FORMULATION AND EVALUATION OF HERBAL OINTMENT ON DIFFERENT EXTRACT OF WHOLE PLANT OF *Indigofera aspalathoides.vahl.ex*.DC., TO TREAT PSORIASIS

A dissertation submitted to

THE TAMILNADU Dr. M.G.R MEDICAL UNIVERSITY

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

MASTER OF PHARMACY

IN

PHARMACOGNOSY

Submitted by

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Under the guidance of

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MAY 2017

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CERTIFICATE

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DR. R. RADHA M.Pharm., Ph.D., M.B.A.,

Place: Chennai-03

Date:

CONTENTS

LIST OF ABBREIVATION

Anti	-	TNF Anti-tumor necrosis factor
BMI	-	Body mass index
BSA	-	Body surface area
CD4	-	Tcells T helper lymphocytes which express the surface protein CD4
CD8+	-	Tcells T cytotoxic lymphocytes which express the surface protein CD8
CsA	-	Ciclosporin
DLQI	-	Dermatology life quality index
HLA	-	Human leucocyte antigen-Cw6
IL	-	Interleukin
PASI	-	Psoriasis area and severity index
PDI	-	Psoriasis disability index
PEST	-	Psoriasis epidemiology screening tool
PLSI	-	Psoriasis life stress index
PT	-	Physiotherapy
PUVA	-	Psoralen-ultraviolet light A
RCT	-	Randomized controlled trial
RR	-	Relative ratio
SF-36	-	Short Form-36
Th	-	T-helper lymphocytes
TNF-α	-	Tumor necrosis factor-alfa
UV	-	Ultraviolet
UVB	-	Ultraviolet light B
WHO	-	World health organisation

DEDICATED TO MY BELOVED

PARENTS

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Introduction

1. INTRODUCTION

Herbalism

Herbalism is the study of botany and use of plants intended for medicinal purposes or for supplementing a diet. Herbalism is also known as botanical medicine, medicinal herbalism, herbal medicine, herbology, herblore, and phytotherapy[1] .

History of Herbal Medicine:

Plants had been used for medicinal purposes long before recorded history. Ancient Chinese and Egyptian papyrus writings describe medicinal uses for plants as early as 3,000 BC. Indigenous cultures (such as African and Native American) used herbs in their healing rituals, while others developed traditional medical systems (such as Siddha, Ayurveda, Unani and TCM) in which herbal therapies were used[2]. The consumption of plant-based medicines and other botanicals in the West has increased manifold in recent years. About two centuries ago, our medicinal practices were largely dominated by plant-based medicines. However, the medicinal use of herbs went into a rapid decline in the West when more predictable synthetic drugs were made commonly available. In contrast, many developing nations continued to benefit from the rich knowledge of medical herbalism. For example, Siddha & Ayurveda medicines in India, Kampo Medicine in Japan, traditional Chinese medicine (TCM), and Unani medicine in the Middle East and South Asia are still used by a large majority of people[3].

2. Herbal Medicine

Herbal medicines are being used by about 80% of the world population primarily in the developing countries for primary health care. They have stood the test of time for their safety, efficacy, cultural acceptability and lesser side effects. Ancient literature also mentions herbal medicines for age-related diseases namely memory loss, osteoporosis, osteoarthritis, diabetes, immune and liver disorders, etc. for which no modern medicine or only palliative therapy is available[4]. The chemical constituents present in them are a part of the physiological functions of living flora and hence they are believed to have better compatibility with the human body[5]. There are over 1.5 million practitioners of traditional medicinal system using medicinal plants in preventive, promotional and curative applications. Medicinal plants have attracted the attention of not only professionals from various systems of medicine, but also the scientific communities belonging to different disciplines, plants are promising source of herbal formulation[6]. The use of herbal drugs due to toxicity and side effects of allopathic medicines, has led to rapid increase in the number of herbal drug manufacturers. For the past few decades, herbal drugs have been more and more consumed by the people with no prescription. These drugs have survived real world testing and thousands of years of human testing. Some drugs have been discontinued due to their toxicity, while others have been modified or combined with additional herbs to counterbalance side effects[7].

Advantages of Herbal Drugs

- ✓ high Low/Minimum cost
- ✓ complete accessibility
- ✓ enhanced tolerance

- ✓ More protection
- ✓ fewer side-effects
- ✓ Potency and efficiency is very high.

Disadvantages of Herbal Drugs

- ✓ Not able to cure rapid sickness and accidents
- ✓ Risk with self-dosing
- ✓ Complexity in standardizations

Role of herbals in modern human era

Many of the currently available to physicians have a long history of use as herbal remedies. The who estimate that 80 percent of the world's population presently use herbal medicine for some aspect of primary health care .in fact ,according to the world health organization ,approximately 25% of modern drugs used in the united states have been derived from plants.

Among the 120 active compounds currently isolated from the higher plants and widely used in modern medicine today, 80 percent shows a positive correlation between their modern therapeutic use and the traditional use of the plants from which they are derived[8].

1. More than two thirds of the world's plant species –at least 35,000 of which are estimated to have medicinal value –come from the developing countries.
2. At least 7,000 medicinal compounds in the modern pharmacopoeia are derived from plats

The Challenges in Herbal Medicines:[9]

A key challenge is to objectively assess conflicting toxicological, epidemiological, and other data and the verification of herbal materials used. The following key issues remain.

- ✓ Management within ranges of risk
- ✓ Communication of uncertainty
- ✓ Pharmacological, toxicological, and clinical documentation
- ✓ Pharmacovigilance
- ✓ Understanding why addition of harmful additives works
- ✓ evaluating "drug" interactions
- ✓ Constraints with clinical trials and people available
- ✓ Standardization
- ✓ Safety, and efficacy assessment

The evaluation of new herbal products consists of six steps:

1. Characteristics of new substances
2. History and pattern of use
3. Any adverse reaction
4. Biological action
5. Toxicity
6. Clinical trial data

The Constraints in Herbal Medicines:

Constraints associated with the handing of medicinal plants[10].

- ✓ Indiscriminate harvesting and poor post-harvest treatment practices.

- ✓ Lack of research on the development of high-yielding varieties, domestication etc.
- ✓ Poor agriculture and propagation methods.
- ✓ Inefficient processing techniques leading to low yields and poor quality products.
- ✓ Poor quality control procedures.
- ✓ Lack of current good manufacturing practices.
- ✓ Lack of R & D on product and process development.
- ✓ Difficulties in marketing.
- ✓ Lack of trained personnel and equipment.
- ✓ Lack of facilities to fabricate equipment locally.
- ✓ In addition, the processing of herbs, such as heating or boiling, may alter the dissolution rate, or even the pharmacological activity of the organic constituents.
- ✓ Similarly, a host of environmental factors, including soil, altitude, seasonal variation in temperature, atmospheric humidity, length of daylight, rainfall pattern, shade, dew, and frost conditions, may affect the levels of components in any given batch of an herb.
- ✓ Other factors, including infections, insects, planting density, competition with other plant species, seeding time, and genetic factors, play an important role.
- ✓ Plant collection for the use in botanicals is one of the factors of concern for quality. Plants collected in the wild may include non-targeted species, especially either by accidental substitution or intentional adulteration.

- ✓ Adulteration of herbal products can be made in various ways; commonly, adulteration is made by substituting other easily available or cheap plant species or sometimes by spiking of a product with synthetic constituents.

Factors affecting quality & purity of Herbal Medicines:[11,12]

1.Drug adulteration

Adulteration may be defined as mixing or substituting the original drug material with other spurious, inferior, defective, spoiled, useless other parts of same or different plant or harmful substances or drug which do not confirm with the official standards.

Adulteration may takes place by two ways:

- Direct or intentional adulteration
- Indirect or unintentional adulteration

Examples for Adulteration,

A. With artificially manufactured materials, e.g. nutmeg is adulterated with basswood prepared to the required shape and size, the colored paraffin wax is used in place of beeswax.

B. With inferior quality materials, e.g. *Belladonna* leaves are substituted with *Ailanthus* leaves, *papaya* seeds to adulterate *Piper nigrum.*

C. With harmful / fictitious substances drugs, e.g. Pieces of amber colored glass in colophony, limestone in asafetida, lead shot in opium, white oil in coconut oil, cocoa butter with stearin or paraffin.

D. Adulteration of powders, e.g. powder liquorice or gentian admixed with powder olive stones, under the name of cinchona. Etc

2. Faulty collection

3. Imperfect preparation Non removal of associated structures e.g. stems are collected with leaves, flowers, fruits. Non-removal of undesirable parts or structures e.g. cork should be removed from ginger rhizome.

Proper drying conditions should be adhered. Improper drying may lead to unintentional adulteration e.g. if digitalis leaves are dried above 65 °C decomposition of glycosides by enzymatic hydrolysis.

4. Incorrect storage Deterioration happens especially during storage, leading to the loss of the active ingredients, production of metabolites with no activity and, in extreme cases, the production of toxic metabolites. Physical factors such as air (oxygen), humidity, light, and temperature can bring about deterioration directly or indirectly.

5. Gross substitution with plant material

6. Substitution with exhausted drugs

The Opportunities in Herbal Medicines:[13]

1. Medicinal plants cultivation.

2. Medicinal plants Exports.

3. In Drug Manufacturing Companies.

4. Teaching profession - Herbal medicine is being taught more in medical schools and pharmacy schools.

5. In the field of Plant monographs

6. Drug inspectors in ISM.

7. Medical taxonomist

8. Pharmacognosist.

9. Herbalist & Chiropractors

10. AYUSH practitioners , Doctors

11. SRF & JRF in Clinical trials.

12. Clinical and Research opportunities- Without doubt, the therapeutic potential of many herbs is yet to be fully discovered. Example, Recent discovery of 'artemisinins', new class of anti-malarial drugs, in Chinese herbs supports this assertion.

13. Carrier options in the various newer fields. E.g. Molecular biology, Nano technology etc.

PSORIASIS

Psoriasis is a long-lasting autoimmune disease which is characterized by patches of abnormal skin[14].These skin patches are typically red, itchy, and scaly. They may vary in severity from small and localized to complete body coverage[15]. Injury to the skin can trigger psoriatic skin changes at that spot, which is known as the Koebner phenomenon[16].

NORMAL SKIN FIG: 1

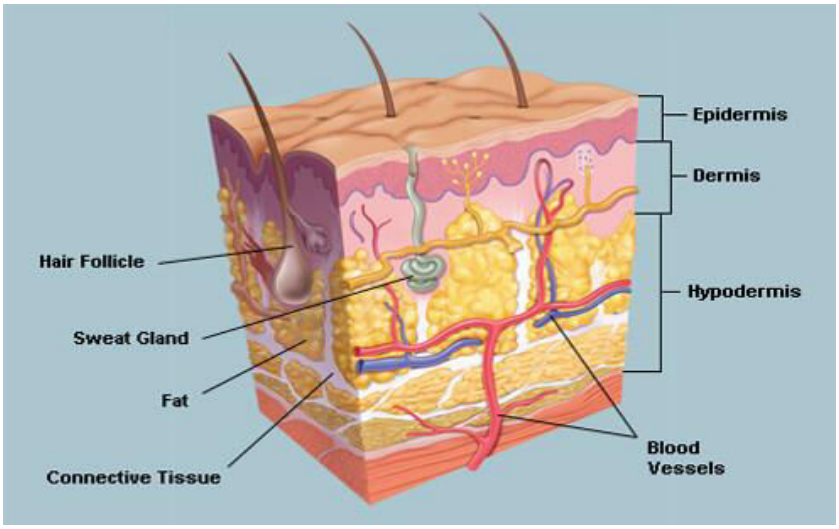
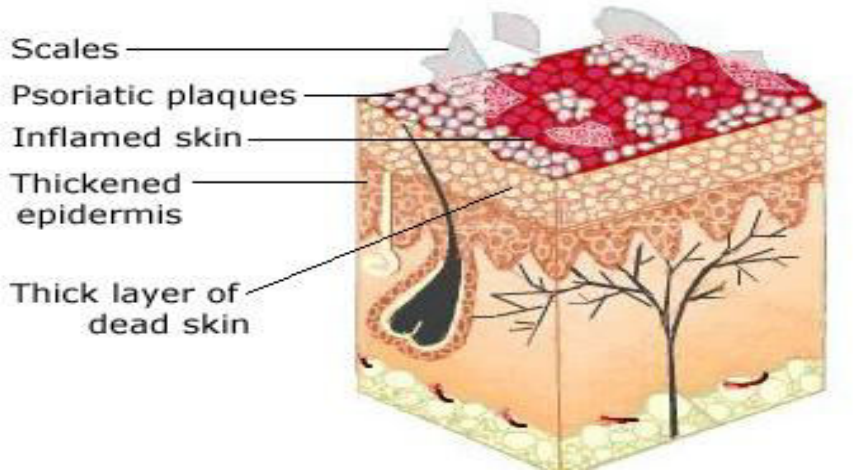


FIG: 2 **PSORIASIS SKIN**



Epidemiology

Psoriasis is found worldwide, affecting approximately 1% to 3% of the population. Men and women are equally affected. Psoriasis exhibits a bimodal distribution with a peak between 15 and 20 years of age and another peak between 55 and 60 years[17]. On the basis of the bimodal distribution of the age at onset and inheritance, two types of psoriasis have been discussed. Type I psoriasis (approximately 65% of the psoriasis population) is associated with onset below the age of 40, a positive family history of psoriasis, a preceding streptococcal sore throat, and guttate lesions. Type II psoriasis (35% of psoriasis patients) appears to be associated with a population with onset after the age of 40 years and with no family history of psoriasis. Type II is not linked to a preceding infectious trigger. The dominate clinical picture is chronic plaques and an association with nail and joint involvement has been described[18].

Clinical features

There are several psoriasis phenotypes. The most common clinical variant is psoriasis vulgaris, which affects approximately 85 to 90% of all patients with the disease. Psoriasis vulgaris is characterised by raised, well-demarcated, erythematous plaques with adherent silvery scale. The areas that are affected the most are the elbows, knees, sacral region and scalp. Other predilection sites include hands, feet, nails and the intertriginous areas (groins, axilla, umbilicus, crena ani, retroauricular folds)[19]. The psoriasis plaques in intertriginous areas are characterised by an oozing, red inflammation without scaling. The true incidence of intertriginous psoriasis is unknown. In a study by Farber et al[20]. it was found that 44 % of the psoriasis patients had perianal involvement, and in a Swedish study by Inerot et al[21] .

Types of Psoriasis[22,23]

Plaque Psoriasis

This is the most common type. About 8 in 10 people with psoriasis have this kind. You may hear your doctor call it "psoriasis vulgaris."

Plaque psoriasis causes raised, inflamed, red skin covered with silvery, white scales. These patches may itch and burn.

Elbow, Knee, Scalp, Lower Back

Guttate Psoriasis

This type often starts in children or young adults. It happens in less than 2% of cases. Guttate psoriasis causes small, pink-red spots on your skin.

- Trunk

- Upper arms
- Thighs
- Scalp

Inverse Psoriasis

This type shows up as areas that are bright red, smooth, and shiny, but don't have scales. It's usually found in these locations:

- Armpits
- Groin
- Under the breasts
- Skin folds around the genitals and buttocks

Pustular Psoriasis

This kind of psoriasis is uncommon and mostly appears in adults. It causes pus-filled bumps (pustules) surrounded by red skin. These may look infectious, but are not. This type may show up on one area of your body, such as the hands and feet. Sometimes it covers most of your body, which is called "generalized" pustular psoriasis. Generalized pustular psoriasis can cause:

- Fever
- Chills
- Nausea
- Fast heart rate
- Muscle weakness

Erythrodermic Psoriasis

This type is the least common, but it's very serious. It affects most of your body and causes widespread, fiery skin that appears burned.

