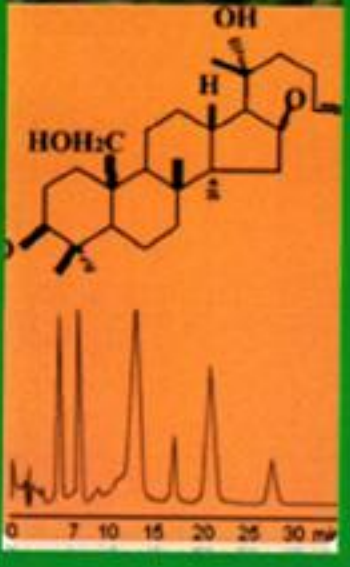

SFRI Technical Bulletin No. 76 (Series- I- Whole plant part)

Quantitative Estimation of Bioactive Compounds Through Chemo-fingerprinting (HPLC) For The Identification of Quality Germplasm

Andrographis Paniculata - Andrographolide

Bacopa monnieri - Bacoside-A

Swertia angustifolia –Swertiamarin

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Contents

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
HPTLC	High Performance Thin Layer Chromatography
TLC	Thin Layer Chromatography
IUCN	International Union for Conservation Natural Resources
DNA	Dioxy Nuclic Acid

Preface

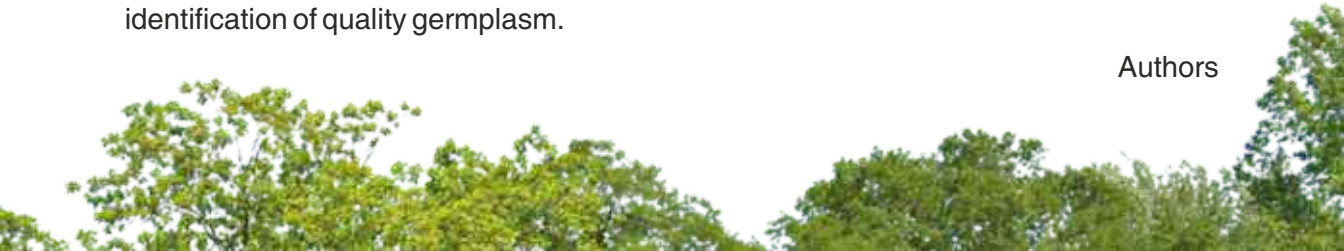
Since the time of civilization medicinal and aromatic plant are consumed by human beings for treatments of their various diseases. Today, in the modern world such group of plants has still significant importance as a raw drug or in a purified form. Large number of herbal based pharmaceutical industries are growing across the world for the formulation of life saving drugs against very server diseases such as cancer etc. WHO has emphasized the need to ensure the quality of medicinal plant products by using of modern control technique and applying suitable standards. Quality assessment of medicinal plants is very much essential for commercial exploitation as well as the medicinal value of the raw drug. Even authenticated plant materials may not be posses desire quality and strength and not conforming to the physiochemical parameters or the concentration of the bioactive constituents or biomarker compound as per the pharmacopoeial standard or the consumer/ industrial requirement. Such plant material is liable to rejected or accepted at very low price causing not only economic loss to the cultivators of collectors of medicinal plants but also entails doubtful efficacy or the potency of raw drug in the alleviation of the human suffering.

Today, identification of quality germplasm of medicinal plants is challenging task while this aspect of the research is vary essential for herbal based value added products. This technical bulletin highlights the Chemo-fingerprinting analysis from whole plant parts for the quantitative estimation of bioactive compounds present in the plant of *Andrographis paniculata, Bacopa monnieri* and *Swertia angustifolia* which are of medicinal importance.

Chemo-fingerprinting analysis was divided into four major parameters – optimization of temperature, solvent, mixture concentration, methods of extraction. The outcome of the work conducted on the basis of above parameters and the results obtained confirm that these methods are an efficient and reproducible analytical way to quantitative determination of the bioactive compounds from crude samples as well as samples.

The standardized chemo-fingerprinting technical bulletin (Series- I- Whole plant part) will help for the persons engaged in the sector of cultivation of medicinal plants, suppliers, traders, manufacturer of herbal drug based industries etc. for the identification of quality germplasm.

Authors



CHAPTER-1

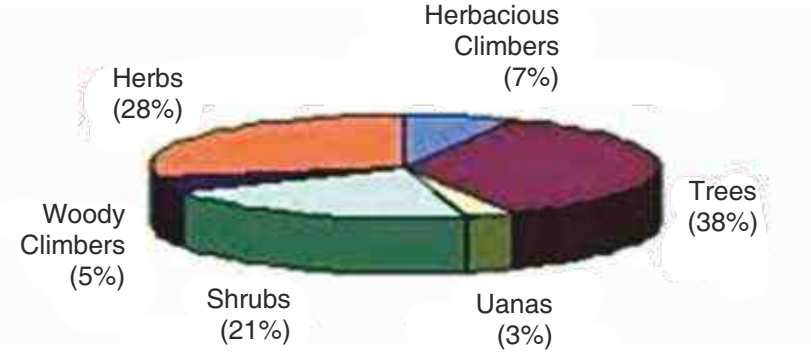
a. Introduction

World is endowed with a rich wealth of medicinal plants. Medicinal plants are the local heritage with global importance. Herbs have always been the principal form of medicine in India and presently they are becoming popular throughout the world, as people strive to stay healthy in the sphere of chronic stress and to treat illness with medicines that work in concert with the body's own defences. People in Europe, North America, and Australia are consulting trained herbal professionals and are using the plant medicines. Medicinal plants also play an important role in the lives of rural people, particularly in remote parts of developing countries with limited health facilities.

It is estimated that around 70,000 plant species, from lichens to towering trees, have been used at one time or another for herbal medicinal purposes. The herbs provide the starting material for the isolation or synthesis of conventional drugs. In ayurveda approximately 2000 plant species are considered to have medicinal value and about 500 herbs are still employed within conventional medicine, although whole plants are rarely used.

