DISCUSSION

The use of natural products as anticancer agents has a long history that began with folk medicine and has been incorporated into the traditional and allopathic medicine. Several drugs currently used in chemotherapy were isolated from plant species or derived form a natural prototype. Most of the research performed today focuses on the development of new drugs for the treatment of cancer, or viral and microbial infections. Cragg and Newman (2000) reported that more than 50% of the drugs in clinical trials for anticancer therapy were isolated from natural sources.

5.1 Short term in vitro cytotoxicity screening of medicinal plants.

Ten medicinal plants belonging to different families were selected for the cytotoxic screening using EAC and DLA cell lines by 3 hrs culture and trypan blue dye exclusion. The plants were selected based on their use in traditional medicine and ethnobotanical literature. The aqueous extracts of these plants were used for the screening and all the plant extracts were used uniformly at the rate of 20mg/ml. Among the 10 plants selected the aqueous extract of the bark of Cassia fistula L. was found to have maximum cytotoxicity in both DLA and EAC cells (95% and 92% respectively). Since cytotoxic screening models provide important preliminary data to select plant extracts with potential antineoplastic properties (Jovita et al.; 1998), Cassia fistula L. was selected for further studies.
5.2. **Short term in vitro cytotoxicity screening of different organic extracts of the bark of *Cassia fistula* L.**

Different organic extracts of the dried and powdered bark of *Cassia fistula* L. was prepared by extracting the powder with different organic solvents with increasing polarity. Dried extracts were dissolved in 0.01% DMSO and used for testing cytotoxicity against EAC, DLA and normal murine lymphocytes. The maximum cytotoxicity was demonstrated by methanol extract in all the test concentrations namely 2.5mg/ml, 5mg/ml, 10mg/ml, 15mg/ml and 20mg/ml. But at concentrations of 15mg/ml and above the normal murine lymphocytes shows cell death with IC\textsubscript{50} value of 15mg/ml. The IC\textsubscript{50} value for murine cancer cell lines DLA and EAC were 2.5mg/ml. The concentrations 2.5mg/ml, 5mg/ml and 10mg/ml could be used without appreciable effect on normal cells. So these concentrations were further selected for in vivo studies using murine cancer models. It was also reported previously that the methanol extract of *Cassia fistula* L. seed had antitumour properties (Gupta *et al.*; 2000).

5.3 **Cytotoxic screening of MEC by MTT assay**

As the next part of the study a panel of cultured human cancer cell lines was used in order to find out the cytotoxic potential of MEC of bark of *C. fistula* in a time and dose dependent manner for an extended period of time. The 4 doses of MEC selected were 37.5, 75, 150 and 300ug/ml, which was, tested for 2 time periods namely 24hrs and 48hrs. The cell lines used were MCF7, HCT116, SW480, IMR32, and HeLa. The IC\textsubscript{50} values of MEC for the cell lines MCF7 (breast carcinoma), and IMR 32 (brain cancer) were around 150mg/ml and 75mg/ml for 24 and 48hrs of incubation respectively. But for the cell lines HeLa (cervical carcinoma), HCT116 (colon cancer) and SW480 (colon carcinoma), IC\textsubscript{50} values were around 300mg/ml and 150mg/ml for 24hrs and 48hrs

In a continuing search for naturally occurring antineoplastic agents from higher plants, cytotoxic activities against human cancer cell lines could be considered as a reliable source of information. Due to the limitations of common methods for determining cell viability namely trypan blue exclusion, incorporation of radioactive nucleotides and clonogenic assays, there was a need for the development of a colorimetric assay, which would have an upper hand over the earlier assay methods. In one of the earliest efforts to develop a practical in vitro drug sensitivity assay, Black and Speer (1953) utilized a tetrazolium/formazan method to assess inhibition of dehydrogenase activity by cancer chemotherapeutic drugs of excised tissue. As an in situ vital staining process, this phenomenon has been used for identifying viable colonies of mammalian cells in soft agar culture (Schaeffer and Friend 1976), and for facilitating in vitro drug sensitivity assays with human tumour cell populations in primary culture (Alley and Lieber, 1984). Since then tetrazolium reagents have become popular as convenient non-radioactive alternatives for determining the number of viable cells in proliferation and cytotoxic assays. Moreover tetrazolium assays can be semi automated with the use of 96 microtitre plates to provide easy and rapid analysis of large number of samples (Finlay et al.; 1984). Based on such a tetrazolium assay the MEC was found to possess appreciable cytotoxicity against different cell lines. Since it is well known that different cell lines might exhibit different sensitivities to a cytotoxic
compound, the use of more than one cell line, is therefore considered necessary in the detection of cytotoxic compounds (Kamaljit et al.; 2005). Here a panel of 5 human cancer cell lines with different origins; morphology and tumourigenicity were selected and used for MTT assay. The results were summarised in Fig. 4.3.1 to Fig 4.3.5 which shows that MEC exhibited a dose and time dependent inhibitory effect on all the human cancer cells examined with varying degree of effect on the different cell lines i.e. the methanolic extract showed maximum cytotoxicity against MCF7 and IMR 32. $IC_{50}$ values of these cell lines were around 75ug/ml for 48hrs of incubation. But for the cell lines HeLa, HCT 116 and SW 480 the $IC_{50}$ value was around 150ug/ml for 48 hrs of incubation. It is clear from this that MEC shows varying activity towards different cell lines.

