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Original Research Article

Heynea trijuga: A Traditionally used Medicinal Plant for Female Reproduction Regulation Possesses Phytocompounds causes Effects on Rodents Ovary and Uterine Functions

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Abstract: *Heynea trijuga*, a plant with medicinal properties, of which bark is traditionally used as an abortifacient for livestock in Arunachal Pradesh, North East India. The present study aims at studying the effect of the crude bark extract (CBE) of *H.trijuga* on reproductive tissues in rodents (rats and mice) under laboratory condition. The crude bark extract was fractionated by Thin Layer Chromatography using estradiol-17â as a reference compound. Albino rats and mice were grouped and treated with methanolic extract orally at doses of 500 mg /kg body weight/ day for eight days (two consecutive cycles). A group of treated females were allowed mating to study the effect of crude extract on early gestation. The effect on ovary, uterus of cyclic females and embryo on day 5 of gestation was done by histological observations using H-E stain. Duration of each phase of estrous cycle was recorded by observing the vaginal cytology. Chromatographic fractionation showed three fractions of the crude bark extract. The animal groups treated with the crude bark extract exhibit pronounce changes in the histoarchitecture of ovary and uterus in comparison to the control group. The ovaries of the treated cyclic females showed degeneration of follicles at various stages and corpus luteum. The uterus showed thinning and detachment of the luminal epithelium from underlying stroma which fails to develop endometrial glands. The CBE exerts effects on the embryo and hinders its growth and successful implantation. The present study showed that the CBE of *H. trijuga* possesses compounds which may act as ovarian steroid receptor modulator in reproductive tissue of rodents.
Key words: Anti-implantation, *Heynea trijuga*, ovary, steroid receptor modulator, uterus

Introduction

Heynea trijuga Roxb. (also known as *Trichilia connaroides* Wight and Arnott) is a plant (Fig. 1) belonging to Meliaceae family which is widely distributed in the countries under the Himalayan terrain region like India, Nepal, Bhutan, China and Myanmar. In India, this plant is found in the north-eastern region including Arunchal Pradesh at an altitude of 700-2400m above mean sea level. *Heynea trijuga* grows upto a height of 20ft and 3-4ft in girth. Leaves are alternate, imparipinnate, 9-18 inch in length. The seeds are generally one (rarely two), ex-albuminous arillate. Different parts of this tree are used as traditional crude drugs in these countries and it is commonly known as Ban-Ritha (Kumaun), Thengare-arong (Assamese) Ankhaataruwaa (Nepal).

The present study has been carried out in order to understand the effect of the crude bark extract of the plant *Heynea trijuga* on the reproductive tissues: the ovaries and the uterus of rodents. This investigation is based on the first hand information of its use by the Adi community of Arunachal Pradesh. Tradition prevails among Adi community of East Siang district for using bark of *Heynea trijuga* (locally

known as Sitil) and *Dysoxylum alliarum* (Locally known as Situ Payu) as abortifacient agent/ medication. Both the tree bark is used for control of the domestic swine population. Crude bark powder is orally administered to the swine post coitum by mixing with the fodder. In our earlier study, effects of crude bark extract of *Dysoxylum alliarum* has been shown in albino rat (Das *et al*, 2014). However, *Heynea trijuga* been used from ages in the tribal community; this is the first scientific laboratory study on animal model system regarding its effects on the female reproductive system (rats and mice).

Materials and methods

Collection of plant materials and preparation of crude bark extract (CBE)

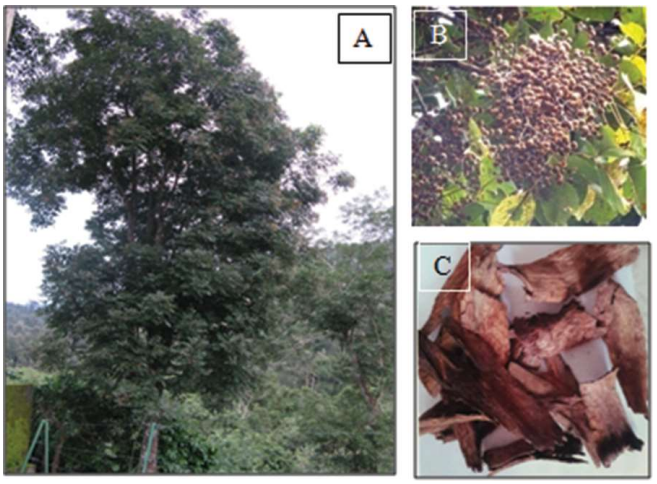


Fig. 1. The tested plant A: *Heynea triguga,* B- *H.trijuga* seeds and C: The bark of *H.trijuga*.