In India, medicinal plants have made a good contribution to the development of ancient Indian Material Medica. One of the earliest treatises on Indian medicine, the Charak Samhitha(1000 B.C.), records the use of over 340 drugs of herbal origin. Most of these continue to be gathered from wild plants to meet the demand of the medical profession. During the past one century there has been a rapid extension of the allopathic system of medical treatment generating commercial demand for pharmacopoeial drugs and their products.

The World Health Organisation (WHO) 2003 estimated that 80% of the population of developing countries being unable to afford pharmaceutical drugs and still relies on traditional medicines, mostly plant drugs, for their primary health care needs. Also, modern pharmacopoeia contains at least 25% drugs derived from plants.

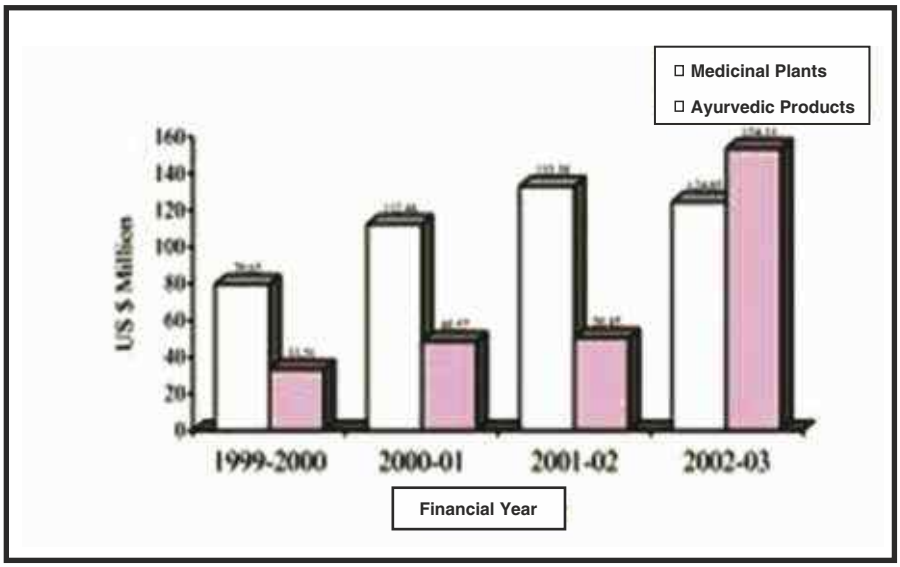


Distribution of herb, shrubs, climbers and trees

Demand for medicinal plants are increasing in both developing and developed countries due to growing recognition of natural products which are being non-toxic, having no side-effects and easily available at affordable prices. Medicinal plants have traditionally occupied an important position in the socio-cultural, spiritual and medicinal arena of rural and tribal lives. Millions of rural households use medicinal plants in a self-help mode. Over 20,000 practitioners of the Indian System of Medicine in the oral and codified streams use medicinal plants in preventive, primitive and curative applications. In recent years, the growing demand for herbal product has led to a quantum jump in volume of plant materials traded within and across the countries. According to an all India ethno biological survey carried out by the Ministry of Environment , Forests & Climate Change, Government of India reported that there are over 8000 species of medicinal plants are being used by the people of India.

Medicinal plant parts used as medicines

With the increasing demand of medicinal plants, their intensity and frequency of harvesting from wild also increased and as a consequence of that some plants came on to the verge of extinction.



Increasing demand of medicinal plants and ayurvedic products

Madhya Pradesh forests are richly endowed with large number of medicinal and aromatic plants including herbs, shrub and trees. The traditional healer of the state consumes these herbal plants as a raw drug for the treatment of several aliments. Since last four decades large number of medicinal and aromatic plants gradually disappearing from their natural habitat and some of them are at the verge of extinction.

As many factors such as soil composition, water stress, temperature and humidity can affect concentration of bioactive compounds present in medicinal plants. These variations in active ingredients of medicinal plants are often noticed, which is due to the synthesis and accumulation of a wide variety of biochemicals that are often plant-specific. These compounds collectively grouped as secondary metabolites are 'high-value low–volume' compounds biosynthetically derived from primary metabolism which help to defend, tolerate, adapt and adjust themselves against abiotic and biotic stresses including insect pests fungal and other pathogenic

diseases. Some of these agents can also act within the human body against microorganisms, other causes of disease and represent an important source of natural drugs.

The concentrations of these compounds found in plants are changes as their collection place changes. These variations recorded as the intra-specific variations of plants are merely due to habitat modifications and adaptation to a particular soil type and agro-climatic zone, rainfall, temperature etc. [The problem of variations is further compounded in medicinal plants which apart from displaying visible variations, synthesize and accumulate an array of plant specific chemicals. A study of variation in the active principles is often an important element in the investigation of variation in such plants. However, as the above factors significantly affect the quality of plants there is an immense need of documentation and development of appropriate methods for harvesting of medicinal plants or plant parts; isolation, extraction, effect of solvent polarity and solubility of phyto -constituents to maximize the phytochemical bioactive compound's yield.]

Alkaloids are an important class of basic organic compounds that occur in higher as well as in lower 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 administrated 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 and are localized in seeds, leaves, bark or root of the plant. 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, ether or chloroform. Most of them are having an intensely bitter taste and are poisons and they all are optically active compounds.

b. About the species

i. *Andrographis paniculata*

Local name- Kalmegh, Kaduchirayta

Andrographis paniculata (Burm. F.) Wall. Ex Nees (AP) also called as Kalmegh or "King of Bitters" belongs to family Acanthaceae. It has been used for centuries in Asia to treat gastro-intestinal tract and upper respiratory infections, fever, herpes, sore throat, and a variety of other chronic and infectious diseases. Indian Pharmacopoeia narrates that it is a predominant constituent of at least 26 Ayurvedic formulations. In Traditional Chinese Medicine (TCM), *Andrographis* is considered as the herb possessing an important "cold property" useful to treat the heat of body in fevers and to dispel toxins from the body.

