

**IDENTIFICATION OF HEPATOPROTECTIVE DRUGS FROM
*SPHAERANTHUS AMARANTHOIDES***

Submitted in partial fulfillment of the requirements for the award of
Bachelor of Technology degree in Biotechnology

By

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INSTITUTE OF SCIENCE AND TECHNOLOGY
(DEEMED TO BE UNIVERSITY)
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BONAFIDE CERTIFICATE

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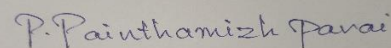
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DECLARATION

We, P.Painthamizh Pavai (37230052) and M.Pavithra (37230054) hereby declare that the Project Report entitled “**Identification of Hepatoprotective drugs from *Sphaeranthus amaranthoides***” done by us under the guidance of Dr. Y. Swarnalatha and Dr. C. Karthikeyan is submitted in partial fulfilment of the requirements for the award of Bachelor of Technology Degree in Biotechnology.

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ABSTRACT

Plant are a good source of medicine. Modern medicines have a little offer for alleviation of hepatic diseases and it is chiefly plant -based preparations which are employed for their treatment of liver diseases. Through the decades many scientists and researchers have reported the hepatoprotective activity of many medicinal plants. *Sphaeranthus amaranthoides* is commonly used in treatment of liver diseases. The plant was reported to wound healing, antimicrobial, anti-inflammatory and hepatoprotective effect. In this study the ethanol extract of the plant *Sphaeranthus amaranthoides* had been taken and subjected to thin layer chromatography for the purification. The isolated compound by TLC was then proceed to the characterization studies for the identification and analysis of compounds. Then the isolated compounds were subjected to spectroscopic studies using NMR and LC-MS analysis and a compound has been identified. Finally the identified compound was performed in vitro studies such as antifungal activity, anti-biofilm activity and microscopic studies.

Keywords: *Sphaeranthus amaranthoides*, Hepatoprotective activity, Spectroscopic studies, in vitro studies.

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LIST OF ABBREVIATIONS

S.NO	ABBREVIATIONS	FULL FORM
1	DILI	Drug Induced Liver Injury
2	CCL4	Carbon Tetrachloride
3	TAA	Tumor Associated Antigen
4	VLDL	Very Low Density Lipoprotein
5	WHO	World Health Organization
6	NADPH	Nicotinamide Adenine Dinucleotide Phosphate Aspartate
7	AST	Aminotransferase
8	ALT	Alanine Aminotransferase
9	ALP	Alanine Phosphatase
10	GLT	Glutamic Oxaloacetic Transaminase
11	Kg	Kilogram

12	mg	Milligram
13	mL	Milliliter
14	μL	Microliter
15	μg	Microgram
16	rf	Retention Factor
17	h	Hour
18	OD	Optical Density
19	BIC	Biofilm Inhibitory Concentration
20	DMSO	Dimethyl Sulfoxide
21	PDA	Potato Dextrose Agar
22	NMR	Nuclear Magnetic Resonance
23	TLC	Thin Layer Chromatography
24	LC	Liquid Chromatography
25	MS	Mass Spectrometry

26	DDPH	2,2-diphenyl-1-picrylhydrazyl
27	IP	Intraperitoneal
28	¹ H	Hydrogen-1
29	C ¹³	Carbon-13
30	MIC	Minimum Inhibitory Concentration
31	RNA	Ribonucleic acid
32	RA	Rosmarinic acid
33	ESI	Electrospray Ionization
34	APCI	Atmospheric Pressure Chemical Ionization
35	m/z	mass to charge ratio
36	g	Gram
37	°C	Celsius

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CHAPTER 1

INTRODUCTION

1.1 HEPATOTOXICITY:

Liver is the main organ responsible for the major metabolic and secretory functions within the body and appears to be a sensitive target site for substances modulating biotransformation (Gram and Gillette, 1969). It is involved in the detoxification from the exogenous and endogenous challenges like xenobiotics, drugs, viral infections and chronic alcoholism (Testa and Jenner, 1981). Due to the above mentioned exposures liver lost its natural protective mechanism and results in liver injury.

Hepatotoxicity is a liver injury caused by drugs or chemicals. Liver disorders caused by chemicals and drugs are:

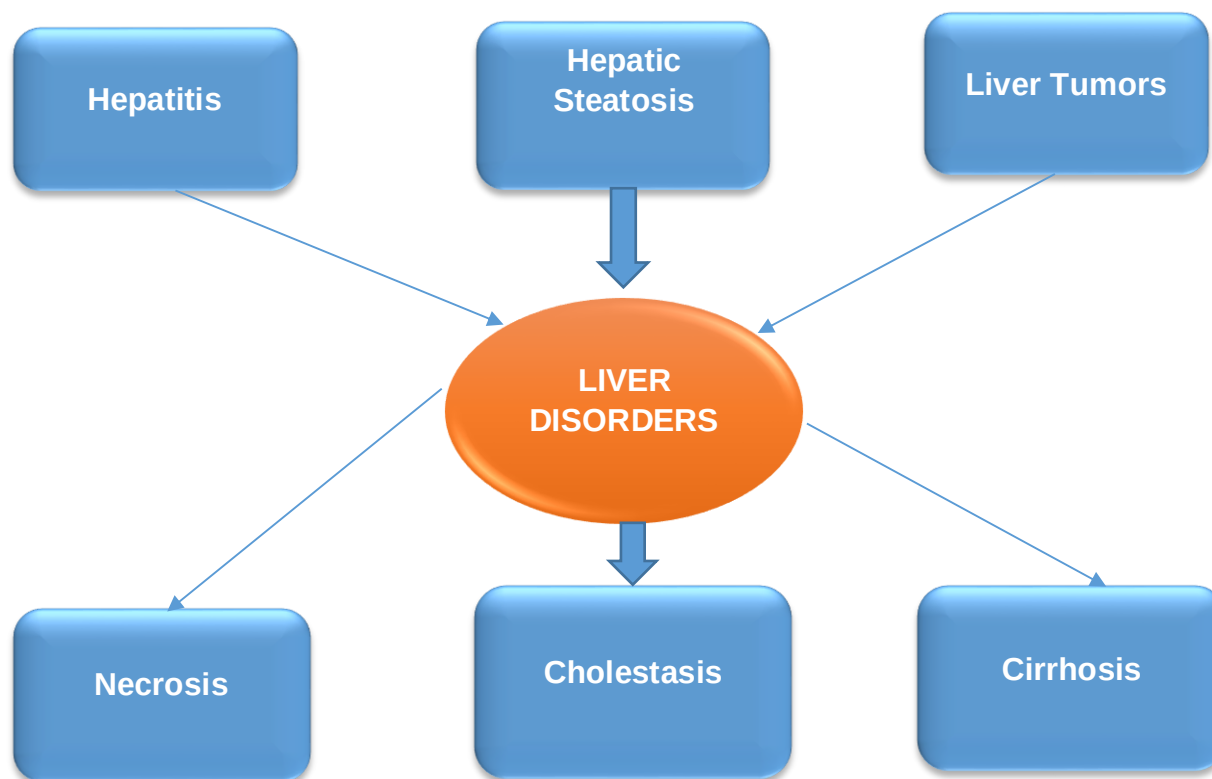


Fig 1.1: Types of Liver Disorders

Drug-induced liver injury (DILI) is a major problem that challenges the health care professionals and the pharmaceutical industry. DILI accounts for more than 50% of acute liver failure including hepatotoxicity caused by overdose of acetaminophen (39%) and idiosyncratic liver injury target by other drugs. Hepatic cell injury caused by various toxic chemicals like certain antibiotics, chemotherapeutic agents, CCl₄, TAA etc., excessive alcohol consumption and microbes (Amit Roy et al., 2014).

