Karyomorphological, cytotoxic, antitumour, RAPD and GC-MS assays in the *in vitro* and *in vivo* plants of *Artemisia nilagirica* (C. B. Clarke) Pamp.

Thesis
Submitted to the University of Calicut
for the award of the degree of
DOCTOR OF PHILOSOPHY in BOTANY

By

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DECLARATION

I, Leeja L., hereby declare that the thesis entitled **"Karyomorphological, cytotoxic, antitumour, RAPD and GC-MS assays in the *in vitro* and *in vivo* plants of *Artemisia nilagirica* (C. B. Clarke) Pamp."** submitted to the University of Calicut, for the award of the degree of DOCTOR OF PHILOSOPHY is a record of original research work done by me under the supervision and guidance of Dr John E. Thoppil, Reader in Botany, University of Calicut and that it has not formed the basis for award of any degree or diploma.

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CERTIFICATE

This is to certify that the thesis entitled **"Karyomorphological, cytotoxic, antitumour, RAPD and GC-MS assays in the *in vitro* and *in vivo* plants of *Artemisia nilagirica* (C. B. Clarke) Pamp."** submitted to the University of Calicut, for the award of the degree of DOCTOR OF PHILOSOPHY is an authentic record of original research work done by LEEJA L., during the period of her study (2002 - 2008) at the Genetics and Plant Breeding Division, Department of Botany, University of Calicut under my supervision and guidance and that it has not formed the basis for award of any degree or diploma.

DR JOHN E. THOPPIL
Supervising Teacher

Dedicated to
My dear sister Preeja Suresh, whom I remember with love at every passing breath .....

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LEEJA L.

CONTENTS

ABBREVIATIONS

2, 4-D	2, 4-Dichlorophenoxy Acetic Acid
ACL	Average Chromosome Length
AP-PCR	Arbitrarily Primed Polymerase Chain Reaction
BA	Benzyl Adenine
BAP	Benzyl Amino Purine
bp	base pair
CHIAS	Chromosome Image Analysis System
CS	Coefficient of Similitude
CTAB	Cetyl Trimethyl Ammonium Bromide
DI	Disparity Index
DLA	Daltons Lymphoma Ascites
DMSO	Dimethyl Sulfoxide
dNTP	deoxy Nucleotide Tri Phosphate
EAC	Ehrlich Ascites Carcinoma
EDTA	Ethylene Diamine Tetra Acetic acid
GC	Gas Chromatography
GC-MS	Gas Chromatography – Mass Spectrometry
HPLC	High Performance Liquid Chromatography
IAA	Indole 3- Acetic Acid
IBA	Indole 3-Butyric Acid
ILS	Increase in Life Span
kb	kilobase
KIN	Kinetin
µg	microgram
µl	microlitre
mM	milli molar
µm	micrometer
µM	micromolar
MS Medium	Murashige & Skoog Medium
NAA	α - Naphthyl Acetic Acid

ng	nanogram
nm	nanometer
OD	Optical Density
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
pg	picogram
RAPD	Random Amplified Polymorphic DNA
RCL	Range of Chromosome Length
RFLP	Restriction Fragment Length Polymorphism
SDS	Sodium Dodecyl Sulphate
SD	Standard Deviation
TAE	Tris Acetic acid EDTA
TCL	Total Chromosome Length
TE	Tris EDTA
TF%	Total Forma percentage
VC	Variation Coefficient

INTRODUCTION

Cancer is an unconquerable giant whose lethal effects are yet to be subdued. Despite many advances in chemotherapy and other modes of treatment, an acceptable formulation for the complete cure for cancer has been eluding scientists since time immemorial. World Health Organisation in its report (WHO, 1990) stated that cancer occupied the second position in the list of killer diseases in the industrially advanced countries, while in the developing world, it ranked the fifth. However, the Indian situation is fast changing and with most of the infectious diseases under control, today cancer as a cause of death occupies a position above that of the other developing countries.

In the Indian scenario, we cannot hope to be able to introduce sophisticated technologies for cancer control in a short time, as envisaged in the advanced countries. Therefore, the most feasible approach for India and other developing countries will be to adopt measures for prevention and early detection of cancer, to find inexpensive drugs for therapy and to improve facilities for palliative care. Unlike in the advanced countries, one of the major challenges of cancer research in the developing countries is to develop cheap and easily available drugs for cancer therapy. Investigation into the natural products and traditional medicine to explore the possibility of developing potent drugs from local resources should be given priority (Umadevi, 2000).

An ideal and effective anticancer agent, free from any side effects has not yet been discovered. Therefore, cancer chemoprevention has become increasingly important in the recent years. Intensive search for cancer chemo preventive agents has been done on medicinal plants and many novel phytochemicals have been discovered which could be successfully utilized by

the pharmaceutical industry for the manufacture of drugs. Plant derived compounds play a vital role in drug discovery programmes.

The plant kingdom is a rich source of drugs that inhibit cell proliferation. During the past quarter century, particularly after 1960, interest in plant and plant products as protectants against cancer has grown tremendously, mainly due to their successful use against some forms of cancer, following the specific use of two derivatives, vincristine and vinblastine from the plant *Vinca rosea* against Hodgkins disease and acute leukaemia in children. Screening of natural isolates for anticancer activity was conducted all over the world during the last five decades by organizations like United States National Cancer Institute (NCI), Eli Lilly, University of Illinois, Research Triangle Institute, Glaxo Wellcome, Shaman Pharmaceuticals *etc*. The drug research and development branch of the NCI of USA, has confirmed after screening over 50,000 plant extracts, representing 8000 species, that over 1000 species could actively inhibit carcinogenesis (Nandi *et al*., 1998).

Chemotherapy is an effective treatment against cancers either singly or in combination with surgery and or radiotherapy. In Chemotherapy, drugs like cisplatin, carboplatin, cyclophosphamide, doxorubicin, melphalan, mitomycin-C, ferncitabine *etc*. have been used for the treatment of cancers (Black and Livingston, 1990a; 1990b). However, the therapeutic efficacy of most of them is limited due to the development of various side effects in the host and or the acquired drug resistance by the cancer cells (Black and Livingston, 1990b; Kortalou and Essigmann, 2001). In an attempt to abate these side effects and to find out a better remedy against various malignancies, many plant derivatives have been used with varying success (Roja and Rao, 2000). Vincristine, vinblastine, podophyllotoxin, peltatins, taxol, camptothecin and colchicine have attained wider acceptability in chemotherapy. Phytochemicals that have undergone screening test and are in

varying degrees of clinical trials are bruccantin, maytansine, colubrinol, indicine-N-oxide, tridiolide, homoharringtonine, ellipticine, bruvardin, forskolin, monocrotaline *etc*. (Vasantha Kumar and Kesavachandran, 2000).

Higher plants, a source of medicinal compounds have been well known to play a dominant role in the health care of human beings (Huang Paul *et al*., 1992). More than 50% of all modern drugs in clinical use are of natural product origin (Roja and Rao, 2000; Huang Paul *et al*., 1992). Many natural products have been recognized to have the ability to induce apoptosis in various tumour cells of human origin (Taraphdar *et al*., 2001). A variety of plant extracts *i.e*., turmeric (*Curcuma longa*) and its active constituent -curcumin, roots of tea plant (*Camellia sinensis* var. *assamica*) and betel leaf have been reported to have potential antitumour or anticarcinogenic activities (Kuttan *et al*., 1985; Sakagami *et al*., 1987; Azuine *et al*., 1991; Chaudhuri *et al*., 1998; Sur and Ganguly, 1994; Roja and Rao, 2000; Sharma *et al*., 2000).

The use of traditional herbal medicines and or direct use of some parts of plants against various ailments are very common among the tribes of the North-Eastern States of India (Syiem *et al*., 1999). India is one of the greatest emporia of medicinal and aromatic plants. References to miracle herbs or wonder drugs are often found in old literature. Medicinal plants constitute a very important national resource because India has one of the richest plant based ethnomedical traditions in the world (Rajasekharan and Ganesan, 2004). The Central Council of Research in Ayurveda and Siddha medicine has drawn a list of 243 commonly used medicinal plants having bulk demand for manufacture of gelanicals, mixtures, compound formation and patent medicines (Gupta, 1998). The global market for medicinal plants and herbal medicine is estimated to be worth US $800 billions a year. International export trade in medicinal plants has been dominated by China, which exports 1,21,900 tons a year and India, which exports 32,600 tons a year

(Rajasekharan and Ganesan, 2004).

The large-scale use of medicinal plants and herbs in the preparation of drugs are increasing due to the growing concern about the side effects of chemicals and synthetic substances. The medicinal plant-based drugs have the advantage of being simple, effective and a broad spectrum of activity with an emphasis on the preventive action. Because of these factors, the demand for plant-based medicine (phytomedicines or phytopharmaceuticals) is increasing worldwide. Plants are also found to contain disease specific curative properties and extracts of such plants are increasingly being used to manufacture effective drugs.

Many traditional plant-based remedies are back in use and find increasing applications:-

- as a source of direct therapeutic agents.
- as the raw base material for the elaboration of more complex semi-synthetic chemical compounds.
- as models for new synthetic compounds.
- as taxonomic markers for the discovery of new compounds.

The production, consumption and international trade in medicinal plants and phytomedicines are growing and expected to grow in future quite significantly. The progress in medicinal plant research has undergone a phenomenal growth during the last two decades.

Herbal preparations are being used in alleviating several diseases. Countries such as India and China have a vast array of traditional medicines, which are not yet explored significantly. These medicines are prescribed not only to reduce suffering but also to prevent diseases produced by the patho-

physiological changes. Eventhough cancer is one of the most difficult diseases for treatment, it is probably one of the most preventable diseases (Block *et al*., 1992). This prevention could be achieved by:

- avoidance of cancer causing substances.
- chemopreventive agents that can inhibit the metabolism of carcinogens or cause its detoxification.
- immunostimulators which can destroy the cancer cells by augmenting the immune response.
- inhibition of signal transduction pathway which can either inhibit the conversion of normal cells to cancer cells or reduce its growth capability and destroy the cells by increasing the recognition by the immunocompetent cells.

Several types of compounds numbering to more than 2000 chemicals, among which many of plant origin, have been known to inhibit the chemically induced carcinogenesis (Soudamini and Kuttan, 1989; Unnikrishnan and Kuttan, 1990).

Search for pure phytochemicals as drug is expensive and time consuming. A phytotherapeutic approach to develop herbal anticancer agents is relevant to Indian condition. We have a rich resource of medicinal plants in traditional use. Simple animal cancer models can be used for screening anticancer activity in extracts or active fractions of medicinal plants selected based on traditional use. Search for immunomodulation and cancer cell specific apoptosis inducers among medicinal plants is promising to develop attractive drugs with minimal toxicity. Knowledge gained in the area of signal transduction in cells can also be applied to discover phytomedicine (Subramonian, 2000). A database was constructed with ethnopharmacological

information about plants used for signs and symptoms frequently related to a variety of cancers associated with cancer cell lines available at the South American Anticancer Development Office (SOAD).

Plant materials have been used in the treatment of malignant diseases for centuries. This clearly indicates the potentiality of phytochemicals as an anticancer therapeutic agent. A large number of plants and plant parts have been screened for their antitumour properties (Herout and Sorm, 1959; Zheng, 1994). Among them, the plants that belong to the family Asteraceae play a significant role. The biological and therapeutic applications of the plants of the Asteraceae are the result of popular tradition and of systematically conducted chemical and pharmacological research. In addition to drugs known since antiquity from plants such as *Chamomilla*, *Cynara* and *Sylibum*, there are many other species in the family, which have found therapeutic applications due to their antihepatotoxic, choleretic, spasmolytic, anthelmintic, antibiotic or antimicrobial activity.

The rich accumulation of essential oils and other terpenoids in certain Asteraceae members is responsible for the use of various taxa such as tansy (*Tanacetum vulgare*) and wormwood (*Artemisia absinthium*) for flavouring foods or liquors. Terpenoids and certain phenolic compounds are responsible for the value of many species of Asteraceae in pharmacy and medicine. Wagner (1977) in his detailed review on the pharmaceutical properties of Asteraceae, has also pointed out the eminent role played in this regard by the genus *Artemisia.*

The genus *Artemisia* is one of the largest and most widely distributed one of nearly 100 genera in the tribe Anthemidae of the Asteraceae (Mucciarelli and Maffei, 2002). Asia seems to show the greatest concentration of species with 150 accessions for China (Hu, 1965), 174 in Russia (Poljakov, 1961) and about 50 reported to occur in Japan (Kitamura,

1939; 1940).

The geological history of the Asteraceae is strongly linked to that of the genus *Artemisia* and of particular interest here is that the most convincing early fossils of the Asteraceae include *Artemisia* pollen of the late oligocene of Central Europe, *Artemisia* fruits and seeds from Poland (middle miocene) and *Artemisia* pollen from Eastern and Western North America of the late miocene and late oligocene respectively (Mucciarelli and Maffei, 2002).

Until recently, consensus placed the origin of the genus *Artemisia* in Central Asia with subsequent migration to North America through the Bering Land Bridge (Clements and Hall, 1923; Mc Arthur and Plummer, 1978; Stebbins, 1974; Mc Arthur, 1979). However, biological evidences point Eurasia as the centre of origin. Shah (1996) describes the genus *Artemisia* as one of the largest and most difficult taxa to understand under an ethnobotanical point of view. The medicinal use of *Artemisia* species was introduced into the Indian Himalayan region by different cultural and ethnic groups who entered this region in the past, coming from the Mediterranean and Arabian regions.

The genus *Artemisia* L. was named after Artemis, daughter of Jupiter and Latona of Greek mythology. Artemis was also the virgin Goddess of Moon and of hunting, supposed to kill without pain, in allusion to the soothing but harmful properties of the plant (Nayar, 1985). Artemis was also considered as one of the names of Diana, the goddess of nature on account of it being used in bringing on precocious puberty (Hereman, 1868).

The genus *Artemisia* includes a large number of species and some have been cultivated as commercial crops with a wide diversity of uses. Some better known uses include antimalarial activity (*A. annua* or sweet wormwood), as culinary spices (*A. dracunculus* - French tarragon), for liquor

flavouring (*A. absinthium* - absinthe), as garden ornamental (*A. abrotanum* - southernwood) and as an insect repellent (*A. vulgaris* - mugwort) (Laughlin *et al*., 2002).

Several *Artemisia* species are used medicinally and hence are of more commercial value. In Western herbal medicine, they include *A. abrotanum*, *A. absinthium*, *A. cina*, *A. dracunculus*, *A. maritima*, *A. pontica* and *A. vulgaris* (Frohne and Jensen, 1992; Evans, 1996). In traditional Chinese herbal medicine, the following *Artemisia* species are used: *A. annua*, *A. argyi*, *A. scoparia* and *A. capillaris* (Tang and Eisenbrand, 1992). In addition, *A. annua* is a source of artemisinin, which is the mother compound of a novel class of antimalarial drugs (Woerdenbag *et al*., 1994).

Among the different species, *Artemisia nilagirica* (C. B. Clarke) Pamp., a less exploited species, was selected for the present study. It is distributed in the South Indian hills, chiefly on the Nilgiris, Khasia hills and Darjeeling in India (Agarwal, 1997).

The plants are bitter, aromatic, gregarious herbs or shrubs (Warrier *et al*., 1994), usually characterized by much divided, oblong, lanceolate leaves with white tomentoes below (Rajan *et al*., 2000), inconspicuous flowers and absence of pappus, a feature which is uncommon in other members of Asteraceae (Govil *et al*., 1993).

A. nilagirica is the most common species found in earlier Indian literature. It was used as a decoction and infusion for the relief of nervous and spasmodic afflictions by Himalayan people (Shah, 1996). It provides us medicinal principles for the amelioration of human suffering not only sufficient for our own but also for the purpose of export.

The plant is used to cure various human ailments such as coughs, bronchitis, cephalalgia, leprosy, anorexia, dyspepsia, flatulence, fever, colic,

anaemia (Warrier *et al*., 1994), asthma, skin diseases and measles (Agarwal, 1997). An infusion of the leaves and flowering tops of *A. nilagirica* is administered in nervous and spasmodic affliction (Chopra *et al*., 1956).

A. nilagirica along with other plants have been used for the treatment of specific human ailments such as allergies, burns, cuts, wounds, inflammation, leucoderma, scabies, smallpox and sexually transmitted diseases (Begum and Nath, 2000). It can be used as a substitute for *Cinchona* in fevers (Govil *et al*., 1993; Agarwal, 1997). It is also used as an emmenogogue, diuretic, aphrodisiac, appetizer, febrifuge, alexiteric (Warrier *et al*., 1994), anthelmintic (Chopra *et al*., 1969; Agarwal, 1997; Warrier *et al*., 1994), expectorant and antiseptic (Chopra *et al*., 1969).

Roots of *A. nilagirica* are used as tonic and antispasmodic (Agarwal, 1997). Leaves and flowering tops are used for asthma (Chopra *et al*., 1956; 1969) and they are bitter, astringent, acrid, thermogenic, aromatic, anodyne, antiinflammatory, digestic, febrifuge and haematinic (Warrier *et al*., 1994).

The plant is reported to possess antibacterial (Agarwal, 1997; Samaiya and Saxena, 1986; Mehrotra *et al*., 1993) and antidermophytic (Kishore *et al*., 2001) activities. The essential oil of *A. nilagirica* is fungistatic in nature and has a broad fungitoxic spectrum (Mehrotra *et al*., 1993; Kishore *et al*., 2001). An ointment of the essential oil, prepared in polyethylene glycol showed pronounced efficiency as herbal antifungal agent against dermatomycosis induced in guinea pigs within 14 days of application (Kishore *et al*., 2001). Thoppil *et al*. (2002) reported antimicrobial properties of essential oil of *A. nilagirica*. Leeja and Thoppil (2004a) reported the cytotoxic potential of extracts of *A. nilagirica*. Essential oil composition and mosquito larvicidal activity of *A. nilagirica* from South India has been studied (Leeja and Thoppil, 2004b).

The plant was used also for magical purposes. It was traditionally kept at front doors and under pillows to discourage evil spirits and ghosts and the aerial parts were used in festivals for worshipping or offered to the local divinity (Shah, 1996). The essential oil is also finding its place in the indigenous perfumery industry.

Artemisia, a herb for cancer has been studied by many workers. Many species of *Artemisia* such as *A. agri* (Seo *et al*., 2003), *A. princeps* (Hwang *et al*., 1999), *A. iwayomogi* (Koo *et al*., 1994) *etc.* are reported to possess cytotoxic and antitumour activities. Among the different species of *Artemisia*, anticancer activity of *A. nilagirica* has not been yet reported. Hence, the present study.

Since all known *A. nilagirica* are sterile, they can be propagated vegetatively, which has prevented the production of new cultivar by plant breeding. Assessment of genetic variability is basic to any plant breeding programme (Farooqi *et al.,* 1990). An alternative method for creating new forms of the plant is by selecting somaclonal variants from tissue culture material. Somaclonal variation is a term coined by Larkin and Scowcroft (1981) to cover all types of variations, which occur in plants regenerated from cultured tissues. Plant tissue culture has the potential to induce genetic variability in *Artemisia* genotypes through somaclonal variants, somatic hybrids or transgenic plants. However, a pre-requisite to applied plant biotechnology is the development of a suitable and reproducible plant regeneration system (Jullien *et al*., 1998). Potential use of cell culture (Drupeau *et al*., 1987), multiple shoots (Constabel *et al*., 1982; Endo *et al*., 1987; Hirata *et al*., 1987) and improvement of various cultivation conditions (Facchini and Dicosmo, 1991) have been attempted to scale up production of secondary metabolites. The exploitation of tissue culture technique in medicinal plants for the extraction of important chemical compounds is

indeed more advantageous (Tabata, 1977). A wide variety of compounds have been shown to be produced in shoot, callus or cell suspension cultures at levels equal to or higher than the levels in the intact plant sources (Brodelins, 1988; Dodds and Roberts, 1995).

Plant tissue culture has been extensively used to exploit the secondary metabolite it can produce. Growth of a cell in a totally controlled environment of physical and chemical factors provides an excellent system for studying changes in the production of secondary metabolites, which are always present in small quantities. The basic information has provided significant clues about genes and their functioning, leading to genetic manipulation of biosynthetic pathways to obtain desired products by either blocking a pathway or enhancing the metabolic reaction (Merillon and Ramawatt, 1999). Exploitation of possible somaclonal variation, which has been observed in *in vitro* cultures, could be used to widen the genetic pool from which to select desirable traits. Application of modern biotechnology can complement conventional breeding techniques and it helps in the development of improved varieties (Chomchalow and Sahavacharin, 1981; Vasil, 1988; Bajaj, 1981).

In vitro propagation can yield a large number of clonal plants for continuous plant establishment. It is also important for germplasm conservation (Kathiravan and Ignacimuthu, 1999; Kukreja and Dhawan, 2000). Variation is a ubiquitous phenomenon associated with tissue culture (Carlson and Polacco, 1975; Green, 1977). Induced variation is an alternative source to naturally occurring variability for crop improvement (Ansari and Siddiqui, 1995). Tissue culture induced variation is defined as the variation that arises de novo during the period of dedifferentiated cell proliferation that takes place between culture of an explant and production of regenerants (Munthali *et al*., 1996). Plantlets derived from *in vitro* culture might exhibit

somaclonal variation (Larkin and Scowcroft, 1981), which is often heritable (Breiman *et al*., 1987). Other reports claim that useful morphological, cytological and molecular variations may be generated *in vitro* (Larkin *et al*., 1989).

Undifferentiated and differentiated *in vitro* tissue culture techniques have been recently developed, concerning the economically valuable *Artemisia* species. The main efforts have been devoted to the *in vitro* selection of highly yielding clones and cell lines, producing secondary metabolites with pharmacological and industrial application. In this regard, major attention has to be paid to *A. nilagirica*, owing to its medicinal properties and moreover for its value as an aromatic plant employed in fragrances, perfumery and cosmetic production.

Thus, the present study aims at an attempt to develop a protocol for the regeneration of medicinally important *A. nilagirica* through tissue culture for large scale multiplication and for secondary metabolite production. We also aim at developing somaclonal variants of *A. nilagirica* with higher levels of secondary metabolites.

Chromosome variability is of well-known occurrence in cells of cultured tissues as well as in regenerants (Bayliss, 1973; Sacristan and Melchers, 1969). Changes can take place at the ploidy level like the production of aneuploids (Taliaferro *et al*., 1989), polyploids (Mariotti *et al*., 1984) and mixoploids (Mariotti *et al*., 1984; Taliaferro *et al*., 1989). Karyological studies can bring to light the variations in chromosome number and their size and suggest the direction of chromosomal evolution in specific taxa (Jones, 1978b). Chromosomal differences may also cause changes in the quality and composition of the essential oils (Guenther, 1949). Moreover, chromosome instability in cultured cells can be useful for the production of plantlet with novel genotypes including chromosomal aberrants (Larkin and

Scowcroft, 1981).

Computer aided Chromosome Image Analysis System (CHIAS) is a modern technique for karyomorphological analysis. Ordinary karyotype analysis has provided a limited success in chromosome identification. Possibility of making errors is much greater in the conventional method of measuring and characterizing by visual evaluation. These difficulties can be overcome by computer aided Chromosome Image Analysis System. It allows an accurate chromosome pairing mainly in those cases where the chromosome size is very small (Fukui and Kakkeda, 1994). This technique gives a better knowledge of the cytogenetic constitution of the material under study (Fukui and Kakkeda, 1994; Fukui and Lijima, 1992; Fukui and Kamisugi, 1995).

Genomic analysis is a prerequisite for establishing the genetic stability and uniformity of a desired clone (Ikeda and Ono, 1967). Several strategies can be used to assess the genetic fidelity of *in vitro* derived clones but most of them have limitations. Karyological analysis cannot reveal alterations in specific genes or small chromosomal rearrangements (Isabel *et al*., 1993). The Polymerase Chain Reaction (PCR) (Saiki *et al*., 1988) has been the basis of a growing range of newer techniques. PCR allows the specific amplification of DNA sequences making it ideal for the identification of plant genotypes. Amplification of a genotype-specific sequence can take advantage of some of the many features of PCR like speed, simplicity, specificity, sensitivity and cost (Henry, 1997). Molecular markers such as Restriction Fragment Length Polymorphism (RFLP) and Random Amplified Polymorphic DNA (RAPD) (Welsh and Mc Clelland, 1990; Williams *et al*., 1990) appears to be good, but when compared to RFLP, RAPD appears to provide a better basis for genetic characterization because of simplicity of the necessary procedures (Baird *et al*., 1992).

The approach of using molecular markers including RAPD profiles is a

powerful tool not only for the identification of genotypes but also to quantify the extent of genetic variation in any given population. While on one hand the approach of RAPD profiling has been useful in tissue culture methods for detection and selection of somaclonal variants (Munthali *et al*., 1996). The molecular technique, with the same logic, is directly utilizable for assessing the population of micropropagated clones from any given explant for genetic uniformity. Using PCR with short primers of arbitrary sequences, RAPD markers were recently shown to be sensitive for detecting variations among individuals between and within species (Carlson *et al*., 1991; Roy *et al*., 1992). This is an alternative approach for finding new DNA based polymorphic markers among closely related genotypes (Welsh and Mc Clelland, 1990; Nymbom *et al*., 1990; Lindout *et al*., 1999). RAPD analysis using PCR with arbitrary oligonucleotide primers (Williams *et al*., 1990) has the advantage of being non-radioactive, rapid and is a convenient assay of polymorphism that requires only a small amount of crude DNA. Today, RAPD technique has been adopted most widely.

The main issues associated with the use of these techniques are the problem of ensuring reproducibility of amplification profiles. The nature of the amplification process with short primers is such that many sites in the genome are potential templates and the profile obtained may be influenced by any variation in the method used to prepare the DNA template and the exact reaction composition and conditions used in the PCR (Muralidharan and Wakeland, 1993). Obtaining reliable results depend upon standardizing the conditions or identifying combination of conditions that give consistent results, even when variations in the key variables are encountered. A key requirement for reliable and reproducible RAPD results is a consistent approach to sample preparation and DNA isolation. Both the quality and quantity of the template DNA preparation have the potential to substantially influence the result.

Polymorphism results from either base changes at the primer binding site (point mutation) or chromosomal changes in the amplified regions (insertions, deletions or inversions) which alter the size or prevent the successful amplification of a target DNA. Southern hybridizations are not required and polymorphisms can also be detected in fragments containing highly repeated sequences, which are recalcitrant to RFLP analysis. The extent of polymorphism detected by RAPDs is therefore greater than that is observed by RFLPs (Williams *et al*., 1990).

Plants represent an unlimited source of phytochemicals such as the metabolites of primary and secondary metabolism. Secondary metabolites are compounds that are biosynthetically derived from the primary metabolites and their distribution in the plant kingdom is restricted. These compounds are generally detected in a lower volume compared to the primary metabolites and possess significant biological activities. Therefore, they are also termed as the higher value - lower volume products or speciality chemicals (Roja and Rao, 1998).

Secondary metabolism in a plant not only plays a role for its survival by producing attractants for pollinators, chemical defense against predators and diseases but also is an important trait of our food, taste, colour and scent. Others such as alkaloids, anthocyanins, flavonoids, quinines, lignans, steroids and terpenoids have a commercial application in the pharmaceutical and biomedical fields and are part of drugs, dyes, flavours, fragrances and insecticides (Veerpoorte *et al*., 2002).

Volatile oil containing drugs and essential oils has been used for a long time both in folk medicines and in therapeutics, both traditional and alternative. Essential oils, the volatile secondary metabolites responsible for the odours of aromatic plants are used in perfumery, as aroma products, flavouring agents in food and beverages, in cosmetic products and as drugs.

There is an increasing global trend in the consumption of self-prescribed herbal and natural products for treating numerous ailments such as cancer and even by healthy individuals as a preventive (Teixeira da Silva, 2004).

Essential oils are frequently referred to as the "life force" of plants. Unlike fatty oils, these essential oils are volatile, highly concentrated substances extracted from flowers, leaves, stems, roots, seeds, bark, resin and fruit rinds. The amount of essential oils found in these plants can be anywhere from 0.01% to 10% of the total. That is why tons of plant material are required to obtain a few hundred pounds of oil. These oils have potent antimicrobial factors, having 200-300 therapeutic constituents. Essential oils cannot be substituted with synthetics. Only pure oils contain a full spectrum of compounds that cheap imitations simply cannot duplicate. Essential oils have unique properties that are prized worldwide for thousands of years, being used therapeutically in early Roman, Greek, Egyptian, Indian and Chinese civilizations ([1]http).

Essential oils are effectively used in aromatherapy. Aromatherapy is the use of pure essential and absolute oils for psychological and physical well being. Essential oils are believed to stimulate the olfactory nerves and exert influence on the brain centre that controls emotion (Mabey, 1988). They are used as natural rejuvenating and antiwrinkle agents in aromatherapy (Varshney, 1991). It is suitable in the treatment of pain, psychological disturbances, allergies, skin diseases, gastrointestinal disorders, cardiovascular problems, urinary disorders, gynaecological disturbances, cancer *etc*. (Jamil, 1997).

The versatile use of several aromatic plants in food, cosmetic and pharmaceutical industries demand an extensive screening of essential oils and their components. Individual chemicals isolated from essential oils are more often used than the oils (Brud and Gora, 1989). Therefore, identification of

trace components is very helpful to reveal the quality of the oil. Analysis of the essential oils can be easily done using the technique of Gas Chromatography and Mass Spectrometry (GC-MS). GC is a tool for separating the volatile components while analysis depends upon retention characteristics under standard conditions. The mass spectrometer can be used as a detector for gas chromatography in which, the high degree of specificity of the mass spectrometer is an aid to the identification of the sample. The large number of spectra obtained in a short time from the GC-MS technique and the routine nature of much of the data obtained make the computer a very useful accessory to the GC-MS unit. With the help of GC-MS technique, it has now been possible to analyze directly the fragrances of natural or artificial materials without the use of heat or solvents and directly by the use of head space analysis (Thappa *et al*., 1982). GC-MS differs from other types of spectral analysis in that the sample does not absorb radiation from the electromagnetic spectrum. It is highly sensitive and only a small quantity of the sample is required. When coupled with separation techniques like GC or HPLC (High Performance Liquid Chromatography), it is a highly specific way to identify organic compounds (Smith and Busch, 1999). A GC-MS machine with computerized library search discs is regarded as the best tool for essential oil analysis (Jose and Rajalakshmi, 2005).

Albeit, a common plant in the country, a purview of literature on *A. nilagirica* reveals that its potentials are yet to be exploited. The present study is an attempt to find out the effectiveness of this plant in the field of cancer treatment and to generate somaclonal variants of *A. nilagirica* by *in vitro* techniques, that differ from the parent plant in quality and quantity of the essential oil and to reveal the genetic basis of variation in them by using the Chromosome Image Analysis System and RAPD technique.

Thus, the present study aims to fulfil the following objectives:-

* To find out the efficacy of *Artemisia nilagirica* in the field of cancer treatment and research.

* To establish a protocol for the regeneration of medicinally important *A. nilagirica* through tissue culture for large scale multiplication and for secondary metabolite production.

* To develop a somaclonal variant of the medicinal and aromatic plant, *A. nilagirica* with higher levels of secondary metabolites.

