CHAPTER-V

DISCUSSION

Although a wide range of synthetic drugs for treating different types of reproductive diseases is available, these cannot be continuously used because of their side effects. In recent times, relatively more stress is being laid on the use of natural products including substances of plant origin for their post-coital anti-fertility activity. So, plants possessing meek intrinsic estrogenic and anti-estrogenic properties present themselves as an effective non-conventional source of contraception with fewer side effects. Evaluation of plants for anti-fertility property has been in progress worldwide for the last several decades to identify safe and effective substances for control of population explosion. In spite of the presence of internationally accepted guidelines for the evaluation of reproductive toxicity or anti-fertility potential of test substances, many published articles or critical reviews seem to be deficient in reproducibility and thus are likely to mislead both the researchers and the general public. Medicinal plants in India and abroad have been screened for contraceptive potential and an anti-fertility effect, as the country and the world is concerned about population explosion. With the advent of Chemistry, the interest in plants as medicines is now aimed at producing therapeutically semi-purified forms, which are quantifiable, with no serious toxicities and cost effective. The focus of the present research was to find out the presence of biologically active compounds present in the selected plant and its effect on fertility. If found to be present, these would be considered to be of great value therapeutically and would also provide the initial material for laboratory synthesis of new compounds of even greater value. This would be of great significance to countries that are financial poor in resources, but are rich in biodiversity (Williams et al., 2006). This renewed interest in the medicinal usefulness of herbs has also resulted in the development of a new type
of therapeutic agents called “Nutraceutical”, a term coined by DeFelice in 1989. He defined nutraceuticals as “foods or part of foods that provide medical or health benefits, including prevention and treatment of disease”. Medication with plant extract has long been used by the people to address problems such as contraception, hormonal imbalance and fertility. These anti-fertility remedies are extracted out from special plants and their products that are believed to have a negative effect on the reproductive organs and its associated hormonal system. These products from plants are taken by men and women who wish to limit the number of members in the family. Herbal extracts for anti-fertility can help to address reproductive problems such as birth control, relieve from post menopausal symptoms and other reproductive problems. The ovaries are responsible for production of estrogen and progesterone, during a woman's reproductive years. The level of these two hormone fall at the time of menopause, causing well-known Menopausal symptoms such as hot flashes, abnormal vaginal bleeding, urinary symptoms, mood changes, fatigue, stress, tiredness, vaginal dryness and itching. Complications that a woman develops after menopause include osteoporosis and heart disease. These complications can be relieved by administration of postmenopausal hormone therapy which includes the two reproductive hormone, estrogen and progesterone. Estrogen alone can be given to women whose uterus has been removed. For others, both synthetic and natural progesterone and estrogen are given. However, women with breast cancer cannot take estrogen, while others may choose not to take hormone therapy. Fortunately, there are some other alternatives to hormone therapy to treat these symptoms. Although they may not be as effective as estrogen but they do provide some relief. Not all women require treatment for hot flashes since they are mild in some women. Some examples include: - Paroxetine (in the United States), an agent that has been used for treating depression; Gabapentin a drug that is primarily used to treat seizures, can
also be taken at a lower dose for hot flashes. For vaginal dryness, non-estrogen lubricants such as Replens, KY Jelly, Astroglide and moisturizers are available. A number of treatments derived from plants have been acknowledged as a natural remedy for hot flashes and other post-menopausal symptoms. Plant-derived estrogens (phytoestrogens) have been used as a natural and safe alternative to hormone therapy for women with menopausal symptoms (Nedrow et al., 2006; Pinkerton and Santen, 1999). Phytoestrogens are abundantly found in many foods, including soybeans, lentils, chickpeas, lentils, grains, fruits, vegetables, and red clover. Lifestyle changes like a healthy diet, regular exercise, avoiding smoking and drinking too much, abstaining from spicy food and caffeine will help in minimizing the menopausal symptoms and helping to maintain overall good health. These complications can be minimized by administration of anti-fertility drugs of herbal origin. Now-a-days many pharmaceutical companies have reported a number of plants which are found to have contraceptive properties.

North Eastern region harbors a rich resources of plant species most of which are have medicinal as well as economic values and are used by the local tribes for treating different types of ailments since time immemorial. Traditional medicinal practice is a routine phenomenon in this part of the country and these practices provides tremendous information about the medicinal properties of different plants. Exploration on the medicinal value of many of these plants has been done by different workers. More need to be done on the scientific confirmation and experimental proof so that effective drugs formulation could be made from these plants.

To assess the quality, purity and pharmacological evaluation of a plant extract, the first and the foremost step is to identify and determine the chemical constituents present in it. Many earlier workers have already reported about the plant possessing different types of phytochemical constituents. Nearly every part of the plant P. acuminata Ait. have been screened for
their preliminary phytochemical analysis as described in Chapter-II. Qualitative estimation of the plant sample is done to verify the presence or absence of different types of phytochemical constituents. In the present investigation, qualitative phytochemical analysis was done using the standard protocol available in literature. The study reveals the presence of saponin, flavonoids, phenolic compounds, alkaloids and terpenoids in addition to carbohydrate and proteins. This study resembles the study undertaken by Gupta et al., (2006); Mahajan and Badgujar (2008) who also reported the presence of these compounds in the powdered leaves of the plant. However, tannin and glycosides were not detected in the present investigation which was found to be present in the powdered leaf extract. Ramesha and Srinivas, (2014) also reported the presence of certain chemical groups in the whole part of the plant such as steroids, phenols, flavonoids, quinines, terpenoids, peptides, xantones, cytotoxiclasins, aliphatic compounds, alkaloids and phenyl propanoids. Other compounds like Stigmast-7-enol, Lupeol carboxylic acid, lupeol acetate and Urosolic acid was also found to occur from ethanolic extract of leaves of Plumeria acuminata (Guevara et al., 1996). In the present study, quantitative estimation of certain plant metabolites was also done using different procedures. The total flavonoid estimation was done by Aluminum Chloride colorimetric method using quercetin as standard. Results of the analysis showed that the value of total flavonoid content was found to be 201.936±8.41 μg/gm quercetin equivalent of the plant extract. Quantitative estimation for total Phenol content was also measured using Folin reagent method. The amount of total phenolic content present in the plant extract was found to be 24.4±2.00 μg/gm gallic acid equivalent.

Flavonoids are low molecular weight bioactive polyphenols that are present in a variety of foods and found to have a varied effect on fertility in both male and female. The different types of flavonoids exerting a varied range of biological activities includes anti-allergic, anti-
viral, cytotoxic anti-tumour, ailing neurodegenerative diseases and vasodilator action (Williams et al., 2006; Chebil et al., 2006; Tsuchiya, 2010), anti-bacterial, anti-inflammatory (Cook and Samman, 1996; Cushine and Lamb, 2005). Flavonoids are found to inhibit lipid-peroxidation, platelet aggregation, capillary permeability, cyclo-oxygenase and lipoxygenase enzyme activities (Middleton et al., 2000). They are found to exert their effect as chelators of divalent cation, free radical scavengers and as anti-oxidants. They also help to inhibit the action of a variety of enzymes such as alkaline phosphatase, hyaluronidase, hydrolases, cAMP phosphodiesterase, kinase (Narayana et al., 2001). Flavonoids are also reported to have an influence on the sex organs and reproduction in animals. A number of workers evaluating the effect of plant extract reported that different types of flavonoids have anti-fertility property in female. Some of such study includes Rivea hypocrateriformis (Shivalingappa et al., 2001), Anethum graveolens (Monsefi et al., 2006), Physalis alkekengi (Montaserti et al., 2007), Terminalia belerica (Vishwanatha et al., 2009), Aspilia Africana (Oyesola et al., 2010), Momordica charantia (Ifeanyi et al., 2011), Acacia leucophloea (Ahirwar, 2011), Ocimum gratissimum (Sripriya et al., 2011), Dactyloctenium aegyptium (Naik et al., 2016), Tephrosia purpurea (Luhadadia and Mali, 2016), Ocimum sanctum (Verma et al., 2016) etc. These researchers are of the opinion that flavonoids do have an influence on the hormones, by binding to 17 beta-hydroxy steroid dehydrogenases, which regulates the androgen and estrogen levels and to 3 beta-hydroxy steroid dehydrogenase, which controls androgen and progestin levels in humans (Melzig, 1996). Thus the presence of flavonoids as revealed from the present study points to the fact that the plant (P. acuminata), might be responsible for acting as an anti-fertility agent.