Botanical Description

Andrographis paniculata is an annual, branched, herbaceous plant erecting to a height of 30-110 cm in moist shady places with stem acutely quadrangular, much branched and fragile texture. Leaves are simple, opposite, lanceolate, glabrous, 2–12cm long, 1–3cm wide with margin acute and entire or slightly undulated and upper leaves often bract form with short petiole.

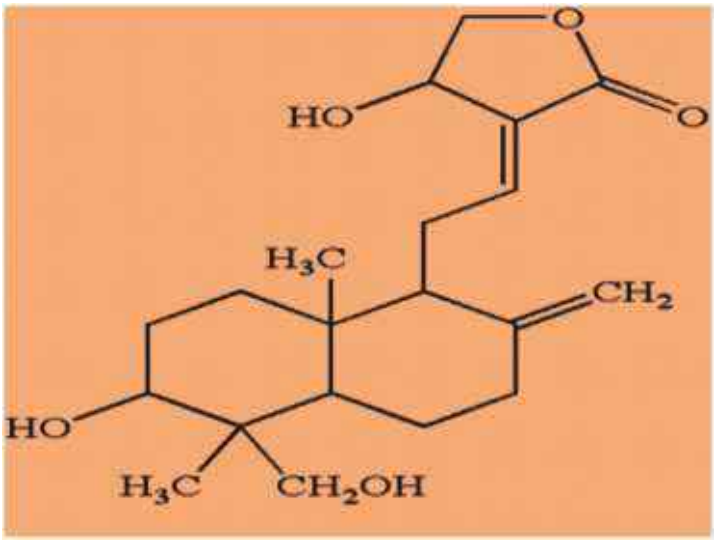
View of *Andrographis paniculata*

Habitat

It grows abundantly in India and Sri Lanka, Pakistan and Indonesia but it is cultivated extensively in China and Thailand, the East and West Indies, and Mauritius etc. *Andrographis* is normally grown from seeds where it grows in evergreen and deciduous forest areas. In India, it is cultivated during rainy phase of summer season in Kharif crop. Any soil having fair amount of organic matter is suitable for commercial cultivation of this crop. In Madhya Pradesh it is found in almost in all agro-climatic zones.

Phyto-chemistry

It is one of the most widely used plants in Ayurvedic formulations and whole



Molecular Structure of *Andrographis paniculata*

plant part known as "*Panchang*" (stem, leaf, flower, seed and root) is being used in various formulation of Indian system of medicine. It was recommended in *Charaka Samhita* (175 BC) for treatment of many diseases along with other plants in multi plant preparations. The characteristic secondary metabolites encountered in the *andrographis* for which it has [importance in the arena of medicinal plants and medicines. It is specifically rated very high in therapeutic action in curing liver disorders and common cough and cold in humans.]

A number of diterpenoids and diterpenoid glycosides of similar carbon skeleton have been isolated from *Andrographis*, mainly the most bitter compounds among them are andrographolide, neoandrographolide, deoxyandrographolide. Other such phytochemicals amassed by the plant are 14-deoxyandrographolide, 14-deoxy-11 12 didehydroandrographolide, andrographiside, deoxyandrographiside, homoandrographolide, andrographan, andrographon, andro-graphosterin and stigmasterol.

Molecular structure of andrographolide

Twelve other minor andrographolide related compounds have also isolated from the plant. There are some other minor constituents as well, which include seven flavonoid compounds, two long chain hydrocarbons, four dimmers of diterpenoids bis andrographolides A,B,C,D and sitosterol, tannin, traces of essential oil and two acidic polysaccharides. Four xanthones are also isolated from the roots of *Andrographis.* Two flavonoids, identified as 5,7,2,3 tetramethoxyflavonone and 5 hydroxy 7,2,3 trimethoxy flavones, as well as several other flavonoids are obtained from the whole plant.

Medicinal importance

Abortifacient, analgesic, antiperiodic, antipyretic fever reducer, antithrombotic, blood clot preventative, antiviral inhibiters, cancerolytic kills cancer cells, cardioprotective -protects heart muscles, coleretic- alters the properties and flow of bile, depurative- cleans and purifies the system, particularly the blood, digestive- promotes digestion, expectorant- promotes mucus discharge from the respiratory system, hepatoprotective- protects the liver and gall bladder , hypoglycemic- blood sugar reducer, immune Enhancement- increases white cell phagocytosis, inhibits HIV-1 replication, and improves $CD4^{+}$ and T lymphocyte counts, laxative- aids bowel elimination and vermicidal- kills intestinal worms etc.

ii. *Bacopa monnieri*

Local name- Brahmi

Bacopa monnieri belongs to family Scrophulariaceae is an critically endangered medicinal herb, found throughout the Indian subcontinent in wet, damp and marshy areas. It is a creeper perinnial herb bitter in taste. The whole plant is used

as a source of brain tonic medicine. *Bacopa* is used in Ayurveda, for the treatment of anxiety, and in improving intellect and memory for several centuries. It is mainly used as cardiac tonic, splenic disorder, digestive aid, anti inflammatory agent and bronchodilator. In addition to memory boosting activity, it is also claimed to be useful in the treatment of cardiac, respiratory and neuropharmacological disorders like insomnia, insanity, depression, psychosis, epilepsy and stress. It is reported to possess anti-inflammatory, analgesic, antipyretic, sedative free radical scavenging and anti-lipid per oxidative activities also.

Botanical Description

Bacopa monnieri is a perennial, creeping herb. It whose habitat includes wetlands and muddy shores. Common name is (English) brahmi. This plant is also known as thyme-leafed gratiola, "figwort" or "moneywort". The leaves of this plant are succulent relatively thick oblanceolate and are arranged oppositely on the stem. The flowers are small and white, with four or five petals.

