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Original Research Article

The Estrogenic Property of *Cynoglossum zeylanicum* a Traditional Female Reproductive Medicine Induces ER-β Expression in Mice Ovary and Uterus

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Abstract: In the present study methanolic crude root bark extract (CRBE) of the plant *Cynoglosum zeylanicum* has been tested on adult ovary intact cyclic and ovariectimized mice. The root bark of this plant has been traditionally used by the folk women of north east India for successful conceive and gestation. In the present study uterine histomorphology and expression of ER-β has been carried out by H&E staining and immunofluorescence using ER-β antibody. In contrast to the control group, the CRBE treated females showed considerable alterations in the histoarchitecture of uterus. The treated mice showed a significant ($P< 0.5$) increase in luminal surface area and glandular cell proliferation of the uterus. This indicates that the extract may specifically target the epithelial cell to cause its effect and this proliferation supports the estrogenic potency of the plant. ER-β has been expressed in a cell-specific manner in both the uterus and the ovary. ER-β signals has been found strong in stromal cells of uterus in the extract treated group. In the ovary, the receptor has been localized with high intensity in thecal cells of extract treated group. Thus, the extract could modulate the estrogen responsiveness in the stromal epithelium. The present research work is the first in vivo and mechanistic insights of *Cynoglossum zeylanicum* on female reproductive tissue to demonstrate the existence of estrogenic compounds (phytoestrogens) that modulate the expression of ER-β across uterine and ovarian cell types.

Keywords: *Cynoglossum zeylanicum*, Estrogen receptor β, ovariectomy, Phytocompounds, uterine histoarchitecture,

Introduction

Cynoglossum zeylanicum, a biannual shrub belonging to the Boraginaceae family, has traditionally been used to regulate female reproduction in the Mishmi tribe of Arunachal Pradesh. The traditional healers recommend water suspension of the freshly collected plant material paste orally to desired women who fail to conceive normally. The dose is prescribed in specific amount to be orally taken during morning hours from day 4 to day 7 (3 doses) of menstrual cycle. This approach prompts us to hypothesize the influence of the extract on features of either uterine pre-receptivity status or ovarian follicular recruitment events. The intent of this study was to assess the effect of root bark extract of *Cynoglossum zeylanicum* on the histoarchitecture and estrogen receptor-β (ER-β) expression in the uterus and ovary of female albino mice.

The uterus is the final target tissue of female reproductive functions for ovarian hormones and is made up of a variety of cell types. Viz. stroma, luminal epithelium, glandular epithelium, myometrium, perimetrium and smooth

muscle. The uterus undergoes constant synchronized changes in proliferation in distinct cell types due to changing levels of circulating estrogen and progesterone (Weihua *et al.*, 2000). Estrogen administration stimulates uterine epithelial cell proliferation and estrogen withdrawal results in apoptotic death (Kodaman and Taylor 2004). Estrogen receptors (ER) are members of a large superfamily of nuclear receptors that mediate all of the estrogen's biological effects (Mangelsdorf *et al.*, 1995). Both the receptor and are unique proteins encoded by different genes on different chromosomes, but not isoforms of one another. The Estrogen receptor (ER) mediates the genomic effect of the hormone 17-β estradiol in mammalian tissue (Couse *et al.*, 1997). With the different levels of circulating estrogen, the uterine endometrium undergoes proliferation in different cell types and continuous synchronized changes (Weihua *et al.*, 2000). The primary site for manufacturing estrogen in females is the ovary, although little is known about how estrogen affects follicle formation in the mammalian ovary. Numerous estrogenic effects on ovarian cells have been reported in animal research. The purpose of this research aimed to determine if there were any variations between the ER-β expression in the uterine endometrium, ovarian granulosa cells, and theca cells in response to the oral administration of crude root bark extract of *Cynoglossun zeylanicum* to adult cyclic female mice. In the present work, ER-β expressed in ovarian stromal cells, including granulosa cell proliferation and the receptor expression in thecal cell. This finding suggests that the phytocompounds present in the crude root bark extract functions to alter the ability of the thecal cell to express ER, which aids in follicular development and granulosa cell proliferation. Ovarian follicular growth during the proliferative phase upregulate the secretion of estrogen which results in regeneration and new growth of endometrial glands, stroma, and endothelial cells of the uterus (Kodaman *et al.*, 2004). The pathway of estrogen action in tissue through ER was examined in ERα and ERβ knockout mice models (Chen *et al.*, 2009; Jayachandran *et al.* 2010). These experiments provide attention to the priority of estrogen Receptor β in female fertility concerns.

