Chapter 4

Effect of Aqueous Extract of *Phyllanthus fraternus* and *Aegle marmelos* against Cyclophosphamide-Induced Reproductive Toxicity

### 7.1. Introduction

CPA is a drug with a wide spectrum of clinical uses. The cytotoxicity of CPA is mediated by alkylation of DNA at the N7 position of guanine and the formation of DNA-DNA cross-links; DNA-protein cross links and single strand breaks (Hemminki and Kallama, 1985; Crook *et al*., 1986; Devi *et al*., 2014). The impact of the chemotherapy treatment on fertility and gamete quality is of concern as a number of these patients are of reproductive age (Meistrich, 2009). Testes are the male gonad in animals, provided with functions such as, production of sperms and androgens. Internally, testis contains very fine coiled tubes called as seminiferous tubules. These tubules, internally and externally lined with many cells, such as, germ cells, sertoli cells, leydig cells, etc. In CPA therapy, there is a common continuing problem in the treatment of a variety of glomerular diseases, which leads to gonadal toxicity as a side effect of the drug (Elangovan *et al*., 2006). Adult male patients treated with CPA have demonstrated, diminished sperm counts and an absence of spermatogenic cycles in their testicular tissue (Jalali *et al*., 2012). In the testis, CPA disrupts meiotic events during spermatogenesis before pachytene stage, thus emphasizing the potential for adverse progeny outcomes due to genotoxic damage. To maintain genomic stability, eukaryotic cells respond to genetic damage by arresting or delaying cell cycle progression (Elangovan *et al*., 2006).
Mammalian spermatozoa are particularly vulnerable to oxidative damage because of high concentration of polyunsaturated fatty acids and low antioxidant capacity (Vernet et al., 2004). Based on this concept, combination of the drug delivery together with the potent and safe antioxidant may be an appropriate approach to ameliorate CPA-induced reproductive toxicity.

CPA generates ROS such as superoxide anions, hydroxyl radicals, etc., that induces oxidative stress and inhibit the activity of antioxidant enzymes in several tissues. This insufficiency of antioxidant system may result in oxidative stress. Oxidative stress may be defined as an imbalance between the oxidant production and oxidative capacity of the cell to prevent oxidative injury (Verma and Vinayak, 2008).

In recent years, considerable attention has been devoted to medicinal plants, particularly rich in polyphenols mainly flavonoids and phenolic acids, such as Cuscuta reflexa (Amar bel), Emblica officinalis (Amla), Glycyrrhiza glabra (Sweet wood), Solenum nigrum (Makoy), Daucus carota (Carrot), etc. which exhibit antioxidant properties due to their hydrogen-donating and metal-chelating capacities as potential chemo preventive agents (Gupta and Sharma, 2006). The protective role of plant is due to the presence of antioxidative constituents, which are able to delay or inhibit the oxidative stress. In recent study, it has been reported that, medicinal plants such as Carya illinoensis (Pecan nut shell), Zingiber officinale (Ginger) and Achillea millefolium (Rojmaari) with antioxidant activity, might be useful in the prevention of side effects of CPA-induced testicular toxicity (Benvegnu et al., 2013; Jalali and Hasanzadeh, 2013; Mohammadi et al., 2013).

Considering the diverse medicinal properties of P. fraternus and A. marmelos, the present study was undertaken to evaluate the CPA-induced changes in sperm characteristics and testicular oxidative damage in mice.
7.2. Materials and Methods

7.2.1. Animals and Treatment: Adult (age 12-15 weeks) male laboratory mice of Parke’s strain weighing 30 ± 3 gm were used in the investigation. Mice were allocated into 10 groups (Group I-X), each group comprising six animals. In a group, all animals had more or less identical body weight (bw). Mice in each group were housed separately in polypropylene cage (430 mm X 270 mm X 150 mm) and the treatment was done as shown below:

Group I  Control mice received distilled water (intraperitoneally) once in a week for 5 weeks.
Group II  AEPF (400 mg/kg bw, orally) once in a week for 5 weeks.
Group III  AEAM (600 mg/kg bw, orally) once in a week for 5 weeks.
Group IV  Received CPA 200 mg/kg bw, for 5 weeks (once in a week) by intraperitoneal injection.
Group V-VII  Received CPA (200 mg/kg bw, intraperitoneally) and AEPF 200, 300 and 400 mg/kg bw, orally for 5 weeks (once in a week).
Group VIII-X  Received CPA (200 mg/kg bw, intraperitoneally) and AEAM 400, 500 and 600 mg/kg bw, orally for 5 weeks (once in a week).

