EXPLOITATION OF *IN VITRO* CULTURES OF INDIAN MADDER (*Rubia cordifolia*. Linn) FOR ANTICANCEROUS COMPOUNDS.

By

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THESIS

Submitted in partial fulfilment of the requirement for the degree of

Master of Science in Agriculture

(PLANT BIOTECHNOLOGY)

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2009

DECLARATION

I hereby declare that the thesis entitled **"Exploitation of *in vitro* cultures of Indian Madder (*Rubia cordifolia.* Linn) for anticancerous compounds."** is a bonafide record of research work done by me during the course of research and the thesis has not previously formed the basis for the award to me of any degree, diploma, associateship, or other similar title, of any other University or Society.

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CERTIFICATE

Certified that the thesis entitled **"Exploitation of *in vitro* cultures of Indian Madder (*Rubia cordifolia.* Linn) for anticancerous compounds."** is a record of research work done independently by Mr. Labade Dinesh Sitaram (2006-11-108) under my guidance and supervision and that it has not previously formed the basis for the award of any degree, diploma, fellowship or associateship to him.

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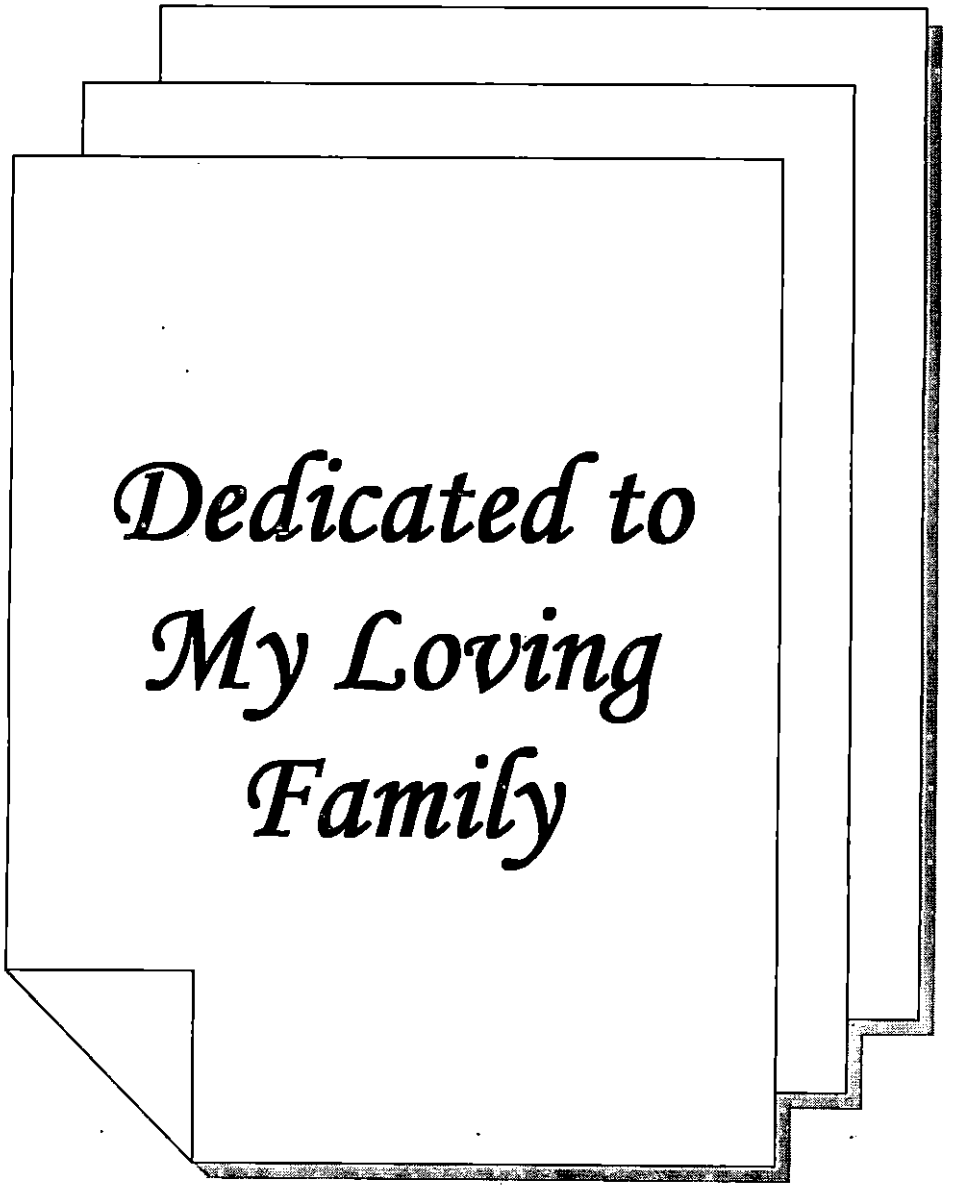
Dedicated to
My Loving
Family

Acknowledgement

I humbly bow my head before the lord ***Almighty*** *who blessed me with the power and courage to complete this endeavour successfully in spite of the most difficult times faced by me during the period of my study.*

Words cannot express my deep sense of gratitude and indebtedness to ***Dr. M. Asha Sankar****, Professor, Department of Plantation crops and Spcies and chairperson of my Advisory Committee. I wish to place my heartfelt thanks to her for her inspiring guidance, untiring help, encouragement, constructive criticism and valuable suggestions during the period of the investigation and preparation of the thesis. My sincere and heartfelt gratitude ever remains with her.*

I owe a great debt of gratitude to ***Dr. P.A. Nazeem****, Professor and Head, CPBMB and member of my advisory committee for her unwavering support and enthusiasm, relevant suggestions and whole hearted cooperation during my academic career.*

I am ever grateful to ***Dr. R. Keshavachandran****, Professor, CPBMB and member of my advisory committee for his invaluable help, guidance and critical assessment throughout the period of the work. I thank him for all the help and cooperation he has extended to me.*

I express my sincere gratitude to ***Dr. Jose Padikkala****, Professor, Amala Cancer research centre and member of my advisory committee for his valuable suggestions and guidance rendered me for the completion of the research programme and preparation of the thesis.*

I also avail this opportunity to pay my sincere obligations and heartfelt thanks to ***Valsala*** *madam,* ***Augustin*** *sir,* ***Rajendran*** *sir,* ***Sujatha*** *madam of CPBMB and* ***Kuttan*** *sir of Amala Cancer Research Centre for their encouragement and kind help offered at different stages of the study.*

I acknowledge the whole hearted co-operation of ***Sri. Krishan Nair,*** *Head of Microbiology, College of Veterinary and Animal Science, for the help rendered in*

taking fluorescent photographs associated with DCFH staining technique employed in my thesis work.

*I sincerely thank Sri. S. **Krishan**, Assistant Professor (Sel. Grade), Department of Agricultural statistics, for his immense help in the statistical analysis of the data.*

*I thank Mr. P. K. **Sreekumar**, Farm Assistant, CPBMB, for the help in photography.*

I wish to express my sincere thanks to all the non-teaching staff members and labourers of CPBMB for their whole hearted cooperation and timely assistance.

*Special thanks go to **Santhosh chettan**, Students Computer Club, COH, for rendering necessary help whenever needed.*

*I express my sincere thanks to **Fijesh. P. Vijayan, Vineesh. V. R., Adil, A., Jaimsha, R., Reshmi, R., Ann Geogre** for their timely and valuable help during my thesis work at Amala Cancer research Centre.*

*I express my deep sense of gratitude to research workers of Dept. of Plant Pathology, Department of Plantation Crops and Spices CPBMB and Bioinformatics Centre, **Gleena chechi, Suma chechi, Amrathaa chechi, Anu chechi, sunanda chechi, Shruthi chechi, Alisha chechi, Reena chechi, Shailaja chechi, Jini chechi, Shiba chechi, Soumya chechi, Sherin chechi, Simi chechi, Omana chechi, Firoz chettan, Sunil chettan** and **Shajahan**, who helped me in several ways for the completion of this venture. Words of gratitude fall short of expressing the care and concern rendered to me by my most esteemed **Praveena chechi** and **Rekha chechi**.*

*Words fall short as I place on record my indebtedness to my seniors and class mates, **Neema chechi, Likhitha chechi, Sameera chechi, Anjali chechi, Geena chechi, Ramya chechi, Saisree chichi, Liffey chettan, Fayas chettan, Shiji, Asha, Jusna, Ragina, Renu, Rashmi, Sindhu, Kimi, Hema, Nisha, Swetaleena, Sathish, Thiagarajan, Rahul, Jaba, Alok, Suja, Kiran,** and **Shivaji** for their prompt help and cooperation for the entire period of study.*

*I am in dearth of words to thank my friends **Tushar, Prashant, Santosh, Sunil, Atul, Rahul, Abhijit, Dinkar, Girish, Raj, Supriya, Meenal and Niharika** for their encouragement and lively support.*

With gratitude and affection, I recall the boundless affection, constant encouragement, warm blessings and motivations from my parents, sisters and brothers without which this endeavour would never have become a reality.

Labade Dinesh Sitaram

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INTRODUCTION

Throughout history, plant derived products and their modified analogues have been rich sources of clinically useful drugs. Modern pharmacopoeia contains at least 25 per cent drugs which are derived from plants or that are semi-synthetic analogues built on prototype compounds isolated from plants (Chabner, 1991). Cancer, one of the most dreaded diseases of the 20th century, responsible for 12 per cent of world's mortality.

Global estimates have indicated that about 10 million new cases and 67 million deaths occur due to cancer. All approaches for prevention and cure of cancer, may be complemented by chemoprevention strategies that could significantly reduce cancer incidence in high risk population (Hofseth *et al.*, 2001). High cost of synthetically derived cancer drugs and their hazardous side effects often pose problems in chemotherapeutic regimes for cancer cure. Hence plant originated cancer chemotherapeutic drugs have to be prioritized in cancer cure regimes, rendering them readily available in domestic market at fairly affordable prices, wherein many indigenous medicinal flora are likely to give good yields.

Among the lesser exploited medicinal species, in modern medicine, Indian Madder (*Rubia cordifolia.* Linn) is a prospective species, with anticancerous properties. It is a climber, known as 'Manjistha' in Sankrit and is seen in evergreen forests of Peninsular India and in the hilly tracts of North West Himalayas, eastward upto an altitude of 2500 M (Chatterjee and Prakashi, 1997). In indigenous medicine, it is reputed as an efficient blood purifier, extensively used against inflammations. It has a large history in skin care and treatment and is administered internally as well, for disorders of the urinary tract (Deshkar *et al.*, 2008). It improves complexion and is used against cough, hepatic obstruction and indigestion.

Antiproliferative property of ethanol extract of whole plant of *Rubia cordifolia* against epidermal carcinimoid cells has also been reported (Tripathi and

Shukla, 1998). Koyama *et al.* (1992) have isolated naphthoquinones from the roots of this species, which exhibited cytotoxicity property. In view of the threatened status of the species due to indiscriminate exploitation by destructive harvesting and habitat destruction and the limited amount of antitumour principles present in the plant, biotechnological interventions are viable options for isolation and scaling up of anticancerous principles from the species.

Also, the crop is amenable to *in vitro* manipulations, as has been revealed in our earlier attempts to propagate and conserve the crop *in vitro*, as a part of a DBT funded collaborative project, with 'Arya Vaidyasala, Kottakkal'. In view of the preliminary reports regarding the cytotoxic property of the plant, investigations on the possibility of *in vitro* synthesis of the anticancerous principles, naphthoquinones, from the species assumes importance.

Hence the present study on "Exploitation of *in vitro* cultures of Indian Madder (*Rubia cordifolia.* Linn) for anticancerous compounds" was taken up at the Centre for Plant Biotechnology and Molecular Biology, College of Horticulture, Vellanikkara from 2006 - 2008 with the following objectives:

1. To standardize *in vitro* techniques for initiation and proliferation of static and suspension cultures of *Rubia cordifolia.*
2. To screen *in vitro* cultures and *in vivo* plants for the synthesis of naphthoquinone.
3. To enhance production of naphthoquinones in *in vitro* cultures employing biotechnological interventions.
4. To assess the anticancerous activity of plant/ callus, through investigations on their cytotoxicity and antioxidant activity.

REVIEW OF LITERATURE

REVIEW OF LITERATURE

2.1 Importance of natural products in anticancer therapy

Nature is an attractive source of new therapeutic compounds as tremendous chemical diversity is found in millions of species of plants, animals, marine organisms and microorganisms. Chemo diversity in nature offer a valuable source, like secondary metabolites from the both hemisphere (Evans, 1996).

The development of novel agents from natural sources present obstacles that are not usually met with, when one deals with synthetic compounds. For instance, there may be difficulties is accessing the source of sample, identification and isolation of the active compound in the sample and problem in synthesizing the necessary amount of the compound of interest.

The drug discovery process increasingly requires the availability of a large number of compounds. Basically there are two different approaches for drug discovery, one is screening and other is to improve the efficacy of traditional medicine and to resolve the mode of action.

Higher plants produce a large number of diverse organic chemicals, which are of pharmaceutical and industrial interest (Dholwani *et al.*,2008). Hence high throughput screening of plant for their antitumor property has played important role in drug discovery and numerous useful drugs have been developed from lead compounds isolated from medicinal plants.

2.2 Anticancerous compounds from plants

Cragg and Newman (2006), in their review on plants used against cancer, listed more than 3000 plant species that have reportedly been used in the treatment of cancer. In recent years, extensive screening of antitumor agents from plants has been undertaken. National Cancer Institute in USA who began the project as early as in

1956, after screening nearly 114000 plants of about 1500 genera, has reported that 4.3% of the plants showed antineoplastic activity (Suffness and Douros, 1982).

Some of the major anticancerous compounds and their sources reported by Misawa and Endo (1995) are given below.

Table 1. Major anticancerous compound and their sources

Compound	**Plants**
Baccharine	*Baccharis megapotamica*
Bruceantine	*Brucea antidysenterica*
Cesaline	*Caesalpinia gillisesii*
3-Deoxycolchicine	*Colchicum speciosum*
Ellipticine, 9-methoxellipticine	*Ochrosia moorei*
Fagaronine	*Fagara zanthoxyloides*
Harringtonine, homoharringtonine	*Cephalotaxus harringtonia*
Holacanthone	*Holacantha emoryi*
Indicine N-oxide	*Heiotropium indicum*
Maytansine	*Maytenus bucchananii*
Podophyllotoxin	*Podophyllum peltatum*
Taxol	*Taxus brevifolia*
Thalicarpine	*Thalictrum dasycarpum*
Tripdiolide,triptolide	*Tripterygium wilfordii*
Vinblastine, vincristine	*Catharanthus roseus*
Camptothecin	*Camptotheca accuminata*

Two of the most significant chemotherapeutic agents, vincristine and vinblastine are isolated from periwrinkle, (*Catharanthus roseus*). The Vinca alkaloid vincristine was responsible for an increase in the curing rate of Hodgkin's disease and

some forms of leukemia (Noble, 1990). Another example of a highly active agent derived from a natural product is etopside, which has produced high cure rate in testicular cancer, when used in combination with bleomycin (Willimas *et al.*,1987). The Taxanes- 'Paclitaxel' and 'Docetaxel' show impressive antitumour activity against breast, and ovarian cancer, leading to mitotic arrest.

A number of additional plant derived agents are currently under investigation. A brief overview of the potential anticancerous compounds isolated from plants is given below.

2.1.1 Flavopiridol

Flavopiridol is one of the most important plant based antineoplastic agents currently in development, representing the first cyclin dependent kinase inhibitor to enter the clinic (Kelland, 2000). It is a synthetic flavone derived from the plant alkaloid rohitenkine which was isolated from the leaves and stem of *Amoora rohitaka* and later, from *Dyoxylum binectariferium*. The mechanism of action of flavopiridol involves interfering with the phosphorylation of cyclin dependent kinases, hampering their activation and blocking cell cycle progression at growth phase.

2.1.2 Camptothecin

Camptotheca acuminata, a native of North China, was found to produce a potent antitumor quinoline alkaloid, camptothecin(CPT) as reported, by Wall *et al.*. in 1966. Hsiang and Liu, (1988) reported that CPT and derivatives are potent inhibitors of topoisomerase, an enzyme essential for the replication of DNA. According to Takimoto and Arbuck, (1996) the topoisomearase activity of this agent is stereospecific, with the naturally occurring S-isomer of CPT being much more potent inhibitor than the R-isomer. It is highly active in Walker 256 rat carcinosarcoma and mouse leukemia, p388 and L1210. The clinical trials in patients with gastrointestinal cancer were at first very promising but subsequent trials showed

toxicity. Several natural and synthetic derivatives, including 9- amino and 10-hydroxy camptothecin, topotecan and irinitocen (CPT-11) are also potent antitumor and DNA topoisomerase I inhibitory agents (Dholwani *et al.*, 2008).

2.1.3 Homoharringtonine

Homoharringtonine, together with harringtonine and isoharringtonine, were isolated from *Cephalotaxus harringtonia* by Powell *et al.* in 1969. Homoharringtonine is an alkaloid and has shown efficiency against various leukemia. The mechanism of action is the inhibition of protein synthesis, blocking cell cycle progression etc. A racemic mixture of harringtonine and homoharringtonine (HHT) has been used successfully in treatment of acute myelogenous leukemia and chronic myelogenous leukemia (Cragg and Newman, 2006).

2.1.4 Ipomoeaonol

4-Ipomoeaonol is a pneumotoxic furan derivative isolated from the *Ipomoea batata* (Rehm and Devor, 1993) and has been under clinical evaluation as a lung cancer specific antineoplastic agent. This compound is converted to DNA binding metabolites upon metabolic activation by cytochrome beta 450 enzymes that are present in cells of the lung. Similarly, beta lapachone is a DNA topoisomerase I inhibitor, that induces cell cycle decay at G_1 or Synthesis phase before inducing either apoptotic or cell death in a variety of human carcinoma cells, inducing ovary , lung and breast cancer.

2.1.5 Podophyllotoxin

Podophyllum pelatatum, May apple, which is a common herb in eastern North America contains an antitumor lignan, podophyllotoxin. It is active to KB cells and is used against certain virus diseases and skin cancer. A semi-synthetic derivative of podophyllotoxin, etoposide (V-16) and VP-26 (teniposide,) was found to be active

against brain tumor, lymphosarcoma and Hodgkins' disease and was approved by the Food and Drug Administration in the U.S. Hande, (1998) reported the role of etoposide in inhibiting topoisomerase II leading to DNA strand breaks and hence cytotoxicity.

2.1.6 Vinca Alkaloids

The dimeric indole alkaloids, vinblastine and vincristine have become highly valued drugs in cancer chemotherapy due to their potent antitumor activity against various leukemias, Hodgkin's disease and solid tumors. They are currently produced commercially by extraction from *Catharanthus roseus* plants, but the process is not efficient because of very low concentrations of the alkaloids in the plant. Fahy, (2001) reported that modification in vinca alkaloids resulting in structural analogues have implications on tubulin interaction activity.

2.1.7 Taxol

Under the intensive National Cancer Institute screening program of antitumor compounds in the U.S., Wall *et al.* (1966). began to isolate an active principle against KB cells from a tree, *Taxus brevifolia*. Pure taxol was first isolated by Wani *et al.*, (1971). It is a diterpene amide and has shown activity against B16 mouse melanoma tumor, the MX-1 human mammary xenograft and CX-1 colon xenografts. The mode of action of taxol is rather unique because it stabilizes microtubules and inhibits depolymerization. The clinical trials begun in 1983 have shown positive results in the treatment of advanced ovarian cancer and breast cancer as well.

Plants play a vital role in maintaining human health and contribute towards improvement of human health. There are thousands of plant species having good potential of offering direct therapeutic effect individually or in combinations. Plants are considered as state of art chemical laboratories capable of biosynthesizing number of biomolecules of different chemical classes (Deshkar *et al.*, 2008). In the present

study, the experimental plant species chosen, *Rubia cordifolia* is screened for the presence of anticancerous compounds in *in vivo* plants as well as in *in vitro* systems derived from the plant. An exhaustive review of the species, its morphology, distribution, habitat, active principles and uses, is attempted.

2.2.1 *Rubia cordifolia*

Rubia cordifolia, L. of the family Rubiaceae, is an important herbal drug used in the Indian systems of medicine. The root of the plant is commonly known as Manjistha and sold in the market under the commercial name 'Manjith'. The plant drug has a number of vernacular names like Aruna, Bhandi, Bhandiralatik in Sanskrit, Mandar, Majathi in Assamme, Manjith, Manjistha in Bengali, Indian Madder in English, Manjatty in Malayalam, Manjestha in Marathi, Manjit in Hindi (Sharma, 1969). The roots of the plant are sweet, and acrid and are used as anti-inflammatory agents (Antarkar, *et al.*, 1984) and as haemostatics (Kosuge *et al.*, 1982). The roots have antidysentric, antipyretic, analgesic and anthelmentic properties. The crude drug obtained from the plant improves voice, and complexion and cures Kapha, inflammation of the uterus, vagina, eye, ear and also acts as a blood purifier. It is also used in curing leucoderma, ulcers, urinary discharges, jaundice, and piles (Sivarajan and Balachandran, 1997).

2.2.2 Distribution

Common throughout the hilly tracts of India from the Northern western Himalayas, eastward ascending to 2500m. the occurrence of *Rubia cordifolia* is also reported from Greece, Africa and other Asiatic countries like China, Japan, Afghanistan, Vietnam and Malaysia (Chatterjee and Pakrashi, 1997).

2.2.3 Morphology and reproductive biology

Rubia cordifolia, as perennial is a climber with very long, cylindric, flexuose roots with thin red bark (Kirtikar and Basu, 1980). Stems are long, rough,

grooved and become slightly woody at the base. Bark is white; branches are scandent, quadrangular, glabrous and shining. Leaves are heart shaped, 2-6 cm long, 1-4 cm wide arranged in four whorls. Flowers are in terminal panicled glabrous cymes, branches trichotomous, spreading bracts are ovate, acute and leafy. Calyx is 0.85 mm long, tube globose and glabrous. Corolla greenish and are divided nearly to the base, 5-lobed, ovate, acute and 3 mm long. Styles are 2 and stigmas globose. Fruits is 4-6 mm in diameter, didymous or globose, smooth, shining purplish black when ripe. (Deshkar *et al.*, 2008).

2.2.4 Anticancerous activity of *Rubia cordifolia*

Son *et al.*, (2005) found that the methylene chloride fraction from the roots of *Rubia cordifolia* L. (the methanol extract of this root was subsequently fractionated into four fractions; methylene chloride, ethyl acetate, n-butanol and water) showed strong cytotoxicity against HT-29 and MCF-7 cell lines, as well as DNA topoisomerase I and II inhibitory activities.

This novel bicyclic hexapeptide (RA-XVII) was isolated from the roots of *Rubia cordifolia* L. showing significant cytotoxicity activities on P-388 murine leukemia cells and two more compound isolated from roots were D-2-aminobutyric and as residue 1, but longer carbon side chain at D-Ala-1 of peptide 1 decreased the activity (Hitotsuyanagi, 2004),

The antiproliferative property of *Rubia cordifolia* (Rubiaceae) extract was also tested on A-431 cells (epidermal carcinomoid cells) and 3T3 fibroblast cells. It was observed that a fraction of *Rubia cordifolia* significantly inhibited the incorporation of [3H]-thymidine, induced by fetal bovine serum, in a dose dependent manner (Tripathi and Shukla, 1998).

2.3 Active principles in *Rubia cordifolia*

The chemistry of *Rubia* species has been widely investigated, and phytochemical investigations in the species from both hemispheres led to the isolation of a number of physiologically active compounds viz., anthraquinones

and their glycosides, naphthoquinones and their glycosides, terpenes, bicyclic hexapeptides, iridoids and carbohydrates. (Singh *et al.*, 2004). The following groups of compounds are reported to be present in the plant.

2.3.1 Anthraquinones

Plants belonging to the family Rubiaceae are known to contain substantial amounts of anthraquinones, especially in the roots. *Rubia cordifolia* is a dye yielding plant of the family Rubiaceae, producing reddish orange dye from roots, stem and leaves (Singh *et al.*, 2004).

Anthraquinones are a class of natural products encompassing several hundreds of compounds, differeing in nature and position of substitutent groups (Schripsema *et al.*, 1999). Anthraquinones have been identified to be the colouring compounds present in the roots of *R.cordifolia.* Purpurin and manjistin are the two anthraquinones present in *Rubia cordifolia* (Mischenko *et al.*, 1999). Murti *et al.*, (1997) reported the presence of glycoside of lucidin and lucidin primeveroside, along with ruberythric acid from roots of *R.cordifolia* (Singh *et al.*, 2004). In one of the studies, a preparative high speed countercurrent chromatography (HSCCC) method, for isolation and purification of the bioactive component mollugin directly from ethanol extract of *Rubia cordifolia* was successfully established (Yanbin *et al.*, 2001).

2.3.2 Naphthoquinones and their glycosides

The first naphthoquinone to be isolated from *Rubia* species was 5- or 8-methoxy-3- 1,4 naphthoquinone (Singh *et al.*, 2004). The ethyl acetate soluble fraction of methanol extract of *Rubia cordifolia* also led to isolation of 2-carbamoyl -3-hydroxy-1,4- naphthoquinone and its 3-methoxy derivative (Koyama *et al.*,1992). The hexane soluble fraction of the same methanol extract gave mollugin and dehydro-alpha lapachone (Koyama *et al.*, 1992).

2.3.3 Triterpenoids and their glycosides

Rubia courmaric acid and rubiafolic acid were the first triterpene

constituents isolated from the chloroform extract of the defatted whole plant of *R. cordifolia* (Talapatra *et al.*, 1981). Later, three oleanolic type triterpenoids, rubiaprassin A-C, were isolated from the chloroform soluble fraction of methanolic extract of the roots of *Rubia cordifolia* (Itokawa *et al.*, 1989). Six arboran type triterpenoids, rubiarbonol A-F, were isolated from the methanol extract of *R. cordifolia* (Itokawa *et al.*, 1990).

2.3.4 Bicyclic hexapeptides

Cyclic hexapeptides of RA seriesfound in *Rubia* and are characterized by a bicyclic structure including a unique cycloisodityrosine unit. The bicyclic hexapeptides RA-III to RA-VII have been obtained from the benzene soluble fraction of the methanol extract of *Rubia cordifolia*. Similarly, RA-I and RA-II have been isolated from chloroform/methanol (1:1) extract of *Rubia cordifolia* as minor constituents (Itokawa, *et al.*, 1986). Further, butanol fraction of chloroform /methanol extract of *Rubia cordifolia* yielded RA-XI and three unique bicyclic hexapeptide glucosides RA-XII to RA-IV (Morita *et al.*, 1992). Hexapeptides isolated from roots of *Rubia cordifolia* were found to be cytotoxic. A novel antitumour bicyclic hexapeptide RAXVII was also isolated from roots of *Rubia cordifolia*. By spectral studies and synthetic approach, its structure was reported to be deoxybouvrdin (Hitotsuyanagi *et al.*, 2004).

2.3.5 Miscellaneous compounds

Beside the above mentioned groups of compounds, several other constituents are also reported in the species.

2.3.5.1 Iriodoids: 6-methoxy geniposidic acid is found along with manjistin, garancin and alizarin (Wu and Wang, 1991).

2.3.5.2 Naphthoic acid esters: Ester of naphthoic acid are isolated from the roots of *R. cordifolia* as suggested by Hua *et al.* (1992).

2.3.5.3 Other compounds

Many of flavonoids and carbohydrates like rhamnose, sucrose, arabinose,

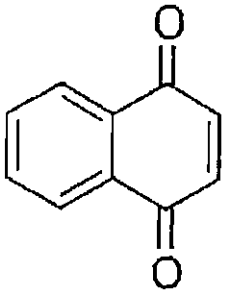
xylose, mannose etc have also isolated from Rubia species. were isolate from *Rubia cordifolia.*

2.4 Quinones and Naphthoquinone

Quinones are widely distributed in nature and constitute an important class of naturally occurring compounds. They are found in plants, fungi, and bacteria (Thomson, 2005). They have also been found in ambient particulate matter (Fraser *et al.*, 1998), automotive exhaust emissions (Schuetzle *et al.*, 1981), and wood smoke particles (Fine *et al.*, 2001). Plants are the repositories for bioactive organic molecules, among which quinones represent a class of ubiquitous secondary metabolites (Thomson, 1971). Quinones, notably naphthoquinones and anthraquinones, are among the most widely distributed natural products. Quinone compounds are aromatic rings with two ketone substitutions (Cowan, 1999). The largest sub group of natural quinones are the naphthoquinones, of which over 350 found in nature, the majority being microbial origin.

Naphthoquinones, compounds of natural origin, mostly appeared as chromatic pigments. They are deposited in cell vacuoles, where are dissolved in the form of glycosides. Naphtoquinones as a group of secondary metabolites occur in number of plant families (*Plumbaginaceae,Juglandaceae, Ebenaceae, Boraginaceae, Dioncophyllaceae Ancistrocladaceae, Iridaceae, Verbenaceae, crophulariaceae, Avicenniaceae, Balsaminceae, Bignoniaceae, Gentianaceae,Droseraceae, Nepenthaceae, Lythraceae, Euphorbiaceae*, fungi and microorganisms (*Streptomyces, Fusarium*) (Babulao *et al.*, 2005).

The majority of them exist as coloured phenolic compounds, useful as dyes and as pigments. The natural red dye including the anthraquinnones, naphthoquinones, and benzoquinones could be also found in a variety of plants (Koyama *et al.*, 2001). A significant number of these naphthoquinones possess the naphtha (2,3-c) pyran ring system and occur commonly as the 5,10-quinones with carbon substituent at C-1and C-3.



Naphthoquinone/1,4-Naphthoquinone/α-Naphthoquinone

1,4-Naphthoquinones and 1,2- Naphthoquinones are widely distributed in nature, containing a quinone nucleus, such as anthracyclines and mitoxantrones that show excellent anticancer activity. Naphthoquinones are very toxic for which antimicrobial, antifungal, antiviral and antiparasitic effects were observed. A kind of naphthoquinone, plumbagin has insect antifeedant (Kubo *et al.*, 1980), cardiotonic (Itoigawa *et al.*,1991), anticancer (Parimala and Sachdanandam, 1993), antimicrobial (Didry *et al.*, 1994), and antimalaria activities (Likhitwitayawuid *et al.*, 1998). Quinone occurring in higher plants have a good to moderate antifugal activity against phytopathogenic fungi (Lee and Lee, 2005).

A number of 1, 4- naphthoquinone derivatives have been found to possess powerful pharmacological effects and are also associated with marked antimicrobial and antitumor activities (Kelker *et al.*, 1986). Naphthoquinones, lawsone and 2-methoxy-1,4-naphthoquinone, were found to be a group of the main constituents, exhibiting antifungal and antibacterial activities (Kang & Moon, 1992). Quinones and their reduction products, semiquinones and hydroquinones, are of toxicological interest because they generate reactive oxygen species and to form covalent bonds with tissue macromolecules (Cadenas *et al.*, 1992).

The present investigation was carried out to assess the efficacy of *in vitro* cultures of the experimental species, *Rubia cordifolia,* to synthesize the naphthoquinones present in the plant, which is responsible for its anticancerous activity. Since literature pertaining to synthesis of secondary products in the target

species, is scanty, attempts to review secondary product synthesis *in vitro*, in other plant species is made here under. Presented below, is a comprehensive review on the biosynthetic potential of cell cultures with emphasis on medicinal plants and the methodologies adopted for *in vitro* synthesis of secondary products.

2.5 Cell cultures and secondary metabolite production

Plant cell cultures contribute in three major ways to the production of secondary product viz. formation of known secondary products in cultured cells, as biotransformation systems utilizing the enzymatic potential of cells and as source of novel compounds, not previously detected in culture (Mathur and Ahuja, 1990).

Production of shikonin from *Lithospermum erythrorhizon* cultures was the first successful case of *in vitro* production of secondary metabolite on commercial scale. This was a remarkable breakthrough, as the *in vivo* plant roots take 3-4 years to grow and the yield 1-2% shikonin, in comparison to *in vitro* cultures which yield 15-20 % shikonin in just 23 days (Fujita, 1988). Plants like *Coscinium* and *Coptis* take 4 to 6 years for cultivation which limit the utility of these plants in the pharmaceutical industry. Further, they are endemic and cannot be grown everywhere, and hence exploitiation of *in vitro* cultures of these crops for secondary products, offers great scope. *In vitro* cultures, producing known secondary products are also capable of synthesing novel compounds (Rao *et al.*, 2008).

Bhalsing and Maheswhari, (1998) have described the present status of secondary metabolite production by *in vitro* techniques. They have listed plants giving high yields of secondary metabolites in cultures.

Table. 2 *In vitro* yields of secondary metabolites

Secondary metabolites	Source Plant species	Yield (per cent of dry wt) in *in vitro* culture
Alkaloids		
Benzophenanathridine alkaloids	*Escholtzia californica*	1.7
Protoberberine alkaloids	*Berberis stolonifera*	10
Berberine	*Cotpus japonica*	8.2
	Thalictrum minus	12.1
Steroids and terpenoids		
Diosgenin	*Dioscorea deltoidea*	7.8
Sterols	*Delphinium ajacis*	8-10
Ferruginol	*Solvia miltiorrhiza*	1.3
Solasodine	*Solanum khasianum*	2.07
Quinones		
Naphthoquinone	*Echium lycopsis*	12.3
Anthraquinone	*Galium spp*	27

Sindhu, (1999) reported higher yield of berberine from *in vitro* cultures (10.079µg g^{-1} callus) than from plant parts (0.013 ug g^{-1}) of *Coscinium fenestratum.* Sankar, (1998) reported significantly higher yield of ephedrine from *in vitro* cultures (0.02 per cent) than from field grown plants (0.008 per cent) of *Sida* spp.

The methology and salient achievements made in the production of secondary product *in vitro,* in medicinal plant species, in particular is reviewed here under.

2.6 Establishment of callus cultures

2.6.1 Selection of explants

Explants are the potential source for *in vitro* cultures. Different explants likes leaves, internodes, stems segment and roots are used for establishing *in vitro* culture. Kadkade, (1982) induced *Podophyllum peltatum* callus from rhizome, leaf, stem, and root segments of the plant and found that the podophyllotoxin content of callus tissues derived from the rhizome was higher than those from the *in vivo* plant parts.

Sehrawat *et al.* (2002) revealed that different explants (leaves, stems and nodes) of *Rauwolfia serpentina* successfully initiated calli in MS basal media supplemented with different growth regulators. Callus of *Rubia tinctorum* was induced from the roots of a germ-free plant, grown on LS medium supplemented with 2, 4-D (10^{-5} M) and 0.2 per cent gellan gum as reported by Odake *et al.* (1991) of San-Ei Chemical Industries in Japan. Margherita *et al.* (1996) reported plant regeneration from thalamus derived callus of *Ramunculus asiaticus.* In *Tylophora indica* Nadha, (2006) reported, callus induction from different explants viz leaves, nodal segments and stem explants on MS medium with combination of auxin and cytokinin. Medium supplemented with NAA (4 mg^{-1}) and kinetin (1 mg^{-1}) was optimal for callus initiation.

Zhang *et al.* (1996) used two explants, leaf and petiole segments, from asceptic plants of *Panax ginseng,* generated from seeds, to induce calli. Callus induction from petiole explants was better. In *Hyoscyamus muticus*, petiole explants gave good callus on medium supplemented with 2,4-D and kinetin (El-Bahr *et al.,* 2001).

Agarwal *et al.* (2001) used leaves petiole and immature stem segments as explants for inducing callus in *Ginkgo biloba.* Seeds of *Solanum platanifolium* were

germinated *in vitro* and hypocotyls, stem and leaves separated from *in vitro* seedling were used as explants for callusing (Jaggi and Singh, 2001).

2.6.2 Surface sterilization

Plant tissue culture media is rich in inorganic salts and organic nutrients including sucrose, which is a good substrate for the growth of many saprophytic bacteria and fungi. Contamination of *in vitro* cultures by microbes presents a major challenge to the initiation and maintenance of viable cultures. Other potential sources of contamination in tissue cultures, are culture vessels, nutrient media, plant tissue itself, instruments and transfer area. To minimize microbial contamination, sterilization of explants has to be done without damaging the plant tissue. The procedure for preparing sterile explants varies with the nature of explant tissues (Webster *et al.*, 2003).

Dodds and Roberts, (1982) suggested that use of antibiotics for sterilization may be discouraged since they cause unpredictable results. Sudha and Seeni, (1994) reported that treatment with 0.1 per cent $HgCl_2$ for 5 minutes gave contamination free cultures in *Adhatoda beddomeii*. Kannan and Jasrai, (1998) reported that washing of explants in ethanol (90 per cent v/v) followed by soaking in mercuric chloride, 0.1 percent for 1 minute gave contamination free cultures in *Vitex negundo*. Kulkarni and Rao, (1999) identified Teepol washing followed by 70 per cent ethanol treatment for 2 minutes) and 0.1 per cent mercuric chloride for 5 minutes, as an effective surface sterilization method for *Acorus calamus*. Amin *et al.* (2003) reported that explants treated with savlon (5 per cent v/v) for 10 minutes followed by surface sterilization with $HgCl_2$ (0.1 per cent w/v) for 10 minutes is ideal for establishing contamination free cultures of *Paederia foetida* .

In *Trigeonella foemum graceum*, for the production of hairy root cultures, seeds were surface sterilized with sodium hypochlorite solution (15 per cent w/v available chlorine) supplemented with two drops of Triton X-100 for 6 minutes

(Merkli *et al.*, 1997). Sudha and Seeni, (1994) reported that treatment with 0.1 per cent $HgCl_2$ for 5 minutes gave contamination free cultures in *Adhatoda beddomeii.*

2.6.3 Basal media for callus establishment and proliferation

Selection of tissue culture medium depends on the plant species and purpose of culture initiation. A wide variety of plant tissue culture media for establishment of *in vitro* cultures have been reported by many researchers. The earliest and widely used media were proposed by White (1943) and Heller (1953). Since 1980, MS (Murashige and Skoog, 1962) medium is most commonly used in plant tissue culture. B_5 medium by Gamborg *et al.*, (1968), SH (Schenk and Hildeberandth, 1972) medium, Linsmair and Skoog (LS) medium, Woody plant medium (WPM) developed by Lloyed and Mc Cown (1980) and other basal media employed, for establishing *in vitro* cultures.

Tabata *et al.* (1974) had observed that callus tissue of *Lithosperium erythrorhizon* produced shikonin derivatives on LS agar medium. Joseph (1997) induced profuse callusing on pseudostem explants of kacholam in MS medium supplemented with 2,4-D (1 mg l^{-1}) and BAP (0.25 mg l^{-1}). Ilahi and Ghauri, (1994) induced calli in *Papaver bracteatum* seedlings, cultured on half MS medium with NAA 1.0 mg l^{-1} and BAP 0.5 mg l^{-1}. Gamborg 's B_5 medium was reported as best, for callus initiation and growth of *Sida cordifolia* (Nair *et al.*, 1992) while half the concentration of MS medium was reported as ideal, for callus initiation in *Sida* spp. (Sankar and Nair, 1997).

Ginkgo biloba cells could be grown on either MS or Gamborg B5 mineral salt medium supplemented with sucrose (3 per cent and 2 per cent respectively) and NAA and kinetin. Growth and maintenance of callus cultures were best achieved using MS medium supplemented with 2 mg l^{-1} and 1 mg l^{-1}, kinetin (Carrier *et al.*, 1990). According to Chen *et al.* (1994) callus was induced from petiole explants of *Angelica dahurica* on MS medium supplemented with 2,4-D (2 g l^{-1}) and kinetin (0.5 mg l^{-1})

Rao *et al.* (2008) reported that callus of *Tinospora cordifolia* established on MS medium was subcultured to LS medium supplemented with NAA(19.8μM) and BA (1.7μM). The *Tinospora cordifolia* cell growth was almost same in both the basal media. However, the content of berberine was better in LS medium (5.5 mg g^{-1} dry wt) compared to MS medium (4.2mg g^{-1} dry wt).

2.6.3 Establishing suspension cultures

Plant cell suspension cultures have been considered as an alternative to agriculture processes, for producing valuable secondary metabolites (Kim *et al.*, 2001). Cell suspension cultures, offer the possibility to establish nearly homogenous culture condition for all cells .Typically, the *in vitro* culture is initiated by the isolation of small pieces of plant tissue, that upon culturing, develop a mass of unorganized cells. The calli initiated from suitable explants are suspended in agitated nutrient solution that are contained in enclosed vessels. As the cells grow and subdivide, they result in a suspension of unorganized and viable plant cells, that can carry out various chemical transformations to produce primary or secondary metabolites or to produce more biomass.

Gamborg and Shyluk, (1981) have reported in detail, the growth stages of cultured plant cells viz; lag phase, log phase, exponential phase, deceleration phase and stationary phase. Bhojwani and Razdan, (1996) have enlisted the general protocol for initiating and maintaining cell cultures. Cell suspension cultures of *Rudgea jasminoides* were successfully obtained from callus derived from petiole explants (Stella and Braga, 2002). Gholba, (2000) used calli derived from leaves to initiate suspension cultures in *Gymnema*. Cells suspension cultures derived from *Ginkgo biloba* leaves produced ginkgolide B whose, production reached a maximum by the 13th day of subculture and was followed by a sharp decrease (Jeon *et al.*, 1995).

Although the undifferentiated cells of a plant suspension culture are generally totiptent many genes including those involved in secondary metabolism, are rerepressed and as a consequence, yields of the desired compound in such cultures are very low (Sham-Ardakani, 2005). However, contrary reports are also cited. For instance, cell suspension cultures derived from the leaf explants of *Tinospora cordifolia* show maximum production of biomass (9.9g dry cell wt l^{-1}) on day 36, while berberine content was highest (5.5 mg g^{-1} dry weight) at day 24. The yield of berberine in cell suspensions of *Tinospora* was 5-14 folds higher than that of intact plant (Rao *et al.*, 2008). Cell suspensions of *Morinda citrifolia* are able to produce large amount of anthraquinones when they are cultivated on B_5 medium containing 1 mg $^{-1}$ NAA (Van der plas *et al.*, 1995).

2.6.4 Growth regulators for initiation and proliferation of *in vitro* cultures

Plant growth regulators affect cell growth, metabolism, cell differentiation and induction or repression of biosynthesis pathways. Auxins promote cell elongation and root initiation, while cytokines promote cell division and shoot differentiation Their effect depends on the rate of uptake from the medium, stability in the medium and on the sensivity of the target tissue (Rozita, 2004).

Murashige (1974) has given a detailed account on the influence of phytohormone on the growth of *in vitro* cultured tissues. He identified 2,4-D, NAA and IAA as the popularly used auxins. He concluded that IAA has less potential than other auxins regarding callus initiation and proliferation. Gold smith (1997) reported 2,4-D to be very potent to produce growth in cultures as it has little polar transport. Hence it accumulates at one point and promotes growth at that point. In *Plumbago rosea*, Sateeshkumar and Bhavananda (1998) reported callus induction by adding 2,4-D to the medium. 2,4-D effectively stimulate the formation of friable callus in combination with kinetin (Panichayapakaramant, 2001). Roy *et al.* (2008) observed

that incorporating 2,4-D into basal medium was not effective, to maintain callus, where as callus remain healthy increase growth scores in varying concentrations of 2,4-D and kinetin combination.

Sankar and Nair, (1997) reported that (NAA 1 mg $^{-1}$ and kinetin 0.5 mg l^{-1}) was ideal for initiating callus of *Sida spp.* and BAP is unsuitable for callus initiation and growth.

Gibberellic acid is also effective on plant cell cultures. DiCosmo *et al.* (1995) recently reported that growth of callus of a taxol-producing plant, *Taxus cuspidata*, was significantly promoted by addition of gibberellic acid into the solid medium.The highest callus induction rate (91.66 per cent) in leaf explants of *Rauwolfia serpentina* was observed in Murashige and Skoog's (MS) basal medium containing 2,4-D (2.0 mg l^{-1}) and benzyladenine (0.2mg l^{-1}). for stem and nodal explants, the highest callus induction was achieved in NAA (1 mg l^{-1}) and benzyladenine (0.5 mg l^{-1}) and 2,4-D (2.0 mg l^{-1}) and benzyladenine (0.5 mg l^{-1}) respectively, (Sherawat *et al.*, 2002).The effect of different concentration of NAA (0.5-2 mg l^{-1}) in combination with BA (0.5-2 mg l^{-1}) on induction of callus from leaf explants of *Rauwolfia caffra* on MS basal medium was investigated. The best response for callus induction and growth was obtained on a medium containing NAA (2 mg l^{-1}) and BA (2 mg l^{-1}). The callus was compact, whitish yellow and fast growing (Upadhyay, 1992)

Roy *et al.* (2008), studied the efficient of different plant growth regulator like 2,4 -D, Kinetin, IAA and BAP on growth of calli of and observed the highest efficiency of callus formation in the medium containing 2,4-D and kinetin.

Callus cultures of *Taxus wallichiana* young leaves were established on Gamborg's (B5) medium supplemented with dicamba (2.0 mg l^{-1}), benzyladenine (2.0 mg l^{-1})and glutamine (292 mg l^{-1}) (Chittimalla *et al.*, 2000).

2.6.5 Other organic compounds

In *Cinhona ledgeriana,* Hunter (1979) found that the addition of phloroglucinol to the medium promoted the culture growth. The cultures, without phloroglucinol showed oxidative browning and necrosis of cut surface and eventually died. Phloroglucinol increased the growth rate of cultures, which overtook the rate of browning and hence the cultures survived for longer time spans. Conger (1981) reported the role of complex organic compounds like casein hydrolysate, coconut water, yeast, malt extract and fruit and vegetable juice for successful growth of tissues and organs. Role of these organic compounds is usually unpredictable and repeatability is also very poor, therefore it has been recommended to avoid their use as far as possible (Gamborg and Shyluk, 1981). Sankar (1998) reported promotion of callus induction in *Sida* spp. at 100 and 125 mg l^{-1} concentrations of phloroglucinol.

2.7 Standardization of production medium for secondary metabolites production

2.7.1 Basal medium

Fuijta, (1988) found that calli of *Lithosperium erythrorhizon* showed varying respone in different media. LS media gave good growth of calli, but low shikonin production, while White's medium produced considerable amount of shikonin, but calli growth was restricted. So, a two phase culture system was developed, wherein growth was first achieved in LS medium and later on shikonin production in White media. Zenk *et al.* (1997) tested various well-known basal media for the production of serpentine, an indole alkaloid. The results indicate that the amount of serpentine depends on the composition of the basal medium used. Among them, MS medium was recognized to be the most suitable one for the production of serpentine alkaloid by *Catharanthus roseus* suspension. Pauthe-Dayde *et al.* (1990) used Gamborg's B5 and MS media to raise cultures of *Gypsophila* spp. to get triterpenoid saponins.