The bark of *Heynea trijuga* had been collected from Mebo village of East Siang district of Arunachal Pradesh, India. The bark were cleaned, shadedried and cut into small pieces and then ground to a fine 60 mesh powder. The powder was immersed in methanol for a period of 72 hours at room temperature (25±2°C) for cold extraction. The solvent was filtered using Whatman filter paper (Cat no. 1001125,125 mm). The filtered crude extract was allowed to dry at room temperature for removal/ dispersal of methanol and further dried in a vacuum evaporator. The dried extract was stored in -40^{0}C for further use in experiments.

Chromatographic fractionation of crude bark extract

The crude bark extract of *Heynea trijuga* was subjected to Thin Layer Chromatography (TLC) for fractionation of compound(s). This technique was done by using TLC plates coated with silica gel having 60µ pore size (MERCK, Germany Cat no. 1.05641). n-Butanol, Glacial acetic acid and Water was taken as solvent system in a ratio 100:10:10. Estradiol-17 β (SIGMA, E8875-1G) was used. Iodine was used as a developing agent for fractions on the TLC plate.

Experimental animal

Adult cyclic female albino rats and mice (60 -70 days old) were used in the present investigation. Animals were kept in the Animal facility of Rajiv Gandhi University under healthy and uniform husbandry conditions were fed with routine diet (Bengal gram, corn) and water *ad libitum*.

Monitoring of estrous cycle

Monitoring of estrous cycle was done by identifying the cell types in the vaginal smear everyday at 8:00hrs-9:00hrs following the methods of Montes and Luque (1988). Distilled water taken in the tip of a glass dropper was flushed into the vagina. The collected fluid was placed on the glass slides, spread to make a smear and allowed to air-dry. on the dried vaginal smear and the slides were stained with Giemsa stain for 5 minutes. Stained slides were washed with distilled water and observed under the Leica DM 5000B microscope. Cell types of different stages of estrous cycle and duration of each phase were considered as one of the parameter to study the effects of crude extract on female reproduction.

Administration of Crude Bark Extract (CBE) and sample collection

The crude bark extract of *H. trijuga* was administered to female rodents at a dose of 500mg/kg body weight per day following the previous experiments on *Dysoxylum alliarum* performed in our laboratory (Das *et al*., 2014). The extract was suspended in 100µl distilled water to prepare the final doses for oral administration in both rats and mice. Adult cyclic female rats/mice were divided into two different groups, each group containing six females. The control rat females (Group-I) received the vehicle (distilled water) orally. The females were treated at the dose of 500 mg/kg body weight/

day (Group-II) for 8 consecutive days (two cycles) begining with proestrus. Female were sacrificed on next morning of last treatment during 8:00 to 9:00 hrs and tissue samples (ovary and uterus) were collected and processed for histological observations following the method as mentioned elsewhere of the present study

Female mice were allowed to mate at a ratio of (2:1) with male mice of proven fertility. The copulation of the mice was confirmed by observing the vaginal smear on the very next morning. Female mice exhibiting fern pattern mucus along with the sperms (Fig. 7 A) validate the estrus phase of the cycle and confirmed copulation and it is considered as day 1 of gestation. These pregnant females were divided into two groups containing six animals in each . Oral administration of crude extract in a dose of 500mg/kg body weight / day by was done to females (Group-III) for 4 consecutive days starting from day 1 of gestation. The control group of female (Group-IV) were maintained in similar manner without treatment of bark extract. The pregnant females of both the control and the treated group were sacrificed on day 5 of gestation (evening in between 20.00 – 22.00 hrs) and uterus were collected to study its effects on embryo and uterine tissues. Among the control group, only the representative females were sacrificed to study the implantation sites. Other control females were allowed to complete the full gestation period and number of pups gave birth was recorded.. Prior to sacrifice, females were injected with Chicago blue dye at the tail vein to determine the site of implantation in uterus. The implantation sites were identified as the blue spots in the uterine horns (Fig.7B).

