CHAPTER 6: DISCUSSION

In the present study the some specific karyomorphological characteristics of the aromatic grasses belonging to the genus Cymbopogon (C. martini, C. winterianus, C. citratus) and Vetiver (Vetiveria zizanioides) are reported along with the evaluation of protective role of the essential oils against well known mutagens (methyl methanesulphonate and hydrogen peroxide) in vitro in human lymphocytes. The results of in vitro studies were then validated by the in vivo studies. Amelioration of cisplatin-induced toxicity by the essential oils was studied in depth with various end points including those of oxidative stress in Swiss albino mice. Taking into consideration of the available literature, the three sections have been discussed in the following pages.

SECTION I

Karyomorphological studies from the root tips of aromatic grasses (Cymbopogon- C. martini, C. winterianus, C. citratus and Vetiver- Vetiveria zizanioides or Chrysopogon zizanioides)

SECTION II

In vitro study for safety evaluation and antioxidant properties of the essential oils in human lymphocytes

SECTION III

In vivo study for evaluation of the essential oils and ameliorating effects on cisplatin-induced toxicity in Swiss albino mice
Cymbopogon and Vetiveria are commercially important genus of aromatic grasses growing in the tropics of the world. Most of the species of Cymbopogon occur in wild and a few of them are under cultivation as a source of important essential oils, such as lemongrass oil, citronella oil, palmarosa oil and ginger grass oil that are extensively used in perfumery, aromatherapy, pharmaceutics and other chemical industries. The aromatic roots of Vetiveria zizanioides commonly known as ‘Khus’ yield the well known 'oil of vetiver', which has been highly valued by perfumery industry since ancient times. The plant grows wild throughout India and is also cultivated for its aromatic roots and aromatic oil. The various plant types grown at various geographic locations are known to vary with respect to oil content and oil quality. The south Indian types are rich in oil percentage but contain more of hydrocarbons and less oxygenated constituents and are therefore inferior in olfactory value compared to the north Indian types. The various north Indian types too vary with respect to the percentage of the various oxygenated compounds present in them. Therefore, it is important as well as necessary to isolate or develop a clone with higher oil content and superior oil quality with wide geographic adaptability (Husain 1982, Sethi 1982). In breeding program involving intraspecific hybridization, chromosome identification becomes imperative. The work conducted so far on this genus has been confined to chromosome counts only mainly from meiotic studies. No systematic attempt has been made to study the chromosome morphology with a view to understand the chromosomal details.

Our karyomorphological data revealed somatic chromosome number 2n=20 from all the collections belonging to the genera Cymbopogon and Vetiveria. Detailed analysis showed that the karyotypes are nearly symmetrical with chromosomes having median to sub-median centromeres. The total chromatin length (haploid complement) is largest in Vetiveria zizanioides (41.57 µm) whereas it is smallest in Cymbopogon winterianus (24.13 µm). In general, there is not much variation in the overall chromosome morphology in the various collections as revealed by the comparison of TF % data ranging from 37.82 to 42.77 %. According to the degree of asymmetry, the karyotypes are classified as group 1A (Stebbins 1971) representing symmetrical karyotype.
Karyotype asymmetry is a good expression for the general morphology of karyotypes in plants. Changes in morphological characters of the genome have been frequently related to evolution in higher plants. The quantitative estimation of the intrachromosomal asymmetry, which is due to centromere position was first addressed by Huziwara (1962) and denoted by TF %. Later, Stebbins (1971) in his masterpiece ‘Chromosomal evolution in higher plants’, summarized a system of twelve categories to measure and classify the characters and proposed a general trend of increasing asymmetry associated with increasing specialization. Four classes (1 to 4) were defined according to the increasing proportion of chromosomes with arm ratio < 2:1, to be combined with three classes (A to C) defined according to the increasing ratio between largest and smallest chromosome in a complement.

