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DISCUSSION
The present investigation was carried out for in vivo validation of a traditional herbal medicine for fertility control. The bark of the plant *Dysoxylum alliarium* (locally known as "Situ Payu" among the ‘Adi’ group of tribal people of Arunachal Pradesh) has long been used for fertility control of domestic animals (e.g. dogs and pigs). The information came into focus, when the present investigator went through the ‘Adi’ villages and villagers (especially the herborists) for collection of first hand information on the traditional abortifacient medicines.

Arunachal Pradesh is known for its huge forest coverage (84% of the total area of the state) where the native people live in the beautiful valleys of the rouged hills and mountains and bank of the mighty rivers. Located on the eastern range of great Himalayan Mountain, Arunachal Pradesh is known to the world as a Biodiversity ‘Hot Spot’. Due to rouged hills, torrential rapids and rivers and dense forest, this region is hitherto unexplored for the world community. The indigenous people collect their food stuffs from neighboring forest, cultivate rice and corn, domesticated dogs and pigs and maintain a peaceful life in the organized villages in remote areas of this hill state. With the passage of time, the indigenous group developed their repository of traditional medicines to cure and prevention of diseases and ailments of human and animals as well. The bark of *Dysoxylum alliarium* is a traditional herbal medicine prevails among the ‘Adi tribe’ of Arunachal Pradesh for fertility control mainly for domesticated dogs and pigs and occasionally refereed to for women.

It has been considered as wonderful information, when the present investigator came across the herborists and traditional medicine practitioners in the ‘East Siang’ district of Arunachal Pradesh. The information revealed that this traditional medicine is applied to the dogs and pigs only during post coital period to get rid of unwanted pregnancies of the animals. The ‘Adi’ people have a cultural identity of maintaining colonies of dogs and
pigs in every family. While pigs are reared as source of meat, dogs are maintained as their domestic pets. The bark of ‘Situ Payu’ is used for the control of population size of these domestic animals. As per the oral literature collected from the herbarists, the bark powder is fed to the females as soon as house owner observed the mating of the female. The dry bark powder of the plant is mixed with the fodder of the animal in specific dose (approximately 50 -100gm) and fed to the mated females for 3 to 4 consecutive days immediately following the mating to avoid give birth of pups and thus maintaining a manageable colony of the animal.

This field observation raised queries to the investigator – does bark of the plant ‘Situ Payu’ possesses anti implantation and/or abortifacient properties for these farm animals? This query leads to hypothesize – “the bark of ‘Sity Payu’ possesses potential component(s) for fertility control in mammals targeting fetal maternal unit and embryonic development during early pregnancy”. It has been speculated that the potential compound in the CBP may involve in the disruption of endometrial maturation and decidualization antagonising blastocyst(s) attachment to the maternal wall and further embryonic development during early pregnancy. The compound(s) present in the bark does not involve in the disruption of ovulatory function of the ovary unlike antiovulatory contraceptive hormonal ‘pills’ available in the modern medicine. The compound(s) may have potentiality either for luteolysis or disruption of endometrial decidualization, which has been considered as ‘epitheloid reprogramming’ of the normal stromal tissue required for growth and development of blastocysts.

The contraceptive and the hormonal properties of stem bark of another species under the genus Dysoxylum namely “Dysoxylum binecteriferum” has been reported recently (Govind Keshri et al., 2007). Administration of ethanol extract of the bark of this plant through oral route in different doses for different time schedule during post coital period
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ted implantation in albino rats. However, the detail study on the effects of the extract
astocyst(s) or/and the implantation sites of maternal tissue has not been studied. The
r reported the presence of an alkaloid ‘rohitukine’ from the bark of this plant which
ented the implantation following post coital oral administration.

Thin layer Chromatographic fraction of methanol extract of bark of
Dysoxylum alliarium and its estrogenic effects on uterus

A preliminary investigation on the nature of the active compound with potential for
duction control present in the bark of Dysoxylum alliarium has been carried out
ng thin layer chromatography. The result as shown in Fig.2 yielded two fractions
imilar with Rf value of estradiol-17β. Although, the detail of the nature and structure of
ese compounds yet to be elucidated, it has been expected that the bark of the tested plant
ay contain compounds having steroidogenic properties. These compounds may
agonize the functions of the native ovarian steroids required for establishment of
regnancy during perilimplantation period. In recent years, many plants having
ytosteroids have been reported which involves as reproduction and sexual function
modulator in laboratory animals as well as in women (Milligan et al., 1999; Takeshi Usui,
006; Ladislav & Dagmar, 2009).

The phytoestrogens which cover wide groups of non steroidal estrogenic
ompound including isoflavones and lignans are widely distributed in nature. The
hemical structures of the phytoestrogens are similar with the structure of estradiol an
ogenous estrogen in mammal. The structural similarities account for the binding
ability of phytoestrogens with the estrogen receptors and thus exert various estrogenic
or antiestrogenic effects on the organism. In the present investigation, it has been assumed
that the bark of the plant Dysoxylum alliarium could possesses the phytoestrogens causing
verse effects on the reproduction and termination of early pregnancy in rats. However,
existence of other compounds (non steroidal) in the bark can not be ruled out, as because in recent years aqueous extract of certain plants have been reported to exert effects on reproductive performance of female rats (Chukwuka N. Uchendo and Thomas Isek, 2009).

The technique of thin layer chromatography has a wide use for the detection and estimation of steroids in phytocompounds (Dawidar and Fayez, 1972). In the present investigation, the methanol extract of the Crude Bark powder of Dysoxylum alliarium was subjected to the high performance thin layer chromatographic (HPTLC) fractionation. The similar migration of the compound of Dysoxylum alliarium with the estradiol-17β indicates the presence of estrogen like compound or some phytoestrogen that is similar to estradiol-17β. Goswami et al., (2008) investigated the steroidogenic property of TLC fraction of root extract of Polygonum hydropiper showing similar Rf value with the estradiol -17β and found that the TLC fraction (similar Rf value with Estradiol -17β) of root extract of Polygonum hydropiper contains compound(s) which has functional similarities with ovarian estrogen in female albino rats. Steroidal lactones present in Ashwagandha and steroidal saponins present in Ginseng were identified with Thin Layer Chromatographic method (Gandhi et al., 1994). Vessel et al., (1999) analysed the ethanolic extract of winter cherry (Physalis alkekengi) for the presence of steroid by thin layer chromatographic fractionation in solvent N-butanol-glacial acetic acid-water at the ratio of 100:10:10 with the use of estradiol-17β, progesterone and cholesterol as reference compound. Taking into consideration, the similar migration rate of the plant compounds of Dysoxylum alliarium with the estradiol-17β, makes the present investigation worthwhile for validation of the plant products' anti reproductive potentiality.

Effects of Threshold dose of CBP on Implantation site and litter size:

In the present investigation, the CBP dose 500mg/kg body weight/day was considered as the threshold dose of CBP, which can suppress the pregnancy by way of
abortion as well as, hindered the process of implantation. It was observed in the present study that the administration of CBP can exert an abortifacient effect and at the same time inhibits the process of implantation also. The presence of few implant sites at the periimplantation period (day 6, 7 and 8) despite of CBP treatment, but the complete absence of litters at the end of gestation in the 15 days treated females supports the possible abortifacient property of the crude powder. Although the normal implantation was stunned due to the CBP treatment, yet it was (implantation) not completely stopped up. However, the possibility of implant resorption cannot be ignored at the same time. The absence of litters at the end of the full term gestation might be resulted due to the possible reason that the implants observed in the periimplantation period, might have not survived as viable fetus due to the continuity of treatment (day 15 of gestation) further the periimplantation period. The implants might reabsorb and/or aborted as the days of treatment increased. The fetus might have been failed to recover from the effects of the crude powder as the duration of exposure to the drug increased.