After recording the body weight, animals were sacrificed by dislocation of cervical vertebrae. The testis was excised, cleared of the adhering tissues and fixed in Bouin’s solution for histopathological analysis by H & E staining. The epididymis was removed and used for sperm analysis. The sperm count, motility and viability of spermatozoa in the cauda epididymis were assessed by WHO Laboratory Manual (World Health Organization, 2010). Details of the procedure of the sperm parameters and histopathology are described in materials and methods.

7.2.2. Statistical Analysis: The results were expressed as Mean ± SE (SEM), analyzed through one-way ANOVA, followed by the post hoc Dunnett’s test for
comparison of various treatments using the SPSS 16.0. Differences were considered statistically significant at \( p < 0.05 \).

7.3. Results

7.3.1. Effect of AEPF and AEAM on the Gonadosomatic Index (GSI) against CPA-Induced Toxicity in Mice: GSI is the calculation of the gonad mass as a proportion of the total body mass. It is a tool for measuring the sexual maturity of animals in correlation to testis development. The treatment of male mice with CPA caused a significant \( (p < 0.001) \) loss in the GSI compared with the control group (Figure 7.1). There is a percent increase in GSI in a dose dependent manner with CPA + AEPF treated groups, as compared to the CPA-treated group. At low doses of AEPF (200 and 300 mg/kg) the effect was only marginal, whereas at the higher dose of AEPF (400 mg/kg) effectively prevented the CPA-induced testis damage. The treatment of CPA + AEAM (400, 500 and 600 mg/kg) caused a significant decrease \( (p < 0.001) \) in GSI (Figure 7.1).

7.3.2. Effect of AEPF and AEAM on the Sperm Parameters against CPA-Induced Toxicity in Mice: The effect of CPA and co-administration of AEPF on epididymal sperm count, sperm motility and sperm viability are presented in Figure 7.2. No significant changes in sperm parameters were observed in mice after AEPF alone administration when compared to the control group. On the other hand, significant decrease \( (p < 0.001) \) was observed in sperm count, sperm motility and sperm viability in mice exposed to CPA-treated group as compared to the control. Co-administration of AEPF with CPA treated animals resulted in significant increase \( (p < 0.001) \) in sperm parameters (sperm count, motile and viable sperm) when compared with CPA-treated mice. Co-administration of AEPF caused a significant increase in semen quality and minimized toxic effects of CPA. At low doses of AEPF (200 and 300 mg/kg) the effect was only marginal, whereas at the higher dose of AEPF (400 mg/kg) effectively prevented the CPA-induced testis damage. Administration of
AEAM caused a reduction in sperm count, motility and viability (Figure 7.2). In AEAM treated mice the cauda epididymal sperm parameters showed evidence of dose dependent toxicity. At higher dose of AEAM (600 mg/kg), the sperm count, motility and viability were very much reduced as compared to the lower doses (400 and 500 mg/kg) treated group as well as control group.

7.3.3. Effect of AEPF and AEAM on the Antioxidants Activity against CPA-Induced Toxicity in Testis of Mice: Intoxication of mice with CPA was followed by a significant decrease ($p<0.001$) in antioxidant enzyme activities such as SOD and CAT in the testis (Figure 7.3). The administration of AEPF restored activity of these enzymes near to normalcy compared to the CPA-treated group. It was evident from Figure 7.3, that CPA-treated mice have significantly higher ($p<0.001$) level of malondialdehyde (MDA) in relation to the control mice. The CPA-induced increased level of MDA was found significantly compensated by administration of AEPF in dose dependent manner. In AEPF alone administration showed no significant changes when compared with control group, indicating that it did not show any adverse effects. The results demonstrated that AEPF treatment provided significant protection against the MDA production induced by CPA in the mice testis. At low doses of AEPF (200 and 300 mg/kg) the effect was only marginal, whereas at the higher dose of AEPF (400 mg/kg) effectively prevented the CPA-induced testis damage. On the other hand, there was a decline in the activity of antioxidant enzymes with AEAM treatment in comparison to control group.

