SCREENING AND IDENTIFICATION OF PHYTOPHARMACEUTICALS FOR MITIGATION OF *PSORIASIS VULGARIS*

A Thesis submitted to Gujarat Technological University

for the Award of

Doctor of Philosophy

in

PHARMACY

by

Ishita A. Basera
169999901002

under supervision of

Prof. (Dr) Mamta B. Shah

GUJARAT TECHNOLOGICAL UNIVERSITY
AHMEDABAD

September–2022

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I declare that the thesis entitled **Screening and identification of phytopharmaceuticals for mitigation of *Psoriasis vulgaris*** submitted by me for the degree of Doctor of Philosophy is the record of research work carried out by me during the period from **2017** to **2021** under the supervision of **Prof.** (**Dr) Mamta B. Shah** and this has not formed the basis for the award of any degree, diploma, associateship, fellowship, titles in this or any other University or other institution of higher learning.

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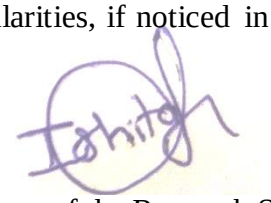

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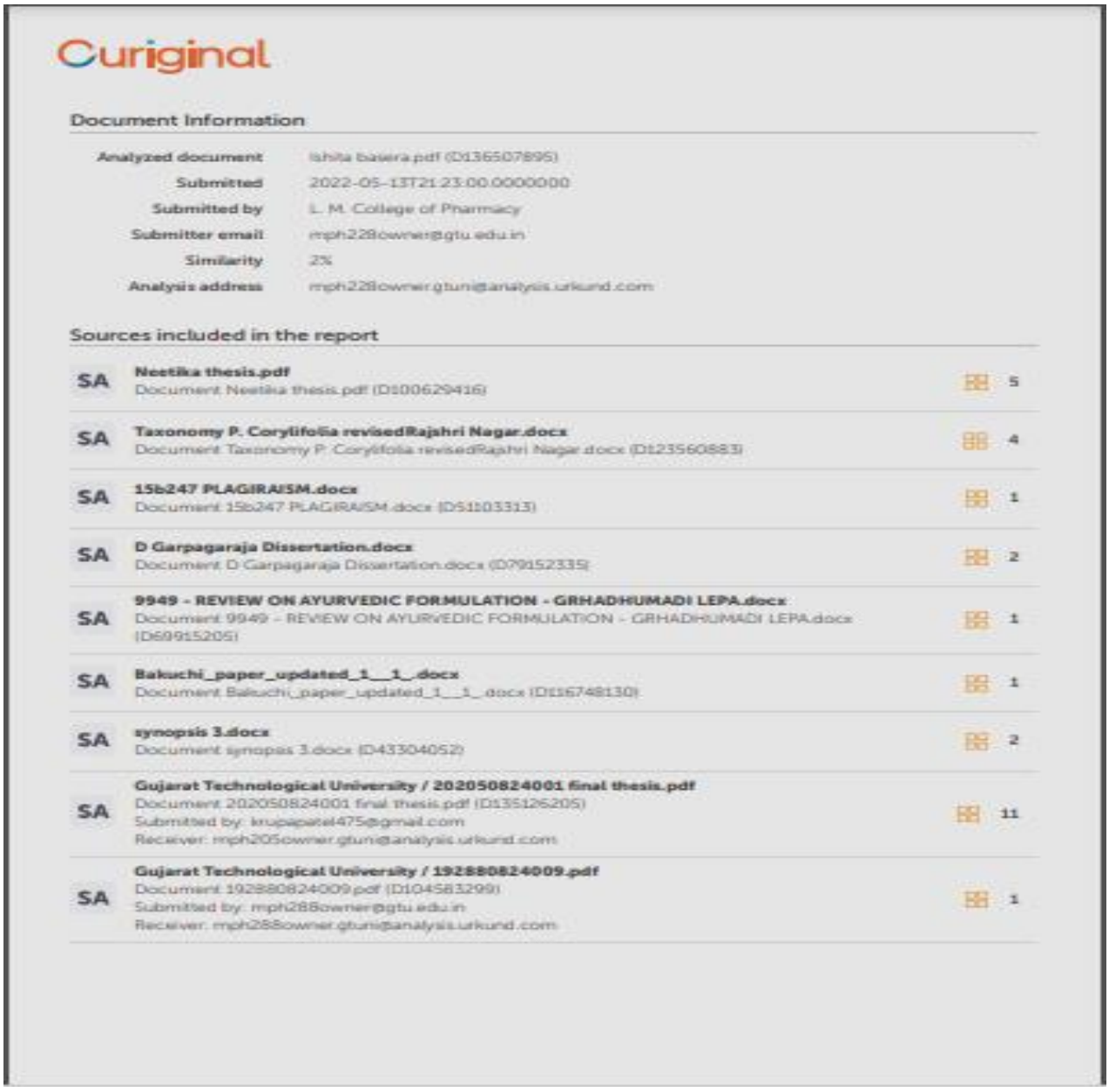

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ABSTRACT

Psoralea corylifolia (Leguminosae) is an annual herb, found throughout India, commonly known as Bakuchi. The whole plant, especially its seed or fruit, has significant medicinal properties for the treatment of many diseases. The plant contains coumarins, meroterpenes, flavonoids, sterols and volatile oil. *Berberis aristata* is an important multipurpose shrub native to hilly parts of Northern India, belonging to the Berberidaceae family and commonly known as Daruhaldi. It is reported to contain isoquinoline alkaloids, flavonoids and resins.

Both the plants were evaluated for pharmacognostic study which includes macro and microscopic evaluation, determination of physicochemical parameters in a systematic way. New isolation methods have been developed for the active principles present in *P. corylifolia* using flash chromatography technique. A simple, validated HPTLC and UHPLC–PDA with ESI–MS-MS methods were developed to estimate the major bioactives from both the plant. *In-vitro* studies using THP-1 cell lines using MTT assay was conducted. Preclinical for safety and IMQ (imiquimod) induced psoriatic lesion model was studied for *Psoralea corylifolia* and *Berberis aristata* using different extracts, combination of extracts and bioactives.

The characteristic features for morphological, microscopical and physico-chemical parameters that are essentially required for the quality evaluation of both the plants are recorded systematically. Psoralen, bakuchiol, 5-hydroxy xanthotoxin and methyl 4-hydroxy benzoate were isolated by column chromatography and purified by flash chromatography.

Validated and Simultaneous HPTLC methods designed for the quantitative and qualitative analysis of the markers in both the medicinal plants revealed the presence of bakuchiol (1.20 %w/w), psoralen (0.585 %w/w) and bavachin (0.285 %w/w) in *P. corylifolia*, and berberine (1.17 % w/w), palmatine (0.84 % w/w), berbamine (0.77 %w/w), jatrorrhizine (0.12 % w/w) and magnoflorine (0.10 % w/w) in B. aristata. Additionally, UHPLC MS/MS methods developed and validated separately for both the plants with an aim to estimate and to corroborate the aforementioned bioactives concurrently showed bakuchiol as the major marker in *P. corylifolia* and berberine in *B. aristata*.

To check the effect of the extracts and bioactives on cell viability and proliferation MTT assay was performed using THP-1 cell line. The methanloic extracts of *P. corylifolia* and *B. aristata* and berberine showed stronger effect (IC50: 60 to 80 ug/ml) than psoralen and hexane extract of *P. corylifolia* (IC50:100 to 200 ug/ml).

In *in vivo* studies performed in imiquimod (IMQ)-induced psoriasis model the methanolic extracts of both the plants singly and in combination exhibhited ameliorative effects but the later (mix of both) showed strong effects similar to that of clobetasol standard where almost complete recovery from the IMQ-induced hyperplasia of the epidermal tissue with only minor inflammatory reaction was observed. Thus, it can be concluded that both the drug extracts (phytopharmaceuticals either alone or in combination) can be used for mitigation of psoriatic lesions.

Key words: *Psoralea corylifolia*, *Berberis aristata*, Psoriasis, Phytopharmaceuticals, Leguminosae, Berberidaceae.

Acknowledgement

Pursuing a Ph.D Dissertation is a both afflictive and gratifying experience. It is just like climbing a high peak, step by step, accompanied with acrimony, asperities, frustration, encouragement and trust and with so many people's kind help. When I foundmyself at the top enjoying beautiful scenery, I realized that it was, in fact, teamwork that got me there. Though it will not be enough to express my gratitude in words to all those people who help me, I would still like to give my many, many thanks to all those people. Words are always poor approximation of what one intends to say.

At first I thank God for giving me the ability, the patience and the drive that reached me to this destination in my life and for showering his blessings.

It is my privilege to express deep sense of gratitude to my guide ***Prof. (Dr) Mamta B. Shah****, Professor, L.M College of Pharmacy, Ahmedabad for her invaluable suggestions, constant encouragement and untiring endeavour throughout the course of this research work, I am greatly thankful to her for her help, without which this work could not be possible. Her love and support always motivated meto do my best.*

Getting the chance to oursue a doctoral degree is a very rewarding experience for me, and I want to thank ***Gujarat technological University, Ahmedabad*** *for this. As part ofr my entire program, I am also grateful to* ***L.M college of Pharmacy, Ahmedabad*** *and especially* ***Department of Pharmacognosy*** *for providing infrastructure and facilities to allow me to do the research.*

I am very much thankful and grateful to my Doctoral Progress Committee members Dr. ***Kilambi Pundrikakshudu and Dr. Niranjan Kanaki*** *for mentoring me and providing me valuable guidance from time to time.*

I express my gratitude to ***Dr. Mahesh Chabbria****, Principal, L.M college of Pharmacy, Ahmedabad, for providing necessary facilities for the completion of this project work.*

I owe sincere gratitude to ***Dr. V.P Bhatt,*** *Scientist, Herbal Research and Development institute for collection and primary authentication of plant.*

I am obliged to ***Dr. Gaurang B. Shah****, Head – M. Pharm. & Pharm. D for providing test animals,* ***Dr. Anita Mehta,*** *Head - Department of Pharmacology for granting permission to perform animal testing experiments,* ***Dr. Anuradha Gajjar,*** *Head - Department of Pharmaceutical Analysis for permission to perform HPTLC, HPLC and for guidance in performing analysis*

Dr. Praful Bharadiya, *deserves special mention; he has guided me throughout the project and was instrumental in getting my project sanctioned under SSIP, leading to financial assistance becoming available for completion of the project.*

I am thankful to the CEO of ***AIC-LMCP****,* ***Dr. Shrinivas Savle*** *from of L.M. College of Pharmacy, for providing necessary facilities & guidance for flash chromatography.*

*Special thanks to **Dr. Mehul Chorawala** for helping me during my entire animal experiment work. I am also thankful to faculty members of Department of Pharmacognosy, L.M. Collage of Pharmacy - **Dr. Preeti Verma, Dr. Karuna Modi,** and **Dr. Krupa Gadhvi** for helping me during my entire research work, **Mrs. Divya Teli Mam** from the department of pharmaceutical chemistry helping me perform the flash chromatography experiment.*

*I would like to thank my friends **Ankur Tilva, Parth Purani, Vinendra Parmar, Vandana Thakur, Vanessa James, my juniors Bhoomi soni, Shailja Jhala, Shambhu Tatma, Nidhi Fefar, Ankita Chandwani, Priyanka Yadav, Vinisha Dudhat** for a cherished time spent together in the lab, and in social settings. My appreciation also goes out to my senior Aruna Rajaprara, Ph.D Scholar for her encouragement and support all through my studies. I express my thankfulness to **Hasil Navliwala**, M. Pharm Student, L. M college of Pharmacy. Ahmedabad, for helping with HPTLC analysis of my extracts. I am very much thankful to **Dr. Aboli Girme**, Senior manager and her team, Pharmanza Herbal PVT. LTD., Anand for helping me in UHPLC-MS-MS analysis of my extracts.*

*Special thanks to non-technical staff **Shagunbhai** of Pharmacology, **Ramchandra Yadavji, Sureshbhai** and **Siddhnathji** for helping me during my entire work and study and also thankful to **Bharatbhai, Prakash kaka, Amitbhai, Kamleshsir, Chiragbhai, Vinu kaka, Raju kaka, Dexterbhai, Yogeshbhai, and Dharmeshbhai** for their help.*

In preparing this dissertation I have received great help from many of my professors, friends, and colleagues in a number of ways, whom I might have missed inadvertently. I take this opportunity to thank all of them.

I dedicate this research work to ***My Father Mr. Arvindsingh Basera, who always pushed me to achieve new heights throughout my life****. Words can never express the love of my mother* ***Mrs. Nandini Basera****, for her constant emotional support during the hardship for this project. They have always supported and encouraged to complete my education. I am ever grateful to my siblings* ***Neha Basera*** *and* ***Jay Basera*** *for always been there for me, as and when needed. I am extremely thankful to my beloved husband* ***Mr. Jignesh Prajapati*** *for his continuous support and help throughout my Ph.D jouney, this would have never been possible without his help, loving son* ***Dhruvish*** *and My* ***in-Laws*** *for their patience and time so that I could complete the present work.*

I am indebted to all my family members, friends and well-wishers who directly or indirectly involved in the completion of this endeavor.

Last but not the least I owe all those silent animals used in the present work for the welfare of human race.

Ishita A. Basera

Table of Content

List of Abbreviation

THP-1:	Human monocytic cell line
ANOVA:	Analysis of Variance
PASI:	Psoriasis Area and severity Index
CPCSEA:	Committee for the Purpose of Control and Supervision of Experiments on Animals
ELISA:	Enzyme Linked Immunosorbent Assay
GLC:	Gas Liquid Chromatography
HPLC:	High Performance Liquid Chromatography
HPTLC:	High Performance Thin Layer Chromatography
i.p.:	intra peritoneal route of administration
IAEC:	Institutional Animal Ethics Committee
IC50:	50 percentage inhibitory concentration
SEM:	Standard Error of Mean
TLC:	Thin Layer Chromatography
WHO:	World Health Organization

List of Symbols

%	Percentage
µ	Micro
µl	Microliter
α	Alpha
β	Beta
A	Absorbance
C	Celsius
g	Gram
h	Hour
IU	International Unit
Kg	Kilogram
mg	Milligram
min	Minute
ml	Milliliter
Nm	Nanometer
º	Degree
ppm	Parts per million
w/v -	Weight by volume
w/w -	Weight by weight

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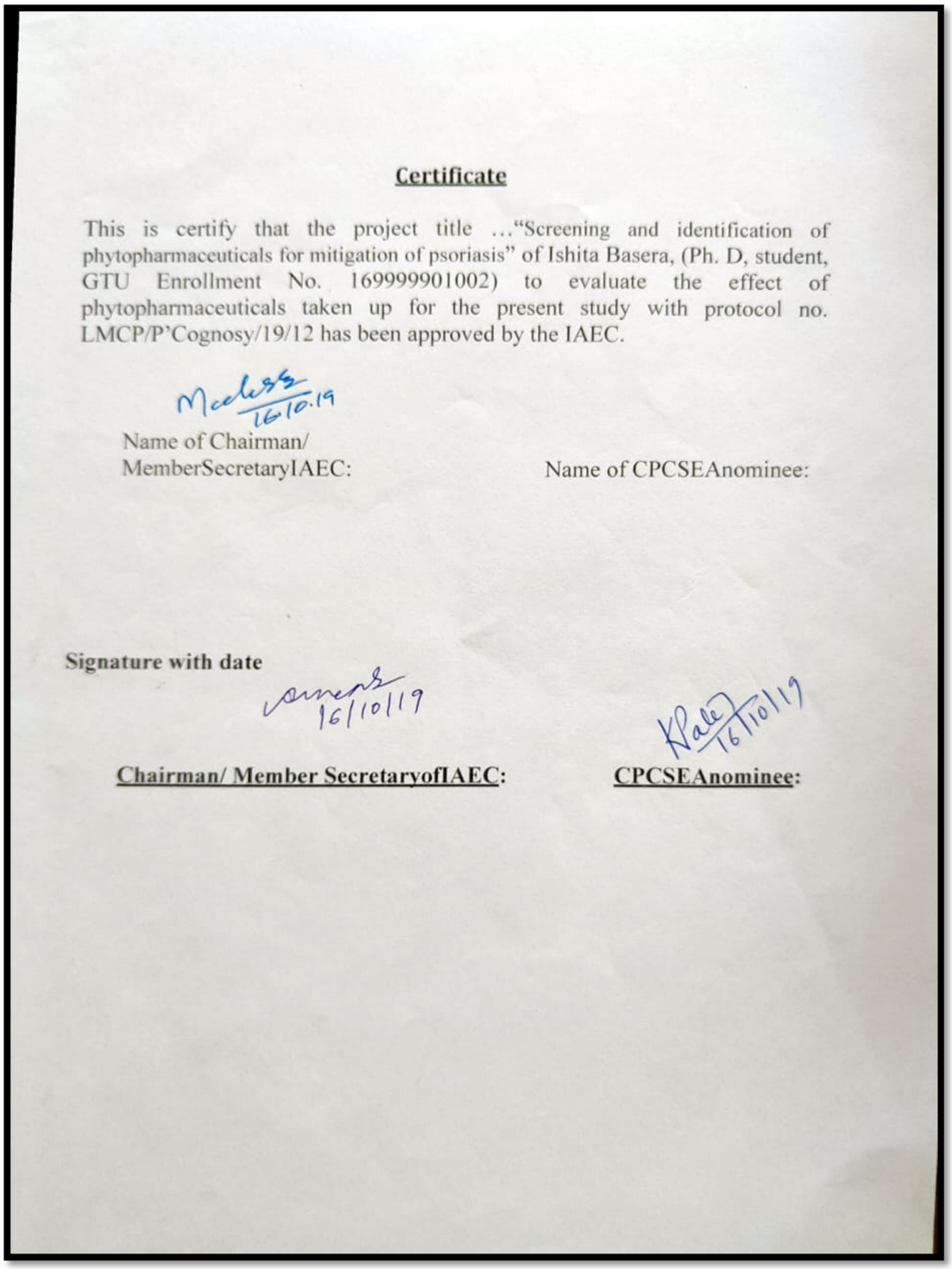
APPENDIX-1

Certificates of approval from Institutional Animal Ethics Committee



Certificate

This is certify that the project title …"Screening and identification of phytopharmaceuticals for mitigation of psoriasis" of Ishita Basera, (Ph. D, student, GTU Enrollment No. 169999901002) to evaluate the effect of phytopharmaceuticals taken up for the present study with protocol no. LMCP/P'Cognosy/19/12 has been approved by the IAEC.

16.10.19

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CHAPTER 1

Introduction

1.1 Introduction to *Psoriasis vulgaris*:

Psoriasis is a chronic inflammatory skin disease with a strong genetic predisposition and autoimmune pathogenic traits. The global incidence is estimated to be around 2%, however it varies by region[1]. Asian and some African ethnicities have a lesser frequency, while Caucasian and Scandinavian populations have up to 11% prevalence[2-5]. *Psoriasis vulgaris*, often known as plaque-type psoriasis, is the most common kind with a wide range of dermatologic symptoms. In the scientific literature, the terms psoriasis and *Psoriasis vulgaris* are used interchangeably; nonetheless, there are considerable variations between the numerous clinical subtypes. Psoriasis has a wide range of dermatological symptoms. Plaque psoriasis is characterised by red skin lesions that are coated by silvery scales at the base. Symptoms of guttate psoriasis include tiny, drop-shaped lesions on the trunk, limbs, and scalp. Upper respiratory infections are the most common cause of guttate psoriasis (for example, a sore throat caused by streptococcal bacteria). Pustular psoriasis is characterised by the formation of noninfectious pus blisters on the skin. Medication, infections, stress, or exposure to certain substances can all trigger pustular psoriasis. Smooth, red spots appear in the folds of the skin near the genitals, under the breasts, or in the armpits in inverse psoriasis. Friction and sweating might aggravate it. Erythrodermic psoriasis is characterised by extensive skin reddening and scaling, which could be caused by a reaction to extreme sunlight, corticosteroids (cortisone), or other drugs. It could also be the result of psoriasis that has been poorly controlled for a long time. Psoriatic arthritis is joint inflammation that causes arthritic symptoms in those who have or will develop psoriasis[1-6].

Psoriasis vulgaris, often known as plaque-type psoriasis, is the most common kind. Approximately 90% of psoriasis cases are chronic plaque-type psoriasis. Sharply defined, erythematous, pruritic plaques coated in silvery scales are the characteristic clinical symptoms. Large amounts of skin might be covered by the plaques. The trunk, extensor surfaces of the limbs, and the scalp are all common sites. 7-8. Psoriasis is characterised by persistent

inflammation, which results in uncontrolled keratinocyte growth and defective differentiation. The inflammatory infiltrates of dermal dendritic cells, macrophages, T lymphocytes, and neutrophils are overlain by acanthosis (epidermal hyperplasia) in the psoriatic plaque. Neovascularization is also a prominent feature. The inflammatory pathways active in plaque psoriasis and the rest of the clinical variants overlap, but also display discrete differences that account for the different phenotype and treatment outcomes[7-9].

The genesis and maintenance of psoriatic inflammation is due to disruptions in the innate and adaptive cutaneous immune responses. In some cases, activation of the innate immune system triggered by endogenous danger signals and cytokines coexists with autoinflammatory perpetuation and T cell-driven autoimmune reactions. As a result, psoriasis has autoimmune characteristics on a (auto) inflammatory backdrop, with both pathways overlapping and even potentiating one another[10-12].

The principle clinical signs of psoriasis are visible in the keratinocyte-rich outermost layer of the skin. The interaction of keratinocytes with many various cell types (innate and adaptive immune cells, vasculature) spanning the dermal layer of the skin shapes the development of the psoriatic plaque, which is not limited to inflammation in the epidermal layer. Psoriasis aetiology can be divided into two phases: an initiation phase initiated by trauma (Koebner phenomenon), infection, or medicines, and a maintenance phase defined by chronic clinical development[10].

Plaque-type psoriasis is characterised by the TNFα–IL-23–Th17 inflammatory cascade. There are six members of the IL-17 cytokine family: IL-17A–F. They are essential regulators of inflammatory responses and are generated by several cell types. IL-17A and IL-17F, which both operate through the same receptor but have differing potencies, have mostly caused clinically meaningful signalling in psoriasis thus far. The effect of IL-17A is stronger than that of IL-17F, while the effect of the IL-17A/IL-17F heterodimer is in the middle. When IL-17A binds to its trimeric receptor complex, which consists of two IL-17RA subunits and one IL-17RC subunit, the ACT1 adaptor protein is recruited. Extracellular signal-regulated kinase (ERK), p38 MAPK, TGF-beta-activated Kinase 1 (TAK1), I-kappa B kinase (IKK), and glycogen synthase kinase 3 beta are among the intracellular kinases activated when ACT1 interacts with the IL-17 receptor complex (GSK-3 beta). These kinases facilitate the transcription of pro-inflammatory cytokines, chemokines, and antimicrobial peptides by NFB, AP-1, and C/EBP. Th1 and Th2 cytokines

activate Janus kinase (JAK)-STAT signalling, whereas Th17 responses are mediated by ACT1 and NFB. T cells, on the other hand, can generate IL-17A without being stimulated by IL-23[12-15]. Psoriasis is a recurring chronic illness that requires long-term treatment. The severity of the disease, comorbidities, and access to health care all influence the treatment options for psoriasis. Depending on the clinical severity of the lesions, the percentage of afflicted body surface area, and the patient's quality of life, psoriatic patients are typically divided into two groups: mild or moderate to severe psoriasis. Topically, glucocorticoids, vitamin D analogues, and phototherapy can be used to treat mild to moderate psoriasis. Psoriasis that is moderate to severe generally necessitates systemic treatment[6, 16, 18-20].

1.2 Herbal medicines and Phytopharmaceuticals:

Natural products have been used since the dawn of civilization and the most common sources of medications include minerals, plants, and animals. Traditional medicines are the foundation of phytomedicines or phytopharmaceuticals, and approximately 365 plants, animals, and minerals have been reported to be useful as medication since ancient times. According to a World Health Organization (WHO) report, approximately 70% to 95% of citizens in the majority of developing countries still rely on traditional medicine as their primary source of medication. Herbal medicine's use began to diminish after the 1960s, as enormous sums of money and resources were spent to promote synthetic medications and their quick effects. Synthetic pharmaceuticals provide symptomatic relief in a dose-dependent way; but, due to the numerous negative side effects associated with them, herbal medicines are gaining wider acceptability in the community due to their superior therapeutic effects. New lead drug discoveries as well as safe and effective plant-based medications are being developed in this field. In the new category of drugs, enrich fraction containing at least four specific chemical markers with one biomarker is to be used as a phytopharmaceutical drug and which is not a part of Ayurvedic literature. Plant-based enriched fractions comprising varied compounds such as terpenoids and steroids, flavonoids, glucosinolates, coumarins, alkaloids, polyphenols, omega-3 fatty acids, phytoestrogens and many more with unique pharmacological effects in human health are known as phytopharmaceuticals. Many of these substances possess various therapeutic properties against inflammation, oxidative stress, microbial infection, diabetes, ageing and many more[21-23].

As the demand for regulated herbal medications, knowledge of precise chemical composition, quantity of pharmacologically active substances and standardisation of herbal formulations

grows, this category has become increasingly significant. Taking this into account, standards for the creation of phytopharmaceutical have been established to meet the growing need for science-based drugs from the box of traditional medicines that have a long history of usage but are not regulated.[24].

In contrast to AYUSH regulation, 2015 regulatory requirements for phytopharmaceuticals are under the purview of the Central Drugs Standards Control Organization (CDSCO). This gazette notification establishes regulatory provisions for phytopharmaceuticals, as well as regulatory submission requirements for scientific data on quality, safety, and efficacy, in order to evaluate and permit the marketing of herbal drugs in the same way that synthetic, chemical moieties are marketed. When conventional pharmaceuticals suspect everything and AYUSH medicines trust everything, phytopharmaceutical is a balanced method that trusts everything while revalidating the plant material specification. Phytopharmaceutical drug is defined as purified and standardized fraction with defined minimum four bioactive or phytochemical compounds (qualitatively and quantitatively assessed) of an extract of a medicinal plant or its part, for internal or external use of human beings or animals for diagnosis, treatment, mitigation, or prevention of any disease or disorder but does not include administration by parenteral route[25].

Herbal medications are commonly utilised as systemic and/or topical therapies, either as a replacement for or in addition to conventional approaches, however research on herbal medicines is primarily focused on inflammation, with little emphasis paid to how they affect immunological dysfunction. Both over-expression and under-expression of key proteins in psoriatic lesions can be linked to the biochemical basis for psoriasis pathogenesis, which is as diverse as the genetic basis. Anomalies in protein expression can be grouped into three categories: aberrant keratinocyte differentiation, keratinocyte hyperproliferation, and inflammatory infiltration. Various markers of aberrant keratinocyte differentiation have been discovered, all of which have significance for the disease's development. Signal Transducer and Activator of Transcription 3 (STAT3), Dihydrofolate reductase (DHFR), Aryl hydro carbon receptor (AHR), Heat shock proteins 70 (HSP70), interleukin 17a, 218 interleukin 17f, interleukin 22, tyrosine kinase, epidermal growth factor, interleukin 23, interleukin 6, interleukin 2, interleukin 2, interleukin 8, Psoriasis was previously treated as a skin condition. However, because it is an auto-immune illness, treatment must be site specific and immune system suppressing. As a result, the goal of this research is to produce phytopharmaceuticals with anti-psoriatic properties.

1.3 HPTLC

The HPTLC method is widely utilised in the pharmaceutical sector for process development, adulterant recognition and detection in herbal products, pesticide content, mycotoxins identification, and quality control of herbaceous plants and health foods. It has been well established that multiple samples can be run simultaneously using less mobile phase than in HPLC. HPTLC can be performed with mobile phases that have a pH of 8 or higher. Another benefit of HPTLC is that the chromatogram can be exposed to the same or different conditions multiple times. Following that, HPTLC was used to test various components in a multi-component formulation at the same time. It is also possible to authenticate many plant species using this procedure[29-32].

1.4 UHPLC-MS

UHPLC refers to liquid chromatography separations that use columns that encapsulate particles smaller than the 2.5–5 m sizes commonly used in high-performance liquid chromatography (HPLC). UHPLC works on the same premise as HPLC, with the controlling principle that efficiency and therefore resolution increase as column packing particle size decreases. Separations with smaller particles in the column have a higher efficiency per unit time, but the efficiency cannot be reduced at higher mobile phase flow rates or linear velocities. Smaller particles, speed and peak resolution can be absolute to new limits after attribute[33-37].

The hyphenated methodology has shown to be an invaluable tool for assessing drugs in a variety of biological samples. The hyphenated technology, such as LC-MS/MS, is an attachment of a chromatographic system to a spectroscopic system with the appropriate interface. It is well known that the performance features of UHPLC technology benefit detection significantly. Because chromatography dispersion is minimised with higher analyte concentration, improved source ionisation efficiency is encouraged[37, 38].

For estimating pharmaceuticals in bulk and pharmaceutical formulations, as well as their metabolites in biological fluids, UHPLC-MS/MS technique detection is the method of choice. Several approaches for drug analysis utilising UHPLC-MS/MS have been documented in the literature. Electrospray ionisation (ESI) and atmospheric chemical ionisation (APCI) ion sources were widely used in all ionisation procedures[39].

1.5 Introduction to Plant: *Psoralea corylifolia*

Psoralea corylifolia L. (Leguminosae) is an erect herb that grows every year. This plant grows to a height of 30 to 180 cm, does not grow in the shade, and requires a warm environment to thrive. Clay, sand and loam types of soil are required for this plant. The plant can thrive in a variety of environments, including acidic, basic and neutral. March through April is the optimal time to plant this plant. In November, the seeds reach maturity. If properly cared for, the plant can live for 5–7 years. Psoralea has a perennial fruit. The fruit has a bitter, caustic, and disagreeable flavour. The leaves are arranged in racemes. The simple leaves are large, oval, and have dented margins. The leaf base has around five main nerves. The upper and lower surfaces of the leaves are pubescent, with white hairs. The blossoms turn purple with blue undertones when it rains. In the axils, they are more crowded. The peduncles have lengthy heads, and each raceme might have 10–30 blooms. The pods have a black chocolate tint to them. The pods are tiny (3.5–4.5 mm x 2.0–3.0 mm x 2.0–3.0 mm). Pods are ovoid to oblong in form and may be flat. There is only one seed in Psoralea. The seed has an elongated shape with a smooth surface. The seed is hairless, compacted, and pitted tightly. The color of the seed is dark brown. The seed is dark brown in hue. The seeds are starch-free, with an oily feel and no endosperm. In 7–8 months, the crop is fully grown. Because the seeds require time to mature, collection can be done 4–5 times between December and March[40-42].

Vernacular names of *Psoralea corylifolia* [43-48]:

Sanskrit	:	Aindavi, Avalguja, Bakuchi
Hindi	:	Babachi, Babchi, Bavanchiyan, Bhavaj, Bakuci
English	:	Babchi seeds, *Psoralea* seeds, Malay tea, Scurf-pea, Fountain bush, West Indian Satinwood
Gujarati	:	Babchi, Bavacha, Babichi, Bawchi

Taxonomic Classification [49]:

Kingdom : Plantae

Division	:	Angiospermae
Class	:	Dicotyledoneae
Order	:	Rosales
Family	:	Leguminosae
Genus	:	*Psoralea*
Species	:	*P. corylifolia*

Figure 1.1, Plant of *Psoralea corylifolia*

Geographical Distribution[50, 51]**:**

It is a small, erect, annual herb growing up to 60–120 cm in height throughout sandy, loamy plains of Central and East India

Description[43, 52-55]**:**

Habit: It is a large shrub, with scattered lateral thorns; the end of main branches is often thorny.

Stem: They have grooved and gland-dotted stems.

Leaf: Leaves are simple, broadly elliptic, rounded, and mucronate at apex, clothed with white hairs on both surfaces, covered with numerous black dots, 5 main nerves springing from the base

Flowers: Flowers are dense, corolla yellow or bluish purple, axillary, 10–30 flowered racemes. Flowering time is from August to December. Flowering: It occurs after 3 to 4 years.

Fruits: Fruit is small, 5 mm long, subglobular, slightly compressed, pitted black, beaked without hairs, indehiscent, one-seeded pod, which is adhering to the pericarp.

Seeds: Seeds are brownish black in color, oblong, and flattened. Das, described the seeds as kidney shaped, 2–4 mm long, 2–3 mm broad, and 1–1.5 mm thick, hard, smooth, exalbuminous with straw-colored testa, with an agreeable aromatic odor and a pungent-bitter taste.

Parts used[56]: Seeds.

Medicinal Uses:

Seeds are useful in bilious disorders and to make perfumed oil.

The extracts have found to possess antitumor, antihyperglycemic, antidepressant, and antioxidant activities.

It has antimicrobial properties in its water extract. Diuretic, anthelminthic, laxative, and wound healing are all uses for the seed and extract powder. Seeds are used as a diaphoretic, stomachic, stimulant, and aphrodisiac. It is used to treat a variety of ailments, including asthma, cough, nephritis, and others. Psoralen and isopsoralen, the two main components, exhibit anticancer, antibacterial, and antiviral activities. It's a good hair tonic, thus it's used to treat alopecia and hair loss.

Impotence, menstrual abnormalities, and uterine bleeding can all be treated with it. It is used to treat gynaecological haemorrhage. It can also aid with early ejaculation and spermatorrhea. It shows coronary artery dilatation. The seeds are deobstruent and can be used to treat ulcers, cardiac problems, blood abnormalities and elephantitis.

Enuresis, pollakiuria, a painful feeling of cold in the waist and knees, and a weak kidney have all been treated with the crude medicine.

The root is beneficial in the treatment of dental cavities. *P. corylifolia* promotes bone calcification, making it effective in the treatment of osteoporosis and bone fractures. To treat diarrhoea, leaves are utilised. The bitterness of the fruit helps to avoid vomiting, cures micturition problems, treats piles, bronchitis, and anaemia and improves complexion.

P. corylifolia seeds are sweet, bitter, acrid, and astringent. They boost vigour and vitality while also improving digestion and mental receptivity. The seeds have antipyretic and alexiteric properties.[41, 43, 49,54,57-71].

1.6 Introduction to Plant: *Berberis aristata*

Berberis aristata (Berberidaceae), commonly known in Hindi as “Daruhaldhi” and “Citra” is spinous herb native to mountainous parts of Northern India and Nepal. These bushes can be found all over the Himalayas, as well as in the Nilgiri Hills of Southern India and Sri Lanka. It has been utilised widely in Ayurvedic medicine since the beginning of time. Its characteristics are reported to be similar to turmeric's. Indian barberry and its extract "rasot" are considered alterative and deobstruent, and are used to cure skin illnesses, menorrhagia, diarrhoea, jaundice, and a variety of eye ailments. A root-bark decoction is used as a wash for ulcers, and it is supposed to improve the look and induce cicatrization. The plant extract is one of the ingredients of the herbal cream Dermocept, which is used to treat sarcoptic mange lesions and has an inhibiting effect against *Salmonella typhi in-vitro* and also exhibited antiamoebic activity against *Entamoebic histolytica*. *B. aristata* also possesses stomachic, astringent, antiperiodic, antipyretic, antidiabetic and diaphoretic properties. Hepato-biliary diseases are said to be treated with the leaves. In rabbits, the berries, together with other plant-based substances, showed hypocholesterolemic effects. Berberine is a significant bioactive ingredient of isoquinoline alkaloids found in the plant. Traditional uses for the plant include inflammation, wound healing, skin illness, menorrhagia, diarrhoea, jaundice, and eye affliction. This plant produces the valuable ayurvedic preparation 'Rashut' [72-77].

Taxonomical classification[78]

Kingdom	Plantae
Division	Phanerogamea
Sub-division	Angiospermea

Class	Dicotyledonae
Sub-class	Polypetalae
Group	Thalamiflorae
Order	Ranunculales
Family	*Berberidaceae*
Genus	*Berberis*
Species	*aristata*

Figure 1.2, Plant of *Berberis aristata*

Vernacular Names [79, 80]

Sanskrit: Katamkateri, Dirvi

English: Indian Berberry

Gujrati: Daruharidra, Daruhuladur

Hindi: Daruhaldi, Darhald

Geographical Distribution:

In the Himalaya region, the plant can be found from the Himalayas to Sri Lanka, Bhutan, and mountainous portions of Nepal. Himachal Pradesh is where you'll find it. It grows at elevations of 2000-3000 metres, primarily in Himachal Pradesh's Kumaon and Chammba regions. It's also found in South India's Nilgris hills [81-85].

Description:

Habit: It is an erect spiny shrub, ranging between 2 and 3 meters in height wood, hard and yellow; bark, yellow to brown from outside and deep yellow from inside, removable in longitudinal strips by hand; spines (which, in fact, are modified leaves), three-branched and 1.5 cm long.

Root: Thick woody covered with a thin brittle bark

Leaf: Leaves are in tufts of 5 to 8, phyllotaxy verticillate, simple spiny, lanceolate, toothed, leathery, sessile, acuminate, with reticulate pinnate venation, 4.9 cm. long, 1.8 cm. broad, deep green on the dorsal surface and light green on the ventral surface

Flowers: Flowers are stalked, yellow, hermaphrodite, cyclic, actinomorphic, and perigynous, with an average diameter of 12.5 mm when fully opened; inflorescence is a simple to corymbose raceme with 11 to 16 flowers per cluster. Yellow calyx with 6 sepals (3 little, 3 big), actinomorphic caducous, 4 to 5 mm long. The polypetalous corolla has six petals, is yellow, actinomorphic, and is 4 to 5 mm in length. Androecium is polyandrous, with six adnate stamens measuring 5 to 6 mm in length, and gynoecium is a single 4 to 5 mm long stamen with a short style while large stigma.

Fruits: Fruits are globose to ovoid, usually covered with bloom as in plums. Fruits are 7 mm long, 4 mm in diameter. Fruit colour is aconite violet.

Seeds: Seeds are 2 to 5 in number, varying in colour from yellow to pink[76, 77].

Part used: Root

Medicinal Uses:

Berberis aristata or Daruharidra is an important herbal plant that has been utilised as a traditional medicine in numerous communities. For more than 2500 years, it has been used as a herbal treatment. Ancient Egyptians utilised it to predict plague outbreaks. During the early middle ages, European herbalists utilised this plant to cure liver and gall bladder diseases. *B.*

aristata was utilised by Russian therapists to treat inflammation, high blood pressure, and irregular menstrual cycles. American Indians use it to treat intermittent fever, neuralgia, and menorrhagia as a bitter tonic, stomachic, cholagogue, antiperiodic, and alternative. It is used to treat leprosy in the Unani medicinal system, and the plant's root extract is used to treat skin disorders like ulcers and abrasions, as well as acting as a blood purifier[86].

It is used as a laxative and to treat allergies, ophthalmia, metabolic abnormalities, and eye illnesses in India. It is traditionally regarded as one of the most important plants among 73 plant species used to treat skin disorders in Nepal and its neighbouring areas. In rural India, the formulation of multi-herbal medications incorporating *B. aristata* is utilised to treat bleeding piles condition.[87, 88].

Reported studies have revealed that the aqueous methanolic extract of the plant is used traditionally to treat osteoporosis diseases, joint pain and menopause. Cholera, diarrhoea, menorrhagia, jaundice, ear and eye infections, and urinary tract diseases are all treated using a decoction of *B. aristata* leaves known as Rasaut, according to ethnobotanical investigations. Antimicrobial, anti-inflammatory, antifungal, analgesic, antibacterial and antipyretic effects have also been observed in the Rasaut formulation[89-93].