The adverse effect of the crude powder in the implantation process is evident from the reduction in the number of implant sites. The plant product might have created an unfavorable environment for the initiation of implantation of the embryos. It can be speculated that prolong treatment with the CBP might have induced changes in the biochemical components and cellular milieu of the uterine endometrium making it non receptive to the embryo. During the preimplantation and/or early pregnancy in the rat, endometrial glands synthesized and secrete several proteins required for establishment of uterine receptivity and embryo implantation (Stewart et al., 1997, Carson et al 2000 and Gray et al., 2001). Effect of the herbal extract on the uterine receptivity and implantation rate was reported earlier. Post coital contraceptive effect of Gracilaria corticata and Gelidiella acerosa was reported by Ratnasooriya et al., (1994). They suggested the
possibility that the crude extract of *Gelidiella acerosa* induces death of embryos indirectly by progesterone receptor blocking activity as seen with RU-486. The higher dose (1000 mg/kg body weight/day) of the crude extract of *Gracilaria corticata* significantly reduced the number of implantation site and littered pups in rat. The crude extract of the red algae administered on day 7-8 of pregnancy significantly reduced the number of viable implantation sites and significantly increased the number of resorption sites and post implantation loss (by 89%). The fruit of *Lagenaria breviflora* Robert., a plant used as abortifacient in Nigeria showed significant result when tested for its anti-implantation activity (Elujoba *et al.*, 1985) causing 60% anti-implantation activity by methanol extract of 20g/kg whole fruit while 5g/kg fruit pulp gave 100% activity. Yuehchukene, an alkaloid isolated from the root of *Murraya paniculata* exerts 100% antiimplantation effect in mice at a dose of 2 or 4 mg/kg on day 1-3 of gestation (Wang *et al.*, 1990). However, it is not clear in all the cases that a single compound or multiple compounds of these plants exerts effect on implantation loss in laboratory animals.

**Effects of CBP on Reproductive organs: Effects on Ovarian follicle of cyclic rats**

The field observations carried out during the present course of research revealed that the crude bark powder of the plant *Dysoxylum alliarium* is capable of termination of early pregnancy in domestic animals (e.g. pigs). This observation lead to the primary objective of the present investigation that “the bark of the plant has the potentiality of pregnancy interception in –vivo in laboratory animal model system”. Therefore, crude bark powder has been orally administered to the female albino rats in a very low dose compared to that applied in the field for higher animals (Pigs). The dose 500mg/kg/day was determined as the threshold dose, through trial and error and previous reports from the present laboratory (Hazarika and Sarma, 2006). The test was carried out on the cyclic females to determine
if, the compounds present in the CBP targets its effects on the reproductive organs: the ovary and uterus.

In the present investigation, it has been observed that the herbal preparation (bark powder of *Dysoxylum alliarium*) had retarded the normal follicular growth and induces pyknosis in the both granulosa and theca cells in certain ovarian follicles during cyclic stage of the females. The alternation of the normal growth and development of granulosa and theca cells can attribute to a decrease functional integrity of these cells. It is well known that the Granulosa and theca cells secrete steroid hormones. The receptors for follicle stimulating hormone (FSH) are exclusively present in the Granulosa cells, while the receptors for LH are located predominantly on the cells of the theca interna. In case of the CBP treated rats, it became obvious that the structural disparity that is noticed in its ovarian follicle is leading to affect the granulosa cells functional coordination, hindering their normal endocrine function. In normal endocrine function, during each ovarian cycle 6-12 primary follicles develops to secondary follicle in rats. In this process an increase in the size of the oocyte takes place and also an increase in the number of granulosa cell layers that surrounds the oocyte. The formation of the primary follicle in the first half of the cycle is independent of the gonadotropin stimulation, but their further development to antral follicle and beyond requires FSH. The development of follicles from primary to secondary are associated with process, such as secretion and deposition of zona pellucid and follicular fluid which also indicate a state of enhanced cellular activity (Zamboni, 1980). It is also established that a close relationship between the Granulosa and theca cell layers is essential for normal steroidogenic function of the follicle (Richards, 1980 and Erickson, 1982). In the present investigation, in certain antral follicles of CBP treated females, the theca layers and the granulosa layers are not in close contact. These cell layers are detached in some portions as shown in the Fig.3.2C. These structural changes of
the theca cells may be accompanied by functional aberration of the theca layers. It is also reported that the reducing number of cell layers in the theca interna is the symptom of follicular atersia (O’ Shea et al., 1978). The major function of the theca cells is to secrete the steroidal compounds (Guraya, 1980 and 1982). The LH receptor of the theca interna stimulated to differentiate and to secrete the hormone testosterone. The testosterone diffuses through the basal lamina of the follicle to its expanding granulosa layer where it is taken up by the granulosa cells to convert it to estradiol-17β. As mentioned earlier the CBP has exert its effect on the granulosa layers also, hence the disorganized, pyknotic granulosa layers of the graffian follicle indicated the occurrence of atresia by effect of crude bark powder of *Dysoxylum alliarium*. This type of follicular atresia induced by herbal extract of flower of *Hibiscus rosa sinensis* was reported by Kholkute et al., (1976).

Retarded follicular development, shrinkage of the follicle and pyknosis of the nuclei of both granulosa and theca cells was reported by Sarma and Mahanta (2000), following treatment of composite root extract to albino rats. Rat ovary treated with crude root extract of *Polygonum hydropiper* induced follicular recruitment, while in the later stages it promoted follicular atresia (Hazarika and Sarma, 2006). In the present research work, it has been assumed that the follicular degeneration in all stages of follicular development in CBP treated females is induced by the estrogenic compound(s) present in the bark of *Dysoxylum alliarium*. The phytosteroids which are nonsteroidal compound bind with estrogen receptors exerts either agonistic or antagonistic effects to the target organ. Miksíček (1993) reported that several commonly occurring flavonoids mimic the biological effects of estradiol-17β by virtue of their ability to bind to and activate the nuclear estrogen receptor. According to Laurence (1964) any compound possessing estrogenic activity may exhibit the antifertility activity; they act by gonadotropin secretion with consequence inhibition of ovulation. The occurrence of antral follicular degeneration
and/or atresia in CBP treated non pregnant rats’ ovary may be an indication of such effect, where follicles are degenerated before reaching the mature graffian follicular stage. Plant estrogens are also known to inhibit enzymes involved in steroidogenesis (Workineh et al., 2006). Phytoestrogens have been shown to interfere in estrogen negative feedback by binding to estrogen receptors in anterior pituitary or hypothalamus and indirectly alter ovarian steroidogenesis. Administration of exogenous androgen to estrogen treated hypophysectomised rats increases the incidence of pyknosis and enhances the morphological disintegration of membrana granulosa (Richards, 1980). In the present investigation, while many of the ovarian follicles showed degeneration and atresia following CBP treatment, a number of follicles remain healthy and shown to be unaffected by the CBP treatment. At the same time, the corpora lutea are found to be unaffected by the CBP in cyclic females. Moreover, the functional period of this traditional medicine (bark of *Dysoxylum alliarium*) has been considered only during post coital period, which is the dominant luteal phase of ovary. This observation in histological studies lead to the speculation that effects of bark of *Dysoxylum alliarium* in pregnancy interception (in pigs, as observed in field) may be mediated through certain other mechanism involving the uterine function. Follicular atresia has been recognized as a normal process during prepubertal, pubertal and active reproductive age of rodents (Baker, 1972; Coucouvanis et al., 1993; Ratts et al., 1995; Reviewed by Melissa E. Pepling, 2006). Therefore, a further study shall be required to elucidate the mechanism of action of CBP of *Dysoxylum alliarium* on ovarian follicle.

