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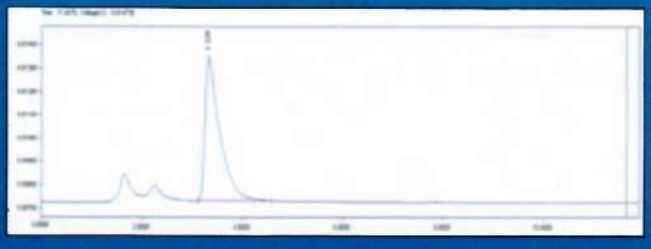
Quantitative determination of bio-active compounds of critically endangered and rare medicinal plants Alectra chitrakutensis and Butea superba through chemoprofiling

Alectra chitrakutensis (Kanda)

Butea superba (Belapalash)



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Author

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List of Abbreviations

^{0}C	Degree Celsius
mg	milligram
ml	millilitre
µ	micron
nm	nanometre
mv	milli volte
RT	Retention Time
WHO	World Health Organisation
HPLC	High Performance Liquid Chromatography
IUCN	International Union for Conservation of Natural Resources

Chapter-1

a. Introduction

Madhya Pradesh and its adjoining forest area provide a suitable environment for the growth and development of large number of medicinal and aromatic plants includes herb, shrub, tree and climber etc. These medicinal plants are greatly consumed by the local tribes for their day to day needs. Since, last four decades due to over exploitation and creation of big herbal based industries the natural population of several medicinal and aromatic plants are drastically declining day by day due to which number of medicinal plant species comes under RET category. On the other hands due to unsustainable harvesting practices of commercially important medicinal plant by the local people also causes declining of their populations. The Medicinal plant poses bioactive compounds which are commonly known as secondary metabolites (alkaloid). These bioactive compounds are used in the preparation of several kinds of life saving drugs by the herbal based industries. India is the second largest herbal drug producing country in the world after China. The herbal based medicines are comparatively cheaper and as far as biological activities are concern they do not show any side effects in the human body. Several herbal based industries such as Zandu, Dabur, Patanjali and so many more industries are coming rapidly for the production of herbal based drugs.

Alkaloids are an important class of basic organic compounds that occur in higher plants. They are known to contain one or more nitrogen heterocyclic rings as an integral part of their structure. They produce striking physiological effects when administered to humans. Thus, some alkaloids stimulate the central nervous system, while others cause paralysis, some alkaloids act as pain relievers, others as local anaesthetics and still others act against infectious microorganism. Most alkaloids are toxic and yet many find use in medicine. They occur chiefly in plants of the dicotyledons family and are localized in seeds, leaves, bark or root of the plants. They are colourless, crystalline solids while few are liquids. In general they are insoluble in water but the liquid ones are soluble, they dissolve readily in organic solvents such as ethanol, or chloroform. Most of them are having an intensely bitter taste and are poisons. They all are optically active compounds and most of them are laevorotatory.

Today, identification of quality planting material in case of medicinal plants is of the great concern of research and development. At country level, National Medicinal Plants Board, New Delhi launched a National Level Network Programme for the identification and multiplication of quality planting material (QPM) under which several institutions in the country are actively engaged. Particularly in MAPs, the identification of quality planting material (QPM) on the basis of morphological traits has no relevance, because they contain alkaloids which can only be estimated by phytochemical analysis through chemoprofiling.

Today, there are several tools, techniques and parameters are available for the identification of quality planting material of medicinal plants. Phenotypic selection is not an appropriate parameters because the phenotypic traits doesn't reflect the availability of bioactive compound in the given plant population hence alternatively modern chemical fingerprinting such as High Performance Liquid Chromatography (HPLC), High Performance Thin Layer Chromatography (HPTLC) are now available through which quality germplasm of medicinal plants can be easily be screen out on the basis of available bioactive compounds. These techniques are commonly known as quantitative determination techniques for the estimation of bioactive compounds. These tools and techniques are scientifically more reliable with more scientific precision. In India large number of institutions such as Central Drug Research Institute (CDRI), Central Institute of Medicinal and Aromatic Plants (CIMAP) Lucknow, ICAR Institute at Anand (Gujarat) and so many more private laboratories such as Zandu, Chavanprash, Patanjali etc. are actively engaged in this aspect.

b. About the species

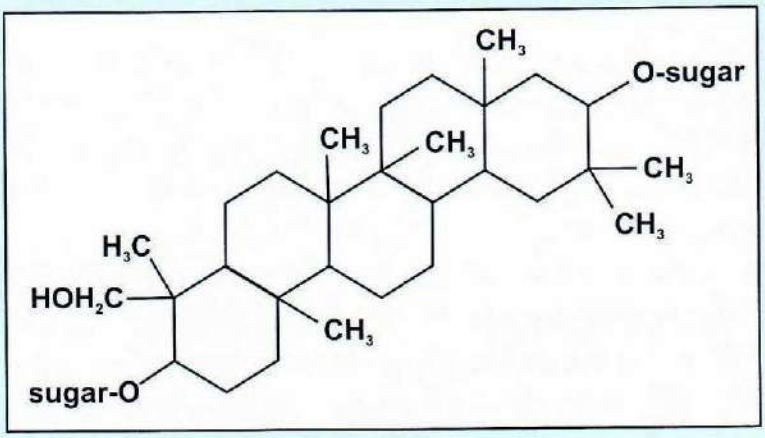
1. ***Alectra chitrakutensis* (Rau) Prasad and Dixit** – The species is commonly known as Kanda, Medkikand belongs to family Serophulariaceae is a critically endangered annual herb includes more than 50 species of parasitic herbs distributed in India, Africa, South America and Australia. A majority of the species is found in tropical Africa and some are common to Australia. Two species are recorded from Brazil, one from Arabia and three from Madagascar. The three members from India are (1) *Alectra indica.Benth* (2) *A. thomsonii Hook.f.* and (3) *A. parasitica* A.Rich var.chitrakutensis (Rau) R.Prasad and R.D. Dixit. Vegetatively it can be distinguished by strongly rhizomatous development of the underground part of the stem. The perennial whole plant strands up shoots from the numerous buds in the axils of closely set scale leaves.