1.1.1 Hepatotoxic Chemicals:

One of the major causes of liver diseases is exposure to hepato-toxic chemicals. Some of the prescription drugs also induce liver damage depending on quality of intake and duration of treatment. The lesion caused by the chemicals depends not only on the chemical agent involved but also on the period of exposure. After acute exposure one usually finds lipid accumulation within the hepatocytes, cellular necrosis or hepatobiliary dysfunction whereas cirrhotic or neoplastic changes are usually considered to be the results of chronic exposure. Difference biochemical alterations may lead to the same end point, no single mechanism seems to govern the appearance of degenerative changes in the hepatocytes or alterations in its function. Some forms of liver injury have been found to be reversible while others result in damaged organ (Mehendale, 1987).

1.1.2 Hepatitis:

Hepatitis is an inflammation condition of the liver. This is the most common type of liver disease caused by virus, toxic chemicals and abnormal immune reactions. This may be acute or chronic. If the inflammation is not cured this can lead to cirrhosis, necrosis and even tumours. Viral hepatitis is the major cause of hepatitis. There are several known hepatitis viruses include Hepatitis A, Hepatitis B, Hepatitis C, Hepatitis D, Hepatitis E, Hepatitis F and Hepatitis G or GBV-C. In addition to the hepatitis viruses, other viruses such as cytomegalovirus, Epstein-Barr virus and yellow fever virus can also cause hepatitis (Minuk et al., 2005).

1.1.3 Steatosis:

Accumulation of lipids in the liver is called fatty liver or steatosis. There are several potential chemical effects that can give rise to accumulation of lipids in the cell

(Umalakshmi et al., 1992). Fatty liver can be developed due to inhibition of lipoprotein synthesis, decreased conjugation of triglycerides with lipoproteins, interference with very low-density lipoprotein (VLDL) transfer, impaired oxidation of lipids by mitochondria and increased synthesis of fatty acids (Ambrose et al., 2012).

1.1.4 Cirrhosis:

Liver Cirrhosis is the last stage of progressive hepatic fibrosis characterized by distortion of the hepatic architecture and therefore the formation of regenerative nodules. Fibrotic tissue with repeated hepatic insult making difficult for the liver to exchange damaged cells. Fibrous tissue begins to form walls separating cells. Distortions in hepatic microcirculation cause cells to become hypoxic and die, resulting to more fibrotic scar tissue. Then the organization of the liver is reduced to nodules of regenerating hepatocytes. This condition is called cirrhosis. Blood flow through the liver becomes obstructed resulting in hypertension. To relieve the pressure blood is diverted through various shunts. It is common for vessels associated with these shunts to rupture resulting in internal hemorrhage. Even without hemorrhagic episodes the liver may still decline until hepatic failure occurs. Hepatic cirrhosis is believed to be irreversible. Examples of chemicals that cause cirrhosis are carbon tetrachloride, trinitrotoluene, tetrachloroethane and dimethylnitrosamine (Scinella and Becker, 1975).

1.1.5 Liver Tumors:

Many chemicals are capable of producing tumors within the liver particularly in laboratory rodents. Liver is the most common site of tumorigenicity in cancer rodent bioassays. Hepatic tumors may be benign or malignant. Conceptually the distinction between them is that benign tumors are well circumscribed and do not metastasize whereas Malignant tumors are poorly circumscribed and are highly invasive. Benign tumors are capable of producing morbidity and mortality. Nitrosoureas and nitrosamines are examples of hepatocarcinogens (Zimmerman and Maddrey, 1993).

1.1.6 Cholestasis:

The decreased or arrested bile flow is known as cholestasis. Many drugs and chemicals are able to produce cholestatic injury, Some drug or chemicals are related to loss of

integrity of the canalicular system that collects bile and carries it to the gall bladder while others are related to the formation and secretion of bile. For example, a 1-Naphthyl isothiocyanate disrupts the tight junctions of the hepatocytes that help form the canaliculi, the smallest vessels of the bile collection system. This causes a leakage of bile contents. Other toxicants impede bile flow by damaging the bile ducts (Witzleben, 1972).

1.1.7 Necrosis:

Hepatotoxic chemicals can cause necrosis of liver cells within distinct zones in the liver. Inflammation of the liver may result in damage to hepatocytes with necrosis. Confinement of the lesion to a particular zone is thought to be a consequence of the mechanism of toxicity of these agents and therefore the balance of activating and inactivating enzymes or cofactors. Liver is able to withstand moderate zonal or diffuse necrosis due to its remarkable ability to regenerate itself. Over a period of several days necrotic cells have been removed and replaced with new cells. If the number of damaged cells is too great however the liver's capacity to restore itself becomes overwhelmed leading to hepatic failure and death (Farber, 1975).

1.2 HEPATOPROTECTIVE ACTIVITY:

DILI can be prevented by using hepatoprotective drugs which possess hepatoprotective as well as antioxidant activities. Many natural agents possess antioxidant activity has been proposed to prevent and cure hepatotoxicity. Several plants have been shown to possess protective role due to the presence of polyphenolic compounds, flavonoids, poly hydroxy organic compounds and terpenoids (Swarnalatha et al., 2012).

The report showed that 160 phyto-constituents from 101 plants have hepatoprotective activity. In spite of modern medicine there are several formulations contains herbal drugs are sold in market for treating liver diseases (Somnath et al., 2017).

1.3 PLANTS AS A SOURCE OF DRUGS:

Nature stands as a resource for drug development to cure several diseases. Particularly Plants will continue to be extremely important for the discovery and synthesis of drugs.

Until recently plants are the major source of novel pharmacologically active compounds with many drugs being derived directly from plants (Veeresham, 2012).

Pharmaceutical companies have produced an enormous number of crude therapeutic formulations by utilizing plant extracts (Bhuwan B 2011). According to the reports most of the developing countries depends on traditional medicines and mostly the traditional medicines are plant based drugs (WHO Global Report on Traditional and complementary Medicine, 2019).

Upto 50% the approved drugs during the last 30 years from either directly or indirectly from natural resources (Newman and Cragg, 2012). The plant derived compounds have an extended report of clinical use, better patient tolerance and acceptance. 80% of 122 plant drugs were related to their original ethno pharmacological purposes (Fabricant and Farnsworth et al., 2001).

1.4 MEDICINAL PLANTS:

The allopathic system of medicine has adopted a number of plant derived drugs which plays an important role in modern pharmacopoeia. Some drugs are even prepared from the excretory plant products such as gum, resins and latex. Many secondary metabolites of plants are broadly used in pharmaceutical compounds (Joy PP 1998).

Nowadays there is an increasing scientific interest in herbal medicine in research field all over the world (Wiley 1990). Some medicinal plants proved to be effective than synthetic medicine and with minimal side effects or no side effects. The first semi synthetic pure drug aspirin, based on a natural product salicin isolated from *salix alba* was introduced by Bayern in 1899 (C Veeresham, 2012). According to the reports one of such medicinal plant *Sphaeranthus amaranthoides* also possess hepatoprotective activity, so in the present investigation the *Sphaeranthus amaranthoides* plant extracts have been screened. Then to isolate and characterize the active constituents from the extract to identify its hepatoprotective activity.

1.5 SPHAERANTHUS AMARANTHOIDES:

Sphaeranthus amaranthoides is an annual herb found in moist places probably in salty water. *S.amaranthoides* is commonly called as sivakaranthai. (Gayatri, 2015).

1.5.1 Taxonomical Hierarchy:

Sphaeranthus amaranthoides belongs to:

Kingdom: Plantae

Phyllum: Angiosperms

Class: Magnoliopsida

Subclass: Gamopetalae

Series: Inferae

Order: Asteracea

Genus: Sphaeranthus

Species: amaranthoides

Scientific name: *Sphaeranthus amaranthoides* Burm.f.



Figure 1.2: *Sphaeranthus amaranthoides* Burm.f. (POWO, 2019)

1.5.2 Distribution:

Sphaeranthus amaranthoides were found only in India and Sri Lanka. Mostly it has been found in southern part of India especially in Tamil Nadu such as Tanjore, Tirunelveli, Madurai, Coimbatore, Tiruchirappalli, Kanyakumari. In Southern Mysore and Travancore area it occurs as a weed of paddy field (Thanigavelan et al., 2012).