**Effects of CBP on Reproductive organs: Uterotropic activity on cyclic rats’ uterus**

The results of the thin layer chromatographic fraction of the methanolic crude extract of bark powder of *Dysoxylum alliarium* indicated the presence of steroidogenic compound(s) in the bark of the tested plant. A detail research work on the nature and
structure of the compound(s) is yet to be carried out. It has been expected that, the steroidogenic compound(s) present in the bark exerts its estrogenic effects on the reproductive organs of the female rats, thus causing pregnancy interception during early gestation period. Estrogenic effects of phytosteroids on female reproduction have been extensively studied in last few decades. Many of these phytoestrogens especially isoflavones and lignanas exerts uterotropic effects (LeeCole L. Legette et al., 2009; Rachon D et al., 2007; J L Burton and M Wells, 2002; Charles DN. Humfrey, 1998), while others are used for addressing climacteric syndrome including vasomotor symptoms and postmenopausal health risks, as well as their anticarcinogenic, neuroprotective and cardioprotective activities and prostate health and bone formation promoting properties (Bagchi D et al., 2001).

It is well established that exact equilibrium of estrogen and progesterone is necessary for fertility and pregnancy. Compounds disturbing the hormonal function can cause infertility (Blye 1970). While the role of Gonadal steroid estrogen and progesterone is crucial for proliferation and differentiation of endometrial tissue during follicular and secretory phase of menstrual cycle; administration of exogenous sex steroids produces changes in classical histological features such as glandular structure, mitotic status of the glandular cells and luminal glands secretion of uterus (Noyes et al., 1950; Habiba et al., 1998; reviewed by Jabbour et al., 2006). In addition, the endogenous estrogen involved in the development of male and female genital tract, neuroendocrine tissues as well as secondary sexual characters (development of breast tissues). It is established that at cellular level the gonadal estrogen promote cellular proliferation, hypertrophy of the female reproductive tract and secondary sexual organs and induce synthesis and secretion of cell type specific proteins (Sonnenschein C and Solo AM, 1998). In the present investigation, it has been speculated that the bark of the tested plant 'Dysoxylum alliarium'
contains phytoestrogens which has been responsible for endometrial cellular proliferation and hypertrophy in non pregnant uterus as shown in Fig.3.5 as well as interception of pregnancy in rats. Increased height of luminal epithelium and cellular proliferation following CBP (of *Dysoxylum alliarium*) treatment was observed both in cyclic and pregnant rats’ uteri. This speculation of presence of estrogenic compound in the bark has been made on basis of thin layer chromatographic fractionation of the methanol extract of CBP. As noted earlier, the phytoestrogens are naturally occurring phytochemicals found in plants and plant products which are structurally and functionally similar the to the estradiol-17β (isoflavone) or the synthetic estrogen, such as diethyl stilbestrol (lignins) (Aldercreutz H. et al., 1982). Similar to the effects of bark powder of *Dysoxylum alliarium*, many phytoestrogens have been reported in recent years for its uterotropic properties. Effects of certain phytoestrogens like coumestrol, genistein, β-sitosterol etc. on maturational and morphological aspects of female reproductive organs of laboratory animals have been well documented (Reviewed by Burton and Wells, 2002). The uterotropic activities of coumestrol (Whitten et al., 1992) and genistein (Goldin et al., 1982) have been reported to increase the uterine fluid content and hyperplasia of the endometrium. Similarly, the uterotropic activity of racemic equol, a soy derivative phytoestrogen is characterized by greater epithelial height of the endometrial surface epithelium, increase thickness of uterine stroma and myometrium (Rachon et al., 2007). Oral administration of the ethanol extract of *Balanites roxburghii* increased the thickness of the endometrium, and height of endometrial epithelium (Padmashali et al., 2006). The results of the biochemical and histological studies of the extract *Balanites roxburghii* reported the presence of mild estrogenic activity of the extract. In the present investigation, the bark powder of *Dysoxylum alliarium* found to be exerts uterotropic effects on the cyclic rats’ uteri. The uterine luminal epithelium appeared hypertrophied
with greater epithelial height, but with lesser areas of uterine stroma. The endometrial surface epithelium showed an increased proliferation which extend finger like projections into the stromal zone. It has been appeared that the number of endometrial glands was lesser in CBP treated females than the controls. In rodents increased luminal epithelial cell height and the density of endometrial glands are reported to be the hallmark of estrogen action on the reproductive organs (Evrim Erdemoglu et al., 2009). In the present investigation, the lesser areas of endometrial stroma and decrease number of endometrial gland in CBP treated females is a deviation from the classical phytoestrogenic effects on female reproductive organs. Therefore, elucidation of the mechanism of action and the nature of active compound present in the CBP of *Dysoxylum alliarium* needs further investigation.

**Effects of CBP of *Dysoxylum alliarium* on pregnant rats’ uterus and ovary during early gestation and possible mechanism of pregnancy interception**

**Effects on decidualization**: In the present investigation the histological studies of uterus during periimplantation period (day 4 to day 8 of gestation) showed abrogated decidualization and embryonic degeneration in the CBP treated rats. A proper decidualization of the receptive uterus is the *sine quo non* for embryo implantation. A galaxy of research article has been published in last few decades on the mechanism of decidualization and associated morphological and physiological changes of the stromal fibroblast into the decidual cells. The classic article of Noyes et al., (1950) described the decidualization process as the differentiation of human uterine stromal cells into decidual phenotype during the menstrual cycle. This finding and description on decidualization has been further elucidated as the elongated fibroblast like stromal cells of the uterine proliferative tissue begins to differentiate around the endometrial blood vessels into larger, rounder and often binucleated decidual cells (Dallenbach-Hellweg, 1987). The human decidual cells have been reported to be induced and affected by large number of bioactive
compounds like cytokines (IL, TGF-β) hypophyseal hormone (Prolactin), ovarian hormones, neuropeptides, extracellular matrix components and enzymes which are not detectable in the stromal cells of proliferative endometrium (Baiqing Tang et al., 1994). In contrast to the stromal cells’ physiological roles in the endometrium, the decidual cells synthesize large number of other compounds like prostaglandins, prolactin like compounds, hyaluronate, alkaline phosphatise (Carson et al., 1987; Gu et al., 1994 and Bany et al., 1998). Cytokines, signaling molecules and factors, homeobox genes, cell cycle molecules, extracellular matrix remodeling factors and certain lipid mediators have been reported to be expressed by the decidual cells and important for the decidualization process (Reviewed by Dey et al., 2004; Wang and Dey, 2006). As observed in the present investigation, CBP exerts its effects on receptive uterus on day 4 of gestation (Fig.4.2). The stromal tissue appeared vacuolated in the CBP treated females from the day 4 onward of the gestation. The structurally abrogated receptive uterus on day 4 and day 5 (Fig.5.2) of gestation further undergoes morphological changes inducing vacuole formation in the cytoplasm of the decidual cells during subsequent days (day 6 to day 8 of gestation) as shown in the Figures 6.2, 7.2 and 8.2. The mechanism of action of CBP on the decidual cells needs further investigation at the cellular level. The present investigation showed the effects of CBP of *Dysosyllum alliarium* on the cellular structure of decidual cells starting from the receptive period of rats’ gestation. This structural aberration may lead to the physiological changes leading to the pregnancy interception during early gestation. Recent researches on mammalian gestation has been defined the process of decidualization as the numerous morphological changes of the endometrial stroma into decidua and is an example of physiologic transdifferentiation (Griselda Vallejo et al., 2010). The differentiation of the endometrial cells into decidual tissues which is also called decidual cell reaction (DCR) or the decidualization involved changes in extracellular components
modulating the matrix either with decrease or increase in the number of collagen fibrils (Mulholland et al., 1992). In the present investigation a drastic changes in the morphologic structure of the cellular arrangement of the myometrial region along with decidual zone has been observed following administration of CBP from day 1 of gestation. As shown in the Fig 4.2D, the myometrium appears loose following CBP treatment indicates the effect of tested plant on cellular matrix. This deleterious effect of CBP may be mediated through the inhibition of ‘moesin’ synthesis which has been considered as the actin binding protein and involved in cytoskeletal remodeling of rat decidual cells (Laura Venuto, 2008). This effect has been appeared continued to the post implantation period on day 7 (Fig 7.2D) and day 8 (Fig. 8.2C) of gestation showing gap between the decidual cells and the myometrial fibrils. It has been speculated that the CBP of Dysoxylum alliarium may be involved in the abrogation of decidualization process affecting both genetic and epigenetic factors of the endometrial cells. In mouse large number of genes has been reported to be involved in the process of rat endometrial cells decidualization (Griselda Vallejo et al., 2010). Many of these genes are involved in protein synthesis, while others are related to chromatin structure and encoding transcription factors.

