PHARMACOGNOSTIC, PHYTOCHEMICAL AND PHARMACOLOGICAL EVALUATION OF *Phyllanthus acidus(L.)skeels*(EUPHORBIACEAE)

A dissertation submitted to

The Tamil Nadu Dr. M.G.R. Medical University
Chennai-600 032

In partial fulfilment of the requirements
for the award of the degree of

MASTER OF PHARMACY
IN
PHARMACOGNOSY

Submitted by

261220708

DEPARTMENT OF PHARMACOGNOSY

COLLEGE OF PHARMACY

MADURAI MEDICAL COLLEGE

MADURAI - 625 020

APRIL 2014

Dr . A. ABDUL HASAN SATHLI, M.Pharm., Ph.D.,
PRINCIPAL i/c,
College of Pharmacy,
MaduraiMedicalCollege,
Madurai-625020

CERTIFICATE

This is to certify that the dissertation entitled **"PHARMACOGNOSTIC, PHYTOCHEMICAL AND PHARMACOLOGICAL EVALUATION OF THE LEAVES OF *Phyllanthus acidus(L.)skeels*(EUPHORBIACEAE)''** submitted by **Miss. D.SUGANYA (Reg. No. 261220708)** in partial fulfilment of the requirement for the award of the degree of **MASTER OF PHARMACY** in **PHARMACOGNOSY**by The Tamil Nadu Dr. M.G.R. Medical University is a bonafied work done by her during the academic year 2012-2014 under my guidance at the Department of Pharmacognosy, College of Pharmacy, Madurai Medical College, Madurai-625 020.

(Dr . A. ABDUL HASAN SATHLI)

Mr.T.Venkatarathinakumar M.Pharm., (Ph.D).,
Assistant Reader,
Department of pharmacognosy,
College of pharmacy
MaduraiMedicalCollege,
Madurai-625020

CERTIFICATE

This is to certify that the dissertation entitled **"PHARMACOGNOSTIC, PHYTOCHEMICAL AND PHARMACOLOGICAL EVALUATION OF THE LEAVES OF *Phyllanthus acidus(L.)skeels* (EUPHORBIACEAE)''** submitted by **Miss. D. SUGANYA (Reg. No. 261220708)** in partial fulfilment of the requirement for the award of the degree of **MASTER OF PHARMACY** in **PHARMACOGNOSY** by The Tamil Nadu Dr. M.G.R. Medical University is a bonafied work done by her during the academic year 2012-2014 under my guidance at the Department of Pharmacognosy, College of Pharmacy, Madurai Medical College, Madurai-625 020.

Station:

Date

(T.VENKATARATHINA KUMAR)

Project supervisor

- **Part B**

Protocol form for research proposals to be submitted to the committee / institutional animal ethics committee, for new experiments or extensions of ongoing experiments using animals other than non – human primates

Project title: EFFECT OF EXTRACT OF *PHYLLANTHUS ACIDUS* AGAINST DIMETHYLNITROSAMINE INDUCED LIVER CIRRHOSIS IN RATS

Chief Investigator

a. Name : Mr. N. Chidambaranathan, M.Pharm, Ph.D.,

b. Designation : Head, Department of Pharmacology,

K. M. College of Pharmacy,

Uthangudi, Melur road,

Madurai – 625 107

c. Telephone Number : 0452 – 2424652, 2423452

- List of names of all individuals authorized to conduct procedures under this proposal.
  A. Mrs. G. Nalini, M.Pharm., (Pharmacological Supervisor).

  B. Mr. N.Jegan.M.Pharm, (Pharmacological Supervisor).

- Funding source: Nil
- Duration of the project
  a. Number of months : Five months

  b. Date of initiation : 01.10.2013.

  c. Date of completion : 28-02-2014.

- If date by which approval is needed is less than six weeks from date of submission, justification for the same.

  NIL

A] To evaluate the effect of extract of *phyllanthus acidus* against dimethylnitrosamine induced liver cirrhosis in rats

Animals required:

- Species: Albino Wistar rats.
- Age/weight/size: 6 months, Rat (180-220gms) Medium.
- Gender: Male and Female in equal ratios.
- Numbers to be used: Total Numbers:30

(For IAEC / CPCSEA usage)

Proposal number	: D.Suganya/M.Pharm/IAEC/124 /KMCP/ 261220708/2013-14
Date first received	:01.10.2013
Date received after modification (if any)	: NA
Date received after second modification (if any)	: NA
Approval date	: 10.10.2013
Expiry date	: 28.02.2014
Name of IAEC / CPCSEA chairperson	: N.CHIDAMBARANATHAN

Date:10.10.2013

CPCSEA NOMINEE
INSTITUTIONAL ANIMAL ETHICS COMMITTEE
K.M. COLLEGE OF PHARMACY
MADURAI-625 107

Signature

I. A. E. C. CHAIRMAN
INSTITUTIONAL ANIMAL ETHICAL COMMITTEE
K. M. COLLEGE OF PHARMACY
MADURAI-625 107.

ANNEXURE

Investigator declaration

- I certify that I have determined that the research proposal herein is not unnecessarily duplicate of previously reported research.
- I certify that all individuals working on this proposal and experimenting on the animals have been trained in animal handling procedures.
- For procedures listed under item 11, I certify that I have reviewed the pertinent scientific literature and have found no valid alternative to any procedure described herein which may cause less pain or distress.
- I will obtain approval from the IAEC / CPCSEA before initiating any significant changes in this study.
- Certified that performance of experiment will be initiated only up on review and approval of scientific intent by appropriate expert body (institutional scientific advisory committee / funding agency / other body (to be named)
- Institutional biosafety committee (IBC) certification of review and concurrence will be taken (required for studies utilizing DNA agents of human pathogens)
- I shall maintain all the records as per format (Form D)

D. Suganya

Signature

(D.suganya)

N. Chandrasekaran

Name of Investigator

I. A. E. C. CHAIRMAN
INSTITUTIONAL ANIMAL ETHICAL COMMITTEE
K. M. COLLEGE OF PHARMACY
MADURAI-625 107.

Dr. (Mr.) D. STEPHEN., M.Sc., Ph.D.,

ASSISTANT PROFESSOR,

DEPARTMENT OF BOTANY,

THE AMERICAN COLLEGE,

MADURAI - 625002.

CERTIFICATE

This is to certify that the specimen brought by **Miss. D.SUGANYA** II.M. Pharmacy, Department of Pharmacognosy, College of Pharmacy, Madurai Medical College, Madurai is identified as ***Phyllanthus acidus*(L.)** skeels belonging to the family.Euphorbiaceae

SSTATION : Madurai.

DATE :

(Dr. D. STEPHEN)

Dr. D. STEPHEN, Ph.D.,
LECTURER IN BOTANY
THE AMERICAN COLLEGE
MADURAI-625 002
TAMILNADU - INDIA

ACKNOWLEDGEMENTS

I first and foremost express my revered regard and obeisance to the **ALMIGHTY GOD** with whose blessings I was able to complete my project work.

I am grateful to express my sincere thanks to **Dr.B.SANTHAKUMAR.M.Sc (F.Sc), M.D(F.M).,** Dean,Madurai Medical College for giving me an opportunity to carry out my project work.

I am very thankful to **Dr.L.SANTHANALAKSHMI M.D, D.G.O, M.B.A.**,vice principal,Madurai Medical College for giving me an opportunity to carry out my project work.

It is my privilege to express a deep and heartfelt sense of gratitude and my regards to our respected **Dr.Mrs. AJITHADAS ARUNA, M.Pharm., Ph.D,** joint Director of Medical Edication (Pharmacy) and former Pricipal,College of Pharmacy,Madurai Medical College and for active guidance,advice,help ,support and encouragement.

I owe a great debt of gratitude and heartful thanks to **Dr. Mr.A.ABDUL HASAN SATHALI, M. Pharm., Ph.D.,** Principal (In-charge)and Head, Department of Pharmaceutics College of Pharmacy, Madurai Medical College, Madurai.

I wish to place on record here my indebtedness and heartfelt thanks to **Mr.T.VENKATARATHINAKUMAR M.Pharm.,Ph.D.,** Assitant Reader.Department of pharmacognosy ,College of pharmacy,Madurai Medical College,Madurai for his constant valuble suggestion and encouragement to improve and complete the project work.

I express my sincere thanks to**, Dr. K. PERIYANAYAGAM, M. Pharm., Ph.D.,** Assistant Readers in Pharmacy, Department of Pharmacognosy, College of Pharmacy, Madurai Medical College for his support and valuable suggestions.

It's my pleasure to thanks **Ms.R. Gowri, M.pharm** ,Assistant Reader ,Department of pharmacognocy,College of pharmacy Madurai Medical College, Madurai for the extended encouragement, during the project work.

I thank **Prof. Mrs.R. THARABAI, M.Pharm,** Professor and Head of Department of Pharmaceutical chemistry, College of Pharmacy, Madurai Medical College for her guidance during the course of my study.

I thank **Mrs. A. SETHURAMANI, M. Pharm., (Ph.D)** and **Mrs. A. KRISHNAVENI, M. Pharm.,Ph.D.,** Tutors in Pharmacy, Department of Pharmacognosy, College of Pharmacy, Madurai Medical College for their help.

I bring forth my sincere thanks to **Dr.Mrs.Sobana** Veterinary Assistant Surgeon, Central Animal House,Madurai Medical College , Madurai, for her support in getting the valuble suggestion regarding pharmacological study needed for this dissertation.

I owe my special thanks to **Mr.Arunachalam Muthuraman M.pharm Ph.D** Deparment of pharmaceutical science and Drug Research,Punjab University, Whose Valuble suggestion regarding the pharmacological studies and timely help made this work as a successfully.

I thank **Mr. Chithambaram M.Pharm.,Ph.D.,** KM college of pharmacy ,Madurai, for his supporting getting the ethical clearance Whose valuable suggestion regarding the pharmacological studies and timely help made this work as a successfully.

I thank **Mr. P. SIVAKUMAR M.Sc., DMLT.,** Lab Supervisor for helping me in carrying out the anticancer activity.

I thank **Mr.S.SELVAKUMAR** Dept. of Pharmacology for helping me in carrying out the anticancer activity.

I extend my thanks to all the teaching staff of College of Pharmacy and of Madurai Medical College who have rendered their help towards completion of the project.

I am thankful to **Mr. Jones** Universal Scientific Supplier for his timely supply of chemicals utilized during the project work.

I place on record my gratitude to **Dr. P. JAYARAMAN, M.Sc., Ph.D.,** Director, Plant Anatomy Research Centre, Chennai 600 045 who helped me in the microscopic studies and **Dr. D. STEPHEN, Ph.D.,** Senior Lecturer, Department of Botany, American College, Madurai who helped me in the identification of my plant.

I thank **Mr. Magudeeswaran DMLT**, and **Mrs. P. Ellayee,** Attender Deptment of Pharmacognosy , for their help throughout my study.

I also thank my ever loving classmates, **Ms.P.Anitha, Ms.P.Bala, Ms.R.Jancy, Mr.S.Jegadeesh, Ms.M.Kalayarasi, Mrs.S.R.Nandhini, Mrs.S.Nadhiya, Ms.K.Vijayalakshmi,** and all my juniors of 2013-2015 batch and other friends for their constant motivation and help.

This is dedicated to my family members and my brothers **Mr.S. MANIKANDAN, Mrs. M.KANIMOZHI, Mr.S.VINOTH, Mrs.V.SARARNYA, Mr.D.MAILVALEN, Mr.T.KANNAN,** and my sisters **Mrs.T.RAMANI,MS.T.JENSI** and my uncle **U.THIRUPATHI , S.SAMIAYYAN INBAMANI, T.BANUMATHI, E.THIRUPATHI, S.KANNAKI** for their understanding, endless patience, help and encouragement which made me to complete this work.

This is special dedicated to **my lovable parents My Father P.DHIRAVIDAMANI,** and **My Mother D.NAVAMANI I Lot of thanks.**

D.SUGANYA

CONTENTS

CHAPTER	TITLE	PAGE NO.
I	**INTRODUCTION**	1
II	**REVIEW OF LITERATURE**	10
III	**AIM AND SCOPE OF THE PRESENT STUDY**	13
IV	**PHARMACOGNOSTICAL STUDIES**	14
	Section –A : Plant Profile	
	Section- B : Microscopical studies of the leaf	
	Section- C : Quantitative microscopical of leaves	
	Section-D : Physical parameters	
V	**PHYTOCHEMICAL STUDIES**	23
	Section-A : Organoleptic evaluation	
	Section-B : Qualitative chemical examination	
	Section-c : Quantitative estimation of Phytoconstituents 1)Total Phenol determination 2)Total flavonoid determination 3)Total tannin determination **Section- D** : HPLC Studies	
VI	**PHARMACOLOGICAL SCREENING**	35
	Section –A : *In-vitro* antioxidant activity DPPH assay Hydrogen radical scavenging activity	
	Section-B: *In -vivo* DEN induced Hepatocellular carcinoma in rats	
VII	**RESULTS AND DISCUSSION**	42
VIII	**SUMMARY AND CONCLUSION**	65
IX	**REFERENCES**	

INTRODUCTION

Herbal medicine are being used by about 80% of the world population primarily in the developing countries for primary health care. They have stood the test for their safety, efficacy, cultural acceptability and lesser side effects. The chemical constituents present in them are a part of physiological functions of living flora and hence they are believed to have better compatibility with the human body. Ancient literature also mentions herbal medicines for age-related disease namely memory loss, osteoporosis, diabetic wounds, immune and liver disorders.

Herbal medicine scenario in India

India is one the 12 mega biodiversity centres having over 45,000 plant species,its diversity is unmatched due to the presence of different agroclimatic zones 10 vegtable zone and 15 biotic provines. The country has 15,000-18,000 flowering plants, 23,000 fungi, 2500 algae, 16,000 lichens,1800 bryophytes and 30 million micro-organism.

The turnover of herbal medicine in India as over –the counter products,ethical and classical formulation and home remedies of Ayurveda,unani,and siddha system of medicine is about 1 billion with a meager export of about 80 million.(Psyllium seed and husk ,castor and opium)80 % of the export to developed countries are of crude drugs and not finished formulations leading to low revenue for the country.

Herbal medicine and WHO

WHO it helped the inclusion of proven traditional remedies in national drug policies and regulatory approvals by developing countries .The world health assembly continued the debate and adopted a resolution (WHA 42.43) in 1989 that herbal medicine is of great importance to the health of individuals and communities. In 1991 WHO

developed guidelines for the assessment of herbal medicine,and same was ratified by the 6th international conference of drug regulatory authorities held at Ottawa in the same year.The salient feature of WHO guidelines are:(1)Quality assessment,ofcrude plant materials;plant preparations;finished products.(ii)stability;shelf life.(iii)safety assessment and Toxicological studies.(IV)Assesment of efficacy.[1]

Standardization of herbal medicine

In indigenous /traditional system of medicine,the drugs are primarily dispensed as water decoction or ethanolic exracts of fresh plant parts, juice and crude powder .Thus medicinal plant parts should be authentic and free from harmful materials like pesticides,heavy metals,microbial or radioactive contamination etc.The medicinal plant is subjected to a single solvent extraction once or repeatedly or water decoction or as described in ancient texts. The extract should then be checked for indicated biological activity in an experimental animal models. The bioactive extracts should be standardized on the basis of active principle or major compound along with finger prints.The next important step is stabilization of the bioactive extract should undergo regulatory or limit safety studies in animals.Determination of the probable mode of action will explain the therapeutic profile.The safe and stable herbal extract may be marketed it its therapeutic use is well documented in indigenous system of medicine ,as also viewed by WHO. A limited clinical trial to establish its therapeutic potential would promote clinical use.The herbal medicine developed in this mode should be dispensed as prescription drugs or even OTC products depending upon disease consideration . [1,2]

Cancer

Cancer cells divide and grow uncontrollably forming malignant tumours, and invade near by parts of the body . The cancer may also spread to more distance of the

body through the lymphatic system(or)blood stream. There are over 200 different known cancers that affect humans.

The causes of cancer are diverse complex and only partially understood.Many things are to increase the risk of cancer

including ;Tobacco, Dietary factors, Certain infections,Radiation,lack of physical activity ,Obesity and environmental pollutants. Appoximately 5-10 % of cancers can be traced directly to inhertited genetic defects. Many cancers could be prevented by avoid smoking, eating more vegetable, fruits and whole grains eating less meat and refined carbohydrates, maintaing a healthy weight exercising minimizing sunlight exposure and being vaccinated against some infectious diseases.

Cancer can be detected in number of such ways including the presence of certain signs and sysmptoms screening tests medical and imaging-microscopic examination of tissue sample. cancer is usually treated with chemotherapy radiation therapy and surgery.

Types of cancer

- Breast cancer/cervical cancer
- Ovarian cancer
- Leukemia , lymphoma multiple myeloma cancer
- Bone cancer
- Oral cancer
- Mouth cancer
- Tongue cancer
- Thyroid cancer
- Throat cancer
- Brain cancer

- Skin cancer
- Kidney cancer
- Urinary Bladder cancer
- Liver cancer
- Hepato cellular carcinoma cancer
- Gall bladder cancer
- Hodgkin's lymphoma and Non-Hodgkin's lymphoma[3]

Hepatocellular Carcinoma-[4 5 6]

The liver is the largest organ in the body.cancer affecting the liver may be primary or secondary. Hepatocellular carcinoma (HCC) is the commonest primary cancer of the liver. It is the sixth commonest malignancy worldwide and the third leading cause of cancer-related death.

Hepatocellular carcinoma has a heterogeneous geographical distribution determined mainly by the presence of the risk factors in different parts of the world. It is more common in sub-saharan Africa and South-East Asia than North America and western Europe.

Hepatitis B virus is the major hepatocarcinogen in Asia and Africa, where the infection is acquired early in life and thus becomes persistent up to 75% of individuals. In the western world, Hepatitis C virus (HCV)is the leading cause of HCC, which develops in older,and cirrhotic patients. Globally, about 80% of cases of HCC are due to underlying chronic hepatitis B and C infection.

A distinct feature of HCC is the fact that prognosis is not determined only by the tumoral disease but also by the severity of the underlying liver disease. Many patients who are diagnosed with HCC have advanced disease and are only candidates for

palliative care, contributing to a relatively low reported 5-year survival rates of approximately 10%.In high prevalence areas ,many patients die within 6 months of diagnosis.

Causes

The most common risk factor in the United States is alcohol abuse; in the rest of the world, Hepatitis B and Hepatitis C are the risk factors responsible for most cases of hepatoma. Although these are preventable problems, the incidence of hepatoma is actually rising in many countries.In the United States ,due to a large increase in Hepatitis C infection several decades ago,the incidence has doubled to over 17,000 cases each year. Recent Research has demonstrated that a large part of the increase in hepatoma is due to the rise in obesity and diabetes over the past few decades.

Clinical Features

Patients who develop HCC usually have no symptoms other than those related to their chronic liver disease. Suspicios for HCC should be heightened in patients with previously compensated cirrhosis who develop decompensation such as ascites, encephalopathy and jaundice.These complications are often associated with extension of the tumor in to the hepatic or portal veins or arteriovenous shunting induced by the tumor.

Some patients may have mild to moderate upper abdominal pain, wight loss, early satiety or a palpable mass in the upper abdomen. These symptoms often indicate an advanced lesion.

Symptoms and Sings

Symptoms such as weakness, fatigue ,weight loss or loss of appetite are common.Signs of liver damage may appear as the tumorgrows,such as an increase in

abdominal size due to accumulation of fluid around the liver and intestines(called ascites)and jaundice,a yellowing of the skin and eyes along with dark urine. Jaundice occurs due to the accumulation of bilirubinin in blood(more than 2 mg%)a breakdown product of red blood cells that is usually handled by the liver.

More severe liver failure may cause internal bleeding and mental changes, including confusion or uncontrollable sleepiness(encephalopathy)as the liver is no longer able to handle all the harmful chemicals in the blood. Rarely, affected people may develop fevers, night sweats or pain.

Causes

Cancers are primarly an environmental disease with 90-95% of cases attributed to environmental factors and 5-10 % due to genetics.

Factors: causes of cancer

1.Environmental

2.Inherited genetically

3.Tobacco 25-30%

4.Obesity 30-35%

5.infection15-20%

6.Radiation(both ionizing and non-ionizing) 10%

7.stress and lack of physical activity

Chemicals

- Alcohol and cancer
- Smoking and cancer
- DENA(Diethyl nitrosamine)

The incidence of lung cancer is highly correlated with smoking.