7.3.4. Histopathological Study of Testis: The spermatogonia, spermatocytes, spermatids and spermatozoa could be clearly identified in the seminiferous tubules (Figure 7.4). The well preserved sertoli cells and tubular basement membrane were observed in group I (control) and group II (AEPF). However, in the CPA-treated group, differences were observed in histopathology of testis such as complete atrophy of seminiferous tubules, degenerated germ cells, shrunken seminiferous tubules and widening of interstitial space (group IV). This indicated that, the testis tissues were
damaged by CPA as compared with the normal architecture of the control group. In the CPA + AEPF-treated groups, toxic effects were ameliorated in a dose dependent manner and restored these changes towards normalcy (group V to VII). The results showed better protective effect on the damaged testis in the AEPF at 400 mg/kg bw group than the rest of AEPF groups (200 and 300 mg/kg bw). In contrast, marked degenerative changes were observed in the histoarchitecture of the testis in CPA + AEAM-treated mice, showing disorganization and decreased amount of mature spermatozoa in seminiferous tubules as compared to control (group VII to X). In lower dose (400 mg/kg) group, seminiferous tubules exhibited active spermatogenesis, but there were loosening of germinal epithelium in seminiferous tubules (group VII). By contrast, marked alterations were noticed in testis of mice treated with higher doses of the AEAM. Intraepithelial vacuolation, loosening of germinal epithelium, marginal condensation of chromatin in round spermatids and degeneration of cell types were observed in the seminiferous tubules of AEAM treated (500 and 600 mg/kg) mice (group IX and X).

7.4. Discussion
Cancer chemotherapy drugs are known to produce toxic side-effects in multiple organ systems, including the testis (Jalali and Hasanzadeh, 2013). In a clinical context, testicular germ cells are damaged in patients exposed to chemotherapeutic drugs for a limited duration. It could result in long-term infertility or genetic alterations (Sawada et al., 1994). CPA is an effective anticancer drug but its use is limited due to its physiological side effects. It’s possible induction of reproductive toxicity in non-tumor cells has been documented in humans as well as in a variety of animal species (Delbès et al., 2010; Meistrich, 2009). An oxidation mechanism may be responsible for reproductive toxicity, wherein CPA and its metabolite acrolein cause an inactivation of cellular antioxidants system which results in increased ROS generation and lipid peroxidation (Shanmugarajan et al., 2008). Our present study revealed marked
protective effect of AEPF against CPA-induced oxidative stress and reproductive toxicity.

The observed reduction in GSI in CPA treated group may be explained by decline number of germ cells and a significant lower rate of spermatogenesis because the weight of the testis mainly depend on the mass of the differentiated spermatogenic cells (Oh et al., 2007). In the present study, decreased number of sperm in the epididymis may reflect less bioavailability or production of androgen in CPA treated mice (Katoh et al., 2002). It was established earlier that CPA causes testicular toxicity by germ cell degeneration and inhibits androgen production in adult male mice, probably by affecting pituitary luteinizing hormones and thereby inhibits Leydig cell testosterone production and serum testosterone levels, which in turn compromises spermatogenesis. Declining sperm count may be a consequence of ROS induced damage in infertile males. Decrease in sperm count is due to the generation of ROS by CPA (Selvakumar et al., 2005). It is believed that oxidative stress is a critical factor that should be considered in addition to hormonal and enzymatic factors in the CPA-induced testicular alterations observed here. Hence, the decrease in testicular sperm count in CPA treated mice reflects spermatogenic cell death. The present data shows that the administration of AEAM with CPA brought about significant loss in GSI, which is related to the number of spermatids and spermatozoa in the testis. Oral administration of Aegle marmelos to male mice caused a decrease in the GSI which could be attributed to the loss of germ cells (D’Souza and Narayana, 2001). Reduction in the GSI might be due to low level of androgen, which was not enough to maintain the weight of gonads (Anitha et al., 2013). The decreasing weight of the reproductive organs in the AEAM-treated male mice clearly indicated that the extract caused structural and functional alteration in the testes (Banerji et al., 2000).