* To analyse the possible variations of somaclonal variant from the parent plant by comparing various aspects such as :-

    - Cytological and Karyomorphological analysis
    - Random Amplified Polymorphic DNA (RAPD) analysis
    - Essential oil analysis
    - Cytotoxic assays
    - Antitumour assays

REVIEW

Micropropagation

Aromatic plants are an important source of materials for flavour and cosmetic industries mainly in the form of essential oils and their use in pharmaceutics cannot be underestimated (Balandrin *et al*., 1985). Aromatherapy, a branch of herbal medicine using essential oils and aromatic principles of plants to cure specific diseases has an added advantage and has been a turning point in recent days. Therefore, modern approaches like biotechnological investigation involving *in vitro* culture studies of aromatic plants could be of immense help to circumvent some of the application oriented problems especially for novelty and to boost the production of essential oils and their components.

An upsurge interest of natural drugs and aroma – a sort of "green wave" for phytoproducts currently is most visible across the world (Sharma *et al*., 1996). Plant cell culture offers potential for *in vitro* technology, as a form of "living factory" for the further exploitation of the plant kingdom as a chemical resource. It has become increasingly significant in the field of biotechnology as a potential source of commercially useful compounds. In addition, this technique offers means not only for the rapid and mass multiplication of the existing stocks of plant germplasm, but also the conservation of their biodiversity (Bajaj, 1986).

Genetic variability, which forms the basis of selection in crop improvement, can be generated through hybridisation, polyploidy and induced mutagenesis. Nowadays attention has been focussed on inducing genetic variability through tissue culture and the plants regenerated from undifferentiated callus cultures have now become a new and useful source of

genetic variation. These variations are of particular interest because it may provide hints as to the basic mechanisms underlying genotypic and phenotypic stability in normal development. Plants regenerated from relatively undifferentiated callus cultures possess a vast array of genetic changes. Such variations can result in useful agricultural, horticultural and biochemical products. Any step made towards understanding the basics of tissue culture induced genetic variation should be helpful in developing a more stable protocol and to manipulate somatic cell system (Phillips *et al*., 1994).

Techniques are now available to induce and select stable genetic variants arising from the cultured tissues and these can be applied for producing high amounts of vitamins, pigments, alkaloids, food flavours and useful metabolites in plant species (Collin and Watts, 1983; Constabel and Vasil, 1987).

In spite of the importance of several species of *Artemisia* as valuable source of anthelmintic drug- santonin, antimalarial drug- artemisinin and their essential oils, the *in vitro* studies on *Artemisia* are comparatively meagre to realize far reaching applications. The first ever trial of using tissue culture techniques such as callus induction and regeneration of plantlets from *A. annua* and changes of qinghaosu contents has been reported by He *et al*. (1983).

Micropropagation techniques have been applied in the flavour industry, for the isolation and growth of *Artemisia* clones of the "genepi group" (*A. umbelliformis* and *A. genipi*), having a high degree of variability in their essential oil GC patterns and organoleptic characteristics (Gautheret *et al*., 1984). Various workers have reported successful *in vitro* propagation of *A. annua via*. shoot cultures (Nair *et al*., 1986; Martinez and Staba, 1988).

Benjamin *et al*. (1990) was able to establish three types of cultures using germinated seedlings of *A. pallens* on MS liquid medium supplemented with different plant growth regulators. On medium containing BA (1.0 mgl^{-1}) and BA (0.1 mgl^{-1}) + 2,4-D (0.2 mgl^{-1}), seed tissues produced unorganized callus. On medium with BA (1.0 mgl^{-1}) + IAA (0.1 mgl^{-1}), semi-organized callus was produced. While on medium supplemented with BA (1.0 mgl^{-1}) + NAA (0.1 mgl^{-1}) + IAA (0.1 mgl^{-1}), profuse callus proliferation accompanied by shoot differentiation was achieved. Umer Sharief and Jagadish Chandra (1991) standardized the protocol for micropropagation of *A. pallens* using MS medium. Seedling explants exhibited maximum number of shoot regeneration capacity on MS medium with NAA (1 mgl^{-1}) and BAP (0.3 mgl^{-1}). Fulzele *et al*. (1991) have successfully achieved the callus initiation from aerial shoots of *A. annua* on MS medium with BA (1 mgl^{-1}) + NAA (1 mgl^{-1}) obtained from different geographical zones. The European variety gave multiple shoots in BA (0.1 mgl^{-1}) + IAA (1 mgl^{-1}). Successful rooting has also been reported on MS media with NAA (1 mgl^{-1}) + KIN (0.1 mgl^{-1}). They have also detected artemisinin production in these cultures, besides, achieving complete shoot differentiation.

Micropropagation techniques have been applied to *A. annua* in order to obtain *in vitro* multiple shoot cultures from which desirable clones may be selected and conserved (El-Hag *et al*., 1992). There are several reports on the biosynthetic pathway leading to artemisinin as well as biotechnological approaches for the production of this sesquiterpene lactones *in vitro* (El-Hag *et al*., 1992; Sangwan *et al*., 1993; Brown, 1994b; Chen and Xu, 1996; Gulati *et al*., 1996). Increased artemisinin production has been obtained in transformed roots of *A. annua* after infection with *Agrobacterium rhizogenes*, in percentage suggesting a feasible commercial production of the active compound (Weathers *et al*., 1994).

Dedifferentiated and differentiated tissue cultures of *A. annua* for artemisinin production were carried out by Paniego and Giulietti (1994). The calluses were initiated on MS medium supplemented with sucrose (30 gl^{-1}), myoinositol (10 mgl^{-1}) and vitamins. The best results were obtained with 2,4-D and NAA. Cell suspensions were established on the same media without regulator supplied.

Various environmental factors such as artificial light quality, sucrose concentration and hormonal cultural media supplements have been shown to be effective in promoting *in vitro* essential oil accumulation as occurred in the test tube plants of *A. balchanorum* (Bavrina *et al*., 1994).

Usha and Swamy (1994) reported the enhancement of organogenesis by manipulating the medium and growth regulators in *A. pallens*. Modified MS medium with combination of NAA (1 mgl^{-1}) + BAP (0.3 mgl^{-1}) induced proliferation of more callus and MS (Murashige and Skoog, 1962) medium supplemented with kinetin (1 mgl^{-1}) induced more number of multiple shoots than reported earlier. *In vitro* shoots developed roots, in the medium devoid of growth hormones.

Valuable metabolites have also been obtained in undifferentiated callus and cell suspension cultures of *A. annua*, including the coumarin, scopoletin (Brown, 1994a) and artemisinin from callus grown on MS medium, supplemented with 2,4-D and NAA. Other than the callus and multiple shoot cultures producing terpenoids, *in vitro* protocols for plantlet cultures in bioreactors have also been developed for different *A. annua* geographical varieties, thus resulting in the production of camphor, 1,8-cineole and β-caryophyllene (Fulzele *et al*., 1995).

Callus cultures (Nin *et al*., 1996) and protoplast isolation (Xu and Jia, 1996) have also been obtained from other *Artemisia* species such as

A. absinthium and *A. sphaerocephala*.

Sharief *et al*. (1997) revealed the propagation of *A. pallens* by encapsulated *in vitro* grown shoot buds. Effect of gibberellic acid on hairy root cultures of *A. annua*, its growth and artemisinin production has been studied by Smith *et al*. (1997). *Agrobacterium* mediated transformation of *A. absinthium* (worm wood) and production of secondary metabolites has been reported by Nin *et al.* (1997).

The different components of the medium such as macro and microelements, carbon source, growth regulators, vitamins and gelling agent affect the growth and metabolism of cells. Higher content of phosphate (as KH_2PO_4) in medium, enhanced the growth of the tissue and production of secondary metabolites. The quality and types of cytokinins and auxins (both endogenous and exogenous) have a marked effect on primary and secondary metabolism and play a major role in determining the potential of a given culture (Tejavathi and Nagashree, 1998).

In vitro micropropagation of sweet worm wood (*A. annua*) has been reported by Usha and Swamy (1998). Isolation and production of artemisinin and stigmasterol in hairy root cultures of *A. annua* has been reported by Xie *et al*. (2000). Liu *et al*. (2002) studied the effects of light irradiation on hairy root growth and artemisinin biosynthesis of *A. annua*. *A. annua* callus could be cryopreserved in a cryoprotectant containing 15% ethylene glycol, 15% dimethyl sulfoxide, 30% glycerol and 13.6% sucrose, a simplified and effective method for long term storage of callus without an effect on regeneration (Chenshu *et al*., 2003). Teixeira da Silva (2003) reviewed the advances of tissue culture, genetics and biotechnology of plants of Anthemidae.

Micropropagation and antioxidant activity of *A. judaica* has been

reported by Liu *et al.* (2004). Sujatha and Ranjitha Kumari (2007) studied the effect of phytohormones on micropropagation of *A. vulgaris.* Mass propagation and essential oil analysis of *A. vulgaris* has been reported by Sujatha *et al.* (2008). Of the different concentrations of BA and flask capacities tested, 4.44 µM of BA and 500 ml flask capacity were found to have produced a maximum of 85.5 shoots after 30 days of culture. Shoot proliferation was found to increase with increasing flask capacity whereas, shoot number decreased with increasing BA concentration (> 4.44 µM).

Cytological Analysis

The genus *Artemisia* of the tribe Anthemidae of Asteraceae has been explored cytologically by many workers (Darlington and Wylie, 1955) and from their study it is evident that only polyploidy has played a major role in the evolution and perpetuation of its species. Numerous cytotypes of *A. vulgaris*, varying in phenotype have been shown to exist in North America by Keck (1964), in North Western Himalayas of India by Khoshoo and Sobti (1958) and Koul (1964a) and in Kashmir, Mukherjee (1932) reported many ecads. Diploids, triploids and tetraploids have been reported from India by Khoshoo and Sobti (1958) and Koul (1964b) in *A. vulgaris.*

Tissue culture is considered to be a useful method for the clonal propagation of economically important plants. However in many instances serious attention has not been paid to determine the ploidy status of regenerants and the extent of somaclonal variation.

The study of chromosomal behaviour in cultures has proved to be an important parameter of investigation in recent years. Lack of genetic stability under culture conditions in response to physical and chemical composition of

the medium is the most serious hindrance for progress in the study of cultured plant cells (Torrey, 1959; Partanen, 1963). Chromosome variability is of well-known occurrence in cells of cultured tissues as well as in regenerants (Sacristan and Melchers, 1969; Bayliss, 1973). Instability of chromosomes in culture has been reported to be influenced by a number of different factors including composition of media (D'Amato, 1978) or by the nature of explant (Bajwa and Wakhlu, 1986). This instability however can be useful for the production of plantlets with novel genotypes including chromosome aberrants (Larkin and Scowcroft, 1981).

When cultured, the plant cells were known to exhibit variation in chromosome number and structure (Partanen, 1965; Sacristan, 1971; Sekera, 1977; Gupta and Ghosh, 1983; Bajwa and Wakhlu, 1986). Regenerability of a callus was often ascribed to numerical and structural changes in the chromosomes (Muir, 1965; Murashige and Nakano, 1965; Torrey, 1967).

Variations in callus culture had been observed to be expressed within the regenerants of several plants (Sacristan, 1971; Orton, 1980). Chromosome abnormalities such as laggards, sticky bridges, anaphasic bridges and formation of micronuclei are possible mechanisms for the origin of *in vitro* aneuploidy (Torrey, 1959; Bayliss, 1973; D'Amato *et al*., 1980).

Numerical changes had been examined most often among flowering plants (Evans and Reed, 1981; Mohanty, 1990) in tissue culture. Chromosome variation and frequency of spontaneous mutation associated with *in vitro* culture and regeneration had been reported by Edallo *et al*. (1981). It had been observed that plant regeneration in callus culture was obtained mainly from cells with diploid chromosome number (Larkin and Scowcroft, 1981; Vasil, 1983). Variation within regenerated plants was often reported (Mc Coy *et al*., 1982; Scowcroft, 1984). Mutant and variable cell lines selected from cultured cells have immense potential for recovering into

new variant types and in cloning of desired genotypes (Bajwa and Wakhlu, 1986).

Cells with chromosome clumps, multipolar spindles, multinuclei, asynchronous divisions and laggards were observed at various subcultures (Wakhlu and Barna, 1988). Such nuclear aberrants are known to form cells with variable chromosome numbers in callus culture of various plants. The possibility of minor genetic reshuffles in the form of minor structural changes in chromosomes without affecting their original chromosome number in regenerants could not be ruled out. Structural alterations of chromosomes in cultured cells were also reported by Mohanty *et al*. (1991).

Many workers had carried out detailed analyses of the mitotic and meiotic behaviour of chromosomes of different species and population of *Artemisia.* The cytomorphology of 3 species of Himalayan *Artemisia* such as *A. roxburghiana*, *A. moorcroftiana* and *A. bengalensis* has been studied by Bakshi and Kaul (1984). The nature of polyploidy in *Artemisia glauca* was studied by Bakshi (1985). Two variants from a wild population in Kashmir, accessions 16238 (2n=36) and 16241 (2n=27) were studied. From the study it was concluded that 16238 is a segmental allotetraploid and 16241 is an allotriploid. Mendelak and Schweizer (1986) reported the Giemsa C-banded karyotypes of some diploid *Artemisia* species. Bands were mostly confined to distal chromosome regions. Intercalary banding was virtually absent and centromeric heterochromatin was scarce.

Bakshi *et al*. (1987) reported the occurrence and behaviour of B -chromosomes in *A. frigida.* Their number was limited to two in all cells examined. Analysis of meiotic behaviour in the variant and in a control, which lacked B-chromosomes, showed that the accessory chromosomes reduced the mean chiasma frequency. They also appeared to increase leaf size, floret number and essential oil content.

The chromosome numbers of 58 accessions of *Artemisia* representing 13 native and introduced taxa found in Canada and USA was reported by Stahevitch and Wojtas (1988). Chromosome numbers observed were n = 8, 9, 18 and 27. A number of 2n = 18 is reported for the first time in *A. pacifica*. A new tetraploid cytotype (2n = 36) is reported for *A. frigida*. Supernumerary chromosomes (n = 9+3) and mixoploidy (n = 18, 36) were observed in the plant for the first time. In some taxa morphological and ecological differences were detected between races, which differed in chromosome number, but in other cases no differences were noted. It is argued from karyological and phylogenetic evidence that the original chromosome number in *Artemisia* was x = 9.

A karyotype study of the root tip cells of 20 species of *Artemisia* reported by Qiao *et al*. (1990) showed that there were two basic chromosome numbers, x = 8 and 9. There were fourteen diploids with four having 2n = 16 and ten having 2n = 18, five tetraploids (2n = 36) and one mixoploid (2n = 50). The mixoploid, *A. argyi* var. *gracilis*, is probably a natural polymorphic hybrid or hybrid variety. Chromosomes of the genus were intermediate or small in size, 2.37- 6.04 μm in length with at least one pair of SAT chromosomes.

Tyagi *et al*. (1990) studied pachytene chromosome morphology of *A. annua*. In all chromosomes of *A. annua* (2n = 18), heterochromatin was clustered around the centromere during pachytene. An idiogram of the 9 bivalents is depicted, showing euchromatic and heterochromatic regions, relative length position of the centromere and characteristic chromomeres. The nucleolus-organizing chromosome exhibited a secondary constriction on its short arm. Meiotic and somatic chromosome studies of *A. pallens* (2n = 16) were reported by Rekha and Kak (1993).

Studies on the chromosome numbers and karyotypes of six species of

Artemisia were reported by Xiong *et al*. (1995). Chromosome numbers and karyotype formulae were given for *A. japonica* (2n = 18), *A. parviflora* (2n = 36), *A. dubia* (2n = 36), *A. myriantha* (2n = 36), *A. lavandulaefolia* (2n = 54) and *A. velutina* (2n = 54).

A karyosystematic study of Ferchichi (1997) on various populations of the *A. herba-alba* from Presaharam of Tunisia identified 2 cytotypes, one diploid with n = 9 and the other tetraploid with n = 18. Morphological, biological and ecological characterization of the 2 races emphasized their difference in terms of geographical distribution, ecological demands, vegetative vigour and flower morphology. These differences allowed the races to be classified as distinct taxa, diploid var. *communis* and tetraploid var. *desertii*. Xirau and Yakovlev (1997) reported flurochrome banded karyotypes of five taxa of *Artemisia,* including the Iberian endemic species *A. barreleri.*

Torrell and Valles (2001) studied the genome size in twenty one *Artemisia* species. Genome size was estimated by flow cytometry. 2C nuclear DNA content values ranged from 3.5 pg (*A. annua*) to 25.65 pg (*A. canna*), which represent a more than seven fold variation. DNA content per haploid genome ranged from 1.75 pg (*A. annua*) to 5.76 pg (*A. judaica*). The DNA amount was highly correlated with karyotype length and ploidy level. Some variations in genome size had systematic and evolutionary implications, whereas others were linked to ecological selection pressures.

New and rare chromosome counts in fourteen species of *Artemisia* from Uzbekistan were reported by Valles *et al*. (2001). All the studied taxa (except *A. scoparia*, x = 8) have x = 9. *A. diffusa* (4x) was more densely tomentose than its sympatric species *A. turanica* (2x). Widespread species in the desert and semidesert zones (*A. diffusa* and *A. sogdiana*) were tetraploids (2n = 36), whereas species more restricted to xerophilous habitats in the

desert (*A. turanica, A. santolina* and *A. leucoides*) or to more humid places in the mountains (*A. tenuisecta, A. juricea, A. serotin* and *A. porrecta*) were diploids (2n = 18).

Torrell *et al*. (2001) studied metaphase chromosome number of twelve *Artemisia* species from Armenia and Iran. Of the twelve *Artemisia* species, four (*A. incana, A. austriaca, A. splendens* and *A. scoparia*) have the chromosome number x = 8 and eight species (*A. armeniaca, A. biennis, A. chamaemelifolia, A. marschalliana, A. ciniformis, A. fragrans, A. gypsacea* and *A. kopetdaghensis*) have x = 9. Chromosome analysis in Asteraceae (Tribe: Inuleae) using Image Analysis System was made by Rajalakshmi and Jose (2002).

Chromosome counts in sixteen populations of five *Artemisia* species (*A. annua, A. dracanculus, A. abrotanum, A. absinthium* and *A. campestris* ssp. *sericea*) from Poland were published by Kreitschitz and Valles (2003). The decaploid level (2n = 90) is described for the first time in noncultivated populations of *A. dracunculus* and several cases of aneusomaty (intra individual aneuploid variations in chromosome number 2n = 87, 88 and 89) have been detected in this species. In addition to the already known diploid number (2n = 18), the tetraploid level (2n = 36) has been detected in *A. absinthium*. The relevance of polyploidy for the evolution of the genus and other cytotaxonomic or cytobiogeographical aspects were briefly discussed.

Random Amplified Polymorphic DNA (RAPD) Analysis

Molecular markers are effective tools used to detect the genetic basis and breeding applications of somaclonal variation (Evans, 1989). Bousquet *et al*. (1990) obtained DNA amplification from vegetative and sexual tissues of plants by PCR. It was reported that molecular markers could be used for differentiation of nearly identical germplasm accessions (Carlson *et al*., 1991;

Potter and Jones, 1991; Weycott and Fort, 1991). RAPDs were used for identification of germplasm (Fukuorka *et al*., 1992; Vidal *et al*., 1999) and in other gene introgression breeding programmes (Frederic *et al*., 1992). Shenoy and Vasil (1992) reported that micropropagation through meristem culture was generally associated with low risk of genetic instability because the organized meristems were generally more resistant to genetic changes that might occur during cell division or differentiation under *in vitro* conditions. Bouman *et al*. (1992), Bouman and Kuijpers (1994) and Al Zahim *et al*. (1999) also found intraclonal RAPD polymorphism among micropropagated plants.

Many authors using RAPD and RFLP have failed to observe intraclonal variations in various plant species (Shenoy and Vasil, 1992; Isabel *et al*., 1993; Valles *et al*., 1993; Choudhury and Vasil, 1993; Rout *et al*., 1998). Recently DNA markers were utilized effectively in plant improvement programmes (Paterson *et al*., 1991; Waugh and Rowell, 1992; Rafulsky *et al*., 1994).

Random Amplified Polymorphic DNA (RAPD) analysis has proved to be useful in determining genetic relationships and variation in many plant species (Yu and Pauls, 1993; Liu *et al*., 1994; Swoboda and Bhalla, 1997; De Bustos *et al*., 1999). The results of RAPD analysis can be used to determine genetic distances and relatedness within or among the species (Van Buren *et al*., 1994; Liu and Musial, 1997), which can then be utilized in the construction of phylogenies.

Choudhury *et al*. (1994) conducted molecular analysis of plants regenerated from embryonic cultures. Virk *et al*. (1995) used RAPD for the study of diversity within plant germplasm. Rani *et al*. (1995) found RAPD variations among 23 micropropagated plants originating from the same clone that were morphologically similar. The variation of monomorphic bands in

micropropagated plants by using different primers had been reported earlier (Potter and Jones, 1991; Angel *et al*., 1996).

The Anthemidae is the seventh largest tribe in the Asteraceae and it is monophyletic and composed of 109 genera and 1740 species (Bremer and Humpries, 1993). Watson (1996) has undertaken a study of the restriction sites of the chloroplast genome (cp DNA phylogeny) to evaluate phylogenetic relationships among the tribe. Watson's results support the monophylogeny of the sub tribes, with a strong concordance between the molecular and morphological phylogenies.

RAPD markers were generated and used in plant improvement programmes (Rafulsky *et al*., 1996) and in assessment of botanical diversity (Karp *et al*., 1996). Identification of plants using sequence characterized amplified region DNA markers were also reported (Xong and Bakalinsky, 1996; Zhou *et al*., 1996; Shoyama *et al*., 1997). Similarly from the conservation point of view, Parani *et al*. (1997) used RAPD approach to compare the mother plant and micropropagated progenies to maintain the fidelity of elite genotypes that were to be conserved.

Several strategies can be used to assess the genetic stability of *in vitro* derived plants such as karyological analysis or isozyme markers, but they have their own limitations (Isabel *et al*., 1993). RAPD analysis is one of the techniques, which uses a single arbitrary sequence of 10 base oligonucleotides (Welsh and Mc Clelland, 1990; Williams *et al*., 1990) to generate DNA fragments that can be used as genetic markers to determine genetic variation of regenerated plants. It has been used in *Allium* (Al Zahim *et al*., 1999), *Lolium* (Wang *et al*., 1993), *Triticum* (Brown *et al*., 1993), *Beta* (Munthali *et al*., 1996) and *Oryza* (Yang *et al*., 1999) to detect somaclonal variation.

RAPD polymorphism in callus-derived plants has been reported in

wheat (Brown *et al*., 1993), poplar (Rani *et al*., 1995), beat (Munthali *et al*., 1996), peach (Hashmi *et al*., 1997) and garlic (Al Zahim *et al*., 1999).

Sangwan *et al*. (1998) developed a protocol to obtain high molecular weight, restrictable and amplifiable genomic DNA from the antimalarial plant *A. annua*. The method is a CTAB procedure that includes a rapid micro column chromatography through DE-52 ion exchange resin. The pure consistency of the template may also offer a reliability advantage for PCR and RFLP based applications. Restriction of the purified DNA with Dra I and Hind III resulted in discrete bands that may have arisen due to some repeat sequences equally spaced between the restriction sites.

RAPD analysis of *Artemisia* subgenus Tridentatae species and hybrids has been recorded ([2]http). Sangwan *et al*. (1998) reported the RAPD profile based genetic characterization of chemotypic variants of *A. annua*. Taxonomy of *Sphaeromeria*, *Artemisia* and *Tanacetum* (Asteraceae, Anthemidae) based on randomly amplified polymorphic DNA has also been studied ([3]http). Mc Arthur and Stewart (1999) reported the contributions of RAPD to *Artemisia* systematics.

RAPD analysis had also been used for the confirmation of hybridity (Patra *et al*., 2001c). Molecular characterization of several other plants have been done successfully by RAPD technique (Ayana *et al*., 2000; Pradeepkumar *et al*., 2001; Dwivedi *et al*., 2001c; Patra *et al*., 2001a). Molecular genome analysis using RAPD proved to be suitable for screening genetic variation in *Stachys sieboldii* regenerants obtained at various phytochrome concentrations. High DNA polymorphism was demonstrated for two types of *S. sieboldii* callus cultures and for plants regenerated from a callus culture (Kochieva *et al*., 2002).

RAPD is referred as an appropriate tool for examining the clonal

identity and for certifying genetic fidelity of *in vitro* propagated plants (Gupta and Rao, 2002; Carvalho *et al.*, 2004). Kim *et al.* (2003) demonstrated the sensitivity of RAPD for revealing the genetic basis for somaclonal variation and detected significantly higher level of polymorphism between regenerants derived from a single genotype. Genome characterization through RAPD showed distinct variation in profiles to confirm menthol tolerance and high menthol content character of the genotype that favoured the *in vitro* selection of *Mentha arvensis* clones (Dhawan *et al*., 2003). The changes in the banding pattern obtained *via.* RAPD analysis in basil plants regenerated *in vitro* suggested the existence of genetic variation that might affect the biochemical synthesis of phytoproducts (Rady and Nazif, 2005).

Essential Oil Analysis

The essential oil composition of various species of *Artemisia* has been reviewed from time to time by many workers (Table 1). Chopra *et al*. (1956) was the first who studied the volatile constituents from the aerial parts of *A. nilagirica*.

Uniyal *et al*. (1985) studied the constituents of *A. nilagirica* by capillary gas chromatography, IR, HNMR and GC-MS assays. The main constituents of the oil were camphor, β-eudesmol, 1,8-cineole, borneol, artemisia alcohol, camphene, α-gurjunene, p-cymene, terpinen-4-ol and α-pinene.

The production of artemisinin in tissue cultures of *A. annua* has been reported by Nair *et al*. (1986). Misra (1986) isolated arteannuin-C, a sesquiterpene from *A. annua*. Banthorpe and Brown (1989) isolated two unexpected coumarin derivatives from tissue cultures of Compositae species. Thakur *et al*. (1990) investigated the chemical composition and use of essential oils of various *Artemisia* species such as *A. absinthium*, *A. annua*,

A. roxburgiana, A. scoparia, A. sieversiana and *A. vestita*. Essential oils were analyzed by GC and GC-MS and constituents were identified by NMR. Thujone, camphene and 1, 8- cineole along with the irregular monoterpenes was found to be the major and most widespread constituents. Caryophyllene and cadinene together with several closely related derivatives were also found to be widespread throughout the genus.

Terpenoid production through tissue cultures of *A. pallens* has been studied by Benjamin *et al*. (1990). Pestchanker *et al*. (1990) isolated a sesquiterpene lactone, dihydroleucodin, from tissue cultured plants of *A. douglasiana*. The effect of NAA and BAP on the accumulation of volatile oil components in cell cultures of tarragon (*A. dracunculus*) has been studied by Cotton *et al*. (1991).

Kim *et al*. (1992) have conducted work related to the production of secondary metabolites by tissue culture of *A. annua*. Volatile oils from normal and transformed root of *A. absinthium* were reported by Kennedy *et al*. (1993). Essential oil production by *Anthemis nobilis* tissue culture has been explained by Fauconnier *et al*. (1993).

There are several reports on the biosynthetic pathway leading to artemisinin as well as biotechnological approaches for the production of sesquiterpene lactone *in vitro* (El-Hag *et al*., 1992; Sangwan *et al*., 1993; Brown, 1994b; Chen and Xu, 1996; Gulati *et al*., 1996). Various environmental factors such as artificial light quality, sucrose concentration and hormonal culture media supplements have been shown to be effective in promoting *in vitro* essential oil accumulation, as occurred in the *in vitro* plants of *A. balchanorum* (Bavrina *et al*., 1994).

Various metabolites have also been obtained in undifferentiated callus and cell suspension cultures of *A. annua,* including the coumarin, scopoletin

(Brown, 1994a) and artemisinin from callus grown on MS medium, supplemented with 2,4-dichlorophenoxy acetic acid and naphthalene acetic acid. However some times superior cultures failed to accumulate any of the terpenoids found in the parent plant (Brown, 1994a; Paniego and Giulietti, 1994). Other than callus and multiple shoot cultures producing terpenoids *in vitro*, protocols for plantlet cultures in bioreactors have been developed for different *A. annua* geographical varieties, resulting in the production of camphor, 1,8-cineole and β-caryophyllene (Fulzele *et al*., 1995).

Genetic transformation of *A. annua* by *Agrobacterium tumefaciens* and artemisinin synthesis in transformed cultures has been studied by Ghosh *et al*. (1997). Nin *et al*. (1997) isolated secondary metabolites from *A. absinthium* (wormwood) cultures.

Liu *et al*. (1998) reported the production of artemisinin by shoot cultures of *A. annua* in a modified inner-loop mist bioreactor.

Sy and Brown (1999) isolated coniferaldehyde derivatives from tissue culture of *A. annua* and *Tanacetum parthenium*. Isolation and production of artemisinin and stigmasterol in hairy root cultures of *A. annua* has been reported by Xie *et al*. (2000). The effect of light irradiation on hairy root growth and artemisinin biosynthesis of *A. annua* has been studied by Liu *et al*. (2002).

Essential oil obtained from aerial parts of *A. nilagirica* var. *septentrionalis* harvested during different growth phases were analysed by GC and GC-MS (Haider *et al.*, 2007). The oil yield was highest (0.6 %) during flowering stage. During the vegetative, budding and fruiting stages, camphor was the main constituent, while during the flowering stage, it was replaced by β-caryophyllene. Other important constituents of the oil were germacrene-D, α-humulene and 1, 8- cineole.

GC - MS studies of essential oil of *A. vulgaris* revealed the presence of 88 components and the extracted oil was rich in camphor (16.8 %), α-thujone (11.3%), germacrene-D (72 %), camphene (6.5%), 1, 8- cineole (5.8%) and β-caryophyllene (5.4%) (Sujatha *et al.*, 2008).

