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Development of micropropagation and suspension culture methods for some medicinal plant species of north-east India and study of their antimicrobial potentials

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Dedicated

To my

Beloved Parents

And

Brothers

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CERTIFICATE

This is to certify that the thesis entitled "**Development of micropropagation and suspension culture methods for some medicinal plant species of north-east India and study of their antimicrobial potentials**" submitted to Tezpur University for the award of the degree of **Doctor of Philosophy** in **Molecular Biology and Biotechnology** is a bonafide research work carried out by **Miss Sangeeta Nath (Regd. No. 171, 2001)** under my supervision and guidance.

To the best of my knowledge no part of this work has been submitted to any other University or Institute for the award of any degree or diploma.

A. K. Buragohain

Dated 11.08.2003 **(A.K. Buragohain)**

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Date 11-08-2003 **(SANGEETA NATH)**

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CONTENTS

ABSTRACT

Emergence of resistance against many of the antibiotics commonly used for treatment of a variety of infectious diseases has assumed an alarming proportion. Infections with multiple drug resistant (MDR) *Staphylococci, Enterococci, Mycobacteria* and *Salmonella typhi* have become a major concern in the health care sector. The pandemic of Human Immunodefeciency Virus (HIV) infections has compounded the problem of MDR-tuberculosis and other pulmonary infections. This has necessitated the development of new drugs to effectively contain the problem.

Plants have been the major source of therapeutic agents that have been used in a variety of human ailments. A large number of plant species from Assam and other parts of the north-east India is known to have medicinal properties. Most of these species are not yet explored scientifically for assessment of their potentials as useful drugs. Wanton destruction of habitats and also collection of these medicinal plants have made some of these species rare, threatened or endangered. Essentially a two pronged strategy - one aimed at developing modern biotechnology based conservation methodology and the other for screening plant metabolites for discovering useful drugs from these plant species, can be adopted for scientific exploitation of these valuable bioresources.

The present study was conceived against this perspective with the following objectives

a) to establish tissue culture methods for efficient micropropagation of three medicinal plant species of Assam *viz, Adhatoda vasica, Asparagus*

racemosus and *Centella asiatica* which are ethnobotanically known to possess antituberculosis activities.

b) To develop suspension cultures for these species to generate secondary metabolites having anti-microbial and specifically anti-*Mycobacterium tuberculosis* activities.

c) To examine the anti-microbial and anti-*Mycobacterium tuberculosis* activities of the crude and partially purified fractions derived from these species.

Micropropagation protocols have been developed through determination of the optimum culture conditions for rapid micropropagation of *Adhatoda vasica, Asparagus racemosus* and *Centella asiatica*. The tissue cultured plants of each of these species has been successfully established in the field condition.

Biochemical investigations have been conducted to resolve, detect and quantify the known active principles in each of these species *viz*, vasicine, L-asparagine and asiaticoside. Biochemical assays were performed using various chromatographic techniques to quantitate the amounts of these active principles from micropropagated plant tissue, calli and suspension cultured cells.

Protocols for suspension culture of these species have been developed using callus derived cells. The objective of developing suspension culture for these species was to find out whether the calli derived cells retain the potential to generate the known active principles of these species. A most significant and important finding of these experiments was that, not only the suspension cultured cells synthesized these bioactive molecules but these were synthesized in relatively higher quantities in comparison to that in the

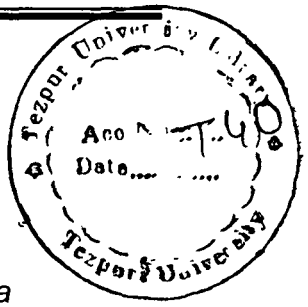
intact plants. There is thus, enough scope for designing appropriate bioreactors for large scale production of these compounds.

Assays were performed to determine whether any of these compounds present in the crude and partially purified fractions of the plants have any inhibitory effect on the growth of *Bacillus subtilis, Psuedomonas* sp, *Mycobacterium tuberculosis* and the eukaryotic microbe *Saccharomyces* sp. The crude extracts from root and callus derived from nodal explants of *Asparagus racemosus* have been found to inhibit completely the growth of *Mycobacterium tuberculosis* while there was no such antimicrobial activity against *Psuedomonas* sp, *Bacillus subtilis* and *Saccharomyces* sp. The leaf extracts from *Adhatoda vasica* completely inhibited the growth of *Mycobacterium tuberculosis* and *Bacillus subtilis*. The extract induced moderate inhibition of growth in *Puedomonas* sp and had no such effect against *Saccharomyces* sp. While the extracts of *Centella asiatica* inhibited the growth of *Pseudomonas* sp and *Bacillus subtilis* to varying extents, there was no antimicrobial activity against either *Saccharomyces* sp or *Mycobacterium tuberculosis*.

The study has proved that the two species, *Asparagus racemosus* and *Adhatoda vasica* are potential sources of drugs against *Mycobacterium tuberculosis* and there is scope for further pharmocogenomic explorations in this direction.

LIST OF TABLES

LIST OF FIGURES



18. Dried plant samples

19. Inhibition on the growth of *Psuedomonas* spp

20. Absence of inhibitory effect of the ethanol leaf extract of *Centella asiatica* and acetone leaf extract of *Adhatoda vasica* on the growth of *Saccharomyces* spp.

21. Inhibition on the growth of *Bacillus subtilis* depicted by the inhibition zone

LIST OF ABBREVIATIONS

ABA	Abscissic acid
AC	Activated charcoal
NH_4OH	Ammonium hydroxide
NH_4NO_3	Ammonium nitrate
ANOVA	Analysis of Variance
BA	6-benzyl adenine
H_3BO_3	Boric acid
$CaCl_2.\ 2H_2O$	Calcium chloride
CO_2	Carbon dioxide
cm	Centimetre
CDRI	Central Drug Research Institute
$CHCl_3$	Chloroform
$CoCl_2.\ 6H_2O$	Cobalt chloride
CM	Coconut milk
$CuSo_4.\ 5H_2O$	Copper sulphate
CSIR	*Council for Scientific and Industrial* Research
DAI	Days after inoculation
^{O}C	Degree Celsius
DNA	Deoxy ribonucleic acid
2,4-D	2,4-Dihydro phenoxy acetic acid
DMSO	Dimethyl sulphoxide
DW, dw	Dry weight
DMRT	Duncan Multiple Range Test
Na_2 EDTA.$2H_2O$	Ethylene diamine tetra acetic acid disodium salt
$FeSo_4.7H_2O$	Ferrous sulphate
FW, fw	Fresh Weight
GLC	Gas Liquid Chromatography

GA_3	Gibberelic acid
g	Gram
ha	Hectare
HTS, uHTS	High Throughput Screening, ultra High throughput Screening
HPLC	High Performance Liquid Chromatography
hrs	Hours
HIV	Human Immunodeficiency Virus
ICAR	Indian Council of Agricultural Research
IMTECH	Institute of Microbial Technology
IBA	Indole-3-butyric acid
IAA	Indole-3-acetic acid
2-ip	N^6-(2-isopentynyl)-adenine
INH	Isoniazid
Kg	Kilogram
KIN	Kinetin
λ	Lambda
LS	Linsmeir and Skoog
L	Litre
$MgSO_4.7H_2O$	Magnesium sulphate
$MnSO_4.H_2O$	Manganese sulphate
λ_{max}	Maximum absorbance
$HgCl_2$	Mercuric chloride
MeOH	Methyl alcohol
MTCC	Microbial Type Culture Collection
μ	Micron
mg	Milligram
ml	Millilitre
mm	Millimetre
MIC	Minimum Inhibitory Concentration
min	Minute
M	Molar

MDR, MDR-TB	Multuple Drug Resistance, Multiple Drug Resistance Tuberculosis
MS	Murashige and Skoog
nm	Nanometer
ng	Nanogram
NAA	α-naphthalene acetic acid
NA	Nutrient Agar
OTA	Ochratoxin A
ε	Optical density
PCV	Packed Cell Volume
PAS	Para amino salicylic acid
PABA	Para amino benzoic acid
%	Percentage
K	Potassium
KH_2PO_4	Potassium dihydrogen phosphate
KI	Potassium iodide
K	Potassium
KNO_3	Potassium nitrate
Lb/inch2	Pound per square inch
PEMs	Proembryogenic masses
RBD	Random Block Design
R_f	Resolving front
RH	Relative humidity
R-plasmid	Resistance plasmid
RO	Reverse osmosis
rpm	Revolution per minute
RNA	Ribonucleic acid
1N NaOH	1N sodium hydroxide
Thiamine HCl	Thiamine hydrochloric acid
TDZ	Thiadiazuron
TLC	Thin layer chromatography
TNTC	Too numerous to count
TFTC	Too few to count

UV	Ultraviolet
VIS	Visible
v/v	Volume by Volume
w/v	Weight by Volume
YPD	Yeast Peptone Dextrose
$ZnSO_4.7H_2O$	Zinc sulphate

Chapter I

INTRODUCTION

Chapter I

Introduction

1.1 Plants in medicine

Plants have been a major source of therapeutic agents for alleviation or cure of human diseases since time immemorial. India has a unique position in the world where a number of traditional systems of medicine are practised such as Ayurveda, Siddha, Unani, Yoga and Naturopathy for total health care. These systems of medicine are heavily dependent upon the medicinal plants (Kumar *et al.*, 1997). Even today, 75% of the total populations rely on the medicinal plants in the rural and remote areas of India by way of traditional systems of medicine (Singh *et al.*, 2001). So far, 500 plant species out of the 25,000 to 30,000 known plant species have been studied exhaustively for medical applications. Most of these plant species that have not been analysed are found in India. Some of these plants are rare and require proper strategies for their conservation before they become extinct.

The reasons for the poor contribution of medicinal plants to modern medicine are, firstly many plant derived chemicals, though biologically active, did not get entry as drugs in modern medicine because they have not been subjected to the required clinical trials because of the very long time and the huge expenditures involved in the process. Another reason is that majority of the main pharmaceutical industries which sponsor the introduction of new drugs are located in highly developed countries whereas the plant biodiversity

mostly exists in developing countries where the information about the traditional uses of the plants is available. The industries were therefore not willing to invest in a big way to procure the plant materials from these countries (Sukhdev, 1997). In spite of the above mentioned situation modern pharmacoepia still contains 25% drugs derived from plants and many others are synthetic analogues built on prototype compounds isolated from plants.

1.2 Multiple drug resistance and the need for new drugs

Emergence of resistance (multiple as well as single) against many of the antibiotics commonly used for treatment of infectious diseases has been on phenomenal rise among the microbes that cause human diseases. Indeed, infections with multiple drug resistant (MDR) *Staphylococci*, *Enterococci*, *Mycobacteria* and *Typhoid* bacteria have become major concern in the health care sector (Kumar Santha *et al.*, 1999). The recent pandemic of HIV infection has compounded the problem of MDR- tuberculosis and other pulmonary infections. The optimal duration of therapy with the existing second line drugs is not definitely known. The response rates are dismal and a recent study showed only 65% response and 56% cure rates (New England J. Med., 328, 527-532).

Resistance to therapeutic drugs arises primarily due to new spontaneous mutations occurring in the genome of the pathogens. These genetic changes alter the sensitivity functions of the pathogens by changing the target of the drug (Spratt, 1994).The genes for drug resistances are present on both the bacterial chromosome and plasmids. Spontaneous mutations in the bacterial chromosome, although they do not occur very often

make bacteria drug resistant. Usually such mutations result in a change in the target molecule disabling the antibiotic-receptor binding process. Frequently a bacterial pathogen becomes drug resistant because of the presence of one or more plasmid borne resistance genes; such plasmids are called R plasmids (resistance plasmids). Plasmid resistance genes often code for enzymes that destroy or modify drugs e.g. the hydrolysis of penicillin or the acetylation of chloramphenicol and aminoglycoside drugs. Once a bacterial cell possesses R plasmid, the plasmid may be transferred to other cells quite rapidly through normal gene exchange processes such as conjugation, transduction and transformation. Because a single plasmid may carry genes for resistance to several drugs, a pathogen population can become resistant to several drugs simultaneously, even though the infected patient is being treated with only one drug.

Drug resistance is an extremely serious public health problem. Much of the difficulty arises from drug misuse. About 50% of the antibiotics prescriptions in hospitals are given without clear evidence of infection or adequate medical indication. Toxic, broad-spectrum antibiotics are sometimes given in place of narrow-spectrum drugs as a substitute for culture and sensitivity testing, with the consequent risk of dangerous side effects, superinfections, and the selection of drug-resistant mutants. The situation is made worse by patients not completing their course of medication, resulting in resistance to the two principal antituberculosis drugs, isoniazid and rifampicin-so called multidrug resistance (Zumia and Grange, 2001).

Molecular diagnostic techniques and automated culture systems have reduced turn around times in the modern mycobacteriology laboratory, and

the continuing evaluation and development of such techniques is increasing the use of molecular technology in developed nations. Simple phenotypic methods for the detection of resistance to first-line drugs and genotypic kit-form assays for detection of rifampicin resistance have been developed that have become key tools in the containment of MDR-TB (Caws and Drobniewski, 2001). Rapidly progressive multidrug-resistant tuberculosis is well documented in human immunodeficiency virus (HIV) positive subjects, but it is not fully recognised in HIV-negative subjects in the familial environment (Sofia *et al.*, 2001).

1.3 Plants as a source of antimicrobial compounds

Plants, because of omnipresence of microbial diversity in the biosphere have always exhibited natural resistance to microbial attack. Plants appear to have evolved complex defence mechanisms against pathogenic bacteria through production of anti-microbial compounds (Delaney *et al.*, 1994). In such an ever evolving interaction, plants as a component of their active defence mechanism, produce anti-microbial compounds (antibiotics) while microbes tend to develop resistance against these antibiotics for survival and perpetuation. Thus, in this competition plants evolve to produce newer anti-microbial compounds. The antimicrobial substances of plant origin vary in their antimicrobial activity. Some have a limited spectrum of activity, being effective against only one group of organisms. Others exhibit broad-spectrum activity against a range of microorganisms. The necessity for screening the plants for detecting their antimicrobial potency therefore becomes the first priority before any further evaluation. Considerable work on the antimicrobial

activity of medicinal plants has been reported from different parts of the world (Ahmad *et al.*, 1995; David, 1997; Desta , 1993).

However, the success of discovering a new or novel candidate drug molecule depends upon the number of chemical compounds screened or examined against a particular target molecule. The dictum being: more the compounds examined, more likely is one to succeed. Thus, we are confronted with the need to screen thousands of compounds for their biological activity in the shortest possible time frame. It becomes imperative therefore, that the assay process used should be suitably miniaturised and automated for testing which can result into reduced costs and time. Such assay systems are referred to as High Throughout Screening (HTS) assays. A rational approach to the indentification of relavant drug targets involves the characterization of gene products that participate in the diseases processes. Comparative genomics involving sequencing and analysis of the nucleotide sequence of the whole genome of disease causing pathogens and of human being has massively extended the number of molecules to be tested by identifying potential new gene target (Spence, 1998). An integrated approach involving combinatorial chemistry, high throughout assays and functional genomics will ensure that new drug molecules keep flowing down the discovery pipeline in future.

With the changing global scenario and to keep pace with an annual industry growth rate of 10-12 %, there is need to fix priorities in areas of research by the Indian pharmaceutical industry and the National laboratories for creating capabilities for developing new drug molecules, advancing drug delivery system, biotechnologies including establishing different

micropropagation protocols for large scale production of plants, suspension culture protocols for secondary metabolite production, fermentation technologies and emerging approaches such as advanced genomics, high throughout screening, combinatorial chemistry, biology and computer assisted *de novo* drug design. In addition to these, there is also need to embrace bold new research culture into a high – performance paradigm.

1.4 Application of biotechnology

1.4.1 *Plant tissue culture for medicinal plants*

Advanced biotechnology like *in vitro* multiplication of plants, also called micropropagation is an advantageous method for obtaining plantlets more rapidly in a short span of time within a limited space. In addition there are now many technologies for the genetic modification of plants *in vitro*, which also depends upon micropropagation for the regeneration, and multiplication of the plants with the novel characteristics. The major constraints to the mass propagation of medicinal plants are the climatic conditions and mode of propagation. Recent biotechnological advances made in the field of plant science, however hold great promise in providing solutions to a number of basic and applied problems associated with the domestication, cultivation and propagation of medicinal plants. They can serve as scientific tools in extraction, identification and processing of chemical compounds from such plants

By the use of tissue culture techniques, various problems in plant biotechnology such as mass multiplication of desired individuals or clones could be obtained. Even establishing tissue culture protocols for the rare and

extinct medicinal plants could serve as a good means of conservation. This will also fulfil the increasing demand of the pharmaceutical industries. The biosynthesis and biotransformation of biologically active compounds *in vitro*, the creation and selection of variants in cultures, storage of plant cells and organs and genetic engineering of higher plants can also be solved by tissue culture. However, in medicinal plants much of the research work is on the production and transformation of pharmacologically active principles by tissue culture. Suspension culture technology is preferred over field cultivation of plants for generation of secondary metabolites despite of its expensiveness and its susceptibility for genetic mutations because suspension cultures are initiated from callus cells under *in vitro* condition which requires limited space and also many rare plants which are of medicinal values are saved from extinction. Most often very few species of medicinal value have been included in dealing with tissue culture and other advanced biotechnological methods, and much is yet to be done in many other species to commercially exploit their pharmaceutical potential on an industrial scale.

1.4.2 *Suspension culture for secondary metabolite production*

Plants usually have a great variety of secondary products. Though they play a minor role in the basic life processes of the plants these have been used as sources of a large number of industrial products, including agricultural chemicals, pharmaceuticals and food additives. These natural products are produced in plants in very small quantities and because of their structural complexity, these compounds are difficult to synthesize. During the last 30 years there has been an increasing interest among scientists to produce high

value natural plant products by cell culture, which can overcome many of the problems associated with industrial production of these phytochemicals by extraction from field grown plants (mass cultivated or natural populations). Even though the generation of secondary plant metabolites through plant cell suspension has been proved to be a feasible proposition, several problems are encountered during large-scale suspension cultures for commercial production of secondary products. A particular characteristics of plant cell suspension is the requirement for a high inoculum density in order to obtain growth. The slow growth of plant cells in suspension culture, sensitivity of the plant cells in culture to shearing stress due to large cell size and rigid cell wall, clump formation, and aeration present are the problems during plant cell suspension culture necessitating careful planning and optimization. Moreover since secondary metabolites are usually produced in differentiated tissues. Therefore in suspension cultures some of these metabolites may not be produced, as the cultures are derived from dedifferentiated callus cells.

Cell cultures not only provide means for synthesis of natural products but also serve as 'factories' for bioconversion of low value compounds into high value products. Since the early 1950s, when the concept of tissue culture production of natural compounds was conceived, many technological advances have been made and in several cases cell cultures have been shown to produce higher amounts of the products than the intact plants from which they are derived. Of the various plant products produced by plant tissue culture, pharmaceuticals have received maximum attention.

Callus culture of plant tissue gives way to more than one possibility for harnessing the inherent genetical potential of the plant cells. It is the growth

and maintenance of largely unorganised cell mass which arise from the uncoordinated and disorganised growth of small plant organs, pieces of plant tissues or previously cultured cells. Somatic embryogenesis, through this route of plant cell culture technology has made "artificial seed" production a reality. Callus culture is the most effective way of generating dedifferentiated cells which can be used for the purpose of suspension culture. Suspension culture of plant cells not only allows study of the underlying mechanism of plant developmental biology at the cellular and molecular levels but there is also enormous biotechnological opportunities through appropriate scaling up of the suspension culture technology upto industrial scale bioreactor levels for generation of valuable secondary metabolites. However it must be emphasized again that the basic studies related to the establishment of a feasible cell suspension culture system constitutes the premise on which such large-scale biotechnological ventures are possible.

1.5 Objectives

Three plant species viz., *Adhatoda vasica* (L.) *Centella asiatica* (L.) and *Asparagus racemosus* Willd have been known to have medicinal properties. Parts of these three plants are traditionally being used by the people to get relief from various pulmonary ailments.

The present work has been defined in the context of the preceding discussion and has the following objectives

1) To establish tissue culture methods for efficient micropropagation of
   (i) *Adhatoda vasica* (L.) (ii) *Centella asiatica* (L.) (iii) *Asparagus*

racemosus Willd

2) To develop suspension cultures for these species to generate secondary metabolites having anti-microbial and specifically anti-mycobacterial activities.
3) To examine the anti-microbial and anti-*Mycobacterium tuberculosis* activities of the crude and partially purified fractions derived from these species.

The following strategies will be used for achieving these objectives

1) Micropropagation protocol will be developed for the three plant species through the conventional route of organogenesis.
2) Suspension culture of appropriate plant cells from the species will be developed.
3) Assay will be done for resolution of the active principles in plant materials derived from various sources *viz.*, wild plants, plants raised through tissue culture, callus tissue and cells from suspension culture.
4) Assays will be done to quantify the potential active compounds in plants derived from wild plants, tissue culture raised plants, callus tissue and cells from suspension culture.
5) Assays will be done to monitor the effect of crude extracts containing the potential active compounds from the plant species in cultures of *Psuedomonas* spp., *Bacillus subtilis* and *Saccharomyces* spp.
6) Culture of *Mycobacterium tuberculosis* in media containing extracts of the leaves, roots, callus and suspension cultured cells for determining the antimycobacterial activity of the three plant species.

Chapter II

REVIEW OF LITERATURE

Chapter II

Review of Literature

2.1 Plants-a major source of drugs

Plants have been used by mankind as sources of therapeutic agents to combat diseases since time immemorial in the conventional systems of medicine such as ayurveda, siddha, unani and homeopathy. With the development of scientific methods and techniques, a number of medicinal plants from various countries were subjected to biochemical and pharmacological investigations and the active constituents were isolated and characterized. Subsequently, these compounds-either in pure state or in the extracts containing them, were included in the pharmacopoeias of several countries. According to one estimate about 35,000-70;000 species have been used in various cultures of the world for medicinal purpose (Farnsworth and Soejarto, 1991). These have been used in the form of crude drugs which were dried parts of the medicinal plants (root, stem, wood, bark, leaves, flowers, fruits, seeds and in some cases whole plants) or their extracts. The active principles from these plant parts or their extracts were introduced as drugs in modern medicines following pharmacological, toxicological investigations and clinical trials. Although the number of plant based drugs used in modern medicine is low compared to the synthetic products, the plant based drugs have been extremely valuable and helpful in the alleviation of human sufferings. Some of the claims made by traditional medicinal systems for their drugs have received clinical support from modern medicinal science (Sukhdev, 1997). As the synthetic drugs cause several side effects, the plant

based medicine has become popular throughout the world now a days. The contribution of medicinal plants in discovery of new drugs has been enormous in terms of value and activity for treating diseases like cancer, hypertension and several other ailments.

The species used for isolation of active ingredients may be indigenous species growing wild or cultivated or hybrids or other cultivated varieties that have been developed through selection for particular characteristics (Anonymous, 1992). In India, about 90% collection of medicinal plants is from wild sources and since 70% of collections involve destructive harvesting, many plants have become endangered or vulnerable or threatened (Anonymous, 1992). The increasing demands of the pharmaceutical industry have created problems of supply. One of the major difficulties being experienced by the industry is that of obtaining sufficient quantities of medicinal plants for the manufacture of genuine medicines. In our country there are only a few herbal drugs which are cultivated on large scale. One of the major difficulties of large-scale medicinal plant cultivation is the lack of scientific and appropriate agrotechnology for different climatic zones of the country. However several organisations *viz*, Indian Council of Agricultural Research (ICAR), Council for Scientific and Industrial Research (CSIR) laboratories, various Indian Universities have taken up the work towards development of appropriate agrotechnologies and of high yielding varieties of medicinal plants. But much efforts are still needed keeping in view the demands of trade and industry. Enormous importance should be given in mounting research efforts to develop *in vitro* mocropropagation protocols for rapid regeneration of these plants on a large scale. There is also the need for

generating somaclonal variants of these species through plant tissue culture approaches for selection of elite clones having features fulfilling specific industrial requirements.

In the present study the three chosen plant species *viz. Centella asiatica*, *Adhatoda vasica* and *Asparagus racemosus* have high demand in the indigenous drug industry (Singh *et al.*, 2001). The drugs from these plant species have also received pharmacological/clinical support from contemporary medical system (Sukhdev, 1997).

Centella asiatica (L.) Urban is a member of Apiaceae family, commonly known in India as 'Indian pennywort' or 'Mandookaparni'. In the traditional system of Indian medicine, *Centella asiatica* is a nervine tonic and is used in the treatment of leprosy, asthma, bronchitis, dropsy, leucorrhoea, skin disease and urethritis (Kakkar and Gomez, 1988). The wound healing activity of the species is ascribed to a triterpenoid saponin, asiaticoside (Fig 1) while antitumour properties are attributed to madecassic acid. *Centella* extracts have antitumour activity *in vitro* and *in vivo* (Babu *et al.*, 1995).

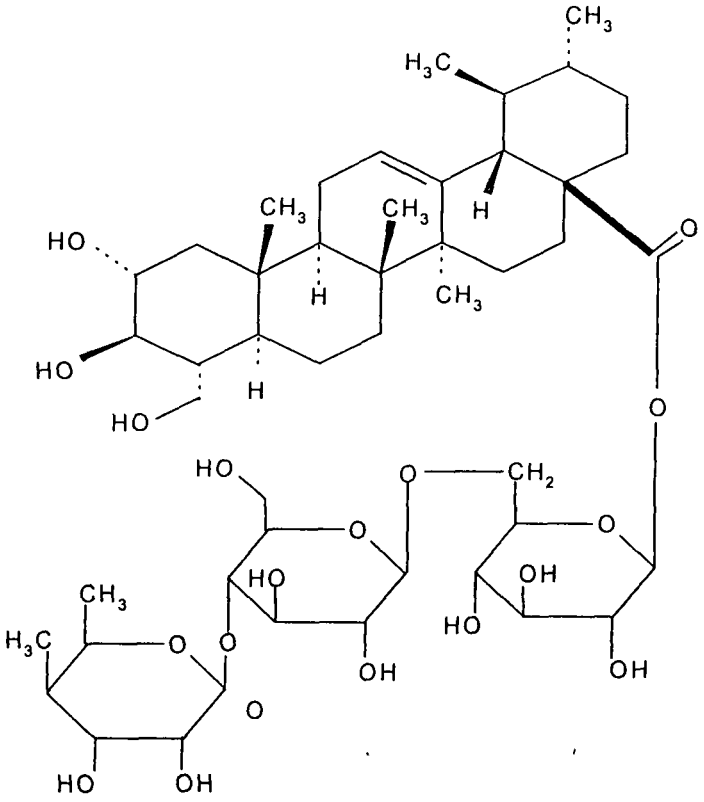
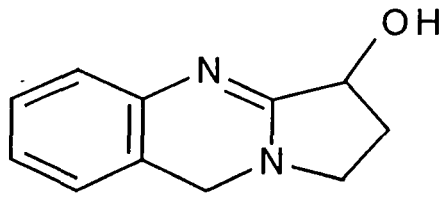


Fig 1 Structure of the triterpenoid saponin, asiaticoside in *Centella asiatica*

Adhatoda vasica Nees (Acanthaceae) is an important medicinal plant. The medicinal importance of *Adhatoda vasica* had been reported in the ancient Indian medicinal treaties of Ayurveda. The plant grows in the wild and is being exploited for extraction of its principal alkaloid vasicine (Fig 2) which is used in the preparation of 'vasaka', a well known drug in the Ayurvedic system of medicine. This drug is recommended by the Ayurvedic medicine for a range of ailments *viz.*, bronchitis, asthma, jaundice, diseases of the respiratory system, diphtheria and gonorrhoea (Kapoor, 2001). The pharmaceutical investigations of vasicine and vasicinone- alkaloids derived from *Adhatoda vasica* had been reported (Gupta *et al.*, 1977). The efficacy of vasicine as a uterotonic abortifacient had also been proved (Gupta *et al.*, 1978). Several alkaloids

from *Adhatoda vasica* have been found to have pronounced protective activity against allergy induced bronchial obstructions (Dorsch and Wagner, 1991). Extract of the plant *Adhatoda vasica* has been used as an effective Ayurvedic medicine in the treatment of tuberculosis. Bromohexine and ambroxol which are the semisynthetic derivatives of vasicine are also found to have growth



Vasicine

Fig 2 Structure of the alkaloid, vasicine in *Adhatoda vasica*

inhibitory effect against *Mycobacterium tuberculosis* (Grange and Snell, 1996). The roots and leaves of the plant *Adhatoda beddomei* C.B. Clarke, an allied species are also used in both traditional and modern systems of medicine for its alkaloid vasicine (Sudha and Seeni, 1994).

Asparagus racemosus Willd. (Liliaceae), commonly has known as 'satawar'or 'shatavari' is a scandent climber. The root of this plant is known to possess anti-diarrhoeal, anti-ulcer, refrigerant, tonic, nutritive, demulcent, diuretic, galactagogue, aphrodisiac and antispasmodic actions and has gained importance by its its use in Ayurveda, Siddha and Unani systems of medicine (Nadkarni, 1954). The alcohol, ethyl acetate and acetone extract of powdered dry roots of *Asparagus racemosus* yield pharmacologically active

(antioxytocic)saponins, one of which is L-asparagine (Fig 3)(CRC Handbook of Ayurvedic Medicinalal plants).

The dry roots of the plants are used as drug (Krinck, 1978).The roots are

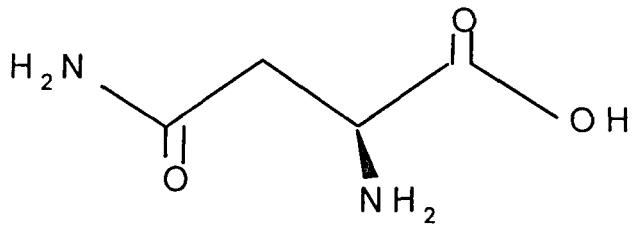


Fig 3 Structure of the saponin,

L-asparagine in *Asparagus racemosus*

said to be tonic and diuretic (Tewari *et al.*,1993). The cultivation of *Asparagus* is not extended at the commercial scale and its demand is met through the wild resources from the forest. Looking at the high demand of shade dried roots of *Asparagus* a study was undertaken to determine the optimum plant density for obtaining high root yield of *A. racemosus* and *A. adscendens* in sandy soils of the north Indian plains. Significantly higher yield of roots were recorded with greater plant density of 1.11×10^5 plant/ha in *A. racemosus* by 1.6 fold as compared to *A. adsendens* (Ram *et al.*, 2001). The aqueous extracts of both fresh and dried roots of *Asparagus racemosus* were found to have amylase and lipase activities (Dange et *al.*, 1969).

2.2 Micropropagation

In recent years, there has been an increased interest in *in vitro* culture techniques which offer viable tools for mass multiplication and germplasm

conservation of rare, endangered and threatened medicinal plants (Ajitkumar and Seeni, 1998 ; Sahoo and Chand, 1998).Plant tissue culture can also be used to generate variants (mutants) having industrially relevant features. Another significant aspect with respect to these species is that in spite of their immense potentials as sources of important drugs, there are some limitations in propagation of some of these species through the conventional methods as they are cross pollinated with high degree of genetic variability. Besides, seed viability is also very low in many a cases. Tissue culture techniques play a crucial role in overcoming these problems.

