Chapter 5

Discussion
DISCUSSION

Fruits and vegetables are rich source of beneficial phytochemicals that can offer enormous advantage to medicine and public health, in prevention of several diseases. The beneficial effects of these food supplements are known from the ancient period. Our Ayurveda, the ancient traditional medicinal and folklore practices in our country prescribe many of these food supplements. Chemists and biologists all over the world have focused their attention on plants that are of ethnobotanical importance. The results are very encouraging, indicating that some herbs, fruits and vegetables of our daily diet can actually reduce the incidence of certain type of cancer in human population (Block et al., 1992; Kohlmeir, 1995).

*Garcinia*, belonging to the family Clusiaceae (Syn - Guttiferae) is a large genus of polygamous trees or shrubs, distributed in tropical Asia, Africa and Polynesia. It is a relative new comer to the ranks of western herbalism, but was apparently used as a food supplement for thousands of years in the different countries of tropical Asia, Africa and Polynesia. The fruit of *Garcinia indica* (*kokam*) is used as a garnish to give an acid flavour to curries and also for preparing syrups during hot months. Dried rinds of *kokam* are widely used all over South India for culinary purposes in place of tamarind or lemon and in ‘Colombo curing’ of fish (Lewis and Neelakantan, 1965; Jena et al 2002). The organic acid present in the fruit, have been held responsible for the bacteriostatic effect of the pickling medium by lowering the pH (Sreenivasan et al., 1959).

Ayurveda and folkloric medication prescribe *Garcinia indica* for the treatment of oedema, delayed menstruation, constipation, intestinal parasites, rheumatism and bowel complaints and heart complaints (Wealth of India, 1976; Jena et al.2002). Syrup from the fruit juice is given in bilious infections (Jena et al, 2002). Mainly the fruit rind is used (Jayaprakash *et al* 2002), but leaves, roots and barks reported to have the similar medicinal properties (Xu *et al* 2001, Permana *et al* 2001). The active principle of *Garcinia indica* fruit rind is (-) erythro hydroxy citric acid [(-) HCA], which comprises about 12.5-15.1% (w/w) of the fruit rind (Jayaprakash and Sakariah, 2002). Another important constituent is a polyisoprenylated benzophenone derivative, garcinol, which comprises about 2-3% of the fruit rind (w/w) (Krishnamurthy et al., 1981, 1982). The other constituents are different biflavoniods, xanthones, benzophenones, polyrenylated depsidones and heptacyclic xanthonoids.
Recent studies have focused on the anti-HIV, anti-microbial, anti-inflammatory, anti-ulcerogenic, anti-tumor, anti-hepatotoxic, anti-obesity and anti-oxidative properties of the different species of *Garcinia*, particularly *G. mangostena*, *G. kola*, *G. atroviridis*, *G. cambogia* and *G. indica* (Grosvenor et al, 1995; Iinuma et al., 1996; Murakami et al., 1995; Mackeen et al., 2000; Permana et al., 2001; Terashima et al., 2002; Tamil Selvi et al., 2003). In view of the traditional usage, recent scientific findings, and paucity of genotoxic reports, the present study was undertaken to evaluate the genotoxic potential of the fruit rind-extract of *Garcinia indica*.

The *Garcinia indica* fruit rind extract (GE) was prepared according to the method of Harborne (1998). All experiments were performed after dissolving the lyophilized powder in double distilled water. The investigation was initially concentrated on the areas related to the genotoxicity viz. clastogenicity, mutagenicity, cytotoxicity. Based on the findings further studies on the antigenotoxicity and antimutagenicity was carried with reference to the role of its antioxidant properties.

Genotoxicity:

To evaluate the genotoxic potential, a preliminary screening of GE was performed using *Allium* assay. *Allium* assay can be utilized to find clastogenicity, through anaphase-telophase aberrant cells, micronuclei in interphase cells, and cytotoxicity by observing the divisional frequency. The *Allium* bulbs were exposed to the different concentrations of GE and the root tips were processed after 3 hours. In another set, following 3 hours exposure, the cells were allowed to grow in absence of the test compound for two successive cell cycles. The number of anaphase-telophase aberrant cells gave a significant dose dependent response (table-2b). There are a few metaphase cells with chromosome breaks. The number of binucleated cells were significantly higher than that of the negative control. Analysis of variance test confirms that this increase was significant for all the exposure periods. The number of cells with micronuclei at interphase cells were few and could be correlated with that of the low number of metaphase cells with chromosome breaks. There was a good correlation between the incidence of binucleated cells and the number of anaphase- telophase aberrations.