Lavania (1988) reported the chromosomal number of numerous species and chemotypes of the genus *Cymbopogon* from various parts of India. The different species revealed 2n=20, or its multiple with the range of total chromatin length (haploid complement) varying from 17.46 to 28.7 µm in diploid, 38.9-42.7 µm in 2n=40 type and 81.75 µm in 2n=60 type and the occasional presence of satellite chromosomes. The total form % data ranged from approximately 36 to 44 %. With the exception of the presence of satellite chromosomes, our findings on the study of the three species of *Cymbopogon* (*C. martini*, *C. winterianus* and *C. citratus*) are similar to the previous reports in relation to the chromosome number, total chromatin length, types of centromeric constrictions and the total form percentage. Twenty different collections representing different plant types of *Vetiveria zizanioides* L. Nash have been studied and reported by Lavania (1985). The somatic chromosome number in all the collections was found to be the same i.e. 20, as reported by earlier workers and cited by Fedorov (1969). The karyotypes were nearly symmetrical with chromosomes having median to submedian centromeres in all the cases, total haploid chromatin length varying from 25.6-38.7 µm with total form (TF %) ranging from 40 to 46 % approximately. Cytogenetic studies of *Vetiveria* germplasm in Thailand by Kongprakhon et al., (2003) showed that all the clones/ecotypes had the same chromosome number, 2n = 2x = 20.
Our data revealed similar observations in karyomorphological analysis of *Vetiveria zizanioides* L. Nash. The karyological observations mentioned in this work could be helpful in chromosome identification, assessment of karyological relationships and ploidy variations.

**SECTION II**

Essential oils extracted from the aromatic grasses like *Cymbopogon martini*, *Cymbopogon winterianus*, *Cymbopogon citratus* and *Vetiveria zizanioides* are of enormous commercial value for environmental, agricultural, food and medical applications as well as in perfumery and aromatherapy. There is an increasing demand for screening of new natural active constituents with applications in pharmaceutical and food industry. A number of essential oils and their components have been registered and classified as GRAS (Generally Recognized As Safe) by the US FDA and approved for use as food additives. Nevertheless, report by Naik et al., (2003) mentioned there is an inverse relationship between the dietary intake of antioxidant rich foods and incidence of human disease. Currently data regarding the toxicity studies of these four essential oils is scarce. Tripathi et al., (2006) showed that oral administration of vetiver oil in rats for 45-90 days elicited mild hematotoxic effect. Hepatotoxic and nephrotoxic effects in mice treated with the extracts of *Cymbopogon citratus* (30 and 80 %) were observed by Guerra et al., (2000). Recently Sousa et al., (2010) reported cytogenotoxicity of the extract from *Cymbopogon citratus* (DC) Stapf inducing chromosome aberration and cell death in roots of *Lactuca sativa*. In addition, estragole and methyl eugenol were found to be genotoxic and were delisted from GRAS (Commission Decision of 23 January, 2002). Considering this, our study was focused on the safety evaluation of the palmarosa, citronella, lemongrass and vetiver acetate essential oils. The present investigation suggested that human lymphocytes exposed to the essential oils significantly modified mitochondrial activity, induced DNA damage, generated reactive oxygen species and lead to death primarily by apoptosis.
In the present work an effort was made to study the cytotoxicity and genotoxicity of the essential oils in human lymphocytes. Human lymphocyte cells incubated with the essential oils revealed reduction in cell viability at higher concentrations as assessed by the trypan blue dye exclusion test and MTT assay. The four essential oils were found to be significantly cytotoxic at high concentrations as shown by the MTT assay. Results of the trypan blue dye exclusion test differed from the MTT assay which did not reflect significant reduction in cell viability. As trypan blue dye reflects cell death only by a loss of plasma membrane integrity associated with necrosis (Bonfoco et al., 1995), the results could be correlated to the effect on metabolic activity or apoptosis as evidenced by the MTT assay. To establish the mode of cell death in human lymphocytes, we conducted Annexin/PI double staining method using flow cytometry that showed the percentages of cells undergoing apoptotic and necrotic cell death. Flow cytometry analysis of lymphocytes exhibited predominant early and late apoptosis in lemongrass and vetiver acetate oils in comparison with the other two essential oils. The number of lymphocyte cells undergoing death by necrosis was negligible in comparison to the number apoptotic cell death for all the four essential oils.

In addition to cytotoxicity, DNA damage was evaluated in human lymphocytes by alkaline comet and DNA diffusion assays. The genotoxicity assays confirmed DNA damages in lymphocytes at concentrations reflecting significant cytotoxicity. Similar to the results of alkaline comet assay, the DNA diffusion assay demonstrated formation of DNA fragments at higher concentrations which might be due to apoptosis/necrosis. The results of the alkaline comet assay and DNA diffusion assay revealed a dose dependant increase in the genotoxicity. Palmarosa and citronella oils reflected weaker genotoxic potential than lemongrass and vetiver acetate oils. The palmarosa and citronella oils were found significantly genotoxic to human lymphocytes at concentration 1000 µg/ml whereas lemongrass and vetiver acetate oils induced significant genotoxicity at concentration 100 µg/ml.

