CHAPTER 2

REVIEW OF LITERATURE
2.1 Plant as a source of alternate healthcare products

The herb and human relationship might be as old as the human civilization itself or even older than that. Mankind has been using plants as a natural source of food and healthcare regularly from that thousands of years ago. In due course of time this searching has been carried out for better healthcare product of human civilization. The searches gradually developed the herbal remedial system. The herbal therapy might be providing a good alternate therapeutic system to treating many health related problems and disorders and hence continues to grow in popularity.

Of the earth’s estimated 250000 to 350000 plant species identified so far, around 35000 are used for medicinal purpose (Kong jin-ming et al., 2003). The number of medicinal plant user and practitioner is highest among the people of developing countries (WHO Report, 1995). In China for example, the annual herbal drug production is worth US $48 billion with export of US $3.6 billion. In the year 2005 sales of these herbal medicinal products increased to total US $14 billion (WHO, 2008). There is 350000 staff are working at more than 2500 hospitals of traditional medicines in China (WHO report, 2001). In Japan, from the year 1974 to 1989, there was a 15 fold increase in ‘Kampoh’ (Chinese method) medicinal preparation in comparison with only 2.6 fold increase in the sales of mainstream pharmaceutical products. The Japanese per capita consumption of herbal medicine appears to be highest in the world (WHO 1996). Herbal medicine revenue in Brazil was US $160 million in the year 2007 (WHO fact sheet, 2008). In India there are currently about 250000 registered medical practitioners of the Ayurvedic system as compared to about 700,000 of the modern medicine system (Reviewed by Seth and Sharma, 2004). The department of Indian system of medicine and homeopathy increased efforts to standardize and promote quality control of Ayurvedic, Unani, Sidha and homeopathic medicines during the year 1998-99. The central cabinet for Unani medicine
also has published two volumes on single plant drug standardization covering 100 medicinal plants. The EXIM bank of India, in its report has reported the value of medicinal plants related trade in India of the order of US $ 5.5 billion and is growing rapidly (Purohit et al., 2005).

Use of herbal remedies in the western world can be marked from the days of Greek physician Hippocrates (460-377 BC) known as the father of medicine, who uses various herbal remedies for treatment. ‘Dioscorides’ a Roman Military physician mentioned over 600 species of plant with medicinal value in his book De Materia Medica (Kong Jin-ming et al., 2003). Meanwhile, an interest has been developing in the developed countries in the use of alternate and complementary therapeutic system. The United States spent US $17.8 billion on dietary supplements and US $4.2 billion of it for botanical remedies in the year 2001 (CDC National Health Statistics Report, 2008). A poll in France in the year 1992, reports that between 20% and 30% of the population had used alternative and complementary medicine (Maddalena, 1999). Plants are the original source materials for as many as 40% of the pharmaceuticals in use in the United States today. This is to say that either the drugs currently contain plant-derived materials, or synthesized materials from agents originally derived from plants (www.medicine hunter.com). Around 12000 metric tons of dried herbal plant material was processed by an Italian company ‘Indena’ for the preparation of 1139 medicinal plant extracts and for isolation of 202 chemicals in mid 1990s. Now among the 25 best-selling drugs in the world, 30% come from natural products (Kong Jin-ming et al., 2003).

Thus, the timeless quest of human being to explore the herbal world for healthcare had culminating in the development of many pharmaceutical products. Like many other parts of the world the indigenous people of the north east India practiced a good number of medicinal plants for various therapeutic uses. Arunachal Pradesh is one of the provinces of
north east India. This state is situated on the eastern Himalayan Mountain range and harbor 26 major tribal group of population. These indigenous tribal people have their own traditional medicinal system using locally available plants in this eastern Himalayan Mega Biodiversity. The *Dysoxylum alliarium* is one amongst the many plants use by these people. The ‘Adi’ is one of the major tribal group of people of Arunachal Pradesh uses the bark powder of *Dysoxylum alliarium* to terminate the pregnancies of their pet dogs and pigs. The plant *Dysoxylum alliarium*, is mainly distributed in the part of north east India being wildly grown in states like Arunachal Pradesh, Assam, Sikkim and Meghalaya. Though very little literature is available on the medicinal use of the *alliarium* species of the genus *Dysoxylum* (Malhotra et al., 1987), the use of other species from this genus like *Dysoxylum malabarium* and *Dysoxylum binectiferum* for cure of rheumatism, treatment of ear and eye disease (wood oil) is widely mentioned. *Guarea alliaria* (Buch-Ham in Mem. Wern Soc 67:57; 1970) and *Dysoxylum hamltoni* (hiern. vern) is mentioned as the synonyms of *Dysoxylum alliarium* in various literature. The *Dysoxylum hamltoni* is reported for its use to get relief in stomach pain in Assam (Kirtikar et al., 1993). Recent works on the ethanolic extract of the bark of *Dysoxylum binectiferum* showed the pregnancy interceptive activity (Keshri et al., 2007). The ethanolic extract intercepted pregnancy in rats at a daily dose 500mg/kg on day 1-7 postcoitum. *Dysoxylum gaudichaudianum* is reported to possess spasmolytic activity (Bourdy et al., 1996).

**2.2 Plants uses as female anti reproductive drug**

Use of plant resources for fertility regulation is not a new practice. To trim the family size as well as to maintain a gap between child births or to avoid unwanted pregnancies, people across the world are practicing the knowledge of herbal medicine even before the access of modern medicine. Various literatures around the world as well as our own ancient Indian literature tell us about the wide use of herbs to solve various
reproductive health problems. The use of herbs on reproductive problem had received great emphasis after the World Health Organization’s (WHO) stress on the use of nonsteroidal drugs as oral contraceptive; with the changing concept of population control but with better reproductive health also focused to minimize the exposure of steroids in women. The WHO has set up task force on plant research for fertility regulation to find out new orally active nonsteroidal compound having antiimplantation property (Griffin, 1988). WHO unveiled its first global strategy for traditional and complementary alternative medicine (TM and CAM) within the year 2005. WHO also intends to integrate traditional medicine into "National Health System Globally" (WHO policy perspective on traditional medicine, 2002). While the status of plant medicine gaining a great attention among modern scientific community through WHO’s initiative, the records of herbal medicine practice has been found to be centuries back. Silphium a plant from genus Ferula was used as contraceptive and abortifacient in the city of Cyrene on the Liban coast of North Africa in 5th century BC (John M. Riddle, 1992). It was one of the best contraceptive of that ancient time that the city’s coins had carried the image of the plant (John M.Riddle, 1992). Dioscorides also recommended Silphium for contraceptive and abortive purposes in his book Materia Medica. The plant became extinct in part because of its value and restricted habited. Later on Galen (AD 129-199), the foremost physician in the Roman world, and Dioscorides also recommended the use of pomegranate (Punica granatum) for birth control (www.islamonline.net.)