Bhotiya populations in India's Himalayan areas utilise the root decoction to treat eye ailments. It is also used to treat skin issues, jaundice, malaria, and piles in the Malani tribal people of Himachal Pradesh, India. The fruit of the plant is also anti-scorbutic and laxative. In India's Garhwal Himalaya, the plant's root is used to cure jaundice and as psychomedicine to treat exorcism in children. The fruit and leaf juice are used to treat diarrhoea and dysentery in Nepal, while a decoction of the root and bark is used to treat fever and jaundice.[94-97].

The plant extract is used to treat diabetes and serves as an anti-hepatopathic in Sikkim and Darjeeling, India. *Berberis aristata* is used to treat acute dysentery, wound healing, skin illness, inflammation, diarrhoea, jaundice, menorrhagia, and eye problems in Ayurvedic medicine[80, 98].

CHAPTER 2

Review of Literature

2.1 Pharmacognostical review of *Psoralea corylifolia*:

- Shrestha et al., 2018 studied the pharmacognosticalcharacters of*Psoralea corylifolia*seed[99].

2.2 Phytochemical review of *Psoralea corylifolia*:

The chemical constituents reported in different parts of *P. corylifolia* are coumarins, meroterpenes, flavones, chalcones, stigmasteroids, lipids, resins, andvolatile oil.

- **Lu et al., 2020** isolated two new flavonoids, 5,4'-dihydroxy-6,7-furanbavachalcone, 1″-methoxy-6,7-furanflaanone and 5 known compounds identified as isobavachalcone, bavachin, psoralenol, corylifol E, corylifol A from the dried seeds of *P. corylifolia* from the methanolic extract of ethyl acetate soluble fraction[100].
- **Ali et al., 2015** reported presence of aromatic, sesquiterpenes, furocoumarins, sterols, fatty acid, and their methyl esters. The major compounds identified were epoxycaryophyllene, isopsoralen, psoralen, and bakuchiol by GC–MS of a low polar fraction of the methanolic seed extract[101].
- **Huang et al., 2014** isolated 10 meroterpenes of which four known compounds, included delta (1),3-bakuchiol, and six new compounds, included 12,13-dihydro-13-hydroxybakuchiol, delta(10)-12,13-dihydro-12-(R,S)-methoxyisobakuchiol, 13-methoxy-iso-bakuchiol, 13-ethoxyiso bakuchiol and 15-demetyl-12,13- dihydro-13-ketobakuchiol from the seeds[102].
- **Kim et al., 2013** recorded presence of psoralen, isopsoralen, isobavachromene, bavachinin (7-*O*-methylbavachin), corylin, bakuchiol and 6-prenyl naringenin in the chloroform fraction of seeds[103].
- **Limper et al., 2013** identified7,2′,4′- trihydroxy-3-arylcoumarin and psoracoumestan from the seeds by HPLC-DAD technique[104].
- **Song et al., 2013** reported the presence of isoflavones, corylifol D and corylifol E, based on a bioassay-directed fractionation of the ethyl acetate extract[105].
- **Xiao et al., 2012** isolated psoracorylifol F from the fruits and two known meroterpenesguided by TLC bioautography against $O_{(2)}^{(-)}$ radicals. All the three

metroterpenes possessed potential inhibitory activity against LPS-induced NO production in RAW 264.7 cells with IC (50) values ranging from 7.71 to 27.63μM[106].

- **Chen et al., 2011** isolated isoflavones, 7-*O*-methylcorylifol A and 7-*O*-isoprenylcorylifol A from n-hexane- and ethyl acetate-soluble fractions of the fruits[107].
- **Tewari et al. 2010** isolated a trans isomer ofbavachromene named psorachromene, along with psoralester from the plant[108].
- **Yang et al., 2009** isolated coryfolia D and bavarigenin from the ethyl acetate-soluble fraction from the ethanolextract[109].
- **Choi et al., 2008** reported an isoflavone neocorylin, which significantly inhibited baculovirus-expressed BACE-1 *in vitro*from the seeds[110].
- **Wu et al., 2008** isolated two meroterpenoids, 12, 13-dihydro-12,13-dihydroxybakuchiol and (120 S)-bisbakuchiol C along with four reported compounds 12, 13-dihydro-12,13-epoxybakuchiol, Acetyl bakuchiol, *O*-methyl bakuchiol and *O*-ethyl bakuchiol from the seeds[111].
- **Matsuda et al., 2007** reported 1,3-hydroxybakuchiol; 3,2-hydroxybakuchiol; and 6-prenylnaringenin from the ethyl acetate soluble fraction of methanolic extract from the seeds[112].
- **Ruan et al., 2007** reported raffinose, psoralen, and isopsoralen from the leaf whereas, isoflavones, corylinin and diadzein were shown to be present in the petroleum ether extract of roots[113].
- **Wu et al., 2007** studied structures of bisbakuchiols A and B and noted to possess dimeric monoterpenoid skeletonhaving two monoterpenes connected through a dioxane bridge[114].
- **Yang et al., 2006** isolated bavadin from the fruits and purified by column chromatography with normal phase silica gel and polyamide[115].
- **Yin et al., 2006** reported psoracorylifols A–E and chalcone and bavachromanol using an isolation procedure involving spectroscopic methods and crystal X-ray diffraction[116].
- **Lee et al., 2005** isolated two dihydroflavones, 6-prenylnaringenin and chromenoflavanone along with 4-hydroxylonchocarpinfrom methanol extracts of *P. corylifolia*[117].
- **Yadava et al., 2005** isolated and identified an antimicrobial flavonol glycoside, 3,5,30,40-tetrahydroxy-7-methoxyflavone-30-*O*-α-L-xylopyranosyl(1→ 3)-*O*-α-L arabinopyranosyl (1→4)-O-β-D-galactopyranoside[118].
- **Yin et al., 2004** isolated flavonoids,corylifols A, B, and C, along with bavachinin, bavachin, isobavachin, isobavachalcone, bavachalcone and bakuchicinfrom the seeds[119].

- **Khatune et al., 2002** isolated a coumestanderivative 6-(-3-methylbut-2-enyl)-6 N-7-dihydroxycoumestan from the chloroform soluble portion of the seeds[120].
- **Hsu et al., 2001** identified three isoflavonoids, genistein, daidzein, and biochanin A, in the fruit of *P. corylifolia* through the optimized high-performance liquid chromatographic method[121].
- **Shah et al., 1997** isolated and characterized three oxygenated derivatives of bakuchiol, as 2,3-epoxybakuchiol, Delta(1),3-hydroxybakuchiol, and Delta(3),2-hydroxybakuchiol[24].
- **Lin et al., 1992** separated two benzofuran derivatives; corylifonol and isocorylifonol along with astragalin, *p*-hydroxy benzoic acid from seed extract[123].
- **Zaka et al.,1989** analyzed the purified crude lipids of *P. corylifolia* seeds by thin layer and gas chromatographic techniques and noted C18:1 as the major polar lipid component along with the mono and diacylglycerol portions containing huge amounts of C14:0 and C18:0 and the hydrocarbons, and the wax ester portions abundant in C22:0. Further, through GC–MS analysis presence of β-caryophyllene, limonene, linalool, terpin-4-ol, and geranyl acetate was decoded in volatile oil[124].
- **Banerjiet al., 1983** reported bakuchiol has and noted that it is a derivative of phenylpropane pathway[125].
- **Suri et al., 1980** reported a chalcone, bavachromanol from the seeds[126].
- **Bajwa et al., 1974** isolated a flavonoid, isoneobavaisoflavonefrom the seeds[127].
- **Mehta et al., 1973** isolated bakuchiol, a meroterpeneusing column chromatography and TLC on SiO2 gel layers (0.3 mm) containing 15% gypsum[128].
- **Bajwa et al., 1972** reported neobavaisoflavonefrom the seeds of *P. corylifolia*[129].
- **Bhalla et al., 1968** isolatedbavachin, bavachinin and isobavachin along with bavachalcone and isobavachalcone from the chloroform extract of the seeds [130].
- **Khastgir et al., 1961** reported psoralidin(natural phytoestrogen), having a coumestrol skeleton which can mimic the biological activity of estrogens[131].
- **Chopra et al., 1958** considerable resin acids (21.5%) along with glycerides of oleic, stearic, palmitic, myristic, myristolic, linoleic, and linolenic acids from the petroleum ether extract of the seeds[132].
- **Jois et al., 1936** isolated angelicin from the seeds which possess antioxidant property along with anti-depressant, anti-inflammatory and anti-microbial potential[133].
- **Jois et al., 1933** isolated the first furocoumarin, psoralen from the fruit part of the plant[134].

2.3 Pharmacological Review of *Psoralea corylifolia*

- **Seo at el 2018; Li et al., 2017** reported that bakuchiol when administered at a dose of 52.5 and 262.5 mg/kg for 6 weeks in rats, caused abnormalities that included suppression of weight gain and food intake, change of some parameters in serum biochemistry, and increased weight of liver. The mRNA expression of CYP7A1, HMG-CoA reductase, PPARα, and SREBP-2 also was decreased in bakuchiol-treated group. Moreover, the expression of BSEP increased at low dose of bakuchiol-while with high dose the expression of BSEP decreased. It was concluded that bakuchiol could induce cholestatic hepatotoxicity, suggesting potential hepatotoxicity. Bakuchiol was also reported to inhibit the formation of reactive oxygen species and mitochondrial dysfunction induced by oxidative stress in hepatocytes HepG2[135,136].
- **Kim et al., 2016** reportedthatbakuchicin inhibited the cytochrome1A-mediated phenacetin *O*-deethylation with an IC_{50} value of 0.43 μM in Human liver microsomes and wasstrong and a selective competitive inhibitor of CYP1A1 and CYP1A2 in HLMs[137].
- **Park et al., 2016; Chen 2010; Liu 214; Guo et al 2011** reported ripe fruits showed an anticancer potential against human colorectal cancer. It suppressed the proliferation of human colorectal cancer cell lines, such as SW480, HCT116, LoVo and HT 29, by decreasing in the protein expression of cyclin D1 and CDK4. In another study, bakuchiol was found to be effective against liver cancer. Psoralidin was also found active against stomach carcinoma cell lines. Psoralidin is an ER agonist also has revealed its activityin MCF 7 cancer cells (isolated from human breast) by induction ofgene pS2 activity. EC_{50} values of ERE reporter gene transcriptionactivities by psoralidin in cell lines MCF 7 was 1.85 μM. Psoralen also showed potential to invade the breast cancer cells MDAMB 231BO in another *in vitro* study. It also stimulates osteoblast differentiationin an *in vivo* study. Psolaren when tested in Human hepatocarcinoma cells showed inhibitory activity by inducingapoptosis. Bakuchiolis shown to suppress the testosterone induced cell proliferation and geneexpression in androgen dependent LNCaP cells. Bakuchiolalso showed strong anticancer action against human lungadenocarcinoma cell line A549 (IC_{50}: 9.58 ± 1.12 μmol/L)and the results were better thanits analogue resveratrol(IC_{50}: 33.02 ± 2.35 μmol/L) [138-141].
- **Sun et al., 2016 and Li et al., 2015** reported concentration dependent inhibitory effect of neobavaisoflavone, corylifolinin, coryfolin, psoralidin, corylin and BCN on the human carboxylesterase 1 (hCE1) enzyme. The ethanol extract of *P. corylifolia* inhibited the enzymatic activity of hCE2, suggesting presence of strong hEC2 inhibitory components. Of the five major hCE2 inhibitors identified, including isobavachslcone,

neobavaisoflavone, corylifol A, bavachinin and bakuchiol, corylifol A was more potent with least IC_{50} values[142, 143].

- **Aiyaz et al., 2015; Hosamani et al., 2012; Satish et al., 2009; Vonshak et al., 2003** reported that *P. corylifolia* significantly reduced the incidents of seed-borne fungi, for example, *Fusarium verticillioides* and *Aspergillus flavus*. In another study, bakuchiol is shown to exhibit antifungal activity against many strains of pathogenic fungi, including *Microsporum gypseum, Epidermophyton floccosum, Trichophyton rubrum, Trichophyton mentagrophytes, Alternari brassicae, Aspergillus niger, Fusarium oxysporum*, and *Rhizoctonia cerealis*[144-147].
- **Dang et al., 2015** studied effects of bavachalcone in cardiovascular diseases and demonstrated that it increased the luciferase activity of the manganese superoxide dismutase (MnSOD) promoter, enhanced MnSOD mRNA and protein expressions, lowered mitochondrial oxidative stress and enhanced the AMPK activity[148]**.**
- **Lee et., al 2015; Khatune et al., 2004; Newton et al., 2002; Katsura et al., 2001** reported bakuisoflavone and bakuflavanone exhibited antibacterial activity against the strains of MRSA481 and MRSA584. In another study, extracts of seeds and aerial part showed activity against *S. eidermidis* and *P. morganii*. Bakuchiol were found to be effective against *Staphylococcus aureus* and *Staphylococcus. epidermdis*. Corylifolinin and neobavaisoflavone, exerted significant antibacterial activity against *S. aureus*, methicillin-resistant *Staphylococcus aureus* (MRSA), and *β*-lactamase positive *Staphylococcus aureus* (ESBLs SA). In another study, psoralidin and bakuchicin showed significant inhibition of Gram negative bacteria, including *Shigella sonnei* and *Shigella flexneri*, whereas psoralen and angelicin compounds showed potential activities against Gram-positive bacteria, *S. aureus*.Bakuchiolhas been found to be effective against *Mycobacterium aurum* (MIC = 62.5 μg/ml) and other strainsincluding *S. mutans, S. sanguis, S. salivarius, S. sobrinus, Enterococcus faecalis, Enterococcus, Lactobacillus acidophilus, L. casei, L. plantarum, Actinomyces viscosus and Porphyromonas gingivalis*, with MICs ranging from1 to 4 μg/ml. [149-152].
- **Liu et al., 2015; Takizawa et al., 2002; Pathak et al., 1963** reported the plant extract and bioactives such as psoralen and isopsoralen caused the inhibition of concentration CYP3A4 activity in a dose dependent manner with different potency *in vitro*when incubated with the recombinant CYP3A4 enzyme or differentiated HuH-7 and HepaRG cells. In another study, bavachin, corylifol A, neobavaisoflavone, isobavachalcone and bavachinin showed potent inhibitory effect against human UDP-glucuronosyltransferase

1A1 (UGT1A1) and thus are considered as main stimulants for *P. corylifolia* hepatic injury and raised bilirubin levels. The plant extract containing psoralen and isopsoralen have been reported to possess potent sensitizing action. Another study reported that the extract caused toxicity at the hypothalamus pituitary gonadal axis[153-155].

- **Mendam et al., 2015; Maurya et al., 2014; Gidwani et al., 2010; Qamaruddin et al., 2002** reported that of the water and alcohol extracts of the seeds and the leaves of *P. corylifolia*, the seed extractsshowed anti anthelmintic potential which is clinically proven on roundworm and flatworms. Moreover, results of clinical trials performed usingformulation of seeds of *P. corylifolia* on patients having eczema revealed it to be effective [156-159]
- **Song et al., 2015; Ling et al., 2013** reported antiprotozoal activity of the methanolic extract *P. corylifolia*, isopsoralen and psoralidin against protozoan parasite *Ichthyophthirius multifiliis*. In another study, methanolextractis shown to exhibit excellent activity against *I. multifiliis* theronts in concentration of 1.25 mg/L or more when was exposed for a period of 4 hr[160-161].
- **Weng et al., 2015; Siu et al., 2013; Cho et al., 2001; Miura et al., 1996** reported osteoblastic property (bone protective role) of bakuchiol and bavachin and suggested them as safe estrogen supplement. Both the compounds prevented the bone loss caused by deficiency of estrogen in overictomized animal models and also in an *in vitro* study by inducing the primary human osteoblast differentiation by up-regulation the Wnt signaling pathway.In another study, it is also found to be effective against hyperosteoidosis, by increasing the serum inorganic phosphate level at a dose of 30 mg/kg. The flavonoids of corylin and bavachin were shown to have the osteoblastic stimulating activity in UMR106 cell line cultured *in vitro*. Another study showed that formulation containing *Psoraleae* has been shown to give remarkable results in women with osteoporosis by suppressing the adipogenesis in mesenchymal stem cells[162-165].
- **Xu et al., 2015** reportedthatpsoralen tested at different concentrations (1, 10, and 100 μM) on ratchondrocytes at 3- and 9-day intervals, was safe the low dose concentration; however, at higher dose suppression of chondrocytes, proliferation was seen. Psoralen increased the synthesis of type II collagen at 100 μM, by 0.48-fold on day 3 and 0.56-fold on day 9. Psoralen caused the inhibition of the type I collagen in gene expression and also in protein synthesis. Thus, psoralen can possibly trigger the cartilaginous cellular functions of chondrocytes[166].

- **Asad et al., 2014** reported the plant extract neutralized the coagulation caused by *Naja naja karachiensis* snake bite when compared against antidote used as a standard. The snake venom was experimented on human plasma (citrated) to estimate its effect on prothrombin time (PT), activated partial thromboplastin time (aPTT),), and thrombin time (TT). Results showed that, PT and TT were prolonged, and it suggested theoccurrence of thrombin-like or plasminogen activating enzymes[167].
- **Im et al., 2014 and Lee at al.** suggested use of the seed extracts as therapeutic agents against neurodegenerative diseases based on its ability to stimulate mitochondrial respiration with uncoupling and inducing an increased bioenergetic reserve. The seed extract significantly attenuated 3-NP induced cell death, reduced ATP levels, and lowered the mitochondrial membrane potential[138].In another study, it was revealed that isobavachalconehas the ability to ameliorate the neuronal injury in braindiseases related to inflammation, and this was proficient through inhibition of lipopolysaccharide induced intercellular adhesion molecule- 1 expression and leukocyte adhesion to brain endothelial cell by blocking toll-like receptor 4 signaling[168-169].
- **Kim et al., 2014** reported activity of the crude ethanol extract of the seeds against the severe acute respiratory syndrome corona virus (SARS-CoV), papain-like protease (PLpro), the main enzyme having a vital role in SARS virus replication with IC_{50} of value of 15 μg/ml[170].
- **Liu et al., 2014 and Lim et al., 2011;** in different studies reported**,** bakuchiol was found to be effective in estrogen receptor binding affinity (ERβ)that inhibits human breast cancer cell proliferation and tumor formation by causing a G2 cell cycle check. Whereas, ERα agonists might facilitate bone protecting properties. Thus, it is shown to be useful in the treatment of osteoporosis or postmenopausal conditions. The compound psoralidin isolatedfrom the ethyl acetate fraction of *P. corylifolia* seeds proved as novel ERmodulator with 92.3% of (estradiol) E2-induced ERE-luciferase activity, In another experiment psoralidin activity as ER agonist has been reported in endometrial and human breast cell lines[171-172].
- **Behloul et al.,** 2013 showed that genestein to have antiobesity and obesity related low grade inflammation activities through multiple mechanisms and cell signaling pathways[173].
- **Chen et al., 2013; Somani et al.** showed that isobavachalcone and bavachinin modulated amyloid β (Aβ) peptides, especially the peptides with 40 (Aβ40) or 42 (Aβ42) residues, that are responsible for the development of amyloid plaques in Alzheimer's disease and reported that these two compounds were effective but gave activity in a different way. IBC

significantly inhibited both oligomerization and fibrillarization of Aβ42, whereas BCN converted Aβ42 into large unstructured aggregates in neuroblastoma cells[174]. In another study, psoralen was reported to inhibitAChE enzyme in a dose dependent way in adult male Wistar rats [175].

- **Jeong et al., 2013** demonstrated thatthe extracts, psoralen, p-hydroxybenzaldehyde, psoracorylifol D,angelicin, BCN, isobavachalone, and bakuchiol hydroxybakuchiol,caused significant inhibition (*in vitro*) of the proliferation of temperature sensitive rat lymphatic endothelial (TR LE) cells. Among these, compounds such as psoracorylifol D, isobavachalone, BCN, hydroxybakuchiol,and bakuchiol inhibited proliferation and the formation of the capillaryliketube of TR-LE cells. Other compounds tested showed selectiveactivity[176].
- **Kang et al., 2013; Chen et al., 2011** reported antioxidant activity of coryfolin (IC50 4.97 mg/L), daidzin (IC50 10.47 mg/L), daidzein (IC50 34.22 mg/L), and astragalin (IC50 31.27 mg/L). Psoralidin proved to be a better scavengerof DPPH free radical with IC50 values of 43.85 mg/L. In another study, psoralen showed promisingantioxidant activity (IC50 value = 1.10 ± 0.60 μg/ml) against the superoxideanion production by human neutrophils in response to formyl-Lmethionyl-L-leucyl-L-phenylalanine/cytochalasin B[107, 177].
- **Kim et al., 2013** reported utility of whole plant extract in the treatment of leucoderma and its protective effect against retinal damage caused by oxidative stress[103].
- **Dua et al., al 2013 and Khatune et al., 2002** reported strong toxicity of volatile oil extracted from the seeds against both larvae and adult of the southern house mosquito, *Culex quinquefasciatus*. In another study coumestan derivative 6-(3-methylbut-2-enyl)-6 N-7-dihydroxycoumestan was found active against larvae and adult *Culex quinquefasciatus*[178-179].
- **Limper et al., 2013** showed that arylcoumarin and psoracoumestan exerted strong anticancer potential by inhibiting the enzyme system of MAPL/ERK kinase phosphorylation[104].
- **Wang et al., 2013; Chen et al., 2013; Ghosh et al., 2009** reported psoralidin and bakuchiol, showed protein tyrosine phosphatase 1B inhibitory activity Similarly, the compounds isolated from *P. corylifolia* were shown to possess alpha-glucosidase inhibitory activity, among them psoralidin was shown to have more potency with IC50 values of 40.74 mg/L followed by coryfolin with IC50 values of 45.73 mg/L, and daidzein with IC50 values of 49.44 mg/L. It was concluded that these compounds have the potential

to be used against type 2 diabetes. Genestein exerted anti-diabetic activity by its action as the protective effects on pancreatic β cells. The aqueous extract of seed was shown to cause significant recovery in the activities of hexokinase, glucose-6-phosphatase, and glucose-6-phosphate dehydrogenase and antioxidant enzymes such as peroxidase, catalase, and superoxide dismutase, along with the lipid peroxidation level in liver tissue and serum transaminase, and corrected the fasting blood glucose level in streptozotocin induced diabetic rats at a dose of 20 mg/0.5 ml water/100gm body weight[173,177,180].

- **Yi et al., 2008** reported that psoralidin exhibited antidepressant activity probably by changing the hypothalamic-pituitary-adrenal axis[181].
- **Sun et al., 2003 reported** the crude extract was found to inhibit DNA polymerase inhibitor of DNA replication enzyme in an activity directed isolation assay that resulted in the purification of novel compound corylifolin, bakuchiol, neobavaisoflavone, and resveratrol. In a similar enzyme assay, daidzein and bakuchicin was shown to inhibit some topoisomerase II inhibitors[182].
- **Latha et al. 2000** recorded stimulant activity against natural killer cells when tested in mice using seed extracts. Extract was also reported to inhibit the antibody complement mediated cytotoxicity when study conducted on male mice[183]**.**
- **Iwamura et al. 1989** attributed anti-acne potential of formulations to the presence of phenolic compound bakuchiol[184].

2.4 Pharmacognostical review of *Berberis aristata*:

- **Rathi et al.,2013** studied the pharmacognostical characters of *B. aristata* root[185].

2.5 Phytochemical reviewof *Berberis aristata*:

- **Lect et al., 1983**recorded a secobisbenzlisoquinoline or simple isoquinoline alkaloid from the root of *B. Aristata*[186].
- **Atta-ur-Rahman et al., 1983** reported presence of oxyberberine, aromoline from the ethanolic extract of root[187].
- **Blaskoet al., 1982** reported dihyrokarachine, taximaline from the root[188].
- **Bhakuniet al., 1968** reported four alkaloids, pakistanine, 1-*O* methyl pakistanine, pseudopalmatine chloride and pseudoberberine chloride from *Berberis aristata*[189].

- **Chatterjiee et al.,1951** reported alkaloids which are berbamine, berberine, oxycanthine, epiberberine, palmatine, dehydrocaroline, jatrorhizine and columbamine from the root of plant[190].

2.6 Pharmacological Reviewof *Berberis aristata*:

- **Das et al., 2020 and other** reported that the methanoilc extract showed a promising result against *H. pyroli*. In another study, antimicrobial activity of hydroalcoholic extracts were reported against *Micrococcus luteus, Bacillus subtilis, Berberis cereus, Enterobactor aerogenus*. The hydroalcoholic extracts of root also showed significant antifungal activity against *Aspergillus terreus* and *Penicillium citrinum*[191, 192].
- **Singh et al., 2009**reported thatroot extract possessed anti hyperglycemic and anti oxidative properties in alloxan induced diabetic rats, by reducing blood glucose level,restoring antioxidant status, reducing oxidative stress and modulating enzymes for glucose metabolites[193].
- **Semwal et al., 2009**demonstrated the anti-diabetic activity of stem bark in alloxan induced diabetic rats and reported that the ethanolic extract reduced blood glucose level in diabetic rats[194].
- **Gupta et al., 2008**showed that topical instillation of aqueous extracts *B. aristata* exerted potent anti-inflammatory activity against endotoxin-induced uveitis in rabbit. Anterior uveitis was induced in rabbits by intravitreal injection of lipopolysaccharide from *Escherichia coli* after pretreatment with *B. aristata* aqueous extracts[195].
- **Mazumdar et al., 2009** reported that methanolic extract of stems showed promising results against breast and colon cancer cell lines by inhibiting HT29 cells[196].
- **Sabnis mukund et al., 2006** demonstratedthat berberine (5, 10, 20 mg/kg, i.p.) inhibited the immobility period in mice in both forced swim and tail-suspension test, however, the effect was not dosedependent. Further, berberine (5 and 10 mg/kg, i.p.) was also shown to reverse the reserpine-induced behavioral despair indicating its antidepressant-like effect in various behavioural paradigms of despair possibly by modulating brain biogenic amines (norepinephrine, serotonin and dopamine)[197].
- **Anis KV et al., 2006** demonstrated that berberine couldsignificantly inhibit the carcinogenesis induced by 20-methylcholanthrene or N-nitrosodiethylamine, in a dose-dependent manner in small animals[198].

- **Anonymous 2001** reported fruit extract exhibits a positive inotropic actionby involving—in the form of the modulatory effect on actin myosin cooperativity—a novel mechanism of action[199].
- **Sharma et al., 2000** reported immunomodulatory activity of a crude extract by showing that T-cell counts remained unaffected in the animals treated with the formulation but cell-mediated immune response was stimulated as observed in the leukocyte migrant ion inhibit ion (LMI) tests[200].
- **Sack et al., 1982** reported the clinical effectiveness berberine in treating acute diarrheal disease [201].
- **Singhal et al., 1976** showed hepatoprotective potential of the crude extract of leaves and fruits possibly through inhibitory action on hepatic drug metabolizing enzymes[202].

2.7 Imiquimod induced psoriasis like dermatitis Model:

- **Parmar et al., 2021** studied psoriatic-like skin inflammation in rat model and successfully developed psoriatc like lesions on Wistar rats[203].
- **Husna et al., 2019** evaluated antipsoriatic activity on aqueous extract of *Brassica oleraceavar*. *capitata* and ethanolic extract of *Mentha spicata* leaves using imiquimod-induced Psoriasis-like dermatitis model[204].
- **Arora et al., 2015 reported** inhibition of imiquimod-induced psoriasis-like dermatitis in mice by herbal extracts[205].
- **Leslie et al., 2009** reported increase in epidermal expression of IL-23, IL-17A, and IL-17F in imiquimod-induced mice[206].

CHAPTER 3

Material & Methods

3.1 Identification, Collection and Authentication of the Plant Material:

The seeds of *Psoralea corylifolia* were procured from local market of Ahmedabad, Gujarat in the month of November 2018. Authentication of the plants was done by comparing morphological and microscopical features with the literature.

The roots of *Berberis aristata* were collected from Uttarakhand. Authentication of the plants was done by Dr. V. P. Bhatt, Taxonomist, Herbal Research and Development Institute (HRDI), Gopeshwar, Uttarakhand.

Fresh samples of *B. aristata* were washed to remove soil, mud and other adhering material and dried at room temperature under shade. 60# powder was prepared from dried samples separately and stored properly in airtight containers.

3.2 Assessment of quality of plant materials:

The plant materials were assessed as per WHO guideline[207-208].

3.2.1 Determination of foreign matter

Both the plants were subjected to determination of any contamination by mould or insects and other animal contamination.

3.2.2 Macroscopic evaluation

Fresh plant parts of *Psoralea corylifolia* and *Berberis aristata* were subjected to color, odor and taste, determination of shape, size, surface characteristics and appearance etc[3].

3.2.3 Microscopical Evaluation

The seed of *P. corylifolia* and root of *B. aristata* were studied for macroscopical characters. The powdered material was subjected to microscopical study also.

3.2.4 Proximate analysis:

Proximate analysis of powdered plant material of of *P. corylifolia* and root sample of *B. aristata* was carried out using reported methods.

3.2.4.1 Loss on drying:

In a previously tarred flat weighing vial, accurately weigh roughly 2 gram of air dried plant material. Heat the sample in an oven at 100-105o C for 5 hours to dry it. Unless otherwise stated in the test procedure, dried until two consecutive weighings did not deviate by more than 5 mg. The weight loss in mg/gm of the air dried material is then calculated.

3.2.4.2 Determination of Ash Values:

Three separate methods were used to determine how much ash remained after powder plant material was ignited: total ash, acid insoluble ash, and water soluble ash.

3.2.4.2.1 Determination of Total Ash

In a muffle furnace, 2 g of accurately weighed powder of both plants were burnt in a silica crucible at a temperature not exceeding 450 °C until white, demonstrating the absence of carbon. If you can't get carbon-free ash this way, cool the crucible and wet the residue with roughly 2 mL water or a saturated ammonium nitrate solution. It was dried in a water bath before being burned to maintain a steady weight. It was then cooled, weighed, and the percentage of ash estimated using the air-dried powdered medication as a reference.

3.2.4.2.2 Determination of Acid Insoluble Ash

The ash from both of the plants was heated in 25 mL of weak hydrochloric acid for 5 minutes. The ash-free filter paper was used to collect the insoluble materials. The ash was cleaned with hot water before being burned to a consistent weight in a muffle furnace at 450 degrees Celsius. The proportion of acid-insoluble ash was estimated using the air-dried medication as a reference.

3.2.4.2.3 Determination of Water Soluble Ash

The total ash from both plants was cooked in 25 mL of water for 5 minutes. The ash-free filter paper was used to collect the insoluble materials. The ash was cleaned in hot water for 15 minutes at a temperature of not more than 450 °C. The ash's weight was removed from the weight of the insoluble substance. The water soluble ash was represented by the weight difference. The proportion of water-soluble ash was estimated using the air-dried powdered medication as a reference.

3.2.4.3 Determination of Extractive Values

Determination of Alcohol Soluble Extractive Value

4 g of air-dried powdered material from both plants was macerated in 100 mL of water for 6 hours in a closed flask with regular shaking. It was then let to stand for 18 hours before being filtered quickly to prevent any evaporation loss. In a porcelain dish, 25 mL of the filtrate was evaporated to dryness and dried at 105 °C to a constant weight. With reference to the air-dried powdered medication, the proportion of alcohol-soluble extractive was estimated.

Determination of Water Soluble Extractive Value

4 g powdered material from both plants was soaked in 100 mL water for 1 hour in a covered flask with regular shaking. The weight was then readjusted after it was gently cooked for 1 hour on a water bath. In a porcelain dish, 25 mL of the filtrate was evaporated to dryness and dried at 105 °C to a constant weight. The proportion of water-soluble extractive was estimated using air-dried powdered medication as a reference.

3.3 Phytochemical Studies

3.3.1 Preliminary Profile

The powder of the air dried plant of *Psoralea corylifolia seed and Berberis aristata* were extracted with 100 mL of methanol (100mL X 5) separately, using reflux, maceration, soxhlet percolation, ultrasonication and autoclave methods. % Yield was calculated.

3.4 Extraction of *P. corylifolia* with different solvents:

Extraction is a process used to obtain compounds which may be diffused out from plant material, while isolation is separation process to obtain a pure compound from plant extract.

5 g of the dried powder of *P. corylifolia* was exhaustively extracted using Diethyl ether by soxhlet method to yield a semi-solid extract **(Extract A).**

100 g. *P. corylifolia* seed powder was successively extracted with benzene for 5 hrs in reflux condition. Filtrate was evaporated to obtain benzene extract (**Extract B**). Extract was dissolved in hexane & ppts were obtained which is filtered and filterate was subjected to Biphasic system (*n*-hexane:ethylacetate:methanol:water). Aqueous Layer was collected to obtain white precipitates which were subjected to flash chromatography. Organic layer was collected & subjected to flash chromatography.

100 g. *P. corylifolia* seed powder was extracted with benzene

for 5 hrs in reflux condition

Filtrate was evaporated to obtain benzene extract (Extract B)

Extract was dissolved in hexane & ppts were obtained which is filtered and filterate was subjected to Biphasic system (*n*-hexane :ethylacetate:methanol:water

Aqueous layer (0.8 g.) was collected to obtain white ppts & subjected to flash chromatography

Organic layer (0.38 g.) was collected & subjected to flash chromatography

FIGURE 3.1, Scheme for extraction of *P. Corylifolia*

10 g of the dried powder of *P. corylifolia* was exhaustively extracted using *n*-Hexane by soxhlet method to yield a semi-solid extract **(Extract C).**

10 g of the dried powder of *P. corylifolia* was exhaustively extracted using methanol by soxhlet method to yield a semi-solid extract **(Extract D).**

3.5 Column Chromatography of Extract A (Dietylether extract):

A cylinder-shaped glass column holding a stationary phase (silica gel) is slowly approached from the top by a liquid solvent (mobile phase) that flows down the column with the assistance of gravity or external pressure. Purification of chemicals from a mixture is accomplished using this method. The sample is loaded into the top of the column once the column is ready. After that, the mobile solvent is allowed to flow down the column. Because the compounds in the mixture interact differently with the stationary phase (silica gel) and the mobile phase, they will flow along the mobile phase at varying times or degrees. The separation of chemicals from the mixture is accomplished in this manner, which are then studied further for structure elucidation[209].

200 mg of the diethyl ether extract i.e. Extract A was loaded on a glass column and gradient elution was performed using different proportions of *n*-Hexane: Ethylacetate. A total of 145 fractions, each of 10 mL, were collected in test tubes. Based on TLC study, similar fractions were pooled together and allowed to concentrate.

FIGURE 3.2, Column chromatography of *P. corylifolia* seed extract

Column condition:

Column length: 52 cm

Column diameter: 1.8cm (inner), 2.3cm (outer)

Stationary phase: Silica gel 60-120 mesh

Mobile phase: *n*-Hexane: Ethyl acetate

Type of elution: Gradient

Number of fractions collected: 145 (10 each mL)

TABLE 3.1. Details of Fractions Collected by Gradient Elution

No	Fraction (10 mL each)	Polarity of Eluent
1	1-40	0.1% EtOAc in n-Hexane
2	41-100	0.15% EtOAc in n-Hexane
3	101-130	0.2% EtOAc in n-Hexane
4	131-145	0.3% EtOAc in n-Hexane

Fractions 41 to 100 were evaporated to give Compound A (White shiny crystals). Fractions 101 to 130 were evaporated to give orange colored fraction and were subjected to flash chromatography for further purification.

3.5.1 Identification of Isolated Phytoconstituents by Thin layer chromatography (TLC)

3.5.1.1 Identification of Compound-A by TLC in fractions 41-100

Test sample :Compound-A

Reference sample :Psoralen (SIGMA)

Stationary phase :Silica gel G60 F_{254}

Mobile phase :Toluene: ethylacetate: formic acid (5:4:1)

Visualization :Under U.V light 254 nm and 366 nm

3.5.2 Automated flash chromatography PuriFlash®XS520 plus INTERCHIM:

Flash chromatography is used for purification of compound mixtures to obtain the single component which cannot be easily purified by distillation, recrystalization or other methods. Flash chromatography has become a popular method of normal phase separation through purification. While flash chromatography is typically a low-pressure technique, scientists are using vacuum or pumps at medium pressure to speed up the separation processes[210].

Automated flash chromatography puriFlash® XS520 plus INTERCHIM was used to purify the mixture of the compound. Designed for routine flash purifications, the technology and unique qualities of pump takes us further. On increasing pressure, the puriFlash® XS520 plus offers the same precision, linearity, repeatability and performs complex and sophisticated purification.

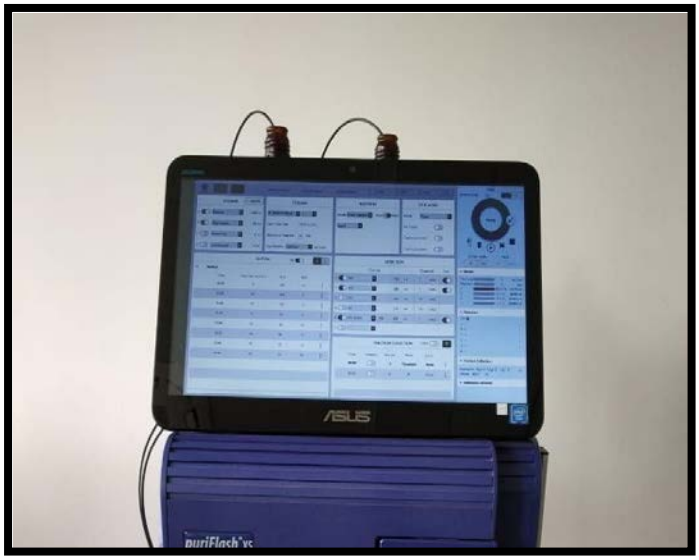


FIGURE 3.3, Automated flash chromatography

Conditions for Flash Chromatography for the purification of isolated compound from Extract A mentioned below:

Preparation of sample: 100 mg of the sample was dissolved in minimum amount of solvent (HPLC grade Methanol).

TABLE 3.2. Conditions for Flash Chromatography

Sr. No.	Parameters	Conditions
1	Crude weight	0.1 g.
2	Solvent	*n*-Hexane (A): Ethyl acetate(B)
3	Flow rate	15. mL/min
4	Pressure	22 Bar
5	Mode	Direct injection
6	Wavelength	254 and 366 nm
7	Loaded on	Silica gel column (60-120#) 24g (PF-30SIHP-F0025)
8	Elution	Gradient

Method: Sample was very carefully loaded onto the top of the Automated Flash Chromatography puriFlash® XS520 plus column, Using a syringe or pipette, the sample was carefully put onto the top of the Automated Flash Chromatography puriFlash® XS520 plus column, right above the sand. To rinse any residual sample from the flask, the operation was repeated with a small amount of pure eluent. As a result, the sample formed a thin, even zone on the silica.

After each individual fraction was collected, the separated sample eluents were monitored by the UV detector after passing through the column. In comparison to highly polar molecules, low polarity molecules spent proportionally longer time in mobile phase. Hence, eluents which were eluted were in order of increasing polarity by the help of gradient mobile phase composition for flash chromatography as mention in table 3.3.

Each fraction was collected separately in a test tube and numbered consecutively for further analysis on thin layer chromatography. The individual compounds were collected as fractions and analyzed further for structure elucidation.

TABLE 3.3. Gradient Mobile Phase Composition

Sr. No.	Time (min.)	% A (*n*-Hexane)	% B (Ethyl acetate)
1	00:00	100	00
2	01:40	90	10
3	05:40	80	20
4	10:40	70	30
5	15:40	50	50
6	20:40	30	70
7	25:40	20	80
8	30:40	20	80
9	35:40	0	100

Fraction 37, which was obtained during elution period of 30 minutes and 40 seconds were evaporated to give orange colour compound B.