1.5.3 Morphology:

Stout, slender and smooth herbs 8 to 12 inches erect and glabrous. Branchlets are very narrowly winged. Leaves are 9 x 1.5 cm linear, oblong, spathulate, apex acute, black dotted, obtuse and distantly serrate. Flower heads are 1 inches long and red in colour (Thanigavelan et al., 2012).

1.5.4 Medicinal uses:

Sphaeranthus amaranthoides possess various medicinal uses such as it is used in the treatment of eczema, blood disorders, fever etc., This plant were even used to cure skin diseases, urethral discharges and jaundice. The activities reported in this plant are antidiabetic activity, antimicrobial activity, antimicrobial activity, hepatoprotective activity, analgesic and anti-inflammatory activity (Gayatri et al., 2013).

CHAPTER 2

LITERATURE SURVEY

2.1 ANTIFUNGAL ACTIVITY:

In some cases plant extracts with biological properties, i.e., antifungal, require such high concentrations to be effective that it might be toxic to healthy cells and cause many side effects. The same situation is additionally applicable to antifungals already utilized in clinical therapy, i.e., amphotericin B that is considered nephrotoxic. Therefore, if these plant extracts could be used combined with antifungals, both at lower concentrations, it could lead on to increased activity and reduce their negative side effects. To examine the activity of the combinations checkerboard assays in which different concentrations of the free extract were used in combination with different dilutions of fluconazole, caspofungin or amphotericin B, both against *Candida albicans* and *Candida glabrata* (Wall et al., 2019).

The results for citral's antifungal activity against *Candida albicans* strains were determined by using the MIC and MFC in broth microdilutions. The MIC of citral was 64 $\mu\text{g/ml}$, inhibiting the growth of tested fungal strains. Amphotericin B has a lesser MIC than the phytoconstituent at 2 $\mu\text{g/mL}$ MIC. The results were showed no fungal growth inhibition (Leite et al., 2014).

2.2 ANTI BIOFILM ACTIVITY:

The free extract activity was tested against *Candida albicans* and *Candida glabrata* biofilms under two different treatment (inhibition of biofilm formation and activity against preformed biofilms) was evaluated using the 96-well microtiter plate of *Candida* biofilm formation and susceptibility testing (Pierce et al., 2008).

2.3 HEPATOPROTECTIVE ACTIVITY:

Acute toxicity studies of ethanolic extract of *Sphaeranthus amaranthoides* didn't show any mortality or toxic symptoms up to 2000 mg/kg. Hence, 1/5th and 1/10th of the highest dose tested is fixed as the dose for further pharmacological activity. The elevated level of AST, ALT, ALP, bilirubin, cholesterol and triglycerides in animals treated with

paracetamol (2 g/kg body weight) which is the indicative of severe liver necrosis. Administration of ethanolic extract at the doses of 200 and 400 mg/kg remarkably prevented paracetamol induced hepatotoxicity during a dose dependent manner. There was a significant ($P<0.001$) restoration of these enzyme levels on administration of ethanol extract (Gowri et al., 2015).

The study of hepatoprotective activity of ethanol extract of *Sphaeranthus amaranthoides* in D-galactosamine induced hepatitis in rats has been reported that the protective role of ethanol extract of *S. amaranthoides* was assessed by measuring the levels of serum and liver enzymes. D-galactosamine induced liver damage resembles viral hepatitis and the hepato necrotic process may involve different pathogenic mechanism such as inhibition of RNA synthesis free radical dependant pathway and modification of phospholipid composition of mitochondria membrane. Ethanolic extract significantly ($P<0.001$) reduced the elevated levels of ALT, AST and ALP and increased the reduced glutathione level in the liver tissues of D-galactosamine intoxicated rats. Since the plant is effective in scavenging the free radicals generated by both D-galactosamine and paracetamol induced liver toxicity. *Sphaeranthus amaranthoides* can be effectively used to treat drug induced hepatotoxicity (Swarnalatha et al., 2012).

2.4 ANTIMICROBIAL ACTIVITY:

Aqueous extract of the leaves of the plant *Sphaeranthus amaranthoides* was effective against both *E. coli* and *P. vulgaris*. Various organic fractions of the plant were found to be effective against *E. coli* and *P. vulgaris* (Sumithra et al., 2011).

Silver nanoparticles prepared with ethanol extract of the *Sphaeranthus amaranthoides* was tested by agar well diffusion assay against microbes. The result showed the maximum zone of inhibition with *E. coli* (3.2 ± 0.1 cm) followed by *P. vulgaris* (2.4 ± 0.05 cm) and then *Bacillus subtilis* (1.3 ± 0.1 cm) (Swarnalatha et al., 2013).

2.5 WOUND HEALING:

Methanolic extract and flavonoid fraction of whole plant *Sphearanthus amaranthoides* was also studied for their wound healing activity. Hydroxyproline level and collagen level of wounded area was found to be increased and the results were comparable with the

standard silver sulfadiazine. Both the extract and the flavonoid rich fractions accelerated the wound healing (Geethalakshmi et al., 2013).

Ethanol extract of the plant *Sphaeranthus amaranthoides* is tested for its protective nature on excision wound model in wistar rats. Epithelization and wound contraction were studied. The results indicated reduction in wound area by the plant extract applied on the dorsal thoracic region of the wistar rat. The rate of wound contraction is compared with that of standard framycetin sulfate (Swarnalatha et al., 2009).

2.6 ANTI-INFLAMMATORY ACTIVITY:

Anti-inflammatory activity was evaluated by carrageenan on wistar albino rats. At 1000 mg/kg/p.o, 95 % inhibition was produced in the second phase of acute inflammation. The inflammation may be due to the inhibition of enzyme cyclooxygenase and subsequent prostaglandin synthesis. Therefore the plant *Sphaeranthus amaranthoides* exhibited a delayed anti-inflammatory activity (Thanigavelan et al., 2012).

2.7 ANTIOXIDANT ACTIVITY:

Antioxidant activity by ferric thiocyanate method for various extracts showed that methanolic extract was having more antioxidant potential in comparison to ethanolic and aqueous extract. The effect produced by methanolic extract was comparable to that of ascorbic acid (Prabakaran M 2011).

The antioxidant activity of the extract was evaluated by using DPPH free radical scavenging method and methanolic extract was found to possess activity comparable to that of butylated hydroxy anisole then followed by chloroform and acetone. Methanolic extract was found to contain more phenolic compounds and diethyl ether extract the least (Geethalakshmi et al., 2013).

2.8 ANTIHELMINTHIC ACTIVITY:

Sillaman technique was used for the screening of anthelmintic activity. The standard used was piperazine citrate at two different concentrations. The time taken by the worm to become motionless was noted as paralysis time and the death of worm was confirmed by transferring to hot water bath at 40 °C. The effect produced by methanolic

extract of *Sphaeranthus amaranthoides* at the dose level 80 mg/kg was comparable with that of the standard (Shankarananth et al., 2009).

The methanolic extract revealed significant dose dependent anthelmintic activity against *Pheretima posthuma* (Indian earthworm) at the highest concentration of 100 µg/mL as compared with albendazole as standard reference and normal saline solution as control. When observed the response of worm in case of paralysis & death, there was significant variations of results produced by the methanolic extract of *Sphaeranthus amaranthoides* at different concentration. The methanolic extract exhibited anthelmintic activity in dose dependent manner varying from loss of movement (paralysis) to loss of response to external stimuli which eventually advanced into death (Somnath 2014).

2.9 SEDATIVE ACTIVITY:

Sedative activity of the methanolic extract of the plant *Sphaeranthus amaranthoides* was carried out by three methods. By standard drug method, after the administration of drug by IP route, the onset and duration of action were compared with the reference drug diazepam. In muscle grip strength method, the animals were placed on a rotarod apparatus and the fall off time before and after drug administration were compared. The third method was Y-maze method in which the time spent by the mice in darkness and number of entries etc., were evaluated (Shankarananth et al., 2009).