Madhya Pradesh is the most suitable for the growth and development of this species particularly in the natural vicinity of Chitrakoot along the borders of Satna (M.P.) and Banda District of Uttar Pradesh. For Madhya Pradesh it is reported to be critically endangered medicinal plant (http://envis.frlht.org/index.php/bot_search).

It is always found growing in a parasite on the roots of *Vitex negundo* Linn which is called midaki in Chitrakut region. The stem of the Nirgundi are extensively lopped by the local people for the use of roofing material for making houses. As a result of this constant lopping the main stem is never allowed to develop and the plants in Chitrakoot region always remains bushy growth of coppice shoots which attain a height of 4 to 8 feet.

Alectra chitrakutensis is traditionally used for the treatment of leprosy for centuries in traditional Ayurveda (Bedi, 1967). It is also used in the treatment of various ailments such as tuberculosis, paralysis, piles, intestinal worms, constipation, leucorrhoea, fever, spermatorrhoea and also as blood purifier.

The whole plant is used as medicinal purpose which contains steroids, glycosides, flavonoids, carbohydrates and important alkaloids, saponin, azafrin and mannitol. Due to its high economic value and used in pharmaceutical industries the natural population of this species is declining rapidly due to which this genus is at the verge of extinct. During the survey under this study it was noticed that this species is only available in few pockets in Chitrakoot region. Therefore, it is an urgent need to domesticate, protect, conserve and multiply this species immediately.



Saponin

Alectra chitrakutensis

2. ***Butea superba* (ROXB) –** It is commonly known as Belapalash belongs to family Fabaceae. It is climber in nature. It is mainly found in Malaysia, Indonesia, Vietnam, Sri Lanka and India. In India it is found in Uttar Pradesh to West Bengal, Madhya Pradesh, Chhattisgarh, some part of Maharashtra, Assam and southwards to Peninsular India (http://envis.frlht.org/index.php/bot_search).

In Madhya Pradesh, it is distributed throughout in the state. However, due to over exploitation and unsustainable harvesting practices of this species since last few decades its natural population from the states is declined rapidly, due to this the species comes under near threat (NT) category. (http://envis.frlht.org/index.php/bot_search).

The roots of *Butea superba* are used as a source of medicine. Traditionally people take it for sexual performance problems (erectile dysfunction, ED) and lack of interest in sexual activity. The roots are also used in treatment of diarrhea, painful difficult urination and fever. Still it is not known how *Butea superba* might work as a sexual stimulant medicine. Some evidence suggests that the bio chemicals which are present in *Butea superba* may act similarly to hormones that regulate sexual function. Due to presence of several important alkaloids in its root which includes flavonoids such as butenin and butin, sterols including beta-sitosterol and campesterol, medicarpin formononetin, Pururain, 7, 4-dimethoxyisoflavone the plant has highly potent for its medicinal value.

Butea superba

c. Quality germplasm

It is a term used to describe the genetic resources or more precisely the DNA of an organism. In the case of medicinal plants, quality germplasm is described on the basis of quantity of its active ingredients (phyto-constituents) *i.e.* the plant having maximum content of bioactive ingredients is considered as the quality germplasm.

Chapter-2

Objectives

1. To collect wild germplasm of designated species from different forest area of Madhya Pradesh.
2. To find out an appropriate solvent, mixture of solvents and their ratio for the isolation of bioactive compounds from raw material.
3. To standardize chemo-fingerprinting methods for quantitative determination of bio-active compounds present in the species.
4. To identify quality planting material.

Chapter-3
Material and Methods

a. Collection of wild germplasm:

The concentration of active ingredients in plants varies along with 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 seasonal variations in the concentration of active ingredients, plants were collected at different environmental conditions and soil types. To perform collection process experiment were design by defining the sample size 20 plants from potentially rich areas of the sate were collected.

As per the available literature regarding their availability in Madhya Pradesh reconnaissance survey was made in different forest areas of M.P. for the collection of plant material in the form of whole plant, cuttings and roots for further studies. Their occurrence is presented in the following map and Table 1.

