In-vitro plant regeneration and comparative studies of secondary metabolite from non-transformed and transformed plants of *Andrographis paniculata* *Wall.Ex Nees*.

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BY

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2013

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A parents love is whole no matter how many times divided.

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CERTIFICATE-I

This is to certify that the thesis entitled **"*In-vitro* plant regeneration and comparative studies of secondary metabolite from non-transformed and transformed plants of *Andrographis paniculata Wall.Ex Nees.*"** submitted in partial fulfilment of the requirements for the award of the Degree of **Master of Science in Agricultural Biotechnology** to the Orissa University of Agriculture and Technology, Bhubaneswar is an authentic record of bonafide research work carried out by **Ms. Thorat Shital Bansi (05 ABT/11)** under my guidance and supervision. No part of the thesis has been submitted for any other degree or diploma or published in any other form. All sorts of help and sources of information availed during this investigation have been duly acknowledged.

GRRout

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Place: Bhubaneswar
Date: 19.08.13

CERTIFICATE-II

This is to certify that the thesis entitled "***In-vitro* plant regeneration and comparative studies of secondary metabolite from non-transformed and transformed plants of *Andrographis paniculata Wall.Ex Nees*.**" submitted by **Ms. Thorat Shital Bansi** to the Orissa University of Agriculture and Technology, in partial fulfilment of the requirements for the degree of **Master of Science in (Agricultural Biotechnology)** has been approved by the student's advisory committee after an oral examination in collaboration with external examiner.

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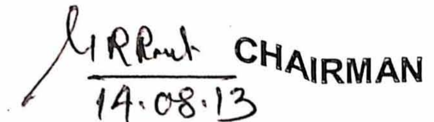

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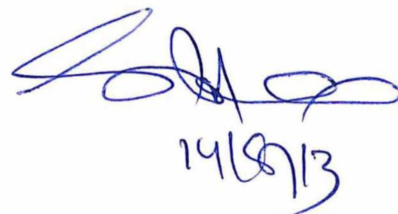

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ABSTRACT

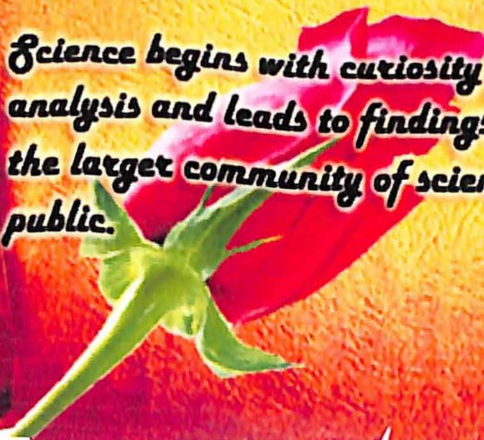
Medicinal and Aromatic plants form a numerically large group of economically important plants which provide basic raw materials for medicines, perfumes, flavours and cosmetics. These plants and their products not only serve as valuable source of income for small holders and entrepreneurs but also help the country to earn valuable foreign exchange by way of export. Medicinal plants are those plants which are rich in secondary-metabolites and arc potential source of drugs. These secondary metabolites include alkaloids, glycosides, coumarins, flavonoids, steroids etc. Medicinal plant like *Andrographis paniculata* family *Acanthaceae* which is rich in secondary metabolite andrographolide, which possess antipyretic, antidiabitic ,analgesic, antihepatotoxic, antimalarial, antibacterial, antifertility, anti- inflammatory ,immunosuppressive and treatments of many diseases including HIV, AIDS, immune disorders. It is also known as "King of Bitters". The aim of the present study was to enhance the secondary metabolite content in the plants by using *Agrobacterium*-mediated transformation and compare the andrographolide content in transformed and untransformed tissues. *Agrobacterium rhizogenes*, a soil pathogen which elicits adventitious, genetically (*Ri* T-DNA) transformed roots are used for these purpose.

Apical meristems were isolated from *in vitro* raised seedlings and cultured aseptically for induction of multiple shoots. Amongst the various cytokinins and auxins tested, BAP in combination with NAA proved to be the most effective. Shoot multiplication and proliferation was achieved on MS (Murashige and Skoog, 1962) medium supplemented with 2.5 mg/l 6-benzylaminopurine (BAP), 1.0 mg/l NAA (1-napthalene acetic acid), and 100 mg/l adenine sulphate with 3 % (w/v) sucrose within 4-6 weeks of culture. Compact and white friable calli were developed from stem and leaf explants of in vitro seedlings on MS medium supplemented with either 3.0 mg/l NAA or 2,4-D in combination with 1.0 mg/l BAP, GA_3 0.1 mg/l within 4 weeks of culture. High frequency shoot bud (6.5 shoots per culture) regeneration from leaf & stem derived calli were achieved on MS medium supplemented with 2.5 mg/l BAP and 1.0 mg/l NAA. Inclusion of 100 mg/l adenine sulphate in the medium favoured higher rate of shoot multiplication and elongation. Significant differences were recorded in terms of average number of shoots per explants and different concentration of BAP and NAA. Sub-culturing was made every 6 weeks interval to enhance the production of multiple shoots. Elongated shoots were isolated and cultured on ½ strength MS medium supplemented with 0.25 – 0.5 mg/l NAA or IBA (0.5 – 1.5 mg/l) for induction of rooting. About 60% of excised shoots were rooted within 3 weeks of culture. This protocol will helpful for mass propagation as well as *in vitro* conservation. Wild strain of *Agrobacterium rhizogenes* 532 (MTCC) harbouring Root inducing plasmid (Ri- plasmid) was used for *in vitro* transformation. Various factors influencing transformation such as duration of co-cultivation (48 hrs) and infection time (20 minutes) and inhibition of growth of *Agrobacterium rhizogenes* (400 mg/l carbenicillin) and bacterial inoculum density (0.6 O.D at 620 nm wavelength) were optimised. Crude plant extract derived from both control and transformed plants were prepared by soxhlet extraction. The comparison between transformed and untransformed plants was also done with the help of HPTLC method. The percentage of increase in andrographolide *content* in transformed callus was observed as 2.6%. The antimicrobial activity was tested against different bacteria and fungi by using crude extract with positive results.

A thesis is a written record of the work that has been undertaken by a candidate. It constitutes objective evidence of the author's knowledge and capabilities in the field of interest and is therefore a fair means to guage them.

The thesis is therefore not merely a record of technical work, but is also an attempt to communicate it to a larger audience.

Science begins with curiosity follows on with experiment and analysis and leads to findings which are then shared with the larger community of scientists and perhaps even the public.



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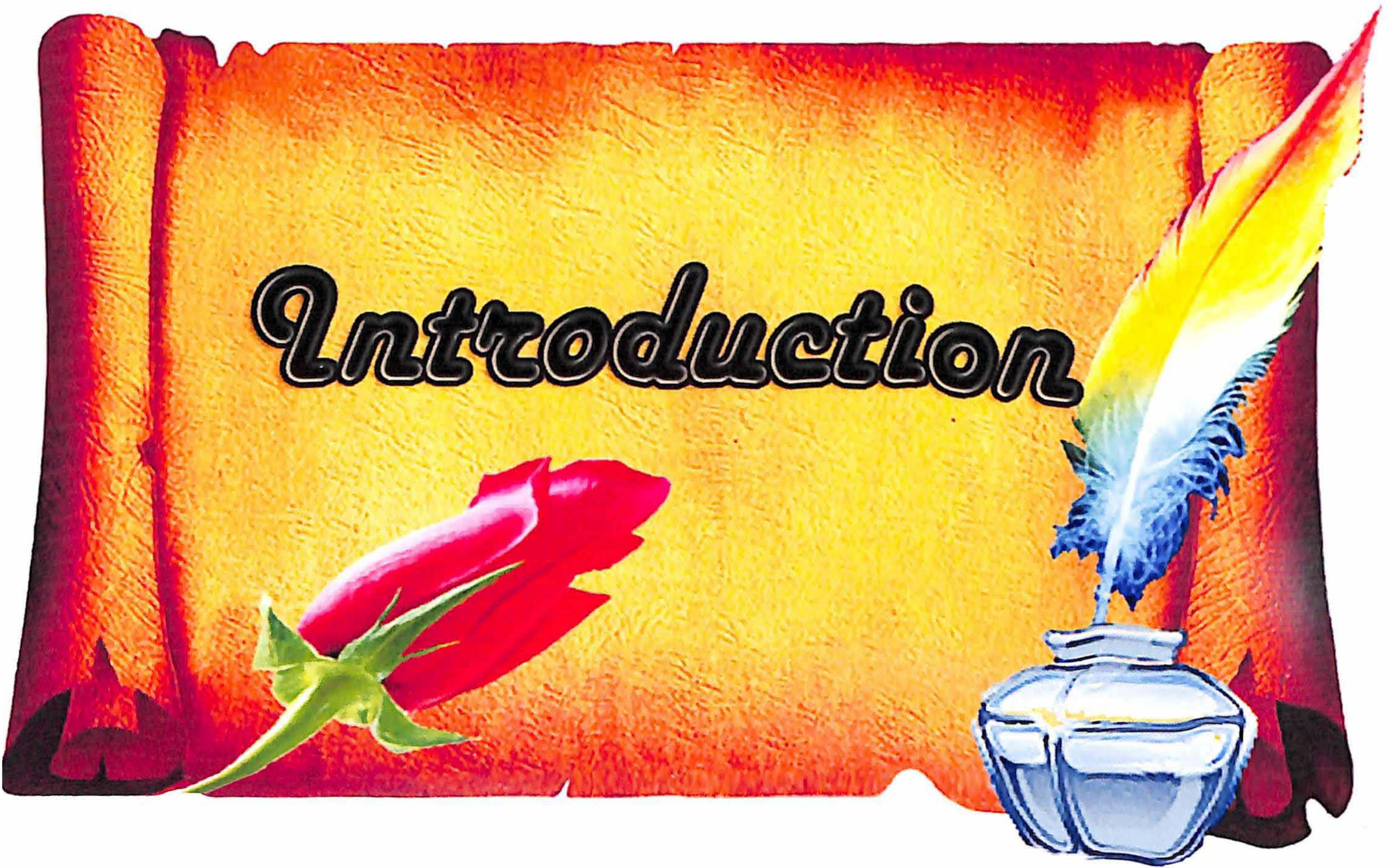
LIST OF ABBREVIATIONS

Abbreviations	Full Name
%	Percentage
μg	microgram(s)
μl	Microliter
μm	Micromole
^{0}C	degree centigrade
2, 4-D	2,4-Dichlorophenoxy acetic acid
Ads	Adenine sulphate
ANOVA	Analysis of variance
AU	Area Unit
AUC	Area Under Curve
BAP	6-benzyl amino purine
Bp	Base pair
$CaCl_2 .2H_2O$	Calcium chloride
CD value	Co-efficient of deviation
cDNA	Complementary deoxyribonucleic acid
cm	centimetre
Conc.	Concentrated / concentration
CTAB	N-Cetyl-N-trimethyl ammonium bromide
$CuSO_4.5H_2O$	
CV	Co-efficient of Variation
d	Day
DCW	Dry cell weight
DDW	Double distilled water
dil.	dilute
DMSO	Dimethyl Sulfoxide
DNA	Deoxy ribose nucleic acid
dw	Dry weight
E. coli	*Escherichia coli*
EDTA	Ethylene Diamine Tetra Acetic Acid
et al	and others
F	Fluorescence
FeSO4.7H2O	Ferrous sulphate
Fig.	Figure
FYM	Farm yard manure
FW	Fresh weight
GA_3	Gibberellic Acid
Gly	Glycine
gm	gram
Ha	Hectors
H_2O_2	Hydrogen peroxide

H_2SO_4	Sulphuric acid
H_3BO_3	Boric acid
HCl	Hydrochloric acid
$HgCl_2$	Mercuric chloride
HPLC	High Performance liquid Chromatography
HPTLC	High Performance Thin layer Chromatography
hrs	hours
IAA	Indole-3-acetic acid
IBA	Indole butyric acid
IR	Infra-Red
kg	kilogram
$KH_2 PO_4$	Di-potassium hydrogen phosphate
KI	Potassium iodine
KN	Kinetin
KNO_3	Potassium nitrate
l	liter/liter(s)
mM	Millimolar/molar
MBC	Minimum bacterial concentration
MAPs	Medicinal and aromatic plants
MeOH	Methanol
mg/l	milligram per litter
$MgSO_4$ .7H2O	Magnesium sulphate
MIC	Minimum inhibitory concentration
min	Minute/minutes
ml	milliliter
mm	millimeter
$MnSO_4$.7H2O	Manganese sulphate
MS	Murashige and Skoog's, 1962
MTCC	Microbial type cell culture
MW	Molecular weight
N	Normal
NA	Nutrient Agar
NAA	α-naphthalene acetic acid
NaCl	Sodium chloride
NaOH	Sodium hydroxide
NB	Nutrient broth
ng	nanogram
OD	Optical density
OUAT	Orissa university of Agriculture and Technology
PCR	Polymerase chain reaction
PDA	Potato dextrose agar
PDB	Potato dextrose broth
pH	Hydrogen ion concentration
ppm	parts per million

Rf	Retardation factor
rpm	Revolution per minute
RNA	Ribose nucleic acid
RT	Retention time
Ri	Root inducing
T	Treatment
T-DNA	Transfer DNA
TAE	Tris acetate ethylene diamine tetra acetic acid
TBE	Tris borate ethylene diamine tetra acetic acid
TDZ	Thidiazuron
TE	Tris - ethylene diamine tetra acetic acid
TLC	Thin Layer Chromatography
Tris	Tris hydroxyl methyl amino methane
UV	Ultraviolet
UV/Vis	Ultraviolet / visible
v	volume
v/v	volume per volume
vir	virulence
w	weight
w/v	Weight per volume
YEMA	Yeast extract mannitol agar
YEMB	Yeast extract mannitol broth

CHAPTER-I



1. INTRODUCTION

1.1 Overview of Medicinal plant

Medicinal plants sector has traditionally occupied an important position in the socio cultural, spiritual and medicinal area of rural and tribal lives. As per World Health Organisation (WHO) estimates, almost 80% of the population of developing countries relies on traditional medicines, mostly plant drugs, for their primary health care needs. The Government of India established the Department of Indian System of Medicine and Homoeopathy, and more recently the Medicinal Plants Board to develop, promote and regulate the sector for maximizing the benefits to the people as well as to ensure sustainable growth. Medicinal plants have been identified as one of the thrust areas by the Ministry and different programmes have been initiated for conservation of medicinal plants found in the forests and protected areas as well as cultivation of these plants in the degraded forest areas. Medicinal plant gaining great interest in pharmaceutical industry for the production of high value secondary metabolite (Rout *et al*; 2000; Das and Rout, 2002). Approximately 6000-7000 species of medicinal plants out of about 17000- 18000 flowering plant are known to be in used in folk and officially recognized system of medicine in India, namely, Ayurveda, Siddha, Unani and Homeopathy. Use of herbal medicines in Asia represents a long history of human interactions with the environment. The earliest reference to use of medicinal herb to cure the disease find in manuscript of 'Eber Papyrus' which is dates back about 16th century B.C. the use of poppy, caster oil, squills, aloe's, etc. are recorded in this book. Number of herbal with medicinal properties appears in the "Athervaveda", "Rigveda", and also in the work of "Charaka" and "Susruta". The importance of medicinal herb was recorded in the Ayurveda part of Athervaveda and even in the old Egyptian, Greek and European literature.

Plants used for traditional medicine contain a wide range of substances that can be used to treat chronic as well as infectious diseases. A vast knowledge of how to use the plants against different illnesses may be expected to have accumulated in areas where the use of plants is still of great importance.

The medicinal value of plants lies in some chemical substances that produce a definite physiological action on the body. The most important of these bioactive compounds of plants are alkaloids, flavonoids, tannins and phenolic compounds (Edeoga, *et al.*, 2005). Rural communities, depend on plant resources mainly for herbal medicines, food, forage, construction of dwellings, making household implements, sleeping mats, and for fire and shade.

During the past decade, traditional systems of medicine have become increasingly important in view of their safety. Current estimates suggest that, in many developing countries, a large proportion of the population relies heavily on traditional practitioners and medicinal plants to meet primary health care needs. Although modern medicine may be available in these countries, herbal medicines (phyto-medicines) have often maintained popularity for historical and cultural reasons. During the course of biological and Cultural Revolution, the value and potential of biodiversity, which encompasses innumerable life forms has been duly recognized by human beings for meeting the basic needs of life such as food, fuel feed, medicines, condiments, clothing and shelter, etc. India is one of the top twelve mega-bio-diversity centres of the world with two hotspots located in the Eastern Himalayas and Western Ghats containing 3,500 and 1,600 endemic species of higher plants, respectively. These medicinal plants are used both for primary health care and for treating chronic diseases such as cancer, hepatic disorders, heart and old age related diseases namely memory loss, osteoporosis, and diabetes. Over 80,000 species of plants are in use throughout the world. In India, more than 95% of the total medicinal plants used in preparing medicines by various industries are harvested from wild. There is a great need to recognise the potential of bioresources at their fullest. 270 medicinal plants belonging to 84 families and 197 genera were recorded. Maximum medicinal plants were reported in the altitudinal zone, 2000-2800 m and decreased with increasing altitude. Out of the total, 162 medicinal plants were native and 98 were endemic to the Himalayan region. The medicinal plants based industry is growing at the rate of 7-15% annually. According to a conservative estimate, the value of medicinal plants related trade in India is to the tune of about Rs 5,000 crores per annum while the world trade is about 62 billion US dollars and is expected to grow to the tune of 5 trillion US dollars by the year 2050. The present

international situation in the production and trade of essential oils and aroma chemicals is very complex and has been rated as third with 16-17% share in terms of production (quantity) and second with 21 to 22% share in value in the world production. The tribal/ethno or folk medicines are still quite prevalent in the remote and far flung areas where above facilities are not available. All these system so medicine are predominantly dependent upon plant drugs and some of these plants are also source of certain clinically useful prescription drugs used in allopathic. The present scenario shows a decline in these traditional, plant-based health-care practices. These age-old practices are conservation oriented and have tremendous potential to uplift the state economy. The excessive extraction of medicinal plant resources for use in the pharmaceutical industry has resulted in ruthless destruction of natural populations of medicinal plants. This work attempts to assess the current status of knowledge of medicinal plant resources. It also focuses on the importance of documenting traditional knowledge and practices related to conservation and sustainable utilization of medicinal plants Traditional health-care, as practiced in the region, consists of two systems, viz. folk medicine and the codified systems of medicine. Folk medicine is ecosystem and ethnic community-specific. Through scientific studies, only about 1% of the total known medicinal plant species (folk, ethno and traditional medicine) is acknowledged to have therapeutic value for human beings.

Table 1.1. Status of traditional medicine in various countries

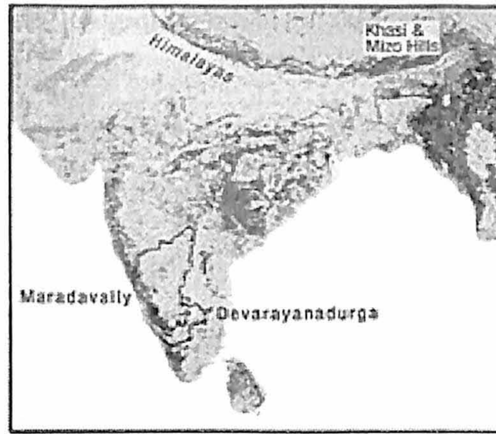
Country/ Region	% Used Traditional Medicine	Forms Of Traditional Medicine Recognized	Regulatory Situation
Africa	70-80%	Africa traditional forms of healing,chiropractic,osteopathy	None in most countries. Some countries such South Africa, Congo, Ethiopia, Zimbabwe etc. have included traditional medicine in legislations governing practice and insurance
USA	42%	Traditional Native North American medicine, Homeopathy, Acupuncture, Naturopathy, Hypnosis, Biofield therapy	Highly evolved: Regulatory controls cover licensing, scope of practice, malpractice, professional discipline, third party reimbursements and access to treatments
Canada	70%	Traditional Native North American medicine Manipulative therapy, Traditional Chinese medicine and Acupuncture, Naturopathy	Coverage by health insurance is selective and minimal. No formal recognition to traditional medicine, only Guidelines for physicians/ practitioners
France	49%	Homeopathy, acupuncture, water cure, chiropractic, osteopathy, thalasso therapy, iridology	Only licensed allopathic physicians allowed to practice medicine
Germany	40-50%	Homeopathy, acupuncture, procaine injection therapy, chiropractic, ozone and oxygen therapy, herbal medicines, massage and cell therapy	Use of traditional medicine allowed only where allopathic treatment is not available or has been unsuccessful or has side effects
Italy	24%	Homeopathy, acupuncture, herbal remedies, prana therapy, anthroposophic and chiropractic therapy	Only licensed allopathic physicians allowed practicing medicine. Chiropractic is recognized as a profession.
Netherlands	Over 50%	Homoeopathy, manipulative medicine, acupuncture, naturopathy	Homeopathic and anthroposophic medicines are reimbursed by insurance
United Kingdom	12% using and 70% in favour of using	Herbal medicines, osteopathy, homeopathy, acupuncture, hypnotherapy and spiritual healing	Only licensed allopathic physicians are recognized officially.

India	70%-widely used in almost all households	Ayurveda, Unani, Siddha and homeopathy, naturopathy, yoga	All are recognized under the Central Council of Indian Medicine Act.
Pakistan	70%	Unani, Tibb, Ayurveda and Homeopathy	Integrated into the national health System
UAE	Very popular	Herbal preparations and Products	Registration criteria for herbal Medicines
Saudi Arabia	Widely used in almost all households	Acupuncture, herbal/ nutritional/ health food products and homeopathy	All herbal preparations require registration
China	Widely used in almost all households	Chinese traditional medicine	Integrated with the national health care system (equality in policies related to traditional and allopathic medicine)
Japan	70-75%	Kampo medicine, acupuncture, moxibustion, massage/finger pressure, judo therapy	Only allopathic physicians can practice
Indonesia/ Malaysia	40% (70% in rural areas)	Siddha, Ayurveda, Unani, acupuncture, massage, spiritual healing	Recognized as an integral part of curative and nursing care
Korea	Very popular	Oriental medicine, acupuncture, moxibustion, chiropractics	Dual system of medical treatment (Oriental and allopathic). Also covered under insurance
Australia	48%	Traditional Chinese systems, homeopathy, chiropractic, osteopathy, naturopathy	Only registered allopathic physicians are allowed to practice
Thailand	Very popular	Herbal medicines, massage, acupressure, reflexology	Traditional practitioners and Ayurvedic doctors are integrated into the healthcare system

India is a treasure chest of biodiversity which hosts a large variety of plants and has been identified as one of the eight important "Vavilorian" centres of origin and crop diversity. The total land area is only 2.4 percent of the total geographical area of the world, the country accounts for eight percent of the total global biodiversity with an estimated 49000 species of plants of which 4900 are endemic (Kumar and Asija, 2000). The ecosystems of the Himalayas, the Khasi and Mizo hills of northeastern India, the Vindhya and Satpura ranges of northern peninsular India, and the Western Ghats contain nearly 90 percent of the country's higher plant species and are therefore of special importance to

traditional medicine. The Indian System of medicine today uses across the various systems i.e. folk and codified around 8,000 species of plants. The maximum numbers of medicinal plants are utilized by the folk traditions, followed by Ayurveda, Siddha, Unani, Homeopathy, Tibetan and Modern respectively. Studies also showed that a large percentage of known medicinal plants occur in the dry and moist deciduous vegetation area compared to evergreen and temperate regions. Habit-wise classification showed that about 33% are trees, 32% herbs, 20% shrubs, 12 % creepers and 3% others. Very small proportions of the medicinal plants are lower plants like lichens, ferns, and algae. The majority of medicinal plants are higher flowering plants.

The medicinal plants are distributed across diverse habitats and landscape elements. Around 70 per cent of India's medicinal plants are found in the tropical zone, mostly in the forests of the Western and Eastern Ghats, the Vindhyas, Chotta Nagpur plateau, Aravalis, the Terai region in the foothills of the Himalayas and the North East. Less than 30 per cent of these medicinal plants are confined to the temperate and colder zones although species of great medicinal value occur in some of these habitats. Peninsular India extending downwards from Gujarat, Madhya Pradesh and Southern Bihar was once dominated by a continuum of tropical forests, namely: thorn forests, dry deciduous forests, moist deciduous forests, dry evergreen forests, wet evergreen forests and semi-evergreen forests. The complexity with respect to soils, topography and climate has created an exceptional variety of bio-mass and specialized habitats within this region. The ecosystems of southern peninsular India including the southern Western Ghats contain more than 6000 species of higher plants including an estimated 2000 endemic species. Of these, 2500 species representing over 1000 genera and 250 families have been used in Indian systems of medicine (Jain, 1991).



The medicinal and aromatic plant species are gaining importance and are promoted for commercial cultivation in order to meet the increasing demand within the domestic and export markets. The medicinal plants find application in pharmaceutical, cosmetic, agricultural and food industry and herbal industry. As per FRLHT's database around 6200 plant species are recorded in medicinal use in India fifty thousand herbal formulations based on around 8000 species, estimated to be known to folk tradition. Paradoxically, there is hardly any reliable data available on area, production and productivity of cultivated medicinal species since these are not recorded at the grass root level for want of adequate knowledge and interest by the record keeping authorities. International annual trade in medicinal plants more than a billion USD. Out of 8,000 plant species recorded in medicinal use in India, about 1000 species are estimated to be in commercial trade as plant raw drugs. All species are considered as one commodity and sometimes some data are taken by some states. The year wise cultivation of medicinal plants in India has been illustrated in (Table 1.2).

Table 1.2. Area under cultivation of important medicinal plants.

S. No.	Botanical Name	Vernacular Name	Area (Hac)	Cultivating States
1.	*Acoruscalamus*	Bhoja, Bauj	500	M.P, Bihar, Haryana and A.P
2.	*Adathodavasica*	Adusa,Ardusa	1,000	-
3.	*Aeglemarmelos*	Bael, Bili	3,000	Bihar, W.B, U.P and Maharashtra.

4.	*Aloevera*	Gritkumari	1,000	Rajasthan, Gujarat, Maharashtra, A.P and T.N
5.	*Androraphis paniculta*	Kalmegh	2,000	Manystates
6.	*Artemisaannua*		100	Gujarat,Maharashtra,
7.	*Asparagus recimosus*	Satavari	1,000	Gujarat,Maharashtra,,W.B,U.P
8.	*Azadirachta indica*	Neem	2,000	Gujarat,Maharashtra,, W.B,A.P, Rajasthan, Karnataka
9.	*Bacopamonnieri*	JalBrahmi	100	W.B,U.P,A.P
10.	*Baliospermum montanum*	Danhtimul	50	-
11.	*Cassiaabsus*	Chaksu	50	-
12.	*Cassiaangustifolia*	Senna	25,000	Gujarat, Maharashtra, A.P, Rajasthan, Karnataka,T.N
13.	*Centellaasiatia*	Brahmi	150	Assam, W.B, Bihar, U.P
14.	*Chlorophytum borivilianum*	Safedmusli	40,000	At present almost all the states Have sizable area
15.	*Coleusforskohlii*		1,000	T.N, Karnataka and Maharashtra
16.	*Commiphora wightii*	Guggal	2,000	Gujarat, M.P, Maharashtra and Rajasthan
17.	*Curculigo orchioides*	KaliMusli	100	Kerala, T.N, M.P and Karnataka
18.	*Daturametal*	Dhatura	200	-
19.	*Desmodium gangeticum*	Salparni	1,000	T.N, Kerala, Maharashtra, W.B and U.P
20.	*Emblica officinalis*	Amla, Amlokior Anwala	20,000	U.P, Gujarat, Rajasthan, A.P, Karnataka and Maharashtra
21.	*Garcinia gummigutta*	Bilatti-Amla	50	Kerala, T.N and Maharashtra
22.	*Gloriosa superba*	Kalihari Vachnag	1,000	T.N and H.P
23.	*Hemidesmus indicus*	Ananthamul	100	-

24.	*Kaemperia galanga*	Chandramula	300	Kerala and W.B
25.	*Lawsoni ainermis*	Mehandi	35,000	Rajasthan and to some extend A.P
26.	*Mucuna pruriens*	Kaunch	1,000	A.P, Karnataka, H.P, Gujarat and Maharashtra
27.	*Ocimum sanctum*	Tulsi	1,000	U.P J and K and Karnataka
28.	*Phyllanthus niruri*	Jar-amla,Bhuni Amla	100	Tamil Nadu and Karnataka
29.	*Piper longum*	Pipal, Pipli	1,500	Maharashtra, Kerala, T.N Assam and W.B
30.	*Plantago ovata*	Isobgul	1,20,000	Gujarat, Rajasthan and M.P
31.	*Plumbago zeylanica*	Chitrak Chitawar	100	Kerala and T.N
32.	*Psoralea corylifolia*	Babchi	50	U.P, M.P and Rajasthan
33.	*Rauvolfia serpetina*	Chota Chand, Sarpagandha	1,000	W.B, Orissa, M.P, Bihar, and U.P
34.	*Solanum surattense*	Kateli, Bhoiringini	100	-
35.	*Trachyspermum ammi*	Ajowan	100	Rajasthan, M.P and Gujarat
36.	*Vetiveria zizanioides*	Khas-Khas, Khas	500	Border crop in U.P, Rajasthan and Bihar
37.	*Withania somnifera*	Ashwagandha	10,000	M.P, U.P, Gujarat, Rajasthan, Maharashtra and A.P
Total			**2,72,150**	

T.N.-Tamil nadu, U.P.-Uttar Pradesh, M.P.-Madhya Pradesh,

W.B.-West Bengal, A.P. - Andhra Pradesh., H.P.-Himachal Pradesh

1.1.1. Importance of medicinal plants

In India, medicinal plants are widely used by all sections of the population and it has been estimated that, in total over 7500 species of plants are used by several ethnic communities (AICEP 1994; Anthropological survey of India 1994) Medicinal and Aromatic plants form a numerically large group of economically

important plants which provide basic raw materials for medicines, perfumes, flavours and cosmetics. These plants and their products not only serve as valuable source of income for small holders and entrepreneurs but also help the country to earn valuable foreign exchange by way of export. Presently, medicinal plants play a very important role in the modern economy. NTFPs account for 70% of India's forest product exports and the demand for phytochemicals is expected to increase in future as a new frontier for trade. India has probably the oldest, richest and most diverse cultural traditions in the use of medicinal plants. Medicinal plants are those plants which are rich in secondary -metabolites and arc potential source of drugs. These secondary metabolites include alkaloids, glycosides, coumarins, flavonoids, steroids etc.