Table 1

Previous reports on the chemical components of *Artemisia* essential oil

Sl. No.	Name of taxa	Part used	Chemical component	Authority	Year
1	*A. capillaris*	Aerial parts	Capillanol	Miyazawa and Kameoka	1975
2	*A. vulgaris*	Flowering tops	Vulgarole	Nano *et al.*	1976
3	*A. judaica*	Flowering tops	Piperitone (40%)	Karawya *et al*.	1977
4	*A. capillaris*	Aerial parts	α and β-thujone	Miyazawa and Kameoka	1977
5	*A. herba-alba* ssp. v*alentina*	Aerial parts	Dihydroreynosin torrentin	Gomis *et al*.	1979
6	*A. heptapotamica*	Flowers and leaves	α-pinene camphene carene cineole n-cymene artemisia ketone camphor bornyl acetate borneol	Gorgaev *et al.*	1980
7	*A. capillaris*	Seeds Stem Leaves and Roots	p-cymene 5-phenyl-1, 3-pentadyne dehydrofalcarinone	Harada and Iwasaki	1982
8	*A. subdigitata*	Aerial parts	β-pinene (35.7%) limonene (11%) sabinene (8.6%) estragole (7.8%)	Shi and Yuan	1982

Sl. No.	Name of taxa	Part used	Chemical component	Authority	Year
9	*A. arborescens*	Aerial parts	β-thujone chamazulene caryophyllene oxide	Hurabielle *et al.*	1982
10	*A. jacutica*	Inflorescence	Prochamazulene α-pinene camphene limonene n-cymene α-thujone creosol n-creosol formic acid propionic acid Isovalerianic acid	Berezovskaya *et al.*	1983
11	*A. abyssinica*	Inflorescence	Camphene α-phellandrene	El-Nasr *et al*.	1983
12	*A. arborescens*	Leaves and flowers	Chamazulene thujone camphor	Sacco *et al.*	1983
13	*A. caerulescens*	Leaves and flowers	α-thujone β-thujone camphor	Sacco *et al*.	1983
14	*A. caerulescens*	Aerial parts	Camphor (47.60%) thujone α-pinene camphene β-pinene sabinene	Mishurova *et al.*	1984

Sl. No.	Name of taxa	Part used	Chemical component	Authority	Year
			limonene cineole o-cymene p-cymene borneol terpineol		
15	*A. monosperma*	Aerial parts	Vicenin-2 lencenin-2 acacetin 7-glucoside acacetin-7-rutinoside glycosides	Saleh *et al.*	1985
16	*A. nilagirica*	Aerial parts	Camphor (9.74%) β-eudesmol (7.98%) 1,8-cineole (6.57%) borneol (5.29%) artemisia alcohol (3.41%) camphene (2.59%) α-gurjunene (0.92%) p-cymene (1.6%) terpinen-4-ol (1.24%) α-pinene (1.20%) α-thujone (0.58%) β-thujone (0.23%) α-santalol (1.35%)	Uniyal *et al.*	1985
17	*A. fragrans*	Aerial parts	1, 8-cineole (36%) α-thujone (28%) β-thujone (12%)	Aleskerova *et al.*	1986

Sl. No.	Name of taxa	Part used	Chemical component	Authority	Year
			camphor (7%)		
18	*A. spicigera*	Aerial parts	Camphor (48%) 1, 8-cineole (46%) n-cymol (0.2%) α-pinene (0.1%)	Aleskerova *et al.*	1986
19	*A. szowitziana*	Aerial parts	β-thujone (75%) α-thujone (9%) 1, 8-cineole (9%)	Aleskerova *et al.*	1986
20	*A. dracunculus*	Aerial parts	Estragole limonene myrcene ocimene α-phellandrene anisaldehyde	Bayrak *et al.*	1986
21	*A. vulgaris*	Aerial parts	α-thujone (56.3%)	Misra and Singh	1986
22	*A. vestita*	Aerial parts	α, β and γ-himachalene caryophyllene germacrene –D himachalol allohimachalol 1,8-cineole yomogi alcohol santolina alcohol thujone thujanol	Weyerstahl *et al.*	1987
23	*A. herba-alba*	Aerial parts	Chrysanthenyl acetate chrysanthenol	Yashpe *et al.*	1987

Sl. No.	Name of taxa	Part used	Chemical component	Authority	Year
			xanthoxylin 1,8-cineole α-thujone β-thujone camphor borneol		
24	*A. abrotanum*	Aerial parts	Umbelliferone scopoletine isofraxidine hydroxydavanone	Bergendorff and Sterner	1995
25	*A. annua*	Aerial parts	Artemisinin deoxyartemisinin artemisinic acid arteannuin-B stigmasterol	Zarga *et al.*	1995
26	*A. arborescens*	Aerial parts	Artemitin arborescin sesamin lirioresinol β-dimethyl ether chrysoeriol apigenin β-sitosteryl glucoside dihydroridentin chrysoeriol-4-glucoside	Zarga *et al.*	1995
27	*A. keiskeana*	Aerial parts	Artekeiskeanin-A	Kwak *et al.*	1997

Sl. No.	Name of taxa	Part used	Chemical component	Authority	Year
28	*A. glabella*	Aerial parts	1, 8-cineole (12%) linalool (8.2%) trans-verbanul (5.4%)	Atazhanova *et al.*	1999
29	*A. feddei*	Aerial parts	Scopoletin achillin	Kang *et al.*	1999
30	*A. vulgaris*	Aerial parts	Caryophyllene oxide (31.1%) hexadecanoic acid (6.3%) iso borneol 2-methyl -butyrate (5.3%) 2-heptadecanone (5.1%)	Pino *et al.*	1999
31	*A. giraldi* var. *giraldi*	Aerial parts	β-sitosterol α-amyrin stigmasterol daucosterol sesamine luteolin eupafolin hispidulin eupatalin belamcanidin pinitol artemin ridentin santolinylol	Tan *et al.* Tan *et al.*	1999 1999
32	*A. mongolica*	Aerial parts	Sesamine eupafolin eupatilin matricarin	Tan *et al.*	1999

Sl. No.	Name of taxa	Part used	Chemical component	Authority	Year
			3 - oxo - 11 - α - H - germacral (10) E 4 z - dien - 12, 6 - α - olide		
33	*A. vestita*	Aerial parts	Stigmasterol daucosterol umbelliferone scopolin scoparone iso scopoletin-o-glucoside	Tan *et al.*	1999
34	*A. scoparia*	Leaves	Eugenol (20.38%) eugenyl valerate (5.49%) limonene (4.98%) p-cymene (4.59%) eugenyl isovalerate (4.24%) eugenyl butyrate (2.85%) caryophyllene (5.27%)	Mohammed Ali *et al.*	2000
35	*A. gypsacea*	Aerial parts	1, 8-cineole (36.5%) β-thujone (28.4%) α-thujone (8.9%)	Rustaiyan *et al.*	2000
36	*A. santolina*	Aerial parts	Lavandulol (37.2%) 1, 8-cineole (15.9%) linalool (13.6%) lavandulyl acetate (9.5%)	Rustaiyan *et al.*	2000
37	*A. dracunculus*	Leaves and stem	Cis-nerolidol (17.1%) sabinene (13.23%) methyl eugenol (13.10%) spathulenol (4.8%)	Sanduin *et al*.	2000

Sl. No.	Name of taxa	Part used	Chemical component	Authority	Year
			terpinen-4-ol (4.76%) γ -muurolene (4.38%) caryophyllene oxide (3.16%) β-caryophyllene ((2.76%) δ-cadinene (2.33%) α-thujone β-pinene p-cymene β-ocimene γ -terpinene		
38	*A. vulgaris*	Aerial parts	Limonene (20%) chrysanthenone (15%) α-thujone (9.4%) germacrene-D (5.3%) myrtenol (4.9%) neryl acetate (4.7%) isopinocarveol (4.0%) β-caryophyllene (3.2%) eudesmol (3.2%) pinocarvone (3.1%) β-bisabolol (3.0%) δ-carene (2.8%)	Singh and Pandey Singh and Pandey	2000 2000
39	*A. annua*	Aerial parts	Arteannuin-B artemisin oleanolic acid β-sitosterol stigmasterol	Tang *et al.*	2000

Sl. No.	Name of taxa	Part used	Chemical component	Authority	Year
			artemetin bonanzin eupatalin chrysosplenetin		
40	*A. sieversiana*	Aerial parts Fruits Flowers	Guaianolides chrysosplenetin 15-hydroxy-3, 4,6,7-tetramethoxy flavone α-thujone (16%) artemisia alcohol (17.7%) camphor (38.7%)	Tang *et al.*	2000
41	*A. verlotiorum*	Leaves	α-thujone (47%) β-thujone (10%) 1, 8-cineole (21%) β-caryophyllene (3.4%)	Vernin	2000
42	*A. princeps*	Leaves	Borneol α-thujone artemisia alcohol verbenone yomogi alcohol germacren-4-ol 1,8-cineole artemisia acetate α-thujone β-caryophyllene bornyl acetate	Umano *et al*. Umano *et al*.	2000 2000

Sl. No.	Name of taxa	Part used	Chemical component	Authority	Year
			farnesene		
43	*A. deserti*	Aerial parts	Piperitone (52%) camphor (15.7%) 1, 8-cineole (11.8%)	Ahmad and Mirza	2001
44	*A. diffusa*	Aerial parts	Camphor (57.5%) verbenone (13%)	Ahmad and Mirza	2001
45	*A. campestris*	Aerial parts	β-pinene (27.9%) p-cymene (22.3%) α-pinene (11%)	Akrout *et al.*	2001
46	*A. diffusa*	Aerial parts	Camphor (35.0%) 1, 8-cineole (25.7%)	Khazraei and Rustaiyan	2001
47	*A. vulgaris*	Aerial parts	Artemisia lactone-1 vulgaris lactone-2	Ravi and Lakshmanan	2001
48	*A. judaica*	Aerial parts	Piperitone (53.5%) Chrysanthenone (9.8%) cis-chrysanthenyl acetate (7.4%)	Charchari	2002
49	*A. annua*	Aerial parts	Artemisia ketone(53%) 1, 8-cineole (8.4%) camphor (6.0%)	Jain *et al.*	2002
50	*A. absinthium*	Aerial parts	2-epoxy ocimene chrysanthenyl acetate β-thujone	Juteau *et al.*	2002
51	*A. fragrans*	Aerial parts	1, 8-cineole (5.2%) α-thujone (34.8%)	Barazandeh	2003
52	*A. khorassanica*	Aerial parts	α-thujone (43.4%) β-thujone (16.2%)	Barazandeh	2003

Sl. No.	Name of taxa	Part used	Chemical component	Authority	Year
			camphor (12.6%) 1, 8-cineole (6.6%) verbenone (6.4%)		
53	*A. campestris*	Aerial parts	Spathulenol (9.2%) 4-hydroxy-9-epi-β-caryophyllene (3.0%) β-pinene (9.1%) α-pinene (3.4%) limonene (2.5%) germacrene-D (3.3%)	Chalchat *et al.*	2003
54	*A. vulgaris*	Leaves	1, 8-cineole (12.2%) α-thujone (11.4%) camphor (23.1%) isoborneol (20.9%)	Haider *et al.*	2003
55	*A. iwayomogi*	Aerial parts	Camphor (19.3%) 1,8-cineole (19.3%) borneol (18.9) camphene (4.6%) β-caryophyllene (3.5%)	Yu *et al.*	2003
56	*A. nilagirica*	Leaves	α-thujone (41.9%) borneol (10.8%) β-thujone (9.1%) 1, 8-cineole (6.2%) β-eudesmol (1.6%) β-caryophyllene (1.9%) terpinen-4-ol (3.3%) pinocarvone (0.7%)	Mohammed Shafi *et al.*	2004

Sl. No.	Name of taxa	Part used	Chemical component	Authority	Year
			α-terpineol (0.8%) caryophyllene oxide (2.6%) eugenol (0.3%)	Mohammed Shafi *et al.*	2004
57	*A. vulgaris*	Aerial parts	1, 8-cineole (24.3%) camphor (14.1%) α-terpineol (8.8%) borneol (5.8%) myrtenol (3.3%) carveol (2.7%) β-elemene (3.9%) spathulenol (1.8%) caryophyllene oxide (1.6%) germacr-1- (10), 5-dien-4-β-ol (2.2%) presilphiperfolan - 9 - α - ol (1.4%)	Thao *et al.*	2004

Table 2

Previous reports on biological activities of some phytochemicals detected in the essential oil analysis of the present study

Component	Biological activity	Authority	Year
Sabinene	Perfumery	Teixeira da Silva	2004
β- pinene	Flavour	Sternberg and Duke	1996
	Herbicide	,,	,,
	Insectifuge	,,	,,
	Perfumery	,,	,,
p- cymene	Analgesic	,,	,,
	Antiflu	,,	,,
	Antirheumatalgic	,,	,,
	Bactericide	,,	,,
	Flavour	,,	,,
	Fungicide	,,	,,
	Herbicide	,,	,,
	Insectifuge	,,	,,
	Viricide	,,	,,
1,8-cineole	Acaricide	Teixeira da Silva	2004
	Allelopathic	,,	,,
	Antiallergic	,,	,,
	Anesthetic	,,	,,

Component	Biological activity	Authority	Year
	Anthelmintic	Harbone and Baxter	1983
	Antiacetylcholine esterase	Harbone and Baxter	1983
	Antiallergic	,,	,,
	Antibacterial	,,	,,
	Antibronchitic	,,	,,
	Anticarcinogenic	,,	,,
	Anticholine	,,	,,
	Esterase	,,	,,
	Antiulcer	,,	,,
	Cytochrome-P- 50	,,	,,
	Inducer	,,	,,
	Fungicide	,,	,,
	Flavour	,,	,,
	Herbicide	,,	,,
	Hypotensive	,,	,,
	Inflammatory	,,	,,
	Neurotoxic	,,	,,
γ-terpinene	Antioxidant	Sternberg and Duke	1996
	Insectifuge	,,	,,
	Perfumery	,,	,,
	ACE Inhibitor	Duke	2003
	Antiacetyline esterase	,,	,,

Component	Biological activity	Authority	Year
	Antioxidant	Duke	2003
	Aldose-reductase inhibitor	Okamura *et al*.	1992
	Irritant	Zebovitz	1989
	Pesticide	Duke	1992
α- thujone	Abortifacient	Teixeira da Silva; Sternberg and Duke	2004 1996
	Antibacterial	Sternberg and Duke	1996
	Anthelmintic	,,	,,
	Bactericide	,,	,,
	Convulsant	,,	,,
	Emmenagogue	,,	,,
	Epileptogenic	,,	,,
	Insecticide	,,	,,
	Larvicide	,,	,,
	Perfumery	,,	,,
	Spasmolytic	,,	,,
β- thujone	Abortifacient	Teixeira da Silva	2004
	Antibacterial	,,	,,
	Convulsant	,,	,,
	Emmenagogue	,,	,,
	Epileptogenic	,,	,,
	Insectifuge	Sternberg and Duke	1996

Component	Biological activity	Authority	Year
	Pesticide	,,	
L- camphor	Allelopathic	Teixeira da Silva	2004
	Analgesic	,,	,,
	Anesthetic	,,	,,
	Antiacne	,,	,,
	Antidiarrhoeric	,,	,,
	Antidysenteric	,,	,,
	Antiemetic	,,	,,
	Antifeedant	,,	,,
	Antifibrositic	,,	,,
	Antineuralgic	,,	,,
	Antioxidative	,,	,,
	Antipruritic	,,	,,
	Antispasmodic	,,	,,
	CNS- stimulant	,,	,,
	Cancer preventive	,,	,,
	Anticarcinogenic	Sternberg and Duke	1996
Camphene	Antilithic	Harbone and Baxter	1983
	Hypocholesterolemic	,,	,,
	Antioxidant	Sternberg and Duke	1996
	Flavour	Sternberg and Duke	1996
	Insectifuge	,,	,,

Component	Biological activity	Authority	Year
	Spasmogenic	,,	,,
	Ovicidal	Singh *et al.*	2000
Terpinen-4 ol	Antiallergic	Sternberg and Duke	1996
	Antiasthmatic	,,	,,
	Antiseptic	,,	,,
	Antitussive	,,	,,
	Bactericide	,,	,,
	Bacteriostatic	,,	,,
	Diuretic	,,	,,
	Fungicide	,,	,,
	Herbicide	,,	,,
	Insectifuge	,,	,,
	Nematicide	,,	,,
	Vulnerary	,,	,,
Cuminal	Bactericide	,,	,,
	Fungicide	,,	,,
	Flavour	,,	,,
	Larvicide	,,	,,
	Perfumery	,,	,,
Limonene	Allergenic	Mitchell and Rook	1923
	Antialzheimeran	Sternberg and Duke	1996
	Anticancer	,,	,,

Component	Biological activity	Authority	Year
	Antiflu	,,	,,
	Antilistic	,,	,,
	Antimutagenic	,,	,,
	Antitumour	Yu *et al.*	1995
	Antiviral	Sternberg and Duke	1996
	Bactericide	,,	,,
	Cancer preventive	‘’	‘’
	Candidistat	Kang *et al.*	1992
	Entero contractant	Sternberg and Duke	1996
	Expectorant	Harbone and Baxter	1983
	Fungiphilic	Sternberg andDuke	1996
	Fungistat	Kang *et al.*	1992
	Herbicide	Keeler and Tu	1991
	Insecticide	Sternberg and Duke	1996
	Insectifuge	,,	,,
	Irritant	,,	,,
	Nematicide	Nigg and Seigler	1992
	Termitifuge	,,	,,
	Viricide	Spring	1989
	Ovicidal	Singh *et al*.	2000
β- ocimene	Perfumery	Sternberg and Duke	1996
4-perillaldehyde	Bactericide	,,	,,

Component	Biological activity	Authority	Year
	Candidicide	,,	,,
	Fungicide	,,	,,
	Nematicide	,,	,,
	Sedative	,,	,,
α- terpinene	Anticarcinogenic	,,	,,
	Antiseptic	Harbone and Baxter	1983
	Bactericide	Sternberg and Duke	1996
	Flavour	,,	,,
	Motor Depressant	,,	,,
	Nematicide	,,	,,
	Sedative	,,	,,
	Perfumery	Harbone and Baxter	1983
	Termiticide	,,	,,
Eugenol	Insecticide	Singh *et al.*	2000
	Repellent	,,	,,
	Ovicidal	,,	,,
	Allergenic	Mitchell and Rook	1923
	Analgesic	Sternberg and Duke	1996
	Anesthetic	,,	,,
	Antiaggregant	,,	,,
	Antiedemic	,,	,,
	Antifeedant	,,	,,

Component	Biological activity	Authority	Year
	Antiinflammatory	,,	,,
	Anticonvulsant	Harbone and Baxter	1983
	Antimitotic	,,	,,
	CNS Depressant	,,	,,
	Antinitrosating	Sternberg and Duke	1996
	Antiprostaglandin	Thomas *et al*.	1986
	Antiradicular	Lamaison *et al.*	1988
	Fungicide	Bezanger	1990
	Candidicide	Sternberg and Duke	1996
	Ulcerogenic	,,	,,
	Antiseptic	,,	,,
	Antithromboxane	,,	,,
	Trypsin enhancer	,,	,,
	Antitumour	Zheng *et al.*	1992
	Antiulcer	Sternberg and Duke	1996
	Apifuge	,,	,,
	Cancer preventive	Sternberg and Duke	1996
	Carminative	,,	,,
	Choleretic	,,	,,
	Cytotoxic	,,	,,
	Dermatogenic	Mitchell and Rook	1923
	Entero relaxant	Sternberg and Duke	1996

Component	Biological activity	Authority	Year
	Febrifuge	,,	,,
	Flavour	,,	,,
	Herbicide	Bezanger	1990
	Insectifuge	Sternberg and Duke	1996
	Irritant	,,	,,
	Juvabional	,,	,,
	Larvicide	Spring	1989
	Motor Depressant	Sternberg and Duke	1996
	Nematicide	‘’	‘’
	Neurotoxic	Hixtable	1992
	Perfumery	Sternberg and Duke	1996
	Sedative	,,	,,
	Trichomonistat	Nigg and Seigler	1992
	Vermifuge	Sternberg and Duke	1996
α- copaene	Antialzheimeran	,,	,,
	Insecticide	Sternberg and Duke	1996
β- caryophyllene	Anticarcinogenic	Muroi and Kubo	1993
	Antiedemic	Shimizu	1990
	Antitumour	Zheng *et al*.	1992
	Insectifuge	Sternberg and Duke	1996
	Perfumery	,,	,,
	Spasmolytic	,,	,,

Component	Biological activity	Authority	Year
	Termitifuge	,,	,,
	Antibacterial	Cobos *et al*.	2001
	Antinemic	Srivastava *et al*.	2000
Farnesene	Antimicrobial	Arambewela *et al.*	1999
	Antifungal	Croteau *et al.*	2001
	Insect control	,,	,,
	Insectifuge	Sternberg and Duke	1996
Germacrene- D	Pheromonal	,,	,,
δ- cadinene	Anticarcinogenic	Muroi and Kubo	1993
	Bactericide	,,	,,
Caryophyllene oxide	Antiedemic	Shimizu	1990
	Antiinflammatory	,,	,,
	Antifeedant	Bettarini and Borgonovi	1991
	Insecticidal	,,	,,
	Antitumourous	Zheng *et al*.	1992
	Perfumery	Chowdhury and Kapoor	2000
	Flavour	,,	,,
β- eudesmol	Antianoxic	Sternberg and Duke	1996
	Antipeptic	,,	,,
	CNS inhibitor	,,	,,
	Hepatoprotective	,,	,,

Cytotoxic and Antitumour Assays

Nature has been the main source of medicinal treatments for thousands of years and plant based systems continue to play an essential role in the primary health care of 80% of the world's population. Crude natural products have been used for the treatment of cancer since ancient times. The use of plant materials for curing diseases like cancer has been reported in ancient Egyptian medical records dating back to 1550 BC and such references are available in the Chinese records as well. However, it is only during the last 35-40 years that serious efforts have been made to study more scientifically the naturally occurring plant derived anticancer agents. The complexity and peculiarity of the secondary metabolism of plants make it possible to search for drugs in any plant species.

Although more than 5, 00,000 plant species exist on earth, only a few have been considered for the production of medicinal drugs. The National Cancer Institute has taken up a major screening programme to isolate potential anticancer drugs from natural products since 1960. In the United States, under the NCI (National Cancer Institute) programme, over 35,000 plants were screened for anticancer activity between 1960 and 1986 and 2,000 crystalline plant-derived compounds were isolated and tested for activity against p388 lymphocytic leukaemia and KB carcinoma in cell culture. Some of the drugs that have been more widely studied for anticancer activity are taxol, camptothecin, vincristine, vinblastin and podophyllotoxins *etc*.

Comprehensive review of the technical and folkloric literature have been published by Ramakrishna *et al*. (1984) where citations are recorded for the use of different plant species which have been used for the treatment of cancer and other complications such as warts and tumours.

Anticancer activity is the best known type of biological activity possessed by sesquiterpene lactones. Computerized prediction of the anticancer activities of repin, acroptilin and contaurepensin found in some species of Asteraceae indicated that the sesquiterpene lactones are the materials with potential anticancer activity. They exhibit cytotoxic, cytostatic, interferon inducing and antiandrogenic activities (Pogrebnyak *et al*., 1998).

Artemisia species (Asteraceae), widespread throughout the world, are important medicinal plants, which are attracting the attention of phytochemists due to their biological and chemical diversity. These species are frequently used for the treatment of diseases such as malaria, hepatitis, cancer, inflammation and infections by fungi, bacteria and viruses (Tan *et al*., 1998).

Members of the Asteraceae are known to contain considerable amounts of sesquiterpene lactones. Amongst the properties associated with the sesquiterpene lactones, their cytotoxicity and antitumour activity are the most valuable. Costunolides (sesquiterpene lactones) with antitumoural activity have been isolated from *A. balchanorum* (Herout and Sorm, 1959). Rodriguez *et al*. (1976) described the different properties of sesquiterpene lactones. Sesquiterpene lactones exhibit cytotoxic and antibiotic properties, act as phytotoxins (plant growth regulating) and insect feeding deterrents and cause allergic contact dermatitis in humans and livestock poisoning.

The antitumour and cytotoxic activity of some sesquiterpene lactones has been previously reported (Gill *et al*., 1981; Hartwell, 1982; Lavault and Bruneton, 1979; Ulubelen *et al.*, 1986). Klayman (1985) reported the *in vitro* cytotoxic activity of the sesquiterpene lactone endoperoxide artemisinin and some chemically prepared derivatives, which have been found to be cytotoxic to cloned murine Ehrlich Ascites Tumour (EAT) cells and human HeLa cells and against murine bone marrow using a clonogenic assay for committed

progenitor cells of the granulocyte-monocyte lineage. Woerdenbag *et al*. (1986) reported the *in vitro* cytotoxicity of some sesquiterpene lactones on Human Lung Carcinoma cell line.

Antitumour activity of flavonoids from *Artemisia* species has been studied by Chemesova and Belenovskaya (1987). The essential oil of *A. caerulescens* ssp. *gallica* was observed to have analgesic, antipyretic and antiinflammatory actions when administered intraperitoneally to rats and mice at doses one fourth to one third that of its LD 50 of 1.35 ml/kg. Lysine acetyl salicylate was used as a reference compound (Moran *et al*., 1989).

Gribel and Pashinskii (1991) reported new data on the antitumour activity of *A. absinthium* extract. Experiments were carried out with mice infected with sarcoma 180, Ehrlich carcinoma, melanoma B-16, Louis's lung carcinoma and Pliss lymphosarcoma.

Monoterpenes are the class of compounds widely distributed in a variety of plants and their oils. Terpenoid compounds are one of the promising groups of compounds in the field of cancer prevention (Steinmetz and Potter, 1991). They have shown to prevent carcinogenesis by initiation and promotion/progression (Gould, 1997). Among the terpenoid compounds, limonene and carvone have shown to inhibit stomach tumour formation and pulmonary adenoma formation (Wattenberg *et al*., 1989). Limonene has shown to block mammary cancer formation in rats (Elegbede *et al*., 1984). Limonene and perillic acid are being evaluated in phase I clinical trials in cancer patients (Mc Namee, 1993).

Cytotoxicity of artemisinin related endoperoxides to Ehrlich Ascites Tumour cells was reported by Woerdenbag *et al*. (1993). The artemisinin related endoperoxides showed cytotoxicity to EAT cells at higher concentrations than those needed for *in vitro* antimalarial activity. Astins A

and B as well as cyclic pentapeptides from *Aster tataricus* showed strong antitumour activity against sarcoma 180 ascites in mice (Morita *et al*., 1993).

Several terpenoids and flavonoids, isolated from *A. annua*, showed significant cytotoxic activity when tested *in vitro* on several human tumour cell lines, among them artemisinin and quercetagetin proved to be responsible for the action observed on five of the tumour lines tested (Zheng, 1994). Camptothecin is a well known antitumour alkaloid first isolated from the bark of a Chinese tree *Camptotheca accuminata*. It is used for the treatment of colon, head and neck tumours and bladder cancer (Verotta and Baltis, 1994).

Immunomodulatory activity of solvent extracts of the air-dried aerial parts of *A. annua* has been demonstrated, through a study of their *in vitro* effects on human complement and T-lymphocyte proliferation (Kroes *et al*., 1995). *A. annua* "Qing Hao" is a herb used in traditional Chinese Medicine for the treatment of malaria, intestinal worms, febrile diseases and malignancy. Artemisinin is the active ingredient of *A. annua* and it is the most effective treatment so far known, against malaria. Scientific studies have also proven that artemisinin has selective cytotoxic action against iron overloaded abnormal cells (Lai and Singh, 1995).

Dimers of dihydroartemisinin were selected by Beekman *et al*. (1998) as potential antitumour compounds and subjected to the National Cancer Institute drug screening programme consisting of about 60 human cancer cell lines derived from nine different tissues. Ethnopharmacology, as a tool for the selection of medicinal plants for screening antitumour activity has been reported by Santos *et al*. (1999). A database was constructed with ethno pharmacological information about plants used to signs and symptoms frequently related to a variety of cancers associated with cancer cell lines available at the South American Anticancer Drug Development Office (SOAD). The database had 1430 records of use for 855 plant species.

The plant families such as Asteraceae, Fabaceae, Apocynaceae, Euphorbiaceae and Rubiaceae contributed the greater number of potential plant species. The species that received the highest scores were *A. absinthium, A. vulgaris, Biden pilosa, Spilanthes ocymifolia* and *Cassia alata etc.*

Aqueous extracts of 66 desert plants of the Negev and Bedouin were tested for antitumour, antimalarial and growth inhibition activities by Sathiyamoorthy *et al*. (1999). *Achillea fragrantissima, Solanum elaeagnifolium, Urginea maritima* and *Gypsophila arabica* exhibited strong cytotoxicity (above 97%) against cultured melanoma cell lines.

The antitumour activity of *A. princeps* (mugwort) was studied by Hwang *et al*. (1999) in ICR mice inoculated with Sarcoma-180 cells. When the acetone fraction was intraperitoneally injected into mice, which had been subcutaneously inoculated in the left groin with sarcoma-180, the tumour growth rate was inhibited by 30% and the average life span was prolonged by 20%.

NMR studies on novel antitumour drug principles, deoxoartemisinin and carboxypropyldeoxoartemisinin were done by Lee *et al*. (2000). The Chinese herbal preparations, denoted as 'PC SPES', a mixture consisting of extracts from 8 herbs, is being used with increasing frequency by prostrate cancer patients worldwide was explained by Darzynkiewicz *et al*. (2000). The 8 herbs include *Dendranthema morifolium, Ganoderma lucidum, Glycyrrhiza glabra, Isaris indigotica, Panax pseudoginseng, Robdosia rubescens, Scutellaria baicalensis* and *Serenoa repens*. Evidence has emerged that PC SPES is an effective modality that alleviates some symptoms in advanced prostrate cancer in a significant proportion of patients including the cases that were failed after the conventional therapy.

Studies conducted with *A. capillaris* aqueous extract shows that treated cells exhibit morphological changes typical of apoptosis, inducing condensed chromatin and a reduction in volume. Cell cycle analyses revealed that the extract induced cell cycle arrest at the G0 / G1 phase (Hu *et al.*, 2000).

Chromatographic separation studies of *A. stolonifera* helped to isolate a triterpene, a sesquiterpene, two aromatic compounds and a benzoquinone, which showed *in vitro* cytotoxicity against non small cell lung adenocarcinoma, ovarian, skin melanoma, central nervous system and colon tumours (Kwon, 2001).

One new triterpene and four new lignans, together with six known compounds were isolated from *A. caruifolia*. Most of the isolated lignans were moderately cytotoxic to Meth-A cells (5-10 µg /ml), but not to Louis's Lung Carcinoma (LLC) cells (Ma *et al.*, 2001).

Dias *et al*. (2001) reported the antiulcerogenic activity of crude ethanol extract and some fractions obtained from aerial parts of *A. annua*. Antioxidant activity of the essential oils of *A. afra*, *A. abyssinica* and *Juniperus procera* were studied by Burtis *et al.* (2001).

Information on the secondary metabolites of *A. annua* and their biological activity was reviewed by Bhakuni *et al*. (2001). GC-MS analysis of the essential oil characterized a large number of monoterpenoids. Biological activities of compounds isolated from *A. annua* were antimalarial, antibacterial, antiinflammatory, angiotensin converting enzyme inhibitory, plant growth regulatory and antitumour activities.

The amazing herb, *A. annua* was also examined for its activity against 55 cancer cell lines. It was found to be most active against leukaemia and

colon cancer and active against melanomas, breast cancer, prostrate cancer, CNS and renal cancer. It was also reported that artemisinin's effectiveness was comparable with other standard drugs used to combat cancer. As such, these results and the low toxicity of artemisinin had made this herb a potential candidate for cancer therapy (Efferth *et al*., 2001).

Singh and Lai (2001) reported that artemisinin effectively killed radiation resistant breast cancer cells *in vitro*.

Rowen reported the remarkable success of using the herb *Artemisia* to treat cancer patients ([4]http). The World Health Organization has recommended it for the treatment of malaria, since it is safe and inexpensive. The mechanism of action makes it closely related to enhanced oxygen therapy, similar to hydrogen peroxide therapy. Efferth *et al*. (2002) documented that *Artemisia* had significant anticancer activity in a wide variety of laboratory cultured cancer cells, but cancers that were resistant to chemotherapy drugs were not resistant to artemisinin. Hoang, whose family physicians have used artemisinin for 10 years reported that he has had a 50-60% long term remission rate with 400 cancer patients when artemisinin is used with a comprehensive integrative cancer strategy ([4]http). There are 3 common *Artemisia* derivatives, with distinct properties. Artesunate is water soluble and may be the most active and least toxic, but it has the shortest life within the body. Artemether is oil or lipid soluble and has the longest half life and the highest toxicity, but that is related to the high dosages, which are not necessary. Its advantage is its ability to cross the blood - brain barrier to reach cancer of the brain and nervous system. Artemisinin is the active potent compound of the plant. It has an intermediate half-life, is very safe and also crosses the blood - brain barrier ([4]http).

