DISCUSSION

DMBA is one of the most potent tumor initiating agents known. Besides the initiation and cause of tumors in skin, mammary tissue, kidney, etc., it also causes tumors in lungs (Bottinger et al. 1997). Application of 3-methylcholanthrene also causes tumors in lungs (Kinoshita et al. 1979). When a single carcinogen is applied to the animals at a time, the rate of development of tumor is slow and the number of tumor developed is small. In the present study, it is found that if the two carcinogens are applied at the same time, then the rate of tumor development is fast as well as number of tumor developed is large. All the animals exposed to the two carcinogens developed tumors in the lungs while no tumor is developed in the control animals. The weight as well as the volume of the tumors developed in the carcinogen exposed M. grandiflora bark extract untreated animals, both male and female, are larger than the tumors developed in the carcinogen exposed plant extract treated animals. Traditionally, the soup of the flower of this plant is used in the treatment of many diseases including liver cancer. The present study shows the anti-tumor activity of the 50% ethanol extract of the M. grandiflora bark. The treatment of the animals with M. grandiflora bark extract reduced the volume of the tumors induced by carcinogens. In the control group, the average tumor volume is $0.555\pm0.072\ cm^3$ in male but it is reduced to $0.375\pm0.051\ cm^3$ and $0.228\pm0.10\ cm^3$ in the animals treated with the plant extract at a dose of $21\mu g$ and $42\mu g$ plant extracts/gm body weight (50 mg/Kg body weight and 100 mg/Kg body weight respectively) of the animals
respectively showing thereby that significant reductions can be effected in the volume of the tumors in animals by 32.43% (p<0.001) and 58.91% (p<0.001) respectively. Similarly, in the females, the average volume of the tumor in the control animals is 0.604±0.075 cm$^3$ which is reduced to 0.186±0.039 cm$^3$ in the 21μg plant extract/ 25 gm body weight treated groups and 0.091±0.31 cm$^3$ in the 42μg plant extract/ 25 gm body weight treated groups which shows significant reductions in the tumor volume by 69.20% (p<0.001) and 84.93% (p<0.001) respectively in the administered two doses. The weight of the tumor is also reduced in the plant extract treated group. In the 21 μg plant extract/25 gm body weight treated group, the tumor weight is reduced by 22.38% (p<0.05) compared to the control group in the male mice, and this reduced to 47.32% when the dose of the plant extract becomes 42μg plant extract/25 gm body weight. The reduction in the tumor weight in the plant extract treated groups is also found in the females. From 224.3±7.65 mg of average tumor weight in the control groups in the female animals, 21μg plant extract/ 25 gm body weight and 42 μg plant extract/ 25 gm body weight treated groups show a reduction by 35.22% (p<0.001) to 145.28±14.14 mg and 54.31% (p<0.001) to 102.47±8.22 mg, respectively. The above results indicate that the 50% ethanol extract of bark of *M. grandiflora* is active in the reduction of the tumor volume as well as tumor weight induced by carcinogens DMBA and 3-MC, and the results obtained corroborates the results obtained by Park *et al.* (2008), Lee *et al.* (1991) and Roomi *et al.* (2009). The two carcinogens induced tumors in the lungs of the animals both in male and females. The average weight of the lungs is greater in the
carcinogen-treated control animals than the untreated control ones. The average weight of the lungs in the plant extract treated animals is reduced significantly from the carcinogen-treated animals but higher than that of the carcinogen untreated control ones. In the plant extract treated males, there is a reduction in the average weight of the lungs by 30.78% (p<0.001) in the 21μg plant extract/25 gm body treated group than the carcinogen-treated control group and this reduction is increased to 48.81% (p<0.001) in the 42μg plant extract/25 gm body weight treated group. In the females, treatment of the animals with 21μg plant extract/25 gm body weight reduced the lung weight by 28.88% (p<0.05) while 57.06% (p<0.001) is reduced in the 42μg plant extract/25 gm body treated groups. There is no significant change in the average body weight of the carcinogen treated control groups and plant extract treated groups in both male and female. Since there is no significant difference in mean body weights of the mice, these findings indicate that the observed difference in the average lung weight among the groups of mice is due to difference in the tumor growth. As mentioned in the results, to determine whether there is a relationship between the tumor volume and lungs weight, data from all the mice in the study have been pooled and compared. In the analysis significant positive correlations has been observed (correlation coefficient r=0.5660, p<0.0001 in males and r=0.8133, p<0.0001 in females) between tumor volume and lungs weight. A significant positive correlation is also observed (correlation coefficient r=0.7685, p<0.0001 in male and r=0.5218, p<0.0004 in females) between the tumor weight and lung weight. As discussed earlier, all the organs have been examine and found to be
free of tumors, except in the lungs. The average weight of the kidney, heart, spleen
and liver do not differ significantly.