Centella asiatica L (Apiaceae) is an important medicinal plant used in several ayurvedic preparations. The requirement of this plant is now met from the natural populations, leading to their gradual depletion. Micropropagation techniques and application of biotechnology have given a wide scope for improvement of this important medicinal plant. A simple and a rapid method for the *in vitro* multiplication of *C. asiatica* from leaf explants had been established (Banerjee *et al.*, 1999). Leaf explants without the petioles were found to be more responsive than those with petioles. BA (2mg/l) along with IBA (0.1 mg/l) caused maximum sprouting where 80% of the leaf segments without petioles showed initiation along the margins and cut ends within two weeks, and only 30% of leaves with petioles responded, showing initiation near the distal cut ends of petioles after 4 weeks of culture initiation. In another attempt nodal segment harbouring single,axillary buds proliferated well in ½ strength MS medium supplemented with 2.0 mg/l BA, 0.5 mg/l KIN and 0.25 mg/l IBA (Josekutty, 1998). Prolonged culture on this medium or transfer to ½ strength MS medium without hormones resulted in profuse

rooting. A protocol was also described for rapid and large scale *in vitro* clonal propagation of *C. asiatica* L. through enhanced axillary bud proliferation in nodal segments isolated from mature plants (Tiwari *et al.*, 2000). MS medium supplemented with 6.7 μM BA and 2.88 μM IAA was found most suitable for shoot elongation. Rooting was highest (90%) on full strength MS medium containing 2.46 μM IBA.

However, initiation of *Centella* nodal culture proved rather difficult due to heavy fungal and bacterial contamination (Tiwari *et al.*, 2001). Treatment with solution of systemic fungicide (Bavistin) and antibiotic (Trimethoprim) were required which resulted in drastic reduction in contamination with nearly 80% cultures were contamination free .Shoot tips have been a source material for obtaining virus-free and genetically abberant plants. Plantlets from shoot tip explants have been described by several workers in different species (Deshpande *et al.*, 1999; Philomina and Rao, 2000).The suitability of shoot tip explant for regeneration and its sensitivity to various hormones is due to the activity of meristematic cells, which are actively dividing and are known to have dense cytoplasm with much more uniform and homogeneous composition (Mathur *et al.*, 2002).

Adhatoda vasica Nees is an important medicinal plant for the presence of bitter crystalline alkaloid called vasicine and an organic acid – adhatodic acid, another alkaloid and an odorous volatile principle (Iyengar, 1984).It is an evergreen woody perrenial bush. The influence of callusing at the excised ends and the effect of browning and phenolic exudation in woody perrenial plant species preventing multiple shoot induction was reported earlier (Komalavalli and Rao, 2000; Reddy *et al.*, 1998; Patnaik and Debata, 1996).

These problems were overcome by modification of the media. Even in *Adhatoda beddomei* C.B. Clarke there was evidence of decline due to browning followed by necrosis of the cultures after 4 weeks irrespective of the concentrations and combinations of the cytokinins tried (Sudha and Seeni, 1994). However an optimum combination of 3.0 mg/l BA, 0.5 mg/l 2-ip and 1.0 mg/l NAA favoured the differentiation of 2-3 shoot buds in 3 weeks and development of 5-10 shoots (0.5-2.0 cm) in 85%of the explants in 6 weeks. Combinations of cytokinins thus found to activate the axillary meristems to form shoot buds but sustenance of the shoot growth depended on the synergistic influence of the auxin.

Efficient tissue culture method for rapid multiplication of *Adhatoda vasica* through nodal segment culture was established (Jaiswal *et al.*, 1989) for the production of vasicine. The vasicine content also varied with varying concentrations of BA. Shoots with broad leaves and multiple shoot buds cultured on medium with 0.5 mg/l BA synthesized the highest amount of vasicine (Jaiswal *et al.*, 1989). However there was persistent contamination problem when nodal explants were used for micropropagtion of *Adhatoda vasica.* The use of nodal explants may also accentuate the problem of phenolic exudates. In the present study therefore, shoot tip were used as explants for micropropagation of *Adhatoda vasica. Adhatoda beddomei* is also as important source of drug *vasaka* in the Indian indigenous system of medicine (Ayurveda) where it is considered therapeutically superior to the allied species, *Adhatoda vasica* .It rarely set seeds and natural vegetation and conventional propagation through vegetative cuttings were also slow and insufficient for conservation. Therefore an attempt was made to establish a

rapid propagation protocol through axillary bud proliferation (Sudha and Seeni, 1994).

Efforts towards development of *in vitro* micropropogation technique is rather meagre for the genus *Asparagus*, despite its horticultural and medicinal importance. Vegetatively propagated *Asparagus* plants are likely to accumulate pathogenic viruses and the latent viruses can be seed transmitted. Yang and Clore (1976) compared shoot tips (0.1-0.3 mm explants with 1-3 leaf primordia) with meristem tip (less than 0.1 mm with no leaf primordia) culture and found that 91%of the plants derived from meristem tip explants were virus free compared to only 43% of those which came from shoot tips. In another attempt, Dan and Stephens, 1991 developed a protocol for culture and plant regeneration of callus derived protoplasts of *Asparagus officinalis* cultivar Lucullus 234. *Asparagus officinalis* Cultivar Lucullus 234 is a highly disease resistant species against *Fusarium moniliforme* (sheld) and *F. oxysporum*. But due to incompatibility barriers sexual crosses were not possible between the resistant species *A. officinalis* and *A. densiflorus* 'sprengeri'. This problem was overcome by protoplast fusion (Davey and Kumar, 1983) for which the above attempt of protoplast regeneration of *A. officinalis* was made.

Murashige *et al* (1972 b) and Hasegawa *et al* (1973) developed a method for propagating *Asparagus* officinalis plants by shoot culture. Multiple shoots were obtained from shoot apex or lateral bud explants on Murashige *et al.*, (1972 b) medium augmented with 0.3 mg/l NAA, 0.1 mg/l KIN and 40 mg/l adenine sulphate dehydrate. Even lateral buds from *Asparagus officinalis* spears were grown by Yang and Clore (1973) into single shoot on a semi-

solid basal medium (3% sucrose) containing 0.05- 0.1 mg/l NAA and 0.05- 0.1 mg/l KIN. Shoot arising from cultured nodes were cut into single node segments, which were recultured on the substrate or rooted. However, no report on efficient micropropagation protocol has been established in *A. racemosus* till date.

2.3 Callus culture and cell suspension culture

Callus is produced on explants *in vitro* in response to wound and growth substances either endogenous or supplied to the medium. Continuous subculture at 3-4 week intervals of small cell clusters taken from these calli can maintain the callus cultures for long periods. Initiation of cell division and subsequent callus production require a supply of cytokinin and auxin in the medium at correct proportion (Skoog and Miller, 1957). Auxin at a moderate to high concentration is the primary growth substance used to produce callus. Cytokinin is supplied in a lesser amount if not adequate within the explant. Ghosh and Sen, 1991 induced callusing in *Asparagus officinalis* using spear sections 3 mm below apex when cultured in MS medium supplemented with 1 mg/l NAA and 1 mg/l KIN. Even Levi and Sink, 1992 obtained callus in the medium supplemented with 0.1 mg/l NAA and 0.28 mg/l 2-ip.

Although callus tissue cultures may appear outwardly to be uniform masses of cells, in reality these structures are relatively complex with considerable morphological, physiological and genetic variation within the callus. Cell divisions do not take place throughout the culture mass but are localised primarily in a meristematic layer on the outer periphery of cells. Organogenesis begins with dedifferentiation of parenchyma cells to produce

centres of meristematic activity (meristemoids)(Sharp and Flick, 1981; Thorpe, 1979). Shoot and root initiation from the meristemoids follow when an appropriate ratio between the auxin and cytokinin is maintained. Reinert J, 1959 discovered the formation of miniature embryo like structures called embryoids in carrot grown on agar using high auxin concentration as the inducing agent. Since then, specific tissues in various species have been found to have either a capacity (competence) for somatic embryogenesis in culture systems or can be induced to develop competency for the same in culture by specific treatments to the medium. In *Asparagus cooperi* somatic embryogenesis was induced from callus following KNO_3 treatment (Ghosh and Sen, 1989). Such somatic embryos provide ideal materials for rapid propagation of stable regenerants. However complete plantlets could not be obtained in MS basal medium only. The optimum results were secured when embryos were cultured in the basal medium (MS) supplemented with Zeatin or GA_3 alone (Ghosh and Sen, 1991).

Reuther, 1977 induced callus by culturing shoot tip or shoot segment explants on LS medium supplemented with 1 mg/l NAA and 1 mg/l KIN. The calli when subcultured at stage II to LS medium with 1 mg/l IAA and 0.1 mg/l BA, the callus became organogenic and gave rise to shoot initials and somatic embryos. Even embryogenic calli were obtained in a variety of *Asparagus* explants when cultured on MS medium in which vitamin content has minor amendments. Ghosh and Sen, 1991 obtained somatic embryos in calli when induced from spear sections 3 mm below apex of *Asparagus officinalis* following transfer to medium with extra 1g/l KNO_3 or 1g/l casein hydrolysate.

Somatic embryos can be induced even by cell suspension culture in liquid culture media. A cell suspension culture is initiated by placing a piece of friable callus or homogenized tissue in liquid medium on a shaker so that the cells dissociate from each other and single cells can be obtained. Somatic embryos in cell suspensions can be obtained if the tissue is subjected to conditioning to induce embryogenic competence. Although embryogenic cell suspension cultures outwardly appear to be callus, on closer inspection these cell mases are well organised as proembryogenic masses (PEMs). PEMs continue to develop in suspension cultures until they are transferred to a stationary medium to develop somatic embryos. In *Acacia catechu* Willd. (Leguminaceae) callus proliferation occurred on cotyledon explants cultured on MS medium supplemented with 2, 4-D (3 mg/l and BA (0.5 mg/l) (Kaur and Kant, 1999). On transferring and agitating callus clumps in liquid MS medium containing 3 mg/l BA and 0.5 mg/l NAA, shoot bud differentiation was obtained.

The proembryogenic masses are often passed through sizing screens to get uniformity and synchrony of development. In *Asparagus officinalis* (Levi and Sink, 1992) the calli obtained in the medium supplemented with 0.1 mg/l NAA and 0.28 mg/l 2-ip were transferred to a suspension culture with continuous agitation and after 3 weeks the suspensions were sieved through 600 μm mesh and re suspended in medium with 10-20 mg/l NAA to get uniform embryogenic cell clusters and globular embryos. The growth of the embryos were promoted when transferred to medium with 0.1 mg/l NAA and 0.22 mg/l 2-ip and complete plantlets were obtained with 8.2 % sucrose initially, followed by transfer to medium with 8 % sucrose.

Plant tissue culture techniques like callus cultures were widely employed to obtain a stable supply of bioactive secondary products (Bajaj *et al.*, 1988; Yeoman *et al.*, 1996), including anti-cancer drugs like taxol (Edgington,1991). Iyer *et al.*, 1998 raised calli from cotyledons, hypocotyls, roots, leaves and internodes of *Nyctanthes arbor-tristis* in MS medium supplemented with 2,4-D, NAA and coconut milk. The alcohol extract of the callus showed the presence of iridoid glycosides by thin layer chromatography. The iridoid arbor-tristoside A had been reported to have pronounced anticancer activity (Mathuram *et al.*, 1991; Stuppner *et al.*, 1993; Mathuram *et al.*, 1994). Castellar and Iborra, 1997 attempted to induce calli from stems of *Crocus sativus* L., the source of saffron which is a high value flavouring agent in MS medium supplemented with 5 mg/l BA and 10 mg/l NAA and the calli differentiated into stigmas when transferred to MS medium with 5 mg/l BA and 1 mg/l NAA. The saffron stigmas contain a secondary metabolite called crocin, a yellow pigment freely soluble in water. The yellow pigmentation due to presence of crocin progressively increased with calli growth, which was analysed by HPLC.

So far, there is no report on the use suspension culture for production of vasicine, asiaticoside and L-asparagine.

2.4 Antimicrobial activity of secondary metabolites of plant origin

The importance of medicinal plants in providing healthcare against various ailments including infectious diseases is well documented (Chopra *et al.*, 1992; Iyengar, 1984). Moreover, the development of multi-drug resistance in the pathogenic bacteria and parasites has created major clinical problems in

the treatment of infectious diseases. This and other problems like toxicity of certain antimicrobial drugs on the host tissue (Idose *et al.*, 1968; Maddux and Barrere, 1980) and non-availability of a suitable antifungal drug for systemic mycoses have triggered interest in search for new antimicrobial substances/drugs of plant origin. Considerable work on the antimicrobial activity of medicinal plants has been reported from different parts of the world (Ahmad *et al.*, 1995; David, 1997; Desta, 1993).

The antimicrobial drugs injure microbes through several different mechanisms. Antimicrobial drugs either kill microorganisms directly (bactericidal) or simply prevent them from growing (bacteriostatic). They function specifically in one of the following ways-

1. inhibition of cell wall formation- The cell walls of most bacteria contain a rigid girdle of peptidoglycan. This structure, which is many layers thick in gram positive species and quite thin in gram negative ones, protects the cell against rupture from hypo tonic environments. Cells actively engaged in enlargement or binary fission must constantly synthesize new peptidoglycan and transport it to its proper place in the cell envelope. Drugs such as penicillin and cephalosporin react with one or more of the enzymes required tocomplete this process, causing the cell to develop weak points at growth sites and to become osmotically fragile (Fig 4). Antibiotics that produce this affect are considered bactericidal, because the weakened cell is subject to lyses.

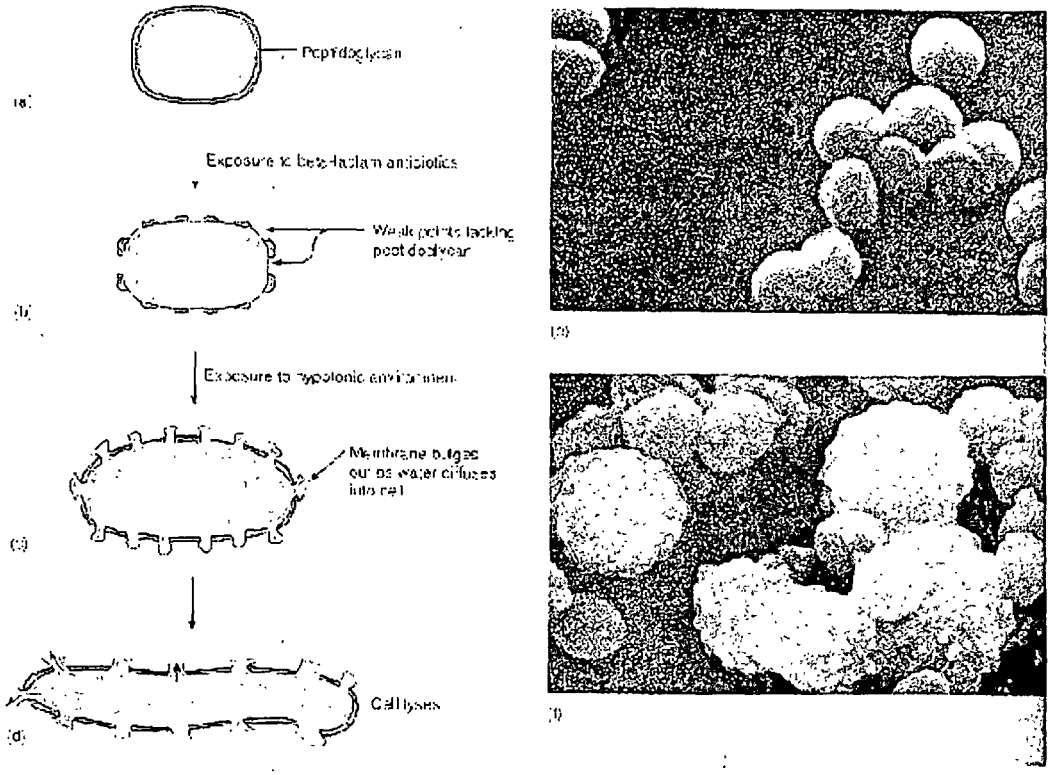


Fig 4 Effect of antibiotics on cell wall synthesis of a growing cell (a) coccus is exposed to betalactam agent (cephalosporin) (b) weak points develop where peptidoglycan is incomplete (c) the weakened cell is exposed to a hypotonic environment (d) the cell lyses (e) scanning electron micrograph of bacterial cells in their normal state (× 10,000) (f) SEM of the same cells in the drug affected state showing surface bulges (× 10,000) (Talaro K and Talaro A, 1996)

(2) inhibition of nucleic acid synthesis- Antimicrobial drugs interfere with nucleic acid synthesis by blocking the synthesis of nucleotides, inhibiting replication or stopping transcription. Sulfonamides act as structural or metabolic analogs that mimic the natural substrate of an enzyme and vie for its active sites. In practice, sulpha drugs are similar to natural metabolic compound PABA (para amino benzoic acid) required by bacteria to synthesize folic acid. Folic acid in turn is a component of the co-enzyme tetrahydrofolic acid that participates in the synthesis of purines and certain amino acids. A sulfonamide molecule has extremely affinity for the PABA site

on the enzyme that synthesizes folic acid, thus it can successfully compete in a " chemical race" with PABA for the same site (Fig 5). Sulfonamides ultimately cause inadequate supply of folic acid for purine production, which invariably halts nucleic acid synthesis and prevents bacterial cells from multiplying.

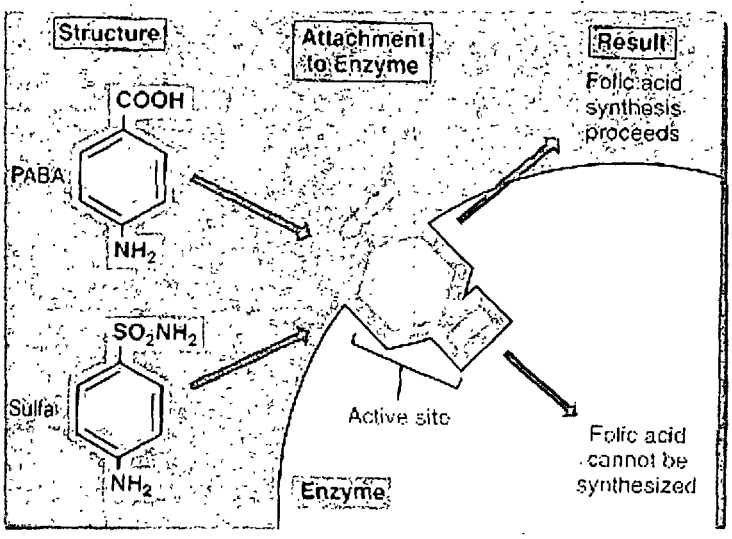


Fig 5 Competetive inhibition of a pathway in nucleic acid synthesis. Sulfonamides are structural analogs of PABA, a chemical required to synthesise folic acid. The similar configuration of the two means that sulfonamides can bind to the active site on an enzyme involved in folic acid synthesis. Although it binds, sulfa still cannot complete the required synthesis (Talaro K and Talaro A, 1996)

(3) inhibition of protein synthesis- Antibiotics like streptomycin, gentamycin, tetracyclines inhibit protein synthesis by binding with the prokaryotic ribosome. Some drugs bind to the 30S (small) subunit, while others attach to the 50S (large) ribosomal subunit. Thus different steps in the protein synthesis mechanisms are affected: aminoacyl-tRNA binding, peptide bond formation, mRNA reading and translocation.

(4) Interference with the function of the cell membrane-Isoniazid (INH) and Ethambutol are examples of effective synthetic antimicrobial drugs against *Mycobacterium tuberculosis,* which inhibit synthesis of mycolic acid, which are components of cell walls restricted only to the mycobacteria. The cells of the genus *Mycobacterium* are long, slender, or curved rods with a slight tendency to be filamentous or branching. The high lipid content of the cell wall imparts the characteristic of acid-fastness and is responsible for the resistance of the group to drying, acids and various germicides. It produces no exotoxins or enzymes effecting infectivity. The majority (85 %) of tuberculosis (TB) cases are contained in the lungs, even though disseminated tubercle bacilli can give rise to tuberculosis in any organ of the body (extrapulmonary TB). Clinical tuberculosis is divided into primary, secondary (reactivation or reinfection) tuberculosis, and disseminated tuberculosis. Treatment of TB therefore involves administering drugs for a sufficient period of time to annihilate the bacilli in the lungs, organs and macrophages usually for 6-24 months. In the conventional treatment of patients with tuberculosis a combined therapy with at least two drugs including isoniazid, rifampin, ethambutol, streptomycin, pyrazinamide, thioacetazone or para aminosalicylic acid (PAS) are administered to avoid drug resistance. However other factors such as drug addiction, HIV pandemic and various socio-economic factors have contributed to the spread of the TB bacillus and led to the growing cases of MDR-TB. An estimate of 273,000 (95 % confidence limits, 185,000 and 414,000) new cases of MDR-TB occurred worldwide in 2000, 3.2 % of which all new TB cases were reported by a survey conducted over 64 countries (Dye *et al.*, 2002). The treatment regime for HIV and MDR-TB is complicated by the fact

that most of the drugs used have not been studied for interactions with antiviral agents. Hence overlapping toxicities require intensive management and monitoring of these patients (Poznaik, 2001). Informations gained from sequencing the *Mycobacterium tuberculosis* genome will enable scientists to accelerate the development of reagents for improved tuberculosis control. Cloning and expressing the genes encoding the enzymes involved in cell wall biosynthesis will provide the tools for screening millions of novel compounds (Young, 2001) having inhibitory effects. Even an approach to target metabolic processes that are essential in nondividing bacteria will be effective and the latent diseases through a drug that acts in synergy with the immune response could be targeted (Young D, 2001).

Considering the rich diversity of Indian medicinal plants, it is expected that screening and scientific evaluation of plant extracts for their antimicrobial activity may provide new antimicrobial substances. Ahmad I *et al.*, 2000 has determined the MIC values against several bacteria and yeast, *Candida albicans* of the extracts (800-9000 mg/ml) of five plants *Emblica officinalis* L., *Plumbago zeylanica* L., *Holarrhena antidysentrica* Rox. bex Fleming, *Terminalia belerica* (Gaertner) Roxb. and *Terminalia chebula* Retz. Maximum potency (lowest MIC) was recorded in *Emblica officinalis* against *Staphylococcus aureus*, *Staphylococcus epidermis* and *Salmonella typhimurium*. Growth inhibition using agar diffusion assays against *E. coli*, *Psuedomonas aeruginosa*, *Staphylococcus aureus* and *Candida albicans* were also studied using the crude extracts from 59 species representing mostly plant families of *Scrophulariaceae* and *Acanthaceae* (Grimes *et al.*,

1996). Growth inhibitory activity against one or more of the microbial species was detected in over 40 % of the samples.

The herb, *Centella asiatica* L. has been used in the treatment of leprosy patients from very early times in India. The *in vitro* effect of an indigenously produced dry powder of *Centella asiatica* on the acid fastness and viability of *Mycobacterium tuberculosis* was investigated and found that it has no direct action on the acid fastness or viability of *M. tuberculosis* H37Rv *in vitro* (Herbert *et al.*, 1994). Even the antitumour effect of the crude extract of *Centella asiatica* as well as its partially purified fractions from chromatographic procedures through both *in vitro* short and long term chemosensitivity and *in vivo* tumour model test system were studied (Babu *et al.*, 1995). The partially purified fractions from the crude extracts of *Centella asiatica* has been reported to inhibit the proliferation of transformed cell lines (L-929) significantly. Hausen, 1993 also found the *C. asiatica* raw extract and its 3 acids namely triterpenic constituents asiaticoside, asiatic acid and madecassic acid to be weak sensitizers. However, the alcohol extract of *C. asiatica* increased cellular proliferation and collagen synthesis at the wound site of rats as evidenced by increase in DNA, protein and collagen contents of granulation tissues (Suguna *et al.*, 1996) which suggests that some kind of anti-tumour properties are present in this plant.

The presence of antiasthamatic activity of *Adhatoda vasica* plant extracts was also supported by Singh *et al.*, 2001. The leaves and roots are antispasmodic and efficacious in cough (CRC Handbook of Ayurvedic Medicinal Plants). The unknown alkaloids from *Adhatoda vasica* showed pronounced protection against allergen induced bronchial obstruction in

guinea pigs (Dorsch *et al.*, 1991). The abortifacient effect of vasicine, the prime alkaloid of *Adhatoda vasica* like its uterotonic effect was more marked under the primary influence of oestrogens which are known to enhance the synthesis of prostaglandin indicating that the action of the vasicine was mediated through the release of prostaglandin (Gupta *et al.*, 1978). The effects of even aqueous or 90 % ethanol extracts of *Adhatoda vasica* were studied in rats orally dosed for 10 days after insemination with special reference to foetal development. Leaf extracts of *Adhatoda vasica* were found to be 100 % abortive at doses equivalent to 175 mg/Kg of starting dry material (Nath *et al.*, 1992). Grange *et al.*, 1996 found that the benzylamines, bromhexins and ambroxol which were semi-synthetic derivatives of vasicine from the Indian shrub, *Adhatoda vasica* had a pH-dependent inhibitory effect on *Mycobacterium tuberculosis*. The plant extracts of *Adhatoda vasica* were found to be rich in enzymic and nonenzymic antioxidants such as catalase, peroxidase, total carotene, ascorbic acid, tocopherol and polyphenols. The plants extracts were found to possess antitubercular activity which were also good sources of antioxidants and therefore capable of preventing tissue damage by reactive oxygen species (Usha *et al.*, 2001).

Alcoholic extract of the root of *Asparagus racemosus* was found to inhibit ochratoxin A (OTA)- induced suppression of production of cytokines. The extract also significantly inhibited the OTA-induced suppression of chemotactic activity (Dhuley, 1997). The claim of the use of *Asparagus racemosus* root in Ayurveda in the treatment of jaundice was scientifically validated by Muruganandan *et al.*, 2000 by evaluating the immunomodulatory and antihepatoxic activities of *Asparagus racemosus* root extracts. He found

that the ethanolic root extract (100 mg/ Kg) enhanced the humoral and cell-mediated immunity in mice and exhibited antihepatotoxicity in rats.The crude saponins from the shoots of *Asparagus* were found to have antitumour activity (Shao *et al.*, 1996). The saponins inhibited the growth of human leukaemia HL-60 cells in culture and macromolecular synthesis in a dose-dependent manner. The inhibitory effect of crude saponins on DNA synthesis was also found to be irreversible. In another attempt, Shao *et al.*, 1996 isolated two oligofurostanosides from the seeds of *Asparagus officinalis* which also showed the same anti-tumour activity. However, so far no report has been found on anti *Mycobacterium tuberculosis* activity of the crude extract of *Asparagus* plant.

In another species of the genus *Asparagus* like in *Asparagus adscendens*, the aqueous and alcoholic extracts of the roots was studied on the muscle preparation of *Setaria cervi, in vitro* and on the survival of microfilariae *in vitro*. It was found that both alcoholic as well as aqueous extracts caused death of microfilariae *in vitro,* LC50 and LC90 being 8 and 16 ng/ml for aqueous, 3 and 12 ng/ml for alcoholic extracts respectively (Singh *et al.*, 1997). Two anti protozoal compounds have been also isolated from the roots of *Asparagus africanus* Lam, which potently inhibit the growth of *Leishmania major* promastigotes, while moderately inhibits *Plasmodium falciparum* schizonts in 12 μM and 49 μM respectively (Oketch-Rabah *et al.*, 1997). These concentrations only moderately affect the proliferation of human lymphocytes.

2.5 Approaches to the discovery of drugs from plant sources

Natural products have been the most successful source of drugs ever (Harvey, 2000; Newman *et al.*, 2003). Historically the most important natural sources have been plants. Research progressed along two major lines: ethnopharmacology (medicinal herbs, substances of abuse, ordeal poisons) and toxicology (poisonous plants, venomous animals, arrow and fish poisons) (Heinrich and Gibbons, 2001). These strategies have produced many valuable drugs and are likely to continue to produce lead compounds (Tulp and Bohlin, 2002).

The interest in the use of ethnopharmacological information was for several reasons. At the beginning of the 1990s, there has been greater cross-cultural interest in the practices of native people than was formerly the case (de smet and Rivier, 1989). Farnsworth and colleagues showed that of the 119 important plants derived drugs used in one or more countries, 88 (77 %) were regarded as having been discovered as a result of being derived from a plant used in traditional medicine (Farnsworth and Soejarto, 1991). In addition, several additional contributions have appeared in the literature recently that report on the value of using an ethnopharmacological approach to the discovery of natural product drugs from plant sources. Another approach is chemotaxonomy which relies upon the fact that taxonomically related plants often biosynthesise chemically similar secondary metabolites. The discovery of digoxin from *Digitalis lanata* is a good example of how a chemotaxonomic approach can lead to a new drug, since this species was investigated phytochemically, based on the known existence of cardiac glycosides, such as digitoxin in another member of the same genus, *D.*

purpurea (Farnsworth and Soejarto, 1991). This approach is potentially useful in that, a higher yield of a given compound of interest can often be found for example, in other member of the same genus of a species under consideration (Anonymous, 1989).

Once significant activity is detected and confirmed for a given plant extract, the plant materials should be recollected under the same conditions, purification and structural characterization of the active constituents were done. Activity-guided fractionation is carried out using a combination of bulk solvent extraction and chromatographic procedures, which vary from lab to lab depending upon individual preference. Compound characterization and structure determination is performed routinely by the interpretation of physical and spectroscopic data, with compound degradations and total or partial synthesis carried out as deemed necessary.

High performance liquid chromatography (HPLC) has become one of the most widely used methods for the analysis of different compound mixtures. Simple, quick and accurate analytical procedures through HPLC with photodiode array detector was used for purity determination, wavelength optimization, similarity curves and component identification by spectral libraries in *Catharanthus roseus*, *Papaver somniferum*, *Adhatoda vasica* for their important alkaloids (Gupta *et al.*, 2000). Brain and Thapa, 1983 studied the degradation of vasicine in various solvents under normal laboratory lightning conditions and continuous UV irradiation at 365 nm. Quantification and detection of vasicine and vasicinone in *Adhatoda vasica* by HPLC method was also reported (Choudhury and Hirani, 1987) which involves the ion-pairing technique and uses an internal standard for quantification.

Fifteen collections of *Centella asiatica* L. were analysed for asiaticoside and madecassocide composition. The estimations were carried out by HPLC using C18 column, acetonitrile-water (3:7) as solvent and UV detector at 220 nm (Singh *et al.*, 1999). A pharmacologically important plumbagin in *Plumbago zeylanica* was estimated by rapid, accurate and simple HPLC method (Gupta *et al.*, 1999). The assay combines the isolation and seperation of plumbagin on silica gel $60F_{254}$ TLC plates followed by scanning of the spot at 265 nm detection modes using a CAMAG Scanner 3.

The alkaloids ajmaline, serpentine, reserpine and ajmaciline from *Rauwolfia serpentina* benthe ex kurz were identified by TLC and quantified by HPLC from tissue culture derived plants and wild plants. The identity of the alkaloids was confirmed by co-chromatography with authentic samples of ajmalicine, reserpine, ajmaline and serpentine (Roja and Heble, 1996). Similarly, quantitative determination of aristolochic acid in *Aristolochia indica* was also done by a rapid, sensitive and reproducible HPLC method based on photo-diode array detector (Singh *et al.*, 2001). The analysis of tropane alkaloids in solanaceous plants is of importance because of the extensive use of atropine and scopolamine in the pharmaceutical preparations. Various spectrophotometric (Worrel and Booth, 1953), HPLC (Verpoort and Svendsen, 1976) methods had been reported for quantitative determination of the tropane alkaloids.

2.6 Bioreactor raised secondary metabolites

Mass culture of plant cells *in vitro* has been proposed as a viable alternative for the production of vast arrays of high value, low volume phytochemicals. It

is greatly influenced by the culture conditions of which culture medium is the most important. The production of secondary metabolites generally occurs in the late stationary phase when the meduim gets depleted of some of its omportant constituents. In such cases a 'dual culture system' is preferred. It involves biomass production in a medium for cell proliferation (growth medium) followed by transfer of healthy cells to a different medium (production medium) which does not support good growth of the cells but is favourable for product yield. The production of an anticancer drug paclitaxel from the plant *Taxus baccata* has been studied by Tabata, 2004 following this strategy. The study revealed that paclitaxel reaches a maximum level of 295 mg l^{-1} in a large scale culture of T.x media cells.

