PHARMACOGNOSTICAL, PHYTO-CHEMICAL AND ANTILEUCODERMIC STUDIES ON THE BARK OF *Dalbergia sissoo Roxb.,*

A Dissertation submitted to

THE TAMILNADU DR. M.G.R. MEDICAL UNIVERSITY

CHENNAI- 600 032.

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

MASTER OF PHARMACY

IN

PHARMACOGNOSY

Submitted By

Reg. No.261220654

Under the guidance of

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DEPARTMENT OF PHARMACOGNOSY
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APRIL 2014

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CERTIFICATE

This is to certify that the Dissertation entitled **"PHARMACOGNOSTICAL, PHYTOCHEMICAL AND ANTILEUCODERMIC STUDIES ON THE BARK OF *Dalbergia sissoo* Roxb,"** submitted by the candidate bearing the **REG.NO 261220654** , in partial fulfillment of the requirements for the award of the degree of **Master of Pharmacy in Pharmacognosy** by The Tamil Nadu Dr. M.G.R. Medical University, Chennai, is a bonafide record of work done by her during the academic year 2013-2014 under the guidance of

Dr. R. Radha, **M.Pharm, Ph.D.,** Department of Pharmacognosy, College of Pharmacy, Madras Medical College, Chennai - 600003.

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Place: Chennai - 03.

Date:

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Assistant Professor,

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Dr. R.RADHA

Place: Chennai - 03.

Date:

V.Chelladurai,
Research Officer- Botany (Scientist –C)
Central Council for Research in Ayurveda & Siddha,
Govt. of India.
(RETIRED)

Authentication Certificate

Certified that the plant material submitted by **T.Muthu Lakshmi** was carefully

Examined and botanically identified as **Dalbergia sissoo Roxb.ex Dc.**

Of the family **Fabaceae. (Papilionaceae)**

Date: 15-9.2013.

V.Chelladurai

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Participation Certificate

This is to certify that Mr./Ms./Dr. T. MUTHULAKSHMI

has participated/presented (poster) in this conference, held at Triple Helix Auditorium, CLRI, Chennai, on the 4th and 5th January 2013.

Dr. Narasimhan Srinivasan
(Convener)

Dr. S. P. Thiyagarajan
(Chairman)

AHRF

CERTIFICATE

This is to certify that Miss.T.MUTHU LAKSHMI, Post graduate student, Department of Pharmacognosy, College of Pharmacy, Madras Medical College, Chennai-03 had submitted her protocol (Part B Application) Vide: A/243/CPCSEA..... For Dissertation programme to the Animal Ethical Committee, Madras Medical College, Chennai -03.

TITLE: **PHARMACOGNOSTICAL, PHYTOCHEMICAL AND ANTILEUCODERMIC STUDIES ON THE BARK OF *Dalbergia sissoo* Roxb.**

The Animal Ethical Committee experts screened her proposal..Vide: A/243/CPCSEA..... and have given clearance in the meeting held on ...22.11.13... at Dean's chamber in Madras Medical College.

17.2.14.

Signature,

SPECIAL OFFICER
ANIMAL EXPERIMENTAL
MADRAS MEDICAL COLLEGE
CHENNAI

64TH INDIAN PHARMACEUTICAL CONGRESS

IPC

CHENNAI. DECEMBER 7-9, 2012

64th INDIAN PHARMACEUTICAL CONGRESS

Theme: Pharmacy Education: Innovation, Strategies and Globalization

64th IPC Chennai
Theme Pharma vision 2020
Pharmacy Education - Innovation, Strategies & Globalisation

Certificate of Participation

This is to certify that

Dr./Prof./Mr./Ms. T. MUTHU LAKSHMI

participated in the 64th Indian Pharmaceutical Congress held at

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Certificate

This is to certify that Ms. / ~~Mr.~~ T. MUTHU LAKSHMI

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Faculty of Pharmacy, PHARMACOGNOSY *Department, has been awarded scholarship of Rs.* 8,000/- *for securing* SECOND *rank in the M.Pharm project for the academic year* 2013 - 2014

SECRETARY

Date 23.11.2013

CHAIRMAN

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First and foremost, I thank ***GOD*** for successful completion of this work.

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I extremely happy to place on record our sincere gratitude and thanks to our beloved Professor and Guide **Mrs. Dr. R.RADHA M.Pharm., Ph.D,** Department of Pharmacognosy, Madras Medical College, Chennai without her gracious guidance, innovative ideas, with constant inspiration, encouragement, suggestion and infinite help our work, would not come in a bound form. We greatly thank her valuable support and endless consideration for the completion of the project work.

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I extend our thanks to **Mrs. T.S.LAKSHMI& Mrs.M.KUMUDHAVALLI,** Lab Technician, Department of Pharmacognosy, Madras Medical College, Chennai for her help during this work.

I feel proud to express my hearty gratitude to all my classmates. Also I want to thank all of those, whom I may not be able to name individually, for helping me directly or indirectly.

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I wish to thank my father Mr. G. Thilagar, who taught me perseverance in life and my mother T.Rajeswari, her affection, prayer, moral support and encouragement throughout my course period.

DEDICATED TO OUR BELOVED

PARENTS,

HUSBAND,TEACHERS,

ALMIGHTY & FRIENDS ....

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INTRODUCTION

Through the years , the world and its people have undergone many changes with lot of invention and growing technology, the world has also seen a number of unknown disease in increasing numbers, some being endemic and others affecting almost whole of the world population.

Medicines have been successfully found out for most of the disease and are in use presently. Synthetic drugs though useful for immediate relief and easily available, they lead to a number of side effects and adverse affects. And more drugs are needed to be taken to treat this adverse affects.

On the other hand, herbal drugs though of a little higher cost, low availability when compared to synthetic drugs, but herbal drugs are more effective and show least or no side effects. More over permanent cure can be obtained by use of herbal medicines therapeutically.

So many researches are being conducted in recent years in order to develop herbal medicines.

Plants have been major contributors to human welfare since the dawn of cicilization. Besides food, shelter and clothing, they are an important source of fine chemicals which find their applications in pharmaceutical industries across the globe.

HISTORY OF TRADITIONAL HERBAL MEDICINE

Herbal medicine is defined as branch of science in which plant based formulations are used to alleviate the disease. By definition ″traditional″ use for herbal medicine implies substantial historical use, and this is certainly true fro many products that are available as traditional herbal medicines. (The traditional system of India).

In many developing countries a large proportion of the population relies on traditional practitioners and there armamentarium of medicinal plants in order to meet health care needs.

Although modern medicine may exist side by side with such traditional practice, herbal medicines have often maintained the popularity for historical and cultural reasons.

IMPORTANCE OF HERBAL MEDICINES

Herbal drugs referred as plant materials or herbalism, involves the use of whole plants or parts of plants, to treat injuries or illness[1].

Herbal medicines are the oldest remedies known to mankind. Herbs had been used by all cultures throughout history but India has one of the oldest, richest and most diverse cultural living traditions associated with the use of medicinal plants[2].

Herbal drug is a chief constituent in traditional medicine and a common constituent in ayurvedic, homeopathic, naturopathic and other medicine systems[3].

Millions of Indians use herbal drugs regularly, as spices, home-remedies, health foods as well as over the counter as self medication or also as drugs prescribed in the non allopathic systems. The more than 500,000 non allopathic practitioners are trained in the medical colleges (> 400) of their respective systems of health and are registered with the official councils which monitor professionalism. Hence, these systems are not folklore or traditional herbal practices. There are basic axioms of these systems leading to a logical and systematic structure of pathogenesis and diagnosis, which serves also as a determinant for therapy [4,5,6] .

Herbal products show extensive beneficial potency like antimicrobial, antidiabetic, antifertility, antiageing, antiarthritic, sedative, antidepressant, antianxiety, antispasmodic, analgesic, anti-inflammatory, anti-HIV, vasodilatory, hepatoprotective, treatment of cirrhosis, asthma, acne, impotence, menopause, migraine, gall stones, chronic fatigue, alzheimers disease [7].

Leucoderma is the most common chronic depigmentation disorder or hypopigmentation. It includes the loss of functioning melanocytes which causes the appearance of white patches on the skin [8,9].

Leucoderma is a miserable acquired skin disorder, making skin white due to loss of the melanin pigment. It is a non contagious disease. **It is** otherwise **termed as Vitiligo**.

Incidences and risk personalities

Leucoderma is mostly restricted to the epidermis layer. It can occur in any age group , sex and races. Females are more commonly affected than males. The most commonly affected areas are face, neck, back, wrist, and hand, groin, genitals, and armpits etc. i.e. dark places , places where folds occur or where friction takes place.

Causes

Till now researchers have not identified any causative factor for leucoderma. It can also be familiar. i.e. hereditary factor also has some role in the prevalence of leucoderma. Being emotionally upset may also precipitate or aggravate the complaint in certain circumstances. Some suspect skin , lack of sun exposure , infection , etc. to be the reason for the problem. All these risk factors only, but the real cause is still obscure or unknown.

The main causes of leucoderma are said to be excessive mental worry, chronic or acute gastric disorder, and impaired hepatic function such as jaundice, worms or other parasites in the alimentary canal, typhoid and defective perspiratory mechanism and burn injuries.

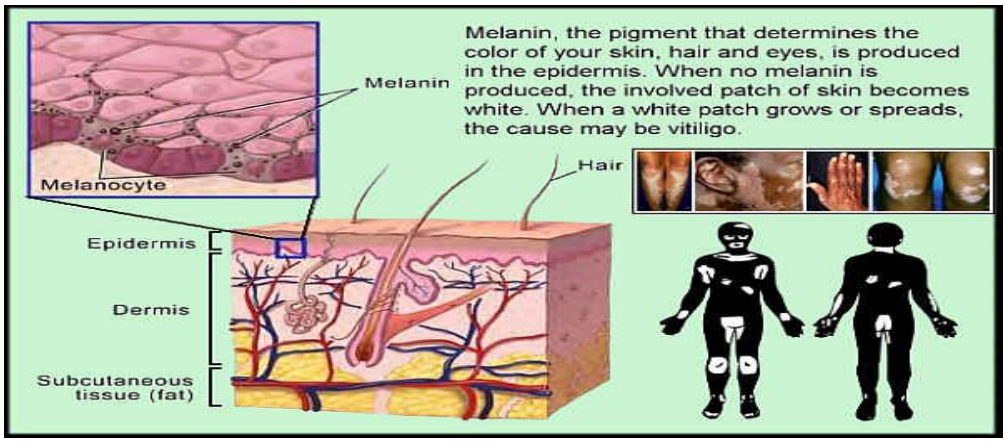


Fig. 1. Skin structure

Behavior of melanocytes in Vitiligo

It is generally agreed that there are no longer functional melanocytes in vitiligo skin and that this loss of histochemically recognizable melanocytes is the result of their destruction. However, the possibility those melanocytes are still present in vitiligo skin but in an undifferentiated state without melanogenic activity has been proposed. The possibility that either inactive melanocytes with no identifiable melanosomes or Dopa negative melanocytes remain in the white macules could not be ruled out. In fact, it is very unusual to observe ultrastructural evidence of melanocyte destruction in vitiligo skin. In this context, several puzzling observations should be quoted.

A recent study, using the split-Dopa rection reports that Dopa positive cells ranging from 102± 9 to 229±17 cells / mm of depigmented epidermis were found in all six patients studied(Kao et al.,1992). An antimelanosome associates monoclonal antibody has been found to selectively reveal antimelanosome antibody positive dendritic cells in melanocytes absent complete vitiligo (Hori et al.,1986; Mishima et al 1988). It was suggested that these cells could be inactive melanocytes that have lost their ability to synthesize melanosome. In addition, tyrosianse activity has been found in vitiligo skin (Husain et al., 1982). Furthermore, homogenates from vitiligo skin could synthesize melanin from labeled tyrosine. This was interpreted as evidence that tyrosinase, and thereby melanocytes, are present in vitiligo epidermis. In a further study on the in vitro growth characteristics of melanocytes obtained from vitiligo subjects, it was found that vitiligo subjects, it was found that vitiligo skin gives rise to a few dendritic cells in culture. Although, no attempt was made to characterize these cells, it was suggested that they could be effete melanocytes (Puri et al., 1987). Although fascinating, these observations do not provide evidence for the persistence of melanocytes in vitiligo macules. They would be most interesting if the absence of melanocytes in the tissues studied was strongly estabilished. Unfortunately, in most of these reports, the precise type of the vitiligo macules used in the experiments is not specified. It is well known that besides the absolute type of vitiligo in which there are no Dopa- positive melanocytes (Jarrett and Szabo, 1956), there are relative types of vitiligo in which melanocytes remain in the lesions with a decreased Dopa positivity.

It may be suggested that the relative types of vitiligo are considered possible forerunners of the absolute type. It would not be surprising to find melanocytes markers or tyrosinase activity

in the epidermis of white macules of relative vitiligo. This stresses the need to use very well characterized vitiligo lesions for these experimental studies. A recent immunohistological study of vitiligo involving epidermis using a panel of melanocytes markers related and unrelated to the melanogenic activity could not detect identifiable melanocytes in the white macules of vitiligo(Le Poole et al.,1992). Another recent study performed on vitiligo patients using a Dopa bluidine Blue complex stain reports that no active melanocytes are present in vitiligo epidermis. Thus, from the presently available data, it is likely that melanocytes are absent from most vitiligo macules.

Vitiligo is an autoimmune disease

Vitiligo is frequently associated with autoimmune diseases, such as multiple glandular insufficiencies, thyroid diseases, pernicious anemia, type I diabetes mellitus, Addison's disease and autoimmune hypoparathyroidism(cunliffe at al., 1968). In addition, various circulating antiorgan antibodies are found in patients with vitligo. This raised the possibility that vitligo might also be an antibody associated autoimmune disease.

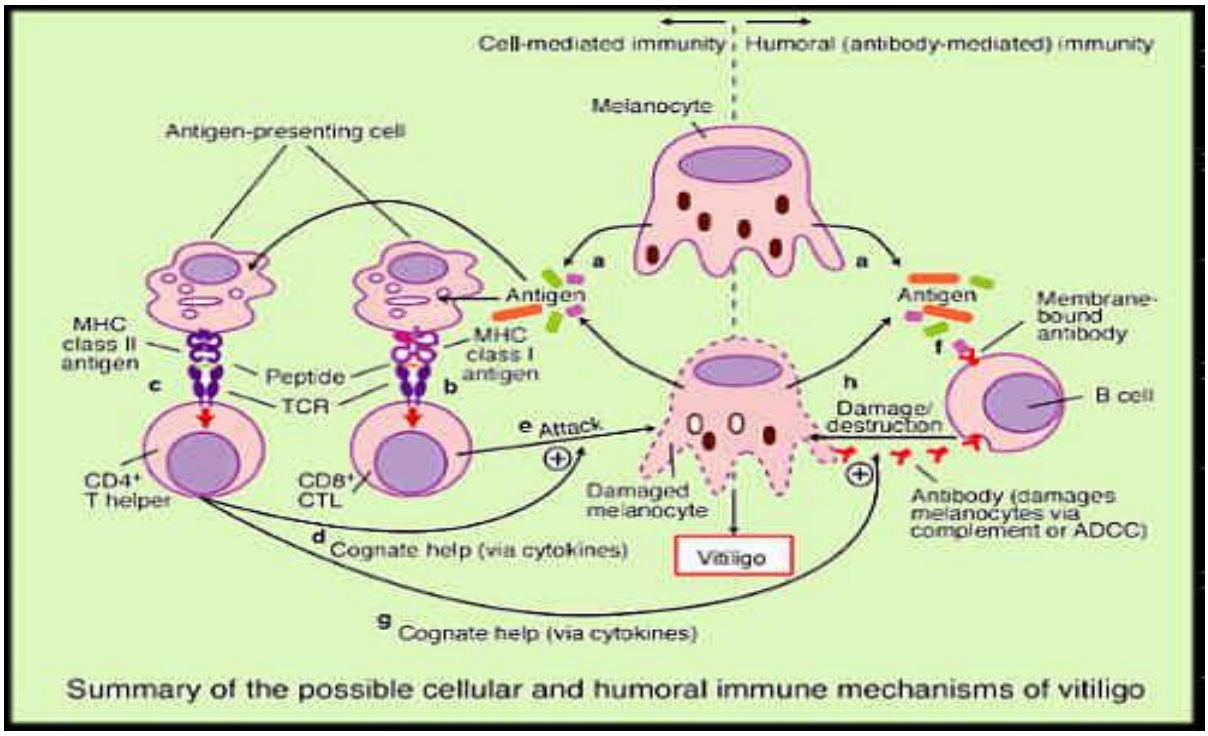


Fig.2. Autoimmune theory of vitiligo showing both cell mediated and humoral autoimmune responses.

Various alterations in peripheral mononuclear cells, especially T-cells and T-cell subsets have been described, not only in patients with vitligo, but also among their first degree relatives (D'Amelio et al., 1990). However, the results are contradictory, the CD4+, CD8+, and NK cells subpopulations being reported either as normal, decreased, or increased (Grimes et al., 1986; Ghoneum et al., 1987). More recently, a significant decreases of the CD4/CD5 RA+ subset (suppressor inducer lymphocytes), together with a significant increase of the circulating HLA-DR^+ cells, has been found in a group of 16 patients with nonsegmental vitligo, suggesting a T-Cell dysregulation in this disorder (Abdel Nasser et al., 1992). These contradictory results may arise from differences in the techniques used to evaluate T-cell subpopulations, as well as from differences in the vitiligo patients studied(i.e., clinical types, disease activity). The main conclusion from these studies is that circulating T-cell subpopulations have rarely been found to be normal in vitiligo patients. The significance of these findings must be interpreted with caution, as many different factors are known to influence these parameters. Furthermore, they show nyctemeral variations and this parameter has obviously not been considered in most available studies (Mozzanica et al., 1989,1990).

Few reports are available on the functional activities of T-lymphocytes in vitiligo patients. An increased release of leukocyte migration inhibition factor from patients lymphocytes has been reported suggesting an hyper functioning of these cells in vitiligo (Zaman et al., 1992). Other studies showed abonrmalities including elevated numbers of peripheral monocytes (OKM5) (Baumer et al., 1990) or depressed interleukin 2 activity (Halder et al., 1986). In situ immunophenotyping of the inflammatory infiltrate present at the margins of active vitiligo macules provides more interesting information and suggests an ongoing local immune reaction. There is a selective accumulation of CD3+, CD4+, and CD8+ T-lymphocytes in the epidermis and in the upper dermis (Abdul Nasser et al.,1991). Many lymphocytes express the IL2 receptor (Gross et al., 1987). There is also strong HLA-DR and intracellular adhesion molecule-1 (I-CAM-1) expressing at the edges of vitiligo macules (Abdul Nasser at al., 1991). A reduction of Factor XIIIa positive dermal dendroctes is also fpund in the skin of vitiligo patients regardless of disease status, but the significance of this findings is unknown (Aronson et al., 1990). During many years, various attempts to identify specific auto antibodies in vitiligo patients failed. Antimelanin antibodies were described a long time ago but the results of this study could not be reproduced (Langhof et al., 1965).

Later, circulating antimlelanocyte antibodies were described a long time ago but the results of this study could not be reproduced (Langhof et al., 1965). Later, circulating antimelanocyte antibodies were described in patients with the complex syndrome associating hypoparathyroidism, addison's disease, chronic mucocutaneous candidiasis, and vitiligo (Hertz et al., 1977).