The use of the medicinal herbs for curing disease has been documented in history of all civilizations. Recently research has supported biological activities of some medicinal herbs. Some medicinal plants have shown significant tumour inhibiting effect. The concept of antioxidants is fast catching up and latest research has shown that a number of herb all derivatives have excellent antioxidant action. The medicinal plant is a biosynthetic laboratory, not only for chemical compounds, but also a multitude of compounds like glycosides, alkaloids etc. The compounds that are responsible for medicinal properties of the drug are usually secondary metabolites. These compounds either act on different systems of animals including man, or act through interfering in the metabolism of microbes infecting them. The microbes may be pathogenic or symbiotic. In either way the bioactive compounds from medicinal plants play a determining role in regulating host-microbe interaction in favour of the host. So, the identification of bioactive compound in plants, their isolation, purification and characterization of active ingredient since crude extracts by various analytical methods is also most important. The medicinal properties of plants could be based on the antioxidant, antimicrobial, antipyretic effects of the phytochemicals in them (Cowman, 1999; Adesokan *et al.*, 2008).

Before onset of synthetic era, man was completely dependent on medicinal herbs for prevention and treatment of diseases. With introduction of scientific procedures the researchers, were able to understand about toxic

principles present in the green flora. The scientists isolated active constituents of the medicinal herbs and after testing some were found to be therapeutically active. Aconitine, Atisine, Lobeline, Nicotine, Strychnine, Digoxin, Atropine, Morphine are some common examples.

Andrographis paniculata important medicinal plants which possess wide range of medicinal and pharmacological application *such as* antipyretic, antidiabitic, analgesic, antihepatotoxic, antimalarial, antibacterial, antifertility, anti-inflammatory, immunosuppressive and treatments of many diseases including HIV, AIDS, immune disorders Calabrese, (2000). The aerial part of *Andrographis paniculata* is commonly used in Chinese medicine. The herb contains diterpenoids, flavonoids and polyphenols as the major bioactive components (Rao *et al*; 2004 and Xu *et al*; 2010). Andrographolide is the secondary metabolites present in this plant. *Andrographis paniculata* also contain other constituents include 14-deoxy-12-methoxyandrographolide, 14-deoxy-11, 12 didehydroandrographolide, 14 deoxyandrographolide. It is used in various ailments like throat infections, dysentery, cancer, hepatotoxicity, cough, cold, headache, edema, pain conditions, inflammation and muscular pain, arthritis, rheumatism, fibro myalgia, multiple sclerosis, depression, diarrhea, dysentery, cholera, candida, lupus, diabetes, piles, fevers, fatigue, hepatitis, herpes, leprosy, loss of appetite, swollen lymph nodes and other lymphatic conditions, jaundice, malaria, dyspepsia, dermatitis, eczema, burns, pneumonia, bronchitis, tuberculosis, chicken pox, mumps, sluggish liver, spleen, kidneys and adrenal glands, sleeplessness, vaginitis, and constipation.

1.2. *Andrographis paniculata.*

1.2. 1. Synonyms

Andrographis paniculata Wall ex. Nees (DMPRD (Division of Medical Plants Research and Development 1990), Justicia latebrosa Russell ex Wall., J. paniculata Burm. f., J. stricta Lam. Ex Steud (Hooker, 1885).

1.2. 2. Common Names

Kalmegh, Kalafath, Kan-jang, Alui, Charita, Cherota, Chiraita, Cheretta, Kariyat, Green chiretta, Halviva, Kreat, Sinta, Rice bitters, Sambilata, Sambiloto, Andrographidis, Kraut (Hsu *et al.*, 1986).

Kalmegh(*Andrographis paniculata* Wall. Ex Nees) belong to family *Acanthaceae* has wide range of medicinal and pharmacological application. It is also known as "King of Bitters". As an Ayurveda herb it is known as *Kalmegh* or *Kalamegha*, meaning "dark cloud". It is also known as *Bhui-neem*, meaning "neem of the ground", since the plant, though being a small annual herb, 'King of bitters', Maha-tita or bhui neem because of its similarity in appearance and bitter taste as that of neem (*Azadirachta indica* A. Juss) though it is much smaller in size (Niranjan *et al.*, 2010).

In Malaysia, it is known as *Hempedu Bumi*, which literally means 'bile of earth' since it is one of the bitterest plants that are used in traditional medicine. 'King of bitters', Maha-tita or bhui neem because of its similarity in appearance and bitter taste as that of neem (*Azadirachta indica* A. Juss) though it is much smaller in size (Niranjan *et al.*, 2010). Kalmegh is one of the nineteen species of the genus Andrographis, which is indigenous to India. It is an active constituent in majority of Ayurvedic preparations and is official in the Ayurvedic pharmacopoeia (Rammohan *et al.*, 2011). *Andrographis paniculata* has been used for centuries in India, Chaina, Thailand, and other Asian countries and is present in 26 different polyherbal formulations in the Ayurvedic tradition health system. Kalmegh is listed in the 1992 pharmacopoeia of the people's republic of chaina as cold property herb used is rid the body of fevers and dispel toxins. An immunostimulant preparation known as Kan Jang, which contains kalmegh and eleutherococcushus, been used in Scandinavian countries for 20 years. *Andrographis paniculata* (Burm. f.) (*A. paniculata*, *Chuanxinlian*), native to Taiwan, Mainland China and India, is a medicinal herb with an extremely bitter taste used to treat liver disorders, bowel complaints of children, colic pain, common cold and upper respiratory tract infection (Kligler *et al.*, 2006).

1.2.3. Morphological Description

Andrographis paniculata grows erect to a height of 30–110 cm in moist, shady places. The slender stem is dark green, squared in cross-section with longitudinal furrows and wings along the angles. The species is also reported to be perennial shrub (Hancharnlerd *et al*; 1994). The lance-shaped leaves have hairless blades measuring up to 8 centimetres long by 2.5 wide. The small flowers are borne in spreading racemes. The fruit is a capsule around 2 centimetres long and a few millimetres wide. It contains many yellow-brown seeds. It reaches a height of 30 to 100 cm. With square stems and lanceolate leaves. In India it is also called Kalmegh or Kiryat.

1.3.4. Habitat

The herb is found in a variety of habitat viz. plains, hill slopes (Lattoo, 2006), waste lands (Zhou, 1987) , farms, dry or wet lands (Muniramappa, 1997), sea shore and even in the road side.

1.3.5. Soil Condition

Andrographis paniculata grown in all types of soil which explains its wide distribution. It grows in that types of soil where almost no other plant can be cultivated, such as 'serpentine soil', which is relatively high in metals such as aluminum, copper and zinc (Samantaray *et al.,* 2001) . However, soil that is flooded or wet throughout the year may be avoided for its cultivation. The species was also observed to grow luxuriously in mild humid locations with tropical temperature and high rainfall (DMPRD (Division of Medical Plants Research and Development, 1990 and MPRI (Medicinal Plant Research Institute, Department of Medical Science, Ministry of Public Health 1999) . Vijaya *et al.* (2008) recommended the use of vermicompost coir pith for the reclamation of soils from industrial sites for the cultivation of *Andrographis paniculata* in a small scale nursery.

1.2.6. Distribution

Andrographis paniculata is distributed in tropical Asian countries, often in isolated patches. It can be found in a variety of habitats, such as plains, hillsides, coastlines, and disturbed and cultivated areas such as roadsides, farms, and wastelands. Native populations of *Andrographis paniculata* are spread throughout south India and Sri Lanka which perhaps represent the center of origin and diversity of the species. The herb is an introduced species in northern parts of India, Java, Malaysia, Indonesia, the West Indies, and elsewhere in the Americas. The species also occurs in Hong Kong, Thailand, Brunei, Singapore, and other parts of Asia where it may or may not be native. The plant is cultivated in many areas, as well. Widely found and cultivated in tropical and subtropical Asia, south-east Asia and India (Chang and But, eds. 1986) . The species is also reported from different phyto geographical and edaphic zones of China, America, West Indies and Christmas Island in Indian Ocean (Lattoo *et al;* 2006)

Unlike other species of the genus, *Andrographis paniculata* is of common occurrence in most places in India, including the plains and hilly areas up to 500 m, which accounts for its wide use. Since time immemorial, village and ethnic communities in India have been using this herb for treating a variety of ailments. It is distributed in tropical Asian countries having hot and humid climatic conditions but it can be cultivated in subtropical regions during the monsoon season (Niranjan *et al.* 2010; Kumar, 2011). *Andrographis paniculata,* known on the Indian subcontinent as Chirayetah and Kalmegh in Urdu and Hindi languages, respectively, is an annual plant, 1-3 ft. high, that is one of the most commonly seed plants in the traditional systems of Unani and Ayurvedic medicines. It grows in hedge rows throughout the plains of India and is also cultivated in gardens (Kabeeruddin and Kitabul Advia 1937; Dymock, 1972). It also grows in many other Asian countries and is used as a traditional herbal medicine in China, Hong Kong, the Philippines, Malaysia, Indonesia, and "ailand." The aerial parts are most commonly used; however, the whole plant or roots are mentioned for certain limited purposes in some manuscripts. The herb

is having a preventive effect from many diseases, due to its powerful immune strengthening benefits (Chauhan, 2009). The therapeutic activities of this plant are attributed to andrographolide and related diterpens i.e., deoxyandrographolide, 14- deoxy-11, 12- didehydro-andrographolide and neo-androrapholide (Bahn, 2006). The demand of Kalmegh is increasing day by day (Chauhan, 2009). The propagation of kalmegh generally occurs through seeds, inspite of several germination problems. The production of any crop heavily relies on quality of planting seeds and for producing good quality seeds it would be desirable to have information regarding germination and associated germination parameters like germination energy, germination period etc (Kumar, 2011).

During their life-cycle, plants experience a variety of abiotic stresses. Among the major abiotic stresses that affect the plant growth and yield are water-logging, drought, high or low temperature, excess soil salinity, heavy metals, inadequate mineral nutrients in the soil and too little or too much light. They cause considerable (up to 80%) economic losses in agriculture. Water-deficit, salinity and heavy metal stress affect the water relations of a plant on the cellular as well as whole plant level causing damages and reduction in growth rate and development. Processes such as seed germination, seedling growth and vigour, vegetative growth, flowering and fruit set are adversely affected by abiotic stresses. In general, the seeds and seedlings may be less stress tolerant than adult plants. The plants respond in a species specific manner to different stresses. The present study was an attempt to standardize germination parameters under varying abiotic conditions.

Andrographis paniculata is a plant that has been effectively used in traditional Asian medicines for centuries. It's perceived "blood purifying" property results in its use in diseases where blood "abnormalities" are considered causes of disease, such as skin eruptions, boils, scabies, and chronic undetermined fevers. "The aerial part of the plant, used medicinally, contains a large number of chemical constituents, mainly lactones, diterpenoids, diterpene glycosides, flavonoids, and flavonoid glycosides. Controlled clinical trials report its safe and effective use for reducing symptoms of uncomplicated upper respiratory tract

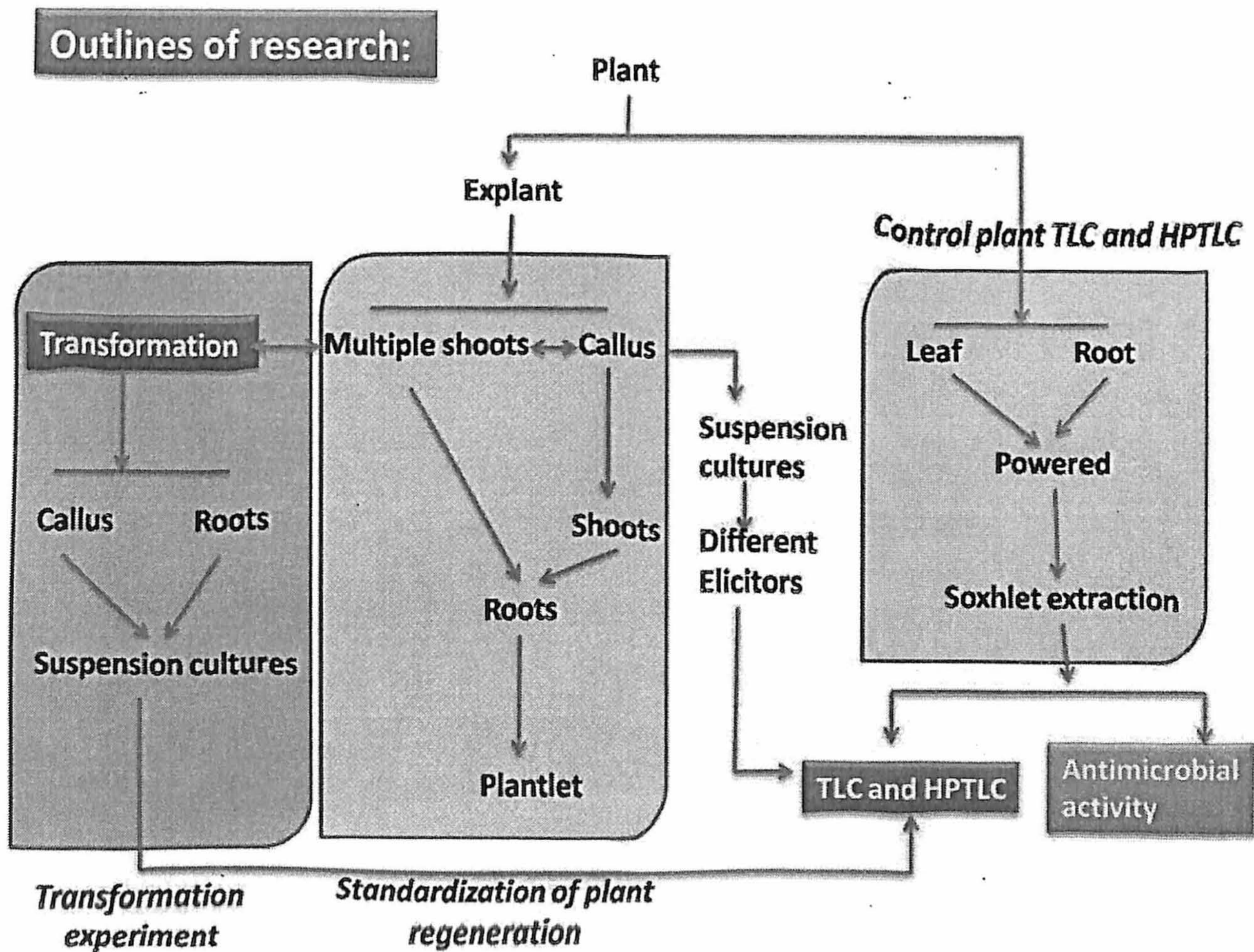
infections. Many of the disease commonly treated with *Andrographis paniculata* in traditional medical systems are considered self-limiting, its purported benefits need critical evaluation. "Traditionally, the plant was used as an infusion, decoction, or powder, either alone or in combination with other medicinal plants. In modern times, and in many controlled clinical trials, commercial preparations have tended to be standardized extracts of the whole plant. *Andrographis paniculata*, endemic to Peninsular India, used as a herbal medicine by local communities. . It is laxative, bitter and overcomes difficulty in breathing, burning sensation, cough, edema, thirst, skin diseases, syphilitic ulcers, worms, acidity and liver complaints (Sivarajan and Balachandran, 1994). *Andrographis neesiana* is a potential alternative source of andrographolide. The heavy demand of andrographolide in Indian as well as international markets has motivated Indian farmers to start commercial cultivation of this medicinal plant (Kanjilal *et al.,* 2002).

1.2.7. Harvest

Harvesting period of *Andrographis paniculata* leaves (Anonymous., 2000) is at 3-5 months old or at 50% blossom whereupon the highest quantity of active lactone compound was found followed by final harvesting after next 2-3 months. As biotechnological tools play an important role for production of secondary metabolite. *Agrobacterium rhizogenes* has been regularly used for gene transfer in many dicotyledonous Plants (Tepfer *et al.,* 1990). Plant infection with this bacterium induces the multibranched adventitious roots at the site of infection so called 'hairy roots' (Chilton, 1982). Transgenic root system offers tremendous potential for introducing additional genes along with the Ri T-DNA genes for alteration of metabolic pathways and production of useful metabolites or compounds of interest. The aim of the present study was to *In-vitro* plant regeneration and comparative studies of secondary metabolite from non-transformed and transformed plants of *Andrographis paniculata with* the following objectives

- To standardisation of protocol *Andrographis paniculata* via. Shoot tip and auxiliary bud culture.

- To standardisation of protocol on genetic transformation on *Andrographis paniculata* via. *Agrobacterium rhizogenes* to enhance the root biomass and secondary metabolite.
- To selection of transformed tissues trough antibiotics and biochemical analysis.
- To analyse the biochemical and molecular characterization of transformed and non-transformed plants of *Andrographis paniculata.*
- To compare the content of secondary metabolite present in non-transformed and transformed plant trough TLC and HPTLC



CHAPTER-II

2. REVIEW OF LITERATURE

2.1 *Andrographis paniculata:*

Andrographis paniculata belonging to the family *Acanthaceae*, annual branched, erect-running ½ to 1 meter in height. The aerial parts of the plant (leaves and stems) are used to extract the active phytochemicals reported by Kiritikar and Basu (1975). In Thailand, this plant was selected by the Ministry of Public Health as one of the medicinal plants to be included in "The National List of Essential Drugs A.D. 1999" (List of Herbal Medicinal Products) (Pholphana *et al.*, 2004).

Andrographis paniculata (Burm. F.) *Nees* is commonly known as Kalmegh (King of Bitters) is an important plant species in Ayurvedic medicine (prominent in 26 Ayurvedic formulations). An overview is conducted in the species considering all essential aspects namely, therapeutic uses, synonyms, common names, distribution, habitat, soil condition, cultivation, harvest, post-harvest and storage, plant description, reproductive biology, cytological aspects, cytogenetical study, molecular genetics, extraction and detection of phytochemicals, assay, photochemistry, insecticidal activity, clinical trials, contraindications and drug interactions, clinical implications, regulatory mechanism; in vitro studies and mutational studies to provide unabridged repository of references to researchers for its effective biological utilization (Ghosh *et al.*, 2012).

It grows abundantly in south-eastern Asia including India, Sri Lanka, Pakistan and Indonesia (Jarukamjorn and Nemoto, 2008). *Andrographis paniculata Nees (Acanthacae)* commonly known as king of bitters, is a perennial herb widely cultivated in China, South Asia, South Africa, India, Pakistan and Srilanka. *Andrographis paniculata* Nees (Family *Acanthaceae*) was available abundantly in India, Pakistan and Srilanka, growing in hot and shade places. It is known by various names such as Kalmegh, Kalupnath, Kiriat and Mahatila meaning the "King of bitters". It is one of the most widely used plants in Ayurvedic formulations.

2.1.1. Uses

The use of *Andrographis paniculata* is increasing day to day as result over-exploitation from natural habitat reported by Chauhan, (2009). The whole plant is used extensively as an anti-inflammatory and antipyretic drug for the treatment of fever, cold, laryngitis, diarrhoea, and inflammation and traditionally for sluggish liver as an antidote in case of colic dysentery and dyspepsia. Dastur, (1959) reported It has been employed with benefit in case of general debility in convalescence after fevers, disorders of liver and advanced stages of dysentery. It is used as a bitter tonic, antispasmodic, antiperistaltic, stomachic and antihelmintic. It has also been employed with benefit in case of general debility in convalescence after fevers, disorders of liver and advanced stages dysentery. It was recommended in Charaka Samhita (175 B.C.) for treatment of jaundice along with other plants in multi plant preparations (Sharma, 1983.). It is one of the most widely used plants in Ayurvedic formulations (Hooker, 1885.) and whole plant part known as "Panchang" (stem, leaf, flower, seed and root) is being used in various formulation of Indian system of medicine. The therapeutic benefit of this herb has been attributed to andrographolide (alkaloid) and its related diterpenoid compound, i.e. deoxyandrographolide and neoandrographolide (ICH Committee 1996; Bunyaprapatrasara and Chokecharocopons, 1997). Pharmaceutical studies suggest anti-inflammatory (Shen *et al.* 2002; Amroyan *et al.* 1999), antipyretic (Madav *et al.* 1995), antiviral (Chang *et al.* 1991), immune stimulatory (Puri *et al.*, 1993), potential cancer therapeutic agent (Rajgopal *et al.*, 2003), antihyperglycemic (Bu-Chin *et al.*, 2003) and antioxidant (Zhang and Tan, 2000) properties.

Mishra *et al* (2010) reported that juice of fresh leaves, which generally contains andrographolide, is used as a domestic remedy in the treatment of colic pain, loss of appetite, irregular stools and diarrhoea. *Andrographis paniculata* is known in traditional Asian medicine as an immune system booster, to treat infections in gastrointestinal tract and upper respiratory tract, fever, herpes, sore throat, and a variety of other chronic infectious diseases (Wangboonskul *et al.*, 2006) and also used for sluggish liver, as antidote in

case of colic dysentery and dyspepsia (Handa and Sharma, 1990). Asian medicine as an immune system booster, *Andrographis paniculata* has demonstrated significant activity in fighting common cold, flu and upper respiratory infections reported by Melchior *et al* (2000).

2.1.1.1. Uses in traditional medical systems

Andrographis paniculata has been reported as having antibacterial, antifungal, antiviral, choleretic, hypoglycemic, hypocholesterolemic, and adaptogenic effects reported by Bhatnagar *et al* (1961). In the Unani system of medicine, it is considered aperient, anti-inflammatory, emollient, astringent, diuretic, emmenagogue, gastric and liver tonic, carminative, antihelmintic, and antipyretic. Due to its "blood purifying" activity, it is recommended for use in cases of leprosy, gonorrhea, scabies, boils, skin eruptions, and chronic and seasonal fevers. In China, the herb derived from the leaves or aerial parts of *Andrographis paniculata* is known as Chuanxinlian, Yijianxi or Lanhelian. It is described as bitter and cold, is considered to be antipyretic, detoxicant, anti-inflammatory, and detumescent, and is thought to remove "pathogenic heat" from the blood. *Andrographis paniculata* is used for the treatment of pharyngolaryngitis, diarrhea, dysentery, cough with thick sputum, carbuncle, sores, and snake bites reported by Chang and But (1987). Various preparations and compound formulas of the herb have been used to treat infectious and non-infectious diseases, such as epidemic encephalitis B, suppurative otitis media, neonatal subcutaneous annular ulcer, vaginitis, cervical erosion, pelvic inflammation, herpes zoster, chicken pox, mumps, neurodermatitis, and eczema, significant effect as burns reported by Chang and But (1987).

2.1.1.2. Modern Uses

The modern use of *Andrographis .paniculata* is for the prevention and treatment of the common cold, cardiovascular disease reported by Pharmacological and clinical studies suggest the potential for beneficial effects in diseases like cancer (Amroyan *et al.,* 1999; See *et al.*, 2002; Sheeja, 2007; Shi, 2008; Yang 2009) and HIV infections (Calabrese *et al.* 2000).

2.1.1.3. Medicinal use

Andrographis paniculata is used in traditional Siddha and Ayurvedic systems of medicine as well as in tribal medicine in India and some other

countries for multiple clinical applications. From a biomedicinal perspective, the therapeutic value of Kalmegh is due to its mechanism of action which is perhaps by enzyme induction. The plant extracts exhibits antityphoid and antifungal activities. Kalmegh is also reported to possess antihepatotoxic, antibiotic, antimalarial, antihepatitic, antithrombogenic, and antiinflammatory as reported by Thiyagarajan *et al* (2011) anti-snake venom, and antipyretic properties to mention a few, besides its general use as an immunostimulant agent (Burgos *et al.*, 2009). The herb has shown an ability to reduce inflammation and viral infection, and is used as a principal ingredient in traditional Chinese medicinal formulas against cold infection reported by Kate Wright (2009). The herb is the well-known as Kalmegh 'green chiretta', and forms the principal ingredient of a household medicine ('alui'), used as a bitter tonic and febrifuge. In Siddha medicine, *Andrographis Paniculata* is widely used to treat fevers like chikenguinea, swine-flu, typhoid etc. A recent study found that effective as mesalazine (mesalamine) in ulcerative colitis. (Tang and Targan, 2011). Andrographolide inhibits interleukin- expression and suppresses prostate cancer cell growth studies *in vitro* (Hun and Tummala, 2010).

Andrographis paniculata has been shown to be a safe traditional remedy for upper respiratory tract diseases as reported by (Coon and Ernst, 2004) Ko and Wei BL, (2006) reported that the herb has been shown to inhibit RANTES secretion in inflamed bronchial cells. RANTES is a chemo attractant for eosinophil, monocytes and lymphocytes that is stored in, and released by, platelets and activated T-cells. Extracts of *Andrographis paniculata* exhibit potent inflammatory modulating and antioxidant actions in mouse models reported as by Sheeja and Shihab, (2006). Andrographolide is extensively used as a stimulating agent for liver enzymes and hepatoprotective agent. Studies showed that andrographolide is a potent hepatoprotective agent when compared with silymarin (Jarukamjorn and Nemoto, 2008). Methanolic extract of *Andrographis paniculata* was found to have significant toxicity against KB (human epidermoid leukemia) and P388 (lymphocytic leukemia) cell lines. It has been observed that the alcohol extract of the andrographolide was able to induce significant stimulation of both “antigen specific” and “antigen nonspecific” types of immune responses in mice.(Verma, *et al*). Andrographolide

administration was reported to cure 91 percent of acute bacillary dysentery cases (Akbar, 2004). According to Chinese medicine theory, *Andrographis paniculata* relieves internal heat, inflammation and pain and also used for detoxication (Chao WW and Lin BF 2010) and beneficial for preventing heart diseases, helps protection from liver diseases, and stimulates gall bladder contraction reported by Zhang and Tan, (2000).

Table 2.1. The Traditional Uses of *Andrographis paniculata* (Zhang, 2004)

	Native Names	Traditional Uses
Traditional Chinese Medicine (TCM)	Chuan-Xin-Lian Chunlianqialio Yiqianxi Si-Fang-Lian Zhanshejian	Fever, Common cold Laryngitis,Pharyngitis, Tonsillitis Pneumonia Respiratory infections Hepatitis
Traditional Indian Medicine	Kalmegh Kiryato Maha-tikta Bhunimba	Diabetes Dysentery, Enteritis Helminth infection Herpes Peptic ulcer Skin infections (topical use) Snake-bites (topical use)
Traditional Thai Medicine	FahTha Lai Nam RaiPangpond	Fever, Common cold Non-infectious diarrhea
Malaysia	Hempedubumi Sambiloto	Diabetes Hypertension
Japan	Senshinren	Fever, Common cold
Scandinavian	Green Chiretta	Green Chiretta

2.1.2. Chemical constitute of *Andrographis paniculata*

The active constituent of *Andrographis paniculata* is andrographolide and generally extracted from leaves and aerial parts of the plant. Andrographolide is colourless diterpene lactone, with bitter taste and its Melting point of is 230°-239°C (Niranjan *et al.*, 2010). The chemical formula of andrographolid is 3 - (2 - (Decahydro - 6 - hydroxyl - 5 - (hydroxymethyl) - 5. *Andrographis paniculata* contains diterpenes, lactones, and flavonoids. Flavonoids mainly exist in the root, but have also been isolated from the leaves.

"The aerial parts contain alkanes, ketones, and aldehydes. Four lactones– chuanxinlian A (deoxyandrographolide), B (andrographolide), C (neoandrographolide) and D (14-deoxy-11, 12-didehydroandrographolide) were isolated from the aerial parts as reported (Chang, But; 1986). A diterpene glucoside (deoxyandrographolide-19beta-D-glucoside) has been detected in the leaves (Weiming and Xiaotian, 1982) and six diterpenoids of the entlabdane type, two diterpene glucosides and four diterpene dimers (bis-andrographolides A, B, C, and D) have been isolated from aerial parts (Matsuda *et al.*, 1994). Two flavonoids identified as 5, 7, 2', 3'-tetramethoxyflavanone and 5-hydroxy-7, 2', 3'-trimethoxyflavone were isolated from the whole plant, (Koteswara Rao *et al.*, 2004). 12 new flavonoids and 14 diterpenoids have been reported from the aerial parts (Chen *et al* 1982). Two new flavonoid glycosides and a new diterpenoid (andrographic acid) were recently reported, (Li *et al.* 2007).

2.1.2.1. Other chemical constituents of *Andrographis paniculata*

Other chemical constituents of *Andrographis paniculata* is 14-deoxy11, 12 didehydroandrographolide, 14 deoxyandrographolid3, 14 didehydrographolide, 14-deoxy-11-oxoandrographolide, neoandrographolide, andrographside and 14-deoxyandrographiside.

