* antioxidant activity of methanolic extract of ***Halodule uninervis*** was evaluated using 1, 1-Diphenyl-2-Picrylhydrazyl (DPPH), hydrogen peroxide and xanthine oxidase inhibition assay.

5. 10.1 .1 DPPH free radical scavenging activity[161]

The DPPH assay is based on the reduction of DPPH a stable free radical. Radical scavenging activity of compounds against stable DPPH was determined spectrophotometrically. When antioxidant reacts with DPPH which is a stable free radical becomes paired off in the presence of a hydrogen donor and it is reduced to DPPHH as a result the absorption decreases from DPPH which is clearly identified by colour change from deep violet to yellow colour measured by reading the absorbance at 517 nm using a UV spectrophotometer. The solution of DPPH is mixed with that of a substance that can donate a hydrogen atom will give rise to the reduced form with the loss of violet colour. A series of test tubes labeled from 1- 5, was taken. To each tube a concentration of 0.1, 0.2, 0.3, 0.5 and 1.0 microgram of test solution (extract) was added to 2.0 ml of 0.004% solution of DPPH in methanol. 1ml of methanol and 2 ml of

0.004% DPPH solution was used as experimental negative control. After 30 min of incubation at room temperature, the reduction in the number of free radicals was measured by reading the absorbance at 517 nm using a UV spectrophotometer. Radical scavenging activity was expressed as the inhibition percentage. The scavenging activity of the samples corresponded to the intensity of quenching DPPH. The standard (positive control) used here was quercetin .

$$\text{Percentage of inhibition} = \frac{\text{Absorbance of control} - \text{Absorbance of test sample}}{\text{Absorbance control}} \times 100.$$

5. 10.1.2 Xanthine oxidase inhibition assay[162]

The Xanthine oxidase activity was assayed spectrophotometrically under aerobic conditions. The assay mixture, consisted of 50 microlitres of test solution, 35 micro liter of 0.1mM phosphate buffer (pH=7.5), and 30 microlitres of enzyme solution (0.01 units/ml in 0.1mM phosphate buffer, pH=7.5) and was prepared immediately before use. After pre incubation at 25^0C centigrade for 15 minutes, the reaction was initiated by the addition of 60 microlitre of substrate solution (150mM xanthine in the same buffer). The assay mixture was incubated at 25degree centigrade for 30minutes. Absorbance at 290nm was measured with a spectrophotometer blank was prepared in the same manner. One unit of Xanthine was defined as the amount of enzyme required to produce 1N mole of uric acid/minutes at 25^0C. Xanthine oxidase inhibitory activity is expressed as the percentage inhibition of Xanthine in the above system, calculated as (1-B/A) X 100, where A and B are the activities of the enzyme without and with test material .

5. 10.1.3 Hydrogen peroxide scavenging assay[163]

A solution of H_2O_2 (40mM) was prepared in phosphate buffer. The extracts and the isolated compounds at the concentration of 10mg/10µl were added to H_2O_2solution (0.6ml) and the total volume was made up to 3ml. The absorbance of the reaction mixture was recorded at 230nm in a spectrophotometer (Genesys 10-S, USA). A blank solution containing phosphate buffer, without H_2O_2 was prepared. Ascorbic acid is used as positive control. The extent of H_2O_2 scavenging of the plant extracts was calculated as

$$\frac{(A_0 - A_1) \times 100}{A_0} \text{ \% scavenging of hydrogen peroxide}$$

A_0=Absorbance of control
A_1- Absorbance in the presence of plant extract

DPPH free radical scavenging activity, Xanthine oxidase inhibition assay, Hydrogen peroxide scavenging assay.

The results of methanolic extract and isolated compounds were tabulated in the **table 28 to 34.** The obtained results were expressed in graphical presentation from **fig. 34-37**

5. 10.2 Anti microbial activity of isolated active constituents from *Halodule uninervis*

Bacterial diseases are widespread worldwide. Antibiotics used for the treatment of these infections. In recent years, a number of antibiotics have lost their effectiveness due to the development of resistant strains, mostly through the expression of resistance genes. In addition to this problem, antibiotics are sometimes associated with adverse effects including hypersensitivity, immune-suppression and allergic reactions [163]. Therefore, there is a need to develop alternative antibacterial drugs for the treatment of

infectious diseases from various sources such as medicinal plants. Undoubtedly, medicinal plants are the prime source of drugs in both developing and developed nations, as drugs or herbal extracts for various chemotherapeutic purposes. There are about 2000+ plant species known to possess medicinal value in the traditional Asian system of medicine[164] The use of plant derived natural compounds used as alternative sources of medicine continues to play major roles in the general wellness of people all over the world. The curative properties of medicinal plants are due to the presence of various complex chemical substances of different composition which occur as secondary metabolites Over 50% of all modern clinical drugs are of natural origin and play an important role in development of drugs [165].

5. 10.2.1 Anti Bacterial activity:

Bacterial strains *staphylococcus aureus*, *Escherichia coli*, *Salmonella* and fungal strains *Aspergillus niger*, *Aspergillus clavatus*, *Candida albicans* were chosen based on their clinical and pharmacological importance. The bacterial strains obtained from Bose Clinical Laboratory, Madurai were used for evaluating antimicrobial activity. The bacterial and fungal stock cultures were incubated for 24 hours at 37°C on nutrient agar and potato dextrose medium respectively, following refrigeration storage at 4°C. The bacterial strains were grown in Muller-Hinton agar (MHA) plates at 37°C (the bacteria were grown in the nutrient broth at 37°C and maintained on nutrient agar slants at 4°C), whereas the yeasts and molds were grown in potato dextrose media, respectively, at 28°C. The stock cultures were maintained at 4°C. Results were tabulated in **Table- 35.**

5. 10.2.2 Antimicrobial Activity - Determination of zone of inhibition method:

The crude Methanolic extract and isolated compounds ME-HU-C-1 and ME-HU-C-2 were screened for antibacterial activity using the agar well diffusion method with sterile cork borer of size 6.0mm. The cultures of 24 hours old cultures, grown on nutrient broth are used for inoculation of bacterial strain on Muller Hinton agar plates. The Methanol extract and the compound after concentration were weighed and dissolved in DMSO (1mg in 1ml). Each microorganism was diluted in sterile saline solution and adjusted to 0.1 OD reading. All the extracts were screened for their antibacterial activity against *Staphylococcus aureus* Gram (+), *Escherichia coli* Gram (-), *Salmonella* Gram (-). The above said microorganisms were then flooded on the surface of the pre sterilized Muller Hinton Agar plate. Three wells, each 10mm in diameter, were cut from the agar and various extracts of same dilution was loaded in to each well. The plates were incubated for 24 hours at 37C. The complete antibacterial analysis was carried out under strict aseptic conditions. The zones of inhibition were measured with the zone scale in mm and the experiment was carried out in single plate **(Fig.38).** The Dilutions of the compounds is 2000ppm[166].

5. 10.2.3 Minimal Inhibitory concentration:

The minimal inhibitory concentration (MIC) was determined by both broth dilution assay and agar diffusion assay methods. For broth dilution assay method, a 24 hour pure culture of *E.coli* was grown in Muller Hinton broth. From the broth, 1000µl of culture was taken and inoculated individually into a set of 5 test tubes that already contained 10ml of fresh Muller Hinton broth.5 different concentrations (1ppm, 10ppm, 100ppm, 1000ppm, 2000ppm) of compounds (1 & 2) were prepared for testing against

E.coli. One ml volume of each concentration of compounds was transferred to the tubes accordingly. The contents in the tubes were mixed thoroughly and incubated overnight at room temperature (**Fig.39**). For agar diffusion assay method, a pre sterilized Muller Hinton agar plate seeded with E. coli was taken. To the plate, 5 holes were plucked with agar borer. To the holes 100µl of each concentration of compounds was transferred accordingly and incubated overnight at room temperature. The MIC end point is the lowest concentration of compounds at which there is no visible growth in the form of zone or turbidity is noticed in the tubes (**Fig.40**). Results were given in **table-36[167].**

5.10.2.4 Anti Fungal activity:

The crude Methanolic extract and isolated compounds I and II were screened for antifungal activity using food poison technique method. The fungal cultures from the periphery region of the grown culture are used for inoculation of fungal strain on Potato dextrose agar plates[168]. All the extracts were screened for their antifungal activity against Aspergillus sp and Candida albicans. The samples were taken as such and 1ml of the sample was mixed with the sterilized cool potato dextrose medium. Then the medium mixed oil sample was poured in the pre-sterilized petriplate. After the medium was solidified, the fungal cultures were inoculated on the middle of the surface of the pre sterilized Potato dextrose agar plate. The plates were incubated for five days under observation. Based on the diameter of the fungal colonies grown on the petriplate, the percentage of inhibition was measured. All the plates were kept for incubation and the following result was observed.

Percentage of inhibition = diameter of the petriplate - diameter of the fungus grown the obtained results from were tabulated in the **Table 33**.

Chapter VI
Results and Analysis

RESULTS AND ANALYSIS

6.1 PHYSICO-CHEMICAL STUDIES OF *Halodule uninervis* PLANT POWDER:

Based on the literature survey it was identified that the *Halodule uninervis* having various phytoconstituents and pharmacological properties, we resolved to work on *Halodule uninervis plant*, which are widely used in medicines of natural products.

The whole plant were shade-dried and ground to coarse powder and extracted in series, using Petroleum ether AR, Chloroform AR, Ethanol AR in an increasing order of polarity.

Phytochemical Screening of *Halodule uninervis* was undertaken to study, isolate and characterizes the chemical constituents and the Pharmacological and Microbiological activities. The results are presented below

Table-2: Physico-chemical studies of *Halodule uninervis*

S.no	Parameter	Observation
1	**Ash values**	
	Total ash (%)	10.15±0.60
	Acid insoluble ash (%)	1.32±0.26
	Water soluble ash (%)	3.52±0.32
2	**Extractive values (%)**	
	Ethanol soluble	16.24±0.56
	Chloroform soluble	14.11±0.53
	Petroleum ether soluble	11.02±0.25
3	**Loss on drying (%)**	69.29±1.14

Table-3: Fluorescence analysis of *Halodule uninervis*

S.NO	PARAMETER	OBSERVATION		
		Ordinary light	UV light	
			254nm	366nm
1	Powder as such	Brownish green	Brown	Brownish green
2	Powder+ Nitrocellulose	Brown	Brown	Brown
3	Powder+1N NaOH in methanol	Dark green	Brown	Green
4	Powder+1N NaOH in methanol+ Nitrocellulose in amyl acetate	Brown	Black	Green
5	Powder+1NHCl	Brownish green	Black	Brownish green
6	Powder+1NHCl+ Nitrocellulose in amyl acetate	Black	Brown	Brownish red
7	Powder+1N NaOH in water	Greenish brown	Black	Green
8	Powder+1N NaOH in water dried and mounted in Nitrocellulose in amyl acetate	Greenish brown	Black	Reddish brown
9	Powder+HNO_3	Reddish brown	Brown	Reddish black
10	Powder+H_2SO_4	Dark green	Black	Black

6.2 EXTRACTION OF POWDER

The dried powder of whole plants was extracted in Soxhlet apparatus using different solvents. The resultant extracts were concentrated and freeze dried and the yield obtained for different solvents are depicted in **table 5.**

Table-4: The percentage yield of successive solvent extractive values of *Halodule uninervis*

S.No	Extracts	Colour	Yield % (W/W)
1.	Petroleum ether (PE-HU)	Dark Green	0.97
2.	Chloroform(CE-HU)	Greenish brown	1.23
3.	Ethyl acetate (EAE-HU)	Yellowish Brown	1.68
4.	Methanol(ME-HU)	Brownish green	6.53

6.3 TLC EVALUATION OF CRUDE EXTRACT OF *Halodule uninervis* IN DIFFERENT SOLVENT SYSTEM:

The obtained extraction from different solvents were evaluated in terms of TLC to identify the presence of constituents qualitatively and the results are represented below.

Fig. 11 TLC evaluation of crude extract of *Halodule uninervis in different solvent system :*

Table:5 Rf values of TLC in different solvent systems composition extract of *Halodule uninervis*

S.No	Extract name	Solvent system I		Solvent system II		Solvent system III		Solvent system IV		Solvent system V	
		No. of spots detected	Rf Value	No. of spots detected	Rf Value	No. of spots detected	Rf Value	No. of spots detected	Rf Value	No. of spots detected	Rf Value
1	PE-HU	3	0.26 0.47 0.57	1	0.87	1	0.86	1	0.09	-	-
2	CE-HU	2	0.24 0.49	3	0.16 0.80 0.88	2	0.22 0.85	2	0.11 0.76	2	0.28 0.92
3	EAE-HU	2	0.23 0.44	2	0.79 0.87	1	0.83	2	0.14 0.79	3	0.03 0.52 0.91
4	ME-HU	2	0.24 0.46	1	0.80	2	0.23 0.82	2	0.15 0.80	2	0.12 0.90

6.4 PRELIMINARY PHYTOCHEMICAL ANALYSIS

The various extracts of whole plants were subjected to chemical tests for detection of various groups of phyto-constituents present. The results obtained are presented in **Table 7.**

Table-6: Results of qualitative chemical analysis of various extract of *Halodule uninervis* :

S.No.	Test	Whole plant powder	Petroleum ether	Chloroform	Ethyl acetate	Methanol
1.	Alkaloids	+	-	+	+	+
2.	Carbohydrates	+	+	+	+	+
3.	Glycosides	+	-	-	+	+
4.	Terpenoids	+	-	-	-	+
5.	Proteins	+	+	-	+	+
6.	Amino acids	+	+	-	+	+
7.	Steroids	+	-	-	-	+
8.	Flavonoids	+	-	-	+	+
9.	Phenols	+	-	-	-	+
10.	Tannins	+	-	-	+	+
11.	Quinones	+	-	+	-	-
12	Anthraquinones	+	-	-	-	+
13	Saponins	+	-	-	+	+

+ = Positive - = Negative

6.5 HEAVY METAL ANALYSIS OF *Halodule uninervis* PLANT EXTRACT BY AAS

Contamination levels of heavy metals in *Halodule uninervis* Plant tested by atomic absorption spectrophotometer (AAS) and the results were given below.

Table 8: Results Obtain from atomic absorption spectrophotometer (AAS)

Name of extract	Cd	Pb	As	Hg	MDL[a]
Petroleum ether (PE-HU)	ND	ND	ND	ND	0.01mg/kg
Chloroform(CE-HU)	ND	ND	ND	ND	
Ethyl acetate (EAE-HU)	ND	ND	ND	ND	
Methanol(ME-HU)	ND	ND	ND	ND	

ND: Not detected; MDL: Minimum detection limit; [a]n=3.

6.6 TOTAL FLAVONOID CONTENT IN THE VARIOUS PLANT EXTRACTS OF *HALODULE UNINERVIS*

Total flavonoid content in the various plant extracts of *Halodule uninervis were* determined and the results were tabulated below

Table 9: Total flavonoid content in the various plant extracts of *Halodule uninervis*

Extract	Total Flavonoid content (mg of quercetin/g)
Petroleum ether extract (PE-HU)	2.843mg/g
Chloroform extract(CE-HU)	3.0426 mg/g
Ethyl acetate extract (EAE-HU)	4.8432 mg/g
Methanolic extract (ME-HU)	7.1232 mg/g

6.7 EVALUATION OF *IN-VIVO* PHARMACOLOGICAL ACTIVITY STUDIES OF METHANOLIC EXTRACT OF *HALODULE UNINERVIS*.

Antidiabetic activity study against the streptozotocin induced diabetic in mice:

Effects of *Halodule uninervis* on serum glucose levels in Streptozotocin -induced diabetic rats :

Effects of *Halodule uninervis* extract in the single dose study on blood glucose levels was estimated after Streptozotocin administration on the 3rd day. There is no reduction in glucose level due to *Halodule uninervis* extract (50 mg/kg). At dose levels of 150 and 250 mg/kg, glucose levels were decreased by 24.8% and 29.9% at the 6th hour, respectively. Antidiabetic effect of the extract was slightly decreased at the 8th hour, but remained statistically significant the below table shown the Effects of *Halodule uninervis* on serum glucose levels in Streptozotocin -induced diabetic rats.

Table 11: Effects of *Halodule uninervis* on serum glucose levels in Streptozotocin - induced diabetic rats

Group	Serum glucose level (mg/dL)				
	0 hour	2 hour	4 hour	6 hour	8 hour
I	70.5 ± 5.6	67.2± 6.9	64.0± 7.9	67.4 ± 7.1	69.2 ± 6.9
II	267.9 ± 10.5$_a$	274.1 ± 9.7 $_a$	277.0 ± 0.9 $_a$	273.1 ± 8.0 $_a$	267.4 ± 7.6 $_a$
III	261.4 ± 9.6	264.4± 8.6	258.2± 8.5	255.2 ± 9.9	252.5 ± 8.7
IV	258.2 ± 8.5	253.0 ± 8.1	237.4± 7.6	204.9 ± 9.2 $_b$	214.2 ± 9.0$_c$
V	259.9 ± 10.9	241.9 ± 9.9	230.5± 7.9	191.2 ± 8.4 $_b$	198.3 ± 8.5 $_b$

After administration of Streptozotocin the serum glucose levels were obtained from fasted rats. Data are expressed as mean ± S.E; (n=6); ap<0.0001 (compared to normal group with the corresponding hours). bp<0.01 and cp<0.05 (compared to control group with the corresponding hours)

In parallel experiments, *Halodule uninervis* extract was administered to diabetic rats for 18 days as per the protocol (Table-11). The glucose level reduction up to 26% on 9th day of *Halodule uninervis* (Forsk.) (50 mg/kg) treatment started to lower serum glucose on the 9th day. An overall reduction of 26% was observed on the 18th day by 50mg/kg administration ($p<0.01$). 52.5% reduction of glucose level in the serum absorved at 18th day administration at dose level of 150 mg/kg ($p<0.0001$). 250mg / kg extract administration is more effective from 6th day onwards with a reduction rate of 18.9% ($p<0.01$) and maximum reduction of serum glucose level by 61.9% on the 18th day. Between the 12th and 18th days, antidiabetic effect of *Halodule uninervis* extract was in a concentration dependent manner the below shown the effects of *Halodule uninervis* (Daily treatment) on serum glucose levels in Streptozotocin -induced diabetic rats

Table 12: Effects of *Halodule uninervis* (Daily treatment) on serum glucose levels in Streptozotocin -induced diabetic rats

Group	Serum glucose (mg/dL)					
	3 days	**6 days**	**9 days**	**12 days**	**15 days**	**18 days**
I	74.9 ± 7.3	72.8± 8.7	71.3 ± 10.6	74.1 ± 8.8	70.8 ± 6.8	75.0 ± 8.3
II	272.4 ± 13.0$^{\#}$	279.4 ± 11.6 $^{\#}$	271.5 ± 11.6$^{\#}$	273.7 ± 11.0$^{\#}$	268.6 ± 11.0$^{\#}$	263.0± 9.5$^{\#}$
III	269.9 ± 10.9	249.6±11.0	230.5 ± 9.4^{+}	218.5 ± 9.1^{+}	208.7 ± 9.9^{+}	199.7 ± 9.1^{+}
IV	275.3± 9.7	231.4± 10.5	211.1 ± 12.3^{++}	174.3 ± 10.0^{+++},a	151.0 ± 10.7^{+++},b	131.4 ± 11.4$_{*,c}$
V	271.5± 10.6	220.7 ± 9.3^{++}	166.4 ± 11^{+++}	141.4 ± 8.8$_{*,d}$	130.8 ± 9.6$_{*,e}$	104.6 ± 12.3$_{*,f}$

Values of serum glucose levels were obtained from Streptozotocin induced diabetic rats in the absence and in the presence of 18 days of *Halodule uninervis* extract treatment (from the 3rd to the 18th day) and expressed as mean ± S.E; n=6; #p<0.0001 (compared to normal group with corresponding day); +p<0.05, ++p<0.01, +++p<0.001 and *p<0.0001 (compared to control group with corresponding day). ap<0.05, bp<0.01 and cp<0.001 (compared to Group III with corresponding day). d,e,f p<0.05 (compared to Group IV with corresponding day)

The effects of *Halodule uninervis* extract on hepatic and renal function in Streptozotocin-diabetic rats. As seen, treatment with 50 mg/kg did affect neither the significantly high levels of ALP, GPT, BUN and creatinine, nor the overall oxidative status. Conversely, rats treated with higher doses of the extract (150 and 250 mg/kg) showed significant improvements in hepatic and renal function the below shown Effects of *Halodule uninervis* extract on liver and kidney functions.