Clinical presentation of vitiligo

Localized type

a) Focal - One or more macules in two single areas but not segmented.

b) Segmental - One or more macules in dermatomal pattern

c) Mucosal - Involvement of mucous membrane alone.

Genaralised type

a) Acrofacial - Involvement of face and distal extremities.

b) Vulgaris - Scattered mascules in symmetrical or asymmetrical distribution [10]

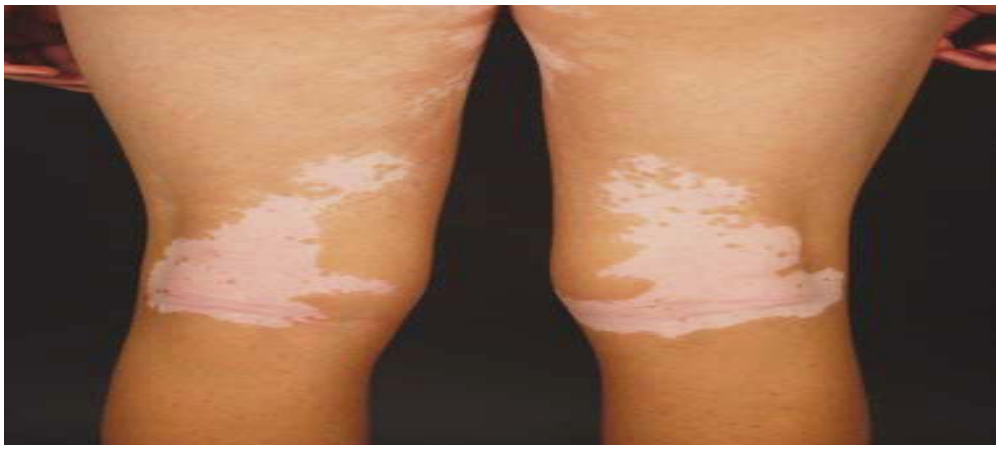


Fig. No.3. Symmetrical distribution of depigmentation in Generalized Vitiligo

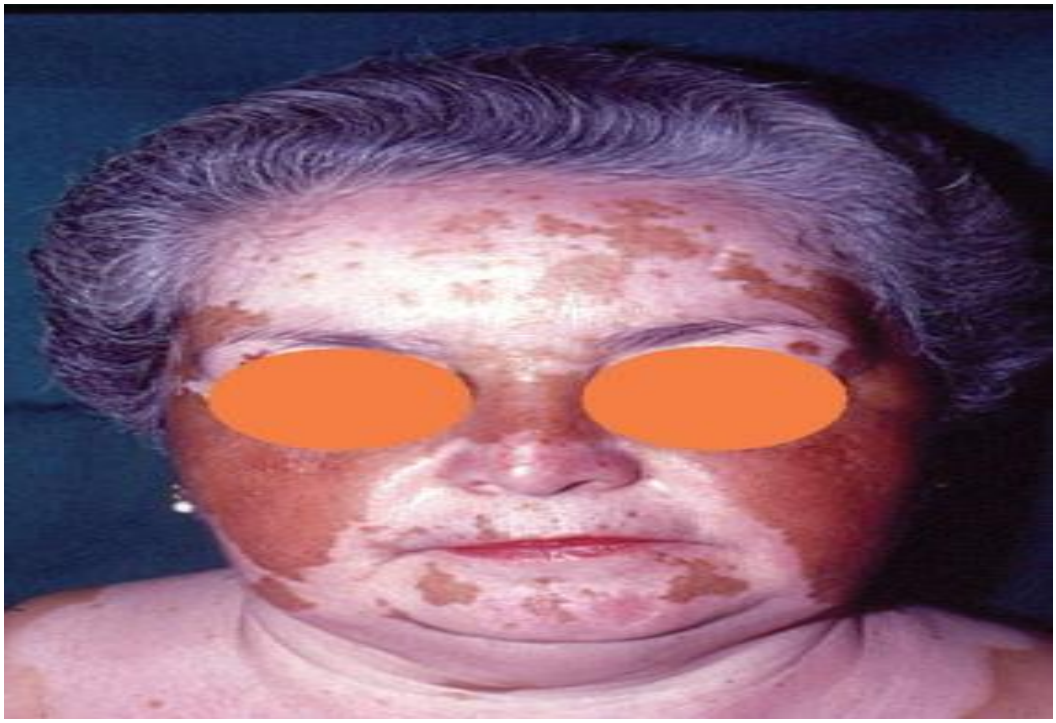


Fig. No.4. Picture of Universal Vitiligo

About 0.5 to 1 percent of the world's population , or as many as 65 million people, have Vitiligo [11].

Most develop vitiligo before their fortieth birthday. The disorder affects both sexes and all races equally. However , it is more noticeable in people with dark skin [12].

Vitiligo seems to be somewhat more common in people with certain autoimmune disease. These include hyperthyroidism, adrenocortical insuffiency and pernicious anemia. People who develop Vitiligo usually first notice white patches on their skin. These patches are more commonly found on sun exposed areas of the body, including the hands, feet, arms, face, and lips. Other common areas where these white patches appear are the armpits and groin, and around the mouth, eyes, nostrils, navel, genitals, and rectal areas. In addition to white patches on the skin, people with Vitiligo may have premature graying of the scalp hair, eyelashes, eyebrows, and beard.

Systemic phototherapy induces cosmetically satisfactory repigmentation in up to 70% of patients with early or localized disease.

Narrow band UV-B phototherapy is widely used and produces good clinical results. Narrow-band fluorescent tubes with an emission spectrum of 310-315 nm and a maximum wavelength of 311 nm are used. Treatement frequency is 2-3 times weekly, but never on consecutive days. This treatment can be safely used in children, pregnant women, and lactating women. Short term adverse effects include pruritus and xerosis. Several studies have demonstrated the effectiveness of narrow band UV B therapy as monotherapy [13].

Vitiligo treatment

In general, topical monotherapy is indicated for mild to moderate vitiligo. Current treatment options for vitiligo include medical, surgical and adjunctive treatments [14].

Medical treatment targets the immune system and helps to arrest the spread of depigmentation. In cases of vitiligo, repigmentation can be achieved by dermatosurgical techniques and adjunctives includes use of cosmetics. Both surgical and medical treatment have their own limitations. Adjunctive can only cover the patch and be used along with surgical or medical treatments.

Medical therapies

The best studied and most commonly used medical treatment options are corticosteroids, Psoralen and depigmenting agents.

Corticosteroids : topical steroids are often first line therapy, especially in children or for localized disease. Moderately potent topical corticosteroids are used [15].

However, vitiligo requires prolonged use of these agents, often much longer than the usual "safe" recommended periods of use for inflammatory dermatoses. This results in significant , therapy limiting side effect like atrphy, hypertrichosis, peri-lesional hypopigmentation etc. It is obvious that currently available dosage formulations do not provide site-specific drug delivery.

Phototherapy: Phototherapy is one of the oldest forms of treatment of vitiligo and remains the bedrock of vitiligo therapy even today. Out of three [5-MOP (bergapten), 4,5,8-trimethylpsoarlen and 8-MOP(methoxsalen) different types of psoralens, 8-MOP is most commonly used. Topical Psoralen photochemotheraphy is often used for people with limited disease. It is also used for children and older, who have localized patches for vitiligo [16].

Severe sunburn, blistering and abnormally dark repigmentation are major potential side effects of topical PUVA therapy. This is due to the uncontrolled photo reaction of psoarlen with UV A irradiation in the epidermis because drug is freely available from currently available dosage formulations, for reaction on surface after topical application [17].

In recent years, narrow band UVB (311 nm) has become the preferred phototheraphy for vitiligo.

Calcium modulators: Recently, calcium modulators, the vitamin D3 analogues(calcipotriol and tacalcitol) have also been tried in the treatment of vitiligo [18].

Defective calcium transport has been shown in melanocytes and keratocytes harvested from patients with vitiligo. Further vitamin D3 has been shown to activate melanin synthesis [19].

There are contradictory reports of their efficacy in vitiligo, both as monotherapy and in combination with both PUVA and NB-UVB phototherapy.

Calcineurin inhibitors: Calcineurin inhibitors (tacrolimus, pimecrolimus) are some of the newest topical drugs in dermatology. However, tacrolimus and pimercrolimus are effective only in vitiligo lesions on the head and neck region. The main difficulty with topical calcineurin, inhibitors, and one that is probably responsible for lack of efficacy in non-facial vitiligo, is their high molecular weight [20].

Adjunctive therapies

Depigmentation: Depigmentation is a more drastic form of treatment, when vitiligo is extensive i.e. vitiligo universalis. Depigmentation involves fading the rest of the skin on the body to match the already white areas by using permanent melanocytotoxic agents such as Monobenzyl ether of hydroquinone cream (Benoquin). A more recent method of depigmentation utilizes topical 4-methoxyphenol and the Q switched ruby laser(QSR) [21].

Sunscreens : People who have vitiligo are more susceptible to sunburn and long term photodamage. To prevent sun induced darkening of the surrounding normal skin, sunscreens which provide protection from both UVB and UVA light should be used.

REVIEW OF LITERATURE

PHARMACOGNOSTICAL REVIEW:

- ✓ Josephin Nerling Rashida.G et al (2012) [ ]–Reported that Pharmacognostical and preliminary phytochemical evaluation of the leaves of *Dalbergia sissoo*[22] .

PHYTOCHEMICAL REVIEW :

- ✓ S.K.Mukerjee et al(1971)- Reported that Dalbergichromene : A new neoflavonoid from stem-bark and heartwood of *Dalbergia sissoo* [23].

PHARMACOLOGICAL REVIEW:

- ✓ **Mohammad Asif et al** conducted a study in 2009, Anti-inflammatory activity of ethanolic extract of *Dalbergia sissoo* (Roxb.) bark. It can be concluded that the ethanolic extract of *Dalbergia sissoo* bark at 1000 mg/kg showed the most potent anti-inflammatory activity compared to the other groups (300 and 500mg/kg) throughout the observation period [24.]
- ✓ **Pankaj singh niranjan et al** conducted a study in 2010; Anti-diabetic activity of ethanolic extract of *Dalbergia sissoo* L. leaves in alloxan induced diabetic rats. They concluded that the ethanolic extract of the *Dalbergia sissoo* leaves are 12% more effective in reducing the BGL compared to standard Glibenclamide [25].
- ✓ **Mallinath H. Hugar et al** conducted a study in 2010, phytochemical and pharmacological studies of ethanol extract of *Dalbergia sissoo* seeds. An approach for the *in-vivo* analgesic and antipyretic activities. It concluded that *Dalbergia sissoo* seeds extract has moderate analgesic and remarkable antipyretic activities [26].
- ✓ **Nitinkumar Upwar et al** conducted a study in 2011, Evaluation of anthelmintic activity of *Dalbergia sissoo* Roxb. The study indicated the potential usefulness of *Dalbergia sissoo* Roxb .against helminthic infections [27].
- ✓ **Arvinder Kaur et al** conducted a study in 2011, Evaluation of antioxidant potential of stem barkextract of *Dalbergia sissoo.* Finally results bark of the plant *Dalbergia sissoo,* chloroform extract possesses marked antioxidant activity, whereas methanolic extract shown moderate activity in different in vitro anti-oxidant assays [28].

- ✓ **Mohammad Asif et al** conducted a study in 2011, phytochemical investigation and evaluation of anti-nociceptive activity of ethanolic extract of *Dalbergia sissoo* (Roxb.) bark. They concluded that (300, 500 and 1000mg/kg) doses of extract exhibited significant and dose dependent anti-nociceptive activity which may be due to presence of flavonoids [29].
- ✓ **Shazia Sultana et al** conducted a survey on,Indigenous knowledge of folk herbal medicines by the women of district Chakwal, Pakistan. This survey describes that, crush the leaves and boiled in water and the filtrate obtained is used to wash hair for removing dandruff and for long hair [30].
- ✓ **S Chandra et al** conducted a study on Antiinflammatory activity of *Dalbergia sissoo* leaves.They concluded that the *D. sissoo* leaf extract possessed significant anti-inflammatory activity (in acute, sub-acute and chronic models of inflammation) without any side effects on gastric mucosa [31].
- ✓ **Harsha Kharkwal et al** conducted a study in 2012, Anti-termite activity of heartwood of *Dalbergia sissoo* Roxb. Ex.Dc. It concluded that the plant extracts can be used as an alternative for synthetic pesticides for termite control in buildings [32].
- ✓ **Neeraj S. Vyawahare et al** Conducted a study in 2012, Anti-diabetic Evaluation of *Dalbergia sissoo* against alloxan induced diabetes mellitus in wistar albino rats. They concluded that ethanolic extract of *Dalbergia sissoo* bark possesses significant anti-diabetic activity [33].

- ✓ **Jaspreet Kaur Sidana et al** conducted a study in 2012, Analgesic and anti-inflammatory activities of *Dalbergia sissoo* leaves extract. They concluded that the extract possesses both analgesic and anti inflammatory properties [34].

AIM AND OBJECTIVE OF THE STUDY

- ✓ The ethnobotanical information reveals that the plat *Dalbergia sissoo* belonging to the family Fabaceae is used for diarrhea, dysentery, dyspepsia, Menorrhea, inflammation, leprosy, ulcers,gout.

- ✓ To standardize the bark Dalbergia sissoo by carrying out the Pharmacognostical, physicochemical and phytochemical parameters.

- ✓ To evaluate the antioxidant activity of various extracts of the bark of Dalbergia sissoo.

- ✓ To evaluate the hyperpigmentant activity of the various extracts of the bark *Dalbergia sissoo* by the method of Tsuboi et al suing B16F10 melanoma cell line. To formulate and evaluate a topical gel from the best extract from cell line study.

- ✓ Evaluation of antileucodermic activity on the bark of *Dalbergia sissoo* by *invivo* method using 6JBLC57 black mice.

- ✓ From the literature survey it was found that no pharmacognostical work has been carried out on the bark *Dalbergia sissoo* so far. An attempt was made to investigate the Pharmacognostical, Phytochemical and antileucodermic studies on the bark of *Dalbergia sissoo.*

PLANT PROFILE [35,36,37,38,39]

ETHNOBOTANICAL REVIEW:

Botanical name : *Dalbergia sissoo*

Spathodea : Monotype genus

Campanulata : shape like a bell

Family : Fabaceae

Synonym :Amerimnon sissoo

English : African tulip tree, flame of forest, nandi flame

TAXONOMICAL CLASSIFICATION

Kingdom :Plantae

Division :Magnoliophyta

Class :Fabales

Family :Fabaceae

Subfamily :Faboideae

Genus :*Dalbergia*

Species :*D.sissoo*

Scientific name :*Dalbergia sissoo*

VERNACULAR NAMES

Hindi : Sisam

Tamil : nukku kattai, yette.

Sanskrit : Kapila-sinsapa

Telugu :Sissukarrha

Bengali **:** Shisu

English :Black wood

HABIT AND HABITAT:

Dalbergia sissoo grows naturally in Baluchistan, Waziristan, Western Himalaya, Nepal, Sikkim, extensively planted throughout India.

PARTS USED:

Bark, Wood, Root, Leaves.

Botany

Dalbergia sissoo is a medium to large tree of about 25 meters high with grey-yellow trunk, longitudinal crack, and downcast twig.

Leaves

Leaves are leathery, pinnately compound, with about five alternate leaflets. Leaf stalk measures about 15 cm long, each leaflet widest at the base to 6 cm long with a fine pointed tip.

Flowers

Flowers are whitish to pink, fragrant, nearly sessile up to 1.5 cm long and in dense clusters 5-10cm in length. Pods are oblong, flat, thin, strap like 4-8cm long, 1cm wide and light brown. They contain 1-5 flat bean shaped seeds 8-10 mm long.

Seeds

Seeds are 6-8x4-5mm, kidney shaped,thin and flat, light brown

Chemical constituent

Leaves contain Isoflavone-O-glycoside

Flowers contain Biochenin A, tectorigenin, 7,4 dimethyle tectorigenin and 7-O-methyle tectorigenin.

Mature pods contain Isocaviumin, tectorigenin, dalbergin, caviunin and tannins.

Stem bark contains Dalberginone, dalbergin, methyl dalbergin and dalbergichromene.

Folklore claim

Decoction of the leaves is helpful in gonorrhea and leprosy. Decoction of the bark used in leprosy. Leaf extracts has been used to treat sore throats, heart problems, dysentery, syphilis and gonorrhea. Sissoo oil is used to treat itching, burning on the skin and scabies.

It is an Indian medicinal plant which has a variety of uses in folk medicine

- Aphrodisiac
- Abortifacient
- Expectorant
- Anthelmintic
- Antipyretic
- Emesis
- Ulcers
- Dysentery
- Stomach troubles

Fig. 5. Tree of *Dalbergia sissoo* Roxb.

Fig. 6.Leaves and Flowers of *Dalbergia sissoo*

Fig. 7. Dalbergia sissoo the whole plant picture

PLAN OF WORK

The present study on the bark of *Dalbergia sissoo* includes collection of the plant and authentication followed by

I. PHARMACOGNOSTICAL STUDIES:

1. **MACROSCOPY**
2. **MICROSCOPY**
   - Sectioning.
   - Staining
   - Photomicrographs

Microscopic features

- Cork
- Fibre
- Medullary rays
  - ✓ **Powder microscopy**

3. **DETERMINATION OF PHYSIOCHEMICAL CONSTANTS**
   - Ash value
     - Extractive value Total ash value
     - Acid insoluble ash value
     - Water soluble ash value
     - Sulphated ash value

Extractive value

- Alcohol soluble extractive
- Water soluble extractive
- Ether soluble extractive

II.PHYTOCHEMICAL STUDIES:

- Preparation of extracts
- Preliminary phytochemical screening of powder and extracts.
- Quantitative estimation of Phytoconstituents.
- Fluorescence analysis of powder and extracts.
- Thin layer chromatography of Extracts.
- HPTLC Finger prints analysis of extract.

III.SELECTION OF ACTIVE EXTRACT BY *IN-VITRO* STUDIES

Evaluation of bark extract of *Dalbergia sissoo* Roxb. for the antileucoderma activity.

- Cytotoxicity studies done using MTT assay.
- Increased melanin content determination by Tsuboi et al method.
- Antioxidant activity- Reducing Power Assay
  Hydrogen Peroxide Scavenging Assay

IV.FORMULATION AND EVALUATION OF GEL

- Appearance
- pH
- Viscosity
- Spreadability
- Extrudability

V.ACCELERATED STABILITY TESTING OF GEL

VI.*IN-VIVO* ANTILEUCODERMIC ACTIVITY.

Using black mice as an animal model.

PHARMACOGNOSTICAL STUDIES

Evaluation of drug means confirmation of its identity and to determine its quality and purity and detection of nature of adulteration. Evaluation of a crude drug can be attempted by different methods which include morphological and microscopical studies of the crude drugs or their physical, chemical and biological behavior. Systematic identification of crude drugs and their quality assurance gives an integral part of drug description.

Pharmacognostical studies basically deals with the identification, authentication and standardization of herbal medicinal plants through Organoleptic character, histological character, powder microscopy, histochemical analysis and physico-chemical observations as prescribed by an authoritative source such as World Health Organization (WHO) . As no pharmacognostical work has been done so far on bark of *Dalbergia sissoo* the present work was taken up, to study the pharmacognostical parameters.