Tobacco smoking is associated with many forms of cancer and causes 90% lung cancer. Decades of research has demonstrated the link between tobacco use and cancer in the lung, larynx, head, neck, stomach, bladder, kidney, esophagus and pancrease. Carcinogens including nitrosamines and polycyclic aromatic hydrocarbon. Millions of workers run the risk of developing cancers such as lung cancer and mesothelioma from inhaling asbestos fibers and tobacco smoke or leukemia from exposure to benzene at their work places.

Diet and exercise

Diet physical inactivity and obesity are related to approximately 30-35% cancer deaths. Physical inactivity is believed to contribute to cancer risk not only through its effects on body weight but also through negative effects on immune system and endocrine system.Diet that are low in vegetables fruits and whole grains and high in processed (or)red meats are linked with a number of cancers.

A high salt diet is linked to gastric cancer, aflatoxin B1, a frequent food contaminate causes liver cancer ,and betalnut chewing with oral cancer. Gastric cancer is more common with intakes of high salt diet .

Infection

Viruses are the usual infectious agents that cause cancer but bacteria and parasites may also have an effect. A virus that can cause cancer is called an oncovirus, These include human papilloma virus β-cell lympho - proliferactive disease and naso pharyngeal carcinoma. herpavirus, hepatitis B, and hepatitis c viruses and human T-cell leukemias.bacterial infection may also increase the risk of cancer as seen in helicobactor

pylori-induced gastric carcinoma. Parasitic infections stongly associated with cancer include schistosoma haematobium.

Physical agents

Prolonged exposure to ultraviolet radiation f the sun lead to melanoma and other skin malignancies. Non-ionozing radio frequency radiation from mobile phones and electric power transmission.

Heredity

Some of these syndrome include certain in heredity mutations in the genes BRCA1,BRCA2 with a more than 75% risk of breast and ovarian cancer.

Hormones

Some hormones play a role in the development of cancer by promoting cell proliferation .Insulin like growth factors and their binding protein play a key role in cancer cell proliferation ,differentiation and apoptosis suggesting possible involvement in carcinogenesis.

Diagnosis

Most cancer are initially recognized either because of the appearance of signs or through screening, medical tests .These commonly include blood tests x-rays, CT scans and endoscopy..

Diethyl nitrosamine

Diethyl nitrosamine is one of the important environmental carcinogen that primarily induced tumors of the liver. because of its relatively simple metabolic pathway and potent carcinogenic activity.DEN confers its carcinogenicity through the metabolic

activation in the liver microsome causing the releasing of alkylating agents that bind to the DNA forming adducts. It has been reported that their biotransformation produces the promutagenic adducts O6-ethyldeoxyguanosine,O4 and O6-ethyldeoxythymidine and 8-hydroxyguanine (8-OHG)that play a role in the initiation step of rat liver carcinogenesis and generation of superoxide radicals, paralleled by lipid peroxidation reaction. lipid peroxidation (LPO) is regarded as one of the basic mechanism of cellular damage caused by freeradicals. Free radicals react with lipid causing peroxidation resulting in release of products such as malandiodehyde (MDA),hydrogen(H_2 O $_2$) and hydroxyl radicals (OH).ROS including oxygen, hydrogen peroxide and hydroxyl radical(OH) play in important role in carcinogenesis.

CHAPTER II

LITERATURE SURVEY

Ethno medicinal uses:

1. Leaves are used to treat cough with, nausea, cancer, mouth sore, liver disease hypertension, lumbago and sciatica
2. Root is used for purgative
3. leaves are used in cancer treatment
4. Decoction of leaves are used externally for urticaria. Decoction of the bark is used for bronchial catarrh.(7)

Tai *et al.*,(2013) have reported the antiviral activity of *Phyllanthus reticulates* against human immuno deficiency virus type1(HIV-1) which is the major cause for AIDS. **(8)**

Chouhan *et al.*,(2013)have reported the photochemical analysis, antioxidant and anti-inflammatory activities of *Phyllanthus simplex*. **(9)**

Hansakul *et al*,(2012have reported the anti tumor and Induction of cancer cell apoptosis effect of *Phyllanthus* emblica.***(10)***

Patel *et al.*, (2012) have reported the ethano medicinal uses phytochemistry and ,pharmacological evaluation of *Phyllanthus* amaraus.**(11)**

Dang *et al.*,(2012) have reported the anti-inflammatory activity of *Phyllanthus emblica*, and Cyprus rotundus in acute models of inflammation.**(12)**

Zhu *et al* .,(2012) have studies on Isolation and x-ray crystal structure of securinega-tybe alkaloid from *Phyllanthus* niruri *Linn*. Studied on analgesic activity, antipyretic potential of *Phyllanthus amarus. (***13)**

Mazumder *et al.*,(2010)) have reported new phthalates from *Phyllanthus muellerianus* (Euphorbiaceous) **(14)**

Pagare *et al.*,(2010) studied on protocol for callus culture of *Phyllanthus amarus schum* and Thonn and possibility of use of callus culture in production of phyllanthin and hypophyllanthin.[15]

Chattopadhyay p et *al.*,(2010) have reported the Increase of insulin activity by *Phyllanthus amaras* Linn on liver cell regeneration in albino rats.[16]

Fan *et al.*,(2009) have reported antioxidant and inflammatory mediator's growth inhibitory effect of compound isolated from *Phyllanthus urinaria*.[17]

Garg *et al.*,(2008) have reported anti diabetic potential of *Phyllanthus urinaria* in albino rats.[18]

kumar *et al.*,(2008)have reported the antidiabetic potential of *Phyllanthus reticulates* in alloxan-induced diabetic in mice.[19]

Karthikeyan *et al.*,(2008)have reported the regeneration of *Phyllanthus niruri L*.from shoot tip and nodal explants.[20]

Harikumar *et al.*,(2007) have reported the antibacterial activity of *Phyllanthus* and *curcumin.*[21]

Kumaran *et al.*,(2007) have reported the anti oxidant activity of polyphenol from *Phyllanthus debilis wild*.[22]

Garg *et al.*,(2007) have reported the antidiabetic and antioxidant potential of Phyllanthus *fraternus* in alloxan induced diabetic in animals.[23]

Bos *et al.*,(2006) have reported the lignans from cell suspension culture o fP*hyllanthus niruri* an Indonesian medicinal plant.[24]

Balakumbahan. et *al.*,**(2006)** have reported the effect of inorganic and biofertilizers on biomass and alkaloid yield of Phyllanthus *amarus*.[25]

Sailaja et *al.*,(2006) have reported the protective effect of *Phyllanthus fraternus* against allyl alcohol- induced oxidative stress in liver mitochondria.[26]

Zhang Y et *al.*,(2004) have reported the antiproliferative activity of the main constituents from *Phyllanthus emblica*.[27]

Srinivasa et *al*.,(2004) have reported the anti cancer and antiviral activity of *Phyllanthus maderasapatensis* . HPTLC method was used for the quantification of gallic acid and ellagic acid content this plant.[28]

Al-re haily et *al*.,(2003) have reported the gastroportective effect of *Phyllanthus emblica in-vivo* test model in rats [29]

Rajeskumar et *al*.,(2001) have reported the *Phyllanthus amarus* extract of administration increase the life span of rats with hepatocellular carcinoma.[30]

Suganya L.*et al*.,(2001)have reported the *Phyllanthus emblica* extract of influecnce on dermal wound healing in rats. [31]

Dep S. *et al.*,(1998) have reported the *Phyllanthus amarus* extract of simple thinlayer chromatograhy-densiometric methods for the estimatio of the two lignans phyllanthin, hypophyllanthin and polyherbal formulation [32]

AIM AND SCOPE OF THE PRESENT STUDY

The ethanomedicinal information of *Phyllanthus acidus* indicates that it was used as an emetic cathartic,antihypertensive,anti asthmatic, liver tonic and also to treat cancer.*Phyllanthu acidus* as well as other species of *Phyllanthus emblica* were used in traditional medicine for the treatment of various type of cancer.The root of this plant was used to treat psoriasis .The leaf of this plant was also found to posses anti-inflammatory ,antinociceptive antioxidantant,hepatoprotective ,hypotensive and antidiabetic activity. Antibacterial and cytotoxicactivity were also reported in this plant.[33-34]

Phyllanthosols A and B from fruits of this plant has already been reported for anticancer activity.

Pharmacognostical studies have not yet been reported in the leaves of this plant. Leaves of this plant have not yet been reported for liver cancer studies.

Hence the present work has been designed to carry out the following studies on the the leaves *Phyllanthus acidus*.

- Pharmacognostical studies
- Preliminary phytochemical screening of crude drug and ethanolic extract of*Phyllanthus acidus*(EEPA)
- Estimation of total phenolic,total tannin and flavonoid content of EEPA
- Phytochemical evaluation of EEPA by TLC and HPLC
- Quantification of active principles present in this plant extracts.
- Screening of the EEPA for the following pharmacological activities;
- *In-vitro* antioxidant activity by DPPH radical and Hydrogen peroxide scavenging assay .*In vivo* anticancer effect of EEPA against DEN induced hepatocellular carcinoma in rats.

CHAPTER - IV
PHARMACOGNOSTICAL STUDIES
SECTION A
PLANT PROFILE [35-36]

Biological source : *Phyllanthus acidus L*

Family : Euphorbiaceae

Synonyms : *Phyllanthus distichus mull Arg, Cicca acida merr, Cicca disticha L , Averrhoa acid L.*

Systemic Position:

Kingdom	:	Plantae
Subkingdom	:	Tracheobionta
Superdivision	:	Spermatophyte
Division	:	Magnoliophyta
Class	:	Rosidae
Order	:	Euphorbiales
Family	:	Euphorbiaceae
Genus	:	*Phyllanthus*
Species	:	*acidus*
English name	:	Star goose berry, Malay goose berry
Common name	:	Star goose berry
Hindi	:	Harfarauri
Marathi	:	Harpharori
Konkani	:	Raiamvali
Manipuri	:	Gihori
Tamil	:	Aranelli
Urudhu	:	Harfarauri

FIG : 1 . Herbarium of *Phyllanthus acidus*

FIG : 2 . Leaves of *Phyllanthus acidus*

FIG:3 . ***Phyllanthus acidus*** **of tree**

FIG :4 . Leaves *of Phyllanthus acidus* Dorsal view

FIG:5 . ventral view

FIG:6 . Fruits of *Phyllanthus acidus*

FIG :7 . Fruits of *Phyllanthus acidus*

FIG:8 .Flowers of *Phyllan*thus acidus

Geographical distribution& occurrence

This tropical (or) subtropical species is found throughout Asia and also in the Caribbean region. The tree is common in Guam in Indonesia. In South Vietnam this plant is called as chum ruot and in India it is called as chalmeri and harpharoi.

Description of the plant

This plant bears alternate leaves which are ovate with short petiole. flowers are in little clusters and bears pale yellow colour. the fruit is sub-spherical with 6 to 8 ribs and pale yellow color**.**

SECTION-B
MICROSCOPICAL STUDIES OF THE LEAVES
MATERIALS AND METHODS [37-38]

Phyllanthus acidus as collected from Vadakkikottai village, Thanjavur and identified by Taxonomist.

Petiole and leaf were fixed in FAA solution (70% ethyl alcohol, formalin and acetic acid in the ratio of 90 ml: 5 ml: 5 ml). The materials were left in the fluid for three days and then they were washed in water and dehydrated with tertiary butyl alcohol. Paraffin wax was filtered and the specimens were embedded in wax for sectioning.

Transverse sections of petiole and leaf were taken using microtome and stained with toluidine blue. All slides, after staining in toluidine blue, they were dehydrated by employing graded series of ethyl alcohol (70 %, 90 %, 100 % alcohol) and xylol-alcohol (50-50) and passed through xylol and mounted in DPX mountant (Johansen, 1940).

Clearing of leaves for studying stomatal number and stomatal index was done by using 5% sodium hydroxide along with chlorinated soda solution supplemented with gentle heat. Quantitative microscopy was carried out and values were determined as per

the procedure given in Wallis (1997). Photomicrographs were taken with the help of Nikon Eclipse E200 Microscope.

The microscopic features of the various parts of the leaf are presented in Fig. 1 to 7

SECTION-C

QUANTITATIVE MICROSCOPY

This is useful for identification, characterization, and standardization of crude drugs. A clear idea about the identity and characteristic features of the drug can be obtained after several numbers of determinations; the characteristic's number obtained is noted and compared with a standard value to find out whether it is within the range.

STOMATAL NUMBER AND STOMATAL INDEX [39-40]

Stomatal number

The average number of stomata present in 1 square millimeter area of each surface of a leaf epidermis is termed as stomatal number .[41]

Stomatal index

The stomatal index is the percentage of the number of stomata formed by the total number of epidermal cells including stomata, each stoma being counted as one cell.

Determination of stomatal number and stomatal index

` To study the stomatal morphology (type of stomata), stomatal number and stomatal index of leaf, the leaf was subjected to epidermal peeling by partial maceration employing the Jeffrey's maceration fluid.

A fragment was transferred in to microscopic slide and the mount of lower and upper epidermis was prepared with a small drop of glycerol solution at one side of the cover slip to prevent the slide from drying. The slide was examined under 45X objective

and 10X eye piece to which a microscopical apparatus was attached. Circle (O) like mark was marked on the drawing paper for each stoma. The average number of stomata/square mm for each surface of the leaf was calculated and their values are tabulated in table 1**.**

For stomatal index, the glycerin mounted leaf peeling as mentioned above was made and circle (O) like mark for each stomata and a cross (X) like mark for each epidermal cells was marked on the drawing paper. The stomatal index was calculated by using the formula,

Stomatal index $\quad S.I = \frac{S}{E+S} \times 100$

Where S = Number of stomata in 1 sq mm area of leaf and E = Number of epidermal cells (including trichomes) in the same area of leaf. The values are tabulated in table 1.

VEIN ISLET NUMBER AND VEIN TERMINATION NUMBER

The term vein islet is used to denote the minute area of photo synthetic tissue encircled by the ultimate division of the conducting strands. The number of vein islets per square mm area is called vein- islet number.

Vein termination number may be defined as the number of vein terminations present in one square mm area of the photosynthetic tissue. [42]

Determination of Vein Islets and Vein Terminations

The fragment of leaf lamina with an area of not less than 1 sq mm excluding the midrib and the margin of the leaf was taken. The fragments of leaf lamina were cleared by heating in a test tube containing chloral hydrate solution on a boiling water bath until clear. The cleared fragments were stained with safranin solution and a temporary mount was prepared with glycerol solution. The stage micrometer placed on the microscopic

stage, examined under 10X objective and 10X eye piece and an area of 1 sq mm square was drawn. The cleared leaf piece was placed on the microscope stage, the vein islets and vein terminals included in the square was drawn.

The number of vein islets and terminals within the square including those over lapping on two adjacent sides and excluding those intersected by others two sides were counted. The results obtained in the number of vein islets and terminals in1 sq mm were tabulated in table 1.

PALISADE RATIO

Palisade ratio is the average number of palisade cells under one epidermal cell. It is another important criterion for identifications and evaluations for crude drugs. Since it is constant for a plant species which is useful to differentiate the species and does not altered based on geographical variation [40].

Determination of Palisade Ratio

Epidermal peeling was done by partial maceration by Jeffery's maceration fluid were prepared. A fragment was transferred into a microscopical slide and the mount of upper epidermis was prepared with a small drop of glycerol on one side of the cover slip to prevent the preparation from drying. The same was examined under 45X objective and 10X eye piece. Four adjacent epidermal cells were traced; focusing gently downward to bring the palisade cells into view and sufficient palisade cells to cover the outlined four epidermal cells were then traced. The palisade cells under the epidermal cells were counted and calculate the palisade ratio by using the following formula and the results were tabulated in table 1.

Palisade ratio = Avarage number of palisade cells beneath the 4 epidermal cells/4

SECTION – D
PHYSICAL PARAMETERS

POWDER ANALYSIS

The behavior of the powder with different chemical reagents was carried out as per standard procedure [43]. The observations are presented in table 2.

Fluorescence analysis

The fluorescent analysis of the drug powder as well as the plant extracts of *Phyllanthus acidus* were carried out and the observations are tabulated in tables 3 & 4**.**

STANDARDIZATION PARAMETERS [40]

Ash values, loss on drying, foreign organic matter and extractive values etc. are the important physical parameters for the standardization of plant materials. The method given in Indian Pharmacopoeia, 1996 and WHO guidelines, 1998 were followed to determine total ash, water-soluble ash, acid-insoluble ash and loss on drying. The percentages of extractive values for different solvents were also determined for the leaves of this plant.

Determination of foreign organic matter

The part of organ or organs other than those specified in the definition or description of the crude drug is defined as foreign organic matter.

An accurately weighed 100 g of air dried coarse drug was spread out as a thin layer. The sample drug was inspected with the unaided eye or with the use of 6X lens and the foreign organic matter was separated manually as completely as possible and weighed. The percentage of foreign organic matter was calculated with reference to the weight of the drug taken. The result is presented in table 5.

Determination of Moisture Content (Loss on Drying)

An accurately weighed 10 g of coarsely powdered drug was placed in a tarred evaporating dish. Then the dish was dried at 105^{o}C for 5h and weighed. The drying and weighing was continued at one hour intervals until the difference between the two successive weighing is not more than 0.25%. The loss on drying was calculated with reference to the amount of powder taken. The readings are tabulated in table 5.

Determination of Ash values [44]

Ash Content

The residue remaining after incineration is the ash content of crude drug, which simply represents inorganic salts naturally occurring in the drug or adhering to it or deliberately added to it as a form of adulteration.

Determination of Total Ash (45)

An accurately weighed 3 g of air dried coarsely powdered drug was taken in a tarred silica crucible and incinerated at a temperature not exceeding 450^{o}C, until free from carbon then allowed to cool and weighed. The percentage of ash was calculated with reference to the air dried drug.

Determination of Acid Insoluble Ash

The total ash obtained from the previous procedure was mixed with 25mL of 2M hydrochloric acid and boiled for 5min in a water bath, and then the insoluble matter was collected in an ashless filter paper and washed with hot water, dried and ignited for 15min at a temperature not exceeding 450^{o}C, cooled in a desiccator and weighed. The percentage of acid insoluble ash was calculated with reference to the air dried drug.

Determination of Water Soluble Ash

The total ash obtained from the previous procedure was mixed with 25mL of water and boiled for 5min in a water bath, and then the insoluble matter was collected in an ashless filter paper and washed with hot water, dried and ignited for 15min at a temperature not exceeding 450^{o}C, cooled in desiccators and weighed. The insoluble matter was subtracted from the weight of the total ash; the difference in weight represents the water soluble ash. The percentage of water soluble ash was calculated with reference to the air dried drug.

The values in respect of the total ash values, acid insoluble ash, water soluble ash and water insoluble ash are tabulated in table 5.

Determination of Extractive Values

Extractive values used to determine the amount of active principle or phyto constituents present in the given amount of plant materials, when extracted with suitable solvents. Determination of extractable matter determines the amount of active constituents extracted with solvents from a given amount of medicinal plant material and herbal formulation. The extraction of crude plant materials with suitable solvents yields a solution containing different phyto constituents. Composition of the phyto constituents in a particular solvent depends upon the nature of drugs and solvents used. This is used to provide preliminary information on the quality of particular sample.

Determination of ethanol soluble extractive

An accurately weighed 5 g of the air dried coarsely powdered drug was macerated with 100mL of ethanol in a closed flask for 24h, shaking frequently during the first 6h and allowed to stand for 18h. Thereafter filtered rapidly, taking precautions against loss

of ethanol. Then evaporate 25mL of the filtrate to dryness in a tarred flat bottomed shallow dish dry at 105^{o}C and weighed. The percentage of ethanol soluble extractive was calculated with reference to the air dried drug.

Determination of water soluble extractive

The procedure adopted for ethanol soluble extractive value was followed for the

determination of water soluble extractive, Chloroform soluble extractive, benzene soluble extractive, acetone soluble extractive value, and ethylacetate soluble extractive value and the reported presented in the table.

Microscopical characters of Powder of *Phyllanthus acidus*

Showe the presence of characters Paracytic stomata, Paraenchyma, collenchyma, Phloem, vessels, fibres.

CHAPTER V
PHYTO CHEMICAL STUDIES

Phytochemical studies are carried out for the separation, purification and identification of the secondary metabolites present in medicinal plants.