Cynoglossum zeylanicum Lehm is a commonly grown herb that has been documented to have wound-healing, hepatoprotective, antioxidant, and anti-inflammatory activities (Malla *et al.*, 2012, Anitha *et al.*, 2012, Anitha *et al.*, 2013). These properties have been reported mainly from studies on the aerial part of the plant. The plant is a well-known traditional medicine in Arunachal Pradesh for the treatment of female reproductive disorders. The root bark of the plant has been used for the correction of irregular menstruation and related female infertility. This indigenous knowledge gave rise to the concept of phytosteroids, which are plant compounds that can have either agonistic or antagonistic effects on gonadal steroid receptors in female reproduction. One of our earlier research studies regarding cornification assays of the crude root bark extract of *Cynoglossum zeylanicum* generated an enhanced high rate of cornification in mice due to estrogenic actions (Miyu *et al.*, 2017). Many of the actions of estradiol are known to be mediated by its high-affinity binding to a particular receptor that activates transcription factors in response to ligands. Granulosa cells in particular, has been shown to bind estrogen in the ovary in early experiments (Anderson *et al.*, 1989). One of our recent study has been subjected to compounds separation, identification and molecular docking analysis of the methanolic crude root bark extract of *Cynoglossum zeylanicum* (Gowala *et al.*, 2020). In this study a total of 41 phytocompounds has been listed. The enlisted phytocompounds were docked *in silico* with gonadal steroid hormone receptors, viz., human ERα, ERβ, and PR and targeted for their binding affinity. Molecular docking studies revealed that the most potent compounds present in the extract were hexadecanoic acid, octadecanoic acid, acetyllycopsamine, demecolcine, and gamma-sitosterol. These compound showed strong binding affinity towards steroid receptor (ER and PR) similar to the native ovarian steroids with remarkable biological/ pharmaceutical action. On the basis of this finding, it has been hypothesized that the phytosteroids present in the root bark extract of *Cynoglossum zeylanicum* could modulate the in vivo expression of ER functioning. This hypothesis also corroborates with our another experimental

work on *Cynoglossum zeylanicum* on endometrial proliferation using H&E staining conducted in ovatiectomized mice (Sarma *et al*., 2021). This study shows that, ovariectomized group treated with plant extract display high rate proliferation in luminal and glandular epithelium when compared with control group. The data provide a strong evidence of estrogenic property of the plant targeting endometrial cells by the plant extract. On this background it has been hypothesized that the estrogenic effects of the phytosteroids may cause endometrial growth and receptivity by enhancing the expression of ER-β in the reproductive tissues uterus and ovary. The present research work provide the first mechanistic insights into how the phytocompounds present in the plant *Cynoglossum zeylanicum* trigger the expression of ER-β across several uterine and ovarian cell types.

This article specifically focused on endometrial proliferation and estrogen receptor beta expression induced by the endocrine-modulating properties of compounds present in the ethnomedicinally important plant *Cynoglossum zeylanicum*. This study also highlights the findings on the site of synthesis and target tissue of Estrogen receptor.

Materials and methods

Collection of plant and extract preparation

The plant *Cynoglossum zeylanicum* (Fig.1) was collected from the Mayodia region, Roing town, Lower Dibang district, Arunachal Pradesh, at GPS coordinates 28° 14' 1"N, 95° 54' 36"E. After collection, the samples were washed in running tap water, the root bark peeled off and dried in the shade at room temperature. A coarse powder was made from the dried bark. The plant extract was made using the same process previously standardized in our laboraratory (Das *et al*., 2013). Briefly, the dried root bark was grinded to a 60 mesh powder and macerated in methanol in a ratio of 25gm:100ml (W/V) for 72 hours. The solvent extract was filtered by a Whatman filter paper (125 mmØ) and allowed to evaporate either in room temperature or vacuum evaporator. The prepared concentrated extract was stored in screw-capped vials at -40 °C until usage in a semi-solid mass.