Plant collection and Authentication

The fresh bark of the plant *Dalbergia sissoo* was collected from Komaneri, Tuticorin district, Tamil Nadu, India and it was botanically identified and authenticated by Dr.V.Chelladurai, Research Officer-Botany (Scientist-C), Central Council for Research in Ayurveda and Siddha, Government of India.

1.MACROSCOPY [40]

Macroscopical character which includes Organoleptic characters and morphological features of various parts of the plant was studied.

2. MICROSCOPY [41-49]

Staining method

The required samples of different organs were cut and removed from the plant and fixed in FAA(Formalin-5ml+Acetic acid -5mi+70% Ethyl alcohol-90ml).After 24 hrs of fixing , the specimens were dehydrated with graded series of tertiary –butyl alcohol as per the schedule given by **Sass. 1940**. Infiltration of the specimens was carried by gradual addition of paraffin wax (melting point 58-60°C) until TBA solution attained super saturation. The specimens were cast into paraffin blocks.

Sectioning

The paraffin embedded specimens were sectioned with the help of Rotary microtome, The thickness of the sections was 10-12µg. Dewaxing of the sections was by customary procedure (Johasnsen, 1940). The sections were stained with Toluidine blue as per the method published by O'Brien et al.(1946). Since Toluidine blue is polychromatic stain. The staining results were remarkably good; and some Phytochemical reactions were also obtained.

The dye rendered pink colour to the cellulose walls, blue to the lignified cells, dark green to suberin, violet to the mucilage, blue to the protein bodies etc. Wherever necessary sections were also stained with saffranin and Fast-green and KI (forStarch) .

Photomicrographs

Microscopic descriptions of tissues are supplemented with micrographs wherever necessary. Photographs of different magnifications were taken with Nikon lab photo 2 microscopic Unit. For normal observations bright field was used.

For the study of crystals, starch grains and lignified cells, light microscope was employed. Since these structures have briefringent property, under polarized light they appear bright dark background. Magnifications of the figures are indicated by the scale-bars. Descriptive terms of the anatomical features are as given in the standard Anatomy books (Esau, 1946).

3. POWDER MICROSCOPY [50]

The shade dried powdered plant material was used for powder microscopical analysis. The Organoleptic characters were observed and to identify the different characteristic features various staining reagents were used. Powder was stained with 1% Phloroglucinol in 90% ethanol, concentrated hydrochloric acid and glycerin and observed through microscope. All the lignified stained pink colour. Calcium oxalate crystals were observed under the polarized light microscope.

4. DETERMINATION OF PHYSICO-CHEMICAL CONSTANTS [51,52,53]

Shade dried, powdered material of the bark *of Dalbergia sissoo* was used for the determination of physico-chemical constants in accordance with WHO guidelines and the ayurvedic Pharmacopoeia of India.

DETERMINATION OF ASH VALUE

Ash values of a crude drug is defined as the inorganic residue remaining after incineration , which simply represents inorganic salts, naturally occurring in drug or adhering to it or deliberately added to it as a form of adulteration. Hence the ash values are helpful in determining the quality and purity of a crude drug in the powdered form.

METHOD

Total ash

2-3 g of the sample was weighed in a tarred silica dish and was incinerated at a temperature not exceeding 450 C until the sample was free from carbon. The ash obtained was cooled and weighed. The percentage of total ash was calculated.

$$\text{Total ash value} = \frac{\text{weight of residue obtained}}{\text{weight of the sample taken}} \times 100$$

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 silica crucible containing the total ash obtained in the earlier test, 25 ml of hydrochloric acid was added, covered with a watch glass and boiled gently for 5 minutes on a burner. The watch glass was rinsed with 5 ml 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 this filter paper was rinsed repeatedly with hot water until the filtrate in neutral/free from acid . the filter paper containing the insoluble matter to the original crucible , dried on a hot plate and ignited to a constant weight in the muffle furnace at 450-500° C. The silica crucible was removed from the muffle furnace and allowed to cool in dessicator for 30 minutes, and then weighed without delay. The content of acid insoluble ash was calculated.

$$\text{Acid insoluble ash value} = \frac{\text{weight of residue obtained}}{\text{weight of the sample taken}} \times 100$$

Water soluble ash

The ash was boiled for 5 minutes with 25ml of water. The insoluble matter was then collected in ash less filter paper. It was washed with hot water and ignited at a temperature not exceeding 450 ° C. The weight of the insoluble matter was subtracted from the weight of the total ash and the difference in weight represents the water soluble ash and then the percentage of water soluble ash with reference to the dried substance was calculated.

$$\text{Water soluble ash value} = \frac{\text{weight of residue obtained}}{\text{weight of the sample taken}} \times 100$$

Sulphated ash

3 g of substance was ignited gently at first in a crucible until the substance was thoroughly charred. Then the residue was cooled, moistened with 1 ml of sulphuric acid, heated gently until the white fumes were no longer evolved and ignited at 800±25° C, until all the black particles 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 dried substance was then calculated.

$$\text{Sulphated ash value} = \frac{\text{weight of residue obtained}}{\text{weight of the sample taken}} \times 100$$

DETERMINATION OF EXTRACTIVE VALUES (INDIAN PHARMACOPOEA 1996)

Extractive values are useful for the evaluation of phytoconstituents, especially when 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.

Water soluble extractive value:

Procedure:

5 g of the air dried drug was macerated with 100 ml of chloroform water (95 ml distilled water and chloroform) in a glass stoppered conical flask for 24 hours, shaking the contents frequently during the first 6 hours and then allowed to stand for 18 hours. The solution was filtered rapidly taking precautions again loss of solvents. 25 ml of filtrate was evaporated to dryness on a water bath in a tarred flat bottomed Petri plate /shallow dish 2 ml of alcohol was added to dry the residue. The contents were shaken and dried again on water bath. The petriplates were dried at 105°C for 1 hour in the hot air oven and cooled in a dessicator for 30 minutes and weighed. The

process was repeated till a constant weight was obtained. The percentage of water-soluble extractive with reference to the air-dried drug was calculated.

$$\text{Water soluble extractive value} = \frac{\text{weight of dried extract}}{\text{weight of the sample taken}} \times 100$$

Alcohol soluble extractive value

The determination of water and alcohol soluble extractive value is used as means of evaluating the quality and purity of drugs the constituents which cannot be readily estimated by other means. Extraction of the drug can be done by maceration with cold water or by a continuous extraction process in a soxhlet extractor.

Procedure:

5 g of the air -dried coarsely powdered drug was macerated with 100 ml of alcohol in a glass stoppered flask for 24 hours, shaking the contents frequently during the first 6 hours and allowed to stand for 18 hours. The solution was filtered rapidly taking precautions against loss of alcohol. 25 ml of the filtrate was evaporated to dryness on a water bath in a tarred flat bottomed petri plate /shallow dish. Dried at 105°C for 1 hour in a hot air oven. The dish was removed cooled in a dessicator and weighed. The process was repeated till the constant weight was obtained. The percentage of alcohol-soluble extractive with reference to the air-dried drug was calculated**.**

$$\text{Alcohol soluble extractive value} = \frac{\text{weight of dried extract}}{\text{weight of the sample taken}} \times 100$$

Determination of non-volatile ether soluble extractive (Fixed oil content)

A suitably weighed quantity of the crushed air dried drug was transferred to an extraction thimble and extracted with solvent ether or petroleum ether B.p. 40-60°C in a soxhlet for 6 hours. The extracts was filtered into a tarred evaporating dish, evaporated and dried at 105°C to constant weight. The percentage of non-volatile ether soluble extractive value with reference to the air drug was calculated**.**

$$\text{Non-volatile ether soluble extractive value} = \frac{\text{weight of dried extract}}{\text{weight of the sample taken}} \times 100$$

Determination of volatile ether soluble extractive

2 gm of dried powdered drug was accurately weighed and extracted with anhydrous ethyl ether in a continuous extraction apparatus for 20 hours. The ether solution was transferred porcelain dish and evaporated spontaneously. Then it was dried over phosphorous pentoxide for 18 hours and the total ether extract was weighed. The extract was heated gradually and dried at 105° C to constant weight. The loss in weight represents the volatile portion of the extract.

$$\text{Volatile ether soluble extractive value} = \frac{\text{weight of dried extract}}{\text{weight of the sample taken}} \times 100$$

Loss on drying

Specified quantity of the substance was transferred in a previously ignited and cooled silica crucible and the substance was evenly distributed by gentle side wise shaking. The crucible with the contents and the lid were weighed accurately. The loaded uncovered crucible and the lid were placed in the drying chamber(105° C). the substance was heated for a specified period of time to constant weight. The crucible was covered with the lid and allowed to cool in a dessicator at room temperature before weighing. Finally the crucible was weighed to calculate the loss on drying with reference to the air dried substance.

$$\text{LOD} = \frac{\text{Wt. of the sample before drying} - \text{Wt .of the sample after drying}}{\text{Wt. of the sample before drying}} \times 100$$

Determination of foaming index

One gm of the coarsely powdered drug was weighed and transferred to a 500 ml conical flask containing 100 ml boiling water. The flask was maintained at moderate boiling at 80-90° C for about 30 minutes. Then it was cooled and filtered into a volumetric flask and sufficient water was added through the filter to make up the volume to 100 ml. the decoction was poured into 10

stoppered test tubes (height 16 cm, diameter 16 mm) in successive portions of 1ml, 2ml, 3ml and up to 10 ml and the volume of the liquid was adjusted in each tube with water to 10 ml. The tubes were stoppered and shaken in a length wise motion for 15 seconds, two shakes per second. They were allowed to stand for 15 minutes and measure the height of the foam. The result were assesses as follows. If the height of the foam in every tube is less than 1 cm the foaming index is less than 100. If a height of 1 cm 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. If the

height of the foam is more than 1 cm 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. The foaming index is calculated using the following formula :

$$1000/a$$

Where, a= 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 taken up by the swelling of 1 gm of plant material under specified conditions. A specified quantity of the plant material previously reduced to required fineness and accurately weighed was transferred into a 25 ml glass stoppered measuring cylinder. The internal diameter of the cylinder should be about 16 mm, the length of the graduated portion about 125 mm, marked in 0.2 ml divisions from 0 to 25 ml in an upwards direction.unless otherwise indicated in the test procedure, 25 ml of water was added and the mixture was shaken thoroughly every 10 minutes for one hour. It was allowed to stand for 1 hours at room temperature, or as specified. The volume in ml occupied by the plant material, including any sticky mucilage was measured. Average of the individual determination related to 1 gm of the plant material was calculated.

5. QUALITATIVE AND QUANTITATIVE ESTIMATION OF HEAVY METALS AND INORGANIC ELEMENTS [54]

Plant minerals play a vital role in metabolism and osmolity. Presence of elements vary with the soil, climate conditions etc, there are essential and non essential elements which may be beneficial or harmful to living things. Non –essential elements like lead, arsenic, cyanide, chromium, cadmium, aluminum, silver bring about toxic effects results in intoxication. Hence, qualitative and quantitative estimation of inorganic elements in the plant *Dalbergia sissoo* were carried out.

QUALITATIVE ANALYSIS OF INORGANIC ELEMENTS AND HEAVY METALS

To the ash of the drug material, 50% v/v HCL was added and kept for 1 hour. It was filtered and the filtrate is used for the following tests.

Aluminium:

White gelationous precipitate of aluminium hydroxide ($Al(OH)_3$) is formed on addition of ammonia solution. It is slightly soluble in excess of the reagent. When freshly precipitated, it dissolves readil;y in strong acid and base but after boiling it becomes difficult to dissolve.

Arsenic:

Arsenious salts in neutral solution react with solution of copper sulphate to form green precipitate (Scheele's green) which on boiling gives a red precipitate of cupric oxide.

Borate:

The mixture obtained by the addition of sulphuric acid and alcohol (95%) to a borate when ignited, burns with flame tinged with green.

Calcium:

Solution of calcium salts, which when treated with ammonium carbonate solutions, yield a white precipitate after boiling and cooling the mixture, is insoluble in ammoniun chloride solution.

Carbonate:

Carbonates when treated with dilute acid produce effervescence due to liberation of CO2 which is colourless and produces a white precipitate in calcium hydroxide solution.

Chlorides :

Chlorides, when treated with silver nitrate solution yield a white curdy precipitate which is insoluble in nitric acid but soluble after being well washed with water in dilute ammonia solution, from which it is reprecipitated by the addition of nitric acid.

Copper:

An excess of ammonia TS ,added to a solution of cupric salt, produces first a bluish precipitate and then a deep blue coloured solution.

Iron:

Solution of ferric salts, when treated with potassium ferrocyanide solution , yields an intense blue precipitate which is insoluble in dilute HCl.

Lead:

Strong solution of lead salts, when treated with HCl, yield a white precipitate which is insoluble in boiling water and is re-deposited as crystals when the solutions is cooled.

Magnesium:

Solution of magnesium salts, when treated with ammonium carbonate solution and boiled , yield a white precipitate , but yield no precipitate in the presence of ammonium chloride solution.

Mercury:

Solution of mercuric salts, when treated with sodium hydroxide solution, yields a yellow precipitate.

Phosphate :

Solution of phosphate when treated with silver nitrate solution yield yellow precipitate of normal silver orthophosphate Ag3PO4(distinction from meta and Pyrophospahte) solution in dilute ammonia solution and in dilute nitric acid.

Potassium:

Moderately strong solution of potassium salts, which have been previously ignited to remove ammonium salts , when treated with Perchloric acid (60 %) yield a white crystalline precipitate.

Sulphate :

Solution of sulphate , when treated with lead acetate solution yields a white precipitate which is insoluble in ammonium acetate solution and in sodium hydroxide solution.

QUANTITATIVE ESTIMATION OF INORGANIC ELEMENTS [55]

Instrumentation parameters:

Instrument Name: Inductively Coupled Plasma Optical Emission Spectrometry.

Instrument Model: PE Optima 5300 DV ICP-OES.

Optical System Dual View-axial or radial

Detector system :Charge coupled detector,(UV-VISIBLE detector which is maintaining at 40°C) to detect the intensity of the emission line wavelength range from 165 to 782 nm.

TORCH (Light source):

Positioned horizontally in the sample compartment along the central axis of the spectrometer optics. Changing from axial to radial reviewing is simple software Command and is accomplished by computer control of a mirror located in the optical path. The torch assembly of this system comprises of two concentric quartz tubes.

Standard alumina injector: 2.0mm inner diameter.

Spray chamber: Scott type.

Nebulizer: Cross flow Gem tip.

Preparation of samples by acid digestion method :

Weighed 50mg of powdered mixture of powder treated with acid mixture of sulphuric acid:Water in the ratio of 4: 1 in the kjeldhal flask and heated continuosly till the solution is colorless. The sample mixture was then transferred in a 25 ml volumetric flask and made upto the volume with distilled water.

Blank solution was prepared as above without sample. The standards were prepared as per the protocol and the calibration curve was developed for each of them.

Detection

Samples were analyzed for the detection of Quantification of the Arsenic(As), Cadmium(Cd) , Copper(Cu), Iron (Fe),Potassium(K), Magnesium(Mg) and Molybdenum(Mo) by Inductively Coupled Plasma Optical Emission Spectrometry.

PHYTOCHEMICAL STUDIES

Phytochemical evaluation is used to determine the nature of phytoconstituents present in the plant by using suitable chemical tests. It is essential to study for its pharmacological activities. It can be done by qualitative analysis using specific reagents followed by confirmation with different chromatographic techniques like TLC and HPTLC. Therefore a complete investigation is required to characterize the phytoconstituents qualitatively and quantitative.

1. MATERIALS AND METHODS

PREPATATION OF EXTRACTS

Extraction is the preliminary step involved in the phytochemical studies. It bring out the primary and secondary metabolites into the extracting solvent depends upon its polarity.

CONTINUOUS HOT PERCOLATION METHOD

The dried coarsely powdered plant material of bark of *Dalbergia sissoo* Roxb were successively extracted using a soxhlet apparatus with solvents of increasing polarity such as Hexane, Ethyl acetate, Ethanol at 60-70°C for 18 hours. All the extracts were redistilled and concentrated under rotary vacuum evaporator. The resulted extracts were tested for qualitative and quantitative analysis.

2. PRELIMINARY PHYTOCHEMICAL EVALUATION [56,57]

2.1.Qualitative analysis

1.**Detection of Carbohydrates**

a. Molisch test :

The extracts and powder were treated with a few drops of alcoholic α-naphthol, then add few drops of concentrated sulphuric acid through sides of test tube. Formation of purple to violet colour ring appears at the junction of test tubes indicated the presence of carbohydrates.

b. Fehling's test

the extracts and the powder were treated with Fehling's A and Fehling's B solution and heat it. Formation of red coloration indicated the presence of sugar.

2. **Detection of Alkaloids**

a. Dragendorff's reagent

The extracts and powder were treated with Fehling' A and Fehling's B solution and heat it. Formation of red coloration indicated the presence of alkaloids.

b. Mayer's reagent

The extracts and the powder were treated with a few drops of Mayer's reagent. Formation of creamy white precipitate indicated the presence of alkaloids.

c. Wagner's reagent

The extract and the powder were treated with a few drops of wagner's reagent. Formation of brown or reddish brown precipitate indicated the presence of alkaloids.

3. Detection of Flavonoids

a. Shinoda test

The extracts and the powder were treated with a few magnesium turnings and concentrated hydrochloric acid drop wise, formation of pink, scarlet, crimson red or occasionally green to blue colour appears after few minutes indicated the presence of flavonoid.

b. Alkaline reagent test

The extracts and the powder treated with a few drops of sodium hydroxide solution, formation of intense yellow colour indicated the presence of flavonoid.

4. Detection of glycosides

a. Borntrager"s test

Boil the extracts and powder with 1ml of sulphuric acid for 5 minutes filter while hot. Cool the filtrate and shake with equal volume of chloroform. Separate the lower layer of chloroform and shake it with half of its volume of dilute ammonia. Formation of rose pink to red colour is produced in the ammoniacal layer, indicated the presence of glycosides.

b. Test for Hydroxy-anthraquinones

The extracts and the powder were treated with a few drops of potassium hydroxide solution. Formation of red colour indicated the presence of glycosides.

5. **Detection of Saponin**

a. Froth formation test

The extracts and the powder were shake well with water. Formation of stable froth indicated the presence of saponin glycoside.

6. Detection of Tannins (Phenolic compounds)

a. Ferric chloride test

The extracts and the powder were treated with a few drops of ferric chloride solution. Formation of green colour indicated the presence of tannins.

7. Detection of Phytosterols

a. Libermann-Burchard test

The extracts and the powder were treated with a few drops of acetic anhydride, boil and cool. Then add concentrated sulphuric acid from the sides of the test tube, 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 colour indicated presence of triterpenoids.

8. Detection of proteins and Amino acids

a. Biuret test

The extracts and the powder were treated with a few drops of Biuret reagent. Formation of violet colour indicated the presence of proteins.

b. **Xanthoprotein test**

The extracts and the powder were treated with a few drops of conc. Nitric acid and boiled, yellow precipitate is formed. After cooling it, add 40% sodium hydroxide solution. Formation of orange colour indicates the presence of proteins.

c. Ninhydrin test

The extracts and the powder were treated with a few drops of 0.25 % ninhydrin reagent and boiled for few minutes. Formation of blue colour indicates the presence of proteins.

9.Gums and mucilage

The extracts and the powder were treated with ruthenium red solution. Formation of pink colour indicates the presence of gums and mucilage.

The extracts and the powder were dissolved in 5 to 10ml of acetic anhydride by means of gentle heat, cool and add 0.05ml of conc. Sulphuric acid. Formation of bright purplish red colour indicates the presence of gums and mucilage.