Collection and aunthentication

The leaves of *Phyllanthus acidus* was collected in an around Thanjavur and authenticated by taxonomist. The shadow dried leaves were powdered and then subjected to the following preliminary phytochemical studies.

SECTION- A

ORGANOLEPTIC EVALUATION

- **Nature** - Coarse powder
- **Color** - Green
- **Odour** - Characteristic odour
- **Taste** - Bitter followed by astringent taste

SECTION-B

QUALITATIVE CHEMICAL ANALYSIS [45-46]

The various extracts of *Phyllanthus acidus* were subjected to qualitative chemical analysis. in order to detect the presence of steroids, terpenoids, flavones,anthraquinones sugars, quinones,phenols,and tannins . The results were tabulated in table -7

1. Test for sterols

The powdered leaf was first extracted with petroleum ether and evaporated to a residue. Then the residue was dissolved in chloroform and tested for sterols.

a. Salkowski's test

A few drops of concentrated sulphuric acid were added to the above solution, shaken well and set aside. The lower chloroform layer of the solution turned red in color indicating the presence of sterols.

b. Liebermann – burchard's test

To the chloroform solution a few drops of acetic anhydride and 1 mL of concentrated sulphuric acid were added through the sides of the test tube and set aside for a while. At the junction of two layers a brown ring was formed. The upper layer turned green indicating the presence of sterols.

2. Test for carbohydrates

a. Molisch's test

The extract of the powdered leaf was treated with 2-3 drops of 1% alcoholic α-naphthol and 2 mL of concentrated sulphuric acid were added along the sides of the test tube.

A purple color indicating the presence of carbohydrates.

Fehling's test

The extract of the powdered leaf was treated with Fehling's solution I and II and heated on a boiling water bath for half an hour.

Red precipitate was obtained indicating the presence of free reducing sugars.

c. Benedict's test

The extract of the powdered leaf was treated with equal volume of Benedict's reagent A red precipitate was formed indicating the presence of reducing sugar.

3. Test for proteins

a. Millon's test

A small quantity of acidulous – alcoholic extract of the powdered drug was heated with Millon's reagent.

White precipitate turned red on heating indicate the presence of proteins.

b. Biuret test

To one portion of acidulous – alcoholic extract of the powdered drug one ml of 10 % sodium hydroxide solution and one drop of dilute copper sulphate solution were added.

Violet color was obtained indicating the presence of proteins.

4. Test for alkaloids

a. About 2 gm of the powdered material was mixed with 1gm of calcium hydroxide and 5 mL of water into a smooth paste and set aside for 5 minutes. It was then evaporated to dryness in a porcelain dish on a water bath. To this 200 mL of chloroform was added, mixed well and refluxed for half an hour on a water bath. Then it was filtered and the chloroform was evaporated. To this 5 mL of dilute hydrochloric acid was added followed by 2 mLof each of the following reagents.

a) Mayer's test:

To small quantity of the extract Mayer's reagent was added. No cream colour precipitate indicates the absence of alkaloids.

b) Dragen dorff"s tesr

To small quantity of the extract Dragendorff's reagent was added. No Orange brown precipitate indicates the absence of alkaloids.

c) Wagner's test:

To small quantity of extracts Wagner's reagent was added . No Reddish brown precipitate indicates the absence of alkaloids.

d) Hager's test:

To small quantity of extract Hager's reagent was added . No Yellow precipitate indicates the absence of alkaloids.

e) Test for purine group (murexide test)

The residue obtained after the evaporation of chloroform as described in (a) was treated with 1mL of hydrochloric acid in a porcelain dish and 0.1 gm of Potassium chlorate was added and evaporated to dryness on a water bath. Then the residue was exposed to the vapour of dilute ammonia solution. No purple Color was obtained indicating the absence of purine group of alkaloids.

5. Test for glycosides

a) Borntrager's test

The powdered leaf was boiled with dilute sulphuric acid, filtered and to the filtrate benzene was added and shaken well. The organic layer was separated to which ammonia solution was added slowly.

Ammoniacal layer showed the pink colour indicating the presence of anthraquinone glycosides.

b) Modified borntrager's test

About 0.1g of the powdered drug was boiled for 2 minutes with dilute hydrochloric acid and few drops of ferric chloride solution, filtered while hot and cooled. The filtrate was then extracted with benzene and the benzene layer was separated. Equal volume of dilute ammonia solution was added to the benzene extract.

Pink color was observed in ammoniacal layer showing the presence of anthraquinone glycosides.

c) Test for cardiac glycosides(for deoxysugar)

i) Keller kiliani test

About 1g of the powdered leaf was boiled with 10 mL of 70 % alcohol for 2 minutes, cooled and filtered. To the filtrate 10 mL of water and 5 drops of solution of lead subacetate were added and filtered, evaporated to dryness. The residue was dissolved in 3 mL of glacial acetic acid. To these 2 drops of ferric chloride solution was added. Then 3 mL of concentrated sulphuric acid was added to the sides of the test tube carefully and observed.

Reddish brown layer was observed indicating the presence of deoxysugars of cardiac glycoside

d) Test for cyanogenetic glycosides

Small quantity of the powder was placed in a stoppered conical flask with just sufficient water, to cover it. A sodium picrate paper strip was inserted through the stopper so that it was suspended in the flask and it was set aside for 2 hours in a warm place.

No brick red color was produced on the paper indicating the absence of cyanogenetic glycosides.

6. Test for saponins

About 0.5 g of the powdered drug was boiled gently for 2 minutes with 20mL of water and filtered while hot and allowed to cool. 5 mL of the filtrate was then diluted with water and shaken vigorously. Frothing was produced indicating the presence of saponins.

7. Test for tannins

A small quantity of the powdered drug was extracted with water. To the aqueous extract few drops of ferric chloride solution was added.

Bluish black color was produced indicating the presence of tannins.

8. Test for flavonoids

a. Shinoda's test

A little of the powdered drug was heated with alcohol and filtered. To the test solution magnesium turnings and few drops of concentrated hydrochloric acid were added. Boiled for five minutes.

Red colour was obtained indicating the presence of flavonoids.

b. Alkali test

To the small quantity of test solution 10 % aqueous sodium hydroxide Solution was added. Yellow orange color was produced indicating the presence of flavonoids.

c. Acid test

To the small quantity of test solution, few drops of concentrated sulphuric acid were added. Yellow orange colour was obtained indicates the presence of flavonoids.

9. Test for terpenoids

A little of the powdered leaf was extracted with chloroform and filtered. The filtrate was warmed gently with tin and thionylchloride. Pink color solution appeared which indicated the presence of terpenoids.

10. Test for the absence of volatile oil

Weighted quantity (250 gm) of fresh leaves were extracted and subjected to hydro distillation using volatile oil estimation apparatus.

Volatile oil was not obtained indicating the of absence of volatile oil.

11. Test for mucilage

Few ml of aqueous extract prepared from the powdered crude drug was treated with ruthenium red. Red colour was produced indicating the presence of mucilage.

SECTION – C

QUANTITATIVE ESTIMATION OF PHYTO-CONSTITUENTS

In quantitative estimations, Secondary metabolites like phenolic compounds tannin and flavonoids present in the extracts were quantified using standard Procedure reference marker compounds.

Preparation of extracts

Preparation of ethanolic extract of *Phyllanthus acidus*

The air dried powdered plant material of leaves of *Phyllanths acidus* was passed through seive no- (10/40) Coarsely powdered Plant material was defatted with petroleum ether (60-80˚c) for three days by triple maceration. The defatted marc was extracted with 70% ethanol by triple maceration and filtered. The filtrate was concentrated under reduced pressure to obtain a solid residue which was dark green in colour.

I) Determination of total phenolic Content[47-48]

Phenolic compounds comprises of Secondary metabolites like tannins, anthraquinones, flavonoids and coumarins. These compounds have at least one hydroxyl group attached to a benzene ring. Phenolic compounds have been reported to have multiple pharmacological effects, including antioxidant activity.

Principle

All the phenolic compounds are oxidized by the Folin-Ciocalteu Reagent. This reagent, which is reduced during oxidation of the phenolic substances, into a mixture of blue molybdenum and tungsten oxides.

The blue colour produced has a maximum absorption at about 750-760 nm. The absorption is proportional to the quantity of oxidized phenolic compounds.

Instrument

Shimadzu UV Visible spectrophotometer, Model 1800

Reagents required

a) **Folin Ciocalteu Reagent (1N)**

The Folin Ciocalteu reagent is a mixture of phosphomolybdate and phosphotungstate. Commercially available Folin Ciocalteu Reagent (2N) was diluted with an equal volume of distilled water. The resultant solution was kept in a brown color bottle and stored in refrigerator at 4 ºc.

b) **Sodium carbonate solution (10 %)**

c) **Standard gallic acid solution.**

Procedure

About 1mL (1 mg/ mL and o.5 mg/mL) of ethanolic extracts of EEPA and 0.5ml of Folin Ciocalteu reagent (1N) were added and allowed to stand for 15 minutes. Then 1mL of 10% sodium carbonate solution was added to the above solution. Finally the mixtures were made upto 10 mL with distilled water and allowed to stand for 30 minutes at room temperature and total phenolic content was determined by spectrophotometrically at 760 nm wavelength.

The calibration curve was generated by preparing Gallic acid at different concentrations (2, 4, 6, 8, and 10µg/mL). The reaction mixture without sample was used as blank. Total Phenolic content is expressed in terms of mg of Gallic acid equivalent per gm of extract (mg GAE/g). The results are tabulated in table(9) and the calibration graph was presented.(FIG.20)

II) Total flavonoid determination [49-44]

The different classes of flavonoid are catechins, leucoanthocyanidins, flavanones, flavones, anthocyanidins, flavonols, chalcones, aurones and isoflavones. They are reported to have various pharmacological activities like antioxidant, antiiflammatory and anticancer.

Principle

The aluminum chloride colorimetric technique was used for estimation of total flavonoid estimation. Aluminum ions form stable complexes with C_4 keto group and either to C_3 or C_5 hydroxyl groups of flavones and flavonols in acidic medium. It also forms acid labile complexes with ortho hydroxyl groups in the A or B rings of flavanoids. These complexes showed a strong absorption at 415nm which is used for the estimation of flavonoids.

Instrument

Shimadzu UV Visible spectrophotometer, Model 1800

Reagents required

- ✓ 10% aluminum chloride
- ✓ 1M potassium acetate

Procedure

1mL(Img/ImL and 0.5mg/mL) of ethanolic extracts of EEPA was taken and 0.1mL of aluminum chloride solution, 0.1mL of potassium acetate solution and 2.8mL of ethanol were added and the final volume was then made up to 5mL with distilled water. After 20 min the absorbance was measured at 415nm.

A calibration curve was constructed by plotting absorbance reading of quercetin at different concentrations. The sample without aluminium chloride was used as a blank. The total flavonoid content in the extract was expressed as milligrams of quercetin equivalent per gram of extract and the results are tabulated (10)(FIG.21)

Total tannin determination [50-51]

Principle

Total tannin content of extract was determined by Folin Denis reagent method.

Tannin like compounds reduces phosphotungstomolybdic acid in alkaline solution to produce a highly coloured blue solution. The intensity of which is proportional to the amount of tannins and it was estimated by spectrophotometer at 700 nm.

Instrument

Shimadzu UV Visible spectrophotometer, Model 1800

Reagents required

a) Folin Denis Reagent (sodium tungstate 100 g and phospho molybdic acid20gwere dissolved in distilled water 750 ml along with phosphoric acid 50 ml.

b) The mixture was refluxed for 2 hours and volume was made upto 1 litre with distilled water)

c) Sodium carbonate solution (10%)

d) Standard tannic acid solution.

Procedure

0.2mL of (1mg/mL) of ethanolic extract of EEPA was made upto1ml with distilled water. Then add 0.5 mL of Folin Denis reagent and allowed to stand for 15 mins, then 1mL of sodium carbonate solution was added to the mixture and it was made upto 10mL with distilled water. The mixture was allowed to stand for 30mins at room temperature and the tannin content was determined spectrophotometrically at 700 nm.

The calibration curve was generated by preparing tannic acid at different concentration (4, 8, 12, 16, 20µg/ml). The reaction mixture without sample was used as blank. The total tannin content in the leaf extract was expressed as milligrams of tannic acid equivalent per gm of extract. The results were tabulated in table 11. (FIG 22.)

SECTION –D

Identification and estimation of Apigenin in EEPA by HPLC

HPLC studies of EEPA was carried out in order to identify and quantify the active principles present in the extract.[52]

Materials and Methods:

Instrument	:	Agilent technologies 1220 infinity LC (USA)
Coloumn	:	Agilent Zorbax SB –C 18
Flow rate	:	1ml/minutes
Injection volume	:	10 µL
Detector	:	UV-362 nm
Sample	:	EEPA (0.1gm in 100 mLof methanol)
Standard	:	Apigenin (5 mg in 100 mL of methanol)
Temperature	:	28 ºC
Mobile phase	:	Methanol:acetonitrite:water (60:35:5)

The pH of the mobile phase was a adjusted to pH 2.8 with the addition of phosphoric acid

Soft ware : Open LAB ,CDS Chemistation work station VL

Linearity graph was genareted by plotting the different concentration of the standard in the X axis (2.07, 4.13, 6.20, 8.26 ,10.33µg/mL)and the area of the peak in Y axis .The observed area of the standard was presented in the fig (23-29)

The linear regression equation was calculated by Y= 101.4893 X 4.5210 and R^2= 0.9999.The result of the standard and sample chromatogram are presented in fig(23-29)

CHAPTER-VI

PHARMACOLOGICAL SCREENING

SECTION- A

ANTIOXIDANT ACTIVITY.

Plant derived antioxidants such as tannins, lignans, stilbenes, coumarins, quinones, xanthones, phenolic acids, flavones, flavonols, catechin, anthocyanins and proanthocyanins could delay or prevent the onset of degenerative diseases because of their redox properties which allow them to act as hydrogen donors, reducing agents, hydroxyl radical scavengers and nitric oxide scavengers.

Hydroxyl radical scavenging activity, β-Carotene – linoleic acid assay, DPPH method, Superoxide radical scavenging activity, Nitric oxide radical inhibition assay, Reducing power method, Phosphomolybdenum method, Peroxy nitrile radical scavenging activity, Xanthine oxidase method, Ferric reducing ability of Plasma and Thiobarbituric acid assay are some of the methods for the evaluation of antioxidant activity.[53-54]

Free radical scavenging activity using diphenyl picryl hydrazyl (DPPH) method [55-56]

Principle

Total antioxidant assays are used to compare the antioxidant activities of different molecules. DPPH is a stable free radical with a distinctive ESR signal. Its reaction with antioxidants can be followed by the loss of absorbance at 517 nm wavelength. It is widely accepted that DPPH can accept an electron or hydrogen radical to become a stable diamagnetic molecule. Due to its odd electron, the ethanol solution of DPPH, which is purple in colour shows a strong absorption at 517 nm. DPPH radicals react with suitable

reducing agents and then electrons become paired off and the solution loses colour stochiometrically with the number of electrons taken up.

$$DPPH^{\cdot} + AH \rightarrow DPPH\text{-}H + A^{\cdot}$$

Instrument

Shimadzu UV Visible spectrophotometer, Model 1800

Reagents

0.1mM diphenyl picryl hydrazyl in ethanol

Procedure

A stock solution of DPPH was prepared in 100mL of ethanol. To the 1ml of test samples of different concentrations, 4mL of DPPH was added. Control without test compound was prepared in an identical manner. In case of blank, DPPH was replaced by ethanol. The reaction was allowed to be completed in the dark for about 30min. Then the absorbance of test mixtures was read at 517 nm wavelength. The percentage inhibition was calculated and expressed as percent scavenging of DPPH radical. Vitamin C was used as standard.

The percentage scavenging was calculated using the formula [(Control-Test)/Control] x 100 and the concentration of the sample required for 50% reduction in absorbance (IC_{50}) was calculated using linear regression analysis.

Determination of scavenging activity against hydrogen peroxide [57]

Principle

The radical scavenging activity against hydrogen peroxide of plant extract was determined by using standard procedure .The principle is based the capacity on the extracts to decompose the hydrogen peroxide to water.

Instrument

Shimadzu UV Visible spectrophotometer, Model 1800

Reagents

6% Hydrogen peroxide diluted with water in the ratio of 1:10

0.1M, pH 7.4 phosphate buffer .

Procedure

To the 1mL of test solutions at different concentrations, 3.8mL of 0.1M phosphate buffer solution (pH 7.4) and 0.2mL of hydrogen peroxide solution were added. The absorbance of the reaction mixture was measured at 230 nm wave length after 10min. The reaction mixture without sample was used as blank. Sample blank was also prepared without reagents. Ascorbic acid was used as standard. The percentage inhibition of hydrogen peroxide was calculated using the formula

$$\% \text{ inhibition} = (A_{control} - A_{sample}) / A_{control} \times 100$$

The concentration of the sample required for 50 % reduction in absorbance (IC_{50}) was calculated using linear regression analysis.The results and presented in table 13.

SECTION- B

ANTICANCER EFFECT OF EEPA AGAINST DEN INDUCED HEPTOCELLULAR CARCINOMA IN RATS.

Materials and methods

CPSCEA approval

The necessary approval for carrying out the study was obtained from the Animal Ethical Committee, K.M Collage of Pharmacy, Uthankudi ,Madurai.

Animals

Wistar albino rats weighing 180-200 g were used for the study. The animals were housed individually in polypropylene cages. Animals were acclimatized under standard laboratory conditions at 25± 2 °C and normal photoperiod (12h light: dark cycle). They were fed on normal pellet diet and water *ad libitum*.

Chemicals and Reagents

- Diethylnitrosamine (DEN)
- Tris HCL buffer
- Thiobarbituric acid (TBARS)
- 5,5 Dithiobis -2-nitro benzoic acid (DTNB)

Diethylnitrosamine (DEN) was purchased from Sigma Chemicals and it was dissolved in normal saline and injected to rats as a single dose (200 mg/kg, *i.p*) to initiate hepatic carcinogenesis.

Experimental protocol

The animals were divided into 4 groups and each group consists of six animals(n=6). Group I animals were administered 1% carboxy methyl cellulose(CMC) for 7 consecutive days by oral route. On the fifth day of experiment a single dose of DEN (200 mg/kg /i.p) was administered to DEN control (Group-II) animals. Group-III and Group-IV animals were administered with EEPA (as a suspension in 1% CMC) at the dose of 300 mg/kg b.w and 400 mg/kg b.w respectively for 7 consecutive days by oral route. Group- III and Group- IV animals were also administered a single dose of DEN (200mg/kg */i.p.*) on the fifth day of the experiment.

Experimental Design:

Group	Treatment
I-Control	Rats were administered 1ml of 1% CMC through oral route for 7 consecutive days.
II-DEN Control	Rats were administered a single dose of DEN (200mg/kg, *i.p*) on the fifth day of the experiment.
III -DEN + Extract(300 mg/kg/oral)	Rats were administered EEPA suspension (300 mg/kg b.w *p.o*) for 7 consecuvite days and a single dose of DEN(200mg/kg,*i.p*) was administered on the fifth day of the experiment.
IV-DEN+ Extract(400 mg/kg/oral)	Rats were administered EEPA suspension (400 mg/kg b.w *p.o*) for 7 consecuvite days and a single dose of DEN(200mg/kg,*i.p*) was administered on the fifth day of the experiment.

At the end of the experiment, Blood sample was withdrawn for biochemical estimation from the retro orbital plexus and the animals were sacrificed by cerivical dislocation (as per CPCSEA guidelines) and the liver was removed and used to study the biochemical ,antioxidant and histopathological parameters.

PARAMETERS TO BE EVALUATED

Serum biochemical parameters

- ✓ Aspartate amino transaminase (AST)
- ✓ Alanine transaminase (ALT)
- ✓ Alkaline phosphatase (ALP)
- ✓ Totalbilirubin and total protein
- ✓ Lactate dehydrogenase(LDH)

Estimation of *in-vivo* antioxidants in liver tissue homogenate

- ❖ Thiobarbituric Acid Reactive Substances(TBARS)
- ❖ Reduced Glutathione(GSH)

Histopathology of liver

ESTIMATION OF *IN-VIVO* ANTIOXIDANTS

Preparation of tissue homogenate

The isolated liver was washed and blotted in a filter paper. A 10 % tissue homogenate was prepared by using chilled 0.15 M Tris Hcl (PH 7.4) buffer and centrifuged at 5000 rpm for 5 minutes. The clear supernatant liquid was used for the estimation TBARS, GSH and total protein.