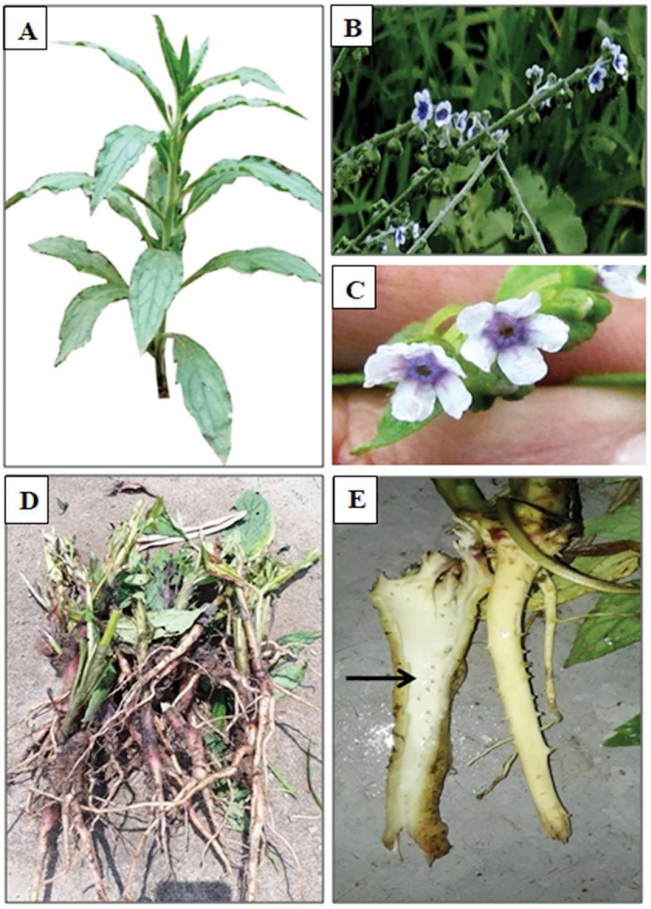

Fig. 1. Plant *Cynoglossum zeylanicum* A- Plant *Cynoglossum zeylanicum* aerial part; B, C- Flower of the plant; D- Root Part of the plant; E- Root bark

Experimental animal

LACA strain Swiss albino mice (25-30g) were used as the experimental animal. The animals were reared in the Rajiv Gandhi University (Central Animal Facilities) as per IAEC (RGU), approval for animal maintenance.

Adult female mice 60-70 days old were used in this study. All of the experimental animals were housed in polypropylene cages measuring 29x22x14 cms or 43x27x15 cms and maintained conditions of natural light dark cycle throughout the experimental period with a standard mice diet. Experiments were performed on cyclic ovary intact and ovariectomized (OVX) females. Ovariectomy was done following standard method (Hogan *et al*., 1986). Briefly, under mild diethyl ether anesthesia the ovaries have been removed with fine forceps making a small dorsolateral incision through

the skin and muscle of the back of the mice. The ovariectomized (OVX) mice were kept in intensive care for three weeks and allow them to recover before being evaluated for in vivo investigations. Oral administration was done between 7:00 and 9:00 hrs. beginning with the proestrus of the cycle.

In vivo experimental design and dose of crude root bark extract

Adult female mice 60-70 days old were divided into two groups. One group of animals (n=6) has been administered crude root bark extract of *Cynoglossum zeylanicum* considered as extract treated group (Group-I) while another group (n=6) has been given only vehicle (Group-II), and considered as vehicle-treated control group. Females were given the vehicle and extract orally for eight days in a row, for a total of two cycles.

The ovariectomized mice with similar age group has been divided as the OVX-vehicle-treated (Group-III; n=5) and the OVX-extract-treated group (Group-IV; n=5). The OVX females were treated with crude root bark extract (CRBE) for four consecutive days (duration of one estrous cycle) in a similar manner of the cyclic treated females. Control OVX females received the vehicles only for the similar period of the treated group. The crude root bark extract (CRBE) of *Cynoglossum zeylanicum* was admisistered to the treated groups of female (both cyclic and OVX) at a dose of 500mg/ kg body eight/day. It is the threshold dose for the CRBE of *Cynoglossum zeylanicum* that has been determined in our earlier research (Miyu *et al*., 2017).