5.4 Antitumour effect of methanol extract of *Cassia fistula* L. in Balb/c mice

5.4.1 Ascites tumour studies

The different parameters studied in this experiment were, increase in lifespan, percentage of viable cells in the ascites fluid, and increase in the body weight of the animals, for both EAC and DLA ascites tumour bearing animals. It was found that there is a significant increase in lifespan of MEC treated animals in all the three concentrations namely 50, 100 &200mg/kg body wt. as compared to untreated control, animals bearing the same ascites tumours (Table 4.4.1.1).

The reliable criterion for judging the value of any anticancer drug is the prolongation of life span of animals (Clarkson and Burchenal, 1965). The results obtained here clearly demonstrate the antitumour effects of MEC against EAC and DLA, as evidenced from increase in the survival potential of the animals.
Table 4:4:1:2 shows that there was a significant decrease in the viable tumour cell count in the ascites tumour bearing animals, treated with MEC at the rate of 200mg/kg/body weight as compared to control animals. This was found out by aspirating the ascites fluid on the 14th day of tumour transplantation and estimating the viability by trypan blue dye exclusion test. This shows that the MEC is cytotoxic and antiproliferative towards tumour cell lines in *in vivo* condition. The *in vitro* cytotoxicity assay performed earlier had also showed the same results (4.1., 4.2, 4.3).

Table 4.4.1.3 shows the increase in body weight of EAC and DLA tumour bearing animals. There was a significant decrease in body weight in the MEC treated animals as compared to control animals, except in the case of EAC tumour bearing animals receiving MEC at 50mg/kg body weight *i.p.* The increase in body weight of tumour bearing animals may be due to accumulation of peritoneal fluid as an abnormal enlargement of peritoneal cavity. Treatment with MEC reduces body weight of ascites bearing animals in comparison to control animals. This shows that the MEC prevents the proliferation of tumour cells in the peritoneal cavity and thereby reduces peritoneal fluid accumulation and as a result increase in body weight is restricted. A reliable criterion for judging the value of any anticancer agents is the prolongation of life span of animals (Hogland, 1982). A decrease in tumour volume as evidenced by reduced body weight and viable cell count as mentioned above finally reduces the tumour burden and enhances the lifespan of EAC and DLA bearing mice. Other medicinal plants like *Abrus precatorius* (Ramnath *et al.;* 2002), *Tinospora cordifolia* (Leyon and Kuttan, 2004), *Withania somnifera* (Christina *et al.;* 2004), *Indigofera asphalthoides* (Rajkapoor *et al.;* 2004), and *Piper longum* (Sunila and Kuttan, 2004) also reported to have antitumour activity.
5.4.2 DLA and EAC solid tumour studies

Here solid tumours were induced on the right hind leg of Balb/c mice by implanting $5 \times 10^5$ DLA and EAC cells. The tumour bearing animals were injected with MEC at doses 50, 100, and 200mg/kg body wt. at alternate days for 14 days either i.p or i.l. On 14th day of tumour implantation mice were sacrificed and results of the parameters studied are discussed below.

