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Quantitative Estimation of Bioactive Compounds of 5 commercially important medicinal plants through Chemofingerprinting (HPLC) for the identification of quality planting material

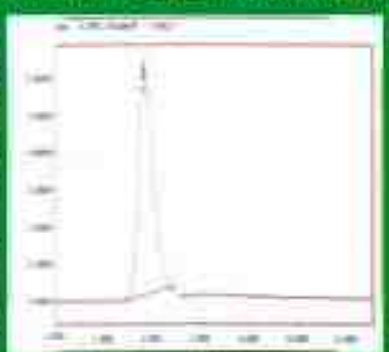
Litsea glutinosa (Lour.) C.B.Rob - **Laurotetanine**

Berberis aristata **DC - Berberine**

Commiphora wightii (Arn.) Bhandari - **Guggulsterone (E&Z)**

Embelia tsjerium-cottom - A.DC - **Embelin**

Saraca asoca (Roxb. Willd) - **Gallic acid**



HPLC Chromatogram

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INDEX

CHAPTER-1

Introduction

The bark and seeds of some medicinal plants contain principal bioactive compounds particularly shrub and tree species. The bark and seeds of such species are commonly used in the formulation of herbal drugs to cure several diseases. Due to unsustainable harvesting of the bark and seeds, large number of such medicinal plants species are disappearing rapidly from their natural habitat. The classical examples of such medicinal plant species are Kullu, Maida, Daruhaldi, Arjun, Salai, Guggal, Baibidang, etc.

The detailed introductory part about medicinal plants, alkaloids, chemo-fingerprinting, techniques of HPLC are already described in SFRI technical bulletin number 76 of series -I for whole plant parts-2017. In this technical bulletin the chemofingerprinting technique is mainly focused on quantitative estimation of bioactive compounds from bark and seeds of 5 commercially important medicinal plants.

About the species

1. Litsea glutinosa (Lour.) C.B.Rob

Local name- Maida, Maidachhal

Litsea glutinosa chinensis (Maida) family Lauraceae is an critically endangered evergreen tree. According to Ayurveda, the bark is useful in treatment of burning sensation. The traditional healers are aware of its unique medicinal properties and uses. Maida alone or in combination is used for treatment of joint pains. The bark of this species plays a key role for the survival of the agarbatti (incense stick) industry in India. Powdered bark of *L. glutinosa* known as IIGAT in trade, functions as an adhesive or binder in agarbatti manufacture. When mixed with water, it forms an ideal material to bind wood charcoal, aromatic roots and herbs to the bamboo splint. Due to increased demand of bark by Agarbatti industry, the trees are completely stripped for the extraction of bark.

Habitat -

Litsea is found throughout in India and outer Himalayas. It grows in moist climatic conditions. In Madhya Pradesh, it is found in some patches of Amarkantak, Balaghat, Dindori, Pachmari, Rewa, Shahdol and Satna forest Division.

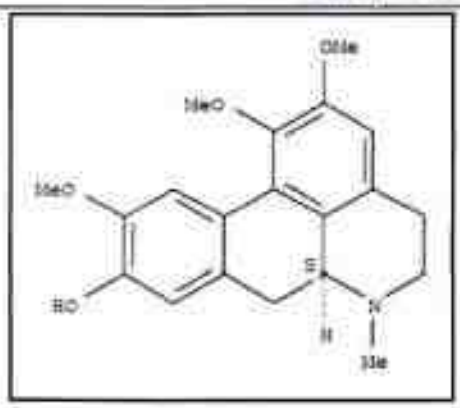
Botanical description -

It is an evergreen tree upto 25 m in height and 60cm in girth. Bark is brownish grey, somewhat corcky, Leaves are variable in size and shape, elliptic ovate or oblong, pubescent and aromatic.

A view of *Litsea glutinosa*

Phytochemistry -

Litsea contains several chemical constituents in different plant parts. Leaves of this species contains alkaloids & volatile oils and seeds also contains aromatic oils which are used in making soaps & cosmatics. Bark of the tree is a rich source of laurotetanine which is a tannic acid derivative. Tannin, β-sitosterol and actinodaphnine are the common constituents of *L. glutinosa*. Other constituents are boldine, norboldine, N-methyllaurotetanine, N-methylactinodaphnine, quercetin, sebiferine, litseferine, etc. Seeds of this plant contains nearly 20 percent fatty oil.



Molecular structure of Laurotetanine

Medicinal Uses -

- It is used to cure inflammations, bruises and boils.
- The bark and the leaves are more frequently used than the fruit.
- The bark is one of the best known and most popular of native drugs and to be capable of relieving pain, arousing sexual power and producing a soothing effect on the body.
- Bark is also considered to be mildly astringent.
- The mashed bark (fresh), which is mucilaginous or sticky, is applied on wounds and bruises. If not fresh, the dried bark is powdered and turned into a paste by adding some water for application on the body and wound by an iron axe would work as an antiseptic. This paste is also applied as plaster on broken limbs. It is used as a medicine or sometimes, as an adhesive that would keep other medicinal ingredients attached to the affected part of the body.
- The bark is largely consumed in making agarbatties.

2. *Berberis aristata* DC.

Local name- Daruhaldi

Berberis aristata commonly known as 'Indian Barberry' is a critically endangered medicinal plant. Locally it is also known as 'Daru haldi'. It belongs to the family Barberideceace. The genus consists of 500 species. It is an erect spiny shrub ranging from 2 to 3 meter in height. Stem wood is hard and brownish from outside

and yellowish from inside. The stem bark is covered with three-branched thorns, which are modified forms of the leaves. The stem, roots and fruits of *B.aristata* has been used traditionally in ayurvedic treatments of several ailments such as ENT infections, wound healing, dysentery, indigestion, uterine and vaginal disorders, anti-inflammatory and immune potentiating property. It is also useful for diabetes, fever, jaundice, stomach disorders, malarial fever, skin disease and used as tonic. Phytochemical studies showed that *Berberis aristata* contains a yellow coloured alkaloid 'Berberine', which is highly potent for the medicinal properties and is mainly found in its stem bark. According to the International Union for Conservation of Nature and Natural Resources (IUCN), the *Berberis aristata* is declared as critically endangered medicinal plant.