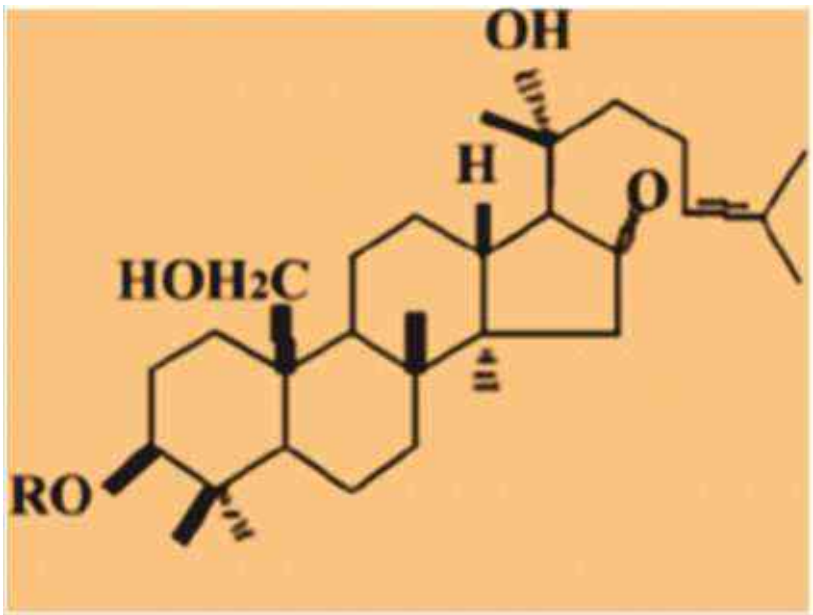
View of *Bacopa monnieri*

Habitat

It is commonly grows in marshy areas throughout India, Nepal, Sri Lanka, China, Taiwan, Vietnam and is also found in Florida and other southern states of the USA. In Madhya Pradesh the natural habitats of this species are very rare due to over exploitation, it is found in Pachmari, Amarkantak and Satna district.

Phyto-chemistry

This plant has a number of uses in Ayurveda. The pharmacological properties of *Bacopa* include saponins, alkaloids, herpestine, mixture of three bases and sterols. Some important active constituents like betulic acid, stigmasterol, beta-sterol, as well as numerous bacosides and bacopa saponins are also found in *Bacopa.* The pharmacological properties of *Bacopa monnieri* are studied extensively and the activities are attributed mainly due to the presence of



Molecular structure of bacoside-A

characteristic saponins called as bacosides. Bacosides are complex mixture of structurally closely related compounds, glycosides of either jujubogenin or pseudojujubogenin. This bacoside is further differentiated into bacoside-A and bacoside-B.

Traditionally it is used for treatment for epilepsy, asthma improving memory capacity and motor learning ability and antianxiety effects. It is listed as a drug that enhances cognitive ability. In India, this plant has also been used traditionally to consecrate newborn babies in the belief that it helps to open the gateway of intelligence. Recent studies suggest *Bacopa* may improve intellectual activity. It has antioxidant properties, reducing oxidation of fats in the bloodstream.

Medicinal Uses

Several research findings conducted in past decades have confirmed that *Bacopa*, if properly administered, has a surprisingly broad range of pharmacological effects, some of them are extremely beneficial such as, analgesic pain killer reduces swelling and cuts down exudation from capillaries, antiflammatory action probably mediated in part by adrenal function, antipyretic fever reducer, sedative rlaxing herb, cognitive enhancing nerve impulse transmission, anti- ischemic it exerts a powerful relaxing effect on the pulmonary arteries, anti- inflammatory, anti-convulsive, antiallergic, anticarcinogen, bronchiolitic, thyroid-stimulant,anti-stress, abortifacient, anxiety reduces mental stress, insomnia sleeplessness reduces sleeplessness, insanity relaxing herb reduces depression., psychosis,epilepsy reduces unconsciousness.

iii. *Swertia angustifolia*

Local name- Chirayta

Swertia angustifolia an critically endangered medicinal plant belongs to family Gentianaceae and commonly known as Chirayta. This species is used in traditional medicine for a wide range of diseases. The plant contains useful bioactive compounds which are used in the treatment of several aliments.

Botanical Description

It is an annual herbaceous plant. *Swertia angustifolia* has an erect, about 2–4 ft long stem, the middle portion is round, while the upper is four-angled, The stems are soft and greenish in colour , and contain white flowers with purple spots on petals. Leaves are long lanceolate greenish in colour.

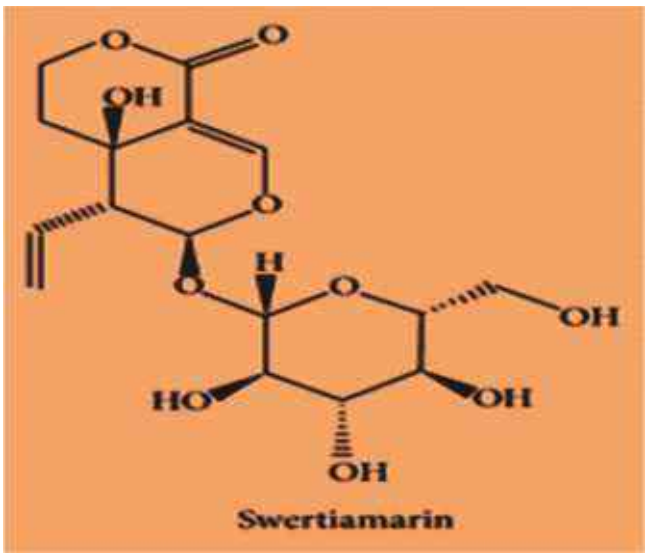
View of *Swertia angustifolia*

Habitat

Globally it is distributed in Nepal, Pakistan, Bhutan, South and Southwest China and India. In India it is found in Jammu & Kashmir, Himachal Pradesh, Punjab, Uttarakhand, Sikkim, Assam and some part of Tamilnadu and Madhya Pradesh in moist and hilly tracks.