Table 13: Effects of *Halodule uninervis* extract on liver and kidney functions

Group	Liver		Kidney	
	ALP (KA/dL)	**GPT (U/mg protein)**	**BUN (mg/dL)**	**Creatinine (mg/dL)**
I	34.9 ± 4.0	160.6 ± 11.3	10.4 ± 1.8	1.9 ± 0.2
II	51.5 ± 7.9$_{a}$	336.5 ± 23.1b	20. ± 3.4c	3.3 ± 0.6.c
III	43.8 ± 6.0	286.8 ± 21.0	17.6 ± 12.3	2.9 ± 0.4
IV	36.5 ± 3.9$_{e}$	245.4 ± 13.5 $_{e}$	14.8 ± 1.9$_{f}$	2.7 ± 0.8$_{e}$
V	32.3 ± 13.3$_{f}$	207.3 ± 10.3 $_{f}$	12.7 ± 1.8$_{g}$	2.4 ± 0.6$_{g}$

ALP: alkaline phosphatase; GPT: glutamate pyruvate transaminase; BUN: blood urea nitrogen. Liver and kidney markers were measured on the 18th day after Streptozotocin administration. Data are expressed as mean ± S.E; n=6; ap<0.01, bp<0.001 and cp< 0.0001; compared to normal group. ep<0.05, fp<0.01 and gp<0.001 (compared to control group)

Additionally, these two dose levels recovered the weight loss and low white blood cell count observed in Streptozotocin-diabetic rats while decreasing liver glycogen. The below table shown the Effects of *Halodule uninervis* extract on body weight, total leucocyte count and liver glycogen.

Table 14: Effects of *Halodule uninervis* extract on body weight, total leucocyte count and liver glycogen

Group	Body Weight		Total leukocyte count (mm3)	Liver glycogen (µg/g tissue)
	Initial	Final		
I	240.5 ± 6.6	258.2 ± 7.9	13233.4 ± 455.5	75.7 ± 3.4
II	244.1 ± 8.4	204.1 ± 20.7$_a$	8566.0 ± 388.0$_c$	56.9 ± 3.6$_d$
III	248.4 ± 8.9	244.4 ± 18.6$_b$	9451.4 ± 510.0	66.6 ± 2.9
IV	242.2 ± 6.9	248.0 ± 11.1	11102.4 ± 656.9$_e$	73.3 ± 3.9$_e$
V	246 ± 10.9	261.9 ± 7.9	13001.3 ± 701.9$_f$	76.4 ± 4.3$_e$

Data were expressed as mean ± S.E; n=6; ap<0.001 (compared to initial body weight of the same group), bp<0.05 (compared to initial body weight of the same group), cp<0.0001 and dp<0.05 (compared to normal group), ep<0.05 and ^{f}p<0.001 (compared to control group)

Halodule uninervis extract (150 and 250 mg/kg) also showed a protective effect on liver oxidative status the below table shown the Effects of *Halodule uninervis* extract on oxidative status

Table 15: Effects of *Halodule uninervis* extract on oxidative status

Parameter	Group I	Group II	Group III	Group IV	Group V
GSH (nmol/mg protein)	9.6±1.6	5.4±1.4$_c$	5.7±1.5	7.1±1.6$^+$	7.5±1.6^{+++}
GSSG (nmol/mg protein)	1.5±0.2	2.1±0.3c	1.9±0.3	1.8±0.6$^+$	1.5±0.4^{+++}
GPx (U/mg protein)	1.4±.0.16	1.3±0.5$_a$	1.3±0.3	1.2±0.1$^+$	1.1±0.1$^+$
MDA (nmol/mg tissue)	355.4±11.4	443.4±3.1$_b$	421.5±14.3	391.4±10.4$^+$	356.8±13.8^{++}
SOD (U/mg protein)	7.5±0.6	4.5±0.5$_c$	4.8±0.5	5.6±0.8^{++}	6.8±0.7^{+++}
Catalase (U/mg protein)	154.6±7.9	99.5±8.2$_b$	120.4±9.5	127.4±8.7$^+$	143.9±11.8^{++}

GSH: reduced glutathione, GSSG: oxidized glutathione; GPx: glutathione peroxidase, MDA: malondialdehyde; SOD: superoxide dismutase. Data were expressed as mean ± S.E; n=6; ap<0.01, bp<0.001, cp< 0.0001 and $^{++}$p<0.01 (compared to normal group); $^+$p<0.05 and $^{+++}$p<0.001 (compared to control group)

Antioxidants namely GSH, GPx, SOD and catalase were increased by *Halodule uninervis* extract administration. When compared to Streptozotocin-diabetic rats, MDA formation, as an indirect measure of lipid peroxidation, was found to be significantly low in high dose *Halodule uninervis* extract-treated rats. **Fig.12** shown the effects of methanolic extract of *Halodule uninervis* extract on glucose tolerance. Glucose levels obtained from Group I (control) (♦); Group II (Streptozotocin treated control) (■); Group III (*Halodule uninervis* extract 50 mg/kg) (▲); Group IV (*Halodule uninervis* extract 150 mg/kg) (X); and Group V (*Halodule uninervis* extract 250 mg/kg) (*)are shown. Data are represented as mean ± S.E. (**p<0.01, compared to control at 60 min and *p<0.05 compared to control at 120 min; n=6

Fig.12: Effects of *Halodule uninervis* on serum glucose levels in Streptozotocin - induced diabetic rats (Hourly basis)

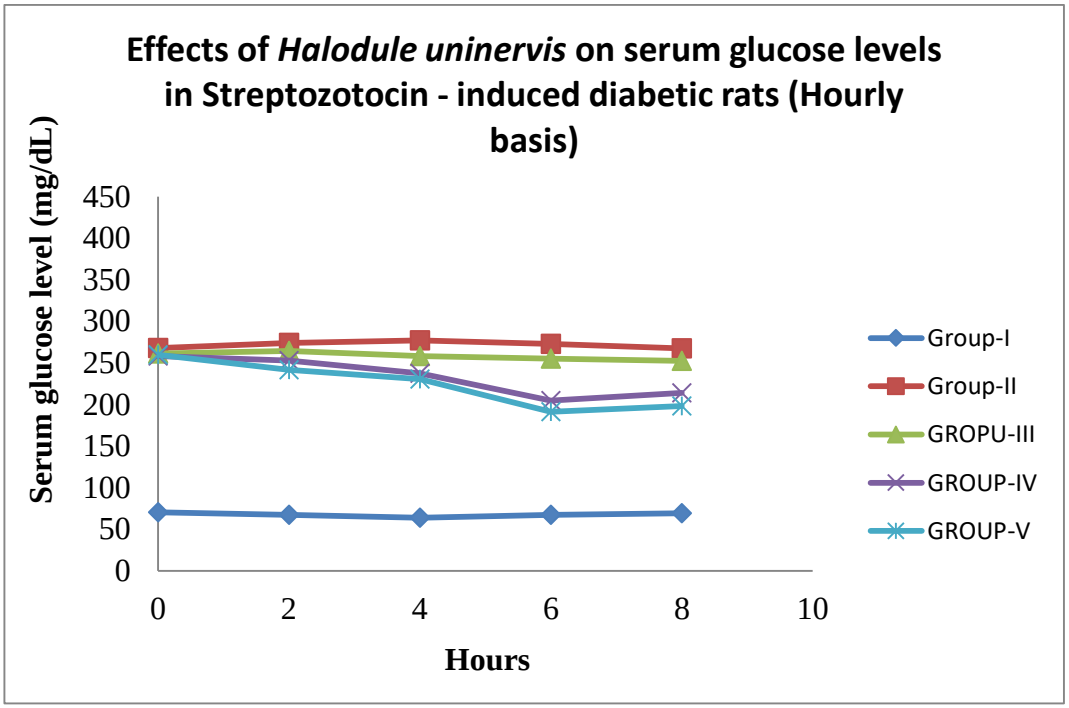


Fig.13 express the effects of *Halodule uninervis* extract on acetylcholine relaxations in streptozotocin diabetic rats. Concentration–response curves derived from Group I

(control) (♦); Group II (Streptozotocin treated control) (■); Group III (*Halodule uninervis* extract 50 mg/kg) (▲); Group IV (*Halodule uninervis* extract 150 mg/kg) (**X**); and Group V (*Halodule uninervis* extract 250 mg/kg) (*) are shown. Data are expressed as mean ± S.E; (+p<0.0001; compared to normal group, ap<0.01, bp<0.001 and cp<0.0001; compared to diabetic control group; n=6.

Fig.13: Effect of *Halodule uninervis* extract on serum glucose levels in Streptozotocin -induced diabetic rats

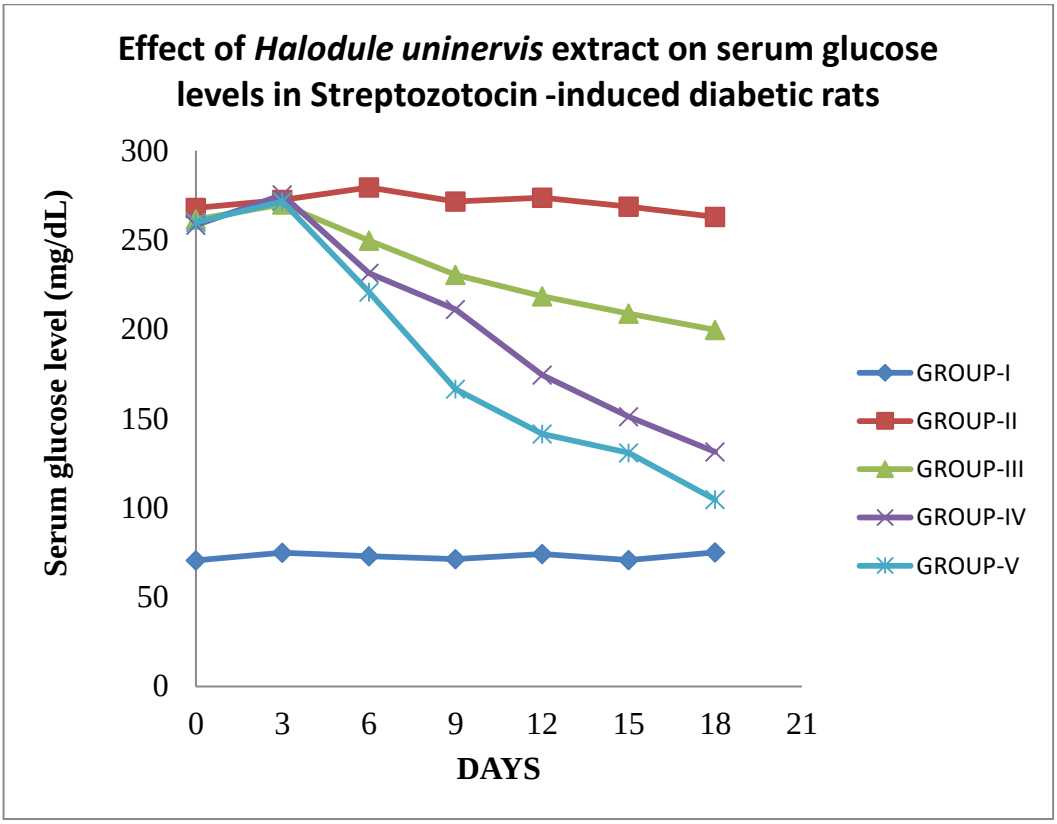


Fig.14 and Fig.15 represent The effects of *Halodule uninervis* extract on hepatic and renal function in Streptozotocin-diabetic rats. As seen, treatment with 50 mg/kg did affect neither the significantly high levels of ALP, GPT, BUN and creatinine, nor the overall oxidative status. Conversely, rats treated with higher doses of the extract (150

and 250 mg/kg) showed significant improvements in hepatic and renal function the below shown Effects of *Halodule uninervis* extract on liver and kidney functions.

Fig.14: Effects of *Halodule uninervis* extract on liver functions [ALP (KA/dL) & GPT (U/mg protein)]

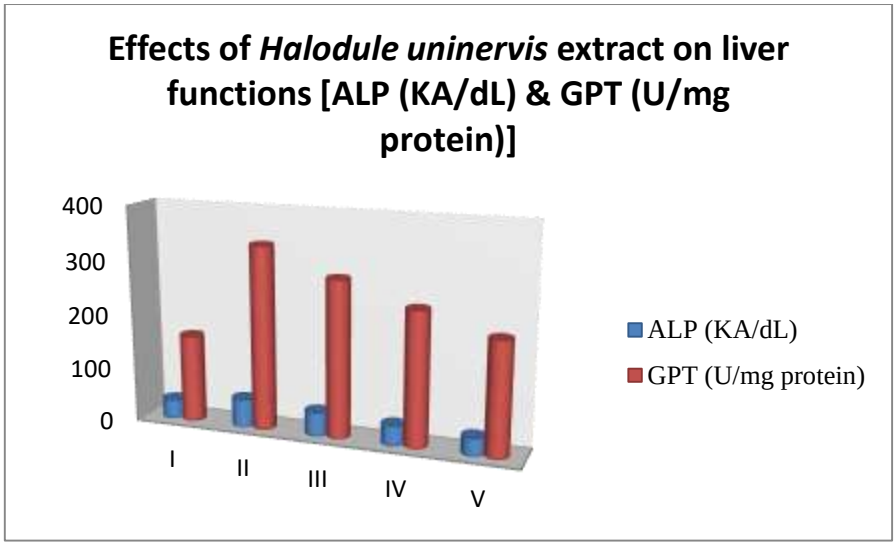
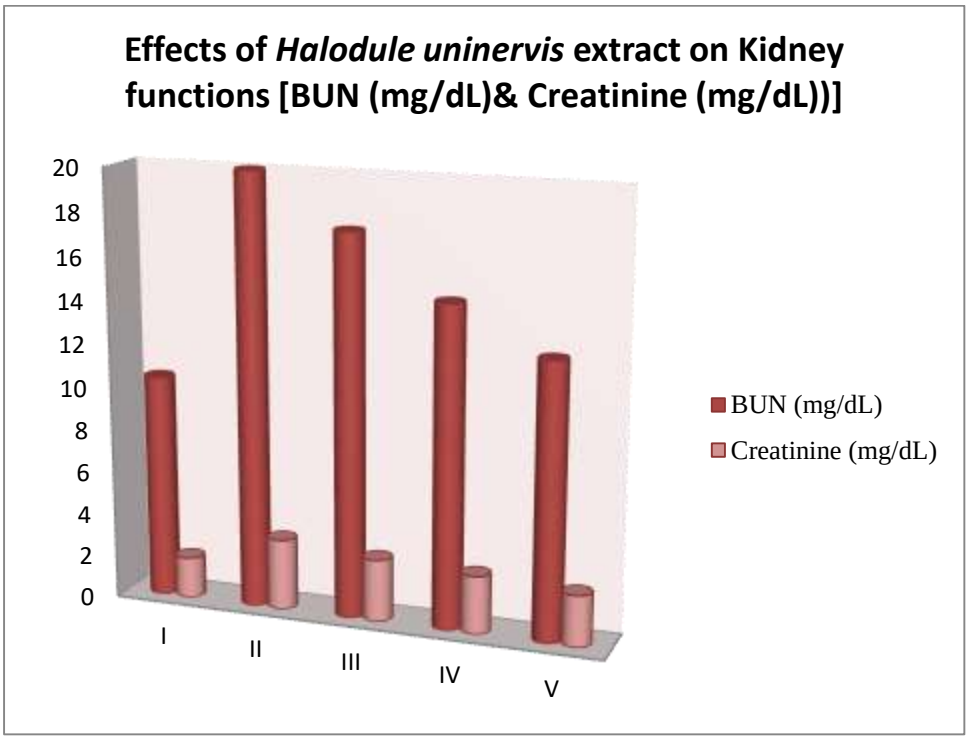


Fig.15: Effects of *Halodule uninervis* extract on Kidney functions [BUN (mg/dL)& Creatinine(mg/dL))]



HISTOPATHOLOGY OF PANCREAS

Images were taken at the magnification of 400x

Plate	Group	Description
19	I	It showed normal architecture no degenerative Changes
20	II	Destruction of c cells
21	III	Mild regeneration of c cells compared to group-II
22	I V	Mild regeneration of c cells
23	V	Regeneration of c cells near to control

Fig.16: histopathology of pancreas

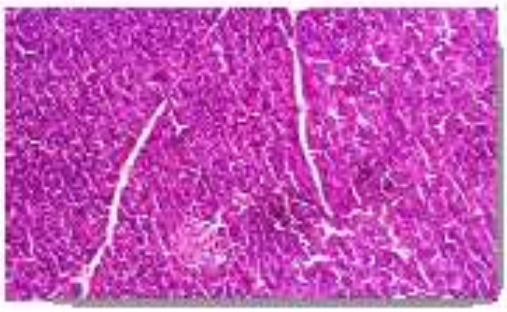


PLATE 19

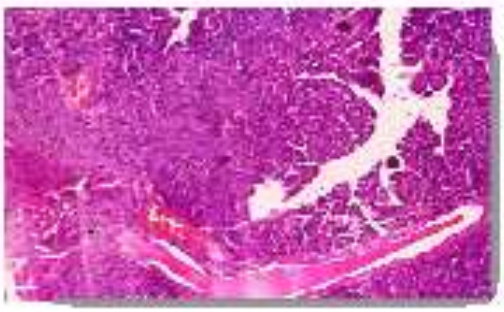


PLATE 20

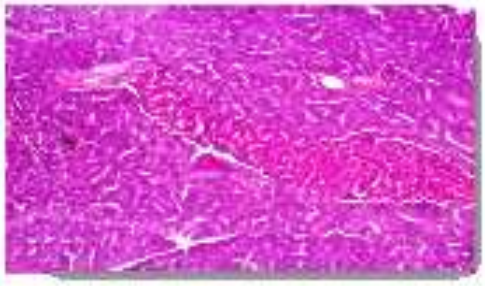


PLATE 21

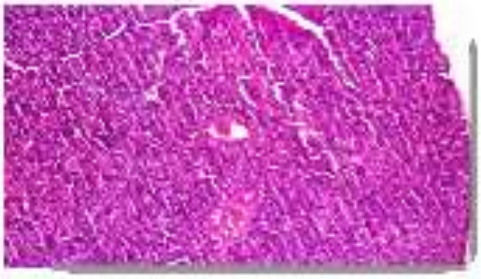


PLATE 22

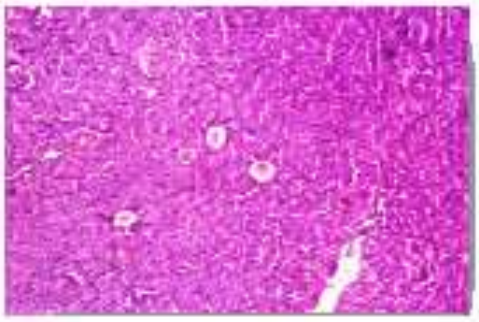


PLATE 23

HISTOPATHOLOGY OF KIDNEY

Images were taken at the magnification of 400x

Plate	Group	Description
27	I	Control/ Normal architecture of mesangial cells and glomeruli
28	II	STZ-induced diabetic control rats showed severe epithelial atrophy and mild sclerotic changes in glomeruli and moderate congestion of capillaries.
29	IV	Mild atrophy and sclerotic changes, congestion with inflammatory cells
30	V	Very mild atrophy and sclerotic changes in glomeruli , congestion with inflammatory cells
31	VIII	Near to normal architecture of mesangial cells and Glomeruli