Habitat -

Berberis aristata is a herb, native to Himalayas in India. It is also widely distributed in wet zone of Sri Lanka and also found in temperate and sub-tropical regions of Asia, Europe and America, Bhutan and hilly areas of Nepal. In Himachal Pradesh it is mainly found especially in Kumaon & Chamba region and also widely distributed in Nilgris hills in South India. However, in Madhya Pradesh it also found in Central Narmada Valley agro-climatic region of Madhya Pradesh. Particularly in satpuda Tiger Resevce (Pachmarhi)

Botanical description -

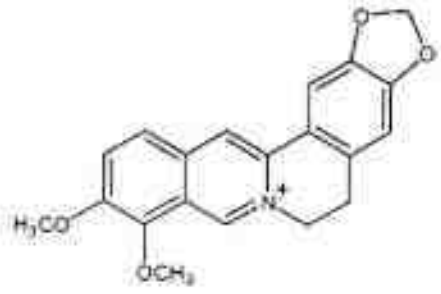
The leaves are arranged in tufts of 5-8 and are approximately 4.9 cm long and 1.8 cm broad. The leaves are deep green on the dorsal surface and light green on the ventral surface. The leaves are simple with pinnate venation. The leaves are leathery in texture and are toothed, with several too many small indentations along the margin of the leaf. The yellow flowers are 1.2 cm long in a racemose inflorescence, with 11-16 flowers per raceme, arranged along a central stem. The flower is polysepalous, with 3 large and 3 small sepals, and polypetalous, with 6 petals in total. Flowers have 6 stamens, 5-6 mm long. The plant produces bunches of succulent, acidic, edible berries that are bright red in colour and have medicinal properties. The berries are approximately 7 mm long, 4 mm in diameter and about 227 mg in weight.

A view of *Berberis aristata*

Phyto-chemistry -

B. aristata contains a valuable isoquinoline alkaloid berberine and berbamine. Berberine, hydrochloride, an alkaloid isolated from *Berberis aristata*, is found to have significant anti-inflammatory activity on acute, subacute and chronic types of inflammations produced by immunological and non-immunological methods.

Berberine, a traditional plant alkaloid, is used in Ayurvedic and Chinese medicine for its antimicrobial and anti-protozoal properties. Interestingly, current clinical research on berberine has revealed its various pharmacological properties and multi-spectrum therapeutic applications.



Molecular structure of berberine

Medicinal uses -

- Paste of this herb is used externally to reduce pain and inflammation and is in eye related disorders.
- It is also used for quick healing of wounds. The paste of this herb is also useful in syphilis ulcers and anal fistula.
- It helps to cure spasmodic pain and it is very beneficial for patients of menorrhagia and leucorrhoea.
- Roots of this plant are used in jaundice and liver toxicity.
- It is also anti-diabetic in nature. Root powder of this herb triggers the extra formation of insulin and protect body from harmful effects of diabetes.
- The bark is very effective herb with anticancer properties which fights against human colon cancer.
- This herb fights against the various infections caused by microbes in both males and females. In females it is also used to cure uterine infections.
- This herb helps to stimulate sweating malaria and in intermittent fever.
- Fruit of this herb is appetizer, helps to stimulate digestive fire and it is very effective in patients of dysentery.
- Leaves of this herb are used in the form of gargles in throat infections and to improve voice.

3. *Commiphora wightii* (Arn.) Bhandari Synonym *Commiphora mukul* (Stocks) Hook.

Common name: Guggal.

Commiphora wightii is an endangered medicinal plant of herbal heritage of India belongs to family Burseraceae. In Indian language, it is known by various names like guggul in Hindi, gukkulu and maishakshi in Tamil, guggulu in Sanskrit and Indian bdellium in English.

Habitat -

This plant is critically endangered and distributed in arid areas of India, Bangladesh, Afghanistan, Sri Lanka and Pakistan. In India it is found in arid, rocky tracts of Rajasthan, Gujarat and northern parts Madhya Pradesh.

Botanical description -

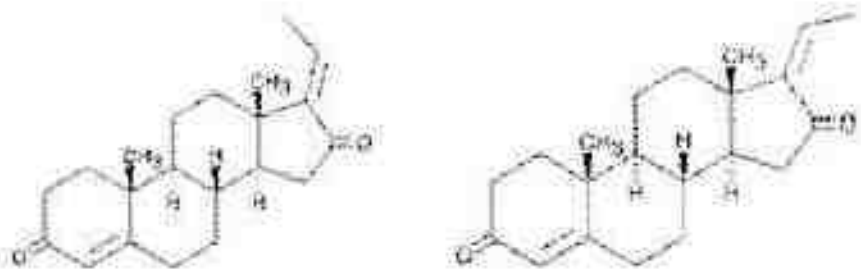
Commiphora wightii is a small tree or shrub. It is a slow growing plant and takes 8 to 10 years to reach to a height of 3 to 4 meters. The plant is dimorphic, one having male flowers and the other having female flowers. The fruits are green berry like drupe. Size of the fruit varies from 6 to 8 mm in diameter. Fruit parts exposed to sun develop pinkish tinge. Fruits remain on the plant for several months. Seeds show polyembryonic nature.

Phyto-chemistry -

The use of plants in the treatment of diseases occupies an important place in Ayurveda, the traditional medicine system of India. The Atharva Veda, one of the four well known Holy Scriptures (Vedas) of the Hindus, is the earliest reference to the medicinal and therapeutic properties of guggul. Sushruta Samhita (600 B.C.), a well-known Ayurvedic medical text, describes the usefulness of the gum resin from the tree *C. wightii* in the treatment of a number of ailments, including obesity and disorders of lipid metabolism. The plant *C. wightii* provides oleogum resin mentioned by Sushruta (3000 year ago) as being a valuable drug. The oleo-gum resin commonly known as "gum guggul" or "Indian myrrh" is the economically important product of Indian bdellium. The oleo-gum is collected as exudates from woody stem.