Sperm count, motility and viability are also important parameters in respect to the fertility of the male. Sperm analysis was carried out to investigate the effect of CPA with AEPF and AEAM on male fertility. Deformity of sperm in mammals is used as an important parameter to access male fertility. Although, it is not possible to find a cause
of infertility in a large percentage of infertile men. An alteration of the spermatidic parameters such as decreased sperm count, diminished motility and viability in CPA administered mice, somewhat indicates reproductive toxicity (Shanmugarajan et al., 2008). The significant reduction in sperm count, motility and viability may be due to the toxic effect of CPA on the flagellum, the important machinery for motility of sperm cells. CPA treated mice showed decrease in testicular tricarboxylic acid cycle enzyme activities and thus impaired energy metabolism. It has been suggested that ATP may serve as an energy source for sperm motility and decrease in energy metabolism may be one of the limiting factors responsible for loss of sperm motility in CPA administered mice. A direct toxicity of CPA to the spermatogenic compartment may be considered as one of the mechanisms of action of CPA in producing the abnormal and dead sperms (Lui et al., 1986). The amount of testicular antioxidant enzymes declined significantly after CPA-treatment. It is well known that antioxidant enzymes are necessary for normal differentiation and development of spermatogonial cells to mature spermatozoa, possibly via protection from ROS injury. Present study indicates the beneficial effects of AEPF against CPA induced germ cell toxicity in mice. AEPF treatment improved the GSI, sperm count, motility and viability. In the present study, AEPF substantially attenuated the testicular spermatogenic cell damage induced by CPA treatment. Sperm count, motility and viability in the cauda epididymis were adversely affected in the CPA + AEAM treatment. It is known that the structure and function of the epididymis are dependent on androgens (Cooper, 1992). In the present investigation, a dose-dependent suppression of cauda epididymal sperm count, motility and viability suggested an undersupply of testosterone to epididymis and caused impaired epididymal function. This impairment may also be due to the reduced activity of the testis, which affects the normal passage of testicular fluid into the epididymis (Ansari et al., 1998). This is also confirmed by reduced GSI. Treatment with AEAM possibly inhibited the activity of ATP in spermatozoa by uncoupling oxidative phosphorylation from the respiratory chain and preventing phosphorylation of ADP to ATP (Sharma and Jacob, 2002), thus rendering the spermatozoa immotile. Inadequate concentration and
immotility of the spermatozoa means that they cannot penetrate the cervical mucus and thus fail to fertilize the ova (Lohiya and Goyal, 1992). The reduction in sperm parameters in cauda epididymis is of importance with regard to fertilization (Bedford 1983).

ROS are chemically reactive molecules containing oxygen. Examples include oxygen ion, peroxides and hydroxy radicals. Lipid peroxidation (LPO) is one of the main manifestations of oxidative damage initiated by ROS and it has been linked to alter membrane structure and enzyme inactivation. It is initiated by abstraction of a hydrogen atom from the side chain of polyunsaturated fatty acids in the membrane. Increased LPO is observed by MDA, the most frequently used biomarker (Oh et al., 2007).

Antioxidants such as SOD and CAT are involved in counteracting the toxicity of ROS. Under normal conditions these enzymes protect the cells and tissues from oxidative damage (Attia et al., 2012). SOD constitutes an important link in the biological defence mechanism through dismutation of endogenous cytotoxic superoxide radicals to H₂O₂ and molecular oxygen that are deleterious to polyunsaturated fatty acids and proteins. The activity of CAT is to degrade H₂O₂ into H₂O and O₂ (Acharya et al., 2008). In the presence of inadequate CAT activity, more H₂O₂ could be converted to toxic hydroxyl radical that may contribute to the oxidative stress of CPA toxicity. Decline in the activities in these enzymes might be due to their inactivation caused by excess ROS production. Thus the balance of this enzyme system is essential to dispose the superoxide anion and peroxides generated in testis (Latchoumycandane and Mathur, 2002). The present study showed the increased MDA level and decline activity of SOD and CAT in CPA-treated group. AEPF treatment significantly reversed the upsurge in the MDA level and normalizes the antioxidant activities. Almost restoration of the activity of SOD and CAT indicates that AEPF have good antioxidative/free radical scavenging property. It has been noted that, the activity of SOD and CAT in CPA + AEAM treated group, were decreased along with the elevation in testicular MDA level in comparison to the control. The reduced level of SOD and CAT might be due to the excess production of anions in response to the aqueous extract of A. marmelos. It is
possible that an increased rate of ROS production may inhibit the action of these antioxidant enzymes or the decreased expression of these antioxidant enzymes may cause increased oxidative stress (Anitha et al., 2013). SOD protects dehydratases against free radical, superoxide (Lenzi et al., 1993) and low CAT activity closely relate to low motility of ejaculated spermatozoa (Kawakami et al., 2007). AEAM showed antitesticular activities by inhibiting pituitary gonadotropin secretion. The extract has some direct effect on testicular tissue and sperm cell for induction of oxidative injury that may suppress the testicular steroidogenic and gametogenic activities (Das et al., 2009). Testicular MDA levels were significantly increased in CPA + AEAM treated group. The increased MDA level by AEAM through the generation of free radicals causes a disturbance in the antioxidant status of the testis. The lipid composition of the sperm membrane exerts a significant effect upon the functional quality of spermatozoa (Zalata et al., 1998).

