PLANT TISSUE CULTURE AND PRODUCTION OF TRITERPENOIDS FROM MEDICINAL PLANTS *AZADIRACHTA INDICA* A. JUSS. AND *LANTANA CAMARA* L.

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PRIYANKA SRIVASTAVA

DEPARTMENT OF BIOTECHNOLOGY
INDIAN INSTITUTE OF TECHNOLOGY GUWAHATI
GUWAHATI- 781039
ASSAM, INDIA

...Dedicated

to

My Parents...

INDIAN INSTITUTE OF TECHNOLOGY GUWAHATI

Department of Biotechnology

STATEMENT

I do, hereby, declare that the matter embodied in this thesis is the result of investigations carried out by me in the Department of Biotechnology, Indian Institute of Technology Guwahati, India, under the guidance of Dr Rakhi Chaturvedi.

In keeping with the general practice of reporting scientific observations, due acknowledgements have been made wherever the work described is based on the findings of other investigators.

Date: May 09, 2010 **Priyanka Srivastava**

INDIAN INSTITUTE OF TECHNOLOGY GUWAHATI

Department of Biotechnology

CERTIFICATE

It is certified that the work described in this thesis entitled "***Plant Tissue Culture and Production of Triterpenoids from Medicinal Plants: Azadirachta indica A. Juss. and Lantana camara L.***" by Priyanka Srivastava for the award of degree of Doctor of Philosophy is an authentic record of the results obtained from the research work carried out under my supervision in the Department of Biotechnology, Indian Institute of Technology Guwahati, India, and this work has not been submitted elsewhere for a degree.

Date: May 09, 2010

Dr Rakhi Chaturvedi
Associate Professor
(Thesis Supervisor)
Department of Biotechnology
IIT Guwahati
Guwahati-781039
Assam, India

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ABSTRACT

Medicinal plants are indispensible to human health. From times immemorial, the therapeutic benefits offered by these plants have been exploited by the common people and medical professionals alike. This is the reason why most of them have made their ways to scientific laboratories throughout the world as their large scale production and enhancements of their bioactive constituents have become a matter of fascination and interest to the scientists worldwide.

Azadirachta indica A. Juss. (commonly known as Neem), an evergreen tropical tree of family Meliaceae, is one of the most fascinating trees of Asian subcontinent. It has many important medicinal, agrochemical and economic uses to its credit. Today, due to its remarkable biopesticidal properties shown by azadirachtin and other related triterpenoids, the tree has attained global importance. Though this property is not unique to neem, it holds a distinction as it provides a highly effective antifeedant, nontoxic and environment-friendly means of controlling or eliminating insect pests. Due to commercial potential and renewed worldwide research interest, the tree has now been introduced to other regions of the globe including Africa, South America and Australia. In spite of having valued properties, improvement of Neem by conventional methods is very limited owing to its highly heterozygous nature, long reproductive cycle and recalcitrant and poor seed yield. In this context, it is noteworthy that, studies utilizing gametophytic cells are in infancy, in this tree species. *In vitro* haploid production from gametophytic cells enables the establishment of completely homozygous lines in a shortened time frame compared to conventional methods and has many potential applications in plant improvement to establish inbred lines rapidly and to observe recessive traits. Therefore, the aim of the present study was to establish *in vitro* androgenic lines of Neem from anthers and check the contribution of these haploid cultures in the production of medicinally important azadirachtin, a tetranortriterpenoid, chiefly present in seeds. The presence of compound has been confirmed by chromatographic and spectroscopic techniques. Bioassays carried out further strengthen the aim of this investigation.

Lantana camara L., commonly known as Red or Wild Sage, is an evergreen, strong-smelling woody shrub belonging to the family Verbenaceae. About 150 species

are known to represent this genus. The plant is a native of tropical America but is now naturalized in many parts of India. All parts of the plant have been used traditionally for several ailments throughout the world. Several triterpenoids, napthaquinones, flavonoids, alkaloids and glycosides isolated from this plant are known to exert diverse biological activities including cytotoxic and anticancer properties. A number of potential uses of *Lantana* plant have been suggested but none has been exploited on the large scale. There is a wide scope of research on this plant. Owing to the prevalent heterozygosity in this genus and seasonal variation in secondary metabolite content, tissue culture offers a solution through which cell biomass can be raised and important compounds can be harvested from its biomass all the year round unaffected by the seasonal variation. The purpose of large scale production of pharmaceutically important metabolites can alone be served with the establishment of callus and cell suspension cultures derived from its various parts. Present study, examines the biotechnological worth of the plant through tissue culture. The cultures obtained have been analyzed for the production of three very potent anticancerous triterpenoids: Betulinic, Oleanolic and Ursolic acids, through chromatographic and spectroscopic procedures. Moreover, their practical utility has also been checked in bioassays.

The thesis is divided into five chapters. Chapter 1, introduces and reviews all the major contributions and studies taken up till date, with regard to tissue culture, secondary metabolite production and bioassays, in both the plants. Chapter 2, clubs together all the protocols and methodologies adopted for the present work. Chapter 3, presents the results obtained in the current investigation. The tables and graphs are included within the text while all the figures have been compiled at the end of the thesis, in the form of plates. Inferences drawn from the results are discussed in chapter 4, in the light of other reports available on related aspects. Chapter 5, throws light on the major highlights of the present work and its future scope. This is followed by the appendix that mentions the taxonomic classification of the plants, studied. The thesis concludes with the list of bibliography and visible research output in terms of peer-reviewed journal publications, book chapters and conference proceedings.

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B. *LANTANA CAMARA*

TABLES

GRAPHS

LIST OF ABBREVIATIONS

AO	☞	Acridine orange
ATCC	☞	American type culture collection
B_5	☞	Gamborg's medium
BA	☞	Betulinic acid
BAP	☞	6-benzylaminopurine
BHK-21	☞	Baby hamster kidney fibroblast cell line
CAD	☞	Caspase-activated deoxyribonuclease
CH	☞	Casein hydrolysate
CLSM	☞	Confocal laser scanning microscopy
CM	☞	Coconut milk
2,4-D	☞	2,4-dichlorophenoxyacetic acid
DKW	☞	Driver's and Kuniyuki medium
DMAPP	☞	Dimethylallyl diphosphate
DMEM	☞	Dulbecco's modified eagle medium
DMSO	☞	Dimethyl sulphoxide
DOXP	☞	1-deoxy-D-xylose 5- phosphate
DW	☞	Dry weight
EB	☞	Ethidium bromide
FBS	☞	Fetal bovine serum
FDA	☞	Fluorescein diacetate
FPP	☞	Farnesyl diphosphate
FW	☞	Fresh weight
GA_3	☞	Gibberellic acid
GPP	☞	Geranyl diphosphate
HeLa	☞	Human cervical adenocarcinoma cell line
HLE	☞	Human leucocyte elastase
HMG-CoA	☞	3-hydroxy-3-methylglutaryl-coenzyme A
HPLC	☞	High performance liquid chromatography

2-iP	☞	2-isopentenyl adenine
IAA	☞	Indole-3-acetic acid
IBA	☞	Indole-3-butyric acid
ICAD	☞	Inhibitor of CAD
IPP	☞	Isopentenyl diphosphate
Kinetin	☞	6-furfurylaminopurine
LM	☞	Light microscopy
MEP	☞	Mevalonate pathway
MS	☞	Murashige and Skoog medium
MS	☞	Mass spectroscopy
MTCC	☞	Microbial type culture collection
MTT	☞	Dimethylthiazol-2-yl-2,5-diphenyltetrazolium bromide
NAA	☞	α-naphthaleneacetic acid
NLN	☞	Lichter's medium
NSO	☞	Neem seed oil
OA	☞	Oleanolic acid
PBS	☞	Phosphate buffered saline
pCPA	☞	para-Chlorophenoxyacetic acid
PVP	☞	Polyvinylpyrrolidone
RAPD	☞	Random amplified polymorphic DNA
R_f	☞	Retention factor
Rt	☞	Retention time
SEM	☞	Scanning electron microscopy
TBA	☞	Tertiary butyl alcohol
TDZ	☞	Thidiazuron
TLC	☞	Thin layer chromatography
TPA	☞	12-*O*-tetradecanoylphorbol-13-acetate
UA	☞	Ursolic acid

LIST OF UNITS

amu	☞	atomic mass unit
µg/g	☞	microgram per gram
µl	☞	microlitre
µm	☞	micrometer
µM	☞	micromolar
°C	☞	degree Celsius
cm	☞	centimeter
mm	☞	millimeter
g	☞	gram
h	☞	hour
l/g	☞	litre per gram
mg/l	☞	milligram per litre
mg/ml	☞	milligram per millitre
min	☞	minute
mm	☞	millimeter
mM	☞	millimolar
nm	☞	nanometer
rpm	☞	revolution per minute
SD	☞	standard deviation
SE	☞	standard error
SED	☞	standard error of difference
SS	☞	sum of Squares
MS	☞	mean square
μ	☞	specific growth rate
% RSD	☞	percent relative standard deviation

Chapter 1

Introduction and Literature Review

1.1 GENERAL

***Azadirachta indica* A. Juss.** or Neem, an evergreen, tropical tree belonging to the family Meliaceae, has emerged as one of the most popular and sought after trees both in India and worldwide. The tree grows well in tropical and subtropical areas of the world where it can attain a height of 30 m with a trunk girth of 2.5 m and can live for over two centuries (Verkerk et al. 1993). It can survive hot and dry climate where shade temperatures often reach 50°C and annual rainfall ranges from 400 to 1200 mm. The tree can also withstand many environmentally adverse conditions like drought, infertility, stony, shallow and acidic soils. However, it is very sensitive to water-logging.

The tree has numerous important medicinal, agrochemical and economic uses to its credit. Almost each and every part of the tree, particularly the leaves, the bark and the seeds possess multiple uses. It has been an integral part of Indian Ayurveda since ages. This broad spectrum bioactivity of Neem can be attributed to numerous secondary metabolites in the genus, that belong to various chemical classes like triterpenoids, phenols, carotenoids, steroids and ketones. Among all, azadirachtin, is one of the most prominent triterpenoids obtained from Neem seed kernels (National Research Council, 1992). Apart from this, polar and non-polar extractions yield about 24 compounds, other than azadirachtin, that have at least some biological activity (Schmutterer 1990; Jacobson 1990). This cocktail of compounds significantly reduces the chance of tolerance or resistance developing in any of the affected organisms. azadirachtin is a mixture of seven isomeric compounds labeled as azadirachtin-A to azadirachtin-G with azadirachtin-A being present in the highest quantity and azadirachtin-E regarded as the most effective insect growth regulator (Verkerk et al. 1993). Its demand has been on rise in industries due to its immediate application as an ecofriendly, biodegradable pesticide and various other significant biological activities. Concerted efforts are being made for its extraction

in higher quantities in an economically feasible manner. However, fulfillment of this objective has long been overdue owing to high qualitative and quantitative variation of azadirachtin in seed kernels due to enormous heterozygosity prevalent in the genus, its long reproductive cycle, recalcitrant and poor seed yields. Also, the reproductive stage in Neem begins at about three to five years of age but the trees do not become fully reproductive until they are ten years old. From this time on, the tree yields an average of about 20 kilograms of fruits per year, with maximum production reaching 50 kilograms per year (National Research Council 1992). Of the fruit yield, only about 10% is attributed to seed kernels, and desired biologically active compounds comprise only 10 grams per kilogram of kernel weight. Based on this estimate, an adult Neem tree produces only about 20 grams of pesticidal compounds in a season (Schmutterer 1990).

The genus ***Lantana*** (Family: Verbenaceae) is mostly native to subtropical and tropical America, but a few taxa are indigenous to tropical Asia and Africa. It was introduced in India in the nineteenth century and there are about 150 species of this plant (Sharma et al. 1988; Ghisalberti 2000). Each and every *Lantana* species is reported to possess a plethora of secondary compounds that exhibit a range of bioactivities. Of these, *Lantana camara L.*, commonly known as wild or red Sage, is the most widespread species of this genus. It has been a subject of rave discussions among the scientific community over a past few decades and is a cause of concern to some due to its ever-spreading nature. At other times it is prominent because of numerous bioactive compounds it harbors in its various parts. Several therapeutic compounds present in the genus account for its use in traditional medicines all over the world, since long. Although a few reports have described chemical constituents of *Lantana* growing in wild, the biotechnological side is still unnoticed.

The plant is a vigorous, low erect (1.2-2.4 m tall), aromatic, woody shrub with a very strong root system. It gives out a new flush of shoots even after repeated cuttings. Its flower flourishes throughout the year and each flower head is made up of 20-40 flowers, ranging in color from white, cream or yellow to orange, pink, purple and red (Ghisalberti 2000). The fruits of *Lantana* are small, greenish to blue-black to blackish, drupaceous, shining, with two nutlets, almost throughout the year and are dispersed by birds. Seeds germinate very easily (Sastri and Kavathekar 1990).

The *L. camara* has been listed as one of the important medicinal plants and has been used in the treatment of various diseases including cancers. Extract from its leaves exhibit antimicrobial, fungicidal, insecticidal and nematicidal activity (Chavan and Nikam 1982; Sharma and Sharma 1989; Begum et al. 2000; Day et al. 2003). Root extract is used for the treatment of malaria, rheumatism and skin rashes (Taoubi et al. 1997). The extract from other plant parts are used in the treatment of fevers, cold, asthma, high blood pressure, rheumatism, ulcers, chicken pox, leprosy and scabies (Anonymous 1962; Kirthikar and Basu 1981; Ghisalberti 2000; Day et al. 2003). The plant is shown to have allelopathic effects on the neighboring vegetation. These allelopathic molecules, therefore, have the potential to be developed as weedicides. Inspite of being a plant of potential medicinal interest, there are a very few reports that document the properties of this plant scientifically. Also, no perceptible biotechnological advances have been made in this genus to explore or enhance its utility.

An overview of tissue culture and secondary metabolite studies, taken up till date in both the plants, is being discussed in the following sections.

1.2. TISSUE CULTURE TECHNIQUES

The totipotency of plant cells has already been predicted in 1902 by Haberlandt and the first true plant tissue culture on agar was established. Since then plant tissue culture techniques have greatly evolved. The technique has developed around the concept that a cell has the capacity and ability to develop into a whole organism. The principles involved in plant tissue culture are very simple and primarily an attempt, whereby an explant can be to some extent freed from inter-organ, inter-tissue and inter-cellular interactions and subjected to direct experimental control. A brief overview of various tissue culture techniques is presented below:

1.2.A. Somatic Tissues

(i) Axillary Bud Proliferation

Shoot proliferation from axillary buds is desirable where generation of true-to-type or clonal plants is of interest, like in case of propagation of plus trees of Neem. The

best explant for this purpose is the nodal segments. Micropropagation by induction of shoots from pre-existing meristems is a most popular approach to clonal propagation of plants because the cells of the shoots-apex are uniformly diploid and least susceptible to genotypic changes under culture conditions. Thus, it guarantees that the characteristics of the source plant are conserved (Rao and Venkateswara 1985). It offers many advantages over the conventional methods of vegetative propagation: (1) The rate of multiplication is extremely rapid and can continue round the year, independent of the season. Thus, over a million plants can be produced in a year starting from a small piece of tissue. (2) The enhanced rate of multiplication can considerably reduce the period between the selection of plus trees and raising enough planting material for field trials.

(ii) Adventitious Shoot Proliferation

Adventitious shoot proliferation in cell and tissue cultures, in response to hormonal manipulation of the culture medium, require *de novo* differentiation of meristematic region, randomly, all over the tissue other than the pre-existing meristem. It is a multistep process and a series of intracellular events, collectively called induction that occurs before the appearance of morphologically recognizable organs. Micropropagation via adventitious shoot regeneration may occur directly or indirectly via an intervening callus phase. Indirect regeneration often results in somaclonal variations, making this strategy less desirable for large-scale clonal multiplication (Marcotrigiano and Jagannathan 1988; Thorpe et al. 1991). Therefore, regeneration of shoots directly from the explants is regarded as the most reliable method for clonal propagation. Various explants like leaf, cotyledon, embryo and root have been tried with different media combinations by the scientists to obtain adventitious shoot proliferation.

(iii) Somatic Embryogenesis

In tissue cultures, plant regeneration via somatic embryogenesis may offer many advantages over organogenesis, such as single cell origin, possibility to automate large scale production of embryos in bioreactors and their field planting as synthetic seeds. Moreover, the bipolar nature of embryos allow their direct development into a plantlet without the need of a rooting stage as required for plant regeneration via organogenesis

(Bhojwani and Razdan 1996). It can also be used for the production of metabolites in species where embryos are the reservoir of important biochemical compounds. Furthermore, epidermal single cell origins of embryos favor the use of this process for plant transformation. This prompted many scientists to achieve regeneration via somatic embryogenesis using various explants, most popular ones being zygotic embryo, or excised cotyledon or hypocotyls.

(iv) Triploid Production

Endosperm is a unique tissue in its origin, development and ploidy level. It is a product of double fertilization but unlike the embryo it is triploid and develops into a formless tissue (Bhojwani and Bhatnagar 1999). It is, therefore, an interesting tissue for morphogenesis. Cellular totipotency of endosperm cells was first demonstrated by Johri and Bhojwani in 1965. To date, differentiation of shoots/embryos/plantlets from endosperm tissue has been reported for more than 64 species belonging to 24 families (Thomas and Chaturvedi 2000). In many of these reports the regenerants were shown to be triploid. A key factor in the induction of cell divisions in mature endosperm cultures is the association of embryo. The embryo factor is required only to trigger cell divisions; further growth occurs independent of the embryo. Triploid plants are usually seed-sterile. However, there are many examples where seedlessness caused by triploidy is of no serious concern or, at times, even advantageous. Some of the crops where triploids are already in commercial use include several varieties of apple, banana, mulberry, sugar beet and watermelon (Elliott 1958). Natural triploids of tomato produced larger and tastier fruits than their diploid counterparts (Kagan-Zur et al. 1990). Traditionally, triploids are produced by crossing induced superior tetraploids and diploids. This approach is not only tedious and lengthy (especially for tree species) but in many cases it may not be possible due to high sterility of autotetraploids (Esen and Soost 1973; Gupta 1982). In contrast, regeneration of plants from endosperm, a naturally occurring triploid tissue, offers a direct, single step approach to triploid production. The selected triploids, expected to be sexually sterile, can be bulked up by micropropagation.

(v) Protoplast Culture

The application of protoplast technology for the improvement of woody plants offers fascinating option to complement conventional breeding programs. The ability of isolated protoplasts to undergo fusion and take up macromolecules and cell organelles offers many possibilities in genetic engineering and crop improvement (Bhojwani et al. 1977). However, to fully explore the potentials for protoplast-technology on woody crops, efficient and reproducible methods for protoplast isolation and purification must first be established. Since leaf tissue is a readily accessible source of genetically uniform cells, it is often desirable to use mesophyll protoplasts in somatic hybridization studies, but, leaf tissues, in general, do not yield large number of protoplasts owing to the difficulty in removing the lower epidermis (Chaturvedi 2003c). An alternative, therefore, is the cultured cell material where protoplasts can show greater potential to divide (Bhojwani and Razdan 1996).

1.2.B. Gametic Tissues

(vi) Haploid Production

(a) Anther Culture

In vitro androgenesis is the most efficient, quick and dependable technique to produce haploid plants. The technique becomes an indispensable tool for producing double haploids, more so for tree species, because majority of the trees are outbreeding, highly heterozygous and have long generation cycles. The prevalent heterozygosity and absence of pure lines in woody plants make selection and genetic studies rather difficult to conduct. Conventional breeding programmes are both unpredictable and time consuming where several generations of selfing is required to obtain homozygous pure lines, which is difficult to realize in woody plants due to long generation cycle. The perspective of raising haploid plants through *in vitro* androgenesis offers scores of foreseeable advantages like, shortening of breeding period, production of homozygous diploid lines in a single step through chromosome doubling and isolation of valuable recessive traits at sporophytic level which otherwise, remain accumulated and unexpressed in natural heterozygous diploid population (Srivastava and Chaturvedi 2008).

Anther culture has been successfully applied to many plant species to produce haploids **(Table 1)**; its single biggest advantage is its simplicity. Alternatively, isolated microspore culture can be carried out, which also has several important advantages over anther culture (Bonga et al. 1997; Radojevic et al. 2002). By removing the anther wall, proliferation of diploid sporophytic tissues are avoided. More importantly, homogenous population of microspores at the developmental stage, most suitable for androgenesis can be obtained. In addition, as the development of microspores is independent of the sporophytic tissues, the media components and culture treatments have direct access to the microspores. Thus, under optimal conditions, more microspores can be induced to convert into embryos. In spite of these positive attributes, microspore culture cannot yet compete with anther culture for double haploid production, especially in tree species. Pelletier and Ilami (1972) introduced the concept of "Wall Factor", according to which the somatic tissues of the anther play an important role in the induction of sporophytic divisions in pollen.

(b) Ovary Culture

Gynogenic development of plants from unfertilized cells of female gametophyte (embryo-sac) in ovary/ovule cultures is one of the available alternatives for haploid production. It was first reported in barley San Noeum (1976). This method of haploid production is more tedious than androgenesis. The reasons for this being the indefinite numbers of microspores (male gametes) within the anther wall for androgenesis as against single egg cell (female gamete) per flower for gynogenic haploid production, which too, is deep seated within the embryo-sac (female gametophyte), thus making the entire process very cumbersome. The technique is very useful where anther culture has been unsuccessful, plants are male sterile or androgenesis is confronted with the problem of albino or non-haploid formation.

Table 1: Reports on androgenesis in tree species

S.No.	Taxa/ Family	Explant	Stage of microspore at culture	Medium		Response	References
				Induction	Regeneration		
1.	***Aesculus hippocastanum*** (Hippocastanaceae)	A, Im	Un	Mod. MS (2% Suc) + PA (10 mg/l) + NA (5 mg/l) + Vit B1 (2 mg/l) + AdS (2 mg/l) + CH (200 mg/l) + kinetin (4.6 µM) + 2,4-D (4.52 µM)	MS + Gln (400 mg/l)	E → Pl	Calic et al. 2003/4. 2005a
2.	***Albizzia lebbeck*** (Leguminosae)	A	Un/ Bn	B_5 + 2,4-D (2.26 µM) + kinetin (9.2 µM)	B_5 + BAP (4.4 µM) + IAA (2.86 µM)	C → Sh/E → Pl	Gharyal et al. 1983a
3.	***Annona squamosa*** (Annonaceae)	A	Un	N (2% Suc) + IAA (28.55 µM)	N + BAP (8.8 µM) + NAA (5.37 µM)	C → Pl	Nair et al. 1983
					N + BAP (8.8 µM) + IAA (0.57 µM)	C → Sh	
4.	***Azadirachta indica*** (Meliaceae)	A	Un	MS (9% Suc) + 2,4-D (1 µM) + NAA (1 µM) + BAP (5 µM)	MS + BAP (5/ 7.5 µM)$^{MM-AI}$	C → Sh →Pl	Chaturvedi et al. 2003
5.	***Camellia sinensis*** (Theaceae)	A	Un	N6 + kinetin (9.2 µM) + 2,4-D (2.26 µM) + Gln (800 mg/l) + Ser (100 mg/l)	N6 + ZEA (9.12 µM) + Ad (20 mg/l) + LAH (10 mg/l)	C → Sh → Pl (in 1 out of 9 cv.)	Chen & Liao (1982)
		Im	?	MS + 2,4-D (4.52 µM) + kinetin (0.46 µM)	MS + BAP (2.2 µM)	EC	Pedroso and Pais (1994) Shimokado et al. 1986
		A	?	N6 (7% Suc) + NAA (0.54 µM)+ 2,4-D (0.45 µM) + kinetin (0.46 µM) + L-Gln (400 mg/l)		E→ Pl Ed	Raina and Iyer (1992) Saha and Bhattacharya (1992)

6.	***Carica papaya*** (Caricaceae)	A	Un	MS + BAP (0.44 µM) + NAA (0.54 µM)		E→Pl	Rimberia et al. 2005
			Un	MS + BAP (2 µM) + NAA (0.5 µM) + AC (1%)		E→Pl	Litz and Conover (1979)
			Un	MS BM		E→Pl	
			Un/ Bn	MS (half strength of salts, vitamins & full strength of Na-Fe EDTA; 6% Suc) + NAA (10.74 µM) + BAP (4.4 µM)		C	Tsay and Su (1985)
7.	***Cassia siamea*** (Leguminosae)	A	Un/ Bn	B5 + CM (15%) + 2,4-D (9.04 µM) + kinetin (2.3 µM)		C	Gharyal et al. 1983b
8.	***Ceratonia siliqua*** (Leguminosae)	A	Un/ Bn	MS + 2,4-D (2.26 µM) + TDZ (18.16 µM)		C→ PE	Custódio et al. 2005
9.	***Citrus clementina*** (Rutaceae)	A	Un	N6 Chu with N & N Vit + Lac (18000mg/l) + Gal (9000mg/l) + CW (5%) + CH (500 mg/l) + L-Gln (200 mg/l) + B (0.5 mg/l) + AA (500 mg/l) + NAA (0.11 µM) + 2,4-D (0.09 µM) + kinetin (4.6 µM) + BAP (2.2 µM) + ZEA (2.28 µM) + TDZ (0.45 µM) + GA_3 (1.45 µM)	MS + GA_3 (2.89 µM) + NAA (0.05 µM)$^{MM-CC}$	C → E → Pl E	Germana & Chiancone 2003
10.	***Eriobotrya japonica*** (Rosaceae)	A	Un	N6 Chu with MS vitamins + Lac (18000 mg/l) + Gal (9000 mg/l) + CW (5%) + CH (500 mg/l) + Gln (200 mg/l) + B (0.5 mg/l) + AA (500 mg/l) + NAA (0.11 µM) + 2,4-D (0.09 µM) + kinetin (4.6 µM) + BAP (2.2 µM) + ZEA (2.28 µM) + TDZ (0.45 µM) + GA_3 (1.45 µM)		C	Germana et al. 2006
11.	***Hevea brasiliensis*** (Euphorbiaceae)	A	?	MCC (9% Suc) + kinetin (4.6 µM) + 2,4-D (4.52 µM) + NAA (5.37 µM) + CM (5%)	MS (7-8% Suc) + kinetin (2.3-4.6 µM) + NAA (1.07 µM) + GA_3 (1.45 µM) ^{HB}	C → E→Pl	Chen et al. 1982

12.	***Malus domestica*** (Rosaceae)	A, Im	Un	MN6mac KNO_3 (1950 mg/l) + $(NH_4)_2SO_4$ (277 mg/l) KH_2PO_4 (400 mg/l) + $CaCl_2.2H_2O$ (166 mg/l) + $MgSO_4.7H_2O$ (185 mg/l) + MS mic + B_5 vit + Gln (1256 mg/l) + MES (1950 mg/l) + Mal (90000 or 180000 mg/l)	MS + TDZ (0.45 µM)	E → Pl	Hofer et al. 1999, Hofer 2004
13.	***Morus indica*** (Moraceae)	A	Un	MB + NAA (2.69 µM) and BAP (4.4 µM)	MB + NAA (2.69 µM) + BAP (2.2 or 4.4 µM) + 2,4-D (2.26 or 4.52 µM) + PVP (600)	C → E → Pl	Jain et al. 1996
14.	***Peltophorum pterocarpum*** (Leguminosae)	A	Un	Mod. MS + KNO_3 (2500 mg/l) + NH_4NO_3 (800 mg/l) + Th (10 mg/l) + I (1000 mg/l) + Gln (800 mg/l) + NAA (5.37 µM) + Kin (4.6 µM)	Mod. MS + NAA (0.54 µM) + kinetin (2.3 µM)	C →E → Pl	Rao & De 1987
15.	***Populus sp.*** (Salicaceae)	A	Tr/Un	MS + 2,4-D (2.26 µM) + kinetin (4.6 µM)	MS + NAA (0.54 µM) + BAP (4.4 µM)	C→ Ed → AdSh → Pl	Stoehr & Zsuffa 1990
				MS (2% Suc) + 2,4-D (2.26 µM) + kinetin (4.6 µM)	MS or WPM (2.5% Suc) + NAA (0.54 µM) + BAP (4.4 µM)	C →Sh→ Pl	Kiss et al. 2001
16.	***Quercus sp.*** (Fagaceae)	A	?	Smac + MS mic + cofac + AC (1% w/v)	Same as induction with Gln (500 mg/l)	E → Pl	Bueno et al. 1997

Before being transferred to regeneration media, calli and embryos were multiplied on MM (Multiplication medium): [AI] MS + 2,4-D (1) + kinetin (10); [CC] MS + NAA (0.11).

?- Not mentioned; A- Anther; AA- Ascorbic acid; AC- Activated charcoal; Ad- Adenine; AdS- Adenine Sulfate; AdSh- Adventitious shoots; B- Biotin; Bi- Binucleate; C- Callus; CM-Coconut milk; CPPU- N-(2-chloro-4-pyridyl)-N-phenylurea; CW- Coconut water; E- Embryos; EC- Embryogenic calli; Ed- Embryoid; Gal- Galactose; I- Inositol; LAH- Lactoalbumin hydrolysate; Lac- Lactose; L-Gln- L-Glutamine; M- Isolated microspore; Mal- Maltose; MB- Modified Bourgin; MC- Modified Chen et al. (1978); MES- 2-(N-morpholino)-ethanesulfonic acid; MN6mac- Modified N6 macrominerals; MSmic+cofac- MS micronutrients and cofactors; Nicotinic acid- NA; N6- Chu (1978), Chu et al. (1990); PA- Pantothenic acid; PE- Proembryos; Pl- Plantlet; Smac- Sommer et al. (1975) macronutrients; Ser- Serine; Sh- Shoot; Th- Thiamine; Uni- Uninucleate; Vit B1- Vitamin B1; WPM- Woody Plant Medium.

1.3. *IN VITRO* STUDIES

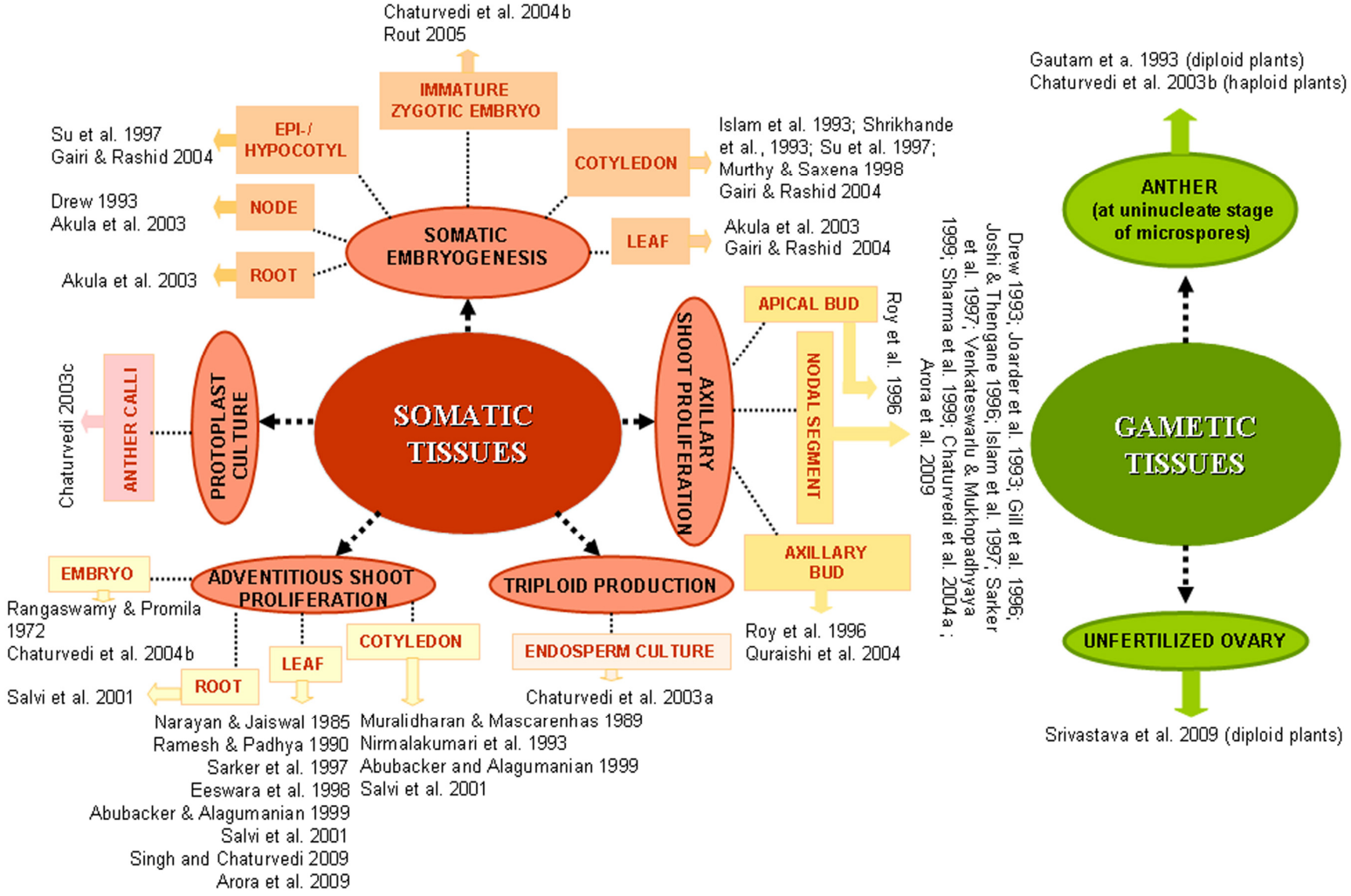
1.3.A. *Azadirachta indica* A. Juss.

Of late, numerous papers were published on *in vitro* plant regeneration of Neem **(Scheme 1)**. This section will illustrate how various plant tissue culture techniques have been applied to the Neem tree, and focuses on potential areas where biotechnological intervention may play a key role in the improvement of this important tree.

(a) Axillary Shoot Proliferation

Neem trees show wide genetic variation in terms of tree size, morphology, fruit size, and fruit production within the natural population (Kumaran et al. 1993; Srivasuki et al. 1993; Ermel et al. 1984, 1987; Benge 1989; Schmutterer 1990; Ketker and Ketker 1993; Ermel 1995; Wewetzer 1998). Considerable variability in the azadirachtin content of their seeds, irrespective of the habitat, is also observed (Ermel 1995; Sidhu et al. 2003). Improvement in azadirachtin production can be achieved by clonal propagation of elite trees. Vegetative propagation of Neem by the conventional methods is possible but difficult (Dogra and Thapliyal 1996). Propagation from seeds yield a heterogeneous population due to strict cross-pollinating nature of the plant. Moreover, the seeds loose viability within two weeks (Mohan Ram and Nair 1996).

Consequently, several investigators have attempted clonal propagation of Neem tree. Drew (1993) cultured apical and nodal segments from 6-12 months old seedlings of Neem. The axillary shoots, developed *in vitro,* were cut into one and two node segments for further multiplication. Two node cuttings gave better response than single node cuttings. On the best medium, MS (Murashige and Skoog 1962) with 6-benzylaminopurine (BAP; 0.1 µM), 100% cultures produced two shoots (probably one from each node) with a mean height of 10.8 mm. Recurrent multiplication of shoots was not attempted. Gill et al. (1996) cultured 0.5-1 cm long nodal segments and found that BAP (4.4 µM) alone induced shoot proliferation in 72% cultures (5-7 shoots per culture), which was slightly improved (80%) by further addition of indole-3-butyric acid (IBA; 2.5 µM) to the medium. The auxin did not affect the number of shoots per explant. Roy et al. (1996) also found a combination of an auxin (α-naphthalene acetic acid; NAA) and a cytokinin (BAP) to be better than BAP alone for shoot proliferation in the cultures of



Scheme 1: Major contributions in neem tissue culture

nodal segments from adult trees. However, the shoots produced on a medium containing BAP and NAA were very small and unsuitable for rooting. Good multiplication and growth of shoots (6 cm long) occurred with the addition of 10% Coconut Milk (CM) and 0.15% Casein Hydrolysate (CH) to MS + BAP (4.4 µM) and NAA (0.5 µM). Islam et al. (1997) reported synergism between BAP and kinetin (6-furfurylaminopurine) for shoot proliferation. Sarker et al. (1997) reported nodal explants to be the best for micropropagation of Neem among various explants like hypocotyl, leaf and shoot tip. He observed that the nodal explants responded best on MS + BAP (8.8 µM) + NAA (1.0 µM) medium. Clonal multiplication of elite Neem trees *via* axillary shoot proliferation has also been reported by Venkateswarlu and Mukhopadhyaya (1999). The micropropagated plants flowered after 25 months of transplantation and appeared true-to-type in terms of morphology, growth habit and azadirachtin content. Joarder et al. (1993) found that the pre-culture of nodal segments, taken from 30-year-old mature Neem tree, on MS basal medium for 2 weeks followed by 4-weeks on MS + BAP (6.6 µM) medium was essential/ beneficial for bud-break to occur. Although, the paper lacks details regarding the rate of shoot multiplication in subsequent subcultures, initially two shoots developed per node and the number of shoots increased with the increasing number of subcultures and then declined after five to six subcultures. Joshi and Thengane (1996) cultured nodal segments from 2-5-year-old juvenile trees as those from 15-20-year-old mature trees showed negligible bud-break. Sharma et al. (1999) cultured nodal segments from 3-, 7- and 40-year-old trees and found the explants from younger trees to be more responsive. Shoot multiplication at a rate of five-fold in 30 days was achieved, generally, after the fifth passage on MS medium supplemented with BAP (1.1 µM) and Indole-3-acetic acid (IAA; 0.29 µM) and adenine sulphate (81 µM). However, in the explants taken from 3- and 7-year-old trees, this rate of shoot multiplication was achieved even by the third subculture. Islam et al. (1997) recorded 4.5 shoots per explant upto fourth subculture from nodal segments of 25-year-old tree on MS medium supplemented with BAP (4.4 µM) and kinetin (4.7 µM). Quraishi et al. (2004) accomplished the micropropagation of Neem by culture of nodal segments from crown branches of a mature tree, basal-sprouts of another mature tree and a single juvenile plant. A significant variation was observed in *in vitro* response of explants from these three sources. In case

of crown and basal-sprouts explants, addition of polyvinylpyrrolidone (PVP-40; 12.5 µM) in the establishment medium was required to control the leaching of phenols into the medium; juvenile explants did not show phenolic leaching. DKW medium (Driver and Kuniyuki 1984) with BAP (0.22 µM) was found to be significantly better than MS medium for shoot proliferation. Shoot cultures of crown branch origin did not survive and eventually died after the third subculture. In the presence of IBA (4.9 µM) in half strength DKW, 90% of the shoots from basal sprout and 100% of the shoots of juvenile origin formed roots, and plantlets survived transplantation.

Chaturvedi et al. (2004a) reported a recurrent method of clonal propagation of a 50-year-old mature Neem tree through axillary shoot proliferation. In this case, multiple small shoots were formed on ½ MS medium (major inorganic salts reduced to half strength) supplemented with BAP (1 µM) and gibberellic acid (GA_3; 0.5 µM). The number of small shoots enhanced further when cultures were transferred to ½ MS + BAP (1 µM) + CH (500 mg/l). Further elongation and recurrent multiplication of shoots were achieved on full MS + BAP (1 µM) + CH (250 mg/l) through single node segment cultures, at a rate of 7-8-fold every 5 weeks, on fresh medium of the same composition. This rate of shoot multiplication was maintained for almost 5 years. The shoots were readily rooted on ¼ MS (major inorganic salts reduced to quarter strength) supplemented with IBA (0.5 µM), with a frequency as high as 82% and transplantation survival of these plantlets was more than 87%. Recently, Arora et al. (2010) established clonal propagation of adult Neem tree, more or less, of same maturity (40-year-old) through nodal segment cultures and ascertained the clonal fidelity of *in vitro* raised field grown plants by random amplified polymorphic DNA (RAPD). azadirachtin content of the progeny was found comparable to the mother tree, thus, proving their chemical stability as well.

(b) Adventitious Shoot Proliferation

Shoot regeneration from leaf explants derived from adult Neem trees was first reported by Narayan and Jaiswal (1985). Leaf discs formed unorganized callus on a medium containing 2,4-dichlorophenoxy acetic acid (2,4-D) and BAP. Transfer of callus to a medium containing BAP (0.44 µM) induced shoot-bud differentiation from them. On

this medium, 5-8 shoots appeared after 6-7 weeks. Addition of NAA (0.3 µM) in conjunction with BAP enhanced the frequency of shoot differentiation from 54% to 62.5%. Ramesh and Padhya (1990) cultured leaf discs from an "elite" tree. The explants formed green nodular callus on Wood and Braun's medium (Wood and Braun 1961) supplemented with 4 µM each of kinetin and BAP. After 4 weeks on the same medium, the callus differentiated 10-12 shoot-buds adventitiously. Further, addition of adenine sulphate (16 µM) to the medium raised the number of buds per culture to 18-20. Healthy shoots were obtained when the shoot-buds were transferred to GA_3 containing medium. Origin of the leaf discs affected the regeneration frequency. Discs from middle of the leaf were more regenerative than those from the basal and apical portions. Eeswara et al. (1998) tested the regeneration potential of leaf explants from 18-month-old seedlings raised from seeds obtained from Niger, Ghana and Srilanka. Direct differentiation of adventitious shoot-buds occurred on MS + BAP (4.4 µM) + kinetin (3.7 µM) + adenine sulphate (32.6 µM). These authors have highlighted the importance of dark incubation for shoot-bud differentiation. Cultures incubated in light, right from the beginning, did not form any shoot-bud. Two weeks of dark incubation was necessary for shoot-bud induction in most of the clones tested. For recurrent multiplication of shoots, it was essential to reduce the concentration of cytokinin ten times the original concentration. These authors also observed synergism between BAP and kinetin for growth and multiplication of shoots. Individually, BAP or kinetin in the medium caused increased abnormalities and vitrification of shoots. The medium recommended by the authors for recurrent shoot multiplication is MS + BAP (0.44 µM) + kinetin (0.4 µM) + adenine sulphate (3.3 µM). Arora et al. (2009) emphasized the superiority of BAP over other cytokinins for shoot organogenesis in Neem. They adopted a two-step procedure whereby the leaflet segments were given a pulse treatment of high concentration of BAP (8.88 µM) along with adenine hemisulphate (81.43 µM) followed by culture of explants in one-tenth concentration of BAP (0.88 µM) along with adenine hemisulphate at (81.43 µM). Singh and Chaturvedi (2009a) reported organogenesis from leaf explants and found the presence of at least one auxin or one cytokinin to be obligatory for the induction of organogenic calli from explants. Whereas BAP (5 µM) was found to be most favorable for shoot organogenesis in these calli, root organogenesis could be achieved on NAA (5

µM) supplemented medium. Thus, the kind of growth regulator had a significant effect on shoot/root organogenesis in these differentiating calli. Compact calli on shoot regeneration medium turned nodulated and, subsequently, an average of 7 green shoots per explant were developed in more than 76% cultures after 10 weeks. These shoots were elongated at a lower concentration of BAP (0.5 µM) and further multiplied by forced axillary branching on MS + BAP (1 µM) + CH (250 mg/l). Rooting of the shoots was achieved on ¼ strength MS medium supplemented with IBA (0.5 µM).

