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:
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 arising 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). Polysomy 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 Wakhu, 1986).

Chennaveeraiah and Habib (1966) reported the structural rearrangements of chromosomes in cultures of *Capsicum annuum*. 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 arise 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 fingerprinting 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, gramaacrene-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.
ilagirica 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,
esquiterpenes and phenylpropanes 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 shikimate 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 O₂ 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 appearance 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, sulphhydryl 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 II 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 Zingiberaceae 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 cm³, while that of 250 µg/ml of *in vivo* and *in vitro* plant extracts treated animals were only 0.138 cm³ and 0.097 cm³ on the same day. For 500 µg/ml gum acacia, the tumour volume of control animals was 2.6 cm³ and that of 500 µg/ml of *in vivo* and *in vitro* drug treated animals were 0.074 cm³ and 0.058 cm³ 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 (L929) 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 A549 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**