

“Studies on callus and root cultures of *Rubia cordifolia* and identification of marker constituent using HPTLC”

Submission of *Final Technical Report*

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INTRODUCTION

In developing countries 80% of the population use herbal medicines in primary health care. They have been culturally accepted and have stood the test of time for their safety, efficacy, and lesser side effects. The chemical constituents present in them are a part of the physiological functions of living flora and hence they are believed to have better compatibility with the human body. Ancient literature mentions herbal medicine for age related diseases namely memory loss, osteoporosis, diabetic wounds, immune and liver disorders etc., for which no modern medicines, only palliative therapy is available (Kamboj, 2000).

India is a largest producer of medicinal herbs and is rightly called the "Botanical Garden of the world". Medicinal herbs have been in use for thousands of year, in one form or another, under the indigenous system of medicines like Ayurveda, Siddha, Unani and homeopathy (Choudhary, 1996). Traditional medicine is defined as the sum total of all the knowledge and practices, whether explicable or not, use in diagnosis, preventive and elimination of physical, mental and social imbalances and relying exclusively on practical experiences and observations handed down from generations to generations whether verbally or in writing (Saxena, 2001). India has one of the 12 mega biodiversity centers having over 45,000 plant species. About 1500 plants with medicinal uses are mentioned in ancient texts and around 800 plants have been used in traditional medicine (Kamboj, 2000). Some important medicinal plants with its activity are mentioned in Table 1 (Natesh, 2001).

The Indian herbal market is about \$ one billion and the export of herbal crude extracts is about \$ 80 million, which is less compared to other countries. A report prepared by the Export-Import Bank of India (Anonymous, 1997) has estimated that the international market of medicinal plant related trade is in the region of US \$ 60 billion, and growing annually at the rate of 7%. The current global market is pegged at US \$ 62 billion (Fig. 1). According to estimates by Ayurvedic Drug manufactures Association (ADMA), the current value of trade

Table 1: Some important crude drugs that have received clinical support for their therapeutic claims.

Botanical name	Vernacular name	Key constituent	Type of activity
<i>Adhatoda zeylanica</i>	Vasa	Vasicine	Bronchodilator
<i>Andrographis paniculata</i>	Bhuinimba	Andrographolide	Hepatoprotective
<i>Bacopa monnieri</i>	Brahmi	Bacosides	Memory enhancer
<i>Boerhavia diffusa</i>	Punarnava	Punarnavine	Diuretic, anti-inflammatory
<i>Cassia angustifolia</i>	Senna	Sennosides	Bowel stimulator, antiabsorptive
<i>Centella asiatica</i>	Mandukparni	Asiaticoside	Skin diseases
<i>Curcuma longa</i>	Turmeric / Haridra	Curcumin	Anti-inflammatory
<i>Ephedra</i> sp.	Ephedra	Ephedrine	Bronchodilator
<i>Ginkgo biloba</i>	Ginkgo	Bilobalide, ginkgolides, flavonoids	Anti-ischemic, antihypoxidotic.
<i>Hollarrhena antidysentrica</i>	Kutaja	Conessine	Antidysentric.
<i>Plantago ovata</i>	Psyllium	Mucilages, hemicelluloses	Laxative
<i>Psoralea corylifolia</i>	Bakuchi	Psoralen, bakuchiol	Antileucoderma, antibacterial
<i>Rubia cordifolia</i>	Manjistha	Munjistin	Blood purifier
<i>Valeriana officinalis</i>	Valerian	Valepotrites	Hypnotic.

in Indian Systems of Medicine (chiefly Ayurveda, Siddha and Unani) and Homeopathy is around rupees 4205 crores, roughly close to US \$ 1.0 billion (Fig. 2). However, this figure does not take account of other streams tribal / folk, allopathy, beauty and personal care industry as well as nutraceutical market (Natesh, 2001).

The herbal medicine market worldwide is shown in table 2. A missionary approach is the need of the hour to exploit our resources in herbal drugs to earn billions of dollars from global market. The need is the proper identification of the herbal source available to us, correct estimate of demand and supply, encouragement of value addition process, standardization, introducing effective mechanism for certification and promoting herbal extraction units to take advantage of the massive industrial approach in the area of herbal drug.

The herbal drug in south Asia mainly comes from cultivated or collected medicinal plants and there is a great variation in the quality, chemical authenticity and shelf life, depending on the method/season of harvesting, storage, transportation and handling, habitat and drying conditions. The therapeutically active constituent of the phytomedicinal drugs are not known unless marker technique can be employed to characterized them.

Most of the raw material used by the herbal drug industry is procured from wild sources and this results in inconsistency in quality, irregularity in supply, adulteration and substitution. Standardization of drugs of well-defined consistent quality is needed for reliable clinical trial/ beneficial therapeutic use. The standardization of an herbal drug is not only an analytical separation. It does not end with the identification and assay of an active principle rather it embodies total information and controls necessary guarantee consistency of composition. Indian herbal drug industry needs to ensure procurements of standardized authentic raw material, free from microbial load and heavy metal, improving processing techniques and maintaining in process quality control which will ensure standardized herbal product with proven therapeutic efficacy, safety and shelf life.