Histological studies

Uterine horns and ovaries collected were immediately fixed in Bouin's solution for 72hrs, dehydrated, cleared in xylene and embedded in paraffin. Histological sections of 5µ thickness were obtained by cutting the block in a rotary microtome and stretched on poly-L-Lysine (Sigma Cat.No. P8920) coated glass slides. Tissue sections were stained using standard Eosin-Hematoxylin stain method (Culling, 1974) and mounted with DPX. Sections were observed under LeicaDM5000B (LAS V4.4) microscope and the appropriate areas were photomicrographed to collate the significant alteration in the ovarian and uterine histoarchitecture following oral administration of crude bark extract in treated groups.

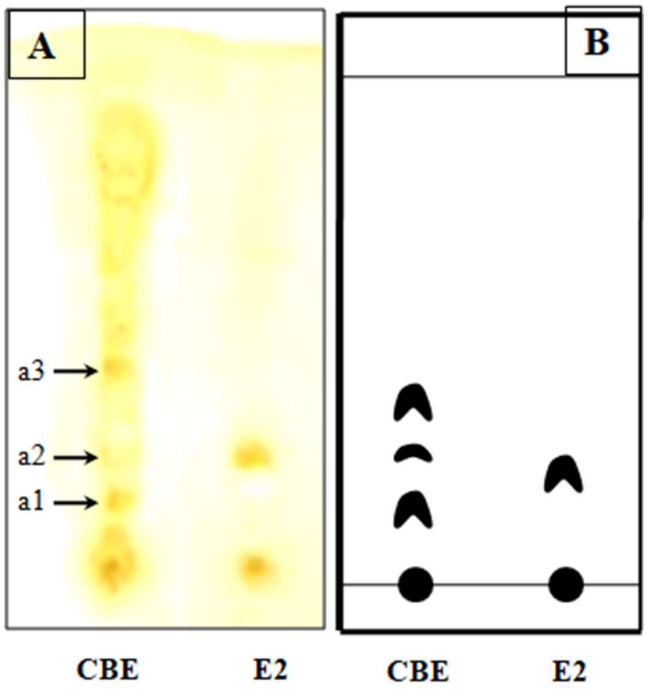


Fig. 2. Thin Layer Chromatography of methanolic bark extract of *Heynea triguga* (A) and its iconographic representation (B). a1, a2, a3 denotes 3 separeted fractions of extract. Estradiol 17 β (E2) was taken as reference compound.

Results

Chromatographic fractionation of Crude bark extract

The potential chromatographic fractionation of active component(s) in crude bark extract of *H. trijuga* is presented in Fig.2 . The thin layer chromatographic separation gives three separated fraction (a1, a2, a3) with solvent system n-Butanol: acetic acid: water (100:10:10). The fraction showed two concentrated spot (a1 & a3) flanking the spot of reference compound Estradiol-17 β (E2). A faint blot (a2) showed similar migration (Rf value of 0.30) with reference compound (E2). This indicates that the plant *H. trijuga* could have some steroidogenic compound(s) either estrogen agonist or antagonist properties, need further investigation.

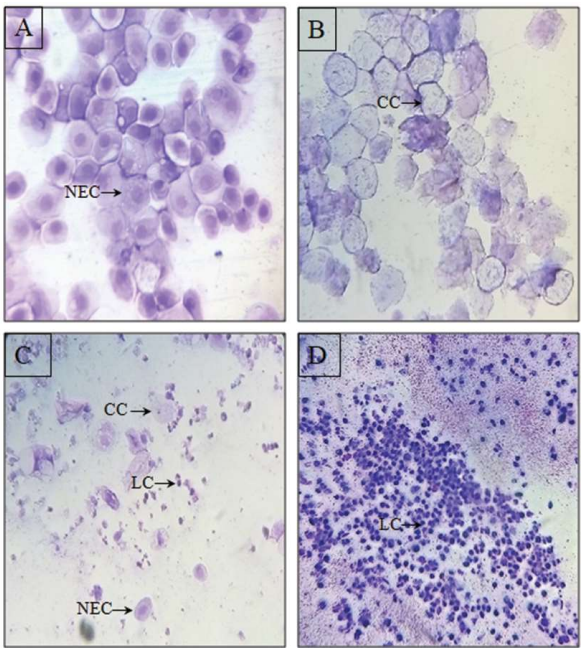


Fig. 3. Cell types of different stages of estrous cycle of female rat. A. Proestrus, B. Estrus, C. Metestrus, D. Diestrus. NEC-Nucleated epithelial cells, CC- Cornified cells, LC- Leucocytes.