Due to the presence of great number of constituents, essential oils seem to have no specific cellular targets (Carson et al., 2002). As typical lipophiles, they can pass through the
cell wall and cytoplasmic membrane, disrupt the structure of their different layers of polysaccharides, fatty acids and phospholipids and permeabilize them in various prokaryotic and eukaryotic systems. Essential oils can coagulate the cytoplasm and damage lipids and proteins. In eukaryotic cells, the essential oils can provoke depolarization of the mitochondrial membranes by decreasing the membrane potential, affect calcium ions cycling (Richter and Schlegel, 1993; Novgorodov and Gudz, 1996; Vercesi et al., 1997) and other ionic channels and reduce the pH gradient, affecting the proton pump and the ATP pool. They change the fluidity of membranes, which become abnormally permeable resulting in leakage of radicals, cytochrome C, calcium ions and proteins, as in the case of oxidative stress and bioenergetic failure. Permeabilization of outer and inner mitochondrial membranes leads to cell death by apoptosis and necrosis (Armstrong, 2006; Yoon et al., 2000).

Therefore to elucidate the possible cause of apoptosis and DNA damage among the various other mechanisms the intracellular redox status was probed. It recent years, antioxidants and prooxidants have been extensively studied. It seems that most of the antioxidants can behave as prooxidants depending on their concentration and the nature of the neighboring molecules (Villanueva and Kross 2012). Therefore, we determined the ability of the essential oils to generate reactive oxygen species. Generation of reactive oxygen species and cell death was low in human lymphocytes treated with palmarosa oil (~ 1.2 fold) whereas it was found to be highest in lemongrass oil (~ 2.5 fold). The degree of reactive oxygen species generation was in the decreasing order of lemongrass>vetiver acetate>citronella>palmarosa oils. The result of DCFH-DA staining may lead to the speculation that the test substances can behave as a prooxidant at certain concentrations. Similarly, well known antioxidants such as vitamin C, α-Tocopherol, carotenoids, flavonoids and phenols were found to become prooxidants at high concentrations (Cillard et al., 1980; Duarte and Lunec 2005; Galati and O’Brien 2004, Yordi et al., 2012; Young and Lowe 2001).

Natural antioxidants are in high demand for application as nutraceuticals, as cosmetics and as well as food additive because of consumer preferences. Oxidative stress
and DNA damage are related to various diseases and pathological conditions such as carcinogenesis, atherosclerosis, cardiovascular, neurodegenerative disease as well as inflammation and ageing (Bonomoni et al., 2008; Ishii, 2007; Klaunig et al., 2004; Laviano et al., 2007). As shown by numerous studies, plant-derived natural chemicals have protective effects against genotoxicity induced by oxidative stress (Ruberto and Baratta, 2000; Glei et al., 2006; Plazar et al., 2008).

Further, investigations were carried out at low concentrations at which the essential oils were found to be non cytotoxic and non genotoxic. The essential oils exhibited both antigenotoxic and antioxidant activities. Our findings showed that the essential oils conferred higher protective effect against an oxidizing agent (hydrogen peroxide) than an alkylating agent (methyl methanesulphonate), indicating their protective efficacy against cellular oxidative stress. Soltani et al., (2008), reported the antigenotoxic effects of umbelliprenin on human peripheral lymphocytes exposed to oxidative stress induced by hydrogen peroxide. Similarly DNA protective effects of few plant volatiles such as eugenol and borneol were investigated against hydrogen peroxide (Slamenova et al., 2009). Borneol and eugenol differed with respect to their DNA-protective effects. While borneol protected HepG2 and, to a lesser extent, VH10 cells (but not Caco-2) against hydrogen peroxide-induced DNA damage, eugenol either did not change the cellular sensitivity to hydrogen peroxide (HepG2 cells) or it even increased the sensitivity (Caco-2 and VH10 cells).