Guggulsterone is a plant steroid found in the resin of *Commiphora wighttii*. Guggulsterone can exist as either of two stereoisomers, E-guggulsterone and Z-guggulsterone. In humans, it acts as an antagonist of the farnesoid X receptor, which was once believed to result in decreased cholesterol synthesis in the liver. Several studies have been published that indicate no overall reduction in total cholesterol occurs using various dosages of guggulsterone and levels of low-density lipoprotein ("bad cholesterol") increased in many people. Z-guggulsterone, a constituent of

Indian Ayurvedic medicinal plant *Commiphora mukul,* inhibits the growth of human prostate cancer cells by causing apoptosis. The ring structure of Guggulsterone E&Z is presented below.



Molecular structure of

(E) - Guggulsterone **(Z) - Guggulsterone**

The plant generally takes 5 to 6 years to reach tapping maturity under the dry climatic conditions. The thick branches are incised during the winter to extract the oleo-gum resin. Guggul gum is a mixture of 61% resins and 29.3% gum, in addition to 6.1% water, 0.6% volatile oil and 3.2% foreign matter. Unfortunately the plant *Commiphora wightii* has become endangered because of its slow growing nature, poor seed setting, lack of cultivation, poor seed germination rate and excessive unscientific tapping for its gum resin by the pharmaceutical industries and religious prophets. This plant is incorporated in Data Deficient category of IUCN's Red Data list.

Medicinal uses -

- Obesity and disorders of lipid metabolism.
- Gum used in agarbatti industries.
- Skin diseases.
- Control cloistral.
- Inhibits the growth of human prostate cancer cells by causing apoptosis.

4. *Embelia tsjeriam-cottam* A.DC -

Common Name - Baibrang

Embelia is a shrub commonly known as babrang or baibidang belongs to family Myrsinaceae.

Habitat -

It is found in peninsular India, particularly on the Malabar region. It is also found in other parts of the country such as Chhattisgarh, Karnataka, Maharashtra, Kerala, Tamil Nadu, Andhra Pradesh and Himalayas from Kashmir to Sikkim, at altitudes of 400-1600 m.

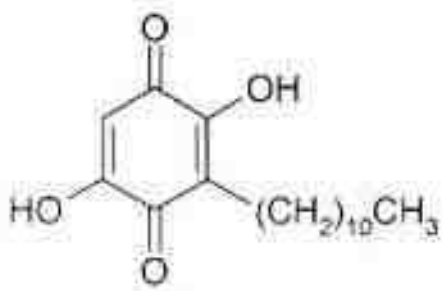
Botanical description -

Leaves are generally at the end of branches, ovate, pointed, with entire margins. Flowers are borne in lateral racemes, which are 3 times shorter than the leaves. Flowers are very small, greenish yellow, with petals expanded, pointed. Berries are round, red when ripe, sweet tasting. The plant flowers in summer. The seeds are very useful as a source of medicinal properties

Phyto-chemistry -

Embelin (2,5-dihydroxy-3-undecyl-2,5-cyclohexadiene-1,4-benzoquinone) is a phenolic compound found in the fruits of *Embelia tsjeriam-cottam* and is responsible for the medicinal properties of the plant. The fruit is bitter in taste, good appetizer, cures tumors, ascites, bronchitis, jaundice and mental disorders. Seeds are used as antibiotic, anthelmintic, antituberculosis, alterative and stimulative.

Leaves are astringent, demulcent, depurative and useful in pruritus, sore throat, mouth ulcers, indolecent, skin diseases and leprosy. A gum obtained from the plant is being used to treat dysmenorrhoea. A decoction of the leaves of this plant has been reported to be a blood purifier.



Molecular structure of Embelin

Medicinal uses -

- It is widely used in the treatment of intestine and heart diseases.
- The fruits are anthelmintic, antispasmodic and carminative.
- The finely powdered of berries are formulated as an ointment for treating pleuritis.
- The bark of the root is reportedly used to treat toothache.
- The dried bark of the root is used as a remedy for toothache.
- The plant contains Embelin 1, a benzoquinone, gallic acid 2 and a polyphenol. Both the compounds have antioxidant and anticancer properties.
- Seeds are used as a vermifuge (an agent that destroys or expels parasitic worms) the bark of the root in toothache and decoction of leaves as a gargle in sore-throat and in making a soothing ointment.

5. *Saraca asoca* (Roxb. Wild) -

Common name - Sita Ashoka

Saraca asoca is commonly known as Asoka, Sita Asoka belongs to family Caesalpinaceae is a medicinally important and globally vulnerable plant spices. It is considered as one of the sacred tree of India and is highly priced for its beautiful foliage and fragment flowers. Almost all parts of the tree are known to have important medicinal properties including antiviral, oxytotic, menorrhagic, anti-HIV. The flower extract is commonly used in diabetes and cancer treatment.

Habitat -

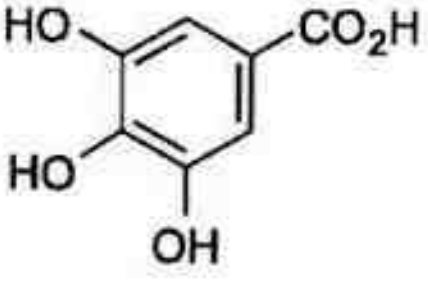
It is distributed in evergreen forests in India upto elevation of about 750 meters. It is found thought out in India especially in Himalaya, Tamil Nadu, Kerala, Karnataka, Andhra Pradesh and some part of Maharashtra. Over harvesting of *Saraca asoca* due to its high medicinal value alongwith high deforestation rate, habitat fragmentation and illegal engrossments of its natural habitats have been resulted in severe reduction in natural population of this species. This species is currently listed as a globally vulnerable species by IUCN (http://www.iucenredlist.org/apps/redilist/details/34623/0).