2.7.2 Supplementing growth regulators to basal medium

It is well known that plant hormones have very important roles in secondary metabolite formation by plant ultured cell. Increasing concentration of 2,4 –D and NAA along with BA in LS liquid medium containing 3 per cent sucrose not only increased growth of suspension but also berberine over a period of four week(Rao *et al.*, 2008). A combination of auxins and cytokinins (NAA, 2,4-D and BA, kinetin) enhanced the production of plumbagin in plants such as *Drosophyllum lusitanicum* (Nahalka *et al.*, 1996) and *Plumbagin rosea* (Komaraiah *et al.*, 2003) Shrivastav and Padhya, (1995) reported that increase in the IAA level from 0.5 to 2 μ M l^{-1} in the basal medium, reduces the number of roots produced as well as alkaloid accumulation (0.05 per cent to 0.15 per cent) in regenerated roots of *Boerhaavia diffusa.*

In *Sida* spp. NAA and kinetin at 1.0 mg l^{-1} each, resulted in higher ephedrine yield whereas incorpation of 2, 4-D resulted in complete inhibition of alkaloid production (Sankar, 1998). Sindhu (1999) observed that MS medium at half strength with phosphate ions reduced to 25 per cent supplemented with IAA 2 mg l^{-1} and BA 1 mg l^{-1} gave high callus index for immature fruit explants of *Coscinium fenestratum.*

When cultures of *Camptotheca acuminata* were grown on MS medium containing 4 mg l^{-1} NAA, accumulation of camptothecin reached 0.998 mg l^{-1} (Van Hengal *et al.*, 1992) compared to medium containing gibberellins and L-tryptophan, which yielded camptothecin at about 0.0025 per cent on dry weight basis.

The highest concentration of DOPA was obtained when *Mucuna hassjoo* were cultivated in MS medium with 0.025 mg l^{-1} 2, 4-D and 10 mg l-1 kinetin. The level of DOPA in the cells was about 8mmol/g on fresh weight basis (Vanisree *et al.*, 2004).

2.7.3 Media additives

For manipulating the growth and secondary metabolite production, many chemical compounds are added to the media, which are collectively grouped as media additives. These supplements include casamino acid, peptone, yeast extracts, malt extracts and coconut milk. Coconut milk is also known as a supplier of growth regulators. Mizukami *et al.* (1992) reported a transient increase in rosmarinic acid content in cultured cells of *Lithospermum erythrorhizon* after addition of yeast extract to the suspension cultures wherein a maximum was reached in 24 hr. When the plant cells were treated with yeast extract on the 6^{th} day of cultivation, the level of rosmarinic acid increased 2.5 times and the activity of phenylalanine ammonia-lyase in the cells rapidly increased before synthesis of rosmarinic acid. Funk *et al.* (1987) used yeast extract to enhance production of secondary metabolites, which gave a steep rise in glyceollin production followed by fall, in *Glycine max* cultures, while in *Thalicturm rugosum*, they found a continous rise in berberine production.

2.8 Strategies to improve secondary metabolite production

Even though plant cell culture techniques offer an attractive alternative for the production of secondary metabolites, one of the key problems with plant cell culture as a source of phytochemicals is that the cultures initially show a productivity which is far below that needed to make any exploitation economically feasible (Yeoman *et al.* 1990), except in a few cases like accumulation of rosmarinic acid in *Coleus blumei* and glutathione in *Nicotiana tabacum*, where the culture produced secondary products nine times and ten times respectively, more than the plant (Fowler, 1986). Much effort has been put into the development of methods to enhance the productivity of plant cells. A number of techniques have been identified which potentially influence the productivity in plant cell cultures resulting in enhanced production of secondary metabolites, which include inducing nutrient limitation, creating stress condition, addition of elicitor compounds, supply of precursors, immobilization and hairy root culture (Zhong, 2002).

2.8.1 Modifying media constituents of basal medium

Cultured plant cells are usually supplied with all essential minerals, vitamins and carbon sources for vigorous growth and active primary metabolism. However, secondary metabolites are almost invariably produced most vigorously by senescent or slow growing cells (Dziezak, 1986). Hence, the general approach is to slow down growth by limiting the supply of sugar, phosphorus or nitrogen (Johnson, 1993).

2.8.2 Reducing nitrate and phosphate concentrations

The culture conditions which promote high rate of cell division are commonly not suitable for maximum rate of secondary product formation (Rokem and Goldberg, 1985). Anthraquinone formation in *Morinda citrifolia* is sensitive to levels of nitrate in the medium, being optimal at 25-40 mM (Zenk *et al.* 1975).The highest oleandrin yield was obtained when the nitrogen concentration was lowered to two third and phosphate concentration to one third (Ibrahim *et al.*, 2007). High concentrations of nitrogen sources inhibited or retarded shikonin derivative formation in *in vitro* cultures of *Lithospermum erythrorhizon* (Hajime *et al.*,1997).

Pedro *et al.* (1990) compared two sources of nitrogen viz, casamino acid and ammonium nitrate in callus and suspension cultures of *Geranium robertianum*. In both the cases, the suspension produced secondary metabolites identical to those produced in *in vivo*. Van Gulik *et al.* (1990) studied the effect of mixed nitrogen sources on the growth of *Catharanthus roseus* cell cultures where it was observed unnecessary to use a mixed nitrogen source. On modifying the ammonium to nitrate ratio from 1 to 2 to 1 to 1, an increase in indole alkaloid production up to levels of 500$\mu g\ g^{-1}$ was noted in cell suspension culture of *Tabaernae montana divaricata* by Schripsema and Verpoorate (1992).

Zhang *et al.* (1996) reported that the ratio of nitrate to ammonium had great influence on the cell growth of *panax notoginseng* and consumption of C and N sources. In the same study it was reported that the dry cell weight increased from 4.9 to 10.9 g/l with an increase of initial nitrate concentration from 0 to 60 mM.

Prathumpai *et al.* (2007) reported highest production of naphthoquinone (5.03g dw^{-1}) in *Cordyceps unilateralis* when ammonium sulfate was used as a sole nitrogen source.

In *Panax notoginseng,* by increasing the initial phosphate concentration in medim, in the range of 0-1.25 mM, both cell growth and saponin accumulation were greatly improved (Zhong and Zhu, 1995). Low phosphate level stimulated cinnamoyl putrescine accumulation in *Nicotiana tabaccum* cell suspension by 3-4 fold (Schiel *et al.*, 1984).

2.8.3 Modifying sucrose concentration

Many plant genes are controlled by sugars that are involved in a variety of processes such as photosynthesis, storage of protein/ starch/lipid and production of homo and hetero polysaccrides (Winter and Huber, 2000). Sugars are also known to interact with several growth regulators, leading to the changes in array of morphological events (Kraemer *et al.*, 2002).

The optimal yield of *Ginkgolide B* was obtained in MS medium supplemented with 30 g sucrose in *Gingko biloba* (Jeon *et al.*,1995). Stimulation of taxol production at elevated levels of sucrose in nodal cultures of *Taxus* was reported by Ellis *et al.* (1996). In addition to taxol higher sucrose levels also enhanced production of 7-epi-10 acetyl cephalomannine and 7-epi-10- deacetyl cephalomannine, so that total content of these taxanes was approximately 39 µg g -1 dry weight. High sucrose concentrations increased the content of shikonin derivatives, but neither glucose nor fructose was effective for shikonin derivative formation. Higher concentrations of sucrose (6-18 per cent) inhibited the growth in the presence of phytohormones (5µM IAA or 0.5µM NAA). In contrast, in phytohormone free medium, 12 per cent sucrose resulted in maximal growth and anthraquinone production (Sato, 1991).

Asaka *et al.* (1994) reported that ginseng embryoids produced maximum amount of saponins when raised in media containing 30 g sucrose and 30 g glucose per litre. Jeon *et al.* (1995) reported that high yield of *Ginkgolide B* was obtained in MS medium supplemented with 30 g sucrose in *Ginkgo biloba*. Jaggi and Singh,

(2001) used 2 per cent sucrose in modified MS medium for solasodine production in culures of *Solanum platanifolium.*

As a single carbohydrate source in medium, fructose exhibited better cell growth when compared to sucrose or glucose. In a combination media of two hexoses (glucose and fructose) at different concentrations, the best proliferation of cell was obtained at the combination of 30 g l^{-1} glucose and 30 g l^{-1} fructose. Low content of sugar had significant impact in shortening lag phase in *Taxus wallichiana* cell suspension cultures (Duong *et al.,* 2003).

2.8.4 Supply of precursors

Under some conditions, the flux through a particular biosynthetic pathway may be limited by the availibity of precursors or particular metabolic intermediates wherein supply of these precursor, externally, increased the rate of product biosynthesis (Johnson, 1993). Addition to the culture media, of appropriate precursors or related compounds sometimes stimulates secondary metabolite production. This approach is advantageous, if the precursors are inexpensive.

Tabata *et al.* (1975) reported that addition of 500 mM tropic acid to the medium of *Scopolia japonica* increased the amount of alkaloids by upto14 times. The level of an anticancer compound, tripdiolide, produced by *Triptorygium wilfordii* cultured cells was increased by addition of 100 µg l^{-1} farnesol which is dephosphorylated farnesyl pyrophosphate, and an intermediate in the biosynthesis of terpenoids (Kupchan *et al.*, 1972). The addition of α-ketoglutaric acid, a biosynthetic precursor of anthraquinones,to the culture of *Rubia cordifolia* var.*pratensis* and *Rubia akane* enhanced the production of alizarin and purpurin (anthraquinone pigments) remarkably, under various conditions. (Soon-Hee, 1989).

Endress, (1994) reported that *Rauwolfia serpentina* formed a new indole alkaloid, 6-hydroxytaumacline, in significant amounts when the cells were cultivated in the presence of ajmalicine..

Namdeo, (2004) reported that the favorable effect of tryptophan addition on ajmalicine in *C. roseus* cells cultured in Zenk's production medium was evident, wherein maximum ajmalicine (310 μg g^{-1} dry weight) was recorded in medium with 100 mg l^{-1} tryptophan, followed by 292 and 140 μg g^{-1} dry weight ajmalicine in medium with 250 and 50 mg l^{-1} tryptophan respectively. Higher concentrations of tryptophan inhibited ajmalicine production. In suspension cultures of *C. roseus* synthesized higher concentrations of ajmalicine in 20l airlift bioreactor by addition of tryptophan as a precursor in culture medium (Fulzele and Heble, 1994).

The production of phenylethanoid glycosides (PeG) was enhanced by feeding precursors L-phenylalanine, L-tyrosine, sodium acetate and phenyl acetic acid at appropriate concentrations to cell cultures of *Cistanche deserticola*. Feeding 0.2 m mol l^{-1} phenylalanine on the eighth day resulted in the highest production of phenylethanoid glycosides (1.10 g l^{-1}), which was 75 per cent higher than that obtained in the cell culture without precursors (Ouyang *et al.*, 2004).

2.9 Employing special techniques for enhanced production of secondary products *in vitro*

2.9.1 Creating conditions of stress

Change in osmotic pressure modifies the properties of cell membrance and hence influences the uptake and exchange of chemicals by the cell wall. Osmotic pressure is increased by adding mannitol to the culture medium. Zhang *et al.* (1995) observed that, in *Panax notoginseng*, addition of mannitol increased the initial osomotic pressure of the culture medium, thereby increasing saponin production. In *Datura stramonium, Atropa belladonna* and *Hyoscyamus muticus,* reduced cell growth was noticed due to stimulating effect of osmotic stress caused by mannitol, which resulted in rapid accumulation of alkaloids (Saker *et al.*, 1997). Accumulation of the alkaloid curine was more in the callus cultures of *Cissampelos pareira* in MS

medium supplemented with 2 mg l^{-1} NAA and 0.5 mg l^{-1} abscissic acid (ABA) compared to these cultures on MS supplemented on 2 mg l^{-1} NAA (Gokul *et al.*, 1998).

2.9.2 Elicitation

Elicitors can strictly be defined as compounds of biological origin involved in plant/ microbial interaction (biotic elicitors). This definition has, broadened to include abiotic elicitors which include UV light(Jones, 1984), pH changes(Wink, 1985), osmotic stress (Rudge and Morris, 1986), wounding (Wink, 1984) and heavy metal ions (Watson and Brooks, 1984) which have been showing to induce change in plant secondary metabolism. Treatment of plant/ tissue cultures with elicitors frequently leads to accumulation of phytoalexins which are thought to play a role in disease resistance (Bailey and Mansfield, 1982) and which are not present or present in very low concentration in untreated plants or cultures.

The accumulation of secondary metabolites in plants is a part of the defense response against pathogenic attack, which is triggered and activated by elicitors, the signal compounds of plant defense responses (Zhao *et al.*, 2005a). When cell cultures are subjected to biotic and abiotic elicitation, some genes are repressed, and as a result, among other things secondary metabolites are formed .Therefore, the treatment of plant cells with biotic and/or abiotic elicitors has been a useful strategy to enhance secondary metabolite production in cell cultures.

2.9.2.1 Biotic elicitors

Biotic elicitors are substance of biological origin, which include polysaccharides derived from plants cell walls (pectin or cellulose) and microorganisms (chitin or gulucan) and glyoproteins or G-protein or intercellular proteins whose functions are coupled to receptor. They act by activating or inactivating a number of enzymes or ion channels (Namdeo, 2007).

The elicitor prepared from *Fusarium conglutinans* enhanced thiophene production on *Tagetes* spp. (Mukundan and Hjortso, 1990) and solavetivone production was increased by *Rhizoctonia solani* in *Hyosyamus muticus*.

Catharanthus roseus cell cultures elicited with different enzyme (*Rhizopus spp cellulase, Trichoderma viride* macerozyme and *Aspergillus niger* polygalaturonase) exhibited increased accumulation of alkaloids (Garnier *et al.*, 1996)

A rapid induction and highest oleandrin production was obtained with 2ml dry cell powder solution of *Aspergillus niger* prepared elicitor (with concentration of 10 g l^{-1}). The oleandrin concentration reached the maximum in 25 days, upon employing *Aspergillus niger*. It was 8.8 fold higher than control cultures that reached the maximum 0.35 mg/l (Ibrahim *et al.*, 2007).

2.9.2.2 Abiotic elicitors

The most frequently used elicitors in previous studies were fungal carbohydrates, yeast extracts, MJ and chitosan. MJ, a proven signal compound, is the most effective elicitor of taxol production in *Taxus chinensis* Roxb (Wu and Lin, 2003) and ginsenoside production in *Panax ginseng* (Thanh *et al.*, 2005) cell/organ culture.

When cells were cultured with the addition of 0.05 mM VSO_4, the excretion of Taxol and baccatin III increased 120 and 97 per cent, respectively, in relation to the value obtained in the control (Cusido *et al.*, 1999).

Recent studies have shown that addition of methyl jasmonate or dihydro-methyl jasmonate to suspension coultures of *Panax ginseng* increases the production of ginsenosides (Wang and Zhong, 2002). Also, jasmonic acid improves the accumulation of ginsenosides in the root cultures of *Panax ginseng* (Yu *et al.*, 2002).

Addition of 200 mg l^{-1} chitosan as an elicitor, to the immobilized cells, resulted in eight and two folds higher accumulation of plumbagin over control and immobilized cell respectively. Also, more than 70 per cent of the plumbagin was

released into the medium, which is highly desirable for easy recovery of the product (Komaraiah *et al.*, 2003).

2.10 Immobilization

The use of immobilized plant cells offer distinct advantage for the production of secondary metabolites by plant cell cultures (Brodelius, 1985). Immobilization of plant cells has been an alternative to suspension cultures to provide cell to cell contact and favourable cell environment to improve secondary metabolite production (Johnson, 1993). The primary limitation of plant cell culture is that the product must leach out to the exterior or be extracellular. Most of the plant cell products are located in vacuoles and their excretion can be affected by using permeability agents and altering the pH. Physical restraining of cells on a fixed support exerts stress on plant cells leading to restricted growth, which is a pre-requisite fro metabolite production (Choi *et al.*, 1996). They achieved 20 fold increase in taxol production from *Taxus brevicola* by immobilization. Biotransformation of codeinone to codeine with immobilized cells of *Papever somniferum* has been reported by Furuya *et al.* (1988). The conversion yield was 70.4 per cent and about 88 per cent of the codeine converted, was excreted into the medium.

Immobilization of cells in calcium alginate enhanced the production of plumbagin by three, two and one folds compared to that of control, un-crosslinked alginate and $CaCl_2$ treated cells respectively. Cell loading at a level of 20 per cent to the polymer volume (Na-alginate) was optimal and maximum plumbagin was obtained. At higher cell loading (40-50 per cent), lower plumbagin accumulation was noticed (Komaraiah *et al.*, 2003).

Biotransformation of externally fed protocatechuic aldehyde and caffeic acid to capsacin in freely suspended cells and immobilized cells cultures of *Capsicum frutescens* was also reported by Ramachandra and Ravishankar, (2000). More than ten fold increase of capsaicin from immobilized cell cultures of *Capsicum spp* was noted, when nitrate was eliminated from the culture medium (Johnson, 1993). Immobilization of Datura innoxia cells in calcium alginate beads resulted in increased

secondary metabolite production (Gontier *et al.*, 1994). Sankar (1998) observed failure of immobilized cells to synthesis any alkaloid as revealed by qualitative and chromatographic test in *Sida* spp.

2.9.3 Establishment of hairy root cultures

2.9.3.1 Hairy root culture

Though cultured plants remain a major source of pharmaceuticals and fine chemicals, despite considerable efforts, only a few commercial processes have been achieved using cell cultures (e.g. shikonin, berberine). The major constraint with cell cultures is that they are genetically unstable and cultured cells tend to produce low yields of secondary metabolites. A potential route for enhancing secondary metabolite production is by transformation using the natural vector system *Agrobacterium rhizogenes*, the causative agent of hairy root disease in plants. Genetically transformed hairy roots obtained by infection of plants with *Agrobacterium rhizogenes*, a gram-negative soil bacterium, offers a promising system for secondary metabolite production (Kim *et al.*, 2002). The fast growing hairy roots are unique in their genetic and biosynthetic stability and their fast growth offers an additional advantage. These fast growing hairy roots can be used as a continuous source for the production of valuable secondary metabolites (Flores *et al.*, 1999). Moreover, transformed roots are able to regenerate whole viable plants and maintain their genetic stability during further subculturing and plant regeneration.

2.9.3.2 Agrobacterium and Ri T-DNA genes

Agrobacterium rhizogenes recognizes some signal molecules exuded by susceptible wounded plant cells and becomes attached to it (chemotactic response). Infection of plants with *A. rhizogenes* causes development of hairy roots at the site of infection. The rhizogenic strains contain a single copy of a large Ri plasmid. On infection, part of the DNA (T-DNA) from the Ri- (root inducing) plasmid is transferred from the bacterium into the nuclear genome of the host plant (Chilton *et*

al., 1982). The delivery of the T-DNA into the nucleus of the host plant cell is achieved by the action of the *vir* genes (Zupan *et al.*, 2000). In the Agropine Ri plasmid, T-DNA is referred to as left T-DNA (TL-DNA) and right T-DNA (TR-DNA). TR T-DNA contains genes homologous to Ti plasmid tumor inducing genes. Genes of Ri TL -DNA direct the synthesis of a substance that recruits the cells to differentiate into roots under the influence of endogenous auxin synthesis (Shen *et al.*, 1988). With the exception of border sequences, none of the other T-DNA sequences are required for the transfer. The transformed T-DNA causes the host plant to form adventitious roots at the infection site and to produce opines that serve as nutrients for the bacteria. Hairy root induction and morphology are controlled by the *rol* (A, B, C and D) genes from the *A. rhizogenes* Ri-plasmid (White *et al.*,1985). The *rol* genes have also been found to affect secondary metabolite production. Modern *Agrobacterium* mediated gene transfer to plants utilizes binary vectors in which the T-DNA and the *vir* region can reside on separate plasmids (Hellens *et al.*, 2000).

2.9.3.3 Hairy root culture for secondary metabolite production

Hairy root cultures are characterized by a high growth rate and are able to synthesize root derived secondary metabolites. Normally, root cultures need an exogenous phytohormone supply and grow very slowly, resulting in poor or negligible secondary metabolite synthesis. However, the use of hairy root cultures has revolutionized the role of plant tissue culture for secondary metabolite synthesis (Rhodes *et al.*, 1994).

For most alkaloid producing plants like *Hyoscyammus, Datura, Atropa, Nicotiana, Catharanthus, Cinchona* and *Peganum,* hairy roots culture have been initiated Because of their rapid growth and the potential of genetic engineering, hairy roots or transformed cells have been shown to be less sensitive to optimization procedures such as medium optimization (Berlin *et al.*, 1990) and elicitation (Eilert *et al.*,1987). Hairy root cultures of *Cassia obtusifolia* clones transformed with *Agrobacterium rhizogenes* strain 9402 were established to investigate *in vitro*

synthesis of anthraquinone production. Seven anthraquinones, together with betulinic acid, stigmasterol and sitosterol were isolated from the hairy roots (Hongzhu, 1998).

The accumulation of total anthraquinones in transformed roots was found to be approximately 2-fold higher than that found in one year-old field grown roots (2.12 ± 0.12 and 1.23 ± 0.12 mg g^{-1} dry weight, respectively). Alizarin was found to be the major anthraquinone in transformed root cultures and was found to be approximately 3-fold higher than in field grown roots (Lodhi and Charlwood, 1996).

Liu *et al.* (1998) reported high artemisinin content in hairy roots of *Artemisia annua*. Hairy root cultures of *Tropaeeolum majus* produced glutatropaeolin in higher amount as compared to callus and cell suspension cultures (Marenza and Henryk, 1999). No artemisinin was detected in roots from plants grown in the field, in vitro roots and hairy root induced by MTCC 2364. But rooting of *in vitro* shoots enhanced the artemisinin content in *Artemisia annua* (Shaneeja, 2007). For example, lawsone normally accumulates only in the aerial part of the plant, but hairy roots of *Lawsonia inermis* grown in half- or full-strength MS medium can produce lawsone in dark.

When a bacterial isochorismate synthase gene was cloned in a binary vector and then mobilized into *Agrobacterium rhizogenes*, the transgenic hairy root cultures of *Rubia peregrina* containing this gene expressed twice as much isochorismate synthase activity as the roots of control plants and accumulated 20 per cent higher levels of total anthraquinones (Lodhi *et al.*, 1996)

In *Lupinus mutabilis* Babaoglu *et al.*, (2004) reported that transformed roots produced twice the concentration of isoflavones compared to non transformed plants, indicating that Ri plasmid Y-DNA gene modified isoflavone concentration and also pattern of biosynthesis.

Withaferin A content was more in *W. somnifera in vivo* root, followed by by hairy root and normal *in vitro* root respectively, as reported by Varghese, (2006).

2.10 Biochemical estimation of naphthoquinones

2.10.1 Extraction of quonines

A variety of quinonoids and their derivatives are found in plants of which most are of 1,4- type which comprise some of the benzo and naphthoquinones. Extraction of quinonoids was generally carried out using organic solvents like hexane, chloroform, acetone, acetonitrile, ethyl acetate, methanol etc, either at ambient temperature or in refluxing condition. However, in some cases, homogenization of the plant material in cold followed by sonication is performed to isolate sensitive compounds like shikonin (Yamamoto, 2000) or aloe-emodin (Okamara *et al.*, 1996). Ultrasonic bath was used to extract phylloquinone (Dolnikowski *et al.*, 2002) and naphthodianthrone (Jensen and Hansen, 2002). Samples of rhubarb, senna and herbal mixture were also sonicated to extract the anthraquinonoids constituents (Lizuka *et al.*, 2004).

Many anthraquinonoid metabolites exist along with their glycosides in roots of madder and rhubard and leaves and fruits of *Senna.* These samples were first extracted with aqueous alcohol, followed by hydrolysis to separate the aglycon part (Metzger and Reif, 1996) in which the two steps could be performed simultaneously

Although quinonoids have been extracted by using various solvents, chloroform or dichloromethane would be the solvent of choice for getting the maximum yield of the lipophilic constituents. However, for an exception like lawsone, the tautomeric form is to be extracted preferentially with aqueous ethanol (Lobstein *et al.*, 2001). Other recent extraction technologies applied to quinonoids are the use of a non CFC solvent like phytosol (Dean *et al.*, 1998) and liquid carbon dioxide for supercritical fluid extraction in *Salvia* and *Hypericum spp.*

Brigham *et al.* (1999) reported that shikonin derivatives were extracted from the roots and liquid media with chloroform. Chloroform was employed as the solvent

for extraction of naphthoquinone from the oven dried roots of *Plumbago scanden* (Pakuski and Budzianowski, 1996).

Extraction of plumbagin in soxhlet apparatus for 5 hours demonstrated a high efficiency, yielding almost 50 per cent over the extract obtained in 2 hours of extraction (Selma *et al.*, 2004)

The leaves of *Impatiens balsamina* separately collected from 40 parent plants and dried at 50 °C were extracted with methanol under reflux for a hour (Panichayupakarant, 2001).

2.10.2 Separation of naphthoquinones

Most of the naturally occurring quinonoids could be separated by conventional methods like TLC, HPTLC, and CC (Thomson, 1971) which have now largely been supplemented by HPLC, one of the most useful techniques for the analysis of natural product in complex biological matrix. HSCCC (High speed countercurrent chromatography) is another method applied for analysis as well as preparative separation of a few quinonids of scientific and commercial importance (Lu *et al.*, 2004)

Quinines are separated, based on the number of isoprenoid unit, by alternating the developing solution with acetone and water containing 5 per cent $AgNO_3$. Hydrogenated menaquinones can be separated from other menaquinones. Fujita *et al.* (1983) reported separation of seven shikonin derivatives, all naphthoquinones, by HPLC with mobile phase consisting of acetonitrile -water- trethylamine- acetic acid (70:3.0:0.3:0.3 v/v) at 40° C.

Bozan *et al.* (1997) found that by modifying HPLC method, four derivatives of alkannin, found in roots of *Arenbia densiflora* can be determined quantitatively. The naphthoquinone was eluted within 12 minute by RPLC (Reverse phase liquid chromatography) using an ultracarb ODS C_{20} column with UV detection sets at 520

nm. The mobile phase was methanol-water-formic acid (9.5:5.9:0.1 v/v) at ambient temperature.

Lu *et al.* (2004) achieved the purification of shikonin from crude extract of *Lithospermum erythrorhizon* by preparative HSCCC (High speed countercurrent chromatography). *Lithospermum erythrorhizon* root extract, obtained by shaking with benzene at room temperature, was treated with 2 per cent NAOH. The alkaline layer was separated and acidified with hydrochloric acid. The resultant residue was processed to get shikonin for HPLC analysis which was done with a RP symmetry C_{18} column using an isocratic mobile phase composed of methanol-water- acetic acid (70:28:2 v/v). The crude extract contained several compounds along with shikonin.

Itokawa *et al.* (1993) reported that methanol extract of dried roots of *Rubia cordifolia* was partitioned between chloroform and water. The chloroform soluble fraction was subjected to column chromatography on different adsorbents, including Diaion Hp-20, silica gel and RP-18 to give compounds like anthraquinones, naphthohydroquinone dimers and naphthoquinone.

2.10.3 Estimation of nahthoquionone

Naphthoquinones are quantified by several methods. Panichayupakaranant, (2001) separated naphthoquinone from other components by using a silica gel F_{254} plate developed in chloroform. The identity of 2-methoxy-1,4-naphthoquinone was assessed by comparison of its Rf and U absorption spectrum with authentic standard. The amount of 2-methoxy-1,4- naphthoquonine was quantified by densitometry.

More recently, gas chromatography was used to quantify vitamin K_1 to a limit of 2 pg ml^{-1} of plasma (Fauler *et al.*, 1996). HPLC coupled to fluorescence detection (after reduction to hydroquinone form) also offered the requisite sensitivity and selectivity for its estimation, in small amount of analyst obtained from biological material (Dolnikowski *et al.*, 2002).

High performance liquid chromatography with UV detection and electrochemical techniques are the most commonly used methods for determination and characterization of single derivates of 1,2 and 1,4 quinone in biological samples. Babula *et al.*, (2005) analysed the content of naphthoquinones in leaves, roots and flowers of *Dionaea muscipula,* employing soxhelt method. The highest amounts of plumbagin were determined in leaves (5338 $\mu g.g^{-1}$), followed by roots (4230 μg.g–1) and flower (3698 $\mu g\ g^{-1}$).

2.11 Assessment of antioxidant and anticancerous activity of *in vitro* cultures and *in vivo* plant extracts

2.11.1 Evaluation of antioxidant activity of *in vivo* and *in vitro* cultures and *in vivo* plant extracts

Any chemical species that possessess an unpaired electron or odd number of electron may be labeled as a free radical. Free radicals exist in a state of thermodynamic. They are chemical species, which contain one or more unpaired electrons due to which they are highly unstable and cause damage to other molecules by extracting electrons from them, to attain stability (Ali *et al.*, 2007).

In the last two decades, there has been an explosive interest in the role of oxygen of free radicals, more generally known as reactive oxygen species (ROS) and reactive nitrogen species (RNS) in experimental and medicine.

It is well known that the exposure to certain noxious factors, such as xenobiotics, infecting agents, air pollution, UV light, cigarette smoke and radiation, may lead to the production of ROS (Gomes *et al.*, 2005).

ROS and RNS are known to play a dual role in biological systems, since they can be either harmful or beneficial to living system (Valko *et al.*, 2006). Beneficial effects of ROS involve physiological roles in cellular responses to noxia as, for example in defense against infectious agents and in the function of a number of

cellular signaling systems. One further beneficial example of ROS at low concentration is the induction of a mitogenic response. In contrast, at increased concentrations, ROS can be important mediators of damage to cell structures, including lipids and membranes, proteins and oxidative stress (Poli *et al.*, 2004). The harmful effect of ROS is balanced by the antioxidant action of non enzymatic antioxidants in addition to antioxidant enzyme (Halliwell, 1996). Despite the presence of antioxidant defense system of the cells to counteract oxidative damage from ROS, oxidant damage accumulates during the life cycle and radical related damage to DNA, to proteins and to lipids has been proposed to play a key role in the development of age dependent diseases such as cancer, artherosclerosis, arthiritis, neurodegenerative disorders and other conditions (Halliwell, 1999).

2.11.1.1 Reactive oxygen species

The reactive oxygen species are superoxide, hydrogen peroxide and hydroxyl radicals. Superoxides are important as the products of the one electron reduction of dioxide, which occurs widely in nature. With one unpaired electron, the superoxide ion is a free radical and is paramagnetic. It is biologically quite toxic and is deployed by the immune system to kill invading organisms. In phagocytes, superoxide is produced in large quantities by the enzymes NADPH oxidase for use in O_2 dependent killing mechanism of invading pathogen. Superoxides are also produced as a byproduct of mitochondrial respiration, as well as several other enzymes like Xanthine oxidase. It can cross cell membranes via anion channels (Mc Bride and Kraemer, 1999).

The biological toxicity of SO_2 is due to its capacity to inactivate iron- sulphur cluster containing enzyme, thereby liberating free iron in the cells, which can undergo ferton chemistry and generate the highly reactive hydroxyl radicals. In its H_2O_2 form, (hydroperoxy radicals) SO_2 can also initiate lipid peroxidation of polyunsaturated fatty acid. It also reacts with carbonyl compounds and halogenated carbon to create toxic peroxy radicals. Superoxide readily reacts with nitric oxide (NO) to form

peroxyl nitrite ($ONOO^-$), which is unstable at physiological pH and rapidly decomposes to form potent nitrating and oxidizing species (Beckam, 1990).

The oxidizing capacity of H_2O_2 is so strong that the chemical is considered a highly reactive O_2 species. H_2O_2 is widely regarded as a cytotoxicity reagent. In fact, H_2O_2 is poorly reactive in the absence of transition metal ions. Levels of H_2O_2 in the body may be controlled, not only by catabolism, but also by excretion, and H_2O_2 could play a role in the regulation of renal function and as an antibacterial agent in the urine. Urinary H_2O_2 levels are influenced by diet, but under certain conditions might be a valuable biomarker of oxidative stress. H_2O_2 peroxide is freely miscible with H_2O and is apparently able to cross cell membrane readily, high levels being cytotoxic. It is therefore widely thought that H_2O_2 is very toxic *in vivo* and must be rapidly eliminated employing enzyme such as catalases, peroxidases (especially gluthione peroxidases) and thioredoxin linked system (Halliwell, 2000).

The hydroxyl radical has a very short *in vivo* half life of approximately 10-9 seconds and a high reactivity. This makes it a very dangerous compound in organism. Unlike superoxide dismutase, the hydroxyl radical cannot be eliminated by an enzymatic reaction, as this would require its diffusion to the enzyme active site. As diffusion is slower than half life of the molecule, it will react with any oxidizable compound in its vicinity. It can damage virtually all types of macromolecules: carbohydrate, nucleic acid (mutations), lipids (lipid peroxidation) and amino acids (e.g. conversion of phe to m-tyrosine and o- tyrosine). The only means to protect important cellular structure is the use of antioxidants such as glutathione and of effective repair system (Kaur and Perkins, 1991).

2.11.1.2 Reactive nitrogen species

Together with ROS, RNS, in particular nitric oxide (NO) is now considered as major components of oxidative burst and redox state regulation (Pauly *et al.*, 2006).

Nitric oxide (NO) is a signal transducting free radical, synthesized from five electron oxidation of the guanidine nitrogen of L- arginine by nitric oxide synthase (NOS), in the reaction O_2 and NADPH act as CO substrate. Flavin nucleotide (FNS),

FAD and thiols serve as enzyme cofactors. Increased release of nitric oxide is found during lung inflammation. They also serve as pulmonary vasodilator. At high concentration, they inactivate critical enzymes by interacting with their iron –sulfur centre, causes DNA strand breaks that result in the activation of the nuclear enzyme poly-ADP ribose transferase and inhibits both DNA and protein synthesis (Zhu, *et al.*, 1998).

The most toxic effects of NO have been attributed to its reaction with O_2 with a rate constant of about 7 X 10^9 M^{-1} sec $^{-1}$ to form ONOO-. They can initiate iron dependent lipid peroxidation and oxidizes thiols, damage mitochondria electron transport chain and cause lipid peroxidation of human low density lipoproteins because of its high reactivity ONOO will attack biological targets even in the presence of antioxidant substance (Zhu *et al.*, 1998).

2.11.1.3 Antioxidant activity of medicinal plants

The herb *Plumbago zeylanica* is known for inhibitory effects against superoxide and nitric oxide production (Natarajan *et al.*, 2006). Natarajan *et al.* (2006) confirmed antioxidant property of the plant, using 2, 20-azinobis 3-ethylbenzothiazoline 6-sulfonate (ABTS) assay.

Rubia cordifolia has been shown to exert cell/neuroprotective properties via preventing the depletion and increasing GSH (glutathione) levels by inducing GCLC (c-glutamylcysteine ligase) expression, reducing oxidant levels by direct scavenging, and decreasing iNOS expression. The protective ability may be attributed to the GSH and vitamin C content of the herb (Rawal *et al.*, 2004).

Tripathi *et al.* (1998) reported that Rubiadin, a dihydroxy anthraquinone, isolated from alcoholic extract of *Rubia cordifolia*, possesses potent antioxidant property. It prevents lipid peroxidation induced by Fe_2SO4 and t-butylhydroperoxide (t-BHP) in a dose dependent manner. The per cent inhibition was more in the case of

$Fe2^+$ induced lipid peroxidation. The antioxidant property of the preparation has been found to be better than that of EDTA, Tris, mannitol, Vitamin E and p-benzoquinone.

Tripathy and Sharma, (1998) found that the inhibition of Fe_2SO4 induced lipid peroxidation in rat liver by alcoholic extract of *Rubia cordifolia* and by one of its constituent rubiadin (1, 3-dihydroxy-2-methyl anthraquinone) in pure form. Both have been found to inhibit lipid peroxidation in a dose dependent manner. Whereas the former shows both oxidising and reducing properties with $Fe2^+$ and $Fe3^+$, the latter shows oxidising property only by converting $Fe2^+$ to $Fe3^+$. The former inhibits the oxidation of reduced glutathione while the latter does not.

The methanolic extract of *Ricinus communis* showed significant free radical scavenging activity by inhibiting lipid peroxidation initiated by carbon tetrachloride and ferrous sulphate in rat liver and kidney homogenates. The extract enhanced free radical scavenging activity of stable radical 2, 2-diphenyl-1-picryl-hydrazyl (DPPH•), nitric oxide and hydroxyl radical in *in vitro* assay methods (Ilavarasan *et al.*, 2006).

Tripathi *et al.* (1996) studied the effect of the alcoholic and hexane fractions of *Bacopa monniera* on $FeSO_4$ and cumene hydroperoxide induced lipid peroxidation. They observed that alcoholic fraction showed greater protection against both the inducers and the results were comparable to known antioxidants like vitamin C. Probable mechanism of action could be through metal chelation at the initiation level and also as a chain breaker suggesting that *Bacopa monniera* is a potent anti-oxidant. The responses with *Bacopa monniera* were found to be dose-dependent. At low doses, it only slightly protected the auto-oxidation and Fe_2SO4 induced oxidation of reduced glutathione, but at higher concentrations, it enhanced the rate of oxidation. The antioxidant action of *Panax ginseng* has been attributed to enhanced nitric oxide synthesis in the corpus cavernosum and in the endothelium of the lung, heart, and kidney (Gillis, 1997). The extract of *Panax ginseng* has been tested for *in vitro* and shown to inhibit hydroxyl radical activity induced by the a fenton reaction system,

and to protect unsaturated fatty acid from decomposition caused by iron mediated lipid per oxidation (Zhang *et al.*, 1996). Administering extracts in rats have been demonstrated to reduce oxidative stress in certain tissue by altering specific antioxidant enzyme activities, so that end product of tissue peroxidant reaction are reduced (Voces *et al.*, 1999).

2.11.2 Prooxidants and Antioxidants

A chemical compound and a reaction capable of generating potential toxic oxygen species free radicals are referred as to as a prooxidant. Reactive oxygen species (ROS) formed *in vivo*, such as superoxide anion, hydroxyl radical and hydrogen peroxide, are highly reactive and potentially damaging transient chemical species. These are continuously produced in the human body, as they are essential for energy supply, detoxification, chemical signaling and immune function. ROS are regulated by endogenous superoxide dismutase, glutathione peroxidase and catalase but due to over- production of reactive species, induced by exposure to external oxidant substances or a failure in the defense mechanisms, damage to cell structures, DNA, lipids and proteins (Valko, 2006) occur, which increases risk of more than 30 different disease processes (Aruoma, 1998). The most notorious among them are neurodegenerative conditions like Alzheimer's disease (AD) (Smith *et al.*, 2000), mild cognitive impairment (MCI) (Guidi, 2006) and Parkinson's disease (PD) (Bolton *et al.*, 2000). Other neurodegenerative diseases significantly associated with oxidative stress include multiple sclerosis, Creutzfeldt–Jacob disease and meningoencephalitis. Valko *et al.* (2007) have done an extensive review on the effect of free radicals and antioxidants in normal physiological functions and human disease. The hydroxyl radical is known to react with all components of the DNA molecule, damaging both the purine and pyrimidine bases and also the deoxyribose backbone (Halliwell & Gutteridge, 1999). Besides DNA, ROS also attack other cellular components involving polyunsaturated fatty acid residues of phospholipids (Siems *et al.*,1995) side chains of all amino acid residues of proteins, in particular cysteine, and methionine residues (Stadtman, 2004).

Interestingly the body possesses defence mechanisms against free radical-induced oxidative stress, which involve preventive mechanisms, repair mechanisms, physical defenses and antioxidant defenses. Enzymatic antioxidant defenses include superoxide dismutase, glutathione peroxidase and catalase (Gutteridge, 1994). Non-enzymatic antioxidants are ascorbic acid (McBride and Kraemer,1999) a-tocopherol (Guyton, 1993) glutathione (GSH), carotenoids (Kanter *et al.*, 1993), flavonoids, etc.

Compounds scavenging free radicals suppressing their formation or opposing their action are called antioxidant (Irshad *et al.*, 2002). The term scavenger refer to their ability to provide electrons, so that the free radicals can attach itself to the antioxidant electron and be rendered harmless before it can attach itself to a so called healthy radical and begin the destructive chain reaction (Khan, 2002).

Antioxidants help organisms deal with oxidative stress, caused by free radical damage. Antioxidants block the process of oxidation by neutralizing free redicals. Their mechanism of action can be classified as chain breaking and preventive (Sherwin, 1978).

In chain breaking action when a free radical releases an electron, a second radical is formed, whereas this molecule continues the reaction to form more unstable products. The process continues until termination occur, either the radical is stabilized by a chain breaking antioxidant such as beta carotene ,vitamin C and vitamin E or it simply decays into a harmless product (Shang *et al.*,2003).

In preventive action, antioxidant enzymes like super oxide dismutase, catalase and glutathione peroxidase, prevent oxidation by reducing the rate of chain initiation. They can also prevent oxidation by stabilizing transition metal radicals such as copper and iron (Reif *et al.*, 2001).

2.11.2.1 Prooxidant activities of plants in relation to cancer prevention

Apoptosis, sometimes called "guardian angel" or "cell policeman," is a cell suicidal altruistic mechanism, targeted to selectively eliminate cancerous and other cells that threaten our health and life (Kerr *et al.*, 1994). The sacrifice of the "bad"

cells occurs to save the integrity and life of the whole organism (Blackstone and Green, 1999). Apoptosis is carried out by a multistage chain reactions in which ROS act as triggers and essential mediators (Johnson *et al.*, 1996). Recently, it became evident that mitochondria play a critical role in apoptosis. Apoptotic signals, which arise in cancer cells, promote accumulation of the p35 protein that triggers the release of ROS, cytochrome C and a few other regulators from mitochondria. The latter activates a cascade of proteolytic enzyme, called caspases, that activated deoxyribonuclease. Cleavage of critical proteins and DNA results in apoptotic cell death. Importantly, most anticancer drugs and radiation kill cancer cell by inducing apoptosis (Salganik *et al.*, 2001). Mutation in the p 35 gene make cancer cells resistance to apoptosis and accordingly, to anticancer drugs.

2.11.2.2 Proxidant activity of naphthoquinones

The biochemistry and cytotoxicity of naphthoquinones have been extensively studied *in vitro*. By reaction with cellular reducing agents, naphthoquinones undergo redox cycling, with concomitant formation of reactive oxygen species (ROS) as superoxide anion and hydrogen peroxide (Inbaraj and Chignell, 2004). Furthermore, the carbon atoms adjacent to the carbonyl groups are electrophilic, and naphthoquinones that are not fully substituted in the quinone ring are alkylating agents. Most chemotherapeutic agents, including alkylating agents, induce cell death in cancer cells by apoptosis. These classes of antineoplastic agents cause cells to over generate ROS, and, thus, are capable of inducing apoptosis, and causing oxidative damage to DNA (Lopaczynski and Zeisel, 2001).

2.11.3 Cytotoxicity in relation to anticancer activity

Plant-derived natural products with antitumor properties can be classified into thirteen distinct chemical groups (Kintzios and Barberaki, 2004). Among them, best documented for their tumor-cytotoxic activity are alkaloids (Facchini, 2001), phenylpropanoids (Dixon and Paiva, 1995), terpenoids (Trapp and Croteau, 2001) and flavonoids (such as baicalin from *Scutellaria baicalensis*) which not only inhibit

cancer cell proliferation by modulating the activity of cyclin-dependent kinases (Chang *et al.*, 2004), but also demonstrate a cytotoxic estrogen-like activity in high concentrations (Woo *et al.*, 2005). *Huanglian* is an herbal widely used in traditional Chinese medicine. It has found that the whole herbal extract potently inhibits the growth of gastric, breast, and colon cancer cells by directly suppressing cyclin B1 protein expression and inhibiting cyclin-dependent kinase1 (cdc2 kinase) activity (Li *et al..,* 2000). It has been suggested that *Ganoderum lucidum* has an antitumour effect medicated by cytokines released from activated macrophage and T lymphocytes (Wang *et al.*, 1997). Epigallocatechin -3 gallate is the main component identified in green tea to posses anti carcinogenic properties (Nie *et al.*, 2002). PC – SPES is an herbal mixture extract commercially available and widely used for the treatment of various cancers, such as pancreatic cancer (Schwarz *et al.*, 2003), prostate cancer and colon cancer (Huerta *et al.*, 2002). Itokawa *et al.* (1997) reported that bioactive peptides from medicinal plants, i.e a series of unique bicyclic peptides, celogentins isolated from *Celocia argentea* (Amaranthaceae) remarkably inhibit the tubulin polymerization, a series of RA peptides isolated from *Rubia* species (Rubiaceae) exhibit potent antitumor activities *in vitro* and *in vivo*, and a series of astins isolated from *Aster tataricus* (Compositae) show antitumor activities *in vivo.*

Rao *et al.* (1997) reported that pergularinine (PgL) and tylophorinidine (TPD) isolated from *Pergularia pallida* are potently toxic and inhibit the growth of *Lactobacillus leichmannii* cells by binding to thymidylate synthetase. The binding led to significant inhibition of thymidylate synthetase activity making them potential anti-tumor agents.

The anti-tumor effect of the crude extract of *Centella asiatica* as well as its partially purified fraction was studied in both, *in vitro* short and long term chemosensitivity test systems and *in vivo* tumor models. The purified fraction inhibited the proliferation of transformed cell lines of Ehrlich ascites tumor cells and Dalton's lymphoma ascites tumor cells more significantly than the crude extract. It also significantly suppressed the multiplication of mouse lung fibroblast cells in long

term culture. *In vivo* administration of both extracts retarded the development of solid and ascites tumors and increased the lifespan of the tumor bearing mice. Tritiated thymidine, uridine and leucine incorporation assays suggest that the purified fraction acts directly on DNA synthesis (Babu *et al.*, 1995).

2.11.3.1 Anticancerous activity of naphthoquinone

Siripong *et al.* (2006) reported that rhinacanthins active components of *Rhinacanthus nasutus*, had a antiproliferative activity against human cancer cell lines. They studied the growth inhibitory mechanism of rhinacanthus-C,-N and –Q three main naphthoquinones component were isolated from the plant, in human cervical carcinoma ($HeLaS_3$) cells by means of TUNEL staining, DNA feagmentation assay, flowcytometry, and cleavage assay of Asp-Glu-Val-Asp-peptide-nitroanilide, a caspase-3 subtrate.

Lapacone a naturally produced compound from naphthoquinone compound having similar basic structure to rhinacanthone showed potent inhibition of DNA topoisomerase I and II and induced apoptosis in various cancer cells (Krishnan *et al.*, 2001).

Rodrigo *et al.* (2004) reported the isolation 1,4-naphthoquinone from *Paepalanthus latipes* an alkylating agent, induces cell death in cancer cells by apoptosis. These classes of antineoplastic agents cause cells to over generate ROS, and, thus, are capable of inducing apoptosis, and causing oxidative damage to DNA.

Plumbagin, a naphthoquinone present in the Drosera genus, is known to generate ROS and has been found to inhibit the activity of topoisomerase II (Topo II) through the stabilization of the Topo II-DNA cleavable complex. Plumbagin induced DNA cleavage in HL-60 cells, and ROS generated by plumbagin at low concentrations (3 mM) act as signal-ling molecules mediating apoptosis through Topo II inactivation rather than through direct DNA damage (Kawiak *et al.*, 2007).

Kitagawa *et al.* (2004) evaluated the *in vitro* cytotoxicity of the 1,4-naphthoquinone isolated from the capitula of *Paepalanthus latipes,* on McCoy cells using the microculture MTT-tetrazolium assay. The new compound showed a significant cytotoxic index of 35.8m g ml^{-1} when compared to cisplatin (IC_{50} value of 41.9m g ml^{-1}), a cytotoxic substance used in antineoplasic therapy, used as reference compound on the same cellular system.