Alteration of steroid hormone profile and its receptors: In the present study the uterine endometrium of control rats at various stages of their gestation showed columnar epithelium with regularly shaped cells and healthy nucleus. The implanted embryo also seemed to be showed normal growth and development in the control groups. The administration of crude bark powder evidently induced prominent histological changes in the pregnant uterus. Changes in the endometrium of the day 4 receptive uterus were apparent from the CBP treated luminal epithelium with hypertrophied and degenerated cells. The disparity of the glandular epithelium of endometrial glands must be directed to a functional breakdown of these glands. In mouse the uterus became most receptive to
blastocyst on day 4 (Paria et al., 1993) as implantation takes place on day 4.5 onwards. Recent researches suggest that concentration of estrogen is very crucial for uterine receptivity at this period. Uterine non receptivity is also induced at high estrogen level with aberrant uterine expression of implantation related genes (Dey et al., 2004). Ovariectomy before the periimplantation estrogen secretion on the morning day 4 of pregnancy induces delayed implantation (Yoshinaga et al., 1966 and Paria et al., 1993). Such delayed implantation can be turned on by activating the blastocyst by a single injection of estrogen in the progesterone primed uterus (Psychoyos et al., 1973 and Yoshinaga et al., 1966). Thus, the two hormones act in a coordinated manner establishing the favorable environment to open 'window of implantation' by regulating proliferation and/or differentiation of uterine cells in a spatiotemporal manner (Dey et al., 2004). The estrogen acts via their receptors (ER) α and β, and progesterone receptor (PR), A and B. Expression of these receptors has been identified in the endometrial glandular epithelium and stromal cells (Critchley et al., 2001 & 2002). Therefore, any endogenous or exogenous changes in these hormonal level may create an unfavorable environment for implantation and progression of pregnancy. Accordingly, in the present study it has been speculated that changes in the endometrial glandular epithelium and/or stroma region induced by CBP of *Dysoxylum alliarium* might interfere in the efficiency of hormone uptake by these receptors. As a consequent, this might hinder the process of normal blastocyst implantation. Moreover, the surface epithelia of the endometrium of the CBP treated day 4 rats showed typical 'estrogenic' pattern of morphology (Kamboj et al., 1969). Appearance of such estrogenic pattern of morphology exerted by the CBP can be attributed to the possible estrogen like property of the plant material.

Although the plant material exhibited noticeable effect on the receptive uterus on day 4 of gestation acting directly on the uterus or indirectly via ovarian function, the
uterus however, succeed to some extent in the duo event of 'receiving the blastocyst' and the 'blastocyst implanting itself' in the uterus. Likewise the day 4, the day 5 treated uterus histoarchitectural changes resulted in the degeneration of the stromal cells underlying the epithelial cell layer. Such modulation of stromal cell layer indicates the retarded stromal cell proliferation and differentiation due to CBP action. Following the event of implantation the stromal cells under the influence of steroid hormones proliferate and differentiated to transform in to decidual cells through the process called decidualization. Secretions from the decidual cells critically regulate the uterine remodeling, early embryonic growth, uterine angiogenesis etc (Das et al., 2009). Thus, the decidualization is essential for the proper growth and development of implanting embryo. Another investigation reported that progesterone through its receptors plays a vital role in decidualization (Lydon et al., 1995). However, the expression of P450 aromatase, a key enzyme which converts the testosterone to biologically active estrogen is markedly expressed in the decidual uterus on day 6 and 7 of pregnancy. The expression of aromatase mRNA is initiated on day 5, though undetectable on day 4. Very recent findings demonstrated that the aromatase-driven intrauterine Estrogen (E) synthesis plays an important role in stromal cell differentiation. Letrozole, a specific aromatase inhibitor when treated in an ovariactomised mice on day 5 of pregnancy with progesterone showed a significant reduction of decidual mass and majority of the embryos failed to developed and reabsorbed by day 10 of gestation (Das et al.,2009). A severe reduction in the intensity of 'Connexin 43' (Cx43), a gap junction protein also evident when pregnant mouse treated with Letrozole. The decreased expression of Cx43, a critical regulator of stromal differentiation indicated that decidualization is impaired when the inhibitor Letrozole blocks aromatase activity. In connection with the above discussed findings it can be assumed that the CBP may exert effect on the uterine estrogen production either by
inducing or inhibiting action, thereby unbalancing the endogenous estrogen level. However, this point of discussion is a mere explanation only to understand the possible working pathways of CBP; the actual mechanism behind the CBP action is unknown. Therefore, it can be concluded that hindrance of the stromal cell differentiation can be a possible reason for resorption of the embryo in later stages of development in this investigation. The formation of vacuole and degeneration of the epithelium in the pregnant day 6 treated uteri signifies the deleterious impact of CBP. Dietary exposure of ethynil estradiol (EE2 at a dose of 0.03μg/kg) and zearalenone (ZEA at dose of 0.1mg/kg) have also been reported to cause oedema and vacuolation in immature rat uterus (Heneweer et al., 2007).

The infiltration of neutrophils around the site of degraded endometrial glands indicates inflammatory reaction induced by the administration of the CBP. Inflammatory reaction also results in a large increase in vascular permeability and allows fibrin to pass through the blood vessels. This might be one possible reason of increase in the vascularity in the stromal and myometrial zone. The degenerated epithelial layer as well as vacuole formation in the underlying decidua zone showed the declining property of the endometrium to nourish and support the implanted embryo. Degeneration of embryos and postimplantation embryonic resorption in the uteri are also known to be caused by massive leukocyte infiltration (Mandal et al., 2007). In contrast, infiltration of large number of polymorphonuclear leucocytes in the endometrial stroma may not have a primary role in preventing conception (Schulten et al., 1975). Yet, there has been reports that this factor can do damage to the blastocyst (Kar, 1968; Corfman and Segal, 1968). The CBP certainly exerted its effect on day 7 and day 8 embryos bringing shrinkage of the implanted blastocyst unlike the control embryos where the normal growth and development of the later was apparent. The loss of implanted embryos in the CBP treated
females is also evident from the litter size counted at the end of full term of gestation (Table 1). Such results unveil the possibility of many unseen events happened in the preimplantation and post implantation period as a result of CBP treatment. As it is noted above that the effect of CBP creates a supposed unfavorable environment for embryo implantation in the day 4 receptive uterus. Nevertheless, it could not stop completely the embryos from implantation. The CBP had succeed to an extent from blocking the normal pace of implantation which was much obvious from the significantly lower implantation sites on day 7 and day 8 (Fig. 2.3) of gestation as compared to control. Likewise, the results of the zero litter size counted after full term of gestation revealed that the CBP stunned the sustenance of pregnancy possibly thereby exerting a supposed abortifacient effect. Since, the implanting embryos were present up to day 7 and 8, but no pups were given birth, it indicates the chance of being aborted the embryos at mid gestation in the CBP treated females. However, the underlying mechanisms of work path of the plant products are not clear. It can be assumed from its effect that it may perhaps works via disturbing normal endogenous endocrine levels either in an agonist or antagonistic way.