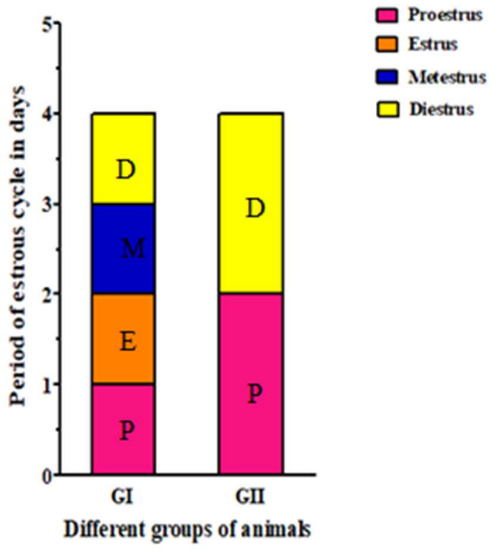


Fig. 4. Graphical presentation of duration of different phases of estrous cycle in GI (Control/vehicle treated) and Crude bark extract treated rats GII(500mg/kg body wt/day).The normal cycle is disturbed in the treated groups.

Effects of crude bark extract on estrous cycle

The cyclic control females (Group-I) showed normal estrous cycle with proestrous, estrus, metestrus and diestrus stages with their characteristic cell types (Fig.3) and duration. Oral administration of crude bark extract for two consecutive cycles (Group-II) causes abnormal cyclicity, with decreased rate of conversion of nucleated epithelial cell to cornified cell. It causes absence of estrus and metestrus stage, but with prolonged proestrus and diestrus. The duration of the different stages in control and treated groups of female rats is represented in the graphical form in Fig.4.

Effects of crude bark extract on cyclic females' ovary

The histological structure of ovary of control adult cyclic females (rat/mice) has been presented in Fig.5. The ovary appeared with different stages of follicular development and with corpora lutea. Preantral and antral follicles with oocytes and multiple numbers of granulosa and theca cells were present in control females' ovary. The Graafian follicles are characterized by the presence of oocyte guarded by cumulus oophorus and multiple layers of granulosa cells within fluid filled antrum. The oocytes with germinal vesicle was covered by zona pellucida was found in the antral follicles in control females' ovary. The cells in the cumulus oophorus were organized and compact in control follicles. The granulosa cells layering with the theca cells, surrounding the oocyte were compact and closely attached with the underlying theca cell layer.

Oral administration of the crude bark extract of *H. trijuga* to the adult cyclic females induced structural changes of the ovarian follicles. The treated rats' ovary failed to show follicular growth. Most of the follicles were found to be degenerated at different level of development (Fig.5C & C1). The preantral follicles showed unorganized and degenerating oocyte with no germinal vesicle and no distinct zona pellucida. The histology showed the degenerating primary follicle and degenerating Graafian follicles. Unlike that of the control females, the antral follicle of the CBE treated ovary showed disorganized granulosa cells. No distinction can be made between the granulosa cells and the cumulus oophorus as the degenerating oocyte is surrounded by loose clump of granulosa cells dispersed in whole of the antrum. Moreover the corpus

luteum is also seen in a degenerating state in the ovary of the treated females. This presence of degenerated ovarian follicles with retarded growth of oocyte and follicular cells indicated follicular atresia.

