Standardization of some Hepatoprotective medicinal plants

A Thesis submitted to Gujarat Technological University
for the Award of

Doctor of Philosophy

In

Pharmacy

By

Hiteksha Sharadchandra Panchal

[119997290031]

under supervision of

Dr. Mamta B. Shah

GUJARAT TECHNOLOGICAL UNIVERSITY
AHMEDABAD

[December - 2018]

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ABSTRACT

In modern day therapeutics, hepatoprotective activity of various medicinal plants and their formulations are gaining renewed interest. In Ayurveda, an ancient system of Indian medicine, numerous plant formulations and combined extracts of plants are used as drug of choice rather than individual for the treatment of various liver diseases. These herbal remedies not only support natural healing phenomena through blocking the progession of the degenerative pathological processes but also are safe, effective, inexpensive, accessible and convenient for many patients.

Liver diseases viz. chronic viral hepatitis B and C, alcoholic liver diseases, non-alcoholic fatty liver diseases are serious health problems in current scenario. Despite advances in conventional medicine in the last decades, there is no effective drug available that stimulates liver function, offer protection to the liver from damage or help to regenerate hepatic cells. Herbal hepatoprotective products with better effectiveness and safe profiles may be beneficial as a substitute for chemical therapeutics in the treatment of liver diseases.

Standardization is the basic need for the establishment of a quality control and quality assurance for production and manufacturing as well as for reliable clinical trials and to provide consistent beneficial therapeutic effects. For polyherbal formulations, standardization is necessary as every ingredient need to be standardized, increase of constituent's load, inconsistency of finished formulations, overlapping chemical and chromatographic patterns during analysis, and difficulty in developing standards along with stability of individual ingredient vs formulation stability. In the recent era, the concept of marker (chemical or biomarker) based standardization of medicinal plants is gaining momentum. Modern chromatographic methods of identification of active principles are most widely used for marker based standardization which includes HPLC and HPTLC.

The aerial parts of *Achillea millefolium L.* (Compositae) and aerial parts of *Thespesia populnea L.* (Malvaceae) are known for their traditional use in treatment of Liver disease. Moreover aerial parts of .*A. millefolium L.* and aerial parts of *T. populnea L.* are ingredients of some of well known Herbal formulations used in the treatment of hepatic disorders. The objective of present study was to perform Pharmacognostical study, Phytochemical study, to standardize the crude extracts with respect to chemical markers and to perform pharmacological study.

The key features of macroscopy and microscopy were in conformity with those referred in standard texts, thus indicating that both the samples used in our study were authentic. Additionally, proximate analysis and quantitative analysis tests were carried out for authentication of plant samples.

After having authenticated the samples of aerial parts of *A. millefolium* and aerial parts of *T. populnea*, hydrolyzed methanolic extracts were prepared for carrying out the standardization of both plants using sophisticated instrument HPTLC and LC-MS/MS. A simple, rapid and precise HPTLC method was developed for quantitative estimation of apigenin, luteolin and quercetin for aerial parts of *A. millefolium*. Quercetin, kaempferol, lupeol and β-sitosterol for aerial parts of *T. populnea*. Optimization of mobile phase was performed on silica gel 60 F_{254} HPTLC plates using toluene: Ethyl acetate: Formic acid (7:3:0.3, %v/v/v) as mobile phase for marker, apigenin, luteolin, quercetin, kaempferol, lupeol and β-sitosterol. The method developed was validated according to ICH guideline. Linear concentration range was found to be 400-1400 ng/band for luteolin, quercetin and 40-140 ng/band for apigenin using hydrolyzed extract of *A. millefolium*. Limit of detection of developed method was found to be for luteolin 42.72 ng/band, for quercetin 96.49 ng/band and for apigenin 4.67 ng/band and Limit of quantitation was found to be for luteolin 129.46 ng/band, for quercetin 292.42 ng/band and for apigenin 14.17 ng/band. Linear concentration range was found to be 600-1400 ng/band for quercetin, kaempferol, lupeol and β-sitosterol using hydrolyzed extract of *T. populnea*. Limit of detection of developed method was found to be quercetin 58.03 ng/band, for kaempferol 90.79 ng/band, for β-sitosterol 59.46 ng/band and for lupeol 62.16 ng/band and Limit of quantitation was found to be quercetin 175.87 ng/band, for kaempferol 275.13 ng/band, for β-sitosterol 180.20 ng/band and for lupeol 188.38 ng/band indicating acceptable sensitivity of the method. This developed validated method can be used as a quality control method for fingerprint profiling and quantitative evaluation of aerial parts of *A. millefolium* and aerial parts of *T. populnea*.

A simple and rapid and sensitive gradient reverse-phase liquid chromatography–tandem mass spectrometry method was equipped with Electro Spray Ionization (ESI) source, operating in the positive ion and multi reaction monitoring (MRM) acquisition mode was devised to quantify apigenin, luteolin and quercetin for aerial parts of *A. millefolium*. Quercetin, kaempferol, lupeol and β-sitosterol for aerial parts of *T. populnea*. This method yielded extremely clean extracts with very good recovery. The method developed was

validated according to ICH guideline. Linear concentration range was found to be 1.25-5000 ng/ml for luteolin, quercetin and apigenin using hydrolyzed extract of *A. millefolium*. Limit of detection of developed method was found to be for luteolin 17.03 ng/ml, for quercetin 27.22 ng/ml and for apigenin 24.04 ng/ml and Limit of quantitation was found to be for luteolin 51.61 ng/ml, for quercetin 82.48 ng/ml and for apigenin 84.98 ng/ml. Linear concentration range was found to be 25-2500 µg/ml for quercetin and kaempferol. Limit of detection of developed method was found to be quercetin 4.11 µg/ml, and for kaempferol 8.56 µg/ml, and Limit of quantitation was found to be quercetin 12.45 µg/ml and for kaempferol 25.96 µg/ml. Linear concentration range was found to be 10-100 µg/ml for β-sitosterol and lupeol. Limit of detection of developed method was found to be lupeol 2.60 µg/ml, and for β-sitosterol 1.96 µg/ml, and Limit of quantitation was found to be lupeol 7.90 µg/ml and for β-sitosterol 5.94 µg/ml. This developed validated method can be used as a quantitative evaluation of aerial parts of *A. millefolium* and aerial parts of *T. populnea*.

Two doses 250 mg/kg and 500 mg/kg p.o of EAMTAM and EAMTTP extracts were subjected for the evaluation of Hepatoprotective activity against CCl_4 (250 mg/kg s.c.), Silymarin (50 mg/kg p.o) was used as a standard drug. Biochemical parameters & histopathological studies were evaluated in the study. Both EAMTAM and EAMTTP showed dose dependent significant reduction in SGPT, SGOT, bilirubin & total protein. Hepatoprotective effect was also confirmed by histopathology of Liver which showed less necrosis in extracts treated rats. The results obtained were comparable with that of the standard

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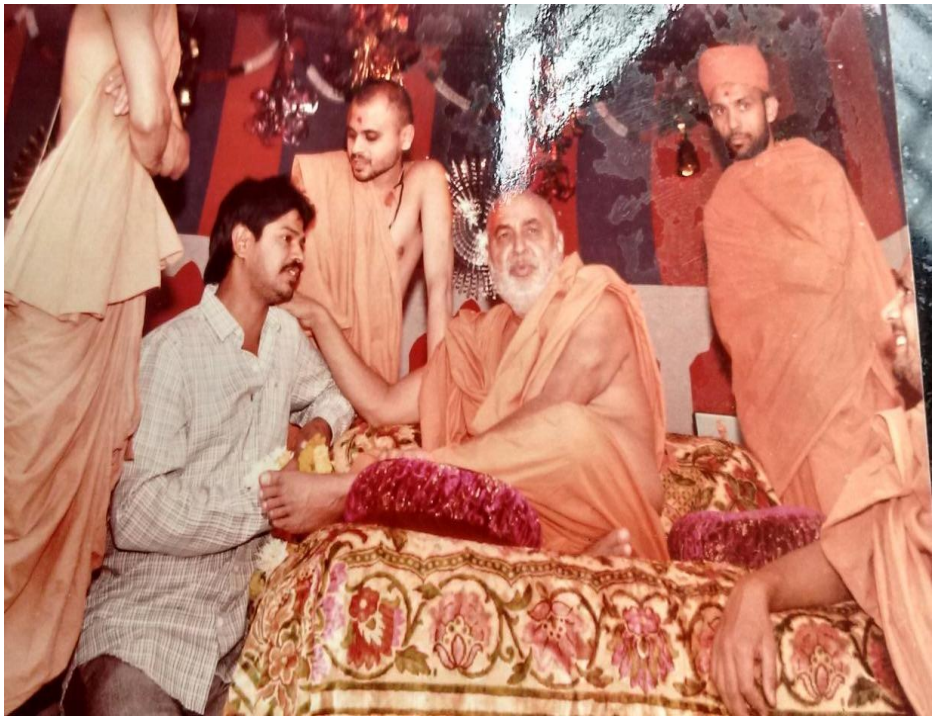

Pramukh Swami Maharaj

&

My beloved Father

Acknowledgement

Research project is like a mammoth tree, which has grown from a small seed which requires optimum conditions to grow. Wonderful gift of Almighty, trees are full of miracles in which all the cellular and enzyme systems unite together to make various parts, which have their own functions. These systems work synchronously to live a healthy life. Similarly, my project work was like a tree where many people helped me in some or the other way for its successful completion.

"God is always with us, above us to bless, below us to support, before us to guide, behind us to protest, besides us to comfort and inside us to sustain"

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Hiteksha Sharadchandra Panchal

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List of Abbreviation

ALP	Alkaline phosphate
ALT	Alanine aminotransferase
a.m	*Achillea millefolium*
ANOVA	Analysis of variance
API	Apigenin
AR	Analytical grade
AST	Aspartate aminotransferase
ATP	Adenosine triphosphate
BHC	Benzene hexachloride
Ca^{+2}	Calcium
CCl_4	Carbon tetrachloride
DDT	Dichloro diphenyl trichloroethane
EAMTAM	Ethyl acetate hydrolyzed extract of *Achillea millefolium*
EAMTTP	Ethyl acetate hydrolyzed extract of *Thespesia populnea*
ESI	Electron spray ionization
FAO	Food and Agricultural organization
FEH	Fingerprint evaluation of Herbals
GC	Gas chromatography
GI	Gastrointestinal tract
GSH	Glutathione S-transferase

HPLC	High Performance Liquid Chromatograhy
HPTLC	High Performance Thin Liquid Chromatograhy
IAE	International Atomic energy
ICH	International Conferrence of Harmonization
i.p	Intraperitoneal
K^+	Potassium
KAE	Kaempferol
LC-MS/MS	Liquid Chromatography- Mass spectophotometry
LDL	Low density lipoprotein
LOD	Limit of Detection
LOQ	Limit of Quantitation
LUP	Lupeol
LUT	Luteolin
MTAM	Methanol extract of *Achillea millefolium*
MTTP	Methanol extract of *Thespesia populnea*
Na^{+2}	Sodium
NH_2	Amide
NH_3	Ammonia
ng	Nanogram
nm	Nanometer
O_2	Oxygen
p.o	Post oral

QUE	Quercetin
RSD	Relative Standard Deviation
s.c	Subcutaneous
S.D	Standard Deviation
SGOT	Serum Glutamate oxaloacetate transaminase
SGPT	Serum Glutamate pyruvate transaminase
SIT	β-sitosterol
STD	Standard
TLC	Thin Layer Chromatography
t.p	*Thespesia populnea*
U.V	Ultraviolet
VLDL	Very low density lipoprotein
WHO	World health organization

List of Symbols

Symbol	Description
°C	Celcius
μ	Micro
β	Beta
α	Alpha
λ	Lamda

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Chapter - 1

INTRODUCTION

CHAPTER-1

1. Introduction

Study of diseases and their treatment must also have been contemporaneous with the down of the human intellect. Since ancient time people have applied herbs and their components as medicines. It is an evolving practice recorded in both folklore and books of early practitioners. At present, despite the abundance and advancement of synthetic drugs, a significant proportion of the population of developing countries still depend on traditional medicines for their health care needs (1).

Medicinal plants have played an important role in world health and are circulated worldwide, found mostly in tropical countries. It is noted that about 25% of all modern medicines are indirectly or directly obtained from higher plants. World Health Organization (WHO) has individual herbal drugs as whole, labeled medicinal products that have robust ingredients, aerial or secret parts of the whole plant or other plant material or mixture of them. World Health Organization (WHO) has a set of specific Guidelines for the evaluation of the safety, efficacy and quality of herbal drugs or herbal medicines. WHO states that 80% of the world people currently use herbal medicine or drugs for the most important health cares (2). Herbal drugs are a main constituent in usual medicine and a general ingredient in Homeopathic, Ayurvedic, Naturopathic and in other medicine system. Herbs are usually measured as safe toxicity, side effects of allopathic drugs, has led to more increased in number of herbal drugs manufacturers. For the past few years, herbal drugs have been mostly used by the people with no prescription, Leaves, stem, bark, flower, seeds, roots and extract of all these have been used in herbal drugs over the thousands of their use (3,4). The long tradition of herbal medicine continues to the present day in China, India, and many other countries. Medicinal herbs continue to contribute significantly to modern prescription drugs by providing lead compounds upon which the synthesis of new drugs can be made.

In India knowledge of medicinal plants is very old and medicinal properties of plants are described in Rig-Veda and in Atharvaveda (3500-1500 B.C) from which Ayurved has developed in Ayurveda. The ancient well-known treatises are Charak Samhita dealing with mostly plants and Susrut Sanhita in which surgery is also mentioned. In Egypt, people were

familiar with medicinal properties of plants and animals. They were familiar with human anatomy and knew of embalming the dead and preserving their bodies as described in Ebers Papyrus (1550 B.C) an ancient book found in one of the mummies. Greek scientists contributed much to knowledge of natural history. Hippocrates (460-370 B.C) is referred to as father of medicine. Theophrastus (370- 287 B.C) described medicinal plants, some of which like belladonna, ergot, opium, colchicum are used even today (5).

Antioxidant agents of natural origin have attracted special interest because they can protect human body from free radicals(6). Theyare essential for good health and are found naturally in wide variety of foods including many vegetables and fruits(7). Therefore, several attempts have been made to prevent and treat hepatotoxicity due to various drugs and chemical by using several antioxidant principles. Antioxidants play important role in hepatoprotective ability and hence, search for crude drugs of plant origin with this property has become a central focus of studies for hepatoprotection(8).

Standardization is the basic need for the establishment of a quality control and quality assurance for production and manufacturing as well as for reliable clinical trials and to provide consistent beneficial therapeutic effects. Polyherbal formulations, standardization is necessary as every ingredient need to be standardized, increase of constituents load, inconsistency of finished formulations, overlapping chemical and chromatographic patterns during analysis, and difficulty in developing standards along with stability of individual ingredient *vs* formulation stability (9,10). In the recent era, the concept of marker (chemical or biomarker) based standardization of medicinal plants is gaining momentum. Modern chromatographic methods of identification of active principles are most widely used for marker based standardization which includes HPLC, HPTLC etc. (11,12).

Identification of purely active moiety is an important requirement for Quality control and dose determination of plant related dugs. Moieties for example, Alkaloids are nitrogenous principle organic moiety combine with acid to form crystalline salt and also herbal plants contains Resin, Oleoresins, lactones, saponin and volatile oils. Complete phytochemical screening of most of the medicinally essential herbs are not done in India. This would be helpful in standardization and dose determination of herbal drugs (13).

Liver is one of the largest organs in human body and the chief site for intense metabolism and excretion, so it has a surprising role in the maintenance and regulating homeostasis of the body. It is involved with almost all the biochemical pathways to growth, fight against disease, nutrient supply, energy provision and reproduction (14). To maintain a healthy liver is a crucial factor for overall health and well being (15).

Since liver is involved with almost all biochemical processes it is no wonder that there are many different diseases that will affect it. Liver disease can manifest itself in many different ways. Manifestation of liver diseases that are particularly important include jaundice, cholestasis, liver enlargement, portal hypertension, ascites, hepatic encephalopathy and liver failure. Liver damage is always associated with cellular necrosis, increase in tissue lipid peroxidation and depletion in the tissue GSH levels. In addition, serum levels of many biochemical markers like SGOT, SGPT, ALP triglycerides, cholesterol & bilirubin are elevated.

Silymarin, a flavonoid ligand mixture extracted from the *Silybum marianum* (milk thistle) is a popular remedy for hepatic diseases. However, there are several herbs/herbal formulations claimed to have possess beneficial activity in treating hepatic disorders. Herbal extracts have yielded molecules, often related to flavonoids, with proven antioxidative, antifibrotic, antiviral or anticarcinogenic properties, including glycyrrhizin, phyllanthin, silibinin, picroside and baicalein, which derive from Liquorice root, *Phyllanthus amarus*, milk thistle, *Picrorhiza kurroa* and shosaiko-to respectively, that can serve as primary compounds for the development of specific hepatoprotective drugs (16).

Various herbal formulations are used in Ayurvedic system of medicine. The most widely used formulations are: Jawarish amla, Jawarish al- tursh, Jawarish al-sirin, Jawarish mastagi, Dawa-e- jigarpith, Dawa-e-Karim, Salajin bajuru and Sharbat bajuri. Most of these formulations contain *Andrographis paniculata* Nees, *Asteracatha longifolia* Nees, *Boerrhaavia diffusa* Linn, *Cinchorium intybus* Linn, *Eclipta alba* Hassk, *Oldenlandia corymbasa* Linn., *Picrorrhiza kurroa*, *Rolex* Benth., *Solanum nigrum* Linn., *Terminalia chebula* Retz., *Tinosphora cordifolia* (Wild.) Miers, etc. are widely used for liver complications. The marketed formulations such as Mandoor Bhasma and Loha Bhasma having single constituents are bitters and vegetable tonics, e.g. gentian, are useful in functional disorders. Torpid liver with accompanying headache is treated with Livomyn. *Ipecachunha* is reported to promote the flow of bile and given in large doses to dysenteric patients suffering also with hepatitis. A formulation prepared by mixing nux vomica, podophyllum and mecury is useful in small doses in hepatic diseases. The important formulations are Acilvan (17), Hep-10 (18,19), Liva-16(20), Livodin (21), Livosin, Livotrit, Livocin (18), Vilmliv, Livomycin, Liv-52 (22), Livex (15), Amlycure, Sanliv etc. Livin (23), Livokin, Livomin and Livosin formulations are composed of excessive number of herbal constituents while least number of drugs are present in Livertone, Stimuliv, Tefroli

(24) and Vimliv. The preparation 'Trisoliv' possesses only *Andrographis paniculata* Nees. The quantity of each herbal drug varies in each formulation.

In spite of phenomenal growth of modern medicine, there are few synthetic drugs available for the treatment of hepatic disorders. In allopathic system of treatment, corticosteroids and immunosuppressants are commonly used. But these drugs have adverse effects such as bone marrow depression and success rate of treating liver diseases is low (25).

In light of above mentioned facts, medicinal plants selected for the present investigation are:

- *Achillea millefolium L.* Family Compositae is a common plant that has naturalized in all counties of Illinois. The variety of Yarrow that occurs in Illinois is probably native to Eurasia, although there is a variety of this plant that is native to western North America. This latter variety tends to be smaller in size and its foliage is more heavily covered with woolly hairs. Habitats include mesic to dry prairies, pastures, fallow fields, grassy waste areas, and edges of paths, yards, or hedges. Disturbed areas are preferred; Yarrow persists in native habitats (e.g., prairies) to a limited extent. Yarrow is often cultivated in flower and herbal gardens, from where it occasionally escapes.

- *Thespesia populnea*, Family Malvaceae, commonly known as Paras-pipalo

  Durjara snigdha shukraprada krumiprada kaphaprada

  prameha asra kushta yonigada vrana

  Portia tree fruits are sweet and sour in taste, hard to digest, unctuous, oily, improves sperm and semen quantity and quality, may cause intestinal worms, increases Kapha Dosha. Root and bark of Indian tulip tree is astringent in taste, Urinary tract disorders, diabetes, blood disorders such as abscess, skin disorders, bleeding disorders such as menorrhagia, nasal bleeding etc. skin diseases, Vaginal infection, excessive bleeding, uterine disorders, Ulcers, wounds.

Chapter - 2

REVIEW OF LITERATURE

CHAPTER-2

2. Review of literature

2.1. Standardization of herbal dugs

Standardization is the process of developing and agreeing upon technical standards. Specific standards are worked out by experimentation and observations, hence standardization is a tool in the quality control process (26). American Herbal Product association defines: "Standardization refers to the body of information and control necessary to product material of reasonable consistency (27). Standardization encompasses the entire field of study from birth of a plant to its clinical application (28). Evaluation means confirmation of its identity, quality, purity and detection of nature of adulteration (29). The fingerprint profiles serve as guideline to the phytochemical profile of the drug in ensuring the quality, while quatification of the marker compounds would serve as an additional parameter in assessing the quality of the sample (30).

2.2. Need of standardization

Need of Quality control and standardization of herbal products can be summarized as follows:

1. When traditional medicines were developed technology and concept of standardization was quite different.
2. During past thousand years' dynamic process of evolution may have changed the identity of plant material.
3. Due to commercialization, supply of genuine raw material has become a challenge.
4. Properties of botanicals may have undergone change due to time and environmental factors (31).

World Health Organization (WHO) stresses the importance of the qualitative and quantitative methods for characterizing the samples, quantification of the biomarkers and/ or chemical markers and the fingerprint profiles. Where active ingredients contributing to therapeutic efficacy are known botanical preparations should be standardized to these compounds (32).

2.3. Current Regulations for Standardization of Crude Drugs

Internationally several pharmacopoeias have provided monographs stating parameter and standard of many herbs and some product made out of these herbs.

Lay down monograph for herbs and herbal products to maintain their quality in their respective nations. Government of India too has brought out Ayurvedic Pharmacopoeia India, which recommends basic quality parameters for eighty common Ayurvedic herbal drugs (33,34).

2.4. Role of WHO in Herbal Medicine

WHO Guidelines for Quality Standardized Herbal Formulations

a. Quality control of crude drugs material, plant preparations and finished products.
b. Stability assessment and shelf life.
c. Safety assessment; documentation of safety based on experience or toxicological studies.
d. Assessment of efficacy by ethnomedical information's and biological activity evaluations.

The bioactive extract should be standardized on the basis of active principles or major compounds along with the chromatographic fingerprints (TLC, HPTLC, HPLC and GC) (35).

2.5. Standardization and Quality Control of Herbal Crude Drugs (36)

It is the process involving the physicochemical evaluation of crude drug covering the aspects, as selection and handling of crude material, safety, efficacy and stability assessment of finished product, documentation of safety and risk based on experience, provision of product information to consumer and product promotion. These include many approaches as

2.5.1. Physical Evaluation

It includes botanical, macroscopic and microscopic descriptions with detailed illustrations and photographic images which provide visual documentation of accurately identified material.

2.5.2. Chemcial Evaluation

Chemical analysis covers screening, isolation, identification, and purification of the chemical components. It helps to determine the identity of the drug susbstance and possible adulteration.

2.5.3. Biological Evaluation

Pharmacological activity includes of evaluation and standardize them by screening invivo and invitro methods.

2.5.4. Analytical Methods

It helps in determining identity and quality of herbal drugs which include sample preparations and other basic steps such as pre-washing, drying of plant materials and grinding, to obtain a homogenous sample and often improving the kinetics of extraction of the constituents. To reduce or eliminate the use of organic solvents and improve the extraction processes, newer sample preparation methods, such as microwave-assisted extraction (MAE), supercritical fluid extraction (SFE), and accelerated solvent extraction (ASE) or pressurized liquid extraction (PLE) have been introduced for the extraction of targeted constituents present in plant materials.

2.5.5. Chromatography

Separation of marker component from herbal extracts is the key step for identification and bioanalytical evaluation. Chromatography is widely used analytical method for separation and quantitative determination of active compounds, even from a complex matrix. These include paper chromatography (PC), thin-layer chromatography (TLC), gas chromatography (GC), HPLC, and capillary electrophoresis (CE).

TLC is widely used as it enables rapid analysis of extracts with minimum sample clean-up requirement, It provides qualitative and semi quantitative information of the resolved compounds. In TLC fingerprinting, the data that can be recorded using a high performance

TLC (HPTLC) scanner includes the chromatogram, retardation factor values, the color of the separated bands, and their absorption spectra λ max. All of these, together with the profiles on derivatization with different reagents, represent the TLC fingerprint profile of the sample.

HPTLC has been investigated for simulataneous assay of several components in a multicomponent formulation. It has been well reported that several samples can be run simultaneously by use of a smaller quantity of mobile phase than in HPLC. HPTLC technique is widely employed in pharmaceutical industry in process development, identification and detection of adulterants in herbal product and helps in identification of pesticide content, mycotoxins and in quality control of herbs and health foods.

LC-MS has become ethod of choice in many stages of drug development. The chemical standardization of an aqueous extract of the mixture of the herbs provided chemical compounds serving as reference markers using LC-MS.

UV absorption has been the most commonly used detection method for the preliminary identification of the separated components (37,38).

2.6. **Liver** (39–41)

Liver is a largest gland in the body, weighing between 1 to 2.5 kg situated in the right upper quadrant of the abdomen, just below the diaphragm. Its upper and anterior surface are smooth and curved to fit the under surface of the diaphragm and posterior surface is irregular in outline.

A thick capsule of connective tissue called Glisson's capsule covers the entire surface of the liver. The liver is multi-lobed organ i.e., it has four distinct lobes, divided into a large right lobe and a smaller, wedge- shaped left lobe, the other two, the caudate and quadrate lobes. The falciform ligament divides the two lobes of the liver. Each lobe is further divided into lobules that are approximately 2mm high and 1mm in circumference (Figure 2.1)

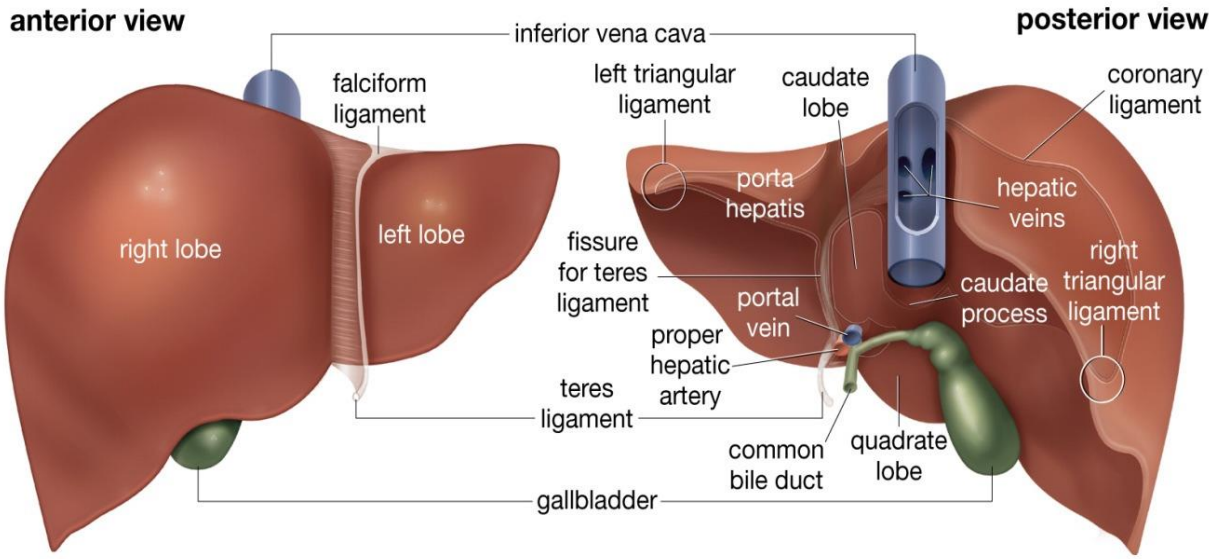


Figure 2.1: Liver Anatomy

These hepatic lobules are the functioning units of the liver, each of them have approximately 1 million lobules that consist of a hexagonal row of hepatic cells called "hepatocytes". They secrete bile into the bile channels and also perform a variety of metabolic functions. Between each row of hepatocytes are small cavities called "sinusoids" and each sinusoid is lined with kupffer cells, phagocytic cells that remove amino acids, nutrients, sugar, old red blood cells, bacteria and debris from the blood that flows through the sinusoids. The main functions of the sinusoids are to destroy old or defective red blood cells, to remove bacteria and foreign particles from the blood and to detoxify toxins and other harmful substances (Figure 2.2).

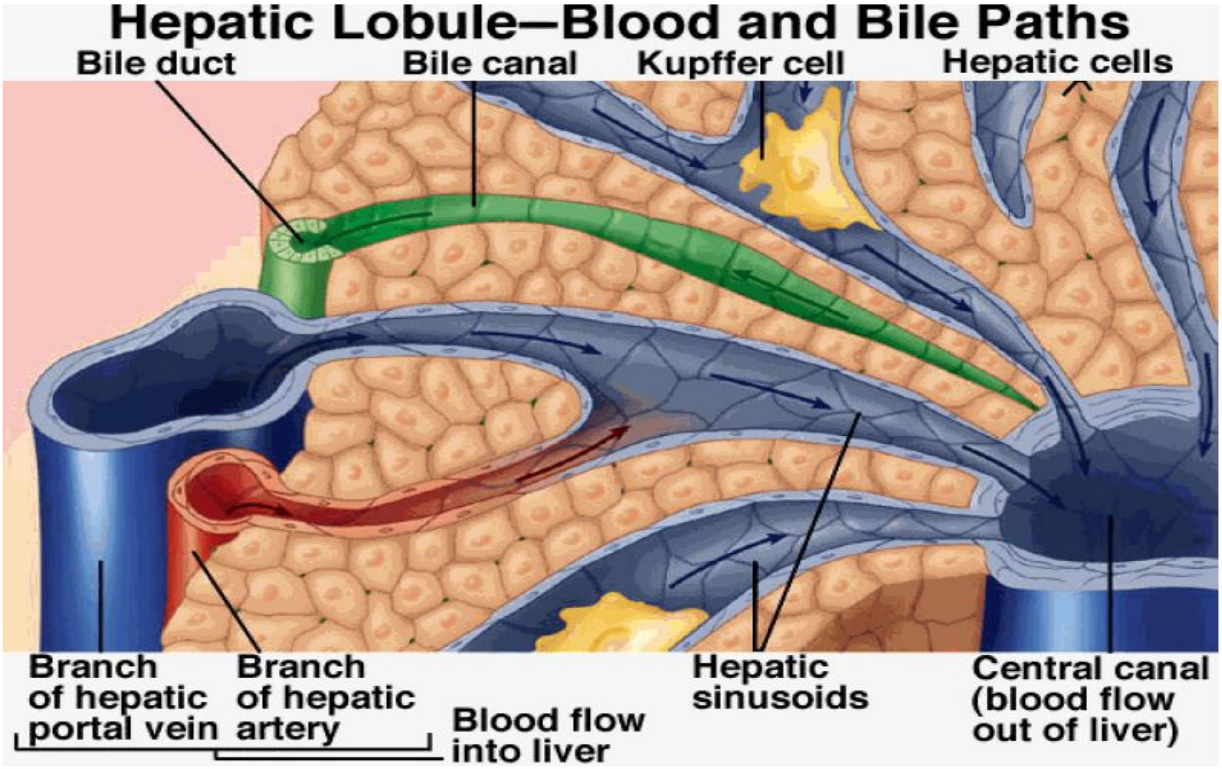


Figure 2.2: A single liver lobule and its cellular components

Almost all blood that enters the liver via the portal tract originates from the gastrointestinal tract as well as from the spleen, pancreas and gallbladder. Total human liver blood flow represents approximately 25% of the cardiac output up to 1500ml/min. Hepatic flow is subdivided in 25-30% for the hepatic artery (500 ml/min) and the major part for the portal vein (1000 ml/min). A second blood supply to the liver comes from the hepatic artery, branching directly from the celiac trunk and descending aorta.

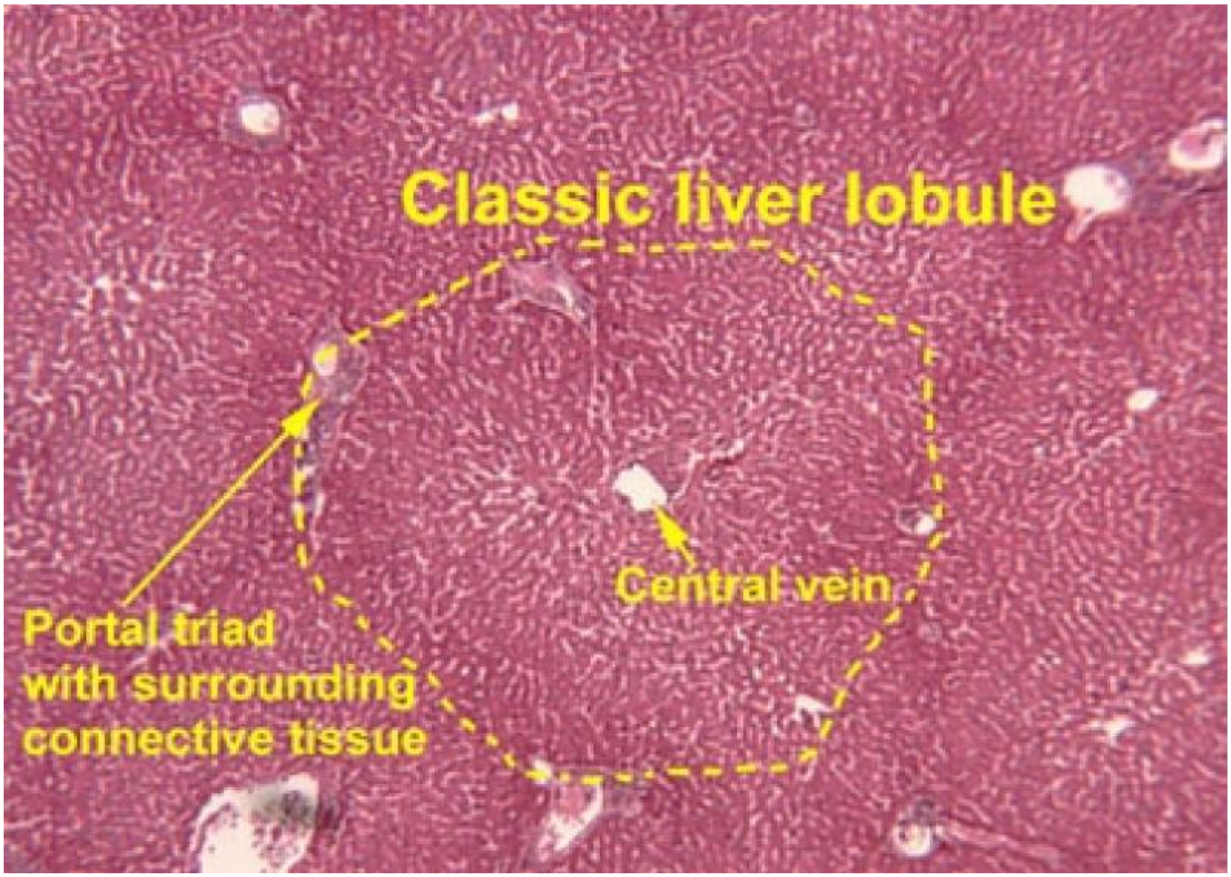


Figure 2.3: Normal liver lobule

The portal vein supplies venous blood under low pressure conditions to the liver, while the hepatic artery supplies high-pressured arterial blood. Since the capillary bed of the gastrointestinal tract already extracts most O_2, portal venous blood has a low O_2 content. Blood from the hepatic artery on the other hand, originates directly from the aorta and is, therefore saturated with O_2. Blood from both vessels joins in the capillary bed of the liver and leaves via central veins to the inferior caval vein (Figure 2.3).

2.7. Functions of liver (39,42)

2.7.1. Secretion and excretion of bile

Bile is partially an excretory product and partially a digestive secretion. The principle bile pigment is bilirubin. When worn out red blood cells broken down, iron, globins and bilirubin (derived from haem) are released.

2.7.2. Metabolic function

a) Carbohydrate metabolism: Maintains glycogenesis and glycogenolysis depending upon blood sugar levels.

b) Lipid metabolism: Liver maintains oxidation of fat into acetyl coenzyme A, and converts excess to ketone bodies (ketogenesis) & stores some triglycerides.

c) Protein metabolism: Liver removes the NH_2 from amino acids so can be used for ATP production & converts the resulting toxic NH_3 into the much less toxic urea for excretion in urine.

d) Metabolism of ethanol: This follows consumption of alcoholic drinks

2.7.3. Haematological functions (haematopoiesis and coagulation)

Production of fibrinogen, prothrombin, heparin, and other clotting factors VII, VIII, IC and C & destruction of erythrocytes (at the end of life span).

2.7.4. Circulatory function

Transfer of blood from portal to systemic circulation & blood storage.

2.7.5. Detoxification and protective functions

Removes foreign bodies from blood (phagocytosis). Detoxication by conjugation, methylation, oxidation and reduction.

2.7.6. Drug metabolism and detoxification

It converts drug molecule from non- polar to polar. Non polar drugs can be conjugated with more polar compounds, which make it water soluble for the urinary excretion.

2.7.7. Storage functions

Storage of Fat soluble vitamins: A, D, E and K, Iron, Copper and other minerals & Storage of glycogen.

2.7.8. Synthetic functions

Synthesis of Carotene, some water soluble vitamins: riboflavin, niacin, pyridoxime, folic acid and Vit B_{12}.

2.7.9. Inactivation of hormones

These include insulin, cortisol, aldosterone, thyroid & sex hormones.

2.7.10. Production of heat

The liver uses considerable amount of heat energy has a high metabolic rate and produces a great deal of heat. It is the main heat production organ of the body.

2.8. Susceptibility of the liver (43)

Liver is the largest organ in the body, is often the target organ for chemically induced injuries. Several important factors are known to contribute to the liver susceptibility**.** First, most xenobiotics enter the body through the gastrointestinal (GI) tract and after absorption, are transported by the hepatic portal vein to the liver, thus the liver is the first organ perfused by chemicals that are absorbed in the gut. A Second factor is the high concentration in the liver of xenobiotic metabolizing enzymes, primarily the cytochrome P450-dependent monooxygenase system. Although most biotransformations are detoxication reactions, many oxidative reactions produce reactive metabolites that can induce lesions within the liver. Often areas of damage are in the centrilobular region, and this localization has been attributed, in part, to the higher concentration of cytochrome P450 in that area of the liver.

2.9. Types of liver injury

The types of injury to the liver depend on the type of toxic agent, the severity of intoxication and the type of exposure, whether acute or chronic. The main types of liver damage are discussed briefly.

2.9.1. Fatty Liver

Fatty liver refers to the abnormal accumulation of fat in hepatocytes. At the same time there is a decrease in plasma lipids and lipoproteins. Although many toxicants may cause lipid accumulation in the liver, the mechanisms may be different. Basically lipid accumulation is related to disturbances in either the synthesis or the secretion of lipoproteins. Excess lipid can result from an oversupply of free fatty acids from adipose tissues or more commonly, from impaired release of triglycerides from the liver into the plasma. Triglycerides are secreted from the liver as lipoproteins (VLDL, LDL) (Figure 2.4) the onset of lipid accumulation in the liver is accompanied by changes in blood biochemistry and for this reason blood chemistry analysis can be a useful diagnostic tool.

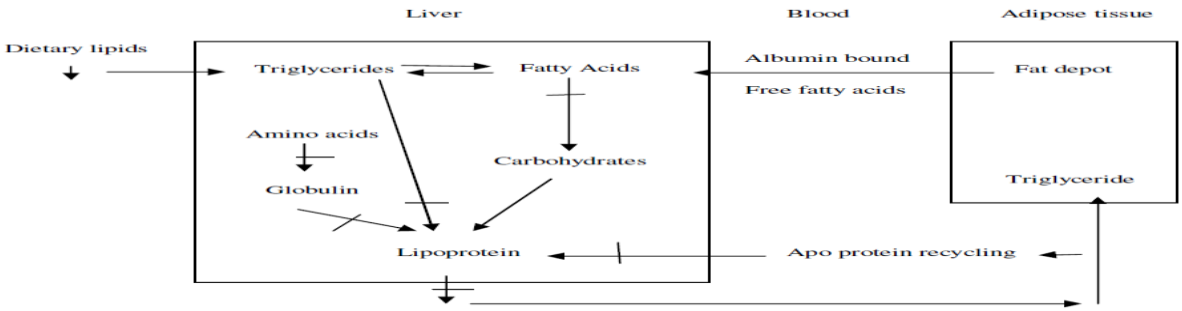


Figure 2.4: Triglyceride cycle in pathogenesis of fatty liver

2.9.2. Necrosis

Cell necrosis is a degenerative process leading to cell death. Necrosis, usually an acute injury, may be localized and affect only a few hepatocytes (focal necrosis), or it may involve an entire lobe (massive necrosis). Cell death occurs along with rupture of the plasma membrane and is preceded by a number of morphologic changes such as cytoplasmic edema, dilation of the endoplasmic reticulum, disaggregation of polysomes, accumulation of triglycerides, swelling of mitochondria with disruption of cristae and dissolution of organelles and nucleus. Biochemical events that may lead to these changes include binding of reactive metabolites to proteins and unsaturated lipids (inducing lipid peroxidation and subsequent membrane destruction) disturbance of cellular Ca^{+2} homeostasis, metabolic pathways, Na^{+} and K^{+} balance, and inhibition of protein synthesis. Changes in blood chemistry resemble those seen with fatty liver.

2.9.3. Apoptosis

Apoptosis is a controlled form of cell death that serves as a regulation point for biologic processes and can be thought of as the counterpoint of cell division by mitosis. Although apoptosis is a normal physiological process, it can also be induced by a number of exogenous factors, such as xenobiotic chemicals, oxidative stress, anoxia and radiation. (A stimulus that induces a cell to undergo apoptosis is known as an *apogen*). If, however, apoptosis is suppressed in some cell types, it can lead to accumulation of these cells. Apoptosis can be distinguished from necrosis by morphologic criteria.

2.9.4. Cholestasis

Cholestasis is the suppression or stoppage of bile flow and may have either intrahepatic or extrahepatic causes. Inflammation or blockage of the bile ducts results in retention of bile salts as well as bilirubin accumulation, an event that leads to jaundice. It can be also due to

change in membranes permeability of either hepatocytes or biliary canaliculi. Cholestasis is usually drug induced. Again, changes in blood chemistry can be a useful diagnostic tool.

2.9.5. Cirrhosis

Cirrhosis is a progressive disease that is characterized by the deposition of collagen throughout the liver. In most cases cirrhosis results from chronic chemical injury. The accumulation of fibrous material causes severe restriction in blood flow and in the liver's normal metabolic and detoxication processes. This situation can in turn cause further damage and eventually lead to liver failure.

2.9.6. Hepatitis

Hepatitis is an inflammation of the liver and is usually viral in origin, however, certain chemicals, usually drugs, can induce a hepatitis that closely resembles that produced by viral infections. Fortunately, the incidence of this type of disease is very low.