- Severe itching, burning, or peeling
- A faster heart rate
- Changes in body temperature

Nail Psoriasis

Up to half of those with psoriasis have nail changes. This is even more common in people who have psoriatic arthritis, which affects your joints.Common symptoms include:

- Pitting of your nails
- Tender, painful nails
- Separation of the nail from the bed
- Color changes (yellow-brown)
- Chalk-like material under your nails

Psoriatic Arthritis

This is a condition where you have both psoriasis and arthritis (joint inflammation). In 70% of cases, people have psoriasis for about 10 years before developing psoriatic arthritis. About 90% of people with it also have nail changes. The most common symptoms are:

- Painful, stiff joints that are worse in the morning and after rest
- Sausage-like swelling of the fingers and toes

- Warm joints that may be discolored

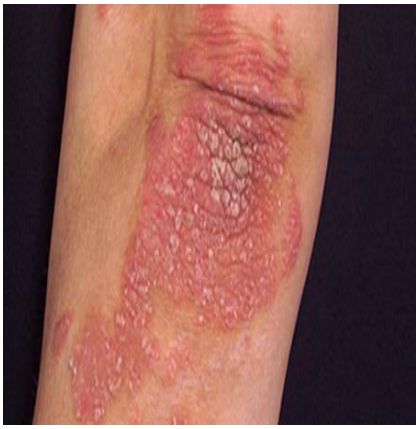


FIG :3 PLAQUE PSORIASIS

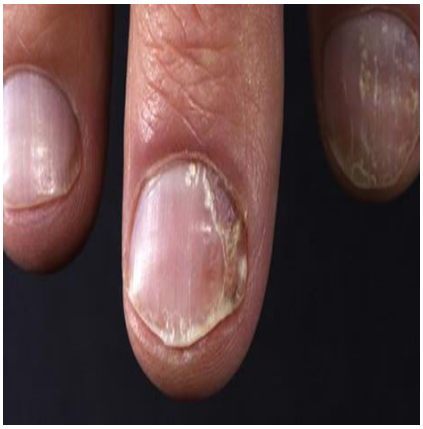


FIG:4 NAIL PSORIASIS

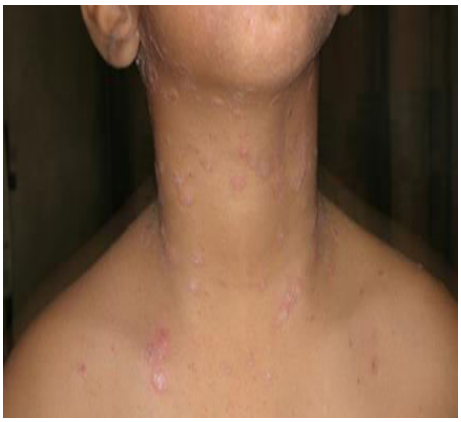


FIG:5 Guttate Psoriasis

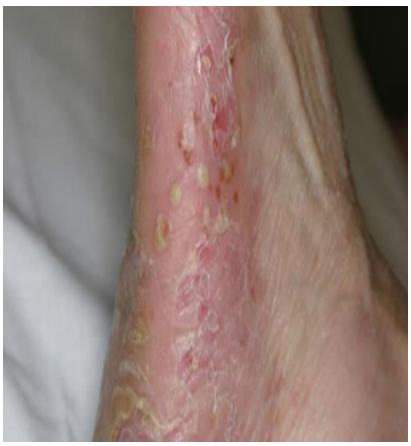


FIG:6 Pustular psoriasis

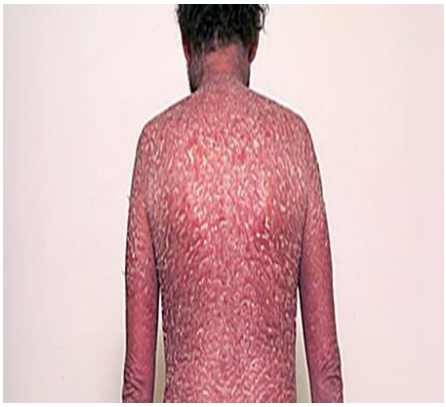


FIG:7 Erythrodermic Psoriasis

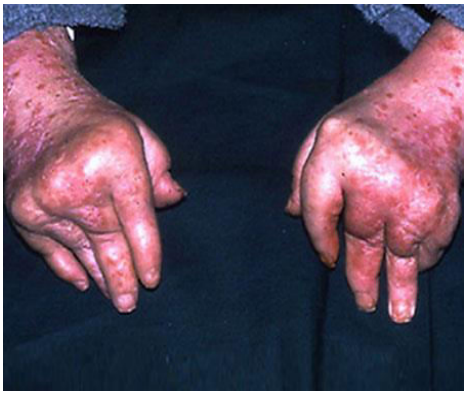


FIG:8 Psoriatic Arthritis

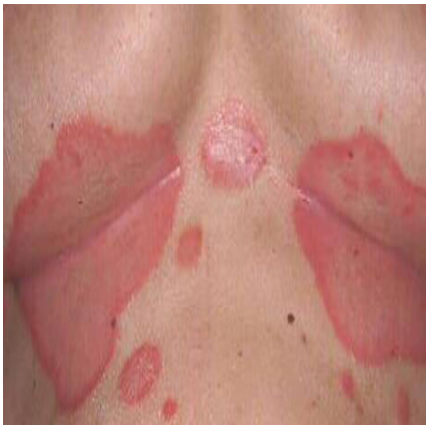


FIG:9 Inverse psoriasis

Histological features

The psoriasis scales are a result of a hyperproliferative epidermis with premature keratinocyte maturation and incomplete cornification with retention of nuclei within the cells of the stratum corneum (parakeratosis). The mitotic rate of the basal keratinocytes is increased and causes thickening of the epidermis. The redness of the lesions is due to increased numbers of tortuous capillaries that reach the skin surface. There is an immune cell infiltrate composed of dendritic cells and CD4+ Th cells within the upper papillary dermis, and neutrophils and CD8+ Th cells within the epidermis[24]. Neutrophilic granulocytes form characteristic Munro's microabscesses.

Immunopathogenesis

The pathogenesis of psoriasis is a complex interaction among genetic, immunological, and environmental components. It was previously assumed that Th1 cells played the dominant role in the initiation and maintenance of psoriasis but, in recent years, the view has changed in favour of a Th17 mediated disease. Innate immune cells produce key cytokines (TNF-α, IFN-α, IFN-γ, IL-1β, and IL-6) that activate dendritic cells. Activated dendritic cells present antigens and secrete mediators such as IL-12 and IL-23, leading to the differentiation of Th1 and Th17. IL-23 serves as a key master cytokine regulator. T cells secrete mediators (e.g., IL-17 and IL-22) that activate keratinocytes and induce the production of antimicrobial peptides, proinflammatory cytokines and chemokines. These mediators feed back into the proinflammatory disease cycle and shape the inflammatory infiltrate[25].

Genetics

The mode of inheritance of psoriasis is complex. Several susceptibility loci for psoriasis vulgaris (*PSORS*) have been identified, but the major genetic determinant of psoriasis is *PSORS1*, which is located within the major histocompatibility complex (MHC) on chromosome 6p. Current data suggest that HLA-Cw6 is the susceptibility allele within *PSORS1*. This association is particularly strong in patients with early onset psoriasis. One of the most important features of HLA-C is its capacity to regulate both innate and adaptive responses at the levels of both antigen presentation and natural killer cell regulation[26].

Environmental triggers

Psoriasis can be provoked or exacerbated by a variety of different environmental factors, particularly infections and drugs. Streptococcal infection is strongly associated with guttate psoriasis. In a study of Mallbris et al[27]. acute streptococcal

pharyngitis was verified in 63% of the patients with guttate phenotype at disease onset. The use of various drugs such as lithium, β-blockers, angiotensin-converting enzyme inhibitors, antimalarial agents and IFN-α has also been associated with induction or deterioration of the disease[28].

Severe acute mental stress can also precede the debut of psoriasis. Smoking has been discussed as a risk factor for psoriasis. Several studies have shown a link between psoriasis and cigarette smoking; patients with psoriasis are at least twice as likely to smoke cigarettes than the general population, and occasional reports have shown that smoking has a negative effect on psoriasis. Heavy tobacco intake also confers an increased risk of more clinically severe disease. Physical trauma (e.g. surgical incisions and tattoos) can give rise to the Koebner phenomenon[29]. The Koebner phenomenon constitutes psoriasis plaques that form at the site of a skin injury, and usually occurs within one to two weeks of injury to the dermis.

Microorganisms

Various microorganisms have been associated with the provocation and/or exacerbation of psoriasis. Certain strains of *Staphylococcus aureus* can produce enterotoxin and one theory is that exacerbation of psoriatic lesions is most likely mediated via toxin secretion[30]. The enterotoxins are highly potent activators of T cells. Due to the ability of the staphylococcal enterotoxins to activate a high frequency of T cells, they have been designated as Superantigens. Superantigens simultaneously bind to MHC class II on APCs and to the TCR on T cells. This cross-linking of APCs and T cells results in a polyclonal activation of CD4+ and CD8+ T cells. This leads to a massive T cell proliferation and an excessive production of cytokines[31].

Assessment tools

In clinical trials, a large variety of assessment tools have been used to evaluate the severity of psoriasis, but there is a lack of standardization[32]. In recent years, the introduction of quality of life instruments has improved psoriasis evaluation, but there is a need for consensus in order to make valid comparisons between studies. In a review article it was found that in randomised controlled trials, the Psoriasis Area and Severity Index (PASI) was the most commonly used measure to describe the extent of psoriasis and the Dermatology Life Quality Index (DLQI) was the most common tool for measuring quality of life[33].

Psoriasis Area and Severity Index

The Psoriasis Area and Severity Index (PASI) is a widely used tool for the measurement of the severity of psoriasis[34]**.** The PASI combines the assessment of the severity of lesions and the area affected, into a single score within the range of 0 to 72. The body is divided into four sections: head (10% of the body area), arms (20%), trunk (30%) and legs (40%). Each of these areas is scored separately, and the four scores are then combined. For each section, the percentage of the area of skin involved is estimated and then transformed into a grade from 0 to 6. The PASI is the most validated objective method to measure the severity of psoriasis and has a high intra-rater reliability and a good interobserver correlation when used by trained assessors. The PASI system is sensitive to changes and reflects disease improvement or deterioration, although the sensitivity to change for small areas of involvement is poor[35]. PASI 75 is a widely used concept, meaning the percentage of patients achieving a 75% improvement in PASI from baseline to the primary endpoint, usually 12 to 16 weeks of treatment. Achieving a 75% improvement in the PASI is considered

to be successful treatment. PASI 50 (50% improvement) and PASI 90 (90% improvement) are sometimes also used.

Body Surface Area

The Body Surface Area (BSA) is an instrument to estimate the extent of psoriasis involvement, calculating one palm of the hand represent 1% of the total body surface area. The advantages of BSA are that it is quick and convenient to use, with a low test-retest variability for the same observer. However, there is moderately high interrater variability and the method is likely to overestimate the extent of psoriatic lesions[36].

Dermatology Life Quality Index

The Dermatology Life Quality Index (DLQI) is a ten-item questionnaire evaluating the quality of life in patients with dermatological diseases. It consists of six subscales: symptoms and feelings, daily activities, leisure, work and school, personal relationships and treatment satisfaction. The DLQI can give a total score of 30 with a higher score indicating a poorer quality of life. An estimate of the minimal clinically important difference of the DLQI total score is a 5 point improvement. However, if patients score less than 5 points at baseline, the definition of a clinically meaningful response is expanded to include patients who achieved a DLQI total score of 0. A set of intervals of DLQI scores is proposed: 0-1=no effect at all on patient's quality of life, 2-5=small effect, 6-10=moderate effect, 11-20=very considerable effect and 21-30=extremely substantial effect. The reliability and validity of the DLQI is well-established[37].

Short Form -36

The Short Form-36 (SF-36) is a general health status instrument and includes one multi-item scale that is applicable to research, general population surveys and health policy evaluations. The SF-36 is used in clinical trials and has shown good reliability and validity for psoriasis[38]. The SF-36 is divided into physical health, subdivided into physical functioning (PF), role-physical (RP), bodily pain (BP), general health (GH) and into mental health subdivided into vitality (VT), social functioning (SF), role-emotional (RE) and mental health (MH). The SF-36 consists of eight scaled scores, which are the weighted sums of the questions in their section. Each scale is directly transformed into a 0-100 scale on the assumption that each question carries equal weight.

Visual Analogue Scale

The Visual Analogue Scale (VAS) is a 100-millimetre horizontal line with descriptive phrases representing extremes of sensation placed at either end. The subject places a mark on the 100 mm line at the most appropriate point. The VAS is a tool that is often used to measure subjective phenomena. It has shown a high level of reliability and validity in terms of assessing pain. The VAS has previously been used in different psoriasis studies, mainly to reflect the intensity of itching and in one study it was also used to measure the patient's self-assessment of the severity of his/her psoriasis and its impact on quality of life[39].

Treatment goals

The treatment strategy is based on disease severity. The European consensus states the definition of disease severity and treatment goals for psoriasis. Mild psoriasis is defined as BSA ≤10, PASI ≤10 and DLQI ≤10. Moderate to severe psoriasis is defined as BSA >10 or PASI >10 and DLQI >10. Treatment goals (assessed after 10-

16 weeks) are a reduction of PASI ≥75% and DLQI 0 or 1. If a treatment regimen results in a reduction of PASI ≥75% or PASI ≥50% to <75% combined with a DLQI ≤5, treatment is successful and therapy should be continued. When there is a reduction in PASI <50% or PASI ≥50% to <75% combined with a DLQI >5, treatment modifications should be considered, including increasing the drug dose, reducing intervals between drug doses, combining therapies or changing the drug[40].

Topical treatment

Emollients

Emollients are used to soften scaling and reduce irritation. The treatment has a positive effect on skin hydration and acts as a barrier function in psoriasis patients[41].

Corticosteroids

Corticosteroids have an anti-inflammatory and immunomodulating effect. Corticosteroids inhibit different proinflammatory cytokines such as TNF-α. Corticosteroids with a low to mild potency are used for intertriginous psoriasis and face lesions. Potent and super potent corticosteroids are used on the body and the scalp. There has been concern regarding the long-term use of corticosteroids. Side-effects that may occur include cutaneous atrophy and the development of striae. There is also a possibility of hypothalamic–pituitary–adrenal axis suppression occurring with prolonged use of excessive quantities of corticosteroids[42].

Calcipotriol

Calcipotriol is a vitamin D analogue affecting epidermal proliferation and differentiation. Calcipotriol is used for plaque psoriasis. Calcipotriol in a fixed combination with betamethasone dipropionate has a faster onset of action than monotherapy Calcipotriol can cause irritant reactions[43].

Phototherapy

Ultraviolet B

The mechanism of action of Ultraviolet B (UVB) treatment is not fully understood. The number of epidermal T lymphocytes and dendritic cells (DCs) decrease and there is a reduction in keratinocyte proliferation. UVB treatment is a standard treatment for moderate to severe plaque psoriasis and guttate psoriasis. The former use of broad-band UVB (BB-UVB) (290–320 nm) is now often replaced by narrow-band UVB (NB-UVB) (311±2 nm). The most common side effects of UVB therapy are erythema and burning. BB-UVB is not thought to lead to a risk of developing skin cancer but the risk of NB-UVB is under debate. No significant association between NB-UVB treatment and BCC, SCC or melanoma has yet been seen, but ongoing risk assessments are essential[44].

Psoralen + Ultraviolet A

PUVA treatment is psoralen (oral or bath) in combination with Ultraviolet A (320-400 nm). Psoralen is a compound in a family of natural products known as furocoumarins. Psoralen intercalates into the DNA and, on exposure to ultraviolet UVA radiation, form covalent interstrand cross-links with thymine, inducing apoptosis. Exposure to more than 350 oral PUVA treatments greatly increases the risk of developing squamous cell carcinoma (SCC) and PUVA treatment has therefore declined over the past few years. However, no risk of developing skin cancer has been seen with bath-PUVA treatment[45].

Climate therapy

Sun exposure has an immunomodulating effect with local and systemic reduction of T cells and cytokines[46]. Climatotherapy is the oldest form of phototherapy.

Grenz rays

The exact mechanism of action of Grenz rays (Bucky) is unknown but it has effects on the Langerhans cells in the epidermis. Grenz rays have wavelengths of around 20 nm, lying between x-rays and ultraviolet rays. Grenz rays are used mainly for scalp psoriasis, but also for psoriasis in the intertriginous areas and for hand and foot psoriasis. Side effects are erythema and hyperpigmentation. One concern is skin malignancy, but the risk is considered to be low if the cumulative dose is less than 100 Gray[47].

Traditional systemic treatment

Methotrexate

Methotrexate is a synthetic folic acid analogue with anti-proliferative and anti-inflammatory properties. Polyglutamate, which is the primary metabolite in methotrexate, competitively inhibits dihyrofolate reductase, preventing the reduction of folate cofactors. This results in preventing pyrimidine and purine synthesis and DNA methylation. Methotrexate empties the intracellular stores of activated folate. Cell replication is disrupted and this leads to the inhibition of epidermal cell proliferation. At low doses, methotrexate has potent anti-inflammatory actions that appear to be mediated via pathways that are separate from folate antagonism. The inhibition of polyamines is thought to contribute to its anti-inflammatory effects. Methotrexate is the first line treatment for moderate to severe psoriasis when systemic treatment is needed. Methotrexate can be administered orally, subcutaneously or intramuscularly. Two different dosage regimes have been proposed. A single, once-weekly dose and a triple dosage schedule given at 12-hours intervals, with the latter regimen based upon cell cycle kinetic studies[48].

Ciclosporin

Ciclosporin is a cyclic polypeptide consisting of eleven amino acids. It suppresses the activation of the calcium-dependent phosphatase calcineurin, inhibiting lymphokine secretion (e.g.,IL-2, IFN- γ, GM-CSF, IL-3, IL-4, TNF-α and IL-17) which leads to diminished activation of T lymphocytes. Ciclosporin also inhibits antigen presenting cells. Ciclosporin is used for severe psoriasis. In recent years, the use has diminished since the introduction of biologic therapies. However, it does still have its place when there is a need for a rapid effect. Ciclosporin is nephrotoxic and functional kidney damage can occur quickly after treatment has started. With intermittent treatments, the kidney function can be normalised between treatment periods. The risk of irreversible kidney damage increases during long-term treatment (more than two years) or ciclosporin doses of >5 mg/kg per day Hypertension is another side effect, but is reversible after reducing the dose or after starting antihypertensive treatment. Ciclosporin treated patients who were previously given high doses of UV and especially PUVA, are at greater risk of developing skin malignancy, especially SCC[49].

Acitretin

Acitretin is a retinoid (synthetic vitamin A derivate) and has antiproliferative and immunomodulatory properties. In the epidermis, acitretin reduces the proliferative activity and favours the differentiation of epidermal keratinocytes. Acitretin inhibits the induction of Th17 cells and promotes the differentiation of T-regulatory cells. Acitretin is used for plaque psoriasis (especially in combination with UVB and PUVA) and also for pustulous psoriasis, hyperkeratotic hand- and foot psoriasis and erythrodermia. Side effects are mainly hyperlipidemia and elevated liver enzymes[50].

Biologics

Biologics are drugs derived from living material and that interfere with the immune system. Biologic therapies for psoriasis were introduced in Sweden in 2004. They are used for the treatment of moderate to severe psoriasis when traditional systemic therapies are contraindicated or cannot be used due to side effects or have not led to satisfactory treatment result[51]. There is a greater risk of developing serious infections during treatment, and screening for tuberculosis and hepatitis is mandatory before treatment starts. To date, there is no robust evidence of an increase in the risk of malignancy, but a possible future risk of lymphoma or other malignancies cannot be ruled out.

Etanercept

Etanercept is a human soluble TNF receptor fusion protein, binding free circulating TNF-α which competitively blocks TNF-α to bind to TNF-receptors. It is administered through subcutaneous injections[52].

Adalimumab

Adalimumab is a fully human anti TNF- α monoclonal antibody and it is administered through subcutaneous injections[53].