Estimation of Lipid Peroxidation (TBARS)[58]

Lipid peroxidation was estimated by using standard procedure method described by Rajkumar *et al.*

To 1mL of tissue homogenate, 2.5mL of trichloroacetic acid 20 %(TCA) was added and centrifuge about 2.5mL of 0.05M sulphuric acid and 3.5mL th iobarbituric acid were added to the supernatant liquid and incubated at 37^0 C for 30 minutes. It was extracted with n-butanol. Intensity of the chromogen in the layer was measured at 530 nm wavelength using uv spectrophotometer.

Estimation of reduced glutathions [59]

Reduced glutathione was estimated by using standard procedure.

1ml of tissue homogenate was precipitated with 1ml of 10 % TCA and was centrifuged to remove the precipitate. 0.5 ml of the supernatant , 2ml of 0.6 mM 5,5 Dithiobis -2-nitro benzoic acid(DTNB) in 0.2 M sodium phosphate was added and the total volume was make upto 3ml with 0.2M phosphate buffer(pH 8). The absorbance was read at 412 nm wavelength.

Histopathological studies

Statistical analysis

All the datas were expressed as standard error of mean (SEM). Data of biochemical parameters were analysed using one way ANOVA followed by Tukey's multiple Kramer Comparison test. A value of $p<0.05$ was considered to be statistically significant.

CHAPTER VII

RESULT AND DISCUSSION

PHARMACOGNOSTICAL STUDIES

Macroscopic studies

The shape of the leaves are ovate. It is about 9 cm in length and 4cm in width. It bears entire margin and acute apex . It has reticulate venation . Dorsal surface of the leaves showed dark green colour and the ventral surface bears light green colour.Taste is astringent and it has characteristic odour.

MICROCSOPICAL STUDIES

Leaf

The leaf consist of a thick plano-convex midrib and thin lamina (fig.9) The midrib is slightly raised on the adaxial side and prominently semi circular on the abaxial side. The midrib is 580 µm thick and 750 µm wide. The epidermal layer of the midrib consists of squarish , thick walled cells. The ground tissue consists of angular thin walled compact parenchyma cells (fig.10). The vascular strand is wide and bowl shaped; it is collateral and includes several short parallel radial lines of xylem elements and thin continuos layer of abaxial phloem strands (fig.10). The xylem strands comprises four or five xylem elements arranged in radial multiples. The phloem elements are in small groups, distributed along this lower part of the xylem arc (fig11) .The meta xylem elements are about 15µm in diameter .

FIG. 9

T.S. of Leaf through Midrib

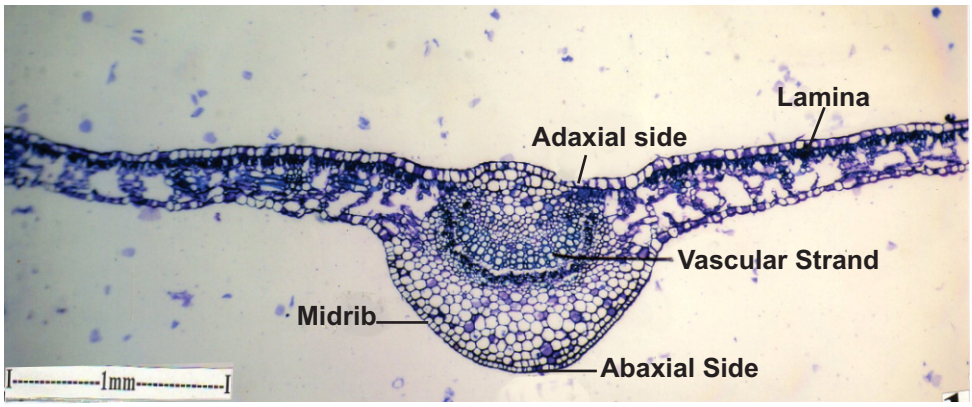


FIG. 10

T.S. of Midrib - Enlarged

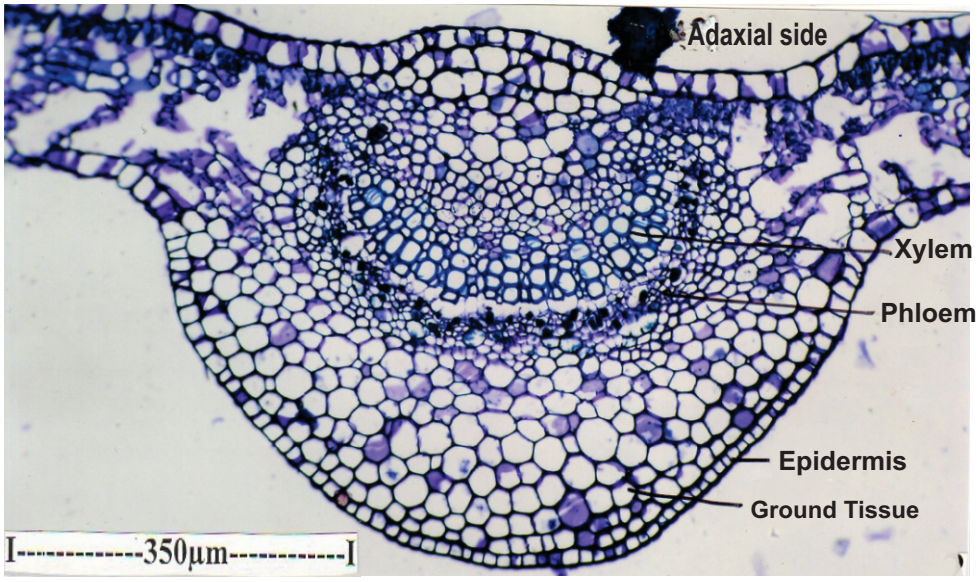


FIG. 11

T.S. of Vascular Bundles of the Midrib

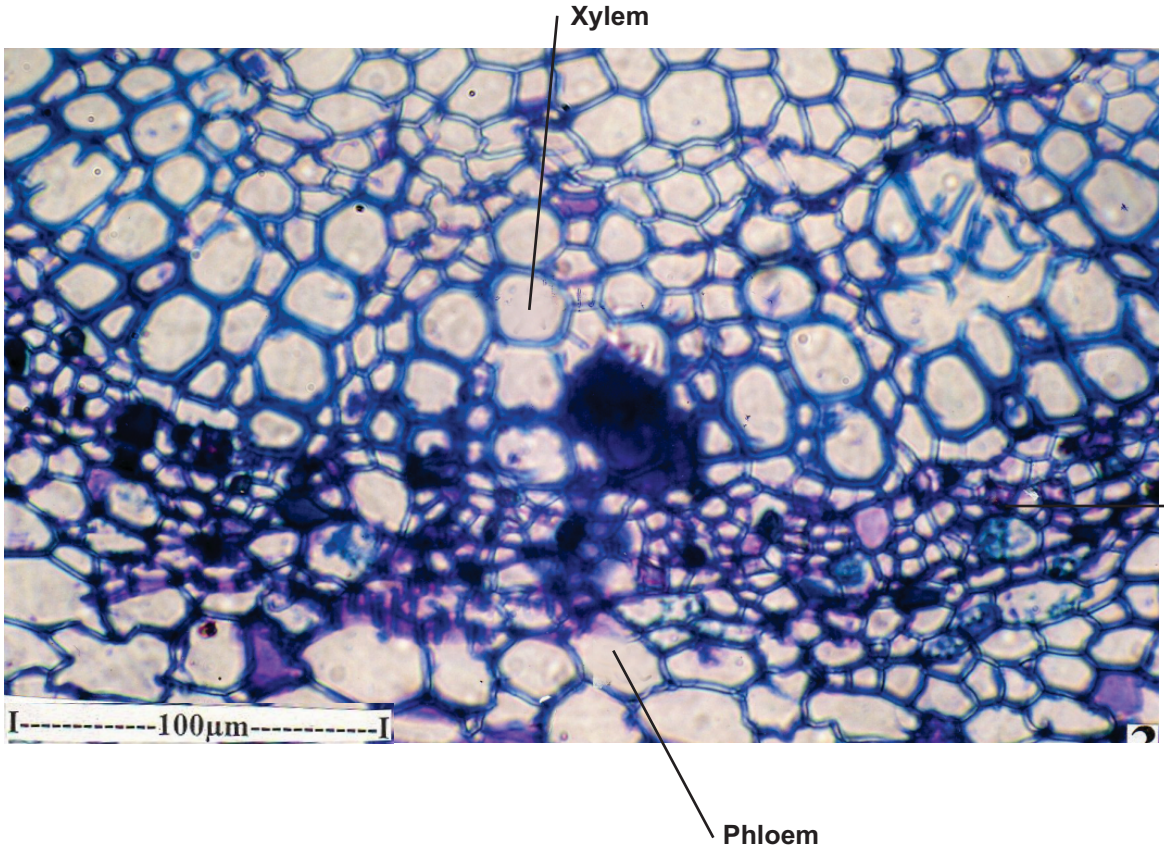


FIG. 12

T.S. of Lamina

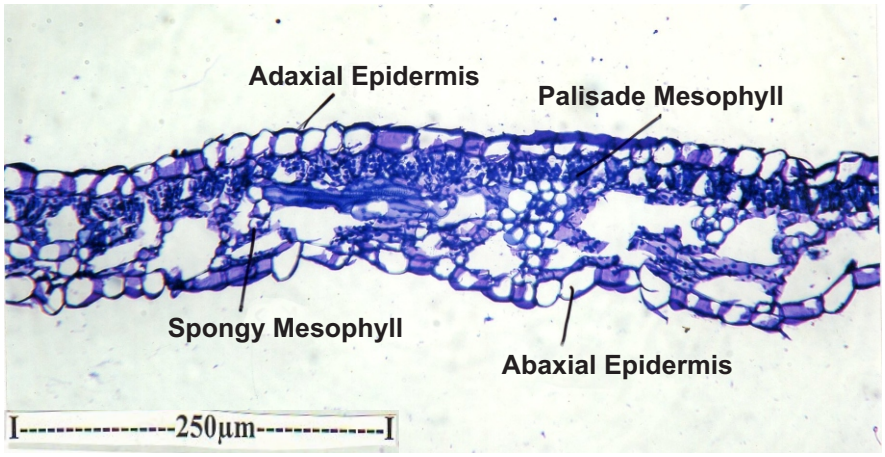


FIG. 13

T.S. of Lamina - Enlarged view

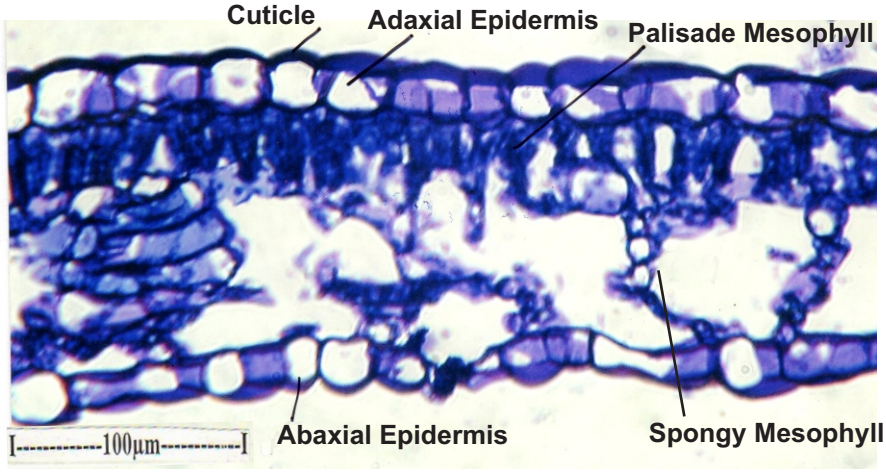


FIG. 14

T.S. of Paradermal section of the lamina showing stomata

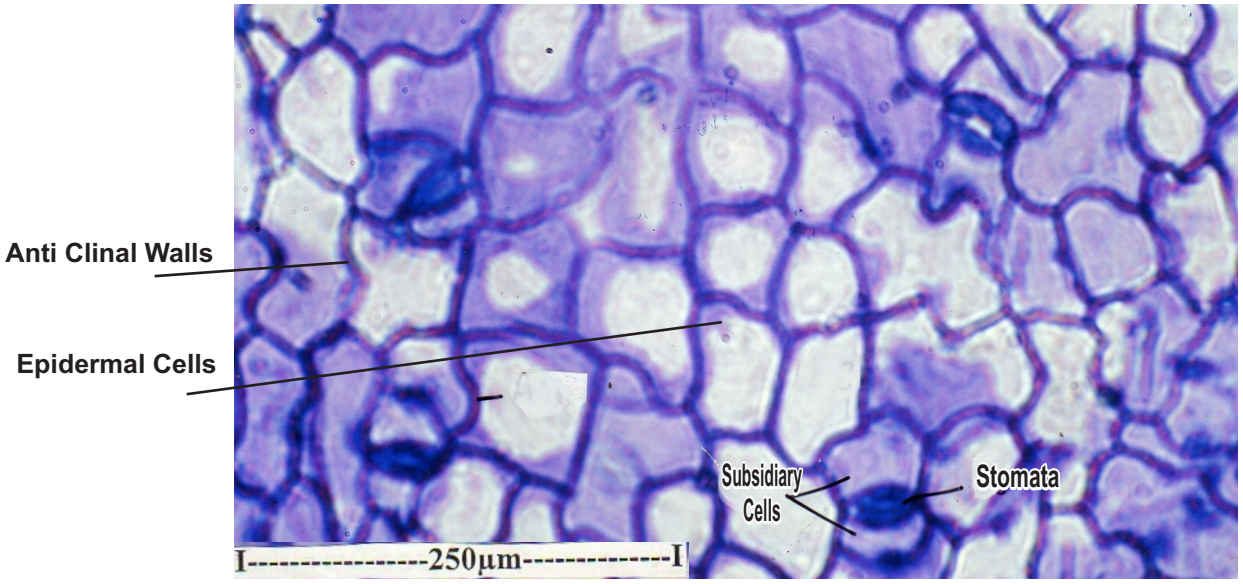


FIG. 15

T.S. of Stomata - Enlarged

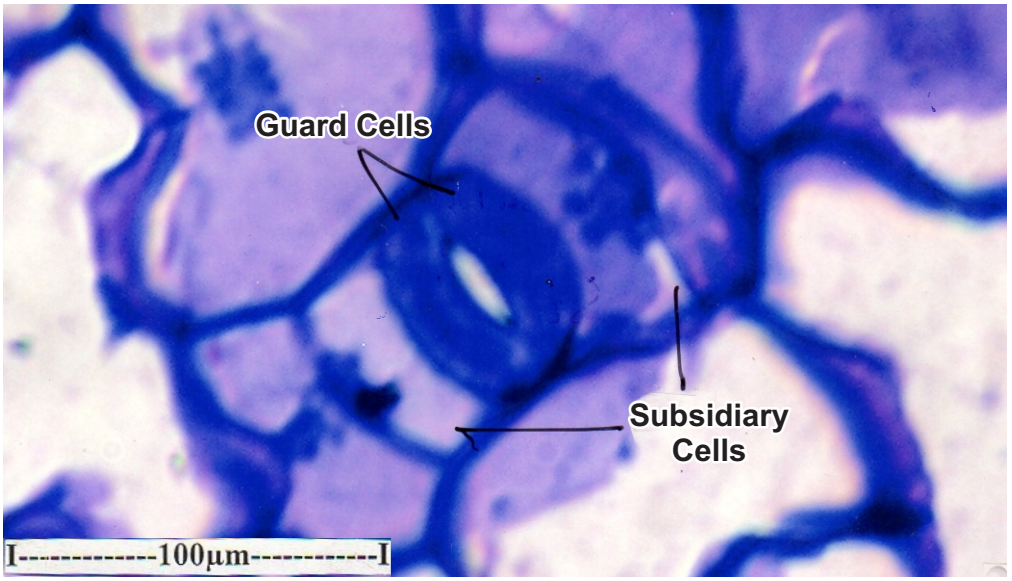


FIG. 16

T.S. of Paradermal section of Adaxial part of the Lamina

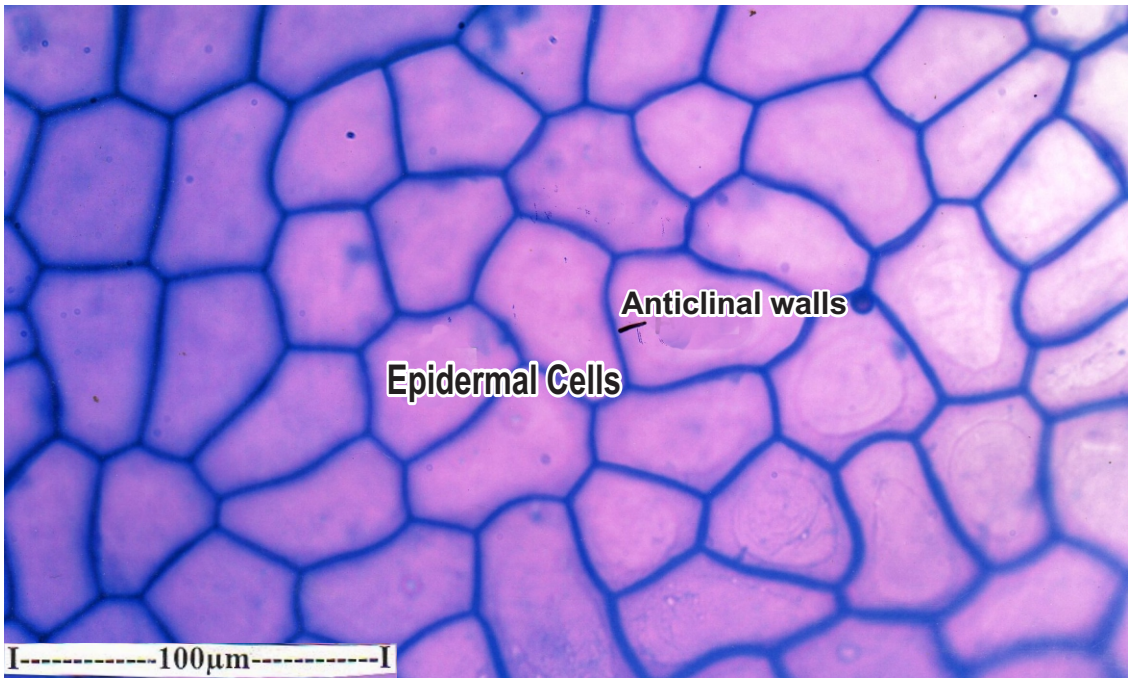


FIG. 17

T.S. of Venation Pattern - Reticulate type

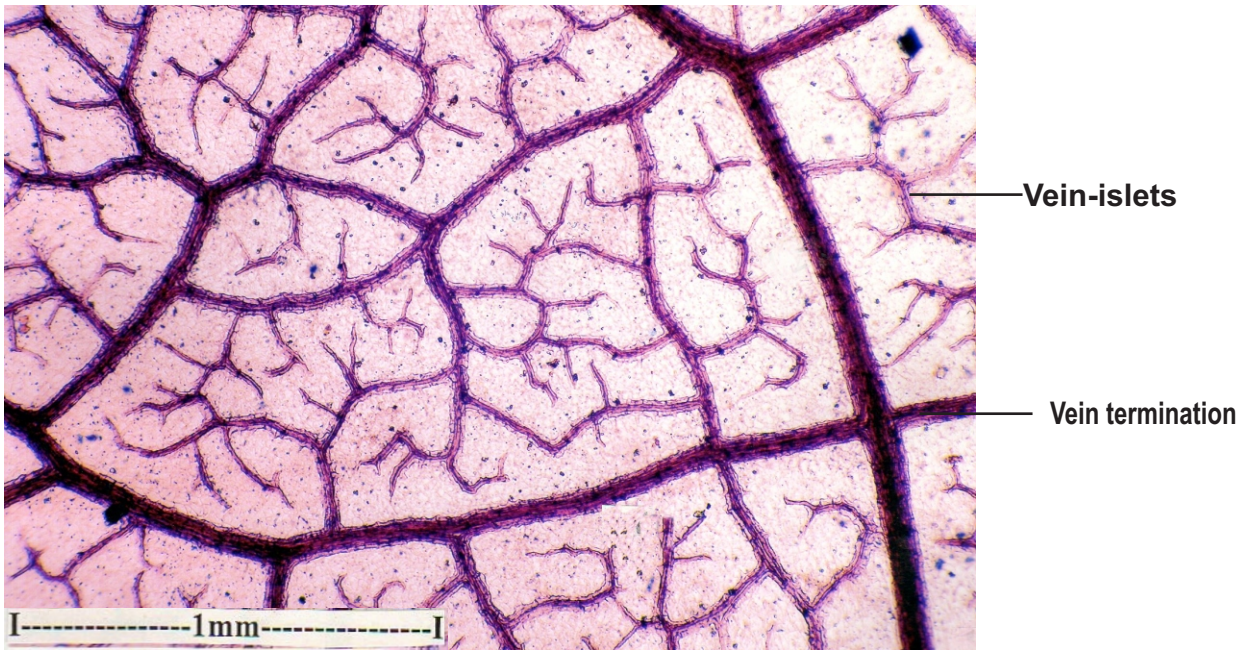
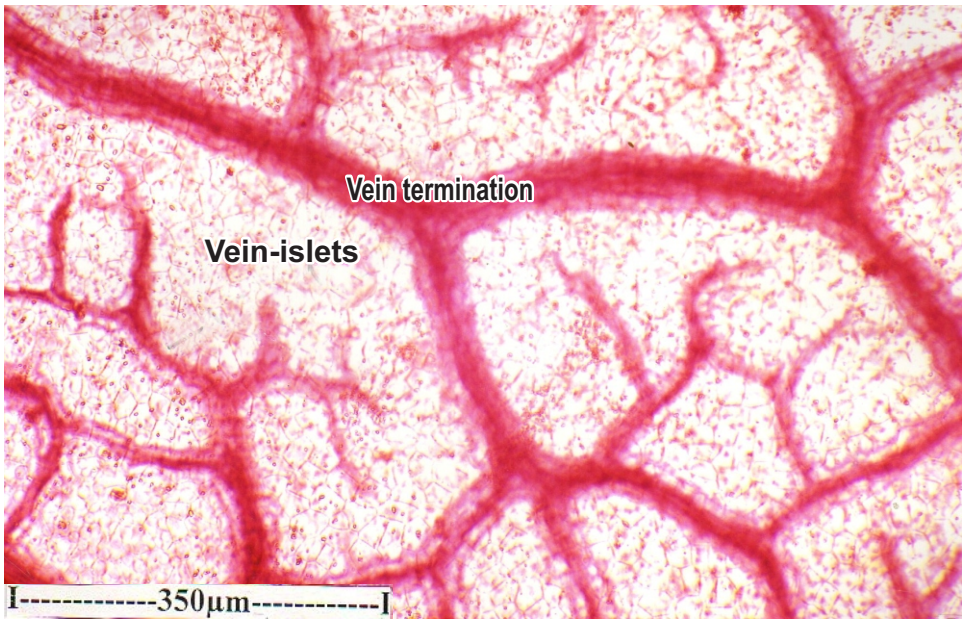