Species Distribution

Table 1. Source of collection of germplasm along with areas

S. No.	Source of Germplasm	Species occurrence	
		Alectra chitrakutensis (Y/N)	*Butea superba* (Y/N)
1.	Bori, Hosangabad	N	Y
2.	Sarni, Betul	N	Y
3.	Kalpi, Mandla	N	Y
4.	Mundwara,Satna	N	Y
5.	Hardua, Satna	N	Y
6.	Pukharwar, Satna	N	Y
7.	Bharatkoop, Satna	N	Y
8.	Bagra, Kalibheet, Khandwa	N	Y
9.	Battu, khandwa	N	Y
10.	Bhesadehi, Betul	N	Y
11.	Bsukhera, Sagar	N	Y
12.	Mangarh, Sagar	N	Y
13.	Hari ki toriya, Sagar	N	Y
14.	Jaruwakhera, Sagar	N	Y
15.	Rootheri, Sagar	N	Y
16.	Bona chor ki gupha (Baregaon), Chhatarpur	N	Y
17.	Singrampur, Damoh	N	Y
18.	Sangakhera, Bhurabhagat (Chhindwara)	N	Y
19.	Chhaladhar, Chitrakoot, Satna	Y	N
20.	Kalinger, Satna	Y	N
21.	Bhanwar, Satna	Y	N
22.	Aseergarh, Burhanpur	N	Y
23.	Raipura, Burhanpur	N	Y
24.	Kherda, Khandwa	N	Y
25.	Fafrisarkar, Khandwa	N	Y
26.	Choral, Khandwa	N	Y
27.	Bistan, Khargone	N	Y
28.	Jasalpur, Hosangabad	N	Y
29.	Balu, Betul	N	Y
30.	Teekamgarh	N	Y
31.	Dhar	N	N

b. Standardization of quantitative determination technique through HPLC–

The concentration of active ingredients in plants varies along with locality and part of the plant collected. To perform HPLC process, experiment were design by defining the sample size.

c. Preparation of standard solution–

5.0 mg or 5 ml standard of designated species were 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 between 0.1-5.0 mg/ml were prepared from the stock solution and stored at 2-8^{0}C and brought to room temperature before use. 5.0µl from each standard solution was injected in six replicates. Calibration graph was generated based on peak areas.

d. Sample preparation–

Preparation of sample for quantitative estimation of bioactive compound through HPLC (General Method for sample extraction)

Different plant parts collected separately from field and washed with tap water, shade dried for different 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 80-85^{0}C. The remaining concentrated material was alkaloid with some impurities.

↓

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

↓

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

↓

The solution was filtered, heated in water bath and 25% ammonia solution was added. pH of the solution was 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 was transferred in a conical flask.

↓

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

↓

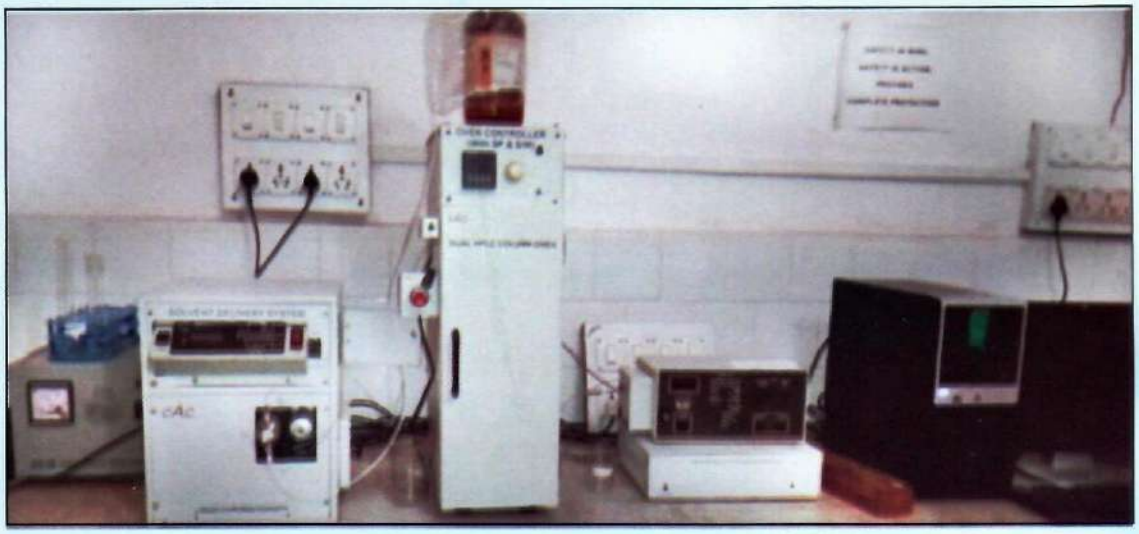
Extracted alkaloids are confirmed by Dragon Droff's reagent and then 20 ml 30% Acetonitrile or methanol was added and filtered with Millipore.

↓

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

e. Chromatographic conditions -

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. The wavelength was recorded through UV detector. Column temperature was ambient at 35°C, Flow rate was 1 ml/min. The mobile phase was Methanol or Acetonitrile and were degassed with ultra sonicater.



High Performance Liquid Chromatography (HPLC) system

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

f. Analytical Instruments, Solvents and 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 and Reagents were use for quantitative estimation of bioactive compounds.

List of equipments

S.No.	Name of the instrument	Manufacturer /Specifications
1.	Soxhlet	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 and Instrument Company, Baroda Column length- C-18. Column's pore size - 40 Å.

Table cont....

6.	UV Detector	Linear
7.	Syringe	Knaver, Hegauerweg, Berlin
8.	Semimicro weighing balance	Sartorius, Ag Germany
9.	pH meter	Eutech

List of Solvents and Reagents

S.No.	Name of the chemical	Specification of the chemical	
1.	Methanol	Acronym Specific density Percentage purity Manufacture	CH_3OH 20^0C 99.9% E-Merck, Rankam, India
2.	Acetonitrile	Acronym Specific density Percentage purity Manufacture	CH_3CN 20^0C 99.9% E-Merck, Rankam, India
3.	HPLC grade water	Acronym Specific density Percentage purity Manufacture	HOH 20^0C 99.9% E-Merck, Rankam, India
4.	Hexane	Acronym Specific density Percentage purity Manufacture	CH_3OH 20^0C 99.9% E-Merck, Rankam, India
5.	Hydrocholric acid	Acronym Specific density Percentage purity Manufacture	HCl 20^0C 99.9% E-Merck, Rankam, India
6.	Ammonia	Acronym Specific density Percentage purity Manufacture	NH_3 20^0C 99.9% E-Merck, Rankam, India
7.	Chloroform	Acronym Specific density Percentage purity Manufacture	CH_3CN 20^0C 99.9% E-Merck, Rankam, India
8.	Sodium sulphate	Acronym Specific density Percentage purity Manufacture	Na_2SO_4 20^0C 99.9% E-Merck, Rankam, India

Table cont...

