DEVELOPMENT AND EVALUATION OF SOME BIOFLAVONOIDS FROM INDIGENOUS PLANTS

Thesis Submitted to

THE TAMILNADU DR. M.G.R. MEDICAL UNIVERSITY. GUINDY, CHENNAI

As a partial fulfillment of the requirement for the award of the degree of

DOCTOR OF PHILOSOPHY

(IN PHARMACY)

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OCTOBER– 2013

CERTIFICATE

This is to certify that the thesis entitled "**DEVELOPMENT AND EVALUATION OF SOME BIOFLAVONOIDS FROM INDIGENOUS PLANTS"** submitted to The Tamil Nadu Dr. M.G.R. Medical University, Chennai-32 for the award of the Degree of Doctor of Philosophy is a bonafide original work and independent research carried out by me under the guidance of **Prof. Dr. MADHIRA GEETHA, M. Pharm., Ph.D.,** and that the thesis or any part thereof has not formed the basis for the award of any degree, diploma, fellowship or any other similar titles.

Date:

Place: Chennai.

(A. VIJAYALAKSHMI)

CERTIFICATE

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Date:

Place: Chennai.

Prof. Dr. MADHIRA GEETHA, M. Pharm., Ph.D.,

Dedicated

to my

Beloved parents

My Husband &

My loveable Kids

ACKNOWLEDGEMENT

This thesis is by far the most momentous accomplishment in my life. It would have been impossible to complete this journey without people who supported me and believed in me. It is a pleasant aspect that I have now the opportunity to express my gratitude to all of them.

First and foremost I express my deepest sense of gratitude and faith fullness to God's grace which has enabled me to complete this project work successfully.

I am very grateful and thankful to **Mr. Vinod Khanna,** Chairman, C.L. Baid Metha College of Pharmacy, Chennai, for providing me support and necessary facilities in time and when needed, with a stimulating environment in which the experiment could be carried out.

It is my proud privilege in thanking our Dynamic Principal **Dr. Grace Rathnam, M. Pharm., Ph.D.,** Principal, C.L. Baid Metha College of Pharmacy, Chennai, for her valuable contribution, enlightening suggestion and excellent facilities provided regarding my dissertation work.

It is difficult to overstate my gratitude to my Ph.D. guide, **Prof. Dr. Madhira Geetha, M. Pharm., Ph.D.** I sincerely thank for all her trust, great ideas, support knowledge, and guidance rendered in designing this work.

I am grateful to **Prof. Dr. V. Ravichandiran, M. Pharm., Ph.D.,** for his expert guidance and necessary advice for my entire work.

I greatly thank **Prof. Dr. P. Jayaraman,** founder of **Plant Anatomy research Centre (PARC)** for his expertise guidance in anatomical references, microphotography and timely suggestions.

I deliver my due thanks to **Dr. P. Amudha, M. Pharm., Ph.D.,** C.L. Baid Metha College of Pharmacy, for providing all necessary guidance while pursuing the pharmacological work. I would also like to gratefully acknowledge the support of **Dr. P. Muthusamy, M. Pharm., Ph.D.**, Madras Medical College.

My renewable thanks to **Dr. S. Jayakumari, M. Pharm., Ph.D., Mrs. Malarkodi Velraj, M. Pharm., Ms. S. Nirmala, M. Pharm., and Dr. J. Anbu, M. Pharm., Ph.D.** for their intelligent decisions, constant inspiration and constructive suggestions regarding my dissertation work.

I exaggerate my personal sense of gratitude to **Dr. V. Naganathan, IFS, Dr. K. Masilamani, M. Pharm., Ph.D.,** for all their help and support during the entire course of my research work.

I extend my gratitude towards **Prof. Dr. R. Sridhar**, Head, Department of Veterinary Pathology, Madras Veterinary College, for the interpretation of histopathological study.

I exaggerate my personal sense of gratitude to **Prof. Dr. P. Kumar, M. Pharm., Ph.D.,** for all his help and support during the course of my research work. I have immense pleasure in thanking all my dear friends **Mrs. M. Sumithra, Mrs. Sakthipriyadarsini, Mrs. V. Rajalakshmi, Mrs. Priya**, for all the emotional support, entertainment, and caring they provided.

I sincerely thank **Mr. Ramadass** for his help in identification of the plants collected for primary screening during my research work. I also thank **Mrs. Geetha** for her help whenever required.

The graceful execution of this dissertation work was possible because of the untiring efforts, moral support, active cooperation, motivation and encouragement of my husband **Mr. G. Baskaran,** from the beginning to the end. He has supported my dreams and aspirations, provided financial support whenever required.

I am cordially indebted to my beloved parents **Late Mr. R. Arumugam** and **Mrs. A. Rajeevi**, my loveable kids, **B.V. Pavithra, B.V. Dineshkumar,** my Brothers **Late Mr. A. Veerapandiyan, B.E.**, **Dr. A. Vijayaragavan**, Scientist-B, Institute of Forest Genetics and Tree Breeding, In-laws, **Mr. T. Ganapathy** and **Mrs. G. Suseela** and other family members for being on my side whenever I needed them. Lastly and most importantly I wish to say a big thanks to my mother **Mrs. A. Rajeevi**. My mother has been an inspiration throughout my life. I'd like to thank her for all she is and all she has done for me that has made my journey possible.

Thanking you

A. VIJAYALAKSHMI

LIST OF ABBREVATIONS

$AlCl_3$	-	Aluminium chloride
ANOVA	-	Analysis of Variance
C. tora	-	*Cassia tora*
C	-	Celsius
Cm	-	Centimeter
CMC	-	Carboxy Methyl Cellulose
Con	-	Concentrated
COX	-	Cyclo-oxygenase
CPCSEA	-	Control and Supervision of Experimentation on Animals
DMEM	-	Dulbecco's modified Eagle's medium
DMSO	-	Dimethyl sulphoxide
DNA	-	Deoxyribose nuleic acid
DPPH	-	1,1-Diphenyl, 2- picryl hydrazyl
EDTA	-	Ethylene diamine tetra acetic acid
ESI MS	-	Electrospray Ionization Mass Spectrometer
FTIR	-	Fourier Transform Infrared ***Spectrometer***
G. rottleriformis	-	*Givotia rottleriformis*
gm	-	Gram

GM-CSF	-	granulocyte-macrophage colony-stimulating factor
H_2O_2	-	Hydrogen peroxide
H_2S	-	Hydrogen Sulphide
Hb	-	Haemoglobin
HCl	-	Hydrochloric acid
HPLC	-	High Performance Liquid Chromatography
hr	-	Hour
i.p.	-	Intra peritoneal
IAEC	-	Institutional Animal Ethics Committee
IKI	-	Iodine and Potassium iodide
IL	-	Interleukin
IR	-	Infra Red
KBr	-	Potassium bromide
kg	-	Kilogram
L	-	Litre
mg	-	Milligram
MHz	-	Mega Hertz
Min	-	Minute
mL	-	Millilitre

mM	-	Millimole
MTD	-	maximum tolerated dose
NaOH	-	Sodium hydroxide
nm	-	Nanometer
OECD	-	Organization of economic Co-operation and Development
ppm	-	Parts per million
R_f	-	Retention factor
SEM	-	Standard Error Mean
TLC	-	Thin Layer Chromatography
UV	-	Ultra Violet
v/v	-	volume/volume
w/v	-	weight/volume
w/w	-	weight/weight
β	-	Beta
µl	-	Microlitre
µg	-	Microgram
%	-	Percentage
^{13}C	-	Carbon-13
^{1}H NMR	-	Proton Nuclear Magnetic Resonance Spectroscopy

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1. INTRODUCTION

Biodiversity of natural resources like plants, animals, microbes, minerals and marine sources has served not only the primary human needs but also health care since time immemorial.[1] In every culture, in every age, there are different theories of disease and different systems employed in medicine, but botanical remedies are universal. For thousands of years all the cultures have utilized herbs for health care. The widespread use of herbal remedies and healthcare preparations, as those described in ancient texts such as the Veda and the Bible has been traced to the occurrence of natural products with medicinal properties. Civilized societies have bequeathed myths and compendiums of healing herbs and the herbal remedies from people of preliterate societies continue to surprise us with their extensive green pharmacy.[2]

MEDICINAL PLANTS: INDIAN WEALTH AND HERITAGE

India is a varietal emporium of medicinal plants and is one of the richest countries in the world as regards to genetic resources of medicinal plants. In rural areas, 75 percent of the population is dependent on herbal medicines for healthcare. In the last few decades, herbal medicine has been found to have some impressive credentials. In India, over 2600 plant species have been considered useful in the traditional system of medicine like Ayurveda, Unani, Siddha and Home remedies. Number of herbal drugs and their compositions are recommended for combating human ailments in the ancient texts as well as in modern medicine.[3]

REVIVAL OF TRADITIONAL MEDICINE

The history of interest in phytochemicals reveals that crude drugs are the dominant therapy until the time of World War II. During the late 19th century, Western medicine began to supersede the folk and learned medicine that had been gathered and traded between cultures since the time of the Ancient Egyptians and moved away from any interest in folk knowledge of

medicinal plants. Today, pendulum is swinging back to an interest in the value of traditional medicine. There is currently a rising recognition of the value of experience and historical knowledge gathered by indigenous cultures with medicinal plants.

Herbal medicines are an important part of the culture and traditions worldwide. It is therefore no surprise that medicinal plants have raised their importance all over the globe. Recently, the renewed interest in medicinal plants as a re-emergent health aid has been fuelled by the extensive antimicrobial resistance along with rising costs of prescription drugs in the maintenance of personal health, well-being and the bioprospecting of new plant-derived drugs.[4]

The revival of interest in herbal medicines is firstly due to increased awareness of the limited horizon of synthetic pharmaceutical products to control major diseases and secondly due to the current widespread belief that 'green medicine' is safe and more accessible and affordable than the costly synthetic drug many of which have adverse side effects. The past decade has witnessed a tremendous resurgence in the interest and use of medicinal plant products especially from developed countries. According to a WHO estimate, about 80% of the world population relies on traditional systems of medicines for primary health care, where plants form the dominant component over other natural resources.[5] Today, the renewed interest in traditional pharmacopoeias reveals that researchers are concerned not only with determining the scientific rationale for the plant's usage, but also with the discovery of novel compounds of pharmaceutical value. Many plant components are now synthesized in laboratories for use in pharmaceutical preparations.

Today, rather than using a whole plant, pharmacologists identify, extract, isolate and synthesize individual components to capture the active properties. Natural products to be used in pharmaceutical preparations are either pure compounds or extracts. With the development of

various analytical methods of high precision, and advances in molecular biology and genetic engineering, it is now possible to isolate compounds in extremely small quantities, study their chemical structure and therapeutic potentialities and then to alter the molecule to be suitable for production of novel and more selective new therapeutic agents.

PHYTOMEDICINAL ACTIONS

The beneficial medicinal effects of phytomedicine typically result from synergistic actions of secondary products present in the plants. In contrast to synthetic pharmaceuticals based upon single chemicals, many phytomedicine exert their beneficial effects through the additive or synergistic action of several chemical compounds acting at single or multiple target sites associated with a physiological process. This synergistic or additive pharmacological effect can be beneficial by eliminating the problematic side effects associated with the predominance of a single xenobiotic compound in the body.[6]

PHYTOMEDICINE: A STRATEGY FOR FUTURE

For future drug development in the 21st century, research should focus not only on bioactive principles (lead compounds), but also on active fractions and active formulations from medicinal herbs. The plant species may be considered as a biosynthetic laboratory for a multitude of compounds such as alkaloids, glycosides, terpenoids, steroids, flavonoids, saponins and tannins. These chemical compounds are responsible for the desired therapeutic properties.

Phenolic compounds

Phenolic compounds constitute one of the main classes of secondary metabolites. They display a large range of structures and are responsible for the major organoleptic characteristics of plant-derived foods and beverages, particularly color and taste properties and they also contribute to the nutritional qualities of fruits and vegetables. The most important natural

pigments are carotenoids which are tetrapyrrole derivatives of naturally occurring phenolic compounds ubiquitously distributed in plant kingdom. Among these compounds, flavonoids constitute one of the most ubiquitous groups of all plant phenolics. So far, over 8,000 varieties of flavonoids have been identified. Until ~50 years ago, information on the working mechanisms of flavonoids was scare. In 1930, Szent-Gyorgyi isolated a new substance from oranges and classified it as vitamin P but later, it became clear that this substance was actually a flavonoid. Flavonoids drew greater attention from researchers with the discovery of the French Paradox, i.e., the decrease incidence of cadio-vascular disease observed in the Mediterranean population which was associated with red wine consumption, and a greater amount of saturated fat in the average diet than in other countries.[7]

Flavonoids

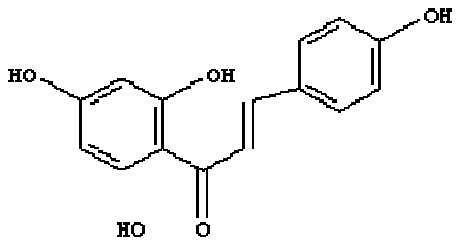
Flavonoid, (or bioflavonoid), also collectively known as Vitamin P and Citrin, are a class of plant secondary metabolites of over 6,000 different substances found in virtually all plants and are responsible for many of the plant colors that dazzle us with their brilliant shades of yellow, orange, red and mostly emit brilliant fluorescence when they are excited by UV light. They are classified as plant pigments that are synthesized from phenylalanine. Flavonoids are discovered in 1938 when a Hungarian scientist named Albert Szent-Gyorgyi used the term "vitamin P" to describe them.

Flavonoids are secondary metabolites characterized by flavan nucleus and C6-C8-C6 carbon-skeleton. These are group of structurally related compounds with a chromane-type skeleton having phenyl substituent in C2-C3 position.[8] The basic structural feature of flavonoid is 2-phenyl-benzo-γ-pyrane nucleus consisting of two benzene rings (A and B) linked through a heterocyclic pyran ring (C).

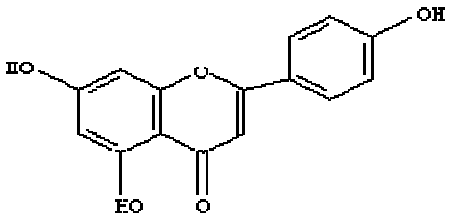
FLAVONOID CLASSIFICATION

Over 5000 naturally occurring flavonoids has been characterized from various plants. Various subgroups of flavonoids are classified according to the substitution patterns of ring C. Both the oxidation state of the heterocyclic ring and the position of ring B are important in the classification. They have been classified into six subgroups.[9,10]

1. Chalcones: Chalcones are a subclass of flavonoids. They are characterized by the absence of "ring C" of the basic flavonoid skeleton structure. Hence, they can also be referred to as open chain flavonoids. Major examples of Chalcones include Phloridzin, Arbutin, Phloretin and Chalconaringenin. Chalcones occur in significant amounts in tomatoes, pears, strawberries, bearberries and certain wheat products.

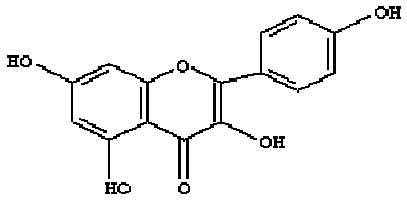


2. Flavones: Flavones are widely present in leaves, flowers and fruits as glucosides. Celery, parsley, red peppers, chamomile, mint and ginkgo biloba are among the major sources of flavones. Luteolin, apigenin and tangeritin belongs to this sub-class of flavonoids.

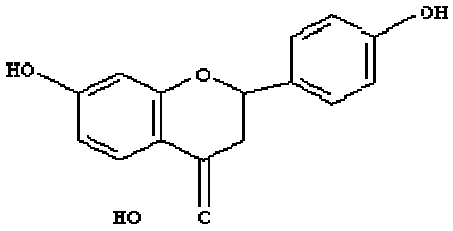


3. Flavonols: They are building blocks of proanthocyanins. Flavonols occur widely in a variety of fruits and vegetables. The most studied Flavonols are Kaempferol, Quercetin, Myricetin and Fisetin. Onions, kale, lettuce, tomato, apple, grape, and berries are among the other good sources.

Apart from fruits and vegetables, beverages such as tea and red wine are also important sources of Flavonols. Intake of Flavonols is found to be associated with wide range of health benefits which includes antioxidant potential and reduced risk of vascular disease.

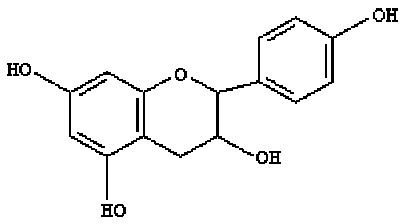


4. Flavanones: Flavonone is another important class of flavonoids which is generally present in all the citrus fruits such as oranges, lemons, grapes etc. Hesperitin, Naringenin, Eriodictyol are examples of this class of flavonoids. Flavonones are associated with a number of health benefits because of their free radical scavenging properties. Hesperidin and Naringin are two glycosides of flavonones Hesperetin and Naringenin. These compounds are responsible for the bitter taste of the juice and peel of citrus fruits. Citrus flavonoids exert interesting pharmacological effects as antioxidant, anti-inflammatory, blood lipid and cholesterol lowering agents.

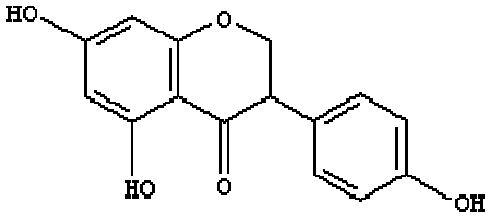


5. Anthocyanins: Anthocyanins are pigments responsible for colors in plants, flowers and fruits. Cyanidin, Delphinidin, Malvidin, Pelargonidin and Peonidin are the most commonly studied Anthocyanins. They occur predominantly in the outer cell layers of various fruits such as cranberry, black currant, red grape, merlot, raspberry, strawberry, blueberry, bilberry and blackberry. Stability coupled with health benefits of these compounds enable them to be used in the food industry in a variety of applications. Anthocyanins display wide range of biological activities including anti-oxidant, anti-inflammatory, anti-microbial and anticarcinogenic

activities. In addition, they exhibit significant effects on blood vessels and blood platelets, and reduce the risk of coronary heart disease.



6. Isoflavonoids: Isoflavonoids are a large and very distinctive subgroup of flavonoids. Isoflavonoids enjoy only a limited distribution in the plant kingdom and are predominantly found in soyabeans and other leguminous plants. Most of these (flavanones, flavones, flavonols, and anthocyanins) bear ring B in position 2 of the heterocyclic ring. In isoflavonoids, ring B occupies position 3. All the isoflavonoids are colourless. Some Isoflavonoids have also been reported to occur in microbial organisms. Isoflavonoids are a type of phytoestrogen, with chemical structure similar to plant hormone estrogen. Isoflavones such as Genistein and Daidzein are commonly regarded to be phytoestrogens because of their estrogenic activity in certain animal models. In addition, consumption of Isoflavones is known to reduce the buildup of arterial plaques, which further reduces the risk of coronary heart disease and associated cardiovascular complications. In addition, they also help reduce breast cancer by blocking the cancer causing effects of human estrogen. They may also prevent prostate cancer and hinder cell growth.



THERAPEUTIC POTENTIAL OF FLAVONOIDS

Flavonoids are a group of natural products with many biological and pharmacological activities; antibacterial, antiviral, antioxidant, and antimutagenic effects and inhibition of several

enzymes have been demonstrated.[11,12] Flavonoids possess potential pharmacological effect such as antioxidant activity, vitamin C sparing activity and the activities of cyclo-oxygenase, 5-lipoxygenase, protein kinase-C, tyrosine kinase, genetic toxicity etc. Flavonoids have free radical scavenging and antioxidant properties, which are useful for their pharmacological activities including anticancer and antiageing properties. Flavonoids show interaction with cytochrome P 450, antileukemic properties and mild vasodilator properties useful for the treatment of vascular diseases. It has been reported that flavonoids inhibit xanthine oxidase and have superoxide scavenging activities. Xanthine oxidase is considered to be an important biological source of superoxide radicals. These and other reactive oxygen species (ROS) contribute to the oxidative stress on the organism and are involved in many pathological processes such as inflammation, atherosclerosis, cancer, ageing, etc. Therefore, flavonoids could be a promising remedy for human gout and ischemia by decreasing both uric acid and superoxide concentrations in human tissues.[13]

Antioxidant activity: The best described property of almost every group of flavonoids is their capacity to act as antioxidants. The scavenging activity of flavonoids has been reported to be in the order: Myrcetin > quercetin > rhamnetin > morin > diosmetin > naringenin > apigenin > catechin > 5,7-dihydroxy-3',4',5'-trimethoxyflavone > robinin > kaempferol > flavone.[14]

Antibacterial activity: Antibacterial activity has been displayed by a number of flavonoids. Quercetin has been reported to completely inhibit the growth of *Staphylococcus aureus*. Most of the flavonones having no sugar moiety showed antimicrobial activities.[15]

Antifungal activity: A number of flavonoids isolated from the peelings of tangerine orange, when tested for fungistatic activity towards *Deuterophoma tracheiphila* were found to be active; nobiletin and langeritin exhibited strong and weak activities, respectively, while hesperidin could

stimulate fungal growth slightly. Chlorflavonin was the first chlorine containing flavonoid type antifungal antibiotic produced by strains of *Aspergillus candidus*.[16]

Antiviral activity: Quercetin, morin, rutin, taxifolin, apigenin, catechin, and hesperidin have been reported to possess antiviral activity against some of the 11 types of viruses.[17] The antiviral activity appears to be associated with the nonglycosidic compounds, and hydroxylation at the 3-position is apparently a prerequisite for antiviral activity. It has been found that flavonols are more active than flavones against *Herpes simplex* virus type 1 and the order of importance was galangin>kaempferol>quercetin.[18]

Antiulcer activity: Isoliquiritin, a flavonoid glycoside from *Glycyrrhiza glabra* Linn with antigastric effects, is used in peptic ulcer in the form of deglycyrrhized liquorice (DGL). Quercetin, rutin, and kaempferol administered intraperitoneally (25-100 mg/kg) inhibited dose-dependent gastric damage produced by acidified ethanol in rats.[19]

Hepatoprotective activity: In a study carried out to investigate the flavonoid derivatives silymarin, apigenin, quercetin, and naringenin, as putative therapeutic agents against microcrystin LR-induced hepatotoxicity, silymarin was found to be the most effective one.[20]

Anti-inflammatory activity: Hesperidin, a citrus flavonoid, possesses significant anti-inflammatory and analgesic effects. Recently apigenin, luteolin and quercetin have been reported to exhibit anti-inflammatory activity.[21]A number of reports have been published which demonstrate that flavonoids can modulate arachidonic acid metabolism via the inhibition of cyclo-oxygenase (COX) and lipooxygenase activity (LO).[22]

Antidiabetic effects: Flavonoids, especially quercetin, have been reported to possess antidiabetic activity. Vessal *et al*[23] reported that quercetin brings about the regeneration of pancreatic islets and probably increases insulin release in streptozotocin-induced diabetic rats.

Anti-atherosclerotic effects: Oxidative modification of low density lipoproteins (LDL) by free radicals is an early event in the pathogenesis of atherosclerosis. The rapid uptake of oxidatively modified LDL via a scavenger receptor leads to the formation of foam cells. Flavonoids may directly scavenge some radical species by acting as a chain breaking antioxidant.[24]

Antithrombogenic effects: Platelet aggregation plays a pivotal role in the physiology of thrombotic diseases. Activated platelets adhering to vascular endothelium generate lipid peroxides and oxygen free radicals which inhibit the endothelial formation of prostacyclin and nitrous oxide. It was shown in the 1960s that tea pigment can reduce blood coagulability, increase fibrinolysis, and prevent platelet adhesion and aggregation.[25]

Antineoplastic activity: A sufficient number of flavonoids have exhibited antineoplastic activity. Detailed studies have revealed that quercetin exerted a dose-dependent inhibition of growth and colony formation.[26] The flavonoids, kaempferol, catechin, taxifolin, genistein and fisetin, also suppressed cell growth.[27]

PSORIASIS

Psoriasis is a common chronic inflammatory T-cell-mediated immune disorder characterized by circumscribed, red, thickened plaques with an overlying silver-white scale. Person of all ages may develop the disease. Psoriasis is sometime associated with arthritis, myopathy, enteropathy, spondylitic heart disease or the AIDs. Psoriatic arthritis may be mild or may produce severe deformities resembling the joint changes seen in rheumatoid arthritis. Clinically, psoriasis most frequently affects the skin of the elbow, knees, scalp, lumbosacral areas, intergluteal cleft and glans penis.[28]

Psoriasis is a medical condition that occurs when skin cells grow too quickly. Faulty signals in the immune system cause new skin cells to form in days rather than weeks. (**Fig.1**) The body

does not shed these excess skin cells, so the cells pile up on the surface of the skin and lesions form.[29]

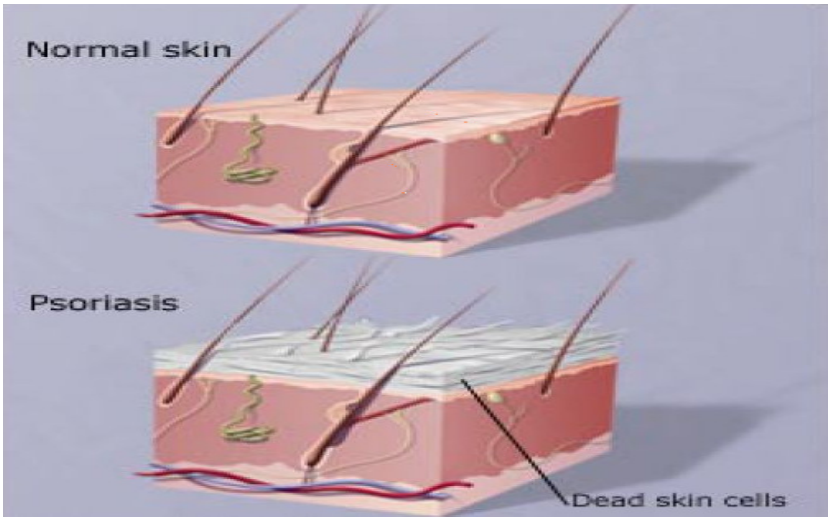


Figure 1: Normal skin and Psoriatic skin

The most typical lesion is a well demarcated, pink to salmon colored plaque covered by loosely adherent scales that are characteristically silver white in color. Psoriasis can be one cause of total body erythema and scaling known as erythroderma. Nail changes occur in 30% of cases of psoriasis and consist of yellow brown discoloration, with pitting, dimpling, separation of the nail plate from the underlying bed (onycholysis), thickening and crumbling. Psoriasis is either benign or localized (hands and feet) or generalized or life threatening, with associated fever, leukocytosis, arthralgias, diffuse cutaneous and mucosal pustules, secondary infection and electrolyte disturbances.[30]

Epidemiology

The prevalence of psoriasis varies widely depending on ethnicity. Psoriasis occurs most commonly in Caucasians, with an estimated occurrence of 60 cases per 100,000/year in this population. Its prevalence in the United States is 2-4 percent, although it is rare or absent in

Native American and certain African-American populations. While common in Japan, it is much less common in China, with an estimated incidence of 0.3 percent. The prevalence in the general population of Northern Europe and Scandinavia is 1.5-3 percent. The observation that latitude affects prevalence is most likely related to the beneficial effect of sunlight on the disease. Although psoriasis can occur at any age, the mean age of onset for chronic plaque psoriasis is estimated at 33 years, with 75 percent of cases initiated before age 46**.** The age of onset appears to be slightly earlier in women than men. Longitudinal studies suggest spontaneous remission may occur in about one-third of patients with psoriasis.[31]

Pathophysiology

Psoriasis is an immune disorder, and a growing body of evidence indicates that abnormal T-cell activity is a critical component of disease pathogenesis. Three lines of evidence implicate T cells in the pathophysiology of psoriasis.

- First, T lymphocytes have been identified in psoriatic plaques.
- Second, it has been demonstrated that the initiation and maintenance of lesions requires activated T cells.
- Finally, clinical studies have shown that drugs that suppress T-cell activity contribute to the improvement of psoriatic plaques.[32,33]

In psoriasis, it is believed that an unknown antigen causes antigen presenting cells (APCs) to be activated in the epidermis. The APCs internalize and process the antigen, which is then presented on the APC surface. Activated APCs then travel to the lymph nodes and activate naive T cells. During this activation, a T cell and an APC bind to each other at many points on their surfaces by means of receptor-ligand pairs.[34] These binding events are critical to any subsequent immune response. The first such event is the recognition of intracellular adhesion molecule-1 (ICAM-1)

on the surface of the APC by lymphocyte function-associated antigen-1 (LFA-1) on the surface of the T cell. This interaction sends a necessary but insufficient activation signal to the T cell.

- Additional co stimulatory signals are sent to the T cell as a result of several other interactions.[34] These include the binding of leukocyte function antigen-3 (LFA-3) on the APC to the CD2 antigen on the T cell. The net effect of all of these signals is an activated T cell with enhanced affinity for endothelial cells.
- The activated T cell rolls along the microvasculature toward peripheral tissues, a process mediated in part by the binding between T-cell LFA-1 and endothelial ICAM-1.[35] Persons with psoriasis have activated T cells that traffic into the dermis and then into the epidermis.
- Once in the skin, activated T cells undergo a second activation (reactivation) similar to the previous encounter with APCs in the lymph node. Reactivated T cells are then able to produce cytokines (soluble proteins that can exert both direct and indirect effects on other cells). These cytokines include interleukin-2 (IL-2) and interferon-gamma (IFN). Such factors may induce other cells to produce the post secretory cytokines-tumor necrosis factor-alpha (TNF), interleukin-8 (IL-8), and granulocyte-macrophage colony-stimulating factor (GM-CSF) causing excessive proliferation of the keratinocytes which are clinically evident as plaques. (**Fig. 2-4**)
- An agent that disables the psoriatic cascade of immunological events at any point from T-cell activation to cytokine binding would have the potential for clinical benefit.

Some of the specific strategies that have been employed in the development of biologic agents include:

- Blockade of interactions leading to T-cell activation or migration to tissue

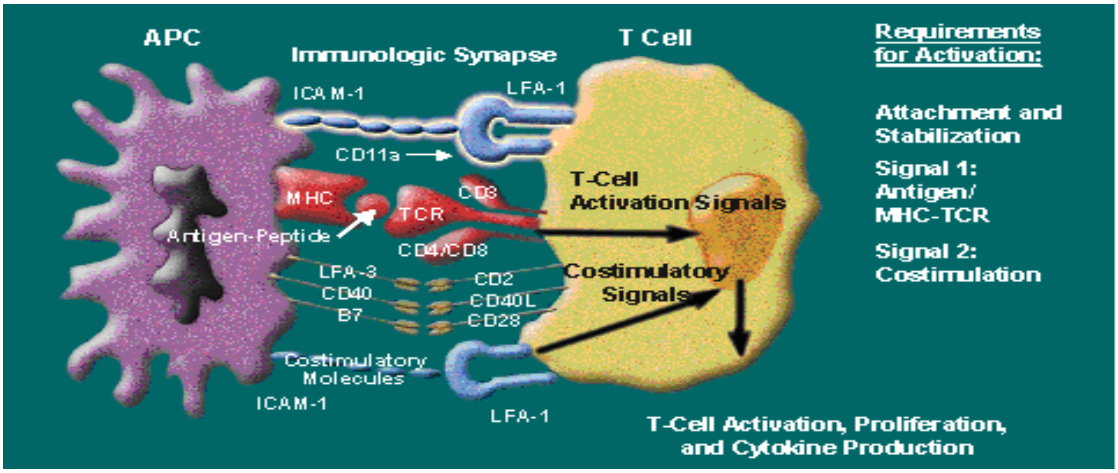


Figure 2: T cell activation in the lymph nodes

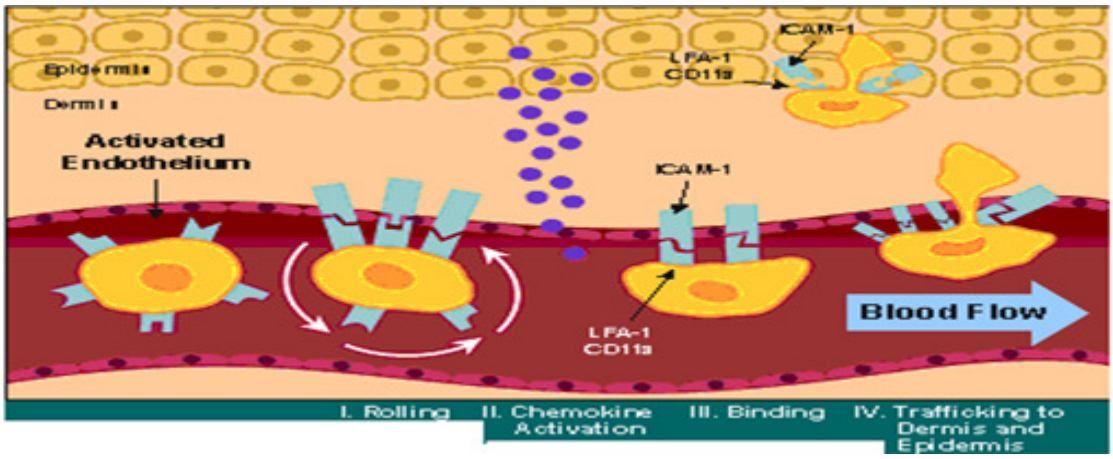


Figure 3: T-cell binding and trafficking into the dermis and epidermis

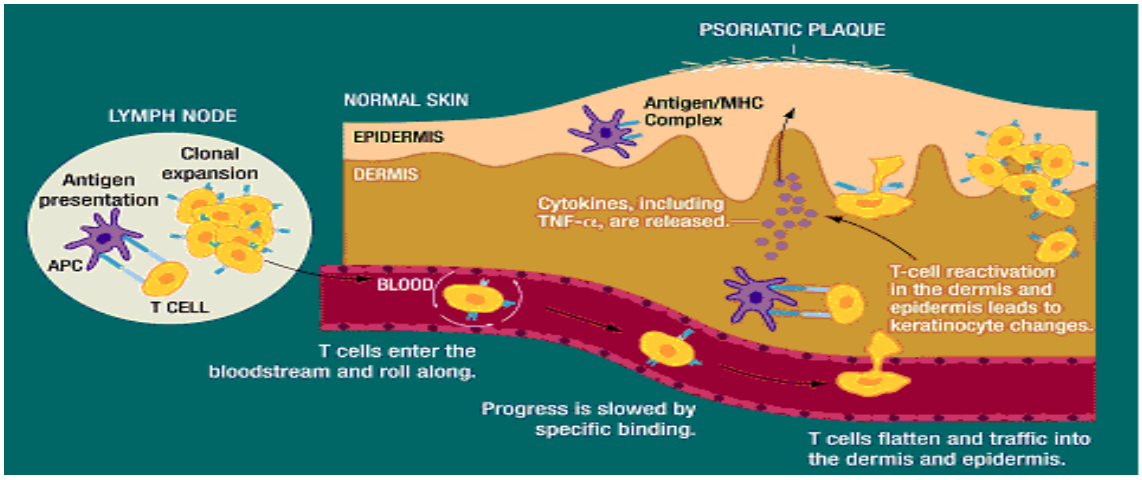


Figure 4: The Psoriatic cascade

- Elimination of the pathologic T cells by therapies targeted against activated T cells
- Alteration of the balance of T-cell types, for example, by redirecting T-cell differentiation away from the T_H1 type implicated in psoriasis toward the T_H2 type
- Binding of cytokines following their secretion by T cells before they can act on keratinocytes to drive the formation of psoriatic plaques
- Interfering with, or activating binding proteins for, pathways responsible for the expression of cytokines (eg, blockade of interferon receptors or IL-18)

Types of psoriasis

Psoriasis presents as a papulosquamous disease with variable morphology, distribution, severity, and course. The five types of psoriasis include plaque (psoriasis vulgaris), guttate, inverse (flexural), pustular, and erythrodermic. Typically, only one type of psoriasis will appear at a time. After an outbreak has cleared, either the same or a different type of psoriasis can appear when triggered.[36,37]

- **Plaque psoriasis** (psoriasis vulgaris) is the most prevalent form of the disease, affecting approximately 80% of individuals with psoriasis. It commonly occurs on the elbows, knees, scalp, and lower back, as well as at sites of trauma. The lesions of plaque psoriasis are characterized by well-demarcated round or oval plaques. The plaques appear scaly, thick, silvery, and erythematous, and are surrounded by normal skin. The scales are usually loosely cohesive and removal may cause small bleeding points, known as the Auspitz sign.
- **Guttate psoriasis** is the second most common form of the disease. It impacts about 10% of those with psoriasis. The lesions have a rapid onset and are small, tear-shaped papules that typically present on the trunk and extremities. The lesions are less thick than those of plaque psoriasis. The number of lesions can range from less than 10 to over 100. An initial outbreak

of guttate psoriasis is often associated with a recent streptococcal respiratory or throat infection.

- **Nummular psoriasis** is characterized by rounded plaques which are several centimeters in diameter. Their shape and size are more or less the same as a coin.
- **Pustular psoriasis** is characterized by white blisters of non infectious pus surrounded by reddened skin. There are three distinct forms of pustular psoriasis, including von Zumbusch, palmoplantar pustulosis, and acropustulosis (acrodermatitits continua of Hallopeau). Von Zumbusch psoriasis has a rapid onset that begins with reddened, painful patches of skin over large areas of the body, followed quickly by the development of the pustules. Within 24 to 48 hours, the pustules will dry and peel and a new eruption of pustules will form. The cycle may continue for days or weeks. Von Zumbusch psoriasis is usually accompanied by fever, chills, dehydration, and muscle weakness and often requires hospitalization for treatment. Palmoplantar pustulosis is characterized by pustules that develop on the palms of the hands and soles of the feet. The pustules appear in a speckled pattern throughout reddened plaques, then turn brown, and crust over. Acropustulosis is a rare form of psoriasis in which the lesions are located on the ends of the fingers or toes. The lesions are extremely painful and cause deformity of the nails, and in some cases, bone changes. This type of psoriasis affects less than 10% of patients. The palms of the hands, soles of the feet, fingers and nails are most affected.
- **Erythrodermic psoriasis** can affect most of the body's surface, and skin becomes erythemato-squamous (covered by red, scaling patches). Limited patches of pustules may appear.

- **Inverse psoriasis:** Inverse (flexural) psoriasis involves lesions that develop in the axilla, groin, or folds of the skin. This type of psoriasis is more prevalent in obese and overweight individuals and those with deep skin folds. The lesions of inverse psoriasis are large, shiny, smooth, and have a deep red color. Inverse psoriasis lacks the scales associated with plaque psoriasis.
- **Psoriasis arthritis:** Psoriatic arthritis is characterized by inflammation affecting joints and in some cases entheses (the point at which a tendon inserts into the bone). It is estimated that approximately 1% of the world population is affected by psoriatic arthritis.

Triggering factors

There are numerous triggers that are associated with psoriasis outbreaks. Psoriasis triggers are highly individualized and can vary from person to person. Established triggers of psoriasis include certain types of infections, skin injuries, stress, and the use of specific medications.[38,39]

- Genetic predisposition and withdrawal of systemic steroids and oral corticosteroids
- Factors that may aggravate psoriasis include stress, excessive alcohol consumption, smoking, exposure to cold weather, sunburn and other injuries to the skin
- Drugs, including salicylates, iodine, lithium, phenylbutazone, oxyphenbutazone, penicillin, hydroxychloroquine, calcipotriol, INF-α, and rINF-β
- Strong, irritating topicals, including tar, anthralin, steroids under occlusion, and zinc pyrithione in shampoo
- Infections, phototherapy, Cholestatic jaundice, Hypocalcemia, Idiopathic in many patients
- Specific medications including lithium, antimalarials and interleukin II are shown to be triggers of erythrodermic psoriasis.

Drug Therapies

Treatment can be topical, systemic or with ultraviolet light. Phototherapy and systemic agents should be used only when topical treatments are inadequate. Novel systemic treatments for psoriasis include a rapidly expanding range of biological therapies. The decision to employ a particular treatment is based on the type of psoriasis, its location, extent and severity. The patient's age, sex, quality of life, co morbidities, and attitude toward risks associated with the treatment are also taken into consideration. Medications with the least potential adverse reactions are preferentially employed.[40]

As a first step, medicated ointments or creams, called topical treatments, are applied to the skin. If topical treatment fails to achieve the desired goal then the next step would be to expose the skin to UV radiation called phototherapy. The third step involves the use of medications which are taken internally by pill or injection called systemic treatment.[41,42]

Diet

The first step is reducing the severity of the psoriasis is "**Drink lots of water**" at least 2 liters a day. The second step is to "Improve the diet" and eat lots of green leafy vegetables. This will not cure the psoriasis, but it may dramatically reduce it. The following foods are popular triggers; Coke-a-cola, red meat, chili, hot spices, junk foods, oily foods, berries (strawberries) tomato, most acidic food and Vit-C. So their consumption needs to be controlled. Ingestion of alcohol has been reported to be a risk factor for psoriasis in men. Fumaric acids, fish oil, triglycerides, folic acid, flaxseed oil, Vit-D are found to be effective against psoriasis.[43]

Topical Treatments

Topical treatments are usually the first to be tried when fighting psoriasis. They involve applying lotions or moisturizers to the skin that can help to reduce the accelerated production of

skin cells and reduce inflammation. Varieties of externally applied preparations used are petroleum jelly, liquid paraffin, tar, ointment, psoralen (photosensitive drug), salicylic acid, steroid ointment, & creams etc to care for skin dryness and infection.[44]

- **Topical steroids:** They may be used as single agents or in combination with other agents in moderate to serious disease. They act by their antimitotic, immunosuppressant and anti-inflammatory effects. Therapy is usually started with a potent steroid (clobetasol propionate or belathasone dipropionate) applied once or twice daily.[45]
- **Salicylic acid:** Helps to slough off dead skin cells and used along with topical creams or coal tar.[46]
- **Coal tar:** Coal tar is used in many forms of treatment and can be purchased in crude or refined form for treating all levels of psoriasis. Coal tar is often combined with ultraviolet B phototheraphy. Coal tar only treats the inflammation, not the cause, and will do nothing to prevent the psoriasis occurring. The FDA says a coal tar solution of between 1-5% has been proven as safe products.[47]
- **Dithranol:** It is an effective agent for treating thick plaques of psoriasis. It is a derivative of a traditional medicine chrysarobin and has been in use for a century. Dithranol causes skin irritation and brownish discoloration of skin. It is best used as short contact therapy to avoid these side effects. In a concentration of 0.1-1%, it is applied once a day and washed off thoroughly after a contact period of 10 minutes to one hour. It may stain clothing.[28]
- **Topical vitamin D3 analogs:** Calcipotriol, a synthetic Vit-D3 analog, is both safe and effective. It blocks epidermal proliferation, enhances maturity of cells, and has anti-inflammatory effects. It is no more effective than the moderately potent topical steroids,

but combination of calcipotriol with topical steroids or oral agents is more effective than either agent alone. Unlike topical steroids, skin atrophy or tolerance is not problem, but irritation may occur on application particularly on areas like face.[48]

- **Tazarotene**: Tazarotene is a synthetic retinoid with properties similar to that of Vit-A. In the treatment of psoriasis, it may be used as a single agent or in combination with a corticosteriod cream or ointment, calcipotriol or phototherapy. It should not be used on the genitals or in the skin folds. It is contraindicated in pregnancy.[48]
- **Tacrolimus:** It is an immunosuppressant and useful in the management of atopic dermatitis, can also benefit psoriasis. It may be beneficial over sensitive areas like the face where topical steroid application may have troublesome side effects.[46]

Phototherapy

It has long been recognized that daily, short, non burning exposure to sunlight helped to clear or improve psoriasis. Phototherapy involves exposure to ultraviolet radiations by means of special equipment using fluorescent light source emitting specific wavelength of radiation. UV acts by reducing cellular proliferation and modifying the immune response. Regular exposure to sun or artificial UV lights can cause the symptoms to subside. Approaches include UVB i.e. exposure to ultraviolet B light and PUVA i.e. exposure to UV rays combined with the drug psoralen, which increases the light sensitivity of skin.[49]

Systemic drugs: Systemic drugs, used for more severe symptoms, are taken by mouth (orally). All can have serious side effects with long-term use. They include:

- **Methotrexate** helps to block the growth of skin cells and reduce inflammation. May also be used to treat psoriatic arthritis. Long-term use can cause serious side effects.[50]

- **Cyclosporine** is a cyclic polypeptide widely used as an immunosuppressant in organ transplantation. It acts in psoriasis through its inhibitory effects on T-cells.[51]
- **Oral retinoids** are synthetic compounds having Vit-A like cellular activities. Oral retinoids act by their anti-inflammatory actions as well as by regulation and maturation.[48]
- **Biologics** may be given to people who fail traditional therapy or who have psoriatic arthritis, given by injection or infusion (IV).
    - Alefacept (Amevive)
    - Etanercept (Enbrel)
    - Infliximab (Remicade)
    - Ustekinumab (Stelara)
    - Adalimumab (Humira)

Herbal medicines

Traditional medicines hold a great promise as source of easily available effective therapy for skin diseases to the people, particularly in tropical developing counties, including India. Herbal remedies for psoriasis are increasingly popular and mainstream.[52]

- **Oregon grape *(Mahonia aquifolium)*:** Skin ointments made with Oregon grape bark has the active ingredients berberine, berbamine and oxycathine, have demonstrated antioxidant and anti inflammatory effects as well as anti proliferative properties in the treatment of psoriasis.
- **Milk thistle *(Silybum marianum)*:** Milk thistle is thought to be good herb for psoriasis because it has shown the ability to hinder the human T-cell activation that happens while encountering psoriasis but it has not been clinically tested on patients.[53]

- **Oregano *(Origanum vulgare):*** Another herbal remedy for psoriasis is oregano. This is due to the fact that it contains not only antibacterial properties, but antifungal properties as well which can be helpful with infections that are induced by psoriasis.[52]
- **Cayenne *(Capsicum annuum):*** Cayenne contains capsaicin which has been demonstrated to reduce itching and pain in psoriasis suffers. It appears to work by depleting neurotransmitters in the sensory nerves. A clinical study performed by the American Academy of Dermatology found that capsaicin, topically applied, was an effective remedy for pruiritic psoriasis.
- **Aloe vera*:*** *Aloe vera* is an herbal remedy frequently used to treat minor injuries and irritations of the skin.[54]
- **Neem *(Azadirachta indica):*** Neem is effective in the treatment of diseases caused by fungus, viruses and bacteria. It has been shown to enhance the production of T-cells which fight infections. The active principles are gedunin and nimbidol which have effective fungicidal properties are thought to be effective as an herbal treatment for psoriasis and eczema.
- **Arnica montana *(Arnica Montana):*** *Arnica montana* is thought to increase the flow of blood in the capillaries under the skin and it is considered helpful in healing wounds and reduces inflammation.
- **Barberry *(Berberis vulgaris):*** Barberry has been used as a natural herb for psoriasis as well as many other inflammatory associated ailments.

Other natural herbs used for the treatment of psoriasis

- Turmeric - (*Curcuma longa*)
- Sarsaparilla - (*Aralia nudicaulis*)

- Cat's Claw - (*Uncaria tomentosa*)
- Coleus forskohlii - (*Plectranthus barbatus*)
- Tea Tree Oil - (*Melaleuca alternifolia*)
- Burdock root - (*Arctium lappa*)
- Juniper - (*Juniperus communis*)
- Red Clover - (*Trifolium pratense*)
- Chickweed - (*Stellaria media*)
- Picrorhiza kurroa - (*Picrorhiza kurroa*)
- Black Walnut - (*Juglans nigra*)
- Dandelion - (*Taraxacum officinale*)

The therapeutic potential of flavonoids and the necessity for scientific validation in popular medicine have prompted increased interest in the field. Currently available allopathic drugs have been associated with a number of side effects. Since flavonoids exihibits several biological effects, in the present work, an attempt has been made to isolate flavonoids from flavonoid rich medicinally potent herbal source and to develop a new lead to treat psoriasis.

2. REVIEW OF LITERATURE

The literature review encompasses systematic information on pharmacognosy, phytochemistry and pharmacological studies of *Givotia rottleriformis* Griff. Ex Wight and *Cassia tora* Linn. It was found that very little information was available for *Givotia rottleriformis*.

GIVOTIA ROTTLERIFORMIS GRIFF. EX WIGHT

2.1. PLANT PROFILE[55]

Botanical name : *Givotia rottleriformis* Griff. Ex Wight (Euphorbiaceae)

Synonyms : *Givotia moluccana* (L.) Sreem, *Croton moluccanus* L.

Part used : Bark

2.1.1. VERNACULAR NAMES[55]

English : White Catamaran Tree

Kannada : Polike, bellitalai

Marathi : Polki

Telugu : Tella poliki

Tamil : Thaala maram, Kottai thanuku, Vellai Poothalai, Vendalai

2.1.2. TAXONOMICAL STATUS[55]

Kingdom : Plantae

Division : Angiospermae

Class : Magnoliopsioda

Order : Euphorbiales

Family : Euphorbiaceae

Genus : Givotia

Species : rottleriformis

Botanical name : *Givotia rottleriformis*

GIVOTIA *ROTTLERIFORMIS* GRIFF. EX WIGHT

Figure 5: Entire Plant of *Givotia rottleriformis*

2.1.3. PLANT DESCRIPTION[56]

Habit and Habitat: The moderately sized tree is commercially valuable in building Catamarans and hence the English name, White Catamaran Tree. The white wood of the tree is exceedingly light, soft, even grained and durable. It is also used in carving figures, toys and fancy items. The distribution of this tree is noticed only in limited areas of the forests of Tamil Nadu, Andhra, Karnataka, West Bengal and coastal Sri Lanka. The commercial value and poor germination of its seeds affecting propagation have made the Puththalai tree an endangered species.

Morphological Characters

Leaves: Leaves are alternate, broadly ovate or orbicular, coarsely dentate, acuminate, glabrous above yellowish tomentose below 5-nerved.

Flowers: The flowers are in sub-terminal pendulous panicles, flowering from April-July.

Fruits: Fruits are a drupe, subglobose or ellipsoid fulvous-tomentose.

Seed: Seed, globose or ellipsoid with a bony testa, fruiting from May-June.

Bark: Bark smooth brown, peeling off in circular scales. Bruised bark yields a blood red sap.

2.1.4. PROPAGATION

Germination of seeds is very poor because of 3 layers of a thick seed coat, which contains a high percentage of phenolics that are mainly responsible for a long dormancy period of 1 to ½ years under natural conditions. The difficulty in seed germination and absence of proper vegetative propagation techniques have rendered non-availability of this timber in required quantities to toy making manufacturers, which is posing severe problems for these communities. In vitro propagation offers promise in achieving rapid propagation of *Givotia*.

2.1.4. ETHNOBOTANICAL USES

The bark and seeds of the tree are used in indigenous medicine in the treatment of rheumatism, dandruff and psoriasis.[57,58] The juice is extracted from the bark and administered to cure jaundice. The Palliyar tribes of southern Tamil Nadu give endosperm of the seeds mixed with milk (10gms to 100ml) for three days to improve digestion in children. Its stem-bark paste is applied and its leaves are used as bandage during deep cuts by ethnic people of Krishna district, Andhra Pradesh. Oil extracted from the seeds is used in lubricating machinery.[59]

2.2. LITERATURE REVIEW

- **Arivoli S *et al*., (2000)[60]** determined the larvicidal activity of: (1) bark extracts of *Bischofia javanica, Cinnamomum zeylanicum* and *Givotia moluccana*; (2) leaf extracts of *Morinda umbellata, Trichopus zeylanicus, Erythroxylum monogynum, Oxalis corniculata, Solanum verbasicum* and *Vitex negundo*; and (3) extracts of shoots with leaves of *Leucas aspera*. Residues of these solvent extracts procured from shoot with leaves of *L. aspera* and leaves of *V. negundo* were found to have significant effects at such dosages, resulting in 100% mortality of the larvae within 24, 48 or 72 h of treatment.
- **Samuel *et al*., (2009)[61]** studied the micropropagation of *Givotia rottleriformis* Griff. as a result of long seed dormancy associated with poor seed germination. Best germination frequency (78.3%) was achieved from mature zygotic embryo axes isolated from acid-scarified fresh seeds when cultured on Murashige and Skoog (MS) medium. However, acid scarification of 1-yr-old seeds decreased the germination frequency of zygotic embryo axes in comparison to those obtained from non-acid-scarified seeds which germinated (96.2%) and converted into plants (80.3%) on MS basal medium.

- **Muthukumarasamy et al., (2009)**[62] reported that the Palliyar tribe of southern Tamil Nadu give endosperm of the seeds mixed with milk (10gms to 100ml) for three days to improve digestion in children.
- **Hari Babu et al., (2011)** [63] identified the phytochemicals present in *Givotia rottleriformis* leaves. Dried and powdered leaves of *Givotia rottleriformis* extracted successively with hexane, ethyl acetate and methanol and screened for phytochemicals and antimicrobial activity by agar-well diffusion method. Ethyl acetate and methanol showed positive result for the presence of flavonoids and steroids. Ethyl acetate extract showed significant antimicrobial activity for selected organism.
- **Baskar ananda raj et al., (2011)** [64] carried out pharmacognostic investigations in terms of organoleptic, percentage yield, extractive value, fluorescence analysis, physical parameters, chemical evaluation, microscopic and macroscopic analysis using powder stem and extract. Preliminary qualitative chemical tests for the extract shows the presence of alkaloids, carbohydrates, proteins, amino acids, flavonoids, phenolic and tannins.

CASSIA TORA LINN.

Plant *Cassia tora,* formally regarded as cassia tora is God's gift to man. Cassia (from Arabic Sana), is a large genus of flowering plants in the family Fabaceae, subfamily Caesalpiniaceae. The number of species is usually estimated to be about 360 but believe that there are many as 350.

2.3. PLANT PROFILE[65]

Botanical name : *Cassia tora*

Synonym : *Senna tora* (L.) Roxb

Part used : Leaves

2.3.1. VERNACULAR NAMES[66]

English : Foetid cassia

Hindi : Cakunda, Cakvat

Kannada : Tagace, Taragasi

Malayalam : Takara

Sanskrit : Cakramardah, Prapunnatah

Telugu : Tagirisa, Tantiyamu

Tamil : Tagarai

2.3.2. TAXONOMICAL STATUS

Kingdom : Plantae

Division **:** Angiospermae

Class **:** Magnoliopsioda

Sub class **:** Rosidae

Order **:** Fabales

CASSIA TORA LINN.

Figure 6: Entire Plant of *Cassia tora*

Family : Caesalpiniodeae (fabaceae)

Sub family : Caesalpiniodeae

Tribe : Cassieae

Sub tribe : Cassiinae

Genus : *Cassia*

Species : *Tora*

2.3.3. PLANT DESCRIPTION[67]

Habit and Habitat: The plant is an herbaceous annual foetid weed, almost an undershrub, upto 90 cm in height. It is grown in dry soil throughout tropical parts of India. In India it occurs as wasteland rainy season weed. It is grown in hot, wet, tropical climates both wild and commercially. *Cassia tora* now occurs throughout Florida and the southeastern United States.

Morphological characters

Leaves: The leaves are pinnately compound and the leaflets are opposite. Rachis grooved with a conical gland between each of the two lowest pairs of leaflets. The leaflets are three pairs, obovate-oblong, membranous with somewhat oblique base and has 8-10 pairs of main nerves. The petioles are 2.5 mm long and pubescent.

Flowers: Flowers are yellow usually subsessile pairs in the axils of the leaves and upper flowers are crowded. It possesses common peduncle of not exceeding 4 mm long. The calyx is glabrous, divided at the base; segments 5 mm, long, ovate, acute, spreading. The flowers have 5 petals, pale yellow, oblong, obtuse, spreading, the upper petal (standard) 2-lobed and the others entire. The stamens are 10, of which 3 upper stamens are reduced into minute staminodes and the remaining 7 stamens are perfect and sub equal.

Fruits: Fruits are subtetragonous obliquely separate pods, much curved when young, obliquely septate, 15-23 cm long with very broad sutures.

Seeds: Seeds rhombohedral 25-30 per pod, with the long axis in the direction of the pod.

2.3.4. CHEMICAL CONSTITUENTS[68]

The roots of *Cassia tora* reported to contain a anthraquinone pigments 1,3,5-trihydroxy-6,7-dimethoxy-2-methylanthraquinone and leucopelargonidin-3-O-α-L-rhanmopyranoside, β-sitosterol, palmitic, stearic and linoleic acid.

The seeds of *Cassia tora* reported to contain naphtho-α-pyrone-toralactone, rubrofusarin-6-β-gentiobioside (III), chrysophanol, physcion, emodin, chrysophanic acid-9-anthrone and rubrofusarin.

The leaves of *Cassia tora* reported to contain emodin, triacontan-1-ol, stigmasterol, β-sitosterol-β-D-glucoside, friedelin, palmitic, stearic, succinic acid, uridine, myo-inositol, d-ononitol, kaempferol, quercetin, juglanin, astragalin, quercetrin and isoquercetrin.

2.3.5. ETHNOBOTANICAL USES[66]

- The leaves and seeds are acrid, thermogenic, laxative, anthelmintic, liver tonic, ophthalmic, expectorant and cardiotonic. The leaves and seeds are useful in ringworm, leprosy, pruritus, skin diseases, hepatopathy, helminthiasis, laxative, flatulence, colic, dyspepsia, constipation, ophthalmopathy, cough, bronchitis, cardiac disorders and haemorrhoids.
- *Cassia tora* leaves and seeds are useful in treating skin diseases like ring worm, itching or body scratch and psoriasis.
- In china, the seeds are used for all sorts of eye diseases, for liver complaints and boils. In Indo China, the pods are used in dysentery and in eye diseases. In Nigeria, the leaves are used as a mild laxative and in Nepal are used externally in treating leucoderma, leprosy and itchy skin.

2.4. LITERATURE REVIEW

Pharmacognostic Review

- **Takahashi S *et al*., (1978)[69]** studied plant hormonal requirement for the tissue culture of seeds of *Cassia tora* by dual-wavelength TLC zig-zag scanning. From the callus of *C. tora,* chrysophanol and physcion have been detected chromatographically.
- **Khosla SN and Dnyanasagar VR (1980)[70]** studied that the Sodium arsenite, MH and 2,4-D inhibited the growth of *C. tora* plants by causing abnormal mitosis of stopping it altogether and chromosomal abnormalities.
- **Trivedi et al., (1982)[71]** subjected the dry ripe seeds of *Caesalpinia pulcherrima, Cassia fistula, Mimosa pudica* and *Albizzia lebbek* to SEM studies. The results indicate that the seeds show rugose pattern. Others such as *Cassia sophara, C. occidentalis* and *C.tora* show reticulate pattern and the seeds of *Tamarindus indica* and *Bauhinia tomentosa*, show tuberculate pattern under SEM. However, the ornamentation of each seed coat is distinct from the other, which helps in its identification.
- **Sexena and Ramakrishnan (1983)[72]** studied growth, resource allocation pattern, nutrient uptake and use efficiency of *Borreria articularis, Cassia tora, Ageratum conyzoides* and *Erigeron linifolius*. In all the species, reproductive allocations of N and P were significantly higher than that of biomass and potassium. Resources allocation strategy and the co-existance of these weeds in the same environment have also been discussed.
- **Pachkhede et al., (1984)[73]** reported that the fungal species of Fusarium and Drechslera causing leaf sport disease in *Tecoma Strans, Ipomoea fistulosa, Cassoa tora, Bauhinia diphyll* and *Dalbergia sissoo* respectively.

- **Jain SP and Puri HS (1984)**[74] described botanical name, family, local name and uses of about 100 plants *Abutilon indicum, Adhatoda zeylanica, Berberis lyceum, Cannabis sativa, Cassia tora, Plumbago zeylanica, Tectoria Fuscipes, Vernonia cineria* and *Vitex negundo*.
- **Mathur M (1985)**[75] distinguished *Cassia tora* and *C. obtusifolia* on the basis of their epidermal structure and seed analysis.
- **Avijit Mazumder et al., (2005)**[76] studied macroscopic characters of *Cassia tora* leaves, ash values, extractive values, behavior on treatment with different chemical reagents and fluorescence characters under ultraviolet light. Preliminary phytochemical studies on different extractives of the leaves were also performed.
- **Chandan das et al., (2011)**[77] evaluated the pharmacognostical and phyto-chemical standards of the leaf of *Cassia tora* Linn. The study shows the presence of paracytic stomata, unicellular, uniseriate covering trichomes with swollen base and acute apex, prisms crystals of calcium oxalate and fibre elements.
- **Yogesh Shakywar *et al*., (2011)**[78] reviewed traditional uses and properties of *Cassia tora* Linn. (Caesalpiniaceae), a small annual herbs or under shrub growing as common weed in Asian countries in rainy season containing anthraquinone.