Botanical description -

Saraca asoca is a small evergreen tree upto 7-10 meter height. Leaves are parpinnate, 15-20 cm long, leaflets 6-12, oblong and rigidly sub-coriaceous. The barks is dark brown or grey or almost black with warty surface and rough. Flowers are fragrant. Flowers are Polygamous apetalous, yellowish orange turning to scarlet in short laterally placed corymbose.

Phyto-chemistry -

Saraca asoca is known to produce tannin, essential oil, catechol, hematoxylin, phenolic glycosides, saponnins and a good amount of gallic acid. These constituents are believed to impart the plant its for characteristic medicinal property. These constituents shows antioxidant, antimutagenicity and antigenotyoxic properties.



Molecular structure of Gallic acid

Medicinal uses -

1. Stem Bark and seeds are usually used in decoction (Kashayam) form.
2. The Side effect of asoca tends to worsen amenorrhoea. People with cardiac disorders should seek medical consultation before taking this herb.
3. The bark is used as a source for antibacterial diseases.
4. A very famous liquid medicine used in bleeding disorders, menorrhagia, diarrhea etc Ashokarishtam prepared from Saraca asoca.
5. The herbal green medicine known as Ashoka Ghrita is used for menstrual pain.

CHAPTER -2

Objectives

1. To collect wild germplasm of designated species from different forest areas of India.
2. To standardized appropriate technique for the isolation of bioactive ingredient (alkaloid) from raw material.
3. To standardize chemofingerprinting methods for quantitative estimation of bio-active compound present in the medicinal plant species.
4. To identify chemomarker zones on the basis of available bioactive compound for the identification of quality planting material.

CHAPTER -3

Material and Methods

A. Collection of wild genetic resources-

The concentration of active ingredients in plants varies along with locality, time, season and part of the plant collected. Seasonal variations also play an important role in the occurrence and concentration of biologically active compounds. To find out these variations in the concentration of active ingredients, plants were collected from different environmental conditions and soil types. To perform collection process, experiments were designed by defining the sample size. 20 plants were collected from each areas. Following five medicinal plant species were selected for the study.

i. *Litsea glutinosa* -

Bark of the tree is used for the treatment of various diseases. For the collection of bark from mature trees having minimum GBH 60cm (8-10 years) were selected. Bark harvesting was done at quarterly interval between January to December because regeneration of bark is a slow process, so if it was harvested at each month then it damages trunk of the tree. The bark samples of this species were collected from following forest areas of M.P.

Forest areas	Candidate plus tree	Girth (cm)	Height (mt)
Pachmarhi	16	60-70	12-15
Amarkantak	5	120-130	15-18
Dindori	9	50-60	8-10
Rewa	3	35-45	3-5
Balaghat	3	45-60	5-8
Chitrakoot	2	38-49	6-7
Mandla	3	45-60	5-7

Litsea glutinosa

ii. *Berberis aristata* -

Stem bark of *Berberis aristata* contains berberin and used for ayurvedic formulations. The bark samples were collected after rainy season. The germplasm were collected from following areas of Hoshangabad forest division.

Forest area
Doopgarh (Pachmarhi, MP)
Foothills (Pachmarhi, MP)

iii. *Commiphora wightii* -

The Bark samples were collected from natural forest areas of Rajasthan, Gujarat and Madhya Pradesh (northern part).

State of Rajasthan	
District/provenance	**Range/village**
Udaypur	Sajjangadh wild life sanctuary Thurmagra Chirvaghat
Rajsamand	Haldighati
Badmar	Kiradu (historical guggal in India)
Jasalmare	Akool Wood Fossil Park

State of Gujarat	
District /provenance	**Range/village**
Bhuj	Daselpur Round (Badhai village) Nakhatrana Round Mathal nursery
Jamnagar	Dwarka Range, Goringa Poshitra

State of Madhya Pradesh	
District /provenance	**Range/village**
Morena	Kemera
Bhind	Bhind

Iv. *Embelia tsjeriam-cottam A.DC -*

The seeds of Embelia tsjeriam-cottam contains Embelin. The seeds were collected from natural forest areas of Chhattisgarh, Maharashtra and Karnataka.

Source of Germplasm
Maharashtra
Borivali forest area
Tekawari, Range - Murbad
Naneghat, Range - Murbad
Maljiwara,Thane
Gambhirgarh,Range - Saywan
Tungreswer, Range - Saywan
Mahabeleswer, Range - Mahabeleswer
Sudhagarh, Range - Sudhagarh
Chhattisgarh
Dugali, Range - dugli, District - Dhamtari
Jabarra, Range - dugli, District - Dhamtari
Nagari -1,Range - dugli, District - Dhamtari
Nagari -2,Range - dugli,District - Dhamtari
Range- Dantewara, District - Dantewara
Karnataka
Gopalaswami hill
Gundalupeth

v. *Saraca asoca* - The bark of *Saraca asoca* contains Gallic acid. The bark samples were collected from potentially rich area of Tamil Nadu, Kerala, Karnataka, Madhya Pradesh and Maharashtra for the estimation of Gallic acid.

Source of Germplasm
Tamil Nadu
Kodaikanal-1
Kodaikanal-2
Madisasollai
Melpallum
Palani
Satyamagalum-1
Satyamagalum-2
Satyamagalum-3
Satyamagalum-4
Kerala
Munnar road forest area-1
Munnar road forest area-2
Karnataka
Gundalupeth, tample
Madhya Pradesh
Veterinary college, Campus, Jabalpur
Gwarighat, Jabalpur
Maharashtra
Borivali forest area

b. Preparation of standard solution -

5.0 mg or 5 ml standard of designated species were accurately weighted and dissolved in 5 ml solvent used for samples preparation to obtain concentrated stock solution in 10.0 ml volumetric flask (Borosil). Various concentration ranges between 0.1-5.0 mg/ml were prepared from the stock solution and stored at 2-8°C and brought to room temperature before use. 5.0µl solution from each standard solution was injected in six replicates. Calibration curve was generated based on peak areas.

c. Sample preparation for quantitative estimation of bioactive compound - General Method for sample extraction

Bark and seeds collected separately from the field and washed with tap water, shade dried for time duration and finely powdered.