MATERIALS AND METHODS

MATERIALS AND METHODS

The study entitled 'Exploitation of *in vitro* cultures of Indian Madder (*Rubia cordifolia.* Linn) for anticancerous compounds' was carried out at the Plant Tissue Culture Laboratory of the Centre for Plant Biotechnology and Molecular Biology, College of Horticulture, Vellanikkara from December 2006 to September 2008. The materials used and the methodology adopted in this study, are described below.

3.1 Culture Media

The medium suggested by Murashige and Skoog, 1962 (MS medium with full strength of all salts) was used for callus induction and establishment of suspensions, in *Rubia cordifolia.*

3.1.1 Materials

All the chemicals, used as ingredients and additives in the MS medium, are of AR grade and were procured from M/s Merck India Ltd., Sisco Research Laboratories, Pvt. Ltd., British Drug House and Sigma Ltd. Borosilicate glassware of Corning, Vensil and Borosil brands were used. The glassware was cleaned by soaking in solution of potassium dichromate in sulfuric acid for half an hour. Later, the glass containers were washed with jets of tap water to remove all traces of potassium dichromate solution. Then, they were further cleaned with 0.1per cent Teepol detergent solution and were washed thoroughly with water and rinsed twice with double distilled water. These were then dried in hot air oven at more than 100°C for 24 hours and later stored in cup boards, free of dust till further use.

3.1.2 Method

Medium was prepared by following the standard procedure adopted by Gamborg and Shyluk (1981). Stock solution of major and minor elements were prepared and stored in precleaned amber coloured bottles in refrigerated condition.

A cleaned steel vessel, rinsed with distilled water, was used to prepare the medium. Aliquots from all stock solutions were pipetted in proportionate volume in the vessel. A little amount of distilled water was added to it and later on required quantities of sucrose

(carbohydrate source) and inositol were added and dissolved in it. The desired volume was made up by adding distilled water. The pH of medium was adjusted between 5.5 to 5.8 using NaOH.

For solid medium, agar from M/s Sigma Research Laboratories Pvt. Ltd. was added at 0.75 per cent (w/v) concentration, after adjustment of the pH. The medium was stirred and heated to melt the agar and was poured when hot, into vessels which were plugged with cotton. For solid media, test tubes (15 cm x 2.5 cm) were used, while for liquid media, jam bottles and conical flasks were used as culture vessels. About 15 ml medium was poured into each test tube and 30 ml medium in each jam bottle. Vessels containing media were sterilized in an autoclave by applying a pressure of 15 Psi for 20 minutes. After this, the culture vessels were kept in media store rooms, till further use.

3.1.3 Inoculation procedure

Inoculation was carried out under strict aseptic conditions inside a laminar airflow chamber procured from Kirloskar Pvt. Ltd. The working floor, inside the chamber, was wiped thoroughly with 70 per cent ethanol to remove any traces of dust or adhering dirt. Sterilized forceps, petridishes, surgical blades, scalpels and blotting papers were kept inside the chamber and were sterilized with UV light for 30 minutes. Surface sterilized explants were inoculated in the culture tubes. One explant per test tube was placed horizontal on the surface of medium.

For inoculating the liquid media, callus was used as initial inoculum. Calli grown in test tubes were removed inside the laminar airflow chambers and were made free of any adhering piece of media. The calli were cut into pieces of approximately one gram and one such piece was transferred to each conical flask.

3.1.4 Culture condition

The cultures were incubated in a closed room providing 3000 lux fluorescent light for a period of 16 hours followed by 8 hours of dark period daily. The temperature was maintained at 26 ± 2°C and humidity between 60 and 80 per cent.

The liquid cultures were kept on rotary shakers at 105 rpm and cultured at 28±2°C temperature in normal light.

3.1.5 Subculture interval

Subculturing was done in aseptic condition similar to those employed during inoculation of explants. Subculturing of calli was done every 25 to 30 days, after in fresh medium, having respective composition and growth hormones and media supplements. Approximately 0.5 gram of callus was subcultured in each tube. The liquid cultures were subcultured after every 27 days into respective fresh media. The conical flasks were allowed to stand for 5 minutes for settling the cell aggregates and then 5 ml of the liquid culture was poured into another sterilized conical flask with 25 ml of fresh media.

3.2 Collection and preparation of explants

3.2.1 Source of explants

Explants for *in vitro* culture establishment were collected from potted plants of *Rubia cordifolia* maintained in the green house of College of Horticulture, Vellanikkara (Plate 1). These plants were maintained free from pest and diseases by regular spraying of fungicides, like Bavistin 0.1 per cent, at weekly intervals.

From the potted plants of *Rubia cordifolia*, green shoots of 2.5 mm in diameter and long terminal portion were selected. These shoots were cut with a sharp surgical blade and were enclosed in a sterile glass container. The explants in closed sterile glass container were taken to the Plant Tissue Culture Laboratory.

For obtaining root explants, *in vitro* roots from axenic plants of *Rubia cordifolia*, were used for callus initiation (Plate 1).

3.2.2 Preparation of explants.

Leaves and nodal segments from *in vivo* grown plants were used as explants. Leaves and stems were cut into pieces of 0.5 to 1 cm length. They were separately washed in frothing solution of detergent. To remove all traces of the detergent, they were thoroughly washed 3 to 4 times, with running tap water.

Leaves and nodal segments were separately wiped with clean swabs of cotton and soaked in 70 per cent ethanol. After that, leaf segments and nodal segments were washed with

Plate 1. Source plants of *Rubia cordifolia.* Linn

a) *Ex vitro* plants

b) *In vitro* plants

millipore water to remove the traces of alcohol. Explants free from adhering water were trimmed at edge to remove all dried tissues. The explants tested for callus induction were:

1. Leaf explants: leaf lamina (one cm^2 area around midrib)
2. Stem explants: Nodal segments (1.5 cm long with one node and axillary bud)
3. Roots explants: Roots segments (1 to 1.5 cm long).

3.2.3 Standardization of surface sterilization

Explants employed in the study were pretreated with the fungicide Bavistin at various concentrations (Table 3) and then surface sterilized under aseptic conditions maintained inside a laminar air flow cabinet. The nodes and leaves were separately dipped in 0.1 per centmercuric chloride solution for varying duration and the containers were shaken intermittently. The leaves and nodal segments were retained for 1 minute. Later on, they were thoroughly washed 3 to 4 times with sterile distilled water and were spread on sterile filter paper to drain, inside the laminar air flow chamber.

Table No 3: Fungicide/surface sterilants used for pretreatment and surface sterilization of *Rubia cordifolia*

Surface sterilants (Per cent)	Duration of treatment (min)
Fungicide(Bavistin)	
0.1	10
	15
0.5	10
	15
1	10
	15
2.5	10
	15
	25
3	10
	15
	25
$HgCl_2$ 0.1	3
	2.5
	1
	0.5

3.3 Standardization of media for callus induction and proliferation

Different plant growth regulators of varying concentrations were added to the basal MS medium. The stock solution of growth regulators at 1000 $mg^{-1}$was prepared and stored under refrigeration and aliquots were taken from them for use after dilution. These aliquots were added to the medium, before the pH was adjusted.

3.3.1 Growth regulators

Auxins like 2, 4-D, NAA, and IAA and cytokinin like BA and kinetin of varying concentrations, were added to basal MS medium for induction and proliferation of calli. The various concentrations in which the phytohormones were used, are given in Table 4.

Table 4. Growth regulators employed for induction and proliferation of calli in *Rubia cordifolia*

Plant growth regulators	Concentrations in mg^{-1}
2, 4- D	1, 2
NAA	1, 2
IAA	1, 2
Kinetin	1, 2
BA	1, 2
NAA + BA	2, 1, 0.5
NAA + Kinetin	2, 1, 0.5
2, 4- D + BA	2, 1, 0.5
2, 4- D + Kinetin	2, 1, 0.5

3.3.2 Media additives

Treatments, incorporating the media supplement phloroglucinol at 100 mg l^{-1} are listed in Table 5. The additive was weighed and added directly to the medium before the final volume was made up.

Table 5. Media additive employed for induction and proliferating calli in *Rubia cordifolia*

Sl. No	Media compostion
1	MS + NAA 1 mg l^{-1} + Phloroglucinol 100 mg l^{-1}
2	MS + NAA 2 mg l^{-1} + Phloroglucinol 100 mg l^{-1}
3	Ms + 2,4 -D 1 mg l^{-1} + Phloroglucinol 100 mg l^{-1}
4	MS + 2,4 –D 2 mg l^{-1} + Phloroglucinol 100 mg l^{-1}
5	MS + IAA 1 mg l^{-1} + Phloroglucinol 100 mg l^{-1}
6	MS + IAA 2 mg l^{-1} + Phloroglucinol 100 mg l^{-1}

Twelve cultures were maintained per treatment.

3.3.3 Observations on growth of callus

The following observations were made on various parameters, influencing induction and proliferation of calli.

(a) Percentage of cultures initiating calli

Of all the inoculating tubes, those, which showed signs of callusing, were counted and were expressed as percentage of total number of inoculated tubes.

(b) Number of days for callus initiation

The times interval between the day of inoculation to the day when first visible signs of callus growth was seen, was counted as number of days for callus initiation.

(c) Percentage of tubes producing full-tube callus

Of all the inoculated tubes, those which produced full tube callus, were counted and this was expressed as percentage of total number of inoculated tubes.

d) Callus growth rate

Based on the extent of callus proliferation, growth of callus per test tube was scored and numerical scores, 0 to 4, were assigned to cultures, indicative of callus growth and proliferation as detailed in Table No 6.

Table 6 Growth scores indicating callus proliferation rate in *Rubia cordifolia*

Extent of callus growth at end of culturing period (30 days after initiation)	Growth score (G_i)
No callus initiation	0
Callus covering about ¼th surface of medium	1
Callus covering about ½nd surface of medium	2
Callus covering about ¾th surface of medium	3
Callus covering full-tube	4

e) Callus index

Callus index (CI) was calculated as CI= P x G.

Where P = percentage of cultures initiating calli

G = Growth score

Since the growth score ranged from 0 to 4, the value of callus index ranged between 0 to 400. Hence the maximum obtainable value of the callus index was 400. The treatment having callus indices near 400 were considered as the best, while those with values near 0 were considered to have poor callusing ability.

3.3.4 Influence of culture environment on proliferation

Effect of culture condition on callus initiation and proliferation was observed by incubating the cultures in dark and under illumination. The incubated cultures were observed for callus initiation and proliferation.

3.4 Biochemical estimation of naphthoquinones

Total naphthoquinones were estimated from callus grown on basal medium supplemented with various growth regulators at different levels and media supplements, as a measure of anticancerous compound synthesized *in vitro*. A thin layer chromatographic detection and relative quantification were conducted for qualitative as well as quantitative estimation of the naphthoquinone,

3.4.1 Extraction of crude quonine from *in vitro* cultures

3.4.1.1. Preparation of extracts

a) *In vitro* culture extract

Calli were collected from test tube without any adhering pieces of nutrient media. The cleaned calli (10 g) callus per sample was ground using mortar and pestle with 30 ml of 70 percent methanol. The ground calli were retained in solvent retained for 6 hours and then, filtered through Whatman No. 4 filter paper and evaporated in a water bath. The original volume was maintained by addition of distilled water. It was mixed with an equal volume of ethyl acetate and agitated in a separating funnel, facilitating separation of extract into two layers. The upper layer of ethyl acetate was separated and the solvent was evaporated to get a fraction, which contained quinone with minimum impurities. The non quinone fraction in the aqueous layer, was discarded. The extract from the ethyl acetate fraction was taken for estimation of napthoquinones.

b) *In vivo* plant extract

In vivo plants were uprooted from the pot and the adhering soil was removed. After that, plants samples (whole plant and root samples) were the n air dried and ground. The clean 10 g ground sample of whole plant as well as roots, was extracted with 30 ml of 70 per cent methanol. The samples were extracted in a continuously stirring condition for 24 hours, filtered through Whatman No. 4 filter paper and were evaporated in a water bath. The original volume was maintained by addition of distilled water. It was mixed with an equal volume of ethyl acetate and agitated in a separating funnel, facilitating separation of extract into two layers. The upper layer of ethyl acetate was separated and the solvent was evaporated to get a fraction, which contained quonine with minimum impurities. The non quonine fraction in the aqueous layer, was discarded. The extract from the ethyl acetate fraction was taken for estimation of naphthoquinones.

3.5 Screening of *in vitro* and *in vivo* extract for the presence of naphthoquinone

3.5.1 Preparation of sample

The ethyl acetate fractions of calli and cell suspension were taken in test tubes and shaken well till a clear golden yellow solution was obtained. This solution was used for spotting on TLC plates.

Besides preparing the extracts of calli and cell suspensions cultures, possibility was explored for secretion of naphthoquinone by the plant cells, into the culture medium. For this, five ml of liquid culture was centrifuged at 7000 rpm for 5 minutes, by which cells settled at the bottom. Cell free liquid medium constituted the supernatant. This liquid medium was partitioned with ethyl acetate and the ethyl acetate fraction was used for estimation of naphthoquinone.

3.5.2 Qualitative tests for detection of quinone

The detection of quinone was done by Borntrager's test. One gram of callus was extracted with methanol (2 ml). To this extract equal volume hexanes was added and shaken well. Then, the upper hexane layer separated. To the lower layer, added 25 per cent ammonia solution. Appearance of a red colour indicated the presence of quinones.

3.5.3 Preparation of TLC plates

TLC plates were prepared by coating 300 µm thick layer of silica gel G on glass plates of 20 X 20 cm size. Forty gram of silica gel G of SRL brand was weighed and added to 85 ml of distilled water. Immediately, it was shaken vigorously for 30 seconds to get a homogenous slurry which was poured in the trough of the TLC plate gel applicator of CAMAG brand. The plates were passed, one by one, beneath the trough and each was coated with a 300 µm thick coat of silica gel G. About eight plates were coated with a slurry made from 40 grams of silica gel G. The plates were quickly coated, within 2 minutes, to avoid setting of the silica within the applicator itself. The plates were allowed to air dry for about ten minutes at room temperature, after which they were placed in aluminum racks and kept in a chromatographic oven for heating, at110 °C for 30 min. This activated the plates for further use.

3.5.4 Standardization of solvent systems and running the chromatogram

3.5.4.1 Standardization of running solvent systems

Running solvent systems were prepared by mixing various solvents in desired ratios (Table No 8). For evaluating the performance of various combinations of running solvent systems, samples were eluted in each of them and the chromatograms were compared. The samples were extracted from callus grown in *in vitro* cultures. Twenty µl volume of the sample

solution of 1 mg ml^{-1} concentration was spotted on the plates. The plates were eluted and developed and the chromatograms were evaluated on the following lines:

(a) Capacity to elute the applied sample

The capacity of a running solvent to elute more compounds from a sample was judged by estimating the quantity of sample remaining uneluted. The uneluted sample which remained at the point of application was graded based on the intensity of colour produced by it.

When the entire sample was eluted, and no colour was seen at the point of application the running solvent was graded as 'very good'. When small quantity of the sample remained uneluted, faint red colour was seen, and the running solvent was graded 'good'. When more sample remained uneluted, shades of reddish color were noticed, based on the intensity of which grading was done as 'satisfactory', 'poor' and 'very poor' with increasing shades of red.

(b) Clarity of the chromatogram

The distinctness of naphthoquinone spots was noted and its extent of mixing with other organic compound was observed. Observations on whether these organic compounds masked or interfered with the visibility of naphthoquinone spots, were made.

For purpose of quantifying the naphthoquinone, the elution was done in a solvent mixture of chloroform : methanol in the ratio(8.5:1.5). About 70 ml of this solvent system was used to elute at a time in a CAMAG TLC developing glass chamber. The solvent system was poured in the tank and lid was placed tightly. Adjacent to the walls of the tank thin filter paper sheets soaked in the solvent were placed for proper saturation of the tank. The tank was then shaken once, vigorously. It was retained as such for 30 min to saturate the tank with the vapours of the volatile components of the solvent system. Ethyl acetate extract of *in vivo* test samples and four week old calli grown on production medium were employed for spotting, along with the reference sample, 1,4-naphthoquinone obtained from $^{M}/_{S}$ Spectrochem Ltd. Twenty microliter each of test sample as well as reference sample were applied with a capillary on a precoated plate in a line, 2cm apart and about 2 cm from the lower end and allowed to dry. Then the spotted plate was placed in the tank such that the edge of the plate below the spots was immersed in the solvent system, and that the spots were above the surface of solvents. All chromatograms were eluted between temperatures of 29 to 30 °C and at 72 to 75 per centrelative humidity, to maintain uniformity in the elution

pattern. After approximately 30 minutes, when the solvent eluted the spots vertically upto $^{2}/_{3}$ rd length of the plate, the plates were removed for developing.

Table 7. Assessment of running solvent systems for eluting the naphthoquinone from callus/cell suspension of *Rubia cordifolia*, on thin layer chromatograms.

Sl. no	Running solvent systems	Ratio of solvents used
1	Water	100 per cent
2	Methanol	100 per cent
3	Acetone	100 per cent
4	Chloroform	100 per cent
5	Hexane	100 per cent
6	Acetone : Water	9.5 :0.5
7	Hexane : Benzene	1:1
8	Hexane : Benzene	2.5 : 7.5
9	Hexane : ethyl acetate	3 : 7
10	Hexane : ethyl acetate	8 : 2
11	Hexane : ethyl acetate	9 : 1
12	Hexane : ethyl acetate	5: 5
13	Hexane : ethyl acetate	6 : 4
14	Disopropyl ether : petroleum ether	1 : 9
15	Hexane : Butyl ether	9 : 1
16	Benzene : ethyl acetate	9.5 : 0.5
17	Petroleum ether : ethyl acetate : formic acid	75 : 24:1
18	Chloroform : ethyl acetate	3 :7
19	Ethyl acetate :toluene	5 : 5
20	Methanol : water	5: 5
21	Methanol : ethyl acetate	5: 5
22	Chloroform : hexane : ethyl acetate	8 : 1 : 1
23	Chloroform : methanol	5 : 5
24	Chloroform : methanol	9 : 1
25	Chloroform : methanol	8 : 2

26	Chloroform : methanol	7 : 3
27	Chloroform : methanol	8.5 : 1.5
28	Chloroform : methanol : Benzene	40 : 7.5 : 2.5
29	Hexane : acetone : Acetic acid	15 : 5 : 0.3

3.5.5 Preparation of the spray reagent

3.5.5.1 Evaluation of spray reagents in *in vitro* and *in vivo* extract of *Rubia cordifolia*

The following spraying reagents were evaluated for detecting naphthoquinone in thin layer chromatograms of *in vitro* cultures of *Rubia cordifolia*:

1) KOH (10 per cent)

Added 10 g of KOH to 100 ml methanol, which was used for detection of naphthoquinone spots on TLC plates (Wagner and Bladt, 1986).

2) Methylene blue (reduced)

To 20 ml distilled water, added 0.001M methylene blue, 2 ml of concentrated sulfuric acid and 1 g Zinc dust. This mixture was filtered and used for spraying the TLC plates.

3) Ammonia solution

In a chromatography chamber, 5 ml of ammonia vapour was added and is allowed to saturate, to develop the naphthoquinone spot on thin layer chromatogram.

3.5.5.2 Developing the chromatogram

The eluted plates were placed under an exhaust flow to evaporate the solvent from the silica gel coated on the plate. When the uncoated side of the plate became free of all moisture droplets, the plates were considered to be free of running solvent. Using a vensil reagent sprayer, each plate was sprayed with a minimum of 10 ml of 10 per cent KOH spray reagent. The spraying was done in an exhaust chamber. The eluted chromatograms were uniformly sprayed all over, with fine droplets of spray reagent.

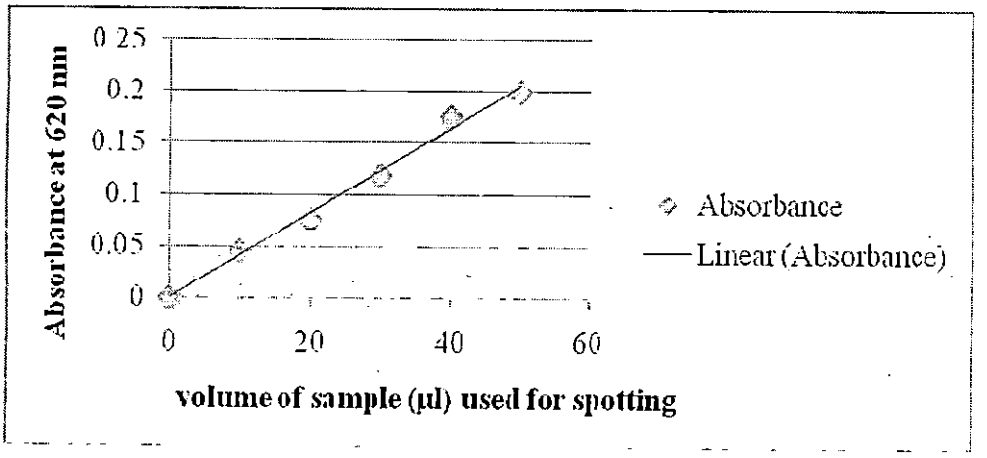
3.5.5.3 Quantification of naphthoquinones

Quantification of naphthoquinone was done by employing relative quantification technique. The spots formed on the developed plate were scrapped before the colour of

naphthoquinone spots faded. Scraped spots were extracted with chloroform and centrifuged to remove silica gel material. Chloroform extract was then read spectrophotometrically at 620 nm.

Different volumes of whole plant samples ranging from 10, 20, 30, 40, 50 µl were spotted and the plate was run with the standardized solvent system. After developing the plate, the spots were scrapped and read spectrophotometrically at 620 nm. A standard graph was plotted with absorbance against known volume of samples. Absorbance of known volumes of test samples were recorded and relative amount of naphthoquinone in the test samples was determined based on the standard graph.

Fig. 1 Standard graph of whole plant sample for relative quantification of naphthoquinone



3.5.5.4 Observation on the naphthoquinone production in *in vitro* cultures and *in vivo* plant of the experimental species were made as follows.

Detection of naphthoquinone in *in vitro* and *in vivo* extracts of target species, was done by observing the following.

(a) R_f values of naphthoquinone spots

The R_f values of the coloured naphthoquinones spots were recorded for each sample against reference sample by estimating the ratio of distance of the target compound from the origin to distance of solvent from the origin.

(b) Colour of naphthoquinone spots

The colour of the spots developed by test sample and reference sample was recorded. The naphthoquinone was identified by the presence the of red spots.

3.6. Standardization of production medium

Leaf, nodal segment and root derived calli were subcultured to basal medium (MS) supplemented with various combinations of growth regulators as detailed in Table 7 and incubated in light. The incubated cultures were analyzed for naphthoquinone after 4 weeks to identify the best growth regulator combination for naphthoquinone production and it was identified as production medium.

Table 8. Growth regulators employed for inducing synthesis of naphthoquinone in *in vitro* cultures of *Rubia cordifolia*.

Sl. No.	Media composition
1	MS + NAA 2 mg l^{-1} + kinetin 0.5 mg l^{-1}
2	MS + NAA 2 mg l^{-1} + kinetin 1 mg l^{-1}
3	MS + NAA 2 mg l^{-1} + BA 0.5 mg l^{-1}
4	MS + NAA 2 mg l^{-1} + BA 1 mg l^{-1}
5	MS + NAA 1 mg l^{-1} + kinetin 0.5 mg l^{-1}
6	MS + NAA 1 mg l^{-1} + kinetin 1 mg l^{-1}
7	MS + NAA 1 mg l^{-1} + BA 0.5 mg l^{-1}
8	MS + NAA 1mg l^{-1} + BA 1 mg l^{-1}
9	MS + 2,4 – D 2 mg l^{-1} + kinetin 0.5 mg l^{-1}
10	MS + 2,4 – D 2 mg l^{-1} + kinetin 1mg l^{-1}
11	MS + 2,4 - D 2 mg l^{-1} + BA 0.5 mg l^{-1}
12	MS + 2,4 - D 2 mg l^{-1} + BA 1 mg l^{-1}
13	MS + 2,4 – D 2 mg l^{-1} + phloroglucinol 50 mg l^{-1}
14	MS + 2,4 – D 2 mg l^{-1} + phloroglucinol 100 mg l^{-1}
15	MS + 2,4 – D 2 mg l^{-1} + phloroglucinol 150 mg l^{-1}
16	MS + 2,4 – D 1 mg l^{-1} + phloroglucinol 50 mg l^{-1}
17	MS + 2,4 – D 1 mg l^{-1} + phloroglucinol 100 mg l^{-1}
18	MS + 2,4 – D 1 mg l^{-1} + phloroglucinol 150 mg l^{-1}
19	MS + NAA 1 mg l^{-1}
20	MS + NAA 2 mg l^{-1}
21	MS + 2,4-D 1 mg l^{-1}
22	MS + 2,4-D 2 mg l^{-1}

3.6.1. Modification of production media in *Rubia cordifolia* cell cultures for *in vitro* production of naphthoquinone.

The proliferating calli were transferred on the production medium modified as detailed below and *in vitro* production of naphthoquinone was assessed after four weeks, employing the method described as in 3.5.5.3.

3.6.1.1 Modification of carbon source

Composition of production medium was modified by increasing level of sucrose to five per centto subculture the experimental calli, for subquent screening of naphthoquinone.

3.6.1.2 Modification of nitrate concentration

Effect of modifying the nitrate concentration of basal media, on naphthoquinone yield of callus of *Rubia cordifolia* was evaluated under two conditions – 1) 50 per cent concentration of nitrate (NO_3) in MS medium and 2) 25 per cent of the concentration of nitrate (NO_3) in MS medium.

3.6.1.3 Modification of phosphate concentration

Effect of modifying the phosphate concentration of basal medium on naphthoquinone yield of callus of *Rubia cordifolia* was evaluated under two conditions – 1) 50 per cent concentration of phosphate (PO_4) in MS medium and 2) 25 per cent of the concentration of phosphate (PO_4) in MS medium.

3.6.1.4 Incorporation of media additive

The media additive like yeast extract at 1 and 2 per cent were added to the basal medium supplemented with NAA at 2 mg l^{-1} and BA at 0.5 mg l^{-1}, to which experimental calli were subcultured for subsequent screening.

3.6.1.5. Precursor feeding

Tyrosine and phenylalanine, the identified indirect amino acid precursors of naphthoquinone (Egoun,1962) was added to production medium at 50, 100 and 150 mg l^{-1} concentrations, in which 1 month old calli were subcultured, for subsequent detection of the naphthoquinone.

3.6.2 Regulation of culture condition

The effect of photoperiod on napthoquinone synthesis was observed by incubating the cultures in dark and under illumination.

To assess the effect of low temperature on naphthoquinone production, *in vitro* derived calli were maintained under refrigerated condition at 6 to 7 °C temperature

3.7 Employing special techniques for synthesis of naphthoquinone in *in vitro* cultures

3.7.1. Creating conditions of stress

3.7.1.1 Addition of osmoregulants

Sorbitol at 1 and 1.5 per centwas added singly to the basal medium supplemented with NAA (2 mg l^{-1}) and BA (0.5 mg l^{-1}), to which experimental calli were sub cultured for subsequent screening and detection of naphthoquinone.

3.7.2 Elicitation

a) Biotic elicitation

The fungus (*Pythium aphanidermatum)* was grown in a 250 ml flask containing 50 ml sterile Potato Dextrose Broth at pH 3.5. The cultures were incubated at room temperature (30 °C) for 20 days. Then the fungal cultures were autoclaved and the fungal mat was separated by filtration. The mat was then washed several times with distilled water and the aqueous extract was made by homogenizing in a mortar and pestle using acid washed neutralized sand. This extract was filtered through muslin cloth and the volume was made equal to that of filtrate with distilled water. The extracts and filtrate were sterilized by autoclaving before use.

Effect of fungal elicitor on naphthoquinone production was tested by incorporating the mycelial extract along with the filtrate at two concentrations (2 per cent and 5 per cent v/v) into the production medium. The elicitor preparations were added to the medium directly and naphthoquinone production was assessed.

b) Abiotic elicition

Salicyclic acid at two concentrations (10 µM and 100 µM) was added to the production medium. The elicitor was added to the medium directly and naphthoquinone production of *in vitro* cultures was assessed.

3.7.3 Immobilization

Naphthoquinone production by *Rubia cordifolia* cells under immobilized condition was assessed as described below.

3.7.3.1 Preparation of cell aggregates

Actively growing callus was collected and cut into small aggregates in aseptic condition.

3.7.3.2 Preparation of support material (Gel)

Five grams of dry powder of sodium alginate was slowly added to 100 ml of MS basal medium, while being continuously stirred with a magnetic stirrer. The stirring was continued till complete sodium alginate got dissolved in the medium. Sodium alginate solution was autoclaved at 15 psi for 20 min in an autoclave.

3.7.3.3 Preparation of calcium chloride

Calcium chloride (1.42gm) was dissolved in 100 ml of distilled water to obtain a solution of concentration 100mM and sterilized at 15 psi for 20 min in autoclave.

3.7.3.4 Preparation of beads

Under sterilized conditions, small aggregate of callus was mixed with sodium alginate solution and mixed thoroughly with sterile glass rod. The prepared sodium alginate solution was extruded drop wise into a sterilized solution of 100 mM $CaCl_2$ from a height of approximately 7 to10 cm. Beads were formed in $CaCl_2$ solution entrapping the calli aggregates and were kept for 30 minutes in the same solution and then dried on blotting paper. After blotting, the entrapped calli in calcium alginate beads were transferred to half MS medium in an orbital shaker (90 rpm) at 25± 2 °C.

3.8 Induction of hairy root in *in vitro* cultures of *Rubia cordifolia* by *Agrobacterium rhizogenes*

3.8.1 Culturing of *Agrobacterium rhizogenesis* strains

The bacterial strains (MTCC-532 and MTCC-2364) were cultured on the growth medium, (Plate 6) Yeast Extract broth (YEB). The solid media was melted and cooled to 40-50 °C and poured into sterilized pertiplates. Each strain was streaked on plates containing the respective media. The growth rate of bacteria on each medium was observed. The strains were cultured at 28 °C.

3.8.2 Isolation of single cell colonies

The bacterial strains were streaked on appropriate culture medium so as to isolate single cell colonies. To streak the bacteria onto the plate, the transfer loop was flamed and cooled repeatedly for three times. The loop was then plunged into a well grown bacterial colony. The lid of the petriplate containing sterilized, solid growth medium was lifted from one side and the loop

loaded with bacteria was drawn gently on about 1/3 rd of the plate surface, to make three lines close together, but separate from each other. The loop was again flamed, cooled and drawn across one end of the second streaked area and the remaining 1/3 rd area of the plate was streaked. Similarly the third side was streaked as well. The plate was closed, sealed with paraffin and kept in the culture room on a rack. Observations regarding growth of bacteria were recorded.

3.8.3 Screening of Agrobacterium rhizogenes strains for antibiotic sensitivity.

The Agrobacterium rhizogenes strains used for the study were tested antibiotics sensitivity. The antibiotics used for testing resistance were cefotaxime, ampicillin, and streptomycin. Nutrient agar medium was selected for study. The sterilized media was melted and cooled to 40-50 °C and supplemented with 50,100, 200, 300, 400, and 500 mg l^{-1} of each antibiotic separately. The media was poured into petriplates and allowed to solidify. The petriplates containing solidified medium were divided by marker lines into two parts. The bacteria from single cell colony were streaked on the respective parts in each petriplate. Observation regarding growth of bacteria were recorded.

3.8.4 Evaluation of the sensitivity of explants to various antibiotics

Different explants like leaf segments and nodal segments were tested for their sensitivity to various concentration of antibiotics. The antibiotics used were cefatoxime and ampillicin. The wounded explants were cultured in solid MS medium containing 100, 250, 500, 1000 mg l^{-1} either ampillicin and cefatoxime. The explants were cultured in MS medium without antibiotics to act as control. Observation regarding the growth and response of explants were recorded.

3.8.5 Maintenance of strains

The strains were maintained as stabs and as glycerol stocks

a) Preparation of stabs

The growth medium (YEB) containing the selective antibiotic for each strain was sterilized poured into sterilized screw cap culture tubes and allowed to solidify. The transfer loop was flamed, cooled and plunged in an isolated single cell bacterial colony. The loop loaded with bacteria was used to stab the solid medium in the culture tube. Similarly, stabs for all the

bacterial strains were prepared following the procedure mentioned above, and allowed to stand in the culture room on racks for growth of bacteria in the medium. The stabs showing good growth of bacteria were further stored at 4-6° C till further use.

b) Preparation of glycerol stocks

The strains were cultured in suitable liquid media (YEB) on a rotary shaker at 180 rpm until the optical density of culture reached approximately one (O.D~1.0). Then 800 µl of the culture was pipetted out into sterile eppendrof tubes aseptically, to which 200 µl autoclaved cooled glycerol was added and then mixed thoroughly. The tubes were then stored at - 20°C.

3.9 Explant for transformation

Explants viz. leaves and nodal segments were used for transformation studies.

3.9.1 Preparation of explants

In vitro plants of the experimental species were raised in MS media supplemented with IBA at 1 mg l^{-1}. Seedlings (1 to 1.5 month old) were taken out of the culture tube in a laminar airflow cabinet on pre-sterilized steel plates. Bases of seedlings were cut and leaf and nodal segments were separated using a sterile blade. Leaves and nodal segments were collected from the *in vitro* plants. The leaf margins were cut from all sides, leaving the middle portion with a small petiole at the proximal end. Nodal segments were dissected such that each segment carried one node.

3.9.2 Pre culturing of explants

The prepared explants were cultured on MS solid medium in petriplates for two days prior to their infection with bacteria. The medium was prepared in required volume in conical flasks and kept in the culture room until use. On the day of use, the media was melted and then cooled to 40-50 °C. Twenty five ml media was transferred to each petriplate and allowed to solidify at room temperature. The explants were inoculated on media contained in the petriplates and were sealed with parafilm and further cultured for two days in the culture room.

3.9.3 Wounding of explants

Wounds were made on the explants using a sterile blade and an injection needle. The leaf discs were pricked in the midrib and lamina with a sterile injection needle. A fresh cut was made at the base of shoot tips with a sterile blade. The shoot tips were then pricked with a sterile injection needle throughout their length. Both ends of the nodal segment were freshly cut and the segments were pricked with a sterile needle. Ten pricks were made on each explant.

3.9.4 Inoculation method

a) Suspension method

Pre cultured explants were wounded first, using a sterile blade and an injection needle. Agrobacterium rhizogenes suspension was prepared (O.D 600~ 1.0) in a sterile jam bottle. Wounded explants were immersed in the suspension for 10 to 20 min with gentle agitation. Explants were then blotted dry using sterile blotting paper and placed on solid MS medium in petriplates devoid of growth regulators.

b) Direct inoculation method

In this method bacterium from isolated single cell colonies was used as the inoculum. Here wounds were made on the explants using sterile blade and injection needle dipped in the inoculum. Cuts were made on all sides in the case of leaf disc and opposite end surface of nodal segment using sterile blade loaded with inoculum. The pricks were made on the explants with injection needle dipped in a single colony. The explants were then blotted with sterile blotting paper and then placed on MS medium contained in the petriplates. As control, explants were wounded with sterile blade and needle dipped in MS medium blotted dry and placed on MS medium.

3.9.5 Co- cultivation of the explants with *Agrobacterium*

The cultures were then co-cultured with *Agrobacterium* in dark at 26± 2°C for 1to 3 days in the culture room. Darkness was provided in the culture rack using black cloth. Observations regarding the growth of bacterium on media were noted.

3.9.5 Influence of co culture period on hairy root induction

The explants infected by suspension cultures were co-cultured for 1-3 days at 26±2 °C in dark for efficient transformation. Transformation percentage obtained and the response of different explants under different co-culture periods was recorded.

3.9.6 Culture media and conditions for hairy root induction

The explants after bacterial co-culture were washed three times successively with MS medium containing 500 mg l^{-1} cefotaxime. To the sterilized MS liquid medium prepared in the jam bottle 500 mg l^{-1} cefotaxime was added aseptically. Co-cultured explants were washed by immersing in the media containing antibiotic with intermittent shaking.

After washing, the explants were blotted dry using sterile blotting paper. The explants were then transferred to MS solid medium containing the bacteriostatic agent, cefotaxime for the complete elimination of bacteria (MS + 500 mg l^{-1} cefotaxime). The explants were further cultured at 26±2 °C under diffused light. If the bacterial growth was seen after a few days, the explants were again washed with antibiotic containing liquid medium. The was repeated until no bacterial growth was seen on the media. The control explants were also treated similarly.the cultures were observed infection by *Agrobacterium* for a period of two months. Biosafety guideline were followed for the work involving *Agrobacterium rhizogenes.*

3.10 Establishment of suspension cultures

Ms basal medium without agar, supplemented with the best growth regulator combination for callus proliferation was distributed to 100 ml conical flask @ 30 ml in each flask. The flasks were plugged with cotton and autoclaved as described in 3.1.2

Calli from leaf, nodal segment and roots, of 1 gm each, were inoculated to the liquid medium. The conical flasks were incubated at 100 rpm at 28±2 °C with a 16 hour photoperiod in an orbital shaker. Subculturing intervals and cell density were established after observing the cell counts at specific intervals, employing a haemocytometer. For subculturing, the supernatant liquid was poured into fresh liquid media at the ratio of 1:4 (v/v) and cultured in an orbital shaker.

3.10.1 Observation on growth of cell suspension cultures

The observations recorded on the growth of cell suspension cultures were as indicated below.

a) Cell number per ml of medium

Cell suspension cultures were homogenized by agitation and about 1 ml of the suspension was poured in an eppendroff tube and diluted in the ratio 1:10. To this, Evan's blue was added and kept for 10 minutes. From this, about 0.1 ml was placed on the grid on each side of a haemocytometer. A cover slip was placed on it and the numbers of cells were counted in all 9 squares on the grid of the hemocytometer (in a volume of 1 µl). Readings from both the grids were noted and their average was computed to per ml basis.

b) Packed cell volume

The conical flasks containing the suspensions were allowed to stand for 5 minutes and then 5 ml of the supernatant culture was removed and centrifuged at 200 rpm for 5 minutes in a graduated centrifuge tube. The cell mass accumulated at base of the tube. Its volume was noted and expressed on percentage basis.

3.10.2 Estimation of naphthoquinones from suspension cultures

To determine the amount of total naphthoquinone produced by cell cultures, 5 ml of liquid culture was extracted by grinding in 70 per centmethanol followed by extraction and fractionation with ethyl acetate similar to the method described in section 3.5.5.3. The ethyl acetate extract thus obtained, was used for the estimation of naphthoquinone

3.11 Determination of anticancerous activity of plant / callus extracts through *in vitro* experiments

3.11.1 Sample:

1) *In vivo* plants

Whole plant and root segments of *Rubia cordifolia*. plants obtained from Department of Plantation Crops and Spices, College of Horticulture, Vellanikkara, Thrissur, formed the *in vivo* samples.

2) *In vitro* culture extracts

Assessment of anticancerous activity of *in vitro* samples yielding, higher amounts of naphthoquinone was done. Calli from leaves, nodal segments and roots of *Rubia cordifolia* grown on production media as well as calli obtained by employing techniques like elicitation (biotic and abiotic), precursor feeding and addition of yeast extract were employed for assessment of cytotoxic property.

3.11.2 Animals

Male swiss albino mice (20 to 25gm) supplied by the small animal breeding station, College of Veterinery and Animal Sciences, Mannuthy, Thrissur were used for the study. The animals were maintained under sterilized environmental conditions (22-28^{0}C, 60-70 per centrelative humidity, 12 h dark/light cycle)and fed with standard rat feed (Lipton India Ltd)and water adlibitum. All animal experiments conducted during the present study have received prior permission from Institutional Animal Ethics Committee (IAEC) and following the guidelines of IAEC.

3.11.3 Preparation of the extract of *in vivo* and *in vitro* samples

Air-dried plant and callus samples were powdered and extracted with 70 per centmethanol. The extracts were concentrated and evaporated to dryness.

3.11.4 Assessment of short term *in vitro* cytotoxicity

Short-term cytotoxicity of *in vivo* plant extract and *in vitro* test sample was studied using DLA cells and EAC tumour cells. The extracts were suspended in the respective reaction buffer solution at various concentrations (1-5000 µg ml^{-1}) and were incubated with tumor cells (10^6) suspended in phosphate buffered saline (PBS, pH 7.2). Cytotoxicity was determined after 3 hrs using trypan blue exclusion method (Babu *et al.*,1995) based on which antitumor activity of test samples was evaluated.

3.12 Determination of *in vitro* antioxidant properties of *in vivo* plant and *in vitro* callus extracts:

a) Superoxide scavenging activity

This was determined by the Nitro Blue Tetrazolium (NBT) reduction method of Mc Cord and Fridovic (1969) which was dependent on light induced super oxide generation by riboflavin and corresponding reduction of NBT. Different concentrations of the test material ranging from 1µg to 1000µg were added to the reaction mixture containing 0.1M EDTA (200µl), 0.12 mM riboflavin (50 µl) and 0.6M phosphate buffer (pH 7.8) in a final volume of 3ml. The tubes containing the reaction mixture were continuously illuminated with incandescent lamp for 15 minutes. The optical density measurements were taken at 530 nm before and after illumination. The effect of test material to inhibit super oxide generation was evaluated by comparing the OD of control and treated tubes.

per cent of inhibition = (Control OD – Treated OD/ Control OD) X100

b) Hydroxyl scavenging activity:

This was determined by the thiobarbituric acid reacting substances (TBARS) method. The efficacy of the test materials to inhibit TBARS formation in the system induced by the Fe^{3+}/ ascorbate / H_2O_2 degenerating deoxyribose was assessed. The reaction system contained deoxyribose (2.8 mM), $FeCl_3$ (0.1 mM), KH_2PO_4 KOH buffer (20 mM; pH7.4) and from 1 µg to 1000µg /ml of the test material in a final volume of 1ml. The reaction mixture was incubated at 37° C for 1hour. The TBARS formed was measured by the method suggested by of Okhawa *et al*, (1979) and the percentage of inhibition was calculated.

per cent of inhibition = (OD of Control – OD of treated/ OD of Control) X 100

c) Nitric oxide scavenging activity:

Nitric oxide, generated from sodium nitroprusside in aqueous solution at physiological pH, interacts with oxygen to produce nitrite ions, which were measured by Griess reaction (Green *et al.*, 1982; Marcocci *et al.*, 1994). The reaction mixture (3 ml) containing sodium nitroprusside (10 mM) in phosphate buffered saline (PBS) and the compound (from 1µg to

1000µg ml^{-1}) was incubated at 25°C for 150 minutes. After incubation, 5ml of Griess reagent (1 per cent sulphanilamide, 2 per centH_3PO_4 and 0.1 per cent naphthylethylene diamine di hydrochloride) was added. The absorbance of the chromophore formed was recorded at 546 nm.

a) Lipid peroxidation Assay

Lipid peroxidation was induced in rat liver homogenate by the method described by Bishayee and Balasubramonian, (1971) in the presence of the test material and estimated by thio barbituric acid reactive substances by the method of Ohkawa *et al.*, (1979). Different concentrations of the test materials were incubated with 0.1 ml of rat liver homogenate (25 per cent) containing 30 mM KCl, Tris- HCl buffer (0.04 M, pH 7.0), ascorbic acid (0.06 mM) and ferrous ion (0.16 mM) in a total volume 0.5 ml for 1 hour. After incubation period, 0.4 ml of the reaction mixture was treated with 0.2 ml SDS (8.1 per cent), 1.5 ml thio barbituric acid (0.8 per cent) and 1.5 ml acetic acid (20 per cent, pH 3.5). The total volume was made up to 4ml by adding distilled water and kept in water bath at 95°C for 1 hour. After cooling, 1ml distilled water and 5ml of butanol-pyridine mixture (15:1 v/v) was added. After vigorous shaking, the tubes were centrifuged and the upper layer containing the chromophore was read at 532 nm. The percentage of inhibition was calculated and the concentration required for 50 per cent inhibition was determined.

3.12.1 Determination of prooxidant activity *in vivo* and *in vitro* cultures

Pro-oxidant activity of *in vivo* and *in vitro* plant extracts was determined by assessing ROS (reactive oxygen species) generation assay. ROS generation in the cells by naphthoquionone was assessed by DCFH-DA staining. The production of intercellular ROS, especially H_2O_2, was detected with the oxidative sensitive fluorescent dye, DCFH-DA (Le Bel *et al.*, 1992; Rosenkranz, 1992). The DLA cells were seeded at a concentration of 5 x 10^6 and the extracts were added to reaction buffer. After incubation of 12 hrs, the cells were treated with DCFH-DA dye at a final concentration of 10µM for 45 minutes. The green fluorescing cells indicating ROS generation was detected by a fluorescent microscope.

3.12 Statistical analysis

The data obtained from the different treatments were subjected to statistical analysis as per Conver (1980). The treatments were grouped into homogenous groups using Friedman test.

RESULTS

RESULTS

The results of the study on 'Exploitation of *in vitro* cultures of Indian Madder (*Rubia cordifolia* L.) for anticancerous compounds' carried out at the Centre for Plant Biotechnology and Molecular Biology, College of Horticulture, Vellanikkara and Amala Cancer Research Centre, Thrissur are presented in this chapter.

4.1 Standardization of pre treatment and surface sterilization of explants

4.1.1 Pretreatment of explants with fungicides

Effect of pretreatment with the fungicide Bavistin at varying levels was studied, for obtaining cultures free from systemic fungal contamination and the results are presented in Table 9. Among the treatments tried, Bavistin at 2.5 per cent concentration for 15 minutes, was most effective in reducing contamination of leaf and nodal segments, yielding 100 per cent healthy, contamination free cultures. Longer duration of treatment and higher concentration of Bavistin reduced microbial interference but resulted in substantial scorching of leaf explants (66.66 per cent). However, stem explants, withstood higher concentration of the fungicide and longer treatment durations, registering cent percent healthy cultures.

4.1.2 Surface sterilization of explants

Effect of surface sterilization of pretreated explants, with $HgCl_2$ at 0.1 per cent concentration, for varying durations, is presented in Table 10. Among the treatments tried, treating explants with $HgCl_2$ at 0.1 per cent concentration for 1 min was effective in yielding cent per cent contamination free cultures for leaf and nodal segments. However, the treatment induced scorching in 8.33 per cent leaf explants, while cent per cent of nodal explants remained healthy, after the treatments. Treatment of leaf explants with 0.1 per cent $HgCl_2$ for 30 seconds however yielded cent per cent healthy and contamination free cultures.

On enhancing the duration of surface sterilization, extent of scorching of explants also increased (Table 10). Based on the results, $HgCl_2$ at 0.1 per cent concentration for 1 min was selected as the treatment for effective sterilization of nodal explants and the same sterilant at 0.1 per cent concentration for 30 seconds was identified as the most effective treatment for leaf explants.

Table No 9. Effect of pre treatment with fungicides on establishment of *in vitro* cultures in *Rubia cordifolia.*

Fungicide (Bavistin)	Duration of treatment (min)	Percentage of cultures contaminated after inoculation.		Scorched (per cent)		Establishment of cultures (Per cent)	
		Leaf	Nodal segment	leaf	nodal	leaf	Nodal
Per cent							
0.1	10	83.33	91.66	-	-	16.1	8.33
	15	75	66.66	-	-	25	33.33
0.5	10	50	58.33	-	-	50	41.66
	15	41.6	58.33	-	-	58.33	41.66
1	10	41.66	41.66	-	-	58.33	58.33
	15	33.33	41.6	-	-	66.66	58.33
2.5	10	8.33	-	-	-	91.6	100
	15	-	-	-	-	100	100
	25	-	-	8.33	0	91.6	100
3	10	-	-	8.33	0	91.6	100
	15	-	-	16.33	0	83.3	100
	25	-	-	33.33	0	66.66	100

All explants were surface sterilized with $HgCl_2$ (0.1 per cent).