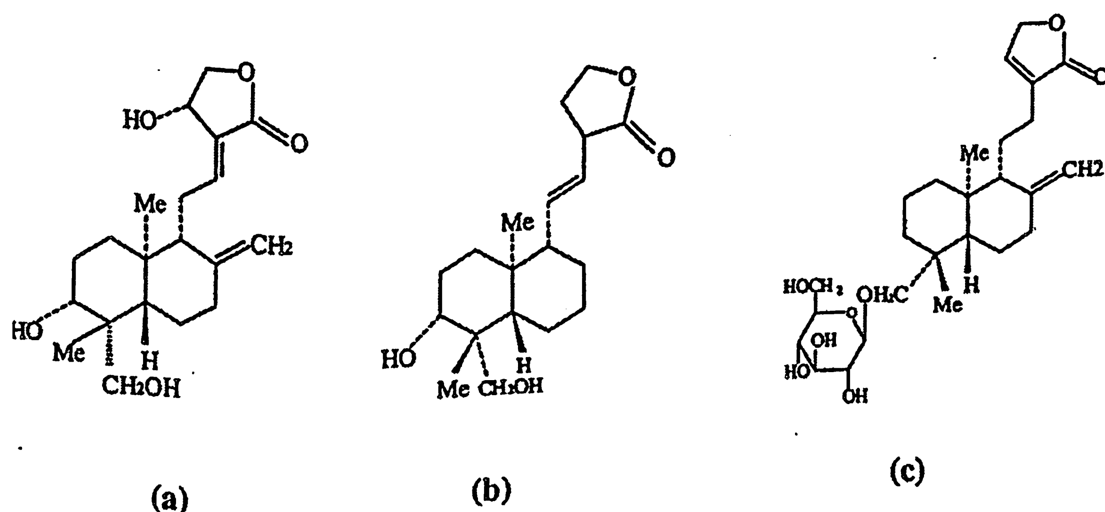


Fig. 2.1 a) Chemical structures of the three main diterpenoids in Andrographis Paniculata. (a) Andrographolide, (b) Deoxyandrographolide and (c) Neoandrographolide.

2.1.3. Andrographolide

The major bioactive constituents of *Andrographis paniculata* are a group of diterpene lactones reported by Wongkittipong *et al* (2000). Leaves of the plant contain several derivatives of diterpene lactones, out of which andrographolide and neoandrographolide are important as reported by Akowuah *et al* (2006). concentration of andrographolid and neoandrographolide is varies within plant parts and with the geographical distributions of the species. The structure of andrographolide has been analysed by using X-ray, 1H, 13 C-NMR, and ESI-MS (Fujita *et al.*, 1984). Andrographolide is in soluble in water; it is soluble in acetone, chloroform, ether, and hot ethanol. Crystalline andrographolide was reported to be highly stable (Lomlim *et al.,* 2003).

Rajani *et al* 2000 reported a simple and rapid method for isolating andrographolide from the leaf of *Andrographis paniculata*. They extracted it using a 1:□1 mixture of dichloromethane and methanol and then isolated the andrographolide directly from the extract by performing recrystallization. The purity of the compound has been evaluated with thin-layer chromatography (TLC), UV absorption spectrum, high-performance liquid chromatography (HPLC), liquid chromatography-mass spectrometry (LCMS), and differential scanning calorimetry (DSC), with the melting point of andrographolide at 235.3°C (Du *et al.*, 2003).The Andrographolide being secondary metabolites are often influenced by the environmental, seasonal factors and its distribution in between leaves and whole plant. It is evident that there is wide variation in the andrographolide present in leaves and whole plant.

Andrographolid is present in more amount in the part of leaf than other part. The leaves normally contain 2.5 - 3% w/w of andrographolide (Ramesh, 1994). Other than these active compounds, other chemical constituents include andrograpanoside, andrographanin, 14-deoxy-12-methoxyandrographolide, and deoxyandrographolide

Table-2.2. Reported chemical constituents and bioactivities of *Andrographis paniculata*

S. NO.	Reported chemical constituent(s)	Pharmacological action (s)	Reference (s) no
1.	Andrographolide	Anticancer, hepatoprotective	Verma *et al.*,
2.	14-deoxyandrographolide	Enhanced proliferation and interleukin-2 induction in human peripheral blood lymphocytes	Kumar *et al.*, (2004)
3.	14-deoxy-11,12-didehydroandrographolide	Anti-cancer	Kumar *et al.*, (2004)
4.	14-deoxy-11-oxoandrograholide	Antilesminasis and antiparasitic dieases	Lala *et al.*, (2003)
5.	Neoandrographolide	Anti-inflammatory	Liu *et al.*, (2007)
6.	Andrographide	Liver cleansing and Hepatitis	Dahanuka *et al.*, (200)
7.	Kalmeghin	Fever and Cold	Koul and Kapil, (1994)
8.	Andrographiside	Anti-oxidant, Anti-Lipoperoxidant, Carcinogenic Detoxification	Balachandranm *et al.*, (2005)

2.1.3.1. Biological Activities of Andrographolide

Andrographolide has been reported to have a wide range of biological activities, such as those that are anti-inflammatory (Shen *et al.*, 2002), anti-allergic (Xia *et al.*, 2004), antiplatelet aggregation (Amroyan, *et al.*, 1999), hepatoprotective (Trivedi and Rawal, 2001), and anti-HIV (Reddy *et al.*, 2005). In biological systems, andrographolide can interact with many other inter- and intracellular constituents as a bipolar compound, thus ensuing in many biological responses. A recent study demonstrated that polysaccharides combined with andrographolide can ease the recovery of diabetic nephropathy (Xu *et al.*, 2012).

2.1.3.2. Structure and Chemical Properties

Andrographolide is a labdane diterpenoid that is the main bioactive component of the medicinal plant *Andrographis paniculata* reported by

Chakravarti and Chakravarti, (1951). Andrographolide is an extremely bitter substance extracted from the stem and leaves of *Andrographis paniculata.* It is used as a wonder drug in traditional Sidha and Ayurvedic system of medicine as well as tribal medicine in India for multiple clinical application (Chadha, 1985 and Raina *et al.,* 2007). The diterpene lactone andrographolid was first isolated as a major constituent 6 and later characterized as a lactone (Gorter *et al.*, 1911). Its full structure was determined in the 1960s (Cava *et al.*, 1965 and Chan *et al.*, 1971). A number of related minor diterpens and their glycosides have since been identified (Reddy *et al.*, 2005). Active compounds extracted with ethanol or methanol from the whole plant, leaf and stem (Cheung *et al.*, 2001). Andrographolide ($C_{20}H_{30}O_5$) is the major diterpenoid in *Andrographis paniculata*, making up about 4%, 0.8~1.2% and 0.5~6% in dried whole plant, stem and leaf extracts respectively (Cheung *et al.*, 2001). The other main diterpenoids are deoxyandrographolide, neoandrographolide, 14-deoxy-11, 12-didehydroandrographide and isoandrographolide (Cheung *et al.*, 2001).

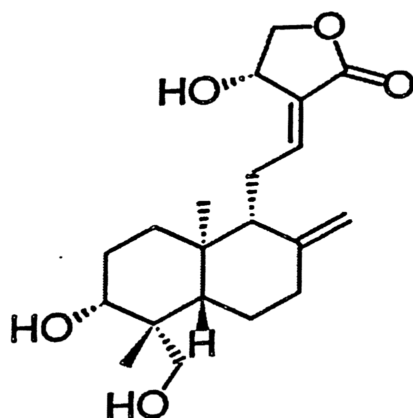


Fig: 2.2. Structure of Andrographolide

2.1.3.3. Natural occurrence andrographolide

Andrographolide found naturally and also researchers have been successful in synthesizing andrographolide derivatives that have also displayed anti-cancer activities. Naturally occurring andrographolide contains: (i) an *α*-alkylidene-c-butyrolactone moiety, (ii) two double bonds $\delta^{8\,(17)}$ and $\delta^{12\,(13)}$ and (iii) three hydroxyls at C-3, C-19 and C-14. Of the three hydroxyls, the one at C-14 is allylic, while others at C-3 and C-19 are secondary and primary, respectively

2.2. *In vitro* regeneration

Plant regeneration through somatic embryogenesis from stem, petiole and leaf explants of Indian chicory has been achieved (*Cichorium intybus* L.) (Abdin and Ilah, 2007 and Rehman, 2003). Micropropagation is the proven method for efficient in vitro propagation of medicinal and aromatic plants and for commercial exploitation of valuable plant-derived pharmaceuticals (Rout, 2002 and Faisal *et al.*, 2005). Micropropagation has been defined as '*in vitro*' regeneration of plants from organs, tissues, cells or protoplasts (Beversdorf, 1990) and as' the true to type propagation of a selected genotypes using *in vitro* culture techniques' (Deberghand Read, 1991). *In-vitro* propagation of plants holds tremendous potential for the production of high-quality plant-based medicines reported by Murch, *et al., (*2000). *In vitro* generated nodal explants to fresh shoot multiplication medium was reported in Leptadenia reticulata (Arya *et al.*, 2003), Sophora flavescens (Zhao *et al.*, 2004), and Vitextrifolia (Hiregoudar *et al.*, 2006), Gardenia jaminoides (George *et al.*, 1993) and Cross and rain fundi buliformis (Girija *et al.*, 1999).

In-vitro propagation achieved through different methods including Micropropagation, excepting a preliminary report on plant regeneration via somatic embryogenesis (Martin, 2004). Micropropagation of various plant species, including many medicinal plants, has been reported (Murashige, 1978; Skirvin *et al.*, 1990 and Withers *et al.*, 1986). Micropropagation through somatic embryogenesis is another option for rapid production of uniform plants of true to type nature of embryo derived plantlets (Jayanthi and Mandle, 2001; Tokuhara and Mii, 2001). Plant regeneration from shoot and stem meristems has yielded encouraging results in medicinal plants like *Catharanthus roseus, Cinchona ledgeriana* and Digitalis *spp*, Rehmannia *glutinosa*, Rauvolfia *serpentina*, *Isoplexis canariensis* (Paek *et al.*, 1995; Roy *et al.*, 1994; Perez- Bermudez *et al.*, 2002). Numerous factors are reported to influence the success of *in-vitro* propagation of different medicinal plants (Hussey, *et al.,* 1980; Bhagyalakshmi *et al.*, 1988; Short *et al.*, 1991). The stimulatory effect of BAP on multiple shoot formation has been reported earlier for several medicinal plant species including Ocimum basilicum (Begum *et al.*, 2002), Feronialimonia (Hiregoudar *et al.*, 2003), Sesbania drummondii (Cheepala *et al.*, 2004) and Vitextrifolia

(Hiregoudar *et al.,* 2006). Similar differential response was observed when combinations of different cytokinins were tested in Garciniaindica (Malik *et al.*, 2005). Different plant growth regulators used for induction, modification, and suppression of morphogenesis has been reported for a variety of plant species. TDZ has stimulating the multiple shoot formation in several medicinal and aromatic plant species reported by Faisal, (2006). The formation of stunted shoots, or the fasciation of the shoots formed on BAP-supplemented medium, has been reported for several plant species such as Rhododendron spp. (Preece and Imel, 1991) and Dalbergiasisoo (Pradhan *et al.*, 1998).

2.2.1. Plant growth regulator for callus initiation

The first report of viable callus culture was reported by Gautheret (1939) and White (1939) in tobacco and carrot, respectively. An *in vitro* propagation involving an unorganized callus phase may produce variant plants *i.e.* somaclonal variants, clonal *in vitro* propagation is achieved by using meristems and axillary buds(Kumar and Kumar,1998).Callus formation is controlled by the level of plant growth regulators (auxin and cytokinins) in the culture medium (Smith,1992).Auxin (IAA,NAA,2,4-D,orIBA) is required by most plant but 2,4-D is widely used for callus induction (Smith,1992).

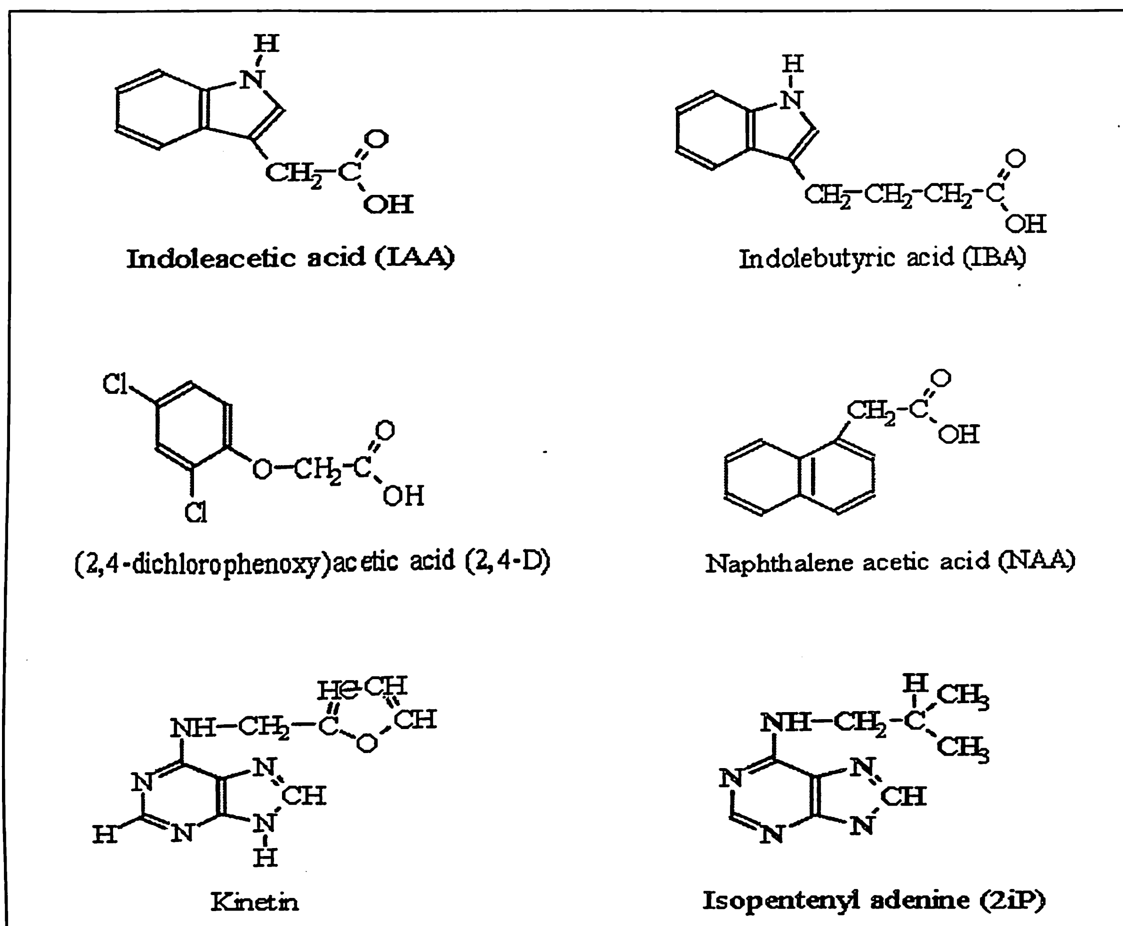


Fig: 2.3 the examples of auxin (IAA, IBA, 2, 4-D and NAA) and cytokinin (Kinetin and 2iP)

The effects of auxins and cytokinins on shoot multiplication of various medicinal plants have been reported by many researches. Benjamin, *et al.*, (1987) has shown that 6-Benzylaminopurine (BA), at high concentration (1–5ppm), stimulates the development of the axillary meristems and shoot tips of *Atropa belladonna.* Lal, *et al.,* (1996) observed a rapid proliferation of callus in *Picrorhiza kurroa* using kinetin at 1.0–5.0mg/l. Borthakur *et al.,* (2002) reported the direct plantlet regeneration from male inflorescences of medicinal yam on medium supplemented with 13.94mM kinetin has also been reported. Similarly, it has been observed that cytokinin is required, in optimal quantity, for shoot proliferation in many genotypes but inclusion of low concentration of auxins along with cytokinin triggers the rate of shoot proliferation. (Rout *et al.*, 1997; Rout *et al.*, 1999; Shasany *et al.*, 1998)

2.2.2. Callus-mediated organogenesis

Callus induction and subsequent differentiation and organogenesis is accomplished by using the differential application of growth regulators and the control of conditions in the culture medium. With the stimulus of endogenous growth substances or by addition of exogenous growth regulators to the nutrient medium, cell division, tissue growth and differentiation are obtained. There are many reports on the regeneration of various medicinal plants via callus culture. Satheesh and Bhavanandan, (1988) have reported the regeneration of shoots from callus of *Plumbago rosea* using appropriate concentrations of auxins and cytokinins. Mantell and Hugo, (1989) have also reported a high frequency of shoot, root, and micro tuber production from *Dioscorea alata* depending on the culture medium used, the type of explant from which the calli originated, and the photoperiod. Ghosh and Sen, (1994) established plant regeneration via callus cultures from different explants of *Asparagus cooper. In vitro* organogenesis of *Zingiber officinale* via callus culture has been reported by Rout *et al.*, (1997).

2.2.3. *In vitro* culture of *Andrographis paniculata*

Conventional vegetative propagation is very limited and slow to meet the demand required and also variability among seed derived progenies (Jayanthi and Mandal, 2001). *In vitro* propagation of *Andrographis paniculata* (Burm.f.) Wall ex Nees through somatic embryogenesis, and influence of 2, 4-dichlorophenoxyacetic acid (2, 4-D) on induction, maturation, and conversion of somatic embryos were investigated. The concentration of 2, 4-D in callus induction medium determined the induction, efficacy of somatic embryogenesis, embryo maturation, and conversion. Friable callus initiated from leaf and internode explants grown on Murashige and Skoog (MS) medium supplemented with 2.26, 4.52, 6.78, and 9.05 mM 2, 4-D started to develop embryos at 135, 105, 150, and 185 d, respectively, after explant establishment. Callus initiated at 13.56 mM 2,4-D did not induce embryos even after 240d, whereas those initiated on MS medium with 4.52 mM 2,4-D was most favourable for the formation and maturation of somatic embryos. Callus subcultured on the medium with reduced concentration of 2, 4-D (2.26 mM) became embryogenic. This embryogenic callus gave rise to the highest number of embryos (mean of 312 embryos) after

being transferred to half-strength MS basal liquid medium. The embryos were grown only up to the torpedo stage. A higher frequency of embryos developed from callus initiated on 2.26 or 4.52 mM 2,4-D underwent maturation compared to that initiated on higher concentrations of 2,4-D. The addition of 11.7 mM silver nitrate to half-strength MS liquid medium resulted in 71% of embryos undergoing maturation, while 83% of embryos developed into plantlets after being transferred to agar medium with 0.44 mMN 6-benzyladenine and 1.44 mM gibberellic acid. Most plantlets (88%) survived under field conditions and were morphologically identical to the parent plant. (Martin., 2003). Conventional propagation of *Andrographis paniculata* is limited to vegetative means, which is difficult and show in meeting the commercial quantities required reported by Martin, (2004). Propagation of medicinal crops where there are limitations of conventional propagation. The modern tools of biotechnology can be exploited to improve quality and content of bioactive compounds of medicinal crops once a protocol for its in vitro regeneration is available. The use of *Andrographis paniculata* is increasing day to day as result over exploitation from natural habitat (Chauhan, 2009).

Kanjilal *et al* (2002) reported that the heavy demand of andrographolid in India as well as international market has motivated Indian farmers to start commercial cultivation of this medicinal plant. Except for the preliminary study of micropropagation by Martin (2004); Purkayastha *et al.* (2008); Kataky, (2010) and Handique (2011) and Basu and Yogananth (2011), no comprehensive *in vitro* studies have been reported on *Andrographis paniculata*. Behera *et al* (2010) reported similar composition of trace elements among in vivo and *in vitro* regenerated roots of *Andrographis paniculata*.

A rapid and efficient method for the large-scale propagation of an endemic medicinal plant, Andrographis neesiana Wight, through in vitro culture of nodal explants obtained from 30-d-old aseptic seedling has been developed. High frequency direct shoot proliferation was induced in nodal explants cultured on Murashige and Skoog's medium supplemented with thidiazuron. Amongst the various cytokinins tested (BAP, kinetin, thidiazuron and 2-isopentyl adenine), thidiazuron proved to be the most effective. The shoot forming capacity of the nodal explants was influenced by the thidiazuron

concentration (1-12.5 μM) and the optimal response was observed at 10 μM thidiazuron, which induced an average of 34 shoots in 94% of the cultures within 4 week. Significant differences were recorded in terms of average number of shoots per explant (8.6-34.1) among the different concentrations of thidiazuron investigated. Concentrations of all cytokinins tested reach a level that can be considered above the optimum level, as marked by a reduced frequency of shoot proliferation. The multiple shoots obtained on various concentrations of thidiazuron failed to elongate even after transfer to hormone-free MS medium. Elongation of the induced shoots was achieved on MS basal medium supplemented with 1.0 μM GA_3 within 2 week. A proliferating shoot culture was established by repeatedly 3 subculturing the original nodal explants on shoot multiplication medium after each harvest of the newly formed shoots. The explants retained their morphogenic potential even after three harvests. Therefore, in 90 d, about 60-70 shoots were obtained from a single nodal explant and the nodal explants from primary shoots further regenerated equivalent number of shoots, depicting their high frequency regeneration potential in *Andrographis neesiana.* Rooting was best induced in 94% of shoots cultured on MS medium supplemented with 2.5 μM indole-acetic acid (IAA), within a week. The plantlets were successfully transferred to soil after hardening with a 92% survival rate. The system is rapid: the initiation of shoot buds to the transplanting of regenerants to soil is completed in 8-9 wk. (Karuppusamy and Kalimuthu, 2010)

The efficiency of shoot regeneration in *Andrographis paniculata* was tested on the Murashige and Skoog (MS) medium supplemented with 6-benzylaminopurine (BAP), thidiazuron (TDZ), kinetin (Kn) and 2-isopentenyl adenine (2-iP) at concentrations of 0.5, 1.0, 2.0, 5.0 and 10.0 μM and BAP (1.0 μM) in combination with other cytokinins like TDZ, Kn and 2-iP (0.5, 1.0, 2.0, 5.0 and 10.0 μM) by using nodal explants. Maximum number of 39 shoots per explant was recorded on MS medium supplemented with BAP (1.0 μM) and Kn (5.0 μM). An anatomical study confirmed shoot regeneration via direct organogenesis. Regenerated shoots were cultured on MS medium supplemented with 1-naphthaleneacetic acid (NAA), indole-3- acetic acid (IAA) and indole-3-butyric acid (IBA) at concentrations of 0.5, 1.0, 2.0 and

5.0 μM for the induction of roots. Were rooted after transfer to half strength MS medium supplemented with IBA (2.0 μM). The rooted plantlets were successfully acclimatized and established in soil. Randomly amplified polymorphic deoxyribonucleic acid (DNA) (RAPD) analysis was carried out to check for possible genetic alterations in regenerated plants and the results revealed that the recovered plants did not exhibit any type of polymorphism. The andrographolide content was determined in regenerated plants using high performance liquid chromatography (HPLC) and regenerated plants had considerable amount of andrographolide, so regenerated plants could be used as raw material for andrographolide extraction (Vijayalaxmi and Hosakatte, 2012).

The roots were induced from the shoots maintained on full strength MS medium without auxins but the roots were very thin. The rooting was best on half strength MS medium supplemented with IAA, IBA and NAA, in *Andrographis paniculata* reported by Purkayastha *et al* (2008), Kataky, (2010) and Handique (2011), Basu and Yogananth (2011). Praveen *et al* (2009) reported that the adventitious roots were induced directly from leaf segment of *Andrographis paniculata* on MS medium with 5.3 μM α-naphthalene acetic acid (NAA) and 30 gm/l sucrose and highlighted the great potentiality of adventitious root cultures for the production of andrographolide.

Micropropagation is the proven method for efficient in vitro propagation of medicinal and aromatic plants and for commercial exploitation of valuable plant-derived pharmaceuticals reported by Rout, (2002) and Faisal *et al.*, (2005.). Micropropagation through somatic embryogenesis is an option for the rapid production of uniform plants. True-to-type nature of the somatic embryo-derived plantlets has been reported (Jayanthi and Mandal, 2001; Tokuhara and Mii, 2001). The preliminary study of micropropagation first reported by Prathanturarug *et al*; (1996), no comprehensive *in vitro* studies has been reported on *Andrographis paniculata*. Rapid propagation protocol for *Andrographis paniculata* through somatic embryogenesis, the present study emphasizes the influence of 2, 4-D concentration on somatic embryogenesis, with respect to induction, maturation, and conversion of embryos into plantlets reported for a variety of plant species.

2.3. Production of secondary metabolites

Higher plants produce a great variety of secondary products which often have an ecological role, such as attractant of pollinators and chemical defense against microorganisms, insects and higher predators. Many of these natural products have been used as sources of large number of industrial products, including agricultural chemicals, pharmaceuticals and food additives. The last 50 years there has been an increasing interest among scientists to produce high value natural plant products through cell and organ culture to overcome many problems associated with industrial production of these phytochemicals by extraction from field grown plants.

Different strategies, using *in vitro* systems, have been extensively studied with the objectives of improving the production of valuable secondary metabolites. Plant cell culture technologies were introduced at the end of the 1960s as a tool for both studying and producing plant secondary metabolites. Many studies have been undertaken with the objective of improving the *in vitro* production of plant secondary compounds Adventitious roots have merit as production system with stable productivity, being in a differentiated state, and being genetically more stable than callus cells. Undifferentiated cell cultures such as callus and cell suspension have been mainly studied, but a large interest has also been shown in hairy roots and other organ cultures (Bourgaud *et al.*, 2001). Among these techniques employed, manipulation of nutrient media, optimization of culture conditions, identification of the most effective elicitors and the use of hairy root culture have been given considerable attention.

2.3.1 Production of secondary metabolites from medicinal plants

Plants are the traditional source of many chemicals used as pharmaceuticals. Most valuable phytochemicals are products of plant secondary metabolism. The production of secondary metabolites *in-vitro* can be possible through plant cell culture (Barz *et al.,* 1981; Deus, 1982). Successful establishment of cell lines secondary compound since cell suspension cultures has been reported by Zenk *et al.,* (1978). The accumulation of secondary products in plant cell cultures depends on the composition of the culture medium, and on environmental conditions (Stafford *et al*, 1986). Strategies for

improving secondary products in suspension cultures, using different media for different species, have been reported by Robins, (1994). The production of secondary metabolites in plant cell suspension cultures has been reported from various medicinal plants. The production of so lasodine from calli of *Solanum eleagni folium*, and pyrrolizidine alkaloids from root cultures of *Senecio* sp. Are examples (Nigra, 1987; Toppel, 1987). Cephaelinan demetine were isolated from callus cultures of *Cephaelisipe cacuanha* (Jha, 1988). Scragg, *et al* (1992) isolated quinolone alkaloids insignificant quantities from globular cell suspension cultures of *Cinchonal edgeriana.* Enhanced in alkaloid biosynthesis in the suspension culture of *Catharanthus roseus* has also been reported by Zhao (2001). Ravishankar and Grewal, (1991) reported that the influence of media constituents and nutrients tress influenced the production of diosgenin from callus cultures of *Dioscorea deltoidea*. Parisi *et al.*, (2002) achieved high yields of proteolytic enzymes from the callus tissue culture of garlic (*Alliumsativum* L.) on MS medium supplemented with NAA and BAP. Pradel *et al* (1997) observed that the biosynthesis of cardenolides was maximal in the hairy root cultures of *Digitalis lanata as* compared to leaf. The production of *Azadirachta* and nimbin has been shown to be higher in cultured shoots and roots of *Azadirachta indica* compared to field grown plant reported by Srividya, (1998). Pande *et al* (2002) reported that the yield of lepidine from *Lepidium sativum* Linn depends upon the source and type of explants.

2.4. *In vitro andrographolide* production

Bhattacharya, (2012) reported that due to climatic changes and different abiotic stresses which leads to reduction in growth and development, process of germination,. seedling growth and also the variation of secondary metabolite production. Praveen *et al.,* (2009) reported that induced adventitious roots from leaf explant of *Andrographis paniculata* and developed the methodology for multiplication of adventitious root biomass in suspension cultures. Root biomass produced from suspension cultures contained 3.5-fold higher andrographolide content compared to natural plants. The study focuses great potentiality of adventitious root cultures for the production of andrographolide.

Unorganised plant tissue cultures are frequently unable to produce secondary metabolite at the same level as intact plant. Various factor are most important to produce secondary metabolites such as carbon source and it's concentration, the ionic concentration of medium, light phytoharmone, the pH of the medium are known to influence growth and secondary metabolism. (Christen *et al.*, 1992; Toivonem *et al.*, 1991; Rhodes *et al.*, 1994; Arroo *et al.*, 1995. Heavy metal ions and concentration of phosphate, nitrate, and ammonia have also important for secondary metabolite production (Christen *et al.*, 1992; Toivonem *et al.*, 1991; Payen, *et al.*, 1987.) The addition of auxin and elicitor often increase the level of secondary metabolite (Dymove *et al.*, 1997; Pittaalvarez *et al.*, 1998).