Antitumour activity of flavones isolated from *A. argyi* was reported by Seo *et al*. (2003). Leyon and Kuttan (2003) explained the role of some

synthetic curcuminoid derivatives in the inhibition of tumour specific angiogenesis.

Immunomodulatory activity of naturally occurring monoterpenes-carvone, limonene and perillic acid in Balb/c mice were studied by Raphael and Kuttan (2003). Administration of terpenoids increased the total antibody production, antibody producing cells in spleen, bone marrow cellularity and α-esterase positive cells significantly, compared to the normal animals indicating its potentiating effect on the immune system.

A series of low (Tang *et al*., 2003a) and high molecular compounds (Tang *et al*., 2003b) from Chinese herbal medicines have proved to be highly effective in tumour therapy. Hwang *et al*. (2003) reported that the polysaccharide fraction from the extract of *A. iwayomogi* suppresses apoptotic death of the mouse spleen cells in culture.

Antitumour activity of some plants from Meghayala and Mizoram against murine ascites Dalton's lymphoma was reported by Rosangkima and Prasad (2004). Inhibition of *Helicobacter pylori* adhesion to human gastric adenocarcinoma epithelial cells by acidic polysaccharides from *A. capillaris* and *Panax ginseng* was reported by Lee *et al*. (2004).

Anticancer and antiviral activities of *Youngia japonica* (Asteraceae) has been reported by Ooi *et al*. (2004). Ji *et al*. (2005) isolated a carbohydrate fraction, AIPI, from *A. iwayomogi*, which down-regulates 'Fas' gene expression and suppresses apoptotic death of the thymocytes induced by 2, 3, 7, 8-tetrachlorodibenzo-p-dioxin (TCDD). Thippeswamy and Bharathi (2006) studied the antiangiogenic and apoptotic properties of *Curcuma aromatica* extract and suggested that the plant extract can be further developed into potential anticancer drugs.

MATERIALS AND METHODS

Plant Material

Healthy young plants of *Artemisia nilagirica*, collected from Wyanad district of Kerala were grown in the net house of Botany Department, University of Calicut. The plant was authenticated at the Herbarium of Botany Department, University of Calicut, where a voucher specimen was deposited (CALI 86002).

Micropropagation

Nodal cuttings (1-1.5 cms), internodal cuttings (1-1.5 cms), leaf cuttings (1 cm^2) and inflorescence axis (1-1.5 cms) from the potted plant of *A. nilagirica* were used as explants to initiate the cultures. Explants were collected in water and were washed with labolene detergent for 5 min. The explants were again thoroughly washed in running water followed by double distilled water. Surface sterilization was done using 0.1% mercuric chloride for 2 min. in the case of nodal explants and 1 min. in the case of leaf cuttings and inflorescence axis. The explants were then washed 2-3 times in double distilled water to remove the traces of mercuric chloride. The explants were trimmed again and implanted onto the culture medium.

Murashige and Skoog (1962) basal medium (Table 3) with 3% sucrose, 100 mg/l myoinositol and 0.8% agar was used. MS basal medium was supplemented with different concentrations of auxin, cytokinin and combination of both. The pH of the medium was adjusted to 5.8. The media were sterilized at 120°C for 20 min. 10-15 replicates of each hormonal combination were tried. The cultures were grown at 25 $\pm$ 3°C with 55-60% humidity under fluorescent day light tubes emitting 2000 lux for 16/8 h. light/

dark period and were subcultured every 4-6 weeks.

Establishment of plants in soil

Four to seven weeks old micropropagated plants were subcultured in 0.5 mg/ml IBA for rooting. Root systems with 6 - 12 roots (2-6 cm long) were developed after 3 weeks growth in 0.5 mg/ml IBA for 7-10 days. The rooted plants were taken out from the cultured tubes, washed gently to remove agar and planted in a mixture of sand and soil (2:1) in plastic cups and initially covered with polythene bags to control humidity (Das, 1993). The plants were initially irrigated with tap water for 7-8 days. Well established plants were transplanted to plastic bags and later to earthern pots, kept in the net house and watered regularly.

Table 3

Murashige and Skoog basal medium (1962)

Stock chemicals		mg/l	Stock concentration	Stock g/l
I.	NH_4NO_3	1650.00	50 X	82.50
	KNO_3	1900.00		95.00
	KH_2PO_4	170.00		8.50
	$MgSO_4.7H_2O$	370.00		18.50
II.	$CaCl_2.\ 2H_2O$	440.00	50 X	22.00
III.	Na_2EDTA	37.30	100 X	3.70
	$FeSO_4.7H_2O$	27.80		2.80
IV.	$MnSO_4.4H_2O$	22.30	100 X	2.23
	$ZnSO_4.7H_2O$	8.60		0.860
	H_3BO_3	6.20		0.620
	Kl	0.83		0.083
	$Na_2MoO_4.2H_2O$	0.25		0.025
	$CoCl_2.6H_2O$	0.025		0.0025
	$CuSO_4.5H_2O$	0.025		0.0025
V.	Vitamins		100X	
	Glycine	2.00		0.200
	Nicotinic acid	0.50		0.050
	Pyridoxine-HCl	0.50		0.050

Thiamine-HCl	0.10		0.010

Cytological Analysis

Mitotic squash preparation

Cytological preparations were made using improved techniques (Sharma and Sharma, 1990). Young healthy root tips were collected at the time of peak mitotic activity (9 a.m. - 10 a.m.) from both the parent plant and the field transplanted micropropagated plants. Two weeks old white friable callus was also used for squash preparation. The root tips and callus were washed thoroughly with distilled water and pre-treated with cytostatic chemicals. Chilled saturated solution of para-dichlorobenzene with a trace of aesculin was used as pre-treatment chemical.

The pre-treatment solution was initially chilled at 0-5^{o}C for 5 min. and root tips and callus were dipped in it. This was kept at 12-15^{o}C for 3 h. After this, the root tips and callus were washed thoroughly with distilled water and fixed in modified Carnoy's fluid (1 acetic acid: 3 ethanol) for 24 h. The fixed root tips and callus were washed in distilled water and hydrolysed with 1N HCl for 20 min. and 30 min. respectively at room temperature. Traces of acid were removed by thorough washing in distilled water. The hydrolysed root tips and callus were stained in 2% aceto orcein for 3-4 h. The stained root tips and callus were washed in 45% acetic acid to remove excess stain and squashed in 45% acetic acid. All the slides were scanned under REICHERT MICROSTAR IV binocular research microscope and photographs were taken with the NIKON COOL PIX 5000 digital camera system attached to it.

Karyomorphological analysis

Karyograms were prepared from the microphotographs using computer based programs such as Adobe Photoshop, AutoCAD and data based analysing system (Microsoft Excel). Photographs were scanned and stored as

digital images. These digital images were converted to grayscale images using photoshop program. Identification numbers were allotted to each chromosome and then loaded to AutoCAD for karyomorphometrical analysis. After determining the centromeric position, arm lengths of each chromosome were measured and centromeric indices were calculated. On the basis of arm ratio and centromeric indices, homologous chromosomes were identified and were classified (Table 4) according to Abraham and Prasad (1983). The images were loaded to photoshop and karyograms were generated.

Karyotype formula was calculated depending upon the length of the chromosome, position of centromere and presence or absence of secondary constriction.

Disparity Index (DI) of the chromosomes was calculated with the method of Mohanty *et al*. (1991) by using the formula,

DI =

The Variation Coefficient (VC) among the chromosome complements was determined after Verma (1980) as follows:

VC =

The Total Forma percentage (TF%) or mean centromeric index value was calculated after Huziwara (1962) by the formula:

TF% =

Table 4

Details of chromosome nomenclature in relation to centromere location based on arm ratios and centromeric indices (Abraham and Prasad, 1983).

Nomenclature	Notation	R_1= s/l	R_2 = l/s	I_1 = 100 s/c	I_2 =100 l/c
Median	M	1.00	1.00	50.00	50.00
Nearly Median	Nm	0.99 to 0.61	1.01 to 1.63	49.99 to 38.01	50.01 to 61.99
Nearly Submedian	Nsm(-)	0.60 to 0.34	1.64 to 2.99	38.00 to 25.01	62.00 to 74.99
Submedian	SM	0.33	3.00	25.00	75.00
Nearly Submedian	Nsm(+)	0.32 to 0.23	3.01 to 4.26	24.99 to 18.20	75.01 to 81.80
Nearly Subterminal	Nst (-)	0.22 to 0.15	4.27 to 6.99	18.19 to 12.51	81.81 to 87.49
Sub Terminal	ST	0.14	7.00	12.50	87.50
Nearly Subterminal	Nst (+)	0.13 to 0.07	7.01 to 14.38	12.49 to 5.01	87.51 to 94.99
Nearly Terminal	Nt	0.06 to 0.01	14.39 to 19.99	5.00 to 0.01	95.00 to 99.99
Terminal	T	0	α	0	100.00

R_1 = arm ratio 1
R_2 = arm ratio 2
I_1 = centromeric index 1
I_2 = centromeric index 2
s = short arm length
l = long arm length
c = chromosome length

Random Amplified Polymorphic DNA (RAPD) Analysis

RAPD is an improved technique used to reveal sequence polymorphism between template DNAs based on selective amplification of DNA sequences. The template DNA (genomic DNA) can be prepared with any purification protocol appropriate for the biological sample under study.

Isolation of DNA

In the present study CTAB method (Ausubel *et al*., 1995) was followed to isolate DNA from the parent and *in vitro* plants of *A. nilagirica*. The stepwise protocol used for extraction of DNA is given below.

- Fresh young leaves of the parent and 12 *in vitro* plants of *A. nilagirica* were collected in ice boxes.
- Grind 2 g of young leaves in liquid nitrogen with a pre-chilled mortar and pestle and add 8 ml of preheated (60^0C) CTAB buffer. Add 0.2% β - mercaptoethanol prior to use. Incubate at 60 ^{0}C for 1h. in a water bath.
- Extract with equal volume (8 ml) of chloroform: isoamyl alcohol (24:1) at 10,000 rpm for 10 min. at room temperature.
- Take the aqueous phase and add 2/3 rd volume of ice cold isopropanol and mix by gentle inversion. The DNA threads were visible.
- Incubate at –20 ^{0}C for 30 min. to enhance the precipitation of DNA and centrifuge at 10,000 rpm for 15 min. at 4 ^{0}C.
- Discard the supernatant and invert the tube on tissue paper for a few min. and dissolve the pellet in 1.5 ml of TE buffer. Store at room temperature over night.

Purification of DNA (Removal of RNA and Protein)

- Add 10 µg/ml of RNase to 200 µl of dissolved unpurified DNA and incubate at 37 ^{0}C for 30 min.
- Add equal volume of tris saturated phenol, mix it well and centrifuge at

10,000 rpm for 10 min.

- To the aqueous phase add equal volume of phenol : chloroform : isoamyl alcohol (25:24:1), shake and centrifuge at 10,000 rpm for 10 min.
- Take the aqueous phase and add equal volume of chloroform : isoamyl alcohol (24:1), shake and centrifuge at 10,000 rpm for 10 min.
- To the aqueous phase add one-tenth volume of 3 M sodium acetate (pH 5.2) and 0.8 volume of isopropanol, mix gently and incubate at 4^0C for overnight.
- Centrifuge at 10,000 rpm for 10 min. and wash the pellet in 70% ethanol (10,000 rpm for 5 min.)
- Air dry the pellet and dissolve in 500 µl TE and estimate the yield.

The composition of various stock solutions and buffers are given in tables 5 and 6.

Quantification of DNA

The isolated DNA was quantified using UV scanning Shimadzu Spectrophotometer. OD at 260/280 nm was recorded and the amount of DNA was calculated based on its OD at 260 nm. DNA was visualized on agarose gel (0.8%) for its quality and stored at -20^0 C.The DNA samples were loaded along with λ DNA standard and DNA quantity was estimated by comparing with that of the standard.

The reagents and buffers for DNA extraction were prepared as per Sambrook *et al.* (1989).

Optimization of PCR protocol for RAPD analysis

RAPD profiles were developed as per the method suggested by Williams *et al*. (1990) with minor modifications. The dNTPs, Taq polymerases and other chemicals were procured from Genei, Bangalore, India. Arbitrary primers from Operon Technologies Inc., Alameda, California were used for PCR reactions. Each primer contains at least 60 – 70% GC content and do not possess self complementary ends.

Table 5

Buffers and the methods of preparation for genomic DNA extraction

Buffer	Method of preparation
CTAB extraction buffer-1litre, 100 mM Tris-HCl (pH 8.0) (Sigma), 20 mM EDTA (pH 8.0)(Sigma), 1.4 M NaCl (Sigma), 2% CTAB (w/v) Merck & 0.2% β- mercaptoethanol (v/v) Merck	Measure 100 ml Tris (1 M), 280 ml of NaCl, 40 ml of EDTA (0.5 M). Mix with about 400 ml of hot distilled water, add 20 g of CTAB to this. Adjust final volume to one litre. Dispense to reagent bottles and autoclave. Just before use, add 0.2% β-mercaptoethanol.
TE (0.1 mM) buffer -100 ml, 100 mM Tris HCl (pH 8.0), 0.1 mM EDTA (pH 8.0)	Take 1ml of Tris HCl (1 M), 20 ml of EDTA (0.5 M). Mix with 99 ml of sterile distilled water taken in a reagent bottle, mix thoroughly and autoclave.
Gel loading buffer (6x) – 100 ml, 0.25% bromophenol blue (BPB) (Sigma), 30% glycerol (Merck)	Dissolve 0.25 g of BPB in 99 ml of 30% glycerol. Keep on magnetic stirrer for several hours to get the dye completely dissolved. Dispense to reagent bottles and store at 4^0C.
RNase A (10 mg/ml)	Make up 10 mg/ml RNase in distilled water. Boil for 10 min. to destroy DNase. Divide into 1 ml aliquots and store at -20^0C.
50x TAE stock – 1 litre	Tris base 242 g, 0.5 M EDTA 100ml, glacial acetic acid 57.1 ml. Adjust the volume to 1 litre with distilled water.
10x DNA loading dye	Ficoll (Type 400) 25% (9 w/v),

	bromophenol blue 0.4 % (w/v), xylene cyanol FF 0.4% (w/v).

Table 6

Stocks for the reagents (Sambrook *et al*., 1989)

Solution	Method of preparation
1 M Tris(pH 8.0) 500 ml	Dissolve 60.55 g of Tris base (Sigma) in 300 ml distilled water adjust pH to 8.0 by adding conc. HCl. Make up the volume to 500 ml. Dispense into reagent bottles and sterilize by autoclaving.
0.5 M EDTA (pH 8.0)	Dissolve 93.05 g of EDTA-disodium salt (Sigma) in 300 ml of water. Adjust pH to 8.0 by adding NaOH pellets. Adjust the final volume to 500 ml. Dispense into reagent bottles and autoclave.
5 M NaCl - 500 ml	Weigh 146.1 g NaCl (Merck) add 200 ml of water and mix well. When the salts get completely dissolved, adjust the final volume to 500 ml. Dispense into reagent bottles and autoclave.
3 M sodium acetate (pH 5.2) 250 ml	Dissolve 61.523 g of sodium acetate (Qualigens) in 200 ml of distilled water. Adjust the pH to 5.2 and with glacial acetic acid, make up the volume to 250 ml. Sterilize by autoclaving.
EtBr (10 mg/ml) – 100 ml	Add 1 g of ethidium bromide to 100 ml of distilled water. Keep on magnetic stirrer till the dye gets dissolved. Dispense to amber coloured reagent bottle and store at 4^0C.
70% ethanol - 500 ml	Take 350 ml of ethanol, mix with 150 ml of distilled water. Dispense to reagent bottle and store at 4^0C.
Chloroform: isoamyl alcohol (24:1) - 500 ml	Measure 480 ml of chloroform and 20 ml of isoamyl alcohol. Mix and store in reagent bottles at room temperature.
1 M $MgCl_2$ - 100 ml	Weigh 20.33 g of $MgCl_2$, dissolve in double distilled water, make up to 100 ml, dispense into reagent bottles and autoclave.

Template DNA concentration

One of the most appealing features of PCR is that the quantity needs to be optimum. The effect of different concentrations of DNA *viz.*, 1, 5 and 10 ng per reaction volume of 25 µl is studied using one primer (Innis and Gelfand, 1990).

Assay buffer concentration

The standard buffer contains 50 mM KCl, 10 mM Tris HCl (pH 8.4), 1.5 mM $MgCl_2$ and 100 µl/ml of gelatin for genomic PCR (Innis and Gelfand, 1990). 2.5 µl buffer (10x) from Genei, Bangalore, India was used for each reaction.

$MgCl_2$ concentration

Magnesium concentration plays an important role in the amplification of DNA by polymerase chain reaction. 2.5 mM concentration of $MgCl_2$ (Genei, Bangalore, India) was used.

Deoxyribo Nucleotide Triphosphates concentration

Two different concentrations (100 µM and 200 µM of dNTPs (dATP, dTTP, dCTP and dGTP) were tried. All the four dNTPs were used at the same concentration to minimize errors (Innis and Gelfand, 1990).

Primer concentration

Primer concentrations between 0.1 µM, 0.5 µM, 1.0 µM and 2.0 µM per reaction volume of 25 µl were studied using 10 ng of DNA, of these 0.1 µM - 0.5 µM were optimal concentration. Higher primer concentrations may promote mispairing and accumulation of non-specific product and may increase the probability of generating a template, independent artifact, termed primer dimer. Non specific products and primer dimer artifacts are themselves

substrates for PCR and compete with the desired product for enzyme, dNTPs and primers, resulting in a low yield of the desired product (Innis and Gelfand, 1990).

Taq DNA polymerase enzyme concentration

Two different concentrations of Taq DNA polymerase *viz.*, 2 U/25 µl and 3 U/25 µl were tried. As a rule, concentrations in excess of 4 U tend to result in the accumulation of non specific amplification products, whereas amounts less than 1 U usually reduce yield of the desired product (Innis and Gelfand, 1990). Taq polymerase from Genei, Bangalore, India having a concentration of 2 U/µl was used in the study.

Optimization of PCR programming

The reaction mixture for PCR was prepared as given in Table 7.

The optimized PCR reaction profile is given below:

35 cycles:

1st	94^0 C for 3 min.	1 cycle
IInd	94^0 C for 1 min.	
	37^0 C for 1 min.	34 cycles
	72^0 C for 1 min.	
IIIrd	72^0 C for 15 min.	1cycle

Table 7. Reaction mixture for PCR

Component	Volume (1x)
Sterile distilled water	11.75 µl
10x PCR buffer	2.50 µl
dNTPs (1mM)	3.75 µl
Primer (5 pmoles/ µl)	2.00 µl
$MgCl_2$ (10 mM)	1.50 µl
Taq polymerse (2U/ µl)	0.50 µl
Template DNA (10 ng/µl)	3.00 µl
Total reaction volume	**25.00 µl**

Amplification protocol

The reaction volume of 25 µl containing, 2.5 µl PCR buffer (10x), 1.5 µl $MgCl_2$ (10 mM), 3.75 µl dNTPs (1 mM), 2.0 µl primer (5 pmoles/µl), 0.5 µl Taq polymerase (2 U/µl), 3.0 µl Template DNA (10 ng/µl) and the volume was made up with nuclease-free water. PCR machine used was Peltir thermocycle MJ Research PTC- 200, USA.

Primer screening

Fifteen decamer oligonucleotides (Operon Technologies Inc., Alameda, California) were screened for polymorphism. Among them, five nucleotide primers showed good polymorphism (Table 8).

Table 8. Operon primers used for developing RAPD profiles

OPA-01	5’CAGGCCCTTC 3’
OPA-02	5’TGCCGAGCTG 3’
OPB-17	5’AGGGAAGGAG 3’
OPB-18	5’CCACAGCAGT 3’
OPC-01	5’TTCGAGCCAG 3’

The PCR reaction mix was prepared using all the seven components in 0.2 ml sterile thin walled microfuge tube. The final reaction volume was 25 µl.

Electrophoresis

PCR products were visualized in 2% agarose gel containing 0.5 µg ml^{-1} ethidium bromide and documented in Alpha imager 2220 gel documentation system. In all case λ phage DNA digested with Eco R1 and Hind III was used as size marker.

Essential Oil Analysis

Essential oil extraction

Shade dried aerial plant parts of both the *in vivo* and *in vitro* plants of *A. nilagirica* were hydrodistilled separately in a Clevenger apparatus (Clevenger, 1928) at 100^{o}C for 4h. as prolonged extraction normally increases the yield (Gildemeister and Hoffman, 1961). The aromatic greenish essential oil was collected and dried over anhydrous sodium sulphate. The pure oil was transferred into a small amber coloured bottle and stored at 4-6^{o}C.

Gas Chromatography – Mass Spectrometry (GC-MS)

GC-MS of the oil was performed on HP 6890/5973-GC-MSD-D5 at 75 eV and at 250°C. The GC column condition used was: HP-5 (DB5), fused silica capillary – 0.32 mm x 30 m with film thickness 0.25 μ; carrier gas – helium, length of the column – 30 m, flow rate – 1.4 ml/min. Temperature programme: initial temperature 60°C for 1 min. and then heated at the rate of 3°C/min to 246°C. Run time is 56 min. The components were analysed and structures of various components were ascertained with the help of Wiley Library 275 combined with the analyser.

Chemotaxonomic evaluation

The data obtained from the qualitative analysis of both *in vivo* and *in vitro* developed plants were subjected to numerical analysis to understand the chemical affinity of both by arriving at a numerical constant, the coefficient of similitude (CS), using the following formula proposed by Sokall and Sneath (1963).

CS =

Cytotoxic and Antitumour assays

Buffer and stain

PBS (Phosphate Buffered Saline) pH 7.2: 0.1 M

NaCl	:	8 g
KI	:	0.2 g
Na_2HPO_4	:	1.44 g
KH_2PO_4	:	0.2 g

Dissolve in 1 L double distilled water and pH is adjusted to 7.2.

Trypan blue

Dissolve 1% trypan blue in saline, filter and use.

Experimental animals

Female Swiss albino mice (6-8 weeks old) were purchased from small Animal Breeding Station, Veterinary College, Kerala Agricultural University, Kerala. The animals were kept in air controlled room, fed with normal mice chow (Sai feeds, India) and water *ad libitum*. All the animal experiments were performed according to the rules and regulations of the Institutional Animal Ethics Committee (IAEC), Government of India and followed the guidelines of IAEC.

***In vitro* cell lines**

Dalton's Lymphoma Ascites (DLA) cells (spontaneous carcinoma of thymus) and Ehrlich Ascites Carcinoma (EAC) cells (spontaneous tumour of mammary gland) of mice were obtained from Amala Cancer Research Institute, Thrissur. Both the cell lines were maintained by transplanting the cells every two weeks as ascites in the peritoneal cavity of Swiss albino mice.

Drug Preparation

Preparation of methanol extract of *A. nilagirica*

Aerial part of the parent and somaclonal variant were dried at 45^{o}C and pulverized. 25 g of powdered plant material was extracted in 250 ml of 100% methanol and stirred overnight using a magnetic stirrer. The supernatant collected after centrifugation at 10,000 rpm at 4^{o}C was concentrated and evaporated to dryness in a water bath for the complete removal of methanol and stored at 4^{o}C in a refrigerator.

Drug preparation for *in vitro* studies

Stock solution of the drugs were prepared by dissolving 50 mg of methanol extracts of both parent and *in vitro* plants of *A. nilagirica* in 1 ml dimethyl sulfoxide (DMSO). Various dilutions of the stock solutions of both the parent and *in vitro* plant extracts were prepared (1000, 750, 500, 250, 100, 50 and 10 μg/ml) in PBS.

Drug preparation for *in vivo* studies

For *in vivo* studies, stock solutions of the drugs were prepared by suspending 100 mg of methanol extract of both parent and i*n vitro* plants of *A. nilagirica* in 10 ml of 1% gum acacia. It was done by dissolving 100 mg of both the extracts in minimum volume of methanol and was poured into the beaker containing 100 mg gum acacia dissolved in 10 ml PBS by boiling. The stock solutions were further diluted to 250 μg/ml and 500 μg/ml in PBS.

Drug administration

Both these drugs in 2 doses (250 μg/dose/animal; 500μg/dose/animal) were administered intraperitonealy to female Swiss albino mice (6-8 weeks old) for 20 consecutive days.

Cytotoxicity (*In vitro* studies)

In vitro cytotoxic activity of parent and micropropagated plant extracts of *A. nilagirica* were analysed with Dalton's Lymphoma Ascites and Ehrlich Ascites Carcinoma cells. The DLA and EAC cells were aspirated from the peritoneal cavity of ascites tumour bearing mice and transferred to tubes containing PBS. Tumour cells were washed 3 times using PBS and adjusted to 10 x 10^6 cells per ml in PBS.

The DLA cells and EAC cells (0.1 ml from stock) were incubated with

various concentrations of both the drugs (10-1000 µg/ml) separately in a final volume of 1 ml for 3 h. at 37°C. After incubation, the viability of cells was determined by the trypan blue dye exclusion method (Talwar, 1974) after adding 0.1 ml of trypan blue (1%). The viable cells exclude the dye, while non-viable cells take up the dye and thus appeared blue in colour. The stained and unstained cells were counted separately using haemocytometer and the percentage of cell death was calculated. Controls were kept as untreated and as DMSO treated cells respectively.

Antitumour activity (*In vivo* studies)

Effect of *A. nilagirica* extracts on the solid tumour development

Dalton's Lymphoma Ascites tumour cells aspirated from Swiss albino mice were washed 3 times in PBS and suspended at a concentration of 10 x 10^6 cells/ml. These cells (0.1 ml containing 1 x 10^6 cells) were injected subcutaneously to the right hand limb of 3 groups (6 mice/group) of female Swiss albino mice for the development of solid tumours. Groups 1 and 2 were treated with *A. nilagirica* extracts separately in 1% gum acacia (250 µg/dose/animal and 500 µg/dose/animal) respectively, simultaneously with the tumour implantation for 20 consecutive days. The 3rd group was kept as untreated control which received the vehicle of 1 % gum acacia during the same period. The radii of developing tumour were measured using vernier callipers on 7th day after the tumour induction and on every third day thereafter for one month. Tumour volume was calculated using the formula

V = , where r_1 and r_2 are the radii of tumour along 2 directions (Kuttan *et al*., 1988). This was compared with untreated control.

Effect of *A. nilagirica* extracts on the ascites tumour development

Ehrlich Ascites Carcinoma cells were aspirated, washed and suspended in PBS. Three groups (6 mice/group of female Swiss albino mice) were induced with ascites tumour by injecting $1x10^6$ cells/animal to the peritoneal cavity. Groups 1 and 2 were inoculated with *A. nilagirica* extracts separately in 1% gum acacia (250 µg/dose/animal and 500 µg/dose/animal) respectively, simultaneously with the tumour implantation and continued for 20 consecutive days. Group 3 was kept as control, which received 1% gum acacia in PBS during the same period. The death pattern of the animals due to tumour burden was noted and the percentage of increase in the life span was

calculated using the formula ILS = , where T and C represents the mean survival time of treated and control groups of animals (Kuttan *et al*., 1988).

Statistical analysis

The results were expressed as mean S.D. The statistical analysis was done by one way analysis of variance (ANOVA) followed by Dunnett's t-test using Instat 3 software. $P < 0.01$ and $P < 0.05$ were considered to be significant.

RESULTS

Micropropagation

Murashige and Skoog (MS) basal medium (Tables 9, 10 & 11) with different hormonal combinations was used for micropropagation. Among the different explants used (leaves, nodes, internodes, inflorescence axis and roots) positive response was exhibited by leaves, nodes and axis of inflorescence. For callus induction and multiple shoot regeneration, medium with auxins (NAA, IAA, IBA and 2, 4-D) alone, cytokinins (BAP and KIN) alone and different combinations of both these were used (Tables 9, 10 & 11).

All the explants such as leaves, nodes and inflorescence axis, produced callus and multiple shoots in the medium supplemented with different combinations of BAP and NAA.

Of the three explants, the leaf explants produced white powdery friable callus after 2-3 weeks in 70-80% of the culture medium when supplemented with BAP (1 mg/l) + NAA (1 mg/l) and BAP (0.5 mg/l) + NAA (1.5 mg/l). 90% of callus production was noticed in medium having BAP (0.5 mg/l) + NAA (0.5 mg/l). Cream coloured friable callus was noticed in BAP (1.5 mg/l) + NAA (0.5 mg/l) containing medium. Brownish white callus was noticed in 30% of the culture when NAA (0.5 mg/l) was used. Medium with BAP (1 mg/l), BAP (1 mg/l) + NAA (2 mg/l) and BAP (0.1 mg/l) + 2,4-D (0.1 mg/l) produced little callus and could help only in the rooting of explant. Medium with BAP (0.1 mg/l) + IBA (0.1 mg/l), BAP (0.1 mg/l) + IBA (0.5 mg/l) and NAA (0.5 mg/l) + 2,4-D (0.5 mg/l) produced hard callus with roots. KIN (0.5 mg/l) + NAA (0.5 mg/l) combination resulted in swelling and crumbling of leaf tissue (Table 9; Plate 1).

Multiple shoot initiation was noticed after 3-4 weeks in 90-100% of the callus after subculturing it in the medium with BAP (0.5 mg/l) + NAA (0.5

mg/l). The medium with BAP (1 mg/l) + NAA (1 mg/l) and BAP (0.5 mg/l) + NAA (1.5 mg/l) also produced multiple shoots in 70-80% of the callus. Some of the calli developed from the leaf explant in the medium containing BAP (0.1 mg/l) + IBA (0.1 mg/l), NAA (0.5 mg/l) + 2, 4-D (0.5 mg/l) showed rhizogenesis, but failed to produce shoots (Table 9; Plates 1- 2).

The nodal cutting and inflorescence axis produced multiple shoots both directly and indirectly. Multiple shoots were developed directly from nodal cuttings in 90-100% of the medium containing BAP (0.5 mg/l) and 60-70% in BAP (1 mg/l). The medium with BAP (0.5 mg/l) + NAA (1.5 mg/l) produced only little callus at the base and axillary development of shoots were noticed (Table 10; Plates 8-9).

Callus formation was profuse when nodal cutting was inoculated in the medium containing BAP (1 mg/l) + NAA (1 mg/l). 80-90% of multiple shoots were developed indirectly from the callus when subcultured in the same medium (Table 10; Plates 3- 4).

Inflorescence axis produced 80-90% multiple shoots directly in BAP (1 mg/l) medium (Table 11; Plates 10 - 11) and indirectly from callus in the medium with BAP (1 mg/l) + NAA (1 mg/l) (Table 11; Plates 5-7).

In vitro shoots developed roots in the medium devoid of growth hormones. However, the medium supplemented with IBA (0.5 mg/l) also developed maximum number of roots (Figs. 13, 14, 26 - 28, 43, 44, 54, 55, 63 & 64). These were later transferred to the sterilized soil - sand mixture and about 95 -100% field survival was obtained in pots (Plate 12).