↓

2 gm of dried material with 20 ml of 30%acetonitrile or Methanol takes in soxhlet apparatus and refluxed for 10 hours.

↓

It then loaded on Rotor-vapour and heated approximately at 80-850C. The remaining concentrated material is alkaloid with some impurities.

↓

It then defatted with hexane 3-4 times to remove fatty acids.

↓

The hexane extract is discarded and the aqueous portion washed 3-4 times with solution of 97 ml double distilled water + 3 ml conc. Hydrochloric acid.

↓

The solution is filtered, heated in water bath and 25% ammonia solution is added. pH of the solution adjusted to 7.0-7.5.

↓

The solution extracted with chloroform through separatory funnel 3-4 times. The dark portion discarded and the combined aqueous extract is transferred in a conical flask.

↓

Anhydrous Sodium Sulphate added to this extract then filtered and washed with chloroform.

↓

Extracted alkaloids are confirmed by Dragon Droff's reagent and then 20 ml 30% Acetonitrile is added and filtered with Millipore.

↓

After that 5µl of finally extracted sample injected to HPLC system for analysis.

d. Chromatographic conditions for all 5 species

A chromatography Instrument Company (CIC, Baroda, India) modular HPLC system was used. Analysis was performed on a reverse phase C-18 ODS-2 column having particle size 400 Å. Wavelength was recorded through UV detector. Column temperature was ambient at 35°C, Flow rate was 1 ml/min. The mobile phase was Methanol, Acetonitrile and HPLC grad water degassed with ultra sonic cleaner.

The analysis of collected samples were performed with the help of above mentioned instruments and chemicals followed by given parameters-

List of solvents and their mixtures tried for sample preparation

S. No.	Solvent and their mixtures
1	Pure Methanol
2	90% Methanol
3	80% Methanol
4	70% Methanol
5	60% Methanol
6	Pure Acetonitrile
7	90% Acetonitrile
8	80% Acetonitril
9	70% Acetonitrile
10	60% Acetonitrile

e. Analytical Instruments, Solvents & Reagents -

To standardize best analytical method for quantitative determination of bioactive ingredients present in the species, it is necessary to search out and analyze all the factors affecting the analysis. These factors can be categorized into moisture content in the plant, temperature of drying, isolation techniques, method of extraction including solvents and different polarities of the solvents as well as different mixture of solvents having different ratios and HPLC analysis with different parameters. Following Instruments, Solvents & Reagents were used for quantitative estimation of bioactive compounds.

List of equipments		
S.No.	**Name of the instrument**	**Manufacturer /Specifications**
1	Soxhlet (Plate-I)	E-Merck, India.
2	Rota vapour	Popular India Pvt. Ltd.
3	Millipore filtration unit	Millipore Instrument Company, Bangalore Pore size of filter paper 0.45µm.
4	Ultra sonicater	Flexit, Pune
5	HPLC	Chromatography & Instrument Company, Baroda (India) Column length- C-18. Column's pore size - 40 Å.
6	UV Detector	Linear
7	Syringe	Knaver, Hegauerweg, Berlin
8	Semimicro weighing balance	Sartorius, Jarmany
9	pH meter	EUTECH

List of Solvents & Reagents- (Chemicals and reagents used in the extraction process)

S. No.	Name of the chemicals	Specification of the chemicals	
1	Methanol	Acronym	CH_3OH
		Specific density	20°C
		Percentage purity	99.9%
		Manufacture	E-Merck, Rankam, India
2	Acetonitrile	Acronym	CH_3CN
		Specific density	20°C
		Percentage purity	99.9%
		Manufacture	E-Merck, Rankam, India
3	HPLC grade water	Acronym	HOH
		Specific density	20°C
		Percentage purity	99.9%
		Manufacture	E-Merck, Rankam, India
4	Hexane	Acronym	

		Specific density	20°C
		Percentage purity	99.9%
		Manufacture	E-Merck, Rankam, India
5	Hydrocholric acid	Acronym	HCl
		Specific density	20°C
		Percentage purity	99.9%
		Manufacture	E-Merck, Rankam, India
6	Ammonia	Acronym	NH_3
		Specific density	20°C
		Percentage purity	99.9%
		Manufacture	E-Merck, Rankam, India
7	Chloroform	Acronym	CH_3CN
		Specific density	20°C
		Percentage purity	99.9%
		Manufacture	E-Merck, Rankam, India
8	Sodium sulphate	Acronym	Na_2SO_4
		Specific density	20°C
		Percentage purity	99.9%
		Manufacture	E-Merck, Rankam, India
9	Dragon droff's reagent	Acronym	
		Specific density	20°C
		Percentage purity	99.9%
		Manufacture	CDH
10	Standards	Laurotetanine, Berberin and Embelin-Natural Remedies, Bangalore	

f. Standard formula was used for the estimation of bioactive compounds (%).

$$\%\text{Concentration} = \frac{\frac{\text{Peak area of sample}}{\mu\text{l injection}} \times \text{Wt. of Standard gm/ml}}{\frac{\text{Peak area of Stadard}}{\mu\text{l injection}} \times \text{Wt. of sample gm/m}} \times 100$$

g. Extraction Process -

***Litsea glutinosa* - (Stem Bark)**

(i) Drying of collected germplasm - Temperature for drying of collected samples -

Different methods of drying were applied on the bark after its harvesting. Collected bark left for drying by spreading on filter paper under sun light and shade at room temperature to avoid fungal infection as the sample contains lots of mucilage. After this the samples were dried under hot air oven.

After the drying process the dried material were powdered by mortar pestle and filtered with fine sieve. After this the samples were ready for extraction process.