2.4.1. *Agrobacterium*-mediated transformation and hairy root culture

The hairy system based on inoculation with agrobacterium rhizogenes has become popular in the last decades as method of producing secondary metabolite synthesized in roots. The recent advances and developments in plant genetics and recombinant DNA technology have helped to improve and boost research in to secondary metabolite biosynthesis. A major line of research has been to identify enzymes of a metabolic pathway and then manipulate these enzymes to provide better control of that pathway. Transformation is currently used for genetic manipulation of more than 120 species of at least 35 families, including the major economic crops, vegetables, ornamental, medicinal, fruit, tree and pasture plants, using *Agrobacterium* mediated or direct transformation methods (Birch *et al.*,1997) However, *Agrobacterium*-mediated transfomation offers several advantages over direct gene transfer methodologies (particle bombardment, electroporation, etc), such as the possibility to transfer only one or few copies of DNA fragments carrying the gene so if interstate higher efficiencies with lower cost and the transfer of very large DNA fragments with minimal rearrangement (Gheysen *et al* 1998; Hansen *et al* 1999; Shibata *et al*, 2000). The gram-negative soil bacteria, *Agrobacterium tumefaciens,* and the related species, *Agrobacterium rhizogenes,* are causal agents of the plant diseases crown gall tumour and hairy root, respectively. These species, which belong to the *Rhizobiaceae*, are natural engineers that are able to transform or

modify, mainly dicotyledonous plants, although there are reports on the infection of monocotyledonous plants (Tepfer *et al*, 1990; Hiei *et al* 1994; Ishida *et al.*, 1996). Virulent strains of *A. tumefaciens* and *A. rhizogenes* contain a large mega plasmid (more than 200 kb) which plays a key role in tumor induction and for this reason it was named Ti plasmid, or Ri in the case of *A. rhizogenes*. During infection the T-DNA, a mobile segment of Ti or Ri plasmid, is transferred to the plant cell nucleus and integrated in to the plant chromosome. *Agrobacterium tumefaciens* transfers the T-DNA in to the nucleus of infected cells where it is then stably integrated in to the host genome and transcribed, causing the crown gall disease (Nester *et al.* 1984; Binns *et al.,* 1988) T-DNA contains two types of genes: the oncogenic genes, encoding for enzymes involved in the synthesis of auxins and cytokinins and responsible for tumor formation; and the genes encoding for the synthesis of opines. *Agrobacterium rhizogenes* has been used regularly for gene transfer in many dicotyledonous plants (Tepfer *et al.*, 1990.) Plant infection with this bacterium induces the formation of proliferative multi branched adventitious roots at the site of infection; the so called 'hairy roots' (Chilton, 1982).The hairy root phenotype is characterised by fast harmone independent growth, lack of geotropism, lateral branching and genetic stability. This infection is followed by the transfer of a portion of DNA i.e. T-DNA, known as the root inducing plasmid (*Ri* plasmid), to the plant cell chromosomal DNA. The research is going for the application of plant transformation and genetic modification using *A. rhizogenes*, in order to boost production of those secondary metabolites, which are naturally synthesized in the roots of the mother plant. Transformed hairy roots mimic the biochemical machinery present and active in the normal roots, and in many instances transformed hairy roots display higher product yields. Genetic transformation would be a powerful tool for enhancing the productivity of novel secondary metabolites of limited yield. Hairy roots, transformed with *Agrobacterium rhizogenes*, have been found to be suitable for the production of secondary metabolites because of their stable and high productivity in hormone-free culture conditions. A number of plant species including many medicinal plants have been successfully transformed with *Agrobacterium rhizogenes*. Genetic transformation has been reported for various medicinal plants. Naina *et al* (1989) reported that the successful regeneration of

transgenic neem plants (*Azadirachta indica*) using *Agrobacterium tumefaciens* containing a recombinant derivative of the plasmid pTi A. The hairy root culture system of the medical plant *Artemisia annua* L. was established by infection with *Agrobacterium rhizogenes* and the optimum concentration of artimisin was 4.8mg/L (Cai *et al*, 1995). Giri *et al*., (2001) induced the development of hairy roots in *Aconitum heterophyllum* using *Agrobacterium rhizogenes.* Pradel *et al.* (1997) developed a system for producing transformed plants from root explants of *Digitalis lanata* by evaluating different wild strains of *Agrobacterium rhizogenes* for the productions of secondary products obtained from hairy roots and transgenic plants and reported higher amounts of anthraquinones and flavonoids in the transformed hairy roots than in untransformed roots. An efficient protocol for the development of transgenic opium poppy (*Papaver somniferum* L.) and California poppy (*Eschscholzia californica* Cham.) root cultures using *Agrobacterium rhizogenes*is reported by Park *et al* (2000) Bonhomme *et al.* (2000) has reported the tropane alkaloid production by hairy roots of *Atropabelladonna* obtained after transformation with *Agrobacterium rhizogenes.* Argolo *et al*., (2000) reported the regulation of solasodine production by *Agrobacterium rhizogenes*-transformed roots of *Solanuma viculare*. The genetic transformation study of *Atropabell adona* has been reported by using *Agrobacterium tumefaciens* for higher alkaloid composition (Yun *et al*., 1992; Cucu *et al*., 2002). *Agrobacterium* mediated transformation of *Echinacea purpurea* has been demonstrated by using leaf ex plants by Koroch *et al*., (2002). Souret *et al*., (2002) have demonstrated that the transformed roots of *A. annua* are superior to whole plants in terms of yield of the sesquiterpene artemisinin. Shi and Kintzios, (2003) have reported the genetic transformation of *Pueraria phaseoloides* with *Agrobacterium rhizogenes* and puerarin production in hairy roots. The content of puerarin in hairy roots reached a level of 1.2 mg/g dry weight and was 1.067 times the content in the roots of untransformed plants. Thus, these transformed hairy roots have great potential as a commercially viable source of secondary metabolites.

Methyl jasmonate has been shown to enhance the production of taxol and its analogues, rosmarinic acid, indole alkaloids, anthocyanin etc. in cell cultures of taxus sp, litho spermumery throrhizon and vacciniumpahale (Yukimune *et al.*, 1996), *Azadirachta indica* (Ramesh *et al* 2006). Ramesh *et al.*, (2006) has reported that azadirachitin production enhanced by the signal compounds in hairy root cultures techniques than hairy root cultures. The secondary metabolite produced by hairy roots arising from the infection of plant material by agrobacterium rhizogenes are same as those usually synthesized in intact parent root, with similar or higher yield reported by Sevon *et al.*, (1992).

3.4.2. Agrobacterium-mediated transformation

The conventional method of regenerated plants *Andrographis Paniculata* yield limited level of andrographolide. (0.7 - 2.3%) reported by Sharma *et al*, (1991). Hairy root cultures help to induce the signal compound on andrographolide production. Praveen *et al.*, (2009) demonstrated that the use of cell cultures techniques to increase the yield of andrographolide by adventitious root culture method.

Due to this immense value, it required more level in pharmaceutical to satisfy the global need. Because it present in a very low level in normal regenerated plant of *Andrographis paniculata.* Micro propagation is a proven method for efficient *in vitro* propagation of medicinal plants and for commercial exploitation of valuable plant derived pharmaceuticals. Hence in this present study aimed that to enhance the andrographolide content through hairy root culture techniques and elicitation process. Hairy root cultures induced in *Andrographis paniculata* used for determining the effect of signal compound on andrographolide production. Methyl jasmonate and salicylic acid were used in varying concentration and introduced into the 10d old hairy root cultures. HPLC studies are revealed that methyl jasmonate at 100 mM concentration shows higher efficacy (5 folds / 25d) than other increasing concentrations of same. Salicylic acid shows 8 fold (100mM) enhancements than hairy root cultures (control) (Sharmila and Subburathinam, 2013)

Zid *et al.*, (2005) has reported that the role of elicitation of andrographolide in the suspension culture of Andrographis paniculata. Hairy roots induced by *Agrobacterium rhizogenes* have received a lot of attention from plant biotechnologists for the production of secondary metabolites. The hairy roots can be indefinitely propagated on a synthetic medium without phytoharmone (Doran, 1997; Eapen and Mitra, 2001).

The main advantage of using hairy root cultures is due to their ability to grow in defined basal media without supplementation of phytoharmone and due to their differentiated nature, they show genetic stability and tend to produce high levels of secondary metabolites characteristic of the species. Effect of signal compounds on production of Andrographolide treatment with biotic or abiotic elicitors has been a useful strategy to enhance secondary metabolite production in plant tissue and cell cultures. The induction mechanism of elicitors

is generally regarded as inducing the expression of defense-related genes and activating defense-related secondary metabolic pathways reported by Namedo, (2007). Methyl jasmonate and salicylic acid have different effects on the production of secondary compounds, so that these elicitors operate in distinct plant signaling pathways and respond differently to abiotic and biotic stresses. In addition, the response to a particular elicitor may vary from plant to plant and between different cell lines; therefore, it becomes crucial to determine suitable concentrations of elicitor because increasing concentration of signal compounds inhibit the production of andrographolide. Appropriate level of signal compounds can produce more concentration of elicitors for product optimization (Qian *et al.*, 2006).

2.5. Chromatographic techniques

Chromatographic techniques are extensively used in bioanalysis for the separation, isolation and purification of drugs and their metabolites. Such techniques can provide useful preliminary information concerning the physicochemical properties of the metabolites in relation to those of the drug. Although they give little information concerning specific chemical structures, comparison of the chromatographic properties of an analyte with those of an authentic reference compound may provide sufficient information to establish the identity of a particular metabolic product. HPTLC is an improved method of TLC which utilizes the conventional technique of TL Cinmore optimized way. It is also known as planar chromatography or Flatbed chromatography.

2.5.1. TLC and HPTLC

Thin- layer chromatography (TLC) is relatively cheap, easy to use, rapid and robust. These features account for the widespread use of the technique in metabolic studies, particularly for the isolation and purification of an alytes prior to their characterization by spectroscopic techniques. A wide variety of stationary phases is available; these include silica gel, alumina and a number of bonded hydrocarbon phases (e.g. C2, C8, C18) for reversed-phase and ion exchange separations. Such phases are coated onto plastic, aluminium foil or glass supports. A recent innovation in TLC stationary phase technology involves the introduction of high performance thin-layer chromatographic (HPTLC) plates which are coated with a layer (200 μm) of 5μm particle size silica which offers

improved performance in terms of resolution and speed of chromatographic development. A number of chiral TLC phases have also been introduced. However the application of these phases in bio analytical studies has been limited. The chromatographic phases can be prepared with fluorescent indicators which facilitate the detection of ultraviolet absorbing analytes. The visualization of analytes may also be achieved by the use of a wide range of chromogenic spray reagents. The colour reactions observed with appropriate assistance in the determination of class of metabolite, e.g. glycine conjugates yield characteristic red orange colours on treatment with *p*-dimethyl amino benzaldehyde, naphtha resorcinolis used for the detection of glucuronides, potassium dichromate silver nitrate for sulphur (II) and ninhydrin for glutathione conjugates. The main application of TLC in metabolic studies is in the isolation of metabolites, for subsequent identification by spectroscopic techniques. In order to separate inorganic ions, Meinhard and Hall, (1949) used as starch binder to give some firmness to the layer and described as surface chromatography. Advances were made by Kirchner *et al*; (1951) who used the now conventional ascending method using as or bent composed of silicic acid. Reitsema, (1954) used much broader plate sand was able to separate several mixtures in one run. However, from 1956 a series of papers from Stahl appeared in the literature introducing thin layer chromatography as an analytical procedure. Since then, silica gel nach Stahl became well known as a stationary phase (Zlatkis and Kaiser, 1977). The use of TLC/HPTL Chave expanded considerably due to the development of forced flow (FF) and gradient TLC methods, stationary and mobile phase selection, as well as new quantisation methods (Poole and Poole, 1994; Sherma, 1994).

A rapid and simple high-performance thin-layer chromatographic (HPTLC) method has been developed for the simultaneous quantitative estimation of the biologically active diterpenoids, 14-deoxy-11, 12-didehydroandrographolide, andrographolide, neoandrographolide and andrographside in *Andrographis paniculata*. The assay combines the isolation and separation of andrographolide derivatives on silica gel 60 F 254 HPTLC plates with spot visualization and scanning at 540 nm. Methanol was found to be the most appropriate solvent for the exhaustive extraction of andrographolide derivatives (Sudhanshu Saxena *et al*., 2000)

A simple, rapid, selective and quantitative HPTLC method has been developed for determination of andrographolide in different samples of *Andrographis paniculata*. The alcoholic extract of *Andrographis panic ulata* (wholeplant)samples were applied on TLC Aluminium plate pre coated with Silca gel60 GF254 and developed using Toluene : Ethyl acetate : Formic acid (5 :4.5:0.5) v/v as a mobile phase. The plate was sprayed (derivatized) with Anisald ehyde- Sulphuric Acid reagent followed by heating at 110^{0}C for 10 minutes and detection and quantification were carried out densitometrically using an UV detec tor at wavelength of 235 nm. Content of marker compound in the samples were f ound similar (Pawar *et al.*, 2010)

It is widely found and cultivated in tropical and subtropical Asia, south-east Asia and India. It is wide range of pharmacological effects and some of them extremely beneficial such as anti-inflammatory, anti-diabetes, antidiarrhoeal, antiviral, antimalarial, hepatoprotective, anticancer, antihuman immunodeficiency virus (HIV), immune stimulatory and antisnakebite activity. In Physicochemical parameters studies LOD was found to be 4.54 %, Total ash content was 28.57 % and acid-insoluble was 5.45%. The water-soluble and alcohol soluble extractive values were found to be 88.27, 55.47% respectively. Other parameters like density, solvent residue were also analysed. The levels of toxic heavy metals and microbial contamination was indicated in such herbal drugs was in permissible limit as per WHO specification. The data indicated suggest that there is requirement of in process improvement to provide better quality for consumer health in order to be competitive in international markets (Arjun Singh *et al.*, 2012).

A simple, rapid, economical, precise and accurate HPTLC method has be en established or the determination of Andrographolide in *Andrographis paniculat a* whole plant powder. HPTLC methods are used for the quantitation of andrographolide reported by Handa, (1990) TLC-UV spectrophotometric determination of andrographolide in the leaves and stems of *Andrographis paniculata*. Talukadar and Dutta, (1969) reported the quantitative estimation of andrographolide by TLC. HPTLC and TLC used during crop improvement programme, quick, sensitive and accurate analytical method was required for the analysis of large numbers of plant samples for andrographolid and its derivatives. Few methods (Gaind *et al.*, 1963; Talukadar and Dutta, 1969; Zhu *et al.*, 1984;

Sharma *et al.*, 1992) have been applied for the quantitative determination of the major compound andrographolide, many of these procedures are time consuming and lack precision. Various techniques can be used for the analysis of andrographolide such as thin layer chromatography (TLC). Thin layer chromatographic methods were used for estimation of andrographolide in *Andrographis paniculata* extracts (Srivastava, 2004 and Saxena, *et al* 2000). In the present study, accurate, simple, specific and reproducible HPLC and HPTLC methods have been developed and validated ("ICH Topic Q2B Validation of Analytical Procedures: Methodology", London, 1996). The analysis of andrographolide can be done by thin layer chromatography (TLC) reported by Rajani, (2000). Choudhury, *et al.* (1987) and Puri, *et al*; (1993) reported that various techniques can be used for the analysis of andrographolide such as thin layer chromatography (TLC). Raina, (2007) reported HPTLC analysis of hepatoprotective diterpenoid Andrographolide from *Andrographis paniculata.* Vijay kumar *et al.,* (2007) also quantitated and validated andrographolide content following HPLC and HPTLC analysis.

2.6. Antimicrobial activity:

In recent years focus on use of non-traditional approaches to treat diseases has been revived worldwide. The evidence collected till now shows immense potential of medicinal plants used in traditional systems. Bacterial skin infections are common outpatient problems and the 28th most common infections diagnosis in hospitalized patients (Elixhauser *et al.*, 2001). Studies have stated that it may account for up to 17% of clinical visits (Sadick, 1997). Therapies of bacterial skin infections are frequent problems due to the emergence of resistant bacterial strains to numerous antibiotics (Marimoto et al., 1999). Some plants have shown the ability to overcome resistance in some organisms and this has led to researchers' investigating their mechanisms of action and isolating active compounds from them (Ncube et al., 2007). Antimicrobial drugs have caused a dramatic change not only in the treatment of infectious diseases but to the fate of mankind. Antimicrobial chemotherapy has made noteworthy advances, resulting in positive observations that infectious diseases might be dominated in the near future. However, in reality, emerging and remerging infectious diseases have indicated a counter charge from

infections. Infections with drug-resistant organisms hang back an imperative problem in clinical practice that is complicated to explain. If an unsuitable antimicrobial agent is preferred over the treatment of infection with drug-resistant microorganisms, the therapy may not achieve beneficial effects and may lead to a worse prognosis.

Medicinal plants represent a rich source of antimicrobial agents. Plants are used medicinally in still widely used in ethno medicine around the world different countries and area source of many potent and (Thomson, 1978; Stockwel, 1988) powerful drugs (Srivastava .*et al.*, 1996). Although compounds of natural or synthetic origin have been the hundreds of plant species have been tested for source of innumerable therapeutic agents. Random antimicrobial properties, the vast majority of have not screening as tool in discovering new biologically active been adequately evaluated (Balandrin *et al.*, 2006). Secondary metabolites synthesized by plants serve as defence mechanisms against predation by microorganisms (Cowan,1999) The effects of plant extracts on bacteria have been studied by a very large number of researchers indifferent parts of the world (Reddy *et al.*, 2001; Ateb and Erdo Urul, 2003). Much work has been done on ethno medicinal plants in India (Maheshwari *et al.*, 1986; Negi *et al.*, 1993). A number of evidences have been accumulated to demonstrate the promising potentials of medicinal plants used in various traditional, complementary and alternative systems (Kanokwan et al., 2008). As medicinal plants are gaining more importance in Pharmaceutical industries for the preparation of new phytomedicines, this study was undertaken to check its properties as a drug (Sule *et al.*, 2011).

2.6.1. Antimicrobial activity of *Andrographis paniculata*

An aqueous and two ethanolic extracts of *Andrographis paniculata,* used in traditional Chinèse, Thai and Indian medicine and andrographolide, an active principle of *Andrographis paniculata,* were investigated for their antimicrobial activity against nine bacterial spècies including *Salmonella typhimurium, Escherichia coli, Shigella sonnet, Staphylococcus aureus, Pseudomonas aeruginosa, Streptococcus pneumoniae, Streptococcus pyogenes, Legionella pneumophila* and *Bordetella pertussis,* using the disc diffusion method. Of all tested concentrations, direct antimicrobial activity of the two ethanolic *Andrographis paniculata* extracts was observed for only two

human pathogens *and Bordetella pertussis.* Given that the TLC of *Andrographis paniculata* extracts showed that andrographolide was present in all the three *Andrographis paniculata* extracts, it was concluded that the observed antimicrobial activity was due to other active principle(s) present in the extracts used in this investigation (Youhong Xu, *et al.*, 2006).

Non-polar (dichloromethane) and polar (MeOH and aqueous) extracts of *Andrographis paniculata* (whole plant) were evaluated for *in vitro* antibacterial activity against 10 skin disease causing bacterial strains (6 gram positive strains; *Staphylococcus saprophyticus, Staphylococcus. epidermis, Staphylococcus aureus, Streptococcus pyogenes, Bacillus anthracis, Micrococcus luteus*) and 4 gram negative strains (*Proteus mirabilis, Proteus vulgaris, Neisseria meningitis, Pseudomonas aeruginosa*) using disc diffusion method at three different concentrations; 1000, 500 and 250 µg/disc respectively. The extracts showed significant antibacterial activities against both Gram-positive and Gram-negative bacterial strains tested. Highest significant antibacterial activity was exerted by the aqueous extract against *M. luteus* at 1000 µg/disc and the least activity was exhibited by the DCM extract against *N. meningitis* at 250 µg/disc. The minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) observed were between 150 to 300 µg/ml and 250 to 400 µg/ml respectively, depending on microorganism and the nature of various extracts. *Andrographis paniculata* extracts have bactericidal characteristic against most of the gram -positive bacteria and bacteriostatic activity against both gram-negative and gram positive bacteria. These results candidly suggest the presence of promising antibacterial substances in the polar as well as non-polar extracts which could be the source of potential phytomedicines for the treatment of skin infections caused by the pathogenic bacterial strains. Our findings explicitly support its traditional claims and form a strong basis for further sincere efforts to explore *Andrographis paniculata's* antibacterial potential to treat skin frailties efficaciously (Abubakar Sule, *et al.*, 2010).

The present study has been designed with the objective to examine the petroleum ether, acetone, chloroform and methanol extracts of *Andrographis paniculata* leaves and stems, in order to evaluate the chemical composition, investigate it's *in vitro* antimicrobial potential against strains of *Enterococcus faecalis, Streptococcus pyogenes, Klebsiella pneumonia, Proteus vulgaris,*

Candida albicans and Aspergillus flavus. Phytochemical analysis revealed the presence of flavonoids, alkaloids, glycosides, steroids, phenols, tannins and saponins. The antibacterial activity is more significant against Gram positive bacterium Enterococcus faecalis whereas the antifungal activity is more significant against *Aspergillus flavus*. These results may justify the popular use of this species as it has antimicrobial activity. However, in order to evaluate possible clinical application in therapy of infectious diseases, further clinical trials are required (Radha *et al.*, 2011).

Samples of *Andrographis paniculata* extracts, obtained by extraction in methanol, respectively, were used for their antimicrobial activity. The antibacterial activities were assessed by measuring the diameter of the inhibition zones, MIC and MBC values. This is the first report on analysis of antimicrobial components from *Andrographis paniculata*, and our results confer the utility of this plant extract in developing a novel broad spectrum antimicrobial agent. Antimicrobial activity of leaf extract of *Andrographis paniculata* was studied using different solvent like chloroform, acetone, ethanol and water against bacterial strains Like *Staphylococcus aurous, Escherichia coli, Pseudomonas aeruginosa, Streptococcus sp., Micrococcus luteus, Bacillus sp.,* and two trains of fungi which are *Saccharomyces cerevisae* and *Aspergillus niger*. The antimicrobial activity was determined by disc diffusion method. Out of the four extract used, acetone and ethanol extracts were found to be highly active against *Staphylococcus aureus* and *Bacillus subtilis* (Ravi *et al*) (www.ijrrpas.com)

The ethanolic extract was able to inhibit *Escherichia coli, staphylococcus aureus and micrococcus uteus*. The ethanolic extracts were screened for their in vitro antioxidant potential. Inhibition of oxygen derived free radicals, viz., assay for free radical scavenging by 2, 2- diphen 1 picryl hydrazyl (DPPH), reducing power ability and nitric oxide scavenging were performed. The antioxidant activity was compared with standard antioxidant such as D- ascorbic acid. The ethanolic extract elucidated agreeable antimicrobial activity against four human pathogenic bacterial strain experimented (Vijayakumar and Kalaichelvan, 2011).

The antifungal activity of extracts of *Andrographis paniculata* was evaluated by Agar well diffusion method against five selected fungal species. Stem extracts of *Andrographis paniculata* showed high antifungal activity against *Aspergillus oryzae*, *Penicillium sp* and C.albicans. The root extracts showed high

antifungal activity against *Aspergillus niger, Aspergillus flavus, Candida albicans, Penicillium sp* and *Aspergillus oryzae* and also leaf extracts showed high antifungal activity against *Penicillium sp* and *Aspergillus flavus* but did not show antifungal activity against *Candida albicans, Aspergillus niger, Aspergillus oryzae.* The results obtained in the present study suggest that *Andrographis paniculata* plant can be used in treating various diseases caused by the test organisms (Rajalakshmi *et al.*, 2012).

Antimicrobial activity of leaf extract of *Andrographis paniculata* Wall. was studied using different solvent like chloroform, acetone, ethanol and water against bacterial strains like *Bacillus subtilis, Staphylococcus aureus, Pseudomonas aeruginosa* and fungal strains *Aspergillus niger and Penicillium chrysogenum.* The antimicrobial activity was determined by disc diffusion method. Out of the four extract used, acetone and ethanol extracts were found to be highly active against Staphylococcus aureus and Bacillus subtilis. Highest in acetone (12 mm) and lowest in ethanol (10 mm). The MIC values were obtained by serial dilution method (Hosamani *et al.,* 2011*)*

Tapsell *et al* (2006) reported that wide use by the traditional clerics in treating some infections of the skin have prompted us to choose and confirm this plant for further evaluation in order to ascertain its antibacterial potential to treat skin infections caused by some pathogenic bacterial strains. Ethanolic extract of the leaves of *Andrographis paniculata* was reported to inhibit growth of *Escherichia coli* and *Staphylococcus aureus*; while, Methanolic extract was effective against *Proteus vulgaris.* Komwatchara 1996 and Rassameemasmaung (1996) reported that *Andrographis paniculata* has inhibitory effect against *Porphyromonas gingivalis.* Prajjal *et al* (2003) reported significant antimicrobial activity of aqueous extract of the species containing andrographolide and arabinogalactan proteins. Roy *et al* (2010) also assessed the antimicrobial activity from inhibition zones, minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) values of *Andrographis paniculata* extracts (chloroform and chloroform+HCl) on four gram positive bacteria (*Staphylococcus aureus, Bacillus subtilis, Enterobacter faecalis, S. epidermidis*) as well as five gram-negative bacteria (*Escherichia coli, Pseudomonas aeruginosa, Klebsiella pneumoniae, Salmonella typhimurium, Enterobacter cloacae*) and suggested the utility of the species

in development of novel broad spectrum antimicrobial agents. Extracts of this plant and andrographolide exhibit pharmacological activities such as antibacterial (Singha, 2003). Singha *et al* (2003) found the significant antibacterial activity in an aqueous extract with andrographolide. A similar result was found in a crude aqueous extract of leaves that exhibit significant antimicrobial activity against gram-positive *S. aureus, methicillin-resistant S. aureus,* and *gram-negative Pseudomonas aeruginosa* (Zaidan *et al.*, 2005). Significant activity against *enterohemorrhagic* strains of *E. coli* was found in the ethanol extract of *Andrographis paniculata* (Voravuthikunchai and Limsuwan, 2006). The virucidal activity of andrographolide has been reported against herpes simplex virus 1 (HSV-1) without having any significant cytotoxicity (Wiart *et al.*, 2005). At a concentration of 0.05□mg/mL of a chloroform extract of *Andrographis paniculata*, the plant completely inhibits malarial parasitic growth within 24□h of incubation; and the same inhibition has been noted within 48□h with methanol extract concentration of 2.5□mg/mL (Rahman *et al.*, 1999). A methanol extract was found to inhibit Plasmodium falciparum substantially at a 50% inhibitory concentration (IC50) of 7.2 µg/mL (Mishra, 2009). Antibacterial activity of phytochemical andrographolide, a labdane diterpenoid (Xu *et al.*, 2006) isolated from plant materials. *In vitro* study corroborates the antibacterial activity of *Andrographis paniculata* used in folkloric medicine to treat skin infections reported by Jain (1991) and Ahmed *et al.* (1998). All of *Andrographis paniculata* extracts were shown to exhibit inhibitory activity against most of the pathogenic bacteria which cause chronic bacterial skin infections. Mishra *et al.* (1992) reported antimalarial activity of *Andrographis paniculata* (kalmegh) against *Plasmodium berghei* NK65 Singhal, *et al* (2003) Antibacterial activity of *Andrographis paniculata, Fitoterapia*, natural products from plants, are being investigated because medicinal plants have been widely used for treatment of many types of acute and chronic diseases in Asia and many plants with antimicrobial activity have been reported (Cowan, 1999). The antibacterial activity of aqueous, ethanol or methanol extracts of *Andrographis paniculata* has been tested *in vitro* against *Escherichia coli, Staphylococcus aureus, Proteus vulgaris, Salmonella typhi, Shigella* species and *Pseudomonas aeruginosa in vitro* (George and Pandalai, 1949; Nakanishi *et al.*, 1965; Prajjal *et al.*, 2003) with no antibacterial activity observed with the aqueous extract tested

against *E. coli,* S. *aureus,* S. *typhi*or *Shigella* species (Leelarasamee *et al,*1990). Subramanian *et al.*, (2008) reported that extracts of *Andrographis paniculata* were used for antibacterial studies. Limsong et al (2004) in their findings suggested that ethanolic extract of plant inhibits adherence of *Streptococcus mutans ATCC 25175* and *Streptococcus mutans TPF-1 in vitro* at the effective concentrations (0.5%). Bauer *et al.* (1966) reported that the antimicrobial activity was determined by disc diffusion method in *Andrographis paniculata.* Komwatchara, 1996 and Rassameemasmaung 1996 reported that *Andrographis paniculata* has inhibitory effect against Porphyromonas gingivalis. Prajjal *et al.* (2003) reported significant antimicrobial activity of aqueous extract of the species containing andrographolide and arabinogalactan proteins.

Many fungi are harmful as they are pathogens of plants, animals and human beings or produce metabolites that are toxic to plants and animals reported by Richard *et al.*, (1993); Bowers and Locke, (2000). Pathogenic fungi are the main infectious agents in plants, causing alterations during developmental stages including post-harvest. In fruit and vegetables, there is a wide variety of fungal genera causing quality problems related to nutritional value, organoleptic characteristics, and limited shelf life (Agrios, 2004). The bioactive compound andrographolide has been reported to be effective in the treatment of upper respiratory tract infection (Gupta *et al.*, 1990; Yin and Guo, 1993; Xie *et al.*, 1983; Chang and But, 1986; Hancke *et al.*, 1995; Melchior *et al.*, 1997; Melchior *et al.*, 2004; Thamlikitkui *et al.*, 1991; Poolsup *et al.*, 2004; Coon and Ernst, 2004). Antifungal activities of the *Andrographis paniculata* extracts via ethanol were determined, using the agar well diffusion assay method reported by Perez *et al.*, (1990). The antifungal activity was significantly recorded in stem and root extracts. This might be due to the better solubility of the active components in the organic solvents (De Boer et al., 2005). The root extract revealed the higher degree of antifungal activity for five fungal spp. (*Candida albicans, Aspergillus niger, Aspergillus flavus, Aspergillus oryzae and Penicillium fluorescence*).

Roy *et al* (2010) also assessed the anti-microbial activity from inhibition zones, minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) values of *Andrographis paniculata* extracts (chloroform and chloroform+HCl) on four gram positive bacteria

(*Staphylococcus aureus, Bacillus subtilis, Enterobacterfaecalis, S. epidermidis)* as well as five gram-negative bacteria (*Escherichia coli, Pseudomonas aeruginosa, Klebsiella pneumoniae, Salmonella typhimurium, Enterobacter cloacae*) and suggested the utility of the species in development of novel broad spectrum antimicrobial agents. An ethanol extract of the leaves inhibited the growth *in vitro* of *Escherichia coli and Staphylococcus aureus* reported by George and Pandalai (1949). Leelarasamee, (1990) reported that no *in vitro* antibacterial activity was observed when dried powder from the aerial parts was tested against *E. coli, Staphylococcus aureus, Salmonella typhi or Shigella* species.

Andrographis paniculata has significant inhibition for a gram positive microbes, *Staphylococcus aureus* and *Bacillus subtilis*. The good antimicrobial activity of the *Andrographis paniculata* leaf extract against *Staphylococcus aureus* and *Bacillus subtilis* is an indication that the leaf extract is beneficial as a cure for skin diseases. wide spectrum of inhibition against both Gram-positive bacteria leaf extract of *Andrographis paniculata* is worthy of further investigation as a natural wide spectrum antibacterial agent in the treatment of infectious disease reported by Sule *et al* (2011). Zaidan et *al*., (2005) who found crude aqueous extract of leaves exhibit significant antimicrobial activity against gram-positive *Staphylococcus aureus*, methicillin-resistant *Staphylococcus aureus* (MRSA), and gram-negative *Pseudomonas aerugi-nosa*, but had no activity against *Escherichia coli or Klebsiella pneumoniae*. The ethanol extract was also devoid of significant activity against *enterohe-morrhagic* strains of *E. coli* reported by Voravuthikunchai and Limsuwan (2006). The minimum inhibitory concentration was determined by serial dilution method (Rollins and Joseph, 2000).