At the given dose of the *M. grandiflora* bark extract which is administered orally, the
animals do not show any sign of toxicity. Neither the life span nor the body weight of
the plant extract given animals do decrease compared to the carcinogen exposed
control group. There is no significant change in the morphology as well as the
weights of other vital organs between the carcinogen exposed plant extract treated
group and carcinogen unexposed control group. The *in vitro* studies of Mohamed *et
al.* (2009) show that the bio-active compounds magnoflorine and lanuginosine
present in this plant have cytotoxic activities in cancer cell lines HEPG2 (human
hepatocellular carcinoma cell line) and U251 (human brain tumor cell). This perhaps
suggest that the 50% ethanol extract of the *M. grandiflora* bark has the cytotoxic
activity against tumor cells *in vivo*.

The present study shows positive result of the leave extract of the *Croton caudatus* in
all the genotoxic testing protocols based on the chromosomal aberration analysis and
SC damages. High frequencies (16.55%) of seven types of somatic chromosome
aberrations (Table 7 and Figures 30 & 31) in mouse bone marrow cells and high
frequency of the 15 types of SC damages (Table 8 and Figure 32) induced by
aqueous extract of *Croton caudatus* Geiseler leaves indicate presence of strong
genotoxic agent(s) in the aqueous extract of leave. According to Hsu (1982), the
traditional chromosome aberration analysis is considered to be the most authentic test among various clastogenicity tests. According to Bochkov et al. (1976), Sharma (1984) and Sarkar & Manna (1989), an agent can be claimed to be clastogenic if it shows positive result in more than one test protocol. Accordingly, it may be concluded that the aqueous extract of *Croton caudatus* leave has clastogenic potentials.

The MTT assay shows good correlation between the spectrophotometric absorbance and cell number (Mosmann 1983). MTT is cleaved by all living, metabolically active cells but not by dead cells or erythrocytes. The amount of formazan generated is directly proportional to the cell number over a wide range of homogenous cell population. Activated cells produce more formazan than resting cells which would allow the measurement of activation even in the absence of proliferation. These properties are all consistent with the cleavage of MTT only by active mitochondria. The present investigations reveal that there are significant growth inhibitions of cancer cells by the elite plant extracts. As shown elsewhere, 50% ethanol extract of *N. indicum* root bark and its different fractions show inhibition of the growth of the human cervical cancer cell Bu25Tk⁻ and mouse myeloma cancer cell Sp2/01 Ag-14. Earlier studies report the various bioactivities of leave, flower and stem extracts including anti-cancer and anti-proliferative potentials on different types of cancer cells (Fu et al. 2005, Zibbu & Batra 2010). The literature available also reports the presence of anti-microbial activity of the root bark extract of this plant (Hussain &
Gorshy 2004). The present work is focused on the activity of the root bark extract on the growth of cancer cells. The whole extract of the root bark of this plant shows 85.03% inhibition of growth of human cervical cancer cell Bu25Tk− at a concentration of 80µg/100µl while the fraction 3 shows 100% inhibition. In mouse myeloma cancer cell Sp2/01 Ag-14, the whole root bark extract of N. indicum and its fraction 1 at a concentration of 80 µg/100 µl culture shows growth inhibition of 96.3% and 83.1% respectively. The rhizome extract of the Belamcanda chinensis also shows active inhibitory effect on the growth of the cervical cancer cell Bu25Tk− and mouse myeloma cancer cell Sp2/01Ag-14. It yields 55.9% inhibition of the growth of Bu25Tk− cells when the concentration is 80µg/100µl of culture. The inhibition of the growth of the Sp2/01 Ag-14 by the rhizome extract of this plant is 79% when the concentration is 80µg/100µl of culture. Earlier in vitro and in vivo study reports the inhibitory effect of the rhizome extract of the plant on prostate cancer cell LNCaP cells and tumor growth on the nude mice (Thelen et al. 2005). The presence of anti-oxidant and anti-mutagenic activities of the rhizome extract of this plant has been already reported (Wozniak et al. 2010). Thus, the results of the present investigations showing the growth inhibitory potential of the 50% ethanol extract of the B. chinensis rhizome corroborates the results obtained earlier. The presence of cytotoxic property of leave extracts of Magnolia grandiflora on various cancer cells including HEPG2 (human hepatocellular carcinoma cell) and U251 (human brain tumor cell) have been shown (Mohamed et al. 2009). The leave extract
of this plant has also the ability to induce apoptosis on B-CCL (B cell chronic lymphocytic) cells (Marin & Mansilla 2010). The present work indicates the growth inhibition potential of the 50% ethanol extract of the bark of this plant on the human cervical cancer cell Bu25Tk−. The plant extract at 80μg/100μl concentration results growth inhibition of this cell by 62.84%. The in vivo study which shows the inhibition of tumor growth in mice substantiates the in vitro results. But, its effect on the growth of the mouse myeloma cancer cell Sp2/01 Ag-14 is very low. At 80μg/100μl concentration of the extract, only 28.8% growth inhibition occurs on Sp2/01 Ag-14 cell. While the bark extract shows a little inhibitory effect on the growth of Sp2/01 Ag-14 cells, the flower extract at the same concentration show good inhibitory effect. It shows 63.6% inhibition of the cell growth when the concentration is 80μg/100μl.