Twelve explants were used for each treatment.

Table 10. **Effect of surface sterilization on survival of leaf and nodal segment explants of *Rubia cordifolia***

Surface sterilant	Duration of treatment (min)	Culture survival without contamination after inoculation (Per cent)		Scorched (Per cent)		Establishment of cultures (Per cent)	
		Leaf	Nodal segment	leaf	nodal	leaf	Nodal
$HgCl_2$ (0.1 per cent)	3	100	100	83.33	33.33	16.66	66.66
	2.5	100	100	66.66	25	33.33	75
	1	100	100	8.33	0	91.66	100
	0.5	100	100	0	0	100	100

Twelve explants were used for each treatment

4.2 Induction and proliferation of calli

4.2.1 Effect of auxins in inducing and proliferating calli

Responses of leaf, nodal segment and root explants, to varying levels of different auxins, in initiating and proliferating calli, are presented in Table 11(Plate 2)

The three auxins employed in the study, NAA, IAA and 2,4-D at varying levels, did not exert a significant influence, on the performance of leaf, nodal segment and root explants of the experimental species, with respect to percentage of cultures initiating calli, callus growth score and mean callus index. Days to callus induction varied significantly, in leaf, nodal segment and root cultures under the influence of various auxins, employed in the study. However, no significant variation was observed between various levels of each auxin, with respect to days to callusing.

MS medium enriched with NAA at 2 mg l^{-1} recorded the highest mean percentage of callusing in 90.83 per cent of cultures. The same medium recorded maximum percentage of callusing in leaf (97.50 per cent) and root (89.58 per cent) cultures. For nodal segments, NAA at 1 mg l^{-1} recorded highest percentage of callusing. NAA, at 2 mg l^{-1} recorded significantly early callusing in nodal segment (7.39 days) and root (18.48 days) explants and at 1 mg l^{-1}, recorded early callusing in leaf explants.

Callus growth rate as measured by callus growth score, was significantly higher, in media supplemented with NAA @ 2 mg l^{-1}, with the highest mean growth score of 2.18. Leaf and root cultures registered highest callus growth score of 2.72 and 1.88 respectively in the same medium. However, nodal cultures registered highest callus growth scores of 1.97 in media enriched with 2,4-D at 2 mg l^{-1}.

MS medium supplemented with NAA at 2 mg l^{-1} recorded the highest mean callus index value of 199.76. Leaf (265.2) and root (168.41) explants, recorded maximum callus index values in the same medium. However, the same auxin, at 1.0 mg l^{-1} registered the highest callus index value (178.07), for stem explants.

Irrespective of the levels tried, the auxin IAA was least effective in inducing callusing in *Rubia cordifolia*, recording lowest values, for all the parameters studied (Table 11). The auxin 2,4-D at 1.0 mg l^{-1} and 2.0 mg l^{-1}, was intermediate in

response, with respect to the various parameters influencing callusing, in the experimental species.

4.2.2 Suitability of explants for initiating and proliferating calli under the influence of auxins.

Leaf explants surpassed nodal segments and roots, registering a mean callus index value of 189.85 with a callus growth score of 2.06 (Fig. 2), wherein, 90.43 per cent of cultures, initiated calli (Plate.3). Next to leaf explants, nodal segments performed better, wherein, 90.43 per cent of cultures, initiated calli. Next to leaf explants, nodal segments performed better, wherein, 85.03 per cent cultures callused, with a mean callus growth score of 1.66 and a mean callus index value of 142.81. Root explants recorded the lowest values for all parameters with respect to callusing. Superiority of leaf explants was evident for earliness in callusing as well (8.9 days).

4.2.3 Effect of cytokinins in inducing and proliferating calli

The influence of cytokinins in inducing and proliferating calli are presented in Table 12. As compared to auxins, the major cytokinins, BA and kinetin, at varying levels, significantly influenced all the parameters studied, with respect to auxins. When cultured in MS medium with BA at 2 mg l^{-1}, leaf explants callused in 8.32 days, nodal segments in 8.27 days and root explants in 19.43 days of culture.

MS medium enriched with BA at 1 mg l^{-1}, recorded higher percentage of callusing in leaf (89.45) and nodal (87.5) segments whereas, for root cultures, kinetin at 1 mg l^{-1} registered higher percentage of callusing (84.33). Maximum callus growth scores of 1.67, 1.41 and 1.03, were observed for leaf, nodal segment and root explants, respectively, when cultured in basal media, supplemented with BA at 1 mg l^{-1}. Highest mean callus index values of 149.38, 123.28 and 84.72 were obtained, on incubating leaves, nodal segments and roots respectively on basal media supplemented with BA at 1 mg l^{-1}. Among the treatments with cytokinins, kinetin at 1 mg l^{-1}, registered lowest values, for all the parameters studied, in all the explants.

As observed for treatments with auxins, among the various explants cultured, leaf explants showed highest mean percentage of callusing (87.35).

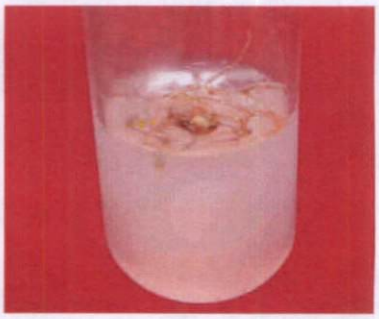
Plate 2. Initiation and proliferation of callus from leaf, nodal segment and root explants of *Rubia cordifolia*. Linn.

Stage I

a) Leaf

b) Nodal segment

c) Root



Stage II

a) Leaf

b) Nodal segment

c) Root

Stage III

a) Leaf

b) Nodal segment

c) Root

Table 11. Effect of auxins on callusing of leaf, nodal segment and root explants of *Rubia cordifolia*

Basal medium - MS

Auxins (mg l^{-1})	Days to initiate callus					Percentage of cultures initiating callus in 30 days					Callus growth score in days					Callus index				
	Leaf	Nodal	Root	Mean	Mean Rank	Leaf	Nodal	Root	Mean	Mean Rank	Leaf	Nodal	Root	Mean	Mean Rank	Leaf	Nodal	Root	Mean	Mean Rank
NAA 1.0	7.25	7.35	18.62	11.07	1.33	91.6	91.6	87.4	90.2	4.67	2.47	1.94	1.39	1.93	4.17	226.25	178.07	121.92	175.41	4.67
NAA 2.0	7.27	7.39	18.48	11.04	1.67	97.5	85.41	89.58	90.83	5.17	2.72	1.94	1.88	2.18	5.5	265.2	165.69	168.41	199.76	5.33
2,4-D 1.0	8.25	8.4	20.65	12.43	4	92.5	85.41	83.33	87.08	3.5	2.66	1.88	1.20	1.91	3.67	246.05	160.57	100.66	169.09	3.67
2,4-D 2.0	8.16	8.27	20.43	12.28	3	95.4	86.1	84.3	88.6	4.67	2.11	1.97	1.67	1.91	4.67	201.29	169.61	140.78	170.56	4.33
IAA 1.0	11.12	11.27	23.89	15.42	5.33	79.5	79.5	72.65	77.21	1	1.25	1.05	0.92	1.13	1.33	99.85	83.43	67.49	87.92	1.33
IAA 2.0	11.43	11.36	23.09	15.29	5.67	86.1	82.21	79.5	82.60	2	1.16	1.25	1.02	1.07	1.67	100.47	99.72	81.09	89.35	1.67
Mean	8.91	9.00	20.86	12.92	-	90.43	85.03	82.79	86.08	-	2.06	1.66	1.35	1.69	-	189.85	142.81	113.39	148.68	-
CD					1.73					4.96					5.06					5.4

Table 12. Effect of cyokinins on callusing of leaf, nadal segment and root explants of *Rubia cordifolia*

Basal medium - MS

Cytokinins (mg l^{-1})	Days to initiate callus					Percentage of cultures initiating callus in 30 days					Callus growth score in days					Callus index				
	Leaf	Nodal	Root	Mean	Mean Rank	Leaf	Nodal	Root	Mean	Mean Rank	Leaf	Nodal	Root	Mean	Mean Rank	Leaf	Nodal	Root	Mean	Mean Rank
KIN 1.0	10.87	11.02	23.15	15.01	4	89.5	87.5	84.33	87.11	3.83	1.47	1.25	0.83	1.18	2	131.56	109.37	70.162	103.70	2
KIN 2.0	10.34	10.91	23.04	14.76	3	86.36	84.09	77.08	82.51	1.33	1.04	1.02	0.66	0.90	1	89.90	85.77	50.87	75.51	1
BA 1.0	8.54	8.65	19.65	12.28	2	89.45	87.5	82.5	86.48	3.17	1.67	1.40	1.02	1.36	4	149.38	123.28	84.72	119.13	4
BA 2.0	8.32	8.27	19.43	12.00	1	84.09	86.36	79.54	83.33	1.67	1.63	1.39	0.91	1.31	3	137.73	120.47	72.85	110.35	3
Mean	9.51	9.71	21.31	13.51	-	87.35	86.36	80.86	84.85	-	1.45	1.26	0.85	1.19	-	127.14	109.72	69.65	102.17	-
C D					0.15					3					0.15					0.15

Maximum mean growth score of 1.45 and mean callus index value of 127.14 were registered by leaf explants. Early callusing (9.52 days) was also observed in the case of leaf explants. For all the parameters studied, root derived cultures, recorded lowest values.

4.2.4 Effect of cytokinins in combination with auxins in inducing and proliferating calli in *Rubia cordifolia*

Influence of cytokinins in combination with auxins in inducing and proliferating calli in *Rubia cordifolia* is detailed in Table 13. The effects of various combinations of auxins and cytokinins at varying levels, employed in the study for inducing callusing in the experimental species, did not vary significantly.

However, the results revealed that the most favourable response was obtained with NAA (2 mg l^{-1}) in combination with BA (0.5 mg l^{-1}) which induced callusing in 97.5, 92.45 and 89.45 per cent of leaf, nodal segments and root explants, registering a mean value of 93.15 per cent of cultures initiating calli. The same medium also registered highest callus index values of 284.70, 213.67 and 168.16 for leaf, nodal segment and root cultures respectively, with a mean callus index of 222.17. Callus growth scores of 2.92, for leaf explants, 2.31 for nodal explants and 1.88 for root explants were recorded in the same medium, with a mean callus growth score of 2.37. The cytokinin, kinetin, at 0.5 mg l^{-1}, when incorporated to the NAA enriched basal medium, recorded a mean callus index value of 172.3 initiating calli in 89.56 per cent cultures with a mean callus growth score of 1.90, all values being adjudged as the second best. BA or kinetin in combination with 2,4-D at varying levels, were ineffective in accelerating callusing in *Rubia cordifolia.*

Leaf explants confirmed their superior performance of explants with respect to callusing was confirmed, as was evident in media supplemented with auxins and cytokinins singly, initiating calli in 91.53 cultures, in 8.95 days, with a mean callus growth score of 1.84 and a mean callus index value of 170.22. Next to leaf explants, nodal segment cultures performed better, registering a mean callus index of 140.59. Inferior performance of root explants, with respect to callusing was evident, for all parameters studied on enriching basal medium with auxins and cytokinins at varying levels (Fig. 2).

Table 13. Effect of auxins and cytokinins on callusing of leaf, nodal segment and root explants of *Rubia cordifolia*

Basal medium - MS

Hormonal combinations (mg l^{-1})	Days to initiate callus					Percentage of cultures initiating callus in 30 days					Callus growth score in 30 days					Callus index				
	Leaf	Nodal	Root	Mean	Mean Rank	Leaf	Nodal	Root	Mean	Mean Rank	Leaf	Nodal	Root	Mean	Mean Rank	Leaf	Nodal	Root	Mean	Mean Rank
NAA 1 + KIN 0.5	9.23	9.12	18.62	12.32	7.67	93.75	89.45	83.33	88.84	6.67	1.77	1.22	1.02	1.341	4	166.40	109.93	84.99	120.44	4.67
NAA 1 + KIN 1	9.12	9.16	18.48	12.25	7.17	93.18	81.24	72.5	82.30	2.67	1.57	1.29	0.96	1.27	2.67	146.75	104.88	70.03	107.22	2.33
NAA 1 + BA 0.5	9.33	9.02	18.34	12.23	6.83	91.6	87.5	89.45	89.51	7.67	1.81	1.40	1.11	1.44	6.5	165.97	123.11	99.55	129.54	6
NAA 1 + BA 1	9.45	9.1	18.2	12.25	7.67	93.75	84.33	72.65	83.57	4.5	1.62	1.20	0.97	1.27	2.67	152.34	101.87	70.97	108.39	2.67
NAA 2 + KIN 0.5	8.23	8.65	17.43	11.43	1.67	93.75	91.6	83.33	89.56	7.33	2.27	2.11	1.33	1.90	9.67	212.81	193.27	110.82	172.3	9.67
NAA 2 + KIN 1	8.12	8.54	17.92	11.52	1.83	95.5	83.33	86.36	88.39	7.5	2.08	1.88	1.47	1.81	9.33	198.64	156.66	126.94	160.74	10
NAA 2 + BA 0.5	8.43	8.85	17.78	11.68	2.67	97.5	92.5	89.45	93.15	11.83	2.92	2.31	1.88	2.37	12	284.7	213.67	168.16	222.17	12
NAA 2 + BA 1	8.74	9.16	17.92	11.94	5.33	91.6	91.6	84.09	89.09	6.83	2.11	1.94	1.5	1.85	10.33	193.27	177.70	126.13	165.7	9.67
2,4 -D 2+ KIN 0.5	9.25	9.4	20.65	13.1	11	86.36	84.33	86.36	85.68	5.33	1.47	1.67	1.40	1.51	6.33	127.38	140.83	121.68	129.96	6.67
2,4 –D 2 + KIN 1	9.16	9.27	20.43	12.95	9.5	82.92	86.36	84.3	84.52	4.67	1.40	1.39	1.20	1.33	4	116.17	120.47	101.83	112.82	4
2,4 - D 2+ BA 0.5	9.02	9.02	20.12	12.72	6.17	94.4	89.45	85	89.61	8.83	1.63	1.40	1.40	1.48	7	154.62	126.03	119.76	133.47	7
2,4 –D 2 + BA 1	9.34	9.23	20.43	13	10.5	84.09	86.34	84.09	84.84	4.17	1.47	1.37	1.10	1.31	3.5	123.61	118.71	92.66	111.66	3.33
Mean	8.95	9.04	18.86	12.28	-	91.53	87.33	83.40	87.42	-	1.84	1.60	1.28	1.57	-	170.22	140.59	107.79	139.53	-
C D					10.41					10.34					9.73					9.58

4.2.5 Effect of media supplements on callusing

Data pertaining to the effect of the media additive, phloroglucinol, on callusing in leaf, nodal segment and root cultures of *Rubia cordifolia* are presented in Table 14.

Incorporation of phloroglucinol at 100 mg l^{-1} to MS medium supplemented with 2,4-D at 2 mg l^{-1} initiated calli in 88.41 per cent cultures. However, the initiated calli proliferated best in MS medium supplemented with NAA 2 mg l^{-1} and phloroglucinol 100 mg l^{-1} registering a mean callus growth score of 1.78 and a mean callus index value of 154.34. Inferior performance of the auxin IAA with respect to callusing, as observed in earlier experiments, was repeated, on addition of the phloroglucinol as well, recording lowest values at 1 and 2 mg l^{-1} of the auxin, for percentages of cultures initiating calli (79.50 and 79.45 per cent respectively), callus growth scores (1.07 and 1.32 respectively) and mean callus index values (87.32 and 105.07 respectively).

As to the performance of explants, other than earliness in callusing, where, nodal explants performed better (9.11 days), leaf explants registered highest mean per cent of callus initiating cultures (85.51 per cent) with the highest mean callus growth score (1.73) and mean callus index (148.99). Incorporation of phloroglucinol, did not bring about a favourable response, with respect to callusing, in root explants.

4.2.6 Standardization of growth medium for induction and proliferation of static cultures in *Rubia cordifolia*

Comparative mean performance of the various media employed, for initiating and proliferating calli in *Rubia cordifolia* is presented in Tables 11, 12, 13.

From the data, it is evident that the media combinations employed, did not exhibit significant differences among one another, with respect to mean days to callusing, mean percentage of cultures initiating calli, mean callus growth score and mean callus index values (Table 11, 12, 13). However, the highest values for the parameters influencing callusing, such as percentage of cultures initiating callusing (93.15 per cent), mean callus growth score (2.37) and mean callus index values (222.17) were recorded for various explants employed in the study, in MS medium enriched with NAA at 2.0 mg l^{-1} and BA at 0.5 mg l^{-1}. Based on this observation, MS

Table 14. Effect of phloroglucinol with auxins on callusing of leaf, nodal segment and root explants of *Rubia cordifolia*

Basal medium - MS

Media compostion	Days to initiate callus					Percentage of cultures initiating callus in 30 days					Callus growth score in days					Callus index				
	Leaf	Nodal	Root	Mean	Mean Rank	Leaf	Nodal	Root	Mean	Mean Rank	Leaf	Nodal	Root	Mean	Mean Rank	Leaf	Nodal	Root	Mean	Mean Rank
MS + NAA 1 mg l^{-1} + Phloroglucinol 100 mg l^{-1}	7.89	7.23	19.12	11.41	1.67	84.3	82.92	81.1	82.77	3.33	1.94	1.66	1.104	1.56	4.33	163.54	137.64	89.53	130.24	4
MS + NAA 2 mg l^{-1} + Phloroglucinol 100 mg l^{-1}	7.54	8.17	18.94	11.55	1.33	89.4	85.41	84.3	86.37	5	2.03	2.01	1.291	1.78	6	181.92	172.27	108.83	154.34	6
Ms + 2,4 -D 1 mg l^{-1} + Phloroglucinol 100 mg l^{-1}	8.76	8.4	20.65	12.60	4	83.3	83.33	81.3	82.64	3	1.66	1.63	1.208	1.49	3.67	138.27	135.82	98.21	124.10	3.67
MS + 2,4 –D 2 mg l^{-1} + Phloroglucinol 100 mg l^{-1}	8.16	8.27	20.43	12.28	3	89.45	89.45	86.34	88.41	6	1.943	1.48	1.052	1.49	3.67	173.80	132.92	90.82	132.51	4
MS + IAA 1 mg l^{-1} + Phloroglucinol 100 mg l^{-1}	11.12	11.27	23.89	15.42	5.33	83.33	79.5	79.5	80.77	2.17	1.167	1.05	1.02	1.07	1	97.24	83.63	81.09	87.32	1.33
MS + IAA 2 mg l^{-1} + Phloroglucinol 100 mg l^{-1}	11.43	11.36	23.09	15.29	5.67	83.33	79.45	72.65	78.47	1.5	1.67	1.27	1.028	1.32	2.33	139.16	101.37	74.68	105.07	2
Mean	9.15	9.11	21.02	13.09	-	85.51	83.34	80.86	83.24	-	1.73	1.52	1.11	1.45	-	148.9	127.28	90.53	122.26	-
C D					2.57					4.64					4.83					5.21

Table 15. Effect of culture environment on callusing in leaf, nodal segement and root explants of *Rubia cordifolia*

Medium - MS + NAA (2 mg l^{-1})

Treatment	Days to initiate callus				Percentage of culture initiating callus in 30 days				Callus growth score in days				Callus index			
	Leaf	Nodal	Root	Mean	Leaf	Nodal	Root	Mean	Leaf	Nodal	Root	Mean	Leaf	Nodal	Root	Mean
Dark	7.86	7.92	18.76	11.51	86.36	84.09	84.09	84.84	1.053	1.056	0.967	1.02	90.93	88.79	81.31	87.01
NAA 2.0	7.27	7.39	18.48	11.04	97.5	85.41	89.58	90.83	2.72	1.94	1.88	2.18	265.2	165.69	168.41	199.76

medium fortified with NAA (2 mg l^{-1}) and BA (0.5 mg l^{-1}) was identified as the growth medium chosen for induction and proliferation of calli in the experimental species.

4.3 Effect of culture environment on callusing in *Rubia cordifolia*

Influence of modified culture environment (culturing in dark) on callusing in *Rubia cordifolia*, as compared to control (culture under 10 hours illumination at 26 ± 1°C), is given in Table 15.

Marked difference was exhibited between culturing under illumination and culturing in dark, with respect to percentage of cultures initiating calli, callus growth score and callus index values, in all the three explants, employed in the study (Table15). Incubating cultures under illumination, with 10 hour photoperiod, at 26 ± 1°C was superior with respect to callusing in *Rubia*, registering a mean callus growth score of 2.18 with a mean callus index value of 199.76, when cultured in MS medium fortified with NAA at 2 mg l^{-1}.

4.4 Biochemical estimation of naphthoquinones

4.4.1 Detection of naphthoquinones employing thin layer chromatography

4.4.1.1 Selection of solvent systems

Relative efficacy of various solvent systems in the detection of naphthoquinones on thin layer chromatograms is illustrated in Table 16. As is evident from the results, the solvent system hexane : ethyl acetate in the ratio 9:1 and chloroform : methanol in the ratio 8.5:1.5, produced maximum clarity of the spot comforming to naphthoquinone, on thin layer chromatograms as indicated by the score (++++). Between these two solvent systems, the solvent system chloroform : methanol (8.5:1.5) was identified as the ideal one for detection of naphthoquinones in *in vitro* and *ex vitro* samples of the experimental species, since, compared to the solvent system hexane- ethyl acetate, this solvent system resulted in complete elution of the applied sample, coupled with maximum clarity (Plate 3).

4.4.1.2 Standardization of spray reagent

Three types of spraying reagents were tried separately for detection of the target compound, on thin layer chromatograms. Among the three spray reagents (Methylene blue, Ammonia solution and Alcoholic KOH) tried for developing the

Table 16. Evaluation of efficacy of solvent systems for detection of naphthoquinone in *in vivo* and *in vitro* extracts of *Rubia cordifolia*

Sl. No	Running solvent system	Ratio of solvents	Clarity
1	Water	100 per cent	+
2	Methanol	100 per cent	+
3	Acetone	100 per cent	+
4	Chloroform	100 per cent	++
5	Hexane	100 per cent	+
6	Acetone : Water	9.5 :0.5	+
7	Hexane : Benzene	1:1	++
8	Hexane : Benzene	2.5 : 7.5	++
9	Hexane : ethyl acetate	3 : 7	++
10	Hexane : ethyl acetate	8 : 2	+++
11	Hexane : ethyl acetate	9 : 1	++++
12	Hexane : ethyl acetate	5: 5	++
13	Hexane : ethyl acetate	6 : 4	++
14	Disopropyl ether : petroleum ether	1 : 9	++
15	Hexane : Butyl ether	9 : 1	+
16	Benzene : ethyl acetate	9.5 : 0.5	+
17	Petroleum ether : ethyl acetate : formic acid	75 : 24:1	+++
18	Chloroform : ethyl acetate	3 :7	++
19	Ethyl acetate :toluene	5 : 5	+
20	Methanol : water	5: 5	+
21	Methanol : ethyl acetate	5: 5	+
22	Chloroform : hexane : ethyl acetate	8 : 1 : 1	+++

23	Chloroform : methanol	5 : 5	++
24	Chloroform : methanol	8.5 : 1.5	++++
25	Chloroform : methanol	8 : 2	+++
26	Chloroform : methanol	7 : 3	++
27	Chloroform : methanol : Benzene	40 : 7.5 : 2.5	+++
28	Hexane : acetone : Acetic acid	15 : 5 : 0.3	++

(++++) -Best (+++) - Good (++) Moderate (+) Mixed

chromatograms with respect to detection of naphthoquinones, the spray reagent, alcoholic KOH (10 per cent in methanol) was adjudged as the best. By using this reagent the spots produced by the test sample corresponding to reference samples, appeared red in colour with good clarity. Hence alcoholic KOH was used as the spray reagent for detection of naphthoquinone.

4.4.1.3 Developing the chromatogram

The thin layer chromatographic plates spotted with the test samples along with the reference sample, gave a red spot with an Rf value of 0.47 in conformity with that of the reference compound (1,4-naphthoquinone), when sprayed with alcoholic KOH, employing the solvent system, chloroform : methanol (8.5 : 1.5) (Table 17). Besides the spot conforming to naphthoquinone, four other spots of varying colours and Rf values as detailed in Table were also visualized on the chromatograms, prior to spraying, alcoholic KOH (Plate 3).

4.5 Regulation of *in vitro* metabolite production

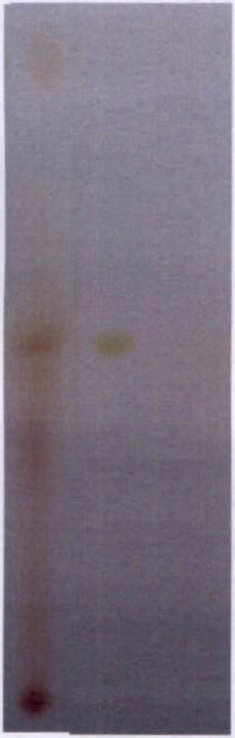
4.5.1 Standardization of production media

4.5.1.1 Regulation of growth factor combinations

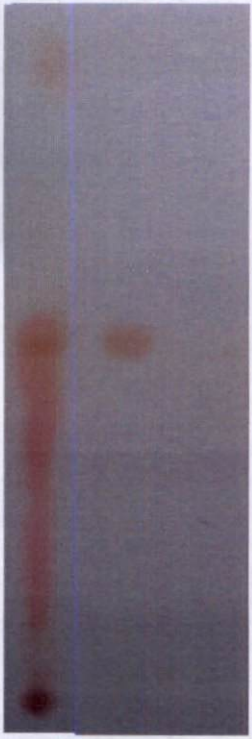
Results of the influence of growth regulators and media supplements on naphthoquinone production in *in vitro* cultures of *Rubia cordifolia* are presented in Table 19.

Four week experimental callus cultures on MS medium supplemented with growth hormones and their combinations at varying levels and the additive phloroglucinol, reacted positively to the presence of naphthoquinones as revealed by the presence of red colour of varying intensities on conducting Borntreger test (Table 18). Intense red colour was recorded for all treatments, wherein, the growth regulator NAA was incorporated into the basal MS medium, either singly or in combination with the cytokinins, BA and kinetin at varying levels (Table 18). The auxin 2,4-D at 2 mg l^{-1} singly resulted in faint red colour, when the test calli, was subjected to Borntrager test. From the Table 19 it is inferred that all the treatments employed, resulted in synthesis of naphthoquinones in leaf, nodal and root derived callus cultures of *Rubia cordifolia*. Among the combinations tried, MS basal medium supplemented with NAA (2.0 mg l^{-1}) and BA (0.5 mg l^{-1}) synthesized higher quantities of

Plate 3 Chromatogram pattern of root callus extract of *Rubia cordifolia* on thin layer chromatography



Before spraying



After spraying

Table 17. Pattern of chromatogram of ethyl acetate extract of root callus of *Rubia cordifolia* in chloroform : methanol (8.5:1.5) mobile phase.

Treatment	No. of spots	Colour of spots	*Rf* value of spot
Test sample (*in vitro* sample)	5	1- Pink	0.05
		2 - Light brown	0.16
		3 – Red	0.47
		4 - yellow orange	0.59
		5 – Yellow	0.93
Reference compound (1,4-naphthoquinone)	1	Red	0.47

Table 18. Influence of combinations of growth regulators and media supplements on expression of naphthoquinone in callus cultures of *Rubia cordifolia*.

Growth regulators/ Media supplement	Concentration (mg l^{-1})	Response to Borntrager test		
		Leaf	Nodal	Root
NAA + BA	2.0 + 1.0	+++	+++	+++
	2.0 + 0.5	+++	+++	+++
	1.0 + 1.0	+++	+++	+++
	1.0 +0.5	+++	+++	+++
NAA + KIN	2.0 + 1.0	+++	+++	+++
	2.0 + 0.5	+++	+++	+++
	1.0 + 1.0	+++	+++	+++
	1.0 +0.5	+++	+++	+++
2,4-D + BA	2.0 + 1.0	+	+	+
	2.0 + 0.5	+	+	+
2,4-D + KIN	2.0 + 1.0	+	+	+
	2.0 + 100	+	+	+
2,4-D + Phloroglucinol	2.0 + 50	+	+	+
	2.0 + 100	+	+	+
	2.0 + 50	+	+	+
2,4-D + Phloroglucinol	1.0 + 50	++	++	++
	1.0 + 100	++	++	++
	1.0 + 50	++	++	++
NAA	1	+++	+++	+++
	2	+++	+++	+++
2,4-D	1	++	++	++
	2	+	+	+

naphthoquinone in leaf (2.32 units per gram of calli), stem (2.64 units per g of calli) and root (3.72 units per gram of calli) cultures with a mean value of 2.89 units per gram of calli. Kinetin at 0.5 mg l^{-1} when added to MS medium along with NAA at 2 mg l^{-1} registered a mean naphthoquinone production of 2.72 units per gram of calli. The auxin NAA (2 mg l^{-1}) when incorporated to the basal medium singly, registered a mean value of 2.72 units as well, with respect to naphthoquinone production. The auxin 2,4-D yielded lower amounts of naphthoquinone, when incorporated singly or along with BA and kinetin at varying levels (Table 19). However, incorporation of phloroglucinol along with 2,4-D at varying levels, resulted in a marginal improvement in content of naphthoquinones, as compared to incorporation of the auxin 2,4-D singly into the culture media. From the data generated, MS medium supplemented with NAA (2 mg l^{-1}) and BA (0.5 mg l^{-1}), yielding a mean value of 2.89 units of naphthoquinone per gram of calli, was designated as the production medium, hereafter referred to as medium P.

4.5.2 Modification of carbon source

Increasing levels of sucrose in the basal medium to 5 per cent did not have a favourable influence on the expression of naphthoquinones in the test calli, as compared to control i.e. sucrose at 3 per cent (Table 20). Media supplemented with sucrose at 3 per cent registered 2.32, 2.64 and 3.72 units of naphthoquinone per gram of leaf, nodal and root calli respectively. But when sucrose level was increased to 5 per cent, leaf, nodal and root derived cultures yielded only 2.11, 2.41 and 3.23 units of naphthoqhinone per gram of leaf, nodal and root derived calli respectively.

4.5.3 Withdrawal of inorganic nutrients

Reducing the nitrogen content of the basal medium to half and one fourth of its original strength, exerted a beneficial effect on naphthoquinone production in *in vitro* cultures of *Rubia cordifolia*, and derived from leaves, nodal segments and roots (Table 21). Reduction of nitrogen to one-fourth the original concentration registered a marked increase in naphthoquinone content (3.21, 4.01 and 6.79 units per gram of leaf, nodal and root calli, respectively) as compared to control (2.32, 2.64 and 3.72 units per gram in leaf, nodal and root callus cultures, respectively). However, when phosphorous content of the basal medium was reduced to half and one-fourth the

Table 19. Influence of growth regulators and media supplement on naphthoquinone production in *in vitro* cultures *Rubia cordifolia.*

Growth regulators / Media supplement	Concentration (mg l^{-1})	Naphthoquinone yield (unit g^{-1})			Mean
		Leaf	Nodal	Root	
NAA + BA	2.0 + 1.0	2.12	2.45	3.51	2.69
	2.0 + 0.5	2.32	2.64	3.72	2.89
	1.0 + 1.0	2.01	2.1	3.42	2.51
	1.0 +0.5	1.96	2.02	3.38	2.45
NAA + KIN	2.0 + 1.0	1.97	2.42	3.56	2.65
	2.0 + 0.5	2.09	2.48	3.61	2.72
	1.0 + 1.0	1.62	2.17	3.46	2.41
	1.0 +0.5	1.7	2.31	3.56	2.52
2,4-D + BA	2.0 + 1.0	1.76	2.23	3.12	2.37
	2.0 + 0.5	1.89	2.39	3.28	2.52
2,4-D + KIN	2.0 + 1.0	1.69	2.81	2.81	2.43
	2.0 + 100	1.74	1.96	2.96	2.22
2,4-D + Phloroglucinol	2.0 + 50	1.99	2.18	3.39	2.52
	2.0 + 100	1.87	2.03	3.19	2.36
	2.0 + 50	1.72	1.96	3.03	2.23
2,4-D + Phloroglucinol	1.0 + 50	2.14	2.51	3.51	2.72
	1.0 + 100	2.08	2.47	3.39	2.64
	1.0 + 50	1.94	2.31	3.25	2.5
NAA	1	2.13	2.44	3.61	2.72
	2	2.09	2.37	3.48	2.64
2,4-D	1	1.87	2.14	3.04	2.35
	2	1.75	1.88	2.72	2.11
Mean		1.92	2.28	3.31	

original strength, no positive effect on naphthoquinone expression was noted in root derived calli, as compared to control, though a marginal increase was noted in leaf and nodal segment derived calli, as compared to control (Table 21).

4.5.4 Supplementing medium P. with media additives

4.5.4.1 Yeast extract

Incorporation of 2 per cent yeast extract into the production medium (MS + NAA 2 mg l^{-1} + BA 0.2 mg l^{-1}) revealed marked increase in naphthoquinone content in *in vitro* cultures of *Rubia cordifolia* registering 4.23, 6.29 and 6.78 units per gram of leaf, nodal segment and root derived calli respectively, as compared to control (2.32, 2.64 and 3.72 units per gram of calli in leaf, nodal and root callus cultures respectively). At one per cent level as well, this media supplement yielded higher amounts of the target compound in *in vitro* cultures of the experimental species as revealed in Table 22.

4.5.5 Feeding of precursors

Response of experimental calli, subcultured into the medium P, to addition of the indirect precursors of the target compound, tyrosine and phenyl alanine, with respect to naphthoquinone production in *in vitro* cultures of the experimental species, are presented in Table 23. The data revealed the favourable influence of both phenyl alanine and tyrosine on production of naphthoquinone in leaf, nodal and root derived calli, and the response was better in phenyl alanine supplemented medium. Phenylalanine at 150 mg l^{-1} produced higher amounts of naphthoquinone in nodal (4.98 units per gram of calli) and root (5.08 units per gram of calli) derived calli as compared to the respective control cultures. The same precursor at 100 mg l^{-1} registered highest production of naphthoquinone in leaf derived callus cultures (3.36 units per gram of calli).

Tyrosine, the other amino acid precursor employed in the study, also registered increased amounts of naphthoquinones in *in vitro* cultures of *Rubia cordifolia*, as compared to control, at all the levels tested (Table 23). However, the response was less as compared to that of phenylalanine, which at all levels, surpassed the effect of tyrosine, with respect to naphthoquinone synthesis in leaf, nodal and root derived calli of *in vitro* cultures.

Table 20. Effect of modifying sucrose concentration on naphthoquinone production in *in vitro* cultures of *Rubia cordifolia*

Carbon Source	Concentration (Per cent)	Naphthoquinone yield (Unit g^{-1})		
Sucrose		Leaf callus	Nodal callus	Root callus
	3	2.32	2.64	3.72
	5	2.11	2.41	3.23

Table 21. Effect of modifying nitrate and phosphate concentrations on naphthoquinone production in *in vitro* cultures of *Rubia cordifolia*

Sl.No	Reduction of inorganic nutrients	Naphthoquinone yield (Unit g^{-1})		
		Leaf callus	Nodal callus	Root callus
1	½ NO_3	2.98	3.39	6.08
2	¼ NO_3	3.21	4.01	6.79
3	½ PO_4	2.45	2.69	3.72
4	¼ PO_4	2.59	2.76	3.56
5	Control	2.32	2.64	3.72

Table 22. Influence of yeast extract on naphthoquinone production in *in vitro* cultures of *Rubia cordifolia*

Concentration (per cent)	Naphthoquinone yield (Unit g^{-1})		
	Leaf callus	Nodal callus	Root callus
1	3.49	5.32	5.99
2	4.23	6.29	6.78
Control	2.32	2.64	3.72

Root callus cultures synthesized more amounts of naphthoquinone at all levels of the two precursors employed, as compared to leaf and nodal segments derived *in vitro* cultures (Table 23).

4.6 Influence of culture conditions on naphthoquinone production in *in vitro* cultures of *Rubia cordifolia*.

4.6.1 Incubation in dark

No significant difference in naphthoquinone production was noticed, on incubating *in vitro* cultures of *Rubia cordifolia* in the dark as compared to incubation in light (Table 24). Incubating leaf, nodal and root derived cultures of the experimental species in the dark, recorded 2.43, 2.78 and 3.89 units respectively per gram of calli, registering a marginal increase in content of the target compound on dark incubation, as compared to control.

4.6.2 Incubation at low temperature

Incubating at low temperature did not bring an increase in naphthoquinone production, in in vitro cultures of Rubia cordifolia as compared to control (Table 24). Incubating leaf, nodal and root derived cultures of the experimental spcies at low temperature, recorded 1.48, 1.55 and 2.95 units respectively per gram of calli.

4.7 Employing special techniques to enhance naphthoquinone content in *in vitro* cultures of Rubia cordifolia.

4.7.1 Elicitation

4.7.1.1 Biotic elicitation

Eliciting the production media with autoclaved mycelia of *Pythium aphanidermatum*, (Plate 4) along with the culture filtrate, at levels of 2 per cent and 5 per cent, yielded encouraging results with respect to production of naphthoquinones *in vitro* by leaf, nodal and root derived calli (Table 25). Addition of 5 per cent of autoclaved extract, recorded substantial increase in naphthoquinone synthesis in all experimental calli (5.02, 7.18 and 8.13 units of naphthoquinone in leaf, nodal and root derived calli respectively. Root calli recorded the maximum amount of naphthoquinone (8.13 units per gram of calli at 5 per cent level and 7.26 units per gram of calli at 2 per cent level of autoclaved mycelia).

Plate 4 Fungal elicitor (*Pythium aphanidermatum*) on Potato Dextrose Broth

Table 23. Influence of precursor feeding on naphthoquinone synthesis in *in vitro* culture of *Rubia cordifolia*

Sl No.	Amino acid	Conc. (mg l^{-1})	Naphthoquinone yield (Unit g^{-1})		
			Leaf	Nodal	Root
1	Phenylalanine	50	3.01	3.46	3.87
2	Phenylalanine	100	3.36	4.02	4.26
3	Phenylalanine	150	3.76	4.98	5.08
4	Tyrosine	50	2.33	2.78	3.73
5	Tyrosine	100	2.58	2.83	3.83
6	Tyrosine	150	2.89	3.04	3.94
7	Control		2.32	2.64	3.72

Table 24. Influence of culture conditions on naphthoquinone production in *in vitro* cultures of *Rubia cordifolia*

Treatments	Naphthoquinone (unit g^{-1})		
	Leaf callus	Nodal callus	Root callus
Incubation in Light	2.32	2.64	3.72
Incubation in Dark	2.43	2.78	3.89
Incubation at low temperature (4°C)	1.41	1.55	2.95

Table 25. Influence of fungal elicitation on naphthoquinone production in *in vitro* cultures of *Rubia cordifolia*

Sl no	Level of autoclaved mycelia *P. aphanidermatum* (per cent)	Naphthoquinone acid yield (Unit g^{-1})		
		Leaf callus	Nodal callus	Root callus
1	2	*3.62*	*5.64*	*7.26*
2	5	*5.02*	*7.18*	*8.13*
3	Control	2.32	2.64	3.72

4.7.1.2 Abiotic elicitation

Eliciting the medium P. with the abiotic elicitor, salicylic acid, at 10 µM and 100 µM, registered increased production of naphthoquinone in leaf (3.85 and 4.86 units per gram of calli respectively) nodal (5.96 and 6.76 units per gram of calli respectively) and root (7.66 and 8.76 units per gram of calli respectively) derived callus cultures. At 100 µM, salicylic acid registered the highest content of the target compound hitherto obtained in this study, in root derived *in vitro* cultures (Table 26).

4.7.2 Immobilization

A 5 per cent solution of sodium alginate, on complexing with 100 mM calcium chloride solution, yielded round firm beads, ranging from 1.0 to 1.3 cm in diameter (Plate 5). On immobilization, no positive response was obtained, with respect to naphthoquinone production, in *in vitro* cultures of the experimental species, as compared to control (Table 27). Leaf, nodal and root derived calli, synthesized 1.37, 1.46 and 2.82 units of naphthoquinone and thus registered a marked reduction in the quantity synthesized as compared to control.

4.7.3 Creating conditions of stress on incubating cultures

4.7.3.1 Addition of osmoregulants

Addition of the osmoregulant sorbitol did not bring about an increase in naphthoquinone production in *in vitro* cultures of *Rubia cordifolia* as compared to control (Table 28). On addition of sorbitol, at 1.0 and 1.5 per cent, naphthoquinone production, registering 2.19 units and 1.92 units, 2.43 units and 2.03 units and 3.19 units and 2.96 units respectively per gram of calli were obtained for leaf, nodal segment and root cultures respectively.

4.7.4 Establishment of hairy root cultures

4.7.4.1 Screening of *Agrobacterium* strains for antibiotic sensitivity

The response of *Agrobacterium rhizogenes* strains to different concentrations of the antibiotics, ampicillin, cefotxime and streptomycin is given in Table 29. The strain MTCC 2364 and MTCC 532 exhibited resistance to the antibiotic ampicillin, at all levels tested, as revealed by good growth of the strains employed. In streptomycin, the strain MTCC 532 showed good growth at all levels tested while, at 400 mg l^{-1}, the antibiotic inhibited growth of the strain MTCC 2364. As to the

Plate 5. Immobilised calli of *Rubia cordifolia*

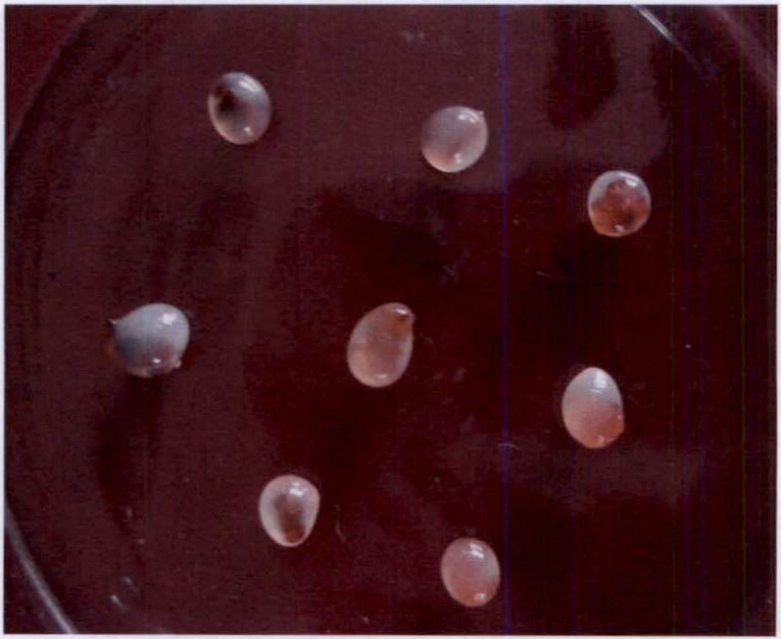
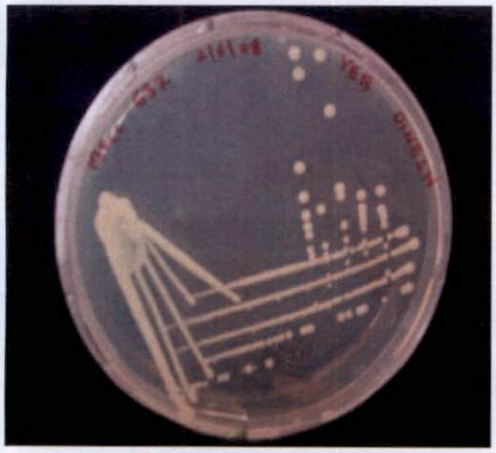
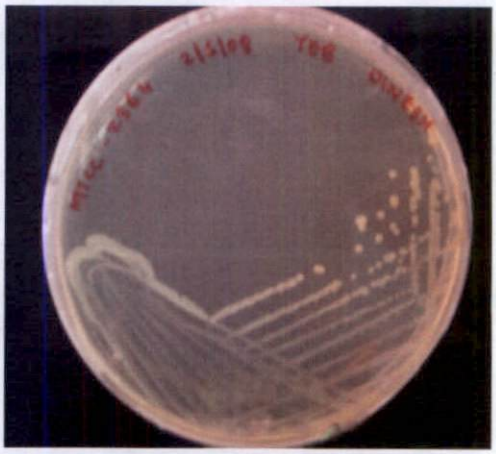


Plate 6. ***Agrobacterium rhizogenes*** **strains employed for induction of hairy root in** ***Rubia cordifolia***



MTCC 532



MTCC 2364

Table 26. Influence of salicyclic acid on naphthoquinone production in *in vitro* cultures of *Rubia cordifolia*

Concentration (µM)	Naphthoquinone yield (Unit g^{-1})		
	Leaf callus	Nodal callus	Root callus
10	3.85	5.96	7.66
100	4.86	6.76	8.76
Control	2.32	2.64	3.72

Table 27.Effect of immobilization on naphthoquinone production in *in vitro* cultures of *Rubia cordifolia*

Treatments	**Naphthoquinone yield (Unit g^{-1})**		
	Leaf callus	Nodal callus	Root callus
Immobilized cells	1.37	1.46	2.82
Free cells	2.32	2.64	3.72

Table 28. Effect of stress inducing compound on naphthoquinone production in *in vitro* cultures of *Rubia cordifolia*

Sl no.	**Stress inducing compounds**	**Conc. (per cent)**	**Naphthoquinone yield (Unit g^{-1})**		
			Leaf	**Nodal**	**Root**
1	Sorbitol	1	2.19	2.43	3.19
2	Sorbitol	1.5	1.92	2.03	2.96
3	Control		2.32	2.64	3.72

Table 29. Screening of *Agrobacterium rhizogenes* strains for antibiotic sensitivity for induction of hairy root in *Rubia cordifolia*.

Antibiotics	Concentration (mg l^{-1})	Strains	
		MTCC 2364	MTCC 532
Ampicillin	0	++	++
	50	++	++
	100	++	++
	200	++	++
	300	++	++
	400	++	++
	500	++	++
Streptomycin	0	++	++
	50	++	++
	100	++	++
	200	+	++
	300	+	+
	400	-	+
	500	-	+
Cefotaxime	0	++	++
	50	+	++
	100	+	+
	200	+	+
	300	+	+
	400	+	+
	500	-	-

(++) Good growth (+) Restricted growth (-) No growth

antibiotic cefotaxime, both the strains employed in the study, survived at levels from 0 to 400 mg l^{-1} (Plate 7). At 500 mg l^{-1}, growth of both the strains was inhibited in cefotaxime. Hence cefotaxime at 500 mg l^{-1} was considered as the optimum concentration of the antibiotic required to inhibit the *Agrobacterium rhizogenes* strains, MTCC 2364 and MTCC 532.