Salvi et al. (2001) observed shoot regeneration from various seedling explants (cotyledon, cotyledonary node, epicotyl, hypocotyl, leaf, root-shoot zone, root) on MS + BAP (8.9 µM) + IAA (0.6 µM). However, leaf explants produced the highest numbers of shoot buds per explant and the roots the least. Earlier, Abubacker and Alagumanian (1999) found cotyledons to be the best juvenile explant for regeneration. All cultures of cotyledon explants developed green, compact and nodular callus on MS + 2,4-D (6.8 µM) + IAA (2.9 µM) + BAP (6.6 µM) and on the same medium shoots were differentiated from the callus. Chaturvedi et al. (2004b) obtained plantlets from immature zygotic embryo cultures via neomorph formation and adventitious shoot bud formation. Maximum direct shoot bud differentiation (57%) occurred from early dicotyledonous stage of embryo on MS + BAP (5 µM) whereas maximum neomorph formation (66%) occurred in cultures of torpedo shape embryos on MS + 2,4-D (5 µM). It was possible to regenerate full plants from the neomorphs via organogenesis on MS + BAP (5 µM).

The limitation associated with immature zygotic embryo culture or other juvenile tissues of seedling origin is that the plants regenerated by this approach are derived from unselected bulked seeds, representing different genotypes. At this stage it is difficult to predict which of these seeds will eventually give rise to an elite tree. In contrast to this, micropropagation by *de novo* differentiation of shoot buds from excised leaves is more suitable for large scale propagation of selected elite mature trees. Again, the initial explant will not be a limiting factor because the leaves are available in abundant numbers throughout the year as compared to zygotic embryos, hypocotyl and cotyledons in case of short viable seeds.

(c) Somatic Embryogenesis

The majority of the publications have described regeneration in Neem via somatic embryogenesis, however, in many cases the structures described as embryos do not appear and/or behave like embryos. Muralidharan and Mascarenhas (1989) were probably the first to describe embryo-like structures in the cultures of cotyledon segments of Neem. On B_5 medium containing BAP (4.4 µM), the explants formed nodular structures, which appeared bipolar. Upon transfer to hormone-free B_5 medium, the nodular structures separated from the parent explant and developed leafy structures and a tap root. The nodular structures never showed bipolar germination. Shrikhande et al. (1993) reported *in vitro* plant regeneration via somatic embryogenesis in the cultures of immature cotyledons on MS (with 5% sucrose) + BAP (4.4 µM) + IAA (2.9 µM) + CH (1000 mg/l). Although these authors have furnished histological data in support of embryogenesis, the structures labelled as somatic embryo appear more like shoots, with their vascular traces running into the callus. None of the structures resembled a bipolar embryo with closed radicular and plumular poles. Moreover, on germination medium the "embryos" developed only a shoot, which had to be transferred to hormone-free ½ MS medium for rooting. Under the culture conditions Shrikhande et al. (1993) got somatic embryos, Su et al. (1997) observed only shoot bud formation. The calli initiated on MS + BAP (4.4 µM) + NAA (2.7 µM) + CH (1000 mg/l), differentiated greenish, globular detachable structures when transferred to MS + BAP + IAA + CH, in suspension cultures. In further subcultures on the same medium, the globular structures differentiated shoots and roots. However, if transferred to MS (5% sucrose) supplemented with only CH (1000 mg/l) the globular structures produced embryos on the spherical head. Maximum embryo differentiation (73%) occurred on MS (10% sucrose) + Zeatin (0.9 µM) and embryos germinated on ½ MS (1% Sucrose).

Islam et al. (1993) reported very high incidence of embryogenesis in the cultures of cotyledons, on MS + NAA (8 µM). However, the germination frequency of the embryos on MS + BAP (0.9 µM) + GA_3 (0.03 µM), was very poor (maximum 11.7%) and germination was monopolar. The embryos formed only shoots. Murthy and Saxena (1998) observed somatic embryo formation in the cultures of mature seeds directly as well as via callusing. The cotyledonary callus differentiated nodular structures in

suspension cultures in MS medium with or without Thiadiazuron (TDZ; 1 µM). When planted on hormone-free, semi-solid medium, these structures differentiated embryos, which germinated (60%-70%) on the same medium. Akula et al. (2003) reported induction of somatic embryogenesis in four out of seven selected clones of Neem. Direct induction of somatic embryogenesis was achieved from both nodal and root explants within eight weeks of culture on MS medium without growth regulators. When these embryos were left on the induction medium, approximately 15% of the somatic embryos developed into whole plantlets after passing through a series of developmental stages. Indirect somatic embryogenesis was obtained from leaf explants grown on MS medium supplemented with a combination of TDZ (2.3-4.4 µM) and 2,4-D (0.5 µM). Gairi and Rashid (2004) reported direct differentiation of somatic embryos on different regions of hypocotyl, epicotyl, cotyledonary-node, cotyledons and leaves of intact seedlings of *Azadirachta*. Individual embryos on transfer to hormone-free medium regenerated readily into plantlets.

To identify the most responsive stage of zygotic embryos for morphogenesis, Chaturvedi et al. (2004b) cultured immature embryos at different stages of development-globular, heart-shape, torpedo-shape and early dicotyledonous stage (early dicotyledonous embryos were 2.5 times smaller than fully developed dicotyledonous embryos. Globular and heart-shape embryos turned brown without showing any morphogenic response. Older embryos germinated, formed calli or differentiated three types of organized structures: shoots, somatic embryos and neomorphs (abnormal structures with varied morphology). The same explant often differentiated more than one kind of regenerants. Maximum somatic embryogenesis and shoot-bud differentiation occurred directly from the explant on MS + BAP (5 µM) medium, and the most responsive embryo stage was early dicotyledonous, followed by torpedo shape. The former showed differentiation of shoots and somatic embryos at higher frequency (57%). In the combined presence of BAP (5 µM) and 2,4-D (1 µM) in MS medium, early dicotyledonous embryos showed a fairly high degree of somatic embryogenesis and neomorph formation (50%) from the callused explants. Irrespective of the treatment and the stage of zygotic embryo, somatic embryos exhibited morphological abnormalities, such as pluricotyledony, fusion of cotyledons and absence of cotyledons. The occurrence

of normal dicotyledonous embryos was extremely rare; only 3% of the embryos developed a long tap root on MS basal medium but, plumular shoots did not appear. Later these somatic embryos developed secondary somatic embryos directly or indirectly via an intervening callus phase on MS + GA_3 (5.0 µM) + IAA (2.5 µM) and MS + BAP (1 µM) + IAA (0.5 µM), respectively. Like primary somatic embryos, secondary somatic embryos exhibited morphological abnormalities, and failed to germinate on any of the treatments tested. Rout (2005) also reported somatic embryogenesis from immature zygotic embryos that were cultured 40 days after anthesis. Embryogenic callus was proliferated on MS medium fortified with BAP (1.11 µM) and 2,4-D (4.52 µM). When these calli were transferred on same medium containing reduced auxin (2,4-D; 0.45 µM), numerous embryos proliferated from the surface of callus. Maturation and germination of the somatic embryos occurred on half strength MS salts and vitamins supplemented with ABA and 2% sucrose; the maximum percentage (64.2%) of germination was observed with ABA (0.94 µM) within 2 weeks of culture.

(d) Haploid Production

i. Anther Culture

Androgenesis is highly desirable in Neem trees to overcome the prevalent self-incompatibility, heterozygosity and long reproductive cycle. In spite of this, a limited effort has been made for the improvement of this valuable tree through *in vitro* haploid production. Gautam et al. (1993) cultured anthers at the uninucleate stage of microspores and observed some multicellular pollen on a medium containing NB (Nitsch 1969) with BAP (10 µM) or MS + BAP (10 µM) + NAA/IAA (10 µM). However, all the plants regenerated from anther calli were diploid. Chaturvedi et al. (2003b) for the first time achieved androgenic haploids of Neem by anther culture at early-to-late uninucleate stage of pollen. A three-step procedure was adapted by them to obtain haploids of Neem via callusing. In the first step, the calli were induced from anthers on MS basal medium (9% sucrose) supplemented with 2,4-D (1 µM), NAA (1 µM) and BAP (5 µM). Histological sections revealed that in 4-week-old cultures the anther-wall cells had started dividing while the microspores appeared to be unchanged. However, in 8-week-old cultures the anther locules were also filled with the calli. The second step was the multiplication of

callus on MS medium supplemented with either 2,4-D alone or 2,4-D + kinetin. The interaction of 2,4-D with kinetin proved better for sustained and massive callus multiplication. In the third step, the calli were transferred to a medium containing BAP for shoot differentiation. For young calli BAP (5 µM) was optimum (75% cultures differentiated shoots) while older calli showed the best regeneration with BAP (7.5 µM). The composition of the callus maintenance medium had a definite effect on the regeneration potential of calli. Calli maintained on medium containing 2,4-D (0.5 µM) exhibited good regeneration initially, but calli multiplied on a medium containing 2,4-D (1 µM) and kinetin (10 µM) retained the regeneration potential for a longer period. Elongation of shoots was achieved at a lower concentration of BAP at 0.5 µM. These shoots were multiplied by forced axillary branching on MS + BAP (1 µM) + CH (250 mg/l) and rooted *in vitro* on ¼ MS (major salts reduced to ¼ strength) + IBA (0.5 µM). The plants were subsequently established in soil. Of the plants that regenerated from anther callus 60% were haploid (2n=x=12), 20% were diploid (2n=2x=24) and 20% were aneuploid (2n=2x-2=22).

ii. Ovary Culture

To date, there is single report on *in vitro* ovary culture of Neem by Srivastava et al. (2009). They obtained unfertilized ovaries, from closed flower buds of an adult 54-year-old Neem tree. Ovaries were excised from flower buds of four sizes, 2, 3, 4 and 5 mm which correspond to immature ovule at integumentary primordia stage, megaspore mother cell, 2-nucleate embryo-sac and 4-nucleate embryo-sac stage, respectively. The best medium for inducing calli from unfertilized ovaries was MS medium with 9% sucrose, 2,4-D (1 µM) and BAP (5 µM); calli were further maintained and multiplied on MS medium supplemented with 2,4-D (0.5 µM) either alone or in combination with kinetin (4.5 µM). The callus maintenance medium, concentration of BAP in the regeneration medium and stage of ovary at culture were found to be critical factors for shoot differentiation from callus. Maximum shoot regeneration (78%) was observed when calli, induced from ovaries of 4 mm size flower buds (4-nucleate embryo-sac stage) and proliferating on MS + 2,4-D (0.5 µM), were subcultured to MS containing BAP (5 µM). The shoots were multiplied by forced axillary branching on MS medium

supplemented with BAP (1.0 µM) and CH (250 mg/l). Later, the shoots were rooted on ¼ strength MS medium supplemented with IBA (0.5 µM) at a frequency of 79%. The plants were subsequently hardened with transplantation rate of 81.8%. The cytological analysis by root-tip squash preparation revealed that all the plantlets were diploid.

(e) Triploid Production

In Neem, azadirachtin is obtained from the kernels and, therefore, triploids may not be good for the elites selected for this tetratriterpenoid. However, the elites of Neem selected for other secondary metabolites such as Nimbin, Nimbinin, Nimbidin, Nimbindiol and tannins, produced in the bark, and quercetin, nimbosterol and gedunin obtained from leaves (Thengane et al. 1995), triploidy may offer advantages. To date, there has been a single published report on *in vitro* production of triploid plants from endosperm tissues of Neem by Chaturvedi et al. (2003a). Since Neem bears non-endospermous seeds, therefore, they obtained triploid plants by immature endosperm culture. Immature seeds, at the early dicotyledonous stage of embryo development, was observed to be the best explant to raise endosperm callus on MS + NAA (5 µM) + BAP (2 µM) + CH (500 mg/l). The association of the embryo proved essential to induce callusing of immature endosperm. After two weeks of culture initiation, seeds burst open, releasing green embryo and white fluffy callusing endosperm. The calli later turned green, compact and nodulated. Shoot-bud differentiation, at high frequency, occurred only when the calli were subcultured on MS + BAP (5 µM). They observed the preponderance of multicellular glands, a characteristic feature of the shoots of endosperm origin. These glands, occasionally stalked, were found in large numbers on younger leaves close to the shoot apex. Shoots were multiplied by forced axillary branching and rooted *in vitro*. The plants were established in soil. Cytological analysis revealed that over 66% of the plants were triploid with 2n=3x=36 and rest were diploids with 2n=2x=24.

(f) Protoplast Culture

In Neem, this aspect is largely unexplored and there is a single report on protoplast isolation and culture by Chaturvedi (2003c). They isolated protoplasts from

Neem callus raised from anthers on MS + para chlorophenoxy acetic acid (pCPA; 5 µM), MS + 2,4-D (1 µM), MS + 2,4-D (1 µM) + kinetin (1 µM) and MS + 2,4,5-T (1 µM). The soft and friable callus, obtained after four subcultures, was found to yield more protoplasts than earlier subcultures. Maximum yield (5.8×10^6) protoplasts/g fresh weight of viable protoplasts was obtained from granular, friable, light brown callus grown on MS + pCPA (5 µM). Besides cycles of subcultures, the age of the callus also appeared to influence protoplast production. Calli from 3-week-old subcultures consistently gave maximum yields of protoplasts than the calli from 2- or 5- week-old subcultures. As compared to mesophyll protoplasts, those isolated from cultured tissues exhibit a great variation in their size, which the author suggested is chiefly due to heterogeneity of the cultured cell population. The optimum duration for enzyme treatment, containing a mixture of 2% cellulase R-10 and 0.2% Macerozyme R-10, was 4 h. Longer incubations in the enzyme solution resulted in clumping of the protoplast. Further, the author observed that protoplast cultured as thin layer were better in terms of sustained division than as flattened drops. Most of the protoplasts cultured on MS + pCPA (5 µM) liquid medium became oval after 4 days, indicating new cell wall regeneration, approximately 10% of the protoplasts, cultured on MS + pCPA (5 µM), entered division after 11 days.

1.3.B. *Lantana camara* L.

Till date, no reports are available on tissue culture of *Lantana*. The list of new chemical structures from the plant is increasing day by day, but, this alone will not serve the purpose. Structural identification of unknown compounds only point towards the further utility of the plant economically or medicinally. In order to exploit its potential to the fullest, there has to be a constant source from where these compounds can be extracted, on a regular basis. There is an immense scope of *Lantana*'s foliage being used for this purpose, owing to the fact that large biomass of this plant is available. However, there are several disadvantages with this option since the plants growing in wild face a lot of climatic and other environmental fluxes that lead to changes in their chemical profile. In contrast to this, plant cell cultures are an attractive alternative to whole plant extraction for the production of high-value secondary metabolites (Rao and Ravishankar 2002;

Ravishankar et al. 1999; Dornenburg and Knorr 1997; Scragg, 1997; Alfermann and Petersen 1995; DiCosmo and Misawa 1995; Stockigt et al. 1995; Endress 1994; Ravishankar and Venkataraman 1990). As each plant cell in culture retains complete genetic information, which means, it is biosynthetically totipotent, and is able to produce a range of chemicals found in the parent plant. The advantages of this technology are:

- It is independent of geographical and seasonal variations and various other environmental factors.
- It offers a defined production system, which ensures continuous supply of products, uniform quality and yield.
- It is possible to produce novel compounds that are not normally found in parent plant.
- Efficient downstream recovery and product.
- Rapidity of production.

In addition to the points listed above, plant cell can perform stereo- and regiospecific biotransformations for the production of novel compounds from low cost precursors. All these factors make plant cell cultures an attractive option as factories producing important and novel metabolites all the year round. In this context, it is important to note that no initiatives have been taken as far as tissue culture of *Lantana* is concerned. The main reason for this is the plant is considered an obnoxious weed and, therefore, has never been viewed from this point of view. A new outlook that can have immense and immediate implications of *Lantana's* foliage is tissue culture, especially, from leaves that are a reservoir of many active compounds. Present study, therefore, is an attempt to realize the potential of *Lantana* by biotechnological means using tissue culture techniques. The biochemical studies that follow the tissue culture reports demonstrate the potential of cell cultures as source of important secondary compounds. Bioassays further strengthen the significance of preceding studies.

1.4 PLANT SECONDARY METABOLITES AND THERAPEUTIC APPLICATIONS

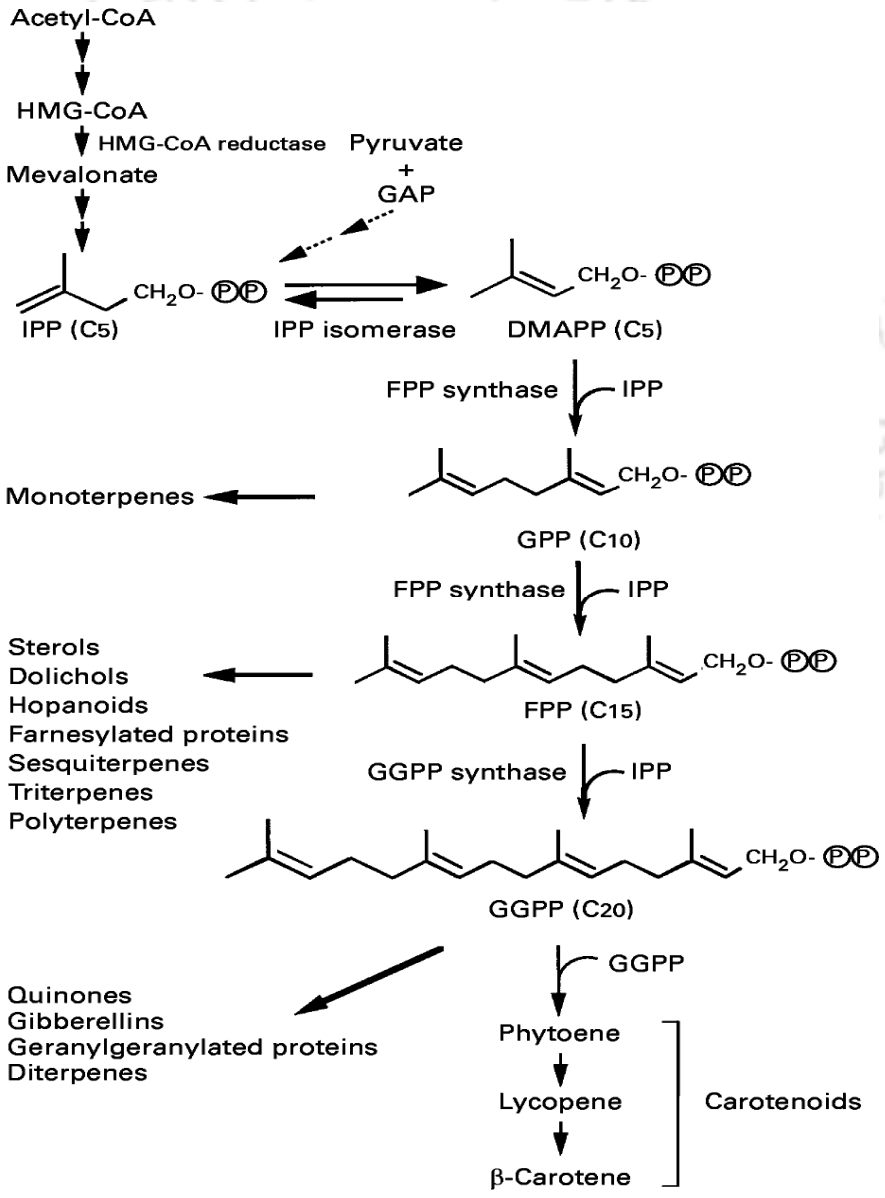
Plant secondary metabolites are frequently regarded as extravagances that serve no obvious biological purpose for the plant that produces them. However, it is becoming increasingly clear that these molecules may play important roles in plant signalling and

defense mechanisms and, thus, play a major role in adaptation of plants to their environment. Besides, they constitute important UV absorbing compounds, thus, preventing serious leaf damage from the light (Li et al. 1993). They have been described as being antibiotic, antifungal and antiviral and, therefore, are able to protect plants from pathogens (phytoalexins). They are also anti-germinative or toxic to other plants (allelopathy). They act on insects as anti-feeders or even cattle for which forage grasses can express estrogenic properties and interact with fertility (Bourgaud et al. 2001). Due to their diverse biological activities, plant secondary metabolites have been used for centuries in traditional medicine. Based on their biosynthetic pathways these compounds are usually classified into three large families (Harborne 1999):

- Phenolics
- Terpenes and steroids
- Alkaloids

Among the three classes, terpenoids are by far the largest group of secondary metabolites. While the enzymes for their synthesis are found in all classes of microorganisms, plants and animals, the widest array of terpenes and terpene-derived structures are synthesized by higher plants. They are isoprenoids synthesized from C_5 isoprene units linked end-to-end. In some terpenes, the isoprene backbone is easily identified but most of them have been enzymatically rearranged and substituted to form highly complex ring structures. The basic building blocks or precursors of all terpene constructs are isopentenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP). Two major pathways postulated for their synthesis are mevalonic acid pathway in the cytoplasm of plant cells, where they are synthesized from acetyl coenzyme A. Another independent pathway for synthesis of isoprenoids precursors is the DOXP-MEP pathway, proposed by Rohmer (1999) that is expressed in the plastids of plants, green algae, some bacteria and apicomplexan parasites such as *Plasmodium*. The initial step in this pathway is the conversion of pyruvic acid and D-glyceraldehyde-3-phosphate to 1-deoxy-D-xylose 5-phosphate (DOXP) by DOXP synthase. The biosynthesis of major terpenoid classes is shown in **Scheme 2**. The first step in the synthesis of terpene involves the condensation of IPP and DMAPP by geranyl diphosphate (GPP) synthase to form the GPP precursor from which monoterpenes are synthesized. Farnesyl diphosphate synthase adds an IPP

moiety to GPP to form farnesyl diphosphate (FPP), the precursor of the sesquiterpenes and the triterpenes. The addition of IPP to FPP forms geranylgeranyl diphosphate, the precursor of diterpenes and carotenoids. Monoterpenes, sesquiterpenes, diterpenes and carotenoids are primarily products of the plastid DOXP-MEP pathway. The triterpenoids and their derivatives, the phytosterols and saponins are produced via the cytoplasmic mevalonic acid pathways and are often the components of cell membranes. The plants chosen for the present study are very rich in terpenoid content especially the triterpenoids.



Scheme 2: Biosynthetic pathway of major terpenoid classes

Nowadays, these metabolites correspond to valuable compounds such as pharmaceutics, cosmetics, fine chemicals, or more recently nutraceutics. The use of natural products with therapeutic properties is as ancient as human civilization (De Pasquale 1984) but with the advent of industrial revolution and development of organic chemistry, a penchant for synthetic products went on rise for pharmacological treatment. The reasons for this were that pure compounds were easily obtained and their structural modifications to produce more active and safer drugs were easy. Until now, drugs from natural sources have been considered an option for poor, uneducated and low income class people. This mindset has changed lately and the importance of natural products has increased enormously. Of the 252 drugs considered as basic and essential by the World Health Organisation (WHO), 11% are exclusively of plant origin and a significant number are synthetic drugs obtained from natural precursors. Examples of important drugs obtained from plants are digoxin from *Digitalis* sp., quinine and quinidine from *Cinchona* sp., vincristrine and vinblastine from *Catharanthus roseus*, atropine from *Atropa belladonna* and morphine and codeine from *Papaver somniferum*. It is estimated that 60% of anti-tumour and anti-infectious drugs already in the market or under clinical trial are of natural origin (Yue-Zhong Shu 1998; Rates 2000). The vast majority of these cannot yet be synthesized economically and are still obtained from wild or cultivated plants.

Azadirachta indica contains numerous compounds that exhibit a wide range of bioactivity. The spectrum of compounds chiefly comprise of nimbin, nimbidin, nimolinone, nimbolide, salanin, dihydronimbin and azadirachtin **(Scheme 3A)**. Among these the most significant compound is azadirachtin, a complex tetranortriterpenoid that makes up the major proportion of the seed kernels. It is a highly oxidized tetranortriterpenoid which boasts a plethora of oxygen functionality, comprising enol ether, acetal, hemiacetal and tetra-substituted oxirane as well as a variety of carboxylic esters. It has been a focal point of research for the last several years. Due to the complex molecular structure, its first synthesis was not published for over 22 years after the compound's discovery. Recently, Veitch et al. (2007) realized a major breakthrough in first total synthesis of this compound. The well-known pesticidal activity of Neem, largely attributed to azadirachtin, spans a wide spectrum, having repellent,

phagodeterrent (antifeedant), insect growth regulatory (IGR), anti-ovipositional, fecundity and fitness reducing properties on insects. Schmutterer and Singh (1995) listed 413 insect pest species sensitive to Neem products. These principles act as ecdysteroid analogues, which affect corpus cardiacum and block reproductive and growth processes in most insects causing sterility in females and degenerative changes in male testis due to disturbance in insect metabolism (Krauss et al. 1987). Formulations like: Margosan O (R), Neemix(™), Azatin (R), NIM-20 and NIM-76, gave negative result with respect to toxicity effect on mammals (Schmutterer 1990, 1997; Govindachari et al. 2000). Hence, Neemix(™), was registered for use on vegetables in US for its inherent safety. In most tests, Neem products performed equally or sometimes better than synthetics like Pirimiphos-methyl (Actellic 25 EC), Permethrin and Lindane (γ-BHC) (Ogunwolu and Oddunlami 1996; Lale and Mustapha 2000). Several "active principles" from Neem have demonstrated high efficacy against most pathogens. As fungicides, over 14 common fungi species are sensitive to Neem preparations (Khan & Wassilew 1987). They include the genera *Trichophyton* (athletes foot), *Epidermophyton* (ringworm of skin & nails), *Microsporum* (ringworm of skin & hair) and *Candida* (thrush). SaiRam et al. (1997, 2000) reported protection against systemic candidiasis (*Candida albicans*) by NIM-76. The mechanism is simply antifungal and immunomodulatory. In *Aspergillus flavus,* Neem leaf extract fails to inhibit growth, but reduces formation of aflatoxin by blocking 'polyketides' production, which is commonly converted to toxins (NRC 1992). As antibiotics, pathogenic bacteria like *Staphylococcus aureus, Salmonella typhi* are significantly suppressed by Neem Seed Oil (NSO). Trials with NIM-76 significantly suppressed *Escherichia coli* and *Klebsiella pneumoniea*, which hitherto were insensitive to whole NSO (SaiRam et al. 2000).

As antiviral agents, experiment with Small pox, Chicken pox and Fowl pox viruses show biological efficacy of Neem extracts; crude Neem extracts adsorbed viruses by blocking entry into uninfected cells. NIM-76 suppressed Polio virus replications and inhibited DNA polymerase of Herpes virus with no potency once infection is established *in vivo* (Rao et al. 1989). In West Africa, India and Burma, both aqueous and alcohol extracts of bark and leaves of Neem are effective anti malarial agents, particularly on chloroquine resistant strains (Badam et al. 1987). Furthermore, Neem bark and leaves

posses strong antiseptic property warranting use as active ingredient in tooth paste in India and Germany. Several herbalists opined that Neem products have broad spectral chemotherapeutic effect on the Flat, Tape and Round worms (Devakumar et al. 1985). Furthermore, NSO possess anti-implantation and abortifacient properties. Sinha et al. (1984) found spermatozoa of human and Rhesus monkey were immotile and die within 30 min of contact with NSO in an intravaginal dose of 1 ml. Vaginal biopsy revealed no side effect, while radio-isotope studies indicate non-absorption in the vagina and non-antiovulatory (Sinha et al. 1984). These findings enabled Neem oil formulation 'sensal' to be used in India as a powerful contraceptive.

Azadirachtin is abundant in the seed kernels but as discussed earlier, the seeds show a lot of variation in azadirachtin content owing to the heterozygosity prevalent in the genus. Moreover, the storage quality of seeds is poor due to high moisture content and they lose viability within a few weeks. Sidhu et al. (2003) reported that the variability in azadirachtin content and other triperpenoids like Nimbin and Salanin is due to the individual genetic differences between the plants. However, variability with respect to azadirachtin content of seed kernels and other seed related parameters among Neem populations growing in the same area, due to both climate and ecotype interaction has also been reported recently by Kaushik et al. (2007). Keeping all this in mind and hardships associated with chemical synthesis of the molecule, researchers all over the world are focusing on tissue culture and scale up studies to harvest this important compound from *in vitro* raised cultures **(Table 2)**. These aseptic cultures offer an advantage over conventional sources by providing consistent supply of chemicals all the year round, unaffected by environmental fluctuations and geographical barriers, once an elite source is selected.

The first report on the application of plant tissue culture techniques for the production of secondary metabolites from Neem is of 1983 (Schulz 1984) where callus proliferation from various parts of the plant by improving culture media was obtained. Subsequently, Sarkar and Datta (1986) studied the relationship between the biosynthesis of Nimbin and β-sitosterol in bark and bark-originated callus of increasing age. They also studied the effect of glycine on *in vitro* biosynthesis of Nimbin and β-sitosterol in tissues (Sanyal et al. 1988). In cotyledons, which contained Nimbin, glycine, other amino acids

and β-sitosterol, it was observed that glycine affected the synthesis of both. Nimbin was also isolated from leaves and callus cultures by Ramesh and Padhya (1988). IAA and IBA interaction showed a linear increase in Nimbin content. This was the first indication to demonstrate that the expression of tetranortriterpenoids produced by Neem in tissue culture could be under hormonal control. The first reports of secondary metabolites production in suspension cultures are of 1993 and 1994 (Van der Esch et al. 1993, 1994a,b). These suspension cultures were raised from seedling hypocotyl derived callus cultures. A sure chemically acceptable proof that azadirachtin A was produced in suspension cultures of Neem was reported by Jarvis et al. (1997).

Other compounds also produced by suspension cultures were azadirachtin B, azadirachtin I, Nimbin 6-desacetylnimbin, Salannin 3-desacetylnimbin and 3-acetyl-1-tigloylazadirachtin. Kearney et al. (1994) could not detect the presence of azadirachtin in callus, suspension culture and shoot culture derived from leaves. Similarly, Zypman et al. (2001) demonstrated the anti-feedant effects of extracts both from whole plants as well as callus cultures but they did not establish which substances were present in cultures. azadirachtin production from callus cultures of leaf and bark from trees of different origin, has been reported by Wewetzer (1998). Analysis showed that the production of azadirachtin A in callus cultures of *A. indica* depended on age, cell line, the medium and the carbohydrate source employed. The main objective was to determine if differentiation was necessary for the expression of azadirachtin in culture and it could be shown that morphological differentiation is not a prerequisite for azadirachtin A production; the highest concentrations were detected in completely undifferentiated cells. Another article reporting production of azadirachtin in callus cultures (Veeresham et al. 1998) was surprisingly high in terms of content. They established callus cultures from both leaves and flowers. Such levels have never been reported either for seed or tissue culture material. Azadiarchtin was also detected in hairy root cultures of leaves and stem (Sundaram et al. 1996), bark derived callus (Bajagopal and Ramaswamy 1996), *in vitro* roots and shoots from embryo (Srividya et al. 1998), callus and suspension culture from leaves (Kuruvilla et al. 1999) and callus from shoot-tips (Schaff et al. 2001). Besides azadirachtin, Nimbin and Gedunin were detected in callus cultures of bark by Bajagopal and Ramaswamy (1996). Raval et al. (2003) studied the effect of basal media on growth

and production of azadirachtin-related limonoids in plant cell culture of Neem with an aim to enhance their yield. Two years later, Prakash and Srivastava (2005) came out with statistically optimized media for cell growth and azadirachtin production in Neem suspension cultures, obtained from seed kernels. The studies were extended further to establish the kinetics of cell growth/ azadirachtin formation and substrate consumption of *A. indica* suspension culture in low shear steric impeller bioreactor with statistically optimized conditions (Prakash and Srivastava 2006). Further, Prakash and Srivastava (2007) compared azadirachtin production in stirred tank bioreactor with different types of impellers. Prakash and Srivastava (2008) also studied the role of elicitors like salicylic acid, chitosan, jasmonic acid, methyl jasmonate, and yeast extract at different concentrations in shake flask suspension culture of *A. indica*. Babu and Nair (2007) reported an excellent protocol for enhanced production of three triterpenoids azadirachtin-A, Nimbin, Salannin and two flavanoids, quercetin and kaempferol in cell suspension cultures of *Azadirachta indica*, by extending the stationary phase. Singh and Chaturvedi (2009b) studied the effect of morphogenesis and explant source on azadirachtin production from calli. Recently, Rafiq and Dahot (2010) studied the effect of explant source, carbohydrates and major nutrients on production of azadirachtin related limonoids in cell suspension cultures of Neem.

As far as ***Lantana camara*** is concerned, several classes of compounds like mono- and sesquiterpenes, triterpenes, iridoid glycosides, flavonoids, furanonaphthoquinones and phenyl ethanoid glycosides are informed to be present in the wild growing plants of this genus (Ghisalberti 2000). The plant has been used in different parts of the world to treat a broad range of disorders. It is used in folk remedies for cancers and tumours. In Central and South America, the paste of leaves has been used to treat sores, chicken pox and measles. In Ghana, infusions of whole plant were used for bronchitis and the powdered root in milk was given to children for stomach-ache (Irvine 1961). In Asian countries, leaves have been used to treat cuts, rheumatisms, ulcers and as a vermifuge. Decoctions were applied topically for leprosy and scabies. Lancamarone, a steroid, from the leaves has been claimed to exhibit cardiotonic properties, and lantamine, an alkaloid, from the stem bark and roots show antipyretic and antispasmodic properties comparable to those of quinine (Sastri 1962).

Table 2: Reports on secondary metabolite production from tissue culture of Neem

S.No.	Explant	Culture Type & Ploidy	Metabolites Identified	Yield	Authors (Year)
1.	Young stem bark	Callus with differentiating roots (diploid)	Nimbin	0.025% of DW	Sanyal et al. (1981)
2.	Young stem bark	Callus grown with low kinetin concentrations (diploid)	Nimbin ß-sitosterol	0.25% of DW	Sanyal and Datta (1986)
3.	Cotyledons	Callus (diploid)	Nimbin, glycine, other aminoacids and ß-sitosterol	Not determined	Sanyal et al. (1988)
4.	Leaves	Callus (diploid)	Nimbin	Not determined	Ramesh and Padhya (1988)
5.	Shoots apex	Cell suspension (diploid)	Azadirachtin A	0.66 µg/mg of DW (grown in light) 0.19 µg/mg of DW (grown in dark)	Van der Esch et al. (1993)
6.	Shoots apex	Cell suspension (diploid)	Azadirachtin A	0.66 µg/mg of DW (grown in light) 0.19 µg/mg of DW (grown in dark)	Van der Esch et al. (1994a) (1994b)
7.	Leaves	Callus (diploid)	Azadirachtin	7 µg/g DW	Allan et al. (1994)
8.	Leaves	Callus/ Suspension/ Shoot culture (diploid)	Azadirachtin	Not detected	Kearney et al. (1994)
9.	Leaves	Hairy roots (diploid)	Azadirachtin	3.6 µg/g DW	Sundaram et al. (1996)
10.	Stem	Hairy roots (diploid)	Azadirachtin	2.7 µg/g DW	Sundaram et al. (1996)
11.	Leaf	Callus (diploid)	Azadirachtin	0.0007% DW	Allan et al. (1994)
12.	Bark	Callus (diploid)	Azadirachtin Nimbin Gedunin	0.12% DW 0.21% DW 0.13% DW	Bajagopal and Ramaswamy (1996)

13.	Shoot apex	Cell suspension (diploid)	Azadirachtin A Azadirachtin B Azadirachtin I Nimbin 6-desacetylnimbin Salannin 3- desacetylnimbin 3-acetyl-1-tigloylazadirachtin	No yields determined but structures confirmed through spectral data	Jarvis et al. (1997)
14.	Embryo	*In vitro* roots (diploid)	Azadirachtin	4 µg/g DW	Srividya et al. (1998)
15.	Embryo	*In vitro* shoots (diploid)	Azadirachtin	8 µg/g DW	Srividya et al. (1998)
16.	Leaves	Callus (diploid)	Azadirachtin	0.0268g/g DW	Veersham et al. (1998)
17.	Flowers	Callus (diploid)	Azadirachtin	0.0246g/g DW	Veersham et al. (1998)
18.	Leaves	Callus (diploid)	Azadirachtin	64 µg/g DW	Wewetzer (1998)
19.	Bark	Callus (diploid)	Azadirachtin	44 µg/g DW	Wewetzer (1998)
20.	Leaves	Callus/ Suspension culture (diploid)	Azadirachtin	5.36 mg/l	Kuruvilla et al. (1999)
21.	Shoot tip	Callus (diploid)	Azadirachtin	0.5 µg/g DW	Schaaf et al. (2000)
22.	Parts of whole plant	Callus (diploid)	Azadirachtin	Not determined	Zypman (2001)
23.	Shoots	Callus/ Suspension culture (diploid)	Azadirachtin related limonoids	0.25 mg/g DW	Raval et al. (2003)
24.	Seed kernels	Callus/ Suspension culture (diploid)	Azadirachtin	2.98 mg /g DW	Prakash and Srivastava (2005)
25.	Seed kernels	Callus/ Suspension culture (diploid)	Azadirachtin	0.05 g/l	Prakash and Srivastava (2006)
26.	Seed kernels	Callus/ Suspension culture (diploid)	Azadirachtin	0.071 g/l	Prakash and Srivastava (2007)
27.	Seed kernels	Callus/ Suspension culture (diploid)	Azadirachtin	15.9 mg/g DW	Prakash and Srivastava (2008)
28.	Different plant parts	Undifferentiated and differentiated callus cultures (diploid)	Azadirachtin	2395.7 µg/g DW	Singh and Chaturvedi (2009b)

Among others, the pentacyclic triterpenes- Betulinic (BA), Oleanolic (OA) and Ursolic acids (UA) **(Scheme 3B),** found in this plant are reported to be of immense pharmacological interest (Hsu et al. 1997; Laus, 2004; Pereira et al. 2007). Betulinic acid is most highly regarded for its anti-HIV-1 activity and specific cytotoxicity against a variety of tumor cell lines (Cichewicz and Kouzi 2004). Oleanolic and Ursolic acids have been used in cosmetics and in health products. In China, Oleanolic acid is marketed as an oral drug for human liver disorders and Ursolic acid is used in antitumour therapy in Korean traditional medicine (Wojciak-Kosior 2007). These molecules also act as inhibitors of human leucocyte elastase (HLE), an enzyme that participates in the destruction of elastin and plays a role in chronic disorders such as pulmonary emphysema, cystic fibrosis, hepatitis and rheumatic arthritis. Other important compounds are Lantadenes. Lantadenes A and C, have been shown to inhibit Epstein Barr virus activation induced by 12-*O*-tetradecanoylphorbol-13-acetate (TPA). Lantadene B and C even at very low concentrations of TPA, could be considered valuable inhibitors of tumor promoters *in vivo* (Inada et al. 1995). Lantadenes A and B were shown to have inhibitory effects on the two-stage carcinogenesis of mouse skin papillomas. Euphane lactone triterpenes have been found to be associated with the thrombin inhibitory activity that inhibits the blood clotting cascade via acylation of Ser 195 residue present in the active site of thrombin (O'Neill et al. 1998). Iridoid glycosides are also well represented in plants and used in folk medicine for the preparation of bitter tonics, sedatives, febrifuges, cough medicines, remedies for wounds and hypotensives. Individual compounds are known to exhibit various pharmacological activities including cardiovascular, antihepatotoxic, choleretic, hypoglycemic, anti-inflammatory, antispasmolytic, antitumour and antiviral activity (Ghisalberti 1998). Furanonaphthoquinones present in *Lantana*, show a pronounced cytotoxicity to a range of tumour cell lines. Also, numbers of furanonaphthoquinones have been shown to possess antimicrobial activity against Gram-positive bacteria and fungi, inhibitory effects on the Japanese encephalitis virus and pronounced activity against *Trypanosoma* parasites (Nagata et al. 1998). Phenylethanoid glycosides particularly, Verbascoside is represented in several plant families and has been shown to display an interesting spectrum of biological activity. It is an inhibitor of protein

kinase C (PKC), the Calcium phospholipid dependent protein kinase that plays a crucial role in signal transduction, cell proliferation and differentiation (Herbert et al. 1991).

Research on *Lantana* is still in its infancy. Only a few reports have described the chemical constituents from its various parts. Huang and Huang (2004) reported the presence of oleanolic acid, lantadene A, lantadene B, 22β-angeloyloxylantanolic acid, lantanilic acid, betulonic acid, betulinic acid, pomolic acid, 3β-hydroxystigmast-5-en-7-one, β-sitosterol-3-*O*-β-D-glucoside as well as a mixture of campesterol, stigmasterol and β-sitosterol from the stems of the yellow flowering taxa of *Lantana camara* L. Similarly, Juang et al. (2005) reported eight triterpenoids from the leaves of this plant. A few others reported the presence of other terpenoids, their derivatives and flavonoids in the plant (Sharma et al. 1990; Siddiqui et al. 1995; Wollenweber et al. 1997; Yadav and Tripathi 2003). However, no quantification studies have been performed in any of these studies except for Lantadenes by Sharma et al. (2000). A few other reports demonstrated the cytotoxic effect of methanolic extract from various parts of the plant on cancerous cell lines (Raghu et al. 2004).