Drying temperatures and time period of drying

S. No.	Method of drying of the bark	Time taken for drying
1	Spreading bark on filter paper and dry at room temperature	15-20 days
2	Drying in oven at 25°C	15-20 days
3	Drying in oven at 30°C	15-20 days
4	Drying in oven at 35°C	13-18 days
5	Drying in oven at 40°C	12 days
6	Drying in oven at 45°C	10 days
7	Drying in oven at 80°C for 30 min, followed by drying at 35°C in oven	5 days

(ii) Extraction process -

Extraction process for *Litsea glutinosa*

Soxhlet extraction

5.0 gm dried bark of *Litsea glutinosa* was crushed and 500 ml of solvent mixtures was taken in soxhlet apparatus and refluxed for 20 hours. It was then loaded on Rotor-vapour and heated approximately till their boiling point. The remaining concentrated material with some impurities treated with hexane extract was discarded and the aqueous portion was washed 3-4 times with chloroform through separatory funnel. The dark portion was discarded and the combined aqueous extract was transferred in a conical flask. Anhydrous Sodium Sulphate was added to this extract then filtered and washed with chloroform and then 20 ml appropriate solvent or solvent mixture was added and filtered with Millipore.

List of solvents and their mixtures tried for sample preparation

S. No.	Solvent and their mixtures
1	Pure Acetonitrile
2	90% Acetonitrile
3	80% Acetonitrile
4	70% Acetonitrile
5	60% Acetonitrile

Folin-Denis method:

5.0 gm of the powdered bark was taken in a 250 ml conical flask and 75-ml HPLC grade water was added in it. The flask was gently heated and boiled for 30 minutes, centrifuged at 2000 rpm for 20 minutes and filtered. The supernatant liquid was collected in 100 ml volumetric flask and the volume was made up 100 ml. 1ml of the sample extract was transferred to a 100ml volumetric flask containing 75-ml HPLC grade water. Added 5 ml of Folin-Deins reagent, 10 ml of 35% sodium carbonate solution and diluted to 100 ml with HPLC grade water.

As the literature is concerned standard of *Litsea* is not available in the market. Therefore, standard was prepared from natural resources. The bark sample of *Litsea* was dried and purified as the soxhlet process mentioned above. The extracted material was evaporated under reduced pressure to obtain dark brown extract. This extracted material was dissolved in acetonitrile chilled and scratched until crystallized precipitate was formed. This extract of the sample was monitored by TLC (Silica gel plate, mobile phase, chloroform: acetonitrile 9:1). 5.0 mg of extracted material was accurately weighed and dissolved in 5 ml solvent used for samples preparations to obtain concentrated stock solution in 10.0 ml volumetric flask (Borosil). Various concentration ranges 0.1-5.0 mg/ml were prepared from the stock solution and stored at 2-8°C and brought to room temperature before use. 5.0µl from each standard solution was injected in six replicates. Calibration curve was generated based on peak areas. Further standard solutions were prepared freshly each day by appropriate dilution of stock solution with solvent for intraday as well as interday analysis.

Extraction process for *Berberis aristata* (Stem pieces with bark), *Saraca asoca* (bark) and *Embelia tsjeriam-cottam* (seed) -

(I) Drying of collected germplasm - Temperature for drying of collected samples

Different methods of drying were applied on the collected samples after their harvesting. Collected samples were left for drying by spreading on filter paper under sun light and shade at room temperature to avoid fungal infection. After this, the samples were dried under hot air oven. After drying, the samples were powdered with mortar pestle and filtered with fine sieve. After this the samples were ready for extraction process.

Drying temperatures and time period of drying *Berberis aristata*

S No	Method of drying of the bark	Time taken for drying
1	Spreading of various samples on filter paper and dry at room temperature	15-20 days
2	Drying in oven at 25°C	15-20 days
3	Drying in oven at 30°C	15-20 days
4	Drying in oven at 35°C	13-18 days
5	Drying in oven at 40°C	12 days
6	Drying in oven at 45°C	10 days
7	Drying in oven at 50°C for 30 min, followed by drying at 35°C in oven	5 days

Drying temperatures and time period of drying *Saraca asoca* (Stem with bark)

S No	Method of drying of the bark	Time taken for drying (days)	% Gallic acid Mean ± SD
1	Spreading of various samples on filter paper and dry at room temperature	15-20	2.21±0.08
2	Drying in oven at 25°C	15-20	2.02±0.22
3	Drying in oven at 30°C	15-20	2.25±0.07
4	Drying in oven at 35°C	13-18	2.31±0.08
5	Drying in oven at 40°C	12	2.36±0.05
6	Drying in oven at 45°C	10	**3.39**±0.36
7	Drying in oven at 50°C for 30 min, followed by drying at 35°C in oven	5 days	2.28±0.60

Drying temperatures and time period of drying *Embelia tsjeriam-cottam*

S.No.	Method of drying	Time taken for drying
1	Spreading material on filter paper and dry at room temperature	20-25 days
2	Drying in oven at 35°C	15-20 days
3	Drying in oven at 45°C	10-15 days
4	Drying in oven at 60°C	3-5 days

(ii) Extraction process -

Sample preparation for quantitative determination:

After the drying process of *Embelia tsjeriam-cottam* (seed), *Saraca asoca* (bark) and *Berberis aristata* (Stem pieces with bark) were powdered separately and were prepared for extraction process.

Extraction process for *Embelia tsjeriam-cottam*

Soxhlet extraction

2 gm dried samples of above mentioned species were crushed and 500ml of various solvent mixtures were taken in soxhlet apparatus and refluxed for 5-6 hours then samples were loaded on Rota-vapour and heated approximately till their boiling point. The remaining concentrated material with some impurities treated with hexane extract was discarded and the aqueous portion was washed 3-4 times with chloroform through separatory funnel. The dark portion was discarded and the combined aqueous extract was transferred in a conical flask. Anhydrous Sodium Sulphate was added to this extract then filtered and washed with chloroform and then 20 ml appropriate solvent or solvent mixture were added and filtered with Millipore for the species. The mobile phase consisting of Acetonitrile:HPLC grade water (10:90) was used.