Phytochemical Review

- **Koshioka M and Takino Y (1978)**[79] established a method for quantitative estimation of anthraquinones and the change of anthraquinone contents in seeds of *C. obtusifolia* and *C. tora* with various grade of maturity and ageing.
- **Koshioka M *et al*., (1979)**[80] studied the amounts of total anthraquinones in the seeds of *C. obtusifolia, C. tora, C. torasa* and *C. occidentalis.* Anthraquinones were detected in all parts

(root, stem, leaf, legume and seed) of the plants; the quantity being in the following order: root>stem>leaf.

- **Kapoor *et al*., (1980)[81]** determined water soluble mucilage content of *C. absus* (14.6%) *C. fistula* (36.8%), *C. grandis* (38.8), *C. hirsuta* (24.0%) *C. occidentalis* (30.9%), *C. renigera* (20.0%), *C. siamea* (24.7%) *C. tora* (25.8%) and *C. sophera* (20.0%). The mucilage present only in the endosperm is a galactomannan type of polysaccaharide. Protein and pentosan content of the mucilages and viscosity of 5 percent solutions have also been estimated. Viscosity of *C. grandis* solutions is highest (5600cps).
- **Singh RP *et al*., (1981)[82]** described the physico-chemical characteristics of the oil (yield 10.5%) isolated from the petroleum ether extract of the seeds of *Cassia tora*. Glucose, galactose, xylose and raffinose have been isolated from the seeds and identified. The seed ash (yield 10.38%), contains following constituents: calcium (0.35%), sodium (0.19%), potassium (10.0%) and phosphorus (2.90%).
- **Pal M and Pal PR (1984)[83]** isolated emodin from the leaves of *C. tora* and exhibited purgative activity in mice.
- **Wong SM *et al*., (1989)[84]** isolated three new anthraquinone glycosides, from the seeds of *C. tora*. Their structures were elucidated on the basis of chemical and spectral data.
- **Wong SM *et al*., (1989)[85]** isolated two new naphtha-pyrone glycosides 5 and 6 together with cassiaside (3) and rubrofusarin-6-beta-gentiobioside (4) from the seeds of *C. tora*. Their structures were elucidated on the basis of chemical and spectral data. The naphtho-gamma-pyrone glycosides (except 5) were found to have significant hepato-protective effects against galactosamine damage.

- **Ga Young Lee *et al*., (2006)[86]** isolated three naphthopyrone glucosides, cassiaside (1), rubrofusarin-6-O-beta-D-gentiobioside (2), and toralactone-9-O-beta-D-gentiobioside (3), from the BuOH-soluble extract of the seeds of *Cassia tora* as active constituents, using an in-vitro bioassay based on the inhibition of advanced glycation end products (AGEs) to monitor chromatographic fractionation and all the isolates (1-3) were evaluated for the inhibitory activity on AGEs formation in vitro.
- **Cho *et al*., (2007)[87]** isolated soluble fibers from the seeds of *Cassia tora* Linn. (SFC) and investigated the effects of SFC on lipid metabolism in male Sprague–Dawley rats feed a normal diet, a high-cholesterol diet, or a high-cholesterol diet with 5% SFC, for 5 weeks. The serum concentration of total cholesterol in rats fed SFC was 27% lower ($p < 0.05$) compared to that of the control group, but the serum high-density lipoprotein cholesterol level was increased in the SFC group. Liver total cholesterol and triglyceride levels were reduced significantly ($p < 0.05$) in rats fed the SFC diet. These results indicate that SFC enhances fecal lipid excretion and may cause a reduction in serum and hepatic lipid concentrations in rats.
- **El-Halawany *et al.,* (2007)[88]** isolated two new phenolic triglucosides, torachrysone 8-O-beta-D-glucopyranosyl (1,3)-O-beta-D-glucopyranosyl(1,6)-O-beta-D-glucopyranoside] (1) and toralactone 9-O-[beta-D-glucopyranosyl-(1,3)-O-beta-D-glucopyranosyl-(1,6)-O-beta-D-glucopyranoside] (2), along with seven known compounds from 70% ethanolic extract of *Cassia tora* seeds and investigated the estrogenic activity of the fractions and the isolated compounds using the estrogen-dependent proliferation of MCF-7 cells. The basic nucleus 1,3,8-trihyroxynaphthalene was found to play a principal role in the binding affinity of these compounds to estrogen receptor alpha (ER).

- **Sudarshan Singh *et al*., (2010)[89]** isolated mucilage from *Cassia tora* seed. Phytochemical characteristics of mucilage such as carbohydrate, protein, fat and flavonoids and physiochemical characteristics of mucilage such as solubility, swelling index, loss on drying, viscosity, hydration capacity, powder porosity, microbiological properties and pH were studied. The mucilage was evaluated for its granulating and binding properties in compressed tablet using Zidovudine as model drug. The tablets had good physicochemical properties and the drug release was more than 85% within 4 hour. It was observed that increasing the concentration of mucilage increases hardness and decreases the disintegration time.
- **Harshal A. Pawar and Priscilla MD Mello (2011)[90]** estimated total polysaccharide content of the gum obtained from *Cassia tora* seeds spectrophotometrically collected from Jalgaon region (Maharashtra). The gum was isolated by precipitation method & evaluated for total polysaccharide content using Phenol- sulphuric acid method. The total polysaccharide content in *Cassia tora* gum was found to be 77 %w/w.

Antimicrobial Review

- **Chatterjee et al., (1980)[91]** tested the seed extracts of 14 plants, viz., *Albizzia lucida, Bauhinia acuminate, B. racemosa, Cassia tora, Calliandra haematocephala, Cicer arietinum, Crotalaria striata, Dalbegia cultrate, Delonix regia, Desmodium cephalotes, D. gageticum, Pongamia glabra, Ricimus communis and Saraca indica* for the agglutination reactions with eleven different isolates of *P. aeruginosa.* The results indicate the possibility of differentiating the *P. aeruginosa* organisms by plant seed extracts.
- **Young-Mi Kim et al., (2004)[92]** determined the fungicidal activities of *Cassia tora* extracts and their active principles against *Botrytis cineria*, *Erysiphe graminis*, *Phytophthora infestans*, *Puccinia recondita*, *Pyricularia grisea*, and *Rhizoctonia solani* with synthetic

fungicides and three commercially available anthraquinones. The results demonstrate the fungicidal actions of emodin, physcion, and rhein from *C. tora*.

- **Haritha and Uma Maheswari (2007)**[93] evaluated the antimicrobial activity of *Cassia tora* seeds in order to assess the feasibility of utilizing the weed seeds as a rich food source of protein. Anti bacterial activity was not observed in *Cassia tora* raw seed extract or in extracts from different processing methods at the concentrations of 5%, 7.5%, and 10% against *Staphylococcus aureus, Proteus vulgaris and Escherichia coli.*
- **Roopashree *et al.*, (2008)**[94] studied the antibacterial activity of *C. tora*, *C. officinalis* and *M. charantia* against both gram positive and gram negative organisms *S. aureus*, *B. subtilis P. aeruginosa* and *E. coli* by cup plate method. Aqueous extracts of seeds of *Cassia tora* and *Momordica charantia* and flowers of *Calendula officinalis* exhibited better antibacterial activity as compared to their petroleum ether, methanolic and ethanolic extracts. Among the organisms tested *S. aureus* was more susceptible to the aqueous extracts of all the three herbs**.**
- **Chavan *et al.*, (2011)**[95] investigated the antibacterial activity of the ethanolic and aqueous extracts from the leaves of *Cassia tora* at the concentrations 0.15mg, 0.31mg ethanolic and aqueous extracts respectively using ciprofloxacin as standard reference. Both the extracts exhibited significant antibacterial activity.
- **Chavan *et al.*, (2011)**[96] investigated antibacterial activity of ethanolic and aqueous extracts from the stem of *Cassia tora* at the concentrations 0.10mg, 0.25 mg ethanolic and aqueous extracts respectively. Both the extracts exhibited significant antibacterial activity. Streptomycin used as standard reference.

Pharmacological Review

- **Hemadri K and Rao SS (1984)[97]** identified seventeen plant drugs used for jaundice by tribal people in Dandakaranya area such as *Andrographis paniculata, Benincasa hispida, Cajanus cajan, cassia tora, Cordia dichotoma, Curcuma augustifolia, Cucumis melo var. agrestis, Diospyros Montana, Ficus hispida, Legenaria siceraria, Lawsonia inermis, Oroxylum indicum, Oryza sativa, Phyllanthus asperulatus, Ricinus communis, Solanum nigrum, Tinospora cordifolia, Eclipta alba* and *Cuminum cyminum.*
- **Gow-Chin Yen and Da-Yon Chuang (2000)[98]** investigated antioxidant properties of water extracts from *Cassia tora* L. (WECT) prepared under different degrees of roasting. The water extracts of unroasted *C. tora* L. (WEUCT) showed 94% inhibition of peroxidation of linoleic acid at a dose of 0.2 mg/mL, which was higher than that of α-tocopherol (82%). Water extracts prepared from *C. tora* L. roasted at 175°C for 5 min and at 200°C for 5 min exhibited 83% and 82%, respectively, inhibition of linoleic acid peroxidation.
- **Patil *et al*., (2004)[99]** studied hypolipidemic activity of ethanolic extract of seeds of *Cassia tora* L. and its fractions on triton induced hyperlipidemic profile. Ethanolic extract and its ether soluble and water soluble fraction decreased serum level of total cholesterol by 42.07, 40.77 and 71.25%, respectively. On the other hand, ethanolic extract, ether soluble fraction and water soluble fraction increased the serum HDL-cholesterol level by 6.72, 17.20 and 19.18%, respectively. Ethanolic extract, ether fraction and water fraction decreased triglyceride level by 26.84, 35.74 and 38.46%, respectively. The reduction in LDL-cholesterol level by ethanolic extract, ether soluble fraction and water soluble fraction were 69.25, 72.06 and 76.12%, respectively.

- **Cherng JM *et al*., (2008)[100]** evaluated the immunostimulatory activities of four anthraquinones of *C. tora* (aloe-emodin, emodin, chrysophanol, and rhein) on human peripheral blood mononuclear cells (PBMC). The results showed that at non-cytotoxic concentrations, the tested anthraquinones were effective in stimulating the proliferation of resting human PBMC and/or secretion of IFN-γ. However, at the concentration of 10 μg/ml (35 μM), rhein significantly stimulated proliferation of resting human PBMC, but inhibited IFN-γ secretion (74.5% of control).
- **Nam J and Choi H (2008)[101]** studied the effects of *Cassia tora L.* seed butanol fraction (CATO) on postprandial glucose control and insulin secretion from the pancreas of the normal and Streptozotocin induced diabetic rats. The results indicated that constituents of *Cassia tora* L. seeds have beneficial effect on postprandial blood glucose control.
- **Deore SL *et al*., (2009)[102]** investigated anthelmintic activity of alcohol and aqueous extracts from the seeds of *Cassia tora* against *Pheretima posthuma* and *Ascardia galli*. Both the extracts exhibited significant anthelmintic activity at highest concentration of 100 mg/ml using piperazine citrate as standard reference.
- **Vetrivel Rajan *et al*., (2009)[103]** determined the hepatoprotective effects of *C.tora* against CCl_4 induced liver damage in albino rats. The results of this study reveal the remarkable increase of marker enzymes in induced rats and decreased level in *C. tora* treated rats.
- **Hyun *et al*., (2009)[104]** screened the bioactivity of seeds from raw and roasted *Cassia tora* via angiotensin converting enzyme (ACE) inhibitory assays. The results showed that both of the MeOH extracts from the raw and roasted *C. tora* exhibited significant inhibitory properties against ACE, demonstrating more than 50% inhibition at a concentration of 63.93 μg/mL.

- **Vijaya Lobo *et al*., (2011)[105]** investigated the antioxidant and antiradical activities of *Cassia tora* Linn. by DPPH, ABTS, nitric oxide, superoxide and hydroxyl radical scavenging assay, FRAP, reducing power and TAC. Results showed that aqueous extract possessed stronger antioxidant and antiradical activities than ethanol extract in nitric oxide, DPPH, ABTS radical scavenging assay with IC_{50} values 769.23±2.63, 1059.78±2.23, 490.08±2.45µg/ml in aqueous extract and 1000.51±2.21 µg/ml, 1700.03±1.18 and 943.23±1.38 µg/ml in ethanol extract respectively.
- **Yuvraj Gulia and Manjusha Choudhary (2011)[106]** evaluated the antiulcer activity of hydroalcoholic extract of *Cassia tora* leaves in albino rats using ethanol induced gastric ulcer model. The extract of leaves of *Cassia tora* showed dose dependent antiulcer activity with maximum activity at 500 mg/kg body weight. The effect at this dose was found to be comparable with that of reference standard, Omeprazole 20 mg/kg.
- **Neelima Janardan et al., (2011)[107]** evaluated cardiotonic activity of alcoholic and petroleum ether extract of *Cassia tora* Linn seeds by using isolated guinea pig heart perfusion technique and digoxin as a standard. The present results indicated that a significant increase in height of force of contraction with decrease in heart rate was observed as the dose of both the text extract increased however, alcoholic extract of *Cassia tora* produced little more positive inotropic effect then petroleum ether extract.
- **Bhaskar Chaurasia *et al*., (2011)[108]** screened for antidiabetic activity for the methanol extract and their ethyl acetate, n-butanol and dichloromethane fraction of seeds of *Cassia tora* in normal and alloxan induced diabetic albino rats at a dose 50, 100, and 200 mg/kg b. w. for fractions and 100 mg/kg b. w for their sub fraction of active extract. Among all fractions of methanol extract, the n-butanol fraction had more significantly reduced the blood glucose

level after single dose and prolonged treatment and nearly equal to standard glibenclamide after prolonged treatment. The napthopyrone glycoside was isolated from the n-butanol extract of the seed of *Cassia tora* as active constituent.

- **Thamhane adesh *et al*., (2012)[109]** investigated anti-asthmatic activity of the extract of *Cassia tora* leaves using different concentration of aqueous extract at 50,500, 1000, 1500 µg/ml in isolated Goat trachea chain preparations by using standard drugs histamine and acetyl choline respectively. The modified physiological salt solution containing hydro alcoholic extract of *Cassia tora* (1.5 mg/ml) significantly inhibited the contractile effect of histamine thus produces significant bronchodilation.

Miscellaneous

- **Rai MK and Upadhyay, S (1988)[110]** identified the organism *Phoma sorghina* responsible for causing fungal disease of medicinal plants such as severe leaf spot disease of *C. tora* in South.
- **Baveja SK *et al*., (1989)[111]** evaluated twenty-two natural gums and mucilages for their ability to sustain the release of freely soluble drug propranolol hydrochloride from tablets. Mucilages from fruits of *Cordia oblique,* seeds of *Cassia tora,* and seed flour of *Phaseolus mungo,* corms and rhizomes of *Colocasia esculenta*, and *Commiphora mukul* and *Sterculila urens* gums were found to have sustaining ability comparable to the widely used synthetic cellulose ethers.
- **Vinayak R Tripathi et al., (2011)[112]** reported the protease inhibitory activity of *Cassia tora* (Seeds) against trypsin, *Aspergillus flavus* and *Bacillus* sp. proteases. *Cassia tora* seed extract has strong protease inhibitory activity against trypsin, *Aspergillus flavus* and *Bacillus* sp. proteases. The inhibitor in *Cassia tora* may attenuate microbial proteases and also might be used as phytoprotecting agent.

3. AIM AND OBJECTIVE OF THE STUDY

Psoriasis, a common chronic inflammatory dermatosis, is an immuno disorder associated with over expression of proinflammatory cytokines. Scientific information supports the view that an insufficient antioxidant system contributes to the pathogenesis of psoriasis. Psoriatic skin is also characterized by an advanced state of lipid peroxidation. Thus, it has been suggested that the antioxidant treatment could be part of a more specific and effective therapy for the management of this skin disease.

Flavonoids, a natural polyphenols, recognized as potent antioxidants are multifunctional molecules that can act as anti-inflammatory and antiproliferative agents through the modulation of multiple signaling pathways. This characteristic could be advantageous for the treatment of multi-causal diseases, such as psoriasis. The therapeutic potential of flavonoids and the necessity for scientific validation in popular medicine have prompted increased interest in the field.

In the light of the above facts, the main objective of the present study has been directed

- To establish pharmacognostical standards and phytochemical investigation for anti-psoriatic plants *Givotia rottleriformis* bark and *Cassia tora* leaves.
- Isolation and characterization of the flavonoids from the plants
- Evaluations of *in-vitro* antioxidant potency, *in-vitro* and *in-vivo* anti-psoriatic activity for the plants extract and isolated flavonoids.
- Bioactive flavonoids are promoted to formulation and evaluation for their product performance.
- The developed formulation is evaluated for anti-psoriatic and cytokine inhibition assay.

4. PLAN OF WORK

- Collection of plant material
- Plant identification and authentication

PHARMACOGNOSTICAL STUDIES

- Microscopical characters
- Histochemical studies
- Physicochemical analysis
- Fluorescence analysis
- Inorganic Mineral analysis

PHYTOCHEMICAL STUDIES

- Preparation of Extract
- Qualitative phytochemical analysis
- Quantitative phytochemical analysis
- HPTLC profile
- Isolation of active constituent by column chromatographic technique
- Characterization of the compound by analytical techniques
- Quantification of compounds with known marker compound using HPLC and HPTLC analysis

***IN-VITRO* ANTIOXIDANT ACTIVITY**

- Hydroxyl radical scavenging method
- DPPH radical scavenging method
- Nitric Oxide radical scavenging method

IN-VITRO ANTI-PSORIATIC ACTIVITY

HaCaT cell line inhibition assay

PHARMACOLOGICAL STUDIES

- Toxicity study
- *In-vivo* Anti-psoriatic activity - Perry scientific mouse tail model

PRODUCT DEVELOPMENT OF BIOFLAVONOIDS

- Formulation of tablets comprising of bioflavonoids
- Evaluation of formulated tablets
- Bio activity evaluation of the formulation for
    - Anti-psoriatic activity - Rat ultraviolet ray B photodermatitis model
    - Cytokine inhibition Assay

5. PHARMACOGNOSTICAL STUDIES

5.1. INTRODUCTION

The study of plant drugs from the pharmacognostical stand point would include the study of the habitat, general characters of the plant from which the drug is derived, its place in the botanical system, the organ or the organs of the plant used, their gross, minute structures in the whole and in the powdered conditions and the chemistry of the constituents especially of those which may be used in therapeutics.

The macroscopic and microscopic description of a medicinal plant is the first step towards establishing the identity and the degree of purity of such materials. This should be carried out before any tests are undertaken. Lack of proper standards of medicinal plants may result in the usage of improper drugs which in turn will cause damage not only to the individual using it, but also to respect gained by the well known ancient system of medicine and the entire work on the plant becomes invalid. Thus, in recent years there has been an emphasis in pharmacognostical standardization of medicinal plants of therapeutic potential. The present work therefore attempts to report various necessary pharmacognostical standards of the bark of *Givotia rottleriformis* and *Cassia tora* leaves.

5.2. MATERIALS AND METHODS

5.2.1. Collection of Plant Materials

The Plant specimen *Givotia rottleriformis* bark was collected from the forest of Athoor, Tamil Nadu and the plant *Cassia tora* leaves was collected from Padappai, Chennai, during the month of January 2011. It was identified and authenticated by the botanist Dr. P. Jayaraman, Director, Plant Anatomy Research Center, (PARC) Tambaram, Chennai. A voucher specimen No. PARC/2011/2140 for *Givotia rottleriformis* bark and PARC/2011/2141 for *Cassia tora* leaves has been deposited for further references.

. **5.2.2. Morphological studies**

The bark of *Givotia rottleriformis* and the leaves of *Cassia tora* were studied individually for its morphological characters such as shape, colour, dimension, upright or creeping, smooth or ridged and organoleptic characters like color, odour, taste etc.

5.2.3. Microscopical studies

Among the selected plants under study, microscopic characters of *Cassia tora* leaves was reported by Chandan Das *et al*[77] and hence, only *Givotia rottleriformis* bark was subjected to microscopic evaluation.

Preparation of histological specimen

The bark of *Givotia rottleriformis* was cut and immediately immersed in fixative fluid FAA (Formalin: Acetic acid: 70% ethyl alcohol in the ratio of 5:5:90). After 24 hr of fixing, the specimens were dehydrated with graded series of tertiary butyl alcohol and ethyl alcohol mixtures as per the schedule given by Sass. Infiltration of the specimen was carried by gradual addition of paraffin wax (melting point 58-60^{o}C) until tertiary butyl alcohol solution attained super saturation. The specimen was transferred to pure paraffin wax and casted into paraffin blocks.

Sectioning[113]

The paraffin embedded specimen was sectioned with the help of rotary microtome. The thickness of the section was 10-12 µm. Section was stained with toludine blue (0.25%, pH of 4.7). Since, toludine blue is a polychromatic stain, different colours of the cells were obtained depending upon the chemical nature of the cells. The dye rendered pink color to the cellulose walls, blue to the lignified cells, dark green to suberin, violet to the mucilage, blue to the protein.

Photomicrographs[114,115]

Microscopic descriptions of tissues were supplemented with photographs wherever necessary. Photographs of different magnifications were taken with Nikon lab photo 2 microscopic units. For normal observations bright field was used. The study of crystals, starch grains and lignified cells were done by using polarized light. Since, these structures have birefringent property; under polarized light they appear bright against dark background. Magnifications of the figures were indicated by the scale bars. Descriptive terms of the anatomical features are as given in the standard anatomy books.

5.2.4. Powder Microscopic Observations[116]

Well shade dried, chopped crude leaves were powdered, sieved and used for powder organoleptic and microscopic analysis. The powder was stained with safranin and observed through microscope. Starch grains were observed by staining with iodine in potassium iodide (IKI) and calcium oxalate crystals of the powder was observed under the polarized microscope.

5.2.5. Histochemical Analysis[117]

The sections embedded in paraffin were used for histochemical studies. The sections were deparaffinised and hydrated before staining them for the tests. The chemicals localized in bark were studied using the standard procedure.

5.2.6. Physico-Chemical Constants[118,119]

The procedures recommended in Indian Pharmacopoeia and WHO guidelines were followed to calculate the physico-chemical constants.

5.2.6.1. Determination of Ash values

Ash values are helpful in determining the quality and purity of a crude drug especially in the powdered form. The residue remaining after incineration of the crude drug is designated as ash. It usually represents the inorganic salts naturally occurring in the drug and adhering to it, but it may also include inorganic matter fielded for the purpose of adulteration. It varies within definite limits according to the soils. There is a considerable difference within narrow limits in the case of the same individual drug. Hence an ash value determination furnishes a basis of judging the identity and cleanliness of a drug and gives information relative to its adulteration with inorganic matter.

The total ash of a crude drug reflects the precaution taken during its preparation. The acid insoluble ash is a part of the total ash that is insoluble in dilute hydrochloric acid. A higher limit of acid-insoluble ash imposed, especially in cases where silica may be present or when the calcium oxalate content of the drug is higher. Ash standards have been established for a number of official drugs. Usually these standards set a maximum limit on the total ash or on the acid insoluble ash permitted. Dried coarsely powdered bark of *Givotia rottleriformis* and *Cassia tora* leaves were subjected to the following analysis.

Total ash value

The total ash was determined by incinerating 2-3gms of accurately weighed air dried coarsely powdered drug in a tarred silica crucible which was previously ignited and cooled before weighing, at a temperature not exceeding 450^{o}C. The ignition was repeated and the percentage of ash with reference to air-dried drug was calculated.

Water soluble ash

The total ash was boiled for 5 min with 25 ml of water. The residue was washed with hot water, ignited for 15 min at a temperature not exceeding 450^{o}C, cooled and weighed. This weight was subtracted from the weight of total ash and the difference in weight represents the water soluble ash. The percentage of water soluble ash was calculated with reference to air-dried drug.

Acid insoluble ash

The total ash obtained was boiled with 25 ml of 2N dilute hydrochloric acid for 5 min and filtered through an ashless filter paper. The residue was washed with hot water, ignited, cooled in a dessiccator and weighed. The percentage of acid insoluble ash was calculated with reference to air dried drug.

Sulphated ash

The sulphated ash was determined by incinerating 1 gm of accurately weighed air dried coarsely powdered drug in a tarred silica crucible which was previously ignited and cooled before weighing at a temperature not exceeding 450^{o}C. The residue was moistened with 1 ml of concentrated sulphuric acid, ignited at 800±25^{o}C until all black particles have disappeared. It was then cooled; again sulphuric acid was added and ignited. It was cooled and the percentage of sulphated ash was calculated with reference to air dried drug.

5.2.6.2. Determination of Extractive values

Extractive values of crude drugs are used to determine the amount of active constituents extracted with solvents from a given amount of medicinal plant material. It is employed for materials for which no suitable chemical or biological assay exists. These values are the indicative of approximate measures of their chemical constituents and the

nature of the constants present in crude drugs. The coarsely powdered *Givotia rottleriformis* bark and *Cassia tora* leaves were subjected to the determination of extractive values.

Procedure

About 5 grams of the powder was macerated with 100 ml of petroleum ether, chloroform, alcohol and distilled water of the specified strength respectively in a closed flask for 24 hours, shaking frequently for 6 hours and allowed to stand for 18 hours. It was filtered rapidly; and 25 ml of the filtrate was evaporated to dryness at 105^{o}C and weighed. The percentage of alcohol soluble extractive was calculated with reference to the dried drug.

5.2.6.3. Loss on Drying

Loss on drying is the loss of mass expressed as percent w/w. About 2 gm of drug was weighed and transferred to a dry stoppered weighing bottle. The weight of the bottle and the drug was taken accurately. After removing the stopper, the bottle containing drug was placed in an oven for 1 hour at 120^{o}C. After 1 hr, the bottle was removed and cooled in a desiccator and weighed by replacing the stopper which was continued until difference between two successive weighing is not more than 0.25% of constant weight.

5.2.6.4. Determination of crude fibre content

About 2 gms of the drug was accurately weighed and extracted with ether. Then 200 ml of 1.25% sulphuric acid was added and boiled for 30 min under reflux. It was filtered and washed with boiling water until free of acid. The entire residue was rinsed back into flask with 200 ml of boiling 1.25% NaOH solution and again boiled under reflux for 30 min. The liquid was quickly filtered and the residue was washed with boiling water until neutral, dried at 110^{o}C to constant weight. It was then ignited to 30 min at 600^{o}C, cooled and weighed. The percentage of crude fibre content was calculated with reference to the air dried drug.

5.2.7. Fluorescence Analysis

The fluorescence analyses of the drug powder as well as extracts were carried out by using the method of Chase and Pratt**.**[120] The powder and extracts were treated with different chemical reagents with color changes under ordinary day light and UV light by the standard method.

5.2.8. Inorganic Mineral Analysis

Chemical analysis of higher plants in general has revealed the presence of 40 or more elements. Plant physiologists have proved that 18 of these elements are indispensable to plants and human beings require 28 or more elements. Of these elements, carbon, hydrogen, oxygen and nitrogen are present in larger quantities than others. Sulphur and phosphorous are present in protoplasm and has constituents of proteins or other important organic compounds. A study of inorganic constituents of plants is of interest to research workers in several fields, such as nutrition medicine and others because plants constitute direct or indirect sources of many of the elements, which are essential to animals including man.[121] Therefore; the plant material was subjected to inorganic mineral analysis.

Preparation of sample solution for inorganic mineral analysis

The plant material (10gms) was digested with 10 ml of nitric acid and left over night. It was then heated on a hot plate until the reddish brown colour ceased and cooled. A small volume of perchloric acid was added and transferred to a 50 ml standard flask and made up to volume with double distilled water.[122]

Determination of Sodium and Potassium by flame photometry

Flame photometry is based on the measurement of intensity of light emitted when a metal is introduced into the flame. The material when introduced into the flame is converted

into gaseous state. The gaseous molecules are progressively dissociated to give free neutral atoms or radicals, which are excited by thermal energy of the flame. The excited atoms which are unstable quickly emit photons and return to the lower energy state, evenly reaching the unexcited state. The measurement of the emitted photons forms the basis of flame photometry. A plot of emission versus concentration in micrograms is prepared. The given unknown solution was diluted suitably and aspirated into the instrument. From the emission intensity produced, concentration of metal is determined by the interpolation of calibration curve. The instrument used was Systronics Flame Photometer.[118]

Procedure

A series of standard solutions containing the element to be determined in increasing concentrations within the concentration range recommended for the instrument were prepared. Nitric acid and perchloric acid used for the preparation of sample solution of the plant material were also added in the same concentration to the standard solution. The appropriate filter was chosen, water was sprayed into the flame and the galvanometer reading was adjusted to zero. The most concentrated solution was then sprayed into the flame and the galvanometer reading was recorded. Again, water was sprayed till the galvanometer reading was zero. Then the standard solution was sprayed into the flame and the procedure was repeated three times for each concentration. A calibration curve was prepared by plotting the mean of three readings of each standard against the concentration. The sample solution prepared as above was then aspirated into the flame three times, the galvanometer reading was recorded and the apparatus was washed thoroughly with water after each aspiration. Using the mean of three readings, the concentration of the element being examined was determined from the calibration curve. To confirm the concentration thus obtained, the operation was

repeated with the standard solution of the same concentration as that of the solution being examined.

Determination of mineral composition by atomic absorption spectroscopy

This technique is based on the fact that when atoms, ions or ion complexes of an element in the ground state are atomized in a flame, the absorbed light has the characteristic wavelength of that element. If the absorption process takes place under reproducible conditions the absorption is proportional to the number of absorbing atoms. The concentration of the metal is determined by interpolation of the calibration curve. The instrument used was Perkin Elmer Atomic Absorption Spectrophotometer.

Procedure

Three standard solutions of the element to be determined covering the concentration range recommended for the instrument was prepared. Nitric acid and perchloric acid used in the preparation of the substance being examined was also added to the standard solutions in the same concentration. After calibration of the instrument, each standard solution was introduced into the flame three times and the steady reading was recorded. The apparatus was thoroughly washed after each introduction. A calibration curve was prepared by plotting the mean of each group of three readings against concentration. The plant extract prepared above was then introduced into the flame and the reading was recorded. The sequence was then repeated twice. Using the mean of the three readings, the concentration of the element was determined from the calibration curve. The process was repeated for the determination of other elements using different lamps.

5.3. RESULTS

5.3.1. Macroscopy oc *Givotia rottleriformis* Bark

- The surface of the bark is rough with dense, irregular wide vertical fissures. The outer surface is pale brown or grey. The inner surface is creamy. (**Fig. 7**).
- The bark is brown colour and has no distinct odour. It is fibrous in texture.

5.3.2. Microscopy

Transverse section (TS), Tangential longitudinal section (TLS) and Radial longitudinal section (RLS) of the bark *Givotia rottleriformis* was studied.

Transverse section of Bark

The bark is very thick and extremely hard and brittle. In cross sectional view, the bark exhibits outer periderm and inner secondary phloem.

Periderm: The periderm includes two or more narrow tangential bands of phloem cells; the phellem is undulate forming lens shaped gaps in between the inner and outer periderm layers. These gaps have non-peridermous tissue, which is mostly cortical tissue and sclerides. This type of periderm which comprises periderm layers with lens shaped non periderm tissue located in between the periderm layers is called rhitidome. (**Fig. 8.1; 8.4**)

Secondary Phloem: It is the major part of the bark and it extends from the inner border of the rhytidome upto the cambial zone. The secondary phloem can be divided into two zones, namely outer wider collapsed phloem and inner narrow non collapsed phloem. (**Fig. 8.2; 8.3**) The collapsed phloem includes highly dilated phloem rays, scattered irregular masses of sclerenchyma cells and dark thick tangential streaks of crushed sieve elements. (**Fig. 8.5-8.7**)

Non Collapsed Phloem: The non collapsed (intact) phloem zone is much narrow and it is 700µm thick. It includes wide rectangular sieve tubes which occur in radial parallel rows. The

companion cells are prominent and they are located at lateral part of the sieve tube members. (**Fig. 8.3; 8.7**) The phloem parenchyma cells are mixed within sieve tubes; the parenchyma cells are smaller than the sieve elements. The phloem rays are wavy and run straight penetrating the phloem tissue.

Tangential Longitudinal View of Bark

In TLS of the bark, the features such as phloem rays height, thickness and frequency can be studied. The rays are exclusively uniseriate, narrow and hetero cellular with terminal upright cells and middle procumbent cells. The upright cells are much elongated and conical in shape. The middle procumbent cells are rectangular and vertically elongated. The range is 250-650 µm in height and 30 µm in thick. Ray frequency is 13-15/mm.

The sieve tubes are long and thick walled straight. They have very oblique sieve plate which is nodulated due to the deposition of cellulose on the pores of the sieve plate. (**Fig. 9.1-9.3**) Phloem parenchyma cells are vertically rectangular and they occur in vertical strands.

Crystal distribution

Two types of calcium oxalate crystals are sparsely seen in the bark. The crystals located in the phloem rays are druses. (**Fig. 9.4**) The crystals associated with sclerenchyma elements of the collapsed phloem are prismatic type. (**Fig. 9.5**)

Radial Longitudinal View of Bark

In RLS, the phloem rays appear in horizontal lines. (**Fig. 10.1**) The rays are heterocellular and consist of upper rows of vertically elongated upright cells and middle part of procumbent cells. These cells are either square shape or horizontal elongated. (**Fig. 10.2**)

5.3.3. Powder Microscopy

The following elements were observed in the powder.

Sclereids: Brachy sclereids of different shape and size were seen in the powder. (**Fig. 11.1, 11.2, 11.3**) The brachy sclereids are generally isodiametric in shape, but they are elongated, rectangular and cubical. The sclereids have very thick secondary lignified walls with long narrow canal like pits and wide cell lumen. (**Fig 11.2**) The sclereids also have thin walls with short pits. (**Fig.11.3**) Prismatic calcium oxalate crystals are sometimes seen with in the sclereids. This is unique features for the sclereids.

Fibres: Short, narrow thick walled libri form fibres are common in powder. (**Fig. 11.4**) They have thick walls and narrow lumen. They are 250 - 370 µm long. The fibres are also seen in thick bundles (**Fig. 11.5**) or in small broken pieces. (**Fig. 11.6**)

Parenchyma cells: Rectangular thin walled parenchyma cells are seen mixed with other elements. The parenchyma cells have some storage products. (**Fig. 11.4**)

5.3.4. Histochemical Colour Reactions

Localisation of flavonoids with NaOH solution

Some of the cortical parenchyma cells possess yellow cell inclusions which indicate presence of flavonoids (**Fig. 12.1(a), 12.1(b)).** The flavonoids containing cells are abundant and cells diffuse in distribution. In longitudinal section, flavonoids are found located mostly in axial parenchyma cells of the phloem. (**Fig. 12.1(c)**)

Localisation of alkaloids by Dragendroff's reagent

Some of the cortical parenchyma cells possess brownish red cell inclusions which indicate presence of alkaloids. (**Fig. 12.2(a), 12.2(b))** The alkaloids containing cells are diffuse in distribution. Alkaloids occupy entire lumen of the cortical parenchyma. In longitudinal section of bark, alkaloids are found to occupy some of the cells of phloem rays. (**Fig. 12.2(c))**

5.3.5. Physico-Chemical Constants

The observed values for the physico-chemical constants were given in Table 1.

Table 1: Physico-chemical constants of *Givotia rottleriformis* and *Cassia tora*

S. No	Parameters	Results (%w/w)	
		G. rottleriformis	***C. tora***
1.	**Ash values**		
	Total ash	9.87	18.46
	Water soluble ash	2.43	5.80
	Acid insoluble ash	1.82	1.12
	Sulphated ash	15.72	20.47
2.	**Extractive values**		
	Petroleum ether extractives	1.26	3.50
	Chloroform extractives	2.70	2.18
	Alcohol soluble extractives	10.25	12.20
	Water soluble extractives	8.20	7.50
3.	**Loss on drying**	3.84	4.32
4.	**Crude fibre content**	12.46	17.60

5.3.6. Fluorescence Analysis

The fluorescence analysis of powder with various reagents and extracts are given in the Table 2a and 2b.

Table 2a: Fluorescence analysis of the powder of *G. rottleriformis* bark and *C. tora* leaves

S.No	Reagents	*G. rottleriformis*		*C. tora*	
		Day light	**UV (254 nm)**	**Day light**	**UV (254 nm)**
1	Powdered drug	Reddish brown	Light brown	Dull green	Greenish yellow
2	Powder + 1 N HCl	Brown	Black	Green	Yellow
3	Powder + 1 N H2SO4	Brown	Black	Dull green	Yellow
4	Powder + 1 N NaOH	Reddish Brown	Black	Light green	Greenish yellow
5	Powder + 50% HCl	Orange	Dark brown	Green	Light yellow
6	Powder + 50% H2SO4	Brown	Black	Green	Yellow
7	Powder +50% HNO3	Reddish brown	Dark brown	Brown	Black
8	Powder + Methanol	Reddish brown	Greenish yellow	Dark green	Reddish yellow
9	Powder + 5 % KOH	Dark brown	Black	Dark green	Yellowish green
10	Powder + Con HNO3	Reddish orange	Black	Brown	Green

Table 2b: Fluorescence analysis of various extracts of *G. rottleriformis* and *C. tora*

S.No	Reagents	*G. rottleriformis*		*C. tora*	
		Day light	**UV**	**Day light**	**UV**
1	Petroleum ether	Light yellow	Yellow	Green	Green
2	Chloroform	Yellowish brown	Brown	Yellowish green	green
3	Ethyl acetate	Light brown	Yellow	Yellowish green	Yellow
4	Methanol	Reddish brown	Light yellow	Dark green	Reddish yellow

5.3.7. Inorganic Mineral Analysis

The amount of sodium and potassium present in 1 gm of plant material was estimated by flame photometry. The amount of other metals present was estimated by Atomic absorption spectroscopy and the results were given in Table 3.

Table 3: Inorganic mineral analysis of *G. rottleriformis* bark and *C. tora* leaves

S. No	Parameters	Concentration/ g sample	
		G. rottleriformis	***C. tora***
1	Cadmium	0.0002 µg	0.001 µg
2	Calcium	0.452 µg	1.094 µg
3	Chromium	0.0011 µg	0.002 µg
4	Copper	0.804 µg	0.530 µg
5	Iron	2.54 µg	1.45 µg
6	Lead	0.0026 µg	0.001 µg
8	Magnesium	0.145 µg	0.225 µg
9	Nickel	0.0006 µg	0.0004 µg
10	Zinc	0.0043 mg	0.0061 mg
11	Phosphorus	Nil	Nil
12	Potassium	1.0158 mg	Nil
13	Sodium	0.0231 mg	Nil

Figure 7: Macroscopy of *Givotia rottleriformis* Bark

Fig. 8: Transverse Section of the bark of *Givotia rottleriformis*

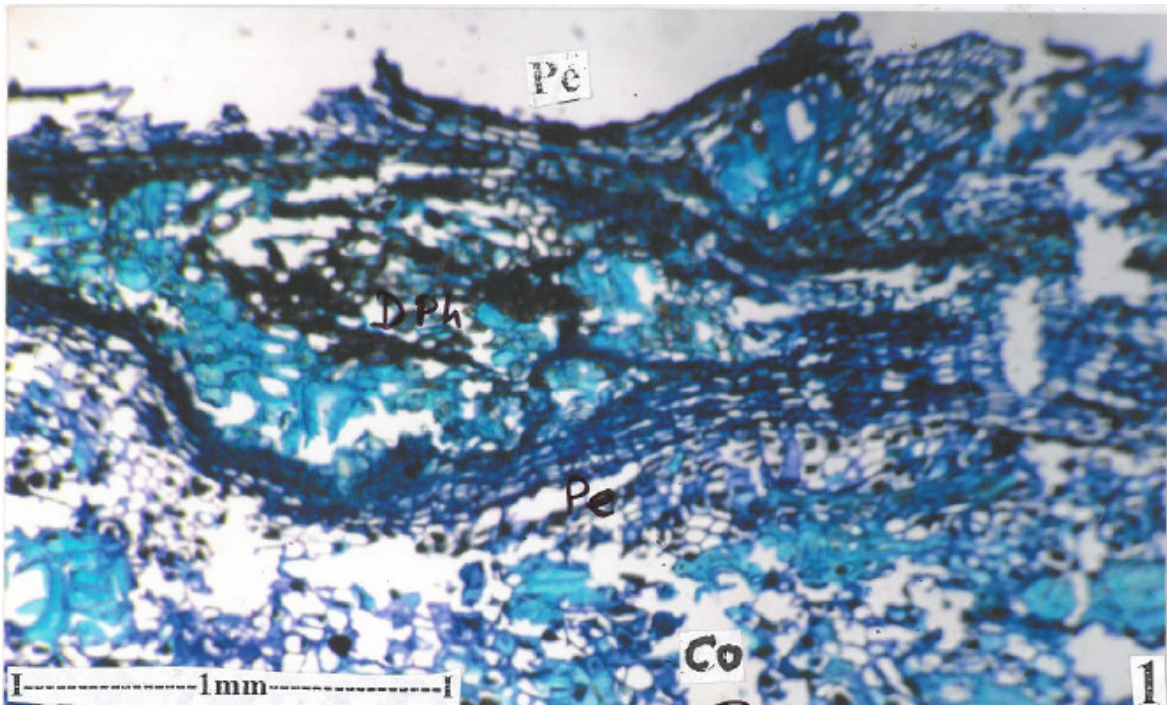


Figure 8.1: TS of Bark: Periderm (Rhytidome) – Portion enlarged

(Co: Cortex; DPh: Dead Phloem tissue; Pe: Periderm)

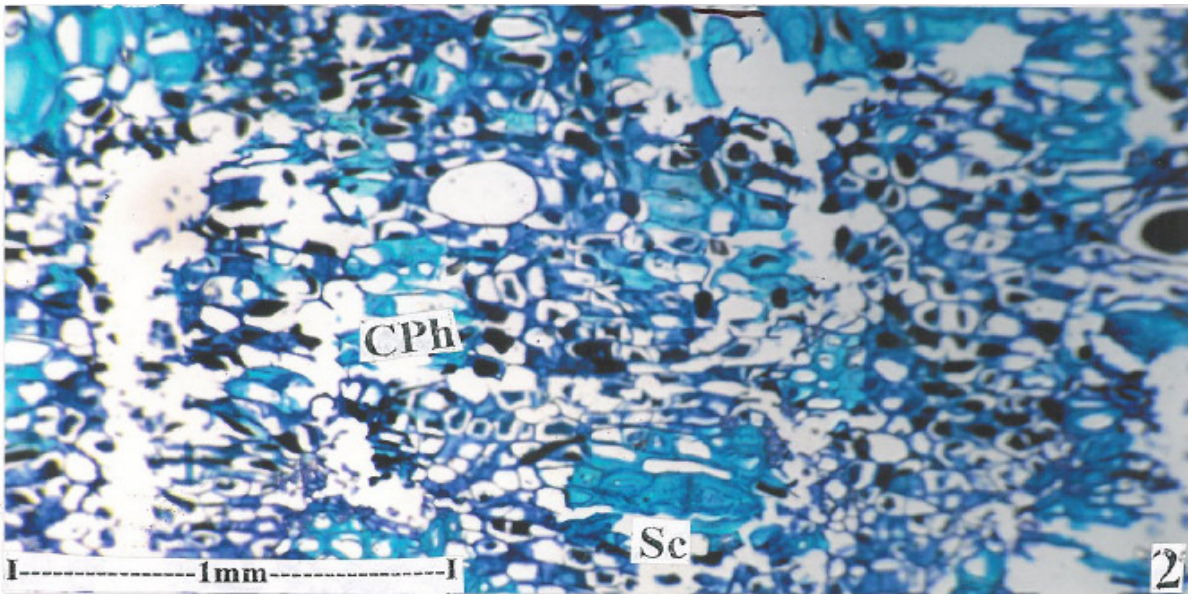


Figure 8.2: TS of Bark: Collapsed phloem with scattered Sclerenchyma cells

(CPh: Collapsed phloem; Sc: Sclereids)

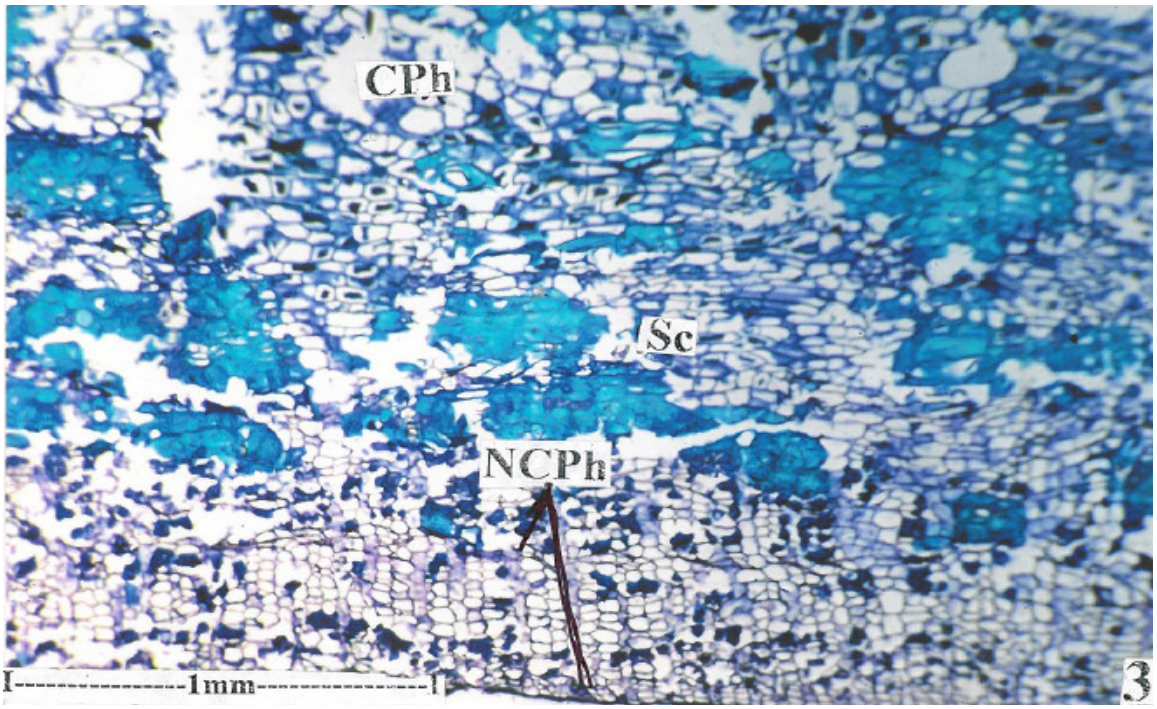


Figure 8.3: TS of Bark: Outer Collapsed Phloem and inner Non Collapsed Phloem

(CPh: Collapsed phloem; NCPh: Non-Collapsed Phloem; Sc: Sclereids)

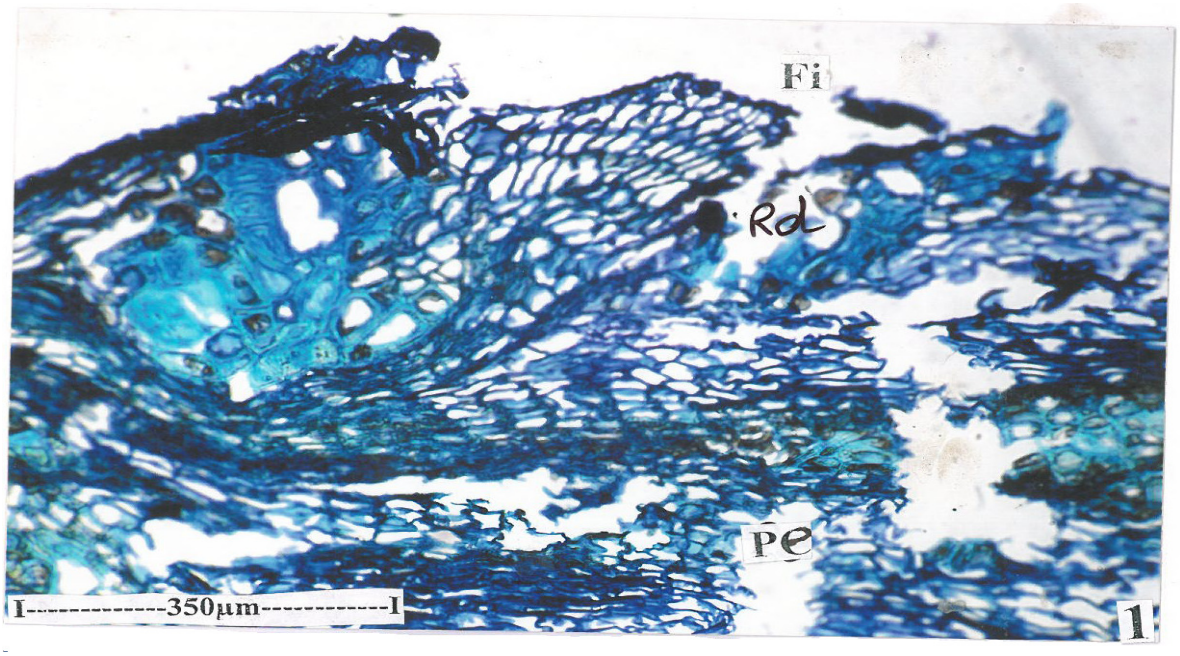


Figure 8.4: TS of Bark: Rhytidome Portion - enlarged

(Fi: Fissure; Pe: Periderm; Rd: Rhytidome)

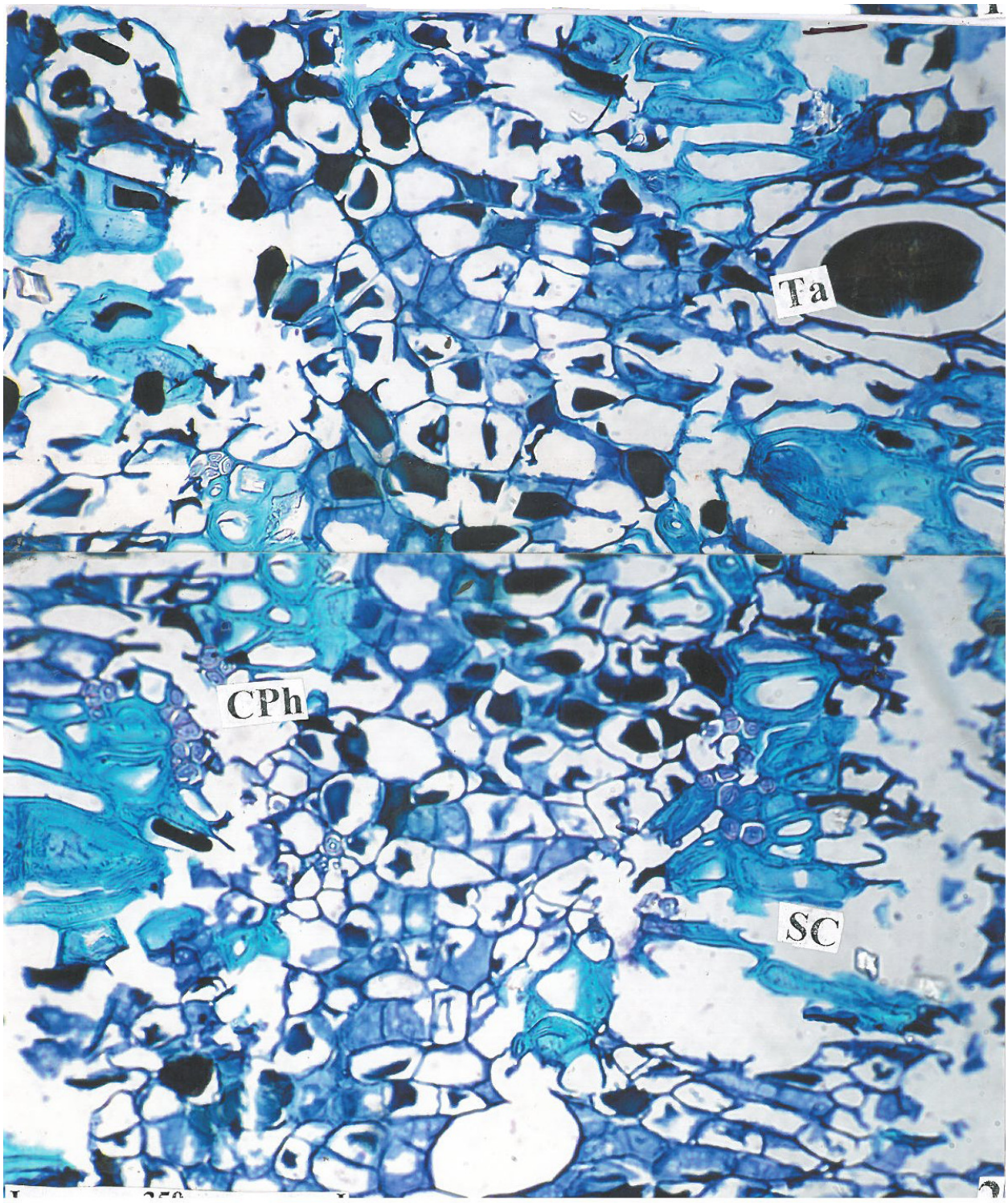


Figure 8.5: TS of Bark: Collapsed phloem tissue

(CPh: Collapsed phloem; Sc: Sclereids; Ta: Tannin content)

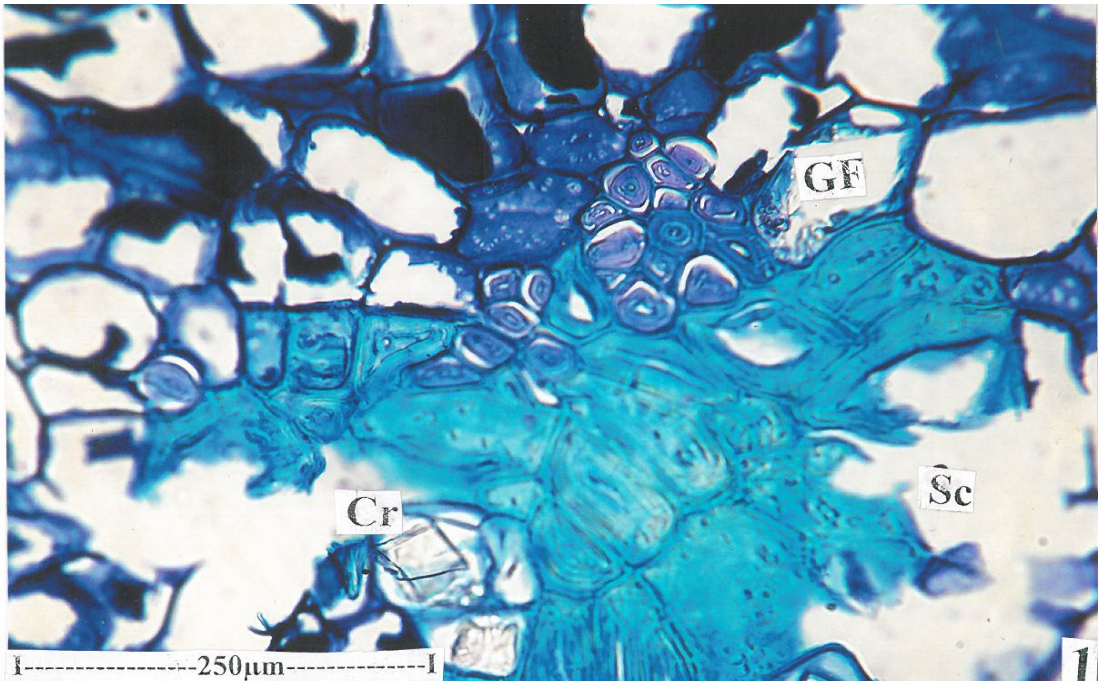


Figure 8.6: Structure of Phloem: Sclerenchyma elements of the Collapsed Phloem

(Cr: Crystals; GF: Gelatinous Fibre; Sc: Sclereids)

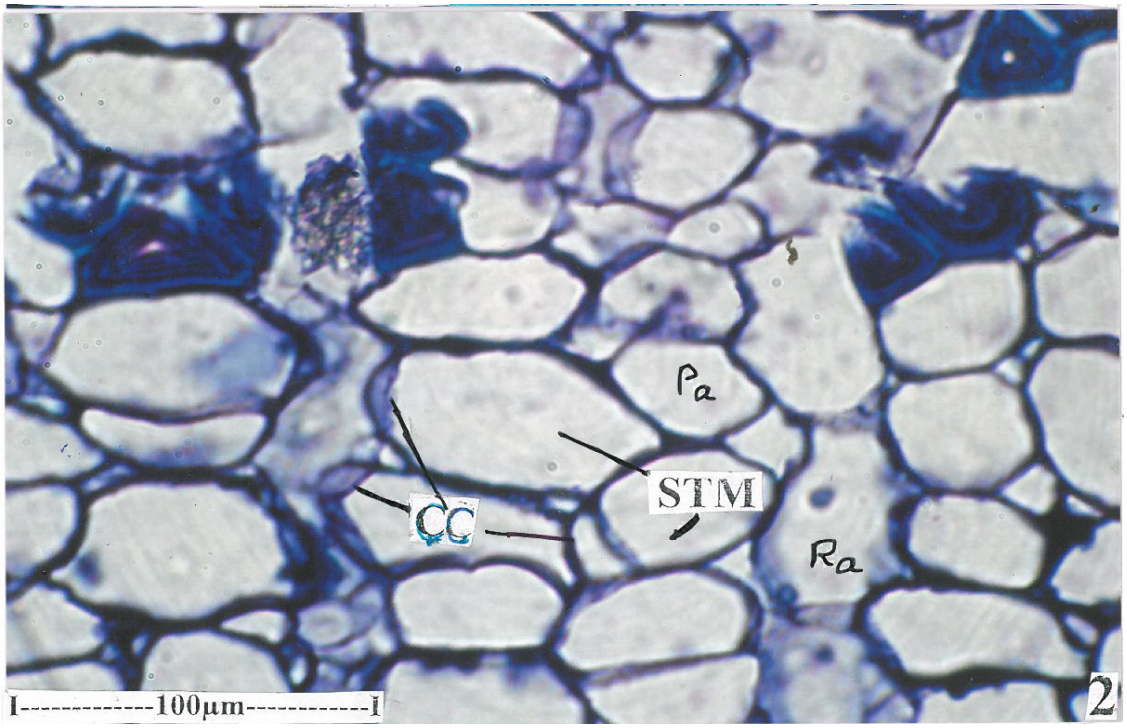


Figure 8.7: Structure of Phloem: Sieve elements of the Non-Collapsed Phloem

(CC: Companion Cells; Pa: Parenchyma cells; Ra: Ray cells; STM: Sieve Tube Member)