Tissue collection and morphometric analysis

The females were sacrificed between 16.00 and 18.00 hrs on the last day of treatment of respective cyclic and OVX females. The tissue samples were collected and processed for routine histological study following the method of Culling *et al*., (1974). From the cyclic groups both ovary and uterine horns were collected while from the OVX females only the uterine horns collected for further study. Briefly, the tissues were rinsed in 0.09 % saline water and promptly fixed in Bouin's fixative for 72 hours. The fixed tissues were thoroughly washed to eliminate excess fixatives and dehydrated using graded alcohol. At 56^{0}C-58^{0}C, dehydrated tissues were embedded in molten paraffin. Paraffin-embedded tissues were cut into 5-µm thick serial sections and stretched on slides coated with poly-L-Lysine. All of the stretched slides were incubated at 37^{0}C overnight. Routine H & E staining of uterine and ovarian tissue sections were carried out for study of the histological structure.

The morphometric study of endometrial proliferation has been analyzed in both control and treated groups of females. The morphometric study took into account of parameters such as the length of lumen (the area occupied by lumen), endometrial gland circumference, and luminal epithelium thickness.

In situ immunolocalization of Estrogen receptor β

Immunofluorescence was performed for immunolocalization of ER-β following the procedure of Eminaga *et al*., 2016. The paraffinized tissues were sectioned to a thickness of 5 µm, stretched on Poly -L- Lysine coated glass slides and incubated at 37°C for overnight. Tissue sections were deparaffinized in xylene and rehydrated in graded ethanol to distilled water. The antigen retrieval process was accomplished by boiling the sections in 10 mM citrate buffer (PH 6.0) for 15 minutes followed by three 5-minute washes in 1 TBS. The blocking was done using a 5% NGS (Normal Goat Serum- Sc-2043) followed by incubation of tissue sections overnight with ERβ-primary antibody (Santa Cruz ER mouse monoclonal Sc-53494) and then for 1 hour with FITC conjugated secondary antibody (Sc-516140). Sections were mounted in Ultra Cruz TM Mounting Medium (SC-249410) and observed under DM 5000 B Leica fuoescence imaging microsystem.

Immunofluorescence signal analysis

The quantitative analysis of immuno-signal intensity of ER-β in different cell types of the uterus and ovary of has been statistically analysed using ImageJ software. This program recognizes and distinguishes the various staining intensities of the selected areas. The intensity has been calculated in terms

of low (+), moderate (++), strong (+++) and intense (++++) following the method of Saikia *et al.*, 2017. Low intensity (+) has been assigned to values below 20%; moderate (++) has been assigned to values between 20% and 40%; strong intensity (+++) has been assigned to values between 40% and 80%; and intense (++++) has been assigned to values above 80%.

Statistical analysis

To compile the major histoarchitecture of the uterus, measurements were taken using the LAS V4.4 software provided with the Leica DM 5000B microscope. Measurements were taken in three regions of the same uterine section taking three replicates, and the values were statistically computed using GraphPad Prism software. Tukey's multiple comparison test at $p<0.05$ has been used to determine the significance of the values.

Results

Hiatoarchitectural study of the uterus and ovary of Ovary intact cyclic females

The effect of the extract of *Cynoglossum zeylanicum* on histoarchitecture of mice uterus and ovary has been studied using different parameters. The endometrial epithelium in the vehicle treated control uterus was intact and had a homogeneous layer of epithelial cells without any discernible proliferation. The luminal epithelium lacks finger like projections. Endometrial glands have entrenched in the stromal area and displayed a thin lining of luminal epithelium. The uterine histological structures of the control female have been presented in Fig 2 A, B. In the CRBE treated mice the endometrial epithelium has seen proliferated extensively. There is a significant proliferation of endometrial epithelium in CRBE treated mice, with the proliferation being most noticeable in the endometrial glands and luminal epithelial region. The luminal epithelium has proliferated forming finger-like projections, and the endometrial glands grew in size and numbers in extract treated group of mice (Fig. 3 A, B). The luminal epithelium is highly proliferative, resulting in an increase in the surface area of the luminal endometrium. In comparison to the control group, the surface