Vigorous vegetative propagation by stem cutting was noticed in the cultured plant also. Since no remarkable morphological variation was noticed among the field established plants, further analysis at cytological, molecular and phytochemical levels were conducted to search for the possible somaclonal variations.

Table 9

Effect of phytohormones on callus induction and regeneration from leaf explant *in vitro*

Sl. No.	Cytokinins (mg/l)		Auxins (mg/l)				% frequency of response			Observation
	BAP	KIN	NAA	IAA	IBA	2,4-D	Callus induction	Formation of		
								multiple shoots	roots	
1	1	-	-	-	-	-	20	-	20	Callus with rooting
2	-	-	0.5	-	-	-	30	-	-	Brownish white callus
3	-	-	1	-	-	-	30	-	-	Hard callus
4	0.5	-	0.5	-	-	-	90	90-100	-	Profuse multiple shoots from cream friable callus
5	0.5	-	1	-	-	-	70	60-70	-	Multiple shoots developed from friable callus
6	1	-	1	-	-	-	80	70-80	-	White friable callus with multiple shoots
7	0.5	-	1.5	-	-	-	80	70-80	80	White powdery callus and rooting of multiple shoots
8	1.5	-	0.5	-	-	-	50	40-50	-	Cream friable callus and multiple shoots
9	1	-	2	-	-	-	20	-	-	Callus from leaf and rooting
10	-	-	-	-	0.5	-	-	-	95	Profuse rooting of multiple shoots
11	-	-	-	-	1	-	10	-	50	Little callus and rooting of multiple shoots
12	0.1	-	-	-	0.1	-	30	-	10	Hard callus with rooting
13	0.1	-	-	-	0.5	-	40	-	10	Hard callus with rooting
14	0.1	-	-	-	-	0.1	10	-	-	Little callus and dried
15	-	-	-	1	-	-	20	10-20	-	Friable callus with shooting
16	-	0.5	0.5	-	-	-	-	-	-	Swelling and crumbling of leaf tissue
17	-	-	0.5	-	-	0.5	20	-	20	Hard callus with rooting
18	0.1	0.1	0.1	-	-	-	10	-	-	Little callus initiation

Table 10

Effect of phytohormones on callus induction and regeneration from nodal explant *in vitro*

Sl. No.	Cytokinins (mg/l)		Auxins (mg/l)				% frequency of response			Observation
	BAP	KIN	NAA	IAA	IBA	2,4-D	Callus induction	Formation of		
								multiple shoots	roots	
1	0.5	-	-	-	-	-	-	90-100	-	Multiple shoots developed directly
2	1	-	-	-	-	-	-	60-70	-	Multiple shoots developed directly
3	-	-	0.5	-	-	-	40	-	-	Green friable callus
4	-	-	1	-	-	-	50	-	-	Green callus
5	0.5	-	0.5	-	-	-	30	30-40	-	Callus with multiple shoots
6	0.5	-	1	-	-	-	50	60-70	-	Multiple shoots developed from friable callus
7	1	-	1	-	-	-	80	80-90	-	Multiple shoots well developed from white friable callus
8	0.5	-	1.5	-	-	-	20	50	-	Little callus at base and axillary development of shoots
9	1.5	-	0.5	-	-	-	30	30-40	-	Callus with multiple shoots
10	1	-	2	-	-	-	-	10-20	-	Direct development of shoots
11	-	-	-	-	0.5	-	-	-	95	Profuse rooting of multiple shoots
12	-	-	-	-	1	-	-	-	50	Rooting of multiple shoots
13	0.1	-	-	-	0.1	-	-	-	30	Rooting of multiple shoots
14	0.1	-	-	-	0.5	-	20	30	20	Little callus with rooting of multiple shoots
15	1	-	-	1	-	-	10	-	20	Little callus with shoots
16	-	0.5	0.5	-	-	-	10	-	-	Hard callus
17	-	0.5	-	-	-	0.5	10	-	10	Little callus and rooting
18	0.1	0.1	0.1	-	-	-	-	20	-	Axillary shoot development
19	0.1 0.1	-	-	-	-	0.1	-	-	-	No response

Table 11

Effect of phytohormones on shoot multiplication and callus induction from inflorescence axis explant *in vitro*

Sl. No.	Cytokinins (mg/l)		Auxins (mg/l)				% frequency of response			Observation
	BAP	KIN	NAA	IAA	IBA	2,4-D	Callus induction	Formation of		
								multiple shoots	roots	
1	0.5	-	-	-	-	-	-	50-60	-	Multiple shoots developed directly
2	1	-	-	-	-	-	-	80-90	-	Direct development of multiple shoots
3	-	-	0.5	-	-	-	30	-	20	White powdery callus and rooting
4	-	-	1	-	-	-	50	-	-	White friable callus
5	0.5	-	0.5	-	-	-	30	30-40	-	Callus with multiple shoots
6	0.5	-	1	-	-	-	40	50	-	Cream friable callus with multiple shoots
7	1	-	1	-	-	-	85	80-90	-	Green callus with profuse multiple shoots
8	0.5	-	1.5	-	-	-	-	40	-	Multiple shoots developed directly
9	1.5	-	0.5	-	-	-	40	50	-	Callus with multiple shoots
10	1	-	2	-	-	-	-	30-40	20	Direct development of shoots and rooting of multiple shoots
11	-	-	-	-	0.5	-	-	-	95-100	Profuse rooting of multiple shoots
12	-	-	-	-	1	-	-	-	80	Rooting of multiple shoots
13	0.1	-	-	-	0.1	-	30	30	30	Callus and rooting of multiple shoots
14	0.1	-	-	-	0.5	-	40	40	30	Callus and rooting of multiple shoots
15	1	-	-	1	-	-	20	20-30	-	Little callus and shoots
16	-	-	0.5	-	-	0.5	10	-	-	Little callus
17	0.1	0.1	0.1	-	-	-	-	-	-	No response
18	0.1	-	-	-	-	0.1	-	-	-	No response

Cytological Analysis

The *in vitro* plant was analyzed for any karyomorphological changes and to detect the cytological basis of any variation, if present. The ploidy level of the parent plant, callus and the cultured plant was found to be different. The parent plant was found to be tetraploid (2n=4x=36) (Plate 13), while the somaclonal variant was octaploid (2n=8x=72) (Plate 17). The callus showed three types of cells, cells showing tetraploidy (2n=4x=36), octaploidy (2n=8x=72) and hexadecaploidy (2n=16x=144) (Plates 14-16). The frequency of callus cells containing the octaploid number was found to be more when compared to the other two types of the cells observed in the callus.

The number of secondary constrictions also showed variations. The parent plant has 4 chromosomes with secondary constrictions, whereas in hexadecaploid callus it was 16 and in somaclonal variant, it was only 8. In tetraploid callus it was only 4 and in octaploid callus, it was 8.

Changes in chromosome length, disparity index, variation coefficient and total forma percentage were noticed (Tables 12 - 17).

The total chromosome length of the parent was only 36.6378 µm and of somaclonal variant was 59.3116 µm. The total chromosome length of tetraploid callus was 43.2618 µm, octaploid callus was 67.9254 µm and hexadecaploid callus was 135.4540 µm. The disparity indices observed in the parent and variant were 32.0546 and 49.7210 respectively. The disparity indices were 35.8231, 31.4554 and 48.3631 for the three types of calli respectively. The variation coefficient of parent and variant was 17.3187 and 26.0546 respectively. The variation coefficient was found to be 21.3698 for the tetraploid, 18.0199 for the octaploid and 23.7143 for the hexadecaploid calli. The total forma percentage

of somaclonal variant was higher (41.2182) than that of parent (40.8120). The tetraploid (44.1054), octaploid (42.2266) and hexadecaploid (41.1287) calli showed variations in their TF % values.

The disparity index, variation coefficient and total forma percentage of the somaclonal variant were higher than those of the parent. But the average chromosome length was higher in the parent (1.0177 µm) than that of the variant (0.8238 µm).

Karyotype formulae deduced for the karyotypes of the parent plant, calli and the tissue cultured plant showed variation in the type of chromosomes.

The general description of the common chromosome types found in the parent, variant and calli are given below.

Type A: Chromosome with secondary constriction with a total length ranging from 1.6191 µm to 0.6089 µm with nearly median / nearly sub median (-) primary constriction.

Type B: Chromosome with length ranging from 1.6711 µm to 0.4325 µm with nearly median primary constriction.

Type C: Chromosome with length ranging from 1.3784 µm to 0.6440 µm with nearly sub median (-) primary constriction.

Type D: Chromosome with length ranging from 1.3268 µm to 0.9533 µm with nearly sub median (+) primary constriction.

The chromosomal nomenclatures were depicted according to the system followed by Abraham and Prasad (Table 4).

The karyotype formula of the parent plant was $A_4 B_{22} C_8 D_2$, and that of

the variant was A_8 B_{44} $C_{20.}$ The karyotype formula differs for three types of calli, *viz*. A_4 B_{28} C_4 for the tetraploid, A_8 B_{56} C_8 for the octaploid and A_{16} B_{90} C_{34} D_4 for the hexadecaploid calli.

Detailed karyotype description (Tables 12- 17), microphotographs of mitotic metaphase stages, computer scanned images of karyotypes, karyograms and idiograms of the parent, somaclonal variant and the calli are shown (Plates 13-17).

Cells of the calli also showed various clastogenic and non-clastogenic abnormalities. The major clastogenic abnormalities observed include chromosome bridge, nuclear lesion, chromosome fragments, sticky bridge and sticky anaphase. The non-clastogenic abnormalities detected were ball metaphase, micronucleus, laggard and stratified anaphase (Plate 18).

***Artemisia nilagirica* (C. B. Clarke) Pamp.**
(2n = 4x = 36 = $A_4B_{22}C_8D_2$)

Parent Plant

Normal somatic chromosome number	:	36
Chromosomes with secondary constriction	:	4
Total chromosome length	:	36.6378 µm
Range of chromosome length	:	1.2771 µm - 0.6571 µm
Average chromosome length	:	1.0177 µm
Disparity index	:	32.0546
Variation coefficient	:	17.3187
TF value	:	40.8120

Table 12

Detailed karyomorphometrical data of *Artemisia nilagirica* – Parent plant (2n = 4x = 36)

Chromos-ome Type	No. of Pairs	Total Length c (µm)	s (µm)	l (µm)	R_1 (s/l)	R_2 (l/s)	I_1 (s/c %)	I_2 (l/c %)	Nature of Primary constriction
B	1	1.2771	0.549	0.7281	0.754	1.3262	42.988	57.012	Nm
B	1	1.2413	0.5189	0.7224	0.7183	1.3922	41.8029	58.1971	Nm
C	1	1.1735	0.4002	0.7733	0.5175	1.9323	34.1031	65.8969	Nsm(-)
B	1	1.1682	0.4778	0.6904	0.6921	1.445	40.9005	59.0995	Nm
B	1	1.1459	0.5014	0.6445	0.778	1.2854	43.756	56.244	Nm
A*	1	1.132	0.4748	0.6572	0.7225	1.3842	41.9435	58.0565	Nm
B	1	1.1073	0.4861	0.6212	0.7825	1.2779	43.8996	56.1004	Nm
C	1	1.0824	0.3902	0.6922	0.5637	1.774	36.0495	63.9505	Nsm(-)
B	1	1.0751	0.5131	0.562	0.913	1.0953	47.7258	52.2742	Nm
C	1	1.0532	0.3819	0.6713	0.5689	1.7578	36.2609	63.7391	Nsm(-)
A*	1	1.0453	0.51	0.5353	0.9527	1.0496	48.7898	51.2102	Nm
D	1	0.9533	0.2225	0.7308	0.3045	3.2845	23.34	76.66	Nsm(+)
B	1	0.9210	0.4437	0.4773	0.9296	1.0757	48.1759	51.8241	Nm

Chromos-ome Type	No. of Pairs	Total Length c (µm)	s (µm)	l (µm)	R_1 (s/l)	R_2 (l/s)	I_1 (s/c %)	I_2 (l/c %)	Nature of Primary constriction
C	1	0.8900	0.2787	0.6113	0.4559	2.1934	31.3146	68.6854	Nsm(-)
B	1	0.8729	0.4056	0.4673	0.868	1.1521	46.4658	53.5342	Nm
B	1	0.8114	0.3445	0.4669	0.7378	1.3553	42.4575	57.5425	Nm
B	1	0.7119	0.289	0.4229	0.6834	1.4633	40.5956	59.4044	Nm
B	1	0.6571	0.2889	0.3682	0.7846	1.2745	43.9659	56.0341	Nm

s : Short arm
l : Long arm
R_1 & R_2 : Arm ratios
I_1 & I_2 : Centromeric indices
* : Chromosome pair with secondary constriction

Artemisia nilagirica (C. B. Clarke) Pamp.
(2n = 4x = 36 = $A_4B_{28}C_4$)

Tetraploid Callus

Normal somatic chromosome number	:	36
Chromosomes with secondary constriction	:	4
Total chromosome length	:	43.2618 µm
Range of chromosome length	:	1.6711 µm - 0.7896 µm
Average chromosome length	:	1.2017 µm
Disparity index	:	35.8231
Variation coefficient	:	21.3698
TF value	:	44.1054

Table 13

Detailed karyomorphometrical data of *Artemisia nilagirica*- Tetraploid Callus (2n = 4x = 36)

Chromos-ome Type	No. of Pairs	Total Length c (µm)	s (µm)	l (µm)	R_1 (s / l)	R_2 (l / s)	I_1 (s /c%)	I_2 (l / c%)	Nature of Primary Constriction
B	1	1.6711	0.8337	0.8374	0.9956	1.0044	49.889	50.1107	Nm
A*	1	1.6191	0.7322	0.8869	0.8256	1.2113	45.223	54.7773	Nm
A*	1	1.5064	0.5614	0.9450	0.5941	1.6833	37.268	62.7323	Nsm(-)
B	1	1.4202	0.6295	0.7907	0.7961	1.2561	44.325	55.6753	Nm
B	1	1.3577	0.6651	0.6926	0.9603	1.0413	48.987	51.0127	Nm
B	1	1.3402	0.6434	0.6968	0.9234	1.0830	48.008	51.9922	Nm
C	1	1.2971	0.4926	0.8045	0.6123	1.6332	37.977	62.023	Nsm(-)
B	1	1.2603	0.6258	0.6345	0.9863	1.0139	49.655	50.3452	Nm
B	1	1.2306	0.4969	0.7337	0.6773	1.4766	40.379	59.6213	Nm
B	1	1.1525	0.5506	0.6019	0.9148	1.0932	47.774	52.2256	Nm
B	1	1.1253	0.4961	0.6292	0.7885	1.2683	44.086	55.914	Nm
B	1	1.1025	0.4195	0.6830	0.6142	1.6281	38.05	61.9501	Nm
B	1	1.0608	0.4802	0.5806	0.8271	1.2091	45.268	54.7323	Nm
B	1	0.9958	0.4416	0.5542	0.7968	1.2550	44.346	55.6537	Nm
B	1	0.9516	0.4128	0.5388	0.7661	1.3052	43.38	56.6204	Nm
B	1	0.9136	0.3650	0.5486	0.6653	1.5030	39.952	60.0482	Nm

Chromos-ome Type	No. of Pairs	Total Length c (µm)	s (µm)	l (µm)	R_1 (s / l)	R_2 (l / s)	I_1 (s /c%)	I_2 (l / c%)	Nature of Primary Constriction
B	1	0.8365	0.4005	0.4360	0.9186	1.0886	47.878	52.1219	Nm
C	1	0.7896	0.2935	0.4961	0.5916	1.6903	37.171	62.8293	Nsm(-)

s : Short arm
l : Long arm
R_1 & R_2 : Arm ratios
I_1 & I_2 : Centromeric indices
* : Chromosomes with secondary constriction

Artemisia nilagirica (C. B. Clarke) Pamp.
(2n= 8x= 72= $A_8B_{56}C_8$)

Octaploid Callus

Normal somatic chromosome number	:	72
Chromosomes with secondary constriction	:	8
Total chromosome length	:	67.9254 µm
Range of chromosome length	:	1.3603 µm - 0.7093 µm
Average chromosome length	:	0.9434 µm
Disparity index	:	31.4554
Variation coefficient	:	18.0199
TF value	:	42.2266

Table 14

Detailed karyomorphometrical data of *Artemisia nilagirica*- Octaploid Callus (2n= 8x= 72)

Chromosome Type	No. of Pairs	Total Length c (µm)	s (µm)	l (µm)	R_1 (s / l)	R_2 (l / s)	I_1 (s / c %)	I_2 (l / c %)	Nature of Primary Constriction
C	1	1.3603	0.4372	0.9231	0.4736	2.1114	32.1400	67.8600	Nsm(-)
*A	1	1.2343	0.4563	0.7780	0.5865	1.7050	36.9683	63.0317	Nsm(-)
B	1	1.2032	0.5532	0.6500	0.8511	1.1750	45.9774	54.0226	Nm
B	1	1.1996	0.5473	0.6523	0.8390	1.1919	45.6235	54.3765	Nm
C	1	1.1993	0.4146	0.7847	0.5284	1.8927	34.5702	65.4298	Nsm(-)
*A	1	1.1475	0.5024	0.6451	0.7788	1.2840	43.7821	56.2179	Nm
B	1	1.1385	0.4882	0.6503	0.7507	1.3320	42.8810	57.1190	Nm
C	1	1.0649	0.3586	0.7063	0.5077	1.9696	33.6745	66.3255	Nsm(-)
B	1	1.0636	0.4851	0.5785	0.8385	1.1925	45.6093	54.3907	Nm
B	1	1.0380	0.4415	0.5965	0.7402	1.3511	42.5337	57.4663	Nm
B	1	1.0358	0.4158	0.6200	0.6706	1.4911	40.1429	59.8571	Nm
C	1	1.0224	0.3476	0.6748	0.5151	1.9413	33.9984	66.0016	Nsm(-)

Chromosome Type	No. of Pairs	Total Length c (µm)	s (µm)	l (µm)	R_1 (s / l)	R_2 (l / s)	I_1 (s / c %)	I_2 (l / c %)	Nature of Primary Constriction
*A	1	0.9990	0.3252	0.6738	0.4826	2.0720	32.5526	67.4474	Nsm(-)
*A	1	0.9870	0.3250	0.6620	0.4909	2.0369	32.9281	67.0719	Nsm(-)
B	1	0.9749	0.4146	0.5603	0.7400	1.3514	42.5274	57.4726	Nm
B	1	0.9724	0.4712	0.5012	0.9401	1.0637	48.4574	51.5426	Nm
B	1	0.9382	0.4260	0.5122	0.8317	1.2023	45.4061	54.5939	Nm
B	1	0.9067	0.3602	0.5465	0.6591	1.5172	39.7265	60.2735	Nm
B	1	0.9004	0.4376	0.4628	0.9455	1.0576	48.6006	51.3994	Nm
B	1	0.8940	0.4200	0.4740	0.8861	1.1286	46.9799	53.0201	Nm
B	1	0.8891	0.4040	0.4851	0.8328	1.2007	45.4392	54.5608	Nm
B	1	0.8687	0.4059	0.4628	0.8771	1.1402	46.7250	53.2750	Nm
B	1	0.8569	0.3962	0.4607	0.8600	1.1628	46.2364	53.7636	Nm
B	1	0.8312	0.3705	0.4607	0.8042	1.2435	44.5741	55.4259	Nm
B	1	0.8304	0.4040	0.4264	0.9475	1.0554	48.6513	51.3487	Nm
B	1	0.8193	0.3586	0.4607	0.7784	1.2847	43.7691	56.2309	Nm
B	1	0.8181	0.3696	0.4485	0.8241	1.2135	45.1779	54.8221	Nm

Chromosome Type	No. of Pairs	Total Length c (µm)	s (µm)	l (µm)	R_1 (s / l)	R_2 (l / s)	I_1 (s / c %)	I_2 (l / c %)	Nature of Primary Constriction
B	1	0.7965	0.3369	0.4596	0.7330	1.3642	42.2976	57.7024	Nm
B	1	0.7857	0.3369	0.4488	0.7507	1.3321	42.8790	57.1210	Nm
B	1	0.7702	0.3319	0.4383	0.7572	1.3206	43.0927	56.9073	Nm
B	1	0.7666	0.3593	0.4073	0.8822	1.1336	46.8693	53.1307	Nm
B	1	0.7629	0.3812	0.3817	0.9987	1.0013	49.9672	50.0328	Nm
B	1	0.7361	0.3028	0.4333	0.6988	1.4310	41.1357	58.8643	Nm
B	1	0.7236	0.3257	0.3979	0.8185	1.2217	45.0111	54.9889	Nm
B	1	0.7181	0.3369	0.3812	0.8838	1.1315	46.9155	53.0845	Nm
B	1	0.7093	0.2933	0.4160	0.7050	1.4183	41.3506	58.6494	Nm

s : Short arm
l : Long arm
R_1 & R_2 : Arm ratios
I_1 & I_2 : Centromeric indices
* : Chromosomes with secondary constriction

Artemisia nilagirica (C. B. Clarke) Pamp.
(2n = 16x = 144 = $A_{16}B_{90}C_{34}D_4$)

Hexadecaploid Callus

Normal somatic chromosome number	:	144
Chromosomes with secondary constriction	:	16
Total chromosome length	:	135.4540 µm
Range of chromosome length	:	1.4728 µm - 0.5126 µm
Average chromosome length	:	0.9407 µ m
Disparity index	:	48.3631
Variation coefficient	:	23.7143
TF value	:	41.1287

Table 15

Detailed karyomorphometrical data of *Artemisia nilagirica* – Hexadecaploid Callus (2n = 16x = 144)

Chromosome Type	No. of Pairs	Total Length c (µm)	s (µm)	l (µm)	R_1 (s/l)	R_2 (l/s)	I_1 (s / c %)	I_2 (l / c %)	Nature of Primary Constriction
B	1	1.4728	0.6784	0.7944	0.8540	1.1710	46.0619	53.9381	Nm
B	1	1.4568	0.7172	0.7396	0.9697	1.0312	49.2312	50.7688	Nm
B	1	1.3826	0.6362	0.7464	0.8524	1.1732	46.0148	53.9852	Nm
C	1	1.3784	0.5030	0.8754	0.5746	1.7404	36.4916	63.5084	Nsm(-)
C	1	1.3354	0.3776	0.9578	0.3942	2.5365	28.2762	71.7238	Nsm(-)
D	1	1.3268	0.3290	0.9978	0.3297	3.0328	24.7965	75.2035	Nsm(+)
A*	1	1.3124	0.6172	0.6952	0.8878	1.1264	47.0283	52.9717	Nm
C	1	1.3066	0.4320	0.8746	0.4939	2.0245	33.0629	66.9371	Nsm(-)
B	1	1.2814	0.5210	0.7604	0.6852	1.4595	40.6587	59.3413	Nm
C	1	1.2160	0.4118	0.8042	0.5121	1.9529	33.8651	66.1349	Nsm(-)
C	1	1.1818	0.4144	0.7674	0.5400	1.8518	35.0652	64.9348	Nsm(-)
B	1	1.1458	0.4710	0.6748	0.6980	1.4327	41.1067	58.8933	Nm
A*	1	1.1376	0.4404	0.6972	0.6317	1.5831	38.7131	61.2869	Nm
C	1	1.1256	0.4058	0.7198	0.5638	1.7738	36.0519	63.9481	Nsm(-)
B	1	1.1230	0.4338	0.6892	0.6294	1.5888	38.6287	61.3713	Nm
B	1	1.1194	0.5480	0.5714	0.9590	1.0427	48.9548	51.0452	Nm
C	1	1.1156	0.3662	0.7494	0.4887	2.0464	32.8254	67.1746	Nsm(-)
B	1	1.1124	0.5048	0.6076	0.8308	1.2036	45.3794	54.6206	Nm

Chromosome Type	No. of Pairs	Total Length c (µm)	s (µm)	l (µm)	R_1 (s/l)	R_2 (l/s)	I_1 (s / c %)	I_2 (l / c %)	Nature of Primary Constriction
A*	1	1.0798	0.5366	0.5432	1.0123	0.9878	49.6944	50.3056	Nm
C	1	1.0636	0.2774	0.7862	0.3528	2.8342	26.0812	73.9188	Nsm(-)
B	1	1.0142	0.4008	0.6134	0.6534	1.5304	39.5188	60.4812	Nm
A*	1	1.0074	0.4320	0.5754	0.7508	1.3319	42.8827	57.1173	Nm
C	1	1.0072	0.3522	0.6550	0.5377	1.8597	34.9682	65.0318	Nsm(-)
C	1	1.0062	0.3526	0.6536	0.5395	1.8537	35.0427	64.9573	Nsm(-)
B	1	1.0060	0.4804	0.5256	0.9140	1.0941	47.7535	52.2465	Nm
B	1	0.9872	0.4352	0.5520	0.7884	1.2684	44.0843	55.9157	Nm
A*	1	0.9790	0.3764	0.6026	0.6246	1.6010	38.4474	61.5526	Nm
C	1	0.9614	0.3418	0.6196	0.5516	1.8128	35.5523	64.4477	Nsm(-)
B	1	0.9598	0.4310	0.5288	0.8151	1.2269	44.9052	55.0948	Nm
D	1	0.9590	0.1980	0.7610	0.2602	3.8434	20.6465	79.3535	Nsm(+)
B	1	0.9550	0.4494	0.5056	0.8888	1.1251	47.0576	52.9424	Nm
B	1	0.9416	0.4542	0.4874	0.9319	1.0731	48.2370	51.7630	Nm
B	1	0.9384	0.4474	0.4910	0.9112	1.0975	47.6769	52.3231	Nm
B	1	0.9306	0.4648	0.4658	0.9979	1.0022	49.9463	50.0537	Nm
A*	1	0.9192	0.4100	0.5092	0.8052	1.2420	44.6040	55.3960	Nm
C	1	0.9176	0.2836	0.6340	0.4473	2.2355	30.9067	69.0933	Nsm(-)
B	1	0.9162	0.3516	0.5646	0.6227	1.6058	38.3759	61.6241	Nm
B	1	0.9024	0.3892	0.5132	0.7584	1.3186	43.1294	56.8706	Nm
B	1	0.9006	0.3692	0.5314	0.6948	1.4393	40.9949	59.0051	Nm
B	1	0.8860	0.3948	0.4912	0.8037	1.2442	44.5598	55.4402	Nm

Chromosome Type	No. of Pairs	Total Length c (µm)	s (µm)	l (µm)	R_1 (s/l)	R_2 (l/s)	I_1 (s / c %)	I_2 (l / c %)	Nature of Primary Constriction
C	1	0.8764	0.2786	0.5978	0.4660	2.1457	31.7891	68.2109	Nsm(-)
B	1	0.8702	0.3912	0.4790	0.8167	1.2244	44.9552	55.0448	Nm
B	1	0.8694	0.4328	0.4366	0.9913	1.0088	49.7815	50.2185	Nm
B	1	0.8650	0.3556	0.5094	0.6981	1.4325	41.1098	58.8902	Nm
B	1	0.8390	0.3716	0.4674	0.7950	1.2578	44.2908	55.7092	Nm
A*	1	0.8364	0.3776	0.4588	0.8230	1.2150	45.1459	54.8541	Nm
B	1	0.8296	0.3788	0.4508	0.8403	1.1901	45.6606	54.3394	Nm
B	1	0.8260	0.3170	0.5090	0.6228	1.6057	38.3777	61.6223	Nm
C	1	0.8260	0.3004	0.5256	0.5715	1.7497	36.3680	63.6320	Nsm(-)
B	1	0.8232	0.3360	0.4872	0.6897	1.4500	40.8163	59.1837	Nm
B	1	0.8138	0.3736	0.4402	0.8487	1.1783	45.9081	54.0919	Nm
B	1	0.8024	0.3376	0.4648	0.7263	1.3768	42.0738	57.9262	Nm
A*	1	0.7896	0.3012	0.4884	0.6167	1.6215	38.1459	61.8541	Nm
B	1	0.7796	0.3694	0.4102	0.9005	1.1104	47.3833	52.6167	Nm
B	1	0.7776	0.3658	0.4118	0.8883	1.1258	47.0422	52.9578	Nm
B	1	0.7744	0.3538	0.4206	0.8412	1.1888	45.6870	54.3130	Nm
B	1	0.7542	0.3770	0.3772	0.9995	1.0005	49.9867	50.0133	Nm
B	1	0.7516	0.3442	0.4074	0.8449	1.1836	45.7956	54.2044	Nm
B	1	0.7298	0.3360	0.3938	0.8532	1.1720	46.0400	53.9600	Nm
C	1	0.7284	0.2468	0.4816	0.5125	1.9514	33.8825	66.1175	Nsm(-)
C	1	0.7240	0.2272	0.4968	0.4573	2.1866	31.3812	68.6188	Nsm(-)
B	1	0.7230	0.3462	0.3768	0.9188	1.0884	47.8838	52.1162	Nm

Chromosome Type	No. of Pairs	Total Length c (µm)	s (µm)	l (µm)	R_1 (s/l)	R_2 (l/s)	I_1 (s / c %)	I_2 (l / c %)	Nature of Primary Constriction
B	1	0.7190	0.3216	0.3974	0.8093	1.2357	44.7288	55.2712	Nm
B	1	0.7046	0.3168	0.3878	0.8169	1.2241	44.9617	55.0383	Nm
C	1	0.6978	0.2582	0.4396	0.5874	1.7026	37.0020	62.9980	Nsm(-)
B	1	0.6456	0.3036	0.3420	0.8877	1.1265	47.0260	52.9740	Nm
B	1	0.6380	0.2950	0.3430	0.8601	1.1627	46.2382	53.7618	Nm
B	1	0.6268	0.2882	0.3386	0.8512	1.1749	45.9796	54.0204	Nm
B	1	0.6250	0.3016	0.3234	0.9326	1.0723	48.2560	51.7440	Nm
B	1	0.6248	0.2974	0.3274	0.9084	1.1009	47.5992	52.4008	Nm
B	1	0.5544	0.2636	0.2908	0.9065	1.1032	47.5469	52.4531	Nm
B	1	0.5126	0.2544	0.2582	1.0149	0.9853	49.6293	50.3707	Nm

s : Short arm
l : Long arm
R_1 & R_2 : Arm ratios
I_1 & I_2 : Centromeric indices
* : Chromosomes with secondary constriction

Artemisia nilagirica (C. B. Clarke) Pamp.
(2n = 8x = 72 = $A_8B_{44}C_{20}$)

Somaclonal variant

Normal somatic chromosome number	:	72
Chromosomes with secondary constriction	:	8
Total chromosome length	:	59.3116 µm
Range of chromosome length	:	1.2879 µm - 0.4325 µm
Average chromosome length	:	0.8238 µm
Disparity index	:	49.7210
Variation coefficient	:	26.0546
TF value	:	41.2182

Table 16

Detailed karyomorphometrical data of *Artemisia nilagirica*- somaclonal variant (2n = 8x = 72)