Among the cellular molecules, lipids that contain unsaturated fatty acids with more than one double bond are particularly susceptible to the action of free radicals. The resulting reaction known as lipid peroxidation, disrupts biological membranes and is thereby highly deleterious to their structure and function (Bakkali et al., 2008). Therefore, lipid peroxidation is being studied extensively in relation to disease and modulation by antioxidants. Our results suggested that the use of the essential oils may afford a cytoprotective effect as they can inhibit lipid peroxidation. Palmarosa, citronella and lemongrass oils significantly inhibited lipid peroxidation at all the tested concentrations in a dose dependant manner. Vetiver acetate oil could inhibit lipid peroxidation at all the concentrations except at the lowest concentration (10 µg/ml).
The antioxidant defense system of our body comprises of several endogenous antioxidants, such as superoxide dismutase, catalase, glutathione peroxidase, glutathione reductase, glutathione, ascorbic acid, uric acid etc. which act either independently, co-operatively or even synergistically against free radicals. These antioxidants protect against the deleterious effects of reactive oxygen species either by scavenging them or converting them to non toxic compounds or chelating the ions required for their activation (Carocho et al., 2013). The free radical scavenging capacity of the essential oils was evaluated by means of DPPH assay. Decolorization of DPPH free radical reflects the ability of the antioxidant species to donate electrons or hydrogen atoms to inactivate this free radical cation. Methanolic solutions of the essential oils palmarosa, citronella, lemongrass and vetiver acetate significantly quenched DPPH free radical in a dose-dependent manner.

The antioxidant activity of palmarosa essential oil has also been evaluated by Lawrence et al., (2012) using DPPH assay, Nitrogen oxide assay, reducing power assay, β-carotene bleaching assay and ferric reducing antioxidant power (FRAP) method. Their results indicated that palmarosa essential oil is effective in scavenging free radical and has the potential to be a powerful antioxidant. There was a constant increase in the reducing activities with the increase in concentrations in both reducing activity and FRAP methods. Antioxidant properties of lemongrass and vetiver essential oils have been investigated previously. Methanol, methanol/water extracts, infusion, and decoction of Cymbopogon citratus (lemongrass) were assessed for free radical scavenging effects measured by the bleaching of the DPPH radical, scavenging of the superoxide anion, and inhibition of the enzyme xanthine oxidase (XO) and lipid peroxidation in human erythrocytes (Cheel et al., 2005). The extracts presented effect in the DPPH and superoxide anion assay, with values ranging between 40 and 68 % and 15-32 % at 33 and 50 µg/ml, respectively, inhibited lipid peroxidation in erythrocytes by 19-71 % at 500 µg/ml and were inactive toward the XO at 50 µg/ml. Isoorientin, isoscoparin, swertiajaponin, isoorientin 2”-O-rhamnoside, orientin, chlorogenic acid, and caffeic acid were isolated from lemongrass extract and identified by spectroscopic methods. Isoorientin and orientin presented similar activities toward the DPPH (IC50, 9-10 µM) and inhibited lipid peroxidation by 70 % at 100 µg/ml. Caffeic and chlorogenic acid were active superoxide anion scavengers with IC50 values of 68.8 and 54.2
µM, respectively, and a strong effect toward DPPH. Caffeic acid inhibited lipid peroxidation by 85 % at 100 µg/ml. Antioxidant capacities of vetiver (Vetiveria zizanioides) oil were evaluated by two different in vitro assays, the DPPH free radical scavenging assay and the Fe²⁺ metal chelating assay (Kim et al., 2005). Their results showed that the vetiver oil possessed a strong free radical scavenging activity when compared to standard antioxidants such as butylated hydroxytoluene (BHT) and alpha-tocopherol. However, its metal chelating capacity was relatively weak. Vetiver oil (10 µl/ml) dissolved in methanol exhibited about 93 % free radical scavenging activity in the DPPH assay and about 34 % Fe²⁺ chelating activity in the metal chelating assay. Among the complex constituents in the crude vetiver oil, β-vetivenene, β-vetivone, and α-vetivone, which had shown strong antioxidant activities, were isolated and identified using various chromatographic techniques including silica gel open column chromatography, silica HPLC, and GCMS. The results showed that vetiver oil and some of its inherent components can be potential alternative natural antioxidants. From our experimental results, it can be said that the palmarosa, citronella, lemongrass and vetiver acetate oils are safe. Thus, they can be used as antioxidant in therapeutic uses for human being. The protective activity of the essential oils was further validated in Swiss albino mice against cisplatin-induced toxicities in kidney and bone marrow cells.