4.7.4.2 Sensitivity of explants to antibiotics

The pattern of sensitivity of explants to the antibiotics, ampicillin and cefotaxime at varying concentrations is presented in Table 30. Explants like leaf and nodal segments, employed for induction of hairy roots, survived in the antibiotic cefotaxime at levels of 0-500 mg l^{-1}. At 1000 mg l^{-1} paling and yellowing were noted for leaf explants in cefotaxime. Identical results were observed with respect to sensitivity screening of the explants in the antibiotic ampicillin.

Hence considering the growth inhibition revealed by the *Agrobacterium rhizogenes* strains MTCC 2364 and MTCC 532 and the healthy survival of the explants employed in the study (leaf and nodal segments), cefotaxime at 500 mg l^{-1} was employed as the antibiotic for eliminating the bacterial strains, at the end of co-culture period (Plate 8).

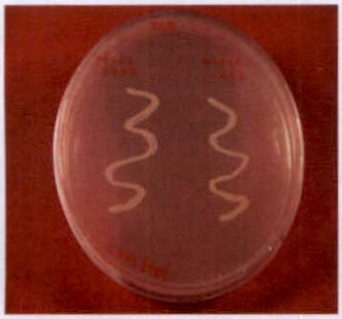
4.7.4.3 Induction of hairy roots

None of the explants employed in the study registered induction of hairy roots at the end of 1 month and 2 months, when cultured in basal MS medium in light as well as dark incubation, after co-culturing with *Agrobacterium rhizogenes* strains MTCC 2364 and MTCC 532 for periods ranging from 1-3 days.

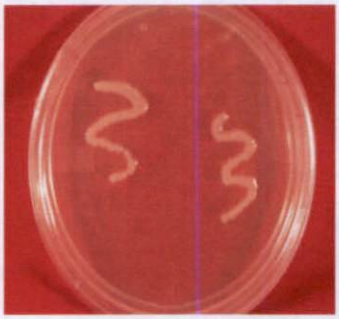
4.8 Influence of age of calli on naphthoquinone synthesis in *Rubia cordifolia*

Trends in expression of naphthoquinones in *in vitro* cultures of *Rubia cordifolia,* in relation to age of the calli are presented in Table 31. Eight week old calli, incubated on medium P, recorded greater amounts of naphthoquinones in leaf (2.93 units), nodal segment (3.29 units) and root (4.53 units) derived callus cultures. Beyond 8 weeks, the naphthoquinone content started decreasing in *in vitro* test samples.

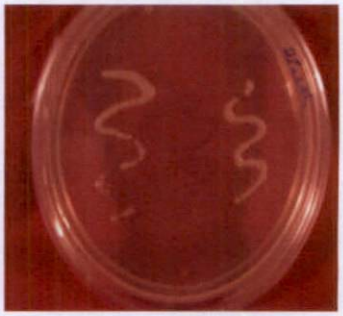
Plate 7. Sensitivity of *Agrobacterium rhizogenes* strains to cefatoxime (mg l^{-1})



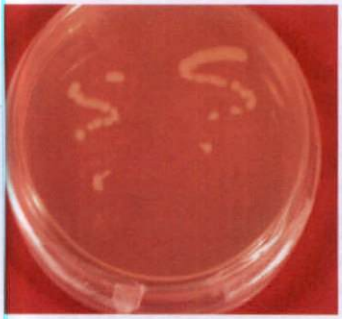
Control



100



200



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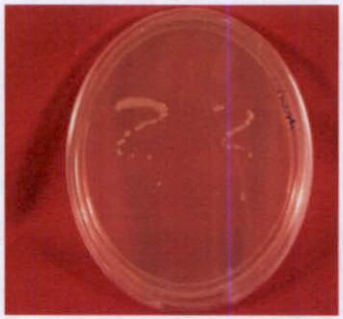
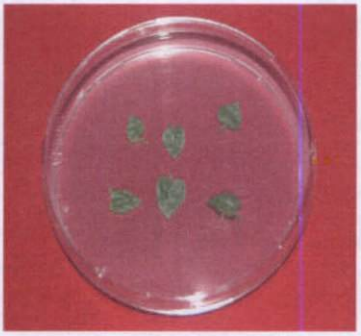


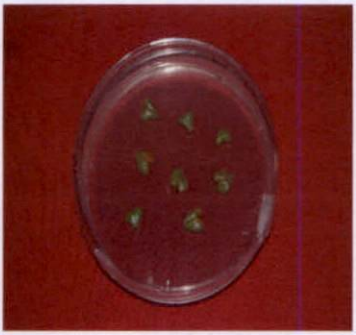
400

500

Plate 8. Sensitivity of leaf explants to cefatoxime (mg l^{-1})



Control



Low concentration (100)

High concentration (1000)

Table 30. Screening of explants of *Rubia cordifolia* for antibiotic sensitivity for induction of hairy roots

Antibiotics	Concentration (mg l -1)	Explants	
		Leaf	Nodal
Cefotaxime	0	H	H
	100	H	H
	250	H	H
	500	H	H
	1000	P	H
Ampicillin	0	H	H
	100	H	H
	250	H	H
	500	H	H
	1000	P	H

H- Healthy P – Pale

4.9 Establishment of suspension cultures

4.9.1 Standardization of subculturing intervals

Figure 10. depicts the cell counts observed in the suspension cultures of *Rubia cordifolia*, over a period of 30 days. The cell count showed a progressive and sustained expontial growth upto 27th day, which declined thereafter (Plate 9). Suspension cultures initiated in basal medium enriched with NAA (2 mg l^{-1}) and BA (0.5 mg l^{-1}) produced a mean cell count of 4650 cells, 4550 cells and 4535 cells per 10 µl of suspensions respectively (Table 32).

4.9.2 Determination of packed cell volume.

The production media in liquid cultures gave a mean packed cell volume of 0.93, 0.83 and 0.80 per cent for leaf, nodal and root derived suspensions, wherein suspension cultures initiated with leaf calli, registered maximum packed cell volume.

4.9.3 Quantification of naphthoquinone in suspension cultures.

Suspension cultures established in production medium (MS + NAA (2 mg l^{-1}) + BA (0.5 mg l^{-1}) produced 2.73 unit per gram, 2.88 unit per gram and 3.94 unit per gram in leaf, nodal and root suspension cultures respectively (Table 33).

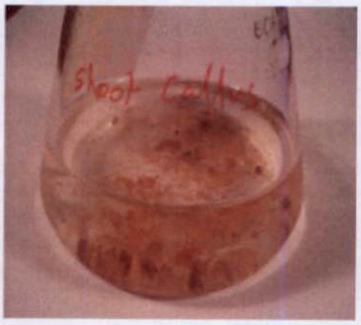
From the Table it is infered that only marginal increase was noted with respect to content of naphthoquinone in suspension cultures as compared to static cultures.

4.10 Qualification of naphthoquinone in *ex vitro* plants of *Rubia cordifolia*

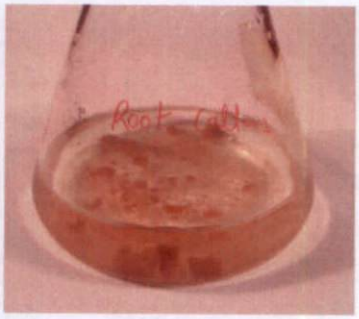
Content of naphthoquinone in whole plant and root samples of *Rubia cordifolia* at varying stages of maturity are presented in Table 34. Compared to whole plant samples, root samples recorded higher amounts of naphthoquinones at all stages of maturity. At 15 months after planting, root samples recorded 2.74 units per gram of sample as compared to 0.76 units per gram of whole plant sample. Both whole plant and root samples recorded increasing trends in the levels of naphthoquinone with increase in age.

Plate 9. Suspension cultures derived from leaf, nodal segment and root calli of *Rubia cordifolia*

Leaf



Nodal segment



Root

Table 31. Influence of age of calli on naphthoquinone production in *in vitro* cultures of *Rubia cordifolia*

Treatments	Naphthoquinone (unit g^{-1})		
	Leaf callus	Nodal callus	Root callus
Four week	2.32	2.64	3.72
Eight week	2.93	3.29	4.53
Twelve week	2.62	3.02	4.12

Table 32. Callus growth and proliferation in liquid suspensions of *Rubia cordifolia* at the stage of subculture.

Production media (mg l^{-1})	Cell count (No. of cells 10 μl^{-1})			Packed cell volume		
	Leaf callus	Nodal callus	Root callus	Leaf callus	Nodal callus	Root callus
NAA (2.0)+ BA (0.5)	4650	4550	4525	0.93	0.83	0.80

Table 33. Comparison of static and suspension cultures of *Rubia cordifolia* for *in vitro* synthesis of naphthoquinone

Source of calli	Naphthoquinone yield (unit g^{-1})	
	Static cultures	Suspension cultures
Leaf	2.32	2.73
Nodal	2.64	2.88
Root	3.72	3.94

Table 34. Influence of stage of harvest on naphthoquinone yield of methanol extract of *ex vitro* samples of *Rubia cordifolia*

Age of plant	Naphthoquinone Yield (unit g^{-1})	
	Whole plant	*In vivo* root
9 months	0.61	2.64
12 months	0.69	2.69
15 months	0.76	2.74

4.11 Evaluation of anticancerous activity of *in vivo* and *in vitro* extracts of *Rubia cordifolia*

4.11.1 Assessment of *in vitro* cytotoxicity of *in vitro* and *ex vitro* samples of *Rubia cordifolia*.

Extent of *in vitro* cytotoxicity of whole plant, root extracts of calli of *Rubia cordifolia* grown on production medium on DLA and EAC cell lines, as expressed by percentage of cell death are presented in Table 35.

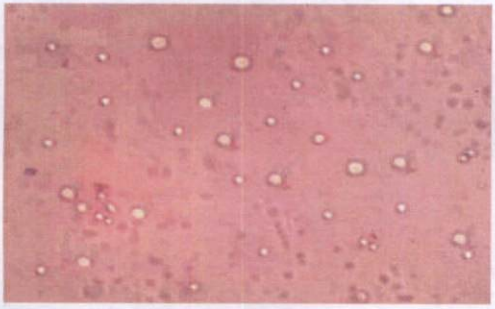
Viable cells counted in a haemocytometer, using tryptan blue (1 per cent) exclusion method revealed that clear evidence of cytotoxicity was expressed by *in vitro* extracts as well as whole plants and root extracts of *Rubia cordifolia* on both DLA and EAC cell lines, as compared to control (plate 11). From the Tables it is also evident that cytotoxicity of *ex vitro* and *in vitro* samples of the experimental species is dose dependant, increasing with increase in the concentration of the test extracts. In both cell lines, *ex vitro* root extracts exhibited maximum cytotoxicity (56 per cent cell death each, on DLA and EAC cell lines at 1000 µg concentration) followed by root callus extract (52 per cent death and 53 per cent cell death on DLA and EAC cell lines respectively, at 1000 µg concentration). But at a concentration of 800 µg, cytotoxic effect of *in vitro* derived root callus extract was marginally higher then *ex vitro* extract, on DLA cell line, while on EAC cell line, *in vitro* root derived calli performed better than *ex vitro* root samples, in terms of percentage of cell death, at all concentrations tried, except at 1000 µg. All *in vitro* cultures exhibited greater cytotoxicity on the cell lines employed as compared to whole plant extract (Table 36).

Between DLA cell line, which is a solid tumour cell line and EAC cell line which is a muscle tumour cell line, varying trends were expressed by the root derived test samples, with respect to extent of cytotoxicity, indicated by percentage of cell death, in that, on EAC cell line, cytotoxic effect of *in vitro* root calli surpassed that of *in vivo* root extract at most of the concentrations tried. Leaf and shoot derived callus extract and whole plant extract displayed similar trends with respect to expression of cytotoxicity, on both the experimental cancer cell lines.

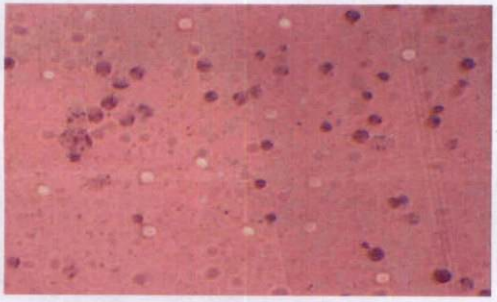
IC_{50} values calculated, on the basis of extent of cytotoxicity, expressed by percentage of cell death, revealed that on DLA cell line maximum IC 50 values were

Plate 11. *In vitro* cytotoxicity of *in vitro* and *ex vitro* samples of *Rubia cordifolia* on DLA and EAC cell line

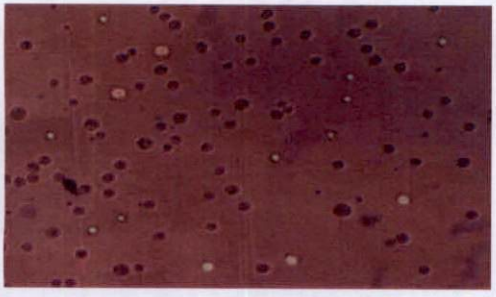
DLA cell line



Control (Without extract)

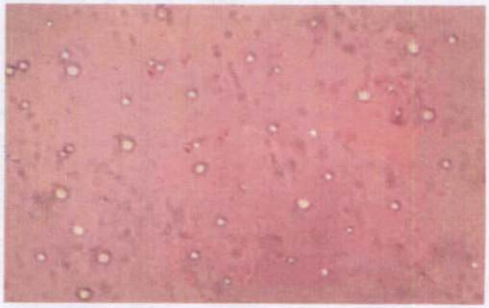


Lower concentration (200 µg)

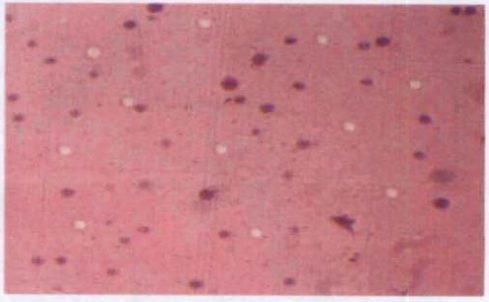


High concentration (1000 µg)

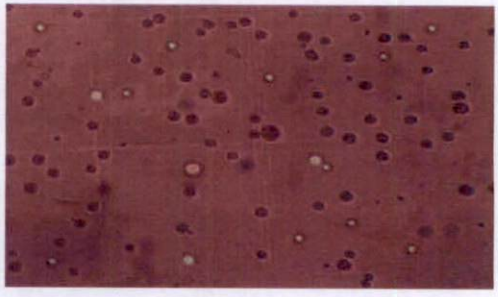
EAC cell line



Control (Without extract)



Lower concentration (200 µg)



High concentration (1000 µg)

Table 35. *In vitro* cytotoxicity of whole plant and callus extract of *Rubia cordifolia* on DLA cell line

Concentration of Drug (10 mg/ml)	Percentage of cell death (Per cent)				
	Leaf callus	Shoot callus	Root callus	Whole plant	normal root
200 µg	4	8	12	6	26
400 µg	6	11	21	9	33
600 µg	9	13	38	13	39
800 µg	12	16	47	16	44
1000 µg	15	20	52	18	56

Table 36. *In vitro* cytotoxicity of whole plant and callus extract of *Rubia cordifolia* on EAC cell line

Concentration of Drug (10 mg/ml)	Percentage of cell death (Per cent)				
	Leaf callus	Shoot callus	Root callus	Whole plant	normal root
200 µg	9	4	19	6	16
400 µg	11	8	29	10	23
600 µg	13	12	40	13	31
800 µg	16	15	48	17	46
1000 µg	18	19	53	20	56

Table 37. IC_{50} values of test extracts of *Rubia cordifolia* with respect to cytotoxicity

Test sample	IC_{50} value (mg)	
	DLA cell line	EAC cell line
Whole plant extract	2.74	2.56
In vivo root extract	0.85	0.89
Leaf callus extract	3.42	2.91
Nodal callus extract	2.64	2.64
Root callus extract	0.89	0.85

registered by *in vitro* derived leaf callus extract (3.42 mg) followed by nodal segment derived callus extract (2.64 mg) and whole plant extract (2.74 mg). Least IC_{50} values were registered by *in vitro* derived root callus extract (0.89 mg) and *ex vitro* root extract (0.85 mg). Similar trends were noted on EAC cell line as well wherein *in vitro* root callus extract and *in vivo* root extract recorded lower IC_{50} values, indicative of their enhanced cytotoxicity activity (Table 37)

4.11.2 Estimation of *in vitro* antioxidant activity of *in vitro* and *ex vitro* extracts of *Rubia cordifolia*

Results of estimation of *in vitro* antioxidant activity of test samples of *Rubia cordifolia* as measured by superoxide radical scavenging activity, inhibition of hydroxyl radicals, inhibition of nitric oxide radicals and inhibition of lipid peroxidation are presented in Tables 38a, 38b, 38c and 38d. The results indicate that scavenging activity of samples is dose dependent. At a concentration of 1000 µg, whole plant extract was observed to scavenge the superoxides generated by riboflavin photoreduction method, to the extent of 52.41 percent. *In vitro* extracts of leaf, nodal segments and roots exhibited less superoxide scavenging activity at the same concentration (20.04 per cent for leaf callus extract, 19.78 per cent for nodal segment derived callus extract and 22.14 per cent root callus extract. At other concentrations, also *ex vitro* samples recorded maximum scavenging activity. The IC_{50} value calculated on the basis of percentage of scavenging activity indicates that for obtaining 50 per cent superoxide radical scavenging activity 2.38 mg of root callus extract is required as against 0.91 mg of whole plant extract.

Data on inhibition of hydroxyl radicals generated by degradation of deoxyribose by H_2O_2 system, revealed that the activity was dose dependent. At 800 µg concentration, *ex vitro* sample exihibited 17.44 per cent of inhibition, followed by root callus extract (17.22 per cent), leaf callus extract (16.63 per cent) and nodal segment de rived callus extract (14.49 per cent). Marginal deviations in the trends were noted at concentrations of 1000 µg and 400 µg, where the leaf callus extracts exhibited higher hydroxyl radical inhibition activity.

Table d. Evaluation of lipid peroxidation inhibition activity

Extract	Concentration of Drug (10 mg ml^{-1})				
	Percentage of inhibition				
	200 µg	400 µg	600 µg	800 µg	1000 µg
Whole Plant	6.66	16.66	23.8	36.09	48.8
Leaf callus	2.48	8.7	14.17	17.91	20.39
Nodal callus	6.66	10.47	15.23	18.8	20.95
Root callus	0.78	11.81	12.44	14.64	15.59

Table 39. IC_{50} values of test extract of *Rubia cordifolia* with respect to antioxidant activity

Test extract	IC $_{50}$ values (mg)			
	Superoxide scavenging activity	Hydroxyl radical inhibition activity	Nitric oxide radical inhibition activity	Lipid peroxidation inhibition activity
Whole plant extract	0.91	2.37	3.36	1.07
Leaf callus extract	2.45	2.46	5.24	2.26
Nodal callus extract	2.43	2.59	3.86	2.32
Root callus extract	2.38	2.46	6.98	2.88

The IC_{50} values of hydroxyl scavenging activity for *ex vitro* samples and *in vitro* leaf, nodal and root callus extracts as presented in Table 39. are 2.37 mg, 2.46 mg, 2.59 mg and 2.46 mg respectively.

Dose dependant inhibition of nitric oxide radicals, by the test samples as revealed in Table 38c indicate that at all concentrations tried, whole plant extract exhibited higher percentage of inhibition of nitric oxide radicals as compared to *in vitro* callus extracts. Among the *in vitro* test extracts, root callus extracts showed least inhibition of nitric oxide radicals (Table 38c).

The IC_{50} values for nitric oxide radical inhibition activity as estimated for *ex vitro* samples, leaf callus extract, nodal callus extract and root callus extract are 3.36 mg, 5.24 mg, 3.86 mg and 6.98 mg respectively.

As to inhibition of lipid peroxidation, whole plant extract exhibited maximum inhibition at all concentrations tried (Table 38d). As in the case of nitric oxide radical inhibition activity, root callus extracts exhibited least inhibition of lipid peroxidation, registering low values for all concentrations tried. The IC_{50} values for *ex vitro* samples and *in vitro* leaf, nodal and root callus extracts with respect to inhibition of lipid peroxidation are estimated as 1.07 mg, 2.26 mg, 2.32 mg and 2.88 mg respectively.

4.11.3 Assessment of pro oxidant activity of *ex vitro* and *in vitro* test samples of *Rubia cordifolia*

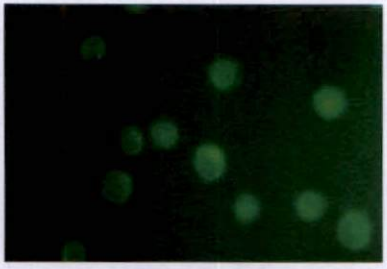
The extent of pro oxidant activity of test extracts exhibiting enhanced levels of cytotoxicity (*ex vitro* root extract and *in vitro* root callus extract), as detected by the intensity of fluorescence obtained with the oxidative sensitive fluorescent dye, revealed that *in vitro* root callus extract exhibited enhanced pro oxidant activity (Plate 12)

Plate 12. **Cytotoxicity effect of *Rubia cordifolia* by ROS generation in DLA and EAC cell lines : DCFH-DA staining method.**

DLA cell line

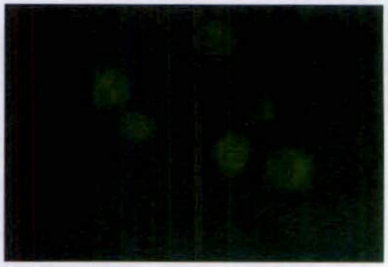
Control (without extract)

Lower concentration (100 µg)

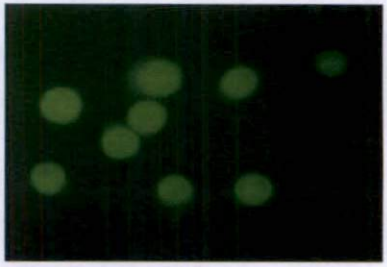


High concentration (1000 µg)

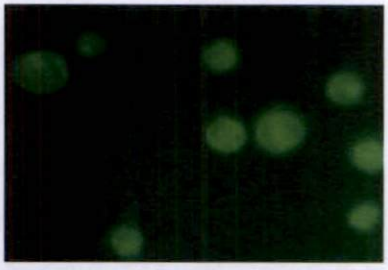
EAC cell line



Control (without extract)



Lower concentration (100 µg)



High concentration (1000 µg)

DISCUSSION

DISCUSSION

5.1 Standardization of pre treatment and surface sterilization in *Rubia cordifolia*

Use of field grown plants as a direct source of explants often requires pretreatments with suitable fungicides/antibiotics to yield contamination free cultures (Webster *et al.*, 2003). Also, systemic contaminants are not destroyed by surface sterilization alone necessitating pretreatment of explants with fungicides.

In the present study, initial observations on contamination free culture establishment confirmed the inevitability of a two step sterilization process combining pretreatment of explants with a fungicide at appropriate levels for varying periods followed by surface sterilization. In view of this observation, standardization experiments on the appropriate concentration of the fungicide and period of treatment of the explants revealed that treatment of leaf, and nodal explants with 2.5 per cent Bavistin for 15 minutes was the best with respect to yielding healthy cultures, (91.6 per cent for leaf explants and 100 per cent for nodal segments) free of contamination (Cent per cent contamination free cultures for both leaf and nodal explants). On increasing the concentration of the fungicide to 3 per cent, leaf explants exhibited scorching at varying intensities at all treatment durations tried (Table 9).

Earlier, Matthews and Duncan (1993) have subjected nodal segments from field grown cocoa plants to treatment with the fungicide, benlate and the antibiotic, rifampicin, to obtain contamination free cultures after sterilization.

Surface sterilization, an inevitable procedure for successful culture and incubation of *in vitro* systems was carried out with the commonly employed surface sterilant, mercuric chloride at 0.1 per cent for varying lengths of time, wherein, sterilization of pretreated leaf and nodal segments for 1 min resulted in 91.66 per cent healthy contamination free leaf derived cultures and 100 per cent healthy, non-contaminated stem derived cultures (Table 10). Mercuric chloride has been used with advantage to generate contamination free cultures with maximum survival in a vast

number of medicinal plant species such as *Vitex negundo* (Kannan and Jasrai, 1998), *Acorus calamus* (Kulkarni and Rao, 1999) and *Paederia foetida* (Amin *et al.*, 2003). Increasing the duration of surface sterilization to 3.5 min and 3.0 minutes resulted in scorching of explants (66.66 per cent and 25 per cent for leaf and nodal explants respectively at 2.5 minutes duration)and 83.33 per cent and 33.33 per cent for leaf and nodal explants, respectively at 3 minutes duration).

Increasing duration of exposure of explants to sterilants and scaling up levels of sterilant had an adverse effect on the survival of cultures in *Tinospora cordifolia* as well as observed by Kalimuthu (2002).

5.2 Induction and proliferation of calli

5.2.1 Effect of auxins in inducing and proliferating calli

Trends in initiation of calli on full strength MS medium supplemented with the three auxins, NAA, 2,4-D and IAA at varying levels were examined (Table 11). MS medium supplemented with NAA at 2 mg l^{-1} recorded highest percentage of callus initiation in leaf (97.50%), stem (89.50%) and root (85.41%) cultures, though the differences were not significant. The same auxin at 1 and 2 mg l^{-1} recorded early callusing in all the explants tried, but with significant differences compared to the other auxins employed (Table 11). NAA at 2 mg l^{-1} resulted in maximum callus growth score and mean callus index values for leaf (2.72 and 265.21 respectively), nodal segment (1.94 and 168.41 respectively) and root (1.88 and 165.69 respectively) derived callus cultures (Plate 2).

Favourable effects of NAA on callusing was observed in various crops like *Papaver bracteatum* (Ilahi and Ghauri, 1994) and *Catharanthus roseus* (Akram and Yurekhi, 1995). Rao *et al.* (1999) obtained maximum percentage of callus initiation in leaf base cultures of the medicinal species *Centella asiatica* in MS medium supplemented with NAA, 2 mg l^{-1}. In the present study, the other auxin 2,4-D, which resulted in values for the parameters studied on par with those for NAA, is also known to be a very potent auxin, to induce callus growth as it has little polar transport and trends to accumulate at one point (Goldsmith, 1977). However, in

Tinospora cordifolia. Kalimuthu (2002) reported that performance of 2,4-D with respect to callus induction and proliferation was not encouraging.

In the present study, performance of the auxin IAA, with respect to callus initiation and proliferation was poor, recording the lowest values, though not significantly different from other auxins for the parameters studied (Table 11). Murashige (1974) has concluded that among the common auxins employed, IAA is the least potent with respect to callus initiation and proliferation. IAA being a highly polar auxin is more conducive to differentiation (Goldsmith, 1977). That IAA was the least beneficial auxin with respect to proliferating calli, was observed in *Gymneama sylvestre* as well by Anu (1993).

5.2.2 Effect of cytokinins on callusing

Incorporation of the major cytokinins, kinetin and benzyladenine at varying levels, into the basal media, were not promising, when compared to supplementing with auxins (Table 12). Leaf, stem and root segments responded in an inferior manner for all the parameters studied with respect to callusing (Table 12).

The observation that cytokinins favour cell elongation and shoot bud initiation, as compared to callusing, holds good in the present study. Compared to kinetin, benzyladenine (BA) performed better with respect to days to initiate calli, callus growth score and callus index, the values being significantly different (Table 12). Favourable influence of BA over kinetin in inducing and proliferating calli was noted in *Gymnema sylvestre* by Anu (1993), in *Coscinium fenestratum* by Sindhu (1999) and in *Tinospora cordifolia* by Kalimuthu (2002).

5.2.3 Effect of auxins and cytokinins on callusing

In the present study, NAA and BA at 2 mg l^{-1} and 0.5 mg l^{-1} respectively, resulted in highest percentage of callusing, callus growth score and mean callus index values in leaf, stem and root derived callus cultures (Table 13). However, on comparison with the other combinations tried, the differences in values for the parameters studied, were not significant. Suryanarayana and Pai, (1998) reported that in *Coleus forskohlii*, callus cultures were initiated and proliferated on MS medium

with NAA (2.0 mg l^{-1}) and BA (0.5 mg l^{-1}). Zhang *et al.* (2007) observed that a low ratio of cytokinin to auxin, as is evident in the present study as well, promoted callus induction in *Erigeron breviscapus*, wherein 100 per cent callus induction was noticed with NAA and BA. Rao *et al.* (2008) obtained callus proliferation from leaf explants of *Tinospora cordifolia* in MS medium fortified with BA and NAA.

5.2.4 Effect of media supplements on callusing

In the present investigation, the phenolic hormone synergist, phloroglucinol at a level of 100 mg l^{-1}, when incorporated into the basal media, along with the auxins, NAA, 2,4-D and IAA, failed to elicit maximum callus growth response in all the explants tried, as compared to incorporation of auxins, singly or in combination with cytokinins (Fig.2).

However, promotive effects of phloroglucinol on callusing have been noted in *Tinospora cordifolia* by Kalimuthu (2002). Phloroglucinol, which is an auxin synergist, when provided at a concentration of 25 mg l^{-1} concentration, exhibited more callusing than control, but at 50 and 100 mg l^{-1} concentrations exhibited less callusing, as compared to control (Gholba, 2000).

5.2.5 Comparative performance of explants to callus induction and proliferation

Cultures originating from leaf explants were better performing with reference to parameters indicating efficient callusing, initiating calli earlier (8.95 days), and registering highest mean callus growth rate (1.84) and callus index value (170.22) in MS media, supplemented with auxins and cytokinins. For the rest of the media combinations also the same trend was noticed. Root explants were inferior with respect to induction and proliferation of calli, in the experimental species, at all media combinations tried (Fig.3).

Differences in explant performance with respect to callusing is evident in many crop plants. Reddy *et al.* (2005) reported that, in *Rauvolfia serpentina*, compared to hypocotyls and roots, leaf bits recorded the highest fresh and dry weight

Fig 2. Comparative response of *Rubia cordifolia* to growth regulators, their combinations and media supplement with respect to callus index.

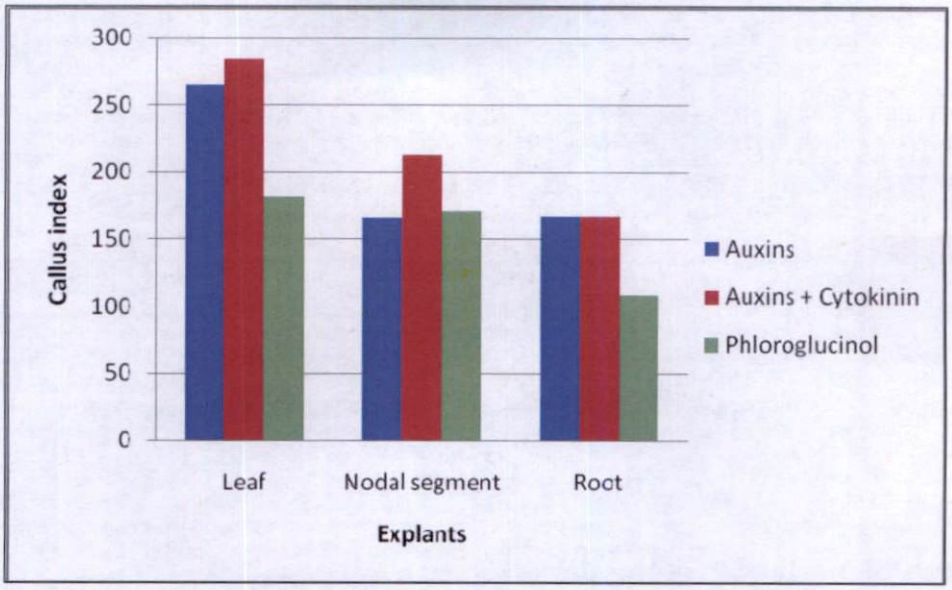
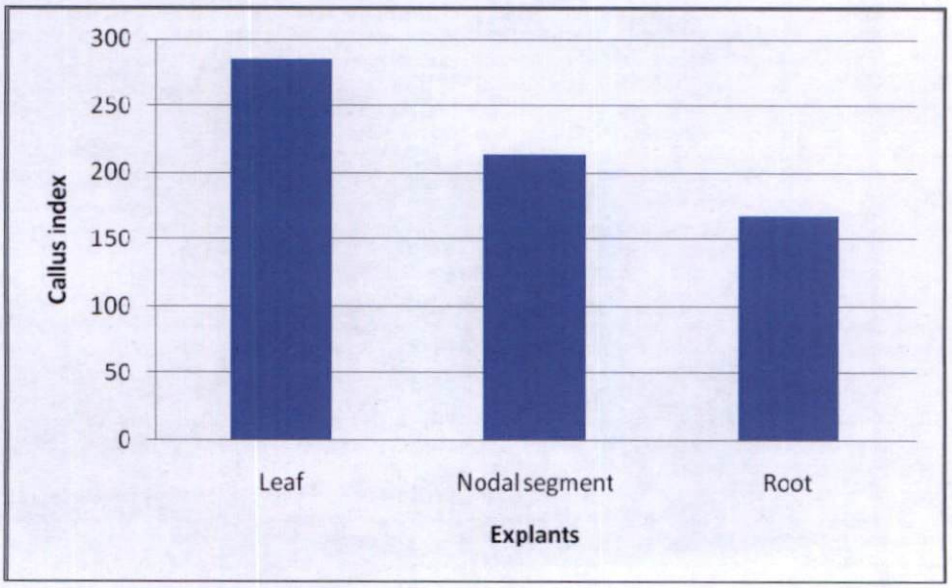


Fig 3. Relative response of explants of *Rubia cordifolia* with respect to callus index
Medium - Ms + NAA (2mg l^{-1}) + BA (0.5 mg l^{-1})



of calli. Suitability of using leaf segments as explants to induce calli, was observed in *Tinospora cordifolia* by Kalimuthu (2002).

5.2.6 Standardization of growth media for callus growth and proliferation

Comparison of effect of incorporating auxins along with cytokinins in induction and proliferation of calli in the experimental species, with incorporation of auxins and cytokinins singly, reveal the favourable response of explants to a combination of auxins and cytokinins, for all the parameters studied with respect to callusing (Fig. 4, 5, 6). Auxins are primarily known to induce cell elongation while cytokinins induce cell division, both the processes being efficient for culture growth. Hence a combined stimulus from auxins and cytokinins may have enhanced the rate of growth of calli, as observed by Balandrin and Klocke (1988).

5.3 Influence of culture environment on callusing

In *Rubia cordifolia* culturing explants under illumination at 26 ± 1°C proved superior to culturing them under dark (Table 15). Leaf, stem and root explants, when cultured in dark registered low callus index values of 90.93, 88.79 and 81.31 respectively. Sankar (1998) and Kalimuthu (2002) obtained similar results in *Sida* spp. and *Tinospora cordifolia* respectively. However, dark incubation resulted in better callusing in *Kaempferia galangal* (Joseph, 1997) and *Coscinium fenestratum* (Sindhu, 1999). However dark incubation resulted in better callusing in leaf explants of *Agastache rugosa* (Xu *et al.*, 2008). The best ratio of production per growth was obtained in callus cultures treated with glucose 75 g l^{-1} under light exposure (Francoise *et al.*, 2007).

5.4 Biochemical estimation of naphthoquinones.

5.4.1 Detection of naphthoquinones employing thin layer chromatography

5.4.1.1 Selection of solvent systems

In the present study, among the various solvent systems employed for detection of naphthoquinone chlororform : methanol was identified to be the ideal solvent system. Hassanean *et al.* (2000) has also reported chloroform : methanol as the solvent system for detection of naphthoquinone anhydride. The same solvent

Fig 4. Relative response of *Rubia cordifolia* to growth regulators and their combinations with respect to percentage of cultures initiating callus.

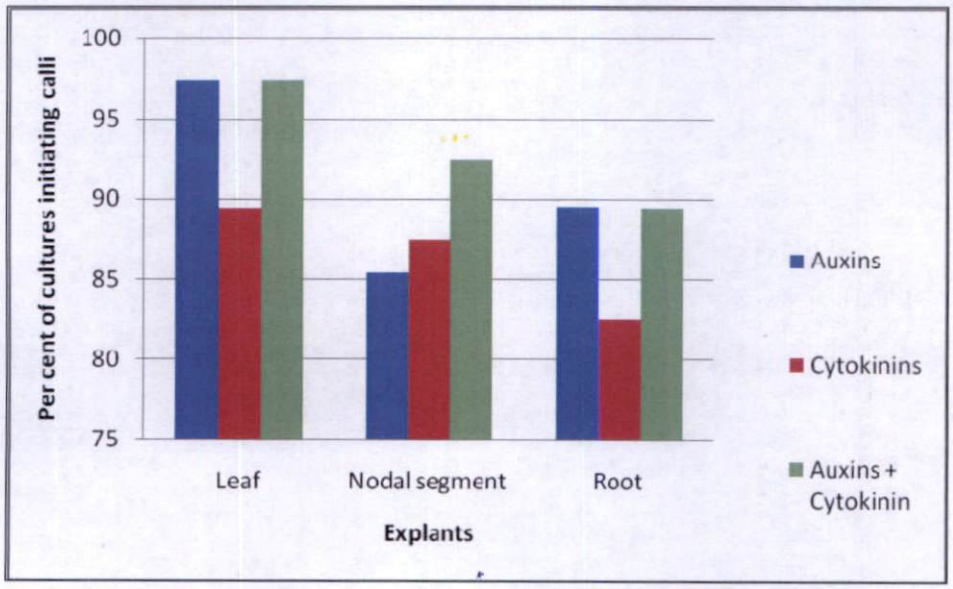


Fig 5. Relative response of *Rubia cordifolia* to growth regulators and their combinations with respect to callus growth scorce.

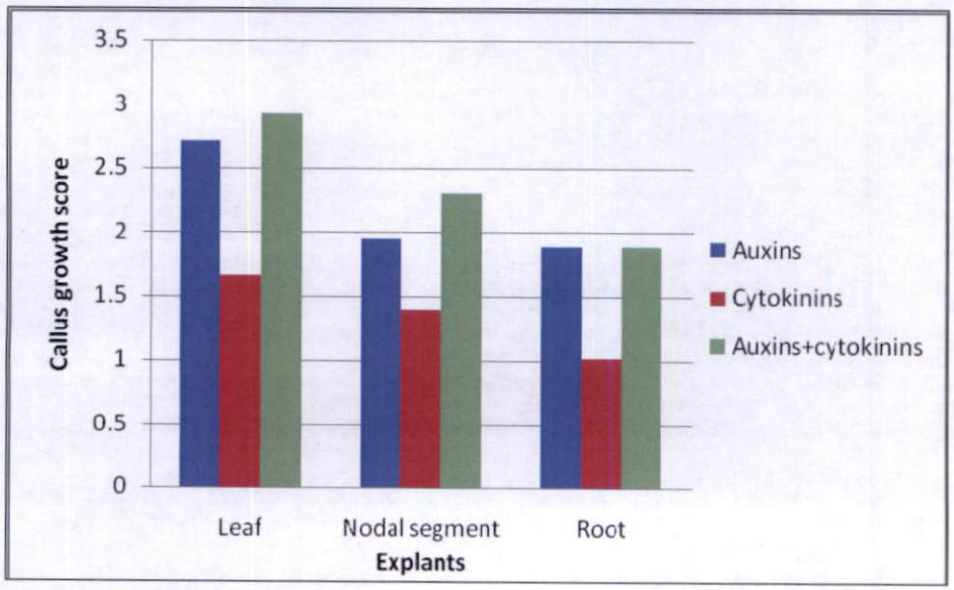
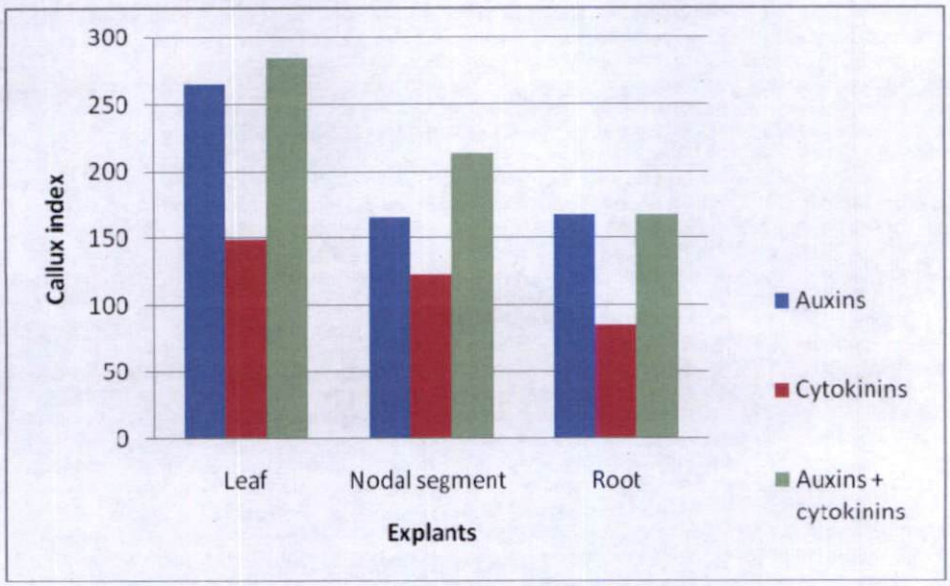


Fig 6. Relative response of *Rubia cordifolia* to growth regulators and their combinations with respect to callus index.



system has been used with advantage for detection of anthraquinones in *Morinda citrifolia* as well by Aobchey *et al.,* (2002). However for shikonin which is also a naphthoquinone, benzene: ethylacetate in the ratio (9.5:0.5) was employed for detection as reported by Singh and Geetanjali (2005). This difference in solvent systems among naphthoquinones may be due to chromatographic properties of naphthoquinones (Sun *et al.*, 1968) and also eluotropic properties of solvent from a given adsorbent (Kirchner,1978).

5.4.1.2 Standardization of spray reagent

In the present investigation, 10 per cent solution of KOH in methanol was employed as the detecting agent from among three detecting agent tried. Wagner and Bladt, (1986) have identified naphthoquinone in *in vitro* system of *Rudgea jasminodes* employing the same detecting agent. In this study, use of ammonia solution (25 per cent) (Meena and Patni, 1985) and reduced methylene blue (Dilley,1964) did not yield detectable and clear spots.

5.4.1.3 Developing the chromatogram

The detection of spots of the target compound, was based on the Rf value of the reference compound (1,4-naphthoquinone) and vitamin K_1 which is a synthetic naphthoquinone, as suggested by Lefevere *et al.*, (1985). Irrespective of the constituent side chains, relative mobility of compounds with same basic chemical structure is found to be the same. Based on this hypothesis 1,4-naphthoquinone and Vitamin K_1 were employed as reference compounds and their Rf values compared with that of the target compound of the present study.

5.5 Regulation of *in vitro* metabolite production

5.5.1. Standardization of production medium

5.5.1.1 Regulation of growth factor combinations

Among the growth factor combinations attempted in the study, the auxin NAA at 2.0 mg l^{-1} in combination with BA at 0.5 mg l^{-1} synthesized the highest amount of naphthoquinones in leaf (2.32 units per gram of calli), nodal (2.64 units per gram of calli) and root (3.72 units per gram of calli) cultures in *Rubia cordifolia*

(Plates 10A). Positive response with respect to secondary product formation was noted in other growth factor and media supplement combinations tried as well. However, incorporation of auxins alone resulted in lesser production of the target compound in *in vitro* cultures (Table 19).

Cytokinins in combination with auxins are known to stimulate production of secondary metabolites in *in vitro* systems. Kalimuthu, (2002) obtained maximum production of berberine in *in vitro* systems of *Tinospora cordifolia*, with NAA and BA, each at 2 mg l^{-1}. A combination of auxins and cytokinins enhanced production of plumbagin in *Plumbago rosea* (Komaraih *et al.*, 2003). Rao *et al.* (2008) observed that NAA along with BA, when incorporated to LS medium, increased berberine accumulation in *in vitro* systems, over a period of 4 weeks.

In the present investigation, incorporation of the growth hormone 2,4-D singly to the basal medium, proved inferior with respect to product formation (Table 19). This result correlates well with the observation by Rhodes *et al.*, (1994) that 2,4-D is less effective for product synthesis. That the quonines and quonine derivatives are better expressed in *in vitro* systems incorporated with auxins like NAA and cytokinins like BA, is evident with respect to anthraquinone production in *in vitro* cultures of *Rubia cordifolia* (Mischenko *et al.*, 1999) and *Morinda citrifolia* (Aobchey, 2002).

In the present study, higher amounts of naphthoquinones were detected in root derived cultures of the target species, in all media combinations tested (Table 19). Naphthoquinones have been detected in maximum amounts in the roots of *in vivo* plant of *Rubia cordifolia* as reported by Koyama *et al.*, (1992), which probably explains the superiority of root derived *in vitro* systems with respect to naphthoquinone production.

5.5.2 Modification of carbon source

Increasing the sucrose concentration to 5 per cent did not exert a beneficial influence on synthesis of naphthoquinones *in vitro* (Table 20). At 5 per cent level, leaf, nodal and root derived calli synthesized 2.11, 2.41 and 3.23 units of

naphthoquinone per gram of calli, which were less than the respective figures in production media with 3 per cent sucrose. In this study, at elevated levels of sucrose suppression of callus growth resulted, which, did not favourably influence synthesis of napthoquinone, which is a growth related product (Plate 10B). However, Ellis *et al.* (1996) could stimulate taxol production in *Taxus* spp. on increasing sucrose concentration to 8 per cent. Similar observations have been put forth by Panichayupakaranant and Tewtrakul (2002), who reported that increase in sucrose concentration, resulted in reduced plumbagin accumulation in *Plumbago rosea* root cultures.

5.5.3 Withdrawal of inorganic nutrients

5.5.3.1 Modification of nitrate concentration

Reducing nitrate concentration to half and one-fourth of the original, resulted in increased naphthoquinone synthesis in leaf, nodal and root derived cultures of the experimental species (Fig.7).

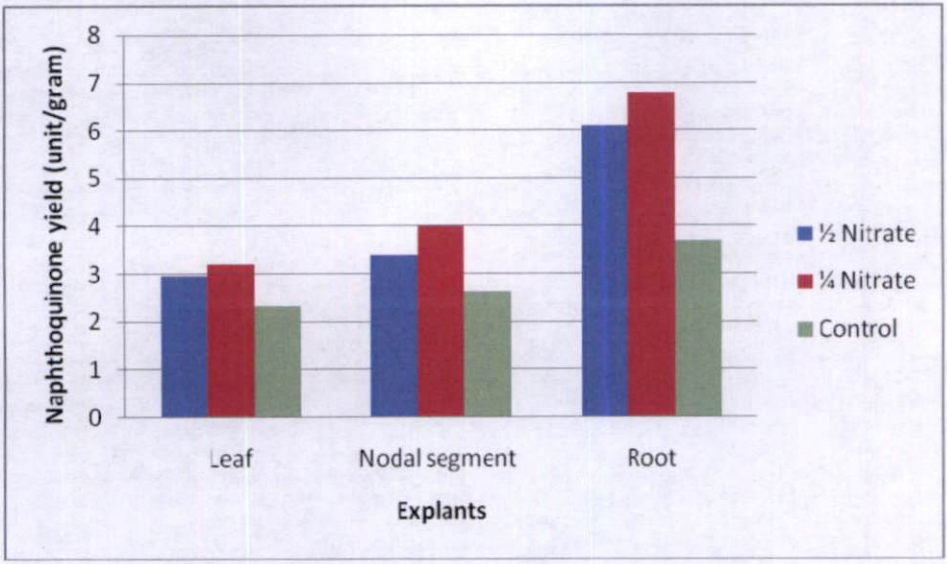
The result again confirms the fact that nitrogen free metabolites like naphthoquinone tried to accumulate at reduced levels at increased in nitrogen concentrations (Plate 10C). This observation was also put forth by Mizukami *et al.*, (1992) regarding synthesis of shikonin, also a naphthoquinone derivative, in *in vitro* cultures of *Lithospermium erythrorhizon*, where reduced nitrogen concentration stimulated shikonin synthesis.