Table 2
Market size of herbal medicines.
(Kamboj, 2000)

Country	Drug sales in US \$ (billion)
Europe (1991)	
Germany	3.0
France	1.6
Italy	0.6
Others	0.8
Europe (1996)	~ 10.0
USA (1996)	4.0
India	1.0
Other countries (1996)	5.0
All countries (1998).	~ 30.0 - 60.0

Fig 1

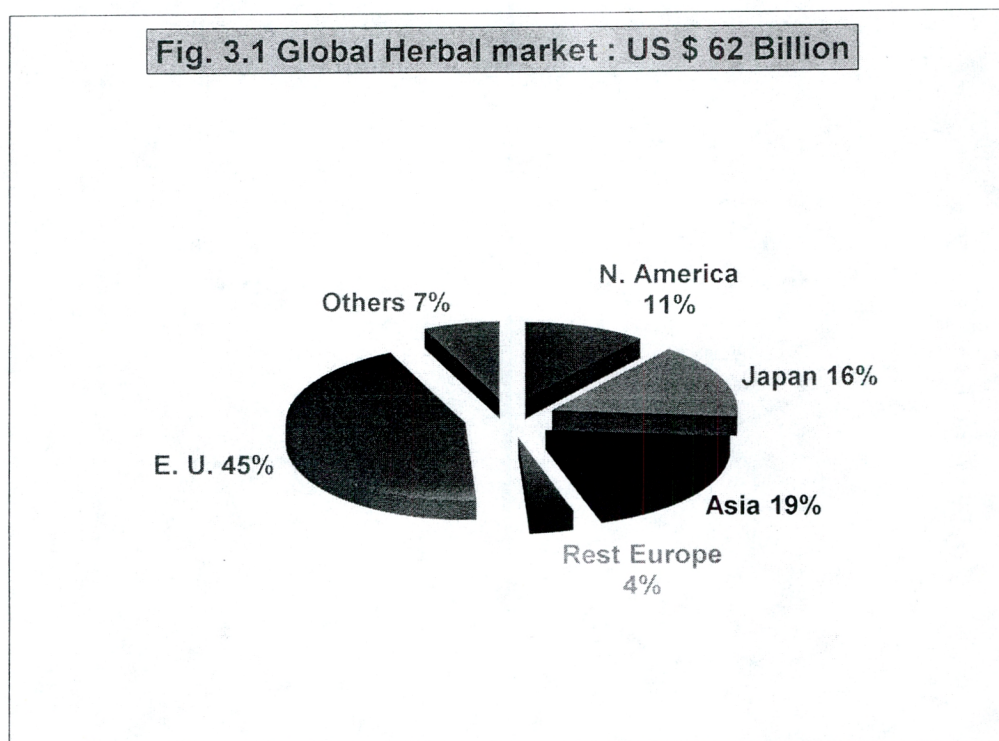
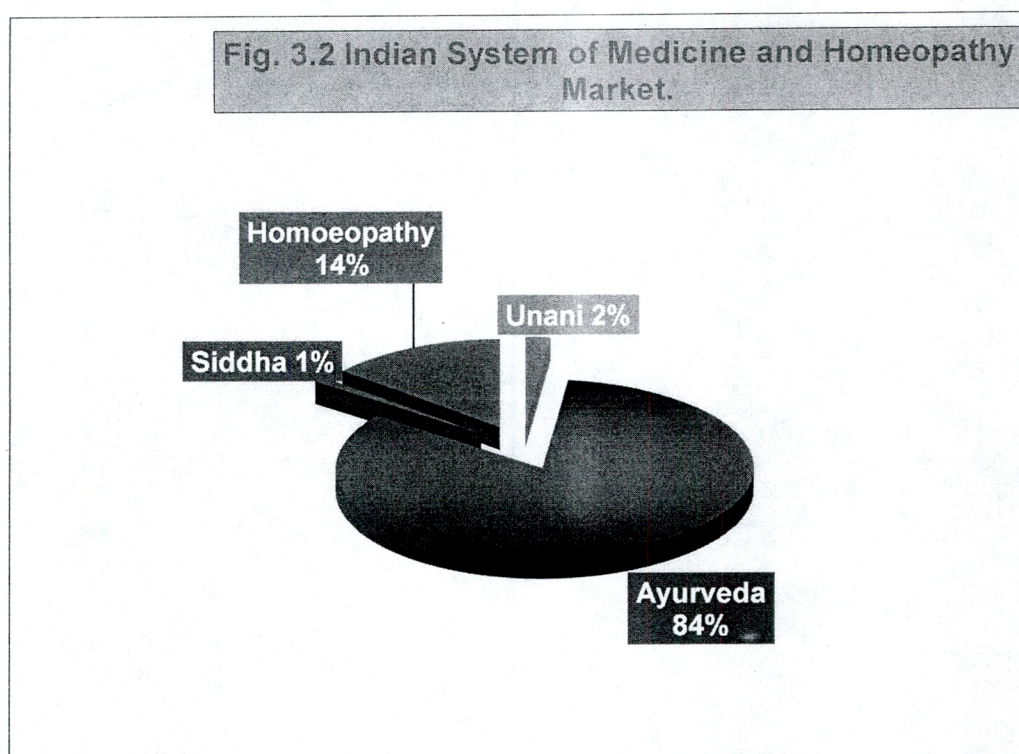


Fig 2



Foreign importers are not sure of quality of the herbal drugs due to the absences of reliable or official agencies for certification about quality (Choudhary, 1996). Another reason for standardization is to convince the Indian market. There is a prevalent belief, both among consumers and practitioners, that drug prepared from plants collected in the wild are somehow more efficacious. This preference for natural product has had adverse impact on wild populations of medicinal

plants. Hence the conventional and modern techniques like tissue culture techniques are not accepted. So to ensure the quality of the herbal products, authentication of the raw material plays a crucial role (Natesh, 2001.)