Table 4.4.2.1 and Table 4.4.2.2 show the tumour volumes of control and treated DLA and EAC tumour bearing animals respectively. In the case of DLA solid tumour bearing animals irrespective of the mode of administration i.e. i.p or i.l solid tumour volumes were reduced significantly in MEC treated groups in all three concentrations namely 50, 100 and 200mg/kg/ body weight. But in the case of EAC tumour solid bearing animals, the mode of administration was found to have a significant effect in the reduction of solid tumours. The treated animals, which received the MEC intralesionally, showed a significant reduction in tumour volume when compared to control animals in a dose dependent manner. But the animals, which received the MEC intraperitonially, did not show any significant reduction in tumour volume in comparison to control animals. This shows that DLA cell line is more sensitive to MEC than EAC cell line. In the case of DLA cell line irrespective of mode of administration there was significant reduction in tumour volume. But in the case of EAC cell line intralesional administration was found to be more effective. Since EAC cell line is more resistance to MEC, the direct application of the extract is found to be more effective than the introduction of the extract through i.p mode. It is suspected that in i.p mode the drug losses some of its activity due to metabolic conversion. But in the case of DLA cell line, since it is more sensitive to MEC,
unmodified MEC as well as metabolically converted MEC was equally effective in reducing tumour volume.

Table 4.4.2.3 and Table 4.4.2.4 show the spleen weights of normal control and MEC treated animals bearing DLA and EAC solid tumours respectively. Control animals showed a significant increase in spleen weight as compared to normal. But DLA tumour bearing animals receiving MEC in either intraperitonially or intralesionally did not show any significant increase in spleen weight as compared to normal animals. Similarly in the case of EAC solid tumour bearing mice, the untreated control animals and all the groups receiving the MEC intraperitonially a significant increase in spleen weight was observed. During tumourigenesis stress of tumour burden usually leads to spleenomegaly (Rivenson et al.; 1981). Boyd (1988) reported that many of the pathological conditions in acute cases, there do occur splenic swelling. So increase in spleen weight in the case of control as well as certain MEC treated groups may be indicative of tumour burden. Since spleen weight of the entire DLA and EAC solid tumour bearing animals receiving MEC treatment (intralesionally) showed a marked reduction, which may be indicative of a reversal of tumour condition. A reduction in spleen weight is accompanied by a reduction in tumour volume as well. Therefore, one can assume that MEC has appreciable tumour reducing property, especially i.l mode of MEC administration is found to be superior to i.p mode.

Table 4.4.2.5 and 4.4.2.6 shows the total Hb content in normal, control and MEC treated animals. In MEC treated animals bearing DLA and EAC solid tumours and receiving MEC i.l mode there was no significant difference in Hb content as compared to normal animals. However, animals bearing EAC tumours and receiving MEC through i.p route had a significant decrease in Hb content as compared to normal
animals. Untreated control animals also show a decrease in Hb content in comparison to normal.

Table 4.4.2.7 and 4.4.2.8 shows the total RBC count of normal, control and MEC treated animals. In DLA tumour bearing animals treated with MEC, the RBC count was on par with normal values. But control animals were showing a significant reduction in total RBC. But in the case of animals bearing EAC solid tumours, only the animals receiving MEC intralesionally had their RBC count on par with normal value. All other MEC treated groups receiving MEC in i.p mode as well as control had a significant reduction in RBC count in comparison to normal. This proves that the MEC is more effective against DLA than EAC solid tumours.

Table 4.4.2.9 and table 4.4.2.10 shows the total WBC count of normal, control and treated animals bearing DLA and EAC solid tumours respectively. In the case of DLA solid tumour bearing animals, control animals showed a significant increase in WBC counts as compared to normal. But in the case of animals receiving the MEC there was no significant enhancement in WBC count in comparison to normal animals. Animals bearing EAC solid tumour showed a different trend. The difference was that the animals receiving the highest dose of MEC intraperitonially i.e. 200mg/kg body wt. did not show significant increase in WBC count. Similarly the treated animals receiving MEC intralesionally did not show a significant increase in WBC counts as compared to normal animals. The increase in WBC count observed in control animals as well as in some treated groups might be due to the induction of tumour. The treated groups, which received the drug intralesionally, generally showed a significant decrease in tumour burden and decrease in tumour burden is found to be associated with general improvement in haematological parameters. Reliable criteria for judging the value of any
anticancer drug are prolongation of life span and decreasing WBC counts from blood (Oberling and Guerin 1954, Brown, 1980). Since MEC treatment when done i.l was able to reduce significantly the WBC count and made the WBC count on par with normal is suggestive of the fact that MEC has appreciable tumour reducing properties.