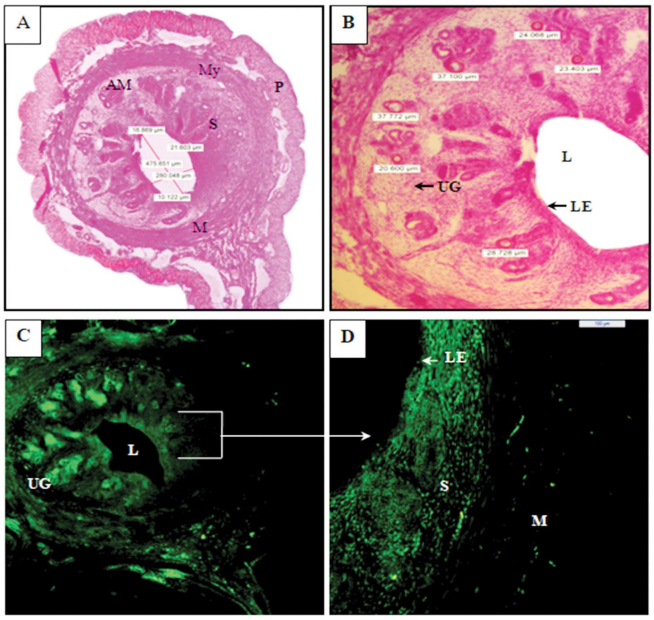

Fig. 2. Photomicrographs of uterine histology (H & E staining) and *in situ* localization of ER-β in vehicle treated control mice uterus. A- Area measurement occupied by luminal epithelium and thickness of Luminal epithelium. B- Measurement of circumference of uterine gland. C,D- Immunolocalization of ER-β in cyclic vehicle treated female mice uteri using FITC-conjugated antibody. The protein has been localized in the cells specifically in the endometrial glands and in stromal cells. L-Lumen, LE-Luminal Epithelium, UG- Uterine Gland, S- Stroma, P-Perimetrium, My-Myometrium., M-Mesometrial pole, AM- Antimesometrial pole. Origininal magnification: A-5X, B-20X, C-5X, D-20X.

area of the uterine lumen, the circumference of uterine glands and the thickness of luminal epithelium have increased significantly in CRBE treated cyclic group of mice. As evidenced by the histological study and measurement, the measured values of considered parameters of uterine endometrium, has been statistically analyzed using Tukey's multiple comparison ($p<0.05$) test presented in Fig. 4.

In the ovarian histology, the control females showed the regular structure with follicles and the cells of the stromal region compactly arranged. Primary, secondary, and tertiary follicles as well Graafian follicles has been seen as presented in Fig. 5 A. Numbers of primary follicles has been observed in vehicle treated control mice ovary. In the CRBE treated group, the stromal area is primarily occupied by secondary, tertiary, Graafian follicle and corpus luteum as presented in

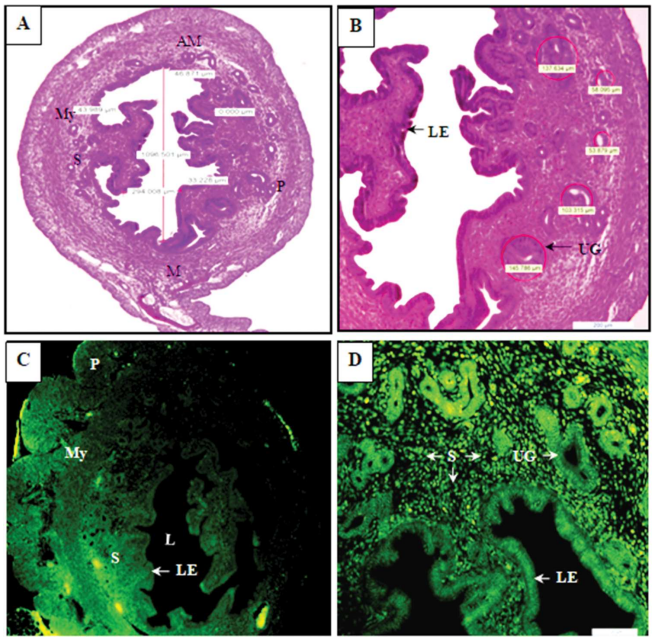

Fig. 3 Photomicrographs of uterine histology (H & E staining) and *in situ* localization of ER-β in CRBE treated mice uterus. A- Area measurement occupied by luminal epithelium and thickness of luminal epithelium. B- Measurement of circumference of uterine gland. C,D- Immunolocalization of ER-β in extract treated cyclic mice uteri using FITC-conjugated antibody. The protein signal is strong specially in stroma (S) and endometrial gland (EG). L-Lumen, LE-Luminal Epithelium, UG- Uterine Gland, S- Stroma, P-Perimetrium, My-Myometrium., M-Mesometrial pole, AM- Antimesometrial pole. Original magnification: A-5X, B-20X, C-5X, D-20X.