Infliximab

Infliximab is a chimeric human-mouse antibody that binds to both soluble TNFα and TNFα on the cell wall and is administered through intravenous infusions[54].

Ustekinumab

Ustekinumab is a human monoclonal antibody that binds with high affinity and specificity to the p40 protein subunit that is used by both the interleukin (IL)-12 and the IL-23 cytokines. It is administered through subcutaneous injections[55]

Ayurvedic drug treated in psoriasis

Herbs may be used In the treatment of psoriasis in different formulations like dried extracts ,tinctures, decoctions, topical cream, gel, ointment and oral formulations like tablets. these herbs have potent activity against psoriasis inflammation similar to allopathic drugs with minimum or no side effects even on long term use.

The following are some of the herbs used for the treatment of psoriasis are as follows[56]

1.silybum marianum

2.rumex crispus

3.trifolium pretense

4.smilax sarsaparilla

5.coleus forskohli

6.stellaria medica

7.calendula officinalis

8.astragalus memranaceus

9.thespesia populnea

10.momordica charanta

REVIEW OF LITERATURE

2. REVIEW OF LITERATURE

PHARMACOGNOSTICAL STUDIES:

1. Tamilselvi et al (2011) Anatomical studies of *Indigofera aspalathoides* Vahl (Fabaceae)[57].

2. Kumar and Ramayya (1982) The morphology of the inflorescence and leaf in *Indigofera aspalathoides* Vahl ex DC[58].

PHYTOCHEMICAL STUDIES:

1. Ariharan et al (2015) Qualitative phytochemical analysis of chloroform extracts of Sivanar Vembu(*Indigofera aspalathoides*)[59].

2. Raaman et al (2015) Micropropagation, qualitative phytochemical analysis and antioxidant potential of *Indigofera aspalathoides* Vahl. ex. DC[60].

3. Tamilselvi et al (2012) Analysis of total phenols, total tannins and screening of phytocomponents in *Indigofera aspalathoides* (Shivanar Vembu) Vahl EX DC[61]

4. Abirami and Rajendran (2011) GC-MS determination of bioactive compounds of *Indigofera aspalathoides*[62].

5. Subhashini et al (2011) Preclinical studies on the phytochemical, antimicrobial, and wound healing properties of *Indigofera aspalathoides* leaves[63].

PHARMACOLOGICAL STUDIES:

1. Swarnalatha et al (2015) Immunomodulatory activity of kaempferol 5-O-beta-D-glucopyranoside from *Indigofera aspalathoides* Vahl ex DC[64].

2. Ariharan et al (2015) Antibacterial activity of Sivanar Vembu (*Indigofera aspalathoides*) against some human pathogenic bacteria[65].

3. Arunachalam et al (2013) Green Synthesis of Crystalline Silver Nanoparticles Using *Indigofera aspalathoides*-Medicinal Plant Extract for Wound Healing Applications[66].

4. Rajendran et al (2013) Preliminary antidiabetic studies on aqueous extract of *Indigofera aspalathoides* Vahl ex DC[67].

5. Claimer et al (2012) Protective Effect of *Indigofera aspalathoides* Roots on N-Nitrosodiethylamine-induced Hepatocarcinogenesis in Mice[68].

6. Balasubramanian et al (2007) Cytotoxic activity of flavone glycoside from the stem of *Indigofera aspalathoides* Vahl[69].

7. Rajkapoor et al (2006) Protective effect of *Indigofera aspalathoides* against CCl4-induced hepatic damage in rats[70].

8. Rajkapoor et al (2004) Antitumor activity of *Indigofera aspalathoides* on Ehrlich ascites carcinoma in mice[71].

9. Amala Bhaskar et al (1982) Anti-inflammatory activity of Indigofera aspalathoides Vahl[72].

AIM AND OBJECTIVE

3. AIM AND OBJECTIVES

Indigofera aspalathoides plant are used in the treatment of skin disease in ethanomedicine. The literature survey indicates that no scientific data on the anti psoriatic activity of this plant.

Various parts of these plants have been claimed to be effective in a wide range of disease .The scientifically state therapeutic properties for *Indigofera aspalathoides* have reported antimicrobial, anti-inflammatory, anticancerous, anti-oxidant, hypoglycemic, hepatoprotective, antiviral, Antitumor activity Antidiabetic ,Wound Healing[64-71] .

Rationale of the study

The allopathic drugs like methotrexate, corticosteroids and immuno suppressant shows effective response in psoriasis by reducing the scale ,plaque ,cell proliferation and inflammation but the adverse reaction is severe than the efficacy of the drug .the long term usage of drug causes serious adverse reaction like hepatotoxicity, renal failure sometimes leads to fatality.So the herbal drugs are safe in psoriasis treatment since the adverse effect is mild or nil and lower the cost of therapy as comparable to the allopathic medicine.

The present investigation was therefore proposed to:

1. To evaluate the anti-microbial activity related to psoriasis from the different extract of whole plant of *Indigofera aspalathoides.*

2. To formulate herbal ointment with different extract of whole plant of indigofera aspalathoides.

3. To evaluate the formulated herbal ointment

4. To evaluate the anti-psoriatic activity of ointment by mice skin test.

ETHNOBOTANICAL SURVEY

4. ETHNOBOTANICAL SURVEY

4.1 PLANT PROFILE

Plant name: *Indigofera aspalathoides* vahl ex dc

Tamil name : Iraivan vembu, shivanar vembu

Family : Fabaceae

4.2 VERNACULAR NAMES:[73,74]

English name : Shiva ‘s name , wiry indigo

Kannada : shivamalli-gida, neelamalligida

Malayalam : manali

Sanskrit : ratakohomba, sivanimba

Sinhalese : ratkohomba

Marathi : Shiva nimb

Punjabi : mil

4.3 TAXANOMY CLASSIFICATION:

Kingdom: Plantae

Order : Fabales

Family : Fabaceae

Subfamily: Faboideae

Tribe : *Indigofereae*

Genus : *Indigofera.L*

species *: Indigofera aspalathoides vahl*

4.4 DISTRIBUTION

india , deccan plains of Carnatic and ceylon .

4.5 HABITAT

A low much branched erect under shrub,branches rigid, divaricately spreading.

4.6 BOTANICAL CHARACTERS:[75]

Leaves : 1-5 foliolate, digitate, sessile, croweded on the young branches but soon decideous stipules minute subulate.

Flowers : solitary, axillary ,corolla red or dark pink,exserted.

Pedicles : 1/8-1/4 inch , filiform.

Leaflets :2.5-6mm long , 1mm wide pale green in colour ,linear,apiculate.

Calyx : 1.5mm long ,incised more than half way .

Pods : 1.2-1.5cm long, straight, glabrous with a few scattered hairs.

Stem : dark brown, branched 0.7 to3.0 cm

Root : brown colour, woody, lateral roots present 0.5 to 3.0 cm width

4.6 PLANT CHEMICALS:[76]

Alkaloids
flavonoids
carbohydrate
phenolic compound
saponins
steroids
tannins
β-sitosterol

4.7 ETNOMEDICAL INFORMATION:[77,78]

1. Leaves, flowers, and tender shoots **:** cooling demulcent, employed in decoction, leprosy and cancerous affections.
2. Whole plant **:** oedematous tumours.
3. Leaves **:** applied to abscesses.
4. Root **:** chewed as a remedy for toothache and apthae of the mouth.
5. Decoction of entire plant **:** given as an alternative in secondary syphilis, psoriasis etc.
6. Oil from root **:** used to anoint the head in erysipelas and skin diseases.

FIG :10 LEAVES

FIG:11 FLOWER

FIG: 12 FRUIT

FIG:13 WHOLE PLANT

PLAN OF WORK

5. PLAN OF WORK

1. **COLLECTION OF PLANT MATERIAL**

2. **AUTHENTICATION**

3. **PHARMACOGNOSTICAL STUDIES**

   MACROSCOPY

   MICROSCOPY

   - Histochemical studies
   - Powder microscopy
   - Quantitative microscopy – Linear measurement
   - Physiochemical constants

4. **PHYTOCHEMICAL STUDIES**

   - Preparation of extracts
   - Preliminary phytochemical analysis
   - Quantitative estimation of phytoconstituents
   - Fluorescence analysis
   - Thin layer chromatography
   - High performance thin layer chromatography

5. ***IN -VITRO*** **STUDIES**

   1. Anti-oxidant activity

   2. Anti- microbial activity

6. FORMULATION AND EVALUATION OF HERBAL OINTMENT

Formulation and evaluation of herbal ointment of extracts of whole plant of *Indigofera aspalathoides*

1. Physical Examination
2. Determination of pH
3. Extrudability:
4. Spreadability
5. Skin sensitivity test

7. IN-VIVO ANTI- PSORIASIS ACTIVITY

Evaluation of anti-psoriatic activity with extract of whole plant of *Indigofera aspalathoides* herbal ointment by mice skin test.

PHARMACOGNOSTICAL STUDIES

6. PHARMACOGNOSTICAL STUDIES

6.1 MATERIAL AND METHODS

COLLECTION OF PLANT MATERIAL

The whole plant of *Indigofera aspalathoides* was collected in 2016 July at panakudi village ,tirunelveli district , tiruneveli -627011.

AUTHENTICATION

The collected specimens was botanically identified and authenticated by V. Chelladurai, Research Officer-Botany (Scientist) , Central Council for Research in Ayurveda and Siddha , Govt Of India, tiruneveli -627011.

MACROSCOPIC EVALUATION

The morphological feature of the plant was evaluated and recored.

MICROSCOPIC EVALUATION [79-82]

Fixation of leaf, stem and root

The whole plant was cut and fixed in FAA solution (Formalin 5ml + Acetic acid 5ml + 90ml of 70% Ethanol). The specimen was dehydrated after 24 hours of fixing. The whole plant was graded with series of tertiary butyl alcohol, as per the standard procedure.

It was carried out by gradual addition of 58 – 60º C of melting pointed paraffin wax until Tertiary butyl alcohol (TBA) solution attained super saturation. The specimens were cast into paraffin blocks.

The paraffin embedded specimens were sectioned with the help of Rotary Microtome. The thickness of the sections was 10 - 12µ. De-waxing of the sections

was done by customary procedures. The sections were stained with haematoxilin. The stained sections were viewed under microscope.

POWDER MICROSCOPY[83,84]

The shade dried whole plant was powdered and used for powder microscopic analysis. The organoleptic characters were observed and to identify the different microscopical characteristic features various staining reagent were used. Powder was stained with 1% phloroglucinol in 90% ethanol, concentrated hydrochloric acid and observed under microscope. Powder analysis is used for the detection of characteristic structures and various cell components.

QUANTITATIVE MICROSCOPY[85]

LINEAR MEASURMENT OF FIBRES

The length and width of the fibers present in the whole plant were observed under microscope. This quantitative analysis will be helpful in the identification of the drug.The first step involved in this is calibration of the eyepiece micrometer using the stage micrometer. For determining the calibration factor, the eyepiece is removed from the microscope, then the lens is unscrewed and in the ridge the eyepieces micrometer is placed. The lens is then replaced. The stage micrometer is then placed on the stage of the microscope and focused under high power with the eyepiece coincides with each division of stage micrometer and calculate the calibration factor using the standard formula. The stage micrometer is replaced with the slide containing the powdered drug. The slide is prepared by using the whole plant powder on a slide is treated with a drop of phloroglucinol and conc.

Hydrochloric acid and viewed under microscope. The width and length of fibers is measured by focusing them on the lines of the eyepiece micrometer. Note the no. of divisions covered by the length and width of the fibers.

PHYSIOCHEMICAL ANALYSIS[86,87]

The shade dried powdered whole of *Indigofera aspalathoides*, was used for the analysis of various physiochemical parameters which is useful in the determination of quality and purity of crude drugs. Total ash, extractive values, loss on drying, foaming index, swelling index and foreign organic matters were determined as per the standard WHO guidelines which is very much useful in the determination of quality and purity of the crude drugs.

DETERMINATION OF ASH VALUES

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

TOTAL ASH

Silica crucible was heated to red hot for 30 minutes and it was allowed to cool in desiccators. About 2gm of powdered sample was weighed accurately and evenly distributed in the crucible. Dried at 100 – 105ºC for 1 hour and ignited to constant weight in a muffle furnace at 600±25ºC. The crucible was allowed to cool in a desiccator. The percentage yield of ash with reference to the air dried substance was then calculated by the formula

Water soluble ash

The total ash was boiled for 5min with 25ml of water. The insoluble matter was then collected in an ash less filter paper. It was washed with hot water and ignited for 15min at a temperature not exceeding 450ºC. The weight of the insoluble matter was subtracted from the weight of the ash and the difference in weight represented the water soluble ash, the percentage of water soluble ash with reference to the air dried substances was calculated with reference to the air dried material.

Acid insoluble ash

Acid insoluble ash is the residue obtained after boiling the total ash with dilute hydrochloric acid, and igniting the remaining insoluble matter. This measures the amount of silica present, especially as sand and siliceous earth

Procedure

To the total ash obtained previously, 25ml of dilute hydrochloric acid was added, covered with a watch glass and boiled gently for 5min on a burner. The watch glass was rinsed with 5ml of hot water and these washings were added to the crucible. The insoluble matter was collected on an ash less filter paper by filtration and the filter paper was rinsed repeatedly with hot water until the filtrate is neutral and free from acid. Filter paper containing the insoluble matter was transferred to the crucible, dried on a hot plate and ignited to a constant weight in the muffle furnace at 450- 500ºC. The silica crucible was removed from the muffle furnace and allowed to cool in a desiccator for 30min, and then weighed without delay. The content of acid insoluble ash was calculated.

Sulphated ash

About 3gm of air-dried substance was ignited gently at first in a crucible, until the substance was thoroughly charred. Then the residue was cooled, moistened with 1ml of sulphuric acid, heated gently until the white fumes were no longer evolved and ignited at 800 ± 25ºC, until all the black particles were disappeared. The crucible was allowed to cool, a few drops of sulphuric acid was added and heated. Then it was ignited as before, cooled and weighed.

The percentage of sulphated ash with reference to the air- dried substance was then calculated.

DETERMINATION OF EXTRACTIVE VALUES

This method is used to determine the amount of active constituents in a given amount of plant material when extracted with solvents. Extractive values are useful for the evaluation of phytoconstituents especially when the constituents of a drug cannot be readily estimated by any other means. Further these values indicate the nature of the active constituents present in a crude drug.

Determination of water soluble extractive

About 5gm of the powder was weighed and macerated with 100ml of chloroform water (95ml distilled water and 5ml chloroform) in a closed flask for 24 hours. It was shaken frequently for six hours and allowed to stand for eighteen hours. It was then filtered rapidly, taking precautions against loss of solvent and 25ml of the

filtrate was evaporated to dryness in a tarred flat bottomed shallow dish. 2 ml of alcohol was added to the residue and it was dried at 105ºCfor 1 hour in the hot air oven and cooled in desiccators for 30min and weighed. The process was repeated till a constant weight was obtained; the percentage of water soluble extractive value with reference to the air dried drug was calculated.

Determination of alcohol soluble extractive

The alcohol soluble extractive value is also indicative for the same purpose as water soluble extractive value. The solvent strength of alcohol varies from 20- 90%v/v. The solvent strength has to be chosen depending upon the strength of alcohol used for the extraction of powdered drug.

Procedure

About 5gm of the powder was weighed and macerated with 100ml 90% ethanol in a closed flask for 24 hours. It was shaken frequently for six hours and allowed to stand for eighteen hours. It was then filtered rapidly, taking precautions against loss of solvent and 25ml of the filtrate was evaporated to dryness in a tarred flat bottomed shallow dish. It was dried at 105ºC for 1hour in a hot air oven. The dish was cooled in desiccator and weighed. The process was repeated till the constant weight was obtained. The percentage of alcohol soluble extractive value with reference to the air dried drug was calculated.

LOSS ON DRYING

Accurately weighed quantity of the substances was taken in a previously ignited and cooled silica crucible and the substance was evenly distributed by gentle side wise

substance was heated for a specified period of time to a constant weight. The crucible was covered with the lid and allowed to cool in a desiccator at room temperature before weighing. Finally the crucible was weighed to calculate the loss on drying with reference to the air dried substance.

DETERMINATION OF FOAMING INDEX

About 1gm of the coarsely powdered drug was weighed and transferred to 500ml conical flask containing 100ml boiling water. The flask was maintained at temperature 80-90ºC for about 30min. It was then cooled and filtered into a volumetric flask and sufficient water was added through the filtrate to make up the volume to 100ml. The decoction was poured into 10 stopper test tube (height 16cm, diameter 16mm) in successive portions of 1ml, 2ml, 3ml, 4ml up to 10ml and the volume of the liquid in each tube was adjusted with water to 10ml. The tubes were stoppered and shaked in a length wise motion for 15 seconds, two shakes per second. Allowed to stand for 15min and the height of the foam were measured. The results are assessed as follows: if the height of the foam in every tube is less than 1cm, the foaming index is less than 100. If a height of 1cm is measured in any tube, the volume of the plant material decoction in the tube (a) is used to determine the index. If this tube is the first or second tube in a series, prepare an intermediate dilution in a similar manner to obtain a more precise result. The height of the foam is more than 1cm in every tube the foaming index is over 1000. In this case repeat the determination using a new series of dilution of the decoction in order to obtain a result. Calculate the foaming index using the following formula.

Foaming index = 1000/a

Where, a is the volume in ml of the decoction used for preparing the dilution in the tube where foaming to a height of 1cm is observed.

DETERMINATION OF SWELLING INDEX

The swelling index is the volume in ml occupied by the swelling of 1gm of plant material under specified conditions.