The SRB assay provides a sensitive method for measuring drug cytotoxicity in culture. The SRB is a protein binding dye which binds to the basic amino acid residues in TCA fixed cells to provide a sensitive index of the cellular protein contents. The amount of protein stained which is measured spectrophotometrically is directly proportional to the number of living cells (Skehan et al. 1990). Among the four plants studied for their cytotoxic activity using SRB assay in MCF-7 cell, 50% ethanol extract of root bark of N. indicum shows highest activity. With the increase concentration of this plant extract, there is gradual decrease in the growth of MCF-7 cells in vitro. There is 8.4% growth of the cells when the concentration of the extract
is 10 μg/100 μl and 0.5% growth when the concentration of the plant extract is 80 μg/100 μl. *B. chinensis* and *M. grandiflora* both show good cytotoxic effects of 18.2% and 27.3% on the growth of MCF-7 cells when the concentration of these extracts reach 80 μg/100 μl. At this dose give only 18.2% and 27.3% growth of the MCF-7 cell. The three fractions of the *N. indicum* as well as *C. caudatus* do not show significant inhibitory activities on the growth of this cell. The plant extracts also do not show a significant inhibitory effect on the growth of human cervical cancer cell ME180. Among the four plants tested for their cytotoxic activity on ME180 cells by SRB assay, the highest inhibitory activity is given by *M. grandiflora* bark extract giving 40.1% growth when the extract concentration of 80 μg /100 μl is used.

Cancer is frequently considered to be a disease of the cell cycle and the hallmark of cancer is deranged growth control (Pardee *et al.* 1978). As such, it is not surprising that the deregulation of cell cycle is one of the most frequent alterations during tumor development. Cell cycle progression is a highly ordered and tightly-regulated process that involves multiple checkpoints that assess extracellular growth signals, cell size and DNA integrity. The development of cancer is frequently associated with mutations or abnormalities in the expression of various genes involved in the regulation of cell cycle and defective in the cell cycle checkpoints (Baker *et al.* 1989, Vogelstein 1990, Hartwell & Kastan 1994, Hollstein *et al.* 1990, Nigro *et al.* 1989). The chronic myelogenous leukemia cell K562 is a continuously growing cell. In the
present investigation, deviation in the cell cycle profile of the K562 cell has been checked by exposing the cells to three different plant extracts. The results show that when the cells are exposed to 50% ethanol extract of *N. indicum* root bark at different concentrations for 24 hrs., the percentage of cells in G₂ as well as in G₀-G₁ are increased indicating the arrest of cells at these phases. In the cell cycle profile of plant extract unexposed K562 cells, 23.76% and 1.16% cells are found in G₂ and G₀-G₁ phases respectively and the number of cells in these two phases increase to 38.60% and 12.22% respectively when the cells are exposed to plant extracts at 40μg/ml concentration for 24 hrs corroborating the results obtained by Cheng *et al.* (2000). The increase in the G₀-G₁ is also observed in the cell cycle profile of K562 which is exposed to *N. indicum* root bark extract for 48 hrs but other extracts, viz., *B. chinensis* rhizome and *M. grandiflora* bark extract do not show significant increase in the percentage of cells.

The present investigations, therefore, reveals the presence of cytotoxic activities of 50% ethanol extracts of *N. indicum* root bark, *B. chinensis* rhizome and *M. grandiflora* bark on various cancer cell lines, viz., human breast cancer cell MCF-7, Human cervical cancer cells Bu25Tk and ME180 and mouse myeloma cancer cell Sp2/01 Ag-14. *N. indicum* root bark extract has the ability to arrest the chronic myelogenous leukemia cell K562 at G₀-G₁ stage which is very crucial for the development of cancers and its treatment. Moreover, *M. grandiflora* bark extract has
the ability to reduce the volume as well as weight of the lung tumors in the mice exposed to carcinogens DMBA/MC. The above three plants, hence, may be promising candidates for the extensive search of anti-cancer compounds for subsequent application in the treatment of cancers of various types.