The frequency of dividing cells decreased following 3 hours treatment with GE and there was a dose dependent response. In absence of the test compound, the number of the dividing cells was more or less same after one/two cell cycles. Analysis of variance test was carried to observe if any significant variation in the number of dividing cells among the treatment groups.
The result shows that except for the 3 hours period of treatment, there was no significant (p<0.05) differences in the recovery sets. Reports on the genotoxicity of *Garcinia* on plant test systems are scarce. Earlier publications from this laboratory have shown that *Allium* assay system provides a valuable system for genetic toxicology testing of unknown compound (Roy Choudhury and Giri, 1989). They have been by far the assay systems of choice in developing countries throughout the world (Reddy et al., 1995). Of the end-points, the micronucleus and anaphase- telophase aberrations are the simplest, rapid and highly validated by different agencies (Ennever et al., 1986; Gichner and Plewa, 1998; Panda and Panda 2002). The results of the present investigation are in line with those reported for other weak clastogens.

The initial screening on *Allium* suggests that GE may be clastogenic, but not cytotoxic. Preliminary toxicity tests with the crude extract of *Garcinia indica* were carried out according to the guidelines of the report from the working group to conduct in vivo mammalian bone marrow chromosomal test (Tice et al, 1994). The highest dose administered here in mice (i.e. 240mg/kg b. wt.) is half of the LD50 dose of 480mg/kg b. wt. Progressive dilutions of this dose were included to determine the minimum effective dose capable of inducing significant number of chromosomal aberrations, sister chromatid exchanges and DNA damage. The volume of the test compounds to be administered per test animal for oral and intraperitoneal administrations was 10 ml /kg b. wt. (Tice et al, 1994). After 6 hours and 18 hours treatment, the animals were routinely processed. The slides were scored for chromosomal and chromatid breaks and chromosomal rearrangements. The percentage of aberrant (damaged) cells and chromosomal aberration per cell were calculated. Two sampling times of 6, and 18 h following a single treatment was used for evaluating the ability of *Garcinia* to induce chromosomal aberrations in bone marrow cells of treated mice. The rationale for these sampling times is that: (1) it was necessary to sample cells at their first mitosis after treatment for the most accurate measure of induced aberration frequency to be made; (2) the selected sampling time was optimum to obtain cells in different stages of the cell cycle. 6 h sampling time provide data on first division cells treated in the S phase and 18 h sample includes first division cells, in the absence of substantial cell cycle delays. The aberrations were mainly of the chromatid type (Table 6A and 6B). The number of aberrations per cell and the frequency of damaged cells (DC) gave a positive dose related increase (p ≤ 0.05). The dose 240 and 120 mg of *Garcinia indica* extract/kg b. wt. was the minimum effective concentrations to induce a significant number of chromosomal aberrations at 6 and 18 h respectively. A high frequency of cells with multiple aberrations were recorded in the
set of animals treated with mitomycin C, whereas in the sets exposed to *Garcinia indica* in each aberrant cell, a single type of aberration was recorded.

*Garcinia* could be classified as positive for clastogenic activity since the frequency of aberrant metaphase cells was significantly increased in more than one dose group and also the increase was dose dependent. The clastogenic activity was however, recorded at the doses much higher than that recommended for human consumption.

The results of the present investigation revealed that *Garcinia per se* could directly act on the DNA and induce genotoxic effects. Since these endpoints have different sensitivities of detection and are induced by both common and unique mechanisms, a positive response for any single end-point would indicate a potential for the *Garcinia* -extract to induce genotoxic damage *in vivo*. There was a significant dose- dependent enhancement of chromosomal aberrations, aberrant cells and SCEs. The minimum effective concentration to induce significant number of CAs and SCEs was 120 and 30 mg/kg of b. wt., respectively. The increased occurrence of chromosomal aberrations, concomitant with an increase in SCE frequency in bone marrow cells is consistent with the induction of DNA damage by an S-phase dependent clastogen (Latt et al, 1983). In addition to examining SCEs, the BrdU-differential staining technique was utilized to assess the effect of *Garcinia* on cell replication. Cells that have replicated once, twice or thrice or more times in the presence of BrdU can be unequivocally identified (Schneider et al, 1977). For the analysis of cell proliferation kinetics data, the proportion of cells among the first, second, and third generations in each bone marrow sample were transformed into an average generation time AGT (Ivett and Tice, 1982).