Procedure

A specified quantity of the plant material was accurately weighed and transferred into a 25ml glass stoppered measuring cylinder. The internal diameter of the cylinder should be about 16mm, the length of the graduated portion about 125mm, marked in 0.2ml divisions from 0 to 25ml in an upward direction. Unless otherwise indicated in the test procedure, add 25ml of water and shake the mixture thoroughly every 10min for 1hour, allowed to stand for 3 hours at room temperature. The volume in ml occupied by the plant material was measured including any sticky mucilage. Calculate the mean value of the individual determination, related to 1gm of plant material

. 6.2 RESULTS AND DISCUSSION

The results of pharmacognostical studies are as follows.

ORGANOLEPTIC CHARACTER

Leaf – pale green

Stem – dark brown

Root – brown colour

MICROSCOPY OF LEAFLET, STEM AND ROOT

T.S. OF LEAFLET

The leaflet is either flat or folded adaxially forming V Shaped outline. The midrib part is 170µm thick vertically and 200µm horizontally. The epidermis along the adaxial groove is smooth and has rectangular cells. The xylem elements are narrow, angular, thick walled and compact. The leaf margin is slightly dilated and it is semicircular in sectional view with 130µm thick. The epidermal layer of the leaf margin is thick . The mesophyll tissue is differentiated into palisade and spongy parenchyma similar to the middle part of the leaflet. **AdS: Adaxial side, PM – Palisade Mesophyll, SM-Spongy Mesophyl**

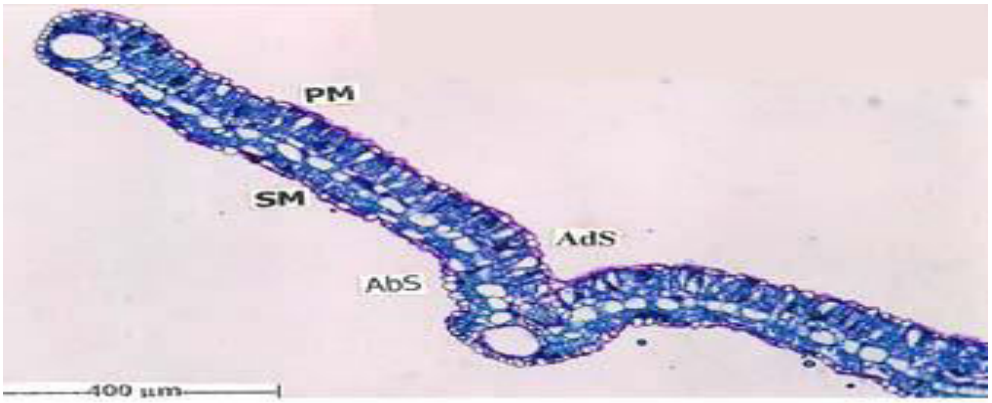


FIG:14 T.S. OF LEAFLET

T.S. OF STEM

The stem is circular in sectional view with smooth and even surfaces with 900 µm thick. The epidermal cells are 5 µm thick. The cortical zone is 100 µm wide. It is heterogeneous comprising of outer part of four or five layers of small compact cells, in addition to wide isolated group of two or three cells; the middle part has prominent, discrete, circular masses of gelatinous or mucilage fibres, arranged in a ring, all around the stem . The inner cortex has four or five layers of fairly wide, compact parenchyma cells of varying dimensions. Phloem occurs in continuous sheath encircling the xylem. The vessel lines extend from the inner to outer boundaries of the cylinder.

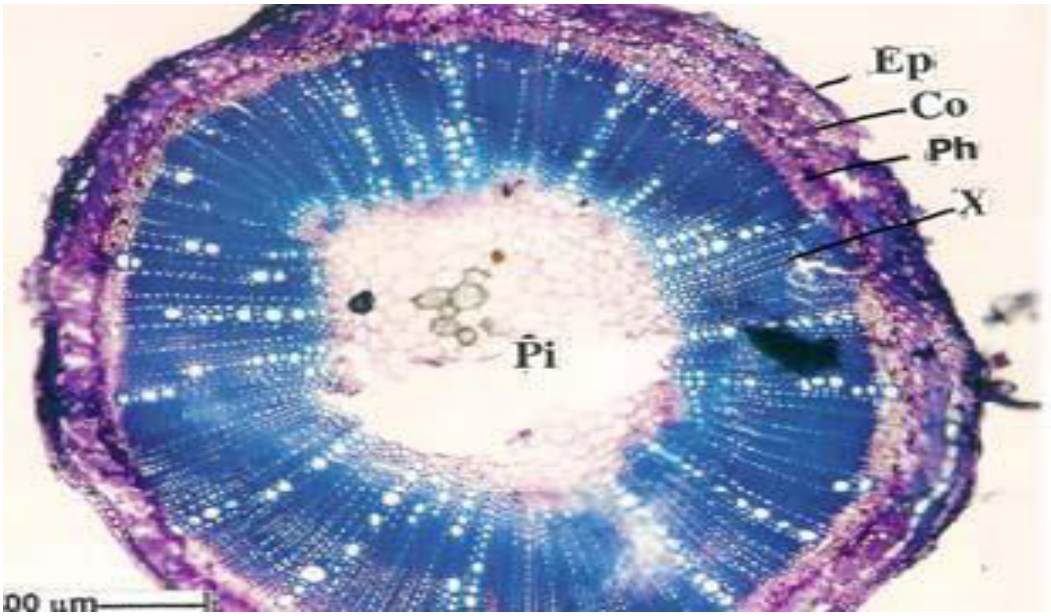


FIG :15 T.S OF STEM

Ep - Epidermis,

Co – Cortex, Ph – Phloem, X: Xylem, Pi: Pith.

T.S. OF ROOT

The thin root consists of four or five layers of tubular, suberized phloem cells. Secondary phloem consists of outer dark cylinder of crushed phloem and sclerenchyma elements. inner phloem has non collapsed, radial files of elements without sclerenchyma cells. Secondary xylem is circular. The wide vessel in the outer zone is 50 μm in diameter. Secondary phloem is differentiated into outer collapsed phloem and inner non collapsed phloem. The collapsed phloem is much wider and consists of funnel shaped dilated rays alternating with triangular cones of fibres and crushed phloem elements. The fibres are gelatinous type; they have inner gelatinous unlignified walls and outer lignified walls.

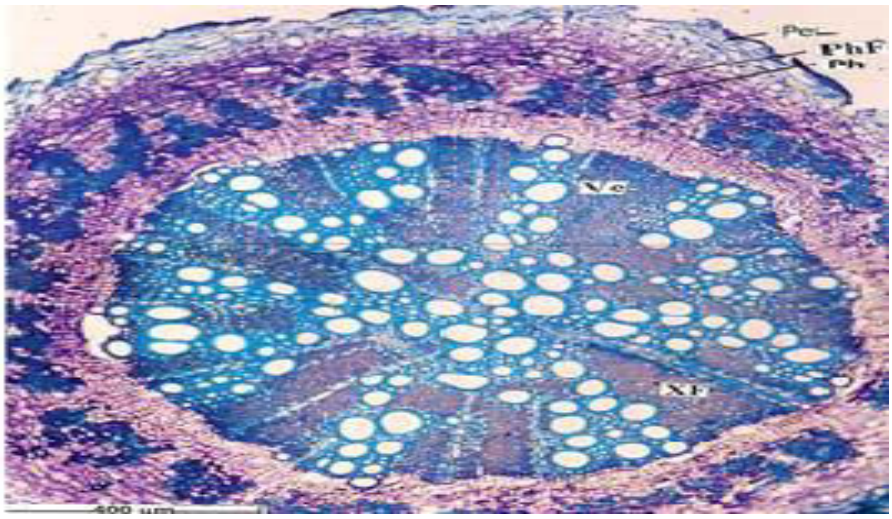


FIG:16 T.S OF ROOT

Ph – Phloem, X: Xylem, PE:Periderm,

Ph F – Phloem Fibre, XF – Xylem fibre, Ve – Vessel.

POWDER MICROSCOPY

Powder microscopy revealed the presence of the following structures

1. Epidermal cells: These cells were present in outer region; the walls may be straight and brownish in colour.

2. Non-lignified fibre: They were present in mid rib region (sclerenchyma region). They are thin walled narrow lumen, and pointed ends.

3. Trichomes: Two types of trichomes were present. (A) Uni cellular covering trichomes: they were found to be long, slender and bent at the base and pointed apex. (B) Knee shaped trichomes: they were present at lamina region, these types of trichome are also present in vasaka leafs.

4. Xylem vessels : spiral

5. Calcium oxalate : prism of calium oxalate

FIG:17 POWDER CHARACTERS

Epidermal cell **Non-lignified fibre** **Unicellularcovering**

Knee- shaped trichomes

Calcium oxalate crystals

Xylem vessels

Spiral nature xylem vessels

Collenchymas cells

Stone cells

QUANTITATIVE MICROSCOPY

Linear measurement of fibres

The length and width of the fibres were measured in the powdered whole plant of *Indigofera aspalathoides* and the results were shown in table no: 1

PARAMETERS	MINIMUM (µm)	AVERAGE (µm)	MAXIMUM (µm)
Length	87	205	420
Width	3.5	8.27	11.38

The length of the fibers were in the range of 87µ m - 420 µ m and the width of the fibers was found in the range of 3.5 µ m – 11.38 µ m.

PHYSIOCEMICAL PARAMETERS

Physiochemical constants like total ash values, acid insoluble ash, water soluble ash, extractive values, loss on drying, swelling index, foaming index were studied and reported in table no: 2

Table no: 2 Physiochemical parameters of *Indigofera aspalathoides* whole plant

S.NO	PARAMETERS	VALUES (%W/W)
1.	Total ash	11.5±0.56
2.	Acid insoluble ash	8.0±0.25
3.	Water soluble ash	9.3±0.45
4.	Sulphated ash	4.2±0.65
	EXTRACTIVE VALUE	
5.	Alcohol soluble extractive	7.3±0.26
6.	Water soluble extract	6.9±0.12
7.	Loss on drying	12.6±0.74
8.	Foaming index	Nil
9.	Swelling index	Nil

The total ash, acid insoluble ash, water soluble ash and sulphated ash were found to be11.5±0.56, 8.0±0.25% w/w, 9.3±0.45% w/w, 4.26±0.65% w/w respectively. The water soluble extractive, alcohol soluble extractive values were found to be 6.9±0.12% w/w, 7.3±0.26% w/w, respectively. Loss on drying was found to be 12.06±0.74% w/w. The foaming index and swelling index was found to be nil.

This detailed Pharmacognostical investigation on the whole plant of *Indigofera aspalathoides* will be useful for the identification of the drug in crude as well as powder form in order to differentiate the plant from its allied species and adulterants.

7. PHYTOCHEMICAL STUDIES

7.1 MATERIAL AND METHODS

Phytochemical evaluation is used to determine the nature of phytoconstituents present in the plant by using suitable chemical tests. Phytochemical analysis is very much important because the therapeutic activity is based on the constituent present in the drug. It can be done by confirmation with different chromatographic techniques like TLC and HPTLC. Therefore a complete investigation is required to characterize the Phytoconstituents qualitatively and quantitatively.

PREPARATION OF EXTRACTS

Whole plant was collected, dried in shade, coarsely powdered and successively extracted with solvents of increasing polarity like chloroform, ethyl acetate and ethanol by continuous percolation process using soxhlet apparatus. After extraction each extracts were concentrated by using rotary vacuum evaporator. It is dried and the percentage yield was calculated. Appearance and consistency of the extract were also noted.

PRELIMINARY PHYTOCHEMICAL SCREENING[88,89]

The whole plant powder and extracts were subjected to qualitative chemical analysis for the identification of active constituents in each extracts and the powdered whole plant.

DETECTION OF ALKALOIDS

Dragendorff's reagent

To the sample 5ml of 2M HCl was added. Then 1ml of Dragendorff's reagent was added and examined for an immediate formation of an orange red precipitate.

Mayer's reagent

To the substance little quantity of dilute hydrochloric acid and Mayer's reagent were added and examined for the formation of white precipitate.

Wagner's reagent

The test substance was treated with little amount of Wagner's reagent and examined for the formation of reddish brown precipitate.

DETECTION OF GLYCOSIDES

Borntrager's test

The powdered material was boiled with 1ml of sulphuric acid in a test tube for five minutes. Filtered while hot, cooled and shaken with equal volume of chloroform. The lower layer of solvent was separated and shaken with half of its volume of dilute ammonia. A rose pink to red colour is produced in the ammonical layer indicates the presence of anthroquinone glycosides.

Modified Borntrager's test

The test material was boiled with 2ml of the dilute sulphuric acid. This was treated with 2ml of 5% aqueous ferric chloride solution (freshly prepared) for 5 minutes, and shaken with equal volume of chloroform. The lower layer of solvent was

separated and shaken with half of its volume of dilute ammonia. A rose pink to red colour is produced in the ammonical layer.

Legal's test

The test sample when treated with sodium nitropruside in pyridine and methanolic alkali. Formation of a pink red colour indicates the presence of cardiac glycosides.

DETECTION OF STEROIDS AND TRITERPENOIDS

LibermannBurchards Test

The powdered drug was treated with few drops of acetic anhydride, boiled and cooled. Conc.sulphuric acid was added from the sides of the test tube formation of brown ring is formed at the junction of two layers and upper layer turns green which shows presence of steroids and formation of deep red color indicates presence of tri terpenoids.

Salkowski Test

The extract was treated with few drops of concentrated sulphuric acid, red color at lower layer indicates presence of steroids and formation of yellow colored lower layer indicates presence of tri terpenoids.

DETECTION OF FLAVONOIDS

Shinoda's test

Small quantity of extract was dissolved in alcohol to this pieces to magnesium followed by concentrated hydrochloric acid were added drop wise and heated. Appearance of magenta colour shows the presence of flavonoids.

Alkaline reagent test

Small quantity of the extract was dissolved in aqueous sodium hydroxide and appearance of yellow colour indicates the presence of flavonoids.

DETECTION OF CARBOHYDRATES

Molisch's test

To the test solution few drops of alcoholic alpha napthol and few drops of conc. sulphuric acid were added through the sides of test tube, purple to violet color ring appears at the junction indicates the presence of carbohydrates.

Fehling's test

The test solution was mixed with Fehling's I and II, heated and examined for the appearance of red coloration for the presence of sugar.

DETECTION OF TANNINS

Lead acetate test

The test solution was mixed with basic lead acetate solution and examined for formation of a white precipitate.

Ferric chloride test

A few drops of 5% aqueous ferric chloride solution was added to 2ml of an aqueous extract of the drug and examined for the appearance of bluish black color.

DETECTION OF PROTEINS

Biuret test

The sample was treated with 5-8 drops of 10% w/w copper sulphate solution and observed for the presence of violet color.

DETECTION OF SAPONINS

A drop of sodium bicarbonate solution was added to the sample and the mixture was shaken vigorously and left for 3 minutes. Development of any honey comb like froth was examined.

DECTECTION OF GUMS AND MUCILAGE

The small quantities of test substance were dissolved in 5 to 10ml of acetic anhydride by means of heat, cooled and added 0.05ml of conc. sulphuric acid. Formation of bright purplish red color indicates the presence of gums and mucilage.

DETECTION OF FIXED OILS AND FATS

Small quantities of extracts were pressed between two filter papers. An oily stain on the filter paper indicates the presence of fixed oils and fats.

QUANTITATIVE ESTIMATION OF TOTAL FLAVONOID CONTENT[90]

Total Flavanoid content

Total flavanoid content was determined by calorimetric method, using quercetin as a standard. The test samples were individually dissolved in DMSO. Then the sample solution (150 µl) was mixed with 150 µl of 2% aluminium chloride. After 10min of incubation at ambient temperature, the absorbance of the supernatant was measured at 435nm using spectrophotometer. Three replicates were made for each test sample.

The total flavanoid content was expressed as quercetin equivalent in mg/gm extract (mg QRT/gm extract).

FLUORESCENCE ANALYSIS[91,92]

Many crude drug show Fluorescence when the sample is exposed to UV radiation. Evaluation of crude drugs based on fluorescence in day light is not much used, as it is usually unreliable due to the weakness of the fluorescent effect. Fluorescent lamps are fitted with suitable filter, which eliminate visible radiation from the lamp and transmits UV radiation of definite wavelength was used for the study several crude drugs show characteristic fluorescence which is very much useful for their evaluation.

THIN LAYER CHROMATOGRAPHY[93]

Principle

The principle of separation is adsorption. It is reliable technique in which solute undergoes distribution between two phases, stationary and mobile phase. The mobile phase flows through because of capillary action (against gravitational force). The compounds having higher affinities towards the stationary phase eluted slower were as the compound having lesser affinities towards stationary phase eluted faster.

TLC Plate Preparation

The plates were prepared using Stahl TLC spreader. 40gm of silica gel G was mixed with 85ml of water to prepare homogenous suspension and poured in the spreader. 0.25mm thickness of plates was prepared, air dried until the transparency of the layer disappeared, then dried at 110ºC for 30 minutes and kept in desiccators**.**

Selection of mobile phase

Solvent mixture was selected on the basis of the phytoconstituents present in each extract. Factors such as nature of components, stationary phase, polarity, influence the rate of separation of constituents was considered. From the vast analysis, best solvents were selected which showed good separation with maximum number of components.

$$\text{Rf value} = \frac{\text{Distance travelled by solute from the baseline}}{\text{Distance travelled by solvent from the baseline}}$$

HPTLC- FINGER PRINT PROFILE[94]

HPTLC is one of the versatile chromatographic method which helps in the identification of compounds and thereby authentication of purity of herbal drugs. The time required in this method for the demonstration of most of the characteristic constituents of a drug is very quick and short. In addition to qualitative detection, HPTLC also provides semi- quantitative information on major active constituents of a drug, thus enabling an assessment of drug quality.