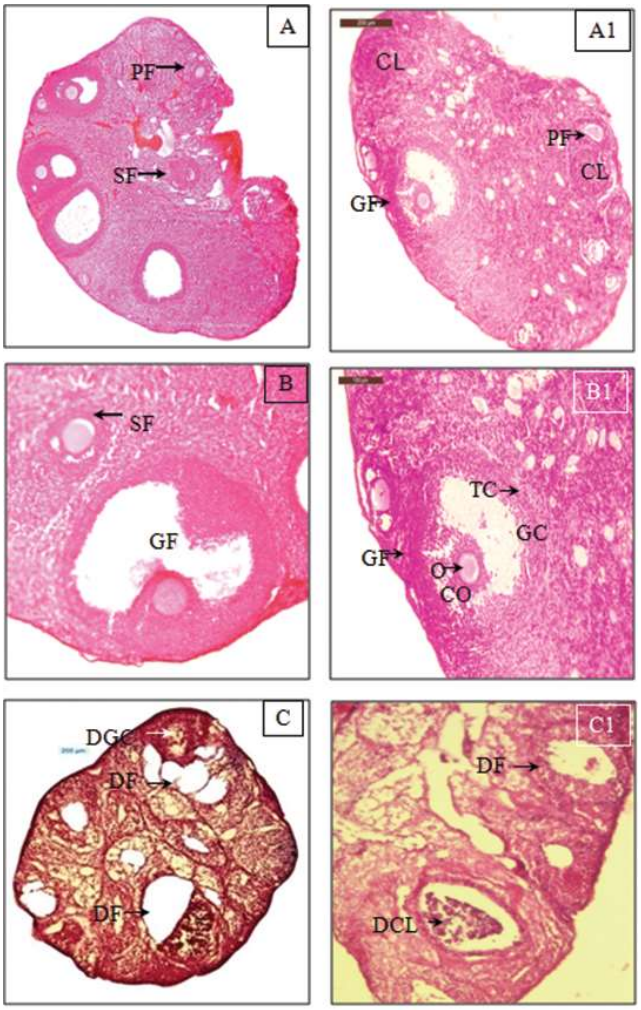


Fig. 5. Control cyclic mice ovary (A & B) and control cyclic rat ovary (A1 & B1)showed follicles in various stages of development (SF: Secondary follicle, GF: Graafian follicle, CL: Corpora lutea, O: Oocyte, CO: Cumulus cells, TC: Thecal cells, GC: Granulosa cells). The treated females in both mice (C) and rats (C1) showed Degenerated follicles (DF) and degenerated corpora lutea (DCL)

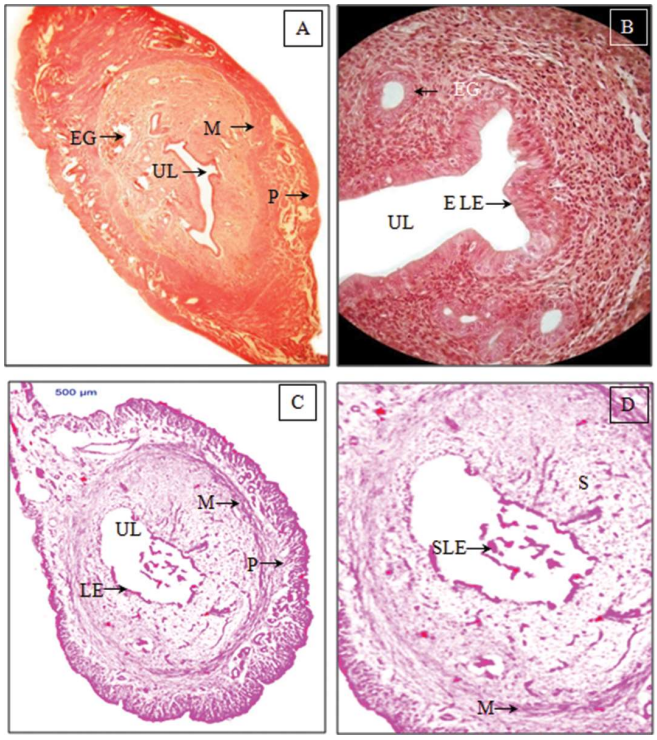


Fig. 6. (A-D). The uterus of control female rat (A & B) showed uterine lumen (UL),the endometrial glands (EG),the myometrium(M), perimetrium (P) and stroma (S) and smooth endometrial luminal epithelium. Females treated with crude bark extract (C & D) showed uterine lumen (UL), but stripped luminal epithelium (SLE) dispersed into uterine lumen. The stroma fails to show endometrial gland.