Effects on ovary during gestation: In the present investigation the CBP was administered to the female rats from day 1 of gestation. During this period, the ovarian structure is dominated by the corpora lutea. The corpora lutea synthesize the progesterone required for endometrial receptivity and decidualization. The result of the present investigation showed that the ovary on day 8 of gestation appeared with multiple number of corpora lutea (Fig. 3.7). A noticeable structural change of the corporal lutea in the CBP treated rats' ovary has not been observed. It is to be mentioned here that the ovarian histology was studied on day 8 of gestation following day 1 to day 8 of CBP treatment. The objective was to detect maximum possible effects of the CBP on ovarian functional structure for pregnancy support. The healthy corpora lutea with the normal lutein cells of
CBP treated females suggested the possible synthesis and secretion of progesterone required for the gestational support by the endometrium. Progesterone has been known to be critical for uterine receptivity and embryo implantation in mice (Lydon et al., 1995). This effect of progesterone on the receptive endometrium is possible only in an estrogen primed environment (Ma WG et al., 2003). Recent findings suggested that ovarian estrogen may not necessary for estrogen priming of the endometrial tissues; rather de novo synthesis of estrogen by the morphologically and physiologically altered stromal cells is essential for progesterone action (Amrita Das et al., 2009). The histological findings of the present investigation reaffirms that, CBP does not interfere in the steroid synthetic machinery of the ovary, rather mediate its antireproductive effects modulating endometrial cellular environment. It has been expected that the potential compound present in the CBP may inhibit steroid hormone binding with the receptor in uterus resulting failure of decidualization and pregnancy interception. Further investigation on the cellular level of ovarian follicular cells and lutein cells and the endometrial tissues shall be required to elucidate the mechanism of action of CBP of Dysoxylum alliarium on the reproductive functions of rats.

**Effects of CBP of Dysoxylum alliarium on uterine protein and vascular endothelial growth factor (VEGF) during peri implantation**

**Effects on uterine proteins** : Structural and physiological regulation of uterine luminal environment is important for successful attachment and implantation of the blastocyst. A number of hormones, growth factors, and cytokines are involved in this process of uterine remodeling during early pregnancy and embryonic development. Multiple numbers of genes are expressed producing structural and functional (transcriptional) proteins for cellular remodeling and embryo implantation. Estrogen and progesterone play pivotal roles during this early stage of gestation promoting the 'epitheloid reprogramming' of stromal cells to decidual cells and the embryo implantation.
The luteal hormone progesterone stimulates the proliferation and function of estrogen primed uterus. A drastic change of the serum concentration of these two gonadal hormone estrogen and progesterone take place during the gestation period depending on its requirement and functions (Yoshinaga, 1976). The mechanism whereby the steroid hormones influence the blastocyst development, supportive endometrium and other fetal-maternal interactions during gestation is poorly understood. The close proximity of the uterine secretory proteins and the growing blastocyst has drawn the attention on the potential role of the uterine proteins in nidation. In this stage disappearance of many inhibitory proteins and/or emergence of activation proteins have been suggested earlier (Surani, 1975; Lejeune et al., 1985). The changes of the uterine protein profile reflect the genetic control of embryonic development from maternal gene pool. In rat multitude of newly synthesized proteins has been identified throughout the pregnancy. The protein molecule having molecular weight 115kDa has been reported to be expressed during first two days of gestation in rat similar to one noted at estrus. This has been followed by synthesis of protein having molecular weight 43kDa on day 5 to 6 and a new protein of molecular weight 160 kDa have been detected on the invasive phase on day 6 of gestation. The appearance of a 43,kDa (MW) protein has been considered as one of the most marked changes at the time of blastocyst invasion of the uterine epithelium. (Michael H. Jacobs and Richards Lyttle, 1987). Generally, during pregnancy despite of large increases (13-fold) in uterine size, the fractional rate of protein synthesis (measured in vivo) remained unchanged when compared with nonpregnant tissue (Morton et al., 1986). In ovariectomized females the proteins of higher molecular weight were virtually undetectable by Day 10 of gestation (Surani, 1976).