2.9.7. Oxidative Stress

Oxidative stress has been defined as an imbalance between the prooxidant/antioxidant steady state in the cell, with the excess of prooxidants being available to interact with cellular macromolecules to cause damage to the cell, often resulting in cell death. Former primarily as a by-product of mitochondrial electron transport. Superoxide, hydrogen peroxide, singlet oxygen and hydroxyl radicals can all arise from this source. Other sources include monooxygenases and peroxisomes. If not detoxified, reactive oxygen species can interact with biological macromolecules such as DNA and protein or with lipids. Once lipid peroxidation of unsaturated fatty acids in phospholipids is initiated, it is propagated in such a way as to have a major damaging effect on cellular membranes. The formation, detoxication by superoxide dismutase and by glutathione-dependent mechanisms, and interaction at sites of toxic action are illustrated in (Figure 2.5).

2.9.8. Carcinogenesis

The most common type of primary liver tumor is hepatocellular carcinoma, other types include cholangiocarcinoma, angiosarcoma, glandular carcinoma and undifferentiated liver cell carcinoma. Some naturally occurring liver carcinogens are Aflatoxin, Cycasin and Safrole. A number of synthetic chemicals have been shown to cause liver cancer in animals, including the dialkylnitrosamines, dimethylbenzanthracene, aromatic amines such as 2-naphthylamine and acetylaminofluorene and vinyl chloride.

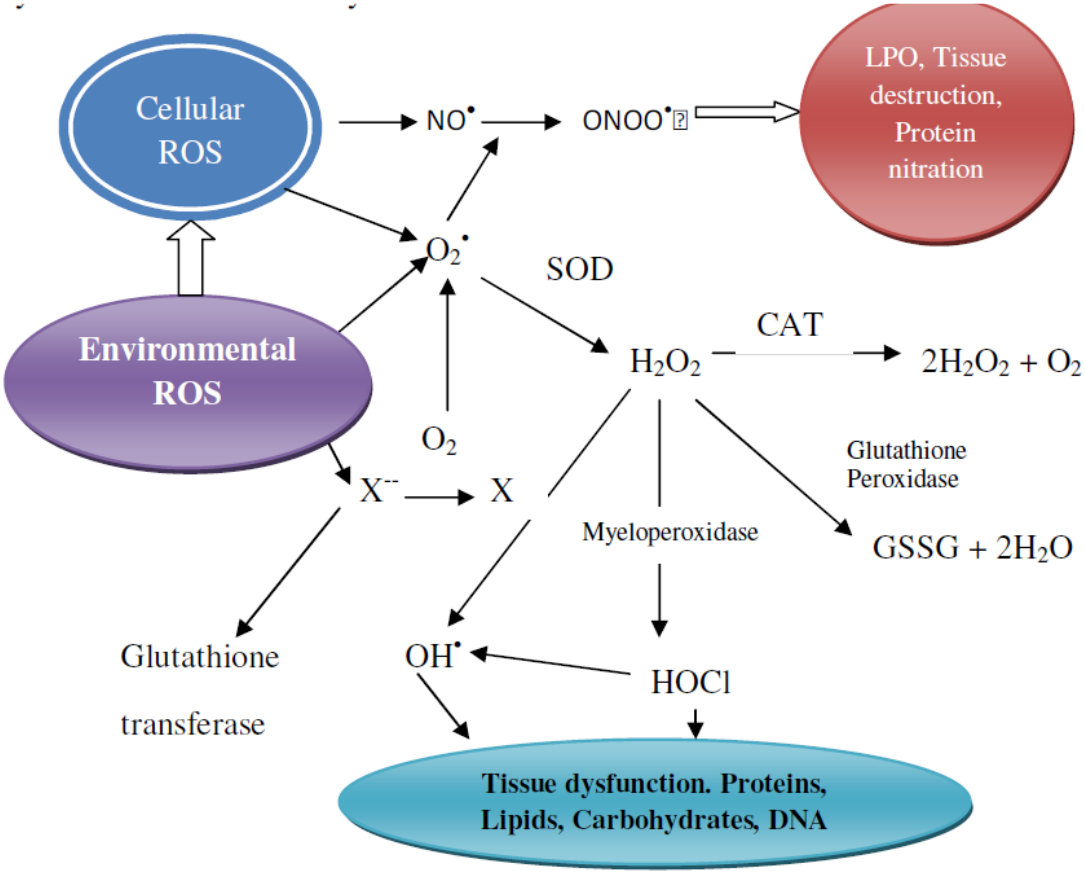


Figure 2.5: Molecular targets of oxidative injury

2.10. Carbon tetrachloride Induced Hepatotoxicity

Carbon tetrachloride has probably been studied more extensively, both biochemically and pathologically, than any other hepatotoxicant. First, CCl_4 in presence of P450 isozyme is converted to the trichloromethyl radical ($CCl_3^{\bullet}$) and then to the trichloromethylperoxy radical ($CCl_3O_2^{\bullet}$).

Such radicals are highly reactive and generally have a small radius of action. For this reason, the necrosis induced by CCl_4 is most severe in the centrilobular liver cells that contain the highest concentration of the P450 isozyme responsible for CCl_4 activation free radicals generated may participate in a number of events, such as covalent binding to lipids, proteins or nucleotides as well as lipid peroxidation. It is now thought that $CCl_3^{\bullet}$, which forms relatively stable adducts, is responsible for covalent binding to macromolecules and the more reactive $CCl_3O_2\bullet$, which is formed when $CCl_3\bullet$ reacts with oxygen, is the prime initiator of lipid peroxidation. Lipid peroxidation is the initiating reaction in a cascade of events, starting with the oxidation of unsaturated fatty acids to form lipid hydroperoxides, which then break down to yield a variety of end products, mainly aldehydes, which can go on to produce toxicity in distal tissues. For this reason, cellular damage results not only

from the breakdown of membranes such as those of the endoplasmic reticulum, mitochondria and lysosomes but also from the production of reactive aldehydes that can travel to other tissues. It is now thought that many types of tissue injury, including inflammation, may involve lipid peroxidation (Figure 2.6)

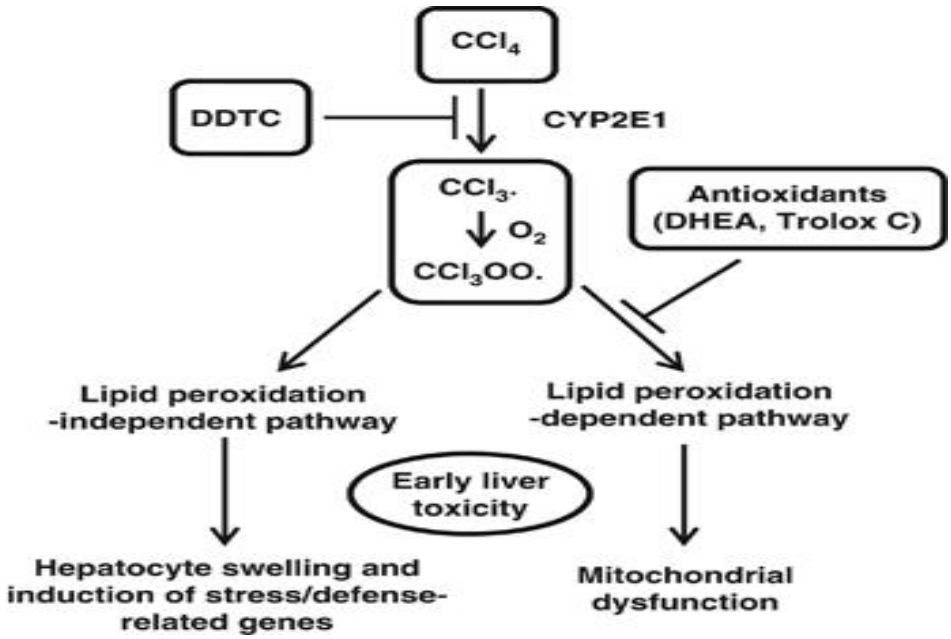


Figure 2.6: Mechanism CCl_4 cause Liver Toxicity

Administration of a single dose of CCl_4 to a rat, within 24 hrs it produces, a centrilobular necrosis and fatty changes(44). The poison reaches its maximum concentration in the liver within 3 hrs of administration. Thereafter, the level falls and by 24 hrs there is no CCl_4 left in the liver. The development of necrosis is associated with leakage of hepatic enzymes into serum. Dose of CCl_4: 0.1 to 3ml/kg i.p.

2.11. Hepatoprotective Medicinal Plants

In Ayurveda about 77 herbal drugs are used as hepatoprotective agents. There are different plants and their parts used for liver treatment (Table 2.1). A lot of medicinal plants, traditionally used for thousands of years, are present in group of herbal preparation of the Indian traditional health care system. In India, over 40 polyherbal commercial formulations reputed to have hepatoprotective action are being used. Scrutiny of the literature indicates that 160 phyto-constituents from 101 plant families have antihepatotoxic activity (Table 2.2).

Table 2.1: Hepatoprotective Medicinal Plants Mentioned In Ayurveda (45)

Sr. no.	Scientific Name	Family	Parts used
1	*Aconitum heterophyllum* wall.	Ranunculaceae	Root
2	*Aegal marmelos* Corr.	Rutaceae	Leaves
3	*Aegiceras corniculatum*	Aegicerataceae	Stem
4	*Allium sativum* Linn.	Liliaceae	Bulb
5	*Aloe barbadensis* Mill.	Ranunculaceae	Plant
6	*Andrographic paniculata* Nees.	Acanthaceae	Plant
7	*Aphanamixis polystachya* Wall. Parkar	Meliaceae	Bark
8	*Apium graveolens* Linn.	Umbelliferae	Seeds
9	*Berberis lycium* Royle.	Berberidaceae	Leaves
10	*Boerhaavia diffusa* Linn.	Nyctaginaceae	Root
11	*Calotropis gigantea* (Linn)R.Br	Asclepiadaceae	latex, flower, stem
12	*Carapa Guianensis* Aublet.	Meliaceae	latex, flower, stem
13	*Cephaelis ipecacuanha* Rich.	Rubiaceae	Draught
14	*Colchicum luteum* Baker.	Liliaceae	Corma
15	*Desmodium biflorum* Linn.	Fabaceae	Whole plant
16	*Eclipta alba* Hassk	Compositeae	Plant juice
17	*Euphorbia neriifolia* Linn	Euphorbiaceae	Fruit
18	*Ficus asperrima* Roxb.	Moraceae	Juice and bark
19	*Flacoutia indica* Merr.	Flacourtiaceae	Bilangra
20	*Fumaria parviflora* Lam.	Fumariaceae	Whole plant
21	*Garcinia indica* chois.	Guttiferae	Fruit
22	*Gymnema sylvestre* R. Br.	Asclepiadaceae	Leaves

23	*Hemidesmus indicus*	Asclepiadaceae	Leaves
24	*Hyssopus officinalis* Linn.	Labiatae	Plant
25	*Jatropha gossypifolia* Linn.	Euphorbiaceae	Leaves
26	*Lawsonia inermis* Linn.	Lythraceae	Bark
27	*Mentha longifolia* Linn.	Labiatae	Leaves
28	*Myristica fragrans* Houtt.	Myristicaeae	Seed
29	*Nelumbo mucifera* Gaertn.	Nymphaceae	Flower
30	*Phyllanthus niruri* Linn.	Euphorbiaceae	Plant
31	*Prunus armeniaca* Linn.	Rosaceae	Fruit
32	*Rhem emodi* Wall.	Polygonaceae	Rhizome
33	*Solanum indicum* Linn.	Solanaceae	Fruit, plant
34	*Swertia chirata* Buch-Ham.	Gentianaceae	Plant
35	*Terminalia chebula* Retz.	Combretaceae	Fruit
36	*Tinospora cordifolia* Willd.	Menispermacea	Stem

Table 2.2: Liver Protective Herbal Drug Along With Their Main Phytoconstituents

Sr. no.	Main phytoconstituent	Liver protective	Part used	References
1	Phenols	*Arnica Montana* Linn	Plant	(46)
		Cichorium intybus Linn.	Plant	(47,48)
		Picrorriza kurroa Royle	Plant	(49)
		Syzygium aromaticum Linn.	Plant	(50)
2	Coumarin	*Armillaria tabescens* Scop	Fungus	(51)

		Artemisiae capillaries herba	Plant	(52)
		Hemidesmus indicus	Roots	(53)
3	Lignans	*Schisandra chinensis* Turcz.	Fruit	(54)
		Schisandra sphenanthera	Fruit	(55)
		Silybum marianum Gaertn	Seed	(22,56)
		Thujopsis dolabrata	Leaves	(57)
4	Essential oil	*Anethum graveolens* Linn.	Fruit	(58)
		Apium graveolens Linn.	Seed	(59,60)
		Azadirachta indica	Leaves	(61)
		Carapa guianensi Aublet	Seed	(62)
		Cynara scolumus Linn.	Leaves, Flower	(63)
		Foenuculum vulgare Mill.	Plant	(64,65)
		Petroselinum sativum Hoffm.	Plant	(66)
		Pimpinella anisum Linn.	Plant	(67)
5	Monoterpens Sesquiterpens	*Murraya koenigii* Linn.	Rhizome	(68)
		Atractylodis lanceae	Root	(69)
		Lindera strychnifolia	Leaves	(70)
6	Diterpens	*Andrographis paniculata* Nees	Whole plant	(71,72)

7	Triterpens	*Glycyrrhiza glabra* Linn.	Root	(73,74)
		Hedyotis corymbosa Linn.	Whole plant Trunkwood	(75)
		Protium heptaphyllum Aubl.	Plant	(76)
		Sambucus chinesis Lindley	Leaves	(77)
		Tetrapanax papyriferus	(74)	(78)
8	Carotenoids	*Gardenia florida*	Fruit	(79)
9	Glycosides	*Aloe barbadensis* Mill	Leaves	(79)
		Dianthus superbus Linn	Plant	(73)
		Panax ginseng	Rhizome	(73)
		Polygonum cuspidatum	Root	(80)
		Polygonum multiflorum Thunb.	Root	(80)
10	Flavonoids	*Acacia catechu* Willd.	Hard wood	(81)
		Aegiceras corniculatum	Stem	(82)
		Artemisia capillaries Thunb.	Plant	(51)
		Calotropis gigantean R. Br.	Leaves	(83)
		Canscora decussate Roxb.	Plant and Juice	(84)
		Cassia occidentals Linn.	Leaves	(85)
		Clausena dentate Willd.	Plant	(86)

		Garcinia kola Heckel	Inflorescences	(87)
		Helichrysum arenarium Linn.	Plant	(88)
		Mentha longifolia Linn.	Leaves	(85)
		Phyllanthus emblica Linn.	Leaves	(89)
		Scrophularia grossheimi	Plant	(86)
		Tagetes patula Linn.	Seeds	(87)
		Uncaria gambir (Hunter)Roxb	Heartwood	(88)
11	Alkaloids	*Aristolochia clematis*	Plant	(90)
		Fumaria parviflora Lam.	Plant	(91)
		Fumaria officinalis Linn.	Plant	(91)
		Herniaria glabra Linn.	Whole Plant	(92)
		Peumus boldus Molina.	Plant	(93)
		Physalis peruviana	Plant	(94)
12	Xanthines	*Coffea Arabic*	Seed	(95)
		Thea sinensis	Leaves	(67,96)

2.12. Polyherbal medication

Combination of different plant extracts as polyherbal medication is new approach in treatment of liver disease, for example Liv 52 (Himalaya drug co. product), Livergen (Standard Pharmaceuticals), Tefroliv (TTK Pharma Pvt. Ltd), etc are generally use in India for hepatotoxicity treatment (97). Multiherbal formulation is used widely by different pharmaceuticals companies for treatment of liver toxicity.

2.12.1. Liv-52 (Himalaya drug co. India)

Liv 52 have been, product of Himalaya drugs co, reported for protection activity against liver paracetamol, ethanol, tert-butyl hydroperoxide and CCl_4 induced hepatotoxicity and other liver disease (98,99). Hepatoprotective mechanism of this drug is its antiperoxidative and free radical scavenging activity, due to this liv-52 has ability to restore activity of antioxidants, glutathione and superoxide dismutase which makes first line defense system against ROS and free radicals (100). Several in vivo and in vitro model (Hep G2 cell line for tert-butyl hydroperoxide induced toxicity) on liv-52 demonstrate that it play significant role in detoxification of drug and other xenobiotics (101,102). Plant that use in formulation of liv-52 medicine are *Achillea millefolium*, *Capparis spinosa*, *Cassia occidentalis*, Cinchorium *intybus*, *Solanum nigrum*, *Tamarix gallica*, *Thespesia populnea*, *Eclipta alba*, *Phyllanthus niruri*, *Boerhavia diffusa*, *Phyllanthus embilica*, *Fumaria officinalis*, *Terminalia chebula*, *Tinospora cordifolia*, *Andrographis paniculatamain* which is rich of phenol component mainly polyphenols that serve protective role in damaged hepatic cells via free radical salvage (98,103).

2.13. Profile of plants under investigation

2.13.1. Phytopharmacology of *Achillea millefolium L.*

2.13.1.1. Synonyms: (104)

- **Guj:** Biramjasif
- **Hindi:** Gandana
- **Eng:** Milfoil, Yarrow
- **Bombay:** Rojmari
- **Himachal Pradesh:** Biranjasif
- **Kashmir:** Akarkhara, chopandiga, momadru

2.13.1.2. Taxonomical Classification

- **Kingdom:** *Plantae*
- **Phylum:** *Tracheophyta*
- **Class:** *Magnoliopsida*
- **Order:** *Asterales*
- **Family:** *Compositae*
- **Genus:** *Achillea*
- **Specific epithet:** *millefolium - L.*
- **Botanical name:** *Achillea millefolium*

2.13.1.3. Distribution

Commonly distributed in the Himalayas from Kashmir to Kumaun at altitudes of 1,050-3,600 m; it has been seen growing in Bombay and Belgaum areas (105).

2.13.1.4. Description

Habit: An erect, perennial, pubescent herb

Stems: Erect, simple or branched, densely tomentose to glabrate.

Leaves: Petiolate or sessile; blades oblong or lanceolate 1-2-pinnately lobed faces glabrate to sparsely tomentose.

Heads: 10-100+, in simple or compound, corymbiform arrays. Receptacles convex; Ray florets 5-8, pistillate, fertile; corollas white or light pink to deep purple; Disc florets 10-20; corollas white to grayish white.

Fruit: Cypselae

Flowering: late Apr-early Jul (south), mid Jul-mid Sep (north).

Parts used: Whole plant (106).

2.13.1.5. Chemical constituents

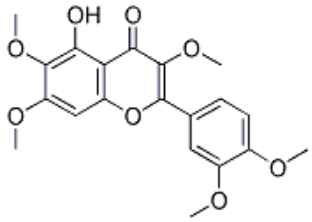
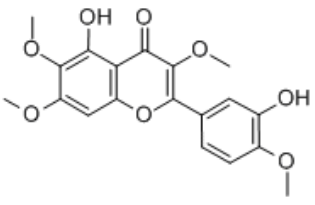
Herb contains an alkaloid, achilleine which has haemostatic principle reducing the blood-clotting time in rabbits. It also contains salicylic acid, β-sitosterol and its acetate, inositol, dulcitol, mannitol, betaine, choline, trigonelline, betonicine and stachydrine. Flavanoids, viz. luteolin-7-D-glucoside, apigenin, luteolin, cosmosin and luteolin-7-O-β-D-glucopyranoside and sesquiterpenic lactone, austricin. The presence of folic acid, quercetin, rutin and ascorbic acid in abundance. Leaves contain presence of rutin, apigenin, cosmosin, luteolin, and its glucoside (107).

2.13.1.6. Therapeutic uses

It is mild aromatic tonic, diaphoretic, stimulant, antispasmodic, emmenagogue and astringent. It is used in colds, obstructed perspiration and at the commencement of fevers. It is antiseptic, used in flatulent, heart burn, hysteria, epilepsy and rheumatism. It suppresses haemorrhage and profuse mucous discharge, its tincture in small and dilute doses, stops bleeding from lungs, kidney or nose, but in larger dose aggrevates bleeding. Decoction of fresh juice applied to cuts, bruises, piles, varicose veins and ulcer. Herb used as anti-inflammatory, stimulates gastric secretion, regulatory and antiseptic for menstrual troubles. In large doses, it produces headache and vertigo (105,108).

2.13.1.7. Review of *Achillea millefolium L.*

Sr. no	Details	Reference
	Pharmacognostical review	
1	Ultrastructure of the glandular cells of the floret secretory trichomes was examined before and after anthesis and compared with the ultrastructure of the cells from the cell suspension culture.	(109)
	Phytochemical review	
2	An extract of *Achillea millefolium* herb (YE) was investigated for antioxidant activity using chemical and biological assays. Qualitative and quantitative analysis of some major phenolics was carried out by HPLC which possesses significant antiradical activity which is due to the presence of active phenolic components.	(110)
3	Production of *Achillea millefolium* L. at different locations in Norway was investigated with regard to the developmental stage. The oil content differed greatly between the vegetative stage (0.13%) and the stage of full bloom (0.34%). Changes in the composition of yarrow essential oil were found to be related to maturation of the plant, with increasing amounts of monoterpenes in relation to the sesquiterpene. However, a clear trend could be detected only for the monoterpenic compounds with	(111)

	increasing levels of pinene thujone and decreasing levels of sabinene, borneol, and bornyl acetate.	
4	From ground parts proline, betaine, stachydrine, betonicine and choline were isolated as the major nitrogen containing compounds. The TLC screening of 11 different species showed qualitatively identical betaine patterns but quantitative differences were observed.	(112)
5	Volatile constituents of *Achillea millefolium* growing wild in Greece were studied by means of GC and GC-MSIE. The main constituents were borneol, camphor, 1,8-cineole and lavandulol. Thirty-eight constituents have not been previously reported in the essential oil of the species, whereas 20 are new for the genus. A comparison of the main volatile constituents were done in which oils revealed great infraspecific variation occurs.	(113)
6	Column chromatography on silica gel using flowering heads petroleum ether extract of *Achillea millefolium* L. allowed three flavones to be separated and identified. Spectral studies (PMR, mass spectrometry, and UV) and a comparison with data for compounds reported in the literature established the flavones as 5-hydroxy-3,6,7,4'-tetramethoxyflavone, artemetin, and casticin.  Casticin  Artemetin	(114)
Pharmacological review		
7	The effects of Achillea millefolium on wound healing in second-degree burns formed in diabetic rats was carried out using 20 Sprague Dawley rats were divided into 2 groups. Group I (n=10 rats) was the Control group and contained the diabetic rats with burn injury. Group II (n=10 rats) was the group where burn injury was created and Achillea	(115)

	millefolium was administered to diabetic rats. Achillea millefolium contributes to wound healing in burn injury due to its antioxidant and anti-inflammatory properties	
8	The volatile oil of the *A. millefolium* wild plant grown in France is quite an effective antioxidant in sunflower oil oxidation; it also possesses inhibitory effects against famous bacteria and fungi.	(116)
9	The effect of salicylic acid foliar application on biomass production and the synthesis of secondary compounds in yarrow (*Achillea millefolium L.* - Asteraceae). The experiment was conducted in potted plants under greenhouse conditions.	(117)
10	Different extracts of yarrow - inflorescences and upper leaves were investigated for their total polyphenolic content and antioxidant activity using several reliable assays, namely DPPH-, ABTS-, FRAP- and CUPRAC assays. The phytochemical profile of the extracts was assessed by RP-HPLC methods as well in order to evaluate the influence of the single constituents.	(118)
11	Effects of *Achillea millefolium* extract on growth of primary rat vascular smooth muscle cells also role of estrogen receptors in this process showed vasoprotective effect.	(119)
12	The cytotoxic and genotoxic effects of aqueous extracts from yarrow leaves on *Lactuca sativa* (lettuce) root tip meristem cells by cytogenetic studies was carried out.	(120)
13	The crude extract was studied for its hepatoprotective activity against D-galactosamine and lipopolysaccharide induced hepatitis and antispasmodic effect in mice.	(121)
14	Solid Phase Microextraction and Gas chromatography were used to determine volatile oil composition of the *A. millefolium* L. Human skin fibroblasts viability based on Neutral Red and spectrophotometrical-3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide methods while morphological analysis was performed in vitro cell culture. Free	(122)

	radical scavenging activity of ethanol, ethyl acetate and water extracts of *A. millefolium* L. was also measured.	
15	*Achillea millefolium* L. is widely used not in treatment of hepato-biliary disorders, gastro-intestinal and antiphlogistic drug. Various proteases, matrix metalloproteinases (MMP-2 and -9) and human neutrophil elastase (HNE), are associated with the inflammatory process, the purpose of study was to evaluate crude plant extract in-vitro-protease inhibition assays for understanding the mechanisms of anti-inflammatory action.	(123)
16	Evaluated of aqueous extract from leaves of *Achillea millefolium* L. on reproductive endpoints in Wistar rats.	(124)
17	The in vitro antimicrobial and antioxidant activities of the essential oil and methanol extracts of *Achillea millefolium* (Asteraceae) were investigated. GC-MS analysis of the essential oil resulted in the identification of 36 compounds constituting 90.8% of the total oil.	(125)
18	The effect of flowers ethanolic and hydroalcoholic extract on the spermatogenesis of Swiss mice was studied by evaluating morphologic characteristics with the light and electron microscopes. The alterations were observed in seminiferous tubule vacuolization, germ cell necrosis, and exfoliation of immature germ cells. I showed increase in number of metaphases in the germ epithelium which may be due to cytotoxic substances or substances stimulating cell proliferation.	(126)
19	More than 30 samples of the three subspecies of *Achillea millefolium* have been studied for their leaf and flower head flavonoid composition along with safety and antiulcer efficacy studies.	(127)
20	An aqueous extract of the dry flower heads showed anti-inflammatory activity as measured by the mouse paw edema test. Fractionation has resulted in the isolation of a material which reduces inflammation by 35 %.	(128)

2.13.2. Phytopharmacology of *Thespesia populnea L.*

2.13.2.1. Synonyms

- **Sans:** Parisha, Gardha-bhanda
- **Hindi:** Paras-pipal, Parsipu
- **Guj:** Parusa-pipalo
- **Eng:** Portia tree
- **Beng**: Dumbla, parespipal
- **Mar**: Parsacha-jhada, bhendi-ke-jhar
- **Tel**: Gangaraavi
- **Kan**: Hoovarase, kandarola
- **Mal**: Poovarasu
- **Oriya**: Gujausto, porosopippali(129)

2.13.2.2. Taxonomical Classification:

- **Kingdom:** *Plantae*
- **Class:** *Magnoliopsida*
- **Subclass:** *Rosidae*
- **Superorder:** *Malvanae*
- **Order:** *Malvales*
- **Family:** *Malvaceae*
- **Subfamily:** *Malvoideae*
- **Tribe:** *Gossypieae*
- **Genus:** *Thespesia*
- **Specific epithet:** *populnea* - (L.) Soland. ex Correa
- **Botanical name:** - *Thespesia populnea*

2.13.2.3. Distribution

A common strand plant, extending from the shores of West Bengal to Peninsular India and the Andamans. Coast forests of Burma and India largely grown as a road-side tree in tropical regions. Cultivated in South Gujarat (130).

2.13.2.4. Description

Habit: A fairly large, quick growing, evergreen tree upto 18m in height with greyish brown fissured bark.

Leaves: Simple, alternate, long petioled, cordate, entire, acuminate, prominent nerves 5-7 with peltate scales on both surface.

Flower: Yellow with purple base slowly changing purple on withering solitary, axillary; Pedicel 2.5-6 cm, Cordate, entire, densely scaly. Epicalyx lobes 3-4, filiform-lanceolate, 8-10 mm, usually caducous, scaly. Calyx cup-shaped; Corolla yellow with purple base, campanulate.

Fruits: Brown, globose or oblong brown capsules covered with minute peltate scale, pubescent, channeled along the back (131).

Flowering: Spring.

Part Used: Fruits, Flowers, Leaves, Bark and Root.

2.13.2.5. Chemical constituent

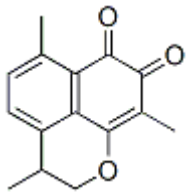
The plant yields kaempferol and its glycosides, hebacetin and its glucoside, populneol, populnin, papulnetin, quercetin, rutin, gossipetin, (+) gossypol, β-sitosterol and its glucoside, lupeol, lupenone, alkanes, myricyl alcohol, calycopetrin, sesquiterpenoidal quinones viz. thespesone, thespone, mansonones C, D, E, and F, amino fatty acid composition of used oil is also reported (132)

2.13.2.6. Therapeutic uses:

The plant is astringent acrid, antiinflammatory, haemostatic, antidiarrhoeal and antibacterial, it is widely used in dermatopathy such as ring worm, guinea worm, leprosy, urethritis, gonorrhoea, haemorrhoids, ulcers, cholesterol, diabetes, cough and asthma (133).

2.13.2.7. Review of *T. populnea L.*

Sr. no	Details	Reference
	Phytochemical review	
1	The plant extract evaluated for phytochemical and chromatographic analysis. HPLC fingerprint was carried out, which can be used for correct identification of the plant. The plant extract contains alkaloids, tannins, terpenoid and steroid. The present study provides evidence that	(134)

	solvent extract of *Thespesia populnea*. Contain medicinally important bioactive compounds.	
2	From dichloromethane extracts of the wood and dark heartwood of *Thespesia populnea* eight new sesquiterpenoids, were isolated named populene A-H (1-8), along with 11 other known compounds by spectroscopic analysis their structures were determined. The cytotoxic activity was also performed using isolated compounds was against four cancer cell lines: MCF-7, HeLa, HT-29, and KB.	(135)
3	Isolation of 7-hydroxycadalene, thespesenone, and dehydrooxoperezinone-6-methyl ether from its red heartwood. Several other sesquiterpene quinones were isolated including mansonone E, mansonone F, mansonone D, mansonone G, mansonone M, and thespesone; the 13C NMR spectra of these compounds are reported for the first time.  Mansonone E	(136)
4	Five sesquiterpenoid quinones were isolated from the aerial parts of *Thespesia populnea* L (Malvaceae) cultivated in Egypt. Their structures were determined on the basis of spectral data. The Mansonones were identified as: Mansonones C, G, E, H and 7-hydroxy-2,3,5,6-tetrahydro-3,6,9-trimethylnaphtho[l,8-b,c]pyran-4,8-dione.	(137)
5	Hepatotoxic activity using the CCl_4 model of liver injury of ethanolic fractions administered orally to groups of rats was evaluated which showed significant activity. A rare flavanoid, quercetin-7-O-rhamnoglucoside, was isolated from this plant and its identity confirmed by spectral studies.	(138)
	Pharmacological review	
6	Seeds were successively extracted unsaponifiable matter and fatty acids were separated from seed oil. A GC–MS analysis of fatty acid methyl esters was carried out. Using carrageenan model dor Acute arthritis	(139)

	activity by sub-plantar injection of carrageenan into the left hind paw of rats. The paw volume was measured using plethysmometer. Analgesic activity was assessed by heat induced pains and antipyretic activity assessed using brewers yeast-induced pyrexia model.	
7	From the aqueous extract three fractions namely ethyl acetate fraction, methanolic fraction and residue fraction were made and studied for antidiarrheal activity. Antidiarrheal activity of the fractions were evaluated in castor oil induced diarrhea, prostaglandin E2 (PG-E2) induced diarrhea and charcoal meal test as in vivo models and the most potent fraction was further evaluated with in vitro models to determine the possible antimotility effect.	(140)
8	The water suspension (500 mg/kg b.wt.) of leaf, flower and stem bark of *T. populnea* showed varying levels of protective action against CCl4-induced liver damage as evidenced from significant reduction in the activities of serum marker enzymes for liver damage (alanine transaminase, aspartate transaminase, and alkaline phosphatase), and bilirubin levels when compared with CCl4-intoxicated control rats. The stem bark suspension showed maximum hepatoprotection compared with leaf and flower. An ethanol extract of the stem bark was more active than n-hexane and water extracts, showing remarkable protection at a dose of 60 mg/kg b.wt. The hepatoprotective effect of this extract was almost comparable to that of silymarin (100 mg/kg), a reference herbal drug.	(141)
9	The ethanolic extract of *Thespesia populnea* bark (TPE) was investigated for anti-inflammatory and analgesic activity at the doses (p.o.) of 100, 200 and 400 mg/kg body weight. For evaluation of inflammation carrageenan, histamine and serotonin-induced paw edema served as acute models and formaldehyde-induced arthritis served as a chronic model in rats. The acetic acid-induced writhing response and formalin-induced paw licking time in the early and late phases of mice were used to assess analgesic activity. The higher doses of TPE (200 and 400 mg/kg, p.o.) were inhibiting carrageenan, histamine and serotonin-	(142)

	induced paw edema as well as formaldehyde-induced arthritis successfully. In addition, TPE (200 and 400 mg/kg, p.o.) significantly attenuated the writhing responses induced by an intraperitoneal injection of acetic acid and late phase of pain response induced by an subplantar injection of formalin in mice.	
10	The ethanolic extract was administered orally in three doses (100, 200 and 400 mg/kg) for 7 successive days to different groups of young and aged mice, diminished cholinergic transmission and increased cholesterol levels appear to be responsible for development of amyloid plaques and dementia in Alzheimer patients.	(143)
11	Ethanol and aqueous extract of *Thespesia populnea* exhibited significant antihyperglycemic and antihyperlipidemic effects on alloxan-induced diabetic rats.	(144)
12	Anti implantation activity was studied using petroleum-ether and ethyl acetate and subsequent crude alcoholic extract of seeds of *T. populnea* in female albino rats.	(84)
13	Antioxidant activity of the aqueous and methanolic extracts of the *Thespesia populnea* bark was investigated in rats by inducing liver injury with carbon tetrachloride: olive oil (1:1). The extracts exhibited significant antioxidant activity showing increased levels of glutathione peroxidase, glutathione S-transferase, glutathione reductase, superoxide dismutase and catalase and decreased level of lipid peroxidation.	(145)
14	Wound healing activity in the excision wound and incision wound models in rats following topical and oral administration was carried out using Aqueous extract of fruit.	(146)
15	Aqueous extract three fractions namely ethyl acetate fraction, methanolic fraction and residue fraction were made and studied for antidiarrheal activity. Antidiarrheal activity of the fractions were evaluated in castor oil induced diarrhea, prostaglandin E2 (PG-E2) induced diarrhea and charcoal meal test as in vivo models and the most potent fraction was	(140,147)

	further evaluated with in vitro models to determine the possible antimotility effect.	
16	Antimicrobial activity of bark was investigated by well diffusion method against four bacteria and two fungi (*Escherichia coli*, *Candida albicans*). Petroleum ether extract showed significant activity against all organisms whereas ethanolic and aqueous extracts showed moderate to mild activity.	(148,149)
17	Anti-steroidogenic activity was screened in female albino mice. Cholesterol and ascorbic acid content in ovaries was evaluated and weight of ovaries and uterus was also reduced.	(150)
18	The shade dried bark powder of *Thespesia populnea* was subjected to successive extraction using the solvents (Pet. ether, chloroform, alcohol and water). The diuretic potential of various extract of the barks was assessed in albino rats. The Volume of urine, urinary concentration of Na+, K+ and Cl- ions were studied.	(151)

Chapter - 3

GOAL

&

OBJECTIVES

CHAPTER-3

3. Goal and Objectives

The study is targeted for the development of quality parameters for aerial part of *Thespesia populnea L.*, aerial part of *Achillea millefolium L.*, which are reported to possess hepatoprotective activity are highly valued and are ingredients of some of well known Herbal formulations used in the treatment of hepatic disorders.

3.1 Procurement and authentication of plant materials: Collection of plant material and authentication of the collected plant was done by the Botanist and even by comparing pharmacognostical parameters of it like morphological evaluation, microscopical evaluation and physical evaluation.

3.2 Preparation of Extracts: Extracts was prepared by hot percolation extraction technique and by using different solvents.

3.3 Standardization of Extracts: Standardization was done by chromatographic technique i.e HPTLC method by performing method development and validation parameters using a marker compounds and also by using LC-MS/MS technique by performing method development and validation parameters using a marker compounds

3.4 Invivo hepatoprotective activity: Hepatoprotective activity was performed using CCl_4-induced hepatotoxicity in rat model in which Male wistar rats was divided into seven groups (Control, Model, Standard, test group of one plant with two dose of it, test group of second plant two dose of it). Biochemical parameters: Aspartate aminotransferase (AST), alanine aminotransferase (ALT), Total bilirubin and total protein. Histophathology Study.

Chapter - 4

MATERIALS AND METHODS

CHAPTER-4

4. Materials and Methods

Sr. No	Chemicals/ reagents/ consumables/ Solvents	Grade/Purity	Manufacturers
1	Methanol	AR	Merck, Mumbai
2	Distill Water	-	Insitu prepare
3	Toluene	AR	S.D. Fine Chem. Ltd., Mumbai
4	Ethyl Acetate	AR	Merck, Mumbai
5	Formic acid	AR	S.D. Fine Chem. Ltd., Mumbai
6	Petroleum ether	AR	S.D. Fine Chem. Ltd., Mumbai
7	Acetone	AR	S.D. Fine Chem. Ltd., Mumbai
8	Chloroform	AR	S.D. Fine Chem. Ltd., Mumbai
9	Apigenin	97.8% HPLC purity	Natural remedies, Banglore
10	Luteolin	98.1% HPLC purity	Natural remedies, Banglore
11	Quercetin	99.0% HPLC purity	Natural remedies, Banglore
12	Kaempferol	97.7% HPLC purity	Natural remedies, Banglore
13	Lupeol	98.2% HPLC purity	Natural remedies, Banglore
14	β-sitosterol	90.5% HPLC purity	Natural remedies, Banglore
15	Methanol	98.99% HPLC	S.D. Fine Chem. Ltd., Mumbai

16	Acetonitrile	Lichrosolv	Merck, Mumbai
17	Hydrochloric acid, pure	AR	Merck
18	Mili-Q Water	-	In house
19	Glacial acetic acid	HPLC	Rankem
20	Diethyl ether	AR	S.D. Fine Chem. Ltd., Mumbai
21	n-hexane	AR	S.D. Fine Chem. Ltd., Mumbai
22	Dichloromethane	AR	S.D. Fine Chem. Ltd., Mumbai
23	Ammonium formate	AR	Merck, Mumbai
24	Ammonia	GR	S.D. Fine Chem. Ltd., Mumbai
25	Sodium hydroxide	AR	S.D. Fine Chem. Ltd.
26	Sulphuric acid	AR	S.D. Fine Chem. Ltd.
27	Nitric acid	AR	S.D. Fine Chem. Ltd.
28	Carbon tetrachloride	AR	S.D. Fine Chem. Ltd.
29	Formaldehyde	AR	S.D. Fine Chem. Ltd.
30	SGPT, SGOT, Total bilirubin, Total protein evaluation kit	I-CHEM-80, CPC-1	Panorama marketing
31	Phloroglucinol	AR	S.D. Fine Chem. Ltd.
32	Glycerin	AR	S.D. Fine Chem. Ltd.
33	Sodium carbonate	AR	S.D. Fine Chem. Ltd.
34	Silymarin	-	Local market
35	Acetic acid	AR	S.D. Fine Chem. Ltd.
36	Picric acid	AR	S.D. Fine Chem. Ltd.
37	Iodine	AR	S.D. Fine Chem. Ltd.

38	Ammonia	AR	S.D. Fine Chem. Ltd.
39	Ferric chloride	AR	S.D. Fine Chem. Ltd.

4.1. Pharmacognostic studies

4.1.1. Identification and storage of Plant materials

Fresh & fully grown plants of *Thespesia populnea L.* collected from, Vallabh Vidhyanagar in the month of March and its authentication was confirmed by taxonomist, Dr. A. S. Reddy, Prof. and Head of Botany Dept., Sardar Patel University, Vallabh Vidyanagar and deposited this plant (voucher specimen no. APC/12-13/01), to Dept. of pharmacognosy, Anand Pharmacy College, Anand. Aerial parts of *Achillea millefolium L.* were collected from Himachal Pradesh in the month of September. Dried aerial parts of *A. millefolium* and *T. populnea* were separately milled into powder with the aid of an electrical grinder, Kenster Super, passed through sieve no. 60 and finally stored in airtight bottles in a dry and dark place before analysis.

4.1.2. Macroscopic and Microscopic examination (29,152)

4.1.2.1. Aerial parts of *A. millefolium L.*

The macroscopic examination was carried out with the help of naked eyes and simple hand lens for the evaluation of shape, size color and fracture. For powder microscopy, the aerial parts were finely powdered and screened for the presence of its own and for vegetative matters (other than the organ selected for the research studies). The powdered was passed through sieve No. 180 to obtain fine powder and then subjected for microscopic examination using projection microscope. The sample was treated with reagents like 50% glycerin as temporary mountant; 2% phloroglucinol in ethanol (90%) and concentrated hydrochloric acid (1:1) for lignin.

4.1.2.2. Aerial parts of *T. populnea.*

The macroscopic examination was carried out with the help of naked eyes and simple hand lens for evaluation of the shape, size, color and fracture. For microscopic evaluation, the fresh leaf, petiole and stem was used and then free hands sections were taken. The resulting section were boiled in chloral hydrate for 10-12 min to clear of interfering pigments in the

tissues. The sections were then treated with phloroglucinol and concentrated hydrochloric acid for 10 min and then observed using projection microscope, according to the method described in (153–155)

4.1.2.3. **Quantitative microscopy** (156)

The important identifying characteristic of leaf constants like Stomatal Number, Stomatal Index, Vein-islet number, Vein termination number were found out.

4.1.2.3.1 Stomatal number

It is the average number of stomata per square mm of the epidermis of the leaf.

Procedure:

Clear lamina portion of leaf piece by boiling with clearing solution. Peel out upper and lower surface bys using forceps. Mount it on slide using glycerin as moisturising agent Calibrate camera lucida and drawing board using stage micrometer, draw square of 1mm. Place the prepared slide on the stage. Trace the stomata and epidermal cell. Count number of stomata seen in the specified area and calculate the average number of stomata per sq. mm.

4.1.2.3.2 Stomatal index

Stomatal index is the percentage which the number of stomata forms to the total number of epidermal cells, each stomata being counted as one cell. Stomatal index can be calculated by using following equation.

$$I = \frac{S}{E + S} * 100$$

Procedure:

Clear lamina portion of leaf piece by boiling with clearing solution. Peel out upper and lower surface bys using forceps. Mount it on slide using glycerin as moisturising agent Calibrate camera lucida and drawing board using stage micrometer, draw square of 1mm. Place the prepared slide on the stage. Trace the stomata and epidermal cell. Count number of stomata seen in the specified area and calculate the the stomatal index using the above formula.

4.1.2.3.3 Vein-islet number

A vein-islet is the small area of green tissue surrounded by the veinlets. The vein-islet number is the average number of vein-islets per square millimeter of a leaf surface. It is determined by counting the number of vein-islets in area of 4 sq. mm. of the central part of the leaf between the midrib and the margin.

Procedure:

Clear lamina portion of leaf piece by boiling with clearing solution for about half hour Mount it on slide using glycerin as moisturising agent Calibrate camera lucida and drawing board using stage micrometer, draw square of 1mm. Place the prepared slide on the stage. Trace the stomata and epidermal cell. Count the number of vein islets in the square millimeter. Find average number of vein-islet from four adjoining square.

4.1.2.3.4 Veinlet termination number

Veinlet termination number is defined as the number of veinlet termination per sq. mm of the leaf surface, midway between midrib of the leaf and its margin. A vein termination is the ultimate free termination of veinlet.