FIG. 18

T.S. of Vein - Islets and Vein Termination Enlarged



Lamina

The lamina is 100µm thick. The adaxial epidermal layer is nearly 20µm thick. The cells are (fig.12,13) squarish, thick walled and have thick cuticle. The abaxial epidermis is slightly thin and the cells are cylindrical or squrish; the abaxial epidermal layer is stomatiferous. The mesophyll tissue is differentiated into adaxial band of a single layer of cylindrical palisade cells. The spongy parenchyma cells are small, spherical or lobed and form wide air spaces (fig.13).

Epidermal Cells And Stomata

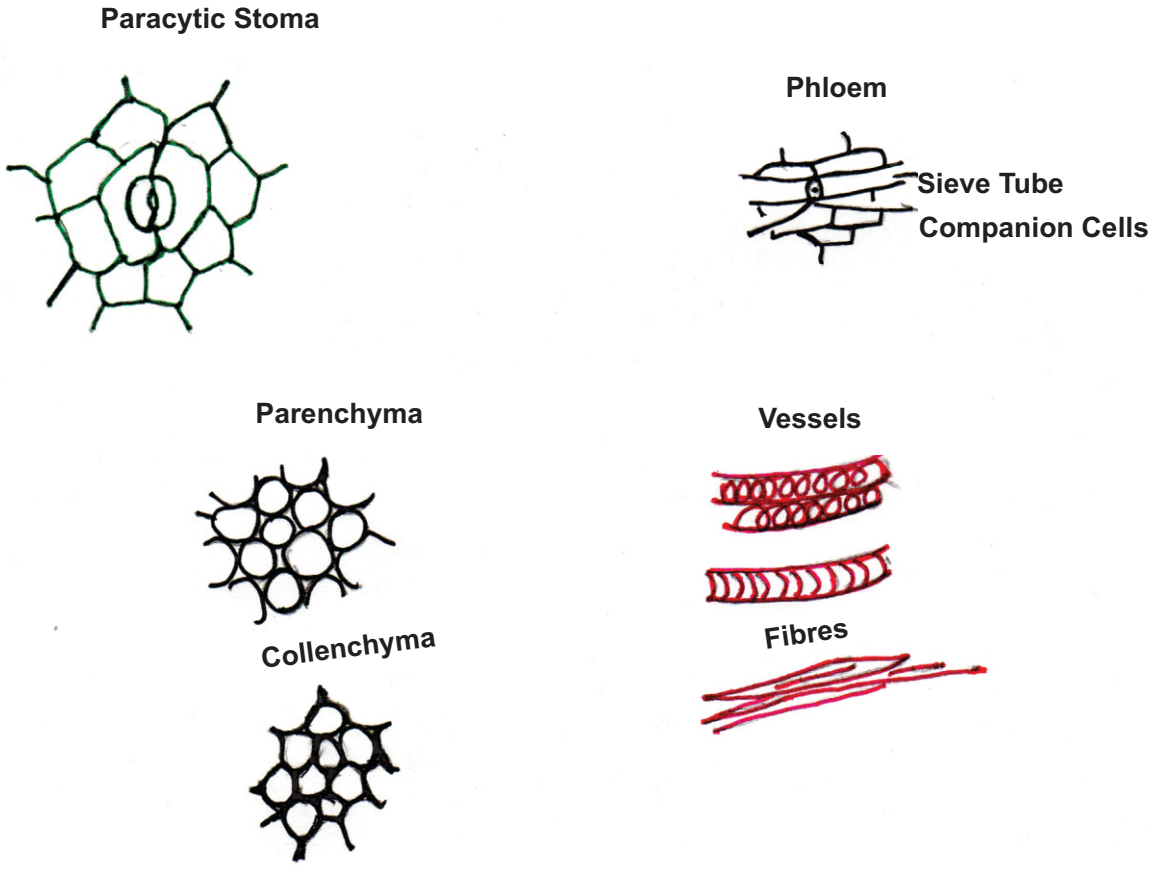
The adaxial epidermal cells are polyhedral in outline. The anticlinal walls are thick and straight. The adaxial epidermis is apostomatic (fig .14,15,16). The abaxial epidermis consists of squarish or polygonal cells. The anticlinal walls are thick, straight or slightly wavy (fig .14). The epidermis is stomatiferous.(fig.16) The stomata are paracytic type. The stomato has two subsdiary cells, which are located, one on either side and paralled to the guard cells (fig.15) The stoma is wide and elliptical is shape.The stomatal pore is narrowly elliptical.The stoma is 40 x 50 µm in size.

Venation pattern

The primary veins are thick and straight. The secondary and tertiary veins become gradually thin and they form distnict vein-islets. The vein-islets are wide and they very in shape and size (fig4:17) In the veins-islets , unbranched or branched once are repeatedly branched and dendroid vein-terminations. The vein terminations are slightly wavy (fig.18) The veins have thin paranchymatous sheath which encloses the veins throughtout their length.

FIG. 19

Microscopical characters of powder of *Phyllanthus acidus*



Leaf- quantitative microscopy

Leaf constants such as stomatal number, stomatal index, vein islet and vein termination were determined for the leaves of this plant and the results were tabulated in table

Table 1 : Quantitative microscopical parameters of the leaf of EEPA

S. No.	Parameters	Values* obtained
1	Stomatal number in upper epidermis	37±0.05
2	Stomatal number in lower epidermis	68±0.62
3	Stomatal index in upper epidermis	19±0.27
4	Stomatal index in lower epidermis	25±0.27
5	Vein islet number	51±1.59
6	Vein termination number	72±1.95
7	Palisade ratio	9±0.27

***mean of three readings ± SEM**

Powder analysis

The behavior of *P.acidus* powder with various chemical reagents is tabulated in table2. It showed the presence of phytostreols, tannins, proteins,starch and compounds.

Table 2: Behavior of the EEPA powder with various chemical reagents

Powder + Reagents	**Colour / Precipitate**	**Presence of active principle**
Picric acid	Yellow precipitate	Protein present
Conc. sulfuric acid	Reddish brown color	Phyto sterols present
Liberman Burchard reagent	Reddish brown color	Phyto sterols present
Aqueous ferric chloride	Greenish black color	Tannins present
Iodine solution	Blue color	Starch present
Mayer's reagent	No cream color	Absence of alkaloids
Spot test	No stain	Fixed oils absent
Sulfosalicylic acid	White precipitate	Protein present

Fluorescence analysis

The organic molecules absorb light usually over a specific range of wave length and many of them emit such radiations. So if the powder is treated with different chemical reagents and seen under UV light, different colours will be produce.(table-3).

Table: 3 Fluorescence analysis of powder of EEPA

Powder +reagent	**Day light**	**UV light (254 nm)**	***UV light (366 nm)***
Drug powder	Green	Green	Brown
Drug powder +aqueous 1M sodium hydroxide	Green	Green	Brown
Drug powder + alcoholic 1M sodium hydroxide	Green	Green	Brown
Drug powder + iodine	Red	Brown	Brown
Drug powder + 50% sulphuric acid	Green	Green	Brown
Drug powder+50% nitric acid	Green	Dark Green	*Brown*

Table: 4 Fluorescence analysis of extracts of EEPA

Extract	**Consistency**	**Colour in Day Light**	**Colour under UV Lamp**	
			366nm	**254nm**
Benzene extract	Semisolid	Yellow	Orange	Yellow
Acetone extract	Semisolid	Yellow	Reddish orange	Green
Ethyl acetate extract	Semisolid	Greenish brown	Green	Greenish - brown
Chloroform extract	Semisolid	Yellowish- brown	Orange	Yellowish brown
Ethanolic extract	Semisolid	Yellowish green	Orange	Green
Aqueous extract	Semisolid	Brown	Green	Dark green

The fluorescence characteristic of the drug powder with different chemical reagent was studied by observing under UV Light at 254 nm and 366 nm. The tests and observations are presented in table . 2 It suggested that a non-fluorescent compound may fluorescent if it mixed with impurities. The results obtained from the present fluorescent studies showed that the leaf powder of *Phyllanthus acidus* is devoid of any impurities.

Loss on drying (LOD)

The results obtained for the determination of LOD for this plant indicate that moisture content is within the limits and presented in table-5

The loss on drying of plant materials should be performed and the water content should also be checked. This is especially important for materials that absorb the moisture easily or deteriorate quickly in presence of water.

Foreign matter

The foreign matter present in the crude plant material of *Phyllanthus acidus* was determined and presented in table-5

Drugs should be free from other than the specified parts, moulds, insects, animal faecal matter and other contaminations such as earth, stones and extraneous material. The amount of foreign matter is not be more than the percentage prescribed in the monograph.

Ash value

Total ash values, acid insoluble ash values and water soluble ash values were determined and presented in table-5. Result of this study showed that ash values are within the limits as prescribed in official texts .The total ash of a crude drug reflects the presence of inorganic salts such as silicates of sodium and potassium salts. The acid insoluble ash is a part of the total ash that is insoluble in dilute hydrochloric acid.

A higher limit of acid-insoluble ash indicates the presence of sand . Water-soluble ash is the water soluble portion of the total ash.

Table: 5 Analytical parameters of EEPA

S. No	Parameters	Values % w/w
1.	**Foreign organic matter**	0.02 ± 0.15
2.	**Moisture content**	3.47 ± 0.02
3.	**Ash values**	
	Total ash	1.77 ± 1.45
	Acid insoluble ash	3.64± 0.45
	Water soluble ash	3.38±0.90

***mean of three readings** ± SEM

Extractive values

Extractive values were determined for the leaves of this plant material and the result are presented in table-6.The result of the study revealed that highest percentage of extractive values were noted as aqueous (19.80%) and hydro-ethanolic(15.56 %) extractives and it indicates the presence of more polar constituents present in the plant material. Table-6.

Table 6: Analytical parameters-Extractive values of EEPA

S.NO	Parameters*	Values* expressed as %
1.	**Extractive Values**	
	Ethyl acetate extract	15.11%
	Acetone extract	13.5%
	Chloroform extract	1.51%
	Hydro-ethanolic extract(Ethanol:water,70:30)	16.56%
	Benzene extract	11.53%
	Aqueous extract	19.80%

PHYTOCHEMICAL SCREENING

Collection, authentication and preparation of extract have been discussed in this chapter. Powdered plant material and various extracts were subjected to preliminary phytochemical studies which indicates the presence of flavonoids, tannins, phenols. The preliminary phytochemical studies also showed that the presence of almost all phytoconstituents viz. flavonoids , phenol, tannins, phytosterols ,triterpenoids and carbohydrates. The 70% ethanolic extract of this drug showed highest extractive value and also showed the presence of therapeutically viable phytoconstituents. Hence it has been chosen for the phytochemical and pharmacological studies.

Table-7 Preliminary phytochemical screening for the leaf powder of EEPA

S.NO	TEST	RESULTS
1.	**Test for sterols**	
	a. Salkowski's test	+
	b. Libermann- burchard's test	+
2.	**TEST FOR CARBOHYDRATES**	
	a. Molisch's test	+
	b. Fehling's test	+
	c. Benedict's test	+
3.	**TEST FOR PROTEINS**	
	a. Millon's test	+
	b. Biuret test	+
4.	**TEST FOR ALKALOIDS**	
	a. Mayer's reagent	-
	b. Dragendroff's reagent	-
	c. Hager's reagent	-
	d. Wagner's reagent	-
	e. Test for Purine group (Murexide test)	-
5.	**TEST FOR GLYCOSIDES**	
	a. Anthraquinone glycosides	
	i) Borntrager's test	-
	ii) Modified Borntrager's test	-
	b. Cardiac glycosides	
	i) Keller Killiani test	-
	c. Cyanogenetic glycosides	-
	d. Coumarin glycosides	
6.	**TEST FOR SAPONINS**	-
7.	**TEST FOR TANNINS**	
	$Fecl_3$ test	+
8.	**TEST FOR FLAVONOIDS**	
	a. Shinoda test	+
	b. Alkali test	+
	c. Acid test	+
9.	**TEST FOR TERPENOIDS**	+
10.	**TEST FOR VOLATILE OILS**	-
11.	**TEST FOR MUCILAGE**	-

(+) indicates positive reaction (-) indicates negative reaction

Table 8: Preliminary Phytochemical Screening for the various extracts of leaf powder of EEPA

Tests	Benzene Extract	Ethyl acetate extract	Chloroform extract	Acetone Extract	Ethanol Extract
1. Test For Sterols					
a. Salkowski's test	+	+	+	+	+
b.Libermann-Burchard's test	+	+	+	+	+
2. **Test for carbohydrates**					
a. Molisch's test	-	-	-	+	+
b. Fehling's test	-	-	-	+	+
c. Benedict's test	-	-	-	+	+
3.**Test for protein**					
a. Millon's test	-	-	-	+	+
b. Biuret test	-	-	-	+	+
3.Test for Alkaloids	-				
a.Mayer's reagent	-	-	-	-	-
b. Dragendroff's reagent	-	-	-	-	-
c. Hager's reagent	-	-	-	-	-
d. Wagner's reagent	-	-	-	-	-
e. Test for purine group (Murexide test)	-	-	-	-	-
4.**Test for glycosides**					
a. Anthraquinone glycosides	-				
i) Borntrager's test	-	-	-	-	-
ii) Modified Borntrager test	-	-	-	-	-
b. Cardiac glycosides	-				
i) Keller Killiani test	-	-	-	-	-
ii) Baljet test	-				-
iii) Cyanogenetic glycosides	-	-	-	-	-
iv) Coumarin glycosides	-	-	-	-	-
VI. Test for Saponins	-	-	-	-	-
VII. Test for Tannins					
i) $FeCl_3$ test	-	-	-	+	+
ii)lead acetate test	-	-	-	+	+
VIII. Test for Flavonoids					
a. Shinoda test	-	+	-	+	+
b. Alkali test	-	+	-	+	+
IX. Test for Terpenoids	+	+	+	+	+

(+) indicates positive reaction (-) indicates negative reaction

Determination of phenolic content

Table 9 Total phenolic content of ethanolic extract of EEPA

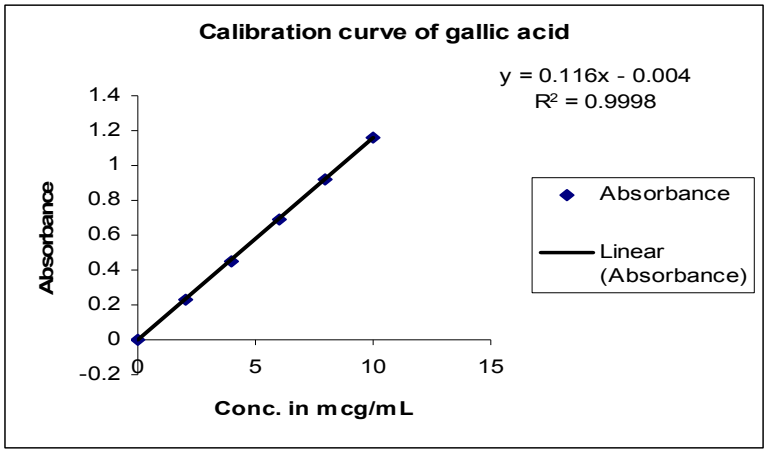
S. No.	Conc. of gallic acid in µg/mL	Absorbance at 760nm	Conc. of ethanolic extract in µg/mL	Absorbance at 760nm*	Amount of total phenolic content in terms mgGAE/g of extract*
1	2	0.229 ± 0.010	50	0.260±0.012	46.39±0.63
2	4	0.452 ± 0.006	100	0.568±0.003	50.3±0.54
3	6	0.695 ± 0.005		**Average**	**48.34±0.5**
4	8	0.918 ± 0.031			
5	10	1.162 ± 0.028			

*** mean of three readings ±SEM**

In the present study, total phenolic content present in EEPA was estimated using modified Folin- ciocalteau method. Values are expressed as gallic acid equivalents and the results were tabulated[60.] The linear regression equation was found to be y=0.116x-0.004 while the correlation was found to be 0.9998. The amount of phenolic content present in the extract in terms of mg GAE/g of extract was found to be **48.34mg/g** by using the above linear regression equation.

Polyphenols are the important secondary metabolites with high level of antioxidant activity. This activity could be due to their ability to absorb, neutralize and to quench free radicals.[57] Their ability to act as free radical scavenger could also be attributed due to their redox properties, presence of conjugated ring structures and carboxylic group which have been reported to inhibit lipid peroxidation. [61]The results indicate that the EEPA showed the significant phenolic content and the phenolic constituents has already been reported for anticancer activity.

.FIG-20Calibratiocurveforgallic acid



Determination of flavonoid content

Table 10 Total flavonoid content of ethanolic extract of EEPA

S. No.	Conc. of quercetin in µg/mL	Absorbance at 415nm	Conc. of ethanolic extract in µg/mL	Absorbance at 415nm	Amt of total flavonoid content in terms of mg quercetin equivalent/ g of extract
1	20	0.589 ± 0.01	50	0.058±0.003	32.38±0.75
2	40	1.151 ± 0.04	100	0.150±0.007	33.16±0.16
3	60	1.710 ± 0.09		**Average**	**32.77±0.4**
4	80	2.390 ± 0.03			
5	100	3.112 ± 0.03			

***mean of three readings ± SEM**

Total flavonoid content for EEPA was found to be **32.77mg/g** . The linear regression equation was found to be y = 0.0307x - 0.0432 while the correlation was found to be 0.9974. Flavonoids play an important role in plants, such as pigmentation, protection against UV-radiation, reproduction, and regulation of plant growth. Flavonoids have shown significant ROS scavenging activity as compared to vitamin C and carotenoids[57] Its antioxidative activity is depends on the chemical structure, such as the number of hydroxyl groups substituted on the B ring. Intake of

flavonoids has been associated with reduced the incidences of cancer, heart disease and various neurological disorders.