CHAPTER-III

Material & Methods

3. MATERIALS AND METHODS

The present study was carried out at Department of Agricultural Biotechnology, College of Agriculture, Orissa university of Agriculture and Technology, Bhubaneswar, Odisha. The details of materials and methods and the experimental techniques are presented below.

Selection of plant:

Medicinal plant was selected for the study namely *Andrographis paniculata*

Taxonomy of plants:

Taxonomic hierarchy

Kingdom	:	Plantae
Division	:	Angiospermae
Class	:	Dicotyledoneae
Order	:	Tubiflorae
Family	:	*Acanthaceae*
Genus	:	*Andrographis*
Species	:	*paniculata Nees*

Morphology of leaves of *Andrographis paniculata*: The leaves of *Andrographis paniculata* are dark in colour, simple, opposite, lanceolate, glabrous, 2–12cm long, 1–3cm wide; acute apex, entire margin. Flower consists of small, linear 5-particle Calyx; tube narrows, about 6 mm long white corolla with violet markings. Two stamens, inserted in the throat and two celled superior ovary. 1–2 cm long, 2–5 mm wide, linear-oblong, compressed, erected capsule. (Bhardwaj *et al*; 2011) (Plate 1) Microscopy of *Andrographis paniculata*. The leaves of *Andrographis paniculata* have diacytic stomata at leaf's lower epidermis, glandular and non-glandular trichomes, fairly large cystoliths, columnar palisade cells, colleen chymas in midrib beneath epidermis; spongy parenchyma cells; vascular bundles of lignified spiral, scalar form and reticulate

xylem vessels in the upper part and lignified phloem in the lower part, small acicular calcium oxalate crystals, a layer of wavy-walled lower epidermis cells, dense collenchyma at the corners of stems, a layer of thick-walled endodermis cells and parenchyma contains chloroplastid. (Bhardwaj *et al*; 2011).

3.1. Materials:

3.1.1 Plant material

The plant *Andrographis paniculata* was obtained from the medicinal garden of Orissa University Agriculture and Technology (OUAT) under All India Coordinated Project on Medicinal Plants in the month of May-June 2012 and was identified and authenticated by Dr. Das, Associate Professor, Dept. of Floriculture and Landscaping, OUAT, Bhubaneswar.

3.1.2. Explants source:

Leaf, shoot tip and nodal explants were collected from the field grown plant of *Andrographis paniculata* and were used for *in vitro* experiments. (plate 2) Matures seeds first soak in LPGM (liquid plant growth medium)(Appendix 6) for two days. After two day, it was grown on the filter paper in sterile condition and then after grown on MS medium but problem is *Andrographis paniculata* seeds not germination is so late up to four week.(Plate3) The 30 days old, *in vitro* grown seedling was used further for *in vitro* studies. (Plate 3) Coteledonary leaves, hypocotyl and coteledonary nodes were used for callus culture, propagation and induction of roots.

3.1.3. Plant nutrient media:

For *in vitro* studies, two basal media were used to carry out the different experiments. Murashige and Skoog (1962) basal salts (MS) were used. (Appendix 1)

3.1.4. Plant growth regulators:

For the standardization of *in vitro* plant regeneration, different auxins like indole-3-aceticacid (IAA), 1-naptheleneacetic acid (NAA) and 2,4- dichloro phenoxy acetic acid (2,4-D) , indol 3-butaric acid (IBA) and cytokinins like 6-

benzyl aminopurine (BAP) and kinetin (Kn) and gibberellic acid (GA_3) at different concentrations were used. (Appendix 2)

3.1.5. Antibiotics (source: M.P. Biomedical)

Carbenicillin and Cefotaxime used for the experiment were purchased From M.P. Biomedicals and HIMEDIA respectively and appropriate stocks were prepared. Stocks of antibiotic were prepared as per the following: (Appendix 2)

Carbenicillin: 1 gm of carbenicillin was dissolved in 50% ethanol. Filter sterilised by using syringe filter in to sterile eppendroff tube aseptically and store at 4^0 c.

Cefotaxime: 1 gm of Cefotaxime was dissolved in double distil water. Filter sterilised by using syringe filter in to sterile eppendroff tube aseptically and store at 4^0 c.

3.1.6. Test microorganisms:

The bacterial strains and the fungal strains were collected from germplasm centre of Dept. of Microbiology and Dept. of plant Pathology OUAT, Bhubaneswar. The bacterium used was *E.coli, Bacillus subtilus, Xanthomonas oyizae, Pseudomonas fluorescence.* The fungal strains used were *Aspergillus niger, Risoctonia solani, Penicillium notatum, Verticillium leucani.*

3.1.6.1. Culture medium

The media used for bacterial test was nutrient agar / broth, yeast extract mannitol agar / broth (Appendix 3 and 4) and media used for antifungal test was Potato dextrose agar (PDA) / broth. (Appendix 5)

3.1.7. Phytochemicals analysis

The HPTLC silica gel 60 F254 plates (Aluminium backed, layer thickness 200μm) and TLC silica gel 60 F254 plates (Aluminium backed, layer thickness 200μm) used for phytochemical analysis and HPTLC, TLC plate was procured from Merck Company.

3.1.7.1. Chemical / Reagents

Various organic solvent like Methanol, Ethyl acetate, Toluene, Glacial acetic acid, Vanillin, Sulphuric acid, Formic acid, p-Anisaldehyde procured from

Spectrochem, HI media company were used for Soxhelet- solvent extraction of phytochemicals.

3.1.7.2. Standards of secondary metabolites:

Andrographolid was received from Dr. M.M.Gupta, Chief Scientist and Dr. Ajit K. Shasany, Scientist-F, CIMAP, Lucknow, Uttar Pradesh.

3.1.8. Agrobacterial strain and plasmid involved:

Agrobacterium rhizogenes 532 (MTCC) harbouring root inducing plasmid (*Ri*-plasmid) received from Microbial Type Cell Cultures, Chandigarh which was used for *in vitro* transformation.

Strain MTCC 532 details:

- Wild type strain
- Media: Nutrient agar pH 7.2
- Incubation time: 24 hours
- Temperature: 25^{0}C
- Subculture: 30 days

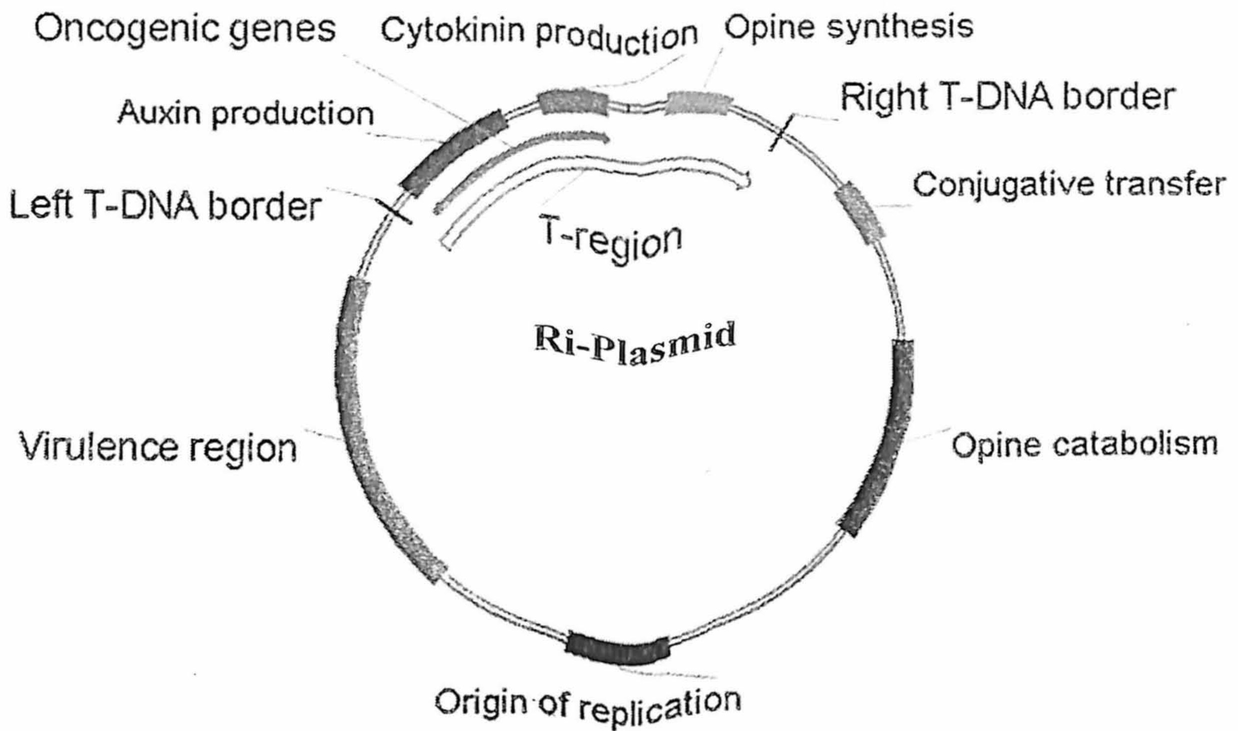
3.1.9. Primers for PCR analysis

Rol B primers-

Fig-Schematic map of *ri* plasmid

Forward: 5'ATGGATCCCAAATTGCTATTCCTTCCACGA 3'

Reverse: 3'TTAGGCTTCTTTCTTCAGGTTTACTGCAGC 5'



3.2 Methodology:

3.2.1. *In vitro* culture and plant regeneration:

3.2.1.1. Viability Studies of Seed

The seeds were kept under running tap water for 25- 30 minute in a beaker with a muslin cloth tied at moth.Then the seeds were surface sterilized with 0.5 % (v/v) Tween-20 detergent. This was followed by bavistin 2% (w/v) treatment for 20 minute. After this all treatment were carried out under laminar air flow. The seeds were treated three to four times with double distil water, then 0.2 % (w/v) $HgCl_2$ 3 minute then wash the seeds with double distil water for three times. Then, seeds again treated with 70% ethanol (Appendix 11) for one minute. Finally seeds again washed with double distil water three to four times. The washed seeds were germinated three different methods:

1. Transferred in petri dishes containing absorbent cotton wool covered with a blotting paper with the help of sterilized forceps, The pad was moistened with double distilled water and sufficient space was maintained in between the seeds to provide sufficient are a for germination.

2. The seeds were transferred in the 10 ml screw cap sterile bottle containing 7 ml LPGM (liquid plant growth media) with 100 µl gibberellic acid (GA_3) after imbibitions of seeds (after 2-3 days for imbibition) the seeds were transferred on to the MS (Murashige and Skoog, 1962) medium containing 100 µl gibberellic acid (GA_3) in jam bottle.

3. The seeds directly transferred on to the MS medium and were inocul-ted in the incubation room with temperature 25± 2^0c and 3000 lux intensity for germination.

3.2.1.2. Explant sterilization:

The seeds, leaf and shoots explants collected from field and were washed thoroughly with tap water and followed by washing with 2% (w/v) Bavistin and tween-20 (10% v/v), for 2-3 minutes . Then different explants were surface sterilized with ethanol (70% v/v) for 1 minute, followed by mercuric chloride (0.1% w/v) for 3 minutes under laminar flow. After this, the explants were washed thrice with sterile double-distilled water and the shoot tips, leaf and stem were dissected using sterilized scalpels and used as explants source.

3.2.1.3. Culture medium and conditions:

3.2.1.3. a) Stock solution preparation for MS media

The macronutrients were dissolved in 500 ml of double distilled water. Micronutrients were dissolved in 250 ml of double distilled water KI was dissolved in 250 ml of double distilled water. Minor nutrients were dissolved in 500 ml of double distilled water. Na_2 EDTA and $FeSO_4$ were dissolved separately in 100 ml of double distilled water. Na_2 EDTA solution was boiled separately and then slowly added with the FeSO4 solution and made up to 250 ml with double distilled water. Vitamins were dissolved separately in 100 ml of double distilled water. (Appendix 1)

3.2.1.3. b) Constitute of media:

MS basal salts supplemented with different growth regulators at various concentrations were used for callus induction , shoot elongation, multiplication and rooting.

Table 3.1. Composition of MS medium (Murashige and Skoog, 1962)

S.No.	Stock	Strength	Amount for 1 lit
1	Macronutrient	20X	50ml
2	Micronutrient	200X	5ml
3	Fe-EDTA	200X	5ml
4	Organic salts	200X	5ml

Appropriate volume was taken out from the prepared stocks to prepare media and volume was made up with distilled water. The media were supplemented with sucrose 2% (w/v) and 3% (w/v) along with adenine sulphate @ 100 mg/l. The pH of the media was adjusted to 5.8 using 0.1 N NaOH and 0.1 N HCl. 0.8 % agar was added to the media and was autoclaved.

3.2.1.3. c) Growth regulators used

Auxins such as NAA (50 mg), IAA (50 mg) and IBA (50 mg) were dissolved separately in 0.1 N NaOH, diluted with double distilled water and made up to 100 ml of stock solution. Similarly 2, 4-D (50 mg) was dissolved in ethanol and diluted with double distilled water and made up to 200 ml of stock solution. Cytokinins such as Kinetin (50 mg), BAP (50 mg) and GA_3 (50 mg) were dissolved separately in 0.1 N NaOH, diluted with double distilled water and made up to 100 ml of stock solution. All these growth regulators were stored in the refrigerator. From this every 2 ml has 1.0 mg/l of hormone in the stock solution. This growth harmone were used for callus induction, shoot elongation, multiplication and induction of rooting.

3.2.1.3. d) Sterilization of medium and inoculation:

The medium were autoclaved at 121^0 c and 1.06Kg cm^2pressure for 20 minutes. The molten media gelled with 0.65 % agar was dispensed (20 ml) into culture tubes (25X150 mm). The tubes were plugged with non-absorbent cotton properly and maintained at 25 ± 2^0C under a 16-h photoperiod and a light intensity of 55 mmol m-2-1 provided by white fluorescent lamps.

3.2.1.3. e) Inoculation of explants

Shoot tip, axillary bud and nodal explant (1 cm size) were inoculated in different culture tubes in vertical position on the medium while other explants like Shoot tip were placed in horizontal position. The leaf explants (1 x 1 cm) were cultured with their adaxial and abaxial surface touching the medium. Explants were sub-cultured at an interval of 4 weeks.

3.2.1.4. Direct regeneration

Shoot tip explants were used for direct regeneration and cultured on MS basal medium supplemented with different cytokinins and auxin such as BAP (1.0 to 3.5 mg/l) in combination with IAA (0.25 mg/l) and Kn (1.0 – 4.0) were used either in combination IAA (0.25 mg/l) or GA_3 (1.5 mg/l), containing sucrose 3% (w/v) agar 0.65%.

3.2.1.4.1. Shoot multiplication

Shoot tip explants were taken from 30 d old seedlings and cut in appropriate size such as 1 cm. selected explants were cultured on the MS medium supplemented with different combinations BAP, NAA, IAA, Kn and sucrose (3% w/v) for shoot multiplication. Subsequent subcultures were made at periodic intervals of 25 to 30 d. The cultures were incubated at 25 ± 2°C with 16 hrs. photoperiod of white fluorescent light (40-50 µmol m^{-2} s^{-1}), irradiance, as provided by cool white fluorescent tubes (1500 lux, Philips, India) and with 55-60% relative humidity (RH). Number of multiple shoot were excised in different explants inoculated.

3.2.1.5. Establishment and maintenance of callus cultures

The surface sterilized explants (leaves and stem) of *Andrographis paniculata* were transferred aseptically onto the MS media supplemented with NAA, BAP, and 2, 4-D at various concentrations in culture tubes. The tubes were plugged properly and incubated in dark at 25 ± 2^0C. The cultures were maintained at 25 ± 2^0C by sub-culturing the fragile globular callus pieces onto the corresponding medium at an interval of 4 weeks.

The results were recorded and callus induction frequencies were evaluated by using the following formula: no. of explants giving callus.

$$\text{Callus induction frequency} = \frac{no.of\ germinatexplant\ giving\ callus}{total\ number\ of\ explant\ inoculated} \times 100$$

3.2.1.5.1. Measurement of growth and frequency of callus induction

The growth in dices of the cultures were determined on dry weight basis (Veeresham, 2006). The growth index (G.I.) of the cultures was calculated by using the following formula:

$$\text{Growth index} = \frac{final\ weight\ of\ biomass}{initial\ weight\ of\ biomass}$$

The frequency of the callus induction was calculated based on the percentage of explants capable of being developed into the callus formation. After four weeks, the callus was subculture on MS with different concentration of 2, 4-D (1.0-4.0mg/l), NAA (1.0-4.0mg/l), BAP (1mg/l). All the treatments were supplemented with 30 g/l sucrose and the PH of the medium were adjusted to 5.7 with 0.1 N NaOH or 0.1 N HCl. The regenerations frequencies were calculated by using the following formulae:

$$\text{Regeneration frequency} = \frac{number\ of\ renerated\ calli}{total\ number\ of\ calli\ incubated} \times 100$$

3.2.1.5.2. Shoot proliferation from callus

For shoot multiplication from stem and leaf callus, MS basal medium supplemented with 3% sucrose used which were supplemented with different

growth hormone like BAP (0.5 - 3.5 mg/ml) along with NAA (1.0 mg/ml) and 50 mg/ml adenine sulphate and also on the MS media supplemented with BAP (1.0 - 3.0 mg/l) along with IAA (1.0 mg/l). Kn (1.0 -2.5 mg/l) along with NAA (1.0 mg/l).The multiple shoots (2 to 3 cm) were excised and subculture on shoot elongation medium supplemented with concentrations of BAP (2.5 mg/l) along with NAA (1.0 mg/ml) and 50 mg/ml adenine sulphate under cultured conditions.

3.2.1.6. Root induction and plant acclimatization

For root induction the explants were transferred to half-strength basal MS media supplemented with different concentrations of NAA (0.25-1.5 ppm) and IBA (0.25-1.5 ppm) and 2% sucrose. The cultures were incubated as previously described. Rooted shoots were thoroughly washed to remove the adhering gel and planted in pot containing mixture of sand, soil and farmyard manure in the ratio of 1:1:1 and kept in green house for acclimatization. About 90% of the plantlets were established well in the green house after 2 weeks of transfer. The plant grew well and attained 10-15 cm height within 8 weeks of transfer. The acclimatized plantlets showed normal growth and no sign of morphological variation were observed.

3.3. Optimization for efficient *Agrobacterium rhizogenes* mediated Transformation method:

3.3.1. Preparation of explants for the co-cultivation

Young leaves was excised from *in vitro* grown shoots cut into small segments (1.0 cm), shoot tips and callus were used as explants for transformation. (Plate 12)

3.3.2. Bacterial culture preparation:

Agrobacterium rhizogenes 532 (MTCC) was used for hairy root induction in both the plants. The bacterium was maintained on nutrient agar medium. The bacteria were streak on fresh plates from the stock plates on first day and kept for 24 hrs. incubation in 37□C in the next day, the single colony was transferred to the broth and cultured for 48 h in the dark on a rotary shaker at 90 rpm. Bacterial count of about (0.6) 108 cells /ml as measured by OD at 620 nm was

used for experiment. The bacterial suspension was centrifuged at 6000 rpm for 10 min and pellet was collected for co-cultivation experiment.

3.3.3. Co-cultivation with explants

The prepared explants were co-cultivated with *Agrobacterium* strain (MTCC 532). The pellet was suspended in LPGM (liquid growth medium) along with 100 μm acetosyringone. The explants were injured with sterile needle and co-cultivated with the pellet and wrapped with aluminium foil and incubated at 25°C for 20 minute at 90 rpm. After 20 minutes, the infected explants were taken out and blotted on sterile filter paper and transferred to the petridishes wrapped with aluminium foil on blotting paper moistened with LPGM (liquid plant growth medium) (Appendix 6) along with Acetosyringone for incubation for a period of 48 hours at 25±2°C.

3.3.4. Explants washing:

After 2 days incubation, the infected explants were washed with LPGM (liquid growth medium) for 5 times followed by washing with cefotoxamine (400mg/l) and carbenicillin (400mg/l). (Appendix 2) Then the washed explants were blotted with sterile filter paper. The individual explants were transferred onto basal MS medium placed in the dark at 25±2°C.

3.3.5. Establishment of hairy roots cultures:

After 4 weeks, the explants develop roots at the infected sites; and were then subculture again on MS basal medium containing 250mg/l carbenicillin. Before transferring to the solid medium, the explants were rinsed with MS liquid medium containing 350 mg/l carbenicillin. Subculture was made at every 14 days interval. Hairy roots was excised and transferred to MS liquid medium and kept in a rotary shaker at 80 rpm in dark or light conditions (12 h photoperiod) for 35 days. The cultures were harvested and secondary metabolite content was determined.

3.3.6. Isolation of plasmid DNA from Agrobacterium rhizogenes: (Alkali lysis method Sambrook and Russel, 2001)

- A single *Agrobacterium rhizogenes* colony was picked up aseptically using a sterile inoculation needle and was grown over night in10ml nutrient agar in a sterile conical flask. (Appendix 4)
- Overnight grown culture was transferred to centrifuge tube and centrifuged for 15 min at 10,000rpm.
- The supernatant was removed and cell pellet was dried mixed with 200µl ice cold suspension buffer, (Appendix 7) the pellet and pellet was dissolved by vortexing.
- Again 200 µl of freshly prepared lysis buffer (Appendix 7) was added, and stored in ice for 5min.
- 200 µl of 1.5 M K-acetate (Appendix 7) was added and mixed well (not by vortexing) and stored in icefor10 minutes.
- Lysate was centrifuged for 15 min at 12,000 rpm. Supernatant was transferred to next tube.
- Equal volume of phenol-chloroform was added, vortexed and centrifuged for 10 min at 10,000 rpm. Aqueous upper layer was transferred to afresh tube. DNA was precipitated by adding 600 µl of isopropanol and kept at -20°C temperature for overnight.
- Suspension was centrifuged at 12,000 rpm for 15 min and DNA pellet was dried.
- Pellet was washed with 1ml of 70 percent ethanol and pellet was dried completely.
- The pellet was dissolved in 30µl of TE buffer. (Appendix 8) 2 µl of RNase was added and incubated at 37°C for 1 hr.
- Purified sample was stored at -20°C temperature for further use.

3.3.7. Isolation of Plant genomic DNA (CTAB Method-Edwards *et al.*, 1991)

- Plant tissue was collected using 1.5ml eppendorf tube lid to ensure uniform size.
- Collected tissue was macerated in chilled pestle and mortar at room temperature without buffer for15 sec.
- Extraction buffer (0.4 ml) (Appendix 11) was added and sample was vortexed for 5 sec (can be kept in room temperature for more than 1hr).
- The solution was centrifuged at 13,000 rpm for 1 minute and 300 µl supernatant was transferred to fresh eppendorf tube.
- Supernatant was mixed with 300µl isopropanol and incubated at room temperature for 2 minutes and centrifuged at 13,000 rpm for 2 min.
- Pellet wasdriedandsuspendedin100µl 1XT10E1.
- 100 µl RNase (1mg/ml) was added to the DNA and incubated at 37^{0}C in water bath for half an hour.
- DNA was precipitated using 1/10 th volume of 3MNa-acetate and ethanol and incubated overnight at 4^{0}C.
- The solution was centrifuged at 13,000 rpm for 2 min and pellet was dried again.
- Pellet was suspended in 50µl 1XTE.

3.3.8. Molecular analysis for the detection of transgene

For the molecular analysis of the transgene in the putative transformants the primer (Forward: 5'-ATGGATCCCAAATTGCTATTCCTTCCACGA-3'

Reverse:3'-TTAGGCTTCTTTCTTCAGGTTTACTGCAGC-5') of *Rol B* gene was used to carry the PCR amplification as the T-DNA portion of the transgene contain *rolB* gene, The total genomic DNA of putative transformants was isolated with the help of DNA isolation methods (CTAB). The isolated genomic DNA is quantified and PCR reaction was carried out under 94°C for 5min (prenaturation), denaturation at 94°C for 1min, annealing 43°C for 1min, 72°C for synthesis for

1min for 45 cycles and final extension at 72°C for 10 min were carried. Then after the reaction the mixture was electrophoresed in 1.5% of agarose gel using 1xTAE (Appendix 10) and the resulted bands were observed under Gel Documentation unit (UVTECH, UK).

PCR reaction mix:

Template DNA -50ng

Primer -20ng

dNTPs -0.1mM

Tag DNA polymerase -0.33µl (1unit)

Tag assay buffer -1xTemplate DNA

Stage	Temperature	Duration(min)	Cycles
Denaturation	92	5	1
Denaturation	92	2	45
Annealing	43	1	
Primer extension	73	2	
Primer extension	73	10	1
Soak	4	1	-

After completion of all cycle sample were stored at 4^0C.

3.3.9. Separation of PCR amplified products by agarose gel electrophoresis

PCR amplified products (25µl) were mixed with 2 µl of loading and were loaded on 1.5% agarose gel. Electrophoresis was carried out at 80 V for 40 mins using 1X TAE buffer (pH 8.0). Marker DNA was run in a separate lane.

3.3.10. Visualization in Gel-Documentation unit:

The gel was visualized under gel documentation unit (UV-TECH, BANGALORE GENEI, INDIA) and analysis of bands was done by comparing it with DNA ladder.

3.4. HPTLC analysis

3.4.1. Materials

Prepared TLC plates were purchased from EMerck (Darmstadt, Germany). The standard *Andrographolid* was obtained from CIMAP, Lucknow.

3.4.2. Collection of sample for extract

Sample was collected from Botanical garden of Orissa University of Agriculture and Technology (OUAT) Bhubaneswar.

3.4.3. Preparation of extract from callus and leaf culture

The callus and leaf stem and root of the plant samples were air-dried and were powdered in grinder and mixer. The powdered plant material (5gm) was loaded in the inner tube of the of Soxhlet apparatus and then fitted in to a round bottomed flask containing methanol. The solvent was boiled gently (60-80°C) over a heating mantle using the adjustable rheostat. The extraction was continued until complete extraction was effected (24-30hrs) and the solvent was removed at the reduced pressure with the help of rotary vacuum evaporator to yield a viscous dark green or brown or blackish residue. The residue was dissolved in methanol and was further used for HPTLC and antimicrobial analysis for presence of standard *Andrographolid.*

3.4.4. HPTLC analysis

3.4.4.1. Calibration of standard graph

Stock solutions (1mg/ml) of standard compounds andrographolide were prepared individually in methanol and different concentrations were spotted on to TLC plates in order to prepare the calibration graphs.

3.4.4.2. Chromatographic Analysis callus and leaf extract

Aluminium plate precoated with silicagel60F254 TLC plates (10 ×10cm) (EMerck) was used as a stationary phase. A Linomat IV (Camag, Muttenz, Switzerland) automatic TLC applicator was used to apply samples and standards on to the TLC plate under a flow of nitrogen gas. The application parameters were identical for all the analysis performed and the delivery speed of the syringe

was 10s/µl. Standard andrographolide solution was prepared in HPLC grade methanol. The extracts and standard solution were applied 1.5 cm away from the lower edge of the plate with the help of syringe. The solvent system used to identify andrographolide was toluene: Ethyl Acetate: formic acid (v/v/v) (7.5 : 2.0 : 0.5) (Appendix 11) under laboratory conditions (25–30°C and 40–50% relative humidity). The loaded plates were then placed vertically in the chamber previously saturated with solvent system for 30 min. After the solvent front moved up to a distance of about 90% of length, the plate was taken out, and the plate was dried at room temperature. Developed plates were dried in a stream of air and then immersed in a freshly prepared mixture of p- anisaldehyde: glacial acetic acid: Sulphuric acid (0.5: 50: 10) After drying, the plates were heated at 110°C for 5 min to develop the colour of the spots. For quantitative determination, spots corresponding to standards were scanned using a Camag TLC Scanner at 245nm wavelength chosen to be appropriate for standards after staining) with a slit size of 6×0.4 mm.

The RF *values* were calculated using formula:

$$Rf = \frac{\text{distance travelled by solute}}{\text{distance travelled by solvent}}$$

3.4.4.3. Determination of andrographolide content

The calibration curve was determined by using the standard solution 1 mg/ml in methanol in different volumes i.e. 0.5, 1.0, 2.0, 3.0, 4.0, 5.0, 6.0, 7.0 µl were applied on a TLC plate and analysed by the proposed method. The amount of andrographolide in the sample was calculated from the calibration curve and *Rf* was calculated. The standard calibration graph was plotted to form a linear line.

3.6. Growth and maintenance of test microorganism for antimicrobial studies

Bacterial cultures of *Escherichia coli (E. coli), Pseudomonas fluorescence, Bacillus subtilus, Xanthomonas oryzae* (Metrology department of OUAT), fungal cultures of verticillium *leucani, Risoctonia solani, Aspergillus niger, Penicillium notatum* were obtained from the Department of

Microbiology, Orissa University of Agriculture and Technology, India, were used for antimicrobial test organisms. The bacteria cultures were maintained on nutrient broth (NB) at 37°C and fungus was maintained on Potato Dextrose Agar (PDA) at 28°C. Cefotaxime purchase from (M.P Biomedicals, Mumbai, India) and Bavistin were used as standards for bacteria and fungus respectively. The strains were subcultured periodically and the cultured strains were allowed to grow for one week and stored at 4°C for further analysis.