Table 4.4.2.11 and Table 4.4.2.12 show the differential count of WBC in normal, control and MEC treated groups bearing DLA and EAC solid tumours respectively. The tumour bearing control mice showed a shift from lymphocytosis, as observed in normal mice to neutrophilia (Gupta et al.; 2000, Rajkapoor et al.; 2004). Thus a shift from lymphoid-myeloid ratios from 3:1 in normal mice was noted in tumour controls. But in the case of MEC treated groups bearing DLA tumour the lymphoid myeloid ratio was 57-61: 25-28 which was on par with normal ratios. But in the case of animals receiving MEC intraperitonially in the case of EAC tumour bearing animals there was significant difference in lymphoid myeloid ratios from normal. The percentage of monocytes did not show any significant difference in the case of control as well as MEC treated groups of both DLA and EAC tumour bearing animals. Roy et al.; (1981) pointed out that the progress of tumourigenesis has been accompanied by (1) gradual decrease in haemoglobin count, erythrocyte counts, and bone marrow cellularity (2) gradual increase in leukocytes, thrombocytes, and spleenic cellularity (3) reversal of the lymphoid myeloid ratio in the differential WBC count.

DLA and EAC solid tumour studies have demonstrated that in tumour bearing animals, there was significant reduction in Hb, and RBC. This was on par with normal values in case of animals receiving the MEC. Similarly there was increase in WBC counts and shift in lymphoid-myeloid ratio in tumour controls, which was made on par with mean normal values.
by MEC in treated animals. It was also found that the improvement of haematological parameters was always accompanied with a reduction in tumour burden. This means that the MEC of *C. fistula* L. does not have any appreciable side effects on haematopoetic system but on the contrary, it protects the haematopoetic system. But at the same time it has significant tumour reducing properties. In cancer chemotherapy, the major problems are myelosuppression and anaemia (Price and Greenfield, 1958, Maseki *et al.*; 1981). The anaemia encountered in tumour bearing mice is mainly due to reduction in RBC or haemoglobin percentage and this may occur either due to iron deficiency or due to haemolytic or myelopathic conditions (Fenninger and Mider, 1954). So it was demonstrated that MEC could reduce tumour burden without causing any side effects. In short MEC reduces solid tumour volumes, spleen weight, WBC counts, and neutrophil counts and make Hb, RBC, and lymphocyte counts comparable to normal in all treated concentrations and in both modes of administration in the case of DLA solid tumour bearing animals. Also many other authors have reported that antitumourals isolated from plants have remarkable ability in making the altered haematological parameters in the tumour bearing animals back to normal (Gupta *et al.*; 2000, Jose *et al.*; 2002, Rajkapoor *et al.*; 2004, Gupta *et al.*; 2004).

The mode of administration of the drug is another factor, which determines its activity. When the MEC had been administered *i. i.* it showed maximum activity as compared to *i. p.* treatment in the case of EAC tumour bearing animals. Similarly MEC was found to be more effective against DLA solid tumours than EAC tumours. This shows that MEC has differential sensitivity towards different tumours. The MEC concentration 100 and 200mg/kg body weight were also comparable.
5.4.3 Evaluation of hepatotoxicity of MEC

In order to evaluate the drug induced hepatotoxicity and nephrotoxicity, the levels of SAKP, SGPT and SGOT were evaluated. Serum levels of these enzymes were found to be significantly elevated in the untreated control mice as well as in the mice treated with MEC through *i.p* route, bearing both DLA and EAC solid tumours. But in the case of treated animals receiving MEC through *i.l* route there was no change in serum enzymes levels as compared to normal mice. It was also found that reduction in tumour volume was accompanied by significant reduction in serum enzyme levels as compared to control animals. So there is every reason to think that tumour reduction has a significant influence on serum levels of SGPT, SGOT and SAKP. This may be due to the reduction in the toxic substances that are formed in the host with increase in tumour stress (Manju Gbosh *et al.*; 1999).

Anticancer drugs such as aminoglutamide, azathioprin, cyclophosphamide, and methotrexate cause hepatotoxicity, and some drugs including cisplatin and cyclosporine produce nephrotoxicity (Menard *et al.*; 1980, Blackley and Hill, 1981, Sherlock, 1986, Kintzel, 2001, Leaker and Cairns, 2001). Transaminases (SGPT and SGOT) and alkaline phosphatase were good indices of liver and kidney damage, respectively (Martin *et al.*; 1981). Since MEC brings about lowering of the serum levels of SGPT, SGOT and SAKP in the animals receiving drug through *i.l* route, it can be surmised that the drug did not cause any kidney or liver damage, when injected directly.