Fig. 9: Tangential Longitudinal Section of the bark of *Givotia rottleriformis*

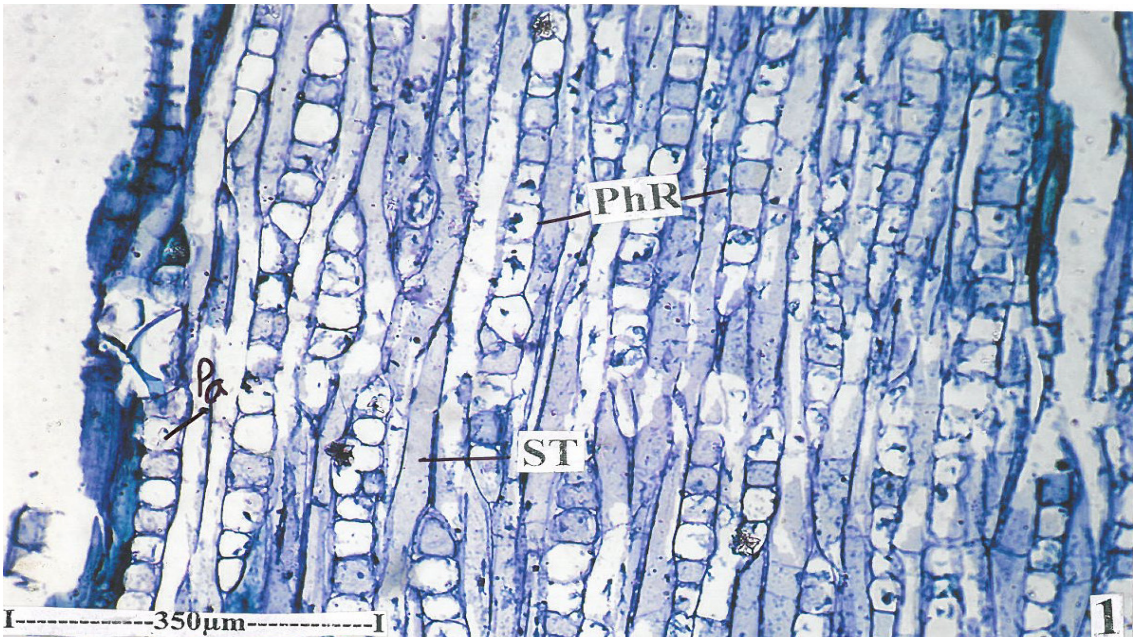


Figure 9.1: TLS of the Phloem

(Pa: Parenchyma cells; PhR: Phloem Ray; ST: Sieve Tube)

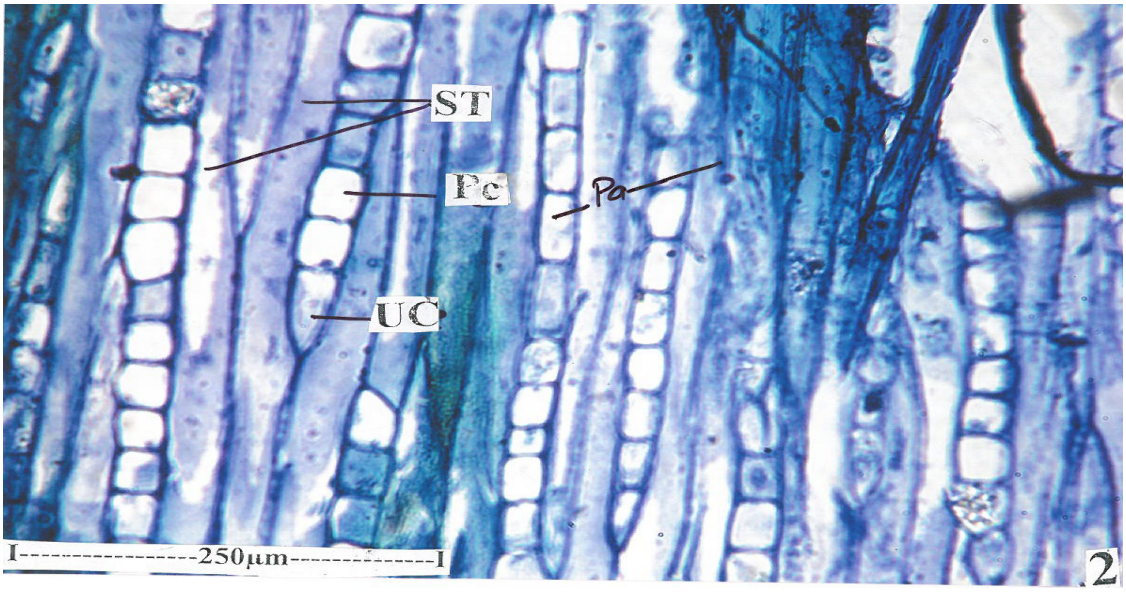


Figure 9.2: TLS of the Phloem

(Pa: Parenchyma cells; PC: Procumbent Cell; ST: Sieve Tube; UC:Upright Cells)

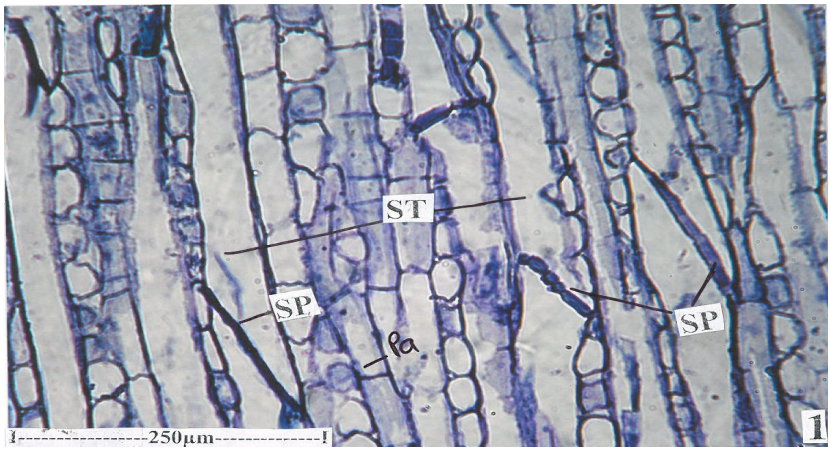


Figure 9.3: TLS of the Phloem: Sieve elements showing Sieve plates
(Pa: Parenchyma; SP: Sieve Plate; ST: Sieve Tube)

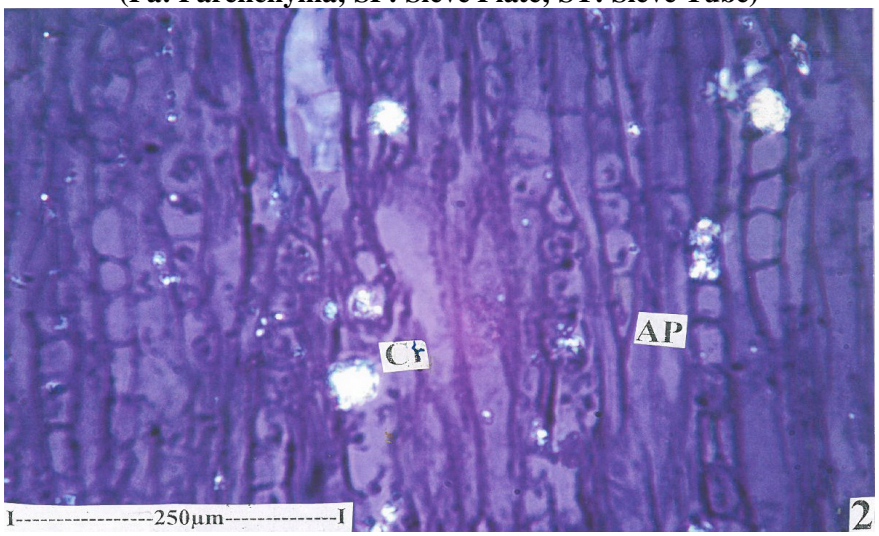


Figure 9.4: Crystal Distribution: Druses in the Phloem ray cells
(Cr: Crystals; AP: Axial Parenchyma)

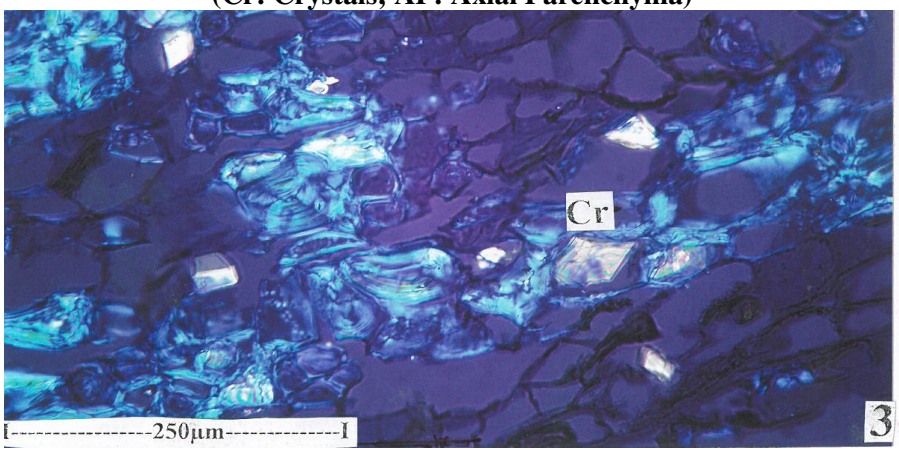


Figure 9.5: Crystal Distribution: Prismatic Crystals in the Phloem Sclerenchyma cells
(Cr: Crystals)

Figure 10: Radial Longitudinal Section (RLS) of the bark of *Givotia rottleriformis*

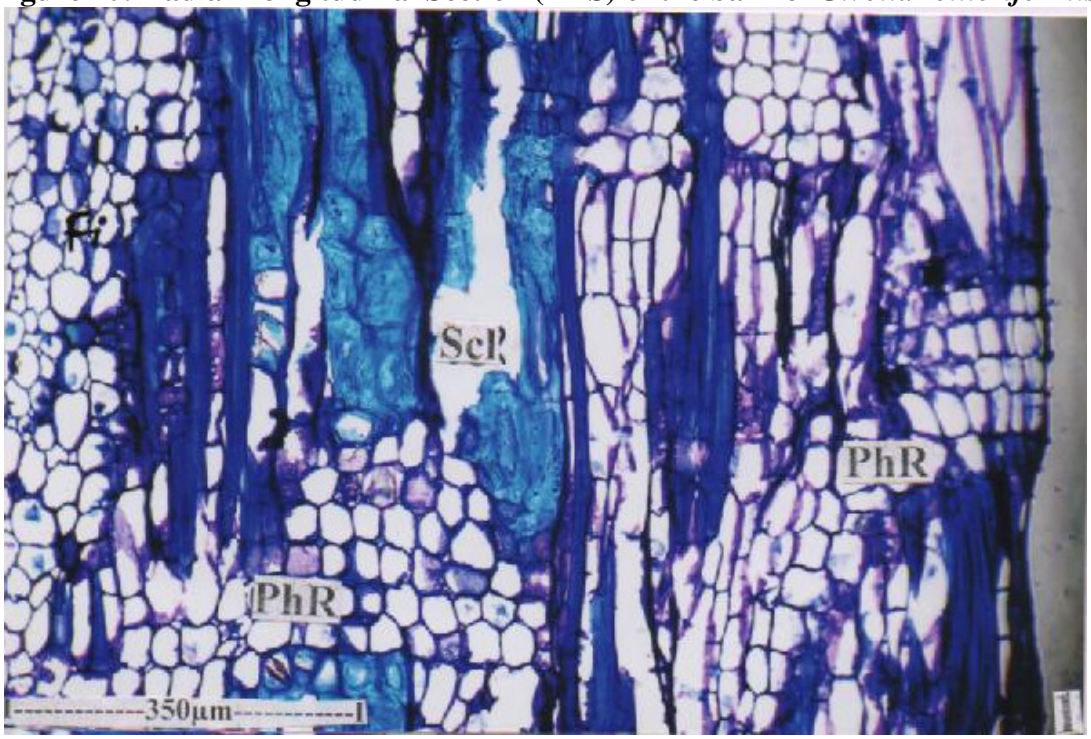


Figure 10.1: Phloem in RLS View: Showing horizontal bands of ray cells

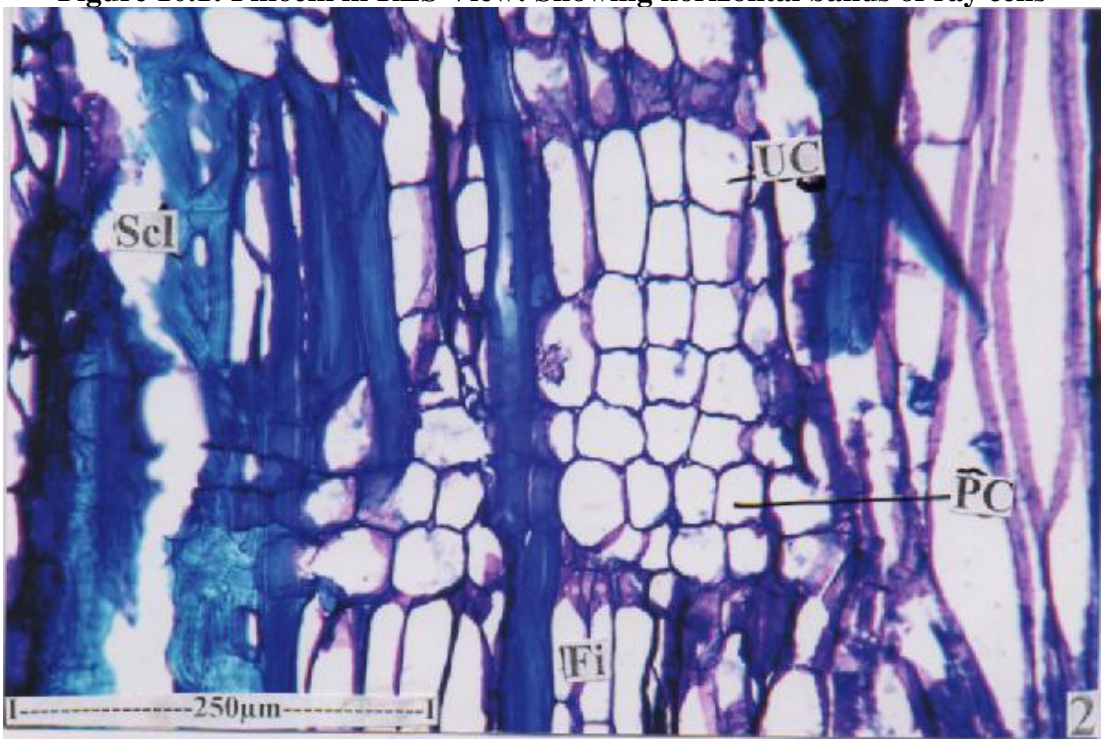


Figure 10.2: Phloem in RLS View: Rays enlarged
(Fi: Fibre; PC: Procumbent cells; PhR: Phloem Rays; Scl: Sclereids; UC: Upright cells)

Figure 11: Powder microscopy of the bark of *Givotia rottleriformis*

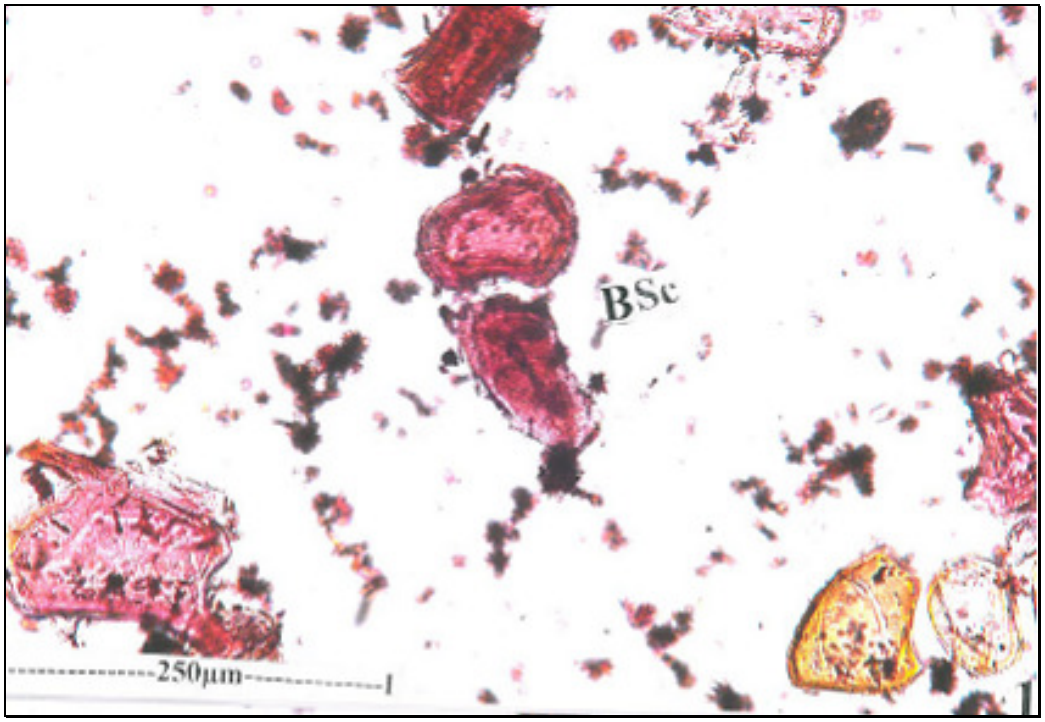


Figure 11.1: Powder microscopy: Brachy Sclereids

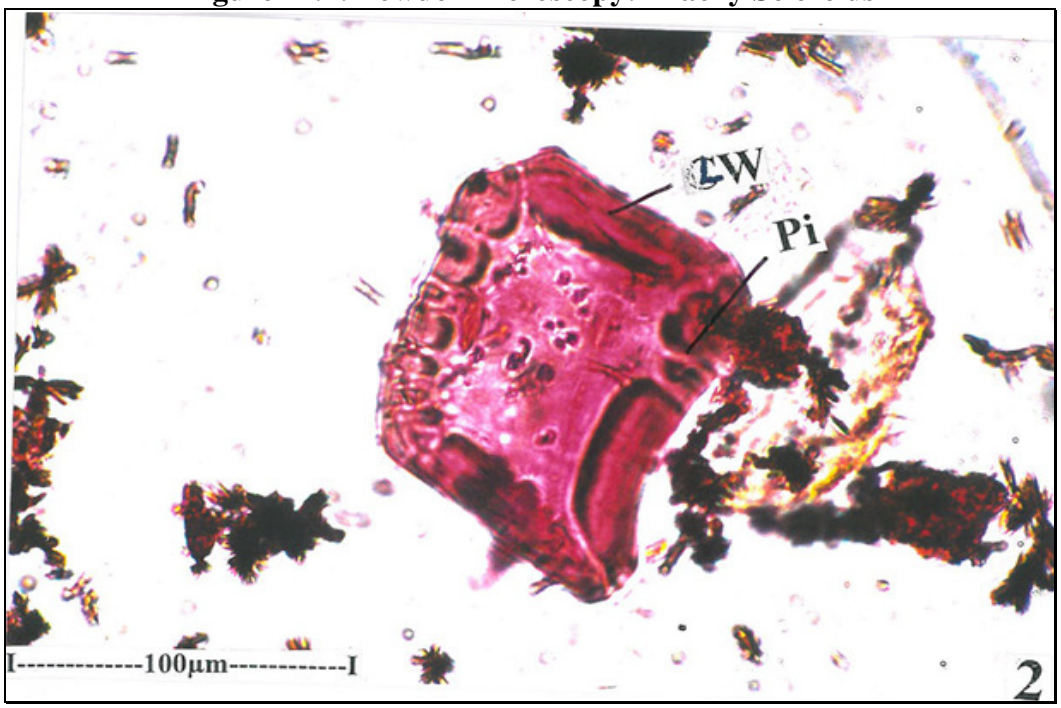


Figure 11.2: Powder microscopy: Sclereids enlarged
(BSc: Brachy sclereids; LW: Lignified Wall; Pi: Pit)

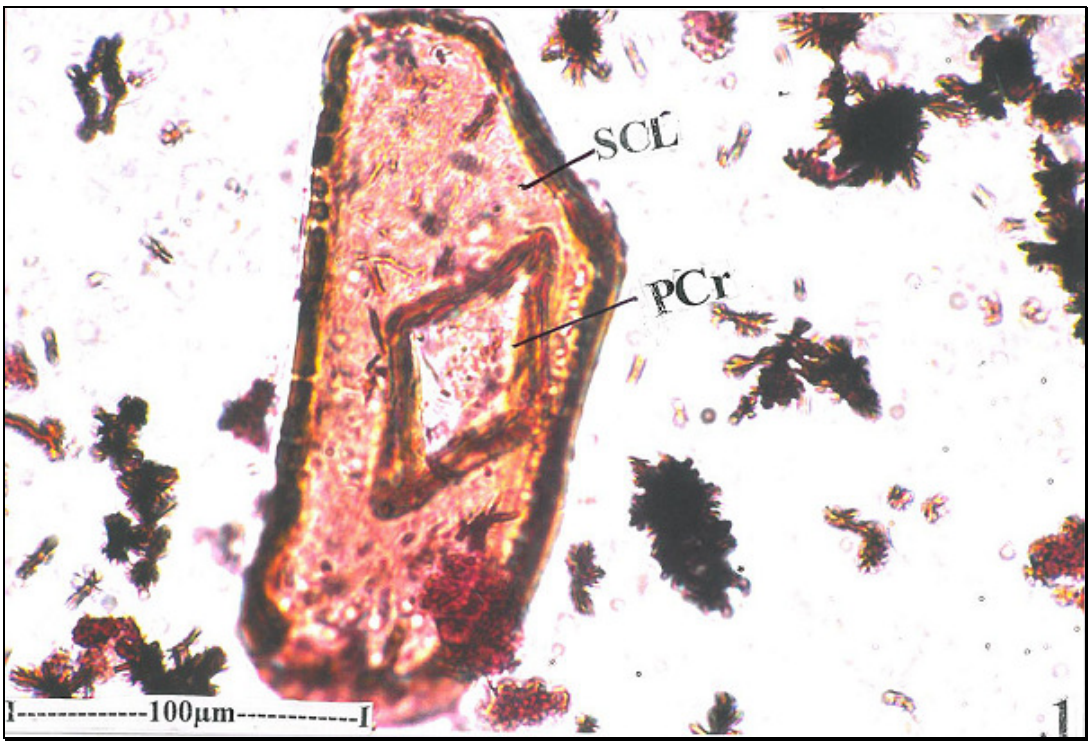


Figure 11.3: Powder microscopy: Thin walled Sclereids with prismatic crystals

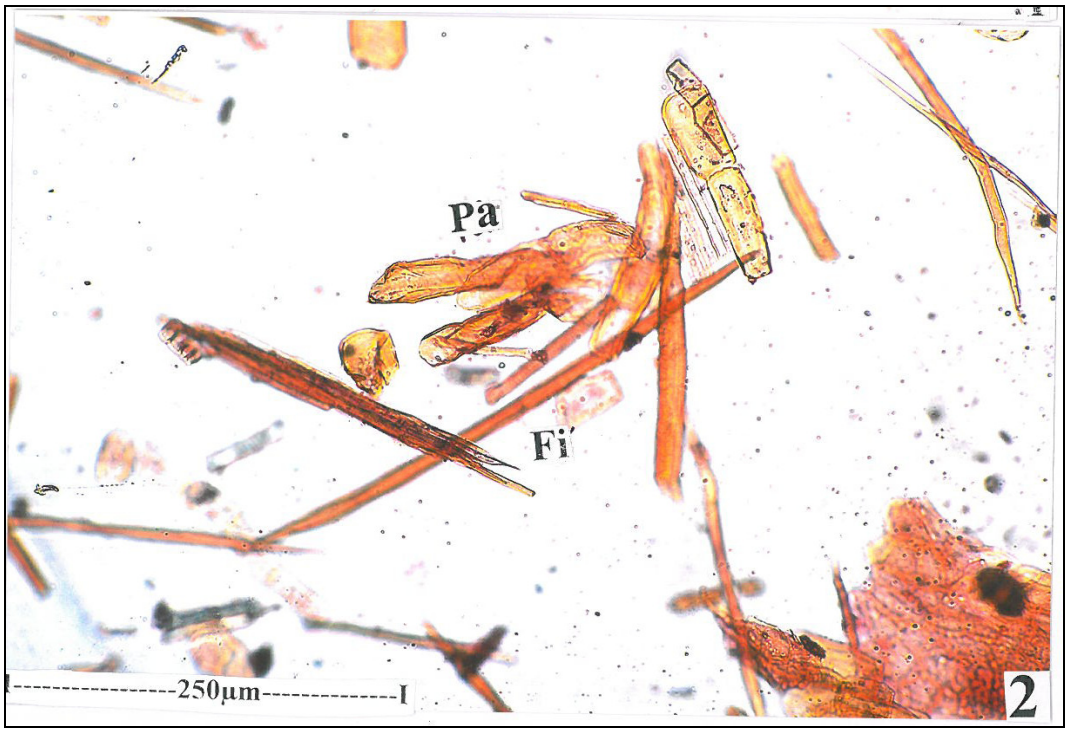


Figure 11.4: Powder microscopy: Fibres and Parenchyma cells

(Fi: Fibre; Pa: Parenchyma; PCr: Prismatic crystal; SCL: Sclereid)

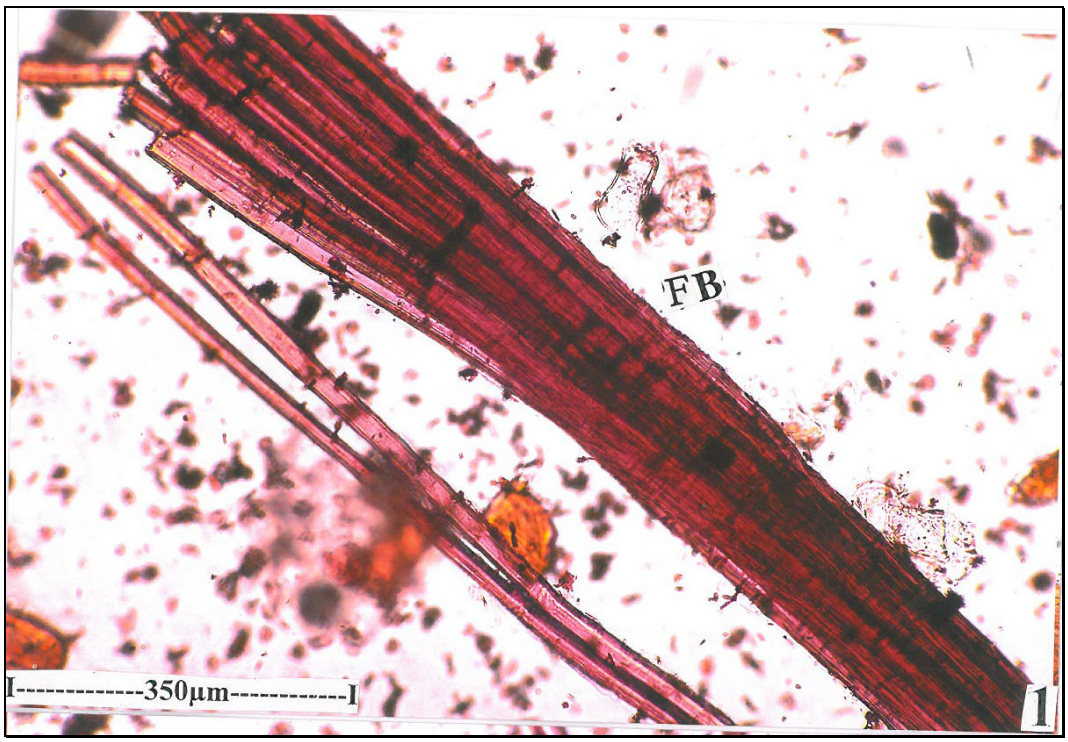


Figure 11.5: Powder microscopy: Bundle of fibres

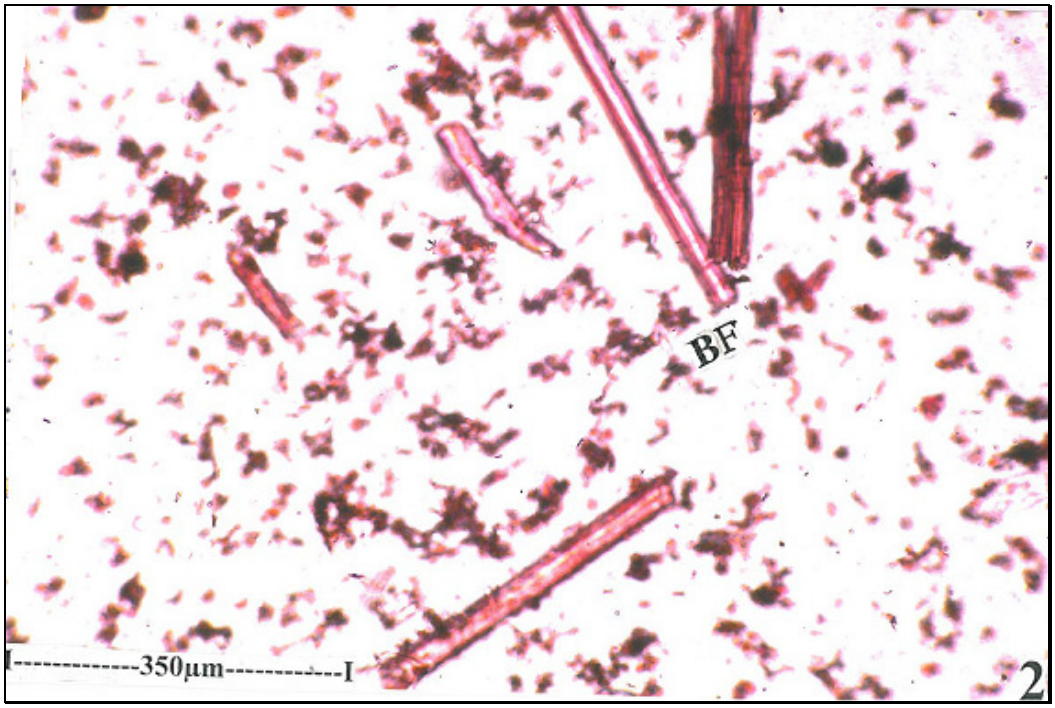


Figure 11.6: Powder microscopy: Broken fibres

(FB: Fibre bundle; BF: Broken fragments of fibres)

Figure 12: Histochemical Colour Reactions

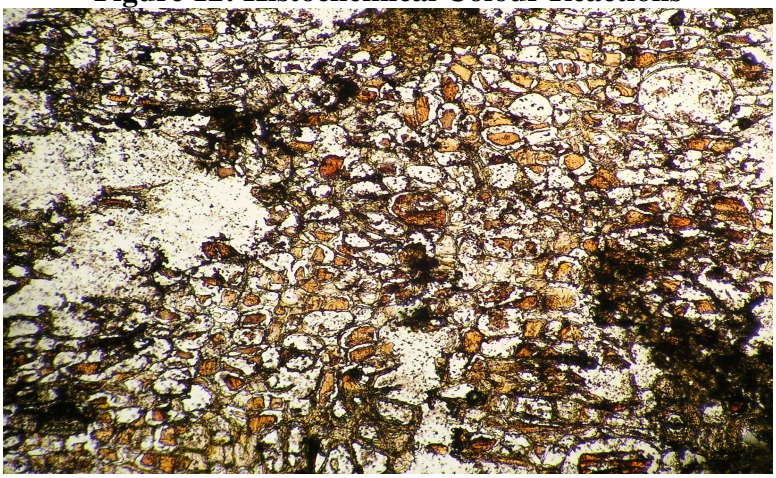


Figure 12.1(a): TS of bark showing flavonoids in cortical parenchyma cells

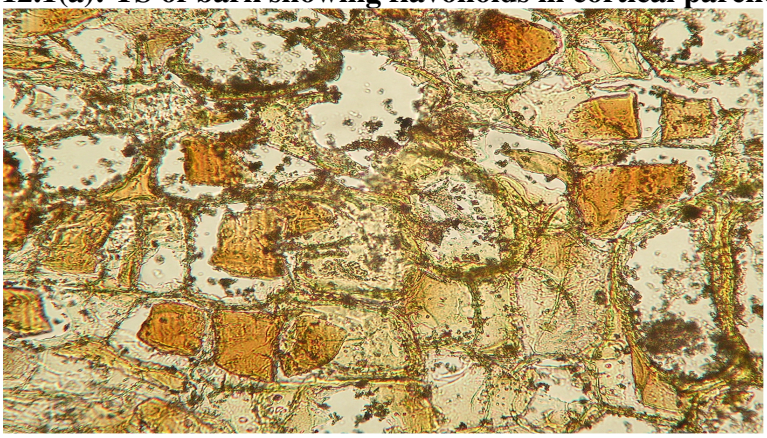


Figure 12.1(b): Flavonoid containing cells - enlarged

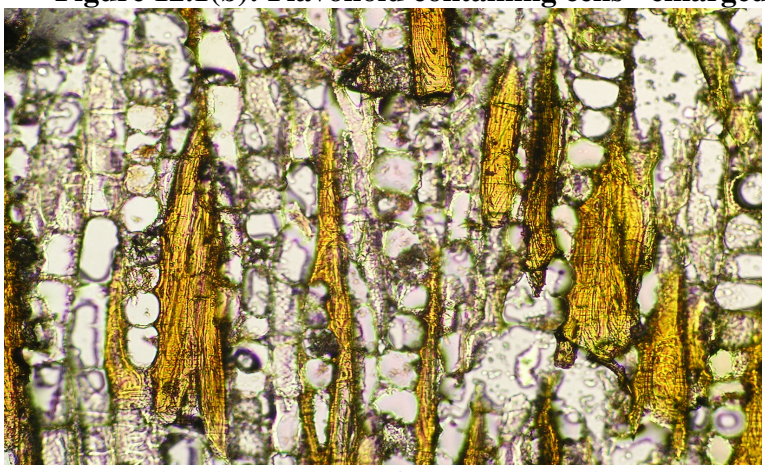


Figure 12.1(c): Longitudinal section of Phloem showing presence of flavonoids

Figure 12.1: Localisation of Flavonoids

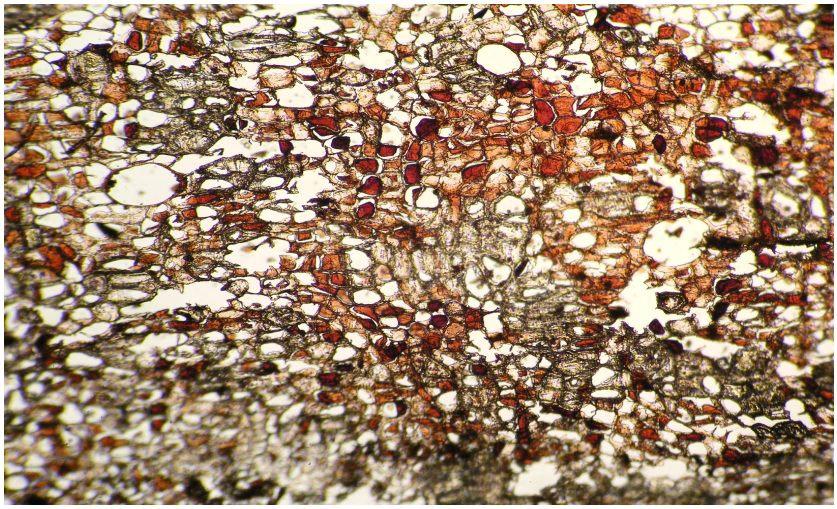


Figure 12.2(a): TS of bark showing Alkaloids in cortical parenchyma cells

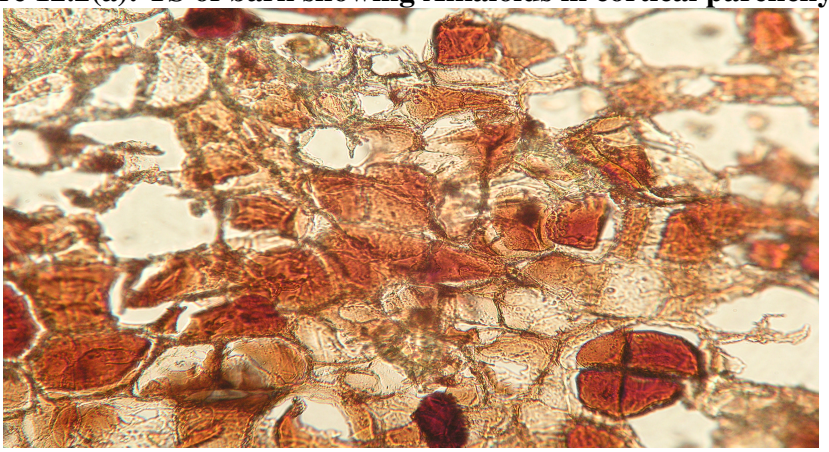


Figure 12.2(b): Alkaloids containing cells - enlarged

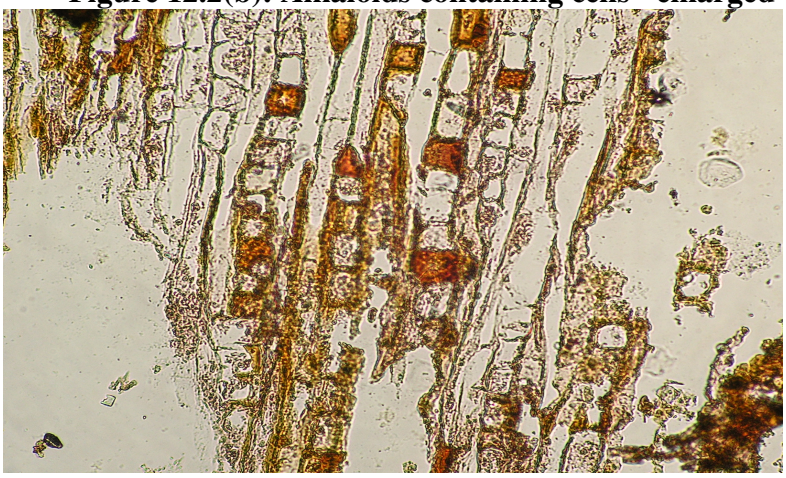


Figure 12.2(c): Longitudinal section of Phloem showing presence of Alkaloids

Figure 12.2: Localisation of Alkaloids

5.4. DISCUSSION

The World Health Organization (WHO), in pursuance of its goal of providing affordable, accessible and culturally acceptable health care to the global population, has encouraged the rational use of traditional medicines by member states of the WHO Health Assembly. In purview of facilitating this process, guidelines for assessment of the quality, safety and efficacy of herbal medicine have been developed.[119] Standardization of traditional plants is a critical and essential issue in ensuring therapeutic efficacy, safety and their rationale use in the health care.

Hence an attempt was made in this work to evaluate pharmacognostical and phytochemical parameters besides carrying out antipsoriatic studies on the bark of *Givotia rottleriformis* and *Cassia tora* leaves. The identification of plant material taxonomically and pharmacognostically is important, in order to avoid the adulteration of drugs and to distinguish the drug from other botanical sources. In the present study, the taxonomical characters dealing with exo-morphology of the plant have been studied which help in identification of the plant in the field. The herbarium specimen was prepared and deposited in the department for future reference. The microscopical and phytochemical studies help in evolving diagnostic characters for the identification of the drug.

Macroscopical features were examined to identify the right crude drug.

Microscopical features

Transverse section of the bark exhibits outer rhitidome periderm and inner secondary phloem that extends from the inner border of the rhytidome upto the cambial zone. The secondary phloem comprises outer wider collapsed phloem and inner narrow non collapsed phloem. The collapsed phloem includes highly dilated phloem rays, scattered irregular masses

of sclerenchyma cells and dark thick tangential streaks of crushed sieve elements. The non collapsed phloem includes wide rectangular sieve tubes, phloem parenchyma cells and wavy phloem rays.

Tangential longitudinal sections of the bark show uniseriate, narrow and hetero cellular phloem rays with terminal upright cells and middle procumbent cell. Prismatic crystals and druses of Calcium oxalate crystals are sparsely seen in the bark.

Radial longitudinal sections of the bark show heterocellular, square shape or horizontally elongated phloem rays in horizontal lines. The rays consist of upper rows of vertically elongated upright cells and middle part of procumbent cells.

Powder Characters

The powder characters of a drug are mainly used in the identification of the drug in the powder form. The bark powder was reddish brown in colour with bitter taste. On micrsocopical examination, the powder showed brachy sclereids sometimes with prismatic calcium oxalate crystals, thick walled libri form fibres in thick bundles or in small broken pieces and rectangular thin walled parenchyma cells.

Histochemical analysis

Histochemistry is mainly used to localise the chemical compounds within the cells and tissues using some chemical reagents which will selectively stain the compounds.

The observations of histochemical analysis showed that the flavonoids are present in cortical parenchyma cells and in axial parenchyma cells of the phloem in longitudinal section.

Alkaloids occupy entire lumen of the cortical parenchyma. In longitudinal section of bark, alkaloids are found to occupy some of the cells of phloem rays.

Physico-chemical constants

The physico chemical constants are important parameters for detecting adulteration or improper handling of drugs. The physicochemical constants like moisture content, total ash, acid insoluble ash, water soluble ash, extractive values using various solvents were carried out which helps in formulating pharmacopoeial parameters for the drug.

The residue remaining after incineration of plant material is the ash content or ash value, which simply represents inorganic salts, naturally occurring in crude drug or adhering to it or deliberately added to it, as a form of adulteration. The ash value was determined by three different methods *viz*. total ash, acid-insoluble ash and water-soluble ash. The total ash method is employed to measure the total amount of material remaining after ignition. This includes both 'physiological ash' which is derived from the plant tissue itself, and 'non-physiological ash', which is the residue of the extraneous matter adhering to the plant surface. Acid-insoluble ash is a part of total ash and measures the amount of silica present, especially as sand and siliceous earth. Water soluble ash is the water soluble portion of the total ash. These ash values are important quantitative standards. Water soluble ash is the water soluble portion of the total ash. These ash values are important quantitative standards. The ash values of the powdered bark and leaves revealed a high percentage of sulphated ash. Any significant deviation in the percentage of ash reported in this work may indicate adulteration of the drug.

Extractive values give an idea about the chemical constituents present in the drug as well as useful in the determination of exhausted or adulterated drugs. The results suggest that the powdered *Givotia rottleriformis* bark and *Cassia tora* leaves have high alcohol soluble extractive value.

The percentage of active chemical constituents in crude drugs is mentioned on air-dried basis. Therefore, the loss on drying of plant materials should be determined and the water content should also be controlled. The moisture content of dry powder of bark was 3.84 % and leaves was 4.32% which was not very high, hence it would discourage bacteria fungi or yeast growth. The crude fibre content which was studied can be implied to determine the nutritive value of the *Givotia rottleriformis* bark and *Cassia tora* leaves.

Fluorescence studies revealed specific fluorescence in visible light and white light with different chemicals treatment; which helps in identification and standardization of the plant within the species. The powder of *Givotia rottleriformis* bark showed light yellow fluorescence with methanol in UV light at 254 nm and *Cassia tora* leaves showed reddish yellow, which indicates the presence of chormophore in the drug.

Inorganic Mineral Analysis

In nutrition, minerals are those elements for which the body requirements is atleast 100 mg/kg and the trace elements that are needed in smaller quantities. The minerals include calcium, chloride, magnesium, phosphorus, potassium, sodium and sulphur. The trace elements are chromium, cobalt, copper, iodine, zinc, molybdenum, nickel, selenium etc. In this study, inorganic analysis of *Givotia rottleriformis* bark and *Cassia tora* leaves showed trace quantity in microgram level of toxic metals (lead, chromium, copper, cadmium, nickel) when compared to beneficial elements such as zinc, manganese, iron. So both the plants were absolutely safe to consume medicinally which is an indicative of nutritional potential of the plants for the treatment of various ailments.

Iron plays an important role in many parts of the body, including immune function, cognitive development, temperature regulation, energy metabolism, and work performance.

The concentration of iron was 2.54 µg in *Givotia rottleriformis* bark and 1.45 µg in *Cassia tora* leaves.

Calcium ions are involved in blood clotting, nerve impulse transmission, muscle contraction. The concentration of calcium was 0.452 µg in *Givotia rottleriformis* bark and 1.094 µg in *Cassia tora* leaves.

Magnesium decreases blood pressure by dilating arteries, and preventing heart rhythm abnormalities. The concentration of, magnesium was 0.145 µg in *Givotia rottleriformis* bark and 0.225 µg in *Cassia tora* leaves.

Zinc is involved in some catalytic reaction and can stabilize the structure of some enzymes. Also studies have shown that low zinc intake exacerbates the effect of low iodine intake. Zinc intake from other food sources or supplementation should be encouraged. Zinc supplementation has been reported to have favorable effect on thyroid hormone levels, particularly total T3, and resting metabolic rate. The concentration of zinc was 0.0043 mg in *Givotia rottleriformis* bark and 0.0061 mg in *Cassia tora* leaves.

6. PHYTOCHEMICAL STUDIES

6.1. INTRODUCTION

Phytochemistry is mainly concerned with enormous varieties of secondary plant metabolites which are biosynthesized by plants. Most of the best plant medicines are the sum of their constituents. Phytochemistry is a distinct discipline which finds a remarkable position in between natural product organic chemistry and plant biochemistry as being closely related to both. It is concerned with the enormous variety of organic substances that are elaborated and accumulated by plants and deals with the chemical structures of these substances, their biosynthesis, and turn over and metabolism of their natural distribution and their biological function. In all these operations, standard methods are needed for separation, purification and identification of the many different constituents present in plants.[123]

The beneficial physiological and therapeutic effects of plant materials typically result from the combinations of these secondary products present in the plant. The information on the constituents of the plant clarifies the uses of the plants but only a small percentage have been investigated for their phytochemicals and only a fraction has undergone biological or pharmacological screening.[123]

In phytochemical evaluation, the powdered bark of *Givotia rottleriformis* and leaves of *Cassia tora* were subjected to phytochemical screening for the detection of various plant constituents, characterized for their possible bioactive compounds, which have been separated and subjected to detailed structural analysis.

6.2. MATERIALS AND METHODS

The plant specimen for the proposed study was collected and after authentification, the fresh, healthy plant bark of *Givotia rottleriformis* and leaves of *Cassia tora* were properly

dried in shade for 2-3 weeks. It was pulverized in a blender, sieved and used for further studies.

6.2.1. Extraction

About 1 kg of air-dried plant material was extracted in Soxhlet assembly using ethanol (70% v/v). The solvent was filtered and the extract was concentrated by using rotary vacuum evaporator. The extract obtained was weighed and the percentage yield was calculated in terms of dried weight of the plant material. All the solvents used for this entire work were of analytical reagent grade (Merck, Mumbai).

6.2.2. Qualitative chemical analysis

The ethanol (70% v/v) extract of *Givotia rottleriformis* bark and ethanol (70% v/v) extract of *Cassia tora* leaves were subjected to the following chemical tests for identification of phytochemical constituents.[124,125]

Test for Alkaloids

A small portion of the extract was stirred separately with a few drops of dilute hydrochloric acid and filtered. The filtrate was carefully tested with various alkaloidal reagents such as Mayer's reagent, Dragendroff's reagent, Hager's reagent and Wagner's reagent.

Test for carbohydrates

The minimum amount of the extract was dissolved in 5 ml of distilled water and filtered. The filtrate was subjected to test for carbohydrates.

❖ ***Molisch's test***

The filtrate was treated with 2-3 drops of 1% alcoholic alpha naphthol and 2 ml of concentrated sulphuric acid was added along the sides of the test tube. Formation of violet colour ring indicates the presence of carbohydrates.

❖ ***Fehling's test***

The filtrate was treated with 1 ml of Fehling's A and B and heated in a boiling water bath for 5-10 min. Appearance of reddish orange precipitate shows the presence of carbohydrates.

Test for Glycosides

A pinch of extract was taken in a watch glass and 2 drops of alcohol was added to dissolve the extract. An equal quantity of anthrone was added and mixed thoroughly and dried. Then one drop of concentrated sulphuric acid was added, spreaded in a thin film with a glass rod in a watch glass and heated over the water bath. Formation of dark green colour confirms the presence of glycosides.

❖ ***Anthroquinone glycosides***

Borntrager's test: To 3 ml extract dilute sulphuric acid was added, boiled and filtered. To the cold filtrate equal volume benzene was added. The organic layer was separated and ammonia was added.Formation of rose pink colour in ammonia layer indicates the presence of anthraquinone glycoside.

❖ ***Cardiac glycosides***

Kellerkillani test: To 2 ml of extract, glacial acetic acid, one drop 5 % ferric chloride and concentrated sulphuric acid were added. Appearance of reddish brown colour at the junction of the two liquid layers indicates the presence of cardiac glycosides.

- ***Saponin glycosides***

  Foam test: The extract and powder were mixed vigorously with water.

- ***Coumarin glycosides***

  Alcoholic extract when made alkaline, shows blue or green fluorescence.

Test for phytosterol

About 1gm of the extract was dissolved in few drops of dry acetic acid; 3 ml of acetic anhydride was added followed by few drops of concentrated sulphuric acid. Appearance of bluish green colour shows the presence of phytosterol.

Test for fixed oils and fats

- Small quantity of the extract was separately pressed between two filter papers. Appearance of oil stain on the paper indicates the presence of fixed oil.
- Few drops of 0.5N alcoholic potassium hydroxide was added to a small quantity of extract along with a drop of phenolphthalein. The mixture was heated on a water bath for 1-2hrs. Formation of soap or partial neutralization of alkali indicates the presence of fixed oil and fats.

Test for tannins and phenolic compounds

Small quantity of extract was taken separately in water and tested for the presence of phenolic compounds and tannins with

(a) Dilute ferric chloride solution (5%) - violet colour

(b) 1% solution of gelatin with 10%NaCl - white precipitate

(c) 10% lead acetate solution - white precipitate

Test for proteins

The extract was dissolved in few ml of water and treated with

- ***Millon's reagent***: Appearance of red colour shows the presence of proteins and free amino acids.
- ***Biuret test***: Equal volume of 5% solution of sodium hydroxide and 1% copper sulphate were added. Appearance of pink or purple colour indicates the presence of proteins and free amino acids.

Test for gums and mucilages

About 10 ml of extract was added separately to 25 ml of absolute alcohol with constant stirring and filtered. The precipitate was dried in air and examined for its swelling properties and for the presence of carbohydrates.

Test for flavanoids

- With aqueous solution of sodium hydroxide blue to violet colour (Anthocyanins), yellow colour (Flavones), yellow to orange (Flavonones).
- With concentrated sulphuric acid yellowish orange colour (Anthocyanins), orange to crimson colour (Flavonones).
- ***Shinoda's test***: The extracts were dissolved in alcohol, to that a piece of magnesium and followed by concentrated hydrochloric acid was added drop wise and heated. Appearance of magenta colour shows the presence of flavonoids.

Test for lignin

With alcoholic solution of phloroglucinol and concentrated hydrochloric acid appearance of red colour shows the presence of lignin.

Test for terpenoids

Noller's test: The substance was warmed with tin and thionyl chloride. Pink coloration indicates the presence of triterpenoids.

Test for steroids

Libermann Burchard Reaction: 2 ml extract was mixed with chloroform. To this 1-2 ml acetic anhydride and 2 drops concentrated sulphuric acid were added from the side of test tube. First red, then blue and finally green colour appears.

6.2.3. Estimation of Phytoconstituents

Plant materials and herbals derived from around 70,000 plant species represents substantial portion of the global market. The W.H.O Assembly currently in number of resolutions emphasized the need to ensure quality control of medicinal plant products by using modern techniques and applying suitable standards.

The purpose of standardization of medicinal plant product is obviously to ensure therapeutic efficacy and to check any adulteration or non deliberate mixing in commercial batches. The phytoconstituents present in dried coarsely powdered bark of *Givotia rottleriformis* and *Cassia tora* leaves were estimated using standard procedures.

Total Phenolic Content

The content of total phenolics in the powdered drug was determined by using Folin-Ciocalteu reagent. About 1 gram of the powder was extracted in an ultrasonic wave bath with 80 ml of aqueous ethanol solution (70% v/v) for 2 hr. After cooling, the volume of the solution was adjusted to 100 ml. The final solution was centrifuged prior to the colorimetric determination. Tannic acid standards (10 - 110 mg/ml) were dissolved in 100 ml of aqueous ethanol solution (70% v/v) respectively. About 10 ml of Folin-Ciocalteu reagent was added to 1 ml of the extract solution and 1 ml of standard solution. After reacting for 3 min, 10 ml of 35% sodium carbonate solution was added and the test solution was diluted to 100 ml with water and mixed. After 45 min, an aliquot was centrifuged for 5 min. The absorption

coefficient for the supernatant was measured at 745 nm. The total phenolic content of the extract was calculated using the mean regression coefficient from the standards.[126]

Determination of Total Flavonoids

Total flavonoid content in dried plant material was estimated by spectrometric method. (Perkin-Elmer UV-Vis spectrometer Lambda 16 (Germany))**.**[127] Dried powdered plant material (10 gm) was extracted by continuous mixing in 100 ml of 70% ethanol, 24 hr at room temperature. After filtration, ethanol was evaporated untill only water remained. Water phase was subsequently extracted with ethyl acetate. The extract was dried over anhydrous sodium sulphate, filtered and concentrated under vacuum up to a concentration of 1 gm/ml of extract. They were further diluted with ethyl acetate to obtain 0.01 gm/ml solutions. About 10 ml of the solution was transferred into a 25 ml volumetric flask, 1 ml of 2% $AlCl_3$ was added and the solution was filled to volume with methanol-acetic acid and was kept aside for 30 min. The absorbance was measured at 390 nm against the same solution without $AlCl_3$ being blank. The total flavonoid content was determined using a standard curve with quercetin (100 - 1000 mg/L) as the standard. Total flavonoid content is expressed as mg of quercetin equivalents (Q) /g of extract.

6.2.4. Thin Layer Chromatography

Of the various methods of separating and isolating plant constituents, thin layer chromatography (TLC) is one of the most powerful techniques used for the separation, identification and estimation of single or mixture of components present in various extracts. All the finely divided solids have the power to adsorb other substances on their surface to a greater or lesser extent. Similarly all substances are capable of being adsorbed, some much more readily than others.

Mechanism employed in this reliable technique is adsorption in which solute adsorbs on the stationary phase according to its affinity. Substances are separated by differential migration that occurs when a solvent flows along the thin layer of stationary phase. The substance which is having more affinity towards mobile phase moves faster when compared to the substance which has less affinity leading to the separation of the compounds. This phenomenon of selective adsorption is the fundamental principle of chromatography. For identification of the components in each extract, the extract was subjected to thin layer chromatography.[128,129]

Stationary Phase

Glass plates coated with silica gel G were used as a stationary phase.

Sample application

The ethanol (70%v/v) extract of *Givotia rottleriformis* bark and *Cassia tora* leaves were diluted with respective solvents and then spotted with the help of capillary tube just 2 cm above its bottom.

Selection of mobile phase

Solvent mixture was selected on the basis of the phytoconstituents present in each extract. Solvents were analyzed as its order of increasing polarity. A number of developing solvent systems were tried and after several trials, the best solvent system was selected which showed good separation with maximum number of components.

Solvent system

Benzene: Methanol: Ammonia (9:1:0.1)

After development, the plates were air dried and R_f values were calculated using Iodine vapour as detecting agent.

6.2.5. High Performance Thin Layer Chromatography

HPTLC method is a modern sophisticated and automated separation technique derived from TLC. Pre-coated HPTLC graded plates and auto sampler was used to achieve precision, sensitive, significant separation both qualitatively and quantitatively.

High performance thin layer chromatography (HPTLC) is a valuable quality assessment tool for the evaluation of botanical materials efficiently and cost effectively. HPTLC method offers high degree of selectivity, sensitivity and rapidity combined with single-step sample preparation. In addition, it is a reliable method for the quantization of nanograms level of samples. Thus this method can be conveniently adopted for routine quality control analysis. It provides chromatographic fingerprint of phytochemicals which is suitable for confirming the identity and purity of medicinal plant raw materials.[130]

HPTLC Profile

Extracts tested

Ethanol (70%v/v) extract of *Givotia rottleriformis* bark and ethanol (70%v/v) extract of *Cassia tora* leaves

Sample application

The samples were dissolved in same solvent and 10 µl quantity of sample was applied on the HPTLC Silica Merck 60F 254 graded plate sized 5cm x 10 cm as narrow bands using CAMAG Linomat V injector.

Chromatogram Development

It was carried out in CAMAG Twin Trough chambers. Sample elution was carried out according to the adsorption capability of the component to be analyzed. After elution, plates were taken out of the chamber and dried.

Scanning

Plates were scanned under UV at 254 nm using Camag TLC scanner 3. The data's obtained from scanning were interpreted using WINCATS-4 software. Chromatographic finger print was developed for the detection of phytoconstituents present in each extract and R_f values were tabulated.

Mobile Phase

Benzene: Methanol: Ammonia (9:1:0.1)

6.2.6. Isolation of Flavonoids by Column Chromatography

Column chromatography is an isolation technique in which the phytoconstituents are being eluted by adsorption. The principle involved in this separation of constituents is adsorption at the interface between solid and liquid. The component must have various degree of affinity towards adsorbent and also reversible interaction to achieve successful separation. No two compounds are alike in the above aspect. Low affinity compounds will elute first.

Since the aim of the present study is to evaluate the pharmacological effects of flavonoids, ethanolic extract (70% v/v) was eluted with solvents of increasing polarity like n-hexane, chloroform, ethyl acetate and methanol to isolate flavonoids.

6.2.6.1. Materials and Methods

Extract: Ethanol extract (70% v/v) of *Givotia rottleriformis* bark and *Cassia tora* leaves

Method: Wet packing method

Stationary Phase: Silica gel G 60 (60-120 mesh)

Preparation of the column

An appropriate column sized 5 cm diameter and 60 cm length was used. It was washed with water and rinsed with acetone and then dried completely. Little of pure cotton was

placed at the bottom of column with the help of a big glass rod. Solvent hexane was poured into the column upto 3/4th level. Defatted ethanol extract with petroleum ether was mixed with equal amount of graded silica gel until it became free flowing powder. When it reached a defined state it was slowly poured into the column containing hexane solvent with slight movement of stirring by glass rod to avoid clogging. Little cotton was placed on top of silica gel- extract mixture pack to get neat column pack. The knob at the bottom was slowly opened to release the solvent.

Isolation of compounds

The defatted 70% v/v ethanol extract (25 g) was subjected to chromatography (Silica gel 120 mesh, 500 g) with gradient elution using solvents of increasing polarity, hexane, chloroform, ethyl acetate and methanol. Shinoda test was carried out for confirming the presence of flavonoids and TLC studies was carried out using Benzene: Methanol: Ammonia (9:1:0.1) solvent system. The spot was visualized by spraying with ammonia, a reagent specific for flavonoids. Fractions with similar spots and positive test for flavonoids were pooled together and concentrated to obtain pure compound. The pure compounds obtained were then subjected to spectral analysis for the determination of the structure of the compound.

Acid hydrolysis of Compound

Each compound was refluxed in 2N HCl (5 mL) for 1 h. The aglycones were extracted with ethyl acetate and identified by co-TLC with authentic standards and UV spectral analysis with the usual shift reagents. Sugar moieties were detected on cellulose TLC plates with ethyl acetate-methanol-water-acetic acid (13:3:3:4) using aniline phthalate as spraying reagent.

6.2.7. Spectral Analysis

The structure of the isolated compounds were determined by instrumental spectral analysis such as

IR spectroscopy

NMR - ^{1}H and ^{13}C

Mass spectroscopy

Infra Red (IR)

Infra Red (IR) spectrum is considered as vibrational-rotational spectra. KBr pellet technique is used for solid compound, for liquid compound Nujol mull method is followed. It is very helpful record which would give information about functional group present in the organic compounds. Mechanism of bond stretching and bending is happened when electromagnetic radiation ranging from 500 cm^{-1} to 4000 cm^{-1} passed through sample. Infra Red (IR) spectra were recorded on a Shimadzu FTIR 8400S Spectrometer.

Nuclear Magnetic Resonance (NMR)

Nuclear magnetic spectrum is the most powerful spectral technique used to detect chemical structure of the molecules. The differences in the chemical environment around the different nuclei are exploited to obtain this information and is expressed in terms of chemical shifts in parts per million. When sample absorbs radiation at different radio frequency region which causes to excite type of proton or certain nuclei contained in the sample against magnetic field. The most commonly used NMR technique is ^{1}H and ^{13}C.

^{1}H NMR

Most commonly used NMR is Proton NMR, because of the sensitivity and wide range of characteristic information. Range of chemical shift (δ) is from 0-14 ppm. Chemical shift of

the test unknown compound was compared with TMS protons which are attributed at 0 ppm. But, the shift extends for the organic compound range δ 0–14 for the component. ^{1}H NMR was obtained on a Bruker (Fallanden, Switzerland) Avance 500 MHz using DMSO as solvent.