Authentification of drug sample is the major area that requires urgent attention. Medicinal plants are traded mostly as bark, roots, twigs, flowers, fruits and seeds. The chances of adulteration both deliberate and unintentional are very high. Also survey of the market samples of herbal drugs in India show that sometimes-different species are sold as the same drug in different parts of country. In such cases, efficacy and even safety of the preparation may be compromised. The popular prescription that herbal preparations are relatively harmless is often misplaced because several plants are known to be potentially hazardous (Tyler, 1989; Betz and Page, 1998). Therefore, there is a need for standardization. The purpose of standardization has to go beyond the morphological level into the identity of the active principle where known, or a biologically active marker compounds.

WHO in its various resolutions emphasized the need to ensure quality control of medicinal plants and their product. Various Government agencies like Department of Indian System of Medicines, Ministry of Health and Family Welfare, Council of Scientific and Industrial Research, Indian Council of Medical Research, Department of Biotechnology and Department of Biotechnology of India and other private organizations and Universities are engaged in standardization and quality evaluation of herbal drugs.

The major traditional sector Pharmas, namely Himalaya, Zandu, Dabur, Hamdard, Maharshi etc and modern sector Pharmas, namely Ranbaxy, Lupin, Allembic etc are standardizing their herbal formulations by chromatographic techniques like TLC/HPTLC/HPLC finger printing. There are about 7000 firms in the small-scale sector manufacturing traditional medicines with or without standardization. However, none of the Pharma has standardized herbal medicines using active compounds as marker linked with confirmation of biodiversity of herbal drugs (Kamboj, 2000).

The therapeutic activity of the medicinal plant lies in the organic molecules possessing pharmacological properties that are produced by them. With the advent of new analytical tools and sophisticated instrumental technology, it has become possible to carry out practicable quality assurance profiles for a crude drug, its bioactive constituents and the formulations themselves. Some modern techniques such as UV -Spectroscopy, IR-Spectroscopy, Atomic absorption Spectroscopy, Thin layer chromatography, high performance thin layer chromatography and high performance liquid chromatography can be very well utilized for the development of chemical markers which, in turn, can be used for identification and quality assurance (Joliffe, 1977).

HPTLC is an advanced versatile chromatographic technique for quantitative analysis with high sample through put and is complementary to HPLC/ GLC. It provides a chromatographic fingerprint. It is therefore, suitable for monitoring the identity and purity of drugs. HPTLC is a versatile separation technique and is official in most of the pharmacopoeias for determining content uniformity, purity profiles, assay values and dissolution rates in unlimited number of monographs. It is precisely for these reasons that almost every laboratory today is equipped with HPTLC system. It is the most simple separation technique today available to the analyst. It can be considered a time machine that can speed your work and allows you to do many things at a time usually not possible with other analytical techniques (Sethi, 1996). The advantages of HPTLC are mentioned below:

1. HPTLC is cost effective and environmentally friendly as compared to HPLC.
2. Solvent consumption per sample is very less in HPTLC.
3. Sample clean up is usually simple. Unlike column chromatography, the stationary phase is used only once.
4. HPTLC is an ideal as a confirmatory method, because it is based on a different separation mechanism, usually reversed phase is used.
5. Densitometric scanning of individual or all fractions can be repeated with the same or different parameters, since all fractions remain stored on the plate. There are many options for evaluating or documenting the fractions both before and after derivatization.

Consistent quality for products of herbal origin can only be assured if the starting point material is defined in a rigorous and detailed manner. Characterization of herbal drug is, therefore essential to allow specifications to be established which are both comprehensive and relevant.

In the present study HPTLC has been chosen as a method of analysis. A standard procedure was developed for evaluation of active constituents from the three medicinal herbs viz: *Bacopa monneria* L., *Centella asiatica* (L.) Urban and *Rubia cordifolia* L. Also the comparison was done between the *in vitro* derived plants and mother plants for the presence of marker constituent.

MATERIALS AND METHODS

Various steps are involved in HPTLC has been represented schematically in Figure 3. All these steps have been described in details.