Phenols, also sometimes called phenolics, are a group of chemical compounds consisting of a hydroxyl group (—OH) bonded directly to an aromatic hydrocarbon group the simplest of
which include phenol/carbolic acid (C6H5OH). These are largely distributed among the plant kingdom and are the most abundant secondary metabolites of plants. They can be divided into two classes: derivatives of benzoic acid eg. gallic acid, and derivatives of cinnamic acid eg. Caffeic, coumaric and ferulic acid. Caffeic acid is the most abundant phenolic acid in most of the fruits and vegetables. Another most abundant phenolic acid is ferulic acid, which can be seen in cereals and is esterified to hemicelluloses in the cell wall of plants (Dai and Mumper, 2010). Recently, plant phenolic acids have been considered to be powerful antioxidants in vitro and proved to be more effective antioxidants than Vitamin E, C and carotenoids (Rice-Evans et al., 1995; Rice-Evans et al., 1996). The inverse relationship between vegetable and fruit intake and the possibility of oxidative stress related diseases such as cardiovascular diseases; osteoporosis or cancer has been moderately ascribed to phenolics (Hollman and Katan, 1999; Scalbert et al., 2005). This compound is also found to have an effect on the fertility in animals. A number of workers have reported about the anti-fertility effect of certain plants having phenolic acids as their secondary metabolite. The names of a few such plants include, *Rivea hypocrateriformis* (Shivalingappa et al., 2001), *Butea monosperma* (Sindhia and Bairwa, 2010), *Momordica charantia* (Ifeanyi et al., 2011) etc. Considering the report of these workers and comparing it with the presence of phenolic acid from our present study on the plant (*P. acuminata*), it can be said that the plant extract might have an effect on the fertility in animals. The present study has estimated the total phenol and flavonoid content in the stem sample but the need of the hour is to find out the particular type of flavonoid or the phenolic group that is responsible for causing the anti-fertility action on the experimental animal.

Alkaloids are a naturally occurring chemical compounds produced by a diversity of living organisms including plants, animals, bacteria and fungi. Well-known alkaloids including
strychnine, morphine, quinine, nicotine and ephedrine are found to have an extensive range of pharmacological activities like anticancer (e.g. homoharringtonine) (Kittakoop et al., 2014), antimalarial (e.g. quinine), analgesic (e.g. morphine) (Sinatra et al., 2010), antibacterial (e.g. chelerythrine) (Cushnie et al., 2014) etc. In the field of reproduction many alkylating agents are found to produce infertility both in animals and man (Miller, 1971; Fairley et al., 1972). There are reports of a number of plants having alkaloids as their phytochemical which influence reproduction in animals. The root of *Tabernaemontana heyneana* wall extract yielded the alkaloids coronaridine, ibogamine voacangin, 19-oxocoronaridine and the pseudoidoxyl of voacangin. Adult female rats when administered orally with coronaridine were found to prevent pregnancies (Meyer et al., 1973). Anti-fertility effect of alkylating agents and vinca alkaloids in male rats were reported by Cooke et al., (1978). Some alkaloids are found to raise the testosterone level in experimental animals (Maggi et al., 2000) suggesting for a possible positive effect on reproduction. Thus it has been seen that alkaloids have both positive as well as negative effect on the reproduction in animals. Thus after evaluating the report of the above researchers and the presence of alkaloids in the extract of the plant (*P. acuminata*) , it can be confirmed that plant under investigation may affect the reproduction in animals.

Saponins, more specifically, the amphipathic glycosides are a group of secondary metabolites found widely distributed in higher plants but are also found in some animal sources, like the marine invertebrates. Saponins exercise a wide range of pharmacological activities like vasoprotective, expectorant, antiinflammatory, hypocholesterolemic, immunomodulatory, hypoglycaemic, antifungal, antiparasitic and many others (Sparg et al., 2004; Sahu et al., 2008). For the production of steroidal hormones the pharmaceutical industry has used steroidal sapogenins as an economically important raw material since many years back (Blunden et al.,
Saponins have been shown to have both positive and negative effect on the viability of human sperm cells with *Sesbania sesban* saponin having spermicidal at 1.0-1.3 mg/ml (Dorsaz *et al.*, 1988) while ginseng saponin are found to increase progression as well as motility of sperm (Chen *et al.*, 1998). Saponin isolated from crude extract of certain plants like *Costus speciosus*, *Phytolacca dodecandra* and *Gleditschia horrida* are known to cause sterility in mice (Chou *et al.*, 1971; Tewari *et al.*, 1973; Stolzenberg *et al.*, 1976). Certain other plants containing saponin in their extract and having a negative influence on the reproduction in animals include *Gleditschia horrid* (Chou *et al.*, 1971), *Azadirachta indica* (Sharma *et al.*, 1987), *Albizia lebbeck* (Gupta *et al.*, 2004), *Trigonella foenum graecum* (Dande and Patil, 2012), *Sesbania sesban* (Dande *et al.*, 2014), *Datura stramonium* (Soni *et al.*, 2016), *Ailanthus altissima* (Mani and Rathore, 2016) etc. Studies on the male animal includes *Ziziphus jujube* (Rekha and Chandrashekhara, 2014), *Dactyloctenium aegyptium* (Naik *et al.*, 2016). The saponin extract from plants that have a negative influence on the reproduction has been long known and has been ascribed to their antizygotic, abortifacient and anti-implantation properties (Tewari *et al.*, 1973; Stolzenberg *et al.*, 1976). These reports of earlier workers have shown that the saponin has a negative effect on the female reproduction. These data clearly indicate that the plant (*P. acuminata*) due to the presence of this compound in its extract might behave in the same way as mentioned above on the experimental animals.

