GENERAL DISCUSSION
Caffeine (chemically 1, 3, 7-trimethyl xanthine) is one of the most widely consumed as beverage (3), present as an active component in tea and coffee. Caffeine is known to be a bronchodilator (1-3), diuretic (1, 47, 71, 72), cardiac stimulant (2, 47-54), skeletal muscle contractor (4, 59-64) and moreover is a potent CNS stimulant (1-4). Its pharmacological activity varies depending on the dose (concentration) and type of target cells (266). Considerable controversy exits in the literature regarding the toxicity of caffeine, including its possible carcinogenic (208-218) and antitumorigenic (228-242) properties. At a very high doses, caffeine appears to have some teratogenic activity in mammals (3) because of its structural similarity with purine bases of DNA (80). Epidemiological studies have shown that consumption of caffeine is associated with cancer of pancreas, kidney, lower urinary tract and ovary (3, 219, 220). There are also evidences, which suggest that caffeine consumption can enhance the development of mammary carcinomas in female rats treated with chemical carcinogen (216, 217). But these effects of caffeine are observed only with the concentrations that are much in excess of those that follow the ingestion of beverages and medicine (3, 219, 220). Welsch et al. (218) have shown that caffeine can modify the tumorigenic process depending on the time span of caffeine treatment, the dose of caffeine used and the animal model examined.

Other studies, on the other hand, have shown that caffeine inhibits cancer formation by delaying induction of chemical carcinogenesis of lung (228), skin tumors (227) and mammary tumor in female mice (232). Caffeine has been found to be synergistic with many DNA-damaging agents such as, x-ray, uv-light and a variety of alkylating agents in causing lethality in mammalian cells (225, 400, 401).

In spite of these informations about the actions of caffeine, no underlined molecular or cellular mechanisms is known to us, though, it has been hypothesized that caffeine inhibit repair processes such as, post replication repair synthesis of DNA (247). Witte and Bohme have established the DNA-caffeine interactions as the basis of the repair inhibition (224).
Considering all these knowledges, it may be stated that no systemic studies have been made on the effect of caffeine at a dose generally consumed by the people per day in relation to the development of carcinoma induced by chemicals or by other means. It is also known that long-term intake of caffeine alone or in combination with other drug(s) inhibits the development of spontaneous and inducible tumors (228, 301). Development of implanted tumors is a result of stress (269, 270). Stress has been found to modulate the central GABA binding to its receptor complex as well as GABA activation of chloride ion influx (271, 369). Further, it is known that stress induces HPA axis and elevates ACTH secretion as well as its level (272) and this stress-induced induction of HPA axis is suppressed by central GABA (273).

The aim and objective of the present investigation is to study the long-term effect of caffeine in the development of EAC cells in female mice at the levels of (a) respiration, viability and \[^{3}H\]-thymidine incorporation in EAC cells, (b) corticosterone status and adrenal ascorbic acid, (c) hepatic antioxidant defense system and (d) changes, if any, in the activity of central GABA.

In the present investigation it has been observed that long-term consumption of caffeine prior to EAC cell inoculation followed by continuation of its treatment during EAC cell growth suppresses the growth of EAC cells (Table 7, Chapter I). This suppression has also been found to be dependent on the duration of caffeine treatment (Table 7, Chapter I). It is known that lowering of cAMP enhances the tumor growth (325). Caffeine is a well known phosphodiesterase inhibitor (158), cAMP degrading enzyme (327). Thus, inhibition of this enzyme may stimulates the cAMP level in the cellular environment and may reduce the EAC cell growth as suggested in Chapter I. Therefore, caffeine may inhibit or delay mitosis in many kind of cells, probably due to variations in cAMP concentration that influence DNA synthesis and mitosis (263). Another possible explanation is that caffeine inhibits DNA repair, such as post replication repair synthesis of DNA. Earlier, it has been shown that caffeine tends to stack on the top of the terminal purine bases or to insert (the single stranded) or to intercalate (the double stranded) between purine bases (402). It has also been reported
that caffeine binds preferentially to single stranded thymus DNA (403) and effectively lowers the melting temperature of this DNA (404). It has been suggested that caffeine binds to DNA, perhaps with higher affinity to damaged regions (405) and thus interferes with the specific binding of repair enzyme (303). It appears that caffeine inhibits photorepair by interfering with the specific binding of photolyase to damage DNA (406). In contrast to photoreactivation, which repairs pyrimidine dimers, nucleotide excision repair entails the removal of a segment of the DNA backbone containing the damaged base(s) followed by filling in the gap by DNA polymerase and sealing by ligase. Damage recognition and removal by ABC excinuclease occurs in several stages (407), the damage recognition subunit, uvrA, binds to DNA containing pyrimidine dimers, delivers the uvrB subunit to the damaged site and dissociates from DNA. The uvrC subunit then binds to the uvrB / damaged DNA complex and incisions are made on both sides of the DNA adduct and at a distance from it. Caffeine inhibits nicking of damaged DNA by the enzyme by trapping the uvrA subunit of ABC excinuclease in nonproductive complexes on undamaged DNA (406). This caffeine-induced suppression of EAC cell growth may be supported by the works of others (227, 301, 302, 320), who in fact have shown that caffeine suppresses both spontaneous and chemically-induced pulmonary adenoma development, apoptosis and cell cycle.