In the scale up of a production process for secondary metabolites in plant cells by suspension culture, a number of basic laboratory-scale experiments have to be performed to yield essential data like growth rate, product formation rate, nutrient uptake, respiration rate and heat production. There are several examples showing changes in growth or productivity of plant cell clusters when scaled up (Scragg *et al.*, 1987; Schiel *et al.*, 1987). It is therefore essential to mimic in the laboratory the conditions occurring on a large scale as carefully as possible, or to study the differences on various scales to pinpoint the responsible factor.

For the last two decades considerable work has been done to design bioreactors for plant cell culture in large scale (Scragg, 1994). Conventional bioreactors like stirred tank reactor, bubble column or airlift bioreactors can be used for cell suspension with free plant cells or their small aggregates because it approximates microbial cultures. The effects that are known to

occur ona large scale can be studied in the same type of bioreactor at a smaller scale, mimicking the critical situations that occur in a large system. Noguchi *et al.*, 1977 cultivated *Nicotiana tobacum* cell suspensions in a 20,000 l stirred tank reactor to produce while Schiel and Berlin, 1987 studied *Catharanthus roseus* in a 5000 l stirred tank reactor.for the production of indole alkaloids.

Efficient mixing of plant cells cultured on large scale is extremely important to provide uniform physiological conditions within the culture vessels. Plant cells are sensitive to the shear stress due to rigid cellulosic wall and extensive vacuole, restricting the use of high agitation for efficient mixing. Plant cells are therfore often grown in modified stirred-tank bioreactors at a very agitation speeds. Air-lift bioreactors may provide even better and uniform enviornmental conditions at low shear. In suspension cultures of *Taxus chinensis* var mairei the shear rates over 719 s^{-1} damaged the cells by decreasing the mitochondrial activity, increasing the membrane permeability and causing cell hypersensitive responses. Consequently phenylalanine ammonia lyase (PAL) were activated and extracellular phenolics were accumulated in the cells leading to lower secondary metabolism (Shi *et al.*, 2003). But Scragg *et al.*, 1988 and Meijer *et al.*, 1993 demonstrated that cells from various plant species are shear tolerant. He explained that recently initiated cell lines may be more susceptible to shear stress than cell lines cultivated for a long time in liquid medium. This conclusion was supported by the observation that the cultivation of freshly initiated (from calli) *Ginseng* cultures on a large scale was troublesome due to shear stress, while the

cultivation of *Echinacea* and *Rauwolfia* cell clusters in 75,000 l stirred tank reactors presented no shear problems (Westphal, 1990).

All plant cells are aerobic and require continuos supply of oxygen. However, plant cells require less oxygen than micro-organisms because of their slow metabolism. It has been observed that carbondioxide level can drastically influences the length of the lag phase and is some cases, higher oxygen concentration even proved to be toxic to the metabolic activities of cells. The cell suspension culture of *Taxus wallichiana* in a 20 l airlift bioreactor accumulated higher amount of paclitaxel and baccatin III (factor of 2.0 and 1.2 respectively) than in the shake flasks, even the cell biomass was at maximum productivity in both the condition. This was mainly due to adequate aeration and mixing of the culture in the bioreactor (Navio osorio *et al.*, 2002).

During the late exponential phase of growth, cells become more sticky because of increased excretion of polysaccharides into the culture vessel, leading to the adhesion of plant cells to the reactor wall, probes and stirring device and formation of large aggregates. Proper mixing is therfore affected leading to lower secondary metabolite production. Even stresses, locations, climates, microenvironments and physical and chemical stimuli often called elicitors; qualitatively and quantitatively alter the content of bioactive secondary metabolites. Enzymatic pathways leading to the synthesis of these phytochemicals are highly inducible (Ebel and Cosio, 1994). This is true for alkaloids (Facchini, 2001), phenylpropanoids (Dixon and Paiva, 1995) and terpenoids (Trapp and Croteau, 2001; Turlings and Tumlinson, 1992) whose levels often increase by two to three orders of magnitude following stress or

elicitation (Darvill and Albersheim, 1984;Dixon, 1986). The enhanced catharanthine production in *Catharanthus roseus* cell cultures in a 20 l airlift bioreactor was due to the synergistic effect of thr elicitors, malate and sodium alginate (Zhao et al., 2001). Even the growth rate and production of paclitaxel and baccatin III in cell suspension culture of *Taxus* increased by 8.3 and 4.0 factors respectively when the production medium was supplemented with an elicitor, methyl jasmonate (220 μg g^{-1} FW) and two putative precursors, mevalonate (0.38 mμ) and N-benzoylglycine (0.2 mμ)(Cusido *et al.*, 2002).

Another important characteristic of plant cell suspensions is the requirement for a high inoculum density in order to obtain growth. This is due to the requirement of plant cells in culture for a factor(s) which is released into the medium from the cells. There is a report on the success of large scale production of oil palm (*Elaeis guineensis*) suspension cells in a bioreactor where the biomass increased approximately by 3.5 fold per month. This was due to the synergistic effect of both inoculum density nd conditioned media respectively (Gorret *et al.*, 2004). Even for high salidroside production, which was identified as the most potent ingredient of the chinese herb, *Rhodiola sachalinensis*, the optimum inoculum amount was 10 %(Wu et al., 2003). With this inoculum amount the optimal concentration for 6-benzylaminopurine and IBA added in the liquid medium was 5 and 2.5 mg l^{-1} respectively. The acidic culture medium and a faster shaking speed favoured the salidroside production. The addition of 2,4-D in the liquid MS medium and the utilization of L-tyrosal for chemical feeding enhanced salidroside production. Using a proper combination of culture condition and treatment, salidroside accumulation could reach 57.72 mg g^{-1} DW that was 5-10 fold higher than that

detected in field grown plants. The corresponding salidroside yield was 555.13 mg l^{-1}, a level suitable for cost effective commercial production to compensate the natural resource shortage of *R. sachalinensis*.

2.6.1 *Optimizing scale-up fermentation processes*

Optimization of the fermentation process takes place once the feasibility of the production in the selected organism has been demonstrated. Before starting long and expensive optimisation work, it is important that the stability of the strain is established, at least for the number of generations necessary for cell banking and largest-scale fermentation, including the pre-cultures. The main aim of optimisation is to maximise the production, so this process can be initiated only once a laboratory-scale purification process and a minimum set of quality control tools are available to quantify and assess the quality of the product (Thiry and Cingolani, 2002).

The factors affected by scale are the number of generations, the mutation probability, medium sterilization, the quality of temperature and pH regulations, agitation, aeration and pressure. The best way to prepare the scaling-up of a process is to first scale-down to the pilot scale of the conditions of culture that will be used at the final scale of production (Kwanmin, 1989). Then, when the scale is increased, the broth will become more and more heterogeneous. In large fermentors, oxygen can be depleted in some area of the reactor (Enfors *et al.*, 2001). Even though there is a trend to standardize as much as possible of the fermentation processes, it has been shown that even subtle changes would powerfully increase the productivity

like, adjunction of a cofactor or substrate can stabilize an enzyme and increase the final productivity (Thiry and Cingolani, 2002).

Chapter III

MATERIALS AND METHODS

Chapter III

Materials and Methods

3.1 Plant Material

Adhatoda vasica, *Centella asiatica* and *Asparagus racemosus* were collected from in and around Tezpur University Napaam campus and also from the campus of District jail at Tezpur, Assam, India.

3.2 Microbial cultures

Cultures of *Psuedomonas* spp (identified in RRL, Jorhat)., *Bacillus subtilis* (MTCC acc. no. 3972) and *Saccharomyces* spp (MTCC acc. no. 3980) were obtained from the Department of Molecular Biology and Biotechnology, Tezpur University. The cultures were indentified at Microbial Type Culture Collection (MTCC), Institute of Microbial Technology (IMTECH), Chandigarh. *Mycobacterium tuberculosis*, H37Rv was received from Central Drug Research Institute (CDRI), Lucknow.

3.3 Purified plant products

The purified plant products which were used as standards in the experiments viz., L-asparagine were procured from Sigma (Product number: A-4159) and Vasicine were received as a kind gift from Central Drug Research Institute, Lucknow.

3.4 Sterilization

The plant materials were washed using a detergent solution (1 % Teepol or Tween 20) and surface sterilisation were carried out asceptically using 0.01 % $HgCl_2$ followed by thorough rinsing (3-4 times) with sterilised Millique water.

The glasswares were cleaned by soaking in a mixture of 100 ml saturated solution of potassium dichromate in concentrated sulphuric acid for 4 hours followed by thorough rinsing under running tap water. The glass wares were then dried in an oven and sterilised in an autoclave (Narang Scientific Works Pvt. Ltd. India) under pressure of 15 lb/inch^2 and temperature of 120° C for 20 min. Sterilisation of the scalpels, tweezers, surgical blades etc., used in micropropagation related work, were done by frequent immersion in 90% ethyl alcohol and flaming during use.

The heat labile compounds such as vitamins and growth regulators were sterilized by filtration using autoclaved GD/X syringe filters [25mm, Whatman nylon filter membrane with pore size 0.2 μm (catalogue number: 68702502)]. Coconut water which was used as media additives was heated to 80° C with continuous stirring, cooled and then filtered through a coarse filter [Standard sheets (Whatman) Grade I, 26 x 31 mm (catalogue number : 1001813) and kept in the refrigerator (4°C)

3.5 Plant tissue culture

Plant cultures were maintained in a purpose designed tissue culture room equipped with a temperature and photoperiod regulator (Larsen and Turbo India Ltd, Design No. 1600041). The desired light intensity was provided by using appropriate number of florescent tube light (Phillips India Ltd. Mumbai).

3.6 Media preparation

3.6.1 *Media preparation for plant tissue culture*

For the preparation of stock solutions required for MS media, the chemicals were dissolved in Millique water. Stock solutions were usually prepared at 10-20 times the concentrations required in the final strength of the medium and stored in a refrigerator (4^0 C). The MS media were prepared from seven stock solutions (each × 100) following the recommendation of Hegelson (1979):

For preparation of 1 litre of MS media the following protocol was used

- 30 gm of sucrose was weighed, added and dissolved in 500 ml double distilled water.
- To it Myo-inositol (100ml), Thiamine HCl (0.1mg), Pyridoxine HCl (0.5 mg), Glycine (2mg) and Nicotinic acid (0.5 mg) were then added.
- 20 ml each of stock solutions of A, B, G and 5 ml each of stock solutions C,D,E and F were then added and the volume was made upto 900 ml.
- The pH was adjusted to 5.8 before it was poured into a graduated cylinder to make up the final volume to 1 litre.
- The media was again poured into a 2 l Erlenmeyer flask, the agar (0.8 %) added and autoclaved.
- The media were swirled to mix properly and cooled to 90^o C.

- The filter sterilized growth hormones and additives were added to the autoclaved media under aseptic condition before it was dispensed in presterilized vessels.

3.6.2 *Media preparation for cell suspension culture*

The composition and the protocol for cell suspension culture media preparation was same as the media preparation for tissue culture except for the addition of agar (0.8 %) in the tissue culture media.

3.6.3 Preparation *of microbial media for culture of Psuedomonas sp. and Bacillus subtilis*

For preparation of 1 litre NA media the following protocol was used

- The various constituents (Annexure II) were weighed in required amounts an dissolved in 900 ml of distilled water in a Erlenmeyer flask.
- The pH was then adjusted to 7.0 and the volume made up to 1000 ml.
- Agar (15g) was added and the media gently heated to boil.
- The media was autoclaved at 15lb/inch^2 pressure for 15 min at 121°C temperature before it was poured into the sterile petriplates or in tubes.

3.6.4 *Preparation of media for culture of Saccharomyces* spp

For preparation of 1 litre YPD media the following protocol was used

- The various constituents (Annexure II) were weighed in required amounts and dissolved in 900 ml of distilled water in a Erlenmeyer flask.
- The pH was then adjusted to 7.0 and the volume made up to 1000 ml .
- Agar (15g) was added and the media gently heated to boil.
- The media were then autoclaved at 15 lb pressure for 15 min at 121°C temperature before it was poured into the sterile Petriplates or were left in tubes.

3.6.5 Preparation of media for culture of Mycobacterium tuberculosis

For preparation of 1 litre Middlebrook 7H10 Agar medium with Middlebrook OADC enrichment the following protocol was used

- Glycerol was added to 900 ml distilled/ deionised water followed by the addition of the remaining components of the Middlebrook 7H10 Agar medium.
- The components of the media were swirled to mix thoroughly and gently heated to boil.
- The media was autoclaved for 15 min at 15 psi pressure at a temperature of 121°C.
- The media was allowed to cool to 50°-55° C and 100 ml of sterile Middlebrook OADC enrichment were then added to the

media and mixed thoroughly before it was dispensed in sterile Petridishes or in sterile tubes.

3.7 Storage of bacteria

Bacterial stocks were prepared by mixing 0.85 ml of bacterial culture with 0.15 ml of sterile glycerol. The mixture was then vortexed briefly before freezing in either liquid nitrogen or solid CO_2. Long term storage of bacterial stocks was accomplished by storing the frozen glycerol stocks at -70° C. For short term (<2 weeks) storage, bacterial cultures were kept at 4° C.

3.8 Micropropagation

3.8.1 Protocol for explant sterilization, inoculation, maintenance for shoot and root development and transfer of plantlets to the field condition and hardening

Centella asiatica

- Young shoot tips of *Centella asiatica* were dipped in 1% teepol for about 10-15 min before it was washed thoroughly under running tap water for about 30 min.
- The explants were then surface sterilized with 0.01% $HgCl_2$ for about 5 min followed by 3-4 rinses with autoclaved Millique water before inoculating into the culture media aseptically to get a near total contamination free cultures.
- The sterilised explants were inoculated into MS media supplemented with different concentration and combinations of 6-benzyladenine (BA)(1.0-4.0 mg l^{-1}), Kinetin (KIN)(1.0-4.0mg l^{-1}) and 6-naphthaleneacetic acid (NAA)(0.05-0.10 mg l^{-1}).

- The cultures were incubated at 25 ± 2°C under 16h photoperiod with a light intensity of 3000 lux and 55-60% RH.
- Two weeks after inoculation into the shooting media the shoot were transferred to rooting media (MS) with different concentration and combinations of indole-butyric acid (IBA) (1.0-3.0 mg l^{-1}) and NAA (0.5-2.0 mg l^{-1}).
- The cultures were maintained under the same conditions as for shooting but under dark for another 3 weeks.
- The rooted microshoots were transferred into the plastic cups containing sterilized mixture of sand and soil in 3:1 ratio and kept under fluorescent light (16h photoperiod) at 25 ± 2°C for a week.
- The plants were kept covered with polythene bags to maintain humidity for another week before these were transferred to the field.

Adhatoda vasica

- The explants were dipped in 1 % teepol for about 10 min before treating with 0.01 % $HgCl_2$ (w/v) for 5-7 min.
- The shoot tips were inoculated in normal MS media with different concentrations of BA (0.5, 1.0, 2.0 and 3.0 mg l^{-1}).
- The cultures were incubated in a culture room at 25±2°C with 60-70% RH and 16h photoperiod with a light intensity of 3000 lux for 30 days.

- After 4 weeks the microshoots were then transferred to secondary MS media supplemented with BA (0.5-1.0 mg l^{-1}) alone for two weeks.
- For vigorous growth of the microshoot the shoot were again transferred to the media (modified secondary MS media) supplemented with BA (0.5 mg l^{-1}) and 15% coconut milk.
- The shoots were then transferred to rooting media (MS) supplemented with different concentration and combinations of activated charcoal (0.5, 0.6 and 0.65 g l^{-1}) and IBA(2.0-3.0mgl^{-1}).
- The cultures were incubated under the same condition as for shooting but under dark condition for another 30 days.
- The rooted microshoots were transferred to full strength MS liquid media and maintained there for a week followed by transfer to half strength MS liquid media for another week.
- The plantlets were then planted in pots containing sterilized mixture of sand and soil (3:1) and kept under fluorescent light (16 h photoperiod) at 25±2°C.
- The plants were kept covered with polythene bags to maintain humidity for a week before these were transferred to the field.

Asparagus racemosus

- The explants were dipped in 1% teepol for about 15 min before these were washed thoroughly under running tap water for about 30 min. Surface sterilization of the explants was done by

treating the explants with 0.01% $HgCl_2$ for 4-5 min followed by 3-4 rinses with sterilized Millique water.

- The sterilised explants were inoculated into the MS media with different concentration and combinations of KIN (1.0-4.0 mg l^{-1}) and NAA (0.1 mg l^{-1}).
- The cultures were maintained under 16h photoperiod with a light intensity of 3000 lux at 25±2°C and 60-70% RH.
- The shoots (1-2 cm) in length were then transferred to rooting media (MS) supplemented with different concentration and combinations of IBA (0.4-0.5 mg l^{-1}) and NAA (0.1-0.2 mg l^{-1}).
- The cultures were maintained under the same conditions as for shooting but under the dark condition for 8 weeks.
- The rooted microshoots were transferred to half strength MS basal liquid media with 10% sucrose for 2 weeks.
- The plantlets were then transferred to plastic cups with a potting mixture of sterilized garden soil and sand in 1:1 ratio.
- The plants were irrigated with liquid MS basal media at 3 days interval. The pots were kept covered under polythene bags for 4 weeks to maintain humidity in the culture room under 16h photoperiod and 25±2°C temperature before they were transferred to the field.

3.9 Callus culture

3.9.1 Source of explants

The choice of explants is an important criterion to bear in mind. The explant must contain living cells, because younger (juvenile) tissue contains a higher proportion of actively dividing cells and is therefore more responsive to a callus initiation program. The parent plant must be healthy and free from any obvious sign of decay or disease, and that the plants from which the explants are obtained should not enter a period of dormancy.

Therefore to obtain a reasonably high amount of calli, young juvenile leaves from the shoot tips which were healthy and free from any diseases were chosen as explants in *Adhatoda vasica* and *Centella asiatica*. In *Asparagus racemosus* nodes with spears have been used as explants for callus initiation.

3.9.2 Medium optimisation, initiation of callus from explants and its maintenance

The treated explants for callus initiation were inoculated in MS media supplemented with different concentrations and in different combinations of plant growth regulators such as BA, NAA, KIN and 2,4-D (Table 8,9,10). The cultures were incubated at 25 ± 2° C at 60-70% RH under complete dark condition. Subculture of the friable calli (2-4 mm in diameter were done at every 4 weeks interval. Calli (< 2mm diameter) plus explant were transferred to fresh medium for better growth.

Each of the media composition was replicated 10-20 times and the response was assessed on a semi-quantitative basis by giving the amount of callus production on each explant a score of '+' for low, '++' for high and '+++' for profuse callusing.

3.10 Suspension culture

Suspension culture in *Centella asiatica* and *Asparagus racemosus* was done by batch culture method. The protocol is as follows-

- Initiation of cell suspension was done by transferring callus to liquid media of the same composition as the callusing media.
- The cultures were incubated at 25 ± 2° C at 60-70 % RH under dark in a horizontal shaker at 150 rpm.
- Maintenance of the cultures was done by routinely transferring a small aliquot (2ml) of the suspension culture to a fresh medium (150 ml) at every 2 weeks interval.
- When large amount of callus was obtained after a few transfers then the culture flasks were allowed to stand for a few seconds to allow the large colonies to settle down. The suspension cultures were then transferred into the fresh medium with the help of a wide-bore pipette to allow small aggregates of cells to pass through.

- Subcultures were carried out routinely at every 3 weeks interval.

3.11 Growth measurement

The growth of callus was measured by assessing the fresh weight (FW) and dry weight (DW) of the culture. The method is as follows

- The fresh callus was transferred to a weighed aluminium foil with a forcep.
- The combined fresh weight was then measured as quickly as possible after the callus was removed from the tissue culture containers because the callus loses water.
- The aluminium foil was then closed carefully over the callus and kept in an oven at 48°C for 2 days.
- The weight of both the foil and the callus were taken and the weight of callus was obtained by substraction. Because of the rapid and uncontrolled weight loss of the fresh callus, the dry weight was the more reliable criterion.

The growth rate of cell suspension was measured by the following methods

- Packed cell volume (PCV): A measured volume of the suspension culture was transferred to a graduated test tube which was centrifuged at a low speed (100 rpm for 10 min).

- Fresh weight (FW) and dry weight (DW) of cell suspension

1) The cells were collected on a pre-weighed circular filter or nylon fabric supported in a Hartley funnel.
2) The cells were then washed with water to remove the medium, drained under vacuum and reweighed.
3) After taking the fresh weight, the cells were then kept in an oven at 48°C for 2 days and reweighed to get the dry weight.

Bacterial and yeast cell growth were measured by Quebec Colony Counter

- Using a Quebec Colony Counter and a mechanical hand counter, all colonies were observed on plates. Plates with more than 300 colonies cannot be counted and are designated as too numerous to count- TNTC. Plates with fewer than 30 colonies are designated as too few to count- TFTC. Only plates containing between 30 and 300 colonies were counted.
- The number of organisms per ml of original culture was calculated by multiplying the number of colonies counted by the dilution factor.

  Number of cells per ml = number of colonies × dilution factor
- The observations and calculated bacterial counts per ml of sample were recorded.

- Since the dilutions plated are replicates of each other, the average of the duplicate bacterial counts per ml of sample were determined and recorded.

3.12 Chromatographic techniques

3.12.1 *Sample preparation*

For chromatographic analyses, the plant samples were prepared following the steps given below.

- The plant samples (2 g) were air dried and grounded in a grinder mixer to fine powder.
- The samples were then soaked in the respective solvents (20 ml) (acetone, ethanol and 1 N NaOH) for 48-72 hrs.
- The extracts were then filtered through a fine Whatman filter paper (125 mm, cat. No. 1001125, Grade 5).
- The filtrates were concentrated in a vacuum dryer and the respective weights recorded.

Species	**Sample**	**Solvent used**
Adhatoda vasica	a) Leaf	Acetone
Centella asiatica	a) Leaf	Ethyl alcohol
	b) Callus	Ethyl alcohol
	c) Callus from suspension	Ethyl alcohol
Asparagus racemosus	a) Leaf	1N NaOH
	b) Root	1N NaOH
	c) Callus	1N NaOH
	d) Callus from suspension	1N NaOH

3.12.2 *Method for Thin layer chromatography*

TLC, which together with paper chromatography constitutes 'planar' or 'flat-bed' chromatography, is the simplest of all widely used chromatographic methods. TLC analyses of the prepared crude extracts of the samples were performed following the method of Mohammad A, 1996 as outlined below.

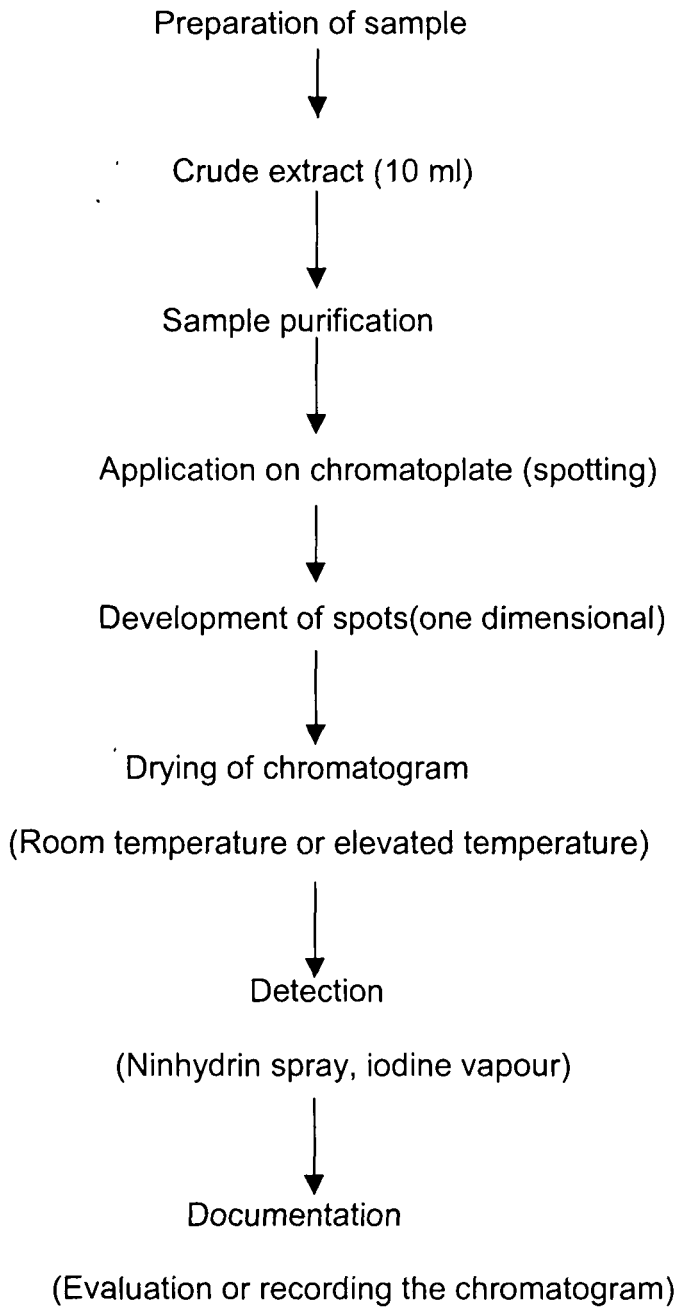


Fig 6: Scheme for thin layer chromatographic analyses

One-dimensional ascending technique was used for the development of chromatograms in a closed chamber (cylindrical or rectangular) at room temperature (20 ± 2°C). About 0.5-10 μl sample solution containing 0.5-5 μg of the solute was spotted on an activated TLC plate [plastic, aluminium or glass sheet coated with an adsorbent layer (Silica-G, Merck, Mumbai) of 3-4 mm thickness} at 1.5-3 cm from the lower edge of the plate. Calibrated micro capillary, micropipette, micro cap, Hamilton syringe, and melting point capillaries were generally used to apply microliter volumes of a sample on the plates. The spots were air dried at room temperature or at elevated temperature using hair driers, fan driers or infrared lamps before the development of TLC plates. The plates were developed in glass jars or chromatographic chambers of appropriate sizes at room temperature. The migration distance for solvent (or mobile phase) had been kept to 10-18 cm on laboratory made plates. The zones were detected by application of a suitable visualization reagent (Ninhydrin, Merk India Ltd, Mumbai) or exposing to a solvent vapour (iodine, Merk India Ltd, Mumbai) in a closed glass chamber.

3.12.2.1 *Qualitative assessment of alkaloids*

Qualitative identification was done on characteristic colours produced by specific detection reagent combined with R_f values. TLC was used for the purpose as a preparative step and the R_f value of zones of samples were compared with the R_f value of standards in more than one type of TLC system with various separate mechanisms. The comparison was made essentially on

the basis of colour characteristics of the respective standards for example with leaf sample of *Adhatoda vasica* the colour characterististics is light gray, with leaf, callus and suspension cultured cells of *Centella asiatica* the color characteristics is light brownish and with leaf, root, callus and suspension cultured cells of *Asparagus racemosus* the colour characteristics is dark pinkish to brown. Alternatively multiple solvent systems were also used with one type of layer in the experiments.

a) *Adhatoda vasica* (Active principle: Vasicine)

Mobile phase: Acetonitrile: 1M phosphate buffer: Glacial acetic acid

15:85:1(v/v/v)

Detection reagent: Iodine vapour.

b) *Centella asiatica* (Active principle: Asiaticoside)

Mobile phase: Ethyl alcohol: MeOH: H_2O

60:12:8 (v/v/v)

Detection reagent: Iodine vapour

c) *Asparagus racemosus* (Active principle: L-Asparagine)

Mobile phase: n-Butanol: acetic acid: water

4:1:1(v/v/v)

Detection reagent: Ninhydrin spray

The alkaloids were analysed and detected by:

- **Visual estimation and spot size measurement method**

    1) The sample aliquots were allowed to run in TLC plates simultaneously along with the standard (pure) containing a known weight of analyte (control).

    2) After detection, the proportion of analyte in the sample was estimated by visual comparison of the size and intensity of the standard and the sample zones.

- **Zone elution and spectrophotometric method**

    1) The layers were dried and the analyte zones were resolved by the respective detection reagents.

    2) The separated zones of both the samples and the standards were then scrapped manually and the analysts were eluted with the respective volatile solvents (acetone, ethyl alcohol and 1N NaOH).

    3) The elutes were concentrated and analyzed by UV/VIS spectrophotometer (Beckman, Model No. DU 530) at 280 nm for vasicine, 227nm, 220nm and 230nm for asiaticoside and 253nm, 225nm for L-asparagine.

    4) In a typical operation of a UV/VIS spectrophotometer, at a single wavelength, measurement was made of the light transmitted by the solvent alone (which may be a buffer or a solution of small molecules), followed by a measurement of that transmitted by

the sample when dissolved in the same solvent. The first value was then subtracted from the second to give the absorbance of the solute. In practice, this subtraction was not done arithmetically, rather the instrument was adjusted to read zero absorbance when the solvent alone was measured (zeroing the instrument). Then, with the instrument so adjusted, the absorbance of the sample was read directly. In the present work, the absorbance of the samples was detected at the range of 200-500 nm wavelength.

3.12.3 *Quantification of the alkaloids*

• High Pressure (or Performance) Liquid Chromatography

High pressure liquid chromatography is an improved version of liquid chromatography. HPLC allows very rapid seperation with extraordinary resolution of peaks.

In the present work, HPLC (Waters) with 2487 Dual λ Absorbance Detector and 515 HPLC pump were used and C_{18} analytical column (Nova-pak) with particle size of 4μm and dimension of 3.9mm x 150mm (part No. WAT086344) were used for seperation.

The following procedure for HPLC analysis was adopted

- The partially purified extract obtained after pre-filtration of the TLC resolved eluent through centrifugation (10,000 rpm) were dissolved in methanol.
- The instrument was then equilibrated with the solvent mixture, methanol : water(60:40) in the isocratic mode.

- The proportions of the various solvents in the solvent systems for use as the mobile phase in the analysis of prepared extracts from the three plant species were determined empirically through several trial and error. Subsequently the following systems were selected as the mobile phases.

Plant samples	**Mobile phase** **Solvent systems**
Adhatoda vasica	acetonitrile:0.1M phosphate buffer: glacial acetic acid (15:85:1)
Centella asiatica	ethyl alcohol :methanol : water (60:12:8)
Asparagus racemosus	n-Butanol : acetic acid : water (4:1:1)

- The prepared plant extracts were then injected (20 µl for *Adhatoda vasica*; 15 µl for *Centella asiatica* and 15 µl for *Asparagus racemosus*) into the HPLC column (C_{18} Nova-pak) at a flow rate of 1 ml min^{-1}.
- The peak detection was monitored at 280 nm, 220 nm and 225 nm wavelength for vasicine, asiaticoside and L-asparagine respectively which was predetermined by UV/VIS spectrophotometer.
- In order to ensure a consistent response, the experiments were repeated for 3 to 4 times.
- The HPLC profile of the samples were compared with that of the standards with respect to the retention time and the quantity of the components were calculated from the peak area of the samples.

3.13 Methods for determining antimicrobial activity

Crude extracts of plants were prepared by the following method for antimicrobial sensitivity tests-

- The plant materials were oven dried at 37° C and grounded in a grinder mixer to fine powder.
- The samples were then soaked in respective solvents (Acetone, ethanol and 1N NaOH) for 48 hrs.
- The extracts were then filtered through a fine whatman filter paper (125 mm, cat. No. 1001125)
- With the help of a vacuum dryer the filtrate was concentrated and the weight was measured.
- The concentrated crude extracts (which was prepared following the same method as for TLC analyses) were dissolved in the respective solvents (acetone, ethanol and 1N NaOH) to carry out the tests for antimicrobial activity.

The Kirby-Bauer method (1960) requires Muellar-Hinton media and the inoculum has to be standardized using a 0.5 McFarland Turbidity standard. In the present work the Kirby-Bauer method has been modified as referred in the Laboratory manual by Cappuccino J.G. and Sherman N (4^{th} edition) for antimicrobial sensitivity test which comprises of the following steps

- Nutrient Agar plates were placed in an incubator heated to 37°C for 10-20 min with the covers adjusted so that the plates were slightly open.