In the present investigation, it has been observed that *Dysoxylum alliarium* generates marked modulation of macromolecules like protein of the uterine tissues of
pregnant rats during early gestation. Day 4 to day 6 has been considered as the critical period of gestation in rats during which a short period of 'implantation window' is formed followed by adhesion of free floating blastocyst to the maternal tissues. A multitude of hormones, proteins, growth factors and cytokines are involved in a coordinated manner in this dynamic event of fetal maternal interaction. In the present study, the CBP of *Dysoxylum alliarium* has been found to stimulate synthesis of a high molecular weight protein (band A Fig. 4.5) on day 4 of gestation. The functional role of this protein in uterus during this uterine maturation phase is not known. The other proteins which have been observed in both control and CBP treated females, but with altered intensity in the treated rats' uteri has been attributed to the estrogenic compound present in CBP. It has been speculated that due to the expression of similar protein molecules in both control and CBP treated females' the uteri of the later did not loose the capability of receiving the embryo. Implantation has occurred during the subsequent days of gestation. Similar to the day 4 of gestation, the protein molecules observed on day 5 of gestation in control females have been expressed following CBP treatment for five consecutive days from day 1 of gestation. In addition, a new protein having molecular weight approximately 125kDa (band C, Fig. 5.6) has been expressed following CBP administration. The control females exhibited similar pattern of protein expression on both day 4 and day 5 of gestation. Synthesis of new proteins and alteration in expression intensity during this phase could lead to physiological changes of uterine stromal cells resulting in structural and functional aberration of the decidualization process. In the present research work, the functional role of these protein molecules expressed during the early gestation either in control or in CBP treated females' uteri is not known. It has been expected that these molecules expressed in the control rats' uteri are inevitable for establishment of pregnancy. Modulation of these protein molecules quantitatively qualitatively may induce deleterious effects either on
maternal tissues or on the embryo. The embryonic degeneration and structural aberration of decidua induced by CBP of *Dysoxylum alliarium* has been evident on day 6 of gestation (Fig. 6.4). On this day of gestation, the control females exhibited as many as nine protein molecules (band B to J, Fig. 6.5) in the uteri. A high molecular weight protein (band A, Fig. 6.5) has been found to be newly synthesized on day 6 of gestation following CBP treatment. The involvement of this CBP induced new protein in the induction and promotion of structural and functional degeneration of decidua and the embryo needs further investigation. The results of the protein profile of the present study on day 7 of gestation showed that requirement of expression of protein may varied on different days of early gestation. This variation may be depending upon the stages of embryonic development and subsequent changes of the maternal tissues to support the embryonic growth. The evidence came from the present observation that protein molecule having molecular weight approximately 66kDa has been expressed in strong intensity on day 5 (band F, Fig. 5.6) and day 6 (band G, Fig. 6.5) of gestation in both control and CBP treated females. In contrast, expression of this protein has been decreased in intensity on day 7 of gestation in control females' uteri indicating decreased physiological role on day 7 of gestation. In the present investigation, CBP of *Dysoxylum alliarium* has been able to alter this normal schedule of protein expression. It has been evident from the observation that this protein molecule (mol wt approximately 66kDa, band E, Fig.7.5) continued to be expressed in similar intensity on day 7 of gestation following CBP administration. Similar to day 6 of gestation, the CBP has been continued to stimulate expression of a high molecular weight protein on day 7 of gestation. The protein molecule having molecular weight > 205 kDa (band A, Fig. 7.5) has been observed as doublet indicating presence of multiple numbers of proteins. The differential expression and requirement of proteins for gestational support has been more pronounced on day 8 of gestation. Results of the present
investigation showed that the expression pattern of certain protein molecules have been shifted on day 8 of gestation in control females. The protein molecules having molecular weight in between 43kDa to 66kDa have observed to express in higher intensity from day 4 to day 7 of gestation. These proteins are selectively down regulated in the control females' uteri on day 8 of gestation (band E & F, Fig. 8.5). In contrast, a low molecular weight protein (band I, Fig. 8.5) has been expressed in greatest intensity among all the proteins expressed on day 8 of gestation. Administration of CBP has reduced this dynamic change of uterine protein profile on day 8 of gestation. This has been evidenced by expression of certain protein molecule (band E, Fig. 8.5) in similar intensity of day 4 to 7 of gestation. The role of these two specific proteins (band E and I, Fig. 8.5) on embryonic development is not known. Histological studies showed increased degree of degeneration of the embryo (Fig.8.4) indicating attenuation of embryonic developmental process by the potential abortifacient compound present in bark of *Dysoxylum alliarium*. In the present investigation, the protein molecules studied during periimplantation period has been considered as the uterine origin. However, it is not clearly known, if the proteins are either synthesized by the maternal tissue or the growing embryo. Therefore, it has been speculated that the CBP may exerts its effects either any one of tissues (maternal or fetal) or both during early gestation. As mentioned above the synthesis and appearance of higher molecular weight protein reported to be increased during early pregnancy (Surani, 1976). Many proteins’ synthesis is up regulated during the period between day 5th and day 6th of pregnancy in rat (Bell *et al.*, 1977). Such increased synthesis of specific protein might be crucial to pregnancy. Thus, the CBP may act on the pregnancy specific high molecular weight proteins to exert its effect. The precise nature and cause of the expression of new proteins and the increased expression of some regular (protein normally appeared in control sample) proteins in response to CBP treatment cannot be detected in considering
any single factor for such effect. However, the estrogen like nature of the methanol extract of the bark powder of *Dyssoxylum alliarum* revealed by the TLC results might be one of the possible explanations of such effect as initiated by CBP. The role of estrogen in inducing uterine protein synthesis during estrous cycle and gestation has been discovered earlier (Benita S. Katzenellenbogen and Nancy G. Greger, 1974; Michael D. Walker et al., 1976; Stefano Iacobelli et al., 1977). Later on these ‘estrogen induced proteins’ have been thought to be involved in the structural and functional modification of uterine stromal tissue for uterine receptivity and decidua formation. It is evident from many previous experiments that estradiol induces the nucleic acid and protein synthesis in the uterus and blastocyst (Mohla et al., 1970) as well as performing many other important reproductive functions. Notides and Gorski (1966) first reported the induction of new protein (induced protein) in the uterus of rats and mice on administration of estradiol-17β. Later on, another investigation (Katzenellenbogen and Gorski, 1975) had shown that the synthesis is induced by a wide variety of estrogens, is dependent on RNA synthesis, and is linearly related to the concentration of nuclear bound estrogen. Furthermore, Hazarika et al., (2002) reported the expression of two high molecular proteins in between 55KD and 68KD in the uterus of OVX female in response to exogenous estradiol-17β (subcutaneous injection). The increased ‘induced protein’ (IP) synthesis in pregnant rat might be associated more directly to uterine cell growth and division (Bell et al., 1980). Recent report (Reviewed by Dey et al., 2004) also mentioned that variety of natural or synthetic xenoestrogen mimics the natural estrogen and thus exerts an estrogen like effect in the uterus including increased DNA and protein synthesis. However, functionally the increased expression of protein and synthesis of new protein may exert an adverse effect in the process of implantation and the maintenance of embryo. The classical concept of steroid hormone-receptor interaction the steroid hormones interact with their target organ.
via specific nuclear receptors. However, recent evidences say that responses to an estrogenic compound in target tissue are not necessarily related to its affinity for the receptor, suggesting the presence of additional pathways. Therefore, the interaction of the xenoestrogen with Estrogen Receptor (ER) or other binding proteins may not result in a similar kind of transaction that normally occurs with natural ligands (Reviewed by Dey et al., 2004). Sometimes failure of the receptor expression may lead to functional impairment of the steroid hormones in the uterus and ultimately culminating in the loss of pregnancy.

In the present investigation, only a few protein molecules have been studied by the one dimensional polyacrylamide gel electrophoresis. Due to low resolution of the protein separation of this technique, further detailed study will be required to determine the effects of CBP on uterine protein during gestation. With the advent of new technologies like cDNA microarray analysis, clusters of gene have reported in recent years involved in the early process of gestation either in the growing embryo or in the maternal endometrial tissues (Griselda Vallejo, 2009). Many of these genes e.g Csdc2, Trim27, BMP1, Wt1, Gna 12, Men1 etc are involved in cell proliferation, signal transduction, matrix/structural proteins, chromatin architecture and remodelling during the early pregnancy. Expression of many other genes like ‘Hedgehog’ (Hh) family genes (Kaiyu Kubota et al., 2008), temporal and spatial expression of heat shock protein 105 (Hsp 105) have been reported to play crucial role in regulating embryo implantation in rat (Jin-Xiang Yuan et al., 2009). However, the key gene and its products required for pregnancy regulation is yet to be identified. The findings of the recent years suggested that embryonic development is a coordinated approach orchestrated by multiple numbers of genes and theirs products.

**Effects on VEGF Expression:** Following the SDS-PAGE separation of uterine protein the qualitative expression of the growth factor VEGF during this early gestation
period has been identified in the uterine protein samples by western blot analysis. The study showed the expression of the growth factor within a molecular weight range 43-66 kDa in uterine tissues from day 4 to day 8 of gestation (Fig.9.1 & 9.2). It has been noted earlier that the estrogen like nature of the plant compound can modulate the regular expression of the growth factor in the uterine tissue. Uterine angiogenesis is known to be influenced by the steroid hormones estradiol-17β (E2) and progesterone (P), through activation of their respective nuclear receptors. VEGF is present in different parts of the uterine tissues and its differential expression is related with ovarian steroid levels. Many investigators have indicated that estrogens regulate VEGF mRNA expression in human endometrial cells (Shifren et al., 1996; Sugino et al., 2002) and rat uterus in vivo (Cullinan-Bove and Koos, 1993). Complete suppression of VEGF expression in the absence of estradiol has been demonstrated in upper compartments of the endometria of hormone-deprived rhesus macaques (Nayak and Brenner, 2002).