3.6.1. Antibacterial susceptibility testing

The antibacterial tests of the leaf, stem, root extracts were tested on the test bacteria i.e. E.coli, *Pseudomonas fluorescence, Bacillus subtilus* and *Xant homonas oryzae* using the paper disc diffusion method. The nutrient agar plates were seeded with the 24 h broth culture (105cfu/ml) of the bacteria was aseptically introduced and was poured in the presterilised petridishes and was allowed to solidify. The sterile paper discs (5mm) were soaked in the leaf, stem, and root extract of different concentrations viz. 5 mg/ml, 20 mg/ml, 50 mg/ml, 70 mg/ml, 100 mg/ml for 2 hours. The paper discs containing the extracts were placed at different areas on the surface of each plate. The plates were incubated at 28°C for 24 h. Cefotaxim was used as standard antibiotic. A disc soaked in methanol was used as control. Antimicrobial activity of the extract against the test bacteria was indicated by growth–free "zone of inhibition" near the respective disc.

3.6.2. Antifungal susceptibility testing

The antifungal tests of the leaf, stem, root extracts were tested i.e. *Aspergillus niger* and *Penicillium, Rizoctonia solani and verticillium* using the paper disc diffusion method. The potato dextrose agar plates were seeded with the fungal spores and were poured in the pre-sterilised petridishes and were allowed to solidify. The sterile paper discs (5mm) were soaked in the leaf, stem, and root extract of different concentrations viz. 5 mg/ml, 20 mg/ml, 50mg/ml, 70 mg/ml, 100 mg/ml for some minute. The paper discs containing the extracts were placed at different areas on the surface of each plate. The plates were incubated at 37°C for 48h. Bavistin was used as standard antifungal agent. A disc soaked in methanol was used as control. Antifungal activity of the extract

against the test fungus was indicated by growth–free "zone of inhibition" near the respective disc. The plates were duplicated in all the experiments.

3.6.3. Minimum inhibitory concentration:

The Minimum Inhibitory Concentrations (MIC) of the extracts was determined according to Elizabeth *et al.*, (1999). A final concentration of 0.5% (v/v) Tween-20 was used to enhance crude extract solubility. A series of two fold dilution of each extract, ranging from 0.2 to 100 mg/ml, was prepared. After sterilization, the medium was inoculated with 3µl aliquots of culture containing approximately 105 CFU/ml of each organism of 24 hours slant culture in aseptic condition and transferred in to sterile 6 inch diameter petridishes and allowed to set at room temperature for about 10 minutes and then kept in a refrigerator for 30 minutes. After the media solidified a number 3- cup borer (6mm) diameter was properly sterilized by flaming and used to make three to five uniform cups/well since each petridish. A drop of molten nutrient agar was used to seal the base of each cup. Different plant crude extracts ranging from 0.2 to100mg/ml were added to the cups/wells of each petridish and the control plates without plant extract. Inhibition of organism growth in the plates containing test crude extracts was judged by comparison with growth in blank control plates. The MICs were determined as the lowest concentration of extracts inhibiting visible growth of each organism on the agar plate.

3.7. Statistical analysis

All experiments were repeated thrice. Data were analysed by analysis of variance (ANOVA) to detect significant differences between means and CD was carried out at (p=0.5). C.V was carried out where the means were significant.

CHAPTER-IV

Result & Discussion

4.1. EXPERIMENTAL RESULTS

Development of plants from single cell or tissue or organ by using in vitro culture on the principle of totipotency. Different type of explants are used for developing the complete plant, such as shoot tips, leaf, internode, root, lateral buds, stem etc. There are some other factor like intrinsic and extrinsic factors influenced the morphogenic responses of the explanted tissue under culture conditions. These factors were either physical or physiological in nature depending upon the type to explants, media composition and the culture environment (Zafar *et al* 1992).

The most commonly used media for tissue culture is Murashige and Skoog (1962) usually referred to acmes in abbreviated form as MS. This media contain various types of major, minor, vitamins and other organic base as well as an energy source (sucrose); growth regulators, and agar to gel the medium is used as nutrient source to plant. The macronutrients like N, P, K, Ca, Mg and S are used in all types of culture but the optimal concentration of each of these added considerably with plant species. Generally plant cell grown in culture medium but sometimes better results are obtained when the medium with a nitrate and nitrogen source. The essential micronutrients such as Fe, Mn, Zn, B, Cu and Mo which are required in traces for culture medium. Fe is added in a chelated from to facilitate the proper dissolution and avoid precipitation. The element like Co, I, Na, and Cl are also used in some media but not very much essential for cell growth. The carbohydrate requirement in the culture media is usually satisfied by the incorporation of source at 2 to 3 % concentration. The function of the carbohydrate like sucrose is partly for maintaining the osmoticum in the medium but it meets the primary carbohydrates needs of the culture tissue, which is not fully capable of assimilating CO_2 like the fully autotrophic plant. Vitamin as a catalyst in various metabolic processes. Three vitamins are like thiamine (B_1), nicotinic and (B_3), pyridoxine (B_6) and myo-inositol are exogenously incorporated into the medium for tissue growth. Growth regulators help in shoot tip elongation, adventitious shoot development and root induction. Agar is used for gelling purpose in the medium and it is most essential for semisolid support to the plant for their growth.

Different types of growth hormone are used in the MS medium which is help to enhance or produce the shoot multiplication, root induction , callus induction from different explant. *In vitro* culture of some important medicinal plants are widely used for enhancement of secondary metabolites. The recent advances and developments in plant genetics and recombinant DNA technology have helped to improve and boost the research into secondary metabolite biosynthesis. One such method was "*hairy root cultures*" because of their stable, high productivity in hormone-free culture conditions, rapidity and technical simplicity. HPTLC is the widely used technique in plant metabolic studies for the presence or absence and comparison of secondary metabolites.

4.1.1. Effect of different plant growth hormone on regeneration

Different growth hormones like auxins, cytokinins and gibberellic acid are utmost important for *in vitro* culture. *In vitro* regeneration of plants form organ, tissue or single cell culture which enhances the secondary metabolite production. By manipulating culture condition and growth regulators, plant regeneration was take place.

4.1.1.1. Direct regeneration

Different explants were used for direct regeneration such as shoot tips, coteledonary leaf and stem explants with various growth regulators like BAP, Kinetin, IAA, GA_3, at different concentration. MS basal media withought growth hormone did not showed any results for shoot induction. At low concentrations of BAP (0.1 – 0.5 ppm) in combination with IAA (0.25 ppm) and adenine sulphate were found to be ineffective for shoot induction. Also Kn (1.0 – 2.0 ppm) in combination with IAA (0.25 ppm) and adenine sulphate showed the low performance of shoot induction. Maximum multiple shoot development were obtained in MS medium supplemented with BAP (2.5 mg/l) along with IAA (0.25 mg/l) with 100 mg/l of adenine sulphate from shoot-tip explants. (Plate 4) Kn (3.5 mg/ml) along GA_3 (1.5 mg/l) was little effect on multiple shoot. Increase in the concentration of BAP from (1.0 - 2.5 mg/ml) showed the higher percentage of shoot multiplication. The average number of shoots varied from 37.20 to 97.25. (Table 4.1)

TABLE 4.1. Effect of growth regulators on shoot induction from meristem explant of *Andrographis paniculata* after 4 week of culture.

S.N	MS+ growth regulator (mg/ml)					Average no. of multiple shoot culture (Mean ± S.E.)*	Days of shoot initiation	Length of the shoot after 1 week
1.	BAP	IAA	Ads	kinetin	GA_3			
2.	0	0	0	0	0	0	0	0
3.	1.0	0.25	100	0	0	37.2	9.00	0.87
4.	1.5	0.25	100	0	0	53.4	9.00	1.83
5.	2.0	0.25	100	0	0	79.21	9.00	3.03
6.	2.5	0.25	100	0	0	97.25	9.00	2.72
7.	3.0	0.25	100	0	0	86.36	7.33	2.27
8.	3.5	0.25	100	0	0	71.21	7.33	2.07
9.	0	0.25	0	1.0	0	32.3	7.33	0.73
10.	0	0.25	0	1.5	0	61.1	7.33	1.43
11.	0	0.25	0	2.0	0	73.4	7.33	1.52
12.	0	0.25	0	2.5	0	92.1	7.33	2.20
13.	0	0.25	0	3.0	0	85.0	7.33	1.95
14.	0	0	0	1.0	1.5	21 .24	10.33	1.51
15.	0	0	0	1.5	1.5	45.60	10.33	0.98
16.	0	0	0	2.0	1.5	74.20	10.33	1.00
17.	0	0	0	2.5	1.5	80.5	9.33	1.47
18.	0	0	0	3.0	1.5	83.40	9.67	1.90
19.	0	0	0	3.5	1.5	89.75	9.67	2.45
20.	0	0	0	4.0	1.5	84.30	9.67	2.10
CD5%							1.24	0.42
CV%							8.27	13.70

*10 explants per treatment, replicated thrice

4.1.1.2. Indirect regeneration

A) Callus induction

The development of callus is greatly influenced and affected by the growth hormones. Generally, a higher concentration of auxin in the growth medium induces callus formation (Skoog and Miller, 1957). Establishment of callus, which retains high morphogenetic potential, was a preliminary step in tissue culture of any species. Proliferation of callus was observed in case of stem explant of *Andrographis paniculata* within 2 week of culture on auxin containing medium. Vigorous growth of callus was observed after 3 weeks of culture. Green friable callus was developed from leaf explant within 3-4 weeks of culture. The combined effect of auxin and cytokinin on callus initiation in *Andrographis paniculata is* shown in Table 4. 2. The maximum proliferation of calli i.e. 90.4 % was noted in the medium containing NAA (3.0 mg/l) and BAP (1.0 mg/l). (Plate 5 and 6) Increasing the concentration of NAA from 3.0 mg/l to 0.4 mg/l decrease the percent callus development. The medium devoid of growth regulators did not help in proliferation of callus. Also subculturing of callus was done on same medium after 1, 2, 4, 6 weeks of interval. (Plate 7) The callus growth was more in subsequent culture period. The percentage of callus growth was maximum in medium having NAA (3.0 mg/l) and BAP (1.0 mg/l) after 4th subculture (Table. 4.3). The well- developed calli was taken for shoot bud regeneration.

Table 4.2 Effect of growth regulators on callus induction from leaf and stem explant of *Andrographis paniculata* after 4 week culture.

S.N.	Ms + growth regulator (mg/ml)			Average % of response (Mean ± S.E.)*		Days of callus initiation from leaf	Days of callus initiation from stem	Colour and texture of callus
	NAA	2, 4-D	BAP	Leaf	Stem			
1.	0.0	0	0.0	0	0	0		0
2.	1.0	0	1.0	20.2	18.5	10.67	11.67	Green friable callus
3.	1.5	0	1.0	30.4	24.6	10.67	11.67	Green friable callus
4.	2.0	0	1.0	50.3	38.4	9.67	11.67	Green friable callus
5.	2.5	0	1.0	70.7	52.6	9.67	11.00	Green friable callus
6.	3.0	0	1.0	90.4	68.4	8.67	10.00	Green friable callus
7.	3.5	0	1.0	80.8	53.8	8.67	10.00	Green friable callus
8.	4.0	0	1.0	60.4	43.2	8.67	10.00	Green friable callus
9.	1.0	1.0	0	9.2	9.6	11.00	12.67	Green compact callus
10.	1.0	1.5	0	20.5	14.2	10.33	12.00	Green compact callus
11.	1.0	2.0	0	50.4	28.6	9.33	10.33	Green compact callus
12.	1.0	2.5	0	65.7	48.6	9.33	9.33	Green compact callus
13.	1.0	3.0	0	89.9	56.8	9.67	9.67	Green compact callus
14.	1.0	3.5	0	86.2	64.2	7.67	9.67	Green compact callus
15.	1.0	4.0	0	75.7	66.8	7.00	9.67	Green compact callus
CD5%						**1.40**	**1.62**	
CV%						**8.73**	**8.84**	

*10 explants per treatment, replicated thrice

Table-4.3. Effect of subculture on callus production of *Andrographis paniculata*. Each subculture having 4 week interval 500 mg of calli subcultured in fresh medium.

Ms + growth regulator (mg/ml)			Subcultured period (% of callus proliferation)			
NAA	2, 4-D	BAP	1st	2 nd	3 rd	4 th
3.0	0	1.0	94.2 ± 0.8	96.8 ±1.2	97.8 ± 1.3	96.8 ± 1.3
3.5	0	1.0	80.6 ± 1.2	90.6 ± 1.0	92.3 ± 1.4	94.5 ± 1.3
1.0	3.0	0	76.8 ± 0.9	82.4 ± 1.2	85.4 ± 0.9	88.36 ± 1.2

*10 explants per treatment, replicated thrice

B) Shoot proliferation from callus

Developed calli from both leaf and stem explants were subcultured on the MS media supplemented with different concentration of BAP (0.5 - 3.5 mg/ml) along with NAA (1.0 mg/ml) and 50 mg/ml adenine sulphate and also on the MS media supplemented with BAP (1.0 - 3.0 mg/l) along with IAA (1.0 mg/l). Kn (1.0 - 2.5 mg/l) along with NAA (1.0 mg/l). Maximum number of multiple shoots culture were observed in leaf derived callus on MS medium containing BAP (3.5 mg/l) in combination with NAA (1.0 mg/l) along with 50 mg/l adenine sulphate. (Plate 8 and 9) Shoot bud regeneration was varied from 32.34 to 87.77 in case of leaf derived callus and 23.09 to 84.98 in case of stem derived calli within 4 week of culture. This shoots were subcultured on MS medium containing different type of cytokinin and auxin concentration .The shoots were proliferated and elongated to 1.0-1.5 cm within 4 week of culture. No shoots were obtained at lower concentrations of either BAP or Kn. Further, increase in the concentration of either BAP or Kn had no effect on the rate of shoot multiplication. (Table 4.4)

TABLE 4.4. Effect of growth regulators on shoot induction from leaf and stem developed callus of *Andrographis paniculata* after four week of culture.

(*10 explants per treatment, replicated thrice)

MS+ growth regulator mg/ml					Average no. of *multiple* shoots (Mean ± S.E.)*		Days of multiple shoot initiation from leaf callus	Days of multiple shoot initiation from stem callus
Kinetin	BAP	NAA	IAA	Ads	Leaf	Stem		
0	0	0	0	0	0	0		
1.0	0	1.0	0	50	32.34	23.09.	30.33	30.33
1.5	0	1.0	0	50	49.56	34.76	29.67	30.67
2.0	0	1.0	0	50	76.00	64.80	28.00	26.33
2.5	0	1.0	0	50	87.77	84.98	24.33	25.33
0	0.5	1.0	0	50	27.54	22.76	30.33	30.67
0	1.0	1.0	0	50	28.78	33.98	28.67	30.67
0	1.5	1.0	0	50	43.09	39.76	26.67	26.67
0	2.0	1.0	0	50	43.33	56.98	26.67	26.67
0	2.5	1.0	0	50	65.67	67.90	26.00	26.33
0	3.0	1.0	0	50	79.98	76.98	25.67	26.33
0	3.5	1.0	0	50	84.07	82.89	25.67	26.67
0	1.0	0	1.0	50	27.78	23.54	30.33	31.33
0	1.5	0	1.0	50	45.98	46.09	28.33	30.67
0	2.0	0	1.0	50	65.09	54.78	28.67	26.67
0	2.5	0	1.0	50	74.89	73.56	25.67	26.33
0	3.0	0	1.0	50	83.65	80.65	25.67	26.33
CD5%							1.48	1.55
CV%							3.12	3.22

4.1.1.3. *In vitro* root induction

Multiple shoots derived from both meristem and from callus was transferred on the different combination of auxins i.e. (NAA 0.25-1.0 mg/l) and (IBA 0.25 - 1.5 mg/l) along with half strength MS media supplemented with 2% sucrose. No rooting was observed in basal MS media. The maximum percentage of rooting (76.34) from excised shoot on half strength MS medium containing IBA or NAA. Maximum percentage of rooting was obtained on half MS medium supplemented with IBA (0.5 mg/l) and 2% sucrose. (Plate 10) (Table 4.5)

Table-4.5 Effect growth hormone on no. of root induction from callus and shoot

MS + growth hormone (mg/ml)		Day to rooting	% of rooting (Mean ± S.E.)*
NAA	IBA		shoot
0.0	0	0	0
0.25	0	11.00	42.36
0.50	0	11.33	67.25
1.0	0	11.00	28.5
0	0.25	11.33	57.23
0	0.5	9.67	76.34
0	1.0	11.67	38.11
0	1.5	11.33	30.43
CD5%		1.39	
CV%		7.08	

*10 explants per treatment, replicated thrice
+ calling at the clonal end of the microshoot

4.1.1.4. Greenhouse establishment

The rooted plantlets were transferred to pots containing mixture of sand: soil: FYM (1:1:1) and kept in green house for acclimatization. About 90 % of the plantlets established well in the green house within 1-2 weeks of transfer. The plant grew well and attained 10-15 cm height within 4 weeks of transfer. (Plate 11)

4.1.2. AGROBACTERIUM MEDIATED TRANSFORMATION

4.1.2.1. Effect different factors for efficient transformation

A) Duration and infection time for transformation

Different explants such as leaf, shoot and callus were used for transformation. (Plate 12) Explants were co-cultivated for different time period i.e. 24 hrs, 48 hrs and 72 hrs by infection them with *Agrobacterium rhizogenes* strain MTCC 532 in LPGM containing 100 mM Acetosyringone. Explant was infected for different time period i.e. 10, 15, 20, 30 minutes. Effective response was found explants infected for 20 minute and co-cultivated for a period of 72 hrs. to obtain maximum survivability. The survivability of explants was maximum in carbenicillin after 48 hours in 20 min. (Plate 13 and 14) (Table 4.6)

Table-4.6. Effect of infection time and co-cultivation period for transformation of *Andrographis paniculata*.

Infection time (min)	Co-cultivation periods (hrs)	Response after 20 days			
		After 2nd washing of cefotaxime		After 2nd washing of carbenicillin	
		alive	Survival (%)	alive	Survival (%)
10	24	2.49	24.9	2.45	46.7
	48	2.68	26.8	3.15	46.7
	72	2.50	25.0	2.67	36.7
15	24	3.23	32.3	3.34	43.3
	48	3.78	37.8	4.38	46.7
	72	3.43	34.3	3.20	36.7
20	24	4.56	46.6	4.78	50.0
	48	5.58	55.8	5.39	60.0
	72	4.50	45.0	4.33	53.3
30	24	3.19	31.9	3.27	46.7
	48	3.13	31.3	3.33	53.3
	72	3.0	30.0	3.19	46.7

*10 explants per treatment, replicated thrice

B) Role of antibiotic for inhibition the growth of *Agrobacterium rhizogenes*.

MS basal medium was supplemented with various concentrations of cefotaxime/ carbenicillin (0-400 mg/l) was used to study positive sensitivity of *Agrobacterium rhizogenes* strain MTCC 532 to explants. Antibiotic was also used to study sensitivity of *Agrobacterium rhizogenes* strain MTCC 532 with explants. Different concentration of Carbenicillin/Cefotaxime differed significantly with respect to inhibition of *Agrobacterium* growth. Concentration of antibiotic was used for washing such as 100, 150, 200, 250, 300, 350, 400 mg/l. *Agrobacterium* was reappeared on explants at low concentration after antibiotic washing. But this reappearance of *Agrobacterium* in cefotaxime and carbenicillin supplemented medium (Table 4.7). The culture having 400 mg/l cefotaxime/ carbenicillin were found to be effective to inhibit growth of *Agrobacterium rhizogenes* with 0% reappearance. (Plate 13 and 14)

Table-4.7. Sensitivity of *Agrobacterium rhizogenes* to various levels of cefotaxime and carbenicillin

S.No.	Cefotaxime/ Carbenicillin concentration (mg/ml)	No. of explants used	Reappearance response %		Reappearance of agrobacterium	
			Cefotaxime	Carbenicillin	Cefotaxime	Carbenicillin
1.	0	25	100	100	++++	++++
2.	100	25	88	84	+++	+++
3.	150	25	80	56	++	++
4.	200	25	68	40	+	+
5.	250	25	40	28	+	+
6.	300	25	12	10	+	+
7.	350	25	8	0	-	-
8.	400	25	0	0	-	-

+ : Slight growth
+++ : Prominent growth
- : Complete inhibition

4.1.2.2. Formation of hairy root through A. rhizogenesis.

For induction of hairy root formation from explant, two strains of *Agrobacterium rhizogenes* was used i.e. MTCC 532 and A4 strain. Out of two strain A4 strain was unable to induce hairy roots from any explant tested, while MTCC 532 induced hairy roots from explant of *Andrographis paniculata.* For co-cultivation shoot, leaf and callus of *Andrographis paniculata* were used. The hairy roots were formed easily from callus. Hairy root also developed from shoot and leaf but frequency very low. In some case the explants become dried very soon as per the long infection time or long co-cultivation period. This co-cultivation and infection time was very important role for hairy root induction .Various factors like type of explants (Dupre *et al.*, 2000), *A. rhizogenes* strains (Giri *et al.*, 2001; Tiwari *et al.*, 2007), phenolic compounds (Kumar *et al.*,2006), growth medium (Azlan *et al.*, 2002), bacterial concentration (John *et al.*,2009), growth hormones (Falasca *et al.*, 2000) and pH (Danesh *et al.*, 2006) showed great influence on root induction. The effective hairy root was found in callus with 20 min. infection and co-cultivation period for 2 days. (Plate 13 and 14)

4.1.3. Detection of transgene by molecular analysis

Explant like callus, leaf and shoot used for co-cultivation to develop transgene. The co-cultivated callus and shoot were used for regeneration by transferring to the MS media containing different growth hormone. Leaf transformed calli were regenerated on MS medium supplemented with BAP (3.0 mg/l), NAA (1.0 mg/l) and 50 mg/l Ads after 4 week of culture. The regenerated shoots were used for the molecular analysis. From these transformants, DNA was isolated by using CTAB method. Pure DNA used for PCR analysis. Primer used for PCR analysis was *rolB* specific forward and reverse primers. Multiple copies of DNA was obtained after PCR analysis. These DNA was subjected to agarose gel electrophoresis to confirm the presence of transgene.

In agropine *Ri* plasmids, the T-DNA region consists of two parts; TL-DNA and TR-DNA. These parts are separated by a non-transferable DNA sequence of approximately18 Kb. Integration of TL-DNA to plant genome was confirmed by the presence of *rol* gene by PCR analysis .Presence of *rol* gene sequence in the genomic DNA of transformed roots confirmed transformation by its integration in plant genome. (Plate 15) No *rol* gene activity was found in normal roots.

4.1.4. Comparison of secondary metabolite content in transformed and untransformed tissues/leaf of *Andrographis paniculata*.

Air dried powdered sample control plant leaf, stem, root, transformed callus and untransformed callus of *Andrographis paniculata* which was used for Soxhlet extraction. These extract were used for TLC and HPTLC, and also antimicrobial analysis.

4.1.4.1. Nature of crude plant extracts

Extraction was made by using methanol as solvent for control leaves, stem, root, and transformed callus i.e. hairy root callus and untransformed tissues. Extraction from transformed callus show blackish sticky and untransformed callus extract was brown sticky. Extract from control leaf show also greenish colour (Table 4.8).

Table 4.8: Nature of crude plant extracts.

Plant species	**Extracting solvent**	**Colour/texture of extract**		
		Leaf	Transformed	Untransformed
Andrographis paniculata	Methanol	Greenish	Black sticky	Brown sticky

4.1.5. HPTLC ANALYSIS:

HPTLC was carried out by HPTLC machine under flow of nitrogen gas. Plant and extract standard was run on the HPTLC plates.(Plate 20) For comparing the presence of andrographolide and tissue in different samples against standard. The calibration graphs of standard *was* obtained to give a straight line with r^2= 0.996 for the compounds.(Plate 16 and 17) Andrographolide was developed in mobile phase toluene: acetic acid: formic acid (7.5: 1.5: 0.5). Developed plate was derivatised with p- anisaldehyde: sulphuric acid: glacial acetic acid (0.5: 1.0: 50). The *Rf* value for andrographolid was found to be 0.13 ±0.01 (Table 4.9).

The percentage of increase the content of andrographolide in the transformed callus extract of *Andrographis paniculata* was found to be 2.6 % as compared to control. Developed HPTLC plate was scanned under 254 nm and 366 nm. The photo was taken under 254 nm and 366nm but under white light bands were not observed on HPTLC plate clearly. Derivatised plate also scanned under 254 nm and 366 nm but only under 254 nm bands were observed clearly. (Plate 18and 19)

Table-4.9. Calibration of standards of andrographolide (Plate 16 and 17)

Standard 1mg/ml	Andrographolide	
	Rf	AU
0.5µl	0.13 ±0.01	689.2
1µl	0.13 ±0.01	1170.6
2µl	0.13 ±0.01	2172.3
3µl	0.13 ±0.01	2963.5
4µl	0.13 ±0.01	3684.1
5 µl	0.13 ±0.01	4419.1
6 µl	0.14 ±0.01	4412.9

Rf- Retardation factor

AU- Area unit

The result show that transformed calli having higher range of andrographolide as compared with untransformed calli (Table 4.10). The unit area of transformed calli was (1911.4) as compared to untransformed (235.0).(Plate 18 and 19)

Table-4.10. Comparison of andrographolide content in control leaf, stem, root, transformed and untransformed callus of *Andrographis paniculata*.

Concentration of sample	Secondary metabolite content expressed in area unit.				
	Explants source				
	Control leaf	Control stem	Control root	Untransformed callus	Transformed callus
20 mg/ml	5951.3	4612.7	797.0	235.0	1911.4

4.1.6 ANTIMICROBIAL ANALYSIS:

The antimicrobial activity was tested as per the zone of inhibition formed by the tissue extract against the bacteria and fungus. All the tissue extracts were tested against the pathogenic bacteria and fungus E.coli, Bacillus subtilus, Xanthomonas oyizae, Pseudomonas fluorescence and fungus *Aspergillus niger,* Risoctonia solani, Penicillium notatum, Verticillium leucani at different concentrations of extract viz. 5, 20, 50, 70 and 100 mg/ml.

Among the different bacteria tested the maximum zone of inhibition recorded in transformed calli against E.coli and Pseudomonas fluorescence in 50 mg/ml and 100 mg/ml respectively (Plate 21) (Table 4.10). The maximum zone of inhibition was observed in case of Xanthomonas oyizae at different concentration. The transformed calli extract showed highest antifungal activity with zone of inhibition against Penicillium notatum and Aspergillus niger at 100 mg/ml.(Plate 21) the zone of inhibition was minimum against Risoctonia solani and Verticillium leucani (Table 4.10). Control root also showed the maximum antibacterial activity with zone of inhibition against Bacillus subtilus (Table 4.10).

Table-4.11. Antimicrobial activity at different concentration of control callus and transformed callus crude extract of *Andrographis paniculata* after 24 hrs. in case of bacteria and 72 hrs. in case of fungus.

Explant source	Concentra-tion (mg/ml)	Zone of inhibition (cm)							
		Bacteria				Fungus			
		E.coli	*Pseudomonas fluorescence*	*Bacillus subtilis*	*Xanthomonas oryzae*	*Aspergillus niger*	*Penecillium notatum*	*Risoctonia solani*	*Verticillium leucani*
CONTROL	100	1.0	0.7	0.9	1.0	1.5	1.0	1.0	0.8
CONTROL CALLUS	5	0.52	0.4	0.7	0.6	0.2	0.5	0.2	0.3
	20	0.57	0.45	0.58	0.34	0.34	0.54	0.23	0.35
	50	0.7	0.5	0.3	0.41	0.36	0.56	0.27	0.43
	70	0.75	0.4	0.12	0.36	0.45	0.60	0.35	0.45
	100	0.9	0.42	0.1	0.22	0.5	0.64	0.4	0.45
TRANSFORM CALLUS	5	0.55	0.4	0.45	0.12	0.3	0.29	0.2	0.2
	20	0.69	0.53	0.44	0.25	0.33	0.42	0.28	0.27
	50	0.77	0.53	0.44	0.27	0.37	0.49	0.32	0.32
	70	0.9	0.67	0.5	0.3	0.48	0.55	0.38	0.54
	100	1.2	0.7	0.59	0.3	0.54	0.67	0.4	0.39
CONTROL	5	0.12	NA	0.76	NA	NA	NA	NA	NA
	20	0.15	NA	1.00	NA	NA	NA	NA	NA

LEAF	50	0.30	NA	0.50	NA	NA	NA	NA	NA
	70	0.42	NA	0.12	NA	NA	NA	NA	NA
	100	0.42	NA	NA	NA	NA	NA	NA	NA
CONTROL STEM	5	0.20	0.32	0.12	0.50	0.20	NA	NA	NA
	20	0.30	0.38	0.90	0.70	0.40	NA	NA	NA
	50	0.40	0.42	0.10	0.75	0.42	NA	NA	NA
	70	0.6	0.47	No	0.76	0.50	NA	NA	NA
	100	0.50	0.50	No	0.90	0.60	NA	NA	NA
CONTROL Root	5	0.44	0.42	1.20	0.20	NA	0.60	NA	0.30
	20	0.47	0.42	1.00	0.34	NA	0.62	NA	0.35
	50	0.70	0.45	0.70	0.70	NA	0.65	NA	0.37
	70	0.73	0.46	0.70	0.90	NA	0.75	NA	0.40
	100	1.15	0.46	1.20	1.20	NA	0.86	NA	0.45

NA : no activity

4.2. DISCUSSION

Medicinal plants sector has traditionally occupied an important position in the socio cultural, spiritual and medicinal area of rural and tribal lives. There has been a significant rise in the number of studies deciphering various aspects of anti-neoplastic activity of andrographolide around the globe. Kalmegh (*Andrographis paniculata Wall.Ex Nees*) belong to family *Acanthaceae* recently has attracted the attention of researchers, because it has wide range of medicinal and pharmacological application. The aerial part of the plant, used medicinally, contains a large number of chemical constituents, mainly lactones, diterpenoids, diterpene glycosides, flavonoids, and flavonoid glycosides. Eves, stem and root contain andrographolide but leaf contains more amount of andrographolid than stem and root, it is also good source of secondary metabolite which is used in various disease treatments. Andrographolide has been reported to prove to reduce inflammation and possess anticarcinogenic properties. The principle phytochemical constituent of the plant, andrographolide, has shown significant anti-neoplastic and immunomodulatory activities in a number of studies performed in recent times. It is a well-established fact that an integrated approach is needed to manage cancer and a compound or a group of compounds that can influence multiple biochemical pathways related to tumorigenesis are of prime interest in cancer chemotherapy. Recently, a greater emphasis has been given towards the researches on complementary and alternative medicine that deals with cancer management. In-vitro cultures for transformation for hairy root induction are used to enhance the production of secondary metabolites which responsible for anticancer activity. These plants are also a rich source of antimicrobial agents. Therefore, such plants have been investigated for better understanding their medicinal properties. Plant contain several derivatives of diterpene lactones out of which Andrographolide (bitter constituent) and neoandrographolide (non bitter constituent) are important one reported by Akowuah *et al* (2006).