2.10 PROTECTIVE ROLE OF ROSEMARINIC ACID:

Rosmarinic acid isolated from *Sphaeranthus amaranthoides* acts as activator having a functional role as antioxidant can help in activation of aurora kinase-A enzymes during phosphorylation. The in vivo and insilico studies proved that Rosmarinic acid can be a potential drug to protect against the toxicity induced by the food colours. The upregulated levels of the aurora kinase-A conclude that rosmarinic acid has a potential role in maintaining the levels and activity of aurora kinase-A (Swarnalatha et al., 2017).

CHAPTER 3

AIM AND SCOPE

3.1 AIM:

The major aim of this study is to evaluate the role of hepatoprotective activity of crude ethanol extract of the plant *Sphaeranthus amaranthoides* in the treatment of liver disorders.

3.2 OBJECTIVES:

- To prepare the crude ethanol extract of the plant *Sphaeranthus amaranthoides*.
- Fractionation and isolation of the bioactive compound from the plant extract using thin layer chromatography.
- Characterization of the isolated compound using analytical techniques like LCMS and NMR.
- To determine In vitro studies of the isolated compound.
- To evaluate the hepatoprotective activity of the compound.

3.3 SCOPE:

Studies with plant extracts are to know for their efficacy and mechanism of action and safety. Natural remedies from medicinal plants are considered to be effective and alternate treatment for hepatotoxicity. Extract of about 25 different medicinal plants have been reported to cure liver disorders. In this project one of such plant *Sphaeranthus amaranthoides* is taken for the study. *Sphaeranthus amaranthoides* possess several medicinal values. Eventhough it is used an energizer in siddha medicine due to the lack of scientific based pharmacological data it is not much explored. This plant has the ability to be considered as an alternate treatment for hepatotoxicity. The novelty of this investigation work is though at a smaller scale, attempts to evaluate the hepatoprotective activity of the plant *Sphaeranthus amaranthoides*.

CHAPTER-4

MATERIALS AND METHODS

4.1 MATERIALS:

4.1.1 Plant Materials:

- A whole plant of *Sphaeranthus amaranthoides* Burm.f.

4.1.2 Chemicals:

- Petroleum ether
- 95% ethanol
- Distilled water
- Silica gel
- N-hexane
- Ethyl acetate
- Formic acid
- Phosphate buffer saline
- Dimethyl sulphoxide

4.1.3 Reagents and Media:

- Agar Agar
- Potato Dextrose Agar
- Yeast Peptone Dextrose Broth
- Vanillin sulfuric acid
- Crystal violet
- Lactophenol Blue

4.1.4 Apparatus:

- Whatman grade 1 filter paper
- Muslin cloth

- Forceps
- Spatula
- TLC plate
- TLC chamber
- Capillary tube
- Glass beaker (250 mL)
- Conical flask
- Test tubes
- Test tube stand
- Micropipettes
- Petriplates
- Swab
- Standard Disc containing Amphotericin B
- 96 well Microtiter plate
- Cuvette

4.1.5 Instruments:

- Rotary evaporator
- Incubator
- Hot air oven
- Iodine Chamber
- Centrifuge
- Scanning Electron Microscope
- Microtiter plate reader
- NMR
- LCMS

4.1.6 Microbial strain:

- *Candida albicans*

4.2 METHODOLOGY:

4.2.1 Collection of plant material:

The fresh plants of *Sphaeranthus amaranthoides* Burm.f. were collected from Tirunelveli district, Tamil Nadu. The collected plant material was identified as they are free from diseases. The plants were identified as they are also free from contamination.



Figure 4.1: *Sphaeranthus amaranthoides*

4.2.2 Drying and grinding of plants:

The fresh plant material were thoroughly washed using distilled water. They were cut into pieces and air dried under shade for a week. Then the dried plant material were finely grinded into a powder form. The powder were also passed through a 40 mesh sieve to get the fine powder and stored for further procedures.



Figure 4.2: Powdered plant material

4.2.3 Preparation of plant extract:

The powdered plant material was soaked in petroleum ether for two days to remove pigments. Then the powdered plant material was soaked in 95% ethanol in the ratio of 1:5 (i.e., 20 g of plant material in 100 mL of ethanol) in a sterile 250 mL conical flask. Flask were then plugged with a cotton and kept in refrigerator at 4°C for 24 h.

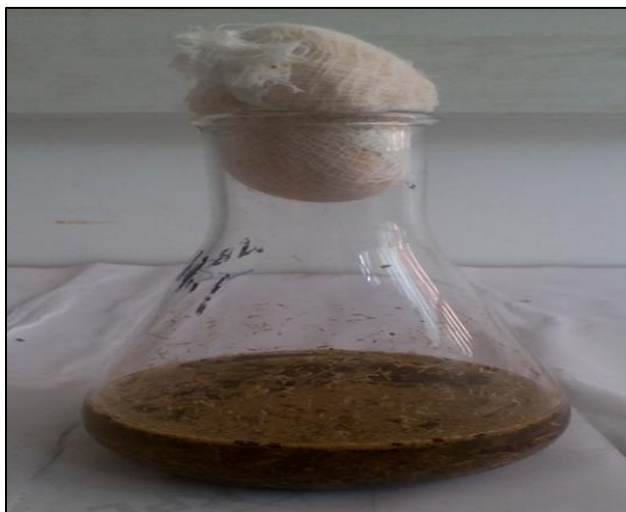


Figure 4.3: Powdered plant extract

4.2.4 Preparation of filtered crude extract:

The collected powder extract were filtered using muslin cloth and Whatman grade 1 filter paper and concentrated using soxhlet extractor to remove the solvent. Thick slurry of crude extract was obtained and it is stored for further analysis.



Figure 4.4: Filtered crude extract

4.2.5 Thin Layer Chromatography:

Thin layer chromatography is particularly valuable for the qualitative determination of the small amounts of impurities. TLC provides partial separation of both organic and inorganic materials by using TLC plates especially for the purity of fractions.

Here TLC was performed on the crude ethanol extracts using TLC plates. Thin layer chromatography was carried out on the ready .made plates. Silica gel 60F₂₅₄ with

fluorescent indicator pre coated aluminum plates was used for the experiment. The required dimension of the plate was cut with the help of a plate cutter. Spotting on the plate with crude extract was done with the help of a capillary tube. The plates were developed in the TLC chamber using n-hexane: ethyl acetate: Formic acid (60:30:10). The development tank consists of a 250 ml beaker, glass cover, solvent and plates spotted with samples. The respective solvent systems were prepared and poured in the chamber and kept for saturation plates were immersed in TLC chamber. 2cm line was drawn from the bottom. The spots were placed on the line. After the solvent run till 5cm the plates were dried and visualized.

4.2.6 Characterization studies:

TLC Plate was developed and required fraction was collected in eppendorf tube. Mixture was dissolved in methanol and undergoes shaking. Centrifuged at 6000 rpm for 5 minutes. Supernatant was collected and filtered. Supernatant was dried under reduced pressure. Filtrate was dissolved in methanol for further analysis.

The isolated compound by TLC was then proceed to the characterization studies for the identification and analysis of compounds. The isolated compounds were subjected to spectroscopic studies using NMR and LC-MS analysis.

4.2.6.1 NUCLEAR MAGNETIC RESONANCE SPECTROSCOPY:

Spectroscopy based on the spin of nucleus is NMR spectroscopy. Since most of the organic molecules contain hydrogen and it has a spin, this is an extremely valuable method for investigating structures of organic compounds. The term nuclear magnetic resonance arises from the fact that energy is transferred from the incoming radio frequency radiation to the nucleus causing a spin change when the frequency of the oscillating electric field component of the radiation just matches the frequency of the electric field generated by the spinning nucleus. NMR is of two types. They are:

- Proton NMR (^1H NMR)
- Carbon NMR (^{13}C NMR)

Proton NMR spectroscopy (^1H -NMR):

NMR is the branch of spectroscopy dealing with the absorption of radio frequency radiation by substances held in a magnetic field. Absorption results from interaction of radiation with magnetic movement of nuclei within the sample and it occurs at different frequencies for nuclei with chemically different environments within a molecule. Proton NMR Spectroscopy essentially provides a means of determining the structure of an organic compound by measuring the magnetic moments of its hydrogen atoms. In most compounds, hydrogen atoms are attached to different groups (as CH_2 , CH_3 , CHO , etc) and therefore the proton NMR spectrum provides the number of hydrogen atoms in these different situations. A ^1H NMR spectrometer of Bruker DR x 500 FT-NMR spectrometer was used to record ^1H NMR.