3.5.2.1 Identification of Compound-B by TLC

Test solution	Compound B
Reference sample	Bakuchiol (Natural Remedies)
Stationary phase	Silica gel G60 F_{254}
Mobile phase	*n*-hexane : ethyl acetate (7:3)
Visualization	Under UV light at 254 nm

3.5.2.2 Isolation of Compound C and Compound D from aqueous layer of Extract B

Aqueous layer obtained from Extract B (Benzene extract) was kept in refrigerator for 24 h, resulting into white ppts **(Compound C)** which was purified using flash chromatography.

Preparation of sample: 800 mg of the sample was dissolved in minimum amount of solvent (HPLC grade Methanol).

TABLE 3.4. Conditions for Flash Chromatography of Compound C and D

Sr. No.	Parameters	Conditions
1	Crude weight	0.8 g.
2	Solvent	*n*-Hexane (**A**): Ethyl acetate (**B**)
3	Flow rate	15. mL/min
4	Pressure	22 Bar
5	Mode	Direct injection
6	Wavelength	254 and 366 nm
7	Loaded on	Silica gel column (60-120#) 24g (PF-30SIHP-F0025)
8	Elution	Gradient

TABLE 3.5. Gradient Mobile Phase Composition of Compound C and D

Sr. No.	Time (min)	% A (*n*-Hexane)	%B (Ethyl acetate)
1	00:00	100	00
2	01:40	90	10
3	05:40	80	20
4	10:40	70	30
5	15:40	50	50
6	20:40	30	70
7	25:40	20	80
8	30:40	20	80
9	35:40	0	100

Fraction 11, which was obtained during elution period of 05 minutes and 40 seconds were evaporated to give white amorphous Compound C.

Fractions 15 to 24, which were obtained during elution period of 10 minutes and 40 seconds were evaporated to give white needle crystals Compound D.

3.5.2.3 Identification of Compound-C by TLC

Test solution	Compound C
Stationary phase	Silica gel G60 F_{254}
Mobile phase	Toluene : ethyl acetate : formic acid (5:4:1)
Visualization	Under UV light at 254 nm

3.5.2.4 Identification of Compound-D by TLC

Test solution	Compound D
Reference sample	Psoralen (SIGMA)
Stationary phase	Silica gel G60 F_{254}
Mobile phase	*n*-hexane : ethyl acetate (7:3)
Visualization	Under UV light at 254 nm and 366 nm

3.5.3 Isolation of Compound E from organic layer of Extract B

Conditions for Flash Chromatography for the purification of isolated compound mentioned below:

Preparation of sample: 380 mg of the sample was dissolved in minimum amount of solvent (HPLC grade Methanol).

TABLE 3.6. Conditions for Flash Chromatography of Compound E

Sr. No.	Parameters	Conditions
1	Crude weight	0.38 g.
2	Solvent	*n*-Hexane (**A**): Ethyl acetate(**B**)
3	Flow rate	15. mL/min
4	Pressure	22 Bar
5	Mode	Direct injection
6	Wavelength	254 and 366 nm
7	Loaded on	Silica gel column (60-120#) 24g (PF-30SIHP-F0025)
8	Elution	Gradient

TABLE 3.7. Gradient Mobile Phase Composition Compound E

Sr. no.	Time (min.)	% A (*n*-Hexane)	% B (Ethyl acetate)
1	00:00	100	00
2	05:00	100	00
3	10:00	100	00
4	20:00	95	05
5	25:00	90	10
6	30:00	80	20
7	35:00	75	25
8	40:00	50	50
9	00:00	100	00

Fraction 24, which was obtained during elution period of 16 minutes and 40 seconds were evaporated to give white colourless crystals Compound E.

3.5.3.1 Identification of Compound-E by TLC

Test solution	Compound E
Reference sample	Methyl 4-hydroxy benzoate (SIGMA)
Stationary phase	Silica gel G60 F_{254}
Mobile phase	*n*-hexane : ethyl acetate (7:3)
Visualization	Under UV light at 254 nm

3.5.4 Characterization of isolated pure compound

Physical methods: The physical characteristics including color, odour, physical stability and state of product.

Melting point: The melting point of isolated compound was recorded and matched with the data given in the literature.

UV-VIS Spectroscopy: The isolated compound was prepared at concentration of 40µg/mL by dissolving in HPLC grade methanol and scanning of the compound was performed at the wavelength range 200-400 nm and compared with reference standard.

Infrared spectroscopy: IR spectroscopy is useful in the identification of functional groups present in a compound. This technique is based on the absorption of electromagnetic radiation at wavelength ranging between 4000 and 400 cm-1. At this range of wavelength, specific functional groups give characteristic vibration, bending and stretching vibrations at characteristic wavelengths. This is recorded in a spectrum by the IR instrument and gives basic information on the structure.

Mass spectroscopy: A chemical is ionised at high energy at a certain voltage in mass spectroscopy. In a magnetic or electric field, the resulting ions are sorted based on their mass to charge ratio (m/z). A plot of the measured m/z ratios against their relative abundance is recorded in the spectra of the separated ions. The parent compound's

molecular weight and fragmentation patterns are revealed by analysing the mass spectrum. The fragmentation patterns are combined to form the compound's molecular skeleton.

3.6 Estimation of Bavachin, Bakuchiol and Psoralen in *P. corylifolia* by HPTLC method[211]:

3.6.1 Preparation of test solutions

20 mg of the methanoilc extract of *P. corylifolia* was dissolved in methanol in 10 mL volumetric flask separately and volumes were adjusted to 10 mL with methanol to get 2 mg/mL concentrations of sample.

3.6.2 Preparation of standard solution

2 mg standard bavachin, bakuchiol and psoralen was dissolved in 2 mL methanol to yield 1000µg/mL concentration of solution.

3.6.3 Instrument

Camag Linomat V (Semi-automatic spotting device).
Camag 100µl HPTLC syringe.
Camag twin trough chambers (20 x 10cm)
Camag TLC scanner 4
Camag Vision-CATS software integration software
Camag Reprostar -3

3.6.4 Materials

Stationary phase	:	Pre-coatedTLCplateofsilicagelG60F_{254}
Mobile phase	:	Toluene: Ether (1:1 saturated with 10% glacial acetic acid)
Reference Standard	:	Bavachin, Bakuciol, Psoralen
Test Samples	:	Methanolic extract
Spotting volume	:	For calibration curve1 µl,3 µl, 5 µl, 7µl, 9 µl and 11µl For test solution15 µland20 µl
Amount/Band	:	1000-11000ng (For calibration curve)
Separation technique	:	Ascending
Developing Chamber	:	Twin trough chambers (20x10cm)
Chamber saturation time	:	30 min
Temperature	:	25ºC
Migration Distance	:	8cm

Spotting Parameters:

Start position	:	8 mm from bottom edge
Band width	:	6 mm
Space between two bands	:	9 mm
Spraying rate	:	6 /sec

Densitometric Scanning:

Mode	:	U.Vlight
Wavelength	:	254 nm
Lampused	:	Deuterium
Slit dimension	:	5 x0.45 mm
Detection	:	Blue colored spot

3.6.5 Calibration Curve of bavachin, bakuchiol and psoralen:

Graded concentrations of standard solution (1 mg/mL) in 1, 3, 5, 7, 9 and 11 µl volume were applied on a pre-coated TLC silica gel 60 F_{254} plate (E. Merck) using Camag Linomat V semi-automatic spotter. The plate was developed in a mobile phase Toluene: Ether (1:1 saturated with 10% glacial acetic acid) and scanned at 254 nm. Data of peak area of each standard spot was recorded. The calibration curve was obtained by plotting area vs. concentration of each peak corresponding to the respective spot.

3.6.6 Quantification of bavachin, bakuchiol, and psoralen:

Test solution of 20 µL (2 $mgmL^{-1}$) was spotted along with 1, 3, 5, 7, 9, and 11 µL of standard bavachin, bakuchiol, and psoralen solutions on the HPTLC plate. The peak areas were noted and quantification of bavachin, bakuchiol, and psoralen were performed using linear regression equations of the respective compounds.

3.6.7 Validation of HPTLC method

Linearity: The capacity of an analytical method to elicit test results that are directly or mathematically proportionate to the concentration of analyte in sample throughout a specific range is known as linearity. Linearity is measured by the linear regression analysis' correlation coefficient. The interval between the upper and lower level of analyte measured with precision and accuracy using the method is known as the analytical method's range.

The linear response was determined by analyzing calibration curve of bavachin, bakuchiol and psoralen in concentration range **1000 - 11000 ng/spot** of linear regression analysis.

Precision: The degree of reproducibility or repeatability of an analytical method is measured by precision. The standard deviation (SD) or relative standard deviation (RSD) is used to express it (RSD). It indicates random error, with the findings reported as RSD or co-efficient of variation.

Repeatability (Precision or Replication): It is the precision involved in analyzing the same sample under same condition, same analyte, same apparatus, short interval of time and identical reagents.

Repeatability of measurement of peak area: (RSD<1%, n=7)

The 5 **µl** of working standard solution was spotted on pre-coated TLC plate. The plate was developed, dried, derivatized and analyzed as described previously. The peak area and height of spot was measured 7 times without changing the position of plate. %RSD was calculated.

Repeatability of sample application: (RSD< 2%, n=7)

The 5 **µl** working **standard** solution was spotted on pre-coated TLC plate seven times. The plate was developed, dried, derivatized and analyzed. The areas of seven spots were measured and % RSD was calculated.

Reproducibility: Variations of result within same day and amongst days are called as reproducibility. It includes following parameters.

Intra-Day Reproducibility (n=3): A variation in results within the same day is called intraday variation. It was determined by repeating calibration curve 3 times on same day at 3 different concentrations.

Inter-Day Reproducibility (n=3): A variation in results across days is called interday variation. It was determined by repeating calibration curve daily for 3 different days at 3 different concentrations.

***Limit of detection (LOD)*:** Under specified conditions, it is the lowest concentration of analyte in a sample that can be identified but not necessarily quantified. LOD proves that the concentration of an analyte is above or below a specific threshold. The regression analysis feature in Excel is used to determine this.

Limit of quantification (LOQ): It refers to an analytical method's ability to detect analyte quantitatively in the presence of other substances. A signal to noise ratio of 10:1 defines it. Different amounts of standard were employed, and the minimum quantifiable limit for acceptable precision and accuracy was identified. The regression analysis feature in Excel is used to determine this.

Accuracy: The accuracy of an analytical method is the degree to which the actual (true) value and the analytical value agree; it is determined by repeating the test process. It is calculated by calculating standard recovery by addition at three different standard concentration levels.

Specificity: The capacity of an analytical method to measure the analyte properly in the presence of other components in the sample, such as synthetic precursors, excipients, degradants, or matrix components, is known as specificity. The purity of spectra was measured at three levels: beginning, middle, and end. The correlation between them was taken into account when determining peak purity. It was determined by spotting a working standard solution on a pre-coated TLC plate, developing, drying, and analysing the plate.

Robustness: The method's robustness was determined by deliberately changing chromatographic parameters such mobile phase composition to see how they affected retention time and quantitative analysis.

Ruggedness: Within laboratories, variables such as various analysts, different days, and different equipment validate the precision.

3.7 Estimation of Berberine, Berbamine, Palmatine, Jatrorrhizine & Magnoflorine in *B. aristata* from HPTLC method:

3.7.1 Preparation of test solutions

Accurately weighed root powder (10.0 g) of sample of *B. aristata* was extracted exhaustively with methanol (3 × 40 mL) under reflux for 2 h/cycle. Methanolic extract was concentrated to dryness under vacuum to yield 0.87 g of extract as dark-brown semisolid. 20 mg of extract was dissolved in 10 mL methanol and an aliquot of 25 µL of methanolic solution was applied to a TLC plate.

3.7.2 Preparation of standard solution

2 mg standard berberine and berbamine was dissolved in 2 mL methanol to yield 1000µg/mL concentration of solution. For palmatine 1 mg standard was dissolved in 5 mL methanol to yield 200 µg/mL concentration of solution. Standard solution of magnoflorine and jatrorrhzine were prepared by dissolving 1 mg standard in 10 mL

methanol and 1.4 mg standard in 10 mL methanol to yield 100 and 140 µg/mL concentration of solution, respectively.

3.7.3 Calibration curve of berberine, berbamine, palmatine, magnoflorine and jatrorrhizine

Calibration plots of peak area against concentration were linear in the range 1000–6000 ng/spot for berberine and berbamine, 200–700 ng/spot for palmatine, while 500–2000 ng/ spot for magnoflorine and 300–1800 ng/spot for jatrorrhizine. 1–6 µL solutions for berberine and berbamine, 1–3.5 µL solutions were applied for palmatine, 5–20 µL solutions for magnoflorine and 3–18 µL solutions jatrorrhizine were applied on a pre-coated TLC silica gel 60 F_{254} plate (E. Merck) using Camag Linomat V semi-automatic spotter. The plate was developed in a mobile phase ethyl acetate–formic acid–glacial acetic acid–water (100:11:11:26, *V/V*) and scanned at 254 nm. Data of peak area of each standard spot was recorded. The calibration curve was obtained by plotting area vs. concentration of each peak corresponding to the respective spot.

3.7.4 Quantification of berberine, berbamine, palmatine, magnoflorine and jatrorrhizine

Test solution of 25 µL (2 mg mL^{-1}) was spotted along with 1, 2, 3, 4, 5, 6 µL of standard berberine and berbamine; 1,1.5, 2, 2.5, 3, 3.5 µL solutions were applied for standard palmatine solutions, 5, 8, 11, 14, 17, 20 µL solutions for magnoflorine and 3, 6, 9, 12, 15, 18 µL solutions for jatrorrhizine on the HPTLC plate. The peak areas were noted and quantifications of berberine, berbamine, palmatine, magnoflorine and jatrorrhizine were performed using linear regression equations of the respective compounds.

3.7.5 Validation of HPTLC method

Linearity: The linearity of an analytical method is its ability to elicit test results that are either directly or by mathematically proportional to the concentration of analyte in sample across the given range. Linearity is expressed in terms of correlation coefficient of the linear regression analysis. The range of the analytical method is the interval

between upper and lower level of analyte including levels that are determined with precision and accuracy using the method.

The linear response was determined by analyzing calibration curve of bavachin, bakuchion and psoralen in concentration range **1000 - 6000 ng/spot for berberine and berbamine, 200 – 700 ng/spot, 300 – 1800 ng/spot & 500 – 2000 ng/spot for** palmatine, jatrorrhizine & magnoflorine, respectively of linear regression analysis.

Precision: The degree of reproducibility or repeatability of an analytical method is measured by precision. The standard deviation (SD) or relative standard deviation (RSD) is used to express it (RSD). It indicates random error, with the findings reported as RSD or co-efficient of variation.

Repeatability (Precision or Replication): It is the accuracy required to analyse the same sample under identical conditions, with the same analyte, same instrument, small time intervals, and identical reagents.

Repeatability of measurement of peak area: (RSD<1%, n=7)

The 3 **µl for berberine and berbamine, 2 µl for palmatine, 9 µl for jatrorrhizine and 11 µl for magnoflorine** of working standard solution was spotted on pre-coated TLC plate. The plate was developed, dried, derivatized and analyzed as described previously. The peak area and height of spot was measured 7 times without changing the position of plate. %RSD was calculated.

Repeatability of sample application: (RSD< 2%, n=7)

The 3 **µl for berberine and berbamine, 2 µl for palmatine, 9 µl for jatrorrhizine and 11 µl for magnoflorine** working **standard** solution was spotted on pre-coated TLC plate seven times. The plate was developed, dried, derivatized and analyzed. The areas of seven spots were measured and % RSD was calculated.

Reproducibility: Variations of result within same day and amongst days are called as reproducibility. It includes following parameters.

Intra-Day Reproducibility (n=3): A variation in results within the same day is called intraday variation. It was determined by repeating calibration curve 3 times on same day at 3 different concentrations.

Inter-Day Reproducibility (n=3): A variation in results across days is called interday variation. It was determined by repeating calibration curve daily for 3 different days at 3 different concentrations.

Limit of detection (LOD): Under specified conditions, it is the lowest concentration of an analyte in a sample that can be identified but not necessarily quantified. LOD proves that the concentration of an analyte is above or below a specific threshold. The regression analysis feature in Excel is used to determine this.

***Limit of quantification (LOQ*)**: It refers to an analytical method's ability to detect analyte quantitatively in the presence of other substances. A signal to noise ratio of 10:1 defines it. Different amounts of standard were employed, and the minimum quantifiable limit for acceptable precision and accuracy was identified. The regression analysis feature in Excel is used to determine this.

Accuracy: The accuracy of an analytical method is the degree to which the actual (true) value and the analytical value agree; it is determined by repeating the test process. It is determined by calculating standard recovery by addition at three different standard concentration levels.

Specificity: The capacity of an analytical method to measure the analyte properly in the presence of other components in the sample, such as synthetic precursors, excipients, degradants, or matrix components, is known as specificity. The purity of spectra was measured at three levels: beginning, middle, and end. The correlation between them was taken into account when determining peak purity. It was determined by spotting a working standard solution on a pre-coated TLC plate, developing, drying, and analysing the plate.

Robustness: Robustness is a measure of a technique's ability to remain unaffected by modest but deliberate changes in the method conditions, and it is an indicator of the

method's reliability.

Ruggedness: It is a measure of the reproducibility of test results under normal, expected operating conditions from instrument to instrument and from analyst to analyst.

3.8 Quantification of Daidzein, Psoralen, Neobavaisoflavone, Bavachin, Psoralidin, Bavachinin and Bakuchiol in *Psoralea corylifolia* by UHPLC–PDA with ESI– MS-MS METHOD

3.8.1 Preparation of test solution

50.0 mg of methanolic extract was dissolved 25.0 mL volumetric flask by adding 15.0 mL methanol and sonicated for 10 min, and volume was made up to 10 mL in each using methanol. Each sample were filtered through the 0.22 µm filter and used for injection.

3.8.2 Preparation of standard solutions

The stock solution for compounds (**1**-**8**) (500 μgmL^{-1}) was prepared by weighing accurately 5.0 mg of each reference standard and adding 5.0 mL of methanol in 10 ml volumetric flask. The solutions were sonicated for 10.0 min and filled volume up to the mark with methanol and were stored at 4°C. The working standard solutions were prepared on daily basis using stock solution with methanol as a diluent; the final concentration for each analyte was kept at 500 μgmL^{-1}.

3.8.3 Chromatographic system

Work Station	**:**	Shimadzu Nexera X2 (Shimadzu Tech., Kyoto, Japan)
auto-sampler	**:**	**SIL-40AC XS**
Detector	**:**	Diode-array detector (SPD-M40IND PDA), coupled with LCMS-8045 (Shimadzu Tech., Kyoto, Japan), and triple-quadrupole mass detector supplied with a thermally aided ESI source.

Integration of data : The Lab Solution software (Version 6.80)

Syringe : Hamilton Shimadzu syringe 25µl

3.8.4 Materials

Stationary phase : A Shim-Pack Velox C18column (2.1 × 100 mm i.d. Shimadzu, USA)

Reagents (AR grade) : Acetic acid, Water and Acetonitrile

Reference standard : Gallic acid

Test samples : Methanolic extract

3.8.5 Chromatographic conditions

Stationary phase : A Shim-Pack Velox C18column (2.1 × 100 mm i.d., ×1.8 µm, Shimadzu, USA)

Mobile phase : Gradient elution mode

Flow rate : 0.80 mL/min

Detection : 245 nm

Injection volume : 1 µl

Retention time : 5.883, 6.676, 10.166, 10.846, 12.555, 16.130 and 19.815 min, respectively

3.8.6 Analytical column:

A Shim-Pack Velox C18column (2.1 × 100 mm i.d., ×1.8 µm, Shimadzu, USA)

3.8.7 Mobile phase

The optimized mobile phase used in the analysis was consisted of gradient elution mode of 0.01 min, 10 % solvent B; 0.01-2.00 min, 10 % solvent B; 2.00-4.50 min, 30 % solvent B; 4.50-8.50 min, 40 % solvent B; 8.50-13.50 min, 45 % solvent B; 13.50-15.50, 55

%solvent B; 15.50-18.00, 70 % solvent B; 18.00-19.00, 80 % solvent B; 19.00-20.00, 80 % solvent B and returned to initial 10 % and held for 3 min.

3.8.8 Detection:

The mode of detection was absorption in the U.V. region and wavelength selected for detection was 245 nm.

3.8.9 Mass spectrometry conditions

Mass confirmation was performed using MS/MS settings in both positive and negative ion modes, with a precursor scan confirming the product ion. The ESI interface was held at 350°C. The heat block and desolvation line were kept at 300 and 400 degrees Celsius, respectively. The flow rates of nebulizing gas, heating gas, and drying gas were set at 3.0 L min-1, 10.00 L min-1, and 10.00 L min-1, respectively, to improve mass spectrometer sensitivity. The bioactives were fragmented at CE 20 eV, and the analysis was carried out in the Q1 scan mode, with a scan range of 50–1000 m/z and a scan speed of 5000 u/sec in both positive and negative modes.

3.8.10 Calibration curve of daidzein, psoralen, neobavaisoflavone, bavachin, psoralidin, bavachinin and bakuchiol

The linearity range for calibration curve ranged between 0.078-5µg/mLfor daidzein, 1.250-80 µg/mLfor psoralen, 0.469-30 µg/mLfor neobavaisoflavone, 0.469-30 µg $m^{-1}$for bavachin, 0.313-20 µg/mLfor psoralidin, 0.625-40 µg/mL for bavachinin, and 6.250-400µg/mLforbakuchiol. Data of peak area of each standard were recorded. The calibration curve was obtained by plotting area vs. concentration of each peak.

3.8.11 Validation of UHPLC Method[211]:

Linearity

The capacity of an analytical method to elicit test results that are proportionate to the concentration of analyte in sample within a certain range, either directly or through a well-defined mathematical transformation. Linearity is measured by the linear regression

analysis' correlation coefficient. The analytical method's range is the distance between the top and lower analyte values, including those that have been shown to be determined with precision and accuracy using the method.

The linear response was determined by analyzing calibration curve in the concentration range 0.078-5µg/mL for daidzein, 1.250-80 µg/mL for psoralen, 0.469-30 µg/mL for neobavaisoflavone, 0.469-30 µg/mL for bavachin, 0.313-20 µg/mLfor psoralidin, 0.625-40 µg/mL for bavachinin and 6.250-400µg/mL for bakuchiol application of linear regression analysis.

Precision

Precision is the measure of either the degree of reproducibility or of repeatability of the analytical method under normal operating conditions. It provides an indication of random error; results should be expressed as relative standard deviation or co-efficient of variation.

Repeatability (Precision or Replication)

It is the precision involved in analyzing the same sample under same condition, same analyte, same apparatus, short interval of time and identical reagents.

Reproducibility

Variations of result within same day and amongst days are called as reproducibility. It includes following parameter.

a) Intra-Day Reproducibility (n=3)

A variation in results within same day is called intraday variation. It was determined by repeating calibration curve 3 times on same day at 3 different concentrations.

b) Inter-Day Reproducibility (n=3):

A variation in results amongst day is called interday variation. It was determined by repeating calibration curve daily for 3 different days at 3 different concentrations.

Limit of detection (LOD)

Under the provided analytical conditions, it is the lowest concentration of an analyte in a sample that can be detected but not necessarily measured. LOD is frequently used to prove that an analyte concentration is above or below a predetermined threshold. According to ICH guidelines it can also be calculated by the following equation.

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

σ = Standard deviation of the Y intercept

S = Slope of the calibration curve equation

Limit of quantification (LOQ)

It refers to an analytical method's capacity to quantitatively detect analyte in the presence of other compounds. A signal to noise ratio of 10:1 defines it. The minimal measurable limit for acceptable precision and accuracy was found using various amounts of standard solution. The following equation can also be used to compute it according to ICH recommendations.

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

σ = Standard deviation of the Y intercept

S = Slope of the calibration curve equation

Accuracy

The accuracy of an analytical method is defined as the degree of agreement or closeness between the actual (true) value and the analytical value achieved by repeating the test method. It was calculated by using the standard addition method to calculate the standard's recovery at three different concentration levels.

Specificity

The capacity of an analytical method to reliably detect the analyte in the presence of other

components such as synthetic precursors, excipients, degradants, or matrix components that may be present in the sample is known as specificity. The purity of spectra was tested on three levels: beginning, middle, and end. The correlation between them was taken into account when determining peak purity. Working standard solution was spotted on a pre-coated TLC plate, which was then developed, dried, derivatized, and analysed.

3.9 Quantification of magnoflorine, berbamine, columbamine, jatrorrhizine, palmatine and berberine in *Berberis aristata* by UHPLC–PDA with ESI–MS- MS Method.

3.9.1 Preparation of test solution

10.0 mg of these extracts were dissolved and sonicated into 5.0 mL of methanol in 10.0 mL volumetric flasks for 10.0 min separately, and volume was made up to 10 mL in each using methanol. The working solution of samples 1,000 mg mL/1 were prepared, filtered through the 0.22 mm filter, and used for injection.

3.9.2 Preparation of standard solution

The stock solution for compounds (1–6) (500mg mL/1) was prepared by weighing accurately 5.0mg of each reference standard in 10mL volumetric flask and adding 5.0mL of methanol. Then sonicated for 10.0min and filled volume up to the mark with methanol and were stored at 4 8C. The working standard solution was prepared daily for the stock solution with diluent; the final concentration was 500 mg mL/1.

3.9.3 Chromatographic system

Work Station	**:**	Shimadzu Nexera X2 (Shimadzu Tech., Kyoto, Japan)
Sampling	**:**	**auto-sampler (SIL-30AC)**
Detector	**:**	a diode-arraydetector (SPD-M20A), coupled with LCMS-8045 (Shimadzu Tech., Kyoto, Japan), and triple-quadrupole

mass detectorsupplied with a thermally aided ESI source.

Integration of data : The Lab Solution software (Version 6.80)

Syringe : Hamilton Shimadzu syringe 25µl

3.9.4 Materials

Stationary phase : Phenomenex Luna 5 µm C8 (2) 100 Å column (150 X 4.6mm X 5 µm)

Reagents (AR grade) : trifluoroacetic acid, Water and Acetonitrile

Reference standard : magnoflorine, berbamine, columbamine, jatrorrhizine, palmatineand berberine

Test samples : Methanolic extract

3.9.5 Chromatographic conditions

Stationary phase : Phenomenex Luna 5 µm C8 (2) 100 Å column (150 X 4.6mm X5 µm)

Mobile phase : 0.1% trifluoroacetic acid in water (A), 0.1% trifluoroacetic acid inacetonitrile (B) with a gradient elution mode

Flow rate : 0.90 mL/min

Detection : 280 nm

Injection volume : 10 µl

Retention time : 4.395, 6.837, 8.320, 8.683, 10.400, and 11.157 min respectively

3.9.6 Analytical column:

Phenomenex Luna 5 µm C8 (2) 100 Å column (150 X 4.6mm X 5 µm)

3.9.7 Mobile phase

The mobile phase used was 0.1% trifluoroacetic acid in water (A), 0.1% trifluoroacetic acid in acetonitrile (B) with a gradient elution mode of 0.01 min, 20% B; 0.01–17.00 min, 20–45% B; 17.00– 17.50min, 45–40% B; 17.50–18.00 min, 40-20% B; 18.00– 20.00min, 20% B. The analysis was done by keeping injection volume and the flow rate 10 mL and 0.90 mL/min respectively.

3.9.8 Detection:

The PDA detector was set to 190–600nm and detection was carried out at 280 nm.

3.9.9 Mass spectrometry conditions

Mass analysis was conducted in scan and MS/MS settings in positive as well as negative ion modes and corroborating the product ion by a precursor scan. The interface for ESI was kept at 350 8C. The heat block and desolvation line temperatures were maintained to 300 and 400 8C, respectively. The other parameters that may enhance sensitivity of the mass spectrometer like flow rates of nebulizing gas, heating gas and the drying gas were set at 3.0 L min/1, 10.00 L min/1 and 10.00 L min/1. The fragmentation of the aforementioned alkaloids was achieved at CE 20 eV and the analysis was performed in the Q1 scan mode, keeping the scan range 50–1,000 m/z and scan speed 5,000 u/sec in a positive and negative mode.

3.9.10 Calibration curve of magnoflorine, berbamine, columbamine, jatrorrhizine, palmatine and berberine

The linearity range for calibration curve ranged between 0.312 and 20 mg/ mL for magnoflorine, 4.68–300 mg mL/1 for berbamine, 0.16– 10 mg/ mL for columbamine, 0.469–30 mg/ mL for jatrorrhizine, 2.19–140 mg/mL for palmatine and 4.68– 300 mg /mL for berberine.. Data of peak area of each standard were recorded. The calibration curve was obtained by plotting area vs. concentration of each peak.

3.9.11 Validation of UHPLC Method[211]

As described in section 3.6.11.

3.10 Pharmacological Screening of Extracts[212, 213]:

MTT assay using THP-1 Cell line:

CELL LINE DESCRIPTION

THP-1 is a human monocytic **cell line** derived from an acute monocytic leukemia patient. It is used to test leukemia **cell lines** in immunocytochemical analysis of protein-protein interactions, and immunohistochemistry. They express complement (C3), Fc receptors and are phagocytic (for both latex beads and sensitized erythrocytes and others) and lack surface and cytoplasmic immunoglobulin. They are weakly responsive to TLR agonists in their undifferentiated state but become more responsive after differentiation. Monocytic differentiation can be induced with the phorbol ester 12-O-tetradecanoylphorbol-13-acetate (TPA)/ phorbol 12-myristate 12-acetate [Sigma P 8139]. The doubling time is approximately 26 hrs.

3.10.1 Method

Receipt of Frozen Cells and Starting Cell Culture

1) Frozen cells were kept immediately in liquid nitrogen freezer storage until ready to culture.

2) At the onset of cell culture, quickly ampoule was thawed in a 37ºC water bath.

3) As soon as ice crystals disappeared, outside surface of the ampoule was swabbed with 70% ethanol, content of the ampoule was dispensed into T25 flask with 2 mL of warm growth media.

4) Cells were allowed to recover overnight in 37ºC, 5% CO2 humidified incubator.

3.10.2 Sub-culture

1) Cells were transfered to a flask containing warmed media, placed the flask in the incubator at an angle or upright in T25.

2) Cells were propagated until to achieve higher density of growth

3) It was collected by centrifugation at 2000 rpm to 10 min.

4) Cells were resuspended in fresh media and PMA at 100uM conc. were added to induce differentiation to macrophages.

5) After count, cells were split. Approx. 1*104 to 2*104 cells/ well are seeded in 96 well plate (for MTT).

6) Inoculants were further cultured at 37ºC, 5% CO2 humidified incubator.

7) Media was changed every alternative days till the cells reached 80% confluency.

8) Test compound was exposed to the cells for 24 hours duration in different concentration as per experimental design.

Dose Selection

The dose concentration of 10 g/mL (G2) to 1000 g/mL (G11) of *test solutions* wasselected.

3.10.3 Experimental Design

The selected groups were assigned to control and different treatment groups as shown below for MTT assay:

TABLE 3.8 Details of Experimental Design

Group No.	Group	Dose Conc. g/mL	No. of replicates
G1	Control	0	3

G2	Dose	10	3
G3	Dose	20	3
G4	Dose	40	3
G5	Dose	60	3
G6	Dose	80	3
G7	Dose	100	3
G8	Dose	200	3
G9	Dose	400	3
G10	Dose	800	3
G11	Dose	1000	3

Exposure of Test Compound Cells were culture in 96 well plate and allowed to reach 80% confluency. Media was replaced with fresh media and test compounds in various dose concentration, and the time was noted as T0. Plate was again incubated in incubator for further 24 hours. After completion of the exposure duration, cells were subjected to parametric analysis.

3.10.4 MTT assay

1) Media containing test compounds was removed from the culture wells and fresh 200µl 1XPBS buffer was added.

2) Two washes was given to the culture to remove traces of test compound from the culture.

3) Further media with (200µl) was added to the culture.

4) 20 µl of MTT (5mg/mL) solution prepared in PBS was added in the culture.

5) Culture was incubated at 37ºC for 3 hours, so as to allow the reaction to take place.

6) After the incubation, media with unbound MTT was aspired out and two washes of 1XPBS was giv0en to cells to remove traces of unbound MTT compound.

7) After washes 100µl of DMSO was added to cells, so the water insoluble compounds can be dissolved in DMSO and reading was taken at 570 nm.

MTT assay were performed on methanolic extract, hexane extract and standard psoralen of *P. corylifolia* while methanolic extract and standard berberine of *B. aristata* samples were taken for study. Each group was exposed to THP-1 cell line in 10 different concentrations ranging from 10 μg/mL to 1000 μg/mL for duration of 24 hours.

3.11 Evaluation of Anti-psoriatic activity in experimental animals[214, 215]

3.11.1 Imiquimod induced psoriasis –Like Dermatitis Rat model

Materials:

Preparation of test extract

Methanolic extract, hexane extract and standard psoralen of *P. corylifolia* and methanolic extract and standard berberine of *B. aristata* were used for the study. Synergestic effects were also observed by combining methanolic extract of both the plants as well as both the bioactives. The extract and bioactives were mixed with vaseline and applied topically in specific dose, expressed as mg/cm^2.

Preparation of standard drug: The dorsal skin of the rat was shaved and a commercially available 5% IMQ cream (Aldara, 3M Pharmaceuticals) was applied

Animals:

6–8 weeks old Albino wistar rats were housed under specific pathogen-free conditions and provided with food and water ad libitum. Prior permission of experiments on Animals had taken from the Institutional Animal Ethics Committee. All the experiments on animal have been conducted as per the guidelines of Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Ministry of Social Justice and Empowerment, Government of India.

TABLE 3.9. Details of different groups

Group		**Receives**
Group I	Normal control (n=6)	Vaseline on shaved dorsal skin surface and right ear pinna.
Group II	Disease Control (n=6)	5% IMQ cream on shaved back 20mg/cm^2 and right ear pinna topically
Group III	Treatment Group-I (n=6)	IMQ plus Clobetasol at 40 mg/cm^2 twice daily topically
Group IV	Treatment Group- II (n=6)	IMQ plus methanolic extract of *P. corylifolia* (MEP) 100 mg/cm^2 twice daily topically.
Group V	Treatment Group- V (n=6)	IMQ plus *methanolic extract of B. aristata* (MEB) 100 mg/cm^2 twice daily topically.
Group VI	Treatment Group-IV (n=6)	IMQ plus Standard Psoralen (SP) 40 mg/cm^2 twice daily topically
Group VII	Treatment Group- V (n=6)	IMQ plus Standard Berberine (SB) 40 mg/cm^2 twice daily topically.
Group VIII	Treatment Group- VI (n=6)	IMQ plus Combination of MEP and MEB 100 mg/cm^2 twice daily topically.
Group IX	Treatment Group- VII (n=6)	IMQ plus Combination of SP and SB 40 mg/cm^2 twice daily topically
Group X	Treatment Group- VIII (n=6)	IMQ plus Hexane extract of *P. corylifolia* 100 mg/cm^2 twice daily topically.

Total rats: 60

Procedure:

Rats were randomly divided into 10 groups (N=6) on day 1 and continuing for 15 consecutive days, rat in all groups except for the normal group received a daily topical dose of 62.50mg of the IMQ cream on the shaved area of their backs. This translates into a daily dose of 3.125mg of the active ingredient. On the right ear, 5% IMQ cream was applied at a dose of 20mg/cm^2. Rat has been received the treatment twice a day for 10 days topically.

Sample Collection. All the rat in groups I to X were sacrificed at the end of the experiment by Co2 asphaxia. Rat in groups III to XVII will receive IMQ treatment for 15 days and either plant extracts or standard compounds or clobetasol treatment from day 7 through day 16. They were then sacrificed on day 17 at the laboratory. Central dorsal skin tissue (approximately 1 cm^2) and right ear pinna from all the groups were excised for histological studies and cytokine production analysis. Blood samples from all the groups were collected in dry vials for cytokine production analysis

3.11.2 Parameters under Study

3.11.2.1 Measurement of Ear Thickness:

At 0, 2, 4, 6, 8, and 10 days, the ear thickness was measured with a thickness gauge. The amount of inflammation was determined by the rise in ear thickness.

3.11.2.2 Scoring Severity of Skin Inflammation:

An objective scoring method based on the clinical Psoriasis Area and Severity Index was created to quantify the severity of ear skin inflammation (PASI). Erythema, scaling, and thickening were all graded on a scale of 0 to 4: 0, none; 1, faint; 2, moderate; 3, marked; 4, extremely marked A grading system with red taints was used to determine the degree of erythema.

3.11.2.3 Histology:

Ear samples and dorsal skin from each rat in each group were fixed in 4 percent paraformaldehyde and embedded in paraffin at the end of the experiment. Hematoxylin and eosin were used to stain sections (thickness = 4 mm) (H&E).

3.11.2.4 Changes of Spleen in Rat:

Before being weighed, each rat's spleen was extracted and a photograph was taken. The weight of the spleen in relation to the bodyweight was used to assess splenomegaly.

3.11.2.5 Assay of Cytokines Production:

To evaluate cytokine levels in serum, rat blood was taken using the cardiac puncture method 24 hours after the final dose and held at 70°C until analysis. The central dorsal skins of the rats were excised and kept at 80°C to evaluate cytokine levels in skin tissue. The skins were homogenised at 4 degrees Celsius in tissue protein lysis buffer, and the supernatants were kept at 80 degrees Celsius until analysis. ELISA was used to assess the levels of IL-17A, IL-23, and TNF- in rat serum and skin tissue.

3.11.2.5.1 Measurement of Cytokine IL-17A[216]:

The Rat IL-17A ELISA Kit (Invitrogen) is an enzyme-linked immunosorbent assay for the quantitative detection of rat IL-17A.

3.11.2.5.1.1 Principle:

An anti-rat IL-17A coating antibody is adsorbed onto microwells. Rat IL-17A present in the sample or standard binds to antibodies adsorbed to the microwells. A biotin-conjugated anti-rat IL-17A antibody is added and binds to rat IL-17A captured by the first antibody. Following incubation unbound biotin-conjugated anti-rat IL-17A antibody is removed during a wash step. Streptavidin-HRP is added and binds to the biotin-conjugated anti-rat IL-17A antibody. Following incubation unbound Streptavidin-HRP is removed during a wash step, and substrate solution reactive with HRP is added to the

wells. A colored product is formed in proportion to the amount of rat IL-17A present in the sample or standard. The reaction is terminated by addition of acid and absorbance is measured at 450 nm. A standard curve is prepared 7 rat IL-17A standard dilutions and rat IL-17A sample concentration determined.

3.11.2.5.1.2 Reagents provided:

Reagents for rat IL-17A ELISA BMS635 (96 tests)

1 aluminum pouch with a Microwell Plate (12 strips of 8 wells each) coated with monoclonal antibody to rat IL-17A

1 vial (70 µL) Biotin-Conjugate anti-rat IL-17A monoclonal antibody

1 vial (150 µL) Streptavidin-HRP

2 vials rat IL-17A Standard lyophilized, 200 pg/mL upon reconstitution

1 bottle (12 mL) Sample Diluent

1 vial (5 mL) Assay Buffer Concentrate 20x (PBS with 1% Tween™ 20, 10% BSA)

1 bottle (50 mL) Wash Buffer Concentrate 20x (PBS with 1% Tween™ 20)

1 vial (15 mL) Substrate Solution (tetramethyl-benzidine)

1 vial (15 mL) Stop Solution (1M Phosphoric acid)

3 adhesive Films

3.11.2.5.1.3 Preparation of solutions:

Buffer Concentrates were brought to room temperature and diluted before starting the test procedure.