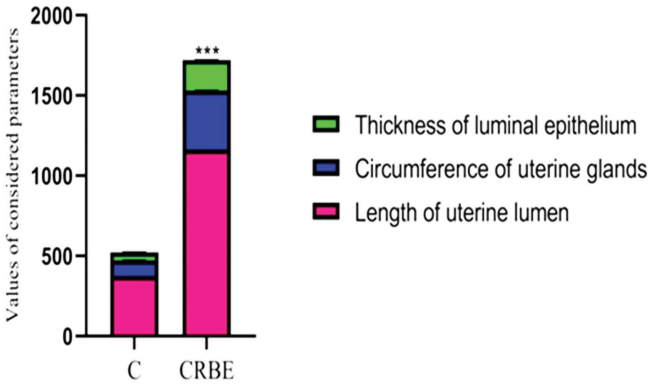

Fig. 4. Graphical presentation of considered parameters in uterine morphometry analysis of both vehicle treated control and CRBE treated mice uterus. The thickness of luminal epithelium, circumference of uterine gland and the length of lumen (mesometrial to antimesometrial pole) or area a of uterine lumen all has increased in CRBE treated mice. The length of uterine lumen ie, area occupied by lumen has increased specially in extract treated uterus. All the values are given as Mean ± SEM., (*p<0.05).

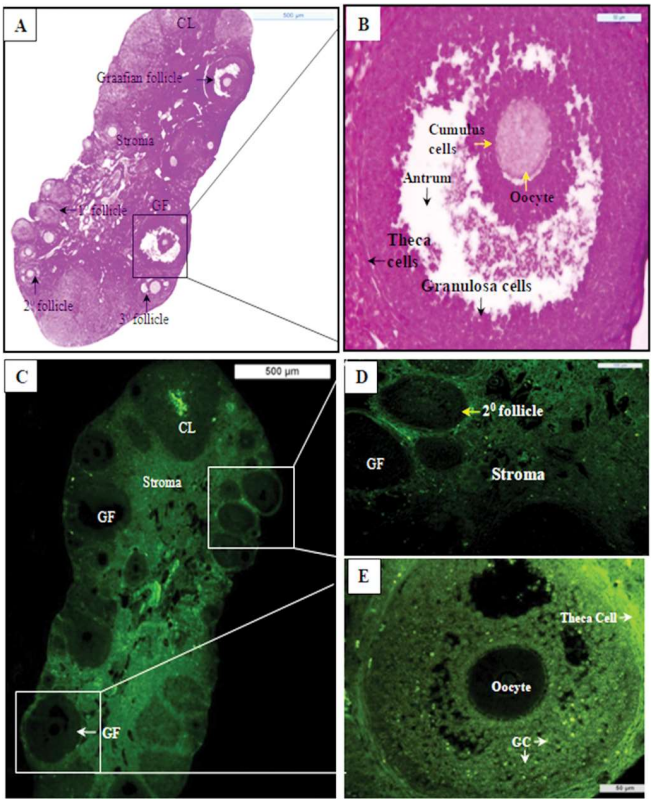

Fig. 5. Photomicrographs of ovarian histology (H & E staining) and *in situ* localization of ERβ of vehicle treated control mice. A- Structure of various stages of follicles and Corpora lutea. B- Histoarchitecture of a Graafian follicle. C,D,E- Immunolocalization of ERβ in different cell types of ovary. GF- Graaffian follicle, GC- Granulosa cells, 10 follicle (Primary), 20 follicle (Secondary) and 30 follicle (Tertiary or Preantral) CL- Corpora lutea. Original magnification: A-5X, B-40X, C-5X, D-20X, E-40X.