Carbon ^{13}C -NMR (^{13}C -NMR) spectroscopy:

^{13}C -NMR spectroscopy is essentially complementary to proton NMR. ^{13}C -NMR provides information about the structure of carbon skeleton of the molecule and the combination of the two techniques provides a very powerful means of structural elucidation of compounds. Small amount of carbon-13 is present along with carbon-12 in natural plant substances. The smaller magnetic moment generated by ^{13}C were amplified, detected and compared with that of proton using NMR apparatus for structural elucidation of compounds (Ewing, 1975).

4.2.6.2 LIQUID CHROMATOGRAPHY AND MASS SPECTROMETRY:

It provides an accurate molecular weight and it may yield a complex fragmentation pattern characteristic of that compound. MS consists of degrading trace amount of an organic compound and recording the fragmentation pattern according to mass. LC-MS analysis of crude ethanolic extract of the plant and the isolated compounds were done by using LC-MS. The column used was Phenomenex RP 18 with column dimension 25×2.5 mm. Mobile phase constitute water : methanol (50:50) variable. 20 microliter sample was injected with flow rate of 2.0 mL/min with column temperature of 25°C . LC detected at 265 and 292 nm using photo diode array detector. The software installed was chemstation integrated.

4.2.7 In vitro studies:

The identified compound was further purified for in vitro studies. The identified compound is Rosmarinic acid. Required fraction of rosmarinic acid was scrapped and collected in eppendorf tube. Mixture was dissolved in methanol. Then it is centrifuged at 6000 rpm for 5 minutes. Pellet was discarded and Supernatant was collected and filtered. Supernatant was dried under reduced pressure. Filtrate was dissolved in methanol for further analysis.

4.2.7.1 ANTIFUNGAL ACTIVITY:

Standard isolates of *Candida* strain i.e *Candida albicans* was taken as a test organism for the study. The test strain were maintained at 4° and subcultured on to PDA broth for 24 h prior to testing. These strains served as test pathogens for both antifungal activity and anti-biofilm activity assays.

The PDA medium was used to make the lawn cultures of fungi for the study of antifungal activity. The media was prepared according to the following constituents below:

Potatoes infusion – 200 g

Dextrose – 20 g

Agar – 15 g

Distilled water – 1000 mL

Final pH (at 25°C) - 5.6±0.2

Suspend 39.0 g in 1000 ml distilled water and sterilized in an autoclave. About 15-20 mL of the above sterile hot medium was poured into previously autoclaved petri plates allowed to solidify within a laminar hood and then subjected to antifungal studies.

4.2.7.2 ANTIFUNGAL SUSCEPTABILITY TEST:

Agar well diffusion method has been used here to determine the antifungal activity. After solidification of the media the inoculums were spread on the plates with sterile cotton swab moistened with the fungal suspension culture of *candida albicans*. Blank discs with 6mm diameter were impregnated by 20 µL of different concentrations (25 µg, 50 µg, 75 µg, 100 µg) of each extract is dissolved in DMSO. After the impregnation the discs were

spread on the PDA plate evenly. Control disc consist of pure DMSO. Amphotericin B (10 µg /disc) were used as a standard. The plates were kept at room temperature for 48-72 h. Then the microbial growth was determined by measuring the diameter of zone of inhibition in millimeter and the assay was carried out for three times.

4.2.7.3 ANTI BIOFILM ACTIVITY:

To assess the anti-biofilm activity of *candida albicans* strain against rosmarinic extract and its purified compounds against clinically important pathogens, microtiter plate assay was carried out using 96-well flat bottom polystyrene titer plates. Each well was filled with 100 µL sterile PDB broth and 50 µL overnight culture for each clinical pathogenic isolate followed by adding 150 µL rosmarinic acid crude extract and 150 µL of its purified compounds separately with concentrations of 1000, 1500, 2000, 2500, 3000, 3500, 4000, and 4500 µg/mL before incubation at 37°C for 24 hours.

After incubation plates were gently washed three times with phosphate-buffered saline and therefore the planktonic cells were discarded while the weakly adherent cells were removed through two rounds of thorough washing with distilled water and allowed to air-dry before being stained. The adherent biofilm was stained by 200 µL of 0.4% crystal violet solution (w/v) for 10 min. The OD of the biofilm was measured at 570 nm (OD₅₇₀) with a microtiter absorbance reader.

4.2.7.4 BIOFILM INHIBITORY CONCENTRATION:

In order to determine the lowest concentration of Rosmarinic acid extract that can cause visible inhibition in the biofilm formation, the biofilm inhibitory concentration (BIC) test was carried out using 6-well flat bottom polystyrene titer plates. A piece of cover slip was placed inside each well to allow the growth of fungi isolates on the surface and to visualize the inhibitory effect of extract on the biofilm formation.

Each well was filled with 300 µL sterile broth followed by inoculation with 150 µL of overnight culture for each clinical pathogenic isolate then addition of 150 µL Rosmarinic acid extract and 150 µL of its purified compounds separately with concentrations ranging from 1000 to 4500 µg/mL before incubation at 37°C for 24 hours.

After incubation biofilm inhibition was determined using a microtiter absorbance reader and visualized using a light microscope.

4.2.7.5 LIGHT MICROSCOPIC STUDIES:

A loop full of the *candida albicans* strain is placed at the center of Potato dextrose agar and incubated three days at 25°C to obtain colony for the morphological identification (Swathi et al., 2013).

Lacto phenol cotton blue was commonly used for semi-permanent microscopic preparations of fungi. It stains the fungal cytoplasm and provides a blue background against which the walls of hyphae can be seen. A drop of Lactophenol Cotton Blue Stain was placed in the center of a slide. The fungus colony 2-3mm from the colony edge was picked using a teasing needle. Then the fungus fragment was placed over the stain gently. A coverslip was placed over it. Then the slide can be viewed under 400× magnification.

CHAPTER 5

RESULTS AND DISCUSSIONS

5.1 THIN LAYER CHROMATOGRAPHY:

The sample was analyzed and visualized using UV 254 nm. Then the plates were dipped in vanillin sulfuric acid. The plates were allowed to dry and then heated in a hot air oven till the colored spots appeared.

In thin-layer chromatography, the retention factor (R_f) is used to compare and help identify compounds. The R_f value of a compound is calculated by the distance travelled by the solute from the point of sample divided by the distance traveled by the solvent from the mobile phase. Finally the sample showed in a dark pink color with 0.55 R_f



Figure 5.1- TLC plate

5.2 NUCLEAR MAGNETIC RESONANCE SPECTROSCOPY:

The isolated compound obtained as off-white powder with RF 0.55. Chemical investigation of this compound indicated the structure of rosmarinic and negative ion mode together with ^1H -NMR and spectral data confirmed the molecular formula to be ($\text{C}_{18}\text{H}_{16}\text{O}_8$).