Procedure:

Clear lamina portion of leaf piece by boiling with clearing solution for about half hour Mount it on slide using glycerin as moisturising agent Calibrate camera lucida and drawing board using stage micrometer, draw square of 1mm. Place the prepared slide on the stage. Trace the stomata and epidermal cell. Count the number of vein termination in the square millimeter. Find the average number of veinlet termination number from the four adjoining squares.

4.1.2.3.5 Palisade ratio

It is the average number of palisade cells beneath each epidermal cell.

Procedure:

Middle part of the leaf was cleared by boiling with chloral hydrate solution. Upper and lower epidermis were peeled out separately with the help of forceps & kept it on slide and mounted in glycerin water. With the help of micrometer, 1mm square was drawn. Number of stomata and epidermal cell which were present in the area of 1 sq.mm were counted.

4.1.3. Flourescence Analysis

Powder of aerial parts of *A. millefolium* and *T. populnea* were treated with various solvents and were examined immediately in visible light and UV lights (254 and 366 nm) using UV cabinet for fluorescence behavior. (157,158)

1. Methanol
2. 1M sodium hydroxide in methanol
3. 1M sodium hydroxide in water
4. 1M hydrochloric acid in methanol
5. 1M hydrochloric acid in water
6. 50% nitric acid in methanol
7. 50% sulphuric acid in methanol
8. Nitric acid
9. Acetic acid
10. 1% picric acid
11. 10% $K_2Cr_2O_7$
12. 5% Iodine
13. Dilute ammonia
14. 5% ferric chloride reagent

4.1.4. Proximate analysis

Proximate analysis included total ash, Insoluble ash, water soluble ash, solvent extractive value (32,153,159).

4.1.4.1. Ash values

4.1.4.1.1 Determination of total ash:

Weigh 2 gm of powdered sample in crucible and incinerate it at 500-600^oC in a muffle furnace till it obtains carbon free ash. It was then cooled, weighed and percentage of ash was calculated with reference to the air-dried drug.

4.1.4.1.2 Determination of acid insoluble ash:

Boil for 5 min total ash obtained with 25 ml of 2M hydrochloric acid and filtered it by ashless filter paper to collect insoluble matter. Wash the ash obtained by hot water and along with the filter paper incinerate it to a constant weight in a muffle furnace. The percentage of acid-insoluble ash was calculated with reference to the air-dried powered drug (60#).

4.1.4.1.3 Determination of water soluble ash:

Boil for 5 min total ash obtained with 25 ml of water and filtered it, the insoluble matter collected on an ash-less filter paper was washed with hot water and incinerated for 15 min at a temperature not exceeded 450ºC in a muffle furnace. Difference in weight of ash and weight of water insoluble matter gave the weight of water-soluble ash. The percentage of water-soluble ash was calculated with reference to the air-dried powered drug.

4.1.4.2. Determination of extractive values:

5 g of the air-dried powdered material were macerated with 100 ml of solvent in a closed flask for 24 hr, shaking frequently at an interval of 6 hr. It was then allowed to stand for 18hr and filtered rapidly to prevent any loss during evaporation. 25 ml of the filtrate was evaporated to dryness in a porcelain dish and dried at 105˚C to a constant weight. The percentage of soluble extractive was calculated with reference to the air-dried drug.

4.2. Phytochemical studies

4.2.1. Determination of Total Phenolic content and total flavonoid content.

4.2.1.1. Preparation of standard and sample solutions

4.2.1.1.1 Standard solution of gallic acid

25 mg of gallic acid accurately weighed was dissolved and diluted using methanol in 25 ml volumetric flask. Finally, the solution was further diluted to prepare a concentration of 100 µg/ml.

4.2.1.1.2 Standard solution of quercetin

25 mg of quercetin accurately weighed was dissolved and diluted using methanol in 25 ml volumetric flask. Finally, the solution was further diluted to prepare a concentration of 100 µg/ml.

4.2.1.1.3 Extract of aerial parts of *A. millefolium* and *T. populnea.*

Macerate for 24 hours using 1 gm of air dried powder with 100 ml methanol and filter it. Make up final volume of the filtrate using methanol. 5 ml of this extract was diluted with an equal volume of methanol

4.2.2. Estimation of Phenolic content (160)

To 01 ml of the methanolic extract were added 10 ml of distilled water and 1.5 ml of diluted (1:2) folin ciocaulteu reagent and the mixture was kept aside for 5 minutes. After adding 4 ml of 20% Na_2Co_3 solution the final volume was adjusted to 25 ml using distilled water. The absorbance was measured at 765 nm at an interval of 30 minutes upto 2 hours using distilled water as a blank.

The data was compared with similarly prepared set of standard substance gallic acid in concentration range of 50 µg to 300 µg per 25 ml.

The total phenol content was measured using following formula:

C = A*282.6 -8.451 (A = absorbance)

The reaction product showed a maximum value at a wavelength of 540 nm.

4.2.3. Total flovonoid content (160,161)

According to colorimetric aluminum chloride method, diluted standard solutions of quercetin (0.5ml, concentration range of 2 to 10 µg/ml) were separately mixed with 1.5 ml of 95% ethanol, 0.1 ml of 10% aluminium chloride, 0.1 ml of 1M potassium acetate and 2.8 ml of distilled water. After incubation at room temperature for 30 min, the absorbance of the reaction mixture was measured at 415 nm with a shimadzu double beam UV/Visible spectrophotometer 1650PC. The amount of 10% aluminium chloride was substituted by the same amount of distilled water in blank. Similarly, 0.5 ml of methanol extracts were reacted with aluminium chloride for the determination of flavonoid as described above. Total flavonoid content was calculated from calibration curve and reported as quercetin equivalent (%w/w).

4.3. HPTLC Analysis of *A. millefolium* and *T. populnea*

4.3.1. Instrument

win CATS version 1.4.6 software:	Camag, Switzerland
Microsoft Excel 2010:	Microsoft Corporation, USA
Linomat 5 applicator:	Camag, Switzerland
Micro-syringe:	Linomat syringe 100 µl, Hamilton-Bonaduz Schweiz, Camag, Switzerland

Pre-coated silica gel 60 F_{254} HPTLC aluminium plates:	10 x 10 cm, 100 μm thickness; Merck, Germany
Twin trough chamber:	10 x 10 cm; Camag, Switzerland
UV chamber and TLC scanner 4:	Camag, Switzerland
Detection:	Camag TLC Scanner 3 with D2 and Hg lamp

4.3.2. Experimental conditions

Stationary phase:	Pre-coated silica gel 60 F254 HPTLC aluminium plates (10× 10 cm, 0.2 mm thick), E. Merck KG a A
Mobile Phase:	Toluene: Ethyl acetate: Formic acid (7:3:0.3 v/v/v)
Application position:	8.0 mm
Band length:	6.00 mm
Preconditioning:	Saturation with mobile phase for 15 min
Mobile Phase volume:	10 ml
Measurement mode:	Absorption/ Flourescence
Scan speed:	20 mm/sec

4.3.3. HPTLC Fingerprint Profile of *A. millefolium L.*

4.3.3.1. Preparation of Standard and Sample Solutions

4.3.3.1.1 Standard solution of apigenin

Standard stock solution (1 mg/ml) of apigenin was prepared in methanol. Working solution of apigenin (1000 µg/ml) was prepared by appropriate dilutions of the stock solution with methanol.

4.3.3.1.2 Standard solution of luteolin

Standard stock solution (1 mg/ml) of luteolin was prepared in methanol. Working solution of luteolin (1000 µg/ml) was prepared by appropriate dilutions of the stock solution with methanol.

4.3.3.1.3 Standard solution of quercetin

Standard stock solution (1 mg/ml) of quercetin was prepared in methanol. Working solution of quercetin (1000 µg/ml) was prepared by appropriate dilutions of the stock solution with methanol.

4.3.3.1.4 Methanol extract of aerial part of *A. millefolium L.* (MTAM)

The aerial part milled to coarse powder (1 g) was macerated with methanol for 3 hr, filtered and filtrate was diluted to 10 ml with methanol in volumetric flask. The solution was filtered through a 0.45 µm membrane filter.

4.3.3.1.5 Ethyl acetate extract of aerial part of *A. millefolium L.* (EAMTAM)

MTAM was dissolved by 2N HCl: toluene (1:1 v/v) and than reflux for three hours in reflux assembly it is than neutralized with 5-10% Na_2CO_3 and refluxed for half hour. Allow the layers to separate. Collect aqueous layer and extract with ethyl acetate. Take ethyl acetate solution and evaporate it. Stock solution was prepared by weighing 100 mg of the sample then quantitatively transferred to 100 ml volumetric flask and volume was adjusted with methanol, kept in the refrigerator and tightly closed.

4.3.3.2. Selection of wavelength

Standard 8µl solution was applied on HPTLC plate. After sample application, the HPTLC plate was transferred to chamber for development of band. The plate was scanned and finally UV spectra were recorded between 200-400 nm.

4.3.3.3. Development of Method

Optimization of various parameters like Mobile Phase Composition, Chamber Saturation Time, mobile phase was carried out for development of analytical method. Various solvent in different ratios like methanol, hexane, toluene, dichloromethane, chloroform, ethyl

acetate, acetonitrile, diethyl ether were tried along with organic modifiers like glacial acetic acid, formic acid, ammonia solution and ammonium formate for minimizing band broadening.

4.3.3.4. Chromatographic procedure

The standard solution of apigenin, luteolin and quercetin (400-1400 ng/band, six bands) was applied using autosampler on plate. Sample of EAMTAM was applied on HPTLC plate (Table 4.1)**.**

Table 4.1: Simultaneous application scheme for Standards mixture and extract

Track no.	Standards mixture/Extract	Volume (µl)
1	Standards mixture	4
2	Standards mixture	6
3	Standards mixture	8
4	Standards mixture	10
5	Standards mixture	12
6	Standards mixture	14
7	EAMTAM	5

This plate was developed in the mobile phase, dried in air and scanned at 264 nm as per the chromatographic conditions mentioned above. The peak areas were recorded for all concentrations. Calibration curve of apigenin, luteolin and quercetin was plotted as peak area versus concentration of apigenin, luteolin and quercetin respectively in µg/band applied five times. The plate was photographed in ultraviolet mode at 264 nm for all standards. The amount of apigenin, luteolin and quercetin in plant extract was quantified using calibration curve.

4.3.3.5. Validation of the developed HPTLC method (162)

The method was validated in accordance with ICH guidelines Q2 (R1) for evaluation of various parameters; linearity, precision, accuracy, LOD, LOQ, specificity and robustness.

4.3.3.5.1 Linearity

Linearity was studied by applying different aliquots of standard stock solution of apigenin, luteolin and quercetin respectively. The calibration curves were developed by plotting peak area versus concentrations. The areas of peak were treated by least square linear regression analysis.

4.3.3.5.2 LOD and LOQ

Limit of Detection (LOD) and limit of quantitation (LOQ) were calculated from the standard deviation (S.D) of densitometric response and slope of curve (s) using the equation,

$$LOD=3.3 (S.D. / S)$$

$$LOQ=10 (S.D. / S)$$

4.3.3.5.3 Precision

The intermediate precision of the method was studied by analyzing aliquots of standard in triplicate at three concentration levels for apigenin, luteolin and quercetin on the same day for intraday precision respectively. The study was also repeated on different days with freshly prepared samples in order to determine interday precision. The results were expressed as relative standard deviation (RSD). Repeatability of the HPTLC instrument was assessed by applying the same sample solution 6 times on a plate with Linomat V using the same syringe and by taking 6 scans of the sample spot for apigenin, luteolin and quercetin.

4.3.3.5.4 Accuracy

The accuracy of the method was determined from recovery studies at three different levels in triplicate by spiking with various concentrations of standard solution of apigenin, luteolin and quercetin respectively. The recovery of spiked solution was calculated**.**

4.3.3.5.5 Specificity

The specificity of the method was ascertained by determining the peak purity the component by overlaying the UV spectra of apigenin, luteolin and quercetin in the EAMTAM with the absorption spectra of reference standards at the start, middle and end positions of the bands respectively.

4.3.4. HPTLC Fingerprint Profile of *T. populnea L.*

4.3.4.1. Preparation of Standard and Sample Solutions

4.3.4.1.1 Standard solution of quercetin

Standard stock solution (1 mg/ml) of quercetin was prepared in methanol. Working solution of quercetin (1000 µg/ml) was prepared by appropriate dilutions of the stock solution with methanol.

4.3.4.1.2 Standard solution of Kaempferol

Standard stock solution (1 mg/ml) of Kaempferol was prepared in methanol. Working solution of Kaempferol (1000 µg/ml) was prepared by appropriate dilutions of the stock solution with methanol.

4.3.4.1.3 Standard solution of Lupeol

Standard stock solution (1 mg/ml) of lupeol was prepared in methanol. Working solution of lupeol (1000 µg/ml) was prepared by appropriate dilutions of the stock solution with methanol.

4.3.4.1.4 Standard solution of β-sitosterol

Standard stock solution (1 mg/ml) of β-sitosterol was prepared in methanol. Working solution of β-sitosterol (1000 µg/ml) was prepared by appropriate dilutions of the stock solution with methanol.

4.3.4.1.5 Methanol extract of aerial part of *T. populnea L.* (MTTP)

The aerial part milled to coarse powder (1 g) was macerated with methanol for 3 hr, filtered and filtrate was diluted to 10 ml with methanol in volumetric flask. The solution was filtered through a 0.45 µm membrane filter.

4.3.4.1.6 Ethyl acetate extract of aerial part of *T. populnea L.* (EAMTTP)

MTTP was dissolved by 2N HCl: toluene (1:1 v/v) and than reflux for three hours in reflux assembly it is than neutralized with 5-10% Na_2CO_3 and refluxed for half hour. Allow the layers to separate. Collect aqueous layer and extract with ethyl acetate. Take ethyl acetate solution and evaporate it. Stock solution was prepared by weighing 100 mg of the sample

then quantitatively transferred to 100 ml volumetric flask and volume was adjusted with methanol, kept in the refrigerator and tightly closed.

4.3.4.1.7 Anisaldehyde sulphuric acid (11)

Take 0.5 ml of Anisaldehyde, add 10 ml of glacial acetic acid, 85 ml of methanol and add 5 ml of sulphuric acid to it.

4.3.4.2. Selection of wavelength

Standard 5 µl solution was applied on HPTLC plate. After sample application, the HPTLC plate was transferred to chamber for development of band. The plate was scanned and finally UV spectra were recorded between 200-800 nm.

4.3.4.3. Development of Method

Optimization of various parameters like Mobile Phase Composition, Chamber Saturation Time, mobile phase was carried out for development of analytical method. Various solvent in different ratios like methanol, hexane, toluene, dichloromethane, chloroform, ethyl acetate, acetonitrile, diethyl ether were tried along with organic modifiers like glacial acetic acid, formic acid, ammonia solution and ammonium formate for minimizing band broadening.

4.3.4.4. Chromatographic procedure

The standard solution of quercetin, kaempferol, lupeol and β-sitosterol (0.6 to 1.6 µg/band, five bands) was applied using autosampler on plate. Sample of EAMTTP was applied on HPTLC plate (Table 4.2).

Table 4.2: Simulataneous application scheme of Standards mixture and extract

Track no.	Standards mixture/Extract	Volume (µl)
1	Standards mixture	6
2	Standards mixture	8
3	Standards mixture	10
4	Standards mixture	12
5	Standards mixture	14
6	Standards mixture	16
7	EAMTTP	5

This plate was developed in the mobile phase, dried in air and scanned before and after derivatization using anisaldehyde sulphuric acid at binary wavelength 264 nm and 600 nm as per the chromatographic conditions mentioned above. The peak areas were recorded for all concentrations. Calibration curve of quercetin, kaempferol, lupeol and β-sitosterol was plotted as peak area versus concentration of quercetin, kaempferol, lupeol and β-sitosterol respectively in µg/band applied five times. The plate was photographed in ultraviolet mode at 264 nm and 600 nm for all standards. The amount of quercetin, kaempferol, lupeol and β-sitosterol in plant extract was quantified using calibration curve.

4.3.4.5. Validation of the developed HPTLC method (162)

The method was validated in accordance with ICH guidelines Q2 (R1) for evaluation of various parameters; linearity, precision, accuracy, LOD, LOQ, specificity and robustness.

4.3.4.5.1 Linearity

Linearity was studied by applying different aliquots of standard stock solution of quercetin, kaempferol, lupeol and β-sitosterol respectively. The calibration curves were developed by plotting peak area versus concentrations. The areas of peak were treated by least square linear regression analysis.

4.3.4.5.2 LOD and LOQ

Limit of Detection (LOD) and limit of quantitation (LOQ) were calculated from the standard deviation (SD) of densitometric response and slope of curve (s) using the equation,

$$LOD=3.3 (S.D. / S)$$

$$LOQ=10 (S.D. / S)$$

4.3.4.5.3 Precision

The intermediate precision of the method was studied by analyzing aliquots of standard in triplicate at three concentration levels for quercetin, kaempferol, lupeol and β-sitosterol on the same day for intraday precision respectively. The study was also repeated on different days with freshly prepared samples in order to determine interday precision. The results were expressed as relative standard deviation (RSD). Repeatability of the HPTLC instrument was assessed by applying the same sample solution 6 times on a plate with

Linomat V using the same syringe and by taking 6 scans of the sample spot quercetin, kaempferol, lupeol and β-sitosterol.

4.3.4.5.4 Accuracy

The accuracy of the method was determined from recovery studies at three different levels in triplicate by spiking with various concentrations of standard solution of quercetin, kaempferol, lupeol and β-sitosterol respectively. The recovery of spiked solution was calculated**.**

4.3.4.5.5 Specificity

The specificity of the method was ascertained by determining the peak purity the component by overlaying the UV spectra of quercetin, kaempferol, lupeol and β-sitosterol in the EAMTTP with the absorption spectra of reference standards at the start, middle and end positions of the bands respectively.

4.4. LC-MS/MS Analysis of *A. millefolium* and *T. populnea*.

4.4.1. Equipments & Accessories

Sr. No.	Instrument	Brand/Model	Manufacturer/ Supplier
1.	Auto-sampler	Shimadzu SIL-20AC	Shimadzu
2.	Column oven	CTO-20AC	Shimadzu
3.	Pump	LC-20ADvp	Shimadzu
4.	Degasser	DGU-20A5R	Shimadzu
5.	System Controller	CBM-20A SPD-M20A	Shimadzu
6.	LC/MS/MS	8030	Shimadzu
7.	Operating Software	Lab Solution 5.53 SP3C	Shimadzu
8.	Micropipette (2-20 µl, 20-200 µl, 100-1000 µl, and 500-5000 µl)	-	Eppendorf
9.	Hot Air Oven	-	Associated scientific technologies

10.	Membrane Filter	0.22 µ	Millipore
11.	Digital Analytical Balance	AUX 220	Shimadzu
12.	Refrigerator (2-8^{o}C)	GL335/388	LG Electronics
13.	Deep Freezer (-20^{o}C)	RQFV-265	REMI Corporation
14.	pH Meter	pH Tutor	Eutech Instruments
15.	Ultra-sonicator	Ultrasonic Steri-cleaner	Cyber-lab
16.	Vortex Shaker	CM-101	REMI
17.	Water Purification System	Elix10, Milli-Q Gradient	Millipore
18.	Incubator	-	Thermolab
19.	Magnetic Stirrer	-	Electro quip
20.	Vacuum Pump	-	Millipore
21.	Vaccum Oven	NV 8510	NOVA instrument Pvt. Ltd.

4.4.2. Glass wares and Apparatus

Sr. No.	Apparatus	Grade/Class	Manufacturer/ Supplier
1.	Centrifuge Tubes	Appropriate volumes	Tarsons
2.	Glass beakers	Appropriate volumes	Borosil Glassworks ltd.
3.	Glass bottles	Appropriate volumes	Borosil Glassworks ltd.
4.	Measuring cylinder	Appropriate volumes (Class A)	Borosil Glassworks ltd.
5.	Ria-vials & caps	-	Tarsons
6.	Volumetric flask	Appropriate volumes (Class A)	Borosil Glassworks ltd.

4.4.3. LC-MS/MS analysis of *A. millefolium L.*

4.4.3.1. Formic acid in water, 0.1 % v/v

Pipette out 0.100 ml of formic acid in to a measuring cylinder containing 100 ml of methanol. Mixed the contents thoroughly and transferred into a reagent bottle. Stored at ambient temperature. This solution was used within 3 days from the date of preparation.

4.4.3.2. Reconstitution solution (Formic acid in water, 0.1% v/v: Acetonitrile 50:50 % v/v)

In measuring cylinder 50 ml of methanol and 50 ml of formic acid in water, 0.1% v/v was taken, then transferred into a reagent bottle and mixed the contents thoroughly. Stored at ambient temperature. This solution was used within 3 days from the date of preparation.

4.4.3.3. Auto sampler Rinsing Solution

In measuring cylinder 500 ml of Methanol and 500 ml of water was taken, then transferred into a reagent bottle and mixed the contents thoroughly. Stored at ambient temperature. This solution was used within 3 days from the date of preparation.

4.4.3.4. Apigenin Stock Solution, 0.4 mg/ml

Apigenin standard was weighed accurately equivalent to 4 mg of apigenin and appropriate volume of Methanol was added to make final concentration of apigenin equivalent to 0.4 mg/ml accounting for its potency and the actual amount weighed. Solution was stored in refrigerator at 5 ± 3°C. Use the solution within 7 days from date of preparation.

4.4.3.5. Luteolin Stock Solution, 0.4 mg/ml

Luteolin standard was weighed accurately equivalent to 4 mg of luteolin and appropriate volume of Methanol was added to make final concentration of luteolin equivalent to 0.4 mg/ml accounting for its potency and the actual amount weighed. Solution was stored in refrigerator at 5 ± 3°C. Use the solution within 7 days from date of preparation.

4.4.3.6. Quercetin Stock Solution, 0.4 mg/ml

Quercetin standard was weighed accurately equivalent to 4 mg of quercetin and appropriate volume of Methanol was added to make final concentration of quercetin equivalent to 0.4 mg/ml accounting for its potency and the actual amount weighed. Solution was stored in refrigerator at 5 ± 3°C. Use the solution within 7 days from date of preparation.

4.4.3.7. Mix intermediate Stock solution:

Pipette out 500 µl of Drug 1 Stock Solution, 0.4 mg/ml, Drug 2 Stock Solution 0.4 mg/ml and Drug 3 Stock Solution 0.4 mg/ml in 10.0 ml volumetric flask and made up the volume to 10.0 ml with Methanol. Solution was stored in refrigerator at 5 ± 3°C. Use the solution within 7 days from date of preparation.

4.4.3.8. Preparation of Standard and Quality Control Samples

4.4.3.8.1 Calibration Curve (CC) Spiking Solutions:

CC Spiking solutions were prepared in Methanol using Mix Intermediate Stock Solution by serial dilution as described below:

Stock Dil. Conc. (ng/ml)	Vol. Taken (ml)	Vol. of methanol (ml)	Total Vol. (ml)	Spiking Solution Conc. (ng/ml)	Spiking Solution ID
40	0.25	1.75	2.000	5	SS STD1
5	1	1	2.000	2.5	SS STD2
2.5	1	1	2.000	1.25	SS STD3
1.25	0.2	1.80	2.000	0.125	SS STD4
0.125	0.2	1.80	2.000	0.0125	SS STD5
0.0125	0.2	1.80	2.000	0.00125	SS STD6

4.4.3.8.2 Accuracy Spiking Solutions:

Accuracy spiking solutions were prepared in Methanol using Mix Intermediate Stock Solution by serial dilution as described below:

Stock Dil. Conc. (ng/ml)	Vol. Taken (ml)	Vol. of Methanol (ml)	Total Vol. (ml)	Spiking Solun Conc. (ng/ml)	Spiking Solun ID
40	0.25	1.75	2.00	5	parent
5	0.4	1.60	2.00	1	SS 80%
5	0.5	1.50	2.00	1.25	SS 100%
5	0.4	1.40	2.00	1.50	SS 120%

4.4.3.8.3 Preparation of herbal sample:

Herbal sample was prepared as described below:

Stock Dil. Conc. (µg/ml)	Vol. Taken (ml)	Vol. of methanol (ml)	Total Vol. (ml)	Spiking Solun Conc. (µg/ml)	Spiking Solun ID
1000	0.600	1.400	2.00	300	
300	0. 050	0.950 (Dilute in RS)	1.00	15	For inject

4.4.4. LC-MS/MS analysis of *T. populnea L.*

4.4.4.1. Preparation of solution for analysis of quercetin and kaempferol

4.4.4.1.1 Formic acid in water, 0.3 % v/v

Pipette out 0.300 ml of formic acid in to a measuring cylinder containing 100 ml of methanol. Mixed the contents thoroughly and transferred into a reagent bottle. Stored at ambient temperature. This solution was used within 3 days from the date of preparation.

4.4.4.1.2 Reconstitution solution (Formic acid in water, 0.3% v/v: Acetonitrile 20:80 % v/v)

In measuring cylinder 80 ml of methanol and 20 ml of formic acid in water, 0.3% v/v was taken, then transferred into a reagent bottle and mixed the contents thoroughly. Stored at ambient temperature. This solution was used within 3 days from the date of preparation.

4.4.4.1.3 Auto sampler Rinsing Solution

In measuring cylinder 500 ml of Methanol and 500 ml of water was taken, then transferred into a reagent bottle and mixed the contents thoroughly. Stored at ambient temperature. This solution was used within 3 days from the date of preparation.

4.4.4.1.4 Kaempferol Stock Solution, 1 mg/ml

Kaempferol standard was weighed accurately equivalent to 10 mg of kaempferol and appropriate volume of Methanol was added to make final concentration of kaempferol equivalent to 1 mg/ml accounting for its potency and the actual amount weighed. Solution was stored in refrigerator at 5 ± 3°C. Use the solution within 7 days from date of preparation

4.4.4.1.5 Quercetin Stock Solution, 1 mg/ml

Quercetin was weighed accurately equivalent to 10 mg of quercetin and appropriate volume of Methanol was added to make final concentration of quercetin equivalent to 1 mg/ml accounting for its potency and the actual amount weighed. Solution was stored in refrigerator at 5 ± 3°C. Use the solution within 7 days from date of preparation.

4.4.4.1.6 Mix intermediate Stock solution, (Kaempferol 100 µg/ml and Quercetin 100 µg/ml)

Pipette out 500 µl of Drug1 Stock Solution, 1 mg/ml and Drug2 Stock Solution, 1 mg/ml in 10.0 ml volumetric flask and made up the volume to 10.0 ml with Methanol. Solution was stored in refrigerator at 5 ± 3°C. Use the solution within 7 days from date of preparation.

4.4.4.1.7 Preparation of Standard and Quality Control Samples for quercetin and kaempferol

4.4.4.1.7.1. Calibration Curve(CC) Spiking Solutions:

CC Spiking solutions were prepared in Methanol using Mix Intermediate Stock Solution by serial dilution as described below:

Stock Dil. Conc. (µg/ml)	Vol. Taken (ml)	Vol. of methanol (ml)	Total Vol. (ml)	Spiking Solution Conc. (µg/ml)	Spiking Solun ID
100000	2.500	2.500	5.000	50000	SS STD1
50000	2.500	2.500	5.000	25000	SS STD2
25000	2.000	3.000	5.000	10000	SS STD3
10000	2.500	2.500	5.000	5000	SS STD4
5000	1.000	4.000	5.000	1000	SS STD5
1000	2.500	2.500	5.000	500	SS STD6

4.4.4.1.7.2. Accuracy Spiking Solutions: Accuracy spiking solutions were prepared in Methanol using Mix Intermediate Stock Solution by serial dilution as described below:

Stock Dil. Conc. (µg/ml)	Vol. Taken (ml)	Vol. of Methanol (ml)	Total Vol. (ml)	Spiking Solun Conc. (µg/ml)	Spiking Solun ID
100000	5.000	5.000	10.000	50000	parent
50000	2.000	3.000	5.000	20000	SS 80%
50000	2.500	2.500	5.000	25000	SS 100%
50000	3.000	2.000	5.000	30000	SS 120%

4.4.4.1.8 Preparation of herbal sample:

Herbal sample was prepared as described below:

Stock Dil. Conc. (µg/ml)	Vol. Taken (ml)	Vol. of methanol (ml)	Total Vol. (ml)	Spiking Solun Conc. (µg/ml)	Spiking Solun ID
1000	0.600	1.400	2.00	300	
300	0. 050	0.950 (Dilute in RS)	1.00	15	For inject

4.4.4.2. Preparation of solution for analysis of β-sitosterol and lupeol

4.4.4.2.1 Formic acid in water, 0.3 % v/v

Pipette out 0.300 ml of formic acid in to a measuring cylinder containing 100 ml of methanol. Mixed the contents thoroughly and transferred into a reagent bottle. Stored at ambient temperature. This solution was used within 3 days from the date of preparation.

4.4.4.2.2 Reconstitution solution (Formic acid in water, 0.3% v/v: Acetonitrile 20:80 % v/v)

In measuring cylinder 80 ml of methanol and 20 ml of formic acid in water, 0.3% v/v was taken, then transferred into a reagent bottle and mixed the contents thoroughly. Stored at ambient temperature. This solution was used within 3 days from the date of preparation.

4.4.4.2.3 Auto sampler Rinsing Solution

In measuring cylinder 500 ml of methanol and 500 ml of water was taken, then transferred into a reagent bottle and mixed the contents thoroughly. Stored at ambient temperature. This solution was used within 3 days from the date of preparation.

4.4.4.2.4 β-sitosterol Stock Solution, 0.4 mg/ml

β-sitosterol standard was weighed accurately equivalent to 4 mg of β-sitosterol and appropriate volume of Methanol was added to make final concentration of β-sitosterol equivalent to 0.4 mg/ml accounting for its potency and the actual amount weighed. Solution was stored in refrigerator at 5 ± 3°C. Use the solution within 7 days from date of preparation

4.4.4.2.5 Lupeol Stock Solution, 0.4 mg/ml

Lupeol was weighed accurately equivalent to 4 mg of lupeol and appropriate volume of Methanol was added to make final concentration of lupeol equivalent to 0.4 mg/ml accounting for its potency and the actual amount weighed. Solution was stored in refrigerator at 5 ± 3°C. Use the solution within 7 days from date of preparation.

4.4.4.2.6 Mix intermediate Stock solution:

Pipette out 500 µl of Drug1 Stock Solution, 0.4 mg/ml and Drug2 Stock Solution, 0.4 mg/ml in 10.0 ml volumetric flask and made up the volume to 10.0 ml with Methanol. Solution was stored in refrigerator at 5 ± 3°C. Use the solution within 7 days from date of preparation.

4.4.4.3. Preparation of Standard and Quality Control Samples for β-sitosterol and lupeol.

4.4.4.3.1 Calibration Curve(CC) Spiking Solutions:

CC Spiking solutions were prepared in Methanol using Mix Intermediate Stock Solution by serial dilution as described below:

Stock Dil. Conc. (µg/ml)	Vol. Taken (ml)	Vol. of methanol (ml)	Total Vol. (ml)	Spiking Solution Conc. (µg/ml)	Spiking Solun ID
400	0.50	1.50	2.00	100	SS STD1
100	1.60	0.40	2.00	80	SS STD2
80	1.00	1.00	2.00	40	SS STD3
40	1.00	1.00	2.00	20	SS STD4
20	1.00	1.00	2.00	10	SS STD5
10	1.00	1.00	2.00	5	SS STD6

4.4.4.3.2 Accuracy Spiking Solutions: Accuracy spiking solutions were prepared in Methanol using Mix Intermediate Stock Solution by serial dilution as described below:

Stock Dil. Conc. (µg/ml)	Vol. Taken (ml)	Vol. of Methanol (ml)	Total Vol. (ml)	Spiking Solun Conc. (µg/ml)	Spiking Solun ID
400	5.000	5.000	10.000	50000	parent
100	2.000	3.000	5.000	20000	SS 80%
100	2.500	2.500	5.000	25000	SS 100%
100	3.000	2.000	5.000	30000	SS 120%

4.4.4.3.3 Preparation of herbal sample: Herbal sample was prepared as described below:

Stock Dil. Conc. (µg/ml)	Vol. Taken (ml)	Vol. of methanol (ml)	Total Vol. (ml)	Spiking Solun Conc. (µg/ml)	Spiking Solun ID
1000	0.600	1.400	2.00	300	
300	0. 050	0.950 (Dilute in RS)	1.00	15	For inject

4.4.4.4. Method validation

4.4.4.4.1 Linearity

To evaluate linearity of this method, calibration standards were prepared and analyzed in triplicate on three consecutive days. Calibration curves were constructed by plotting the peak area ratio versus the spiked concentrations of markers by least square linear regression analysis.

4.4.4.4.2 Precision and accuracy

Precision of developed method were assessed by determining six replicates of concentration 1000 µg/ml on three consecutive days. The precision was expressed as the % relative standard deviation (RSD). Accuracy of developed method was determined at three level (80%, 100% and 120%).

4.4.4.4.3 Limit of Detection and Limit of Quantification

The LOD with S/N of 3:1 and the LOQ with S/N of 10:1 were calculated for both drugs using the following equations according to International Conference on Harmonization (ICH) guidelines:

$$LOD = 3.3 \times \sigma/S$$

$$LOQ = 10 \times \sigma/S$$

Where σ the standard deviation of the response and S is the standard deviation of the y-intercept of the regression line**.**

4.5. Hepatoprotective Activity

4.5.1. Instruments

Instruments like U.V (Shimadzu), Micro centrifuge (REMI), rotary flash evaporator (Superfit, Rotavap), Semiautoanalyser (Mispa-plus) and Microscope (Magnus), were used for the present study.

4.5.2. Calculation of the dose

Following formula was applied to calculate the dose of extract and chemicals:

D= [a ×b ×c /1000] where,

a= weight of the animal

b= dose of the drug (mg/kg)

c= number of animals

D= amount of drug required for specified number of animals.

E.g. now the volume to be administered to each animal through i.p route is 0.1ml.

Therefore, the dilution volume= number of animals × volume to be administered to each animal.

Y= X × 0.1 ml

Thus D amount of drug is to be dissolved in Y ml of the vehicle.

4.5.3. Carbon tetrachloride (CCl_4) induced liver toxicity (141,163,164)

Experimental design:

The different groups were assigned as below.

Experimental model	**Number of animals**
Group-I Normal control (R.O water).	6
Group-II Model control (CCl_4 2 ml/kg; s.c)	6
Group-III Standard control (CCl_4+ Silymarin 50 mg/kg; p.o)	6
Group-IV Test group (CCl_4 + t.p extract 250 mg/kg; p.o)	6
Group-V Test group (CCl_4 + t. p extract 500 mg/kg; p.o)	6
Group-VI Test group (CCl_4 + a.m extract 250 mg/kg; p.o)	6
Group-VII Test group (CCl_4 + a.m extract 500 mg/kg; p.o)	6
Total animals	42

Wistar rats of either sex weighing between 150-200 g were divided into seven groups of six animals each. For the first nine days of study Group I & II were fed with normal feed, water and CCl_4. Group III animals were treated with Silymarin 50 mg/kg and group IV & VII were treated with EAMTAM and EAMTTP 250 mg/kg and 500 mg/kg respectively for 9 days. All the treatment was done post orally. On 9^{th} day, all the animals except Group I and II were intoxicated by the administration of CCl_4 (2 ml/kg s.c. 1:1 of CCl_4 in olive oil). After 48 hrs of intoxication by CCl_4 administration, blood was collected through retro orbital puncture and analyzed for various biochemical parameters. Animals were sacrificed using ether anesthesia and liver was dissected out and used for histopathological studies.

4.5.4. Biochemical parameters estimated includes

Various parameters	Biological sample	Parameters to be investigated
Biochemical Parameters	Serum	Aspartate amino transferase (AST), alanine amino transferase (ALT), Total bilirubin and total protein.
Other parameters	Liver	Histopathology

Chapter - 5

RESULTS AND DISCUSSION

CHAPTER-5

5. Results and Discussion

5.1. Pharmacognostic Studies

5.1.1. Macroscopic and Microscopic examination

5.1.1.1. Aerial parts of *A. millefolium L.*

Stem is erect, simple; Leaves are petiolate or sessile; lanceolate, sparsely tomentose. Flowers are simple, corymbiform arrays, ray florets 5-8, pistillate, corollas white; disc florets 10-20; corollas white to grayish white.
Powder study showed unicellular trichome, spiral xylem vessels, stone cells, anther, mesophyll region, rosette shaped crystal, anomocytic stomata. These characters of powder study are as shown in (Figure 5.1).

5.1.1.2. Aerial parts of *T. populnea L.*

Leaves are simple, alternate, long petioled, cordate, entire, acuminate, prominent nerves 5-7 with peltate scales on both surfaces (Figure 5.2).

A Transverse Section stem is circular in outline. In outermost single layer of Upper epidermis is present. It is cubical in shape and fitting closely along their radial walls with well defined cuticle extending over it. Cortex consists of Outer cortex which consists of tangentially and radially elongated 3-4 layer of parenchymatous cells. Inner cortex consists of 6 to 7 layers of parenchymatous cells. Pericycle consists of scattered pericyclic fibres in group of 6-10 cells pericyclic fibres are lignified. The vascular bundles are arranged in a ring. Phloem lies externally. Phloem is followed by lignified elements of xylem. Uni to tri seriate medullary ray are present. Central stellar region consists of small parenchymatous pith. Rosette crystals of calcium oxalate are present in cortex as well as in the pith region. Starch grains are also present in the cortex and medullary rays (Figure 5.3)

Transverse section of the leaf through the midrib showed bowl shaped abaxial parts and slightly raised adaxial side. Both the epidermal layers are single layered thin walled cubical

cells. The epidermal cells followed by four to five layers of angular collenchyma cells on both the sides. Mucilage cavities are present in the collenchymatous hypodermal region. The vascular strand of the midrib occurs in arc form.

The lamina of the leaf is dorsiventral. Both the epidermal cells are squarish to rectangular, cuticle is thin and smooth. Palisade tissues are single layered. They are cylindrical less compact and occupy one third of the thickness of the lamina. Spongy tissues are three-four layered, spherical and less compact.

Abundant tannin containing cells and calcium oxalate crystals are present in the ground tissues. The calcium oxalate crystals i.e sphaerocrystals are present and are found in mesophyll tissues it contain presence of special type of pellate trichome (Figure 5.4).

A Transverse Section of Petiole is circular in outline. In outermost single layer of Upper epidermis is present. It is cubical in shape and fitting closely along their radial walls with well defined cuticle extending over it. Hypodermis (Hy) is the outer collenchymatous tissue forming a narrow zone of 4 to 5 layers of tangentially elongated or cubical to rectangular cells. General Cortex (Ct) consists of 13 to14 layers of parenchymatous cells. Pericycle region is the region lying in between hypodermis & vascular bundle. Pericyclic fibres are lignified and present in the form of patches. The vascular bundles are arranged in a ring. Phloem lies externally. Phloem is followed by lignified elements of xylem. Metaxylem outer side to the cortex region and protoxylem inner side to the pith so endarch type of vascular bundle. Inner to xylem big parenchymatous pith is present. Rosettes of calcium oxalate crystals are present in cortex as well as in the pith region (Figure 5.5).

Powder study showed peltate trichome, spiral xylem vessels, mesophyll region, anomocytic stomata and spiral shaped xylem vessel. These characters of powder study are as shown in (Figure 5.6).

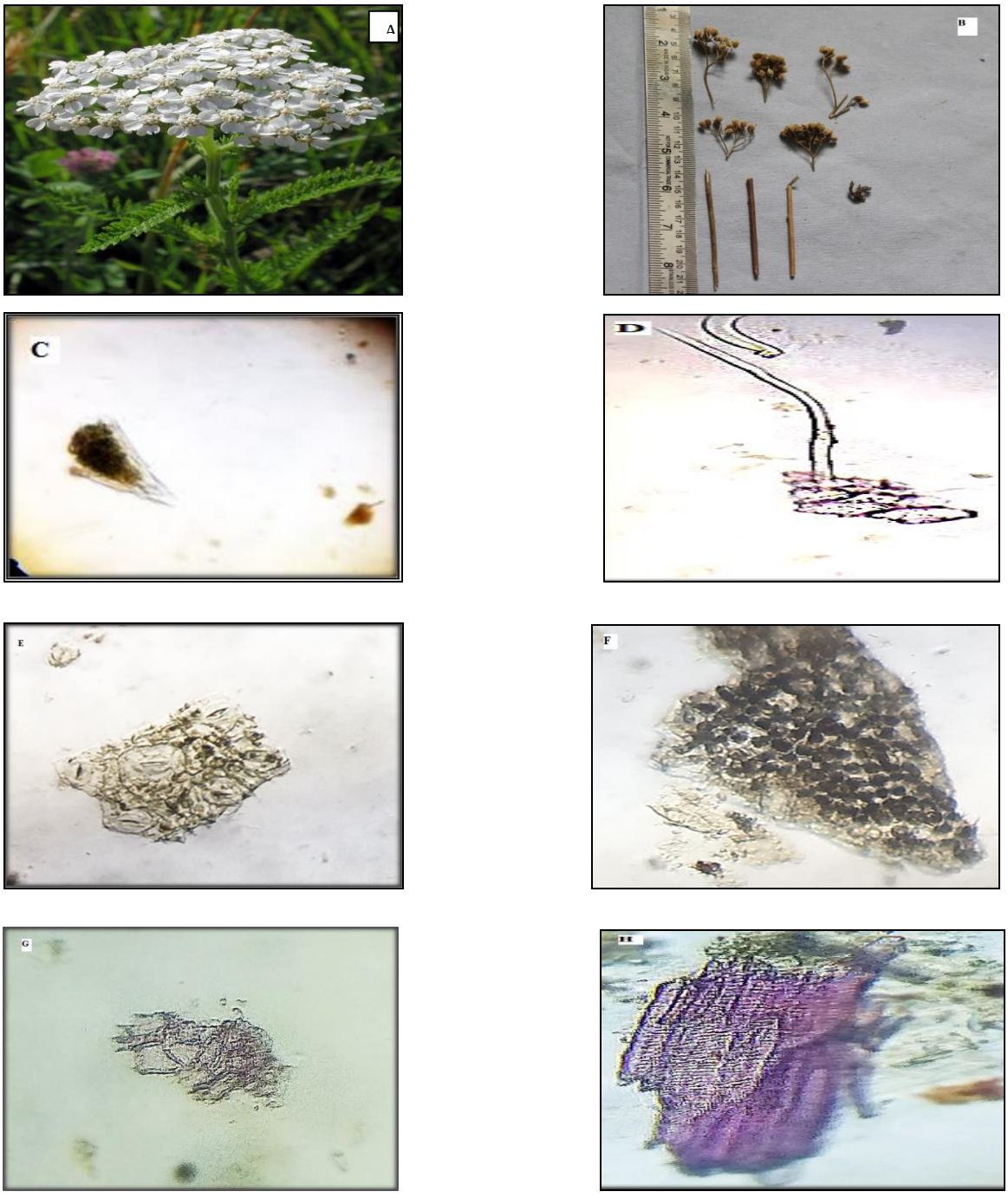


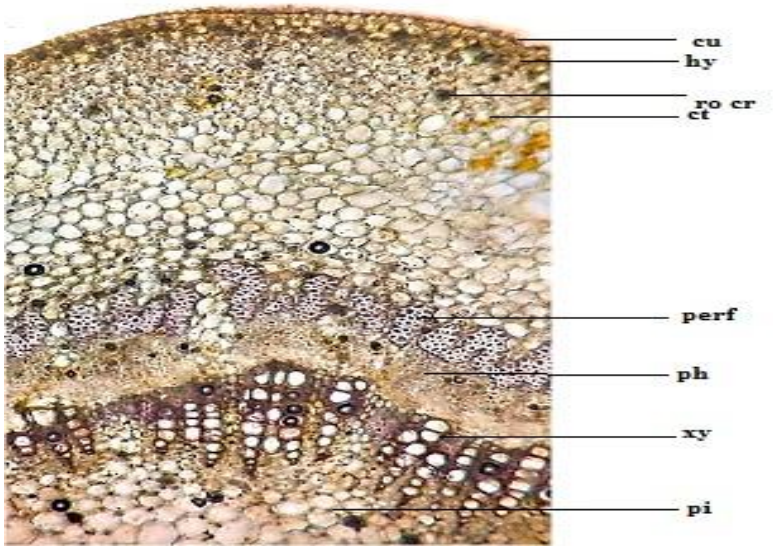
Figure 5.1: (A-B) Macroscopic characteristics of aerial parts of *A. millefolium*, (C-H): Powder characteristics of *A. millefolium*, Mesophyll, Stone cell, Unicellular trichomes, anomocytic stomata, rosette shaped crystal, anther, spiral xylem vessels.