SECTION III

Antineoplastic drugs used in the treatment of cancers exhibit variable renal tolerance profiles. In addition, myelosuppression leading to anemia and thrombocytopenia is a frequent and major complication of cancer therapy (Hoagland 1982; Khynriam and Prasad 2001). Among drugs with a potential for renal toxicity, platinum salts, especially cisplatin is a well known agent that may induce acute and chronic renal failure. Cisplatin is one of the most broadly effective chemotherapeutic agents used for the treatment of many cancers such as head and neck, testicular, ovarian, small cells and non-small cell cancers (Miller et al., 2010). Clinical application of cisplatin at high dosage is limited due to its deleterious side effects often leading to discontinuation of treatment. The major side effects include nephrotoxicity, hepatotoxicity, ototoxicity, myelosuppression and spermatotoxicity (Al-
Kharusi et al., 2013). About 25-30% of the patients administered with cisplatin experiences various degrees of renal dysfunction (Yao et al., 2007). Acute kidney injury remains a significant cause of increased morbidity and mortality among patients, particularly in critical care units. Although several therapeutic strategies have been suggested for prevention of this condition, no specific treatments are currently recommended; except for vigorous hydration with normal saline (Launay-Vacher et al., 2008). Therefore, new and effective therapeutic strategies are needed for the prevention of cisplatin-induced toxicity.

Cisplatin has multiple intracellular effects, including regulating genes, causing direct cytotoxicity with reactive oxygen species, activating mitogen-activated protein kinases, inducing apoptosis, and stimulating inflammation and fibrogenesis (Pabla and Dong 2008; Nagwani and Tripathi et al., 2010). Cisplatin-DNA crosslinks cause cytotoxic lesions in tumors and other dividing cells. DNA damaging agents usually have less toxicity in non proliferating cells, yet the quiescent proximal tubule cells of kidney tissue are selectively damaged by cisplatin (Attia 2010). It induces oxidative stress with increased generation of reactive oxygen species due to depletion of the antioxidant enzymes and proteins (Baliga et al., 1998; Masuda et al., 1994; Matsushima et al., 1998; Sadzuka et al., 1992). In addition, inflammation with enhanced production of proinflammatory cytokines seems to play an important role leading to toxic effects in the normal tissues such as kidney, liver and bone marrow (Yao et al., 2007).

Various treatment strategies have been implicated to reduce the side effects of cisplatin chemotherapy since its clinical use. Several antioxidants and anti-inflammatory agents are proven effective in protecting the normal tissues against the toxic side effects of cisplatin treatment. Antioxidants including quercetin, coenzyme Q10, naringenin, pravastatin, tannic acid and rosiglitazone have shown protection against cisplatin toxicities in animal models (Ahmad and Sultana 2011; An et al., 2011; Badary et al., 2005; Fouad et al., 2010; Lee et al., 2006; Premkumar et al., 2003; Sanchez-Gonzalez et al., 2011). Thus in the present study, we investigated the ameliorating effect of the essential oils against cisplatin-induced nephrotoxicity and bone marrow toxicity (myelosuppression).
In the present investigation, results of the *in vivo* experiments revealed that priming animals with the essential oils can significantly ameliorate cisplatin-induced toxicities. Cisplatin administration significantly decreased the body weight of the animals as a result of reduced appetite and dysfunction of gastrointestinal system. The reduction may also be due to either tubular injury, which affects water reabsorption leading to dehydration and loss of body weight or to cytotoxic effects of cisplatin on the gastrointestinal tract (Al-Kharusi et al., 2013). In addition, cisplatin treatment affected the kidneys as evidenced from significant increase in the organ weight. The reduction in body weight may be due to renal tubular injury affecting water reabsorption and dehydration. A marked recovery from loss in body weight and altered kidney weight was observed by priming animals with essential oils.