9.	Dragon droff's reagent	Acronym Specific density Percentage purity Manufacture	CH_3OH 20^0C 99.9% CDH
10.	Standards	Saponin and Purerain- Natural Remedies, Bangalore	

Following standard formula was used for the estimation of bioactive compounds from plant sample in (%)

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

g. Extraction Process for *Alectra chitrakutensis* – (Whole plant)

(i) Drying of collected sample - Temperature for drying of collected samples :

Different methods of drying were applied on the whole plant after its harvesting. Collected whole plant, 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 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 of *Alectra chitrakutensis*

S.No.	Method of drying of the whole plant	Time taken for drying
1.	Spreading whole plant on filter paper and dry at room temperature	20-25 days
2.	Drying in oven at 25^0C	15-20 days
3.	Drying in oven at 30^0C	15-20 days
4.	Drying in oven at 35^0C	10-12 days
5.	Drying in oven at 40^0C	10 days
6.	Drying in oven at 45^0C	8 days
7.	Drying in oven at 50^0C for 30 min, followed by drying at 35^0C in oven.	3 days

(ii) Extraction process for *Alectra Chitrakutensis*

Soxhlet extraction

5.0 gm dried root powder of *A. Chitrakutensis* were 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

h. Extraction process for *Butea superba* (root) -

(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 the drying process 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 of *Butea superba*

S.No.	Method of drying of the root	Time taken for drying
1.	Spreading of root on filter paper and dry at room temperature	25-30 days
2.	Drying in oven at 25°C	25-30 days
3.	Drying in oven at 30°C	22-25 days
4.	Drying in oven at 35°C	20-25 days
5.	Drying in oven at 40°C	15-20 days
6.	Drying in oven at 45°C	10-15 days
7.	Drying in oven at 50°C for 30 min, followed by drying at 35°C in oven.	8 days

(ii) Extraction process for *Butea superba*

Soxhlet extraction

5.0 gm dried root powder of *Butea superba* were 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 Methonal
2.	90% Methonal
3.	80% Methonal
4.	70% Methonal
5.	60% Methonal

Chapter-4
Result and Discussion

The wild germplasm of designated species were collected from the areas as mentioned in Table no. 1. It was observed that *Butea superba* was found in almost entire area of Madhya Pradesh. However, its occurrence was very rich in the areas as shown in Table no. 1. So these areas were marked as potentially rich areas of *Butea superba*. From the study, it was observed that its natural habitat is declining rapidly so its conservation is very necessary. *Butea superba* contain important bioactive compound which are used for increasing sexual potency in human. As far as *Alectra chitrakutensis* is concern it was found only in few pockets of Satna and Chitrakoot forest areas as shown in Table no. 1. From the study it was observed and as per the discussion with the local people of these forest areas, the population of this species is declining drastically because the species contain important bioactive compound which are used for making several important herbal drugs so that the species is comes under critically endangered category. Hence, its domestication, conservation and propagation require serious attention.

(a) Quantitative determination of bioactive compounds (alkaloids) :-

A number of studies have been conducted to evaluate and test the medicinal properties, evaluation of new compounds and pharmacological effects of medicinal plants. Variations of these biologically active compounds particularly on the basis of their variation in place have been a subject of least investigation. The intra-specific variation of the species is the important factors to identify the superior germplasm for appropriate conditions for finding the maximum yield of biologically active compounds. The complexity of the chemical composition of herbal extracts, quality of the herbal extracts can only be assured by the use of validated analytical methods for identification and quantification of the active ingredients.

The root samples of *Butea superba* and whole plant part of *Alectra chitrakutensis* were collected for chemoprofiling analysis through HPLC technique. The bioactive compound was quantified by HPLC chromatogram which are mentioned below. Various physical factor viz drying temperature of root and whole plant, moisture content, mobile phase, oven temperature, wavelength, solvent delivery solution, injection parameters were standardized for quantitative estimation of saponin in *Alectra chitrakutensis* and Purerain in *Butea superba*.

The species wise chromatographic results and discussion are described below

1. *Alectra chitrakutensis*

Temperature of drying

Different methods were used for drying the root samples of *Alectra chitrakutensis*. The effect of drying temperature were recorded in following table as

Effect of different drying treatments on *Alectra chitrakutensis*

S. No.	Method of drying of the whole plant	Time taken for drying	% Saponin, Mean ± SD
1.	Spreading whole plant on filter paper and dry at room temperature	20-25 days	2.11 ± 0.24
2.	Drying in oven at 25°C	15-20 days	1.42 ± 0.11
3.	Drying in oven at 30°C	15-20 days	2.02 ± 0.08
4.	Drying in oven at 35°C	10-12 days	2.98 ± 0.15
5.	Drying in oven at 40°C	10 days	3.55 ± 0.20
6.	Drying in oven at 45°C	8 days	3.31 ± 0.09
7.	Drying in oven at 50°C for 30 min, followed by drying at 35°C in oven	3 days	0.44 ± 0.11

The observations showed that the effect of storage of roots at different temperatures define that the optimum temperature of drying on which maximum yield of saponin was obtained at 40°C.