In traditional Mexican medicine, ‘Zoapatle’ a decoction, made from Montanoa tomentosa has been used as an oral contraceptive for centuries. Records of the use of this also mentioned in Spanish reports of the year 1529 (www.healthy.net), though its use goes back a lot further. Pharmaceutical observation of Zoapatle were made in Mexico in 1945 and focused on the administration of Zoapatle aqueous crude extract for its anti
reproductive value (Gallegos A.J 1983). Landgren et al., (1979) reported that Oral administration of freshly prepared aqueous extract of *Montanoa tomentosa* (Zoapatele) in early stages of pregnancy for two days (two days prior to the interruption of gestation by vacuum aspiration) at a dose of 1gm to 1.4gm per kg body weight showed a distinct uterotonic effect and induces cervical dilation and uterine bleeding. Chinese scientists have also been making a great effort for the development of fertility regulating agents capitalizing on the rich flora and folklore with the ethno medical experience of their country. A number of plant species contributing to the development of antifertility drug have been mentioned in the pioneering work of Xiao Pei-Gen and Wang Nai-Gong (1991).

Pseudolaric acid A and B were isolated from root bark of *Pseudolarix kaempferi* could terminate pregnancy in rat (Zhao et al., 1983). Pseudolaric acid A showed early pregnancy terminating effect during vaginal administration or subcutaneous injection. Pseudolaric acid B could also terminate the pregnancy in rats while injected subcutaneously in a bicarbonate solution at 15-40 mg/kg body weight. 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). Zhang X et al., 2007 investigated on the bioassay guided fractionation of antifertility components of castor bean (*Ricinus communis* L.) seed extracts in rodents. The active components isolated from the crude extract were a mixture of five components: four phytosterols which were ergost-5-en-3-ol, stigmasterol, gamma-sitosterol, and fucosterol; and one probucol analog. The fractionated compound showed a significant inhibitory activity in the primary cultured rat decidual stromal cells (DSC) viability. It was presumed that the gamma-sitosterol may be the main compound contributing the inhibitory activity. 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).
methanol extract of 20g/kg whole fruit gave 60% anti-implantation activity while 5g/kg fruit pulp gave 100% activity. Ratnasooriya et al., 1994 reported the post-coital contraceptive activity of Sri Lankan marine red algae Gelidiella acerosa in albino rat. 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. Alternatively, uneven distribution of uterine implants resulting from impaired uterine prostaglandin levels may cause embryonic deaths. A Study done by Premakumara et al., (1995) on the post-coital contraceptive mechanisms of crude extract of Gelidiella acerosa demonstrates that CE of G.acerosa mediates its post-coital contraceptive activity primarily via anti-progestational mechanisms.

Belachew Desta (1994) tested about 70 traditionally used Ethiopian plants which are subjected to uterotonic and anti-implantation bioassay. The extract of Papaya latex was tested on the rat uterine preparations in vitro at various stages of estrous cycle and gestation period (Cherian Thomas, 2000). Effect of this preparation at different doses showed a remarkable increase in the uterine contractile activity at proestrus and estrus compared to metestrus and diestrus stages. The maximum contractibility of the uterus was observed in the later stages of gestation corresponding with the higher level of estrogen in the plasma. Rumex studelelii a plant use in Ethiopian traditional medicine reported to be produce an antifertility effect mainly by inhibiting implantation through the possible mechanisms of reduction of estrogen level and increment of progesterone level (Endalk Gebrie et al., 2005). Leonotis ocymifolia, another Ethiopian medicinal plant reported for its antifertility and antiimplantation property (Geremew et al., 2005). The antiimplantation and antifertility activities of the leaves extract were 37% and 20% respectively. Both the ethanol and aqueous extract of leaves and roots of Leonotis ocymifolia were observed to increase acetylcholine induced uterine contraction. Acanthus montanus T. Anderson., a
plant used in folk medicine in Cameroon to treat pain, inflammation and threatened abortion was investigated for antifertility activities in Wistar rats (Asongalem et al., 2008). The aqueous extract of *Acanthus montanus* at a dose of 1000mg/kg/day caused an appreciable effect in periimplantation losses.