^{13}C NMR

It is new technique but natural abundance is very low 1.1%. So, this further reduces the sensitivity of the absorption. The range of chemical shift (δ) was from 0 -180 ppm with the use of TMS internal reference. An advantage is one can directly observe the functional group contained carbon atom. ^{13}C NMR was obtained on a Bruker (Fallanden, Switzerland) Avance 100 MHz using DMSO as solvent.

Mass Spectroscopy

It is an accurate method to determine the molecular mass of the compound. The main advantage is very small amount of sample is required for analysis than any other spectral methods. The mass spectroscopy uses the electric and magnetic fields to produce electrically charged ions of chemical substance under analysis. The Mass spectral data was recorded on a Micro Mass Quattro II (ESI MS). The record spectrum tells about the mass, relative abundance of the molecular ions and positively charged fragments formed by electronic bombardment.

6.2.8. Fingerprint Analysis by High Performance Liquid Chromatography

HPLC, is a chromatographic technique used to separate the components in a mixture by injecting small volume of liquid sample into a tube packed with tiny particles (3 to 5 micron (µm) in diameter called the stationary phase) where individual components of the sample are moved down the packed tube (column) with a liquid (mobile phase) forced through the column by high pressure delivered by a pump. These components are separated

from one another by the column packing that involves various chemical and/or physical interactions between their molecules and the packing particles.

Procedure

The Qualitative and Quantitative analysis of the sample was performed according to the method of Boligon *et al* 2012.[131] The HPLC system of Jasco consists of a pump (model Jasco PU2080, intelligent HPLC pump) with injecting facility programmed at 20 µL capacity per injection was used. The detector consists of a UV/ VIS (Jasco UV 2075) model operated at a wavelength of 270 nm. The software used was Jasco Borwin version 1.5, LC-Net II/ADC system. The column was Thermo ODS Hypersil C18 (250 x 4.6 mm, 5 µm) in isocratic mode. The separation was achieved using a mobile phase of methanol, water and phosphoric acid (100: 100: 1, *v/v/v*) at a flow-rate of 1.5 ml/min. The effluent was monitored using UV detection at a wavelength of 270 nm. The mobile phase was filtered through 0.45 µm nylon filter prior to use.

Sample preparation

Powdered *Givotia rottleriformis* bark was weighed and transferred to a 250-ml flask fitted with a reflux condenser. About 78 mL of extraction solvent (Alcohol: Water and Hydrochloric acid (50:20:8)) was added, refluxed on a hot water bath for 135 minutes, cooled at room temperature and transferred to a 100 mL volumetric flask. About 20 mL of methanol was added to the 250 mL flask, sonicated for 30 min, filtered and the filtrate was transferred to the 100 mL volumetric flask, the residue was washed on the filter with methanol. The washing was collected in the same 100 mL volumetric flask and diluted to volume. The procedure was repeated with *Cassia tora* leaves.

Identification is based on retention times and on-line spectral data in comparison with authentic standards. Quantification is performed by establishing calibration curves for each determined compound, using the standards.

6.2.9. HPTLC analysis with known marker

HPTLC (silica gel G 60 F254 TLC plates of E. Merck, layer thickness 0.2mm) fingerprint profile was established for ethanol extract (70%v/v) of *Givotia rottleriformis* bark and ethanol extract (70%v/v) of *Cassia tora* leaves. HPTLC was performed on 10 cm×10 cm aluminum backed plates coated with silica gel 60F254 (Merck, Mumbai, India).[132] Standard solution of Rutin, Kaempferol, Luteolin, Quercetin and Formononetin were applied to the plates as bands 8.0 mm wide, 30.0 mm apart, and 10.0 mm from the bottom edge of the same chromatographic plate by use of a Camag (Muttenz, Switzerland) Linomat V sample applicator equipped with a 100-µL Hamilton (USA) syringe.

Ascending development to a distance of 80 mm was performed at room temperature (28±2°C), with Benzene: Methanol: Ammonia (9:1:0.1), as mobile phase, in a Camag glass twin-trough chamber previously saturated with mobile phase vapour for 20 min. After development, the plates were dried with a hair dryer and then scanned at 254 nm with a Camag TLC Scanner with WINCAT software, using the deuterium lamp. A stock solution of standard Rutin, Kaempferol, Luteolin, Quercetin and Formononetin (100 µg/mL) was prepared in methanol. Different volume of stock solution 2, 4, 8, 16 and 32 µLwere spotted on to TLC plate to obtained concentration 100 ng, 200 ng, 300 ng, 400 ng and 500 ng/spot of Rutin, Kaempferol, Luteolin, Quercetin and Formononetin.

6.3. RESULT

6.3.1. Extraction

The percentage yield of 70% v/v ethanol extractive values of *Givotia rottleriformis* bark and *Cassia tora* leaves were tabulated in Table 4.

Table 4: Percentage yield of the total extract of *G. rottleriformis* bark and *C. tora* leaves

Parameter	70% v/v Ethanol extract	
	***G. rottleriformis* bark**	***C. tora* leaves**
Percentage Yield (% w/w)	12.50	11.36
Colour	Reddish brown	Dark Green
Consistency	Solid	Semi solid

6.3.2. Qualitative Phytochemical Analysis

Qualitative phytochemical analysis for ethanol extract of *G. rottleriformis* bark and *C. tora* leaves indicates the presence of the various secondary metabolites tabulated in Table 5.

Table 5: Qualitative Phytochemical Analysis of *G. rottleriformis* bark and *C. tora* leaves

S. No	Phytoconstituent	Ethanol (70%v/v)	
		***G. rottleriformis* bark**	***C. tora* leaves**
1	Alkaloids	+	-
2	Carbohydrates	+	+
3	Glycosides	+	+
4	Saponins	-	+
5	Steroids	+	+
6	Phenols	+	+
7	Tannins	+	+
8	Proteins	+	+
9	Terpenoids	+	+
10	Flavonoids	+	+
11	Anthroquinones	-	+
12	Quinones	-	-

Note: + ve indicates presence, whereas – ve indicates absence

6.3.3. Estimation of Phytoconstituents

The total phenol and flavonoid content present in the *G. rottleriformis* bark and *C. tora* leaves were estimated by Folin-Ciocalteu and $AlCl_3$ method respectively.

Table 6: Estimation of phytoconstituents in *G. rottleriformis* bark and *C. tora* leaves

Test	Total phenol content	Total flavonoid content
***G. rottleriformis* bark**	13.80 % w/w	5.7% w/w
***C. tora* leaves**	18.60% w/w	9.5% w/w

6.3.4. Thin Layer Chromatography

The TLC studies of the ethanol extract of *G. rottleriformis* bark and *C. tora* leaves are shown in Table 7 and Fig. 13 & 14.

Table 7: Thin layer chromatography of ethanol extract of *G. rottleriformis* and *C. tora*

Solvent system	*G. rottleriformis* bark		*C. tora* leaves		Detecting agent
	No. of spots	R_f Value	No. of spots	R_f Value	
Benzene : Methanol: Ammonia (90:10:1)	**7**	0.114 0.180 0.229 0.262 0.560 0.642 0.737	10	0.148 0.203 0.240 0.314 0.565 0.629 0.703 0.796 0.833 0.925	Iodine Vapour

6.3.5. High Performance Thin Layer Chromatography

The HPTLC fingerprints of the ethanol extract of *G. rottleriformis* bark and *C. tora* leaves were shown in Table 8, Fig. 15 & 16 and HPTLC chromatogram in Fig. 17 & 18.

Table 8: The HPTLC of *G. rottleriformis* bark and *C. tora* leaves

Detecting Wavelength	***G. rottleriformis* bark**		***C. tora* leaves**	
	No. of spots	**Rf Value**	**No. of spots**	**Rf Value**
254	**8**	0.03 0.20 0.25 0.27 0.34 0.61 0.64 0.73	**9**	0.04 0.13 0.16 0.34 0.53 0.61 0.71 0.81 0.99

6.3.6. Column Chromatography

***Givotia rottleriformis* bark**

Totally 84 fractions were eluted. The eluates chloroform: ethyl acetate (25:75), ethyl acetate: methanol (50:50) and methanol (100) gave positive response for flavonoids producing pink colour with shinoda test. The fractions 1-29 were negative for flavonoid test. The fractions 30-38, 52-59 and 75-84 exhibited single spot with Rf values of 0.18, 0.53, 0.64. Fractions with similar spots and positive test for flavonoids were pooled together and concentrated to obtain compound **I, II, III**. The yield of each compound was **I** (680 mg), **II** (1.12 g), **III** (1.05 g).

Table 9a: Isolation of flavonoids from the ethanol extract (70% v/v) of *G. rottleriformis*

S.No	Fraction	Eluates	Rf value	Nature of the Compound
1	1-29	Hexane (100%) to Chloroform: Ethyl acetate (50 : 50)	-	Negative for flavonoid test
5	30-38	Chloroform : Ethyl acetate (25 : 75) **Broad Band**	0.18	Positive for flavonoid test Yellowish amorphous powder **Compound I**
9	52-59	Ethyl acetate: Methanol (50 : 50) **Broad Band**	0.53	Positive for flavonoid test Yellowish amorphous powder **Compound II**
11	75-84	Methanol (100 %) **Broad Band**	0.64	Positive for flavonoid test Yellowish brown amorphous powder **Compound III**

Cassia tora **Leaves**

Totally 84 fractions were eluted. The eluates chloroform: ethyl acetate (25:75), ethyl acetate: methanol (50:50) and methanol (100) gave positive response for flavonoids producing pink colour with shinoda test. The fractions 1-29 were negative for flavonoid test. The fractions 30-38, 52-59 and 75-84 exhibited single spot with R_f values of 0.53, 0.61, 0.76. Fractions with similar spots and positive test for flavonoids were pooled together and concentrated to obtain compound **IV, V, VI**. The yield of each compound was **IV** (710 mg), **V** (640 mg), **VI** (1 g).

Table 9b: Isolation of flavonoids from the ethanol extract (70% v/v) of *C. tora* Leaves

S.No	Fraction	Eluates	Rf value	Nature of the Compound
1	1-40	Hexane (100%) to Chloroform: Ethyl acetate (10 : 90)	-	Negative for flavonoid test
5	41-46	Ethyl acetate (100) **Broad Band**	0.53	Positive for flavonoid test Yellowish amorphous powder **Compound IV**
9	62-69	Ethyl acetate: Methanol (40 : 60) **Broad Band**	0.61	Positive for flavonoid test Yellowish amorphous powder **Compound V**
11	84-94	Methanol (100 %) **Broad Band**	0.76	Positive for flavonoid test Pale Yellow amorphous powder **Compound VI**

6.3.7. Spectral Analysis

The compound (I, II and III) obtained with the ethanol extract of *G. rottleriformis* bark have identified and spectral data's were depicted in the **Table 10** and **Fig. 19-30**

Compound I (Rutin): A yellow amorphous powder; mp 192-196 °C; Mol. formula $C_{27}H_{30}O_{16}$, Mol wt. 610. TLC R*f*: 0.18 (Benzene: Methanol: Ammonia).

UV λ_{max} (MeOH) 358, 298sh, 266sh, 258 nm, (NaOMe) 410, 328, 202 nm, ($AlCl_3$) 432, 302sh, 272nm, ($AlCl_3$/HCl) 402, 360sh, 300, 270 nm (NaOAc) 385, 327, 274 nm, (NaOAc/H_3BO_3) 376, 290, 264. IR (KBr) λ_{max} 3340 (OH), 1634 (C=C), 1507, 1374 (aromatic ring) cm-1.

^{1}H NMR (500 MHz, DMSO-d_6): 12.74 (s, C5-OH), 7.48 (1H, d, J = 1.8 Hz, H-2′), 7.54 (1H, d, J = 8.1, 1.8 Hz, H-6′), 6.87 (1H, d, J = 8.1 Hz, H-5′), 6.42 (1H, d, J = 2.3 Hz, H-8), 6.74 (1H, s, H-3), 6.26 (1H, d, J = 2 Hz, H-6), 5.30 (1H, d, J = 7.5 Hz, H-1″), 4.41 (1H, d, J = 1.5 Hz, H-1‴), 3.72 (1H, dd, J = 10 Hz, H-6″a), 3.45 to 3.32 (5H, m, H-2″, 3″, H-4″, 5″, 6″b), 3.0 to 3.32 (m, 8H-of sugar moieties), 1.10 (d, 3H, J=6 Hz, CH_3-rhamnose).

^{13}C NMR (100 MHz, DMSO-d_6): δ = 177.92 (C-4), 164.78 (C-7), 160.86 (C-5), 157.54 (C-2), 156.48 (C-9), 148.67 (C-4′), 145.32 (C-3′), 133.60(C-3), 122.68 (C-6′), 121.61 (C-1′), 116.92 (C-5′), 115.56 (C-2′), 104.14 (C-10), 101.85 (C-1″), 101.43 (C-1‴), 99.80 (C-6), 94.14 (C-8), 75.70 (C-5″), 76.46 (C-3″), 74.26 (C-2″), 72.49 (C-4‴), 70.92 (C-2‴), 70.83 (C-4″), 70.60 (C-3‴), 68.63 (C-5‴), 67.67 (C-6″), 18.4 (C-6‴).

Compound II (Luteolin-7-O-β-D-Glucuronide): Mol. formula $C_{21}H_{18}O_{12}$ Mol wt 462.36 m.pt- 260-262^{o}C. TLC R*f*: 0.53 (Benzene: Methanol: Ammonia). UV λ_{max} MeOH nm: 254, 268, 346; + NaOMe: 263, 309 sh, 398; + NaOAc + H_3BO_3: 268, 293 sh, 370; + $AlCl_3$: 276, 298 sh, 386; + $AlCl_3$ /+ HCl: 270, 289 sh, 353, 387; + NaOH: 243 sh, 270, 304 sh, 392.

Negative ESI-MS ($C_{21}H_{18}O_{12}$) *m/z*: 461 [M-H]ō, 285 [M-H-176]ō = [agycone-H]ō IR (KBr) λ_{max} 3420 (OH), 1634 (C=O), 1620, 1507, 1374 (aromatic ring) cm-1.

^{1}H NMR (500 MHz, DMSO-d6): δ = 7.46 (1H, dd, J = 8.2, 2.2 Hz, H-6′), 7.43 (1H, d, J = 2.4 Hz, H-2′), 7.30 (1H, d, J = 8 Hz, H-5′), 6.77 (1H, d, J = 1.9 Hz, H-8), 6.74 (1H, s, H-3), 6.43 (1H, d, J = 1.8 Hz, H-6), 5.14 (1H, d, J = 7.4 Hz, H-1″), 3.74 (1H, br d, J = 10.2 Hz, H-6″a), 3.58 to 3.24 (5H, m, H-2″, 3″, H-4″, 5″, 6″b).

^{13}C NMR (100 MHz, DMSO-d6): δ = 182.4 (C-4), 172.3 (C-6″), 165.7 (C-2), 164.1 (C-7), 161.7 (C-5), 157.6 (C-9), 149.7 (C-4′), 146.2 (C-3′), 121.5 (C-6′), 119.9 (C-1′), 115.5 (C-5′), 112.6 (C-2′), 105.2 (C-10), 103.9 (C-3), 100.4 (C-6), 99.2 (C-1″), 97.9 (C-8), 76.7 (C-3″), 75.2 (C-5″), 72.8 (C-2″), 70.8 (C-4″).

Compound III (Kaempferol-3-O-[2-O-(6-O-feruloyl)-β-D-glucopyranosyl]-β-D-galactopyranoside): A dark yellow amorphous powder; mp 210-214°C; Mol. formula $C_{37}H_{38}O_{19}$, Mol wt. 786. TLC R_f: 0.64 (Benzene: Methanol: Ammonia).

UV (MeOH) λ_{max} 269, 328 nm; + NaOAc: 273, 369; + NaOAc + H_3BO_3: 270, 339; + $AlCl_3$: 259 sh, 278, 295 sh, 351, 387 sh; + $AlCl_3$ + HCl: 256 sh, 280, 293 sh, 345, 387 sh; + NaOH: 269, 302 sh, 379.

IR (KBr) ν_{max} cm^{-1}: IR 3410 cm^{-1} (OH) 3500-3300, 2925 (C-H) 2930-2853, 1610 (C=O in flavon), 1610-1444 (Aromatic rings).

^{1}H NMR (DMSO-d_6, 500 MHz): δ 12.50 (1H, s, C-5, OH), 8.13 (2H, d, *J*= 8.8, H-2′,6′), 7.45 (1H, d, *J*) 15.9, H- γ), 7.14 (1H, d, *J*) 1.6, H-2‴′), 6.87 (1H, dd, *J* = 8.1, 1.6, H-6‴′), 6.85 (2H, d, *J*= 8.8, H-3′, 5′), 6.77 (d, *J*=) 8.1, H-5‴′), 6.32 (1H, br s, H-8), 6.24 (1H, d, *J* = 15.9, H-*â*), 6.14 (1H, br s, H-6), 5.46 (1H, d, *J* = 7.5, H-1″), 4.73 (1H, *J*= 7.8, H-1‴), 3.73 (3H, s, OCH_3), 2.4-4.3 (m, sugar protons).

^{13}C NMR (DMSO-d_6, 100 MHz): δ 177.4 (s, C-4), 166.5 (s, C-α), 164.1 (s, C-7), 161.2 (s, C-5), 159.9 (s, C-4′), 156.2 (s, C-2), 155.4 (s, C-9), 149.2 (s, C-4′′′′), 147.8 (s, C-3′′′′), 145.0 (d, C-γ), 132.9 (s, C-3), 131.0 (d, C-2′, C-6′), 125.4 (s, C-1′′′′), 122.9 (d, C-6′′′′), 120.8 (s, C-1′), 115.3 (d, C-5′′′′), 115.2 (d, C-3′, C-5′), 113.9 (d, C-β), 110.9 (d, C-2′′′′), 104.5 (d, C-1′′′), 103.8 (s, C-10), 98.6 (d, C-6), 98.4 (d, C-1′′), 93.6 (d, C-8), 81.1 (d, C-2′′), 76.2 (d, C-5′′), 75.8 (d, C-3′′′), 74.5 (d, C-2′′′), 74.0 (d, C-5′′′), 73.3 (d, C-3′′), 69.5 (d, C-4′′′), 67.5 (d, C-4′′), 63.3 (t, C-6′′′), 59.8 (t, C-6′′), 55.6 (q, OCH_3).

Table 10: ^{1}H NMR (500 MHz, DMSO-d_6) and ^{13}C NMR (100 MHz, DMSO-d_6) data of Compound I, II and III from *G. rottleriformis* bark

Position Aglycone	Compound I		Compound II		Compound III	
	δC	δH	δC	δH	δC	δH
2	157.5	-	165.7	-	157.4	-
3	133.6	6.74s	103.9	6.74 s	134.6	-
4	177.9	-	182.4	-	177.6	-
5	160.8	12.74	161.7	-	161.8	12.5 s
6	99.8	6.26 d	100.4	6.43 d	99.8	6.14 br s,
7	164.7	-	164.1	-	163.4	-
8	94.1	6.4 d	97.9	6.77 d	95.2	6.32 br s,
9	156.4	-	157.6	-	155.4	-
10	104.1	-	105.2	-	105.8	-
1′	121.6	-	119.9	-	121.4	-
2′	115.5	7.48 d	112.6	7.43 d	131.8	8.13 d
3′	145.3	-	146.2	-	116.6	6.85 d
4′	148.6	-	149.7	-	160.8	-
5′	116.9	6.87 d	115.5	7.30 d	116.6	
6′	122.6	7.54 d	121.5	7.46 dd	131.8	
	Glu		**Sugar**		**β-Galc**	
1″	101.8	5.30 d	99.2	5.14 d	99.2	5.46 d
2″	74.2		72.8	3.24 -3.58	81.3	4.43 m
3″	76.4	3.30-	76.7	3.24-3.58	73.3	4.34 m
4″	70.8	3.80	70.8	3.30 t	67.3	4.26
5″	75.7		75.2	3.00 m	76.4	4.28
6″	67.6	3.72 dd	172.3	3.74 d	59.8	3.73
	Rham			3.49 d	**β-Glu**	
1‴	101.4				104.2	4.73
2‴	70.9	4.41 d			74.8	3.26
3‴	70.6				75.6	3.18
4‴	72.4	1.20 d			69.6	3.10
5‴	68.6				74.2	2.76
6‴	18.4				63.3	2.43
					β-ferulolyl	
1⁗					125.7	7.45 d
2⁗					110.6	7.14 d
3⁗					147.3	3.73 s
4⁗					149.2	6.24 d
5⁗					115.2	6.77 d
6⁗					122.3	6.87 dd

Spectral Analysis

The compounds (**IV, V** and **VI**) obtained with the ethanol extract of *C. tora* leaves have identified and spectral data's were depicted in the Table 11 and **Fig. 31 -42**

Compound IV (Luteolin-7-O-β-glucopyranoside): A yellow amorphous powder with chromatographic properties: R_f 0.53 (Benzene: Methanol: Ammonia); deep yellow fluorescence ($AlCl_3$) and green color with $FeCl_3$ spray reagents. UV λ_{max} MeOH nm: 269, 336; + NaOAc: 273, 369; + NaOAc + H_3BO_3: 270, 339; + $AlCl_3$: 259 sh, 278, 295 sh, 351, 387 sh; + $AlCl_3$ + HCl: 256 sh, 280, 293 sh, 345, 387 sh; + NaOH: 269, 302 sh, 379. IR (KBr) λ_{max} 3340 (OH), 1634 (C=C), 1507, 1374 (aromatic ring) cm-1. Negative ESI-MS ($C_{21}H_{20}O_{11}$) *m/z*: 447 $[M-H]^-$; 285 $[M-H-162]^-$ = $[aglycone-H]^-$.

^{1}H NMR (500 MHz, DMSO-d6): δ = 12.97 (1H, s, H-bounded OH-5), 7.44 (1H, dd, J = 8.1, 1.8 Hz, H-6′), 7.42 (1H, d, J = 1.8 Hz, H-2′), 6.90 (1H, d, J = 8.1 Hz, H-5′), 6.78 (1H, d, J = 2.1 Hz, H-8), 6.74 (1H, s, H-3), 6.44 (1H, d, J = 2.1 Hz, H-6), 5.08 (1H, d, J = 7.2 Hz, H-1″), 3.72 (1H, br d, J = 9.9 Hz, H-6″a), 3.55 to 3.10 (5H, m, H-2″, 3″, H-4″, 5″, 6″b).

^{13}C NMR (100 MHz, DMSO-d_6): δ = 183.82 (C-4), 166.8 (C-2), 165.8 (C-7), 162.96 (C-5), 158.2 (C-9), 149.7 (C-4′), 148.2 (C-3′), 121.9 (C-1′), 119.8 (C-6′), 114.9 (C-5′), 112.6 (C-2′), 105.84 (C-10), 104.74 (C-3), 102.8 (C-1″), 99.4 (C-6), 94.6 (C-8), 78.7 (C-5″), 77.9 (C-3″), 74.82 (C-2″), 70.8 (C-4″), 61.9 (C-6″).

Compound V (Quercetin-3-*O*-β-d-glucuronide): A yellow amorphous powder with chromatographic properties: R_f 0.61 (Benzene: Methanol: Ammonia); It gave a red colour with Mg-HCl, olive green with alcoholic Fe^{3+}, golden yellow colour with NH_3 and NaOH. UV λ_{max} MeOH nm: 256, 275sh, 303sh, 370; +NaOMe 248sh, 325, 414; +A1Cl_3 271, 302sh, 335, 460; +$AlCl_3$-HCl 267, 303sh, 330, 429; +NaOAc 272, 332, 387; and +NaOAc-H_3BO_3, 263,

304sh, 340 nm. IR (KBr) λ_{max} 3058 (OH), 1682 (C=C), 1507, 1334 (aromatic ring) cm-1. Negative ESI-MS ($C_{21}H_{27}O_{13}$) m/z 477 $[M - H]^-$

^{1}H NMR (500 MHz, DMSO-d6): δ= 12.52 (1H, s, H-5), 8.06 (1H, br s, H-2′), 7.38 (1H, d, *J* =8.2 Hz, H-6′), 6.80 (1H, d, *J* = 8.2 Hz, H-5′), 6.37 (1H, br s, H-8), 6.23 (1H, br s, H-6), 5.32 (1H, d, *J* = 6.5 Hz, H-1″).

^{13}C NMR (100 MHz, DMSO-d_6): δ= 177.4 (C-4), 172.2 (C-6′), 164.3 (C-7), 161.1 (C-5), 157.1 (C-2), 156.7 (C-9), 148.4 (C-4′), 144. 6 (C-3′), 133.7 (C-3), 121.3 (C-1′), 122.6 (C-6′), 117.3 (C-2′), 115.2 (C-5′), 104.3 (C-10), 101.6 (C-1″), 99.7 (C-6), 93.1 (C-8), 76.9 (C-3″), 74.7 (C-5″), 74.4 (C-2″), 71.6 (C-4″).

Compound VI (Formononetin-7-O-Glucoside): Pale yellow powder with Mol wt 431, mp 204-206°C; UV (MeOH) λ_{max} 208, 259, IR (KBr) νmax cm^{-1}: IR 3402cm^{-1}(OH) 2924, 2517, 2170, 1625 (C=O in flavon), 1530. TLC R_f: 0.76 (Benzene:Methanol:Ammonia). UV λ_{max} MeOH nm: 254, 268, 346; +NaOMe: 263, 309 sh, 398; +NaOAc + H_3BO_3: 268, 293 sh, 370; + $AlCl_3$: 276, 298 sh, 386; + $AlCl_3$ /+ HCl: 270, 289 sh, 353, 387; +NaOH: 243 sh, 270, 304 sh, 392.

^{1}H NMR (500 MHz, DMSO-d_6): δ = 8.44 (1H, s, H-2), 8.08 (1H, d, J = 8.8 Hz, H-5), 7.54 (1H, d, J = 6.6, 2.3 Hz, H-6′), 7.28 (1H, d, J = 2.2 Hz, H-8), 7.14 (1H,dd, H-6), 7.10 (1H, d, J = 9 Hz, H-5′), 5.14 (1H, d, J = 7.2 Hz, H-1″), 3.79 (3H, s, OCH_3), 3.70 to 3.31 (5H, m, H-2″, 3″, 4″, 5″, 6″).

^{13}C NMR (DMSO-d_6, 100 MHz) δ 173.7 (C-4), 160.2 (C-7), 158.8 (C-4’), 156.9 (C-9), 154.4 (C-2), 130.8 (C-2′, C-6′), 125.6 (C-1′), 125.3 (C-3, C-5), 118.4 (C-10), 115.3 (C-6), 113.7 (C-3′, C-5′), 104.4 (C-8), 102.1 (C-1″), 77.4 (C-5″), 74.6 (C-2″), 73.4 (C-3″), 61.4 (C-6″), 54.7 (OCH_3).

Table 11: ^{1}H NMR (500 MHz, DMSO-d6) and ^{13}C NMR (100 MHz, DMSO-d6) data of Compound IV, V and VI from *C. tora* leaves

Position	Compound IV		Compound V		Compound VI	
Aglycone	**δC**	**δH**	**δC**	**δH**	**δC**	**δH**
2	166.8	-	157.2	-	154.4	8.44 s
3	104.7	6.74s	133.7	6.17 s	125.3	-
4	183.8	-	177.4	-	173.7	-
5	162.9	12.97, s	161.1	12.52, s	125.3	8.08, d,
6	99.4	6.44 br s	99.7	6.23 br,s	115.3	7.14, dd
7	165.8	-	164.3	-	160.2	-
8	94.6	6.78 br s	93.1	6.37 br, s	104.4	7.28, d
9	158.8	-	156.7	-	156.9	-
10	105.8	-	104.3	-	118.4	-
1′	121.9	-	121.3	-	125.6	-
2′	112.6	7.42 br s	117.3	8.06 br,s	130.8	7.54,d
3′	148.2	-	144.6	-	113.7	7.10, d
4′	149.7	-	148.4	-	158.8	-
5′	114.9	6.90 d	115.2	6.80 d	113.7	7.10, d
6′	119.5	7.44 d	122.6	7.38 d	130.8	7.54,d
	β-Glu		**β-**		**β-Glu**	
1″	102.8	5.18 d	**Glucuronic**	5.32 d	102.1	5.14,d
2″	74.8	3.14-3.48	101.6	3.26 -3.60	74.6	3.31,m
3″	77.9	3.14-3.48	74.4	3.26-3.60	73.4	3.32,m
4″	70.8	3.55 t	76.9	3.28 t	68.9	3.17, m
5″	78.7	3.00 m	71.6	3.04 m	77.4	3.44, m
6″	61.9	3.78 d	74.7	3.74 d	61.4	3.46, m
		3.59 d	172.2	3.47 d		3.70, m
4′-OCH_3					54.7	3.79, s

6.3.9. HPLC Fingerprint analysis with known marker

Further the flavonoids present in both the plants were detected and quantified using HPLC by comparing with marker compound. The HPLC chromatograms of *Givotia rottleriformis* bark, *Cassia tora* leaves and marker compound were shown in **Fig. 43-46.**

6.3.10. HPTLC Fingerprint analysis with known marker

The flavonoids Rutin, Luteolin and Kaempferol in *Givotia rottleriformis* bark, were detected and quantified using HPTLC silica gel 60 F254 pre-coated plates using the solvent systems Benzene: Methanol: Ammonia (90:10:1 v/v/v). The flavonoids Luteolin, Quercetin and Formononetin in *Cassia tora* leaves were detected and quantified under similar condition.

The identity of flavonoids in sample chromatograms was confirmed by comparison of the R_f values and chromatogram obtained from the reference standard solution at various concentration 100-500 ng/spot at wave length 254 nm were shown in **Fig. 47-58**.

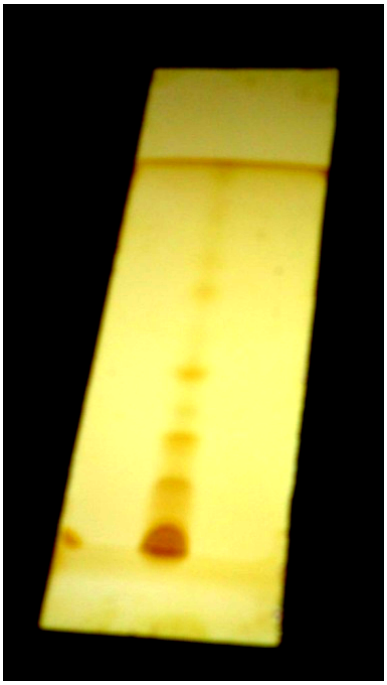


Figure 13: TLC of Ethanol extract (70% v/v) of *G. rottleriformis* bark

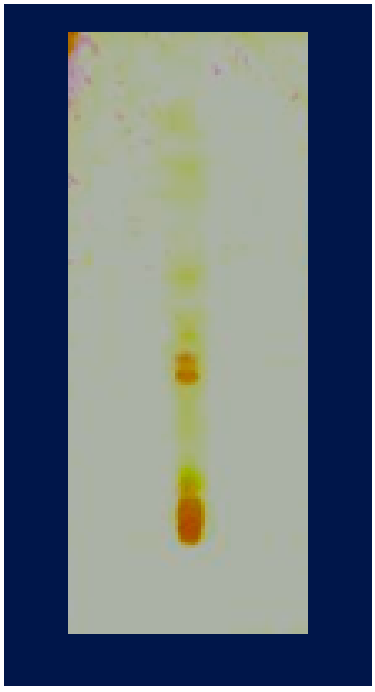
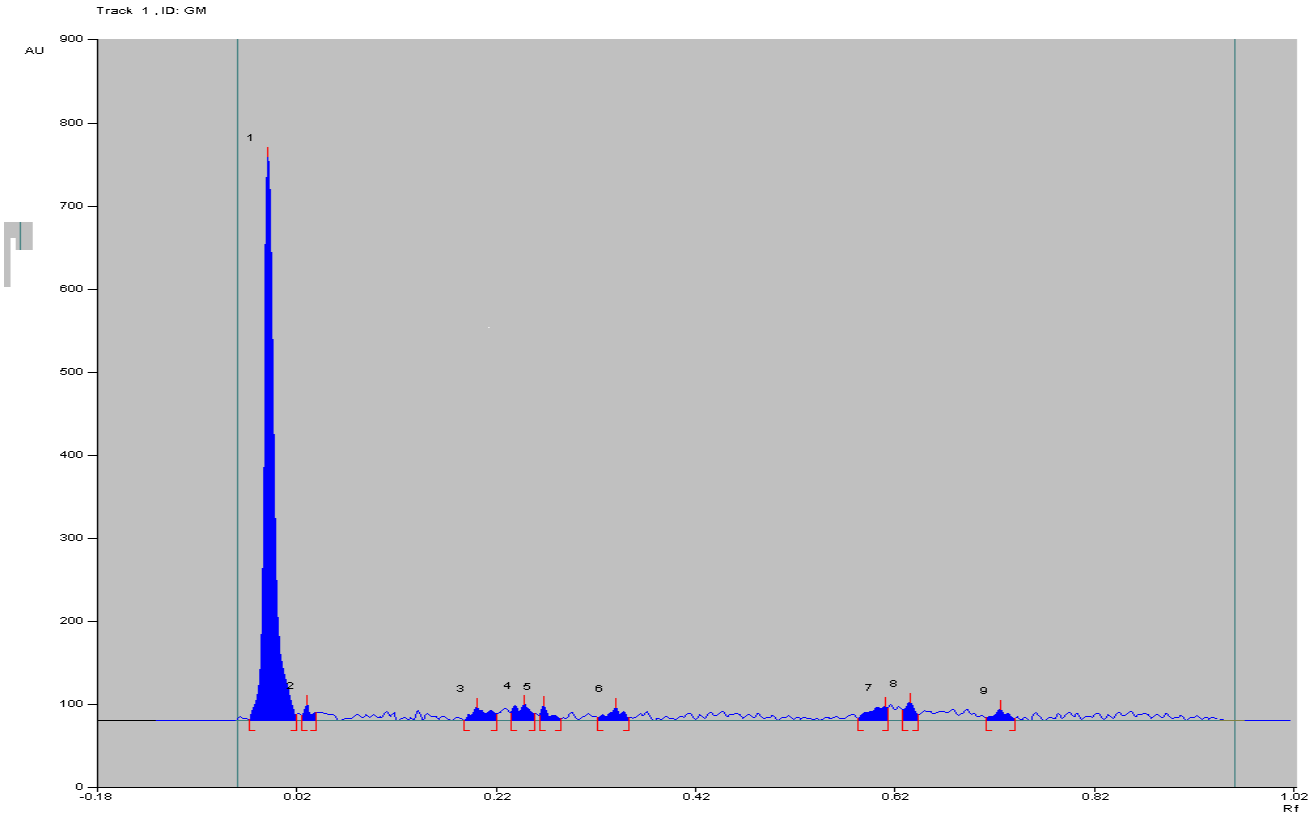
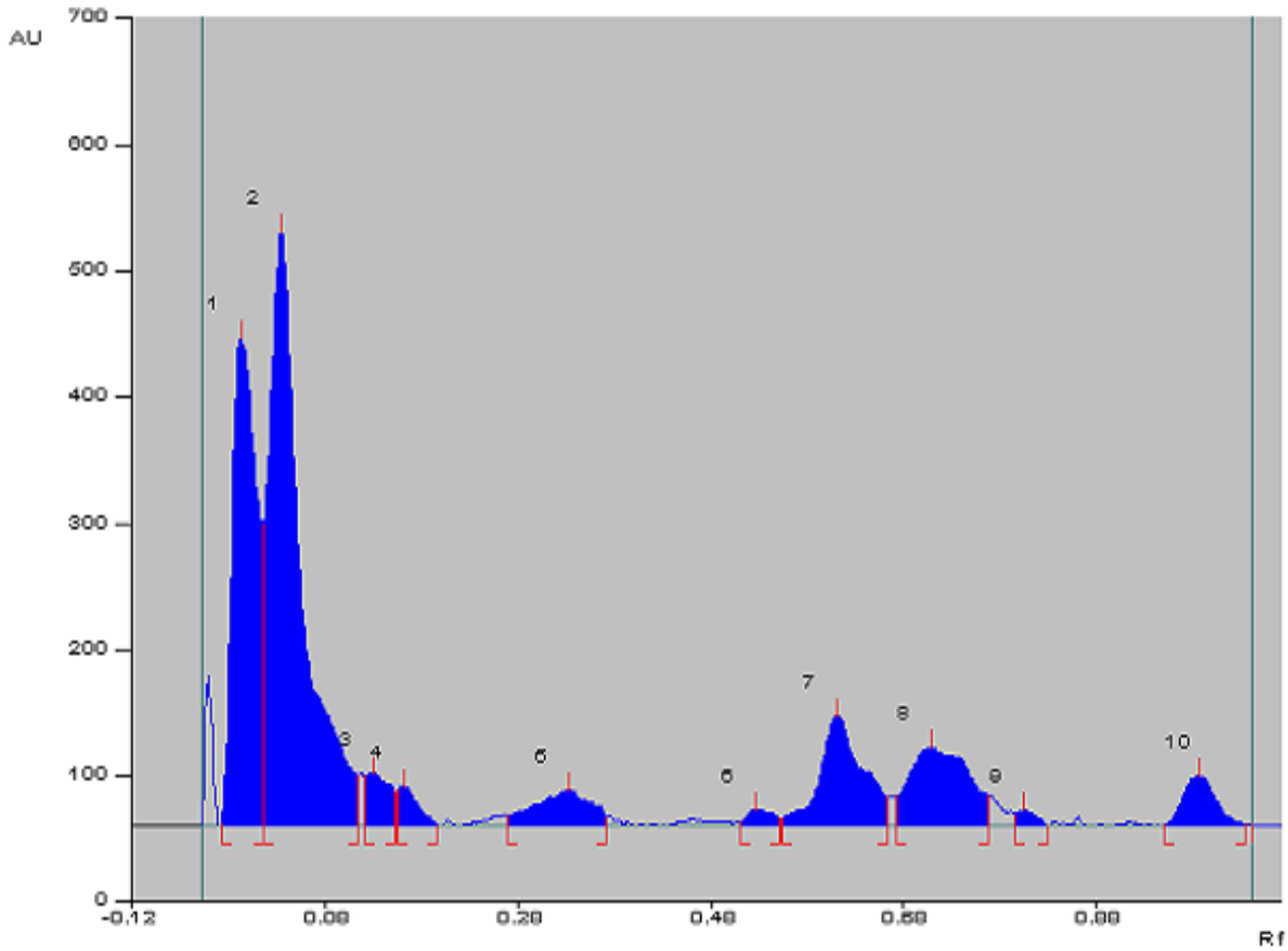


Figure 14: TLC of Ethanol extract (70% v/v) of *C. tora* leaves



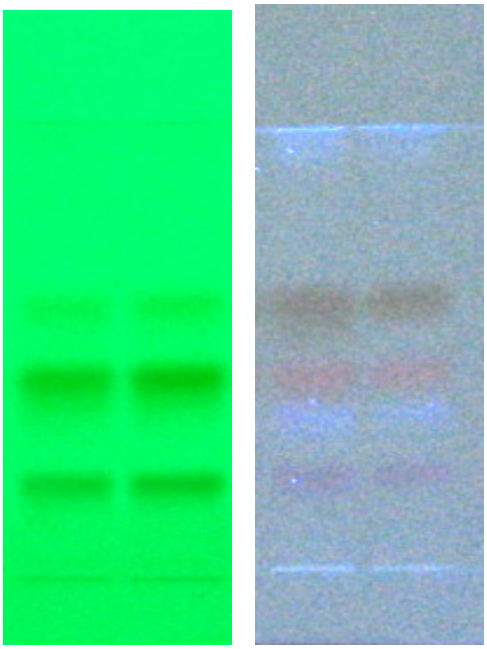
Peak	Start Rf	Start Height	Max Rf	Max Height	Max %	End Rf	End Height	Area	Area %	Assigned substance
1	-0.03	0.8	-0.01	678.5	83.02	0.02	7.1	6414.1	78.90	unknown *
2	0.03	6.2	0.03	18.7	2.28	0.04	9.3	138.2	1.70	unknown *
3	0.19	0.5	0.20	15.7	1.92	0.22	7.6	257.4	3.17	unknown *
4	0.23	8.7	0.25	19.3	2.37	0.26	8.6	287.3	3.53	unknown *
5	0.26	6.1	0.27	17.5	2.14	0.29	2.2	144.3	1.77	unknown *
6	0.32	3.5	0.34	15.8	1.94	0.35	3.2	213.0	2.62	unknown *
7	0.58	2.3	0.61	16.5	2.02	0.61	14.6	303.6	3.73	unknown *
8	0.63	12.4	0.64	21.5	2.63	0.64	6.9	199.1	2.45	unknown *
9	0.71	3.3	0.73	13.8	1.68	0.74	2.4	172.9	2.13	unknown *

Figure 15: HPTLC fingerprint of ethanol (70%v/v) extract of *Givotia rottleriformis* bark



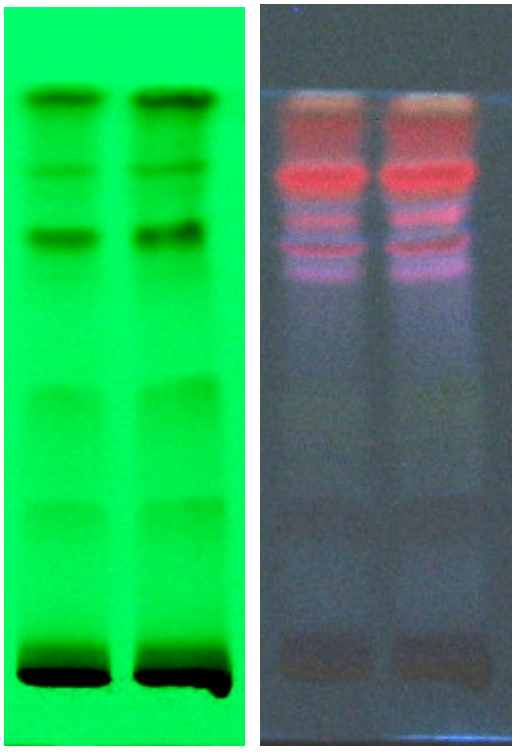
Peak	Start Rf	Start Height	Max Rf	Max Height	Max %	End Rf	End Height	Area	Area %	Assigned substance
1	-0.03	1.3	-0.00	386.6	32.90	0.02	237.6	7418.4	24.82	unknown *
2	0.02	238.6	0.04	471.6	40.14	0.12	40.4	12457.3	41.68	unknown *
3	0.12	39.6	0.13	41.5	3.53	0.16	27.3	831.7	2.78	unknown *
4	0.16	28.4	0.16	31.7	2.70	0.20	0.1	497.9	1.67	unknown *
5	0.27	8.0	0.34	28.6	2.43	0.37	6.2	1255.3	4.20	unknown *
6	0.51	2.4	0.53	13.4	1.14	0.56	6.2	267.3	0.89	unknown *
7	0.56	6.3	0.61	87.5	7.44	0.67	23.2	2957.0	9.89	unknown *
8	0.67	23.5	0.71	62.3	5.30	0.77	23.6	2962.5	9.91	unknown *
9	0.80	9.1	0.81	12.3	1.05	0.83	0.1	201.1	0.67	unknown *
10	0.95	1.6	0.99	39.6	3.37	1.04	1.3	1036.2	3.47	unknown *