1.5 OBJECTIVES OF THE PRESENT STUDY

The discussions in the foregoing sections have adequately revealed that *Azadirachta indica* A. Juss. and *Lantana camara* L., two medicinal plants chosen for the present study, hold important places in Indian and global scenario owing to their economic and medicinal properties. However, in both the cases, the benefits claimed in paper cannot be fully utilized because of certain practical limitations. The bottlenecks in Neem, with regard to extraction and availability of metabolites like azadirachtin lie in its out breeding nature and long reproductive cycle, that makes one to wait for long periods to obtain the seeds. Improvement by conventional methods is also restricted because of the same reason. In this respect, tissue culture techniques can play an important role. Apart from shortening the growth cycle they can also offer varied practical applications. Our aim and interest to raise haploids in *Azadirachta*, therefore, cropped up from the fact that they bring us a step closer to obtain genetically pure, homozygous diploid lines that produce consistent quality and quantity of compounds. Qualitative and quantitative variation in metabolites like azadirachtin due to prevalent

heterozygosity in the genus will diminish. As for *Lantana,* little scientific work has been done on this genus. Inspite of being a plant of potential medicinal interest there are a very few reports that document the properties of this plant scientifically. No tissue culture reports exist, till date, and all the biochemical studies carried out, so far, have been done on plants growing in wild. As we are aware that environmental fluxes cause alterations in type and quantity of metabolites produced, establishment of *in vitro* cultures will help to utilize the biomass and nullify the effect of seasonal variation on secondary metabolite content. Also, studies on secondary metabolites require an in-depth understanding of biosynthetic pathways which is often difficult to conduct in whole plants because the biosynthetic activities may only be expressed in particular cell types within a specific plant organ or at a certain time of season. Cell cultures have a higher rate of metabolism than intact differentiated plants because the initiation of cell growth in culture leads to fast proliferation of cell mass and to a condensed biosynthetic cycle. As a result, secondary metabolite production can take place within a short cultivation time (about 2-4 weeks) with an added advantage of tunability. These cell cultures may further aid in scale up operations, for isolation of desirable compounds in bulk. Therefore, the present study was undertaken with the following specific objectives:

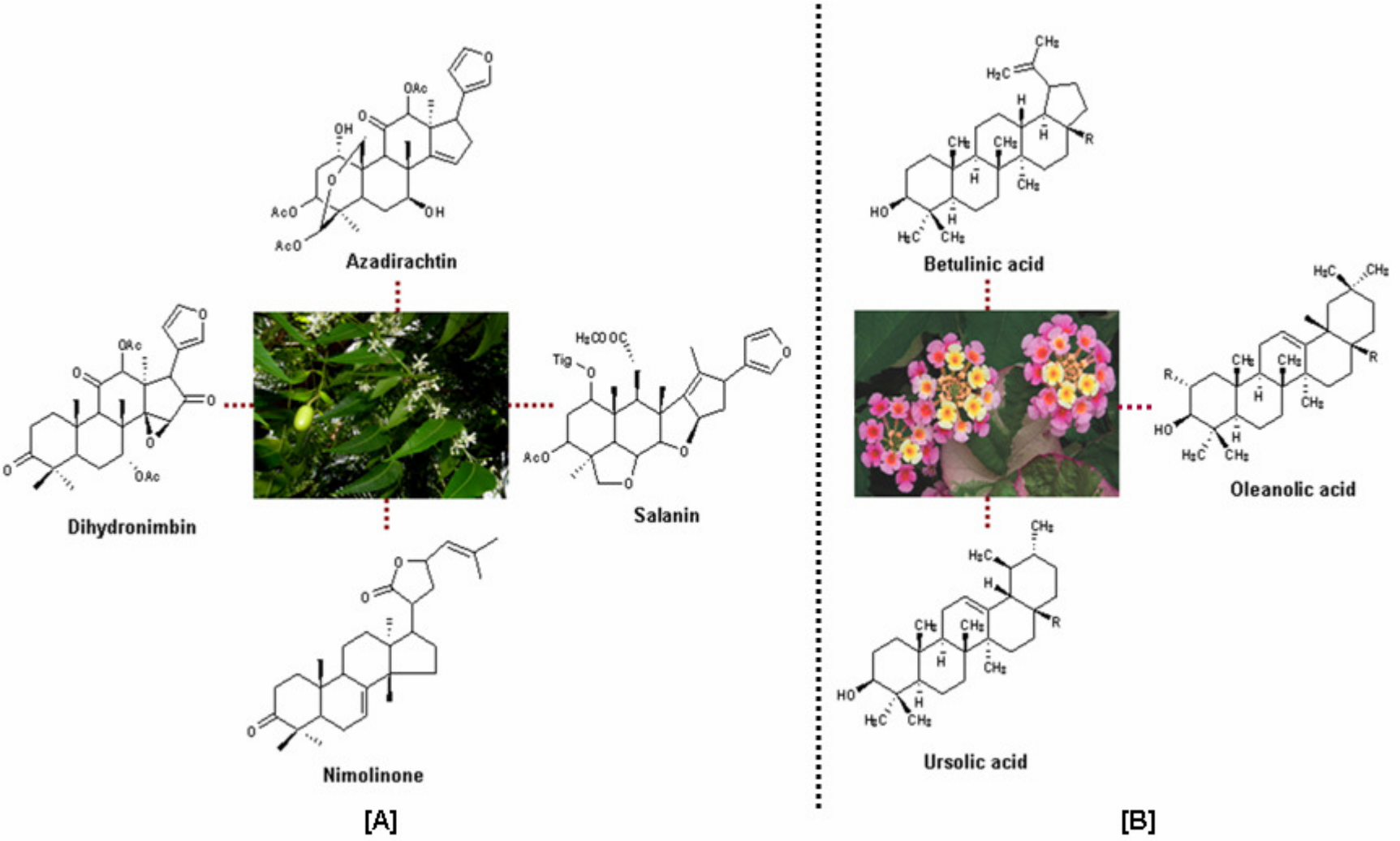
Specific Objectives:

A. *Azadirachta indica* A. Juss.

1. Androgenesis in anther cultures.
2. Cytological analysis for ploidy determination.
3. Analysis of haploids for production of azadirachtin, a tetranortriterpenoid.

B. *Lantana camara* L.

4. Establishment of *in vitro* cultures
5. Bioactivity guided extraction, purification and estimation of triterpenoids from *in vitro* cell lines.



Scheme 3: Chemical structures of medicinal compounds present in [A] *Azadirachta indica and* [B] *Lantana camara*

Chapter 2

Materials and Methods

Materials used in the present study as well as the methodologies followed, have been grouped into two broad sections. **Section 2A** is devoted to *Azadirachta indica* A. Juss. while **Section 2B** is dedicated to *Lantana camara* L. Each section is further divided into three subsections: the first contains all methodologies related to tissue culture studies, second subsection groups together all the biochemical analysis part and the third subsection reports methodologies related to bioassays.

2A *AZADIRACHTA INDICA*

2A.1. Materials

Young inflorescences of neem were collected from a mature 35-year-old neem tree, growing near the campus of IIT-Guwahati, during the months of April-May between 5.30 to 6.30 AM. The anthers from closed flower buds of 2 mm size were selected as explants to raise androgenic cultures (Fig. 5A-C). The various callus lines and plants, obtained from these cultures, were analysed for azadirachtin content.

The plant growth regulators, standard compound of azadirachtin, used in the present study, were procured from Sigma, USA. Constituents of MS (Murashige and Skoog 1962), B_5 (Gamborg et al. 1968) and NLN (Litcher 1982) basal media were purchased from Merck, India. Glassware and plasticware were obtained from Borosil, India and Tarsons, India, respectively.

For chemical analysis, HPLC grade methanol, analytical grade methanol, dichloromethane were purchased from Merck, India. Water used for HPLC analysis was purified by Milli-Q system (Millipore, USA).

The reference strains of *Staphylococcus aureus* (MTCC 7443, ATCC 25923), *Pseudomonas aeruginosa (*MTCC 741*), Klebsiella pneumonia* (MTCC 3348), *E. coli*

(MTCC 324) and *Streptococcus mutans* (MTCC 890) were obtained from IMTECH, Chandigarh. Clinical isolates of *Staphylococcus aureus, Pseudomonas aeruginosa, Klebsiella pneumoniae, Cons* and *E. coli* were provided by Guwahati Medical College, Guwahati, Assam and *Streptococcus mutans* was provided by Regional Dental College, Guwahati, Assam.

2A.2. Methods

2A.2.1. In Vitro Culture

i. Initiation and Establishment of Anther Cultures

The buds from first flush of flowers were used to initiate anther cultures. The stage of microspore development was checked with acetocarmine squashes. Two mm flower buds were surface sterilized with 0.1% mercuric chloride solution for 7 minutes inside the laminar-air-flow cabinet (Saveer Biotech, India) and rinsed thrice with sterile distilled water. The buds were dissected under a stereomicroscope (Nikon, Japan), using pre-sterilized Petriplates, forceps and fine needles. The damaged anthers, if any, were discarded and filament was gently removed. Twenty anthers, from two buds, were cultured in 60 mm x 15 mm pre-sterilized, disposable Petriplates containing 10 ml of medium without or with different combinations and concentrations of growth regulators (2,4-D, NAA, BAP, IAA, kinetin) and sealed with parafilm (Pechiney, USA). The best responding combination was checked at various sucrose concentrations, 3%-12%. Anthers from each treatment were given a temperature preatreatment of 4°C, 12°C, 32°C and 40°C for 1, 7 and 14 days, respectively. All the cultures were kept in dark until 8 weeks, for callus induction.

ii. Callus Multiplication and Shoot Regeneration

The calli obtained from induction media were further multiplied and maintained on media containing completely different sets of auxin (2,4-D) and/ or cytokinin (Kinetin). From now on calli were maintained in diffused light (1000-2000 lux) under 16 h photoperiod.

For experiments on shoot organogenesis, calli (ca. 0.2 g) from maintenance media were utilized. MS medium supplemented with cytokinins like BAP, TDZ, zeatin and

kinetin alone and/ or in combination with auxins like NAA, 2,4-D, IAA and additives like CH were used at different concentrations, to obtain shoot regeneration from callus.

iii. Shoot Elongation, Multiplication and Rooting

For elongation, individual shoots of 0.5 cm length were detached from regeneration media and cultured on MS medium containing a lower concentration of BAP at 0.5 µM or 1.0 µM. BAP was tested alone or in combination with CH (250 mgl^{-1}) or NAA (0.05 µM) or GA_3 (0.5 µM). In another experiment, effect of GA_3 (3 µM) pre-treatment was tested on shoot elongation.

Once the shoots attained sufficient length, they were cut into single node segments and transferred to fresh medium for further multiplication. The number of propagules obtained at the end of a multiplication cycle was regarded as the rate of shoot multiplication.

For rooting, individual shoots, measuring 3 cm and with 3-4 nodes, were excised and cultured on ¼ (major salts reduced to quarter strength) and full strength MS media supplemented with IBA at 0.5 µM.

iv. Culture Media

The anther cultures were raised on MS, B_5 and NLN basal media (for composition see Table 3). For subsequent experiments only MS basal medium was used. The media were variously supplemented with growth regulators and other adjuvants (Table 4). Unless mentioned otherwise, all the media contained 3% sucrose and were gelled with 0.8% agar (HiMedia, India).

Analytical grade (AR) chemicals and Milli-Q water were used to prepare stock solutions and culture media. Stock solutions of macronutrients (x 20), micronutrients (x 200), iron (x 200) and vitamins (x 200) were made separately and stored at 4°C till further use. The stock solutions of growth regulators were prepared at a concentration of $1x10^{-3}$ M and stored in refrigerator. Myoinositol and sugar were weighed and directly added to the culture medium at the time of media preparation. Required quantities of the various stock solutions, growth adjuvants and sugar were added to molten agar and final volume made up with distilled water (Elix, Millipore, USA). After adjusting the pH to

5.8, by using 0.1N HCl or 0.1N NaOH, the medium was dispensed into 150 x 25 mm Borosil rimless glass tubes with 20 ml medium per tube. The culture tubes were plugged with nonabsorbent cotton wrapped in cheesecloth and autoclaved at 121°C at 15 psi for 15 min. For initiation of anther cultures, the media were autoclaved in Erlenmeyer flasks and allowed to cool down to ca. 50°C, before dispensing into 60 mm pre-sterilized Petriplates under aseptic conditions. The thermolabile substances such as IAA, ABA, GA_3 and TDZ were filter sterilized using Millipore filters (0.45 µm pore size) and added to the autoclaved medium cooled to 50°C. The medium was then dispensed into glass tubes inside a laminar-air-flow cabinet.

v. Inoculation

All aseptic manipulations of the plant material, including inoculations, were made inside a laminar-air-flow cabinet. Before starting the work, the platform and the inner sides of the cabinet were swapped with rectified spirit and exposed to UV light for 30-45 min. The instruments used for inoculation (forceps, scalpels and needles etc.) and Petriplates used for dissection and chopping the material at subculture were wrapped in aluminum foil and autoclaved. During inoculation, at regular intervals, the instruments were dipped in 90% ethanol, flamed and cooled before use. At the time of raising fresh cultures or subcultures, the rim of the glass tube was flamed, the plug was removed and after planting the material on the medium the plug was replaced in quick succession.

vi. Culture Conditions

The cultures were generally incubated at 25°C ± 2°C and 50-60% relative humidity. Depending on the experiment, the cultures were maintained in diffuse light (1000-2000 lux) with 16 h photoperiod, provided by cool day light fluorescent tubes (Philips TL 40W) or in continuous darkness. To study the effect of temperature on callusing the anther cultures were kept at 4°C, 12°C, 32°C and 40°C for different periods before being transferred to 25°C temperature.

Table 3: Constituents of MS (Murashige & Skoog 1962), B_5 (Gamborg et al. 1968) and NLN (Lichter 1982) basal media. Concentrations expressed in mg/l.

Constituents	MS	B_5	NLN
NH_4NO_3	1650	-	-
KNO_3	1900	2527.5	125
$CaCl_2.2H_2O$	440	150	-
$Ca(NO_3)_2.4H_2O$	-	-	500
$MgSO_4.7H_2O$	370	246.5	125
KH_2PO_4	170	-	125
$(NH_4)_2SO_4$	-	134	-
$NaH_2PO_2.H_2O$	-	150	-
KI	0.83	0.75	-
H_3BO_3	6.2	3.0	-
$MnSO_4.4H_2O$	22.3	-	25
$MnSO_4.H_2O$	-	10	-
$ZnSO_4.7H_2O$	8.6	2	10
$Na_2MoO_4.2H_2O$	0.25	0.25	0.25
$CuSO_4.5H_2O$	0.025	0.025	0.025
$CoCl_2.6H_2O$	0.025	0.025	0.025
$FeSO_4.7H_2O$	27.8	-	27.8
$Na_2EDTA.2H_2O$	37.3	-	37.3
Sequestrene	-	28	-
Myoinositol	100	100	100
Nicotinic acid	0.5	1.0	0.5
Pyridoxine.HCl	0.5	1.0	0.5
Thiamine.HCl	0.1	10	0.5
Glycine	2	-	2
H_3BO_3	-	-	10
L-Glutamine	-	-	800
Glutathione	-	-	30
L-Serine	-	-	100
Folic Acid	-	-	0.5
Biotin	-	-	0.5
Sucrose	3%	2%	13%

Table 4: Growth regulators, amino acids and other compounds used as additives to basal media.

Additives	Manufacturer
Auxins	
2,4-D	Sigma, USA
NAA	Sigma, USA
IAA	Sigma, USA
IBA	Sigma, USA
2iP	Sigma, USA
Cytokinins	
BAP	Sigma, USA
Kinetin	Sigma, USA
Zeatin	Sigma, USA
TDZ	Sigma, USA
Aminoacids	
L-Glutamine	Sigma, USA
L-Serine	SRL, India
Other compounds	
GA_3	Sigma, USA
Caesin hydrolysate	HiMedia, India
Folic Acid	SRL, India
Glutathione	SRL, India
Biotin	SRL, India
Sucrose	Merck, India
Maltose	Merck, India
Glucose	Merck, India

vii. Observation of Cultures

Unless mentioned otherwise, eighteen cultures were raised for each treatment and all the experiments were repeated at least three times. The cultures were observed periodically, and the morphological changes were recorded at weekly intervals or whenever necessary. Final observations were taken after 8 weeks. Standard error of the mean was calculated and is indicated by ± sign. In primary cultures of anthers, the number of explants showing callus formation was recorded. The degree of callusing was expressed in terms of diameter of callus. Regeneration in the subcultures of callus is expressed as percent response.

viii. Histological Studies

For histological analysis, regenerating calli were sampled, and wax sections were cut to trace the developing vascular strands in the tissue. Material was fixed in FAA (5:5:90 v/v/v Formalin: Acetic acid: 70% Ethanol) for 48 hours and stored in 70% alcohol. The material was passed through the tertiary-butyl alcohol (TBA) series for dehydration, infiltrated with paraffin wax (melting point 60°C, E. Merck, Germany) and, finally, embedded in pure paraffin wax. Paraffin blocks were mounted on wooden stubs and 8-10 µm thick sections were cut using Rotary Microtome (Leica, Germany), attached with a steel knife. The sections were mounted on microslides, dewaxed and double stained with safranin (1%) and astra-blue (1%).

ix. Scanning Electron Microscopy

Nodulated calli from shoot regeneration media were fixed in 2.5% glutaraldehyde and dehydrated through a graded alcohol series. After drying, the samples were sputter-coated with gold and observed under a scanning electron microscope (Leo 1430vp, Carl Zeiss, Germany).

x. Cytological Studies

For cytological analysis, calli from induction, multiplication and regeneration media, root/ shoot-tips from *in vitro* raised plantlets and root tips (approx. 1 cm) from seedlings were excised at around 10.00 AM. After washing the samples with distilled

water, the material were pretreated with 0.02% 8-hydroxyquinoline (BDH, India) at 4°C for 4 h. This was followed by fixation in modified Carnoy's fluid containing absolute alcohol, chloroform, glacial acetic acid and methanol (7:3:1:1) for 48 h. The fixed material was placed in a mixture of nine drops of 1% aceto-orcein (or) 2% acetocarmine and one drop of 1 N HCl in a watch glass and heated gently. After cooling, the material was placed in a drop of fresh stain on a glass slide, and a cover slip was placed over it. The slide was warmed gently and the material squashed. The slides were observed under a light microscope (80i, Nikon, Japan), and the cells showing a good separation of chromosomes were photographed.

2A.2.2. Secondary Metabolite Production

i. Selection of Cell Lines

The androgenic calli, which contained diploid and aneuploid cells as well, were broadly categorized into dedifferentiated (non-regenerative) and redifferentiated (regenerative) lines. The calli on maintenance media remained undifferentiated or non-regenerative throughout the culture period. Eight-week-old calli from three maintenance media and two regeneration media, grown on MS basal medium (containing 3% sucrose) augmented with plant growth regulators such as auxins (2,4-D, NAA, IAA) and cytokinins (BAP, kinetin) were analysed for azadirachtin production profile. Besides, leaves from *in vitro* developed haploid plantlets were also utilized to check azadirachtin production; leaves and seeds from adult parent plant served as control I and control II, respectively.

ii. Preparation of Plant Extract

Cultured plant cells were harvested, washed with distilled water and filtered under vacuum. Thereafter, washed cell lines, leaf and seed samples were dried in an oven at 30°C ± 2°C until a constant weight was achieved. The drying temperature was kept low to prevent thermal decomposition of metabolites. The dried samples were soaked in methanol (analytical grade, Merck, India) overnight followed by sonication for 45 minutes at 35% amplitude (pulser 5 sec on/off cycle). The extract was centrifuged at 10,000 rpm for 15 min and filtered to remove the cell debris. This methanolic extract was

further fractionated by adding water in 60:40 ratio followed by 50:50 dichloromethane (Merck, India). Dichloromethane fractions were pooled, dried in a rotary evaporator (Buchi R-200, Japan) and the extract obtained was further analysed for presence of azadirachtin. Extraction from seeds consisted of an additional step of defattening with hexane prior to extraction with methanol.

iii. Preparation of Azadirachtin Standard

Stock solution of standard azadirachtin (Sigma-Aldrich, USA) was prepared in methanol (HPLC grade, Merck, India) at a concentration of 1 mg/ml and stored at -20°C. Calibration curve was generated by external standard method. The stock solution was serially diluted to five different concentrations and each concentration was run at least thrice to check the repeatability of results.

Linearity of developed method was checked by running the standard compound at five different concentrations. A calibration curve was generated by plotting concentration against peak area on Microsoft Office (Excel) Professional Edition 2003. The standard equation obtained from curve, was used for quantification of the compound in unknown samples. The correlation coefficients (R^2) were also generated in Excel by fitting the linear trendlines to the standard curve obtained. Precision of developed assay was evaluated by running same concentration of standard compounds atleast four times on the same day (intraday) and thrice at one day interval (interday). The values were calculated in terms of percent relative standard deviation (% RSD) which is calculated as: (Standard Deviation/ Mean) x 100.

iv. High Performance Liquid Chromatography

Quantitative estimation of azadirachtin was carried out on Varian Prostar HPLC system (Varian, USA) equipped with a binary pump, UV detector and a 20 µl injection loop. Hypersil BDS RP-C18 column (Thermo, USA) of dimensions 250 x 4.6 mm was used with methanol:water (90:10) (v/v) as the mobile phase at a flow rate of 0.5 ml/min. The eluted samples were detected at 214 nm wavelength. The identification of azadirachtin was done by comparing their retention times with those of authentic standard. The crude and standard samples were filtered through 0.22 µm PVDF

membrane filters (Millipore, USA) prior to analysis and aliquots of 20 µl of clean solution were injected into the HPLC system. System suitability tests were performed by checking linearity and precision of the developed assay.

v. Mass Spectroscopy

MS detection was carried out on Waters quadrapole-Tof premier mass spectrometer with micro channel plate detector (Waters, USA). Samples were analysed in positive mode with a probe temperature of 400˚C and a source block temperature of 150˚C. The source was operated with a corona pin voltage of 3.50 kV, and a cone voltage of 25 V. The MS data were obtained in full scan mode (mass range 100–1000 amu). A comparison of mass spectra of the standard compound obtained from Sigma-Aldrich, with that of sample isolated from HPLC, confirmed the presence of azadirachtin.

2A.2.3. Bioassay

i. Antimicrobial Assays

a. Culture Media

All the reference strains were grown in nutrient agar (agar-agar 20 g) or nutrient broth (without agar) media (Titan Biotech, India). Both the media consisted of peptone 5 g, beef extract 3 g, NaCl 5 g, and distilled water 1000 ml at pH 7.0. The clinical isolates were grown on blood-agar plates (Rankem, India; meat extract 20 g, tryptone 20 g, sodium chloride 5 g, agar 15 g, 5% sheep's blood and distilled water-1000 ml at pH 6.8) and the plates were incubated for 24 h at 37°C. Thereafter, the cultures were passed into peptone broth and incubated for 4-5 hours at same temperature. After adjusting the concentration with McFarland no. standard 0.5, bacterial colonies were swabbed on Muller-Hinton agar plates (Rankem, India; acid hydrolysate of casein 17.5 g, beef infusion 2.0 g, starch 1.5 g bacteriological agar 17 g, distilled water-1000 ml at pH 7.3) in three directions according to CLSI (Clinical and Laboratory Standards Institute).

b. Disc Diffusion Assay

Antibacterial susceptibility test was done by Bauer-Kirby disc diffusion method (Bauer et al. 1966). Filter paper discs of 5 mm diameter were made from Whatman filter

paper number 3 and sterilized. Each disc was about 4.7-4.8 mg in weight. Plant extracts were dissolved in methanol to make a stock concentration of 10 mg/ml. The samples were filtered by 0.22 µm nylon filter membrane (Millipore, USA) prior to use. Each disc was loaded with 5 µl of the extract, allowed to dry and placed on swabbed agar plates with the help of pre-sterilized forceps.

2A.2.4. Statistical Analysis

For culture initiation and induction responses, 20 anthers were placed in one Petridish with 24 dishes for each treatment. Data were collected as number of responding anthers relative to total number of anthers cultured. Regeneration in the subcultures of callus was expressed as percent response. Data were subjected to one-way or two-way analysis of variance (ANOVA) and means were calculated using Duncan's multiple range test by SPSS 16.0 software. p-values less than 0.05 were considered statistically significant. Arcsine transformation was done for the percent values before proceeding for ANOVA. For azadirachtin estimation, all results are an average of three separate analysis. Results are represented as mean ± SD.

2B *LANTANA CAMARA*

2B.1. Materials

Leaves from healthy *L. camara* plants bearing pink-yellow colored flowers, growing in the campus of Indian Institute of Technology-Guwahati, were collected at monthly intervals, over three consecutive years, to initiate cultures.

Constituents of MS medium (Murashige and Skoog 1962), B_5 vitamins (Gamborg et al. 1968) and White's organic (White 1963) were purchased from Merck, India. The plant growth regulators were purchased from Sigma-Aldrich, USA. Glassware and plasticware were obtained from Borosil, India and Tarson, India, respectively.

For chemical analysis, HPLC grade acetonitrile, analytical grade methanol, ethyl acetate and 95% ethanol were purchased from Merck, India. Water used for HPLC analysis was purified by Milli-Q system. The standards of pentacyclic triterpenoids, BA, OA and UA were procured from Sigma-Aldrich, USA.

For cytotoxicity assays, 96 well culture plates were obtained from Corning, Sigma. The reference strains of *Staphylococcus aureus* (MTCC 7443, ATCC 25923), *Pseudomonas aeruginosa* (MTCC 741), *Klebsiella pneumoniae* (MTCC 3348), *E. coli* (MTCC 324) and *Streptococcus mutans* (MTCC 890) were obtained from IMTECH, Chandigarh. Clinical isolates of *Staphylococcus aureus, Pseudomonas aeruginosa, Klebsiella pneumoniae, Cons* and *E. coli* were provided by Guwahati Medical College, Guwahati, Assam and *Streptococcus mutans* was provided by Regional Dental College, Guwahati, Assam.

2B.2. Methods

2B.2.1. In Vitro Culture

i. Initiation and Establishment of Leaf-disc Cultures

Leaves were washed with 1% Tween-20 (Merck, India) for 15 min, followed by three rinses in sterile distilled water (SDW). Thereafter, the remaining steps were carried out inside the laminar-air-flow cabinet (Saveer Biotech, India). Leaves were surface sterilized with 0.1% mercuric chloride solution ($HgCl_2$) for 10 min and rinsed thrice with SDW. Leaf-disc explants were prepared by punching the sterilized leaves with 5 mm sized cork-borer before being cultured with the adaxial side in contact with the media.

ii. Culture Media

Leaf disc explants were incubated on MS (with 3% sucrose) or B_5 (with 2% sucrose) basal medium consisting of macro and microsalts, vitamins, iron, 100 mgl^{-1} myoinositol and solidified with 0.8% agar (HiMedia, India). Additionally, the cultures were raised on MS basal medium where MS vitamins were replaced with B_5 vitamins or White's organics. All the media were enriched with varying combinations and concentrations of auxins and cytokinins, including NAA, IAA, 2,4-D, BAP, 2iP and kinetin at a concentration range of 0.5 µM-10 µM, for induction and multiplication of callus from leaf disc explants. After adjusting the pH to 5.8, 20 ml of the medium was dispensed into each 150 x 25 mm Borosil rimless glass tubes. The culture tubes were plugged with nonabsorbent cotton wrapped in cheesecloth and autoclaved at 121°C at 15 psi for 15 min. Thermolabile compound, such as IAA, was filter-sterilized using

Millipore filters (0.45 µm pore size) and added to the autoclaved medium cooled to 50°C; Medium was then dispensed into test tubes inside a laminar-air-flow cabinet. All the cultures were maintained in diffuse light (1000-1600 lux) and 16 h photoperiod at 25 ± 2°C and 50-60% relative humidity. At least 24-cultures were raised for each treatment and each experiment was repeated at least three times. Observations were recorded at weekly intervals. After callus induction, the biomass was multiplied constantly by inoculating 0.2 g of calli onto fresh media at every 4-week interval.

iii. Establishment of Suspension Cultures

After 7-8 passages of callus subcultures, healthy, green, friable and soft calli maintained on responding semi-solid media were utilized to establish suspension cultures. Cultures were initiated in Erlenmeyer flasks of 250 ml capacity, containing 50 ml of liquid medium and inoculated with 0.2 g of fresh calli. The cultures were incubated in an orbital shaker under shaking conditions at 120 rpm at 25°C ± 2°C and maintained in diffuse light (1000-1600 lux) with 16 h photoperiod or in continuous darkness. The cell biomass was subcultured regularly into fresh medium at every three weeks.

2B.2.2. Secondary Metabolite Production

i. Determination of Dry Cell Weight

The calli maintained on responding semi-solid medium were harvested at the end of the growth period of 4 weeks, washed with distilled water and filtered under vacuum. Subsequently, its fresh weight was taken on high precision analytical balance (Sartorius, India). Thereafter, the cells were dried in oven at 30°C ± 2°C until a constant weight was achieved. The drying temperature was kept low to avert decomposition of thermolabile compounds. The dried cell mass of 50 g was utilized for biochemical studies. For batch kinetic studies, cells were harvested at a regular interval of two days from liquid medium and dried in a similar manner as described above.

ii. Preparation of Plant Extracts

To prepare samples, 50 g of dried cell mass and powdered leaf samples were soaked separately in 200 ml methanol for 48 h and thereafter, sonicated at 30% amplitude

at pulser 5 sec on/off cycle for 40 min, then again for 20 min at pulser 3 sec on/off cycle. The methanolic mixture, thus, obtained was filtered and the filtrate was centrifuged at 10,000 rpm (Sigma 4K15C, Osterode Am Harz, Germany) for 10 min. Supernatant was pooled, filtered and dried in a rotary evaporator at 40°C. The methanolic extract, thus obtained, was further fractionated into an organic (ethyl acetate) and aqueous fractions. The ethyl acetate fraction was dried under reduced pressure in a rotary evaporator at 40°C (Buchi Rotavapor R-200, Japan) and aqueous fraction was freeze-dried in a lyophilizer (Christ, Germany) before being utilized for further studies.

iii. Preparation of Standard Solutions

Stock solutions of BA, OA and UA were prepared, individually, in 95% ethanol at a concentration of 5 mg/ml and the solutions were stored at -20°C. The quantification was performed using 5 levels of external standards, obtained by serial dilutions of stock solutions, at a concentration range of 2.5 mg/ml to 0.16 mg/ml. Each concentration of standards was run at least thrice to check the repeatability and precision of results. Both external and internal standards (spiking) were employed for confirmation of the compounds.

iv. Chromatographic Analysis

a. Thin Layer Chromatography (TLC)

The dried organic extract was dissolved in chloroform and qualitatively analyzed by TLC on the glass plates coated with silica gel. The extract was eluted with chloroform/methanol (24:1) solvent system. Spot visualization was accomplished in an iodine chamber. The Retention factor or the R_f value of the spots was calculated as: Distance traveled by the sample/ Distance traveled by the solvent.

b. High Performance Liquid Chromatography (HPLC)

Quantitative estimation of BA, OA and UA was carried out on Varian Prostar HPLC system (Varian, USA) equipped with a binary pump, UV detector and a 20 µl injection loop. Hypersil BDS RP-C_{18} column (Thermo, USA) of dimensions 250 x 4.6 mm, 5 µm particle size, was used with acetonitrile:water (80:20) (v/v) as the mobile

phase, at a flow rate of 1 ml/min. The eluted samples were detected at 209 nm wavelength. The identification of all three acid triterpenes was done by comparing their retention times with those of authentic standards. The crude and standard samples were filtered through 0.22 µm cellulose nitrate membrane filters (Millipore, USA) prior to analysis, and aliquots of 20 µl of clean solution were injected into the HPLC system. System suitability tests were performed by checking linearity, precision and recovery of compounds in the developed assay. Each of the three peaks, corresponding to the three compounds, were isolated from the crude extract and used for mass spectroscopic analysis.

c. Linearity, Precision and Recovery of Compounds

Linearity of developed method was checked by running the standard compounds at five different concentrations. A calibration curve was generated by plotting concentration against peak area on Microsoft Office (Excel) Professional Edition 2003. The standard equation obtained from the curve, was used for quantification of the three triterpenes in the unknown samples. The correlation coefficients (R^2) were also generated in Excel by fitting the linear trendlines to the standard curves obtained for each of the three compounds.

Precision of developed assay was evaluated by running same concentration of standard compounds atleast four times on the same day (intraday) and twice at one day interval (interday). The values were calculated in terms of relative standard deviation (RSD).

RSD= (SD/average) x 100%

The recovery experiments for all the three compounds were performed by adding known amount of BA, OA, UA standards to the callus cells, which were then extracted in a similar manner as mentioned in the section 2B.2.2.ii. The recovery percentages were calculated as:

$$\textbf{Recovery (\%)} = (A - B / C) \times 100$$

Where, *A* is the quantity of BA, OA and UA in the spiked powder, *B* is the quantity of BA, OA and UA in the powder without added standards and *C* is the quantity of added BA, OA and UA.

v. Mass Spectroscopy (MS)

MS detection was carried out on Waters quadrapole-Tof premier mass spectrometer with micro channel plate detector (Waters, USA) and was operated in the negative ion mode, with collision energy of 5V. The cell entrance and exit voltage was set at 2V and -10V, respectively. The MS data were obtained in full scan mode (mass range 100–1000 amu). A comparison of mass spectra of three standard compounds obtained from Sigma-Aldrich, with those of the individual samples isolated from HPLC, confirmed presence of all the three triterpenic acids.

vi. Batch Kinetics of Cell Suspension Cultures

a. Batch Kinetics Studies

To determine the specific growth rate, cells were harvested from liquid medium at an interval of two days, washed and dried with same procedure as mentioned in the section 2B.2.2.i. The pH and conductivity of the suspension cultures were monitored after every two days. Phosphate was estimated by the standard calibration curve made from dihydrogen sodium phosphate (NaH_2PO_4); to 0.5 ml of standard or sample solution, 4 ml of reagent [Acetone (CH_3COCH_3), Sulphuric acid (H_2SO_4) 2.5 M and Ammonium molybdate tetrahydrate $(NH_4)_2MoO_4.4H_2O$) 10 mM, mixed in the ratio of 2:1:1] was added. After mixing the solutions were thoroughly, 0.4 ml of 1M citric acid was added and absorbance was taken at 355 nm. Similarly, for nitrate estimation, standard curve was made from 0.01N stock solution of Potassium nitrate (KNO_3), preserved in chloroform. After acidification of samples with hydrochloric acid, absorbance was recorded at 275 nm in a UV visible spectrophotometer (Cary 100, Netherlands). All measurements and results are average readings obtained from three flasks.

b. Carbon Source

Three carbon sources, glucose, maltose and sucrose were tested at 3% concentration to understand the growth and production profile of cells in suspension cultures. The *L. camara* cells were inoculated such that each 250 ml Erlenmeyer flasks containing 50 ml of medium had 0.2 g of the cells. The suspension cultures were

incubated as described in the section 2B.2.1.iii. The cell biomass was harvested at the end of the growing phase of 3 weeks, to analyse dry cell weight and triterpenoid content.

c. Agitation Speed

Effect of agitation speeds was evaluated on fresh and dry weight of cells and their viability, at the end of each passage. Callus cells weighing approximately 0.2 g were harvested at the end of growth period and re-inoculated in 50 ml of fresh medium of the same composition. The cultures were incubated in shaking conditions at 60, 120 and 240 rpm, under darkness, for a period of three weeks and their fresh and dry weights were recorded. The viability of cells under each condition was checked with 1% fluorescein diacetate (FDA) solution.

2B.2.3. Bioassays

i. Cytotoxicity Assay

a. Culture and Maintenance of Mammalian Cell Lines

The Human cervical adenocarcinoma cell line- HeLa, and Syrian golden hamster kidney normal fibroblast cell line - BHK-21(C-13) were obtained from National Centre for Cell Science, Pune, India. These were grown as monolayer cultures in Dulbecco's Modified Eagle Medium (DMEM), containing 2 mM L-glutamine, 1.5 g/l sodium bicarbonate, 0.1 mM non-essential amino acids and 1.0 mM sodium pyruvate, supplemented with 10% (v/v) heat inactivated fetal bovine serum (FBS) and 1% antibiotic antimycotic solution (1000 U/ml penicillin G, 10 mg/ml streptomycin sulfate, 5 mg/ml gentamycin and 25 µg/ml amphotericin B). The cultures were maintained at 37^{o}C in 5% CO_2: 95% air atmosphere and 95% relative humidity.

b. Stock Preparation of Plant Extracts for Cytotoxicity Studies

The stock solutions of aqueous and organic extracts were prepared at a concentration of 10 mg/ml. The lyophilized aqueous extract was dissolved in serum free DMEM. The organic extract was dissolved in dimethyl sulphoxide (DMSO) and final volume was made upto 1 ml with serum-free DMEM. The final concentration of DMSO did not exceed 1% in any experiment. Before use, the stocks were filter-sterilized through

0.2 µm filter and appropriate concentrations were prepared with culture medium before each experiment. Curcumin (HiMedia, India), a natural polyphenolic compound from *Curcuma longa*, was used as a positive control in the experiments. The stock solution of curcumin was prepared at a concentration of 1mg/ml in a similar manner as that of the organic extract.

c. Cell Viability Assay

For viability assays, both HeLa and BHK-21 (C-13) cells, grown in T-25 culture flasks, were harvested by trypsinization, plated at a rate of ~$1x10^4$ cells/well in 96 well culture plates and incubated for 24 h in a CO_2 incubator to allow confluent growth. After 24 h, medium from each well was removed and cells were washed twice with Dulbecco's Phosphate Buffered Saline without Ca^{+2} and Mg^{+2}. The cells were then exposed to different concentrations of organic extract ranging from 2.5 to 200 µg/well and aqueous extract ranging from 100 to 300 µg/well. Curcumin was taken at concentrations ranging from 0.1 to 10 µg/well. Each well contains 100 µl of serum free DMEM with the above mentioned amount of the extract. The plates were incubated for different time periods of 24 h, 36 h, 48 h, 60 h and 72 h. The cell viability was estimated using 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (Mosmann 1983). At the end of each incubation period, cells were incubated with 0.5 mg/ml MTT (dissolved in serum free DMEM without phenol red) for 4 h at 37°C for colorimetry based cytotoxicity assay. The yellow colored MTT is reduced to an insoluble purple formazan by mitochondrial reductase that is active only in mitochondria of living cells. Thus, the conversion can be directly correlated to the number of viable cells. The reaction product, formazan, was dissolved in DMSO, and the absorbance of the purple colored solution was quantitatively measured at 570 nm in a UV visible spectrophotometer (Cary 100, Netherlands).

d. Microscopic Observations of Cell Morphology

HeLa and BHK-21 cells, treated at various concentrations of extracts for different durations, were examined under Inverted Light Microscope (TS 100-F, Nikon, Japan), Confocal Laser Scanning Microscope (CLSM; LSM 510Meta, Carl Zeiss, Germany) and

Scanning Electron Microscope (SEM; Leo 1430vp, Carl Zeiss, Germany). For CLSM analysis, the cells were stained with Acridine Orange/Ethidium Bromide (AO/EB) before observation. For SEM analysis, cells were initially allowed to grow on a cover slip and subjected to extract treatment. The samples were fixed, dehydrated, dried under vacuum and finally sputter coated with gold prior to examination.

e. DNA Fragmentation Assay

For laddering experiments, the cells were treated with 44.75 µg/ml extract and incubated for 48 h in CO_2 incubator. Treated cells were harvested, washed with ice cold Phosphate Buffered Saline (PBS) and centrifuged at 3000 rpm for 6 min at 4°C. Cell pellet obtained, was dispersed in 30 µl of lysis buffer (10 mM Tris, pH 7.4; 100 mM sodium chloride; 25 mM ethylene diamine tetra acetic acid; 1% sodium lauryl sulfate) by gentle vortexing. About 4 µl of proteinase K (10 µg/µl) was added to the above mixture and incubated at 45°C for about 1-2 h. Around 2 µl of RNase (10 µg/µl) was added to the cell lysates and further incubated for 1 h at room temperature. After the incubation, cell lysates were mixed with 4 µl of 6X DNA sample dye (50% glycerol; 1 mM EDTA, pH 8.0; 0.25% bromophenol blue) and the samples were finally run on 2% agarose gel electrophoresis at 7 V/cm. The gel was stained with ethidium bromide (0.5 µg/ml) and visualized under gel documentation system (BioRad, USA).

ii. Antimicrobial Assays

a. Culture Media

All the reference strains were grown in nutrient agar (agar-agar 20 g) or nutrient broth (without agar) media (Titan Biotech, India). Both the media consisted of peptone 5 g, beef extract 3 g, NaCl 5 g, and distilled water 1000 ml at pH 7.0. The clinical isolates were grown on blood-agar plates (Rankem, India; meat extract 20 g, tryptone 20 g, sodium chloride 5 g, agar 15 g, 5% sheep's blood and distilled water 1000 ml at pH 6.8) and the plates were incubated for 24 h at 37°C. Thereafter, the cultures were passed into peptone broth and incubated for 4-5 hours at same temperature. After adjusting the concentration with McFarland no. standard 0.5, bacterial colonies were swabbed on Muller-Hinton agar plates (Rankem, India; acid hydrolysate of casein 17.5 g, beef

infusion 2.0 g, starch 1.5 g bacteriological agar 17 g, distilled water 1000 ml at pH 7.3) in three directions according to CLSI (Clinical and Laboratory Standards Institute).

b. Disc Diffusion Assay

Antibacterial susceptibility test was done by Bauer-Kirby disc diffusion method (Bauer et al. 1966). Filter paper discs of 5 mm diameter were made from Whatman filter paper number 3 and sterilized. Each disc was about 4.7-4.8 mg in weight. Plant extracts were dissolved in ethyl acetate to make a stock concentration of 10 mg/ml. The samples were filtered by 0.22 µm nylon filter membrane (Millipore, USA) prior to use. Each pre-sterilized disc was loaded with 5 µl of the extract, allowed to dry and placed on swabbed agar plates with the help of pre-sterilized forceps.

2B.2.4. Statistical Analysis

For triterpenoid estimation from callus cultures, observations are an average of three separate analysis. For batch kinetics studies, all results are an average of two separate analysis and two consecutive experiments with three replicate flasks in each treatment for kinetics of growth and effect of other variables like pH, conductivity and nutrient consumption. Results are represented as mean ± SD. Specific growth rate (μ) was calculated by:

$$\mu = \ln (MT_2\text{-}MT_1) / T_2\text{-}T_1;\ T_2 > T_1$$

where, MT_2 and MT_1 are biomasses at the different time points (T_1 and T_2) respectively.

For cytotoxicity assays, all experiments were done in triplicates, along with positive and negative controls. To minimize experimental errors, exponentially growing cells of the same passage were used for each of the experiments. Results were expressed as mean ± standard deviation. Data on percent cell viability were recorded and subjected to paired t-tests between the treatment groups (HeLa and BHK-21). p-values less than 0.05 were considered statistically significant.

Chapter 3

Results

Two highly valued medicinal plants were investigated and evaluated, in the present chapter, for tissue culture studies and production of bioactive compounds. The results of the work have been divided into two broad sections; **section 3A** presents the results on *Azadirachta indica* A. Juss. and **section 3B** deals with *Lantana camara* L. results. Each section is further divided into three subsections: the first subsection presents results related to tissue culture studies, the second subsection groups together the biochemical analysis part and the third subsection reports results related to bioassays.

3A. *AZADIRACHTA INDICA* A. JUSS.

3A.1. Anther Culture

Androgenic shoot induction in Neem involved three steps:

(1) Callus induction: The anthers were cultured at uninucleate stage of microspores on the induction medium to induce calli.

(2) Callus multiplication: The individual calli, obtained from anthers, were grown on proliferation medium to support sustained growth of callus.

(3) Shoot regeneration: Subculture of calli on regeneration medium for shoot differentiation.

3A.1.1. Callus Induction

Buds of 2 mm size with anthers at early-to-late uninucleate stages of microspores were selected for the study **(Fig. 1A-C)**. Microspores, freshly released from the tetrads (**Fig. 1D-E)** are ideal candidates to induce androgenic calli as their wall is thin and it is easy to divert their gametophytic mode of development to sporophytic pathway. All the cultures were incubated in dark for callus induction, for first 8 weeks. On several treatments, the anthers callused. Two clear patterns of callusing were seen on the responding medium: in one, the anther walls burst open

and pulled apart due to pressure of callusing microspores and shiny, globular masses emerged from within **(Fig. 2A-F)**. In few other cases, callus was developed at the base from anther walls **(Fig. 2H-J)**. The former cultures were utilized in further experiments and for data calculation, while, the latter were discarded. The callusing started after 4 weeks of culture and after 8 weeks excellent callus growth was observed in almost all responding anthers. The calli that emerged from inside of burst anthers were detached from the parental anther wall and subcultured on multiplication medium with a reduced sucrose concentration of 3% and 16/8 h photoperiod regime. Therefore, all later experiments related to regeneration and chemical analysis had been conducted with microspore derived calli.

Several experiments were designed to improve callus induction from microspores in anther cultures:

i. Effect of Growth Regulators on Callus Induction

Effect of MS medium with 3% or 9% sucrose and supplemented with various growth regulator combinations on callusing in anther cultures is presented in **Graph 3A.1.** All the media contained 3% or 9% sucrose. In the absence of growth regulator (controls I and II) or with auxin alone (medium IV), the response was nil. The combined presence of one auxin and one cytokinin induced some callusing but the response was extremely poor. However, the presence of two auxins and one cytokinin improved the number of cultures showing callusing. Overall, 9% sucrose in the medium supported better callus induction than 3% sucrose. Out of the various treatments, MS (with 9% sucrose) + 2,4-D (1 µM) + NAA (1 µM) + BAP (5 µM) gave maximum response (> 62%). On this medium, the callus appeared soft, friable and yellowish brown in color.

ii. Effect of Sucrose Concentration on Callus Induction

MS medium normally contains 3% sucrose. However, in the previous experiments on effect of growth regulators, the media containing 9% sucrose showed excellent results **(*see* Graph 3A.1).** Therefore, to study the effect of sucrose concentration and to improve the callus induction response, various concentrations of sucrose were tested with the best medium, MS + 2,4-D (1 µM) + NAA (1 µM) + BAP (5 µM). The results are presented in **Table 3A.1**. At its different concentrations, 12% sucrose was observed to be significantly better ($p<0.05$) for callus induction from

anthers, followed by 9%, 6% and 3% concentration. Concentrations above 12% were found to be inhibitory. With 12% sucrose, 85.4% anthers developed fresh, soft, shiny and cream calli from inside of burst open anthers, after 8 weeks of dark incubation. The high sucrose concentration was required only during the initial induction phase of 8 weeks which selectively facilitates haploid cells to grow and divide while suppressing the divisions of diploid cells.

iii. Effect of Basal Media on Callus Induction

As MS medium showed best response when supplemented with 2,4-D (1 µM) + NAA (1 µM) + BAP (5 µM) and 12% sucrose, this combination of growth regulators and sucrose was also tested with B_5 and NLN media **(Table 3A.2)**. Maximum (85.4%) responding anthers were found on MS medium followed by NLN (63.5%). No response was observed on B_5 medium. Nature and growth of callus was distinctly better on MS medium than the NLN medium. Therefore, further experiments were conducted with MS medium only. On MS medium callus growth was excellent and the calli were fresh, soft, friable and cream while those on NLN medium it was dark brown and friable with tiny growth, that too mostly from the anther walls.

iv. Effect of Temperature Pre-treatments on Callus Induction

In many species, the incubation of anther cultures at low or high temperatures, for various periods, before shifting them to 25°C enhanced the androgenic response. Therefore, anther cultures of Neem were exposed to low (4°C, 12°C) and high (32°C, 40°C) temperatures for 0, 1, 7 or 14 days, to study their callusing response. The cultures were raised on MS (12% sucrose) + 2,4-D (1 µM) + NAA (1 µM) + BAP (5 µM). Best response was observed in control at 25°C temperature, followed by one day pre-treatments at 12°C and 4°C temperatures **(Table 3A.3).** Temperatures higher than 25°C were found to have adverse effect on callusing and no response was observed when the anthers were exposed to 40°C for 7 and 14 days.