### 2.3 Plant uses as female anti reproductive drug in India

The Ancient Indian medical practice like Ayurveda, Unani and Sidha itself shows a rich and old Indian traditional healthcare system. The Charaka Samhita (1000B.C.), one of the earliest treatises of Indian medicine mentioned the use of around 2000 herbs for medicinal purpose. Many Indian literature of ayurvedic and unani medicine flourished with knowledges and information on the use of plants for contraceptive and abortifacient properties. Avrodhak (*Lawsonia inermis*), a plant with an ancient history for traditional remedy appears to have some contraceptive value under laboratory conditions too, preventing pregnancy in 60% of animals tested (Munshi et al., 1977). In the last few decades Indian scientist have reported many herbal preparations having property of reproduction regulation (Kirtikar and Basu,1935; Chopra and Nayar, 1956; Casey, 1960; Choudhury and Saxena,1970; Prakash and Mathur,1976; Satyavati et al.,1976; Dhawan and Patnaik,1977; Aswal et al.,1984; Satyavati 1984; Prakash et al., 1986; Kamboj et al., 1982 and 1989; Bhakuni et al., 1990). Kholkute et al., (1976) reported that the benzene extract of *Hibiscus rosa sinensis* exerts significant antifertility activity in rats including alternation of estrogen-progesterone balance leading to a transitory and reversible follicular atresia in the ovary, atrophy of uterus and degranulation of gonadotrophs in the pituitary. Recent research on the antifertility property of *Hibiscus rose sinensis* exhibited that the ethanolic extract of the roots of this plant posses a strong antiimplantation(100% inhibition) and uterotropic activity at the dose of 400 mg/kg body weight (Neeru et al., 2007). Chaudhury and Haq (1980) mentioned about 11 plants viz. *Aristolochia indica,*
Curcuma longa, Cumium cymimum, Daucus carota, Embelia ribes, Ensete superum, Hyptis suaveolens, Mentha arvensis, Podocarpus brevifolia, Polygonum hydropiper and Sapindus trifolius with 100% antifertility activity. They also listed nine other plants to have more than 60% antifertility activity namely, Ananas cosmosus, Areca catechu, Butea monosperma, Carica papaya, Grewia asiatica, Hibiscus rosa sinensis, Ocimum sanctum, Plumbago zylancia, Sida cordifolia. Urosolic acid one of the major constituent of the Tulsi (Ocimum sanctum) leaves has been suggested to possess antifertility activity in rats of both sexes (Rajeshwari, 1992). The central drug research institute of India, in collaboration with the US national Institute of health, the world health organization, and the ICMR confirm the antiimplantation activity in Ferula jaeschkeana, Bupleurum marginatum, Lepedium capitatum, Caesalpinia sepiaria, Lonicera japonica, Juniperus communis, Lotus corniculatus, Lamium allum and Acacia farnesiana (Chudhury, 1993). The Ferula jaeschkeana also reported to inhibit the activity of β-Glucoeurimidase in rat uterus during implantation on day 5th of pregnancy (Prakash et al., 1994). Lakshmi et al., (1998) reported the chemical investigation of Verbena bonariensis and its antifertility activity in rat. Another plant Striga orobanchioides shows reversible antifertility effect in albino rats when treated with its ethanolic extract (Hiremath et al., 1994). The shoot extract of the plant Abras precatorious showed the atrophic degeneration of the ovary and oviduct of albino rat and significant fall in serum LH and FSH level (Mukharjee-Monisa, 2000). Sarma and Mahanta (2000) investigated on the use of composite root extracts of the plants, namely: Plumbago rosea, Borassus flabellifer, Carica papaya (Male), Dolichos lablab and Shorea robusta for fertility control and reported to induce sterility in female albino rat. Qureshi et al., (2006) reported about 18 plants for antifertility activity both in males and females. The ethanolic extract of the roots of Calotropis gigantea Linn. exhibited 100% pregnancy interceptive activity in rats when administered as a single oral dose of
100mg/kg on day 1 postcoitum (Srivastava et al., 2007). Satyanarayana et al., (2008) reported on the antifertility activity of the leaves of plant Argyreia speciosa and roots of Amaranthus spinosus in pregnant rats. The alcoholic and aqueous extract of Argyreia speciosa at doses of 150 and 175 mg/kg showed significant pregnancy interceptive effect as evidenced by decreased the number of animals delivered with less number of neonates, when compared to control rats. Pretreatment with the ethanolic extract of Allium cepa Linn. showed significant inhibition in number of implant site at a dose of 300mg/kg for day 1 to 7th of pregnancy (Vishnu et al., 2009).

2.3 Plants uses as abortifacient agent

A variety of plants having anti reproductive properties are reported to induce abortion. People from Spanish and Mexican descent in New Mexico have used a number of plants as abortifacients. The most widely used are Cotton root bark (Gossypium sp.), Asclepias ocicornu Woodson, Hedeoma oblongifolia Heller, Chenopodium ambrosioides L, and three species of Artemisia. The cotton root bark shows lowest toxicity as an abortifacient (Conway et al., 1979). Saksena et al., (1980) mentioned that ‘Trichosanthin’ (TCS), a plant protein obtained from the roots of Trichosanthis kirilowii with prostaglandin-F2 alpha act synergistically, rendering the termination of 10 day pregnancy in rabbit. The plant Momordica angustisepala has been used to induce abortion in humans in some parts of Nigeria. Momordica angustisepala was tested for its abortifacient effect in female albino mice and guinea pig (Aqua CN et al., 1983). Yeung and co workers (1986) isolated two abortifacient proteins designated as α and β momorcharinis from seeds of the Momordica charantia. The abortifacient activity of the proteins, when used at a dose (500μg), effectively induced midterm abortion in mice. Nath et al., (1992) listed about 17 commonly used Indian abortifacient plants. Among these plants Moringa oleifera and Adhatoda vasica showed 100% abortifacient activity. Traditional physicians of
Kotagiri village near Ootacamud in India, uses a mixture of powdered root of *Cassia occidentalis*, *Derries brevipes variety coriacea* and *Justicia simplex* to control female fertility (Badami et al., 2003). In Bangladesh, *Marsdenia tinctoria*, a perennial climber has been used for abortion by the ‘Shoutal’, an aborigine tribe of the country. The Phytochemical investigation revealed that the plant contained tinctoramine, a new steroidal alkaloid and tinctoralactone, a novel steroid as the active constituents. Both the compounds showed the antiimplantation and abortifacient activities in mice and rats (Chowdhury et al., 1994). Goonasekera et al., (1995) observed the effect of *Jatropha curcas* inducing fetal resorption with methanol, petroleum ether and dichloromethane extracts indicating the abortifacient properties of the plant. Another plant Ponderosa pine needles cause abortion when eaten by cattle (Steigelmeier et al., 1996). Ford et al., (1999) reported that a unique class of vasoactive lipids in *Pinus ponderosa* needle (PN) exhibit abortifacient activity in Guinea pigs. The aqueous extract of *Imula viscosa* when administered in day 1 to 6\(^{th}\) of gestation, totally diminished fetal implantation and caused significant (p<0.05) reduction in the number of corpora lutea and blood progesterone level. Administration on day 13-15 of gestation exhibited mid-term abortion (Al-Dissi NM et al., 2001). Badami et al., (2003) investigated the antifertility activity of the three plants, *Cassia occidentalis*, *Derries brevipes variety coriacea* and *Justicia simplex*. A mixture of powdered roots of these three plants and its ethanolic extract were given orally at a dose of 200 and 600mg/kg body weight on day 1-7 of pregnancy. The ethanolic extract exhibited 40\% anti-implantation at the dose 600mg/kg body weight and the rats, which continued their pregnancy did not deliver any litters after their full term. Hence, showed the both combined anti-implantation and abortifacient activity of the ethanolic extract. Investigation on the alcoholic extract of dried flowers of *Woodfordia fruticosa* kurz. also revealed promising result as abortifacient at 100mg/kg body weight (Khushalani et al.,
Muthureddy et al., (2007) reported that *Aristolochia bracteolata* Lam. shows significant and dose-related anti-implantation and abortifacient activity when treated at 20 and 40 mg/kg body weight during pre-coital period.