Instrument Conditions

Sample used : Ethanol Extract

Instrument : CAMAG HPTLC

HPTLC Applicator : CAMAG LINOMAT IV

HPTLC Scanner : CAMAG TLC SCANNER II

Sample dilution : 100mg of sample extracted with 1ml of Ethanol

Volume of injection : 20µl

Mobile phase : Hexane: Ethyl acetate: Chloroform: Methanol: Formic acid (2:3:5:0.5:0.5)

Lambda max : 400 - 800nm

Lamp : Tungsten

Stationary phase : TLC Aluminium coated silica gel 60 F254 (Merck)

Equipment **:** A Camag HPTLC system equipped with a sample applicator Linomat IV, Twin trough plate development chamber, TLCScanner II.

Chromatographic conditions

The estimation has been done using the following chromatographic conditions.Chromatography was performed on a 12 × 3 cm (H x W) pre-activated HPTLC silicagel 60 F254 plate. Samples were applied to the plate as 6mm wide band with an automatic TLC applicator Linomat IV with nitrogen flow (CAMAG, Switzerland), 8mm from the bottom. Densitometric scanning was performed on CAMAG scanner II. The plates were prewashed with solvent ethyl acetate. HPTLC serves as a convenient tool for finding the distribution pattern of phytoconstituents which is unique to each plant. The fingerprint obtained is suitable for monitoring the identity and purity of drugs and for detecting adulteration and substitution. HPTLC technique is helpful in order to check the identity, purity and standardize the quantity of active principles present in the herbal extract.

7.2RESULTS AND DISCUSSION

Phytochemical investigations were carried and the results are as follows.

Table no: 3 Percentage yield extracts of Whole plant of *indigofera aspalathoides*.,

S.NO	**EXTRACT**	**YIELD (%W/W)**
1.	Chloroform	6.8
2.	Ethyl acetate	6.2
3.	Ethanol	7.6

QUALITATIVE PHYTOCHEMICAL ANALYSIS

TABLE NO: 4 PRELIMINARY PHYTOCHEMICAL ANALYSIS

S. N	CHEMICAL CONSTITUENS	**POWDERED DRUG**	CHLORO FORM	ETHYL ACETATE	ETHANOL
1	Flavanoids	+	+	+	+
2	Alkaloids	+	+	-	+
3	Carbohydrates	+	+	+	+
5	Proteins & Amino acids	-	-	-	-
6	Fats and fixed oils	+	+	-	+
7	Steroids	+	+	+	+
8	Glycosides	+	+	-	-
9	Carbohydrates	+	+	+	+
10	Gums & Mucilage	-	-	-	-
11	Phenols	-	-	+	-
12	Saponins	-	-	-	-
13	Volitle oils	-	-	-	-

Note : + - Indicates the presence and absence

From the qualitative analysis, it was observed that the ethanol extract showed the presence of maximum active constituents such as flavonoids, steroids, carbohydrate, and alkaloids. All these compounds were found in the powdered whole plant also. The ethyl acetate extract also showed the presence of more number of active constituents. Chloroform extract showed the

presence of glycoside, fat and fixed oil and alkaloids. All extract showed the presence of steroids and flavonoids

TOTAL FLAVONOID CONTENT

Total flavonoid content was determined and compared with that of standard and thus ethanolic extract was found to have higher absorbance than ethyl acetate. It is represented in table

Table No :5 Determination of Total Flavanoid Content

S.No	Concentration of standard solution(µg/ml)	Absorbance(765nm)
1.	2	0.06
2.	4	0.10
3.	6	0.13
4.	8	0.16
5.	10	0.21
6.	Ethyl Acetate	0.14
7.	Ethanol	0.16

Standard calibration curve for determination of Total Flavonoid Content

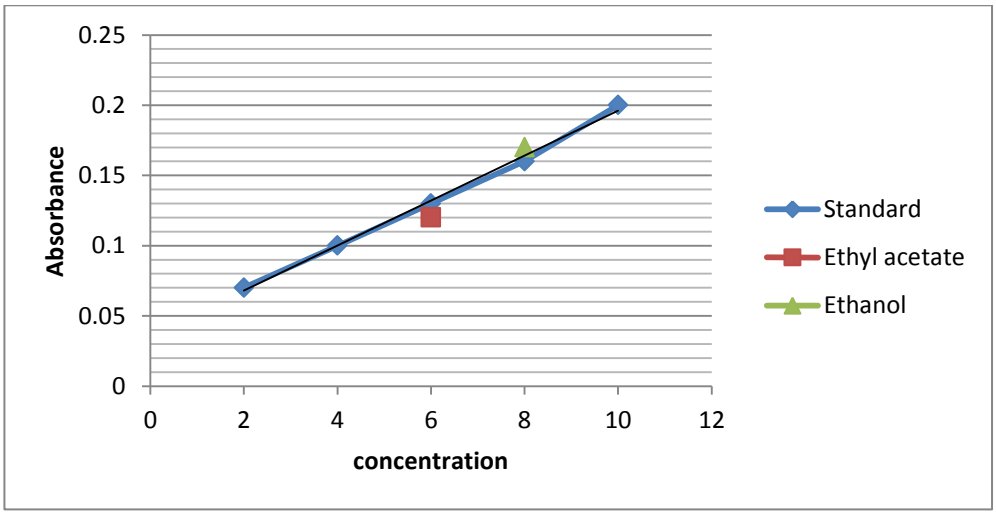


Fig : 1 Total Flavonoid content

FLUORESCENCE ANALYSIS

TABLE NO: 6 FLUORESCENCE CHARACTERISTIC OF POWDEREDWhole plant OF *indigofera aspalathoides*

S.NO	TREATMENT	DAY LIGHT	SHORT-UV (254nm)	LONG-UV(365nm)
1	Powder	Pale brown	Black	Brown
2	Powder + Water	White viscus	Light black	Brown
3	Powder + Ethanol	Light yellow	Brown	Light brown
4	Powder + 1N HCL	Yellow	Yellow	Greenish brown
5	Powder + 1N H_2SO_4	Black	Black	Brown
6	Powder + 1N NaOH	Green	Black	Dark green
7	Powder + 1N alcoholic KOH	Dark green	Brown	Dark brown
8	Powder + $FeCl_3$	Green	Green	Light brown
9	Powder + Acetic acid	Light brown	Green	White viscus
10	Powder + Ammonia	Yellow	Green	light brown
11	Powder + Iodine	Deep brown	Brown	Pale yellow

Fluorescence characteristics of the extracts of the whole plant of *Indigofera aspalathoides* TABLE NO: 7

S.NO	EXTRACTS	DAY LIGHT	SHORT-UV (254nm)	LONG-UV (365nm)
1	Chloroform	Brown	Light brown	Black
2	Ethyl acetate	Brown	Dark brown	Dark brown
3	Ethanol	Light brown	Brown	Brown

There was no characteristic fluorescence were seen with either the powdered whole plant or the extracts

CHROMATOGRAPHIC STUDIES

THIN LAYER CHROMATOGRAPHY

Thin layer chromatography was done with all the three extracts and their R_f values were tabulated in the table no: 8

s.no	extract	Solvent system	No. of spot	Rf value
1	chloroform	Chloroform : methanol(9:1)	5	085, 0.65 0.57,0.52, 0.47
2	Ethyl acetate	Chloroform : methanol(9:1)	4	0.87,0.77,0.70,0.62
3	Ethanol	Chloroform : methanol(9:1)	7	0.85,0.72,0.67,0.62 ,0.57,0.50,0.45

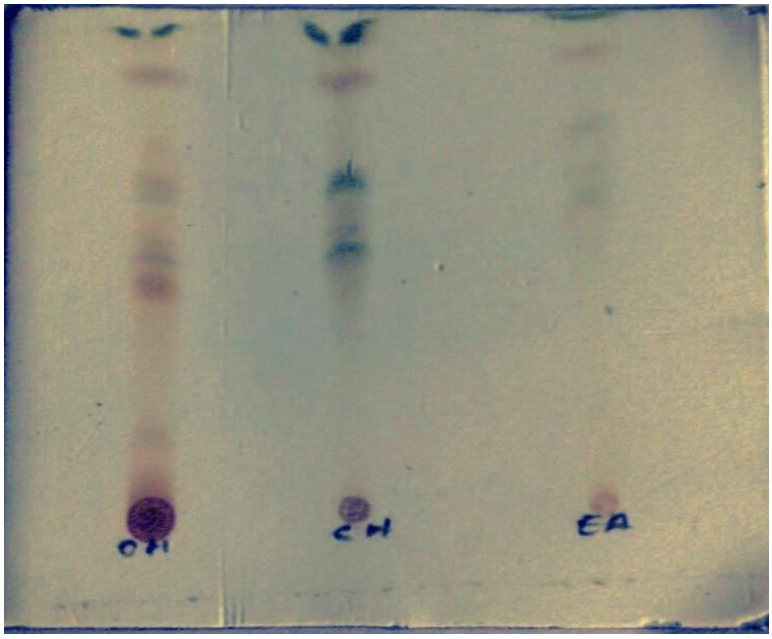


FIG:2 THIN LAYER CHROMATOGRAPHIC STUDIES OF EXTRACT

HPTLC FINGER PRINT PROFILE

HPTLC Finger print Data of Ethanolic Extract of *Indigofera aspalathoides*.High performance thin layer chromatography (HPTLC) finger printing was performed with the ethanol extract of *Indigofera aspalathoides* The chromatographic conditions were carried as detailed in material and method of this study. There were 12 peaks observed with different Rf Values and different heights. Percentage of areas was also obtained from the chromatogram.

FIG :3 HPTLC OF ETHANOLIC EXTRACT

FIG :4 FINGER PRINT DATA ANALYSIS FOR HPTLC

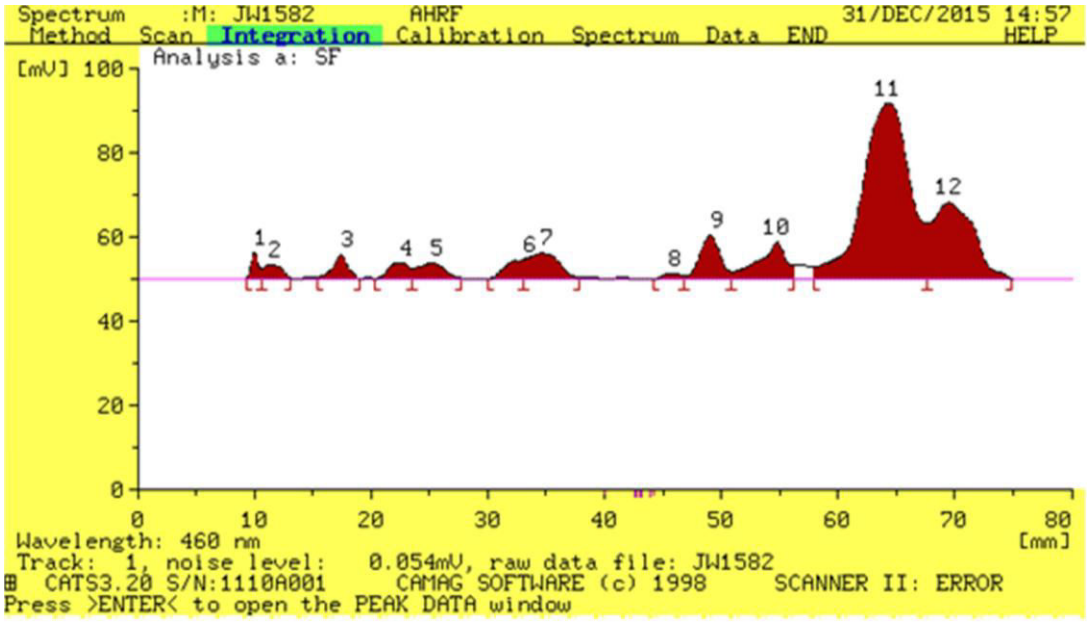
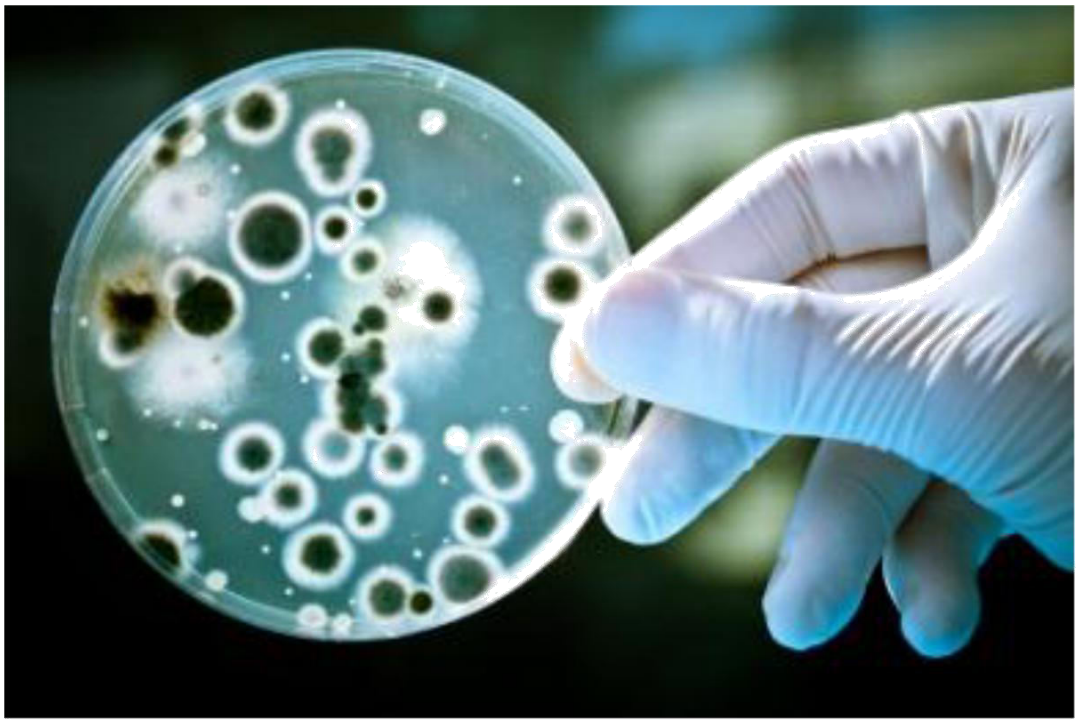


TABLE NO: 9 HPTLC FINGER PRINT DATA

S. No	R_f	Height	Area
1	0.10	6.3	44.1
2	0.11	3.3	62.3
3	0.17	5.8	86.6
4	0.22	4.0	80.6
5	0.25	3.8	100.6
6	0.33	4.6	81.9
7	0.34	6.1	191.9
8	0.45	1.5	24.7
9	0.49	10.3	206.6
10	0.54	8.6	236.2
11	0.64	41.9	1949.5
12	0.69	18.1	753.2



SELECTION OF ACTIVE EXTRACT

8. SELECTION OF ACTIVE EXTRACT

8.1 MATERIALS AND METHODS

In order to select the best extract all the extracts were subjected to *in-vitro* antioxidant activity and anti-microbial studies. These studies are used for the selection of best extract which would take to precede the further activities.

IN VITRO ANTIOXIDANT ACTIVITY[95]

Antioxidant is a molecule that inhibits oxidation of other molecule which produces free radicals. These radicals in turns produce chain reactions there by cause damage to the cells, resulting in development of various ailments. Antioxidant terminates these chain reactions by removing free radicals and inhibiting oxidative reactions. Therefore, antioxidant with free radical scavenging effect will be of greater importance in the prevention and therapeutics of disease.

DPPH ASSAY (2, 2-DIPHENYL -1-PICRYLHYDRAZYL)

Antioxidants react with DPPH and reduce it to DPPH-H and as consequence the absorbance decreases. The degree of discoloration indicates the scavenging potential of the antioxidant compounds or extracts in terms of hydrogen donating ability. Different volumes (1.25-10µ l) of plant extracts were made up to 40µ l with DMSO and 2.96ml DPPH (0.1mM) solution was added. The reaction mixture was incubated in dark condition at room temperature for 20 minutes. After 20 minutes, the absorbance of the mixture was read at 517nm. 3ml of DPPH was taken as control.

$$\% \text{ Inhibition} = \frac{\text{Abs (control) - Abs (test) X 100}}{\text{Abs (control)}}$$

ANTI MICROBIAL STUDIES

ANTIBACTERIAL ACTIVITY

The different extracts of whole plant of *Indigofera aspalathoides* were subjected to antibacterial studies against gram positive and gram negative organism.

The organism used were[96]

Gram positive organism

Enterococcus

Staphylococcus aureus

Gram negative bacteria

E-coli

Pseudomonas aeruginosa

Proteus mirablis

MEDIUM:

Muller Hinton agar.

PREPARATION OF THE BACTERIAL SUSPENSION FOR INOCULATION:

Few colonies of the pathogenic strains were picked up and inoculated into 4ml of peptone water. These tubes were incubated for 2-5hours to produce a bacterial suspension. The suspension was then diluted if necessary with saline solution to

density, which is visually equivalent to that of standard prepared by adding 0.5ml of 1% barium chloride to 99.5ml of 1% sulphuric acid. This suspension was then used as inoculum of bacteria.

PREPARATION OF MUELLER HINTON AGAR (MH AGAR):

1.	Beef infusion	-	30g/I
2.	Casein acid hydrolysate	-	17.5g/I
3.	Starch	-	1.5g/I
4.	Agar	-	17g/I

38g of Muller Hinton agar was weighed and dissolved in 100ml of distilled water and adjusted to pH 7.3±0.2 sterilized by autoclave at 121℃ at 1516 pressure for 15mins and used for the sensitivity test [97].

PREPARATION OF AGAR PLATES:

The extracts were poured into Muller Hinton agar medium such that the final concentration of extraction is in equal volume to that of the original.

PROCEDURE:

MINIMUM INHIBITORY CONCENTRATION:

The plates were prepared using agar and different extracts of various dilutions and it is allowed to solidify and dry. The suspensions of bacterial colonies were inoculated into the plate. Then the plates were incubated at 37°C for 24hours and then results are recorded.