Fig.17: histopathology of Kidney

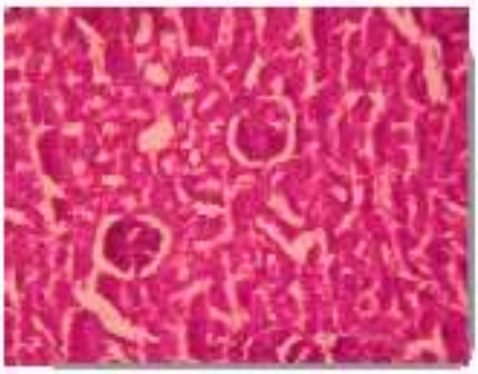


PLATE 27

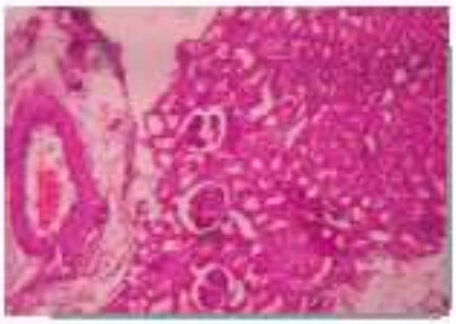


PLATE 28

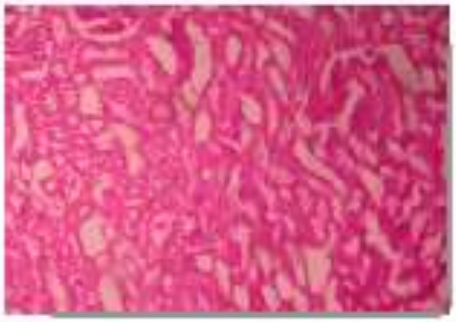


PLATE 29

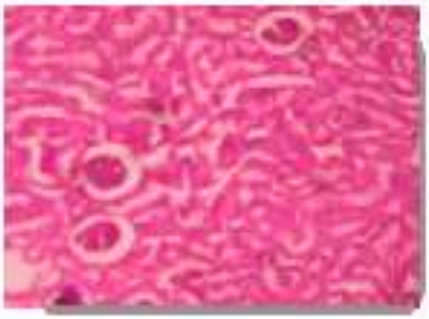


PLATE 30

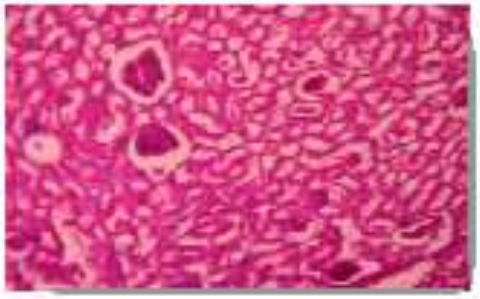


PLATE 31

6.8 CHARACTERIZATION OF METHANOLIC EXTRACT AND COMPOUNDS ISOLATED FROM METHANOLIC EXTRACT OF *Halodule uninervis*.

6.8.1 TLC analysis

TLC analysis were performed for the isolated compounds to identify the rf values and the results were mentioned below

Table 17 : Results Obtain from TLC analysis of isolated compounds

Mobile phase	Detection	Rf value	
Ethyl acetate: Formic Acid: Water (8:1:1).	UV light	ME-HU-C-1 ME-HU-C-2	0.68 0.79

6.8.2 HPTLC analysis:

HPTLC analysis performed to quantify the % of components in the methanolic extract and the results are below.

Fig.18: HPTLC plate seen at 254nm for methanolic extract *Halodule uninervis*

Fig.19 HPTLC Chromatogram of *Halodule uninervis* methanolic plant extract showing different peaks of phytoconstituents.

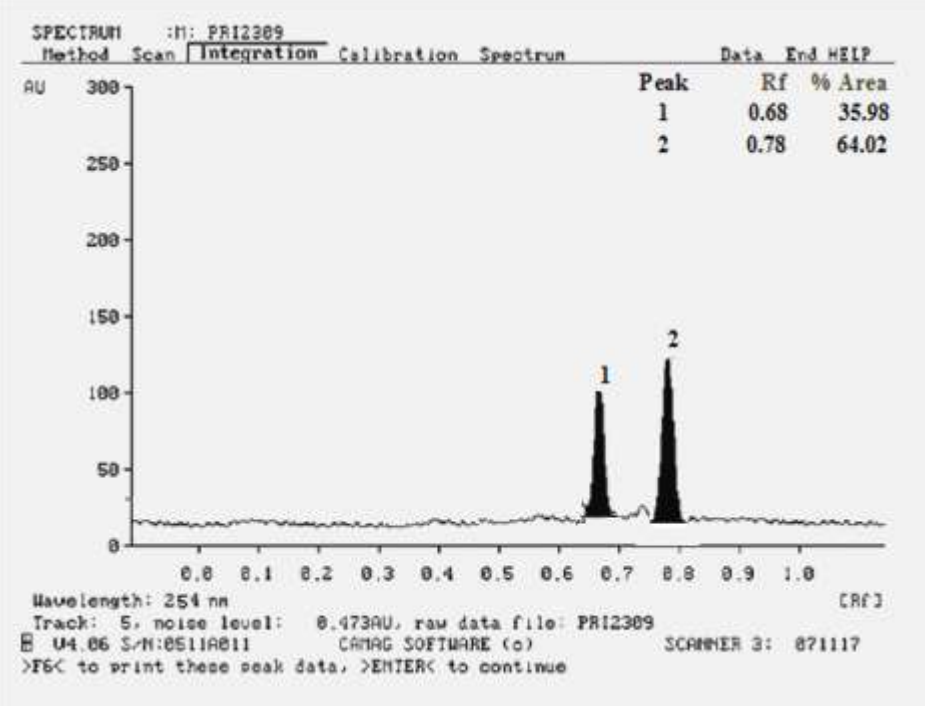


Table 18: HPTLC profile of the methanolic extract of *Halodule uninervis*

Peak	Rf	Percentage Area
1	0.68	35.98
2	0.78	64.02

6.8.3 IR Spectroscopic evaluation of crude extract:

IR Spectroscopic evaluation of crude extract (Methanolic and ethyl acetate extracts) were given prominence and thus the characteristic of the main functional groups (OH, NH2, C=O, C-H, etc.) were observed in this range. Moreover, various relevant peaks were observed in the fingerprint region (< 1250 cm-1). The most intense calculated bands of the IR spectra and the corresponding assignments were summarized in **Table 18 and Table 19**. Examination of the IR spectra showed a broad and very strong O–H stretch peak between 3450- 3300 cm-1 and was the most common

characteristic peak of the methanol and hexane extracts. This could infer the presence of alcohols, phenols and flavonoid in those plants.

Characteristic peaks in the range of 1680 - 1740 cm-1 of carbonyls were also observed in the methanol extracts of *Halodule uninervis* (1737.7 cm-1). The presence of esters might be suggested in the methanol extract of the *Halodule uninervis* due to the presence the characteristic C-O stretching around 1028 cm-1 and 1261 cm-1. Moreover, C-H vibrational frequencies of saturated hydrocarbons were observed in all the extracts ranging from 2950 to 2830 cm-1. In the analysis of IR results, the characteristic signals of fatty acids and their esters at 700 cm-1 and 800 cm-1 (CH2 rocking); 918 (-CH2 wagging), 1096 (OCH2-C), and 1299 (C-CO-O); 1681, 1737 (C=O esters); 2831 cm−1 (CH2 sym. and asym. str.) also observed in the IR spectra.

Fig.20: FTIR spectrum of crude Methanol extract of *Halodule uninervis*

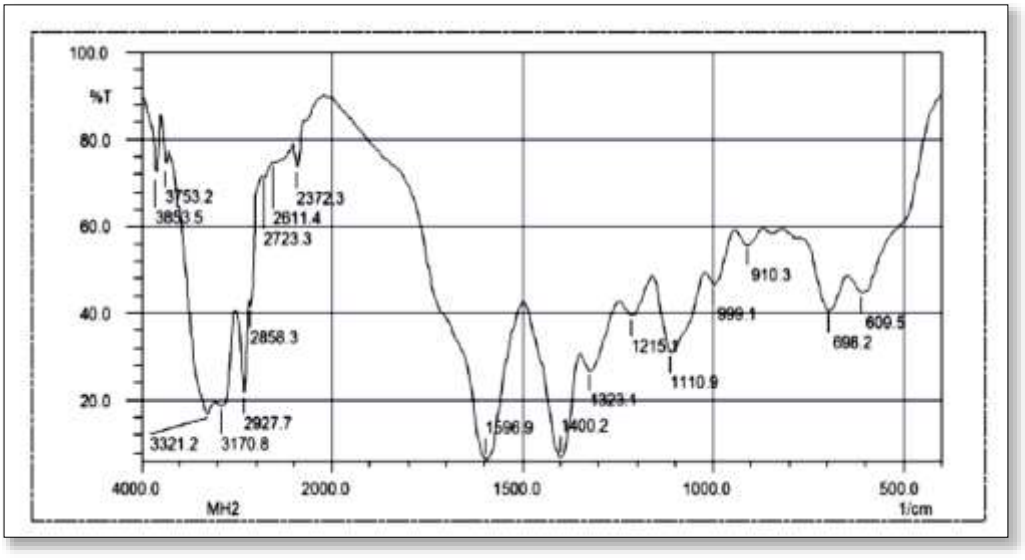


Table 19: IR modes and their corresponding assignments of Methanolic extracts of *Halodule uninervis*

υ (cm-1)	**Corresponding assignments**
3321.2 3170.9	stretching vibration of O–H groups hydrogen bonded (alcohols and hydroxyls)
2927.7 2858.3	C–H stretching (hydrocarbon skeleton)
2372.3	amino acids /acetals
1596.9	aromatic C=C stretch and/or
1400.2	ester skeleton (RCOO-)
1323.1	C–C stretching of phenyl groups
1215.1	C–O stretch (due to hydroxyl)
1110.9	stretching in phenols / alcohols
999.1 910.3	- OH (oop)
698.2, 609.5	C–H bend/ -CH2 rocking

Fig.21: FTIR spectrum of crude Ethylacetate extract of *Halodule uninervis*

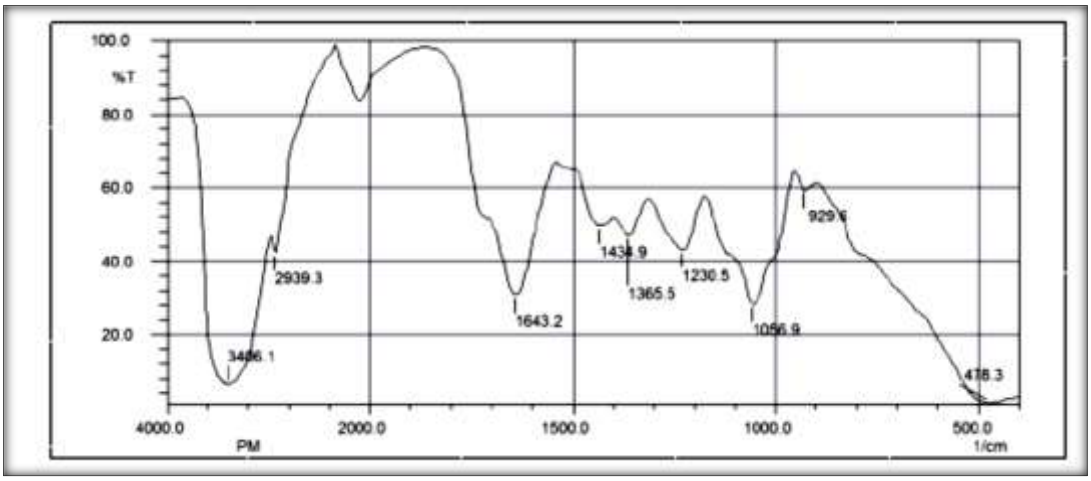


Table 20: IR modes and their corresponding assignments of Ethyl acetate extracts of *Halodule uninervis*

υ (cm-1)	**Corresponding assignments**
3406.1	stretching vibration of O–H groups hydrogen bonded (alcohols and hydroxyls)
2939.3	sp3 C–H stretching (hydrocarbon skeleton)
1643.2	bending vibration in N-H
1434.1 1365.5	C–C stretching of phenyl groups
1230.5	bending of O–H groups and/or C-O stretching in phenols / alcohols
1056.9	C–O stretching (due to hydroxyl)
926.6	C–H bend or OH (oop)

6.8.4 Characterization of isolated compounds

6.8.4.1 Identification of isolated compounds by chemical test

Table 21 : Identification of isolated compounds by chemical test

Test	**Observation**	**Result**
Shinoda Test: To 1 ml of the extract, magnesium turnings were added followed by 1-2 drops of concentrated hydrochloric acid.	red colour formed	Presence of flavanoid group.

6.8.4.2 Evaluation of physical characters:

Physical characters of evaluated compounds were tabulated below

Table 22: Physical characters of isolated compounds

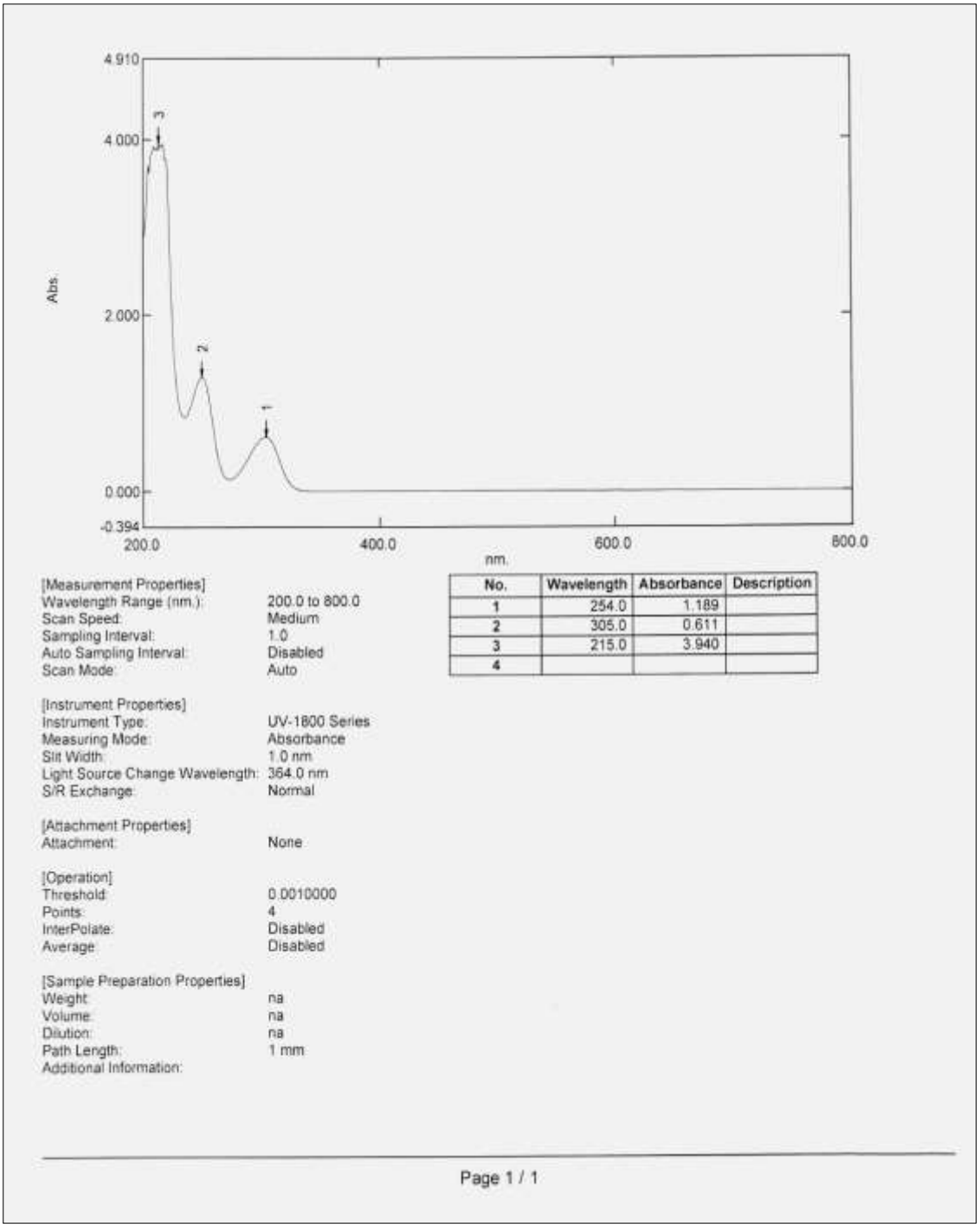
Parameters	Physical characters	
	Compound-I: ME-HU-C-1	**Compound-2: ME-HU-C-2**
Appearance	Pale yellow residue	light brown colour residue
Yield Obtained	148.52 mg	119.29 mg
Solubility	Soluble in Methanol, IPA, n-Hexane and Ethyl acetate	Soluble in Methanol, IPA, n-Hexane and Ethyl acetate
Melting Range	202.23 to 204.56°C	344.6 to 346.3°C
Wavelength maximum	215,254 and 305 nm	225, 254 and 340nm
pH	5.84	4.72

6.8.4.3 Spectroscopic characterization of Isolated compounds:

6.8.4.3.1 UV spectroscpy:

Compound dissolved in methanol and scanned in Shimadzu UV-1800 from200 to 800nm and found the wave length maximum of compound-1 is 254nm.UV spectrum of Compound –I (ME-HU-C-1) **and** Compound –II (ME-HU-C-2) given in **Fig.22** & **Fig.23**.