AGT measures alterations in cellular proliferation kinetics and reflect cytotoxic effects induced either by genotoxic damage or by an independent mechanism(s) (Tice and Ivett, 1985). Increases in the AGT are correlated with the inhibition of bone marrow cell kinetics. If replication were inhibited, one would find a decrease in the frequency of second-replication cycle cells and an increase in the frequency of first-replication cycle cells. In the *Garcinia*- treated animals, the proportion of proliferating cells in the bone marrow of treated animals implanted with BrdU tablets and the rate (AGT-Average generation time) at which these cells divide were not significantly depressed. Mitomycin C (MMC), led to a pronounced increase in the SCE frequencies without affecting the proliferation of cells. The AGT values were statistically non-significant indicating that MMC was not cytotoxic to the bone marrow cells at the dose tested. This could be supported by the results of sister chromatid exchange assay in bone marrow and spleen cells of CD-1 male mice (Krishna et al, 1986).
Thus *Garcinia indica* per se was not cytotoxic to the bone marrow cells under *in vivo* conditions. A similar negative trend of non-cytotoxicity was reported by Mackeen et al., (2000). Crude extracts (methanol) of various parts, viz. the leaves, fruits, roots, stem and trunk bark, of *G. atroviridis* were tested in CEM-SS (human T-lymphoblastoid), Raji (human B-lymphoblastoid) cell lines and in brine shrimp (*Artemia salina* Leach) assays. Except the root extract, the other extracts were not cytotoxic towards the Raji cells and none of the extracts exhibited cytotoxicity towards the CEM-SS cell line or against the brine shrimp. On the other hand, crude methanolic extract (CME) from the pericarp of *Garcinia mangostena*, showed a dose-dependent inhibition of cell proliferation, associated with apoptosis on human breast cancer (SKBR3) cell line as determined by the morphological changes and oligonucleosomal DNA fragments using human breast cancer (SKBR3) cell line (Moongkamdi et al, 2004).

Similar *in vitro* studies were conducted on cell viability and apoptosis in different human cancer cell lines like KB, HL60, U937, K562, and NB4. Cytotoxicity was observed in the cell lines by garcinol isolated from *G. indica* (Pan et al, 2001) *G. bracteata* (Thoison et al, 2000) or *G. purpurea* (Matsumoto et al, 2003) where as Tanaka et al, (2000) found that garcinol from *G.indica* did not cause any cytotoxicity on HL 60 cells at a concentration of 10uM. At this time, there is no apparent explanation for the inconsistency in the results of cytotoxicity. This phenomenon may be related to the selection of cells, differences in cell cycle length, and/or of repair mechanisms in cells. Interestingly these compounds appeared to exert a negligible toxic effect on normal human lymphocytes (Matsumoto et al, 2003). In conclusion, we suggest that the endpoints screened were sensitive indicators of the genotoxic potential of *Garcinia* at doses much higher than used for human consumption.

It was also important to find out, whether GE is mutagenic or not. The bacterial reverse mutation assay was carried using different amino acid requiring strains of *Salmonella typhimurium* and *Escherichia coli*. These bacterial strains are already mutated at an easily detectable locus, when treated with graded doses; a second mutation is occurred to suppress the effect of the existing mutation. The tester strains used were *Salmonella typhimurium* TA 97a, TA 98, TA 100 and TA102 and *Escherichia coli* WP2uvrA (pKM101). Each and every strain facilitates a particular mutation pattern-TA 97a and TA98 frameshift and TA100. TA102 of *Salmonella typhimurium* and WP2uvrA (pKM101) of *Escherichia coli* base-pair substitution. The mutagenic response was considered positive only when the number of revertant colonies was increased by at least a factor of one and half or two over the control. None of the strains displayed any intrinsic mutagenic activity. GE
with or without metabolic activation was found neither to induce frameshift mutation and nor cause base-pair substitution mutations at a significant level.