To understand the metabolic changes which may occur in the different tissues of the tumor-bearing animals, an approach has been made by undertaking a study of the functional activity of endocrine organs of the host in the presence of a growing tumor. The hypersecretion of ACTH causes an increase of plasma corticosterone level as well as tumor growth (265). Development of implanted inducible tumors is a result of stress (269, 270). Further, it is known that stress induces HPA axis and elevates ACTH secretion as well as its level (272). Hilf et al. (267) has also found that a growing tumor induces an adrenal response like a nonspecific chronic progressive stress. Corticosterone has been shown to the major adrenal steroid in mice (312) and adrenal cortical function has been assessed by measuring its concentration in blood as well as in adrenal glands. The plasma corticosterone level in the present study has
been found to be elevated with a decrease of its adrenal level during the development of EAC cells (Figs. 6 and 7, Chapter I). This may be explained by the fact that the secretion of corticosterone in plasma during tumor growth may be much greater than its synthesis in adrenal, due to the hyperactivity of the adrenal gland (267). Though long-term caffeine treatment (which generally produces tolerance to caffeine) did not show any significant change in the level of corticosterone (Figs. 6 and 7, Chapter I), the growth of EAC cells during long-term caffeine treatment on the other hand, shows no appreciable change in the level of both plasma and adrenal corticosterone with respect to their corresponding control (Figs. 6 and 7, Chapter I) suggesting that long-term caffeine treatment may have a negative role over EAC cell growth.

It is well known that corticosterone biosynthesis is stimulated by ACTH with a reduction of adrenal ascorbic acid level (315). Further, it is known that increased secretion of ACTH which may cause an increase of tumor growth (321) results in the depletion of adrenal ascorbic acid by hydrolytic decomposition of dehydroascorbic acid to 2, 3-diketogulonate (322). Thus, the reduced level of adrenal ascorbic acid (both total and dehydro) observed in the present study (Table 9, Chapter I) during the development of EAC cells suggests that EAC cell development may be associated with the enhancement of ACTH secretion followed by elevation of plasma corticosterone level and reduction of adrenal ascorbic acid level (Fig. 6 and Table 9, Chapter I). Long-term caffeine consumption, which has been found to develop tolerance to caffeine (123, 125, 126, 135) does not appreciably change the adrenal ascorbate level (Table 8, Chapter I). Whereas, pretreatment of caffeine and continuation of its treatment during EAC cell development may suppress the EAC cell - induced induction of plasma corticosterone level (Fig. 6, Chapter I) and restores the EAC cell - induced reduction of adrenal ascorbic acid level (Table 10, Chapter I) suggesting that long-term caffeine treatment may resist the EAC cell growth - induced changes at the level of both adrenal ascorbate (Table 10) and corticosterone status (Figs. 6 and 7) as observed in Chapter I. Further, it has been observed that the development of EAC cell resist the long-term caffeine-induced tolerance to LA in mice (Table 15, Chapter III). In other words, long-term caffeine treatment may
suppress the growth of EAC cells. So, the present study demonstrate that long-term pretreatment of caffeine and continuation of its treatment during EAC cell growth may (a) inhibit the growth of EAC cells (b) restore and modulate the EAC cell growth - induced changes in adrenal ascorbate and corticosterone status (Chapter I).

It is well known that induction of superoxide radical and related oxygen species cause cell damage, which has been found to be involvement in the formation of malignancy (274, 276). In carcinogenesis, oxygen active radicals may damage specific genes that control the growth and differentiation of cell during the promotion phase and greater stimulation is expected in the following progression phase, which finally may develop malignancy (350). Therefore, it is not surprising that cell developed several lines of defense against the oxidative attack. The most important defense systems elaborated by the cells are scavenger enzymes of free radicals CAT, GPx and SOD (359). The function of this system keeps free radicals at physiologic level. Reactive oxygen free radicals have been known to damage tissue through LP, resulting in changes in biophysical character of the cell membrane (332). Unchecked peroxidative decomposition of membrane lipids is catastrophic for living systems (278).