ZONE OF INHIBITION:

A suitable dilution of a broth culture or a broth suspension of the test bacterium or fungi is flooded on the surface of a solid medium (MH agar). The plate is tilted to ensure uniform spreading and the excess broth pipetted off. Inoculations may also be performed by spreading with swabs. After drying the plates (37◦C for 30mins) antibiotic are applied with sterile syringe in the well. After overnight incubation, the degree of sensitivity is determined by measuring the zones of inhibition of growth around the well.

STANDARD DRUGS:

1.Ciprofloxacin

2.Gentamycin

3.Cefotaxime

Ciprofloxacin:

It is a fluoroquinolone derivative with their broad antimicrobial activity. The bacteria develop resistance to fluoroquinolones due to the mutation.

Mechanism of action

They act by inhibiting DNA gyrase (topoisomerase II) and DNA topoisomerase IV. The former action is direct and leads to an arrest of DNA replication. The later action also arrests DNA replication but by blocking the enzyme‟s normal function of delinking the daughter DNA molecule.

Gentamycin:

This is the aminoglycoside antibiotic is produced by *Micomonospora purpura.*

Mechanism of action:

It diffuses through outer cytoplasmic membrane and is transported to ribosome. Ribosomes manufacture enzyme as per the direction from messenger RNA. It binds to 30S ribosomes and interferes with initiation of protein synthesis, block the translation of m RNA and prematurely terminate the synthesis**.**

Cefotaxime:

These are cephalosporin, they are active against gram positive and gram negative bacteria. The gram negative organisms susceptible to these antibiotics include E.coli, Proteus, etc.

Mechanism of action:

They act by inhibiting bacterial cell wall synthesis [98].

ANTIFUNGAL ACTIVITY

The different extracts of whole plant of *Indigofera aspalathoides* were subjected to antifungal studies against *Candida albicans*.

MEDIUM:

Muller Hinton agar

PROCEDURE:

MINIMUM INHIBITORY CONCENTRATION:

The plates were prepared using agar and different extracts of various dilutions and it is allowed to solidify and dry. The suspension of fungus was inoculated into the plate. Then the plates were incubated at 37°C for 24hours and then results are recorded. The reports were recorded [99]

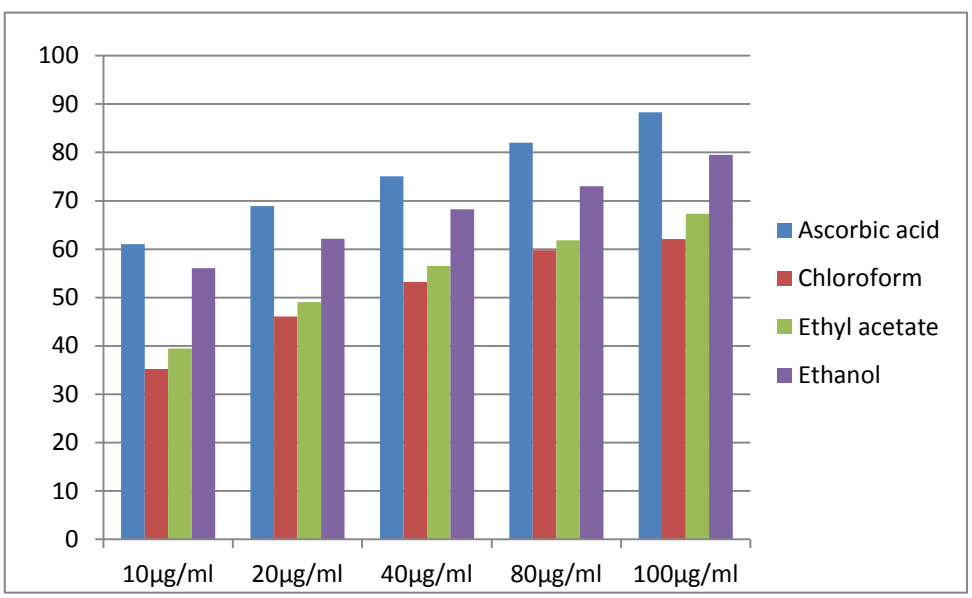
8.2 RESULTS AND DISCUSSION

IN-VITRO ANTIOXIDANT ACTIVITY(DPPH ASSAY)

TABLE NO: 10 PERCENTAGE INHIBITION OF EXTRACTS-DPPH ASSAY

Extract	% Inhibition at various concentrations				
	10	20	40	80	100
Ascorbic acid	61.03	68.88	75.04	82.04	88.31
Chloroform	35.20	46.07	53.24	59.77	62.08
Ethyl acetate	39.48	49.09	56.52	61.84	67.31
Ethanol	56.09	62.15	68.23	73.01	79.53

FIG NO: 22 GRAPHICAL DATA OF PERCENTAGE INHIBITION



In DPPH assay the scavenging potential of the antioxidant compounds or extracts were found. The percentage inhibition of Chloroform, Ethyl acetate and Ethanol was found to be 58.76, 62.08, 67.31 and 79.53 respectively at a maximum concentration of 100µ g/ml. The percentage inhibition of Ascorbic acid (Standard) was found to be 88.31. In that ethanol extract showed the maximum percentage of inhibition which is comparable to the standard.

Minimum inhibitory concentration of various extracts of *indigofera aspalathoides* for antibacterial activity and antifungal activity (table.no.11)

S.No	Extract	Conc. µg/ml	Microorganism used					
			1	2	3	4	5	6
1.	Chlorofom	100	+	+	+	+	+	+
		200	+	+	+	+	-	-
		300	+	-	-	+	-	-
		400	-	-	-	-	-	-
		500	-	-	-	-	-	-
2.	Ethyl Acetate	100	+	+	+	+	+	+
		200	+	+	+	+	-	-
		300	+	-	-	+	-	-
		400	-	-	-	-	-	-
		500	-	-	-	-	-	-
3.	Ethanol	100	+	+	+	+	+	+
		200	+	-	+	+	-	-
		300	-	-	-	-	-	-
		400	-	-	-	-	-	-
		500	-	-	-	-	-	-

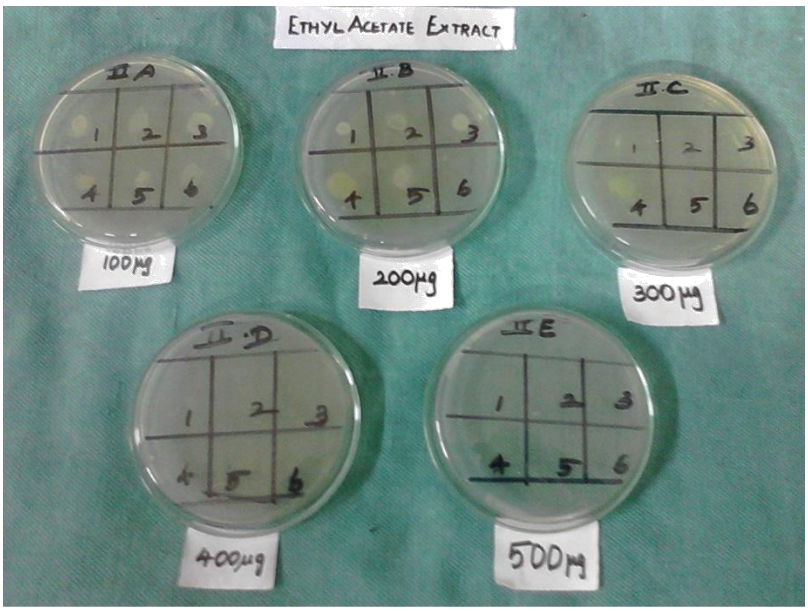
1.Staphylococcus aureus	(+) Presence of colonies
2.Enterococcus	
3.E.coli	(-) Absence of colonies
4.Pseudomonas aeruginosa	
5.Proteus mirablis	
6.Candida albicans	

MINIMUM INHIBITORY CONCENTRATION OF VARIOUS EXTRACTS OF *Indigofera aspalathoides* FOR ANTIBACTERIAL AND ANTIFUNGAL ACTIVITY

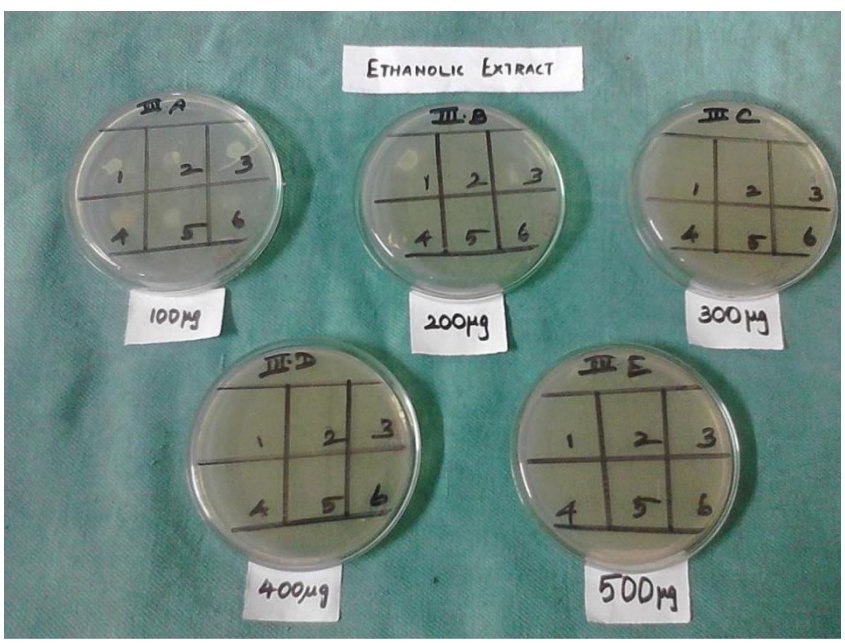
Control and water

Minimum inhibitory concentration of chloroform extract in different concentration (FIG:23)

Minimum inhibitory concentration of ethyl acetate extract in different concentration(FIG :24)



Minimum inhibitory concentration of ethanol extract in different concentration(FIG:25)

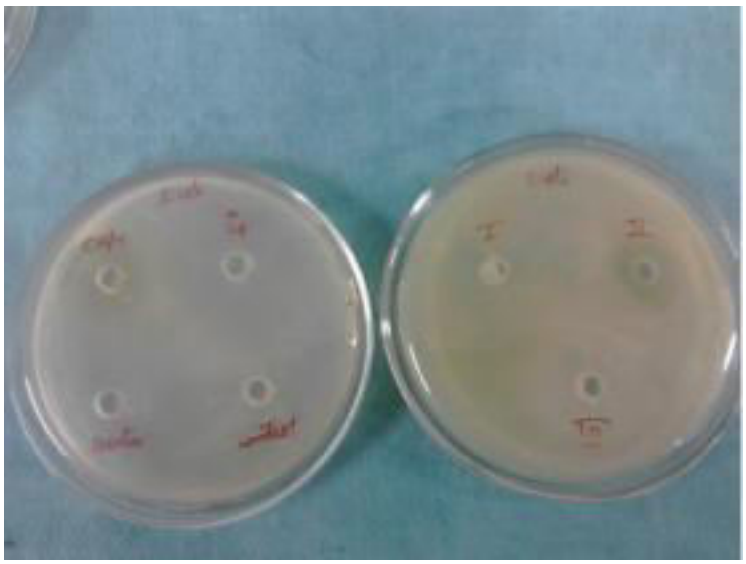


Zone of inhibition of various extracts of *indigofera aspalathoides* for antibacterial activity

(table.no.12

NAME OF THE STANDARD/ EXTRACT	ZONE OF INHIBITION(mm)				
	E-Coli	***Staphylococcus aureus***	***Proteus Mirablis***	***Psuedomonas Aureginosa***	***Enterococcus***
STANDARD					
Cefotaxime	22	37	30	30	37
Ciprofloxacin	18	24	35	50	30
Gentamycin	21	28	36	20	32
EXTRACT					
Chloroform	6	7	6	13	10
Ethyl acetate extract	20	28	13	11	18
Ethyl alcohol extract	7	15	25	20	20
Water	resistant	resistant	Resistant	Resistant	Resistant

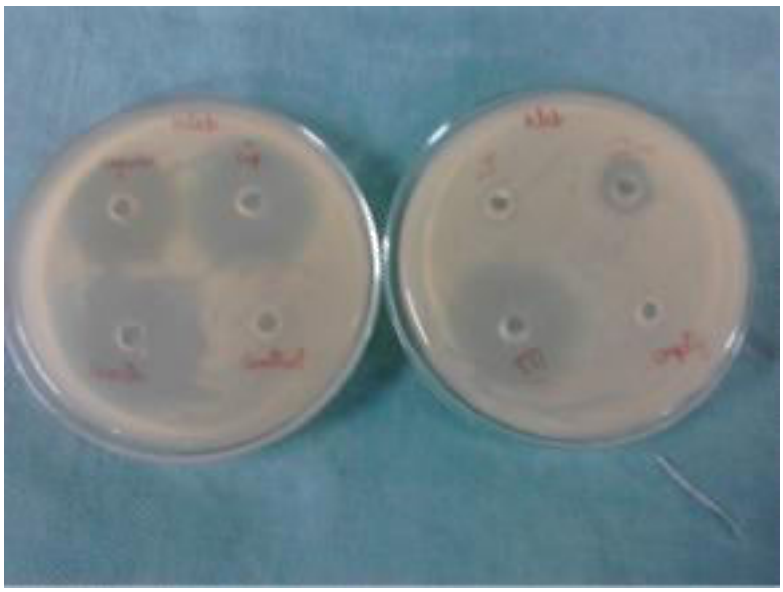
Zone of inhibition of various extracts *of indigofera aspalathoides* for antibacterial activity



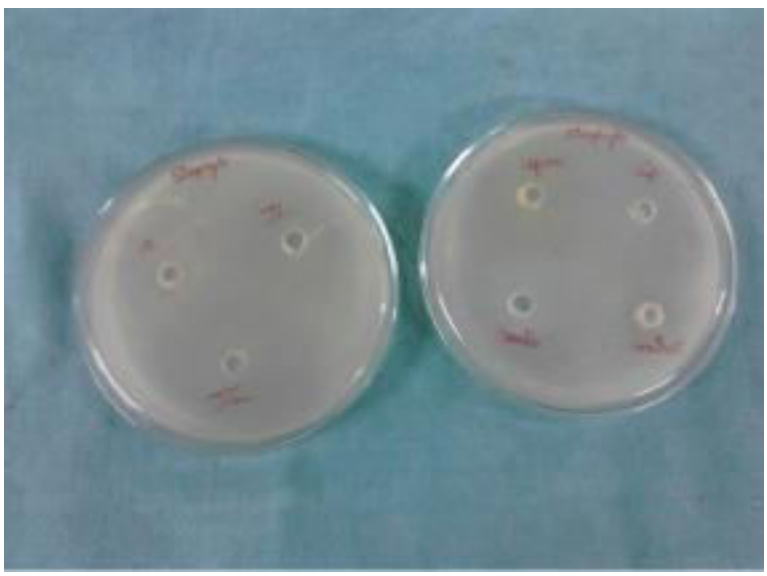
Zone of inhibition against *E-Coli* various extracts of *Indigofera aspalathoides* (FIG :26)

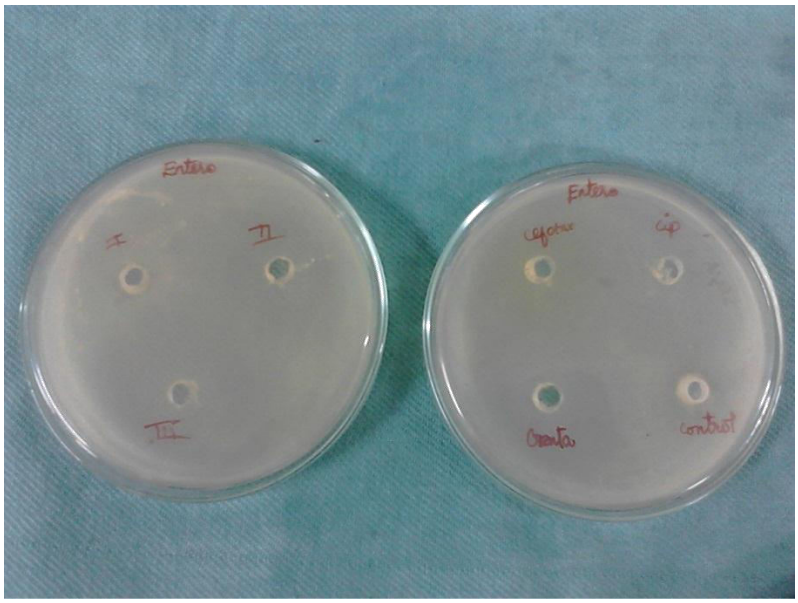
Zone of inhibition against *Pseudomonas* various extracts of *Indigofera aspalathoides* (FIG:27)

Zone of inhibition against *Proteus* various extracts of *Indigofera aspalathoides* (FIG :28)



Zone of inhibition against *Staphylococcus* various extracts of *Indigofera aspalathoides*(FIG:29)





Zone of inhibition against *Enterococcus* in various extracts of *Indigofera aspalathoides* (FIG:30)

The antimicrobial activity of *Indigofera aspalathoides* extracts werecarried out by *in vitro* method in agar plate against 5 bacterial strains and one fungal strain which showed an inhibition of microbial growth of different extracts of whole plant.

The minimum inhibitory concentration of the ethanol extracts was found to be 300µg/ml against the tested microorganism which was found to be more effective when compared to other extracts.

The ethanolic extracts of the plant showed better antibacterial activity against *Pseudomonas, Proteus And Enterococcus* by exhibiting almost equal zone of inhibition compared to that of standard drug Gentamycin.

The plant extracts showed good antifungal activity with minimum inhibitory concentration of about 300µg/ml for the ethanol and chloroform extracts.

The antimicrobial studies of various extracts of the *Indigofera aspalathoides* confirms its benefits to be used in the remedies of the diseases caused by these organisms.