The critical role of endometrial angiogenesis during gestation beginning with stromal cell decidualization, placentation and the ultimate embryo survival has been widely accepted (Maruyama T and Yshimura Y, 2008). Vascular endothelial growth factor (VEGF) is an angiogenic factor which have been attributed to embryonic phenotype that fail to develop different phases of the normal vasculature due to VEGF/VEGFR receptor (VEGFR) specific signaling molecules (Leigh Coulta et al., 2005). Recent researches have shown that expression of VEGF and VEGFR in the fetal-maternal unit during periimplantation period of embryonic development in rats (Krishna P Chennajhi, and Nihar P Nayak, 2009), non human primates (Nataki C Douglas, 2009) and human (Neil Sidell et al., 2010) plays critical role in embryonic survivability and growth through the development of vascularity both in maternal and fetal tissues.
In the present investigation vascular endothelial growth factor-C has been studied during periimplantation period (day 4 to day 8 of gestation) in both control and CBP treated females. The objective was to determine, if the CBP of *Dysosyllum alliarium* mediates its antireproductive effects modulating expression this angiogenic growth factor (VEGF). The results showed that the VEGF-C has been expressed both in fetal maternal tissues in rat during the periimplantation period (day 4 to day 8 of gestation). The uterus on day 4 of gestation rat has been known as the most receptive phase of the preimplantation stage. During this phase, the uterine tissue is characterized by dramatic physiological changes and morphological remodeling of the tissue creating a favorable environment for embryo attachment. In the present study VEGF-C has been observed to be temporally and spatially expressed in the uterus during this dynamic event on day 4 and day 5 of gestation (Fig.4.3 & 5.4). The cells of endometrial surface epithelium and glandular epithelium showed the expression of VEGF in control rats' uteri. The transforming stromal tissues and the myometrium expressed the growth factor on day 4 of gestation. This information suggests the promoting role of VEGF in tissue remodeling and growth during early gestation in rats. On day 5 of gestation, the VEGF has been selectively expressed in the decasualizing areas below the endometrial surface epithelium (Fig. 5.4) in control females. It has been speculated that the site of embryo implantation during this critical has been determined by the expression of VEGF in the decasualized tissues. Administration of CBP has been found to alter the VEGF expression pattern in uterine tissues during day 4 to day 5 of gestation (Fig. 4.4 & 5.5). An uneven expression of VEGF in different cellular parts of the uteri has been observed in a discrete manner in the CBP treated rats' uteri than that of controls. The uterine luminal epithelium of the CBP treated females showed the expression of VEGF in a similar quantum with that of the control, while the decidualized tissue exhibited lesser degree of this growth factor expression during this stage of
periimplantation. Decrease VEGF expression in the decidualizing stromal cells in the CBP treated rats' uteri indicated unfavorable physiological environment for embryonic support. Accumulation of VEGF in the luminal epithelium before implantation was probably due to the estrogen peak during preovulation period (Longjiang et al., 2001). Moreover, as soon as the opening of implantation window, i.e. the preparation of the rat uterus to receive the blastocyst, the increased vascular permeability at implantation site also increased. These processes are believed to be triggered by the timely interplay of estrogen and progesterone (Psychoys et al., 1973 and Chakraborty et al., 1995). During this period the endometrial epithelial and stromal cells undergo coordinated proliferation and differentiation forming decidua promoted by estrogen and progesterone (Stewart and Cullinan, 1997; Carson et al., 2000). The decidual cells in close proximity to the endothelial cells proliferate to form a new vascular network in the uterus during early pregnancy (Wang and Dey, 2006). The role of VEGF in mediating this process as well as the role of gonadal steroids on VEGF expression during angiogenesis has been poorly understood. The VEGF mRNA expression in the luminal and glandular epithelium during the early stages before implantation (D1, D5) was also recorded by Longjiang et al., (2001). Rat endometrial cellular transformation is said to be produced by endothelial cell migratory factor on days 3-4 of pregnancy at a low concentration and this signal increases on day 5 and then decreases on days 6 and 7 of pregnancy (Abberton and Rogers, 1995). Such findings advocates that the VEGF is present and active in rat endometrium on day 5 of pregnancy and thus it support the possibility that the endothelial cell migratory factor could be VEGF (Rabbani et al., 2001).

The control females' uteri on the day 6 of gestation exhibited extensive VEGF expression in the decidual zones and in the implanted embryo (Fig.6.3). This finding forwarded the evidence of requirement of VEGF in embryonic development beginning with the blastocyst stage. Administration of CBP (of Dysoxylum alliariun) results in
moderate expression of VEGF in the primary and secondary decidual zones. The degenerating embryo evidenced by histological observation has been characterized by lesser degree of VEGF expression (Fig. 6.4). In the present investigation, it is poorly understood, if the CBP induced the embryonic degeneration mediating through VEGF down regulation or vice versa. Chakraborty et al., (1995) in their in situ hybridization experiment demonstrated accumulation of VEGF mRNA in the decidual bed at both the mesometrial and antimesometrial poles on days 6–8 of gestation in rodents. Halder et al., (2000) observed more intense expression of the growth factor (VEGF) on days 6-8 of gestation at the mesometrial pole. They hypothesized that the intense expression in the mesometrial pole is, because it is the possible part of heightened angiogenesis and placentation.

In addition of expression of VEGF by the maternal tissues, this growth factor has been expressed by the growing blastocyst in mouse (Karin Aase, 1999). The recent evidences of VEGF studies indicated that the vascular endothelial growth factor plays essential role in vasculogenesis and angiogenesis during embryonic development and in adults (Ferrara and Davis-Smith, 1997; Ferrara et al., 1996). In the present investigation expression of VEGF has been detected both in control and CBP treated females' embryo and the surrounding decidual zones during day 7 and day 8 of gestation. A spatial change in VEGF expression has been observed during the post implantation period (especially day 7 to day 8 of gestation) than the preimplantation (day 4 to day 5 of gestation) phase. Expression of VEGF has found to be more in the embryo and the surrounding trophoblast cells than the maternal decidual tissues. Expression of VEGF by the embryo showed the paracrine role of the growth factor in the vasculature development during the entire events of embryonic development and maternal support during gestation (Karein Asse et al., 1999). The control females of the present investigation exhibited the expression of VEGF
by the embryo and the surrounding maternal tissues on day 7 (Fig. 7.3) and day 8 (Fig. 8.3) of gestation. In contrast, the CBP treated females exhibited altered VEGF expression both in the embryo and surrounding maternal tissues during this post implantation period (Fig. 7.4 and Fig. 8.4). It was observed that expression intensity of VEGF following CBP treatment was restricted in certain cells of embryo and the maternal tissues. The embryonic tissues and surrounding trophoblast cells showed a lesser intensity of VEGF than that of the control females'. The western blot studies of the VEGF showed a higher intensity of protein band in the CBP treated uterine protein samples than that of the control (Fig. 9.2) during day 7 to day 8 of gestation. Possibility of increased VEGF might be due to overall consideration of VEGF expression in the uterine tissue following CBP treatment. It is to be noted that increased VEGF has accompanied by the degeneration of both the embryo and the supporting maternal decidual tissues in the CBP treated females. At this juncture, it is not clearly understood, if the increase VEGF is due to embryonic degeneration or vice versa. It has been speculated that the estrogenic compound present in the CBP induces embryonic degeneration. VEGF has been expressed in higher intensity either to restore the embryonic viability or in response to the estrogenic compound present in the CBP. VEGF is expressed in the trophoblast cells during the initial stages of blastocyst implantation and with advancement in the process of placentation in the primate endometrium also (Gosh et al., 2000). The VEGF expressed in the trophoblast cells act as an endometrial chemical inducer to promote the feto-maternal vascular correlation and thus plays an important role in the feto-maternal signal transduction (Koch et al., 1994). The role of VEGF in implantation and pregnancy can be assessed by its expression and inhibition pattern in various normal or induced unusual physiological conditions. The VEGF absence during heterozygous VEGF- deficient (VEGF<sup>−/−</sup>) mice embryos showed abnormal blood vessels formation, while homozygous VEGF deficient (VEGF<sup>−/−</sup>) mice
died on D11-D12 of pregnancy (Carmeliet P et al., 1996). The mice which lacked a functional vegfr-3 gene, a receptor for VEGF C, showed defective blood vessel development in early stage mouse embryos and dies at midgestation due to failure in remodeling of the blood vessels (Dumont et al., 1998). Mice injected with DC101, (a blocking antibody against VEGFR-2), in the pre (day 3.5) and early postimplantation period (day 6.5) disrupts function of the corpora lutea of pregnancy, resulting in the decrease in organ size, regression of luteal vessels and a fall in progesterone secretion within 24hr post injection. Such declining progesterone secretion level results into arrested normal function of the decidual tissue and uterus as its function is dependent on luteal support. As a consequence there is a complete absence of embryonic structures and of placentas on pregnancy day 13.5 resulting in implant reabsorption. Such findings raise the possibility of terminating the pregnancy at any level when the normal expression of the growth factor is altered.