1. Sample preparation:

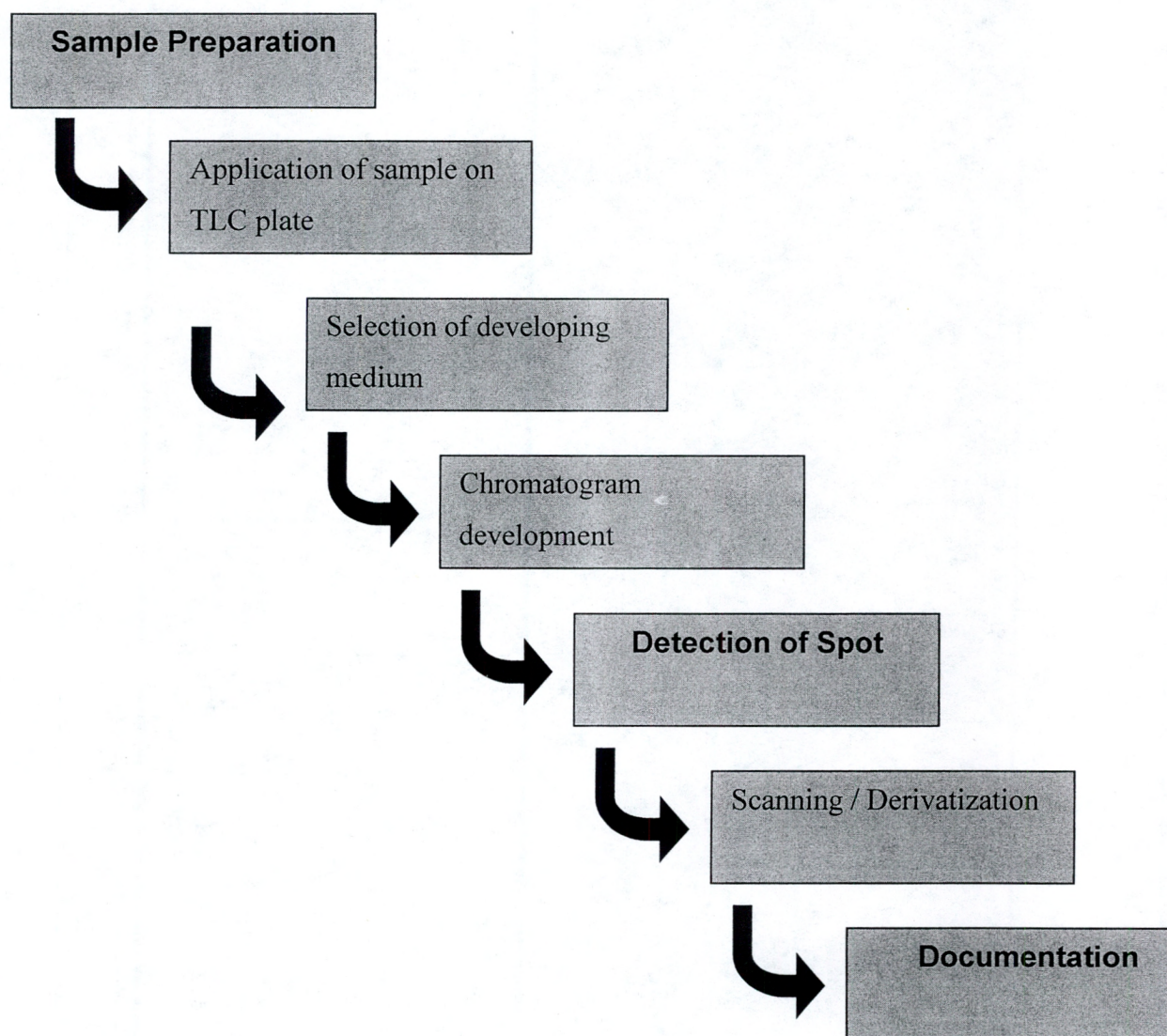
The *in vitro* and field grown plant material of *Rubia cordifolia* (roots) were dried and powdered. Each powdered sample (10 g) was exhaustively extracted in methanol (200 ml) using Soxhlet apparatus (Daniel, 1991). Extracts were concentrated at room temperature, filtered with Whatman's filter paper No. 1 and finally made up to 10 ml with methanol.

Standard Preparation: Standard solutions were prepared in methanol at a concentration of 1µg/1 µL. The standard used for each of the mentioned plant is given below:

<i>Rubia cordifolia</i>	Alizarin
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2. Application of sample: Commercially available precoated plates of Silica gel 60 F 254 (Merck) with 20 cm x 20 cm size were used for the study. *Rubia cordifolia* were applied separately on different tracks of the plate. The application was done by Camag (Switzerland) HPTLC system

Figure 3: Schematic Procedure for HPTLC.



equipped with Linomat IV sample applicator. The loading was done using Hamilton syringe.

3. Selection of developing medium:

The qualitative/quantitative analysis can be done only if an ideal mobile phase, that can resolve the compound of interest. The development should be free from overlapping of spots a tailing, in order to achieve the accuracy in the characterization and further measurement. The selection of a developing medium was based on the maximum resolution of compounds. After trying with various solvent systems, a suitable system for individual plant was selected and used for further study. The solvent system for chromatogram development of individual plant has been standardized.

4. Chromatogram development:

The plates after drying were developed in a previously saturated twin trough chamber using various mobile phases under study. After the solvent front attains a height of 80% the developed plates (chromatogram) were removed, air dried and observed under UV light for detection of spots. The compound, which shows fluroscences, can be seen under UV light at 366 nm whereas non-fluorescent compound, which clearly absorbs UV light, can be seen as dark regions on a green fluorescent background of silica gel F₂₅₄ under UV light 254 nm. The resolved spots on the chromatogram can be easily seen under UV light at 254 as dark spots. So, UV light irradiation is selected for visualization of resolved substances on chromatogram.

5. Post chromatographic derivatization and detection:

Certain chromatograms require derivatization to visualize the fluorescence of compounds. So the chromatograms were derivatized by spraying

reagents like anisaldehyde sulphuric acid or 10% ethanolic KOH (Wagner and Badlt, 1996).

6. Densitometric chromatogram evaluation (Scanning):

The developed chromatograms were scanned using a densitometer (TLC plate scanner - CAMAG). It contains a light source and a photocell, which scans each of the tracks on the plate in regular pattern.

RESULTS AND DISCUSSION

Rubia cordifolia L.

Chromatographic conditions:

Stationary phase : Silica gel 60 F254 (Merck) Precoated TLC plates.

Mobile phase : Benzene (7.5) : Ethyl acetate (1) : Formic acid (0.1) : glacial acetic acid (0.2)

Saturation : 10 minutes.

Lamp : Deuterium

Wavelength : 254nm.

Detection : 10% Ethanolic KOH.