The information mentioned above showed that a large number of plants across the world contain potential compound for fertility control. In recent years, the scientists focused on the isolation of plant derived compound having antifertility property. It has been reported in various literature these natural products exert its' effects mimicking endogenous steroid (estrogen). Therefore, these natural compounds are often regarded as phytoestrogens.

### 2.5 Phytoestrogens and its effects on Female Reproduction

The phytoestrogens are diverse group of biologically active nonsteroidal plant substances with a chemical structure similar to that of estradiol which is a native gonadal steroid. There are three main classes of phytoestrogens: Isoflavones (similar to estradiol), lignans (similar synthetic ethynyl estradiol) and coumestans which occur either in plants or in seed. These plant derived compounds have the capability of bind to estrogen receptor (ER) and exert various estrogenic and antiestrogenic effects. In last two decades researches on phytoestrogens have been increased two hundred folds (Takeshi Usui, 2006). Many of these phytoestrogens like resveratrol and trans-resveratrol demonstrate broad spectrum of pharmacological and therapeutic health benefits (Bagchi et al., 2001). Phytoestrogens are often use as the natural alternative to hormone replacement therapy (HRT) and to reduce menopausal symptoms as well as to reduce other female reproductive abnormalities (Whitten and Naftolin, 1998). The uterotropic property of coumestrol and genestein in laboratory animals has been well documented. At the same time many of the phytoestrogens have been reported to induce irregular estros cycle and infertility in
laboratory animals (Reviewed by Burton and Wells, 2002). Genestein which is present in many plants as a dietary isoflavone can inhibit mouse oocyte maturation, fertilization and sequential embryonic development (Wen-Hsiung Chan, 2009). While the phytoestrogens have been shown to induce both estrogenic and antiestrogenic effects, their biological relevance and potency have not been well characterized (Stark A and Madar Z, 2002).

2.6 Separation of Plant derivatives using thin layer chromatography

Thin layer chromatography (TLC) is widely used in the fields of phytochemistry, biochemistry, and molecular biology for separation of compounds. The advantages of thin layer chromatography technique, such as less expensive, simple and quick results is making it one of the popular techniques used for separation and analyzing bioactive components in plants. Researchers are using this chromatographic technique commonly to separate and isolate active phytocompounds from various plants having different medicinal properties. In addition, this technique is widely used for screening antifungal and antibacterial compounds as well as for screening radical scavengers and antioxidants (Kurt Hostettmann, 1999). Vessel et al., (1999) analyzed the ethanolic extract of winter cherry (Physalis alkekengi) for the presence of steroid and alkaloid glycosides by thin layer chromatographic fractionation using N-butanol-glacial acetic acid-water as solvent at the ratio of 100:10:10. Estradiol-17β, progesterone and cholesterol were used as standard for steroid, while tomontine was used as standard for detection of steroid-alkaloid-glycoside. Using this technique a major steroid glycoside, a free sugar and a glycoalkaloid were identified on the chromatograms. Chokoe et al., (2008) used the thin layer chromatography to analyze the phytocompounds of the Carpobrotus edulis L. (plant used to treat microbial infection) extracts as well as to assay the plants' for antioxidant compounds. The plates were developed separately in CEF (5:4:1, chloroform: ethylacetate: formic acid), EMW (10:1.35:1, ethylacetate: methanol : water), BAW (4:1:5, butanol :
acetic acid : water), and BEA (18:2:0.2, benzene : ethanol : ammonia) solvents. Ahmad et al., (2001) carried out the thin layer chromatographic separation of 11 plants extracts known to have strong antimicrobial activity. Different solvents were used to separate different compounds. Solvent systems used were (i) petroleum ether and benzene, 1:1, (ii) benzene and chloroform 1:1, (iii) benzene and ethyl acetate 2:1, (iv) acetone and alcohol, 1:1, and (v) methanol and water 1:1. Tannic acid, resorcinol, and anthrone were used as control. The majority of the plants tested by TLC showed the phenols and tannins as the most common active constituents. Goswami et al., (2008) fractionated the methanolic root extract of the Polygonum hydropiper in thin layer chromatogram to isolate steroidogenic compound. They used the solvent mixture of n-butanol : acetic Acid : water, in a ratio of 100 : 10 : 10 (v/v/v) and estradiol-17β as reference compound. K Olawole et al., (2006) investigated the efficacy of Bridela erruginea bark extract in reducing the coliform load and BOD of wastewater. They isolated the phyto compounds from the crude extract by using the thin layer chromatography and preparative thin layer chromatography. The chromatographic separation revealed the presence of alkaloids, saponins, steroids, and tannins. In the present investigation, crude bark powder of Dysoxylum alliarium (60 mesh size) was subjected to cold methanol extraction. The extract was concentrated evaporating the solvent and fractionated by thin layer chromatography using estradiol-17β as reference compound.