Histopathological examination of testicular tissue of CPA-treated group revealed disturbed spermatogenesis, severe damage of seminiferous tubules that reached to early atrophic changes with complete loss of the spermatogonial cells together with decreased number of spermatids. The increased oxidative stress results in the lipid peroxidation, which affects the membrane integrity and fluidity. Further, the ROS mediated peroxidation of critical thiol groups in protein could alter the structure and function of spermatozoa (Sikka, 2004). Acrolein induced the formation of ROS, including superoxide anion, hydroxyl radical, hydrogen peroxide and hypochlorite, which could affect the acrosome integrity of human spermatozoa (Ichikawa et al., 1999). Histopathological observations showed that CPA + AEAM treatment disrupted seminiferous tubule architecture and consequently the spermatogenesis process. In AEAM treated mice, most of the seminiferous tubules were azoospermic (Figure 7.4, X). The affected tubules showed loosening of germinal epithelium, intraepithelial vacuolation and mixing of spermatids of different stages of spermatogenesis. Similar histopathological changes have also been described in testis of mice after treatment with aqueous leaf extracts of Azadirachta indica (Mishra and Singh, 2005), Allamanda
cathartica (Singh and Singh, 2008) and hexane extract of flower buds of Syzygium aromaticum (Singh and Chakravarty, 2001) and several other antispermatogenic agents such as gossypol tetra-acetic acid (Singh and Rath, 1990, 2009), nitrofurazone (Singh and Chakravarty, 2001) and 20,25-diazacholesterol dihydrochloride (Singh and Chakravarty, 2003). Furthermore, the treatment also caused significant reductions in the diameter of the seminiferous tubules of the germinal epithelium in testis of extract treated mice compared to controls. These observations are suggestive of the adverse effect of the treatment on spermatogenesis.

ROS such as free radicals may reduce sperm count, motility and viability. Ascorbic acid has been found to help in preventing cell damage by neutralizing free radicals. It is a powerful antioxidant and has been found to increase fertilization rates, enhances sperm quality and prevents sperm agglutination thus making them more motile with more forward progression (Glenville, 2008). In this study, we report that AEPF is used in herbal medicines to promote fertility in male mice. Ascorbic acid would be effective in improving male fertility. Ascorbic acid is moderately present in P. fraternus, which would increase the testosterone production, sperm count, motility, viability and thus enhances male fertility. The extract also contains flavonoids, which are associated with functions related to fertility enhancement. These listed constituents and bioactivities can serve as promoters of fertility in male mice (Glenville, 2008).

In AEPF administered mice, the observations were significantly positive as it improved all parameters used to determine testis injury. The study on the active principles in the aerial part of P. fraternus extract by GC-MS analysis clearly showed the presence of salicylate, chromane, grape seed oil, cineol, tartaric acid etc. The possible way of AEPF protection attributed to its antioxidative and free radical scavenging property, which might be due to these active components.