[A]

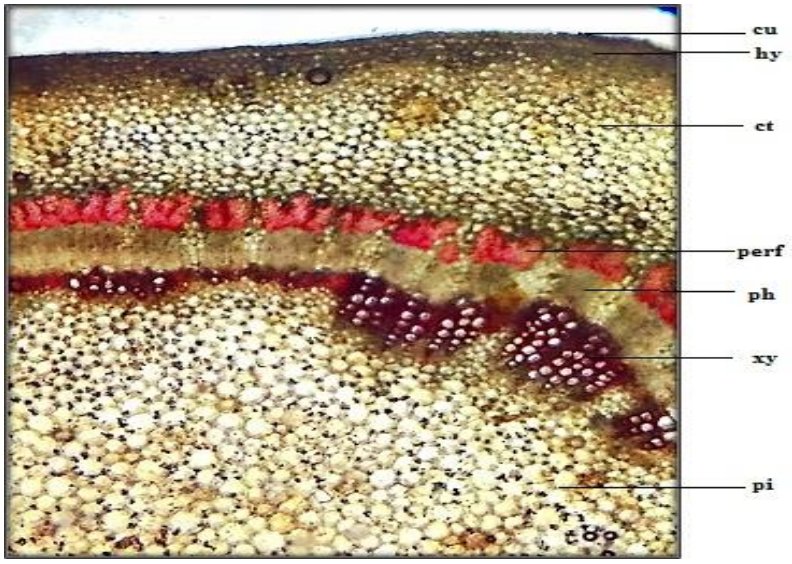
[B]

[C]

Figure 5.2: **(A-C) Macroscopic characteristics of aerial parts of *T. populnea.***

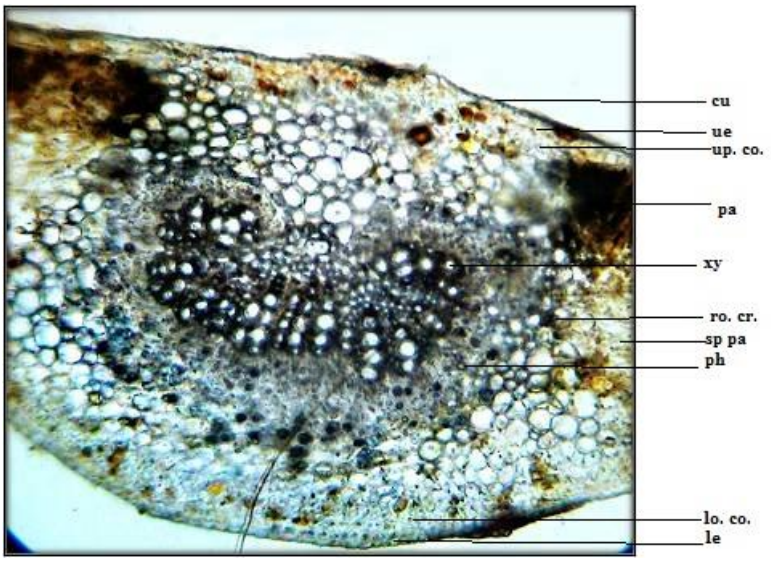


[A]

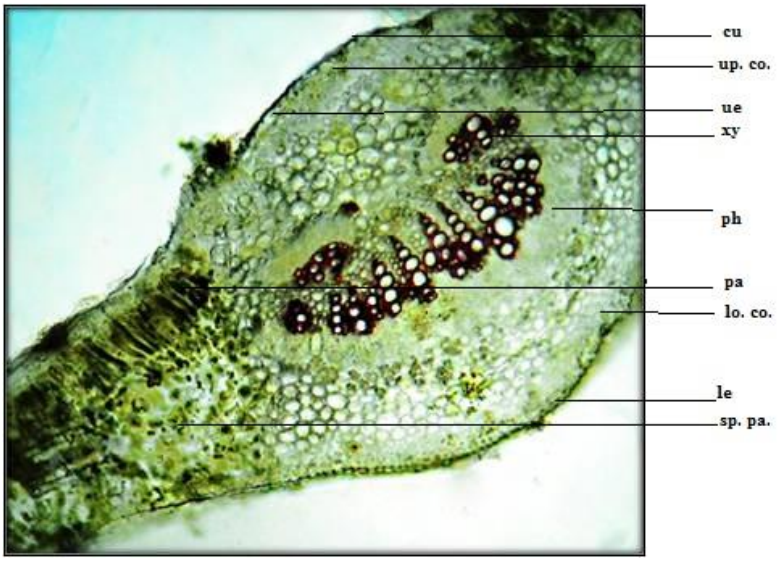


[B]

Figure 5.3: [A] Transverse section-unstained of stem of *T. populnea*, showing presence of different microscopic characters, cu-Cuticle, hy-hypodermis, ct-Cortex, perf-pericyclic fibre, xy-Xylem vessel, ph-Phloem parenchyma, pi-Pith. [B] Transverse section-stained of stem of *T. populnea*

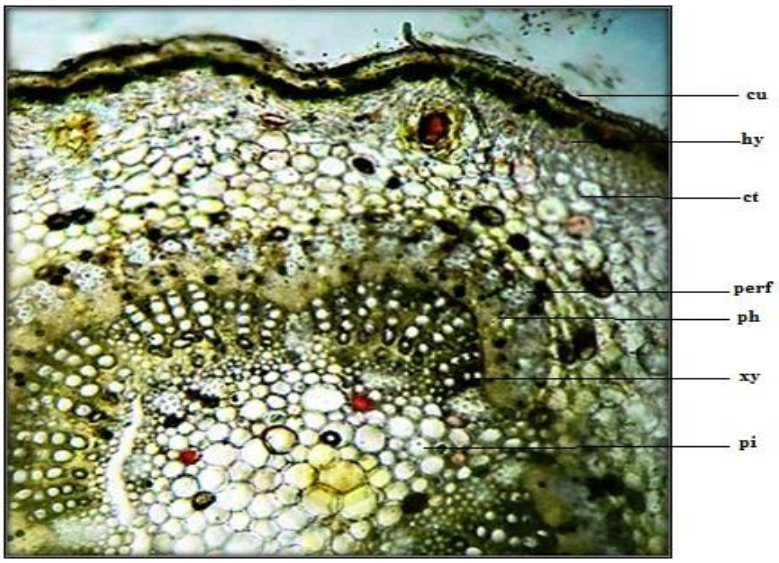


[A]

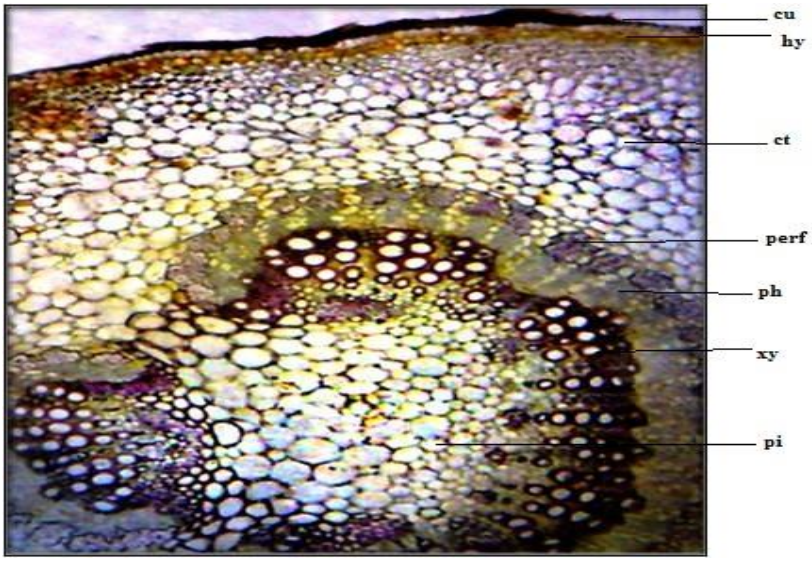


[B]

Figure 5.4: [A]Transverse section-unstained of leaf of *T. populnea*, showing presence of different microscopic characters, pa-Palisade parenchyma, sp. Pa.-Spongy parenchyma, ue-Upper epidermis, up.co.-Upper collecnhyma, cu-Cuticle, xy- Xylem, ph-Phloem, ro.cr.-Rosette crystals, lo.co.-Lower collecnhyma, le-Lower epidermis. [B] Transverse section-stained of leaf of *T. populnea*



[A]



[B]

Figure 5.5: [A]Transverse section-unstained of petiole of *T. populnea*, showing presence of different microscopic characters, cu-Cuticle, hy-Hypodermis, , ct-Cortex, perf-pericyclic fibre, xy-Xylem vessel, ph-Phloem parenchyma, pi-Pith. [B] Transverse section-stained of petiole of *T. populnea*

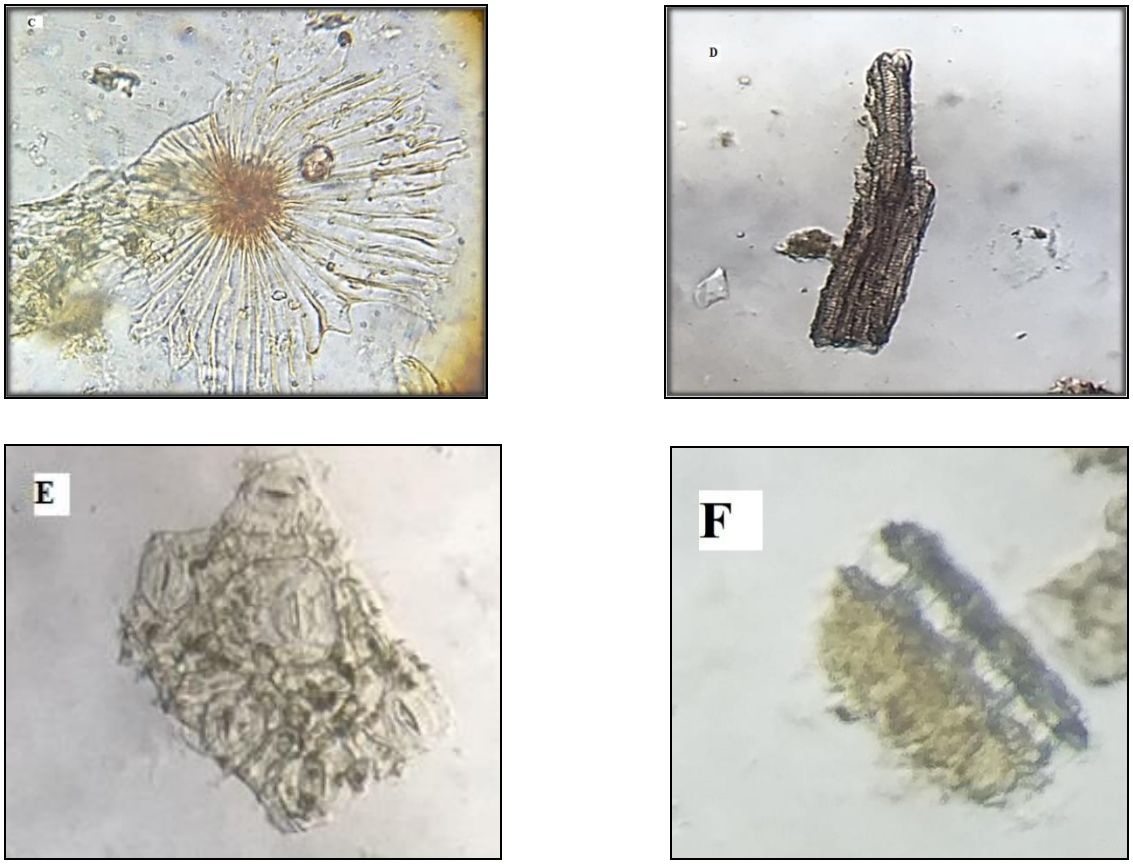


Figure 5.6 (C-F): Powder characteristics of *T. populnea*, Peltate trichome, spiral xylem vessel, anomocytic stomata, mesophyll.

5.1.1.3. Quantitative microscopy

Quantitative microscopy of leaf *T. populnea* was done and parameters for stomatal index, vein-islet number, vein termination number, palisade ratio are shown in (Table 5.1).

Table 5.1: Quantitative microscopy of leaf of *T. populnea*

Sr. No.	Determination	Value per square mm
1.	Stomatal number (Lower surface)	21-24
2.	Stomatal index (Lower surface)	3-17
3.	Vein islet number	12
4.	Vein termination	16
5.	Palisade ratio	15-19

5.1.1.4. Flourescence analysis

The results of the fluorescent properties of the powder of aerial parts of *A. millefolium* and *T. populnea* obtained on treatment with several reagents are as presented in (Table 5.2, Table 5.3). Flourescence analysis revealed the presence of starch and phenolic compounds in aerial parts of *A. millefolium* and *T. populnea.* Reaction with acid and alkali showed fluorescence indicating that phenolic compounds like flavonoids, flavones and coumarins may be present.

Table 5.2: Flourescent properties of aerial parts of *A. millefolium*

Reagent	Daylight	U.V. (254 nm)	U.V. (365 nm)
Methanol	Light greenish yellow	Light yellow	yellow
1M sodium hydroxide in methanol	yellow	yellow	Light greyish flourescent
1M sodium hydroxide in water	Golden yellow	Brown	Greyish brown
1M hydrochloric acid in methanol	Light cream	Light brown	Brownish flourescent
1M hydrochloric acid in water	Transparent	Transparent	Light greenish flourescent
50% nitric acid in methanol	Light yellow	Light yellow	Light greenish yellow
50% sulphuric acid in methanol	Light yellow	Light yellow	Light greenish yellow
Nitric acid	Orange	Orange	Reddish
Acetic acid	Light yellow	Yellowish orange	Yellowish fluorescent
1% picric acid	Yellow	Light yellow	Yellowish fluorescent
10% $K_2Cr_2O_7$	Yellow	Yellow	Greenish yellow flourescent

5% Iodine	Reddish orange	Reddish yellow	Red
Dilute ammonia	Light yellowish brown	Light brown	Greenish
5% ferric chloride reagent	Brown	Grey	Greyish black

Table 5.3: Flourescent properties of aerial parts of *T. populnea*

Reagent	**Daylight**	**U.V. (254 nm)**	**U.V. (365 nm)**
Methanol	Greenish yellow	Brown red	Greenish yellow
1M sodium hydroxide in methanol	Green	Greenish yellow	Orange
1M sodium hydroxide in water	Golden yellow	Grey	Grey
1M hydrochloric acid in methanol	Light greenish yellow	Light yellow	Orange
1M hydrochloric acid in water	Transparent	Transparent	Bluish
50% nitric acid in methanol	Light yellow	Light yellow	Green
50% sulphuric acid in methanol	Yellowish	Grey yellow	Grey yellow
Nitric acid	Yellowish orange	Yellowish orange	Red
Acetic acid	Light green	Cream	Bluish white fluorescent
1% picric acid	Golden yellow	Yellowish orange	Yellowish
10% $K_2Cr_2O_7$	Golden brown	Yellow	Greenish
5% Iodine	Reddish orange	Reddish orange	Orange
Dilute ammonia	Brown	Blackish brown	Greenish yellow fluorescent
5% ferric chloride reagent	Yellowish brown	Purple	Light purple

5.1.1.5. Proximate analysis

Percent content of total ash for aerial parts of *A. millefolium* was found to be 10.87% while that of *T. populnea* was found to be 5.62%. The total ash value indicating percentage of inorganic salts ans acid insoluble ash value due to presence of siliceous matter. Results for standardization parameters of aerial parts of *A. millefolium* and *T. populnea* are presented in (Table 5.4)

Table 5.4: Proximate analysis of aerial parts of *A. millefolium* and *T. populnea*

Sr. No.	Determination	*A. millefolium* (%w/w)	*T. populnea* (%w/w)
1.	Total Ash	10.87 ± 0.65	5.62 ± 0.32
2.	Acid insoluble Ash	2.86 ± 0.14	0.76 ± 0.22
3.	Water Soluble Ash	7.42 ± 0.53	3.20 ± 0.56
4.	Alcohol Soluble Extractive value	38.06 ± 1.49	48 ± 0.12
5.	Water soluble Extractive value	56.34 ± 0.83	44.8 ± 0.36
6.	Ether soluble Extractive value	23.21 ± 0.52	39.2 ± 0.49

Results are mean of triplicate determinations. Each value represents mean ± S.D

Among the various solvent extractive values, water and methanol soluble extractive was found to be higher for aerial part of *A. millefolium* and *T. populnea* indicating the presence of polar and non polar constituents.

5.2. Phytochemical studies.

5.2.1. Total Phenolic study.

Total phenolic content has been reported as gallic acid equivalent with reference to standard curve, $Y=0.003x + 0.002$, $R^2=0.993$ (Figure 5.7). Total phenolic content in aerial parts of *A. millefolium* and *T. populnea* was found to be 18.44 ± 0.89 %w/w and 4.87 ± 0.89 %w/w respectively calculated as gallic acid equivalent.

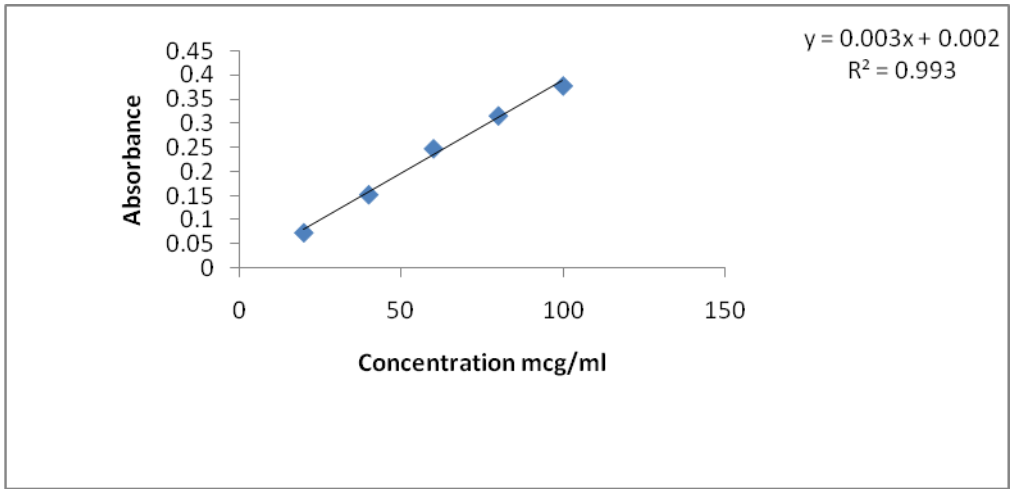


Figure 5.7: Calibration curve of standard gallic acid

5.2.2. Total flavonoid content

The total flavonoid content was calculated as quercetin equivalent with reference to standard curve, Y=0.009x + 0.015, R^2=0.999 (Figure 5.8).

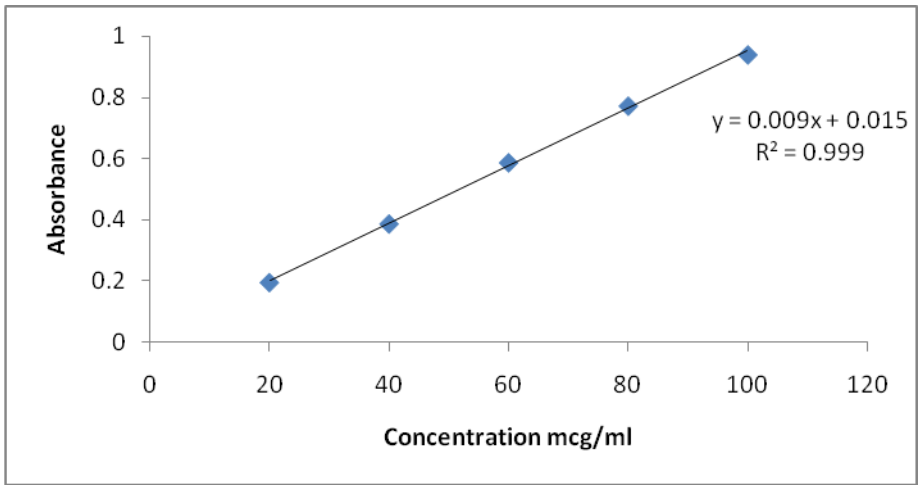


Figure 5.8: Calibration curve of standard quercetin

Total flavonoid content in aerial parts of *A. millefolium* and *T. populnea* was found to be 80.30 ± 5.793%w/w and 16.48 ± 0.63%w/w.

5.3. HPTLC Analysis of *A. millefolium* and *T. populnea*

5.3.1. HPTLC Analysis of EAMTAM

5.3.1.1. Selection of wavelength

An ideal wavelength is the one that gives good response for the drugs that are to be detected. In the present study, a solution containing LUT, QUE and API concentration of

800 ng/band was prepared in methanol and scanned from 200-400 nm. The common detection wavelength selected for analysis of all three drugs were showing optimum response at 264 nm (Figure 5.9).

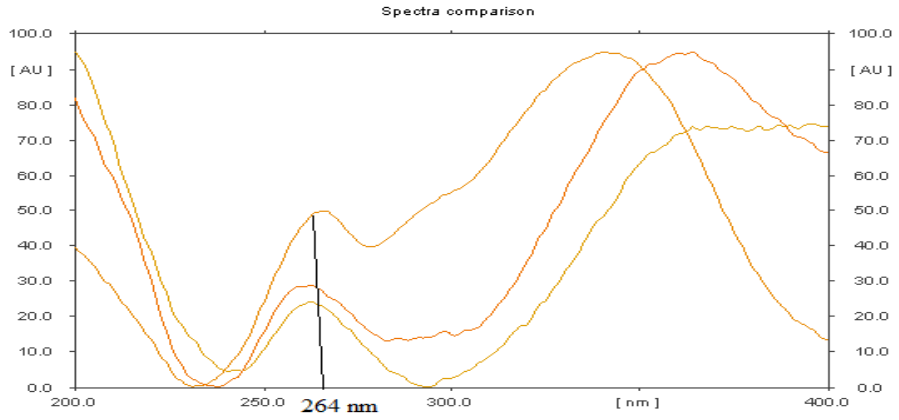
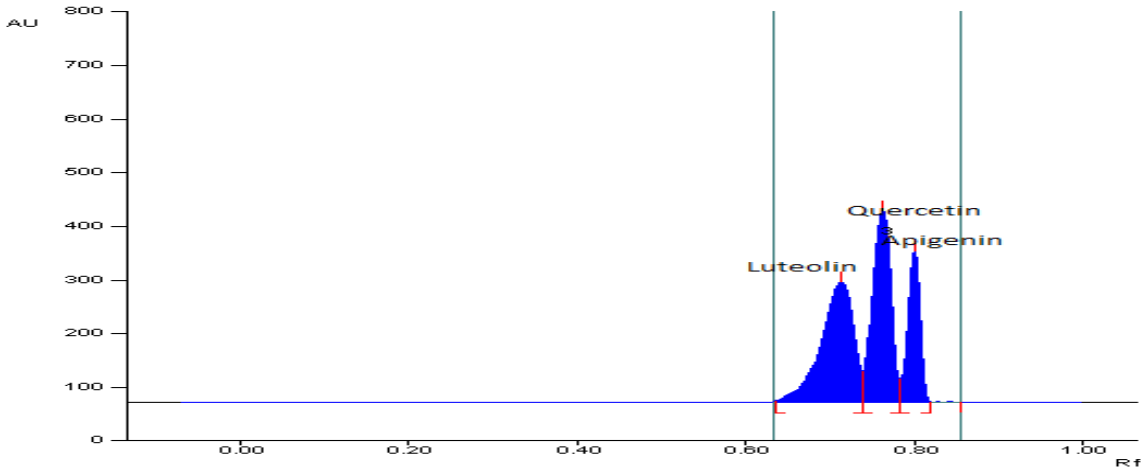


Figure 5.9: Selection wavelength of LUT, QUE and API (264 nm).

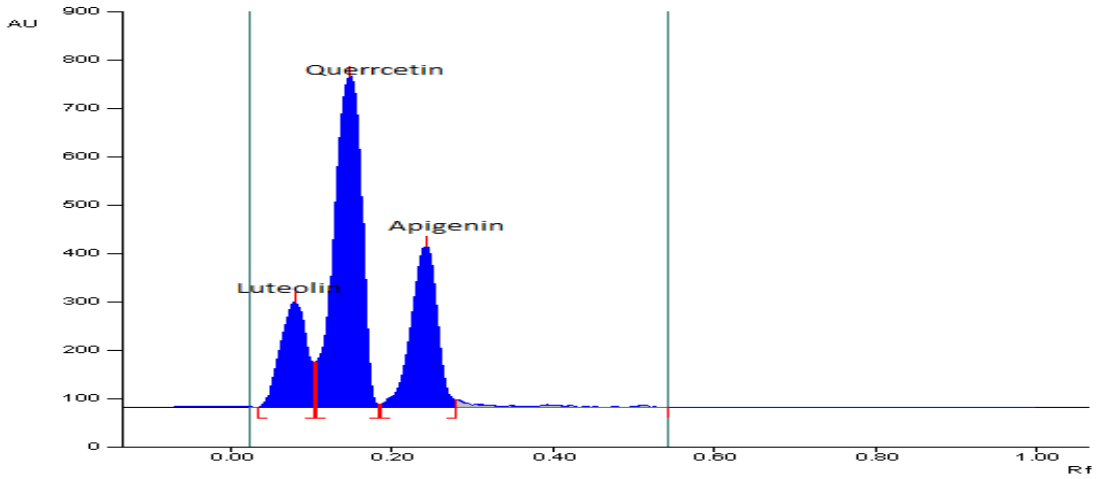
5.3.1.2. Optimization of mobile phase

All the three drugs were spotted on the HPTLC plates and run in different solvent systems and different ratios of various solvents were tried like n-hexane, toluene, methanol, ethyl acetate, acetonitrile, diethyl ether, chloroform, dichloromethane. From these, combination of ethyl acetate, and toluene gave good result and good separation and hence, further trials were initiated for different ratios of methanol, ethyl acetate, toluene with addition of different modifiers like glacial acetic acid, ammonia solution, formic acid, ortho phosphoric acid and ammonium formate. Finally, the mobile phase consisting of ethyl acetate: toluene: formic acid (7:3:0.3 %v/v/v) gave sharp and symmetrical peak. Well defined band of LUT at R_f 0.42, QUE at R_f 0.50 and API at R_f 0.61 (Figure 5.10) was obtained when the chamber was saturated with the optimized mobile phase for 15 min at room temperature.

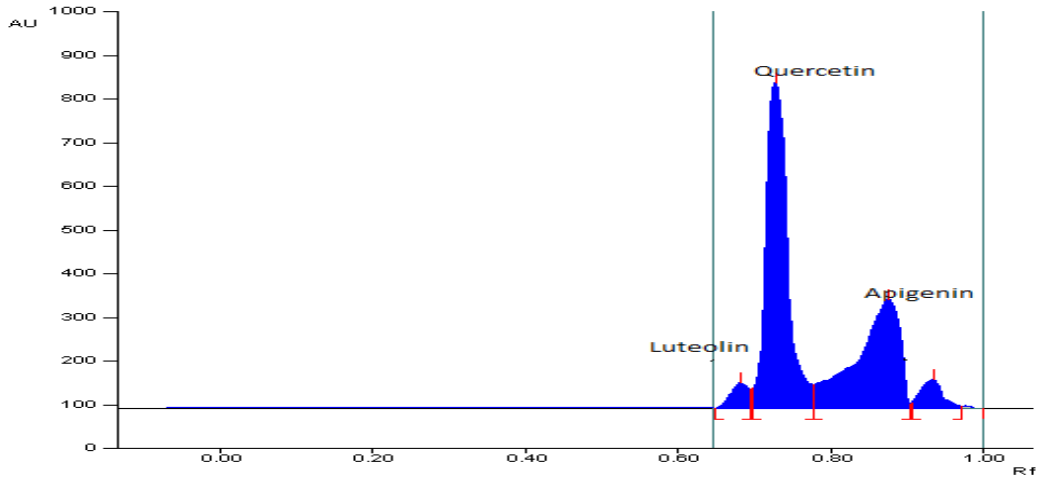
5.3.1.3. Preliminary trials for optimization of mobile phase (Table 5.5)



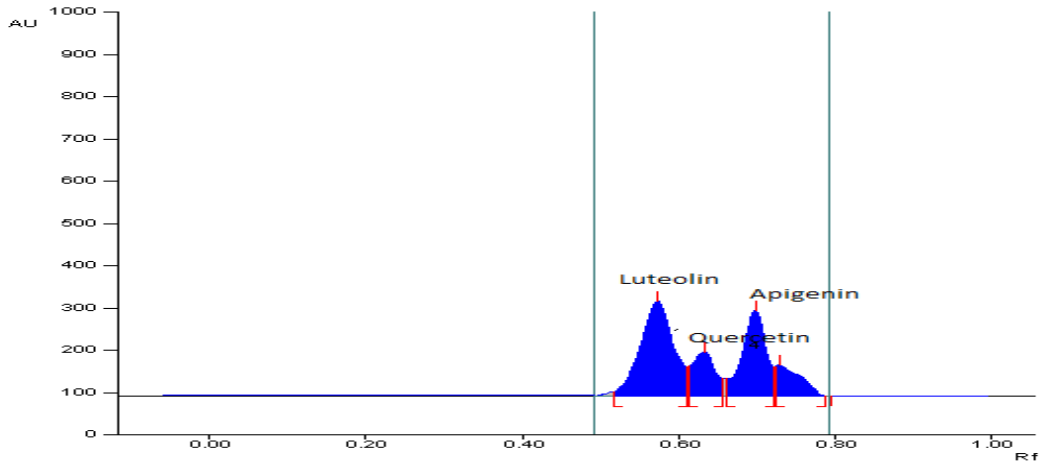
[A]: Toluene: Ethyl acetate: Chloroform: Formic acid (6: 6: 4: 1).



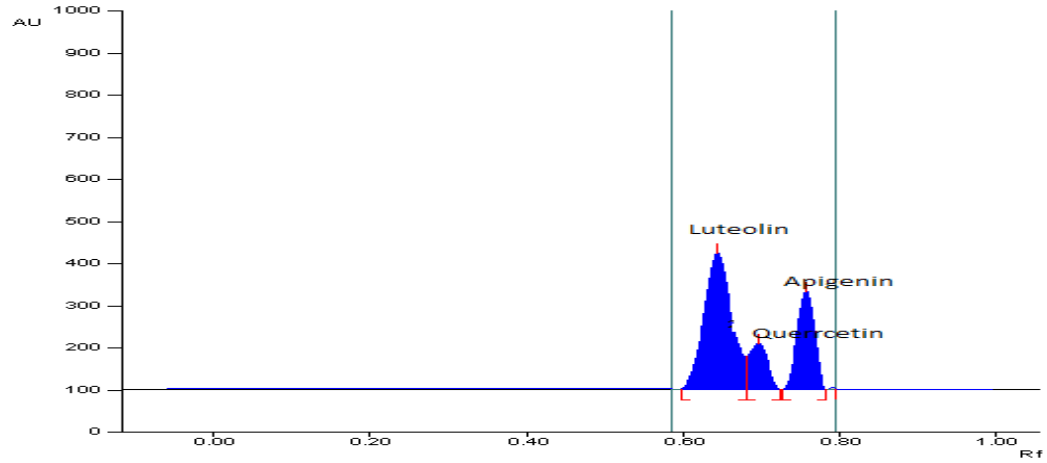
[B]: Chloroform: Methanol: Formic acid (8: 2: 1).



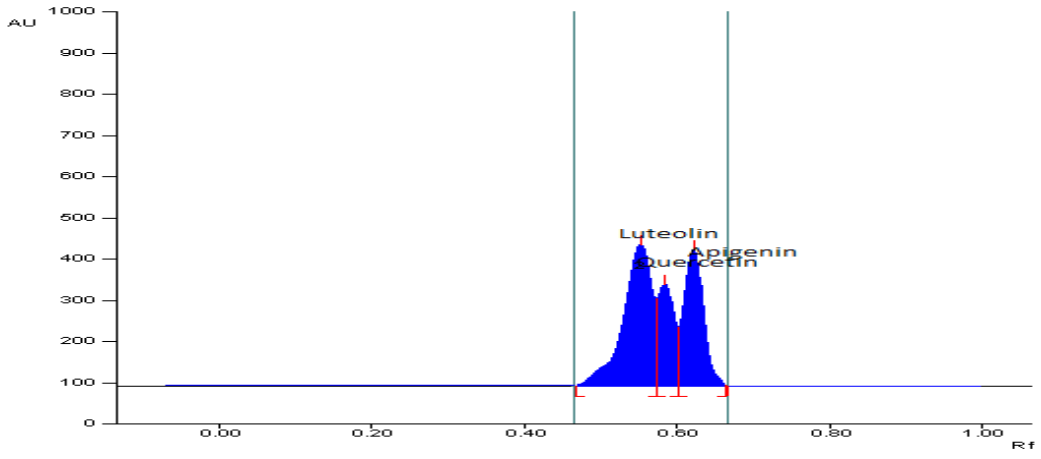
[C]: Hexane: Ethyl acetate (7: 3).



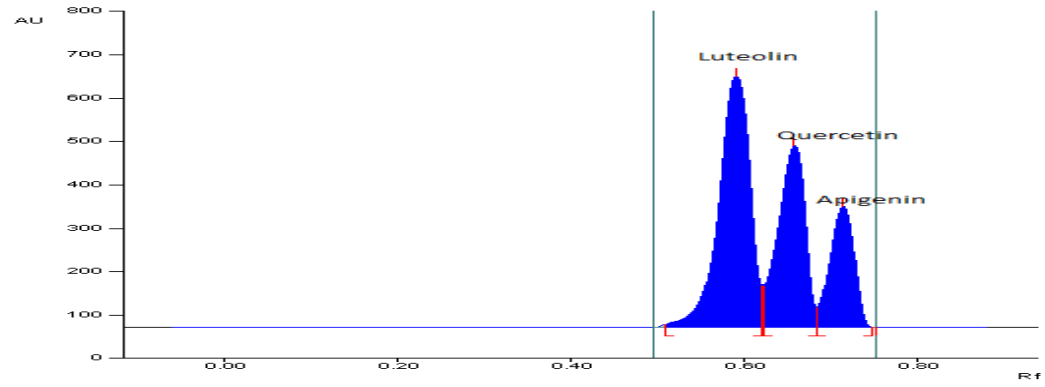
[D]: Toluene: Ethyl acetate: Chloroform: Formic acid (6: 4: 3: 1).



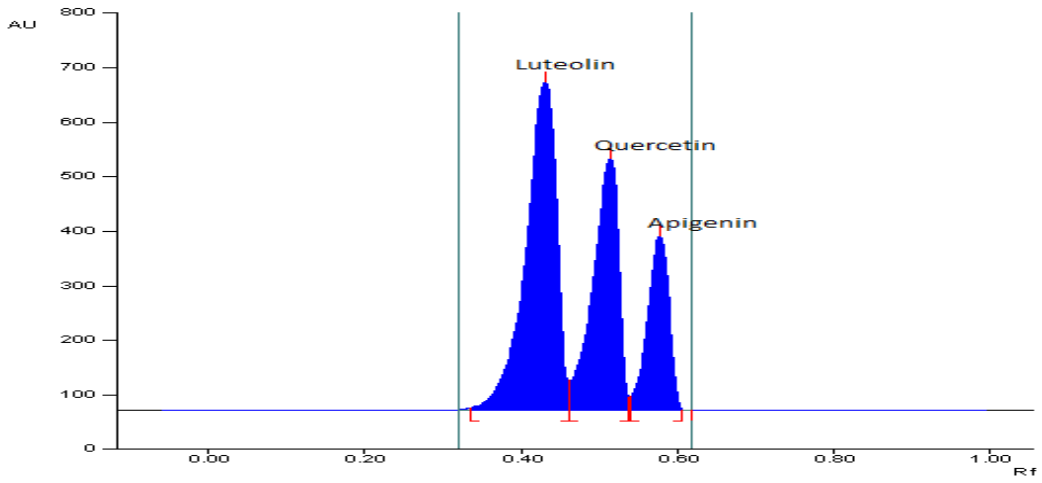
[E]: Methanol: Toluene: Ethyl acetate: Formic acid (0.3: 6: 4: 0.3).



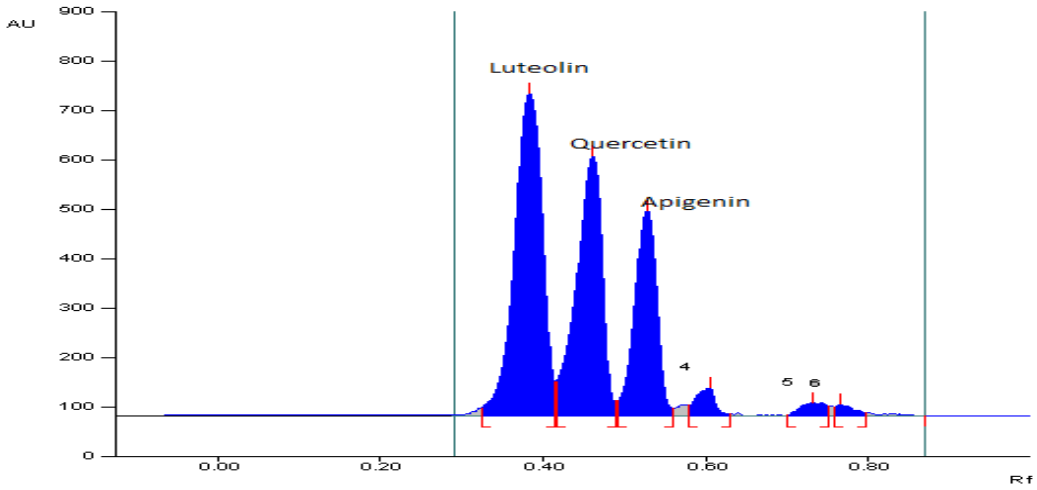
[F]: Methanol: Toluene: Ethyl acetate: Formic acid (0.1: 5: 4: 0.5).



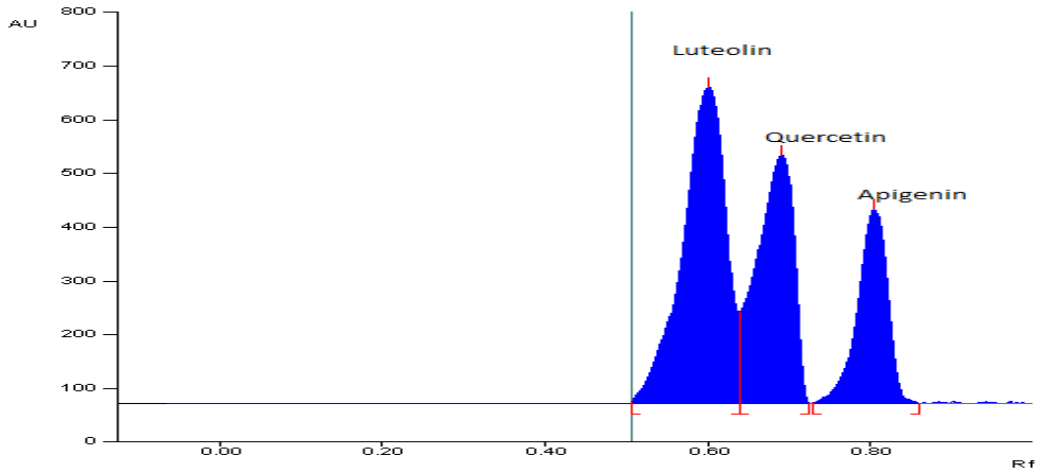
[G]: Toluene: Ethyl acetate: Formic acid (6: 4: 0.3).



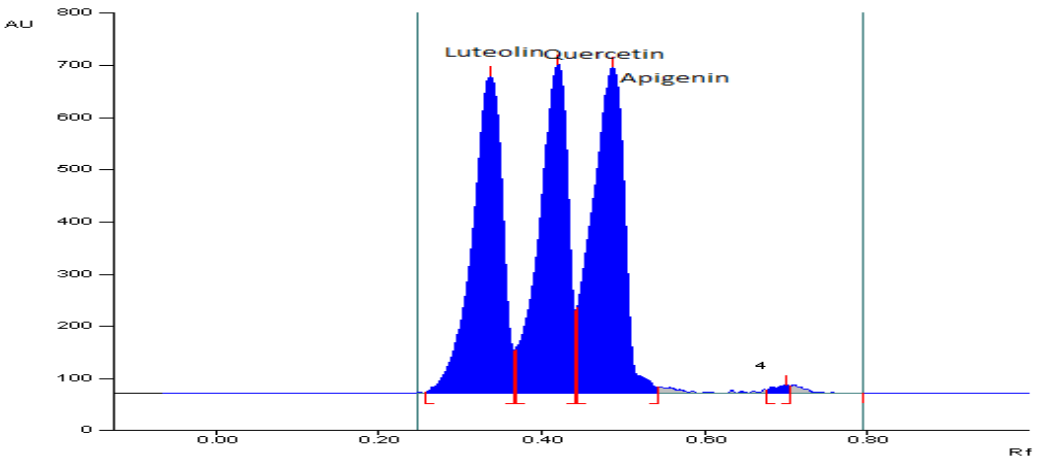
[H]: Toluene: Ethyl acetate: Formic acid (6: 4: 0.2).