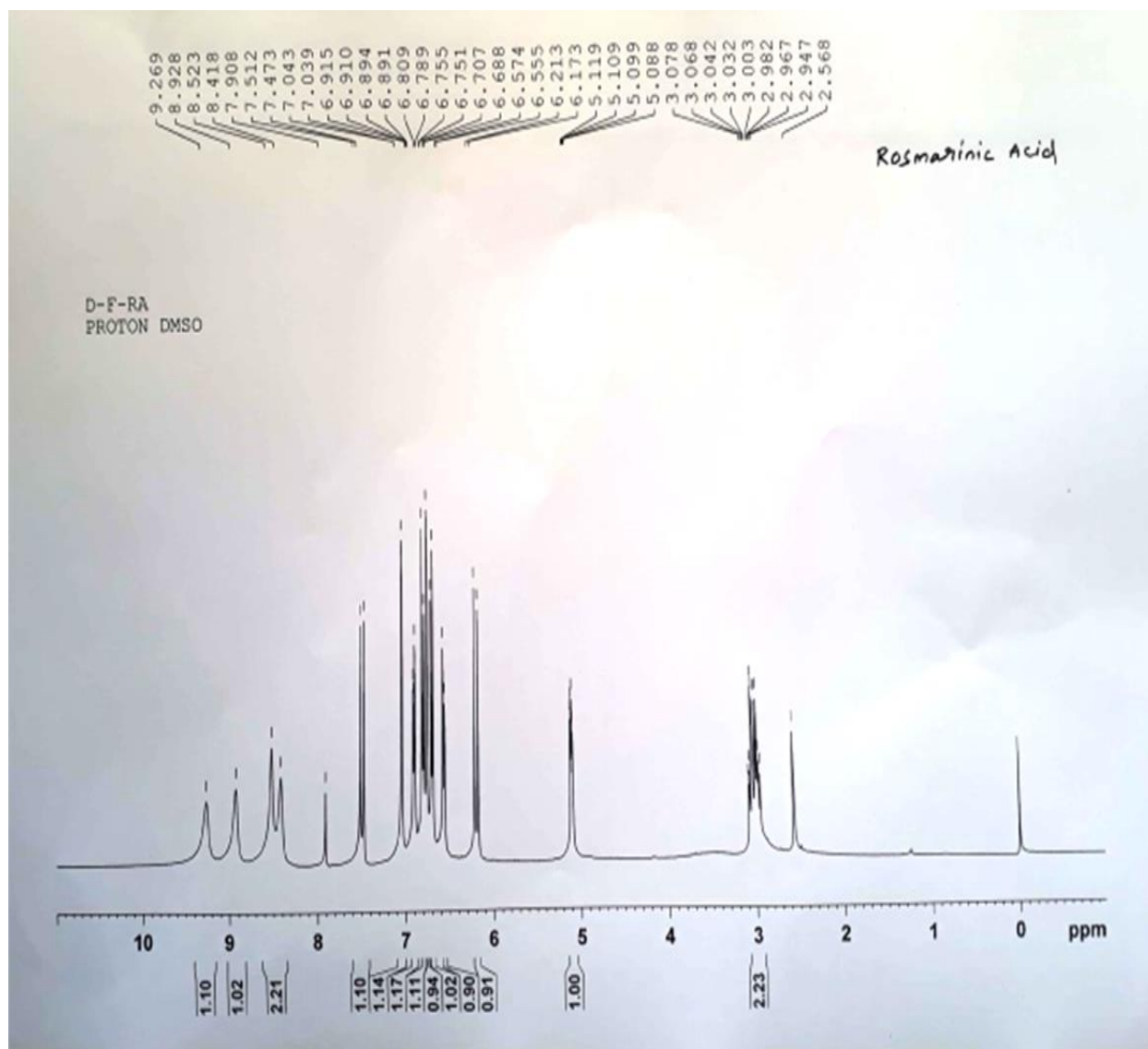


Figure 5.2: Analysis of ^1H NMR

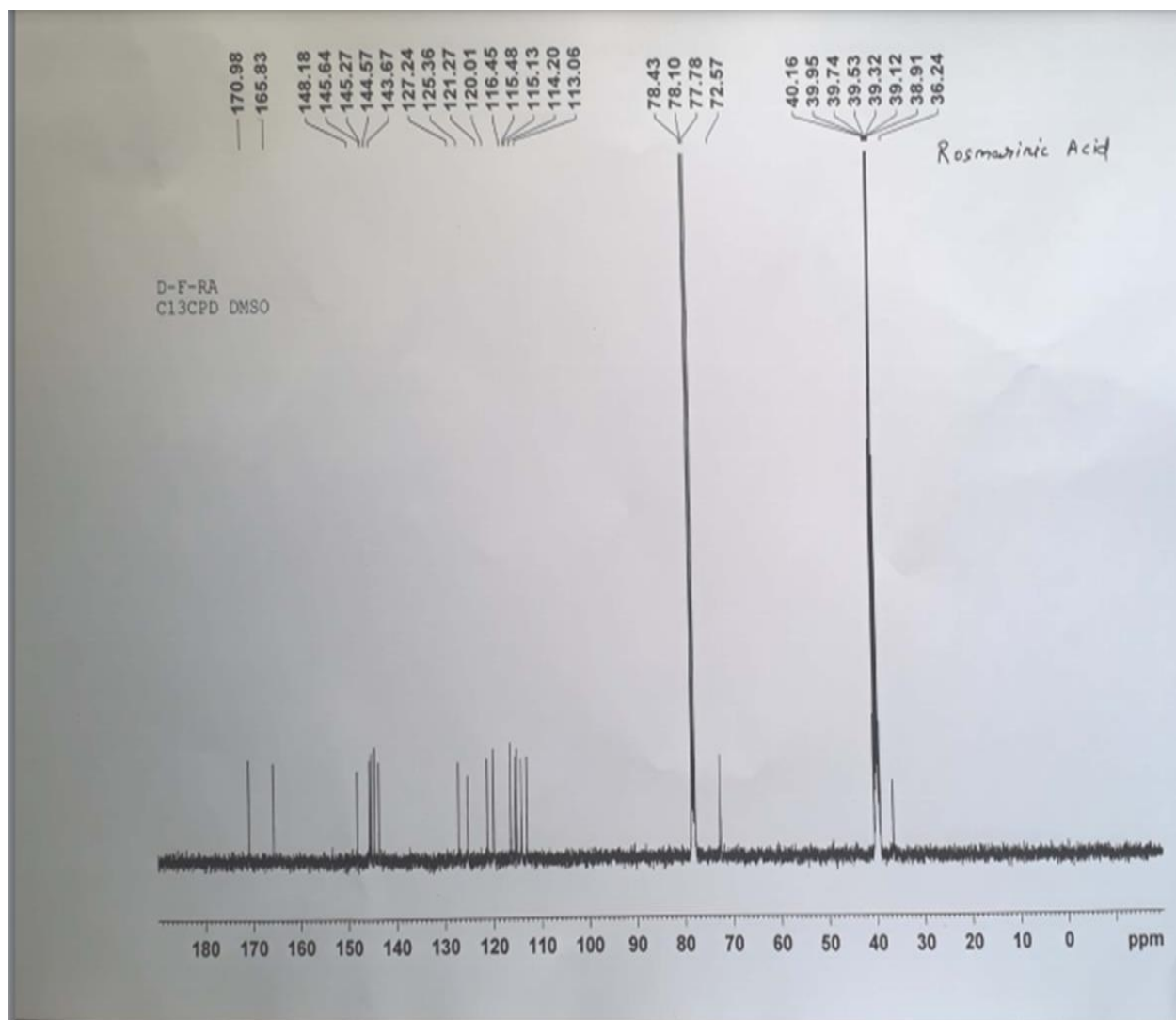


Figure 5.3: Analysis of ^{13}C NMR

The structure of rosmarinic acid ($\text{C}_{18}\text{H}_{16}\text{O}_8$) incorporates two unsaturated 6-membered rings (C1-C6) and $\text{C1}'\text{-C6}'$), a double bond ($\text{C7}'\text{-C8}'$) an ester group ($\text{C9}'$), a carboxyl group (C9) and four hydroxyl groups (C3 , C4 , $\text{C3}'$, $\text{C4}'$).