Phyto-chemistry

The plant contains useful bioactive compounds which are used in the



Chemical Structure of Swertiamarin

treatment of several aliments. The major bioactive compounds of *Swertia angustifolia* is Swertiamarin with enormous pharmacological potentials. It is also reported as is Anti-diabetic activities due to presence of gentinine.

Medicinal Uses

This species is used in traditional medicine for a wide range of diseases such as malarial fever, bronchial asthma, blood purifier and febrifuge due to presence of important alkaloid swertiamarin. It is also use as a substitute of *Swertia chraytia.*

c. Chemo-fingerprinting

Within the context of increased herbal medicines use and lack of effective regulatory control, the safety of herbal medicines has become a key priority issue. For converting botanical materials into medicines, herbal drug technology is used where standardization and quality control with proper integration of modern scientific techniques and traditional knowledge is important. The new Pharmacognosy includes all the aspects of drug development and discovery.

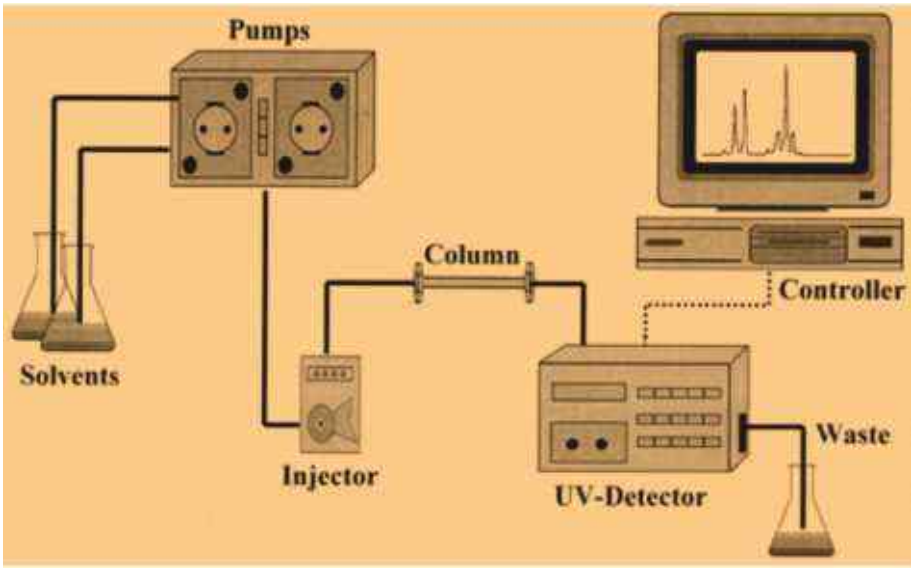
Scientifically validated and technologically standardized herbal medicines may be derived using a safe path of reverse pharmacology approach based on traditional knowledge database. This may play a vital role in drug discovery, development and therapeutics, in addition to dealing with a typical western against Ayurveda. Correct identification and quality assurance of the starting material is therefore, an essential prerequisite to ensure reproducible quality of herbal medicine, which contributes to its safety and efficacy.

Selection of chemical markers is crucial for the quality control of herbal medicines, including authentication of genuine species, harvesting the best quality raw materials, evaluation of post harvesting handling, assessment of intermediates and finished products, and detection of harmful or toxic ingredients .Chemo-fingerprinting has been demonstrated to be a powerful tools and technique for the identification of quality germplasm.

Thin layer chromatography (TLC) and High Performance Thin Layer Chromatography (HPTLC) and High Performance Liquid Chromatography (HPLC) are valuable tools for qualitative determination of small amount of bioactive compounds in medicinal plants. For quantitative studies, specific marker is used. For

example, *Andrographis paniculata* is reported to have three chemotypes depending upon the presence of a class of closely related steroidal lactones like andrographolide, neoandrographolide and 14-deoxyandrographolide etc. The contents of these and other biologically active compounds may vary depending upon place to place due to variation in agroclimatic zones, environment, genotype, time of collection of plant material, etc.

Chemo-fingerprinting should be used at various stages of the development and manufacturing of an herbal medicine, such as authentication and differentiation of species, collecting and harvesting, quality evaluation, identification of quality germplasm , diagnosis of intoxication and discovery of lead compounds.



Diagrammatic representation of HPLC system

Furthermore, there are many technical challenges in the production of bio-chemical markers. For example, temperature, light and solvents often cause degradation and or transformation of purified components, isomers and conformations may also cause confusions of chemical markers. Therefore, it is essential to prepare a data base inventory of chemical profile on the basis of available bioactive chemical compounds of plants which may help in the development and manufacturing of herbal medicine and identification of quality germplasm.

d. 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 areas of Madhya Pradesh.
2. To find out an appropriate solvent, mixture of solvents and their ratio for the isolation of bioactive ingredients from raw material.
3. To standardize chemo-fingerprinting methods for quantitative determination of bio-active ingredients present in the species.
4. To find out quality planting material for further genetic improvement programme.
5. To find out the best harvesting time of plant at different stage of maturity to optimize best collection time.

CHAPTER -3

Material and Method

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 those 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 of 20 plants from potentially rich areas of the sate were collected.