Fig.22: UV spectrum of Compound –I (ME-HU-C-1)



No.	Wavelength	Absorbance	Description
1	254.0	1.189	
2	305.0	0.611	
3	215.0	3.940	
4			

[Measurement Properties]
Wavelength Range (nm.): 200.0 to 800.0
Scan Speed: Medium
Sampling Interval: 1.0
Auto Sampling Interval: Disabled
Scan Mode: Auto

[Instrument Properties]
Instrument Type: UV-1800 Series
Measuring Mode: Absorbance
Slit Width: 1.0 nm
Light Source Change Wavelength: 364.0 nm
S/R Exchange: Normal

[Attachment Properties]
Attachment: None

[Operation]
Threshold: 0.0010000
Points: 4
InterPolate: Disabled
Average: Disabled

[Sample Preparation Properties]
Weight: na
Volume: na
Dilution: na
Path Length: 1 mm
Additional Information:

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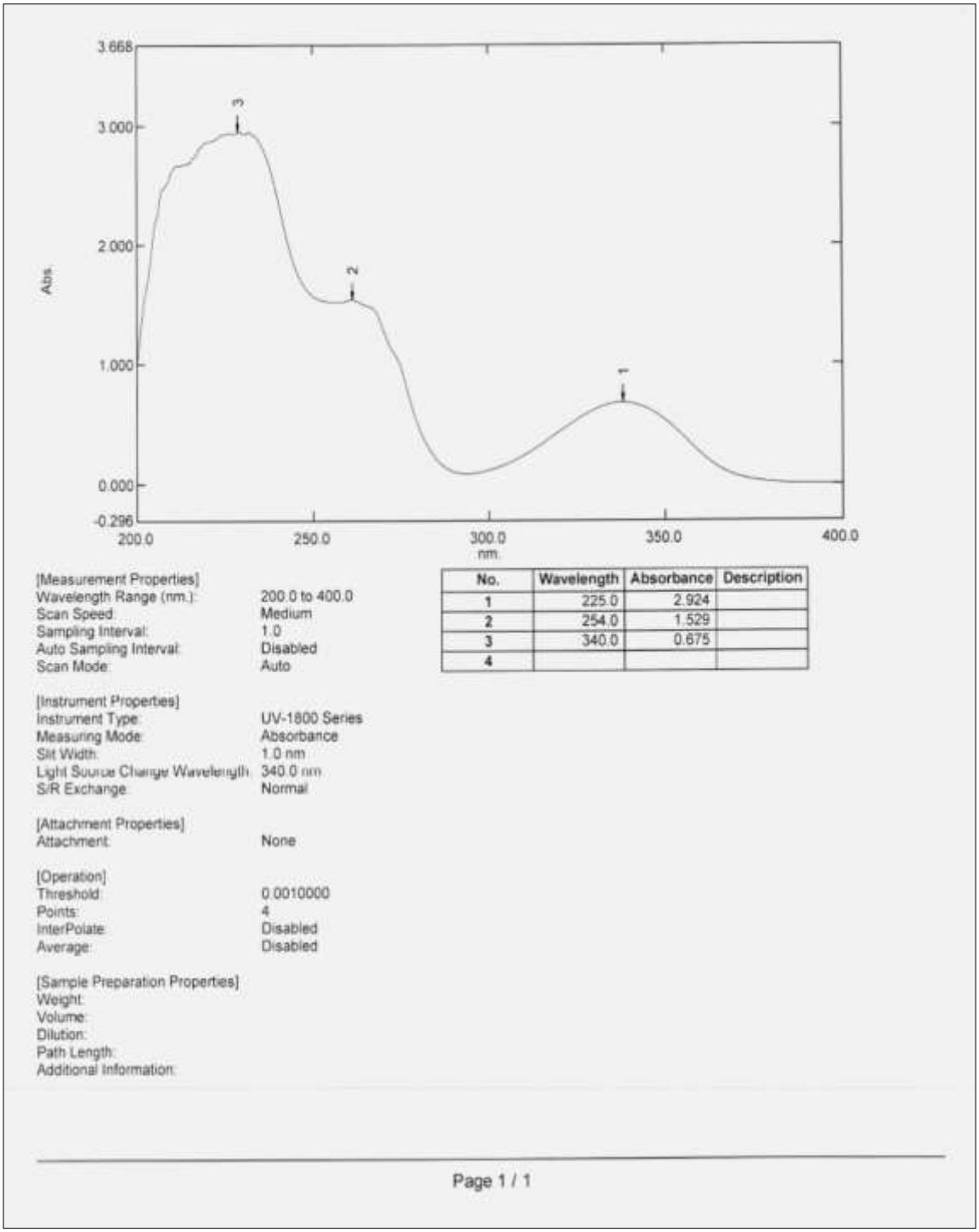
Fig.23: UV spectrum of Compound –II (ME-HU-C-2)



[Measurement Properties]
Wavelength Range (nm.): 200.0 to 400.0
Scan Speed: Medium
Sampling Interval: 1.0
Auto Sampling Interval: Disabled
Scan Mode: Auto

No.	Wavelength	Absorbance	Description
1	225.0	2.924	
2	254.0	1.529	
3	340.0	0.675	
4			

[Instrument Properties]
Instrument Type: UV-1800 Series
Measuring Mode: Absorbance
Slit Width: 1.0 nm
Light Source Change Wavelength: 340.0 nm
S/R Exchange: Normal

[Attachment Properties]
Attachment: None

[Operation]
Threshold: 0.0010000
Points: 4
InterPolate: Disabled
Average: Disabled

[Sample Preparation Properties]
Weight:
Volume:
Dilution:
Path Length:
Additional Information:

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6.8.4.3.2 IR :

IR Spectrum was taken using KBr- media using Alpha- Bruker IR spectrophotometer. The IR spectrum for compound-I and II given in **Fig.23** and **Fig.24** the characteristic peak table given in **Table-22** and **23**

Fig.24: IR spectrum of compound-I isolated from methanolic extract of

Halodule uninervis

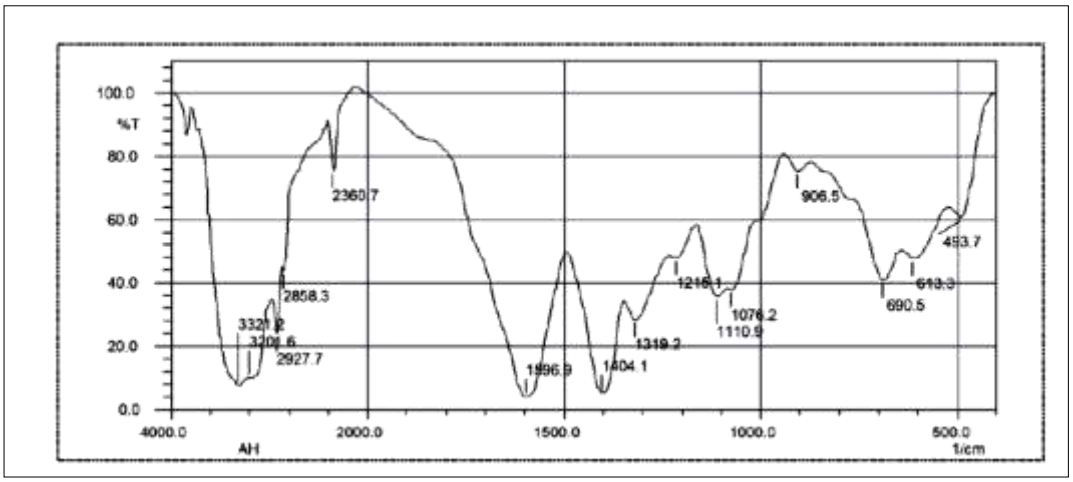


Table 23: IR modes and their corresponding assignments of Compound-I

PM **υ (cm^{-1})**	**Corresponding assignments**
3321.2 3201.6	stretching vibration of O–H groups hydrogen bonded (alcohols and hydroxyls)
2943.2	sp3 C–H stretching (hydrocarbon skeleton)
1681.8	Quinone or conjugated ketone
1596.9	Benzene (C=C benzene)
1400.2 1299.9	C–C stretching of phenyl groups
1222.8 1114.8	bending of O–H groups and/or C-O stretching in phenols / alcohols
1064.6	C–O stretching (due to hydroxyl)
918.1 690.5	C–H bend or OH (oop)

Fig.25: IR spectrum of compound-II isolated from methanolic extract of *Halodule uninervis*

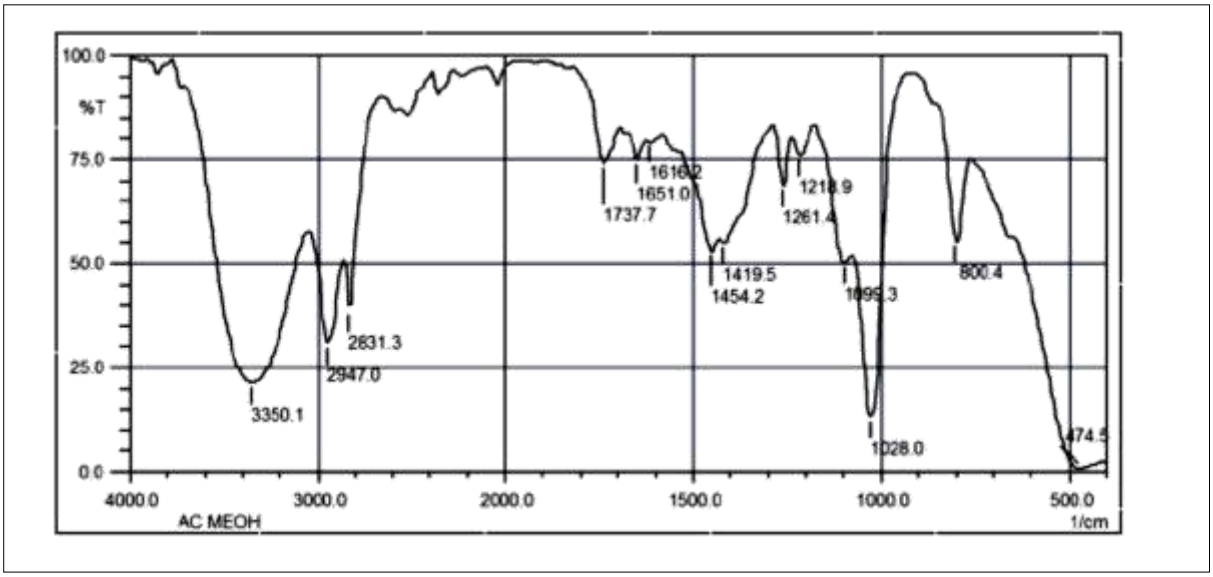


Table 24: IR modes and their corresponding assignments of Compound-II

υ (cm-1)	**Corresponding assignments**
3350.1	stretching vibration of O–H groups hydrogen bonded (alcohols and hydroxyls)
2947.0, 2831.3	sp3 C–H stretching (hydrocarbon skeleton)
1737.7	C=O stretch (could be ester)
1651.0	C=C stretching vibration
1616.2 , 1454.2	Benzene (C=C benzene)
1419.5	C–C stretching of phenyl groups
1261.4 1218.9	bending of O–H groups and/or C-O stretching in phenols / alcohols
1099.3 1028.0	C–O stretching (due to hydroxyl)
800.4	C–H bend or OH (oop)

6.8.4.3.3 NMR:

^{1}H NMR and ^{13}C NMR was taken on (Bruker Advance II 400 NMR spectrophotometer), using CDCl3 as solvent and with tetramethylsilane (TMS) as standard.

Fig.26: ^{1}H NMR (DMSO-*d*6, 500 MHz) spectrum of **COMPOUND-I: ME-HU-C-1**

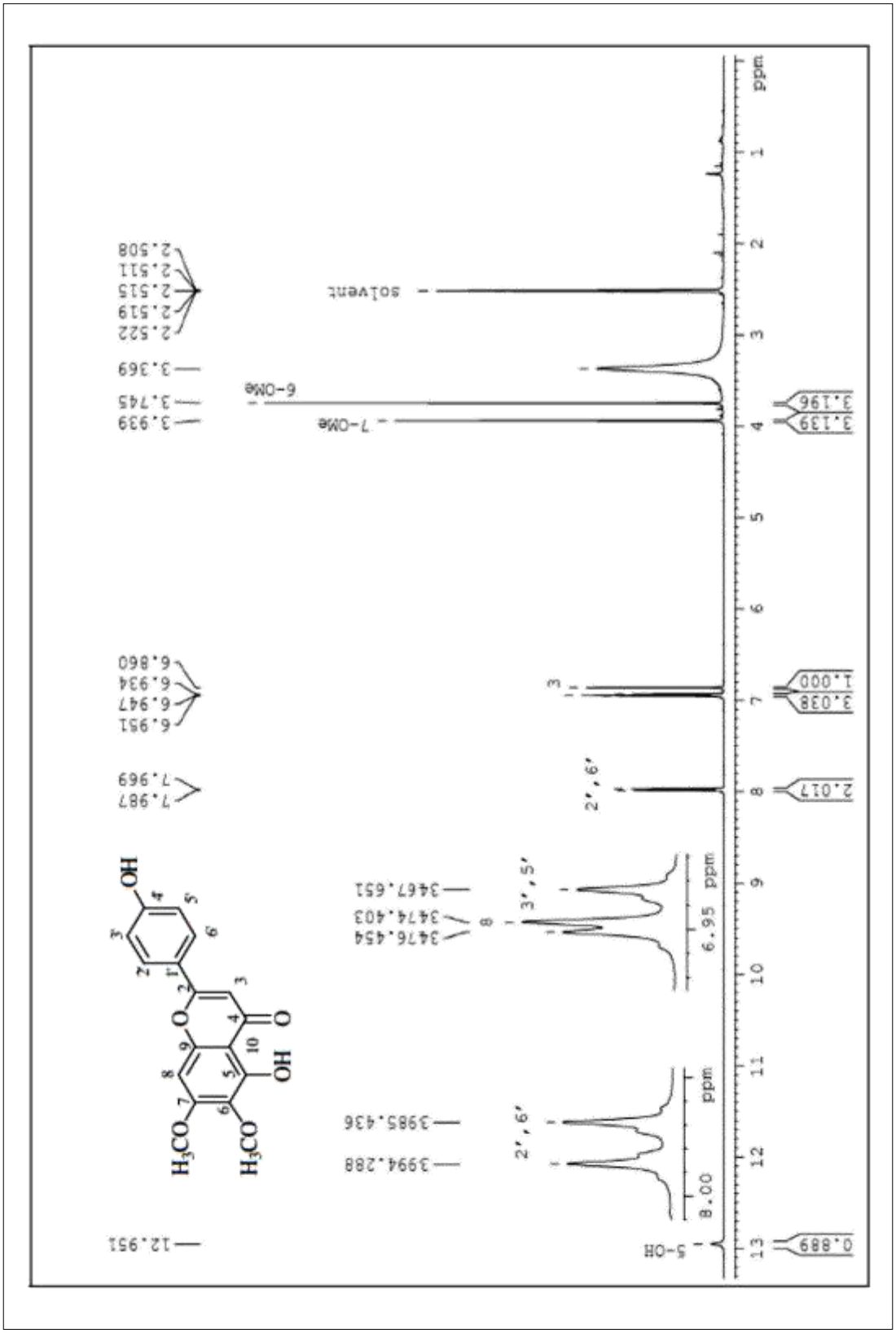
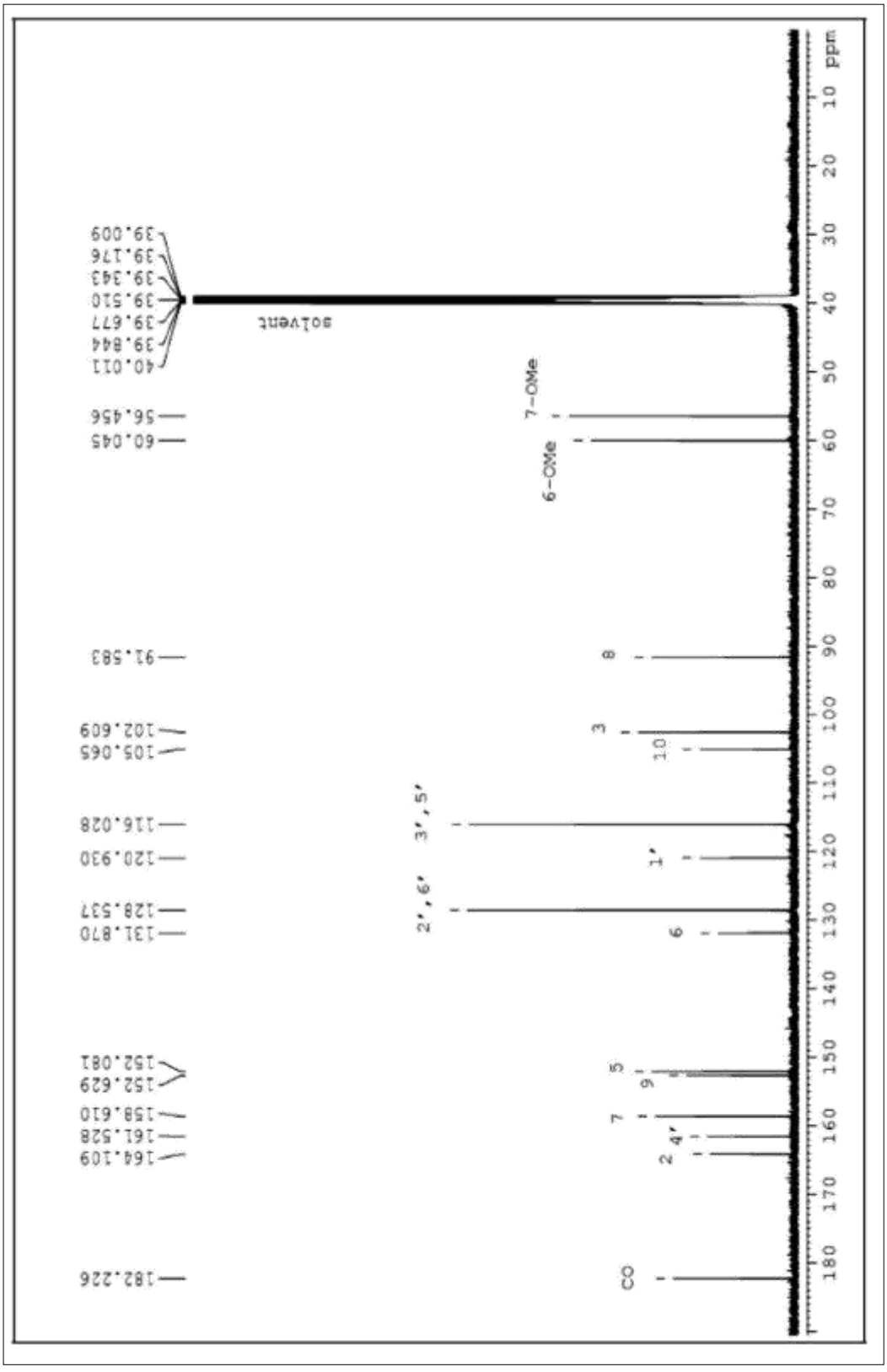
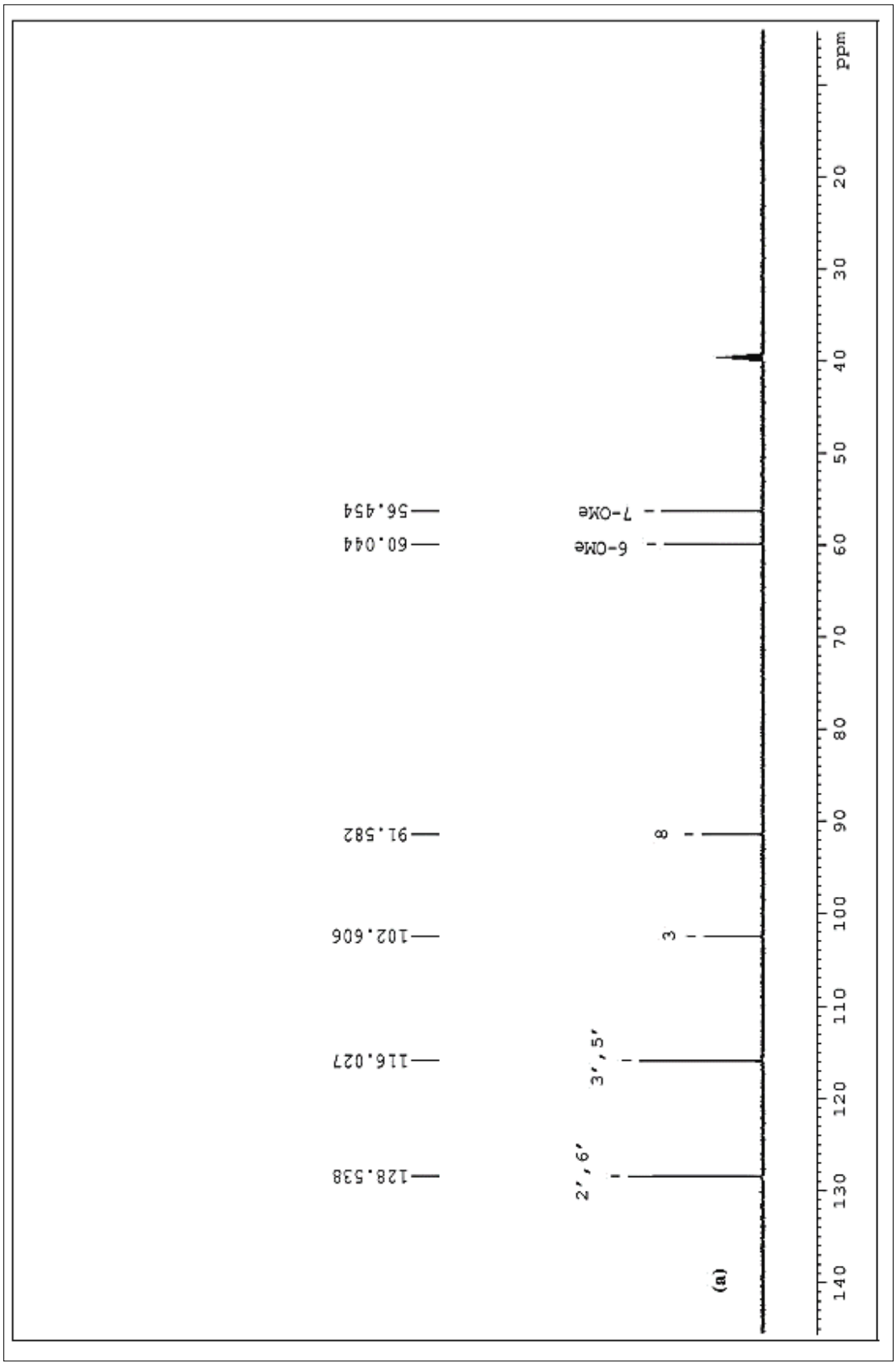


Fig.27: ^{13}C NMR (DMSO-*d*6, 125 MHz) spectrum of **COMPOUND-I: ME-HU- C-1**




(a)
2',6'
3',5'
3
8
6-OMe
7-OMe
128.538
116.027
102.606
91.582
60.044
56.454
ppm

The below characteristic peaks were identified in the ^{1}H NMR and ^{13}C NMR spectrum of isolated compounds of metanolic extract compound-I.