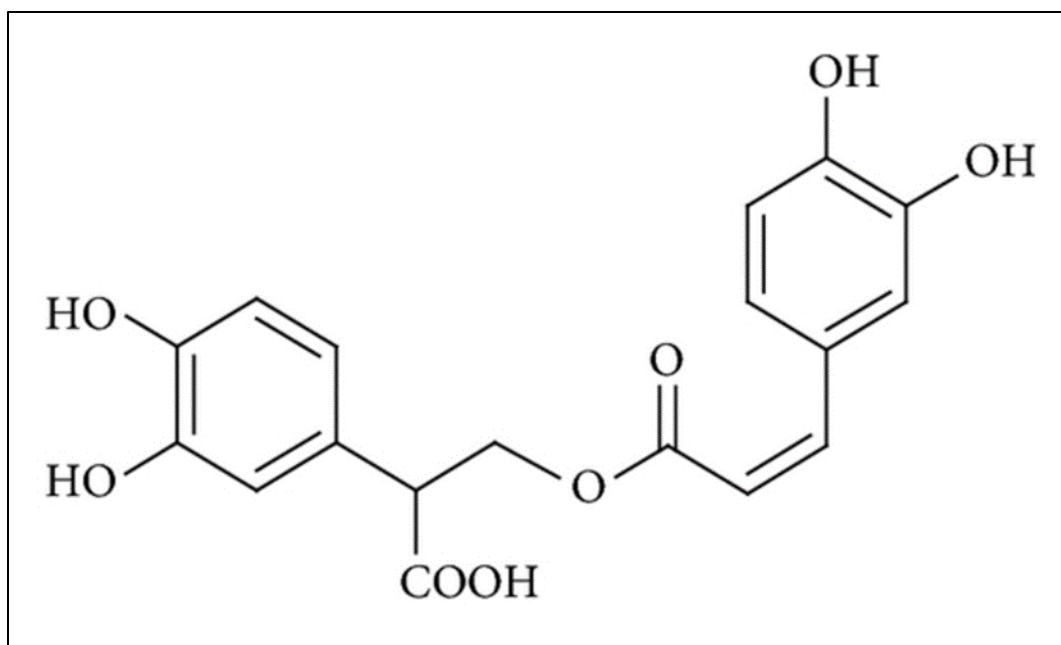


Figure 5.4: Chemical structure of rosmarinic acid

5.3 LIQUID CHROMATOGRAPHY MASS SPECTROMETRY:

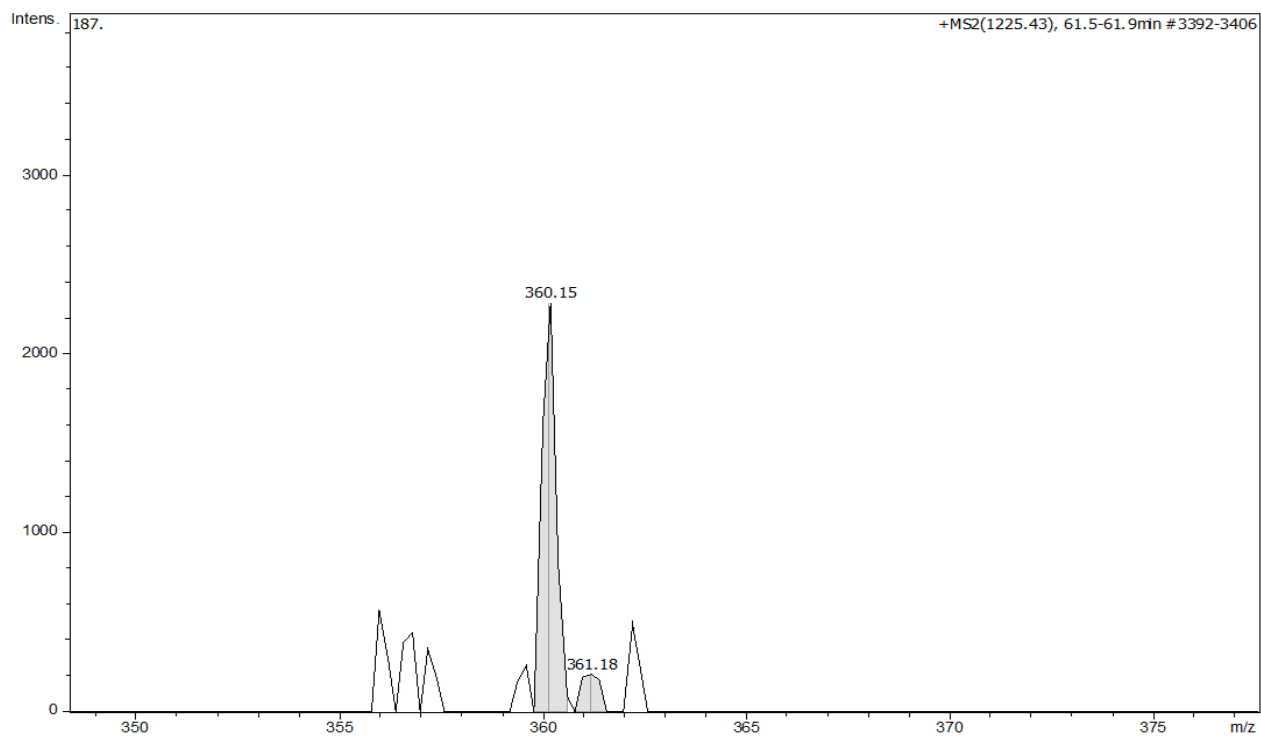


Figure 5.5: LCMS analysis of purified fractions

Fragmentation patterns of the ions are mostly determined by ESI and APCI. Both positive $[M+H]^+$ and negative $[M-H]^-$ ionization modes are used in RA analysis. However, the negative ion mode is more commonly used. Fragmentation of the protonated and deprotonated molecular ions generated from ESI is ordinarily limited and the mass spectra are comparatively simple. In ESI negative ion mode, the common fragments observed for phenolic acids are $[M-H-CO_2]^-$. This is due to the removal of the carboxyl group from the deprotonated molecular ions. Figure 5.5 shows the main mass fragmentation profile of RA, where $m/z = 360$ is the radical ion of RA ($C_{18}H_{16}O_8^+$).

5.4 ANTIFUNGAL ACTIVITY:

In the present study Rosmarinic acid extract is taken against *Candida albicans* to determine the antifungal activity.



Figure 5.6: Antifungal assay of *Candida albicans*

The results shows that the methanolic extract of rosmarinic acid was as effective as the standard Amphotericin B (10 μ l/disc of 100 mg/ml solution). The methanolic extract was very much resistant against the test organism *Candida albicans* showing zone of inhibition 11mm, 12mm, 13mm and 14mm.

5.5 ANTI BIOFILM ACTIVITY:

Compared with planktonic cells, *Candida* biofilms are characterized by increased resistance to conventional antifungal drugs in particular to amphotericin B and fluconazole (Fonseca *et al.*, 2014).