10.Detection of Fixed oils and fats

- **Stain test**

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

11. Detection of resins

- **Acetone-water test**

Extracts were treated with acetone. Small amount of water was added and shaken. Appearance of turbidity indicates the presence of resins.

2.2QUANTITATIVE ESTIMATION OF PHYTOCONSTITUENTS

2.2.1.Total phenolic content (Folin-ciocalteu assay) [58]

Total phenolic content of extract were determined using Folin-ciocalteu assay. Briefly 0.5ml extract solutions were mixed with 2.5ml of 10 fold diluted folin-ciocalteu reagent and 2.5ml of 7.5% sodium carbonate (NA_2CO_3). After incubation at 40°C for 30 minutes, the absorbance of the reaction mixtures was measured at 765nm a spectrometer. Three replicates were made for each test sample. Gallic acid was a standard and the extract were expressed in milligram .

2.2.2.Total flavonoid content [59]

Total flavonoid contents was determined by aluminum chloride colorimetric method, using quercetin as a standard. Briefly , the test samples were individually dissolved in DMSO. Then , the sample solution (150µl) of 2% AlCl3. After 10 min of incubation at ambient temperature , the absorbance of the supernatant was measured at 435nm by using a spectrometer. Three replictaes were made for each test sample. The total flavonoids content was expressed as quercetin equivalent in milligram per gram extract (mg QRT/ g extract).

2.2.3. Total tannin content [60]

Prior the quantitative estimation of the tannin content in the extracts, the presence of tannins was identified using the classic $FeCl_3$ and gelatin tests. The quantitative tannin content in sample was estimated by the method of Price and Butler, with some modifications. 1g of the plant extracts was transferred to 100ml flask; 100 ml water was added, and boiled for 30 min.

After filtration with cotton filter, the solution was further transferred to a test tube and 10 ml of water was added. 0.5ml aliquots were finally transferred to vials, 1ml 1% $K_3Fe(CN)_6$ and 1ml 1% $FeCl_3$ was added. The solution was made upto 10ml with distilled water. After five mins time period , the solutions were measured colorimetrically at 720nm. Gallic acid was used as a standard and calibration was prepared using various concentration 5,10,15,20 µg/ml

3. FLUORESCENCE ANALYSIS [61,62]

Fluorescence analysis was carried out according to the method of Chase and Pratt (1949) and Kokshi et al. (1958) in day light and in UV light. The bark powders and extracts were treated with different solvents and the fluorescence was observed in day light and in near and the far UV light.

4.CHROMATOGRAPHY [63, 64]

Chromatography methods are important analytical tool in the separation identification and estimation of different components present in crude present in crude plant mixture or extract.

4.1.THIN LAYER CHROMATOGRAPHY

Principle

Thin layer chromatography is a technique used for the separation , identification and estimation of single or mixture of components present in the various extracts. It is reliable technique in which solute undergoes distribution between two phases, stationary and mobile phase. The separation is mainly based on the differential migration that occurs when a solvent flows along the thin layer of stationary phase. This may be achieved by partition and adsorption depending on stationary phase used.

TLC plate preparation

The plates were prepared using stahl TLC Spreader. 40 gm of silica G was mixed with 85 ml of water to prepare homogenous suspension and poured in the spreader. 0.25 mm thickness of plates was prepare, air dried until the transparency of the layer disappeared, then dried at 1100°C for 30 mins and kept in desiccators.

Selection of mobile phase

Solvent mixture was selected on the basis of the phytoconstituents present in each extract. Solvents were analysed in the order of increasing polarity. Factors such as nature of components, stationary phase, mobile phase polarity influence the rate of separation of constituents. Several mobile phases were tried for the separation of maximum components. From the vast analysis, best solvent was selected which showed good separation with maximum number of components.

Solvent system

The two extracts were run in the mobile phase

Ethyl acetate-Formic acid-Glacial acetic acid – Water (100:11:11:26)

Detection

Polyethylene glycol reagent –UV-365 nm.

4.2.HIGH PERFORMANCE THIN LAYER CHROMATOGRAPHY-FINGER PRINT PROFILE

HPTLC is one of the versatile chromatographic method which helps in 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-quantitaive information on major active constituents of a drug, thus enabling n assessment of drug quality.

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, purity, and standardize the quantity of active principles present in the herbal extract.

Instrument Conditions

Sample used : ETHYL ACETATE EXTRACT

HPTLC Applicator : CAMAG LINOMAT IV

HPTLC Scanner : CAMAG TLC SCANNER II

Sample dilution : 10 mg of sample extracted with 1 ml of ethyl acetate.

Volume of injection : 20µl

Mobile phase : Ethyl acetate -Formic acid-Glacial Acetic acid-Water(100:11:11:26)

Lambda max : 254nm

Lamp : Deuterium

Stationary phase : TLC Silica gel 60 F_{254} (Merck)

Equipment

A Camag HPTLC system equipped with a sample applicator Linomat IV, twin trough plate development chamber, TLC SCANNER II.

Chromatographic conditions:

The estimation has been done using the following chromatographic conditions. Chromatography was performed on a 10 x 10 cm pre-activated HPTLC silica gel 60 F_{254} 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 pre-washed.

IN-VITRO MELANIN CONTENT DETERMINATION ACTIVITY BY TSUBOI ET AL METHOD USING B16F10 MELANOMA CELL LINE

Materials and method

Plant extract

- Hexane
- Ethyl acetate
- Ethanol

Reagents

- MEM – (Minimal Essential Media) was purchased from Hi Media Laboratories.
- Fetal bovine serum (FBS) was purchased from Cistron laboratories.
- Trypsin, MTT – (3-(4,5- dimethylthiazol-2yl)-2,5- diphenyl tetrazolium bromide), and Dimethyl sulfoxide (DMSO) were purchased from (Sisco research laboraotory chemicals Mumbai).
- All of other chemicals and reagents were obtained from Sigma Aldrich Mumbai.

Cell line and culture

Normal B16F10 melanoma cell line were obtained from National Centre for cell sciences Pune (NCCS). The cells were maintained in Minimal Essential Media supplemented with 10% FBS, penicillin (100 U/ml), and streptomycin (100µg/ml) in a humidified atmosphere of 50µg/ml CO2 at 37 °C.

Maintenance of cultures was passaged weekly, and the culture medium was changed twice a week.

Standard drug

Kojic acid were dissolved in 10ml of DMSO and diluted to 100ml with minimum essential medium. 1000, 500, 250, 125, 62.5, 31.2, 15.6,7.8 µg/ml solutions were prepared by diluting with distilled water.

Sample solutions

100mg of various extracts of test sample such as Hexane, Ethyl acetate and Ethanol were dissolved in 10ml of DMSO and diluted to 100ml with minimum essential medium. 1000, 500, 250, 125, 62.5, 31.2, 15.6, 7.8 µg/ml solutions were prepared by diluting with distilled water.

***In -vitro* assay for Cytotoxicity activity (MTT assay) [65]**

Principle

MTT assay is laboratory tests and standard colorimetric assays, for measuring the activity of enzymes. It can also be used to determine Cytotoxicity of potential medicinal agents and other toxic materials.

The assay is based on conversion of the MTT – (3-(4,5- Dimethylthiazol-2-yl) – 2,5-diphenyl tetrazolium bromide), a yellow tetrazole to a purple coloured formazan crystal by the active mitochondrial reductase (or cellular reductase) present in the viable cells. The purple colour thus formed is directly proportional to the viable cells present. This provides study of the cytotoxic activity of the test compounds. The absorbance of this coloured solution can be quantified by measuring at a wavelength of 500 to 600nm by spectrometer.

Procedure

Cytotoxicity assay

MTT assay

- The monolayer cells were detached with Trypsin – Ethylene diamine tetra acetic acid (EDTA) to make single cell suspensions and viable cells were counted using a hemocytometer and diluted with medium along with 5% FBS to give final density of $1x10^5$ cells /ml.
- Cells ($1x10^5$ / well) were plated in 5ml of medium/well in 96 well plates (Costar Corning, Rochester,NY).
- After 48 hours incubation the cell reaches the confluence. Then cells were incubated with different concentration of Kojic acid, Hexane, Ethyl acetate, Ethanol, for 24-48h at 37°C.

- After removal of the sample solution and washing with phosphate buffered saline (pH 7.4), 1ml/well (5mg/ml) of 0.5% 3-(4,5- dimethyl- 2-thiazolyl)-2,5-diphenyl-tetrazolium bromide cells (MTT) phosphate-buffered saline solution was added.
- After 4h incubation, 0.04m HCL/ isopropanol were added.
- Viable cells were determined by the absorbance at 570nm.
- Measurements were performed and the concentration required for a 50% inhibition of viability (IC 50) was determined graphically.
- The absorbance at 570nm was measured with a UV- Spectrophotometer using wells without sample containing cells as blanks.
- Triplicate was maintained for all concentrations.
- The effect of the samples on the proliferation of Chang Liver Cells was expressed as the % cell viability, using the following formula.

$$\% \text{ Cell Viability} = \frac{\text{A 570 of treated cells}}{\text{A 570 of Control cells}} \times 100$$

Determination of melanin content in melanocytes[66]

Extracts influence on the production of melanin in melanocytes was determined using the modified method of Tsuboi et al method. Cells were added into the wells of a 24 well plate (4×10^4 cells per well). After 48 hour, different concentrations of bark extracts and kojic acid (1mM as a reference drug) were added to the cells and incubated at 37°C in 5% CO2, humidified atmosphere for 4 days. Control group was incubated just with DMEM or DMEM plus extract vehicle (0.02% ethanol). Then, the medium was removed and cells were lysed with 500µl of NaOH 1 N in DMSO at 80°C for 1 hour. The relative melanin content was determined by measuring the absorbance at 490 nm in plate reader.

ANTIOXIDANT ACTIVITY [67]

Reducing power Assay

The reducing power assay was determined according to the method of Oyaizu. Various concentrations of plant extracts were mixed with 1ml of 200mmol/l sodium phosphate buffers (pH6.6) and 1ml of 1% potassium ferricyanide. The mixture was incubated at 50°C for 20mins. To this 1ml of 10%trichloroacetic acid(w/v) was added, the mixture was centrifuged at 2000 rpm for 10mins. The upper layer solution (2.5ml) was mixed with 2.5ml of deionised matter and 0.5ml of fresh ferric chloride (0.1%). The absorbance was measured at 700nm; a higher absorbance value indicates a higher reducing power.

Hydrogen peroxide scavenging assay

The ability of the extracts to scavenge hydrogen peroxide was determined according to the method of (Ruch). A solution of hydrogen peroxide (2mmol/l) was prepared in phosphate buffer (pH7.4). Various concentration of extracts were added to hydrogen peroxide at 230nm was determined after 10min against a blank solution containing phosphate buffer without hydrogen peroxide.

% scavenging activity = Abs (control)-Abs (standard)/ Abs control) x 100.

Formulation of Gel [68]

Gels are transparent or translucent semisolid or solid preparation consisting of solutions or dispersions of one or more active ingredients in suitable hydrophilic or hydrophobic phases. They are made with the aid of gelling agent. Usually gels exhibit pseudoplastic flow properties an those made with synthetic or semisynthetic polymers with a high degree of cross-linking have relative high yield value and low viscosity. Products tend to be smooth, elegant, and produce cooling effect because of evaporation of water. They are also dry out to form films. Films adhere well to the skin and are usually easily removed by washing. Gels are semisolid systems consisting of either suspensions made up of small inorganic particles or large organic molecules interpenetrated by a liquid.

Herbal gel was prepared using Carbopol 934 as a gelling agent in 1% w/w concentration with deionized water using mechanical stirrer. The pH of the gel was adjusted to neutral by addition of small quantities of triethanolomine with continuous stirring.1% w/w ethyl acetate extract of Dalbergia sissoo was added to the gel and stirred for sufficient time for homogenous mixing of extract in gel base. Prepared gel was filled in collapsible tubes and stored at a cool and dry place. Physical parameters such as colour, appearance and feeling on application were recorded. pH of the gel was recorded using a pH meter.

A. Physical Evaluation

Physical parameters such as color and appearance were checked.

B. Measurement of pH

pH of the gel was measured by using digital type pH meter by dipping the electrode completely in the gel so as to cover the electrode. This to confirm that the pH of the formulation is nearer to skin pH.

C. Viscosity

Viscosity of the gel was measured by using Brookfield viscometer with spindle 7. Brook field viscometer was used to measure the viscosity of the gel at a controlled temperature.

D. Spreadability [69]

Spreadability was determined by the apparatus which consist of a wooden block, which was provided by at on end. By this method Spreadability was measured on the basis of slip and drag characteristics of gels. An excess of gel (about 2g) under study was placed on this ground slide. The gel was then sandwiched between these slides for 5minutes to expel air and to provide a uniform film of the gel between the slides. Excess of the gel was scrapped off from the edges. The top plate was then subjected to pull of 80gm. With the help of string attached to the hook and time (in seconds) required by the top slide to cover a distance of 7.5cm noted. A shorter interval Spreadability was calculated using the following formula.

$$S = M \times L / T$$

Where,

S=Spreadability,

M= Weight in the pan(tied to the upper slide)

L= Length moved by the glass slide

T= Time (in sec.) taken to separate the slide completely each other.

E. Extrudability [70]

The gel formulations were filled in standard capped collapsible aluminium tubes and sealed by crimping to the end. The weights of the tubes were recorded. The tubes were placed between two glass slides and were clamped. 500gm was placed over the slides and then the cap was removed. The amount of the extruded gel was collected and weighed. The percent of the extruded gel was calculated (> 90% extrudability: excellent, >80% extrudability: good, >70% extrudability: fair).

F. Stability studies [71]

Accelerated stability studies

Stability

Stability is official defined as the time lapse during which the drug product retains the same property and characteristics that it possessed at the time of manufacture. This process begins at early development phases.

Definition

Stability of a pharmaceutical preparation can be defined as " the capability of a particular formulation in a specific container/ closure system to remain within its physical, chemical, microbiological, therapeutic and toxicological specifications throughout its shelf life.

Instability in modern formulations is often undetectable only after storage period under normal conditions. To assess the stability of a formulated product it is usual to expose it to high stress conditions to enhance deterioration and therefore the time required for testing is reduced. Common high stress factors are temperature and humidity. This will eliminate unsatisfactory formulation.

Purpose of stability testing

To study of drug decomposition kinetics

To develop stable dosage form

To establish the shelf life or expiration date for commercially available drug product.

To ensure the efficacy, safety and quality of active drug substance and dosage forms.

STABILITY CONDITIONS CHART

S.NO	STUDY	STORAGE CONDITION	MINIMUM PERIOD
1	Long-term	25°C±2°C 60%±5% RH	12 months
2	Intermediate	30°C±2°C 65%±5% RH	6 months
3	Accelerated	40°C±2°C 75%±5% RH	3 months

Procedure

The stability study was performed as per ICH guidelines 6. The formulated gel were filled in collapsible tubes and stored at different temparatures and humidity conditions,40 ºC ± 2ºC / 75% ± 5% RH for a period of three months and studied for appearance, pH and Spreadability.

APPLICATION OF HERBAL GEL AND DRAIZE SKIN IRRITATION TEST [72]

The formulated gel was tested for irritancy as evaluation of irritation potential to human skin of any chemicals or formulations is mandatory and hence it is to be checked on rabbits. The protocol was followed according to OECD TG 404.

A protocol hard copy was submitted to the institutional animal ethical committee and got approval (Vide 4/243/CPCSEA dated 22/11/13).The animals were conditioned to the normal diurnal and nocturnal rhythms. The animals were fed with leafy vegetables and water ad libitum. The average weight of rabbits was 2.5kgs

Healthy male Albino Rabbits (white, 2.5 kg) are divided into 2 groups . 3 animals in each group were procured from Animal Experimental Laboratory, Madras Medical College, Chennai-03

The formulated gel was to be tested for irritancy .The animals were kept under standard laboratory conditions, at 25±1°C and 55±5 % relative humidity with a 12 h light/dark cycle.

PROCEDURE

Skin irritation studies were carried out in the presence of skin of rabbit. The hairs on the dorsal side of the rabbit are removed 1 day before performing experiment. Care was taken to avoid abrading the skin and only animals with healthy, intact skin are used for the study. About 0.5 g of test substance was applied to intact skin with patch for 4 hours. The test substance is removed after 4 hour exposure period and the formation for any erythema or edema is observed at 24, 48, and 72 hour thereafter. The observation is made for 14 days to determine any persistent or delayed effects.

GROUP I- Treated with the gel base. (carbopol 934)

GROUP II- Treated with the gel from the best extract.

In vivo STUDIES [73]

The study was carried out with prior approval from the institution animal ethical committee.

Antileucodermic activity was studied in black mice (6J BL C57) obtained from Sri Venkateshwara enterprises, Bangalore, India. Mice were housed separately in a polystyrene cage bedded with rice husk and fed on standard laboratory diet and pure water adlibitum. A protocol hard copy was submitted to the institutional animal ethical committee and got approval (Vide 4/243/CPCSEA dated 22/11/13).

The black mice weighing between 20-25gm ranges were weighed and divided into three groups. Six mice were taken in each group. At most care was taken to ensure that the animals were treated in the most humane and ethically acceptable manner.

Treatment protocol

Group 1- Treated as control

Group 2-Treated with bark extract into gel (1%)

Group 3- Treated with Psoralen ointment.

Induction of leucoderma

Group 1 was served as control. Induction of depigmentation was done in group 1, Group 2 and Group 3 by application of 5% 4-hydroxy anisole ointment using cotton buds. The ointment was prepared by fusion method. After the application of the ointment, on seventh day, exposing the pale coloured skin

A circular area of about 2.5 cm diameter around the back, below the neck was chosen as

the area of application for the ointment. Within a week the transformation of black to white patch along with fur was noticed as irregular patch.

Application of bark extract into gel and Psoralen ointment

After depigmentation on the 7th day, Psoralen ointment and test extract into Gel were applied to Group 2 and Group 3 respectively to the depigmented skin of the mice in the similar way of the application of 4-hydroxy anisole for depigmentation. A thin coat samples were applied with the help of cotton buds on the depigmented area of mice three times per week (but not on 2 consecutive days). After each application the surface was exposed to early morning sunlight for 30 minutes.

RESULTS AND DISCUSSIONS

PHARMACOGNOSTICAL STUDIES

The results of pharmacognostical studies are as follows

MACROSCOPICAL FEATURES

Habit : *Dalbergia sissoo* is a medium to large tree of about 25 meters high with grey yellow trunk, longitudinal crack, and downcast twig. Leaves are leathery pinnately compound, with about five alternate leaflets.

Morphology

Color	: Brown
Odour	: Odourless
Taste	: Bitter
Size	: 0.5 – 1cm thickness.
Shape	: Flat
Surface	:Rough and Hard
Fracture	:Splintery

Fig. 8. Bark of *Dalbergia sissoo*

Transverse section of bark:

- Outer most layer cork composed of several layers of compactly arranged rectangular cells.
- Phelloderm consists of rectangular cells of many layers with scleride cells.
- The inner bark shows the collapsed and non-collapsed phloem cells.
- Outer collapsed phloem composed is made up of irregular masses of dilated phloem rays, scattered sclerides, fibre masses and sieve elements.
- Non collapsed phloem layer composed of sieve tube, companion cells and phloem parenchyma.
- The parenchyma cells show tanniferous contents. Prismatic crystals are seen in phloem fibres.
- Druses are seen in the phelloderm cells.
- Starch grains are present in the cortex region.