1H NMR (500 MHz, acetone*d*6): δ12.94 (s, 1 H, 5-OH), 7.95 (d, *J* = 8.8 Hz, 2 H, H-2', 6'), 7.02 (d, *J* = 8.8 Hz, 2 H, H-3',5'), 6.85 (s, 1 H, H-8), 6.68 (s, 1 H, H-3), 3.98 (s, 3 H, 7-OMe), 3.79 (s, 3 H, 6-OMe);

13C NMR (125 MHz, acetone-*d*6): δ183.61 (C-4), 165.49 (C-2), 162.33 (C-4') 160.13 (C-7),154.13 (C-9), 153.94 (C-5), 133.53 (C-6), 129.33 (C-2', 6'), 123.05 (C-1_), 116.97 (C-3', 5'), 106.51 (C-10), 103.84 (C-3), 92.02 (C-8), 60.62 (6-OMe), 56.89 (7-OMe).

The below characteristic peaks were identified in the ^{1}H NMR and ^{13}C NMR spectrum of isolated compounds of metanolic extract compound-II.

^{1}H NMR (500 MHz, DMSO-*d*6): δ12.97 (s, 1 H, 5-OH), 7.93 (d, *J* = 8.8 Hz, 2 H, H-2', 6'_), 6.93 (d, *J* = 8.8 Hz, 2 H, H-3', 5'), 6.78 (s, 1 H, H-3), 6.48 (d, *J* = 2.0 Hz, 1 H, H-8), 6.19 (d, *J* = 2.0 Hz,1 H, H-6);

13C NMR (125 MHz, DMSO-*d*6): δ181.75 (C-4), 164.29 (C-7), 163.72 (C-2), 161.46 (C-5), 161.20 (C-4'), 157.33 (C-9), 128.48 (C-2', 6'), 121.17 (C-1_), 115.97 (C-3', 5'), 103.65 (C-10), 102.82 (C-3), 98.87 (C-6), 93.99 (C-8).

Fig.28: ^{1}H NMR (DMSO-*d*6, 500 MHz) spectrum of **COMPOUND-II: ME-HU-C-2**

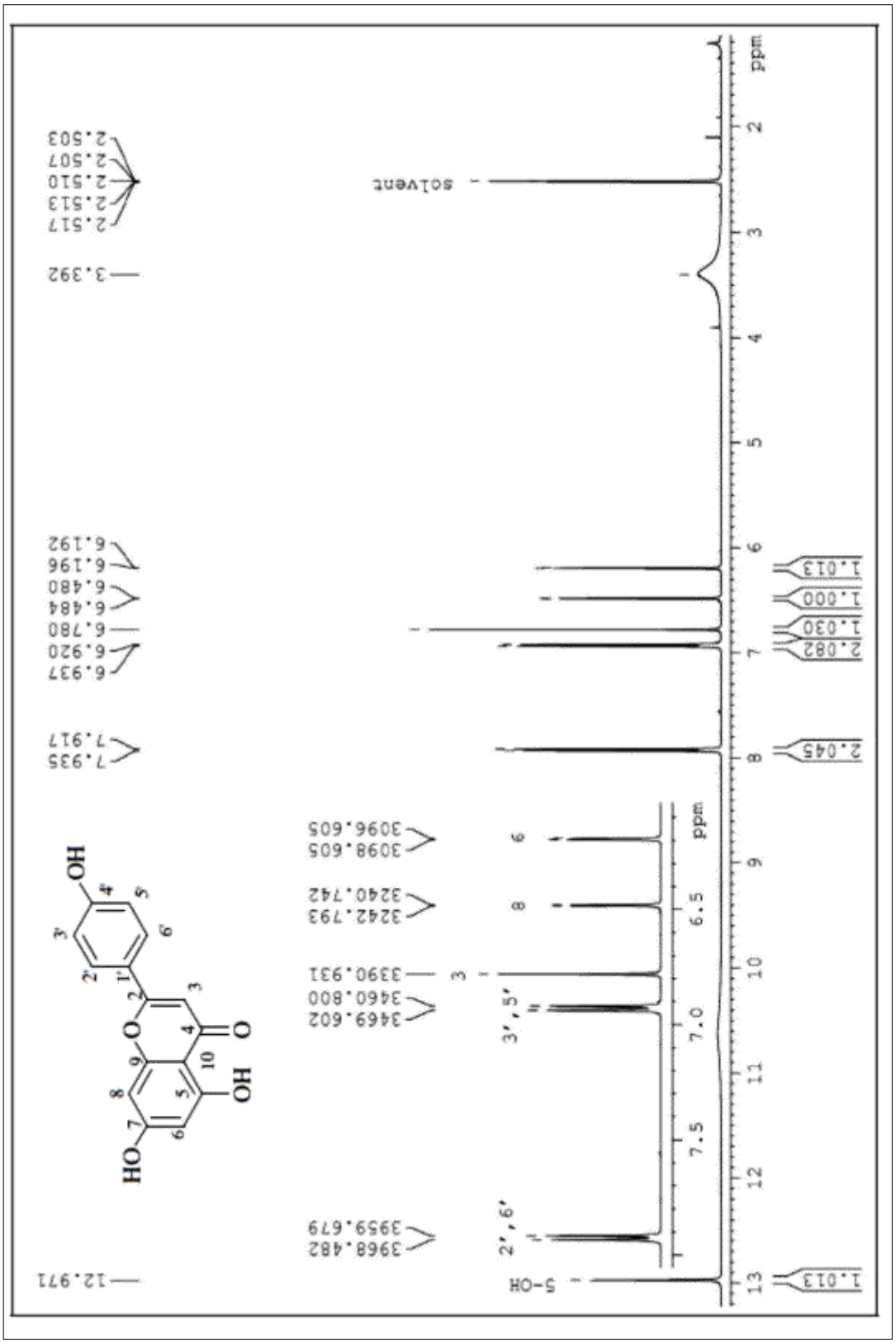
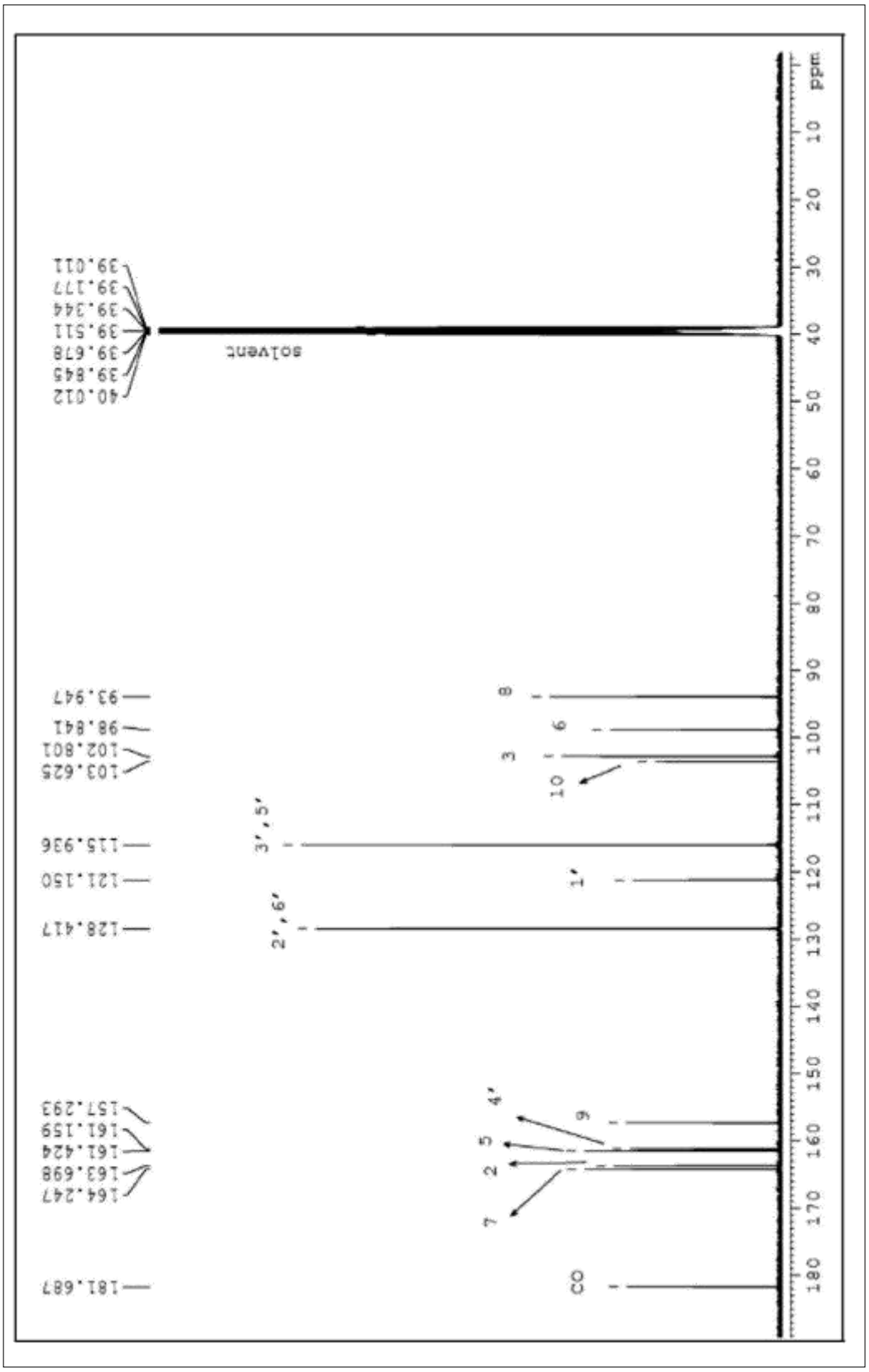
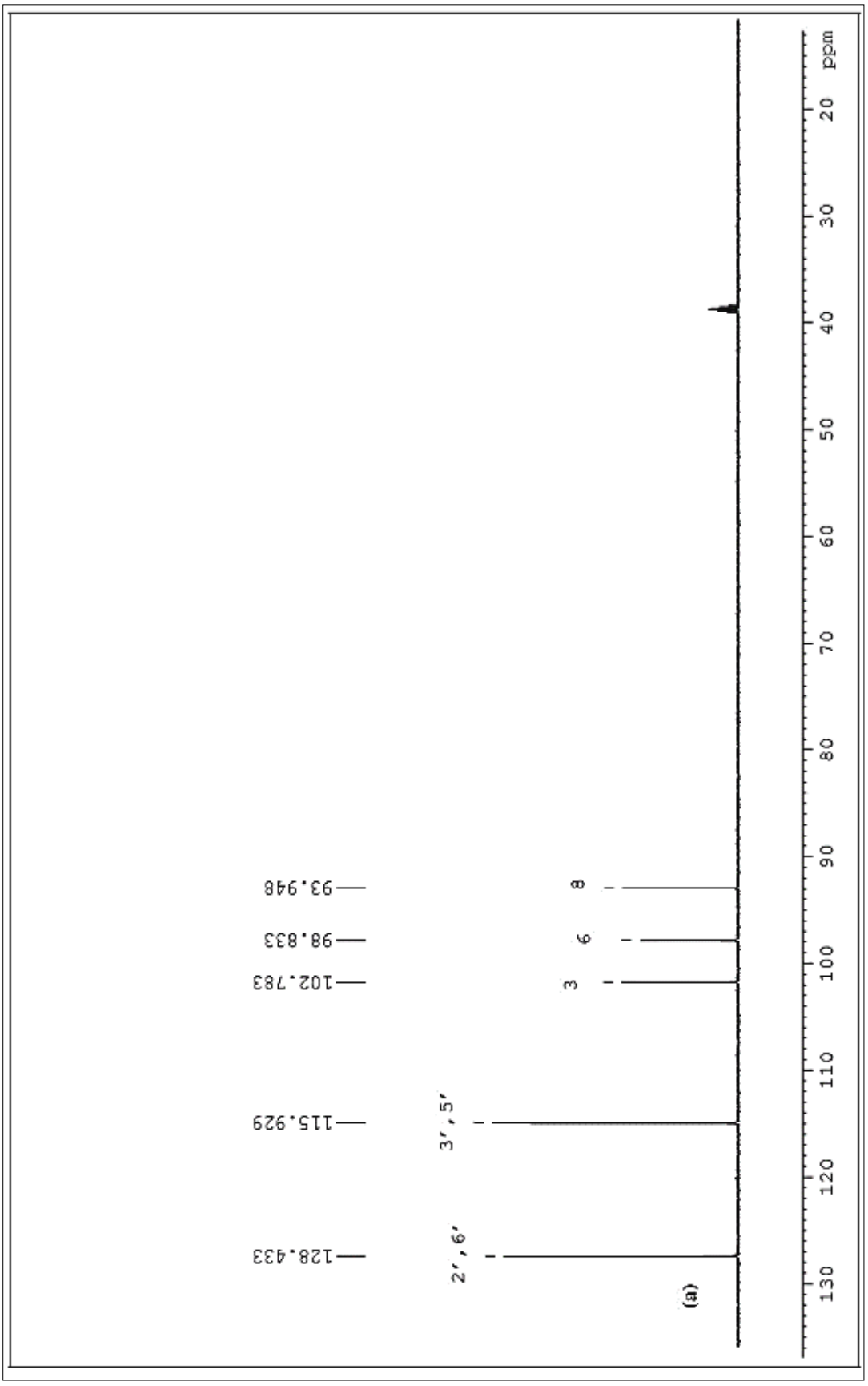


Fig.29: ^{13}C NMR (DMSO-*d*6, 125 MHz) spectrum of **COMPOUND-2: ME-HU-C-2.**

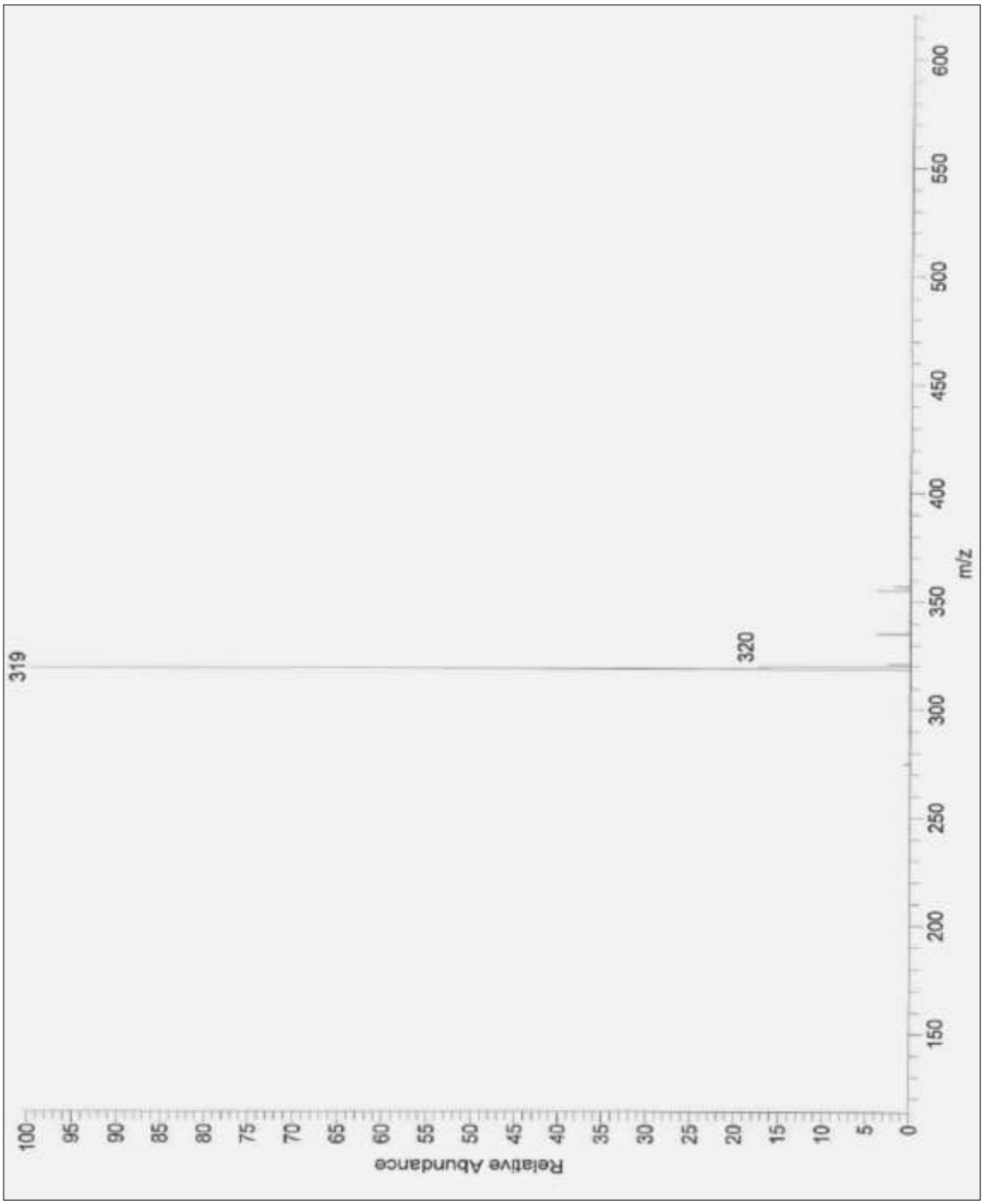



(a)
2′,6′
3′,5′
3
6
8
128.433
115.929
102.783
98.833
93.948
ppm

6.8.4.3.4 Mass spectroscopy:

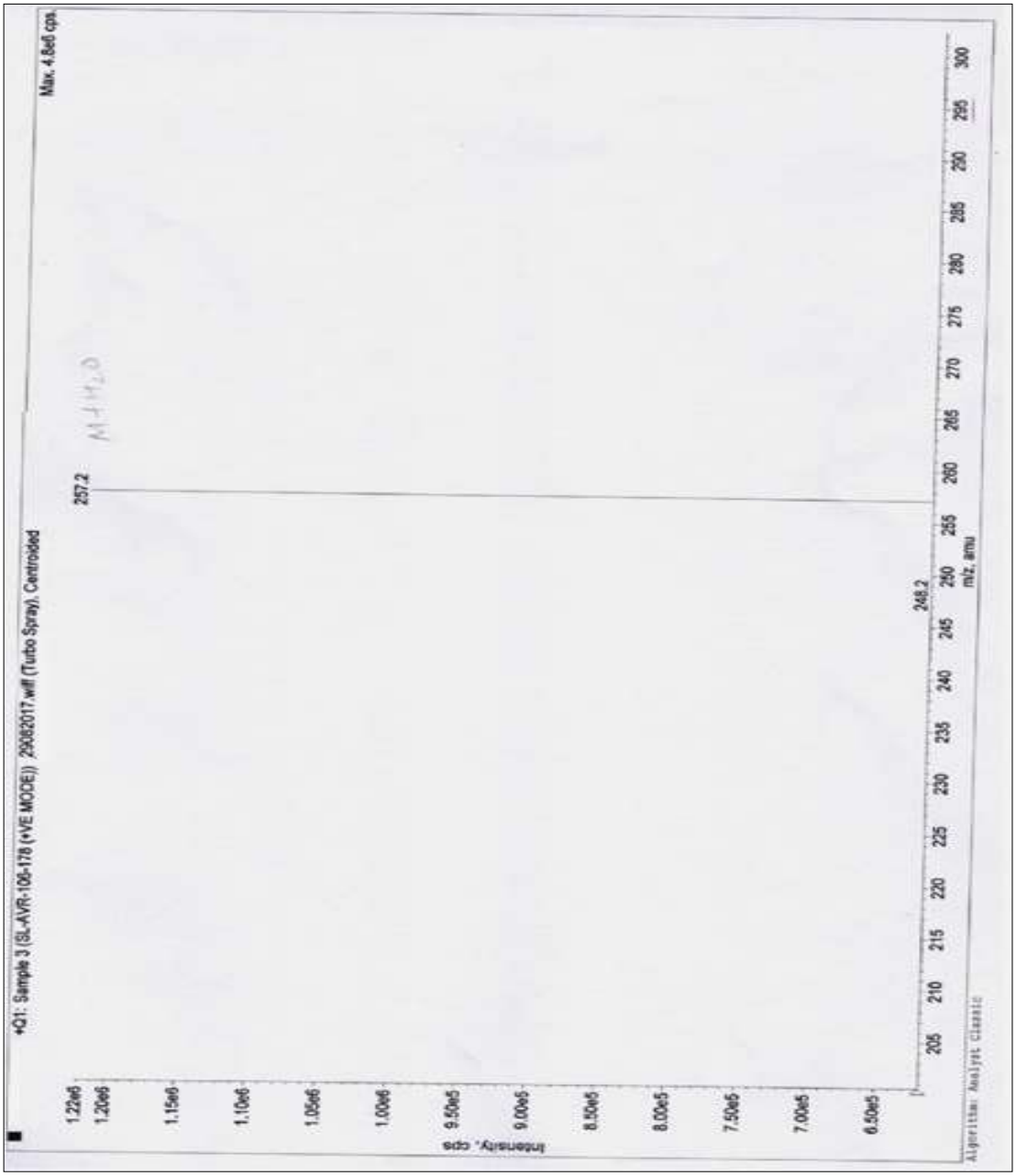
MASS spectrum was taken using neat on (TOF MS ES - 3.26e3 spectrophotometer). The spectrum was given in **Fig. 29 and 30**.