Chromosome Type	No. of Pairs	Total Length c (µ m)	s (µm)	l (µm)	R_1 (s / l)	R_2 (l /s)	I_1 (s / c %)	I_2 (l / c%)	Nature of Primary Constriction
B	1	1.2879	0.5407	0.7472	0.7236	1.3819	41.9831	58.0169	Nm
B	1	1.2207	0.5088	0.7119	0.7147	1.3992	41.6810	58.3190	Nm
C	1	1.1625	0.4128	0.7497	0.5506	1.8161	35.5097	64.4903	Nsm(-)
C	1	1.1291	0.3994	0.7297	0.5473	1.8270	35.3733	64.6267	Nsm(-)
A*	1	1.1103	0.4544	0.6559	0.6928	1.4434	40.9259	59.0741	Nm
B	1	1.1011	0.5272	0.5739	0.9186	1.0886	47.8794	52.1206	Nm
C	1	1.0942	0.3469	0.7473	0.4642	2.1542	31.7035	68.2965	Nsm(-)
B	1	1.0020	0.4634	0.5386	0.8604	1.1623	46.2475	53.7525	Nm
A*	1	0.9897	0.3982	0.5915	0.6732	1.4854	40.2344	59.7656	Nm
C	1	0.9340	0.3517	0.5823	0.6040	1.6557	37.6552	62.3448	Nsm(-)
B	1	0.9300	0.3627	0.5673	0.6393	1.5641	39.0000	61.0000	Nm
B	1	0.8743	0.4002	0.4741	0.8441	1.1847	45.7738	54.2262	Nm
B	1	0.8602	0.3339	0.5263	0.6344	1.5762	38.8166	61.1834	Nm
B	1	0.8583	0.4271	0.4312	0.9905	1.0096	49.7612	50.2388	Nm
C	1	0.8556	0.2997	0.5559	0.5391	1.8549	35.0281	64.9719	Nsm(-)
B	1	0.8538	0.3780	0.4758	0.7945	1.2587	44.2727	55.7273	Nm
B	1	0.8441	0.3967	0.4474	0.8867	1.1278	46.9968	53.0032	Nm
B	1	0.8343	0.3502	0.4841	0.7234	1.3824	41.9753	58.0247	Nm
C	1	0.8128	0.2692	0.5436	0.4952	2.0193	33.1201	66.8799	Nsm(-)

Chromosome Type	No. of Pairs	Total Length c (µ m)	s (µm)	l (µm)	R_1 (s / l)	R_2 (l /s)	I_1 (s / c %)	I_2 (l / c%)	Nature of Primary Constriction
A*	1	0.8027	0.3207	0.4820	0.6654	1.5030	39.9527	60.0473	Nm
B	1	0.7542	0.3332	0.4210	0.7914	1.2635	44.1793	55.8207	Nm
B	1	0.7105	0.3372	0.3733	0.9033	1.1071	47.4595	52.5405	Nm
B	1	0.7071	0.3371	0.3700	0.9111	1.0976	47.6736	52.3264	Nm
C	1	0.7052	0.2625	0.4427	0.5930	1.6865	37.2235	62.7765	Nsm(-)
B	1	0.6909	0.3145	0.3764	0.8355	1.1968	45.5203	54.4797	Nm
C	1	0.6868	0.1824	0.5044	0.3616	2.7654	26.5579	73.4421	Nsm(-)
C	1	0.6694	0.2371	0.4323	0.5485	1.8233	35.4198	64.5802	Nsm(-)
B	1	0.6631	0.3279	0.3352	0.9782	1.0223	49.4496	50.5504	Nm
B	1	0.6508	0.3067	0.3441	0.8913	1.1219	47.1266	52.8734	Nm
C	1	0.6440	0.2326	0.4114	0.5654	1.7687	36.1180	63.8820	Nsm(-)
A*	1	0.6089	0.2465	0.3624	0.6802	1.4702	40.4828	59.5172	Nm
B	1	0.5671	0.2395	0.3276	0.7311	1.3678	42.2324	57.7676	Nm
B	1	0.5609	0.2681	0.2928	0.9156	1.0921	47.7982	52.2018	Nm
B	1	0.5308	0.2464	0.2844	0.8664	1.1542	46.4205	53.5795	Nm
B	1	0.5160	0.2317	0.2843	0.8150	1.2270	44.9031	55.0969	Nm
B	1	0.4325	0.1742	0.2583	0.6744	1.4828	40.2775	59.7225	Nm

s : Short arm
l : Long arm
R_1 & R_2 : Arm ratios
I_1 & I_2 : Centromeric indices
* : Chromosome pair with secondary constriction

Table 17

Summary of karyomorphometric features of parent, calli and somaclonal variant of *A. nilagirica*Random Amplified Polymorphic DNA (RAPD) Analysis

Type	Chromosome Number	No. of Secondary Constrictions	TCL (µm)	RCL (µm)	ACL (µm)	DI	
Parent (2n = 4x = 36 = $A_4B_{22}C_8D_2$)	36	4	36.6378	1.2771 - 0.6571	1.0177	32.0546	1
Callus (2n = 4x = 36 = $A_4B_{28}C_4$)	36	4	43.2618	1.6711 - 0.7896	1.2017	35.8231	2
Callus (2n= 8x= 72= $A_8B_{56}C_8$)	72	8	67.9254	1.3603 - 0.7093	0.9434	31.4554	1
Callus (2n = 16x = 144 = $A_{16}B_{90}C_{34}D_4$)	144	16	135.4540	1.4728 - 0.5126	0.9407	48.3631	2
Somaclonal variant (2n= 8x= 72= $A_8B_{44}C_{20}$)	72	8	59.3116	1.2879 - 0.4325	0.8238	49.7210	2

DNA was isolated from the parent plant (P) and twelve tissue cultured plants designated as number TC1 to TC12. 20-30 ng of template DNA is required to amplify a specific DNA segment by PCR. 3 µl of the template DNA was used for the preparation of reaction mixture.

To detect the somaclonal variation at the molecular level RAPD analysis was carried out using 15 primers of arbitrary sequences. Of the 15 primers used, only 5 successfully amplified the extracted DNA with consistent reproducible bands (Plates 19 & 20). The number of bands resolved per primer ranged from a minimum of one to a maximum of thirteen. The size of the amplification products also differed and ranged from approximately 0.5 kb to 3 kb. The primers and the characteristic bands are

listed (Table 20).

RAPD fingerprints of the tissue cultured plants (TC1-TC12) differed from parent (P) with all five primers (OPA 01, OPA 02, OPB 17, OPB 18 & OPC 01). A few bands were found to be missing in the variants when these five primers were used (Plates 19 & 20; Table 18).

Table 18

Primers	***In vitro* plants of *A. nilagirica* lacking some bands**
OPA 01	TC1, TC2, TC3, TC4, TC5, TC6, TC7, TC8,TC9
OPA 02	TC1, TC3
OPB 17	TC3, TC9
OPB 18	TC2, TC3, TC4, TC5, TC6, TC8, TC11, TC12
OPC 01	TC1, TC2, TC3, TC4, TC7, TC11

Additional bands in the variants were also detected by this marker screening (Plates 19 & 20; Table 19).

Table 19

Primers	***In vitro* plants of *A. nilagirica* having additional bands**
OPA 01	TC3, TC5, TC8
OPA 02	TC2, TC3, TC5, TC6
OPB 17	TC2
OPB 18	Nil
OPC 01	TC2,TC3, TC5, TC6, TC10, TC12

In OPA 01 series, an additional band in TC5 and four bands in TC3 and two in TC8 were observed. One to five bands were found to be missing in TC1 to TC9 plants. OPA 01 primer could not generate any polymorphism in TC10, TC11 and TC12 plants (Plate 20; Table 20).

In OPA 02 series, no band was found above 1.5 kb length. In TC3 three additional bands below 0.5 kb and in TC2, four bands (one above 0.5 kb and three below 0.5 kb length) were noticed. Two additional bands were observed below 0.5 kb in both TC5 and TC6. Intensity of the bands were found to be increased in TC2, TC3, TC4, TC5 and TC6 when compared to the parent (Plate 19; Table 20).

Amplification products with OPB 17 generated no bands above 1.5 kb in both the *in vitro* plants and parent. When compared to parent, TC2 showed one additional band below 1 kb length with OPB 17. Two bands were missing in TC3, which were detected in the parent. In TC9, a band corresponding to 1.5 kb length was absent (Plate 20; Table 20).

In OPB 18, no bands were seen in all the plants above 1.5 kb length. A single band was found missing in TC2, TC3, TC4, TC5, TC6, TC8, TC11 and TC 12 plants (Plate 19; Table 20).

For the OPC 01 primer, additional bands were found in TC2, TC3, TC5, TC6, TC10 and TC12 plants. A band was found to be missing in TC1, TC2, TC3, TC4, TC7, and TC11 plants (Plate 19; Table 20).

The reproducibility of the genomic DNA bands of TC3 was consistent in successive repetition than other TC plants. Since the amplification profile of TC3 plant showed more polymorphism and consistency than other TC plants, it was considered as a somaclonal variant and used for further analysis.

Table 20

Primers and characterization of consistent bands in 12 tissue cultured plants and the parent plant of *A. nilagirica* (C. B. Clarke) Pamp.

Primer	Sequence	Number of bands											
		TC1	TC2	**TC3***	TC4	TC5	TC6	TC7	TC8	TC9	TC10	TC11	TC12	Parent
OPA 01	5'CAGGCCCTTC 3'	6	7	**13**	8	10	7	9	11	10	11	11	11	11
OPA 02	5'TGCCGAGCTG 3'	2	7	**5**	3	5	5	3	3	3	3	3	3	3
OPB 17	5'AGGGAAGGAG 3'	3	4	**1**	3	3	3	3	3	2	3	3	3	3
OPB 18	5'CCACAGCAGT 3'	5	4	**4**	4	4	4	5	4	5	5	4	4	5
OPC 01	5'TTCGAGCCAG 3'	2	3	**3**	2	6	7	1	3	3	5	2	4	3

TC 1-TC12 : Tissue cultured plants

TC3* : Somaclonal variant **Essential Oil Analysis**

RAPD analysis revealed a notable deviation in the genetic make up of the *in vitro* plant (TC3). So the essential oils of the parent plant and the *in vitro* plant (TC3) were analyzed quantitatively and qualitatively to search for biochemical variations in the secondary metabolism. The oil yield of the parent plant was comparatively low (1.1%). The *in vitro* plant contained a higher quantity of oil (1.8%).

Gas Chromatography and Mass Spectrometry Analysis

The results of Gas Chromatography and Mass Spectrometry analysis of the *in vivo* and *in vitro* (TC3) plants are listed (Table 21). In the present investigation, the essential oil of *in vivo* and *in vitro* plants of *A. nilagirica* seems to belong to a terpenoid chemotype. GC-MS analysis revealed 29 components in the parent plant and 21 in the somaclonal variant. There was a clear difference between the compositions of the two oils tested. The percentage of monoterpenes present in the *in vivo* plant was 93.82 while that of *in vitro* plant was 86.04. But the percentage of sesquiterpenes was higher in the *in vitro* plant (13.96) than that of the parent plant (5.99). The major components were mainly α- thujone and camphene in both the oils.

Sixteen essential oil components like α-fenchene, sabinene, 1,8-cineole, α-thujone, β-thujone, bicyclohex-3-en-2-one, thujyl alcohol, L-camphor, camphene, terpinen-4-ol, α-copaene, β-caryophyllene, germacrene-D, δ-cadinene, caryophyllene oxide and β-eudesmol were found in both *in vivo* and *in vitro* plants. Among them, percentage of eight components like α-fenchene, sabinene, terpinen-4-ol, α-copaene, β-caryophyllene, germacrene-D, δ-cadinene and β-eudesmol

were more in *in vitro* plant than *in vivo* plant.

The *in vitro* plant was characterized by new components like ρ-cymene, dl- limonene, α-terpinene, farnesene and bicyclogermacrene.

1-octen-3-ol, β-pinene, γ- terpinene, chrysanthenone, M-mentha-1, 8-diene, cuminal, β-ocimene, 4-perillaldehyde, cyclofenchene, eugenol, β –bourbonene, germacrene-B and γ-cadinene were the unique components detected from parent plant. The mass spectra of the compounds identified in the GC – MS analysis are shown in plates 21-33.

Chemotaxonomic evaluation

The total number of chemical components detected by GC-MS in both *in vivo* and *in vitro* plants were found to be 34. However the number of similar components which occur in both the plants were found to be 16. Coefficient of similitude between the parent and *in vitro* (TC3) plant was found to be 47.05.

Table 21

List of essential oil components detected in the *in vivo* and *in vitro* plants of *Artemisia nilagirica*

Sl. No.	Components	Retention Time	Class	Composition %	
				P	TC3
1	α-fenchene	3.18	Monoterpene	0.41	0.55
2	Sabinene	3.62	"	0.37	0.40
3	1-octen-3-ol	3.74	"	0.16	0.00
4	β-pinene	3.95	"	0.08	0.00
5	ρ-cymene	4.64	"	0.00	0.38
6	1,8-cineole	4.79	"	0.44	0.32

Sl. No.	Components	Retention Time	Class	Composition %	
				P	TC3
1	α-fenchene	3.18	Monoterpene	0.41	0.55
7	γ -terpinene	5.47	"	0.12	0.00
8	α -thujone	7.08	"	45.06	42.75
9	β -thujone	7.29	"	8.74	7.79
10	Bicyclohex-3-en-2-one	7.35	"	0.51	0.45
11	Thujyl alcohol	7.88	"	7.63	6.61
12	L-camphor	8.02	"	1.27	0.61
13	Camphene	9.03	"	26.20	24.65
14	Terpinen-4-ol	9.21	"	0.76	0.79
15	Chrysanthenone	10.25	Monoterpene	0.19	0.00
16	M-mentha-1,8-diene	10.65	"	0.13	0.00
17	Cuminal	11.33	"	0.22	0.00
18	dl- limonene	12.38	"	0.00	0.27
19	β-ocimene	12.41	"	0.34	0.00
20	4-perillaldehyde	12.60	"	0.13	0.00
21	α-terpinene	13.41	"	0.00	0.47
22	Cyclofenchene	13.44	"	1.06	0.00
23	Eugenol	15.95	Phenol	0.19	0.00
24	α-copaene	16.45	Sesquiterpene	0.14	0.40
25	β - bourbonene	16.77	"	0.12	0.00
26	β-caryophyllene	18.09	"	0.36	0.62
27	farnesene	19.92	"	0.00	0.33
28	Germacrene-D	20.54	"	1.51	5.50
29	Germacrene-B	20.94	"	0.16	0.00
30	Bicyclogermacrene	21.13	"	0.00	0.92
31	δ-cadinene	22.27	"	0.59	1.24
32	Caryophyllene oxide	24.34	"	0.97	0.92
33	γ –cadinene	26.16	"	0.36	0.00
34	β-eudesmol	26.89	"	1.78	4.03

P : Parent plant of *A. nilagirica*

TC3: Somaclonal variant of *A. nilagirica*

Cytotoxic Assays (*In vitro* studies)

Methanol extracts of both *in vivo* and *in vitro* plants (TC3) of *A. nilagirica* were found to be cytotoxic towards DLA and EAC cells at different concentration (10-1000 µg/ml). Both the extracts produced a concentration dependent cytotoxic effect to DLA and EAC cells. But the percentage of cytotoxicity was different for the two different extracts. *In vitro* plant extract produced 100% toxicity at a concentration of 250 µg/ml for DLA cells and 500 µg/ml for EAC cells, while that of *in vivo* plant extract caused 100% cytotoxicity only at a concentration of 500 µg/ml for both DLA and EAC cells (Plate 34; Tables 22 & 23).

Table 22

Cytotoxicity of extracts of *in vivo* and *in vitro* (TC3) plants of *A. nilagirica* towards Daltons Lymphoma Ascites (DLA) cell line

Sl. No.	Concentration of drugs (µg/ml)	Tumour cells (µl)	PBS (µl)	% Cytotoxicity	
				A	B
1	1000	100	700	100	100
2	750	100	750	100	100
3	500	100	800	100	100
4	250	100	850	96	100
5	100	100	700	70	98
6	50	100	800	60	80
7	25	100	800	25	40
8	10	100	860	0	15
9	0	100	900	0	0
10	DMSO 100 µl	100	800	0	0

A: *in vivo* plant extract of *A. nilagirica*
B: *in vitro* (TC3) plant extract of *A. nilagirica*

Table 23
Cytotoxicity of extracts of *in vivo* and *in vitro* (TC3) plants of *A. nilagirica* towards Ehrlich Ascites Carcinoma (EAC) cell line

Sl. No.	Concentration of drugs (µg/ml)	Tumour cells (µl)	PBS (µl)	% Cytotoxicity	
				A	B
1	1000	100	700	100	100
2	750	100	750	100	100
3	500	100	800	100	100
4	250	100	850	97	80
5	100	100	700	50	70
6	50	100	800	13	20
7	25	100	800	0	5
8	10	100	860	0	0
9	0	100	900	0	0
10	DMSO 100 µl	100	800	0	0

A: *in vivo* plant extract of *A. nilagirica*
B: *in vitro* (TC3) plant extract of *A. nilagirica*

Antitumour Assays (*In vivo* studies)

Effect of *in vivo* and *in vitro* (TC3) plant extracts of *A. nilagirica* on solid tumour reduction

There was a significant ($\rho < 0.01$) reduction of tumour volume in *in vivo* and *in vitro* (TC3) plant extracts treated animals. The

tumour volume of control animals with 250 µg/ml gum acacia on 31st day was 2.8 cm^3 , while that of 250 µg/ml *in vivo* and *in vitro* plant extracts treated animals was only 0.138 cm^3 and 0.097 cm^3 on the same day. For 500 µg/ml gum acacia, the tumour volume was 2.6 cm^3 and for 500 µg/ml *in vivo* and *in vitro* drug treated animals was 0.074 cm^3 and 0.058 cm^3 respectively (Plates 35 - 37; Table 24).

Effect of *in vivo* and *in vitro* (TC3) plant extracts of *A. nilagirica* on ascites tumour development

Administration of the extracts could enhance the survival days of Ehrlich Ascites tumour bearing animals. Life span of ascites tumour bearing mice, treated with *in vivo* and *in vitro* plant extracts was found to be significantly increased. Control animals survived only 19 days after the tumour induction while the 250 µg/ml extract of *in vivo* and *in vitro* treated animals survived 27 and 29 days with an increase in the life span of 39.47 % and 54.37 % respectively. For 500 µg/ml plant extracts, it was 31 and 33 days with an increase in the life span of 61.37 % and 75.42 % respectively (Plate 38; Table 25).

Table 24

Effect of extracts of *in vivo* and *in vitro* (TC3) plants of *A. nilagirica* on solid tumour reduction

Drug	Concentration (µg/ml)	Tumour volume (cm^3) in different days								
		7	**10**	**13**	**16**	**19**	**22**	**25**	**28**	**31**
Control gum acacia	250	0.157 ± 0.02	0.217± 0.01	0.339± 0.02	0.460± 0.03	0.685 ± 0.05	0.929 ± 0.07	1.44± 0.19	2.13± 0.09	2.8± 0.1
	500	0.156 ± 0.03	0.28 ± 0.008	0.360 ± 0.64	0.478 ± 0.03	0.655 ± 0.02	0.859 ± 0.03	1.13± 0.09	1.94 ± 0.17	2.6 ± 0.11
A	250	0.091 ± 0.01	0.124 ± 0.03	0.202 ± 0.03	0.292 ± 0.04	0.356 ± 0.03	0.277 ± 0.05	0.257 ± 0.05	0.181 ± 0.03	**0.138 ± 0.03
	500	0.132 ± 0.05	0.188 ± 0.05	0.192 ± 0.06	0.245 ± 0.10	0.289 ± 0.09	0.151 ± 0.06	0.123 ± 0.05	0.095 ± 0.03	**0.074 ± 0.02
B	250	0.088 ± 0.01	0.143 ± 0.01	0.215 ± 0.03	0.266 ± 0.04	0.342 ± 0.02	0.229 ± 0.02	0.167 ± 0.03	0.133 ± 0.03	**0.097 ± 0.02
	500	0.119 ± 0.03	0.171 ± 0.09	0.180 ± 0.03	0.208 ± 0.14	0.368 ± 0.16	0.140± 0.09	0.116 ± 0.08	0.086 ± 0.04	**0.058 ± 0.03

A: *in vivo* plant extract of *A. nilagirica*
B: *in vitro* (TC3) plant extract of *A. nilagirica*

All values are mean ± S. D. of 6 animals in each group.
**p<0.01, with respect to control (one way ANOVA followed by Dunnett t-test)

Table 25

Effect of extracts of *in vivo* and *in vitro* (TC3) plants of *A. nilagirica* on ascites tumour development

Drug	Concentration (µg/ml)	Mortality in days						Average	% of increase in life span
Control gum acacia	250	16	17	18	20	21	22	19	_
	500	16	17	17	20	22	22	19	_
A	250	18	20	25	30	32	34	26.5	*39.47
	500	24	25	29	30	36	40	30.66	**61.37
B	250	24	25	26	30	32	39	29.33	**54.37
	500	26	28	33	33	38	42	33.33	**75.42

A: *in vivo* plant extract of *A. nilagirica*
B: *in vitro* (TC3) plant extract of *A. nilagirica*
All values are mean ± S. D. of 6 animals in each group.
**$p < 0.01$, * $p < 0.05$, with respect to control (one way ANOVA followed by Dunnett t-test).

DISCUSSION

Micropropagation

In recent times, people are heeding to the call of nature to explore the possibilities of developing novel green products as remedies for various ailments. Traditional remedies derived from plants continue to be a source of genuine biologically active compounds. Medicinal plants have been used in large scales in the Indian Pharmaceutical Industry. Because of the large scale and unrestricted exploitation of natural resources to meet the demand by the Indian Pharmaceutical Industry coupled with limited cultivation and insufficient attempts at its replenishment, the wild stock of this medicinally important plant species have been markedly depleted. Natural regeneration and conventional propagation of the plants through the vegetative cuttings is slow and cuttings do not survive after the transport and transplantation. Moreover vegetative propagation has prevented the production of new cultivars by plant breeding. An alternative method for creating new forms of a plant is by selection of somaclonal variants from tissue culture.

In vitro culture of plants has gained considerable importance during recent years in view of their possible application to the production of known and new aromatics (Heble and Chadha, 1986; Mulder Krieger *et al.*, 1988). The most economically valuable *Artemisia* species has now gained the focus of *in vitro* tissue culture techniques. The main efforts have been devoted to the *in vitro* selection of highly yielding clones and cell lines, producing secondary metabolites, with pharmacological and industrial applications. In this regard

major attention has been paid to *A. nilagirica* owing to its biological properties and moreover for its value as an aromatic plant, employed in fragrances, perfumery and cosmetic production.

Very little information was found in the literature, concerning the tissue culture of *A. nilagirica.* A beginning has been made in the area of tissue culture of this plant by studies on micropropagation and organogenesis. The protocol developed is simple, rapid and reproducible and may be ideally suited for the mass multiplication of this important aromatic medicinal plant.

Since all known *A. nilagirica* are sterile, they can be propagated vegetatively, which has prevented the production of new cultivar by plant breeding. Assessment of genetic variability is basic to any plant breeding programme (Farooqi *et al.,* 1990). An alternative method for creating new forms of the plant is by selecting somaclonal variants from tissue culture material.

The term somaclonal variation describes any variation that can arise through the culture of plant cells, tissues and organs (Larkin and Scowcroft, 1981). Such variation has been observed among regenerants from a large number of species and various aspects of this process have been the subject of a number of reviews (Karp, 1991; Peschke and Phillips, 1991).

Genetic variation is a common phenomenon associated with plant tissue culture. Plantlets derived from *in vitro* culture might exhibit somaclonal variation which is often heritable (Breiman *et al.,* 1987). Useful morphological, cytological and molecular variation may be generated *in vitro* (Larkin *et al.,* 1989).

According to Larkin and Scowcroft (1985) and Larkin (1987) the origin of the somaclonal variation may be due to the following reasons:

- genetic variation already present in the mother plant tissue
- variation induced by mutagenic action of the culture media
- epigenetic variation
- variation induced by stress (eventually leading to activation of mobile genetic elements)

Plant tissue culture has the potential to induce genetic variability through somaclonal variation (Jullien *et al.*, 1998). Novak (1980) reported phenotypic and cytological variation in *in vitro* plants araising from callus cultures. Almost half of the regenerants being tetraploids, aneuploids or mixoploids.

Variation may arise due to several factors such as genotype used, pathways of regenerants *etc.* (Breiman *et al.*, 1987). Several parameters such as morphology (Swedland and Vasil, 1985), field assessment, molecular studies (Breiman *et al.*, 1989; Shenoy and Vasil, 1992; Choudhury *et al.*, 1994) *etc.* have been employed for assessing the effect of *in vitro* culture.

The morphogenic response of the explant is mainly based on the type and concentration of hormone used. Tissue culture studies on a number of medicinal plants (Irawati and Nyman, 1986; Kumar, 1992; Nirmal Babu *et al.*, 1992) suggest that a fine balance of exogenous auxin and cytokinin are necessary for successful regeneration of plants. The role of cytokinin in shoot organogenesis is well established (Evans *et al.*, 1983). In the present study, it was observed that a combination of BAP and NAA was most effective in inducing callus and multiple shoot initiation from all the explants such as leaf segment, nodes and inflorescence axis (Plates 1-7; Tables 9, 10 & 11).

The role of cytokinins in overcoming the apical dominance of the terminal shoot bud and enhancing the branching of the lateral buds from axils was observed. It is known that BA is the most effective synthetic cytokinin for stimulating axillary shoot proliferation for different plant systems (Bhojwani, 1980; Hasegawa, 1980; Kitto and Young, 1981; Welander *et al.*, 1989; Nadel *et al.*, 1991; Devi *et al.*, 1994; Gangopadhyay *et al.*, 1998). These results agree with those of the present study in the formation of axillary shoots from the nodal and inflorescence axis explants in the medium with BAP (Plates 8-11; Tables 10 & 11).

BAP with IAA produced only little callus and shoot from the node and inflorescence axis explant. IAA alone produced friable callus and shooting from leaf explant whereas, it did not produce any effect on nodal and inflorescence axis explants.

The incorporation of auxin in the medium generally promotes rooting (Gautheret, 1945). Roots have been reported to originate from elaborate callus tissue (Hubakoa, 1986; Hartman *et al.*, 1990). The relative levels of auxin have been known to greatly influence morphogenic responses like rooting (Sitborn *et al.*, 1993). The observation that IBA generally performed better as an auxin for rhizogenesis than IAA emphasized the fact that auxin types differs in their morphogenic ability and organogenic effect on plant tissues in culture (Nagasawa and Finer, 1988). In the present study IBA alone produced rooting of multiple shoots.

BAP along with kinetin and NAA produced axillary shoot development from nodal explant and little callus from leaf explant and no effect from inflorescence axis explant. BAP and 2, 4-D produced little callus followed by

drying of callus from leaf explant whereas, they had no effect on nodal and inflorescence axis explants (Tables 9, 10 & 11).

It has already been reported that the nature of growth regulators used in the medium may result in the occurrence of somaclonal variation (Patel and Berlyn, 1982). High concentrations of growth regulators in the medium and long term culture are thought to be the main causes of variation in plant cultured *in vitro* (George and Sherrington, 1984). It is accepted that *in vitro* manipulation do cause genetic aberration (Vajrabhaya, 1977). The possibility of genetic changes occurring in plants raised from callus cultures can be used as a potential source of somatic variation (Pillai and Hilde Brandt, 1969; Bush *et al*., 1976). The frequency of variation is also influenced by culture duration, concentrations of cytokinins and number of plants produced from each explant (Reuveni *et al.*, 1986; Vuylsteke *et al.*, 1988).

It seems possible that mutation of cells under culture may in some instances resemble somatic mutation that occurred in nature, which must have led to the formation of different clones in the sterile species of plants (Al Zahim *et al.*, 1999). The frequency of genetic changes in somaclones is much higher than the spontaneous genetic changes brought about in the entire plant (Prat, 1983). Genetic variation may be due to a DNA sequence which is susceptible to tissue culture induced mutation (Bohanec *et al*., 1995).

It is well documented that *in vitro* culture conditions induce a genomic stress that might result in chromosome breakage. Many studies have indicated that the break position does not appear to be random, but occurs in the heterochromatic region and could lead to chromosomal translocation, inversion or deletion (Benzion and Phillips, 1988; Lapitan *et al*., 1988). It would be

interesting to access the frequency of somaclonal variation over an extended time course. RAPD polymorphism may have accumulated with time (Al Zahim *et al*., 1999).

One of the possible mechanisms suggested, explaining somaclonal variation is the activation of different mobile genetic elements such as those reported during tissue cultures of different plants (Peschke and Phillips, 1991; Hirochika, 1993; Hirochika *et al*., 1996).

Epigenetic variation is another important cause of somaclonal variation in plants. This aspect of somaclonal variation involves mechanism of gene silencing or gene activation that was not due to chromosomal aberration or sequence change (Kaeppler *et al*., 2000).

Skirvin (1978) studied natural and induced variation and opined that variation is quite ubiquitously associated with *in vitro* propagated plants. Somaclonal variation can provide means of amplifying variability within the existing cultivar, thereby opening new opportunities for clonal selection. Much of the variability found in tissue culture can be directly or indirectly attributed to gross chromosomal changes and other chromosomal abnormalities (Conger, 1987).

Revealing the mechanisms of mutation will lead to a better understanding of genomic changes in response to stress factors thereby contributing to the knowledge of genomic stability and methods to control variation among tissue culture regenerants. Any step made towards understanding the basis of tissue culture induced genetic variation should be helpful in developing a more stable and manipulable somatic cell system.

Cytological Analysis

Cytological analysis is an important tool in systematics since it enhances knowledge to understand interrelationships among taxa and the genetic mechanism involved in species formation. Although conventional methods furnish little information about species evolution when compared with the molecular ones currently employed, there is a great interest in these studies mainly in tropical countries where biodiversity is very high and cytological investigation is scanty. Gene revolution has become a reality due to recent developments in molecular biology and tissue culture (Gholamreza Bakshi, 2002).

The chromosomes seen during mitosis in cells of eukaryotes offer visible evidence of the genetic architecture of the organism as the number of chromosomes, their size and morphology is necessary for a full understanding of genome in plant genetic studies and plant improvement. Karyotype is useful to understand the origin and nature of chromosome variations (Gu *et al*., 1984).

In some members, karyotype analysis for the identification of homologues is unreliable, because not all chromosomes can be distinguished by their length and centromere position and no useful additional cytological markers are available (Koopman *et al.,* 1996). Therefore the karyotypes are established using numerical parameters describing the chromosome length, area, perimeter, uniformity coefficient, variation coefficient, disparity index of chromosomes, total forma percentage and number of discernible satellites. In some groups karyotypic differences between species are largely quantitative and have been difficult to assess by conventional quantitative methods.

Chromosome identification and mapping are indispensable in cytological and genome analysis. There are limitations for conventional measuring and

characterization of chromosome complement by visual evaluation, especially for very small chromosomes. The ordinary karyotype analysis provides only limited success from the view point of chromosome identification (Fukui and Mukai, 1988). Karyomorphometrical studies by computer based image analysis system provide a better knowledge of the cytogenetic constitution of various species over conventional methods (Rajalakshmi and Jose, 2002).

The study of plant tissue culture brings a lot of important cytogenetic problems, namely karyotypic changes and chromosomal instability of cell population during *in vitro* culture (Partanen, 1963; D' Amato, 1964; 1975; 1977; Sunderland, 1973; Sheridan, 1974; Skirvin, 1978).