- Using a spreader, the entire agar surface was spread with the inoculum (200μl) taking care that a uniform growth takes place.
- The culture plates were then allowed to dry for 5 min under the laminar flow hood.
- Solutions of the extract or antibiotic were prepared at 2,4,6,8 & 10 μg/ ml concentration in their respective solvents (Acetone, ethanol and 1N NaOH).
- Sterile filter paper discs of uniform size were then immersed into the extracts with a sterile forcep, allowed to drain the excess and then laid on agar plate surface.
- Agar plates inoculated with only the solvents were taken as negative control for comparison.
- The plates were then put in a refrigerator for 1-2 hr to cause slow growth and diffusion of the samples from the discs.
- Plates were then incubated at 37°C for 24 hr. Diameters of inhibition zone were measured.

Mycobacterium tuberculosis bacteria cannot grow in Muellar-Hinton Agar and moreover growth inhibition test based on OD method is much less reproducible than a disk diffusion test. Therefore for determining the anti mycobacterium activity the following Kirby-Bauer method was used

- A suspension of 1mg/ml concentration of extracts/ compounds was prepared in DMSO.
- This suspension was added to 7H10 Middlebrook medium (containing 1.7 ml medium and 0.2ml OADC supplement) at

different concentrations keeping the volume constant i.e 0.1 ml when it was cooler before getting solidified.

- Medium was then allowed to cool keeping the tubes in slanting position.
- Tubes were then incubated (at 37° C) for 24 hours followed by streaking of *Mycobacterium tuberculosis* H37Rv (5 x 10^4 bacilli/tube, 10 μl).
- Growth of bacilli was seen after 21 days of incubation at 37° C. Extracts containing tubes were compared with control tubes where medium without any sample was inoculated with H37Rv.

3.14 Statistical Methods

The tissue culture experiments were set up in Randomised Block Design (RBD) and the data were analysed and summarised in a analysis of variance (ANOVA) table together with a table of treatment means and their standard errors. The significance of variances between the treatment means was compared by variance-ratio (F) distribution at 5 % probability (Fisher and Yates Statistical tables for Biological, Agricultural and Medical Research, Published by Oliver and Boyd Limited, Edinburgh).

Chapter IV

RESULTS AND DISCUSSION

Chapter IV
Results and Discussion

4.1 Micropropagation of *Adhatoda vasica*

Tissue culture protocols for *Adhatoda vasica* Nees and *Adhatoda beddomei* C.B. Clarke have been developed earlier, (Jaiswal *et al.*, 1989; Sudha and Seeni, 1994) using nodal segment and axillary bud explants respectively. However the problem of tissue browning and exudation of phenolic compounds remained unsolved in the case of these two species. There is also scope for exploring the feasibility of using explants other than the ones already being used in the reported protocols for enhancing shooting efficiency through reduced microbial contamination on one hand and to increase the level of vasicine content in the micropropagated plant tissue. In the context of these issues, experiments have been conducted to establish a protocol for micropropagation of *Adhatoda vasica* using shoot tips as explants.

4.1.1 *Choice of explants*

Establishment of shoots of *Adhatoda vasica* appeared to be rather difficult initially due to heavy fungal and bacterial contamination. Bacterial or fungal contamination was seen even after a gap of one month in some cultures as a result of which only 50% of the cultures were contamination free. This was often experienced with the explants derived from hard woody shrubs. To avoid such contamination, shoot tips were chosen as explants.

4.1.2 *Growth of shoot cultures*

The average number of shoots (3.0 ± 0.15) were seen more in the normal MS media supplemented with 2 mg l^{-1} BA after 2 weeks, but 96.67% of the

Table 1: Effect of plant growth regulators and coconut milk on multiple shoot induction of Adhatoda vasica 30 days after inoculation in primary media.

Media	Growth regulator (mg l^{-1})	Shoot number per culture (Mean ± SD)	Shoots with basal callus (%)	Average number of leaves per shoot (Mean ± SD)
MS	BA			
	0.5	$2.1 \pm .15^{bc}$	90.00	-
	1.0	$2.3 \pm .30^{b}$	93.33	-
	2.0	$3.0 \pm .15^{a}$	96.67	-
	3.0	$2.1 \pm .15^{bc}$	90.10	-
MSp	TDZ			
	0.30 + 5% CM	1.73 ± .21	NB & NC	3.93 ± .46

In each column, mean±SD followed by the same letter were not significantly different (p=0.05) according to Duncan's multiple range test. N=30

MSp =primary MS media
NB = no browning of the media
NC =no callusing at the cut portion

Table 2: Effect of plant growth regulator and coconut milk on 14 day old shoots of Adhatoda vasica in modified secondary media.

Media	Growth regulator (mg l^{-1})	Shoot number per responding explant (Mean±SD)	Average number of leaves per shoot (Mean±SD)
MSs	BA		
	1.0	5.4 ± 1.03^{a}	3.8 ± 0.20^{b}
	0.5	5.2 ± 0.38^{ac}	5.2 ± 0.20^{a}
MSm	BA	Average length of shoot (cm)(Mean±SD)	Average number of nodes per shoot (Mean±SD)
	0.5 +15% CM	4.34 ± 0.28^{bd}	3.2 ± 0.13^{c}

In each column, mean±SD followed by the same letter were not significantly different (p=0.05) according to Duncan's multiple range test. N=30

MSs= secondary MS media
MSm= modified secondary MS media

explants in the above media were seen with basal callus in the cut portion. The leaves later on turned necrotic and fell off.

4.1.3 *Modification of the media*

After 30 days of inoculation in the primary MS media supplemented with thiadiazuron (TDZ) (0.30 mg l^{-1}) and coconut milk (5%) young green shoots were seen with healthy leaves (Table 1). No phenolic exudation and no callusing were seen on the base of the explants (Table 1). The leaves were green, healthy but curly. The curly leaves were due to deficiency in K in the media. To overcome this problem, the explants were transferred to a secondary media [MS basal salts, sucrose-3%, 0.8% agar and BA (0.5mg l^{-1} and 1.0 mg l^{-1}) with pH 5.8]. The shoots in the media supplemented with BA (0.5mg l^{-1}) were stouter, dark green in colour, larger in size and without swelling in the base unlike the results obtained in the media supplemented with 1.0mg l^{-1} BA (Figure 7A). The leaves were soft in the base and tended to fall off in the media supplemented with 1mg l^{-1} BA Though the number of leaves have increased (5.2 ±0.20) (Table 2) the shoot elongation was found to be less (Figure 7A). For further shoot elongation the shoots were subcultured and transferred to MS media with the same composition but supplemented with 15% CM (v/v) 14 DAI. Long elongated shoots (4.34±0.28 cm) were seen after 2 weeks (Table 2) (Figure 7B-C).

Such modifications were also supported by Anderson (1975) where he suggested that by reducing the concentration of KNO_3 to half, browning can be prevented in *Rhododendron* explants. Even in *Dioscorea opposita*,

browning did not occur if callus cultures were initiated on MS medium with 10mM NH_4NO_3 and KNO_3 (Kuginuki and Nishimura, 1989). The activity of polyphenol oxidase also decreased at lower pH and highest at pH 6.5(Ichihashi and Kako, 1977).

4.1.4 *Rooting of in vitro derived shoots and plantlet establishment*

Excised shoot tip derived microshoots, 4.34±0.28 cm long were rooted in only full strength MS basal media supplemented with different concentration of AC (0.5-0.65g l^{-1}) and IBA (2.0-3.0mg l^{-1}). Activated charcoal is used here because it has several stimulatory effects on root growth (George, 1993/1996) as it absorbs inhibitory substances, darkens the culture medium and slowly absorbs the auxins thereby improving the milieu for root initiation. At 3.0mg l^{-1} IBA, only 3.33 ± 1.29 number of roots were seen on an average. However, the number of healthy and vigorous roots increased in the basal media (9.33 ± 1.29) (Table 3). The average length of roots were seen more in media with activated charcoal (0.60 ± 0.21). After 4 weeks, the rooted microshoots were transferred to full strength MS liquid media (Figure 7D) and maintained there for a week followed by transfer to half strength MS liquid media for acclimitization for another week. The plantlets were then planted in pots containing sterilized mixture of sand and soil (3:1)(Figure 7E) and kept under fluorescent light (16 h photoperiod) at 25 ± 2°C. These plants were kept covered with polythene bags to maintain humidity for a week before these were transferred to the field.

Table 3: Effect of plant growth regulators and activated charcoal on rooting of micropropagated shoots of Adhatoda vasica.

Media	**Growth regulator ($g\ l^{-1} + mg\ l^{-1}$)**	**Number of roots per shoot (Mean±SD)**	**Average length of roots per shoot (Mean±SD)**
MS	AC+IBA		
	0.5+2.0	0.00	0.00
	0.6+3.0	0.00	0.00
	0.0+3.0	3.33 ± 1.29^{b}	0.21 ± 0.21^{b}
	0.65+0.0	2.0 ± 1.29^{c}	0.60 ± 0.21^{a}
MSo	0.0 +0.0	9.33 ± 1.29^{a}	0.60 ± 0.21^{a}

In each column, mean±SD followed by the same letter were not significantly different (p=0.05) according to Duncan's multiple range test. N=30

AC= Activated charcoal
Mso= MS media without growth regulator

Fig 7 Micropropagation of *Adhatoda vasica* through shoot tip explants

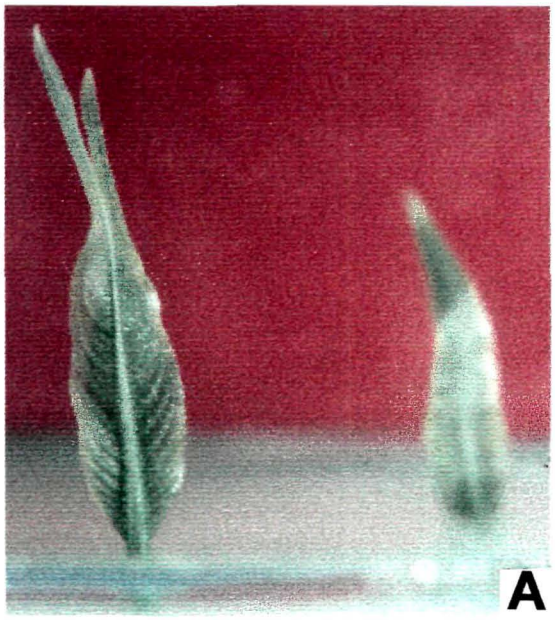
(A-C) Multiple shoot proliferation after 30 days of inoculation

A Primary MS media

B-C Modified secondary MS media

D *In vitro* rooted plantlet in liquid basal MS media for acclimatisation after 30 days of culture in modified secondary MS media

E Normal growth of micro propagated plant measuring 10 cm in soil.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4.2 Micropropagation of *Centella asiatica*

4.2.1 Choice of explants

Fungal and bacterial contamination was a major problem for initiation of culture of *Centella asiatica* with nodal segments as explants (Tiwari *et al.*, 2000). Therefore in the present study, shoot tips were chosen as explants for attempting micropropagation of *Centella asiatica*.

4.2.2 *Shoot regeneration*

Shoot tips cultured in MS medium with BA alone showed increased shoot length although the mean number of shoots per responding explants and average number of leaves was more in cultures with BA (4.0 mg l^{-1}) and NAA (0.1 mg l^{-1})(Fig 8A)(Table 4). An increase in the level of BA and NAA was found to promote callus formation (Banerjee *et al.*, 1999) when leaf was used as explant for shoot regeneration. Even KIN (4.0 mg l^{-1}) in combination with NAA (0.10 mg l^{-1}) had marked influence on shoot length and average number of leaves per shoot but the mean number of shoots per responding explant was less in comparison to those obtained with BA and NAA. The superiority of BA over KIN in multiple shoot induction was also reported in a number of medicinal plants (Sahoo *et al.*, 1997; Tiwari *et al.*, 1998). A comparison of means by DMRT revealed that the optimum number of shoots (3.38) with optimum number of leaves per shoot (4.25) was attained on MS medium

Table 4: Analysis of variance(ANOVA) and comparison by DMRT of the effects of BA, NAA and KIN concentrations on the number of shoots, mean shoot length and average number of leaves per shoot in Centella asiatica.

Source of variation			**Degrees of freedom**	**Mean square**			**F_{cal}**		
				Number of shoots per responding explants	Mean shoot length (mm)	Average number of leaves per shoot	Number of shoots per responding explants	Mean shoot length(mm)	Average number of leaves per shoot
Treatment			7	9.68	0.55	11.55	7.39**	1.83	6.75**
Replication			7	2.60	0.63	-0.16	1.98	2.1	-0.09
Error			49	1.31	0.30	1.71			
Plant growth regulators(mgl^{-1})							**Mean**		
BA	NAA	KIN					Number of shoots per responding explants	Shoot length(mm)	Number of leaves per shoot
1.0	0.0	0.0					3.25^{a}	0.98^{a}	3.25^{ab}
2.0	0.05	0.0					1.50^{b}	0.72^{ab}	2.00^{cd}
3.0	0.05	0.0					2.5^{a}	0.51^{b}	2.38^{bc}
4.0	0.10	0.0					3.38^{a}	0.67^{ab}	4.25^{a}
0.0	0.0	1.0					1.00^{b}	0.57^{ab}	1.38^{cde}
0.0	0.10	2.0					0.75^{b}	0.44^{bc}	1.13^{de}
0.0	0.10	3.0					0.63^{b}	0.08^{c}	1.13^{de}
0.0	0.10	4.0					1.38^{b}	0.71^{ab}	3.63^{a}

Each mean is based on eight replicates each of which is repeated five times. Data recorded after four weeks of culture initiations.

Each treatment mean followed by the same letter were not significantly different from each other ($p<0.05$) according to the Duncan Multiple Range Test (DMRT).

supplemented with 4.0 mg l^{-1} BA and 0.1 mg l^{-1} NAA (Fig 8B-C). Shoots with 3-4 nodes were subcultured every four weeks with a combination of 4.0mg l^{-1} BA and 0.1mg l^{-1} NAA.

4.2.3 Rooting in plantlets

In the present investigation micro shoots were directly transferred to rooting media. No separate media was needed for shoot elongation, by which, plantlet formation was achieved in two steps only. Shoots induced from nodal explants on the optimum BA and NAA media condition failed to elongate rapidly and were required to transfer in MS media supplemented with low level of BA (Tiwari *et al.*, 2000). Analysis of variance showed significant effect on the number of roots per shoot and mean root length (cm). Though MS basal medium showed minimum number of roots per shoot (4.8) with a mean root length of 10.5 cm, profuse rooting per shoot (46.8) was obtained in MS basal medium with 2.0 mg l^{-1} IBA (Fig 8D) with root length of 19.7cm(Table 5). Banerjee *et al.*, 1999 and Tiwari *et al.*, 2000 also found promontory effect of IBA in rooting in *Centella asiatica*. IAA and low level of sucrose have been reported to be the optimum conditions for rooting in *Centella asiatica* microshoots (Patra *et al.*, 1998).

After 4 weeks, the rooted microshoots were transferred from culture tubes into the plastic cups (Fig 8E) containing sterilized mixture of sand and soil in 3:1 ratio and kept under fluorescent light (16 h photoperiod) at 25 ± 2° C for a week. The plants survived in the field with 1-2% mortality rate.

Table 5: Analysis of variance(ANOVA) and comparison by DMRT of the effects of IBA and NAA concentrations on the number of roots per shoot and mean root length in Centella asiatica

Source of Variation	Degrees of freedom	Mean square		F_{cal}	
		No. of roots	Mean root Per shoot length(cm)	No. of roots	Mean root length (cm) per shoot
Treatment	6	2019.2	12.92	15.77**	2.94*
Block	4	217.97	5.31	1.70	1.21
Error	24	127.97	4.39		

Plant growth regulator (mg l^{-1})		Mean	
		No. of roots per shoot	Root length (cm)
IBA	NAA		
1.0	0	9.4^{b}	12.5^{b}
2.0	0	46.8^{a}	19.7^{a}
3.0	0	4.0^{b}	12.5^{b}
0	1	7.4^{b}	6.5^{c}
0	2	0.2^{b}	1.0^{d}
0	0.5	0.2^{b}	4.5^{c}
0	0	4.8^{b}	10.5^{b}

Each mean is based on five replicates each of which is repeated five times. Data recorded after four weeks of transfer into the rooting media.

Treatment means followed by the same letter were not significantly different from each other($p<0.05$) according to the Duncan Multiple Range Test (DMRT).

Fig 8 Micropropagation of *Centella asiatica* using shoot tip explants

A Shoot tips cultured in MS medium supplemented with 4.0 mg l^{-1} BA and 0.1 mg l^{-1} NAA

B-C Regeneration of axillary shoots from shoot tips after 14 and 28 days of inoculation respectively.

D *In vitro* rooted plantlet in MS media containing 2.0 mg l^{-1} IBA

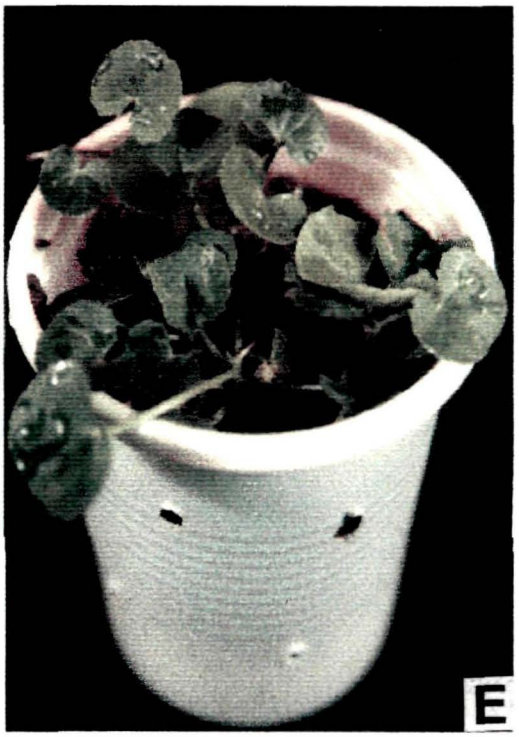
E *In vitro* regenerated plantlet transferred into a plastic cup (Scale=15 cm in 20cm long culture tube)


A


B


C


D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4.3 Micropropagation of *Asparagus racemosus*

Tissue culture protocol for micropropagation of *Asparagus racemosus* has not been reported as yet. There is however a great necessity for such a micropropagation technology in view of the fact that the roots of this species are regularly collected from wild habitats in a rather indiscriminate manner. Considering the importance of developing a suitable micropropagation protocol for *Asparagus racemosus*, a comprehensive strategy involving all aspects of tissue culture technology of the species has been developed in the present study.

4.3.1 *Choice of explants*

Axillary shoots or nodes are frequently used as explants as they are relatively easy to manipulate, they establish rapidly and often proliferate well. The shoot and nodal explants tend to be the largest explants normally used for establishment of cultures ranging from 3 to 20 mm in length (Bowes, 1990). The optimal size of an explant was governed by the effectiveness of sterilization procedures and diffusion of nutrients into the cultured explant. In the present investigation, micropropagation of *Asparagus racemosus* was carried out using nodes with axillary bud (3-15 mm long) as explants.

4.3.2 *Shoot regeneration*

Analysis of variance and by comparison of the means showed increased shoot length (2.08cm) with mean average number of shoots per responding explant (4.33) in cultures supplemented with 3.0 mg l^{-1} KIN and 0.1 mg l^{-1} NAA (Fig 9A-C)(Table 6). No significant effect was seen in mean shoot length

and in the average number of shoots per responding explant in cultures with 4.0 mg l^{-1} KIN and 0.1 mg l^{-1} NAA when compared by DMRT. Yang and Clore (1973) also cultured lateral buds from *Asparagus officinalis* into single shoots on a semi solid basal media containing 3% sucrose, (0.05-0.1 mg l^{-1}) NAA and KIN. The shoots arising from cultured nodes were cut into single node segments and recultured under the same conditions. Alternatively these shoots were induced to develop roots.

4.3.3 *Rooting of microshoots*

MS basal medium was found to have no effect on the length of roots and on the average number of roots. Though by Analysis of variance significant effect was seen on the average length of roots but no significant differences was found between the means of average number of roots per responding explant and average length of roots (cm) when compared by DMRT (Table 7). Profuse rooting (9.67) with effective root length (1.83 cm) were obtained in the full strength MS medium cultured with 0.1 mg l^{-1} NAA (Fig 9D). IBA also did not show any promontory effect on rooting in *Asparagus racemosus* unlike in *Asparagus officinalis* where shoots obtained from embryos when transferred to basal medium supplemented with IBA developed roots and in MS basal medium with ABA containing either Zeatin(1 mg l^{-1}) or GA_3 (Ghosh and Sen, 1990).

Table 6: Analysis of variance(ANOVA) and comparison by DMRT of the effects of KIN and NAA concentrations on the average number of shoots per responding explant and mean shoot length in Asparagus racemosus.

Source of variation	Degrees of freedom	Mean square		F_{cal}	
		Average no. of shoots per responding explant	Length of shoots (cm)	Average no.of shoots per responding explant	Length of shoots (cm)
Treatment	3	10.49	0.36	1.0	0.19
Block	5	10.12	3.79	0.96	1.95
Error	15	10.52	1.94		

Plant growth regulator (mgl^{-1})		Mean	
		Average no. of shoots per responding explant	Length of shoots(cm)
KIN	NAA		
1.0	0.1	2.0^a	1.74^a
2.0	0.1	3.17^a	1.52^a
3.0	0.1	4.33^a	2.08^a
4.0	0.1	6.0^a	1.34^a

Each mean is based on six replicates each of which is repeated four times. Data recorded after four weeks of culture initiations.

Each treatment mean followed by the same letter were not significantly different from each other($p<0.05$) according to the Duncan Multiple Range Test (DMRT).

Table 7 Analysis of variance(ANOVA) and comparison by DMRT of the effects of IBA and NAA concentrations on the average number of roots per responding explant and average length of roots in Asparagus racemosus.

Source of Variation	Degrees of freedom	Mean square		F_{cal}	
		Average no.of roots per responding explant	AverageLength of roots(cm)	Average no.of roots per responding explant	Average Length of roots(cm)
Treatment	4	130.33	5.72	0.40	17.88**
Block	2	11.63	0.17	0.75	0.53
Error	8	15.5	0.32		

Plant growth regulator (mgl^{-1})		Mean	
		Average no. of roots per responding explant	Average Length of roots(cm)
IBA	NAA		
0	0.1	9.67[a]	1.83[a]
0	0.2	7.0	1.73[a]
0.4	0	-	-
0.5	0	-	-
0	0	-	-

Each mean is based on five replicates each of which is repeated four times. Data recorded after four months of culture initiations.

Each treatment mean followed by the same letter were not significantly different from each other($p<0.05$) according bto the Duncan Multiple Range Test (DMRT).

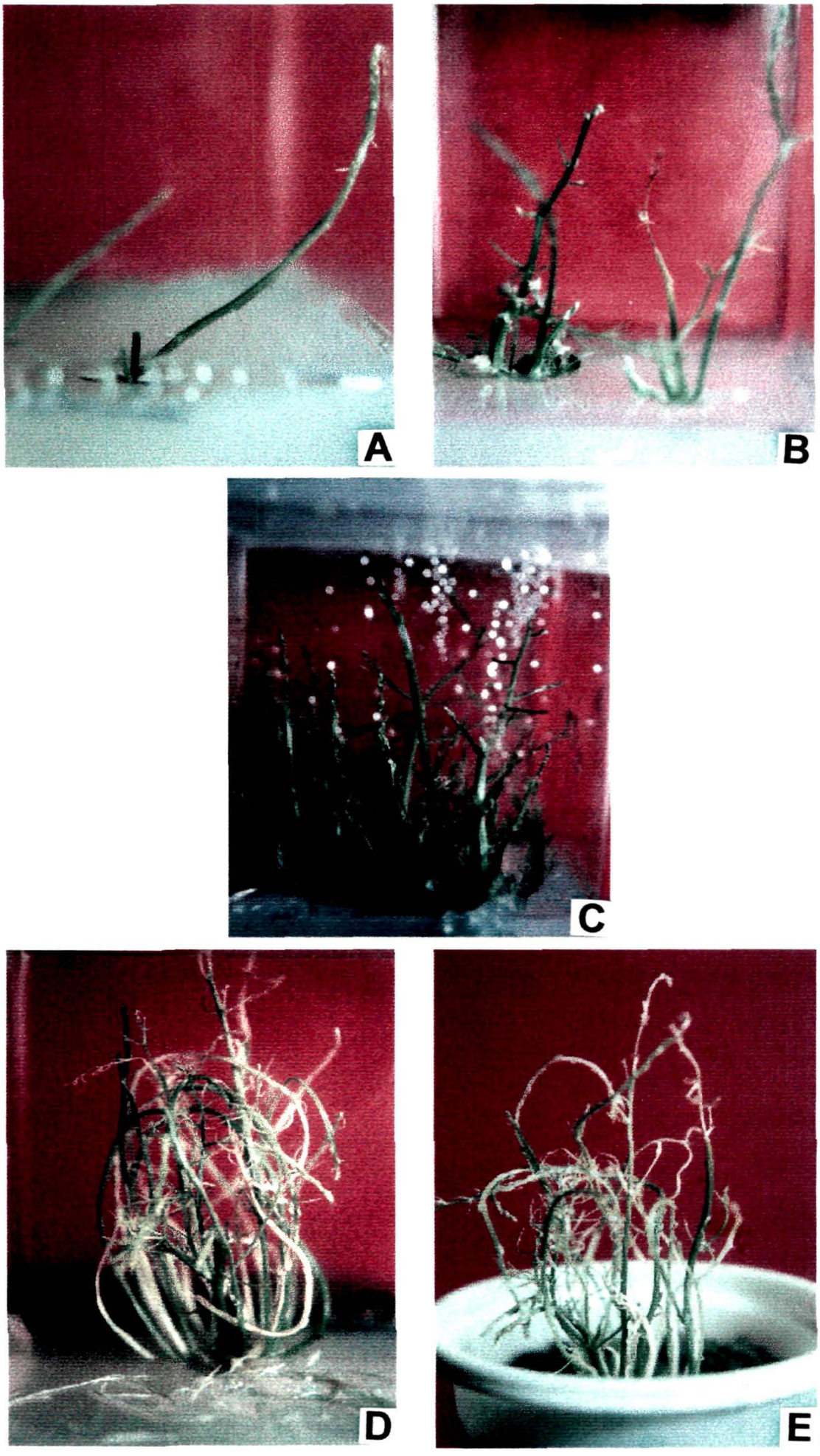
Fig 9 Micropropagation of *Asparagus racemosus* using nodes with axillary bud as explants

A Nodal explant with an axillary bud inoculated in MS media supplemented with 3.0 mg l^{-1} KIN and 0.1 mg l^{-1} NAA

B-C Multiple shoot regeneration after 14 and 28 days of inoculation respectively

D *In vitro* rooted plantlet in full strength MS media containing 0.1 mg l^{-1} NAA

E *Ex vitro* establishment of regenerated plant in a plastic cup (Scale=7 cm in a Casablanca jar)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After four months, the rooted microshoots were transferred in half strength MS basal liquid media with 10% sucrose for 2 weeks for sufficient growth of the roots. After 2 weeks, sucrose was completely removed from the liquid media and allowed to harden for another 2 weeks. The plantlets were then transferred to plastic pots with a potting mixture of sterilized garden soil and sand in 1:1 ratio (Fig 9E).

4.4 Callus culture

One of the major objectives of the present study is to find out whether the dedifferentiated cells of the three plant species drive the metabolic pathways leading to the synthesis of the principle active principles responsible for their medicinal values. This plant tissue culture technique called callus culture are widely employed to obtain a stable supply of bioactive secondary products (Bajaj *et al.*, 1988; Yeoman *et al.*, 1996) including anti-cancer drugs like taxol (Edgington, 1991). Experiments on callus culture of thee three species have been conducted in order to develop feasible suspension culture systems subsequently. So far, there is no report on the use of suspension culture for production of vasicine, asiaticoside and L-asparagine.

4.4.1 *Callus initiation and medium optimisation from explants in Adhatoda vasica*

The growth of the calli was found to be very slow, and these were white and watery eight weeks after inoculation in the medium supplemented with BA and 2,4-D (MS_2 and MS_3). In the MS_4 and MS_5 medium the growth of the callus had rapidly increased upto the eighth week. The calli in the MS_4 medium

gradually changed to white and became friable after four weeks of inoculation (Fig 10A-B), which was suitable for subculture (Table 8). On the other hand, the callus continued to remain green and compact in MS_5 medium even after 8 weeks of inoculation. These green embryogenic calli tended to proliferate as clumps of repetitive embryos but were not amenable to manipulation necessary for the purpose establishing feasible suspension culture. For initiation of embryogenic cultures for any given species, the explant response is highly genotype dependent (Gray and Meredith, 1992). However attempts were made to generate plantlets from these putative embryos. But none of these experiments yielded positive results. These calli was transferred to the maintenance medium to get friable callus. Friable and light coloured calli (2-5 mm in diameter) were subcultured at every 4 wks interval in the MS_4 media containing NAA and KIN (1.0+1.0) mg l^{-1} (Fig 10C).

Table 8 Optimisation of callusing media for Adhatoda vasica

Media	No. of explants	Days after inoculation	Callus score	Callus morphology	Remarks
MS_1	50	30	-	-	Leaves turning slightly pale in colour
		60	-	-	Leaves turned brown and necrotic
MS_2	50	30	+	White	
		60	++	White, watery and soft, slow growth	
MS_3	50	30	-	-	
		60	+	White and slow growth	Turned brown and necrotic
MS_4	50	14	+	Green	
		21	++	Green	
		28	++	Green	
		56	+++	Green callus gradually changes to white and becomes friable	
MS_5	50	14	-	-	
		21	+	Callus green	
		28	++	Green	
		56	++	Green	

"-" = Negative ; "+" = Low ; "++" = Medium ; "+++" = High

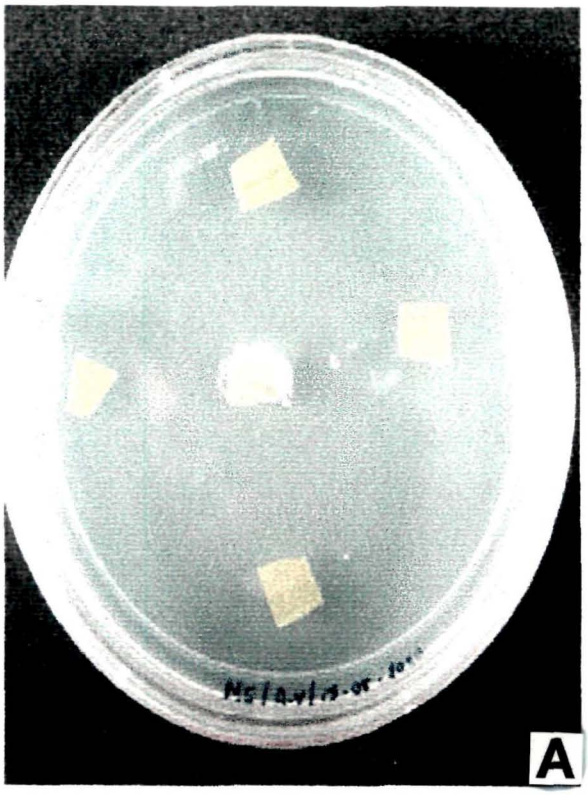
MS_1 = BAP + 2,4-D = (0+1) mg/l ; MS_2 = BAP + 2,4-D = (1+1) mg/l
MS_3 = BAP + 2,4-D = (1+2) mg/l ; MS_4 = NAA+ KIN = (1+1) mg/l
MS_5 = NAA + KIN = (1+2) mg/l

Fig 10 Callusing from leaf explants of *Adhatoda vasica*

A Inoculation of leaf explants in the MS_4 media (NAA + KIN = 1 +1 mg l^{-1}) with the ventral side facing downward

B Swelling of the leaf explants and initiation of callus from the cut surface two weeks after inoculation

C 4 week old white and friable calli after subculture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4.4.2 *Callus initiation and medium optimisation from explants in Centella asiatica*

Swelling of the leaf explants were seen in almost all the media compositions. Though callusing have been achieved 28 and 21 days after inoculation in the MS_6 and MS_7 medium respectively, the growth mass of the callus rapidly increased only in the MS_7 medium (Table 9)(Fig 11A-B). 2,4-D was reported to be an effective hormone to induce callus formation in other members of Apiaceae as well (Sugano H & Hayashi K, 1967). But in this species, the callus in the MS_7 medium supplemented with BA and NAA (1.0+1.0 mg l^{-1}) showed the best result. It turned green and friable 35 days after inoculation, which were later, subcultured at every 3 weeks interval (Fig 11C).