Graph 3A.1: Effect of some growth regulators on callus induction in anther cultures on MS with 3% and 9% sucrose. Growth period: 8 weeks. Control I: MS (3% sucrose); Control II: MS (9% sucrose).

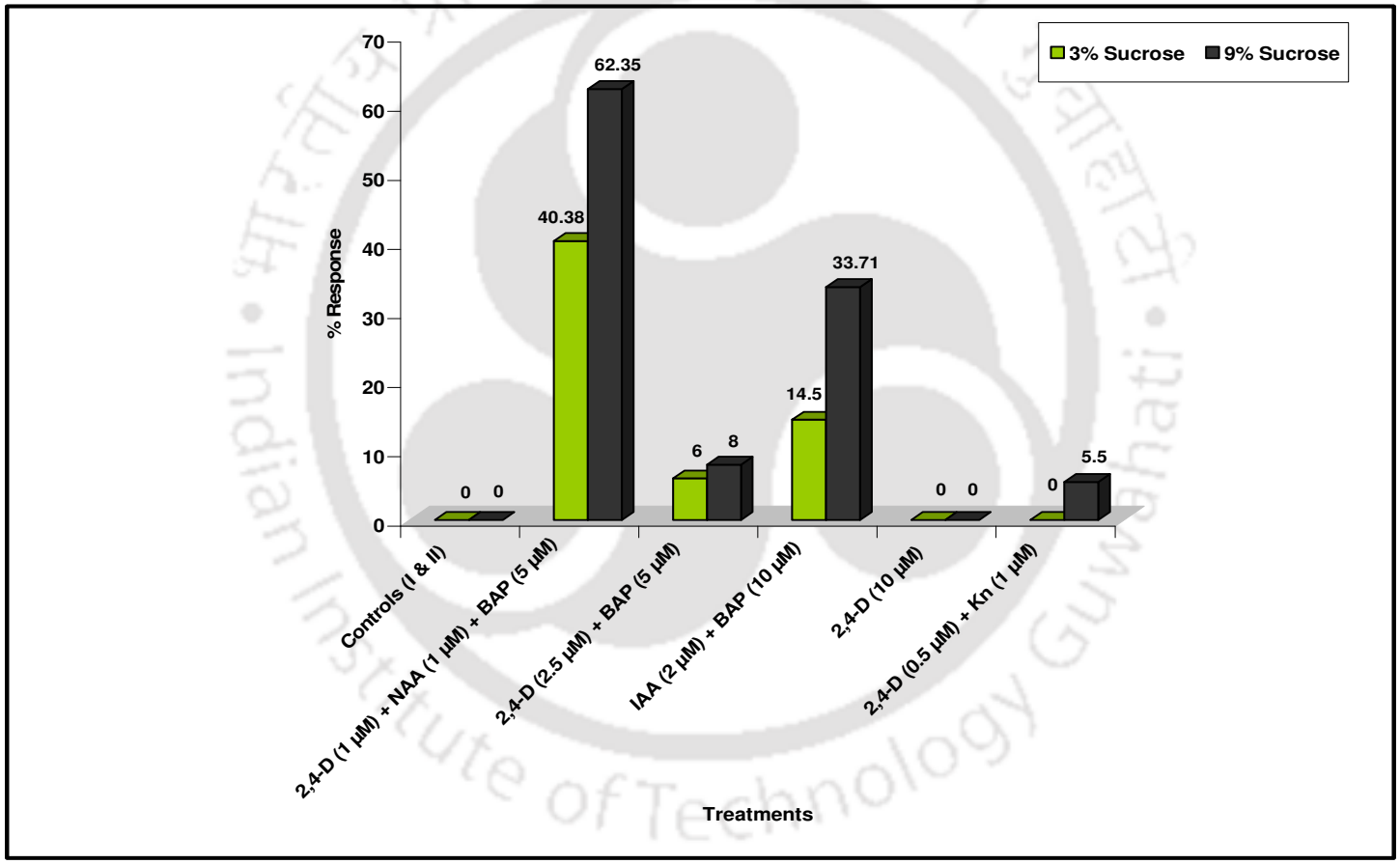


Table 3A.1: Effect of sucrose concentration on callus induction from anther cultures. Growth Period: 8 weeks. Control: MS + 2,4-D (1 µM) + NAA (1 µM) + BAP (5 µM) with 3% Sucrose.

Sucrose (g/l)	Total Anthers Cultured	Anthers Responded	Percent Response(*)
30 (control)	480	193.82	40.38^{ab}
60	480	252.96	52.7^{b}
90	480	299.28	62.35^{ab}
120	480	409.92	85.4^{a}
150	480	168	35^{c}
180	480	24	5^{d}

(*) Those marked with the same letters are not significantly different at $p<0.05$ according to Duncan's multiple range test

Table 3A.2: Effect of different basal media on percent callusing response of anthers cultured on medium with 12 % Sucrose + 2,4-D (1 µM) + NAA (1 µM) + BAP (5 µM). Growth Period: 8 weeks.

Media used	Total Anthers Cultured	Anthers Responded	Percent Response
MS (control)	480	409.92	85.4^{a}
B_5	480	0	0^{b}
NLN	480	304.80	63.5^{a}

(*) Those marked with the same letters are not significantly different at $p<0.05$ according to Duncan's multiple range test.

Table 3A.3: Effect of temperature pretreatments at different duration on callusing from anthers cultured on MS (with 12% sucrose) + 2,4-D (1 µM) + NAA (1 µM) + BAP (5 µM); Growth Period: 8 weeks.

Temperature (°C)	Duration (days)	Total Anthers Cultured	Anthers Responded	Percent Response
25 (control)	**---**	480	409.92	85.4
4	1	120	94.44	78.7
	7	120	67.92	56.6
	14	120	51.24	42.7
12	1	120	98.28	81.9
	7	120	68.64	57.2
	14	120	56.76	47.3
32	1	120	58.18	48.48
	7	120	60.00	50.0
	14	120	74.11	61.76
40	1	120	66.32	55.27
	7	120	0	0
	14	120	0	0

Two-way ANOVA to study the effect of different temperatures and duration of pretreatment on anther cultures.

SUMMARY	COUNT	SUM	AVERAGE	VARIANCE		
Row 1	4	264.35	66.0875	278.7176		
Row 2	4	163.8	40.95	755.93		
Row 3	4	151.76	37.94	705.7011		
Column 1	3	178	59.33333	329.6033		
Column 2	3	186.4	62.13333	317.5433		
Column 3	3	160.24	53.41333	52.82773		
Column 4	3	55.27	18.42333	1018.258		
Source of Variation	**SS**	**df**	**MS**	**F**	**P-value**	**F crit**
Treatment length	1910.981	2	955.4905	3.758117	0.087476	5.143253
Different temperatures	3695.563	3	1231.854	4.8451-05	**0.048185**	4.757063
Error	1525.483	6	254.2472			
Total	7132.027	11				

3A.1.2. Callus Maintenance

The calli on the induction medium turned to wet, paste like dirty brown mass with convulated surface by 20th week, in the third subculture **(Fig. 3A)** and did not support sustained callus growth. Such calli, when transferred to the other medium for regeneration, were not able to survive after a short period.

Therefore, the individual calli that developed on the induction medium, were transferred to MS or MS medium supplemented with single auxin as 2,4-D and single cytokinin in the form of kinetin for sustained callus proliferation, as against two auxins and one cytokinin required during the induction phase. In terms of biomass, best response was observed on MS + 2,4-D (1 µM) + kinetin (10 µM) followed by MS + 2,4-D (0.5 µM) + kinetin (4.5 µM) and MS + 2,4-D (0.5 µM), in a single growth cycle of 8 weeks. The calli obtained on these combinations were cream, soft, friable and moderate to fast growing **(Fig. 3B-D)**. The calli were allowed to multiply on these three media for more than 2 years.

3A.1.3. Regeneration of Shoots from Anther Calli

Since the origin of callus often affects the frequency of regeneration, the calli derived from above three multiplication media were utilized for regeneration experiments **(*see* Table 3A.4)**. Of these, MS + 2,4-D (0.5 µM) + kinetin (4.5 µM) served as the best source**,** as it gave maximum percentage of regenerating cultures. Numerous growth regulator combinations were tried **(Table 3A.4)** but shoot regeneration was achieved on only two combinations: MS + BAP (2.5 µM) + IAA (5 µM) + CH (500 mg/l) and MS + BAP (2.2 µM) + NAA (0.05 µM). The callus in the former medium turned to bright green color with compact nodular structures that differentiated into shoot-buds, after 6 weeks **(Fig. 4A-C)**. On an average 27.8% cultures formed 4.5 shoot-buds per explant on this combination. A considerably better percentage (98.5%) of shoot-bud proliferation was achieved in the latter medium. For this, two step protocol was adopted, in the first step, the calli from multiplication medium were transferred to a combination of high BAP (22 µM) and low NAA (0.5 µM) medium. After 12 weeks of incubation, these calli were transferred to ten times lower concentration of the same growth regulator combination, MS + BAP (2.2 µM) + NAA (0.05 µM). This has remarkably improved the frequency of

shoot-bud proliferation where 98.5% cultures formed an average of 8.5 shoot-buds per explant, after 6 weeks **(Fig. 4D-F)**. On repeated subcultures on the regeneration media, the callus gradually got consumed by shoot-bud differentiation. Therefore, for maintenance of callus MS + 2,4-D (0.5 µM) + kinetin (4.5 µM) was selected. Histological sections of the regenerating calli, passing through the nodular regions, showed the presence of developing vascular strands within the tissue, indicating the points of origin of shoot-buds. Well formed tracheary elements can be seen in the sections **(Fig. 5A, B)**. Development of nodulated areas was also evident in scanning electron micrographs of the regenerating callus **(Fig. 5C, D)**. The shoot-buds further developed into shoots that did not grow beyond 0.5 cm in length while attached to the callus

3A.1.4. Elongation, Multiplication and Rooting of Shoots

The above shoot regeneration media did not support satisfactory shoot elongation. Therefore, the individual shoots were excised and transferred to MS basal medium or basal medium supplemented with a lower concentration of BAP (0.5 µM, 1.0 µM) either alone or in combination with an auxin NAA (0.05 µM) and/or CH (250 mgl^{-1}) or GA_3 (0.5 µM, 3.0 µM). In one of the treatments, MS was tested with GA_3 (3 µM) alone **(Table 3A.5a)**. On all the treatments, yellowing or abscission of leaves was observed with insufficient elongation of shoots. However, pre-culture of shoots for 12 days on MS + GA_3 (3 µM), followed by their transfer to MS + BAP (0.5 µM) alone or supplemented with CH (250 mg/l) remarkably improved the shoot elongation **(Table 3A.5b).** With this procedure, the best shoot growth was observed on MS + BAP (0.5 µM). On an average, the original shoots attained a length of 4.5 cm, with 4 nodes per shoot, after 6 weeks **(Fig. 6A-C)**. Once the shoots acquired sufficient length, further multiplication was achieved on MS medium supplemented with BAP (1 µM) and CH (250 mg/l). At the end of the passage, each elongated shoot was cut into single nodal segments and planted on MS + BAP (1.0 µM) + CH (250 mg/l). Each node again produced a single, 6 cm long, multimodal shoot, which provided 5-6 cuttings after 6 weeks **(Fig. 6D)**. Thus, 6-fold shoot multiplication could be achieved in 6 weeks on MS + BAP + CH by cutting the solitary shoot into single node segments and culturing them on fresh medium.

For rooting, 3 cm long terminal portions of 6-week-old elongated shoots were cut and transferred to MS medium with full (MS) or quarter (¼ MS) strength of major salts. Both the media were supplemented with 0.5 µM IBA. Remaining part of the shoots were further utilized for multiplication by nodal segment culture. MS basal medium did not support rooting. Direct rooting was attained in 100% cultures, from the basal cut end, on both the media **(Fig. 7A-C).** However, on ¼ MS + IBA (0.5 µM), number of roots per shoot was more and the roots were longer with higher number of laterals per root **(Table 3A.6)**. Again, the root initiation was faster on this medium and occurred within three weeks **(Fig. 7B)** while it was delayed by a week on MS medium containing full strength of major salts **(Fig. 7A).** Thus, routinely, the shoots were rooted on ¼ MS + IBA (0.5 µM).

3A.1.5. Ploidy Analysis

Cytological analysis of callus cells in induction, multiplication and regeneration media revealed that majority of the cells had haploid number of chromosomes (2n=x=12). However, by the time entire plantlets were developed, out of the 10 plants, 40% maintained their haploid status **(Fig. 8)**. The rest of the plants were diploids (2n=2x=24) or aneuploids (2n=2x-2=22) or (2n=2x-1=21) which arose from haploid cells either spontaneously or due to manipulation of the cultures.

Table 3A.4. Effect of three multiplication media on shoot-bud differentiation from calli in the regeneration medium. Growth period: 8 weeks. Control: MS basal medium.

Regeneration treatments (µM)	Multiplication media and their effect on nature and growth of calli			Percentage of cultures showing shoot regeneration
	MS + 2,4-D (0.5µM) (I)	MS + 2,4-D (0.5µM) + kinetin (4.5µM) (II)	MS + 2,4-D (1µM) + kinetin (10µM) (III)	
Control	Nil			Nil
BAP (5.0)	bright green, slightly hard, friable			Nil
BAP (7.5)	bright green, slightly hard, friable			Nil
BAP (10)	bright green, slightly hard, friable			Nil
BAP (15)	bright green, slightly hard, friable			Nil
BAP (3.0) + NAA (0.5)	bright green, very hard, compact			Nil
BAP (0.5) + NAA (3.0)	bright green, moderately hard, compact			Nil
BAP (22.0) + NAA (0.5)	dark green, granular, friable			Nil
BAP (2.2) + NAA (0.05)	dark green, granular, friable	**dark green, granular, friable with numerous shoot buds**	dark green, granular, friable	**98.5 (8.5)***
BAP (2.5) + IAA (5.0) + CH (500 mg/l)	**bright green, with massive growth, moderately hard**	**bright green, with massive growth, moderately hard; callus with shoot buds**	bright green, massive, moderately hard	**27.8 (4.5)***
BAP (9.0) + IAA (5.0) + CH (500 mg/l)	Greenish brown, homogeneous and soft			Nil
Zeatin (2.0) + NAA (3.0)	very light green, hard, nodulated			Nil
Zeatin (2.5) + NAA (0.5)	very light green, hard, nodulated			Nil
TDZ (0.5)	Cream, soft and shiny with many globular structures	Nil	bright green, shiny, soft with massive growth	Nil
TDZ (1.0)	Cream, soft and shiny with many globular structures	Nil	bright green, shiny, soft and massive	Nil
TDZ (2.0)	Cream, soft and shiny with many globular structures	Nil	bright green, shiny, soft with massive growth	Nil
TDZ (0.5) + NAA (0.5)	bright green, massive, soft and friable			Nil
TDZ (1.0) + NAA (0.5)	bright green, massive, soft and friable			Nil
TDZ (2.0) + NAA (0.5)	bright green, massive, soft and friable			
TDZ (5.0)	Fluorescent green, moderately friable numerous small shiny nodulated structures			Nil

* Values in parentheses represent average number of shoot buds formed per explants.

Table 3A.5a. Effect of BAP, NAA, GA_3 and CH in various combinations on elongation and nature of shoots in culture. Growth period: 6 weeks. Control: MS basal medium.

Medium used	Nature of shoots	Effect on length (cm)
Control	yellow-brown	0.5
BAP (0.5 µM)	yellowish-green	≤ 2.0
BAP (0.5 µM) + CH (250 mg/l)	bright green	≤ 2.0
BAP (1.0 µM) + CH (250 mg/l)	yellow	< 2.0
BAP (0.5 µM) + NAA (0.05 µM)	yellowing within 2 days of culture	no effect
BAP (1.0 µM) + NAA (0.05 µM)	yellowing within 2 days of culture	no effect
BAP (0.5 µM) + NAA (0.05 µM) + CH (250 mg/l)	white colored within a week of culture	no effect
BAP (0.5 µM) + GA_3 (0.5 µM)	yellowish-green	< 0.7
BAP (0.5 µM) + NAA (0.05 µM) + GA_3 (3 µM)	yellow droopy shoots on prolonged exposure	< 2.5
GA_3 (3 µM)	yellow droopy shoots on prolonged exposure	≤ 2.5

Table 3A.5b. Effect of 12-day long GA_3 pre-treatment on elongation of shoots. Growth period: 6 weeks. Control: MS basal medium.

Medium	Length of Shoot (cm)	No. of nodes per shoot	Nature of shoots
Control	0.5±0.1	Nil	yellow-brown
BAP (0.5 µM)	4.5±0.2	4.0±0.1	healthy, bright-green shoots
BAP (0.5 µM) + CH (250 mg/l)	2.8±0.3	2.1±0.1	Bright-green shoots

Values are represented as Mean ± SE

Table 3A.6. Rooting response of shoots on MS and ¼ MS media supplemented with IBA at 0.5 µM. Growth Period: 4 weeks. Control: MS basal medium.

Parameters	Control	MS + IBA (0.5 µM)	¼ MS + IBA (0.5 µM)
Percentage of rooted shoots	0	100 ± 0.0	100 ± 0.0
No. of roots per shoot	0	3.0 ± 0.5	10 ± 0.8
No. of laterals	0	0	15 ± 0.4
Length of longest and smallest root (cm)	0	1.0 and 0.4	7.0 and 2.2
Length of laterals (cm)	0	0	0.5 ± 0.2

Values are represented as Mean ± SE

3A.2. Analysis of Azadirachtin Production in Anther Callus Lines

3A.2.1. Detection and Estimation of Azadirachtin

i. Standard Curve Analysis of Azadirachtin

With the protocol adopted, azadirachtin eluted at 6.39 min **(Fig. 9)**. Calibration curve for azadirachtin standard showed good linearity at tested concentrations (0.0625 mg/ml to 1 mg/ml) with a correlation coefficient (R^2) of 0.9889. The equation generated from the curve by external standard method **(Table 3A.7)** was used to calculate amount of compounds present in the crude sample. The precision of the developed method, as mentioned in section 2A.2.2.iii, was evaluated by measuring intra- and inter-day variability in terms of relative standard deviation. The standard sample, at same concentration, was analyzed at least five times within the same day (intraday) and the RSD value obtained was 3.99%. Similarly, for interday variability, same concentration of the standard compound was run thrice at one day interval and the value obtained was 3.78% **(Table 3A.7)**.

ii. Identification and Quantification of Azadirachtin by HPLC

From the standard equation obtained, the amount of azadirachtin in different callus lines was calculated and listed in **Table 3A.8**. Presence of azadirachtin was confirmed in all the callus lines tested. However, it was observed that redifferentiated calli on MS + BAP (2.2 µM) + NAA (0.05 µM), possessed highest azadirachtin content (728.41 µg/g DW) while the least was obtained from dedifferentiated line, maintained on MS + 2,4-D (1 µM) + kinetin (10 µM) (49 µg/g DW). *In vitro* grown haploid leaves contained 700 µg/g DW of azadirachtin **(Table 3A.8)**.

Table 3A.7: Standard curve analysis of azadirachtin.

Compound	Retention time (min)	Standard equation	R^2	(% RSD)	
				Intraday	Interday
Azadirachtin	6.39 ± 0.6	y=132.55x + 41.4	0.9889	3.99	3.78

Table 3A.8: Evaluation of callus and leaf samples of Neem for azadirachtin production.

Material	Medium	Amount of azadirachtin (µg/g DW)
Dedifferentiated callus	MS + 2,4-D (0.5 µM)	288 ± 0.32
Dedifferentiated callus	MS + 2,4-D (0.5 µM) + Kinetin (4.5 µM)	79 ± 0.3
Dedifferentiated callus	MS + 2,4-D (1 µM) + Kinetin (10 µM)	49 ± 0.2
Redifferentiated callus	MS + BAP (2.5 µM) + IAA (5 µM) + CH + 500 mg/l)	411.38 ± 0.3
Redifferentiated callus	MS + BAP (2.2 µM) + NAA (0.05 µM)	728.41 ± 0.4
In vitro leaves (haploids)	BAP (0.5 µM)	700 ± 0.52
Control I (Leaves parent plant)	---	5435.6 ± 4.0
Control II (Seeds parent plant)	---	6800 ± 4.1

iii. Analysis of Azadirachtin by Mass Spectrometry

The fraction of crude extract eluted from HPLC, was collected, analysed by mass spectrometry and the fragment characteristics were compared with that of standard azadirachtin procured from Sigma, Aldrich. Spectra were obtained in full scan mode. Base peak of m/z 685 resulted due to loss of two water molecules [MH^+-$2H_2O$], m/z 703 formed due to the loss of one water molecule [MH^+-H_2O] and m/z 743 correspond to the formation of sodium adduct [MH^++Na^+]. Similar m/z fragments in standard compound and HPLC fraction confirmed the presence of azadirachtin **(Fig. 10)**.

3A.3. Antimicrobial Assays

In the present study, the extract derived from *in vitro* raised androgenic callus lines did not show any activity with most of the tested strains, except some strains of *Staphylococcus aureus*. Results are presented in **Table 3A.9.**

Table 3A.9: Antibacterial activity of anther culture lines of *Azadirachta indica* against some pathogenic strains.

S.No.	Strains	Zone of inhibition (cm)
1.	*Staphylococcus aureus* ATCC **25923** *S. aureus* 4079, 4100, 4205, 4354, 4107	**0.9**
2.	*E. coli* MTCC 324 *E. coli* 4350, 4342, 4376, X, Y	--
3.	*Klebsilla* 4320, 4317, 4328, 4318, 4295	--
4.	*Cons* 4322, 4331, 4369, 4360, 4227	--

3B. *LANTANA CAMARA* L.

3B.1. Leaf Culture

3B.1.1. Culture Media

Establishing cell and tissue cultures of *Lantana* is a difficult task to accomplish because of the interference posed by phenolic compounds. In the present study, leaf-disc explants of 5 mm size were cultured on a range of media. The treatments involved two basal media like MS, B_5, additives like CH and varying concentrations and combinations of cytokinins and auxins like BAP, kinetin, 2iP, NAA, IAA, 2,4-D and organics of B_5 and White's media. **(Table 3B.1).**

Most of the media combinations tested for leaf-disc dedifferentiation, resulted in browning and death of the explants soon after inoculation. On few combinations, like, MS + BAP (10 µM) + NAA (5 µM) and MS + 2,4-D (1 µM) + NAA (1 µM) + BAP (5 µM) + CH (250-500 mg/l), cultures responded initially for callusing but did not survive after the first subculture. The best treatment for callusing, in terms of number of explants showing callusing and the degree of callusing, was the combination of MS + 2,4-D (1 µM) + NAA (1 µM) + BAP (5 µM). On the responding medium, 100% of the explants callused, and the callus growth was profuse after the first subculture. On this medium, the leaf-disc explants first turned brown but after a week, bright-green, hard, compact calli started developing from the margins of the leaf-disc **(Fig. 11A,B)**. These compact calli were dissected out and subcultured on the fresh medium of the same growth regulator composition. The rate and degree of

callus proliferation increased with the subsequent subcultures, the nature of callus did not improve substantially. The callus was friable and soft but remained deep brown in nature **(Fig. 11C,D)**. Until 10th subculture, the cells in the callus were a mixture of green and brown cells **(Fig. 11E)**. It took about 26 weeks of regular subculturing, at 4-week intervals, to obtain profusely growing fresh, friable, granulated and cream callus. **(Fig. 11F)**. In another attempt to screen the callus lines, the green and brown portions of the calli were cultured separately. Surprisingly, it was observed that both the cell types again gave rise to a mixture of green and brown cells. Again, the chemical analysis of the two cell types did not show any difference with respect to the triterpenoid profiles.

Table 3B.1: Different media and growth regulator combinations for dedifferentiation from leaf disc explants of *L. camara*.

S.No.	Treatments
1.	MS + BAP (1 µM)
2.	MS + BAP (2 µM)
3.	MS + BAP (3 µM)
4.	MS + BAP (4 µM)
5.	MS + BAP (5 µM)
6.	MS + IAA (10 µM) + BAP (5 µM)
7.	MS + IAA (5 µM) + BAP (10 µM)
8.	MS + NAA (5 µM) + BAP (10 µM)
9.	MS + NAA (1 µM) + BAP (5 µM)
10.	MS + 2,4-D (1 µM) + NAA (1 µM) + BAP (5 µM)
11.	MS + White's organic + 2,4-D (0.5 µM) + kinetin (1 µM) + 2% sucrose
12.	MS + kinetin (5 µM) + IAA (1 µM)
13.	MS + kinetin (10 µM) + IAA (1µM)
14.	MS + kinetin (5 µM) + BAP (5 µM)
15.	MS + kinetin (10 µM) + BAP (10 µM)
16.	MS + 2,4-D (1 µM) + NAA (1 µM) + BAP (5 µM) + CH (250 mg/l)
17.	MS + 2,4-D (1 µM) + NAA (1 µM) + BAP (5 µM) + CH (500 mg/l)
18.	B_5 medium + 2,4-D (1 µM) + NAA (1 µM) + BAP (1 µM)
19.	B_5 medium + 2,4-D (1 µM) + NAA (1 µM) + BAP (2 µM)
20.	B_5 medium + 2,4-D (1 µM) + NAA (1 µM) + BAP (5 µM)
21.	B_5 medium + BAP (5 µM) + NAA (1 µM)
22.	MS + BAP (2 µM) + 2iP (2 µM)
23.	MS + BAP (5 µM) + 2iP (5 µM)
24.	MS + B_5 vit + 2,4-D (1 µM) + NAA (1 µM) + BAP (1 µM))
25.	MS + B_5 vit + 2,4-D (1 µM) + NAA (1 µM) + BAP (2 µM)
26.	MS + B_5 vit + 2,4-D (1 µM) + NAA (1 µM) + BAP (5 µM)
27.	MS + B_5 vit + BAP (2 µM) + NAA (1 µM)
28.	MS + B_5 vit + BAP (2 µM) + NAA (1 µM) + 2iP (2 µM)

3B.2. Analysis of Pentacyclic Triterpenoids in Cell Cultures

3B.2.1. Detection and Estimation of BA, OA and UA

i. Thin Layer Chromatography

By following the protocol as described in the section 2B.2.2.iv.a, the calli (50 g dry cell mass) proliferated on leaf-disc explants of *L. camara* yielded 0.17% of the organic (ethyl acetate) extract and 0.23% of aqueous extract. Qualitative analysis of the organic extract by TLC revealed the presence of three anti-cancerous acid triterpenoids: BA, OA and UA. The compounds were identified by comparing their retention factors (R_f value) with those of authentic standards. BA, OA and UA produced a spot with R_f value of 0.51, 0.28 and 0.17, respectively **(Fig. 12A-C)**.

ii. High Performance Liquid Chromatography

Quantitative estimation by HPLC of the cell culture derived organic extract further demonstrated that all the three triterpenoids BA, OA and UA were produced and accumulated, simultaneously, in the same cultures. On comparison with the authentic standards, it was revealed that the three compounds were eluted at the retention time of 10.8, 12.61 and 13.2 mins, respectively **(Fig. 13A-C)**. When leaves from field growing plant were extracted and analysed, they were found to contain only OA and UA while BA was altogether absent.

(a) Method Optimization for Separation of Acid Triterpenes

The detection wavelength of 209 nm was selected by checking the absorption maxima of the standard compounds, dissolved in ethanol, by a UV-Visible spectrophotometer (Cary, USA). In a preliminary study, methanol was used as one of the mobile phases in HPLC. As the absorption of methanol was higher, high interference was observed especially at a short wavelength of 209 nm. Moreover, as OA and UA are structural isomers, their separation needed a low absorbing solvent that doesn't interfere with the resolution process. Hence, acetonitrile was chosen for the purpose. Acetonitrile and water at 80:20 (v/v) ratio was found to be appropriate as the mobile phase for satisfactory separation of the three components, at a flow rate of 1 ml/min. The chromatograms of BA, OA and UA, obtained by this method, are presented in **Fig. 13A-C**. A fairly short acquisition time of less than 15 min was adequate for good separation of all the three triterpenes.

(b) Linearity and Precision

Calibration curves for all the three standards showed good linearity at tested concentrations (0.16 mg/ml to 2.5 mg/ml) with correlation coefficients (R^2) of 0.9866, 0.9892 and 0.9914 for BA, OA and UA, respectively. The equations generated from the curves by external standard method **(Table 3B.2)** were used to calculate amount of compounds present in the crude sample. Presence of compounds in the crude extract was reconfirmed with the use of internal standards by co-injecting all three together in HPLC **(Fig. 13C)** and then one by one, with the extract **(Fig. 14A-C)**. A very distinct and clear separation of three acids can be observed. The precision of the developed method, as already mentioned in chromatographic conditions of materials and methods section, was evaluated by measuring intra- and inter-day variability in terms of relative standard deviation. The standard sample, at same concentration, was analyzed at least four times within the same day and the RSD values obtained were 1.19%, 0.6% and 0.8% for BA, OA and UA, respectively. Similarly, for inter-day variability, same concentration of the three standards was run at least twice at one day interval and the RSD values for the same figured out to be 1.37%, 0.55% and 0.65% for BA, OA and UA, respectively **(Table 3B.3)**.

(c) Yield and Recovery studies

From the standard equations, the amount of BA, OA and UA in the cell extract were calculated and listed in **Table 3B.3**. The method provides an excellent yield of 3.1%, 1.88% and 4.12% of BA, OA and UA, respectively, in cell cultures of *L. camara*. When leaves from field grown plant (control) were extracted and analysed, they exhibited only marginal increase of OA (ca. 2%) and UA (4.5%), whereas, BA was altogether absent **(Fig. 15)**. The recovery experiments for all the three compounds were performed by adding known amount of BA, OA, UA standards to the cells, which were extracted in a similar manner as described in materials and methods section. The percentage recoveries for all three compounds were observed to be above 95% in all the cases **(Table 3B.3)**.

Table 3B.2: Standard curves and retention times of three triterpenes present in cell cultures of *L. camara*.

Compound	Retention time (min)	Standard equation	R^2
Betulinic acid (BA)	10.80	Y= 51x – 2.4333	0.9866
Oleanolic acid (OA)	12.61	Y= 64.465x – 17.387	0.9892
Ursolic acid (UA)	13.20	Y= 55.734x – 4.5191	0.9914

Table 3B.3: Precision, content and recovery percentages of three triterpenes present in cell cultures of *L. camara*.

Compound	RSD (%)		Content (% per g dry weight)	Recovery (%)
	Intra-day	Inter-day		
Betulinic acid (BA)	1.19	1.37	3.1	96.90
Oleanolic acid (OA)	0.60	0.55	1.88	95.07
Ursolic acid (UA)	0.80	0.65	4.12	97.60

iii. Analysis of Mass Spectra

In the present study, both positive and negative mode electrospray ionization conditions were applied for all the three compounds, but only negative spray ionization mass spectra could be obtained during analysis. **Figure 16A,B** shows the mass spectra of BA standard and sample compound. Since BA has a strong proton donor (carboxyl group) in its structure, the deprotonated ion at *m/z* 455 can be generated more easily than protonated ion. The deprotonated ion peak at *m/z* 455 has been observed for OA and UA also **(Figs. 17A,B and 18A,B)**. The fragmentation pattern was similar for standard and sample compounds, where almost all the fragments present in the standard were present in the sample as well. In the HPLC chromatogram of field grown leaf samples (control), a very prominent peak was observed between 3-4 min **(Fig. 15)**. When this peak was purified and analyzed on a mass spectrometer, it was confirmed to be of porphyrin, which forms the core of chlorophyll molecule in plants. The mass spectrum of porphyrin is given in **Fig. 19**.

3B.2.2. Analysis of Batch Cultures Raised in Shake Flasks

i. Kinetics of Cell Growth and Nutrient Uptake

With the formula mentioned in section 2B.2.4, the specific growth rate (μ) of the suspended cells was found to be 0.1072 d^{-1}. It was observed that the cultures remained in the lag phase till the 2nd day. Biomass increased till the 10^{th} day following which the stationary phase started **(Graph 3B.1)**. The pH of the medium underwent variation during different stages of culture. It was observed that after showing a slight decrease in the value, the pH dropped sharply between 4-6 days, which dropped further after 10 days **(Graph 3B.1)** resulting in poor growth and stationary phase of cells. The drop of pH may be attributed to preferential uptake of NH_4^+ ions which in turn resulted in decreased pH due to liberation of H^+ ions; pH tends to increase if NO_3^- is utilized faster than NH_4^+. Uptake of nitrate was observed at slower rate, in the present study. It was present in the culture medium till the last days of cultivation (16^{th} day). The concomitant synthesis of acid triterpenes in the medium was found to be growth associated and showed an increase with the increase in biomass. Conductivity, as expected, was shown to have an inverse relationship with growth. Among the major inorganic nutrients, it was invariably observed that phosphate was almost completely consumed by the 10^{th} day of culture. Its utilization was very fast in the initial days than in the later stages of growth. Hence, it may be concluded that complete utilization of phosphate and a marked drop in the pH of the culture medium resulted in the onset of stationary phase in cell suspension cultures.

ii. Chromatographic Detection and Effect of Carbon Sources on Triterpenoids

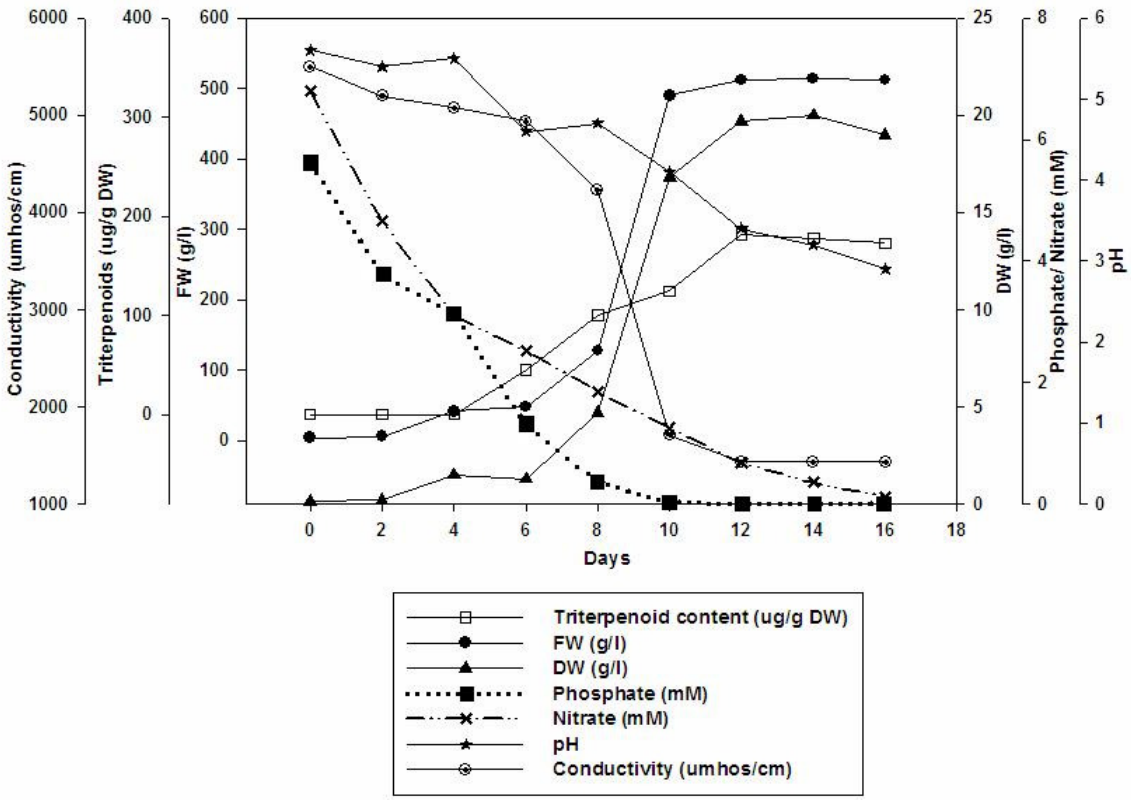
The three acid triterpenes were screened and quantified in the previous sections, from the callus cultures, in the present study. In an extensive study, in this section, where the cells harvested at every alternate day from liquid suspension cultures were extracted and analysed by HPLC. It was revealed that the synthesis of these triterpenoids is concomitant with the log phase of cultures. On comparison with the standard compounds, it was found that BA, OA and UA eluted at 10.8, 12.6 and 13.2 mins, respectively. With the onset of stationary phase, the amount of these acids show a decline in the medium while in the lag phase they were not detectable **(Graph 3B.1)**.

The type of carbon source used in the medium, bears profound effect on quantity of metabolite produced. Maltose gave maximum yield of 31.08 mg/l (293.72 µg/g DW) of triterpenoids, followed by sucrose and glucose with the yield of 21.6 mg/l (183.45 µg/g DW) and 10.69 mg/l (115.21 µg/g DW), respectively **(Graph 3B.2)**. However, it was noteworthy that in maltose and glucose, BA was totally absent and the values represented in the graph were the summation of amounts of OA and UA present in the extract. Sucrose, though second best in terms of amount, favored the production of all the three triterpenoids. However, as far as the growth profile of calli is concerned, among different carbon sources, the maximum biomass was obtained from sucrose (1018.2 g/l FW) and the least from that of glucose (253 g/l FW) after 21 days. The low yield in suspension cultures, as compared to the calli on semi-solid medium, may be due to early onset of stationary phase in the former.

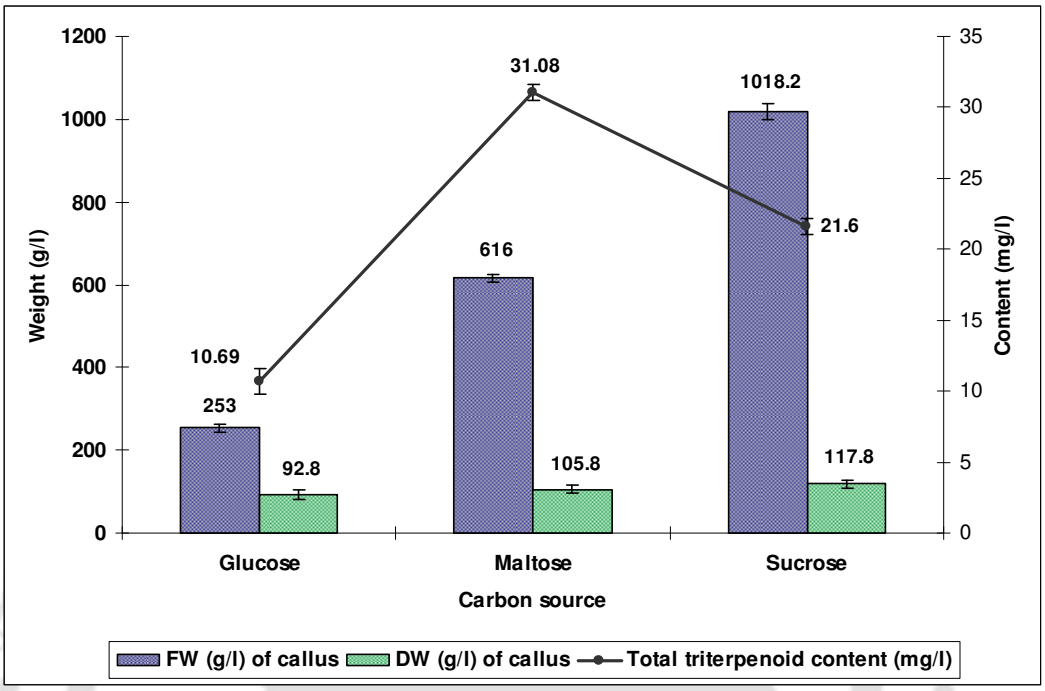
iii. Effect of Agitation Speed on Cell Survival and Viability

Speed of agitation directly affected the growth and viability of cells in suspension cultures due to aeration and shearing effect. The maximum fresh weight (50.91 g) **(Graph 3B.3)** and maximum viability **[Fig. 20B]** was observed at 120 rpm. At the other two rpm, the biomass and viability profile was highly unsatisfactory. At lower agitation (60 rpm), the cells died due to aggregation and clumping; only the cells at the outermost layer of the aggregate were alive and fluorescent green when stained with fluorescein diacetate (FDA) **[Fig. 20A]**. At higher speed (240 rpm), the cells died due to rupturing **[Fig. 20C]**. FDA is a cell permeant dye. Within the cells, the molecule is cleaved by esterase activity to fluorscein which is unable to pass through the cell membrane of live cells while it leaches out from the dead cells. Hence, only the live, intact cells take up the stain and fluoresce green.

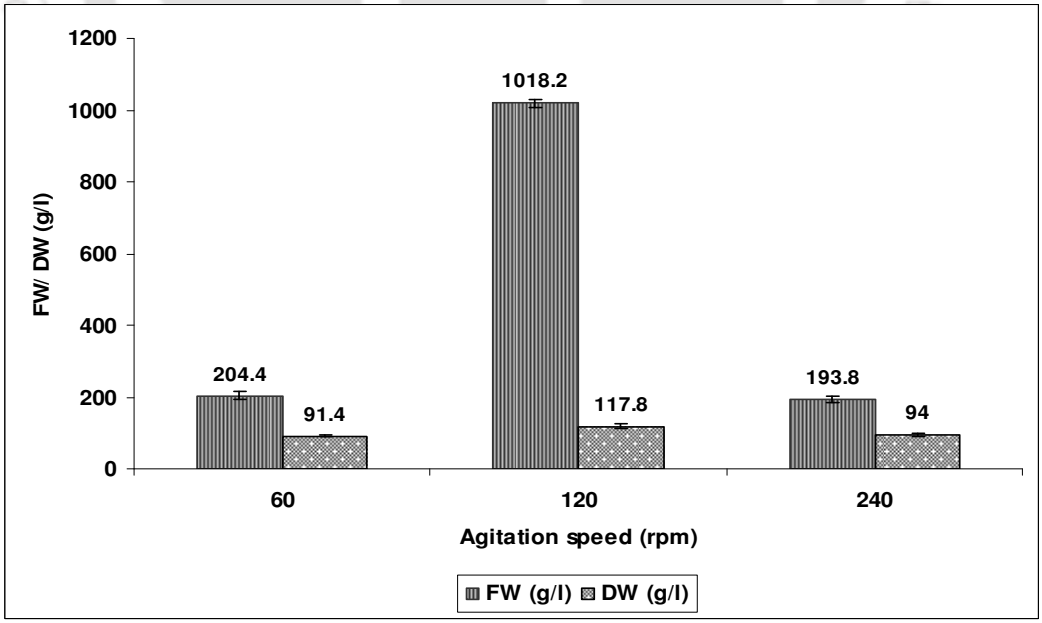
Graph 3B.1: Kinetics of cell growth and nutrient uptake in cell suspension cultures of *L. camara*.