The disproportionate accumulation of cisplatin in kidney tissue contributes to cisplatin-induced nephrotoxicity. Cisplatin is selectively accumulated in the proximal tubular cells that results in nephrotoxicity. The cisplatin concentration in the proximal tubular epithelial cells is 5 times the serum concentration (Kuhlmann et al., 1997). Uptake of cisplatin is mainly through the organic transporter pathway. The kidney accumulates cisplatin to a greater degree than any other organ and is the major route for its excretion. In the rat, cisplatin excretion occurs predominantly by glomerular filtration and to a lesser extent by secretion. There is no evidence of tubular reabsorption. Cisplatin is accumulated by peritubular uptake in both the proximal and distal nephrons (Arany and Safirstein 2003; Kroning et al., 2000). The S3 segment of the proximal tubule accumulates the highest concentration of cisplatin followed by the distal collecting tubule and the S1 segment in the proximal tubule (Kroning et al., 2000). In addition to the transporter mediated process, cisplatin enters the cell through passive diffusion. Transporter mediated uptake is likely the major pathway in renal cells. The organic cation transporter (OCT 2) is the critical transporter for cisplatin uptake in proximal tubules in both animals and humans. The expression of another transporter, copper transporter receptor 1 (Ctr 1) along with the organic cation receptor 2 (OCT 2) is high in the kidney tissues. These two receptors actively transport cisplatin into the kidney tubules contributing to the fact that kidney has the highest concentration of cisplatin compared to any other organ in the body. Metabolism of cisplatin to nephrotoxic molecules in the proximal tubule cells is another cause of cellular injury and
nephrotoxicity (Townsend et al., 2003). Cisplatin is conjugated to glutathione and then metabolized through a γ-glutamyl transpeptidase and a cysteine-S-conjugate β-lyase-dependent pathways to a reactive thiol, a potent nephrotoxin (Yao et al., 2007).

Cisplatin nephrotoxicity is chiefly characterized by tubular damage, mainly affecting the renal proximal and distal tubuli. In animal models, cisplatin damages the proximal tubules, specifically the S3 segment of the outer medullary stripe. Mitochondrial swelling and nuclear pallor occur in the distal nephron. The glomerulus has no obvious morphological changes (Cornelison and Reed 1993; Meyer and Madias 1994; Vickers et al., 2004). Only a few studies have described the pathological results associated with cisplatin-induced nephrotoxicity in humans (Tanaka et al., 1986). The site of injury involves either distal tubules and collecting ducts or the proximal and distal tubules. In patients with acute renal failure, the predominant lesion is acute necrosis located in the proximal convoluted tubules. Tubular damage may range from a mere loss of the brush border of epithelial cells to an overt tubular necrosis in severe cases. The severity of necrosis is dose, concentration and time dependent. Patients with chronic nephrotoxicity have focal acute tubular necrosis characterized by cystic dilated tubules lined by a flattened epithelium showing atypical nuclei and atypical mitotic figures with hyaline casts. Long term cisplatin treatment and injury may cause cyst formation and interstitial fibrosis (Cornelison and Reed 1993). In the present study, histopathological examination of the kidney tissues of mice treated with cisplatin revealed acute tubular necrosis. Priming animals with the essential oils (palmarosa, citronella, lemongrass and vetiver acetate) for a period of 7 days prior to cisplatin, leads to improvement of tubular cell necrosis. Similar results have been observed in murine model by many researchers (Ahmad and Sultana 2011; Ali et al., 2008; Al-Kharusi et al., 2013; Chakraborty et al., 2011; Fouad et al., 2010; Lee et al., 2006; Menon and Nair 2013; Nagwani and Tripathi 2010)

Tubular damage causes impaired reabsorption, which underlies the observed proteinuria, hypomagnesemia and hypokalemia. In addition, cisplatin nephrotoxicity often progresses with reduced glomerular filtration rate and increased serum creatinine, which may result from the onset of tubuloglomerular feedback mechanism and to reduced renal
blood flow resulting from renal vasoconstriction. Cisplatin treatment affects kidney function as reflected by elevated blood urea nitrogen (BUN) and creatinine levels in the serum (Baliga et al., 1999; Chakraborty et al., 2011; Menon and Nair 2013). Blood urea nitrogen measures urea nitrogen which is a waste product of protein metabolism, in the blood. Urea is formed by the liver and carried by the blood to the kidneys for excretion. As urea is cleared from the blood stream by the kidneys, a test measuring the amount of urea nitrogen remaining in the blood can be used as a marker of renal function. Rise in the levels of BUN results from improper and deficient functioning of damaged kidneys. Creatinine levels are also elevated due to deficient filtering capacity of the kidneys. Creatinine is a spontaneously formed cyclic derivative of creatine. Creatinine is chiefly filtered out of the blood by the kidneys. If the filtering of the kidneys is deficient, creatinine levels in the blood rises. Pretreatment with the essential oils provided significant protection against cisplatin-induced nephrotoxicity as evidenced by lowered levels of BUN and creatinine in the serum.