4.4.3 *Callus initiation and medium optimisation from explants* in *Asparagus racemosus*

The swelling of the spears were seen 14 days after inoculation in the MS_{10}, MS_{11} and MS_{12} medium. Callusing was initiated from the cut ends and from the adaxial surface of the explants 21 days after inoculation in the MS_{10} medium (Fig 12A) (Table 10), which gradually turned white and compact in the fourth week. In the sixth week the calli in MS_{11} medium turned brown, hard and necrotic whereas the calli in the MS_{10} medium supplemented with NAA and KIN (1.0+1.0 mg l^{-1}) turned white and friable.

Table 9 Optimisation of callusing media for Centella asiatica

Media	No. of explants	Days after inoculation	Callus score	Callus morphology	Remarks
MS_6	20	14	-	-	
		21	-	-	Swelling of leaves
		28	++	Yellowish	Leaves pale yellowish green
		35	++	Yellowish	Leaves turned brown and necrotic
MS_7	20	14	-	-	Swelling of leaves
		21	++	Slightly yellowish	Leaves pale yellowish green
		28	+++	Green and compact	
		35	+++	White and friable	
MS_8	20	14	-	-	
		21	-	-	
		28	+	Yellowish	Leaves pale yellow
		35	+	Turned necrotic	
MS_9	20	14	-	-	
		21	-	-	
		28	+	Yellowish	Leaves pale yellow
		35	+	-	Leaves brown and necrotic

" –" = Negative ; " +" = Low; "++" = Medium ; "+++" = High

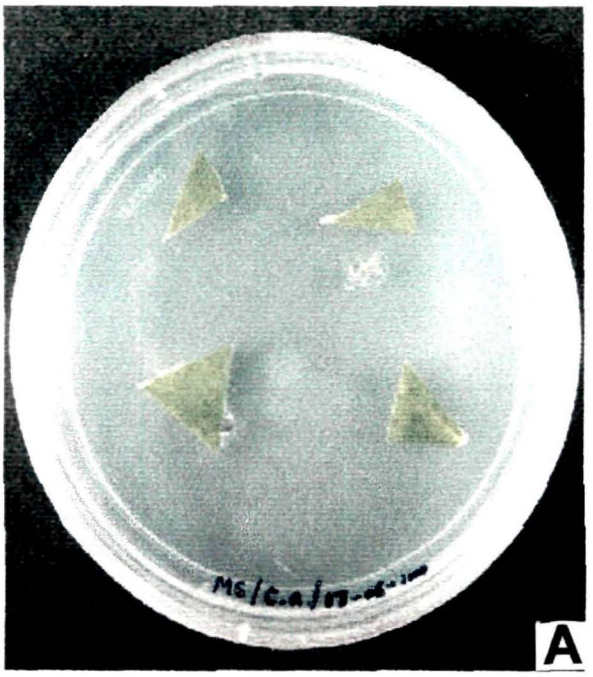
MS_6 = BAP + NAA = (0.5+1) mg/l ; MS_7 = BAP + NAA = (1+1) mg/l
MS_8 = BAP + 2,4-D = (0.5+1) mg/l ; MS_9 = BAP+ 2,4-D = (1.5+1.5) mg/l

Fig 11 Callusing from leaf explants of *Centella asiatica*

A Inoculation of leaf explants in the MS_7 media containing 1.0 mg l^{-1} BA and 1.0 mg l^{-1} NAA

B Rapid growth of the calli from the cut surface of the leaf explants after 21 days of inoculation

C Green and friable calli after subculture


A


B

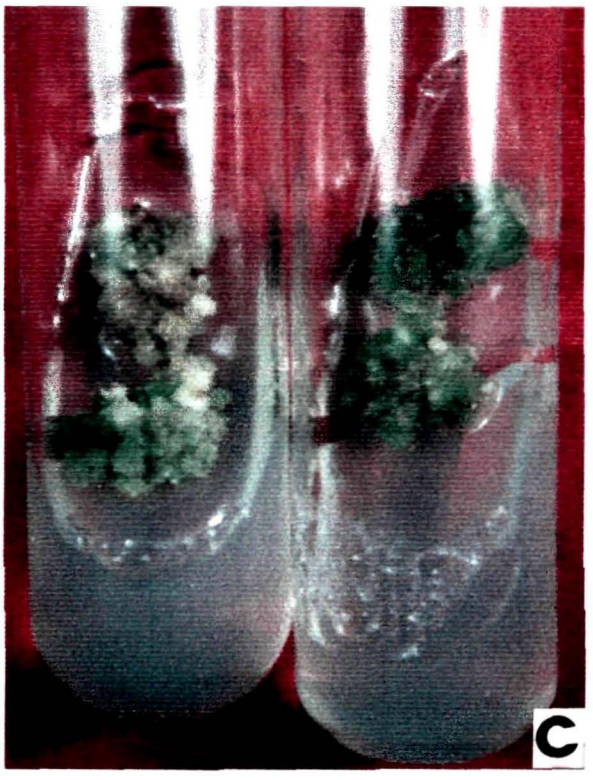

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Table 10 Optimisation of callusing media for Asparagus racemosus

Media	No. of explants	Days after inoculation	Callus score	Callus morphology	Remarks
MS_{10}	30	14	+	Off white	Swelling of explants
		21	++	White and compact	
		35	+++	White and friable	
		42	+++	White and friable	
MS_{11}	30	14	-	-	Swelling of explants
		21	++	Yellowish	
		35	++	White and compact	
		42	+	Hard and brown	
MS_{12}	30	14	+	Off white	Swelling of explants
		21	++	White	
		35	+++	White and friable	
		42	++	Turned brown	
MS_{13}	30	14	-	-	Swelling of explants
		21	+	White and compact	Radicle seen in some calli pieces
		35	++	White and friable	Radicle elongated
		42	++	White and compact	Callus brown and necrotic

"–" = Negative ; "+" = Low; "++" = Medium ; "+++" = High

MS_{10} = NAA + KIN = (1 + 1) mg/l ; MS_{11} = NAA + KIN = (1.5 + 1) mg/l
MS_{12} = NAA + KIN = (1.5 + 1.5) mg/l ; MS_{13} = IAA+ BAP = (1 + 0.1) mg/l

Fig 12 Callusing from spear explants in *Asparagus racemosus*

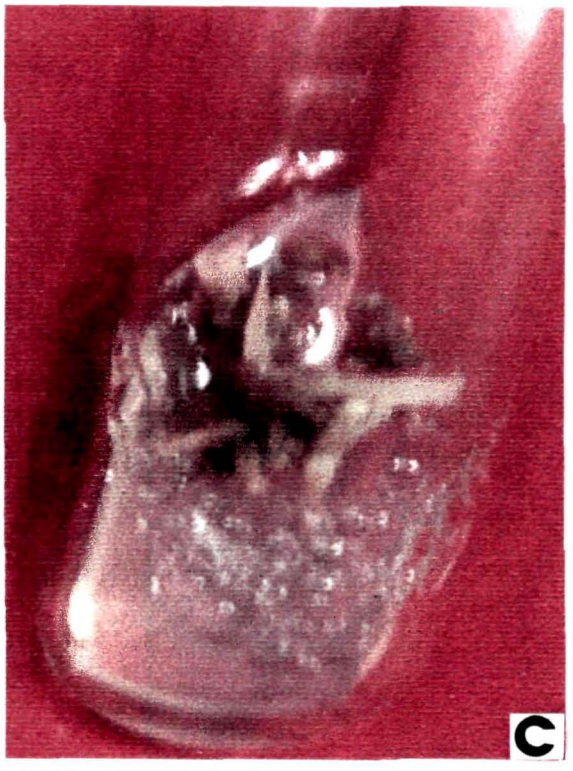
A Swelling of the spear explants and initiation of callus growth from the adaxial surface after 21 days of inoculation in MS_{10} media

B Smooth, glossy and nodule like structures after the third subculture

C Banana and torpedo-like structures after the third subculture in the MS_{10} media containing 1.0 mg l^{-1} NAA and 1.0 mg l^{-1} KIN


A


B


C

These white and friable calli were then subcultured at every 4 weeks interval in the same medium. After the third subculture, the calli became smooth, glossy, nodular and yellowish in colour (Fig 12B). Gradually globular nodular structures appeared fromthecalli, which assumed the shapes of banana and torpedo-like structures (Fig 12C). These types of somatic embryos were also induced from callus in *Asparagus cooperi* following increase in KNO_3 treatment from 1900 mg l^{-1} to 2900 mg l^{-1} in basal media (Ghosh and Sen, 1991). Radicles like structures were also seen to develop from some calli in MS_{13} medium after third week of inoculation but it later turned brown and necrotic.

4.5 Growth measurement

4.5.1 *Growth measurment of callus in Adhatoda vasica, Centella asiatica and Asparagus racemosus.*

Callus formation from explant tissue proceeds with progressively more random planes of cell division, less frequent specialization of cells, and loss of organized structures (Thorpe, 1980; Wagley *et al.*, 1987). When subcultured regularly on agar media, callus cultures ususally exhibit an S-shaped or sigmoidal pattern of growth during each passage. However with respect to *Centella asiatica* the pattern of growth during callus culture did not conform to the usual sigmoidal pattern and showed a somewhat linear one instead. It may be because of the relatively longer culture period that is required for reflecting the typical S-curve for growth in this species. Callus growth can be

monitored by fresh weight and dry weight measurements, which are used as parameters of growth.

In *Adhatoda vasica*, there was a gradual increase in the fresh weight (46.64%) of the callus upto the fourth week with a sudden decline in growth in the 6^{th} week (Fig 13). Maximum growth was recorded after the second week with respect to dry weight of callus in this species, which was maintained at the same level till the 4^{th} week, after which the growth declined.

There was a gradual increase in the fresh weight (124.15%) of the callus upto the sixth week of incubation in *Centella asiatica* with a sudden increase in growth in the eight week (Fig 14). The dry weights of callus tissue also reached the maximum by 270% than the dry weight of the initial inoculum after 8 weeks of incubation. The calli on the fifth week turned green and friable in the MS_7 media, which were suitable for further subculture or for cell suspension culture (Table 9).

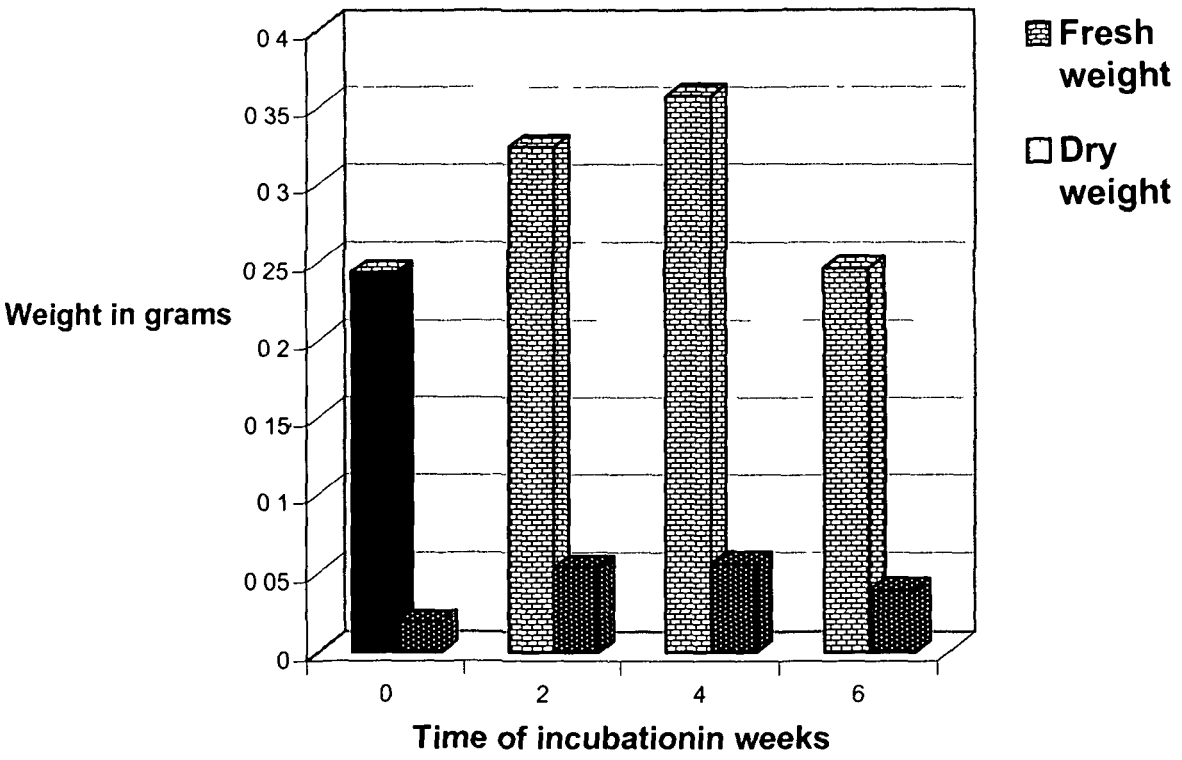


Fig 13 Callusing efficiency in *Adhatoda vasica* during a subculture period

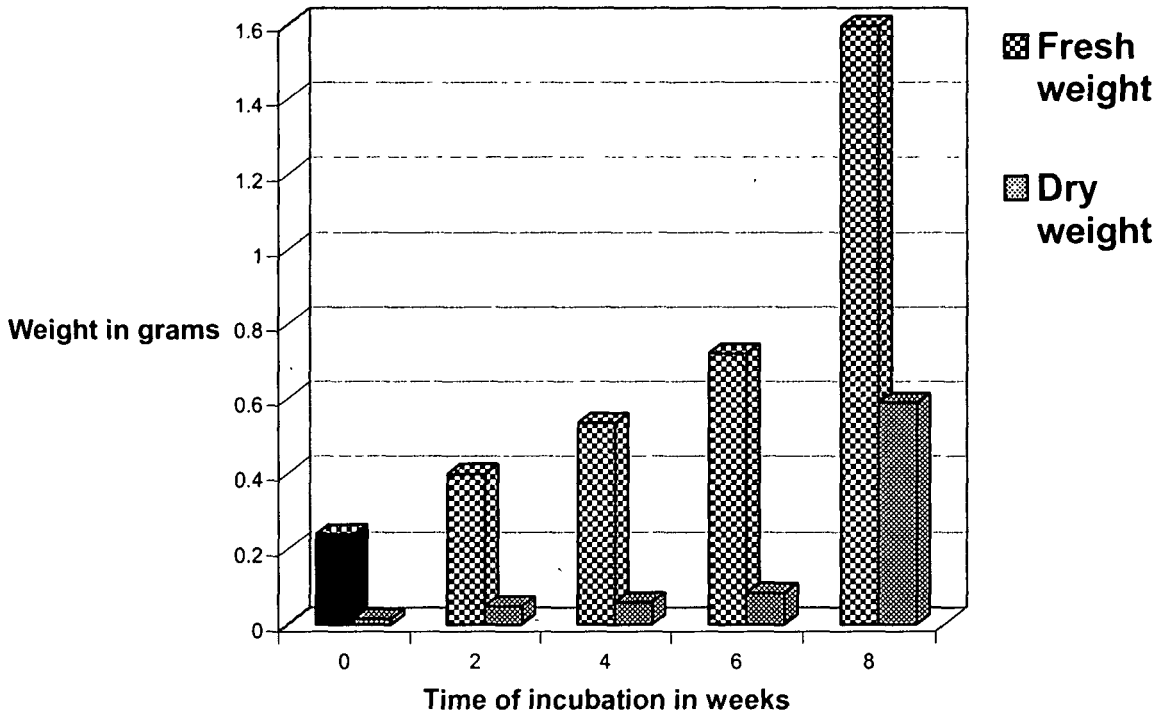


Fig 14 Callusing efficiency in *Centella asiatica* during a subculture period.

The average weight of the 3-week-old callus (fresh and dry weight) in *Asparagus racemosus* was about 8.5 times more than the weight of the one-week-old callus. Though the calli turned white and friable in the fifth week in the MS_{10} medium (Table 10) but the biomass had been maximum in the third week itself. No further growth in the fresh weight was recorded in the 4^{th} week and eventually growth declined in the eight week (Fig 14).

4.5.2 *Growth measurement of Centella asiatica and Asparagus racemosus in suspension culture*

Cells in suspension culture exhibit much higher rates of cell division than do cells in callus culture (Razdan, 2003). Thus, cell suspensions offer advantages when rapid cell division or many cell generations are desired, or when a more uniform treatment application is required such as during the cell selection procedures. Cell suspension growth can be monitored by PCV, which is correlated with fresh weight. Dry weight measurement provides the best estimate of cell doubling. The growth of cells in suspension culture also exhibits a sigmoidal pattern of growth but over a shorter growing period than in callus.

The growth curve of the cell suspension culture in *Centella asiatica* (Fig 16) was seen with an initial lag phase extending upto 10 days of incubation followed by a steep rise in the growth rate by 2.54 mg dry weight per day upto the third week. The growth cycle was completed more rapidly in cell suspension culture in 3 weeks after incubation in MS_7 media (Table 9) in contrast to that in callus culture where the growth cycle was completed in 8 weeks after incubation in the same media (Fig 14). The growth of the cell

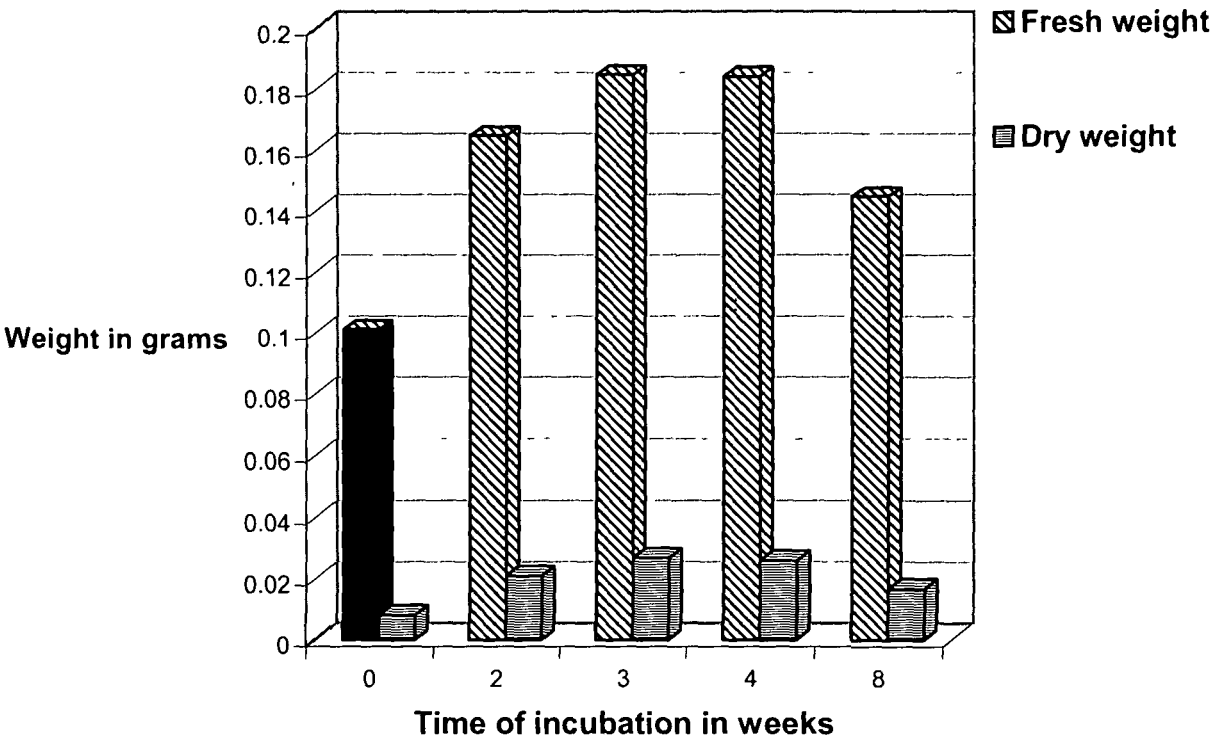


Fig 15 Callusing efficiency in *Asparagus racemosus* during a subculture period.

mass doubled in the 3rd week which increased by 268.54% (FW), 946.78% (DW) and 373.68% (PCV) of the initial fresh and dry weight of the inocula in MS_7 media by cell suspension culture which was 28.5 times more than the weight of the cell mass obtained in the fourth week by callus culture. The growth of the cells in cell suspension culture eventually has been found to decline in the fourth week and the cells started dieing. Therefore subculturing at 3 weeks interval was done to prevent any loss of culture in *Centella asiatica.*

The growth curve of the fresh weight and PCV of cell suspension in *Asparagus racemosus* was found to be in the initial lag phase for more than a week followed by an increase in weight of the cells after 10 days of incubation in the MS_{10} media. But the dry weight of the cells in suspension culture of *Asparagus racemosus* was found to reach the maximum after 21 days of incubation (Fig 17) with a rise in growth rate by 2.92 mg dry weight per day followed by a stationary phase for another week before the cells started dying. The dry weight of the cell mass in suspension culture was found to double nearly 21 days after the day of incubation. The dry weight of the cell mass in suspension culture was found to be 185.59% of that of the initial inoculum which was only 1.22 times less than the cell mass obtained by callus culture. Because of the rapid and uncontrolled weight loss of the fresh callus, the dry weight was considered to be more reliable and accurate. Some differentiation of cells might have occurred in cell cultures of *Asparagus racemosus* during the period of stationary growth, but it was generally less marked and incomplete.

Cultures could not be maintained in the stationary phase for longer periods because cell begins to die. Suspensions are subcultured soon after they reach their maximum dry weight yield (Street, 1977). As the maximum dry weight yield was obtained in the 3rd week after incubation in *Asparagus racemosus*, subculturing became a very crucial step in the suspension culture of *Asparagus racemosus.* Subculturing was done at 3 weeks interval before the process of cell death ensued.

4.6 Isolation and identification of the active principles

The study focussed primarily on the isolation and characterization of the three well known alkaloid components specific to each of the three medicinal plant species

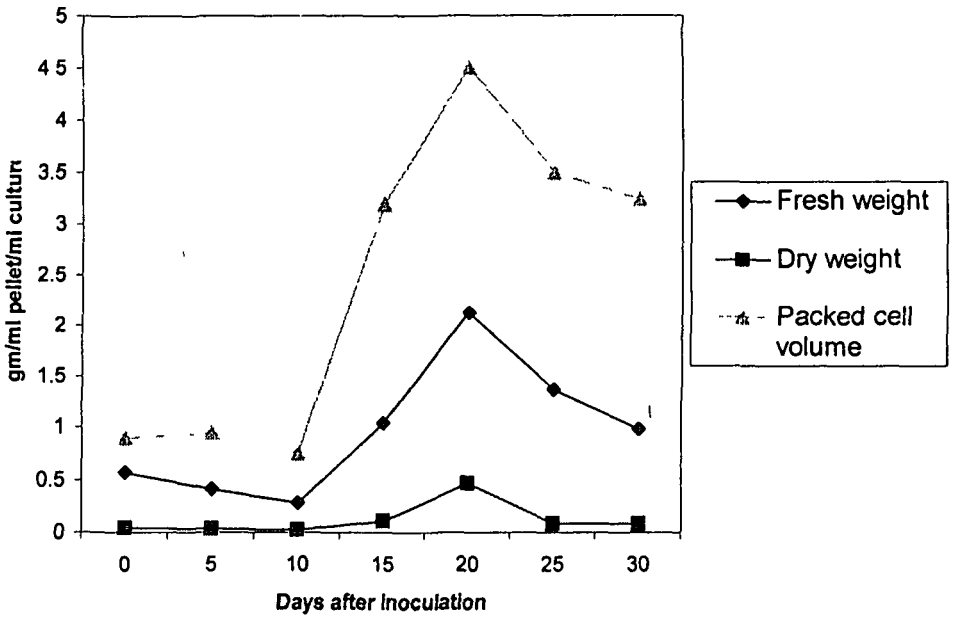


Fig 16 Growth assessment in suspension culture of *Centella asiatica* during a subculture period

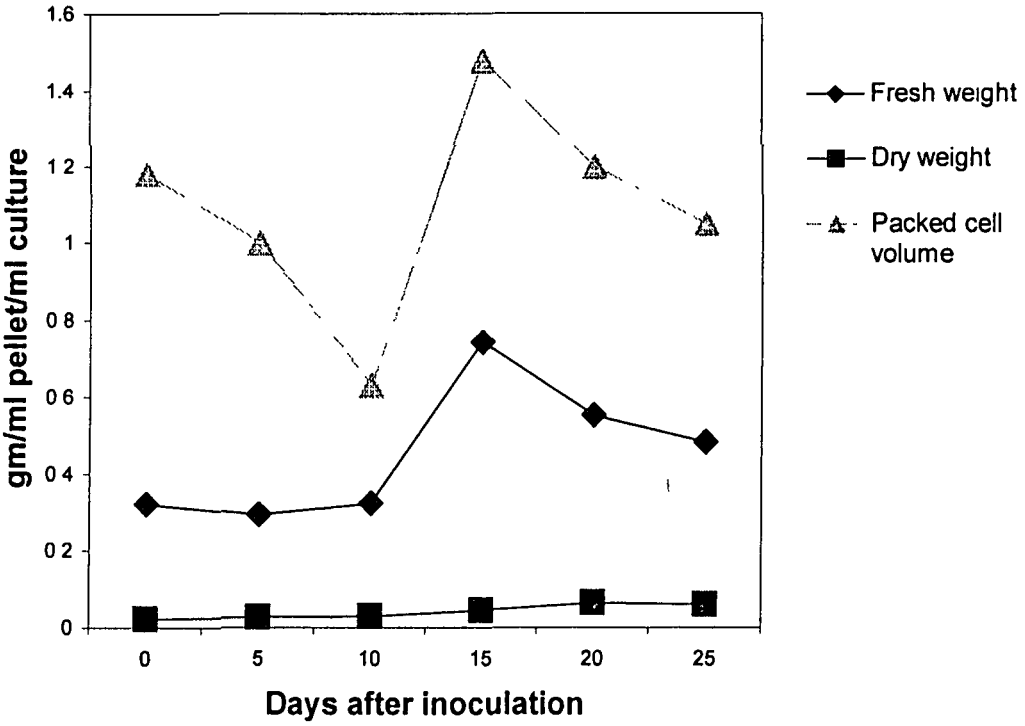


Fig 17 Growth assessment in suspension culture of *Asparagus racemosus* during a subculture period

viz., vasicine in *Adhatoda vasica*, asiaticoside in *Centella asiatica* and L-asparagine in *Asparagus racemosus*.

The alkaloids were indentified by TLC (silica gel 'c'), using the following solvent systems: acetonitrile: 1M phosphate buffer: glacial acetic acid (15:85:1, v/v/v) in *Adhatoda vasica*, ethyl alcohol: methanol: water (60:12:8, v/v/v) in *Centella asiatica* and n-Butanol: acetic acid: water (4:1:1, v/v/v) in *Asparagus racemosus*. The R_f values of zones of samples were compared with standards such as vasicine for *Adhatoda vasica* and L-asparagine for *Asparagus racemosus*. In this context it is to be mentioned that Roja and Heble, 1996 identified indole alkaloids by TLC using $CHCl_3$: MeOH: NH_4OH (95:4.5:0.5, v/v/v) as the solvent system by comparison of R_f values with authentic samples such as reserpine, ajmalicine, ajmaline and serpentine in *Rauwolfia serpentina* Benthe ex Kurz.The vasicine content was analysed using TLC and GLC technique in the *in vitro* grown shoot cultures of *Adhatoda vasica*. The nodal segments was found to contain only traces of vasicine in comparison to the *in vitro* grown shoot cultures where it was reported to synthesize the highest amount of vasicine (Jaiswal *et al.*, 1989). Therefore in the present study leaf was chosen as explants for isolating vasicine.

The R_f value of vasicine (standard) which is 0.58, has been found to be similar to the R_f value of the zone of leaf sample which is 0.57.The R_f values of the zones of root, callus and callus cells from suspension cultured samples of *Asparagus racemosus* are found to be very close to that of the standard (0.28) (Table 11), except for the R_f value of leaf sample (0.35). This is perhaps due to the fact that the cells in the intact leaves of *Asparagus racemosus* do not synthesise this alkaloid. The R_f values of the resolved.

Table 11 Qualitative and quantitative assessment for separation of alkaloids from *the different solvent extracts by thin layer chromatography.*

Plant species	Dry plant parts	Solvent	R_f value	Alkaloid present in the sample (%)	Colour of the spot after treatment
Adhatoda vasica	Leaf	Acetone/ Methanol	0.57 0.58	35.19	Light grey
	Vasicine (standard)	Acetone/ Methanol	0.57	100.00	Light grey
Centella asiatica	Leaf	Ethanol	0.87	27.12	Light brownish
	Callus (4 wks old)	Ethanol	0.84	63.64	Light brownish
	Callus from suspension culture (4 wks old)	Ethanol	0.88	28.70	Light brownish
Asparagus racemosus	L-asparagine (standard)	1N NaOH	0.28	100.00	Dark yellow to brown
	Leaf	1N NaOH	0.35	0.18	Dark pinkish to brown
	Root	1N NaOH	0.29	20.22	Dark pinkish to brown
	Callus (4 wks old)	1N NaOH	0.25	49.20	Dark pinkish to brown
	Callus from suspension culture (4 wks old)	1N NaOH	0.25	16.33	Dark pinkish to brown

* For analysis of asiaticoside the pure sample was not available and hence the result from Singh C *et al.*, 1999 was used as reference.

zones of the samples derived from *Centella asiatica* were found to be similar (0.87-0.88). Even the colour of the resolved compounds in the spots in the three samples were found to be brownish after exposure to iodine vapour. This is indicative of the synthesis of same species of alkaloid molecules in all the three samples

4.6.1 Visual estimation and spot size measurement

From the intensity of the colour of the resolved spots of both the samples and the standards on the TLC plates after treating with the detection reagents, similar type of alkaloids such as vasicine in the leaf samples of *Adhatoda vasica* and L-asparagine in the leaf, root, callus and suspension culture samples of *Asparagus racemosus* were found to be present (Table 11).

The *in vitro* grown leaves of *Adhatoda vasica* in modified secondary MS media supplemented with 0.5 mg l^{-1} BA and 15% coconut milk (Table 2) has been subjected to TLC analyses for detection of vasicine. Analyses of the eluent from the spot apparently containing vasicine, it has been estimated that 35.19% of the resolved contents present in the eluent is that of vasicine. This supports the earlier report by Jaiswal *et al.*, 1989, where *in vitro* grown shoot cultures supplied with 0.5 mg l^{-1} BA were found to contain maximum amount of vasicine (1765.0 μg/g dw) than in cultures supplied with 0.1 mg l^{-1} BA (350.0 μg/g dw).

The totipotency of cells *in vitro* provides the possibility of obtaining all the products that the plant accumulates *in vivo*. The active principle alkaloid of

Centella asiatica, asiaticoside was found to be present in about 63.64% of the resolved contents in the eluent obtained from ethanol extract of callus after subjected to TLC analyses which is about 30 times more than in the *in vitro* grown leaves (27.12%) (Table 11). The secondary metabolite accumulation is believed to be more when the cells in culture are growing slowly, at the stationary phase (Lindsey and Yeoman, 1983). But since in cell suspension cultures of *Centella asiatica* the growth curve has shown declination in the fourth week, the alkaloid accumulation was also found to be in lesser amount (28.70% of the resolved contents in the eluent) in comparison to that in the callus.