5.4.4 Antioxidant potential of MEC

Cancer is always associated with various changes in the functions of tissue. So analysis of antioxidant enzymes and lipid peroxidation is of importance in detecting the effectiveness of a drug. Here the parameters
analyzed were tissue antioxidants like SOD, GSH, GR and lipid peroxidation by TBARS in the serum of DLA and EAC solid tumour bearing mice. Reactive Oxygen Species (ROS) have been suggested to be involved in mutagenesis and carcinogenesis. ROMs can be produced by both endogenous and exogenous sources. Potential endogenous sources include oxidative phosphorylation, P 450 metabolism, peroxisomes and inflammatory cell activation. Exogenous sources include environmental agents (nongenotoxic carcinogens), chlorinated compounds, radiation, metal ions, barbiturates etc (Klaunig and Kamendulis, 2004)

Free radicals and other oxygen derivatives are constantly generated in vivo both by ‘accident of chemistry’ and for specific metabolic purposes. The reactivity of different free radicals vary with, many causing inflammation or even severe damage to biological molecules, especially to DNA, lipids and proteins. Antioxidant defense systems scavenge and minimize free radical formation. The action of free radicals is counteracted by antioxidants, either endogenous or exogenous like sulfhydryl containing compounds, natural herbs etc (Conner and Grisham, 1996). Under normal physiological conditions, cells are capable of counterbalancing the production of ROS with antioxidants. Endogenous cellular antioxidant defenses are mainly enzymatic and include SOD, GPx, and CAT. SOD is localized within the cytosol and mitochondria and may reduce superoxide anion to Hydrogen peroxide and water.

It is well known that induction of superoxide radical and related oxygen species cause cell damage which has been found to be involved in the formation of malignancies (Mc Cord, 1993). In this study the DLA and EAC control have a decrease in SOD activity. However MEC treatment made SOD levels as comparable with normal. Antioxidant
defense system of tumour bearing mice may be impaired through the production of superoxide radicals, oxyradicals and peroxide radicals (Mukhopadhyay et al.; 2003). But the treatment with MEC made SOD levels comparable with normal values. The inhibition of SOD activity as a result of tumour growth was also reported (Gupta et al.; 2004). It was also reported that free radical scavengers can protect against the genotoxicity induced by chemical carcinogens by decreasing oxidative damage. In rats treated with KB,O2 which is a kidney specific carcinogen, it is found that treatments with melatonin, resveratrol, butylated hydroxyl toluene, α – phenyl – N- tert- butyl nitrone (PNB) and vitamin E (all known antioxidants), reduced the oxidative damage (Susana and Gustavo, 1999).

GR is a tripeptide involved in protecting membranes against oxidative damage. Decreased level of GR in tumour control mice may be due to the increased utilization of glutathione to scavenge the oxyradicals produced (Aruna and Sivaramakrishnan, 1990). The level was found to be increased in the drug treated group. Activity of GSH is decreased in the case of tumour control group. MEC treated group, attained normalcy by the administration of drug. Glutathione, a potent inhibitor of neoplastic process plays an important role as an endogenous antioxidant system that is found particularly in high concentration in liver and is found to have key function in the protective process (Gupta et al.; 2004).

Antioxidants are supposed to reduce the risk of cancer and other disease by helping the body to get rid of oxygen free radicals, which are thought to contribute to cancer development by damaging the DNA. Now it has been reported that a free radical (a superoxide) helps a protein called RAS to transmit growth-stimulating message to cell interior and thereby cause multiplication of cancer cells (Rawson and Peto, 1990).
Lipids containing several double bonds like polyunsaturated fatty acids (PUFA) and cholesterol may be subject to oxidative damage. In the case of membrane phospholipids, peroxidative destruction of their PUFA’s generally result in an impairment of the function of cellular or subcellular membranes. Excessive production of free radicals resulted in oxidative damage, which leads to damage of macromolecules such as lipids, can induce lipid peroxidation in vivo (Yagi, 1991). The process of lipid peroxidation is a chain reaction. The C-H bond is activated and therefore vulnerable to oxidation reaction. Hydrogen atoms in such position can react with free radicals and are thus easily split from the C-H bond. This initiation reaction results in the formation of lipid alkyl radical. This is followed by isomerization reaction leading to the transformation of an isolated double bond into a conjugated one. In the propagation reaction sequence that follows the lipid alkyl react with molecular oxygen; generating a lipid peroxy radical. This propagates in an autocatalytical manner. Termination reaction occurs between two radical intermediates resulting in the generation of nonradical products. The peroxyradical can either form a stable Hydrogen peroxide or breakdown to release a wide variety of aldehydes like MDA, ketones and hydroxyl lipids. The aldehyde reacts with thiobarbituric acid forming Thiobarbituric Acid Reactive Substances. (Willis, 1987) Increased lipid peroxidation would cause degeneration of tissues. Lipid peroxide formed in the primary site would be transferred through the circulation and provoke damage by propagating the process of lipid peroxidation. In higher animals lipid peroxidation is known to cause destabilization and disintegration of the cell membrane leading to liver injury, atherosclerosis, kidney damage and ageing and susceptibility to cancer (Osawa et al; 1990). MDA, the end product of lipid peroxidation was reported to be higher in carcinomatous tissue than in nondiseaseous organs.
The cell membrane phospholipids, which containing large amount of PUFA are highly susceptible to peroxidative damage. Lipid peroxidation is determined by TBARS reaction with MDA. The level of TBARS is found to be increased in tumour bearing mice. But the administration of drug lowers the level towards normal.