Graph 3B.2: Effect of different carbon sources on cell growth and production profile of two triterpenoids, OA and UA, in cell suspension cultures of *L. camara* after 21 days.



Graph 3B.3: Effect of different agitation speeds on fresh and dry weight of cells in suspension cultures.



3B.3 Cytotoxicity Assay

3B.3.1. MTT Assay

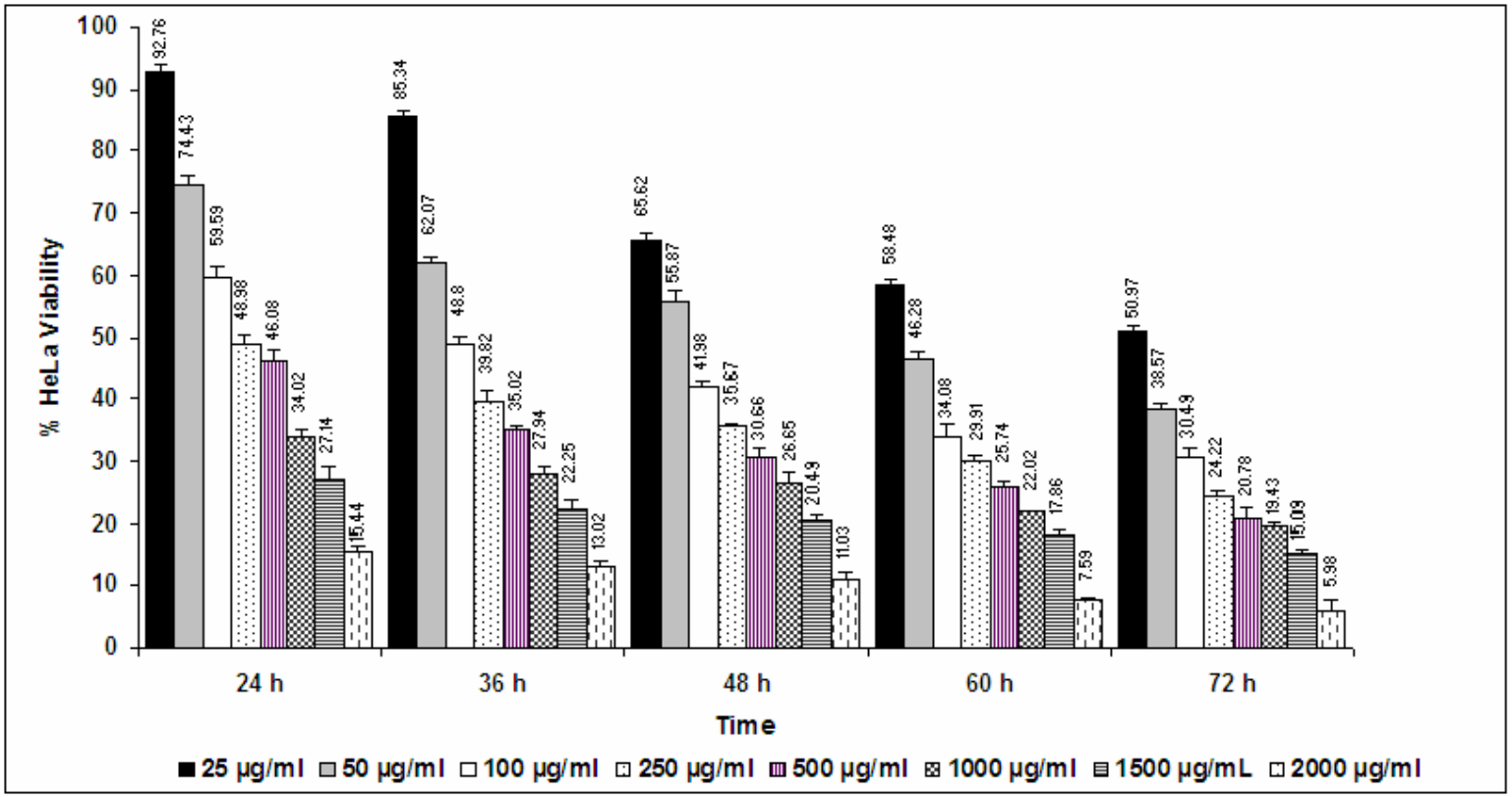
The results of MTT assay of HeLa and BHK-21 cells, treated with the organic extract are shown in **(Graph 3B.4 and 3B.5)**. A noticeable effect of the extract on viability of HeLa cells was observed at concentration range of 25 µg/ml (at 72 h) to 100 µg/ml (at 36 h) **(Graph 3B.4)**, where 50% of the cells were killed. At these concentrations and time periods, the effect on BHK-21 cells was negligible ($p<0.05$ for 24 h to 72 h) **(Graph 3B.5, Table 3B.4)**. In the preliminary experiments, the concentrations of aqueous extract below 1000 µg/ml did not elicit any significant cytotoxic effect and thus, the levels higher than 1000 µg/ml were subsequently used. In this case also, extract had a dose and time dependent activity **(Graph 3B.6)**. After 36 h, aqueous extract levels ranging from 1500-3000 µg/ml showed a significant cytotoxicity against HeLa cells. As the duration of treatment increased, there was a marked decline in percentage survival of HeLa cells **(Graph 3B.6);** however, necrosis was predominant over apoptosis in this case. At a similar concentration range (1500 µg/ml-3000 µg/ml), the effect of the extract on normal BHK-21 cells was negligible ($p<0.05$ for 36-72 h) **(Graph 3B.7).** Curcumin, another plant derived anti-cancer agent, was used as a positive control under same experimental conditions, for both cell types and similar pattern of apoptotic mode of cell death was observed **(Fig. 21)**. Thus, the extracts exhibited selective cytotoxicity for the cancer cells as evident from viability percentages of cells.

Table 3B.4: Results of paired t-tests ($p=0.05^{a}$ and 0.01^{b}) between treatment (organic extract) groups (HeLa and BHK-21) at different time intervals.

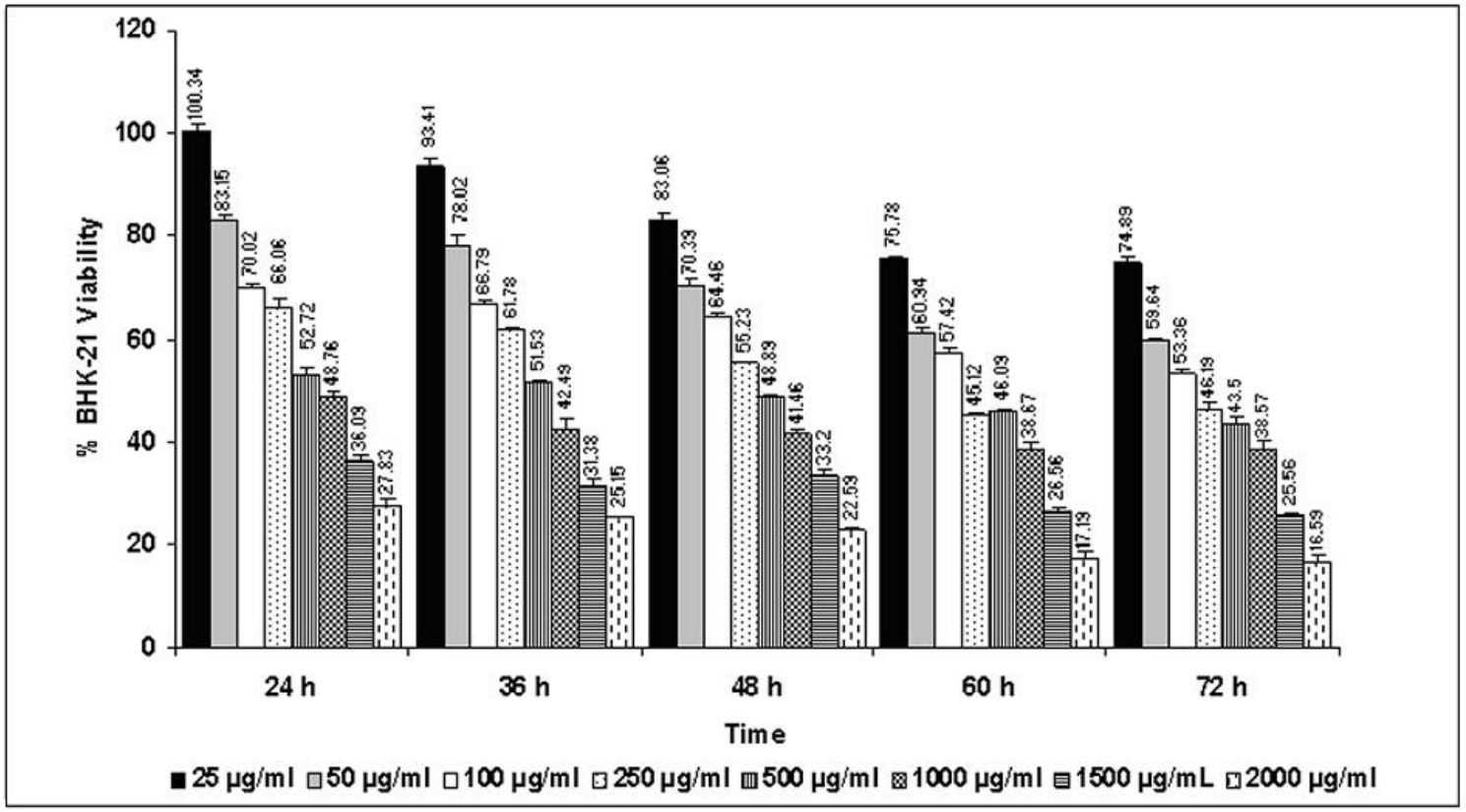
	24 h	**36 h**	**48 h**	**60 h**	**72 h**
t	8.3911	8.8888	12.6363	9.0001	9.8795
df	7	7	7	7	7
SED	1.289	1.635	1.299	1.747	1.933
P value	$< 0.0001^{*a,b}$	$< 0.0001^{*a,b}$	$< 0.0001^{*a,b}$	$< 0.0001^{*a,b}$	$< 0.0001^{*a,b}$

^{*a,b} Significant at both p=0.05 and p=0.01

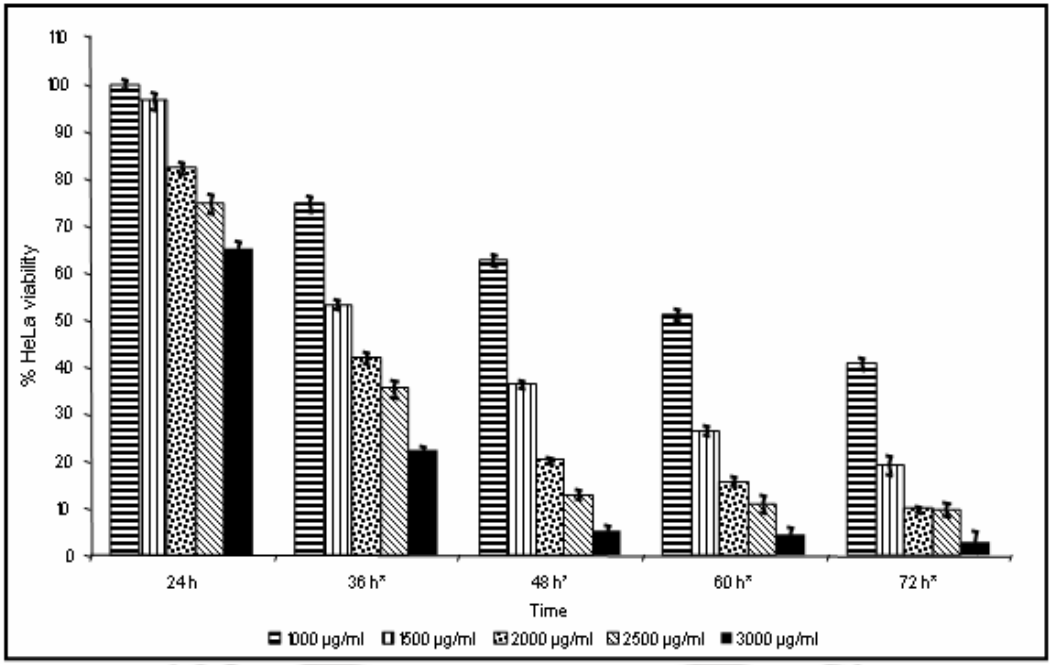
Graph 3B.4: The effect of organic extract on percentage viability of HeLa cells.



Graph 3B.5: The effect of organic extract on percentage viability of BHK-21 cells.

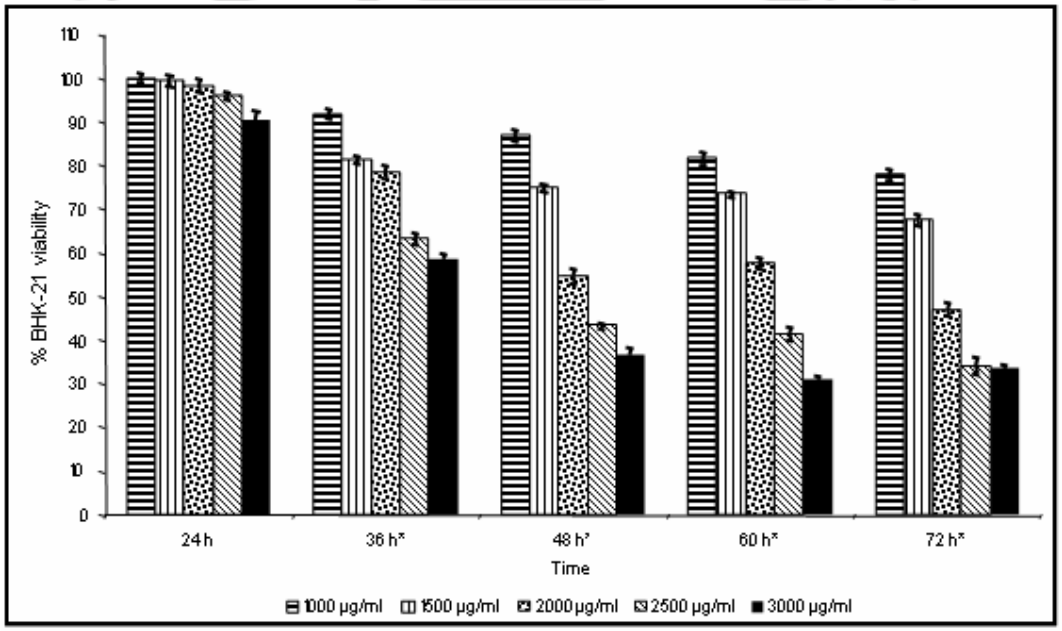


Graph 3B.6: Graphic representation of percentage viability of HeLa cells treated with aqueous extract.



*p< 0.05

Graph 3B.7: Graphic representation of percentage viability of BHK-21 cells treated with aqueous extract.



*p<0.05

3B.3.2. Microscopic Observations of Cell Morphology

Light microscopy revealed that organic and aqueous extracts had an adverse effect on morphology of the cells. A gradual change in the appearance of treated cells was observed in dose and time dependent manner. Most of the dead cells showed characteristic features of apoptosis, viz. reduction in cell volume, cytoplasmic membrane blebbing and formation of apoptotic bodies. Also, there were very few populations of cells which showed the characteristic features of necrotic cell death, viz. increase in cell volume, vacuolization in the cytoplasm and finally dissolution of cytoplasmic membrane leading to the lysis of cells. The morphological features of treated and untreated cells, both HeLa and BHK-21, as observed under inverted light microscope are shown in **Figs. 21 and 22**. From the observations, it was clear that the cells were prone to apoptosis after treatment with the organic and aqueous extracts. Also the pattern of cell death was similar to the one that was induced by curcumin **(Fig. 21E,F and Fig. 22E,F)**, a potent anticancer compound of natural origin, which was taken as positive control.

The morphological pattern of the cell death was further confirmed by CLSM **(Fig. 23 and 24)**, after staining the cells with AO/EB. AO stains both viable and nonviable cells, emits green fluorescence when intercalated into double stranded DNA, and also emits red fluorescence when bound to single stranded RNA. In contrast EB stains only nonviable cells which gets intercalated into DNA and thus, emits red fluorescence. The increase in incubation time of cells with the extract from 24 h to 72 h resulted in decreased number of viable cells **(Fig. 23 and 24)**. The viable cells exhibited bright green nucleus with intact cell structure and the dead cells showed orange to red fluorescence with characteristic features of apoptosis. As the time progressed, the number of viable cells decreased while the number of dead cells increased gradually, as evident from green and red fluorescence. However, this effect was more pronounced with organic extract **(Fig. 23)**.

To further confirm our results, the treated cells were examined under SEM. An overview of treated HeLa cells can be seen in **Fig. 25A**. The cells in the early stages of treatment period were found to exhibit normal morphology, where the cells were spread on the substratum with intact size, shape and cell-cell contacts **(Fig. 25B)**. Subsequent morphological changes include contraction of extra cellular matrix **(Fig. 25C)**, loss of cell-cell contacts **(Fig. 25D)**, detachment of cell from substratum,

membrane blebbing **(Fig. 25E)** and finally the formation of apoptotic body **(Fig. 25F)**. Further, the reduction in the over all cell size, from about 20 µm to about 5 µm, was also evident from the SEM observations.

Thus, the results of light microscopy, CLSM and SEM collectively suggested that there were significant changes in cell morphology of treated cells and showed characteristic features of programmed cell death (apoptosis). A compound's ability to induce apoptosis in tumor cells can be considered as an important criterion to consider it as a potent anticancer compound.

3B.3.3. DNA Fragmentation Assay

DNA laddering was performed to confirm the apoptotic mode of cell death. The gel electrophoresis image is shown in **Fig. 26**, which clearly depicts that the extract caused DNA fragmentation and subsequently leads to the death of proliferating cells. The fragmentation of genomic DNA is a late event during the process of apoptosis. DNA fragmentation is a result of active caspase-3 mediated cleavage of ICAD (Inhibitor of CAD) to activate CAD (caspase-activated deoxyribonuclease), which is responsible for the fragmentation of the DNA. The results obtained in DNA fragmentation analysis conclusively demonstrated the apoptotic activity of the extract.

3B.4. Antimicrobial Assays

A very mild inhibitory activity of the extract against certain pathogenic strains of bacteria was observed in the leaf extract of *Lantana*. Extract derived from *in vitro* raised lines did not show any activity in the disc-diffusion assay. However, in some preliminary experiments by agar streak method, the callus derived extract exhibited activity against *Streptococcus mutans*. Working solutions of 1:1, 1:2, 1:4, 1:8 and 1:16 were obtained by serial dilution 10 mg/ml stock solution of the extract. Maximum inhibition was observed at a dilution of 1:4. Results are presented in **Table 3B.5.**

Table 3B.5: Antibacterial activity of *Lantana* against some pathogenic strains.

S.No.	Strains	Zone of inhibition (cm)
1.	*Staphylococcus aureus* ATCC 25923 *S. aureus* 4079, 4100, 4205, 4354, 4107	0.6
2.	*E.coli* MTCC 324 *E. coli* 4350, 4342, 4376, X, Y	0.7
3.	*Klebsilla* 4320, 4317, 4328, 4318, 4295	--
4.	*Cons* 4322, 4331, 4369, 4360, **4227**	0.7
5.	*Streptococcus mutans*	Activity observed in agar streak method

Chapter 4

Discussion

Medicinal plants are extremely important to human health. According to a recent survey, 93% of the wild medicinal plants, used for making ayurvedic medicines, in the country, are endangered and the government is trying to relocate them from their usual habitat, to protect them (Botanical Survey of India 2010). The officials were of the view that the plant resources in the country are threatened by over exploitation to meet the demand of herbal industries. Apart from this, it is also well known that the conventional methods of plant propagation are very lengthy and time consuming. Moreover, the wild populations are circumvented with the problems of low rate of fruit set, poor seed yield/ germination/ viability, and in most cases the clonal uniformity cannot be maintained through seed due to high heterozygosity in populations. In such situations, there is an urgent need for the conservation of elite germplasms. In this context, the advantages of tissue culture have been widely recognized. Tremendous progress in this technique has been observed in the last few years. As mentioned in the introductory chapter, plant tissue culture comprises of a set of techniques and the choice of technique depends on the results that one wants to retrieve. In plants, where molecule of interest is localized in the seeds, heterozygosity is not a desirable trait. Therefore, production of pure lines would be advantageous and so is the choice of haploid production. In higher plants, the developmental fate of the male gametophyte or its precursors can be experimentally deviated towards haploid androgenic development. By means of *in vitro* procedures, haploid and doubled-haploid plants can be regenerated. This process presents diverse advantages in both basic and applied research (Touraev et al. 2001; Maluszynski et al. 2003), the most important one being related to plant breeding. A doubled-haploid plant constitutes a pure line (100% homozygous), and can be obtained in just one *in vitro* generation. In plants, where leaves are the reservoir of desirable metabolites, establishing *in vitro* cultures from leaves and utilizing them for the extraction of compounds would be

an ideal option. Present report describes a comprehensive evaluation of two medicinal plants, *Azadirachta indica* A. Juss. and *Lantana camara* L. from different perspectives. Androgenic haploids of Neem, obtained in the present study, attach to themselves a lot of importance given to the fact that they are the precursors of homozygous diploid lines which can be a source of uniform quality and yield of important metabolites. In the present study, detection of azadirachtin, an ecofriendly, non-toxic biopesticidal compound, in these haploid lines, further strengthens their position. As for *Lantana*, present study, is an attempt to realize the potential of this plant by biotechnological means, using tissue culture. Leaf disc cultures of *Lantana* were established and examined, for the production of pharmacologically important pentacyclic triterpenoids: Betulinic, Oleanolic and Ursolic acids. These studies, demonstrate the potential of cell cultures as source of important medicinal triterpenoids. Bioassays further strengthen the significance of preceding studies.

4A. *AZADIRACHTA INDICA*

4A.1 Tissue Culture Studies

"Androgenesis", by definition, is the transition of pollen from gametophytic mode of development, normally manifested in pollen-tube growth, to the formation of a sporophyte, which is haploid. Thus, haploids are sporophytic plants carrying gametophytic chromosome number of their parents. Natural occurrence of sporophytic haploids was first described by Dorothy Bergner in the weed species *Datura stramonium* in 1922 (Blakeslee et al. 1922). This was followed by *Nicotiana tabacum* (Clausen and Mann 1924), *Triticum aestivum* (Gains and Aase 1926) and subsequently, in several other plant species (Kimber and Riley 1963). The credit for producing first haploids in laboratory goes to Guha and Maheshwari (1964, 1966) in *Datura,* through anther culture. The number and extent of studies on the induction of androgenesis in trees, however, are limited and results obtained to date are nowhere near those of model crops such as *Brassica* and *Nicotiana*. The major problem encountered is the extreme recalcitrance of tree species in culture. In the past decades, induction of callus from cultured anthers and the production of haploid plants from calli have been reported by researchers only in 16

tree species belonging to 12 families. The most effective and popular technique to obtain haploids is by *in vitro* anther or microspore culture. Anther culture has been successfully applied to many plant species to produce haploids; its single biggest advantage is its simplicity. Large-scale anther culture operations can overcome the limitation of low per-anther yield. However, the presence of extraneous tissues (eg. the anther wall) makes this a messy system for cell biology and other precise studies (Forster et al. 2007). Isolated microspore culture in contrast to anther culture, has several important advantages (Bonga et al. 1997; Radojevic et al. 2002). By removing the anther wall, proliferation of diploid sporophytic tissues are avoided. More importantly, homogeneous population of microspores at the developmental stage, most suitable for androgenesis can be obtained and studied using cell biology, microbiology and functional genomic techniques (Touraev et al. 2001). In addition, as the development of microspores is independent of the sporophytic tissues, the media components and culture treatments have direct access to the microspores. Thus, under optimal conditions, more microspores can be induced to form sporophyte. Inspite of these positive attributes, microspore culture cannot yet compete with anther culture for double haploid production, especially in tree species, but might be used to understand the mechanism on gametophytic to sporophytic transition in pollen, for transformation and for *in vitro* selection systems (Kasha 1989; Tuvesson and Ohland 1992). So far, there is only a single woody species where androgenic haploids have been raised successfully by isolated microspore culture (Höfer et al. 1999; Höfer 2004). In this regard, Pelletier and Ilami (1972) introduced the concept of "Wall Factor", according to which the somatic tissues of the anther play an important role in the induction of sporophytic divisions in pollen. Factors that affect androgenesis have been described elaborately by Atanassov et al. (1995) and Bhojwani and Razdan (1996). It has been observed that physiological status of the donor plant, genotype, environment, developmental stage of microspores at culture, type and concentration carbon source, culture conditions and media, play a pivotal role in induction of androgenesis. Anthers from the first flush of flowers are generally more responsive (Shtereva et al. 1998).

Present study describes androgenesis in Neem trees, growing near the campus of Indian Institute of Technology, Guwahati, India. The first and only report, till date, on this aspect came from Chaturvedi et al. (2003b) from the tree growing at Delhi, India.

The authors reported an efficient protocol for haploid plant production from a 50-year-old Neem tree but no studies related to biochemical characterization of the haploids was taken up. Interactions between the genotype and prevalent environment in any given area greatly influence the morphometric parameters as well as nutritional requirements of trees in natural as well as in *in vitro* conditions (Bhojwani and Razdan 1996; Pattnaik et al. 2006). Guwahati has a mild, humid climate with an annual mean temperature of 24°C and an annual rainfall of 1800 mm while New Delhi has an annual mean temperature of 25°C and an annual rainfall of 714 mm. In April, when anther cultures are established, the average temperature at Guwahati is 30.5°C (max.), 20°C (min.) and average rainfall is 150 mm. In the same month, the average temperature at Delhi is 40°C (max.), 30°C (min.) and an average rainfall is 10 mm for New Delhi. This may account for the different nutritional requirements of anthers, from the two places, under *in vitro* conditions to induce androgenesis.

The high sugar concentration during induction phase is critical as it tends to suppress the divisions of somatic cells, thereby, selectively promoting microspore callusing/ embryogenesis (Clapgham 1973; Wang et al. 1974; Sopory et al. 1978). The later stages grow better at lower sucrose concentrations (2% to 4%) (Sopory 1979). The favorable effect of sucrose was also seen in the present study, throughout the experiments. This could be because the sucrose is easily hydrolyzed by cell wall bound invertase into more efficiently utilizable form of sugars, glucose and fructose, which are incorporated into the cells. Cells exhibit a preference for glucose whereas fructose is utilized as a substrate only after glucose is depleted from the medium (Kretzschmar et al. 2007). Thus, the presence of sucrose facilitated the constant availability of utilizable forms of carbohydrates, in the present study, throughout the long culture period of 8 weeks in the callus induction medium. Again the positive effect of sucrose may be attributed to its contribution in increasing the osmotic pressure of the medium, which stimulates mitochondrial activity and, hence, production of high amount of energy (Bonga and Voan Aderkas 1992) which may be the cause to induce divisions from microspores, in the present study.

Chaturvedi et al. (2003b) emphasized on the requirement of high sucrose (9%) concentration during establishment phase of Neem anther cultures. This was found to be

true in the present investigation as well, as the best callus induction response (100%) was obtained in 12% sucrose. However, beyond a certain level, as in the present study, sucrose above 12% showed a decline in percentage of responding anthers. This reduction can be caused by an excessive osmotic contribution or by toxicity of the carbohydrate due to high degree of hydrolysis (Neto and Otoni 2003). Sucrose being the best carbon source, is also the main sugar translocated in the phloem of many plants (Giaquinta 1980; Strickland et al. 1987). Starch is produced from sucrose supplied in the culture medium (Jasik and De Klerk 1997). It accumulates in the target cells just before regeneration and may be a carbohydrate reserve during meristem formation. Overall, organ initiation is associated with the utilization of accumulated starch and free sugars of the medium (Thompson and Thorpe, 1987; Bahmani et al. 2009). This may explain the requirement of sucrose, though at lower concentration of 3% even in the callus multiplication and regeneration medium, in the present case as well.

It has been postulated that androgenic induction is only possible with immature anthers , containing immature pollens at early-to-late uninucleate stages of development ie. just before or just after pollen mitosis. In fact, the stage of microspore development at which the anthers are cultured is more crucial than the composition of the nutrient medium. During this phase of development, the microspores are non-committal in their developmental potential, as most of the sporophyte-specific gene products are eliminatd from the cytoplasm before meiosis (Porter et al. 1984), and the gametophyte specific genes are generally transcribed only after first pollen mitosis (Scott et al. 1991). After the first mitosis, the cytoplasm gets populated with gametophytic information and it gradually becomes irreversibly programmed, to form the male gametophyte (Mascarenhas 1971). The switch between the two modes can be amplified by different abiotic stress (Barret et al., 2004), like heat in rapeseed (Lichter 1982) and cold in corn (Brettel et al. 1981) and might be related to mechanisms described in somatic embryogenesis (Hecht et al. 2001). Stress, in the form of pre-treatment, can be applied at various levels of explants, such as intact flowers and isolated anthers or microspores at culture. In the present study, the anthers were cultured at early-to-late uninucleate stage of microspores. The best response was observed in control at 25°C temperature followed

by one day cold pre-treatments to anthers, at 12°C and 4°C temperatures. Heat pre-treatment was entirely unfavorable.

Promotory effects of temperature pre-treatments have been observed in many tree species. Rao and De (1987) reported proliferation of haploid calli, embryos and plantlets from anthers, cultured at mid-late uninucleate stages of microspores, in *Peltophorum* after pre-treating the flower buds at 14°C for 8 days. In *Populus*, anthers at uninucleate stage of microspores, taken from cold pre-treated flower buds (at 4°C for 4 days), produced. globular calli after 4-8 weeks of dark incubation at 20°C on MS + 2.4-D (2.26 µM) and kinetin (0.46 µM) (Stoehr and Zsuffa 1990; Kiss et al. 2001). Hidano et al. (1995) observed activated charcoal and cold pretreatment to be favorable for embryogenesis of *M. pumila*. Jain et al. (1996), who evaluated the effect of various physical and chemical pre-treatments on induction of haploid plants in anther cultures of *Morus alba*, revealed that cold pre-treatment to flower buds at 4°C for 24 h, increased the percentage of anther cultures showing callus induction from microspores (9.20%). Germanà and Chiancone (2003) proposed an improved and detailed protocol for haploid induction through anther culture of *C. clementina* Hort. Ex Tan. cv Nules by evaluating a number of factors that affect androgenesis. They observed that temperature pretreatment of flower buds at 4°C and 25°C, for 14 days, were very favorable to induce embryogenic callus and embryoids in anther cultures. Rimberia et al. (2005) investigated the positive effect of high temperature pretreatment to anthers on induction of embryos from microspores in *Carica papaya*.

Pre-treatment to whole anthers results in changes in cell wall properties of anther tissues (Wang et al. 2000). It is, generally associated with rapid loss of chlorophyll from the middle layer, degeneration of tapetal cells and finally damaged innermost layer of locules. Cold treatment kills weak or non-viable anthers and microspores, while arresting many of the viable microspores in the first mitosis stage due to blockage of starch production. Microspore and tapetal development then become asynchronous, influencing the switch from the gametophytic to the sporophytic phase (Pickering & Devaux 1992; Foroughi-Wehr & Wenzel 1993). Heat pre-treatment is associated with the appearance of small heat-shock proteins (smHSP) before induction of androgenesis (Pechan 1991; Smykal and Pechan 2000). Generally, larger the temperature difference between the

donor plant growth conditions and *in vitro* culture conditions, stronger is the smHSP signal. However, no smHSP is produced below 25°C.

The salt composition of media has been found to have negligible effect on frequency of initial divisions, a moderate effect on plating efficiency, but a dramatic effect on embryogenesis and plant regeneration (Mordhorst & Lorz 1993). In the present study, out of the three basal media tested, best response in terms of callusing of microspores was obtained in MS medium and no response was observed on B_5 medium due to low salt concentrations. NLN medium which is almost similar in composition to MS medium but lacks in NH_4^+ source and contains free amino acids, was second best in the study. However, on NLN medium, substantial callusing was observed from anther walls. Various pre-treatments enhance the embryogenic ability of microspores, although this is affected mainly by the endogeneous hormone level of the donor plant tissues (Atanassov et al. 1995). The type and concentration of plant growth regulators in media have been recognized as one of the critical factors in the development of plantlets in anther cultures (Reynolds 1987; Tuvesson et al. 1989; Ball et al., 1993; Chaturvedi et al. 2003b). Basal medium alone did not show any response, at any stage of culture during this study. Mixture of two auxins and one cytokinin; MS + 2,4-D (1 µM) + NAA (1 µM) + BAP (5 µM), was suitable for initial callus induction and selective division from haploid microspores. For maintenance of callus, one auxin and one cytokinin were sufficient; MS + 2,4-D (0.5 µM) + kinetin (4.5 µM) served as the best source, as it gave maximum percentage of regenerating cultures. This interaction of auxin and cytokinin in the induction and multiplication medium bore a profound effect on shoot organogenesis in the regeneration medium. In the previous report by Chaturvedi et al. (2003b), regeneration medium consisted only of a cytokinin (BAP), whereas in the present case, it had to be compounded with an auxin (NAA). However, it is noteworthy that maximum shoot regeneration frequency in their case was 75%, with cultures forming an average of 4.5 shoot-buds/ explant on MS + BAP (5 µM), whereas in this case, it is 98.5% with an average of 8.5 shoot-buds/ explant on MS + BAP (2.2 µM) + NAA (0.05 µM). Conclusively, a right mix of auxin and cytokinin is essential for callus and shoot induction in Neem. In the present study, shoot elongation required a pretreatment of GA_3 (3 µM) before being transferred to MS + BAP (0.5 µM). Rooting of the shoots was

achieved on ¼ MS + IBA (0.5 µM). These results, established once again the conventional roles of auxins and cytokinins. A 6-fold shoot multiplication could be achieved every 6 weeks on MS + BAP (1 µM) + CH (250 mg/l). Ploidy analysis of the in vitro regenerated shoots revealed that 40% of the plants were haploids while the rest were either diploids or aneuploids. It has been established that haploid calli have a tendency to increase in ploidy level and diploidization proceeds at a higher rate than tetraploidization (Zagorska et al. 2004). It was observed, in the present study, that majority of the cells maintained their haploid status for a long period but gradually, by the time of plantlet formation, this frequency reduced. It may be attributed to the unstability of cells in haploid state and tendency to undergo autodiploidization through endomitosis to regain their normal status. As a result, the frequency of diploid cells in culture increases with age (Sacristan 1971; Bhojwani and Razdan 1996).

4A.2 Secondary Metabolite Analysis

Production of secondary metabolites from plant tissue culture has emerged as a promising and feasible option attracting the attention of scientists worldwide. The entire exercise becomes obligatory if we aim at metabolites from an out-breeding tree species, due to the variability inflicted upon by heterozygyosity in the genus. In Neem, numerous reports have been published as of now that establish production of azadirachtin and related limonoids under *in vitro* system. However, as is the case with the metabolite content under in situ conditions, in context to variability, the same limitation is posed if cultures are established from seed or other somatic tissues. No reports, till date have attempted to study the production pattern of this compound in haploid lines. Haploid lines can fix the genetic variability as they are the raw materials for production of doubled haploid pure lines. With this they can also help in conserving the elite germplasm.

Apart from androgenesis, the focal point of this report lies in the assessment of haploid (n) cell lines and their derivatives as source of azadirachtin. Such kind of investigation has been carried out for the first time in Neem. Till date, the available reports on azadirachtin production, have utilized cultures from different somatic parts (2n) like leaves (Kuruvilla et al. 1999; Allan et al. 1994; Kearney et al. 1994; Sundaram et al. 1996; Wewetzer 1998; Veeresham et al. 1998; Prakash et al. 2005), bark (Sanyal et

al. 1981; Sarkar and Datta 1986; Bajagopal and Ramaswamy 1996; Wewetzer 1998), embryo (Srividya et al. 1998), stem (Sundaram et al. 1996), seeds (Prakash et al. 2005), cotyledons (Sanyal et al. 1988), shoot apex (van der Esch et al. 1993; 1994a, b), shoot tip (Schaaf et al. 2000) and flowers (Veeresham et al. 1998). While in leaf derived callus the values ranged from 4 µg/g to 64 µg/g DW in different reports; bark, embryo, and stem cultures yielded 44 µg/g DW, 4-8 µg/g DW and 2.7 µg/g DW azadirachtin, respectively. Veeresham et al. (1998) reported an accumulation of high amount of azadirachtin in leaves (0.0268 g/g DW) and flowers (0.0246 g/g DW). In the present study, the reported azadirachtin values are from the cells that the haploid genome content (n). The highest yield (728.41 µg/g DW) obtained, are comparable to the highest azadirachtin yielding seed-derived cell line (2n) (1890 µg/g DW), an organ which is the main storehouse of this metabolite (Prakash et al. 2005).

Although, numerous reports are available on the isolation of this compound (Butterworth and Morgan 1968; Yamaski et al. 1986; Turner et al. 1987; Butterworth and Morgan 1971). The isolation of azadirachtin in a pure state remained a tedious and time-consuming affair involving repeated partioning between solvents and extensive chromatography. However, time to time different strategies by different authors have been reported. Almost all these reports are based on solvent extraction, flash chromatography, supercritical fluid chromatography and/ or reversed phase high performance liquid chromatography. Colorimetric determination of azadirachtin related limonoids using acidified vanillin solution was reported by Dai et al. 1999. Later, in the year 2001, they identified a multivariate calibration technique for the estimation of azadirachtin related limonoids as well as the simple terpenoids in different parts of the Neem tree. In the present study, a very simple ultrasonication based protocol for azadirachtin extraction, has been adopted. The cells were sonicated in methanol followed by partitioning in dichloromethane and water. The azadirachtin rich dichloromethane fraction was further used for analysis.

Significant progresses have been made by different workers in optimizing conditions for the production of azadirachtin on a large scale in bioreactors (Raval et al. 2003; Prakash and Srivastava 2005, 2006, 2007, 2008). Raval et al. (2003) studied the effect of major nutrients of MS medium on growth and production of azadirachtin-related

limonoids in plant cell culture of neem with an aim to enhance their yield. While maximum biomass was obtained on MS medium, maximum production of azadirachtin related limonoids was observed on White's medium. Their results indicated the non-growth associated production characteristic of azadirachtin related compounds. Two years later, Prakash and Srivastava (2005) came out with statistically optimized media for cell growth and production of azadirachtin in suspension cultures, raised from Neem seed kernels. A maximum of 15.02 g/l biomass and 2.98 mg/g azadirachtin was produced using optimum nutrient concentrations. The studies were extended further to establish the kinetics of cell growth/ azadirachtin formation and substrate consumption of *A. indica* suspension culture in low shear steric impeller bioreactor with statistically optimized conditions. The culture marked a growth of 15.5 and 0.05 g/l azadirachtin within 10 days of cultivation, thereafter azadirachtin concentration showed a decline (Prakash and Srivastava 2006). Further, Prakash and Srivastava (2007) compared azadirachtin production in stirred tank bioreactor with two different impellers. The maximum cell mass (18.7 g/l) and azadirachtin yields (0.071 g/l) were observed with centrifugal impeller, as compared to steric impeller bioreactor. Prakash and Srivastava (2008) studied the role of elicitors like salicylic acid, chitosan, jasmonic acid, methyl jasmonate and yeast extract, at different concentrations, in shake flask suspension culture of *A. indica*. Chitosan, salicylic acid and jasmonic acid stimulated an increase in azadirachtin content, which ranged from 2 to 3-fold greater than the control. The combined effect of these elicitors on azadirachtin content studied by Response Surface Methodology resulted in 5-fold higher azadirachtin production (15.9 mg/g DW) due to synergistic effect of these elicitors. Material used by them is the callus derived from high azadirachtin producing seeds. Variability in content due to heterozygosity is the major concern in such cases where somatic parts are utilized. Haploid plants, obtained in the present study, can be a constant source of homogeneous quality and quantity of important metabolite like azadirachtin. The present study forms a backdrop for further studies involving improvement in constant yield of azadirachtin from haploid lines. Recently, Rafiq and Dahot (2010) analysed calli obtained from various Neem explants. Calli from immature flowers yielded highest dry weight 0.381 g while those from the leaves, the least (0.199 g), after eight weeks of inoculation. Out of these calli cultures, highest azadirachtin

containing limonoids (254.2 and 235.6 µg/g DW) were found in calli raised from immature flowers and the lowest 16.1 µg/g DW from calli raised from leaves. A much higher amount of azadirachtin has been obtained in the present study, with different haploid callus lines of Neem. Redifferentiated cultures on MS + BAP (2.2 µM) + NAA (0.05 µM) yielded the highest (728.41 µg/g DW) which was closely followed by leaves from *in vitro* haploid plantlet (700 µg/g DW). Least amount was observed in an dedifferentiated line maintained on MS + 2,4-D (1 µM) + kinetin (10 µM).

4A.3 Bioactivity

The biological activity of neem needs no introduction. The tree from times immemorial has been used in herbal remedies all over the world to cure various ailments. Different parts of the plant and their extracts have formed a fundamental part of Indian Ayurveda, homeopathic and unani systems of medicine. As mentioned in the introductory section, the diverse activities can be attributed to a spectrum of compounds localized in various parts of the plant. More than 140 compounds have been isolated from different parts of Neem (Subapriya and Nagini 2005). In the present study, extracts derived from different anther culture lines have been utilized to study antibacterial activity against certain clinical isolates and reference strains.

Chander (1995) studied the antimicrobial activity of the neem oil and water extract of the neem leaves against the standard strains of bacteria i.e. *Escherichia coli* (NCTC 10418), *Staphylococcus aureus* (NCTC 6571) and *Pseudomonas aeruginosa* (NCTC 10662). Neem oil inhibited the growth of *Staphylococcus aureus* at a concentration of 10 ml/mL, *Escherichia coli* at 200 ml/mL but the *Pseudomonas aeruginosa* was not inhibited even up to a concentration of 250 ml/mL. The water extract of the neem leaves not only failed to inhibit the growth of these bacteria but also on the contrary enhanced the pigmentation of *Pseudomonas aeruginosa*. SaiRam et al. (2000) studied the anti-microbial activity of a vaginal contraceptive NIM-76 obtained from neem oil and it was observed that NIM-76 has a potent broad-spectrum antimicrobial activity against a range of bacteria like *E. coli* and *Klebsiellia pneumoniae*, antifungal activity against *Candida albicans* and antiviral activity against Polio virus replication in Vero cell lines. Vanka et al. (2001) studied the antibacterial effect of Neem mouthwash against

salivary levels of *Streptococcus mutans* and *Lactobacillus* over a period of 2 months. Also its effect in reversing incipient carious lesions was assessed. While *S. mutans* was inhibited by Neem mouthwashes, with or without alcohol as well as chlorhexidine, *Lactobacillu*s growth was inhibited by chlorhexidine alone. Biswas et al. (2002) reported that oil from the leaves, seed and bark possesses a wide spectrum of antibacterial action against Gram-negative and Gram-positive microorganisms, including *M. tuberculosis* and streptomycin resistant strains. *In vitro*, it inhibits *Vibrio cholerae, Klebsiella pneumoniae*, *M. tuberculosis* and *M. pyogenes*. Antimicrobial effects of neem extract have been demonstrated against *Streptococcus mutans* and *S. faecalis*. Mahfuzul et al. (2007) studied antibacterial activity of guava and neem extracts against food borne pathogens and spoilage bacteria. They observed that extracts from both the plants are more effective against gram positive bacteria compared to the gram negative ones. The ethanolic neem extract was found to be active against *Listeria monocytogenes* ATCC 43256 and *L. monocytogenes* ATCC 49594. However, higher degree of inhibition was observed for *Staphylococcus aureus* JCM 2151 and *S. aureus* IFO 13276. Prashant et al. (2007) studied the effects of mango and neem aqueous extracts on four organisms causing dental caries: *Streptococcus mutans*, *Streptococcus salivavius*, *Streptococcus mitis*, and *Streptococcus sanguis*. Neem extract produced the maximum zone of inhibition on *Streptococcus mutans* at 50% concentration. Even at 5% concentration neem extract showed some inhibition of growth for all the four species of organisms. However, the authors concluded that a combination of neem and mango chewing sticks may provide the maximum benefit. Thakurta et al. (2007) evaluated the antibacterial and antisecretory activity of methanolic neem extract against *Vibrio cholerae*, a causative agent of cholera. The extract was found to have a significant antibacterial activity against the multi-drug-resistant *Vibrio cholerae* of serotypes O1, O139 and non-O1, non-O139.