2.7 Embryo implantation and endometrial decidualization:
Changes of endometrial proteins & involvements of hormones, growth factors and cytokines

Implantation is the process in which the blastocyst establishes an intimate physical contact with the mother’s uterus and begins a physiological relationship with the later. Successful implantation is the result of synchrony between the implantation of competent blastocyst(s) and receptive uterus. This reciprocal interaction involves an intricate
modulation of genetic and cellular activities. The endometrial stromal cells of receptive uterus undergo dramatic morphological and physiological transdifferentiation and convert itself to the large polyploidy decidual cells with epitheloid appearance (Griselda Vallejo et al., 2010). This differentiation of the endometrial stromal cells into decidual cells which is also known as decidual cell reaction is characterized by development of numerous intracellular organelles like rough endoplasmic reticule, lysosomes, accumulation of lipids and glycogens in the cytoplasm, extensive cell to cell contact and junctional complexes (Dey et al., 2004). In contrast to the stromal cells, these decidual cells synthesize prastaglandins, prolactin like protein, vimentin, homeobox transcription factor and cytokines ( Reviewed by Dey et al., 2006). It has been reported that the mouse decidual tissues synthesize estrogen de novo for support of the embryonic development (Amrita Das et al., 2009). Sixty one numbers of genes have reported to involve in the decidualization process of rat endometrial stromal cells, of which 14 are related to chromatin structure and dynamics and 26 encode transcription factors (Griselda Vallejo et al., 2010). Although, a large number of molecules have been reported to be involved in the process of decidualization and subsequent implantation, the mechanism of this process is not well understood. Describing the course of attachment of the blastocyst, Enders and Schlafke (1967 and 1969) have classified the process of implantation into three stages: apposition, adhesion and penetration. Apposition involves the stage where embryonic trophectoderm cells become closely apposed to the uterine luminal epithelium. According to Schlafke and Enders et al., (1975) apposition of the trophoblast to uterus is brought about before adhesion can develop. They therefore, divided this attachment stage of implantation into an initial appositional stage and a subsequent adhesion. Dey et al., (2004) reviewed the implantation mechanism and stated that adhesion is the stage, when sufficient intimation is achieved between the trophectoderm and the luminal epithelium so
as to resists dislocation of the blastocyst by flushing the uterine lumen. Following the establishment of position of the blastocyst, the penetration stage initiates the invasion of the luminal epithelium by the throphectoderm. This stage is evident by the extensive differentiation of stromal cell into decidual cells (Decidualization) and the loss of luminal epithelium. In species like mice, rats and hamsters, an implantation chamber is formed by the invagination of the uterine epithelium, a type of eccentric implantation. (Reviewed by Dey et al., 2004). In both human and mice the extensive decidualization of the stromal cells surrounding the implanting blastocyst makes the embryo to get embedded in the antimesometrial stromal bed. Psychoyos (1973) showed that with the injection of a macromolecular blue dye, the localized increase in the stromal vascular permeability at the site of blastocyst can be demonstrated. The uterine receptivity for implantation lasts for a limited period. At this stage the uterine environment is become supportive to blastocyst growth, attachment and other subsequent events of implantation. The major factors that are specific to this stage are ovarian steroids, progesterone (P4) and estrogen (E2). In mice and rats, ovarian progesterone and estrogen are crucial, but not essential in species like pigs, guinea pigs, rabbits and hamsters (Psychoyos, 1973; Heap and Deanesly, 1967; Harper et al., 1969; Kwun, 1974; McCormack, 1974 and Heap et al., 1981). Evidence suggests that uterine receptivity to implantation in rat or mice is highest on the day 4 of pregnancy which is also called as implantation window, (Paria et al., 1993) and the efficiency is decreases with time (Song et al., 2002). The concentration of estrogen is very crucial for the duration of the window of implantation in mice; uterine receptivity remains exists for an extended period at low estrogen level but rapidly closes at higher levels (reviewed by Dey et al., 2004). Such high estrogen level uterine non receptivity is accompanied by aberrant uterine expression of implantation related genes. These
evidences also suggest that careful regulation of estrogen levels can be a decisive factor in female fertility regulation and fertility improvement.

With the many crucial factors that takes part in the process of implantation, the expression of growth factor and their receptors in the uterus in a temporal and cell specific manner during the periimplantation period suggests their role in the process of implantation. Growth factors like epidermal growth factor (EGF) and its receptor, with others like cyclooxygenase-2(COX-2) and histamine type 2 receptor are associated with blastocyst attachment reaction and are expressed in a normal active blastocyst (Paria et al., 1993; Paria, 1993 and Zhao, 2000). The HB-EGF member of the EGF family is the earliest molecular marker found in the uterus at the sites of active blastocyst appearing several hours before the attachment reaction in mice (Das et al., 1994 and Dey et al., 2004). This induction is followed by the expression of many genes, of which amphiregulin is expressed throughout the uterine epithelium on the morning of day 4, but its expression is absent by the morning day 5. Some of the maternally derived growth factors act as a paracrine manner on the embryo, such as the IGF related factor stimulate growth preferentially, but others such as EGF plays more important role in differentiation (Adamson, 1993). Genes for TGF, IGF, FGF, PDGF and its receptors are expressed by early embryos of several species including mouse, rat, cow and sheep (Harvey, 1995). Recent research reveals that both leukemia inhibitory factor (LIF) and Epidermal growth Factor (EGF) stimulate secretion of urokinase-type plasminogen activator (µPA) and gelatinase B/ matrix metalloproteinase-9(MMP-9) in day 7 mouse blastocyst outgrowths (Harvey et al., 1995). However, another finding suggests that absence of TGF-α results in an increase in cell death within the inner cell mass (ICM), indicating their possible role in cell survival (Brison and Schultz, 1997). As discussed above, the phenomenon of decidualization is associated with many cellular and molecular events as well as with a
start of angiogenesis including increased uterine vascular permeability and development of maternal vessels. For uterine vascular permeability and angiogenesis, the proangiogenic factor VEGF and its receptors is important during the attachment phase of the blastocyst and even before that, whereas the VEGF with the other angioproteins (Ang1 and Ang2) and their receptor Tie-2 directs angiogenesis during decidualization. Ang2 is known to be required for postnatal angiogenesis remodeling, and Ang2 with VEGF takes part in the development of lymphatic vasculature (Gale et al., 2002). Several other proteins and cytokines are crucial to uterine receptivity and implantation. Integrins, the adhesion molecule with its some subunits, α5β1, α6β1, and αvβ3 are expressed in the mouse embryo throughout the periimplantation period; and in later stages of development in the differentiating trophoblast (Sutherland et al., 1993). Western immunoblotting of proteins for COX-1 and COX-2 indicated that rat uteri, cervix and myometrial cells express both COX-1 and COX-2 proteins. During proestrus and estrus, uterine expression of COX-2 is elevated (Yuan-Lin Dong et al., 1996). Immunohistochemical localization of another protein Stathmin, associated with microtubule dynamics revealed that Stathmin is exclusively localized in the decidual zone, surrounding the embryo, on days 7 and 9 of pregnancy (differential localization of decidual stathmin during pregnancy in rat placenta). In mice, two Hox genes, Hoxa10 and Hoxa 11, are expressed in uterine stromal cells during the receptive phase (reviewed by Haibin Wang and Dey, 2006). The expression of the Hoxa10 persists during post implantation decidualization (Benson et al., 1996 and Satokata et al., 1995). Females deficient with this gene (HOXA 10−/−) are infertile, may be due to reduced stromal cell proliferation. Although the process of implantation is an incredibly complex array of cellular and molecular events involving numerous factors, the above discussed events and factors are some of the vital characteristic and sequences of implantation.
2.8 Effects of synthetic abortifacient RU486 (antiprogesterone) on uterus