On the contrary, plumbagin production in cultured roots of *Plumbago rosea* increased at enhanced levels of nitrogen (Panichayupakarenant and Twetrakul, 2002).

5.5.3.2 Modification of phosphate concentration

Maintaining phosphate levels below optimum, is often employed as a strategy to confer a growth limiting environment to *in vitro* systems, that stimulate product synthesis. In the present study, withdrawal of phosphates from the production medium to maintain the phosphate concentration of the medium, to half and one-fourth the original concentration brought about a favourable response on

Fig 7. Effect of modifiying nitrate concentration on naphthoquinone yield in *in vitro* cultures of *Rubia cordifolia*.



naphthoquinone synthesis by *in vitro* leaf and nodal cultures of *Rubia cordifolia* (Table 21).as compared to control.

Typical examples of beneficial effect of phosphate withdrawal include increased berberine synthesis in *in vitro* cultures of *Coscinium fenestratum* (Sindhu, 1999). In *Panax notoginseng*, increase in phosphate levels in medium, considerably improved cell growth and saponin accumulation. However, in root derived cultures in the present study, no enhancement in naphthoquionone yield was observed on withdrawing phosphate from the culture medium (Plate 10D), implying that, such nutrient effects are not predictable and that individual optimum conditions have to be determined experimentally. In *Sida* spp. also, Sankar (1998) did not obtain synthesis of ephedrine, on withdrawing phosphate from the culture medium.

5.5.4 Incorporation of media supplements to production medium

5.5.4.1 Yeast extract

Among the media additives supplied to the culture medium, yeast extract of levels of 1 per cent and 2 per cent, provided encouraging results with respect to secondary product formation in callus cultures of *Rubia cordifolia* (Table 22). At 2 per cent levels, yeast extract synthesized 4.23 units per gram of calli, 6.29 units per gram of calli, and 6.78 units per gram of calli respectively in leaf, stem and root callus cultures of the experimental species (Plate 10E). Positive influence of yeast extract in product synthesis in *in vitro* cultures of members of Rubiaceae family has been evident in several studies, as is seen in detection of anthraquinones in cell suspension cultures of *Rudgae jasminoides* (Oliveira, 2007). The effect of yeast extract is due to its role in increasing the PAL (Phenylalanine ammonia lyase) activity which is a key enzyme of phenyl propanoid pathway forming a link between primary metabolism and secondary one, and formation of secondary products with phenyl propanoid skeleton (Sham-Ardakain, 2005). So, the target compound of this study, the naphthoquinone, being a product with phenyl propanoid skeleton, addition of yeast extract, will undoubtedly elicit a positive response in its production as confirmed by this study.

Plate 10 Thin layer chromatograms of ethyl acetate fraction of *in vitro* cultures of *Rubia cordifolia*

Plate 10A. Effect of growth regulators on naphthoquinone synthesis of leaf, nodal segment and root callus extracts of *Rubia cordifolia*

1. NAA 2 mg l^{-1}+ BA 0.5 mg l^{-1} – L, N, R
2. Standard - s

Plate 10B Effect of modifying carbon source on naphthoquinone synthesis of leaf, nodal segment and root callus extract of *Rubia cordifolia*

1. NAA 2 mg l^{-1}+ BA 0.5 mg l^{-1} – L, N, R.
2. NAA 2 mg l^{-1}+ BA 0.5 mg l^{-1} – L, N, R
3. Standard - S

Plate 10C Effect of reducing nitrate concentration on naphthoquinone synthesis of leaf, nodal segment and root callus extract of *Rubia cordifolia*

1. NAA 2 mg l^{-1}+ BA 0.5 mg l^{-1} + ½ NO_3 – L, N, R.
2. NAA 2 mg l^{-1}+ BA 0.5 mg l^{-1} + ¼ NO_3– L, N, R
3. Standard - S

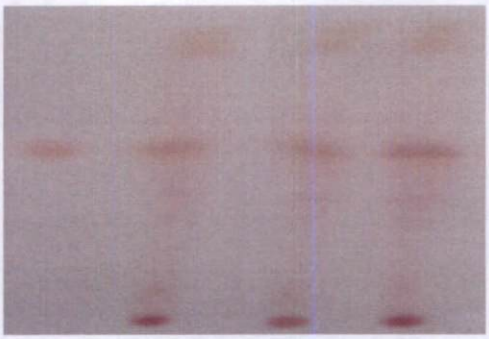
Plate 10D Effect of reducing phosphate concentration on naphthoquinone synthesis of leaf, nodal segment and root callus extract of *Rubia cordifolia*

1. NAA 2 mg l^{-1}+ BA 0.5 mg l^{-1} + ½ po_4 – L, N, R.
2. NAA 2 mg l^{-1} BA 0.5 mg l^{-1} + ¼ PO_4– L, N, R
3. Standard - S

Plate 10E Effect of yeast extract on naphthoquinone synthesis of leaf, nodal segment and root callus extract of *Rubia cordifolia*

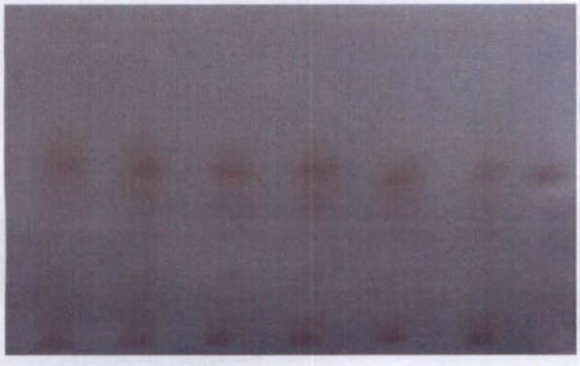
1. NAA 2 mg l^{-1}+ BA 0.5 mg l^{-1} + 1 per cent – L, N, R.
2. NAA 2 mg l^{-1}+ BA 0.5 mg l^{-1} + 2 per cent – L, N, R
3. Standard - S

A



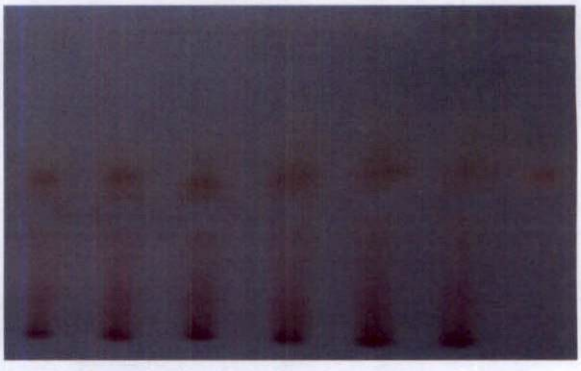
S L N R

B



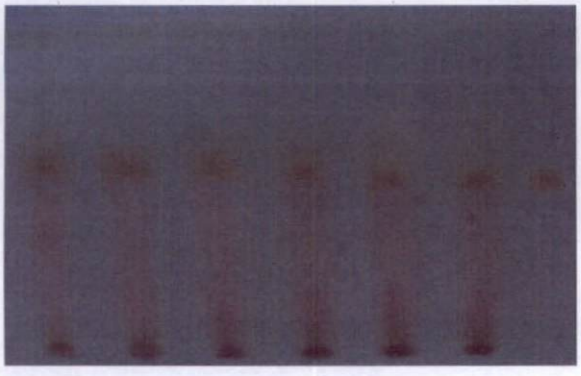
L N R L N R S

C



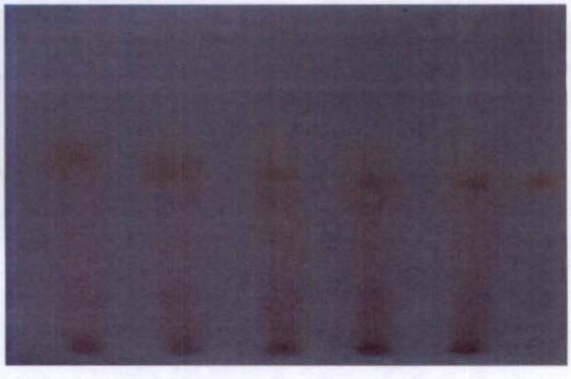
L N R L N R S

D



L N R L N R S

E



L N R L N S

5.5.5 Supply of precursors

The concept of precursor feeding is based on the fact that, any compound, which is an intermediate compound in or at the beginning of a secondary metabolite biosynthetic route, stands a good chance of increasing the yield of the final product (Namdeo *et al.*, 2007). As reported by Arita *et al.* (1999), even without the knowledge of the entire synthetic pathway, the yield of a specific compound can be increased by locating its key precursor.

In the present study, tyrosine the indirect amino acid precursor, of target compound, as reported by Eugon, (1969) at levels of 50, 100 and 150 mg l^{-1} had a desirable influence on the biosynthetic capability of leaf, nodal and root calli of *Rubia cordifolia*, which responded positively, when subjected to appropriate tests employed, to screen naphthoquinones (Table 23 and Plates 10F to 10H). Tyrosine at 150 mg l^{-1} registered maximum yield of naphthoquinone in leaf (3.76 units per gram of calli), nodal (3.74 units per gram of calli) and root (3.94 units per gram of calli) cultures. There findings correlate well with many published reports as detailed herewith. Production of phenylethanoid glycoscides in cell cultures of *Cistanche deserticola* was enhanced by feeding the precursors l-tyrosine and sodium acetate at appropriate concentration (Quyang *et al.*, 2004).

Phenylalanine, the other indirect precursor of naphthoquinone, as suggested by Eugon, (1969) also stimulated naphthoquinone production in *in vitro* cultures of *Rubia cordifolia*, registering maximum yield of 3.19 units, 4.98 units and 5.08 units per gram of calli, respectively in leaf, nodal segment and root cultures at a level of 150 mg l^{-1}. Addition of 1 mM phenyl alanine to the culture medium, significantly increased taxol production in callus lines of *Taxus baccata* (Cusido *et al.*, 1999). At lower levels (50 mg l^{-1} and 100 mg l^{-1}), also the two precursors registered higher quantities of the target compound in *in vitro* cultures as compared to control (Table 23).

There have been instances, where more than one precursor effectively contributed to product synthesis, wherein both phenyl alanine and isocapric acid

stimulated capsaicin synthesis in *Capsicum frutescens* (Lindsey and Yeomann, 1984) and loganin, secolaganin and tryptamine induced ajmalicine production in cell lines of *Catharanthus roseus* (Contin *et al.*, 1998).

Contrary to the above findings, Sankar (1998) reported that between phenyl alamine and methionine, only phenylalanine induced synthesis of ephedrine in callus cultures of *Sida* spp. whereas methionine was not effective.

The results of the present study reveal that root derived callus cultures, registered maximum amount of naphthoquinones on precursor feeding. This observation is in conformity with the results of several other treatments employed in the study, wherein cultures derived from roots, which inherently has more naphthoquinone content, almost always surpassed leaf and nodal segment derived callus cultures with respect to naphthoquinone content.

5.6 Influence of culture condition on naphthoquinone production in *in vitro* cultures of *Rubia cordifolia*

5.6.1 Incubation in dark

Incubating *in vitro* cultures of *Rubia cordifolia* in the dark did not bring about a significant increase in naphthoquinone production though marginal increase is noted in its content in leaf (2.43 unit per gram of calli), nodal (2.78 unit per gram of calli and root 3.89 unit per gram of calli) callus cultures, as compared to control (Plate 10L). Shikonin, also a naphthoquinone, has registered substantial enhanced synthesis on dark incubation since light has an inhibitory influence on its synthesis (Tabata *et al.*, 1974).

In the present study, the target compound, also a naphthoquinone has responded in a similar way though the increase in its content on dark incubation, was not significant. As in the case of shikonin, as suggested by Tabata *et al.* (1974), photodegradation of the target compound of the present study may account for marginal decrease in its content, under illumination. Lee *et al.* (2001) reported that production of gagaminine was significantly higher in *in vitro* cultures of *Cynanchum wilfordii*, in the dark.

Conversely, instances where illumination is considered essential for the expression of certain metabolic pathways have been reported, as in the case of secondary products like flavanoids (Hahlbrock and Greisbach, 1979) and cardenolides (Ohlsson *et al.*, 1983).

5.7 Employing special techniques to upgrade the content of naphthoquinone

5.7.1 Elicitation

5.7.1.1 Biotic eliciation

Synthesis of naphthoquinone in leaf, stem and root derived calli of *Rubia cordifolia* was positively influenced on incorporating autoclaved mycelia of *Pythium aphonidermatum* at levels of 2 and 5 per cent (Table 25). Among the levels tried, the fungal mycelia at 5 per cent elicited maximum napthoquinone synthesis in root callus cultures (8.13 units per gram of calli) of the experimental species (Plate 10I).

Successful report of enhanced secondary product synthesis *in vitro*, through purified biotic elicitors, their culture filtrates or autoclaved mycelia of microbes, dominate in the area of exploiting *in vitro* systems for secondary product synthesis. Kalimuthu (2002) has reported that autoclaved mycelia of *Pythium aphanidematum* at 0.5 g l^{-1} elicited maximum berberine synthesis by leaf callus cultures (19.262 µg) of the Madurai ecotype and stem callus cultures (23.176 µg) of Vellanikkara ecotype respectively.

The elicitor employed in the present study, *Pythium aphanidermatum*, has been used to advantage in several other species and in the genus *Rubia*, as well. Tegelen *et al.* (1999) has observed that elicitation of *Rubia tinctorum* cell cultures with a *Pythium aphanidermatum* elicitor, led to a doubling of anthraquinone content. That, the target compound of the present study napthoquinone, responds positively to elicitation is evident from the observations of Flores *et al.* (1988) who reported that elicitation of *Lithospermum erythrorhizon* cell cultures with mycelia from a two week old culture of *Pythium ultimatum*, produced increased amounts of the naphthoquinone, shikonin. Moreno *et al.* (1996) attributed the success of employing *Pythium* to changes in enzyme activities which channeled intermediates through the

desired metabolic pathway. In the present study also, enhanced enzyme activity responsible for channeling intermediaries through secondary metabolic pathway, might have contributed to the positive effect of the elicitor. Another possible reason for the enhanced effect, is the presence of polygalaturonic acid, as one of the mycelial cell wall components, which hydrolyses the cell walls of *in vitro* cultures, releasing enzymes bound to cell walls, for secondary pathway, as suggested by Rijhwani and Shanks, (1976) in *Catharanthus roseus*. Accumulation of defense related proteins such as chitinases and glucanases during elicitation, also contribute to enhanced product synthesis.

5.7.1.2 Abiotic elicitation

Incorporation of salicylic acid at levels of 10 and 100 µM elicited a positive response on naphoquinone yield from leaf, nodal segment and root callus cultures of *Rubia cordifolia* (Table 26 and Plate 10J). This finding agrees with the observations by Bulgakov *et al.* (2002) who noted the stimulatory effect of salicylic acid on anthroquinone production in *Rubia cordifolia*.

Salicylic acid is considered to be a plant signaling molecule, playing a key role in defense responses in plants, wherein such signal molecules are involved in signal transduction systems, which induce particular enzymes of secondary metabolic pathway (Ding *et al.*, 2002). Salicylic acid, along with methyl jasmonate act as signaling molecules that cause specific changes in gene expression levels that activate PAL (Phenylalanine ammonia lyase) activity, which plays a major role in plant defense (Rao *et al.*, 2000).

Positive role of salicylic acid on naphthoquinone production, as witnessed in the present study, suggests the involvement of salicylic acid mediated pathway in the biosynthesis of napthoquinone. Also, as suggested by Menke *et al.* (1999), abiotic elicitors like salicylic acid and methyl jasmonate mediate signal transduction between an elicitor receptor complex via transcriptional activation of defense genes.

5.7.2 Immobilization

A five per cent solution of sodium alginate, in complexing with 100 mM calcium chloride solution, yielded round firm beads ranging from 1.0 to 1.3 cm in diameter (Plate 5).

Shailajraj (1998), in her studies on *in vitro* synthesis of secondary products from *Sida* spp., also obtained beads of good quality, on employing identical concentrations of sodium alginate and calcium chloride. The immobilized cell cultures of *Rubia cordifolia* synthesized naphthoquiones, but at levels, lower than that produced in free cells (Fig. 8). Root callus cultures in immobilized state synthesized 2.82 units of naphthoquinone compared to 3.72 units in root derived calli in free state.

Instances of immobilized cell systems producing lesser quantities of secondary products as compared to free cell systems, are many. An observation wherein free cells of *Catharanthus roseus* accumulated three times in tryptamines as compared to immobilized systems, is in agreement with the result of the present study (Facchini and Dicosmo, 1991). Another relevant observation pertaining to this investigation is the absence of ephedrine synthesis in immobilized cell cultures of *Sida* spp. (Sankar, 1998).

The consistent observation in the present study that naphthoquinone is a growth related product, justifies the absence of its biosynthesis under conditions of non-growth, imposed as a result of immobilization.

Reports contrary to the above are available as in the case of plumbagin production *in vitro*, wherein immobilization of cell cultures of *Plumbago rosea*, resulted in an enhanced production of plumbagin by three fold as compared to free cell systems (Komaraiah *et al.*, 2003). Instances of inhibition of cell growth resulting in greater accumulation of secondary product are also reported, as in taxol accumulation using immobilized cells of *Taxus cuspidata*, at reduced cell growth rates (Seki *et al.*, 2000).

Fig .8. *In vitro* synthesis of naphthoquinone in immobilized cultures and free cell systems of *Rubia cordifolia.*

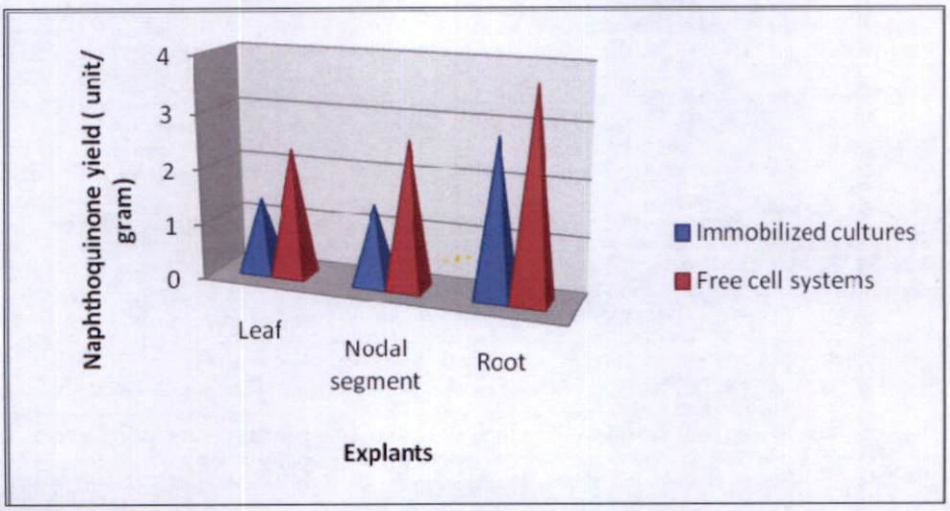
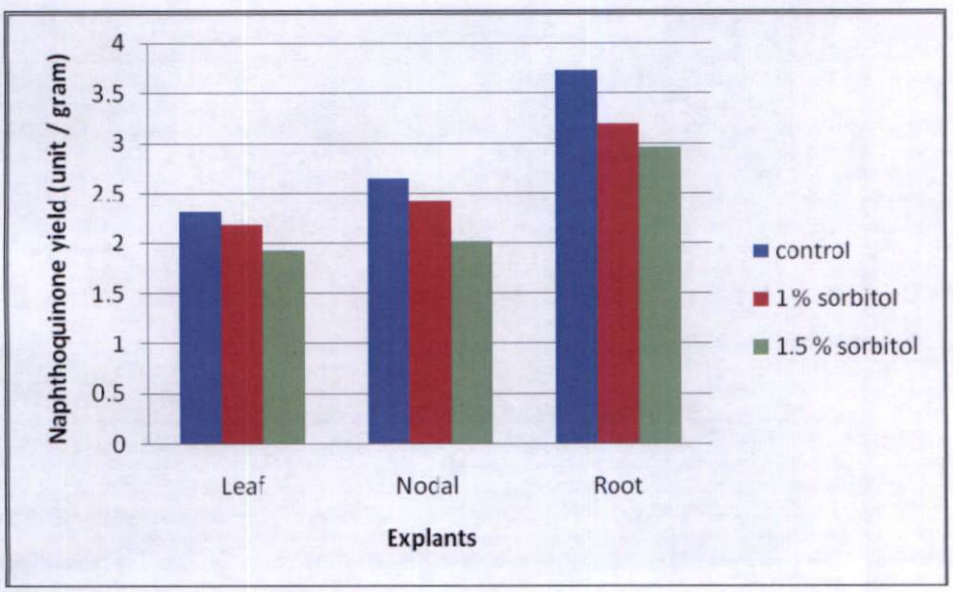


Fig 9. Effect of stress inducing compound on naphthoquinone yield in *in vitro* cultures of *Rubia cordifolia*



5.7.3 Creating conditions of stress.

Adapting cultures of the experimental species to stress conditions by incorporating sorbitol at varying levels did not bring about an increased production of the target compound as compared to control (Fig. 9 and Plate 10K).

This observation is in conformity, with the result obtained in *Nicotiana* by Gangopadhyay *et al.*, (1997), wherein *in vitro* systems failed to respond markedly to stress conditions imposed by sorbitol, in terms of nicotine production. The growth related synthesis of naphthoquinone and nicotine positively render them non-responsive to conditions of stress imposed by sorbitol.

As against the above observations, Zhang *et al.*, (1995) and Saker *et al.*, (1997) obtained increased production of saponins and hyoscyamine, respectively, in cell cultures of *Panax motoginseng* and *Datura stramonium*. Kalimuthu, (2002) also obtained enhanced levels of berberine production in *in vitro* cultures of *Tinospora cordifolia*, on incorporation of mannitol to the culture medium.

5.7.4 Establishment of hairy root cultures

5.7.4.1 Culturing of *Agrobacterium rhizogenes* strains

In the present investigation, Yeast Extract Broth (YEB) media was employed for culturing the various strains of *Agrobacterium rhizogenes*, employed in the study. YEB medium has been used with favourable result for culturing strains of *Agrobacterium rhizogenes* as reported by Varghese, (2006). However, differential response of various strains to different culture media has also been witnessed as in the case of *Agrobacterium rhizogenes* by Shaneeja, (2007).

5.7.4.1 Screening of *Agrobacterium rhizogenes* strains for sentivity to antibiotics

The *Agrobacterium rhizogenes* strains MTCC 2363 and MTCC 532, employed in the study for induction of hairy roots were susceptible to the antibiotic cefatoxime, at a concentration of 500 mg l^{-1}. Several reports suggest the use of the antibiotic, cefatoxime to kill *Agrobacterium rhizogenes* strains. The same antibiotic, at 500 mg l^{-1} was employed for eliminating various strains of *Agrobacterium rhizogenes* by Koile *et al.*, (2003), Zdravkovve-Korac *et al.*, (2004) and Varghese,

Plate 10F Effect of precursor feeding on naphthoquinone synthesis of leaf, nodal segment and root callus extract of *Rubia cordifolia*

1. NAA 2 mg l^{-1} + BA 0.5 mg l^{-1} + Phenyl alanine 50 mg l^{-1} – L, N, R.
2. NAA 2 mg l^{-1} + BA 0.5 mg l^{-1} + Tyrosine 50 mg l^{-1} – L, N, R
3. Standard - S

Plate 10G Influence of precursor feeding on naphthoquinone synthesis of leaf, nodal segment and root callus extract of *Rubia cordifolia*

1. NAA 2 mg l^{-1} + BA 0.5 mg l^{-1} + Phenyl alanine 100 mg l^{-1} – L, N, R.
2. NAA 2 mg l^{-1} + BA 0.5 mg l^{-1} + Tyrosine 100 mg l^{-1} – L, N, R
3. Standard - S

Plate 10H Influence of precursor feeding on naphthoquinone synthesis of leaf, nodal segment and root callus extract of *Rubia cordifolia*

1. NAA 2 mg l^{-1} + 0 BA 0.5 mg l^{-1} + Phenyl alanine 150 mg l^{-1} – L, N, R.
2. NAA 2 mg l^{-1} + BA 0.5 mg l^{-1} + Tyrosine 150 mg l^{-1} – L, N, R
3. Standard - S

Plate 10I Effect of fungal elicitation on naphthoquinone synthesis of leaf, nodal segment and root callus extract of *Rubia cordifolia*

1. NAA 2 mg l^{-1} + BA 0.5 mg l^{-1} + 2 pe r cent – L, N, R.
2. NAA 2 mg l^{-1} + BA 0.5 mg l^{-1} + 5 per cent – L, N, R
3. Standard - S

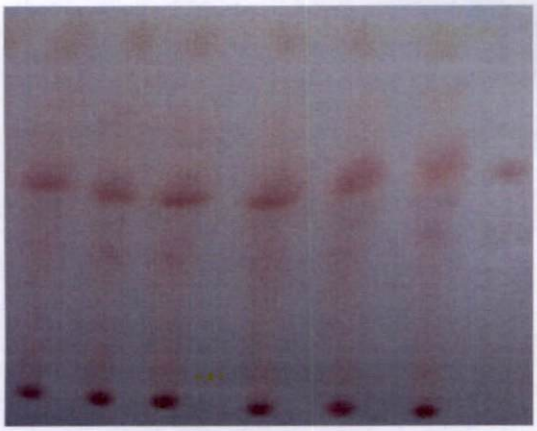
Plate 10J Effect of salicylic acid on naphthoquinone synthesis of leaf, nodal segment and root callus extract of *Rubia cordifolia*

1. NAA 2 mg l^{-1} + BA 0.5 mg l^{-1} + 10 µM – L, N, R.
2. NAA 2 mg l^{-1} + BA 0.5 mg l^{-1} + 100 µM – L, N, R
3. Standard - S

Plate 10K Effect of sorbitol on naphthoquinone synthesis of leaf, nodal segment and root callus extract of *Rubia cordifolia*

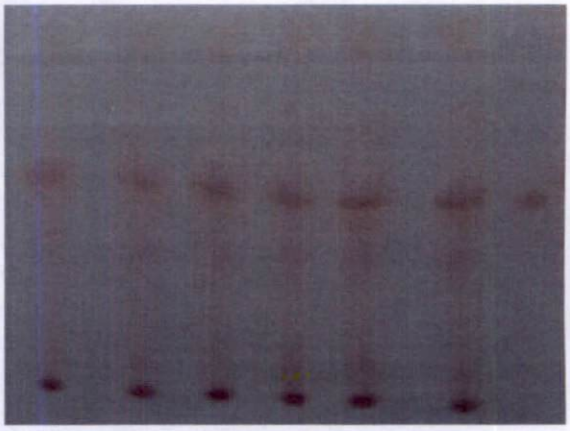
1. NAA 2 mg l^{-1} + BA 0.5 mg l^{-1} + 1 per cent – L, N, R.
2. NAA 2 mg l^{-1} + 0.5 mg l^{-1} + 1.5 per cent – L, N, R
3. Standard - S

F



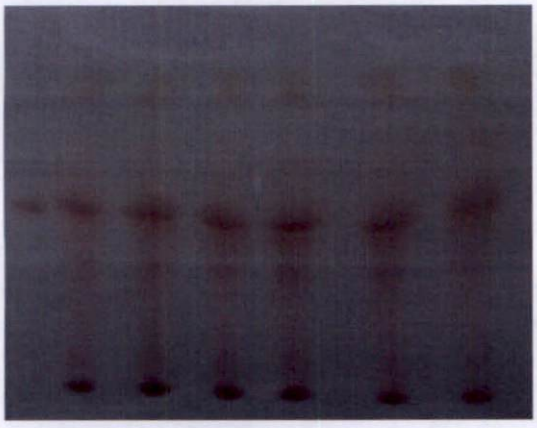
L N R L N R S

G



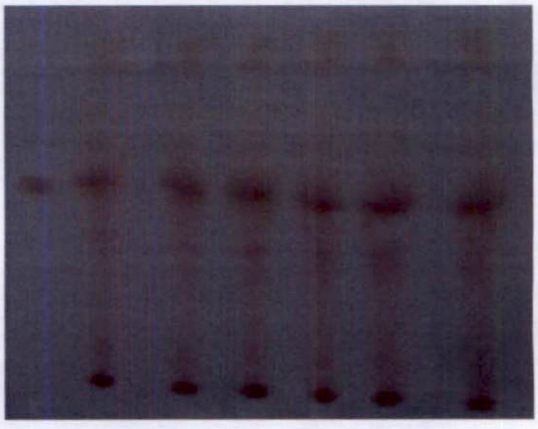
L N R L N R S

H



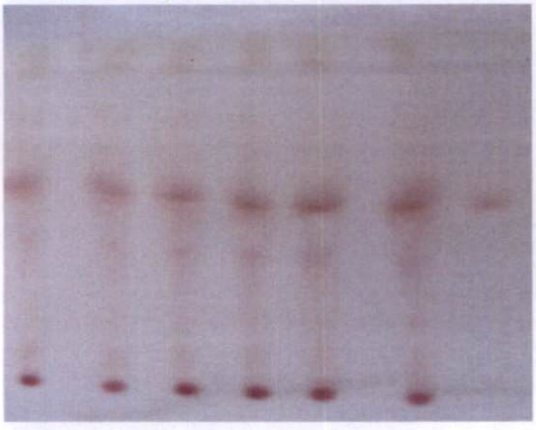
S L N R L N R

I



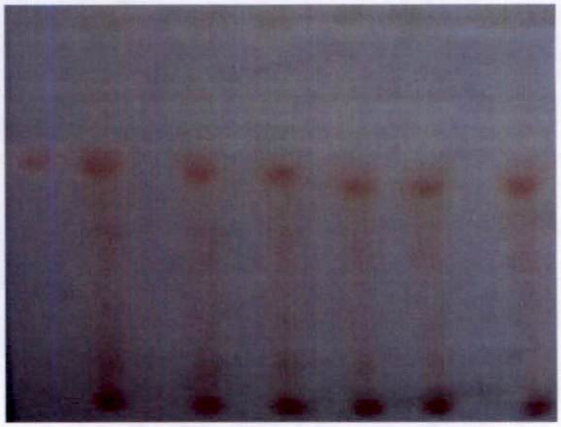
S L N R L N R

J



L N R L N R S

K



S L N R L N R

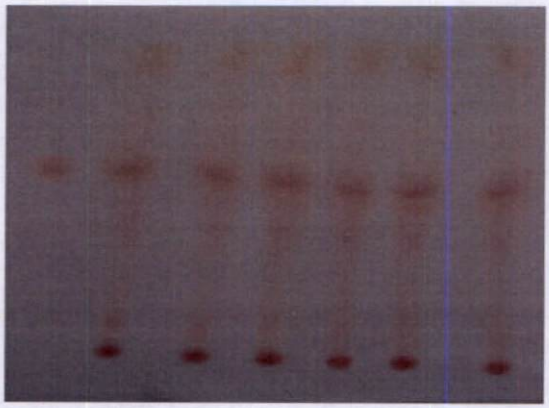
Plate 10L Influence of culture conditions on naphthoquinone synthesis of leaf, nodal segment and root callus extract of *Rubia cordifolia*

1. NAA 2 mg l^{-1}+ BA 0.5 mg l^{-1}+ Dark – L, N, R.
2. NAA 2 mg l^{-1}+ BA 0.5 mg l^{-1}+ Low temperature – L, N, R
3. Standard - S

Plate 10M Influence of age of calli on naphthoquinone synthesis of leaf, nodal segment and root callus extract of *Rubia cordifolia*

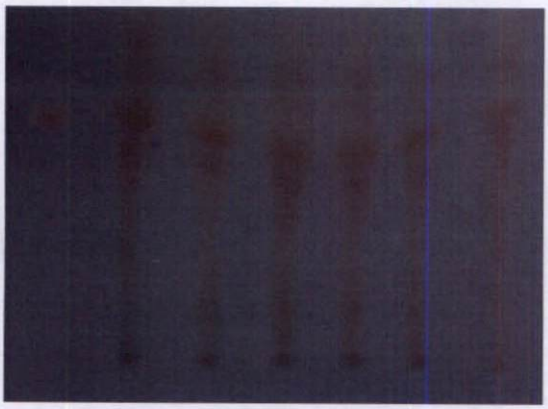
1. NAA 2 mg l^{-1}+ BA 0.5 mg l^{-1}+ 8 week – L, N, R.
2. NAA 2 mg l^{-1}+ BA 0.5 mg l^{-1}+ 12 week – L, N, R
3. Standard - S

L



S L N R L N R

M



S L N R L N R

L - Leaf callus
N – Nodal segment callus
R – Root callus
S – 1,4- naphthoquinone (reference standard)

(2006). Though the strain MTCC 532 was susceptible to cefatoxime at 500 mg l^{-1}, at concentrations ranging from 50 to 400 mg l-1, restricted growth occurred. This observation confirms the fact that growth inhibition of *Agrobacterium rhizogenes* strains by antibiotic was dose dependent, an observation also made by Eracn *et al.*, (1999). The strain MTCC 2364, also responded to the antibiotics employed in the study, is a dose dependent manner.

In present study, the strains MTCC 2364 and MTCC 532, were resistant to ampicillin, at all concentrations. This observation was also put forth by Varghese, (2006), where ampicillin, at all concentrations inhibited the growth of the bacterial strains. The antibiotic streptomycin failed to achieve cent per cent inhibition of the *Agrobacterium rhizogenes* strains MTCC 532, which exhibited restricted, growth at concentrations of 400 mg l^{-1} and 500 mg l^{-1} as against the strain MTCC 2364, which was susceptible to the antibiotic at the above concentration. Differential response of bacterial strains to various concentration of antibiotic has been reported by Shaneeja, (2007), as well, during her investigation on genetic transformation in *Artemisia annua.*

5.7.4.2 Evaluation of explants of *Rubia cordifolia* for sensitivity to antiobiotics.

In the present study, leaf and nodal segments of the experimental species were employed as explants for induction of hairy roots, which remained healthy in both the antibiotics, cefaoxime and ampicillin, at concentration of 500 mg l^{-1}, each. At higher concentration of the antibiotic, leaf explants turned pale. Susceptibility of plant species to *Agrobacterium rhizogenes* strains vary with type of explants (Zu *et al.*, 2003).

5.7.4.3 Induction of hairy roots

The explants employed in the present study failed to induce hairy roots on incubating in MS basal medium, following co-culture with the bacterial strains for 1-3 days, after being precultured in MS basal medium and subjected to wounding and dipping in bacterial suspension for 10 to 25 minutes. The level of tissue

differentiation also determine the ability to give rise to transformed roots after *Agrobacterium rhizogenes* inoculation as suggested by Trypsteen *et al.* (1991).

5.8 Influence of age of *in vitro* derived calli on synthesis of naphthoquinone

In the present investigation, eight week old calli recorded great amount of naphthoquinone in leaf, nodal segment and root derived cultures (Table 31). The productivity diminished as age of calli advanced, wherein 12 week old calli registered lesser amounts of naphthoquinone in *in vitro* cultures, as compared to eight week old calli (Table31). The growth related synthesis of the target compound account for lesser amount of naphthoquinone in 12 week old calli, at which time callus growth and proliferation had diminished (Plate 10M). Sankar (1998) observed ephedrine synthesis in 4-5 week old calli of *Sida* spp. which diminished thereafter.

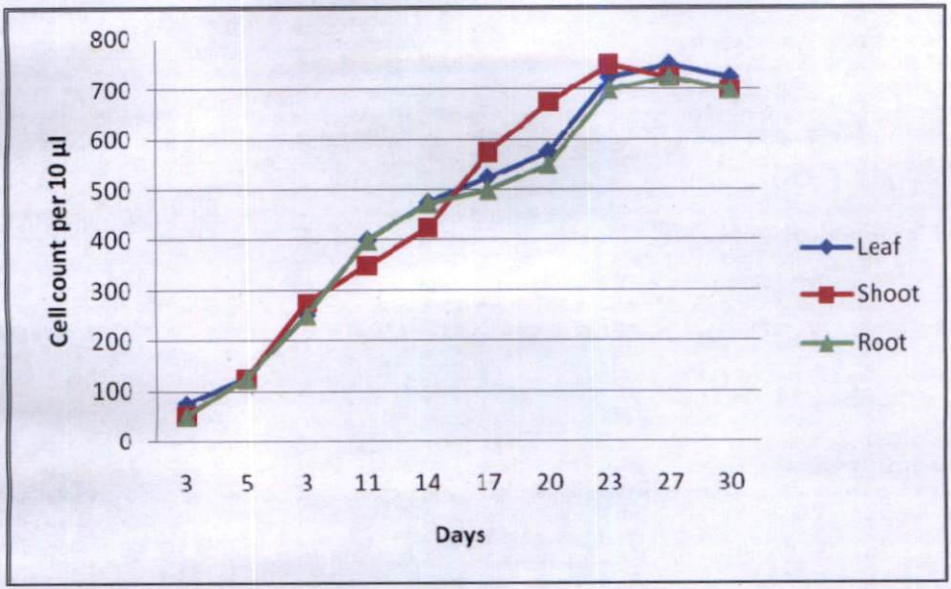
5.9 Establishment of suspension cultures

5.9.1 Standardization of subculturing intervals

In the present investigation, cell growth of target species in liquid MS + NAA (2mg l^{-1}) + BA (0.5 mg l^{-1}) as indicated by serial cell counts, reached the exponential phase at 27 days after initiation of suspensions for leaf calli, 24 days after initiation of suspension for nodal calli and 27 days after initiation of suspension of root calli, declining steadily thereafter, fixing regular subculture interval at 27, 24 and 27 days for leaf, nodal and root derived suspension respectively (Fig.10). The pattern of cell growth in shake culture confirm to the report of Gamborg and Shyluk, (1981) that cells undergo lag, exponential, deceleration and stationary phases and subculturing is done at the end of exponential phase, since cell growth declines thereafter.

However Mizutani, (1997) had obtained good cell growth in *Rubia akane* in MS medium supplemented with NAA and Kinetin at 14 days of initiation of suspensions. Genotypic differences in efficiency of establishment of suspensions cultures has been obsereved, wherein serial subculture intervals of cell suspension of various species of the same genus, differed as reported by Sankar, (1998) in *Sida spp.* Kittipongpatana *et al.* (1998) observed that cell cultures of three Solanaceas crops,

Fig 10. Relative cell proliferation in leaf, nodal and root derived suspensions in *Rubia cordifolia.*



Capsicum annum, *Solanum aculeatissimum* and *Datura fastuosa* exihibited maximum cell growth 24 days after initiation of suspensions.

5.9.2 Determination of packed cell volume

Marked difference in packed cell volume of various explants, was not witnessed in this investigation as against the observation by Kalimuthu, (2002) in his study on berberine production in *Tinospora cordifolia*, wherein leaf and stem explants exhibited marked variation in packed cell volume. Absence of significant variation in callus texture in the experimental species under the influence of various growth regulators, probably explains this outcome.

5.9.3 Detection and quantification of naphthoquinone in suspension cultures

Naphthoquinone synthesis in shake cultures of the experimental species was not enhanced significantly as compared to static cultures, though higher values were registered for leaf, stem and root derived suspensions than their respective static cultures (Table 33). Hook (2001), also could not detect significant increase in naphthoquinone synthesis in cell derived suspension of *Drosera rotundifolia*, as compared to *in vitro* grown plants. Mizutani *et al.* (1997) however, obtained higher amount of anthraquinone in root and stem derived suspension of *Rubia akane* giving indication that secondary compounds differ in their response to product accumulation in shake cultures based on pattern of cell growth to product synthesis.

5.10 Quantification of naphthoquinone in *ex vitro* plants of *Rubia cordifolia*

In the present study, ex vitro plants and root samples of *Rubia cordifolia* positively responded to the presence of naphthoquinone, at all stages of maturity tested (Table 34) though root samples recorded higher content of the target compound. Presence of naphthoquinone in roots of *Rubia cordifolia* has been reported by Koyama *et al.*, (1992). Variation in the content of secondary metabolites, based on maturity of *ex vitro* plants, has been reported in many medicinal species. In *Sida* spp. Sankar, (1998) obtained higher ephedrine content in *in vivo* root samples, with increase in maturity, an observation identical to the one, in the present investigation. At all stages of maturity, *ex vitro* samples recorded lower amount of

naphthoquinones as compared to *in vitro* root callus extract in production medium (3.72 unit per gram of calli). Chemical compounds with quonine moitety are known to be expressed in higher quantities in *in vitro* systems (Suzuki *et al.*, 1982). A classic example of a naphthoquinone, being expressed in tremendous quantities in *in vitro* systems, is shikonin, wherein *in vitro* cultures of *Lithospermum erythrorhizon* produced 15-20 per cent shikonin, in 23 days (Fujita, 1988). In fact, this was the first successful instance of a secondary product being produced *in vitro*, on a commercial scale, which was a remarkable breakthrough. Also other quinones like anthraquinones are also reported to be synthesized in higher quantities in *in vitro* systems, as in the case of *Morinda citrifolia* (Bajaj, 1995). Results of the present investigation support the above observations, implying that perusal of *in vitro* systems for synthesizing and upgrading the content of the root targeted compound naphthoquinone in *Rubia cordifolia* is likely to give good leads in future, considering the endangered status of the plant.

5.11 Evaluation of anticancerous activity of *Rubia cordifolia*

5.11.1 Assessment of cytotoxicity

Many naturally ocuuring quinones like naphthoquinones and related compounds are known to possess cytotoxicity activities (Dholwani *et al.*, 2008). In our study, on evaluation of the *in vitro* cytotoxicity of *ex vitro* samples and *in vitro* callus extracts, all test samples expressed cytotoxicity as expressed by percentage of cell death.

Cytotoxicity effects of naphthoquinone moieties have been reported in many plant species, wherein, nearly 300 naphthoquinones of different structural types have been proved valuable as anticancer agents (Kitagwa *et al.*, 2004). The workers, in their study have confirmed the *in vitro* cytotoxicity effect of 1,4-naphthoquinone from *Paepalanthus latipes* on McCoy cells using MTT-tetrazolium assay. Plant metabolites with naphthoquinone structure are known to induce mammalian topoisomerase II mediated DNA cleavage *in vitro* and this mechanism is through

formation of a cleavable complex, as is seen with other antitumour agents like demythlepipodo phyllotoxin (Fujii *et al.*, 1992).

Though cytotoxicity was expressed by all the test samples, *ex vitro* roots and *in vitro* root callus extract, exhibited maximum cytotoxicty at all levels tried (Table 35 and 36). Whole plant extracts and *in vitro* leaf and shoot derived callus extracts registered lower values for percentage of cell death of DLA and EAC cell lines. This observation is in conformity with the amount of naphthoquinones in the test samples, wherein root extracts (*in vivo* and *in vitro*) revealed enhanced production of the anticancerous principle, naphthoquinone (2.73 units per gram and 3.72 units per gram respectively).

In the present study, percentage of cell inhibition of test extracts increased with a corresponding increase in the concentration of the extract (Plate 11). Increasing concentration of abrin resulted in increased levels of cell death or apoptosis in DLA cell lines as reported by Ramnath *et al.,* (2003). Percentage of cell inhibition exhibited by the root derived test lines on EAC cell line revealed a varying trend, wherein, *in vitro* root derived callus extract exhibited higher percentage of cell death as compared to *in vivo* root extract (Fig 11). Varying response of test compounds to different cancer cell lines, in terms of percentage of cell death, has been witnessed by Chung *et al.* (2004), wherein the solid cancer cell line SNU1 proved to be more resistant to naphthoquinone derivatives as compared to the lymphocytic leukemia cell line LI 210 and the lymphoid neoplasma cell line P 388.

5.11.2 Assessment of antioxidant activity

Antioxidants counteract the cellular byproduct like free radicals by interrupting an oxidation chain reaction to minimize the damage caused by free radicals (Pandey *et al.*, 2005). In the present study, maximum inhibition of superoxide activity was noted for *ex vitro* whole plant samples. As compared to ex vitro samples, *in vitro* callus extracts exhibited comparatively less inhibition of superoxide radicals (Table 38a). The IC_{50} value of all *in vitro* samples, for inhibiting superoxide radicals was higher (Tables 39), indicating that *in vitro* cultures of the

experimental species are not promising antioxidant systems. Also, in the event of the *in vivo* plant sample expressing higher superoxide, hydroxyl, nitric oxide and peroxide inhibition, it could be inferred that, in the *in vitro* systems, probably compounds expressing pro-oxidant activity which counteracts the antioxidant activity of the experimental species, may have been generated. The above reference is based on the report by Rao *et al.,* (2008) who suggested that *in vitro* cultures are capable of synthesizing new compounds.

Other tests employed to assess the antioxidant activity of the test samples, like inhibition of hydroxyl radicals, nitric oxide radicals and lipid peroxidation, also reveal that, compared to *ex vitro* plant samples, less inhibition rates were recorded by *in vitro* samples, which can be substantiated by the assumption of generation of novel pro oxidant compounds, in *in vitro* systems (Rao *et al.*, 2008).

5.11.3 Assessment of pro oxidant activity of *ex vitro* and *in vitro* test samples of *Rubia cordifolia*

The extent of prooxidant activity as measured by the intensity of yellow fluorescence, reveal that, fluorescence of greater intensity was recorded by *in vitro* root callus extract. This observation further substantiates that *in vitro* systems of *Rubia cordifolia* possess least antioxidant activity as is revealed in Plate 12.

Also naphthoquinones form a class of antineoplastic agents that induce cell death in cancer cells by apoptosis, by over generating free radicals like reactive oxygen species by undergoing redox cycling (Inbaraj and Chignell, 2004). Hence test samples employed in the present study which contain more amount of naphthoquinones, like *in vitro* root callus extract, exhibited more pro oxidant activity as well as least inhibition of free radicals.

But being less promising, antioxidant agents, in no way hinder the *in vitro* systems of the experimental species from being potential anticancerous systems, since naphthoquinones, synthesized in appreciable quantities by *in vitro* systems, being alkalyting agents induce cell death in cancer cells by apoptosis (Lopaczynski and Zeisel, 2001).

SUMMARY

SUMMARY

The present investigation entitled "Exploitation of *in vitro* cultures of *Rubia cordifolia* L. for anticancerous compounds" was undertaken at the Centre for Plant Biotechnology and Molecular Biology, College of Horticulture, Vellanikkara, Thrissur, during period from 2006 to 2008. The study was aimed to standardize the *in vitro* techniques for initiation and proliferation of static and suspension cultures of *Rubia cordifolia*, to screen *in vitro* cultures for synthesis of naphthoquinone and quantify it and to enhance the product synthesis in *in vitro* cultures. The results of the investigation are summarized below.