In all the above studies, the antimicrobial activity of Neem has been tested from the extracts of field grown plants and their parts. In the present study, extracts, derived from *in vitro* established anther culture cell lines were evaluated for antimicrobial activity. In accordance with the previous reports, the inhibition of extract against the clinical isolate and reference strain of *Staphylococcus aureus* were observed. Revelation of presence of azadirachtin as the major component of the extract, by chemical analysis,

strengthened the claims of antimicrobial activity of azadirachtin. Additionally, its presence in *in vitro* established haploid lines is very encouraging.

4B. *LANTANA CAMARA*

4B.1 Tissue Culture Studies

Establishing cell and tissue cultures of *Lantana* is a difficult task to accomplish because of browning of explants and culture media, due to phenolic exudation. Present study is the first attempt ever to utilize *Lantana*'s foliage in tissue culture. As of now there are no reports available on this aspect. Tissue culture is an effective alternative to employ the large biomass of this plant in a more productive manner. Browning of explants and the culture media due to phenol exudation has been discussed by several workers. Muhammad and Jaiswal (1987) observed browning in nodal segment explants of guava. They employed polyvinylpyrrolidone (PVP) and ascorbic acid solution to minimize the problem of browning. In Pomegranate, Naik et al. (1999) observed that browning first appeared at the cut ends of explants and gradually spreaded into the surrounding medium. Shoot development was completely inhibited and the explants died within a week. The problem was more intense with explants collected from the older shoots than those from the younger ones. It was overcome by transferring the nodal segments to the fresh medium of the same composition, twice, at 24 h interval. Following this the explants exhibited normal shoot development. Establishment of *in vitro* cultures of woody plants is greatly affected by browning of the medium and necrosis of explants. Browning is generally the result of oxidation of phenolic substances released from the cut ends of the explants by polyphenol oxidases or peroxidases. The methods, commonly employed to overcome the harmful effect of browning, include use of adsorbing agents such as activated charcoal or polyvinylpyrrolidone, inclusion of antioxidants in medium or soaking explants in antioxidant solution, transfer of explants to fresh medium at frequent intervals or sealing the cut ends of explants with paraffin wax (Broome and Zimmerman 1978; Weatherhead et al. 1978; Gupta et al. 1980; Lloyd and McCown 1980; Amin and Jaiswal 1987; Mathur et al. 1988; Bhat and Chandel 1991; Rout et al. 1999). In the present study, repeated subculturing of calli was adopted as a route to eliminate phenolic exudation from culture into the medium. Use of anti-oxidants was avoided to

prevent changes in the secondary metabolite profile of cultured cells. The best treatment to obtain growing callus was MS + 2,4-D (1 μM) + NAA (1 μM) + BAP (5 μM). The rate and degree of callusing increased with increasing subculture. It took about 26 weeks of repeated subculturing to obtain profusely growing, fresh, friable, granulated and cream callus.

It is worth noting that apart from its popularity as a garden plant, the potential for utilization of the large available biomass of *Lantana* was not explored earlier by biotechnological means. In this context, plant cell culture is a promising approach that has been adopted for production of various categories of secondary metabolites (Fowler and Scragg 1988; Alfermann and Petersen 1995). Importance of *in vitro* cultures lies in the uniformity and stability of the metabolites produced by them, which is independent of plant availability and seasonal variations. Over the past decade, a huge research interest is being reflected in isolation, purification and pharmacological studies of three pentacyclic triterpenes, BA, OA and UA from various plants, for the treatment of a range of diseases (Pereira et al. 2007; Ikuta et al. 2003; Liu 2005; Pasqua et al. 2006; Resende et al. 2006). Though the plant has been in use in traditional medicines for curing various ailments, it has been by and large neglected probably because it is a flagrant weed. However, it is noteworthy that there are enough evidences which establish weeds as an important source of medicines for indigenous people and they have a highly significant over representation in indigenous pharmacopoeias in relation to other types of plants (Stepp and Moerman 2001). There is an increasing evidence to support the hypothesis that weeds are relatively high in bioactive secondary compounds and are, thus, likely to hold promise for drug discovery.

4B.2 Secondary Metabolite Analysis

A breakthrough in cell-culture methodology occurred with the successful establishment of cell lines, capable of producing high yields of secondary compounds in cell suspension cultures (Zenk 1978). It had been considered for a long that dedifferentiated cells, such as callus or cell suspension cultures, were not able to synthesize secondary compounds, unlike differentiated cells or specialized organs (Krikorian and Steward 1969). Zenk and co-workers (1975) experimentally demonstrated

that even the dedifferentiated cell cultures of *Morinda citrifolia* could yield good amounts (2.5 g/l medium) of anthraquinones. This finding opened the door for the possible use of plant cultures for the production of secondary compounds of industrial interest.

Stafford et al. (1986) reported that the accumulation of secondary products in plant cell cultures depends on the composition of the culture medium, including the kind and concentration of plant growth regulators, mineral salts and carbon sources. The environmental conditions such as temperature, light, and gas composition also affects the secondary metabolite production. Kargi and Rosenberg (1987) observed that immobilization of plant cells improved the production of secondary metabolites. The production of secondary metabolites in plant cell suspension cultures was reviewed by Rokem and Goldberg (1985). Panda et al. (1989) provided an extensive review on the role of plant cell reactors on the production of secondary metabolites. Various medicinal metabolites, like solasodine, pyrrolizidine, alkaloids, cephalin and emetine, were reported to be isolated from *in vitro* raised callus/ cell suspension cultures (Nigra et al. 1987; Toppel et al. 1987; Teshima et al. 1988). In most of these cases, the amount of secondary metabolites produced from *in vitro* cultures was comparable to those of field grown parent plants. Scragg et al. (1990) reported that quinoline alkaloids were produced in significant quantities from globular cell suspension cultures of *Cinchona ledgeriana* on B_5 medium supplemented with 0.1 mg/L NAA. Appreciable amount of sanguinarine was produced in cell suspension cultures of *Papaver somniferum* using bioreactors (Park et al. 1990). Ravishankar and Grewal (1991) noticed that the influence of media constituents and nutrient stress had a great influence on the production of diosgenin from callus cultures of *Dioscorea deltoidea.*

BA, OA and UA are highly sought after pentacyclic triterpenoids due to their wide spectrum biological activities. BA is most highly regarded for its anti-HIV-1 activity and specific cytotoxicity against a variety of tumor cell lines (Cichewicz and Kouzi 2004). Besides this, their anti-inflammatory, hepatoprotective, antitumour, anti-HIV, antimicrobial, antifungal, anti-ulcer, gastroprotective, hypoglycemic and antihyperlipidemic activities are reported in the literature (Liu 2005). While there are a few incidences where co-occurrence of OA and UA has been observed in plant cell cultures but no *in vitro* source has been identified for BA so far. To the best of our

knowledge, this work is the first report on simultaneous accumulation of three pentacyclic triterpenoids, Betulinic, Oleanolic and Ursolic acids from *in vitro* cultures of *L. camara.* Moreover, the quantification studies have never been performed. Razborsek et al. (2008) reported detection and quantification of BA, OA and UA in five plants of Lamiaceae, growing in wild, by GC-MS. They obtained 0.6% BA, 0.09-0.9% OA and 0.09-1.6% UA, which is considerably less than that reported in the present study. Moreover, the extraction protocol adopted by them was too complicated, which included several steps. Since their studies were carried out on field grown plants, prone to climatic fluctuations and thus, may result in inconsistent production of metabolites. Skrzypek and Wysokinska (2003) identified the presence of OA and UA in cell cultures of *Hyssopus officinalis,* however, quantification studies were not done. Whereas Pasqua et al. (2006) could obtain 1.5% total triterpenoid content (including OA and UA) from *in vitro* callus cultures of *Camptotheca acuminata*, with the best solvent (95% ethanol), Zhao et al. (2007) were able to achieve 1.86% of BA in white birch bark collected from different provinces of China. The present study, provides an excellent reproducible protocol for simultaneous determination of BA, OA and UA in cell cultures of *L. camara* with a yield of 3.1%, 1.88% and 4.12%, respectively.

The detection wavelength of 209 nm was selected for the present study, by checking absorption maxima of the standard compounds, dissolved in ethanol, by a UV-Visible spectrophotometer (Cary, USA). In our preliminary studies, methanol was used as one of the mobile phase instead of acetonitrile. As the absorption of former was higher, high interference was observed especially at a short wavelength of 209 nm. Similar kind of inferences has been reported by Zhao et al. (2007). Moreover, as OA and UA are structural isomers, their separation needed a low absorbing solvent that doesn't interfere with the resolution process. Hence, acetonitrile was chosen for the study. Acetonitrile and water at 80:20 (v/v) ratio was found to be appropriate as the mobile phase for satisfactory separation of the three components at a flow rate of 1 ml/min. Also, the study allows optimum separation and quantification of three pharmacologically active pentacyclic triterpenes in the cultured cells with a small acquisition time of 15 min. This identifies the merit of cell cultures as a constant source of medicinally important compounds, in high amounts, all the year round. Additionally, establishment of *in vitro* cell lines for

production of compounds is justifiable in this genus, seeing that the leaves from parent plant (control) contained only marginally higher OA and UA while BA was entirely absent.

Dynamics of biosynthetic capacity of *L. camara* cells in suspension cultures has also been evaluated, in the present study. The study was focused on production and accumulation of three anticancer triterpenoids: BA, OA and UA. Information concerning the factors regulating secondary metabolism is as important as the selection of high producing cell lines in increasing the production of secondary metabolites. A number of physical and chemical factors that could influence secondary metabolism in plant cell cultures have been found. Optimization of the hormone concentration and combinations are often effective; high auxin levels, although good for cell growth, are often deleterious to secondary metabolite production. Alterations in the environmental factors such as nutrient levels, light, and temperature may also be effective in increasing productivity. Phosphate levels and nitrogen sources may also play an important role in product accumulation in plant cells (Dorenburg and Knorr 1995).

In the present study, complete utilization of phosphate from culture medium was found to result in the onset of stationary phase. Similar kind of kinetic profiles, where phosphate is assimilated faster than the nitrate has been observed and reported by many workers in different plant species (Nigra et al. 1990, Fett-Neto et al. 2004). Growth coupled synthesis of triterpenoids has been observed previously in case of other triterpenoid azadirachtin (Prakash et al. 2005). Moreover, in agreement with the observations of other workers it was found that the growth period in liquid medium is reduced by one week as compared to growth in callus cultures. This might be due to facilitated nutrient transport in liquid medium (Nigra et al. 1990). This shortened growth period, however, had an adverse effect on production of BA, OA and UA, in cell suspension cultures, in the present study. It was observed that the cultures remained in the log phase (growth phase) for a very short period of 2 to 10 days. As the synthesis of these triterpenoids was found to be growth associated, this shortened growth phase (hence quick attainment of stationary phase), resulted in relatively low yield of compounds as compared to the callus cultures maintained in semi-solid media. The log phase was much longer for calli in the semi-solid medium with the growth cycle of 4 weeks. Based on

results, it can be suggested that during scale up, fed-batch cultivation is a better strategy in such cases and can be used to prolong the log phase of cultures with the supply of growth limiting nutrients at regular intervals. This mode of cultivation has shown improvements in productivity of ginseng by *Panax ginseng* and taxane by *Taxus chinensis* (Chattopadhay et al. 2004).

The ability of plant suspension and callus cultures to grow on different carbohydrates has been reviewed by Maretzki et al. (1974).They concluded that sucrose and glucose were the best general carbon sources for growth of cultures and that other carbohydrates could substitute for sucrose or glucose depending on the species of origin and the plant or tissue used (Verma and Dougall 1977). Variation in growth and chemical profile in *in vitro* cell cultures, with manipulation of media components has been previously reported (Arias-Castro et al. 1993). Such effects on biomass production have been observed in cell suspension cultures of *Psoralea corylifolia* (Shinde et al. 2009). In accordance with their results, our studies, also demonstrate the effectiveness of sucrose for biomass production. The results are also in agreement with a recent finding which stated that sucrose being a balanced source of carbon supply favors highest biomass accumulation in *Dendrobium huoshanense* cell suspensions cultures (Zha et al. 2007). Sucrose dissociates to allow a higher osmotic potential within the cells. Thus, the role of sucrose in plant tissue culture media, as an osmoticum and carbohydrate source, has been established (Khury and Moorby 1995). Type and concentration of carbohydrate source has been a crucial factor that affects the accumulation of secondary metabolites. The positive influence of maltose on yield of metabolites has also been reported earlier. Kinnersley (1988) reported twice as high production of anthocyanin in cells grown on maltose as compared to ones grown on sucrose. Choi et al. (2000) reported that intermittent feeding of maltose considerably enhanced the paclitaxel production in cell cultures compared to that of sucrose. In the present study also, we observed that maximum amount of metabolites were produced in maltose, however, it did not support the production of BA. The slow hydrolysis of maltose and the creation of starvation like condition in the medium might act as a stress signal in response to which production of metabolites is stimulated.

The perceived sensitivity of plant cells to hydrodynamic stress associated with aeration and agitation can be attributed to the physical characteristics of the suspended cells, viz. their size, the presence of thick cellulose based cell wall, and existence of large vacuoles (Chattopadhyay et al. 2002). In this context, viability test by dyes like fluorscein diacetate (FDA) is important in determining viability of cells in suspension cultures. In the present study, maximum viable cultures were obtained at 120 rpm as confirmed by bright green color of FDA. Below (60 rpm) and above this speed (240 rpm), the cells died either due to aggregation or rupturing and hence did not take up the stain. FDA is a non-fluorescing, non- polar dye that freely permeates through the plasma membrane. In the living cells it is cleaved by esterase activity releasing the polar fluorescent portion, fluorscein, which is unable to pass through the plasma membrane of living cells while in dead and broken cells it is lost. Hence, only the live, intact cells take up the stain and fluoresce green.

4B.3 Bioactivity

Over 3000 species of plants have been reported to have anticancer properties (Uddin et al. 2009). About 80% of the population in developing countries relies on traditional plant based medicines for their primary health care needs. Extensive study in this area is under progress and extracts from numerous plants are being screened for cytotoxic activity against cancerous cell lines (Steenkamp and Gouws 2006; Suffredini et al. 2007; Ashidi et al. 2010). Based on ethnopharmacological evidences, *in vitro* cytotoxic activity of the cell culture derived ethyl acetate and aqueous extracts of *Lantana* have been evaluated against the cancerous HeLa cell lines, in the present work. Till date, there are only two reports that evaluate the cytotoxic activity of *Lantana camara* extracts obtained from the wildly growing plants (Raghu et al. 2004; Sharma et al. 2007). Raghu et al. (2004) carried out *in vitro* cytotoxic assays from extracts derived from different parts of *Lantana*. Maximum activity was observed in the leaf extract. Sharma et al. (2007) demonstrated the chemopreventive effect of *Lantana camara* methanol leaf extract on 7, 12-dimethylbenz [*a*] anthracene (DMBA)-induced skin cancer in Swiss albino mice. In both the studies crude methanolic extract was used and also no characterization studies were carried out to determine the composition of the extract.

Our results exhibited significant cytotoxicity of organic and aqueous extracts against HeLa cells, former being more effective than the latter. The extracts showed a dose and time dependent activity with very promising IC_{50} values. As compared to this, insignificant activity was observed on the normal BHK-21 cell lines. One of the most notable features of both the extracts was their apoptotic mode of action on the cells. Ability to induce apoptotic mode of cell death is a desirable property for an extract/ drug to be considered as an anticancer agent. Two central pathways have been shown to be involved in the process of apoptotic cell death: one is the direct involvement of caspase proteases (especially caspase-8), and the other is the mitochondrial pathway in which caspases are activated as a downstream event of apoptosis. In addition to these pathways, lysosomes are involved as the primary organelles in apoptotic cell death induced by some signals, such as B cell receptor-induced apoptosis (He et al. 2005; Noda et al. 2007). The p53 tumour suppressor protein is one of the key regulators of apoptosis and plays a pivotal role in mediating DNA damage-induced apoptosis. Several lines of evidence suggest that p53 death signals lead to caspase activation; however, the mechanism of caspase activation by p53 still is unclear (Schular et al. 2000; Lantto et al. 2009). Tumor cells, in general, undergo death by apoptosis and necrosis which can be detected by morphological and biochemical changes. Of these, the morphological study was considered to be the most reliable method (Walker et al. 1988; Cotter et al. 1990; Renvoize et al. 1998; Xiao et al. 2007). The manifestation of molecular events like nuclear fragmentation was detected only after the appearance of the morphological features characteristic of apoptosis (Xiao et al. 2007). Thus, to assess the pattern of cell death, the morphological features of HeLa and BHK-21 cells were examined by microscopic techniques. The morphological pattern of the cell death was further confirmed by CLSM, after staining the cells with AO/EB. AO stains both viable and nonviable cells, emits green fluorescence when intercalated into double stranded DNA, and also emits red fluorescence when bound to single stranded RNA. In contrast EB stains only nonviable cells which gets intercalated into DNA and thus, emits red fluorescence. Apoptotic mode of action was further confirmed by SEM.

Besides cytotoxic activity, antibacterial activity of *Lantana* has also been evaluated in the present study. Extracts from leaves and callus cultures were tested

against a range of clinical isolates and pure strains of bacteria. However, it was noticed that the leaf derived organic extract was more effective and antibacterial to some of the isolates with an average inhibition zone of 9 mm. Extract from the callus was active only on a clinical isolate of *Streptococcus mutans* at a dilution of 1:4, when agar streak method was followed. Several reasons may be cited for this observation. Firstly, the three triterpenic acids detected in the extract are considered as weak antibacterial agents. This was demonstrated by Fontanay et al. (2008), where they reported no antibacterial activity for BA while OA and more particularly UA, did show a moderate to good antibacterial activity, but limited to gram-positive bacteria. Nevertheless, in their study, OA and UA were devoid of antibacterial activities against clinical isolates. Moreover, viability and cytotoxic assays demonstrated that the three compounds induced a significant cytotoxicity. This observation was in agreement with the present study, where the extracts from cell lines demonstrated a very promising cytotoxic activity against a cancerous cell line and a very mild antibacterial activity. Nonetheless, presence and interference of other compounds cannot be ruled out in leaves of an intact plant whichmight be responsible for the observed activity.

Recently, antibacterial activity of *Lantana* has been studied by various workers. Sharma and Kumar (2009) demonstrated antimicrobial efficacy of flavonoids and crude alkaloids of *Lantana camara* L. against three bacteria (*Escherichia coli, Proteus mirabilis,* and *Staphylococcus aureus*) and two fungi (*Candida albicans* and *Trichophyton mentagrophytes*), by disc diffusion assay. Minimum inhibitory concentration, minimum bactericidal/fungicidal concentration and total activity were also studied. Most susceptible microorganism in their study was *C. albicans* followed by *P. mirabilis, S. aureus, E. coli,* and *T. mentagrophytes.* The range of minimum inhibitory concentration of tested extracts was 0.039-0.625 mg/ml while minimum bactericidal/fungicidal concentration ranged from 0.078-1.25 mg/ml. Ganjewala et al. (2009) reported the biochemical composition and antibacterial activities of the leaves and flowers of four *Lantana camara* (Verbanaceae) plants with yellow, lavender, red, and white flowers. Three out of the four plants showed almost similar carbohydrates and lipid compositions. Extracts of leaves and flowers obtained with ethyl acetate were studied by them for antibacterial activities. Ethyl acetate extracts from leaves and flowers exhibited

considerable antibacterial activities and the zone of inhibition ranged from 10-21 and 9-15 mm, respectively, for leaves and flower derived extracts. They concluded that the biochemical parameters viz., lipids, carbohydrates and proteins, revealed similarity among the four different *L camara* plants whereas antibacterial activities varied from one *L. camara* to the other and also according to the type of tissue used. Barreto et al. (2010) studied the antibacterial activities of the ethanolic extracts obtained from leaves and roots of *L. camara* and *L. montevidensis*, against gram-positive and gram-negative strains, standard and multi-resistant bacteria clinical isolates. The extracts demonstrated antibacterial activity against all tested bacteria, but the extract from fresh leaves of *L. montevidensis,* showed the best result against *P. aeruginosa* (MIC 8 µg/ml) and against multi-resistant *E. coli* (Ec 27) (MIC 16 µg/ml). The use of medicinal plants with therapeutics properties represents a secular tradition in different cultures, mainly in underdeveloped countries (Barreto et al. 2010). Plant extracts are known to consist of many chemicals and among them, a few compounds could be acting synergistically. Sometimes, isolation of the compounds from the extract may cause a decrease in desired activity, which underlines the importance of extract screening (Orhan et al. 2009). Ethnopharmacological data has been one of the common useful ways for the discovery of biologically active compounds from plants (Cordell et al. 1991; Cragg et al. 1994). The big advantage of the ethnopharmacological information is that the extensive literature will allow for some rationalization with respect to the biological potential of a reputed use (Cordell et al. 1991; Kamuhabwa et al. 2000)

Chapter 5

Conclusions and Future Prospects

The present study is an attempt to realize the potential of two medicinal plants, *Azadirachta indica* A. Juss. and *Lantana camara* L., by biotechnological means using tissue culture techniques. Biochemical studies demonstrated the potential of cell cultures as source of important secondary metabolites. Bioassays undertaken in both the plants further confirmed the utility of these studies. Key points of the entire work are given in the bulleted list below.

- In ***Azadirachta indica* A. Juss.**, haploid plants were obtained from *in vitro* anther cultures at early-to-late uninucleate stage of microspores. Haploid formation occurred via callusing. The protocols for callus induction, multiplication and regeneration were standardized in the present study.

- For callus induction, MS + 2,4-D (1 µM) + NAA (1 µM) + BAP (5 µM) with 12% sucrose was found to be the best.

- The growth rate of calli was maximum on MS + 2,4-D (1 µM) + Kn (10 µM), followed by MS + 2,4-D (0.5 µM) + Kn (4.5 µM) and on MS + 2,4-D (0.5 µM), in a single growth cycle of 8 weeks. The best shoot regeneration was achieved on MS + BAP (2.2 µM) + NAA (0.5 µM), when the calli were obtained from MS + 2,4-D (0.5 µM) + Kn (4.5 µM).

- Shoot elongation was attained on MS + BAP (0.5 µM), preceded by pre-treatment of shoots on GA_3 (3 µM) medium for 12 days. The shoots were rooted on ¼ MS + IBA (0.5 µM).

- Cytological analysis of plantlets, regenerated from anther callus via organogenesis, revealed that 40% of the plants were haploids with chromosome number 2n=x=12, and the rest were diploids (2n=2x=24) or aneuploids (2n=x-2=22) or (2n=x-1=23).

- Calli and leaves from *in vitro* raised haploid plantlets were analyzed for the production of azadirachtin. HPLC and MS analysis revealed the accumulation of azadirachtin in all the *in vitro* raised cultures. Highest azadirachtin was found to be accumulated in re-differentiated cell lines on MS + BAP (2.2 µM) + NAA (0.5 µM) (728.41 µg/g DW) while the lowest was found in de-differentiated cultures on MS + 2,4-D (1 µM) + Kn (10 µM) (49 µg/g DW).

- Simultaneous presence of three powerful anti-cancer agents: Betulinic acid (3.1%), Oleanolic acid (1.88%) and Ursolic acid (4.12%) were observed from *in vitro* raised cell biomass of *L. camara*, for the first time, in this study. Moreover, the three acid triterpenes, BA, OA and UA could be determined and quantified in a single, short and relatively simple method.

- Analysis of leaves from field grown plants (control) contained only OA and UA; BA was entirely absent. Amount of OA (2%) and UA (4.5%) in leaf extract was only marginally higher than the amount accumulated in *in vitro* cultures (OA 1.88%; UA 4.12%).

- In accordance with the previous reports, it was observed that these compounds show better cytotoxic activity than antimicrobial activity. Significant cytotoxicity of cell derived extract against HeLa cells demonstrated the effectiveness of the extracts. The IC_{50} for organic extract was found to be in the range of 100 µg/ml in 36 h to 25 µg/ml in 72 h while for aqueous extract it was in the range of 1500 µg/ml to 3000 µg/ml starting from 36 h through 72 h.

- In batch kinetics studies, it was concluded that the drop in pH and complete utilization of phosphate from the culture medium, resulted in the onset of stationary phase and it was a major limiting nutrient for growth. The production of the three pentacyclic triterpenoids was observed to be growth associated and increased with an increase in fresh weight of the cells.

FUTURE PROSPECTS:

- Diploidization of haploid plants to generate homozygous diploid lines.
- Further purification and characterization of components from aqueous extract of *Lantana* and determination of their bioactive potential.
- The results of batch kinetics study can serve as a background for further scale-up related aspects in bioreactors.

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APPENDIX

AZADIRACHTA INDICA A. JUSS.

Kingdom Plantae – Plants
Subkingdom Tracheobionta – Vascular plants
Superdivision Spermatophyta – Seed plants
Division Magnoliophyta – Flowering plants
Class Magnoliopsida – Dicotyledonous
Order Rutales
Family Meliaceae
Genus *Azadirchta*
Species *indica*

LANTANA CAMARA L.

Kingdom Plantae – Plants
Subkingdom Tracheobionta – Vascular plants
Superdivision Spermatophyta – Seed plants
Division Magnoliophyta – Flowering plants
Class Magnoliopsida – Dicotyledonous
Order Lamiales
Family Verbenaceae
Genus *Lantana*
Species *camara*

List of Publications

National/ International Journals:

1. **Priyanka Srivastava** and Rakhi Chaturvedi. *In vitro* androgenesis in tree species: an update and prospect for further research. *Biotechnology Advances*, 2008 26 (5): 482-491, **2008**.

2. **Priyanka Srivastava**, Chandana Kalita and Rakhi Chaturvedi. Accumulation of oleanolic acid and ursolic acid in cell cultures of *Lantana camara* L. *Research Journal of Biotechnology*, 361-365, **2008**.

3. **Priyanka Srivastava**, Naresh Kasoju, Utpal Bora and Rakhi Chaturvedi. Dedifferentiation of leaf explants and cytotoxic activity of cell culture derived aqueous extract of *Lantana camara* L. *Plant Cell, Tissue and Organ culture*, 99 (1): 1-7, **2009**.

4. **Priyanka Srivastava**, Mithilesh Singh, Prateek Mathur, Rakhi Chaturvedi. *In vitro* organogenesis and plant regeneration from unpollinated ovary: a novel explant of neem (*Azadirachta indica A. Juss.*). *Biologia Plantarum*, 53 (2): 360-364, **2009**.

5. **Priyanka Srivastava,** Mithilesh Singh and Rakhi Chaturvedi. Effect of casein hydrolysate and Major inorganic salts on clonal propagation from nodal explants of a mature neem tree, *Azadirachta indica* A. Juss. *Research Journal of Biotechnology*, 4 (4): 30-38, **2009**.

6. **Priyanka Srivastava**, Mithilesh Singh and Rakhi Chaturvedi. Production of azadirachtin in anther cultures of *Azadirachta indica* A. Juss. and its bioactivity against *Aspergillus sydowii. Icfai University Journal of Biotechnology*, III (3): 38-45, **2009**.

7. **Priyanka Srivastava,** Rashmi Rekha Hazarika, Mithilesh Singh and Rakhi Chaturvedi. Assessment of age and morphometric parameters of seeds on azadirachtin production in neem seed kernels collected from various ecotypes. *Research Journal of Chemistry and Environment* 14: 24-28, **2010.**

8. **Priyanka Srivastava** and Rakhi Chaturvedi. Detection and estimation of betulinic, oleanolic and ursolic acid from cell cultures of *Lantana camara* L. by RP-HPLC. *In Vitro Cellular and Developmental Biology- Plant (Accepted).*

9. **Priyanka Srivastava**, Naresh Kasoju, Utpal Bora and Rakhi Chaturvedi. Accumulation of betulinic acid, oleanolic acid and ursolic acid in *in vitro* cultures of *Lantana camara* L. and their cytotoxic activity against HeLa cell lines *(Under review).*

10. **Priyanka Srivastava** and Rakhi Chaturvedi. Batch kinetics of cell suspension cultures of *Lantana camara* L. *(Under review).*

11. **Priyanka Srivastava** and Rakhi Chaturvedi. Azadirachtin accumulation in androgenic cultures of *Azadirachta indica* A. Juss. *(Communicated).*

Book Chapter:

12. **Priyanka Srivastava**, Mithilesh Singh and Rakhi Chaturvedi. **2010**. Biotechnological improvement in neem. In: K. Ashwani (Editor) Advances in Plant Biotechnology. I.K. International Publishing House Pvt. Ltd., New Delhi, India (In Press).

National/ International Conferences:

13. **Priyanka Srivastava** and Rakhi Chaturvedi. *In vitro* morphogenesis and biochemical analysis in neem (*Azadirachta indica* A. Juss.). In: Second international symposium on green/sustainable chemistry, January 10-13, 2006. Department of Chemistry, University of Delhi, Delhi, India. Page No. 147, **2006**.

14. Rakhi Chaturvedi and **Priyanka Srivastava**. Effect of casein hydrolysate (CH) on clonal propagation from nodal explants of an elite mature neem tree (*Azadirachta indica* A. Juss.). In: National conference on increasing forest productivity: genetic and breeding options, February 21-23, 2007. Genetics and Plant Propagation Division, Tropical Forest Research Institute, Indian Council of Forestry Research & Education, Jabalpur. Page No. 47, **2007**.

15. Rakhi Chaturvedi and **Priyanka Srivastava**. Synergistic effect of auxin and cytokinin on *in vitro* androgenesis in *Azadirachta indica* A. Juss. In: *In Vitro* Cellular and developmental Biology and 2008 World Congress on *In Vitro* Biology, June 14-18, 2008. Society for *In Vitro* Biology (SIVB), Tucson, Arizona, USA. Vol 44, Page No 37, **2008**.

16. **Priyanka Srivastava** and Rakhi Chaturvedi. Establishment of elite cell lines of *Lantana camara* L. In: International conference on Biotechnology, February 6-8, 2008. School of Biotechnology, Chemical & Biomedical Engineering, Vellore Institute of Technology (VIT) University, Vellore, Tamil Nadu, India. Page No. 131, **2008**.

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18. **Priyanka Srivastava** and Rakhi Chaturvedi. Accumulation of oleanolic acid and Ursolic acid in cell cultures of *Lantana camara* L. In: First international symposium on green/sustainable chemistry, December 28-30, 2008. Department of Chemistry, Gangtok, Sikkim, India. Page No. 147, **2008**.

19. Rakhi Chaturvedi and **Priyanka Srivastava**. Enhanced accumulation of betulinic, oleanolic and ursolic acid in Cell cultures of *Lantana camara*. In: *In Vitro* Cellular and developmental Biology and 2009 World Congress on *In Vitro* Biology, June 14-18, 2009. Society for *In Vitro* Biology (SIVB), South Carolina, USA. Vol 44, Page No 37, **2009**.

20. Priyanka Srivastava, Mithilesh Singh and Rakhi Chaturvedi. Bioproduction of azadirachtin in anther cultures of *Azadirachta indica* and its antifungal activity on*Aspergillus sydowii*. In: International Herbal Conference, February 28-30, 2009, Bangalore, Karnataka, Page No. 152, **2009**.

21 Priyanka Srivastava and Rakhi Chaturvedi. Production of triterpenoids in in vitro cell cultures of medicinal plants. In: *In Vitro* Cellular and developmental Biology and 2010 World Congress on *In Vitro* Biology, June 6-11, 2010. Society for *In Vitro* Biology (SIVB), Missouri, USA, **2010** *(Accepted)*.

Workshop Attended:

22. Workshop on AFLP organized by Tea Research Association, Toclai, Jorhat, Assam; October 29-November 2, **2007**.

FIGURE 1

ANTHER CULTURE

Establishment of cultures

A. A twig of neem with flower buds (x 1X)

B. Flower buds of 2 mm size bearing correct stage of microspores (x 2.2X)

C. An isolated anther at culture, with uninucleate microspores (x 70X)

D. A group of tetrads bearing microspores just before release (x 400X)

E. Uninucleate microspores stained with acetocarmine (x 700X)

FIGURE 2

ANTHER CULTURE

Callus induction

Growth Period: 8 weeks

A. Establishment of anther cultures on MS (12% sucrose) + 2,4-D (1 µM) + NAA (1 µM) + BAP (5 µM) showing emergence of yellow, translucent, soft and shiny calli (x 1X)

B-G. Individual anthers at culture where the anther walls pulled apart with the pressure of callusing microspores (x 55X)

H-J. Anthers callused at the wounded base (x 44X)

FIGURE 3

ANTHER CULTURE

Callus multiplication

Growth Period: 8 weeks

A. A 20-week-old callus on induction medium MS + 2,4-D (1 µM) + NAA (1 µM) + BAP (5 µM) with dull brown, paste like, soggy appearance; the cells were not able to survive on further subcultures to this original medium (x 2.08X)

B-D. Calli on three multiplication media, **B.** MS + 2,4-D (0.5 µM), **C.** MS + 2,4-D (0.5 µM) + Kn (4.5 µM), **D.** MS + 2,4-D (1 µM) + Kn (10 µM), showing healthy, cream, moderately to fast growing, friable calli (x 2X)

FIGURE 4

ANTHER CULTURE

Shoot regeneration

Growth Period: 6 weeks

A-B. Calli on MS + BAP (2.5 µM) + IAA (5 µM) + CH (500 mg/l) exhibiting bright green, compact nodular structures (black arrows) that differentiated into shoot-buds (arrows heads) (x 1.52X)

C. Same as B, an enlarged view of light green shoots, showing stalked glands on their surface (x 11.2X)

D-E. The calli on MS + BAP (2.2 µM) + NAA (0.05 µM), showing numerous, bright to dark green compact nodular structures (black arrows) that differentiated into shoot-buds (arrows heads)
(x 2.5X)

F. Same as E, an enlarged view of bright green shoot showing numerous multicellular glands on its surface (x 7X)

FIGURE 5

ANTHER CULTURE

Shoot regeneration

Histology and Scanning electron microscopy

A-B. Histological section of 8-week-old nodulated callus from MS + BAP (2.2 µM) + NAA (0.05 µM) showing developing vascular strands and distinct tracheary elements (x 260X)

C-D. Scanning Electron Micrograph of 8-week-old callus from MS + BAP (2.2 µM) + NAA (0.05 µM) showing well developed nodular structures (x 200X)

FIGURE 6

ANTHER CULTURE

Shoot elongation and multiplication

A. GA_3 (3 µM) pretreated shoot on elongation medium MS + BAP (0.5 µM), after 2 weeks (x 1.32X)

B. Same as A, after 4 weeks (x 1.32X)

C. Same as B, after 6 weeks showing shoot with bright green leaves (x 1.2X)

D. Single nodal segment from C were cultured on MS + BAP (1.0 µM) + CH (250 mg/l). The axillary bud has developed into single, long multimodal shoot, after 6 weeks (x 1X)

FIGURE 7

ANTHER CULTURE

Rooting of shoots

A. An *in vitro* shoot developed on MS + IBA (0.5 µM), showing unhealthy highly coiled, thin, cream colored root, after 4 weeks (x 1.8X)

B. An *in vitro* developed shoot on ¼ MS + IBA (0.5 µM), showing a shoot with thick, cream colored, long roots, after 3 weeks (x 1.52X)

C. Same as B, after 6 weeks. Healthy, thick, 4-5 cream colored, branched roots have differentiated directly from the cut, basal end (x 1.4X)

FIGURE 8

ANTHER CULTURE

Ploidy analysis

A. A root-tip cell of a seedling, stained with 1% aceto-orcein, showing diploid number of chromosomes (2n=2x=24) (x 3000X)

B. A shoot-tip cell of an *in vitro* haploid plantlet, stained with 2% aceto-carmine, showing haploid number of chromosomes (2n=x=12) (x 4000X)

C. A root-tip cell of an *in vitro* haploid plantlet, stained with 2% aceto-orcein, showing haploid number of chromosomes (2n=x=12) (x 3000X)

FIGURE 9

AZADIRACHTIN PRODUCTION IN ANTHER CALLUS LINES

High performance liquid chromatography of standard azadirachtin

FIGURE 10

AZADIRACHTIN PRODUCTION IN ANTHER CALLUS LINES

Mass spectra of azadirachtin present in anther callus

A. Mass spectrometric profile of standard azadirachtin

B. Mass spectrometric profile of HPLC eluted fraction of crude extract obtained from one of the callus lines on MS + BAP (2.2 µM) + NAA (0.05 µM)

FIGURE 11

LANTANA CAMARA LEAF-DISC CULTURE

Establishment of cultures of *Lantana camara*

A. Leaf disc at culture after one week, green and brown compact calli seen proliferating from the margins of leaf disc (2X)

B. Same as A, after 3 weeks (x 1.28X)

C. 8-week-old, 2nd Subculture of callus from B; showing further proliferation of compact and hard callus (x 2.17X)

D. 13-week-old culture showing soft and friable callus after 7th Subculture, the cells were deep brown in color (x 2.07X)

E. An 18-week-old culture, showing a mixture of brown and green cells after ten passages (x 1.61X)

F. A 26-week-old culture showing healthy, fresh, granulated, friable and cream calli ready for analysis after 14 passages (x 2.4X)

FIGURE 12

ANALYSIS OF PENTACYCLIC TRITERPENOIDS IN CELL CULTURES

Thin layer chromatography

(On each plate, the 1st lane consisted of the crude sample, 2nd and 3rd lanes were a mixture of crude and standard, and the 4th lane consisted of standard sample only).

A. TLC of Betulinic acid (BA) with R_f value as 0.51

B. TLC of Oleanolic acid (OA) with R_f value as 0.28

C. TLC of Ursolic acid (UA) with R_f value as 0.17

FIGURE 13

ANALYSIS OF PENTACYCLIC TRITERPENOIDS IN CELL CULTURES

High performance liquid chromatography

A. Chromatogram of a mixture of standards of BA, OA, UA

B. Chromatogram of organic extract showing the presence of three triterpenoids

C. Chromatogram of organic extract spiked with three standards viz. BA, OA and UA

FIGURE 14

ANALYSIS OF PENTACYCLIC TRITERPENOIDS IN CELL CULTURES

High performance liquid chromatography

A. Chromatogram of organic extract individually spiked with BA

B. Chromatogram of organic extract individually spiked with OA

C. Chromatogram of organic extract individually spiked with UA

FIGURE 15

ANALYSIS OF PENTACYCLIC TRITERPENOIDS IN LEAVES

High performance liquid chromatography

High Performance Liquid Chromatography of organic extract derived from leaves of field grown *L. camara* plants (control), showing the presence of Oleanolic and Ursolic acids; absence of Betulinic acid can be clearly noted. Also, a very prominent unknown peak can be seen between 3-4 min which could be porphyrin.

FIGURE 16

ANALYSIS OF PENTACYCLIC TRITERPENOIDS IN CELL CULTURES

Mass spectroscopy

Comparative negative mode electrospray ionization mass spectra of standard compounds and the purified samples from cell biomass:

A. BA standard

B. BA sample

FIGURE 17

ANALYSIS OF PENTACYCLIC TRITERPENOIDS IN CELL CULTURES

Mass spectroscopy

Comparative negative mode electrospray ionization mass spectra of standard compounds and the purified samples from cell biomass:

A. OA standard

B. OA sample

FIGURE 18

ANALYSIS OF PENTACYCLIC TRITERPENOIDS IN CELL CULTURES

Mass spectroscopy

Comparative negative mode electrospray ionization mass spectra of standard compounds and the purified samples from cell biomass:

A. UA standard

B. UA sample

FIGURE 19

ANALYSIS OF PORPHYRIN MOLECULE FROM LEAVES

Mass spectroscopy

The mass spectra of prominent peak obtained in HPLC chromatogram, between 3-4 min, of the organic extract, derived from field-grown leaves of *L. camara*. The spectrum in the upper panel is of the blank sample that consisted only of mobile phase acetonitrile and water, and solvent ethanol; Spectrum in the lower panel shows the presence of porphyrin that forms the central portion of the chlorophyll molecule.

FIGURE 20

SHAKE FLASK CULTURE

Effect of agitation speed on viability of cells

Cells stained with 1% fluorscein diacetate solution

A. Cellular clump at 60 rpm, showing unstained dead cells in the centre of cell aggregate and live cells fluoresce green at the periphery (x 40X)

B. The cultures maintained at 120 rpm in the cell suspension, showing aggregates of live and healthy, fluorescent green stained cells (x 108X)

C. Same at 240 rpm, showing dead clumps (dark bodies) and sheared cells (x 108X)

FIGURE 21

CYTOTOXICITY ASSAYS

Inverted light microscopic images (Organic extract)

A-B. Images of untreated HeLa and BHK-21 cells (x 40X)

C-D. Images of HeLa and BHK-21 cells treated with organic extract, showing the presence of rounded apoptotic bodies (x 40X)

E-F. Similar pattern of morphological changes is exhibited in both cell types when treated with curcumin (positive control) (x 40X)

FIGURE 22

CYTOTOXICITY ASSAYS

Inverted light microscopic images (Aqueous extract)

A-B. Images of untreated HeLa and BHK-21 cells (x 40X)

C-D. Images of HeLa and BHK-21 cells treated with aqueous extract showing the presence of rounded apoptotic bodies; also, a lesser frequency of apoptotic bodies can be seen as compared to organic extract (x 40X)

E-F. Similar pattern of morphological changes is exhibited in both cell types when treated with curcumin (positive control) (x 40X)

FIGURE 23

CYTOTOXICITY ASSAYS

Confocal microscopy (Organic extract)

A. Healthy untreated HeLA cells (x 50X)

B. Treated HeLa cells, 24 h after treatment with organic extract showing bright green nuclei indicating initiation of chromatin condensation (x 50X)

C. Same as B, after 36 h, showing zones of cleared monolayer (x 50X)

D-F. Same as C, 48 h-72 h after treatment with organic extract showing a gradual increase in percentage of dead cells taking orange stain of ethidium bromide (x 50X)

FIGURE 24

CYTOTOXICITY ASSAYS

Confocal microscopy (Aqueous extract)

A. Healthy untreated HeLA cells (x 50X)

B. HeLA cells treated with extract for 24 h showing initiation of chromatin fragmentation (x 50X)

C. HeLA cells treated with extract for 36 h, showing cleared zones of monolayer (x 50X)

D.-F. HeLA cells treated with extract from 48 h-72 h, showing gradual increase in frequency of dead cells (x 50X); the mode of action is similar to organic extract, however, the effect is less pronounced (x 50X)

FIGURE 25

CYTOTOXICITY ASSAYS

Scanning electron microscopy

A. An overview of treated cells showing a mixture of live/ dead cells; the sequential stage of apoptosis can be seen in figure B-F

B. Cells in the initial state with intact size, shape and morphology

C. Gradual contraction of extra cellular matrix

D. Subsequent loss of cell- cell contacts

E. Membrane blebbing

F. The formation of apoptotic bodies

FIGURE 26

CYTOTOXICITY ASSAYS

DNA fragmentation assay

The gel electrophoresis image obtained after DNA fragmentation assay. The lanes M, T, P, N_1 and N_2, represent marker (lambda DNA Hind III digest), test sample (extract treated), positive control (curcumin treated), negative control– 1 (DMSO treated) and negative control – 2 (without any treatment) respectively. N_1 was performed to check the effect of DMSO (which was used as solvent to dissolve the extract) alone on the cells. The molecular weights of the DNA marker ranged from 23,130 D to 564 D.