MICROSCOPICAL FEATURE

Fig.9. T.S of Outer bark at 40X

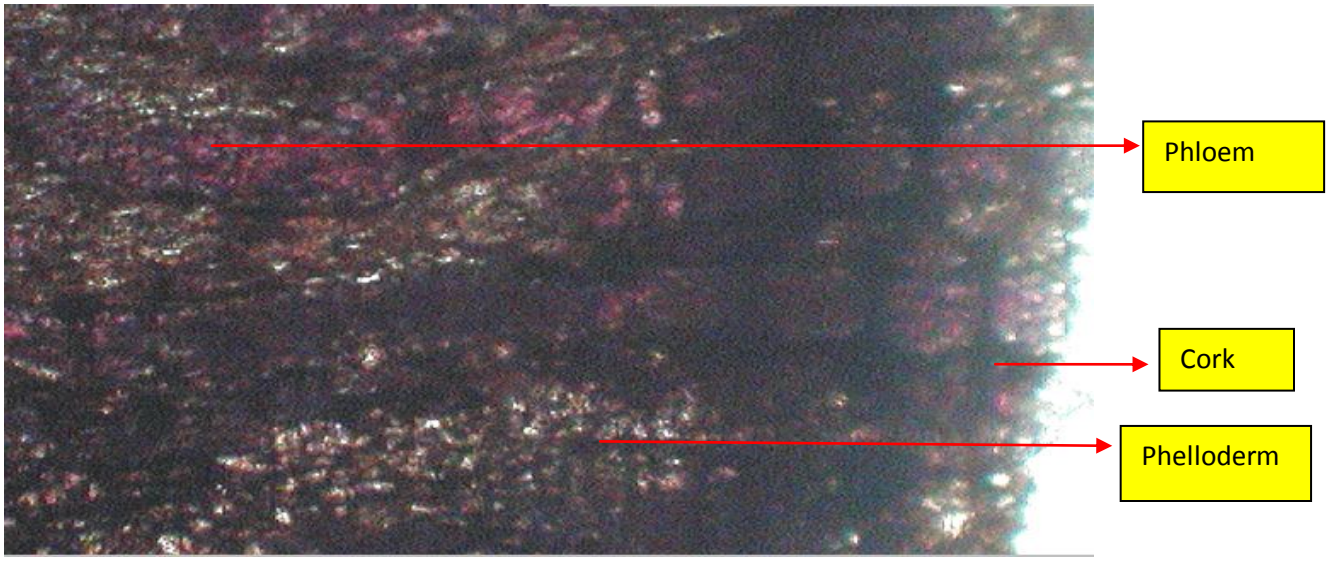


Fig. 10. Phelloderm in 10X view

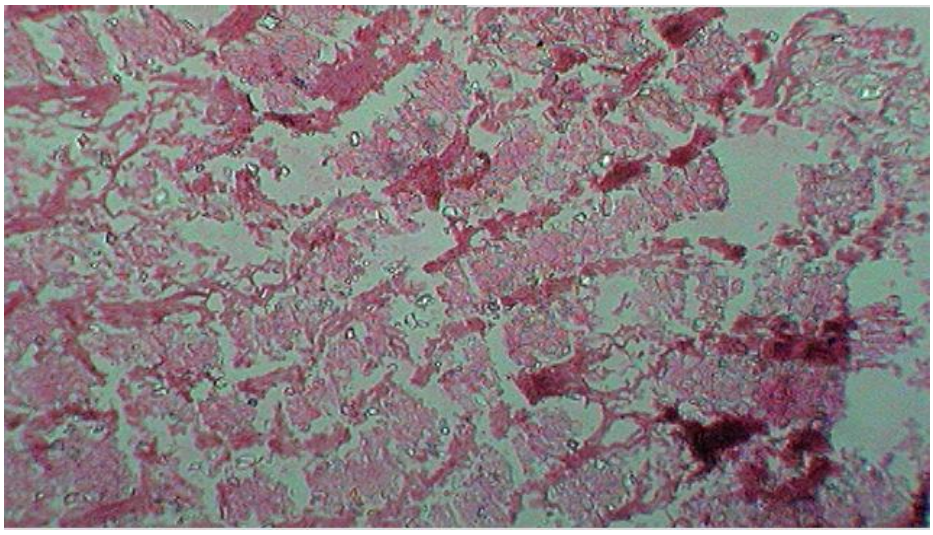


Fig. 11. Phloem region 10X view

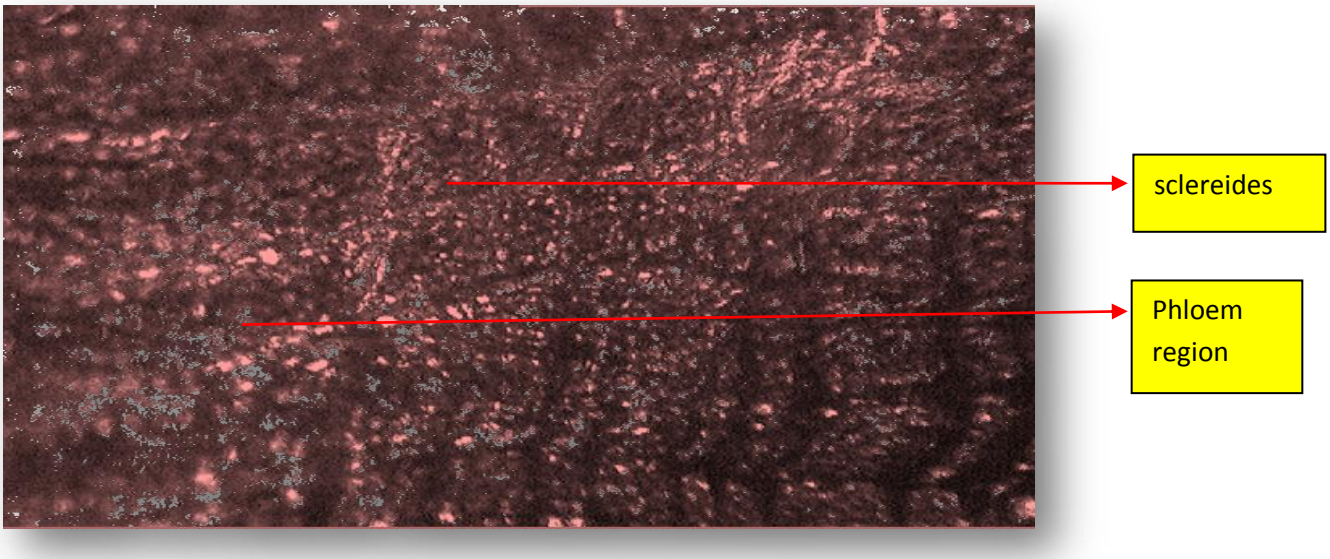


Fig. 12. Phloem region 40X view

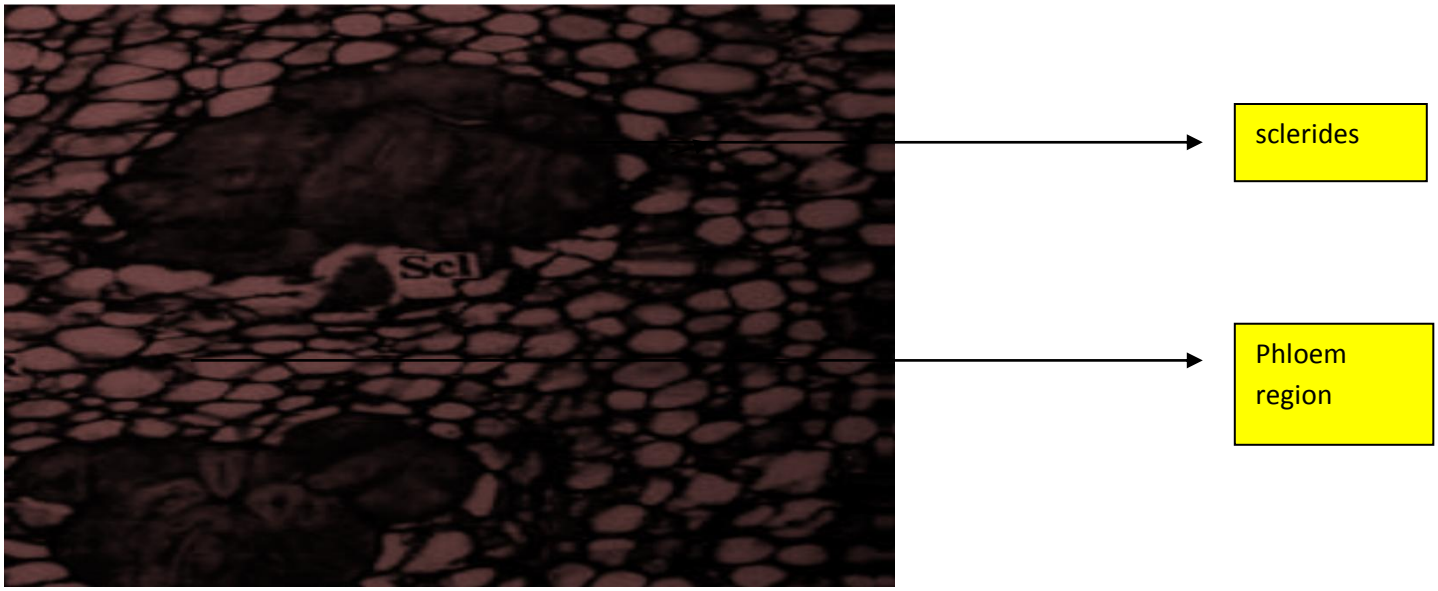


Fig. 13. Crystals in 40X view

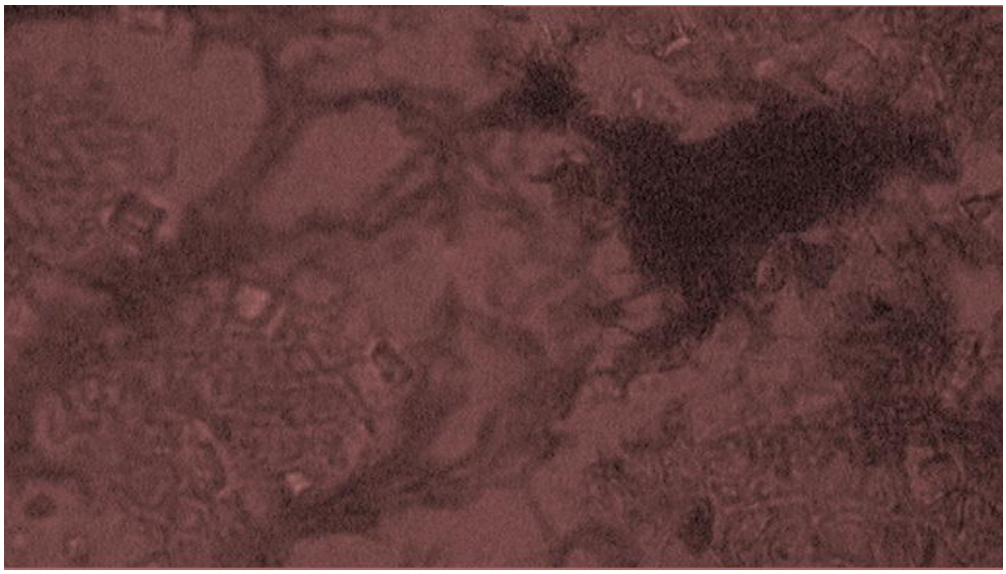


Fig. 14 .Crystals in 10X view

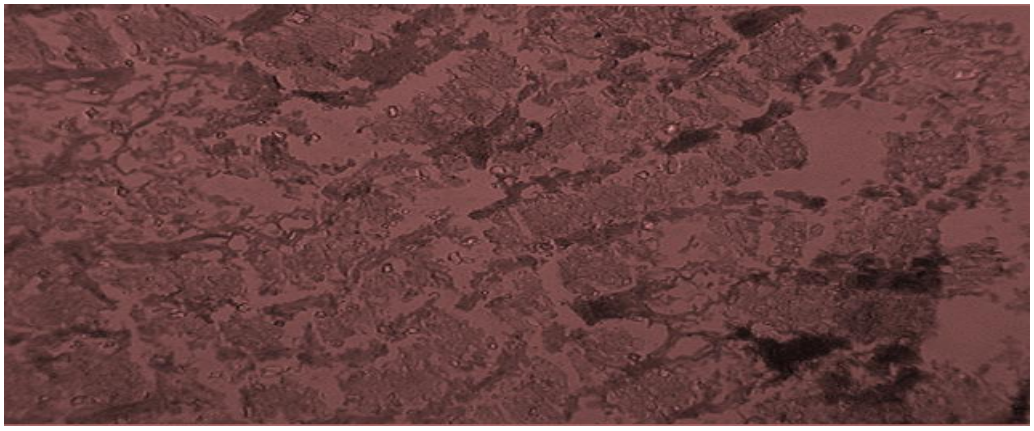


Fig . 15. Sclereides in 40x View

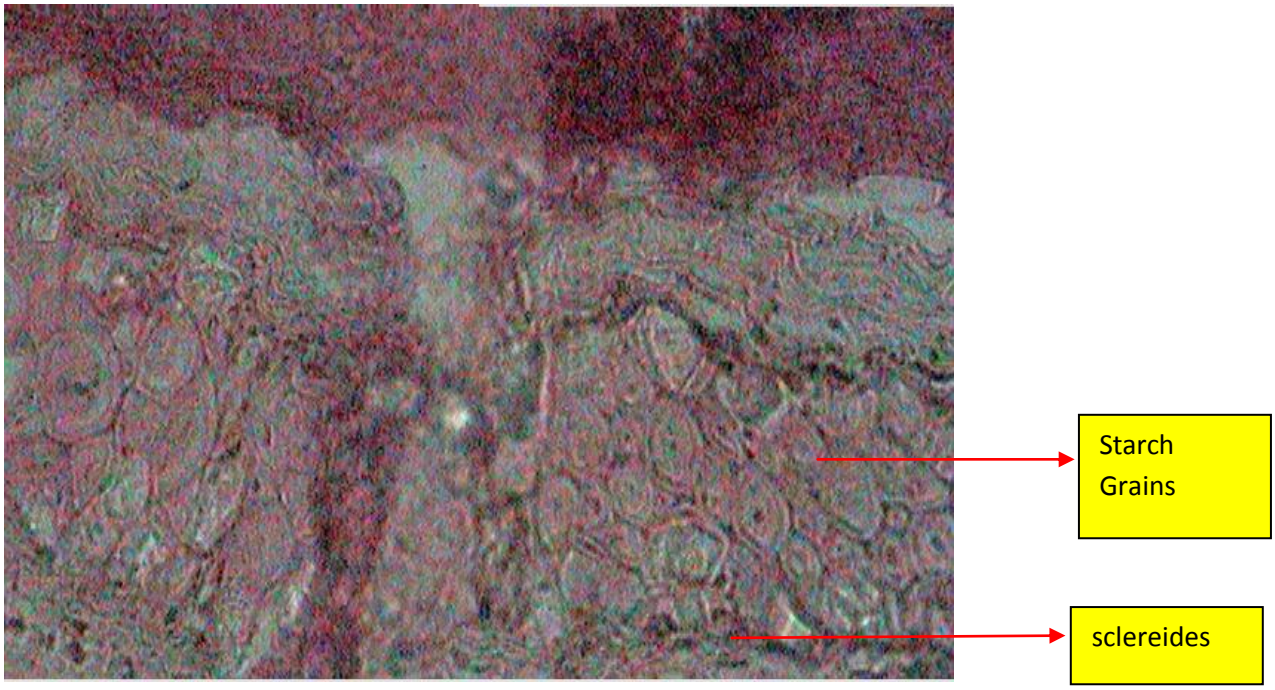
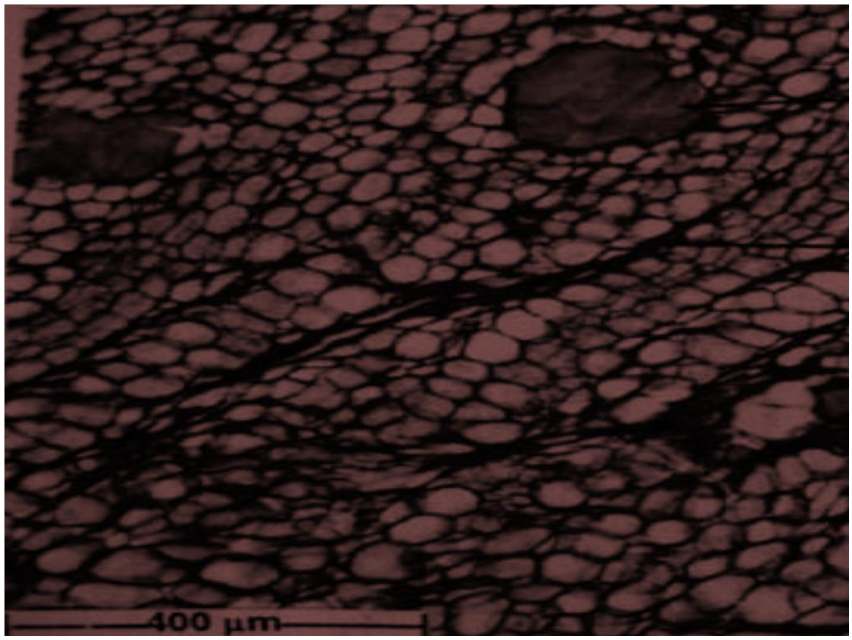


FIG.16.COLLAPSED PHLOEM



Powder microscopy

Description:

- Cork cells with lignified walls.
- Lignified sclerides.
- Phloem parenchyma are present.
- Parenchyma cells showing tanniferous content.
- Unlignified fibers.
- Prismatic calcium oxalate crystals are present.
- Calcium oxalate crystals with cortical parenchyma cells.
- Scattered parenchyma cells.