Similarly in *Asparagus racemosus*, the active principle alkaloid, L-asparagine was found to be present in about 49.20% of the resolved contents in the eluent obtained from 1N NaOH extract of callus cells when subjected to TLC analyses which is 30 times more than in suspension cultured cells (16.33% of the resolved contents in the eluent) (Table 11). Application of precursors or changes in the culture conditions may result in significant increase in the yield of the active principles. Aitchison *et al.*, 1977 also suggested that the increase in the yield of cell cultures could be obtained by selecting high yielding strains and addition of precursors to the culture medium. The alkaloid was present in very less amount (0.18%) in the resolved contents of the eluent obtained from the 1N NaOH extract of the *in vitro* grown leaves after subjecting to TLC analyses in comparison to the amount present in the roots (20.22%). This is in conformation with the findings that the alcohol and acetone extracts of powdered dry roots of *Asparagus*

racemosus yielded pharmacologically active (antioxytocic) saponins (CRC Handbook of Ayurvedic Medicinal plants).

4.6.2 *Spectrophotometric method*

After locating the analyte zones in the chromatographic plates with the help of detecting reagents, the analytes were resolved with a suitable solvent (Acetone, ethanol and 1N NaOH). The eluates were concentrated and their estimation was done by absorbance spectrophotometric method. The analyte zones of leaf samples and the standard (vasicine) of *Adhatoda vasica* were resolved in acetone. The spectra of vasicine exhibited maximum absorbance (λ_{max}) at 280 nm both in the standard (Annexure IV,1A) and in the leaf sample (Annexure IV, 1B).

The λ_{max} for the active principle, asiaticoside was found to be at 227nm (Annexure IV, 2A) in callus extract, 220nm (Annexure IV, 2B) in suspension cultured cells and 230 nm (Annexure IV, 2C) in leaf extract. The slight differences in the λ_{max} values and the optical density (ε) were possibly due to the transition of the polar chromophores present in the molecule in a polar hydroxylic solvent (ethyl alcohol).

Similarly the analyte zones of the samples of *Asparagus racemosus* were resolved in 1N NaOH which were later concentrated in a vacuum dryer and used for analysis. The spectra of L-asparagine (standard) exhibited maximum absorbance (λ_{max}) at 255 nm (Annexure IV, 3A) which was almost similar with the spectra of the active alkaloid present in the callus (λ_{max} = = 253 nm) (Annexure IV, 3B) and suspension cultured cells (λ_{max} = 253 nm) (Annexure IV, 3C) of *Asparagus racemosus* with a slight difference in optical

densities. However, the spectra of the active alkaloid present in the *in vitro* leaf (λ_{max} = 224nm) (Annexure IV, 3D) and in the root (λ_{max} = 225nm) (Annexure IV, 3E) of *Asparagus racemosus* was found to be quite different with the spectra of the standard. The variation in the λ_{max} and ε values in the two samples is perhaps due to the differences in the relative orientation of the chromophores of the alkaloid molecule in the intact plant and in cultured callus cells as was also suggested by Sharma, 2000.

4.6.3 *High performance liquid chromatography*

The solvent system used for vasicine analysis was acetonitrile: 0.1 M phosphate buffer: glacial acetic acid (15:85:1,v/v/v) as reported earlier (Gupta *et al.*, 2001). The injected volume of samples (20 µl) was run for 5 min at a flow rate of 1ml min^{-1}, which provided a good resolution of peaks. The similarity of the peaks in retention time both in standard (Annexure V, 1A) and in the leaf sample (Annexure V, 1B) indicates the presence of vasicine in the sample. On the other hand, the single peak found in the HPLC profile of leaf sample confirms the purity of the product. Seperation of vasicine and vasicinone analogue was also attempted using methanol: water (70:30) as solvent system (Chowdhury and Hirani, 1987) by reversed phase chromatography. However, the vasicine and its analogues appeared as broad tailing peaks which was not suitable for quantitative work and the analysis time was also long. Gupta *et al.*, 2001 obtained good seperation of vasicine and vasicinone with peak purity from: up 0.9986-0.998; down 0.9980-0.9993 using acetonotrile: 0.1M phosphate buffer: glacial acetic acid (15:85:1, v/v/v) as the solvent system at a flow rate of 0.7 ml/min with a photo diode array

detector. Using the same mobile phase the amount of vasicine was found to be 48 mg/g of dried biomass (leaf) (Table 12).

For analysis of asiaticoside, the active principle in *Centella asiatica* different composition of the mobile phase were tested and a good resolution was achieved by using ethyl alcohol: methanol: water (60:12:8, v/v/v) as solvent system. The ethanol extracts of leaf, callus and suspension cultured cells were injected into the HPLC system at 15µl volume for 15 min run time at a flow rate of 1ml min^{-1}. This solvent system provided a good seperation of peaks [Annexure V, 2(A-C)] at 220 nm wavelength showing a retention time of 0.954 min, 1.072 min and 1.015 min respectively. The advantage of known HPLC profile of the concerned compound (asiaticoside) on a C_{18} column, using acetonitrile: water (3:7) as solvent and UV detector at 220 nm (Singh C *et al.*, 1999) has been taken as reference for comparison of the HPLC profile for compounds purified from leaf, callus and suspension culture derived cells of *Centella asiatica*. The similarity in retention time of the peaks indicates the presence of asiaticoside in the samples. Secondary metabolites have also been reported to be synthesized in the undifferentiated calli of *Crocus sativus* (Castellar and Iborra, 1997) and *Nyctanthes arbor-tristis* (Iyer *et al.*, 1998).

Table 12: Qualitative and quantitative assessment of the purified fractions extracted from the three plant species by high performance liquid chromatography.

Plant species	Sample	Alkaloid present	Injection volume (μl)	Retention time (min)	Alkaloid calculated (mg/g of dried biomass)
Adhatoda vasica	Vasicine (standard)	Vasicine	20	1.105	
	Leaf	Vasicine	20	1.055	48.0
Centella asiatica	Leaf	Asiaticoside	15	0.954	125.0
	Callus	Asiaticoside	15	1.072	190.48
	Suspension	Asiaticoside	15	1.015	494.62
Asparagus racemosus	L-asparagine (standard)	L-asparagine	15	1.613	
	Leaf	L-asparagine	15	1.361	107.53
	Root	L-asparagine	15	1.367	253.33
	Callus	L-asparagine	15	1.383	129.03
	Suspension	L-asparagine	15	1.557	250.0

The alkaloid, asiaticoside was found to be about 2.5 times higher in amount in suspension cultured cells (494.62 mg/g of dried biomass) than that in callus cells (190.48 mg/g of dried biomass)(Table 12). Application of precursors or changes in culture condition may also result in increase in the yield of derived compounds (Iyer *et al.*, 1998). This fact was well documented by the increase in synthesis of Diosgenin and Solasodine contents of the cells of *Solanum jasminoides* and *Solanum verbascifolium*, which was found to be in higher amount with increase in cholesterol supplementation in suspension culture (Sahoo *et al.*, 1999). In this regard, bioreactors play a crucial role in scaling up of cell suspension cultures under defined parameters for the production of bioactive compounds. The growth rate and production of Paclitaxel and Baccatin III of *Taxus wallichiana* cell suspension significantly increase in its respective optimum media in the presence of methyljasmonate using a 5 l stirred bioreactor (Cusido *et al.*, 2002). Increasing the activity of metabolic pathways by elicitation, in conjunction with end product removal and accumulation in an extractive phase have also been proved to be the most successful strategy (Brodelius and Pedersen, 1993).

The present investigation therefore provided a feasible opportunity for subsequent increase in productivity of asiaticoside in *Centella asiaticoside*.

Different composition of mobile phase was tested for analysis of L-asparagine in *Asparagus racemosus*. Good resolution of peaks were obtained with mobile phase, n-Butanol:acetic acid:water (4:1:1, v/v/v) when 15µl of each sample of leaf, root, callus and suspension cultured cells were subjected to HPLC seperation for 10min run time at a flow rate of 1ml min^{-1} and UV

detected at 225 nm. Asymmetrical peaks were found in the HPLC profiles of the samples [Annexure V, 3(B-E)] in comparison to the standard (L-asparagine)(Annexure V, 3A). This may be due to the presence of some impurities in the samples. But the similarity in retention time of the peaks in the sample profiles with that of the standard is strongly suggestive of the presence of the alkaloid. The amount of alkaloid was found to be higher in root than in leaf, callus and suspension cultured cells (Table 12). The roots of *Asparagus racemosus* yielded pharmacologically active saponins, one of which is L-asparagine (CRC Handbook of Ayurvedic Medicinal Plants). On the contrary, callus and subsequently suspension culture was established in the present investigation using the spear explants of *Asparagus racemosus*. This is an evidence of the ability of the dedifferentiated cells derived from the spear explants as well, to synthesise L-asparagine. This is the first report on the presence of L-asparagine in the undifferentiated calli derived cells of spear explants in *Asparagus racemosus*.

4.7Determination of antimicrobial activity of the crude extracts

In the present study, preliminary investigation was done to determine the antimicrobial potency of three plant species namely, *Adhatoda vasica*, *Centella asiatica* and *Asparagus racemosus* against *Pseudomonas* spp (gram –ve), *Bacillus subtilis* (gram +ve) and yeast (*Saccharomyces* spp).The antibiotic discs viz., the Norfloxacin (10 μg/ disc), Gentamycin (10 μg/disc) and Ciprofloxacin (5 μg/disc) used as standards in the experiments were broad spectrum antibiotics. The crude extract were prepared from dried plant samples (Fig 18) using different solvents in which their respective active

principles were soluble.The growth of the *Pseudomonas* spp was shown to be inhibited by the acetone leaf extract (1mg/ml) of *Adhatoda vasica* (Fig 19A) (Table 13) whereas the ethanol leaf extract of *Centella asiatica* did not show any inhibitory effect (Fig 19B). The growth rate of the organism had increased with the increase in the dilution factor (Table 13) of the leaf extract of both *Adhatoda vasica* and *Centella asiatica*. Similarly the 1N NaOH leaf and root extracts of *Asparagus racemosus* also did not show any inhibitory effect on the growth of *Pseudomonas* spp (Table 14).

The leaf extracts of *Centella asiatica* and *Adhatoda vasica* did not show any inhibitory effect on the growth of *Saccharomyces* spps (Table 15)(Fig 20). Even the leaf and root extracts of *Asparagus racemosus* had no inhibitory effect on the growth of *Sacchoromyces* spps (Table 16) in YPD media.

The acetone leaf extract of *Adhatoda vasica* (Table 17) and ethanol leaf extract of *Centella asiatica* (Table 18)(Fig 21A-B) had shown comparatively less and minimal inhibitory effect on the growth of *Bacillus subtilis* respectively whereas 1N NaOH root extract of *Asparagus racemosus* had shown inhibitory effect on the growth of *Bacillus subtilis* (Fig 21C) as depicted from the inhibition zone diameter (Table 19).

Table 13 Effect of ethanol leaf extract of Centella asiatica and acetone leaf extract of Adhatoda vasica on the growth of Psuedomonas spp

Micro organism	Crude extract	Dilution factor	Growth		Average colony diameter (mm)	
			C. asiatica	*A. vasica*	*C. asiatica*	*A. vasica*
Pseudomonas spp.	Leaf extract	Sample (1mg/ml)	TNTC	-	2.5	–
		Ethanol	TNTC	TNTC	-	-
		10^{-1}	++	TFTC	3.7	2.4
		10^{-2}	++	TFTC	3.9	2.9
		10^{-3}	++	TFTC	4.0	3.5
		10^{-4}	TNTC	++	5.3	5.0
		10^{-5}	TNTC	TNTC	5.7	Dense smear

* TFTC = Too few to count (< 30 colonies) ; TNTC = Too numerous to count (>300 colonies) ; " ++" = Between 30-300 colonies ; " – " = Complete inhibitiom of colony growth (*After Microbiology a Laboratory Manual by J.G. Cappuccino and N. Sherman, 4th Edition)

Table 14 Effect of 1N NaOH leaf and root extract of Asparagus racemosus on the growth of Pseudomonas spp

Micro-organism	Crude extract	Dilution factor	Growth	Average colony diameter (mm)
Pseudomonas spp.	Leaf extract	Sample(1ml)	++	3.5
		1N NaOH	TNTC	5.2
		10^{-1}	TNTC	5.5
		10^{-2}	++	4.7
		10^{-3}	TNTC	5.08
		10^{-4}	TNTC	5.0
		10^{-5}	TNTC	5.01
	Root extract	Sample (1ml)	TNTC	4.0
		1 N NaOH	TNTC	4.1
		10^{-1}	++	3.4
		10^{-2}	++	2.6
		10^{-3}	TNTC	3.8
		10^{-4}	TNTC	4.5
		10^{-5}	TNTC	4.5

*TFTC = Too few to count (< 30 colonies) ; TNTC = Too numerous to count (> 300 colonies) " ++" = Between 30-300 colonies (*After Microbiology a Laboratory Manual by J.G. Cappuccino and N. Sherman, 4th Edition)

Table 15 Effect of ethanol leaf extract of Centella asiatica and acetone leaf extract of Adhatoda vasica on the growth of Saccharomyces spp

Micro-organism	Crude extract	Dilution factor	Growth		Average colony diameter (mm)	
			C. asiatica	*A. vasica*	*C. asiatica*	*A. vasica*
Saccharomyc -es spp	Leaf extract	Sample (1ml)	++	TNTC	2.0	3.0
		Ethanol/ Acetone	++	TNTC	2.0	3.0
		10^{-1}	TNTC	++	2.5	2.0
		10^{-2}	++	TNTC	2.5	3.0
		10^{-3}	++	++	2.0	2.0
		10^{-4}	++	++	2.0	2.0
		10^{-5}	TNTC	++	2.5	2.0

*TFTC = Too few to count (< 30 colonies) ; TNTC = Too numerous to count (> 300 colonies) ; " ++" = Between 30-300 colonies ; " – " = Complete inhibition of colony growth (*After Microbiology a Laboratory Manual by J G. Cappuccino and N. Sherman, 4th Edition)

Table 16 Effect of 1N NaOH leaf and root extract of Asparagus racemosus on the growth of Saccharomyces spp

Micro-organism	Crude extract	Dilution factor	Growth	Average colony diameter (mm)
Saccharomyces spp.	Leaf extract	Sample (1ml)	++	2.06
		1N NaOH	TNTC	3.50
		10^{-1}	TNTC	2.25
		10^{-2}	TNTC	2.93
		10^{-3}	TNTC	2.0
		10^{-4}	TNTC	2.64
		10^{-5}	TNTC	3.50
	Root extract	Sample (1ml)	++	1.63
		1N NaOH	TNTC	2.70
		10^{-1}	TNTC	2.81
		10^{-2}	TNTC	3.0
		10^{-3}	TNTC	2.71
		10^{-4}	TNTC	2.71
		10^{-5}	TNTC	2.83

TFTC = Too few to count (< 30 colonies) ; TNTC = Too numerous to count (> 300 colonies) ; " ++" = Between 30-300 colonies (*After Microbiology a Laboratory Manual by J.G. Cappuccino and N. Sherman, 4th Edition)

Table 17 Effect of methanol leaf extract of Adhatoda vasica on the growth of Bacillus subtilis

Micro-oganism	Crude extract	Zone diameter, Nearest whole mm				
		Disc concentration (µg/ disc, ml)	Inhibition zone diameter (mm)	Resistant (R)	Intermediate (I)	Susceptible (S)
Bacillus subtilis	Norfloxacin (std)	10	39.3	-	-	S
	Gentamycin (std)	10	37.3	-	-	S
	Ciprofloxacin (std)	5	40.3	-	-	S
	Leaf extract	1(mg/ml)	-	-	-	-
		Methanol	-	-	-	-
		2	34.6	-	-	S
		4	33.6	-	-	S
		6	35.0	-	-	S
		8	34.3	-	-	S
		10	36.3	-	-	S

Resistant (R) = < 12mm ; Intermediate(I) = 13-16 mm ; Susceptible(S) = > 17 mm

Table 18 Effect of ethanol leaf extract of Centella asiatica on the growth of Bacillus subtilis

Micro-organism	Crude extract	Zone diameter, Nearest whole mm				
		Disc concentration (µg/disc,ml)	Inhibition zone diameter (mm)	Resistant (R)	Intermediate (I)	Susceptible (S)
B. subtilis	Norfloxacin (std)	10	33.6	-	-	S
	Gentamycin (std)	10	33.3	-	-	S
	Ciprofloxacin (std)	5	37.6	-	-	S
	Leaf extract	1(mg/ml)	17.6	-	-	S
		Ethanol	17.9	-	-	S
		2	16.0	-	I	-
		4	18.0	-	-	S
		6	21.0	-	-	S
		8	21.5	-	-	S
		10	16.0	-	I	-

Resistant (R) = < 12mm ; Intermediate(I) = 13-16 mm ; Susceptible(S) = > 17mm

Table 19 Effect of 1N NaoH leaf and root extract of Asparagus racemosus on the growth of Bacillus subtilis

Micro-organism	**Crude extract**	**Zone diameter, Nearest whole mm**				
		Disc concentration (µg/disc,ml)	Inhibition zone diameter (mm)	Resistant (R)	Intermediate (I)	Susceptible (S)
B. subtilis	Norfloxacin (std)	10	33.2	-	-	S
	Gentamycin (std)	10	34.5	-	-	S
	Ciprofloxacin (std)	5	37.6	-	-	S
	Leaf extract	1(mg/ml)	8	R	-	-
		1N NaOH	8.1	-	-	-
		2	8	R	-	-
		4	7.3	R	-	-
		6	8.7	R	-	-
		8	8.3	R	-	-
		10	10.3	R	-	-
	Root extract	1(mg/ml)	9.0	R	-	-
		1N NaOH	-	-	-	-
		2	8.0	R	-	-
		4	9.0	R	-	-
		6	-	-	-	-
		8	9.0	R	-	-
		10	7.0	R	-	-

Resistant (R) = < 12mm ; Intermediate (I) = 13-16 mm ; Susceptible (S) = >17mm

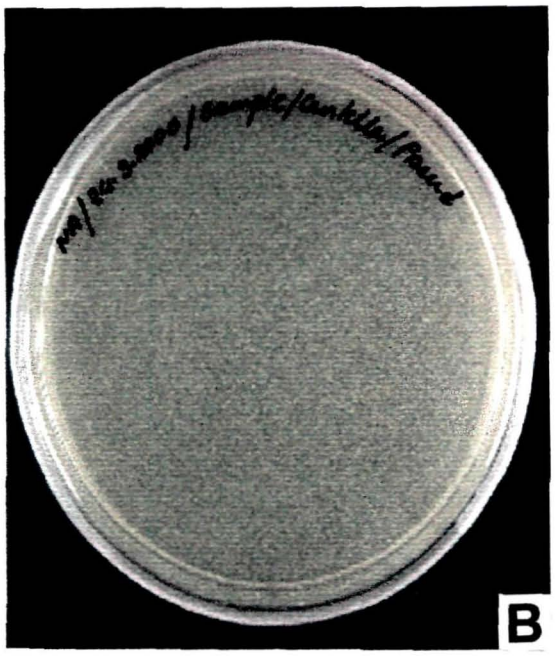
Fig 18 Dried plant samples

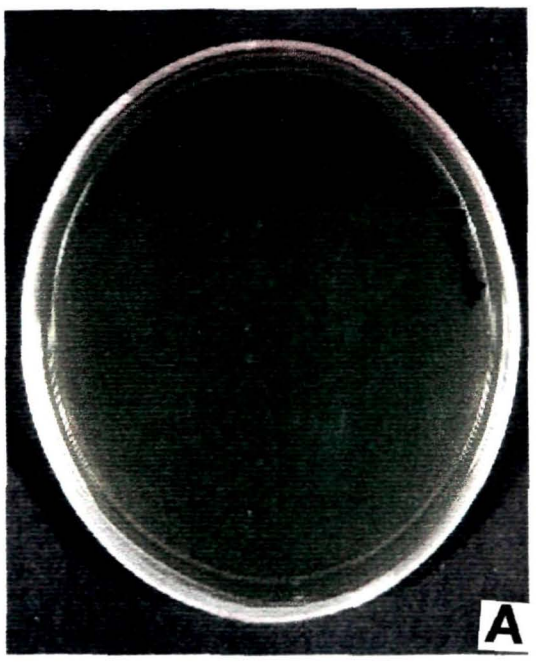
Fig 19(A-B) Inhibition on the growth of *Psuedomonas* spp

A Complete inhibition by the acetone leaf extract (1mg/ml) of *Adhatoda vasica*

B No inhibitory effect of the ethanol leaf extract of *Centella asiatica*

Fig 20 Absence of inhibitory effect of the ethanol leaf extract of *Centella asiatica* and acetone leaf extract of *Adhatoda vasica* on the growth of *Saccharomyces* spp.


B


A

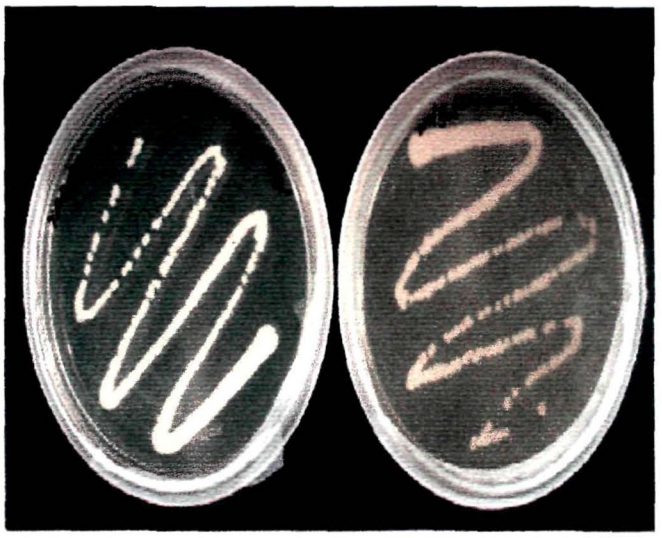
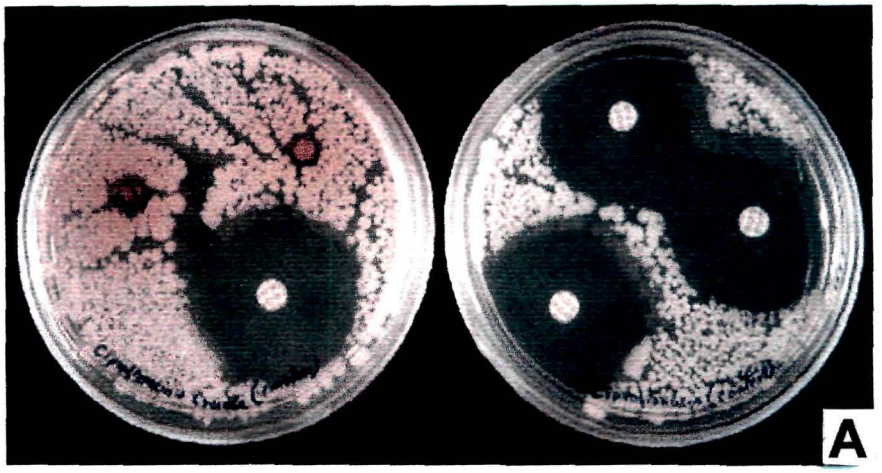


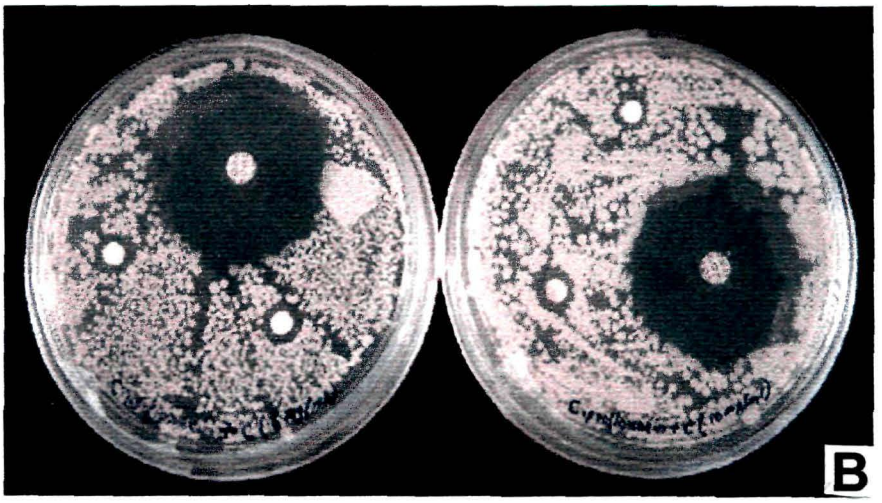
Fig 21 (A-C) Inhibition on the growth of *Bacillus subtilis* depicted by the inhibition zone

A No inhibitory effect of acetone leaf extract of *Adhatoda vasica*

B Intermediate inhibitory effect of ethanol leaf extract of *Centella asiatica*

C Inhibition of 1N NaOH root extract of *Asparagus racemosus*


A


B


C

4.7.1 *Anti-tuberculosis activity of the crude extracts*

In recent years, development of multi-drug resistance in pathogenic bacteria and parasites has created major clinical problems in the treatment of infectious diseases (Davis, 1994). Tuberculosis, gonorrhoea, malaria and childhood earinfections are just a few of the diseases that have become difficult to treat with the conventional antibiotic drugs. Therefore search for new anti-infectives is on (Bhardwaj and Dharmana, 2003). The three plant species namely, *Adhatoda vasica*, *Centella asiatica* and *Asparagus racemosus* used in the present study are known to possess antituberculosis, wound healing property and antiulcer activity respectively in the Ayurvedic system of Indian medicine (CRC handbook of Ayurvedic Medicinal Plants). An attempt was therefore made in terms of minimal inhibitory concentration (MIC) to determine the antituberculosis activity of the crude extracts derived from both *in vitro* and *in vivo* grown tissues of the three plant species.

The benzylamines, bromhexine and ambroxol which are semi-synthetic derivatives of vasicine from *Adhatoda vasica* were used as mucolytics, and has a pH dependent growth inhibitory effect on *Mycobacterium tuberculosis* (Grange and Snell, 1996). Vasicine, the active principle of *Adhatoda vasica* also was a auterotonic abortifacient (Gupta *et al.*, 1978) apart from the presence of antiasthmatic and antibronchial activity in its leaf extract (Kapoor, 2001).The acetone leaf extract of *Adhatoda vasica* was found to completely inhibit the growth of the bacilli at 50 μg/ml concentration of the extract (Table 20). This confirms the presence of anti tuberculosis activity in the leaf extract of *Adhatoda vasica*.

The crude saponins obtained from *Asparagus officinalis* were found to possess anti-tumour activity (Shao *et al*, 1996). Even the oligofurostanosides isolated from the seeds of *Asparagus officinalis* were found to inhibit the growth of human leukaemia HL-60 cells (Shao *et al*.1997). However, no investigation was done to determine the anti tuberculosis activity of the crude extracts of *Asparagus racemosus* till date. In the present study, the 1N NaOH root, callus and suspension cultured cell extract of *Asparagus racemosus* was found to inhibit the growth of *Mycobacterium tuberculosis* bacilli at 50 μg/ml concentration of the extracts. However, the extracts of samples of both *Adhatoda vasica* and *Asparagus racemosus* did not show any growth inhibitory effect at 25 μg/ml concentration of the crude extracts. This indicates that 50 μg/ml of the extracts is the minimal inhibitory concentration which is required for the inhibition of growth of *Mycobacterium tuberculosis* bacilli.

The medicinal herb, *Centella asiatica* has been reported to have asiaticoside in the leaves (Polonoski, 1951) and yielded encouraging results in the treatment of leprosy (Bailey, 1945; Viala *et al*., 1977). The partially purified fractions of *Centella asiatica* inhibited proliferation of transformed cell lines significantly than did the *Centella asiatica* crude extracts (Babu *et al*., 1995). This suggests that the principle alkaloid asiaticoside might have been present in the partially purified fractions inhibiting the growth of the tumour cells. However, the ethanol extract of the leaf, callus and suspension cultured cell of *Centella asiatica* did not show any inhibition on the growth of the bacilli at both 25 and 50 μg/ml concentrations of the crude extracts (Table 20). This indicates that either the synthesis of alkaloid, asiaticoside in the leaf and calli

Table 20 Anti-Mycobacterium tuberculosis H37Rv activity of plant extracts

Name of the plant extracts	Plant sample	Solvent used	Concentration of (μg /ml)	
			50	25
Centella asiatica	Leaf (*in vitro*)	Ethanol	NA (-)	NA (-)
Centella asiatica	Callus	Ethanol	NA (-)	NA (-)
Centella asiatica	Calli suspension culture extract	Ethanol	NA (-)	NA (-)
		Ethanol	NA (-)	NA (-)
Adhatoda vasica	Leaf (*in vitro*)	Acetone	CI (+)	NA (-)
		Acetone	NA (-)	NA (-)
Asparagus racemosus	Leaf (*in vitro*)	1N NaOH	NA (-)	NA (-)
Asparagus racemosus	Root	1N NaOH	CI (+)	NA (-)
Asparagus racemosus	Callus	1N NaOH	CI (+)	NA (-)
Asparagus racemosus	Suspension culture extract	1N NaOH	CI (+)	NA (-)
		1N NaOH	NA (-)	NA (-)

CI (+) = Complete inhibition of growth of the bacilli at this concentration

NA (-) = Not active (Full growth of bacilli at this concentration)

derived cells is insignificant and inadequate for triggering antimicrobial activity or the synthesized alkaloid has no effect at all on the bacilli. This confirms the earlier report that the powder of crude extracts of *Centella asiatica* had no effect on the viability of *Mycobacterium tuberculosis* (Herbert *et al.*, 1994).

Chapter V

CONCLUSIONS AND FUTURE WORK

Chapter V
Conclusions and Future Work

5.1 Conclusion

Comprehensive experimental strategies were developed in the context of the three major objectives of the present study. Development of micropropagation protocols for the three medicinally important plant species- *Adhatoda vasica*, *Centella asiatica* and *Asparagus racemosus* addressed the specific problems related to tissue browning and exudation of phenolic compounds from the explants, enhancement of shooting efficiency and very stubborn microbial contamination in the case of *Adhatoda vasica*. Microbial contamination and selection of explants have been the two principal issues addressed with respect to the micropropagation of *Centella asiatica*. The micropropagation of *Asparagus racemosus* has not been reported earlier and all relevant aspects involved in the development of micropropagation technology for this species have been considered.

The conclusions of the work pertaining to micropropagation of the three plant species can be summarised as follows:

1. The problem of phenolic exudates and callusing at the cut ends of the explants of *Adhatoda vasica* were overcome by culturing the explants in a modified MS media.
2. Tissue culture conditions have been worked out for regeneration of *Adhatoda vasica* and *Centella asiatica* using shoot tip as explants. Shooting efficiency had been enhanced using the same protocol. The

acute problems of microbial contamination with respect to tissue culture of *Adhatoda vasica* and *Centella asiatica* from the commonly used explants viz., foliar and nodal explants have been eliminated by proper selection of explant tissue in the form of shoot tips in both cases.

3. Tissue culture protocols have been developed for regeneration of *Asparagus racemosus* using nodes with axillary bus as explants. This is the first ever instance of micropropagation of *Asparagus racemosus* following tissue culture approaches.

Tissue cultured derived plantlets of *Adhatoda vasica*, *Centella asiatica* and *Asparagus racemosus* have been successfully transferred to and established in pots.

Suspension culture of plant cells for generation of secondary metabolites of importance is a general objective of any plant biotechnological strategy for medicinally important plants. A major objective of the present study centered around the following issues:

- Development of suspension culture protocols for the proliferation of plant cells from the investigated plant species.
- Generation and quantitative assessment of the active principles in the dedifferentiated cells under suspension culture conditions.