List of solvents and their mixtures tried for sample preparation

S No	Solvent and their mixtures
1	Pure Methanol
2	Methanol:Water (80:20)
3	Pure Acetonitrile
4	Acetonitrile:Water (10:90)
5	90% Acetonitrile
6	80% Acetonitrile
7	70% Acetonitrile
8	60% Acetonitrile
9	90% Methanol
10	80% Methanol
11	70% Methanol
12	60% Methanol

Folin-Denis method for *Berberis aristata* and *Saraca asoca*

2 to 5.0 gm of the powdered bark was taken in a 250 ml conical flask and 75-ml HPLC grade water was added in it. The flask was gently heated and boiled for 30 minutes, centrifuged at 2000 rpm for 20 minutes and filtered. The supernatant liquid was collected in 100 ml volumetric flask and the volume was made up 100 ml. 1ml of the sample extract was transferred to a 100ml volumetric flask containing 75-ml HPLC grade water. Added 5 ml of Folin-Deins reagent, 10 ml of 35% sodium carbonate solution and diluted to 100 ml with HPLC grade water.

As per the available literature the standards of active alkaloids of the designated species were procured from Natural remedies Pvt.Ltd. Bangalore with 95% purity.

Commiphora wightii -(Stem Bark)

(i) Drying of collected germplasm - Temperature for drying of collected samples -

The bark/gum of the plant is used for the treatment of various diseases. For the collection of bark/gum from mature trees having minimum GBH 15-20cm were selected. To standardize best analytical method for quantitative determination of active bioactive compound present in the species, it is necessary to search out and analyze all the factors affecting for chemofingerprinting.

These factors can be categorized into moisture content in the plant, temperature of drying, isolation techniques, method of extraction including solvents and different polarities of the solvents as well as different mixture of solvents having different ratios and HPLC analysis with different parameters. Following table provides the details of solvents reagent used to perform the study.

Drying temperatures and time period of drying

S.No.	Method of drying	Time taken for drying
1	Spreading material on filter paper and dry at room temperature	15 days
2	Drying in oven at 40°C	4 days
3	Drying in oven at 45°C	3 days

(ii) Extraction process - 2.0ml standard of Guggulsterone E&Z (Natural Remedies Pvt. Ltd.) was accurately dissolved in 5 ml solvent used for samples preparations to obtain concentrated stock solution in 10.0 ml volumetric flask (Borosil). Various concentration ranges between 0.1-5.0 ml were prepared from the stock solution and stored at 2-8°C and brought to room temperature before use. 5.0µl from each standard solution was injected in six replicates.

2 gm of powered material with 20 ml of solvent mixture was taken in soxhlet apparatus and refluxed for 10 hours. It was then loaded on Rotor-vapour and heated approximately till their boiling point. The remaining concentrated material with some impurities defatted with hexane 3-4 times to remove fatty acids. The hexane extract was discarded and the aqueous portion was washed 3-4 times with 3% HCl solution. The solution was filtered, heated in water bath and 25% NH_4 solution was added, pH of the solution was adjusted to 7.0-7.5. The solution was extracted with $CHCl_3$ through separatory funnel 3-4 times. The dark portion was discarded and the combined aqueous extract was transferred in a conical flask. Anhydrous Sodium Sulphate was added to this extract then filtered and washed with chloroform. Extracted Guggulsterone were confirmed by Dragon Droff's reagent and then 20 ml appropriate solvent or solvent mixture was added and filtered with Millipore.

CHAPTER-4

Result and Discussion

Chemofingerprinting protocol

Species wise details of available bioactive compound (alkaloid) in percent concentration.

The HPLC methods for the quantitative estimation of Laurotetanine, Berberin, Guggulsterone (E&Z), Embelin and Gallic acid were validated with regard to their specificity, precision, accuracy and linearity. All the collected samples were analyzed. Three physical factors viz. Temperature, solvent polarity and extraction methods were studied for the designated species for quantitative of bioactive compounds.

a. Chemo-fingerprinting protocol for *Litsea glutinosa* -

Optimum temperature and condition for drying of plant samples:

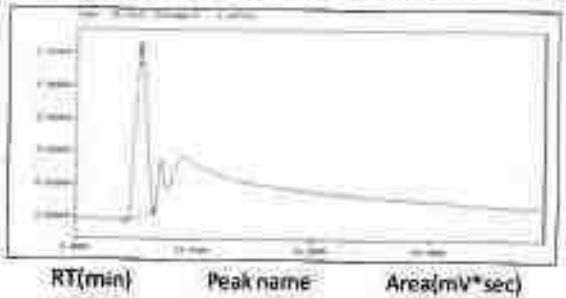
Oven temperature	-	45°C
Number of days for drying of plant samples	-	10
Extraction methods	-	Soxhlet
Detection parameters -		
Solvent fraction	-	CH_3CN : HOH (60:40)
Wavelength	-	700nm
Column temperature	-	35°C
Flow rate	-	1ml/min
Column	-	C-18 ODS2

Range of percent concentration of bioactive compound (Laurotetanine)-

Barks	-	2.71 to 5.18%

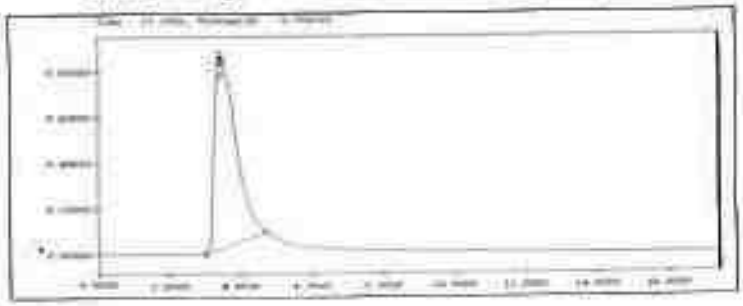
Maximum percent concentration of Laurotetanine was found in Rewa forest Division (5.81%) and minimum was found (2.71%) in Balaghat forest division.