The uterotrophic assay offers the best possible method to assess the estrogenic potency of a chemical or an extract under study. The effect of estrogen on uterus of rodents has established the importance of this organ for the bioassay of estrogen-like substances (Sonnenchein and Soto 1998; Kanno *et al.*, 2001; Yamasaki *et al.*, 2001). In rodent uterotropic assay, ovariectomized rats offer a decent model for evaluating estrogen activity of a substance (either plant extract or
industrially synthesized) in female reproductive organs, along with bone and cholesterol connected parameters (Wronski et al., 1991). This model is an efficient indicator of the changes in low density lipoprotein (LDL) cholesterol and is responsible for monitoring the effects of estrogen on cholesterol (Windler et al., 1980). Further, ovariectomy mimics exact menopausal condition in female subjects thereby, minimizing the circulation of endogenous estrogen (Liu and Bachmann, 1998). In developing mice, the growth and development of the reproductive system largely depends on the level of circulating estrogens. So ovariectomy was performed in the present study, to reproduce exact postmenopausal condition. Ovariectomy raises the level of cholesterol in serum. The absence of estrogen is understood to raise the cholesterol levels, both in animals (Dodge et al., 1996; Lundeen et al., 1997; López-Belmonte et al., 2012) and humans (Stevenson et al., 1993) following induction of a rate limiting enzyme called hepatic HMG CoA reductase, in the cholesterol synthesis (Ness and Chambers, 2000). Moreover, high levels of insulin in ovariectomized rats caused increased dephosphorylation of HMG Co-A reductase, accelerated its activity (Lim et al., 2013). Ovariectomy also is found to increase the body weights of rodents resulting in overweight. The increased body weight is considered as a result of altered energy metabolism caused by estrogen deficiency, favoring fat deposition (Arjmandi et al., 1997). Converse to the enrichment in the body weights, uterine weights were found to decrease. Such a decrease in weight of the uterus is a direct outcome of estrogen deficiency, which is very much essential for the normal maintenance and functioning of the uterus (Stevenson et al., 1993). One of the objectives of the present study was to determine the estrogenic / antiestrogenic nature of the plant extract (*Plumeria acuminata* Ait). So in the present study, adult female mice showing normal estrus cycle were ovariectomized and these animals were then used for the investigation of estrogenic activity of the plant under study.
The rodent uterotrophic assay evaluates a rise in the wet weight of the uterus (Evans et al., 1941). In the present study, oral administration of the plant extract induced positive uterotrophic response in the adult female ovariectomized mice, i.e., there was a significant increase in the wet weight of the uterus (Fig-4.3) in the treated subject when compared with the vehicle treated control group. The increase in wet weight of the uterus was analogous to that induced by E2. However treatment with 17β-estradiol showed many fold (p < 0.01) increases in uterine wet weight thus validating to the well known bioassays of estrogenicity (Jordan et al., 1985). Many researchers have reported that the plant extract that are either used as contraceptive (anti-fertility), as fertility enhancers or in the treatment of post menopausal symptoms and many other gynecological disorders posses either estrogenic or anti-estrogenic properties (Yadav and Jain, 1999; Tinwell et al., 2000; Kayisli et al., 2002; Gebrie et al., 2005). These workers were of the opinion that E2 and estrogenic substance elicit growth response in uterine tissue in a non-genomic mechanism (Grunert et al., 1987; Sonnenchein and Soto, 1998) which includes bringing about changes such as vascular permeability, water imbibitions and cellular infiltration (Sheehan, 1995; Rockwell et al., 2002). The estrogenic compounds bind to the estrogen receptors (ER) in the uterus as an agonist ligand. After binding, this active ligand-receptor complex bind to the responsibe element (i.e., DNA sequence) that are specific for the estrogen reseptor, and up-regulates or down-regulates the transcription of specific genes. This result of gene transcription modulation is manifested as a biological response of the target tissue, such as uterine growth and cell division. This sequence of actions subsequently leads to a raise in uterine weight. The physiologic and genomic response to estrogenic compounds in the uterus has been described as biphasic (Hewitt et al., 2003; Katzenellenbogen et al., 1979). The early event occurs within the first few hours after estrogen administration and includes increased electrolyte and fluid intake.
by the uterus. The water imbibitions results in a rapid increase in the wet weight of the uterus (O’Brien et al., 2006). Moreover, the uterus responds to estrogenic compound by increasing cell division leading to uterine growth. It was found that maximum mitotic division occurs 24 hours after 17β-estradiol administration. The mitotic response is found greatest in the epithelium of the uterus, followed by the stroma and the myometrium (OECD, 2003). In addition, the morphology of the uterine cells is also transformed. These morphological transformations include increased epithelial cell height, stromal thickness, epithelial gland cell height; and differentiation and extension of the luminal epithelium into a columnar shape (Uterotrophic Assay, OCSPP Guideline 890.1600). In ovariectomized mice, circulating level of estrogen is negligible because of the removal of ovary, which is the major source of the steroid hormone. This leads to degeneration of the uterus. The effect of P. acuminata in the uterus of ovariectomized mice as found in the present study suggests that some phytochemicals present in the plant extract led to an increase in uterine weight. This finding was consistent to the report of Kanno et al., 2003, where the immature and OVX female mice were fed with five weak estrogen receptor agonists – genistein, methoxychlor, nonylphenol, bisphenol A and DDT containing diet, all of which cause a marked increase in uterine weight. However, this increase in uterine weight was significantly lower than that of 17β-E2-treated group, which indicates that the plant exhibited only weakly estrogenic property at different doses. Some other studies with daidzen and genistein, the 2 major isoflavones found in many leguminous plants, have also confirmed weak estrogenic effects as reflected by changes in uterine weight (Santell et al., 1997; Picherit et al., 2000). Many other workers also reported similar observations while working with different plant extract. Sheehan (1995) have showed that the increase in the weight of the uterus (a typical estrogen target site) is one of the ways to measure the biological activity of phytoestrogen. Phytoestrogens also
commonly known as dietary estrogens are a diverse group of naturally occurring non-steroidal plant compounds (xenoestrogens). Because of their structural resemblance with estradiol (17-β-estradiol), they have the ability to meekly mimic like estrogen and cause estrogenic or antiestrogenic effects, by accelerating or blocking receptor sites against estrogen (Yildiz, 2005). They are phenolic compounds that bind to the estrogen receptors (ERs). Isoflavones are a major subclass of the phytoestrogen family. Kayisli et al., 2002 conducted an experiment to assess the estrogenicity and/or anti-estrogenicity effect of isoflavones (genistin, geniatein, dadzein and daidzin) in cultured human grandular and endometrial stromal (Ishikawa) cells by MTT colorimetric cell proliferation assay, alkaline phosphatase activity assays and proliferating cell nuclear antigen expression. It was found that, although isoflavones alone have weak estrogenic activity as assessed by alkaline phosphatase activity on the endometrial stroma and glandular cells, but when administered along with E2, they acted as an anti-estrogen. Yadav and Jain (1999), while working on the seed extract of Cassia fistula also reported similar estrogenic effect. Tinwell et al., 2000 while working on the uterotopic effect of cumestrol, reported that the extract induced not only to the increase in uterine fluid content, but hyperplasia of the endometrium was also inevitable. Thus in the present study, an increase in the weight of the uterus may well be attributed to the effect of the extract on hormonal changes that might have taken place in the ovariectomized mice via the effect of the extract on the uterus. The absence of body weight change after seven days treatment with the extract exposed that there is no major negative impact observed on the general metabolic status of the animals. Morphologically, the size of the uterus was also increased in the extract treated mice when compared with that of the control (Plate-4.3). Thus the increase in wet weight of the uterus as observed in our present study
can very well be attributed to the effect of *Plumeria acuminata* Ait. extract on the animal suggesting that the plant possess estrogenic property.

The most responsive tissues to estrogenic regulation are the uterus and ovaries and both the tissues express ERα and ERβ, ERα being the major isoform in uterus while ERβ prevails in the ovaries (Couse *et al.*, 1997; Kuiper *et al.*, 1997). Buchanan *et al.*, 1998 established that vaginal epithelial proliferation was mediated through stromal ERα indirectly, and both stromal and epithelial ERα was required for estrogen-induced stratification and cornification. Estrogenic compounds present in plants compete stronger with 17β-Estradiole for binding to ERβ than to ERα and elicit their estrogenic-like physiological responses by primarily acting through ERβ (Kuiper *et al.*, 1998). Cornification of the vaginal epithelial cells bye estrogenic substances have been reported (Safranski *et al.*, 1993; Cordial *et al.*, 2006). In the present study when the smears from the vagina were examined in the experimental animal, it was found that the smears of the extract treated animals were able to induce some cornified cells (Plate-4.1). This might be due to the weak estrogenic activity exhibited by *P. acuminata* crude extract on the ovariectomized mice. The cornified cells were numerous in the E2 treated groups (Plate-4.1, B), while the cells were much fewer in numbers in the extract treated group (Plate-4.1, C). A higher surge of estrogen level is required for full cornification of vaginal epithelial cells. This could explain the rapid appearance of fully cornified cells at day 3 in smears of mice treated with E2. However, the vehicle treated control group could not produce cornification throughout the entire study period (Plate-4.1, A). Mice treated with 400 mg/kg of the crude extract showed some cornification suggesting the presence of some components of *P. acuminata* which are responsible for estrogen-related changes. But, this effect could not match the pattern seen in mice treated with exogenous E2. However, the mechanism of action remains to be explored. It can be presumed
that the stem extract of the plant under investigation would bind to the estrogen receptor and
induce transcription of specific genes of the vaginal epithelial cells, inducing cell proliferation
and cornification. These results are consistent with the finding of other researchers who are
working on screening potent estrogenic compounds. There has been report that vaginal epithelial
cell cornification in ovariectomized rodents could only be induced by estrogenic compounds
(Drill, 1966). Patil, 2008 also reported that when *Citrus medica* extract were administered to
immature ovariectomized rats for seven consecutive days, induced vaginal cornification and
early vaginal opening in them. Vaginal cornification was also achieved when thin layer
chromatographic fraction of root extract of *Polygonum hydropiper* were fed to adult
ovariectomized female rats for eighteen days (Goswami *et al.*, 2008). Kulkarni *et al.*, 2012 noted
that when seed extract of another species of Citrus, *Citrus limonum* were orally fed to immature
ovariectomized rats for seven days resulted in an increase in wet uterine weight as well as
vaginal cornification. Gray and Ostby (1998) reported about vaginal mucification and vaginal
histologic alterations after 10 weeks exposure to weak estrogenic compounds in ovariectomized
rats. A longer exposure period may be able to achieve full cornification of cells with weak
estrogenic crude extract dose. Thus from the above discussion and relating our work with many
other researchers, it can be concluded that the increase in the wet weight of the uterus and the
induction of vaginal cornification is due to the estrogenic activity of the plant.