Powder Microscopy

Fig.16. cork cells with lignified walls

Cr-cork cells

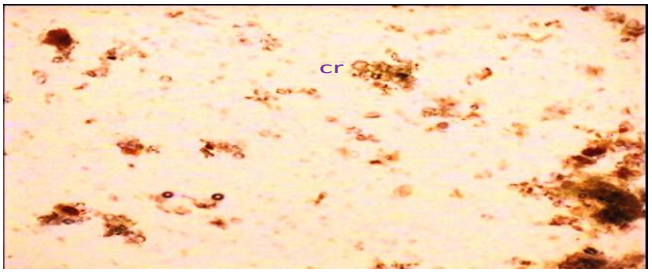


Fig. 17. Lignified Sclereides

Scl- sclerides

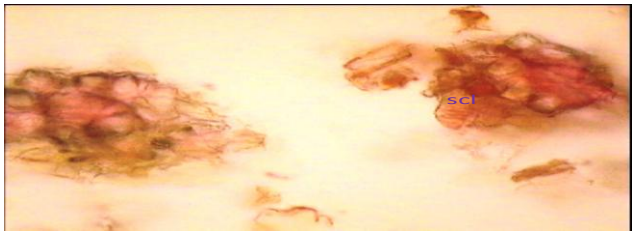


Fig. 18. Unlignified Fibres

F- Fibre

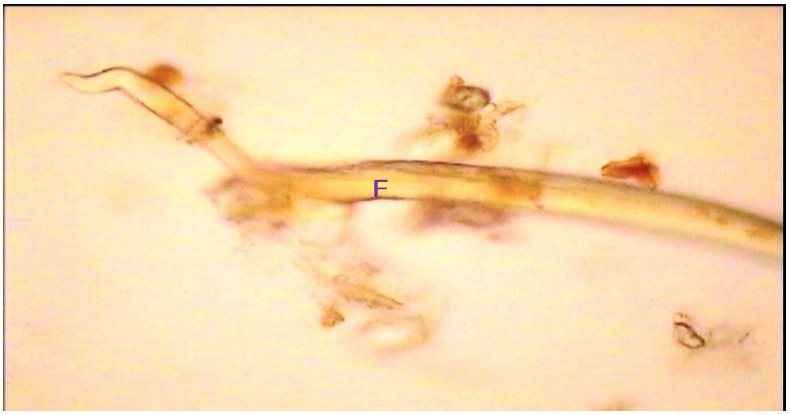


Fig. 19. Phloem tubes

Ph- phloem tubes

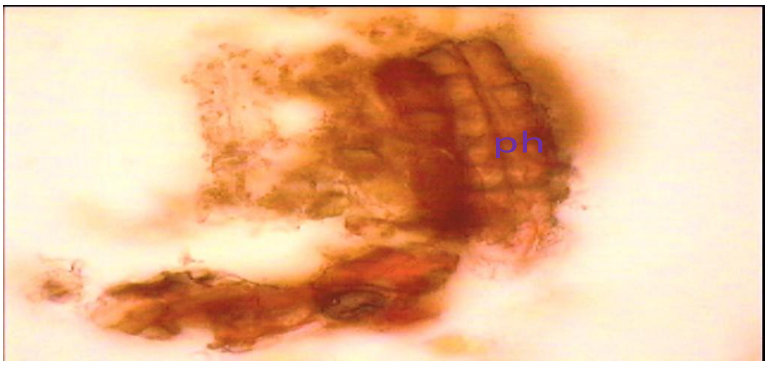


Fig. 20. Tanniferous cells

tn - Tanniferous cells

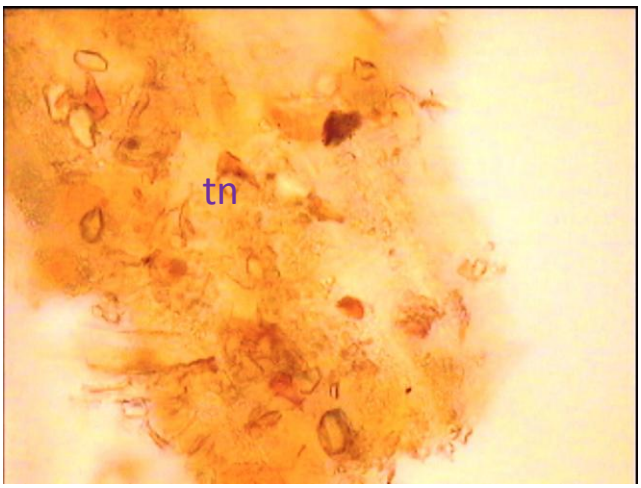


Fig. 21. Medullary ray

Mr- Medullary ray

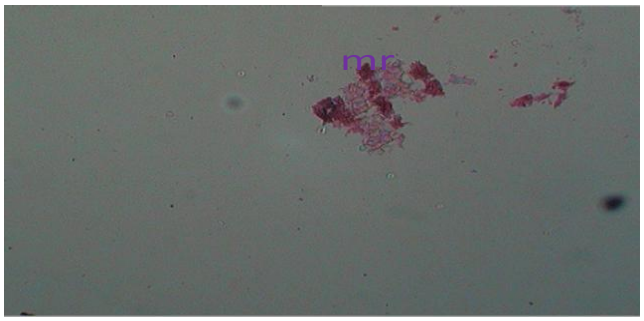


Table 1

The Physico-chemical constant analysis of bark of *Dalbergia sissoo Roxb.*

S.No	PHYSICO-CHEMICAL CONSTANT	Percentage (% W/ W)
I	**ASH VALUES**	
1	Total ash	12.48± 0.18
2	Water soluble ash	3.37±0.04
3	Acid insoluble ash	2.16±0.09
4	Sulphated ash	13.23±0.13
II	**Extractive value**	
1	Alcohol soluble extractive value	7.90±0.40
2	Water soluble extractive value	12.16±0.16
3	Non-volatile ether soluble extractive value	4.13±0.15
4	Volatile ether soluble extractive value	3.05±0.06
III	**Loss on Drying**	15.02±0.05
IV	**Foaming Index**	>100
V	**Swelling Index**	Nil

Values are expressed as Mean ± SD (n=3)

INORGANIC ELEMENTS AND HEAVY METAL ANALYSIS

Qualitative and Quantitative estimation of inorganic metals was analysed using Inductively Coupled Plasma Emission Spectrometry and the results were tabulated.

Table 2 .Qualitative estimation of inorganic elements of *Dalbergia sissoo Roxb*

S.NO	INORGANIC ELEMENTS	TOTAL AMOUNT
1	Aluminium	_
2	Arsenic	+
3	Boron	-
4	Calcium	+
5	Carbonate	_
6	Chlorides	_
7	Copper	+
8	Iron	+
9	Lead	+
10	Magnesium	+
11	Mercury	-
12	Phosphate	+
13	Potassium	+
14	Sulphur	+
15	Sodium	+

Table 3 .Quantitative estimation of inorganic elements of *Dalbergia sissoo* Roxb.

S.NO	INORGANIC ELEMENTS	TOTAL AMOUNT (% W/W)
1	Calcium	0.213
2	Copper	0.176
3	Potassium	0.112
4	Sodium	0.237
5	Magnesium	0.043

Table 4. Heavy metal analysis of *Dalbergia sissoo* Roxb.

S.NO	HEAVY METALS	RESULTS (ppm)	STANDARD LIMITS
1	Arsenic	0.006	5
2	Cadmium	0.007	0.3
3	Iron	0.075	10
4	Lead	0.020	10

PHARMACOGNOSY DISCUSSION

The pharmacognostical studies play an important factor in identifying and authenticating the herbal plant material. The Botanical identity of the plant was established by examining its anatomical features.

Dalbergia sissoo Roxb is the member of the family Fabaceae and is closely related to other species.

The anatomical examination of *Dalbergia sissoo* Roxb, bark exhibited important microscopical characters such as cork, phloem, sclerides, phelloderm, starch grains, calcium oxalate crystals.

Physico chemical constant parameters are used to confirm the purity and quality of the powdered drug. Ash values are helpful in determining the inorganic salts or any extraneous matter adhering to it or plant tissue itself or deliberately added to it.

The total ash involves the oxidation of the component of the powdered crude drug. The total ash consists of carbonate, phosphates, silicates, and silica. An increase in ash value is the indicative of contamination, adulteration, or substitution of the drug.

An increase in acid-insoluble ash indicates that the drug contaminates with siliceous matters or sand. The value was found to be 2.16±0.09%W/W.

The water soluble ash is the good indicator of water soluble salts. The value of water soluble ash was found to be 3.37±0.04 % W/W.

Sulphated ash was obtained by treatement with dilute sulphuric acid where the oxides are converted into sulphates. The value was found to be 13.23±0.13 %W/W.

The solvent extractive values give an idea about the phyto-constituents present in the given drugs as well as useful in the determination of exhausted and adulterated drug. The ethanol and water soluble extractive values obtained are very important because any addition of exhausted material to the crude drug would be reflected on the lowering of these solvent extractive values. The alcohol and water soluble extractive values was found to be 7.90±0.40%W/W and 12.16±0.16 %W/W. Non-volatile ether soluble extractive and volatile ether soluble extractive values was found to be 4.13±0.15% w/w and 3.05±0.06. The constants would help to identify and standardize the plant for future researches.

Loss on drying also termed as moisture content. It was carried out to find out the percentage of moisture content facilitates the enzyme hydrolysis, growth of microbes which leads to deterioration of phytoconstituents. The value was found to be 15.02±0.05%w/w.

Foaming index exhibited the presence of saponin content in the crude drug. The value was found to be >100. The swelling index was absent. The qualitative and quantitative estimation of heavy metal analysis and inorganic elements was carried out. The heavy metals are present within the limits. The percentage composition of different inorganic elements was determined. The presence of calcium at 0.213% and iron at 0.075% shows its nutraceutical importance. Hence this shows the drug is safe to consume it medicinally.

The detailed Pharmacognostical studies on the bark of *Dalbergia sissoo* Roxb have the essential data or it may serve as tool for identification of the plant for validation of the raw material and for standardization of its formulation.

PHYTOCHEMICAL STUDIES

The results of phytochemical studies are as follows

Table 5 .Percentage yield of successive extracts of bark of *Dalbergia sissoo* Roxb.

S.NO	EXTRACT	METHOD OF EXTRACTION	PHYSICAL NATURE	COLOUR	YIELD (%W/W)
1	Hexane	Soxhlet extraction	Semisolid	Greenish black	3.45%
2	Ethyl acetate		Semi sticky	Brown	7.75%
3	Ethanol		Semisolid	Brown	5.25%

Table 6 Preliminary Phytochemical screening on bark of *Dalbergia sissoo* Roxb.

S.NO	PHYTOCONSTITUENTS	POWDER	HEXANE	ETHYL ACETATE	ETHANOL
1	Carbohydrates	+	_	+	+
2	Flavonoids	+	_	+	+
3	Glycosides	+	_	+	+
4	Alkaloid	_	_	_	_
5	Saponin	+	_	+	+
6	Phytosterols	_	_	_	_
7	Phenolic compounds	+	_	+	+
8	Proteins	_	_	_	_
9	Fixed oils and fats	_	_	_	_
10	Tannins	+	_	+	+
11	Triterpenoids	+	+	_	_
12	Gums and mucilage	_	_	_	_

+ve – indicates the presence of Phytoconstituents.

-ve – indicates the absence of Phytoconstituents.

QUANTITATIVE PHYTOCHEMICAL ANALYSIS

Total Phenolic content of bark of *Dalbergia sissoo* Roxb

Table 7.QUANTITATIVE ESTIMATION OF PHENOL

Concentration (µg/ml)	Absorbance
20	0.14
40	0.17
60	0.19
80	0.23
100	0.26
Ethyl acetate	0.22
Ethanol	0.18

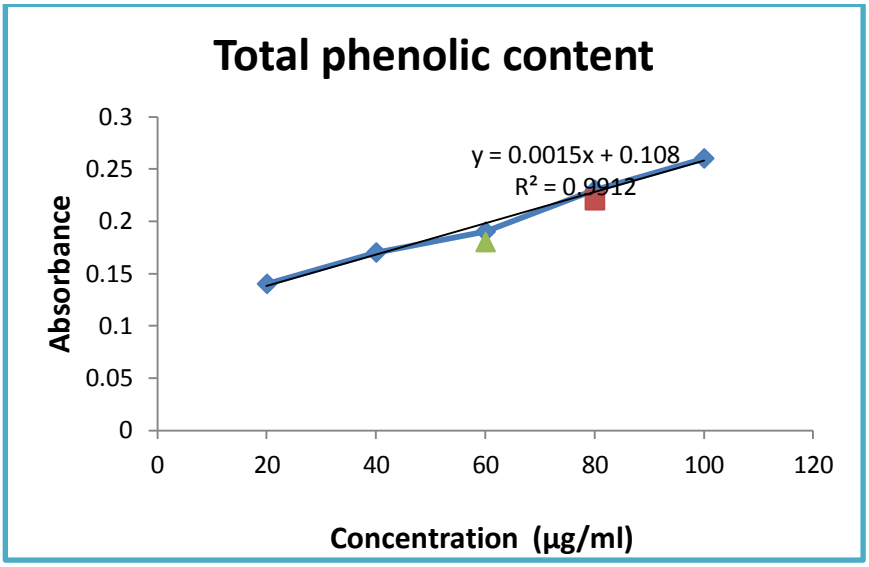


Fig. 22.Calibration curve of Total Phenolic Content

The concentration of phenol present in the ethyl acetate extract and ethanolic extract were found to be 74.65µg/mg and 61.18µg/mg

Quantitative estimation of Tannins

Total tannin content of bark of *Dalbergia sissoo* Roxb

Table 8.Quantitative estimation of tannins

Concentration (µg/ml)	Absorbance
5	0.47
10	0.59
15	0.73
20	0.85
25	0.89
Ethyl acetate	0.86
Ethanol	0.74

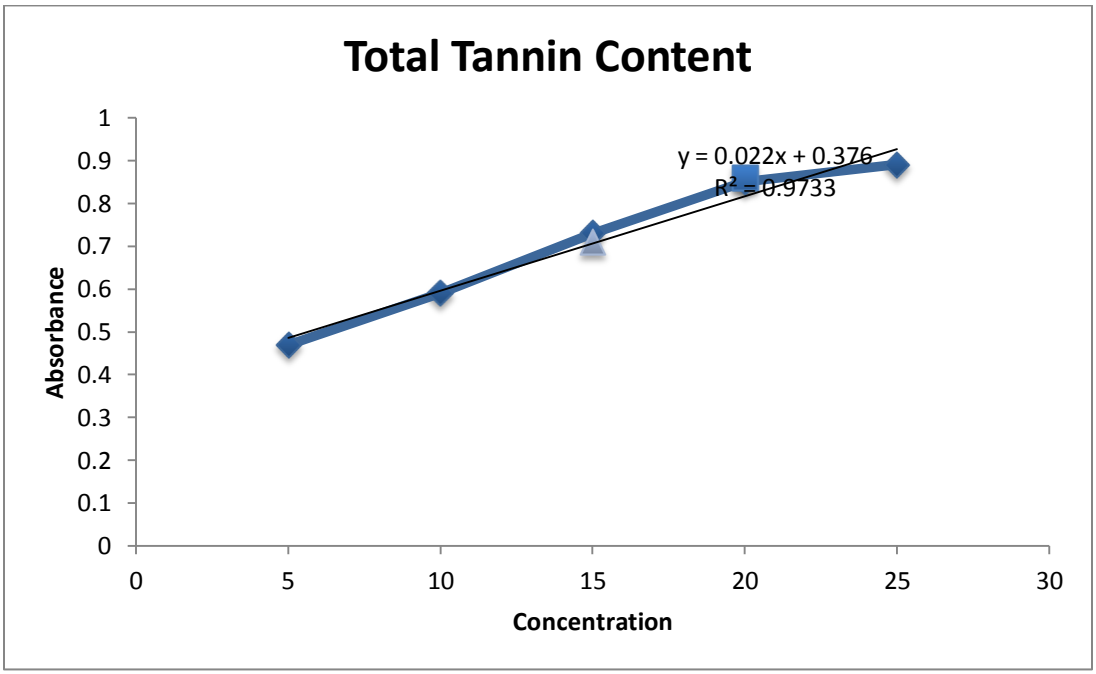


Fig. 23. Calibration curve of Total Tannin Content

The concentration of tannins present in ethyl acetate extract and ethanol were found to be 21.23µg/mg and 18.23 µg/mg .

Quantitative estimation of Flavonoid

Total Flavonoid content of bark of *Dalbergia sissoo* Roxb

Table 9 Quantitative estimation of Flavonoid

Concentration	Absorbance
2	0.06
4	0.10
6	0.13
8	0.15
10	0.19
Ethyl acetate	0.17
Ethanol	0.16

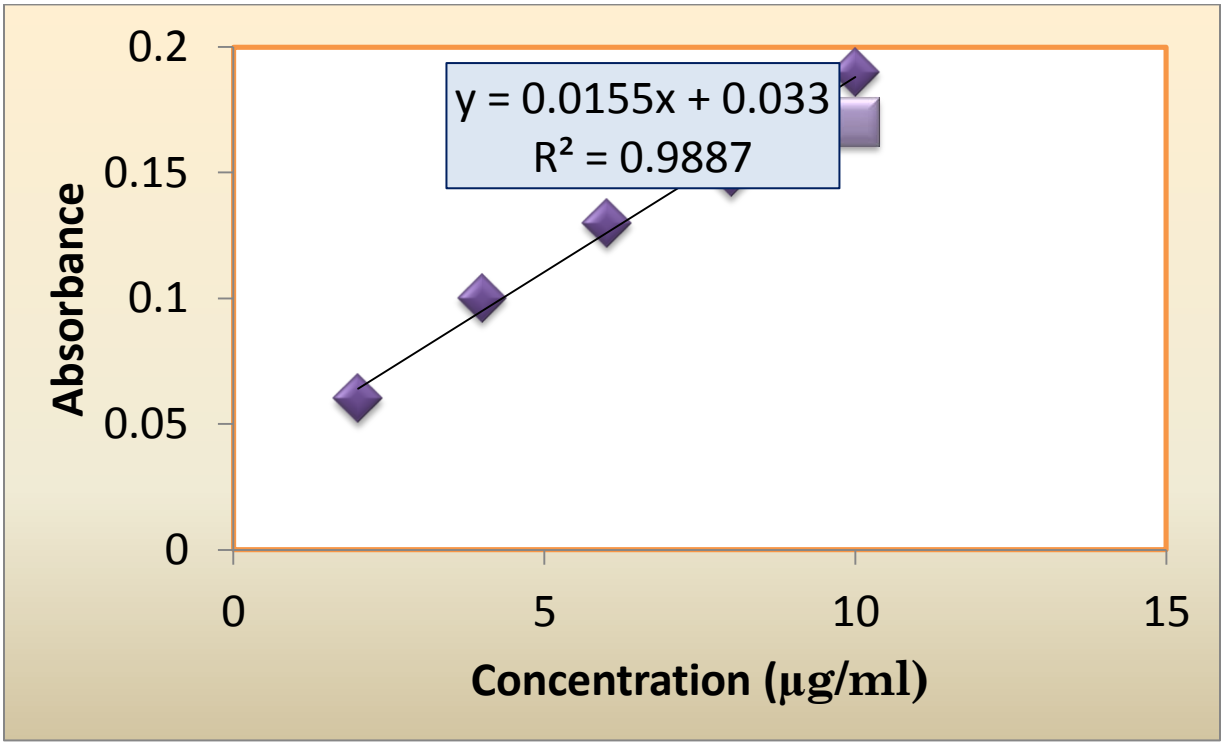


Fig. 24. Calibration curve of Total Flavonoid Content

The concentration of flavonoids present in ethyl acetate and ethanolic extract

was found to be 9.48µg/mg 9.16 µg/mg.

FLUORESCENCE ANALYSIS

The fluorescence analysis of powder drug and extract was reported and tabulated below:

Table 10.Fluorescence analysis of powdered drug of bark of *Dalbergia sissoo* Roxb.

S.NO	POWDERED DRUG	DAYLIGHT	SHORT UV 264nm	LONG UV 365 nm
1	Powder	Brown	Yellowish brown	Brown
2	Powder + Water	Brown	Brownish black	Purple
3	Powder + 1N HCl	Greenish brown	Brownish black	Dark brown
4	Powder + 1N H_2SO_4	Greenish brown	Brownish black	Dark brown
5	Powder + 1 N HNO_3	Yellowish brown	Greenish black	Black
6	Powder + CH_3COOH	Yellow	Pale green	Light brown
7	Powder + 1 N NaoH	Greenish brown	Brownish black	Dark brown
8	Powder + Alc.KOH	Light brown	Greenish brown	Brownish black
9	Powder + 1 N KOH	Light brown	Pale green	Dark brown
10	Powder + Alc.KOH	Light brown	Greenish brown	Brownish black
11	Powder + NH_3	Yellowish brown	Greenish brown	Brownish black
12	Powder + I_2	Dark brown	Greenish brown	Brownish black
13	Powder + $Fecl_3$	Yellow	Greenish brown	Dark brown
14	Powder + Ethanol	Light brown	Greenish yellow	Dark brown

Table 11.Fluorescence analysis of various extracts of *Dalbergia sissoo* Roxb.