Following three species were selected for the study and the collection parameters of these species were as follows-

i. *Andrographis paniculata*

Kalmegh is known as king of bitter and according to traditional knowledge complete plant (panchang) is used for herbal formulations. To quantify the maximum andrographolide content in plant whole plant including leaves, flowers, fruits and roots (panchang) were collected during the month of September to December due to its occurrence after rainy season. The germplasm were collected from whole M.P.

ii. *Bacopa monnieri*

Whole plant of *Bacopa* contains bacosides and used for ayurvedic formulations. Complete or whole plants of the herb were collected after rainy season at quarterly interval. The germplasm were collected from Panchmarhi, Satna and Chhindwara.

iii. *Swertia angustifolia*

Whole plant of *Swertia angustifolia* contains swertiamarin and used for ayurvedic applications. Complete plants of the herb were collected after rainy season from September to December as it dried afterwards. The germplasm were collected from Amarkantak, Umania Rajandra garm (MP.) and Koddainal Tamilnadu

b. Drying of collected germplasm

Temperature for drying of collected samples

Different methods of drying were applied on the herb after its collection. Collected samples were washed under running tap water separately and left for drying by spreading on filter paper under shade in aerated room and under oven at different temperatures as below mentioned table.

i. *Andrographis paniculata*

Drying temperatures and time period of drying

S No	Method of drying of the herb	Season	Temperature(^{0}C)	Time taken for drying (days)
1	Spreading material on filter paper and dry at room temperature	September - December	15 - 25	20 -25
1.	Drying in oven		30	15 -20
2.	Drying in oven		35	10 -15
3.	Drying in oven		40	8 -10
4.	Drying in oven		45	7
5.	Drying in oven		60	1

ii. Bacopa monnieri

Drying temperatures and time period of drying

S No	Method of drying of the herb	Season	Temperature(^{0}C)	Time taken for drying (days)
1	Spreading material on filter paper and dry at room temperature	Jan–March	10 -30	25 -30
		April - June	35 -40	15 -20
		July - Sept.	25 -35	40 -45
		Oct. - Dec.	25 -10	30 -35
2	Drying in oven at	-	25	10 -15
3	Drying in oven at	-	30	10 -12
4	Drying in oven at	-	35	8 -10
5	Drying in oven at	-	40	6 -8
6	Drying in oven at	-	45	5
7	Drying in oven at	-	50	2
8	Drying in oven at	-	60	1

iii. *Swertia angustifolia*

Drying temperatures and time period of drying

S No	Method of drying of the herb	Season	Temperature(°C)	Time taken for drying (days)
1	Spreading material on filter paper and dry at room temperature		15 - 25	20 -25
2.	Drying in oven	September-December	30	15 -20
3.	Drying in oven		35	10 -15
4.	Drying in oven		*40*	8-10
5.	Drying in oven		45	7
6.	Drying in oven		60	1

c. 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, Indi a*
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* *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 were used in the extraction process)

S No	Name of the chemical	Specification of the chemical
1	Methanol	Acronym - CH_3OH Specific density - 20 ^0C Percentage purity - 99.9% Manufacture E - Merck, India
2	Acetonitrile	Acronym - CH_3CN Specific density - 20 ^0C Percentage purity - 99.9% Manufacture E - Merck, India
3	HPLC grade water	Acronym - HOH Specific density - 20 ^0C Percentage purity - 99.9% Manufacture E - Merck, India
4	Hexane	Acronym Specific density - 20 ^0C Percentage purity - 99.9% Manufacture E - Merck, India
5	Hydrocholric acid	Acronym - HCl Specific density - 20 ^0C Percen tage purity - 99.9% Manufacture E - Merck, India
6	Ammonia	Acronym - NH 3 Specific density - 20 ^0C Percentage purity - 99.9% Manufacture E - Merck, India
7	Chloroform	Acronym - CH_3CN Specific density - 20 ^0C Percentage purity - 99.9% Manufacture E - Merck, India
8	Sodium sulphate	Acronym - Na_2SO_4 Specific density - 20 ^0C Percentage purity - 99.9% Manufacture E - Merck, India
9	Dragon droff's reagent	Acronym Specific density - 20 ^0C Percentage purity - 99.9% Manufacture - CDH
10	Standards	Andrographoloid (Sigma, USA) *Bacoside -A*(Sigma, USA) Swertiamarin - Natural Remedies, Bangalore

d. Sample preparation for quantitative determination

After the drying process for *Andrographis paniculata, Bacopa monnieri* and *Swertia angustifolia* species were takes place and samples were powdered separately for extraction process.

Extraction process
Soxhlet extraction

2 gm of powered material with 20 ml of solvent mixtures 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_3 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 triterpenoids were confirmed by Dragon Droff's reagent 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	CH_3OH
2	90% CH_3OH
3	80% CH_3OH
4	70% CH_3OH
5	60% CH_3OH
6	Pure CH_3CH
7	90% CH_3CH
8	80% CH_3CH
9	70% CH_3CH
10	60% CH_3CH

e. General Method for sample extraction

Fresh leaves, roots and whole plants 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.

↓

Then loaded on Rotor-vapour and heated approximately at 80-85^{0}C.The remaining concentrated material is alkaloid with some impurities.

↓

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 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.

f. Preparation of standard solution

5.0 mg standard of designated species were accurately weigh 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°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.

g. 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. The mobile phase was Methanol, Acetonitrile and HPLC grade water degassed with ultra sonic cater, wavelength was recorded through UV detector. Column temperature was ambient at 35^{0}C, Flow rate was 1 ml/min.

h. Standard formula was used for the estimation of percent concentration of bioactive compounds.

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

CHAPTER-4

Chemo-fingerprinting Protocol

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

The HPLC methods for the quantitative estimation of andrographolide, bacoside-A and swertiamarin 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 quantification of bioactive compounds.