Leaf, nodal and root segments of mature plants of the experimental species, were used as explants. Pretreatment of the explants with 2.5 per cent Bavistin followed by surface sterilization with mercuric chloride at 0.1 per cent for 1 minute and 30 seconds was most effective in reducing microbial contamination in nodal and leaf explants of *Rubia cordifolia* respectively registering cent of survival. MS medium enriched with the auxin NAA at 2.0 mg l^{-1} recorded highest percentage of cultures initiating callus, registering callusing in 97.5 per cent leaf derived cultures.

Mean callus index values of 199.76 was obtained on incubating leaf, nodal and root explants on MS medium incorporated with NAA at 2.0 mg l^{-1} which was on par with the values obtained for other auxins, employed in the study. The auxin IAA, singly or in combination with cytokinins, was ineffective in improving callusing in *Rubia cordifolia*. None of the cytokinins employed in the study effectively induced callusing in *Rubia cordifolia*.

On supplementing cytokinins to auxin enriched media, most favourable and consistent response was obtained with BA, which, at a concentration of 0.5 mg l^{-1}, when supplemented to media containing NAA 2.0 mg l^{-1}, initiated calli in 93.15 per cent cultures, consequent to which, it was designated as the growth medium for callus proliferation in *Rubia cordifolia*.

Root explants of the experimental species were inferior to leaf and nodal explants, in initiating and proliferating calli. Leaf explants exhibited callusing in maximum cultures (91.53) with the highest mean callus growth score (1.84) and mean callus index (170.22) in MS medium supplemented with NAA (2.0 mg l^{-1}) and BA (0.5 mg l^{-1}). Incubating leaf, nodal and root cultures under illumination at 26 ± 1°C was significantly superior to incubation in dark.

Among the various solvent systems tried, chloroform : methanol at a ratio of 8.5 :1.5 was indentified as the best solvent system for detection of naphthoquinone in *in vitro* cultures of *Rubia cordifolia*, employing thin layer chromatography. Alcoholic KOH at 10 per cent was designated as the localizing agent for chromatographic tests, for detecting naphthoquinone in *in vitro* cultures of *Rubia cordifolia*. Presence of naphthoquinone in test sample was indicated by the development of red spots of Rf value 0.47, in conformity with the reference sample.

Four week old leaf, nodal and root calli of *Rubia cordifolia* subcultured on MS medium supplemented with NAA (2 mg l^{-1}) and BA (0.5 mg l^{-1}) produced maximum amount of naphthoquinone and was therefore designated as the production medium.

Increasing levels of sucrose in the medium to 5.0 per cent did not attribute a favourable influence on expression of naphthoquinone in the test calli. Reducing concentration of nitrate in the medium exerted a beneficial effect on napthoquinone production in *in vitro* cultures of *Rubia cordifolia*. Withdrawing phosphate from the production medium was not effective in increasing naphthoquinone yield in root callus cultures.

Modified culture environment like culture in dark or at low temperature, at 11 – 12 °C, did not exert a favourable influence on synthesis of naphthoquinone in *in vitro* cultures of *Rubia cordifolia*.

Favourable influence of the precursors, phenyl alanine and tyrosine on the expression of naphthoquinones, in the leaf, nodal and root calli of *Rubia cordifolia*, was evident, at levels of 50,100 and 150 mg l^{-1}. Incorporation of yeast extract at 2.0 per cent to the production medium elicited positive response in *in vitro* cultures of *Rubia cordifolia*, with respect to naphthoquinone synthesis, producing 4.23 units, 6.29 units and 6.78 units in leaf, nodal and root cultures respectively.

The stress inducing agent, sorbitol when supplemented to the production medium did not trigger synthesis of the target compound, registering values of 2.19 units, 2.43 units and 3.19 units in leaf, nodal and root cultures respectively, at 1.0 per cent level.

Incorporation of autoclaved mycelia of *Pythium aphanidermatum* at levels of 2.0 per cent and 5.0 per cent, resulted in substantial enhancement of naphthoquinone synthesis by leaf, nodal and root callus cultures of the experimental species.

Eliciting the in *in vitro* cultures of *Rubia cordifolia* with the abiotic elicitor salicylic acid at 100 µM brought about maximum synthesis of naphthoquinone, registering a yield of 8.76 units in root derived cultures.

Visual assessment of immobilized beads of the experimental calli revealed the suitability of employing 5.0 per cent sodium alginate and 100 mM calcium chloride solution for entrapping the experimental calli. On immobilization, no positive response was obtained with respect to naphthoquinone production in *in vitro* cultures of *Rubia cordifolia*.

Eight week old calli incubated on production medium recorded greater amount of naphthoquinone in leaf (2.93 units gram $^{-1}$ of calli), nodal segments (3.29 units gram $^{-1}$ of calli) and root (4,53 units gram $^{-1}$ of calli) derived callus cultures. Beyond eight weeks, synthesis of the target compound, diminished.

Cefatoxime at 500 mg l^{-1} resulted in maximum inhibition of the *Agrobacterium rhizogenes* strains MTCC 2364 and MTCC 532. Surival of the explants, leaf and nodal segments employed for induction of hairy roots, was maximum in the antibiotic cefatoxime at 500 mg l^{-1}. None of the explants employed in the study, induced hairy roots, when cultured in MS medium, in light as well as dark, after a co-culture period of 1-3 days, with *Agrobacterium rhizogenes* strains MTCC 2364 and MTCC 532.

Suspension cultures initiated with leaf calli registered the highest packed volume of 0.93 per cent. Suspension cultures registered marginal increase in naphthoquinone content as compared to static cultures.

Naphthoquinones were detected in whole plant extract and *in vivo* root extract at all levels of maturity tested. Both whole plant and root extract exhibited increasing trends in levels of naphthoquinone, with increase in age.

Clear evidence of cytotoxicity was expressed by *in vitro* extracts as well as whole plant and *in vivo* root extracts of *Rubia cordifolia.* Extent of cytotoxicity of *ex vitro* and *in vitro* samples of *Rubia cordifolia* was dose dependent. On DLA and EAC cell lines, employed in the study, *ex vitro* root extracts exhibited maximum cytotoxicity indicated by percentage of cell death (56 per cent each) at 1000 µg followed by *in vitro* root derived callus extract (52 per cent cell death on DLA cell line and 53 per cent cell death on EAC cell line) at a concentration of 1000 µg.

Least IC_{50} values in terms of per cent of cell death were expressed by *ex vitro* root extract and *in vitro* root callus extract, on both the DLA and EAC cell lines, indicative of their enhanced cytotoxicity activity.

Estimation of *in vitro* antioxidant activity of *in vitro* and *ex vitro* test samples of the experimental species revealed that whole plant extract exhibited highest

percentage of inhibition of superoxide radical , nitric oxide radical and lipid peroxides.

Root callus extracts exhibited least antioxidant activity as revealed by its least inhibition of superoxide, hydroxyl, nitric oxide radicals and lipid peroxidases, with maximum IC_{50} values for attaining inhibition. Extent of pro oxidant activity was observed to be high in *in vitro* root extract of the experimental species.

REFERENCE

REFERENCE

Agarwal, S., Kumar, A., Gupta, M. M., Verma, R. K., Singh, D. V. and Kumar, S. 2001. Production of bilobalide in cultures of clone GBC-1 of *Ginkgo biloba*. *J. Med. Arom. Plant Sci.* 22(4A) & 23(1A): 194-196

Akram, M. and Yurekhi, A. K. 1995. Effect of different nutrient media and explants source on callus induction of *Catharanthus roseus* L.G. Don plants. *Turkish J. Bot.* 19(6): 569-572

Ali, S. S., Kasoju, N., Luthra, A., Singh, A., Sharanabasava, H., Sahu, A. and Bora, U. 2007. Indian medicinal herbs as sources of antioxidants. *Food Res. Int.* 23: 23-45

Amin, M. N., Rahaman, M. M. and Manik, M. S. 2003. *In vitro* clonal propagation of *Paederifoetida* L. A medicinal plant of Bangladesh. *Plant Cell Tiss. Org. Cult.* 13: 117 -123

Antarkar, D. S., Chinwalla, T. and Bhatt, N. 1984. Anti-inflammatory activity of *Rubia cordifolia*. Linn in rats. *Indian J. Pharmacol.*15: 185-188

Anu, K. I. 1993. Standardization of *in vitro* propogation technique in *Gymnema sylvestre* Br. M.Sc (Hort.) thesis, Kerala Agricultural University, Thrissur, Kerala, India, pp.54-78

Aobchey, P., Sriyam, S., Praharnripoorab, W., Lhieochaiphant, S. and Phutrakull, S. 2002. Production of red pigment from the root of *Morinda angustifolia* Roxb. var. *scabridula* Craib. by root cell culture. *CMU. J.* 1: 66-68

Arita, M., Asai, K. and Nishika, T. 1999. Finding precursor compounds in secondary metabolism. *J. Japan Soc. Artif. Intel.* 32(2): 678-688

Aruoma, O. I. 1998. Free radicals, oxidative stress and antioxidants in human health and disease. *J. Am. Oil Chem.Soc.* 75: 199–212

Asaka, I., Hirotani, M., Asada, Y., Yoshikawa, T. and Furaya , Y. 1994. Mass production of ginseng (*Panax ginseng*) embryoids on media containing high concentration of sugar. *Planta. Med.* 60(2): 146 -148

Babaoglu, M., Davey, M. R., Power, J. B., Sporer, F. and Wink, M. 2004. Transformed roots of *Lupinus mutabilis*: induction, culture and isoflavone biosynthesis. *Plant Cell, Tiss. Org. Cult.* 78(1): 29-36

Babu, T.D., Kuttan, G. and Padikkala, J. 1995. Cytotoxic and anti-tumor properties of certain taxa of Umbelliferae with special reference to *Centella asiatica* (L.) Urban. *J. Ethnopharmacol.* 48:53-64

Babulaa, P., Mikelovab, R., Potesilb, D., Adamb, V., Kizekb, R., Haveld, L. and Sladkya, Z. 2005. Simultaneous determination of 1,4-Naphtoquinone, lawsone, juglone and plumbagin by liquid chromatography with UV detection. *Biomed. Papers.* 149: 23-29

*Bailey, J. A. and Mansfield J. W. 1982. Pytoalexins. Blackie, Glasgow Gontier, E., sangwan, B.S., and barbolin, L. N. 1994. Effect of calcium alginate immobilization on growth and tropane alkaloids off a stable suspension cell line of *Datura innoxia* Mill. *Plant Cell Rep.*13(9): 533-536

Bajaj, Y.P.S. 1995. Cryopreservation of germplasm of medicinal and aromatic plants. *In* Y.P.S.Bajaj (ed.), *Biotechnology in Agriculture and Forestry, Cryopreservation of Plant Germplasm* I, vol. 32. Springer-Verlag, Berlin, pp. 419-434.

*Balandrin, M. F. and Klocke, J. A. 1988. Medicinal Aromatic and Industrial Materials from plants. *Biotechonlogy in Agriculture and Forestry.* Vol.4. *Medicinal and Aromatic Plants.* I (ed Bajaj, Y. p. S.), Springer Verlag, London, pp. 3-30

*Berlin, J. 1990. Screening and selection for various cell lines with increased levels of secondary metabolites. In secondary products from plant tissue culture. (eds) Charlwood, B. V. and Rhodes, M. J. C. Clerendon Press, Oxford, pp. 119-137

Bhalsingh, S. B. and Maheshwari, V.L. 1998. Plant Tissue Culture-A Potential Source of Medicinal Compounds. *J. Sci. Indust. Res.* 57: 703-708

*Beckam, S. 1990. Apparent hydroxyl radical production by peroxinitrite: implication for endothetial injury from nitric oxide and superoxide. *Pro. Natl. Acad. Sci.* 87: 1620-1624

Bhojwani, S. S. and Razdan, M. K. 1996. *Plant Tissue Culture: Theory and Practice.* Elsevier Science, Amsterdam. pp. 246-150

Bishayees, S. and Balasubramanian, A. S. 1971.assay of lipid peroxide formation. *J. Neurochem.* 18: 909-920

Blackstone, N. W. and Green, D. R. 1999. The evolution of a mechanism of cell suicide. *Bioessays.* 21: 84-88

Bolton, J.L., Trush, M.A., Penning, T. M., Dryhurst, G., and Monks,T. J. 2000. Role of quinones in toxicology. *Chem. Res. Toxicol.* 13: 135–160

Bozan, D., Krenn, L., Blaeser, U. and Hausknost-Chenicek, N. 1997. Determination of Naphthoquinones in *Arenbia densiflora* by Reversed-Phase High Performance Liquid Chromatography. *Journal Liquid Chromatography & Related Technologies.* 21(2): 3149 - 3160

Brigham, L. A., Michaels, P. J. and Flores, H. E. 1999. Cell-specific production and antimicrobial activity of naphthoquinones in roots of *Lithospermum erythrorhizon. Plant Physiol.* 119: 417–428

*Brodelius, P. 1985. In: *Application of isolated enzymes and immobilized cells to Biotechnology* (Ed) laskin AI, Addison Wesley Publ., Reading Massachussetts, pp.109-148

Bulgakov, V.P., Tchernoded, G.K., Mischenko, N.P., Khodakovskaya, M.V., Glazunov, V.P., Radchenko, S.V., Zvereva, E.V., Fedoreyev, S.A. and Zhuravlev, Y.N. 2002. Effect of salicylic acid, methyl jasmonate, ethephon and cantharin on anthraquinone production by *Rubia cordifolia* callus cultures transformed with the *rolB* and *rolC* genes. *J. Biotechnol.* 97: 213–221

Cadenas, E., Hochstein, P. and Ernster, L. 1992. Pro- and Antioxidant Functions of Quinones and Quinone Reductases in Mammalian Cells. *Adv. Enzymol. Relat. Areas Mol. Biol.* 65: 97–146

Carrier, D. J., Cosentino, G., Neufeld, R., Rho, D., Weber, M. and Archambault, J. 1990. Nutritional and hormonal requirements of *Ginkgo biloba* embryo-derived callus and suspension cell culture. *Plant Cell Rep.* 8: 635–638

Chabner, B.A., Allegra, C.J., Curt, G.A., and Calabresi, P. 1996. Antineoplastic agents. In *The pharmacological basis of therapeutics.* 9th ed. *Edited by* Hardman, J.G. and Limbird, L.E. McGraw-Hill, New York. pp. 1233–1290

Chang, J. L., Chavez, C.D. and Rubiasins, A.C. 2000. new anthracene derivatives from the roots and stems of *Rubia cordifolia*. *Tetrahedron Lett.* 41(37): 7157-7162

*Chatterjee, A. and Pakrashi, S.C. 1997. *The Treatise on Indian Medicinal Plants* 5: NISCOM, New Delhi.

Chattopadhyay, R. S., Mehra, A. K., Srivastava, S. S. and Bhojwani, V. S. 2003. Effect of major nutrients on podophyllotoxin production in *Podophyllum hexandrum* suspension cultures. *Appl. Microbiol. Biotechnol.* 60:541–546

Chen, C. C., Chang, W. T., Chang, Y. S. and Tsay, H. S. 1994. Studies on the tissue culture of *Angelica dahurica var. formosana* II. Establishment of cell suspension culture and evaluation of cultural conditions. *J. Chinese Medicine.* 5: 123-134

*Chilton, M, Tepfer, D. A., Petit, A., David, C., Casse-Delbart, F. and Tempe, J. 1982. *Agrobacterium rhizogenes* inserts T-DNA into the genomes of the host plants root cells. *Nature.* 295:432-434

Chittimalla, C. Veeresham, R. Mamatha, K. and Srisilam, C. 2000. Biotransformation and Production of the Anticancer Drug Taxol by Plant Cell Cultures. *Indian Drugs.* 37: 86-89

Choi, H. K., Kim, S. I., Son, J. S., Hong, S. S., Lee, H. S., Chung, I. S.,and Lee, H. J. 1996. Intermittent maltose feeding enhance paclitaxel production in suspension culture of *Taxus chinesis* cells. *Biotechol. Lett.* 22: 1793-1796

Chung, Y., Jong Kwan, I., Lee, S. and Cho, H. 2004.Synthesis and Cytotoxicity of nilinomethyl-1,4-naphthoquinones. *Bull. Korean Chem. Soc.* 25(9): 12-16

*Conger, B.V. 1981. Agronomic crops. In: *Cloning Agricultural Plants via in vitro Techniques* (ed. B.V. Conger). CRC Press, Boca Raton, FIorida, USA. pp.165-215

Conover, W. J. 1980. *Practical non parametric statistics.* Second edition pp 299-337

Contin, A., Heijden, R., Lefeber, A.W. M. and Verpoort, R. 1998. The iridoid glucoside secologanin is derived from the novel triose phosphate/pyruvate pathway in a *Catharanthus roseus* cell culture. *FEBS Lett.* 437:413–416

Cowan, M. M. 1999. Plant products as antimicrobial agents.*Clinical Microbiol. Rev.*12(4): 564-582.

Cragg, G. M. and Newman, D. J. 2006. Plants as source of anticancer agents. *J. Ethnopharmacol.* 100: 72-79

Cusido, R.M., Palazon, J., Navia-Osorio, A., Mallol, A., Bonfill, M., Morales, C. and Pinol, M. T. 1999. Production of taxol and baccatin III by a selected *Taxus baccata* callus line and its derived cell suspension culture. *Plant Sci.* 146: 101-107

Dean, J. R., Liu. B. and Price, R. 1998. Extraction of magnolol from *Magnolia Officinalis* using supercritical fluid extraction and phytosol solvent extraction. *Phytochem. Anal.* 9(5): 248 – 252

Deshkar, N., Tilloo, S. and Pande, V, 2008. A comprehensive review of *Rubia cordifolia* linn. *Pharmacognosy Rev.* 2(3): 124-136

Demir, C.H., Foyer, H., Descourvieres, P. and K.J. Kunert, K. J. 2003. Protection against oxygen radicals: important defence mechanism studied in transgenic animal. *Biol. Pharm. Bull.* 17: 507–523

Dholwani, K. K., Saluja, A. K., Gupta, A. R. and Shah, D. R. 2008. A review on plant-derived natural products and their analogs with anti-tumor activity. *Indian J. Pharmacol.* 40 (2):49-58

Didry, N., Dubrevil, L. and Pinkas, M. 1994. Activity of anthraquinonic and naphthoquinonic compounds on oral bacteria. *Die Pharmazie.* 49:681-683

Dicosmo, F. and Misawa, M. 1995. Plant cell and tissue culture: Alternatives for metabolite production. *Biotechnol. Adv.* 13(3): 425-453

Dilley, R. A. 1964. Thin-layer chromatography of naturally occurring quinones and hydroquinones. *Anal Biochem.*7:240–246

Ding, C. K., Wang, C. Y., Gross, K. C. and Smith, D. L. 2002. Jasmonate and salicylate induce expression of pathogenesis-related protein genes and increase resistance to chilling injury in tomato fruit. *Planta Med.* 214: 895–901

Dixon, R. A. and Paiva, N. L. 1995. Stress-induced phenylpropanoid metabolism.*Plant Cell Rep.* 7: 1085–1097

*Dodds, J. A. and Roberts, L. W. 1982. *Experiment in Plant Tissue Culture.* Cambridge University press, London, p. 28

Dolnikowski, G. G., Sun, Z., Grusak, M. A., Peterson, J. W., Booth, S. L., 2002. Observation on the biosynthesis of phytoterpenoid quinone. *J. Nutr. Biochem.* 13:168

Duong, T. N., Nguyen, T.T. and Don, N. T. 2003. Effect of sucrose, glucose and fructose in proliferation of Hymalaya Yew (*Taxus wallichiana* zucc.) cell suspension cultures – a potential source for taxol production.. *J. Plant Physiol.* 28: 11-22

Dziezak, J. D. 1986. Biotechnology and flavor development: plant tissue cultures. *Food Technol.* 40: 122-129

Eilert ,U., Constabel F. and Kurz, W. G. M. 1987. Elicitor stimulation of monoterpene indole alkaloid formation in suspension culrues of *Catharanthus roseus. J. Plant Physiol.* 128: 11-22

Ellis, D.D., Zeldin, E. L., Brodhagen, M., Russin, W. A. and McCown, B. H., 1996. Taxol production in nodule cultures of *Taxus. J. Nat. Prod.* 59: 246-250.

El- Bahr, M. K. Ghanem, S. A., El-Missery, M. M. and El-Nasr, M. M. S. 1997. Production of tropane alkaloids in tissue cultures of *Hyoscyamus muticus. Fitoterapia.* 68(5): 423-428.

*Endress, R. 1994. *Plant Cell Biotechnology.* Springer Verlag, Berlin, Heidelberg pp. 234-356

Ercan, A.G., Taskin, K.M., Turgut, K. and Yuce, S. 1999. *Agrobacterium rhizogenes*-mediated hairy root formation in some *Rubia tinctorum* L. populations grown in Turkey. *Turkish J. Bot.* 23(6): 373-377.

Eugon, S.A. 1969. *Thin layer chromatography- A laboratory handbook.* 2nd ed., SpringerVerlag, New York. pp. 786-830

*Evans, W. C. 1996. *Trease and Evan' s pharmacognosy.* 15th ed., Philadelphia: Elservier. pp. 12

Facchini, P. J. 2001. Alkaloid biosynthesis in plants: biochemistry, cell biology, molecular regulation, and metabolic engineering applications. *Annu. Rev. Pl. Physiol.* 51: 29-34

Facchini, P. J., and DiCosmo, F. 1991. Secondary metabolite biosynthesis in cultured cells of *Catharanthus roseus* (L.) G. Don immobilized by adhesion to glass fibres. *Appl. Microbiol. Biotechnol.* 35:382–392

Fahy, J. 2001. Modifications in the Velbenamine Part of the Vinca Alkaloids have major implications for tubulin interacting activities. *Current Pharmaceutical Design.* 7 (13): 1181-1197.

*Fauler, G., Muntean, W. and Leis, H.J. 1996. Vitamin K. In: *Modern Chromatographic Analysis of Vitamins,* 3 rd ed. De Leenher, A.P., Lambert, W.E. and Van Bocxlaer, J.F. (ed.), Marcel Dekker, Inc., New York. pp. 229-270

Fine, P. M., Cass, G. R. and Simoneit, B. R. 2001.Chemical Characterization of Fine Particle Emissions from Fireplace Combustion of Woods Grown in the Northeastern United States. *Environ. Sci. Technol.* 35: 2665–2675

Flores, H. E., Dai, Y. R., Freyer, A. J. and Michaels, P. J. 1999. Biotransformation of butylated hydroxytoluene in hairy root cultures. *Plant Physiol. Biochem.* 32: 511-519

Flores, H., Pickard, J. and Hoy, M. 1988. Production of polyacetylenes and thiophenes in heterotrophic and photosynthetic root cultures of Asteraceae. *In* J Lamb, H Breteler, T Arnason, L Hansen, eds, *Chemistry and Biology of Naturally-Occurring Acetylenes and Related Compounds* (NOARC). Elsevier, Amsterdam, pp. 233–254

Fowler, M. W. 1986. Plant cell culture: Natural product and industrial application . *Biotechnol. Genet. Eng. Rev.* 2: 41-67

Fraser, M. P., Cass, G. R., Simoneit, B. R. T. and Rasmussen, R. A.1998. Air quality model evaluation data for organics. 5. C6–C22 Non polar and semi polar aromatic Compounds, *Environ. Sci. Technol.* 32:1760–1770

Fujii, N., Yamashita, Y., Arima, Y., Nagashima, M. and Nakamo, H. 1992. Antimicrobial agents in chemotherapy 36: 2589–2594

Fujita, N., Maeda, Y., Suga, C., Morimoto, T. 1983. Production of shikonin derivatives by cell suspension cultures of *Lithospermum erythrorhizon*. III.

Comparison of shikonin derivatives of cultured cells and Ko-shikon. *Plant Cell Rep.* 2: 192–193

*Fujita, Y. 1988. Shikonin: production by plant (*Lithospermum erythrorhizon*) cell culture : In Bajaj, Y. P. S. (ed) *Biotechnology in agriculture and forestry*, Vol 4 *Medicinal and aromatic plants* I Springer, Berlin Heidelberg, New york, pp. 227 -236

Fujita, Y., Hara, Y., Ogino, T. and Suga, C. 1981. Production of shikonin derivatives by cell suspension cultures of *Lithospermum erythrorhizon* I. *Pl. Cell Rep.* 1:59-60

Fulzele, D. P. and Heble, M. R . 1994. Large-scale cultivation of *Catharanthus roseus* cells: production of ajmalicine in a 20-l airlift bioreactor. *J. Biotechnol.* 35(1): 1-7

Funk, C., Gugler, K. and Brodelius, P. 1987. Increased secondary product formation in plant cell suspension cultures after treatment with yeast carbohydrate preparation.(elicitor). *Phytochem.* 26: 401-405

Furuya, T., Ikuta, A., and Syono, K. 1972. Alkaloids from callus cultures of *Papaver somniferum*. *Phytochem.*11: 3041-3044

Francoise, B. Hossien, S., Halimeh, H. and Zahra, N. F. 2007. Growth optimization of *Zataria multiflora* Boiss. Tissue culture and Rosamarinic acid production production improvement. *Pakistan J. Biol. Sci.* 10(19): 3395-3399

Gamborg, O. L., Miller, R. A. and Ojima, K. 1968. Nutrient requirements of suspension cultures of soybean root cells. *Exp. Cell Res.* 50: 151-158

*Gamborg, O.L. and Shyluk, J. P. 1981. Nutrition, media and characteristics of cell and tissue cultures. *Plant Tissue Culture:Methods and Applications in Agriculture* (ed. Thrope, T. A.), Academic press, New York, pp. 21-24

Gangaopadhyay, G., Basu, A. S., Mukherjee, B. R. and Gupta, S. 1997. Effect of salt and osmotic shock on updates and adapted callus lines of tobacco. *Plant Cell Tiss.Org. Cult.* 49:45-52

Garnier, F., Carpin, S., Labelb, P., Joel, C., Rideau, M. and Hamdi, S.1996. Effect of cytokinin on alkaloid accumulation in periwinkle callus cultures transformed with a light-inducible *ipt* gene. *Plant Sci.*120: 47-55

Gholba, D. N. 2000. *In vitro* callus induction in Gurmar (*Gymnema sylvestre* R. Br.) for secondary metabolite synthesis. M.Sc (Hort.) thesis, Kerala Agricultural University, Thrisssur, India. pp. 148-151

Gillis, C. N. 1997. *Panax ginseng* pharmacology: a nitric oxide link?. *Biochem Pharmacol.* 54: 1-8

Goel, H.C., Prasad, J., Sharma, A. and Singh, B.1998. Antitumour and radioprotective action of *Podophyllum hexandrum*. *Indian J .Exp. Biol.*36:583-7

Gokul, H., Sowmya, S. and Tejavathi , D.H .1998. Effect of stress on alkaloid production in the culture of *Cissampelos pareira* L. *Abstract of National seminar on Plant Biotechnology for sustainable Hill Agiculture,* Defence Agricultural Research Laboratory, UP. May 6-8th, p.10

*Goldsmith, M. H. M. 1977. The polar transport of auxin. *Ann. Rev. Plant Physiol.* 28:439– 78

Gomes, A. Peter, M., Håkan, W. and Lisbeth, E. K. 1996. Oxidative stress associated with exercise, psychological stress and life-style factors. *Chemico. Biological Interactions*. 102 (1): 17-36

Gontier, E., Sangwan, B. S., Barbotin, J. N. 1994. Effect of calcium, alginate and calcium alginate immobilization on growth and tropane alkaloid levels of a stable suspension cell line of *Datura innoxia* mill. *Plant cell Rep.* 13 533-536

*Goyton, A.C. 1993. *Textbook of medical physiology*. Philadelphia: W B Saunders company. pp. 675-701

Green, L. C., Wagner, D. A., Glogowski, J., Skipper, P. L. and Tannenbaum, S. R. 1982. Analysis of nitrate, nitrite and 15 N in biological fluids. *Anal. Biochem.* 126: 131-136

Guidi, I., Galimberti, D., Lonati, S., Novembrino, C., Bamonti, F., Tiriticco, M., Fenoglio, C., Venturelli, E., Baron, P., Bresolin, N. and Scarpini, E. 2006. Oxidative imbalance in patients with mild cognitive impairment and Alzheimer's disease. *Neurobiol. Aging.* 27:262–269

Gutteridge, J. M.1994. Biological origin of free radicals and mechanisms of antioxidant protection. Chem. *Biol.Interact.* 91: 133-140

Hahlbrock, K. and Griseback, J. 1979. Enzymatic control in the biosynthesis of lignin and flavanoids. *Ann. Rev. Plant Physiol.*30:105-130

Hajime, M., Konoshima, M., Mamoru, S. Tabata .M. 1997. Effect of nutritional and hormonal factor on growth and production of shikonin in *Lithospermum eythrorhizon. J. Plant Physiol.* 140:2 218-222

Halliwell, B. 1996. Antioxidants in human health and disease. *Annu. Rev. Nutr.* 16: 33-50

Halliwell, B. 2000. The antioxidant paradox. *Lancet.* 355: 1179 – 1180

Halliwell, B. 2006. Reactive species and antioxidants. redox biology is a fundamental themeof aerobic life. *Plant Physiol.* 141: 312-322

Halliwell, B. and Gutteridge, J. M. C. 1999. Antioxidant defenses, *In Free Radicals in Biology and Medicine*, 3rd ed. Oxford Science Publications, Oxford, UK. pp.105-159

Halliwell, B.1999. Antioxidant defense mechanisms: from beginning to the end (of the beginning). *Free Radic. Res.* 31: 261-272

Hande, K. R. 1998. Etoposide: four decade of development of a topoisomerase II inhibitore. *Eur. J.cancer.* 34:1514-1521

Hellens, R. P., Edwards, E.A., Leyland, N. R., Bean, S., and Mullineaux, P. M. 2000. Green: a versatile and flexible binary Ti vector for *Agrobacterium-mediated* plant transformation. *Plant Mol. Biol.* 42: 819–832

Heller, R. 1953. Researches on the mineral nutrition of plant tissues. *Ann. Sci. Nat. Bot. Biol. Veg.*14: 1–223.

Hitotsuyanagi, Y. Ishikawa, H., Hasuda, T. and Takeya, K. 2004. Isolation, structural lucidation, and synthesis of RA-XVII, a novel bicyclic hexapeptide from *Rubia cordifolia,* and the effect of side chain at residue 1 upon the conformation and cytotoxic activity. *Tetrahedron Lett.* 45(5): 935-938.

Hitotsuyanagi, Y., Aihara, T. and Zhou, J. 2004. RA-dimer A, a novel dimeric antitumor bicyclic hexapeptide from *Rubia cordifolia* L. *Tetrahedron Lett.* 41(32): 6127-6130

Hofseth, L. J., Wargovich, M., and Peter, C. 2001. Inflammation, cancer and target of ginseng. *J. Nutr.* 137. 183-185

Hongzhu, G. Zhenzhan , C., Rujun Y., Dean G. and Junhua, Z.1998.Anthraquinones from hairy root cultures of *Cassia obtusifolia. Phytochem.* 49:6:1623-1625.

Hook, I. L. I. 2001. Naphthoquinone contents of *in vitro* cultured plants and cell suspensions of *Dionaea muscipula* and *Drosera species. Plant Cell Tiss. Org. Cult.* 67: 281–285

Hsiang, Y. H. and Liu, L. F. 1988. Identification of mammalian DNA topoisomerase I as an intracellular target of the anticancer drug camptothecin. *Cancer Res.* 48(7): 1722–1726.

Hua, H. M., Wang, S. X., Wu, L. J., Li, X. and Zhu, T. R. 1992. Studies on naphthoic acid esters from the roots of *Rubia cordifolia* L. *Yaoxue Xuebao.* 27: 279-282

Huerta, S., Arteaga, J. R., Irwin, R. W., Ikezoe, T., Heber, D. and Koeffler, P. H. 2002. PC-SPES inhibits colon cancer growth *in vitro* and *in vivo. Cancer Res.* 62: 5204-5209

Hui, X., Kim, Y. K., Jin, X., Lee, S. Y. and Park, S. U. 2008. Rosmarinic acid biosynthesis in callus and cell cultures of *Agastache rugosa* Kuntze. *J. Med. Plant Res.* 2(9): 237-241

*Hunter, C. S. 1979. *In vitro* culture of *Cinchona ledgeriana* L. *J. hort. Sci.* 54(2):111-114

Ibrahim, A. K., Khalifa S., Khafagi, I., Youssef, D., Khan, I. and Mesbah, M. 2007. Stimulation of oleandrin production by combined *Agrobacterium tumefaciens* mediated transformation and fungal elicitation in Nerium oleander cell cultures. *Enzyme Microb. Technol.* 41(3): 331-336

Ilahi, I. and Ghauri, E. G. 1994. Regeneration in cultures of *Papaver bracteatum* as influence by growth hormones and temperature. *Plant Cell Tiss. Org. Cult.* 50(2): 91-95

Ilavarasan. R., Mallika, M. and Venkataraman, S. 2006. Anti-inflammatory and free radical scavenging activity of *Ricinus communis* root extract. *J. Ethnopharmacol.* 103: 478–480

Inbaraj, J. J. and Chignell, C. F., 2004. Cytotoxic action of juglone and plumbagin: a mechanistic study using HaCaT keratinocytes. *Chem. Res. Toxicol.* 17: 55–62

Irshad, M., Chaudhuri, A. and Joshi, Y. K. 2002. Superoxide dismutase and total anti-oxidant levels in various forms of liver diseases. *Hepatol Res.* 23: 178–184

Ito. C., Murata, T., Nakao, K., Furukawa, H. and Itoigoawa, M. 2006 Induction of apoptosis by isoflavanoids from leaves of Milletia taiwania in human leukemia HL-60. *Planta Med.* 72(5): 424-429

Itoigawa, M.,Takeya, K. and Furukawa, H. 1991. Cardiotonic action of plumbagin on guinea- pig papillary muscle. *Planta Med.* 57(4): 317-319

Itokawa, H., Takeya, K., Hitotsuyanagi, Y. and Morita, H. 1999. Antitumor compounds isolated from higher plants. *Yakugaku Zasshi.* 119:529–583

Itokawa, H., K. Takeya, Y. and Qiao, Y. 1986. Studies on antitumor cyclic hexapeptides RA obtained from *Rubiae radix*, Rubiaceae: VI. Minor antitumor constituents. *Chem. Pharmaceutical Bull.* 34(9): 3762-3768

Itokawa, H., Qiao, Y. and Takeya, K. 1989. Anthraquinones and naphthohydroquinones from *Rubia cordifolia. Phytochem.* 28:3465–3468

Itokawa, H., Qiao, Y. and Takeya, K. 1990. Anthraquinones, naphthoquinones and naphthohydroquinones from *Rubia oncotricha. Phytochem.* 30: 637–640

Itokawa, H., Takeya, K., Hitotsuyanagi, H. and Morita, H. 1997. Macrocyclic Peptide Alkaloids from Plants. *Phytochem.* 49: 301-387

Itokawa, H., Ibraheim, Z. Z., Lin, X., Li, X. 1993. Anthraquinones, naphthohydroquinones and naphthohydroquinone dimers from Rubia cordifolia and their cytotoxic activity. *Chem. Pharmaceutical Bull.* 41(10): 1869-1872

Jaggi, K. R. and Singh, J. 2001. Solasodine production in cultures of *Solanum platanifolium. J.Med. Arom. Pl. Sci.* 22(4A) &23(1A): 197-200

Jensen, A. G. and Hansen, S. H. 2002. Separation of hypericins and hyperforins in extracts of *Hypericum perforatum* L. using non-aqueous capillary

electrophoresis with reversed electro-osmotic flow. *J. Pharma. Biomed. Anal.* 27(1): 167-176

Jeong, G.S., Kwon, O.K., Park, B.Y., Ahn, K.S., Kim, J.C., Min, B.S. and Lee, H.K. 2007. Lignans and coumarins from roots of *Anthrescus sylvestris* and their increase of caspase-3 activity in HL -60 cells. *Biol. Pharm. Bull.* 30(7):1340-1343

Joen, M. H., Sung, S. H., Hah, H. and Kim, Y. C.1995. Ginkgolide –B production in cultured cells from *Ginkgo biloba* L. leaves. *Pl. Cell Rep.* 14 (8): 501-504

Johnson, T. M., Yu, Z. X., Ferrans, V. J., Lowenstein, R. A. and Finkel, T. 1996. Reactive oxygen species are downstream mediators of p53-dependent apoptosis. *Proc. Nat. Acad.* 93: 11848–11852

Johnson, T. S. 1993. Studies on production of capsaicin in immobilized cell and placental tissue of *Capsicum annum* and *Capsicum frutescens*. Ph.D Thesis submitted to the University of Mysore. pp 1-191

Jones, D. M. 1984. Phenyl alanine ammonialyase- Regulation of its induction and its role in plant development. *Phytochem.* 23:1344-1359

Joseph, M. 1997. Indirect organogenesis and embryogenesis in *Kaempferia galanga* L. M.Sc (Hort.) thesis, Kerala Agricultural University, Thrissur, India. pp 148-151

Kadkade, P.G. 1982. Growth and podophyllotoxin production in callus tissues of *Podophyllum peltatum. Pl. Sci. Lett.* 25: 107-115

Kalimuthu, M. 2002. Utilization of *in vitro* cultures of *Tinospora cordifolia* Mier. (Chittamrithu) for berberine. M. Sc (Hort.) thesis, Kerala Agricultural University, Thrissur, India. pp 148-151

Kang, S. C. and Moon, Y. H. 1992: Isolation and antimicrobial activity of a naphthoquinone from *Impatiens balsamina. Saengyak Hakhoechi 23*: 240–247

Kannan, V. R. and Jasrai, Y. T. 1998. Micropropogation of medicinal plant –*Vitex negundo. J. Med. Arom. Pl. Sci.* 20:693-696

Kanter, M.M., Nolte, L.A. and Holloszy, J.O. 1993. Effects of an antioxidant vitamin mixture on lipid peroxidation at rest and postexercise. *J. Appl. Physiol.* 74: 965-969

Kaur, H. and Perkins, J. 1991. The free radical chemistry of food additives. In O. I. Aruoma & Halliwell (Eds.), *Free radicals and food additives* London: Taylor and Francis Ltd. pp.17-35

Kawaik, T., Rowe, T. C., Chen, G. L., Hdang, Y. H. and Lu, L.F. 2007.DNA damage by antitumor plumbagin mediated by mammalian DNA topoisomerase II. *Cancer Res.* 46:2021-2026.

Kelkar, V. V., Dhumal, V. R., Bhavsar, V. H. and Mardikar, B. R. 1986. Some aspects of activity profile of sodium lawsonate in mice and rats. *Arch. Int. Pharmacodyn. Ther.* 283(1): 71-79

Kelland, L. R. 2000. Flavopiridol, the first cyclic-dependent kinase inhibitor to enter the clinic: current status. *Expert. Opin. Investig. Drugs*. 9: 2903-2914.

Kerr, J. F. R., Winterford, C. M., and Harmon, B. V. 1994. Apoptosis. Its significance in cancer and cancer therapy (Review). *Cancer.* 73: 2013–2026.

Khan, S. A. 2005. Hyperoxaluria-induced oxidative stress and antioxidants for renal protection. *Urological Res.* 33 (5): 349-357

Kim, B. H., Yoo, J., Park, S. H., Jung, J. K., Cho, H. and Chung, Y. 2002. Synthesis and Evaluation of Antitumor Activity of Novel 1, 4- Naphthoquinone Derivatives (IV). *Arch. Pharm. Res.* 29: 123-130

Kim, S.I. , Choi, H. K., Son, J. S., Yun, J. H., Jang, M. S., Kim, H. R., Son, J. Y., Kim, J. H., Choi, H. J., Hong, S. S. 2001. Cryopreservation of *Taxus chinensis* suspension cell cultures. *Cryo Lett.* 22: 43–50.

Kim, Y. J., Barbara, E., and Pamela J. 2003. Growth dynamics of *Artemisia annua* hairy roots in three culture systems. *Biotechnol. Bioeng.* 83(4): 428-443.

Kim, Y., Barbara E. Wyslouzil, B. E., and Weathers, P. J. 2002. Secondary metabolism of hairy root cultures in bioreactors. *In Vitro Cell. Dev. Biol.Pl.* 38: 1–10.

Kim, Y., Hahn, E., Murthy, D., Hosakatte, N., and Paek, K. 2004. Adventitious root growth and ginsenoside accumulation in *Panax ginseng* cultures as affected by methyl jasmonate. *Biotechnol. Lett.* 26(21): 1619-1622.

*Kintzios, S. and Barberaki, M. 2004. *Plants That Fight Cancer*. CRC Press, Boca Raton, FL

Kirchner, J. G. 1978. Thin layer chromatography 2 nd edition. *Techniques of chemistry*, Vol. 14. Wiley Interscience. pp. 105- 173.

Kirtikar, K. R. and Basu, B. D. 1980. *Indian Medicinal Plants* vol II (International Book Distributors, Dehradun, 1980). Sec edition. pp.1305-1307

Kitagawa, R. R., Raddi, M, S., Santos, L. C., and Vilegas, W. 2004. A new cytotoxic naphthoquinone from *Paepalanthus latipes. Chem. Pharm. Bull.* 52:1487-1496.

Kitanov, G.M. and Pashankov, P.P. 1994. Quantitative investigation on the dynamics of plumbagin in Plumbago europea L. roots and herb by HPLC. *Pharmazie.* 49(6): 462

Kittipongpatana, N., Hock, R. S. and Porter, J. R.1998.Production of solasodine by hairy root, callus, and cell suspension cultures of *Solanum aviculare. Forst. Plant Cell Tiss. Org. Cult.* 52:133–143

Kittipongpatana, I., Tangjai, T., Srimanee, A. and Kittipongpatana, O. S. 2007. Biotransformation of Hydroquinone to Arbutin by Cell-Suspension Cultures of Three Thai Solanaceous Plants. *CMU. J. Nat. Sci. Vol.* 6(2):12-26.

Koile, Y. Hoshhino, Y., Mii, M. and Nakano, M. 2003. Horticultural characterization of *Angelonina salicarifolia* plants transformed with wild type strains of *Agrobacterium rhizogenes. Pl. Cell Rep.*21: 981-987

Komaraiah,P., Ramakrishna, S.V., Reddanna, P. and Kavi , P.B. 2003. Enhanced production of plumbagin in immobilized cells of *Plumbago rosea* by elicitation and in situ adsorption*J. Biotechnol.*101(2): 181-188

Kosuge, T., Yokota, M., Yoshida, M. and Ochiai, A. 1982. Anti Hemorrhagic Principles in the Crude Drugs for Hemostatics 1. Hemostatic Activities of the Crude Drugs for Hemostatics. *Yakugaku Zasshi.* 101: 501-503

Koyama, J., Morita, I., Tagahara, K. and Aqil, M. 2001. Bianthraquinones from *Cassia siamea. Phytochem.* 56: 849-851

Koyama, J., Ogura, T., and Tagahara, K. 1992. Two naphthoquinones from *Rubia cordifolia. Phytochem.* 31(8): 2907-2908.

Kraemer, K. H., Schenkel, E. P. and Verpoorte, R. 2002. *Ilex paraguariensis* cell suspension culture charaterization and response against ethanol. *Plant Cell Tiss. Org. Cult.* 68: 257– 263

Krishnan, P and Bastow, K F. 2001. Novel mechanism of cellular DNA topoisomerase II inhibition by the pyranonaphthoquinone derivatives alpha-lapachone and beta- lapachone. *Cancer Chemother. Pharmacol.* 47(3): 187-198

Kubo, I., Taniguchi, M., Chapya, A. and Tsujimoto, K. 1980. An insect antifeedant and antimicrobial agent from *Plumbago capensis*. *Planta Med. Suppl.* 185-187.

Kulkarni, V. M. and Rao, P S. 1999. *In vitro* propogation of sweet flag (*Acorus calamus,* Araceae). *J.Med. Arom. Plant Sci.* 21:325-330

Kupchan S. M., Court W. A, Richard G. D., Gilmore, J. C. J. and Robert F. B. 1972. Triptolide and tripdioinlide, novel antileukemic diterpenoid triepoxides from *Tripterygium wilfordii. J. Am. Chem. Soc.* 94: 7194- 7195

LeBel, C., Ischiropoulos, H. and Bondy, S. 1992. Evaluation of the probe 2'-dichlorofluoresceinas an indicator of reactive oxygen species formation and oxidative stress. *Chem. Res. Toxicol.* 5: 227-231

Lee, C. H. and Lee, H. S. 2005. Antifungal property of dihydroxyanthraquinones against phytopathogenic fungi. *J. Microbiol. Biotechnol.* 15:442-446

Lee, D., Kim, C. and Lee, D. 2001. Effect of culture conditions on the biosynthesis of gagaminine, a potent antioxidant from the roots of *Cynanchum wilfordii. Biol. Pharm. Bull.* 24(12): 1451—145

Levefere, K., Binkley, S. B., Mckee, R. W., Sidney A. Thayer, K. and Doisy, A. 1985. The constitution of vitamin K.*J.Biol. Chem.* 131: 1-9

Li, Y., Li, C. J., Yu, D. and Pardee, A. B. 2000. Potent induction of apoptosis by ß-lapachone in human multiple myeloma cell lines and patient cells. *Mol. Med.* 6: 1008-1015

Li, D.W., Lee, E.B., Kang, S.S., Hyun, J.E. and Whang, W.K. 2002. Activity guided isolation of saponins from *Kalopanax pictus* with anti-inflammatory activity. *Chem. Pharma. Bull.* 50: 900–903

Likhitwitayawuid, K., Kaewamatawong, R., Ruangrungsi, N. and Krungkrai, J. 1998. Antimalarial naphthoquinones from *Nepenthes thorelii*. *Planta Med.* 64:237-241

Lindsey, K. and Yeoman, M. M. 1984. The synthetic potential of immobilized cells of *Capsicum frutescens* Mill cv. annum. *Planta.Med.* 162:100-127

Liu C. Z., Wang, Y. C., Ouyang, F. , Ye, H. C., and Li, G. F.1998. Technical factors of artemisinin biosynthesis in *Artemisia annua* hairy root culture. *Acta Bot. Sin.* 40: 831-835

Lizuka, A., Iijima, O. T. Kazuo Kondo, K., Itakura, H. and Yoshie, F. 2004. Evaluation of Rhubarb using antioxidative activity as an index of pharmacological usefulness. *J. Ethnopharmacol.* 91(1): 89-94

*Lloyed, G. B. and Mc Cown, B. H. 1980. In : *Proceeding of the international Plant Propagation Society.* 30:421-434

Lobstein, A., Brenne, X., Feist, E., Meth, N., Weniger, B. and Anton, R. 2001. *Phytochem Anal.* 12:202–205

Lodhi, A.H., Bongaerts, R.J.M., Verpoorte, R., Coomber, S.A. and Charlwood, B.V.1996.Expression of bacterial isochorismate synthase (E C 5.4.99.6) in transgenic root cultures of *Rubia peregrina. Plant Cell Rep.*16:54–7

Lodhi, A. H. and Charlwood, B. V. 1996. *Agrobacterium rhizogenes*-mediated transformation of *Rubia peregrina* L.: *in vitro* accumulation of anthraquinones. *Plant Cell Tiss. Org. Cult.* 46(2): 103-108.