S.NO	EXTRACTS	DAYLIGHT	SHORT UV	LONG UV
1	Hexane	Greenish black	Dark green	Greenish black
2	Ethyl acetate	Brown	Reddish brown	Reddish brown
3	Ethanol	Brown	Brownish yellow	Brownish yellow

THIN LAYER CHROMATOGRAPHY

The thin layer chromatography was analysed with the mixture of different mobile phase and its detection was done using UV are tabulated below.

TLC of Plant extract

Table 12.TLC Rf value for various extracts of Dalbergia sissoo Roxb.

S.NO	EXTRACTS	SOLVENT SYSTEM	No. Of spots	R_f VALUE
1	Ethyl acetate	Ethyl acetate : Formic acid : Glacial acetic acid : Water (100:11:11:26)	1	0.63
2	Ethanol		1	0.59

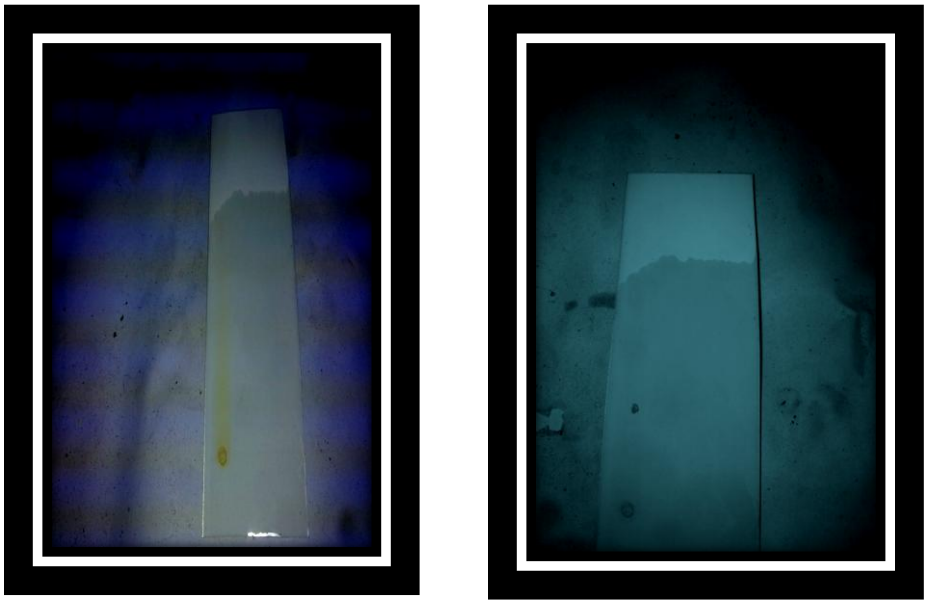


Fig. 25.TLC FOR FLAVONOIDS

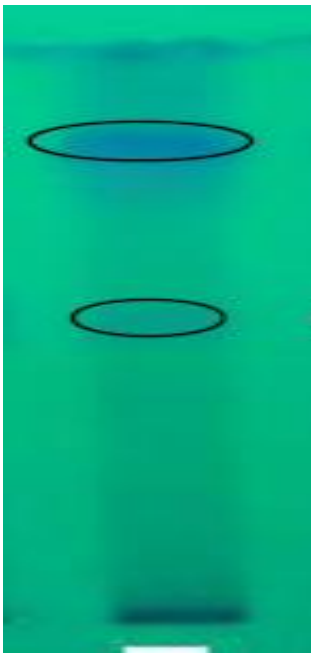


Fig. 26. TLC of ethyl acetate extract

HPTLC Finger Print Data of Ethyl acetate Extract of *Dalbergia sissoo* Roxb.

High Performance Thin Layer Chromatography (HPTLC) finger printing was performed with the ethyl acetate extract of Dalbergia sissoo Roxb. The Chromatographic conditions were carried as detailed in material and methods of this study. There were 10 peaks observed with different Rf values and different heights. Percentage of areas was also obtained from the chromatogram.

Table 13. **Rf values and area of ethyl acetate extract of *Dalbergia sissoo.***

S. No	Rf	Height	Area	Lambda max(nm)
1	0.02	53.2	1933.8	278
2	0.10	16.9	298.9	291
3	0.13	12.7	608.3	291
4	0.21	10.0	378.3	293
5	0.29	6.3	176.0	288
6	0.40	5.5	234.7	288
7	0.53	13.8	652.5	260
8	0.78	16.1	488.4	276
9	0.84	33.0	1060.7	270
10	0.94	2.5	54.9	280

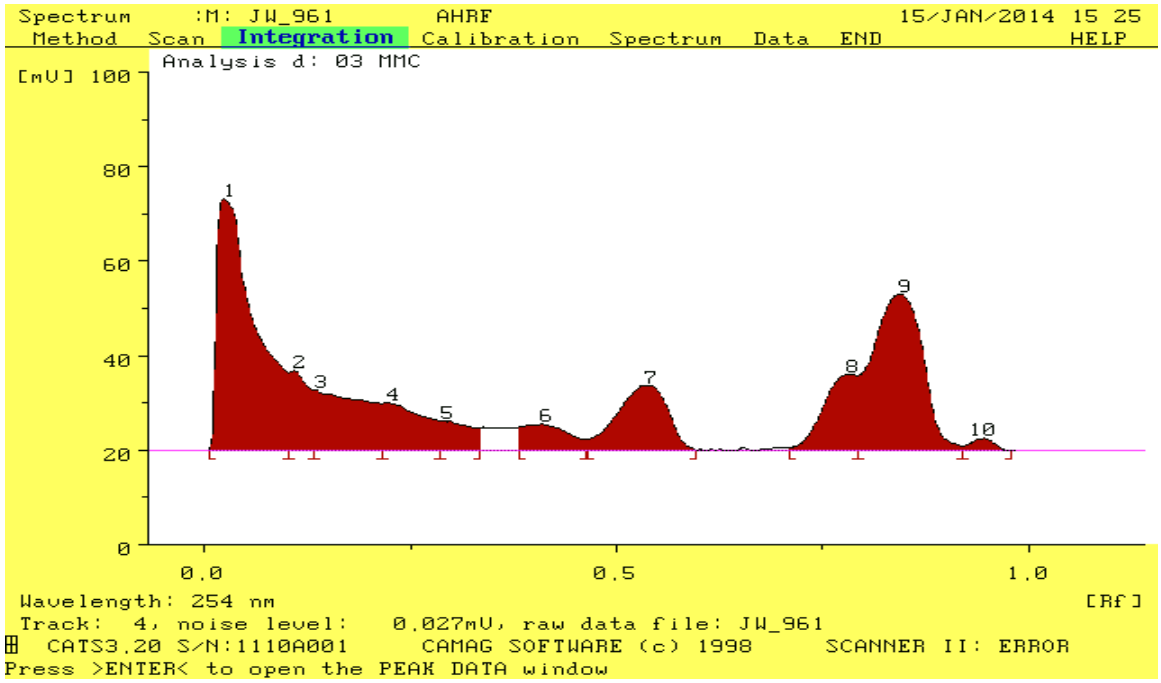


Fig. 27. HPTLC finger printing analysis of ethyl acetate extract

PHYTOCHEMICAL DISCUSSION

Since the herbal medicines are prepared from the plant origin in the form of infusion, decoction or extraction. In such cases they are prone to contamination which leads to deterioration of phytoconstituents and variation in chemical composition. Hence, before proceeding to pharmacological screening or clinical trials, the scientists or research scholars need a tool to authenticate the phyto constituents of herbal plants and to detect their potency. The number of analytical techniques was developed for quality control of crude drugs.

Here the phytochemical investigation plays a major part along with the biological screening to understand the therapeutic dynamics of herbal plants and also to develop the quality parameters. In this study , the different polarity of phyto constituents were extracted from the coarsely powdered bark of Dalbergia sissoo Roxb by using solvents of increasing polarity such as Hexane, Ethyl acetate, and Ethanol by continuous hot percolation method using soxhlet apparatus.

Successive extractive values revealed the solubility and polarity particulars of the metabolites in the powdered drug. The percentage yield of various extracts were given in Table 5. Hexane: Ethyl acetate, Ethanol . Ethyl acetate showed a high percentage yield when compared to other extracts.

Qualitative preliminary phytochemical analysis was performed initially with different chemical reagent to detect the presence of phytoconstituents in each extract and powder. Hexane extract showed the presence of triterpenoids. Ethyl acetate extract showed the presence of Flavonoid, Glycosides, Phytosterols, Phenolic compounds, saponin and tannins. Ethanol showed the presence of Flavonoid, Glycosides, Phytosterols, Phenolic compounds , Saponin and Tannin.

Quantitative estimation of Tannin, Phenolic and Flavonoid content were carried out for Ethyl acetate and Ethanol extracts. The amount of Phenolic content present in Ethyl acetate 74.65 µg/mgwhereas ethanol showed 61.18 µg/mg. The amount of flavonoid content present in Ethyl acetate 9.48 µg/mg whereas ethanol showed 9.16 µg/mg. The amount of tannin content present in Ethyl acetate 21.23 µg/mg whereas ethanol showed 18.23µg/mg.

Fluorescence analysis is an important qualitative diagnostic tool to detect the presence of chromophore in crude powdered drug and extract under UV and day light.

Qualitative chromatographic analysis of these extracts using thin layer chromatography method was performed to separate and identify the single or mixture of phytoconstituents in each extract. The following solvent system were used to separate the phytoconstituents by using mobile phase Ethyl acetate : Formic acid : Glacial acetic acid : Water (100:11:11:26) .

High performance thin layer chromatography fingerprinting was performed with the Ethylacetate extract of the bark *Dalbergia sissoo*. There were 10 peaks observed with different Rf values and different heights. Percentage of areas was also obtained from the chromatogram.

PHARMACOLOGICAL STUDIES

In-vitro Cytotoxicity study by MTT assay

The Cytotoxicity study by MTT assay performed. The results are tabulated below.

In-vitro Cytotoxicity study by MTT assay in B16F10 melanoma cell line.

Table 14.*In-vitro* Cytotoxicity assay

S.NO	Conc. (µg/ml)	Cytotoxicity study of standard(Kojic acid),and Plant extracts (Hexane, Ethyl acetate, Ethanol)							
		Standard		Hexane		Ethyl acetate		Ethanol	
		Abs (O.D)	% Cell Viability	Abs (O.D)	% Cell Viability	Abs (O.D)	% Cell Viability	Abs (O.D)	%Cell Viability
1	1000	0.10	18.52	0.12	22.22	0.09	16.66	0.10	20.37
2	500	0.13	25.92	0.17	31.48	0.21	38.88	0.17	33.33
3	250	0.21	40.74	0.22	40.74	0.25	46.29	0.23	44.44
4	125	0.28	53.70	0.26	48.14	0.28	51.85	0.27	50
5	62.5	0.33	62.96	0.31	57.40	0.35	64.81	0.29	55.55
6	31.2	0.38	72.22	0.33	61.11	0.39	74.07	0.38	72.22
7	15.6	0.43	81.48	0.38	70.37	0.43	81.48	0.42	79.62
8	7.8	0.49	92.59	0.43	81.48	0.48	90.74	0.45	85.18
9	Cell control	0.54	100	0.54	100	0.54	100	0.54	100

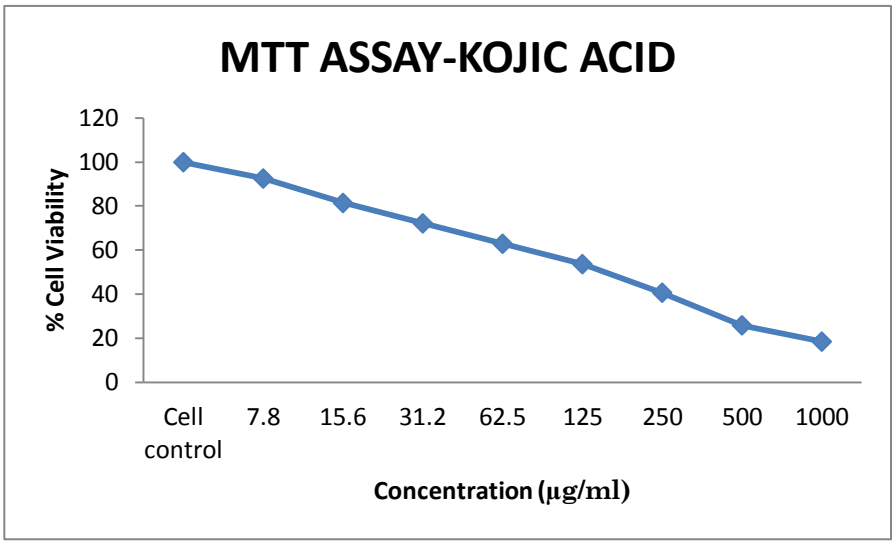


Fig. 28.Cytotoxicity effect of Kojic acid on B16F10 melanoma cell line

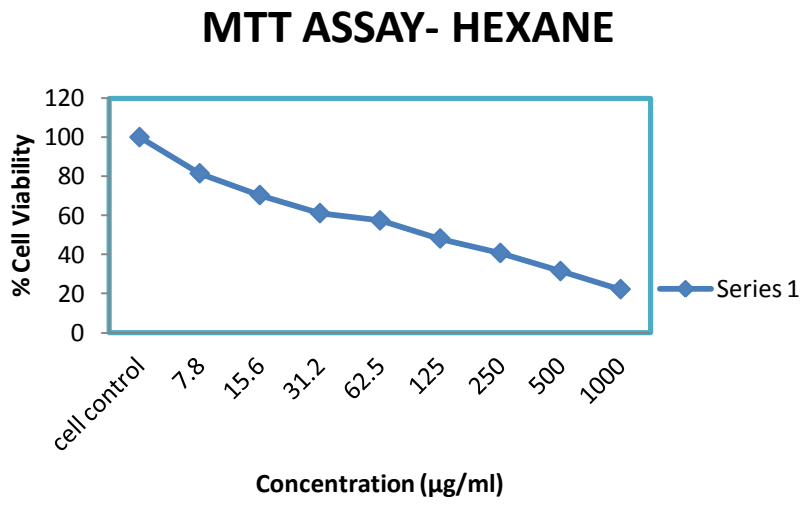


Fig.29.Cytotoxicity effect of hexane on B16F10 melanoma cell line

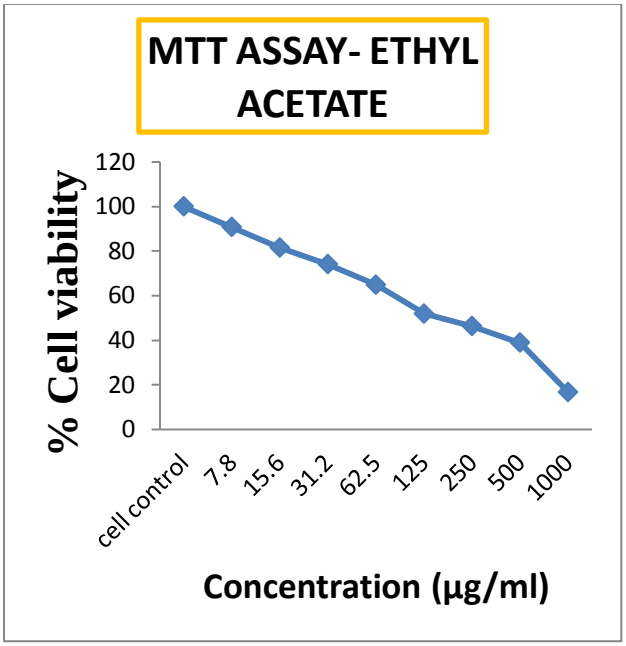


Fig.30.Cytotoxicity effect of ethylacetate on B16F10 melanoma cell line

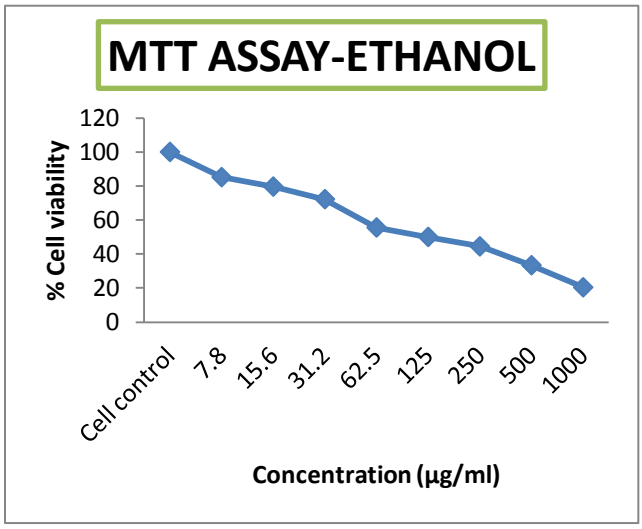


Fig.31.Cytotoxicity effect of ethanol on B16F10 melanoma cell line

Table.15. Effects of various extracts on melanin content production in B16F10 melanoma cell line:

Concentration	Hexane	Ethyl acetate	Ethanol
Control	0.15	0.15	0.15
Vehicle	0.1	0.1	0.1
Standard	0.31		
0.01	0.056	0.058	0.058
0.03	0.072	0.079	0.076
0.1	0.098	0.114	0.103
0.3	0.146	0.163	0.159
1	0.24	0.26	0.26
3	0.29	0.39	0.32

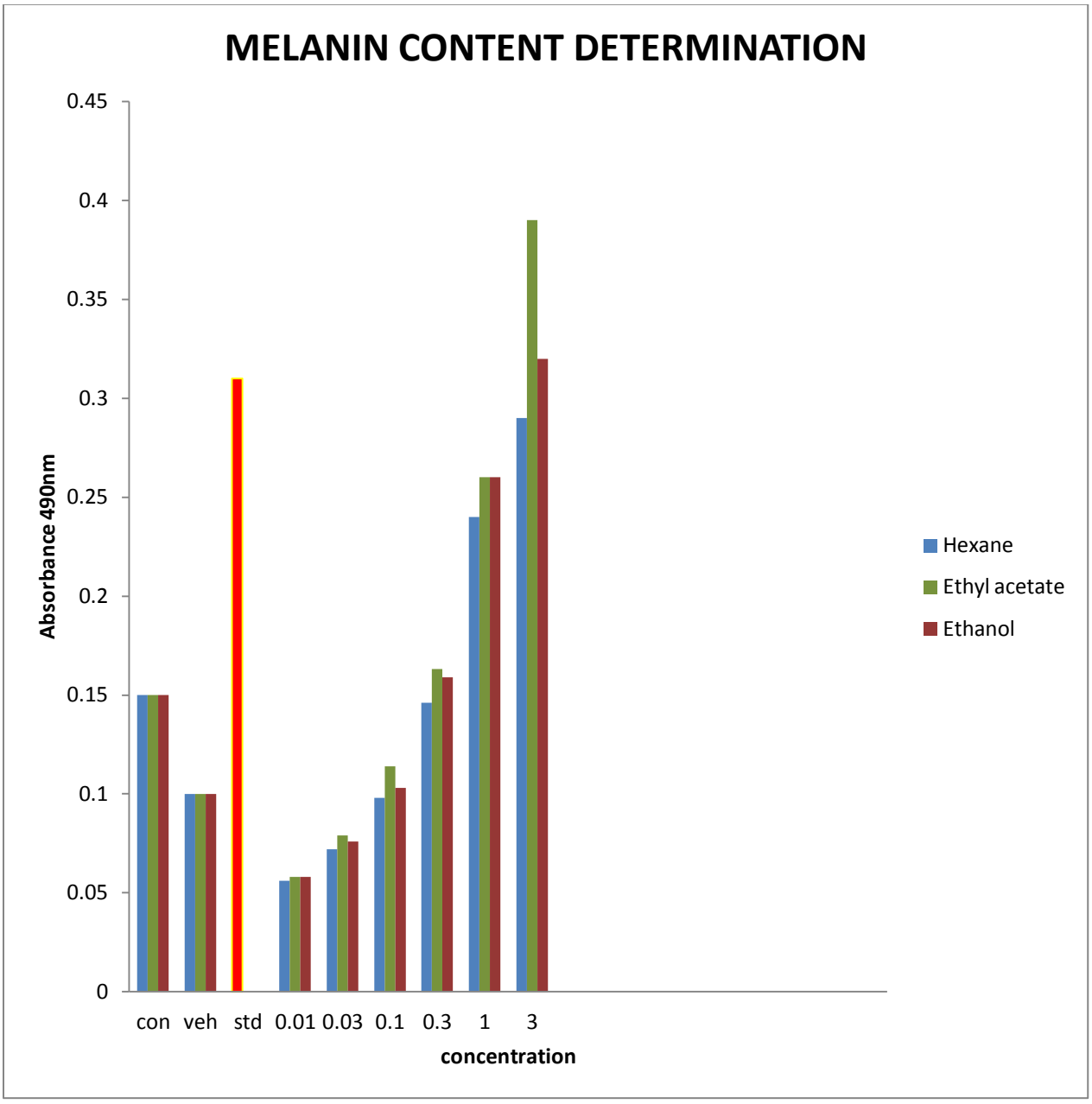


Fig. 32. Graphical representation of bark extracts of *Dalbergia sissoo* on melanin content in B16F10 melanoma cell line

IN-VITRO STUDY DISCUSSION

Cytotoxicity study by MTT Assay:

The Cytotoxicity assay was carried out by MTT assay method in B16F10melanoma cell line for Kojic acid and the bark extracts of Dalbergia sissoo Roxb. These compounds were screened for its Cytotoxicity assay (i.e.,50% cell viability).