The best separation of compounds was obtained in the solvent system consisting of benzene, ethyl acetate, formic acid and glacial acetic acid in the ratio of 7.5:1:0.1:0.2. The plate was scanned at different wavelength. The compound emits fluorescence at 366 nm. The maximum absorption was achieved at 254 nm. The active principle alizarin appeared as a dark red band on visual observation after derivatisation (Photoplate 7). Sharp peaks of alizarin were obtained in all the tracks when the plate was scanned at 254 nm (Fig. 4). It was observed that alizarin moved at R_f 0.42. The amount of alizarin present in the sample was estimated by calibration with the standard alizarin peak area and those present in each sample. The amount of alizarin present in the *in vitro* and field grown material were more or less similar being 1.66 % and 2.03 % on dry weight basis respectively.

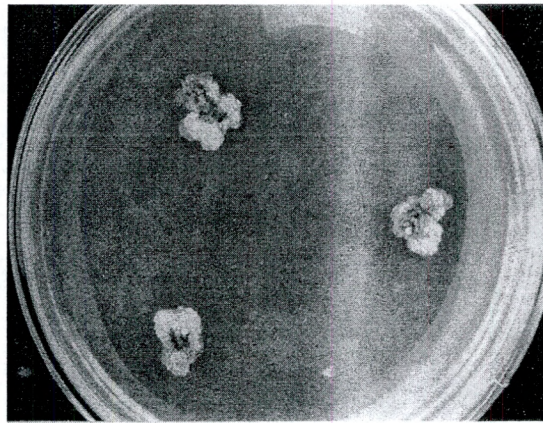
Photoplate 1

Rubia cordifolia L

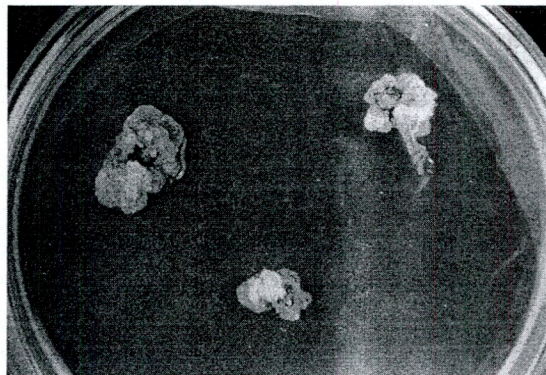


Photoplate 2

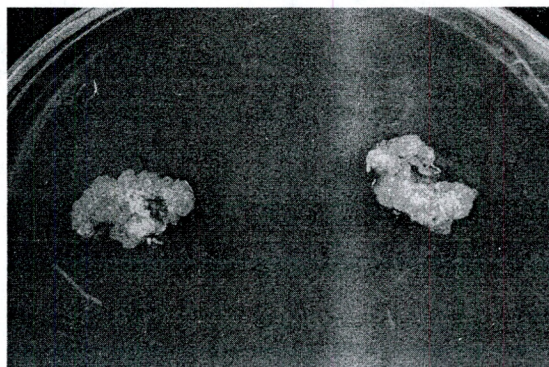
*Callus derived on various media from leaf explants
of Rubia cordifolia.*



A. BA



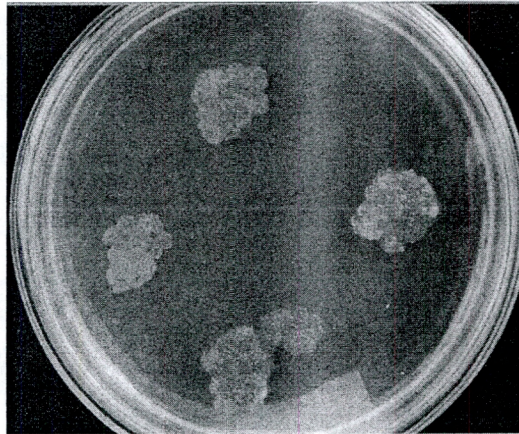
B. TDZ



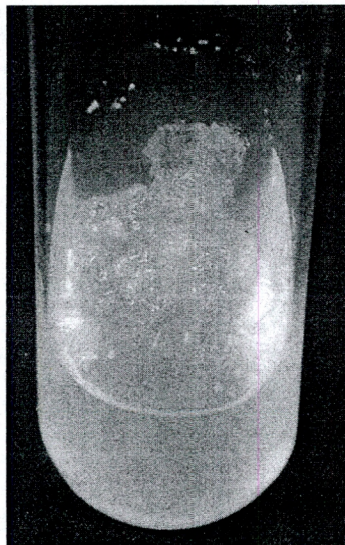
C. ADS

Photoplate 3

*Callus derived on different auxins from
from leaf explants of Rubia cordifolia.*



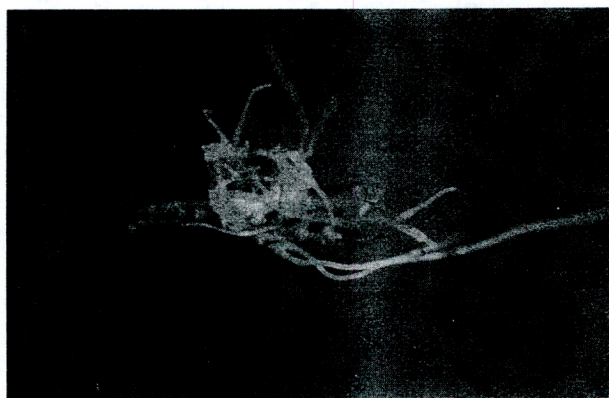
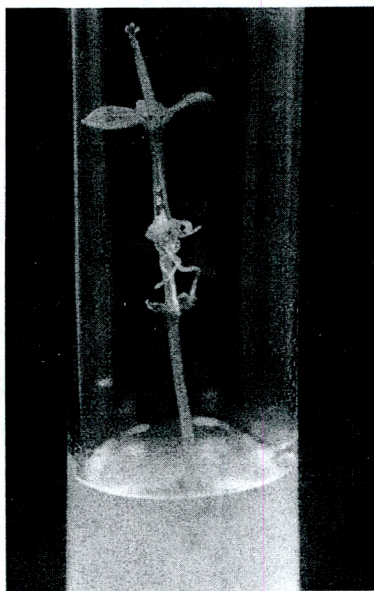
A. Callus derived on NAA