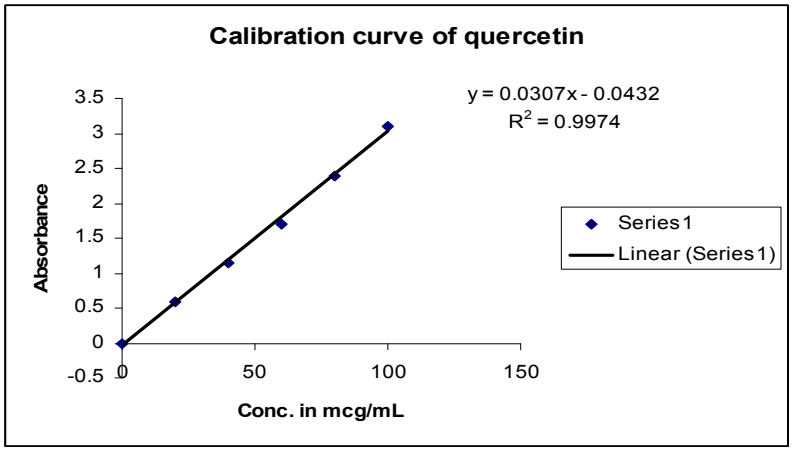


FIG.21: Calibration curve of quercetin

.

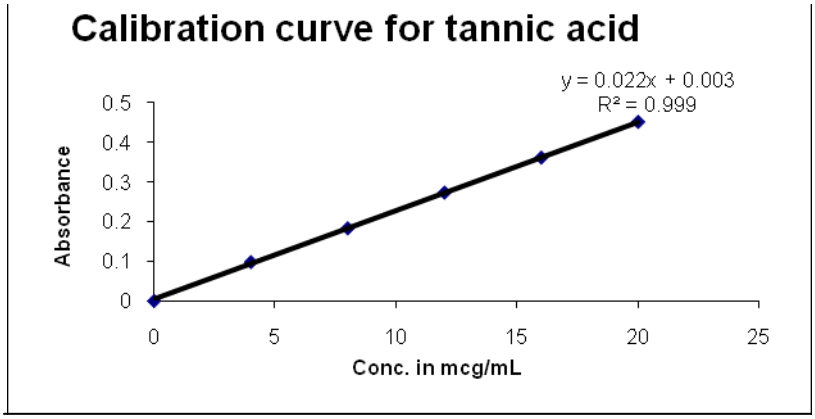
Total Tannin Content:

Table 11 Total tannin content in ethanolic extracts of EEPA in terms of Tannic acid equivalents

S.No.	Conc. of Tannic acid in µg/mL	Absorbance at 700nm	Conc. of ethanolic extract in µg/mL	*Absorbance at 700nm	*Amt of total Tannin content in terms mg tannic acid equivalent/ g of extract
1	20	0.589 ± 0.01	20	0.132±0.04	**197.7±0.12**
2	40	1.151 ± 0.04			
3	60	1.710 ± 0.09			
4	80	2.390 ± 0.03			
5	100	3.112 ± 0.03			
				Average	**98.85±0.06**

Total tannin content of EEPA was found to be **98.85 mg/g** .The linear regression equation was found to be y = 0.022x + 0.003 while the correlation was found to be 0.999. Tannin are antioxidant phytocontituents.

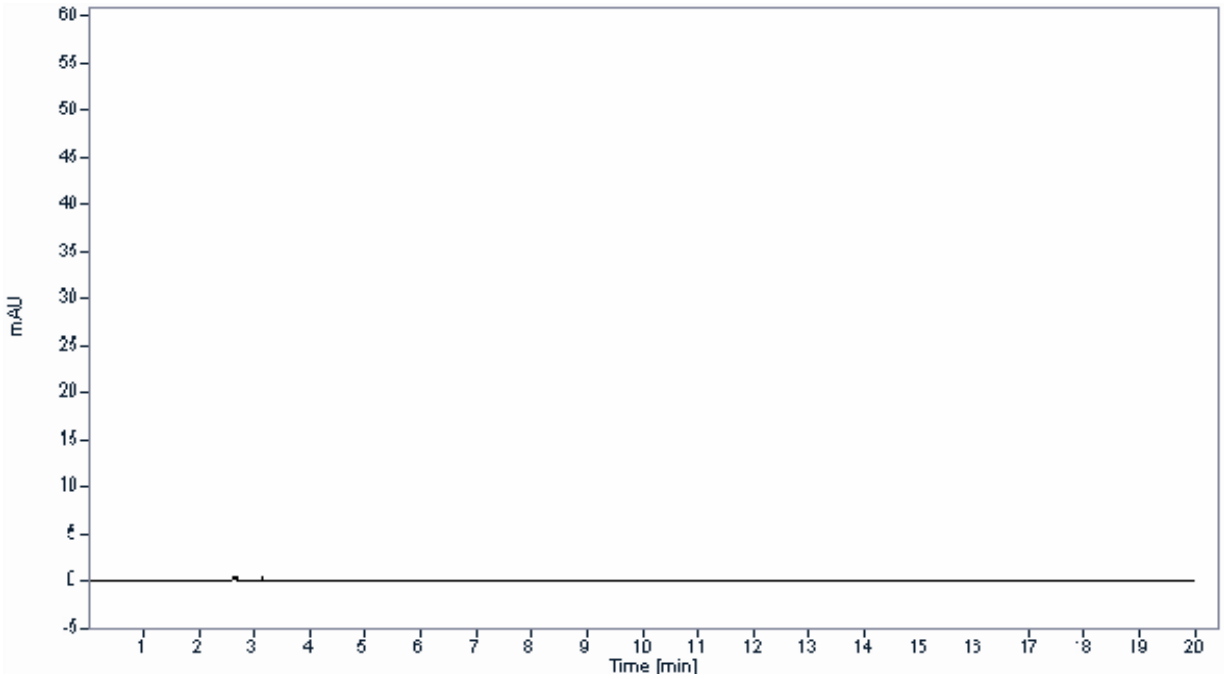
FIG- 22 Calibration curve for Tannic acid



Identification and estimation of apigenin in EEPA by HPLC

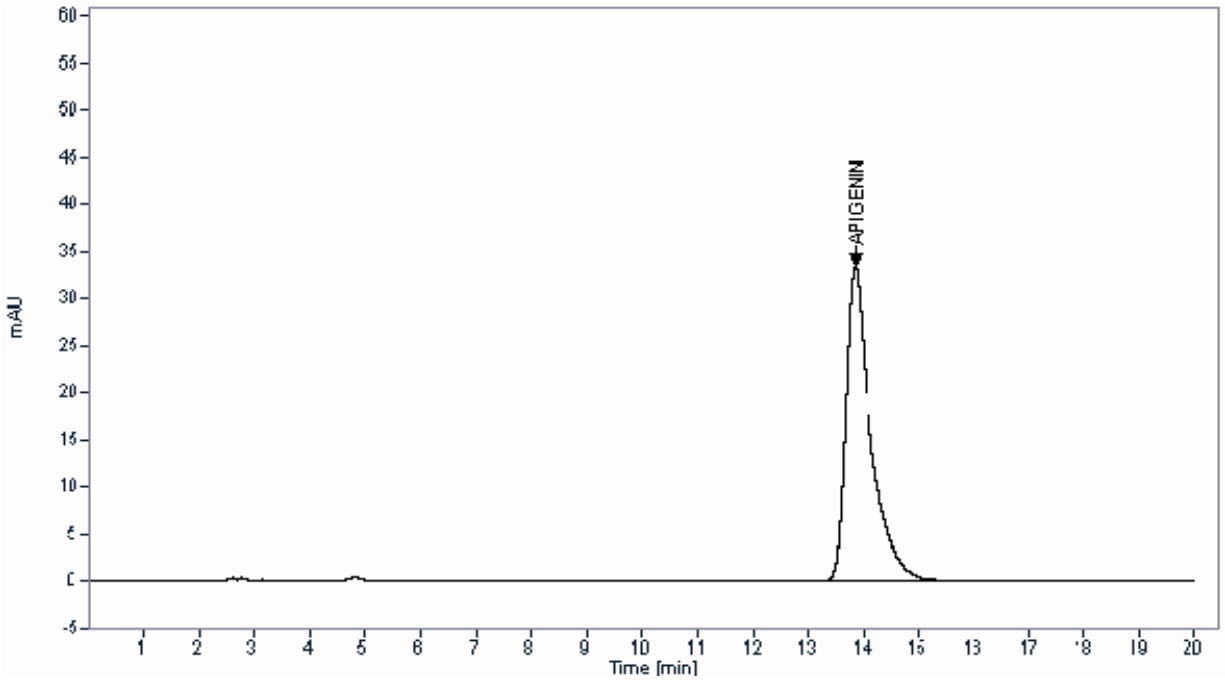
The results of the HPLC chromatogram of standard apigenin and EEPA extract are presented in (FIG.23-29) HPLC chromatogram revealed that the retention time (t_R) of standard apigenin was found to be **13.85 mints** (t_R). The retention of a peak appeared in the chromatogram of EEPA extract was found to be **13.88 mints** (t_R) and it indicates the presence of apigenin in the EEPA extract. The percentage content of apigenin present in the air dried leaf plant material of EEPA was calculated as 0.042% So for apigenin has not been reported in this plant, but it has been reported in other species of this plant.

FIG:23. HPLC chromatogram of EEPA



Name	RT[min]	Area	Area %	TP	TF
	Sum				

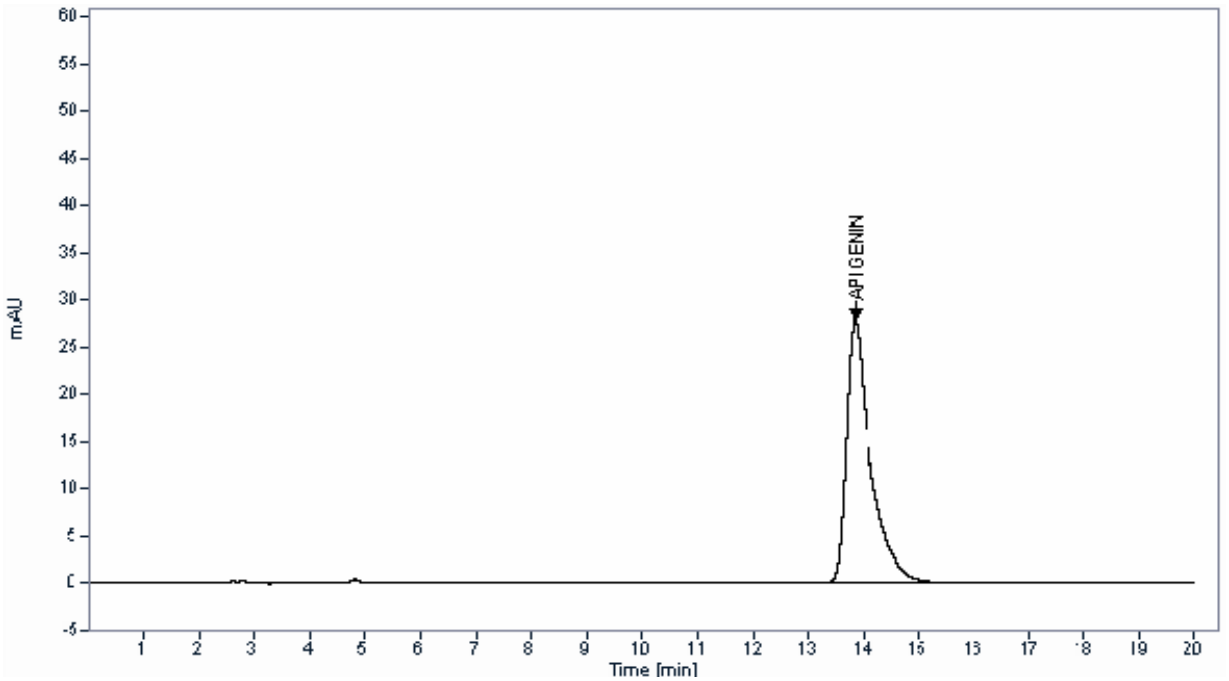
FIG:24. HPLC chromatogram of apigenin(10.33 MCG/ML)



Name	RT[min]	Area	Area %	TP	TF
APIGENIN	13.85	1041.13	100.00	5772	1.73
	Sum	1041.13	100.00		

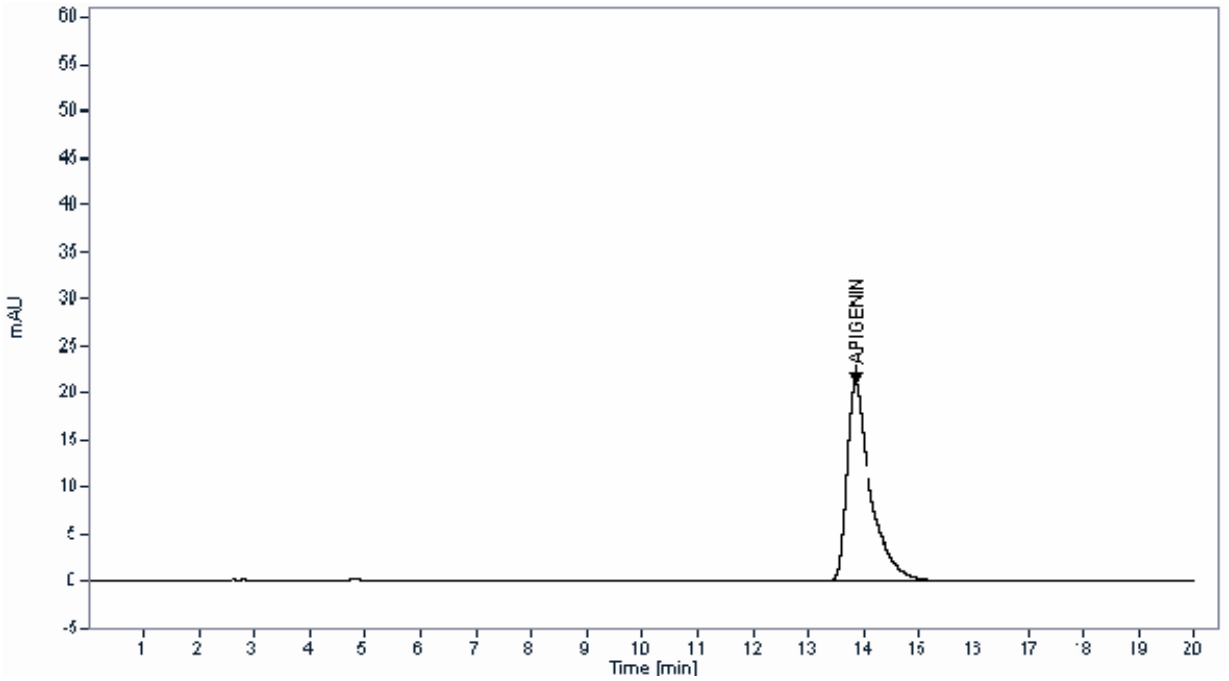
Analysed by: Checked by:

FIG:25. HPLC chromatogram of apigenin (8.26 MCG/ML)



Name	RT[min]	Area	Area %	TP	TF
APIGENIN	13.85	838.12	100.00	6253	1.80
	Sum	838.12	100.00		

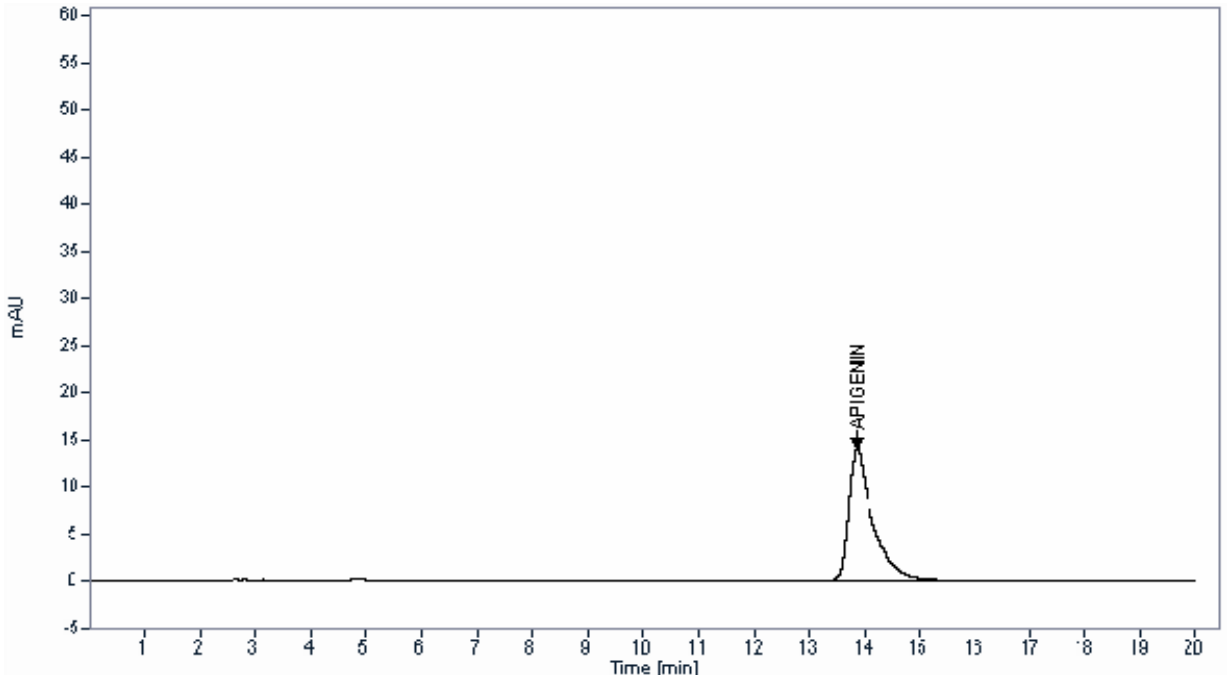
FIG:26 HPLC chromatogram of apigenin(6.20MCG/ML)



Name	RT[min]	Area	Area %	TP	TF
APIGENIN	13.85	624.26	100.00	6435	1.84
	Sum	624.26	100.00		

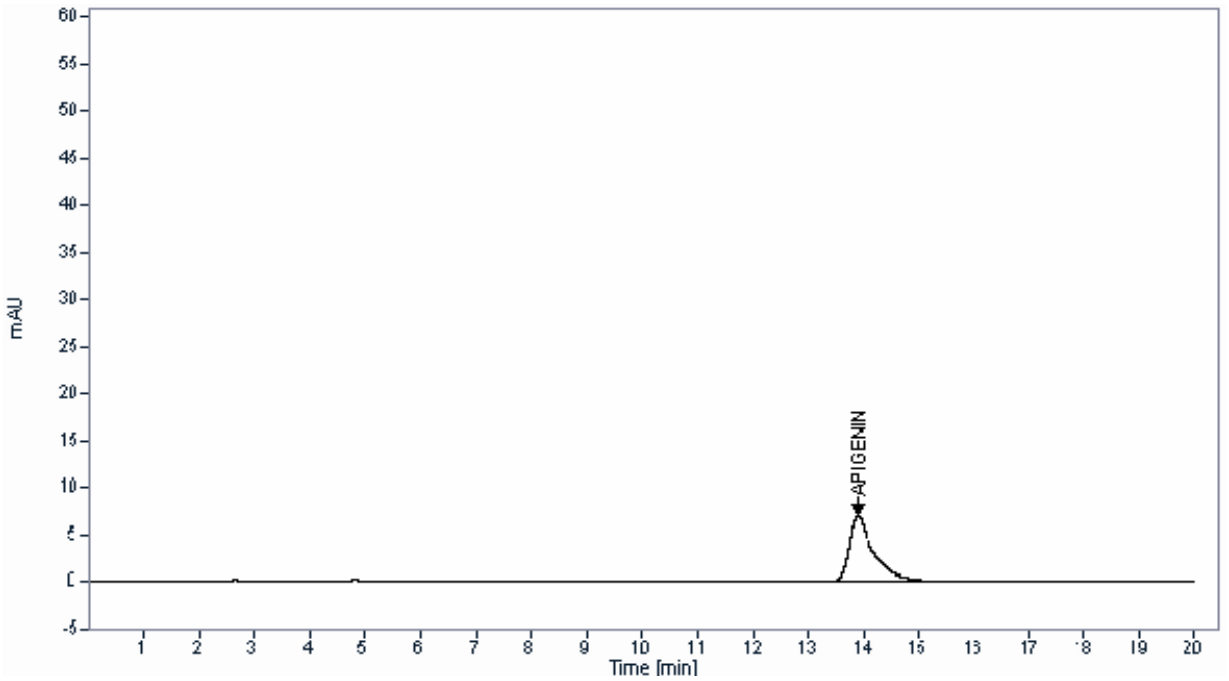
Analysed by: Checked by:

FIG:27. HPLC chromatogram of apigenin (4.13 MCG/ML)



Name	RT[min]	Area	Area %	TP	TF
APIGENIN	13.87	414.87	100.00	6501	1.86
	Sum	414.87	100.00		

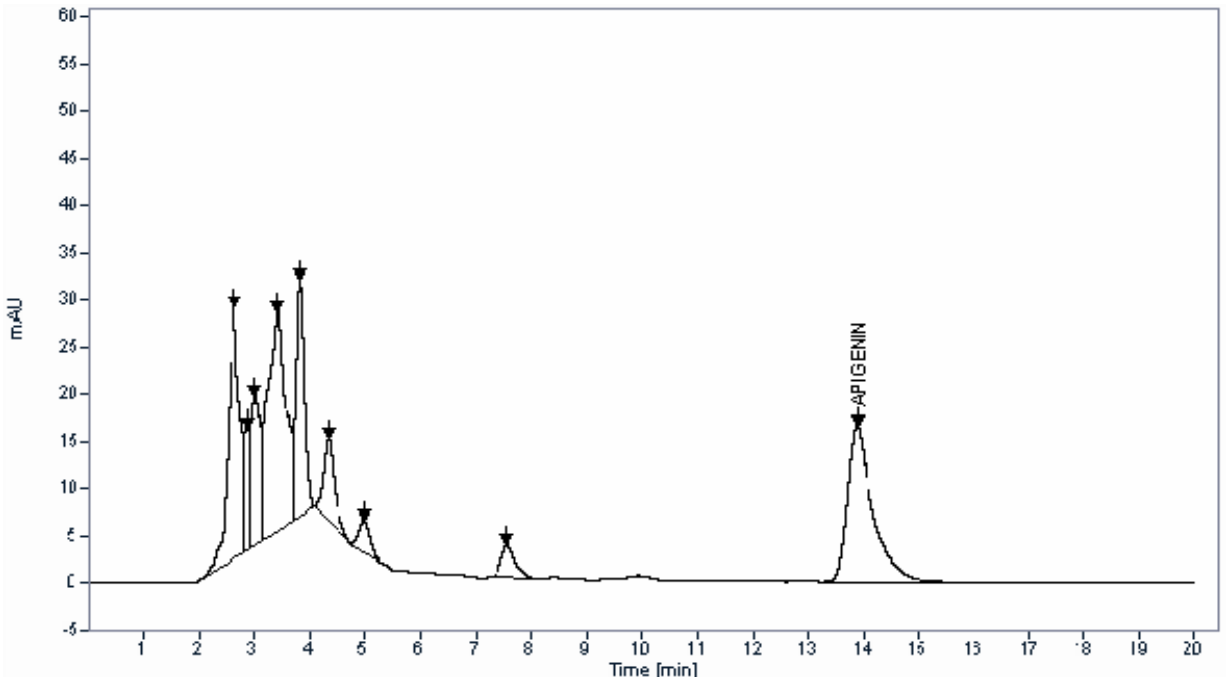
FIG:28. HPLC chromatogram of apigenin (2.07 MCG/ML)



Name	RT[min]	Area	Area %	TP	TF
APIGENIN	13.89	204.32	100.00	6551	1.89
	Sum	204.32	100.00		

Analysed by: Checked by:

FIG:29.HPLC chromatogram of EEPA



Name	RT[min]	Area	Area %	TP	TF
	2.62	391.58	17.73	894	0.85
	2.87	74.48	3.37	19	0.81
	3.00	190.50	8.63	280	1.10
	3.41	538.25	24.37	573	1.03
	3.83	261.84	11.86	3101	1.33
	4.33	123.15	5.58	2535	0.90
	4.97	44.29	2.01	3234	0.86
	7.57	61.56	2.79	4839	1.30
APIGENIN	13.88	522.80	23.67	5509	1.68
	Sum	2208.45	100.00		

Analysed by: Checked by:

PHARMACOLOGICAL SCREENING

SECTION-A

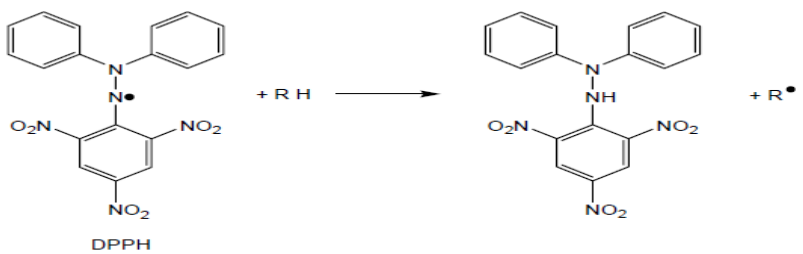
Antioxidant activity

DPPH radical scavenging activity

Table- 12 DPPH radical scavenging activity of (EEPA)

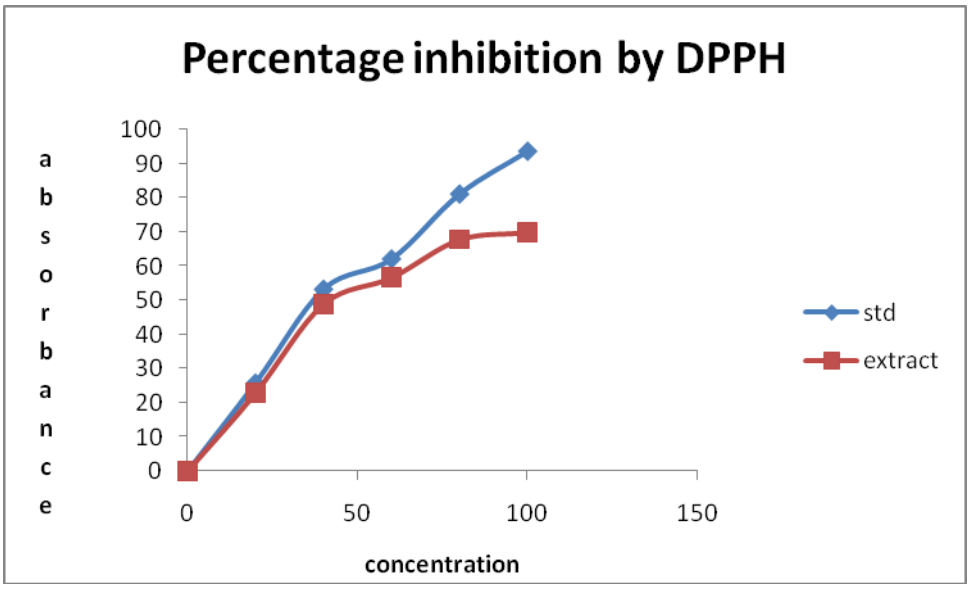
S. No.	Conc. in µg/mL	Percentage inhibition by standard ascorbic acid	Percentage inhibition by ethanolic extract
1	20	25.86 ± 5.63	22.99
2	40	53.32 ± 4.84	48.98
3	60	62.2 ± 7.35	56.71
4	80	81.21 ± 5.87	67.84
5	100	93.73 ± 0.85	69.98
	IC_{50}	**47.04µg/mL**	**51.99µg/mL**

DPPH is a stable free radical and accepts an electron or hydrogen radical to become a stable diamagnetic molecule [61] In the DPPH assay, the antioxidants are able to reduce the stable radical DPPH to non-radical form, DPPH-H. The purple colour alcoholic solution of DPPH radical changes to yellow colour which could be measured at 517 nm.



Ascorbic acid is used as a standard.The radical scavenging activity of ascorbic acid and EEPA increases in a dose-dependent manner and the results are tabulated in table-13. IC $_{50}$ of ascorbic acid and EEPA were found to be 47.04 µg/ml and 51.99µg/ml respectively.

FIG:30. Free radical scavenging activity of EEPA and standard ascorbic acid against DPPH



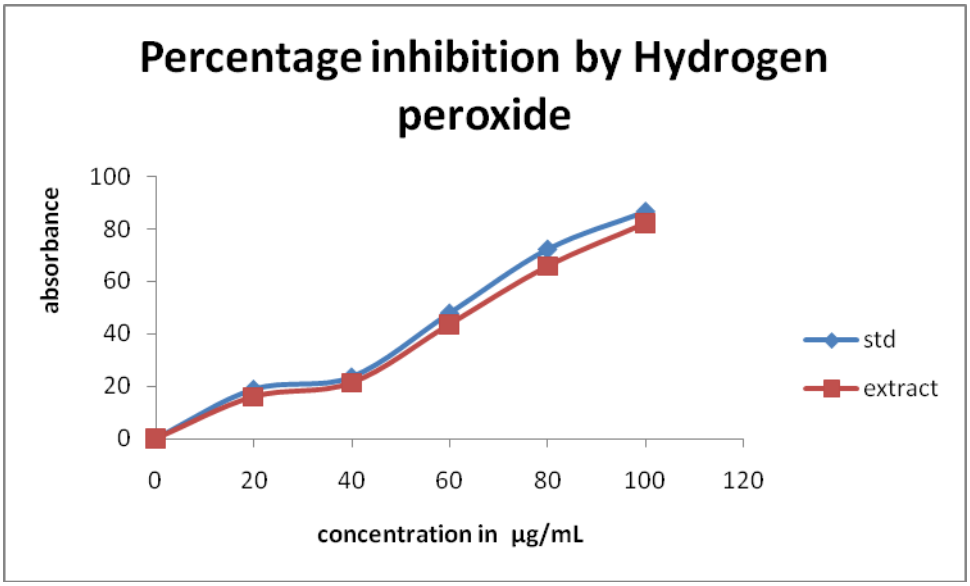
Hydrogen peroxide scavenging activity assay:

Table-13. Free radical scavenging activity of EEPA

S. No.	Conc. in µg/mL	Percentage inhibition by standard ascorbic acid	Percentage inhibition by EEPA
1	20	18.81± 0.12	16.04
2	40	23.52± 0.28	21.31
3	60	47.85± 0.11	43.63
4	80	72.18± 0.54	65.79
5	100	86.56± 0.39	82.14
	IC_{50}	**47.04µg/ml**	**55.97µg/ml**

Hydrogen peroxide has strong oxidizing properties. It can be formed *invivo* by many oxidizing enzymes, such as superoxide dismutase and can cross cellular membranes and may slowly oxidize a number of intracellular compounds. It has been shown that the IC_{50} of EEPA was found to be **55.97µg/ml** when compared to standard ascorbic acid 47.04µg/ml.

FIG:31. Free radical scavenging activity of EEPA standard ascorbic acid against Hydrogen peroxide



Discussion

This study showed the EEPA exhibited significant free radical scavenging activity; phenolic constituents such as flavonoids and tannins present in this extract may be attributed for this activity. Phenolic OH groups present in these phytoconstituents spares hydrogen atoms for free radical scavenging activity.[(61)]This study revealed that the significant anti oxidant activity of these extracts.

SECTION-B

Anticancer activity of EEPA against DEN induced liver cancer

This part of the pharmacological studies has dealt with anticancer effect of EEPA against in DEN induced liver carcinoma in rats. Biochemical parameters such as serum ALT(ALANINE TRANSAMINASE) AST(ASPARTATE AMINO TRANSAMINASE) ALP(ALKALINE PHOSPHATASE) and bilirubin and liver tissue homogenate TBARS and GSH were estimated and the results are presented in table-14. The histopathological studies were carried out in liver tissue in order to ascertain the induction of liver carcinoma in rats by the administration of DEN.

Effect of EEPA on biochemical parameters

Table 14. The effect of EEPA extract on liver tissue biomarker changes

Groups	**AST** (IU/L)	**ALT** (IU/L)	**ALP** (IU/L)	**Bilirubin** (mg/dl)	**LDH** (IU/L)	**Liver weight** (g)	**Total Protein** (mg / g wet tissue)	**GSH** (µg / mg of Protein)	**TBARS** (nmol/mg of Protein)
Control	114.5 ± 1.3	44.7 ± 1.2	189.2 ± 1.8	0.5 ± 0.2	79.4 ± 0.9	4.6 ± 0.7	69.2 ± 1.1	3.2 ± 0.3	0.35 ± 0.02
DEN	259.8 ± 1.1	202.5 ± 0.7	395.2 ± 2.3	3.5 ± 0.6 [a]	147.8 ± 1.4	8.2 ± 1.1 [a]	49.6 ± 1.4 [a]	1.7 ± 0.2 [a]	0.62 ± 0.03 [a]
EEPA (300)	171.2 ± 0.9	142.9 ± 1.2	309.6 ± 2.6	1.1 ± 0.5 [a]	129.5 ± 1.1	6.3 ± 0.9 [a]	64.9 ± 0.9 [b]	2.1 ± 0.4 [a]	0.53 ± 0.02 [a]
EEPA (400)	135.7 ± 1.2[b]	56.9 ± 0.8 [b]	219.9 ± 2.2	0.6 ± 0.4 [b]	83.9 ± 1.5 [b]	5.1 ± 0.8 [b]	67.8 ± 0.6 [b]	3.1 ± 0.3 [b]	0.39 ± 0.04 [b]

Digits in parenthesis indicate dose in mg/kg.

Data were expressed as mean ± SEM, n= 6 rats per group. [a]$P < 0.05$ vs control group.

[b]$P < 0.05$ vs DEN treated group.

a- DEN control compared with control group

b- EEPA 300mg/kg and EEPA 400mg /kg compared with DEN control group

Statistical significance test comparison was done by one way ANOVA followed by Tukey -Kramer multiple comparison test

Serum biochemical parameters

The effect of DEN and EEPA treatment on serum liver function markers like ALT, AST, ALP, LDH ,total bilirubin and total protein of experimental rats are as follows:

Administration of DEN to Group II animals caused significant increase in serum ALT, AST, ALP, LDH and bilirubin and decreases the level of total protein when compared to the(control) Group I animals ($P<0.05$). Treatment of EEPA at 400mg/kg b.w *p.o* to Group IV animals attenuated DEN induced elevated serum ALT, ALP ,AST, LDH and total bilirubin ($P<0.05$)and increases the total protein level($P<0.05$) But treatment of EEPA at 300mg/kg b.w *p.o* to Group III animals did not modify the DEN induced biochemical abnormalities significantly.

***In vivo* antioxidant parameters**

Animals treated with DEN and EEPA caused alteration of oxidative stress biomarker TBARS and endogenus antioxidant GSH in liver tissue homogenate and the results are presented in table-24. DEN administration to group II animals modified the significant increase in TBARS level and a significant decrease in GSH level as compared to the Group I(control) animals ($P<0.05$). Group IV animals treated with EEPA at (400mg/kg b.w *p.o*) caused the significant attenuation of DEN induced elevated TBARS level and augmented the GSH level ($P<0.05$). But treatment of EEPA at (300mg/kg b.w *p.o*) to Group III animals did not restore the DEN induced antioxidant imbalance significantly.

Liver weight

Animals treated with DEN caused changes in liver weight and the results are presented in table-14 DEN administration to group II animals caused an increase in liver weight when compared to the Group I animals (control) ($P<0.05$). Group IV animals treated with EEPA at (400mg/kg b.w *p.o*) significantly decreased the liver weight ($P<0.05$). But treatment of EEPA at 300mg/kg b.w *p.o* to Group III animals did not decrease the liver weight significantly.

Histopathological study

The histopathological changes of liver tissue induced by DEN in normal and EEPA treated rats are as follows;

Group I animals (control) showed normal liver histology with no signs of liver injury and this was indicated by normal hepatic nodules and central vein.

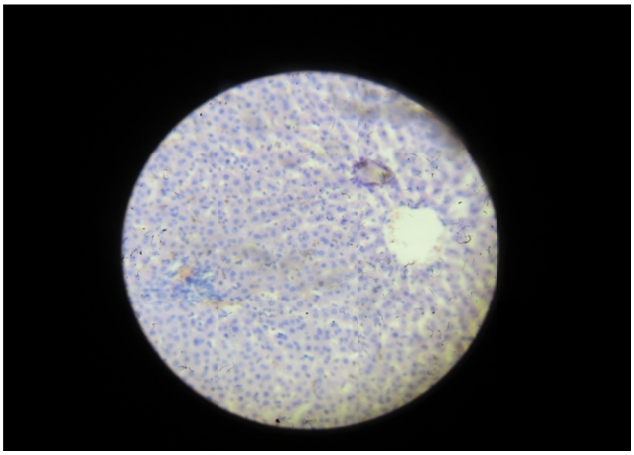
Group II animal treated with DEN showed clear signs of severe hepatic injury. Hepatocytes were manifested with periportal and perivascular inflammatory infiltrates with diffuse ballooning degeneration and proliferation of vascular channels are also noted in several areas. Binucleation and acidophilic bodies were also focussed

Group III animals treated with EEPA (300mg/kg b.w *p.o*) and DEN showed hepatic injury and manifested with periportal inflammation with conspicuously dilated blood vessel and ballooning degeneration.

Group IV animals treated with EEPA (400/kg b.w *p.o*) and DEN showed mild hepatic injury and manifested with minimal periportal inflammation.

FIG: 32 HISTOPATHOLOGICAL STUDIES OF LIVER TISSUE

control



Den control

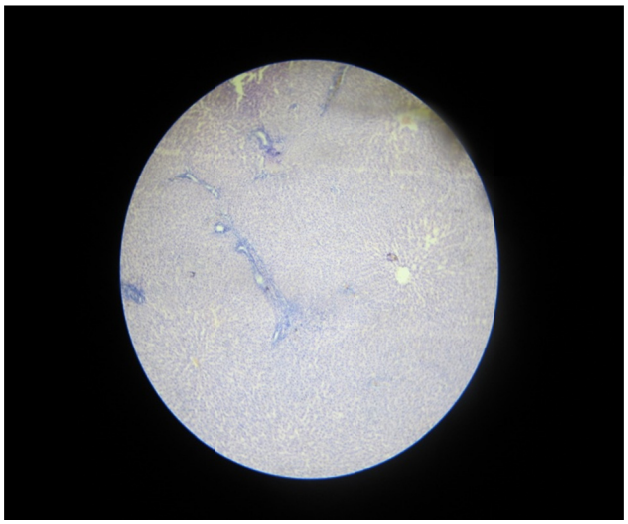


FIG :33

EEPA at 300 mg/kg treated group

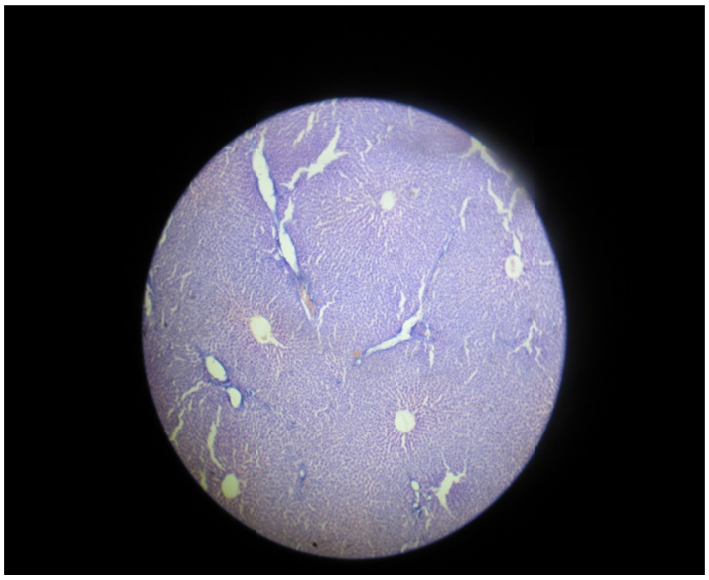
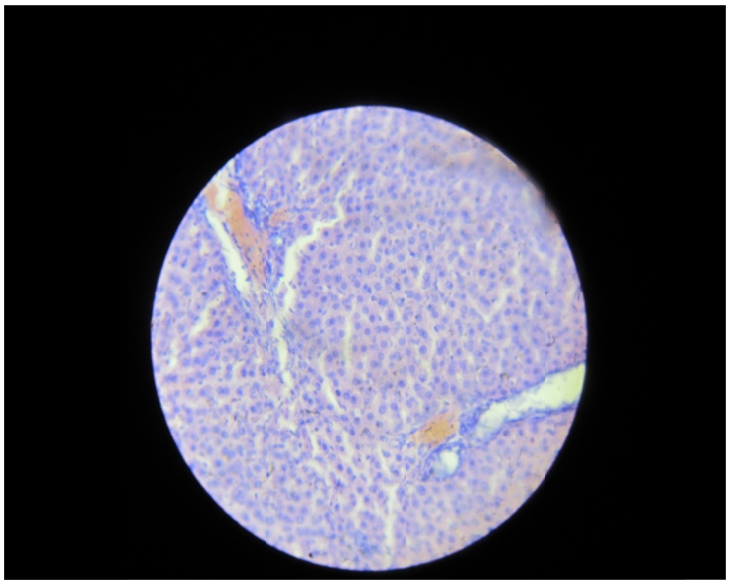


FIG:35 EEPA at 400 mg/kg treated group



Discussion

Diethyl nitrosamine (DEN) is an important environmental carcinogen that primarily induce tumor of the liver, because of its relatively simple metabolic pathway and potent carcinogenic activity[62]. DEN exhibited its carcinogenicity through the metabolic activation in the liver microsomes causing the release of alkylating agents that binds to the DNA forming adducts. [63]Lipid peroxidation (LPO) is considered as one of the basic mechanism of cellular damage induced by free radicals. Free radicals react with lipids causing peroxidation resulting in release of malandioldehyde (MDA), hydrogen peroxide (H_2O_2) and hydroxyl radicals (OH). ROS (Reactive oxygen species) including oxygen, hydrogen peroxide and hydroxyl radicals (OH) play an important role in carcinogenesis .