Figure 16: HPTLC fingerprint of ethanol extract of *Cassia tora* leaves



254 nm **365 nm**

Figure 17: HPTLC chromatogram of ethanol extract of *G. rottleriformis*



256 nm **365 nm**

Figure 18: HPTLC Chromatogram of ethanol extract of *C. tora* leaves

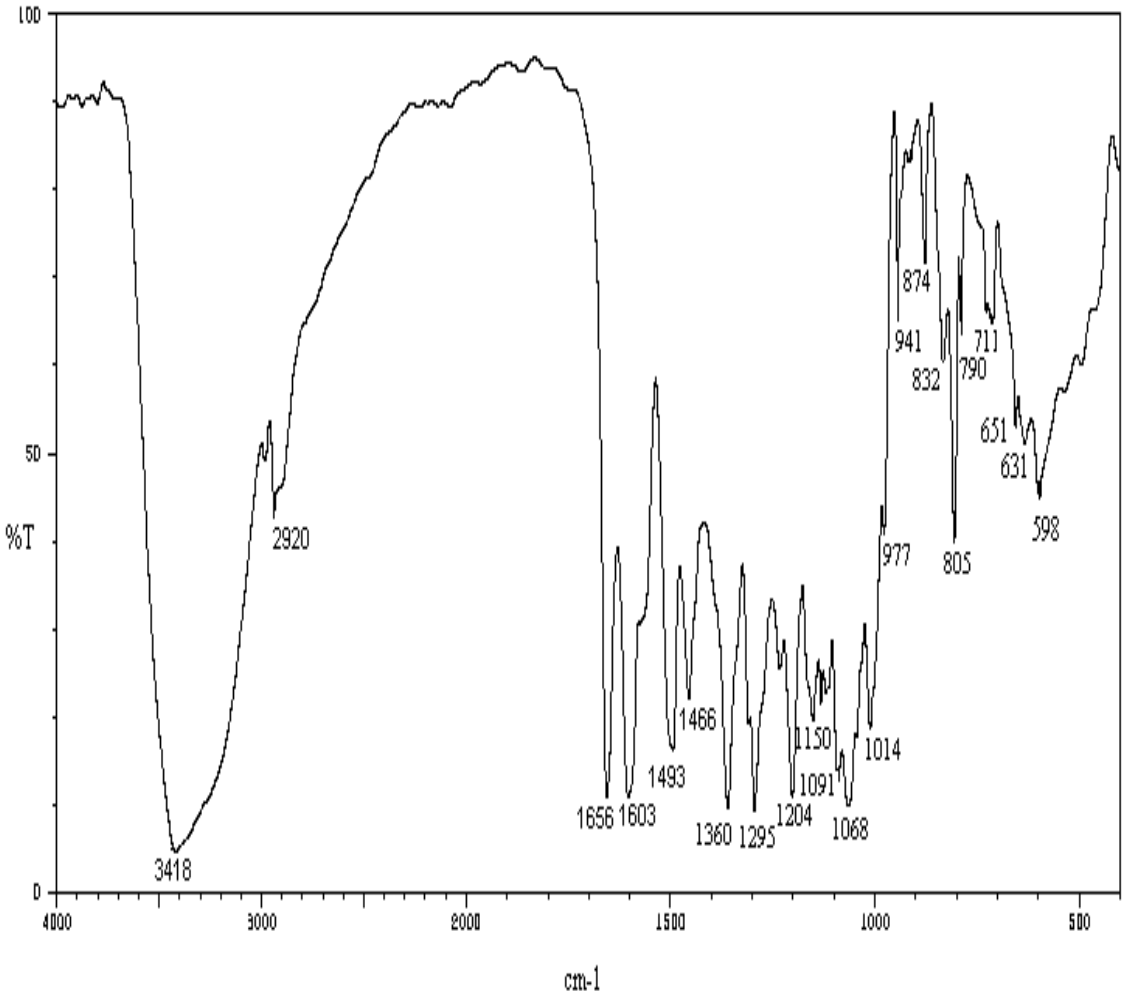


Figure 19: IR Spectrum of Compound I from *G. rottleriformis* bark

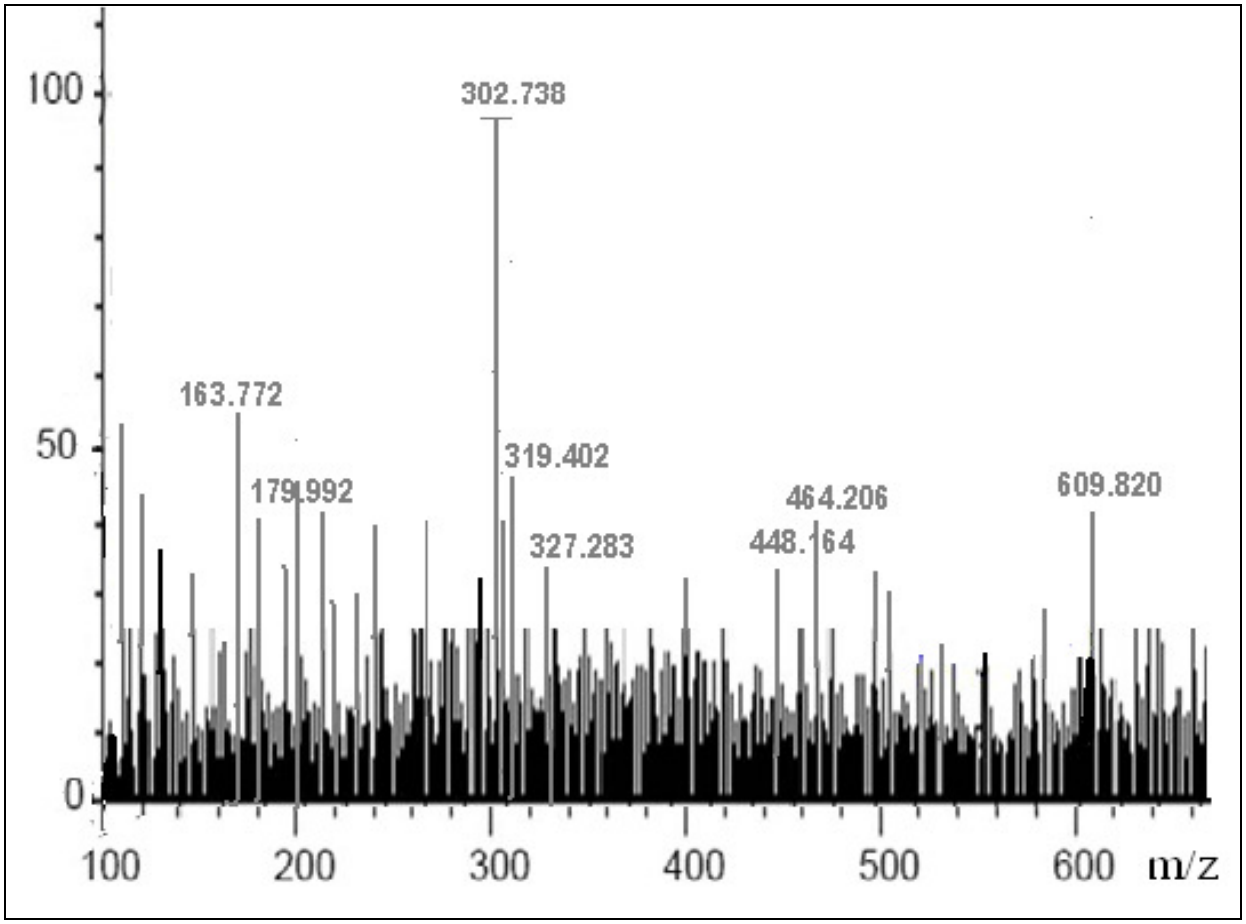


Figure 20: Mass Spectrum of Compound I from *G. rottleriformis* bark

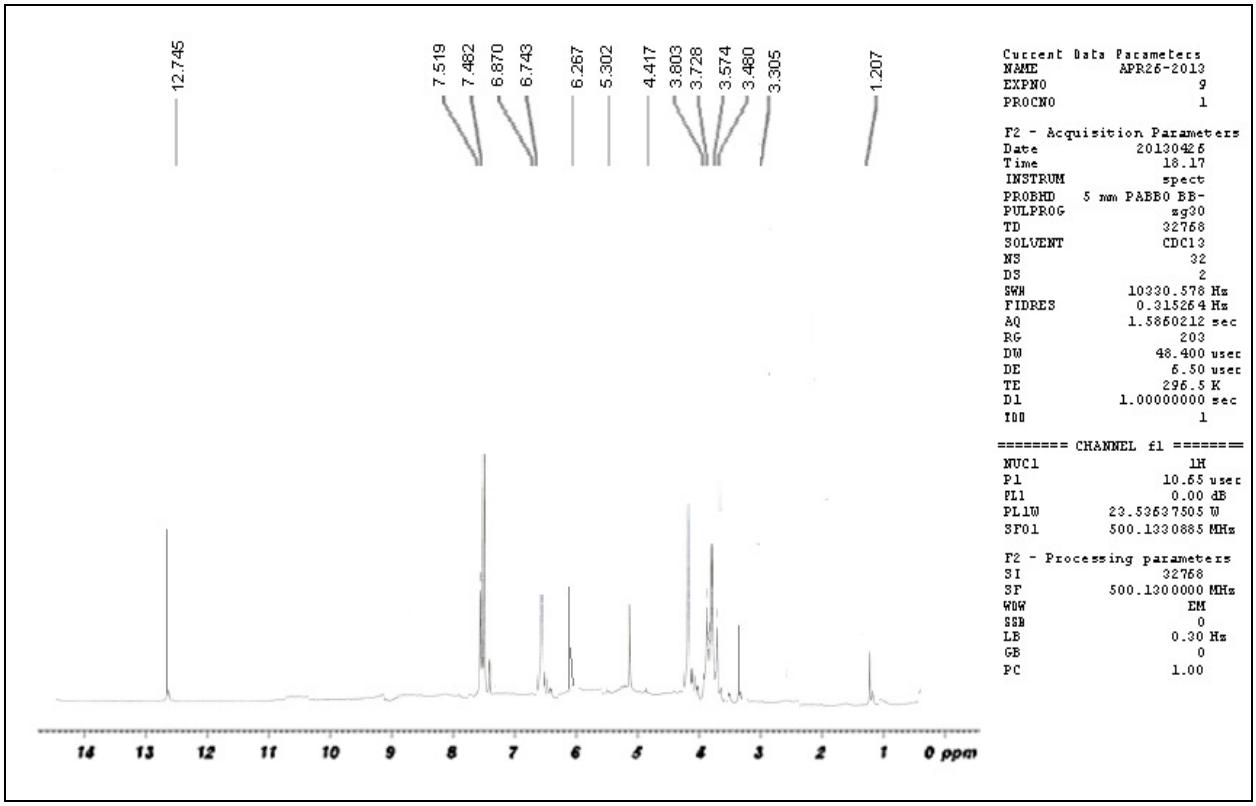


Figure 21: ^{1}H NMR Spectrum of Compound I from *G. rottleriformis* bark

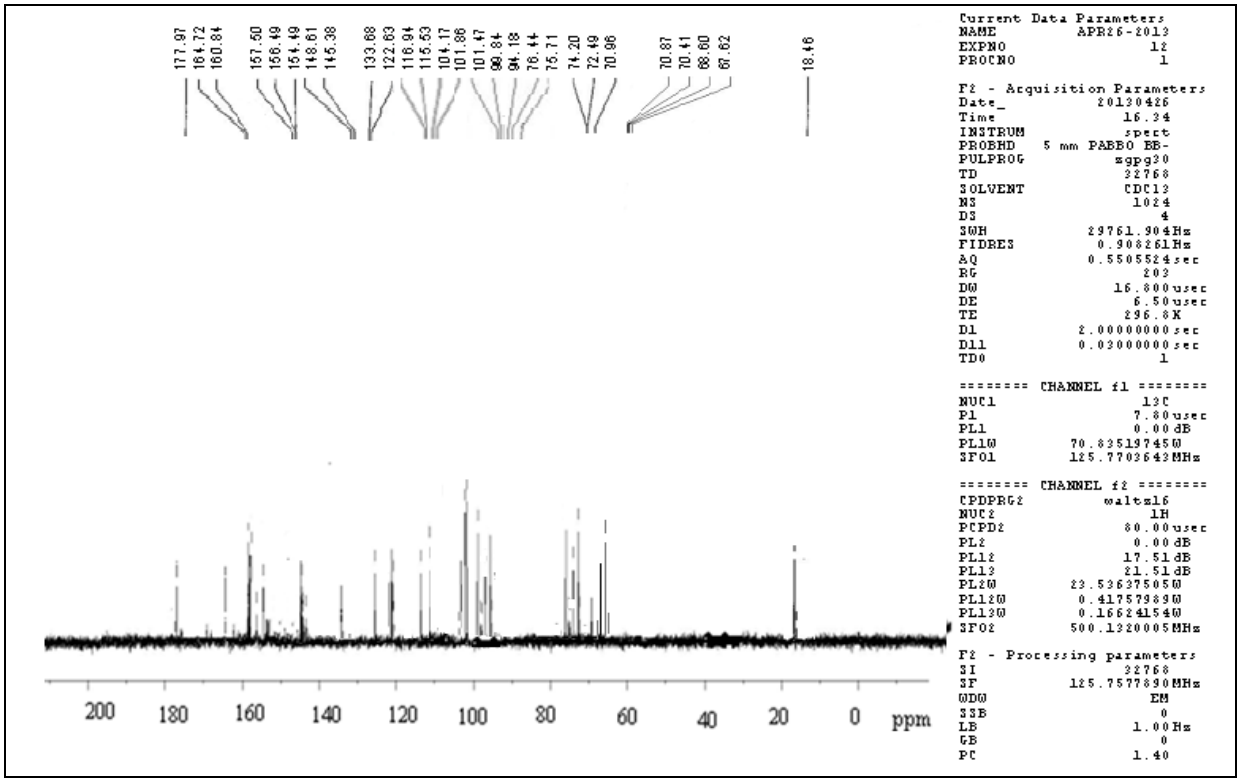


Figure 22: ^{13}C NMR Spectrum of Compound I from *G. rottleriformis* bark

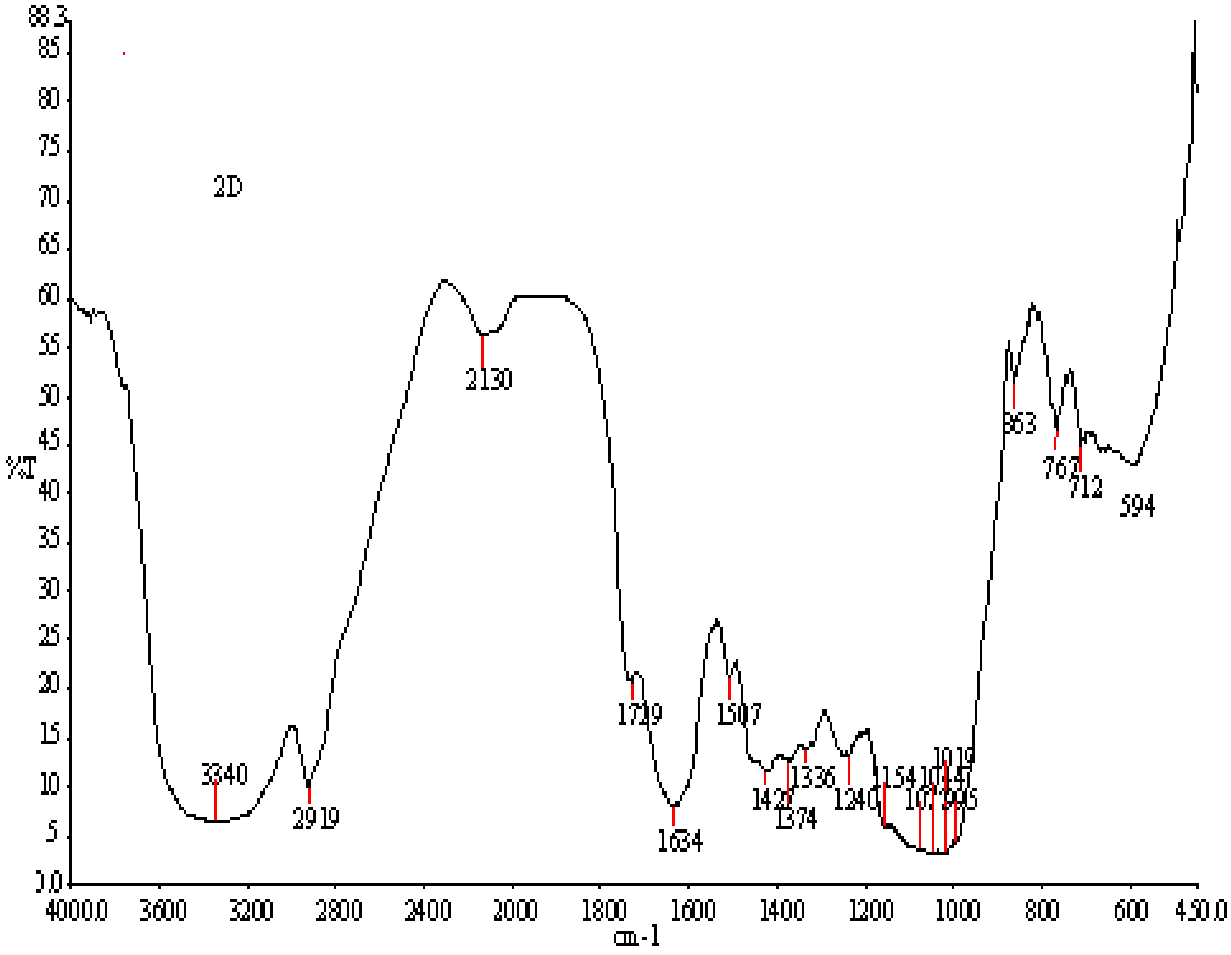


Figure 23: IR Spectrum of Compound II from *G. rottleriformis* bark

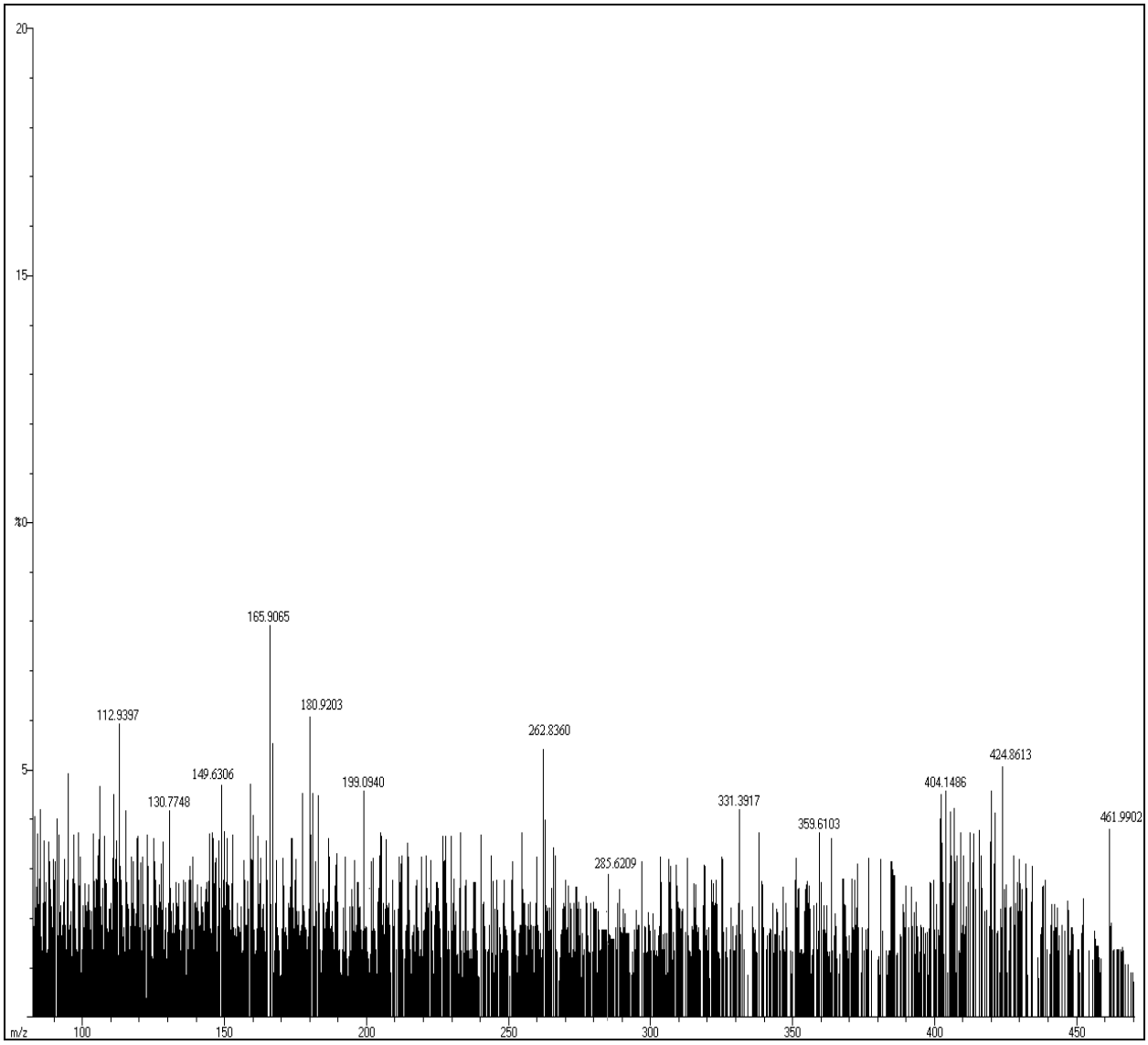


Figure 24: Mass Spectrum of Compound II from *G. rottleriformis* bark

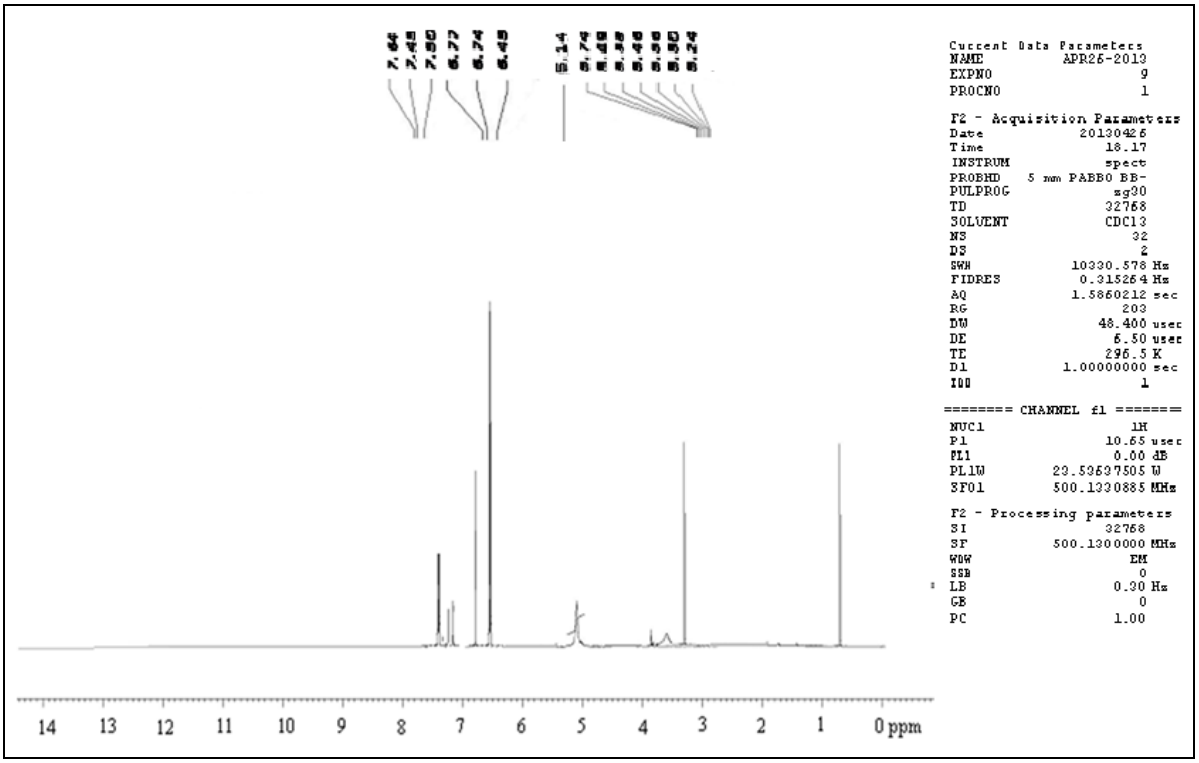


Figure 25: ^{1}H NMR Spectrum of Compound II from *G. rottleriformis* bark

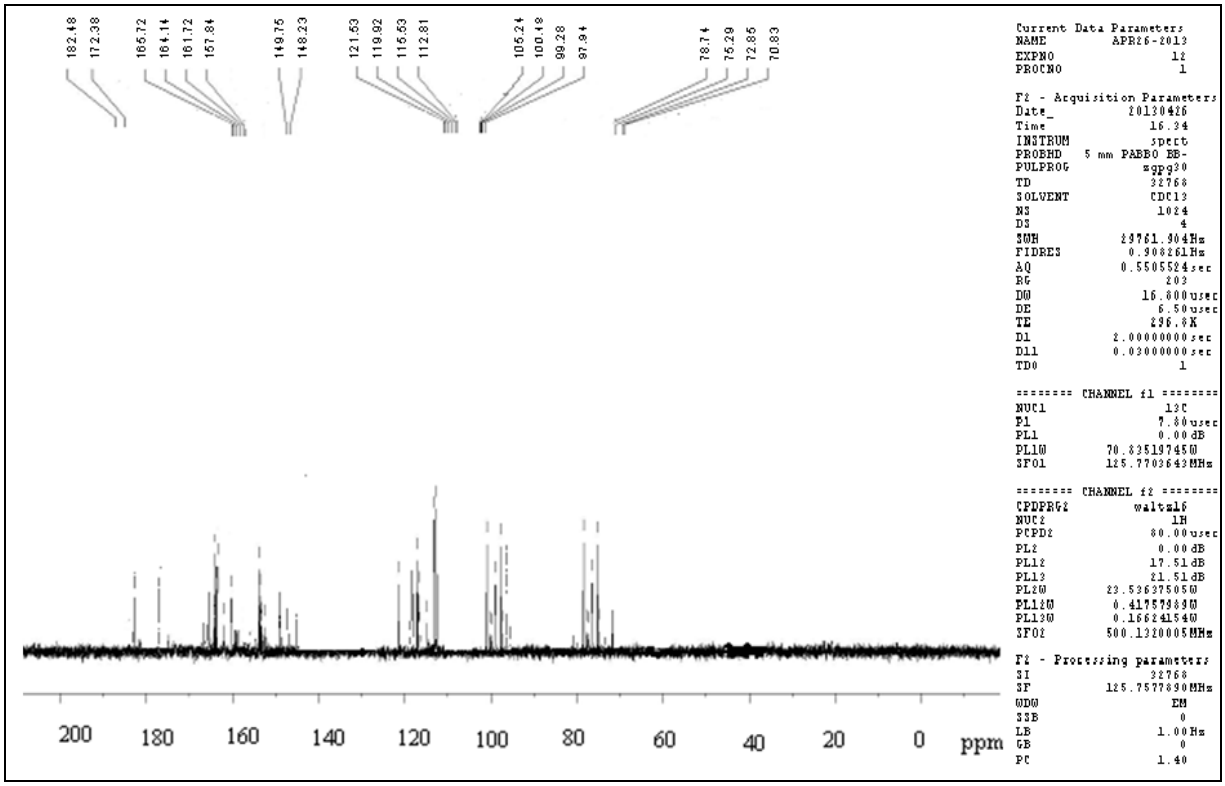


Figure 26: ^{13}C NMR Spectrum of Compound II from *G. rottleriformis* bark

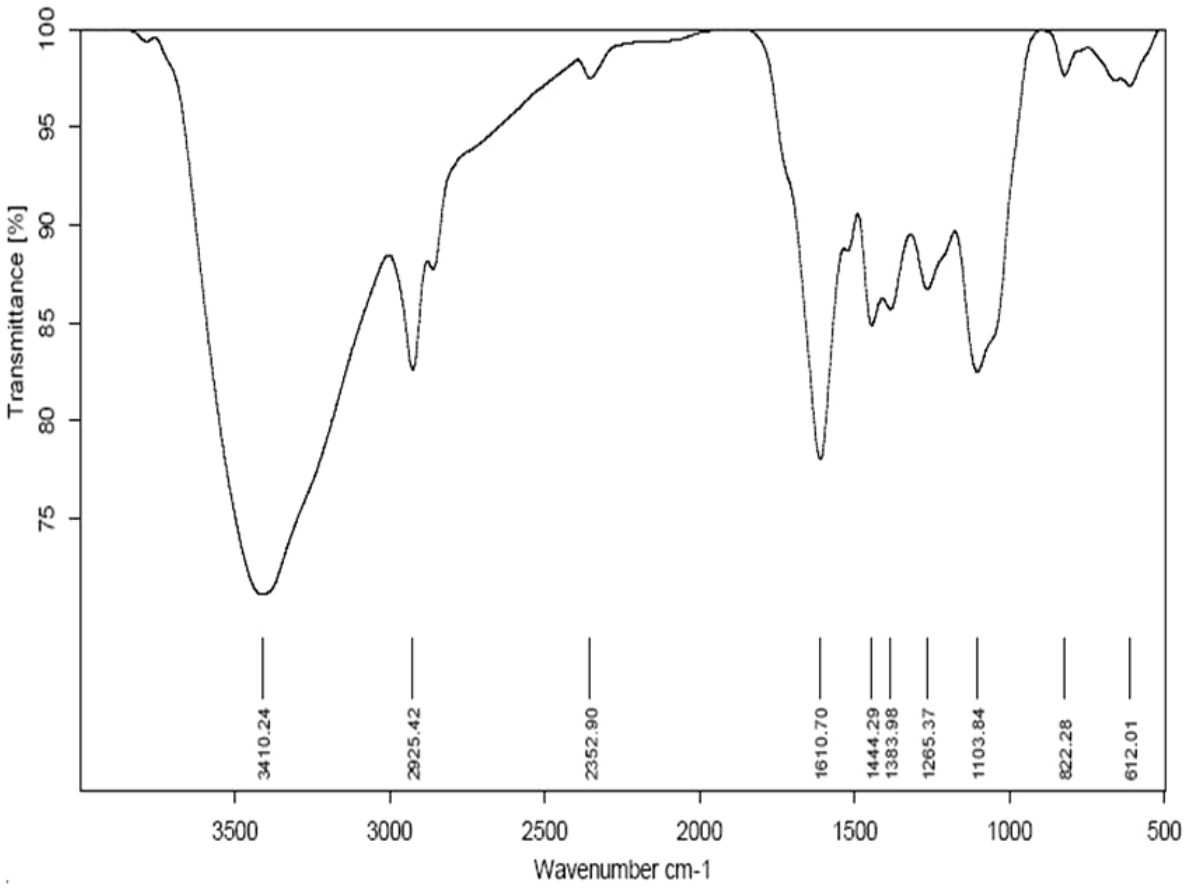


Figure 27: IR Spectrum of Compound III from *G. rottleriformis* bark

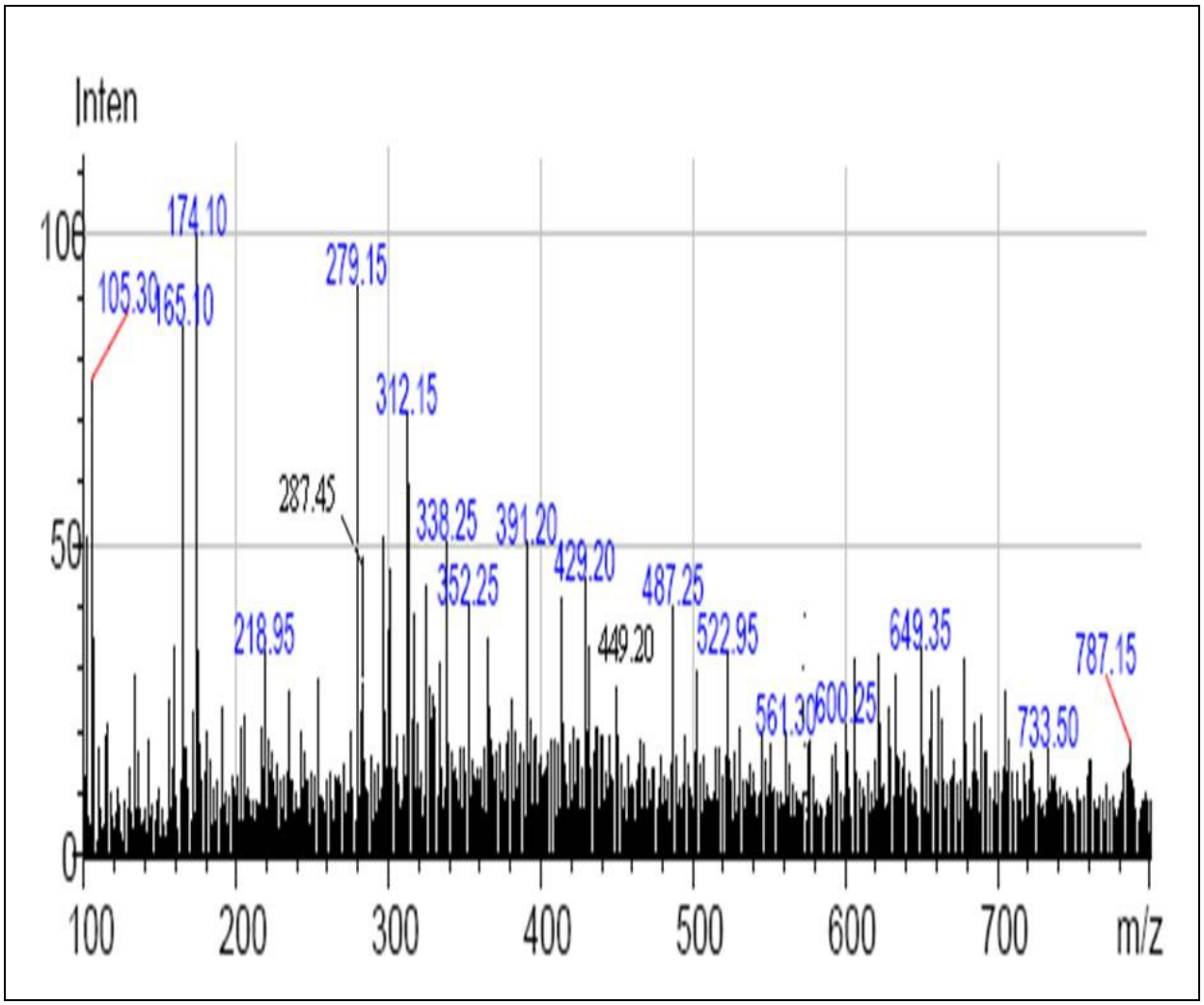


Figure 28: Mass Spectrum of Compound III from *G. rottleriformis* bark

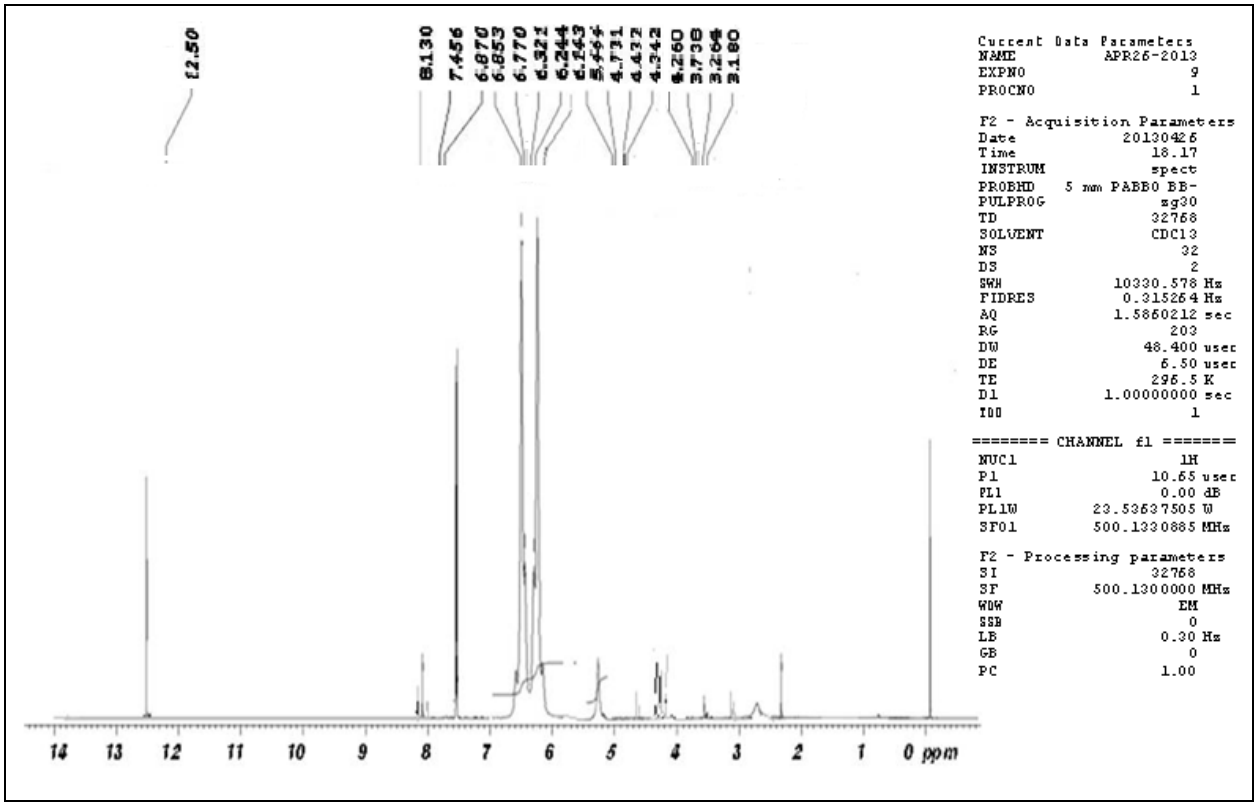


Figure 29: ^{1}H NMR Spectrum of Compound III from *G. rottleriformis* bark

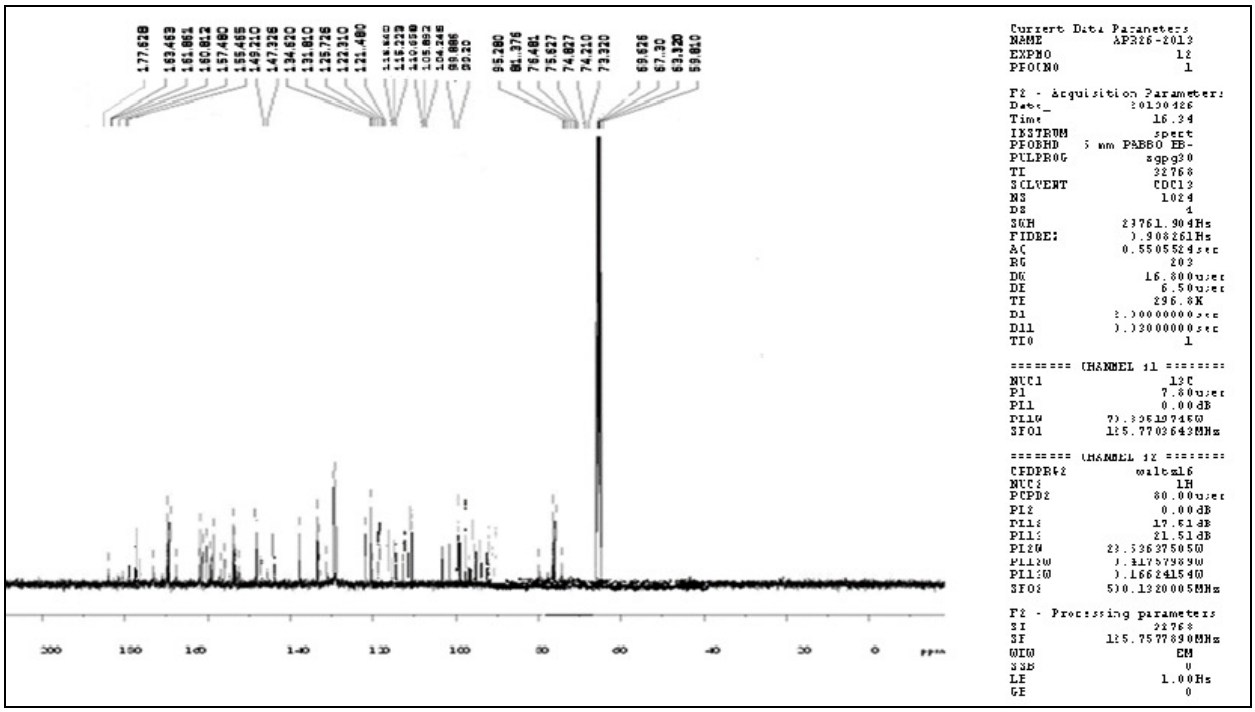


Figure 30: ^{13}C NMR Spectrum of Compound III from *G. rottleriformis* bark

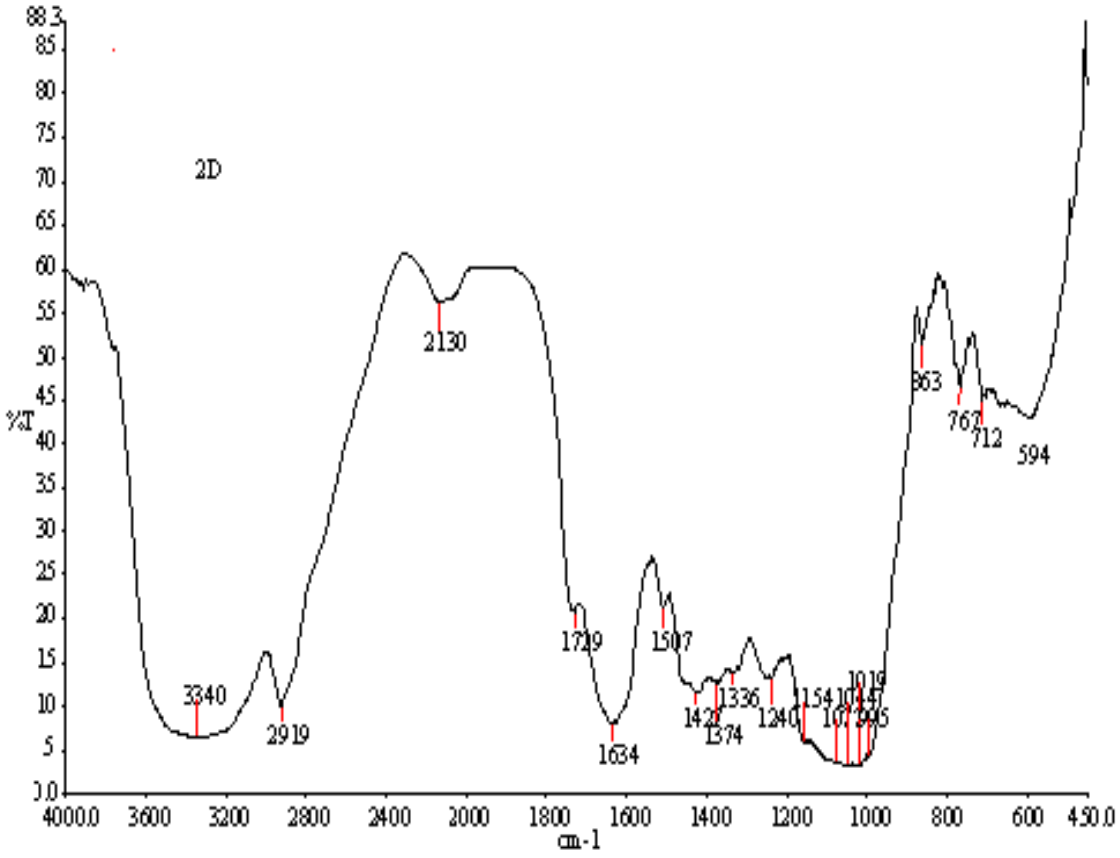


Figure 31: IR Spectrum of Compound IV from *C. tora* leaves

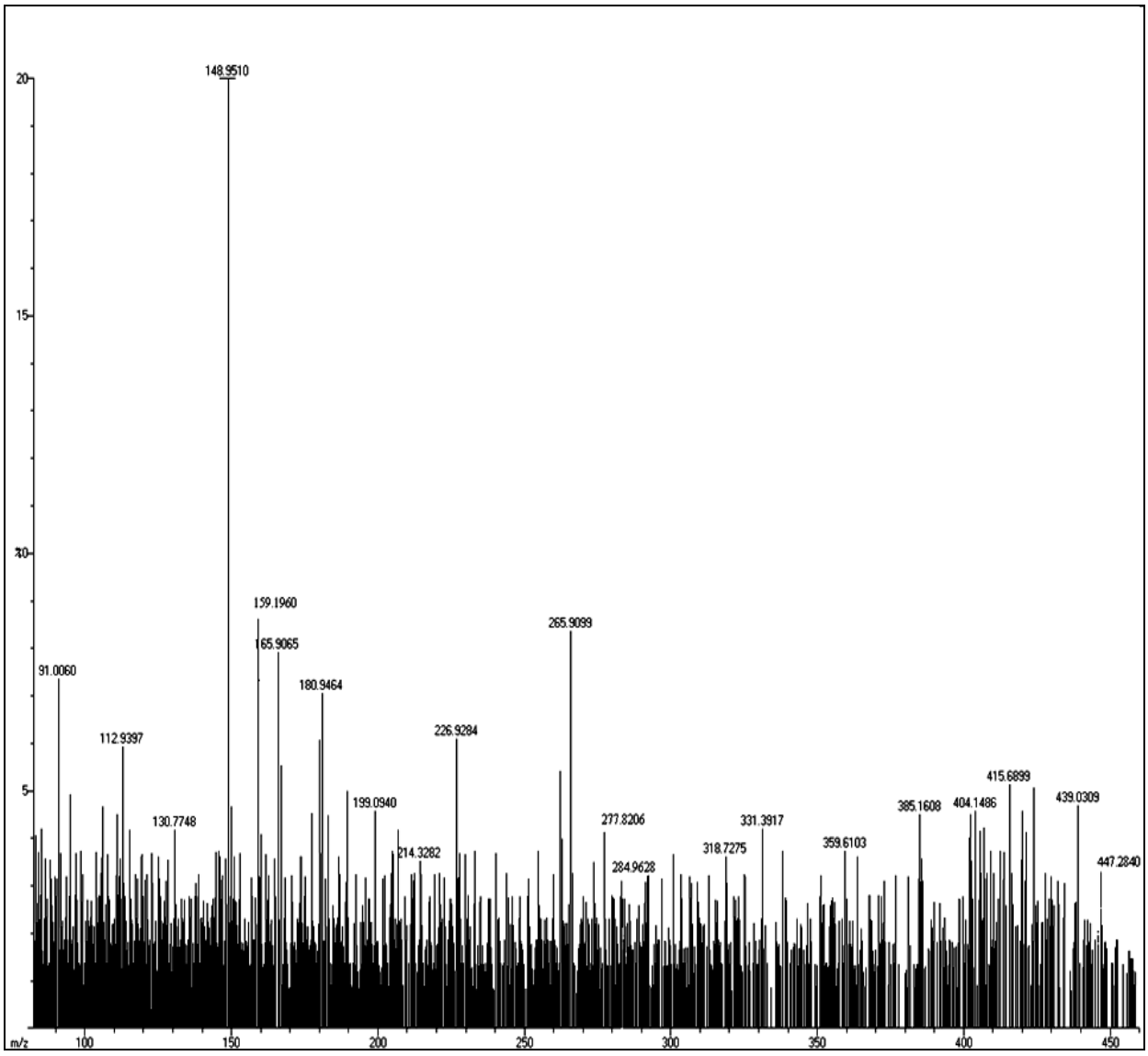


Figure 32: Mass Spectrum of Compound IV from *C. tora* leaves

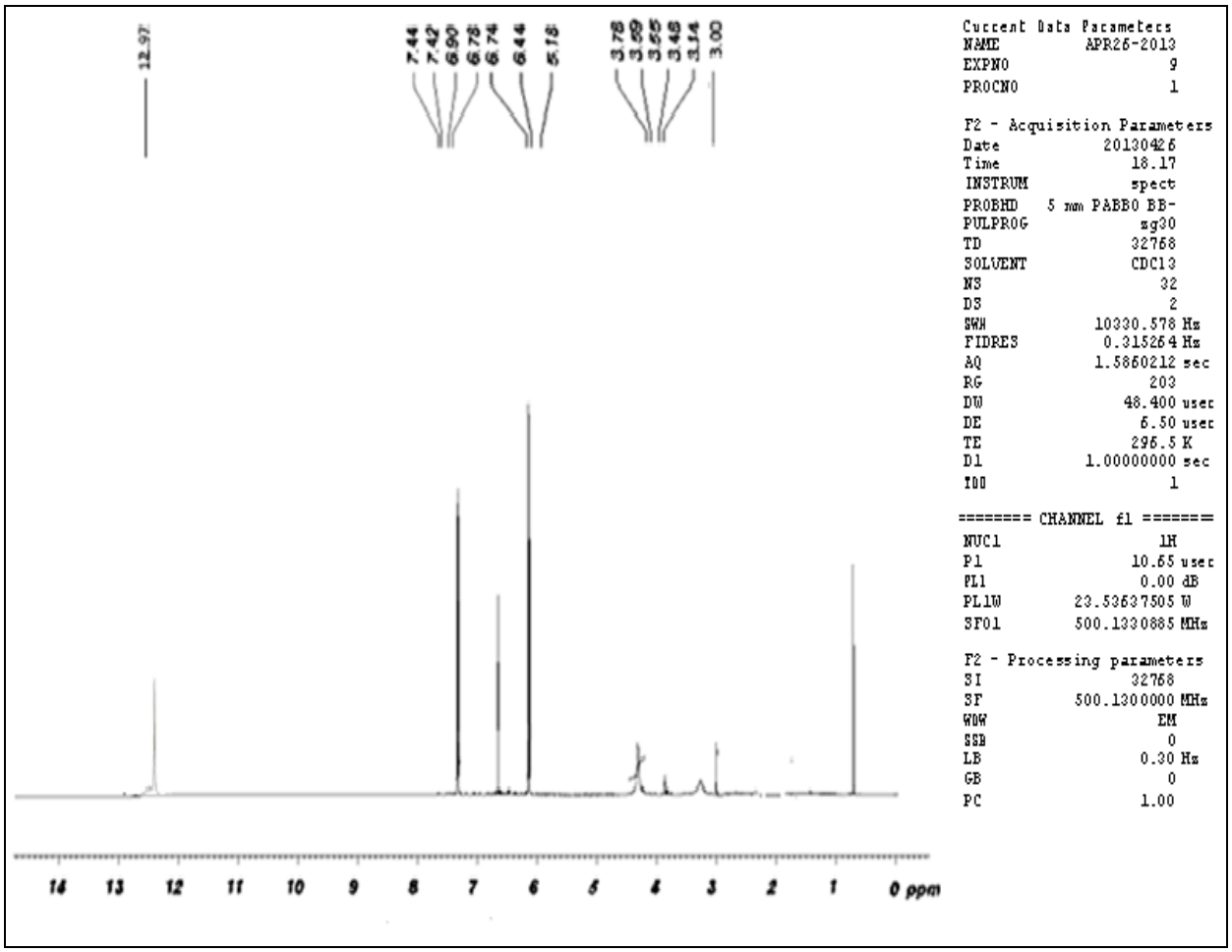


Figure 33: ^{1}H NMR Spectrum of Compound IV from *C. tora* leaves

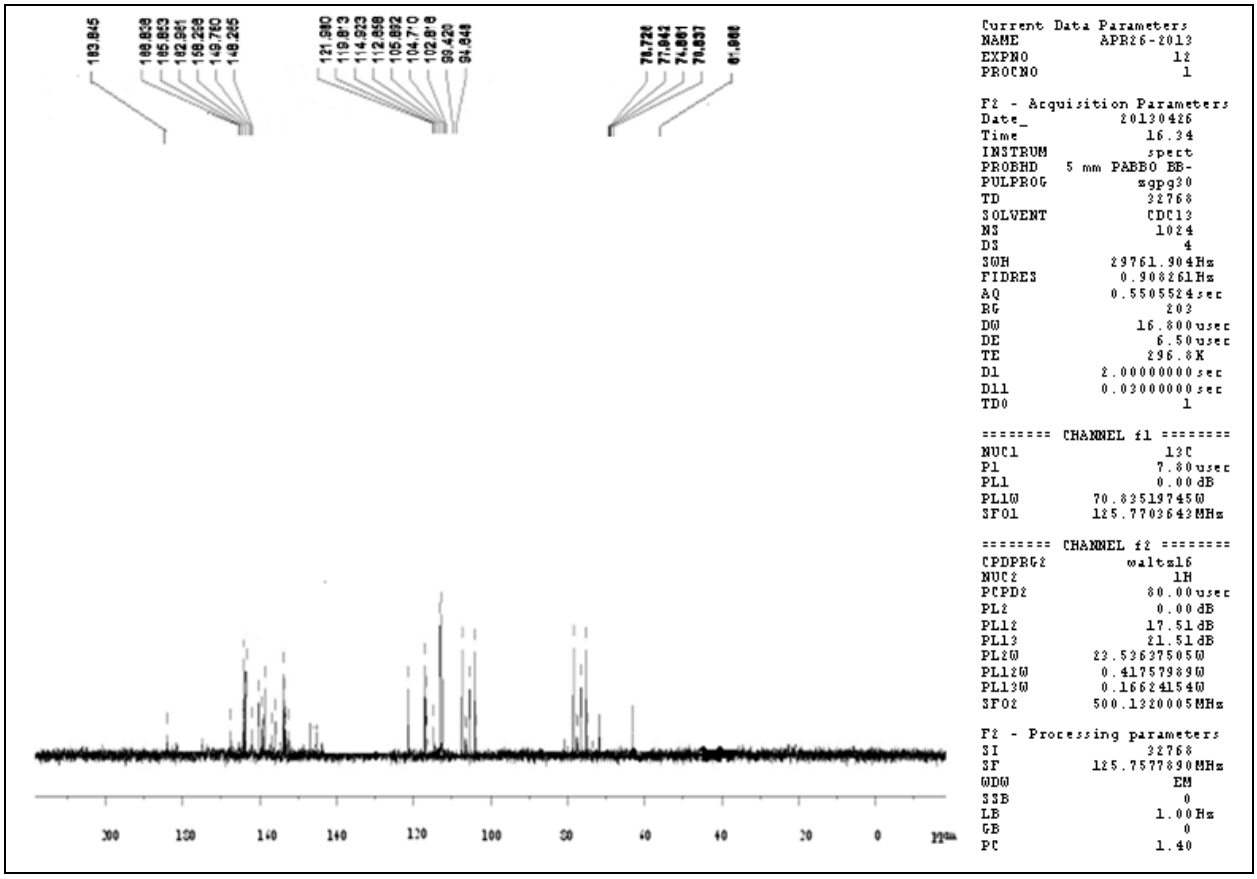


Figure 34: ^{13}C NMR Spectrum of Compound IV from *C. tora* leaves

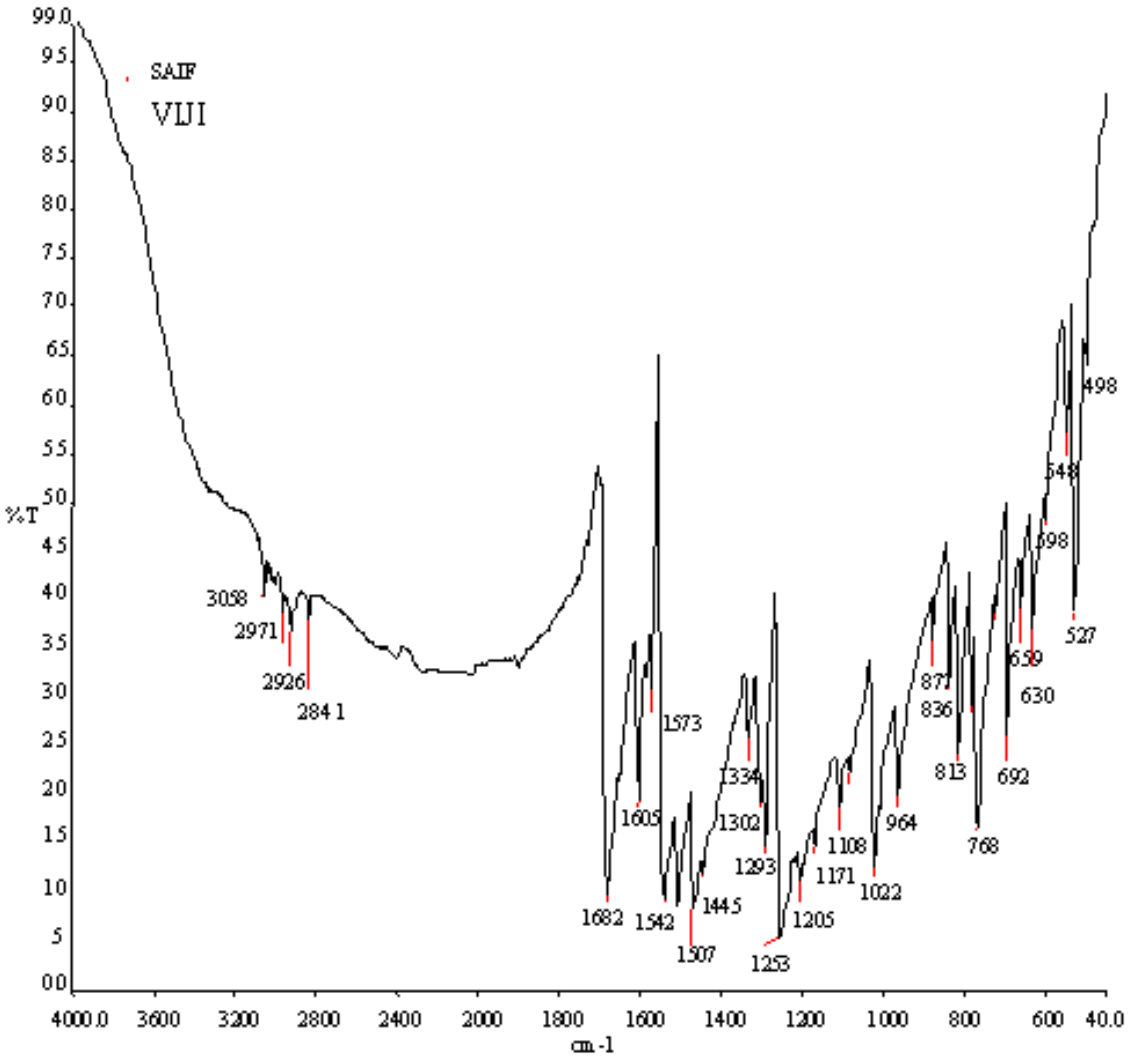


Figure 35: IR Spectrum of Compound V from *Cassia tora*

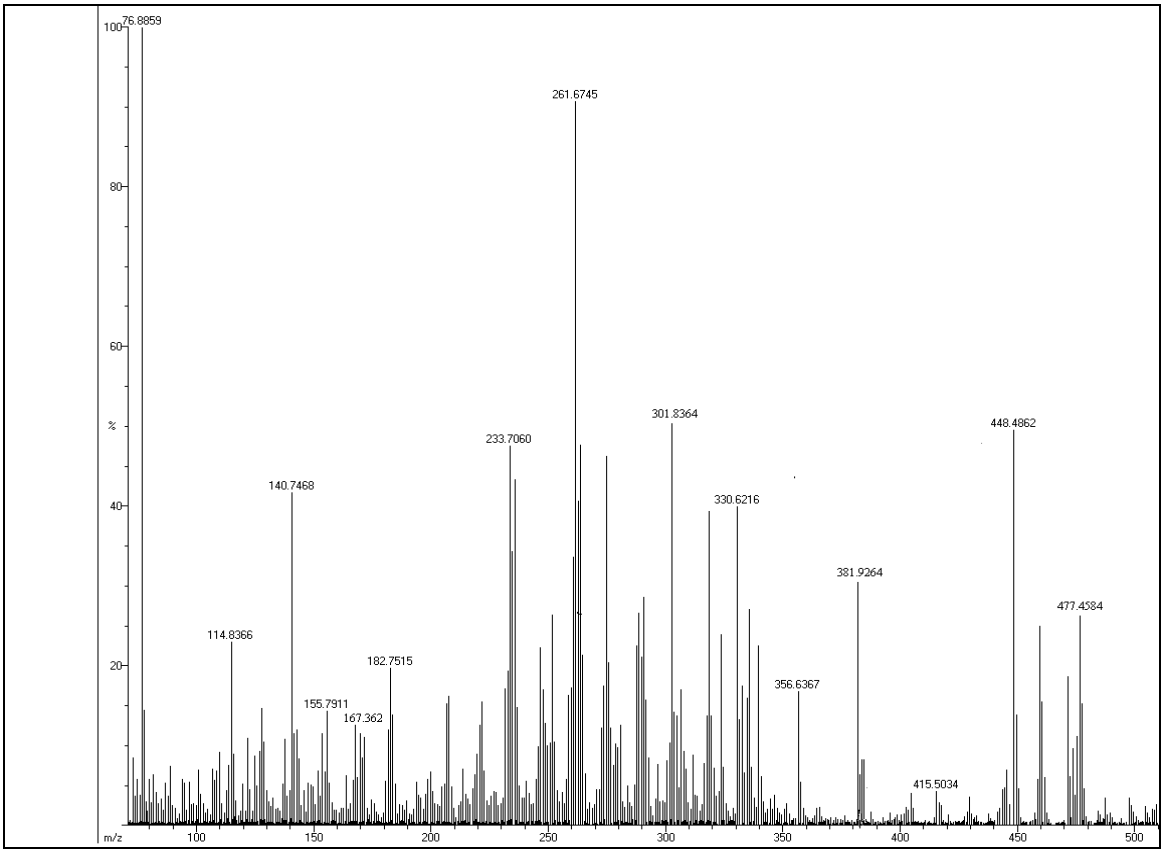


Figure 36: Mass Spectrum of Compound V from *C. tora* leaves

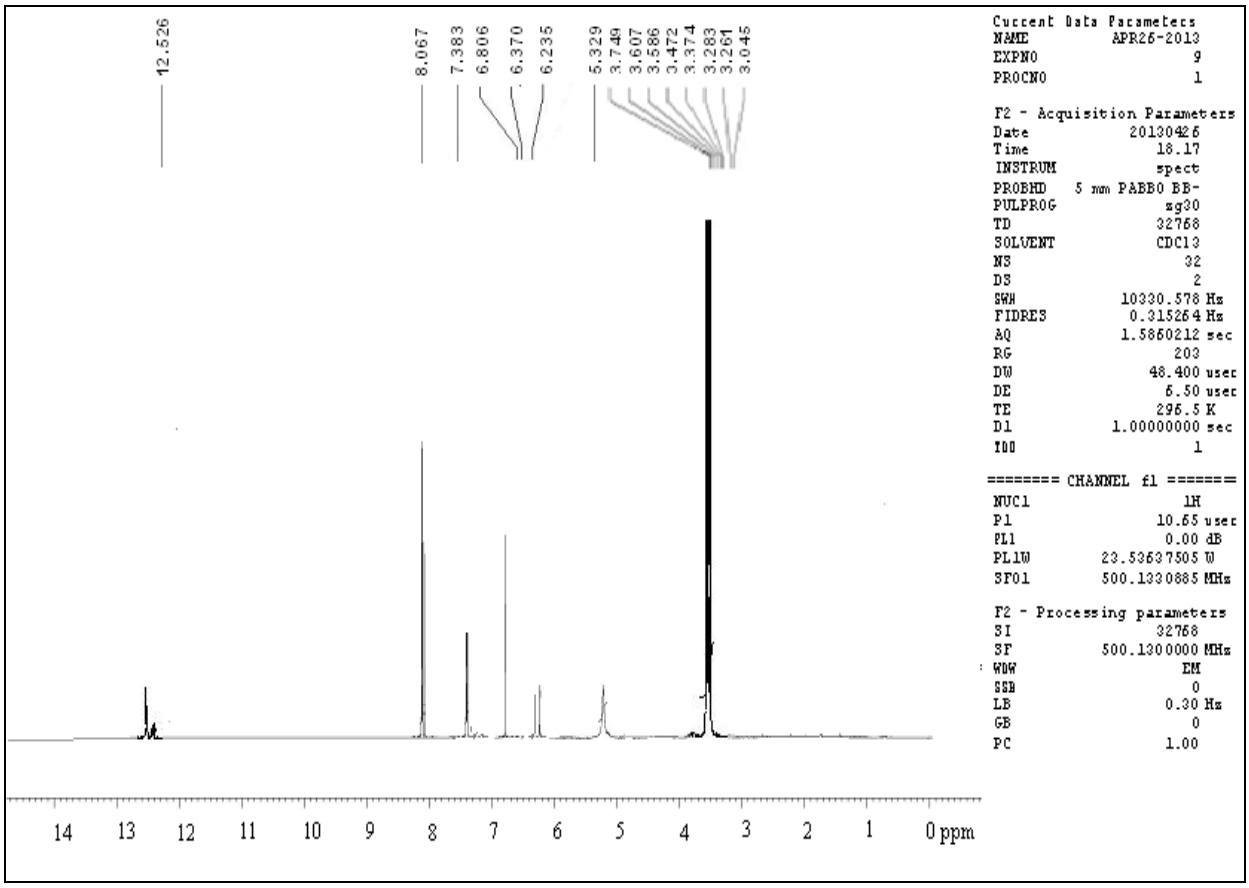


Figure 37: ^{1}H NMR Spectrum of Compound V from *C. tora* leaves

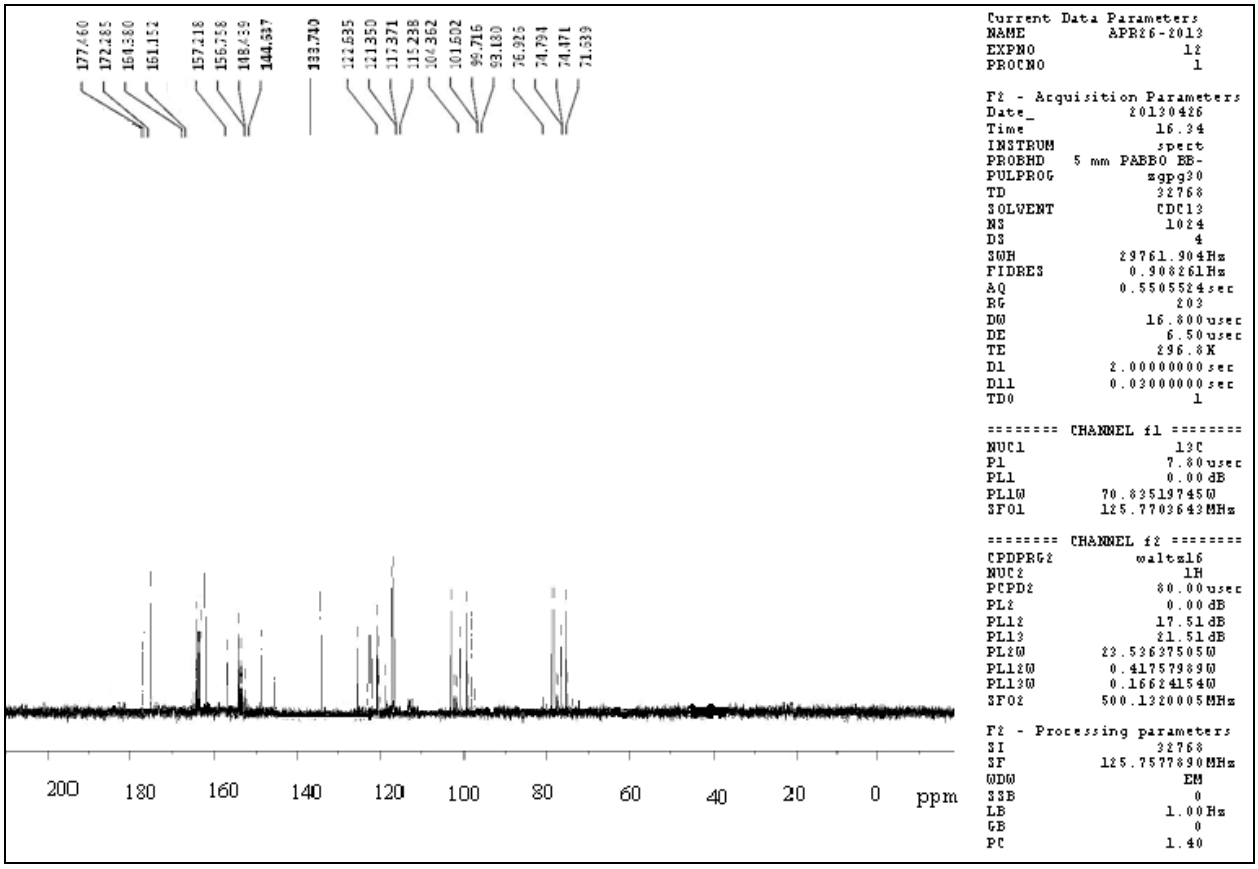


Figure 38: ^{13}C NMR Spectrum of Compound V from *C. tora* leaves

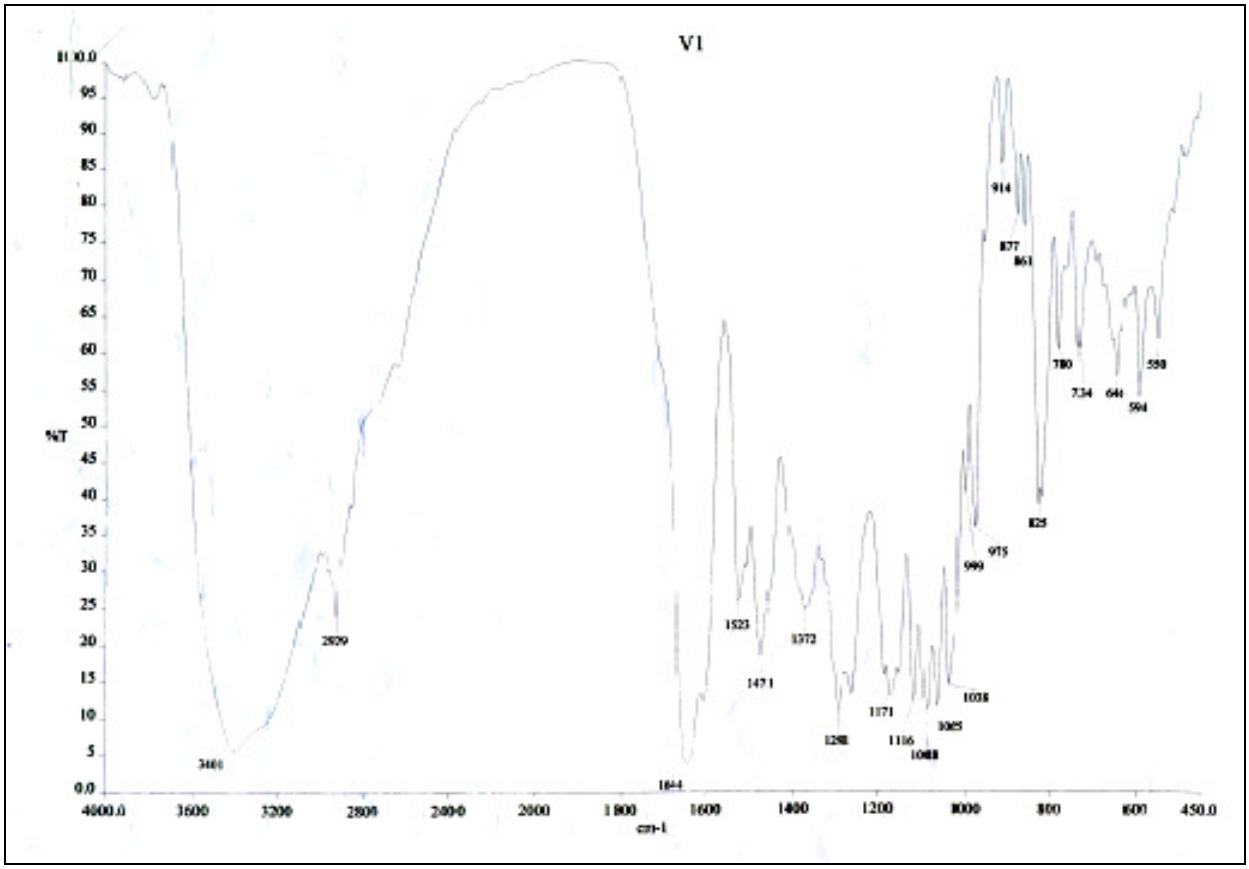


Figure 39: IR Spectrum of Compound VI from *C. tora* leaves

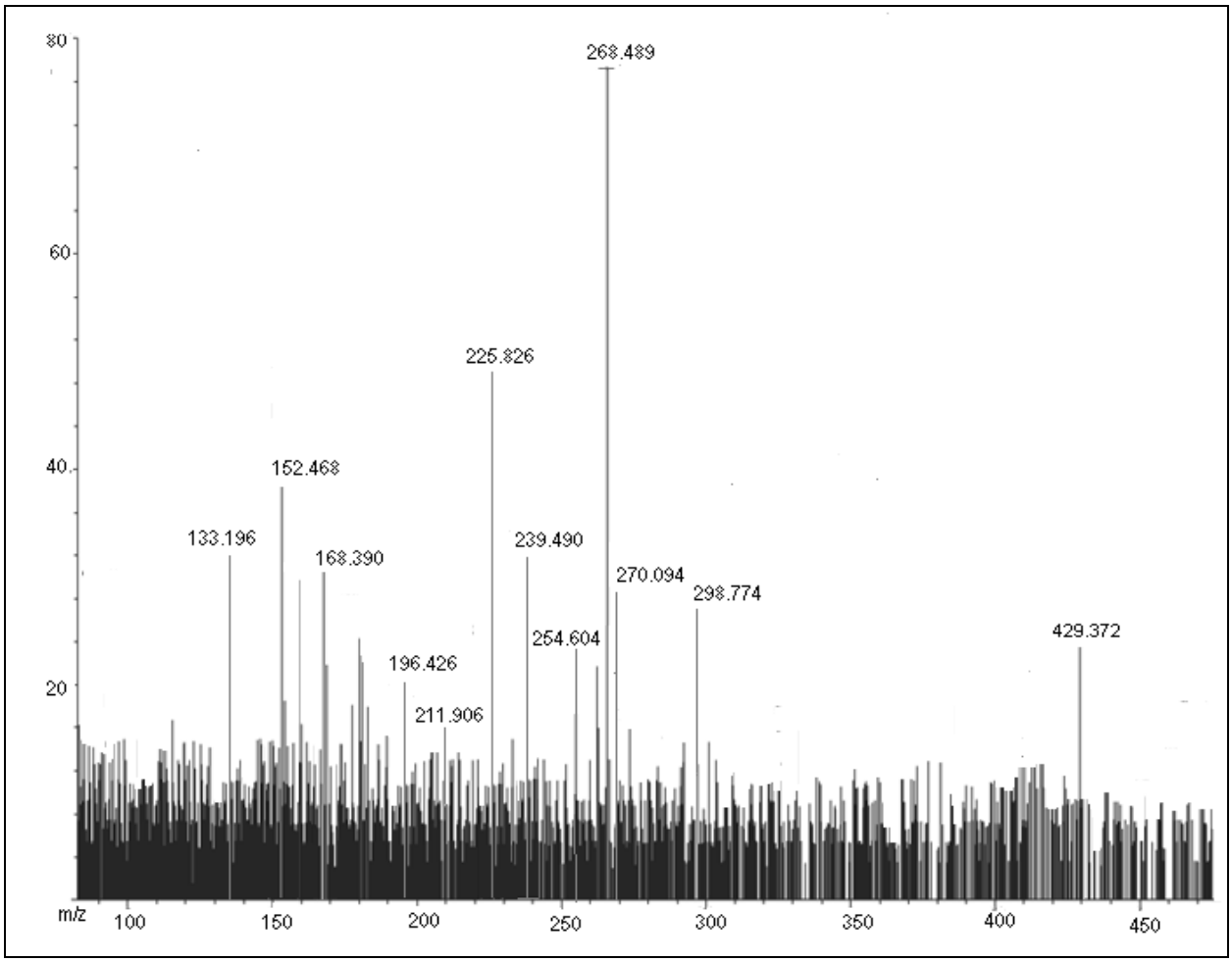


Figure 40: Mass Spectrum of Compound VI from *C. tora* leaves

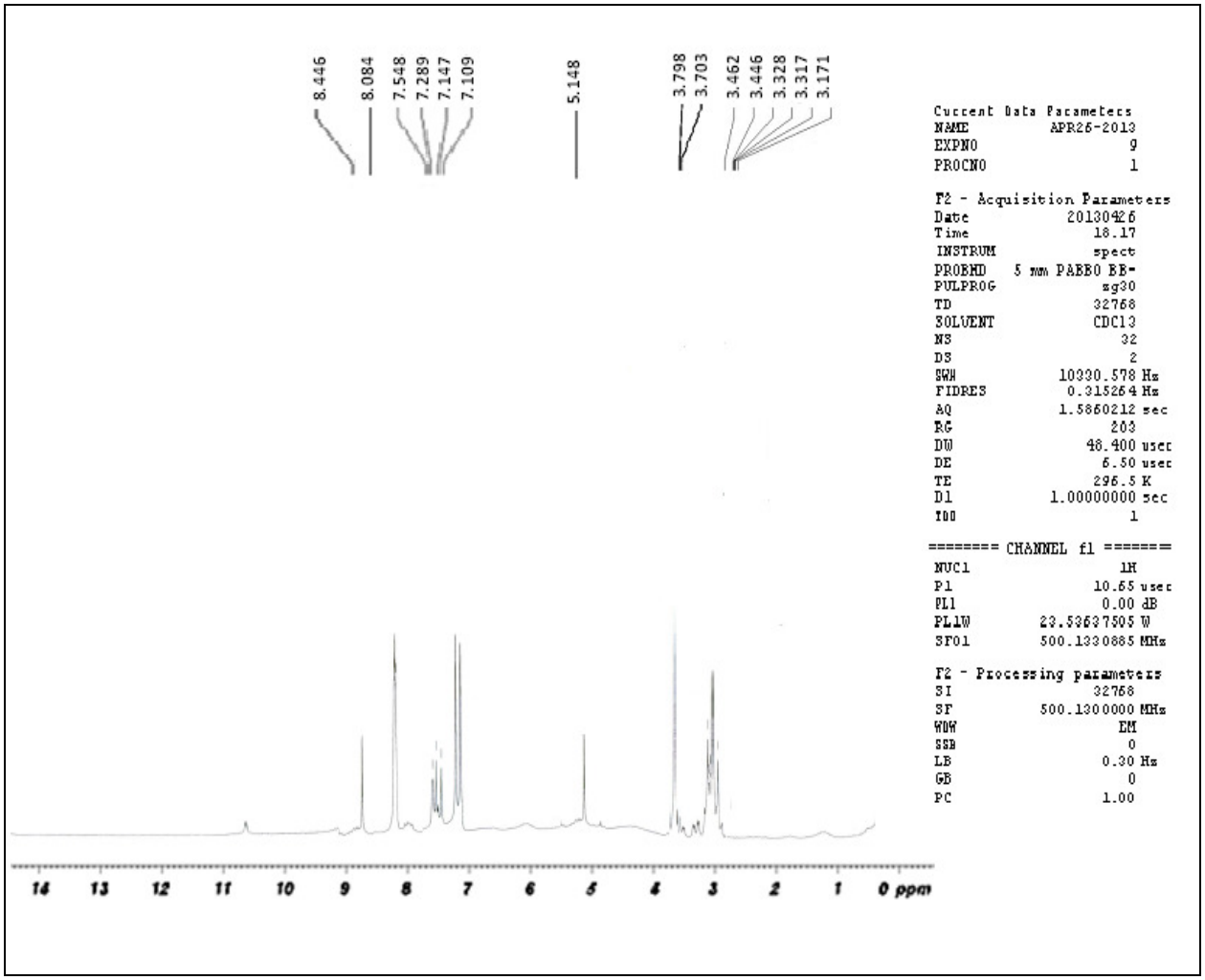


Figure 41: ^{1}H NMR Spectrum of Compound VI from *C. tora* leaves

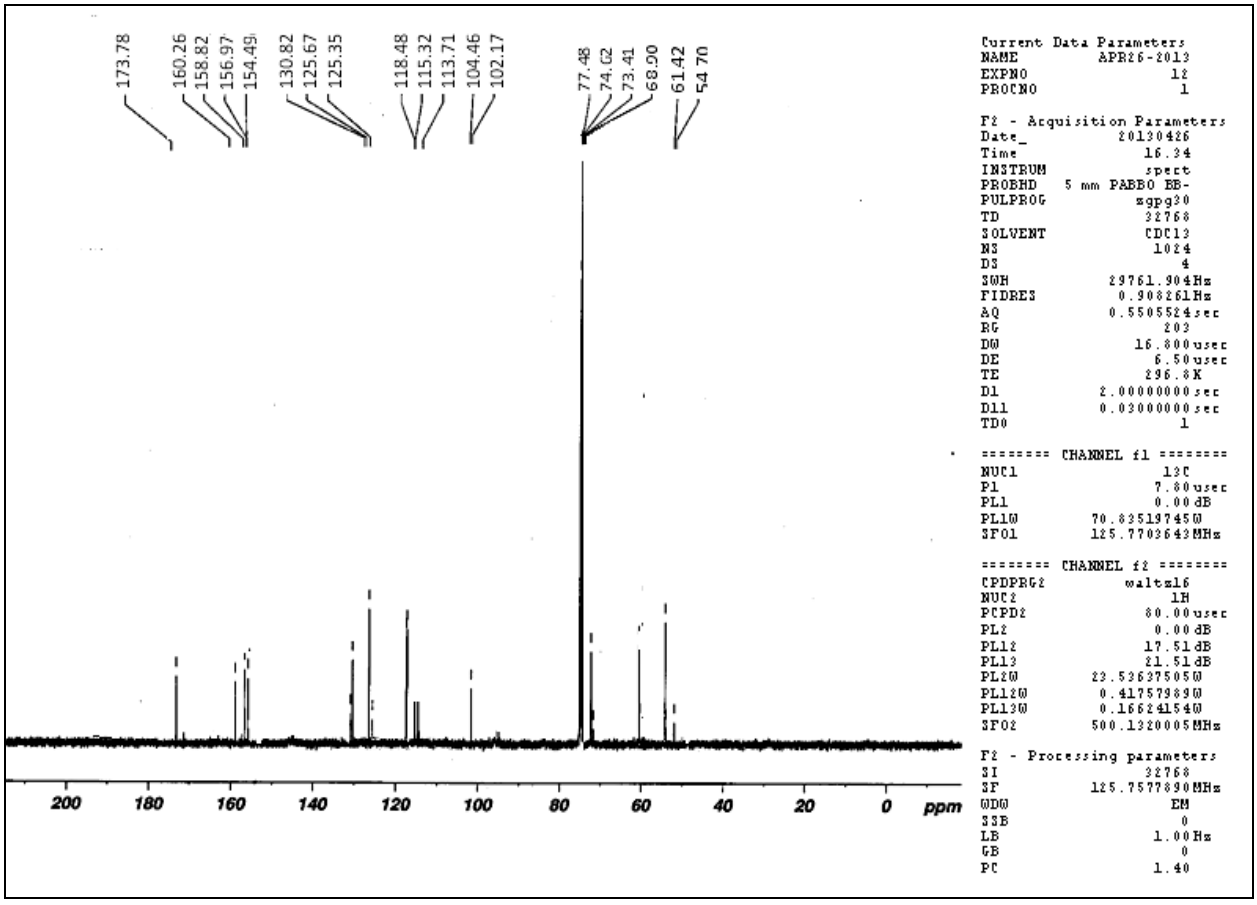


Figure 42: ^{13}C NMR Spectrum of Compound VI from *C. tora* leaves

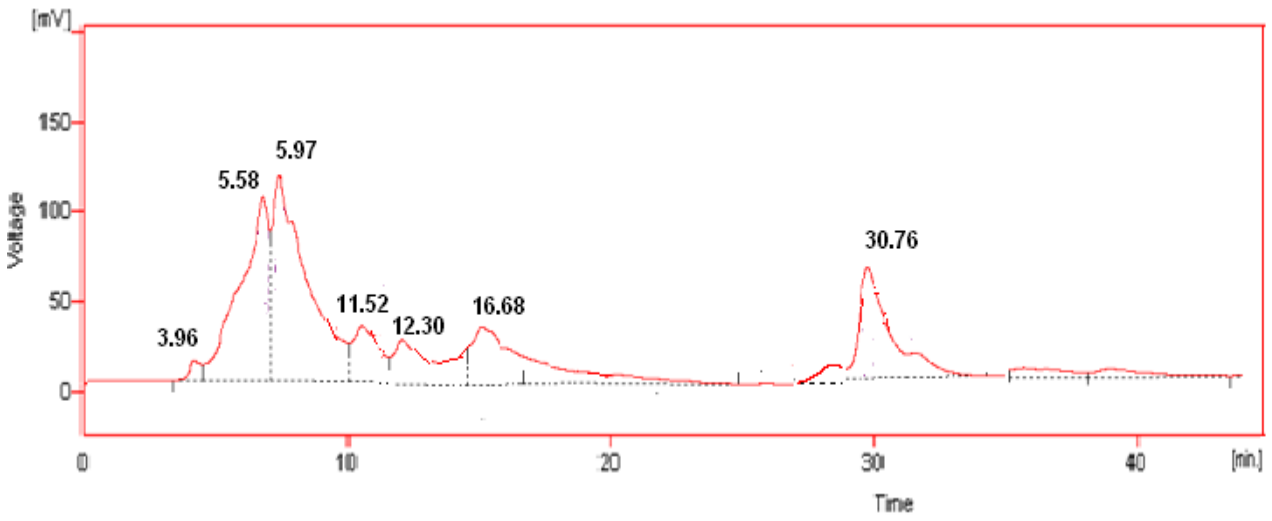


Figure 43: HPLC fingerprint of ethanol extract of *G. rottleriformis* bark

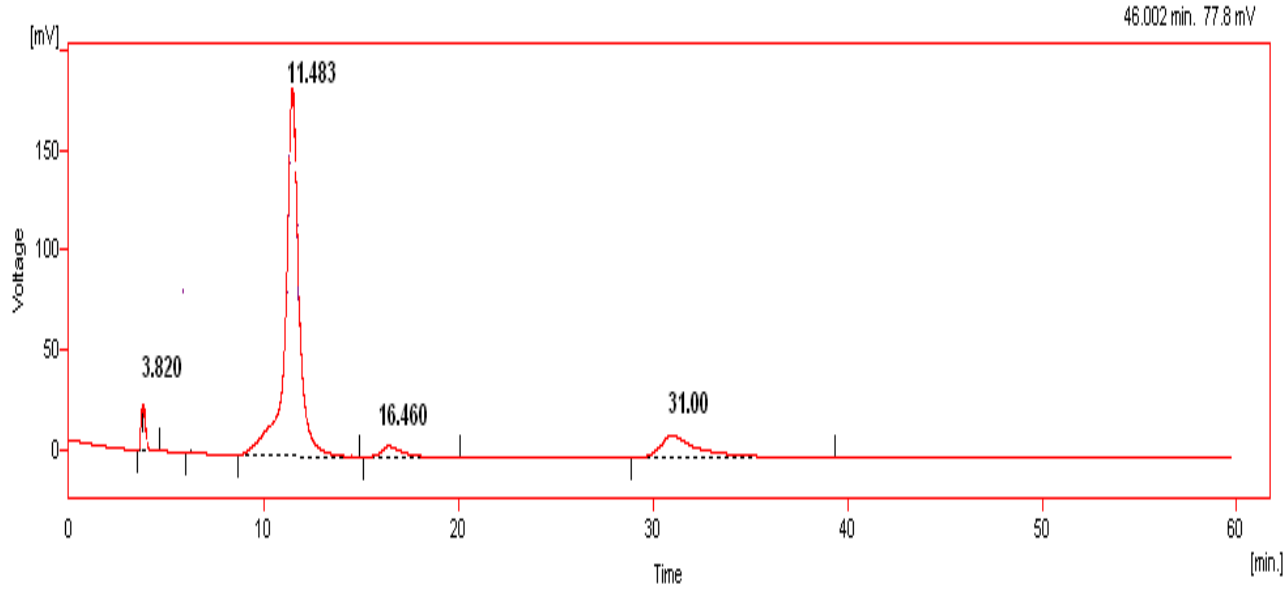


Figure 44: HPLC fingerprint of reference standards

1: Rutin 2: Quercetin 3: Kaempferol 4: Luteolin

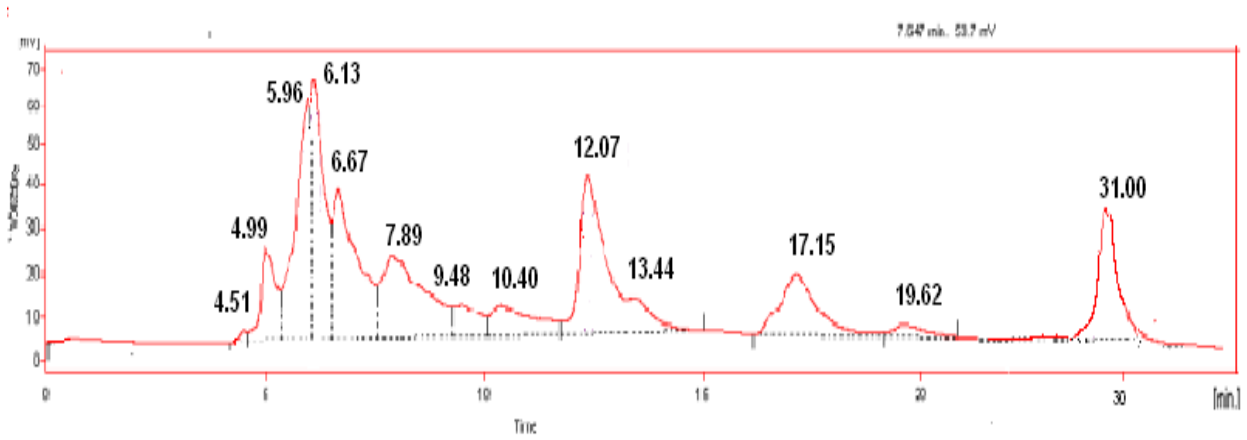


Figure 45: HPLC fingerprint of ethanol extract of *Cassia tora* leaves

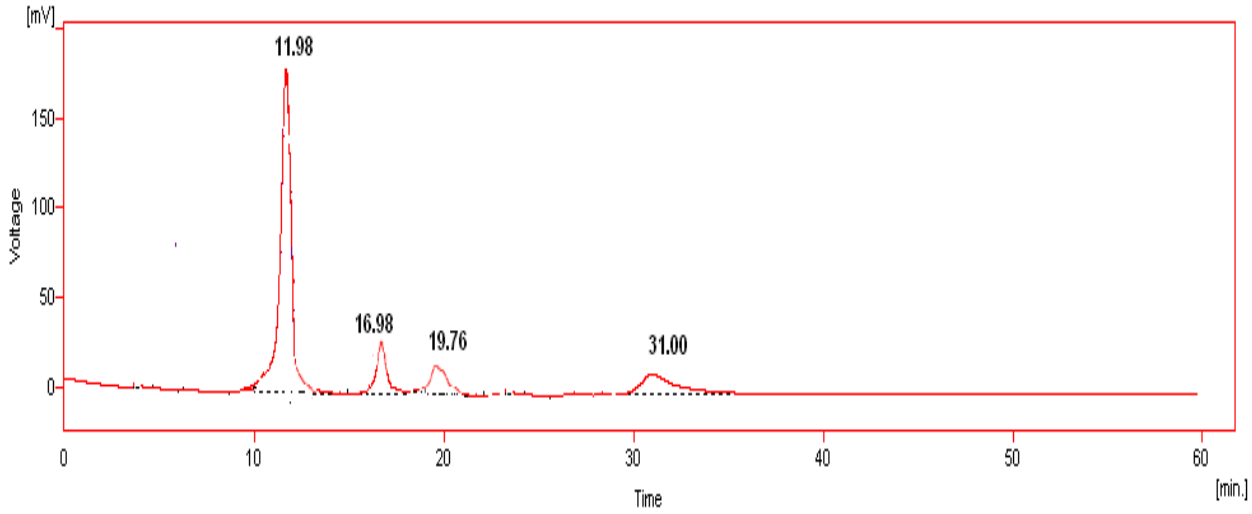
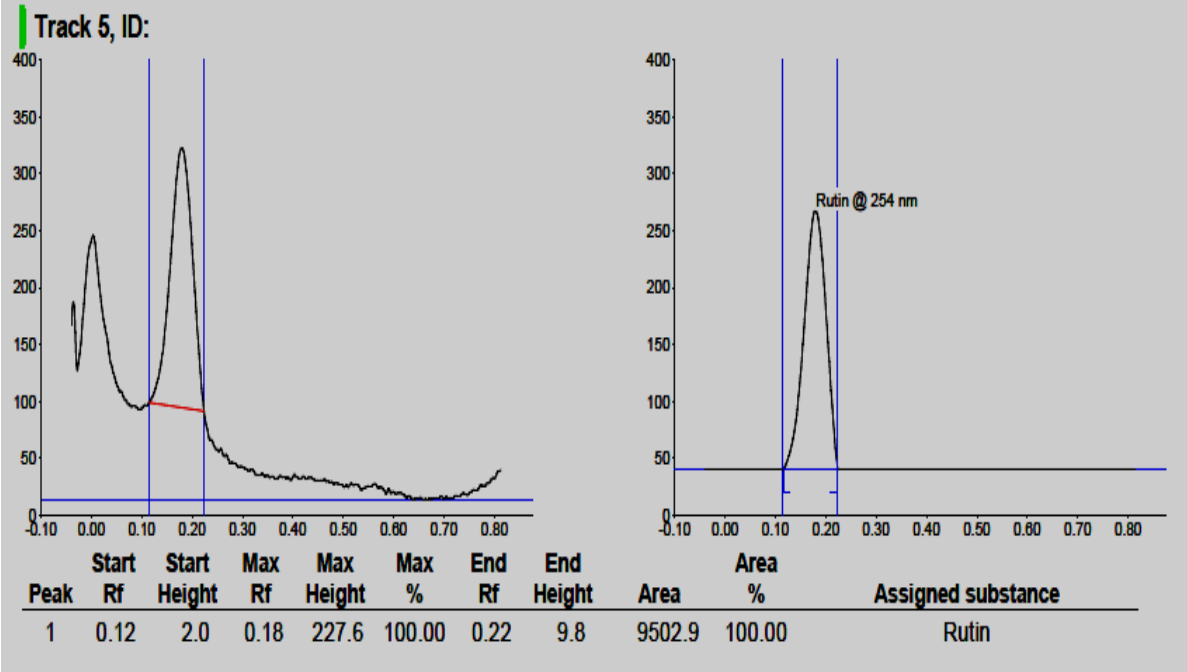


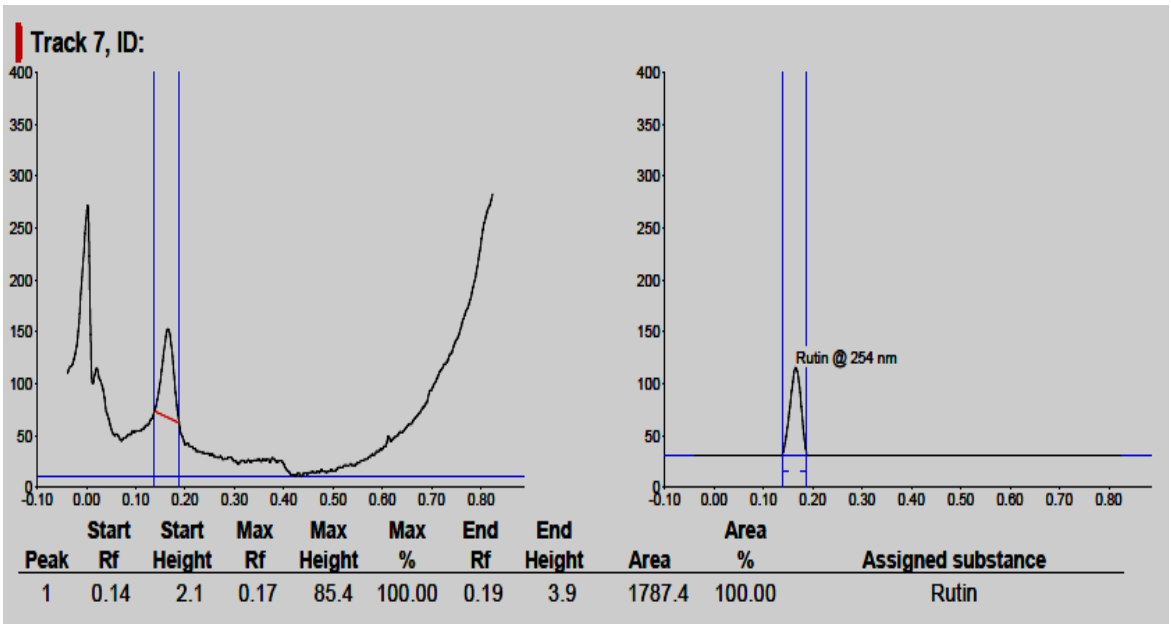
Figure 46: HPLC fingerprint of reference standards

1: Quercetin 2: Kaempferol 3: Formononetin 4: Luteolin



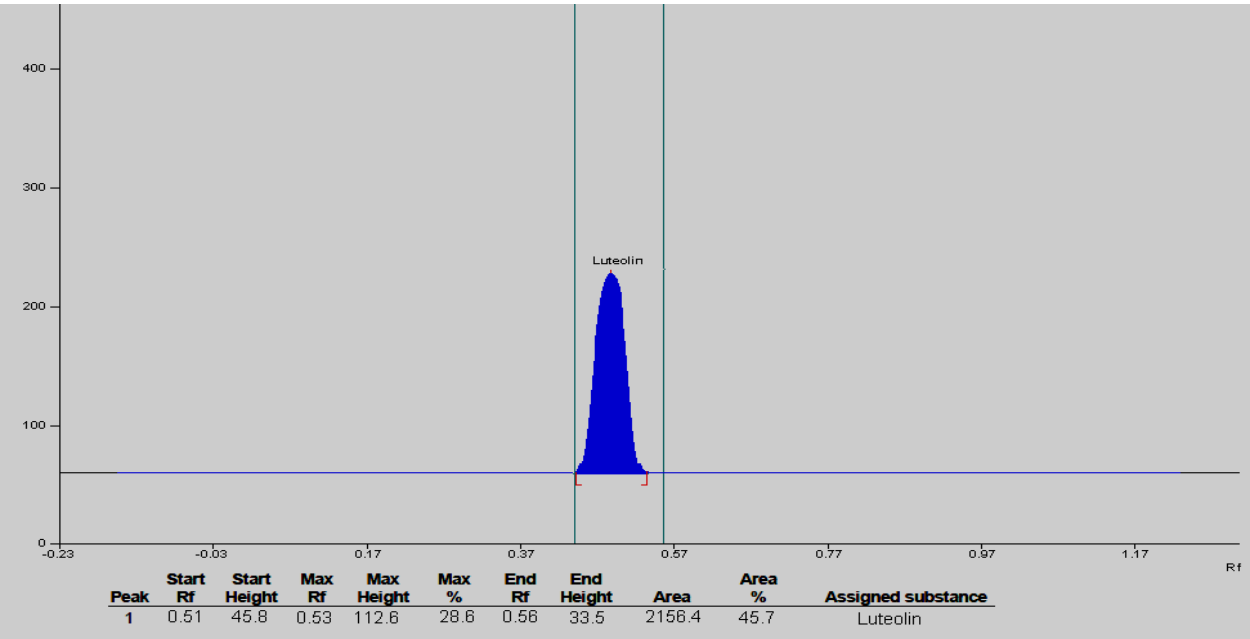
Peak	Start Rf	Start Height	Max Rf	Max Height	Max %	End Rf	End Height	Area	Area %	Assigned substance
1	0.12	2.0	0.18	227.6	100.00	0.22	9.8	9502.9	100.00	Rutin

Figure 47: HPTLC Fingerprint of Marker Rutin



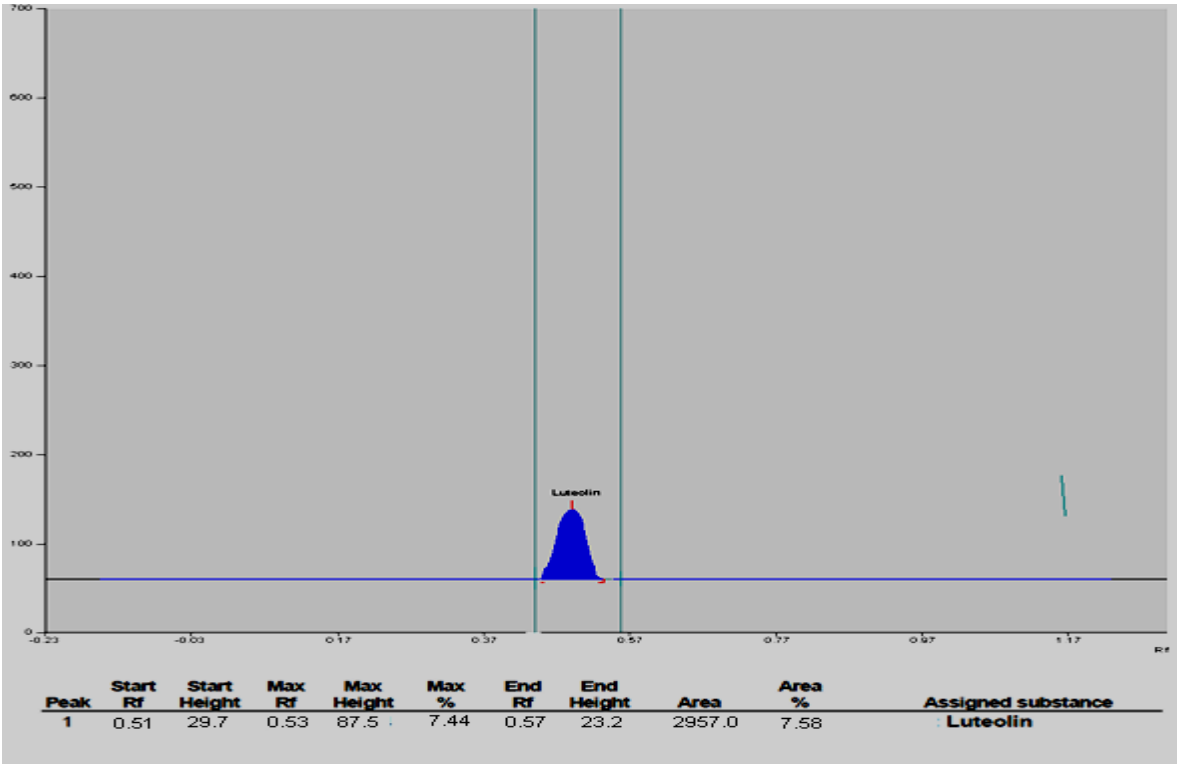
Peak	Start Rf	Start Height	Max Rf	Max Height	Max %	End Rf	End Height	Area	Area %	Assigned substance
1	0.14	2.1	0.17	85.4	100.00	0.19	3.9	1787.4	100.00	Rutin

Figure 48: HPTLC Fingerprint of Rutin in ethanol extract of *G. rottleriformis* bark



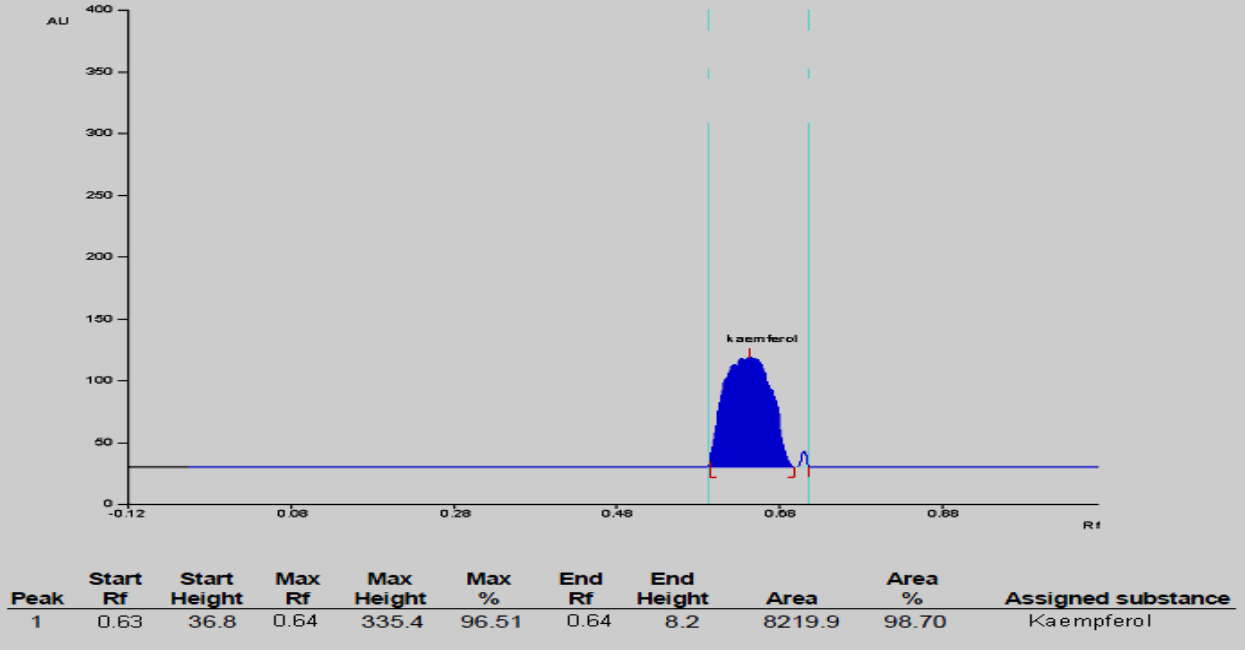
Peak	Start Rf	Start Height	Max Rf	Max Height	Max %	End Rf	End Height	Area	Area %	Assigned substance
1	0.51	45.8	0.53	112.6	28.6	0.56	33.5	2156.4	45.7	Luteolin

Figure 49: HPTLC Fingerprint of Marker Luteolin



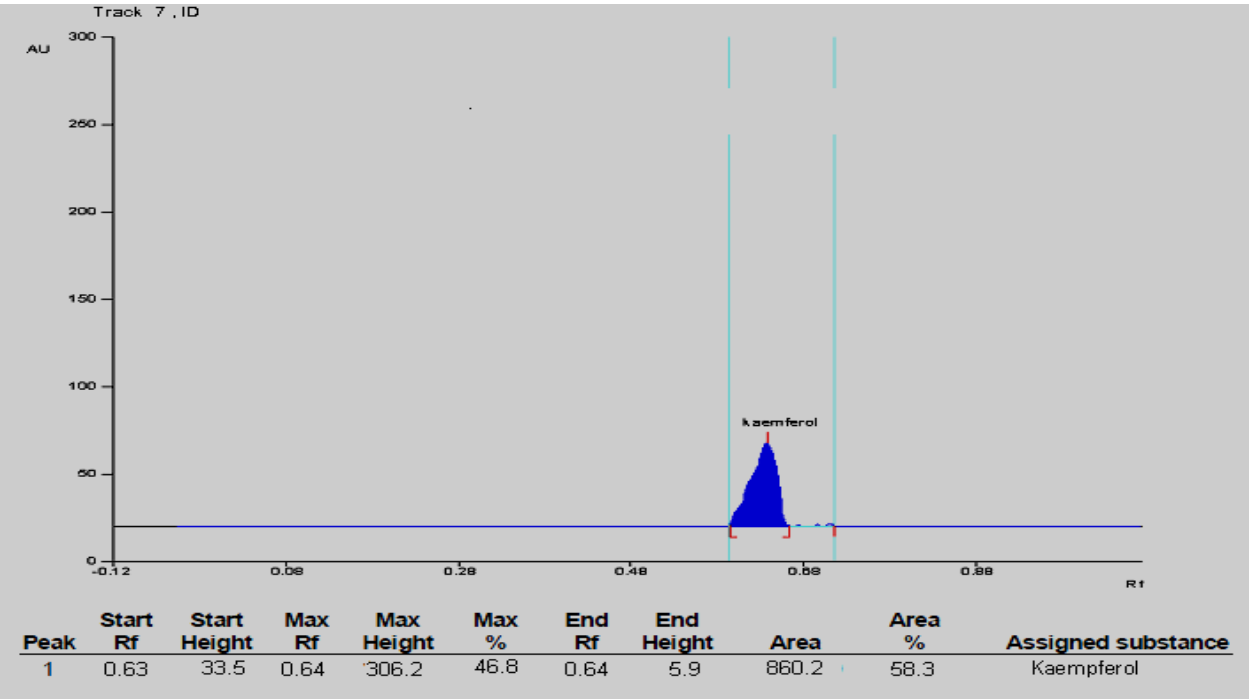
Peak	Start Rf	Start Height	Max Rf	Max Height	Max %	End Rf	End Height	Area	Area %	Assigned substance
1	0.51	29.7	0.53	87.5	7.44	0.57	23.2	2957.0	7.58	Luteolin

Figure 50: HPTLC Fingerprint of Luteolin in ethanol extract of *G. rottleriformis* bark



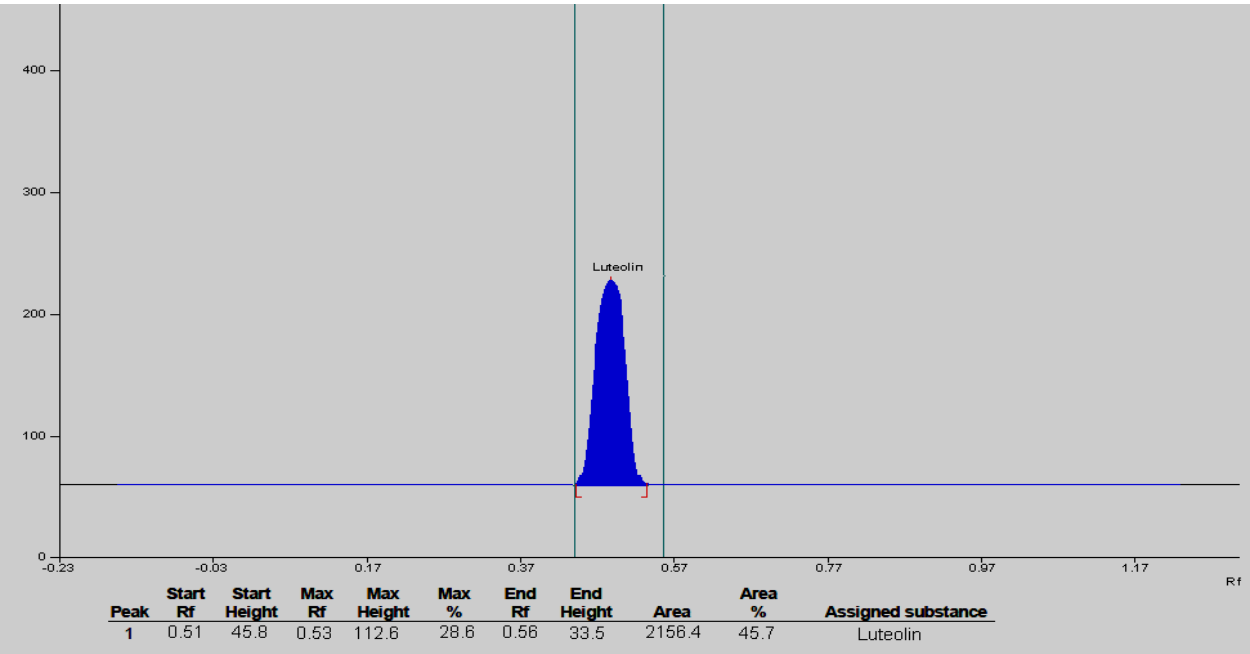
Peak	Start Rf	Start Height	Max Rf	Max Height	Max %	End Rf	End Height	Area	Area %	Assigned substance
1	0.63	36.8	0.64	335.4	96.51	0.64	8.2	8219.9	98.70	Kaempferol

Figure 51: HPTLC Fingerprint of Marker Kaempferol



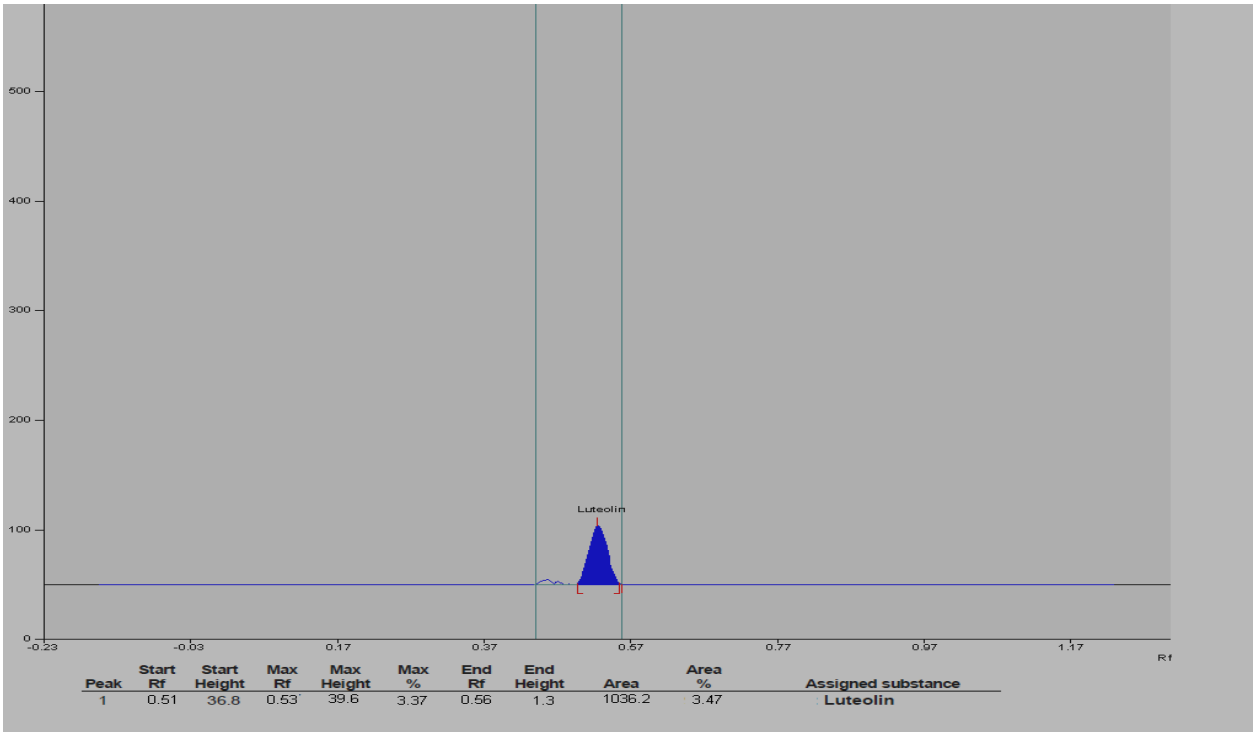
Peak	Start Rf	Start Height	Max Rf	Max Height	Max %	End Rf	End Height	Area	Area %	Assigned substance
1	0.63	33.5	0.64	306.2	46.8	0.64	5.9	860.2	58.3	Kaempferol

Figure 52: HPTLC Fingerprint of Kaempferol in ethanol extract of *G. rottleriformis* bark



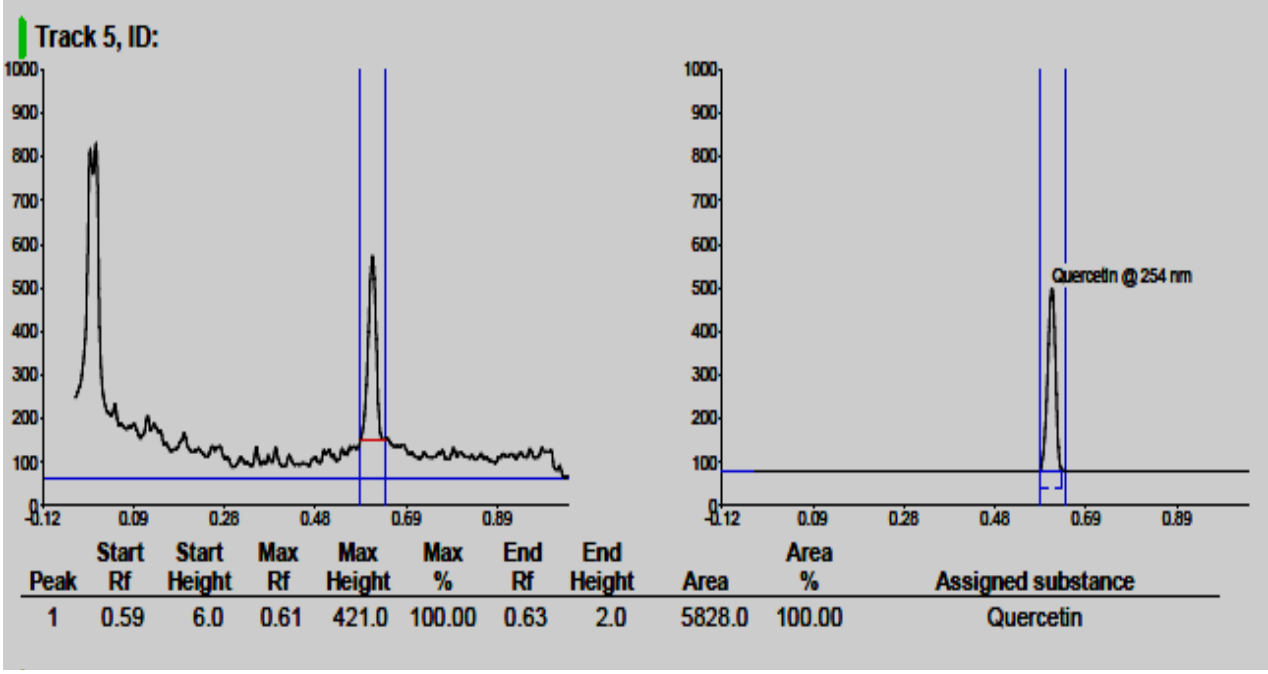
Peak	Start Rf	Start Height	Max Rf	Max Height	Max %	End Rf	End Height	Area	Area %	Assigned substance
1	0.51	45.8	0.53	112.6	28.6	0.56	33.5	2156.4	45.7	Luteolin

Figure 53: HPTLC Fingerprint of Marker Luteolin



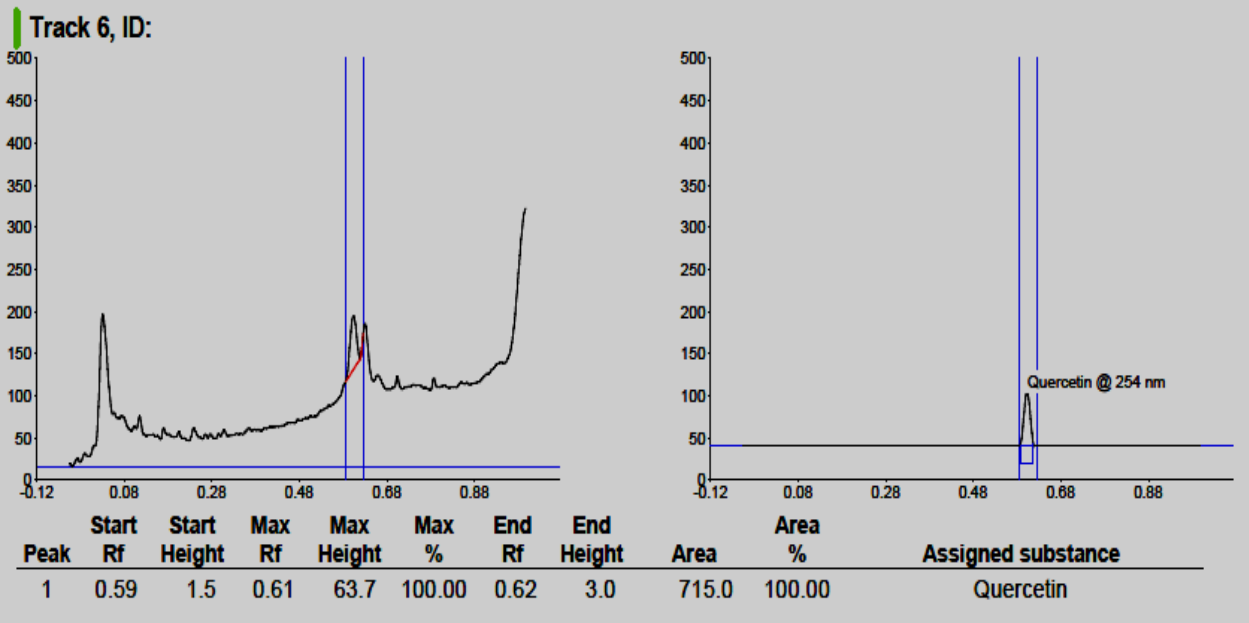
Peak	Start Rf	Start Height	Max Rf	Max Height	Max %	End Rf	End Height	Area	Area %	Assigned substance
1	0.51	36.8	0.53	39.6	3.37	0.56	1.3	1036.2	3.47	Luteolin

Figure 54: HPTLC Fingerprint of Luteolin in ethanol extract of *C. tora* leaves



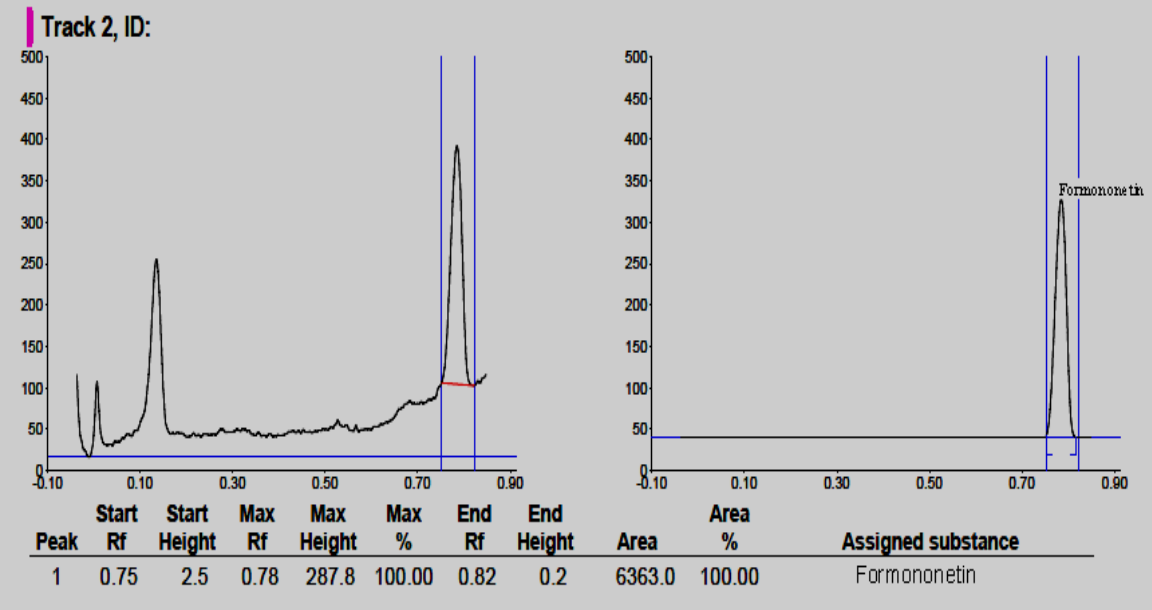
Peak	Start Rf	Start Height	Max Rf	Max Height	Max %	End Rf	End Height	Area	Area %	Assigned substance
1	0.59	6.0	0.61	421.0	100.00	0.63	2.0	5828.0	100.00	Quercetin

Figure 55: HPTLC Fingerprint of Marker Quercetin



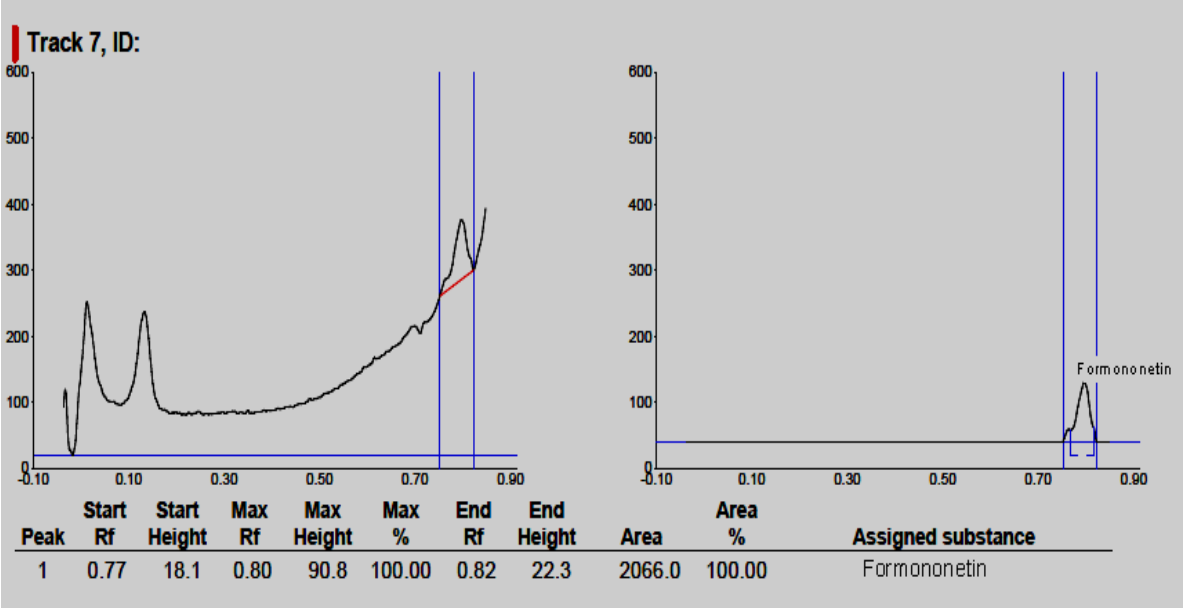
Peak	Start Rf	Start Height	Max Rf	Max Height	Max %	End Rf	End Height	Area	Area %	Assigned substance
1	0.59	1.5	0.61	63.7	100.00	0.62	3.0	715.0	100.00	Quercetin

Figure 56: HPTLC Fingerprint of Quercetin in ethanol extract of *C. tora* leaves



Peak	Start Rf	Start Height	Max Rf	Max Height	Max %	End Rf	End Height	Area	Area %	Assigned substance
1	0.75	2.5	0.78	287.8	100.00	0.82	0.2	6363.0	100.00	Formononetin

Figure 57: HPTLC Fingerprint of Marker Formononetin



Peak	Start Rf	Start Height	Max Rf	Max Height	Max %	End Rf	End Height	Area	Area %	Assigned substance
1	0.77	18.1	0.80	90.8	100.00	0.82	22.3	2066.0	100.00	Formononetin

Figure 58: HPTLC Fingerprint of Formononetin in ethanol extract of *C. tora* leaves

6.4. Discussion

Most of the traditional knowledge about medicinal plant was in the form of oral knowledge. There is no uniform or standard procedure for maintaining the inventory of these plants and the knowledge about their medicinal properties.

Therefore, it is necessary that such procedures to be documented and studied for systematic regulation and widespread application. The leads for a significant number of modern synthetic drugs have originated from isolated plant ingredients since the search for new entities begins from either derivatizing the existing drug or from traditional medicinal system. It is very important to undertake phytochemical investigations along with biological screening to understand therapeutic dynamics of medicinal plants and also to develop quality parameters.

Qualitative preliminary phytochemical analysis was performed initially with different chemical reagents to detect the nature of phytoconstituents and their presence in the extract. Preliminary phytochemical analysis of the ethanol extract of *Givotia rottleriformis* bark indicates the presence of various secondary plant metabolites like alkaloids, phenols, tannins, flavonoids, terpeneoids, steroids, glycoside, protein and ethanol extract of *Cassia tora* leaves indicates the presence of terpeneoids, steroids, saponins, phenols, tannins, flavonoids, anthraquinone glycoside and protein.

The total phenol and flavonoid content present in the plants were estimated by Folin-Ciocalteu and $AlCl_3$ method respectively. The total phenolic content and flavonoid content in *Givotia rottleriformis* bark was found to be 13.80% w/w and 5.7% w/w respectively. The total phenolic content and flavonoid content in *Cassia tora* leaves was found to be 18.60% w/w and 9.5% w/w respectively.