B. Callus derived on 2,4-D

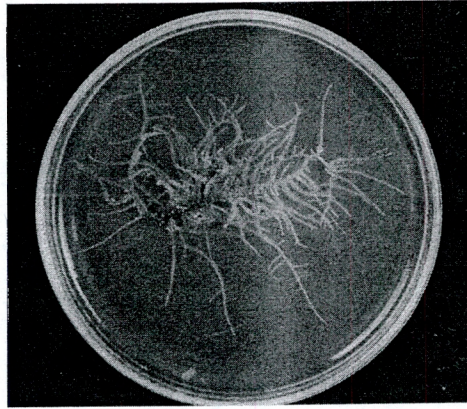
Photoplate 4

Emergence of hairy roots from the inoculated site in Rubia cordifolia plantlet.

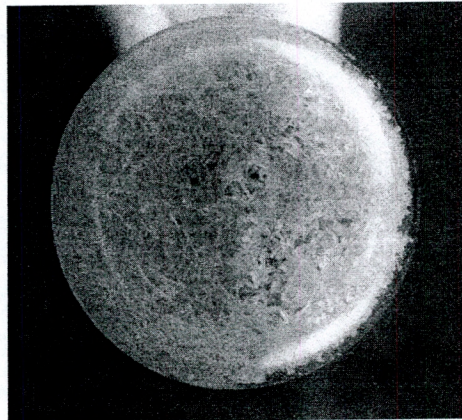


Photoplate 5

*Bacteria free roots of Rubia cordifolia
on solid and liquid medium*



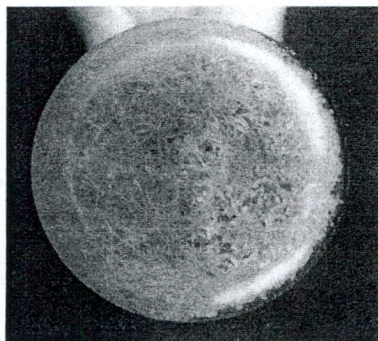
A. Solid Medium



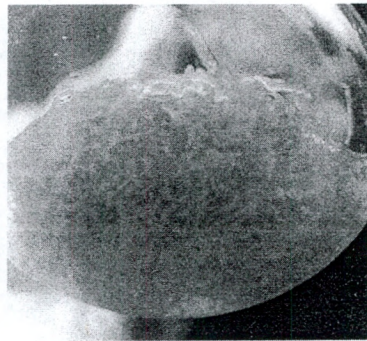
B. Liquid Medium

Photoplate 6

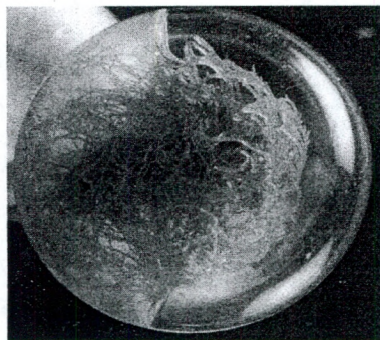
*Hairy roots of Rubia cordifolia
grown on different media*