Since caffeine are structural analogues of purines, the examination of the structure of caffeine reveals that it could readily accept electron (278). The results of the present study provide evidence that long-term caffeine consumption (a) increases the activity of hepatic SOD and CAT (Table 11, Chapter II) and (b) decreases hepatic LP (Table 11, Chapter II) without any significant alteration of its GPx activity (Table 11, Chapter II). Further, it is also observed that these long-term caffeine-induced changes in hepatic antioxidant enzyme activities significantly affected with the increase of duration of caffeine treatment (Table 11, Chapter II). The observed decrease of hepatic LP in mice treated with caffeine under the present long-term condition is likely due to the induction of hepatic CAT and SOD activities. These observed antioxidant effects of caffeine suggest that the quenching of the reactive oxygen species by caffeine may be considered as one of the possible factor
responsible (351). ESR measurements provide evidence that a caffeine-derived oxygen-centered radical is formed in the reaction of caffeine with \( \cdot \text{OH} \) and suggest a biochemical basis for the understanding of anticarcinogenic property of caffeine (351). Thus, caffeine as suggested by others (351, 352), may be considered as an antioxidant against oxidative damage of the biological membrane by potentiating the activities of antioxidant enzymes (such as SOD, CAT etc.).

It is known that under physiological conditions, oxy-radicals are part of normal regulatory circuit and cellular redox state is very much sensitive to the antioxidant (274). However, increase in flux of oxy-radicals and loss of cellular redox homeostasis can cause oxidative stress and lead to tumorigenesis (354-356). The antioxidant enzymes are of vital importance under this environment in an organism defense against oxidative stress (274). The most important one is SOD, whose main function is to remove \( \text{O}_2^- \) radicals (274). It is reported that SOD is absent in tumor mitochondria (327) and a low SOD activity in the cytosol of tumor cells (328, 329). Again, in cancer-bearing mice, there is a marked decrease in the CAT activity of the liver (408). Therefore, the depression of hepatic CAT activity (Table 12, Chapter II) as well as activity in SOD (Table 12, Chapter II) in Ehrlich ascites tumor bearing mice observed in the present study which has also been supported by others observations (274, 357, 358) suggests that hepatic antioxidant defense system of ascites tumor bearing mice may be impaired through the production of superoxide radicals, oxy-radicals and peroxide radicals, which have been shown to be involved in LP (409-411) and this enhanced LP in the carcinoma cell perturb the hepatic cell membrane (339, 357) as well as in hepatic microsomal membrane (Table 12, Chapter II).

The administration of caffeine during the development of EAC cells, on the other hand, antagonizes the activity of the Ehrlich ascites tumor cell-induced decrease of hepatic antioxidant enzymes along with the induction of hepatic microsomal LP (Table 14, Chapter II) and restored to their (enzymes) respective control value, suggesting that caffeine may effectively scavenges \( \cdot \text{OH}, \text{ROO}^- \) and \( \text{O}_2^- \) and plays a potential antioxidant activity against the oxidative damage to the hepatic cellular
system caused by the development of tumor cells. The degree of caffeine-induced antioxidant effect on hepatic cell damage caused by tumorigenesis depends on the duration of caffeine treatment (Table 14, Chapter II).

In addition to the above primary antioxidant defense system, which protects against ROS, there is another enzyme GPx (359). In fact, there are two types of GPx. One is Se dependent and other is Se independent. It has been also reported that in hepatic tissue Se dependent GPx is dominant (334) and it has been further known that Se content and Se dependent GPx activity, both are enhanced in tumorigenesis or in tumor bearing mice (334). Though contradictory reports in hepatic GPx activity in cancerous condition have been cited in the literature (359), the results of the present study show no significant change in caffeine treated or EAC cell developing or treatment of caffeine in EAC cell developing mice liver GPx activity (Tables 11, 12 and 14, Chapter II) suggesting that present observation may be with the Se independent hepatic GPx activity.

Since caffeine has been found to inhibit the generation of ROS by increasing the activities of hepatic antioxidant defense enzymes known from the present study as well as from other studies (277, 278), it is not unlikely that this may be a cause of suppression of the EAC cell growth as observed in the Chapters I and II of the present investigation.

It is known (as mentioned earlier) that development of tumor is a stress (269, 270). Increase in steroid production that occurs in response to stress originates in the CNS and culminates in adrenals via HPA axis (361). There is a general agreement that the CNS plays a major role in the regulation of anterior pituitary function. Synaptic organization of the CNS with regard to regulation of adrenal cortical function, with the hypothalamic secretory neuron is known to functioning as an integer in the sense of a final common pathway (412). No significant change in the steady state level of brain GABA (during the development of EAC cells for 12 and 15 days) with either no appreciable change (12 days EAC cell development) or
significant increase (15 days EAC cell development) in both GAD (58.63%) and GABA-T (46.39%) activities in mice brain or appreciable increase in steady state level of GABA (34.69%) (during 18 days EAC cell development) with a greater increase in GAD activity (104.47%) than that observed in GABA-T activity (68.82%) in mice whole brain under the same period of EAC cell development (Table 18, Chapter III) suggest that development of EAC cell in mice may stimulates the vesicular release of GABA which may increase the GABAergic activity in mice brain. The increase in brain GABAergic activity during the development of EAC cell may be supported by the significant increase in $[^{3}H]$-GABA receptor binding in mice brain (Table 19, Chapter III). Since the growth of implanted tumors is a result of inescapable stress (269, 270) and stress (a) induces the rapid synthesis of GABA binding sites or may induce dissociation of one or more of the endogenous inhibitors of GABA binding which include occluded GABA bound to cryptic receptors, phospholipids and peptides (271, 396) and (b) modulates GABA activation of Cl\(^{-}\) ion influx (369), it may be suggested that development of EAC cell in mice induces stress and may led to activation of the brain GABAergic activity.