It is well documented that cisplatin-induced nephrotoxicity has been associated with oxidative stress, DNA damage and apoptosis (Lee et al., 2006; Chaney et al., 2004; Hannemann et al., 1988). Cisplatin induces the generation of various reactive oxygen species (ROS) through inactivation of cellular antioxidant system, disruption of mitochondrial respiratory chain or interaction with microsomal cytochrome P450. Highly potent ROS appears to target multiple cellular components, such as lipids, proteins and DNA and activate multiple signaling pathways and thereby implicated in the pathogenesis of acute cisplatin-induced renal injury. Various oxidative stress markers such as lipid and protein oxidation products were markedly elevated by cisplatin-challenge. Lipid peroxidation constitutes complex chain reaction of free radicals, which leads to the degradation of polyunsaturated fatty acid in cell membranes. The preservation of cellular membrane integrity depends on protection or repair mechanisms capable of neutralizing oxidative reactions. Cisplatin generated ROS such as superoxide anion and hydroxyl radicals stimulates renal lipid and protein oxidation determined by increased level of MDA and protein carbonyls. In the present study the essential oils were able to reduce cisplatin-induced lipid peroxidation as well as protein carbonyls in kidney tissue of mice.
One of the most important intracellular antioxidant systems is the glutathione redox cycle. Antioxidants particularly glutathione (GSH) is considered a sensitive stress marker as it helps in maintaining the integrity of mitochondria and cell membrane. Glutathione is one of the essential compounds for maintaining cell integrity because of its reducing properties and participation in the cell metabolism. Its high electron donating capacity (high negative redox potential) combined with high intracellular concentration (millimolar levels) generate great reducing power. This characteristic underlies its potent antioxidant action and enzyme cofactor properties, and supports a complex thiol exchange system, which hierarchically regulates cell activity. GSH is an extremely important cell protectant. It directly quenches reactive hydroxyl free radicals, other oxygen-centered free radicals, and radical centre on DNA and other bio-molecules. Cisplatin induced glutathione depletion is a determinant step towards oxidative stress and renal toxicity. Our results agree with the other reports pertaining to cisplatin-induced renal GSH depletion (An et al., 2011; Badary et al., 2005; Hassan et al., 2010; Joy and Nair 2008; Menon and Nair 2013; Mistry et al., 1991).

Antioxidant enzyme GST plays an important role in detoxification/transport of many DNA alkylating agents, carcinogens and xenobiotics by catalyzing the conjugation of GSH with these chemicals or active metabolites (Hayes and Pulford 1995). GSTs are induced under conditions of oxidative stress, and alpha, pi, mu, and theta-class GSTs are active in detoxification of organic epoxiced, hydroperoxides, and unsaturated aldehydes, including reactive purine and pyrimidine bases and lipid peroxides produced by reactive oxidant damage to DNA and lipids, respectively. The antioxidant status of the kidney tissue of mice was found to be decreased after cisplatin administration. Depletion of GSH content and reduced GST activity was restored by administration of the essential oils prior to cisplatin which may be due to its free radical scavenging activity (Ahmad and Sultana 2011; Ali et al., 2008; An et al., 2011; Arhogro et al., 2012; Badary et al., 2005; Chakraborty et al., 2011; Hassan et al., 2010; Menon and Nair 2013).

Previous studies showed that cisplatin induces genotoxicity due to its interaction with DNA, forming cisplatin-DNA adducts (Attia 2010; Ahmad and Sultana 2011; Premkumar et al., 2001; Wozniak et al., 2004). In vitro and in vivo studies have indicated
that cisplatin can induce various types of genotoxic damage, of which DNA cross-linking is the most important one. Direct breakage of the DNA strands occurs when reactive oxygen species interact with DNA (Moller et al., 1998). DNA damage evaluated by alkaline comet assay in both kidney and bone marrow cells revealed significant amount of DNA strand breaks. It is evident from the present study that the essential oils significantly prevented genotoxicity in both kidney and bone marrow cells.