The conclusions from the various experiments on these aspects are

1. Callusing efficiencies of *Adhatoda vasica*, *Centella asiatica* and *Asparagus racemosus* from leaf (in the case of *Adhatoda vasica* and *Centella asiatica*) and spear explants (in the case of *Asparagus*

racemosus) was determined after 30 days of inoculation. Growth studies of the calli in MS_7 media revealed highest growth rates in the case of *Centella asiatica*.

In *Asparagus racemosus* the growth of callus reached the exponential phase in the third week after incubation in MS_{10} media (NAA + KIN = 1+1 mg/l). Callus growth of *Adhatoda vasica* was found to be lowest. Green and friable calli of *Centella asiatica* and *Asparagus racemosus* were obtained after 35 days of inoculation in MS_7 and MS_{10} media respectively unlike in *Adhatoda vasica* where it was obtained after 56 days of inoculation.

2. Suspension culture protocols for culture of calli derived cells of *Centella asiatica* and *Asparagus racemosus* have been developed. Assessment of the growth of suspension cultured cells revealed that in *Centella asiatica* the average weight of the cell mass increased by 268.54 % FW, 946.78 % DW and 373.68 % PCV of the initial inoculum when the growth curve reached the exponential phase after 21 days of incubation; in *Asparagus racemosus* the average weight of the cell mass was 185.59 % DW of the initial inoculum after 21 days of incubation.
3. Chromatographic experiments revealed that the dedifferentiated calli cells retained the ability to drive the metabolic pathways leading to the synthesis of asiaticoside and L-asparagine. The experiments on generation of vasicine in suspension cultured cells could not be performed due to constraints of time as the callusing efficiency of the explants from *Adhatoda vasica* was found to be comparatively less.

A very significant findings of the study was that the active principle, asiaticoside in *Centella asiatica* was found to be about 10 times higher in amount in suspension cultured cells than that in intact callus cells. Higher growth rates of cells under suspension culture conditions accounts for this enhancement.

Another interesting finding with respect to *Asparagus racemosus* was that even the spear derived calli cells were capable of producing the active principle, L-asparagine under suspension culture condition. This compound is generally localised in the root cells in intact plant.This is the first report on the use of suspension culture for production of asiaticoside and L-asparagine.

Scientific validation of the ethnobotanical and traditional knowledge based facts pertaining to effectiveness of various plant products against specific human ailments is a major aspect of study in medicinal plants. The third objective of the present study relates to experimentation to determine the anti-tuberculosis (anti-*Mycobacterium tuberculosis*) activities of the known active principles from *Adhatoda vasica*, *Centella asiatica* and *Asparagus racemosus* which are vasicine, asiaticoside and L-asparagine respectively. Two approaches have been adopted for such determination in the present study. Firstly, experiments were designed to find out the effect of plant extracts (partially purified) of these species on selected microorganisms from the prokaryotic and eukaryotic groups. Secondly, specific tests were performed using *Mycobacterium tuberculosis* H37Rv strains with the same purpose.

Conclusions from these studies are as follows:

1. The acetone leaf extract of *Adhatoda vasica* showed inhibitory effect on the growth of *Pseudomonas* spp at 1 mg/ml concentration.
2. The 1 N NaOH leaf and root extracts of *Asparagus racemosus* inhibited the growth of *Bacillus subtilis*.
3. A very significant finding was that the acetone leaf extract of *Adhatoda vasica* and 1N NaOH root, callus and suspension cultured cell extracts of *Asparagus racemosus* completely inhibited the growth of *Mycobacterium tuberculosis*, H37Rv bacilli at 50 μg/ml concentration.

There is no previous report on the anti-*Mycobacterium tuberculosis* H37Rv activity of *Asparaus racemosus* derived metabolites.

5.2 Future work

The present work can be extended further to address three critical aspects. Firstly, having proved that the dedifferentiated calli derived cells of *Asparagus racemosus* do synthesise the active principles under tissue culture conditions, strategies can be developed for scaling up of the production levels of the compound on a sustained basis using bioreactors.

Secondly, specific studies can be undertaken for identifying the key enzyme (s) regulating the metabolic pathways leading to the synthesis of vasicine and L-asparagine. This would enable development of appropriate molecular biotechnological approaches for subsequent metabolic pathway engineering for enhanced generation of the compounds. Even structure determination and purification of the active compound having anti-tubercolic activity such as vasicine and L-asparagine can be undertaken.

Thirdly, having proved the anti-*Mycobacterium tuberculosis* H37Rv activities of the plant products from *Adhatoda vasica* and *Asparagus racemosus*, specific tests have to be designed and performed on *in vivo* situations involving infected animal model systems.

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ANNEXURE I

Murashige and Skoog's media (After Murashige and Skoog, 1962)

Stock solution (× 100)	Composition	g/l
A	NH_4NO_3	82.5
B	KNO_3	95.0
C	$CaCl_2.2H_2O$	88.0
D	KH_2PO_4	34.0
E	H_3BO_3	1.25
	$Na_2MoO_4.2H_2O$	0.05
	$CoCl_2.6H_2O$	0.005
	KI	0.166
F	$MnSO_4.H_2O$	3.38
	$MgSO_4.7H_2O$	74.0
	$CuSO_4.5H_2O$	0.005
	$ZnSO_4.7H_2O$	1.725
G	$Na_2EDTA.2H_2O$	1.865
	$FeSO_4.7H_2O$	1.39
Vitamins	nicotinic acid	50-mg/100 ml
	Thiamine HCl	50 mg/100 ml
	Pyridoxine HCl	10 mg/100 ml

ANNEXURE II

Nutrient Agar media (After Microbiology, a Laboratory Manual by Cappuccino J.G. and Sherman N. (4th edition)

Composition	**g/l**
Agar	15.0
Peptone	5.0
NaCl	5.0
Yeast extract	2.0
Beef extract	1.0

Yeast Peptone Dextrose media (After Microbiology, a Laboratory Manual by Cappuccino J.G. and Sherman N. (4th edition)

Composition	**g/l**
Yeast extract	10
Peptone	20
Dextrose	20
Agar	1.5 %
pH	7.4 ± 2 at 25 °C

ANNEXURE III

Middlebrook 7H10 Agar with Middlebrook OADC Enrichment (After Handbook of Microbiological media by Atlas R.M., 1993)

Composition	g/l
Agar	15.0
Na_2HPO_4	1.5
KH_2PO_4	1.5
$(NH_4)_2PO_4$	0.5
L-Glutamic acid	0.5
Sodium citrate	0.4
Ferric-ammonium citrate	0.04
$MgSO_4.7H_2O$	0.025
$ZnSO_4.7H_2O$	1.0
$CuSO_4.5H_2O$	1.0
Pyridoxine	1.0
Biotin	0.5
$CaCl_2.2H_2O$	0.5
Malachite green	0.25
Middlebrook OADC Enrichment	100.0
Glycerol	5.0
pH	6.6 ± 0.2 at 25 °C

Middlebrook OADC Enrichment

Composition	**g/100ml**
Bovine albumin fraction V	5.0
Glucose	2.0
NaCl	0.85
Oleic acid	0.05
Catalase	4.0

ANNEXURE IV

UV/VIS (Beckman) spectra of *Adhatoda vasica*, *Centella asiatica* and *Asparagus racemosus* from TLC samples

1. Spectra of vasicine showing maximum absorbance (λ_{max}) at 280 nm in

   A Standard

   B Leaf sample of *Adhatoda vasica*

2. Spectra of asiaticoside exhibiting maximum absorbance (λ_{max}) at

   A 227 nm in callus extract of *Centella asiatica*

   B 220 nm in suspension cultured cell extract of *Centella asiatica*

   C 230 nm in leaf extract of *Centella asiatica*

3. Spectra of L-asparagine exhibiting maximum absorbance (λ_{max}) at

   A 255 nm in standard

   B 253 nm in callus extract of *Asparagus racemosus*

   C 253 nm in suspension cultured cell extract of *Asparagus racemosus*

   D 224 nm in leaf extract of *Asparagus racemosus*

   E 225 nm in root extract of *Asparagus racemosus*

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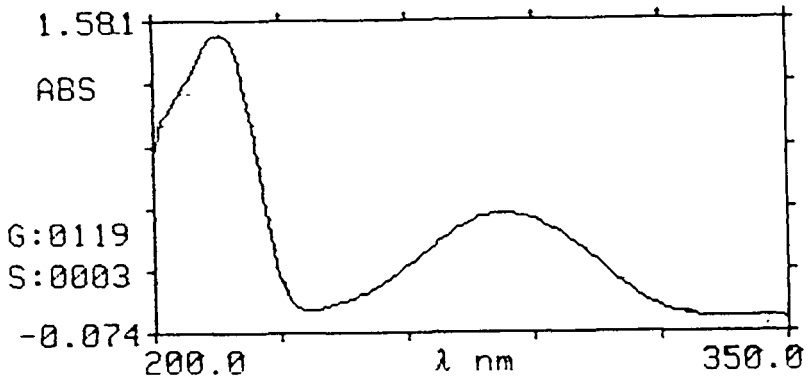

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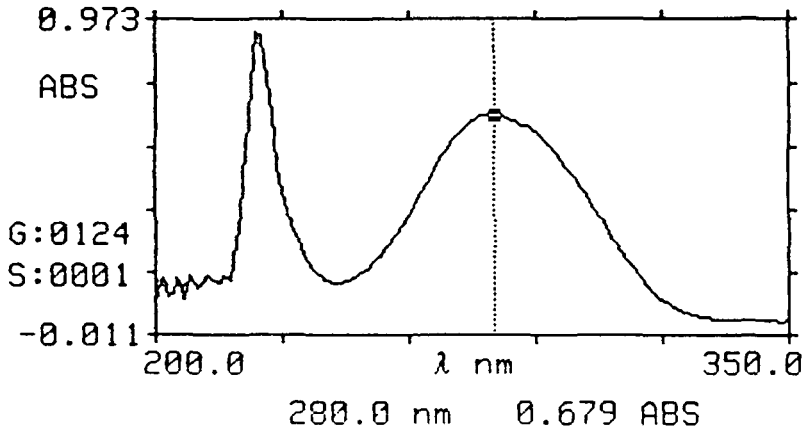

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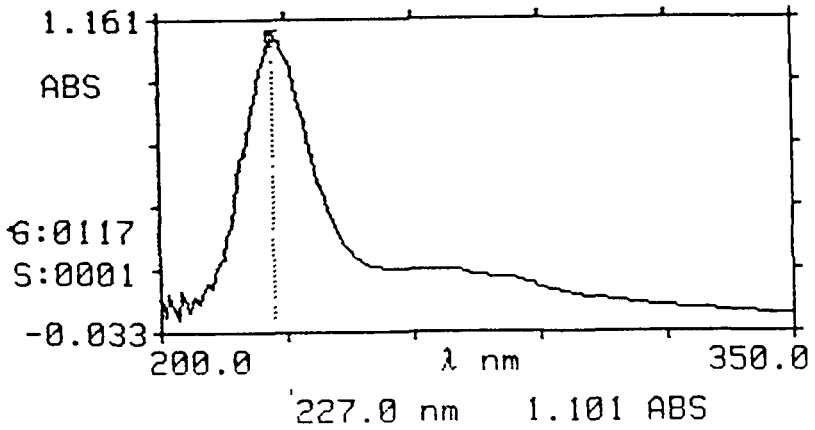

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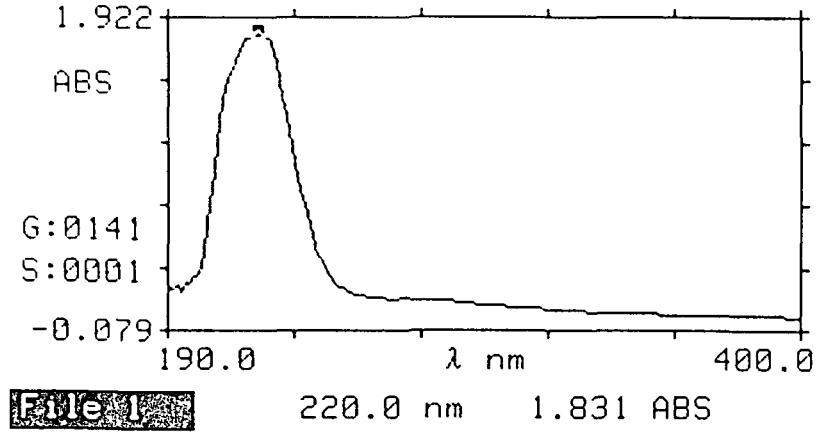

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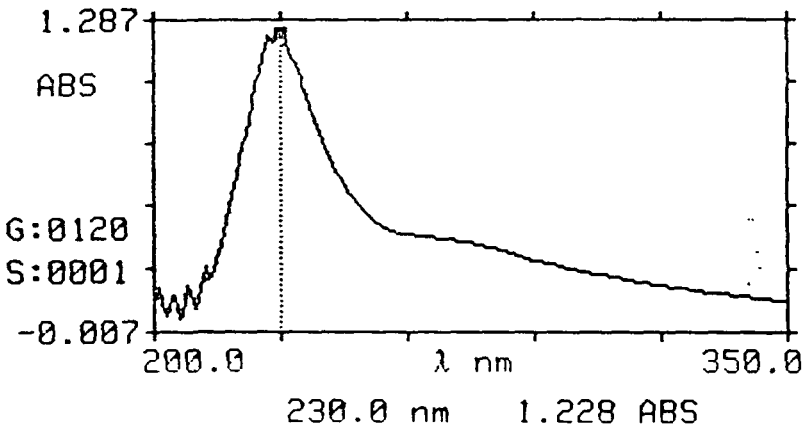

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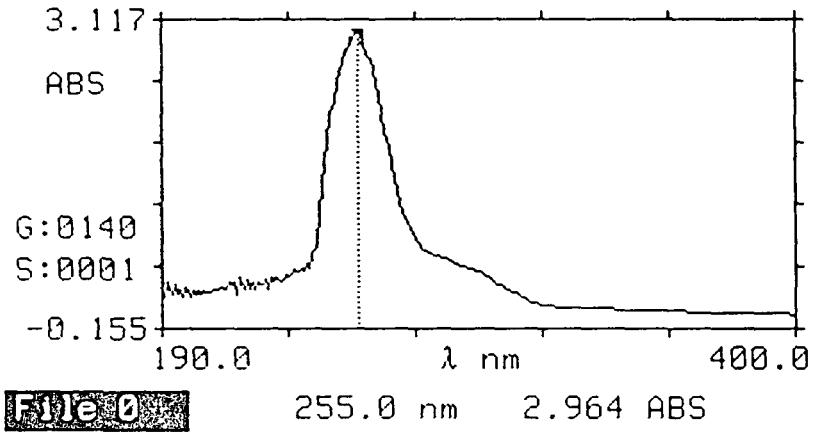

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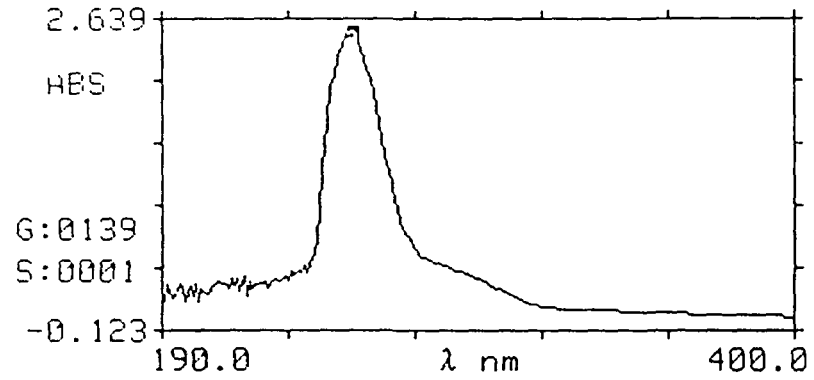

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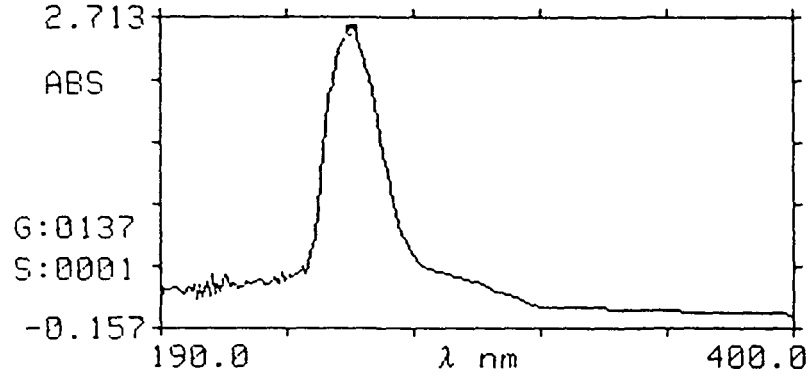

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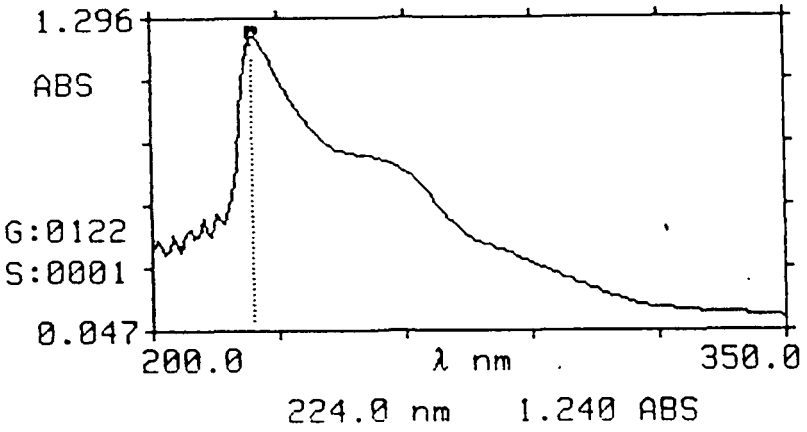

D

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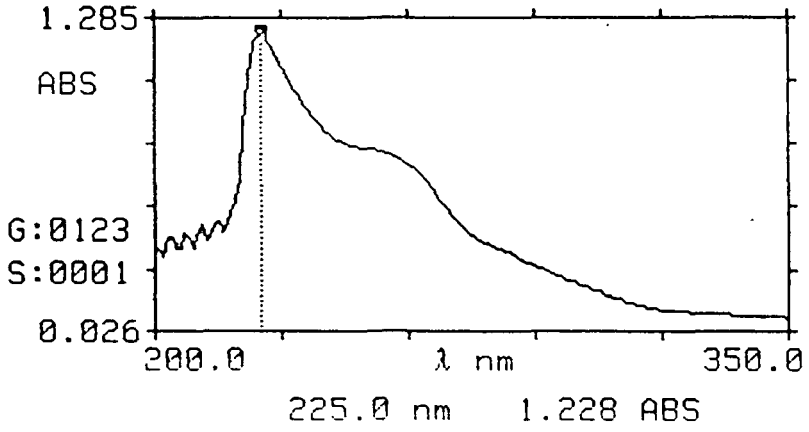
12-MAY-02 12:16:47 SCAN
200-350 nm Step 1.0 nm



E

ANNEXURE V

HPLC (Waters with 2487 Dual λ Absorbance Detector) profiles of *Adhatoda vasica*, *Centella asiatica* and *Asparagus racemosus* for qualitative and quantitative assay of vasicine, asiaticoside and L-asparagine

1. HPLC profile of (A) vasicine (standard) and (B) vasicine from leaf sample of *Adhatoda vasica* at 280 nm wavelength showing retention time of 1.105 min and 1.044 min respectively.

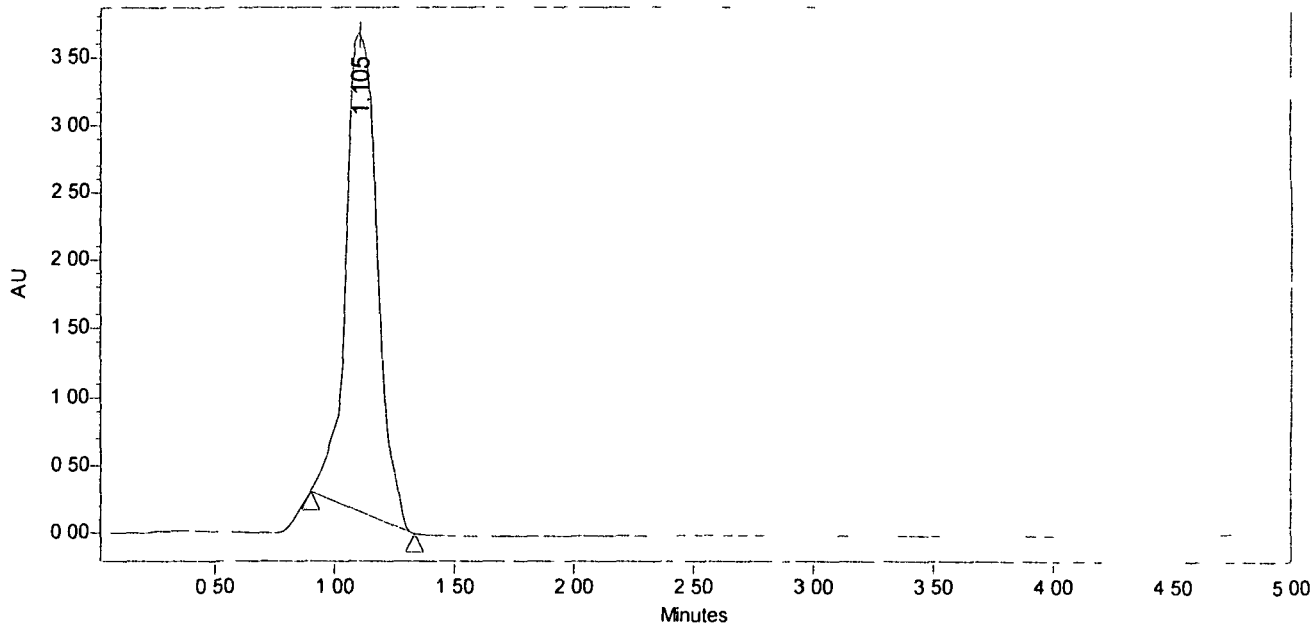
2. HPLC profile of asiaticoside in (A) *in vitro* regenerated leaf (B) callus tissue and (C) suspension cultured cells of *Centella asiatica* using C_{18} column at 220 nm absorbtion mode showing retention time of 0.954 min ,1.072 min and 1.015 min respectively.

3. HPLC profile of (A)L-asparagine (Standard) (B) *in vitro* regenerated leaf (C) root (D) callus tissue and (E) suspension cultured cells of *Asparagus racemosus* using C_{18} column at 225 nm wavelength showing a retention time of 1.338 min, 1.361 min, 1.367 min, 1.212 min and 1.305 min respectively.

Sample Information

SampleName	Vas-std5	Sample Type	Unknown
Vial	1	Date Acquired	5/31/02 12 43 38 PM
Injection	8	Acq Method Set	test1
Injection Volume	20 00 ul	Processing Method	Vas_std5prntout
Channel	2487Channel 2	Date Processed	4/1/03 8 06 13 PM
Run Time	5 0 Minutes		

Auto-Scaled Chromatogram



User K K Hazarika

Software Version 3 05 01

Peak Results

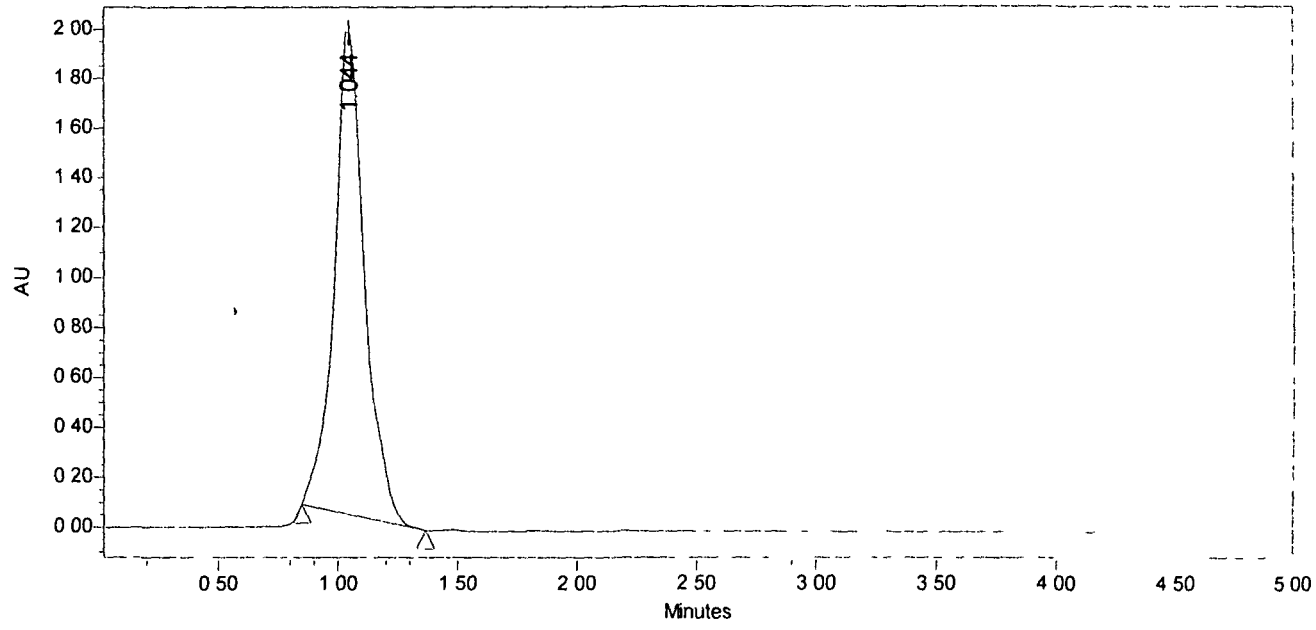
	Name	RT	Area	Height	Amount	Units
1		1 105	32496796	3545990		

A

Sample Information

SampleName	Vas-samp1	Sample Type	Unknown
Vial	1	Date Acquired	5/31/02 12 54 12 PM
Injection	11	Acq Method Set	test1
Injection Volume	20 00 ul	Processing Method	Vas_samp1printout
Channel	2487Channel 2	Date Processed	4/1/03 8 02 32 PM
Run Time	5 0 Minutes		

Auto-Scaled Chromatogram



User K K Hazarika

Software Version 3 05 01

Peak Results

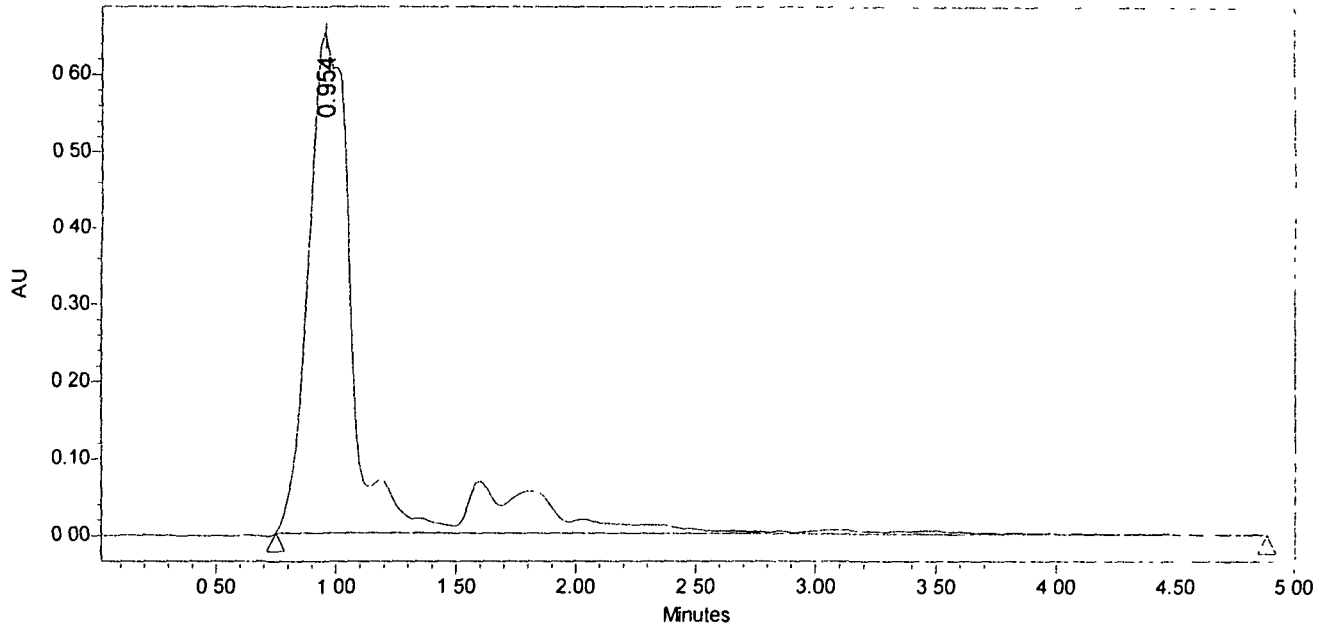
	Name	RT	Area	Height	Amount	Units
1		1 044	17033090	1961635		

B

Sample Information

SampleName	Asiaticoside_L3	Sample Type	Unknown
Vial	1	Date Acquired	7/11/02 11 53 33 AM
Injection	6	Acq Method Set	L_Asp
Injection Volume	15.00 ul	Processing Method	Asiaticoside_L3printout
Channel	2487Channel 1	Date Processed	4/1/03 8:11:36 PM
Run Time	5.0 Minutes		

Auto-Scaled Chromatogram



Software Version 3 05.01

Peak Results

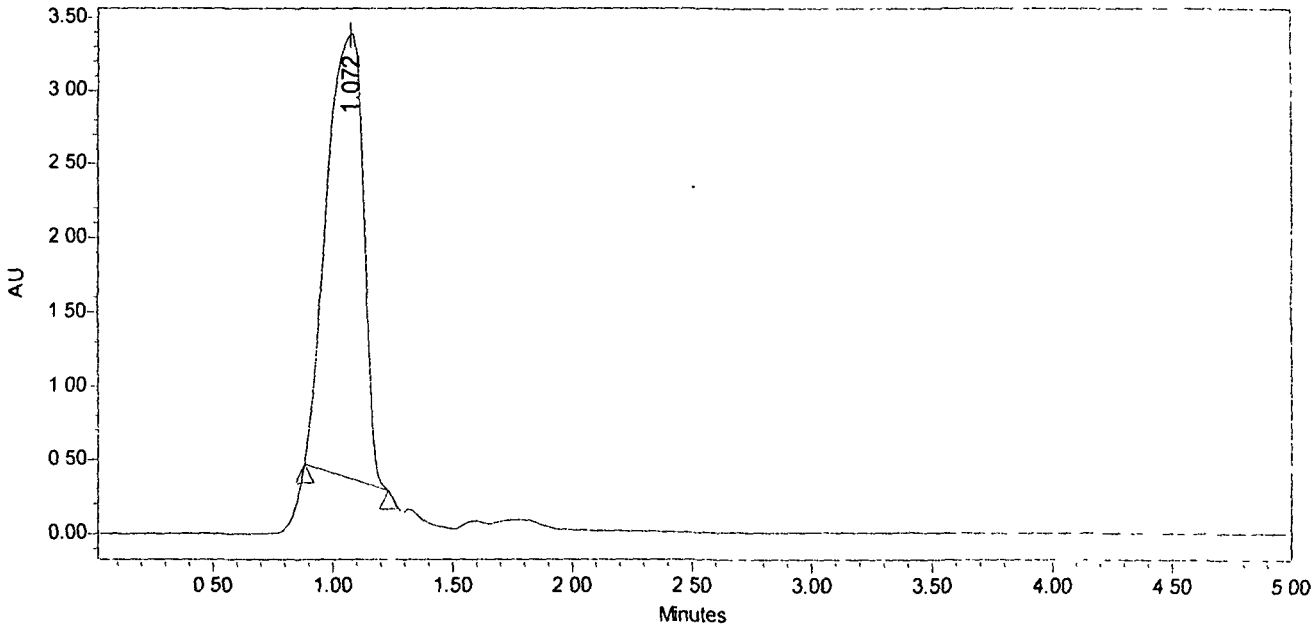
	Name	RT	Area	Height	Amount	Units
1		0 954	9808811	649769		