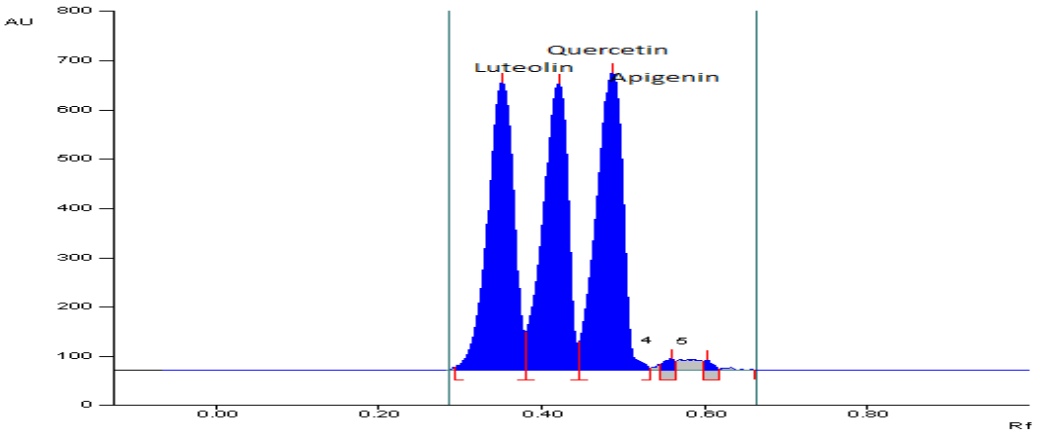
[I]: Toluene: Ethyl acetate: Formic acid (7: 3: 0.3).



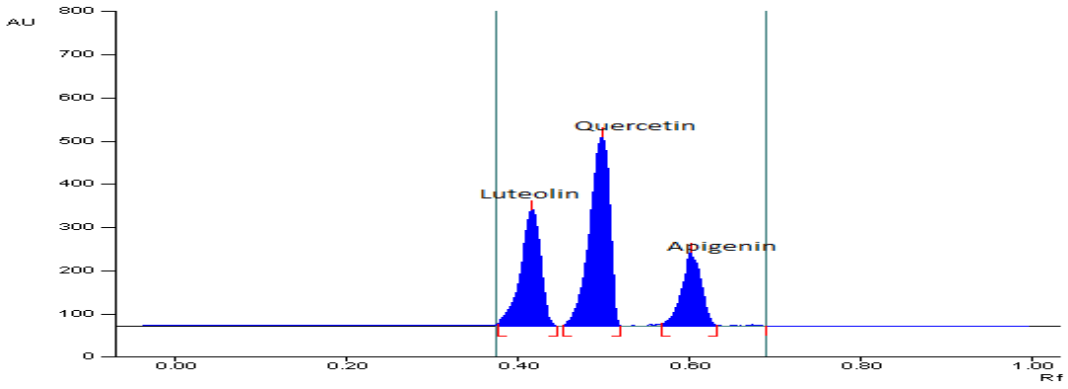
[J]: Chloroform: Ethyl acetate: Formic acid (6: 4: 0.2).



[K]: Methyl tertiary butyl ether: Ethyl acetate: Formic acid (6: 4: 0.2).



[L]: Octanol: Ethyl acetate: Formic acid (6: 4: 0.2).



[M]: Dual run- Toluene: Ethyl acetate: Formic acid (7: 3: 0.3).

Figure 5.10: [A-M] Preliminary trials for optimization of mobile phase

Table 5.5: Preliminary trials for Optimization of mobile phase

Figure 5.10	Solvent	R_f			Peak shape
		LUT	QUE	API	
A	Toluene: Ethyl acetate: Chloroform: Formic acid (6: 6: 4: 1)	0.69	0.70	0.80	High Rf with all three peak merge.
B	Chloroform: Methanol: Formic acid (8: 2: 1)	0.09	0.12	0.23	Less Rf with improper peak resolution.
C	Hexane: Formic acid (7: 3)	0.65	0.72	0.89	Tailing of second peak with high Rf.
D	Toluene: Ethyl acetate: Chloroform: Formic acid (6: 4: 3: 1)	0.57	0.62	0.70	Tailing of third peak and less resolution.
E	Methanol: Toluene: Ethyl acetate: Formic acid (0.3: 6: 4: 0.3)	0.63	0.70	0.76	Two peak merge third get separated.
F	Methanol: Toluene: Ethyl acetate: Formic acid (0.1: 5: 4: 0.5)	0.52	0.56	0.62	All peak merge and no resolution.
G	Toluene: Ethyl acetate: Formic acid (6: 4: 0.3)	0.59	0.66	0.73	High Rf and less resolution.

H	Toluene: Ethyl acetate: Formic acid (6: 4: 0.2)	0.40	0.50	0.59	Rf is less and tailing in first and second peak.
I	Toluene: Ethyl acetate: Formic acid (7: 3: 0.3)	0.38	0.46	0.53	Peak merge decreases but less resolution.
J	Chloroform: Ethyl acetate: Formic acid (6: 4: 0.2)	0.60	0.67	0.80	Tailing of first and second peak while third peak separated.
K	Methyl tertiary butyl ether: Ethyl acetate: Formic acid (6: 4: 0.2)	0.34	0.41	0.48	Split of first peak, second peak merge.
L	Octanol: Ethyl acetate: Formic acid (6: 4: 0.2)	0.35	0.41	0.50	Peak merge, less resolution
M	**Toluene: Ethyl acetate: Formic acid** **(7: 3: 0.3)-Dual run**	**0.42**	**0.50**	**0.61**	**Sharp peak** **Sharp peak** **Sharp peak**

5.3.1.4. Linearity

LUT, QUE and API showed good correlation over a concentration range of 400-1400 ng/band for LUT, QUE (Table 5.6, Table 5.7) and 40-140 ng/band for API (Table 5.8) with respect to peak area (Figure 5.11,Figure 5.12,Figure 5.13,Figure 5.14). The linearity of calibration curve and adherence of system to Beer's law was evaluated by high value of correlation coefficient (Table 5.9).

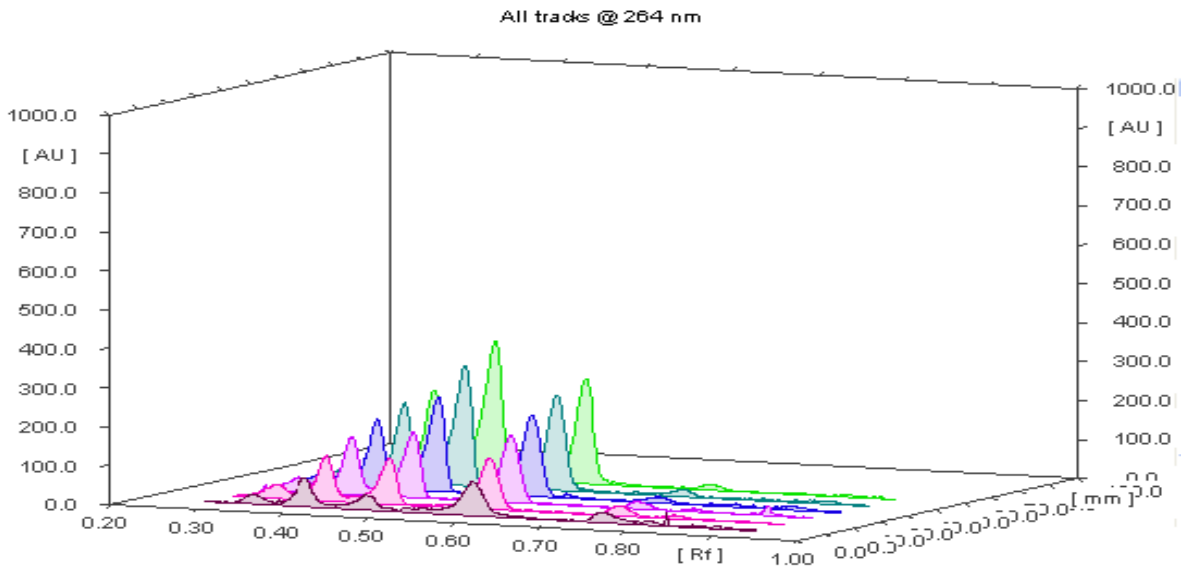


Figure 5.11: Linearity of LUT (400-1400 ng/band), QUE (400-1400 ng/band) and API (40-140 ng/band) standard

Table 5.6: Linearity study for LUT (400-1400 ng/band)

Conc. (ng/band)	Peak area 1	Peak area 2	Peak area 3	Peak area 4	Peak area 5	Avg. peak area[a]	SD	%RSD
400	5692	5692	5512	5517	5612	5605	88.77	1.58
600	6768	6818	6981	6878	6878	6865	79.60	1.15
800	7607	7453	7574	7654	7842	7626	141.75	1.80
1000	8882	8721	8915	8974	8745	8847	109.96	1.25
1200	9866	10115	9902	9987	9847	9943	109.71	1.11
1400	10915	11041	10974	11114	11247	11058	129.19	1.14

[a] n=5 replicates, SD= Standard deviation, % RSD= relative standard deviation

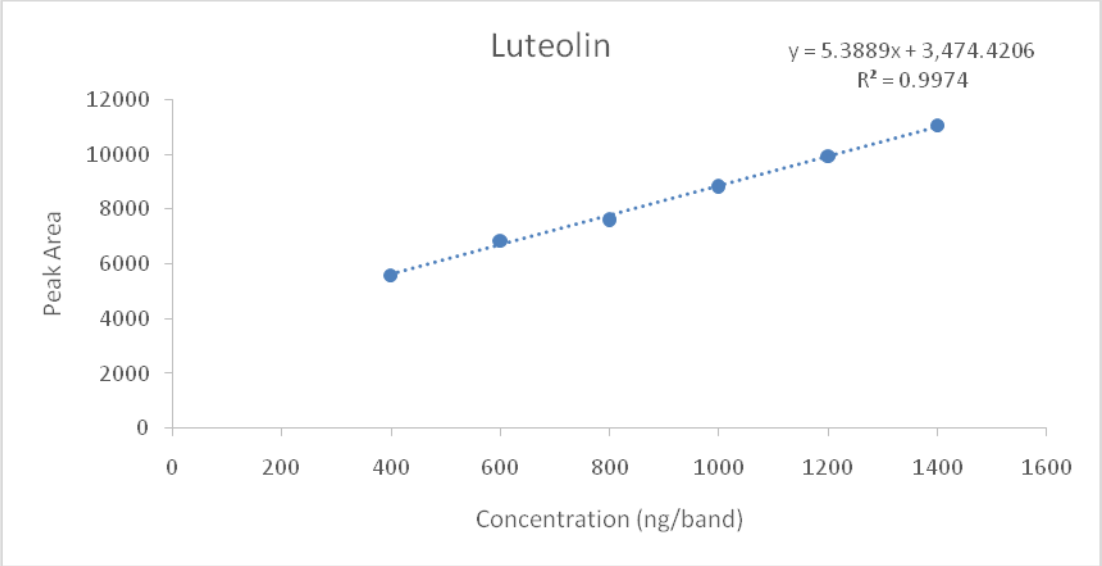


Figure 5.12: Calibration curve of LUT standard (400-1400 ng/band)

Table 5.7: Linearity study for QUE(400-1400 ng/band)

Conc. (ng/band)	Peak area 1	Peak area 2	Peak area 3	Peak area 4	Peak area 5	Avg. peak area[a]	SD	%RSD
400	10112	10215	10457	9987	10158	10185.9	173.31	1.70
600	12337	12396	12302	12378	12362	12355.5	36.76	0.29
800	14415	14489	14887	14498	14975	14653.0	257.84	1.75
1000	16463	16603	16489	16478	16789	16564.7	137.11	0.82
1200	17927	17933	18086	17889	17394.1	17846.1	263.67	1.47
1400	19854	19954	19873	19784	19254	19744.2	280.67	1.42

[a] n=5 replicates, SD= Standard deviation, % RSD= relative standard deviation

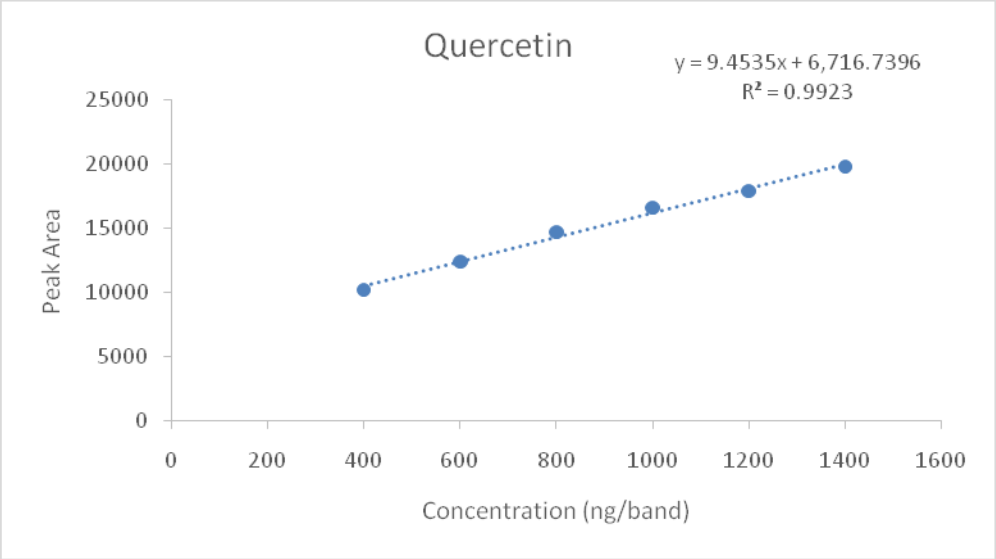


Figure 5.13: Calibration curve of QUE standard (400-1400 ng/band)

Table 5.8: Linearity study for API (40-140 ng/band)

Conc. (ng/band)	Peak area 1	Peak area 2	Peak area 3	Peak area 4	Peak area 5	Avg. peak area[a]	SD	%RSD
40	3879	3791	3848	3947	3920	3877.2	61.27	1.58
60	4968	5046	5102	5214	5142	5094.9	93.45	1.83
80	6109	6308	6117	5998	6247	6156.1	122.88	1.99
100	7642	7718	7885	7521	7845	7722.6	148.74	1.92
120	8939	8997	9145	8975	8798	8971	124.44	1.38
140	9662	9762	9885	9745	9874	9786	93.96	0.96

[a] n=5 replicates, SD=Standard deviation, % RSD= relative standard deviation

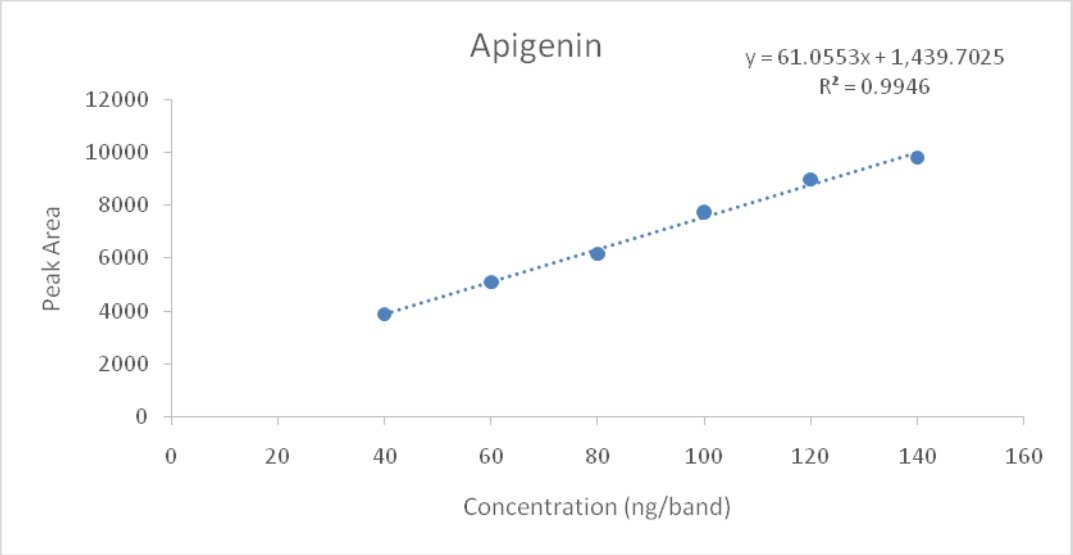


Figure 5.14: Calibration curve of API standard (40-140 ng/band)

Table 5.9: Linear regression parameters for LUT, QUE and API

Linear Regression Parameters	LUT	QUE	API
Calibration range [a] (ng/band)	400-1400	400-1400	40-140
Regression equation	5.3889x + 3474.4	9.4535x + 6716.7	61.0553x + 1439.7
Correlation coefficient	0.9974	0.9923	0.9946
Limit of detection (ng/band)	42.72	96.49	4.67
Limit of quantification (ng/band)	129.46	292.42	14.17

[a] n=5 replicates

5.3.1.5. Analysis of EAMTAM.

Extract when analysed in triplicate using the developed HPTLC method in present study was quantify for LUT, QUE and API (Figure 5.15) showed good recovery (Table 5.10) indicating that the method can be applicable in routine quality control testing of extract. The %RSD value was found to be less than 2.

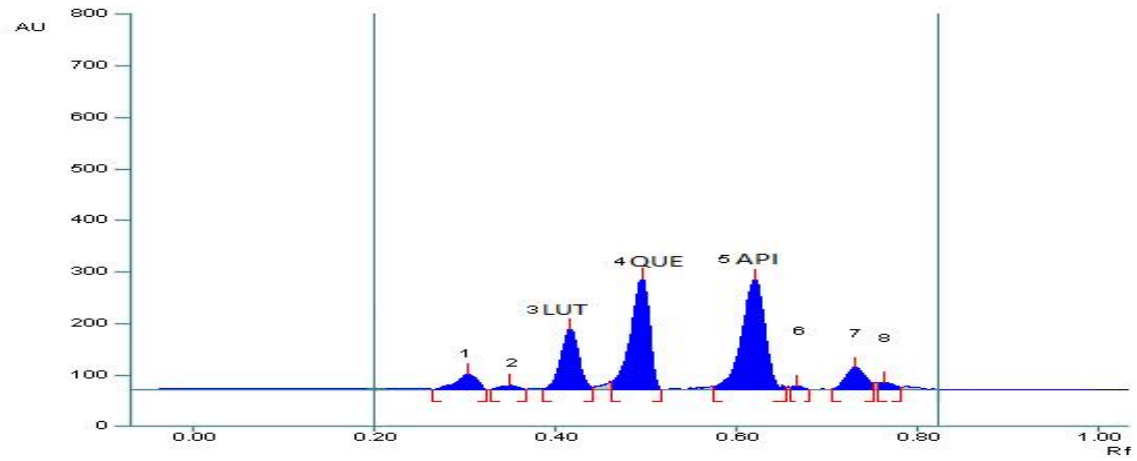


Figure 5.15: HPTLC chromatogram of EAMTAM containing 500 ng/band

Table 5.10: Analysis of EAMTAM

Drug	% amount of drug found[a] ± SD	% RSD
LUT	0.090 ± 0.14	1.48
QUE	0.142 ± 0.16	1.22
API	0.128 ± 0.22	0.81

[a] n=3 replicates, SD=Standard deviation, % RSD= relative standard deviation

5.3.1.6. Precision

Precision of developed method was evaluated by repeatability and interaday precision, and was expressed as %RSD of peak area. Repeatability and intermediate precision was carried out by performing three replicates of three different concentration (600, 800 and 1000 ng/band) for LUT and QUE, (60, 80 and 100 ng/band for API) showed %RSD less than 2% (Table 5.11), indicating acceptable precision in terms of repeatability of peak area measurement and sample application.

Table 5.11: Precision study for HPTLC method

Amount (ng/band)	Repeatability[a]		Interday precision[a]	
	Mean amount of drug found[a] ± SD (ng/band)	%RSD	Mean amount of drug found[a] ± SD (ng/band)	%RSD
LUT				
600	6865.1 ± 12.74	1.15	6860.5 ± 4.25	1.03
800	7746.24 ± 16.66	1.47	7748.04 ± 6.39	1.39
1000	8847.66 ± 38.18	1.25	8857.06 ± 2.78	1.23
QUE				
600	12355.52 ± 36.76	0.29	12513.12 ± 22.12	1.78
800	14653.02 ± 25.82	1.75	14655.02 ± 19.82	1.72
1000	16564.74 ± 13.49	0.82	16584.54 ± 26.45	0.77
API				
60	5094.92 ± 22.45	1.83	5094.72 ± 1.42	1.98
80	6156.18 ± 18.33	1.99	6139.58 ± 6.15	1.64
100	7722.68 ± 10.25	1.92	7718.08 ± 5.76	1.86

[a] n=3 replicates, SD=Standard deviation, % RSD= relative standard deviation

5.3.1.7. Accuracy

The proposed method when used for evaluation of recovery at three concentrations levels, 50%, 100% and 150% after spiking with standard, showed percentage recovery between 95.35 to 100.25% with acceptable % RSD, less than 2 revealed good accuracy of the method (Table 5.12).

Table 5.12: Recovery study for determination of LUT, QUE and API

Drug name	Recovery Level (%)	Amount added (ng/band)	Initial amount (ng/band)	Mean amount found	% Recovery[a]	SD	%RSD
LUT	50	200	400	5039.27	98.26	1.83	1.87
	100	400	400	6145.13	96.33	1.13	1.17
	150	600	400	7749	103.33	2.03	1.97
QUE	50	200	400	12345.7	99.23	0.84	0.84
	100	400	400	14490.6	102.79	1.16	1.13
	150	600	400	16518.9	103.68	0.78	0.75
API	50	20	40	6796.07	102.73	0.81	0.79
	100	40	40	7545.03	94.42	1.88	1.99
	150	60	40	8839.7	99.56	1.93	1.94

[a] n=3 replicates**,** SD= Standard deviation, % RSD= relative standard deviation

5.3.1.8. Limit of detection (LOD) and limit of quantitation (LOQ)

Limit of detection of developed method was found to be 42.72 ng/band for LUT, for QUE 96.49 ng/band and for API 4.67 ng/band and Limit of quantitation was found to be 129.46 ng/band for LUT, for QUE 292.42 ng/band and for API 14.17 ng/band indicating acceptable sensitivity of the method.

5.3.1.9. Specificity

The EAMTAM using the developed method, showed three peaks at R_f value 0.42 for LUT, R_f value 0.50 for QUE and R_f value 0.61 for API that was found to be at the same Rf values for all three standards by comparison of Densitogram (Figure 5.16) and HPTLC plate photo at 264nm (Figure 5.17). The absorption spectra by overlaying their UV absorption spectra (Figure 5.18) with those of respective standards. There were no interfering spots by the plant constituents at the R_f values of the markers.

The absorption spectra of standard marker LUT, QUE and API and the corresponding spot present in extract matched exactly, indicating no interference by the other plant

constituents. The purity of the bands in the sample extract was confirmed by overlaying the absorption spectra recorded at start, middle and end position of the band in the sample tracks respectively.

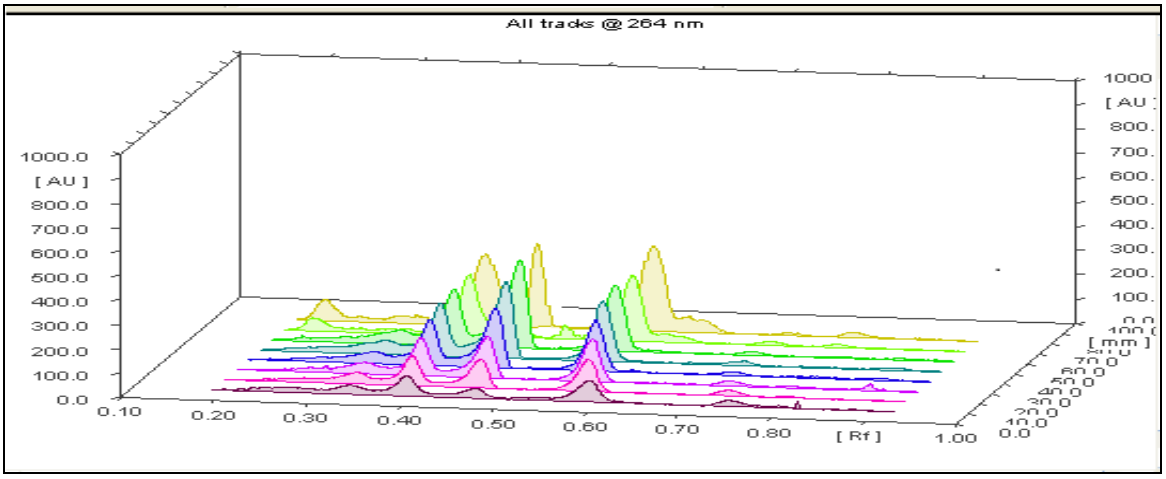


Figure 5.16: HPTLC chromatogram of EAMTAM containing 500 ng/band and standard LUT (400-1400 ng/band), QUE (400-1400 ng/band) and API (40-140 ng/band)

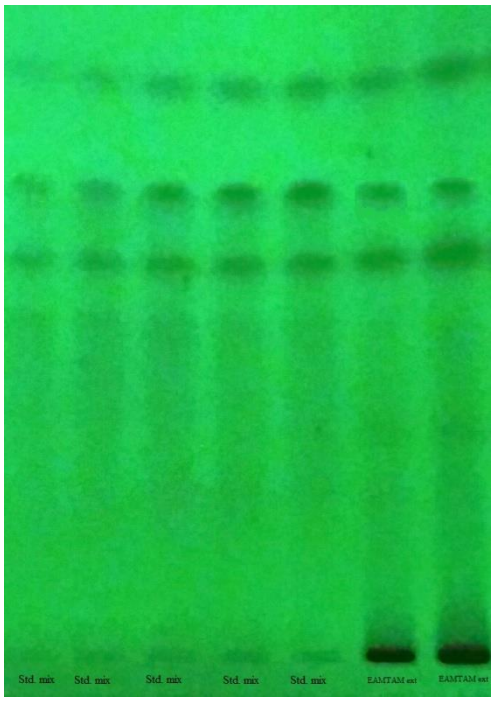
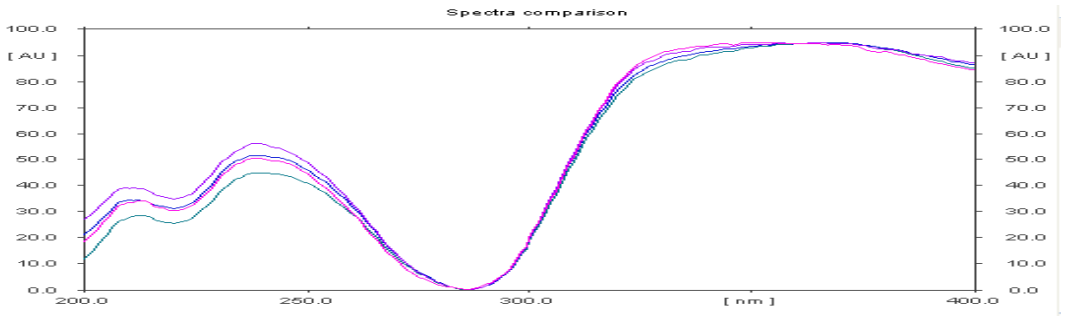
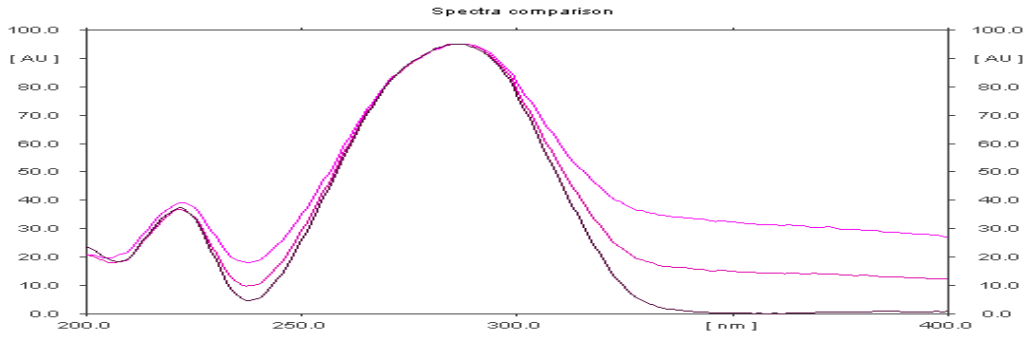


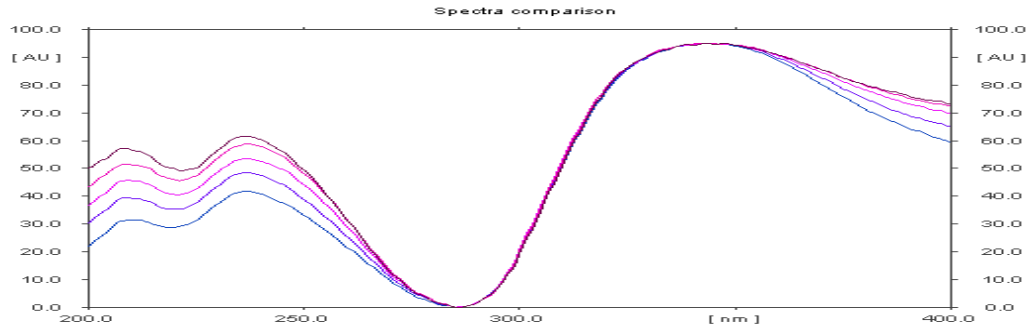
Figure 5.17: HPTLC plate photo at 264 nm containing standard mixture and EAMTAM



[A]



[B]



[c]

Figure 5.18: Overlay spectra of [A] luteolin, [B] quercetin and [C] apigenin

5.3.1.10. Robustness

The effect of small and deliberate variations on method parameters like change in mobile phase ratio (± 0.5 ml of ethyl acetate), solvent front (150 ± 5 cm) saturation time (15 ± 5 min) and wavelength (264 ± 2 nm). The effect of these changes on both the R_f values and peak areas was examined and % RSD calculated for each parameter was found to be less than 2% indicating the robustness of method (Table 5.13**).** From the robustness study it can be concluded that response percentage recovery is robust for all the factors within selected

range but, as percentage RSD of the response R_f and resolution was observed more than 2% on changing the ethyl acetate content in mobile phase, it evinced that R_f was getting affected by varying the variable ethyl acetate volume in the mobile phase from the optimum. Hence, the limits must be strictly defined for the method condition; ethyl acetate content in mobile phase in mobile phase for optimum method performance of standard with acceptable R_f.

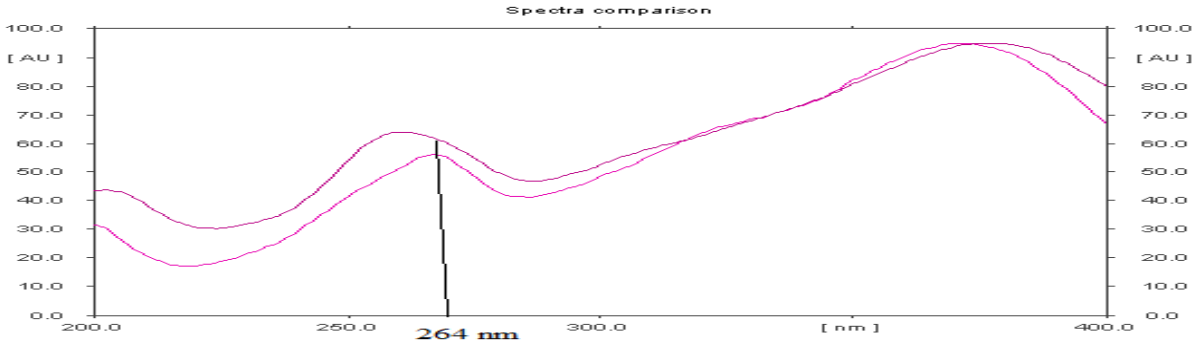
Table 5.13: Effect of changed parameters on R_f, Peak Area and Resolution

Parameters	R_f			Peak Area			Resolution	
	LUT	QUE	API	LUT	QUE	API	R_{12}	R_{23}
Chamber saturation time (min)								
10	1.66	1.96	1.61	1.90	1.26	0.63	0.65	0.59
15								
20								
Wavelength (nm)								
264	1.36	1.15	0.93	1.90	1.26	0.63	0.65	0.91
264								
266								
Ethyl acetate content in mobile phase (ml)								
2.5	8.92	7.61	6.34	1.90	1.26	0.63	7.92	3.95
3.0								
3.5								
Solvent front (cm)								
8.0	1.362	1.96	1.61	0.46	1.39	1.87	1.35	1.24
8.5								
9.0								

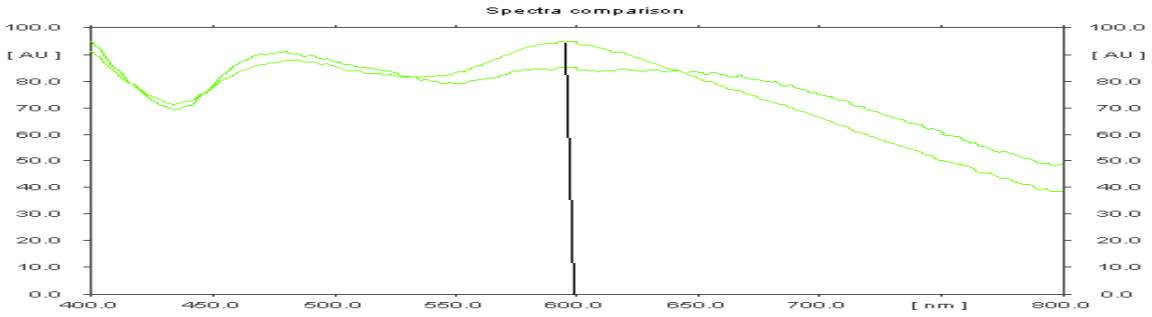
5.3.2. HPTLC Analysis of EAMTTP

5.3.2.1. Selection of wavelength

An ideal wavelength is the one that gives good response for the drugs that are to be detected. In the present study, a solution containing QUE, KAE, SIT and LUP concentration of 500 ng/band was prepared in methanol and scanned from 200-800 nm. The common detection wavelength selected for analysis was 264 nm as QUE and KAE were showing optimum response at 264 nm. While the common detection wavelength selected for analysis was 600 nm as LUP and SIT were showing optimum response at 600 nm after derivatization with Anisaldehyde sulphuric acid (Figure 5.19).



[A]



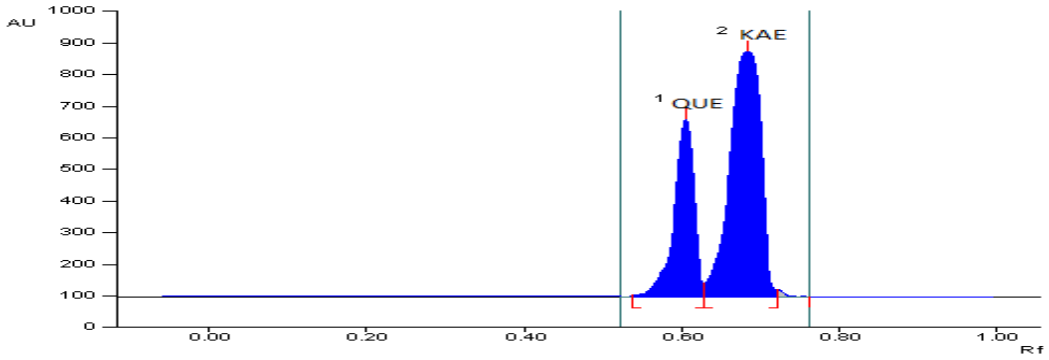
[B]

Figure 5.19: [A] Selection wavelength of QUE and KAE (264 nm), [B] Selection wavelength of SIT and LUP (600 nm)

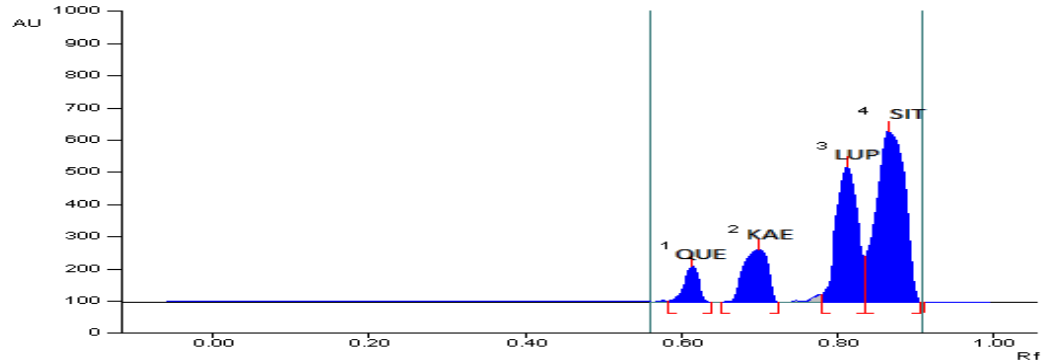
5.3.2.2. Optimization of mobile phase

All the four drugs were spotted on the HPTLC plates and run in different solvent systems and different ratios of various solvents were tried like n-hexane, toluene, methanol, ethyl acetate, acetonitrile, diethyl ether, chloroform, dichloromethane. From these, combination of ethyl acetate, and toluene gave good result and good separation and hence, further trials were initiated for different ratios of methanol, ethyl acetate, toluene with addition of different modifiers like glacial acetic acid, ammonia solution, formic acid, ortho

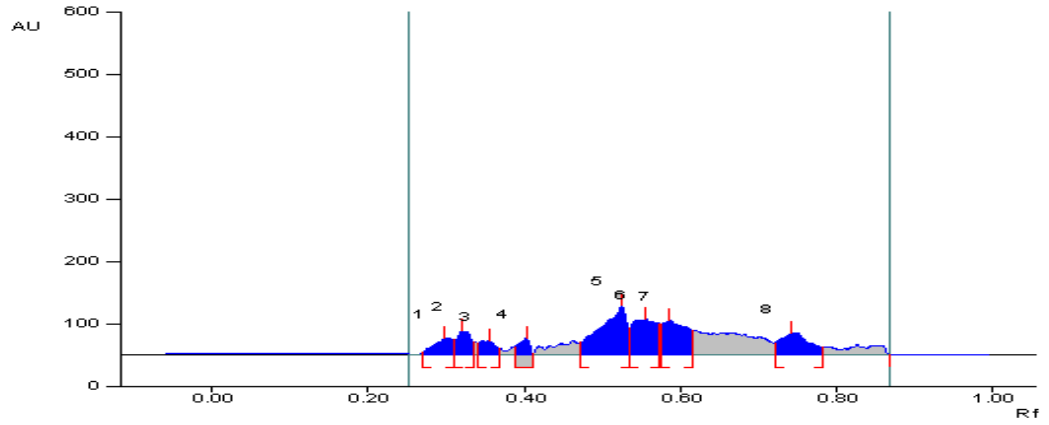
phosphoric acid and ammonium formate. Finally, the mobile phase consisting of ethyl acetate: toluene: formic acid (7:3:0.3 % v/v/v) gave sharp and symmetrical peak. Well defined band of QUE at R_f 0.28, KAE at R_f 0.40, LUP at R_f 0.56 and SIT at R_f 0.67 (Figure 5.20) was obtained when the chamber was saturated with the optimized mobile phase for 15 min at room temperature (Table 5.14)



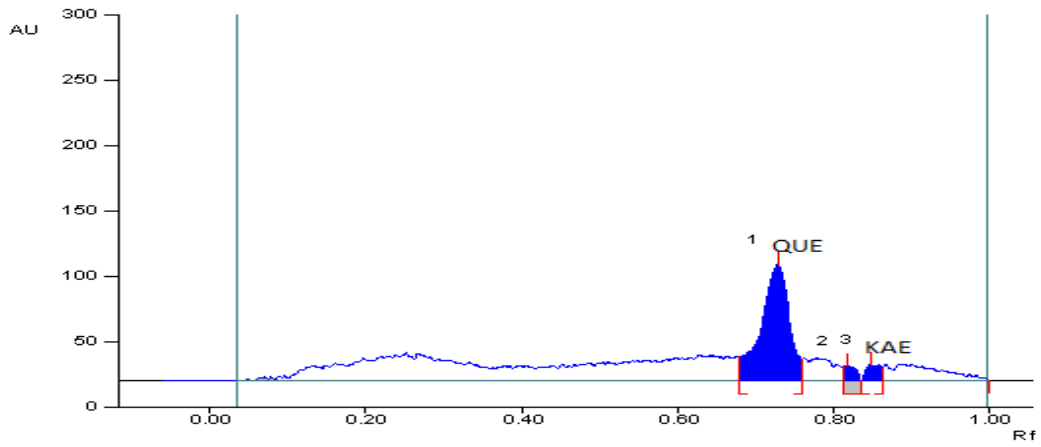
[A]: Toluene: Ethyl acetate: Formic acid (6: 4: 0.2) at 264 nm.



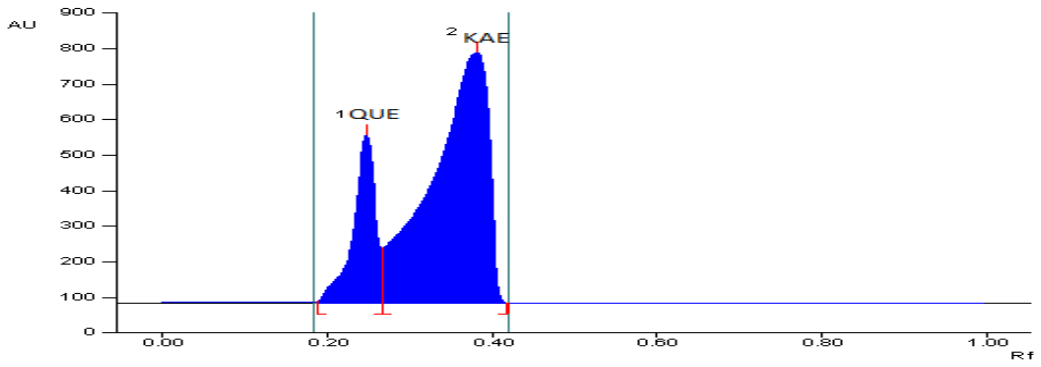
[A]: Toluene: Ethyl acetate: Formic acid (6: 4: 0.2) at 600 nm.



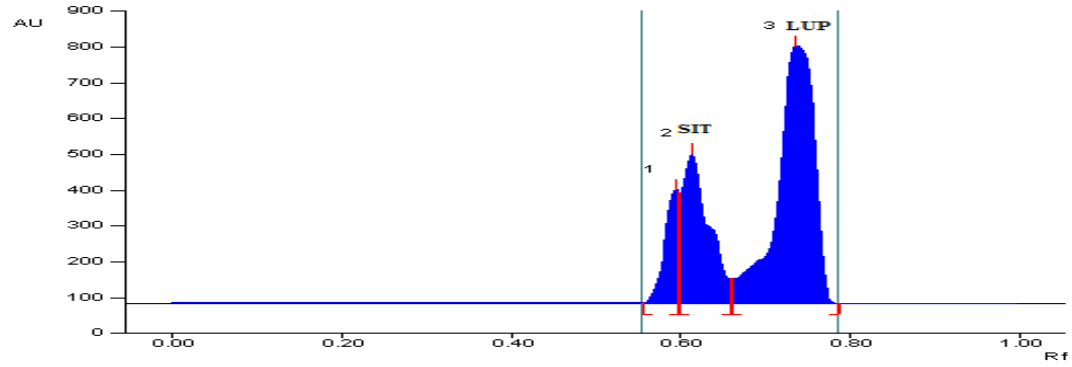
[B]: Toluene: Ethyl acetate: Water: Formic acid (8: 2: 0.6: 0.3) at 264 nm.