Moreover, the clastogenic potential of cisplatin has become of great interest because of its serious effects on the chromosomes of non-tumor cells (Attia 2010). In patients treated with long term cisplatin, genetic damage can be observed during chemotherapy or many years later. In the present investigation, significant incidence of chromosomal aberrations was prevalent in the bone marrow cells of the cisplatin administered mice. This damage, particularly in the population of undifferentiated cells such as bone marrow, is dangerous because it can lead to mutations and DNA rearrangements. If such cells survive and proliferate, the risk of secondary acute myeloid leukemia and other drug related cancers can increase (DeMas et al., 2001; Elsendoom et al., 2001; Osanto et al., 1991). In the current study, the chromosomal aberrations revealed that chromatid breaks and gaps occurred frequently. The divisional index (mitotic index) of the bone marrow cells of cisplatin treated mice exhibited a marked reduction in the number of undergoing mitotic cell division. Essential oils gavaged to mice prior to cisplatin injection significantly protected the bone marrow cells from cisplatin-induced clastogenic effects and myelosuppression at lowest concentration of 5 mg/kg. Previously, amelioration of cisplatin-induced clastogenic effects by prior oral administration of quercetin has been reported by Attia (2010).

Cell cycle analysis demonstrated that cisplatin has the ability to arrest cell cycle progression independent of stages of cell division (da Silva et al., 2010). Incidence of clastogenicity was reduced with restoration of mitotic index and normal cell cycle progression. According to Attia (2010), cisplatin treatment resulted in significant decrease in bone marrow GSH, increased ROS generation with the development of clastogenic effects and apoptosis. Quercetin was effective in restoring GSH levels, reducing ROS generation,
clastogenesis and apoptosis in the bone marrow cells possibly by its free radical scavenging activity.

Various hematological parameters are usually monitored during antineoplastic therapy (Hoagland 1982). Acute dose dependent depression in leukocytes (leucopenia), erythrocytes (anemia) and platelets (thrombocytopenia) has been reported after treatment with cisplatin in Fischer 344 rats (Ohno et al., 1993). In agreement with the previous findings, bone marrow toxicity and subsequent myelosuppression induced by cisplatin was evident from the hematological parameters studied in our experiment. In the present study, cisplatin treatment caused reduction in the number of peripheral leukocytes, RBC, hemoglobin and platelet. The essential oils gavaged to the mice could retrieve the reduced erythrocyte, leukocyte and platelet count to the normal range along with hemoglobin content. It has been suggested that decreased blood antioxidant capacity, indicating impairment of erythrocyte fragility, decreased erythrocyte life span and anemia (Durak et al., 1994).

A possible mechanism by which the essential oils exerted protective effects in this study is by acting as an antioxidant, quenching the free radicals (ROS) generated by cisplatin (Badary et al., 2005; Chakraborty et al., 2011; Menon and Nair 2013; Naziroglu et al., 2004). In our preliminary experiments, the essential oils were administered to mice for seven days and sacrificed for estimation of GSH, lipid peroxidation and GST activity from the kidney tissues. The results indicated that the antioxidant status in kidney was elevated at all the treatment doses as reflected by elevated GSH content and GST activity with simultaneous decrease in lipid peroxidation. The antioxidant capacity in mice was most pronounced at the treatment dose of 5 mg/kg body weight of the four essential oils. Similarly, the ameliorating activity of three different doses of the essential oils (5, 10 and 20 mg/kg body weight) on cisplatin-induced toxicities revealed that the lowest dose of 5 mg/kg was most efficient. Thus, the effective dose of pre-treatment with the essential oils against cisplatin-induced toxicities in mice was found to be 5 mg/kg through oral administration. The probable reason behind the lowest dose of treatment being most effective may be due to the fact that the essential oils has high free radical scavenging capacity at concentration 5 mg/kg body weight and at higher concentrations, they could behave as prooxidants. Al-
Kharusi et al., 2013 have studied the protective effects of ellagic acid against cisplatin-induced nephrotoxicity in rats at different doses. They reported that the lowest treatment concentration (30 mg/kg body weight) of their treatment regimen was most effective against cisplatin toxicity.

Taken together our findings suggested that when cisplatin is used in combination with the essential oils, it can minimize oxidative stress-induced kidney and bone marrow toxicity by recovery from the oxidative stress. This study promises the beneficial use of the essential oils palamarosa, citronella, lemongrass and vetiver acetate and further necessitates experimental (tumor model) and clinical studies.