RU486 or Mifepristone is a synthetic steroid compound used as an abortifacient in the first two months of pregnancy (http://en.wikipedia.org/wiki/Mifepristone). It is an antiprogestin. It binds to progesterone receptors on the wall of the uterus thus blocking the effect of the endogenous progesterone. This triggers the shedding of the uterine wall, much like a normal period to expel the embryo. A number of works has been done to study the underlying mechanisms and pathways of RU486 clinical action. Lockwood et al., (1994) investigated the effect of RU486 on endometrial hemostatic potential. The endometrial stromal cell tissue factor (TF), which is the primary initiator of hemostatis, has been known to be regulated by progestationally in vivo and in vitro. The results showed that exposure to RU486 alone or with E2 plus medroxyprogesterone acetate (MPA) greatly reduced the levels of stromal cells TF protein and mRNA expression compared to those maintained in confluent stromal cell cultures maintained in E2 plus MPA. The result also demonstrated that RU486 not only blocks but also reverses the progestin-enhanced stromal cell TF protein and mRNA expression. Another experiment revealed that treatment with 400mg mifepristone on day 2 of pregnancy in swine delays both uterine protein secretion and conceptus development (Vallet and Christenson, 2002).

Uterine leiomyomata are the tumors which are dependent on steroid hormones and possess receptors for estrogen and progesterone. Patients with this tumor were treated with RU486 hypothesizing that antiprogesterone RU486 may induce the regression of leiomyomata by withdrawing the progesterone action and/or by its interference with estrogen. The results showed a regression of tumors through a direct antiprogesterone effect (Murphy et al., 1993). Treatment in female cynomolgus monkeys on cycle days with RU486 (1 mg/kg/day) resulted in characteristic suppression of endometrium with few mitosis, dense
stroma and simple glands (Heikinheimo et al., 1999). Dibbs et al., (1995) investigated the estrogenic activity of RU486 and concluded that the estrogenic activity of RU486 was dependent on the presence of both estrogen receptor and the promoter's estrogen response element.

2.9 Effect of plant products (Phytoestrogens) on uterine protein expression

It is mentioned in many literature that the herbal crude extract used as medicine works as an endocrine disruptor and thus effects expression of the protein profile of female uterine tissues. Devarshi et al., (1991) mentioned that the administration of dry root powder of Plumbago zeylanica during the first 7 days of pregnancy abolished uterine proteins of 13KD, 19KD, 26KD and 75 KD molecular weights resulting in preimplantation loss. Rats treated with root powder of Plumbago zeylanica from day 6th to day 17 of the pregnancy, results in the loss of the proteins having molecular weight of 55KD and 56 KD and the fetus were aborted. The results suggest that the proteins lost during the treatment are important for implantation and maintenance of pregnancy. Krishman and Daniel (1967) suggested that a uterine specific protein blastokinin, was necessary for inducing and regulating blastocyst formation. However, it was accepted later on that blastokinin may exert its effect on blastocyst growth rather than blastocyst formation (Bazer 1975). Tarachand (1994) also reported the differential rate of protein synthesis by antimesometrial and mesometrial decidual cells of rats. He reported that gel electrophoresis of decidual proteins from day 7th onward of pregnancy showed quantitative changes with number of proteins of both high and low molecular weight. The locally produced growth factors reported to be mediate the effects of sex steroids on cell proliferation and differentiation in the uterus (Stewart and Cullinan, 1997). Hazarika et al., (2006) reported the administration of crude root extract of Polygonum hydropiper in a dose of 1000mg/kg body weight/ day for three consecutive cycles abolished proteins of
55KD, 36KD, 21KD and 19KD from uterine tissues of ovary intact extract treated rats. At the same time administration of CRE induces synthesis of a new protein (49KD) in the ovary intact females.