Fig.30: Mass spectrum of **COMPOUND-I: ME-HU-C-1.**



The mass spectrum of the isolated compound- I is presented in **Fig. 29.** The peak at m/z value of 319 (M^+) of isolated compound corresponded to the molecular ion peak.

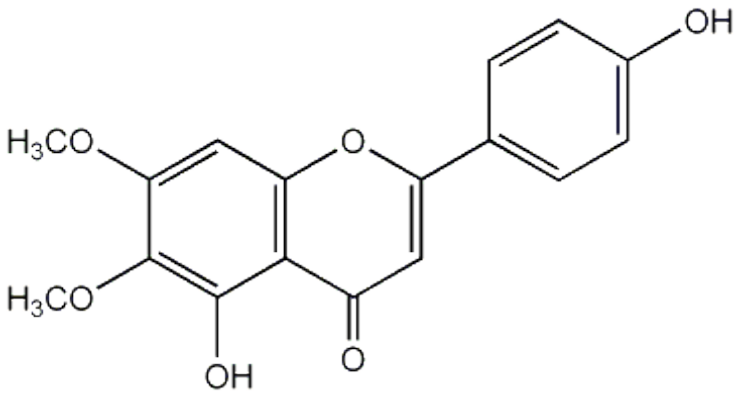
Fig.31: Mass spectrum of **COMPOUND-II: ME-HU-C-2.**



The mass spectrum of the isolated compound- II is presented in **Fig. 30.** The peak at m/z value of 257.2 (M^+) of isolated compound corresponded to the molecular ion peak.

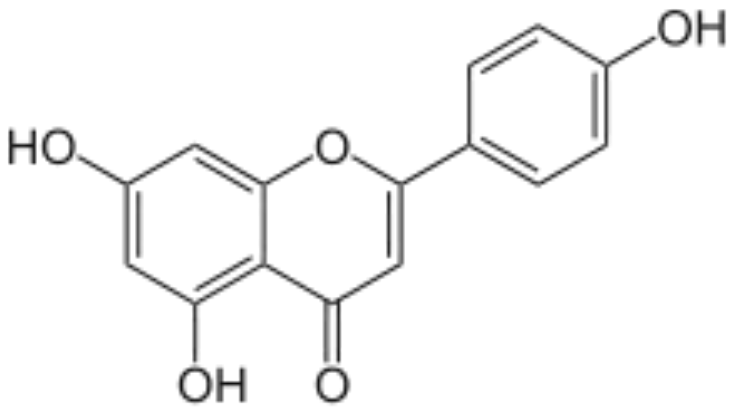
Based on the all spectral data, the tentative chemical name of **compound-I: ME-HU-C-1 to proposed as 4', 5-Dihydroxy-6, 7-dimethoxyflavone**.

The molecular formula of the compound-I was deduced as $C_{17}H_{14}O6$ and the tentative chemical structure proposed as follows



Based on the all spectral data, the tentative chemical name of **compound-II: ME-HU-C-2 to propose as 4′, 5, 7-trihydroxyflavone.**

The molecular formula of the compound-II was deduced as $C_{15}H_{10}O_5$ and the tentative chemical structure proposed as follows



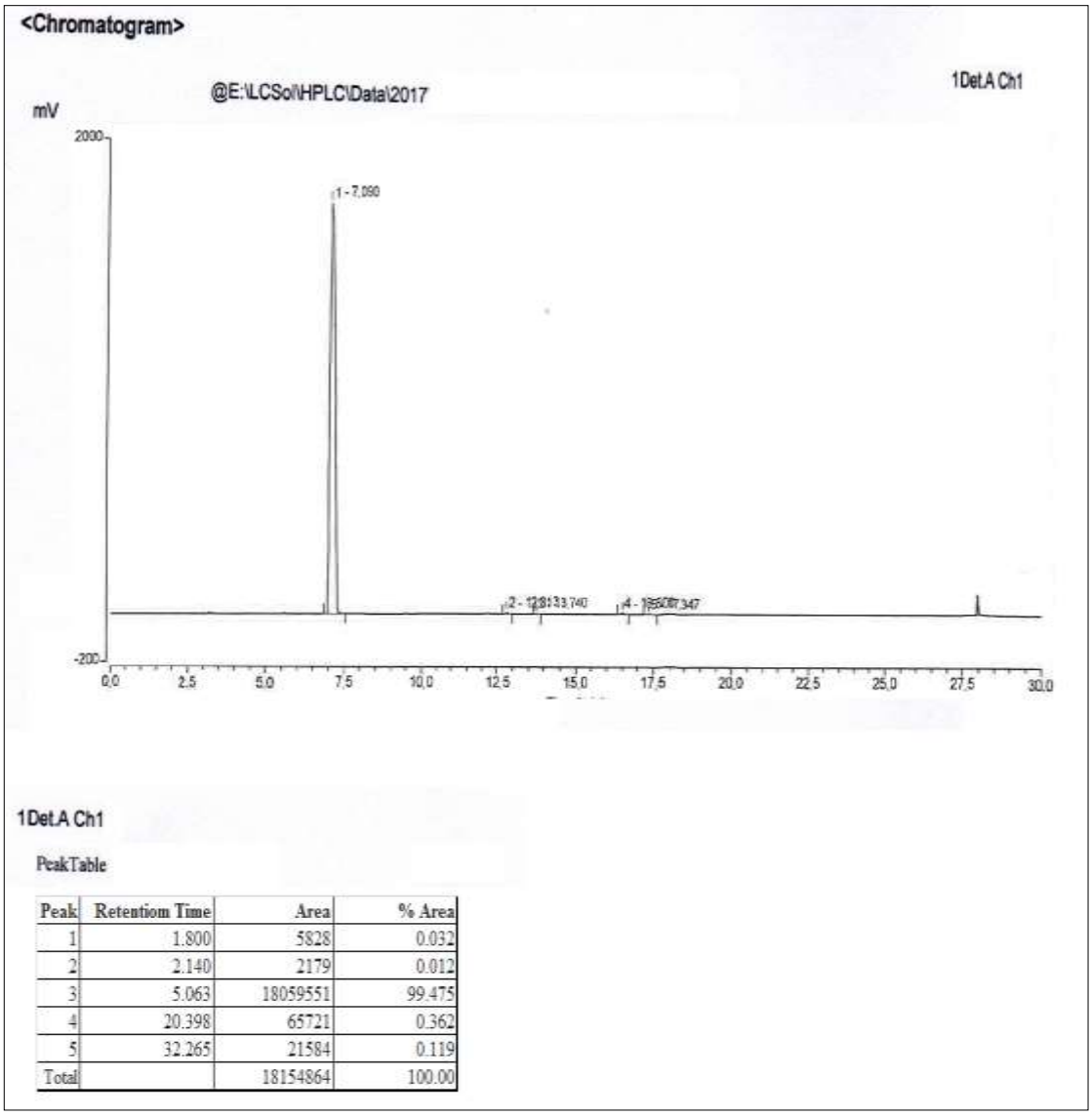
6.8.4.3.5 HPLC purity determination:

The purity of isolated components were determined by HPLC and the results are tabulated in **table-25**. Respective chromatograms are given in **fig.32 and 33**

Table-27: The HPLC purity of isolated components

Compound	HPLC purity
COMPOUND-I: ME-HU-C-1	99.5
COMPOUND-I: ME-HU-C-2	99.5

Fig. 32 HPLC purity chromatogram of COMPOUND-I: ME-HU-C-1

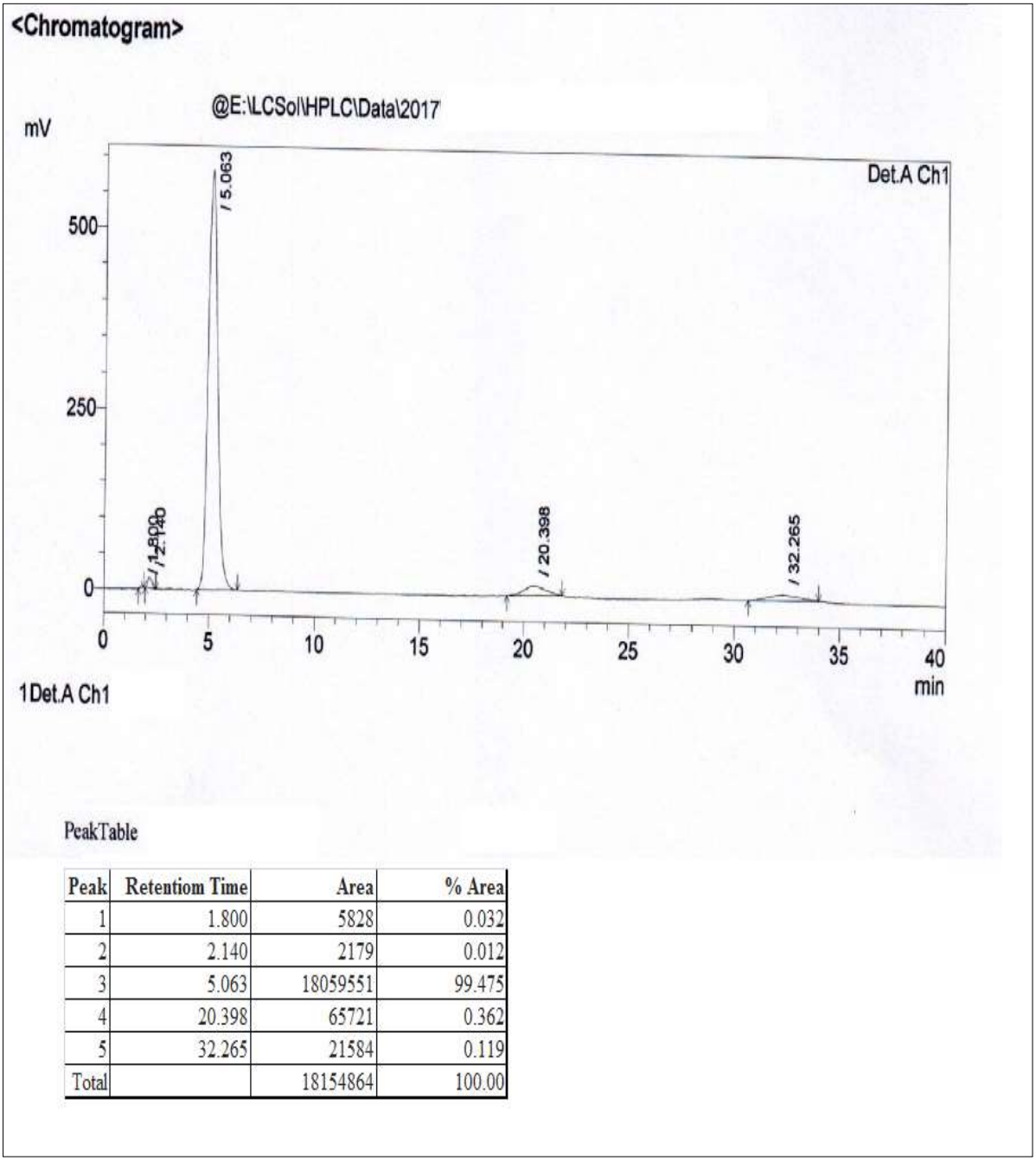


1Det.A Ch1

PeakTable

Peak	Retention Time	Area	% Area
1	1.800	5828	0.032
2	2.140	2179	0.012
3	5.063	18059551	99.475
4	20.398	65721	0.362
5	32.265	21584	0.119
Total		18154864	100.00

Fig. 33 HPLC purity chromatogram of COMPOUND-I: ME-HU-C-2



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2	2.140	2179	0.012
3	5.063	18059551	99.475
4	20.398	65721	0.362
5	32.265	21584	0.119
Total		18154864	100.00

6.9 ANTIOXIDANT ACTIVITY

6.9.1 DPPH free radical scavenging activity

Methanolic extract of *Halodule uninervis* showed up to 62% inhibition of DPPH free radical at 517 nm at high concentration of 1mg/ml. At the concentrations of 0.3mg/ml, 0.5mg/ml and 1mg/ml, quercetin shows similar activity of about 77.6% inhibition of DPPH free radicals. The percentage inhibition of DPPH free radical by Methanol extract in the presence of standard quercetin at 517 nm .

Table-28: Percentage inhibition of DPPH free radicals by extract /standard at 517 nm

Particulars	Concentration (µg/ml)	% of activity(inhibition)	
		Extract	Quercetin
Extract	0.1	9.2	14.9
	0.2	14.8	25.3
	0.3	26.1	51.6
	0.5	55.1	62.2
	1.0	62.3	77.6

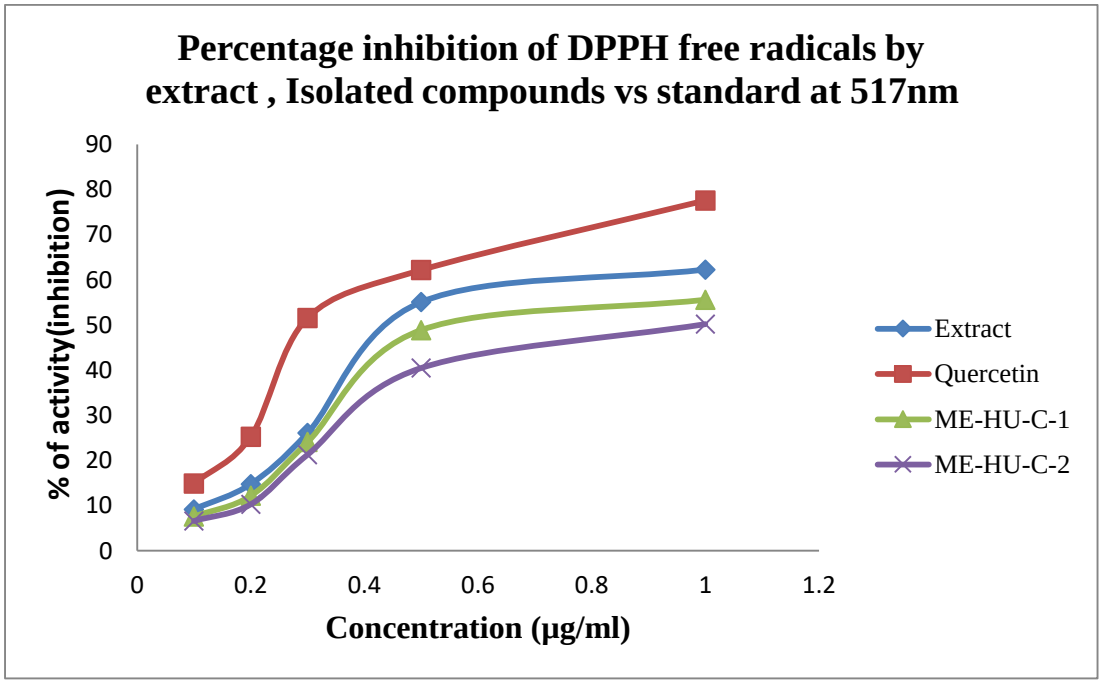
Table: 29: Percentage inhibitions of DPPH free radicals by standard and isolated compound-I at 517 nm

Particulars	Concentration (microgram/ml)	% of activity(inhibition)	
		ME-HU-C-1	Quercetin
COMPOUND I ME-HU-C-1	0.1	7.6	14.9
	0.2	12.2	25.3
	0.3	24.1	51.6
	0.5	48.9	62.2
	1.0	55.6	77.6

Table: 30: Percentage inhibitions of DPPH free radicals by standard and isolated compound-II at 517 nm

Particulars	Concentration (microgram/ml)	% of activity(inhibition)	
		ME-HU-C-2	Quercetin
COMPOUND II ME-HU-C-2	0.1	7.6	14.9
	0.2	12.2	25.3
	0.3	24.1	51.6
	0.5	48.9	62.2
	1.0	55.6	77.6

Fig.34: Percentage inhibition of DPPH free radicals by extract, Isolated compounds vs standard at 517nm



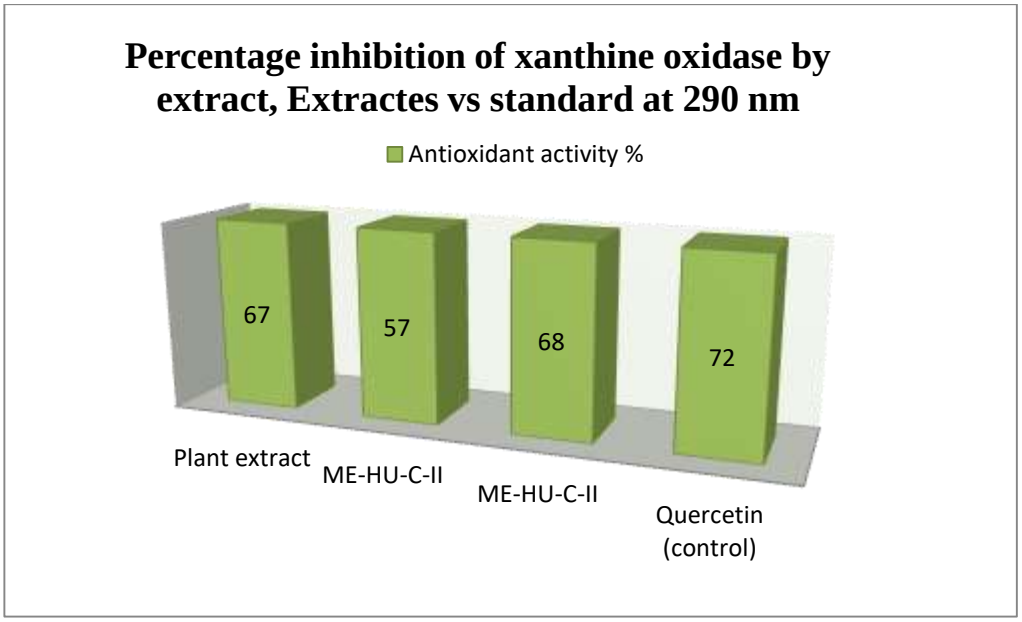
6.9.2: Xanthine oxidase inhibition assay

The *in-vitro* xanthine oxidase inhibition of Methanolic extract revealed 67% of inhibitory activity at 30µg/ml and it was compared with the standard drug quercetin which showed 72% inhibition at 30µg/ml also the isolated compounds showing prominent activity against standard.

Table-31: Percentage inhibition of xanthine oxidase by extract, isolated compounds and standard at 290 nm

Sample	Concentration	Antioxidant activity %
Quercetin(control)	30µg/mL	72
Plant Extract	30µg/mL	67
COMPOUND I	30µg/mL	57
COMPOUND II	30µg/mL	68

Fig.35: Percentage inhibition of xanthine oxidase by extract, Extracts vs standard at 290 nm



6.9.3 Hydrogen peroxide scavenging assay

The *Halodule uninervis* Methanolic crude extract 100, 200, 300 and 400 µg/ml .was capable of scavenging H_2O_2 in an amount dependent manner.