Wash buffer (1x)

1. Entire contents (50 mL) of the Wash Buffer Concentrate (20x) were poured into a clean 1000 mL graduated cylinder. Diluted to final volume of 1000 mL with glass-distilled water.

2. Transferred to a clean wash bottle and stored at 2°C to 25°C.

Assay buffer (1x)

1. The entire contents (5 mL) of the Assay Buffer Concentrate (20x) were poured into a clean 100 mL graduated cylinder. Diluted to final volume of 100 mL with distilled water.

2. Stored at 2°C to 8°C.

TABLE 3.10 Assay buffer concentration

Number of Strips	Wash Buffer Concentrate (20x) (mL)	Distilled Water (mL)
1 – 6	0.03	2.97
1 – 12	0.06	5.94

1:200 dilution of the concentrated Streptavidin-HRP solution with Assay Buffer (1x) was made in a clean plastic tube according to the following table:

TABLE 3.11 Streptavidin Solution

Number of Strips	Wash Buffer Concentrate (20x) (mL)	Distilled Water (mL)
1 – 6	0.03	5.97
1 – 12	0.06	11.94

Preparation of Rat IL-17A standard

1. Reconstituted rat IL-17A standard by addition of distilled water.

2. Mixed gently to ensure complete and homogeneous solubilization (concentration of reconstituted standard = 200 pg/mL).

3. Allowed the standard to reconstitute for 10-30 minutes. Mixed well prior to making dilutions.

Preparation of External standard dilution

1. Labeled 7 tubes, one for each standard point: S1, S2, S3, S4, S5, S6, S7.

2. Then prepared 1:2 serial dilutions for the standard curve as follows: Pipetted 225 µL of Sample Diluent into each tube.

3. Pipetted 225 µL of reconstituted standard (concentration of standard = 200.0 pg/mL) into the first tube, labeled S1, and mixed (concentration of standard 1 = 100.0 pg/mL).

4. Pipetted 225 µL of this dilution into the second tube, labeled S2, and mixed thoroughly before the next transfer.

5. Repeated serial dilutions 5 more times thus creating the points of the standard curve Sample Diluent serves as blank.

3.11.2.5.1.4 Test protocol

1. Determined the number of microwell strips required to test the desired number of samples plus appropriate number of wells needed for running blanks and standards. Each sample, standard, blank and optional control sample were assayed in duplicate.

2. Microwell strips were washed twice with approximately 400 µL Wash Buffer with thorough aspiration of microwell contents between washes. Allowed the Wash Buffer to sit in the wells for about 10 – 15 seconds before aspiration. After the last wash step, wells were emptied and tapped microwell strips on absorbent pad to remove excess Wash

Buffer.

3. Standard dilution on the microwell plate (Alternatively the standard dilution can be prepared in tubes - see "External standard dilution" on page 37.): Added 100 µL of Sample Diluent in duplicate to all standard wells. Pipetted 100 µL of prepared standard (see "Rat IL-17A standard" on page 37 concentration = 200 pg/mL) in duplicate into well A1 and A2. Mixed the contents of wells A1 and A2 by repeated aspiration and ejection (concentration of standard 1, S1 = 100.0 pg/mL) and transferred 100 µL to wells B1 and B2, respectively. This procedure was continued 5 times, creating two rows of rat IL-17A standard dilutions ranging from 100.0 to 1.6 pg/mL.

4. 100 µL of Sample Diluent was added in duplicate to the blank wells.

5. 50 µL of Sample Diluent was added to the sample wells.

6. 50 µL of each sample was added in duplicate to the sample wells.

7. Biotin-Conjugate was prepared

8. 50 µL of Biotin-Conjugate was added to all wells.

9. Covered with an adhesive film and was incubated at room temperature (18°C to 25°C) for 2 hours on a microplate shaker.

10. Streptavidin-HRP was prepared.

11. Adhesive film was removed and wells were emptied. Microwell strips were washed 4 times according to point 2 of the test protocol.

12. 100 µL of diluted Streptavidin-HRP was added to all wells, including the blank wells.

13. Covered with an adhesive film and was incubated at room temperature (18°C to 25°C) for 1 hour on a microplate shaker.

14. Adhesive film was removed and wells were emptied. Microwell strips were washed 4 times according to point 2 of the test protocol.

15. Pipetted 100 µL of TMB Substrate Solution to all wells.

16. The microwell strips were incubated at room temperature (18°C to 25°C) for about 30 minutes. The color development on the plate was monitored and the substrate reaction stopped before positive wells were no longer properly recordable. The substrate reaction was stopped as soon as Standard 1 reached an OD of 0.9 – 0.95.

17. The enzyme reaction was stopped by quickly pipetting 100 µL of Stop Solution into each well. Results were read immediately after the Stop Solution was added.

18. Absorbance of each microwell was recorded on a spectro-photometer using 450 nm as the primary wave length. The absorbance of both samples and the standards were determined.

3.11.2.5.1.5 Calculation of results:

The average absorbance values for each set of duplicate standards and samples were calculated. Duplicates were within 20% of the mean value.

Standard curve was plotted by the mean absorbance for each standard concentration on the ordinate against the rat IL-17A concentration on the abscissa.

The concentration of circulating rat IL-17A for each sample was determined by finding the mean absorbance value on the ordinate and extending a horizontal line to the standard curve. At the point of intersection, extended a vertical line to the abscissa and read the corresponding rat IL-17A concentration.

3.11.2.5.2 Measurement of Cytokine IL-23[217]:

The Rat IL-23 ELISA Kit is a solid-phase sandwich Enzyme-Linked Immunosorbent Assay (ELISA) designed to detect and quantify the level of rat IL-23 in cell culture supernatants, plasma, and serum.

3.11.2.5.2.1 Reagents:

Rat IL-23 Antibody Coated wells, 96-well plate

Rat IL-23 Biotin Conjugate

Rat IL-23 Standard, recombinant rat IL-23

Wash Buffer Concentrate (20X)

Assay Diluent C

Assay Diluent B (5X)

Streptavidin-HRP (400X)

TMB Substrate

Stop Solution

Adhesive Plate Covers

3.11.2.5.2.2 Preparation of solutions:

Preparation of 1X Wash Buffer

1. Wash Buffer Concentrate (20X) was allowed to reach room temperature and mixed to redissolve any precipitated salts.

2. 20 mL of the Wash Buffer Concentrate was diluted into 380 mL of distilled water.

3. Concentrate and 1X Wash Buffer was stored in the refrigerator.

Preparation of biotin conjugate

1. Biotin conjugate was briefly spun down before use.

2. 100 µL of 1X Assay Diluent B was added into the vial to prepare a biotin conjugate concentrate.

3. Pipetted up and down to mix gently.

4. The biotin conjugate concentrate was diluted 80-fold with 1X Assay Diluent B and used in step 2 of ELISA procedure.

Pre-dilute samples

Assay Diluent C was used for dilution of serum, plasma and cell culture supernatant samples.

Serum and plasma were diluted to 2-fold.

400 µL of Assay Diluent C was added into vial to prepare a 200 ng/mL standard. The powder was dissolved thoroughly by a gentle mix. 270 µL of Assay Diluent C was pipette into each tube. The 200 ng/mL standard solution was used to produce a dilution series. Each tube was mixed thoroughly before the next transfer.

Preparation of 1X Streptavidin-HRP solution

1. Mixed gently before use, to remove precipitates formed during storage.

2. Diluted Streptavidin-HRP 400-fold with 1X Assay Diluent B.

For the standard curve

1. 100 µL of standards was added to the appropriate wells (see Dilute standards). For samples, 100 µL of diluted samples was added to the wells.

2. Wells were covered and incubated for 2.5 hours at room temperature or over night at 4°C with gentle shaking.

3. Discarded the solution and washed 4 times with 1X Wash Buffer. Remaining Wash Buffer was removed after the last wash by aspirating.

4. 100 µL of prepared biotin conjugate was added to each well.

5. Incubated for 1 hour at room temperature with gentle shaking.

Streptavidin-HRP addition

a. 100 µL of prepared Streptavidin-HRP solution was added to each well.

b. Incubated for 45 minutes at room temperature with gentle shaking.

Addition of TMB substrate

a. 100 µL of TMB Substrate was added to each well. The substrate began to turn blue.
b.Incubated for 30 minutes at room temperature in the dark with gentle shaking.

50 µL of Stop Solution was added to each well. The solution in the well changed from blue to yellow

Standard curve generation

1. The absorbance was read at 450 nm.

2. Curve-fitting software was used to generate the standard curve.

3. The concentrations for unknown samples and control were read from the standard curve.

3.11.2.5.3 Measurement of Cytokine TNF-α[218]:

The Diaclone Rat TNFα ELISA kit is a solid phase sandwich ELISA for the in-vitro qualitative and quantitative determination of rat TNFα (rTNFα) in supernatants, buffered solutions or serum and plasma samples. This assay will recognise both natural and recombinant rTNFα.

3.11.2.5.3.1 Principle:

A capture Antibody highly specific for rTNFα has been coated to the wells of the microtiter strip plate provided during manufacture. Binding of rTNFα samples and known standards to the capture antibodies and subsequent binding of the Biotinylated anti-rTNFα secondary antibody to the analyte is completed during the same incubation period. Any excess unbound analyte and secondary antibody is removed. The HRP conjugate solution is then added to every well including the zero wells, following incubation excess conjugate is removed by careful washing. A chromogen substrate is added to the wells resulting in the progressive development of a blue coloured complex with the conjugate. The colour development is then stopped by the addition of acid turning the resultant final product yellow. The intensity of the produced coloured complex is directly proportional to the concentration of rTNFα present in the samples and standards. The absorbance of the colour complex is then measured and the generated OD values for each standard are plotted against expected concentration forming a standard curve. This standard curve can then be used to accurately determine the concentration of rTNFα in any sample tested.

3.11.2.5.3.2 Reagents:

Anti-rTNFα Coated Plate

Plastic plate covers

rTNFα Standard: 1000 pg/ml

Standard Diluent

Biotinylated Anti-rTNFα

Biotinylated Antibody

Streptavidin-HRP

Streptavidin-HRP Diluent

Wash Buffer

TMB Substrate

H2SO4 Stop Reagent

3.11.2.5.3.3 Preparation of solutions:

Preparation of Wash Buffer

Diluted the (200X) concentrate Wash Buffer 200 fold with distilled water to give a 1X working solution. Poured entire contents (10 ml) of the concentrated wash buffer into a clean 2,000 ml graduated cylinder. Final volume was made up to 2,000 ml with glass-distilled water. Mixed gently to avoid foaming. Transfered to a clean bottle.

Preparation of Standard Diluent Buffer 1X

Diluted the (10X) concentrated Standard Diluent 10 fold with distilled water to give a 1X working solution. Poured entired contents of the concentrated standard diluents into a clean appropriate graduated cylinder. Final volume was made with glass-distilled water. Transfer to a clean wash bottle.

Preparation of Standard

This reconstitution gave a stock solution of 1000 pg/ml of rTNFα. The reconstituted standard mixed gently by inversion only. Serial dilutions of the standard were made directly in the assay plate to provide the concentration range from 1000 to 31.25 pg/ml. A fresh standard curve was produced for each new assay. Immediately after reconstitution 200µl of the reconstituted standard was added to wells A1 and A2, which provides the highest concentration standard at 1000 pg/ml. 100µl of Standard Diluent Buffer 1X was added to the remaining standard wells B1 and B2 to F1 and F2. 100µl was transferred from wells A1 and A2 to B1 and B2.

Preparation of Biotinylated Anti-rTNFα Diluted the Biotinylated Anti-rTNFα with the Biotinylated Antibody Diluent in an appropriate clean glass vial using volumes appropriate to the number of required wells.

Preparation of Streptavidin-HRP Diluted the 5µl vial with 0.5ml of Streptavidin-HRP Diluent immediately before use.

Standard curve was prepared and added in duplicate to appropriate wells 2. 100µl of each Sample and zero (Standard Diluent Buffer 1X) was added in duplicate to appropriate number of wells 3. 50µl of diluted Biotinylated Anti-rTNFα was added to all wells 4. Covered with a plastic plate cover and incubated at room temperature (18 to 25°C) for 3 hours 5. Removed the cover and washed the plate as follows: a) Aspirated the liquid from each well b) Dispensed 0.3 ml of 1x Wash Buffer into each well c) Aspirated the contents of each well d) Repeated step b and c another two times 6. 100µl of diluted Streptavidin-HRP solution was added into all wells 7. Covered with a plastic plate cover and incubated at room temperature (18 to 25°C) for 30 min 8. Wash step 5 was repeated. 9. 100µl of ready-to-use TMB Substrate was added into all wells 10. Incubated in the dark for 10-15 minutes at room temperature. Direct exposure to light was avoided by wrapping the plate in aluminium foil. 11. 100µl of H2SO4 Stop Reagent was added into all wells.

The absorbance value of each well was read on a spectrophotometer using 450 nm as the primary wavelength and optionally 620 nm as the reference wave length.

The average absorbance values for each set of duplicate standards and samples were calculated. A linear standard curve was generated by plotting the average absorbance of each standard on the vertical axis versus the corresponding rTNFα standard concentration on the horizontal axis. The amount of rTNFα in each sample was determined by extrapolating OD values against rTNFα standard concentrations using the standard curve.

3.11.2.5.6 Statistical Analysis

Results were represented as Mean ± SEM. The test extract, standard and control were analyzed with the help of one-way analysis of variance (ANOVA) followed by Bonferrni-Test. P values < 0.05 were considered as statistically significant.

Chapter 4

Results & Discussion

In this chapter the various results obtained from different experiments carried out are compiled. An attempt has also been made to discuss these results in order to provide convincing reason for the studies performed.

4.1. Collection and Identification of Plant Material Plant collection and Identification

The seeds of *P. corylifolia* were procured from local market of Ahmedabad, Gujarat and *Berberis aristata* were collected from Uttarakhand in the month of November 2018. Authentication was done by Dr. V. P. Bhatt, Taxonomist, Herbal Research and Development Institute (HRDI), Gopeshwar, Uttarakhand.

4.2 Assessment of quality of plant materials

4.2.1 Macroscopic evaluation

4.2.1.1 Morphology of *P. corylifolia* seeds

Seeds of Psoralea (fig. 4.1) are exalbuminous, typically kidney shaped, oval, dorsiventrally flattened, 2-4 mm long, 2-3 mm broad and 1-1.5 mm thick, having straw coloured hard testa, pitted with shiny spikes. Embryo is made up of two elongated kidney shaped planoconvex cotyledons and a small plumule. Testa and tegmen are separable. Strophiole (rim-aril) is adherent to the hilum. Odour and taste are aromatic and characteristic.

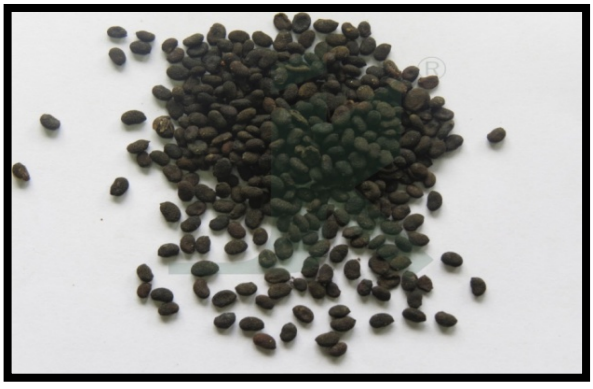


Figure 4.1, Morphology of *P. corylifolia* seed

4.2.1.2 Morphology of *Berberis aristata* root

Roots are cylindrical up to 5 cm or more in diameter, with corky, grayish brown outer surface, showing conspicuous longitudinal and transverse fissures. The wood is lemon yellow and noticeably radiated. The root bark is 4 to 6 mm thick, externally corky, knotty and friable. And internally smooth, closely striated, and yellowish brown in color. Fracture very hard; odour none and taste very bitter (Fig. 4.2).

Figure 4.2, Morphology of *Berberis aristata* root

4.2.2 Microscopical study (Powder Study)

4.2.2.1 Powder characteristics of *P. corylifolia* seed

The powder of *P. corylifolia* is brownish coloured with characteristic odor and taste and showed following microscopical characteristics.

Stone cells

They are irregularly polygonal in shape with prominent lignification. Its wall is showing numerous single and branched pits.

Brown matter

It is abundantly present.

Volatile oil globules

Greenish yellow coloured, big oil drops and numerous oil droplets were present.

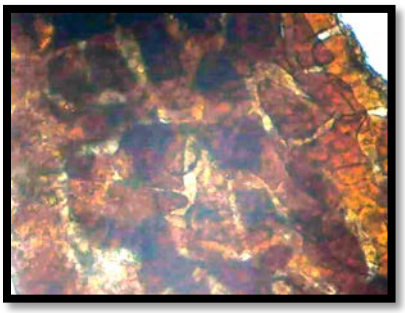
Simple fibre

It has very thick fibre wall with narrow lumen having regularly arranged numerous pits.

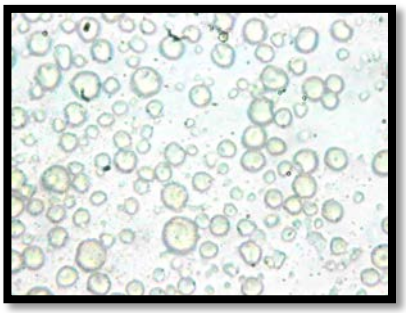
Pollen grains

Pollen grains are transparent, spherical to semi spherical, sometimes shrunken or ruptured. The exine (outer pollen wall) is relatively thin while the intine (inner pollen wall) is thick and it swells in aqueous environment.

Stone cell (45x)

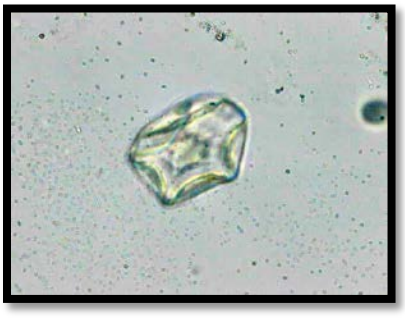


Brown content (45x)



Volatile oil (45x)

Simple fibre (45x)



Pollen grain (45x)

Figure 4.3, Powder characteristics of *P. corylifolia*

4.2.2.2 Powder characteristics of *Berberis aristata root*

The powder of *Berberis aristata* roots appears to be yellowish, fibrous with bitter taste and revealed the following characteristics**.**

Cork

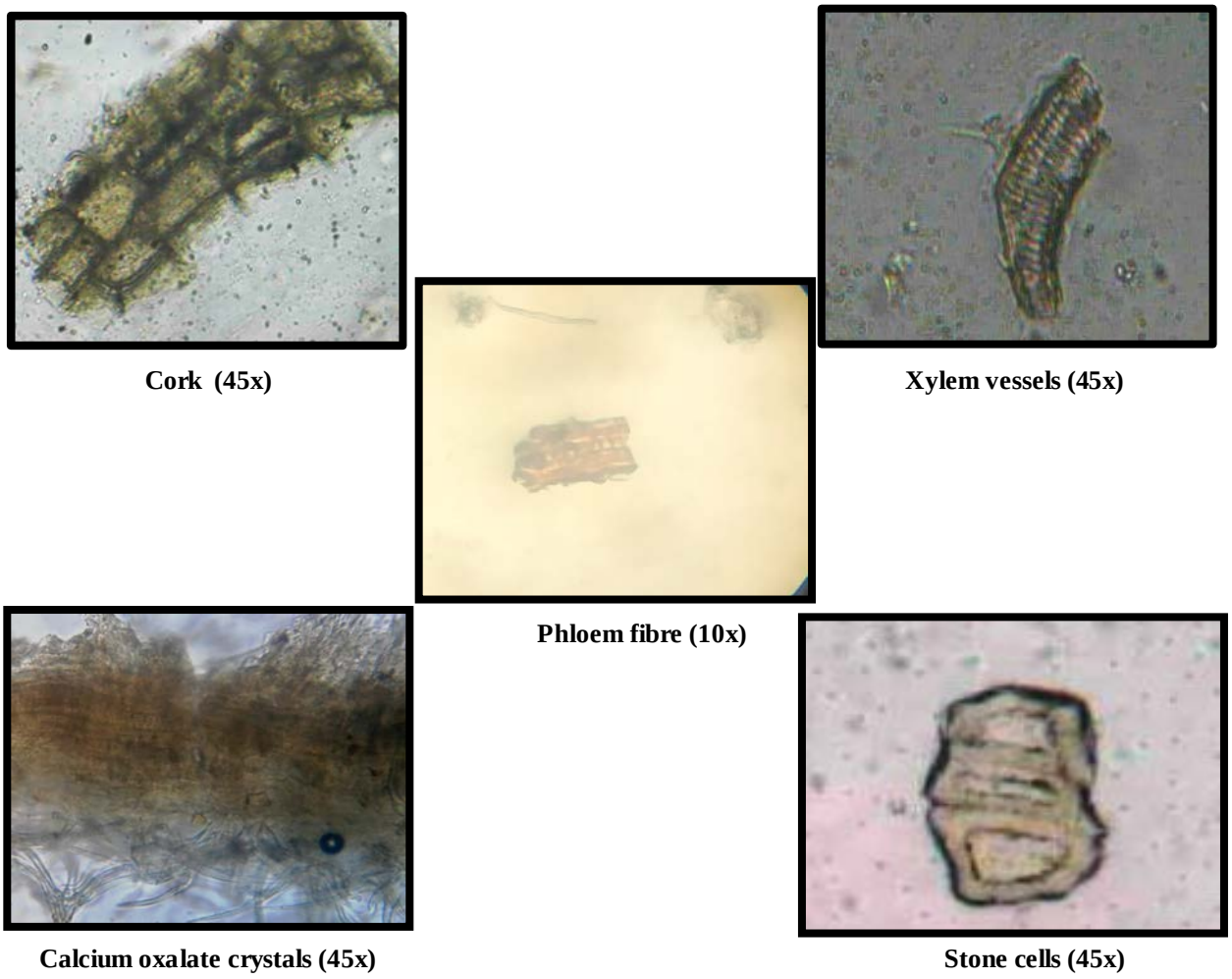
Fragments of rectangular cork cells are present

Xylem vessels

Spiral, pitted and reticulate vessels were present

Phloem fibres

Plain type of phloem fibres were observed



Cork (45x) **Xylem vessels (45x)** **Phloem fibre (10x)** **Calcium oxalate crystals (45x)** **Stone cells (45x)**

Figure 4.4, Powder characteristics of *Berberis aristata*

4.3 Proximate Analysis

The results obtained for various physico-chemical parameters (Table 4.1 and Table 4.2) complied with the data given mentioned in API[219, 220]. The values given here are expressed as percentage of air dried material. Each value is average of three.

TABLE 4.1. Physico-chemical parameters of *P. corylifolia*

Sr. No.	Parameters	*P. corylifolia* seeds Values (%w/w)
1	Loss on drying	6.20%
2	Ash value	
	Total ash	6.00%
	Acid insoluble ash	1.08%
	Water soluble ash	1.50%
3	Extractive value	
	Water soluble extractive	28.80%
	Alcohol soluble extractive	20.25%

.

TABLE 4.2. Physico-chemical parameters of powder of *Berberis aristata*

Sr. No.	Parameters	*Berberis aristata* root Values(%w/w)
1	Loss on drying	7.50%
2	Ash value	
	Total ash	2.20%
	Acid insoluble ash	0.06%
	Water soluble ash	1.90%
3	Extractive value	
	Water soluble extractive	10.04%
	Alcohol soluble extractive	6.64%

The morphological, microscopical and physico-chemical parameters of *P. corylifolia* and *Berberis aristata* will be useful in standardization viz., sample identification, quality, purity and also help to differentiate the drug from their respective other species and adulterants.

4.4 Phytochemical studies

4.4.1 Preliminary profile:

P. corylifolia seed and *Berberis aristata* root powder were extracted with different methods using methanol. The extracts obtained with different methods are recorded inTable-4.3 and Table 4.4 with their %yield, respectively.

TABLE 4.3. Preliminary profile of *P. corylifolia*

Sr. No.	Solvent	*P. corylifolia* seeds Values(%w/w)
1	Reflux	26
2	Maceration	17.03
3	Soxhlet	31.73
4	Percolation	19.53
5	Ultrasonication	10.66
6	Acclerated solvent extraction method	21.46

TABLE 4.4. Preliminary profile of *Berberis aristata* root

Sr. No.	Solvent	*Berberis aristata* root Values (%w/w)
1	Reflux	8.7
2	Maceration	4.7
3	Soxhlet	8.9
4	Percolation	3.9
5	Ultrasonication	3.1
6	Accelerated solvent extraction method	5.8

4.5 Extraction of *P. corylifolia* with different solvents:

In case of both the drugs Soxhlet extraction yielded maximum extractives so, next attempt of extraction was done with various solvents (Table 4.5) using the same technique. The maximum amount of practical yield was found to be using methanol solvent which could be due to its high polarity.

TABLE 4.5. Percentage yield of *P. corylifolia* with different solvents

Sr. No.	Parameters	Extract A	Extract B	Extract C	Extract D
1	Type of extract	Diethyl ether extract	Benzene extract	Hexane extract	Methanolic extract
2	Practical yield of extract	17.3%	15.69%	11.4 %	31.73%

4.6 Identification of Phytoconstituents yielded from Column chromatography and Flash chromatography of *P. corylifolia* extract

Many pharmacologically active compounds have been reported from *P. corylifolia* seeds. So, in the present study an attempt was made to isolate them using flash chromatography (Experimental Section 3.5 & 3.5.2). Diethyl ether extract (Extract A) and benzene extract (Extract B) yielded a total of five bioactives (Compounds A, B, C, D, and E). Compund A and Compound D (Psoralen) was found in both extracts i.e extract A and extract B. In Extract B (benzene extract), the highest yield of psoralen was discovered. Thin layer chromatography (TLC) and spectroscopic analyses were used to identify them.

4.6.1 Identification of Compound-A from Extract A

4.6.1.1 Physical Characteristic of Compound A:

State: Crystalline shiny specks

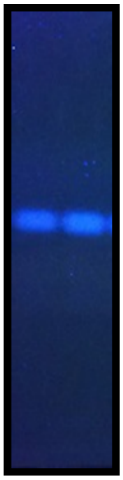
Color: white coloured

M.P.: 166□ C

These data matches with the data given for psoralen in the literature[221].

4.6.1.2 Chemical characteristic of CompoundA:

On TLC study, Compound and standard psoralen resolved at R_f 0. 62 in the mobile phase Toluene: ethyl acetate: formic acid (5: 4:1) as blue fluorescent spot.



A= Compound A
P = Psoralen (Standard)

A P

Figure 4.5, TLC of Compound A

4.6.1.3 Spectroscopical chaacteristics of Compound A[221]:

4.6.1.3.1 UV-VIS Spectroscopy:

U.V overlain spectra of isolated compound was compared with standared psoralen. The presence of coumarin derivative was indicated by the presence of UV absorption at 240, 290, 330 nm which is in accordance with that given in literature.

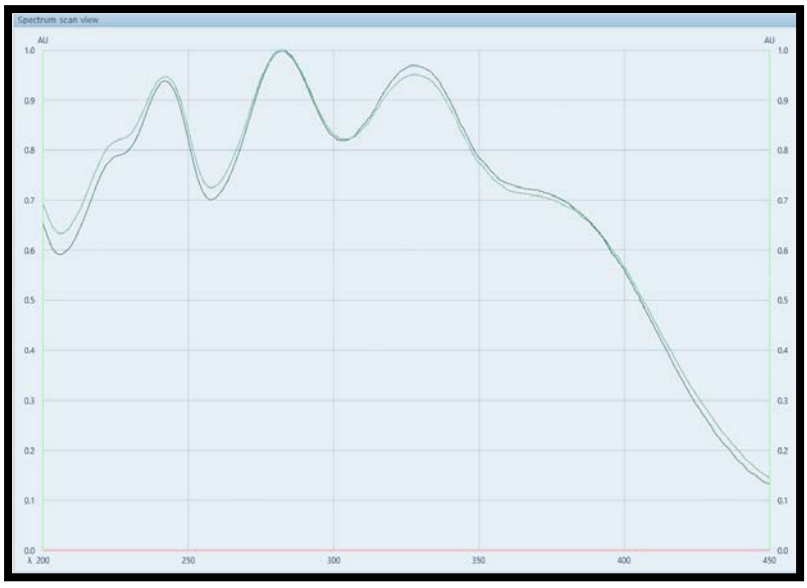


Figure 4.6, UV-overlain spectra of Compound A with Standard Psoralen

4.6.1.3.2 Mass Spectroscopy:

The Mass spectrum of Compound A showed [M + H] $^{+}$ at 187.2 as similar to the molecular weight of psoralen 186.16.

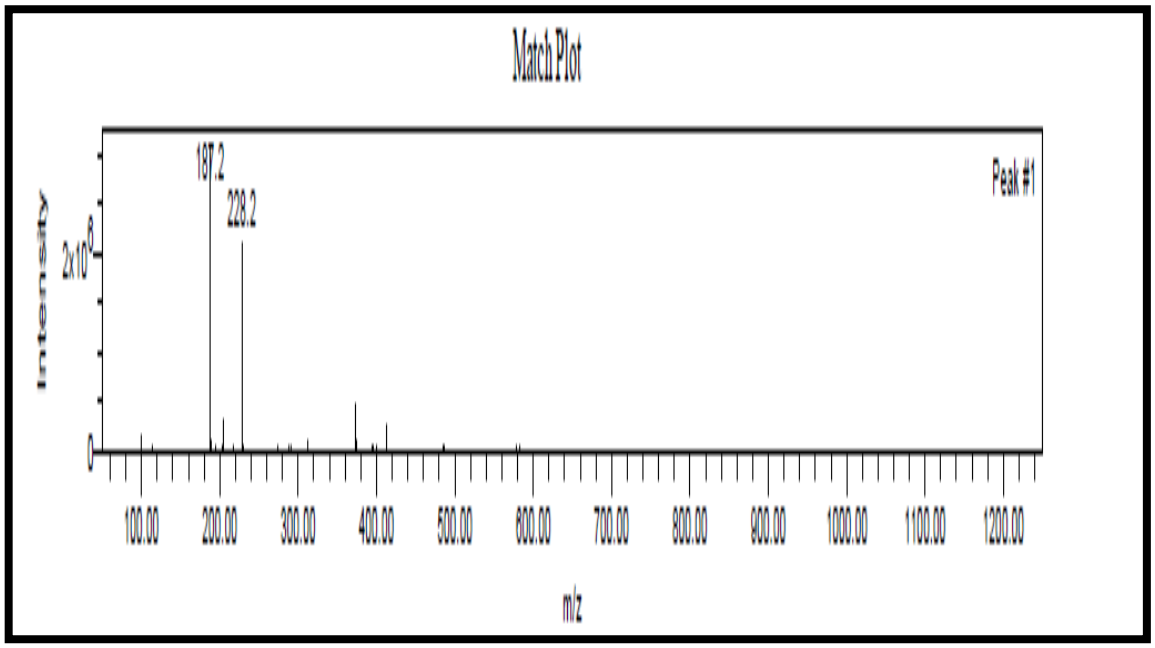


Figure 4.7, Mass spectra of Compound A

4.6.1.4 Structure of isolated compound A:

Based on the above results, the structure of the compound is assigned as Psoralen.

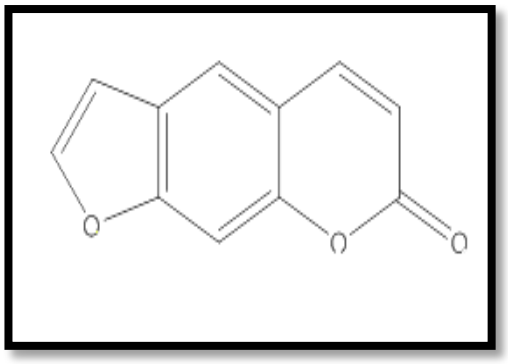


Figure 4.8, Structure of Compound (A) Psoralen

TABLE 4.6. Summary characteristic of Compound A-Psoralen

Parameter	Result
Appearance	White needle shaped compound
Solubility	Methanol, Diethylether
Melting Point (ºC)	166
R_f value	0.64
λmax (UV/Vis)	247 nm
Mass spectra	m/z = 187.2

4.6.2 Identification of Compound-B from Extract A

4.6.2.1 Physical Characteristic of Compound B:

State: Liquid

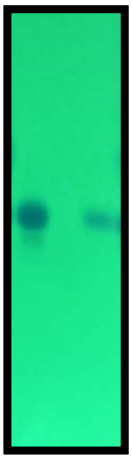
Color: Orange yellowed

M.P.: 391 □ C

These data matches with the data given for bakuchiol in the literature[222, 223].

4.6.2.2 Chemical characteristic Compound B:

Compound B and standard bakuchiol resolved at same R_f 0.76 in the mobile phase *n*-Hexane : ethyl acetate (7: 3).



B S

B= Compound B

S = Bakuchiol (Standard)

Figure 4.9, TLC of Compound B

4.6.2.3 Spectroscopical chaacteristics of Compound B[224-227]:

4.6.2.3.1 UV-VIS Spectroscopy:

U.V overlain spectra of isolated copound was compared with standared bakuchiol. scanning of the compound give maximum absorption at wavelength λ_{max} =267 nm that was similar to reference standard.

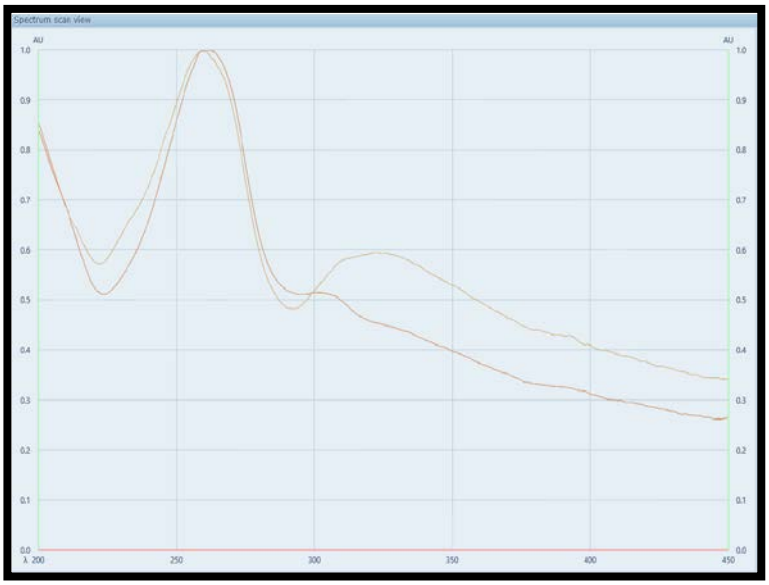
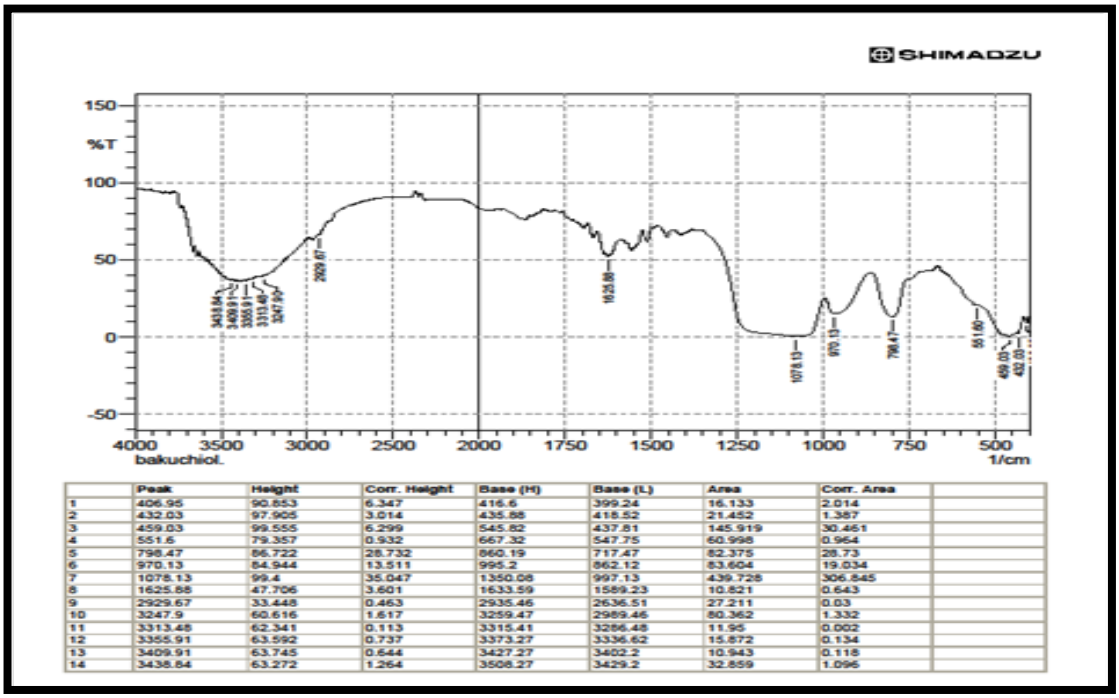


Figure 4.10, UV overlain spectra of Compound B with Standard Bakuchiol

4.6.2.3.2 I.R spectroscopy of Compound B:

The IR spectra show the absorption bands at IR_{vmax} (KBr): 2929.67, 3200, 1645, 1078.13 cm^{-1} which resembles to IR spectra of bakuchiol given in literature.



	Peak	Height	Corr. Height	Base (H)	Base (L)	Area	Corr. Area	
1	406.95	90.853	6.347	416.6	399.24	16.133	2.014	
2	432.03	97.905	3.014	435.88	418.52	21.452	1.387	
3	459.03	99.555	6.299	545.82	437.81	145.919	30.461	
4	551.6	79.357	0.932	667.32	547.75	60.998	0.964	
5	798.47	86.722	28.732	860.19	717.47	82.375	28.73	
6	970.13	84.944	13.511	995.2	862.12	83.604	19.034	
7	1078.13	99.4	35.047	1350.08	997.13	439.728	306.845	
8	1625.88	47.706	3.601	1633.59	1589.23	10.821	0.643	
9	2929.67	33.448	0.463	2935.46	2636.51	27.211	0.03	
10	3247.9	60.616	1.617	3259.47	2989.46	80.362	1.332	
11	3313.48	62.341	0.113	3315.41	3286.48	11.95	0.002	
12	3355.91	63.592	0.737	3373.27	3336.62	15.872	0.134	
13	3409.91	63.745	0.644	3427.27	3402.2	10.943	0.118	
14	3438.84	63.272	1.264	3508.27	3429.2	32.859	1.096	

Figure 4.11, IR Spectroscopy of Compound B

4.6.2.3.3 Mass Spectroscopy:

The Mass spectrum of Compound A showed $[M + H]^+$ at 257.4 as similar to the molecular weight of bakuchiol 256.4.