**Anti-genotoxicity:**

An increasing number of dietary compounds have been shown to have significant chemopreventive actions. Consumption of cruciferous vegetables, vegetables rich in betacarotene content and those rich in anti-oxidative principles are known to be associated with a decreased incidence of human cancers. *Garcinia* is reported to have antioxidative properties. The purpose of the present study was to determine whether and to what extent *Garcinia* would provide a broad spectrum of protection against standard clastogens/ mutagens when administered to mice intraperitoneally or in bacterial mutagenicity assay. The anti-mutagenicity assays in preincubation tests with *Garcinia* extract, using the tester strains TA97a, TA98, TA100 and TA 102 of *Salmonella typhimurium* and WP2uvrA (pKM101) of *Escherichia coli*, with and without S9 metabolic activation manifested significant anti-mutagenic property of *Garcinia*. There are a large number of reports on the antimutagenic effects of natural plant products (Gupta et al, 2002).

Antigenotoxicity tests were carried on Swiss albino male mice. Mice bone marrow cells were chosen as indicator cells for their high sensitivity to the clastogens. Based on our preliminary studies, the minimal effective concentration (MEC) of *Garcinia* to induce chromosomal damage was detected. To study the modulatory activity of *Garcinia* on the two known mutagens, cyclophosphamide (CP) and mitomycin-C (MMC), the animals were primed for 9 successive days with half and one-fourth dose of the MEC. On the 9th day, mutagens were injected intraperitoneally. Animals were killed and slides were prepared from the bone marrow cells. The data on the induction of chromosomal aberrations in the bone marrow cells of mice following exposure to GE, both singly and in combination with cyclophosphamide (CP) and mitomycin-C (MMC) were documented. Treatment with different doses of GE alone induces a weak clastogenic effect and the number of chromosomal aberrations is not more than one per cell. A single administration of CP or MMC gave significant higher percentage of damaged cells and per cell chromosomal aberrations than that of the vehicle control. Chromatid type of aberrations, were pre-dominant in all the experimental sets. Both CP and MMC gave more than one chromosomal damages per cell.

The fruit rind extract of *Garcinia indica* at the doses 30 and 60 mg 10^(-2) lowered the clastogenic effects of the two mutagens. The priming of animals with different doses of GE
significantly antagonizes the CP-/MMC- induced clastogenicity. It may be confirmed that, GE was found to have anti-genotoxic activities. The modulation of cellular responses to the genotoxic damage of the known mutagens/clastogens can be attributed to the bio-activation of P450 enzymes, or to the detoxification of reactive species, or increased repair of damaged DNA by the active principles present in the extract of *Garcinia*. Literature survey reveals a large number of publications on the antioxidant property of the different genus of *Garcinia* (Aruoma et al., 1990; Farombi 2002b; Farombi, 2003; Terashima et al., 2002; Murakami et al., 1995; Mackeen et al., 2000; Grosvenor et al., 1995b; Mackeen, 1995; Murakami et al., 1995; Mackeen et al., 1997a, b).

**Anti-oxidant**

The antioxidant properties of GE were determined by chemiluminescence assay. The superoxide molecules are generated by the action of different enzymatic oxidation reactions. An anti-oxidant scavenges these free radicals. *Garcinia* extract exhibited high antioxidative activities. This could be attributed to the presence of garcinol, which is known for its free radical scavenging property. Since the molecule has phenolic hydroxyl groups and a \( \beta \)-ketone structure.