(i) Chemo-fingerprinting protocol for *Andrographis paniculata*

Optimum temperature and condition for drying of plant samples:

Oven temperature	-	40^0C
Number of days for drying of plant samples	-	8-10
Extraction methods	-	Soxhlet

Detection parameters

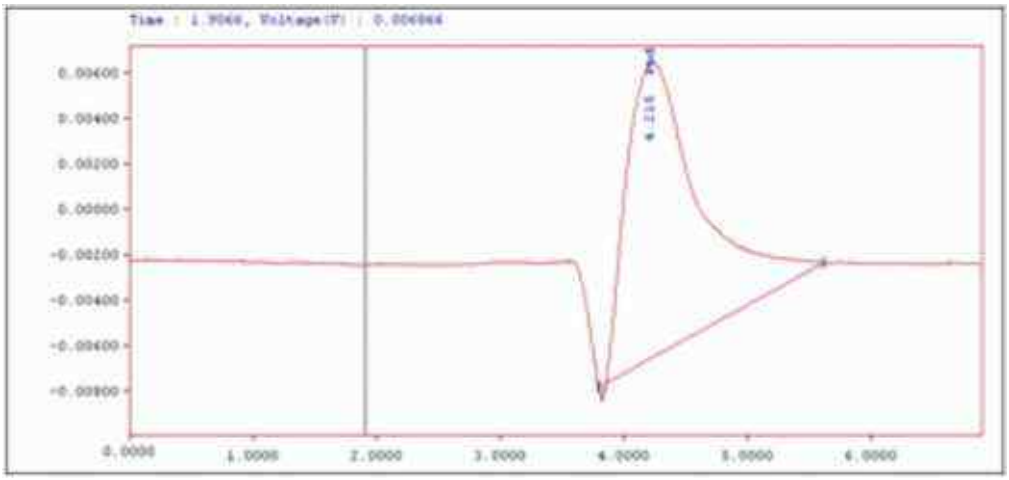
Solvent fraction	-	Methanol (CH_3OH): Hydrogen hydroxide(HOH) (70:30)
Wavelength	-	230nm
Column temperature	-	35^0C
Flow rate	-	1ml/min
Column	-	C-18 ODS2

Range of percent concentration of bioactive compound (andrographolide)-

Whole plant	**-**	**0.331 to 0.831% .**
Roots	**-**	**0.290 to 0.698%.**
Leaves	**-**	**0.64 to 1.840%.**

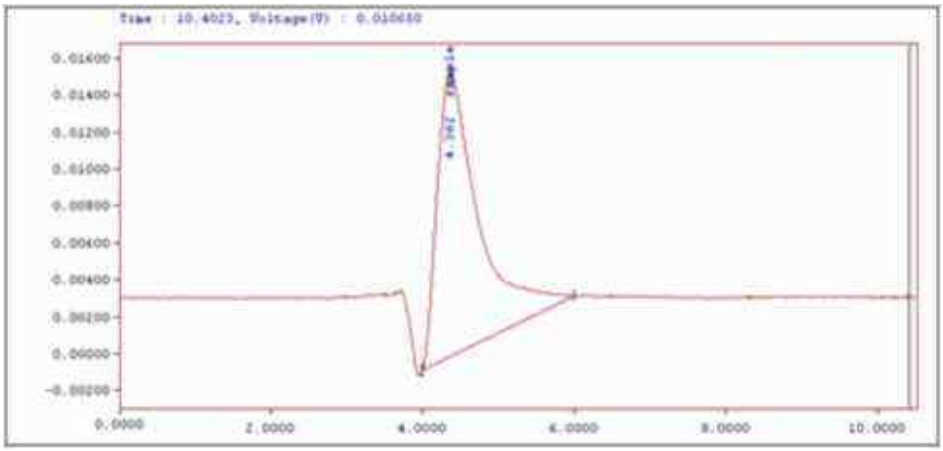
Maximum percent concentration of andrographolide was found in **Chhindwara forest Division (1.840%)**, followed by **Hoshangabad (1.830%)**, and in **Rewa it was found minimum (0.613%)** during November to December.

Standard chromatogram of andrographolide of *Andrographis paniculata*



RT(min)	Peak name	Area(mV*sec)
4.215	std	533.826

Sample chromatogram of andrographolide of *Andrographis paniculata*



RT(min)	Peak name	Area(mV*sec)
4.362	sample	586.508

(ii) Chemo-fingerprinting protocol for *Bacopa monnieri*

Optimum temperature and condition for drying of plant samples:

Oven temperature - 60°C

Number of days for drying of plant samples - 1

Extraction methods - Soxhlet

Detection parameters

Solvent fraction - Methanol (CH_3OH): HPLC grade water (80:20)