(b) Effect of solvent polarity on the sample:

Polarity is the basic criteria for separation of compounds, polarity effect solubility of compounds, so increase and decrease in polarity plays very important role in the extraction of compounds. To overcome this obstacle the experiments were designed to test the different polarities of the solvents and the results are tabulated in the following table.

Different solvents and their mixtures were used to optimize the HPLC technique for the estimation of saponin as mentioned in the following table. The result showed that pure Acetonitrile was better solvent as compared to other mixtures of Acetonitrile for maximum estimation of saponin.

Effect of solvents and their fractions on % concentration of Saponin

S.No.	Solvent and their mixtures	% Saponin ± SD
1.	Pure Acetonitrile	3.55 ± 0.11
2.	90% Acetonitrile (90:10)	3.31 ± 0.09
3.	80% Acetonitrile (80:20)	2.72 ± 0.15
4.	70% Acetonitrile (70:30)	1.88 ± 0.17
5.	60% Acetonitrile (60:40)	0.44 ± 0.08

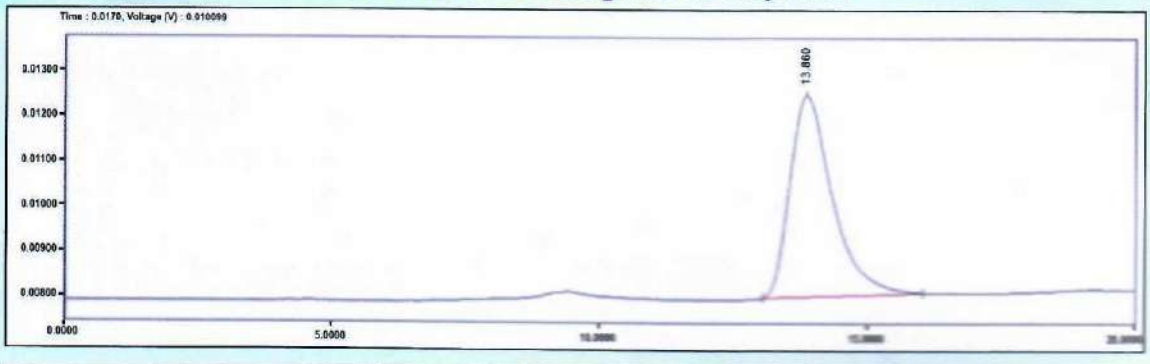
(c) HPLC analysis-

Various physical factors were find out for the maximum estimation of saponin from *Alectra chitrakutensis*. These factors were kept constant and analysis of all the samples were carried out and following results were obtained as given in the table-2. Above experiments showed that the saponin content depends on the temperature variation and optimum temperature of drying of the herb which was 40°C. Solvent polarity also played a very important role and it was concluded that the solvent fraction of pure Acetonitrile gave maximum yield of saponin. By considering these factors all the collected samples were analyzed through High Performance Liquid Chromatography system (HPLC).

Detection parameters

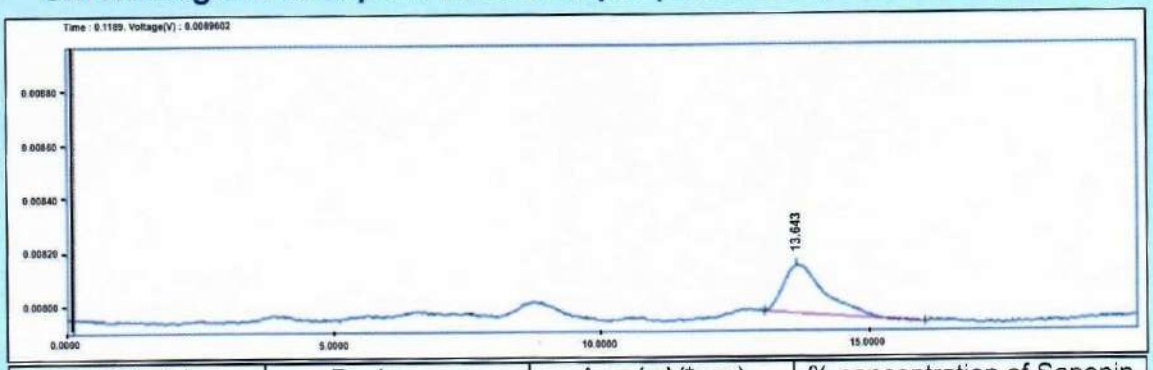
Solvent fraction	Pure Acetonitrile
Wavelength	230nm
Column temprature	35°C
Flow rate	1ml/min

Standard chromatogram of Saponin



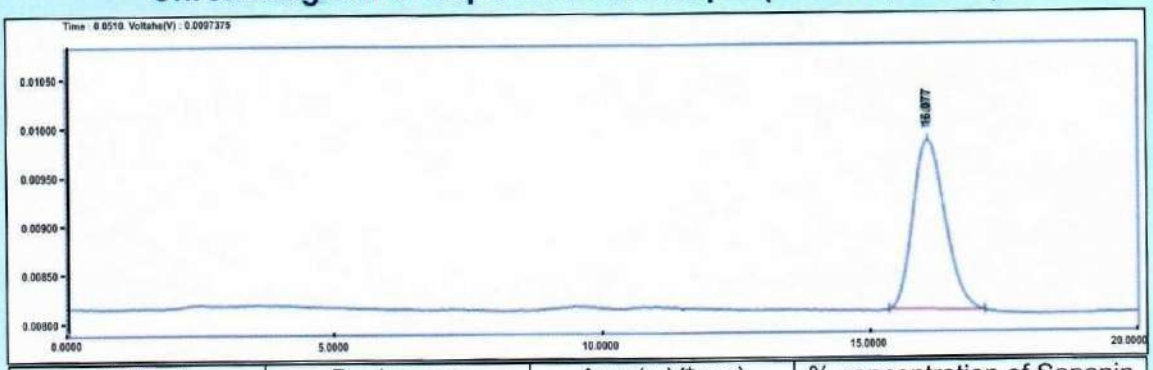
RT(min)	Peak name	Area(mV*sec)
13.860	Standard	246.123