Agrobacterium rhizogenes has been used regularly for gene transfer in many dicotyledonous plants (Tepfer *et al*; 1990.) Plant infection with this bacterium induces the formation of proliferative multibranched adventitious

roots at the site of infection; the so-called 'hairy roots' (Chilton, 1982).

The study was aimed to in vitro regeneration, transformation, HPTLC analysis for presence of secondary metabolite in transformed and untransformed plant and exploring the antibacterial and antifungal activity of plant extract.

4.2.1. *In vitro* regeneration

Micropropagation is presently used as an advanced biotechnological tool for the production of identical pathogen-free plants for commercialization (George,1993).Different growth hormone were used for the regeneration of plant either directly from explant via. callus phase. The activity of cytokinins is essential to maintain undifferentiation cells in shoot apical meristem and to promote cell differentiation in the shoot & root meristems (Kyozuka, 1993). Skoog and Miller (1957) demonstrated that the ability of cytokinins to induce shoot regeneration in number of plant species. Cytokinins have been implicated in the control of shoot meristem activity (Werner *et al*, 2003). Different growth hormone used for In vitro studies with special reference to adventitious root forming for production of secondary metabolite.

4.2.1.1. Culture Medium

MS basal media was used for the propagation of Andrographis paniculata. MS basal medium supplemented with different growth regeneration showed the diffraction of variation plant species as reported earlier (Martin, 2003; Martin, 2004; Karuppusamy and Kalimuthu, 2010; Vijayalaxmi and Hosakatte, 2012; Purkayastha *et al*; 2008; Kataky and Handique, 2010, 2011; Basu and Yogananth, 2011).

4.2.1.2. Effect of different plant growth regulator

The pivotal role of plant growth regulators in controlling in vitro morphogenesis has been well established in different plant species. Organ differentiation in plant is regulated by interplay of auxins and cytokinins (Skoog and Miller, 1957). In case of Andrographis paniculata, different auxins, cytokinin, and gibberellic acid were used for direct and indirect regeneration. Auxin like indole-3-buteric acid (IBA), 1-naptheleneacetic acid (NAA) and 2,4-dichlorophenoxyacetic acid (2,4-D), Indole 3-acetic acid (IAA), cytokinins like 6-

benzylaminopurine (BAP) and kinetin (Kn) and gibberellic acid (GA_3) were used to supplement in the MS basal media. Cytokines like 6-benzylaminopurine (BAP) in combination with Indole 3-acetic acid (IAA) and kinetin (Kn) in combination with GA3 responded significantly for shoot induction. Auxins like indole-3-buteric acid (IBA), and 1- naptheleneacetic acid (NAA) showed better response for in vitro rooting. Addition of NAA in the culture medium proved to be beneficial in callus induction and regeneration of root bud. The low concentration of auxin and high concentration of cytokinin in the culture medium helps the shoot meristem elongation, multiplication and plantlet regeneration. The present investigation showed that the combination of BAP and IAA or NAA along with Ads showed maximum frequency of shoot bud regeneration. Vijayalaxmi *et al* (2012) reported that the maximum number of shoots obtained from meristem explant on Ms medium supplemented with BAP in combination with Kn. Vijayalaxmi and Hosakatte, (2012) reported that the highest number of shoot multiplication frequency obtained on MS medium supplemented with TDZ and for GA_3 was incorporated to develop the maximum growth and shoot elongation.

4.2.1.3. Callus induction and proliferation

Growth regulators plays important role in the callus induction from various explant. In case of *Andrographis paniculata* leaf and stem explant were used for callus induction. Growth medium having NAA or 2, 4-D in combination with BAP and 50 mg/l Ads. Leaf explant develops effective callus formation (about 90%) from MS medium supplemented with NAA (3.0 mg/l) in combination with BAP (1.0 mg/l) along with 50mg/l Ads. The medium having 2, 4-D in combination with NAA along with GA3 and Ads was also better effect on callus proliferation from leaf explant. Increasing the concentration of NAA from 1.0 mg/l to 3.0 mg/l proved to be very responsive in callus development in plants however, the calli become yellowish in subsequent subculture.

Martin, (2003) reported that MS medium fortified with 4.52 mM 2, 4-D was found to be optimal for induction, maturation, and conversion of embryos. A similar promoting effect of 2, 4-D in somatic embryogenesis has been reported in Hemidesmus indicus (Sarasan *et al.*, 1994), Eleutherococcus sessiliflorus

(Choi *et al.*, 2002), and Cuminum cyminum (Tawfik and Noga, 2002). Somatic embryogenesis, which offers great promise for rapid multiplication, has been reported to be under the control of plant growth regulators in several medicinal plants, namely *Tylophora indica* (Jayanthi and Mandal, 2001), *Gymnema sylvestre* (Kumar *et al.*, 2002), *Psoralea corylifolia* (Sahrawat and Chand, 2002), and *Holostemma ada-kodien* (Martin, 2003). The calli developed in the medium having BAP + NAA + Ads has compact globular organogenic form. Whereas, the calli were friable in the medium having 2, 4-D + BAP + Ads. medium.

4.2.1.4. *In vitro* root induction and acclimatization

Growth regulator like normally auxin induces rooting in plant. Root developments from regenerated shoot are very essential for further study. In case of Andrographis paniculata NAA or IBA forming root induction from regenerated shoot. Root induction and proliferation was achieved within 9 –12 days depending on the auxin treatment. Combination of both auxin has no effect on rooting. The maximum root induction and proliferation was achieved in the medium having 0.5 mg/l IBA as compare with NAA. The medium having two kinds of auxins (NAA + IBA) did not favour root induction rather callus induction occur at the basal end the well-developed rooted plantlets were transformed to greenhouse for acclimatisation. About 60% of plantlets were survived in the greenhouse.

Vijayalaxmi *et al* (2012) reported that root induction take place on MS medium supplemented with either NAA or IAA or IBA. Karuppusamy and Kalimuthu (2010) reported that rooting achieved on MS medium supplemented with IAA in case of *Andrographis paniculata*.

4.2.2. *Agrobacterium* mediated genetic transformation

Genetic transformation technology has been proved to be a powerful tool for the production of plants with desired traits in many crops (Sawahel, 1997; Sawahel and Cove, 1992). Different explants such as leaf, shoot and callus were used for transformation. Plant transformation mediated by *Agrobacterium tumefaciens*, a soil plant pathogenic bacterium, has become the most commonly used method for the introduction of foreign genes into plant cells and the subsequent regeneration of transgenic plant (Riva *et al.*,

Falasca, G., Reverberi, M., Lauri, P., Caboni, E., Stradis, A. and Altamura, M.M., 2000. How *Agrobacterium rhizogenes* triggers *de novo* root formation in a recalcitrant woody plant, an integrated histological, ultra structural and molecular analysis. *New Phytol.*, 145: 77-93.

Fujita, T., Fujitani, R. and Takeda, Y., (1984). On the ditrpenoids of *Andrographis paniculata*: X-ray crystallographic analysis of andrographolide and structure determination of new minor diterpenoids. *Chemical and Pharmaceutical Bulletin*, 32(6): 2117-2125.

Gaind, K.N., Dai, R.N., and Kaul, R.N., (1963). Spectro-photometric estimation of andrographolide in Kalmegh. *Ind. J. Pharm.*, 25: 255-256.

Gelvin, S.B., (2003). *Agrobacterium*-mediated plant transformation, the biology behind the gene-jockeying tool. *Microbial Mol. Biol. Rev.*, 67: 16-37.

George, E.F, (1993). Plant propagation by tissue culture, Part 2. Exegetic Ltd, Edington Wesbury, UK.

George, M. and Pandalai, K.M. (1949). Investigations on plant antibiotics. Part IV. Further search for antibiotic substances in Indian medicinal plants. *Indian J. Med. Res.*, 37: 169-181.

George, P.S., Ravishankar, G.A. and Venkataraman, L.V. (1993). Clonal multiplication of *Gardenia jaminoides* through axillary bud culture. Plant. Cell. Rep., 13: 59- 62.

Gheysen, G., Angenon, G. and Montague, M.V., (1998). *Agrobacterium* mediated plant transformation, a scientifically intriguing story with significant application. In: Lindsey K (ed). Transgenic Plant Research. Netherlands: Harwood Academic Press, pp.1-33.

Ghosh, B.E. and Sen, S., (1994). Micropropagation of *Asparagus cooperi* as affected by growth regulators. *Biologia Plantarum.*, 36: 527-534.

Ghosh, K.B., Datta, K.A., Mandal, A., Dubey, K.P. and Halder, S., (2012). An overview on *Andrographis paniculata* (Burm. F.) Nees. *Int. J. Res. Ayur. Pharm.,* 3(6): 752-760.

Giri, A., Ravindra, S.T., Dhingra, V. and Narasu, M.L., (2001). Influence of different strains of *Agrobacterium rhizogenes* on induction of hairy roots and artemisinin production in *Artemisia annua. Curr. Sci.*, 81: 378-382.

Girija, S., Ganapathi, A. and Vengadesan, G., (1999). Micropropagation of *Crossandra infundibuliformis* (L.). *Sci. Hortic.*, 82: 331-337.

Gorter, M.K., (1911). The bitter constituent of *Andrographis paniculata* Nees. *Rec. Trav. Chim.* 30: 151-60.

Gupta, S., Choudhry, M.A., Yadava, J.N.S., Srivastava, V. and Tandon, J.S., (1990). Antidiarrheal activity of diterpenes of *Andrographis paniculata* (KalMegh) against *Escherichia coli* enterotoxin in *in vivo* models. *Int. J. Crude Drug Res.*, 28: 273-283.

Hancharnlerd, O., Babprasert, C. and Phisuksanthiwattana, Y., (1994). Medicinal plants in pakchong research station garden. Faculty of agriculture. Kasetsart. univ. thailand.

Handa, S. S., and Sharma, A., (1990). Hepatoprotective activity of Andrographolide from *Andrographis paniculata* against carbon tetrachloride. *Indian Journal of Medical Research.*, 92: 276-283.

Hansen, G. and Wright, M.S., (1999). Recent advances transformation of plants. Trends Plant Sci., 4: 226-231.

Hiei, Y., Komari, T. and Kubo, T., (1997). Transformation of rice mediated by *Agrobacterium tumefaciens. Plant Mol. Biol.*, 35 (1997) 205-218.

Hiei, Y., Ohte, S., Komari, T. and Kumashiro, T., (1994). Efficient transformation of rice mediated by *Agrobacterium* and sequence analysis of the boundaries of the T-DNA. *J. Plant.*, 6: 271-282.

Hiregoudar, L.V., Murthy, H.N., Bhat, J.G., Nayeem, A., Hema, B.P., Hahn, E.J. and Paek, K.Y., (2006). Rapid clonal propagation of *Vitex trifolia. In vitro Cell. Dev. Biol.*, 50(2): 291-294.

Hiregoudar, L.V., Murthy, H.N., Hema, B.P., Hahn, E.J., Paek, K.Y., (2003). Multiple shoot induction and plant regeneration of *Feronia limonia* (L.). *Sci. Hort.*, 98: 357-364.

Hooker, J.D., (1885). Flora of British India. Vol. IV, L. Reeve & Co. LTD. Ashford, Kent., UK.

Hosamani, P.A., Lakshman, H.C., Sandeepkumar, K. and Rashmi, C., (2011). Antimicrobial Activity of Leaf extract of *Andrographis paniculata* Wall. *Science Research Reporter.*, 1(2): 92-95.

Hsu, H.Y., (1986). Oriental materia medica: a concise guide. long beach, ca, oriental healing arts institute.

Hun, J.Y., Tummala, R., Nadiminty, N., Lou, W., Liu, C., Yang, J., Evans, C.P., Zhou, Q. and Gao, A.C., (2010). Andrographolide an herbal medicine, inhibits interleukin-6 expression and suppresses prostate cancer cell growth. *Genes Cancer.* 1(8): 868-876.

ICH Committee (1996). Step 4 of the ICH Process. ISTA. 10: 11. International rules for seed testing, 1976. *Seed Sci. and Tech.* 4: 52-70.

Ishida,Y., Saito, H., Ohta, S., Hiei, Y., Komari, T., Kumashiro, T., (1996). High frequency transformation of maize (*Zea mays*) mediated by *Agrobacterium tumefaciens.* Nature *Biotechnol.*, 14: 745-50.

Jain, S.K., (1991). Dictionary of Indian folk medicine and ethnobotany-A reference manual of man-plant relationships, ethnic groups and ethnobotanists in India. *Deep Publication*, New Delhi, India.

Jarukamjorn, K. and Nemoto, N., (2008). "Pharmacological aspects of *Andrographis paniculata* on health and its major diterpenoid constituent andrographolide," *Journal of Health Science*, vol. 54, (4): 370–381.

Jayanthi, M. and Mandal, P.K., (2001). Plant regeneration through somatic embryogenesis and RAPD analysis of regenerated plants in *Tylophora indica* (Burm. f. Merril.). *In Vitro Cell. Dev. Biol.*, 37: 576-580.

Jha, S., Sahu, N.P. and Mahato, S.B., (1988). Production of the alkaloids emetine and cephaeline in callus cultures of *Cephaelis ipecacuanha*. *Planta Medica*., 54: 504-506.

John, K.M.M., Joshi, S.D., Mandal, A.K.A., Kumar, S.R. and Kumar, R.R., (2009). *Agrobacterium rhizogenes* mediated hairy root production in tea leaves *Camellia sinensis* (L.) *O. Kuntze*}. *Indian J. Biotechnol.*, 8: 430-434.

Kabeeruddin, M. Kitabul, Advia, (1937). Delhi, India, Aligarh Barqi Press. Vol 2. 148.

Kanokwan, J. and Nobuo, N., (2008). Pharmacological aspects of *Andrographis paniculata* on health and its major diterpenoid constituent andrographolide. *J. Health Sci.*, 54: 370-381.

Karuppusamy, S. and Kalimuthu, K., (2010). 1 Rapid *in vitro* Multiplication and Plant Regeneration from Nodal Explants of *Andrographis paniculata*, A Valuable Endemic *Medicinal Plant Advances in Biological Research.,* 4(4): 211-216.

Kataky, A. and Handique, P.J. (2011). Effect of medium in shoot tips and nodal segments of *Andrographis paniculata*. *Asian J. Sci.Technol.*, 4: 24-28.

Kataky, A. and Handique, P.J., (2010). Micropropagation and screening of antioxidant potential of *Andrographis paniculata*. *J. Hill. Agric.*, 1: 13-18.

Kate Wright (2009). "Natural Anti-Viral Support for Coughs and Congestion" (in English). Nutrition Review 4 (4).

Kate Wright (2009). "Natural Anti-Viral Support for Coughs and Congestion".

Kiritikar, K.R. and Basu B.D., (1975). Indian Medicinal Plants, Periodical experts book agency, Delhi. 3, 1965. 11.

Kligler, B., Ulbricht, C., Basch, E., Kirkwood, C.D., Abrams, T.R., Miranda, M., Khalsa, K.P.S., Giles, M., Boon, H. and Woods, J., (2006). *Andrographis*

paniculata for the treatment of upper respiratory infection. *The Journal of Science and Healing*, 2: 25-29.

Ko, H.C., Wei, B.L. and Chiou, W.F., (2006). The effect of medicinal plants used in Chinese folk medicine on rantes. *Ethnopharmacol*, 17: 166-168.

Komwatchara, T. (1996). The development of *Andrographis paniculata* extract gel for microbial inhibition in adult periodontitis. Graduate School, Mahidol University. Bangkok, Thailand.

Koroch, A.R., Kapteyn, J., Juliani, H.R. and Simon, J.E., (2003). *In vitro* regeneration of *Echinacea pallida* from leaf explants. *In vitro Cell Dev. Biol. Plant.*, 39: 415-418.

Koteswara, Rao, Y., Vimalamma, G., Rao, C.V. and Tzeng, Y.M., (2004). Flavonoids and andrographolides from *Andrographis paniculata*. Phytochemistry., 65: 2317-2321.

Koul, I.B. and Kapil, A., (1994). Effect of diterpenes from *Andrographis paniculata* on antioxidant defense system and lipid peroxidation. *Indian J. Pharmacol.*, 26: 296-300.

Kumar, A.R., Sridevi, K., Kumar, N.V., Nanduri, S. and Rajagopal, S., (2004). Anticancer and immunostimulatory compounds from Andrographis paniculata. *J. Ethnopharmacol.*, 92(2-3): 291-295.

Kumar, B., Verma, K.S., Singh, H.P., (2011). Effect of temperature on seed germination parameters in Kalmegh (*Andrographis paniculata* Wall. Ex Nees.). *Ind. Crops and Products.*, 34: 1241-1244.

Kumar, V. and Asija, (2000). Biodiversity Conservation in: Biodiversity Principles and Conservation. *Agrobiosis* (India), Jodhpur.

Kumar, V., Sharma, A., Prasad, N.C.B., Gururaj, B.H., Ravishankar, A.G., (2006): *Agrobacterium rhizogenes* mediated genetic transformation resulting in hairy root formation is enhanced by ultra-sonication and acetosyringone treatment. Electr. J. Biotechnol. 9: 349-357.

Kyozuka, S., McElroy, J., Hayakawa, T., Xie, Y., Wu, R. and Shimamoto, K.

(1993).*Plant Physiol.* 102: 991-1000.

Lal, N. and Ahuja, P.S., (1996). Plantlet regeneration from callus in *Picrorhiza kurroa* Royle exBenth. An endangered medicinal plant. *Plant Tissue Cult.*, 6: 127-134.

Lala, S., Nandy, A.K., Mahato, S.B. and Basu, M.K., (2003). Delivery *in vivo* of 14-deoxy-11-oxoandrographolide, anti-leishmanial agent, by different carriers. *Indian J. Biochemist. Biophys.*, 40: 169-174.

Lattoo, S.K., Khan, S., Dhar, A.K, Choudhary, D.K. and Gupta, K.K., (2006). Genetic and mechanism of induced male sterility in *Andrographis paniculata* Nees and its significance. *Curr. Sci.*, 9(4): 515-519.

Leelarasamee, A., Trakulsomboon, S. and Sittisomwong, N., (1990). Undetectable anti-bacterial activity of *Andrographis paniculata* (Burma) *wall. Ex ness. J. Med. Assoc. Thai.*, 73: 299-304.

Li, W., Xu, X., Zhang, H., Ma, C., Fong, H., Van Breemen, R., and Fitzloff, J., (2007). Secondary metabolites from *Andrographis paniculata. Chem. Pharm. Bull.*, 55: 455-458.

Limsong, J., Benjavong kulchai, E. and Kuvataanasuchati, J. (2004). Inhibitory effects of some herbal extracts on adherence of *S. mutans. J. Ethnopharmcol.*, 92(2-3): 281-289.

Liu, J., Wang, Z.T. and Ji, L.L., (2007). *In vivo* and *in vitro* anti-inflammatory activities of neoandrographolide. *Am. J. Chin. Med.*, 35(2): 317-328.

Lomlim, L., Jirayupong, N. and Plubrukarn, A., (2003). Heat-accelerated degradation of solid-state andrographolide. *Chemical and Pharmaceutical Bulletin*, 51(1): 24- 26.

Madav, S., Tripathi, H.C. and Tandan, S.K., (1995). Mishra, "Analgesic, antipyretic and antiulcerogenic effects of andrographolide," *Indian Journal of Pharmaceutical Sciences,* 57(3): pp.121-125.

Malik, S.K., Chaudhury, R., and Kalia, R.K., (2005). Rapid in vitro multiplication and conservation of *Garcinia indica*, a tropical medicinal tree species. *Sci. Hort.*, 106: 539-553.

Mantell, S.H. and Hugo, S.A., (1989). Effects of photoperiod, mineral medium strength, inorganic ammonium, sucrose and cytokinin on root, shoot and microtuber development in shoot cultures of *Dioscorea alata* L. and *D. bulbifera* L. *Plant Cell Tissue Organ Cult.,* 16: 23-37.

Marimoto, K. and Fujimoto, M., (1999). Report of questionnaire survey for methicillin- resistant *Staphylococcus aurous* and penicillin-resistant *Streptococcus pneumonia* in the Kinki district. *Kansenshogaku Zasshi,* 73: 584-592.

Martin, K.P., (2003). Plant regeneration through somatic embryogenesis on *Holostemma adakodien*, a rare medicinal plant. *Plant Cell Tiss. Organ Cult.*, 72: 79- 82.

Martin, K.P., (2004). Plant regeneration protocol of medicinally important *Andrographis paniculata* (Burm. F.) *Wall Ex Nees* via somatic embryogenesis. *In vitro* Cell Dev. Pl., 40(2): 204-209.

Matsuda, T., Kuroyanagi, M., Sugiyama, S., Umehara, K., Ueno, A. and Nishi, K., (1994). Cell differentiation inducing diterpenes from *Andrographis paniculata*. *Chem. Pharm. Bull.* 42: 1216-1225.

Melchior, J., S. Palm and Wikman, G., (1997). Controlled clinical study of standardized *Andrographis paniculata* extract in common cold a pilot trial. *Phytomedicine.*,3: 315-318.

Melchior, J., Spasov, A.A. and Ostrovskij O.V., (2000). Double blind placebo controlled pilot and phase III study of activity of standardized *Andrographis paniculata* Herba Nees extract fixed combination (Kanjang) in the treatment of uncomplicated upper respiratory tract infection. *Phytomedicine*, 7: 341-350.

Melchior, J., Spasov, A.A., Ostrovskij, O.V., Bulanov, A.E. and Wikman, G., (2004). Double-blind, placebo controlled pilot and phase III study of activity of standardized *Andrographis paniculata* Herba Nees extract fixed combination (Kanjang) in the treatment of uncomplicated upper-respiratory tract infection. Phytomedicine, 7: 341-350.

Mishra, K., Dash, A.P., Swain, B.K. and Dey, N., (2009). Anti-malarial activities of *Andrographis paniculata* and *Hedyotis corymbosa* extracts and their combination with curcumin. *Malaria Journal.*, 8(1): 26.

Mishra, P., Pal, N.L., Guru, P.Y., Katiyar, J.C. and Srivastava tendon, J.S., (1992). Antimalarial activity of (kalmegh *Andrographis paniculata*) against *Plasmodium bergheink* 65 in *Mastomys natalensis. Int. Jour. Pharmacogn.*, 30: 263-274.

Mishra, S., Tiwary, S.K., Kakkar, A. and Pandey, A.K., (2010). Chemoprofiling of *Andrographis paniculata* (kalmegh) for its andrographolide contain in Mathya predesh, India. *International Journal of Phrama and Biological Science.*, 1(2): 1-5.

MPRI (Medicinal Plant Research Institute, Department of Medical Science, Ministry of Public Health), (1999). Standard of Thai Herbal Medicine: *Andrographis paniculata* (Burm. f.) Nees. The War Veterans Organization Press: Bangkok, Thailand.

Muniramappa, R.P., Farooqi, A.A., Gowda, H.G.R. and Maricapu, S., (1997). Influence of macronutrients on yield and active principle content in Kalmegh. *J. Med. Arom. Plant Sci.*, 19: 1039-1042.

Murashige, T. and Skoog, F., (1962). A revised medium for rapid growth and bioassays with tobacco tissue culture. *Physiologia Plantarum.* 15: 473-497.

Murashige, T., (1978). The impact of plant tissue culture on agriculture. In: Thorpe TA (ed). Frontiers of Plant Tissue Culture, University Offset Printing Service, pp. 15-26.

Murch, S.J., Krishna, Raj. S. and Saxena, P.K., (2000). Tryptophan is a precursor for melatonin and serotonin biosynthesis in *in vitro* regenerated St .John's wort (*Hypericum Perforatum* L.) plants. *Plant Cell Rep.*, 19: 698-704.

Naina, N.S., Gupta, P.K. and Mascarenhas, A.F., (1989). Genetic transformation and regeneration of transgenic neem (*Azadirachta indica*) plants using *Agrobacterium tumefaciens. Curr. Sci.,* 58: 184-187.

Nakanishi, K. (1965). Phytochemical survey of Malaysian plants, Preliminary chemical and pharmacological screening. *Chern. Pharmaceut. Bull.*, 13: 882-890.

Namedo, A.G., (2007). Plant cell elicitation for production of secondary metabolites, A review. *Pharmacogn Rev.*, 21: 69–79.

Ncube, N.S., Afolayan, A.J. and Okoh, A.I., (2007). Assessment techniques of antimicrobial properties of natural compounds of plant origin, current methods and future trends. *Afr. J. Biotechnol.*, 7: 1797-1806.

Negi, K.S., Tiwari, J.K. and Gaur, R.D., (1993). Notes on ethno botany of five districts of Garhwal Himalaya, Uttar pradesh, India. *Ethno Botany*. 5: 73-81.

Nester, E.W., Gordon, M.P., Amasino, R.M. and Yanofsky, M.F., (1984). Crown gall, a molecular and physiological analysis. *Ann. Rev. Plant Physiol.*

Nigra, H.M., Caso, O.H. and Guilietti, A.M., (1987). Production of solasodine by calli form different parts of *Solanum eleaginifolium* Cav. *Plants. Plant Cell Rep.*, 6: 135-137.

Niranjan, A., Tiwari, S.K. and Lehri, A., (2010). Biological activity of kalmegh (*Andrographis paniculata* nees) and its active principle. *Int. J. Nat. Prod. Res.*, 1(2): 125-126.

Paek, K.Y., Yu, K.J., Park, S.I., Sung, N.S., and Park, C.H., (1995). Micropropagation of *Rehmannia glutinosa* as medicinal plant by shoot tip and root segment culture. *Acta. Horticult.*, 390: 113-120.

Pande, D., Malik, S., Bora, M. and Srivastava, P.S., (2002). Rapid protocol for *in vitro* micropropagation of *Lepidium sativum* Linn and enhancement in the yield of lepidine. *In vitro Cell Dev. Biol.*, 38: 451-455.

Parisi, M., Moreno, S. and Fernandez, G., (2002). Characterization of a novel cysteine peptidase from tissue culture of garlic (*Allium sativum* L.). *In vitro Cell Dev. Biol.*, 38: 608-612.

Park, J.M. and Yoon, S.Y., (1992). Production of sanguinarine by suspension culture of *Papaver somniferum* in bioreactors. *J. Ferm, Bioeng.*, 74: 292-296.

Payen, J., Hamill, J.D., Robins, R.J. and Rhodes, M.J.C., (1987). Production of hyoscyamine by 'hairy root' culture of *Datura stramonium. Planta Med.*, 53: 474-478.

Pawar, R. K., Sharma Shivani, Singh K.C. and Sharma Rajeev K.R. development and Validation of HPTLC method for the determination of andrographolide from *Andrographis paniculata* (whole plant) International *Journal of Chemistry Research ISSN-* 0976 – 5689 Vol 1, Issue 2, (2010).

Perez, C., Paul, M. and Bazerque, P., (1990). Antibiotic assay by agar well diffusion method. *Acta Biol. Med. Exp.*, 15: 113-115.

Perez-Bermudez, P., Seitz, H.U., Gavidia, I. A., (2002). Protocol for rapid micropropagation of endangered Isoplexis. *In vitro Cell Dev. Biol.*, 38: 178-182.

Pholphana, N., Rangkadilok, N., Thongnest, S., Ruchirawat, S. and Ruchirawat, M., (2004). Determination and variation of three active diterpenoids in *Andrographis paniculata* (Burm.f.) Nees. *Phytochem Anal.*, 15(6): 365-371.

Pittaalvarez, S.I. and Giulietti, A.M., (1998). Novel biotechnological approachs to obtain scopolamine and hyoscyamine. The influence of biotic elicitors and stress agents on the culture of transformed roots of *Brugmansia candida*. *Phytother Res.*, 18-20.

Poolsup, N., Suthisisang, C., Prathanturarug, S., Asawamekin, A. and Chanchareon, U., (2004). Andrographis paniculata in the symptomatic treatment of uncomplicated upper respiratory tract infection, systematic review of randomized controlled trials. *Journal of Clinical Pharmacy and Therapeutics.*, 29(1): 37-45.

Pradel, H., Dumkelehmann, U., Diettrich, B. and Luckner, M., (1997). Hairy root cultures of *Digitalis lanata*. Secondary metabolism and plant regeneration. J. Plant Physiol. 151: 209-215.

Prajjal, K., Singha, Roy, S. and Dey, S., (2003). Antimicrobial activity of *Andrographis paniculata, Fitoterapia.*, 74(7-8): 692-694.

Prathanturarug, S., Schaffner, W., Berger Buter, K. and Pank, F., (1996). *In vitro* propagation of the Thai medicinal plant *Andrographis paniculata* Nees. Proc. Int. Symp. *Breeding Research on Medicinal and Aromatic Plants.*, 304-306.

Praveen N., Manohar, S.H., Naik, P.M., Nayeem, A. and Jeong, J.H., (2009). Production of andrographolide from adventitious root cultures of *Andrographis paniculata*. Curr. Sci., 96:694-697.

Puri, A.R., Saxena, R.P., Saxena. and Saxena, K.C., (1993). Immunostimulant agents from *Andrographis paniculata. J. Natl. Prod.*, 56: 995-999.

Purkayastha, J., Sugla, T., Paul, A., Solleti, S. and Sahoo, L., (2008). Rapid *in vitro* multiplication and plant regeneration from nodal explants of *Andrographis paniculata*, a valuable medicinal plant. *In vitro Cell. Dev. Biol.*, 44: 442-447.