Wavelength - 230nm

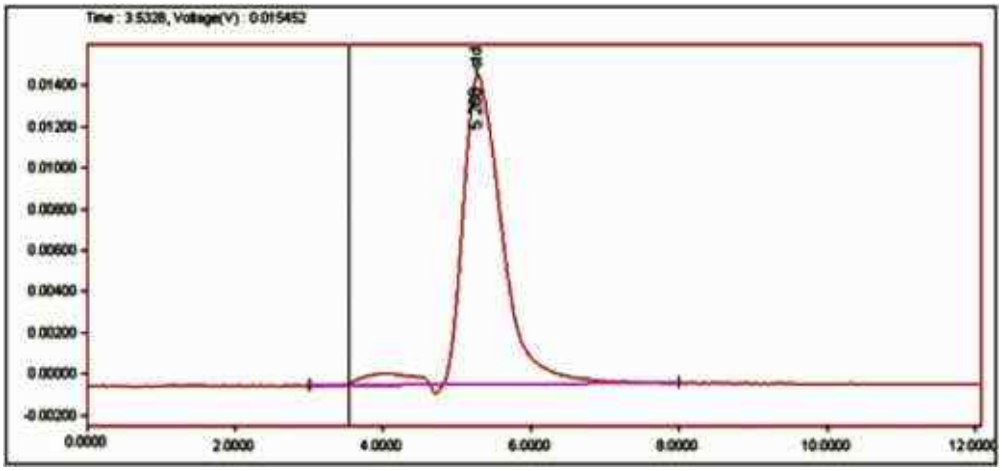
Column temperature - 35°C

Flow rate - 1ml/min

Column - C-18 ODS2

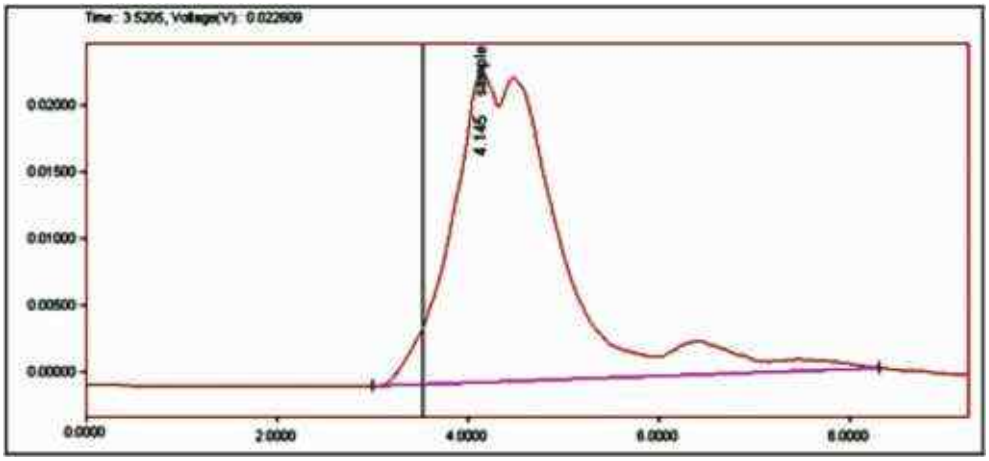
Range of percent concentration of bioactive compound from whole plants - 1.05 to 1.60% .

Maximum percent concentration of **Bacoside-A was found in Panchmari forest area(1.60%)**, followed by **Satna (1.45%)**, and in Chhindwara it was found minimum (1.05%). The maximum concentration of bioactive compound Bacoside-A between **February to Jun**e



RT(min)	Peak name	Area(mV*sec)
5.268	std	571.076

Sample chromatogram of Bacoside-A from *Bacopa monnieri*



RT(min)	Peak name	Area(mV*sec)
4.145	sample	1845.688

(iii) Chemo-fingerprinting protocol for *Swertia angustifolia*

Optimum temperature and condition for drying of plant samples:

Oven temperature	-	40^0C
Number of days for drying of plant sample	-	8-10
Extraction methods	-	Soxhlet

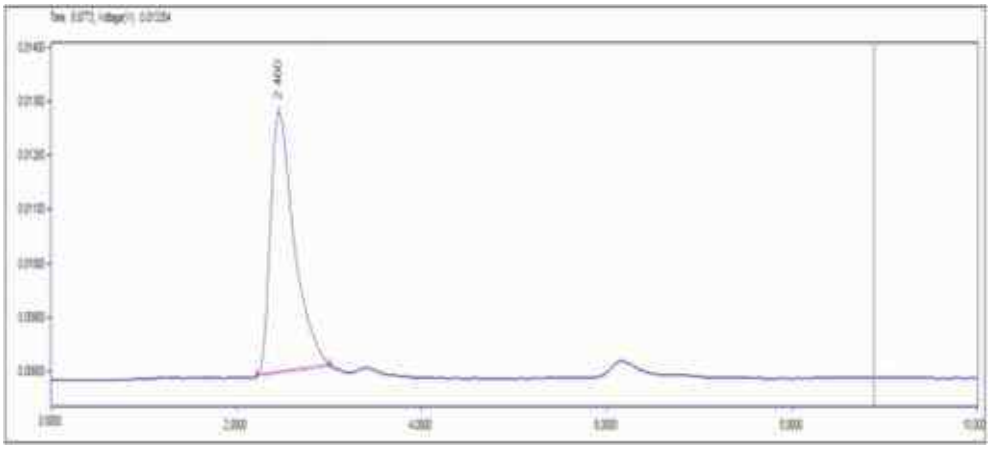
Detection parameters

Solvent fraction	-	Acetonitrile
Wavelength	-	254 nm
Column temperature	-	35^0C
Flow rate	-	1ml/min
Column	-	C-18 ODS2

Range of percent concentration of bioactive compound from whole plants- 2.28 to 4.51%.

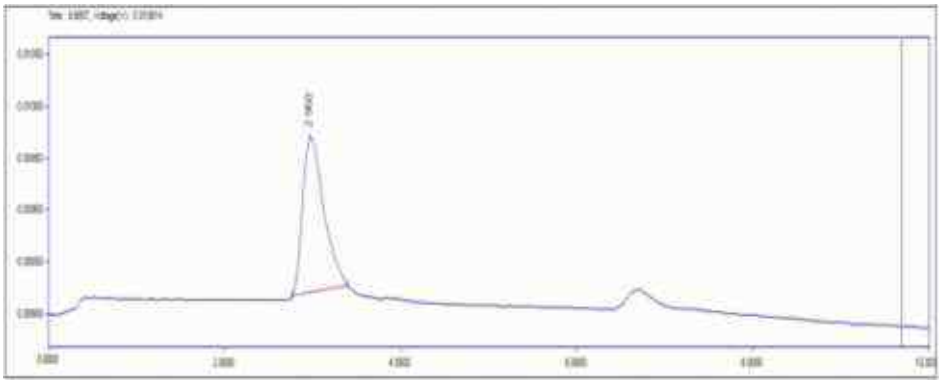
Maximum percent concentration of **Swertiamarin was found in Amarkantak forest area(4.51%)**, **followed by Omaniya, Rajendragram (4.40%), and in Kodaikanal (TN)** it was found minimum (2.28%).As the plant is seasonal, therefore the bioactive compound **swertiamarin** was found maximum during the month of **December.**

Standard chromatogram of Swertiamarin *Swertia angustifolia*



RT(min)	Peak name	Area(mV*sec)
2.460	Standard	85.412

Sample chromatogram of Swertiamarin from *Swertia angustifolia*



RT(min)	Peak name	Area(mV*sec)
2.960	sample	25.554

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