Chromosome variability is of well known occurrence in cells of cultured tissues as well as in regenerants (Bayliss, 1973; Sacristan and Melchers, 1969). Instability of chromosomes in culture has been reported to be influenced by a number of different factors including composition of media (D' Amato, 1978). This instability, however, can be useful for the production of plantlets with novel genotypes including chromosomal aberrants (Larkin and Scowcroft, 1981). Mutant and variable cell lines selected from cultured cells have immense potential for recovering new variant types and in cloning of desired genotypes (D' Amato, 1978). For this reason the study of chromosomes in cultured cells has a special significance.

In the present investigation, the morphology of chromosomes in *in vivo* and *in vitro* cells of *A. nilagirica* has been studied. No detailed *in vitro* cytological analysis on this plant is so far available.

In the present study, chromosome number variation was observed in the somaclonal variant when compared to the parent. The chromosome number of

the parent was 2n = 4x = 36, while that of *in vitro* plant was 2n = 8x = 72. The calli cells were of three types- tetraploid (2n = 4x = 36), octaploid (2n = 8x = 72) and hexadecaploid (2n = 16x = 144), of which the percentage of octaploid cells in callus was more frequent (Plates 13-17). Moreover, plant regeneration was noticed only in octaploid callus.

Grant (1981) proposed that the original base numbers of Angiosperms range from x_1 = 7 - 9. A karyotype study of the root tip cells of 20 species of *Artemisia* reported by Qiao *et al*. (1990) showed that there were two basic chromosome numbers, x = 8 and 9. In addition to the already known diploid number (2n = 18), the tetraploid level (2n = 36) has been detected in *A. absinthium*.

From the present study, the basic chromosome number of the plant was found to be x = 9, which coincides with the previous report. It is argued from karyological and phylogenetic evidence that the original chromosome number in *Artemisia* genus was x = 9 (Stahevitch and Wojtas, 1988). Basic chromosome number is one of the most widely used characters in biosystematic studies and there has been a vast amount of phylogenetic speculation whether this value can be used as a dependable and stable marker of the direction of evolution (Jones, 1970; 1974; 1978a).

According to Fernandes and Leitao (1984), primary, secondary and tertiary basic chromosome number exists in plants. It seems probable that the parent plant of *A. nilagirica* is of polyploid (tetraploid) origin, with the original base number x = 9. The tetraploid chromosome complement (2n = 4x = 36) of *A. nilagirica* reveals proto-autopolyploidy from the primary basic chromosome number of x_1 = 9. Thus in the present investigation there is a probability that the

somaclonal variant of *A. nilagirica* may be an octaploid (2n=8x=72), having evolved from the primary basic chromosome number of $x_1 = 9$.

Reese (1961; 1966) suggested that an increase in the number of chromosomes provides increased possibilities for new gene combinations. Polyploidy also results in increase in the genes controlling characters favourable for natural selection, when these characters are already present in the plant. Polyploidy is of great relevance for the evolution of the genus because of various cytotaxonomic or cytobiogeographical aspects (Qiao *et al*., 1990).

Plant tissue cultures exhibit chromosomal, numerical and structural variability as well as uniformity. A sizable portion of the variability is induced during cell proliferation *in vitro* (Bajwa and Wakhlu, 1986; Bayliss, 1980; D' Amato, 1964; 1978) although some of it may also reflect the variation already present in the primary explant (Cionini *et al.*, 1978; Mathews and Vasil, 1975).

When cultured, the plant cells were known to exhibit variations in chromosome number and structure (Bayliss, 1973; Constantin, 1981; D' Amato, 1978; Gupta and Ghosh, 1983) as observed in the present study.

A study of literature on the behaviour of chromosomes in tissue culture has shown that in many instances the regenerated plantlets contain a normal chromosomal complement. Diploid plants have been recovered from mixoploid callus cultures (*eg.* in *Daucas carota*) suggesting that diploid cells are selectively favoured during plant regeneration.

Nevertheless, reports of regeneration of plants with either aneuploid or polyploidy constitution, as in the present investigation, are not uncommon. *Asparagus officinalis*, *Nicotiana* sps. and *Oryza sativa* (Sacristan and Melchers,

1969) are some of the examples showing a variant chromosome constitution.

Sinha *et al.* (1987) reported numerical variation in chromosomes in the long term callus cultures raised from cotyledons of *Sesbania grandiflora.* Chromosomal instabilities in the *in vitro* cultured somatic cells generally occur in plant cell cultures (Sunderland, 1977). The present results are in agreement with the previous observations, where they recorded polyploidy in three various callus lines of *Allium sativum*, derived from different cultivars (Novak, 1974). A high ploidy level is also typical for cell population of *A. cepa* callus tissue (Sekera, 1977).

Chromosome irregularities may result in alterations of basic chromosome numbers (Jauhar and Joshi, 1969) and repatterning of karyotypes (Jauhar, 1974). The variation in somatic chromosome number among calli may be due to the irregularities in spindle mechanism (Haque and Ghoshal, 1981). Moreover, somatic cells are known to show more variation in chromosome number than meiotic cells as reported in the genus *Commelina* (Patwary *et al*., 1987).

Cytological variability increases with increasing number of subcultures (Novak, 1981). Cells with chromosome clumps, multipolar spindles, multinuclei, asynchronous divisions and laggards were also observed at various subcultures. Such nuclear aberrations may lead to the formation of cells with variable chromosome number in callus cultures of *Zea mays* (Mohanty *et al*., 1986). Chromosomal instability *in vitro* may be influenced by the type of explants which gave rise to the callus, as suggested by Partanen (1965).

The formation of polyploid cells *in vitro* has been attributed to spindle fusion and endomitosis (Bayliss, 1973). Polysomaty existing *in vitro* among plants are suspected as a means of numerical variability in callus cultures (D'

Amato, 1978). It has been reported frequently that plant tissues and cells display a high degree of instability under *in vitro* conditions resulting in the formation of mixoploid tissues (Sunderland, 1977). Higher polyploid cells were also noticed during different subcultures. Several literature reviews dealing with ploidy, instability and related phenomenon in *in vitro* cultured cells are available (D'Amato, 1952; 1977; 1978; Skirvin, 1978; Constantin, 1981).

Among the media components, auxins and cytokinins have been found to contribute to ploidy changes (Torrey, 1961). The sugar, potassium and phosphate concentrations in the medium, the nitrogen sources, the pH of the cultures and the addition of organic acids to buffer media have been found to affect the formation of propagules in plant tissue culture (Dougall, 1981).

Alterations in structure and behaviour of chromosomes *in vitro* have been reported by different authors. Endomitotic replication resulting in increase in chromosome number (Partanen, 1965), polyploidy (Sacristan, 1971), nuclear fusion (Collins *et al*., 1974; Kasha, 1974; Mahlberg *et al*., 1975) as well as somatic reduction (Sunderland, 1973) have been reported in addition to other aberrations. Chromosome breakage, polyploidy and aneuploidy are rather common in suspension cultures, induced by the growth promoters (Heinz *et al*., 1969; Torrey, 1967).

Studies on the mitotic behaviour revealed that split spindles allow the unhampered separation of chromatids to different poles. The larger number of chromatids moving towards one pole than to the other may be due to the passage formed by multispindles. At telophase, in many cells, presumably because of insufficient room, the irregularly distributed groups often fuse. Thus two or more groups become enclosed within one cell wall resulting in higher chromosome number (Chennaveeraiah and Wagley, 1985).

Multipolar separation of chromosomes suggests that polyploidisation may result from anomaly in spindle fibres. Abnormal migration of chromosomes from the mother cell to the daughter cells might be one of the ways to cause numerical chromosomal variation in cultured cells.

Endoreduplication might be another possible reason for chromosomal variations. The diplochromosomes seen at metaphase are perhaps in support of this. Diplochromosomes help in increasing the number due to chromosome duplication (White, 1935), as seen in legumes like red clover, garden peas and common vetch (Wipf and Cooper, 1938). In *Solanum*, schematic coalescence of two mitotic spindles (Jorgensen, 1928) and in *Acer planatoides*, nuclear fusion in a binucleate cell (Meurman, 1933) were said to cause polyploidy.

Structural changes of chromosomes were also observed in the present study. Variations in karyotypes involved total chromosome length, average chromosome length, centromeric positions, disparity index, variation coefficient and total forma percentage (Tables 12-17). Similar reports are available in *Triticum durum* (Gupta and Ghosh, 1983), *Crepis capillaris*, *Haplopappus gracilis* and *Allium cepa* (Bajwa and Wakhlu, 1986).

In the present study, the average chromosome length of the parent plant (1.0177 µm) was found to be higher than that of the somaclonal variant (0.8238 µm). Reduction in chromosome size is apparently a consequence of polyploidy, since it is an adaptation to a decrease in size of the cell or to an increase in number of chromosomes (Darlington, 1958). In the present investigation also average chromosome length decreases with increase in the number of chromosomes. Chromosomal rearrangements may lead to slight changes in the size of the chromosome. The differences in the

chromosome length and volume may be attributed to differential spiralization and condensation of chromosome along with the content of protein and DNA. It may also arise by translocations, duplications and deletions. Robertsonian translocations can also lead to changes in the size of the chromosome. The change in the chromosome length may be the aftermath of cryptic changes, probably duplications, which may arise due to *in vitro* stress produced in the altered culture environment. Moreover retrotransposon activation and inversions may significantly contribute to the change in the physical size of the genome (Olhoft and Phillips, 1999). Similar reports are available in *Allium cepa* (Sekera, 1977) and *Papaver somniferum* (Bajwa and Wakhlu, 1986).

Chennaveeraiah and Habib (1966) reported the structural rearrangements of chromosomes in cultures of *Capsicum annum.* In the present investigation, the difference in the length of chromosomes in the *in vitro* plant and the calli exhibiting different ploidy levels (tetraploid, octaploid and hexadecaploid) (Tables 12 - 17), when compared with the karyotype of the parent plant may be due to any of these above mentioned reasons.

Total forma percentage of *in vitro* plant was slightly higher (41.2182) than that of parent (40.8120). In the present study the chromosome complement of the parent, *in vitro* plant and various calli are characterized by smaller chromosomes. Comparatively smaller chromosomes in the karyotype seem to be an advanced characteristic feature (Das Gupta and Datta, 1976).

The karyotype formulae deduced for all (parent, somaclonal variant, and the different calli) showed variation in the type of chromosomes. In the present investigation, different types of chromosomal categories were deduced, *viz.*, A, B, C and D. The karyotype formulae are as follows:

Parent	=	$A_4 B_{22} C_8 D_2$
Somaclonal variant	=	$A_8 B_{44} C_{20}$
Tetraploid calli	=	$A_4 B_{28} C_4$
Octaploid calli	=	$A_8 B_{56} C_8$
Hexadecaploid calli	=	$A_{16} B_{90} C_{34} D_4$

The number of chromosomes with nearly median primary constriction was higher in the parent, somaclonal variant and the various calli (Tables 12-17). At higher ploidy level of the calli, the sub metacentric chromosomes were found to increase, when compared to lower ploidy levels. Excessive number of submetacentric chromosome reveals an advanced evolutionary status (Levitsky, 1931). Change of the centromere from median to submedian and increased size difference between different chromosomes of the same set are two basic processes responsible for karyotype speciation (Levitsky, 1931) and this is often considered as a potential factor in the evolution of species, especially at the diploid level (Stebbins, 1970; Mathew and Thomas, 1974).

Disparity index of the somaclonal variant was higher (49.7210) than that of the parent (32.0546). High disparity index denotes an advanced heterogenous nature of the karyotype (Mohanty *et al*., 1991).

Variation coefficient is the most reliable karyomorphological parameter because it is calculated considering the length of all the chromosomes. In the present analysis, variation coefficient of the *in vitro* plant was found to be higher (26.0546) than that of *in vivo* plant (17.3187) of *A. nilagirica*. The high variation coefficient value corresponds to the heterogenous assemblage of chromosomes. The karyotype that are heterogenous both cytologically and genetically are important in the evolution of species (Stebbins, 1958).

Structurally changed karyotypes may araise due to deletion and translocations (Lee and Ono, 1999). In general, numerical and structural aberrations in chromosomes are attributed to spindle failure that causes endoreduplication, c-mitosis, nuclear fragmentation *etc*. These changes are induced by media composition, age of callus (morphogenic vs non-morphogenic), genetic background of explants and kinds of media (solid vs liquid) (Bayliss, 1973; 1980; Evans and Reed, 1981; D'Amato, 1985; Ogura, 1990; Geier, 1991). Chromosome aberrations (Plate 18) induced in cultures are the result of the direct influence of chemical substances present in the medium. The reason cited above may be the reason for the chromosome variations observed in the present study.

Li *et al.* (1986) and Heszky *et al.* (1990) postulated that the phenotypic manifestation of molecular and chromosomal changes (somaclonal variation) depends on the origin and ploidy level of initial explant and primary callus. The genetic variability or instability of callus cells is well characterized by the variation in the chromosome number. Several factors are said to play an important role in the chromosome constitution of cultured tissues. They are nuclear conditions of original explants, composition of medium especially kinds and concentration of plant growth regulators, age of culture, variation due to plant species, karyotypic changes *etc*.

Plant cells growing in an artificial culture environment show numerous genetic changes. These alterations are manifested as increased frequencies of gene mutation, chromosome breakage, transposable element activation, quantitative trait variation and modification of normal DNA methylation patterns (Kaeppler and Phillips, 1993). Callus culture can be proposed as a potential source of regenerants bearing structural changes of the chromosomes and this in

turn might result in the generation of somaclonal variants (Mohanty *et al.*, 1991).

Genetic heterogeneity in culture arises mainly due to factors like expressions of chromosomal mosaicism or genetic disorders in cells of the initial explants and new irregularities brought about by culture conditions through spontaneous mutations. Cell or tissue cultures undergo frequent genetic changes such as polyploidy, aneuploidy, chromosomal breakage, deletion, translocation, gene amplification and mutation and these are expressed at biochemical or molecular levels (Ignacimuthu, 1997). The number and form of chromosomes in single cells and the frequencies of primary structural changes are the features by which a proliferating cell population can be characterized in cytogenetic terms (Ford, 1964).

In the present study, cultured cells at metaphase carried structurally altered chromosomes showing three different levels of ploidy in them. Conditions in the artificial environment of cell culture may enhance mutation rate, which may act as a genetic shock. The mutation rate may also be enhanced by leakage of toxic by-products and exudates from the calli into surrounding medium (Olhoft and Phillips, 1999).

The increase in the total chromosome content of the somaclonal variant may be probably due to the genetic stability achieved by the variant after regeneration. So in the present investigation it seems probable that the octaploid somaclonal variant might have originated from the octaploid calli after regeneration and stabilization. Minute and cryptic structural differences and gene alterations and rearrangements are therefore responsible for the origin of new species. Such structural changes might have also contributed to the origin of different cytotypes (Stebbins, 1971).

Chromosome breakage and subsequent alterations in chromosomes were reported in many plants. The changes include cytological aberrations, which are primarily the result of chromosome breakage, single base changes, and changes in the copy number of repeated sequences and alterations in DNA methylation pattern (Benzion *et al*., 1986). Mutations involve loss or gain of a defined enzyme function. Every deleterious change in the cistron controlling these enzymes should result in the mutational event (Szybalski *et al.*, 1964).

Alien cultural environment and chemicals of the medium influence the chromosomal behaviour of the cultured cells (Bajwa and Wakhlu, 1986).

The chemical composition of the culture medium has been shown to affect the cytogenetic behaviour of plant cells *in vitro* (Bennici *et al*., 1970; Karp, 1992). Mineral deficiencies, chelating agents and some heavy metal ions have been reported to have some role in inducing chromosome breakage and rearrangement in plants (Steffenson, 1961). The hormone itself can potentially be toxic to the cell there by directly leading to chromosomal aberrations. The concentration and type of hormone in culture medium also influence the patterns of methylation (Lo Schiavo *et al*., 1989).

Singh (1986) reported a few chromosomal variations in callus cultures of crops, which are produced due to the effect of media components. According to Singh (1976), KIN can cause chromosomal change. Effect of hormones like 2,4-D, IAA, NAA and KIN on chromosome aberration in cultured plants was already reported (Singh, 1993).

High concentration of growth regulators results in karyotypic alterations in cultured cells. Several phytohormones have shown to induce chromosomal variability in cultured cells, leading to the formation of somaclones. Variants

may arise due to single gene mutation in cultured cells. Another aspect of single gene mutation responsible for somaclonal variation relates to transposable elements. Variation has been reported as a result of insertion of plasmid like DNA in the mitochondrial genome of cell cultures of some plants (Ignacimuthu, 1997).

In plants derived from cell and tissue cultures, major changes in chromosome complement often do not appear to be accompanied by corresponding changes in the phenotype of the plant. The gain or loss in chromosomes sometime may not be sufficient to cause a large change in morphological character and it is also possible that changes can also occur that are not visibly expressed (Liu and Chen, 1976). This may be the reason for the absence of considerable morphological variation in the regenerated plant of *A. nilagirica* in the present study.

According to Darlington and Wylie (1955), when the karyological aberrations affect the genic system, the structural changes in the chromosomes act mainly as a means of holding together certain favourable gene combinations and therefore promoting immediate fitness at the expense of flexibility. Chromosomal interchanges have been described to be the basis for obtaining somaclonal variation (Karp and Bright, 1985; Pijnaker and Ferweda, 1987). The epigenetic (Meins and Binns, 1977) and genetic (Ryan and Scowcroft, 1987) alterations of plant species occurring during tissue or cell culture cycle are transmitted by some regenerants into offsprings through gametes (Maliga, 1984). This indicates that cultured tissues are able to produce novel chromosomal, genic and physiological variations caused by mitosis, which are different from those occurring through meiotic cell cycle. Pardue (1991) has hypothesized that genomic stability is not the default state but is the result of a rather finely tuned

system of checks and balances. The tissue culture environment may cause a general disruption of the cellular controls, leading to the numerous genomic changes present in the tissue culture regenerants. Variations could have been induced by *in vitro* process by added biochemicals and stresses (Swartz, 1990).

Although there are various factors for chromosome variations, the role of exact factors causing changes is yet to be studied.

Random Amplified Polymorphic DNA (RAPD) Analysis

Advances in biotechnology have provided several molecular markers useful in crop improvement programmes. There are versatile tools for finger printing and for monitoring variation in plants based on genetic polymorphism obtained from a sufficient number of unbiased markers.

Among the diverse DNA markers identified during the past decades RAPDs with the potentially unlimited number of markers allow finer distinction, especially if too little isozyme diversity exists. The use of molecular markers is becoming widespread for the identification of somaclonal variants and the assessment of micropropagation or *in vitro* regeneration protocols (Heinze and Schmidt, 1995; Wallner *et al.,* 1996).

RAPD appears particularly suitable for the evaluation of genetic integrity during tissue culture (Isabel *et al*., 1993; Taylor *et al*., 1995), the identification of clonal plant material (Castiglione *et al.*, 1993; Rani *et al.*, 1995) and the detection of somaclonal variants (Munthali *et al.*, 1996). The RAPD technique was found to be most effectively assessing the genetic constitution of plants.

Polymerase chain reaction, a key tool in molecular biology provides a rapid and powerful technique for the *in vitro* amplification of DNA sequences

(Mullis *et al.,* 1986). PCR technique developed by Saiki *et al.* (1988) is one of the most significant contributions to the field of DNA technology, facilitating comparative analysis of a large number of genomes in a relatively small period of time. The technique greatly helped DNA researchers to overcome many technical limitations encountered in conventional RFLP analysis. It requires very little amount of DNA and it is very fast, producing millions of copies of DNA fragment within a few hours. One specific advantage of RAPD markers is its capability of detecting polymorphism in both coding and non-coding regions of the genome of interests (Williams *et al.,* 1990).

PCR based RAPD technique (Williams *et al.*, 1990) was applied to assess somaclonal variation since this method has proved effective in a number of cases as in *Lolium* (Wang *et al.*, 1993), *Triticum* (Brown *et al*., 1993), *Picea* (Isabel *et al.,* 1993) and *Beta* (Munthali *et al.,* 1996). In the present study, considerable change in RAPD bands have been observed in the somaclonal variant (TC3).

The use of PCR amplification to detect target DNA sequences has many application in plant genotyping, gene mapping, diagnosis and diversity assessment (Kreader *et al.,* 2001). It is evident from the electrophoretic gels that somaclonal variation at DNA level is also present in the hitherto mentioned variant of *A. nilagirica*. Similar results were already reported in other plants also (Wang *et al.*, 1993; Hashmi *et al*., 1997; Brown *et al*., 1993; Munthali *et al*., 1996) using RAPDs. RAPD profiles were unambiguously used to establish the distinct identity of *in vitro* plants, which are different from the parent plant in many varieties of plants (Khanuja *et al*., 2001a; 2001b; 2001c; Dwivedi *et al*., 2001a; 2001b; 2001c; Patra *et al*., 2001b). In *A. nilagirica,* there is no previous report on this type of analysis.

The tissue culture environment may show a general disruption of the normal cellular controls, leading to numerous genomic changes present in the tissue culture regenerants (Phillips *et al*., 1994). As far as the genetic stability of the proliferated tissue is concerned, RAPD markers are efficient tools for detection of somaclonal variation in tissue culture. Direct analysis of the DNA by use of RAPD markers proved a very sensitive technique for evaluating genetic changes after *in vitro* culture (Piccioni *et al*., 1997). In the present study, RAPD analysis using arbitrary 10-mer oligonucleotide primers was employed in order to investigate the genetic variability of the somaclonal variant (TC3) of *A. nilagirica.*

Polymorphisms in amplified bands were observed in the present study when the parent and TC3 plants were compared (Plates 19 & 20). It represents changes in the sequence of primer binding site (*eg.* point mutation) or change which alters the size or prevents the successful amplification of a target DNA (*eg*. insertions, deletions and inversions) (Rani *et al.,* 1995). DNA amplification products, which represent one allele per locus, could result from changes in either the sequence of the primer binding site or changes which alter the size and prevent the successful amplification of target DNA (Rout *et al*., 1998). Presence of RAPD markers at a specific locus in both genotypes indicates a high level of homology at that site. The sequence difference between two genotypes is expressed as the absence of marker and thus as band differences in the RAPD fingerprint (Williams *et al*., 1993).

Three types of polymorphism were observed in the study, such as the presence of additional bands, absence of existing bands as well as band intensity differences (Plates 19 & 20; Tables 18 - 20).

RAPD fingerprint of the somaclonal variant (TC3) differ from the parent (P) with all five primers (OPA 01, OPA 02, OPB 17, OPB 18 and OPC 01). A few bands were found to be missing in the somaclonal variant (TC3) when all the five primers were used. Additional bands in the variant were also detected by the markers like OPA 01, OPA 02, OPB 17 and OPC 01 (Plates 19 & 20; Tables 18 -20).

RAPD analysis of *Allium cepa* revealed a novel band in independent gametoclones and it was suggested that this was due to a DNA sequence which was highly susceptible to tissue culture induced mutation (Al Zahim, 1999). The occurrence of some novel bands in independent regenerants has also been observed in wheat (Brown, 1993).

Polymorphism between genomic DNA are considered to be produced through several different processes such as nucleotide substitution, insertion, deletion or inversion in primer annealing sequences or creation / alteration of new primer sites or changes in the length of DNA segments between primer sites (Williams *et al.,* 1993). Failure of amplification of different lines may be due to a single base change or completely different sequences (Vierling and Nguyen, 1992).

In the present study, certain intensity differences in the bands were also noticed in the amplification products of OPA 02 and OPB 18 primer tested (Plate 19). The same types of results were also reported in other plants also (Yang and Quiros, 1993: Hashmi *et al.*, 1997). Varied fragment intensity on gels is also considered as a way of analyzing polymorphism from a RAPD profile. The relative intensity of bands is also affected by magnesium ion concentration and annealing temperature (Williams *et al.,* 1993). However such variations are

constant for a particular study.

The reproducibility of the genomic DNA bands of TC3 was consistent in successive repetition than other TC plants. Since the amplification of TC3 plants showed more polymorphism and consistency than other plants, it was considered as a somaclonal variant and used for further analysis.

This is the first report on the molecular basis of variation detected by RAPD in the micropropagated plants of *A. nilagirica*. The results suggest that RAPDs are useful for establishing the genetic basis of somaclonal variation and strengthens the idea of variant development by tissue culture.

Essential Oil Analysis

All medicinally used *Artemisia* are fragrant plants which contain essential oils. The medicinal use is sometimes based upon the oil. The essential oil content as well as the composition therefore becomes a valid criterion for the quality of the crude drug (Woerdenbag and Pras, 2002).

Several secondary metabolites characterize the chemical composition of the genus *Artemisia*. Survey of literature indicates that almost all classes of compounds are present in the genus with particular reference to terpenoids and flavonoids. However, wax constituents, polyacetylenes and to a lesser extent, nitrogen containing molecules have also been found in several species. The wide array of molecules present in the genus and the distribution of plants in several different habitats provide the opportunity for the study of genotypic and phenotypic variation as well as chemotaxonomic relationships among species (Mucciarelli and Maffei, 2002).

The rich accumulation of essential oils and other terpenoids is responsible

for the use of various members *Artemisia* for flavouring food and liquors. Terpenoids and certain phenolic compounds are also responsible for the value of many species of Asteraceae in pharmacy and medicine (Wagner, 1977).

Plant tissue culture has the potential to perform, biochemical reactions when organic compounds are added to the medium. It is possible to transform a substance from a lower to a higher scientific, commercial or economic value and also to produce a new compound (Kukreja *et al.*, 2000).

The capacity of cultured plant cells to serve as catalyst for biochemical reactions such as epoxidation, esterification, glycolsylation, methylation, isomerisation and dehydrogenation of organic compounds was comprehensively reviewed (Reinhard, 1974; Furuya, 1978). Variations for four major constituents of essential oils were recorded in somaclonal variants of mints (Kukreja *et al*., 1991). Over the years, *de novo* syntheses of many commercially important chemical compounds have been reported (Nair *et al*., 1986; Calleboutet *et al.*, 1990).

Culture stress may induce variation in tissue cultured plants which are sometimes associated with useful agronomic characters such as oil yield, oil content *etc.* (Patnaik *et al.*, 1999).

Various environmental factors such as artificial light quality, sucrose concentration and hormonal culture media supplements have been shown to be effective in promoting *in vitro* essential oil accumulation as occurred in the *in vitro* plants of *A. balchanorum* (Bavrina *et al.*, 1994). Growth medium related oil production was also found for *A. alba* (Turra), as well as for other species of the Belgian flora, where the aim of the research was to develop a conservation programme based on micropropagation (Ronse and De Pooter, 1990).

Micropropagation techniques have also been applied in the flavour industry for the isolation and growth of *Artemisia* clones of the genipi- group (*A. umbelliformis* and *A. genipi*) having a high degree of variability in their essential oil GC pattern and organoleptic characterization (Gautheret *et al*., 1984). A significant genetic variation was reported in accordance with variation in physiological and biochemical traits in different somaclones of other plants also (Tiwari *et al*., 1995).

In the present investigation, the essential oil of *A. nilagirica* seems to belong to a terpenoid chemotype. GC-MS analysis revealed 29 components in the parent plant and 21 in the somaclonal variant (Table 21; Plates 21-33). There was a clear difference between the compositions of the two oils tested. The major components were mainly α- thujone and camphene in both the oils.

The percentage of monoterpenes present in the *in vivo* plant was 93.82 while that of *in vitro* plant was 86.04. But the percentage of sesquiterpenes was higher in the *in vitro* plant (13.96) than that of the parent plant (5.99) (Table 21).

The common monoterpenes were α- fenchene, sabinene, 1, 8- cineole, α- thujone, β- thujone, bicyclohex-3-en-2-one, thujyl alcohol, L-camphor, camphene and terpinen-4-ol. Common sesquiterpenes were mainly α- copaene, β- caryophyllene, germacrene-D, δ-cadinene, caryophyllene oxide and β- eudesmol (Table 21).

The unique compounds present in somaclonal variant were p-cymene, dl-limonene, α-terpinene, farnesene and bicyclogermacrene. The percentage of essential oil components

like α- fenchene, sabinene, terpinen-4-ol, α-copaene, β-caryophyllene, germacrene-D, δ-cadinene and β- eudesmol were more in the somaclonal variant than in the parent plant (Table 21).

The oil investigated in this study is clearly the South Indian thujone chemotype, although levels of α- thujone, camphene, sabinene, β- pinene, germacrene - D and β- eudesmol were higher than those reported previously (Uniyal *et al*., 1985; Thakur *et al*., 1990; Mohammed Shafi *et al*., 2004). Moreover compounds like α-fenchene, thujyl alcohol, chrysanthenone, M-mentha-1,8-diene, cyclofenchene, α-copaene, β-bourbonene, farnesene, gramacrene-B, bicyclogermacrene and δ-cadinene were the novel compounds detected in the present study.

The lesser value of coefficient of similitude (47.05) obtained when essential oils of both the parent plant and somaclonal variant was compared, shows the dissimilar nature of these oils (Table 21). This dissimilarity may be probably due to the variation in the biosynthetic pathway of essential oils which are genetically controlled.

Plant cell culture can produce

- new compounds previously not known in the intact plants
- new derivatives of known compounds
- new compounds by biotransformation of molecules incorporated in the medium (Merillon and Ramawatt, 1999).

It is presumed that the production of new compounds or derivatives might

be due to altered gene function in cultured cells when compared to the mother plant (Merillon and Ramawatt, 1999). Previous reports prove that in *Mentha rotundifolia*, the products synthesized *in vitro* were more oxidized than the natural forms (Kukreja *et al.*, 2000). The type of growth regulators in the culture medium can affect the production of secondary metabolites in cultured cells quite dramatically (Cline and Coscia, 1988). Changes in the constituents of in *vivo* and in *vitro* grown plants are influenced by various genetic and non - genetic factors (Gerhardt, 1972). But there are also reports on tissue cultured plants that match the parent plant in their biosynthetic capacities (Kireeva *et al*., 1978; Charlwood and Charlwood, 1983). However, in the present investigation, the marked differences observed in the essential oil constitution of the parent plant and the somaclonal variant (Table 21) may be due to their respective biosynthetic pathways (Tetenyi, 1973).

Analysis of essential oils of both *in vivo* and *in vitro* plants of *A. nilagirica* revealed α-thujone as the major compound along with a little amount of β-thujone. In both the samples, the major component (thujone) remained unchanged even if there is marked variation in the other components. This indicates that the genetic changes due to culture stresses or hormones used did not affect the biosynthetic pathway of the major component. The absence of some constituents in the somaclonal variant may be due to the triggering of certain diverged biosynthetic pathways.

Almost all the secondary metabolites such as monoterpenes, sesquiterpenes and phenylpropenes arise from one of the three biosynthetic pathways or from a combination of two or more of these pathways. These are known as the acetate, mevalonate, and shikhimate pathways (Waterman, 1993).