Qualitative chromatographic analysis of the extract using thin layer chromatography was performed to separate and identify the single or mixture of constituents in the extract. The ethanol extract of *Givotia rottleriformis* bark showed 7 spots (R_f values 0.11, 0.18, 0.22, 0.26, 0.56, 0.64, 0.73) and ethanol extract of *Cassia tora* leaves showed 10 well separated spots (Rf values 0.14, 0.20, 0.24, 0.31, 0.56, 0.62, 0.70, 0.79, 0.83, 0.92) in the mobile phase Benzene: Methanol: Ammonia (9:1:0.1) using iodine vapour as detecting agent.

The construction of chromatographic fingerprints plays an important role in the quality control of complex herbal medicines. Chemical fingerprints obtained by chromatographic techniques are strongly recommended for the purpose of quality control of herbal medicines, since they might represent appropriately the "chemical integrities" of the herbal medicines and therefore be used for authentication and identification of the herbal products. HPTLC was scanned at 254 nm with the best solvent to detect the maximum number of components and peak abundance qualitatively. The finger print of ethanol extract of *Givotia rottleriformis* showed 8 spots in the solvent system with the R_f values 0.03, 0.20, 0.25, 0.27, 0.34, 0.61, 0.64, 0.73 and ethanol extract of *Cassia tora* showed 9 spots in the solvent system with the R_f values 0.04, 0.13, 0.16, 0.34, 0.53, 0.61, 0.71, 0.81, 0.99. HPTLC fingerprint is one of the versatile tools for qualitative and quantitative analysis of active constituents. It is also a diagnostic method to find out the adulterants and to check the purity.

The defatted ethanol extract of *Givotia rottleriformis* bark and *Cassia tora* leaves was subjected to column chromatography separately and eluted with various solvents in the order of increasing polarity. The isolated compounds were characterized by spectral analysis. From *Givotia rottleriformis* bark, 3 flavonoid glycosides, **Compound I-III** and from *Cassia tora* leaves, 3 flavonoid glycosides **Compound IV-VI**.

Compound I gave yellow colour with alkalis, pink colour with Mg-HCI, olive green with Fe^{3+} and answered Molisch's test. Acid hydrolysis of **I** afforded aglycone - quercetin and sugars-rhamnose and glucose. The UV spectrum of the aglycone in methanol and changes observed after the addition of shift reagents indicated that there is a free hydroxyl group presented at C-5 and C-7 position and the 3-hydroxyl group was substituted.

The ^{1}H NMR spectrum of the compound I exhibited a characteristic proton signal at δ H 12.74 corresponding to a chelated hydroxyl group at C-5. In addition to this, the presence of five aromatic protons were seen in the ^{1}H NMR spectrum; two ortho coupling protons assignable to H-6' (δ 7.54, 1H, d, J = 8.4 Hz) and H-5' (δ 6.87, 1H, d, J = 8.4 Hz); two-meta coupling protons at H-6 (δ 6.26, 1H, d, J =1.6 Hz) and H-8 (δ 6.40, 1H, d, J = 1.6 Hz); a singlet aromatic proton at H-2' (δ 7.48, 1H, d). The ^{1}H NMR spectrum also supported the presence of rhamnose and glucose moieties with the rhamnose anomeric proton signal at δ H 4.41 and glucose H-1 signal at δ H 5.30. A doublet of methyl group of rhamnose was observed at high field δ *H* 1.20 (3H, d, J = 6 Hz). The rest of protons in the sugar moiety resonated between 3.30 and 3.80 ppm.

The ^{13}C NMR spectrum (100 MHz, DMSO-*d*6) showed 27 carbon signals which indicated the presence of 15 carbon signals due to the flavonol skeleton. In the aliphatic region of ^{13}C NMR, 12 carbon resonances are assigned for a rutinoside moiety among which the most downfield signals at 101.4 and 101.8 are assigned for the two anomeric carbons C1'" and C1" of rhamnose and glucose, respectively. The cross-peak between the δ H 4.41 (H-1'", rhamnose) and the δ *C* 67.67 (C6" of the glucose) confirmed that the glycosylation of the glucose unit by the rhamnose took place on the 6″-hydroxyl. The chemical shift of each carbons of the isolated compound in the ^{13}C NMR spectrum (Table 10) was assigned by comparing with the literature data.[133] The structure of the compound **I** was further evidenced by mass spectrum. The mass

spectrum of the glycoside shows prominent peaks at *m/z* 609 $[M-H]^-$ and a typical fragments due to the loss of diglycoside moiety (glucose and rhamnose) at *m/z* 301 aglycone-H. The molecular formula was determined as $C_{27}H_{30}O_{16}$, *m/z* 610 by Negative ESI-MS. Therefore, the compound I was established as Rutin.

Compound II was isolated as yellow amorphous powders, gave yellow colour with alkalis, pink colour with Mg-HCI, olive green with Fe^{3+} and answered Molisch's test. Acid hydrolysis of **II** afforded aglycone - luteolin and sugar - glucuronic acid. The UV spectrum of the aglycone in methanol and changes observed in UV shift with AlCl3/HCl relative to MeOH indicated that there is a free hydroxyl group presented at C-5 position and the 7-hydroxyl group was substituted.

The ^1H NMR spectrum showed the pattern of a luteolin-O-glucoside. Thus, the signals located at δ □6.43 (1H, d, J=1.7 Hz) and 6.77 (1H, d, J=1.9 Hz) could be attributed to H-6 and H-8 of ring A, respectively, while the signals at δ 6.89 (d, J=8 Hz), 7.46 (d, J=2 Hz) and 7.43 (dd, J=2 and 8.7 Hz) was consistent with the signals of a 3′,4′-disubstituted ring B of a flavonol. Further features were signals corresponding to the anomeric proton δ 5.14, (d, J=7.6 Hz) of a β-D-glucuronic unit.

The identity of the sugar was confirmed by ^{13}C NMR (Table 10) was assigned by comparing with the literature data.[134] The ^{13}C NMR spectrum of **II** showed the presence of signals at δ □172.3 for C-6″ of the glucuronic acid, that supported the FAB-mass data (The fragmentation pattern showed a peak at m/z 285 $[M-H-176]^-$ due to the loss of glucuronic acid). The structure of the glycoside luteolin 7-O- substituted aglycone was further evidenced by mass spectrum. The mass spectrum of the glycoside shows prominent peaks at *m/z* 461 $[M-H]^-$ and at 285 $[M-H-176]^-$ aglycone-H. The molecular formula was determined as $C_{21}H_{18}O_{12}$, *m/z* 462 by

Negative ESI-MS. Therefore, the compound **II** was established as Luteolin-7-O-β-D-glucuronide.

Compound III gave gave yellow colour with alkalis, pink colour with Mg-HCI, olive green with Fe^{3+} and answered Molisch's test. Acid hydrolysis afforded aglycone - kaempferol and sugars - glucose, ferulic acid, galactose. The aglycone was yellow under UV and UV/NH_3, characteristic of a flavonol with free 5-OH and had λ_{max} (MeOH) 259, 266, 365nm. The UV spectrum of the aglycone in methanol and changes observed after the addition of shift reagents indicated that there is a free hydroxyl group presented at C-5 position and the 3-hydroxyl group was substituted.

Its ^{13}C NMR spectrum showed 37 carbon signals. The UV and ^{1}H NMR spectra were suggestive of flavonol glycosides. The ^{1}H NMR spectrum revealed H-3′,5′ and H-2′,6′ protons at δ 6.85 (2H, d, *J*= 8.6 Hz) and 8.03 (2H, d, *J*= 8.6 Hz), respectively, suggesting a *para*-substituted B ring. In addition, a 5,7-dihydroxy-substituted A ring was evident from the two broad singlets at δ 6.14 and 6.32 for H-6 and H-8, respectively. These data together indicate that the aglycone moiety is kaempferol, and the ^{13}C NMR spectrum was comparable to that of kaempferol itself. The presence of a *trans* feruloyl moiety was deduced from the ^{1}H NMR spectrum, which displayed three additional 1,2,4-trisubstituted aromatic protons (δ 7.14, 6.87, and 6.77), a pair of double bond protons (δ 6.24 and 7.45), and a methoxy proton (δ 3.73). The fragment at *m*/*z* 449 [aglycone + hexose + H]$^+$ suggested the existence of an acyl moiety since it indicated loss of hexose and acyl moieties from the molecule. A disaccharide unit was suggested by the pair of anomeric carbon resonances at δ 98.4 and 104.5. Together the anomeric protons (δ 5.46 and 4.73) and anomeric carbon signals suggested the δ-configuration of the glycoside bonds. The ^{13}C NMR chemical shifts of the carbohydrate moiety of **III** were very similar to

those of kaempferol 3-*O*-[2-*O*-(6-*O*-caffeoyl)-β-D-glucopyranosyl]- β -D-galactopyranoside isolated from *Hedyotis diffusa*,[135] indicating that the sugar part is a [2-*O*-(6-*O*-feruloyl)-β-D-glucopyranosyl]- β-D-galactopyranoside. The sites of sugar and acyl linkages were further supported by the glycosylation shift (ca. 9.2 ppm) of C-2″ in galactose and acylation shift (ca. 2.4 ppm) of C-6‴ in glucose, respectively.