Fig. 6A. The cells of a mature follicle (Graafian follicle) has been observed around the oocyte, arranged in proper location with formation of the antrum (Fig. 6 B). In majority of histological sections of CRBE treated females' ovary, the number of follicles in early developmental stages (primary) found to be higher than that of the control females. However, looking to the present objective of study of ER-β, number of follicular recruitment has not been accounted. In general, all groups the ovarian histology showed integration of ovarian stromal tissues, presence of follicles at its various stages of development (primary, secondary, preanatarl and antral, thecal cells, granulosa cells, cumulus cells, and oocyte surrounded by the Zona pellucida layer).

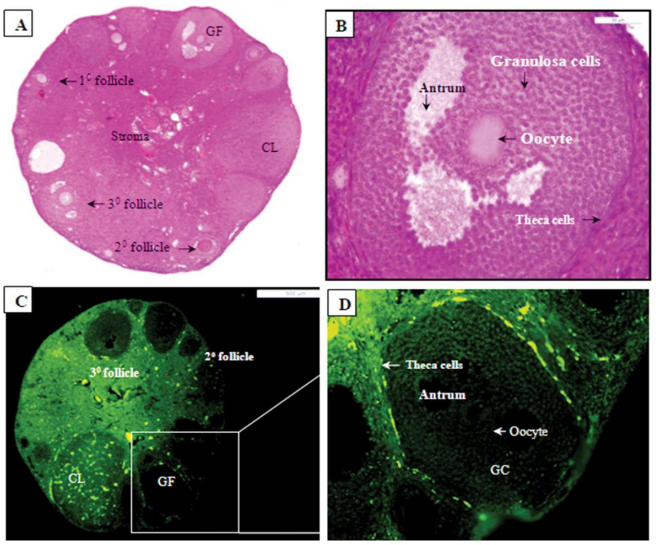

Fig. 6. Photomicro graphs of ovarian histology (H & E staining) and in situ localization of ER-β of cyclic CRBE treated mice. A- Histological section of ovary showing primary, secondary, graafian follicle and Corpora lutea. B- Histoarchitecture of a Graafian follicle. C,D- Immunolocalization of ERβ in different cell types of ovary. The TC expresses intense signal of the receptor while cumulus cells and Granulosa cells GF- Graaffian follicle, CL- Corpus luteum, 10 follicle (Primary), 2^0 follicle (Secondary) and 30 follicle (Tertiary or Preantral). Original magnification: A-5X, B-40X, C-5X, D-40X.

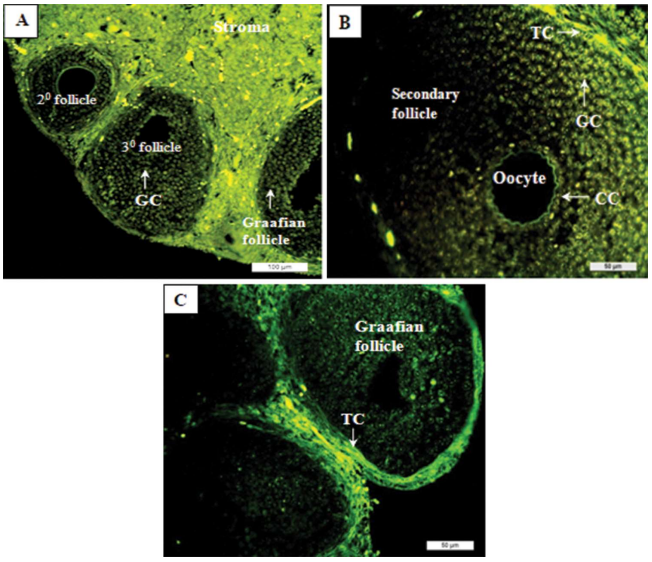

Fig. 7. Photomicrographs of in situ localization of ER-β in different cell types of CRBE treated ovary. A,B, C- Ovary showing ER-β expression in Secondary follicle, Graffian follicle, TC and Stroma. GC- Granulosa cells, TC-Theca cells, GF- Graaffian follicle. CC- Cumulus cells Original magnification: A-20X, B-40X, C-40X, D-40X.