Plants possessing estrogenic property have been reported to increase the uterine protein
content in ovariectomized mice. Synthesis of protein was due to the stimulation of estradiol as
has been reported by different scientist (Ireland *et al.*, 1980; Takeda *et al.*, 1988). Results of the
present study on the effect of PAME on the uterine protein content are shown in Fig-4.4. It was
observed that there was an increase in uterine protein contents after administration of the PAME
but these effects were of lesser in extent than E2 treated group. The result reveals that when PAME are fed to ovariectomized mice, the level of protein content in them increased when compared to the vehicle treated control group. This increase was found to be statistically very significant in the E2 treated group. Ovariectomy, due to the removal of natural source of estrogen, led to a decrease in several different kinds of uterine protein (Sharma and Sarma, 2015). Exogenous estradiol treatment to ovariectomized rodents resulted in increase synthesis of different molecular range proteins (Komm et al., 1985). Sarmah and Sarma (2015) reported that five new proteins have been detected after treatment of tomoxifen and estradiol-tomoxifen together treatment in ovariectomized rats, of which two new proteins having molecular weight \( \approx 86 \) and \( \approx 69 \) kDa respectively were synthesized. These proteins were not found to be present in either estrous or diestrous phase of normal cyclic female rats. Adams (1981) also reported a rise in uterine protein content in phytoestrogen fed ewes. Prakash (1979) reported that protein content of uterine increased when rats were fed with benzene and ethanolic flower extract of *Hibiscus rosa-sinensis* for 18 days. Methanolic extract of *Careya arborea* Roxb. roots when administrated for 14 days to adult ovariectomized mice, increased the uterine protein level (Kalita et al., 2011). Similar finding was also reported by Dabhadkar et al., (2012), while working on the pod extract of *Plumeria rubra* on the reproductive organs of female rats. From this study, we can substantiate that the stem extract of the plant under investigation have estradiol agonist effect on the uterus of mice. Such type of study has been conducted in a variety of plant extract and it has been established that the effect on the increase in the uterine protein content in ovariectomized animals is one of the basis for establishing the estrogenisity of a particular plant species. Therefore, this discussion firmly establishes the fact that the methanolic stem extract of the plant used in this experiment possess estrogenic activity.
The total amount of serum cholesterol level in ovariectomized mice (Fig-4.5) in response to PAME treatment caused a significant reduction in the total cholesterol level in the extract treated group when compared to the control. The hypocholesteromic effect in group-III was statistically significant. However, the hypocholesteromic effect of E2 (17β estradiol) treated mice was found to be highly significant compared to the control. This finding in the present study is in consistent with the findings of some earlier workers. When soy products are fed to both humans and animals, they are found to reduce the level of total cholesterol in blood (Carrol and Kurowska, 1995; Sirtori et al., 1995). The cardio protective effect of soy leads to an increase in T4 level resulting in lower level of serum cholesterol level (Patisaul and Whitten, 1999). Soy food are also reported to have possessed high level of isoflavones, phytic acis, saponins and trypsin inhibitors along with a variety of protein and amino acids which could possibly contribute to the hypocholesteromic effect. Lundeen et al., 1997 studied the effect of 17α-etinyl estradiol, 17 α-estradiol and E2 on overiectomized female rats. They found that when these substances are administrated either orally or sub-cutaneously to the animals, the plasma cholesterol concentration level was found to decrease but an increase in the wet-weight of the uterus was observed. Cholesterol acts as an essential precursor for the production of steroid hormone including estrogen, progesterone, cortisol and testosterone in rabbit, rat and bovine luteal tissues which has been reported earlier (Wilks et al., 1970). Since cholesterol is the precursor molecule in steroidal bio-synthesis, the changed parameters associated with a reduction in the serum cholesterol levels may be attributed to the use of cholesterol for steroidal hormone synthesis resulting in increased circulating levels of estrogen suggesting for an estrogenic property of the extract. This result in hyper-functioning of steroidogenic activity on the uterus of the extract treated mice, leading to an increase in the wet weight of the organ. Therefore,
estrogen or estrogen like compounds has a role on the lipid metabolism and causes lowering of blood cholesterol level. The same effect has been documented by administration of stem extract

*P. acuminata* Ait. in this experiment. This effect may be helpful in reduction of cardio-vascular diseases like ischaemic heart disease, hypertension etc. As the present study reveals the same result as the other workers, it can be concluded that the stem extract of the plant under investigation may possess estrogenic activity.

The role of estrogenic compounds in relation to epithelial cells proliferation was established in mice and rats (Huet-Hudson *et al.*, 1989), which advocates that it is a common phenomenon which affects the uterus. The increase in luminal epithelial cell is due to the rise in the number and size of cells and conversely to the amplification in cell proliferation. The present study revealed a wide range of structural changes that have occurred in the uterus of adult female mice when they are treated with PAME extract. Histological evidences supporting the above finding shows that the luminal epithelial cells height, endometrium, myometrium and the diameter of the uterus increased (Plate-4.3, Table-4.4) in the mice with PAME treated group, on seven day of administration in comparison to that of the vehicle treated control groups. Similar findings were also reported by Pollard and Finn (1974) which suggests that estrogen induce rapid yield of the luminal and glandular epithelial cells and in the deficiency of the hormone, the pace of division was found to be very slow in the endometrial cells. They are also of the opinions that even after long period of ovariectomy, the uterus can still responds to exogenous hormone and treatment of estrogen to these animals causes remarkable morphological changes in the endometrium. Another report suggest that coumestrol exposure to ovariectomized mice, led to a remarkable increase in the labeling index of the uterine epithelium, stroma of endometrium and the uterine glands. They reported that there was a significant rise in the epithelial cell height and
thickness of the endometrium, signifying that these changes were direct evident of increase in wet weight of the uterus (Tinwell *et al.*, 2000). The present study reveals that treatment with the PAME extract could also bring about remarkable changes to the uterine morphology, causing an increase in the number of glands as well as mitotic figures. These findings prove that high dose of PAME extract stimulates hyperplastic changes in the uterus. This epithelial hyperplasia may also affect normal development of the endometrium which could interfere with the implantation process (Nikas and Makrigiannakis, 2003). Endometrial morphological changes have been proposed to affect fertility as reported in Polycystic Ovarian Disease, which was featured by a lengthened proliferative phase (Rudnicka *et al.*, 2009). We consider that hyperplastic changes in the endometrium may restrain its transformation into a receptive state, which therefore, may interfere with the processes of implantation. In addition to such estrogenic compounds of plants, other environmental oestrogens for examples p-tert-octylphenol (OCT) and bisphenol-A (BPA), have also been found to bring about changes in the uterine morphology thus affecting normal fertility. Treatment of the subjects with PAME extract also revealed a significant increase in the thickness of the endometrium (Plate-4.3). The endometrial thickness increased with administration of the plant extract. Estrogenic substances are found to have an influence on the growth and development of the endometrium. These substances aid in activation of cell genome of the uterus, through its receptor in the nucleus of target cells (Horne and Blithe, 2007). Analogous findings were also revealed by Murray about the epithelial cells and uterine endometrium of the ovariectomized sheep which experience morphological alternations in protein-synthesizing organelles (Murray, 1992). Oral administration of an extract, in many previous experiments have demonstrated that the effect on the organs are lower when compared to subcutaneous treatment, as because the latter was found to result in better tissue response and
higher plasma bioavailability (Jefferson and Williams, 2011). Our present findings with PAME treatment on ovareictomized mice, resulted in a significant rise in the endometrial layer (S), the epithelial cell heights (E) and the number and size of the glands (G) (Plate-4.3, E & F) when compared to vehicle treated control group (Plate-4.3, A & B), which indicate that the plant possess potent estrogenic property.