MS Modified



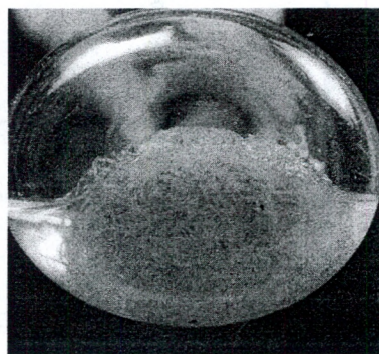
MS Original



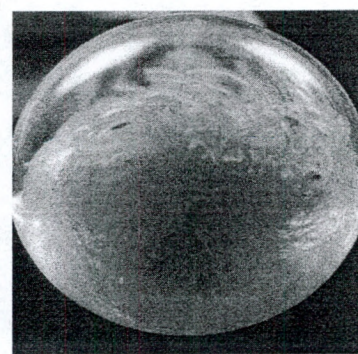
N&N



White



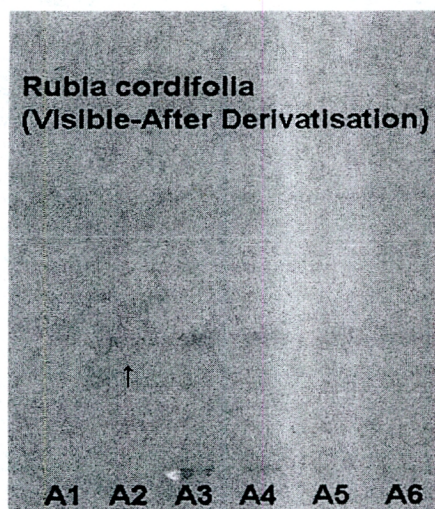
SH



B5

Photoplate 7

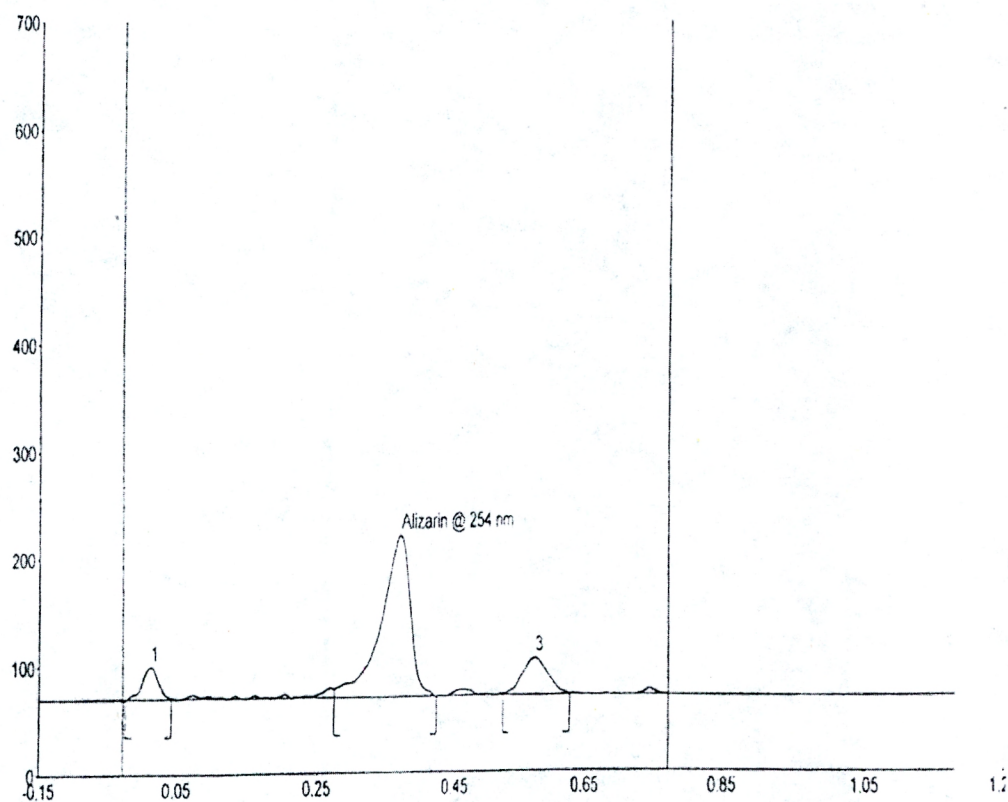
HPTLC analysis of alizarin from *in vitro* and field grown
Rubia cordifolia plants



A1	:	Standard	0.1 µg
A2	:	Standard	0.1 µg
A3	:	<i>In vitro</i>	10 µg
A4	:	<i>In vitro</i>	10 µg
A5	:	<i>In vivo</i>	5.0 µg
A6	:	<i>In vivo</i>	5.0 µg

Fig. 4
HPTLC chromatogram of alizarin.

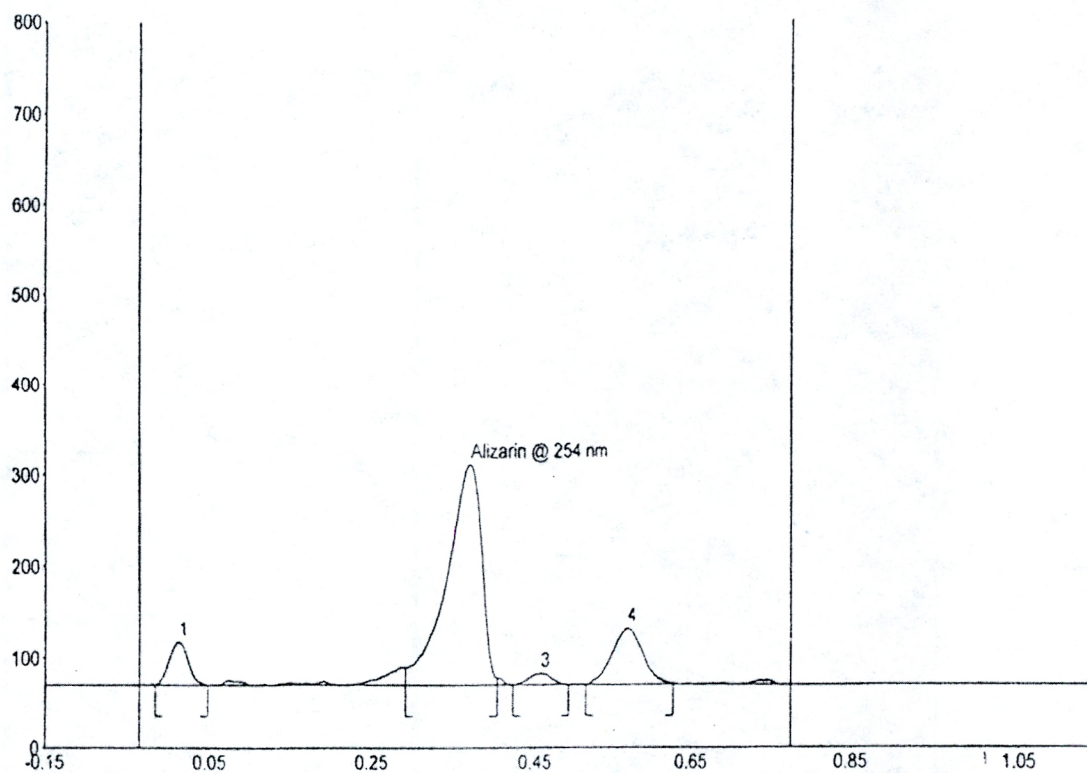
Track 6: *Rubia cordifolia*. In vivo: 5 µg