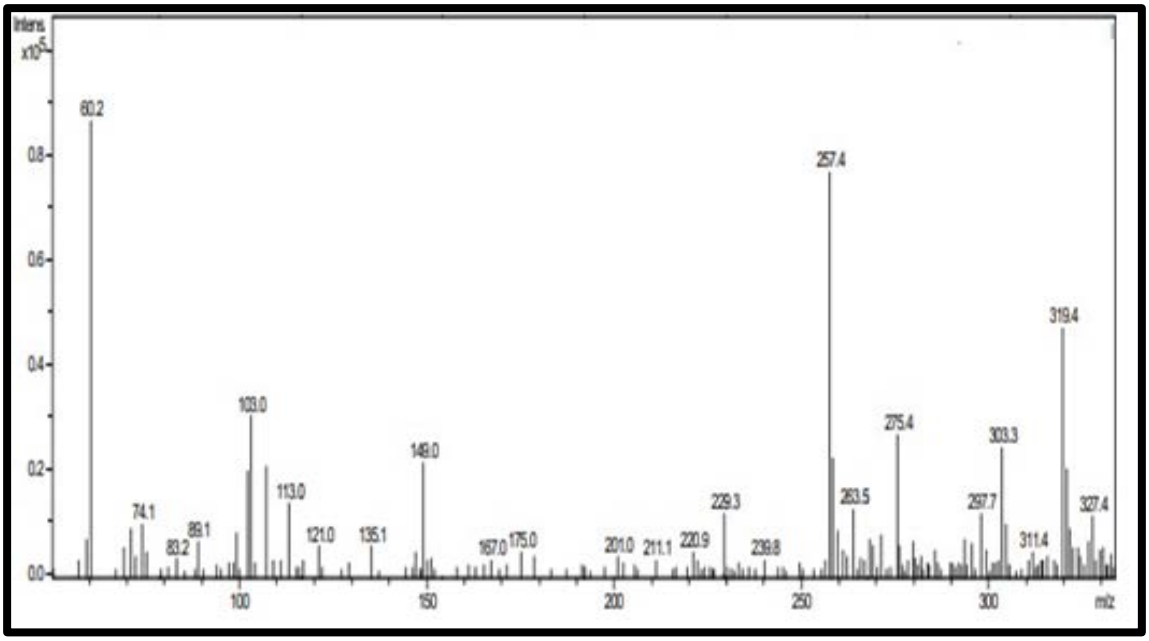


Figure 4.12, Mass Spectroscopy of compound B

4.6.2.4 Structure of isolated compound B:

Based on the above results, the structure of the compound is assigned as Bakuchiol.

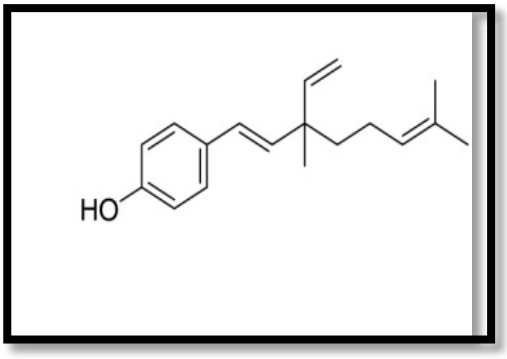


Figure 4.13, Structure of isolated Compound Bakuchiol

TABLE 4.7. Summary of characteristic of Compound B-Bakuchiol

Parameter	Result
Appearance	Orange color oily compound
Solubility	Methanol, Diethyl ether
Boiling Point (°C)	390
R_f value	0.76
λmax(UV/Vis)	267 nm
IR spectra	3200-3600cm-1(-OH)2929.67cm-1(-CH3), 1620-1680cm-1(C=C), 1078.13cm-1,(C-O)
Mass spectra	m/z = 257.4

4.6.3 Identification of Compound C from aqueous layer of Extract B:

4.6.3.1 Physical Characteristic of Compound C:

State: Solid amorphous

Color: white colored

M.P.: 146 □ C

These data matches with the data given for 5-Hydroxy xanthotoxin in the literature[227].

4.6.3.2 Chemical characteristic of Compound C:

Compound C was found to have R_f0.68 in the mobile phase *n*-hexane: ethyl acetate (7: 3) as Blue colored spot under U.V. (254 nm),

C= Compound C (Extract B)

C

Figure 4.14, TLC of Compound C

4.6.3.3 Spectroscopical analysis of Compound C:

4.6.3.3.1 I.R spectroscopy:

The IR spectra show the absorption bands at IR_{vmax} (KBr): 2248, 1442, 1469, 3564.21 and 1128 cm^{-1} which resembles to IR spectra of 5-Hydroxy xanthotoxin given in literature[227].

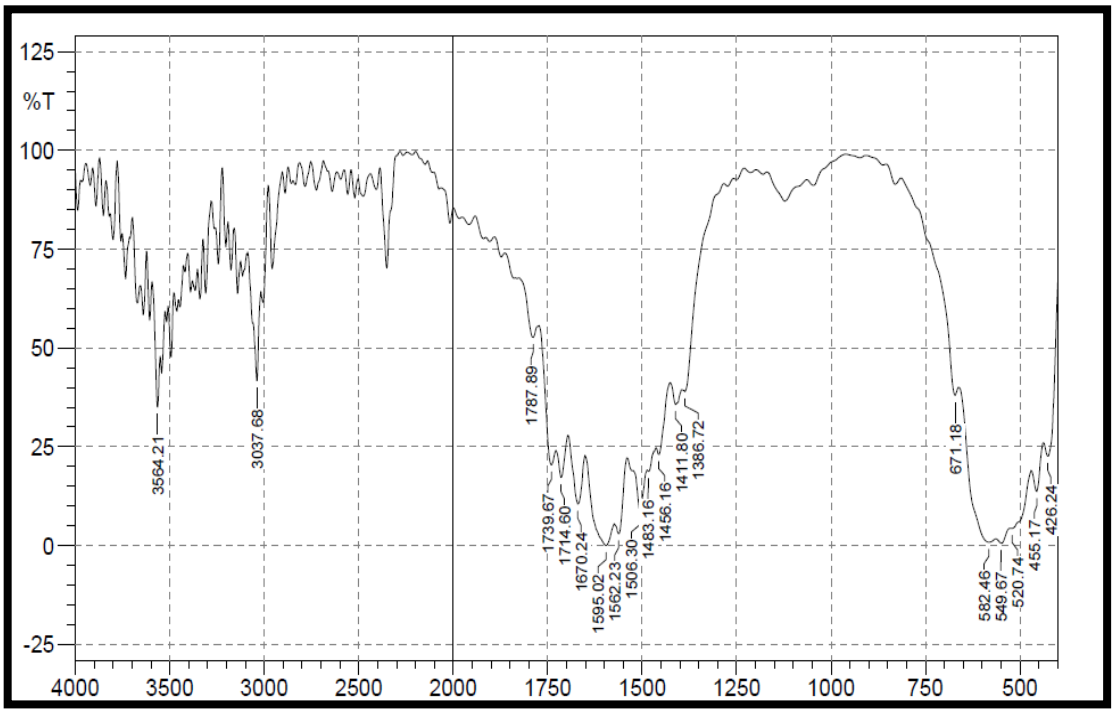


Figure 4.15, IR spectrum of Compound C

4.6.3.3.2 Mass Spectroscopy:

The Mass spectrum of Compound C showed $[M + H]^+$ at 233.1 as similar to the molecular weight of 5-Hydroxy xanthotoxin which is 232.

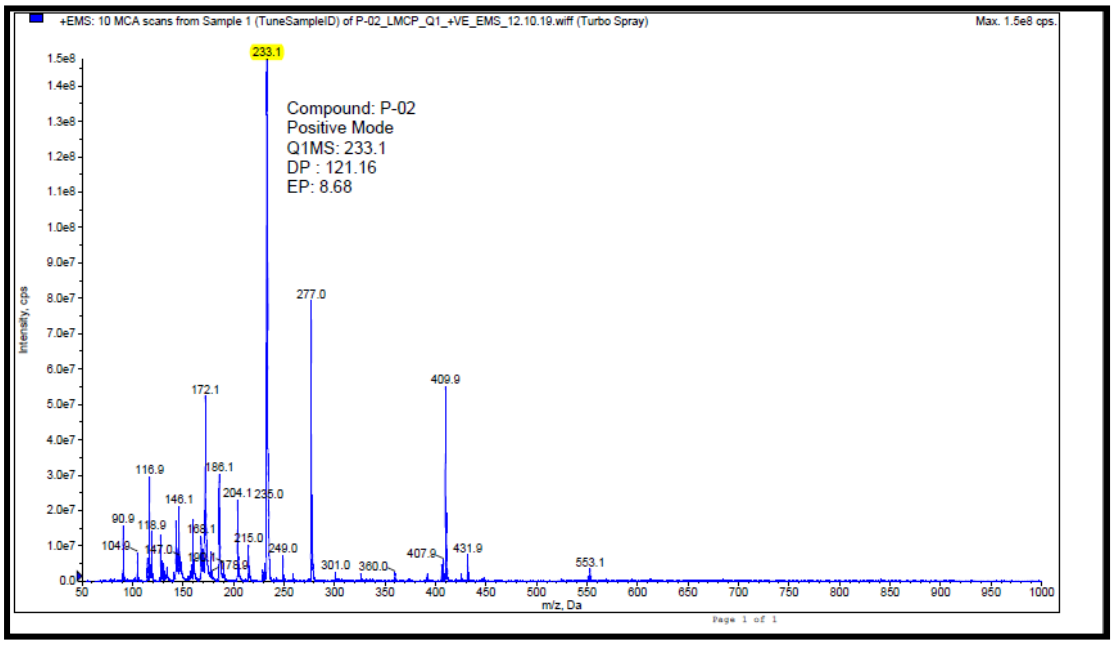


Figure 4.16, Mass Spectra of compound C

4.6.2.4 Structure of isolated compound C:

From the above results, compound C was identified as 5-Hydroxy Xanthotoxin

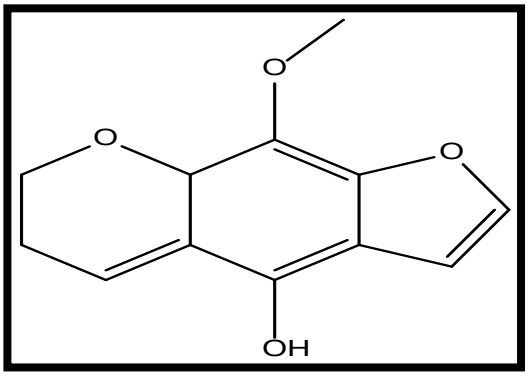


Figure 4.17, Structure of 5-Hydroxy Xanthotoxin

TABLE 4.8. Summary of characteristic of Compound C-5-Hydroxy Xanthotoxin

Parameter	Result
Appearance	White colour compound
Solubility	Methanol
Melting Point (ºC)	146
R_f value	0.64
IR spectra	2248- CH3, 1442-1469- aromatic, 3564.21 - OH 1128-CO
Mass spectra	m/z = 233.1

4.6.4 Identification of Compound E from organic layer of Extract B:

4.6.4.1 Physical Characteristic of Compound E:

State: Solid crystalline

Color: white

M.P.: 124 □ C

These data matches with the data given for Methyl 4-hydroxy benzoate in the literature[228].

4.6.4.2 Chemical characteristic of Compound E:

On TLC study, Compound E (Methyl 4-hydroxy benzoate**)** from extract and standard Methyl 4-hydroxy benzoate resolved at same R_f 0.48 in the mobile phase *n*-hexane: ethyl acetate (7: 3) as Blue coloured spot.

E= Compund E

M=Methyl 4-hydroxy benzoate (Standard)

E M

Figure 4.18, TLC of Compound E

4.6.3.3 Spectroscopic analysis of Compound E:

4.6.3.3.1 I.R spectroscopy:

The IR spectra show the absorption bands at IR_{vmax} (KBr): 2964.39, 3288.40 and 1676.03 cm^{-1} which resembles to IR spectra of Methyl 4-hydroxy benzoate given in literature[230].

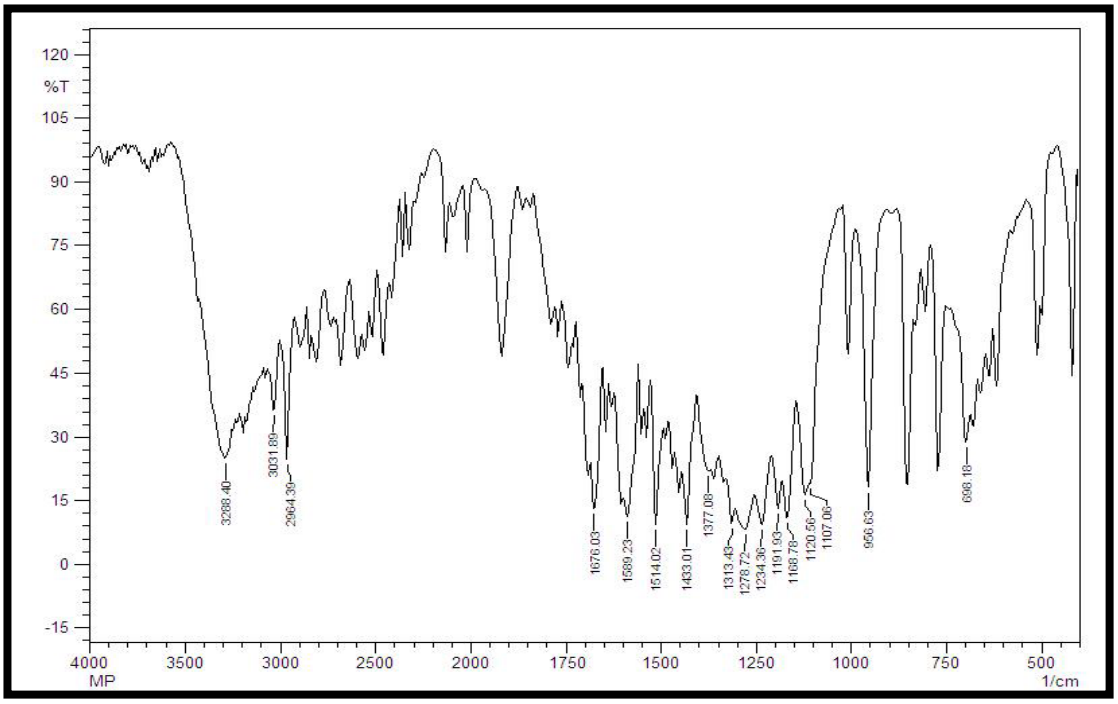


Figure 4.19, IR Spectrum of Compound E (Methyl 4-hydroxy benzoate)

4.6.2.4 Structure of isolated Compound E:

From the above results, compound E was identified as Methyl 4-hydroxy benzoate

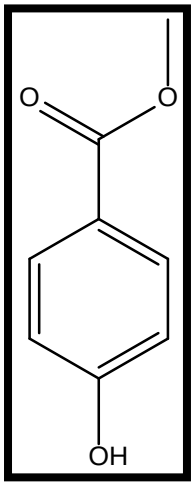


Figure 4.20, Structure of Methyl 4-hydroxy benzoate

TABLE 4.9. Summary of characteristics of Compound E- Methyl 4-hydroxy benzoate

Parameter	Result
Appearance	White colour compound
Solubility	Methanol
Melting Point (ºC)	124
R_f value	0.48
IR spectra	2964.39 - CH3, 3288.40-OH, 1676.03- C_6H_5COO

P. corylifolia is considered as one of the most important medicinal plants worldwide. Psoralen, bakuchiol, 5-hydroxy xanthotoxin and methyl 4-hydroxy benzoate were isolated using column as well as flash chromatography. Isolation of these compounds was targeted from non-polar solvent extracts (benzene and hexane) for getting these compounds in quantity to be used for rest of the study. The isolated compounds specifically psoralen and bakuchiol have several pharmacological activities. The method developed gave pure substances and was simple and cost effective.

4.7 Estimation of Bavachin, Bakuchiol and Psoralen in *P. corylifolia* by HPTLC Method

HPTLC chromatogram of standard Bavachin, Bakuchiol and Psoralen were taken with methanolic extract and it was confirmed by scanning at 254 nm.

4.7.1 Calibration curve of Bavachin, Bakuchiol and Psoralen:

The calibration data ranged between 1000-11000 ng/spot for bavachin, bakuchiol and psoralen. The calibration curve was obtained by plotting concentration *vs*. peak area.

TABLE 4.10. Calibration curves of Bavachin, Bakuchiol and Psoralen

Sr. No.	Bioactive	Range (ng/spot)	Linear regression equation	r^2
1	Bavachin	1000-11000	2.726x - 335.8	0.999
2	Bakuchiol	1000-11000	1.995x + 613.4	0.999
3	Psoralen	1000-11000	3.573x - 148.9	0.999

4.7.2 Validation of HPTLC Method for Bvachin, Bakuchiol and Psoralen:

Linearity

The calibration curves showed linearity for all compounds with r^2>0.99. Linear regression analysis of the calibration curves of these compounds are provided in Table 4.10.

Precision

The interday precision and intraday precision coefficient of variation for compounds bevachin, bakuchiol and psoralen varied from 0.10 to 1.29 and 0.41 to 0.89 respectively.

TABLE 4.11. Results of Precision

Bioactive	Interday Precision % RSD	Intraday Precision % RSD
Bavachin	1.29	0.94
Bakuchiol	0.10	0.41
Psoralen	0.60	0.89

Specificity

The developed and validated method was found to be specific for quantifying compounds bavachin, bakuchiol and psoralen as distinct from their peak purity values and the absence of any other co-eluting peaks.

Limit of detection

The LODs were 273.6, 317.42 and 108.46 ng/spot for bavachin, bakuchiol and psoralen, respectively

Limit of Quantification

The minimum quantification limit was found to be 832.14, 961.88and 328.66 ng/spot for bavachin, bakuchiol and psoralen respectively.

Recovery:

The recovery percentage was found to be in the range of 98.18 – 99.50% for three different concentrations in triplicates for each compound.

TABLE 4.12. LOD, LOQ and Recovery

Sr. No.	Bioactive	LOD (ng/spot)	LOQ (ng/spot)	Recovery (%)
1	Bavachin	273.6	832.14	98.18
2	Bakuchiol	317.42	961.88	99.31
3	Psoralen	108.46	328.66	99.50

Quantification:

An external calibration procedure applied for the quantification**.** The peak of compounds in all samples was identified by comparing the retention time and UV spectra obtained in reference standard. All eight compounds were detected in extract.

TABLE 4.13.Quantification (*n*=3) data of *P. corylifolia* extract

Sr. No.	Analyte	% content (w/w)
1	Bavachin	0.285 %
2	Bakuchiol	1.20%
3	Psoralen	0.585%

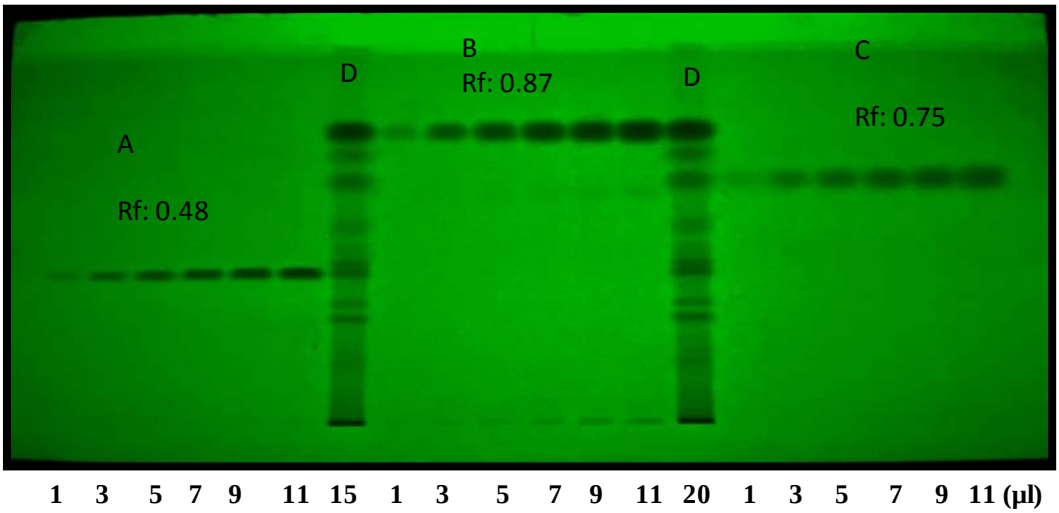


Figure 4.21, HPTLC chromtograms of standards Bavachin (A), bakuchiol (B)& Psoralen (C) and test extract (D)

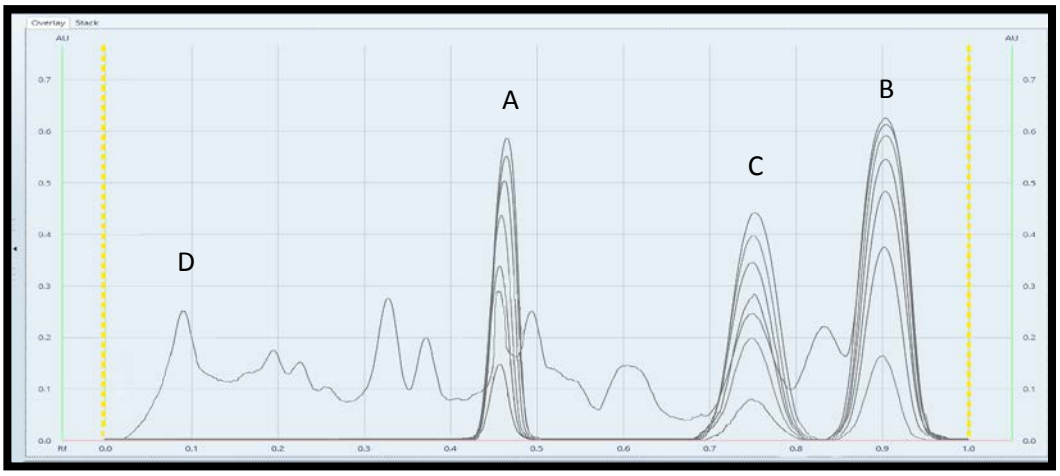
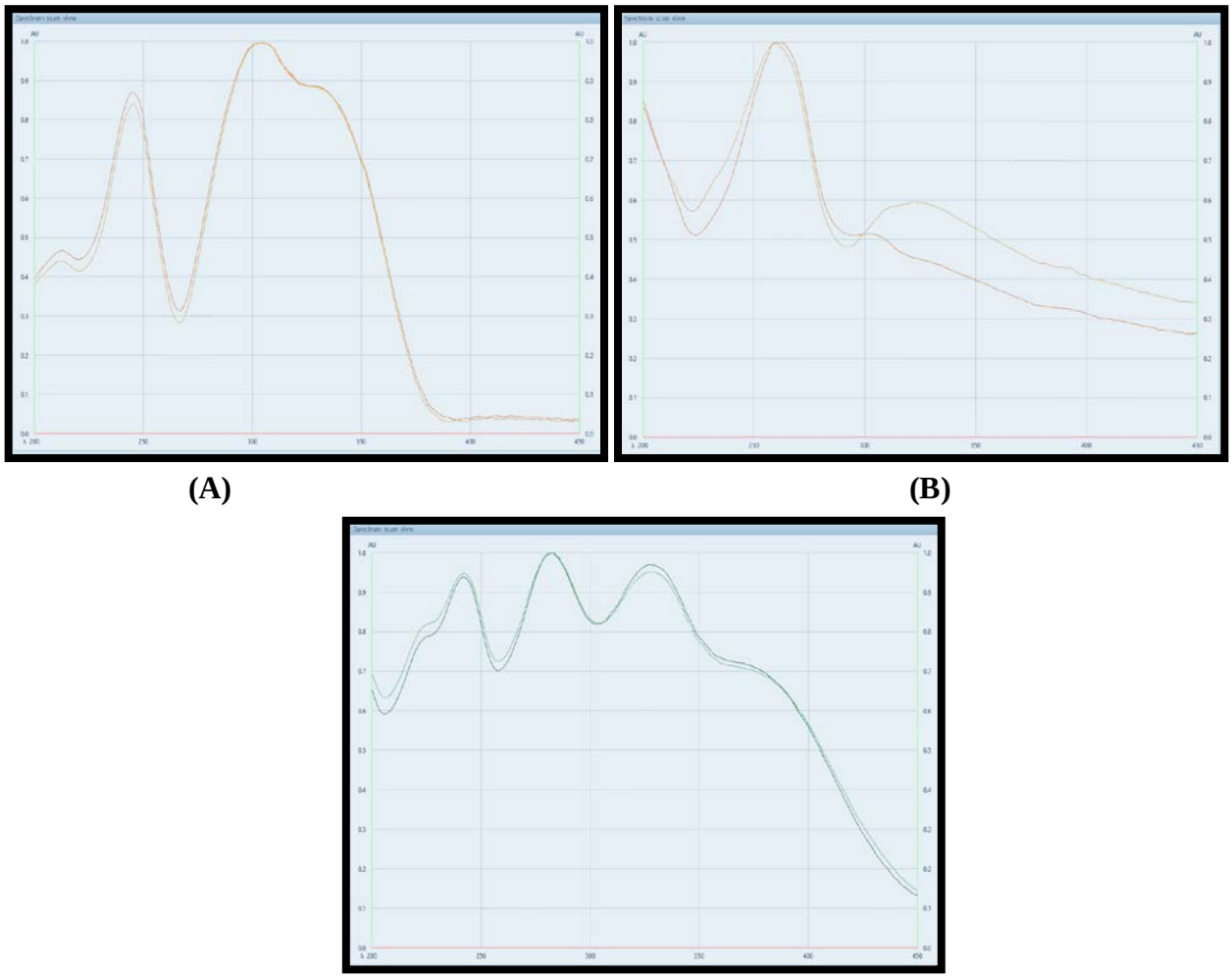


Figure 4.22, Densitometric chromtograms of standards Bavachin (A), Bakuchiol (B) & Psoralen (C) and test extract (D)



(A) **(B)**

(C)

Figure 4.23, Overlain spectra of Bavachin (A), Bakuchiol (B) & Psoralen (C) with test extract

TABLE 4.14. Results of Robustness Studies in the seeds of *P. corylifolia* samples

Compounds	Amount (ng/spot)	Mobile phase	% CV
Bavachin, Bakuchiol Psoralen	5000	toluene: ether, 0.9:1.1 saturated with 10% acetic acid (*v/v/v*)	0.68
Bavachin, Bakuchiol Psoralen	5000	toluene: ether,1:1 saturated with 9% acetic acid (*v/v/v*)	0.85

***Ruggedness*:**

Expression of the precision is confirmed with variations like different analyst, different days, and different equipment within laboratories. Ruggedness of the method was performed by spiking the standard 6 times on two different days with different Analysts.

TABLE 4.15. Results of Ruggedness Studies in the seeds of *P. corylifolia* samples

Bioactives	% Recovery	
	Analyst-I	Analyst-II
Bavachin	98.59	98.72
Bakuchiol	99.07	99.31
Psoralen	98.82	98.76

The methanolic obtained from the seed part of *P. corylifolia* was analyzed by the proposed HPTLC method. The amount of bavachin, bakuchiol and psoralen was computed from the calibration data. The HPTLC chromatogram of bavachin, bakuchiol and psoralen are shown in Fig 4.21 to Fig. 4.23.

TABLE 4.16. Summary of validation parameters for Bavachin, Bakuchiol, Psoralen estimation by HPTLC

Sr. No.	Parameters	Bavachin	Bakuchiol	Psoralen
1	Linearity range	1000-11000ng/spot		
2	Correlation co-efficient	0.99	0.99	0.99
3	Repeatability	0.733%	0.94%	0.63%
	Reproducibility	0.696%	0.89%	0.60
	Interday Precision (%RSD)	0.15%-1.29%	0.86%-0.10%	0.13%-0.60%
	Intraday Precision	0.35%-0.94%	0.20%-0.41%	0.28-0.89%

	(%RSD)			
4	Accuracy	98.18- 99.00%	98.75-99.31%	98.33-99.50%
5	Limit of detection	274.60ng/spot	317.42ng/spot	108.46 ng/spot
6	Limit of quantification	832.14ng/spot	961.88ng/spot	328.66 ng/spot
7	Specificity	Specific	specific	specific

The method for simultaneous estimation of bavachin bakuchiol and psoralen was validated according to the ICH guidelines. The linearity study indicated that the area was directly proportional to concentration and, hence, the developed method was considered linear. Quantification was achieved with linear calibration curves at concentration range of 1000–11,000 ng/spot indicating that the method is sensitive. %CV for repeatability and reproducibility study was less than 1.5 showing that the method was precise. In robustness study, %CV was found to be less than 1.5 indicating that small changes in parameters, such as mobile phase ratio, did not show any major changes in results. The LOD and LOQ for bavachin, bakuchiol, and psoralen were found to be at 274.60 and 832.14 ng, 317.42 and 961.88 ng, and 108.46 and 328.66 ng, respectively. Accuracy study was carried out at concentration level of 2 mg. The average recoveries of bavachin, bakuchiol, and psoralen were found close to 98%.

4.8 Estimation of Berberine, Berbamine , Palmatine, Jatrorrhizine & Magnoflorine by HPTLC method in *Berberis aristata*

HPTLC chromatogram of standard Berberine, Berbamine , Palmatine, Jatrorrhizine & Magnoflorine were taken with methanolic extract of *Berberis aristata* root and it was confirmed by scanning at 254 nm

4.8.1 Calibration curve of Berberine, Berbamine, Palmatine, Magnoflorine and Jatrorrhizine

The calibration data ranged between 1000-6000 ng/spot for berberine and berbamine; 200-700 ng/spot for palmatine; 500-2000 ng/spot for magnoflorine and 300-800 ng/spot

for jatrorrhizine.. The calibration curve was obtained by plotting concentration *vs*. peak area.

TABLE 4.17. Calibration curves of Berberine, Berbamine, Palmatine, Magnoflorine and Jatrorrhizine

Sr. No	Bioactive	Range (ng/spot)	Linear regression equation	r^2
1	Berberine	1000-6000	2.811x + 12308	0.997
2	Berbamine	1000-6000	1.503x + 6625	0.999
3	Palmatine	1000-11000	3.573x - 148.9	0.999
4	Magnoflorine	500-2000	4.089x + 3655	0.998
5	Jatrorrhizine	300-1800	5.717x + 5920	0.997

4.8.2 Validation of HPTLC Method for Berberine, Berbamine, Palmatine, Magnoflorine and Jatrorrhizine:

Linearity

The calibration curves showed linearity for all compounds with r^2>0.99. Linear regression analysis of the calibration curves of these compounds are provided in Table 4.17.

Precision

The Interday precision and intraday precision coefficient of variation for compounds berberine, berbamine, palmatine, magnoflorine and jatrorrhizine varied from 0.23 to 0.67 and 0.19 to 0.86 respectively.

TABLE 4.18. Results of Precision

Analyte	Interday Precision % RSD	Intraday Precision % RSD
Berberine	0.23	0.19
Berbamine	0.26	0.25
Palmatine	0.57	0.74
Magnoflorine	0.62	0.86
Jatrorrhizine	0.67	0.60

Specificity

The developed and validated method was found to be specific for quantifying compounds berberine, berbamine, palmatine, magnoflorine and jatrorrhizine as distinct from their peak purity values and the absence of any other co-eluting peaks.

Limit of detection

The LODs were 323.24, 227.42, 55.68, 86.67 and 86.56 ng/spot for berberine, berbamine, palmatine, magnoflorine and jatrorrhizine, respectively.

Limit of Quantification

The minimum quantification limit was found to be berberine, berbamine, palmatine, magnoflorine and jatrorrhizine respectively.

Recovery:

The recovery percentage was found to be in the range of 98.55 – 99.98 % for three different concentrations in triplicates for each compound.

TABLE 4.19. LOD, LOQ and Recovery

Sr. No.	Analyte	LOD (ng/spot)	LOQ (ng/spot)	Recovery (%)
1	Berberine	323.24	979.51	99.35
2	Berbamine	227.42	687.88	99.98
3	Palmatine	55.68	168.74	98.55
4	Magnoflorine	86.67	288.91	98.91
5	Jatrorrhizine	86.56	288.55	98.70

The results obtained for robustness studies, shown in Table 4.20, indicated that the method was robust. Ruggedness of the method was performed by spiking the standard 6 times with different analysts on two different days

TABLE 4.20. Results of robustness studies in the root of *Berberis aristata* samples

Berberine Berbamine	3000	Ethyl acetate–formic acid–glacial acetic acid–water (100:10:10:26, *V/V*)	0.72
Palmatine	500		
Magnoflorine	1100		0.86
Jatrorrhizine	900		0.61
Berberine Berbamine	3000	Ethyl acetate–formic acid–glacial acetic acid–water (100:11:11:24, *V/V*)	0.69
Palmatine	500		
Magnoflorine	1100		0.94
Jatrorrhizine	900		0.43

TABLE 4.21. Results of ruggedness studies in the root of *Berberis aristata* samples

Bioactives	% Recovery	
	Analyst-1	Analyst-II
Berberine	99.35	99.82

Berbamine	99.98	99.31
Palmatine	98.55	98.76
Magnoflorine	99.40	99.61
Jatrorrhizine	98.92	98.43

The simultaneous HPTLC method for berberine, berbamine, palmatine, magnoflorine and jatrorrhizine was validated according to the ICH guidelines. Because it can be utilised for both qualitative and quantitative examination of these biomarkers in *Berberis aristata* root, the suggested HPTLC approach was found to be simple, specific, and robust. Because the recovery rate was over 98 percent, the approach was proven to be exact and dependable. Because berberine is found not only in a variety of Berberis species but also in a variety of plants belonging to a wide range of families, the procedure is also helpful in identifying the species. Its importance is significant because it provides a simple and quick method for analysing the key bioactives of *Berberis aristata* simultaneously.

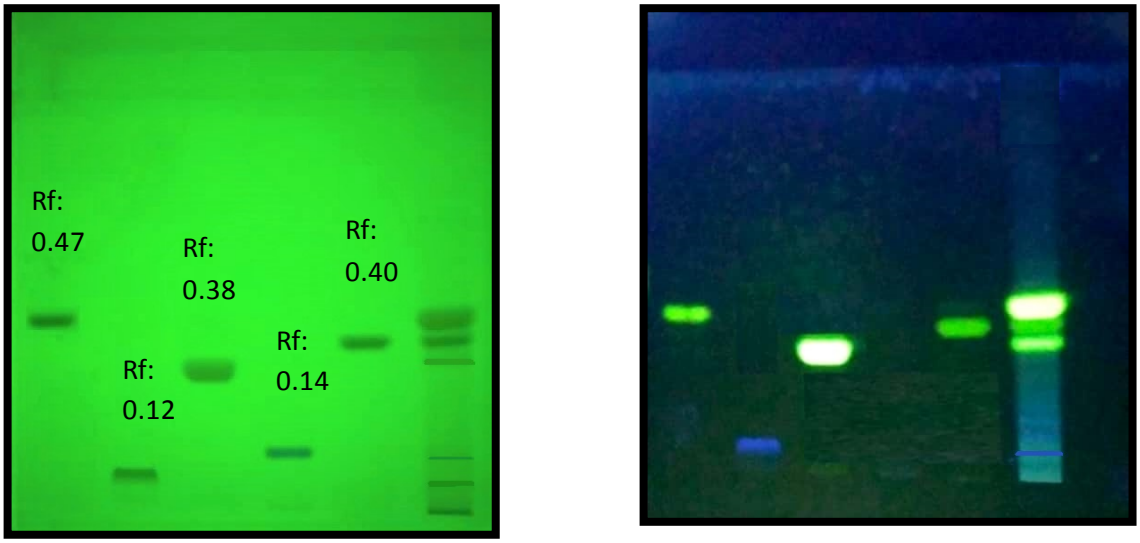
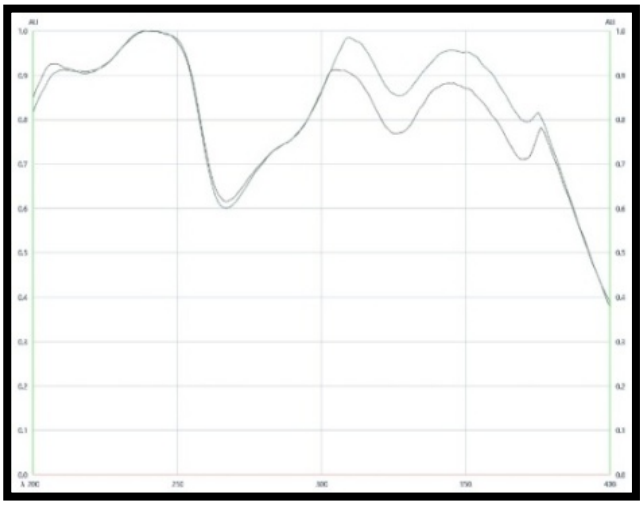
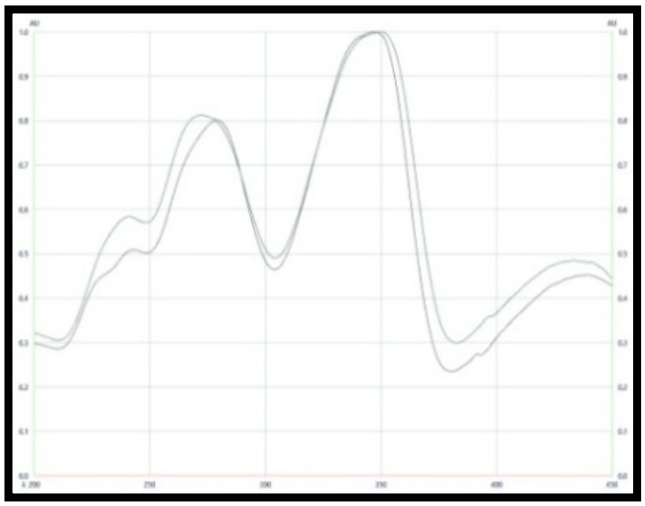


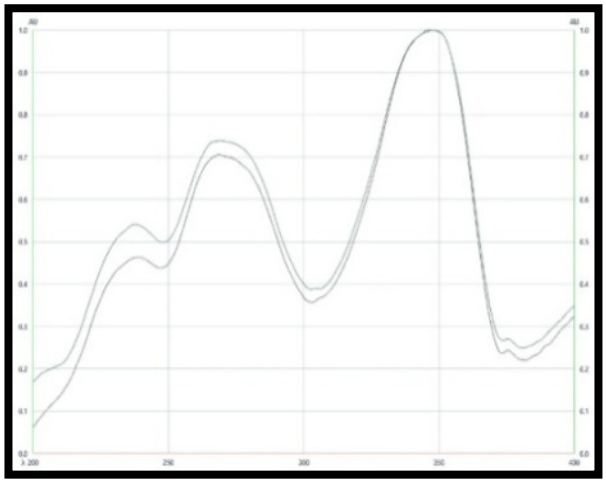
Figure 4.24, HPTLC chromtograms of standards Berberine (A), berbamine (B) Palmatine (C), Magnoflorine (D) & Jatrorrhizine (E) and test extract (F)



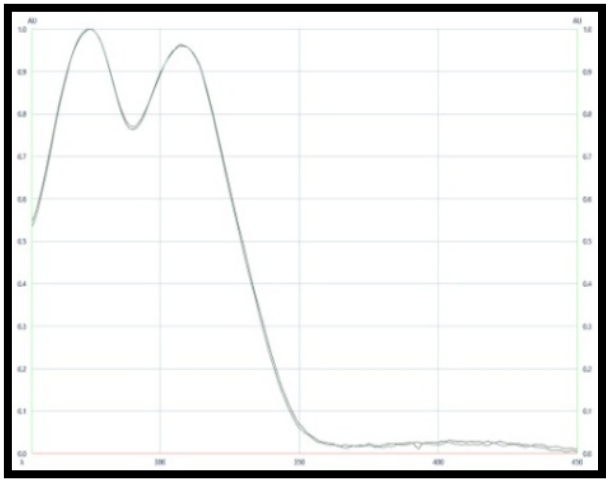
(A)



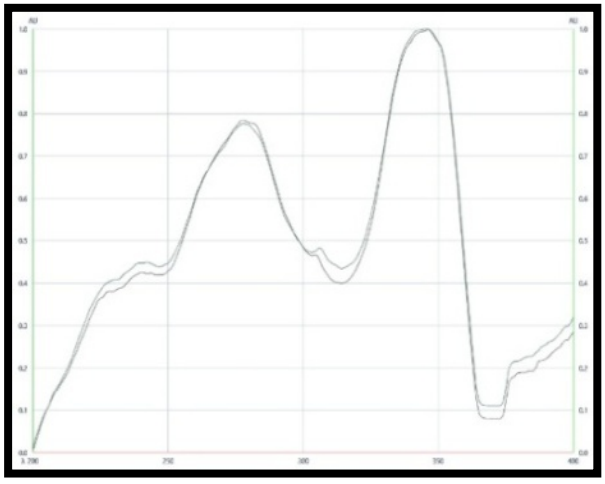
(B)



(C)



(D)



(E)

Figure 4.25, Overlain spectra of Standard Berberine (A), Berbamine (B), Palmatine(C) Magnoflorine (D) & Jatrorrhizine (E) with test Extract

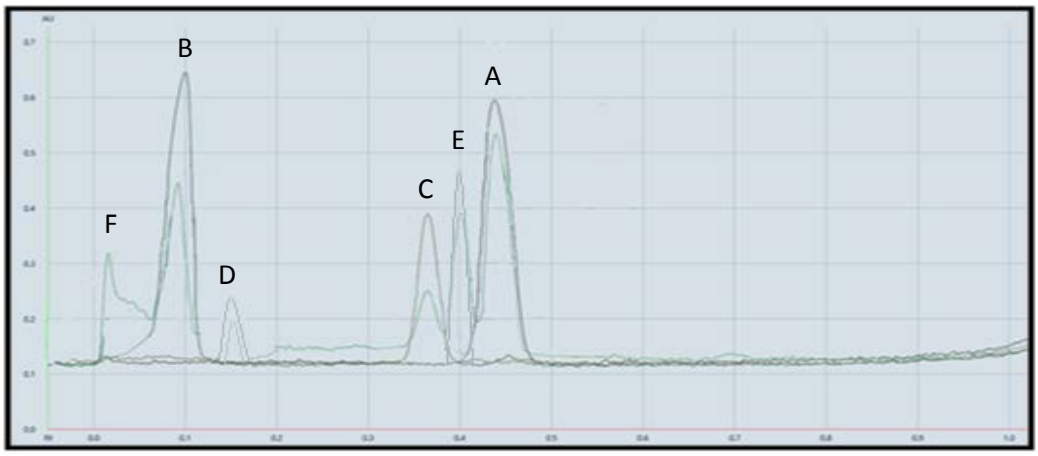


Figure 4.26, Densitometric chromtograms of standards Berberine (A), Berbamine (B), Palmatine (C), Magnoflorine (D) , Jatrorrhizine (E) and test extract (F)

Quantification:

An external calibration procedure applied for the quantification**.** The peak of compounds in all samples was identified by comparing the retention time and UV spectra obtained in reference standard. All eight compounds were detected in extract.