FORMULATION AND EVALUATION

9. FORMULATION AND EVALUATION OF HERBAL OINTMENT

9.1 MATERIALS AND METHOD

Formulation Of Herbal Ointment[100]

Ingredients:

Bess wax - 2g

Liquid paraffin - 1g

Methyl paraben - 0.02

Propyl paraben -0.02

METHOD:

Bees wax and liquid paraffin were heated separately in a china dish at 70℃. Methyl paraben and propyl paraben were mixed separately. Bees wax and liquid paraffin mixture was added drop by drop with constant stirring to form a ointment base.

Preparation of herbal ointment I

Ointment base -3g

Ethanol extract -.02g

The above the ingredients were mixed together and stirred

Preparation of herbal ointment II

Ointment base -3g

Ethanol extract -.04g

The above the ingredients were mixed together and stirred

EVALUATION OF THE OINTMENT[101]

Physical Examination:

The prepared ointment formulations were inspected visually for their colour, homogeneity, consistency.

Determination of pH:

2.5gm Ointment sample was taken in 100 ml dry beaker, 50 ml water was added to it. Beaker was heated on water bath maintained at about 60ºC to 70ºC for 10 minutes, cooled to room temperature and then centrifuged at 3000 rpm for 10 minutes. The pH of water extract was measured by using pH meter. The pH measurements was done by using a digital type pH meter by dipping the glass electrode into the ointment formulation.

3. **Extrudability:**

Ointments were filled into collapsible tubes after formulating them and extrudability was determined.

4. Rubout

It included spreadability and wetness. A 0.1g of ointment was applied on dorsal skin surface of human volunteer and the properties were observed .

5. Skin sensitivity test

The ointment was applied on the shaved intact skin of Albino mice and examined for any changes on the skin after 24hrs.

9.2 RESULTS AND DISCUSSION

1. **Physical Appearance:**

Ointment formulations were pale green viscous preparation with a smooth homogeneous texture. The Physical Appearance of the ointment were given in Table no: 13

Table no :13 Physical Appearance

S.No	Formulations	Colour	Homogeneity	Consistency
1	2%w/w	pale green	Excellent	+++
2	4%w/w	pale green	Excellent	+++

2.Determination of pH:

The pH of the ointment solution was measured with the help of pH meter. 0.5g of ointment was dissolved in 50ml of distilled water and stored for two hours. The measurement of pH each formulation was done in triplicate.

Table no :14 pH of ointment

S.no.	Formulations	pH
1	2%w/w	6.36±0.3
2	4%w/w	6.27±0.1

3.Extrudability:

Ointments were filled into collapsible tubes after formulating them. The extrudability of the formulation has been checked.

Table no :15 Extrudability of ointment

S. No.	Formulations	Extrudability
1	2%w/w	Easily Extrudable
2	4%w/w	Easily Extrudable

4. Spreadability

Spreadability of the ointment on skin surface of human volunteers were givenin table no:16

Table no :16 Spreadability of ointments

S. No.	Formulations	Spreadability
1	2%w/w	Good
2	4%w/w	Good

5. Skin sensitivity test

The skin sensitivity showed no irritation ,redness or erythema indicating that both ointments were non-irritant.

Table no :17 skin sensitivity test of ointments

S. No.	Formulations	Skin test
1	2%w/w	non irritant
2	4%w/w	non irritant

PHARMACOLOGICAL STUDIES

10. ANTI- PSORIATIC ACTIVITY[101,103]

10.1 Materials and Methods

Imiquimod Cream Induced Psoriasis Model

Plant extract

Ethanolic extract ointment of whole plant of *Indigofera aspalathoides*

Animal selection and procurement

Healthy Albino mice (weighing about 18 – 25 g) were procured from the Madras Medical College animal house. The animals used for the entire study was approved by the Institutional Animal Ethical Committee which is certified by the Committee for the purpose of control and supervision of experiments on animals, India.

Approved CPCSEA Registration No : IAEC/MMC/09/2016 Dated: 21/11/2016.

The procured animals were kept in a clean, dry polycarbonate cages and maintained in a well-ventilated animal house. The temperature of experimental animal room was maintained at 22ºC (± 3ºC) and the relative humidity was maintained from 50-60%. Lighting was artificially maintained for 12hrs dark and 12hrs light. All the animals were kept in the cages for at least 5days prior to dosing for acclimatization to the laboratory conditions. The animals were fed with standard pellet diet and water was given ad libitum. Before starting the dose, the animals were fasted overnight but allowed to access water.

EXPERIMENTAL DESIGN:

Grouping of animals:

TABLE NO: 18 Grouping of animals

S.NO	GROUPS	NO.OF ANIMALS
1	Control group	6
2	Disease control	6
3	Standard	6
4	Test group-I	6
5	Test group-II	6
TOTAL		30

Psoriasis induction[104]

5% Imiquimod cream

Procedure

Mice at 8–10 weeks of age were used in this experiment. The mice were divided into five groups such as group I, II, III, IV, V . Back skin of the mice was shaved and

remaining hair were removed using a depilatory cream (Veet). Shaved back skin were daily treated with 62.5 mg Aldara©cream (5% Imiquimod, 3M Pharmaceuticals) for 6 days. Control mice were treated similarly with Vaseline .Severity of skin inflammation was evaluated using a clinical score based on Psoriasis Area Severity Index as erythema, scaling and thickening were scored on a scale from 0 to 4 (0: none; 1: slight; 2: moderate; 3: marked; and 4: severe) used to measure the severity of inflammation.

Treatment of animal groups

GROUPS	**TREATMENT SCHEDULE**	**NUMBER OF ANIMAL**
1. Control group	vaseline for 15day	6
2.Disease control	Imq for 6days	6
3.Standard	Imq(6days) +clobetasol for 15days	6
4.Test group-I	Imq(6days) + low dose ointment(2%w/w) for 15 days	6
5.Test group-II	Imq(6days) + high dose ointment(4%w/w) for 15days	6
Total		30

TABLE NO:19 treatment of animal group

The standard and ointments were applied topically once daily for 2 weeks , drugs were applied topically. Two hours after the last treatment the animal were sacrificed , longitudinal sections of the skin were made.

Histopathological examination

The longitudinal sections of the skin fixed in 10% formalin embedded in paraffin and cut into 5 µm thick section using a microtome. Sections are mounted on glass slides using standard techniques. The sections are stained with Haematoxylin-eosin and are examined under a microscope using 10 × magnifications and photographed under a light microscope equipped for photography.

PARAMETERS EVALUATED[105,106]

1. **Drug activity and percentage orthokeratosis**

Drug activity and percentage of orthokeratosis(ok) in those parts of the adult mouse skin, which normally have a parakeratotic differentiation, was quantified measuring the length of the granular layer (A) and the length of the scale (B).

% Orthokeratosis = (A/B) × 100

$$\%\text{activity} = \frac{\text{Mean OK of treated group - Mean OK of control group} \times 100}{100 - \text{Mean OK of the control group}}$$

OK= orthokeratosis

10 sequential scales per animal were measured and the results were given in % orthokeratosis per scale. Individual orthokeratosis values were obtained per test group.

2. Epidermal thickness

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

$$\text{Epidermal thickness} = \frac{\text{ET of treated group} - \text{ET of control group} \times 100}{100 - \text{ET of the control group}}$$

ET = Epidermal thickness

Statistical analysis

Results were expressed as Mean ± SEM. The data was analyzed using one way analysis of variance (ANOVA) followed by Dennett's test. P values < 0.01 were considered as Significant.

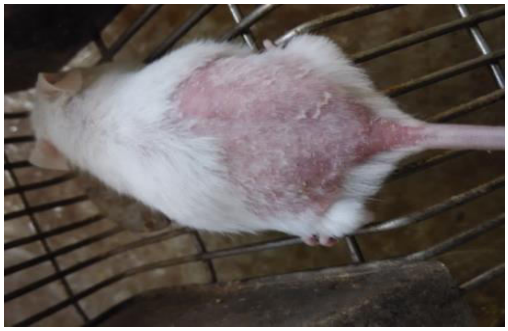
10.2 RESULTS AND DICUSSION

Anti-psoriatic activity of the herbal ointment formulated with whole plant of *Indigofera aspalathoides*. were carried out by mice skin model and result are summarized as follows,

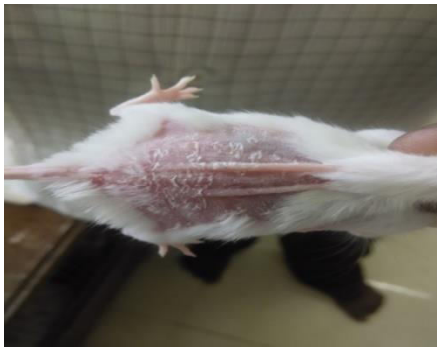
INDUCTION OF PSORIASIS

IMQ-induced skin inflammation in mice phenotypically resembles psoriasis. Erythema, scaling, and thickness of the back skin was scored daily on a scale from 0 to 4. Scores are measured the result are summarized as follows.

Control animal fig:31

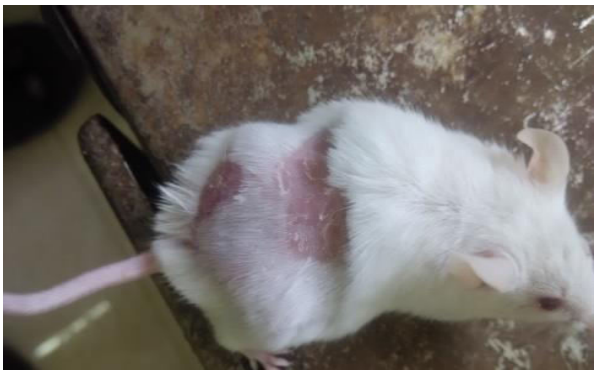


Disease control fig :32



Standard animal fig:33

Test 1 animal fig :34



Test 2 animal fig :35

Table no:20 Scaling of mice skin

Days	Scaling				
	Control group	Disease group	Standard group	Test 1 group	Test 2 group
1	0	0	0	0	0
2	0	1±0.16	1±0.16	1±0.16	1±0.16
3	0	2±0.31	1±0.16	1±0.16	2±0.31
4	0	3±0.50	2±0.31	2±0.31	2±0.31
5	0	4±0.66	3±0.50	4±0.66	3±0.50
6	0	4±0.66	3±0.66	4±0.66	4±0.66

Fig :36 Scaling of mice skin

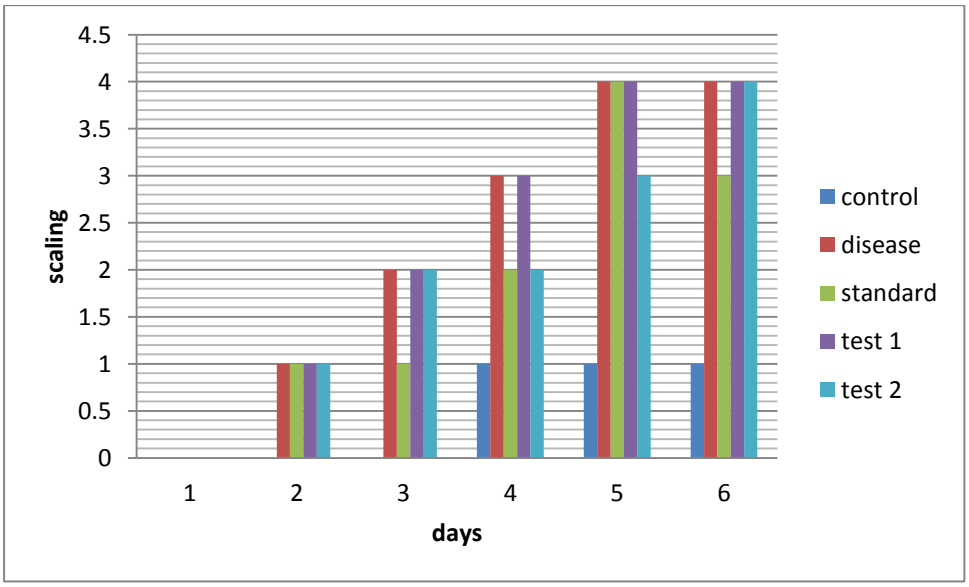
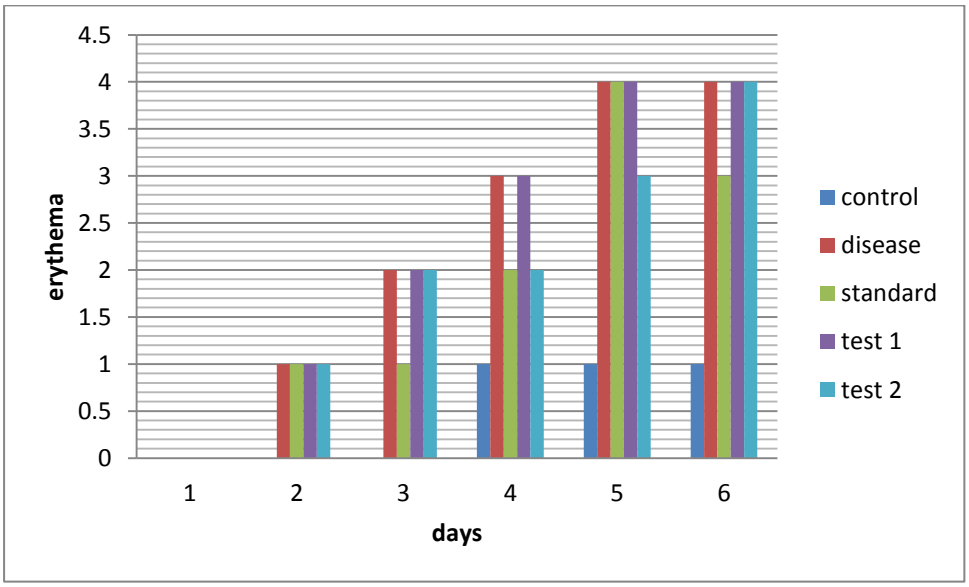


Table no:21 Erythema of mice skin

Days	Erythema				
	Control group	Disease group	Standard group	Test 1 group	Test 2 group
1	0	0	0	0	0
2	0	1±0.16	1±0.16	1±0.16	1±0.16
3	0	2±0.31	1±0.16	1±0.16	2±0.31
4	1±0.16	3±0.51	2±0.31	2±0.31	2±0.31
5	1±0.16	4±0.66	3±0.50	4±0.66	3±0.50
6	1±0.16	4±0.66	3±0.50	4±0.66	4±0.66