Alterations in the sperm parameters and histopathological examination of testis suggested a disturbed testicular and epididymal tissue due to AEAM treatment. Extract affects the sex organs due to some of the chemical compounds like coumarin present in
the extract which is selectively stored in the sex organs. It is well established that active sperm motility is a prerequisite to achieve fertilization. Sperms ejaculated into the vagina must reach to the ovum in the fallopian tube by penetrating the cervical mucus and passing through the uterine cavity. Because an adequate number of sperms possessing normal function are necessary for successful fertilization, any deviation that alters sperm function leads to infertility (Daniel et al., 2011). The results of this investigation showed that treatment with aqueous extract of A. marmelos, inhibiting the sperm motility in mice. This inhibition coincides with a gradual and significant decline in cauda epididymal sperm count and percentage of viable spermatozoa. A. marmelos contains coumarin which is known for selectively inhibiting the calcium channels (Nugroho et al., 2011). These calcium channels blockage might be a vital mechanism through which AEAM is acting as spermicidal agent. Role of calcium channels is implicated in the sperm viability (Brandelli et al., 1996; Goodwin et al., 1997) as it mediate acrosomal reaction of human sperm during fertilization and inhibitors of these channels may prevent sperm–egg fusion (Hershlag et al., 1995; Enders, 1997). Alternatively the plant may be exploited for use as an ‘antifertility drug’.

The protective activity of extracts has been compared against CPA-induced toxicity in testis. The results of administration of different doses of AEPF and AEAM with CPA showed that AEPF significantly normalized the CPA-induced toxicity while AEAM showed testicular damage and spermicidal activity (Sur et al., 1999, 2002). Therefore, it may be concluded that AEPF has a good efficacy while AEAM increased the damage caused by CPA.

### 7.5. Conclusion

The present results exhibited that the AEPF could protect the testis from toxicity induced by CPA. This activity can be partially attributed to the free radical scavenging activity and enhancement of the antioxidant system effectively by AEPF since many of the active ingredients like salicylate, chromane, grape seed oil, cineol, tartaric acid etc. present in extract are potent free radical scavengers. Thus it may be concluded that
AEPF have antioxidant potential, to reduce CPA-induced oxidative stress and reproductive toxicity in male mice. Therefore, AEPF may be supplemented with CPA to improve sperm characteristics and testicular oxidative damage in men. This study therefore, supports the claim on the folkloric use of the aerial part of *P. fraternus* to improve libido and reproductive function in men.
Figure 7.1: Effect of aqueous extracts of *P. fraternus* and *A. marmelos* in GSI against CPA-induced reproductive toxicity. ‘a’ indicates the significant difference between the control and CPA treated groups, ‘b’ indicates the significant difference between the CPA treated and AEPF and AEAM treated groups. Each bar represents the Mean ±SE, n=6.

CPA + P : Cyclophosphamide + Aqueous extract of *P. fraternus*

CPA + A : Cyclophosphamide + Aqueous extract of *A. marmelos*
Figure 7.2: Effect of aqueous extracts of *P. fraternus* and *A. marmelos* in sperm count, motility and viability against CPA-induced reproductive toxicity. ‘a’ indicates the significant difference between the control and CPA treated groups, ‘b’ indicates the significant difference between the CPA treated and AEPF and AEAM treated groups. Each bar represents the Mean ±SE, n=6.

CPA + P : Cyclophosphamide + Aqueous extract of *P. fraternus*

CPA + A : Cyclophosphamide + Aqueous extract of *A. marmelos*
Figure 7.3: Effect of aqueous extracts of *P. fraternus* and *A. marmelos* in LPO, SOD and CAT against CPA-induced reproductive toxicity. ‘a’ indicates the significant difference between the control and CPA treated groups, ‘b’ indicates the significant difference between the CPA treated and AEPF and AEAM treated groups. Each bar represents the Mean ±SE, n=6.

CPA + P: Cyclophosphamide + Aqueous extract of *P. fraternus*
CPA + A: Cyclophosphamide + Aqueous extract of *A. marmelos*
**Figure 7.4:** The effect of AEPF and AEAM on the testicular damage induced by CPA. Sections from control mice testis showed different stages of spermatogenesis (group I). In the AEPF treated testis showed normal spermatogenesis (group II). CPA treated mice testis showed alterations in spermatogenesis, atrophy of seminiferous tubules, degenerated germ cells and shrunken seminiferous tubules, compared with the normal cellular content of the control group (group IV). In the CPA + AEPF showed, the damage to the seminiferous tubules was considerably less severe, showing undamaged spermatogenesis (group V-VII). In the CPA + AEAM showed, the damage to the seminiferous tubules was considerably more severe (group VIII-X). (H & E, 40X)