TABLE 4.22. Quantification of Berberine, Berbamine, palmatine, Magnoflorine and Jatrorrhizine in *Berberis aristata* root extract

Bioactive	**Amount present (%w/w)**
Berberine	1.18%
Berbamine	0.77%
Palmatine	0.84%
Magnoflorine	0.1%
Jatrorrhizine	0.12%

TABLE 4.23. Validation Summary of Berberine, Berbamine, Palmatine, Magnoflorine & Jatrorrhizine

Sr. No.	Parameters	Berberine	Berbamine	Palmatine	Magnoflorine	Jatrorrhizine
1	Linearity range	1000-6000ng/spot		200-700 ng/spot	300-1800 ng/spot	500-2000 ng/spot
2	Correlation co-efficient	0.99	0.99	0.99	0.99	0.99
3	Repeatability Reproducibility Interday Precision (%RSD) Intraday Precision (%RSD)	0.14% 0.16% 0.23% 0.19%	0.14% 0.24% 0.26 % 0.25%	0.62% 0.76% 0.57% 0.74 %	0.85% 0.67% 0.62% 0.86%	0.79% 0.61% 0.67% . 0.60%
4	Accuracy (root)	99.35%	99.98%	98.55%	98.91%	98.7%
5	Limit of detection	323.24 ng/spot	227.42 ng/spot	55.68 ng/spot	86.67 ng/spot	86.56 ng/spot
6	Limit of quantification	979.51 ng/spot	687.88 ng/spot	168.74 ng/spot	288.91ng/spot	288.55ng/spot
7	Specificity	Specific	Specific	specific	Specific	specific

4.9 Quantification of Daidzein, Psoralen, Isopsoralen, Neobavaisoflavone, Bavachin, Psoralidin, Bavachinin and Bakuchiol in *P. corylifolia* by UHPLC–PDA with ESI–MS-MS Method

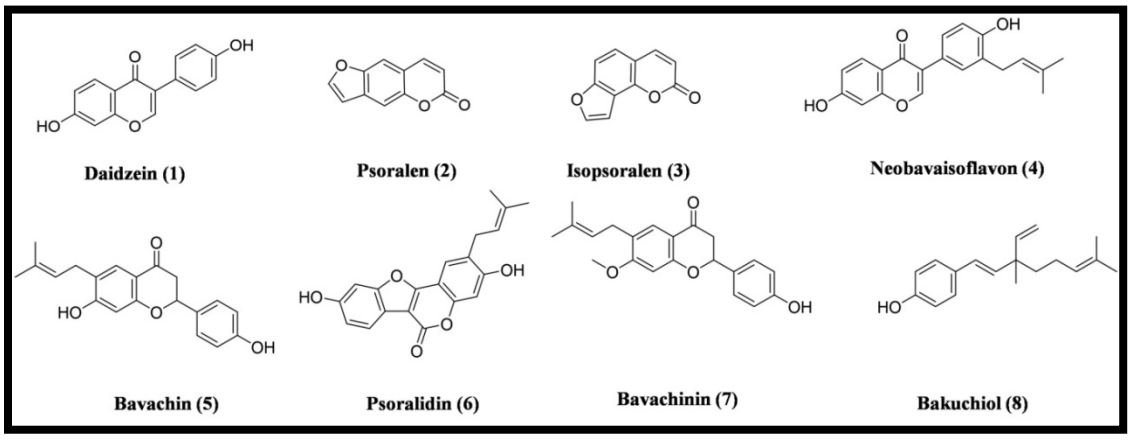


Figure 4.27, Structure of the *P. corylifolia* co compounds Daidzein (1), Psoralen (2), Isopsoralen (3), Neobavaisoflavone (4), Bavachin (5), Psoralidin (6), Bavachinin (7), Bakuchiol (8)

4.9.1 Calibration curve of Daidzein (1), Psoralen (2), Isopsoralen (3), Neobavaisoflavone (4), Bavachin (5), Psoralidin (6), Bavachinin (7) and Bakuchiol (8)

The calibration data ranged between 0.078-5µgmL^{-1}for daidzein (1), 1.250-80 µgmL^{-1}for psoralen (2), 0.938-60 µgmL^{-1}for isopsoralen (3), 0.469-30 µgmL^{-1} for neobavaisoflavone (4), 0.469-30 µgmL^{-1}for bavachin (5), 0.313-20 µgmL^{-1}for psoralidin (6), 0.625-40µgmL^{-1}for bavachinin (7), and6.250-400 µgmL^{-1} for bakuchiol (8). The calibration curve was obtained by plotting concentration *vs*. peak area.

TABLE 4.24. Calibration curves of (1) to (8) Analytes

Sr. No.	Analyte	Range (µg/mL)	Linear regression equation	r^2
1	Daidzein	0.078-5	63708x - 30.236	1.0000

2	Psoralen	1.250-80	52879x + 59267	0.9994
3	Isopsoralen	0.938-60	61980x + 45292	0.9996
4	Neobavaisoflavone	0.469-30	24627x + 357.43	1.0000
5	Bavachin	0.469-30	12776x - 571.19	1.0000
6	Psoralidin	0.313-20	20903x + 105.87	0.9999
7	Bavachinin	0.625-40	24471x + 2399.3	1.0000
8	Bakuchiol	6.250-400	11199x + 66353	0.9993

Linearity

The calibration curves showed linearity for all compounds with r^2>0.99. Linear regression analysis of the calibration curves of these compounds are provided in Table 4.24.

Precision

The Method precision and intermediate precision coefficient of variation for compounds (1−8) varied from 3.802 to 4.914 and 2.448 to 2.863 respectively.

TABLE 4.25. Results of Precision

Analyte	**Method Precision % RSD**		**Intermediate Precision % RSD**
	Day-1	**Day-2**	
Daidzein	4.842	4.376	2.863
Psoralen	3.947	4.914	2.577
Isopsoralen	4.276	4.823	2.611
Neobavaisoflavone	4.809	4.486	2.488
Bavachin	3.802	4.383	2.448
Psoralidin	4.779	3.572	3.294
Bavachinin	4.874	4.812	2.555
Bakuchiol	4.858	4.329	2.599

Specificity

The developed and validated method was found to be specific for quantifying compounds (1−8) as distinct from their peak purity values and the absence of any other co-eluting peaks.

Limit of detection

The LODs were 0.023, 0.384, 0.310, 0.140, 0.135, 0.093, 0.167 and 1.965µgmL^{-1}fordaidzein, psoralen, isopsoralen neobavaisoflavone, bavachin, psoralidin, bavachinin and bakuchiol, respectively

Limit of Quantification

The minimum quantification limit was found to be 0.069, 1.165, 0.940, 0.423, 0.408, 0.282, 0.505 and 5.963µgmL-1respectively for daidzein, psoralen, isopsoralen neobavaisoflavone, bavachin, psoralidin, bavachinin and bakuchiol, respectively

Recovery:

The recovery percentage was found to be in the range of 84.67 - 98.88% for three different concentrations in triplicates for each compound.

TABLE 4.26. LOD, LOQ and Recovery

Sr. No.	Analyte	LOD (µg/mL)	LOQ (µg/mL)	Recovery ± SD
1	Daidzein	0.023	0.069	98.888 ± 0.976
2	Psoralen	0.384	1.165	86.231 ± 0.788
3	Isopsoralen	0.310	0.940	93.751 ± 3.527
4	Neobavaisoflavone	0.140	0.423	98.136 ± 2.266
5	Bavachin	0.135	0.408	91.745 ± 1.318
6	Psoralidin	0.093	0.282	89.773 ± 5.183
7	Bavachinin	0.167	0.505	88.016 ± 3.016

8	Bakuchiol	1.965	5.953	84.673 ± 2.334

Characterization of PC compounds by LC-ESI-MS/MS:

The results obtainedfrom the ESI MS/MS analyzer are presented in Table 4.27**,** which shows MS experimental data, retention time (R_t), and main fragments generated by ESI from seed extracts. The $[M]^+$ ions of the compounds daidzein, psoralen, isopsoralen, neobavaisoflavone, bavachin, psoralidin, bavachinin and bakuchiol eluting at 5.63, 6.16,6.39, 9.28, 9.82, 11.17, 14.55 and 19.18 min were observed.

TABLE 4.27. LC-based ESI-MS/MS identification and mass scan

Sr. No.	Analyte	MW	Mode	RT	MS Fragments *m/z*
1.	Daidzein	254.24	+	5.630	100.2, 105.2, 137.4, 143.4, 222.4, 255.2
			-	5.617	119.2, 141.2, 253.2
2.	Psoralen	186.16	+	6.165	105.2, 137.4, 187.3, 222.3
3.	Isopsoralen	186.16	+	6.391	104.2, 136.4, 188.3, 221.3
4.	Neobavaisoflavone	322.4	+	9.284	105.2, 143.4, 288.5, 323.4, 345.5
			-	9.298	321.3, 322.3
5.	Bavachin	324.37	+	11.170	105.2, 130.8, 137.4, 143.4, 222.2, 288.6, 325.4
6.	Psoralidin	336.3	+	11.170	105.2, 130.8, 137.4, 143.4, 222.2, 288.6, 337.3
7.	Bavachinin	338.4	+	14.550	143.4, 222.4, 288.6, 316.6, 317.7, 339.4, 340.5

			-	14.550	119.2, 141.2, 311.4, 337.4, 338.4
8.	Bakuchiol	256.4	+	19.189	105.2, 122.3, 222.6, 316.6, 257.5, 288.6
			-	19.192	119.2, 255.4, 256.4, 315.4

Quantification:

An external calibration procedure applied for the quantification**.** The peak of compounds in all samples was identified by comparing the retention time and UV spectra obtained in reference standard. All eight compounds were detected in extract.

TABLE 4.28. Quantification (*n*=3) data of *P. corylifolia* extract

Sr. No.	Analyte	% content (w/w)
1	Daidzein	0.039
2	Psoralen	1.301
3	Isopsoralen	1.353
4	Neobavaisoflavone	0.215
5	Bavachin	0.447
6	Psorlidin	0.219
7	Bavachinin	0.977
8	Bakuchiol	4.985

The UHPLC−PDA method is validated as per International Conference on Harmonization (ICH) guidelines (ICHQ2R1). The method was developed by optimizing wavelength, mobile phase, mode of elution and temperature of ESI interface. Similarly, at CE 20 eV in MS was confirmed based on the resolution peaks (free from other interfering molecules) with high intensities and lower background noise for the fragmentation. Moreover, the method was found to be specific for the analysis of these markers as evident from

comparison of the representative chromatogram of standards and spiked samples. Confirmation of compounds namelydaidzein**(1)**, neobavaisoflavone**(4)**, bavachin**(5)**, psoralidin**(6),** bavachinin **(7)**has been done by using ESI−MS/MS coupled with UHPLC−PDA techniques. Positive ionisation sensitivity was found to be higher in the ESI-MS method, and all of the selected compounds displayed extremely strong signal responses in the positive ion mode compared to negative ionisation, therefore the process was performed in positive ionisation. To improve the selectivity of the detection approach, the m/zions were seen in precursor and product-ion scan mode. All eight analytes could be detected in *P.corylifolia* seed extracts by comparing their retention durations and UV and MS data with reference standards in the total ionisation current (TIC) and extracted ion current chromatograms under optimised UHPLC and ESI MS/MS conditions. The positive ionisation mode was used to characterise each analyte in *P. corylifolia* extracts using a combination of UHPLC, PDA, and the ESI-MS method. The chromatographic profile was identified using collision-induced dissociation (CID) fragmentations, which result in the development of molecular formulas for the observed ions. By comparing the retention time, UV, and MS overlay spectra obtained in reference standards, the peaks of substances in all samples were recognised. Bioactive compounds from the class of furanocoumarins, isoflavonoids, and monoterpene phenol of *P. corylifolia* seed were resolved in 19 minutes using a validated UHPLC method. Chemical profiling of bioactive molecules present in the seed was accomplished by assessing the fragment ion patterns corresponding to peaks of reference compounds. The developed method for determining major bioactive compounds by UHPLC-PDA and ESI-MS/MS could be suitable for rapid and precise determination of bioactive compounds like furanocoumarins, isoflavones and monoterpenes of *P. corylifolia*.

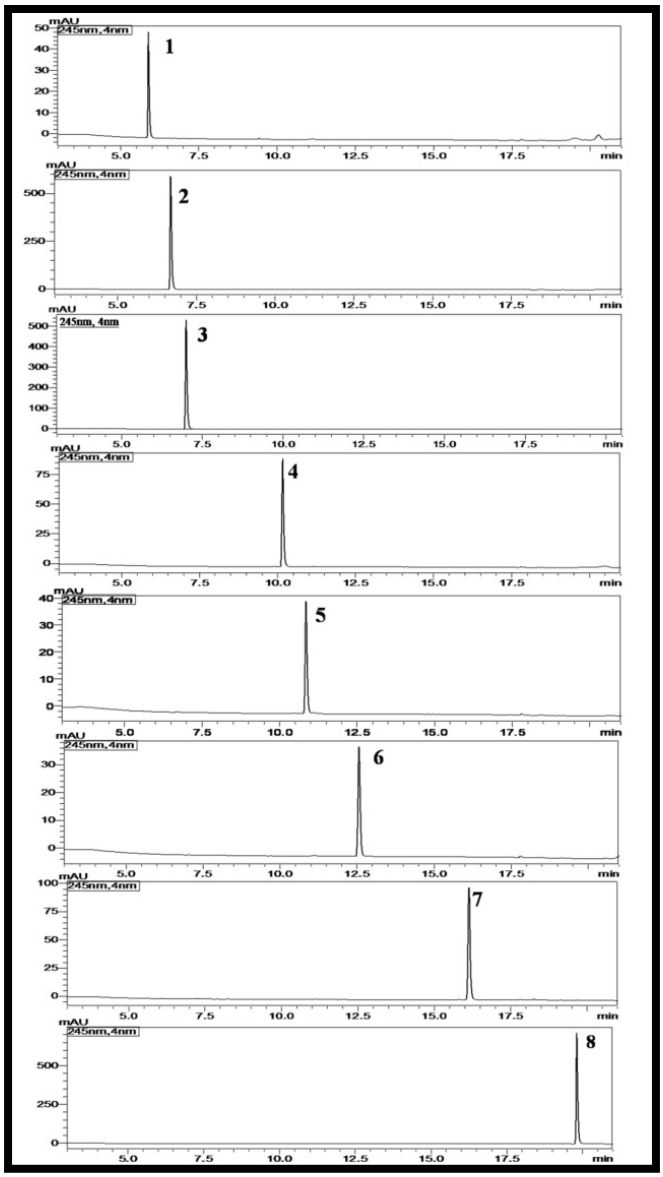


Figure 4.28, UHPLC-PDA chromatogram of reference compounds 1–8, in optimized conditions. Daidzein (1), Psoralen (2), Isopsoralen (3), Neobavaisoflavone (4), Bavachin (5), Psoralidin (6), Bavachinin (7), Bakuchiol (8), with detection at 245 nm.

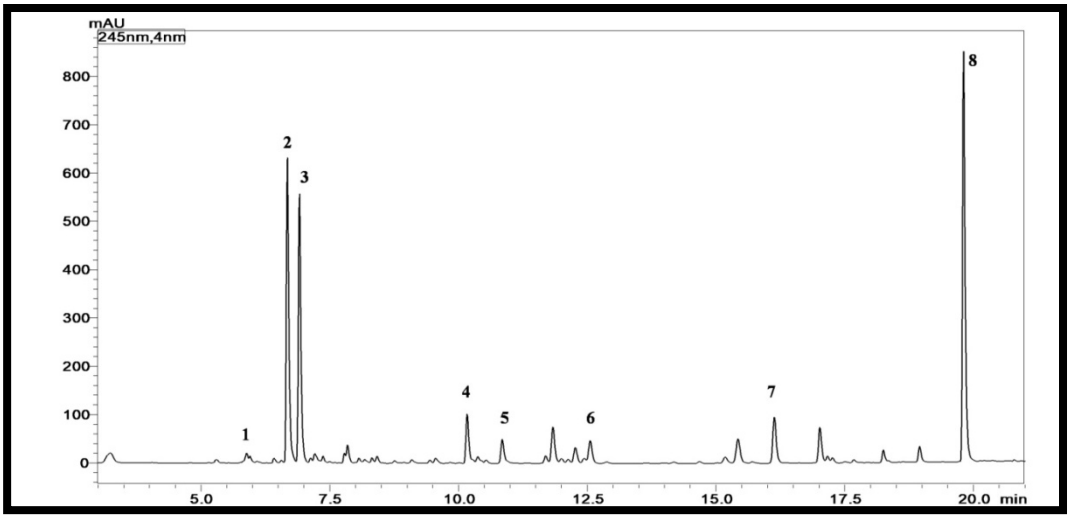


Figure 4.29, Sample chromatograms in RP-HPLC method. compounds 1-8 at 245 nm where peak identifications are as described in Figure 4.29.

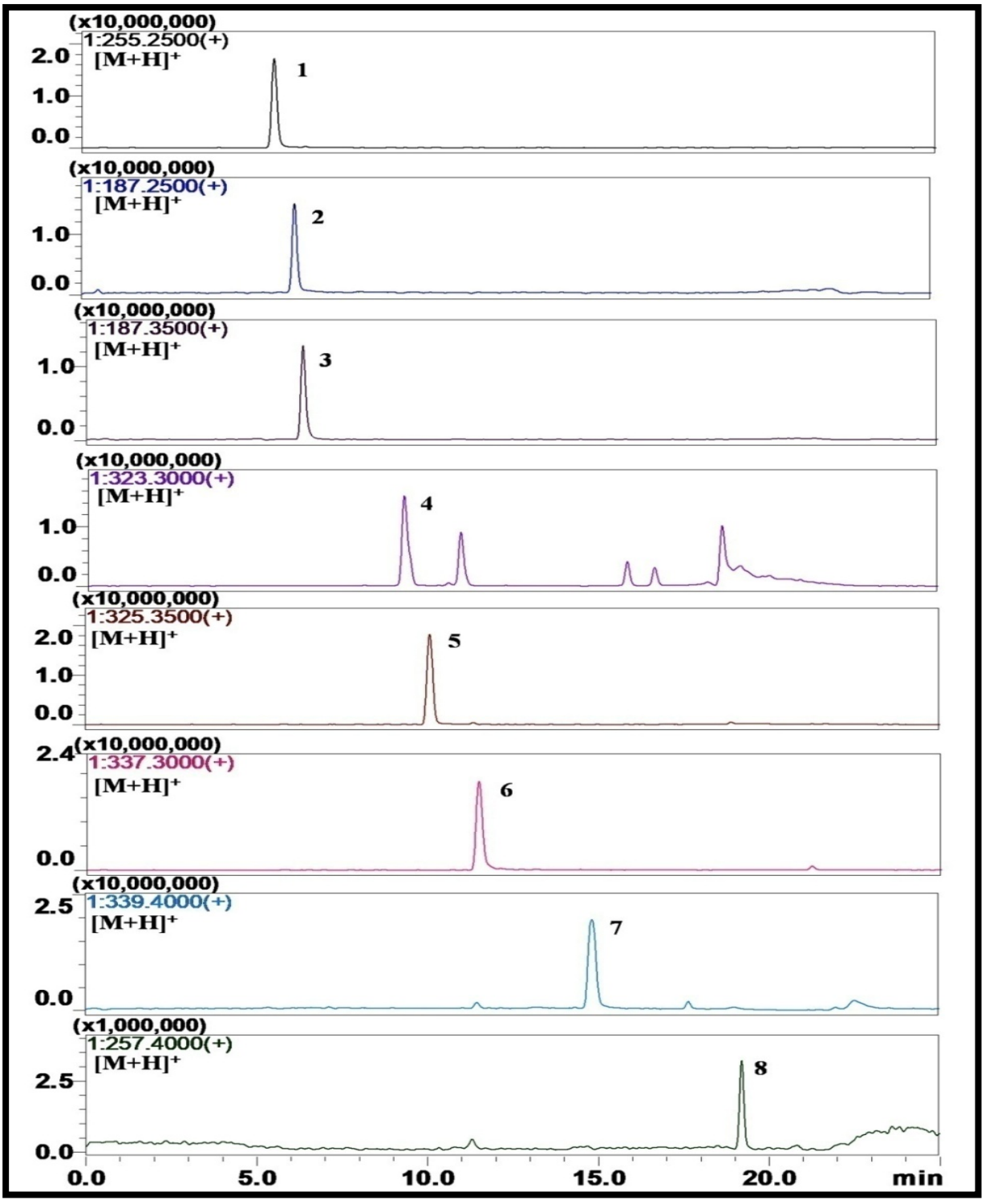


Figure 4.30, LC-ESI-MS/MS confirmation of eight compounds in PCE in full scan (SRM) mode with extracted ion chromatogram (EIC) of compounds and mass spectra at m/z values with either negative or positive ion of each identified compound. Peak identifications are as described in Figure 4.30.

4.10 Quantification of magnoflorine, berbamine, columbamine, jatrorrhizine, palmatine and berberine in *Berberis aristata* by UHPLC–PDA with ESI–MS-MS Method:

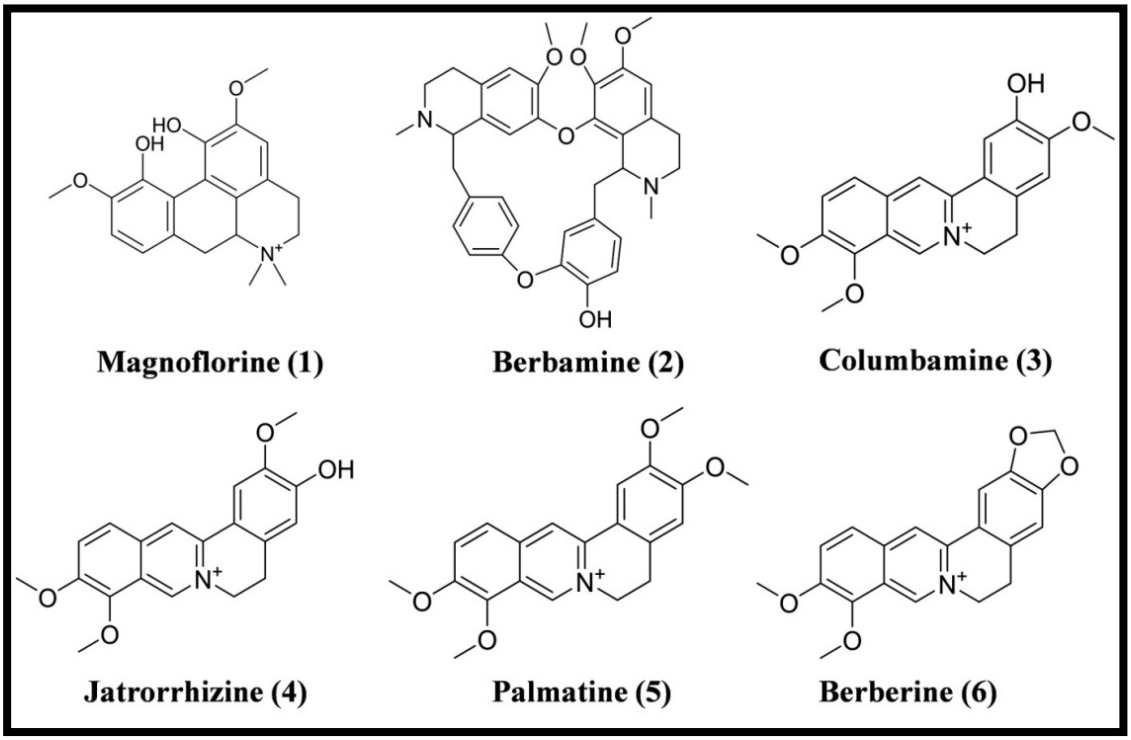


Figure 4.31, Structure of Compounds magnoflorine, berbamine, columbamine, jatrorrhizine, palmatine and berberine

4.10.1 Calibration curve of magnoflorine, berbamine, columbamine, jatrorrhizine, palmatine and berberine

The calibration data ranged 0.312 and 20 µgmL^{-1}for magnoflorine (1), 4.68-300 µgmL^{-1}for berbamine (2), 0.16-10 µgmL^{-1}for columbamine (3), 0.469-30 µgmL^{-1} for jatrorrhizine (4), 2.19-140 µgmL^{-1}for palmatine (5), and 4.68-300 µgmL^{-1} for berberine (6). The calibration curve was obtained by plotting concentration *vs* peak area.

TABLE 4.29. Calibration curves of (1) to (6) Analytes

Sr. No.	Analyte	Range (µg/mL)	Linear regression equation	r^2
1	Magnoflorine	0.312–20	3685.5x + 1029.2	0.9998
2	Berbamine	4.68–300	1883.5x+ 4696.7	0.999
3	Columbamine	0.16-10	11145x + 676.16	0.9998
4	Jatrorrhizine	0.469-30	21129x + 3133.2	0.9997
5	Palmatine	2.19-140	21093x – 15145	0.9997
6	Berberine	4.68-300	18020x + 27872	0.9996

4.10.1 Validation of HPLC Method for magnoflorine, berbamine, columbamine, jatrorrhizine, palmatine and berberine Estimation

Linearity

The calibration curves showed linearity for all compounds with r^2>0.99. The calibration curve slope (CCS) method was used to obtain regression equations.

Precision

The system precision and intermediate precision coefficient of variation for compounds (1−6) varied from 0.312 to 1.295 and 2.822 to 4.671% respectively.

TABLE 4.30. Results of Precision

Sr. No.	Analyte	System Precision % RSD	Intermediate Precision % RSD
1	Magnoflorine	1.295	2.822
2	Berbamine	0.312	3.816
3	Columbamine	0.344	4.671
4	Jatrorrhizine	0.469	3.220
5	Palmatine	0.450	4.111
6	Berberine	0.393	4.096

Specificity

The developed and validated method was found to be specific for quantifying compounds (1−6) as distinct from their peak purity values and the absence of any other co-eluting peaks.

Limit of detection

The LODs were 0.087, 0.727, 0.035, 0.124, 0.782 and 0.794 μgmL^{-1} for magnoflorine, berbamine, columbamine, jatrorrhizine, palmatine and berberine, respectively.

Limit of Quantification

The minimum quantification limit was found to be 0.264, 2.203, 0.105, 0.378, 2.369 and 2.405μgmL^{-1} respectively for magnoflorine, berbamine, columbamine, jatrorrhizine, palmatine and berberine, respectively.

Recovery:

The recovery percentage was found to be in the range of 84.74% and 96.35% for three different concentrations in triplicates for each compound.

TABLE 4.31. LOD, LOQ and % Recovery

Sr. No.	Analyte	LOD (µg/mL)	LOQ (µg/mL)	Recovery ± SD
1	Magnoflorine	0.087	0.264	94.70
2	Berbamine	0.727	2.203	84.74
3	Columbamine	0.035	0.105	96.35
4	Jatrorrhizine	0.124	0.378	88.41
5	Palmatine	0.782	2.369	88.09
6	Berberine	0.794	2.405	88.47

Characterization of PC compounds by LC-ESI-MS/MS:

The results obtainedfrom the ESI MS/MS analyzer are presented in Table-4.66**,** which shows MS experimental data, retention time (R_t), and main fragments generated by ESI from seed extracts. The $[M]^+$ ions of the compounds daidzein, psoralen, isopsoralen, neobavaisoflavone, bavachin, psoralidin, bavachinin and bakuchiol eluting at 5.63, 6.16,6.39, 9.28, 9.82, 11.17, 14.55 and 19.18 min were observed.

TABLE 4.32. LC-based ESI-MS/MS identification and mass scan

Sr. No	Compound Name	RT	Molecular formula	Polarity	Molecular Weight (g/mol)	Precursor ion	MS/MS (*m/z*)
1	Magnoflorine	4.288	$C_{20}H_{24}NO_4^+$	Positive	342.2	342.00	265.05, 297.10, 295.80, 282.05, 58.00, 237.05, 66.10, 94.05, 95.45
2	Berbamine	6.722	$C_{37}H_{40}N_2O_6$	Positive	608.7	609.25	551.75, 573.50, 566.20, 578.10,

							499.65, 465.00, 383.05, 192.05, 176.20, 174.40, 94.80
3	Columbamine	8.068	$C_{20}H_{20}NO_4^+$	Positive	338.1	338.10	322.15, 307.95, 306.00, 293.90, 278.90, 270.60
4	Jatrorrhizine	8.397	$C_{20}H_{20}NO_4^+$	Positive	338.1	338.15	322.15, 308.05, 306.20, 294.00, 280.00,279.00
5	Palmatine	10.089	$C_{21}H_{22}NO_4$+	Positive	352.2	352.10	336.10, 337.05, 322.00, 320.05, 308.05
6	Berberine	10.846	$C_{20}H_{18}NO_4$+	Positive	336.1	336.05	320.10, 321.00, 306.00, 303.95, 292.00

Quantification:

An external calibration procedure applied for the quantification**.** The peak of compounds in all samples was identified by comparing the retention time and UV spectra obtained in reference standard. All eight compounds were detected in extract.

Table 4.33. Quantification (*n*=3) data of *Berberis aristata* extract

Sr. No.	Analyte	% content (w/w)
1	Magnoflorine	1.182
2	Berbamine	0.769
3	Columbamine	0.202
4	Jatrorrhizine	2.204
5	Palmatine	1.036
6	Berberine	1.641

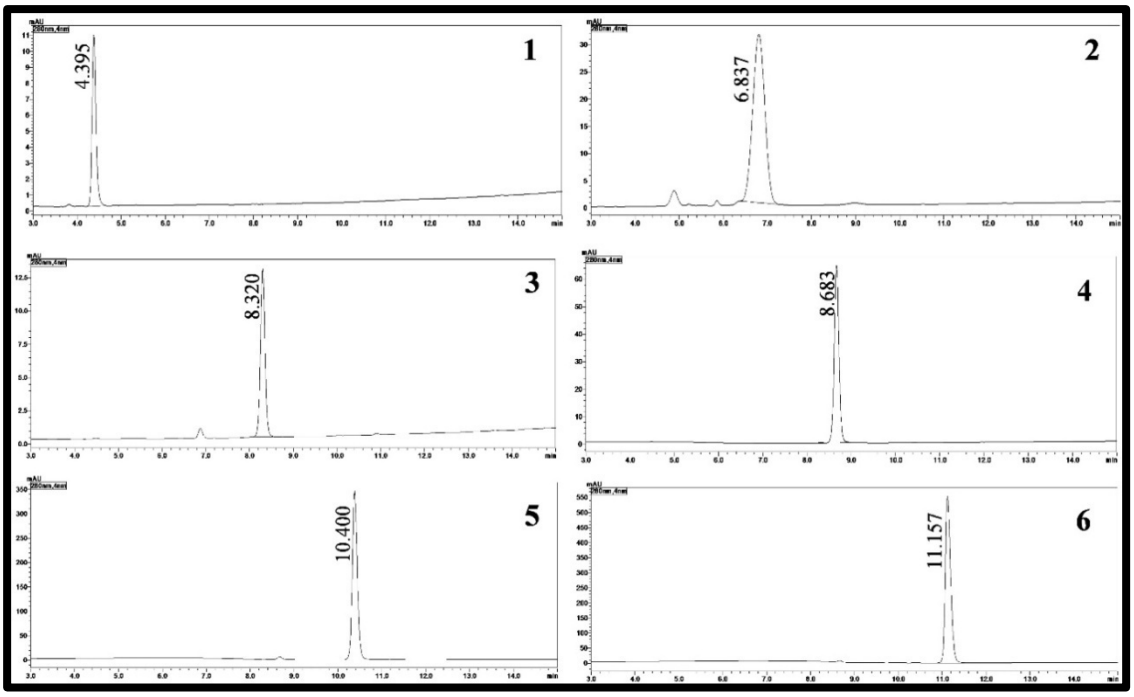


Figure 4.32, UHPLC-PDA chromatogram with detection alkaloids compounds (1-6) at 280 nm in the *Berberi aristata* root extracts as A & B-reference standards; C-sample chromatogram; D- sample chromatogram for compound.

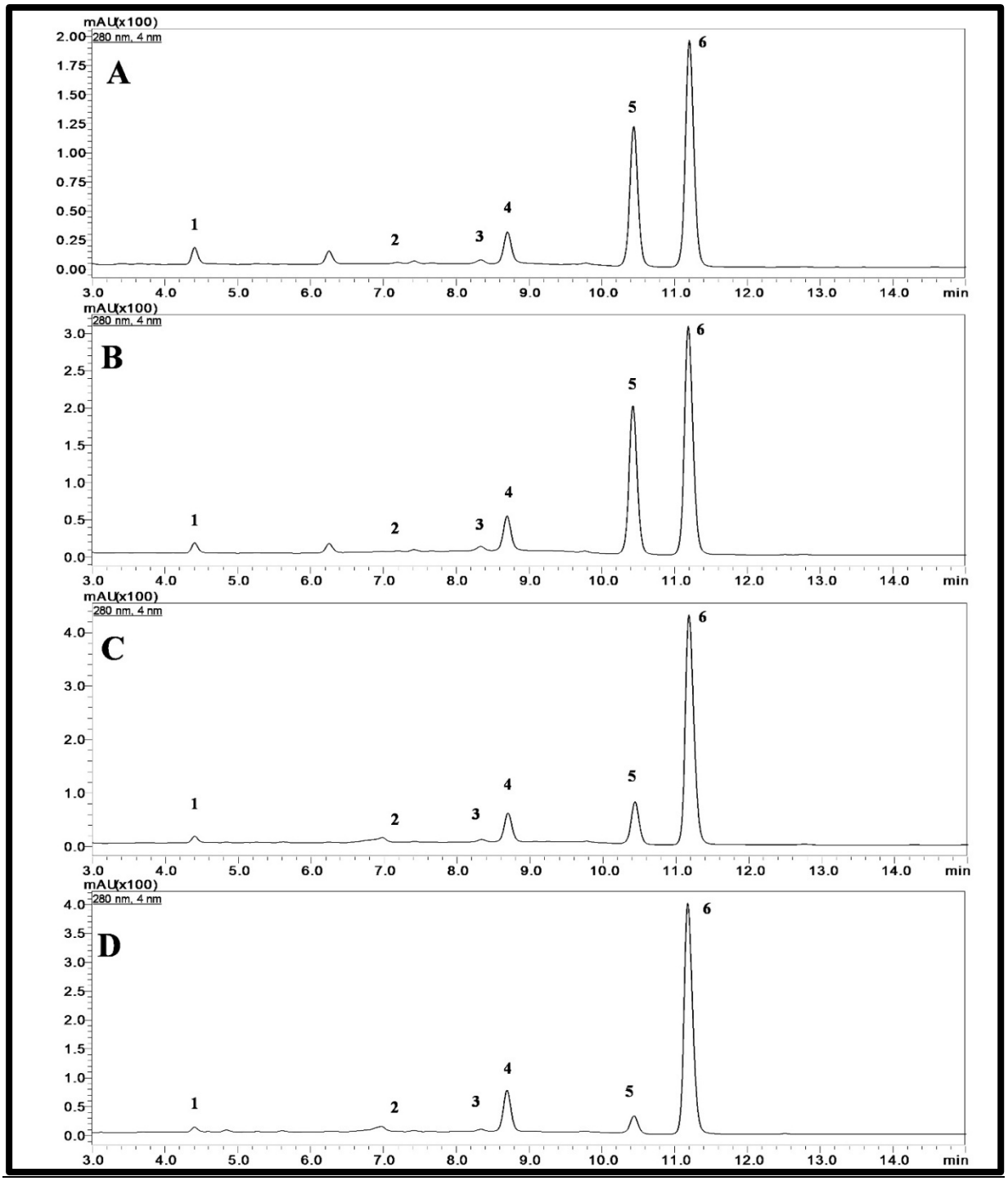


Figure 4.33, UHPLC-PDA chromatogram with detection alkaloids compounds (1–6) at 280nm in the *Berberis aristata* root extracts as A &B-reference standards; C-sample chromatogram; D-sample chromatogram for compound. Where 1, magnoflorine; 2, berbamine; 3,columbamine; 4, jatrorrhizine; 5, palmatine, 6, berberine

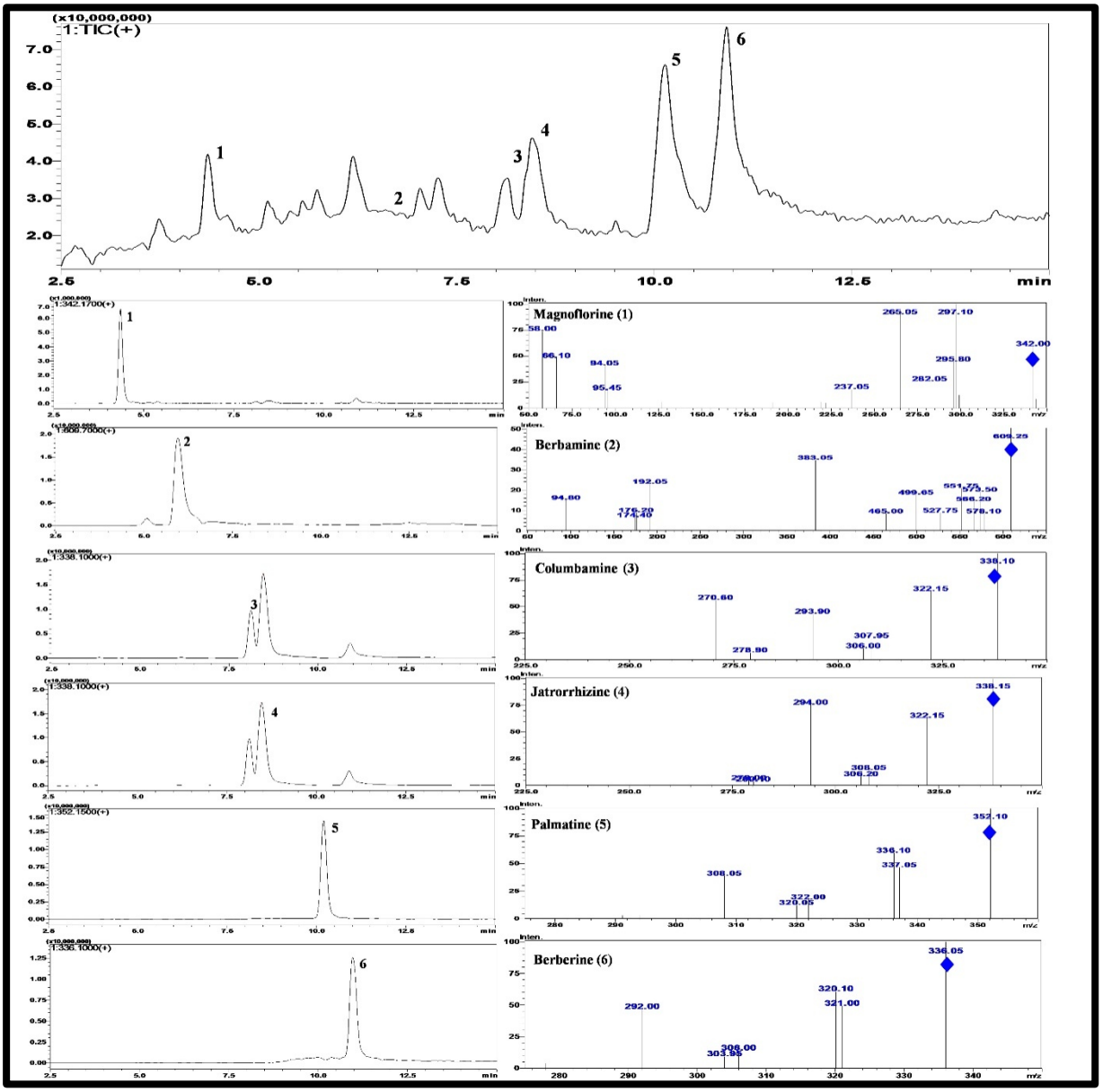


Figure 4.34, TQ-ESI-MS/MS based confirmation of 6 alkaloids in the BA with m/z values in selective ion scan mode. (A) TIC (+ve); (B) ionchromatograms extracted at m/z values with either the negative or positive ion or the fragment ion (C) molecular ion and m/z values of eachof the identified compounds (1–6) in the *Berberis aristata* root extracts

Berberis aristata root extract was prepared by Soxhlet method. Experiments were carried out at 240, 260, 280, and 350nm wavelengths to optimise the biggest peak responses and check reasonably high sensitivity. The best wavelength for compound identification and analysis (chemical profiling) of *Berberis aristata* root extract was

confirmed for the investigation based on the results (peak shape and reaction). After numerous trials, the mobile phases of 0.1 percent trifluoroacetic acid in water and 0.1 percent trifluoroacetic acid in acetonitrile were determined to be effective in separating these alkaloids. Furthermore, when compared to isocratic elution, the gradient elution approach provided improved separation of the extract's chemical elements. The temperature of the electrospray ionisation (ESI) interface at 350°C resulted in improved selectivity and efficiency for selective ion monitoring in both polarities than at 250, 300, and 400°C, according to an MS optimization research for mass ion fragmentation. In contrast to 5, 10, 15, 25, 30, and 40 eV, fragmentation at CE 20 eV had larger peak intensities and lower background noise. The ICH guidelines were used to verify the UHPLC PDA technique. ESI-MS/MS and UHPLC-PDA were used to confirm the presence of the alkaloids magnoflorine (1), berbamine (2), columbamine (3), jatrorrhizine (4), palmatine (5), and berberine (6) in *Berberis aristata* root extracts.