Chromatogram of Saponin from sample (Chilladhar, Chitrakoot, Satna)



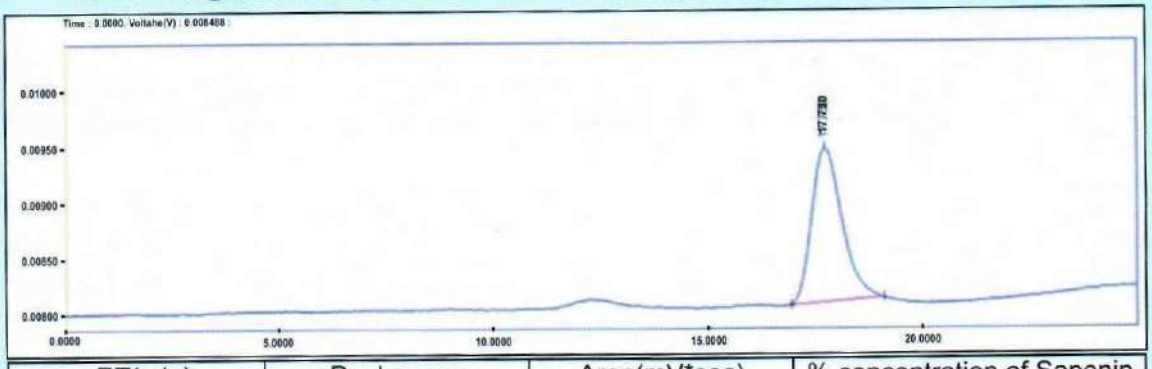
RT(min)	Peak name	Area(mV*sec)	% concentration of Saponin
13.643	Sample-1	9.334	0.44

Chromatogram of Saponin from sample (Bhawer, Satna)



RT(min)	Peak name	Area(mV*sec)	% concentration of Saponin
16.077	Sample-2	72.472	3.55

Chromatogram of Saponin from sample (Kalinger, Chitrakoot, Satna)



RT(min)	Peak name	Area(mV*sec)	% concentration of Saponin
16.730	Sample-3	67.623	3.31

Table-2 % concentration of Saponin in 3 forest ranges of Chitrakoot, Satna Divisions

S.No.	Accession source	Saponin (%) in roots
1.	Chilladhar, Chitrakoot	0.44
2.	**Bhawar, Satna**	**3.55**
3.	Kalinger, Chitrakoot	3.31

From the above table it was interesting to observe that there were great variations of % concentration of bioactive compound, saponin in collected germplasm of *Alectra chitrakutensis* from different forest ranges of Chitrakoot, Satna Forest Division. The *Alectra chitrakutensis* germplasm which were collected from Bhanwar and adjoining areas of Kalinger, Chitrakoot showed maximum 3.55% saponin and the minimum 0.44% saponin was recorded from the germplasm of Chilladhar area. So, the germplasm of this species which were collected from from Kalinger area was reported to be quality planting material because it contain maximum bioactive compound saponin. This germplasm was further multiplied using clonal multiplication technology. Earlier Sharma *et.al.*, 2016 reported different accession collected from Chitrakoot and adjoining areas contain saponin alkaloid but the % concentration is not reported so far in the collected germplasm. This is the first kind of report/information reported for the available saponin contained in this species from Chitrakoot forest areas.

2. *Butea superba*

Temperature of drying

Different methods were used for drying the root samples of *Butea superba*. The effect of drying temperature were recorded in table as mentioned below.

Effect of different drying treatments on *Butea superba*

S.No.	Method of drying of the herb from Oct. to Dec.	Time taken for drying	% Purerain Mean ± SD
1.	Spreading root on filter paper and dry at room temperature 10°C to 25°C.	30-35 days	1.57 ± 0.13
2.	Drying in oven at 25°C	25-30 days	1.78 ± 0.09
3.	Drying in oven at 30°C	22-25 days	2.36 ± 0.10
4.	Drying in oven at 35°C	20-25 days	3.57 ± 0.25
5.	Drying in oven at 40°C	15-20 days	4.41 ± 0.13
6.	Drying in oven at 45°C	10-15 days	2.82 ± 0.10
7.	Drying in oven at 50°C for 30 min, followed by drying at 35°C in oven.	8 days	1.51 ± 0.13

The temperature effects on the plant sample is a vital role for the estimation of bioactive compound. The observations showed from the above table that the effect of storage of roots at different temperatures define that the optimum temperature of drying of plant sample on which maximum yield of Purerain was obtained in 15 to 20 days at 40°C.

Effect of solvent polarity on the sample:

Polarity is the basic criteria for separation of compounds, polarity effect solubility of compounds, so increase and decrease in polarity plays very important role in the extraction of compounds from plant samples. To overcome this obstacle the experiments were designed to test the different polarities of the solvents and the results are tabulated in table-3.