The mass spectrum of the glycoside shows prominent peaks at *m/z* 787 $[M+H]^+$ and typical fragments due to the loss of glucose at *m/z* 577 [M-Glc]+, 449 [(M - feruloyl glucose) + H]+, and at 287 [aglycone + H, kaempferol]+. The molecular formula was determined as $C_{37}H_{38}O_{19}$, *m/z* 786. Therefore, the compound **III** was established as Kaempferol 3-*O*-[2-*O*-(6-*O*-feruloyl)-β-D-glucopyranosyl]-β-D-galactopyranoside.

Compound IV was isolated as a yellow amorphous powder with chromatographic properties: Rf 0.53 (Benzene: Methanol: Ammonia); It gave yellow colour with alkalis, pink colour with Mg-HCl, olive green with Fe^{3+} and answered Molisch's test. Acid hydrolysis afforded aglycone – luteolin and sugar - glucose. The aglycone was yellow under UV and UV/NH_3, characteristic of a flavonol with free 5-OH and had λ_{max} (MeOH) 259, 266, 365nm. The UV spectrum of the aglycone in methanol and changes observed after the addition of shift reagents indicated that there is a free hydroxyl group presented at C-5 position and the 7-hydroxyl group was substituted.

^{1}H NMR showed a singlet at δ 12.97 as an evidence for H- bounded OH-5. The H-3 resonance was assigned at 6.74 (s) as an evidence for ring C in flavone. In the aliphatic region, a β-anomeric proton doublet was located at 5.08 (J= 7.2) and also one of the two diastereomeric CH_2-6″ was assigned at 3.72 (9.9 Hz, H-6″a), proving β–4C-stereostructure for glucoside moiety.

The ^{13}C NMR spectrum showed 15 signals typical for luteolin-7-O-substituted aglycone including five key signals at δ = 183.82 (C-4), 165.8 (C-7), 149.7 (C-4′), 148.2 (C-3′) and 104.74 (C-3). The full assignment of all ^{1}H and ^{13}C-resonances were confirmed by comparing with previously published data.[136]

The structure of the glycoside luteolin 7-O- substituted aglycone was further evidenced by mass spectrum. The mass spectrum of the glycoside shows prominent peaks at *m/z* 447 $[M - H]^-$ and at 285 $[M-H-162]^-$ aglycone-H. The molecular formula was determined as $C_{21}H_{20}O_1$, *m/z* 447 by Negative ESI-MS. Therefore, the compound **IV** was established as Luteolin-7-O-β-D-glucopyranoside.

Compound V was isolated as a yellow amorphous powder with chromatographic properties: R_f 0.61 (Benzene: Methanol: Ammonia); It gave a red colour with Mg-HCl, olive green with alcoholic Fe^{3+}, golden yellow colour with NaOH. The aglycone was yellow under UV and UV/NH_3, characteristic of a flavonol with free 5-OH and had λ_{max} (MeOH) 256, 275sh, 303sh, 370 nm. The UV spectrum of the aglycone in methanol and changes observed after the addition of shift reagents indicated that there is a free hydroxyl group presented at C-5 and C-7 position and the 3-hydroxyl group was substituted.

In the 1H NMR spectrum (500 MHz, DMSO-d_6) of the glycoside quercetin 3-O-glucuronide, the signal appearing at δ 12.5 ppm corresponds to the -OH at C-5. The signal at δ 9.8-10.5 ppm is due to the hydroxyl proton at C-7. The doublet appearing in the region of δ 8.06 ppm (d, J=8Hz) and δ 7.3 ppm corresponds to the proton at C-2′ and C-6′, while the proton of C-3′ appears at δ 6.17 ppm. The signal appearing at δ 6.8 ppm (d, J=8Hz) corresponds to C-5′ proton. C-8 proton due to meta coupling with C-6 proton appears as a doublet at δ 6.3 ppm (d, J=2.2Hz). C-6 proton, due to meta coupling with C-8 proton appears

as a supporting evidence for the structure of the flavonol glycoside quercetin 3-O-glucuronide is provided by the ^{13}C-NMR (100MHz, DMSO-d_6) spectral data. The ^{13}C NMR spectrum of **V** showed the presence of signals at δ □172.2 for C-6″ of the glucuronic acid that supported the FAB-mass data. The structure of the glycoside quercetin-3-O-glucuronide was further evidenced by mass spectrum. These data are identical with those for quercetin-3-*O*-β-d-glucuronide reported elsewhere.[137]

The mass spectrum of the glycoside shows prominent peaks at *m/z* 477 $[M - H]^-$ and at 301 $[M-H-178]^-$ aglycone-H. The molecular formula was determined as $C_{21}H_{18}O_{13}$, *m/z* 478 by Negative ESI-MS. Therefore, the compound **V** was established as quercetin-3-O-glucuronide.

Compound VI was isolated as pale yellow amorphous powder, gave yellow colour with alkalis, pink colour with Mg-HCI, olive green with Fe^{3+} and answered Molisch's test. Acid hydrolysis of **VI** afforded aglycone - formononetin and sugar - glucose. This was confirmed by co-chromatography with authentic samples. The UV spectrum (λ_{max} in MeOH, 261 nm and 330 nm) showed the characteristic absorption of an isoflavone. No UV bathochromic shift was observed with $AlCl_3$ suggesting the absence of free 5-OH group and absence of bathochromic shift on addition of sodium acetate suggests that there is a free hydroxyl group presented at C-7 position and the 7-hydroxyl group was substituted.

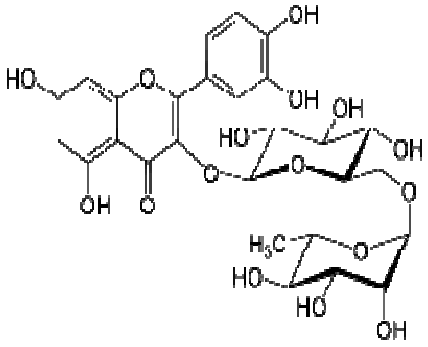
The 1H NMR spectrum of compound **VI** also suggests an isoflavone skeleton with a signal at 8.44 (1H, S, H-2) and one methoxy group with signal at 3.79 (3H, S). The ^{1}H NMR signals of 2 coupled doublets (*J* = 8.8 Hz) at 8.08 and 7.14 were characteristic of 2-ortho-related H-5 and H-6 protons of ring A. ^{1}H NMR signal at 7.28 (1H, S) indicates non-coupled proton at C-8. The ^{1}HNMR spectrum also supported the presence of one sugar moiety with the proton signals at δ H 5.14 (1H, d, *J*=7.8 Hz, Glc-H-1″) related to glucose. ^{1}H NMR signal at 3.79 (3H, s) indicated a methoxy group. Presence of signals at 7.10 (2H, dd, *J* = 6.6, 2.3Hz,

H-3' and H-5') and at 7.54 (2H, dd, *J* = 6.6, 2.3Hz, H-2' and H-6') indicated the presence of the methoxy group at C4' in ring B.

The identity of the sugar was confirmed by ^{13}C NMR (Table 11). The ^{13}C NMR spectrum of **VI** showed the presence of signals at δ □102.1 for C-1″ of the glucose, that supported the FAB-mass data (The fragmentation pattern showed a peak at m/z 268 $[M+H-162]^-$ due to the loss of glucose). The structure of the glycoside formononetin-7-O-substituted aglycone was further evidenced by mass spectrum. The mass spectrum of the glycoside shows prominent peaks at *m/z* 429 $[M-H]^-$ and at 268 $[M+H-162]^-$ aglycone-H. The molecular formula was determined as $C_{22}H_{22}O_9$, *m/z* 430 by Negative ESI-MS. Therefore, the compound **VI** was established as Formononetin-7-O-β-D-glucoside.

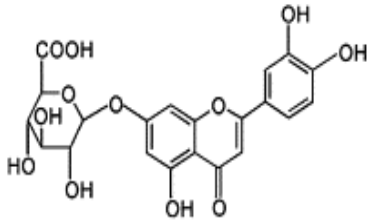
Further the flavonoids present in both the plants were detected and quantified using HPLC using marker compound. The HPLC chromatograms of the bark of *Givotia rottleriformis* showed 7 components. The main difference was in peak eluted at 3.96 min, 11.52 min, 16.68 min and 30.76 min respectively. In the present investigation, 4 flavonoids were quantified at 254 nm using peak area by comparison to a calibration curve derived from the standard, rutin (0.215 mg/gm) quercetin (1.36 mg/gm), kaempferol (6.36 mg/gm) and luteolin (8.64 mg/gm). The HPLC chromatograms of the *Cassia tora* leaves showed 13 components. The main difference was in peak eluted at 12.07 min, 17.15 min, 19.62 min and 31 min respectively. In the present investigation, 4 flavonoids were quantified at 254 nm using peak area by comparison to a calibration curve derived from the standard, quercetin (10.33 mg/gm), kaempferol (7.43 mg/gm), formononetin (0.46 mg/gm) and luteolin (8.56 mg/gm). The results above showed, therefore, that the bark of *Givotia rottleriformis* and *Cassia tora* leaves is a rich source of the important biologically active flavonoids. The described HPLC procedure could be useful for the qualitative and quantitative analysis of flavonoids in plant materials.

High performance thin layer chromatography (HPTLC) is an invaluable quality assessment tool for the evaluation and quantification of botanical materials. The major advantage of HPTLC is that several samples can be analyzed simultaneously using a small quantity of marker compound and mobile phase with very less time. In *Givotia rottleriformis* bark, the flavonoids Rutin, Kaempferol and Luteolin were detected and quantified using HPTLC silica gel 60 F254 pre-coated plates. Solvent systems were optimized to achieve best resolution of the marker compounds from the other compounds of the sample extracts. Of the various solvents tried Benzene: Methanol: Ammonia (90:10:1) gave best resolution for Rutin (R_f 0.18), Luteolin (R_f 0.53) and Kaempferol (R_f 0.61). The identity of Rutin, Kaempferol and Luteolin in sample chromatograms were confirmed by comparison of the R_f values and chromatogram obtained from the reference standard solution at various concentration 100-500 ng/spot at wave length 254 nm were shown in **Fig. 47-52**. Amount of flavonoid present in the ethanol extract was calculated from the calibration curve. The detection of Rutin, Luteolin and Kaempferol was observed to be linear over a concentration range of 100-500 ng/mL and the concentration was found to be Rutin 285 ng/mg, Luteolin 380 ng/mg and Kaempferol 360 ng/mg. Similarly in *Cassia tora* leaves, the flavonoids Luteolin, Quercetin and Formononetin were detected and quantified using HPTLC silica gel 60 F254 pre-coated plates with the mobile phase made of Benzene: Methanol: Ammonia (90:10:1) **Fig. 53-58**. The detection of Luteolin, Quercetin and Formononetin was observed to be linear over a concentration range of 100-500 ng/mL and the concentration was found to be Luteolin 220 ng/mg, Quercetin 160 ng/mg and Formononetin 210 ng/mg.



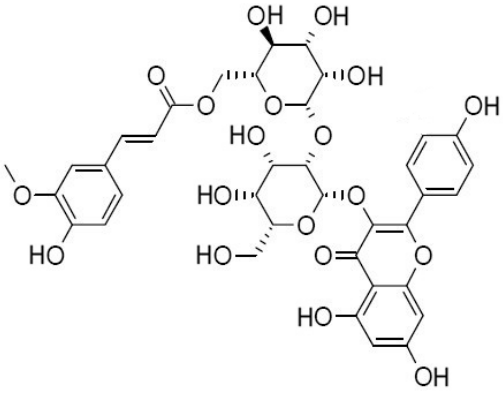
Compound I

(Rutin)



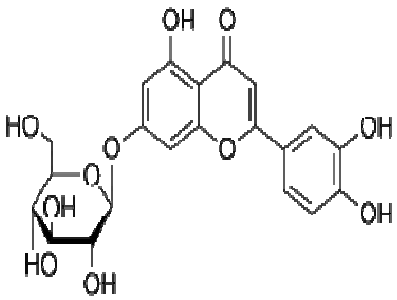
Compound II

(Luteolin-7-O-β-D-glucuronide)



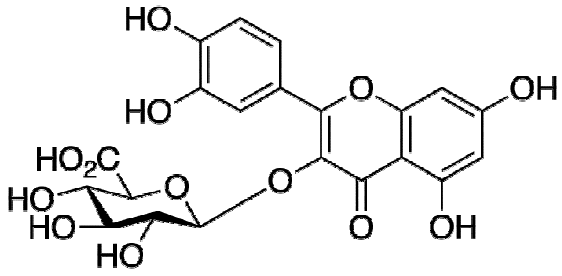
Compound III

(Kaempferol-3-*O*-[2-*O*-(6-*O*-feruloyl)-β-D-glucopyranosyl]-β-D-galactopyranoside)



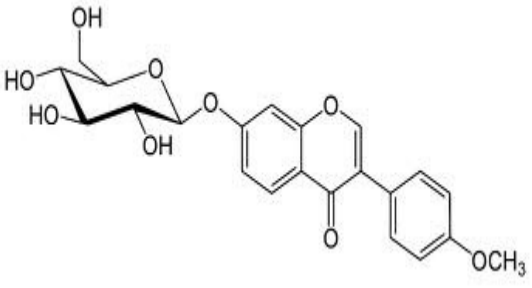
Compound IV

(Luteolin-7-O-β-D-glucopyranoside)



Compound V

(Quercetin-3-O-β-D-glucuronide)



Compound VI

(Formononetin-7-O-β-D-glucoside)

7. ANTIOXIDANT STUDIES

7.1. Introduction

It is increasingly being realized that many of today's diseases are due to the "oxidative stress" which is initiated by free radicals that results from an imbalance between formation and neutralization of pro-oxidants, which seek stability through electron pairing with biological macromolecules such as proteins, lipids and DNA in healthy human cells and cause protein and DNA damage along with lipid peroxidation. These changes contribute to cancer, atherosclerosis, cardiovascular diseases, ageing and inflammatory diseases.[138],[139]

Free radical can be defined as chemical species possessing an unpaired electron, which is formed by homolytic cleavage of a covalent bond of a molecule, by the loss of a single electron from a normal molecule or by the addition of a single electron to a normal molecule. Most of the molecular oxygen consumed by aerobic cells during metabolism is reduced to water by using cytochrome oxidase in mitochondria. However, when oxygen is partially reduced it becomes 'activated' and reacts readily with a variety of bio-molecules. The partial reduction occurs in one electron steps, by addition of one, two and four electrons to O_2, which leads to successive formation of reactive oxygen metabolites (ROMs).[140]

Different Types of Free radicals

The free radicals are species with very short half life, high reactivity and damaging activity towards macromolecules like proteins, DNA and lipids. These species may be either oxygen derived (ROS- reactive oxygen species) or nitrogen derived (RNS- reactive nitrogen species).[141]

The oxygen derived oxidant species: Superoxide anions ($\bar{O}_2$), Hydrogen peroxide (H_2O_2), Hydroxyl radical (•OH), Hydroperoxyl radical (HO_2•), Peroxide ion (HO_2-)

The Nitrogen derived oxidant species: Nitric oxide (NO), Peroxynitrite ($ONOO^-$), Nitrogen dioxide (NO_2), Dinitrogen trioxide (N_2O_3)

All human cells protect themselves against free radical damage by enzymes such as superoxide dismutase (SOD) and catalase, or compounds such as ascorbic acid, tocopherol and glutathione. Sometimes these protective mechanisms are disrupted by various pathological processes, and antioxidant supplements are vital to combat oxidative damage. Recently, much attention has been directed towards the development of ethnomedicines with strong antioxidant properties but low cytotoxicities. Herbs are considered as a store house of enormous antioxidant phytoconstituents out of which flavonoids and polyphenolic compounds are of particular interest.[142]

Flavonoids are powerful antioxidants against free radicals and are described as free-radical scavengers. Flavonoids exhibit several biological effects such as anti-inflammatory, anti-hepatotoxic, antiulcer, anti-allergic, anti-viral and anti-carcinogenic action.[143] These activity is attributed to their hydrogen donating ability. Indeed, the phenolic groups of flavonoids serve as a source of a readily available ‘‘H” atoms such that the subsequent radicals produced can be delocalized over the flavonoid structure. Free radical scavenging capacity is primarily attributed to high reactivities of hydroxyl substituents that participate in the reaction.[144]

$$\mathbf{F\text{-}OH + R^{\cdot} \rightarrow F\text{-}O^{\cdot} + RH}$$

Flavonoids inhibit lipid peroxidation in vitro at an early stage by acting as scavengers of superoxide anion and hydroxyl radicals. They terminate chain radical reaction by donating hydrogen atom to a peroxy radical, thus, forming flavonoids radical, which, further reacts with free radicals thus terminating propagating chain.[145]

The plants *Givotia rottleriformis* bark and *Cassia tora* leaves posess antipsoriatic activity and psoriasis is a chronic inflammatory skin disease. The oxygen free radicals liberated from phagocytes are important in the inflammation process and hence, during the inflammatory response, the increased free radical generation is one of the tissue damaging factors. These undesirable effects of oxidative stress have been found to be controlled by the antioxidant and/or anti-inflammatory effects of compounds and plant extracts. Therefore, the present research work was under taken to investigate the in-vitro antioxidant activity of ethanol extract and isolated flavonoids from the bark of *Givotia rottleriformis* and *Cassia tora* leaves.

7.2. Materials and Methods

In-vitro antioxidant Study

Several concentrations ranging from 50-400 µg/ml of the ethanolic extract of *Givotia rottleriformis* and *Cassia tora* leaves and concentrations ranging from 5-40 µg/ml of isolated flavonoids from the ethanolic extract of *Givotia rottleriformis* and *Cassia tora* leaves were tested for their antioxidant activity in different *in-vitro* models.

7.2.1. Hydroxyl radical scavenging activity

The assay was performed by adding 0.1 ml EDTA, 0.01 ml of ferric chloride, 0.1 ml of hydrogen peroxide, 0.36 ml of deoxyribose, 1 ml of ethanol extract (50-400 µg/ml), 0.33 ml of phosphate buffer (50 mM. pH 7.4), 0.1 ml of ascorbic acid in sequence and incubated at 37^{o}C for 1 hr. The procedure was repeated with isolated flavonoids (5-40 µg/ml), reference standard ascorbic acid (5-40 µg/ml) and control (without the test compound). A 1.0 ml portion of the incubated mixture was mixed with 1.0 ml of 10% tri chloro acetic acid and 1.0 ml of 0.5% thio barbituric acid to develop the pink chromogen, which was measured at 532 nm.[146]

7.2.2. DPPH radical scavenging activity

The free radical scavenging activity was measured in terms of hydrogen donating or radical scavenging ability, using the stable radical, DPPH (2,2-diphenyl-1-picrylhydrazyl). About 0.1 mM solution of DPPH in methanol was prepared and 1 ml of this solution was added to 3 ml of the different concentration of ethanolic extract (50-400 µg/ml), in different test tubes. The procedure was repeated with isolated flavonoids (5-40 µg/ml), reference standard ascorbic acid (5-40 µg/ml) and control (without the test compound, but with an equivalent amount of methanol). The mixture was shaken and allowed to stand at room temperature for 30 min and the absorbance was measured at 517 nm using a spectrophotometer.[147] Decrease in absorbance of the reaction mixture indicates higher free radical scavenging activity.

7.2.3. Nitric oxide radical scavenging assay

Nitric oxide was generated from sodium nitroprusside and measured by Griess reaction. Sodium nitroprusside (5 mM) in standard phosphate buffer saline solution (0.025 M, pH: 7.4) was incubated with different concentrations of ethanolic extract (50-400 µg/ml) dissolved in phosphate buffer saline (0.025 M, pH: 7.4) and the tubes were incubated at 25^{o}C for 5 hr. Control experiments without the test compounds but equivalent amounts of buffer were conducted in an identical manner. After 5 hr, 0.5 ml of incubation solution was removed and diluted with 0.5 ml of Griess reagent (1% sulphanilamide, 2% O-phosphoric acid and 0.1% naphthyl ethylene diamine dihydrochloride).[148] The procedure was repeated with isolated flavonoids (5-40 µg/ml), reference standard ascorbic acid (5-40 µg/ml) and control (without the test compound). The absorbance of the chromophore formed during diazotization of nitrite with sulphanilamide and its subsequent coupling with naphthyl ethylene diamine was read at

546 nm. All determinations were performed in 6 replicates. Percentage inhibition in all the models were calculated by using the formula,

$$\text{Percentage inhibition (\%)} = \frac{\text{Absorbance of control - Absorbance of test}}{\text{Absorbance of control}} \times 100$$

7.2.4. Statistical analysis

Level of significance of all the parameters was expressed as the arithmetic mean ± SEM and was analyzed by one-way analysis of variance (ANOVA), followed by Dunnett's "t" test. *P* value less than 0.05 ($P < 0.05$) was the critical criterion for statistical significance.

7.3. Results

The result showed that the percentage of antioxidant activity of the test sample increases with increasing concentration. The percentage inhibition in various models viz., hydroxyl radical, DPPH, nitric oxide radical were tabulated in Table 12 and Table 13.

Table 12: Free radical scavenging activity of EE and flavonoids from *G. rottleriformis*

Drugs	Concentration (µg/ml)	Hydroxyl radical inhibition (%)	DPPH radical inhibition (%)	Nitric oxide inhibition (%)
Ethanol extract	50	17.18±1.24	14.05±2.48	13.57±0.70
	100	23.62±2.12	22.83±3.26	27.40±2.26
	200	43.24±1.60	48.50±2.82**	56.62±1.35**
	300	65.12±1.36**	68.67±0.90**	74.31±1.79**
	400	87.72±3.27**	92.89±0.68**	88.26±1.28**
	IC_{50}	**230 µg/ml**	**220 µg/ml**	**180 µg/ml**
Compound **I**	5	13.20±1.14	18.23±0.92	18.53±1.10
	10	28.90±2.04	31.07±1.36	34.20±2.86
	20	56.17±1.16**	64.01±1.78**	64.03±1.66**
	30	81.32±1.82**	83.04±1.34**	80.15±2.32**
	40	93.57±1.94**	91.46±1.26**	96.58±4.24**
	IC_{50}	**18 µg/ml**	**16 µg/ml**	**15 µg/ml**
Compound **II**	5	12.38±0.92	14.25±2.20	15.16±2.06
	10	34.92±1.16	24.09±1.84	21.35±1.36
	20	47.23±0.82**	50.12±1.40**	56.17±3.11**
	30	72.07±1.14**	71.10±2.12**	82.68±2.33**
	40	86.41±1.60**	85.20±3.04**	90.90±1.04**
	IC_{50}	**22 µg/ml**	**20 µg/ml**	**18 µg/ml**
Compound **III**	5	16.37±2.02	13.72±0.83	12.58±2.80
	10	21.18±3.20	21.83±0.05	20.92±3.12
	20	40.62±1.18	38.38±1.46	41.48±2.36
	30	61.12±0.68**	54.94±0.78**	64.82±4.68**
	40	81.28±1.06**	76.92±2.28**	83.57±1.26**
	IC_{50}	**25 µg/ml**	**28 µg/ml**	**24 µg/ml**
Ascorbic acid	5	14.31±1.20	12.38±1.80	10.81±1.72
	10	30.46±3.09	20.68±1.28	18.68±0.62
	20	59.68±1.38**	41.36±1.82	28.26±2.31
	30	88.25±1.49**	62.84±1.82**	44.36±1.28**
	40	94.60±2.30**	80.82±2.18**	70.10±2.16**
	IC_{50}	**18 µg/ml**	**24 µg/ml**	**35 µg/ml**

***EE** – Ethanol extract; Values are mean ± SEM of 6 parallel measurement. Statistical significant test for comparison was done by ANOVA, followed by Dunnet's 't' test (n=6). All the values are statistically significant at ***P*< 0.01.

Table 13: Free radical scavenging activity of ethanol extract and flavonoids from *C. tora* leaves

Drugs	Concentration (µg/ml)	Hydroxyl radical inhibition (%)	DPPH radical inhibition (%)	Nitric oxide inhibition (%)
Ethanol extract	50	11.24±1.14	13.36±1.36	14.14±1.29
	100	18.45±1.72	28.26±2.14	30.36±1.40
	200	39.13±1.32	56.25±1.46**	61.02±1.72**
	300	54.24±0.66**	80.14±2.52**	82.31±1.79**
	400	83.24±1.36**	97.17±2.40**	94.15±1.50**
	IC_{50}	**270 µg/ml**	**190 µg/ml**	**130 µg/ml**
Compound **IV**	5	12.68±1.20	11.97±1.67	12.20±1.58
	10	26.46±1.89	22.24±0.05	27.21±1.69
	20	50.90±2.11**	44.90±1.12	54.35±1.50**
	30	74.43±1.45**	68.15±0.93**	69.12±1.40**
	40	88.16±2.36**	85.36±3.17**	90.14±0.75**
	IC_{50}	**20 µg/ml**	**23 µg/ml**	**18 µg/ml**
Compound **V**	5	19.32±0.08	18.43±1.22	11.23±1.36
	10	33.48±1.64	34.21±1.09	28.32±2.20
	20	68.40±2.60**	69.68±0.83**	57.28±1.27**
	30	80.20±3.02**	85.35±1.60**	82.43±1.47**
	40	96.27±1.03**	98.08±0.94**	93.27±2.38**
	IC_{50}	**15 µg/ml**	**14 µg/ml**	**18 µg/ml**
Compound **VI**	5	13.06±1.46	10.90±1.48	19.24±1.37
	10	24.41±2.10	21.69±2.13	34.42±2.15
	20	52.45±1.60**	48.83±1.56**	68.11±3.20**
	30	79.08±2.20**	72.56±3.22**	80.25±0.36**
	40	87.32±3.28**	90.54±1.38**	95.68±2.59**
	IC_{50}	**19 µg/ml**	**21 µg/ml**	**14 µg/ml**

Values are mean ± SEM of 6 parallel measurements. Statistical significant test for comparison was done by ANOVA, followed by Dunnet's 't' test (n=6). All the values are statistically significant at $^{**}P < 0.01$.

7.4. Discussion

Psoriatic skin is also characterized by an advanced state of lipid peroxidation. Thus, it has been suggested that the antioxidant treatment could be part of a more specific and effective therapy for the management of this skin disease.[149] Recent literature data continue to support the fact that polyphenolic compounds, found in most plants, can have a positive effect on many chronic diseases.[150] Natural polyphenols, recognized as potent antioxidants, are multifunctional molecules that can act as anti-inflammatory and antiproliferative agents through the modulation of multiple signaling pathways.[151] This characteristic could be advantageous for the treatment of multi-causal diseases, such as psoriasis.

Inhibition of Hydroxyl radical: Hydroxyl radical is highly reactive oxygen centered radical, formed from the reaction of various hydroperoxides with transition metal ions. It attacks proteins, DNA, polyunsaturated fatty acid in membranes, and most biological molecule it contacts and is known to be capable of abstracting hydrogen atoms from membrane lipids and brings about peroxidic reaction of lipids.[148] The ethanolic extract of *Givotia rottleriformis*, *Cassia tora* and isolated flavonoids exhibited concentration dependent scavenging activity against hydroxyl radical generated in a Fenton reaction system. The IC_{50} value of ethanolic extract of *Givotia rottleriformis*, *Cassia tora* and reference standard ascorbic acid being of 230 µg/mL, 270 µg/mL and 18 µg/mL respectively. The IC_{50} value of flavonoids **I, II, III** isolated from *Givotia rottleriformis* were 18 µg/mL, 22 µg/mL, 25 µg/mL, and flavonoids **IV, V, VI** isolated from the *Cassia tora* leaves were 20 µg/mL, 15 µg/mL, and 19 µg/mL respectively. On a comparative basis, the flavonoid **V** showed better activity in quenching nitric oxide with an IC_{50} value of 15µg/ml.

Inhibition of DPPH radical: DPPH assay is considered to be a valid and easy way to evaluate scavenging activity of antioxidants, since the radical compound is stable and does not have to generate as in other radical assays. DPPH radicals react with suitable reducing agents and then electrons become paired off and the solution loses colour with the number of electrons taken up. Such reactivity has been widely used to test the ability of compound and plant extract to act as free radical scavengers. The antioxidant candidate which proves promising in the DPPH antioxidant assay would provide an optimistic scaffold for prospective in vivo studies.[152] The potential decrease in the concentration of DPPH radical due to the scavenging ability of ethanolic extract of *Givotia rottleriformis* and its flavonoids **I, II, III** showed significant free radical scavenging activity of about 92%, 91%, 85% and 77% respectively at higher doses with the IC_{50} value being 220 µg/ml, 16 µg/mL, 20 µg/mL and 28 µg/mL respectively. Similarly ethanolic extract of *Cassia tora* leaves and its flavonoids **IV, V, VI** showed significant free radical scavenging activity of about 97%, 85%, 98%, and 90% respectively at higher doses with the IC_{50} value being 190 µg/ml, 23 µg/mL, 14 µg/mL and 21 µg/mL respectively.

Inhibition of Nitric oxide radical: Nitric oxide (NO) is a potent pleiotropic mediator of physiological process such as smooth muscle relaxant, neuronal signaling, inhibition of platelet aggregation and regulation of cell mediated toxicity. It is a diffusible free radical which plays many roles as an effectors molecule in diverse biological systems including neuronal messenger, vasodilatation and antimicrobial and antitumor activities.[153] Although nitric oxide and superoxide radicals are involved in host defense, over production of these two radicals contributes to the pathogenesis of some inflammatory diseases. Moreover in the pathological conditions, nitric oxide reacts with superoxide anion O^2 to produce stable potentially cytotoxic molecules peroxynitrite through intermediates NO_2, N_2O_4 and N_3O_4. Nitric oxide inhibitors have

been shown to have beneficial effects on some aspect of inflammation and tissue damage seen in inflammatory diseases.[154] The IC_{50} value of ethanolic extract of *Givotia rottleriformis*, *Cassia tora* and reference standard ascorbic acid being of 180 µg/mL, 130 µg/mL and 35 µg/mL respectively. The IC_{50} value of flavonoids **I, II, III** isolated from *Givotia rottleriformis* were 15 µg/mL, 18 µg/mL, 24 µg/mL and flavonoids **IV, V, VI** isolated from the *Cassia tora* leaves were 18 µg/mL, 18 µg/mL and 14 µg/mL respectively. On a comparative basis, the flavonoid **VI** showed better activity in quenching nitric oxide with an IC_{50} value of 14µg/ml.

8. *IN VITRO* ANTI-PSORIATIC ACTIVITY

8.1. Introduction

The biological experiments like *in-vitro* and *in-vivo* screening models for various diseases may be applied to discover new molecules. These contemporary techniques include bio evaluation, clinical trials, toxicological studies etc. The use of disease induced animal models or the genetically transformed disease models are in use to validate the claims of these medicines, but ethical issues and cost effectiveness of these models have discouraged their common use. Besides, these disease induced models very often get reversed to the normal condition in due course of time as a part of natural healing, which also makes it difficult in interpreting the result of a test drug. Therefore, *in-vitro* mechanism based screening of herbal medicine is mandatory in the initial phases of plant drug research before taking them to *in-vivo* study to evaluate their efficacy.

Keratinocyte is the primary cell found in the epidermis, the outermost layer of the skin constituting 90% of the cells. Keratinocyte is also sometimes found in the basal layer of the skin. The function of the keratinocyte is the formation of the keratin layer that protects the skin and the underlying tissues from the environmental damages such as the heat, UV.[155] Anti-psoriatic activities are reflected by inhibition of keratinocyte proliferation. Hence the potency of the ethanol extract of the *Givotia rotleriformis* bark, *Cassia tora* leaves and isolated flavonoids were screened using HaCaT human keratinocyte cell line.

8.2. Materials and Method

HaCaT Cell Inhibition assay

In vitro antipsoriatic activity was carried out in HaCaT human keratinocyte cell line. Human HaCaT keratinocytes were obtained from NCCS, Pune, India. The cells were seeded

at a concentration of 1.0×10^5 cells/ml in a 96 well microtitre plate and grown in Dulbecco's modified Eagle's medium (DMEM, Gibco) supplemented with 10% fetal bovine serum (BioWest). After 24 h, the supernatant was decanted and the monolayer was washed once. Then 100 µl of test drug dilution, ethanol extract of *Givotia rottleriformis* bark, *Cassia tora* leaves and isolated flavonoids **I-VI** prepared with above media was added per well in microtitre plates. The plates were then incubated at 37°C for 3 days in 5% CO_2 atmosphere. Antiproliferant activity was assessed by performing the Sulphorhodamine B (SRB) assay.[156]

Cells were fixed by adding 25µl of ice-cold 50% trichloroacetic acid on top of the growth medium and the plates were incubated at 4°C for 1 h, after which plates were washed to remove traces of medium, drug and serum. SRB stain (50µl; 0.4% in 1% acetic acid) (Sigma) was added to each well and left in contact with the cells for 30 min after which they were washed with 1% acetic acid, rinsing 4 times until only dye adhering to the cells was left. The plates were then dried and 100µl of 10mM Tris buffer (Sigma) added to each well to solubilise the dye. The plates were shaken gently for 5 min and absorbance read at 550 nm using a micro plate reader (Biorad, USA). Data obtained at different concentrations were used for IC_{50} calculations.

8.3. Result

The cytotoxic effect of ethanol extract and isolated flavonoids **I –VI** were evaluated using HaCaT cells, a rapidly multiplying human keratinocyte cell line, as a model of epidermal hyperproliferation in psoriasis. The results were validated using asiaticoside as positive control and tabulated in Table 14.

Table 14: Inhibitory concentration against HaCaT cell line by Sulphorhodamine B assay

S. No	Sample	IC_{50} value (µg/ml)
1	Ethanol extract of *G. rottleriformis*	310.30
2	Ethanol extract of *C. tora*	290.48
3	Compound **I**	180.70
4	Compound **II**	56.50
5	Compound **III**	76.50
6	Compound **IV**	58.65
7	Compound **V**	146.20
8	Compound **VI**	87.74
9	Asiaticoside	31.50

Discussion

Psoriasis is a chronic inflammatory skin disorder characterized by hyperproliferation and aberrant differentiation of keratinocytes, inflammation in dermis and epidermis and leukocyte infiltration. In normal condition, epithelial turn over takes place in about 311 hrs (12.5 days) and divisions of a single keratinocyte occur in about once in 60 days. In psoriatic state, the epithelialization occurs in about 36 hrs and so the division of keratinocytes which reduces to 10 days (240 hrs). This phenomenon collectively leads to hyperkeratinized state.

Human keratinocyte cell line (HaCaT) has been widely employed as cell models to evaluate anti-psoriatic activities of test molecules. Anti-psoriatic activities are reflected by inhibition of keratinocyte proliferation and down regulation of stimulated levels of cytokines secreted by keratinocyte and monocytes. Among the tested flavonoids, **II, III, IV, VI** showed appreciable antiproliferant activity in HaCaT cell line. Asiaticoside showed a potent activity with IC_{50} value of 31.50 µg/ml.

9. PHARMACOLOGICAL STUDIES

Models of human diseases reproduced in animals have long been a requisite for discovering new modalities in managing diseases therapeutically. Rodents being the animals of choice for modern medical researchers with their short life span (2-3 years) which allows scientists to observe within a short time span, the prognosis and pathogenesis of a disease. Pharmacological screening is essential to evaluate the efficacy and potency of the plant drug.

Givotia rottleriformis bark and *Cassia tora* leaves have been used in the indigenous systems of medicine for the treatment of chronic inflammatory skin disease, psoriasis. The acute and subacute toxicity study of *Cassia tora* leaves was reported earlier by Ambali et al., 2006.[157] Among the isolated compounds, the compound **II** and **V** exhibited similar antiproliferant activity in HaCaT cell line, they were of same flavonoid luteolin and the yield of compound **II** was high when compared with compound **IV**. So compound **II** was subjected to further studies along with compound **III** and **VI**. Hence, the present study was focused to find out the subacute toxicity of the ethanolic extract of *Givotia rottleriformis* bark in rat and acute toxicity of the ethanolic extract and compound **II, III** and **VI** in mice respectively.

9.1. ACUTE TOXICITY STUDY

9.1.1. Materials and Methods

Ethanolic extract (70%v/v) of *Givotia rottleriformis* bark and compound **II, III** and **VI**

9.1.2. Experimental animals

Healthy *Wistar* Albino rats of either sex, weighing about 250-200 g and *Swiss* Albino mice of either sex weighing 25-30 g were procured from Animal House. The entire process was approved by the Institutional Animal Ethical Committee which is certified by the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA) IAEC/52/2012.

The animals were kept in clean and dry polycarbonate cages and maintained in a well ventilated animal house with 12 hrs light– 12 hrs dark cycles. The animals were fed with standard pellet diet and water was given as libitum. For experimental purpose, the animals were kept fasting overnight but allowed for access to water.

9.1.3. Acute toxicity study

Acute toxicity study was performed according to OECD guidelines 423 (Organization of Economic Co-Operation and Development). It is a stepwise procedure with three animals of single sex per step. Depending on the mortality and morbidity status of the animal, on average of 2-4 steps may be necessary to allow judgment on the test substance. The procedure is to fix a minimal number of animals, which allows acceptable database scientific conclusion. The method uses different defined doses (5, 50, 500, 2000 mg/kg body weight) and the results allow a substance to be ranked and classified according to the "Globally Harmonized System" (GHS) for the classification of extracts which cause acute toxicity.[158]

Procedure

Three healthy, Swiss Albino mice weighing 25-30 gm were selected for the study. The mice were fasted over-night and provided with water ad libitum. Following the period of fasting, the animals were treated with the ethanolic extract at the dose of 2000 mg/kg body weight and with compound **II, III** and **VI** at the dose of 500 mg/kg body weight, orally. As most of the crude extracts possess LD_{50} value more than 2000 mg/kg body weight and this was used as starting dose. After oral administration, the mice were observed on hourly basis for 24 hrs to access mortality and to detect any changes in the autonomic or behavioral responses viz. alertness, aggressiveness, spontaneous activity, irritability, tremor, corneal reflex, salvation, urination, respiration and convulsion etc.

The mice were observed regularly for 14 days to note the mortality or toxic symptoms. Since there was no death as per the guidelines, the study was repeated with the same dose to confirm the results. The flow chart depicts the procedure adopted for this method.

9.2. SUB-ACUTE TOXICITY STUDY

9.2.1. Materials and Methods

In a 28-days sub acute toxicity study, eighteen Wistar rats of either sex were divided into three groups of 6 rats each. Group I that served as normal control was administered with distilled water (p.o.) while groups II, III, were administered daily with the ethanolic extract of the *Givotia rottleriformis* bark at a dose 200 and 400 mg/kg body weight based on the acute toxicity results respectively. The animals were then observed daily for gross behavioural changes and any other signs of subacute toxicity. The weight of each rat was recorded on day 0 and weekly throughout the course of the study, food and water consumption per mice was calculated. At the end of the 28 days they were fasted overnight, each animal was anaesthetized with diethyl ether, following which they were then dissected and blood samples were obtained by cardiac puncture into heparinised tubes. The blood sample collected from each rat was centrifuged with 3000 X g at 4^{o}C for 10 min to separate the serum and used for the biochemical assays.

Haematological Parameters

At the end of 28 days, blood samples were collected from overnight fasted animals through retro-orbital sinus puncture in ethylene diamine tetra acetic acid (EDTA) coated vials and plasma was separated by cold centrifugation (Plasto Crafts Superspin-R centrifuge) at 3000 rpm for 10 min. Blood was also collected for the analysis of haematological parameters such as white blood cell (WBC) count, red blood cell (RBC) count, haemoglobin (Hb) levels, mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH), mean corpuscular

haemoglobin concentration (MCHC) using BC 2300 Haematology Analyzer (Shezhen Mindray Biomedical Electronics Co., Ltd., China).

Plasma biochemical parameters

Aspartate aminotransferase (AST), alanine aminotransferase (ALT),[159] total protein (liver damage)[160] alkaline phosphatase (ALP)[161], urea[162] and creatinine (kidney damage)[163] were analyzed using commercially available kits (Recon diagnostic Ltd., Vadodara, India). Also, plasma glucose and lipid profile[163] [total cholesterol (TC), triglyceride (TG) and high density lipoprotein (HDL-C)] were assessed and low density lipoprotein (LDL-C) and very low density lipoprotein (VLDL-C) were calculated.

Relative organ weights and histopathology

Animals were later sacrificed by cervical dislocation under mild ether anesthesia for autopsy and liver, kidney, heart, lung, stomach, brain, ovary, testes and spleen were excised, rinsed in 0.9 % saline and weighed. After sacrifice, organ weights (lungs, heart, liver, kidney, brain, stomach, ovary, testes, and spleen) were recorded and relative organ weights (ROW) were calculated as follows.

$$\text{ROW} = \frac{\text{Absolute organ weight (g)}}{\text{Body weight on the day of sacrifice (g)}} \times 100$$

Tissue pieces of vital organs (heart, liver and kidney) were fixed in 10 % para formaldehyde for paraffin histology and processed in paraffin embedding as per the standard protocol. 7 μm thick sections of each tissue were stained with hematoxylin and eosin, and observed for possible histopathological damages.

9.2.2. Statistical analysis

Values were represented as mean ± SEM. Data were analysed using one-way analysis of variance (ANOVA) and group means were compared using the Tukey-Kramer Multiple Comparison test using Instat-V3 software. *P* values < 0.05 were considered significant.

9.2.3. RESULTS

Acute toxicity study

During the acute toxicity study, the ethanolic extract and compound **II, III** and **VI** was administered orally and animals were observed for mortality and behavioral responses. There was no mortality observed even at 2000 mg/Kg for the extract and at 500 mg/Kg for the compounds. All the animals were found to be normal and there were no gross behavioral changes till the end of the observation period. This observation revealed that the ethanolic extract of the *Givotia rottleriformis* bark was found to be very safe up to 2000 mg/Kg of body weight known as maximum tolerated dose (MTD) by acute toxicity model study as per OECD guidelines 423. Hence from this 1/10th and 1/5th of MTD was selected and the effective doses were fixed as 200 and 400 mg/kg for the further pharmacological studies. Similarly, the isolated compound was found to be very safe up to 500 mg/kg of body weight known as maximum tolerated dose (MTD) by acute toxicity model study as per OECD guidelines 423. Hence from this 1/10th of MTD was selected and the effective doses were fixed as 50 mg/kg for the further pharmacological studies.

Sub acute toxicity study

The results of haematological investigations conducted on day 28, revealed following significant changes in the values of different parameters investigated when compared with those of respective controls; however, the increase or decrease in the values obtained was within normal biological and laboratory limits tabulated in Table 14-17 and shown in **Fig. 60.**

Table 14: The effects of the ethanol extract of the G. rottleriformis on weight changes

Group	Initial Weight	Body Weight (g)			
		1 Week	2 Weeks	3 Weeks	4 Weeks
Normal	158.20±10.20	162.11±7.78	162.66±12.22	163.32±14.02	165.68±9.88
Ethanol extract 200 mg/kg	160.25±8.22	163.55±14.12	172.15±11.45	185.14±12.30	190.20±10.46*
Ethanol extract 400 mg/kg	162.74±10.22	164.32±11.20	175.29±10.25	186.28±11.94	193.34±4.11*

n = 6; values are expressed as mean ± SEM. Data were analyzed by one-way ANOVA followed by Tukey multiple comparison test. The values are $*P< 0.05$ when compared against control.

Table 15: The effects of ethanol extract of the *G. rottleriformis* on haematological studies

S. No	Parameters	Control	Ethanol extract 200 mg/Kg	Ethanol extract 400 mg/Kg
1	RBC ($10^{2/}$µL)	8.32±0.68	8.20±0.38	8.64±0.42
2	Hb (g/dl)	16.22±1.02	15.88±0.24	16.38± 0.16
3	MCV (fL)	64.61±6.66	60.54±5.88	61.22±5.98
4	MCH (pg)	18.98±4.62	17.86±1.68	19.12±2.28
5	MCHC (gm/dl)	36.61±2.86	36.84±2.54	36.08±2.22
6	WBC ($10^{2/}$µL)	8.2±2.08	7.7±1.88	7.9±2.46
7	Neutrophils (%)	22.24±2.54	23.68±1.82	23.72±1.26
8	Eosinophils (%)	1.22±0.4	1.38±0.86	1.28±0.66
9	Basophils (%)	0.0±0.00	0.1±0.02	0.00±0.00
10	Lymphocyte (%)	68.12± 0.24	68.86±0.26	69.02±1.12
11	Monocyte (%)	2.06±0.22	2.46±0.48	2.82±0.98

n = 6; values are expressed as mean ± SEM

RBC: Red blood corpuscle, **Hb:** Haemoglobin; **MCV:** Mean corpuscular volume, **MCH**: Mean corpuscular haemoglobin, **MCHC**: Mean corpuscular haemoglobin concentration, **WBC:** white blood corpuscle.

Table 16: The effect of ethanol extract of the *G. rottleriformis* on Biochemical Profiles

Parameter	Control	Ethanol extract 200 mg/Kg	Ethanol extract 400 mg/Kg
Glucose (mg/dl)	104.61±4.35	105.51±5.42	114.10±4.33
Cholesterol (mg/dl)	44.15±1.42	42.27±1.23	41.50±2.41
Triglyceride (mg/dl)	78.72±2.15	85.84±2.12	86.72±4.14
HDL (mg/dl)	105.12±8.04	104.8±8.71	113.12±7.27
LDL (mg/dl)	78.32±2.31	79.40±3.42	79.58±2.35
Protein (mg/dl)	7.11±0.30	7.82±0.28	7.71±0.32
Albumin(mg/dl)	3.10±0.20	3.18±0.22	3.12±0.24
Globulin(mg/dl)	3.16±0.07	3.15±0.06	3.16±0.08
Creatinine (mg/dl)	0.20±0.04	0.27±0.04	0.24±0.05
Urea (mg/dl)	58.60±0.80	62.47±0.24	61.60±0.56
AST IU/L	54.10±1.12	55.21±2.31	55.10±2.20
ALT IU/L	25.42±1.76	26.10±2.28	22.18±2.36
ALP IU/L	62.26±3.30	65.12±3.11	62.10±1.54

n = 6; values are expressed as mean ± SEM. Data were analyzed by one-way ANOVA followed by Tukey multiple comparison test.

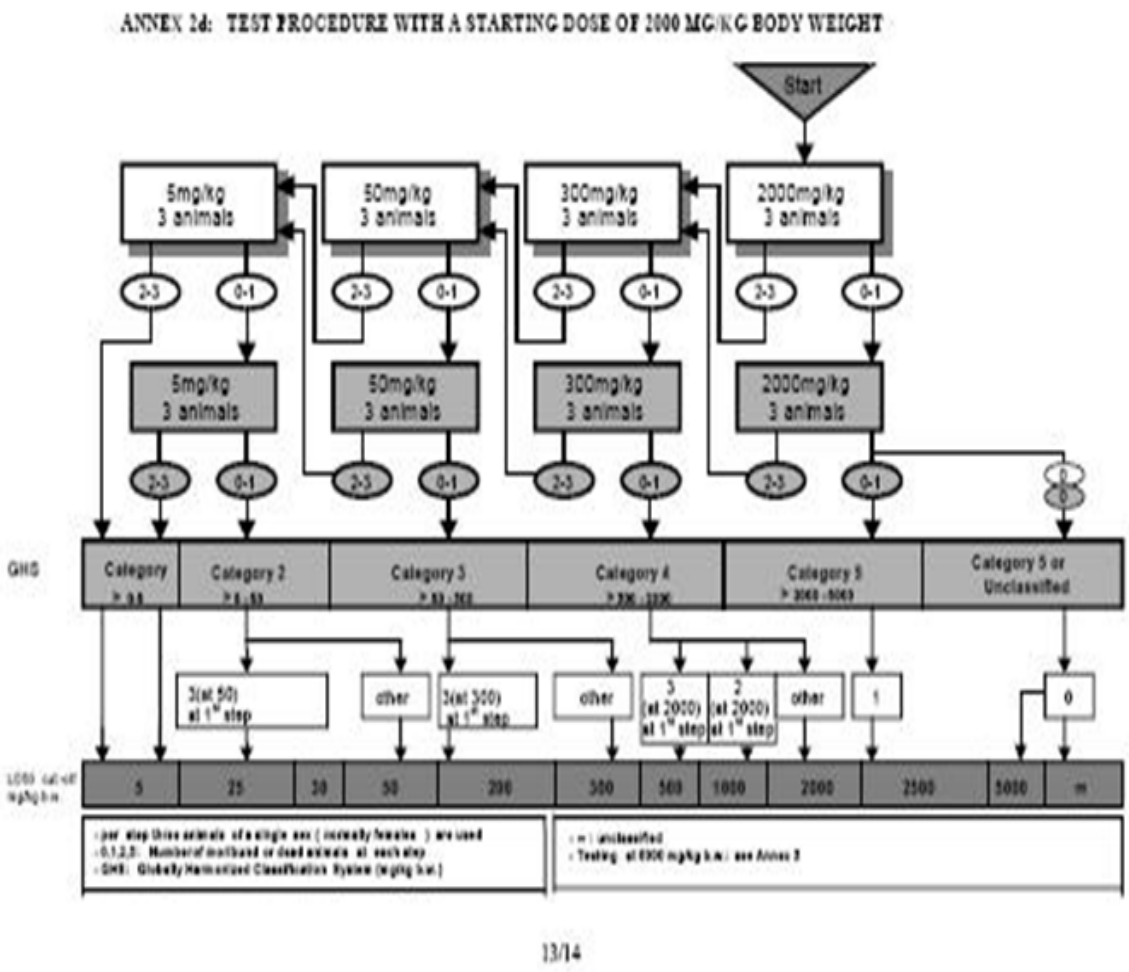
HDL: High density lipoprotein; **LDL**: Low density lipoprotein; **AST**: Aspartate transaminase; **ALT:** Alanine transaminase; **ALP:** Alkaline phosphatase

Table 17: Effect of ethanol extract of the *G. rottleriformis* bark on organ weight

Organ	Control	Ethanol extract 200 mg/Kg	Ethanol extract 400 mg/Kg
Liver (g)	3.00±0.10	3.10±0.12	2.92±0.09
Heart (g)	0.30±0.04	0.30±0.04	0.29±0.02
Lung (g)	0.44±0.12	0.44±0.10	0.45±0.10
Spleen (g)	0.45±0.04	0.46±0.04	0.46±0.05
Ovary (g)	1.62±0.28	1.64±0.20	1.66±0.18
Testes (g)	2.32±0.12	2.34±0.11	2.30±0.10
Brain (g)	1.22±0.14	1.24±0.10	1.32±0.12
Kidney (g)	0.81±0.05	0.78±0.05	0.75±0.05
Stomach (g)	1.12±0.11	1.14±0.12	1.10±0.10

n = 6; values are expressed as mean ± SEM

Data were analyzed by one-way ANOVA followed by Tukey multiple comparison test. The values are NS-Non significant when compared against control.



13/14

Figure 59: OECD Guidelines

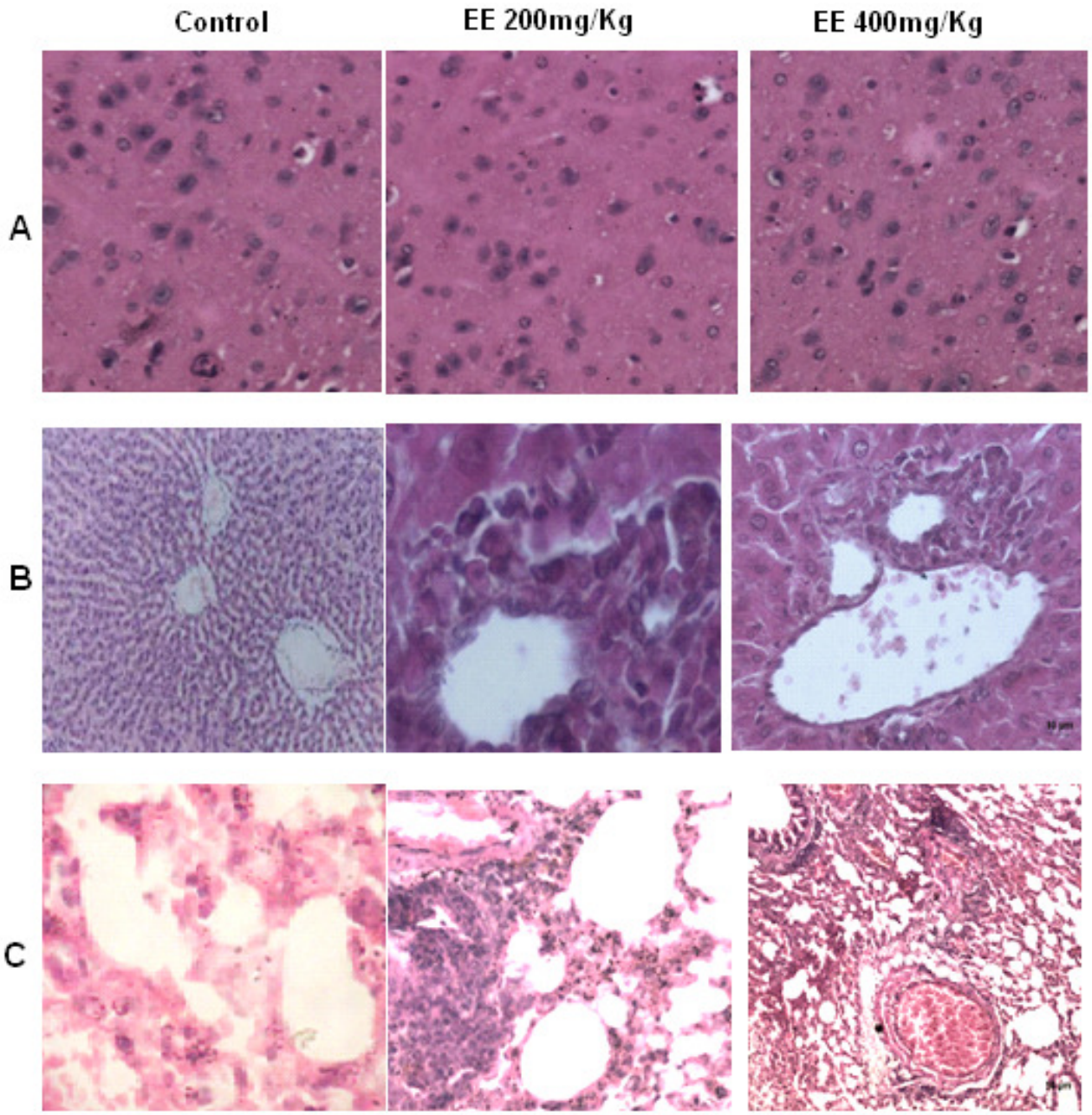


Figure 60: Photomicrographs of the sections of the (A) Brain, (B) liver; (C) Lung of control, ethanol extract (EE 200 mg/Kg); ethanol extract (EE 400 mg/Kg) of *G. rottleriformis* administered mice for 28 days

9.2.4. Discussion

World Health Organization (WHO) estimates that approximately 80 % of the developing world's population is using traditional medicine for primary healthcare.[164] However, there is a prevalent misunderstanding that herbal medicines are devoid of toxic effects (WHO, 2004).[165] Adverse effects of herbs have been reported including allergic reactions, hepatotoxicity, nephrotoxicity, cardiac toxicity, neurotoxicity and even death have been reported.[166,167,168.169] Therefore, a pre-clinical toxicity study is indispensible to validate their safe medicinal use.

Sub-acute oral toxicity studies have provided information on drugs that can possibly pose health risks. Twenty eight days of oral administration of ethanol extract of *G. rottleriformis* did not result in death of the animals. No sign of observable toxicity was detected during the experimental period. Rats treated with various doses of ethanolic extract of *G. rottleriformis* had a progressive weight gained. This increase in weight is significantly different ($P< 0.05$) from that of the control (Table 14). The progressive increase in body weight at doses of 200 and 400 mg/Kg of rats during 28 days of drug administration may indicate the improvement of the nutritional state of the animal. The growth response effect could be as the result of increased food and water intake.

The levels of serum analytes such as glucose, cholesterol, AST and ALT, ALP, total protein, albumin, urea and creatinine were not significantly different between the control and the experimental groups of rats when fed with ethanol extract (Table 16). High levels of AST and ALT are reported in liver diseases or hepatotoxicity.[170] Plasma AST, ALT and bilirubin of ethanol extract of *G. rottleriformis* treated groups were comparable to the control group, thus indicative of normal functional status of liver. Renal dysfunction can be assessed by concurrent measurements of urea and creatinine and their normal levels reflect at reduced likelihood of renal problems.[171]In the present study, changes in plasma urea and creatinine levels in ethanol extract

treated groups showed no significant differences on a dose dependent manner indicating a normal renal function.

Food consumption of control and treated animals was found to be comparable throughout the dosing period of 28 days. Organ weight data of animals sacrificed at the end of the dosing period was found to be comparable with that of respective controls. No abnormal changes were observed in organ mass with respect to body mass of ethanol extract fed rats when compared with control.

The haematopoietic system is one of the most sensitive targets for toxic compounds and hence it is mandatory to record any possible alterations resulting from a test substance.[172] Change in haematological parameters has a higher predictive value, when the data of drug toxicity on animal studies are translated for clinical usage.[173] A normal haematological profile of ethanol extract treated groups also further justified the non-toxic nature of plant extract.

Histopathological examination of brain, liver and lung did not reveal any significant abnormality when compared with control.Based on these findings, it can be concluded that the effect of ethanol extract of *G. rottleriformis* (200 mg/Kg and 400 mg/Kg) treated via oral route over a period of 28 days have no toxic effect on rats. In light of these findings, we may conclude that ethanol extract of *G. rottleriformis* is not toxic in all the doses studied herein. This study is the first report that evaluates toxicity of ethanol extract of *G. rottleriformis* and defines it as non-toxic up to a dose of 2000 mg/kg body weight.

Toxicity profile of *Cassia tora* leaves reported by Ambali et al.[157] revealed that the twenty eight days of oral administration of methanol extract of *Cassia tora* did not result in death of the animals. Clinical signs of toxicity include diarrhea; histopathological findings included congestion of the liver, spleen and kidney.

9.3. *IN VIVO* ANTIPSORIATIC ACTIVITY

9.3.1. Introduction

Psoriasis is characterized by complex and striking alterations in epidermal growth and differentiation.[174] Granular layer of the epidermis is greatly reduced or absent in psoriatic lesions. Parakeratotic condition is seen in the adult mouse tail which is one of the hallmarks of psoriasis. Induction of orthokeratosis in the adult mouse tail is the basis behind the mouse tail test.[175,176] The healing process of psoriasis includes increase in orthokeratotic portion which is non nucleated region lies on uppermost skin layer. i.e. epidermis. Many drugs presently used in the treatment of psoriasis have been evaluated by the mouse tail test and are found to have shown good efficacies. Drug activity is defined by the increase in percentage of orthokeratotic regions. (These are the regions in a cell having no nucleus and involved in protection from invaders like micro-organisms, UV rays, weak acids & bases).

9.3.2. Materials and Method

Perry scientific mouse tail model

The mouse-tail model is based on the induction of orthokeratosis in those parts of the adult mouse-tail, which have normally a parakeratotic differentiation. This is accepted as a screening method for measuring anti psoriatic activity of drugs. Orthokeratotic portions are the regions in a cell having no nucleus and involved in protection from invaders like micro-organisms, UV rays, weak acids & bases.[177] Therefore orthokeratosis is the indication of skin repair in psoriasis. In psoriasis there is a disorder of excessive growth and reproduction of skin epidermal cells due to rapid increase in keratinocyte differentiation. As an indicator of orthokeratosis, the number of scale regions with a continuous granular layer is counted and

expressed as a percentage of the total number of scale regions per section. Drug activity is defined by the increase in percentage of orthokeratotic regions.