DEN administration caused the elevation of liver function markers including ALT, AST, ALP ,LDH and total bilirubin and severe histopathological lesions were noted in liver tissues.

The elevation of ALT, AST and ALP indicated hepatocellular damage in the DEN administered rats. In the present study, the observed increase in serum indices of liver function by DEN could be a secondary event following DEN-induced lipid peroxidation of hepatocyte membranes with the consequent increase in the leakage of ALT, ALP, AST and total bilirubin from liver tissues. An elevated level of serum indices of hepatocellular damage has been previously reported in many models of DEN-induced hepatocellular degeneration. LDH is a tumour marker. Elevated LDH level were noted in the DEN administered rats. Treatment of EEPA at 400mg/kg significantly decrease this tumour marker.

There are evidences to support that oxidative stress is an important component of carcinogenesis and clear examples of the participation of ROS in hepatocarcinogenesis in rats. There was an abnormal increase of LPO in the present study in the cancer bearing rats when compared to control. It was indicated by the elevation of TBARS which is the marker compound of LPO. This might be due to oxidative stress induced free radical[(64)] mediated changes by DEN. The decreased level of LPO in plant extract treated animals may be attributed due to free radical quenching effect of EEPA 400 mg/kg.It was indicated by the decreased level of TBARS.

GSH is a well known non enzymic antioxidant defense system of cells. It has been shown to offer protection against super oxides as well as H_2O_2 and it contributes to membrane stability. It is said to be involved in many cellular processes including detoxification of endogenous and exogenous compounds. Decreased level of GSH has been reported in patients with liver diseases of alcoholic, non-alcoholic, viral and other etiology. DEN, an electrophilic carcinogen may interact with the large nucleophilic pool of GSH thereby reducing the macromolecules and carcinogen interaction. Glutathione is a potent inhibitor of the neoplastic process, plays an important role in the endogenous anti-oxidant systems. The decreased GSH level in DEN administered rats were noted in the present study. Treatment with EEPA (400mg/kg) showed elevation of GSH level.

Preliminary phytochemical studies indicate the presence of phenolic constituents such as flavonoids, tannins,sterol and protein in EEPA. HPLC studies revealed the presence of apigenin in EEPA..Flavonoids are known to possess hepatoprotective activity and apigenin was reported for the treatment of hepatocellular

carcinoma Flavonoids have are already been reported for the elevation of GSH and suppress the TBARS level in various animal model.

EEPA showed the potential *invitro* antioxidant activity by DPPH assay and hydrogen peroxide scavenging activity. These plant extracts also exhibited *invivo* antioxidant activity by elevating endogenus antioxidant GSH and suppress the TBARS in liver tissue homogenate. Both *in vitro* and *in vivo* antioxidant pontential of EEPA may be attributed for the anticancer activity against the DEN induced hepatocellular carcinoma.[65]

SUMMARY AND CONCLUSION

P*hyllanthus acidus* belonging to the family euphorbiaceae was evaluated for pharmacognostic, phytochemical studies including quantification of apigenin by HPLC analysis, *in vitro* anti oxidant activity and DEN induced liver cancer studies in rat model. The macroscopical and microscopical characters, physical standards like ash values, extractive values, loss on drying, foreign organic matter and fluorescence analysis have been studied in order to standardize the leaves of this plant The gist of the finding derived from the investigations of this project is furnished in the following concluding lines.

Pharmacognostical parameters have been determined for the leaf of *phyllanthus* acidus in order to substantiate and identify the plant for future work.

Preliminary phytochemical screening of the leaf *phyllanthus acidu*s confirms the presence of sterols,terpenoids,carbohydrates ,proteins,flavonoids,tannins and phenols.Total phenolic content(48.34mg/g,)total flavonoid content(32.77mg/g) total tannin content(98.85mg/g) were determined for *EEPA extract* by UV Spectrophotometric method.

HPLC study was performed for EEPA extract revealed that the t_R of the standard (apigenin) and sample were found to be **13.88 mins** and **13.85mins** respectively and this study indicates the presence of apigenin in the extract. Apigenin present in this exract was also quantified as 0.042% . The presence of apigenin has not been reported from the leaves of this plant.This is the first time we have reported the presence of apigenin from the leaves of this plant .

Pharmacological screening confirms that EEPA exhibited the potent radical scavenging activity by both DPPH and hydrogen peroxide assay methods and the IC 50 Values were foud to be 47.04 µg/mL and 51.99 µg/mL of EEPA respectively.The free radical scavenging activity may be attributed due to the presence of flavonoid constituents like apigenin.

EEPA exhibited the significant anticancer activity against diethyl nitrosamine induced liver cancer in rats treated at the dose of 400 mg/kg, where as the treatment of 300 mg/kg did not show significant anticancer activity. Anti cancer activity of EEPA against the DEN induced liver cancer may be due to the presence of antioxidant phytoconstiuents like flavonoids. Apigenin present in this extract has been reported for hepatocellular carcinoma. Oxidative stress is the basic mechanism behind carcinogenesis. EEPA exhibited significant anticancer activity by virtue of its anti oxidant activity and this may be due to the presence of apigenin.

Future scope of the study

The active principles responsible for liver cancer may be isolated and evaluated for this activity cancer studies using different animal model.This extract and the isolated active principles may be formulated in suitable dosage form and they may be launched in the market. These herbal formulations will be beneficial to the liver cancer patients those who are not affordable to treat with allopathic chemotherapeutic agents**.** Sorafenib is the only drug which is used for the chemotherapy of liver cancer. It will not cure the liver cancer but increases the survival of the patient for only six months and for this treatment the patient has to spent lakhs of rupees.

REFERENCES

1. .Kamboj V.P Herbal medicine .Current Science. 2000:78(1):35-9
2. Agarwal SS, Paridhavi M.Text book of Herbal drug technology. 1st Edn, University Press Private Ltd,Hyderabad. 2007; 219,625.
3. **www .wikipedia.org.**
4. Kew MC, Dos Santos HA, Sherlock S. Dignosis of primary cancer of the liver .British medical Journal 1971;4:408
5. Schwartz JM, Larson AM, Gold PJ. Hepatocellular carcinoma: A one year experience at a tertiary referral center in the united states .Hepatology 1999;30:278.
6. A new Prognostic system for hepatocellular carcinoma: a retrospective study of 435 patients:the cancer of the liver in italian programme (CLIP)investigators. Hepatology 1999;30:278
7. **www.wikipedia.org**.
8. Tai BMH, Nhut ND, Nhle NT, Quang TH, et al. An evaluation of R Nase H inhibitory effects of Vietnam medicinal plant extract and natural compound. Pharmaceutical Biology 2013; 49(10):1046-1051.
9. Chouhans HS, Singh Sk. Phytochemical analysis, antioxidants and anti-inflammatory activites of *Phyllanthus simplex*. Journal of Ethanopharmacology 2013;137(3):1334-1337
10. Ngamkitidechakul C, Jaijoy K, Hansakul P, Soothornchareonnon N. Antitumour effect of *Phyllanthus emblica L*. induction of cancer cell apoptosis and inhibition of *in vivo* tumour promotion and *in vitro* invasion of human cancer cells. Phytotheraphy Research 2012;24(9):1405-1413.

11. Patel JR, Tripathi P, Sharma V, Chaunan NS, Dixit V. Phytochemistry, Pharmacology and Ethano-medicinal uses and evalution of *Phyllanthus amarus.* Journal of Ethonopharmacology 2012; 138(2):286-313.
12. Dang GK, Parekar RR, Kamal SK, Scindia AM, Rege NN. Anti-inflammatory activity of *Phyllanthus emblica Plumbago zeylanica* and C*yperus rotundus* in acute models of inflammation. Phytotheraphy Research 2012;25(6):904-988.
13. Zhu M, Zhu H, Wang K, Wei W, Zhang Y. Isolation and x-ray crystal structure of a securinega-type of alkaloid from *Phyllanthus niruri*Linn. Natural products Research 2010 ;26(8): 762-764.
14. Mazumder A, Mahato A, Pattnaik K, Mazumader R. Antipyretic potential of *Phyllanthus amarus*. Hamdard Medicus 2010; 51(4): 198-201.
15. Pagare RS, Auti GS, Aglave BA, Nikam TD. Protocol for callus culture of *Phyllanthus amarus* schum and Thonn and possibility of use of callus culture in production of Phyllnthin and hypophyllanthin. Advanced in plant science 2010; 22(20): 433-436
16. Chattopadhyay P, Garg P, Varshey VP, Sharma AK, Agarwal SS. Increase in insulin activity by *Phyllanthus amarus Linn* on liver cell regeneration of partially hepatectomised albino rats. Research Journal of Medicinal Plant 2010; 1(1):17-20.
17. Fang SN, Rao YK, Tzen VM. Antioxidant and inflammatory mediator's growth inhibitory effect of compound isolated *Phyllanthus urinaria.* Journal of Ethano pharmacology 2009; 116(2): 333-340. .
18. Garg M, Dhar VJ, Kalia AN. Antidiabetic potential of *Phyllanthus urinaria in* albino rats. International Journal of Green pharmacy 2008; 6(4): 54-58.

19. Kumar S, Kumar D, Deshmukh RR, Lokhande PD, Rangari VD. Antidiabetic *Phyllanthus recticulates* in alloxon-induced diabetic mice. Fitoterapia 2008 ;79 (1) :21-23.
20. Karthikeyan K, Chandra C, Kolothungan S. Rapid regeneration of *Phyllanthus niruri* L from shoot tip and nodel explants. Indian Journal of applied & pure Biology. 2008; 22(2):337-342.
21. Harikumar KB, Kuttan R. Antibacterial activity of *Phyllanthus* and *curcumin* .Amla Research Bulletin 2007;26:198-205.
22. Kumaran A, Joel karunakaram R. Antooxidant activity of polyphenol from *Phyllanthus deblis Wild*. Journal of Remedies 2007; 6(2): 141-146.
23. Garg M, Dhar VJ. Antidiabetic and antioxidant potential of *Phyllanthus fraternus* in alloxan induced diabetic animals. Pharmacology Magazine 2007; 4(14):138-143.
24. Elfahmi S,Koulman A, Bos. Lignans from cell suspension culture of *Phyllanthus niruri*. an Indonesion medicinal plant. Journal of Natural products 2006; 69(1):55-58.
25. Balakumbahan R, Sadasakthi A, Kumar S, Saravana A. Effect of inorganic and biofertilizers on biomass and alkaloid yield of *Phyllanthus amarus.* Journal of medicinal and aromatic plant science 2006 ;27(3):478-482.
26. Sailaja R, Setty OS. Protective effect of *Phyllanthus fraternus* against allyl alcohol-induced oxidative stress in liver Mitochondria. Journal of Ethanopharmacology 2006;105(1-2):206-209.
27. Zhang YJ, Nagao T, Yang CR, Okabe H, Kouno I. Anti-proliferative activity of the main constituent from *Phyllanthus embilica.* Biological&Pharmaceutical Bulletin 2004;27(2):251-255.

28. Srinivasa H, Bagul MS, Rajini M. Anticancer and antiviral activity of *Phyllanthus madarespatens* was studied by HPTLC method used for the quantification of gallic acid and ellagic acid content . Pharmaceutical edication and research development centre. 2004;173.
29. Al-Rehaily AJ, Al-Howiriny TA, Al-Sohaibani MO, Rafatulla S. Gastroprotective effect of *Emblica officinalis* on *in vivo* test model in rat.Medicinal aromatic and poisonous plant research centre. 2003;9(6):515-522.
30. Rajeshkumar NV, Kuttan R. *Phyllanthus amarus* extract administration increases the life span of rats with hepato-cellular carcinoma. Journal of Ethno pharmacology 2001;73:12
31. Suganya L, Sumitra M, Chandrakasan G. *Phyllanthus emblica* extract on dermal wound in rats. Journal of Medicine and Aromatic Plant Science 2001; 22(1):2-3.
32. Dep S, Mandal SK. *Phyllanthus amarus* was evaluated the pharmacology activity of hepatoproective, phytochemica screening exhibited in the simple thin layer chromatography-denstiometric methods estimation of chemical constituents of two lignin,phyllanthin,hypophyllanthin in polyherbal formulation. Indian Drugs 1998;33(8):415-416.
33. Saraju Devi, Satya B, Paul. Ethano-medicinal uses of *Cicca acida leaf.* Assam university Journal of science &Technology 2011;7:156-160.
34. Mominur Rahman MD, Razibul Habib MD, Raquibul Hasan SM . Antibacterial, cytotoxic and antioxidant potential of methanolic extract of *Phyllanthus acidus fruits.* International Journal of drug development& Research 2011;3(2):154-161.
35. www.wikipedia.org.
36. www.wikipedia.org.

37. Kokate CK, Purohit AP , Gokhale SB .Text book of Pharmacognosy .14th Edn , Nirali Prakashan, New Delhi;2000:74.

38. Agarwal SS, Paridhavi M. Textbook of Herbal Technology .1st Edn 2007 ;University press private Ltd . Hyderabad;2007; 219,625.

39. Almeida , FI . Maharashtra 3:3 .2001; Cook , FI . Bombay 1:600 .1903.

40. Chas CR, Pratt R. Fluorescence of powdered Vegetable Drugs With particular reference to development of a system of identification . J Amer Pharm Assoc Science Edn 1949; 28:324-331.

41. Mukherjee PK. Quality control of herbal drugs:An approach to evaluation of botanicals. 1st Edn. Business Horizons Pharmaceutical Publishers, New Delhi; 2002: 132-133,161,173,186.

42. Mulzer J, Bohlman R. "The role of Natural products in Drug Discovery .Workshop series 32, Springer, New York ;2000:205-214.

43. Mukherjee PK. Quality control of herbal drugs: An approach to evaluation of botanicals. 1St Edn, Business Horizons pharmaceutical publishers, New Delhi; 132-133, 161, 173, 186. .

44. Ya-Li Wang, Xiao-Dang Wang, Bing Zhao,Yu-Chun. Enhancing antioxidative capacity of *lepidium meyenii calli* by addition of methyl salicylate to culture medium.Wang Acta physiol plant 2007 ; 29:417-41.

45. Kokate CK. Practical pharmacognosy, 4th Edn, Vallab prakashan,New Delhi.1994:107

46. Trease,Evans WC. Textbook of pharmacognosy.15 th Edn, Elseivier publishers, New Delhi; 74.

47. Harborne JB. Phytochemical methods:a guide to modern techniques of plant analysis.2nd Edn. Chapman and Hall, London ;1994:1-35.
48. Mabry TJ, Markham KR, Thomas MB. The systematic identification of flavonoids.Springer Verlay ,New York ,USA ;1970.
49. Siddique MA, Mujeeb M, Najim AK, Akram M. Evaluation of antioxidant activity,quantitative estimation of phenols and flavonoids in different parts of *Aegle marmelos*. African J Plant Sci 2010;4(1):1-5.
50. Olennikov DN, Tankhaeva LM. A method for quantitative estimation of total flavonoids in greater plantain leaves. Pharmaceutical Chemistry Journal 2008; 42(4):17-25.
51. Rawat JS, Banerjee SP. The influence of Salinity on growth, biomass production and photosynthesis of *Eucalyptus Camadulensis* Dehnh and *Dalbergia sissoo Roxb*. Seedlings.Plant and Soil 1998;205 (2):163-169.
52. Neeraj Kumar, Pamita Bhandari, Bikram singh. Reverse phase HPLC for rapid determination of polyphenols in flowers of rose species. Natural plant products division. Institution Himalayan Bio-resource technology J.sep.sci.2008;31:262-267.
53. Neuza Mariko ,Aymoto Hassimotto NMR,Maria Inea Genovese S, Franco Maria Lajolo FM.Antioxidant Activity of Dietary Fruits, Vegetables, and Commercial Frozen Fruit pulps . J.Agric. Food Chem 2005 ; 53(1):2928-2953.
54. Nagendran Balasun,Tan Yew Ai, Ravigadevi sambanthamurthi, Kalyana sundram, Samir samman. Antioxidant properties of palm fruit extracts. Asia pac J Clin Nutr 2005;4(4):319-324.
55. Jananie RK , Priya V, Vijayalakshmi K. *in vitro* assessment of free radical scavenging activity of *Cynodon dactylon.* J.Chem.pharm.Res 2011;3(4):647-654.

56. Jelili Badmus A, Temitope Adedosu O, John Fatok O, Victor Adegbite A, Oluwatosin A, Adaramoye , Oyeronke A. Lipid Peroxidation inhibition and antiradical activities of some leaf fraction of *Mangifera indica.* Acta poloniae Pharmaceutical and Drug Research 2011 ; 68(1):1

57. Ruch RJ , Cheng SJ , Klaunig JE. Prevention of cytotoxicity and inhibition of Intercellular communication by antioxidant properties catechins isolated from Chinese green tea . Carcinogenesis 1989 ; 10:1003-08

58. Mohamed M, Abulaziz M, Salim S , AI-Rejaie, Abdulaziz A, AI-Yahya , Othman A, AI-Shabanah, Mohamed M, Hafez, Nagi MN. Thymoquinone attenuates diethyl nitrosamine induction of hepatic carcinogenesis through antioxidant signaling. Oxidative Medicine and Cellular Longevity 2010;3(4): 254-261.

59. Moron, MS, DePierreJW, Manerwik KB. Levels of glutathione, glutathione recductase and glutathione -S- transferase activities in rat lung and liver .Biochimica Biophysica ACTA 1979;582:67-68.

60. Chase CR , Pratt R. Fluorescence of powdered vegetable drugs with particular reference to developed of system of identification .J Amer pharm Assoc Sci Edn 1949;28:324-33

61. Aruoma OI . Free radicals, oxidative stress and antioxidants in human health and disease . J.Am. Oil Chemists Society 1998; 75:199-212 .

62. Archer MC. Mechanisms of action N nitroso compounds.Cancer surviv 1989;8:241-250

63. Nakae D, Kobayashi Y, Akai H, Andoh N, Satoh H and Ohashi k. Involvement of 8-hydroxyguanine formation in the intiation of rat liver carcinogenesis by nitrosodiethylamine. Cancer Res 1997;57:1281-1287.

64. Ray G and Husain SA .Oxidants, antioxidants and carcinogenesis. Indian . J Exp Biol 2002;40:1213-1232.

65. Barbisan LF, Scolastici C, Miyamoto M, Salvadori DM, Riberio LR, Da Eira AF,et al. Effects of crude extract of *Agaricus blazei* on DNA damage and on rat liver carcinogenesis induced by diethyl nitrosamine. Genet Mol Res 2003:2:295-308.