A

Sample Information

SampleName	Asiaticoside_C2	Sample Type	Unknown
Vial	1	Date Acquired	7/11/02 1:31:57 PM
Injection	10	Acq Method Set	L_Asp
Injection Volume	15.00 ul	Processing Method	Asiaticoside_C2printout
Channel	2487Channel 1	Date Processed	4/1/03 8:14:50 PM
Run Time	5.0 Minutes		

Auto-Scaled Chromatogram



Software Version 3 05 01

Peak Results

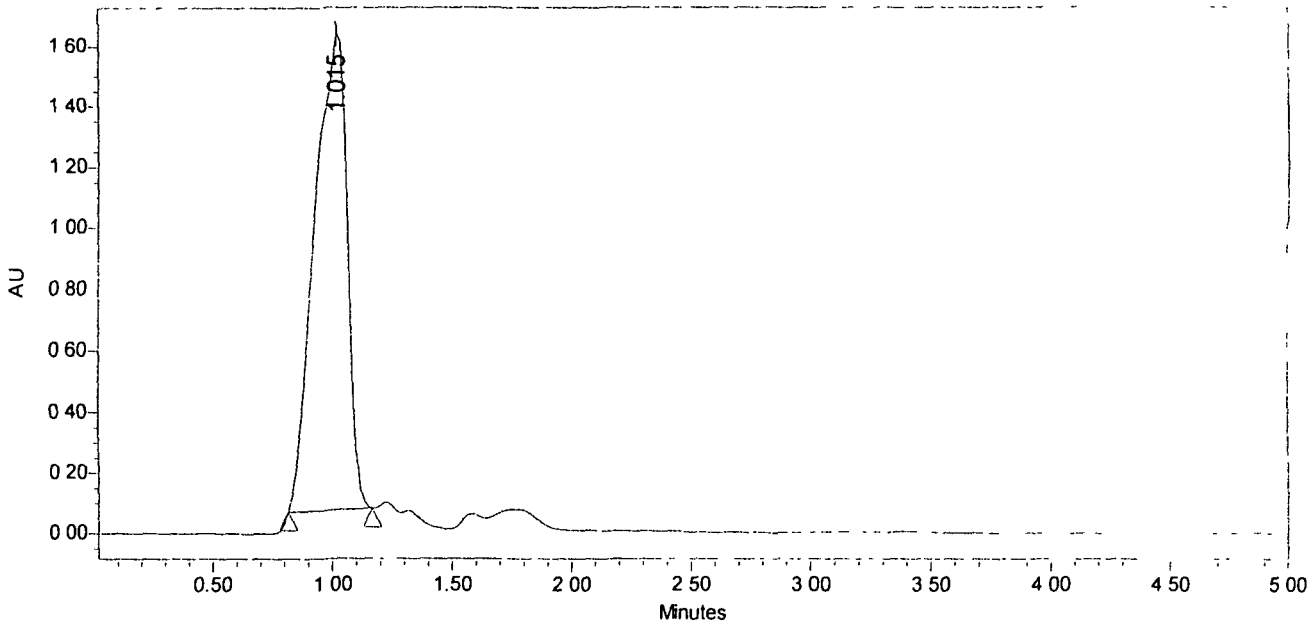
	Name	RT	Area	Height	Amount	Units
1		1 072	31693980	3057124		

B

Sample Information

SampleName	Asiaticoside_S1	Sample Type	Unknown
Vial	1	Date Acquired	7/11/02 1 40 45 PM
Injection	11	Acq Method Set	L_Asp
Injection Volume	15.00 ul	Processing Method	Asiaticoside_S1printout
Channel	2487Channel 1	Date Processed	4/1/03 8:16.32 PM
Run Time	5.0 Minutes		

Auto-Scaled Chromatogram



User K.K.Hazarika

Software Version 3.05.01

Peak Results

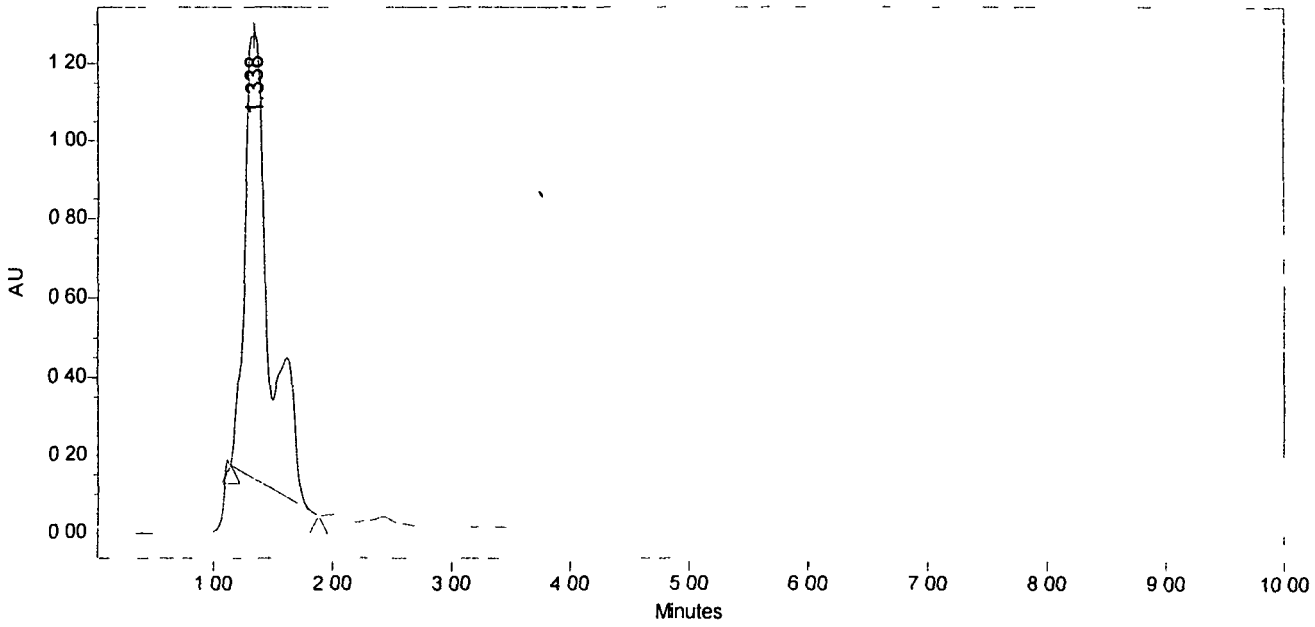
	Name	RT	Area	Height	Amount	Units
1		1 015	14740363	1565934		

C

Sample Information

SampleName	LAsp_Std1	Sample Type	Unknown
Vial	1	Date Acquired	7/16/02 11 40 51 AM
Injection	3	Acq Method Set	L_Asp
Injection Volume	15 00 ul	Processing Method	LAsp_Std1printout
Channel	2487Channel 1	Date Processed	3/31/03 2 55 26 PM
Run Time	10 0 Minutes		

Auto-Scaled Chromatogram



User K K Hazarika

Software Version 3 05 01

Peak Results

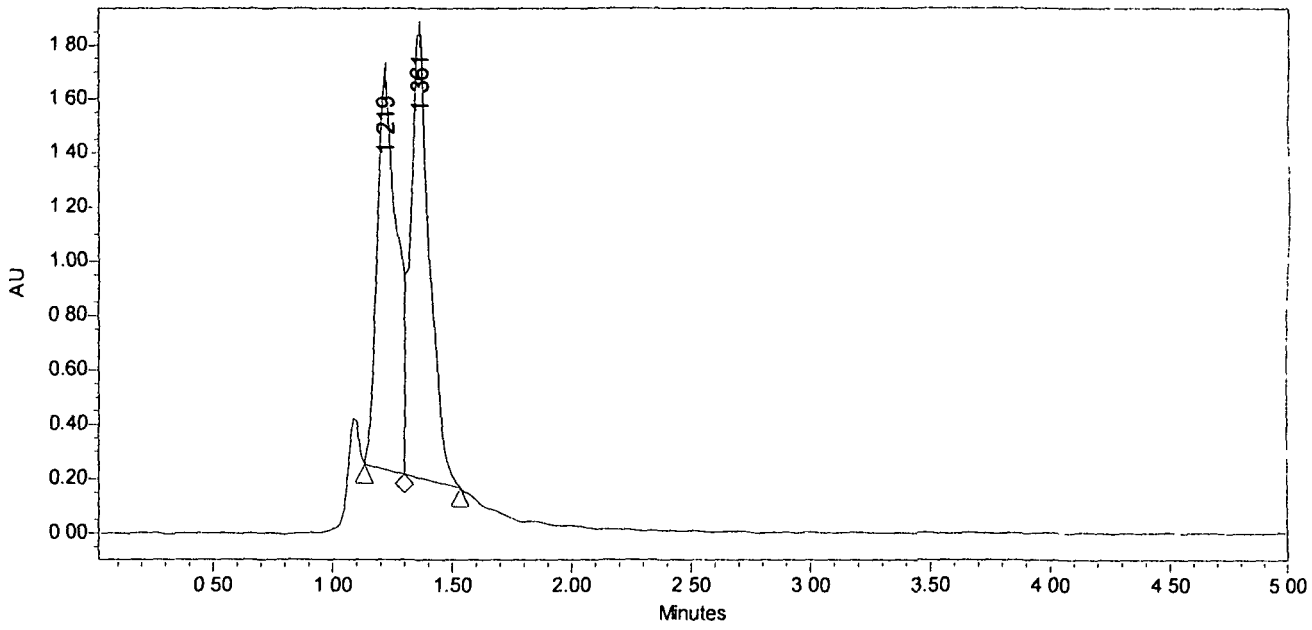
	Name	RT	Area	Height	Amount	Units
1		1 338	16272347	1147183		

A

Sample Information

SampleName	L_asp samLf1	Sample Type	Unknown
Vial	1	Date Acquired	6/4/02 4:30.32 PM
Injection	4	Acq Method Set	L_Asp
Injection Volume	15.00 ul	Processing Method	L_aspsamLf1printout
Channel	2487Channel 1	Date Processed	3/31/03 4:12:18 PM
Run Time	5.0 Minutes		

Auto-Scaled Chromatogram



User : K.K.Hazarika

Software Version 3.05.01

Peak Results

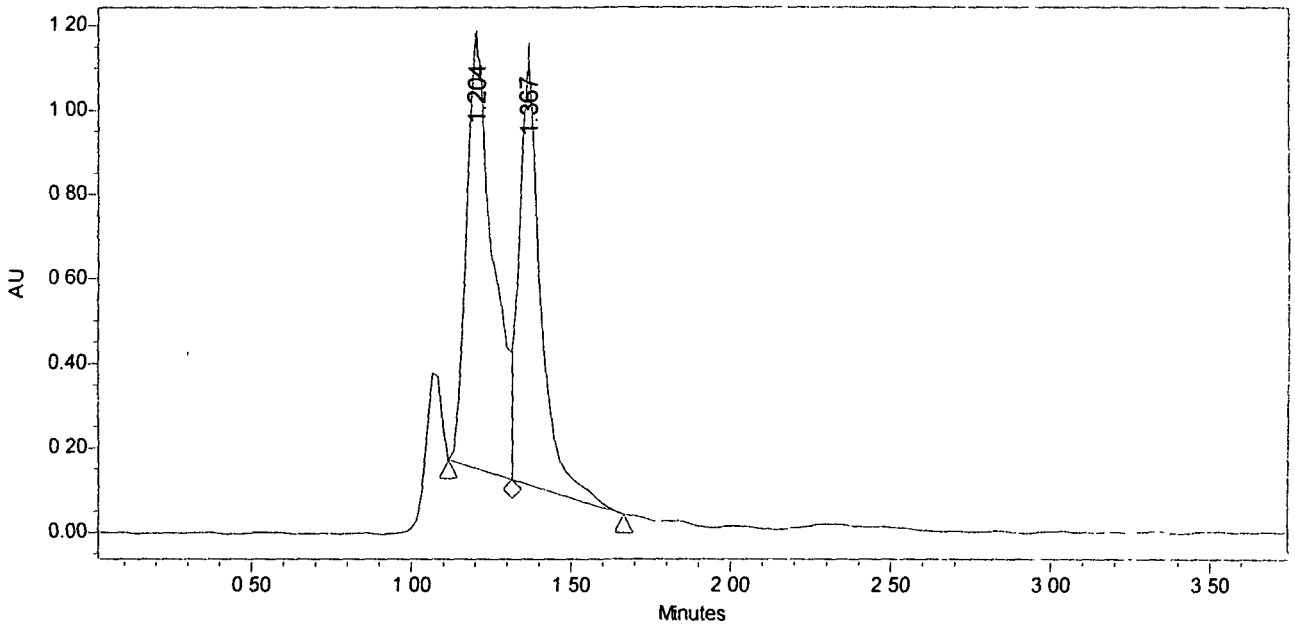
	Name	RT	Area	Height	Amount	Units
1		1 219	8307308	1444133		
2		1 361	9114659	1606947		

B

Sample Information

SampleName	L_asp sam1	Sample Type	Unknown
Vial	1	Date Acquired	6/4/02 4:08:44 PM
Injection	2	Acq Method Set	L_Asp
Injection Volume	15.00 ul	Processing Method	L_asp sam1printout
Channel	2487Channel 1	Date Processed	4/1/03 7 59:34 PM
Run Time	10.0 Minutes		

Auto-Scaled Chromatogram



User · K.K.Hazarika

Software Version 3.05 01

Peak Results

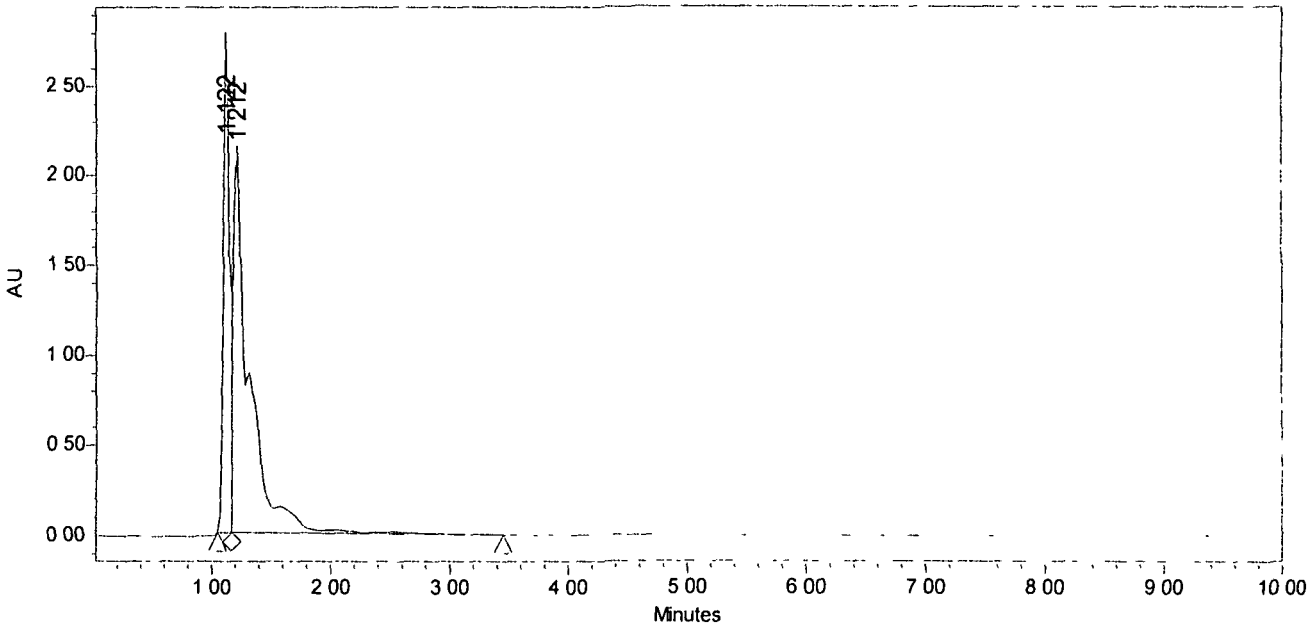
	Name	RT	Area	Height	Amount	Units
1		1 204	5913003	1007711		
2		1 367	4779164	968332		

C

Sample Information

SampleName	LAsp_Cal1	Sample Type	Unknown
Vial	1	Date Acquired	7/16/02 11.02.22 AM
Injection	1	Acq Method Set	L_Asp
Injection Volume	15.00 ul	Processing Method	LAsp_Cal1printout
Channel	2487Channel 1	Date Processed	3/31/03 3:31:58 PM
Run Time	10.0 Minutes		

Auto-Scaled Chromatogram



User : K.K.Hazarika

Software Version 3.05 01

Peak Results

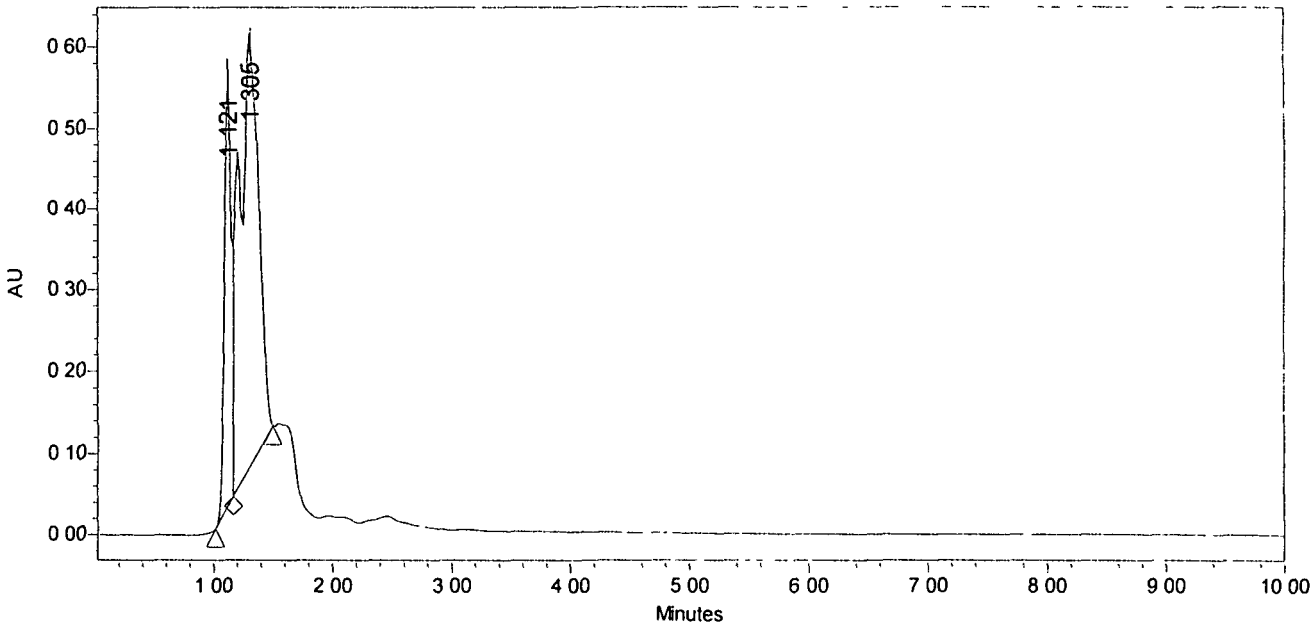
	Name	RT	Area	Height	Amount	Units
1		1.122	9840572	2655037		
2		1 212	20150250	2073491		

D

Sample Information

SampleName	LAsp_Sus1	Sample Type	Unknown
Vial	1	Date Acquired	7/16/02 11 29 09 AM
Injection	2	Acq Method Set	L_Asp
Injection Volume	15.00 ul	Processing Method	LAsp_Sus1pnntout
Channel	2487Channel 1	Date Processed	3/31/03 3 31 12 PM
Run Time	10 0 Minutes		

Auto-Scaled Chromatogram



User . K.K.Hazarika

Software Version 3.05 01

Peak Results

	Name	RT`	Area	Height	Amount	Units
1		1 121	2137056	530524		
2		1 305	6072651	531846		

E

Reprints of the published work from the Ph.D Thesis

J Plant Biochemistry & Biotechnology Vol 12, 167-169, July 2003

Short Communication

In Vitro Method for Propagation of *Centella asiatica* (L) Urban by Shoot Tip Culture

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A rapid clonal propagation system has been developed for the medicinally important herb *Centella asiatica* (L) Urban by shoot tip (2-3 cm long) culture. The shoot tips isolated from mature plants were inoculated on MS medium incorporated with BA alone or in combination with NAA and Kn. The optimum number of shoots (3.38) with optimum number of leaves per shoot (4.25) were attained on MS medium supplemented with 4.0 mg l^{-1} BA and 0.1 mg l^{-1} NAA. On transferring the microshoots on full strength MS medium supplemented with various concentrations of IBA (1.0-3.0 mg l^{-1}) and NAA (0.5-2.0 mg l^{-1}), profuse rooting (46.8 per shoot) was obtained in MS basal medium with 2.0 mg l^{-1} IBA with root length of 19.7 cm. Well rooted plantlets were acclimatized successfully by adjusting the temperature and humidity for 3-4 weeks after transfer to pots filled with sterilized vermiculite soil : sand (1:1)mixture. This micropropgation protocol could be useful for raising a stock of genetically homogenous material for field cultivation within a very short period.

Key words: *Centella asiatica,* shoot tip, clonal propagation, Mandookaparni

Centella asiatica (L) Urban is a member of Apiaceae family, commonly known in India as 'Indian pennywort' or 'Mandookaparni' In traditional system of Indian medicine, *Centella asiatica* is a nervine tonic and is used in the treatment of leprosy, asthma, bronchitis, dropsy, leucorrhea, skin disease and urethritis (1) The wound healing activity of the species is ascribed to a triterpenoid saponin, asiaticoside while, antitumour properties are attributed to madecassic acid *Centella* extracts have antitumour activity *in vitro* and *in vivo* (2)

Considering its medicinal properties and overexploitation from natural population the requirement for application of tissue culture techniques in the rapid multiplication of elite clones and germplasm conservation is a crucial prerequisite Moreover, a stable supply of the bioactive secondary products has become a utmost priority The present study was therefore, attempted to reproduce a rapid method for multiplication of the species by shoot tip culture to meet the requirement of the pharmaceutical industries Earlier regeneration has been derived from leaf derived callus (3,4), stem segments (3) and nodal segments of *Centella asiatica* (5)

Plants of *Centella asiatica* were collected from in and around the Tezpur University Campus, Napaam The plants were washed throroughly under running tap water for 30 min The shoot tips (2-3 cm long) were then excised with a sharp razor and soaked in a beaker with detergent solution (1% Teepol) for about 30 min and washed thoroughly under running tap water Surface sterilization of the shoot tips was done asceptically with 0 01% $HgCl_2$ (Himedia India) for 5 min followed by a final 3-4 rinses with sterile double distilled water The explants were cultured on MS (6) medium supplemented with BA alone or in combination with NAA and Kn (Table 1) for shoot regeneration and various IBA/NAA combinations in full strength MS medium for root differentiation (Table 2) The cultures were incubated at 25 ± 2°C under 16 h photoperiod of 3000 lux intensity and 55-60% RH for shooting and under completely dark condition for rooting. Rooted plantlets were transferred to paper cups containing autoclaved vermiculite soil·sand (1 1) The plantlets were irrigated with MS basal liquid media devoid of Myo-inositol and sucrose at every 3 day interval To maintain the humidity the pots were kept covered with polythene bags and maintained under 16 h photoperiod of 3000 lux intensity at 25 ± 2°C for 4 weeks before it was transferred to the field

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Abbreviations MS, Murashige and Skoog, NAA, α-Naphthalene acetic acid, Kn, Kinetin, BA, Benzyladenine, BAP, 6-benzylamino-purine, IBA, Indole-3-butyric acid, 2,4-D, 2,4- dihydrophenoxy-acetic acid, RH, relative humidity, RBD, Randomised Block Design, ANOVA, Analysis of Variance, DMRT, Duncan Multiple Range Test

Data were analysed following Randomised Block Design (RBD) Each mean was based on eight replicates repeated five times each The analysis of variance (ANOVA) appropriated for the design was carried out to detect the significance of differences among the treatment means The treatment means were compared using Duncan Multiple Range Test (DMRT) at 5 % probability level according to Gomez and Gomez (7)

Shoot regeneration from *Centella asiatica* using shoot tip explants were carried out in MS medium supplemented with various concentration of BA alone or in combination with NAA and Kn (Table 1) Analysis of variance revealed significant effect of treatments($p<0.05$) for number of shoots per responding explant, mean shoot length and average number of leaves per shoot Shoot tips cultured in MS medium with BA alone showed increased shoot length though the frequency of shoots per responding explant and average number of leaves is more in cultures with BA (4 0 mg l^{-1}) and NAA (0 1 mg l^{-1}) An increase in the level of BA and NAA was found to promote callus formation as was previously found by Banerjee *et al* (4) when leaf was used as explant for shoot regeneration Even Kn (4.0 mg l^{-1}) in combination with NAA (0 10 mg l^{-1}) have marked influence on shoot length and average number of leaves per shoot, though the number of shoots was less in comparison to

Table 1. Analysis of variance and comparison by DMRT of the effects of BA, NAA and Kn concentrations on the number of shoots, mean shoot length and average number of leaves per shoot in *Centella asiatica*

Plant growth regulators (mg l^{-1})			Mean		
BA	NAA	Kn	Number of shoots per responding explants	Shoot length (mm)	Number of leaves per shoot
1 0	0 0	0 0	3 25^{a}	0 98^{a-}	3 25^{ab}
2 0	0 05	0 0	1 50^{b}	0 72^{ab}	2 00^{cd}
3 0	0 05	0 0	2 50^{a}	0 51^{b}	2 38^{bc}
4 0	0 10	0 0	3 38^{a}	0 67^{ab}	4 25^{a}
0 0	0 0	1 0	1 00^{b}	0 57^{ab}	1 38^{cde}
0 0	0 10	2 0	0 75^{b}	0 44^{bc}	1 13de
0 0	0 10	3 0	0 63^{b}	0 08^{c}	1 13de
0 0	0 10	4 0	1 38^{b}	0 71^{ab}	3 63^{a}

Each mean is based on eight replicates each of which is repeated five times Data recorded after four weeks of culture initiations Each treatment mean followed by the same letter were not significantly different from each other ($p<0.05$) according to the Duncan Multiple Range Test (DMRT)

BA and NAA The superiority of BA over Kn in multiple shoot induction was also reported in a number of medicinal plants (8,9) A comparison of means by DMRT revealed that the optimum number of shoots (3 38) with optimum number of leaves per shoot (4 25) was attained on MS medium containing 4 0 mg l^{-1} BA and 0 1mg l^{-1} NAA (Fig 1 A,B) Shoots with 3-4 nodes were subcultured every four weeks with a combination of 4 0 mg l^{-1} BA and 0 1mg l^{-1} NAA (Fig 1 C) Elongated shoots of 6-7 cm in length were separated and cultured individually on full strength MS medium supplemented with various concentrations of IBA (1 0-3 0 mg l^{-1}) and NAA(0 5-2 0 mg l^{-1}) alone (Table 2)

In the present investigation micro-shoots were directly transferred to rooting media No separate media was needed for shoot elongation by which plantlet formation could be achieved in two steps only Even shoots induced from nodal explants on the medium containing optimum BA and NAA medium failed to elongate rapidly and required a transfer to MS media supplemented with low level of BA (5) Analysis of variance showed significant effect on

Table 2. Analysis of variance and comparison by DMRT of the effects of IBA and NAA concentrations on the number of roots per shoot and mean root length in *Centella asiatica*

Plant growth regulator (mg l^{-1})		Mean	
IBA	NAA	No of roots per shoot	Root length (cm)
1 0	0	9 4^{b}	12 5^{b}
2 0	0	46 8^{a}	19 7^{a}
3 0	0	4 0^{b}	12 5^{b}
0	1 0	7 4^{b}	6 5^{c}
0	2 0	0 2^{b}	1 0^{d}
0	0 5	0 2^{b}	4 5^{c}
0	0	4 8^{b}	10 5^{b}

Each mean is based on five replicates each of which is repeated five times Data recorded after four weeks of transfer into the rooting media Treatment means followed by the same letter were not significantly different from each other($p<0.05$) according to the Duncan Multiple Range Test (DMRT)

number of roots per shoot and mean root length Profuse rooting was obtained in full strength MS medium supplemented with 2 mg l^{-1} IBA (Fig 1 D) MS basal medium also showed minimum number of roots per shoot (4 8) with a mean root length of 10 5 cm A comparison by DMRT revealed optimum number of roots per shoot (46 8) and mean root length (19 7cm) in MS medium containing

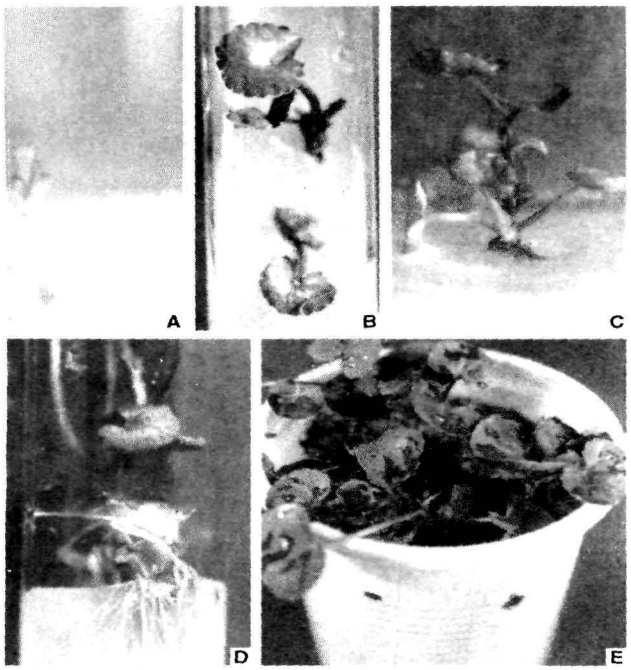

Fig.1. Micropropagation of *Centella asiatica* using shoot tip explants.(**A**) Shoot tips cultured in MS medium supplemented with 4.0 mg l^{-1} BA and 0.1 mg l^{-1} NAA, (**B-C**) Regeneration of axillary shoots from shoot tips after 14 and 28 days of inoculation respectively, (**D**) *In vitro* rooted plantlet in MS media containing 2.0 mg l^{-1} IBA, and (**E**) *In vitro* regenerated plantlet transferred into a plastic cup.

2 mg l^{-1} BA. Banerjee *et al* (4) and Tiwari *et al* (5) also found promontory effect of IBA in rooting in *Centella asiatica,* while IAA and low level of sucrose was reported optimum by Patra *et al* (3). The rooted plants were transferred from culture tubes into the plastic cups (Fig. 1 E) containing vermiculite soil:sand in 1:1 ratio. The acclimatized plantlets were successfully established in field with 1-2% mortality rate.

Shoot tips have been a source material for obtaining virus-free and genetically aberrant plants. Plantlet formation from shoot tip explants has been described by several workers in different species (10,11). The use of shoot tips as explants has overcome the problem of fungal and bacterial contamination in the present investigation which was a real hindrance when nodes were used as explants for micropropagation. Pretreatment of the nodal explants in a solution of systemic fungicide (bavistin) and antibiotic (trimethoprim) was done to get 80 % cultures free from contamination (5) which was not necessary in the present work where shoot tip was used as explants. Thus, the protocol described in this paper could be useful for large scale multiplication of this important medicinal herb within a short period and also will prove a useful method for germplasm conservation.

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