Effects of crude bark extract on uterine histological structure in cyclic females

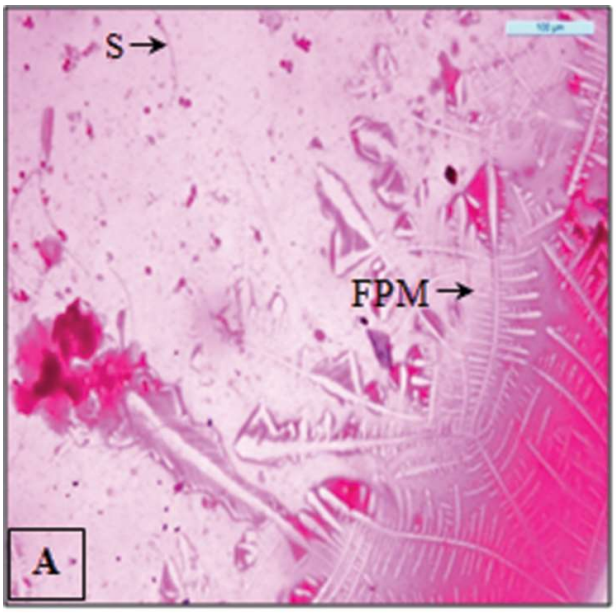
The control females' uteri during the estrus phase showed a proliferated lumen with multiple number of endometrial glands embedded in the stroma (Fig. 6A & B). The endometrial surface epithelium appeared to be smooth, uninterrupted with layer of nucleated epithelial cells (Fig.6B). The stroma is compact with multiple number of endometrial lands. The myometrium and perimetrium layers appeared distinctly separated from each other with compact structure.

The treated females showed widening of uterine lumen with decrease in the luminal folding (Fig.6C & D). Uterine luminal epithelium layer is thin and shows non proliferation of the epithelial cells. Moreover stripping of the luminal epithelium from the basal layer has been observed distinctly in various regions. The epithelial cell layer is found to be separated from the lamina propria. The endometrium

shows redundancy in endometrial glands. The myometrium and the perimetrium are found to be intact and distinct. Endometrial surface epithelium is disintegrated in several regions, however, the stroma remains intact.

Effects of crude bark extract on uterus during early gestation (day5)

Mating was confirmed in all females of both control and extract treated groups by observing the fern pattern mucous and appearance of sperms (Fig. 7A) on the slide or vaginal plug formation. It has been observed that 40% of the females (treated group) showed the implantation sites in both the uterine horns. Remaining sixty percent females failed to show the implantation site (Table 1). The control females completed full term pups. The implantation sites of the control females showed the growing embryo proliferated and differentiated uterine stromal cells. On day 5 of gestation, maternal stromal cells differentiated to decidual cells with largerounded nuclei. The round structure of embryo is enclosed by the trophoblast cells embryo is enclosed within the embryonic cavity within the maternal tissue (Fig. 8 A). The embryo has been oriented in the mesometrial and anti-mesometrial direction of the uterine horn. Cellular apoptosis at both the ends and proliferated multinucleated decidual cells are apparent in the implantation sites of control group of females. Administration



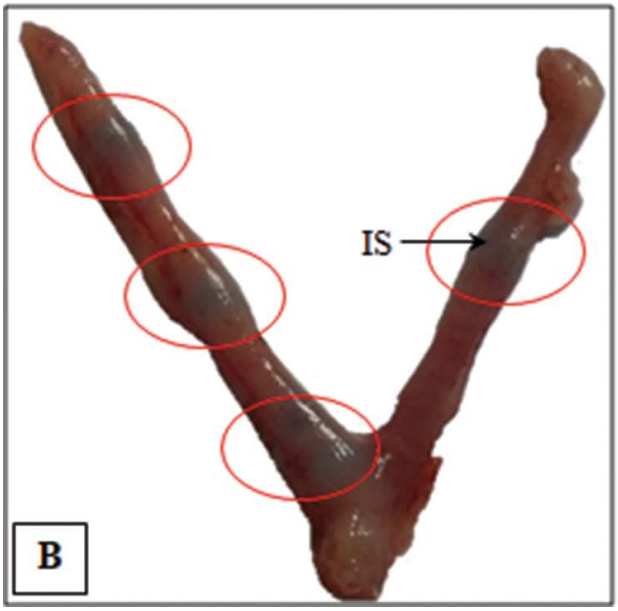
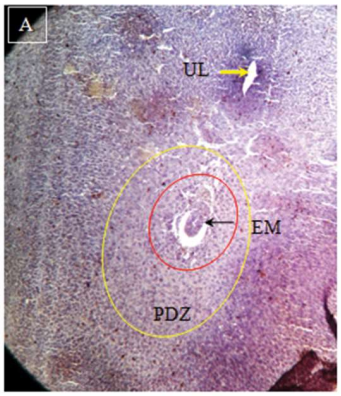