To evaluate the effect of PAME on adult ovary intact female mice, another set of study was carried out. In this study, the weight of the mice in the entire group was measured every day in all the treated days. The weight of the mice showed slight increase, but the dose response increase was not significant in both the control as well as treated group of mice. Similar results are reported earlier by a number of workers. Michel et al., 2012 while studying on the estrogenic potency of the different solvent extract of Passiflora foetida leaves on both immature and matured rats have reported that there was no significant increase in the body weights of the animals in all the different treated groups. The body weight of the rats increased gradually from the first week to the fourth week of treatment but weight gains of the treated groups were not significant from those of the control group. In another study by Cordial et al., (2006), the ethanolic leaf and stem extract of Pueraria phaseoloides Roxb. (a plant having strong estrogenic potency), when administered to rats continuously for 15 days, were found to have an elevated level of weight along with the rise in uterine weight. Watcho et al., 2005 while working on the aqueous and methanol extracts of the dried fruits from Ficus asperifolia to evaluate the fertility and uterotrophic properties, have reported that there were no alterations in the body weight or liver weight in the treated subjects. These results mentioned above and the findings of the present study suggest about the stem extract having no adverse effect on the general metabolic reactions in the body and can be used as a safe drug.
The estrous cycle in female is the time between periods of sexual receptivity. It comprises the several recurring physiological and morphological changes in the ovaries, uterus and vagina that are brought about by reproductive hormones. In rats and mice, this cycle is completed at 4/5 day intervals and reoccurs after that (Maligalig, 2001). The presence of a particular type of cell in the vaginal smear signifies a particular (luteal and follicular) phase of the reproductive cycle in rodents. In females, one of the target organs of gonadial steroid hormone is the uterine endometrium, which is again dependent on the hypothalamus-hypophyseal-gonadial axis (Turner and Bagnara, 1975). These hormones, viz. estrogen and progesterone, can cause uterine tissue modulation, induce maturation in the endometrium and make the endometrium conducive so that implantation of the blastocyst may occur (Dey et al., 2004). Disruption of the estrous cycle may cause inconsistency of endometrial function and vice versa. The structural and functional changes of the endometrium brought about by different chemicals may lead to failure of implantation. In the present study, it was observed that administration of *P. acuminata* affected the estrus cycle. The results as revealed from this study have showed that, this plant extract could alter the ovarian endocrine function resulting in a change in the cyclicity of the treated animals. This has also lead to a thorough change in the characteristic of the different cell types as revealed in the vaginal smears during the post treatment period (Plate-4.5). The loss of cyclicity as displayed by the study indicated that there was a disruption in the ovarian estrogen and progesterone level. These hormones are believed to be very crucial for normal uterine endometrial maturation and receptivity to the embryo (Hazarika and Sarma, 2007). As evident from the study on the effect of PAME on estrous cycle of female mice, results indicated that the extract caused a significant modification in the duration of the different phases of the estrous cycle (Table-4.6). The normal periodicity of the cycle as in
the control group was replaced by an imprecise representation where there were a prolonged
diestrus along with non-significant shortened duration of the proestrus and elongation of the
estrus phase and reduced number of cycles. Since estrus phase is the only matting phase in the
animals, its non alteration in duration at the treated doses and the prolongation in the diestrous
phase, as evident from our study could possibly suggest some antifertility effect of PAME. The
administration of the plant extract to mice might have altered the synthesis of some endometrial
protein which might affect the normal cyclicity in the animals (Hazarika and Sarma, 2006a). A
similar finding was also revealed by Hazarika and Sarma, 2007, while working with the
*Polygonum hydropiper* crude root. A number of earlier workers who have reported similar
effects of different plant extract which caused disruption in the estrous cycle are mentioned
below. Ganguly *et al.*, 2007 while working with *Mimosa pudica* root extract, a plant known to
have anti-fertility property, found that oral administration of the extract at a dose of 300 mg/kg
body weight/day, were seen to prolong the length of the estrous cycle. They showed that there
was significant increase in the duration of the diestrous phase and the number of litters was
reduced in albino mice. The analysis of the principal hormones showed that the root extract
altered the gonadotropin release and estradiol secretion. In another experiment with the seeds of
*Carica papaya* oral administration of chloroform extract at 100 mg/ kg-body weight, prolonged
the dioestrous phase of the oestrous cycle, resulting in increases in the number of atretic follicles
and a significant decrease in the number of pregnancies (60%)( Raji *et al.*, 2005). This plant was
also found to have anti-fertility property. Yet in another experiment, oral administration of the
benzene extract of *Hibiscus rosasinensis* flowers for 30 days at 150 and 250 mg/kg body weight,
increased the number of atretic follicles, and after 21 days all animals were in prolonged
dioestrus phase. However, intraperitoneal administration of the extract at 125-250 mg/kg body
weight induced prolonged oestrus and metoestrus with irregular oestrous cycles. These results suggested an antiovulatory effect of the extract (Kholkute et al., 1976). These workers are of the view that the different factors that govern the shift in different stages in the normal estrous cycle in animals are under the influence of various ovarian hormone levels (estrogen & progesterone), which again, is regulated by the secretion of gonadotropins hormones (FSH and LH) and hypothalamic releasing factor from the pituitary (Lerne, 1969). Thus, the reduction in the number of the estrous cycle as evident from our study corresponds to a decline in the time during which ovulation occurs and hence a reduction in the rate of ovulation resulting in decreased fertility. The persistence of the diestrous phase by the extract indicates that it possibly interferes with the actions of estrogen, progesterone, FSH and LH that are accountable for the control of the estrus cycle in female animals through its action on the pituitary-gonadal axis. For all the reproductive functions like ovulation, implantation and subsequent sustenance of pregnancy, normal estrus cycle is very much essential, which in turn is dependent upon the coordinated action of estrogen and progesterone. A perfect balance in their levels is highly essential for maintaining fertility; any disturbance in their levels will result in unbalanced ovarian functions that affect the estrus cycle and thereby fertility. Prolonged diestrous phase suggest that PAME extract might have interfered with the synthesis of hormones that might have brought about changes in the various phases of the estrus cycle and the number of cycles. Thus as evident from the present study, the prolongation in the diestrous phase and the reduction in the number of estrous cycle is a clear indication that the extract might have interfered with the normal hormone level of the animal which will have a bearing on fertility of the animal.