Fig: 37 Erythema of mice skin



Scores

0: None

1: Slight

2: Moderate

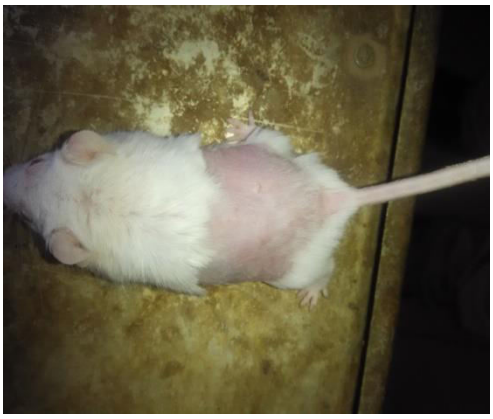
3: Marked

4: Severe

ANTI PSORIATIC ACTIVITY

Control animal fig:38

Disease control animal fig: 39



Standard(clobetasol) animal

Fig:40

Test 1(2% w/w ointment)animal

fig :41

Test2 (4% w/w ointment)animal fig :42

HISTOPATHOLOGICAL EVALUTION OF THE MICE SKIN

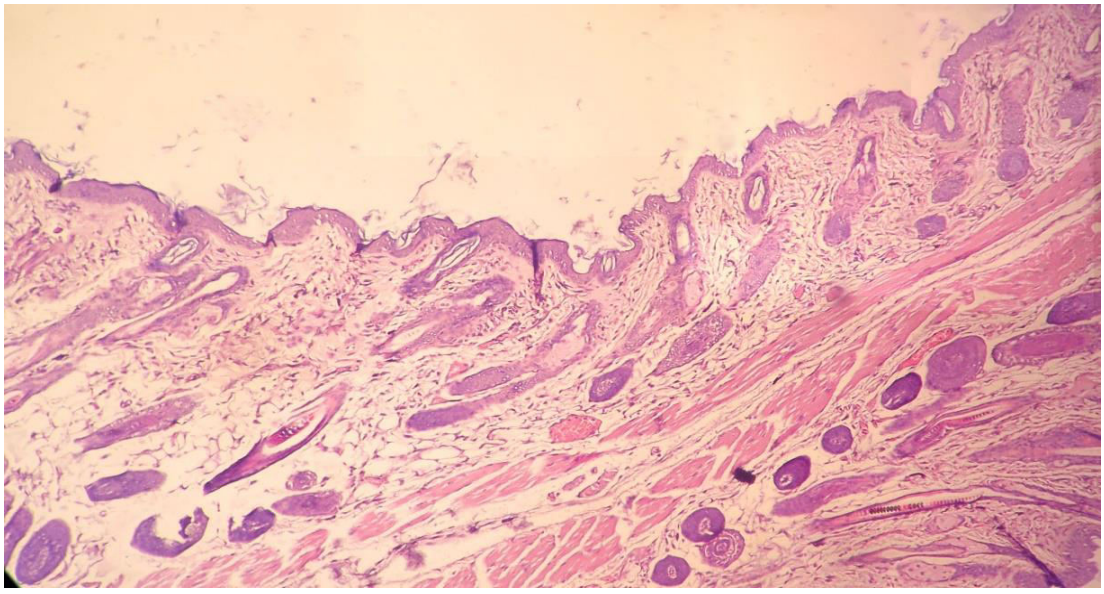


FIG:43 CONTROL MICE SKIN

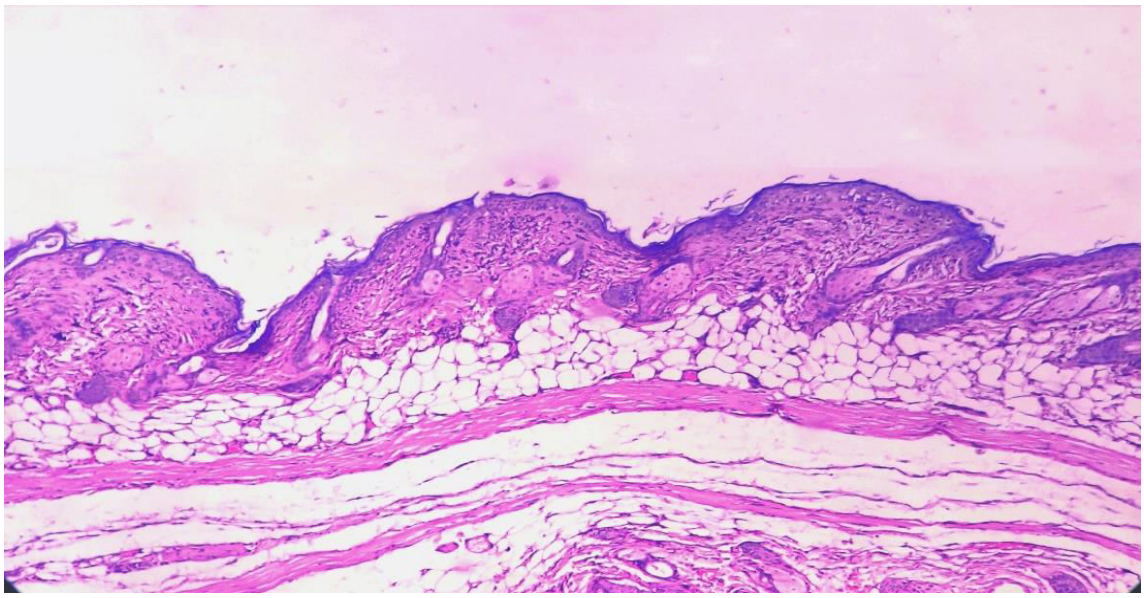


FIG:44 DISEASE CONTROL MICE SKIN

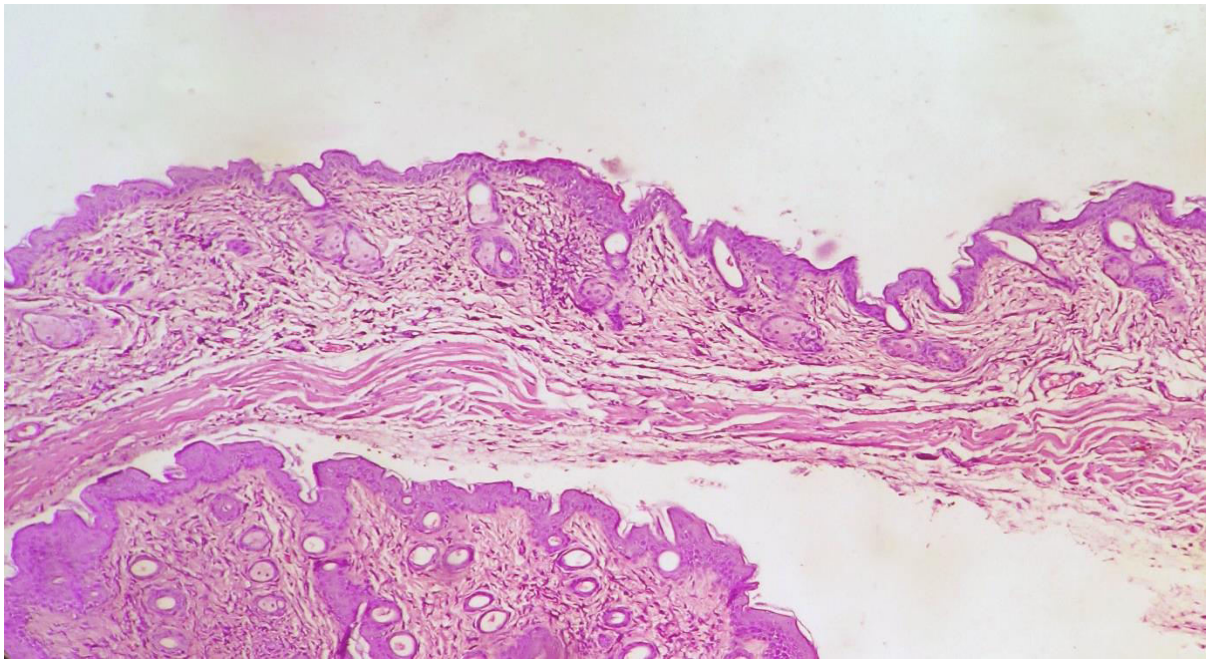


FIG:45 STANDARD MICE SKIN

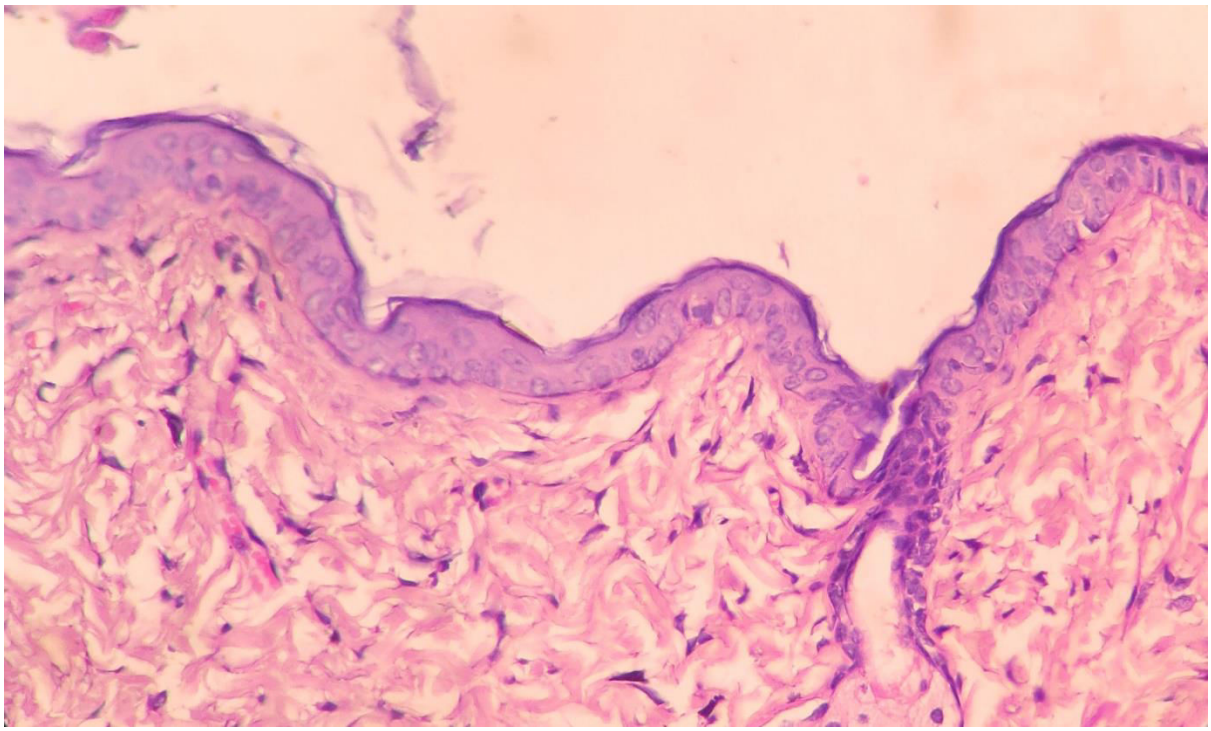


FIG:46 TEST 1(2%W/W OINTMENT) MICE SKIN

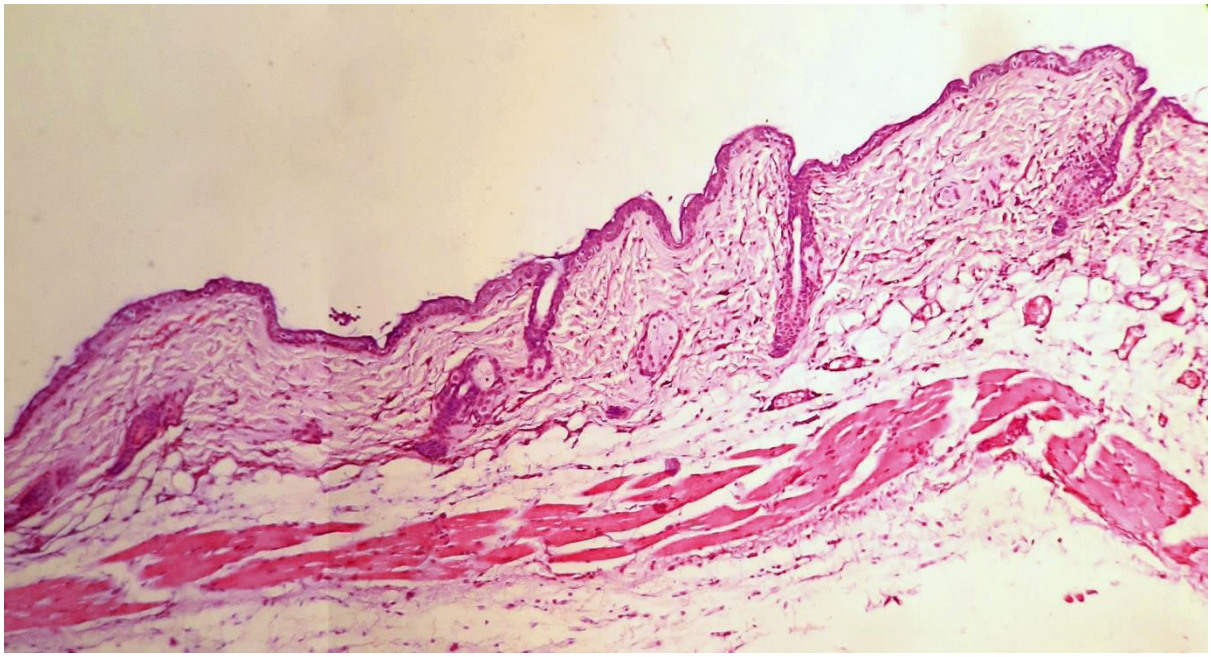


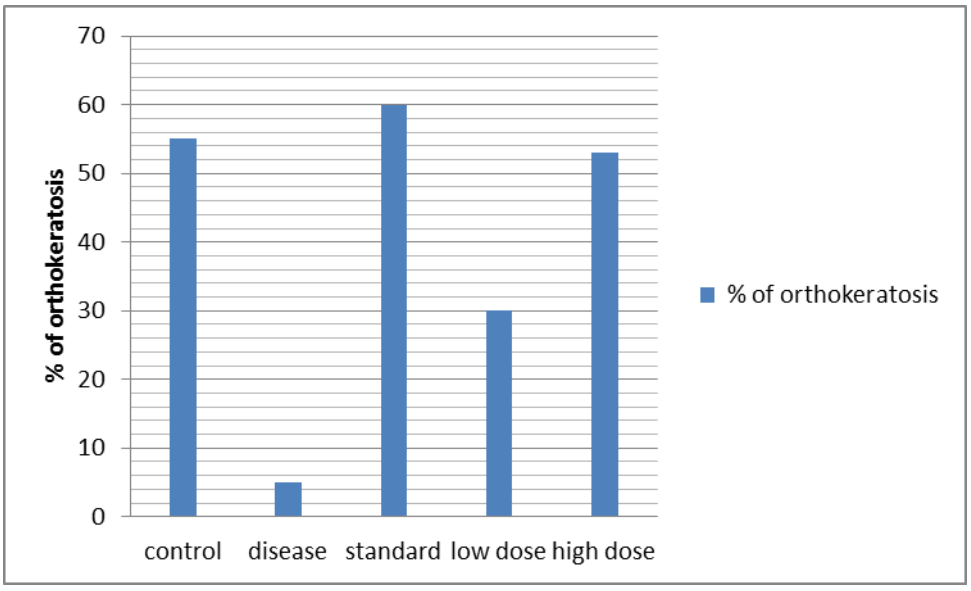
FIG:47 TEST-2(4% W/W OINTMENT) MICE SKIN

% of orthokeratosis in mice skin Table:22

Animal groups	% of orthokeratosis
Control	55 ± 1.2
Disease	5± 2.1**
Standard	60± 1.8***
Test1 low dose (2% w/w)	30± 2.0**
Test 2 high dose(4% w/w)	53±1.5***

Values are mean ± SEM Statistical significant test for comparison was done by ANOVA, followed by Dunnet's 't' test (n=6). **P< 0.01, *P< 0.05 when compared against control.

% of orthokeratosis in mice skin fig:48

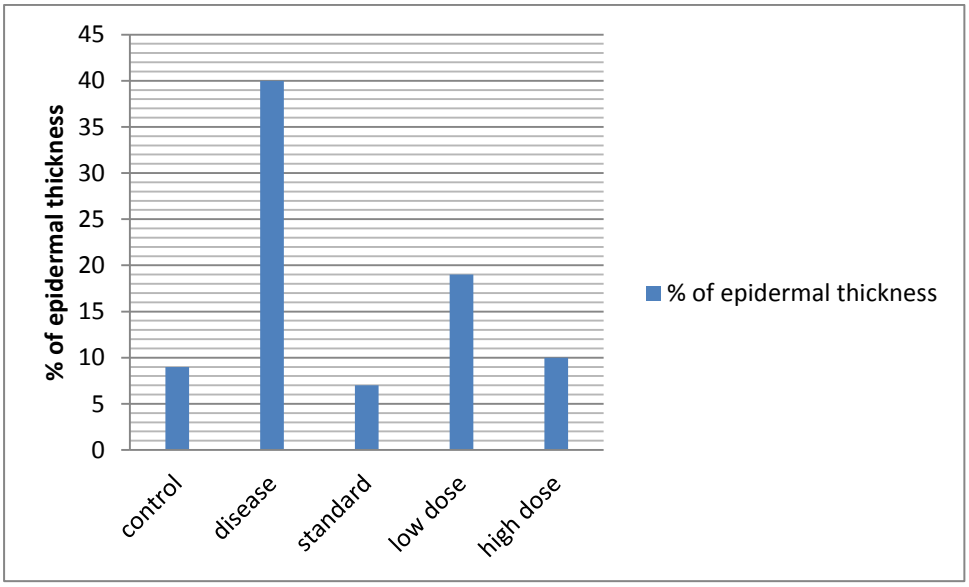


% of epidermal thickness in mice skin Table:23

Animal groups	% of epidermal thickness
Control	9±.052
Disease	40±0.70**
Standard	7±0.42***
Test1 low dose (2%w/w)	19±0.71**
Test 2 high dose(4%w/w)	10±0.32***

Values are mean ± SEM Statistical significant test for comparison was done by ANOVA, followed by Dunnet's 't' test (n=6). ***P*< 0.01, **P*< 0.05 when compared against control.

% of epidermal thickness in mice skin fig:49



Discussion:

The ethanolic extract ointment (high dose4%w/w) showed increased orthokeratosis percentage and decreasd epidermal thickness regions. The standard drug clobetasol showed the increased orthokeratotic and decrease epidermal thickness regions. Respectively in comparison to normal and imq treated skin. At present ,psoriasis remain pathology for which no complete cure is available . Corticosteroid treatment, which lead to a quick remission of symptoms , must be used for a short term because of its serious adverse effects. Therefore, it thus remains as a valuable therapeutic objective to find out a compound with some of the beneficial properties of the anti-psoriatic drugs but without their deleterious effects.

In this aspect ,the herbal ointment formulated with ethanolic extract of *Indigofera aspalathoides* show prominent anti-psoriatic activity with 53 ±1.5% of orthokeratosis and 10 ±0.32% epidermal thickness activity in mice skin which is comparable to the standard (clobetasol).


CONCLUSION

11. SUMMARY AND CONCLUSION

Indigofera aspalathoides plant is used in traditionally in the treatment of skin disease. The literature survey indicates that no scientific data on the anti-psoriatic activity of this plant. Various parts of this plant have been claimed to be effective in a wide range of disease.

The allopathic drugs like methotrexate, corticosteroids and immune suppressants shows effective response in psoriasis by reducing the scales, plaque, cell proliferation and inflammation but the adverse reaction is severe than the efficacy of the drug. The long term usage of drug causes serious adverse reaction like hepatotoxicity, renal failure sometimes lead to fatality. So herbal drugs are safe in psoriasis treatment since the adverse effect is mild or nil and lower the cost of therapy as comparable to the allopathic medicine.

The morphological and physiochemical properties of the whole plant were evaluated. Different extract of whole plant powder were prepared and these extracts were subjected to phytochemical evaluation. The ethanolic extract showed the presence of various phyto constituents such as ,Steroids, flavonoids, alkaloids and carbohydrates.

All the extract was tested for anti-microbial activity. Ethyl acetate and ethanol extract showed good activity even at low concentration and also shows maximum zone of inhibition.

The ethanolic extract were formulated as an ointment in a concentration of 2%w/w and 4%w/w evaluated for anti-psoriatic activity. The ointment were then pharmacologically evaluated for their anti psoriatic activity using mice skin model test. The ointment prepared with ethanolic extract showed antipsoriatic activity which was comparable to the standard clobetasol cream. The activity of ethanol extract cream was seen to be superior to standard ointment.

The ethanolic extract was subjected to HPTLC finger printing to establish an identity for future comparison. An attempt was also made to isolate the active constituents from the ethanol extract.

The present study confirms the anti-psoriatic activity of ethanol extract of *Indigofera aspalathoides.,* whole plant by various studies including *in vitro and in vivo* as mentioned in the study .The efficacy of the ointment could also be evaluated for clinical settings.

FUTURE SCOPE

1. Quantification of major phytoconstituents

2. Isolation of the responsible compounds for the activity

3. Clinical trails may be performed on psoriatic patients

4. Large scale manufacturing and marketing may be taken up based on clinical trails evaluation

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