Fig. 7. A. Vaginal smear with fern pattern mucus (FPM) and sperms(S) confirming copulation. **B.** Implantation sites(IS) of pregnant females on Day 5 of gestation visible as blue spots after Chicago blue injection



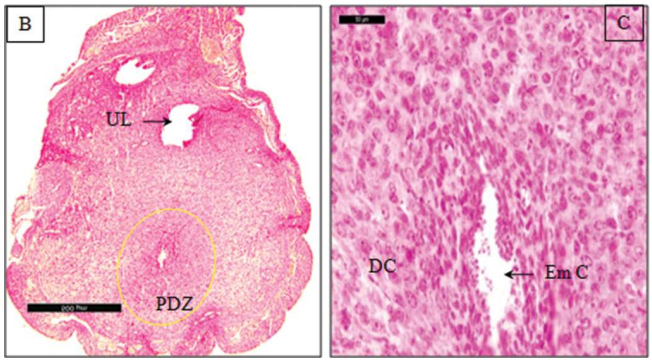


Fig. 8. (A-C). Uterus of the pregnant control mice at Day 5 of gestation (A) shows the presence of embryo (EM) , primary decidual zone (PDZ) and the uterine lumen (UL). Uterus of pregnant female mice treated with crude bark extract (500mg/kg body wt) (B & C) showed primary decidual zone, but without embryo in the embryonic cavity (Em C) surrounded by decidual cells (DC).

Table 1. Number of pregnant females, implantation sites and percentage of implantation and implant resorption. The treated females showed 50% resorption/ fail to implant. All treated females (100%) fails to give birth pups at the end of full term of gestation.

Groups	No. of Pregnant females	Females allowed for completion of full term of gestation	Females sacrificed for study of implantation sites	Females showing implantation sites	Mean number of implantation sites	No. of females having implant resorption/ fail to implant	% of implantation	Total Number of pups given birth
Group-III (Treated pregnant mice) (n=6)	6	2	4	2	1.5	2	50	0
Group-IV (Control pregnant mice) (n=6)	6	4	2	2	9.5	0	100	27

of crude extract to the females hinders the process of implantation as observed on day 5 of gestation. The maternal tissues showed the proliferation and decidual cell reaction with the formation of decidual cells. However, an intact developing embryo was not found in the implantation site of treated group of females. Differentiation of maternal tissues has been observed with the formation of mesometrial and antimesometrial pole. A mass of tissue and cells at the implantation sites indicates the presence of implants. However, a healthy embryonic structure was not observed in the treated group of females.

Discussion

Various uses of different parts of the plant are reported. In India, leaves are made into decoction and are used for treating cholera (Garg, 2011) and in Nepal barks are used as tonic (Watanabe *et al.,* 2013). In Chinese traditional medicine system the roots are used for treating pharyngitis, arthritis, tonsillitis and several other ailments (Agrawal *et al.,* 2010). Tooth problems are treated using fruits and the stem bark juice of the plant is used to treat stomach ailments (Joshi *et al.,* 2000). In addition, several other uses of different parts of *H.trijuga* have shown hypocholesterolemic (Purnima *et al.,* 2003), anti-inflammatory (Purnima *et al.,* 2006), hypotensive (Agrawal *et al.,* 2006), hepatoprotective (Agrawal *et al.,* 2010), antioxidant (Prassanna *et al.,* 2011), analgesic and antihyperlipidemic (Subbarao et *al.,* 2011) activities. Arunachal Pradesh is a state with numerous tribes and so a bundle of traditional system of medicines is being used in this region. The Adi tribe of Arunachal Pradesh uses the plant as abortive agent especially for domesticated animals like pigs and dogs. Crude bark powder is fed to domestic animal (swine) in order to control the population size of livestock. Identification, isolation and characterization of compounds from *Heynea trijuga* were done and it reveals the presence of various compounds like limonoids, steroids and lignans (Wang *et al*, 2013; Yang *et al.,* 2012; Yang *et al.,* 2012; Zhang *et al.,* 2011; Wang *et al,* 2008; Zhang *et al.,* 2011; Ji *et al.,* 2015) and various other phenolic compounds (Devkota *et al.,* 2014).