2.10 Effects of Plant products on Histological structures of ovary and uterus

Plant products that are used for fertility regulation in female are known to target the reproductive organs like ovary and uterus and very often modulate its normal histological architecture. It is evident from the works done by various researchers in the last few years to evaluate the effect of herbal products in reproduction regulation. Prakash et al., (1967) studied on the endocrinological effect of Byakangelicin, a main furanocoumarin constituent isolated and characterized as an aldose reductase [aldehyde reductase] inhibitor from the roots of Angelica dahurica on female rats. The histological examination of the uteri showed the enlarged diameter of the lumen and proliferated endometrial epithelium. Tewari et al., 1970 studied the antifertility effect of betal leaf stalk (Tambul patrabrint). Progestational activity was studied in 12 immature female rabbits injected with stilbestrol then treated with betal leaf stalk. A mild progestational activity was found in immature estrogen primed rabbits but follicle depressant type action was noted as several graffian follicles in the ovary was seen in regressive phase. Kholkute et al., (1976) reported that the benzene extract of Hibiscus rosa sinensis exerts significant antifertility activity in rats leading to a transitory and reversible follicular atresia in the ovary, atrophy of uterus and degranulation of gonadotrophs in the pituitary. The flower extracts of Malva viscus consattii cause degeneration of ovary and uterine structures when treated at a dose of 200mg/day for 20 days (Dixit, 1977). Administration of neem oil at a dose of 0.2 ml/days to cyclic rats caused alternation in the cellular organization of graffian follicles and stroma making it loose (Prakash et al., (1988). Subcutaneous injection of the neem oil also caused significant damage to the endometrium
and the uterine glands. Petroleum ether extract and chloroform extract of whole plant of *Striga lutea* in a dose of 100mg/kg and 50mg/kg in albino rats changes the histological structure of ovary and uterus (Hiremath *et al*., 1990). The Graffian follicles become atretic leading to reduced number of corpora lutea. The uterus indicated partial resorption of the implants. Hexane extract of *Ferula jaeschaena* in a dose of 25mg/kg body weight when administered through oral route increased the ovarian vascularity and a proliferative endometrium with loose stroma and wide lumen in rats (Prakash *et al*., 1991). Ethanolic extract of *Bupleurum marginatum* (Sipil) has been studied to assess its estrogenic activity in immature rats. Rats treated with 100mg/kg body weight showed increase in the height of the luminal epithelium with loose stroma (Jonathan 1995). Sharma and Mahanta (2000) reported the effect of composite root extract of five plants on histological structure of graffian follicle and endometrial epithelium in female albino rat. The composite root extract decreased the follicular recruitment as well as degeneration of ovarian follicle. Oral administration of the ethanol extract of *Balanites roxburghii* in female rats caused increase in the diameter of the uterus, thickness of the endometrium, and the height of endometrial epithelium (P damashali *et al*., 2006).

### 2.11 Role of Vascular Endothelial Growth Factor (VEGF) during implantation and pregnancy

Vascular endothelial growth factor(also designated as VEGF-A), originally known as the vascular permeability factor (VPF), is a heparin- binding, homodimeric glycoprotein with four different isoforms, consisting of 121,165,189 and 206 amino acids (Ferrara Napoletone and Terri Davis-Smyth, 1997). The VEGF family also includes placenta growth factor (PIGF), VEGF-B, VEGF-C, VEGF-D and VEGF-E. VEGF is also a potent mitogen for micro and macrovascular endothelial cells and a principal regulatory growth factor for vasculogenesis (the *de novo* formation of the embryonic circulatory system) and angiogenesis (Dey *et al*., 2004). Amongst the VEGF family members PIGF is
predominantly expressed in placenta and binds exclusively to vascular endothelial growth factor receptor 1 (VEGFR-1). VEGF-B binds to both VEGFR-1 and neuropilin-1. VEGF-B is implicated in angiogenesis by its role in the regulation of extracellular matrix degradation, cell adhesion and migration of endothelial cells (Olofsson et al., 1998). VEGF-C is a ligand for both VEGFR-2 and VEGFR-3. VEGF-D is structurally very similar to VEGF-C and it also binds to VEGFR-2 and VEGFR-3 (Achen, 1998). VEGF-E is the collective term for a group of proteins with homology to VEGF-A that are encoded by certain strains of the orf parapoxvirus, which affects goats, sheep and occasionally humans (Robinson et al., 2001). VEGF-C mRNA is found in several tissues including heart, placenta, ovary, and small intestine, mice which lacked a functional vegfr-3 gene 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). Thus, the VEGF-C/VEGFR-3 system has an essential role not only for lymphatic vessel formation but also for angiogenesis.