Reactive oxygen species (ROS) have been shown to play a critical role in many diseases such as cancer (Muramatsu et al., 1995), arteriosclerosis (Steinberg et al., 1989), gastric ulcer (Das et al., 1997), and other conditions (Oliver et al., 1987; Babizhayev and Costa, 1994; Busciglio and Yankner, 1996; Brown et al., 1996; Smith et al., 1996). Cellular anti-oxidants may not be sufficient to counter all the hazards of today. Therefore, there is a need to add additional anti-oxidants from outside. The intake of antioxidants such as polyphenols in tea and red wine has been seen as very attractive in the prevention of these diseases (Vinson et al., 1995; Teissedre et al., 1996; Leanderson et al., 1997; Wiseman et al., 1997; Lotito and Fraga, 1998; Cohly et al., 1998; Cao et al., 1997). Epidemiological studies suggested that the intake of polyphenols from red wine in particular reduces the risk of cardiovascular disease and arteriosclerosis (Renaud et al., 1992). The ROS scavenging activity of grape seed extract and its pharmaceutical properties has been reported earlier (Yamaguchi et al., 1999; Saito et al., 1998; Yamakoshi et al., 1998; Arii et al., 1998). Grape seed extract is a water-soluble ROS scavenger. Different species of *Garcinia* has shown the free radical scavenging activities. Among them, garcinol, a yellow pigment (Krishnamurthy et al., 1987), one of the main constituent isolated from the species *G. indica*, has the property of free radical scavenging is most important one (Yamaguchi et al, 2000). Mangostin, isolated from *G. indica*...
mangostana, was found to inhibit the oxidation of low-density lipoprotein and thus performing as an antioxidant (Mahausarakam et al., 2000; Williams et al., 1995). Crude methanolic extract of the fruit rind of mangosteen, significantly decrease intracellular reactive oxygen species (ROS) production on SKBR3 cells in dose-and time-dependent manner (Moongkarndi et al., 2004). Kolaviron, isolated from G. kola, has found to involve in the scavenging of reactive oxygen species and thus performing as an antioxidant (Aruoma et al., 1990) in a dose dependent manner (Farombi 2002b). Kolaviron was found to inhibit the peroxidation of rat liver microsomes in a linear manner with the increase of doses. Recent studies have demonstrated the ability of kolaviron to inhibit intracellular ROS induced by H2O2 in HepG 2 cells detected as 2, 7-dichlorodihydrofluoroscein diacetate (DCF) fluorescence (Nwankwo et al., 2000). Thus, the ability of kolaviron to act as antioxidant in this cell indicates its potential role in the chemoprevention of chemically-induced genotoxicity (Firombi, 2003). Another constituent garcinoic acid was found to report as a powerful antioxidative agent (Terashima et al., 2002). The extract of the trunk bark of G. atroviridis was found to exhibit a strong anti-oxidant activity (Mackeen et al. 2000).

Garcinia extract exhibited antioxidative activity when evaluated through chemiluminescent study. This could be attributed to the presence of garcinol. Garcinol is known for its free radical scavenging activity, since the molecule has phenolic-hydroxyl groups and a β-ketone structure (Yamaguchi et al.2000; Terashima et al, 2002).

Garcinol, one of the major constituent of Garcinia indica might exhibit anti-oxidative activity because the molecule has phenolic hydroxyl groups and a β-diketone structure, which is expected to contribute such property. The anti-oxidative activity of garcinol was reported in an emulsified system by Yamaguchi et al (2000) with its free radical scavenging property by Das et al., (1997, 1998). Garcinol can suppress the signal of the superoxide anion: DMPO (5, 5-Dimethyl-1-pyrroline-N-oxide) in a dose dependent manner, in the hypoxanthine/xanthine oxidase system. It may act as an iron chelator, suppress the formation of hydroxyl radical in the system and also inhibit by Fenton reaction (Yamaguchi et al., 2000). Garcinol was found to suppress the formation of all kinds of radicals such as methyl radical, hydroxyl radical and superoxide anion and can suppress hydroxyl radical in the non-Fenton type ROS generating system (Yoshimura et al., 1999).

The antioxidative property of Garcinia indica per se was investigated and the findings are in line with those reported by other workers.
Therefore, it may be concluded that, *Garcinia indica* fruit rind extract can induce a weak cytotoxicity and mitotoxicity and insignificant chromotoxicity. It causes weak dose dependent clastogenicity but per cell damage never more than one. GE does not show any mutagenic effect, but causing significant DNA damage at 0.1% and 1% level but not in 5% level. As we know, 5% level is the base line for biological studies. GE was found to be responsible for weak dose dependent SCE formations, causes insignificant changes in AGT and significantly lowering the effects of 2 mutagens CP and MMC, in number of damaged cells and per cell aberrations. And lastly, GE exhibits a powerful antioxidant property.