The role of steroid hormone in regulation of VEGF expression is still debatable. Estradiol (E2) increases the steady state concentrations of VEGF mRNA in human endometrial cells through a predominately transcriptional mechanism (Mueller et al., 2000). Estradiol (E2) treatment in the ovariectomised rat strongly expressed the VEGF in the luminal and glandular epithelium, while progesterone (P4) has no detectable effect compared with control (oil treatment) ovariectomised females (Longjiang et al., 2001). Other workers also described a modest (1.6 fold) induction of VEGF in human uterine cells in response to estrogen (Soares et al., 2002). The de novo production of Estrogen in the uterus plays a central role in the regulation of uterine neovascularization during early pregnancy (Das et al., 2009). Local estrogen production in the stromal cells facilitates the decidualization process, which in turn promotes the synthesis and secretion of important angiogenic factors to support the expansion of endothelial cell network in the stromal bed.
The intrauterine estrogen controls the stromal expression of HIF2α, a transcription factor that regulates VEGF production (Ema M et al., 1997). VEGF in concert with angioproteins regulate angiogenesis (Dey et al., 2004). Aromatase driven uterine Estrogen controls the expression of both angioprotein 2 and angioprotein 4 in uterine stromal cells (Das et al., 2009). Such findings support the possible role of estrogen in VEGF regulation. Therefore, the increased expression of VEGF in the CBP treated tissues can be a possible outcome of increased estrogen level in the uterus supposed to be induce by the plant material by reason unknown.

**Toxicological effects of CBP of *Dysoxylum alliariam* during gestation**

Drug detoxification is one of the major functions of hepatic lobules. The hepatocytes remove the toxic substances including alcohol and other toxic materials from the blood which then exits the lobule through the central vein or the hepatic venule (Cunninghingham and Van Horn, 2003). Continuous exposure of the liver tissues to the toxicants may results in hepatoctyes injury which may cause chronic hepatitis and fibrosis ultimately leading to the life threatening complications of portal hypertension and liver failure. A number of adverse agents like alcohol, toxic substances, viruses, genetic and epigenetic factors, chronic biliary stasis, metabolic disorder or hypoxia may trigger the hepatic disorder (Sophie Lotersztajn et al., 2005). Injury to the hepatocytes may leak enzymes into the blood where these can be measured as the indicator of cell damage. Alanine amonotransferase (ALT) and the aspartate aminotransferase (AST) are such two enzymes which indicate the elevation of hepatic injury and liver damage. In the present investigation these two enzymes have been studied during periimplantation period (day 4 to day 8 of gestation) and following a long term treatment (day 1-15) during the gestation period. The liver tissues have been studied histologically only on day 15 of gestation in both control and CBP treated females. The objective was to find out the maximum level of
histopathological changes in the hepatic tissue following a long term treatment of CBP during the gestation. The intensity of histopathological lesions of chronic hepatitis and its grading is yet to define clearly (Elizabeth M Brunt, 2000). The ‘knodell histology activity index’ has been widely regarded as the benchmark for reproducible description of the various morphological lesions of chronic hepatitis (Knodell et al., 1981). In recent years the METAVIR scale has been widely used to measure the degree of fibrosis on HE (hematoxyline cosin) stained sections (Pik-Yuen Cheung et al., 2006; Bedossa and Poynard, 1996). This system grades the fibrosis in five point scale: F0 (no fibrosis), F1 (portal fibrosis without septa), F2 (portal fibrosis with a few septa), F3 (numerous septa without cirrhosis) and F4 (cirrhosis). The cellular necrosis characterized by ballooning degeneration and steatosis in HE stained cells have been categorized according to a four point grade scale signifying: Grade 0 (negative), Grade 1 (upto 33%), Grade 2 (33% - 66%) and Grade 3 (> 66%) cell shows the ballooning degeneration (Sanyal, 2002). In the present investigation infiltration of the neutrophils in the liver following long term CBP treatment was observed. These observations lead to the speculation of hepatocyte necrosis resulting in increase level of SGOT (AST) and SGPT (ALT). The necrotic areas are associated with hemorrhage and neutrophil infiltrations mainly in the periportal areas of hepatic vein (Riordan and William, 2000). A higher ordered hepatic damage (F3 and grade 3) has not been observed following CBP treatment for consecutive 15 days during the gestation period.

The toxic effects of CBP on rat hepatic tissues as described above may be induced by the various agents present in the bark of the plant *Dysosxylum alliarium*. In the present investigation the crude bark powder has been tested for its abortifacient property in albino rats. The thin layer chromatographic fraction suggested the presence of phytoestrogen in the bark which may be attributed for the abortifacient property of the plant. A higher order
hepatic fibrosis caused by the phytoestrogens either in human or in laboratory animals has not been reported. The widely publicized and discussed results of the ‘Women’s Health Initiative’ (WHI) and the one million women study considerably increased the pharmaceutical value of phytoestrogens nullifying its toxicological impact (Clemens B. Tempfer, 2007). It has been reported that dietary increase in phytoestrogens in addition to regular diet is associates with increase quality of life and a significant amelioration of the signs and symptoms of climacteric syndrome (Albertazzi and Purdie 2002; Jeri, 2002; Hann et al., 2002; Nagata et al., 2001). Many of the phytocompounds have recently been used as chemopreventive agent rather than a toxic substance (Edwina N. Scott, 2009).

In addition to the phytoestrogens present in the CBP of *Dysosxylum alliarium*, presence of other toxicants and heavy metals can not be ruled out. These additional substances may not be involved in the reproductive performance of the animal, but affects the hepatocytes causing hepatic injury. The heavy metal like ‘lead’ may induce necrosis of the hepatocytes in rat liver (Isabel Corpas et al., 2002). Similarly, oxidative DNA damage by the selenite ion in primary rat hepatocyte culture has been well documented (Yohko Fujimoto et al., 2009). Presence of such type of toxicants in the stem bark of *Dysosxylum alliarium* can not be ruled out without detail investigation. The hepatic necrosis might be due to the lipid peroxidation supposed to be induced by the plant product. Aqueous extract of Eucalyptus globules is known to be stimulate the membrane lipid peroxidation with a significant increase in the malondialdehyde (MDA) is a major product of lipid peroxidation (Arise et al., 2009).

A gradual increase in the enzymes level was observed as the days of treatment gradually increased (Fig. 10.1 and 10.2). This gradual increase in the aminotransferase level might be due to the period of exposure of the rat liver to the crude powder of the plant. Many plant extracts are known to exert adverse effect on liver tissue. Solomon et
al., (1993) reported the toxic effect of the crude root extract of *Plumbago rosea* on rats. A
significant increased in the ALT (SGPT) level was observed in the rats treated with the
plant extract for 30 days at a dose of 50mg/kg body weight. Administration of chloroform
fraction of *Duranta repens* stem to rats at a dose of 2mg/kg/day (0.2ml contained
2mg/kg/day) increased significantly the levels of SGPT and SGOT (Nikkan *et al* 2008).
The authors indicated that such elevated level of these enzymes may be due to cellular
leakage and loss of functional integrity of cell membrane in liver. The alternation of
membrane permeability might also contribute to the release of these aminotransferases
(Nayanatara *et al.*, 2009).

It is to be mentioned here that the CBP of *Dysoxylum alliarium* has been tested for
it abortifacient property. Therefore, the toxicological parameters have not been studied in
it cyclic non pregnant females. A detailed study of toxic effects of potential active
component present in the bark of *Dysoxylum alliarium* shall be required to develop a
health friendly ‘lead’ from this plant. Isolation of the compound(s) and chemical
characterization of this plant derive materiel shall be the future direction of research on
this potential herbal product for development of an alternative health friendly abortifacient
medicine.

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