Drug tested

The ethanolic extract of bark of *Givotia rottleriformis* (100, 200 and 400 mg/kg BW) and the *Cassia tora* leaves (100, 200 and 400 mg/kg BW), compound **II, III** and **VI** (50 mg/kg BW) and combination of compounds (**II, III, VI**) to access synergistic effect of three compounds were selected for the *in-vivo* pharmacological screening based on the *in-vitro* pharmacological study. The control group received normal saline (10 ml/kg, p.o.) and standard group received retinoic acid (0.5 mg/kg, p.o.).

Procedure

Healthy adult albino mice weighing about 25-30 g received their treatment once in a day for five days a week for 2 weeks. Two hours after the last treatment animals were sacrificed; longitudinal sections of the tail skin were made and prepared for histological examination (hematoxylin- eosin staining). As an indicator of orthokeratosis, the number of scale regions with a continuous granular layer is counted and expressed as a percentage of the total number of scale regions per section. Drug activity is defined by the increase in percentage of orthokeratotic regions. Ten sequential scales were examined for the presence of a granular layer induced in the previously parakeratotic skin areas. The induction of orthokeratosis in those parts of the adult mouse tail, which have normally a parakeratotic differentiation, was quantified measuring the length of the granular layer (A) and the length of the scale (B). The proportion (A/B)×100 represents the % orthokeratosis per scale, and the drug activity (DA) was calculated as follows:

$$DA = \frac{\text{mean OK of treated group - mean OK of control group}}{\text{100 - mean OK of control group}} \times 100$$

where OK = orthokeratosis:

The measurements were carried out at the border of the scale with a semiautomatic image evaluation unit. [178]

Measurement of epidermal thickness

It was obtained by measuring the distance between the dermoepidermal borderline and the beginning of the horny layer. Five measurements per animal were made in every 10 scales and the mean of different animals was calculated.

9.3.3. Statistical analysis

Values were represented as mean ± SEM. Data were analysed using one-way analysis of variance (ANOVA) and group means were compared using the Tukey-Kramer Multiple Comparison test using Instat-V3 software. *P* values < 0.05 were considered significant.

9.3.4. Results

Drug activity is defined by the increase in percentage of orthokeratotic regions. Ethanolic extract of *G. rottleriformis* (100, 200 and 400 mg/Kg) has increased the orthokeratotic regions by 26.76%, 34.86 and 45.93 % respectively, where as 66.26% and 57.18% by isolated flavonoid **II** and **III** (50 mg/Kg) in comparison to normal. Ethanolic extract of *C. tora* (100, 200 and 400 mg/kg) has increased the orthokeratotic regions by 28.58, 38.94 and 57.23 % respectively. The compound combined (30 mg/Kg) showed the increase by 66.26%, whereas the standard drug Retinoic acid (0.1 mg/Kg) showed the increase by 68.08%. The results were shown in Table 18 & 19 and **Fig. 61& 62**.

Table 18: Effect of ethanol extract and isolated compounds of *G. rottleriformis* on the degree of orthokeratosis, epidermal thickness and the drug activity in the mouse tail test

Treatment	Orthokeratosis (%)	Activity (%)	Relative epidermal thickness (%)
Control	18.47±4.86	-	100
Retinoic acid (0.1 mg/kg)	68.06±1.09**	60.9**	57.60±2.28**
Ethanol extract (100 mg/kg)	26.76±3.90	9.7	75.18±1.26
Ethanol extract (200 mg/kg)	34.86±5.60**	19.5**	85.07±3.82
Ethanol extract (400 mg/kg)	45.93±7.12**	34.1*	70.50±5.37*
Compound **II** (50 mg/kg)	64.35±7.20***	56.27**	62.26±1.64*
Compound **III** (50 mg/kg)	57.18±6.24**	47.5**	64.26±1.28*

Values are mean ± SEM of 6 parallel measurements.

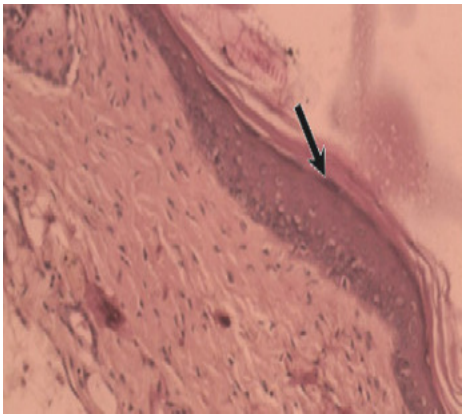
Data were analyzed by one-way ANOVA followed by Tukey multiple comparison test. The values are *P< 0.05; **P< 0.01; ***P< 0.001when compared against control.

Table 19: Effect of ethanol extract and isolated flavonoids of *Cassia tora* on the degree of orthokeratosis, epidermal thickness and the drug activity in the mouse tail test

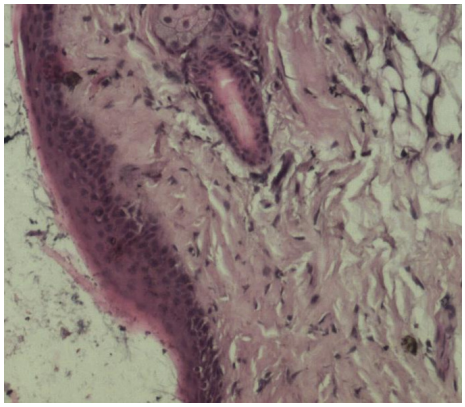
Treatment	Orthokeratosis (%)	Activity (%)	Relative epidermal thickness (%)
Control	18.47±4.86	-	100
Retinoic acid (0.1 mg/kg)	68.06±1.09**	60.9**	57.60±2.28**
Ethanol extract (100 mg/kg)	28.58±4.42	12.40	85.50±3.07
Ethanol extract (200 mg/kg)	38.94±3.80**	25.10*	77.37±4.12
Ethanol extract (400 mg/kg)	57.23±6.30**	47.54**	67.45±0.97*
Compound **VI** (50 mg/kg)	56.48±8.60**	46.62**	71.40±7.35*
Combined Compounds **II, III, VI (1:1:1)** (30 mg/kg)	66.26±5.76**	58.5**	53.83±8.09**

Values are mean ± SEM of 6 parallel measurements.

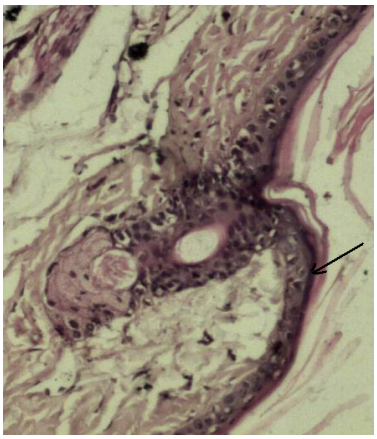
Data were analyzed by one-way ANOVA followed by Tukey multiple comparison test. The values are *$P<$ 0.05; **$P<$ 0.01 when compared against control.



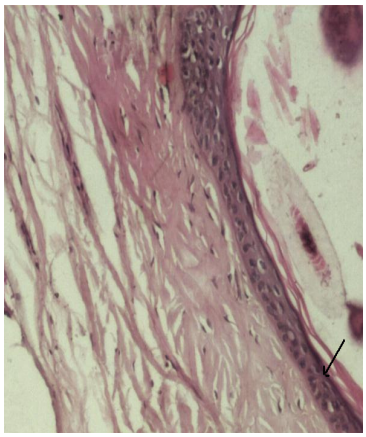
Standard
(68% orthokeratotic region)



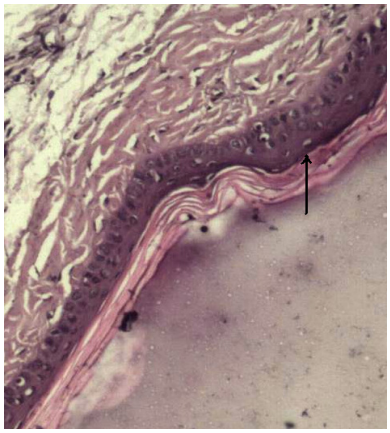
Control
(18% orthokeratotic region)



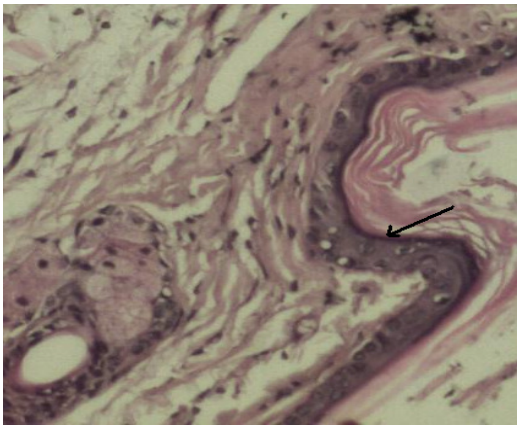
Ethanol extract 100 mg/Kg
(27% orthokeratotic region)



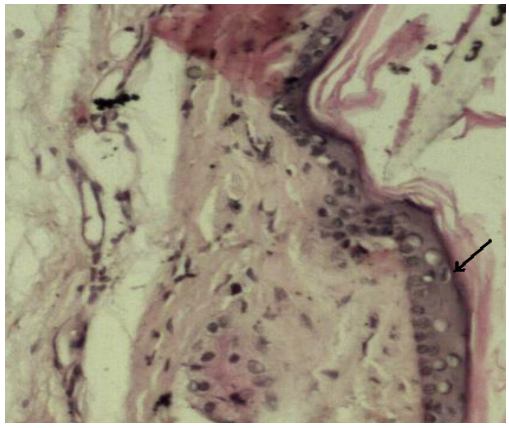
Ethanol extract 200 mg/Kg
(35% orthokeratotic region)



Ethanol extract 400 mg/Kg
(46% orthokeratotic region)

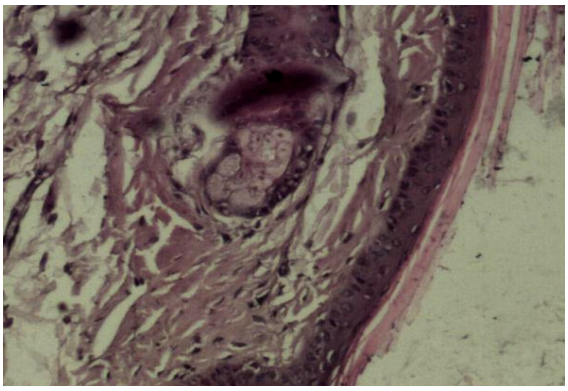


Compound II (50 mg/Kg)
(64% orthokeratotic region)

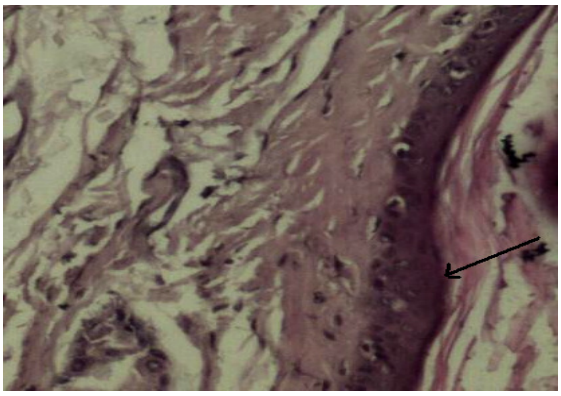


Compound III (50 mg/Kg)
(57% orthokeratotic region)

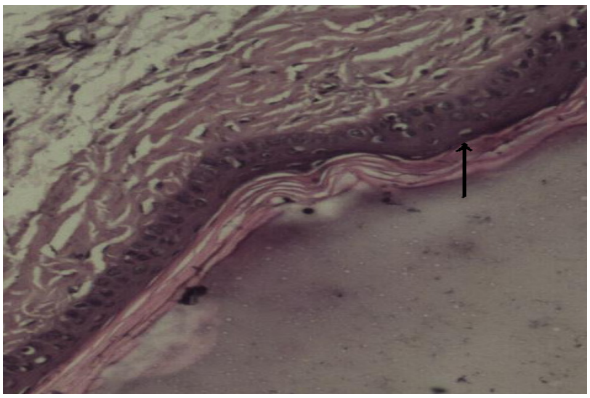
Figure 61: Longitudinal histological sections through the skin of mouse tails treated orally for 2 weeks with ethanol extract and isolated compounds of *G. rottleriformis*, HE staining (original magnification 40×).



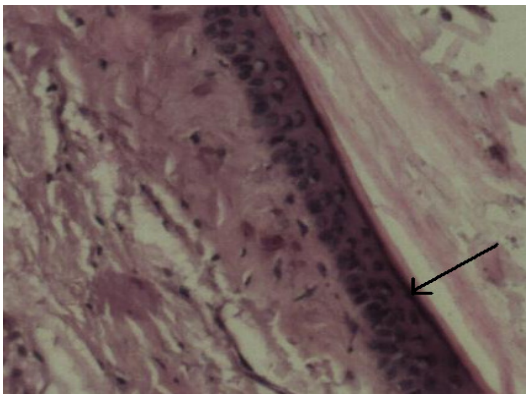
Ethanol extract 100 mg/Kg (29% orthokeratotic region)



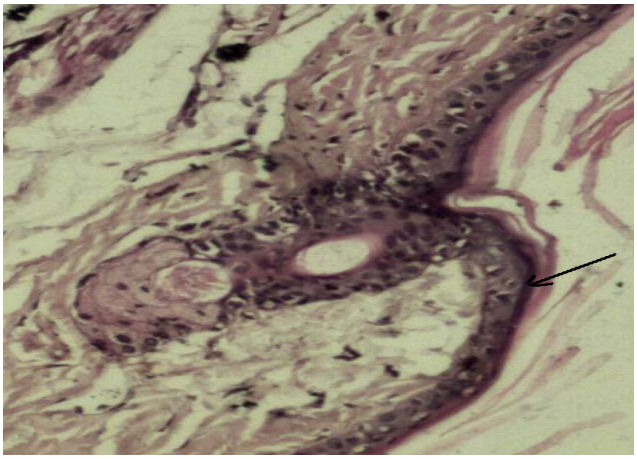
Ethanol extract 200 mg/Kg (39% orthokeratotic region)



Ethanol extract 400 mg/Kg (57% orthokeratotic region)



Compound VI (50 mg/Kg) (56% orthokeratotic region)



Compound combination II+III+VI (30 mg/Kg) (66% orthokeratotic region)

Figure 62: Longitudinal histological sections through the skin of mouse tails treated orally for 2 weeks treated with ethanol extract, isolated compound of *C. tora* and compound combination of (II, III, VI) HE staining (original magnification 40×).

9.3.5. Discussion

Granular layer of the epidermis is greatly reduced or absent in psoriatic lesions.[179] Parakeratotic condition is seen in the adult mouse tail which is one of the hallmarks of psoriasis.[180] Induction of orthokeratosis in the adult mouse tail is the basis behind the mouse tail test.[181] Many drugs presently used in the treatment of psoriasis have been evaluated by the mouse tail test and were found to have shown good efficacies.[176] Hence in the present study, the mouse tail test was used for evaluating the efficacy of the ethanol extract of the bark of *Givotia rottleriformis,* the *Cassia tora* leaves and isolated compound. In the mouse tail test, ethanol extract of *G. rottleriformis* (200, 400 mg/Kg), ethanol extract of *Cassia tora* (200, 400 mg/Kg) and compound **II, III, VI** produced significant orthokeratosis when compared to control (Table 18&19). The ethanol extract of both the plants at higher dose (400 mg/Kg) and compound **II, III, VI** showed significant change in epidermal thickness compared to control while ethanol extract at lower doses did not produce any significant change in epidermal thickness. The group treated with combined compounds (30 mg/Kg) increased the orthokeratotic regions (66.26%) to a greater extent compared with single test compound treatments and showed effect comparable to that of the standard treated group (68.06%). Thus, combined compounds showed significant effect among all tested groups, which may be due to the synergistic effect of three compounds.

10. DEVELOPMENT OF FORMULATION

10.1. INTRODUCTION

Among the isolated compounds **I-III** from ethanol extract of *Givotia rottleriformis* bark and compounds **IV-VI** from ethanol extract of *Cassia tora* leaves, compounds **II, III, VI** showed significant antiproliferant activity in HaCaT cells. These compounds were selected for the screening of *in-vivo* anti-psoriatic activity using Perry's mouse tail model. In this model, ethanol extract of *Givotia rottleriformis* (400 mg/Kg), ethanol extract of *Cassia tora* (400 mg/Kg), compounds **II, III, VI** (50 mg/Kg) and combined compounds **II, III, VI** (30 mg/Kg) has increased the orthokeratotic regions significantly.

The active compound is converted into dosage form for acceptance and palatability. The renowned, safe and effective dosage form tablet was chosen for formulation. The oral route of drug administration is the most important method of administrating drugs for systemic effects. Solid oral dosage forms represent the preferred class of product for orally administered drugs.

10.2. MATERIALS AND METHODS

Tablets are solid dosage form of medicament(s) with or without suitable additives and prepared by compression or moulding. Tablets hold lot of advantages like safe and effective, ease of manufacture, stable, cost effective.

The development of tablet dosage form may lead to:

- Improved patient compliance and convenience due to less repeated dose frequency.
- Unit dosage forms, easy to handle and transport
- Increased safety margin
- Decreased adverse effects and toxicity

Hence, combination of bioactive flavonoids were promoted to formulation of combinational tablet dosage form and evaluation for their product performance using various evaluation parameters such as weight variation, hardness, friability, thickness, disintegration & dissolution study.

10.2.1. FORMULATION OF HERBAL TABLETS

10.2.1.1. Preparation of granules

The tablets were prepared by classic wet granulation method. The compound **II, III** and **VI** (1:1:1) were mixed uniformly in mortar and pestle for 15 minutes. Then microcrystalline cellulose was added and mixed well. Then it was granulated by using starch solution as granulating agent. The granules were sifted through sieve no.20, and then it was dried.[183]

10.2.1.2. Characterization of granules[184,185]

The powder blend was evaluated for its physical characteristics bulk density, tapped density, angle of repose, compressibility index, and Hausner's ratio.

Bulk and tapped densities

About 30 g of the sample of the granules were placed in a 100 ml measuring cylinder. The volume occupied by the sample was noted as the bulk volume. The bulk density (ℓ_B) was obtained by dividing the mass of the sample weighed out by the bulk volume, as shown in Equation.

$$\text{Bulk density} = \frac{\text{Mass of powder (M)}}{\text{Bulk volume of powder (VB) (1)}}$$

The cylinder was tapped on a wooden platform by dropping the cylinder from a height of one inch at 2 s interval until there was no change in volume reduction. The volume occupied by

the sample was then recorded as the tapped volume. The tapped density (ℓ_T) was calculated using the formula:

$$\text{Tapped density} = \frac{\text{Mass of powder (M)}}{\text{Tapped volume of powder (VT) (2)}}$$

Flow rate and angle of repose

A funnel was properly clamped onto retort stand. The funnel orifice diameter, base diameter and efflux tube length were appropriately measured. A 30 g quantity of the granule was weighed out and gradually placed into the funnel with the funnel orifice closed with a shutter. The time taken for the entire sample in the funnel to flow through the orifice was noted. The flow rate was obtained by dividing the mass of the sample by the time of flow in seconds. The dynamic angle of repose was determined by measuring the height of the heap of powder formed using a cathetometer; the radius was calculated by dividing the diameter by two. Angle of repose (θ) for each granule sample was obtained using the Equation.

$$\theta = \tan^{-1} h/r$$

Compressibility index and Hausner's quotient

Carr's compressibility index (%) of the granules was obtained using the Equation

$$\text{Carr's index (\%)} = \frac{\text{Tapped density} - \text{Bulk density}}{\text{Tapped density}} \times 100$$

While Hausner's ratio was obtained using Equation:

$$\text{Hausner's ratio} = \frac{\text{Tapped density}}{\text{Bulk density}}$$

10.2.1.3. Preparation of tablets

The dried granules were lubricated with magnesium stearate. Finally the lubricated granules were compressed into tablets by using Rimek punching machine.

10.2.2. EVALUATION OF TABLETS

The formulated herbal tablets were evaluated for quality control tests such as appearance, thickness**,** hardness**,** friability**,** weight variation test**,** disintegration, drug content and *in vitro* drug release as per British Pharmacopoeia (BP), 2009.

10.2.2.1. Appearance

The appearances of the herbal tablets were visually inspected.

10.2.2.2. Thickness

Thickness of a tablet is measured by using Vernier calipers. Ten tablets were used for this test.

10.2.2.3. Hardness Test

Hardness of a tablet depends on weight of the material utilized, space between upper and lower punches, quantity of excipients used in formulation. Hardness was carried out by Monsanto hardness tester. Ten tablets were used for this test.

8.2.2.4. Friability Test

Friability test indicated the withstand abrasion during handling and transportation. Friabilator used for this purpose. Twenty tablets were weighed and subjected to rotating chamber of the apparatus and rotated for 25 rpm for 4 minutes. After that, the tablets were collected and reweighed. The formula to calculate the friability was

$$F = 100\ \frac{[W_0 - W]}{W}$$

Where W_o = Initial weight of 20 tablets.

W = Final weight of 20 tablets.

10.2.2.5. Weight variation

Twenty tablets were weighed and the average weight was calculated. Then the tablets were individually weighed and the percent weight variation was calculated.

10.2.2.6. Disintegration test

The test equipment consists of 1 litre beaker. Cylindrical basket moves up and down with standard perforation made of acrylic sheet inside. The one litre glass beaker of distilled water unit was fixed on the top for beaker. The temperature maintained by thermostat between 35°C to 37°C. Six tablets were kept in the apparatus and the disintegration time was recorded.

10.2.2.7. Drug content

Twenty tablets were weighed and powdered. An adequate amount of this powder equivalent to 30 mg of the drug was accurately weighed and shaken with 150 ml of 0.1N HCl for 10 minutes. The mixture was diluted with 0.1N HCl to produce 200 ml and filtered. 10 ml of the filtrate was diluted to 100 ml with distilled water and the absorbance was measured at respective maximum wave length. The drug content in the formulation was calculated using the standard curve.

8.2.2.8. *In Vitro* Drug Release

Drug release was assessed by dissolution test under the following conditions: n = 6 (in triplicate), USP type II dissolution apparatus (Lab India, DISSO 2000) at 50 rpm in 900 ml of 0.1N HCl maintained at 37±0.5˚C. The tablet was allowed to sink to the bottom of the flask before stirring. Special precaution was taken not to form air pockets on the surface of the tablet. Five milliliters of the sample was withdrawn by using a syringe filter at regular intervals and

replaced with the same volume of pre warmed (37±0.5°C) fresh dissolution medium. The drug content in each sample was analyzed after suitable dilution using UV spectrophotometer method at respective maximum wave length.

10.2.3. Stability studies

The formulated tablets were subjected to stability studies as per ICH guidelines. Samples were withdrawn at predetermined time intervals and then evaluated.

10.3. RESULTS

10.3.1. Preparation of granules

The type and concentration of additives used were optimized and the final formula was achieved. The composition of herbal tablets was given in Table 20.

Table 20: Formulation of herbal tablets

S. No	**Ingredients**	**1 tablet** (mg)	**100 tablets** (gm)
1	Compound **II**	10	1
2	Compound **III**	10	1
3	Compound **VI**	10	1
4	Starch solution	QS	QS
5	Microcrystalline cellulose	60	6
6.	Magnesium stearate	4	0.4

10.3.2. Characterization of granules

The powder blend was evaluated for its physical characteristics bulk density, tapped density, angle of repose, compressibility index, and Hausner's ratio. The results were given in Table 21.

Table 21: Pre compression parameters of granules

S.No	Parameters	Recorded values
1	Angle of repose (º)	24.44± 0.12
2	Bulk density (g/ml)	0.66 ± 0.04
3	Tapped density (g/ml)	0.72 ± 0.04
4	Compressibility index (%)	11.90±0.04
5	Hausner's Ratio	1.14±0.03

10.3.3. Evaluation of tablets

About 100 tablets were formulated and will be extending it to pilot plant scale in future. The formulated herbal tablets were evaluated for quality control tests such as appearance, thickness**,** hardness**,** friability**,** weight variation test**,** disintegration, drug content and *in vitro* drug release. The results were given in Table 22.

Table 22: Results of Post compression parameters of Herbal Tablets

S. No	Tablet parameter	Recorded values	
1.	Appearance	Tablet surface was smooth and elegant nature	
2.	Thickness (mm)	2.52 ± 0.24	
3.	Hardness (kg/cm^2)	5.2 ± 0.1	
4.	Friability (%)	0.90 ± 0.12	
5.	Avg. wt. (mg)	98.42± 1.49	
6.	% Weight Variation	1.52± 0.02	
7.	Disintegration	2 min 33 sec	
8.	Drug content (%)	Compound **II**	99.55± 0.96
		Compound **III**	98.62± 0.96
		Compound **VI**	98.54± 0.86
9.	% *In vitro* drug release	Compound **II**	98.27± 0.19
		Compound **III**	99.37± 0.19
		Compound **VI**	98.33± 0.19

SD values (n=6)

10.3.4. Stability studies

The result of the stability study of the formulation was given in Table 23.

Table 23: Stability studies as per ICH guidelines

Tablet parameter	Recorded values			
	1st month		3rd month	6th month
Appearance	smooth and elegant nature		smooth and elegant nature	smooth and elegant nature
Thickness (mm)	2.52 ± 0.26		2.50 ± 0.28	2.50 ± 0.32
Hardness (kg/cm^2)	5.2 ± 0.1		5.1 ± 0.1	5.1 ± 0.1
Friability (%)	0.92 ± 0.16		0.94 ± 0.18	0.98 ± 0.20
Avg. wt. (mg)	98.22± 1.69		98.02± 1.80	97.02± 1.82
% Weight Variation	1.58± 0.02		1.62± 0.06	1.68± 0.08
Disintegration	2 min 33 sec		2 min 32 sec	2 min 29 sec
Drug content (%) (Compounds)	**II**	9.53± 0.98	99.32± 0.84	98.32± 0.99
	III	98.52± 0.96	98.12± 0.98	97.18± 0.66
	VI	98.52± 0.82	98.22± 0.86	97.22± 0.88
% In vitro release (Compounds)	**II**	98.17± 0.29	97.87± 0.39	97.22± 0.86
	III	99.27± 0.19	98.96± 0.20	98.06± 0.24
	VI	98.24± 0.29	97.84± 0.39	97.24± 0.39

SD values (n=6). Herbal tablets at Storage Temp 40^{o}C ± 2 ^{o}C /RH75% ± 5%

10.4. DISCUSSION

The beneficial medicinal effects of phytomedicine typically result from synergistic actions of secondary products present in the plants. In contrast to synthetic pharmaceuticals based upon single chemicals, many phytomedicine exert their beneficial effects through the additive or synergistic action of several chemical compounds acting at single or multiple target sites associated with a physiological process. This synergistic or additive pharmacological effect can be beneficial by eliminating the problematic side effects associated with the predominance of a single xenobiotic compound in the body. Hence the combined active compounds (compound **II, III, VI**) is converted into dosage form for acceptance, palatability and evaluated for product performance.

The results showed that granules containing bioactive flavonoids exhibited good flowability and the values obtained fell within the acceptable range for good powder flow. Values of angle of repose were significantly below 35°, which showed that the granules had low interparticle cohesion and hence good flowability. Hausner's ratio less than or equal to 1.25 indicates good flow, while Hausner's ratio greater than 1.25 indicates poor flow. Therefore, the granules were within the specified limits for good flow, Also, Carr's index of 5 to 16 indicates good flow, while 18 to 23 shows fair flow.[184,185] The results of compressibility index indicate that the prepared granules had good flowability and consolidation properties. When the Carr's index (CI) and Hausner's ration (HR) are adequate, the powder flows at minimum bulk density. A high bulk density, that is, a low porosity, will result in a low deformation potential, a lack of space for deformation during compression will cause less intimate contact between the particles within the tablets, resulting in weak tablets.[185] The results showed that the granules had low bulk

and tapped densities and hence, exhibited good properties required for the production of good quality tablets.

The tablets weight had percentage deviation below 5%. Tablets friability results showed that the tablets passed the friability test as the friability values were significantly below 1%. Therefore, the tablets can comfortably withstand handling, packaging and transportation without compromising the properties of the tablets. The results of disintegration time test showed that formulated tablets complied with the official requirements of uniformity of weight. From the results it can be concluded that the newly developed tablet met Pharmacopoeial specifications limit, and the tablets were suitable for oral administration.

11. BIO ACTIVITY EVALUATION OF THE DEVELOPED FORMULATION

11.1. Rat UV ray photo dermatitis model for psoriasis

11.1.1. Introduction

In the "ultraviolet ray photodermatitis model for psoriasis'', the exposure of the rat skin to UV radiation using UV-B bulb (wavelength <290 nm) has induced proinflammatory reaction in the skin that resembles the one observed in psoriasis. This was evident by the altered skin parameters; the most important of which are increase in epidermal thickness to almost double the normal size, absence of stratum granulosum and the movement of neutrophils towards epidermis which are typical of psoriasis. Although the causative factor and the mechanism underlying the chronic manifestation of psoriasis is not clearly understood, there is scientific agreement that the end result is the initiation of a T-cell lymphocyte mediated immunological response that is proinflammatory in nature. The inflammatory reaction is restricted to the area of the skin where the antigen exists. Overall, the irradiation of rat skin with UV-B for 45 min has shown good changes in the epidermis, resembling psoriasis. The close resemblance of inflammatory process produced by ultraviolet radiation to the one exhibited in psoriasis provides us with a good model to investigate drugs that have a potential to reduce the inflammatory reaction associated with psoriasis.[187]

11.1.2. Materials and Method

Irradiation of the depilated rat skin with ultraviolet radiation is known to produce a biphasic erythema. Immediately after irradiation, initial faint erythema appears, and disappears within 30 min. The second phase of erythema starts 6 h after the irradiation and gradually increases, peaking between 24 and 48 h. This reaction is confined to the exposed area and has a sharp boundary. It develops a brownish-red colour. By 48 to 72 h, silvery white

scale appears on the erythematous lesion. These scales are relatively thick and begin to fall beyond 72 h. Although the erythematic reaction is induced artificially, many of the pathological features resemble those seen in psoriasis vulgaris.

Drug tested

The ethanolic extract of bark of *Givotia rottleriformis* (400 mg/kg BW, p.o.) and the *Cassia tora* leaves (400 mg/kg BW, p.o.), formulation (30 mg/kg BW, p.o.) and reference standard retinoic acid (0.5 mg/Kg, p.o.) was selected for the study based on the *in-vivo* anti-psoriatic method, Perry's mouse tail model.

Procedure

The hairs of the rat skin, on one side of the flank, were depilated by clipping with a scissors followed by careful shaving taking precaution to avoid injury to the skin. The animals were then placed on a curved wooden block and their legs tied around it, to avoid contact with the floor. This arrangement prevented the movement of the animal during its subsequent exposure UV radiation. Except for an area of 1.5 × 2.5 cm on the depilated skin, the entire animal was covered with a UV resistant film. The uncovered area of 1.5 × 2.5 cm was then irradiated for 20 min with a UV-B lamp kept at a vertical distance of 20 cm from the skin. Animals were treated orally with the test drug once daily, 5 times a week, 12 h after irradiation for 2 weeks. Two hours after the last treatment animals were sacrificed; longitudinal sections of the tail skin were made and prepared for histological examination with hematoxylin- eosin staining.[177]

Histopathological Examination

1. Sections were examined for presence of Munro's microabscesss, elongation of rete ridges, and capillary loop dilation by direct microscopy.

2. The vertical epidermal thickness between the dermoepidermal junction and the lowest part of the stratum corneum (n = 3 measurements per scale, n = 3 scales per animal, n = 6) were examined. The percentage relative epidermal thickness of all the groups was calculated in comparison to the positive control group (100%; n = 54 measurements per treatment).
3. It was also examined for Mean thickness of stratum corneum and stratum granulosum.

All measurements were made at a magnification of 400X using OLYMPUS microscope having a digital camera attachment and software to take measurements.

11.1.3. Statistical analysis

Values were represented as mean ± SEM. Data were analysed using one-way analysis of variance (ANOVA) and group means were compared using the Tukey-Kramer Multiple Comparison test using Instat-V3 software. P values < 0.05 were considered significant.

11.1.4. Results

Histopathological Examination

Histopathologically, numbers of features are observed in fully developed lesions in psoriasis such as Munro's microabscess, regular elongation of rete ridges, and capillary loop dilation which are shown in Table 24 and in **Fig. 63**. In comparison to positive control group, all other groups led to significantly decreased relative epidermal thickness.

Mean thickness of the epidermis, stratum corneum and stratum granulosum

Mean thickness of the epidermis, stratum corneum and stratum granulosum in the positive control group, and the groups that were treated with the ethanol extract of *G. rottleriformis* and *C. tora*, and formulation were tabulated in Table 25 and **Fig. 64**.

Table 24: Effect of formulations on Histopathological features on UV-B-induced psoriasis in rats

Treatment	Munro's microabscess	Elongation of rete ridges	Capillary loop dilation
Positive control	++	+++	++
Standard	-	-	-
EE of *G. rottleriformis* 400 mg/Kg	-	+	+
EE of *C. tora* 400 mg/Kg	-	-	-
Formulation	-	-	-

Note: + mild or slight grade lesion; ++ moderate grade lesion; +++ severe grade lesion; – no lesion

Table 25: Relative Epidermal thickness and different layers of epidermis on UV-B-induced psoriasis in rats

Treatment	% Relative Epidermal thickness (µm)	Thickness of stratum corneum (µm)	Thickness of stratum granulosum (µm)
Positive control	94.86±6.82	2.95±0.83	Absent
Standard	22.37±2.59***	15.62±1.20	12.83±0.38
EE of *G. rottleriformis* 400 mg/Kg	40.60±3.58**	6.20±1.40	3.86±0.24
EE of *C. tora* 400 mg/Kg	38.57±2.80**	8.43±0.69	4.94±0.80
Formulation 30 mg/Kg	28.53±5.25***	13.84±0.74	8.12±0.44

*** EE-ethanol extract;** n = 6; values are expressed as mean ± SEM. Data were analyzed by one-way ANOVA followed by Tukey multiple comparison test. The values are *$P<$ 0.05; **$P<$ 0.01; ***$P<$ 0.001 when compared against control.

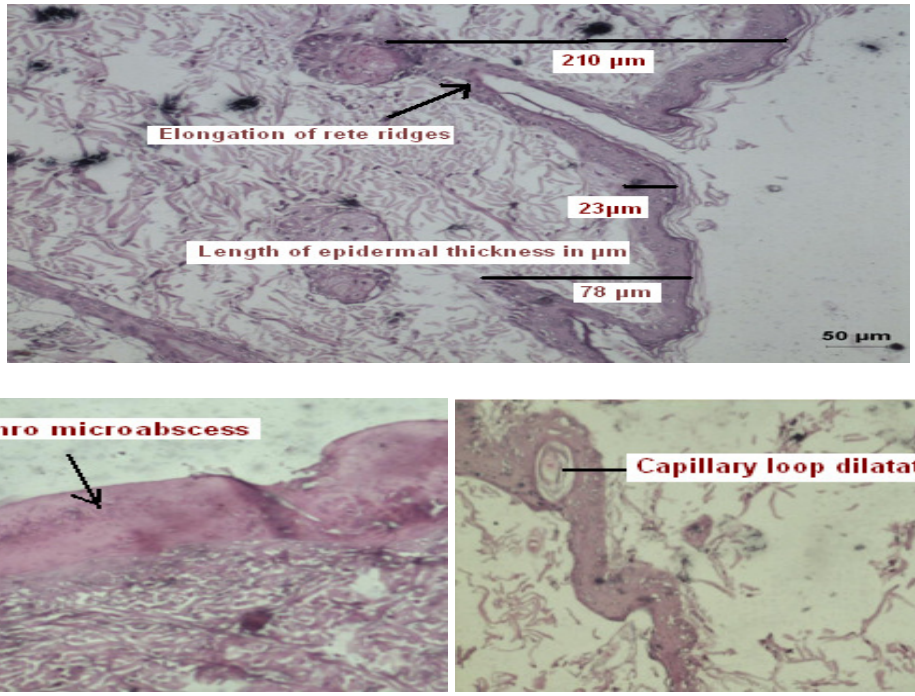
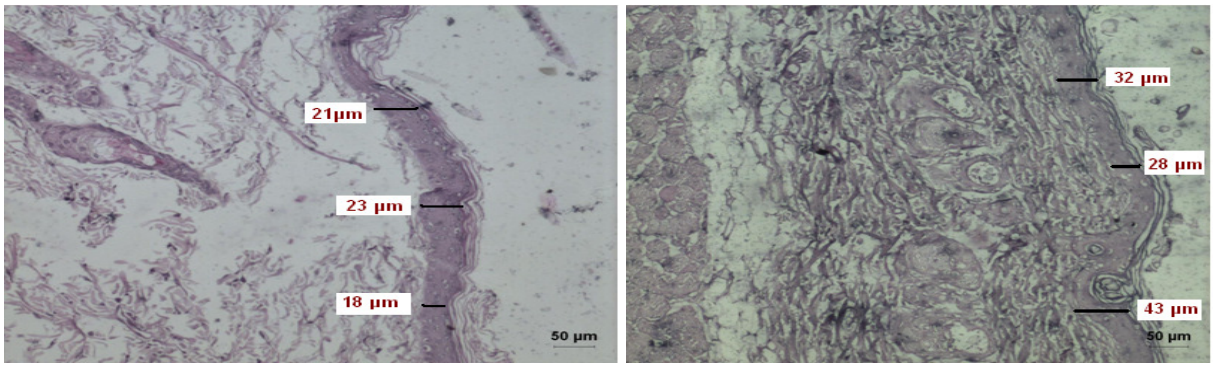
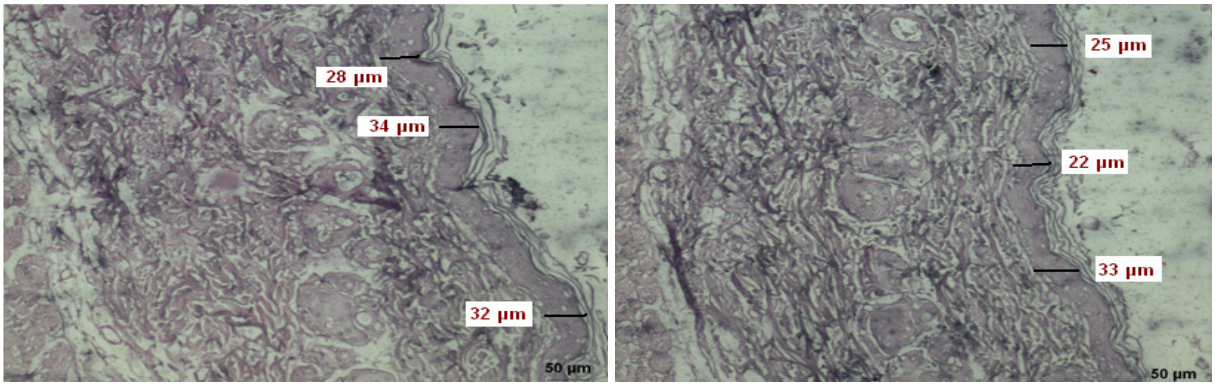


Figure 63: Photomicrographs of positive control rat skin on UV-B-induced psoriasis



Standard **EE of *G. rottleriformis* (400 mg/Kg)**



EE of *C. tora* (400 mg/Kg) **Formulation**

Figure 64: Histopathological features on UV-B-induced psoriasis in drug treated rats

11.1.5. Discussion

In case of positive control group, section showed regular elongation of rete ridges, capillary loop dilation with minimal grade lesion of diagnostic Munro's microabscess and marked increase in relative epidermal thickness as compared to other groups. In case of ethanol extract of *G. rottleriformis* 400 mg/Kg, there was a minimal grade lesion of elongation of rete ridges along with capillary loop dilation in the section and absence of Munro's microabscess. In ethanol extract of *C. tora* 400 mg/Kg, there was no lesion of Munro's microabscess, capillary loop dilation along with elongation of rete ridges in the section of skin of rats. The irradiated rat skin treated with formulation containing flavonoids has shown a significant reduction in the total epidermal thickness and also significant retention of the stratum granulosum, the absence of movement of neutrophils, further substantiates that the formulation containing flavonoids has been very useful in containing the changes that occur in the skin due to irradiation. The significant increase in the thickness of stratum corneum has been observed in the formulation containing flavonoids treated group.

The formulation significantly decreases total thickness of the epidermis indicating that its presence in the formulation has an influence to retard the hyper proliferation of the keratinocytes that occurs when the skin is exposed to UV radiation. The significant increase in the thickness of the stratum corneum is probably due to its ability to enhance the keratinisation process which is a protective strategy adopted by the skin when exposed to penetrating radiation. The presence of the granulosum layer in the skin treated with formulation and is completely absent in the positive control, indicates that the formulation containing flavonoids is probably able to suppress the altered process of differentiation of the keratinocytes. Further, formulation containing flavonoids

has brought about useful changes in the epidermis of the irradiated skin, which shows that the drug may be useful in psoriasis. Hence, the developed formulation has shown antipsoriatic activity by good reduction in the thickness of epidermis, significant retention of the stratum granulosum and the absence of movement of neutrophils in UV-B induced psoriasis.

11.2. CYTOKINE INHIBITION ASSAY

11.2.1. Introduction

Histologically, psoriasis is characterized by marked keratinocyte hyperproliferation, a dense inflammatory infiltrate consisting of T cells and neutrophils, and vascular dilatation and proliferation. The primary defect in psoriasis patients was believed to be abnormal epidermal cells proliferation.[188] The very early lesion of psoriasis is characterized by an inflammatory infiltrate of mononuclear cells in the upper dermis with only minimal changes in the epidermis.[189] Thus, systemic effects of circulating cytokines may play an important role in the induction of epidermal cell proliferation. Cytokines are small, biologically highly active proteins that regulate the growth, function, and differentiation of cells and help steer the immune response and inflammation.[190] It was proposed that in normal skin there could be an interaction between the epidermis and circulating T cells.[191] Recently, abnormal cytokines production is being studied as a possible mechanism in psoriasis.

Keratinocytes secrete a number of cytokines and chemokines that either activate or suppress immune responses.[192] Any local or systemic stimulus may stimulate keratinocyte cytokines production.[193] Cytokine or growth factor secretion by epidermal keratinocytes can be sufficient to recruit immune cells into the skin and induce a hyperplastic epidermis with hyperkeratosis and reproduce features of psoriatic disease.

Psoriatic keratinocytes are able to produce and release IL-1α, IL-1β, IL-6, IL-8, IL-17 and IL-20, all of them involved in the development of different alterations which compose the complex and intricate net of psoriasis pathogenesis. The cellular composition of the

inflammatory infiltrate within the psoriatic plaques as well as hyperproliferation of keratinocytes and so the whole pathogenetic process of psoriasis appears to be mediated by these cytokines. [194] The serum TNF-α, IL-1, IL-6, IL-8, IL-12, and IL-17 levels were significantly higher in active psoriatic patients than in controls. Regulation of the inflammatory events initiated or perpetuated by keratinocytes could so represent an important strategy for the treatment of psoriasis and other chronic inflammatory skin diseases. Thus inhibition of TNF-α, IL-1, IL-6, IL-8, IL-12, and IL-17 could be employed as criteria for the evaluation of anti-psoriatic activity.

11.2.2. Materials and Method

Assay for the inhibitory effects on IL-lα, IL-1β, IL-6, IL-8, IL-17 and TNF-α biosynthesis

Endotoxin (LPS) from *Escherichia coli* 055:B5 was obtained from Difco (Detroit, MI). Heparin was purchased from Takeda (Osaka, Japan) and ELISA kits from RayBio® (RayBiotech, Inc.).

Blood collection

About 20 mL of blood collected from healthy human volunteers after an overnight fast of 10–12 h. containing 20U heparin/ml by venapuncture and 30% solution is prepared by suspending in supplemented RPMI-1640 medium containing 100 U/ml penicillin and 100 mg/ml streptomycin.

Procedure

Lipopolysaccharide stimulated human peripheral mononuclear cells (LPS) (1 µg/ml) was dissolved in the supplemented RPMI-1640 media at a concentration of 3 µg/ml. The test sample was dissolved in DMSO at concentrations of 1, 3, 10, and 30 µg/ml and each of these concentrations was diluted with the supplemented RPMI-1640 media (1:100). Only DMSO was

contained in control suspension. Equal volumes from each of three solutions (whole blood, LPS and test sample) were mixed and the mixture was incubated at 37°C in a humidified atmosphere of 5% CO_2–95% air for 18—24 h. The supernatant of culture prepared by centrifugation was stored at -20°C until the assay of cytokine. The concentrations of the human cytokines (IL-lα, IL-1β, IL-6, IL-8, IL-17, TNF-α) were assayed using an ELISA kits.[196] The ratio (%) of inhibition of the cytokine release was calculated by the following equation:

$$\text{Inhibition } (\%) = 100\times(1\text{-}T/C)$$

where *T* represents the concentration of the cytokine in the culture supernatant with the test compound, and *C* represents the concentration of the cytokine in the culture supernatant with the solvent (control).

11.2.3. RESULT

Inhibitory effects of the test samples on IL-lα, IL-1β, IL-6, IL-8, IL-17, TNF-α biosynthesis are given in Table 25 as the inhibitory percentages. For the interpretation of the results, percentage values are classified under four groups; an inhibition between 70 and 100% is accepted as high, values between 40 and 69% as moderate, 20 and 39% as low and an inhibition less than 20% is considered to be insignificant.

Table 25: Inhibitory effects of the ethanol extracts from *G. rottleriformis, C. tora* and developed formulation on IL-lα, IL-1β, IL-6, IL-8, IL-17, TNF-α Biosynthesis

Treatment	Final concentration (μg/ml)	Inhibitory ratio (%)					
		IL-lα	IL-1β	IL-6	IL-8	IL-17	TNF-α
Formulation	1	0	-1	2	-10	-6	12
	3	6	5	8	3	10	14
	10	11	8	13	12	19	27
	30	22	30	31	23	44	70
EE of *G. rottleriformis*	1	-2	-22	0	-31	-16	-13
	3	3	-9	-8	-16	-13	16
	10	8	11	15	3	2	26
	30	19	24	27	25	13	40
EE of *C. tora*	1	-3	-21	-12	-16	-7	-9
	3	12	-3	-1	5	13	16
	10	14	10	17	11	23	22
	30	38	24	19	27	31	45

11.2.4. Discussion

Psoriasis can be described as a T-cell-mediated disease, with a complex role for a variety of cytokines and other factors. Interaction between T lymphocytes and keratinocytes, via cytokines, is likely to play a pivotal role in the pathogenic process in psoriasis. The Th1 cytokines (TNF-α, IFN-γ, and IL-12) and some proinflammatory cytokines (such as IL-6, IL-8, and IL-18) are influenced in the serum of psoriatic patients.[198]

Th17 cells are stimulated by IL-23 (which shares the p40 subunit with IL-12) to produce IL-17 and also IL-22, which has recently been shown to be a major driver of acanthosis in psoriasis, and so is a novel target for treatment.[195] The exact role of TNF-α in the pathomechanism of psoriasis is still unclear, but anti-TNF-α therapy is highly effective in psoriasis indicating that this cytokine has, together with IFN-γ, a central role in the pathogenesis. IFN-γ and TNF-α induce IL-6, IL-8, IL-12, and IL-18 and constitute an important link in the cytokine network in the pathogenesis of psoriasis.[199] Moreover, the intradermal administration of IFN-γ into nonlesional skin of psoriatic patients causes the appearance of lesions at the inoculation site.[192] IL-6 mediates T-cell activation, stimulates proliferation of keratinocytes.[200] and, at the beginning of acute inflammation, mediates the acute phase responses.[201] In fact, data currently available suggest that this cytokine exerts a critical role as a potent chemoattractant for neutrophils and T lymphocytes, as well as a factor prompting keratinocyte proliferation.

Antje R. Weseler et al., reported that the flavones fisetin, morin, or tricetin attenuated LPS-induced increases in concentrations of TNF-α in blood from COPD patients (chronic obstructive pulmonary disease) and IL-6 in blood from T2D patients (Type 2 diabetes), indicating a potential application as nutraceutical agents for these patient groups.

In the present study, the formulation developed containing flavonoidal glycosides viz., Kaempferol, Luteolin and Formononetin showed remarkable inhibition of IL-17 and TNF- α, key cytokines involved in the pathogenesis of psoriasis at higher concentration. At higher concentration, both the ethanol extract of *G. rottleriformis* and *C. tora* exhibited remarkable inhibition against TNF-α. This result demonstrates that the flavonoidal glycosides Kaempferol, Luteolin and Formononetin exert strong anti-TNFα. and anti-IL-17 effects in ex vivo LPS-stimulated blood. So the study can be concluded that the developed formulation can be effective and safety as dietary supplements with health benefits to psoriatic patients. Therefore, additional clinical investigation of these compounds is indicated to evaluate the efficacy and safety of their application as dietary supplements with health benefits to psoriatic patients.

12. SUMMARY AND CONCLUSION

The thesis entitled development and evaluation of some bioflavonoids from indigenous plants deals with pharmacognostical, phytochemical and pharmacological investigation of medicinal plants *Givotia rottleriformis* and *Cassia tora,* traditionally used for the treatment of chronic inflammatory skin disease psoriasis. A perusal of the literature reveled that only fragmentary information was available on *Givotia rottleriformis* regarding pharmacognosy, phytochemistry and pharmacological activity by any other researchers. This study was designed first time for the isolation of flavonoids from the selected plants, screening of pharmacological activities of the isolated flavonoids in order to establish its folklore claims and the bioactive flavonoids were promoted to formulation and evaluation for their product performance.

Plants are becoming potential source for phytoconstituents with varied pharmacological activities. Identification of such plants of potential use in medicine is of significance and as a prelude to this; it becomes necessary to examine the various pharmacognostical characters of the plant before further investigation.

In pharmacognostical studies, the macroscopy, microscopy, histochemical studies of the bark of *Givotia rottleriformis*, physico-chemical constants, fluorescence analysis and inorganic mineral analysis of *G. rottleriformis* bark and *C. tora* leaves were carried out. Pharmacognostical standards obtained during the observation are valuable tools for the identification of the plant material.

Morphological study had provided a characteristic identity of bark which was smooth brown colour, bitter taste yielding blood red sap from the bruised bark and leaf which was green in colour, bitter taste with characteristic odour.

The various distinguishing features of *G. rottleriformis* bark observed through anatomical studies were

- The transverse section of the bark exhibits outer periderm and inner secondary phloem. Secondary phloem is the major part of the bark. It is differentiated into outer wider collapsed phloem and inner narrow non collapsed phloem or intact phloem.
- The tangential longitudinal sections of the bark exhibits uniseriate, narrow and hetero cellular phloem rays with terminal upright cells and middle procumbent cells and long and thick walled straight sieve tube and nodulated sieve plate. Phloem parenchyma cells are vertically rectangular and they occur in vertical strands. Prismatic Calcium oxalate crystals are seen in sclerenchyma elements of the collapsed phloem and druses located in the phloem rays.
- The micrsocopical examination of the powder showed brachy sclereids, short, narrow thick walled libri form fibres in thick bundles or in small broken pieces and rectangular thin walled parenchyma cells.

Histochemistry is mainly used to localize the chemical compounds within the cells and tissues using some chemical reagents have been done and it showed the presence of alkaloids, and flavonoids.

Various physico-chemical parameters such as ash values, extractive values, loss on drying and crude fibre content were found to substantiate its standard values. Any significant deviation in the percentage of any parameters reported in this work may indicate adulteration or substitution in the drug. Presence of calcium and iron added up its nutritional value, fluorescence analysis is also a part of diagnostic tool for the presence of chromophore in the particular species.

The pharmacognostical details evolved from the present study would help to fix up the standards for *Givotia rottleriformis* Griff. Ex Wight in relation to its identification, authentication and differentiation from other related species and adulterants. This is the first report on the pharmacognostical standardisation on the bark of *Givotia rottleriformis* Griff. Ex Wight. From the above mentioned studies it can be concluded that the pharmacognostical standards generated will be useful for the proper identification of the plant *G. rottleriformis* bark.

In Phytochemical evaluation, the ethanolic extract of *Givotia rottleriformis* and *Cassia tora* were prepared and studied for qualitative chemical analysis, TLC and HPTLC finger print analysis.

The total phenol and flavonoid content present in the plants were estimated by Folin-Ciocalteu and $AlCl_3$ method respectively. The total phenolic content and flavonoid content in *Givotia rottleriformis* bark was found to be 13.80% w/w and 5.7% w/w respectively. The total phenolic content and flavonoid content in *Cassia tora* leaves was found to be 18.60% w/w and 9.5% w/w respectively.

The qualitative preliminary phytochemical analysis was performed to detect the nature of the phyto-constituent present in the plants. The ethanolic extract of *Givotia rottleriformis* showed the presence of alkaloids, glycosides, proteins, phenols, tannins, flavonoids, steroids and terpenoids. The ethanolic extract of *Cassia tora* showed the presence of anthroquinone glycosides, proteins, phenols, tannins, flavonoids, saponins, steroids and terpenoids.

Qualitative chromatographic analysis (TLC) was performed for the identification of different components in the extracts qualitatively.

The HPTLC finger print of ethanolic extract of the plants was also studied. HPTLC was scanned at 280 nm with the best solvent to detect the maximum number of components and peak abundance qualitatively. HPTLC fingerprint is one of the versatile tools for qualitative and quantitative analysis of active constituents. It is also a diagnostic method to find out the adulterants and to check the purity.

The defatted ethanol extract of *Givotia rottleriformis* bark and *Cassia tora* leaves was subjected to column chromatography separately and eluted with various solvents in the order of increasing polarity. The isolated compounds were characterized by spectral analysis. From *Givotia rottleriformis* bark, 3 flavonoid glycosides, *viz*., Rutin, Luteolin-7-O-β-D-Glucuronide, Kaempferol 3-*O*-[2-*O*-(6-*O*-feruloyl)-*β*-D-glucopyranosyl]-*β*-D-galactopyranoside, and from *Cassia tora* leaves, 3 flavonoid glycosides *viz*., Luteolin-7-O-β-glucopyranoside, Quercetin-3-*O*-β-d-glucuronide, Formononetin-7-O-Glucoside were isolated.

The flavonoids present in both the plants were detected and quantified using HPLC. In *Givotia rottleriformis* bark, 4 flavonoids were quantified, *viz*., rutin (0.215 mg/gm), luteolin (8.64 mg/gm), quercetin (1.36 mg/gm), kaempferol (6.36 mg/gm) using peak area by comparison to a calibration curve derived from the standard. Similarly in *Cassia tora* leaves, 4 flavonoids were quantified, *viz.*, quercetin (10.33 mg/gm), kaempferol (7.43 mg/gm), formononetin (0.46 mg/gm) and luteolin (8.64 mg/gm) at 254 nm using peak area by comparison to a calibration curve derived from the standard.

Further the presence of flavonoids was detected and quantified using HPTLC. In *Givotia rottleriformis* bark, the flavonoids Rutin, Kaempferol and Luteolin were detected and quantified using HPTLC silica gel 60 F254 pre-coated plates with the mobile phase made of Benzene:

Methanol: Ammonia (90:10:1). The detection of Rutin, Luteolin and Kaempferol was observed to be linear over a concentration range of 100-500 ng/mL and the concentration of Rutin, Luteolin and Kaempferol was found to be Rutin 285 ng/mg, Kaempferol 360 ng/mg and Luteolin 380 ng/mg. Similarly in *Cassia tora* leaves, the flavonoids Luteolin, Quercetin and Formononetin were detected and quantified using HPTLC silica gel 60 F254 pre-coated plates with the mobile phase made of Benzene: Methanol: Ammonia (90:10:1). The detection of Luteolin, Quercetin and Formononetin was observed to be linear over a concentration range of 100-500 ng/mL and the concentration of Luteolin, Quercetin and Formononetin was found to be Luteolin 220 ng/mg, Quercetin 160 ng/mg, and Formononetin 210 ng/mg.

In vitro antioxidant studies for ethanol extract of *Givotia rottleriformis* bark, *Cassia tora* leaves and isolated flavonoids were done by Hydroxyl, DPPH and Nitric oxide radical scavenging method. The tested extracts and isolated flavonoids showed good dose-dependent free radical scavenging property in all the models.

The cytotoxic effect of the ethanol extract of *Givotia rottleriformis* bark, *Cassia tora* leaves and isolated flavonoids **I-VI** were evaluated using *in- vitro* model - HaCaT human keratinocyte cell inhibition, a rapidly multiplying human keratinocyte cell line, as a model of epidermal hyperproliferation in psoriasis. Among the tested flavonoids, **II, III, V** and **VI** showed appreciable antiproliferant activity in HaCaT cell line. The compound **II** and **V** exhibited similar antiproliferant activity in HaCaT cell line, were of same flavonoid (luteolin), the yield of compound **II** was high when compared with compound **IV** and hence compound **II** was subjected to further studies along with compound **III** and **VI**. The results were validated using asiaticoside as positive control.

The ethanol extract of *Givotia rottleriformis* bark, *Cassia tora* leaves was found to be safe up to 2000 mg/kg body weight and isolated flavonoids **II, III** and **VI** up to 500 mg/kg body weight by acute toxicity model study as per the OECD guidelines 423. In sub acute toxicity study, ethanol extract of *G. rottleriformis* (200 mg/Kg and 400 mg/Kg) treated via oral route over a period of 28 days have no toxic effect on rats. Toxicity profile of *Cassia tora* leaves reported by Ambali et al., revealed that the twenty eight days of oral administration of methanol extract of *Cassia tora* did not result in death of the animals and clinical signs of toxicity include diarrhoea.

The ethanol extract of *Givotia rottleriformis* bark, *Cassia tora* leaves and isolated flavonoids **II, III** and **VI** were selected for the screening of *in-vivo* anti-psoriatic activity using Perry's mouse tail model. The ethanol extract of both the plants at higher dose (400 mg/Kg) and compound **II, III, VI** (50 mg/Kg) and combination of compound (30 mg/Kg) also showed significant change in epidermal thickness compared to control while ethanol extract at lower doses did not produce any significant change in epidermal thickness.

Bioactive flavonoid combination II, III, VI (1:1:1) were promoted to formulation of combinational tablet dosage form and evaluation for their product performance using various evaluation parameters such as weight variation, hardness, friability, thickness, disintegration & dissolution study. From the studies - appearance, weight variation test**,** thickness**,** hardness**,** friability**,** disintegration, dissolution and assay were recorded and it was found that formulated tablet results met Pharmacopoeial specifications limit, and the tablets were suitable for oral administration.

The standardized formulation (30 mg/Kg) along with ethanol extract of *G. rottleriformis* and *C. tora* (400 mg/Kg) was evaluated for anti-psoriatic activity using ultraviolet-B-induced psoriasis in rat model and cytokine inhibition assay against IL-1, IL-6, IL-8, IL-17 and TNF -α and. The developed formulation has shown antipsoriatic activity by good reduction in the thickness of epidermis, significant retention of the stratum granulosum and the absence of movement of neutrophils in UV-B induced psoriasis. The developed formulation containing bioactive flavonoids showed remarkable inhibitory activity against the cytokines TNF-α amd IL-17 at higher concentration (30 µg/ml)

In conclusion, we could demonstrate that the formulation containing flavonoids Kaempferol, Luteolin and Formononetin exert strong anti-TNFα and anti-IL-17 effects in ex vivo LPS-stimulated blood. Therefore, additional clinical investigation of these compounds is indicated to evaluate the efficacy and safety of their application as dietary supplements with health benefits to psoriatic patients.

With the support of *in-vitro* assay and *in-vivo* pharmacological activity the ethanolic extract of both the plants at the dose level of 400 mg/kg and isolated flavonoids **II, III, VI** (50 mg/Kg) and its combination (30 mg/Kg) showed significant anti-psoriatic activity. Further studies were focused on structural activity relationship of phytoconstituents isolated from the ethanolic extract. This scientific study revealed the efficacy of the drug and it would definitely have wide scope in future.

So the study can be concluded that the developed formulation containing bioflavonoids can be effective and safety as dietary supplements with health benefits to psoriatic patients, justifying the use of these plants in traditional medicine.

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