Qian, Z.G., Zhao, Z.J., Xu, Y., Qian, X. and Zhong, J.J., (2006). Novel chemically synthesized salicylate derivative as an effective elicitor for inducing the biosynthesis of plant secondary metabolites. Biotechnol Prog., 22: 331–333.

Radha, R., Sermakkani, M. and Thangapandian, V. (2011). Evaluation of phytochemical and antimicrobial activity of *Andrographis paniculata* nees (*Acanthaceae*) aerial parts *Int. J. of Pharm. and Life Sci.* (IJPLS)., 2(2): 562-567.

Rahman, N.N.N.A., Furuta, T., Kojima, S., Takane, K. and Ali Mohd, M., (1999). Antimalarial activity of extracts of Malaysian medicinal plants. *Journal of Ethnopharmacology*, 64(3) 249-254.

Raina, P., Kumar, A. and Pareek, S.K., (2007). HPTLC analysis of hepatoprotective diterpenoid Andrographolide from *Andrographis paniculata* (Kalmegh). *Indian J. of Pharmaceutical Sciences.*, 69(3): 473-475.

Rajalakshmi, G., Aruna, D., Bhuvaneswari, B., Venkatesan, R.S., Natarajan, A., and Jegatheesan, K., (2012). Prophylactic effect of Andrographis paniculata extracts against fungal species. *J. App. Pharm. Sci.*, 2(9): 058-060.

Rajani, M., Shrivastava, N. and Ravishankara, M.N., (2000). A rapid method for isolation of andrographolide from *Andrographis paniculata* Nees (Kalmegh). *Pharmaceutical Biology.*, 38(3): 204-209.

Rajgopal, S., Kumar, R.A., Devi, D.S., Satyanarayan, C. and Rajgopalan, R., (2003). Andrographolide, a potential cancer therapeutic agent isolated from *Andrographis paniculata. J. Exper. and Therapeutic Oncology.*, 3: 147-158.

Ramesh, P.M., (1994). Standardization of cultural practices in Kalmegh (*Andrographis paniculata* Nees), University of Agricultural sciences, Bangalore.

Ramesh. K., Satdive., Devanand, P., Fulzele, and Susan Eapen., (2006). Production of biuopesticide *Azadirachta indica* by hairy root cultures of neem (*Azadirachta indica.* Juss) BARG newsletter.

Rout, G.R., (2002). Direct plant regeneration from leaf explants of Plambago species and its genetic fidelity through RAPD markers. Annal. Appl. Biol., 140: 305-313.

Rout, G.R. and Das, P., (1997). *In vitro* organogenesis in ginger (*Zingiber officinale* Rosc.). *J. Herbs, Spices and Med.*, 4: 41-51.

Rout, G.R., (2002).Tryptophan is a precursor for melatonin and serotonin biosynthesis in *in vitro* regenerated St. John's wort (*Hypericum Perforatum* L. cv. Anthos) plants. *Plant Cell Rep.*, 19: 698-704.

Rout, G.R., S. Samantaray. and P. Das., (2000). *In vitro* manipulation and propagation of medicinal plants. *Biotech. Adv.,* 18: 91-120.

Rout, G.R., Saxena, C. Samantaray. S. and P. Das., (1999). Rapid clonal regeneration of *Plumbago zeylanica* Linn. *Plant Growth Regul.*, 28: 1-4.

Roy, S., Rao, K., Bhuvaneswari, C., Giri, A. and Mangamoori, L.N. (2010). Phytochemical analysis of *Andrographis paniculata* extract and its antimicrobial activity. *World J. Microbiol Biotechnol.,* 26: 85-91.

Roy, S.K., Hossain, M.Z., Islam, M.S., (1994). Mass propagation of *Rauvolfia serpentina* by in vitro shoot tip culture. *Plant Tissue Cult.*, 4: 69-75.

Sadick, N.S., (1997). Current aspects of bacterial infections of the skin. *Dermatol. Clin.*, 15: 341-349.

Sahrawat, A. K.; Chand, S. (2002)Somatic embryogenesis and plant regeneration from root segments of Psoralea corylifolia L., an endangered medicinally important plant. In Vitro Cell. Dev. Biol. Plant. 38:33–38;.

Samantaray, S., Rout, G.R. and Das, P. (2001). Heavy metal and nutrient concentration in soil and plants growing on a metaliferous chromite minespoil. *Enviro Tech.*, 22: 1147-1154.

Satheesh, Kumar. K. and Bhavanandan, K.V., (1988). Micropropagation of *Plumbago rosea* Linn. *Plant Cell Tissue Organ Cult.*, 15: 275-278.

Sawahel, W. (1997). Plant genetic transformation technology. Daya Publishing House, India.

Sawahel, W. and Cove, D. (1992). Gene transfer strategies in plants. *J. Biotechnol. Adv.,* 10: 393-412.

Saxena, S., Jain, D.C., Gupta, M.M., Bhakuni, R.S., Mishra, H.O., and Sharma, R.P., (2000).High-performance thin layer chromatographic analysis of hepatoprotective diterpenoids from Andrographis *paniculata*. *Phytochemical Analysis*., 11: 34-36.

Scragg, A.H., (1992). Bioreactors for mass cultivation of plant cells. In: Fowler, M.W., Warren, G.S., (eds). Plant Biotechnol. Oxford: Peragmon Press.

Sarasan, V.; Soniya, E. V.; Nair, G. M. Regeneration of Indian Sarsaparilla, Hemidesmus indicus R. Br., through organogenesis and somatic embryogenesis. Indian J. Exp. Biol. 32:284–287; (1994).

Sharma, A., Lal, K. and Handa, S.S., (1992). Standardisation of the Indian crude drug Kalmegh by high-pressure liquid chromatographic determination of andrographolide. *Phytochem. Anal.*, 3: 129-131.

Sharma, A., Singh, R. T., Sehgal, V. and Handa, S. S., (1991). Antihepatotoxic activity of some plants used in herbal formulation. *Fitoterapia*., 62: 131-138.

Sharma, P.V., (1983). Charka Samhita Ed. *Chankhambhia Orientalia*, Vol. II, Varanasi.

Sharmila, R. and Subburathinam, K.M., (2013). Effect of Signal compounds on Andrographolide in the Hairy Root Cultures of *Andrographis paniculata*. *Int. J. Pharm. Sci. Res.*, 4(5): 1773-1776.

Shasany, A.K., Khanuja, S.P.S., Dhawan, S., Yadav, U., Sharma, S. and Kumar, S., (1998). High regenerative nature of *Mentha arvensis* internodes. *J. Bio. sci.,* 23: 641-646.

Sheeja, K. and Kuttan, G., (2007). Antibody dependent complement-mediated cytotoxicity by andrographolide in normal and enrich ascites carcinoma-bearing mice. *Integr. Cancer Ther.*, 6: 66-73

Sheeja, K., Shihab, P.K. and Kuttan, G., (2006). Antioxidant and anti-inflammatory activities of the plant *Andrographis Paniculata* Nees. *Immunophar-macol. Immunotoxicol.*, 28: 129-140.

Shen, Y.C., Chen, C.F. and Chiou, W.F., (2002). Andrographolide prevents oxygen radical production by human neutrophils: possible mechanism(s) involved in its anti-inflammatory effect. *British Journal of Pharmacology.*, 135(2): 399-406.

Shi, M.D., Lin, H.H., Lee, Y.C., Chao, J.K., Lin, R.A.and Chen, J.H., (2008). Inhibition of cell-cycle progression in human colorectal carcinoma Lovo cells by andrographolide. *Chemico-Biological Interactions*, 174(3): 201-210.

Shibata, D. and Liu, Y.G., (2000). *Agrobacterium* mediated plant transformation with large DNA fragments. *Trends Plant Sci.*, 5: 354-357.

Siivarajan, V. V. and Indira Balachandran, (1994). Ayurvedic drugs and their Plant Sources: p.315.

Singh, A., Meena, A.K., Sudeed., Meena., Pant, P. and padhi, M.M., (2012). Studies on standardisation of *Andrographis paniculata* nees and identification by HPTLC using andrographolide as marker compound. *Int. j. pharm. Sci.,* 4(2): 197-200.

Singha, P.K., Roy, S. and Dey, S., (2003). Antimicrobial activity of *Andrographis paniculata. Fitoterapia,* 74(7-8): 692-694.

Siripong, P., Kongkathip, B., Preechanukool, K., Picha, P., Tunsuwan, K. and Taylor, W.C., (1992). Cytotoxic diterpenoid constituents from *Andrographis paniculata* Nees leaves. *J. Sci. Soc.*, 18: 187-194.

Skirvin, R.M., Chu, M.C., Young, H.J., Rose, Ammirato, P.V., Evans, D.R., Sharp, W.R., Bajaj, Y.P.S., (eds), (1990). Handbook of Plant Cell Cultures. *MacMillan*, New York, 5: 716– 743.

Souret, F.F., Weathers, P.J. and Wobbe, K.K., (2002). The mevalonate independent pathway is expressed in transformed roots of *Artemisia annua* and regulated by light and culture age. *In vitro Cell Dev. Biol. Plant.* 38: 581-581.

Srivastava, A., Misra, H., Verma, R.K., and Gupta, M.M., (2004). Chemical fingerprinting of Andrographis paniculata using HPLC, HPTLC and densitometry. *Phytochemical Analysis.* 15: 280-285.

Srivastava, J., Lambert, J. and Vietmeyer, N., (1996). Medicinal plants: An expanding role in development World Bank Technical Paper. No. 320.

Srividya, N. and Devi, B.P.S., (1998). Azadirachtin and nimbin content in in vitro cultured shoots and roots of *Azadirachta indica* A. Juss. *Indian J. Plant Physiol.*, 3: 129-134.

Stafford, A., Morris, P. and Fowler, M.W., (1986). Plant Cell Biotechnology, a perspective. *Enzyme Microbial Tech.*, 8: 578-597.

Stockwell, Bylka., W., Szaufer-Hajdryc, M. and Matalawskan, I., (1988). Natures pharmacy. London, United Kingdom. Century Hutchinson Ltd.

Subramanian, R., Asmawi, M Z. and Sadikun, A., (2008). In vitro α-glucosidase and α-amylase enzyme inhibitory effects of *Andrographis paniculata* extract and andrographolide. *Acta Biochimica Polonica.*, 55(2): 391-398.

Sudhanshu, Saxena., Dharam, C., Jain, Madan, M., Gupta, Rajendra, S., Bhakuni, Hari, O., Mishra, and Ram, P.S., (2000). HPTLC Analysis of Hepatoprotective Diterpenoids from *Andrographis paniculata* phytochemical analysis. *Phytochem.*, 11: 34-36.

Sule, A., Ahmed, Q.U., Samah, O.A. and Omar, M.N. (2011). Bacteriostatic and bactericidal activities of *Andrographis paniculata* extracts on skin disease causing pathogenic bacteria. *J. Med. Plant Res.*, 5(1):7-14.

Talukadar, P. B., and Dutta, A.K., (1969). Quantitative estimation of andrographolide by TLC (thin layer chromatography). *Ind. J. Appl. Chem.*, 32: 25-28.

Tang, T., Targan, S.R., Li, Z.S., Xu, C., Byers, V.S. and Sandborn, W.J., (2011). Randomised clinical trial, Herbal extract HMPL-004 in active ulcerative colitis- A double-blind comparison with sustained release mesalazine. *Alimentary Pharmacology and Therapeutics.*, 33(2): 194-202.

Tapsell, L.C., Hemphill, I., Cobiac, L., Patch, C.S., Sullivan, D.R., Fenech, M., Roodenrys, S., Keogh, J.B., Clifton, P.M., Williams, P.G., Fazio, V.A. and Inge, K.E., (2006). Health benefits of herbs and spices, the past, the present, the future. *Med. J. Aust.*, 21: 14-24.

Tepfer, D., (1990). Genetic transformation using *Agrobacterium rhizogenes. Physiol. Pl.*, 79: 140-146.

Tawfik, A. A.; Noga, G. Cumin regeneration from seedlings derived embryogenic callus in response to amended kinetin. Plant Cell Tiss. Organ Cult. 69:35–40; (2002).

Thamlikitkui, V., Dechatiwongse, T. and Theerapong, S., (1991). Efficacy of *Andrographis paniculata* Nees for pharyngotonsillitis in adults. *J. Med. Assoc. Thailand*, 74: 437-442.

Thiyagarajan, P., Deepak, H.B., and Agarwal, A., (2011). *In vitro* modulation of LPS/calcimycin induced inflammatory and allergic mediators by pure compounds of *Andrographis paniculata* (King of bitters) extract Chandrasekaran. *International Immunopharmacology*. 11(1): 79-84.

Thomson, W.A.R., (1978). Medicines from the Earth. Maidenhead, United Kingdom. McGraw-Hill Book., 158: 353-357.

Tiwari, R.K., Trivedi, M., Guang, Z.C., Guo, G.Q., Zheng, G.C., (2007). Genetic transformation of Gentiana *macrophylla with Agrobacterium rhizogenes*, growth and production of secoiridoid glucoside gentiopicroside in transformed hairy root cultures. *Plant Cell Rep.*, 26: 199-210.

Toivonem, L., Ojala, M. and Kauppinen, V., (1991). Studies on the optimization of growth and indole alkaloid production by hairy root culture of *Catharanthus roseus. Biotechnol Bioenge.,* 37: 673-680.

Tokuhara, K. and Mii, M., (2001). Induction of embryogenic callus and cell suspension culture from shoot tips excised from flower stalk buds of *Phalaenopsis* (Orchidaceae). *In Vitro Cell. Dev. Biol.*, 37: 457-461.

Toppel, G., Witte, L., Riebesehl, B., Von, Borstel, K. and Hartman, T., (1987). Alkaloid patterns and biosynthetic capacity of root cultures from some pyrrolizidine alkaloid producing Senecio spp. *Plant Cell Rep.*, 6: 466-469.

Trivedi, N.P. and Rawal, U.M., (2001). Hepatoprotective and antioxidant property of *Andrographis paniculata* (Nees) in BHC induced liver damage in mice. *Indian Journal of Experimental Biology.*, 39(1): 41-46.

Tzfira, T., Li, J., Lacroix, B. and Citovsky, V., (2004). *Agrobacterium* T-DNA integration, Molecules and models. *Trends Genet*, 20: 375-383.

Verma, A., Padh, H. and Shrivastava, N., Andrographolide- A new plant-derived antineoplastic entity on horizon.

Vijaya, D., Padmadevi, S.N., Vasandha, S., Meerabhai, R.S. and Chellapandi, P., (2008). Effect of vermin composted coir pith on the growth of *Andrographis paniculata*. *Jour. Org. Syst.*, 3(2): 51-56.

Vijayakumar. Arul Doss. and Kalaichelvan, P.T., (2012). *In vitro* antimicrobial and antioxidant activity screening of *Andrographis paniculata* leaf ethanolic extract in Tamilnadu. *int. Jour. Pharm. sci.*, 4(1): 227-229.

Vijayalaxmi, S., Dandin, Hosakatte, Niranjana. and Murthy., (2012). Regeneration of *Andrographis paniculata*, Analysis of genetic fidelity and andrographolide content in micro-propagated plants. *African Journal of Biotechnology.*, 11(61): 12464-12471.

Vijaykumar, K., Murthy, P.B.S., Kannababu, S., Syamasundar, B., and Subbaraju, G.V., (2007). Estimation of andrographolide in *Andrographis paniculata* herb, extracts and dosage forms. Int. J. Appl. Sci. Eng., 5: 27-39.

Voravuthikunchai, S.P. and Limsuwan, S., (2006). Medicinal plant extracts as anti- *Escherichia coli* O157:H7 agents and their effects on bacterial cell aggregation. *Journal of Food Protection.*, 69(10): 2336-2341.

Wangboonskul, J., Daodee, S., Jarukamjorn, K. and Sripanidkulchai, B.O., (2006). Pharmacokinetic study of *Andrographis paniculata* tablets in healthy thai male volunteers. *J. Thai Pharm. Health* Sci., 1(3): 209-218.

Werner T, Motyka V, Laucou V, Smets R, Van Onckle H, Schmulling, T. (2003). Cytokinin-deficient.

Weiming, C., Xiaotian, L., (1982). Deoxyandrographolide- 19 beta-D-glucoside from the leaves of *Andrographis paniculata. Planta. Med.*, 45: 245-246.

White, P.R., (1939). Potentially unlimited growth of excised plant callus in an artificial medium. *American Journal of Botany*, 26(1): 59-64.

Wiart, C., Kumar, K., Yusof, M.Y., Hamimah, H., Fauzi, Z.M. and Sulaiman, M., (2005). Antiviral properties of ent-labdene diterpenes of *Andrographis paniculata* Nees, inhibitors of herpes simplex virus type 1. *Phyto. Res.*, 19(12): 1069–1070.

Withers, L.A., Anderson, P.G., (1986). Plant Tissue Culture and its Agricultural Applications. Butterworths, London.

Wongkittipong, R., Prat, L., Damronglerd, S. and Gourdon, C., (2000). Solid liquid extraction of andrographolide from plants- experimental study, kinetic reaction and model. *Separation and Purification Technology*, 40: 147-154.

Xia, Y.F., Ye, B.Q. and Li, Y.D., (2004). Andrographolide attenuates inflammation by inhibition of NF-KB activation through covalent modification of reduced cysteine 62 of p 50. *Journal of Immunology*, 173(6): 4207-4217.

Xie, Y., Chturvedi, G., Tomar, G.S., Tiwart, S.K. and Singh, K.P., (1983). Clinical studies on Kalmegh (*Andrographis paniculata* Nees) in infective hepatitis. *J. Internat. Inst. Ayurveda.*, 2: 208-211.0

Xu, J., Li, Z. and Caoa, M., (2012). Synergetic effect of *Andrographis paniculata* polysaccharide on diabetic nephropathy with andrographolide. *International Journal of Biological Macromolecule*., 51(5): 738-742.

Xu, Y., Marshall, R. L. and Mukkur, T.K.S., (2006). An investigation on the antimicrobial activity of *Andrographis paniculata* extracts and andrographolide *in vitro*. *Asian Journal of Plant Sciences.,* 5(3): 527-530.

Yang L, Wu D, and Luo K, (2009). Andrographolide enhances 5-fluorouracil-induced apoptosis via caspase-8-dependent mitochondrial pathway involving p53 participation in hepatocellular carcinoma (SMMC-7721) cells. Cancer Lett ., 276:180-188.

Youhong, X.u., Marshall, R.L. and Mukkur T.K.S., (2006). An investigation on the antimicrobial activity of *Andrographis paniculata* extracts and Andrographolide *in vitro*. *Asian J. Plant Sci.*, 5(3): 527-530.

Yukimune, Y., Tabata, H., Higuchi, Y and Hara, Y., (1996). Methyl *jasmonate* induced over production of paclitaxel and baccatin III inTaxus cell suspension cultures. *Nature Biotechno.*, l14: 1129-1132.

Yun, D.J., Hashimoto, T. and Yamada, Y., (1992). Metabolic engineering of medicinal plants, transgenic *Atropa belladonna* with an improved alkaloid composition. *Proc. Nat. Acad. Sci. USA.*, 89: 11799-11803.

Zafar, Y., Wajid, A., Malik. and Gamborg, O.L., (1992). Establishment of regenerating calli and cell suspension line of Basmati rice (*Oryza sativa* L.). *J. Bot.* 24(1): 64-71.

Zaidan, M. R., Noor Rain, A. Badrul, A. R., Adlin, A., Norazah, A. and Zakiah, I., (2005). *In vitro* screening of five local medicinal plants for antibacterial activity using disc diffusion method. *Tropical Biomedicine.*, 22(2): 165-170.

Zenk, M.H., (1978). The impact of plant cell culture on industry. In: Thorpe T.A. (ed). Frontiers of Plant Tissue Culture, University of Calgary, Int. Ass. for Plant Tissue Culture., 1-13.

Zhang X.F. and Tan, B.K., (2000). Anti-hyperglycaemic and antioxidant properties of *Andrographis paniculata* in normal and diabetic rats. *Clinical Experimental Pharmacology and Physiology,* 27: 358-363.

Zhang, X., (2004). WHO monograph on selected medicinal plants, World Health Organization, Geneva, vol. 2.

Zhao, D.L., Guo, G.Q., Wang, X.Y. and Zheng, G.C., (2004). In vitro propagation of a medicinal plant species *Sophora flavescens*. *In vitro Cell. Dev. Biol.*, 47:117-120.

Zhao, J., Zhu, W.H., Hu, Q. and Guo, Y.Q., (2001). Compact callus cluster suspension cultures of *Catharanthus roseus* with enhanced indole alkaloid biosynthesis. *In vitro Cell Dev. Biol.*, 37: 68-72.

Zhu, P.Y., Peng, N.B., and Jiang, W.J. (1984). TLC-UV spectrophotometric determination of andrographolide in the leaves and stems of *Andrographis paniculata*. *Chin. J. Pharm. Anal.*, 4: 34-46.

Zid, S.A. and Orihara, Y., (2005). Polyacetylenes accumulations in *Ambrosia maritime* hairy roots and cell cultures after elicitation with methyl jasmonate. *Plant Cell Tiss. Org. Cult.*; 81: 65-67.

Appendices

APPENDIX- 1

Components	Concentration in medium (mg/l)	Concentration in stock solution (mg/l)
	MS	**MS**
MACRONUTRIENTS		**20 X**
NH_4NO_3	1650.00	33000
KNO_3	1900.00	38000
$MgSO_4.7H_2O$	370.00	7400
KH_2PO_4	170.00	3400
$CaCl_2.2H_2O$	440.00	408800
MICRONUTRIENTS		**200X**
KI	0.83	166
H_3BO_3	6.20	1240
$MnSO_4.4H_2O$	22.30	4460
$ZnSO_4.7H_2O$	8.60	1720
$CuSO_4.5H_2O$	0.025	50
$CoCl_2.6H_2O$	0.025	5
$Na_2MoO_4.2H_2O$	0.25	5
IRON SOURCE		**200 X**
$FeSO_4.7H_2O$	27.80	5560
$Na_2EDTA.H_2O$	37.30	7460
ORGANIC SUPPLIMENTS		**200X**
Thiamine HCl	0.5	100
Pyridoxine	0.5	100
Nicotinic acid	0.5	100
Glycine	2.0	400
Myoinositol	Added freshly	100
Energy source - Sucrose(Added as solid) @30000.00mg/l		
Solidifying agent – Agar @ 8000.00 mg/l		

Iron- EDTA (200X) 500 ml stock

5560 mg of $FeSO_4.7H_2O$ was dissolved in 500 ml of D.W. 7460 mg of Na_2 EDTA was added in 500 ml of D.W and boiled to dissolve it completely and it was then mixed with $FeSO_4.7H_2O$ solution.

Volume to be taken from stocks

Stock	Volume/lit
Macronutrients	50ml
Micronutrients	5ml
Iron EDTA	5ml
Vitamins	10ml
Myoinositol	10mg
Glycine	2mg
Ascorbic acid	100mg
Sucrose	30gm
Agar	6.5gm
D.W	Make up 1000 ml
pH	5.8

APPENDIX - 2

1. Preparation of the phytoharmone stock

a) 1 mM NAA stock solution 100 ml (MW 186.2)

18.62mg of NAA was dissolved in 0.5ml DMSO (Dimethyl Sulfoxide) and adds sterile double distilled water, stir it and the make up to 100ml and store at 4° C

b) 1 M 2,4-D stock solution 100 ml (MW. 221)

22.1mg of 2,4-D dissolved in 0.5ml 1N NaOH and 10ml water by vortexing and make up the volume to 100ml by adding sterile double distilled water, Store at 4° C

c) 1 mM BAP stock solution 100 ml (MW. 224.2)

22.5mg of BAP was dissolved in in 1N NaOH (0.3-0.5Ml) and add double distilled water slowly with stir volume make up to 100ml and store at 4° C

d) 1 mM Kinetin stock solution 100 ml (MW. 215.2)

21.5mg of Kn was dissolved in 0.5ml of 0.5N NaOH and add double distilled water slowly and make up the volume to 100 ml. Store at 4° C.

e) 1 mM IAA stock solution 100 ml

20 mg of IAA was dissolved in 0.5 ml of 0.1N NaOH and add double distilled water slowly while sterile, and make up the volume to 80 ml. Store at 4° C.

f) GA_3

20 mg of GA was dissolved in 0.5 ml of 0.5N NaOH and add double distilled water slowly while sterile, and make up the volume to 80 ml. store at 4o C.

g) IBA

20 mg of IAA was dissolved in 0.5 ml of 0.1N NaOH and add double distilled water slowly while sterile, and make up the volume to 80 ml. Store at 4^{0} C.

2. **Antibiotic Stock Solution**
   1. **Carbenicillin:** 1 gm of carbenicillin was dissolved in 50% ethanol. Filter sterilised by using syringe filter in to sterile eppendroff tube aseptically and store at 4^{0} c.
   2. **Cefotaxime:** 1 gm of Cefotaxime was dissolved in double distil water. Filter sterilised by using syringe filter in to sterile eppendroff tube aseptically and store at 4^{0} c.

APPENDEX- 3

YEMA medium (100 ml)

Chemical	Quantity
Yeast extract	0.1 gm
Mannitol	1.0 gm
KH_2PO_4	0.05 gm
$MgSO_47H_2O$	0.02 gm
NaCl	0.01 gm
Agar	1.5 gm

By adding all chemical in 100 ml of D/W media was autoclave and used for next purpose.

APPENDEX- 4

Nutrient agar (100 ml)

Chemical	Quantity
Beef extract	0.1 g
Yeast extract	0.2 g
Peptone	0.5 g
NaCl	0.5 g
Agar	1.5 g

By adding all chemical in 100 ml of D/W media was autoclave and used for next purpose.

APPENDIX - 5

Potato

Chemical	Quantity
Potato infusion	0.2 g
Dextrose	0.02 g
Agar	0.02 g

Dextrose Agar (100 ml)

By adding all chemical in 100 ml of D/W media was autoclave and used for next purpose.

APPENDIX - 6

LPGM (Liquid Plant Growth Medium)

Liquid MS Medium + 2 mg/l BAP + 200 Mm Acetosyringone

APPENDEX- 7

1. Suspension buffer

Stocks:

- 1 M glucose (Stored at 40C)
- 0.5 M EDTA
- 1 M Tris-HCl (pH 8.0)

Working solutions:

5 ml of 1 M glucose + 2 ml of 0.5 M EDTA + 2.5 ml of 1 M Tri-HCL were combined and 5 mg/ml lysozyme was added to solution 1 buffer use.

2) Lysis buffer

Stocks:

- 10 N NaOH
- 10% SDS

Working solution:

0.8 ml 10 N NaOH + 4 ml 10% SDS + 35.2 ml of sterile distilled water

3) 1.5 M Potassium acetate solution Stock (stored at 40C):

5 M K-Acetate

Working solution:

60 ml of 5 M potassium acetate was mixed with 28.5 ml of glacial acetic acid and 11.5 ml of sterile distilled water. pH of the final solution was adjusted to 4.8 – 5.3 using glacial acetic acid.

APPENDEX- 8

TE BUFFER

Tris-HCL	10 mM
EDTA	1 mM

APPENDIX - 9

DNA loading dye:

Stock (6X):

0.25% bromophenol blue, 0.25% xylene cynol FF, 30% glycerol in water.

Reagent needed:

25 mg bromophenol blue.

25 mg xylene cyanol FF.

3.3 ml glycerol.

6.7 ml dd H_2O.

APPENDIX - 10

TAE buffer 50X pH 8.0 – 100 ml:

Tris base	24.2 gm
Glacial acetic acid	5.71 ml
0.5M EDTA, pH 8.0	10ml

0.5M EDTA pH 8.0 – 100ml:

Weighed 18.61 gm of EDTA in 60-70 ml of D/W and dissolve it on magnetic stirrer by adding NaOH pellet's adjust the pH 7.0 and made the final volume 100ml with sterile d/w.

Ethidium bromide stock

Weighed 10 mg EtBr powder and dissolved in 1 ml of D/W and stock conc.was 10 mg/ml.

Working conc. Of EtBr is 0.5µg/ml.

APPENDEX- 11

REAGENTS AND SOLUTIONS:

A. 1.0 M Tris-HCl (pH 8.0)

Dissolve 12.11gm Tris HCl in sterile de-ionized water, adjust pH to 8.0 with conc. HCl and make up volume to 100 ml with de-ionized water and autoclave at 15 psi for 20 min.

B. 0.5 M EDTA (pH 8.0)

EDTA (dissolved salt; Mw = 372.3) = 18.61gm

Dissolve, 18.61gm EDTA in sterile de-ionized water, adjust pH to 8.0 with 5N NaOH, make up volume to 100 ml with de-ionized water and autoclaved at 15 psi for 20 min.

C. 5 M NaCl

NaCl = 29.2 gm

Dissolve 29.2gm Nacl in sterile de-ionized water, make up volume to 100 ml with de-ionized water and autoclave at 15 psi for 20 min

D. Extraction buffer

1M Tris-HCl (pH 8.0)	- 10 ml
0.5 M EDTA (pH 8.0)	- 2 ml
3 M NaCl	- 46.6 ml
2% CTAB (w/v)	- 2 g
0.2% ß-Mercaptoethanol	- 0.2ml

Dissolve the above and make up to 100 ml with de-ionized water and autoclave at 15 psi For 20 min.

E.Choloroform: Iso-amyl alcohol mixture (24:1)

Choloroform	- 96 ml
Iso-amyl alcohol	- 4 ml

F. 70% ethanol (100ml)

Absolute alcohol	- 70 ml
Double distilled water	- 30 ml

APPENDIX 12

1. Mobile phase for HPTLC

Toluene: ethyl acetate: formic acid (7.5: 2.0: 0.5)

Toluene	- 75 ml
Ethyl acetate	- 20ml
Formic acid	- 5 ml

2. Spraying reagents for HPTLC

Anisaldehyde: glacial acetic acid: Sulphuric acid

Anisaldehyde	- 0.5 ml
H_2SO_4	-1.0 ml
Glacial acetic acid	- 50.0 ml

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