Oxygen radicals and lipid peroxidation, a free radical mediated phenomenon are involved in several pathogenic conditions. In order to combat free radical pathologies, we are looking for natural substances, which might exert a more potent pharmacological effect and be less toxic than synthetic drugs (Williamson et al.; 1978). Luca et al.; (1992) observed that antilipoperoxidant and superoxide scavenging effects of fruits depends on their total polyphenol content. The flavonoid containing drug obtained from Semecarpus anacardium improves the antioxidant defense system in the aflatoxin B1 induced hepatocellular carcinoma conditions. It was also observed that the anticancer property of Semecarpus anacardium extract might be explained by its strong antioxidant capacity and capability to induce the in vivo antioxidant system (Premalatha and Sachdanandam, 1999). Since C fistula L is reported to be rich in flavonoids (Sidhuraju et al.; 2002), the antioxidant property of the MEC could be explained based on this fact. It is also reported dietary intake of flavonoids containing foods potentially lowers the risk of certain free radical related pathophysiologies (Purgas et al.; 2000).

The methanolic extract of Cassia fistula L. reduces the elevated level of TBARS and increases the activity of SOD, GR and GSH in the serum of DLA and EAC bearing mice. This indicated that the plant extract have antioxidant and free radical scavenging activity. Vitamin E and epigallo catechin gallate are antioxidants commonly consumed by cancer patients as dietary supplements. Mice bearing MCA – IV solid tumours
(a breast carcinoma) when fed with vitamin E and epigallocatechin gallate, tumour cell apoptosis was increased (Lawenda et al.; 2007). This proves the antitumour activity of antioxidants. Similarly, melatonin an antioxidant has reported to have antitumour activity in murine lung adenoma model (Vesnushkin et al.; 2006). In addition, ascorbic acid was able to inhibit and redifferentiate cultured malignant human gastric cells (Sun et al.; 2006). Also MEC did not show any harmful effects in the normal mice receiving only MEC.

Plants containing antioxidants can reduce the toxicity of specific chemotherapeutic agents used in the treatment of cancer. Almost all of the present day drugs used in chemotherapy of cancer is toxic to normal cell leading to unwanted side effects. But as per this study, methanolic extract of *Cassia fistula L.* does not show any cytotoxic effect and helps in elevating the tissue antioxidants of the serum and reduces the lipid peroxidation. The tumour reducing properties of the plant may be due to the above mentioned reasons.

### 5.5 Chemo preventive potential of MEC of *C. fistula L.*

Fig 4.4.1 show that group 1 (control) and group II, which received the MEC treatment at the pre-initiational phase of tumourigenesis showed 80% tumour incidence. The animals which received the MEC treatment at the promotional stage i.e. group III showed 50% tumour incidence and the group which received MEC treatment at both pre as well as post initiational phases showed 40% tumour incidence.

It is clear from Fig 4.4.2 that the control group had maximum cumulative number as well as average number of papilloma. Group II that received MEC treatment only in the preinitiational stage had come second in the cumulative number of papilloma as well as average number of papilloma. This demonstrates that the MEC extract possesses some preventive effect on mutagenesis. Similarly the group, which
received MEC treatment in the promotional stage, came third in the cumulative as well as average number of papilloma. Tumour promotion is a process where there is clonal expansion of initiated cells. So mutated cells selectively multiply and accumulate more mutation and cause malignant conversion. The present study emphasizes that the MEC treatment is effective in preventing clonal expansion and malignant conversion, thereby reducing the number of papilloma. The group which received MEC treatment at both pre and post initiation stages developed the least number of cumulative and average numbers of papilloma, showing the effective prevention of mutagenesis and tumour promotion by the MEC. From Fig 4.4.3 it is evident that the MEC treatment delayed the onset of first papilloma. In group II and I the first papilloma appeared in 8th week. But in group III it appeared in 9th week and group IV only in 12th week. Similarly the application of MEC did not significantly reduce the body weight of the animals as per table 4.4.1 showing the nontoxic nature of MEC. The average number of papilloma recorded at 8th, 12th and 15th showed significant difference between the control and group III and group IV as per table 4.4.2. This also demonstrates the antimutagenic and chemopreventive potential of MEC.