Different solvents and their mixtures were used to optimize the HPLC technique for the estimation of Purerain as mentioned in the following table. The result showed that the pure CH_3OH (Methonal) was better solvent as compared to other mixtures of CH_3OH for maximum estimation of Purerain.

Table-3 : Effect of solvents and their fractions on % concentration of Purerain

S.No.	Solvent and their mixtures	% Purerain ± SD
1.	Pure CH_3OH (Methonal)	4.24 ± 0.22
2.	90% CH_3OH :HOH (90:10)	3.42 ± 0.10
3.	80% CH_3OH :HOH (80:20)	2.72 ± 0.17
4.	70% CH_3OH :HOH (70:30)	2.42 ± 0.13
5.	60% CH_3OH :HOH (60:40)	1.78 ± 0.14

From the above table it is observed that the pure methanol extract 4.24% Purerain from plant sample

(d) HPLC analysis-

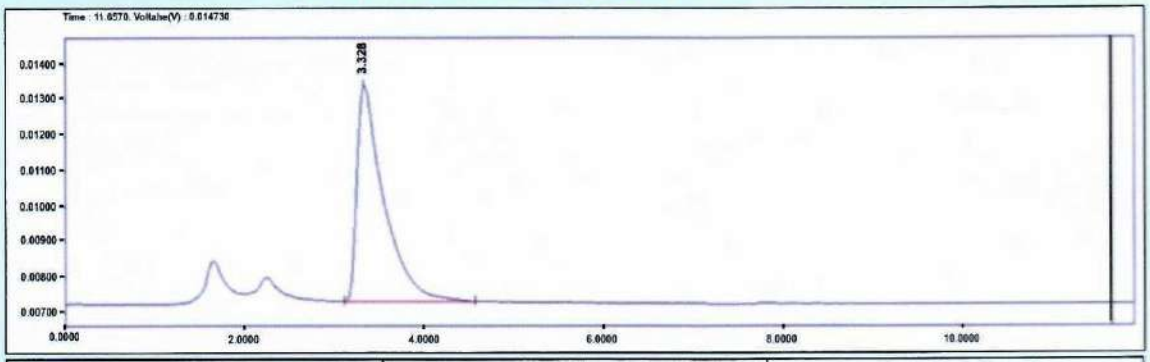
Various physical factors were find out for the maximum estimation of Purerain from *Butea superba*. These factors were kept constant and analysis of all the samples were carried out and following results were obtained as given in the table-4. Above experiments showed that the Purerain content depends on the temperature variation and optimum temperature of drying of the herb which was 40°C. Solvent polarity also played a very important role and it was concluded that the solvent fraction of CH_3OH (pure) gave maximum yield of Purerain. By

considering these factors all the collected samples were analyzed through High Performance Liquid Chromatography system (HPLC).

Detection parameters

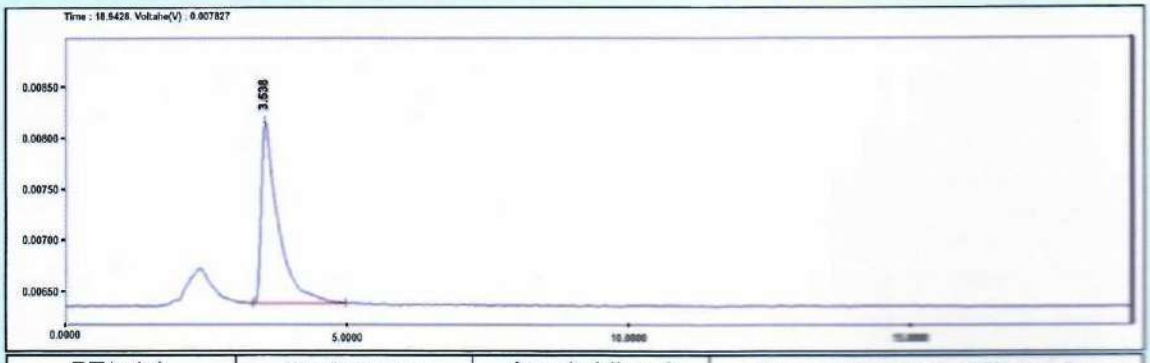
Solvent fraction	CH_3OH (Pure)
Wavelength	230nm
Column temperature	35°C
Flow rate	1ml/min

Standard chromatogram of Purerain:



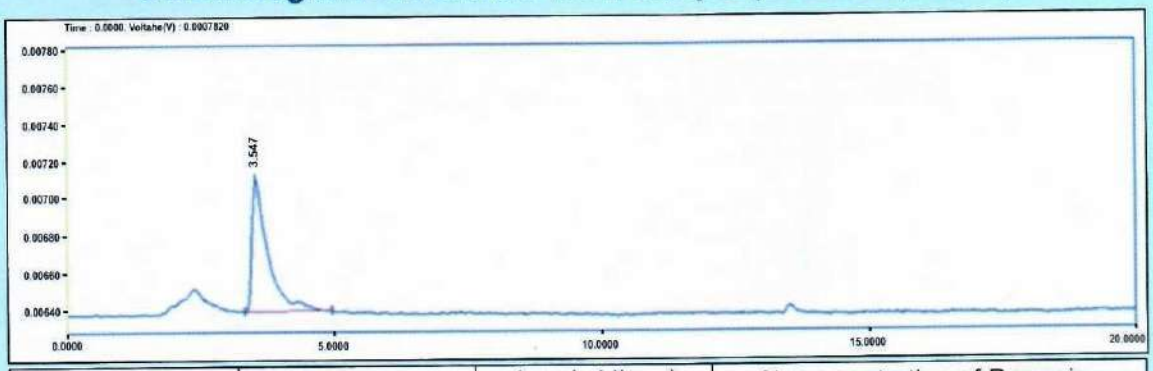
RT(min)	Peak name	Area(mV*sec)
3.328	Standard	127.040