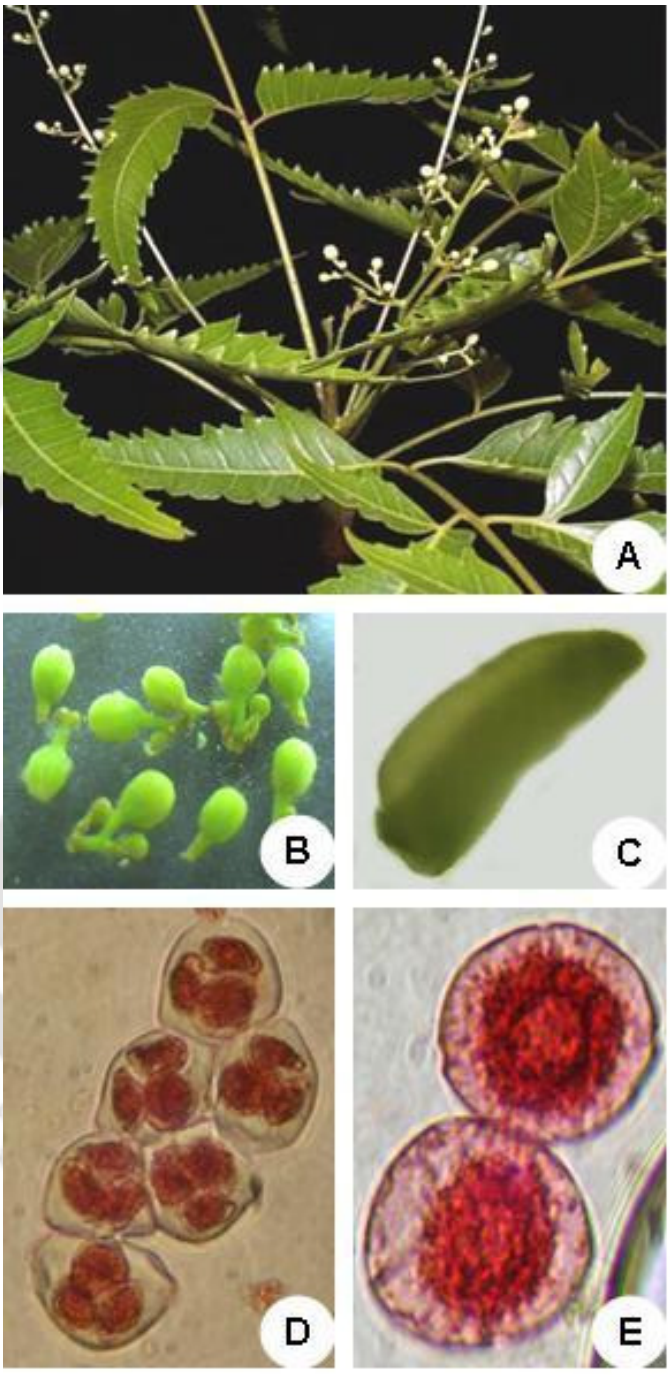
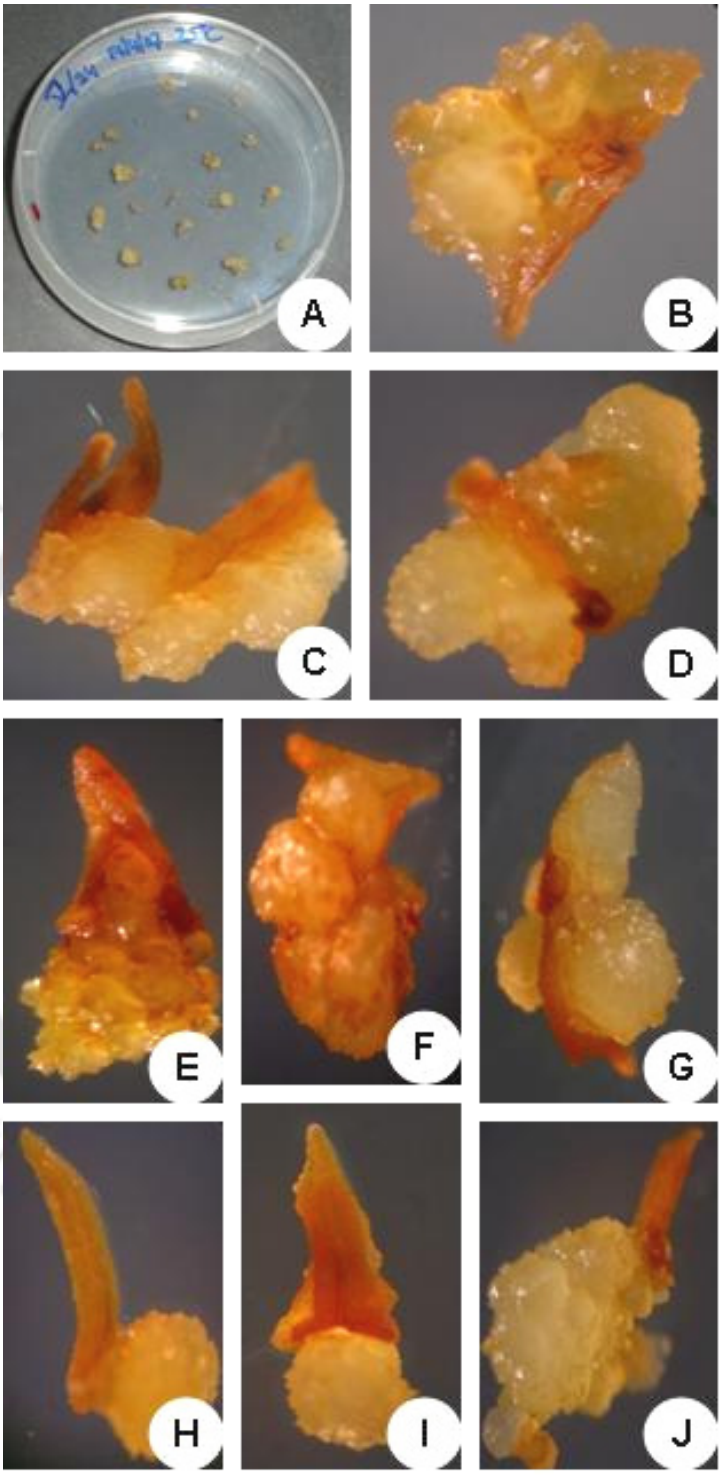


Figure 1



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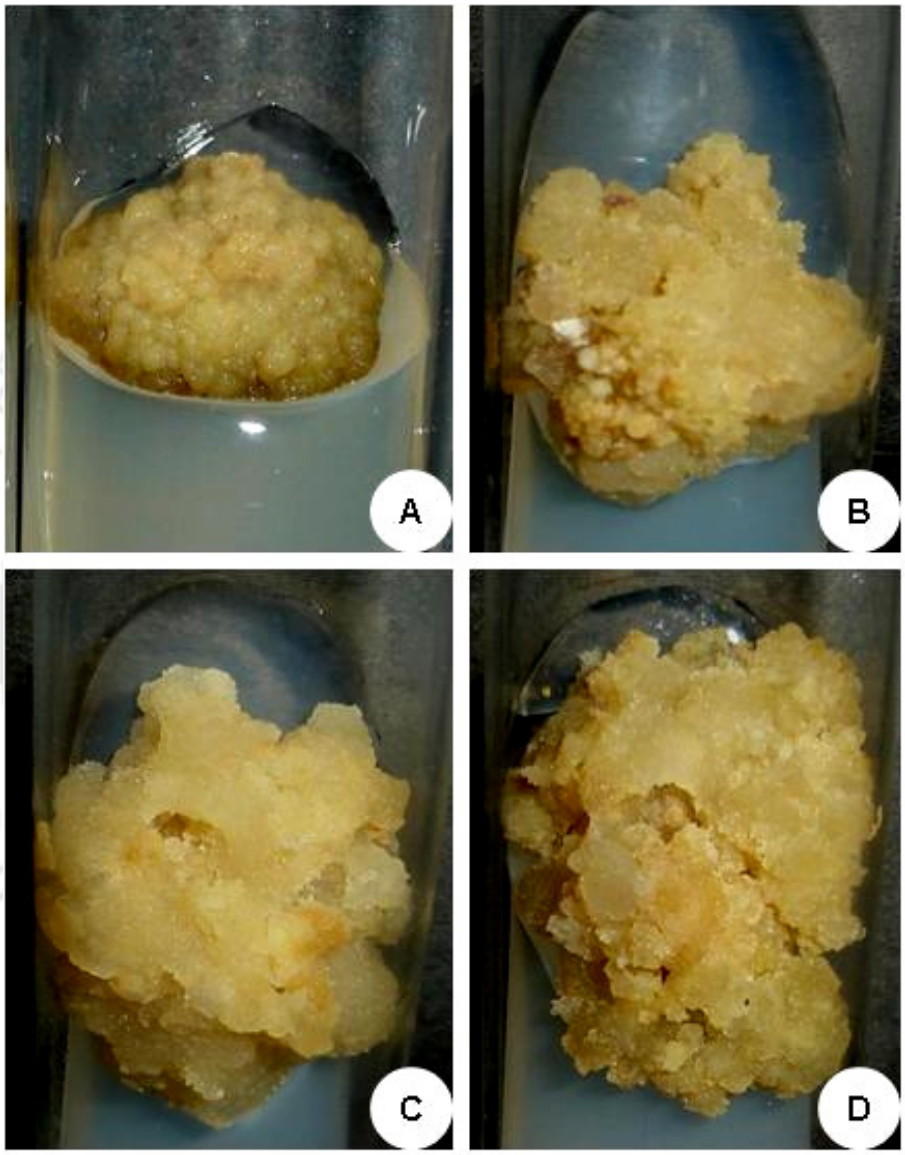