Chromatograms of Laurotetanine (standard)



RT(min)	Peak name	Area(mV*sec)
5.537	standard	34811.348

Chromatograms of Laurotetanine (sample)



RT(min)	Peak name	Area(mV*sec)
3.447	sample	34811.348

b. Chemo-fingerprinting protocol for *Berberis aristata* -

Oven temperature	-	45°C
Number of days for drying of plant samples	-	10
Extraction methods	-	Soxhlet
Detection parameters -		
Solvent fraction	-	CH_3CN : HOH (60:40)
Wavelength	-	700nm
Column temperature	-	35°C
Flow rate	-	1ml/min
Column	-	C-18 ODS2
Range of percent concentration of bioactive compound (Berberine)-		
Barks	-	1.84 to 1.25%

Maximum percent concentration of Berberine was found in Dhoopgarh, Pachmari, M.P. and minimum was found from Foothills (M.P.).

Doopgarh (Pachmarhi, MP)	1.84
Foothills (MP)	1.25

Chromatograms of berberin (standard)

RT(min)	Peak name	Area(Mv*sec)
3.383	Berberin std.	41.926

Chromatograms of Sample

RT(min)	Peak name	Area(Mv*sec)
3.338	Sample-2	46.373

c. Chemo-fingerprinting protocol for *Commiphora wightii* (Arn.) Bhandari -

Optimum temperature and condition for drying of plant samples:

Oven temperature	-	45°C
Number of days for drying of plant samples	-	3
Extraction methods	-	Soxhlet
Detection parameters -		
Solvent fraction	-	Acetonitrile: HPLC Grade water (10:90), (Merck)
Wavelength	-	230nm
Column temperature	-	35°C
Flow rate	-	1ml/min
Column	-	C-18 ODS2

Range of percent concentration of bioactive compound (Guggulsterone)-

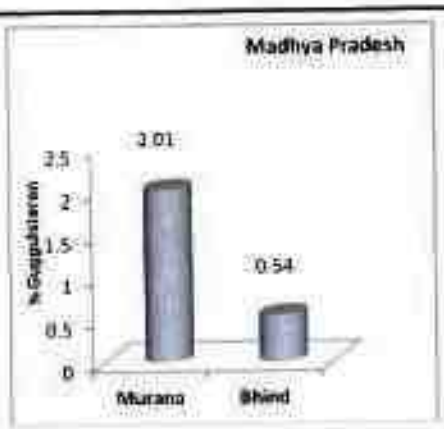
Barks	-	2.32 to 0.54%

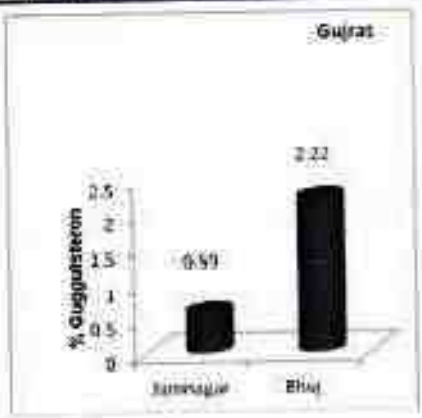
The percent concentration of Guggulsterone in the accessions collected from different geographical locations of Rajasthan, Gujarat and M.P. are presented below:

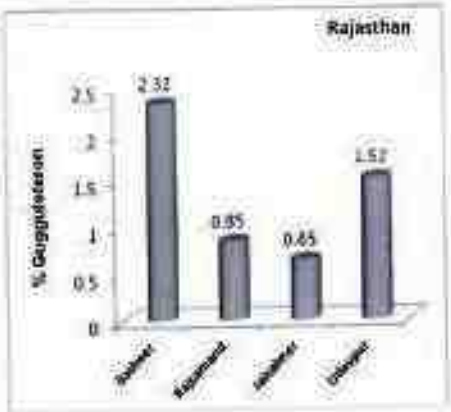
a. Rajasthan: ranges from 0.65 % to 2.32%.

b. Gujarat: ranges from 0.59 % to 2.22%.

c. MP: ranges from 0.54 % to 2.01%.







d. Chemo-fingerprinting protocol for *Embelia tsjeriam-cottom* - A.DC -

Optimum temperature and condition for drying of plant samples:

Oven temperature	-	45°C
Number of days for drying of plant samples	-	15
Extraction methods	-	Soxhlet
Detection parameters -		
Solvent fraction	-	Acetonitrile: HPLC Grade water (10:90), (Merck)
Wavelength	-	230nm
Column temperature	-	35°C
Flow rate	-	1ml/min
Column	-	C-18 ODS2

Range of percent concentration of bioactive compound (Embeline)-

Barks	-	2.29 to 4.88%

Chromatograms of Embelin (Standard)

RT(min)	Peak name	Area(mV*sec)
1.685	Standard	116.145

Chromatograms of sample

RT(min)	Peak name	Area(mV*sec)
1.723	Sample	57.198

Maximum percent concentration of Embeline was found in Tekawari, Range- Murbad, Maharashtra (4.88%) and minimum was found (2.29%) in Sudhagarh, Range-Sudhagarh, Maharashtra.

e. Chemo-fingerprinting protocol for *Saraca asoca (Roxb. Wilid)* -

Optimum temperature and condition for drying of plant samples:

Oven temperature	-	45°C
Number of days for drying of plant samples	-	10-15
Extraction methods	-	Folin-Denis
Detection parameters -		
Solvent fraction	-	Methanol HPLC Grade
Wavelength	-	230nm
Column temperature	-	35°C
Flow rate	-	1ml/min
Column	-	C-18 ODS2

Range of percent concentration of bioactive compound (Gallic acid)-

Barks	-	2.28 to 3.39%

Maximum percent concentration of Gallic acid was found in Satyamagalum-1, Tamil Nadu (3.39%) and minimum was found (2.28%) from Jabalpur (M.P.)

Chromatograms of Gallic acid (Standard)

RT(min)	Peak name	Area(mV*sec)
2.918	Standard	99.117

Chromatograms of sample

RT(min)	Peak name	Area(mV*sec)
2.538	SAMPLE-1	22.225

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