Sitil (*H. trijuga*) and Situ Payu (*Dysoxylum alliarum*) are two plants which are traditionally used for population control of domestic animals (pig, dog) by the Adi community of East Siang district of Arunachal Pradesh. The effects of crude bark extract of *Dysoxylum alliarum* on rat embryo development has been shown earlier from our laboratory (Das *et al*., 2014). In vivo laboratory study of effects of bark extract of *H. trijuga* on female reproduction has not been done so far beyond traditional information and use. Present investigation is the first such scientific approach to show its effects on rodent's female reproduction. It has been observed that methanolic crude extract of *H. trijuga* exerts effects on estrous cycle of rat and mice. Level of cornification of nucleated epithelial cells came down resulting in decreased duration of estrus phase.

It has been seen from the results that the extract showed evident effects on the structure of ovary and uterus. The structures of the preantral and antral follicles were found to be disorganized and disintegrated. The oocytes as well as the theca and granulosa cells were found to be degenerated. The lutein cells constituting the corpus luteum were degenerated in the treated females' ovary. This result suggests the presence of compounds in the CBE which caused the retardation of the ovarian follicular development during their maturation process and checks/blocks the formation of a matured oocyte in the ovary.

The follicular atresia is the degeneration of the ovarian follicle and is a part of ovarian cycle. Although it is an integral part of ovarian cycle, but increase in follicular atresia and decrease in the number of follicles is detrimental to the normal ovarian physiology as because follicular atresia is accompanied by the disintegration of the theca and granulosa cells. The theca interna and the granulosa cells are the target cells of the gonadotrophins LH and FSH. Theca interna is responsible for the expression of LH receptors to produce testosterone, which is the precursor molecule for the synthesis of Estradiol-17 β in the granulosa cells. FSH on the other hand acts upon the granulosa cells to promote the follicular development. The degeneration of the primary follicles in the crude bark extract treated ovary can be related to the functional disintegration of the endocrine response to the cells of the ovary, which causes decrease in the follicular recruitment and development. The disintegration of the theca interna and granulosa cells directly affects the responsiveness towards the gonadotropins which in turn hinders the synthesis of the Estradiol-17 β . Similarly the disintegration of the lutein cells of the corpora lutea due to the effect of the extract causes the hindrance in the production of progesterone hormones. Similar type of abnormalities in the ovary like the theca cell degeneration and granulosa cell degeneration is reported in the wistar albino rats treated with ethanolic root extract of *Rauwolfia vomitaria* (Aquiasia *et al.,* 2014). From the above results it can be speculated that these effects are exerted by the constituent compounds present in the extract, directly by insensitizing the receptors present in the follicle towards the gonadotropins or indirectly by affecting the hypothalamo-hypophyseal-ovarian axis.

The corpus luteum formation is an ovarian process in the preovulatory follicular development leading to the ovulation. In this process of formation of luteotrophic complex, estrogen plays a major role. Its secretion is enhanced by the LH and FSH through promotion of growth of large follicles (Shivalingappa *et al.,* 2002). The decrease in the number of corpora lutea as seen in treated rats indicates that the crude bark extract of *H.trijuga* may have inhibited the process of conversion of the preovulatory follicles into corpora lutea thus arresting the ovulation. Earlier reports on *Dysoxylum alliarium* extract hindering the ovarian function and causing follicular atresia have also been reported and it was also seen that the plant extract acts as abortifacients when tested on pregnant mice (Das *et al.,* 2014).These results are corroborative to the findings of the present investigation and can be related to its use by the folk people in Arunachal Pradesh.

As evidenced by the histological study the bark extract has shown several effects in the uterus of the treated female rats. The hypotrophy of the luminal epithelium of the treated uterus can be correlated to the decrease in the estrogen supplied from the ovaries. Dixon *et al.,* 2014, has discussed that, uterine atrophy is related to declining levels of estrogen and progesterone which is preceded by the suppression of gonadotropins. It can be speculated that certain compounds present in the crude bark extract may affect the release of gonadotropic hormones or interfere with the ovarian steroid production causing the atrophy of uterus of treated rats.

As seen in the results, the extract causes deleterious effect on both uterus and the ovaries and these effects are found to be dose-dependent. The present investigation provides the basic and preliminary understanding of how the crude bark extract of *H.trijuga* may act as abortifacients in the livestock as used by the ethnic communities. With this basis, further study on this plant extract can provide a great scope in understanding . To understand the functioning of the extract further studies can be done in animal model system undergoing gestation. Furthermore, the identification and characterization of the active constituents of the extract may

help in projecting this plant as a potent herbal abortive agent in future.

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