Long, D. J., Jaiswal, A. K. and David , K. 2000. New anthracene derivatives from the roots and stems of *Rubia cordifolia. Interact. chem. Biol* 129: 99-112

Lopaczynski, W. and Zeisel, S. H. 2001. Antioxidants, programmed cell death, and cancer. *Nutr. Res.* 21: 295-307

Lu, H., Yue, J. and Feng, C. 2004. Preparative high-speed counter-current chromatography for purification of shikonin from the Chinese medicinal plant *Lithospermum erythrorhizon. J. Chromatography.* 1023(1): 159- 163

Marcocci, L., Maguire, J. J., Droy-Lefaix, M. T., and Packer, L. 1994. The nitric oxide scavenging property of *Ginkgo biloba* extract EGb 761. *Biochem. Biophys. Res. Commun.* 201:748-755

Margherita B., Paolo, C., and Pierre, D. 1996. Callus growth and somatic embryogenesis in thalamus tissue of *Ranunculus asiaticus* L. Cultivated *In vitro* : Cytokinin effect and phenol metabolism. *In Vitro Cellular & Developmental Biology -Plant.* 32(3): 154-160

Marzena, W. and Henryk, U. 1999. Glucotropaeolin and myrosinase production in hairy root cultures of Tropaeolum majus. *Plant Cell Tiss. Org. Cult.* 57(1): 39-45

Mathur, J. and Ahuja, P. S. 1990. Plant regeneration from callus cultures of *Valeriana wallichii* DC. *Plant Cell Rep.* 9(9): 523-526

Matthews, R. and Duncan, E.J. 1993. A method to reduce microbial contamination in *in vitro* culture. *Third Caribbean Biotechnology Conference.* 160-166

McBride, J. M. and Kraemer, W. J. 1999. Free radicals, Exercise and Antioxidant. *J. strength and conditioning Res.* 13(2): 175- 183

McCord, J. M. and Fridovich, I. 1969. Superoxide dismutase: An enzymic function of erythrocuprein (hemocuprein). *J. Biol. Chem.* 244: 6049-6055

Meazza, G., Dayan, F. E. and Wedge, D. E. 2003. Activity of quinones on *Colletotrichum* species. *J. Agric. Food Chem.* 51:3824-3828

Menke, F. L., Champion, A., Kijne, J. W. and Memelink, J. 1999. A novel jasmonate- and elicitor-responsive element in the periwinkle secondary metabolite biosynthetic gene Str interacts with a jasmonate- and elicitorinducible AP2-domain transcription factor, ORCA2. *EMBO. J.* 18:4455–4463

Meena, M. C. and Patni, V. 1985. Isolation and Identification of "Quercetin" from *Citrullus colocynthis (Linn.)* Schrad. *Asian J. Exp. Sci.* 22(1): 137-142.

Merkli, A., Christen, P., and Kapetanidis, I.1997. production of disogenin by hairy root cultures of *Trigonella foenum*-graecum. L. *Plant Cell Rep.* 16:632-636.

Metzger W. and Reif K. 1996. Determination of 1,8-dihydroxyanthranoids in senna. *J. Chromatography A.* 740(1):133-138

Misawa, M., T. Endo, A., Goodbody, J., Vukovic, C., Chapple, L. Choi, C., and Kutney, J. P. 1988. Synthesis of dimeric indole alkaloids by cell free extracts from cell suspension cultures of *C. roseus. Phytochem.* 27: 1355-1359

Misawa, M. and Endo, J. 1995. Plant cell and tissue culture: Alternatives for metabolite production. *Biotechnol. Adv.* 13(3): 425-453

Mischenko, N. P., Fedoreyev, S. A., Glazunov, V. P., Tchernoded, G. K., Bulgakov, V. P. and Zhuravlev ,Y. N.1999. Anthraquinone production by callus cultures of *Rubia cordifolia. Fitoterapia.* 70:552–557

Mizukani, H., Ogawa, T., Ohashi, H. and Ellis, B. E. 1997. Induction of rosmarinic acid biosynthesis in *Lithospermum erythrorhizon* cell suspension cultures by yeast extract. *Pl. Cell Rep.* 11: 480–483

Mizukami, H., Konoshima, M. and Tabata, M. 1977. Effect of nutrition factors on shikonin derivative formation in *Lithospermum* callus cultures. *Phytochem.* 16: 1183–1186

Mizukami, H., Tabira, Y. and Ellis, B. E. 1992. Methyl-jasmonate-induced rosmarinic acid biosynthesis in *Lithospermum erythrorhizon* cell suspension cultures. *Pl. Cell Rep.* 12: 706–709

Mizutani, H., Hashimoto, O., Nakashima, R. and Nagai, J. 1997. Anthraquinone production by cell suspension cultures of *Rubia akane* NAKAI. *Biosci. Biotech. Biochem.* 61:1743–1744

Monks, C., Freiberg, B., Kupfer, H., Sciaky, N. and Kupfer, A. 1998. Three-dimensional segregation of supermolecular activation clusters in T cells. *Nature.* 395: 82-86

Moreno, P. R. H., Poulsen, V., Vander- Heijden, R. and verpoorte, R. 1996. Effects of elicitation on different metabolic pathways in *Catharanthus roseus* (L) G. Don cell suspension cultures. *Enzyme. Microb. Technol.* 18 (2): 99-107

Morita, H., Yamamiya, T. Kondo, T. 1992. Conformational analysis of antitumor cyclichexapeptides, RA series. *Tetrahedron.* 47(16-17): 2757-2772

Mukundan, U. and Hjortso, M. A. 1990. Thiophene content in normal and transformed root cultures of *Tagetes erecta*: A comparison with thiophene content in roots of intact plants. *J. Exp.Bot. 41*: 1497-150

*Murashige, T, 1974. Plant propogation through tissue cultures. *Ann. Rev. Plant Physiol.* 25:135- 166

*Murashige, T. and Skoog, F. 1962. A Revised Medium for Rapid Growth and Bioassays with Tobacco Tissue Cultures. *Physiol. Planta* 15: 473-497

Murti, V.V.S., Seshadri , T.R. and Sivakumaran, S.1997. Anthraquinones of *Rubia cordifolia* (Rubiaceae) *Phytochem.* 11 (4): 1524

Nadha, H. K.2006. Clonal propagation OF *Tylophora indica*- An important medicinal plant through tissue culture. M. Sc (Biotechnology)Desertation Report, Thapar Institute of Engineering and Technology, Patiala, India, pp-12 -20

Nahalka, J., Blanarik, P., Gemeiner, P., Matusova, E., and Partlova, I. 1996. Production of plumbagin by cell suspension cultures of *Drosophyllum lusitanicum* Link . *J. Biotechnol.* 49: 153-163

Nair, A. J., Sudhakaran, P. R., Rao, J. M., and Ramakrishna, S. V. 1992. Beberine synthesis by callus and cell suspensions of *Coscinium fenestratum. Plant Cell Tiss. Org. Cult.* 29:7-10

Namdeo A. G. 2007. Plant Cell Elicitation for Production of Secondary Metabolites *Pharmacognosy Rev.* 1(1):13

Namdeo, A. G. 2004. Investigation on pilot scale bioreactor with reference to the synthesisof bioactive compounds from cell suspension cultures of *Catharanthus roseus* Linn.Ph.D. Thesis, Devi Ahiya Vishwavidyalaya, Indore, M.P. India.pp. 12-47

Namdeo, A., Patil, S. and Fulzele, D. P. 2000. Influence of fungal elicitors on production of ajmalicine by cell cultures of *Catharanthus roseus. Biotechnol Prog.* 18:159–162

Natarajan, K. S., Narasimhan, M., Shanmugasundaram, K. R. and Shanmugasundaram, E. R. B. 2006. Antioxidant activity of a saltspice-herbal mixture against free radical induction. *J. Ethnopharmacol.*105: 76–83

Neill, S. J, Desikan, R., Clarke, A., Hurst, R. D. and Hancock, J. T. 2002. Hydrogen peroxide and nitric oxide as signalling molecules in plants. *J. experiment. Bot.*53, 1237–1247

Nie, G., Cao, Y., and Zhao, B. 2002. Protective effects of green tea polyphenols and their major component, (-)-epigallocatechin-3-gallate (EGCG), on 6-hydroxydopamine-induced apoptosis in PC12 cells", *Redox Rep.* 1351(2): 171-176

Noble, R. L. 1990. The discovery of the vinca alkaloids--chemotherapeutic agents against cancer. *Biochem. Cell Biol.* 68(12):1344-51

Odake, K., Ichi, T. and Kusahara, K. 1991. In: Komamine A (ed) Plant Cell Culture in Japan. *CMC,* Tokyo, pp 301-325

Ohlsson, A. B. Bjork, L. and Gatenbeck, S. 1983. Effect of light on cardenolide production of pigments by entrapped cells. *J. Biotech.* 2:107-117

Okamura, N., Asai, M., Hine, N., and Yagi, A. 1996 High-performance liquid chromatographic determination of phenolic compounds in *Aloe* species. *J. Chromatography A.* 746(2): 225-231.

Okhawa, H., Ohishi, W., and Yagi, K. 1979. Assay formulation lipid peroxides in animal tissues by thiobarbituric acid reaction. *Anal. Biochem.* 95: 351.

Oliveira, M. D., Negri, G., Salantino, A. and Braga, M. R. 2007. Detection of anthraquinone and identification of 1,4-naphthoquinone in cell suspension cultures of *Rudgea jasminodes* (Cham) Mull.Arg.(Rubiaceae). *Revista Brasil.Bot.* 30(1): 167-172

Ouyang, J., Wang, X., Zhao, B. and Wang, Y. 2004. Enhanced production of phenylethanoid glycosides by precursor feeding to cell culture of *Cistanche deserticola. J. Biotechnol.* 101(2): 181-187.

Pakulski, G. and Budzianowski, J. 1996. Ellagic acid derivatives and naphthoquinones of *Dionaea muscipula* from *in vitro* cultures. *Phytochem.* 4: 775–778

Pandey, M. M., Govindarajan, R., Rawat, A., and Pushpangadan. P. 2005. Free radical scavenging potential of *Saussarea costus. P. Acta Pharm. 55:* 297–304.

Panichayaupakaranant, P. and Twetrakul, S. 2002. Plumbagin production by root cultures of *Plumbago rosea*. *Electronic Journal of Biotechnology*. 5(3): 228-232.

Panichayupakaranant, P. 2001. Naphthoquinones formation in *Impatiens balsamina* cell cultures. *Pharma. Biol.* 39(4): 293-296

Parimala, R. and Sachdanandam, P.1993. Effect of plumbagin on some glucose metabolizing enzymes studied in rats in experimental hepatoma. *Mol. Cell. Biochem.* 125: 59 -63

Pauly, N., Chiara Pucciariello, C., Karine Mandon, K., Gilles I. and Jamet, A. 2006. Reactive oxygen and nitrogen species and glutathione: key players in the legume–*Rhizobium symbiosis* . *J. experiment. Bot.* 57(8): 1769–1776

Pauthe-Dayde, D., Rochd, M. and Henry, M. 1990. Triterpenoid saponins production in callus and multiple shoot cultures of *Gypsophila* spp. *Phytochem.* 29 2: 483-487

Pedro, L., Sousa, M. J., Novais, J. M. and Pais, M. S. S. 1990. Callus and suspension cultures from Geranium robertianum L (Geraniaceae) *Biotechnol. Lett.* 12(6): 439-442

Poli, G., Leonarduzzi, G., Biasi, F., and Chiarpotto, E. 2004. Oxidative stress and cell signaling. *Curr. Med. Chem.* 11:1163-1182.

Powell, R. G., Weisleder, D., Smith, C. R. and Rohwedder, W. K.1969. Structures of harringtonine, isoharringtonine, and homo-harringtonine. *Tetrahedron Lett.* 11: 815-818

Prathumpai, W., Kocharin, K., Phimmakong, K. and Wongsa, P. 2007. Effects of different carbon and nitrogen sources on naphthoquinone production of Cordyceps unilateralis BCC 1869. *Appl.Biochem.Biotechnol.* 136(2): 223-232

Ramachandra Rao, S. and Ravishankar, G. A. 2000. Biotransformation of protocatechuic aldehyde and caffeic acid to vanillin and capsaicin in freely suspended and immobilized cell cultures of *Capsicum frutescens*. *J. Biotechnol.* 76: 137-146

Ramnath, V., Girija, K. and Ramadasan, K. 2003. Inductuion of apoptosis in the Dalton's lymphoma ascites(DLA) cells by abrin. *Amala Res. Bull.*23: 47-51

Rao, M. G. M., Rao, C. V., Pushpangadan, P. 2006. Epatoprotective effects of rubiadin,a major constituent of *Rubia cordifolia* Linn. *J. Ethnopharmacol.*103: 484-490

Rao, R. S., Tripathi, U., Suresh, B. and Ravishankar, G. A. 2001. Enhancement of secondary metabolite production in hairy root cultures of beta vulgaris and *Tagetes patula* under the influence of microalgal elicitors *Food Biotechnol.* 15(1): 35 – 46

Rao, B. R., Kumar, D. V., Amrutha, R. N., Jalaja, N. K., Vaidyanath, A., Rao, M., Rao, S., Polavarapu, R. and Kavi Kishor, P. B. 2008. Effect of growth regulators, carbon source and cell aggregate size on berberine production from cell cultures of *Tinospora cordifolia Miers.Curr. Trends in Biotechnol. Pharma.* 2: 269 -276.

Rao, K.N., Bhattacharya, R.K. and Venkatachalam, S.R.1997. Inhibition of thymidylate synthase and cell growth by the phenanthroindolizidine alkaloids pergularinine and tylophorinidine. *Chem. Biol. Interact.*106:201-212

Rao, M.V., Lee H. I., Creelman, R. A., Mullet, J. E. and Davis, K.R *2000. Jasmonaic acid signaling modulates ozone-induced hypersensitive cell death. Pl. Cell Rep.*12:1633–1646

Rao, P. K., Rao, S. S. and Sadanandam, M. 1999. Tissue culture studies of Centella asiatica. *Indian J. Pharm. Sci.* 23: 392-393

Rawal, A., Muddeshwar, M. and Biswas, S. 2004. Effect of *Rubia cordifolia, Fagonia cretica.* linn, and *Tinospora cordifolia* on free radical generation and lipid peroxidation during oxygen–glucose deprivation in rat hippocampal slices. *Biochemical and Biophysical Research communication.* 34: 588-596

Reddy, G. S., Kumar, D.D., Babu, R.S. and Mathavi, M. 2005. Callus cultures and synthetic seed production in *Rauvolfia serpentina. Indian. J. Hort.* 62 (1): 102-103

Rehm, S. and Devor, D. E. 1993. Acute effects of 4-ipomeanol on experimental lung tumors with bronchiolar or alveolar cell features in Syrian hamsters or C3H/HeNCr mice. *J. Cancer Res. Clin. Oncol.* 120(1-2): 41-50

Reif, A., Luigi, Z., Peter, R., Martin, F., and Harald, H. H. W. 2001. Nitroxyl oxidizes NADPH in a superoxide dismutase inhibitable manner. *Free Radic. Biol. Med.* 30(7): 803-808

Rhodes, M. J. C., Parr, A. J., Giulietti, A., and Aird, E. L. H. 1994. Influence of exogenous hormones on the growth and secondary metabolite formation in transformed root cultures. *Plant Cell Tiss. Org. Cult.* 38 :143–151

*Rijhwani, S. K. and Shanks, J. V. 1996. Effect of fungal wall components on indole alkaloid synthesis by *Catharanthus roseus* hairy root cultures. *Abstract papers of 211 th National meeting of Amenrica Chemical Society*, New Orleans, USA, 25-28 October. p-56

Rodrigo, R. K. Raddi, M. S. G., Lourdes, C. S. and Wagner, V. 2004. A new cytotoxic naphthoquinone from *Paepalanthus latipes, Chem. Pharm. Bull.* 52(12): 1487—1488

Rokem, J. S. and Goldberg, I. 1985. Secondary metabolites from plant cell suspension cultures: methods for yield improvement. *Adv. Biotechnol. Processes.* 4: 241–274

Rosenkranz, A. R., Schmaldienst, S., Stuhlmeier, K. M., Chen, W., Knapp, W. and Zlabinger, G. J. 1992. A microplate assay for the detection of oxidative products using 20,70-dichlorofluorescin- diacetate. *J. Immunol. Meth.* 156: 39–45

Roy, M.K., Kobori, M., Takena, M. Nakahara, K.and Shinomoto, H. 2007. Antiproliferatve effect on human cancer cell lines after treatment with nimbolide extracted from an edible part of *Azadirachta indica. Pytother Res.* 21 (3) 254 -250

Roy, A. Ghosh, S. Chaudhuri, M., and Saha, P. K. 2008. Effect of different plant hormones on callus induction in *Gymnema sylvestris* R.Br. (Asclepiadaceae). *Afr. J. Biotech.* 7 (13): 2209-2211

Rozita, O., Abdullah, M. A., Hasan, M. A. and Marziah, M. 2004. Development of growth medium for *Centella asiatica* cell culture via response surface methodology. *Am. J. Appl. Sci.* 1(3): 215-219

Rudge, K. and Morris, P. 1986. The effect of osmotic stress on growth and alkaloid accumulation in catharanthus roseus. In *Secondary metabolism in plant cell cultures* (eds), Morris P., Scrag g A H., Stafford A., and Fowler M W .Cambridge University Press, Cambridge, pp. 75-81

Saker, M.M., Rady, M. R., and Ghanem, S.A. 1997.Elicitiation of tropane alkaloids in suspension cultures of *Hyoscyamus, Datura* and *Atropa* by osmotic stress. *Fitoterapia.* 68(4): 338-342

Salganik, R. I., Sivashinskiy, M. S., Lopaczinsky, W., Gulij, M., Veisman, A., Bokovanov, V. E., and Zeisel, S. H. 2001. Controlling the anticancer activity of cisplatin by changing the redox state of MCF-7 breast cancer cells. Carcinogenesis, in press.

Sanker, M A. 1998. *In vivo* and *in vitro* screening of *Sida* spp. for ephedrine content. Ph.D. (Hort.) thesis,Kerala Agricultural University, Thrisssur, India. pp 198-206

Sanker, M. A. and Nair, G. S. 1997. Effect of cytokinin on callusing in *Sida* spp. In : *Proceedings of the National Symposium on Emerging trends in plant tissue culture and molecular biology* held at Osmania University from Jan. 20-31, pp 36-37

Sateeshkumar, K. and Bhavanandan, K. 1989. Regeneration of plant from leaf callus *Plumbago rosea* Linn. *Indian J. Exp. Biol.* 27(4): 368-369

Sato, K. T., Yamazaki, E., Okuyama, K., Yoshihira, H. and Shimomura, K.1991. Anthraquinone production by transformed root cultures of *Rubia tinctorum* : influence of phytohormones and sucrose concentration. *Phytochem.* 30: 1507-1509

*Schenk, R.U. and Hildebrandt, A.C. 1972. Medium and techniques for induction and growth of monocotyledonous and dicotyledonous plant cell culture. *Canadian J. Bot.* 50: 199-203

Schiel, 0., Jarchow-Redecker, K., Piehl, G. W., Lehman, J. and Berlin, J. 1984. Increased formation of cinnamoyl putrescines by fedbatch fermentation of cell suspension cultures of *Nicotiana tabacum. Plant Cell Rep.* 3: 18-20

*Schripsema, J and Verpoorte, R. 1992. Search for factors involved in indole alkaloid production in cell suspension cultures of *Tabernaemontana divaricata*. *Planta Med.* 58: 245–249

Schripsema, J., Ramos, V. A. and Verpoorte, R. 1999. Robustaquinones, novel anthraquonines from elicited *Cinchona robusta* suspension culture. *Phytochem.* 51: 55-60

Schuetzle, D., Lee, F. S. and Prater, T. J. 1981. The Identification of Polynuclear Aromatic Hydrocarbon (PAH) Derivatives in Mutagenic Fractions of Diesel Particulate Extracts. *Int. J. Environ. Anal. Chem.* 9:93–144

Schwarz, R. E., Donohue, C. A., Sadava, D. and Kane, S. E. 2003. Pancreatic cancer in vitro toxicity mediated by Chinese herbs SPES and PC- SPES: implications for monotherapy and combination treatment . *Cancer Lett.* 189(1):59-68

Sehrawat, A. R., Sanjogta, U. and Chowdhury, J.B. 2002. Establishment of plantlets and evaluation of differentiated roots for alkaloids in *Rauvolfia serpentina*. *J. Plant Biochem. Biot.* 11(2): 105-108

Selma, R. D., Lucilene, A. L., Figueiredo, M. R ., Auxiliadora C. M. and Kaplan, M. A. 2004. Plumbagin quantification in roots of *Plumbago scandens* L. obtained by different extraction techniques. *Anais da Academia Brasileira de Ciências*. 76(3): 499-504

Shaljaraj, J. 1998. Studies on in vitro production of secondary metabolites from medicinal plants.Thesis (PhD) Cochin University of Science and Technology, Kerala, India, pp. 98-109

Shams-Ardakani, M.R., Mohagheghzadeh, A.,Ghannadi, A. and Barati, A. 2007. Formation of glycyrrhizin by *in vitro* cultures of *Glycyrrhiza glabra* L. *Chem. Nat. Compounds*. 43: 353-354

Seki, M., Ohzora, C., Takeda, M., Furusak, F. 2000. Taxol (paclitaxel) production using free and immobilized cells of *Taxus cuspidate Biotechnol. Bioeng.* 53(2):214 – 21

Shaneeja, V. M. 2007. Genetic transformation in *Artemesia annus.* L. for hairy root induction and enhancement of secondary metabolites. M.Sc. (Hort.) thesis, Kerala Agricultural University, Thrissur, India. pp. 126-130

Shang, F., Lu. M., Edward, D., Reddan, J. and Taylor, A. 2003. Vitamin C and vitamin E restore the resistance of GSH-depleted lens cells to H_2O_2. *Free Radic. Biol. Med.* 34(5): 521-530

*Sharma, P.V. 1969. Dravyaguna Vijnana, Chaukhambha Bharti Academy, Varanasi, 2-3, 928

Shen, W. H., Petit, A., Guern, J. and Temp, J.1988. Hairy roots are more sensitive to auxin than normal roots. *Proc. Natl. Acad. Sci.* 85:3417-3421

Sherwin, E. R. 1987. Oxidation and antioxidants in fats and oil processing. *J. Am. Oil Chem. Soc.* 55: 809-813

Shin, S .W. and Lyu, L. 2000. Effects of compounds related to signal transduction on anthraquinone biosynthesis in transformed cells of *Rubia cordifolia* var. pratensis. *Korean. J. Pharmacognosy.* 31(2): 235-239

Shrivastav, N. and Padhya, M. A. 1995. *Tinospora cordifolia* Miers. A review *Hamdard Medicus* 38(3):85-90

Siems, W. G., Grune, T., & Esterbauer, H. (1995). 4-Hydroxynonenal formation during ischemia and reperfusion of rat small- intestine. *Life Science.* 57: 785–789

Simoneit, B. R. T., Sheng, G. Y., Chen, X. J., Fu, J. M., Zhang, J. and Xu, Y. P.1991. Molecular Marker Study of Extractable Organic-Matter in Aerosols from Urban Areas of China, *Atmos. Environ.* A-25:2111–2129

Sindhu, M 1999. *In vitro* callus induction and its exploitation in *Coscinium fenestratum* (Gaertn) Colebr. M.Sc (Hort) thesis, Kerala Agricultural University, Thrissur, India, pp. 126-130

Singh, R. and Geetanjali, S. 2005. Isolation and synthesis of anthraquinones and related compounds of *Rubia cordifolia*. *J. Serb. Chem. Soc.* 70 (7): 937–942

Singh, R., Geetanjali. S. and Chauhan, S. M. 2004. 9, 10-Anthraquinones and other biologically active compounds from the genus *Rubia*. *Chem Biodiversity*. 1(9): 1241- 1264

Siripong, P. Kanokmedakul, K., Piyaviriyagul, S., Yahuafai, J., Chanpai, R., and Ruchirawat, S. 2006. Antiproliferative naphthoquinone esters from Rhinacanthus nasutus Kurz. roots on various cancer cells. *J. Tradit. Med.* 23(5):166-172

Sivarajan, V. V., & Balachandran, I. 1994. Ayurvedic drugs and their plant sources. In P. Mohan (Ed.) Oxford: IBH Publishing Co Pvt. Ltd. pp. 456-512

Smith, J. L., Smart, N. J., Misawa, M., Kurz, W. G. W., Tallevi, S. G. and Dicosmo, F. 1987.Increased accumulation of indole alkaloids by some cell lines of *Catharanthus roseus* in response to addition of vanadyl sulphate. *Pl. Cell Rep.* 6: 142-145

Smith, M. A., Perry, G., Richey, P. L., Sayre, L. M., Anderson, V. E. and Beal, M. F. 1996. Oxidative damage in Alzheimer's [letter]. *Nature*. 382: 120

Smith, M. A., Rottkamp, C. A., Nunomura, A., Raina, A. K. and Perry, G. (2000). Oxidative stress in Alzheimer's disease. *Biochimica et Biophysica Acta*, 1502, 139–144

Son J. K., Soon J. J., Ji. H. J., Zhe, F., Chong, S. L., Chang, S., Dong, C. 2005. Anticancer constitutents from the roots of Rubia cordifolia L. *Chem. Pharm. Bull.* 23: 1245- 1248

Soon-Hee, S. 1989. Studies on the production of anthraquinone derivatives by tissue culture of *Rubia species. J. Biotech.* 12 (2):99-102

Stadtman, E. R. 2004. Role of oxidant species in aging. *Curr. Med. Chem.* 11:1105–1112

Stalman, M., Koskamp,A. M., Luderer, R., Vernooy, J. H. J., Wind, J. C., Wullems,G. J. and Croes, A.F. 2003. Regulation of anthraquinone biosynthesis in cell cultures of *Morinda citrifolia. J. Plant Physiol.* 160(6): 607-614

Stella, A. and Braga, M. R. 2002 .Callus and cell suspension cultures of *Rudgea jasminodes*, a tropical woody Rubiaceae. *Plant Cell Tiss. Org. Cult.* 68: 271-276

Subramoniam, A., Rajasekharan, S. and Latha, P.G. 1996. Immunomodulatory and antitumour activities of Janakia arayalpathra root. *Fitoterapia.* 67:140-4

Sudha, C. G. and Seeni, S. 1994. *In vitro* multiplication and field establishment of *Adhatoda beddomei* C. B. Clarke, a rare medicinal plant. *Plant Cell Rep.* 13: 203-207

Suffness, M. and Douros, J. 1982. Current status of the NCL plant and animal products program. *J. Nat. Prod.* 45: 1-14

Sun, H. L., Synder, C., Rapoport, D. H. and Pennock, J. F. 1968. Comparative studies on plastoquinones IV, plastoquinone in algae. *Plant Physiol.* 43: 1935-1940

Suryanarayanan, M. and Pai, J. S. 1998. Studies in micro propagation of Coleus forskohlii. *J. Med. Arom. Plant Sci.* 20: 379-382

Suzuki, H., Matsumoto, T., and Obi, Y. 1982. In Fujiwara A (ed.): *Plant Tissue Culture.* Japanese Association for Plant Tissue Culture, Tokyo, pp 285

Tabata, M. 1975. In: Barz W, Reinhard E, Zenk NH (eds) *Plant tissue culture and its biotechnological application.* Springer-Verlag, Berlin Heidelberg New York, p326

Tabata, M., Yamamoto, H., Hiraoka, N., and Konoshima. M. 1972. Organization and alkaloid production in tissue cultures of *Scopolia parviflora. Phytochem.* 11: 949-955

Tabata, M., Mizukami, H. M., Hiraoka, N. and keneshina, M. 1974. Pigment formation in callus cultures of *Lithospermum erythrorhizon. Phytochem.* 13: 927-934

*Takimoto, C.H, and Arbuck, S.G. 1996.The camptothecins, cancer chemotherapy and biotherapy, In: Bruce, A.C and Dan, L. L. (Eds).2nd Lippincott Riven Publishers, Philadelphia, 463

Talapatra, S. K., Sarkar, A. C. and Talapatra, B. 1981. Two pentacyclic triterpenes from *Rubia cordifolia. Phytochem.* 20: 1923–1927

Tegelen, L. Pvan .J., Bongaerts, R.J.M., Croes, A. F., Verpoorte, R., Wullems, G. J. 1999. Isochorismate synthase isoforms from elicited cell cultures of *Rubia tinctorum. Phytochem.* 51(2): 263-269

Thanh, N.T., Murthy, H.N., Yu, K.W., Hahn, E. J., and Paek, K.Y. 2005. Methyl jasmonate elicitation enhanced synthesis of ginsenoside by cell suspension cultures of *Panax ginseng* in 5-l balloon type bubble bioreactors. *Appl. Micro. Biotechnol.* 67 (2): 197-201

Thomson, R. H. 2005. Distribution of naturally occurring quinines. *J. World Sci.* 13(2): 70-*73*

Thomson, R.H., In *Naturally Occurring Quinones,* 2nd Ed., Academic Press, London, 1971

Trapp, S. C. and Croteau, R. B. 2001. Genomic organization of plant terpene synthases and molecular evolutionary implications. *Genet.* 158: 811-832

Tripathi, Y. B. and Shukla, S. D. 1998. *Rubia cordifolia* extract inhibits cell proliferation in A-431 cells. *Phytotherapy Res.*12(6): 454 – 456

Tripathi, Y. B., Sharma, M., and Manickam, M. 1998. Rubidianin, a new antioxidant from *Rubia cordifolia. Indian J. Biochem. Biophys* . 34:523-6

Tripathi, Y.B. and Chaturvedi, P.1995. Assessment of endocrine response to Inula racemosa in relation to glucose homeostasis in rats. *Indian. J .Exp. Biol.*33:686-9

Tripathi, Y.B. and Sharma, M., 1998. Comparison of the antioxidant action of the alcoholic extract of *Rubia cordifolia* with rubiadin. *Indian J. of Biochem. and Biophy.* 35: 313

Tripathi, Y.B., Chaurasia, S., Tripathi, E., Upadhyay, A. and Dubey,G.P.1996. *Bacopa monniera* Linn. as an antioxidant: mechanism of action. *Indian. J. Exp. Biol.*34:523-6

Tripathi, Y.B., Tripathi, E. and Upadhyay, A. 1996.Antilipid peroxidative property of Nardostachys jatamanasi. *Indian. J. Exp. Biol.*,34:1150-1

Trypsteen, M., Van Lijsebettens, M., Van Severen, R., and Van Montagu, M. 1991. *Agrobacterium rhizogenes* mediated transformation of *Echinacea purpurea*. *Pl. Cell Rep.* 10: 85-89

Upadhyay, N., Makoyeyenchuk, A Y., Nikolaeva, L. A. and Batygina, T. B. 1992. Organogenesis and somatic embryogenesis in leaf callus cultures of *Rauawolifa caffra* Sond. *J. Pl. Physiol.* 140(2): 218-222

Valko, M., Leibfritz, D., Moncol, J., Cronin, M. T. D., Mazur, M. and Telser, J. 2007. Free radicals and antioxidants in normal physiological functions and human disease. *Int. J. Biochem. Cell Biol.* 39: 44–84

Valko, M., Rhodes, C. J., Moncol, J., Izakovic, M. and Mazur, M. 2006. Free radical metals and antioxidants in oxidative stress-induced cancer. *Chemico. Biol. Interaction.*160: 1–40

Van der Plas, L. H. W., Eijkelboom, C. and Hagendoorn, M. J. M. 1995. Relation between primary and secondary metabolism in plant cell suspensions. *Plant Cell Tiss. Org. Cult.*43: 111–116

Van Gulik, W. M., Ten Hoopen, H. J. G., and Heijnen, J. J. 1990. Kinetics and stoichiometry of growth of plant cell cultures of Catharanthus roseus and Nicotiana tabacum in batch and continuous fermentors. *Biotechnol. Bioeng.* 40:863–874

Van Hengel, A.J., Harkes, M. P, Wichers, H. J., Hesselink, P.G. M., and Buitelaar, R. M.1992. Characterisation of callus formation and camptothecin production by cell lines of *Camptotheca acuminata. Plant Cell Tiss. Org. Cult.* 28:11–18

Vanishree, M., Lee, C. Y., Lo, S. F., Nalawade, S. M., Lin, C.Y. and Tsay, H. S. 2004. Studies on the production of some important metabolites from medicinal plants by plant tissue cultures. *Bot. Bull. Acad. Sin.* 45: 1-22

Varghese, S. 2006. Genetic transformation for hairy root induction and enhancement of secondary metabolites in Aswagandha (*Withania somnifera.* L. Duanl). M.Sc. (Hort.) thesis, Kerala Agricultural University, Thrissur, India. Pp 132-140

Voces, J., Alvarez, A. I., Vila, L., Ferrando, A., Cabral, C., and Prieto, J. G. 1999. Effects of administration of the standardized *Panax ginseng* extract G115 on

hepatic antioxidant function after exhaustive exercise. *Comparative Biochem. Physiol.* 123: 175-184

*Wagner, H. and Bladt, S. 1996. Plant drug analysis: *A Thin Layer Chromatography Atlas*, Berlin Springer-Verlag, New York. pp

Wall, M.E, Wani, M.C., Mcphail, A.T, and Sim, G. 1966. Plant antitumor agents: I. The isolation an structure of camptothecin, a novel alkaloidal leukemia and tumor inhibitor from camptotheca acuminate. *J. Am. Chem. Soc.* 88:3888 - 3890

Wang, S. Y., Hsu, M. L., Hsu, H. C., Tzeng, C. H., Lee, S.S., Shiao, M. S., and Ho, C. K. 1997. The anti-tumor effect of Ganoderma lucidum is mediated by cytokines released from activated macrophages and T lymphocytes. *Int. J. Cancer.*70(6):699-705

Wang, W, and Zhong, J. J. 2002. Manipulation of ginsenoside heterogeneity in cell cultures of *Panax notoginseng* by addition of jasmonates. *J. Biosci. Bioeng.* 93:48–53

Wani, M.C, Talyor, H.L, Wall, M E. and Coggon, P.1971.Plant antitumor agents VI . the isolation and structure of taxol, anovel antileukemic and antitumor agent from *Taxus brevifolia* . *J. Am. Chem. Soc.* 93: 2325-2327

Watson, D. G. and Brooks, C. J. W. 1984. Formation of caspidol in Capsicum annum fruits in response to non specific elicitors. *Physiol. Plant Pathol.* 24: 231-337

Webster, Seymour, Mitchell, S.A., and Ahmad, M.H. 2003. A novel surface sterilization method for reducing Microbial contamination of field grown medicinal Explants intended for *in vitro* culture. *Crit. Rev. Plant Sci.* 13(2): 139-183

White, F. F., Taylor, B. H., Huffman, G. A., Gordon, M. P. and Nester, E. W. 1985. Molecular and genetic analysis of the transferred DNA regions of the root-inducing plasmid of *Agrobacterium rhizogenes. J. Bacteriol.* 164: 33–44

White, P. R. 1943. *A handbook Of Plant Tissue Culture.* Journal Cattel, Lancaster . pp 128- 214

Wijnsma, R.,Verpoorte, R., and Mulder, K. T. 1984. Anthraquinones in callus cultures of *Cinchona ledgeriana. Phytochem.* 23:2307-2311

Williams, S. D., Birch, R., Einhorn, L. H.1987. Treatment of disseminated germ-cell tumors with cisplatin, bleomycin, and either vinblastine or etoposide. *N. Engl. J. Med.* 316:1435-1440

Wink, M. 1984. Wounding induced increasing quinolozonde alkaloid accumulation in lupin leaves. *Z. Naturforsch.* 38: 905-909

Wink, M. 1985. Evidence for an extracellular lytic compartment of plant cell suspension cultures: the culture medium. *Naturwissensechafen* 71: 635-636

Winter, H. and Huber, S. C. 2000. Regulation of sucrose metabolism in higher plants: localization and regulation of activity of key enzymes.*Crit. Rev. Pl. Sci* 19:31–67

Woo, H. C., Seong, S. H,. Seon, A. L., Xiang, H. H., Kyong, S. L., Myung, K.L., Bang, Y. H.and Jai, S. R. 2005. Monoamine Oxidase Inhibitory Naphthoquinones from the Rootsof *Lithospermum erythrorhizon. Arch. Pharm. Res.* 28(4): 400-404

Wu, J. and Lin, L.2003. Enhancement of taxol production and release in *Taxus chinensis* cell cultures by ultrasound, methyl jasmonate and in situ solvent extraction. *Appl. Micro.Biotechnol.*62 (2-3): 151-155

Wu, L. J., and Wang, S. X. 1991. 6- Methoxygeniposidic acid, an iridoid glycoside from *Rubia cordifolia. Phytochem.*30(5): 1710-1711

Xu, H. Kim, Y. K., Jin, X., Lee, S. Y., and Park, S. U. 2008. Rosmarinic acid biosynthesis in callus and cell cultures of *Agastache rugosa* Kuntze. *J. Med. Plant Res.* 2(9): 237-241

Yamaomoto, H. 2000. Caffeic acid oligomers in *Lithospermum erythrorhizon* cell suspension cultures. *Phytochem.* 53(6): 651-657

Yanbin, L., Rui, L., Cuirong, S. and Yuanjiang, P..2001. An effective high-speed countercurrent chromatographic method for preparative isolation and purification of mollugin directly from the ethanol extract of the Chinese medicinal plant *Rubia cordifolia. Journal of Separation Science.* 30 (9): 1313-1317

*Yeoman, M. M. Holden, M. A. Corchet, P. Holden, P. R. Goy, J. G. and Hoibbs, M. C. 1990. Exploitation of diorganised plant cultures for the production of secondary metabolites. In: *Secondary products from plant tissue culture.* (eds.) Charlwood, B. V. and Rhodes, M. J. C. Clarendon Press, oxford, pp-139-165

Yu, K. W., Gao, W.Y., Hahn, E. J. and Paek, K. Y. 2002. Jasmonic acid improving ginsenoside accumulation in adventitious root culture of *Panax ginseng C.A. Meyer. Biochem Eng J,* 11:211–215

Zdravkovic-Korac, S., Muhovski, Y., Druart, P., Calic, D., and Radojevic, L. 2004. Agrobacterium rhizogenes-mediated DNA transfer to *Aesculus hippocastanum* L. and the regeneration of transformed plants. *Pl. Cell Rep.* 22 :698

Zenk, M. H., El-Shagi, H. and Schulte, U. 1975. Anthraquinone production by cell suspension cultures of *Morinda citrifolia. Planta Med.Suppl.* 79–101

Zenk, M. H., El-Shagi, H., Arens, H., Stockigt, J., Weiler, E. W., and Deus, B. 1997. Formation of indole alkaloids serpentine and ajmalicine in cell suspension cultures of Caratharanthus roseus.In: *Plant tissue culture and its Biotechnological application* (eds.) Barz, W., Reinhard, E., Zenk, M. H. Springer Verlag, Berlin, pp 17-43

Zhang, D., Yasuda, T., Yu, Y., Zheng, P., Kawabata, T., Ma, Y. and Okada, S. 1996. Ginseng extract scavenges hydroxyl radical and protects unsaturated fatty acids from decomposition caused by iron-mediated lipid peroxidation. *Free Radic. Biol. Med.* 20: 145-50

Zhang, Y. H., Zhong, J. J. and Yu, J. T. 1995. Regulation of apparent viscosity and O2 transfer coefficient by osmotic pressure in cell suspensions of *Panax notoginseng. Biotechnol. Lett.*17.1347–1350

Zhang, Y.H., Zhong, J.J., and Yu, J.T. 1996. Effect of nitrogen souce on cell growth and production of ginseng saponin and polysaccharide in suspension cultures of *Panax notoginseng. Biotechnol. Prog.* 12: 567-571

Zhang, L., Liu, C., Lin, L. and Chen, W. 2007. Callus induction and adventitious shoot regeneration from petiole of *Erigeron breviscapus. Plant Prod. Sci.* 10(3): 343-345

Zhao J, Fujita K, Sakai K (2005b) Oxidative stress in plant cell culture: A role in production of b-thujaplicin by *Cupressus lusitanica* suspension culture. *Biotechnol. Bioeng.* 90:621–631

Zhao, J., Davis, L.C. and Verpoorte, R. 2005. Elicitor signal transduction leading to production of plant secondary metabolites. *Biotechnol. Adv.* 23(4): 283-333

Zhong, J. J and Wang, D. J. 1996. Improvement of cell growth and production of ginseng saponin and polysaccharide in suspension cultures of *Panax notoginseng*: Cu^{2+} effect. *Journal of Biotechnology.* 46(1) : 69-72

Zhong, J. J. 2002. Plant cell culture for production of Paclitaxel and other Taxanes. *J. Biosci. Bioeneg.* 94(6): 591-599

Zhong, J. J., and Zhu, Q. X. 1995. Enhancement of ginseng saponin production in suspension cultures of *Panax notoginseng*: manipulation of medium sucrose *Appl. Biochem. Biotechnol.* 55: 241–247

Zhong, J.J. and Zhu, Q. X. 1995. Effect of initial phosphate concentration on cell growth and ginsenoside saponin production by suspended cultures of *Panax notoginseng*. *Appl. Biochem. Biotechnol.* 55: 241-246

Zhu, M., Phillipson, J.D., Yu, H., Greengrass, P.M., Norman, N.G., 1998. Application of radioligand-receptor binding assays in the search for the active principles of the traditional Chinese medicine. *Phytopath. Res.*11:231–236

Zhu, Y., Nam, J., Carpita, N. C., Matthysse, A. G., and Gelvin, S. B. 2003. *Agrobacterium*-mediated root transformation is inhibited by mutation of an *Arabidopsis* cellulose synthase-like gene. *Plant Physiol.* 133: 1000-1010

Zupan, J. R., Citovsky, V., Zambryski, P. 2000. *Agrobacterium* Vir E2 protein mediates nuclear uptake of single stranded DNA in plant cells. *Pro. Natl. Acad. Sci.* 93: 2392- 2397

APPENDIX

ANNEXURE I

Composition of MS basal medium (Murashige and Skoog, 1962)

Components	Quantity (mgl^{-1})
a) Major nutrients (Stock solution I)	
NH_4NO_3	1650
KNO_3	1900
$CaCl_2.2H_2O$	440
$MgSO_4.7H_2O$	370
KH_2PO_4	170
b) Minor nutrients (Stock solution II)	
H_2BO_3	6.20
$MnSO_4.4H_2O$	22.30
$ZnSO_4.7H_2O$	8.60
KI	0.83
$Na_2MoO_4.2H_2O$	0.25
$CuSO_4.5H_2O$	0.025
$CoCl_2.6H_2O$	0.025
c) Stock solution III	
$FeSO_4.7H_2O$	27.8
$Na_2EDTA.2H_2O$	37.3
d) Organic constituents (Stock solution IV)	
Myoinositol	100
Pyridoxine HCl	0.5
Glycine	2.0
Thiamine	0.1
Nicotinic acid	0.5
e) Sucrose	30 gl^{-1}
f) Agar	8 gl^{-1}

ABSTRACT

EXPLOITATION OF *IN VITRO* CULTURES OF INDIAN MADDER (*Rubia cordifolia*. Linn) FOR ANTICANCEROUS COMPOUNDS.

By

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ABSTRACT OF THE THESIS

Submitted in partial fulfilment of the requirement for the degree of

Master of Science in Agriculture

(PLANT BIOTECHNOLOGY)

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2009

ABSTRACT

The present investigation on "Exploitation of *in vitro* cultures of Indian Madder (*Rubia cordifolia* L.) for anticancerous compounds" was carried out at the Plant Tissue Culture Laboratory of the Centre for Plant Biotechnology and Molecular Biology, College of Horticulture, Vellanikkara and Amala Cancer Research Centre, Thrissur during the period 2006-2008. The study was undertaken with the objective to standardize the *in vitro* techniques for initiation and proliferation of static and suspension cultures of *Rubia cordifolia* and to screen the *in vitro* cultures for synthesis of naphthoquinone and quantify it. It was also envisaged to enhance the level of product synthesis in *in vitro* cultures and to assess the anticancerous activity of *in vitro* and *in vivo* extracts in terms of cytotoxicity, antioxidant and prooxidant activities *in vitro*.

Leaf, nodal and root derived callus cultures of *Rubia cordifolia* were established *in vitro*. Explants were pre treated with the fungicide, Bavistin 2.5 per cent for 15 minutes. Surface sterilization with mercuric chloride ($HgCl_2$) at 0.1 per cent for 1 min and 30 sec was effective for yielding healthy, contamination free cultures from nodal segments and leaves, respectively. MS medium at full strength, supplemented with NAA at 2 mg l^{-1} along with BA at 0.5 mg l^{-1} was observed ideal for initiation and proliferation of calli. The auxin synergist phloroglucinol, when supplemented to the medium, did not not yield encouraging results, with respect to callusing in the experimental species. Root derived cultures were inferior with respect to callus initiation and proliferation, registering low values for all the parameters studied. Incubating *in vitro* cultures under illuminated condition at 26 ± 2° C was superior to dark incubation, with respect to callus initiation and proliferation.

Chloroform – methanol at 8.5 :1.5 ratio was indentified as the appropriate solvent system for detection of naphthoquinone on thin layer chromatograms in the

test extracts of the experimental species, with alcoholic KOH (10 per cent) as the spray reagent.

Ms medium at full strength, fortified with NAA and BA at 2.0 mg l^{-1} and 0.5 mg l^{-1} respectively, which recorded maximum naphthoquinone synthesis, was standardized as the production medium.

Enhancing concentration of sucrose to 5 per cent in the production medium, did not elicit a positive response on naphthoquinone production *in vitro*. Reducing nitrate concentration of the production medium, to half and one fourth the original concentration, resulted in enhanced *in vitro* synthesis of the target compound. Supplementing the production medium with yeast extract (1 per cent and 2 per cent) as well as precursor feeding with phenyl alanine and tyrosine each at levels of 50 mg l^{-1}, 100 mg l^{-1} and 150 mg l^{-1} exerted a favourable influence on synthesis of naphthoquonines, *in vitro*. Incubation in dark resulted in marginal increase in *in vitro* production of naphthoquinones.

Incorporation of autoclaved mycelia of *Pythium aphanidermeatum* at levels of 2.0 per cent and 5.0 per cent resulted in enhanced *in vitro* production of naphthoquinone. The abiotic elicitor, salicylic acid at concentration of 10 µM and 100 µM resulted in maximum synthesis of naphthoquinones in *in vitro* root cultures (8.76 units g^{-1} calli) of *Rubia cordifolia*. Immobilization of test calli with sodium alginate – calcium chloride complex as well as subjecting the *in vitro* cultures to stress conditions, as imposed by sorbitol failed to bring about an enhancement in the *in vitro* production of naphthoquinones. None of the explants employed in the study induced hairy roots, when co- cultured with the *Agrobacterium rhizogenes* strains, MTCC 2364 and MTCC 532. Based on cell count, subculturing intervals of leafs, nodal and root derived suspension were fixed as 24, 27 and 27 days respectively with the respective packed cell volume as 0.93 per cent, 0.83 per cent and 0.80 per cent.

Naphthoquinone was detected, in *ex vitro* and *in vitro* test extracts at all levels of maturity tested. Both *ex vitro* and *in vitro* root extracts exihibited maximum cytotoxicity, as revealed by the percentage of cell death on DLA and EAC cell lines as well as their IC_{50} values. As compared to whole plant extract, *in vitro* systems of the experimental species exhibited least antioxidant action. Extent of pro-oxidant activity was higher in *in vitro* root extract of the experimental species.