An increase in the wet weight of the uterus in the PAME extract treated group of mice as compared to the vehicle treated control group (Fig-4.6) was observed from the present study. The
increase in wet weight may be attributed to the estrogenic effect of the plant extract. The effect of different plant extract on the uterus of ovary intact animals was studied to find out the estrogenic/anti-estrogenic, fertility enhancing or anti-fertility property and documented by several workers. Watcho et al., 2009 reported that the aqueous extract of the fruits of *Ficus asperifolia* produced an increase in the weight of the uterus in adult female rats. But the same plant extract was reported by Ngadjui et al., 2013, that it did not alter the uterine as well as the ovarian weight in adult female mice. Lembe et al., 2012, while working on the effect of *Lepidium meyenii* (Black maca) and *Turraeanthus africanus* on fertility and estrogenic activity in female mice found that the uterine weight of ovariectomized as well as ovary intact was higher \((p < 0.05)\) than those observed in controls, but there was no change observed in the *Lepidium meyenii* and *Turraeanthus africanus* combine treated group. The weight of uterus of both treated *Turraeanthus africanus* and \((Lepidium meyenii + Turraeanthus africanus)\) combine extract treated group significantly increased \((p < 0.001)\) in the proestrous and estrus stage when compared to control. Shivalingappa et al., 2002 have showed that ethanolic extract of *Rivea hypocrateriformis*, a plant known to have anti-fertility property, when administered to adult albino rats at dose of 200 and 400 mg/kg body weight orally, induced an increase in the weight of the uterus, indicating the uterotrophic effect of the extract. Vasudeva and Sharma, 2008 reported that administration of *Hibiscus rosa-sinensis* extract caused a significant increase in the weight of the uterus, diameter of the uterus and also the thickness of the endometrium suggesting for a possible estrogenic effect of the plant extract.

As proteins are the main building blocks of new tissues they play a major role in the maintenance of the successful reproductive events beginning right from mating to complete gestation period. In this present investigation, significant increase in the amount of protein
content (Fig-4.7) along with the increase in weight of uterus was well established. All these suggest for a possible estrogenic effect of the plant extract. Similar studies were carried out by different workers. Ireland et al., 1980 have demonstrated that E2 when administrated to immature rats increased the uterine protein content in these animals. Padilla-Blanks et al., 2001 also established the fact that when E2 was fed to both rat and mice, there was strong expression of specially two protein viz. lactoferin and compliment C3 in the uterine luminal epithelial cells. In cell culture method also, the formation of complement C3 protein was induced by E2 (Hopert et al., 1998). Syed et al., 2012 have shown that the aquous and methanolic extract of Flax seeds (Linum usitatissimum) when fed to immature rats showed overall increase in body, ovarian and uterine weights in mice when given in higher doses of extract (200 or 300 mg/kg) compared to control group. The increase was also true for the ovarian and uterine protein contents, while there was an opposite trend found for ovarian cholesterol contents. There are also many instances proved by researchers confirming that plants possessing estrogenic potency tend to increase the amount of protein content of the uterus.

Estrogenic substances are found to affect the different layers of the uterus of animals. The present investigations on the effect of PAME extract have shown that the diameter of the uterus was significantly increased in the extract treated group (Table-4.7). The lumen of the uterus was also found to be enlarged in the PAME treated groups. The endometrial thickness of the uterus too showed an increased. An increasing response of the luminal as well as glandular epithelial cells height along with the number of glands of the uterus was also observed after 7 days administration of the animal with PAME extract (Table-4.7; Plate-4.6). Endometrial cellular proliferation in response to estrogen is well established (Snijders et al., 1992, Tinwell et al., 2000). In the present study, the recommencement of endometrial cellular mitosis and
multiplication in response to PAME treatment is evidence for the estrogenic property of the *P. acuminata* stem extract. The effect of PAME on uteri can be compared with the effect of exogenous E2 on OVX mice uterus. E2 stimulated the enlargement of OVX uterine, endometrial and stromal tissues in a uniform manner. In the same way, the administration of PAME induced the increase in endometrial and stromal cells of the uteri. These findings on the effect of PAME extract is in consistent with the findings of the below mentioned researchers. Alrefaied *et al.*, 2010 working on the outer scales of onion on mice reveals that the extract was capable to induce proliferative and stimulatory changes in the estrogen target tissues that are similar to those brought about by estrogens. The extract concentration at 30 mg/kg body weight induced an increase in the wet weight of uterus, proliferation of luminal and glandular epithelial cells height along with proliferation of the endometrium and myometrium. Similar effects were observed when ethanolic extract of *Rivea hypocrateriformis* were administrated to adult albino rats at 200 and 400 mg/kg body weight orally, a rise was observed in myometrium and endometrial thickness and also the diameter increased indicating the uterotrophic effect of the extract (Shivalingappa *et al.*, 2002). Pare *et al.*, 2013 reported that *Trilliantha portulacastrum* extract when fed to female albino rats caused significant increase in uterine weight, uterine diameter and thickness of endometrium, suggesting mild estrogenic activity of the extract. Cruz-Aquino *et al.*, 1967 showed that at a high dose, high efficacy oral contraceptive produces hyperplasia of the stromal cell of endometrium, decidual reaction and glandular atrophy accompanied by hyperplasia of the endothelia of the blood vessels with thickening of the walls in women. Some of the endometrial glands in the PAME treated mice exhibited cellular degeneration in the present study. The underlying causes behind this glandular degeneration by PAME though are unknown, it can be speculated that the increase in protein content in extracted treated mice may
lead to a synthesis of new protein which might be the factors responsible for such changes in extracted-treated mice (Hazarika and Sarma, 2006). In the present investigation, the chemical compound(s) that is/are responsible for bringing about these changes in the uterus is yet to be determined. These effects exerted by the plant extract on the uterus may be due to either a single compound or multiple of compounds that might be present in the stem extract of *P. acuminata* Ait. It is assumed that the active compound (s) may be an agonist or a partial agonist of E2.