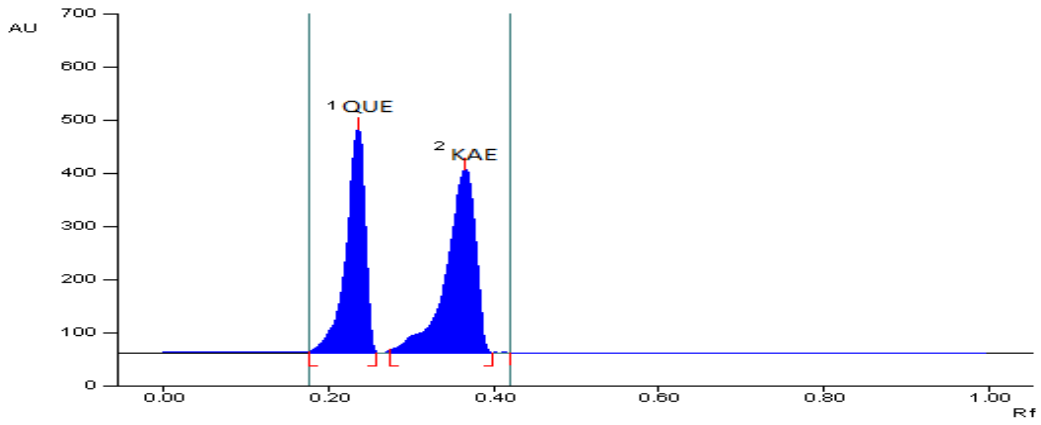
[C]: Methanol: Hexane: Ethyl formate (7: 3: 0.9) at 264 nm.



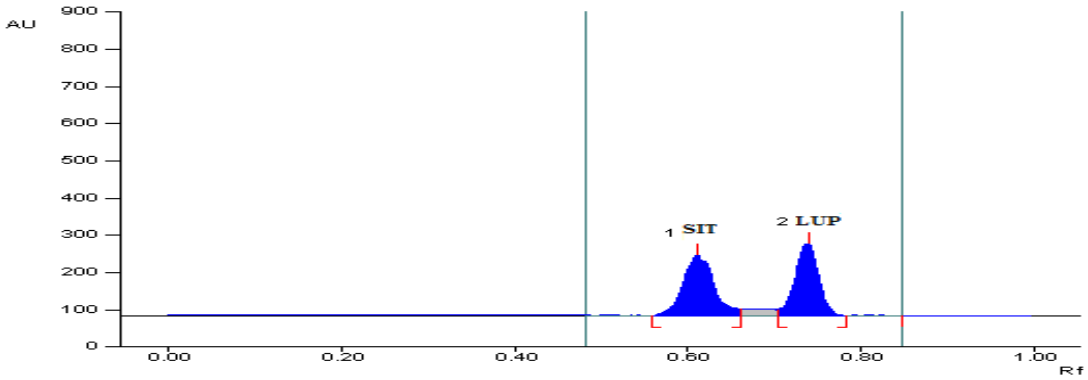
[D]: Chloroform: Methanol (9: 1) at 264 nm.



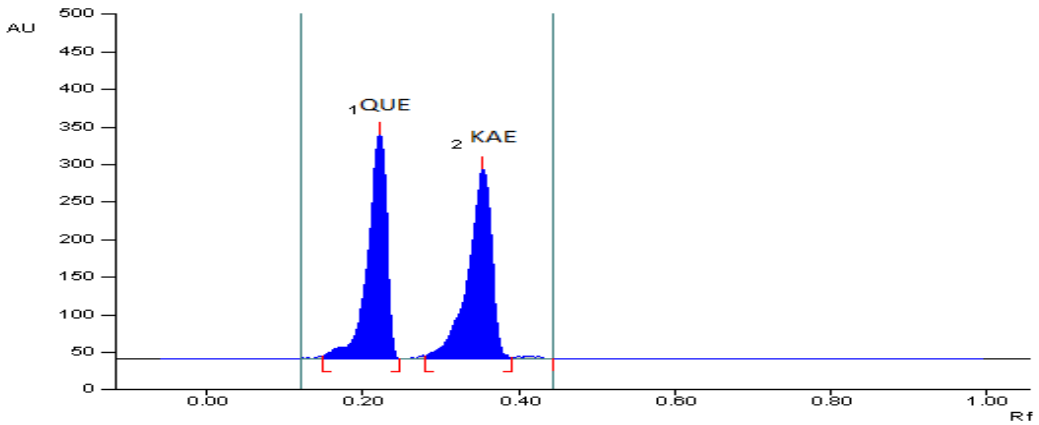
[D]: Chloroform: Methanol (9: 1) at 600 nm.



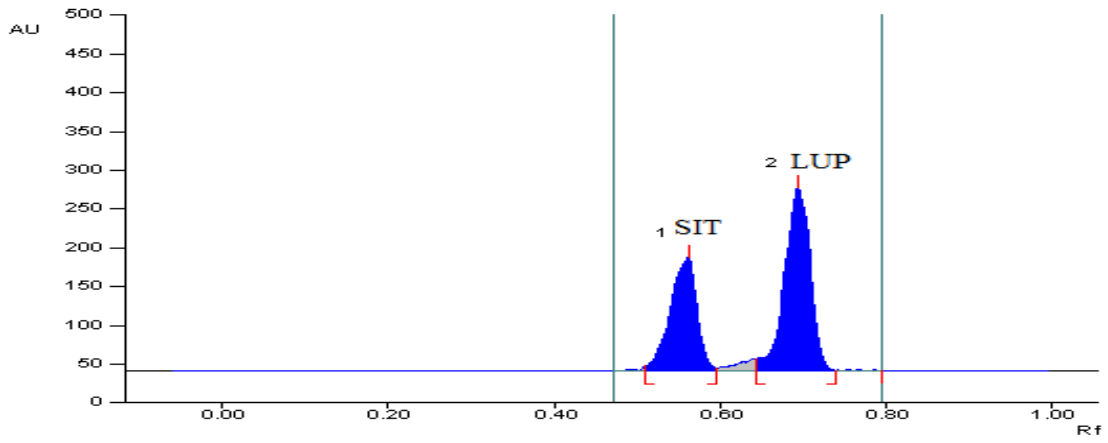
[E]: Toluene: Ethyl acetate: Formic acid (6: 3: 0.3) at 264 nm.



[E]: Toluene: Ethyl acetate: Formic acid (6: 3: 0.3) at 600 nm.



[F]: Toluene: Ethyl acetate: Formic acid (7: 3: 0.3) at 264 nm.



[F]: Toluene: Ethyl acetate: Formic acid (7: 3: 0.3) at 600 nm.

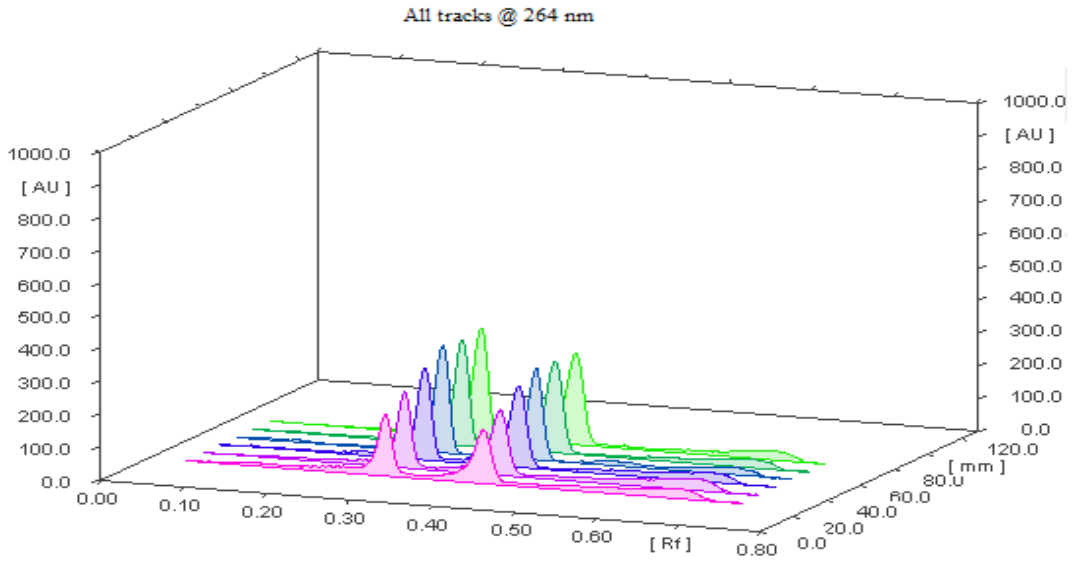
Figure 5.20: [A-F] Preliminary trials for Optimization of mobile phase

Table 5.14: Preliminary trials for Optimization of mobile phase

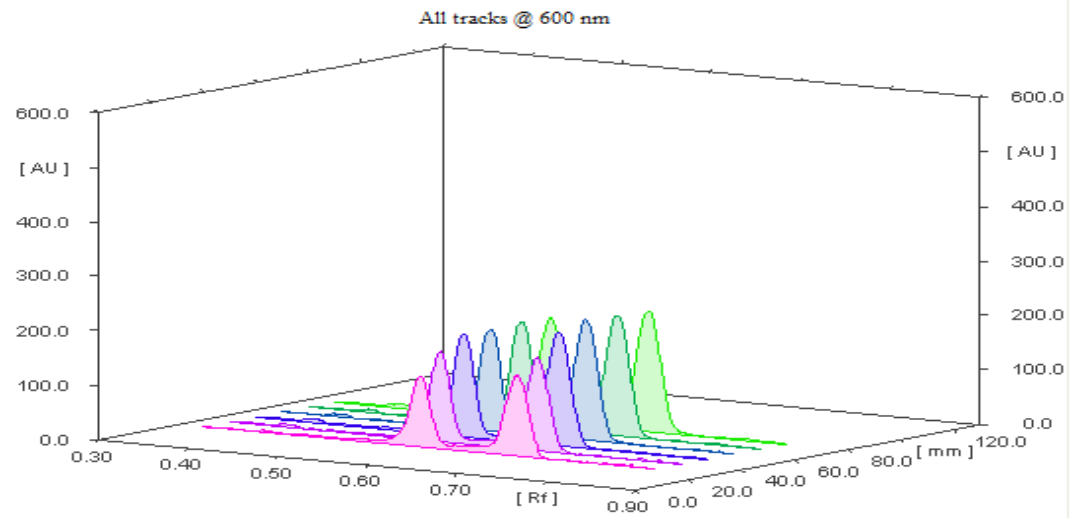
Figure 5.20	Solvent	R_f				Peak shape
		QUE	KAE	SIT	LUP	
A	Toluene: Ethyl acetate: Formic acid (6: 4: 0.2)	0.60	0.65	0.80	0.92	High R_f with last two peak merge
B	Toluene: Ethyl acetate: Water: Formic acid (8: 2: 0.6: 0.3)	-	-	-	-	No proper seperation
C	Methanol: Hexane: Ethyl formate (7: 3: 0.9)	0.73	0.82	-	-	High R_f with last two peak missing.
D	Chloroform: Methanol (9: 1)	0.23	0.38	0.61	0.78	Peak merge with tailing of second and fourth peak
E	Toluene: Ethyl acetate: Formic acid (6: 3: 0.3)	0.22	0.30	0.60	0.78	Peak sharp but improper resolution
F	**Toluene: Ethyl acetate: Formic acid (7: 3: 0.3)**	**0.28**	**0.40**	**0.56**	**0.67**	**Sharp peak** **Sharp peak** **Sharp peak** **Sharp peak**

5.3.2.3. Linearity

QUE, KAE, SIT and LUP showed good correlation over a concentration range of 600-1600 ng/band with respect to peak area (Figure 5.21,Figure 5.22,Figure 5.23,Figure 5.24,Figure 5.25). The linearity of calibration curve and adherence of system to Beer's law was evaluated by high value of correlation coefficient (Table 5.15,Table 5.16,Table 5.17,Table 5.18,Table 5.19).



[A]



[B]

Figure 5.21: Linearity of QUE, KAE, SIT and LUP (600-1600 ng/band standard

Table 5.15: Linearity study for QUE (600-1600 ng/band)

Conc. (ng/band)	Peak area 1	Peak area 2	Peak area 3	Peak area 4	Peak area 5	Avg. peak area[a]	SD	%RSD
600	3930	3829	3775	3881	3847	3852.44	57.89	1.50
800	5025	5036	5089	4979	4912	5008.34	66.25	1.32
1000	6073	6197	6196	6018	6078	6112.4	80.30	1.31
1200	7040	7119	6985	6913	7097	7030.8	83.99	1.19
1400	7629	7657	7729	7884	7887	7757.2	122.67	1.58
1600	8859	8826	8759	8665	8842	8790.22	79.61	0.90

[a] n=5 replicates, SD= Standard deviation, % RSD= relative standard deviation

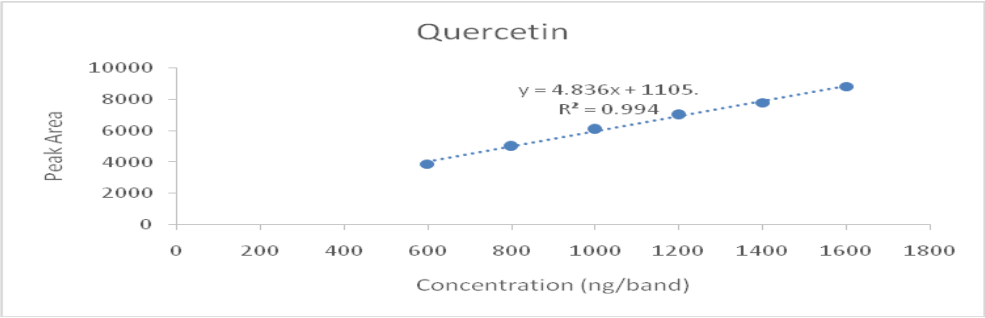


Figure 5.22: Calibration curve of QUE standard (600-1600 ng/band).

Table 5.16: Linearity study for KAE (600-1600 ng/band)

Conc. (ng/band)	Peak area 1	Peak area 2	Peak area 3	Peak area 4	Peak area 5	Avg. peak area[a]	SD	%RSD
600	4305	4390	4295	4378.8	4359	4345.56	43.19	0.99
800	5383	5386	5230	5338.7	5330	5333.54	63.16	1.18
1000	6242	6301	6388	6575	6388	6378.8	125.92	1.97
1200	7710	7681	7417	7609	7645	7612.44	115.65	1.51
1400	8710	8697	8852	8529	8475	8652.66	151.44	1.75
1600	9850	9726	9678	9602	9778	9726.96	94.54	0.97

[a] n=5 replicates, SD= Standard deviation, % RSD= relative standard deviation

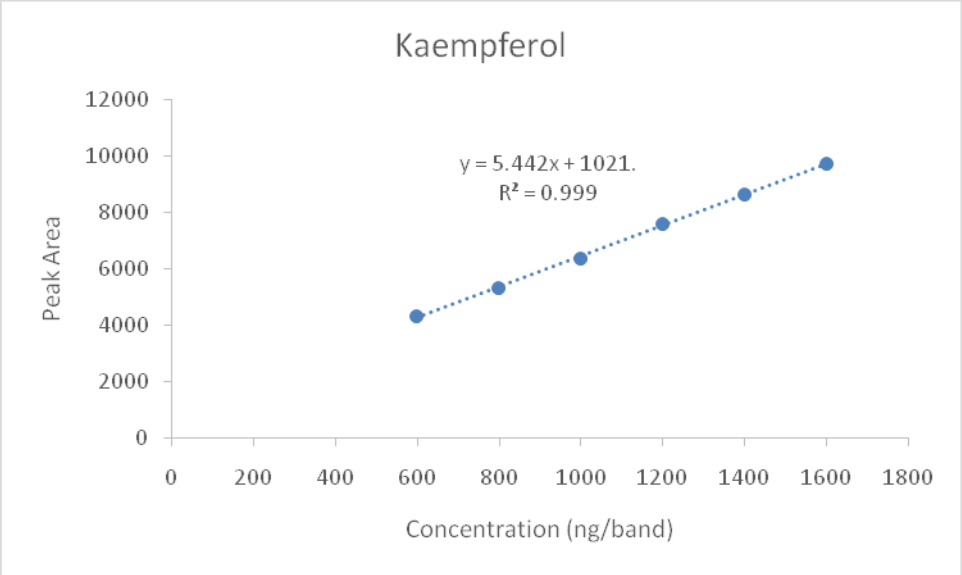


Figure 5.23: Calibration curve of KAE standard (600-1600 ng/band).

Table 5.17: Linearity study for SIT (600-1600 ng/band)

Conc. (ng/band)	Peak area 1	Peak area 2	Peak area 3	Peak area 4	Peak area 5	Avg. peak area[a]	SD	%RSD
600	3114	3025	3198	3119	3120	3115.2	61.30	1.96
800	3878	3899	3912	4010	3902	3920.4	51.63	1.32
1000	4540	4655	4510	4625	4575	4581	59.51	1.30
1200	5158	5177	5138	4995	5124	5118.54	71.85	1.40
1400	5689	5638	5697	5921	5672.3	5723.46	112.72	1.98
1600	6325	6442	6356	6435	6412	6394.24	50.98	0.79

[a] n=5 replicates, SD= Standard deviation, % RSD= relative standard deviation

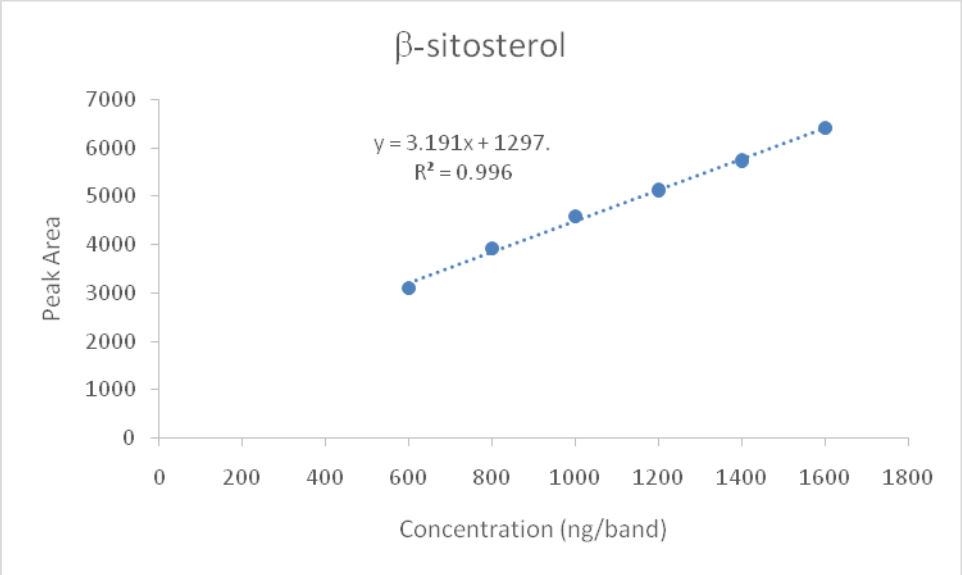


Figure 5.24: Calibration curve of SIT standard (600-1600 ng/band).

Table 5.18: Linearity study for LUP (600-1600 ng/band)

Conc. (ng/band)	Peak area 1	Peak area 2	Peak area 3	Peak area 4	Peak area 5	Avg. peak area[a]	SD	%RSD
600	4228	4192	4172	4117	4145	4170.9	42.55	1.02
800	5194	5218	5138	5026	5139	5143	74.08	1.44
1000	6295	6138	6121	6214	6214	6196.4	69.70	1.12
1200	7147	7155	7116	6955	7155	7105.6	85.70	1.19
1400	7990	8190	8152	8047	8125	8100.9	81.07	0.99
1600	8992	9012	8902	8988	8912	8961.4	50.43	0.56

[a] n=5 replicates, SD= Standard deviation, % RSD= relative standard deviation

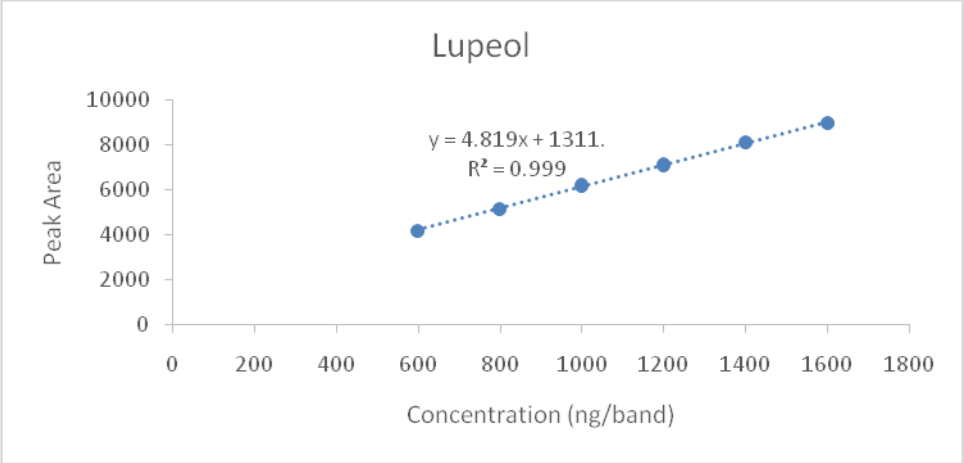


Figure 5.25: Calibration curve of LUP standard (600-1600 ng/band).

Table 5.19: Linear regression parameters for QUE, KAE, SIT and LUP

Linear Regression Parameters	**QUE**	**KAE**	**SIT**	**LUP**
Calibration range [a] (ng/band)	600-1600	600-1600	600-1600	600-1600
Regression equation	4.836x + 1105	5.442x + 1021	3.191x + 1297	4.819x + 1311
Correlation coefficient	0.994	0.999	0.996	0.999
Limit of detection (ng/band)	58.03	90.79	59.46	62.16
Limit of quantification (ng/band)	175.87	275.13	180.20	188.38

[a] n=5 replicates

5.3.2.4. Analysis of EAMTTP

Extract when analyzed in triplicate using the developed HPTLC method in present study was quantify for QUE, KAE, SIT and LUP (Figure 5.26,Figure 5.27) showed good recovery where percentage amount for all the drugs were within the range of 90.00%-99.64% (Table 5.20) indicating that the method can be applicable in routine quality control testing of extract. The %RSD value was found to be less than 2.

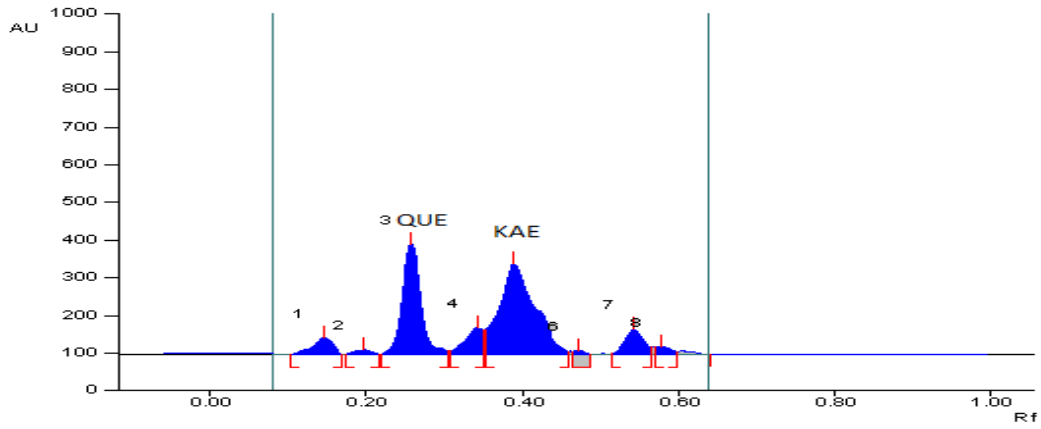


Figure 5.26: HPTLC chromatogram of EAMTTP containing 264 ng/band

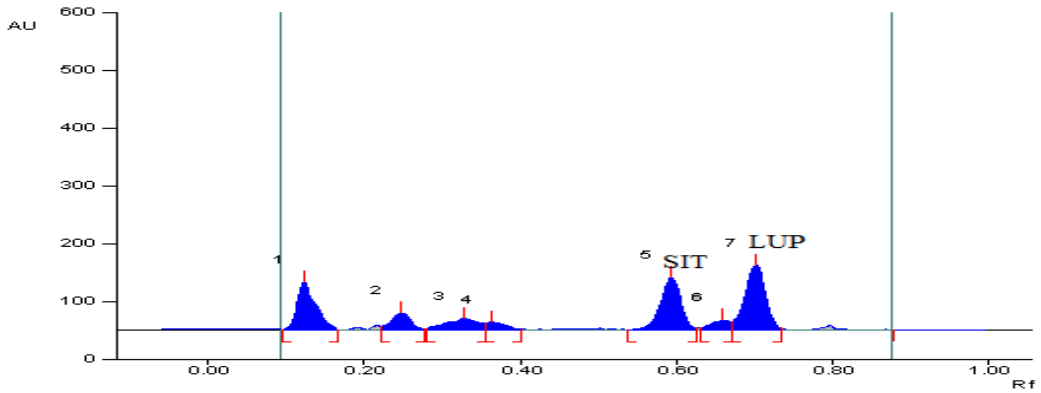


Figure 5.27: HPTLC chromatogram of EAMTTP containing 600 ng/band after derivatization

Table 5.20: Analysis of EAMTTP

Drug	% amount of drug found [a] ± SD	% RSD
QUE	0.122 ± 0.54	1.22
KAE	0.148 ± 0.63	0.81
SIT	0.096 ± 2.21	1.00
LUP	0.095 ± 3.35	0.72

[a] n=3 replicates, SD= Standard deviation, % RSD= relative standard deviation

5.3.2.5. Precision

Precision of developed method was evaluated by repeatability and interaday precision, and was expressed as %RSD of peak area. Repeatability and intermediate precision was carried out by performing three replicates of three different concentration (800, 1000 and 1200

ng/band) for QUE, KAE, SIT and LUP showed %RSD less than 2% (Table 5.21), indicating acceptable precision in terms of repeatability of peak area measurement and sample application.

Table 5.21: Precision study for HPTLC method

Amount (ng/band)	Repeatability[a]		Interday precision[a]	
	Mean amount of drug found[a] ± SD (ng/band)	%RSD	Mean amount of drug found[a] ± SD (ng/band)	%RSD
QUE				
800	815.64 ± 7.07	0.70	815.16 ± 6.82	0.69
1000	1044.19 ± 14.74	1.15	1042.53 ± 5.24	1.31
1200	1228.77 ± 13.92	0.92	1229.59 ± 12.72	1.07
KAE				
800	792.17 ± 16.39	1.68	799.46 ± 15.50	0.77
1000	992.12 ± 17.90	1.47	1003.35 ± 16.52	1.72
1200	1209.19 ± 29.66	2.00	1208.64 ± 28.35	1.60
SIT				
800	814.17 ± 5.44	0.54	815.06 ± 7.44	0.80
1000	1024.66 ± 23.98	1.91	1020.49 ± 20.89	1.21
1200	1209.31 ± 6.11	0.41	1211.92 ± 5.62	0.22
LUP				
800	814.17 ± 5.44	0.86	804.58 ± 9.62	0.80
1000	1024.66 ± 23.98	1.60	1017.13± 18.56	1.51
1200	1209.31 ± 6.11	0.28	1213.42 ± 5.20	0.30

[a] n=3 replicates, SD= Standard deviation, % RSD= relative standard deviation

5.3.2.6. Accuracy

The proposed method when used for evaluation of recovery at three concentrations levels, 50%, 100% and 150% after spiking with standard, showed percentage recovery between 95.35 to 103.25% with acceptable % RSD, less than 2 revealed good accuracy of the method (Table 5.22).

Table 5.22: Recovery study for determination of QUE, KAE, SIT and LUP

Drug name	Recovery Level (%)	Amount added (ng/band)	Initial amount (ng/band)	Mean amount found	% Recovery[a]	SD	%RSD
QUE	50	272	544	815.64	99.95	0.70	0.70
	100	544	544	1096.61	100.79	0.13	0.13
	150	816	544	1361.75	100.12	0.90	0.90
KAE	50	227	454	676.92	99.40	1.75	1.77
	100	454	454	912.37	100.48	0.41	0.41
	150	681	454	1137.76	100.24	1.02	1.02
SIT	50	260	520	779.99	99.99	0.93	0.93
	100	520	520	1042.93	100.28	1.09	1.08
	150	780	520	1293.21	99.47	1.86	1.87
LUP	50	206	412	619.40	100.22	0.72	0.72
	100	412	412	828.98	100.60	1.70	1.69
	150	618	412	1065.32	103.42	1.007	0.973

[a] n=3 replicates, SD= Standard deviation, % RSD= relative standard deviation

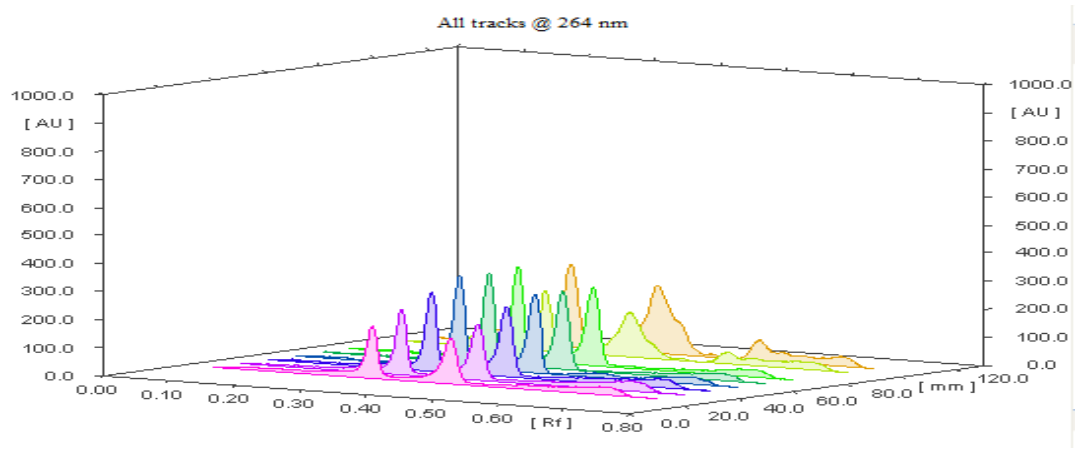
5.3.2.7. Limit of detection (LOD) and limit of quantitation (LOQ)

Limit of detection of developed method was found for QUE 58.03 ng/band, for KAE 90.79 ng/band, for SIT 59.46 ng/band and for LUP 62.16 ng/band and Limit of quantitation was found for QUE 175.87 ng/band, for KAE 275.13 ng/band, for SIT 180.20 ng/band and for LUP 188.38 ng/band indicating acceptable sensitivity of the method.

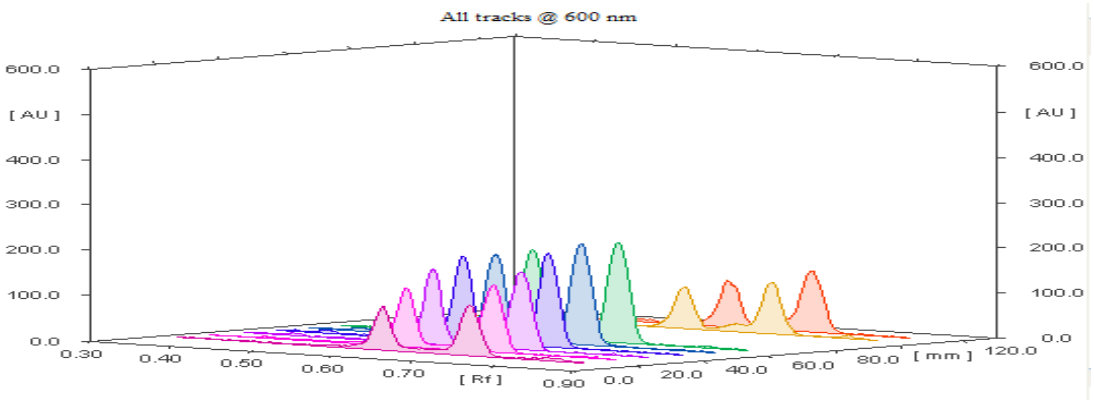
5.3.2.8. Specificity

The EAMTTP using the developed method, showed four peaks at R_f value 0.28 for QUE, R_f value 0.40 for KAE, R_f value 0.56 for SIT and R_f value 0.67 for LUP that was found to be at the same Rf values for all four standards by comparison of Densitogram (Figure 5.28) and HPTLC plate photo at 264 nm and 600 nm (Figure 5.29). The absorption spectra by overlaying their UV absorption spectra (Figure 5.30) with those of respective standards. There were no interfering spots by the plant constituents at the R_f values of the markers.

The absorption spectra of standard marker's QUE, KAE, SIT and LUP and the corresponding spot present in extract matched exactly, indicating no interference by the other plant constituents. The purity of the bands in the sample extract was confirmed by overlaying the absorption spectra recorded at start, middle and end position of the band in the sample tracks respectively.

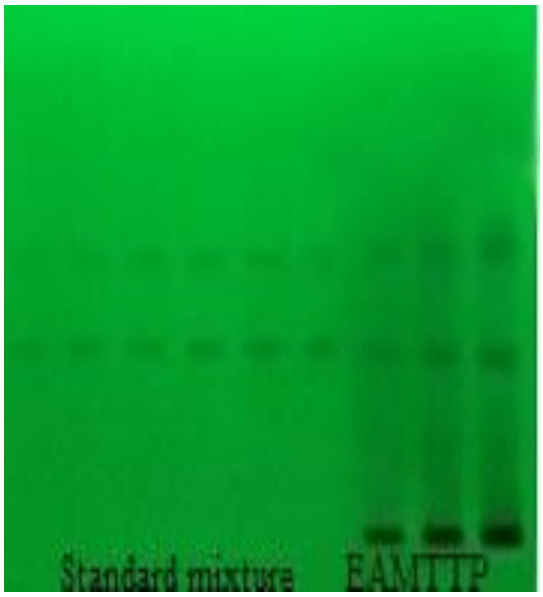


[A]

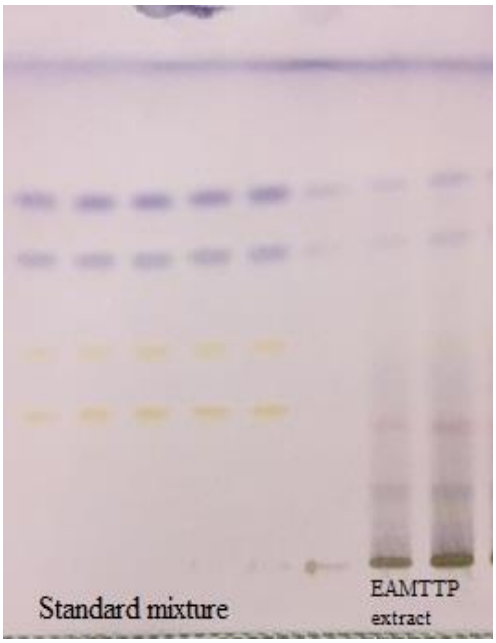


[B]

Figure 5.28: HPTLC chromatogram of EAMTTP containing 500 ng/band and standard QUE, KAE, SIT and LUP (600-1600 ng/band) [A] at 264 nm and [B] 600nm.

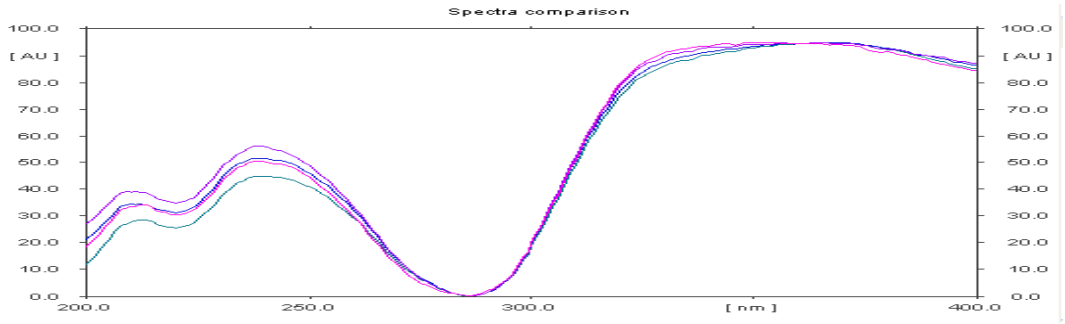


[A]

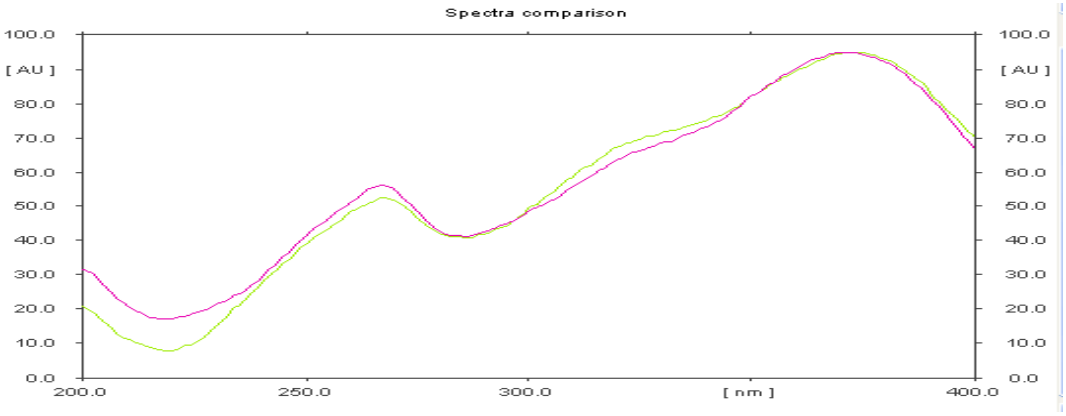


[B]

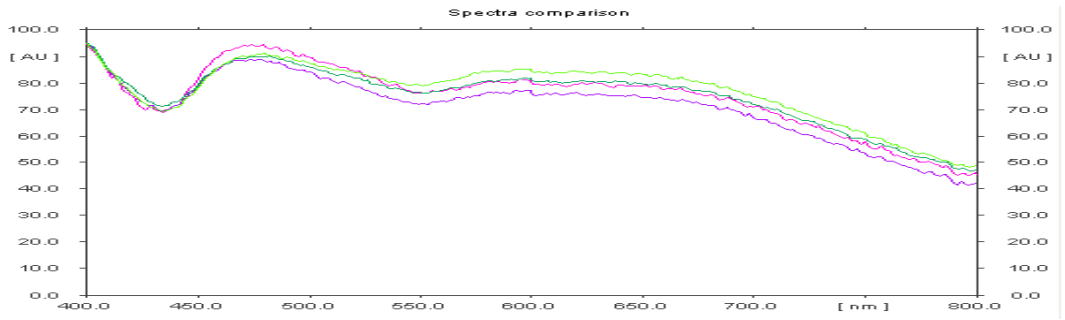
Figure 5.29: HPTLC plate photo at [A] 264 nm and [B] 600 nm containing Standard mixture and EAMTTP



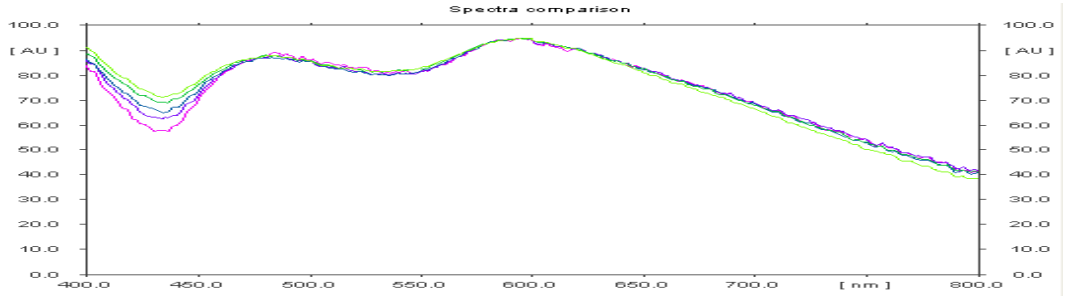
[A]



[B]



[C]



[D]

Figure 5.30: Overlay spectra of [A] quercetin, [B] kaempferol, [C] β-sitosterol and [D] lupeol standards.

5.3.2.9. Robustness

The effect of small and deliberate variations on method parameters like change in mobile phase ratio (± 0.5 ml of ethyl acetate), solvent front (150 ± 5 cm) saturation time (15 ± 5 min) and wavelength (264 ± 2nm). The effect of these changes on both the R_f values and peak areas was examined and % RSD calculated for each parameter was found to be less than 2% indicating the robustness of method (Table 5.23).

Table 5.23: Effect of changed parameters on R_f, Peak Area and Resolution

Parameters	R_f				Peak Area				Resolution	
	QUE	KAE	SIT	LUP	QUE	KAE	SIT	LUP	R_{12}	R_{34}
Chamber saturation time (min)										
10	2.03	1.41	1.78	1.47	0.35	0.49	0.93	0.54	1.39	0.66
15										
20										
Wavelength (nm)										
262/598	2.08	1.45	1.03	0.85	0.75	0.49	1.37	0.54	1.02	1.00
264/600										
266/602										
Ethyl acetate content in mobile phase (ml)										
2.5	7.26	11.17	8.10	9.08	0.58	0.96	1.94	0.26	2.16	7.01
3.0										
3.5										
Solvent front (cm)										
85	2.03	1.43	1.78	1.47	0.35	0.49	1.03	0.54	2.16	1.00
90										
95										

From the robustness study it can be concluded that response percentage recovery is robust for all the factors within selected range but, as percentage RSD of the response R_f and resolution was observed more than 2% on changing the ethyl acetate content in mobile phase, it evinced that R_f was getting affected by varying the variable ethyl acetate volume in the mobile phase from the optimum. Hence, the limits must be strictly defined for the method condition; ethyl acetate content in mobile phase in mobile phase for optimum method performance of standard with acceptable R_f

5.4. LC-MS/MS analysis of *A. millefolium* and *T. populnea*

5.4.1. LC-MS/MS analysis of *A. millefolium L*.

5.4.1.1. Optimized chromatography trial

Better chromatography and response were found with following chromatographic conditions:

Water: 0.1% formic acid and Acetonitrile: Methanol and 0.1% formic acid (50:50 v/v)

0.300 ml/min

Column: Gemini C18 column (50 mm x 2.0 mm, 3 μm).

5.4.1.2. Chromatographic condition

Multiple chromatographic conditions were explored in order to have appropriate retention time, and better resolution and sensitivity. Gemini C18 column (50 mm x 2.0 mm, 3 μm) was evaluated to attain better separation and was finally chosen for the chromatographic separation. The mobile phase consisting of Water: 0.1% formic acid and Acetonitrile: Methanol and 0.1% formic acid (50:50 v/v) 0.300 ml/min was found to be optimal for this study (Table 5.24). In addition, under the optimized conditions, no significant endogenous interference was found.

Table 5.24: Chromatographic conditions.