It is reported that tumourigenesis is initiated by, one subminimal dose of carcinogen and the treatment with croton oil promotes the development of the visible tumour stage by clonal expansion (Berenblum and Shubik, 1947). The application of promoter to the mice skin results in rapid accumulation of inflammatory cells such as neutrophils and macrophages and an increase in the release of active oxygen species (Copeland 1983, Cerrutti, 1985). Klaunig and Kamendulis (2004) were of opinion that there is a strong link between cellular reactive oxygen radicals and pathogenesis of several chronic diseases including cancer. Cellular oxidants (reactive oxygen and nitrogen species) can be
generated from endogenous as well as exogenous sources. When the antioxidant control mechanisms fail, the cellular redox potential may shift towards an oxidative stress, which in turn results in the damage to cellular nucleic acids, lipid and protein. Unrepaired damage to DNA may result in mutation, provided cell replication occurs prior to repair of modified bases. It is evident that agents that can scavenge the free radicals can also prevent the occurrence of cancer.


Sidhuraju et al (2002) reported that all parts of Cassia fistula L. namely stem bark; leaves, flowers and fruit pulp possesses antioxidant activity. Among these maximum antioxidant activity was shown by the methanol extract of stem bark. The stem bark had the highest concentration of phenolics (Sidhuraju et al.; 2002). The presence of polyphenolic compounds, anthraquinones, xanthones, proanthocyanidins and flavonols could be the reason for its good antioxidant activity. Infact this antioxidant activity may be the reason for the antimutagenic and chemopreventive potential of MEC. Similarly, many flavonoids are reported to have strong antioxidant and antitumour activity (Jovanovic et al.; 1994, Aitken et al.; 1994, Pomilio et al.; 1994). The MEC of stem bark of C. fistula L. is reported to have flavonols (Sidduraju et al.; 2002). The present study showed that it had remarkable antioxidant activities in tumour bearing mice. The flavonols of the MEC may be the active constituent imparting its antioxidant chemopreventive and antitumour activity.
5.6 **Apoptogenic effect of C. fistula L.**

In cancer apoptotic process occur both spontaneously as well as by introduction of antitumour principles (Holdenrieder and Stieber, 2004). Qualitative and quantitative changes in cell death along with proliferative alterations are essential determinants in the pathogenesis and progress of malignant disease and responsiveness to therapy. Many qualitative and quantitative methods are available to detect cell death by assaying the cell itself and through identifying apoptotic products released into the circulation.

In the present study the *in vitro* antiproliferative potential of the MEC of *C. fistula* was confirmed and the results were discussed elsewhere. So an attempt was made to find out, whether the antiproliferative action was due to apoptogenic effect of the MEC and for this three different experiments were conducted. Chromatin condensation and apoptotic body formation, which was confirmed by morphological evaluation of the MEC, treated cells by DAPI staining. In MCF 7 cells treated with MEC, there was an increase in the no. of cells showing apoptotic morphology in a dose dependent manner (Fig. 4.6.1). The cells treated with 150 ug/ml of MEC for 24 hours showed Fifty percent cell death with respect to control. Other cell lines also showed the same trend. For SW 480 and HeLa cell lines, fifty percent apoptosis was shown by the MEC concentration of about 300 ug/ml (Fig 4.6.2 and 4.6.4). But HCT 116 and IMR 32 fifty percent apoptosis was shown by the MEC concentration of about 150 ug/ml; as depicted in Figures (4:6:3 and 4.6.5).

Apoptosis usually affects the cells that are aged, dysfunctional, or damaged by external stimuli. It is an active, energy requiring process leading to a well regulated degradation of the cell. Early pathomorphological features are chromatin condensation and
marginalization in the nucleus, DNA fragmentation into mono and oligo nucleosomal units, cellular shrinkage, packing or organelles and dilatation of the endoplasmic reticulum (Holdenrieder and Stieber, 2004). Qualifying cell death and cellular proliferation can provide information about the process of carcinogenesis and the response to antitumour treatment. Since the MEC could induce apoptosis in cancer cell lines as evident from DAPI staining and morphological observation of condensation, it can be said that the MEC has promising anticancer potential. Hengartner (2000) reported that depending on the type and dosage of the chemotherapeutic drugs, the modality of radiotherapy and the sensitivity of tissue, cellular damage might bring about the cell cycle arrest followed by insufficient repair; induction of active apoptotic cell death might result.