The condensation of two molecules of dimethyl allyl pyrophosphate derived from isoprene *via.* the mevalonate pathway, gives rise to geranyl pyro phosphate (GPP), which is considered to be the common precursor of all monoterpenes. The cyclization of GPP by the catalytic action of GPP: (+) - sabinene cyclase yields sabinene. In *A. absinthium*, sabinene has been found to act as a substrate for conversion to (+) – sabinyl acetate and (+) - 3- thujone and microsomal preparations were shown to catalyze the NADPH and O_2 dependent hydroxylation of (+) – sabinene to (+) - *cis* - sabinol. These results indicate that the synthesis of thujone requires a cytochrome P -450 dependent mixed function monooxygenase. In some other *Artemisia* species the NADPH – dependent stereo selective reduction of (+) – sabinone to (+) 3-thujone has been demonstrated (Croteau, 1987).

Another important monoterpene which often occurs in *Artemisia* essential oils is camphor. The formation of camphor involves the conversion of GPP to (+) - bornyl pyrophosphate which, after hydrolysis by a specific bornyl pyrophosphatase is converted to the bicyclic monoterpene alcohol (+) - borneol. An NAD dependent dehydrogenase oxidizes borneol to the ketone camphor (Croteau, 1992).

Volatile oils are complex mixtures often containing more than hundred individual components. In the present investigation, there is a marked change in the minor components in the essential oil of the somaclonal variant. Most oils have one to several major components, which impart characteristic odour and taste, but many minor constituents also play their part in the final product.

The lack of production of some components after *in vitro* culture may have been due to either a loss in genetic ability or due to a repression of the

relevant genes under the culture conditions (Brown and Charlwood, 1986). The absence of some bands in the RAPD analysis in the somaclonal variant can be accounted for this reason, which in turn may be responsible for the absence of certain components in the essential oil of *in vitro* plant of *A. nilagirica.*

The production of each component of essential oil is effected by the genetical and environment factors. The production of secondary metabolites by plant cells *in vitro* is in part, dependent on the culture conditions employed. The nutritional components of various cultural media have been known to effect secondary product formation. The form in which the nitrogen is provided and the concentration supplied have effects on the products of secondary metabolites (Fujita *et al.*, 1981). Since the growth of plant cells depend on the phytochrome content of the corresponding culture media, numerous studies were carried out about the dependence of the secondary metabolism on phytochrome content. High doses of growth promoters can increase the content of secondary metabolites (Misawa, 1985). Direct evidence for the involvement of auxin in enzyme activation was reported by Hino *et al*. (1982). The switching of precursors from primary to secondary pathway is operated *in vitro via.* a change in the organization of the cells. Alternatively the growth rate itself may be the determining factor in secondary product formation by allowing diversion of precursors from primary pathway to secondary pathway (Yeoman *et al.*, 1982). Illumination also is reported to be a controlling factor in the biosynthesis and accumulation of secondary metabolites (Luckner and Diettrich, 1987).

The metabolism of monoterpenes is strongly influenced by environmental factors. It has been shown that the diurnal change in temperature is an important factor of influence regarding the oil composition (Burbott and Loomis, 1967). It is generally accepted that the definition of interspecific chemical races may

concern the presence or absence of a particular compound in the secondary metabolism (Tetenyi, 1973; Harbone and Turner, 1984).

Since there is a close connection between differentiation, developmental process and secondary metabolism in plants, the growth regulators may influence the formation of secondary metabolites (Petri *et al*., 1989). It is clearly evident that the biosynthesis of secondary plant products is controlled by the genetic factors (Franz, 1989). The genetic basis of biosynthesis of monoterpenoids and sesquiterpenoids has already been reported (Lincon *et al*., 1986).

Variation in essential oil composition may be attributed to the segregation of chimeral tissues, polyploid changes and heritable changes, which may involve individual chromosomal aberrations or single gene mutation. Chromosomal differences can cause changes in the quality and composition of essential oils (Guenther, 1949).

The marked deviation in the essential oil composition of somaclonal variant from the parent plant may be due to the genetic changes revealed by chromosomal studies and RAPD analysis.

Cytotoxic and Antitumour Assays

Carcinogenesis is a multistage process which involves initiation, promotion and progression. During initiation, the carcinogen may bind to the genetic material (DNA) and undergo DNA replication to produce an altered expression. These initiated cells remain latent unless acted upon by a promoting agent which induces tumour development by maintaining a high growth potential and keeping other factors constant. Progression is a biological phenomenon related to the sequential appearence of subpopulation of cells that differ from normal ones due to genetic instability. Such genetic imbalance in these cells is

expressed in specific phenotypic behaviour such as invasiveness, rate of growth, immortality, metastatic ability, altered karyotype, hormonal responsiveness and susceptibility to antineoplastic drugs. Thus despite the fact that most malignant tumours are monoclonal in origin, by the time they become clinically evident their constituent cells are extremely heterogenous (Nandi *et al*., 1998).

The cancer control programmes at the National Cancer Institute, USA, aim to eradicate the death and suffering from cancer, to cure cancer once it starts and ultimately to prevent cancer. Therefore, high priority is given to research promoting continuous development of sophisticated molecular technologies and clinical application of these technologies for the prevention, diagnosis and treatment of cancer (Border, 1993).

The plant kingdom is a rich source of drugs that inhibit cell proliferation. Scientists have been looking at nature and plants have been identified as a likely source for such drugs. A recent example is taxol, a diterpenoid isolated from the bark of the pacific yew tree, which has been introduced for cancer treatment. It has been found to be effective especially in breast cancer patients (Donehower and Rowinsky, 1993). Several medicinal plants are being screened for their antitumour properties in India, China, Korea, Brazil and some other countries. Roots of Ashwagandha (*Withania somnifera*), a common ingredient of many Ayurvedic preparations, have shown very promising cancer therapeutic effects in experimental tumours (Umadevi, 1996) and in a preliminary clinical study (Umadevi *et al*., 1998). China is a good example where traditional and modern medicines are being developed side by side and is assuming complementary roles in human health.

Chemotherapy is an effective treatment against cancers either singly or in

combination with surgery and or radiotherapy. In chemotherapy, drugs like cisplatin, carboplatin, cyclophosphamide, doxorubicin, melphlan, mitomycin-c, gemcitabine *etc.* have been used for the treatment of cancers (Black and Livingston, 1990a; 1990b). However, therapeutic efficacy of most of them are limited due to the development of various side effects in the host and/or the acquired drug resistance by cancer cells (Black and Livingston, 1990b; Kartalou and Essigmann, 2001).

In an attempt to abate these side effects and better remedy against various malignancies, many plant derivatives have been used with varying success (Roja and Rao, 2000).

The original proposed definition of chemoprevention (Sporn and Newton, 1979) strictly refers to the prevention of cancer by the use of pharmacological agents which inhibit or reverse the process of carcinogenesis.

Higher plants, a source of medicinal compounds, have been well known to play a dominant role in the health care of human beings (Huang Paul *et al*., 1992). More than 50% of all modern drugs in clinical use are of natural product origin (Roja and Rao, 2000; Haung Paul *et al*., 1992).

A large number of active principles with potential anticarcinogenic property have been isolated and undoubtedly many more will be added to the list (Balandrin *et al*., 1985; Gerber, 1996; Kinghorn and Balandrin, 1993; Masilungan *et al*., 1967). These components may be effective against complete carcinogens or against tumour promoters. Based on their activity, three major types of chemopreventive agents of plant origin have been identified namely, inhibitors of carcinogen formation, blocking agents and suppressing agents (Hartwell, 1976). Their activities however may vary.

The inhibitors of carcinogen formation act mainly by preventing formation of nitrosamines from secondary amines and nitrates in an acidic environment. Plants containing ascorbic acid, phenols such as caffeic acid and ferulic acid, sulphydryl compounds, proline and thioproline are involved in the prevention of mutagenic nitrosamine formation (Nandi *et al*., 1998).

Blocking agents prevent carcinogens from reaching or reacting with target sites. They may act by inhibition of cytochrome P- 450, induction of phase 11 enzymes, induction of DNA repair and by scavenging electrophiles (Block *et al.*, 1992; Correa, 1992a; 1992b; Forman and Webb, 1993; Morse and Stoner, 1993). Various components of plant origin contain chemicals which can act in the blocking of tumour initiation.

The third category of suppressing agents are antipromotion or antiprogression agents which act by inhibition of polyamine or arachidonic acid metabolism or by inhibition of protease, protein kinase C, or oncogene expression (Nandi *et al*., 1998).

No systematic study has been reported earlier on cytotoxic and antitumour activity of *A. nilagirica*. The result of the present study reveals that the methanol extracts of both *in vivo* and *in vitro* plants of *A. nilagirica* produced a concentration dependent cytotoxic effect to DLA and EAC cell lines (Tables 22 & 23; Plate 34).

Zaeoung *et al.* (2005) reported cytotoxic activity of the methanol extract of five species of Zingiberacaeae against colon adenocarcinoma and breast adenocarcinoma cell lines. They have further observed that the monoterpenes, sesquiterpenes and phenyl propanoids could be responsible for the cytotoxic activity. The cytotoxic activity of *A. nilagirica* towards DLA and EAC cell lines

seems to be attributed to the chemical composition of the plant, which may even vary depending on the environmental conditions.

The cytotoxic methanol extracts of *A. nilagirica* were further screened for their efficacy in antitumour assays. The results indicated that both the *in vivo* and *in vitro* plant extracts have considerable antitumour activity. The plant extracts could significantly inhibit the solid tumour formation induced by Daltons Lymphona Ascites tumour cells in mice. The tumour volume of control animals with 250 µg/ml gum acacia on 31st day was 2.8 cm^3, while that of 250 µg/ml of *in vivo* and *in vitro* plant extracts treated animals were only 0.138 cm^3 and 0.097 cm^3 on the same day. For 500 µg/ml gum acacia, the tumour volume of control animals was 2.6 cm^3 and that of 500 µg/ml of *in vivo* and *in vitro* drug treated animals were 0.074 cm^3 and 0.058 cm^3 respectively (Table 24; Plates 35-37). After the 19th day of inoculation, a decrease in the tumour volume was noted and this was correlated with a decrease in cell proliferation (Estrela *et al*., 1992).

Administration of the extract could enhance the survival days of Ehrlich Ascites tumour bearing animals. Control animals survived only 19 days after the tumour induction while the 250 µg/ml of *in vivo* and *in vitro* extract treated animals survived 27 and 29 days with an increase in the life span of 39% and 54% respectively. For 500 µg/ml of plant extracts, it was 31 and 33 days with an increase in the life span of 61% and 75% respectively (Plate 38; Table 25). An enhancement of life of 25% or more was considered as effective antitumour response (Gerum *et al.*, 1972).

Ascites fluid is the direct nutritional source to tumour cells and the factor needed for the faster increase of tumour cells (Prasad and Giri, 1994). The extract treatment increased the mean survival time and lowered the ascites fluid

volume to a considerable extent. Extract affects the tumour volume probably by reducing the ascites nutritional fluid volume. The number of living cells were significantly reduced in tumour-bearing mice after the administration of extract.

Sylvia *et al.* (2003) observed a positive correlation between *in vitro* cytotoxic properties and *in vivo* antitumour activities. Zhang *et al.* (2006) reported the antitumour activity of plant extract may be due to cytocidal properties. Shylesh and Padikala (2000) revealed that the methanolic extract of *Emilia sonchifolia* was found to be cytotoxic to Daltons Lymphoma, Ehrlich Ascites Carcinoma and mouse lung fibroblast cells (L_{929}) but not toxic to normal human lymphocytes under *in vitro* conditions. The administration of the extract reduced the development of solid tumours and ascites tumours and increased the life span of the tumour bearing mice.

Hence it seems probable that the cytotoxic ability of *A. nilagirica* may be due to the chemical components present in the plant extracts, which may induce apoptosis and may be responsible for the antitumour effect.

Various reports on the mechanism behind the antitumour activity of various plant extracts indicate that different plant extracts exhibited their antitumour activities through different mechanism of action in the host (Sakagami *et al*., 1987; Chaudhuri *et al*., 1998; Sllchenmyer and Von Hoff, 1991; Tanaka *et al*., 1996; Das, 2004).

In the antitumour assay of tea plant (*Camellia sinensis* var. *assamica*) root extract, the activity of superoxide dismutase, a free radical scavenger, was found to be increased in the serum of tumour bearing mice, suggesting the involvement of tea root extract in the enhancement of the defence mechanism (Chaudhuri *et al*., 1998).

The decrease in GSH level by the extract treatment seems to play a significant role in antitumour activity of the extract of *Dillenia pentagyna* against Ascites Daltons Lymphoma. GSH is a major non-protein thiol, which is involved in protection against endogenous and exogenous toxic compounds (Meister, 1985) and its role in the detoxification of chemotherapeutic agents is widely acknowledged (Arrick and Nathan, 1984). Depletion of GSH levels could potentiate the cytotoxicity of a variety of antitumour agents (Arrick and Nathan, 1984).

GSH depletion caused by the extract treatment may have role in increasing cell death by enhancing susceptibility of the cells to oxidative stress thereby increasing the host's survivability (Rosangkima and Prasad, 2004).

Resistance of many cells against oxidative stress is associated with high intracellular levels of GSH (Novarro *et al*., 1999). In fact, loss of GSH and oxidative damage has been suggested to play a role in apoptotic cell death also (Kane *et al*., 1993).

Immunosuppression is a major setback in diseases like cancer and AIDS. Use of plant products as immunomodulators is still in a developing stage. Some plant products such as *Viscum album* extract (Kuttan and Kuttan, 1992), *Withania somnifera* extract (Davis and Kuttan, 1998) and herbal preparations (Praveenkumar *et al.,* 1999) are highly suggestive remedies in immunosuppressive condition.

The immunotherapeutic effect of garlic extract on hind limb transplanted transitional cell carcinoma in mice was studied by Lau *et al.* (1986). They attributed the cytotoxic destruction of tumour cells to enhanced production of cytokines. Cisplatin has been used successfully as a potent antitumour compound

against a variety of experimental tumour in mice (Rosenberg *et al.,* 1969; Tally, 1970). It has been suggested that tumour regression by cisplatin is achieved as a result of enhancement of host's immune system (Conran and Rosenberg, 1971; Sodhi, 1972; Sodhi and Agarwal, 1974; Bahadur *et al.*, 1984).

The progressive growth of primary tumours and metastasis depend largely on adequate blood supply, the failure of which retards tumour growth. Tumour cells have mechanisms to overcome this by inducing angiogenesis. Antiangiogenic therapy is a promising diversion in cancer treatment. Identification and purification of natural products or its derivatives will be highly relevant in this regard. Studies on the role of some synthetic curcuminoid derivatives in the inhibition of tumour specific angiogenesis have been reported by Leyon and Kuttan (2003).

Programmed cell death or apoptosis, is important in the homeostatic regulation of many types of immune cell population and thus plays an important role in the regulation of immune response. It also functions in the pathogenesis of a variety of disorders including cancer and autoimmune diseases (Lindsten *et al*., 2000), prompting interest in the natural products that can modulate this process (Kuo *et al*., 2000). Many natural products have been recognized to have the ability to induce apoptosis in various tumour cells of human origin (Taraphdar *et al*., 2001). In the present investigation, the most potent antitumour activity of the methanol extracts of *A. nilagirica* may be due to the above said reasons.

Secondary metabolites of plants seem to play a significant role in cytotoxic and antitumour activity. The phytochemicals, especially terpenoids are known to decrease the risk of cancer. In nature, terpenoids are biosynthesized by random reaction of the phosphorylated isoprene unit bearing five carbons.

According to the number of combined isoprene units, they are classified into mono (C_{10}), sesqui (C_{15}), bi (C_{20}) and triterpenoids (C_{30}) and so on.

Crowell (1999) suggested that terpenoids have anticarcinogenic activities after conducting a variety of rodent experiments. Chung *et al.* (2001) reported that the crude ethanolic extract of *Glycyrrhiza uralensis* accelerate apoptosis of A_{549} cells possibly due to the chemical components in the crude extracts. It also showed the role played by the sample in limiting the initiation of carcinogenesis and results in effectively inhibiting the growth of cancer cells.

Sternberg and Duke (1996) recorded the cytotoxic activity of thujone and eugenol. Anticarcinogenic and antitumour activity of caryophyllene has also been studied (Muroi and Kubo, 1993; Zheng *et al*., 1992; Teixeira da Silva, 2004) (Table 2).

Antioxidant activity of γ-terpinene, L-camphor (Teixeira da Silva, 2004) and camphene (Sternberg and Duke, 1996) has been reported. Antitumour activity of 1, 8 - cineole (Teixeira da Silva, 2004; Sternberg and Duke, 1996), eugenol (Sternberg and Duke, 1996) and caryophyllene oxide (Zheng *et al*., 1992) has also been reported (Table 2).

Compounds like limonene and α-terpinene (Sternberg and Duke, 1996) and δ-cadinene (Muroi and Kubo, 1993) were found to have anticarcinogenic activity.

Sesquiterpene lactones are one among the most prominent natural products found in *Artemisia* species and are largely responsible for the importance of these plants in medicine and pharmacy (Mucciarelli and Maffei,

2002). Sesquiterpene lactones are natural products displaying a variety of biological activities including cytotoxicity (Rodriguez *et al*., 1976; Picman, 1986). It is likely that the sesquiterpenes are pharmacologically relevant because of its modern uses, not just as bitter stimulants, but also because, they have several well documented pharmacological effects. They are found to be cytotoxic, antibacterial, antiinflammatory and anthelmintic (Heinrich *et al*., 1997).

Cytotoxicity of terpenoids and flavonoids isolated from *A. annua* was tested *in vitro* on several human tumour cell lines and showed significant cytotoxicity (Zheng, 1994). Sylvestre *et al.* (2005) suggested the cytotoxic activity of myrcene, limonene and α- phellandrene containing extracts against human lung carcinoma cell lines. Treatment for long duration with these compounds containing extracts showed higher cell growth inhibition due to sesquiterpene enrichment.

Moteki *et al.* (2002) showed that suppression of growth by 1,8- cineole in leukaemia cell lines, results from the induction of apoptosis by this compound. Sylvia *et al.* (2003) indicated that diterpene and sesquiterpene fractions of *Copaifera multijuga* have reduced cell viability when incubated with melanoma cell lines and these fractions have tumouricidal activity against melanoma in both models *in vivo* and *in vitro*.

Hence it seems probable that the cytotoxic and antitumour activity of *A. nilagirica* extracts may be due to the specific effect of the major chemical constituents or due to the combination effect of all the chemical compounds in totality.

A slight increase in the cytotoxicity and antitumour activity of somaclonal

variant (TC3) may be due to the presence of more amounts of sesquiterpenoids (13.96 %) than that of parent (5.99 %). Moreover anticancer compounds like limonene and α-terpinene were found to be present only in the somaclonal variant. The percentage of anticancer components such as β-caryophyllene and δ-cadinene was more in somaclonal variant than in the parent plant. All these account for the slight increase in the antitumour activity of the somaclonal variant than that of the parent.

The highlights of the present investigations are as follows:

- A simple, rapid and reproducible protocol was developed for the production of a somaclonal variant of *A. nilagirica*. MS medium supplemented with growth regulator combination of BAP (0.5 mg/l - 1 mg/l) and NAA (0.5 mg/l - 1 mg/l) produced maximum multiple shoots.

- Cytological analysis reveals the ploidy level of the parent, calli and cultured plant of *A. nilagirica* to be different. The parent was found to be tetraploid (2n = 4x = 36) while that of variant was octaploid (2n = 8x = 72).

- Calli exhibit three different ploidy levels, *viz*. tetraploid (2n = 4x = 36), octaploid (2n = 8x = 72) and hexadecaploid (2n =16x = 144), with only octaploid calli showing successful plant regeneration.

- The karyomorphological features like total chromosome length, average chromosome length, range of chromosome length, number of primary constriction, disparity index, variation coefficient and total forma

percentage in the parent, calli and the variant (TC3) exhibit differences.

- RAPD fingerprint of the somaclonal variant (TC3) that differs from the parent with all the five primers (OPA 01, OPA 02, OPB 17, OPB 18 & OPC 01) indicates the genotypic variation which might have emerged due to *in vitro* culture conditions.

- GC - MS analysis of the parent and the variant (TC3) revealed quantitative and qualitative differences of the essential oil components. The coefficient of similitude was found to be 47.05. The major component (thujone) remains the same for both the oils.

- *In vitro* cytotoxic assays conducted on DLA and EAC cell lines revealed the prominent activity of the methanol extracts of the parent and variant (TC3) of *A. nilagirica*.

- The cytotoxic plant extracts showed remarkable antitumour activity against DLA induced solid tumour. There was significant reduction of tumour volume in *in vivo* and *in vitro* (TC3) plant extracts treated animals.

- The life span of ascites (EAC) tumour harbouring mice, when treated with methanol extracts of parent and variant (TC3) of *A. nilagirica*, were found to increase significantly in a dose dependent manner.

- The cytotoxic and antitumour activity of the *in vitro* (TC3) plant extract was found to be slightly more than that of the parent plant of *A. nilagirica.*

In conclusion, this study revealed a shoot regeneration method for the

production of somaclonal variant having better essential oil profiles and antitumour activity and it also reveals the cytological and molecular basis for the phytochemical variations in them. The protocol reported here could help in the large scale propagation and germplasm conservation of this valuable plant, *A. nilagirica* for continuous production of essential oils for commercial uses. The major and minor components of the essential oil may act synergistically thereby contributing to the cytotoxicity and antitumour activity. Although further studies with more cell lines and *in vivo* assays are needed to determine the efficacy of the plant extract as antitumour agent and also to clarify the mechanism of the antitumour activities of individual components of the extract, the present results suggest that methanol extract of *A. nilagirica* could be a good candidate as antitumour agent. In short, *A. nilagirica* is a promising anticancer plant having better essential oil profiles and needs more insight into the mechanism of its action.

It is needless to reiterate the fact that natural products from plants will continue to be regarded as important sources of biologically active compounds, flavourings, colourings and agrichemicals. Many of the relevant plants are yet to be fully exploited and it is reasonable to expect that even more novel and valuable compounds await discovery. It is imperative to extend the scope of research to exploit the potential of all these plants. Advances being made in analytical techniques, sophisticated bioassays and biotechnological exploitation should serve as the springboard by which these important plants continue to play a key role to the benefit of man and his environment.

It is hoped that the findings of the present investigation will be a stepping stone to open avenues to meet the current need for safe and effective anticancer drugs.

SUMMARY

Plant materials have been used in the treatment of malignant diseases for centuries. The plant kingdom is a rich source of drugs that inhibit cell proliferation. This clearly indicates the potentiality of phytochemicals to be used as anticancer therapeutic agents. Scientists have been looking at nature and plants have been identified as a likely source of such drugs. A large number of plants and plant parts have been screened for their antitumour properties. Among them, the plants that belong to the family Asteraceae play a significant role. The biological and therapeutic applications of the plants of the Asteraceae are the result of popular tradition and of systematically conducted chemical and pharmacological research. Terpenoids and certain phenolic compounds are responsible for the value of many species of Asteraceae in pharmacy and medicine.

The genus *Artemisia* is one of the largest and most widely distributed of the nearly 100 genera in the tribe Anthemidae of the Asteraceae. *Artemisia* species, widespread throughout the world are important medicinal plants, which are attracting the attention of phytochemists due to their biological and chemical diversity.

Among the different species, *Artemisia nilagirica* (C. B. Clarke) Pamp., a less exploited species was selected for the present study. Since all known *A.*

nilagirica are sterile, they can be propagated vegetatively. This has prevented the production of new cultivar by plant breeding. Assessment of genetic variability is basic to any plant breeding programme. An alternative method for creating new forms of the plant is by selecting somaclonal variants from tissue culture material. Plant tissue culture has the potential to induce genetic variability in *Artemisia* genotypes through somaclonal variants, somatic hybrids or transgenic plants. The exploitation of tissue culture technique in medicinal plants for the extraction of important chemical compounds is indeed more advantageous. Potential use of cell culture, multiple shoots and improvement of various cultivation conditions have been attempted to scale up production of secondary metabolites. A wide variety of compounds have been shown to be produced in shoot, callus or cell suspension cultures at levels equal to or higher than the levels in the intact plant sources.

The present study is an attempt to find out the effectiveness of *A. nilagirica* in the field of cancer treatment and to generate somaclonal variants of *A. nilagirica* by *in vitro* techniques, that differ from the parent plant in quality and quantity of the essential oil and to reveal the genetic basis of variation in them by using the Chromosome Image Analysis System and RAPD technique.

Micropropagation

Very little information was found in the literature, concerning the tissue culture of *A. nilagirica.* An efficient protocol was developed for the production of the somaclonal variant of *A. nilagirica*. Murashige and Skoog (MS) basal medium with different hormonal combinations of auxins and cytokinins were used for micropropagation. All the explants such as leaves, nodes and inflorescence axis, produced callus and multiple shoots in the medium

supplemented with different combinations of BAP and NAA.

Of the three explants, the leaf explants produced cream friable callus in the medium having BAP (0.5 mg/l) + NAA (0.5 mg/l). Multiple shoot initiation was noticed after 3-4 weeks in 90-100% of the callus after sub culturing it in the same medium. The nodal cutting and inflorescence axis produced multiple shoots both directly and indirectly. Multiple shoots were developed directly from nodal cuttings in 90-100% of the medium containing BAP (0.5 mg/l). Callus formation was noticed when nodal cutting was inoculated in the medium containing BAP (1 mg/l) + NAA (1 mg/l). 80-90% of multiple shoots were developed indirectly from the callus when subcultured in the same medium.

Inflorescence axis produced 80-90% multiple shoots directly in BAP (1 mg/l) medium and indirectly from callus in the medium with BAP (1 mg/l) + NAA (1 mg/l).

In vitro shoots developed roots in the medium devoid of growth hormones. However, the medium supplemented with IBA (0.5 mg/l) also developed maximum number of roots. These were later transferred to the sterilized soil and sand mixture and about 95-100% field survivals was obtained in pots.

Vigorous vegetative propagation by stem cutting was noticed in the cultured plants also. Since no remarkable morphological variation was noticed among the field-established plants, further analysis at cytological, molecular and phytochemical levels was conducted to search for the possible somaclonal variations.

Cytological Analysis

The cultured plant was analyzed for any karyomorphological changes and to detect the cytological basis of any variation, if present. The ploidy level of the parent plant, callus and the cultured plant was found to be different. The parent plant was found to be tetraploid (2n = 4x = 36), while the somaclonal variant was octaploid (2n = 8x = 72). The calli were of 3 types *viz.*, cells showing tetraploidy (2n = 4x = 36), octaploidy (2n = 8x = 72) and hexadecaploidy (2n = 16x = 144). The frequency of callus cells containing the octaploid number was more when compared to the other two types of cells observed in the callus.

The number of chromosomes with secondary constriction also showed variations. The parent plant has 4 chromosomes with secondary constriction whereas in the somaclonal variant, it was 8. Chromosomes with secondary constriction were found to be 4 in tetraploid, 8 in octaploid and 16 in hexadecaploid calli.

Changes in chromosome length, disparity index, variation coefficient and total forma percentage were also noticed.

Random Amplified Polymorphic DNA (RAPD) Analysis

DNA was isolated from the parent plant (P) and twelve tissue cultured plants designated as number TC1 to TC12 using CTAB method.

To detect the somaclonal variation at the molecular level, RAPD analysis was carried out using 15 primers of arbitrary sequences. Of the 15 primers used, only 5 successfully amplified the extracted DNA with consistent reproducible bands. The number of bands resolved per primer ranged from a minimum of one to a maximum of thirteen. RAPD fingerprints of the tissue cultured plants (TC1-

TC12) differed from parent (P) with all five primers (OPA 01, OPA 02, OPB 17, OPB 18 and OPC 01).

A few bands were found to be missing in the variants when these five primers were used. Additional bands in the variants were also detected by this marker screening. The reproducibility of the genomic DNA bands of TC3 was consistent in successive repetition than other plants. Since the amplification of TC3 plant showed more polymorphism and consistency than other plants, it was considered as somaclonal variant and used for further analysis.

Essential Oil Analysis

RAPD analysis revealed a notable deviation in the genetic make up of the *in vitro* plants. So the essential oils of the parent plant and the *in vitro* plants were analyzed quantitatively and qualitatively to search for biochemical variations in the secondary metabolism. The oil yield of the parent plant was comparatively low (1.1%). The *in vitro* (TC3) plant contained a higher quantity of oil (1.8%).

In the present investigation, the essential oil of *A. nilagirica* seems to belong to a terpenoid chemotype. GC-MS analysis revealed 29 components in the parent plant and 21 in the somaclonal variant. There was a clear difference between the compositions of the two oils tested. The percentage of monoterpenes present in the *in vivo* plant was 93.82 while that of *in vitro* (TC3) plant was 86.04. But the percentage of sesquiterpenes was higher in the *in vitro* (TC3) plant (13.96) than in the parent plant (5.99). The major component was mainly thujone in both the oils.

Sixteen essential oil components were found to be similar in both *in vivo* and *in vitro* (TC3) plants. Among them, the percentage of eight components like α-fenchene, sabinene, terpinen-4-ol, α-copaene, β-caryophyllene, germacrene-D, δ-cadinene and β-eudesmol were more in the *in vitro* (TC3) plant than *in vivo* plant.

The *in vitro* (TC3) plant was characterized by new components like ρ-cymene, dl- limonene, α-terpinene, farnesene and bicyclogermacrene.

The lesser value of coefficient of similitude (47.05) obtained when essential oils of both the parent plant and somaclonal variant (TC3) was compared, shows the dissimilar nature of these oils. This dissimilarity may be probably due to the variation in the biosynthetic pathway of essential oils which are genetically controlled.

Cytotoxicity (*In vitro* studies)

Methanol extracts of both *in vivo* and *in vitro* (TC3) plants of *A. nilagirica* produced a concentration dependent cytotoxic effect to DLA and EAC cells. *In vitro* plant extract produced 100% toxicity at a concentration of 250 µg/ml for DLA cells and 500 µg/ml for EAC cells, while that of *in vivo* plant extract caused 100% cytotoxicity only at a concentration of 500 µg/ml for both DLA and EAC cells.

Antitumour activity (*In vivo* studies)

Effect on solid tumour reduction

There was a significant ($\rho < 0.01$) reduction of tumour volume

in the *in vivo* and *in vitro* (TC3) plant extract treated animals. The tumour volume of control animals with 250 µg/ml gum acacia on 31st day was 2.8 cm^3 , while that of 250 µg/ml *in vivo* and *in vitro* (TC3) plant extracts treated animals was only 0.138 cm^3 and 0.097 cm^3 on the same day. For 500 µg/ml gum acacia, the tumour volume was 2.6 cm^3 and for 500 µg/ml *in vivo* and *in vitro* (TC3) drug treated animals was 0.074 cm^3 and 0.058 cm^3 respectively.

Effect on ascites tumour development

Life span of ascites tumour bearing mice, treated with *in vivo* and *in vitro* (TC3) plant extracts was found to be significantly increased. Control animals survived only 19 days after the tumour induction while the 250 µg/ml extract of *in vivo* and *in vitro* (TC3) treated animals survived 27 and 29 days with an increase in the life span of 39.47 % and 54.37 % respectively. For 500 µg/ml plant extracts, it was 31 and 33 days with an increase in the life span of 61.37 % and 75.42 % respectively. An enhancement of life of 25% or more was considered as an effective antitumour response.

This study revealed a shoot regeneration method for the production of somaclonal variant of *A. nilagirica*, having better essential oil profiles and antitumour activity and it also reveals the cytological and molecular basis for the phytochemical variations in them.

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