<table>
<tr><th>Parameters</th><th colspan="3">Detail</th></tr>
<tr><td>Column</td><td colspan="3">Gemini C18, (50 mm x 2.0 mm, 3 µm)</td></tr>
<tr><td rowspan="9">Mobile Phase</td><td colspan="3">A: Water + 0.1% formic acid
B: ACN (50): Methanol (50) + 0.1% formic acid</td></tr>
<tr><td>Time(min)</td><td>A%</td><td>B%</td></tr>
<tr><td>0.00</td><td>80</td><td>20</td></tr>
<tr><td>1.50</td><td>50</td><td>50</td></tr>
<tr><td>2.50</td><td>0</td><td>100</td></tr>
<tr><td>4.00</td><td>0</td><td>100</td></tr>
<tr><td>4.50</td><td>50</td><td>50</td></tr>
<tr><td>5.50</td><td>80</td><td>20</td></tr>
<tr><td>6.00</td><td>80</td><td>20</td></tr>
<tr><td>Flow rate</td><td colspan="3">0.400 ml/min</td></tr>
<tr><td>Column oven temperature</td><td colspan="3">40 ± 0.3°C</td></tr>
<tr><td>Autosampler temperature</td><td colspan="3">10 ± 3°C</td></tr>
<tr><td>Volume of injection</td><td colspan="3">5.0 µl</td></tr>
<tr><td>Detector</td><td colspan="3">Mass detector (MS/MS)</td></tr>
<tr><td>Retention time</td><td colspan="3">Apigenin at about 3.25 minutes.</td></tr>
<tr><td></td><td colspan="3">Luteolin at about 3.21 minutes</td></tr>
<tr><td></td><td colspan="3">Quercetin at about 3.19 minutes</td></tr>
<tr><td>Run time</td><td colspan="3">5.0 minutes</td></tr>
</table>

5.4.1.3. Mass spectrometric conditions

Electron spray ionization (ESI) is the most commonly used soft ionization sources in mass spectrometry. By investigating the full scan mass spectra of Apigenin, Luteolin and quercetin, it was found that the signal intensity in the positive mode was much higher than that in the negative ion mode (Figure 5.31,Figure 5.32,Figure 5.33). Thus, all detections were carried out using the predominantly positive ion The most suitable mass spectrometric conditions were determined by optimizing all the parameters of the mass spectrometer such as collision energy, nebulizer gas, DL temperature, heat block temperature and drying gas flow to obtain much higher and more stable response (Table 5.25, Table 5.26).

Table 5.25: Mass Spectrometric conditions

Parameters	Apigenin	Luteolin	Quercetin
Ion Source	Electro Spray ionization		
Polarity	Positive		
Parent Ion	271.0	287.0	303
Daughter Ion	121	153.1	229
Dwell Time (msec)	100	100	100
Collision Energy (CE)	42	44	37

Table 5.26: Mass Spectrometric source dependent parameters

Parameters	Used
DL Temperature	250 °C
Nebulizing Gas Flow	3.0 l/min
Heat Block Temperature	450 °C
Drying Gas Flow	15.0 l/min

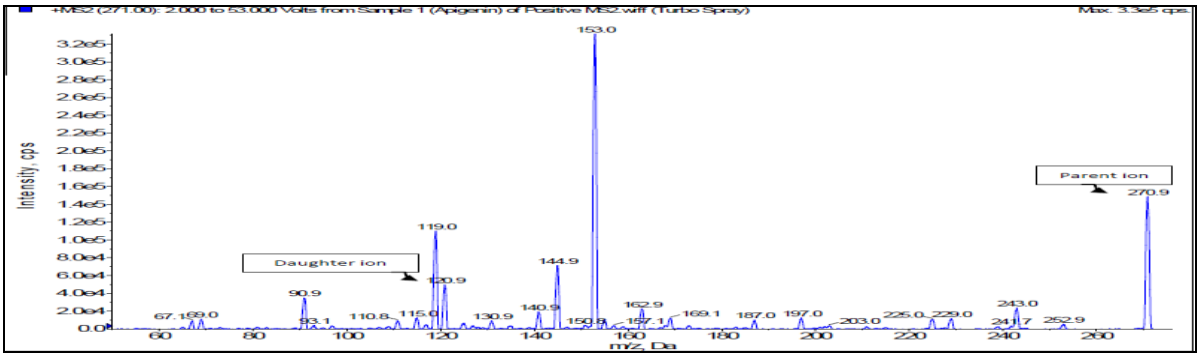


Figure 5.31: MS-MS spectra of Apigenin.

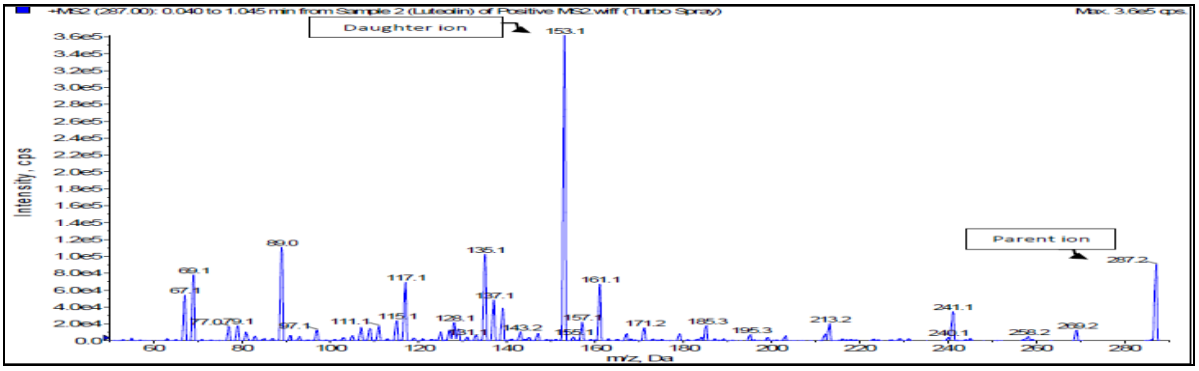


Figure 5.32: MS-MS spectra of Luteolin.

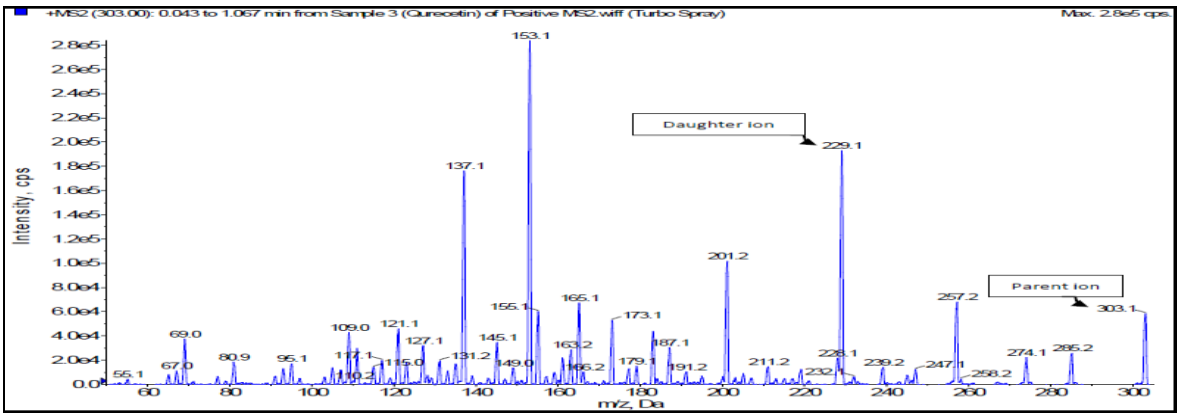


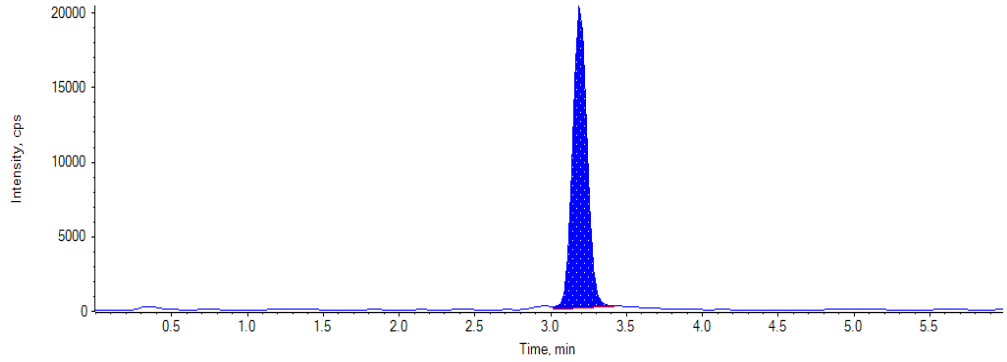
Figure 5.33: MS-MS spectra of Quercetin.

5.4.1.4. Method validation

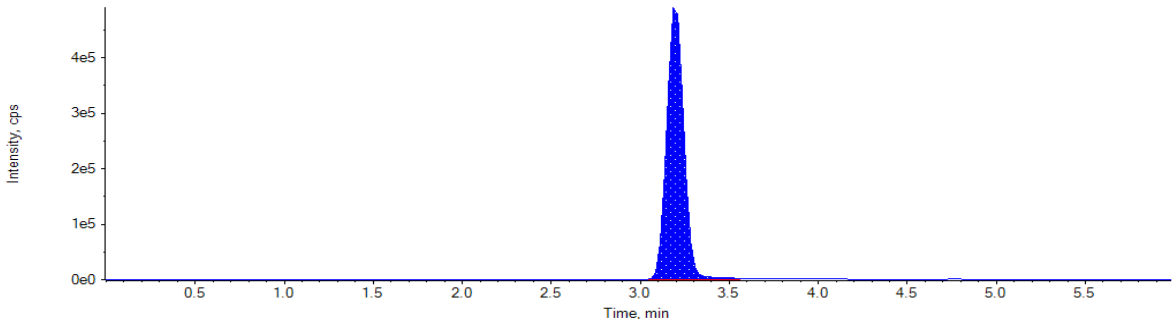
5.4.1.4.1 Selectivity and specificity

The selectivity of the method was investigated by comparing chromatograms of apigenin, luteolin and quercetin. The retention time was 3.22, 3.18 and 3.16 min for apigenin, luteolin and quercetin, respectively. There were no significant endogenous peaks that could

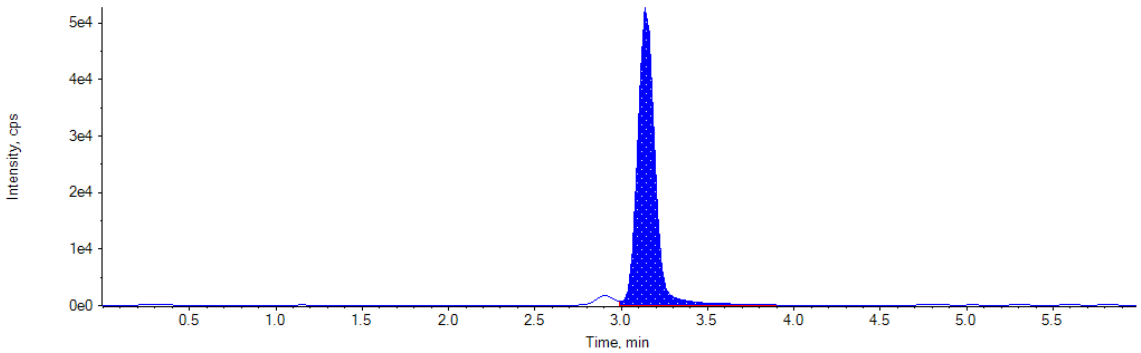
interfere with the analyte (Figure 5.34). The results indicated that the method exhibited good specificity and selectivity.



[A]



[B]



[C]

Figure 5.34: Chromatograms of [A] apigenin, [B] luteolin and [C]quercetin

5.4.1.4.2 Linearity

The linearity was evaluated by analyzing a series of different concentrations of the standard apigenin, luteolin and quercetin where each concentration was applied triplicate. Linear regression data for the calibration curves of standard apigenin, luteolin and quercetin showed a good linear relationship over the concentration range of 1.25-5000 ng/ml with respect to the area (Table 5.27, Table 5.28, Table 5.29). The correlation coefficient (R^2) was 0.996, 0.995 and 0.991 and linear regression equation was found to be: y = 66.81x + 8,762, y = 86.1x + 19,246 and y = 72.89x + 8031 where y is the spot area and x is the concentration of the analyte (Figure 5.35,Figure 5.36,Figure 5.37).

Table 5.27: Linearity study for API (1.25-5000 ng/ml)

Conc. (ng/ml)	Peak area 1	Peak area 2	Peak area 3	Avg. peak area[a]	SD	%RSD
5000	329000	335000	340090	334696.7	5551.2	1.65
2500	184000	187900	191200	187700	3604.1	1.92
1250	104000	99950	99700	101216.7	2413.6	2.38
125	16500	17000	16890	16796.67	262.7	1.56
12.5	4830	5020	4950	4933.333	96.0	1.94
1.25	1165	1150	1170	1161.667	10.4	0.89

[a] n=3 replicates, SD= Standard deviation, % RSD= relative standard deviation

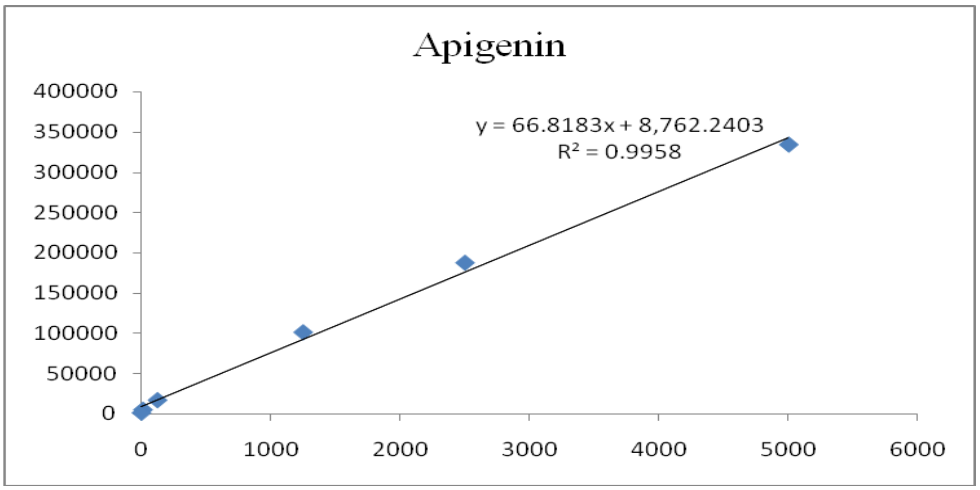


Figure 5.35: Calibration curve of API standard (1.25-5000 ng/ml)

Table 5.28: Linearity study for LUT (1.25-5000 ng/ml)

Conc. (ng/ml)	Peak area 1	Peak area 2	Peak area 3	Avg. peak area[a]	SD	%RSD
5000	365000	362940	359900	362613.3	2565.64	0.70
2500	206000	200800	210000	205600	4613.02	2.24
1250	108000	110000	107800	108600	1216.55	1.12
125	13300	13450	13400	13383.33	76.37	0.57
12.5	4290	4320	4330	4313.33	20.81	0.48
1.25	1640	1690	1660	1663.33	25.16	1.51

[a] n=3 replicates, SD= Standard deviation, % RSD= relative standard deviation

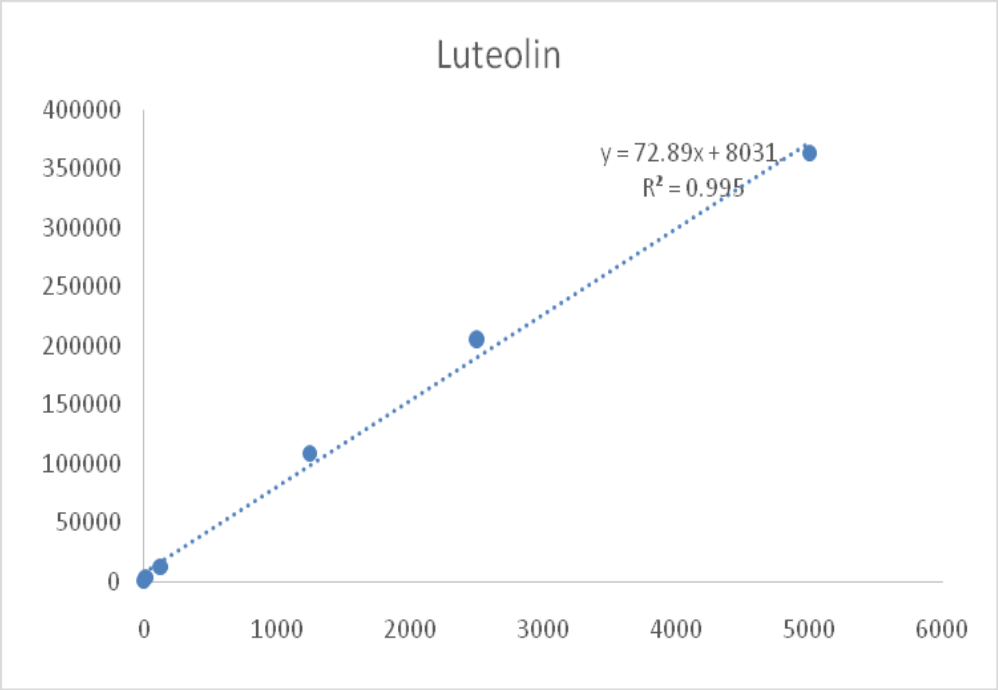


Figure 5.36: Calibration curve of LUT standard (1.25-5000 ng/ml)

Table 5.29: Linearity study for QUE (1.25-5000 ng/ml)

Conc. (ng/ml)	Peak area 1	Peak area 2	Peak area 3	Avg. peak area[a]	SD	%RSD
5000	426000	438100	443900	436000	9132.90	2.09
2500	249000	255000	253000	252333.3	3055.05	1.21
1250	148000	151500	147900	149133.3	2050.20	1.37
125	23800	23750	24350	23966.6	332.91	1.38
12.5	16100	15900	16000	16000	100	0.62
1.25	4150	4050	4225	4141.6	87.79	2.11

[a] n=3 replicates, SD= Standard deviation, % RSD= relative standard deviation

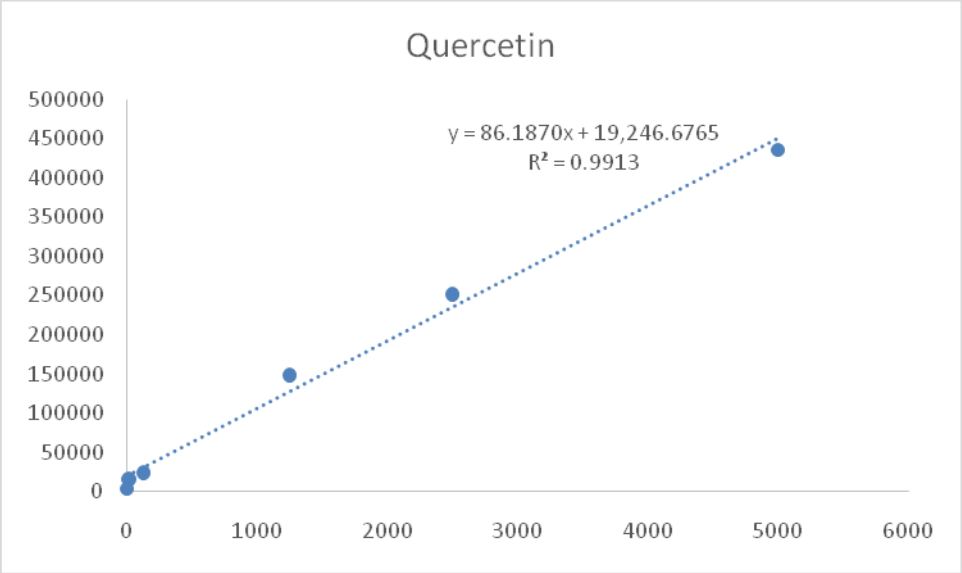


Figure 5.37: Calibration curve of QUE standard (1.25-5000 ng/ml)

5.4.1.4.3 Analysis of EAMTAM.

Extract when analysed in triplicate using the developed method in present study was quantify for LUT, QUE and API (Figure 5.38) indicating that the method can be applicable in routine quality control testing of extract. The % RSD value was found to be less than 2 (Table 5.30).

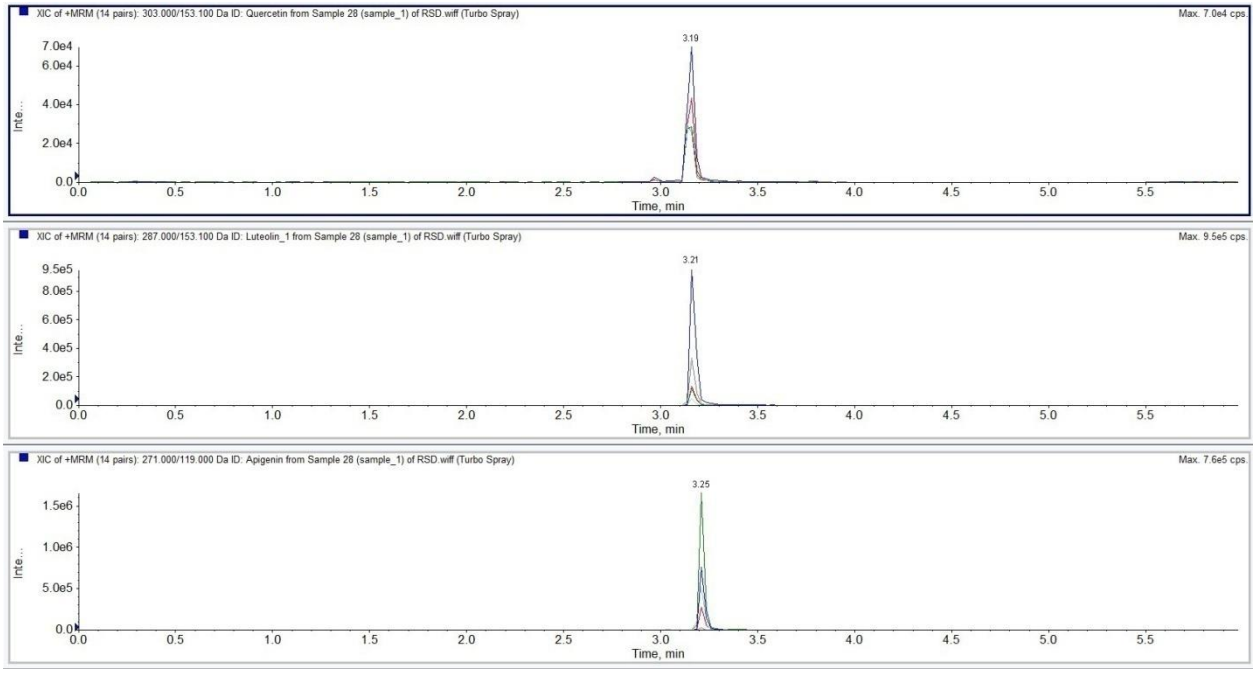


Figure 5.38: Chromatogram of API, LUT and QUE in EAMTAM

Table 5.30: Analysis of EAMTAM

Drug	% amount of drug found [a] ± SD	% RSD
API	0.182 ± 0.18	1.01
LUT	0.163 ± 0.28	1.20
QUE	0.274 ± 0.22	0.91

[a] n=3 replicates, SD=Standard deviation, % RSD= relative standard deviation

5.4.1.4.4 Precision

Precision of developed method was evaluated by repeatability and interaday precision, and was expressed as %RSD of peak area. Repeatability and intermediate precision was carried out by performing three replicates of three different concentration (1250, 12.5 and 1.25 ng for API, LUT and QUE) showed %RSD less than 2% (Table 5.31), indicating acceptable precision in terms of repeatability of peak area measurement and sample application.

Table 5.31: Precision study

Amount (ng/ml)	Repeatability[a]		Interday precision[a]	
	Mean amount of drug found[a] ± SD (ng/ml)	%RSD	Mean amount of drug found[a] ± SD (ng/ml)	%RSD
API				
1250	101102 ± 18.65	1.85	101386 ± 22.33	2.13
125	16888 ± 33.52	1.36	16888 ± 54.12	1.36
12.5	5010 ± 17.42	1.40	5010 ± 12.32	1.40
LUT				
1250	108160 ± 12.26	1.60	107400 ± 14.25	1.44
125	13320 ± 32.78	1.62	13520 ± 17.85	1.71
12.5	4363.8 ± 10.20	1.62	4558 ± 2.32	1.90
QUE				
1250	14984 ± 32.96	1.33	15006 ± 54.12	1.36
125	2380 ± 22.38	1.38	2391 ± 36.78	1.56
12.5	1617 ± 14.12	1.51	1563 ± 18.95	1.56

[a] n=3 replicates, SD= Standard deviation, % RSD= relative standard deviation

5.4.1.4.5 Accuracy

The proposed method when used for evaluation of recovery at three concentrations levels, 80%, 100% and 120% after spiking with standard, showed percentage recovery between 99.35 to 132.40% with acceptable % RSD, less than 2 revealed good accuracy of the method (Table 5.32).

Table 5.32: Recovery study for determination of LUT, QUE and API

Drug name	Recovery Level (%)	Amount added (ng/ml)	Initial amount (ng/ml)	Mean amount found	% Recovery[a]	SD	%RSD
API	80	1250	1000	207800	132.40	2.19	1.66
	100	1250	1250	187700	107.13	2.15	2.01
	120	1250	1500	226666	118.60	0.95	0.80
LUT	80	1250	1000	185666	108.31	2.16	1.99
	100	1250	1250	208000	109.73	1.09	1.00
	120	1250	1500	252166	121.79	1.62	1.33
QUE	80	1250	1000	225100	106.162	0.65	0.62
	100	1250	1250	252333	108.186	1.41	1.31
	120	1250	1500	274000	107.493	0.08	0.07

[a] n=3 replicates**,** SD= Standard deviation, % RSD= relative standard deviation

5.4.1.4.6 Limit of detection (LOD) and limit of quantitation (LOQ)

Limit of detection of developed method was found to be for API 24.04 ng/ml, for LUT 17.03 ng/ml and for QUE 27.22 ng/ml and Limit of quantitation was found to be for API 84.98 ng/ml, for LUT 51.61 ng/ml and for QUE 82.48 ng/ml indicating acceptable sensitivity of the method.

5.4.2. LC-MS/MS analysis of *T. populnea*.

5.4.2.1. LC-MS/MS analysis of *T. populnea* using marker quercetin and kaempferol

5.4.2.1.1 Optimized chromatography trial

Better chromatography and response were found with following chromatographic conditions:

0.3 % Formic acid in water & Acetonitrile

0.400 ml/min

Column: Gemini C18 column (50 mm x 2.0 mm, 3 μm).

5.4.2.1.2 Chromatographic condition

Multiple chromatographic conditions were explored in order to have appropriate retention time, and better resolution and sensitivity. Gemini C18 column (50 mm x 2.0 mm, 3 µm) was evaluated to attain better separation and was finally chosen for the chromatographic separation. The mobile phase consisting of 0.3 % Formic acid in water & Acetonitrile was found to be optimal for this study (Table 5.33). In addition, under the optimized conditions, no significant endogenous interference was found.

Table 5.33: Chromatographic conditions.

<table>
<tr><th>Parameters</th><th colspan="3">Used</th></tr>
<tr><td>Column</td><td colspan="3">Gemini C18, (50 mm x 2.0 mm, 3 µm)</td></tr>
<tr><td rowspan="6">Mobile Phase</td><td colspan="3">A : 0.3 % Formic acid in water & B: Acetonitrile</td></tr>
<tr><td>Time (Min.)</td><td>A %</td><td>B %</td></tr>
<tr><td>0.01</td><td>10</td><td>90</td></tr>
<tr><td>2.00</td><td>90</td><td>10</td></tr>
<tr><td>4.00</td><td>10</td><td>90</td></tr>
<tr><td>5.00</td><td>10</td><td>90</td></tr>
<tr><td>Flow rate</td><td colspan="3">0.400 ml/min,</td></tr>
<tr><td>Column oven temperature</td><td colspan="3">40 ± 0.3°C</td></tr>
<tr><td>Autosampler temperature</td><td colspan="3">10 ± 3°C</td></tr>
<tr><td>Volume of injection</td><td colspan="3">2.0 µl</td></tr>
<tr><td>Detector</td><td colspan="3">Mass detector (MS/MS)</td></tr>
<tr><td rowspan="2">Retention time</td><td colspan="3">kaempferol at about 2.50 minutes</td></tr>
<tr><td colspan="3">Quercetin at about 2.35 minutes</td></tr>
<tr><td>Run time</td><td colspan="3">5.0 minutes</td></tr>
</table>

5.4.2.1.3 Mass spectrometric conditions

Electron spray ionization (ESI) is the most commonly used soft ionization sources in mass spectrometry. By investigating the full scan mass spectra of quercetin and kaempferol, it was found that the signal intensity in the positive mode was much higher than that in the negative ion mode (Figure 5.39,Figure 5.40). Thus, all detections were carried out using the predominantly positive ion The most suitable mass spectrometric conditions were determined by optimizing all the parameters of the mass spectrometer such as collision energy, nebulizer gas, DL temperature, heat block temperature and drying gas flow to obtain much higher and more stable response (Table 5.34,Table 5.35).

Table 5.34: Mass Spectrometric conditions

Parameters	Kaempferol	Quercetin
Ion Source	Electro Spray ionization	
Polarity	Positive	
ParentIon	287.0	303.0
DaughterIon	153.1	153.0
Dwell Time(msec)	100	100
Collision Energy(CE)	-35	-35

Table 5.35: Mass Spectrometric source dependent parameters

Parameters	Used
DL Temperature	250 °C
Nebulizing Gas Flow	3.0 l/min
Heat Block Temperature	450 °C
Drying Gas Flow	15.0 l/min

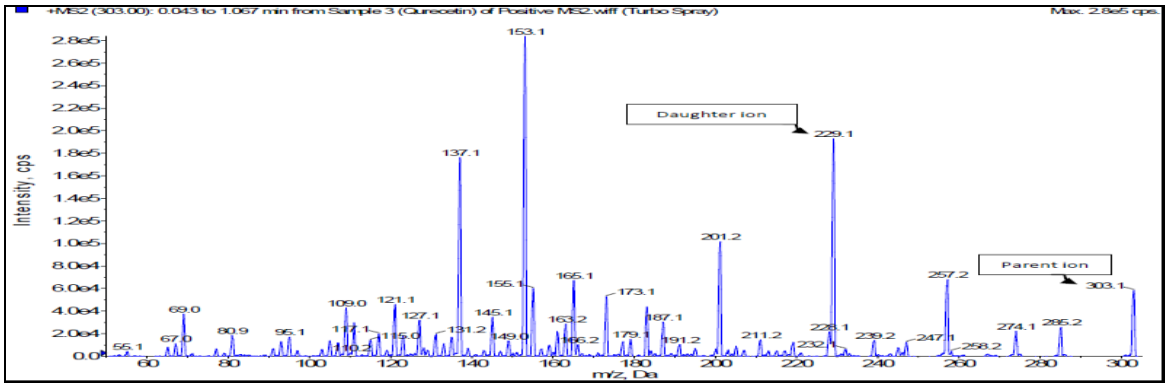


Figure 5.39: MS-MS spectra of Quercetin

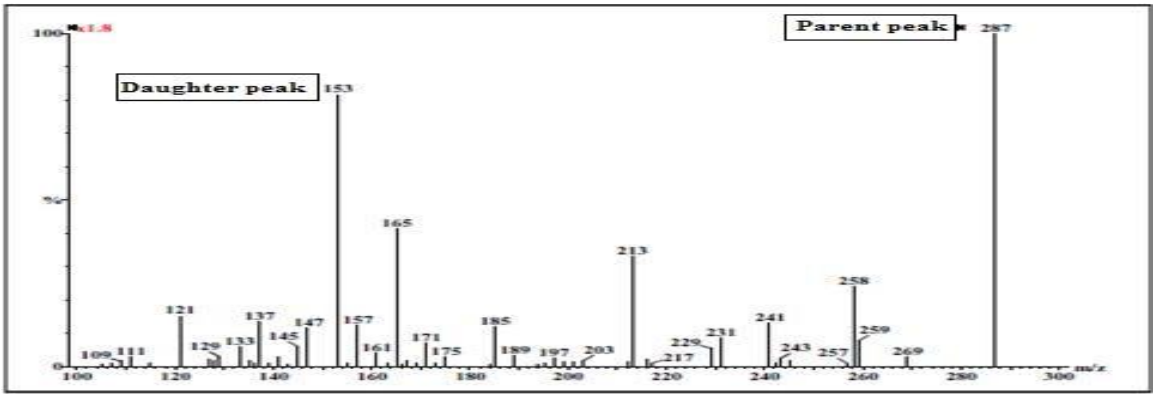
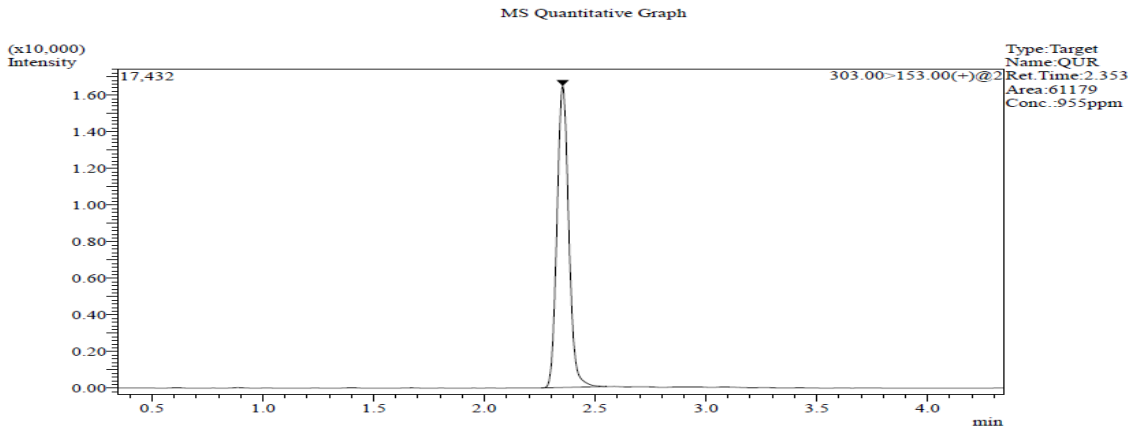


Figure 5.40: MS-MS spectra of kaempferol

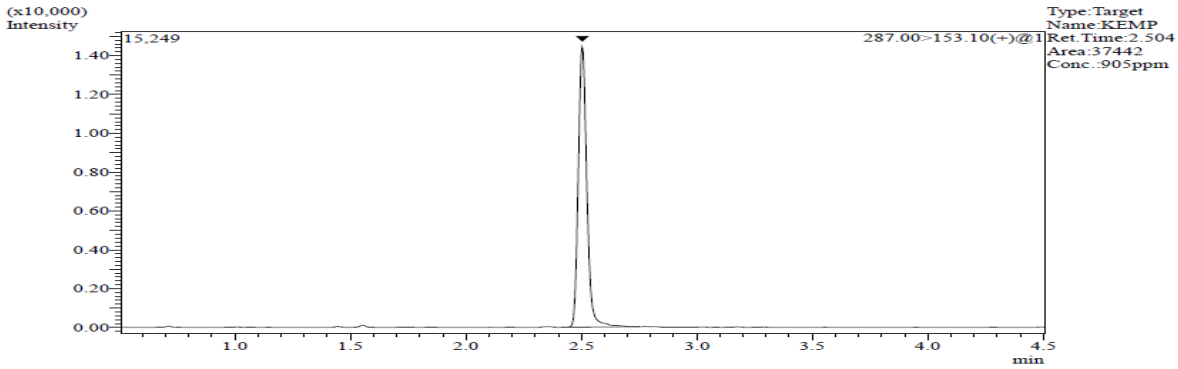
5.4.2.1.4 Method validation

5.4.2.1.4.1. Selectivity and specificity

The selectivity of the method was investigated by comparing chromatograms of quercetin and kaempferol. The retention time was 2.35 and 2.5 min for quercetin and kaempferol, respectively. There were no significant endogenous peaks that could interfere with the analyte (Figure 5.41). The results indicated that the method exhibited good specificity and selectivity.



[A]



[B]

Figure 5.41: **Chromatogram of [A] quercetin and [B] kaempferol**

5.4.2.1.4.2. Linearity

The linearity was evaluated by analyzing a series of different concentrations of the standard quercetin and kaempferol where each concentration was applied triplicate. Linear regression data for the calibration curves of standard quercetin showed a good linear relationship over the concentration range of 25-2500 µg/ml with respect to the area (

Table 5.36). The correlation coefficient (R^2) was 0.997 and linear regression equation was found to be: y = 92.36x + 1453, where y is the spot area and x is the concentration of the analyte and kaempferol showed a good linear relationship over the concentration range of 25-2500 µg/ml with respect to the area (Table 5.37). The correlation coefficient (R^2) was 0.994 and linear regression equation was found to be: y = 53.81x + 3216 (Figure 5.42,Figure 5.43).

Table 5.36: Linearity study for QUE (25-2500 µg/ml)

Conc. (µg/ml)	Peak area 1	Peak area 2	Peak area 3	Avg. peak area[a]	SD	%RSD
2500	224166	225569	223704	224479.7	971.26	0.43
1250	129253	124731	127763	127249	2304.40	1.81
500	49876	49406	48236	49172.67	844.53	1.71
250	22723	21882	21945	22183.33	468.42	2.11
50	4065	3998	3910	3991	77.73	1.94
25	2239	2169	2190	2199.33	35.92	1.63

[a] n=3 replicates, SD= Standard deviation, % RSD= relative standard deviation

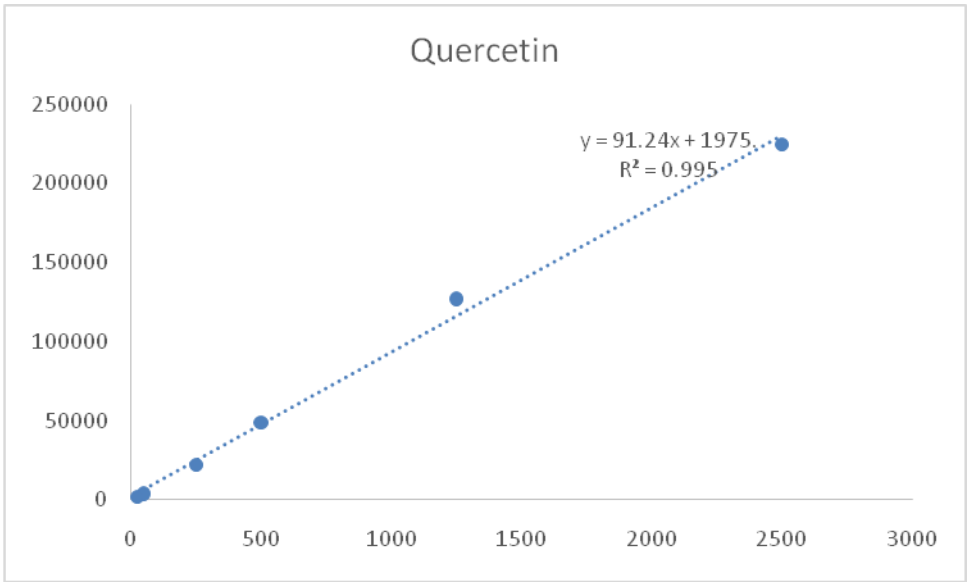


Figure 5.42: Calibration cure of QUE (25-2500 µg/ml)

Table 5.37: Linearity study for KAE (25-2500 µg/ml)

Conc. (µg/ml)	Peak area 1	Peak area 2	Peak area 3	Avg. peak area[a]	SD	%RSD
2500	133138	133443	131924	132835	803.55	0.60
1250	77272	75084	74695	75683.67	1389.22	1.83
500	34636	33898	33927	34153.67	417.96	1.22
250	16372	15831	15956	16053	283.24	1.76
50	3238	3318	3292	3282.66	40.80	1.24
25	1878	1898	1856	1877.33	21.00	1.11

[a] n=3 replicates, SD= Standard deviation, % RSD= relative standard deviation

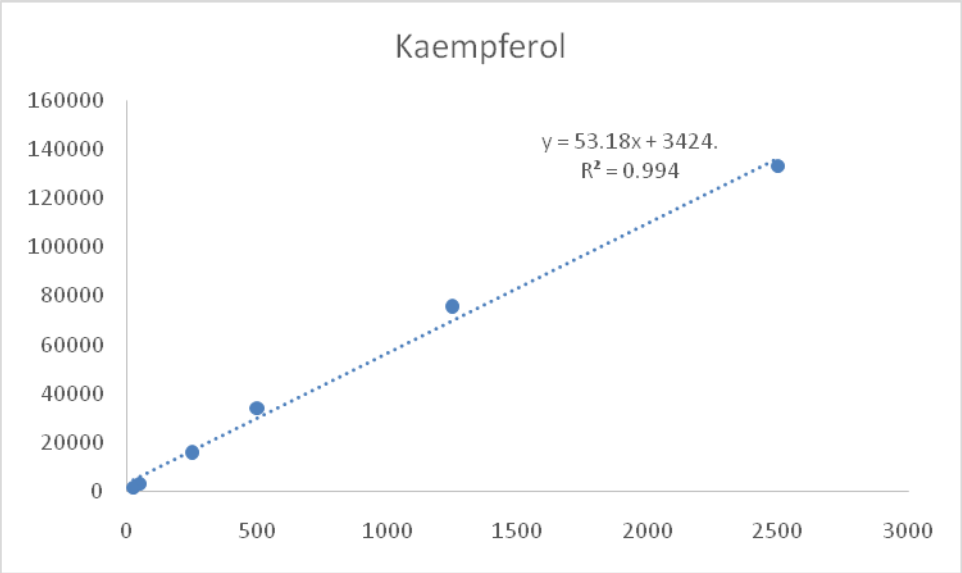


Figure 5.43: Calibration cure of KAE (25-2500 µg/ml)

5.4.2.1.4.3. Analysis of EAMTTP.

Extract when analysed in triplicate using the developed method in present study was quantify for QUE and KAE (Figure 5.44) The %RSD value was found to be less than 2 (Table 5.38).