The Cytotoxicity effect of plant extracts and standard was assessed by cell viability. The cell viability was found to be increased with decreasing in concentration of test compounds. Results are tabulated (Table 14) and graphically represented.

Among these test compounds, the ethyl acetate showed a high percentage of cell viability when compared to Hexane and Ethanol

Determination of melanin content in melanocyte:

Melanogenic activity in cultured murine B16F10 melanoama cells is directly related to the quantity of produced melanin in which is estimated through the amount of melanin retained in the cells. All extracts increased melanin formation. Since melanin content was found to be enhanced in response to several concentrations of Hexane, Ethyl acetate, Ethanolic extracts of bark of Dalbergia sissoo(0.01 to 3µg/ml). Among these test extracts, the ethyl acetate showed a high amount of melanin content production when compared to Hexane and Ethanol.

REDUCING POWER ASSAY

Table 16.Reducing power assay

S.No	Conc (µg/ml)	% Inhibtion		
		Standard	Ethylacetate	Ethanol
1	10	14.12	13.94	10.04
2	20	26.56	24.54	22.12
3	30	42.13	40.03	33.13
4	40	60.23	56.39	53.45.
5	50	80.12	76.14	73.89

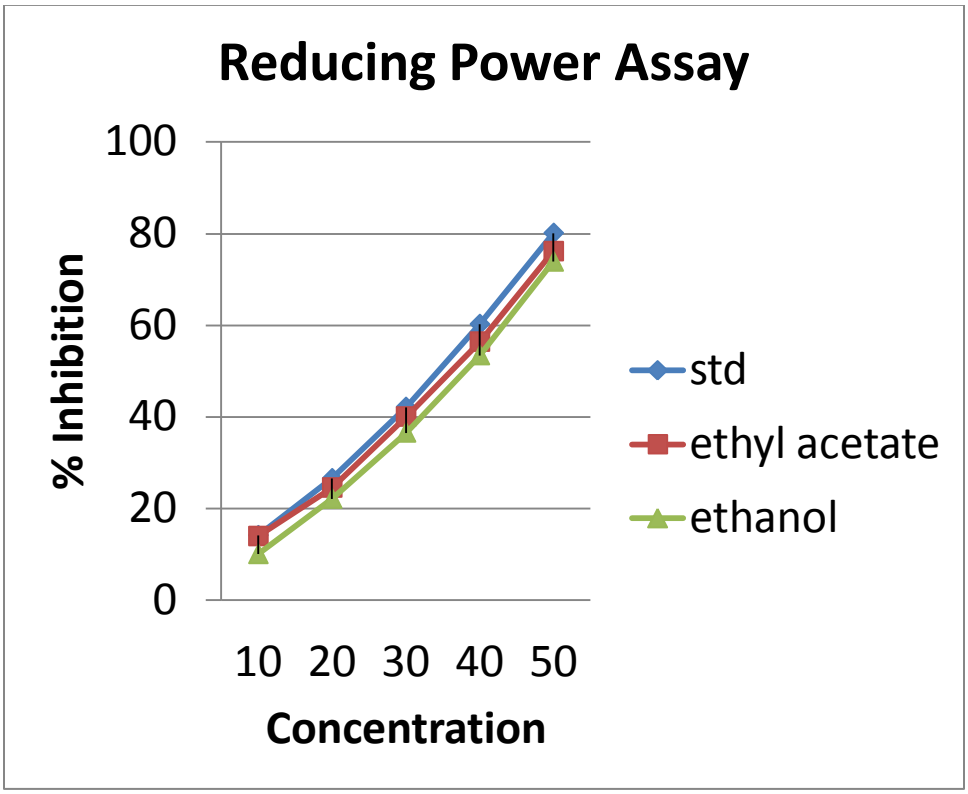


Fig. 33. Reducing power assay

Since the IC50 value of ethyl acetate extract is nearer to that of the standard than the ethanol extract, It is known that ethyl acetate extract has higher antioxidant activity than ethanol.

Hydrogen peroxide scavenging method

Table 17.Hydrogen peroxide scavenging assay

S.No	Conc (µg/ml)	% Inhibition		
		Std(Ascorbic acid)	Ethyl acetate	Ethanol
1	10	13.34	13.24	12.62
2	20	26.44	22.76	20.07
3	30	48.91	47.72	46.42
4	40	59.19	58.65	57.40.
5	50	71.25	70.02	62.29

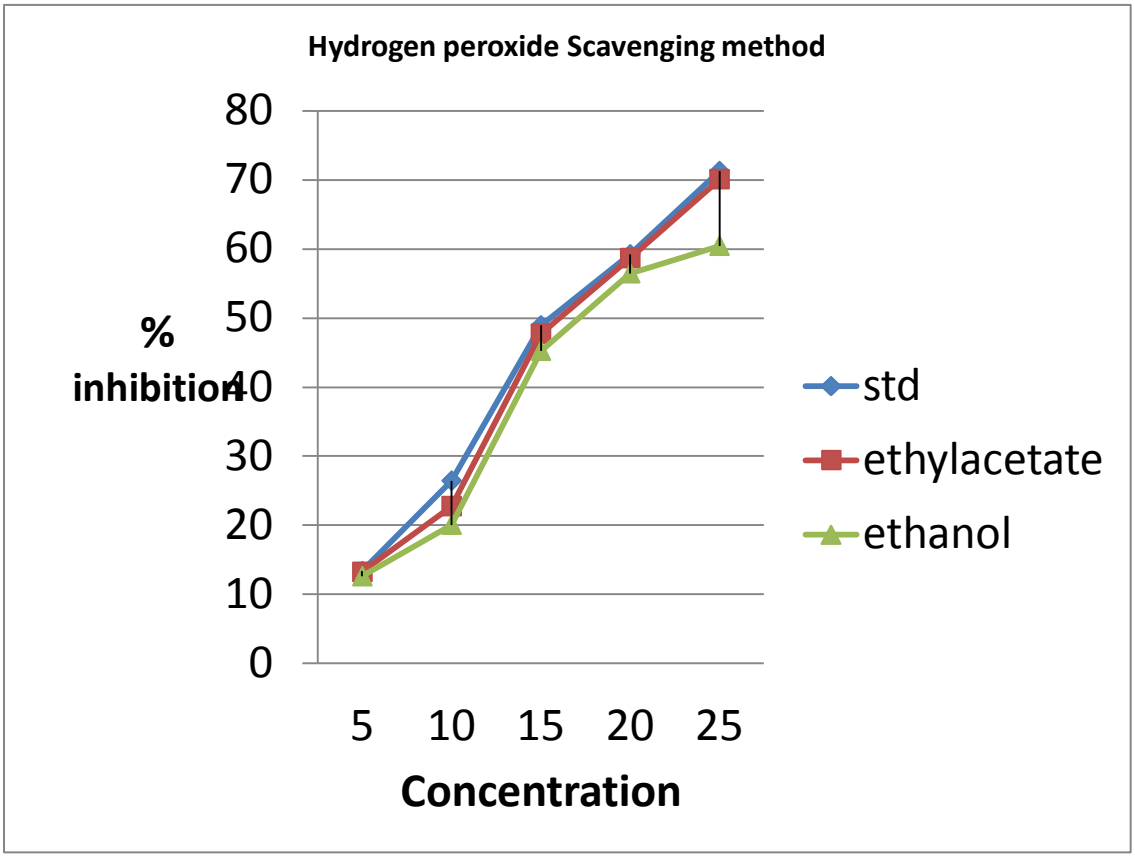


Fig. 34. Hydrogen peroxide scavenging assay

Since the IC50 value of ethyl acetate extract is nearer to that of the standard than the ethanol extract. It is known that ethyl acetate extract has higher antioxidant activity than ethanol.

EVALUATION OF GEL

Table 18.Extrudability of the herbal gel at the time of preparation (Mean± SEM).

Extrudability	**Mean of three tubes (Initial month)**
Net wt of formulation tube (g)	12.33± 0.01
Wt. of gel extruded (g)	11.15±0.011
Extrudability amount percentage	90.34±0.07

Table.19. Spreadability of the Herbal gel during the evaluation period (Mean ± SEM)

EVALUATION CONDITION	**SPREDABILITY (g.cm/sec)**
Initial at 40˚C ±2˚C /75±5% RH	26.37±0.12
At 1month at 40˚C ±2˚C /75±5% RH	26.35±0.32
At 2month at 40˚C ±2˚C /75±5% RH	26.34±0.32
At 3month at 40˚C ±2˚C /75±5% RH	26.29±0.32

Table 20 .Viscosity of the Herbal gel during evaluation period

Evaluation month	**Viscosity (cps)**
Initial	17000
At 1 month	17000
At 2 month	17000
At 3month	16500

The herbal gel was prepared and subjected to evaluation of the various parameters. The herbal gel was brownish yellow in color and translucent in appearance and had a smooth feeling on application. pH also maintained constant throughout the study which was found to be 6.5 to 6.8 and the gel was non-irritant upon application on the skin.

Spreadability was also measured and found to be less variant than the initially prepared gel after performing stability study. Extrudability was excellent after performing stability studies from that of the initially prepared gel. The initial viscosities were recorded at 25°C. Furthermore, the stability study's results revealed the preparation was stable under normal storage conditions.

DRAIZE SKIN IRRITATION STUDY

Table.21. Draize Skin Irritation Study

Name of the Group	Animal numbers	Grading	
Group I		7^{th} day	14^{th} day
	1	0	0
	2	0	0
	3	0	0
Group II	1	0	0
	2	0	0
	3	0	0

0-No erythema 1-Minimally Perceptible Erythema

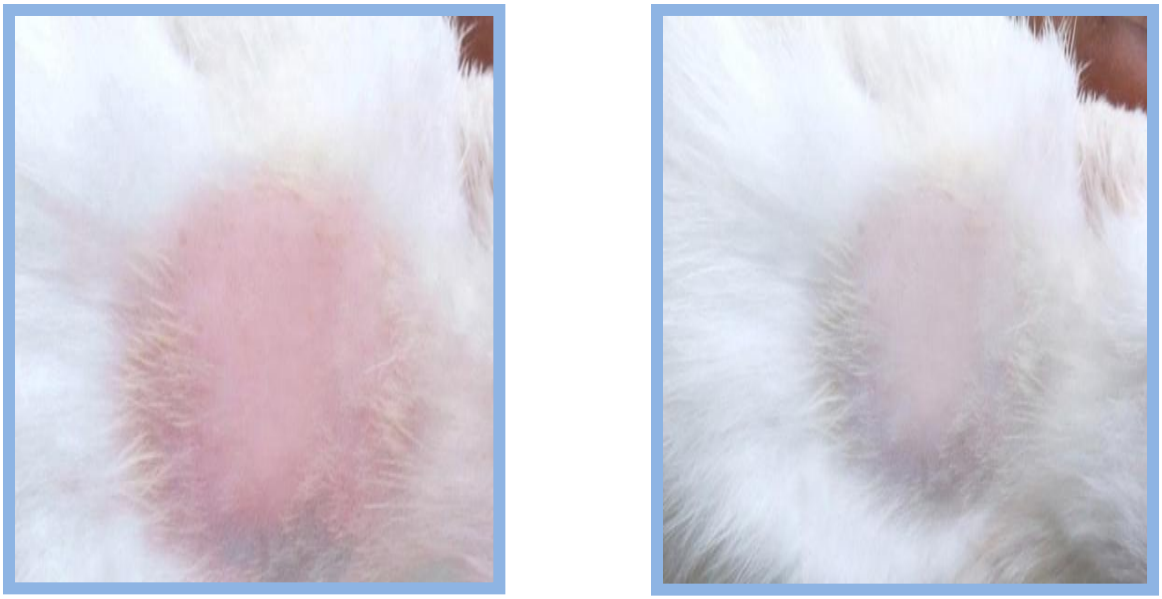
2-Marked erythema 3-Fiery red erythema with edema

Group I

7th day 14th day

Fig.35..DRAIZE SKIN IRRAITATION TEST IN GROUP I

Group II



7th day 14thday

Fig.36..DRAIZESKIN IRRAITATION TEST IN GROUP II

The Draize skin test sensitivity showed that there was no sign of irritation such as redness or erythema indicating that the gel was no irritant.

FIG.37.CONTROL (GROUP I)

Normal black mice

After induction of leucoderma

FIG.38.GROUP II (Treated with standard)

Pigmentation at 1st week

Pigmentation at 2nd week

Pigmentation at 3rd week

Pigmentation at 4th week

FIG.39.GROUP III(Treated with test extract into Gel)

Pigmentation at 1st week

Pigmentation at 2nd week

Pigmentation at 3rd week

Pigmentation at 4th week

Pigmentation at 5th week

The repigmentation developed gradually and complete repigmentation occured at 4^{th} week for standard and 5^{th} week for test extract into gel.

SUMMARY AND CONCLUSION

The dissertation entitled "**Pharmacognostical, Phytochemical and antileucodermic studies on the bark of *Dalbergia sissoo* Roxb**." deals with macroscopic and microscopic features as well as phytochemical and pharmacological aspects of *Dalbergia sissoo*.

In the present work, a medicinally useful plant in folklore claim and in the Indian system of medicine, *Dalbergia sissoo* Roxb was selected.

The literature survey showed that very little work has been done on *Dalbergia sissoo.*

The Macroscopical description of the bark showed that bark are rough and hard with splintery fracture, 0.5-1cm thickness, odour less, bitter taste, flat in shape, brown colour.

The anatomical examination of *Dalbergia sissoo* Roxb, bark exhibited important microscopical characters such as cork, phloem, sclerides, phelloderm, starch grains, calcium oxalate crystals.

Powder microscopy revealed the presence of Cork cells with lignified walls, Lignified sclerides, Phloem parenchyma ,Parenchyma cells showing tanniferous content, Unlignified fibers, Prismatic calcium oxalate crystals.

Determination of Physico-Chemical constants such as Ash values, Extractive values, Loss on drying, Foaming index, Swelling index, Qualitative and Quantitative estimation of heavy metals and inorganic elements was carried out and the percentage composition of different inorganic elements were determined.

Physicochemical constants were evaluated for the bark of Dalbergia sissoo. The total ash, Alcohol soluble ash and water soluble extractive, ethanol soluble extractive value complies with Ayurvedic Pharmacopeia of India. This helps in confirming the identity and purity of this plant to detect adulterants, their nature.

Qualitative estimation of inorganic elements was carried out and this shows the absence of toxic metals and presence of useful elements which could be responsible to its medicinal activity.

In Phytochemical screening, successive solvent extraction was carried out with Hexane, ethyl acetate and Ethanol solvents. Ethyl acetate extract of bark showed maximum yield of 7.75% W/W.

Preliminary Phytochemical analysis aided in identifying the phytoconstituents present in different extracts showed the presence of flavonoids, tannin, phenols, saponins, glycosides.

Quantitative estimation Total Phenolic, Flavonoid & tannin content was carried out. Fluorescence analysis was carried out to detect the fluorescent chromophores present in the extract, no fluorescence was observed.

Thin layer chromatography was carried out for ethyl acetate and ethanol extracts for flavonoids. The ethyl acetate extract of bark showed one spot for flavonoids and ethanol extract of bark showed one spot.

High performance thin layer chromatography –Finger print Profile was carried out to detect the number of phytoconstituents present in ethyl acetate extracts of bark. The HPTLC chromatogram of the ethyl acetate extract shows 10 peaks. This indicates ethyl acetate extract of bark consist of 10 different phytoconstituents. Some of these phytoconstituents may be responsible for antileucodermic activity.

The antioxidant activity was carried out by **H_2O_2 scavenging method** and **Reducing power ability assay.** The IC_{50} value of the **ethyl acetate extract** in both the methods was found to be almost equivalent to that of the standard drug Ascorbic acid and showed the most potent antioxidant activity compared to the other two extracts.

Melanogenic activity in cultured murine B16F10 melanoama cells was directly related to the quantity of produced melanin in which is estimated through the amount of melanin retained in the cells. All extracts increased melanin formation. Since melanin content was found to be enhanced in response to several concentrations of Hexane, Ethyl acetate, Ethanolic extracts of bark of *Dalbergia sissoo*(0.01 to 3µg/ml). Among these test extracts, the ethyl acetate showed a high amount of melanin content production when compared to Hexane and Ethanol.

The best extract from the cell line study was formulated into gel. The gel was formulated and was subjected to accelerated stability studies.

The skin irritation and *in vivo* studies of gel has proved that GEL having reduced side effects to a greater extent and quick repigmentation effect when compared with the conventional therapy**.**

This study substantiates our hypothesis that *Dalbergia sissoo* may be a useful antileucodermic plant. Further studies on isolation of the phytoconstituents responsible for the activity are suggested.

This research work presents a new approach for the preparation of gel.

The prepared gel exhibited characteristics suitable for formulating as gel, an ideal delivery system for topical application.

Future scope

- Melanin estimation by suitable techniques.
- Histopathological studies.
- Clinical Evaluation.
- Isolation of active phytoconstituents responsible for activity.

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