The compounds showed m/z 342.17, m/z 609.15, m/z 338.10, m/z 338.15, m/z 352.10 and m/z 336.05 respectively, for in the *Berberis aristata*. Positive ionisation sensitivity was discovered to be higher during the optimization of the ESI-MS method, and all of the aforementioned alkaloids showed very strong signal responses in the positive ion mode compared to negative ionisation, hence the method was set to positive ionisation. To improve the selectivity of the detection approach, the m/z ions were monitored in precursor and product ion scan mode. In the chromatograms of total ionisation current (TIC) and extracted ion current (EIC). By comparing retention durations and UV and MS data with reference standards under optimal UHPLC and ESI MS/MS settings, all six alkaloids in *Berberis aristata* root extracts could be precisely identified. The approach used consisted of generating the molecular formula of the observed ions in the chromatographic profile and then identifying chemicals in the extracts by collision-induced dissociation (CID) fragmentations. Bererine had the highest concentration in the extract, followed by jatrorrhizine, magnoflorine, palmatine, berbamine, and columbamine.

4.11 Pharmacological Screening of Extracts using MTT assay

MTT method is one of the most widely used methods to analyze cell proliferation and viability. To check the cell viability MTT assay was performed using THP-1 cell line (since psoriasis is considered to be an auto immune disorder). The MTT assay

involves the conversion of the water-soluble yellow dye MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] to an insoluble purple formazan by the action of mitochondrial reductase. Formazan is then solubilized and the concentration determined by optical density at 570 nm. The result is a sensitive assay with excellent linearity up to 106 cells per well.

Results of the test extracts observed to be toxic as per their potential to inhibit cellular dehydrogenase activity.

TABLE 4.34. Data showing % inhibition of methanolic extract of *P. corylifolia*

ug/ml	OD1 570	OD2	OD3	std DEV	Avg	% inhibition P
Control	**0.941**	**0.999**	**0.941**	**0.033486**	**0.960333**	**0**
10	0.897	0.873	0.869	0.015144	0.879667	8.841797589
20	0.746	0.804	0.789	0.030105	0.779667	19.80270369
40	0.676	0.635	0.702	0.033779	0.671	31.71355499
60	0.578	0.566	0.513	0.034588	0.552333	44.72049689
80	0.456	0.484	0.435	0.024583	0.458333	55.02374863
100	0.434	0.422	0.407	0.013528	0.421	59.11582024
200	0.321	0.302	0.304	0.01044	0.309	71.39203507
400	0.267	0.302	0.276	0.018175	0.281667	74.38801608
800	0.223	0.214	0.207	0.008021	0.214667	81.73182316
1000	0.109	0.176	0.142	0.033501	0.142333	89.66021191
PC 5 ug DOX	**0.498**	**0.487**	**0.486**	**0.006658**	**0.490333**	**51.51625868**
PC 10 ug DOX	**0.255**	**0.213**	**0.254**	**0.023965**	**0.240667**	**78.88198758**

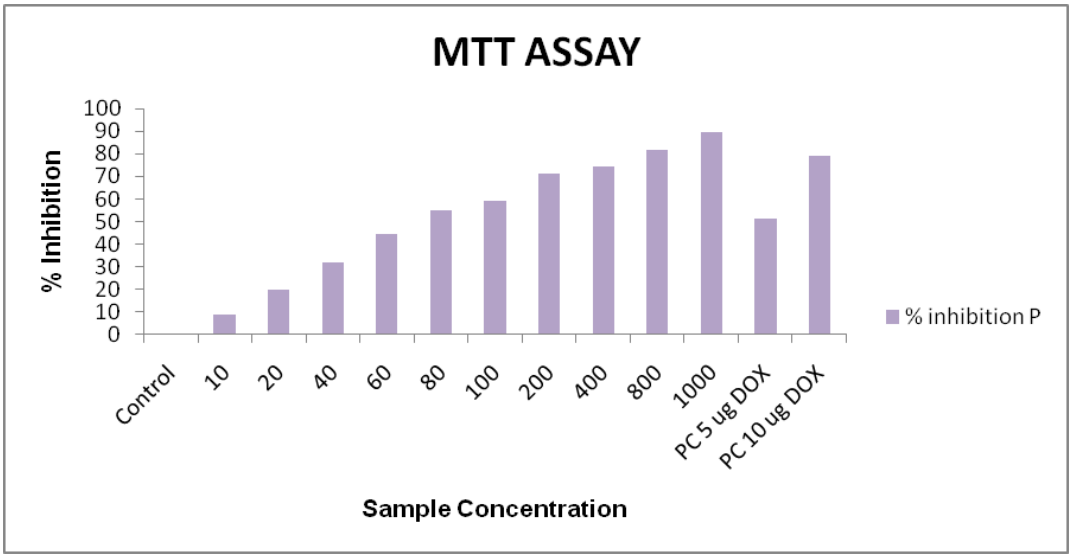


Figure 4.35, Graph of sample concentration vs %inhibition of methanolic extract of *P. corylifolia*

TABLE 4.35. Data showing % inhibition of Standard Psoralen of *P. corylifolia*

ug/ml	OD1 570	OD2	OD3	std DEV	Average	% inhibition P2
Control	**0.941**	**0.999**	**0.941**	**0.033486**	**0.960333**	**0**
10	0.953	0.921	0.904	0.02488	0.926	3.763244428
20	0.902	0.834	0.805	0.04979	0.847	12.42236025
40	0.811	0.743	0.767	0.034487	0.773667	20.46035806
60	0.606	0.723	0.643	0.059802	0.657333	33.21154549
80	0.603	0.6	0.598	0.002517	0.600333	39.45926197
100	0.487	0.423	0.442	0.032868	0.450667	55.86408476
200	0.309	0.322	0.316	0.006506	0.315667	70.661308
400	0.305	0.299	0.294	0.005508	0.299333	72.45158933
800	0.231	0.189	0.205	0.021197	0.208333	82.42601388
1000	0.106	0.162	0.165	0.033232	0.144333	89.44099379
PC 5 ug DOX	**0.498**	**0.487**	**0.486**	**0.006658**	**0.490333**	**51.51625868**
PC 10 ug DOX	**0.255**	**0.213**	**0.254**	**0.023965**	**0.240667**	**78.88198758**

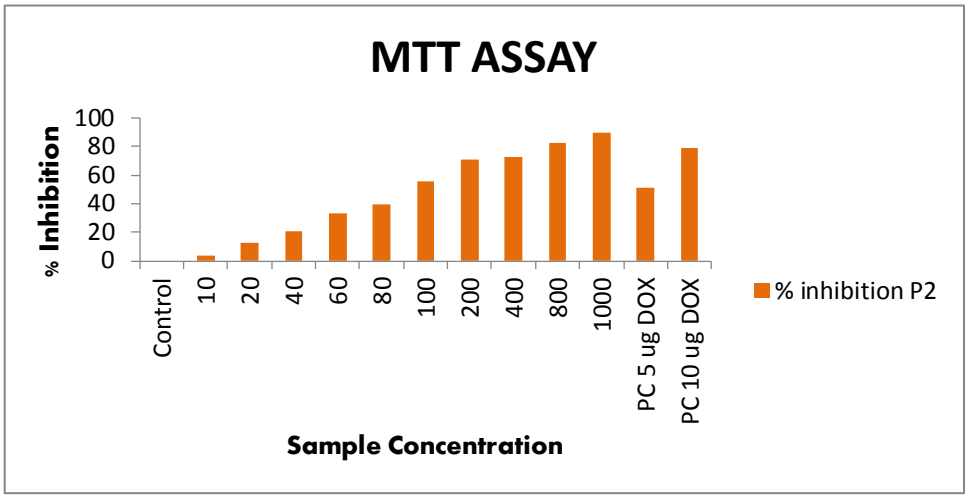


Figure 4.36, Graph of sample concentration *vs* %inhibition of standard psoralen

TABLE 4.36. Data showing % inhibition of hexane extract of *P. corylifolia*

ug/ml	OD1 570	OD2	OD3	std DEV	Average	% inhibition P1
Control	**0.941**	**0.999**	**0.941**	**0.033486**	**0.960333**	**0**
10	0.942	0.956	0.952	0.007211	0.95	1.132626964
20	0.856	0.808	0.865	0.030643	0.843	12.86079649
40	0.754	0.678	0.693	0.040253	0.708333	27.62148338
60	0.688	0.654	0.687	0.019348	0.676333	31.12897333
80	0.621	0.632	0.644	0.011504	0.632333	35.95177201
100	0.508	0.524	0.498	0.013115	0.51	49.36061381
200	0.357	0.377	0.302	0.038837	0.345333	67.40957252
400	0.323	0.298	0.345	0.023516	0.322	69.96711728
800	0.245	0.213	0.217	0.017436	0.225	80.5991962
1000	0.21	0.21	0.216	0.003464	0.212	82.02411399
PC 5 ug DOX	**0.498**	**0.487**	**0.486**	**0.006658**	**0.490333**	**51.51625868**
PC 10 ug DOX	**0.255**	**0.213**	**0.254**	**0.023965**	**0.240667**	**78.88198758**

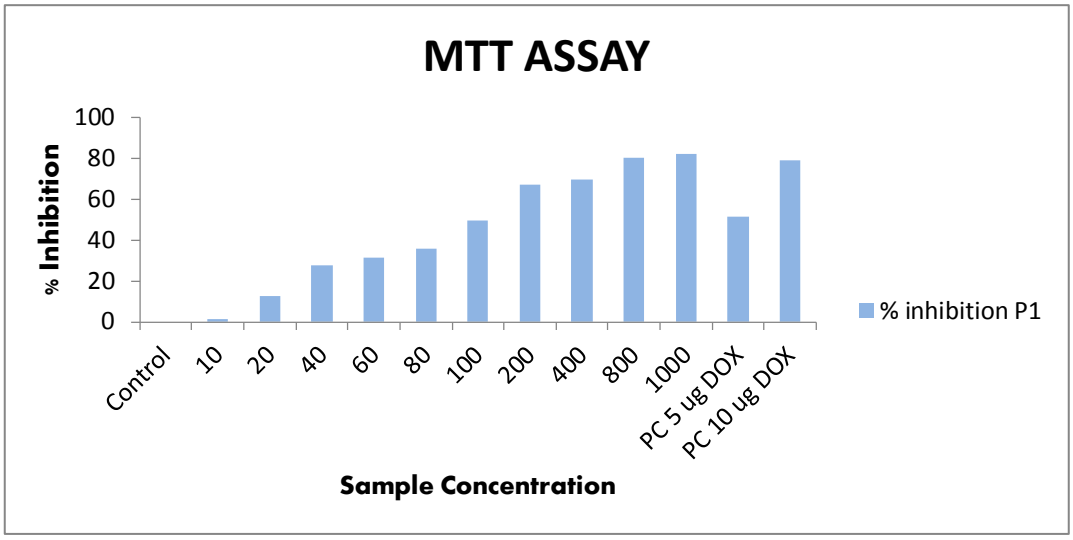


Figure 4.37, Graph of sample concentration vs %inhibition of hexane extract of *P. corylifolia*

TABLE 4.37. Data showing % inhibition of methanolic extract of *Berberis aristata*

ug/ml	OD1 570	OD2	OD3	std DEV	Average	% inhibition B
Control	**0.941**	**0.999**	**0.941**	**0.033486**	**0.960333**	**0**
10	0.899	0.942	0.924	0.021595	0.921667	4.238217026
20	0.833	0.811	0.823	0.011015	0.822333	15.12605042
40	0.72	0.786	0.745	0.033322	0.750333	23.01790281
60	0.612	0.689	0.623	0.041645	0.641333	34.96529046
80	0.532	0.499	0.51	0.016803	0.513667	48.95871392
100	0.469	0.47	0.473	0.002082	0.470667	53.67190354
200	0.319	0.311	0.298	0.010599	0.309333	71.35549872
400	0.267	0.251	0.247	0.010583	0.255	77.31092437
800	0.195	0.189	0.223	0.018148	0.202333	83.08366825
1000	0.143	0.165	0.176	0.016803	0.161333	87.57763975
PC 5 ug DOX	**0.498**	**0.487**	**0.486**	**0.006658**	**0.490333**	**51.51625868**
PC 10 ug DOX	**0.255**	**0.213**	**0.254**	**0.023965**	**0.240667**	**78.88198758**

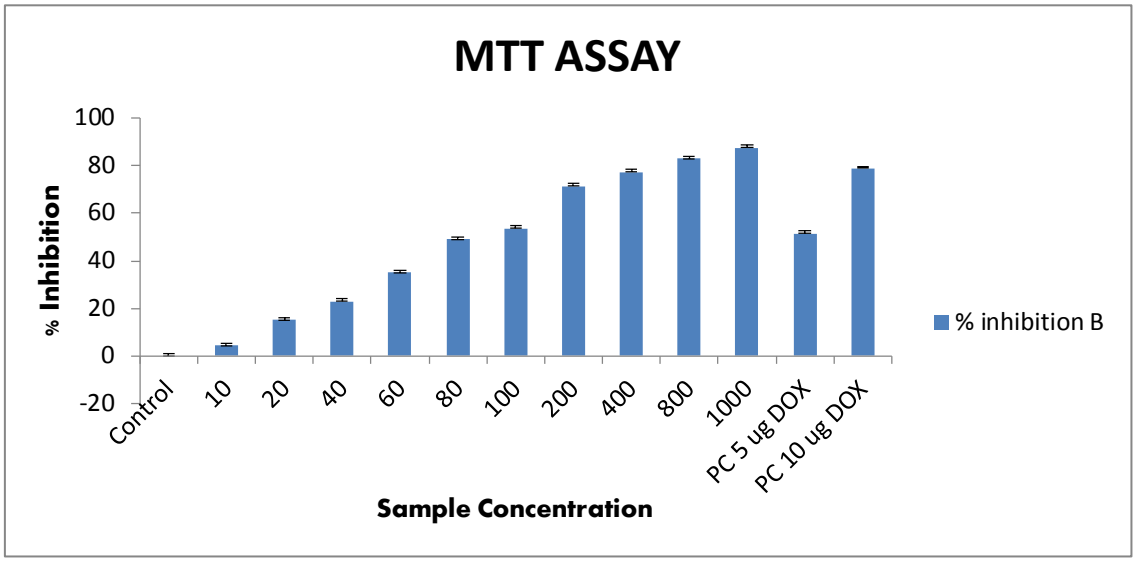


Figure 4.38, Graph of sample concentration vs %inhibition of Std berberine

TABLE 4.38. Data showing % inhibition of Standard berberine of *Berberis aristata*

ug/ml	OD1 570	OD2	OD3	std DEV	Average	% inhibition B1
Control	**0.941**	**0.999**	**0.941**	**0.033486**	**0.960333**	**0**
10	0.865	0.838	0.921	0.042336	0.874667	9.389842894
20	0.811	0.803	0.823	0.010066	0.812333	16.22214103
40	0.743	0.756	0.723	0.016623	0.740667	24.07745707
60	0.623	0.598	0.642	0.022068	0.621	37.19400804
80	0.523	0.51	0.514	0.006658	0.515667	48.7394958
100	0.419	0.421	0.404	0.009292	0.414667	59.81001096
200	0.303	0.289	0.287	0.008718	0.293	73.14578005
400	0.253	0.234	0.203	0.025239	0.23	80.0511509
800	0.178	0.167	0.176	0.005859	0.173667	86.22579467
1000	0.132	0.155	0.156	0.013577	0.147667	89.07563025
PC 5 ug DOX	**0.498**	**0.487**	**0.486**	**0.006658**	**0.490333**	**51.51625868**
PC 10 ug DOX	**0.255**	**0.213**	**0.254**	**0.023965**	**0.240667**	**78.88198758**

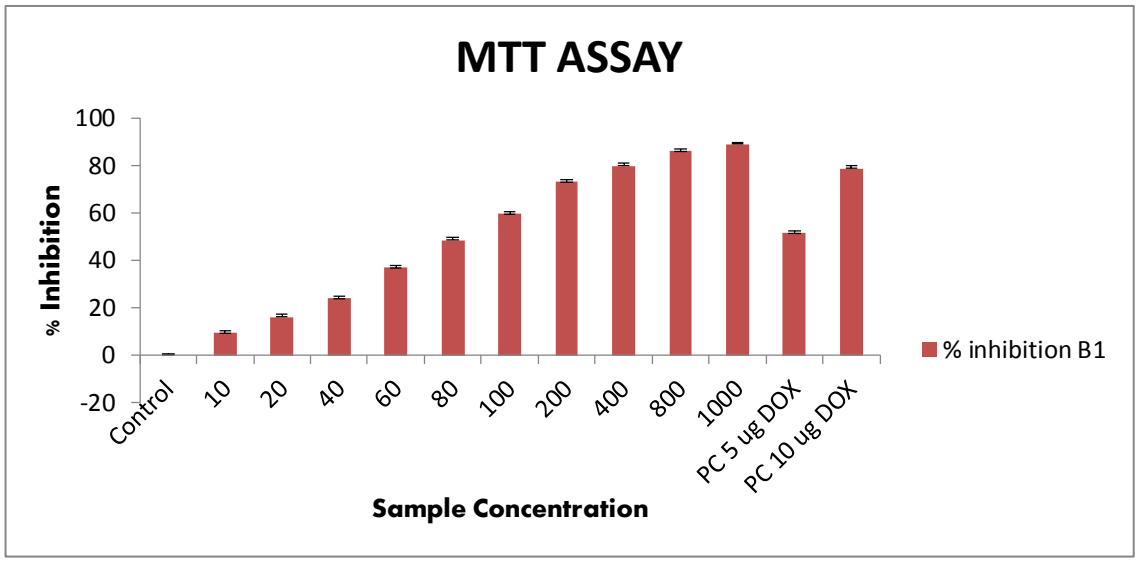


Figure 4.39 Graph of sample concentration vs %inhibition of methanolic extract of *Berberis aristata*

From the data it can be concluded that the methanloic extracts of *P. corylifolia* and *Berberis aristata* and standard berberine proved to be toxic at lower doses (60 to 80 ug/ml). Whereas standard psoralen and hexane extract of *P. corylifolia* proved to be toxic at mid doses (100 to 200 ug/ml).

4.12 Evaluation of Anti-psoriatic activity in experimental animals using Imiquimod induced psoriasis –Like Dermatitis Rat model

Antivpsoriatic Activity

The results of the present investigation showed that hexane and methanol extracts of *P. corylifolia* and methanolic extract of *Berberis aristata* have antipsoriatic activity.

4.12.1 Effect of IMQ-Induced Psoriasis-Like Dermatitis in Rat:

The dorsal skin of rats showed symptoms of erythema, scaling, and thickening two or three days after initiating IMQ administration. Following then, the degree of psoriasis-like symptoms in the groupII (disease control) rat gradually rose till the end of the treatment (day 16). However, rats in the daily Vaseline-treated group showed no symptoms of irritation on the dorsal skin or right ear pinna.

The independent PASI scores depicted in Fig. 4.40-4.42 show the continually increasing levels of inflammation after IMQ application from day 1 to day 7, before

initiation of either clobetasol or phytopharmaceutical treatment. The PASI scores reached peak intensity at the seventh day after IMQ treatment which indicates successful induction of psoriasis-like dermatitis in the IMQ-treated rat.

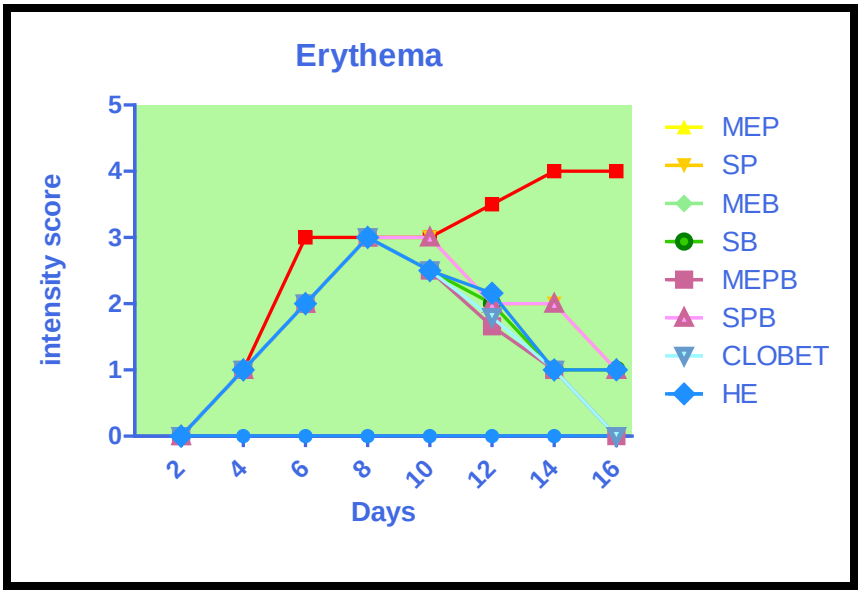


Figure 4.40, Graph of PASI score showing intensity of Erythma in treated groups

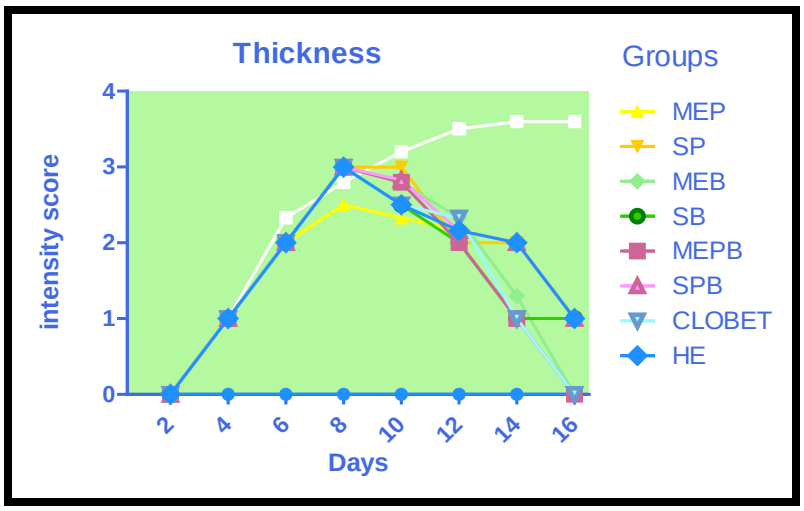


Figure 4.41, Graph of PASI score showing intensity of Thickness in treated groups

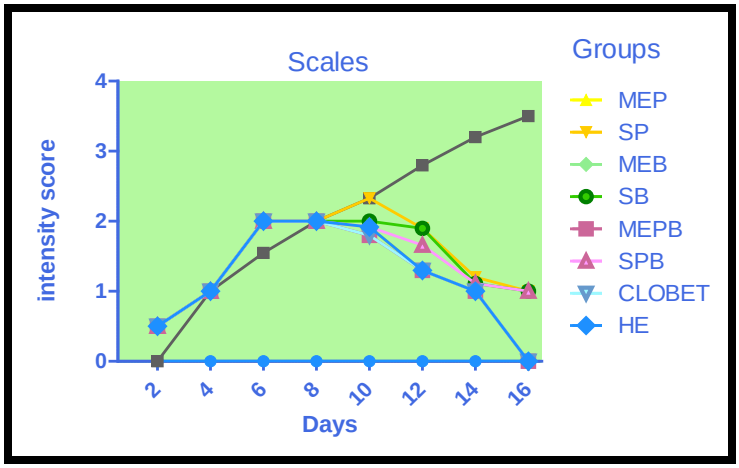
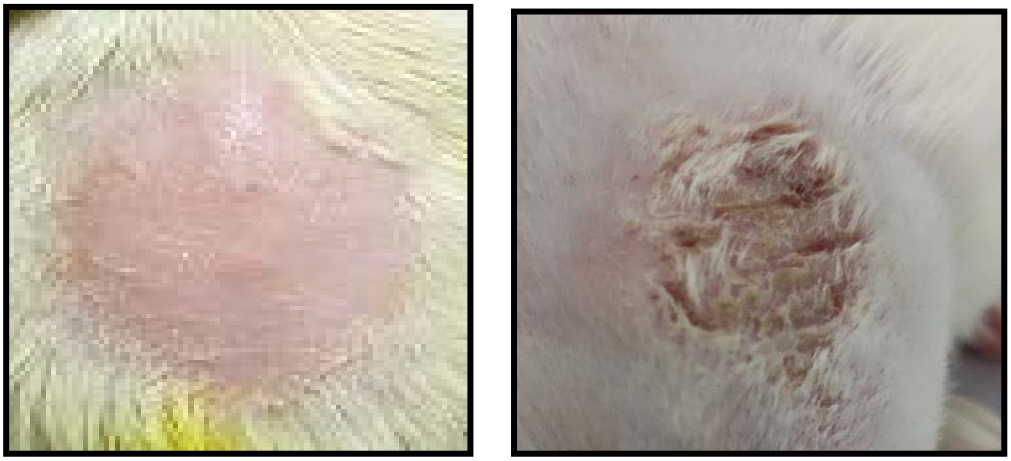


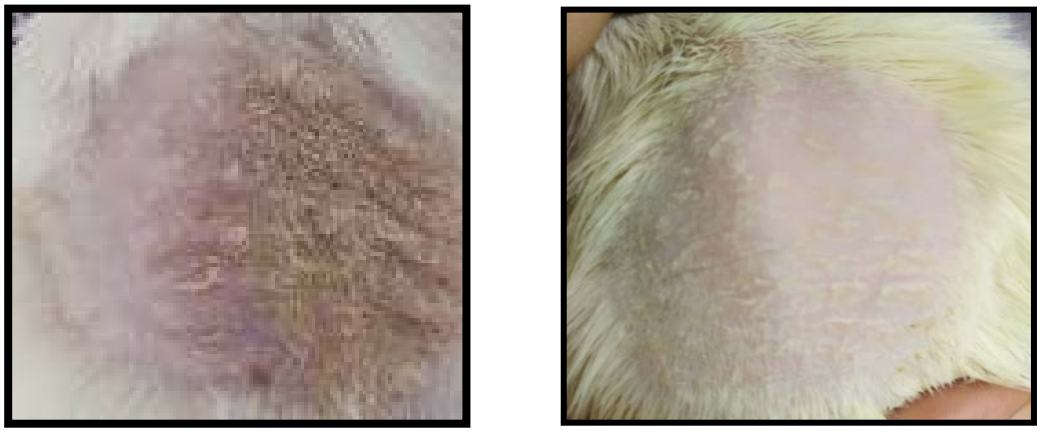
Figure 4.42, Graph of PASI score showing intensity of Scaling in treated groups

Figures are mean ± SD of six rats in each group. PASI intensity scores recorded on days 1 to 16 for all groups



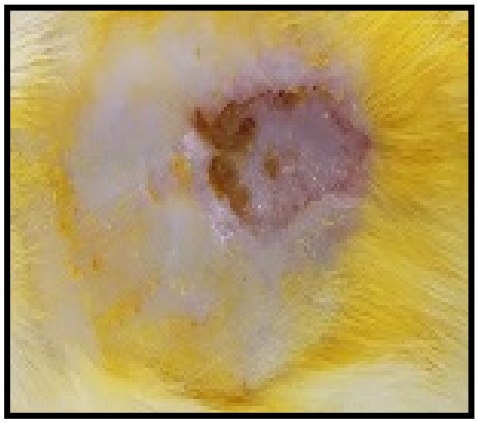
Control (NC) **Disease Control (DC)**

Day 1 **Day 7**

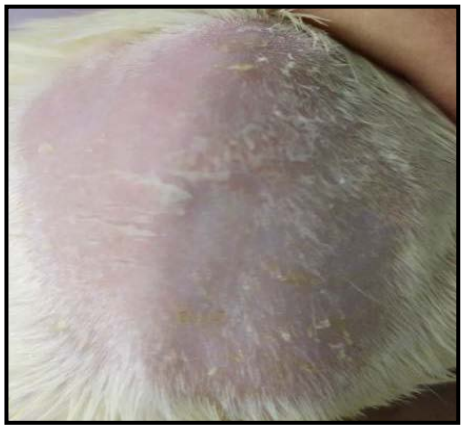


Day 7 **Day 17**

MeOH extract *Berberis aristata* (MEB)

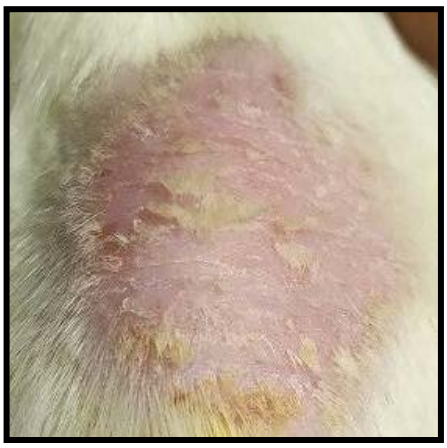


Day 7

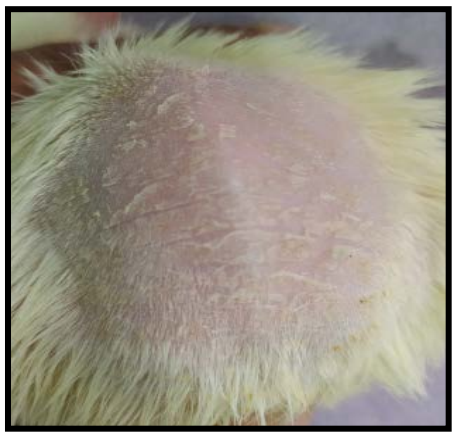


Day 17

STD Berberine (SB)

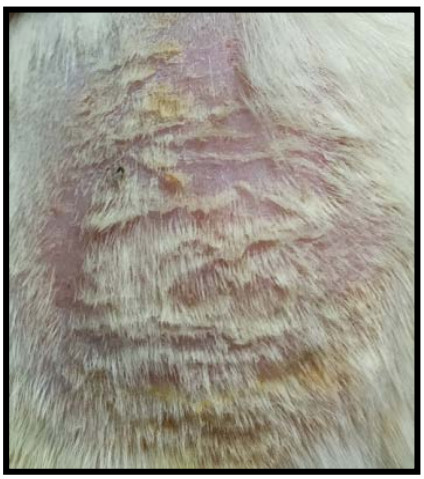


Day 7

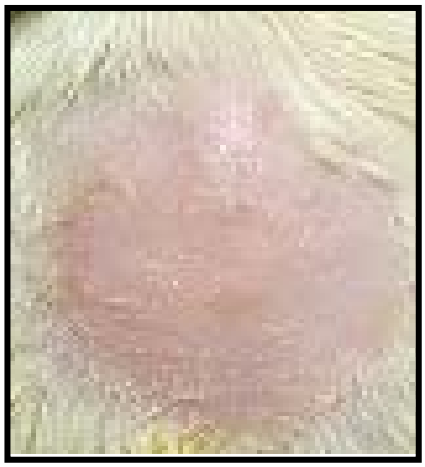


Day 17

Hexane extract of *P. corylifolia* (HEP)

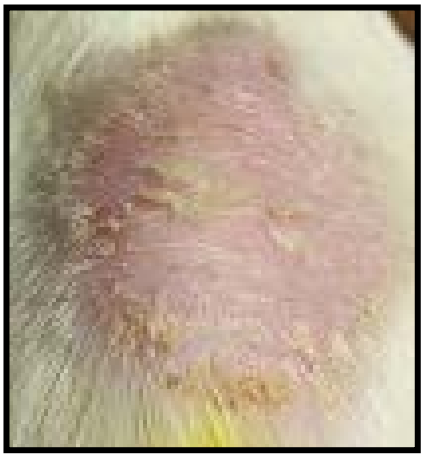


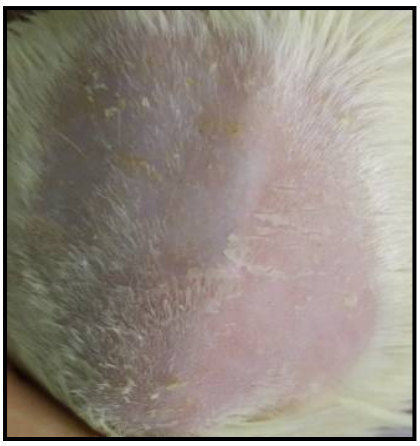
Day 7



Day 17

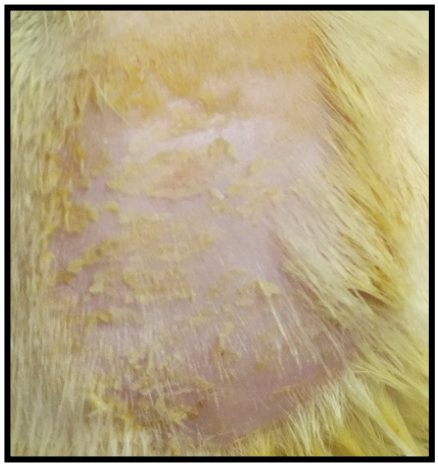
Mixture of MeOH extract of *P. corylifolia* & *Berberis aristata* (MEPB)





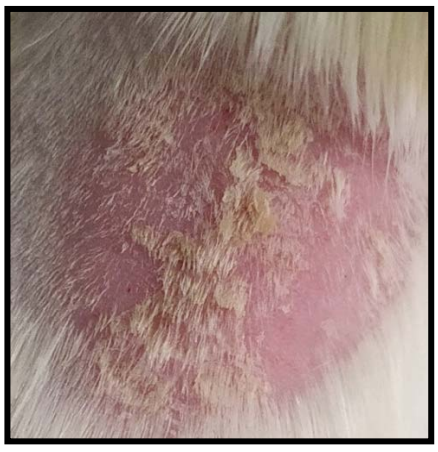
Day 7 **Day 17**

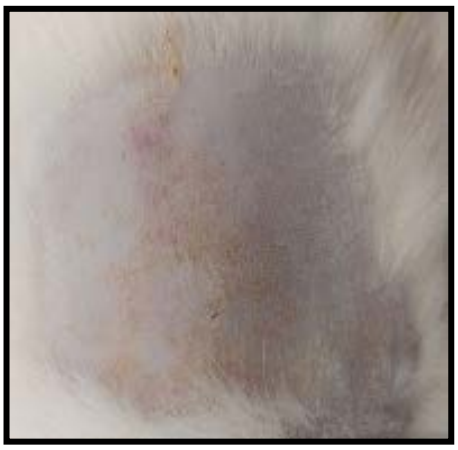
MeOH extract of *P. corylifolia* (MEP)



Day 7 **Day 17**

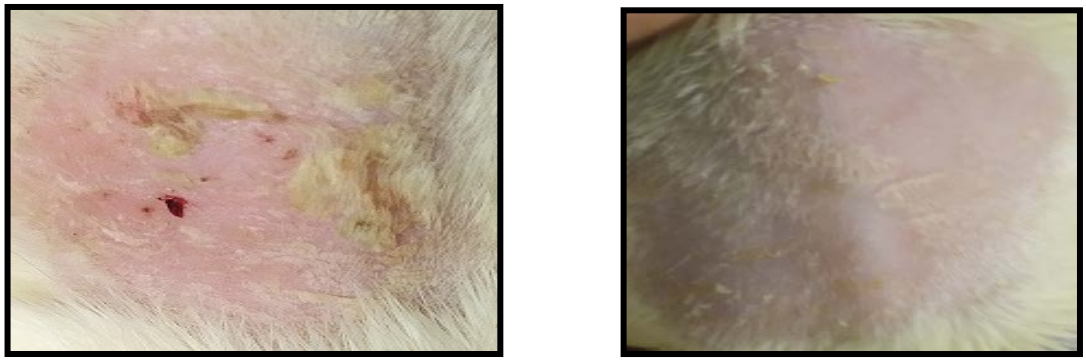
Standard Psoralen (SP)





Day 7 **Day 17**

Reference Standard Clobetasol (CLOBET)



Day 7 **Day 17**

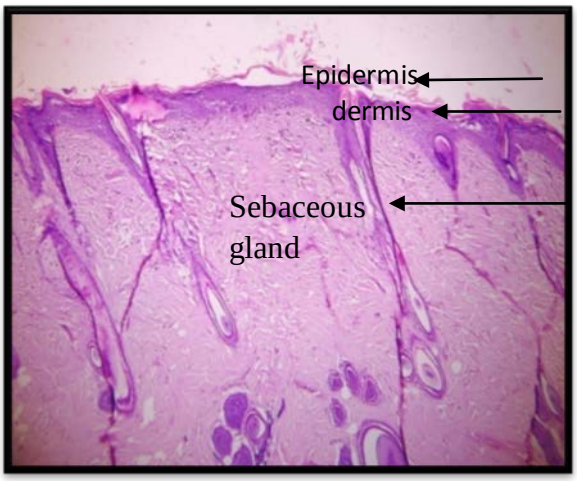
Mixture of Standard Psoralen &Berberine (SPB)

Figure 4.42, Phenotypical Observations of WCR-Treated IMQ-Induced Psoriasis-Like Dermatitis in Rat

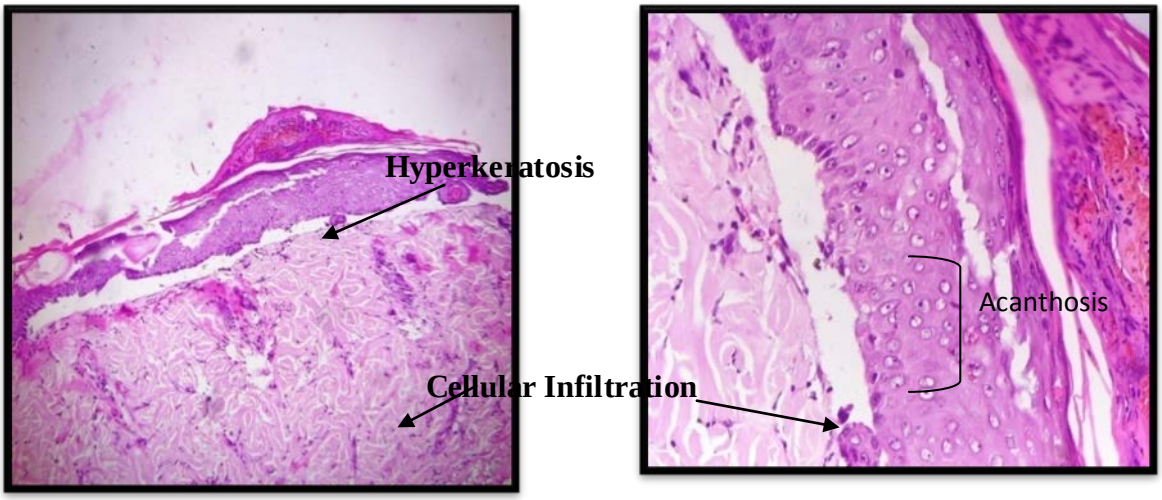
The phenotypic presentations of phytopharmaceutical-treated IMQ-induced inflammation with structural features characteristic of psoriasis show that erythema, thickening, and scaling appeared on the dorsal skin 2 to 3 days after the first IMQ application, with maximum inflammatory severity occurring on days 7 and 8. From day 1 to day 16, the intensity of the psoriasis-like symptoms in the IMQ-only treated group (group II) steadily increased. However, starting on day 9, the second day after starting therapy with extracts or actives, there was a statistically significant reduction in psoriasis-like symptoms. The symptoms consistently declined until day 16, the end of the phytopharmaceuticals or actives treatment. Comparing with the IMQ-treated group, all treated groups showed a significant inhibitory effect on IMQ-induced psoriasis-like dermatitis.