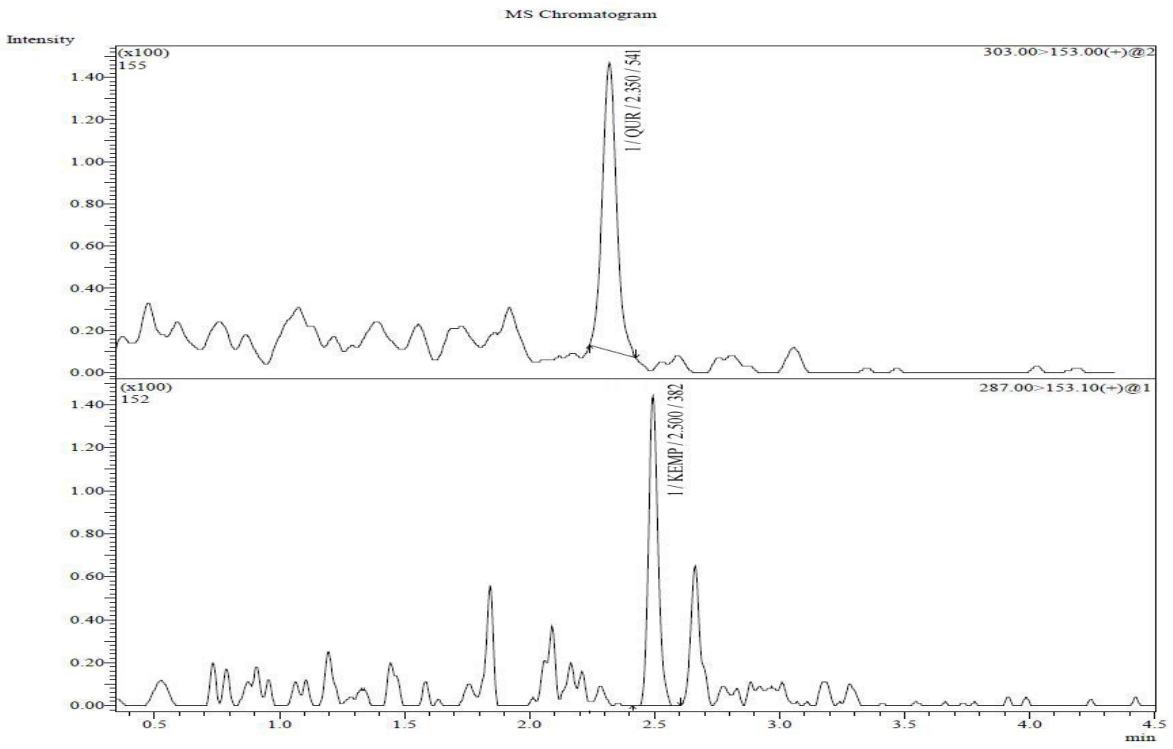


Figure 5.44: Chromatogram of QUE and KAE in EAMTTP

Table 5.38: Analysis of EAMTTP

Drug	% amount of drug found [a] ± SD	% RSD
QUE	0.263 ± 0.87	1.81
KAE	0.246 ± 1.23	1.22

[a] n=3 replicates, SD= Standard deviation, % RSD= relative standard deviation

5.4.2.1.4.4. Precision

Precision of developed method was evaluated by repeatability and interday precision, and was expressed as %RSD of peak area. Repeatability and intermediate precision was carried out by performing three replicates of three different concentration (500, 250 and 50 µg for QUE and KAE)showed %RSD less than 2% (Table 5.39), indicating acceptable precision in terms of repeatability of peak area measurement and sample application.

Table 5.39: Precision study

Amount (µg/ml)	Repeatability[a]		Interday precision[a]	
	Mean amount of drug found[a] ± SD (µg/ml)	%RSD	Mean amount of drug found[a] ± SD (µg/ml)	%RSD
QUE				
500	45383 ± 23.65	2.05	46615 ± 22.45	1.74
250	22236 ± 34.12	1.83	21863 ± 36.78	1.81
50	4014 ± 15.47	1.81	4004 ± 19.17	1.96
KAE				
500	30260 ± 32.45	1.43	31946 ± 34.91	2.18
250	15431 ± 45.13	1.76	15727 ± 26.32	1.60
50	3203 ± 21.69	2.08	3231 ± 24.13	1.92

[a] n=3 replicates, SD=Standard deviation, % RSD= relative standard deviation

5.4.2.1.4.5. Accuracy

The proposed method when used for evaluation of recovery at three concentrations levels, 80%, 100% and 120% after spiking with standard, showed percentage recovery between 99.35 to 115.40% with acceptable % RSD, less than 2 revealed good accuracy of the method (Table 5.40).

Table 5.40: Recovery study for determination of QUE and KAE

Drug name	Recovery Level (%)	Amount added (µg/ml)	Initial amount (µg/ml)	Mean amount found	% Recovery[a]	SD	%RSD
QUE	80	1250	1000	210233	101.44	0.31	0.30
	100	1250	1250	231285	100.53	2.17	2.15
	120	1250	1500	253793	100.36	1.50	1.49
KAE	80	1250	1000	125526	102.04	1.15	1.12
	100	1250	1250	136401	100.02	1.93	1.93
	120	1250	1500	152433	101.89	0.63	0.62

[a] n=3 replicates**,** SD=Standard deviation, % RSD= relative standard deviation

5.4.2.1.4.6. Limit of detection (LOD) and limit of quantitation (LOQ)

Limit of detection of developed method was found to be for QUE 4.11 µg/ml and for KAE 8.56 µg/ml and Limit of quantitation was found to be for QUE 12.45 µg/ml and for KAE 25.96 µg/ml indicating acceptable sensitivity of the method.

5.4.2.2. LC-MS/MS analysis of *T. populnea* using marker lupeol and β-sitosterol.

5.4.2.2.1 Optimized chromatography trial

Better chromatography and response were found with following chromatographic conditions:

Water: 0.1% formic acid and Acetonitrile: Methanol and 0.1% formic acid (50:50 v/v)

0.300 ml/min

Column: Gemini C18 column (50 mm x 2.0 mm, 3 µm).

5.4.2.2.2 Chromatographic condition

Multiple chromatographic conditions were explored in order to have appropriate retention time, and better resolution and sensitivity. Gemini C18 column (50 mmx2.0 mm, 3 µm) was evaluated to attain better separation and was finally chosen for the chromatographic separation. The mobile phase consisting of Water: 0.1% formic acid and Acetonitrile: Methanol and 0.1% formic acid (50:50 v/v) 0.300 ml/min was found to be optimal for this study (Table 5.41). In addition, under the optimized conditions, no significant endogenous interference was found.

Table 5.41: Chromatographic conditions

<table>
<tr><th>Parameters</th><th colspan="3">Detail</th></tr>
<tr><td>Column</td><td colspan="3">Gemini C18, (50 mm x 2.0 mm, 3 µm)</td></tr>
<tr><td rowspan="9">Mobile Phase</td><td colspan="3">A: Water + 0.1% formic acid
B: ACN (50): Methanol (50) + 0.1% formic acid</td></tr>
<tr><td>Time (min)</td><td>A%</td><td>B%</td></tr>
<tr><td>0.00</td><td>80</td><td>20</td></tr>
<tr><td>1.50</td><td>50</td><td>50</td></tr>
<tr><td>2.50</td><td>0</td><td>100</td></tr>
<tr><td>4.00</td><td>0</td><td>100</td></tr>
<tr><td>4.50</td><td>50</td><td>50</td></tr>
<tr><td>5.50</td><td>80</td><td>20</td></tr>
<tr><td>6.00</td><td>80</td><td>20</td></tr>
<tr><td>Flow rate</td><td colspan="3">0.400 ml/min</td></tr>
<tr><td>Column oven temperature</td><td colspan="3">40 ± 0.3°C</td></tr>
<tr><td>Autosampler temperature</td><td colspan="3">10 ± 3°C</td></tr>
<tr><td>Volume of injection</td><td colspan="3">5.0 µl</td></tr>
<tr><td>Detector</td><td colspan="3">Mass detector (MS/MS)</td></tr>
<tr><td>Retention time</td><td colspan="3">Lupeol at about 3.08 minutes</td></tr>
<tr><td></td><td colspan="3">β-sitosterol at about 3.53 minutes</td></tr>
<tr><td>Run time</td><td colspan="3">5.0 minutes</td></tr>
</table>

5.4.2.2.3 Mass spectrometric conditions

Electron spray ionization (ESI) is the most commonly used soft ionization sources in mass spectrometry. By investigating the full scan mass spectra lupeol and β-sitosterol (Figure 5.45,Figure 5.46) it was found that the signal intensity in the positive mode was much higher than that in the negative ion mode. Thus, all detections were carried out using the predominantly positive ion The most suitable mass spectrometric conditions were determined by optimizing all the parameters of the mass spectrometer such as collision energy, nebulizer gas, DL temperature, heat block temperature and drying gas flow to obtain much higher and more stable response (Table 5.42,Table 5.43).

Table 5.42: Mass Spectrometric conditions

Parameters	Lupeol	β-sitosterol
Ion Source	Electro Spray ionization	
Polarity	Positive	
Parent Ion	427	397
Daughter Ion	121	135
Dwell Time (msec)	100	100
Collision Energy (CE)	29	35

Table 5.43: Mass Spectrometric source dependent parameters

Parameters	Used
DL Temperature	250 °C
Nebulizing Gas Flow	3.0 l/min
Heat Block Temperature	450 °C
Drying Gas Flow	15.0 l/min

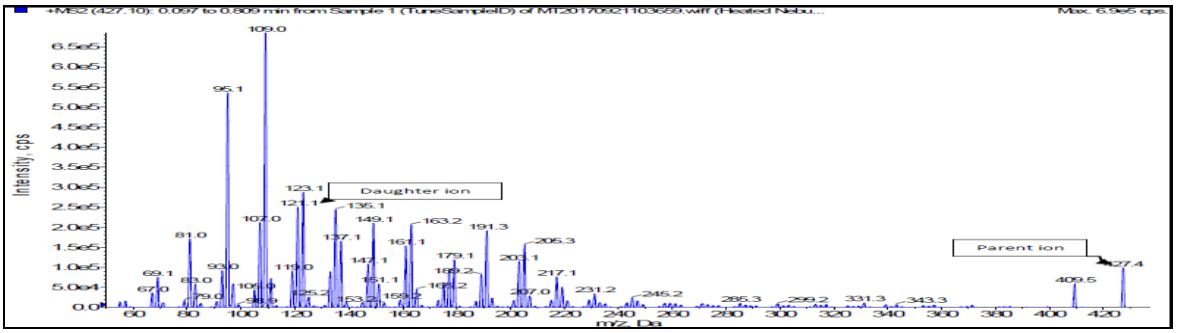


Figure 5.45: MS-MS spectra of lupeol

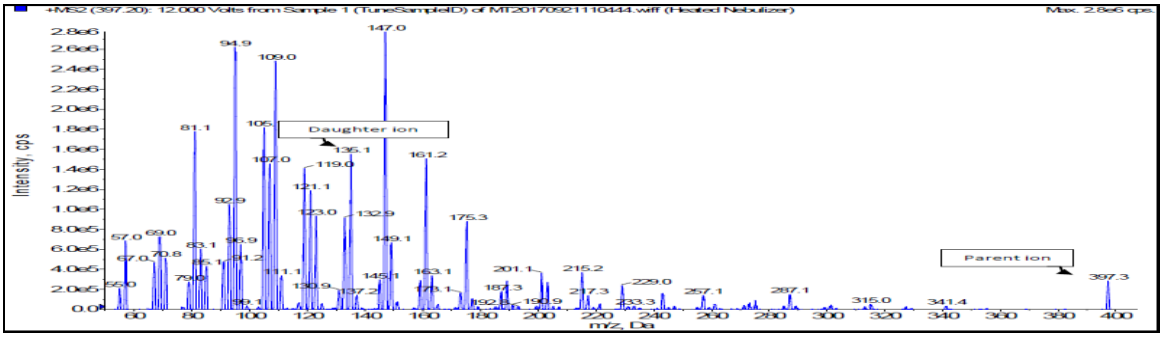
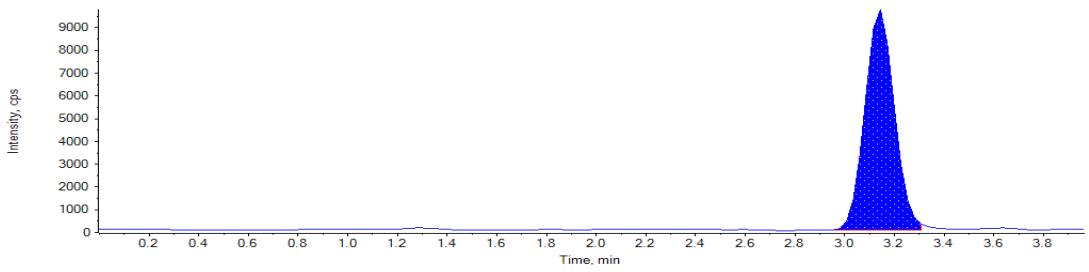


Figure 5.46: MS-MS spectra of β-sitosterol

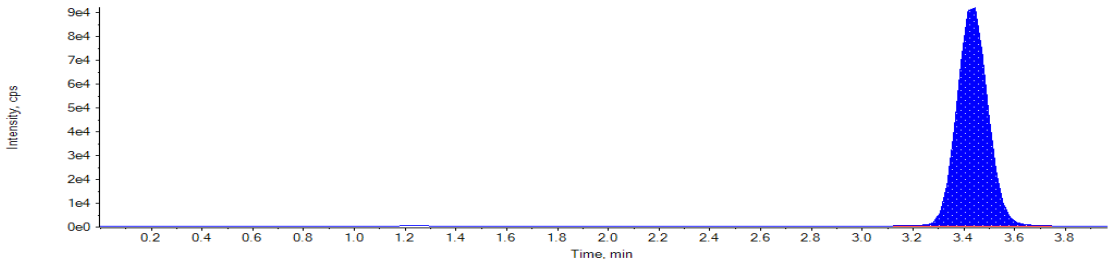
5.4.2.2.4 Method validation

5.4.2.2.4.1. Selectivity and specificity

The selectivity of the method was investigated by comparing chromatograms of lupeol and β-sitosterol. The retention time was 3.08 and 3.53 min for lupeol and β-sitosterol, respectively. There were no significant endogenous peaks that could interfere with the analyte (Figure 5.47). The results indicated that the method exhibited good specificity and selectivity.



[A]



[B]

Figure 5.47: Chromatogram of [A] lupeol and [B] β-sitosterol

5.4.2.2.4.2. Linearity

The linearity was evaluated by analyzing a series of different concentrations of the standard lupeol and β-sitosterol where each concentration was applied triplicate. Linear regression data for the calibration curves of standard lupeol and β-sitosterol showed a good linear relationship over the concentration range of 10-100 µg/ml with respect to the area (Table 5.44,Table 5.45). The correlation coefficient (R^2) was 0.999 and 0.995 linear regression equation was found to be y = 2,097.8x - 1,628 and y = 487.8x – 417 where y is the spot area and x is the concentration of the analyte (Figure 5.48,Figure 5.49).

Table 5.44: Linearity study for LUP (10-100 µg/ml)

Conc. (µg/ml)	Peak area 1	Peak area 2	Peak area 3	Avg. peak area[a]	SD	%RSD
100	210250	208000	209500	209250	1145.64	0.54
80	165000	168000	171000	168000	3000	1.78
60	120500	121000	120950	120816	275.37	0.22
40	80300	80800	81000	80700	360.55	0.44
20	35900	41500	42000	39800	3386.73	8.50
10	21900	22000	22100	22000	100	0.45

[a] n=3 replicates, SD=Standard deviation, % RSD= relative standard deviation

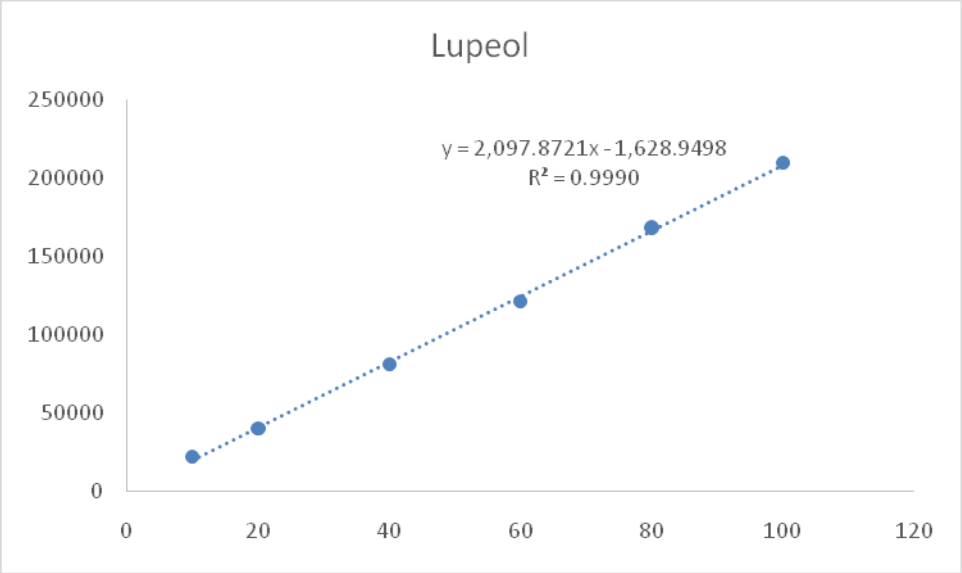


Figure 5.48: Calibration curve of LUP (10-100 µg/ml)

Table 5.45: Linearity study for SIT (10-100 µg/ml)

Conc. (µg/ml)	Peak area 1	Peak area 2	Peak area 3	Avg. peak area[a]	SD	%RSD
100	46700	47550	48600	47616.67	951.75	1.99
80	37400	37800	39000	38066.67	832.66	2.18
60	29000	30000	32000	30333.33	1527.52	5.03
40	19200	21000	21500	20566.67	1209.68	5.88
20	7900	8200	8150	8083.33	160.72	1.98
10	3990	4080	4110	4060	62.44	1.53

[a] n=3 replicates, SD=Standard deviation, % RSD= relative standard deviation

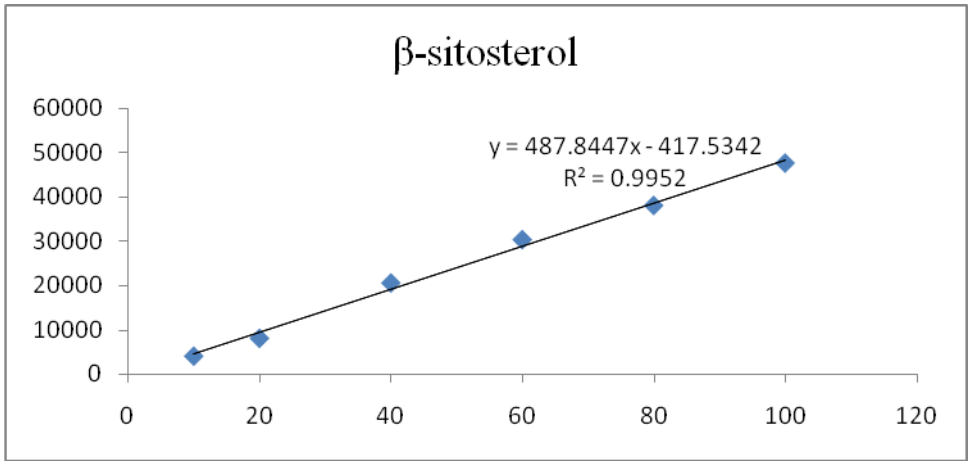


Figure 5.49: Calibration curve of SIT (10-100 µg/ml)

5.4.2.2.4.3. Analysis of EAMTTP.

Extract when analysed in triplicate using the developed method in present study was quantify for LUP and SIT (Figure 5.50). The %RSD value was found to be less than 2 (Table 5.46).

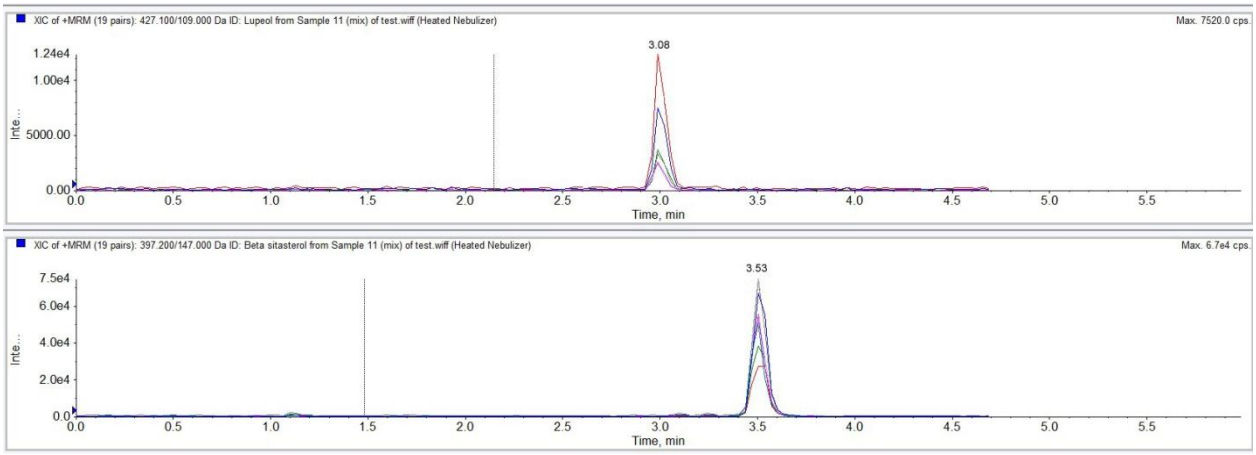


Figure 5.50: Chromatogram of LUP and SIT in EAMTTP

Table 5.46: Analysis of EAMTTP

Drug	% amount of drug found [a] ± SD	% RSD
LUP	0.201 ± 1.96	1.56
SIT	0.198 ± 2.01	1.81

[a] n=3 replicates, SD= Standard deviation, % RSD= relative standard deviation

5.4.2.2.4.4. Precision

Precision of developed method was evaluated by repeatability and interaday precision, and was expressed as %RSD of peak area. Repeatability and intermediate precision was carried out by performing three replicates of three different concentration (80, 60 and 40 µg/ml for LUP and SIT) showed %RSD less than 2% (Table 5.47), indicating acceptable precision in terms of repeatability of peak area measurement and sample application.

Table 5.47: Precision study for determination of LUP and SIT

Amount (µg/ml)	Repeatability[a]		Interday precision[a]	
	Mean amount of drug found[a] ± SD (µg /ml)	%RSD	Mean amount of drug found[a] ± SD µg /ml)	%RSD
LUP				
80	169200 ± 23.15	1.70	168590 ± 36.12	1.54
60	120910 ± 22.36	0.64	122510 ± 12.15	0.94
40	80848 ± 15.26	0.55	81508 ± 27.45	0.83
SIT				
80	38210 ± 35.69	1.65	38390 ± 21.33	1.24
60	29820 ± 45.21	1.92	29820 ± 24.12	1.74
40	19230 ± 24.12	0.91	19370 ± 23.15	0.95

[a] n=3 replicates, SD=Standard deviation, % RSD= relative standard deviation

5.4.2.2.4.5. Accuracy

The proposed method when used for evaluation of recovery at three concentrations levels, 80%, 100% and 120% after spiking with standard, showed percentage recovery between 94.00 to 99.30% with acceptable % RSD, less than 2 revealed good accuracy of the method (Table 5.48).

Table 5.48: Recovery study for determination of LUP and SIT

Drug name	Recovery Level (%)	Amount added (µg/ml)	Initial amount (µg /ml)	Mean amount found	% Recovery[a]	SD	%RSD
LUP	80	40	32	150666	98.67	1.33	1.35
	100	40	40	168000	99.13	1.78	1.80
	120	40	48	182666	98.06	0.41	0.42
SIT	80	40	32	33883	95.28	0.73	0.76
	100	40	40	37833	95.87	1.15	1.20
	120	40	48	42470	97.96	1.42	1.45

[a] n=3 replicates**,** SD=Standard deviation, % RSD= relative standard deviation

5.4.2.2.4.6. Limit of detection (LOD) and limit of quantitation (LOQ)

Limit of detection of developed method was found to be for LUP 2.60 µg /ml and for SIT 1.96 µg/ml and Limit of quantitation was found to be LUP 7.90 µg/ml and for SIT 5.94 µg/ml indicating acceptable sensitivity of the method.

5.5. Pharmacological study

5.5.1. Effect of EAMTAM and EAMTTP on biochemical parameters

The rats treated with CCl_4 (2 ml/Kg; s.c.) for ten days significantly ($P<0.001$) elevated the serum enzyme levels of SGOT and SGPT in CCl_4 control as compared to model control (group I) as shown in (Figure 5.51,Figure 5.52) respectively indicating marked hepatocellular damage. Administrations of EAMTAM and EAMTTP (group IV to VII) at 250 and 500 mg/kg, and silymarin (standard; group III at 50 mg/kg; p.o.) had significantly ($P<0.001$) prevented elevation in enzyme levels as compared to model control. Administration of CCl_4 significantly ($P<0.001$) increased TBL and TP level in comparison with model control as shown in (Figure 5.53,Figure 5.54).

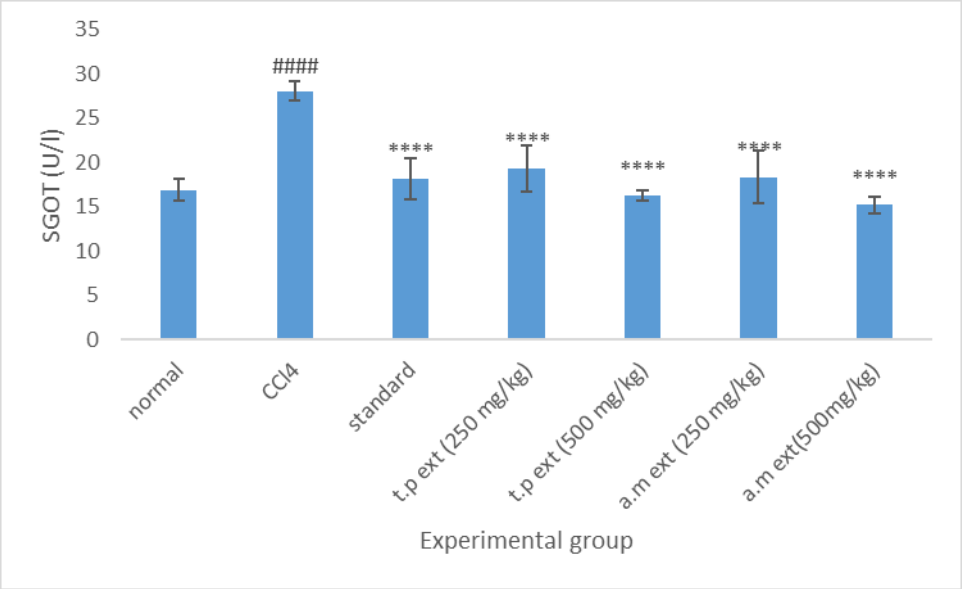


Figure 5.51: **Effect of EAMTAM and EAMTTP on SGOT level on CCl_4-induced liver damage**

The values are expressed as mean ± SEM (n=6). #### P<0.001 is considered significant when compared with normal model group using one-way ANOVA; **** P<0.001 is considered significant when compared with model group using ANOVA followed by Dunnett test.

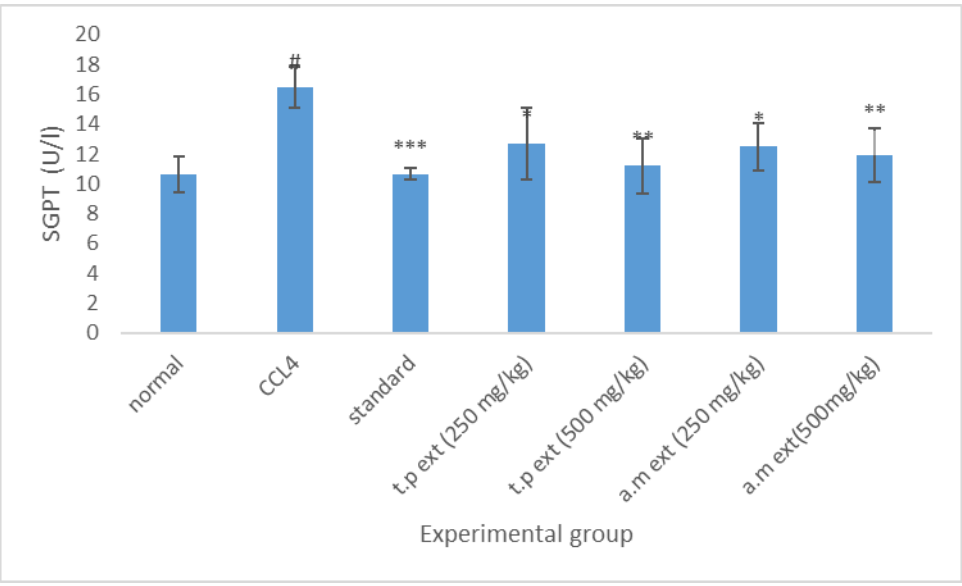


Figure 5.52: **Effect of EAMTAM and EAMTTP on SGPT level on CCl_4-induced liver damage**

The values are expressed as mean ± SEM (n=6). # P<0.05 is considered significant when compared with normal model group using one-way ANOVA; **** P<0.01, ** P<0.05, *

P<0.05 is considered significant when compared with model group using ANOVA followed by Dunnett test.

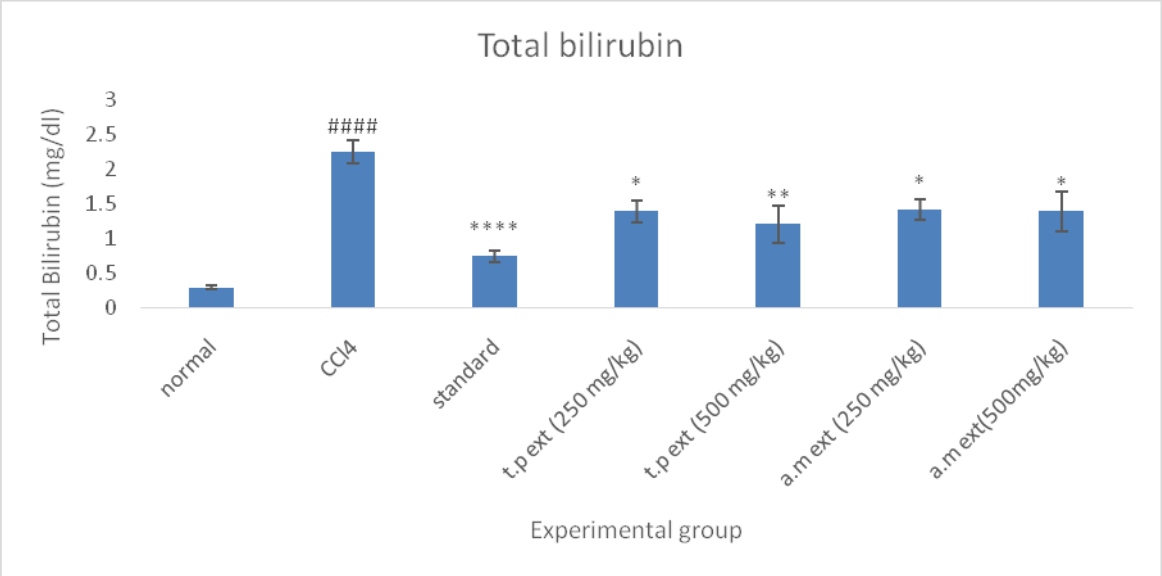


Figure 5.53: Effect of EAMTAM and EAMTTP on TBL level on CCl_4-induced liver damage

The values are expressed as mean ± SEM (n=6). #### P<0.001 is considered significant when compared with normal model group using one-way ANOVA; **** P<0.001, ** P<0.05, * P<0.05 is considered significant when compared with model group using ANOVA followed by Dunnett test.

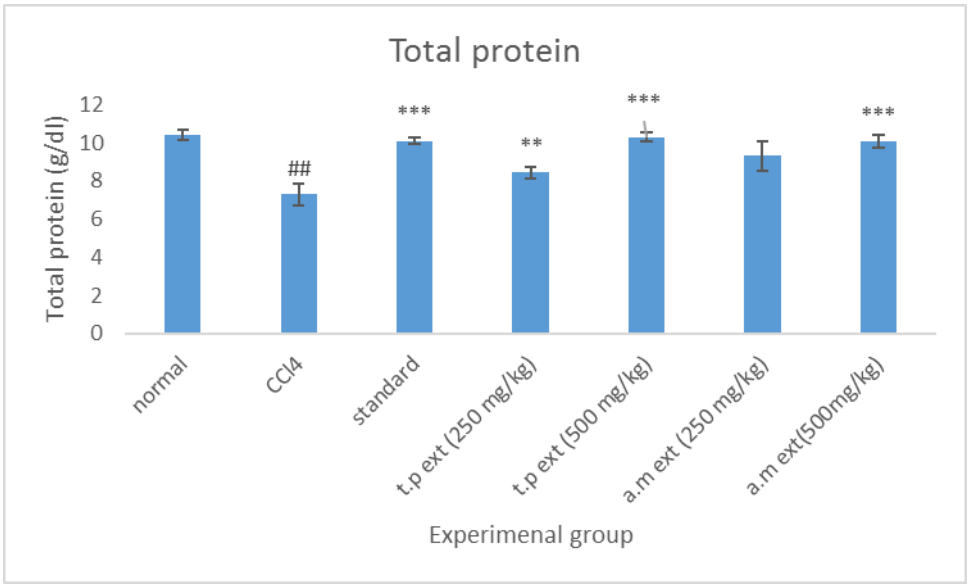
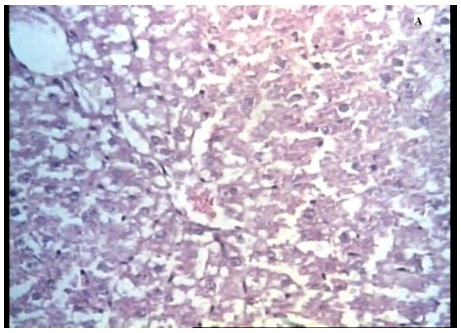


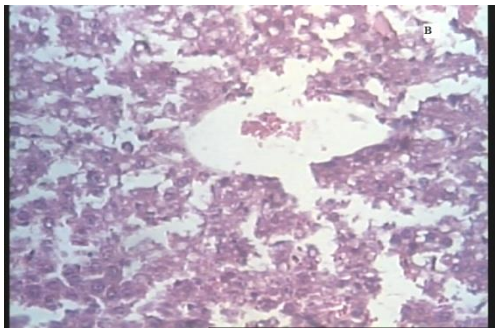
Figure 5.54: Effect of EAMTAM and EAMTTP on TP level on CCl_4-induced liver damage

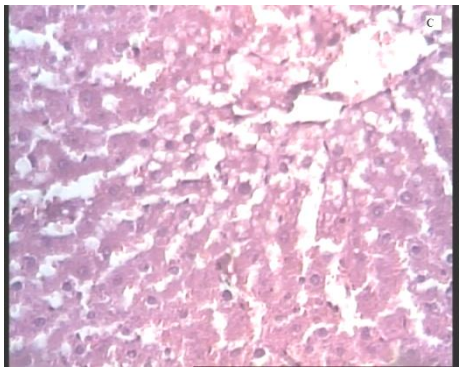
The values are expressed as mean ± SEM (n=6). ## P<0.05 is considered significant when compared with normal model group using one-way ANOVA; **** P<0.001, *** P<0.01, * P<0.05 is considered significant when compared with model group using ANOVA followed by Dunnett test.

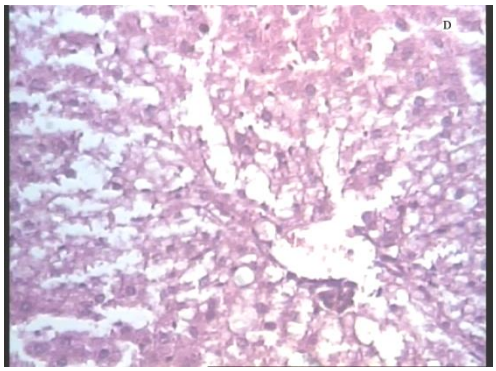
5.5.2. Histopathological examination on liver sections

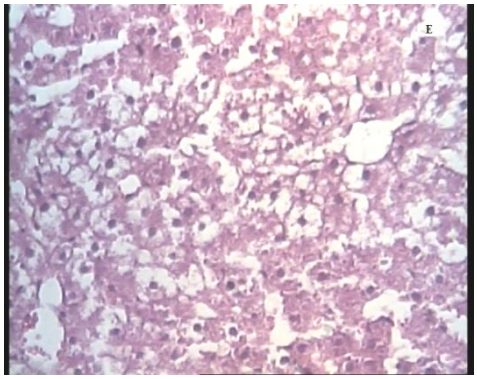
Histopathological examination of the normal control showed a normal architecture of liver with distinct hepatic cells, sinusoidal spaces and central vein(Figure 5.55). There was an intense centrilobular necrosis, vacuolization and macrovascular fatty changes observed in CCl_4 control. Pretreatments with EAMTAM and EAMTTP at 250 mg/kg dose and 500 mg/kg dose showed liver protection as evident from the reduced scored of necrosis and fatty changes. Similarly, administration of silymarin showed a significant protective effect against hepatic injury induced by CCl_4.

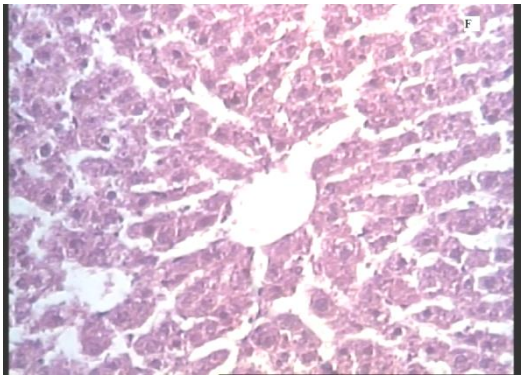












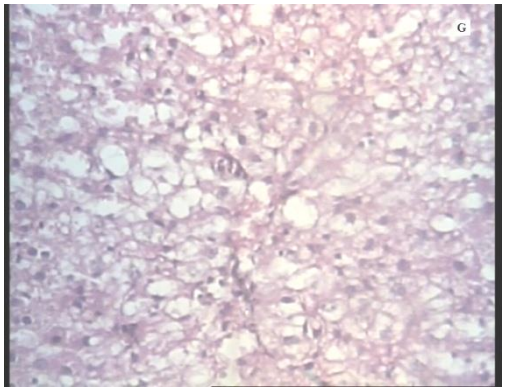


Figure 5.55: [A] Normal: Normal texture of liver tissue [B] Positive control (CCl4 treated): Severe tissue degeneration & necrosis. [C] Standard (Silymarin): Mild tissue degeneration & necrosis [D] t.p 250 mg/ml: Moderate to severe [E] t. p 500 mg/ml: Moderate [F] a.m 250 mg/ml: Moderate to severe [G] a.m 500 mg/ml: Moderate

Chapter - 6

SUMMARY AND CONCLUSION

CHAPTER-6

6. Summary and Conclusion

The aerial parts of *Achillea millefolium* and aerial parts of *Thespesia populnea* were subjected to pharmacognostical evaluation parameters and phytochemical study for quatification of secondary metabolites along with standardization using sophisticated analytical methods. Few RP-HPLC and HPTLC analytical methods were reviewed for the estimation of maker compound in *A. millefolium* and *T. populnea*. For this study three to four marker compounds were selected and they were subjected to analytical estimation including hepatoprotective study. A simple, accurate, economical and precise methods were developed and validated as per ICH guidelines.

A novel and precise HPTLC method was been developed for the simultaneous estimation of apigenin, luteolin and quercetin in *A. millefolium* and were validated for specificity, linearity, accuracy, repeatability, intra-day and inter-day precision, limit of detection and limit of quantification, robustness and system suitability.

Similarly, novel and precise HPTLC method was been developed for the simultaneous estimation of quercetin, kaempferol, β-sitosterol and lupeol in *T. populnea*. The developed method was based upon derivatization of β-sitosterol and lupeol using anisaldehyde sulphuric acid and were validated for specificity, linearity, accuracy, repeatability, intra-day and inter-day precision, limit of detection and limit of quantification, robustness and system suitability. Major advantage of developed HPTLC is that several samples can be run simultaneously using a small quantity of mobile phase, thus lowering the analysis time, sample clean up and cost per analysis.

Even one more analytical LC-MS/MS method was being developed for simultaneous estimation of apigenin, luteolin and quercetin in *A. millefolium* and were validated for specificity, linearity, accuracy, repeatability, intra-day and inter-day precision, limit of detection and limit of quantification and system suitability.

Similarly, LC-MS/MS method was being developed for simultaneous estimation of quercetin, kaempferol, β-sitosterol and lupeol in *T. populnea* and were validated for

specificity, linearity, accuracy, repeatability, intra-day and inter-day precision, limit of detection and limit of quantification, robustness and system suitability. It is a rapid, simple and reliable method which provides an excellent quantitative tool because of it high capacity, high sensitivity, high selectivity and short analysis time. The developed LC-MS/MS method was highly suitable for rapid determination of standards, individual extracts and or with a combination of other extracts in the form of formulations.

The newly developed HPTLC and LC-MS/MS methods can be successfully applied for standardization of herbal formulations. The fingerprint profiles of chromatogram obtained from extracts may be used for comparison and quality control parameters. The developed method of plant extracts may be used further for invivo study.

The EAMTAM and EAMTTP were evaluated for hepatoprotective activity in CCl_4 induced liver toxicity in experimentally induced hepatotoxic rats which showed dose related hepatoprotective activities in hepatotoxic animals and demonstrated significant protective effects on liver. Further hepatoprotective effect was supported by histopathological studies.

6.1. Conclusion

The chromatographic methods i.e HPTLC and LC-MS/MS developed are simple, sensitive, precise, accurate and reproducible for determination of apigenin, luteolin and quercetin for analysis of *A. millefolium* extract and quercetin, kaempferol, lupeol and β-sitosterol for analysis of *T. populnea* extract. Statistical analysis proves that all the methods are repeatable and specific and can be used for routine analysis of markers and plant extracts.

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APPENDIX

List of Publications

Paper Presentation

- **Poster Presentation** entitled 'Evaluation of Pharmacognostical parameters and hepatoprotective activity in *Achillea millefolium* and *Thespesia populnea*' at 22nd National Convention of society of Pharmacognosy and International Conference on "Integrating Ayurveda & Herbal Drugs for next generation therapeutics & Supplements: Opportunities & Challenges", on 20 & 21 January, 2018, organized by Ganpat University, Shree S. K. Patel College of Pharmaceutical Education & research, Gujarat, India.

- **Poster presentation** entitled 'Development of validated high performance thin layer chromatography method for simulatenous determination of apigenin, luteolin and quercetin in *Achillea millefolium*' at 5th Indo-West Indies Conference on "Modern Trends in Pharmaceutical Education and Research" organised by APP Gujarat State Branch and APP West Indies International Branch at Indubhai Patel College of Pharmacy and Research Centre, Dharmaj, Anand, Gujarat on December 10, 2017.

- **Oral Presentation** was given entitled 'Development of Validated High performance thin layer chromatography method for simultaneous determination of apigenin and luteolin in *Achillea millifolium*.' at 3rd International Conference on Dravyaguna and Rasasastra-Bhaisajya Kalpana, at Nadiad on 2nd and 3rd September, 2017.

Paper published

- S. Panchal Hiteksha and B. Shah Mamta. *Thespesia populnea Linn*: A review. International journal of Pharmacognosy., 2017, 4(1); 1-5.

- Hiteksha Panchal and Mamta Shah. Development and Validation of a Rapid LC-MS/MS Method for Simultaneous Determination of Kaempferol and Quercetin in

Thespesia populnea extract. Journal of AOAC International., 2017, 100(4); 971-975.

- Hiteksha Panchal, Aeshna Amin and Mamta Shah. Development of Validated High-performance Thin-layer Chromatography Method for Simultaneous Determination of Quercetin and Kaempferol in *Thespesia populnea.* Pharmacogn. Res., 2017, 9(3), 277-281.

- Hiteksha Panchal, Aeshna Amin, Mamta Shah and Vijay bhatt. Development of Validated High Performance Thin Layer Chromatography Method for Simultaneous Determination of Apigenin and Luteolin in *Achillea Millefolium.* Journal of Chemical and Pharmaceutical Research, 2017, 9(11):39-45