In the second experiment with five different cell lines the loss of mitochondrial membrane potential was assayed by JC-1 dye. JC-1 is a cationic fluorescent dye, staining the mitochondria bright red in healthy cells. The negative charge established by the intact mitochondrial membrane potential allows the lipophilic dye bearing a delocalized positive charge, to enter the mitochondrial matrix where it accumulates (Smiley et al., 1991). When the critical concentration of the dye is exceeded, aggregates of JC-1 form which become fluorescent red. In apoptotic cells, the mitochondrial membrane potential collapses, thereby JC-1 fails to accumulate within the mitochondria. In these cells JC-1 remains in the cytoplasm in a green fluorescent monomeric form. Apoptotic cells showing primarily green fluorescence are easily differentiated from healthy cells, which showed red and green fluorescence.

The mitochondrial permeability transition is an important step in the induction of cellular apoptosis. During apoptosis the electrochemical
gradient referred to as $A\psi$ across the mitochondrial membrane collapses. The collapse is thought to occur through the formation of pores in mitochondria by dimerized Bax or activated Bid, Bak or Bad proteins. Activation of these pro apoptotic proteins is accompanied by the release of Cytochrome C into the cytoplasm (Narita et al.; 1998, Luo et al.; 1998, Basanez et al.; 1999, Desagher et al.; 1999).

It is clear from the plates 4.6.5, 4.6.6, 4.6.7, 4.6.8, 4.6.9 and 4.6.10, that MEC could induce Cytochrome C release by damaging the mitochondrial membrane. In all the five cell lines treated with MEC, there was remarkable release of Cytochrome C into the cytosol, in a dose dependent manner, as compared to the controls. This supports the claim that MEC could induce apoptosis. The pores created by proapoptotic proteins cause the release of Cytochrome C into cytosol (Desagher et al.; 1999). Therefore it is evident that proapoptotic proteins of intrinsic pathway of apoptosis are induced by the treatment with MEC.

Mitochondria play a key role in the apoptotic machinery of the cell by releasing caspase activators such as Cytochrome C, releasing caspase independent death effectors and causing the loss of essential mitochondrial functions (Yuan et al.; 2001, Green and Kroemer, 2004). During apoptosis, the outer mitochondrial membrane becomes permeabilized, allowing inner membrane proteins to be released and activate the downstream apoptotic machinery including Cytochrome (Orrenius, 2004). Mitochondrial membrane permeabilisation alone can trigger apoptosis or necrosis, even in cancer cells (McLahkan et al.; 2005). If the MEC is able to overcome the cancer cells’ resistance to MMP, then the cells will have no choice but to undergo apoptosis.

Induction of apoptosis is an effective mechanism used to eradicate, transformed or deleterious cells. Many chemotherapeutic or
chemopreventive agents act through triggering of apoptotic pathways in tumour cells (Kutuk et al.; 2005). The cellular apoptotic machinery is formed by protein interactions and protein modification. Protein kinases as well as various cysteiny1-specific aspartate proteases or caspases have been proposed to mediate apoptosis induced by cytokines, chemotherapeutics and cellular stress through a highly organized network at different signaling levels (Daniel, 2000, Kaufmann and Earnshaw, 2000, Joza et al.; 2002).

In the third experiment the cell death associated caspases (caspase 2, 3, 7, 8, 9, and 10) were collectively assayed by using the common caspase substrate, in MCF7 breast cancer cell line in a dose dependent manner. As shown in Fig. 4.6.6 the activities of one or more of caspases 2, 3, 7, 8, 9 and 10 were increased significantly on MEC treated cells as compared to the activities in control cells. The MEC dose of 150 ug/ml showed a 50% increase in caspase activity on treated cells as compared to control cells. The increased caspase activity in MEC treated cells as compared to control cells, supported that the treated cell were undergoing apoptotic cell death.

It is clear from the above mentioned studies that DNA damage, MMP and caspase activation is evident and that MEC is able to induce apoptosis in cancer cell lines. Therefore the mechanism by which the MEC produces the cell death in cancer cells and prevents its proliferation may be due to apoptosis.