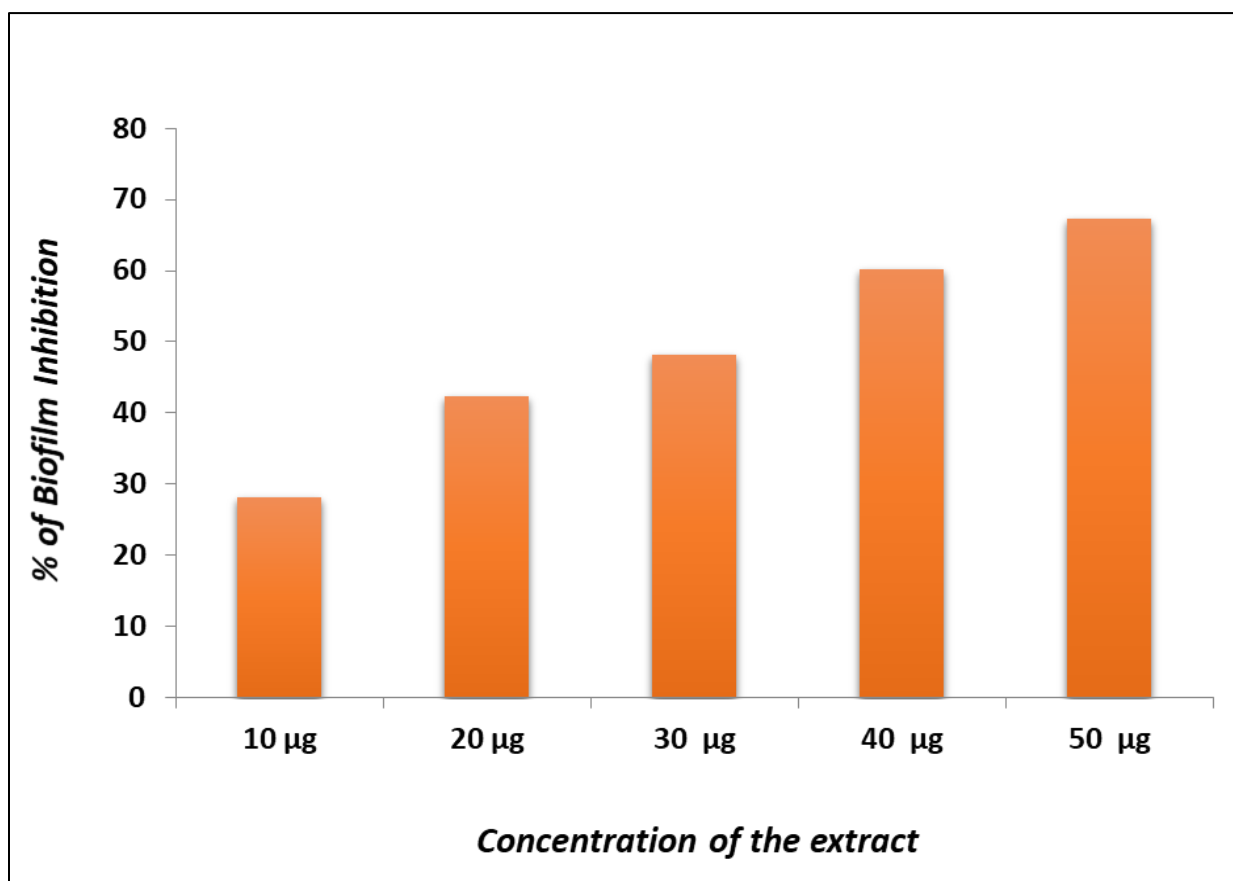


Figure 5.7: Graphical representation of anti-biofilm activity

- Methanol extract of rosmarinic acid showed significant reduction in biofilm formation after 48h of growth in dose dependent manner (10-50 μg) with highest inhibition at 67% at 5 μg .
- At 3 μg methanol extract (50 μg) showed 50 % biofilm inhibition which was fixed as the biofilm inhibitory concentration (BIC_{50})
- From anti-biofilm assay it is resulted that the secondary metabolite of RA (50 μg) at BIC have only anti-biofilm activity against *Candida albicans*.

5.6 LIGHT MICROSCOPIC OBSERVATION OF *CANDIDA ALBICANS* BIOFILM:

- Light microscopic images (400 $\times$) revealed the anti-biofilm activity of rosmarinic acid extract at their BIC_{50} (3 μg) against *Candida albicans*.

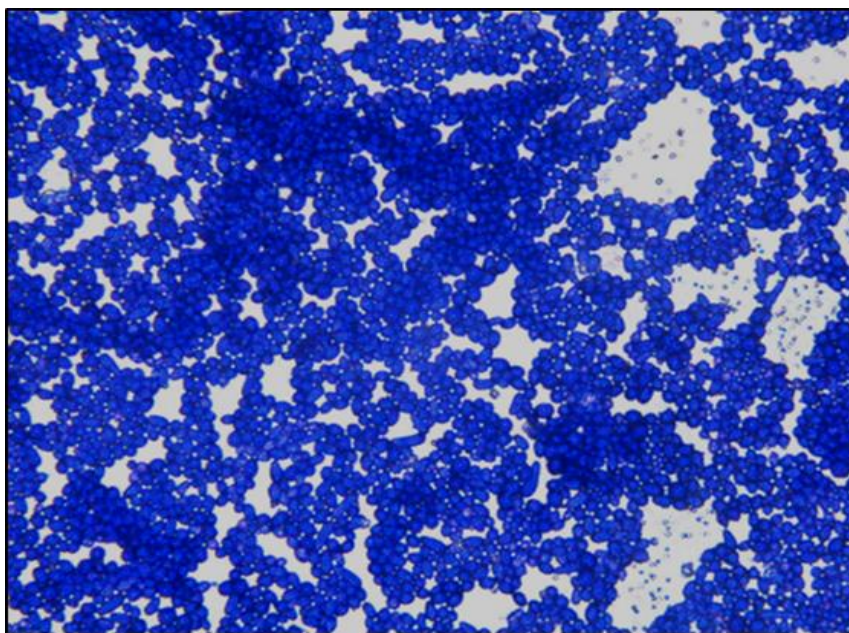


Figure 5.8: Control

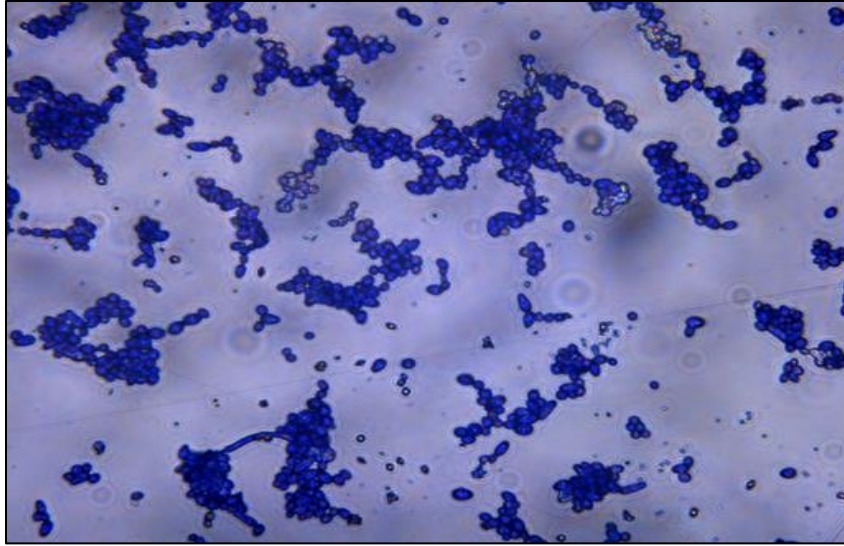


Figure 5.9: Treated

- Light microscopic analysis revealed the presence of increased yeast cells on the surface as shown in Figure 5.8 when compared to treated cells figure 5.9 which shows that the reduction in biofilm formation in treated slides.

CHAPTER 6

SUMMARY AND CONCLUSIONS

6.1 SUMMARY:

In the present study thin layer chromatography was done on the extraction of the plant *Sphaeranthus amaranthoides* for the separation and determination of the compound. Then the separated compound was subjected to LCMS and NMR for the identification and analysis. Through the characterization studies the identified compound is rosmarinic acid. The structure of rosmarinic acid by NMR spectroscopy shed lights on the conformational and dynamic stability of the molecule. Despite the successful purification, an appropriate mobile phase and an adaptable fixed chromatographic bed could be further investigated by LCMS insuring high purity fractions. Finally the rosmarinic acid is tested for in vitro studies to determine its efficiency and activity. Hence in this study we done attempts to grasp highest degrees of purification, to effectively discard by-products to reliably elucidate the chemical structure of rosmarinic acid and to evaluate its property in hepatoprotective activity.

6.2 CONCLUSIONS:

The identified bioactive compound from the study Rosmarinic acid is a natural phenolic compound found in several medicinal plants, herbs and spices. Here we have identified that rosmarinic acid is also present in *Sphaeranthus amaranthoides*. Many reports have proven the hepatoprotective effect of RA through various mechanisms. Several studies shows that cholestatic hepatoprotective effect of RA was due to its improvement in serum biochemical and hepatic histopathological changes, inflammation of liver and oxidative stress. It is also reported that RA inhibited the development of hepatic fibrosis and suppress the activation of matrix producing cells and fibronic changes.

The conclusions drawn from the result of the present study are that the *Sphaeranthus amaranthoides* has endowed with hepatoprotective activity. So the present small scale work supports that further studies in future will harness all the research findings and lead ultimately to the use of rosmarinic acid against liver disorders in a precise and in a big beneficial way.

CHAPTER 7

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