Consumption of caffeine on a regular schedule develops tolerance to many of the effects of caffeine within a few days or weeks depending on the amount consumed and the frequency of consumption per day (122). Recently, it has been observed that long-term consumption of caffeine restore the short-term caffeine-induced induction of LA in rat (123) and this restoration of LA following long-term consumption of caffeine has been considered to be the state of caffeine tolerance (123). The development of tolerance to LA and other behavior to caffeine following its long-term consumption is in accord with the observation of Carney, Holtzmann and others (124-126). In the present study, long-term administration of caffeine to mice restores gradually its (mice) short-term caffeine-induced LA to the control value with the duration of exposure and this may be considered as a state of tolerance (Table 15, Chapter III). Biochemically, this tolerance can be explained by the upregulation of central adenosine receptor activity (by increasing the number of receptors) (394, 395) as well as central GABAergic activity (123). Caffeine, in fact, stimulates the LA by
reducing the central GABAergic activity under its nontolerant condition and this caffeine-induced down regulation of central GABAergic activity has been found to be restored to the control value and developed tolerance to caffeine following its long-term consumption (123). This restoration of central GABAergic activity may be suggested in the present study where whole brain GABAergic activity in mice remain unaltered following long-term caffeine treatment because, there is no significant change in whole brain steady state level of GABA, its metabolizing enzymes activities (GAD and GABA-T) as well as $[^3H]$GABA receptor binding in mice under long-term caffeine treatment (Fig. 11, Tables 16 and 17, Chapter III). It is well known that GABA$_A$ receptor is a ligand gated Cl$^-$ channel, which is open following the calcium dependent release of GABA at the presynaptic end of the neuron (3). The upregulation of central GABAergic activity under caffeine tolerant condition may be supported by the withdrawal of caffeine-induced block of Cl$^-$ channel under its nontolerant condition and ultimately became normal in Ca$^+$ eflux as well as Cl$^-$ influx (200, 392).

The EAC cell-induced induction of GABAergic activity becomes normalized in long-term caffeine administration during the development of EAC cell (Fig. 11, Tables 20 and 21, Chapter III). Inoculation of EAC cells following 12 consecutive days of caffeine treatment resists the development of caffeine tolerance to LA (Table 15, Chapter III), inspite of continuation of caffeine treatment under long-term condition. Further, it is known that in tumor cells adenosinergic activity is increased by increasing the adenosine A$_1$ receptor population (393). Since, both GABA and adenosine are inhibitory neurotransmitters and their activities are increased in tumor cells and caffeine is an antagonist of adenosine receptors (141,142), it is not unlikely to interact the caffeine with the GABAergic system through the interaction with adenosinergic system and affect the tumor growth. Caffeine treatment under the present long-term condition blocks the inhibitory action of adenosine (397-399), which in turn may lower the GABAergic activity and reducing the EAC cell growth (Chapter I). In other words, this may be an effect of inhibition towards the development of upregulation of central GABAergic activity (Fig. 11, Tables 20 and
21, Chapter III) under long-term caffeine treatment during the development of EAC cells by antagonistic effect of caffeine towards adenosine receptor (141, 142).

Therefore, from the present investigation, it may be stated that long-term pretreatment of caffeine and continuation of its treatment during EAC cell growth may (i) inhibit the growth of EAC cells by suppressing the viability, oxygen consumption and [3H]-thymidine incorporation in EAC cells and antagonize the EAC cell-induced stimulation of plasma corticosterone level and inhibition of adrenal ascorbate level, (ii) inhibit the induction of hepatic cell damage in Ehrlich ascites tumor-bearing mice caused by generation of ROS due to its effective scavenging property against oxidative damage to the biological system and (iii) suppress the EAC cell-induced induction of central GABAergic activity.

Finally, from the present investigation, it may be concluded that caffeine acts as an anticarcinogen. Further, it may be stated that reasonable daily intake of caffeine or caffeine-containing beverages is expected to be beneficial in view of negative role observed in EAC cell growth.