Table-32: Percentage inhibition of Hydrogen peroxide by extract /standard at 230 nm

S.No	Concentration(µg/ml)	%of inhibition
1	100	26.55
2	200	41.90
3	300	65.43
4	400	77.5
Ascorbic acid		
1	100	76.5

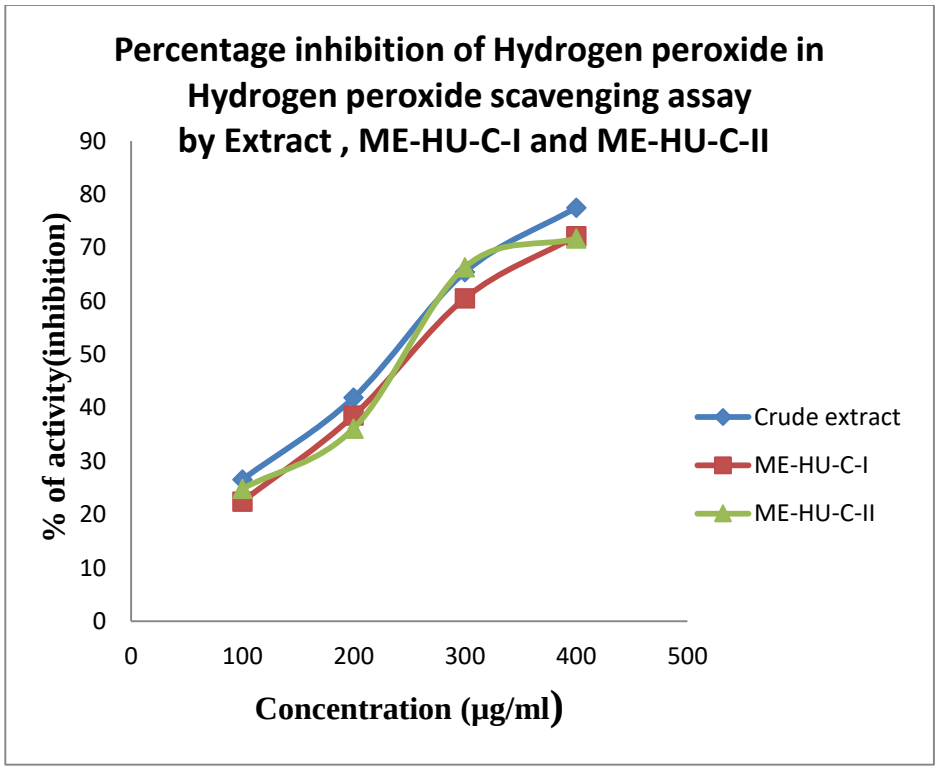
Table-33-Percentage inhibition of Hydrogen peroxide by COMPOUND I /standard at 230 nm

Sl.No	Concentration (µg/ mml)	%of inhibition
1	100	22.43
2	200	38.55
3	300	60.53
4	400	72.18
Ascorbic acid		
1	100	76.5

Table-34: Percentage inhibition of Hydrogen peroxide by COMPOUND II /standard at 230 nm

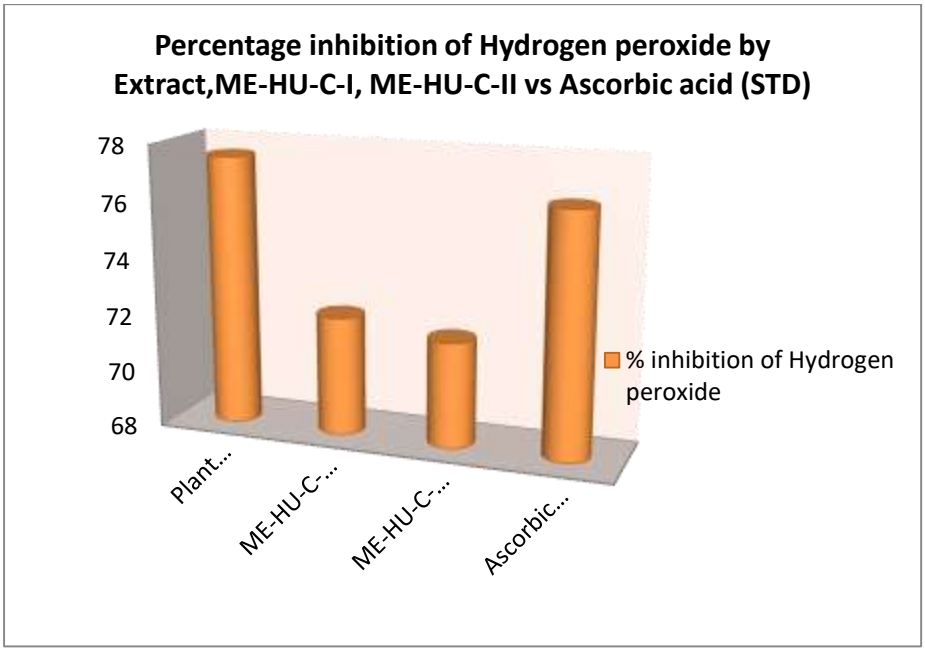
Sl.No	Concentration(µg/ml)	%of inhibition
1	100	24.82
2	200	36.14
3	300	66.35
4	400	71.71
Ascorbic acid		
1	100	76.5

Fig.36: Percentage inhibition of Hydrogen peroxide in Hydrogen peroxide scavenging assay by Extract, ME-HU-C-I and ME-HU-C-II



H_2O_2 scavenging activity was closer to that of standard Ascorbic acid (100µg/ml) at doses 400 µg/ml also the isolated compounds showing prominent activity against standard.

Fig.37: Percentage inhibition of Hydrogen peroxide by Extract, ME-HU-C-I, ME-HU-C-II vs Ascorbic acid (STD)



6.10 ANTI MICROBIAL ACTIVITY OF ISOLATED ACTIVE CONSTITUENTS FROM *Halodule uninervis*

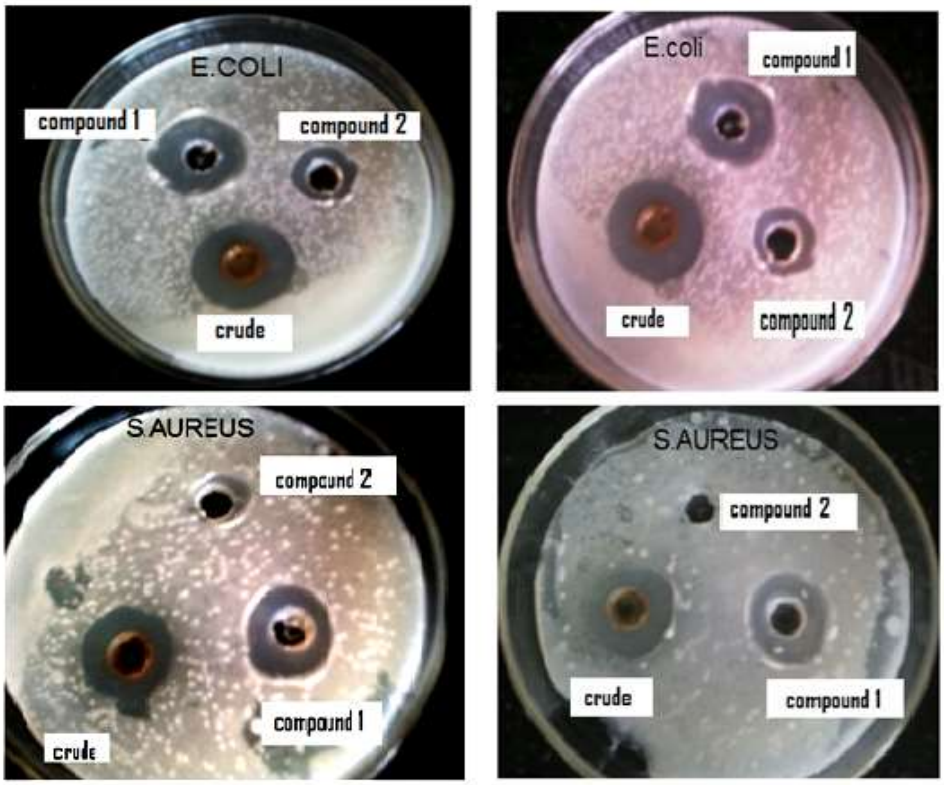
6.10.1Antimicrobial Activity - Determination of zone of inhibition method:

The crude Methanolic extract and isolated compounds ME-HU-C-1 and ME-HU-C-2 were screened for antibacterial activity using the agar plates.

Table-35: Anti bacterial activity for the extract and isolated compounds

Sample	Extracts	Microorganisms			
		Replicates	*E.coli*	*Salmonell*	*S. aureus*
2000ppm	Crude extracts	Replicate 1	15mm	No activity	14mm
		Replicate 2	14mm	No activity	14mm
	ME-HU-C-1	Replicate 1	12mm	No	12mm
		Replicate 2	10mm	No	10mm
	ME-HU-C-2	Replicate 1	11mm	No activity	9mm
		Replicate 2	14mm	No activity	7mm

Fig.38: Anti bacterial activity for the extract and isolated compounds



6.10.2 Minimal Inhibitory concentration:

The minimal inhibitory concentration (MIC) was determined by both broth dilution assay and agar diffusion assay methods.

Table-36: Broth dilution assay for MIC

Sample	*E.coli*					
	Micro organism	**1 ppm**	**10ppm**	**100ppm**	**1000ppm**	**2000ppm**
ME-HU-C-1	***E.coli***	Visible bacterial growth	Visible bacterial growth	Slight growth	No growth	No growth
ME-HU-C-2		Visible bacterial growth	Visible bacterial growth	Slight growth	Slight growth	No growth

Table-37: Antifungal activity for the extract and isolated compounds

Sample	*Aspergillus sp*	*Candida*
Crude Extract	No inhibition	29% inhibition
ME-HU-C-1	No inhibition	No inhibition
ME-HU-C-2	No inhibition	No inhibition

Fig 39 : Anti bacterial activity of MIC (Broth dilution assay)

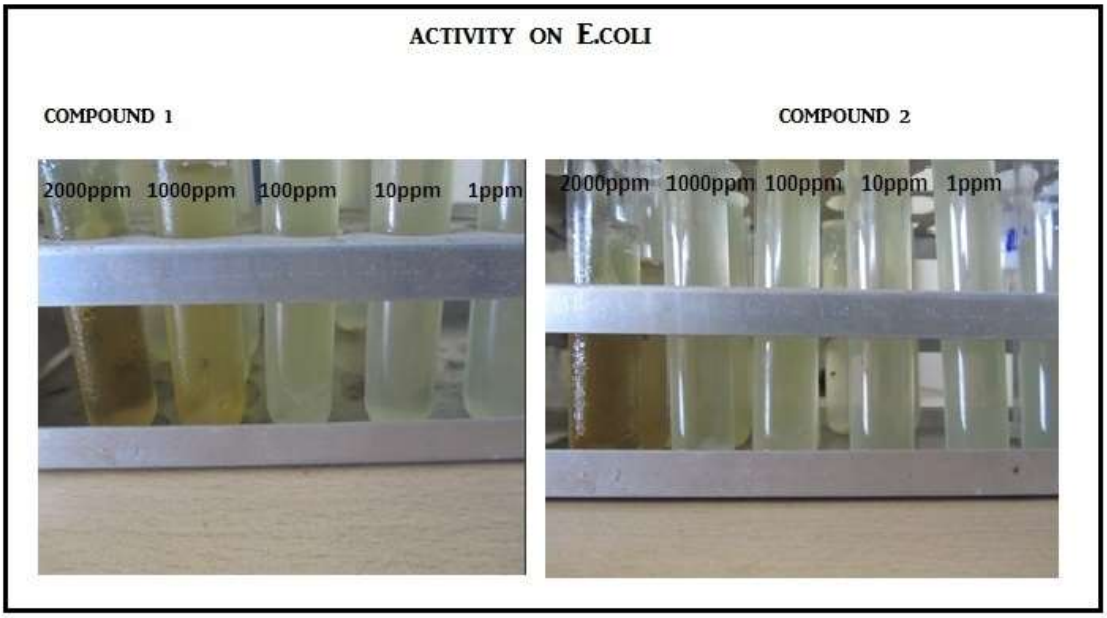
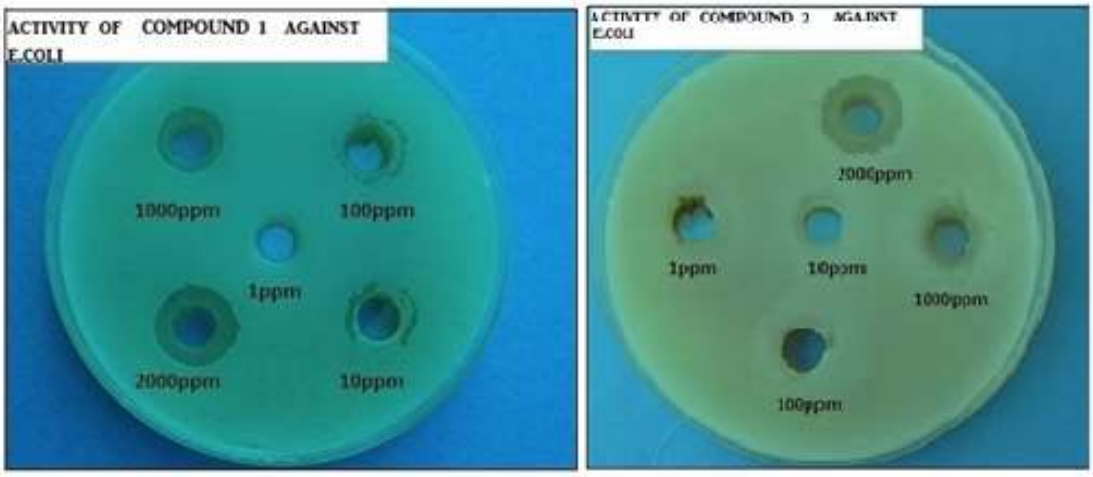


Fig 40: Anti bacterial activity of MIC (Agar diffusion assay)



Chapter VII
Discussion

DISCUSSION

7.1 Seagrasses and its impotents in natural medicines:

In India the use of plant materials and components compounds for medicinal and manufacturing pharmaceutical actives has measurably increased. The kingdom of plants are the reservoir of a largely unexplored biologically active compounds not only as drugs but also the unique components that could serve as a starting material an intermediates for synthetic compounds. Plants kingdom also serve to understanding of biological processes leads to invent the better synthetic routs to produce pharmaceutical active components with cost effective manner.

Among them marine products possess quite novel structures which lead to pronounced biological activities and novel pharmacology. The study of such compounds lead to most successive results for the development of new pharmacological classes with cost effective manner. Indian costal regains hold treasures in terms of natural products which have many pharmaceutical potential values as like the natural products in the land. It is infinite and will remain undiscovered unless research into drug development establishes the medicinal value of the chemical compounds they hold. Marine herbal medicine sometimes referred as herbalism or botanical medicine because for their medicinal or therapeutic value and has been used throughout history by all cultures. Seagrasses are marine flowering plants. They are capable of completing their life cycle when they are submerged in sea water. Ecosystem of Seagrass is one of the most widespread coastal vegetation types as compared to the ecosystems of coral and mangrove. phytochemical study on sea grasses has clearly shown that seagrasses have pharmaceutically active secondary

metabolites. Different studies on these seagrass metabolites proven that the extracted active components from saegrasses have alkaloids, glycosides, galactomannan gum, polysaccharides, peptidoglycan, hypoglycans, guanidine, steroids, carbohydrates, glycopeptides, terpenoids, amino acids and inorganic ions which directly or indirectly have a effective action aganst infection, pharmaceutically active hypolipemic agents, reduce blood pressure, and blood cholesterol levels. Phenolic compounds are effective secondary metabolites of seagresses which play a main role in various bio relevant activities in human body maintenance. The presence of phytoconstituents, such as flavonoids, phenols, and tannin, in seaweeds and seagrasses indicates a possibility that extracts may have anti-oxidant activity and anti diabetic activity.

7.2 Halodule uninervis and its Phytochemical evaluation

To identify the various phytoconstituents of *Halodule uninervis* and its pharmacological properties, The plant shade powder was screened from the multiple analysis it was shown the presence of various Phytoconstituents like alkaloid, carbohydrate, tannin, Saponin, terpenoid, flavonoid, Coumarin and phenol are present .Ash of any organic material is composed of their non-volatile inorganic components. Controlled incineration of crude drugs results in an ash residue consisting of an inorganic material (metallic salts and silica). This value varies within fairly wide limits and is therefore an important parameter for the purpose of evaluation of crude drugs. Therefore percentage of the total ash, acid insoluble ash and water soluble ash were carried out. The determinations such as loss on drying and ash values indicate the status of air-dried drugs used for studies. The total ash values when comes in acceptable range it simply shows that no inorganic adulteration is present. The extraction of any crude drug with a particular solvent yields a solution containing different phyto-constituents.

The Methanol extract obtained from the successive extraction of *Halodule uninervis was* shown a well response for most of the qualitative chemical tests which conforms that the effective extraction of maximum constituents from the plant material and shows effective in-vivo and in-vitro pharmacological activity. The Brownish green methanol extract was taken for the isolation using silica gel packed column by using the solvents with increasing polarities. The elute was collected and concentrated. Extractive value is also useful for evaluation of crude drug, which gives an idea about the nature of the chemical constituents present in a crude drug and is useful for the estimation of specific constituents, soluble in that particular solvent used for extraction.

Based on the data these fractions were tested for the presence of various constituents and nature of the compounds. Upon the investigation of column fraction there are two major components were identified which are Compound 1-ME-HU-C-1 and Compound 2- ME-HU-C-2. Further characterizations of the isolated components were proceeded by IR, NMR and MASS spectroscopy to identify the isolated components and identified the presence of flavonoid moiety.

7.3 Atomic absorption spectrophotometric analysis:

Atomic absorption spectrophotometric analysis of methanolic extract of *Halodule uninervis* conformed the absence of Cd, Pb, As and Hg. The soil and the environment has been the predominant source of the HMs contamination mainly Cd, Pb, As and Hg. This may account for the high incidence and concentrations of Cd, Pb, As and Hg compared with other HMs in the plants. HMs in roots, rhizomes, seeds and fruits were apparently higher than in flowers, indicating that the sample of these parts might be more favourable for HMs contamination.

Cadmium is absorbed by the roots of many plants, cannot be removed by washing and is concentrated particularly in the kidneys, liver, blood forming organs and the lungs. The high Pb value in plants were due to the uptake from the available Pb in the soil and in the above ground parts (leaves, stem and seeds) is due to air born Pb. Mercury has a particular affinity to become deposited in vital organs such as brain, nervous system, heart, liver, kidneys, bone marrow and also known to cause dementia, peripheral neuropathy, Parkinson's disease and

7.4 Pharmacological evaluation:

7.4.1 Anti diabetic activity:

The current research focuses on identification of a effective active component from the marine source for the treatment diabetes mellitus, Insulin insufficiency or insulin dysfunction cause the complex metabolic disorder which is knows as Diabetes mellitus. a major disorder in the world leading to massive economic losses. Diabetes is a chronic disorder of carbohydrate, fat and protein metabolism characterized by increased fasting and fed blood sugar levels in the human body. World health organization predicted that the developing countries will face major burden due to diabetes. Studies conducted in India, highlighted that the prevalence of diabetes is high and also the rapid increase in the urban population. Prolonged medications required for the treatment of diabetes mellitus, considering the limitations and side effect in the synthetic drugs, natural products derived from medicinal plants and marine products are being looked for the treatment of diabetes. However components of medicinal plants and marine products are the base of many conventional drugs. Among 1200 plants and marine products which used for the treatment of diabetes mellitus there 200 pure compounds have showed lowering blood glucose activities considering low toxic,

ready availability and cost effective, usage of natural products for the treatment of diabetes mellitus has recommended and encouraged by WHO (World Health Organization) worldwide.