Figure 3

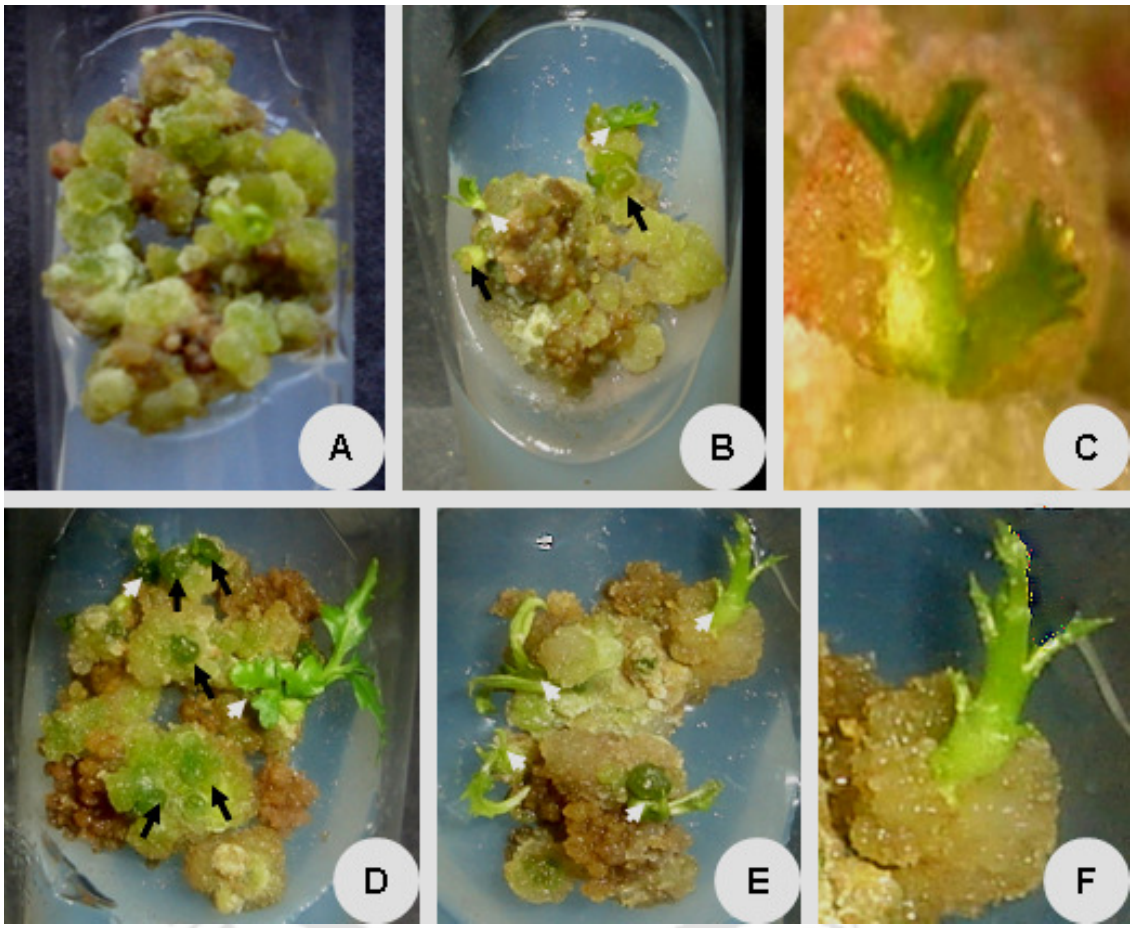

Figure 4

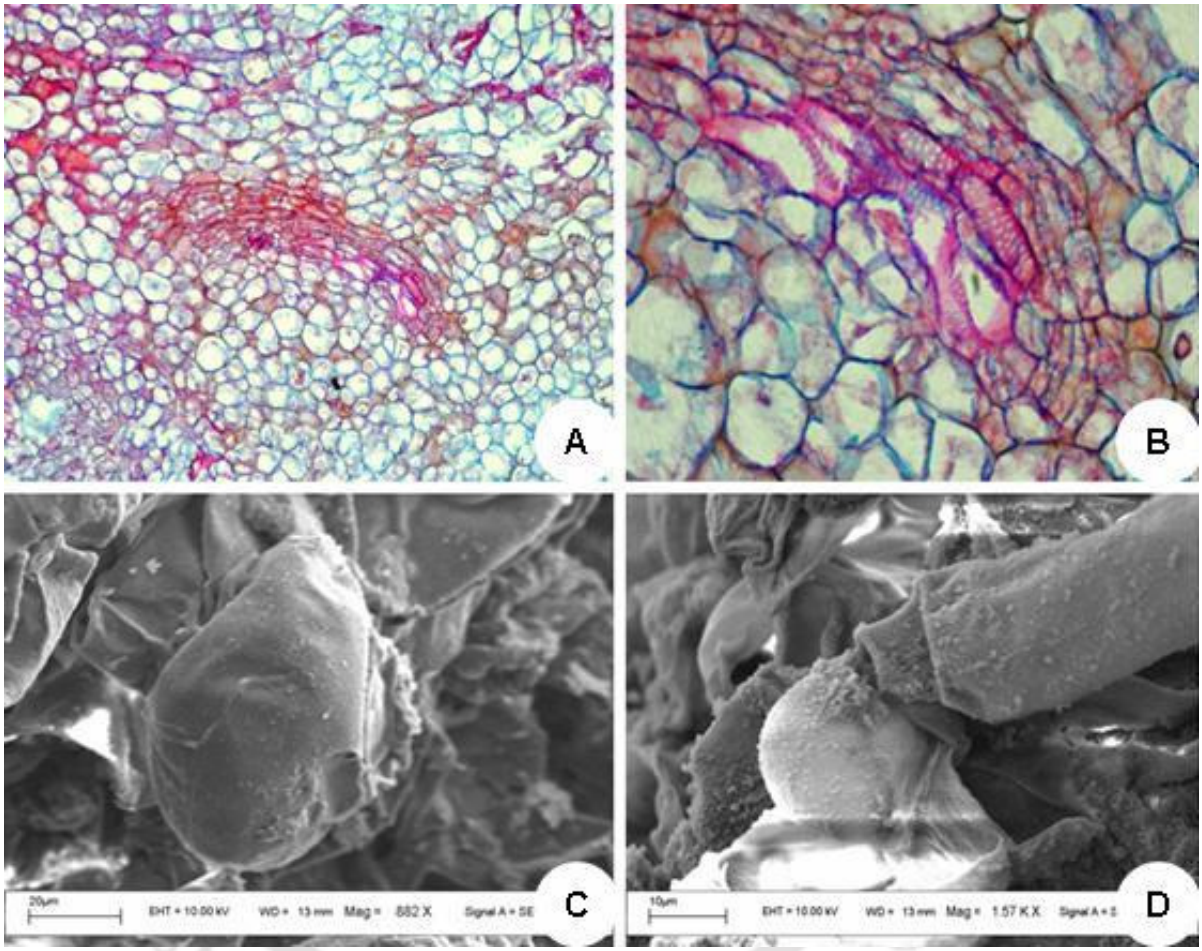

Figure 5

Figure 6

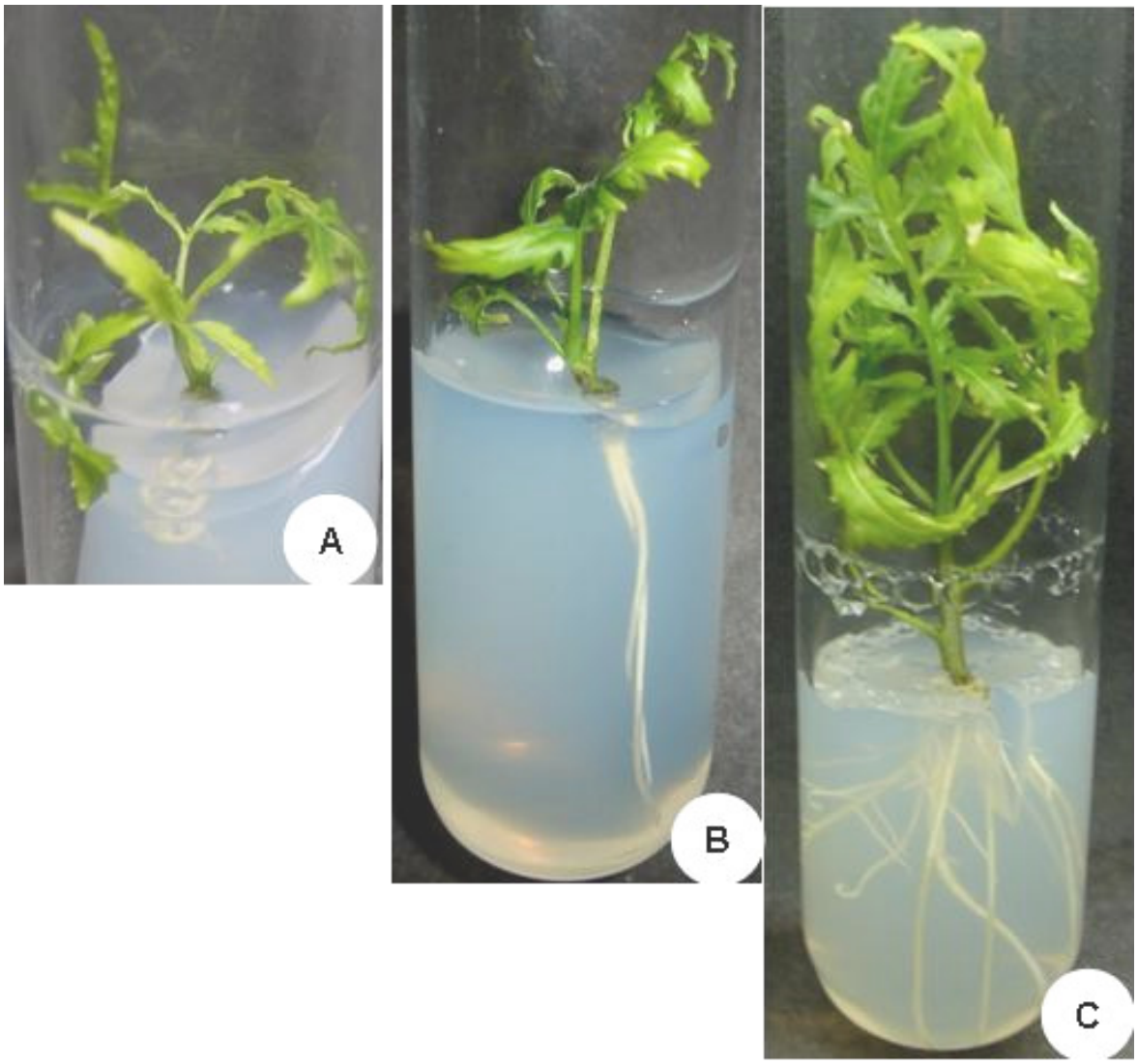

Figure 7

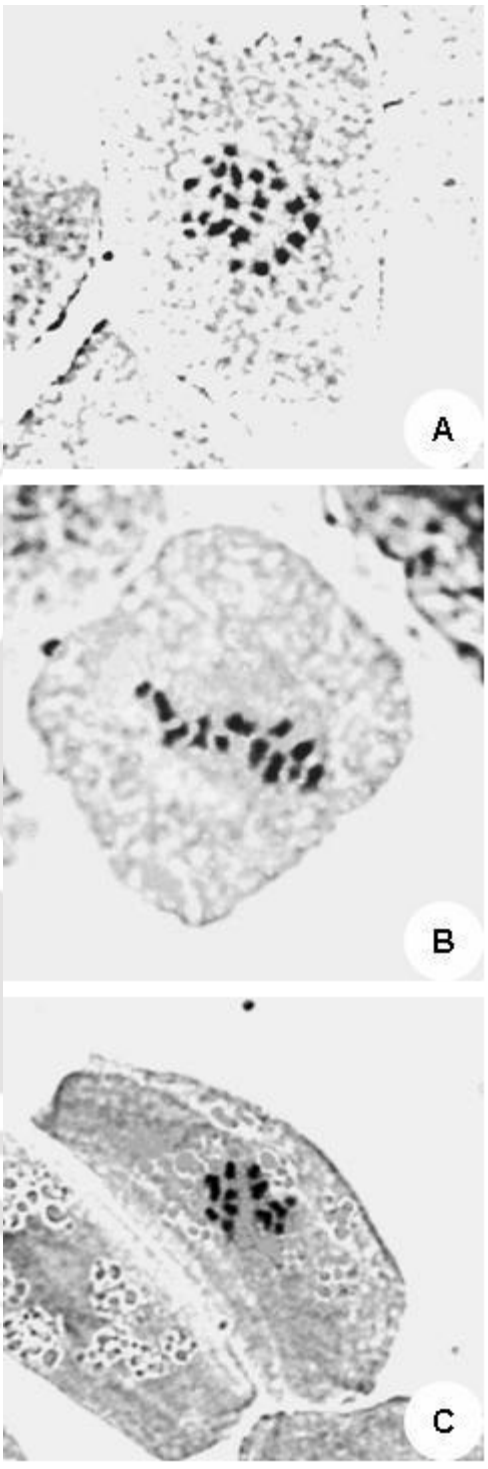

Figure 8

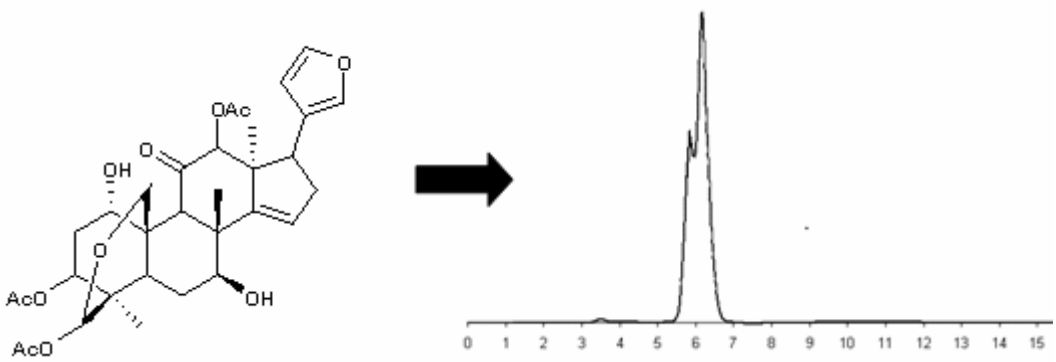

Figure 9

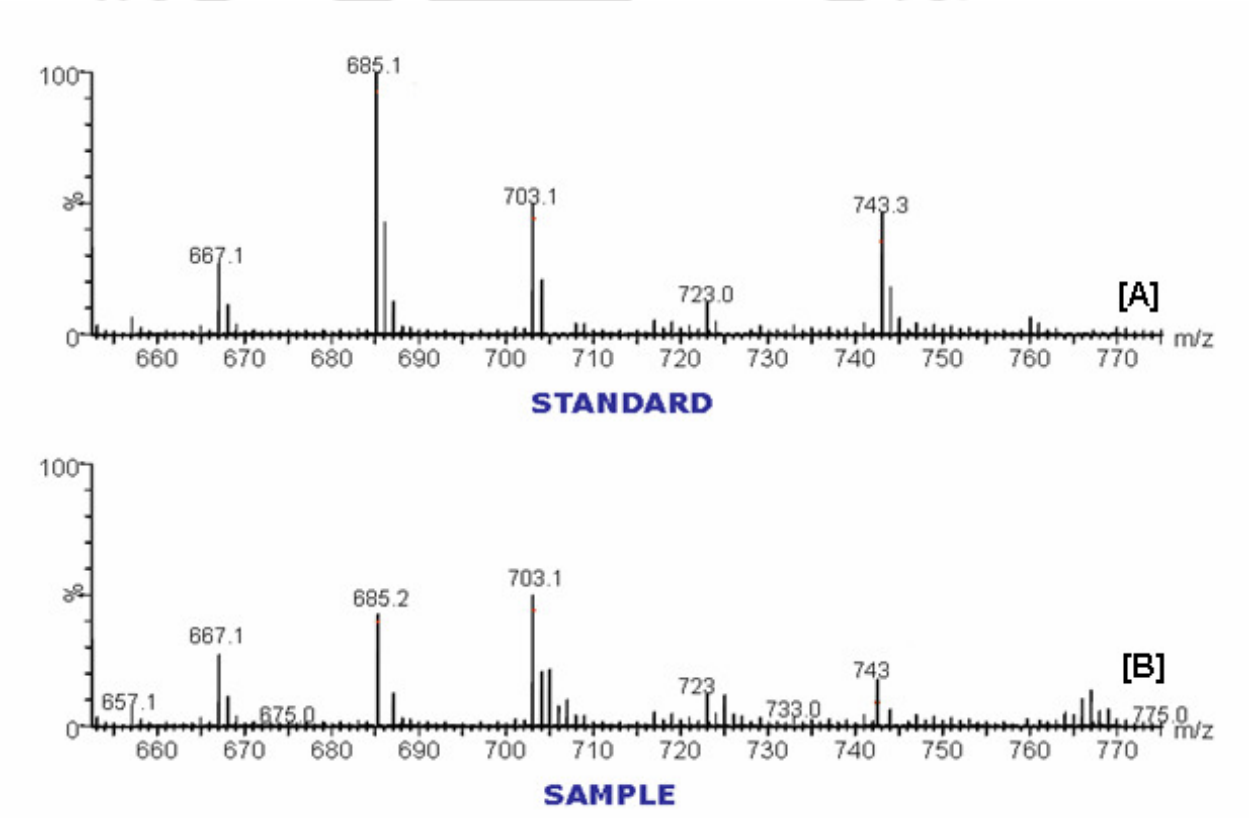

Figure 10

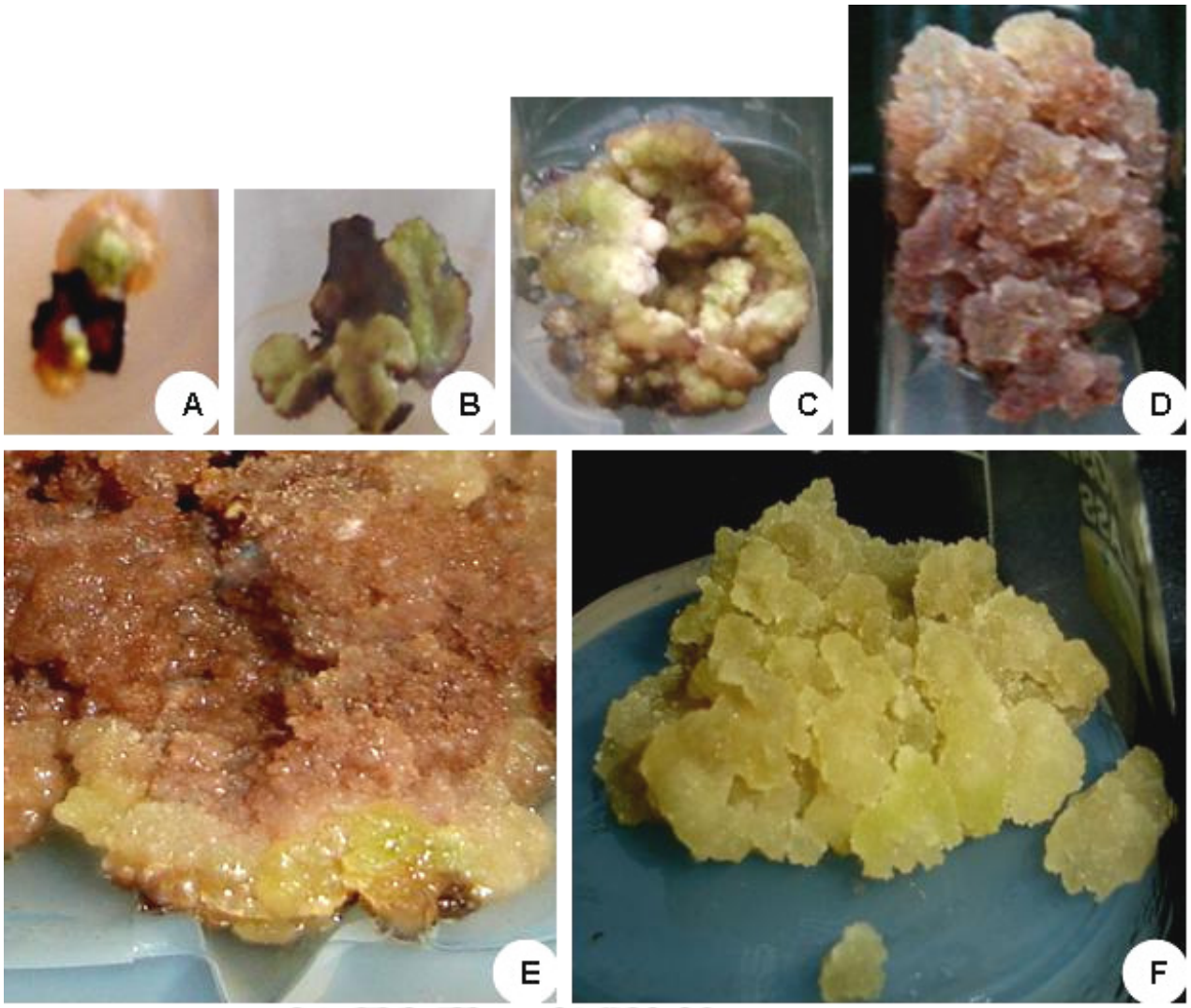

Figure 11

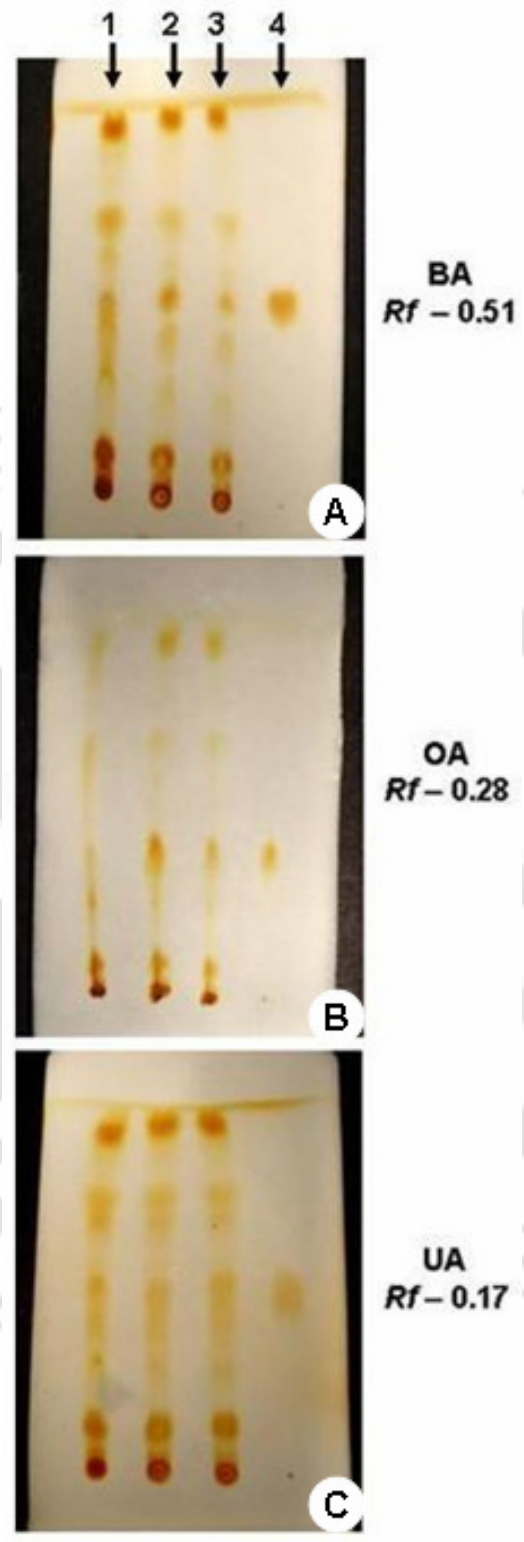

Figure 12

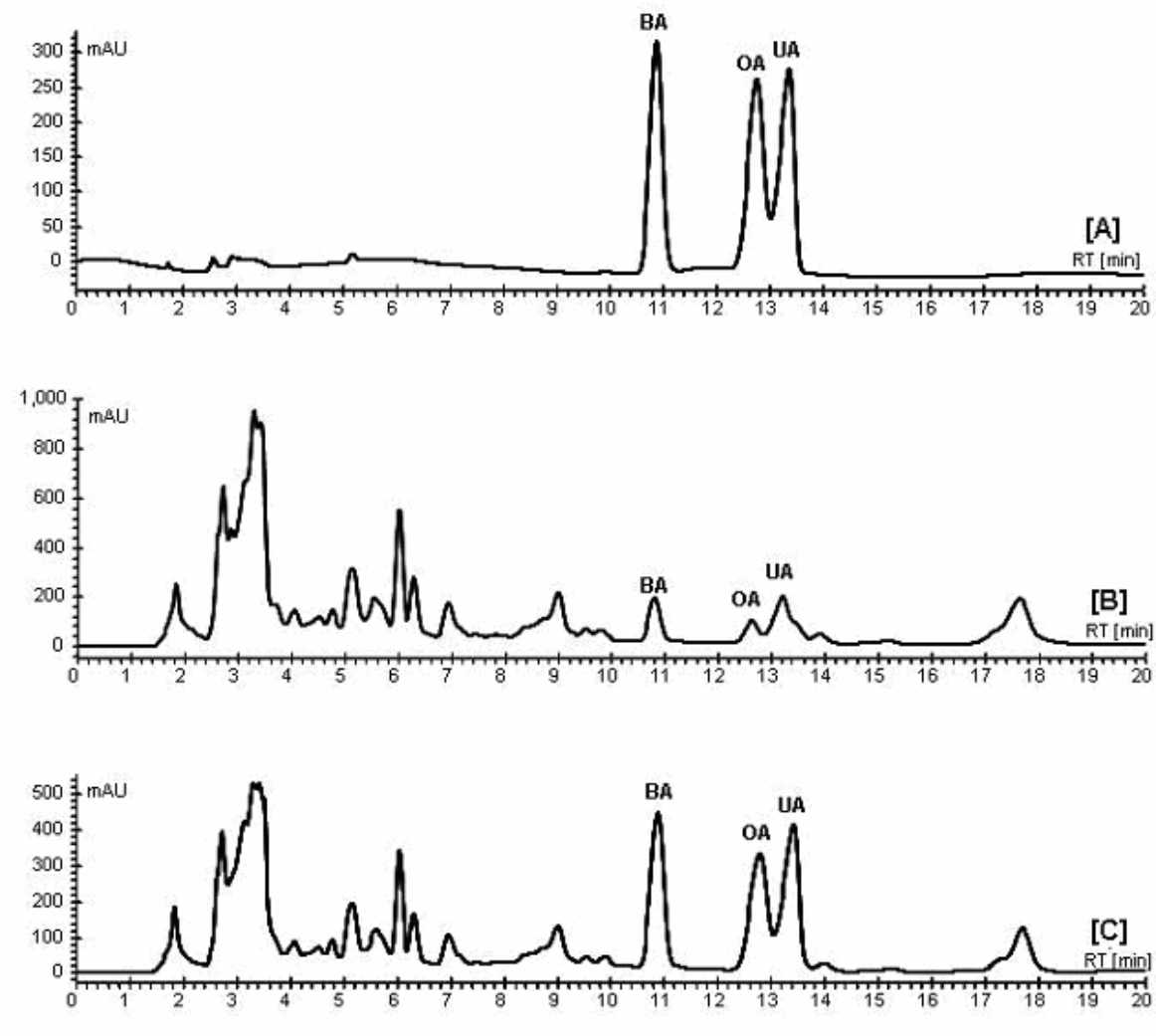

Figure 13

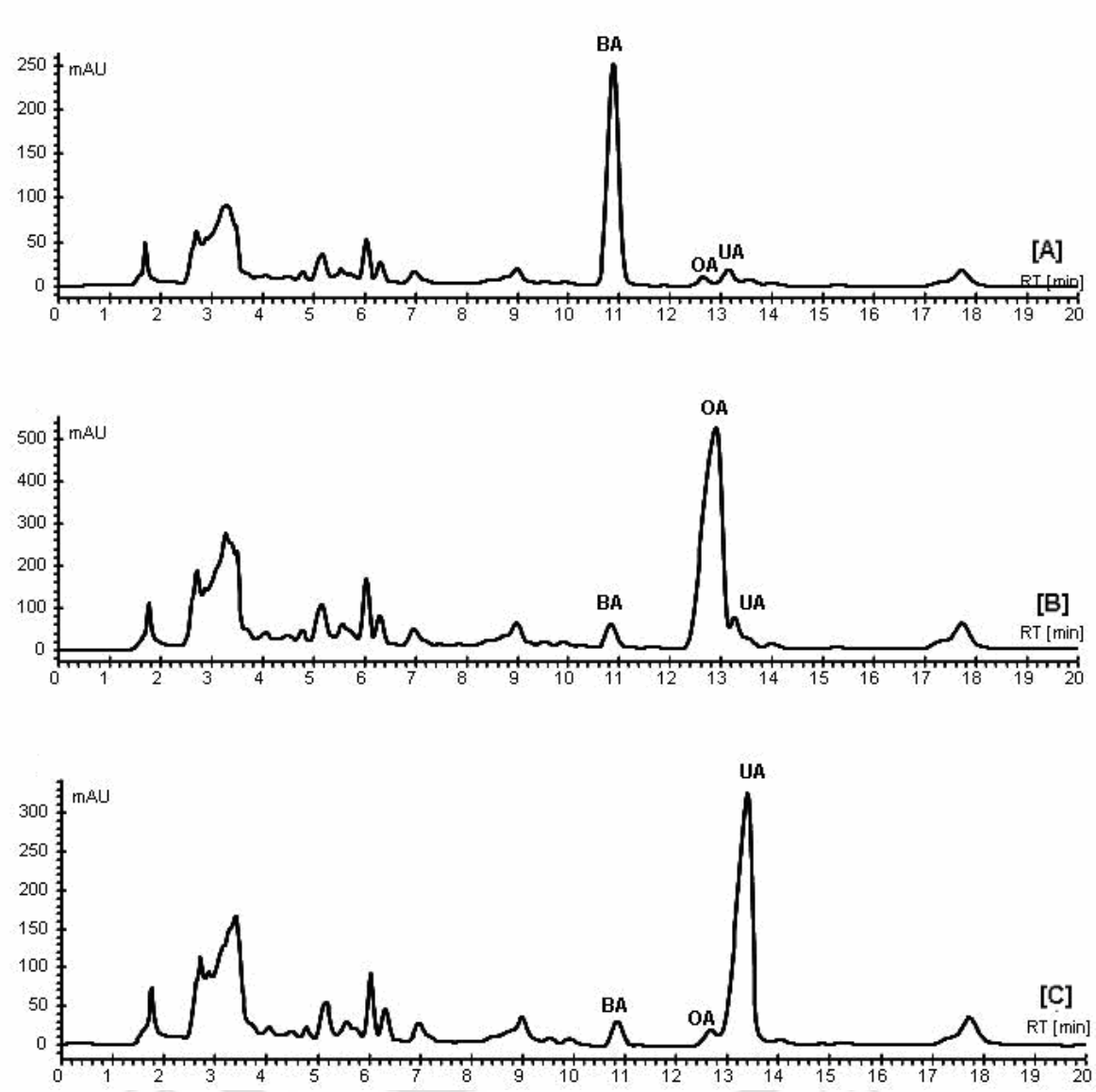

Figure 14

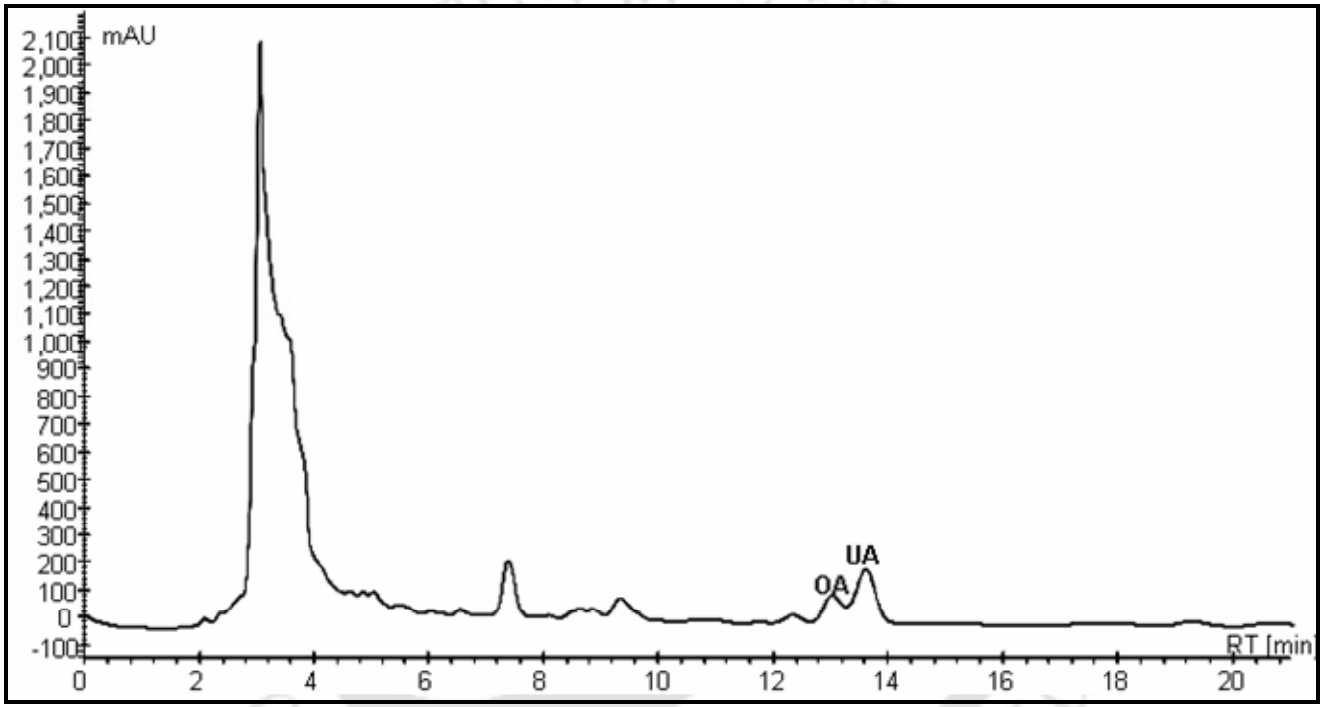
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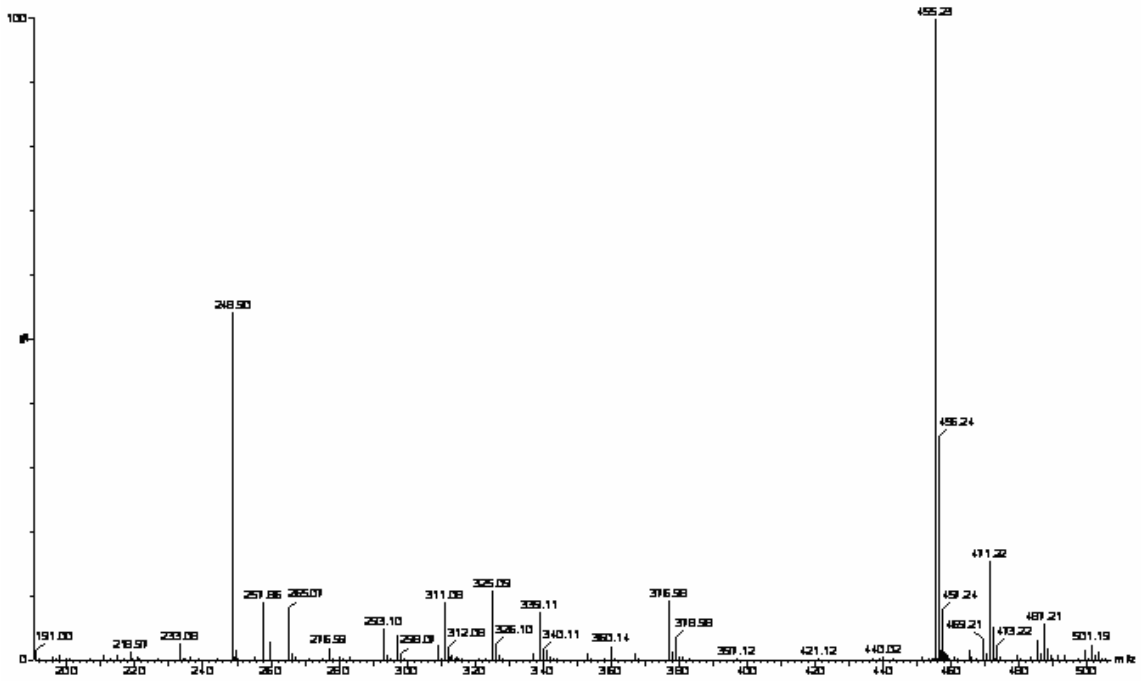
Figure 15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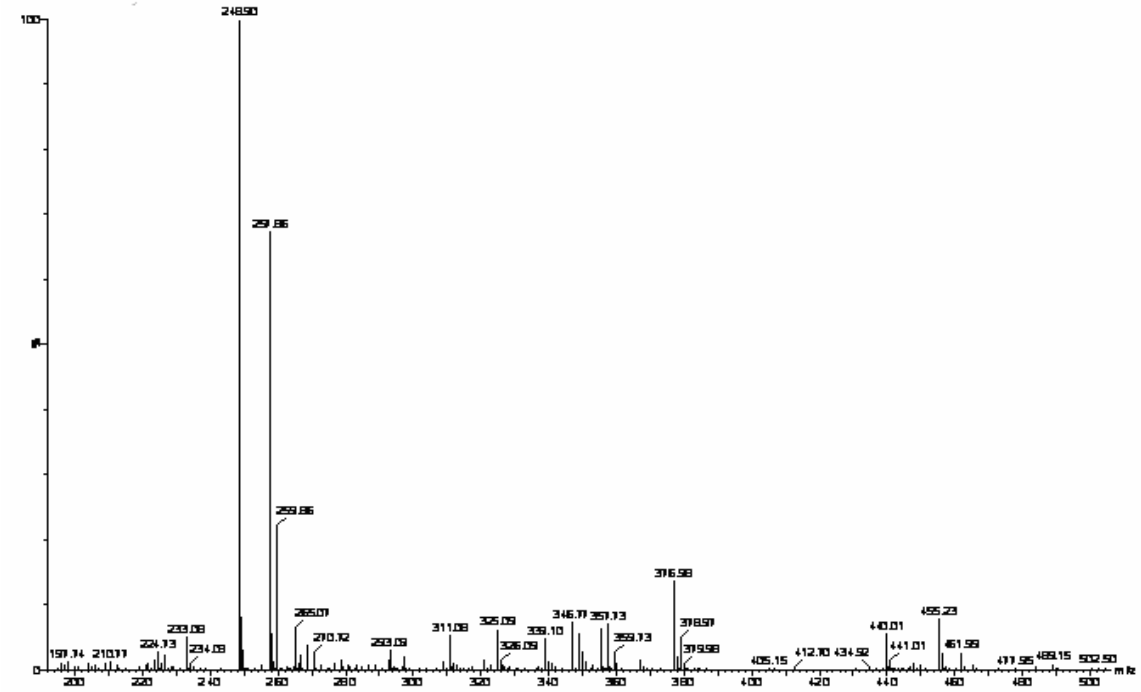
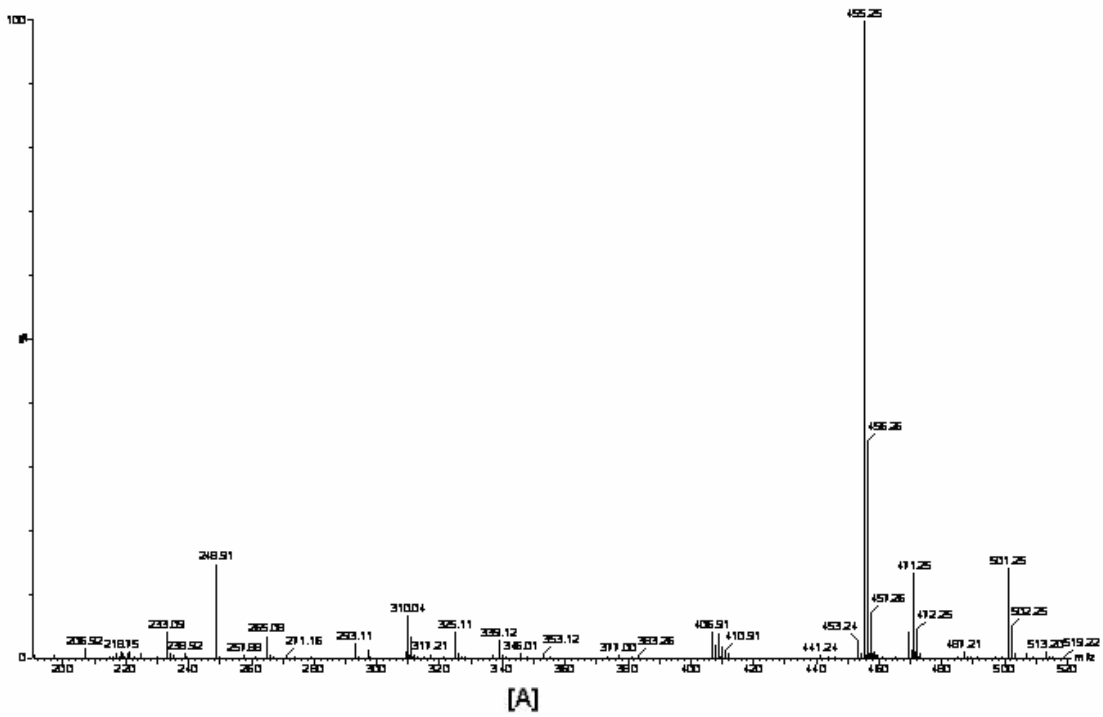
m/z

[A]

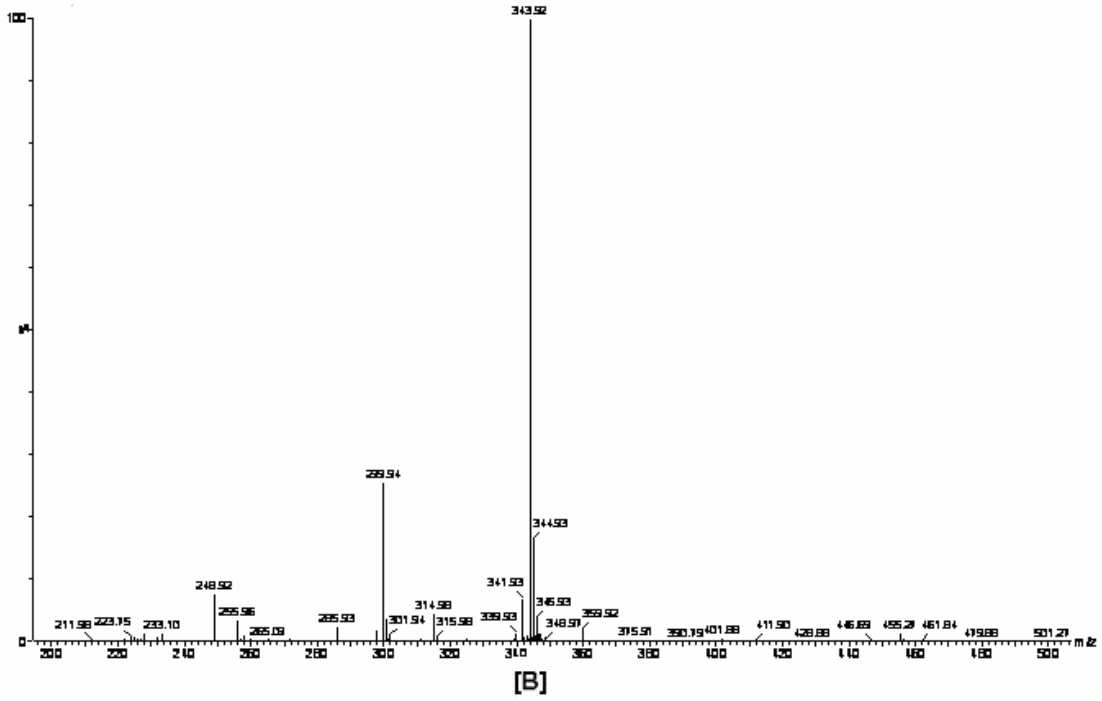

100
%
248.90
259.86
265.07
270.92
293.09
311.08
325.09
326.09
339.10
346.77
351.93
359.93
376.58
378.97
379.98
405.15
412.70
434.52
440.01
441.01
455.23
461.99
471.95
489.15
502.50
159.74
210.97
224.93
233.08
234.08
200
220
240
260
280
300
320
340
360
380
400
420
440
460
480
500
m/z

[B]

Figure 16


100
%
0
455.25
456.26
248.91
501.25
471.25
457.26
472.25
502.25
310.04
325.11
233.09
265.08
271.16
293.11
336.12
353.12
406.91
410.91
453.24
441.24
481.21
513.20
519.22
m/z

[A]


100
%
0
343.92
299.94
344.93
248.92
341.93
314.98
345.93
255.96
265.09
285.93
301.94
315.98
339.93
348.91
359.92
211.98
223.75
233.10
m/z

[B]

Figure 17

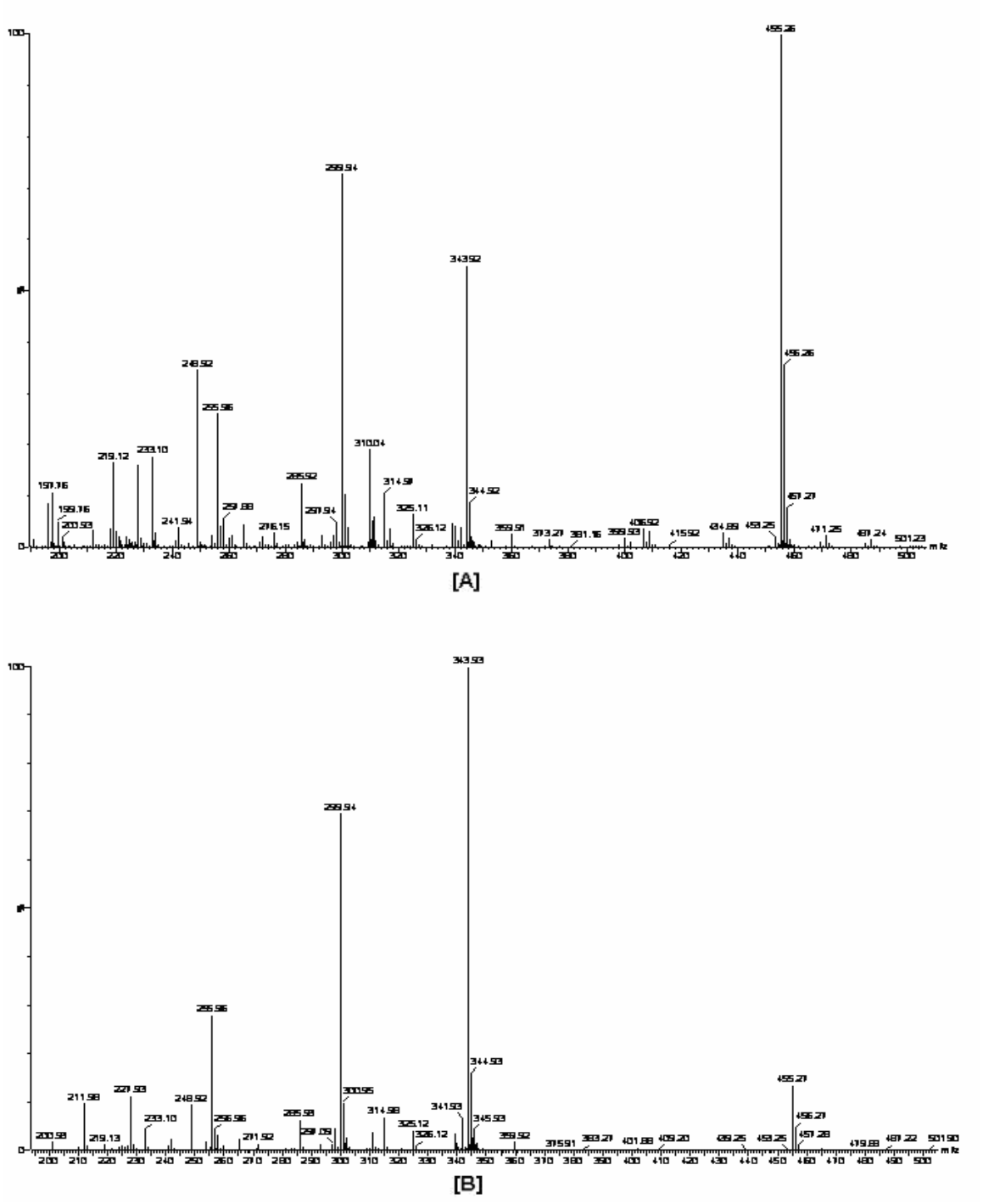


Figure 18

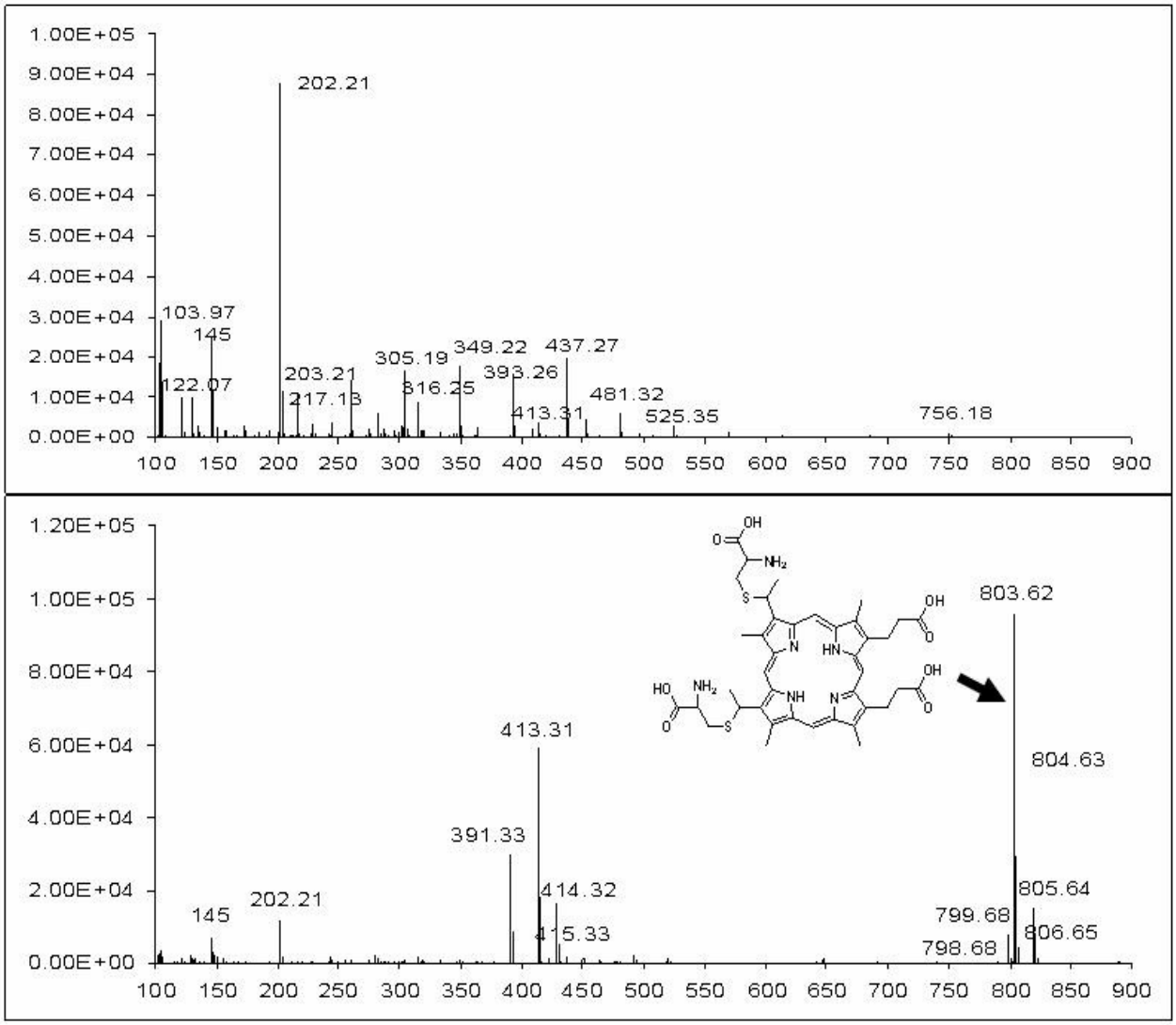

1.00E+05
9.00E+04
8.00E+04
7.00E+04
6.00E+04
5.00E+04
4.00E+04
3.00E+04
2.00E+04
1.00E+04
0.00E+00
202.21
103.97
145
122.07
203.21
217.13
305.19
316.25
349.22
393.26
413.31
437.27
481.32
525.35
756.18
100 150 200 250 300 350 400 450 500 550 600 650 700 750 800 850 900
1.20E+05
1.00E+05
8.00E+04
6.00E+04
4.00E+04
2.00E+04
0.00E+00
803.62
804.63
805.64
806.65
799.68
798.68
413.31
391.33
414.32
415.33
145
202.21
OH
NH2
HO
HN
NH
N
S
O
100 150 200 250 300 350 400 450 500 550 600 650 700 750 800 850 900

Figure 19

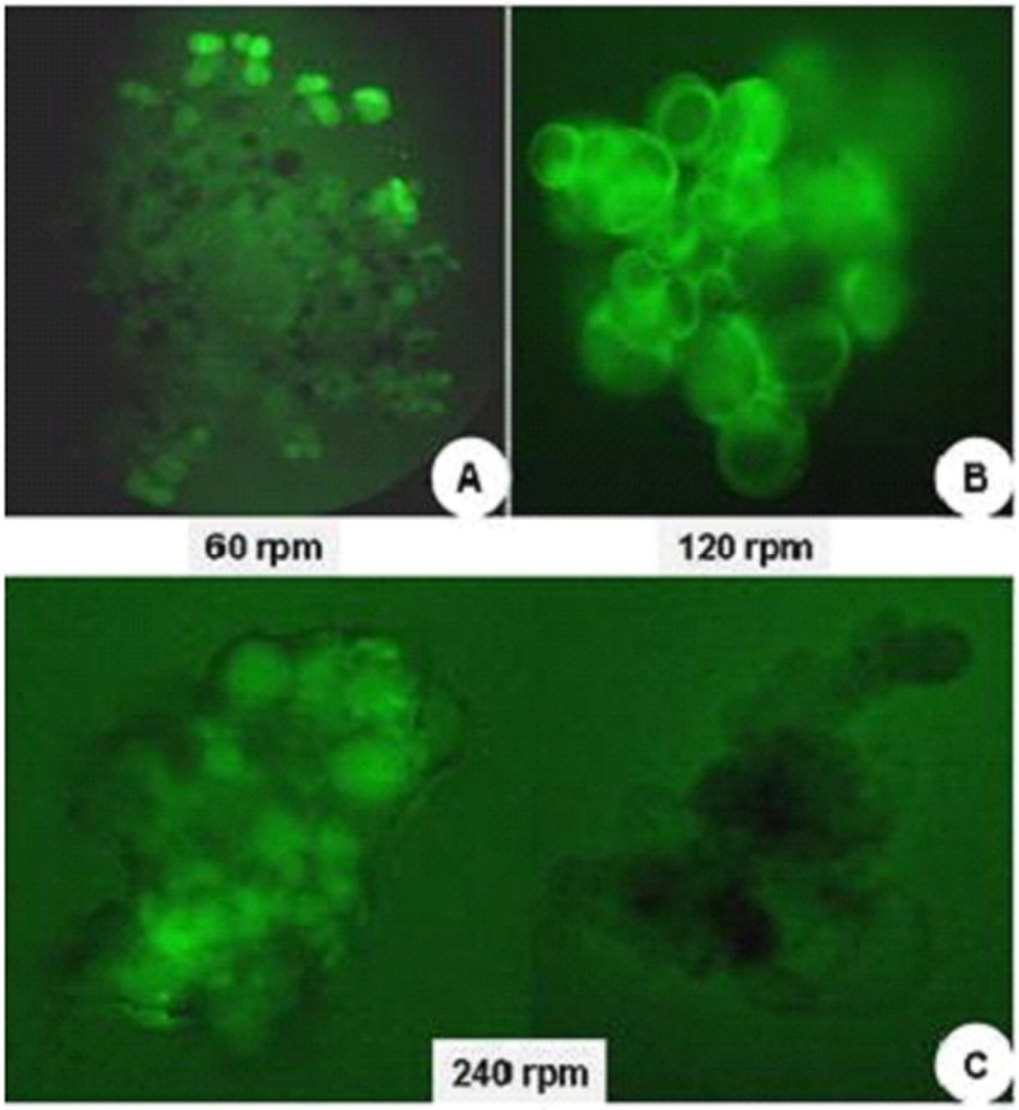


Figure 20

ORGANIC EXTRACT

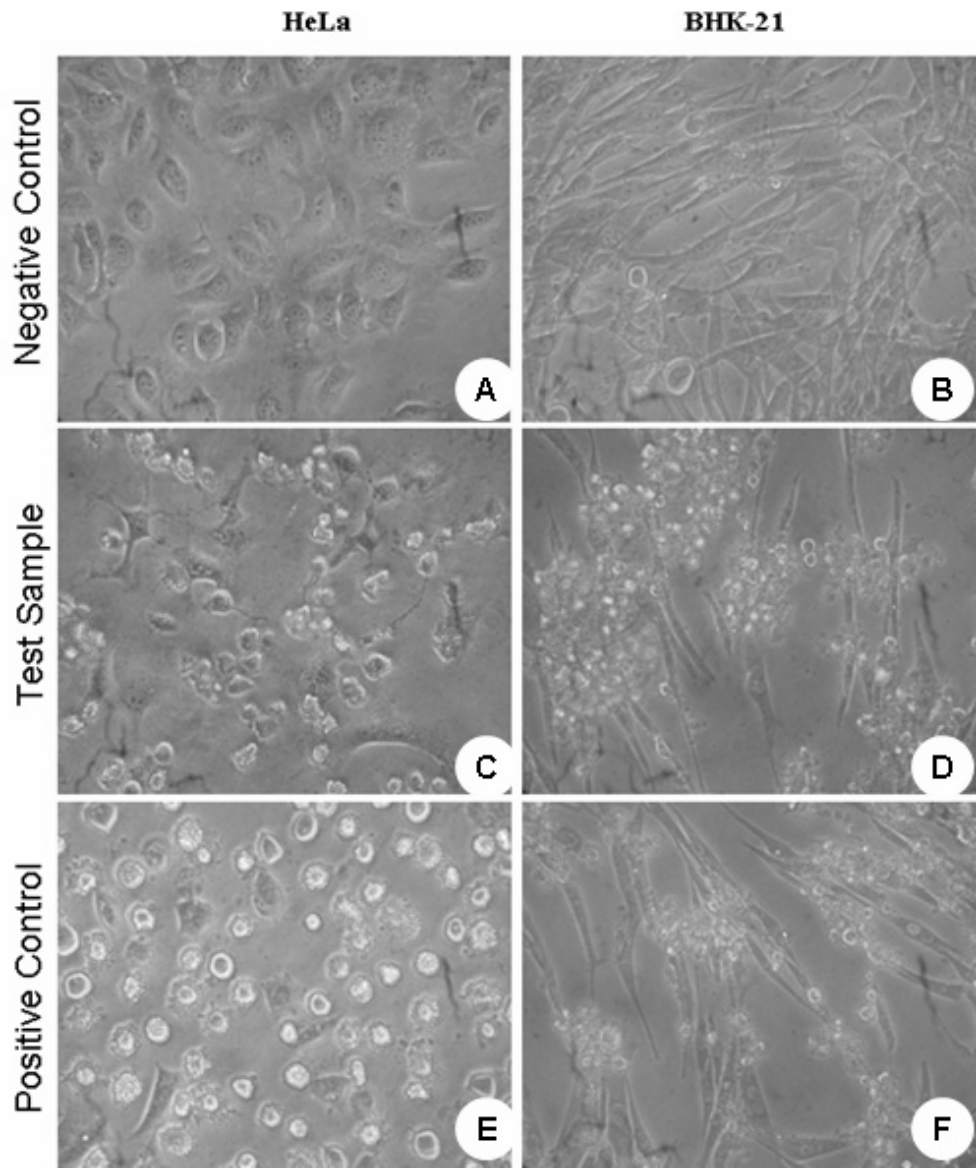


Figure 21

AQUEOUS EXTRACT

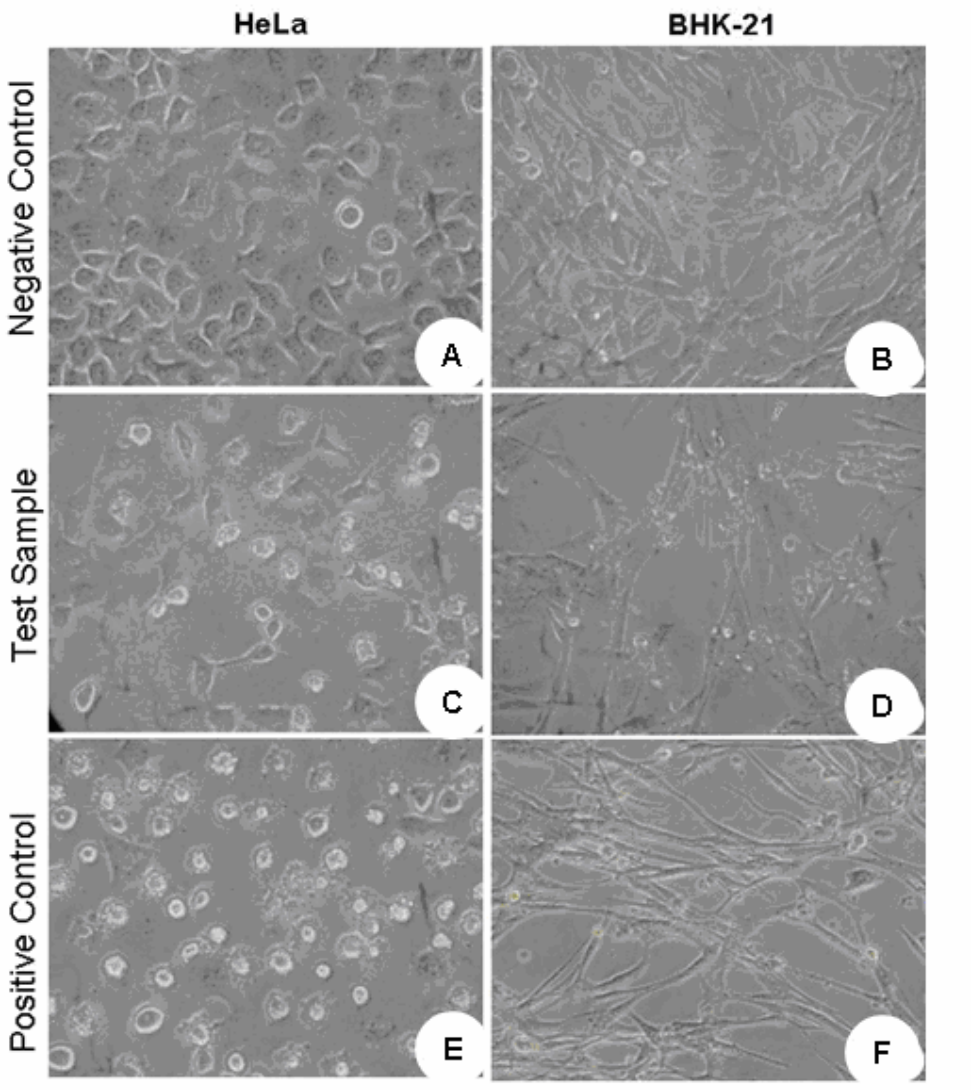


Figure 22

ORGANIC EXTRACT

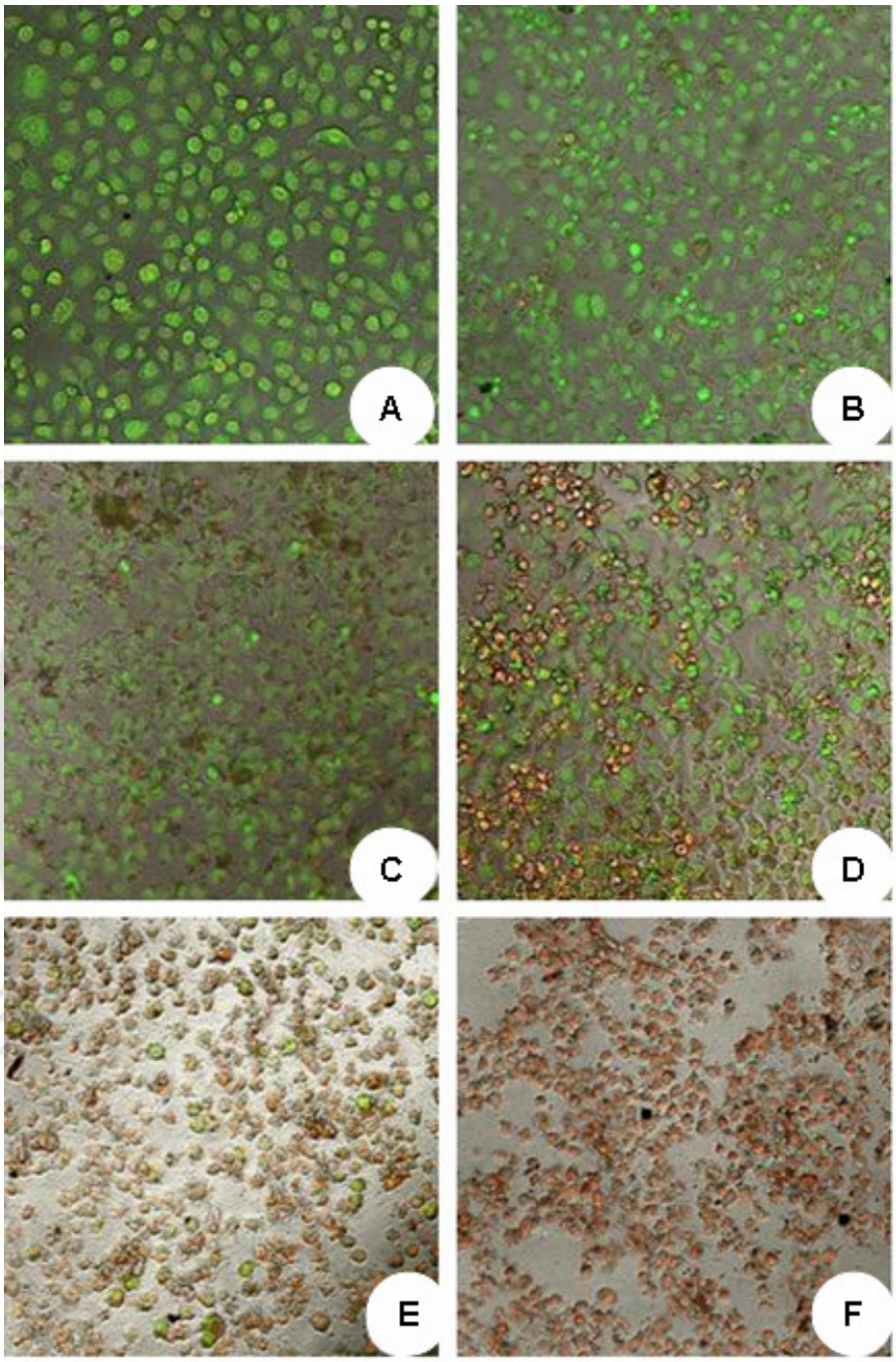


Figure 23

AQUEOUS EXTRACT

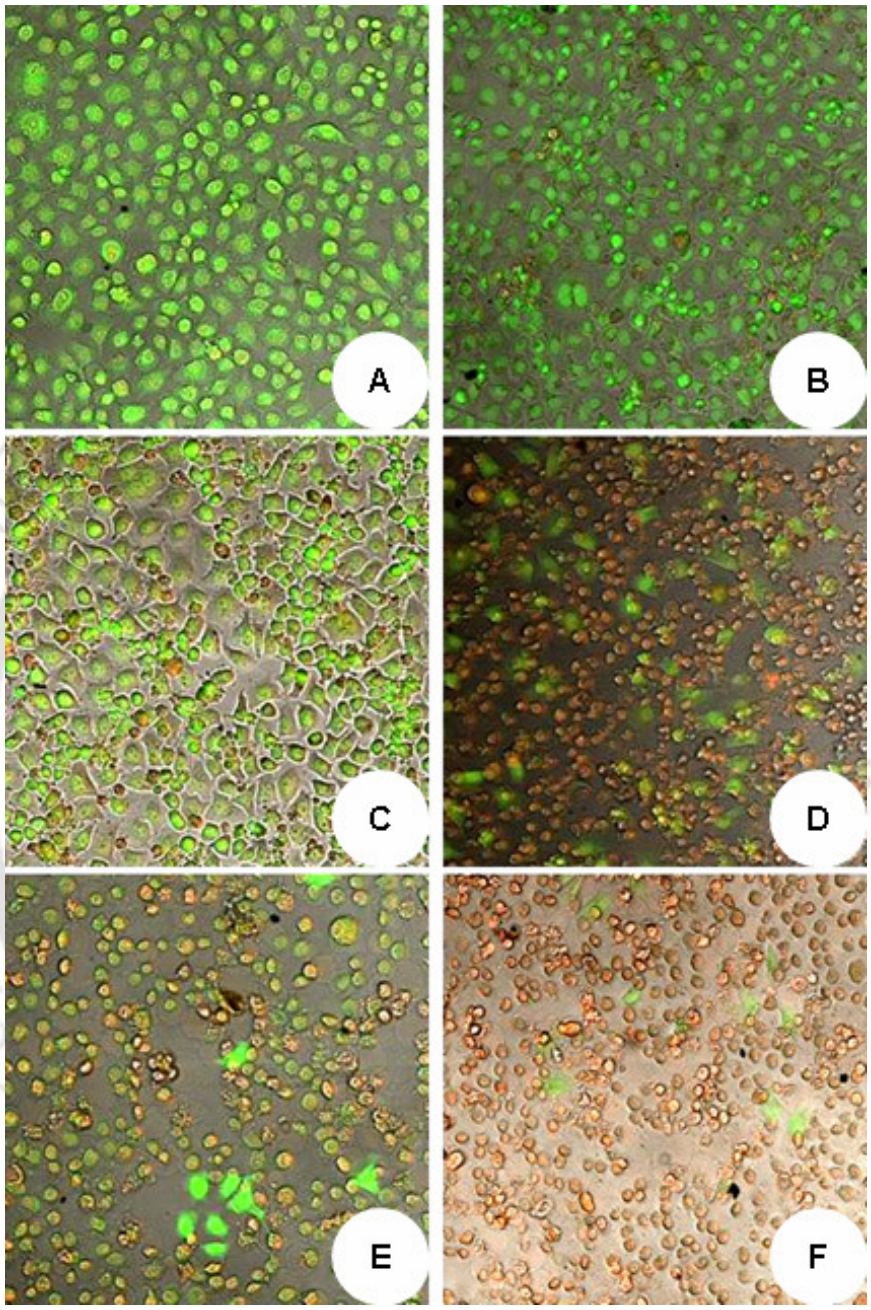


Figure 24

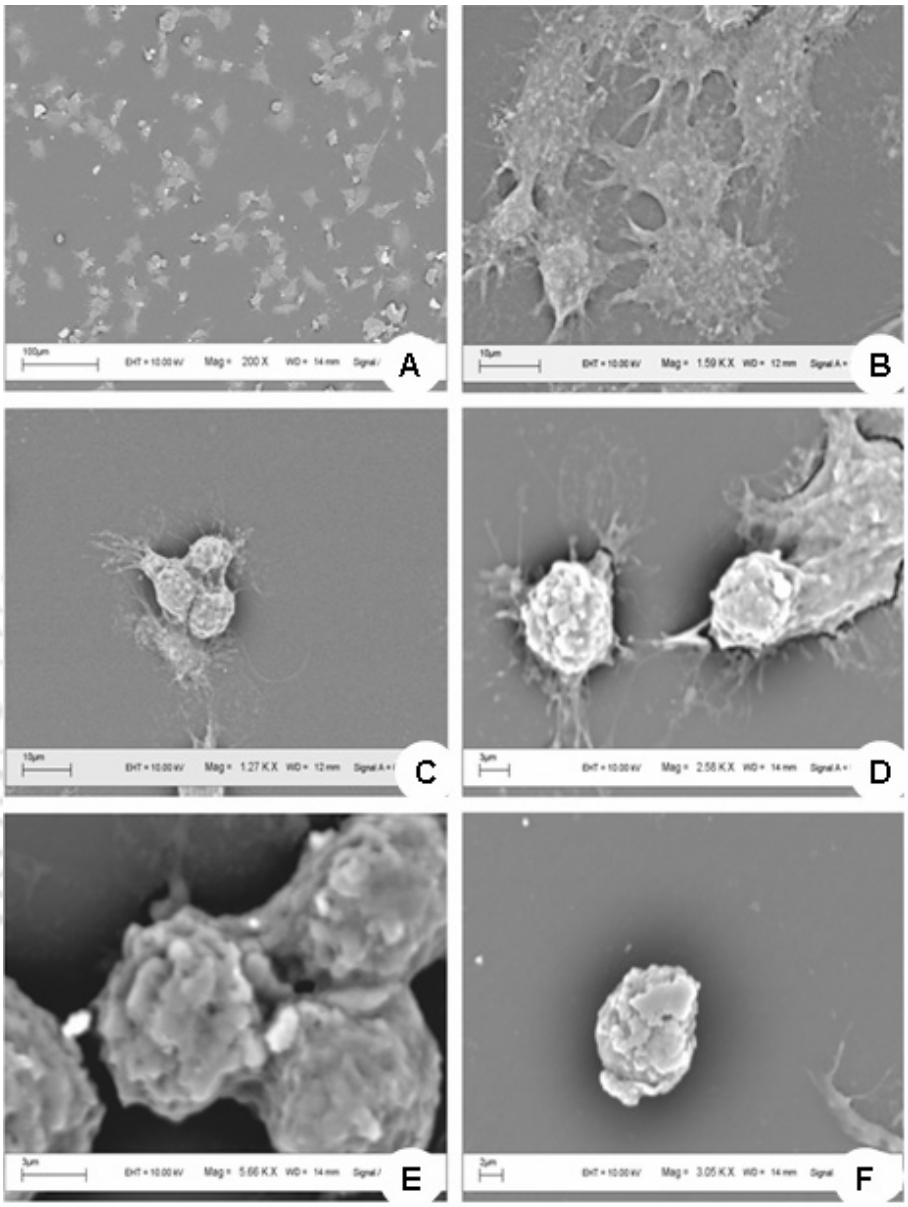


Figure 25

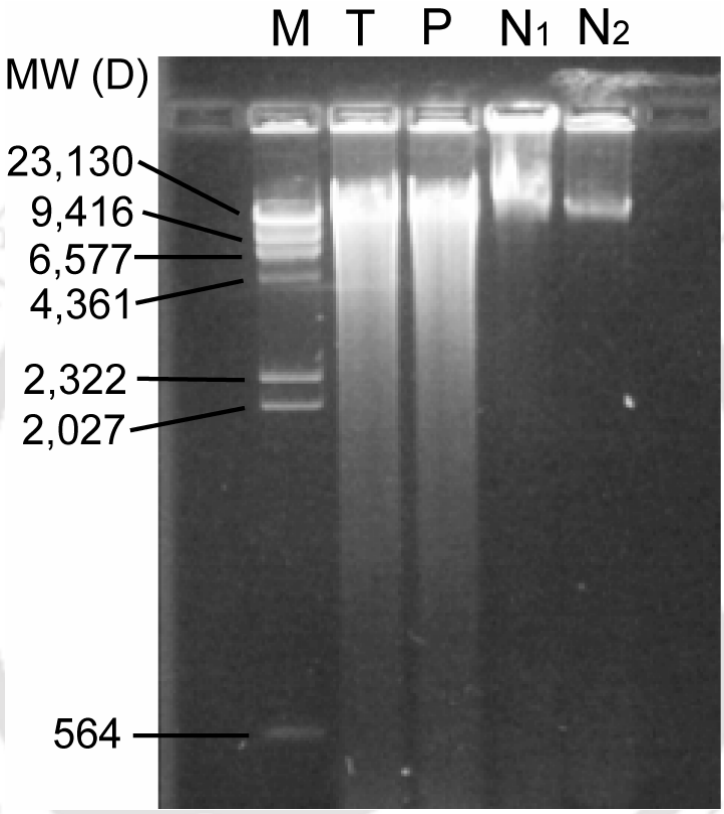


Figure 26