Chromatogram of Purerain from sample (Sarni, Betul)



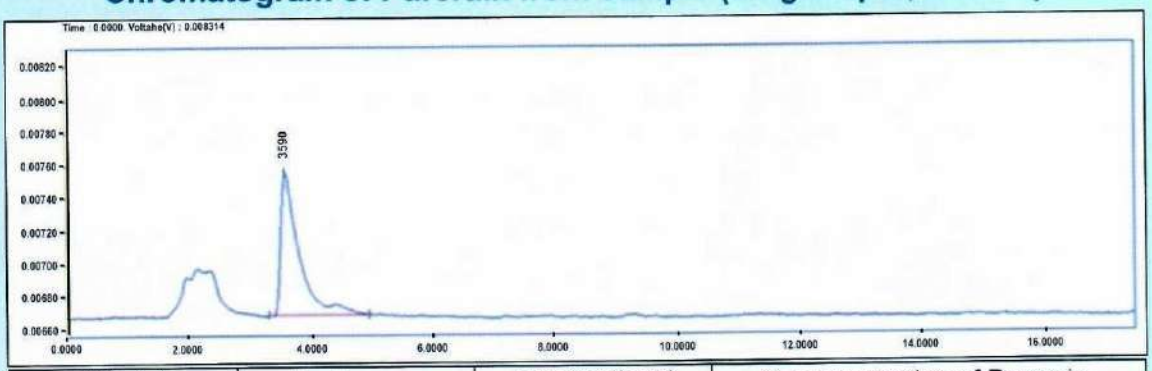
RT(min)	Peak name	Area(mV*sec)	% concentration of Purerain
3.538	Sample-1	37.230	4.41

Chromatogram of Purerain from sample (Mudwara, Satna)



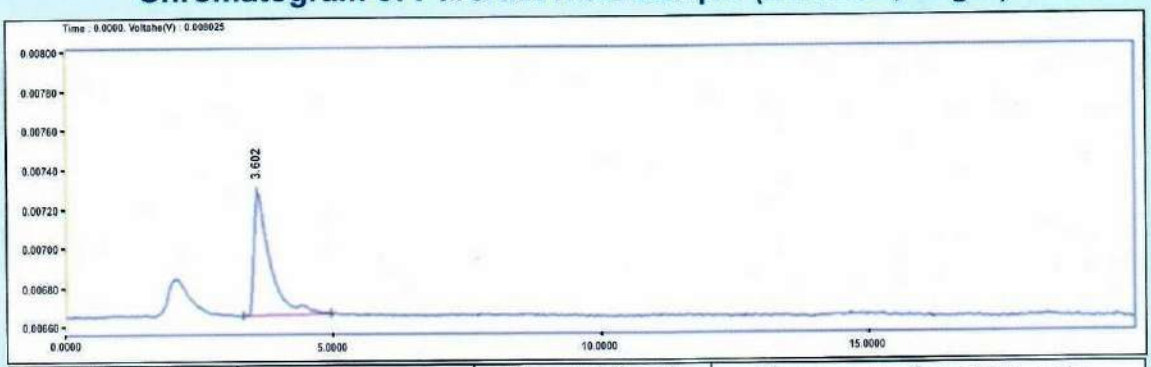
RT(min)	Peak name	Area(mV*sec)	% concentration of Purerain
3.547	Sample-2	14.942	1.78

Chromatogram of Purerain from sample (Singrampur, Damoh)



RT(min)	Peak name	Area(mV*sec)	% concentration of Purerain
3.590	Sample-3	18.161	2.16

Chromatogram of Purerain from sample (Berkheri, Sagar)



RT(min)	Peak name	Area(mV*sec)	% concentration of Purerain
3.602	Sample-4	13.189	1.57

Table-4 : % concentration of Purerain from the 9 forest divisions after chemoprofiling

S.No.	Accession source	Purerain (%) in roots ± SD
1.	**Sarni, Betul**	**4.41 ± 0.13**
2.	Mundwara, Satna	1.78 ± 0.09
3.	Singrampur, Damoh	2.16 ± 0.14
4.	Berkheri, Sagar	1.57 ± 0.13
5.	Bori, Hoshangabad	2.16 ± 0.25
6.	Kalpi, Mandla	2.15 ± 0.16
7.	Khandwa	2.10 ± 0.11
8.	Khargone	2.12 ± 0.12
9.	Teekamgarh	1.92 ± 0.20

From the above table it was interesting to observe that there were great variations of % concentration of bioactive compound, Purerain in collected germplasm of *Butea superba* from different forest areas. The *Butea superba* germplasm which were collected from Sarni and adjoining areas of Betul Forest Division showed maximum 4.41% Purerain and the minimum 1.57% Purerain was recorded from the germplasm of Berkheri, Sagar Forest Division. So, the germplasm of this species which were collected from Betul Forest Division was reported to be quality planting material because it contain maximum bioactive compound Purerain. This germplasm was further multiplied using clonal multiplication technology. As per available literature the chemoprofiling of *Butea superba* for the estimation of bioactive compound have not been reported so far from the state of Madhya Pradesh and this is the first kind of report particularly for *Butea superba*. This helps to establish chemofingerprinting library and Sarni, Betul in confined as chemomarker zone of this species.

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