Effects of Multi concentration level of *Halodule uninervis* methanolic extract (50mg, 150mg and 250mg/kg) on blood glucose levels was estimated after Streptozotocin administration along with Liver and kidney functions, Antioxidant status, Biochemical parameter includes Serum glucose , plasma insulin , serum lipid profile were.

At dose levels of 150 and 250 mg/kg, glucose levels were decreased by 24.8% and 29.9% at the 6th hour, respectively. Antidiabetic effect of the extract was slightly decreased at the 8th hour, but remained statistically significant. In parallel experiments, *Halodule uninervis* extract was administered to diabetic rats for 18 days as per the protocol. The prominent reduction of glucose level shown in concentration dependent manner. The effects of *Halodule uninervis* extract on hepatic and renal function in streptozotocin-diabetic rats were analyses and 150 and 250 mg/kg concentration level shown significant improvements in hepatic and renal function rather than 50 mg/kg extract administration. Additionally, these two dose levels recovered the weight loss and low white blood cell count observed in streptozotocin-diabetic rats while decreasing liver glycogen. *Halodule uninervis.* extract (150 and 250 mg/kg) also showed a protective effect on liver oxidative status. Antioxidants namely GSH, GPx, SOD and catalase were increased by *Halodule uninervis* extract administration. When compared to streptozotocin-diabetic rats, MDA formation, as an indirect measure of lipid peroxidation, was found to be significantly low in high dose *Halodule uninervis* extract-treated rats. Histopathological Investigation shown the improvement of

destroyed cells. At the higher concentration regeneration of c cells and architecture of mesangial cells and Glomeruli observed near to control.

7.4.2 Anti oxidant activity

Free radicals are harmful by-products generated during normal cellular metabolism, which could initiate oxidative damage in the body. Antioxidants are believed to play a significant role in the body's defense system against free radical damage. The antioxidant activity of phenolic compounds is mainly due to their redox properties, which play an important role in neutralizing free radicals, quenching singlet and triplet oxygen, flavonoids are wide spread in all natural compounds and posses a broad spectrum of biological activities. The chemical composition of *Halodule uninervis* indicates the presence of phenolic compounds including tannins and flavonoids, which are known to possess antioxidant activities. The high phenolic and flavonoid content in the ethanolic extract of *Halodule uninervis* may be responsible for its free radical scavenging activity.

DPPH is the agent of choice for many similar studies in evaluating the free radical scavenging activity of natural compounds. DPPH is a stable free radical that accepts an electron or hydrogen radical to become a stable diamagnetic molecule. The reduction capability of the DPPH radical is determined by the decrease in its absorbance at 516 nm induced by antioxidants. The absorption maximum of a stable DPPH radical in ethanol was at 516 nm. The decrease in absorbance of DPPH radical caused by antioxidants, because of the reaction between antioxidant molecules and radical progresses, which results in the scavenging of the radical by hydrogen donation. Xanthine oxidase is a flavoprotein that catalyses the oxidation of hypoxanthine to xanthine and generates superoxide and uric acid. It has been shown that xanthine

oxidase inhibitors may be useful for the treatment of hepatic disease and gout, which is caused by the generation of uric acid and superoxide anion radical. Flavonoids and Polyphenolic crude extracts have been reported to possess xanthine oxidase inhibitory activity. Recent findings show that the occurrence of gout is increasing worldwide, possibly due to the changes in dietary habits like intake of food rich in nucleic acids, such as meat and sea foods. Hypouricemic agents are commonly employed for the treatment of chronic gout arthritis, which includes xanthine oxidase inhibitors and uricosuric agents. Xanthine oxidase also acts as an important biological source of oxygen-derived free radicals that contribute to oxidative damage in living tissues, resulting in many pathological processes such as inflammation, atherosclerosis, cancer and aging.

Polyphenolic compounds such as Flavonoids, Phenolic acids and Tannins are considered to be major contributors to the antioxidant activity. The antioxidant activities of polyphenols were attributed to their redox properties, which allow them to act as reducing agent, hydrogen donators and singlet oxygen quenchers.

Hydrogen peroxide itself is not very reactive, but it can sometimes be toxic to cell because it can give rise to hydroxyl radical in the cells. Thus the removal of H_2O_2 is very important for antioxidant defence in cell or food systems. H_2O_2 can cross membranes and may oxidize a number of compounds. The crude extract of *Halodule uninervis* scavenged H2O2 which may be attributed to the presence of phenolics, which could donate electrons thereby neutralizing it into water. Since phenolic compounds present in the root bark extract are good electron donors, they may accelerate the conversion of H2O2 to H2O. Hence, the crude extract might also help accelerate the conversion of H2O2 to H2O.

7.4.3 ANTIMICROBIAL ACTIVITY

Antimicrobial properties of medicinal plants are being increasingly reported from different parts of the world. The World Health Organization estimates that plant extract or their active constituents are used as folk medicine in traditional therapies of 80% of the world's population. In the present work, the extracts obtained from *Halodule uninervis show* strong activity against most of the tested bacterial and fungal strains. In this screening work, extracts of *Halodule uninervis* were found to be not inactive against any organism, such as Gram-positive, Gram-negative, and fungal strains were resistant to all the extracts of root bark of *Halodule uninervis* . The above results show that the activity of ethanol extract and isolated compound I and II of *Halodule uninervis* shows significant antibacterial and antifungal activities. This study also shows the presence of different phytochemicals with biological activity that can be of valuable therapeutic index. The result of phytochemicals in the present investigation showed that the plant contains more or less same components like alkaloid, carbohydrate, tannin, saponin, terpenoid, flavonoid, coumarin and phenol. Results show that plant rich in tannin and phenolic compounds have been shown to posses antimicrobial activities against a number of microorganisms.

Chapter VIII
Summary and Conclusion

SUMMARY AND CONCLUSION

Marine flowering plants Seagrasses are capable of completing their life cycle when they are submerged in sea water. Ecosystem of Seagrass is one of the most widespread coastal vegetation types as compared to the ecosystems of coral and mangrove. Phytochemical study on sea grasses has clearly shown that seagrasses have pharmaceutically active secondary metabolites. Different studies on these seagrass metabolites proven that the extracted active components from saegrasses have alkaloids, glycosides, galactomannan gum, polysaccharides, peptidoglycan, hypoglycans, guanidine, steroids, carbohydrates, glycopeptides, terpenoids, amino acids and inorganic ions which directly or indirectly have a effective action aganst infection, pharmaceutically active hypolipemic agents, reduce blood pressure, and blood cholesterol levels. Phenolic compounds are effective secondary metabolites of seagresses which play a main role in various bio relevant activities in human body maintenance. The presence of phytoconstituents, such as flavonoids, phenols, and tannin, in seaweeds and seagrasses indicates a possibility that extracts may have anti-oxidant activity and anti diabetic activity.

Shade-dried plant material grounded to coarse powder and the quality of plant powder determined by ash content, acid insoluble ash, acid soluble ash and fluorescence analysis. Positive acceptable results were observed for the total ash values, which show that no inorganic adulteration is present. The Plant powder was treated with various chemical reagents and examined under long UV (254 nm), short UV (366 nm) and visible light.

The active constituents were extracted using solvents-Petroleum ether, Chloroform, Ethyl acetate and Methanol in an increasing order of polarity. Preliminary phytochemical Screening (TLC and chemical tests) for the extract of *Halodule uninervis* was performed to study presence of chemical constituents qualitatively. TLC analysis of methanolic extract of *Halodule uninervis* shown that there was many phytoconstituents with different composition of mobile phase.

Investigation of Petroleum ether, chloroform, ethyl acetate and Methanol extracts have identified the presence of various phytochemical constituents like alkaloid, carbohydrate, glycosides, terpenoids, proteins, amino acids, steroids, tannin, saponin, terpenoid, flavonoid, anthraquinones and phenol. As methanolic fraction responded for the many testes and shown good response against pharmacological activity, This extract tested for the presence of various constituents and nature of the compounds.

Upon the investigation of column fraction there are two major components were identified which are Compound 1-**ME-HU-C-1** and Compound 2- **ME-HU-C-2**. Further characterizations of the isolated components were preceded by IR, NMR and mass spectroscopy to identify the isolated components and identified the presence of flavonoid moiety. The methanolic extract was subjected to Column Chromatography on silica (100-200) mesh. Two chemical components were isolated ME-HU-C-1 and ME-HU-C-2. TLC performed for the isolated components and the Rf were identified 0.68 and 0.79 for compound-1 and compound-2 respectively.

These compounds were eluted with solvents of increasing polarity like hexane, ethyl acetate and methanol. In the course of isolation procedure, the above named 2 compounds were further processed and characterized. The compound ME-HU-C-1 is

Pale yellow residue gave colorless crystalline substance after crystallization with methanol (About 41.25 mg). In TLC chromatogram developed with hexane: Ethyl acetate (8.5:1.5) was homogenous with Rf 0.68. The presence of flavonoid active group is identified by chemical test and identification by IR, ^{1}H NMR, C_{13} NMR and MASS spectral data. The purity was quantified by reversed phase HPLC and the quantified purity is 99.5%. Based on the spectral data the compound-I was identified as

ME-HU-C-1 : 4', 5-Dihydroxy-6, 7-dimethoxyflavone

The compound ME-HU-C-2 is light brown colour residue which after crystallization converted to Pale yellow white crystals (46 mg). In TLC chromatogram was developed with hexane: Ethyl acetate (8.5:1.5) and it was homogenized with Rf 0.79. Melting range was 95.6-98.2°C. The Chemical test and identification by IR, ^{1}H NMR, C_{13} NMR and MASS Spectral data were shown the characteristic feature of flavonoid component. The purity was quantified by reversed phase HPLC and the quantified purity is 99.5%. Based on the spectral data the compound-II was identified as

ME-HU-C-2 : 4′, 5, 7-trihydroxyflavone

Heavy metal analysis performed in selected plant measurements were made for cadmium, lead, arsenic, and mercury and shows absence of heavymetal contents in the extract.

The methanolic extract of plant powder extract of *Halodule uninervis* shows presence of maximum chemical constituents which are responsible for the required *in-vivo* and *in-vitro* pharmacological activity.

Effects of *Halodule uninervis* extract in the single dose study on blood glucose levels was estimated after Streptozotocin administration on the 3rd day. There is no reduction in glucose level due to *Halodule uninervis* extract (50 mg/kg). At dose levels of 150 and 250 mg/kg, glucose levels were decreased by 24.8% and 29.9% at the 6th hour, respectively. Antidiabetic effect of the extract was slightly decreased at the 8th hour, but remained statistically significant.

In parallel experiments, *Halodule uninervis* extract was administered to diabetic rats for 18 days as per the protocol. The glucose level reduction up to 26% on 9th day of *Halodule uninervis* (50 mg/kg) treatment started to lower serum glucose on the 9th day. An overall reduction of 26% was observed on the 18th day by 50mg/kg administration 52.5% reduction of glucose level in the serum absorved at 18th day administration at dose level of 150 mg/kg. 250mg / kg extract administration is more effective from 6th day onwards with a reduction rate of 18.9% and maximum reduction of serum glucose level by 61.9% on the 18th day. Between the 12th and 18th days, antidiabetic effect of *Halodule uninervis* extract was in a concentration dependent manner.

The effects of *Halodule uninervis* extract on hepatic and renal function in Streptozotocin-diabetic rats. As seen, treatment with 50 mg/kg did affect neither the significantly high levels of ALP, GPT, BUN and creatinine, nor the overall oxidative status. Conversely, rats treated with higher doses of the extract (150 and 250 mg/kg) showed significant improvements in hepatic and renal function. Additionally, these two dose levels recovered the weight loss and low white blood cell count observed in Streptozotocin-diabetic rats while decreasing liver glycogen. *Halodule uninervis.* extract (150 and 250 mg/kg) also showed a protective effect on liver oxidative status.

Antioxidants namely GSH, GPx, SOD and catalase were increased by *Halodule uninervis* extract administration. When compared to Streptozotocin-diabetic rats, MDA formation, as an indirect measure of lipid peroxidation, was found to be significantly low in high dose *Halodule uninervis* extract-treated rats.

Methanolic extract of *Halodule uninervis* showed up to 62% inhibition of DPPH free radical at 517 nm at high concentration of 1mg/ml. At the concentrations of 0.3mg/ml, 0.5mg/ml and 1mg/ml, quercetin shows similar activity of about 77.6% inhibition of DPPH free radicals. The percentage inhibition of DPPH free radical by methanol extract in the presence of standard quercetin at 517 nm .

The *in-vitro* xanthine oxidase inhibition of methanolic extract revealed 67% of inhibitory activity at 30µg/ml and it was compared with the standard drug quercetin which showed 72% inhibition at 30µg/ml.

The *Halodule uninervis* methanolic crude extract 100, 200, 300 and 400 µg/ml .was capable of scavenging H_2O_2 in an amount dependent manner. H_2O_2 scavenging activity was closer to that of standard Ascorbic acid (100µg/ml) at doses 400 µg/ml .

Anti microbial activity for crude extract and isolated compounds from the *Halodule uninervis* methanolic extract were studied. The anti bacterial activity of the crude Methanolic extract and isolated compound ME-HU-C-1 and ME-HU-C-2 at 2000 ppm concentration and the activity against 3 pathogenic bacterial strains, *Staphylococcus aureus* Gram (+), *Escherichia coli* Gram (-), *Salmonella* Gram (-).Antibacterial potential of extracts were assessed in terms of zone of inhibition of bacterial growth. The bacterial strains *Staphylococcus aureus* Gram (+), *Escherichia coli* Gram (-) shows the good zone of inhibition in both crude Methanolic extract (15

mm) and isolated compound ME-HU-C-2 (12mm) in the dilution of 2000 ppm in replicate manner.

Both Compounds ME-HU-C-1 and ME-HU-C-2 was shown predominant microbial growth up to 100ppm. The agar diffusion assay method, there was no growth of E.coli in 1000 ppm for compound ME-HU-C-1 and slight growth observed in compound ME-HU-C-2. In Both dilution assay, there was no growth of E.coli in 2000 ppm for Compounds ME-HU-C-1 and ME-HU-C-2.

The antifungal activity of the crude Methanolic extract and isolated compound ME-HU-C-1 and ME-HU-C-2 were mixed with potato dextrose medium and the activity against 2 fungal strains Aspergillus sp and Candida albicans. Antifungal potential of extracts were assessed in terms of percentage of inhibition of fungal growth. The crude Methanol extracts shown 29% inhibition against Candida albicans. No inhibition was observed against Aspergillus sp and Candida albicans due to ME-HU-C-1. ME-HU-C-2 had 6% inhibition against Candida albicans and no inhibition was observed against Aspergillus sp.

The results were showed that the crude Methanolic extract and isolated compound of *Halodule uninervis* were found to be more effective against all the microbes tested.

CONCLUSION

- Marine organisms offer a wide source to discover useful therapeutics with cost effective. Recently, numerous marine metabolites with potent pharmacological properties have been identified.
- Among them seagrass is one of the most widespread coastal vegetation types as compared to the ecosystems of coral and mangrove. Phytochemical study on sea grasses has clearly shown that seagrasses have pharmaceutically active secondary metabolites.
- Different studies on these seagrass metabolites proven that the extracted active components from saegrasses have alkaloids, glycosides, galactomannan gum, polysaccharides, peptidoglycan, hypoglycans, guanidine, steroids, carbohydrates, glycopeptides, terpenoids, amino acids and inorganic ions which directly or indirectly have a effective action aganst infection, pharmaceutically active hypolipemic agents, reduce blood pressure, and blood cholesterol levels. Phenolic compounds are effective secondary metabolites of seagresses which play a main role in various bio relevant activities in human body maintenance. The presence of phytoconstituents, such as flavonoids, phenols, and tannin, in seaweeds and seagrasses indicates a possibility that extracts may have anti-oxidant activity and anti diabetic activity.
- Diabetes mellitus. a major disorder in the world leading to massive economic losses. Diabetes is a chronic disorder of carbohydrate, fat and protein metabolism characterized by increased fasting and fed blood sugar levels in the human body. World health organization predicted that the developing countries

will face major burden due to Diabetes. Studies conducted in India, highlighted that the prevalence of diabetes is high and also the rapid increase in the urban population.

- Prolonged medications required for the treatment of diabetes mellitus, considering the limitations and side effect in the synthetic drugs, natural products derived from medicinal plants and marine products are being looked for the treatment of diabetes. However components of medicinal plants and marine products are the base of many conventional drugs. Among 1200 plants and marine products which used for the treatment of diabetes mellitus there 200 pure compounds have showed lowering blood glucose activities. considering low toxic, ready availability and cost effective, usage of natural products for the treatment of diabetes mellitus has recommended and encouraged by WHO (World Health Organization) worldwide.
- The seagrass *Halodule uninervis* which is easily available from the coastal area of south Tamilnadu and identified that contain rich pharmaceutically effective secondary metabolites and is the evident that the presence of many phytochemical constituents in the marine plant extract. Multiple analyses shown the presence of various active phytoconstituents like alkaloid, carbohydrate, tannin, Saponin, terpenoid, flavonoid, Coumarin and phenols are present.
- The isolated phytochemical components were characterized and the Methanol extract obtained from the successive extraction of *Halodule uninervis was* shown a well response for most of the qualitative chemical tests which confirms that the effective extraction of maximum constituents from the plant material and shows effective *in-vivo* and *in-vitro* pharmacological activity. As the better

response derived from the methanolic extract this extract was chosen for the further investigation and shown significant anti diabetic activity respect to the protocol studies.

- A well response observed against the induced diabetic. Based on the multi concentration against response, higher concentration shown well response against high concentration of 250mg/kg of body weight. Also the extract was well responded for anti-oxidant and antimicrobial activities.
- Atomic absorption spectrophotometric analysis of methanolic extract of *Halodule uninervis* conformed the absence of Cd, Pb, As and Hg. Further investigation of the on the isolation and identification of component of the plant may lead to chemical entities with potential for clinical use with cost effective manner.
- Based on the above study it was concluded that the investigation of sea grass *Halodule uninervis* optained from marine source have active phyto-constituents which are pharmaceutically effective and less toxic. Considering low toxic, ready availability and cost effective, usage of *Halodule uninervis* for the treatment of diabetes mellitus is mostly recommended.

Chapter IX
Recommendations

RECOMMENDATIONS

Based on the analysis of the survey data, identified phytochemicals, characterization data and anti-diabetic profile, the following recommendations were made on the report of this work.

- Majority of the informants involved in the ethnomedicinal survey of seagrasses were explained about their morphology and other uses. There are very few studies which explained towards the pharmaceutical uses of sea grasses. As traditional medical knowledge of sea grasses are orally passed down via lifestyle, thus the informants are dying without transferring the indigenous knowledge of this sea plants to others. Therefore, it is important to exhaustively identify, document and publicise sea grasses medicinal knowledge for diabetes and other diseases in the different Zones of world.
- This study is one of the first reports related to the anti-diabetic profile and phytochemical constituents of sea grass *Halodule uninervis*. Thus, the study was carried out based on the minimal available data and may not be adequate enough to explain about many other pharmacological activity of the plant. Therefore, continuous pharmacological studies are compulsory to provide suggestion for a safe and effective use of this plant.
- The results reported in this paper can be used as basis for the development of evaluation of seagrasses and their pharmaceutical activity studies. Therefore, with the proper and safe formulations can be produced by using this plant and employed as candidates for the development of potential and alternative herbal medicine.

However, preliminary clinical studies are recommended for ensuring a better efficacy and safety.

- The plant included in this research is indigenous; therefore the plants should be preserved and can be cultivated for future use. Any further bioactivity studies and identification of bioactive compounds can be done to supplement this report.
- The high performance liquid chromatography, mass spectrometry (HPLC/QTOF-MS/MS) and GC-MS has proven to be powerful techniques for rapid identification of the constituents of the complex matrix of plant extracts and their application for qualitative and quantitative purposes can be highlighted for several future studies.

Isolation of pure compounds if any present additionally, complete identification and structural elucidation of some to be recommended and of the bioactive evaluation of those compounds would be mandatory for the develpment of drugs from seagrasses. This may give better alternative medicine with cost effective manner.

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