Angiogenesis, the process of growth and formation of new blood vessels is rare in healthy adults, except in the ovary, endometrium and placenta, where intense angiogenesis takes place as the follicle grows, the corpus luteum develops and the endometrium is shed in each ovulatory cycle and in pregnancy (Folkman, 1995). Moreover, increased vascular permeability and angiogenesis are crucial to successful implantation, decidualization and placentation (Dey et al., 2004) as the VEGF is a prime component in the vascular remodeling and the survival of cytotrophoblast in the placenta (Banyasz et al., 2006). Dey et al., (2004) reported in their investigation that the VEGF and its receptor Flt1(VEGFR1) and Flk1 (VEGFR2) are primarily important for uterine vascular permeability and angiogenesis before and during the attachment phase of the implantation process, whereas VEGF together with the other angiopoietins (Ang1 and Ang2) and their receptor ‘Tie-2’
directs angiogenesis during decidualization after implantation. Chakraborty et al., (1995) also showed the expression of VEGF and its receptors in the mouse uterus as a whole during pregnancy and in response to steroid hormones. Their Results of *in situ* hybridization experiments demonstrated accumulation of VEGF mRNA in the luminal epithelium on days 1 and 2 and a modest level of signals in the stromal cells on day 3 of pregnancy. On day 4, luminal epithelial cells and those in the subepithelial stromal bed exhibited the accumulation of VEGF mRNA. On days 6 to 8, the accumulation of VEGF mRNA occurred in cells in the decidual bed at both the mesometrial and antimesometrial poles. The data also suggest that VEGF is involved in trophoblast differentiation and invasion, as well as in decidualization and placentation. Halder et al., (2000) detected the presence of VEGF mRNA in periimplanted mouse uterus. They observed that the expression was low during the first two days of pregnancy followed by increased thereafter. The expression was more intense on days 6-8 at the mesometrial pole, the presumptive part of heightened angiogenesis and placentation. Mammalian endometrial stromal decidualization occurs in the stromal cells surrounding the implanted embryo (Chinese Science Bulletin, 2003). Western blot analysis with endometrial homogenate and blastocyst on nonreducing gels showed that immunoreactions was present in blastocyst and in endometrium at day 6 to 3/4 of pregnancy period at 34kDa, and in both implant and nonimplant samples on day 8th of pregnancy (Das et al., 1997). However, they observed poor immunodetection when reducing SDS-PAGE conditions were employed. They indicated the possibility that VEGF could serve as a local signal between the implanting embryo and vascular structures in the receptive endometrium. Shao et al., (2001) studied the VEGF regulation mechanism and possible role of VEGF in implantation by studying the VEGF expression and angiogenesis in rat uterus during estrous cycle, ovariectomised and peri-implantation stages using in situ mRNA hybridization and confocal laser
scanning techniques. The results showed that during the early stage before implantation on day 1 and day 5, VEGF mRNA was mainly expressed in luminal and glandular epithelium and only a little signal emerged in stroma. On day 5.5 the signals were emerged in stroma, embryo and also in some spatial arteriole endothelium. On day 6 and day 7 the VEGF mRNA was extensively expressed in myometrium and decidualizing regions. Watanabe et al., (1998) examined the temporal and spatial expression of VEGF protein and mRNA in the rat uterine and placental tissues throughout pregnancy to elucidate the physiological roles of VEGF during pregnancy. Northern analysis revealed the existence of VEGF mRNA in the uterus and placenta regardless of the stage of estrous cycle or pregnancy. Furthermore, fetal compartments such as trophoblasts, trophoblast giant cells, vitelline epithelial cells, and amnion were labeled more strongly with VEGF than the maternal compartment. They concluded that the spatial expression of VEGF was relatively constant in rat maternal uterus during the estrous cycle and during pregnancy but with temporal changes of VEGF expression. Koch et al., (1994) also reported that 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. Carmeliet et al., (1996) reported that in heterozygous VEGF- deficient (VEGF\textsuperscript{−/+}) mice embryos showed abnormal blood vessels formation, but the mice did not die at once, while homozygous VEGF deficient (VEGF\textsuperscript{−/−}) mice died on D11-D12 of pregnancy. The reason for death arises from damages of early formation of vessel, including distribution of blood islands and angiogenesis. Dumont et al., (1998) investigated that 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 mid gestation due to failure in remodeling of the blood vessels (Dumont et al., 1998). Rabbani et al., (2001) investigated the effects of systemic administration of blocking antibody to
VEGF on endometrial vascular permeability at the time of embryo implantation in rats. The number of implantation sites (detected with injecting Evans blue dye) was significantly lowered (P<0.05) after injection of 1.0ml blocking antibody (1.8+/−1.56) compared to that with control rats (11.6+/−1.97). The numbers of blue bands were significantly different among rats injected with 1.0(1.8+/−1.56), 0.8(6.0+/−3.67) or 0.6(10.7+/−0.33)ml anti-VEGF antibody, indicating a concentration effect of anti-VEGF antibody. The results suggest that the VEGF is the main factor responsible for increased endometrial vascular permeability at implantation. Chang et al., (2004) concluded from their investigation that VEGF plays an important role in placental development by the induction of VE-cadherin in trophoblasts which in part maintains the survival of labyrinth trophoblast in rat placentas. VE-cadherin is a calcium-dependent homotypic adhesion molecule, contributes to endothelial assembly and VEGF-mediated survival during angiogenesis. Samuel et al., (2005) showed that 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. As the progesterone secretion level declines the decidual tissue and uterus normal function is arrested 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 resorption. They also revealed the possibility to develop emergency contraceptive by developing the agents that disrupts ovarian blood vessel function and ultimately dysfunctional uterus by blocking the VEGF/VEGFR-2 pathway.
2.12 Studies on Serum glutamic oxaloacetic transaminase (SGOT) and Serum glutamic pyruvate transaminase (SGPT) and histological structures of liver

Liver is an important organ for metabolism and detoxification of metabolites and/or other toxicants. Serum glutamic oxaloacetic transaminase (SGOT) and Serum glutamic pyruvate transaminase (SGPT) which are also known as Aspartate aminotransferase (AST) and Alanine aminotransferase (ALT) respectively are considered as marker enzymes of hepatic function. Plasma or serum level of these enzymes indicates the structural and functional status of hepatic parenchymal tissues. Under normal physiological conditions, these enzymes reside within the liver cells. But whenever liver disorders arise, these enzymes are spill out into the blood stream, showing elevated levels in blood indicating liver damage (www.herbalprovider.com). Such conditions are mostly caused by the toxins, overdose of many drugs and acetaminophen (Tylenol). Some mild to moderate elevations of these enzymes are also due to fatty liver. Excess alcohol intake is one of the causes behind such type of fatty liver.

Many literatures mentioned about the elevated levels of such enzymes due to administration of various drugs and toxins and at the same time how does hepatoprotective effect of some other agent reduce such elevated level indicating the lower toxic state. Solomon et al., 1993 investigated on 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 for 30 days at a dose of 50mg/kg body weight. An elevated level of SGOT and SGPT was observed in carbon tetrachloride (CCL₄) intoxicated female rats. However, reduction in the levels of the SGOT and SGPT towards the respective normal value was observed when a herbal formula having hepatoprotective effect, administered in the CCL₄ treated rats at different doses (Anthony et al., 2003). Sharma et al., (2007) investigated on the antifertility efficacy of the Piper betle on female rats and the effect of ethanolic extract
of *Piper betle* on the serum SGOT and SGPT levels. The plant extract revealed an enzyme inhibiting effect evident from the significantly reducing level of serum SGOT and SGPT.

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. Chemical characterization of the chloroform fraction revealed the presence of two oleanene type triterpenes (Nikkan *et al.*, 2008). Mandal *et al.*, (1993) also reported a significant reduction in the CCl₄ induced increased levels of SGOT and SGPT when treated with *Ficus racemosa* leaf extract. Recently Girish *et al.*, (2009) reported the Peracetamol (PCM) induced hepatotoxic effect, where, PCM was administered to mice (500mg/kg) on day 8 following a pretreatment of hepatoprotective six polyherbal formulations for 7 days. The result revealed that PCM toxicity significantly increased ALT and AST level. In the present investigation, SGOT and SGPT have been studied in the control and crude bark powder treated females to determine any toxicity of the later on the female rats.

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