The extracts of different plants are found to have diverse mode of action on the ovary in treated animals. Some of these extract are found to help in the development of follicles while others are anti-ovulatory in nature. Induction of ovulation can be brought about by plant extract have been reported long back (Borasky and Bradbury, 1942). In the present study, increasing follicular recruitment was evident after PAME treatment. A number of follicles were found to develop from primary to secondary structures in response to the extract treatment. This finding signifies the estrogenic nature of the plant under investigation (Plate-4.7, D). Similar findings on follicular recruitment were also reported when adult sheep were exposed to exogenous testosterone during the prenatal period or when they were allowed to graze on phytosteroid-containing plants (Adams, 1977; Padmanabhan *et al.*, 2004). However, these ovarian follicles degenerate as a result of disruption in the growth and differentiation of the growing follicles which forms atretic follicle. The antral follicles also showed changes in the organization of granulosa cells and theca cell layers (Plate-4.7, F). The administration of PAME might bring about obstruction of cellular proliferation resulting in smaller number of theca cell layers. Even though follicular atresia is an essential part of ovarian function, the amplified number of atretic follicles and associated recruitment in follicular number of follicles in the extract treated mice as compared to control may be due to the non availability of an adequate amount of the extra
ovarian regulators, the gonadotrophins (FSH and LH). This could be brought about by the effect of the extract directly by in-sensitizing or over-sensitizing the follicular receptors to the existing gonadotrophins or indirectly at the level of pituitary gland or hypothalamus (Solomon et al., 2010). The presence of two main intra-gonadal regulatory factors which modulate the effect of the major hormones can be distinguished: intra-follicular and intra ovarian (Cortvrindt, 2001). So the biologically active substances that are present in the extract might have increased follicular growth in the ovaries by interfering at the level of receptors and mRNA expression of these intra follicular and intra ovarian regulating factors. PAME in addition to exerting its influence on theca cell layers also are found to changes the structural organization of granulosa cells. The disorganized and loosely arranged granulosa cell layers in the antral follicles (Plate-4.7, F) indicate the generation of atretic condition of the follicles in the PAME treated mice. This effect may not be conducive and lead to toxicity in follicular development in mice. In the present study, pre-ovulatory follicles in the ovaries of the extract treated mice were not observed (Plate-4.7, D). But the ovary in the vehicle treated control group showed distinct pre-ovulatory follicle (Plate-4.7, A). The absence of pre-ovulatory follicles may be due to non-availability of an adequate amount of pituitary gonadotrophins for folliculogenesis (Richards, 1980). The direct continuation of preovulatory follicular development leads to the formation of corpus luteum. In rodents and many other species, the main luteotrophic hormone is estradiol. FSH, LH and Prolactin also contribute to the luteotrophic complex as they increase estrogen secretion by promoting the growth of large follicles (Shivalingappa et al., 2002). A high amount of serum estrogen is very crucial for the luteinizing hormone for inducing ovulation (Adewale et al., 2004). The absence of corpora lutea in extract treated mice indicates that the extract inhibited the transformation of the preovulatory follicles into corpora lutea, arresting ovulation. The findings of the follicular
recruitment in this experiment correlates with the extended diestrous phase of the estrous cycle in the extract treated mice as has been reported in the previous study by Gebrie et al., (2005 a & b). Earlier studies have revealed that the process of ovulation is analogous to an inflammatory process (Akpantah et al., 2005). Blocking the ovulation process can be brought about by administering anti-inflammatory drug (Espey, 1994). So the medicinal plants possessing anti-inflammatory activity may also be employed in blocking ovulation process in animals. The anti-inflammatory activity of flavonoids is supposed to result from inhibition of the enzyme cyclooxygenase (Gaytan et al., 2002). Cyclooxygenase, converts arachidonic acid obtained from cell membrane to prostaglandins (PG), as two isomers, Cyclooxygenase-1 (COX-1) and Cyclooxygenase-2 (COX-2) (Liang et al., 1999). Cyclooxygenase-1 is necessary for the production of PG which is an endogenous form of the enzyme while COX-2 is thought of as being an inducible enzyme related with inflammation. It is thought that COX-2 is essential for ovulation mechanism. It was shown that all traditional non-steroidal anti-inflammatory drugs has an effect on the action of both COX-1 and COX-2 but exerts most of their effect by blocking COX-2 (Tokuyama et al., 2001). COX-2 is expressed in many organs and condition such as in acute inflammation, kidneys and brain, bone resumption, female reproductive organs and can be induced in various cells by stimulation of cytokines or growth factors (Staud, 2000). COX-2 deficient mice are found to suffer from defect in reproductive function such as fertilization and ovulation (Katori and Majima, 2000), implying that COX-2 is important in ovulation. The above studies indicate that COX-2 enzyme is very crucial for follicular rapture through the metabolites of arachidonic acid, which by activating protease play important role in follicular rapture. The anti-inflammatory effect of methanolic extract of P. acuminata has already been established (Gupta et al., 2006). Phyto-chemical analysis of the plant under investigation also revealed the
The presence of flavonoids. The results of present study suggest that the methanolic extract of *P. acuminata* may block the ovulation process leading to non-availability of corpus luteum in the ovary of the extract treated mice. There was an increase in the atretic follicles observed in the animal treated with PAME extract, signifying certain types of atrophic condition in the follicles. The absence of corpora lutea and an increase in the atretic follicles in the ovaries of the treated mice indicates the absence of ovulation, which suggest that the critical balance of the pituitary gonadotrophins (Kobayashi and Tamotsu, 1969) were disturbed due to the extract treatment. There are many reports substantiating the above fact and effect of different plant extract on follicular growth. Shivalingappa *et al.*, 2001 reported that ethanolic extract of *Rivea hypocrateriformis* when administered to adult albino rats orally, the ovary showed a decline in graafian follicles number with a rise in number of atretic follicles indicating the antiovulatory effect of the extract and thus has a good anti-fertility potential. When petroleum ether extracts of *Citrus medica* seeds was fed to rats, there was a marked increase in number of atretic follicles and reduction in number of healthy developing follicles (Patil and Patil, 2013). Monsefi *et al.*, 2006 have reported that the ethanolic extract of *Anethum graveolens* did not divulge any significant changes in the volumes of ovaries, and the number of primary, secondary and graffian follicles. Analogous results was found in the present study, when *P. acuminata* Ait. stem extract was administration orally for seven days to adult female cyclic mice, the ovaries did not show any significant increase in their weight. Solomon *et al.*, 2010 reported that treatment of *Rumex steudelii* methanolic root extract at 3.0 g/Kg dose level significantly decreased the number of small antral, Graffian follicles and corpora lutea with associated significant rise in the number of atretic follicles in a dose dependent manner. Devendra, *et al.*, 2009 reported that *Trichosanthes cucumerina* extract significantly reduced the number of follicles and corpora lutea and raised the
number of regressing follicles. They also observed that there were no significant differences in the body and organ weights and no mortality was recorded between controls and treated rats. Other notable plant species that have been reported to induce significant changes in the number of ovarian follicles and disruption of oestrous cycles includes- *Hibiscus rosasinensis* (Kholkute et al., 1976; Murthy et al., 1997), *Momordica charantia* (Chan et al., 1986; Sharanabasappa et al., 2002), *Carica papaya* (Chinoy et al., 1997; Raji et al., 2005), *Azadirachta indica* (Dhaliwal et al., 1999; Roop et al., 2005) and *Melia azedarach* (Keshri et al., 2003; Mandal and Dhaliwal, 2007). These workers are of the opinion that gonadotrophic hormones (FSH and LH), especially FSH are responsible for accelerating the growth of immature ovarian follicles. It is believed that FSH provides the induction stimulus that initiates the process of follicle growth and development including the graafian follicles. The FSH receptors on the granulose cells are the primary players in this process. This induction leads to an increase secretion of estrogen by the maturing follicles. This rise in estrogen inhabits the secondary rise in FSH by a negative feedback mechanism by acting on the pituitary-gonadal axis. This cause a subthreshold level of FSH in the nondominant cohort follicles, which then under goes atresia (Erickson, 1986). Extracts of all these plant species showed potential anti-fertility property as they induce loss of the various follicle types, most of which appears to be due to increased rates of atresia. Most of these plants are found to inhibit the implantation process and reduction in the litter size in experimental female animals. Different dose level and different parts of the plant like the leaves, stem, root, flowers etc. were used in these study. The present investigation after treatment of the PAME on adult female mice have found changes in ovarian follicular growth and histology which were accompanied by prominent histological changes on the uterus of extract treated mice. The extract caused hypertrophic effect on the uterine tissue as revealed by a significant increase in the epithelial cell
height, myometrial thickness and stromal thickness. These changes may be brought about through the effect of the extract either indirectly by disruption of the hormonal balances on the hypothalamo-hypophysial-ovarian-uterine axis or directly on the uterine tissue as estrogen agonist.

The ovary of adult female mice when administrated with the PAME extract for seven days reveals that there was no significant increase or decrease in the ovarian weight when compared to control. The structures as revealed from histology showed certain abnormality indicating decreased functional identity. The ovary showed follicular recruitment with an increase in the atretic follicles, the results of which can be compared with the findings of the above mentioned researchers. The structural integrity of the granulose cell and the stroma were also found to be different from those of vehicle treated control animals. Similar effect was found by many workers on certain plants which are estrogenic in nature, who reported that the different plant extract when fed to animals increased their ovarian weight and the number of different types of follicles (Al-Mual and Al-Jiboori, 2010; Lienou et al., 2012; Syed et al., 2012). The present study on PAME extract provides evidence that the stem extract may have brought about a contraceptive effect thus reducing the chances of pregnancy.