4.12.2 Histopathological Analysis:

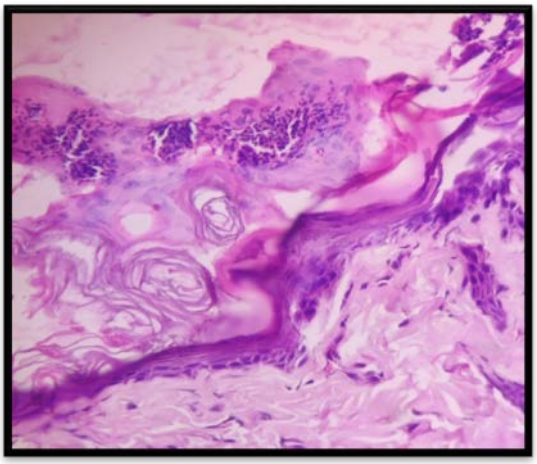
H&E stained slices of IMQ-treated dorsal skin were found to be consistent with phenotypical observations and PASI score values. The IMQ-treated rat's dorsal skin sections showed significantly enhanced acanthosis, epidermal hyperkeratosis, and inflammatory infiltration. The dorsal skin of control rat slices, on the other hand, was normal in both epidermis. In contrast to the IMQ-treated rat, epidermal thickness was significantly reduced in the phytopharmaceuticals or actives-treated rat.



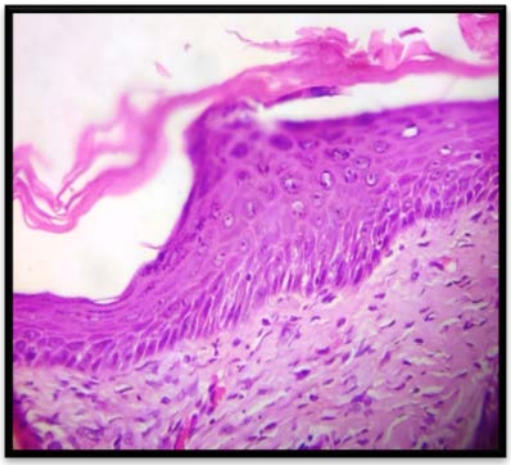
Controlled dorsal skin tissue treated with vaseline showing normal epidermis, dermis and sebaceous gland.



Dorsal skin section of IMQ treated rat showing acanthosis and hyper keratosis of the epidermis with cellular infiltration



MEP



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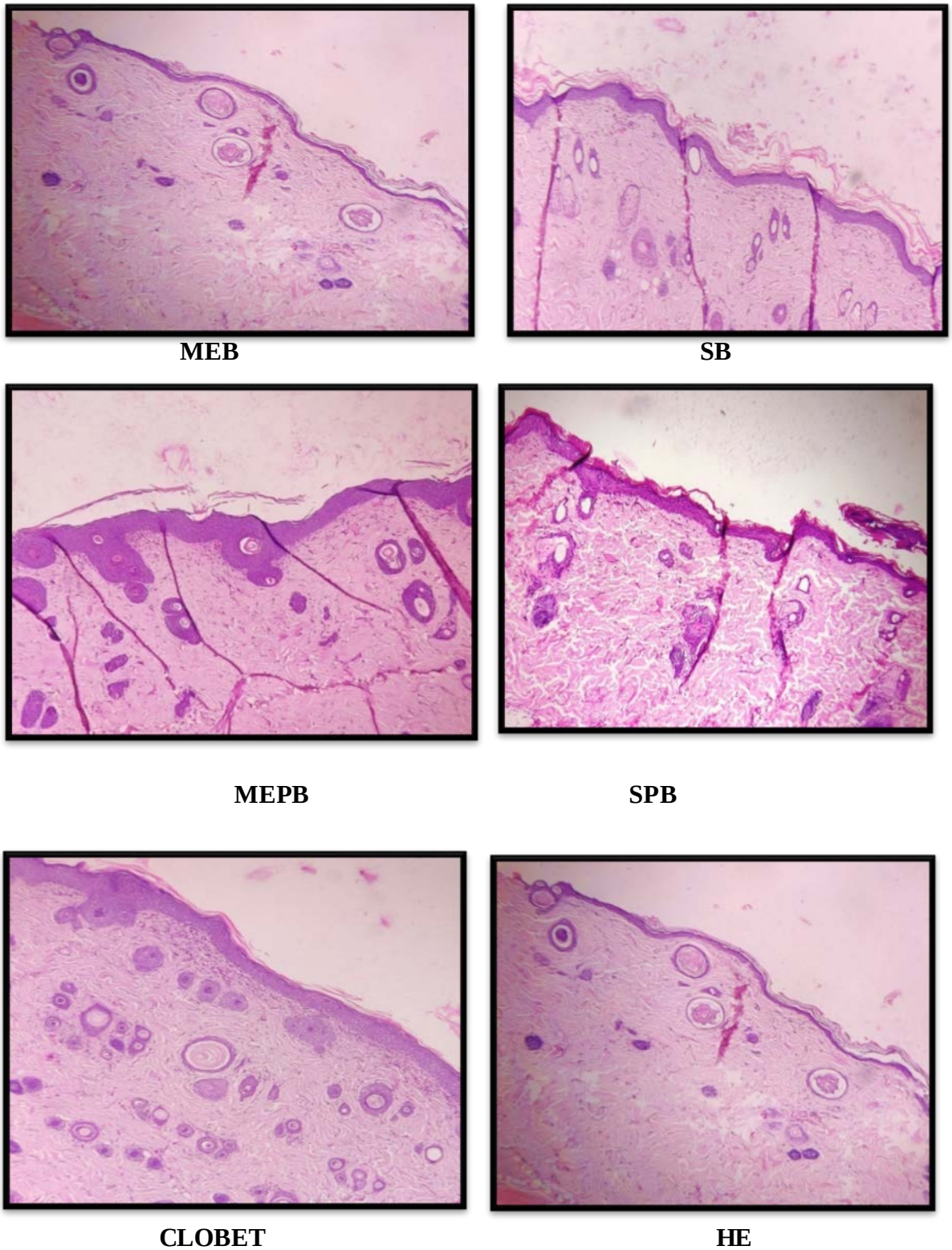

Figure 4.43, Histological examinations of the ten different groups, stained with hematoxylin and eosin (H&E). (a) H&E stained dorsal skin of control and IMQ-treated rat.

Effect of WCR Treatment on the Ratio of Spleen Weight to Bodyweight

The size and weight of the spleen were markedly enlarged in IMQ-induced psoriasis rat. The ratio of spleen weight to bodyweight was significantly decreased in the phytopharmaceutical or actives treatment group, even not to the level of the ratio of the control group.

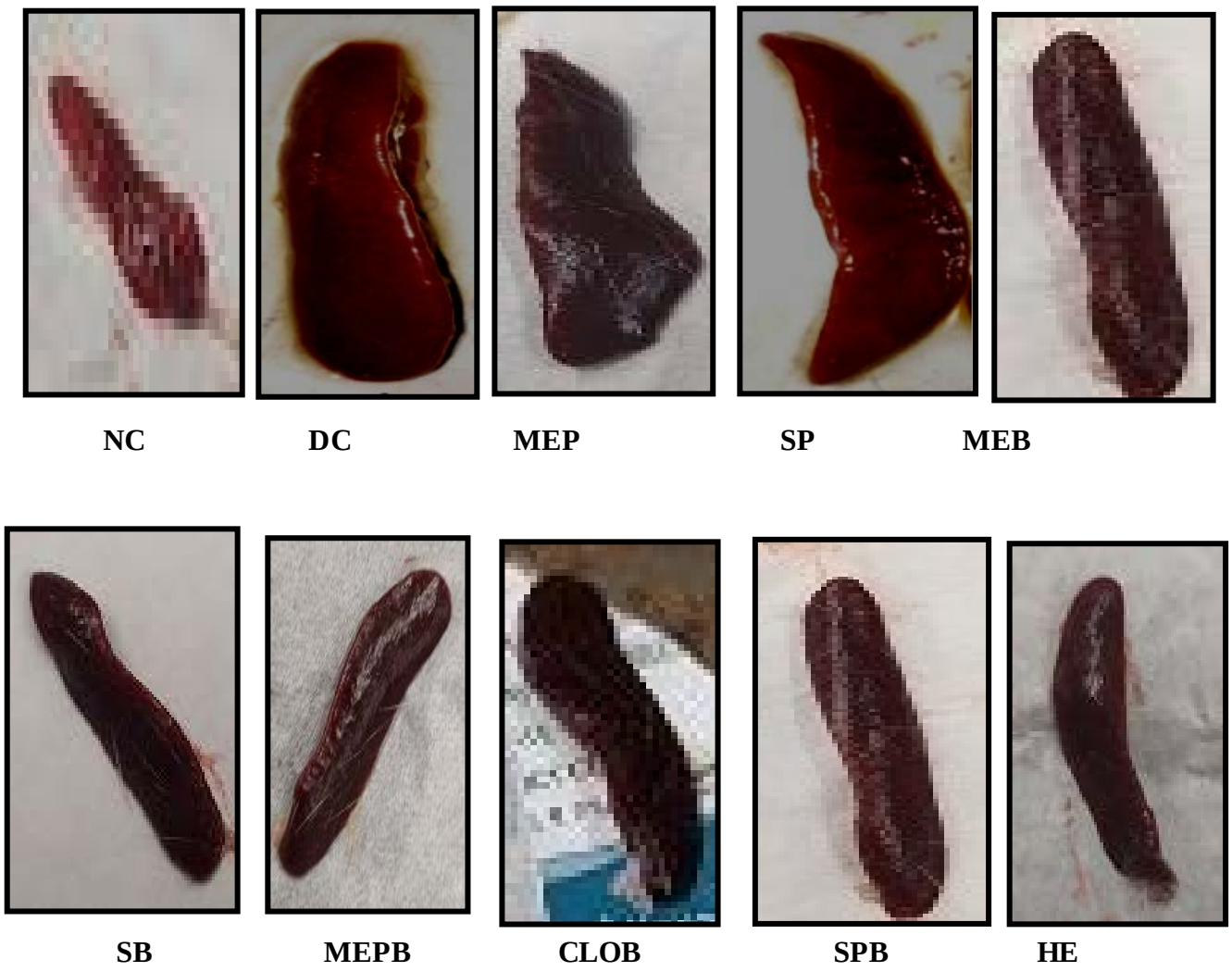

Figure 4.44, Effect of treatment on the ratio of spleen weight to bodyweight. Representative photographs of IMQ-induced rat: normal, untreated (IMQ alone), treated with different treatment groups

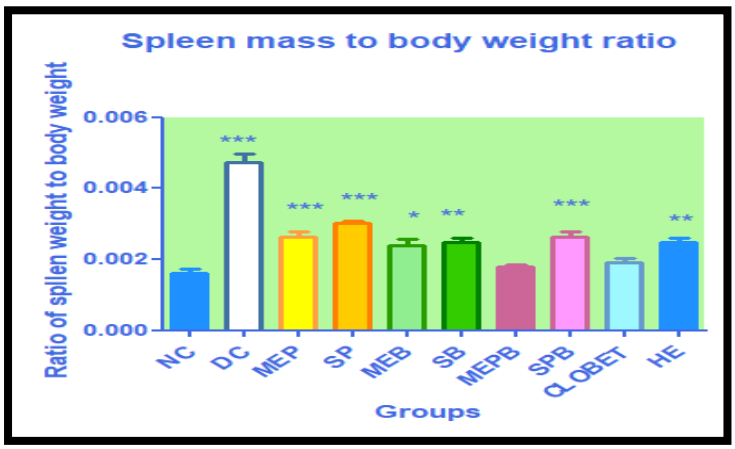

Figure 4.45 The ratio of spleen weight to bodyweight was determined. Data presented are mean ± SD (n = 6) ∗ P< 0.005 indicates a statistically significant difference from the IMQ group.

4.12.3 Effect of WCR Treatment on Increased Inflammatory Cytokines in Serum and Skin of IMQ-Induced Psoriasis- Like Dermatitis in rat:

All the groups were studied for Cytokines analysis (IL-17a, IL-23 and TNF-α) as mentioned in Table 4.72-4.74.

TABLE 4.39. Effect of phytopharmaceuticals on IL-17a cytokines in serum and tissue of IMQ induced psoriasis like dermatitis in rat.

Group no.	Group name	No. of animals	Conc. in serum (Mean)	S.D	Conc. in tissue (Mean)	S.D
1	N	6	3.393	0.976	8.107	0.961
2	DC	6	11.357	1.989	43.702	6.107
3	MEB	6	4.976	2.491	15.583	4.018
4	MEBP	6	7.190	4.209	18.929	5.067
5	HEP	6	7.000	4.153	21.131	4.690
6	MEP	6	8.881	4.072	24.893	5.159
7	SBP	6	9.750	4.733	28.845	6.508
8	SB	6	10.060	5.022	33.714	6.310

9	SP	6	10.714	5.465	38.012	6.137
10	CLOBET	6	5.940	3.075	15.940	4.272

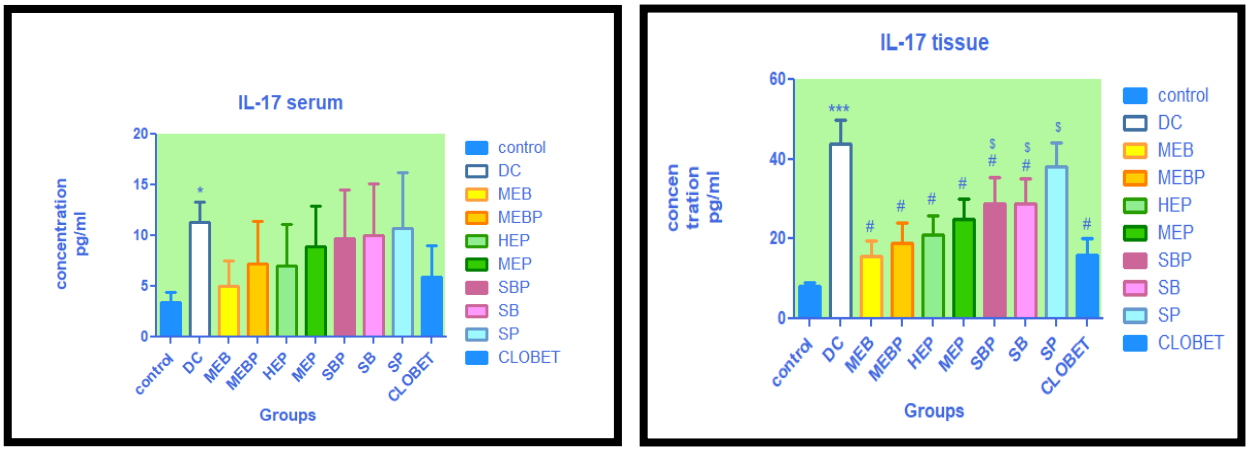

Figure 4.46, IL-17 in serum & Tissue, values are expressed as the mean ± SD (n = 6);P < 0.05 of treatment groups compared to normal control (one-way ANOVA followed by a Bonferrni-test.* Indicates significant difference from control group.# Indicates significant difference from disease control group. $ Indicates significant difference from Standard treatment group

TABLE 4.40. Effect of phytopharmaceuticals on IL-23 cytokines in serum and tissue of IMQ induced psoriasis like dermatitis in rat

Group no.	Group name	No. of animals	Conc. in serum (Mean)	S.D	Conc. in tissue (Mean)	S.D
1	N	6	0.286	0.052	0.316	0.112
2	DC	6	3.013	0.369	2.712	0.540
3	MEB	6	0.710	1.125	0.778	1.077
4	MEBP	6	1.527	1.301	0.981	1.018
5	HEP	6	1.731	1.329	1.340	1.056
6	MEP	6	1.822	1.371	1.325	1.082
7	SBP	6	1.972	1.454	1.544	1.126
8	SB	6	2.064	1.282	1.691	1.320
9	SP	6	2.157	1.356	1.981	1.428
10	CLOBET	6	0.741	1.098	0.740	1.093

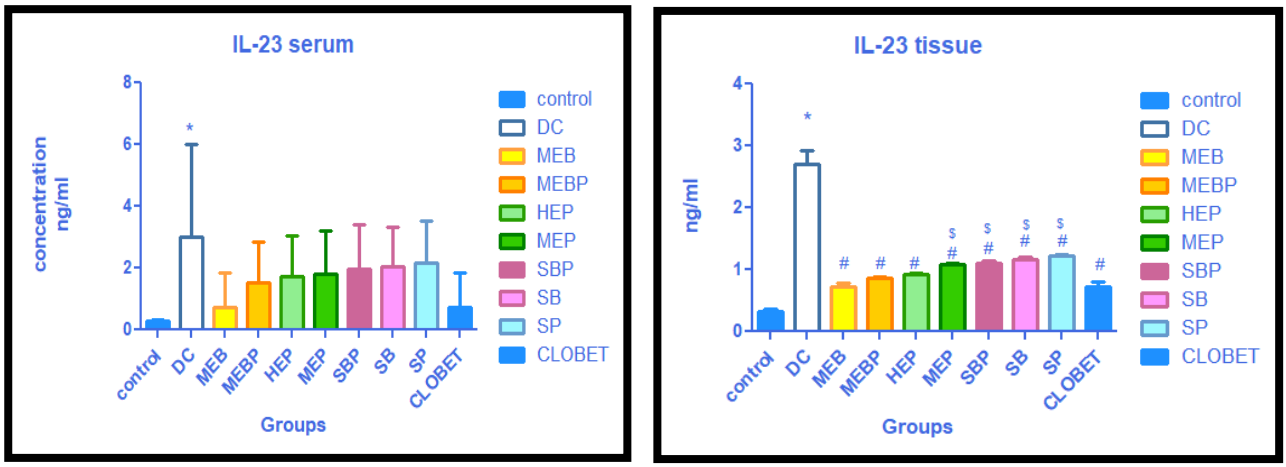

Figure 4.47, IL-23 in serum & Tissue, values are expressed as the mean ± SD (n = 6);P < 0.05 of treatment groups compared to normal control (one-way ANOVA followed by a Bonferrni-test.* Indicates significant difference from control group.# Indicates significant difference from disease control group. $ Indicates significant difference from Standard treatment group

TABLE 4.41. Effect of phytopharmaceuticals on TNF-α cytokines in serum of IMQ induced psoriasis like dermatitis in rat

Group no.	Group name	No. of animals	Conc. in serum (Mean)	S.D	Conc. in tissue (Mean)	S.D
1	N	6	13.889	1.377	17.000	1.825
2	DC	6	40.000	5.370	53.611	14.373
3	MEB	6	19.278	4.144	22.722	6.611
4	MEBP	6	22.167	5.604	26.500	5.089
5	HEP	6	25.056	5.990	30.111	5.455
6	MEP	6	28.389	5.705	34.333	5.545
7	SBP	6	31.111	6.768	37.500	6.130
8	SB	6	35.722	5.174	41.167	5.954
9	SP	6	39.111	6.163	5.882	5.882
10	CLOBET	6	20.444	6.431	22.556	5.344

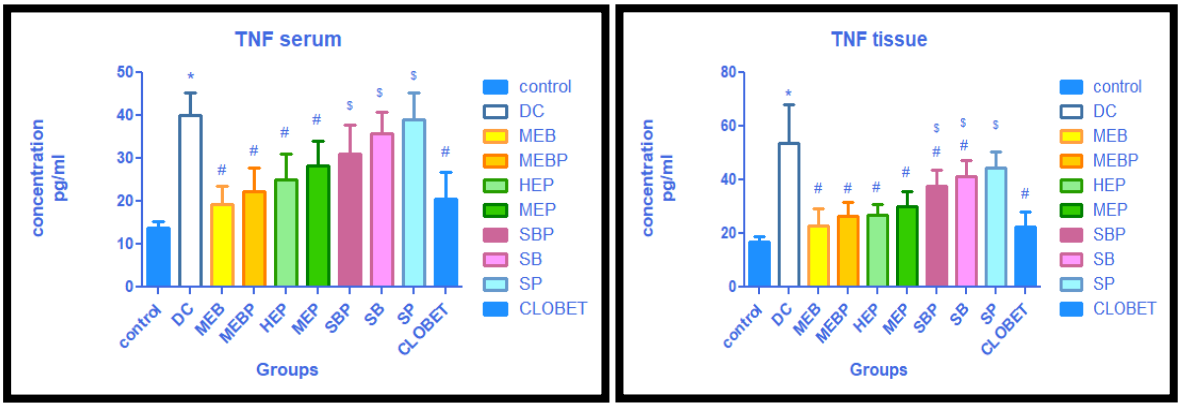

Figure 4.48, TNF-α in serum & tissue, values are expressed as the mean ± SD (n = 6); P < 0.05 of treatment groups compared to normal control (one-way ANOVA followed by a Bonferrni-test.* Indicates significant difference from control group.# Indicates significant difference from disease control group. $ Indicates significant difference from Standard treatment

Inflammatory cytokines responsible for inflammation in psoriasis were measured in the serum and skin samples and reduction in the expression of IL-17A, IL-23 and TNF-α was noted in the normal, IMQ-treated rat and phytopharmaceuticals or actives - treated psoriasis-like dermatitis-induced rat by ELISA assay. Compared to the normal group, the levels of Th17- mediated cytokines, particularly IL-17A, IL-23 and TNF-α were significantly higher in dorsal skin samples from IMQ-treated rat. However, a marked reduction was noted in phytopharmaceuticals and actives treated groups compared to the IMQ-only treated group. There was no significant difference of IL-17A, IL-23 and TNF-α in serum samples compared to normal group.

Psoriasis is a chronic inflammatory skin disease accompanied by excessive keratinocyte proliferation. Corticosteroids, vitamin D3 analogs, and calcineurin inhibitors, which are used to treat psoriasis, have diverse adverse effects[231, 232], whereas natural products are popular due to their high efficiency and relatively low toxicity. *P. corylifolia,* psoralen, *Berberis aristata,* berberine and combination of *P. corylifolia, Berberis aristata,* significantly decreased the Psoriasis Area and Severity Index (PASI), erythema, scaling, reduced epidermal thickness, and decreased the proliferation and differentiation of epidermal cells in psoriasis-like dermatitis rat model induced by imiquimod (IMQ). Attenuation of IMQ-induced upregulation of inflammatory cytokines including IL-17A and IL-23 and tumor necrosis factor-α (TNF-α) was observed in skin lesion of rat treated with them.

P. corylifolia L. is an important medicinal plant used in Asian countries as a kidney-tonifying herbal medicine for treating various diseases such as leucoderma, osteoporosis, and inflammatory skin diseases[233]. Furanocoumarins are considered as important group of chemicals of this plant useful for the photochemotherapeutic treatment of certain skin diseases, lymphomas and autoimmune disorders[234-235]. The ability of these chemicals to photoinduce specific DNA lesions is linked to their antiproliferative activities. Because of their direct antiproliferative impact on keratinocytes, they are effective in the treatment of psoriasis. Psoralens attach to DNA, with pyrimidine nucleotides serving as photochemical apoptosis targets18. Previous research has shown that antiproliferative activity against keratinocytes for compounds like 8-methoxy psoralen, psoralen, isopsoralen, psoralidin, and bakuchiol can reduce psoriasis-like lesions by reducing epidermal thickening and cytokine

release, which can help to alleviate psoriasis-like lesions. The antipsoriatic activity of these compounds was due to a reduction in keratinocyte proliferation, with 8-MOP and isopsoralen inhibiting keratinocyte growth more than the others. Both furonocoumarins could be considered safe for topical application[19]. In the J774A.1 cell line and mouse peritoneal macrophages, Bavachin has been shown to inhibit LPS-induced IL-6 and IL-12p40 production. As a result, it may be useful in reducing LPS-induced inflammatory reactions[238]. Our study on imiquimod induced psoriasis like lesions in rat revealed that the methanolic extract have more potency to cure psoriasis than standard psoralen and hexane extract of psoralen. These could be possibly due to presence of collection of diverse groups of bioactives in it, namely furanocoumarins, meroterpenes, sterols, falvanoids etc. and as mentioned above compounds like 8-methoxy psoralen, psoralen, bakuchiol, bavachin and others.

Berberis aristata is one among many herbs that have been widely used in traditional medicines for skin diseases[239]. Isoquinoline alkaloids, specifically Berberine inhibits PGE2 generation by lowering cyclooxygenase-2 concentrations. Interferon-, interleukin-1 (IL-1), tumour necrosis factor (TNF- α), interleukin-2 (IL-2), interleukin-8 (IL-8), interleukin-6 (IL-6), and monocyte chemotactic protein-1 (MCP-1) are all inhibitors of delayed hypersensitivity (MCP-1). Berberine can treat alloantigen-induced autoimmune nephritis in rats, including primary anti-glomerular basement membrane and Heymann nephritis. It can reduce serum creatinine levels and glomerulus degenerative alterations by inhibiting urine protein excretion[240-242].

Berberine inhibits the activation of nuclear factor kappa-B (NF-B) and inflammatory cytokine transcription. It can also diminish the infiltration of inflammatory cells and lesions by inhibiting the adherence of polymorphonuclear leukocytes and vascular endothelial cells triggered by TNF-α[243, 244]. Berberine along with other isoquninoline alkaloids are present in *Berberis aristata* root methanolic extract and thus, its effect is elevated in imiquimod induced psoriasis –like dermatitis in rat model. When *P. corylifolia* and *Berberis aristata* methanolic extracts were given in combination to the rats, synergistic effects were seen. The study revealed that *P. corylifolia* seeds and *Berberis aristata* roots in combination as a phytopharmaceutical preparation could be used as natural therapeutic agent for the treatment of *Psoriasis vulgaris*.

Chapter 5

Conclusion

Psoralea corylifolia and *Berberis aristata* were collected, authenticated and used for the present study. Important features that be considered for morphological identification in case of seeds of *P. corylifolia* includes its dorsiventrally flattened oval shape, dark brown to black in colour, with shiny pits whereas for *B. aristata* root yellow colour, thick wood covered with a thin brittle barkand granular fracture.

The microscopical characteristics exhibited in powder microscopy of *P. corylifolia* and *B. aristata* powders were in line with the reported data. Of the different extraction techniques opted to get maximum amount of extractives, Soxhlet method yielded the highest percent of 31.73% and 8.9% of methanolic extracts of *P. corylifolia* and *B. aristata* respectively. Plants synthesize numerous organic compounds having complex chemical structures that are known to play a crucial role in their ecological functions. Thus, isolation of the bioactives present in *P. corylifolia* seed was attempted using column chromatography followed by purification by flash chromatography leading to psoralen, bakuchiol, 5-hydroxy xanthotoxin and methyl 4- hydroxyl benzoate.

HPTLC has been explored for simultaneous assay of several components in a multi-component formulation. Through this technique, authentication of various species of plant is also possible. Bavachin, bakuchiol and psoralen that fall under three different groups of chemical categories viz., flavanoids, meroterpenes and coumarins respectively were estimated in the methanolic extract of *P. corylifolia* by a simultaneous HPTLC method tailored and validated according to ICH guidelines. Similarly, estimation of isoquinoline alkaloids namely, berberine, berbamine, palmatine, magnoflorine, jatrorrhizine in the methanolic extract of *B. aristata* was done concurrently by a customized HPTLC method. Both the methods gave good resolution of bands and are simple, precise and reproducible and can be employed for routine analysis of these two commercially highly valued medicinal plants.

The advent of hyphenated analytical techniques like UHPLCTQ-MS/MS has aggrandized

qualitative and quantitative analysis of bioactive phytoconstituents with precision. Thus, UHPLC-PDA with ESI- MS-MS method was developed for both the plants. Daidzein, psoralen, isopsoralen, neobavaisoflavone, bavachin, psoralidin, bavachinin and bakuchiol in *P. corylifolia*, and magnoflorine, berbamine, columbamine, jatrorrhizine, palmatine and berberine in *B. aristata* were quantified and inveterated by MS-MS.

Results of both the methods were in agreement of each other and confirmed bakuchiol as major bioactive in *P. corylifolia*, and berberine in *B. aristata*.

MTT assay using THP-1 cell line for both the extracts and bioactives (psoralen and berberine) of both the plants confirmed their cytotoxic potential with the IC50 values being 98.4 µg/ml, 74.66 µg/ml for psoralen and the methanolic extract of *P. corylifolia*, 83.03 µg/ml and 80.51 µg/ml for berberine and methanolic extract of *B. aristata*.

Psoriasis is a noncommunicable and immune-mediated inflammatory skin disorder, which is characterized by sharply demarcated, red, scaly plaques most often on the elbows, knees, scalp, and lumbar area. Symptoms include scaling, itching, erythema, burning and bleeding. Strong evidence exists that the cell-mediated adaptive immune system, T helper 17 (Th17), plays critical roles in psoriasis, while myeloid cell-produced interleukin-23 (IL-23) functions as a key cytokine for the expansion and maintenance of Th17 cells. Th17 cells and their downstream effector molecules, which include IL-17A, IL-22, and TNF-α, have been shown to induce keratinocyte proliferation and other hallmark features of psoriasis.

P. corylifolia and *B. aristata* contains furanocoumarins and isoquinoline alkaloids that are recorded as strong antioxidants and can significantly reduce the expression of the NF-κB signaling biomarker leading to a decrease of cytokine production by T cells. Psoralen, which is isolated from *P. corylifolia*, has been shown to exert significant inhibitory effects on TNF-α, and IL- 6 production through p65, ERK1/2, and JNK pathways. In addition, anti-inflammatory and antioxidant activities have been well documented for *P. corylifolia* and *B. aristata*. As psoriasis is an immune mediated inflammatory skin disorder, this extensive evidence of pharmacological activity may be indicative of the potential for using of both these plants in treatment of psoriasis. IMQ is an agonist of the

toll-like receptor-7/8 (TLR-7/8) which has been approved for the treatment of actinic. keratosis, external genital warts, and superficial basal cell carcinoma. IMQ-induced psoriasis-like dermatitis in mouse model is mediated through the IL-23/IL-17 axis has been described as closely resembling human plaque-type psoriasis with respect to inflammatory infiltration, redness, thickening and scaling of the skin. To treat psoriasis, established conventional systemic drugs such as methotrexate, cyclosporine, and acitretin have been the first line of treatment. However, these agents seem to cause many serious adverse effects that some patients might be intolerant of during long-term treatment. Therefore, there is a demand for new effective and safe therapeutic methods. Thus, phytopharmaceuticals were designed to overcome these side effects.

Inflammatory cytokines responsible for the inflammation in psoriasis were measured in the serum and skin samples and significant reduction in the expression of IL-17A, IL-23 and TNF-α was noted in the extracts or actives treated experimental IMQ-induced psoriatic rat. Compared to the normal group, the levels of Th17- mediated cytokines, particularly IL-17A, IL-23 and TNF-α were significantly higher in dorsal skin samples from IMQ-treated animals. However, a marked reduction was noted in extracts and actives treated groups compared to the disease control (IMQ-only treated) group.

Animals treated with mix of psoralen and berberine, berberine alone and methanolic extract of *P. corylifolia* were found to be giving ameliorative effects against IMQ induced psoriasis like dermatitis. However, hexane extract of *P. corylifolia* and psoralen had lowest level of anti-psoriatic effect compared to other groups. Interestingly, animals treated with combination of methanolic extracts of *P. corylifolia* and *B. aristata* showed effects similar to those of clobetasol treated rat where almost complete recovery from the IMQ-induced hyperplasia of the epidermal tissue with only minor inflammatory reaction was observed.

The present study is important in the view of the scientific validation for the traditional medicinal claims of both the plants and their bioactives. Further, the indication of the stronger activity potential of the extracts (probably due synergistic action of multi-component nature) over the single bioactives would probably be of more value in terms

of their commercial utility (single markers are costly) and safety for incorporation in to formulations.

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List of Publications

❖ **Poster Presentation:**

1. "Quantification of psoralen from *Psoralea corylifolia* L. using High Performance Thin Layer Chromatography". Basera IA, Shah MB at international conference on herbal medicine; research and commerce-global perspectives at L.M college of Pharmacy, Ahmedabad, Gujarat on 27th – 29th December 2019.

❖ **Oral Presentation:**

1. "Isolation and quantification of bioactives from *Psoralea corylifolia*" Basera IA, Shah MB at AICTE sponsored International Conference on "Biomolecular and biotechnological aspects of medicinal plants" at L.M college of Pharmacy, Ahmedabad, Gujarat on 18th & 19th October 2019. (Won 2nd Prize)

❖ **Publications:**

Research Article:

1. Basera IA, Shah MB, 2020, A validated high–performance thin–layer chromatography method for quantification of bavachin, bakuchiol, and psoralen from *Psoralea corylifolia* seeds, JPC- J Planar Chromat.

2. Basera IA, Girme V, Bhatt V, Shah MB, 2020, A validated high-performance thin-layer chromatography method for the simultaneous estimation of berberine, berbamine, palmatine, magnoflorine and jatrorrhizine from *Berberis aristata*, JPC- J Planar Chromat, 34, 147-155.

3. Basera IA, Girme V, Bhatt V, Saste G, Pawar S, Hingorani L, Shah MB, 2021, Development of validated UHPLC–PDA with ESI–MS-MS method for concurrent estimation of magnoflorine, berbamine, columbamine, jatrorrhizine, palmatine and berberine in *Berberis aristata*, Acta Chromatographica.

Gujarat Technological University

PhD *Viva Voce* Report

TITLE OF THE THESIS: Screening and identification of Phytopharmaceuticals for mitigation of *Psoriasis vulgaris*

Name of the Scholar	Enrollment No.	Day & Date of Public Viva Voce	Discipline/ Branch	Venue
Ms. Ishita ArvindSingh Basera	169999901002	Friday, 02/09/2022	Pharmacy	GTU

Based on the thesis defense of above mentioned PhD Thesis, the overall recommendation on the thesis is as follows (Please tick any one of the following option):

☐ The performance of the candidate was satisfactory. We recommend that he/she be awarded the PhD Degree.

☐ Any further modifications in research work recommend by the panel after 3 months from the date of first *viva-voce* upon request of the Supervisor or request of Independent Research Scholar after which *viva-voce* can be re-conducted by the same panel again. The suggestions for improving the thesis based on the discussions during the oral examination is detailed in a separate sheet to be incorporated in the thesis.

☐ The performance of the candidate was unsatisfactory. We recommend that he/she should not be awarded the PhD Degree. A separate sheet is enclosed describing unsatisfactory performance.

Further, it is certified that the examiner who participated in the thesis defense through electronic medium (if any), have confirmed the above recommendation after the *viva-voce* (through email as attached; if any) and the same may be considered sufficient record for acceptance.

BOARD OF EXAMINERS:

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1.		**Supervisor/ Co-Supervisor***		
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*The Co-Supervisor may sign in place of Supervisor if he/she has been assigned with the academic and administrative affairs/ responsibilities of the above mentioned scholar.

Encl.:

1) Separate sheet for suggestions / comments on the thesis (if any) endorsed by the Supervisor/ Co-Supervisor and the external examiners. The same to be provided to the scholar for revision/ modification in the thesis.
2) Email of external examiner (if any) who participated in the thesis defense through electronic medium.
3) Undertaking for final submission of hard copy of Ph.D. thesis & CD.

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Honorable Vice Chancellor