Peak	Start Rf	Start Height	Max Rf	Max Height	Max %	End Rf	End Height	Area	Area %	Assigned substance
1	0.05	0.2	0.08	30.2	14.15	0.10	1.7	469.8	8.53	unknown *
2	0.30	8.3	0.38	148.9	69.72	0.42	0.2	4111.1	74.63	Alizarin
3	0.51	2.0	0.55	34.4	16.12	0.60	1.6	927.5	16.84	unknown *

Fig. 4

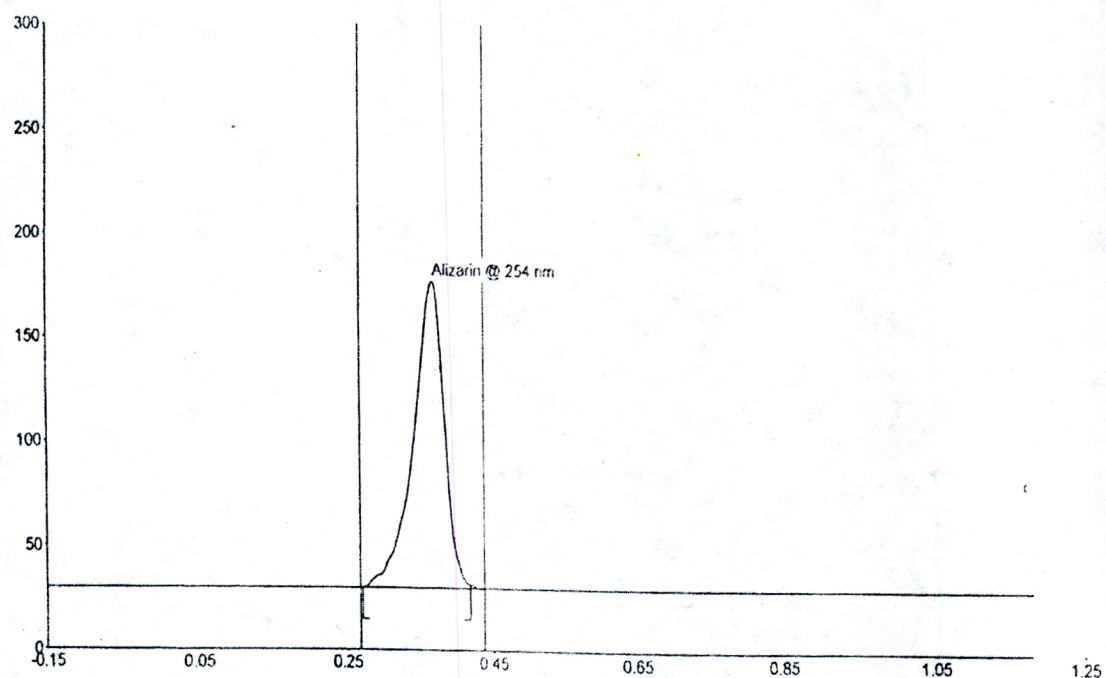
Track 4: *Rubia cordifolia*. In vitro: 10 µg



Peak	Start Rf	Start Height	Max Rf	Max Height	Max %	End Rf	End Height	Area	Area %	Assigned subst
1	0.05	0.8	0.08	48.0	13.17	0.11	0.2	715.2	7.62	unknown *
2	0.32	19.6	0.38	241.4	66.18	0.41	8.0	6742.2	71.85	Alizarin
3	0.43	0.5	0.45	13.0	3.58	0.48	0.0	254.0	2.71	unknown *
4	0.50	0.3	0.55	62.3	17.08	0.60	2.5	1672.9	17.83	unknown *

Fig. 4

Track 1: *Rubia cordifolia*. Standard Alizarin: 0.1 μ g




Peak	Start Rf	Start Height	Max Rf	Max Height	Max %	End Rf	End Height	Area	Area %	Assigned substance
1	0.30	0.5	0.38	147.2	100.00	0.42	2.0	4048.2	100.00	Alizarin

From the results obtained from high performance thin layer chromatography studies, it is obvious that each and every plant is having their own chromatographic profiles based on the nature of the active compound present there in.

If go through the worldwide available pharmacopoeia it is clear that pharmacopoeia in the world is comprehensive enough to cover all the medicinal herbs, and no pharmacopoeia is able to give complete methodology for routine quality control for medicinal herbs. It indicates the limitation of basic approach of developing standards in the line with allopathic system of medicine, that is why for the quality control of herbal medicine it is important to follow some standardization procedures for the raw materials as well as finished products. Based on those approaches our aim was to make a standardization procedure and thereby to evaluate it for proper standardization of selected medicinal plants.

HPTLC is more powerful, reliable and cost effective with respect to accuracy of the results based on both quantitative and qualitative analysis. HPTLC is one of such modern technique, which has so many advantages in characterization of herbal drugs.

In the present investigation *Rubia cordifolia* were subjected to HPTLC study based on chromatogram development and their scanning i.e. densitometric chromatogram evaluations were made which shows that each plant have specific peak with definite R_f values and chromatogram which matches each other. Also the comparison was made between *in vitro* derived and field grown plants that showed the presence of active constituent in both the samples. This shows that these particular HPTLC characteristics may be used as standardization tool for medicinal plants more effectively and more accurately.



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