Implantation is the early stage of pregnancy i.e. after the egg is fertilized by a sperm; it travels through the Fallopian tube towards the uterus where the conceptus adheres to the wall of the uterus so that it can continue to grow properly. The whole process, from fertilization to implantation, usually takes about 4-6 days in rodents. In the present study, the implantation sites were studied in two phase. The first phase includes 7 days treatment after mating and in the second phase treatment was given for 21 days on adult female mice. In both the treatment groups, there was a significant loss of implantation sites in all the treated groups when compared
to control (Table-4.11 & Table-4.12). Analysis of fertility enhancing/anti-fertility property of a plant involves the study of implantation. Al-Said et al., 1987 reported that the seeds of Coriandrum sativum when fed to rats at doses of 250 and 500 mg/kg orally found that the extract produced a dose-dependent significant loss in implantation site, but failed to produce complete infertility. When aqueous extract of Lepidium meyenii was fed orally to adult female mice for 22-28 days (15 days prior to mating and first seven days of gestation), there was an increases in the litter size in the treated group but the implantation sites were found to be similar to that of the control (Ruiz-Luna et al., 2005). Watcho et al., 2009 reported that when reproductive effects of Ficus asperifolia were investigated in female rats, by orally administering with the aqueous and methanol extracts of the plant at 100 and 500mg/kg body weight, for seven days, the study showed a significant increase in the implantation sites and litter size of animals. An investigation was undertaken by Vishwanatha et al., 2009, to determine the activity of benzene and ethanol extracts of the barks of Terminalia bellirica and found a strong anti-implantation (74.20% inhibition) activity at the dose level of 25mg/100g body weight, thus, concluding that barks extract may be used as a female contraceptive. Vasudeva and Sarmah, 2008 reported that the ethanolic extract of the roots of Hibiscus rosa-sinensis Linn. was able to induce a strong anti-implantation (inhibition 100%) and uterotrophic activity at 400 mg/kg body weight dose level. At a lesser dose of 200mg/kg, this effect was found to be lesser in magnitude. Ahmed et al., 2002 also reported about the leaves of Ocimum sanctum which have been found to possess anti-implantation activity in experimental rats. Other plants which induce anti-implantation activities as reported by different scientist includes, the ethanolic extract of Striga orobanchioides (Hiremath et al., 1994), ethanolic extract of Calotropis procera roots (Kamath and Rana, 2002), Lawsonia inermis root extract (Agunu et al., 2011) etc. In the uterine endometrium, implantation
is an extremely complex phenomenon in which the two steroidal hormones, estrogen and progesterone play an important role in the target tissue. These hormones coordinated a set of changes in the uterine endometrium that transforms it from a non-receptive to a receptive stage allowing the implantation of the developing blastocyst. Any disturbances in the equilibrium level of these hormones may lead to unsuccessful implantation resulting in infertility (Ding et al., 1994; Hiremath et al., 1999). This interplay of the two hormone and the requisite changes they bring about in the uterus is still a matter of discussion among the present day researchers. During the processes of implantation, E2 administered rats promotes RNA synthesis in the endometrium and exhibits its mode of action through this RNA thus synthesized. Thus a precise amount of nucleic acid and protein is needed for implantation to occur. Any deviation from this might lead to anti-implantation and anti-fertility activities (Srivastava et al., 1985). In the present study, though the uterine protein was found to increase, but this rise in the amount of protein might not be conducive for the implantation to take place. Moreover, a surge in the amount of progesterone is highly essential for implantation to occur. During the reproductive cycle in females, the circulating level of E2 and P4 produced by the ovaries fluctuates. In rodents, estrous cycle which is divided into four stages called diestrus, proestrus, estrus and metestrus, generally lasts for 4–5 days. In them, the circulating levels of E2 peaks prior to ovulation i.e. it occurs at estrus, while P4 levels increases during diestrus and metestrus, and then declines from proestrus to estrus (Walmer et al., 1992, Fata et al., 2001). The pituitary-gonadal axis is very important for maintenance of the reproductive system; hence any deformation to this axis can be deleterious (Amah et al., 2008; Koneri et al., 2006; Yama et al., 2011). Follicule stimulating hormone is required for stimulating maturation of the Graafian follicles while leutinizing hormone causes it to produce testosterone which is then converted to estrogen by aromatase (Moore and Persaud,
Discussion

Structural and functional attributed to estrogen include promoting the formation of female secondary sex characteristics, accelerates metabolism, stimulates the development of endometrial growth and overall increase in uterine growth. It is also responsible for synthesis of protein and helps in regulating the menstrual cycle. If the egg is fertilized, estrogen works in co-ordination with progesterone and helps to stop ovulation during pregnancy. A high estrogen surge is important for the luteinizing hormone which induces ovulation (Adewale et al., 2014). The resultant formed corpus luteum secretes progesterone. Progesterone plays a very important role in maintaining the normal uterine function. It converts the endometrium of the uterus to its secretory stage, prepares it for implantation and establishment of pregnancy (Sheeja et al., 2012). A non increase or decline in serum estrogen level prevents ovulation hence low level of progesterone. Also a direct toxic effect on the corpus luteum may also be a possible cause for decline in progesterone level (Adewale et al., 2014) resulting in anovulatory dysfunctional uterine bleeding. Earlier studies have reported that alkaloids present in different plant extract have been reported to inhabit the synthesis of cellular progesterone (Gocze et al., 1996). The analysis of *P. acuminata* extract also revealed the presence of alkaloid as one of the phyto-constituent which might be the responsible factor for a decrease in the amount of serum progesterone level thus affecting implantation. From the above finding, conclusion can be drown that the *in vivo* exposure of the uterus to PAME extract may have contributed to induce some modification in the uterine endometrium that transformed the uterus from a receptive to a non-receptive phase, allowing the lose in implantation of the blastocyst.

Pregnancy is the period during which a female carries one or more offspring from implantation through gestation in the uterus. Gestation begins with fertilization and ending at birth. The length of this period varies between different species. Smaller species normally have a
shorter gestation period than larger ones. One of the fundamental components of evolutionary theory is that the number of offspring produced by an individual contributes to their relative and differential wellbeing. In litter-bearing species, litter size contributes heavily to the total number of offspring produced. Both the hereditary and environmental factors influence the litter size (Jaquish et al., 1996). This experiment was done to study the long term effect of the plant extract on female mice. The present investigation reveals that the litter size was found to significantly decrease with respect to the vehicle treated control but there was no decrease in the weight of the litters observed suggesting for the non-toxic effect of the plant extract on the litter health. However, the length of gestation was not affected in the process. Plants having potent fertility property have been reported to have an increase in litter size, mating index and pregnancy index when compared to control (Watcho et al., 2009; Ugwah-Oguejiofor et al., 2011). Hyacinth and Nwocha, (2011) reported that *Hymenocardia acida* a traditionally used herbal medicine with numerous therapeutic benefit in African, when ethanolic extract are dosed at 100, 200 and 400 mg/kg body weight daily for 19 days of gestation showed reduced number of live fetuses and reduction in weight of the litters. Anti-implantation activity of the treatment groups were increased with increase in the dose of the extract. Abdulazeez et al., 2009 treated female rats with fermented seeds of *Carica papaya* from gestation day 6 to gestation day 15, at 500 and 1500 mg/kg, respectively and kept till terms and allowed to litter. They observed that there was no significant difference in litter size and litter body weight in rats within all the groups, compared to the control group suggesting that it may be safe as a food condiment. Raji et al., 2010 treated female albino rats with quassin, a bioactive triterpenoid extracted from stem bark extract of *Quassia amara*. The treated rats had a significantly decreased in mean litter number and weight suggesting for a possible anti-fertility property of the extract, possibly acting by
inhibition of estrogen secretion. Essien and Effiong, (2014) observed that when methanolic extract of *Garcinia kola* seed are fed to female rats with 200 mg/kg and 300 mg/kg of the extract, significantly impaired implantation causing resorption of implantation sites and postponed delivery for 4-5 days. From the studies of the above workers and comparing the data with the present investigation, it can be concluded that the extract at doses 400 mg/kg orally produced a significant anti-implantation effect, but failed to produce complete infertility. The extract might have disrupted the hormonal level, specially caused a decrease in the level of progesterone after treatment, which might lead to this decrease in the litter size. When the litter morphology was observed, it was found that no undesirable affect was noticed either in weight or in external appearance which indicate that PAME extract might not affect fetal development if administered before conception (Garcia *et al.*, 1989). The effect of the plant extract might be restricted only at the implantation period due to the non increase in the level of progesterone. In conclusion, the consequences of the present study suggested that PAME extract possesses anti-fertility and estrogenic properties, which was attributed to reduction in litter size in the entire treated group. This was probably due to certain phyto-chemical present in the plant extract which have lead to a non increase in serum levels of progesterone leading to loss of implantation sites in the treated animals.