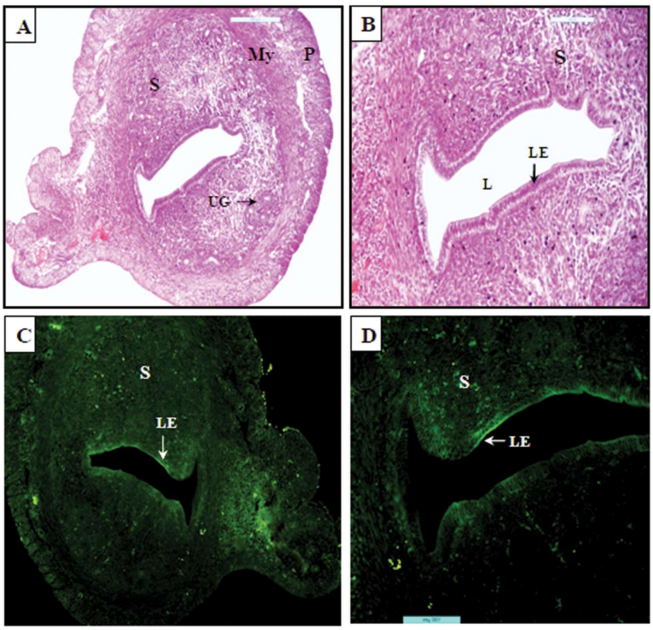

Fig. 8. Photomicrographs of uterine histology (H & E staining) and immunolocalization of ER-β in OVX-vehicle treated control mice. A, B- Histology of OVX-vehicle treated mice uterus. Endometrium showed less proliferation with a reduced number of endometrial glands. C,D- Very low (+) ER-β expression in the luminal epithelium and no expression (—) in stromal cells. L-Lumen, LE-Luminal Epithelium, UG- Uterine Gland, S- Stroma, P-Perimetrium, My-Myometrium, M-Mesometrial pole, AM- Antimesometrial pole. Original magnification: A-5X, B-20X, C-5X, D-20X.

Histoarchitectural study of the uterus of ovariectomized females

The histological structure of the mice that are devoid of endogenous estrogen source (i.e. ovariectomized) has been presented in Fig. 8. In the ovariectomized control group of females (treated with vehicle), the uterine tissue shows less proliferation with reduced number of endometrial glands since there is no source of native steroids (Fig. 8 A, B). The structure of stromal cell in the endometrium and myometrium is compactly arranged. In the ovariectomized group treated with CRBE showed glandular and luminal epithelium proliferation, especially noticeable in the luminal epithelial regions (Fig. 9 A, B). Increased number of uterine gland and glandular epithelium has been observed in OVX- extract (CRBE) treated uterus. In comparison to the OVX-vehicle-treated control group of mice, the investigation on histology affirms significant proliferation in endometrial tissue as well

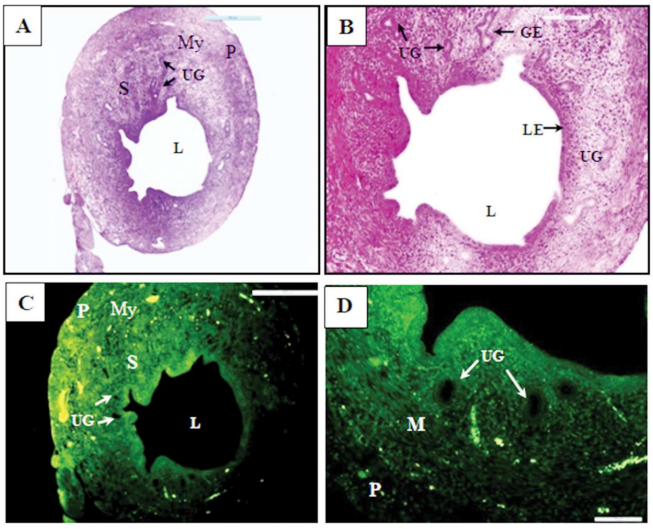

Fig. 9. Photomicrographs of uterine histology (H & E staining) and Immunolocalization of ER-β in OVX-CRBE treated mice. A, B. Histology of OVX- CRBE treated mice uterus. Uterine endometrium showed proliferation of both luminal and glandular epithelium. C, D. Strong expression in stromal cells and moderate expression of ERβ in luminal epithelium. L-Lumen, LE-Luminal Epithelium, UG- Uterine Gland, GE- Glandular epithelium. S- Stroma, P-Perimetrium, My-Myometrium. Original magnification: A-5X, B-20X, C-5X, D-20X.

as glandular cell proliferation in OVX mice treated with CRBE of the plant *Cyoglossum zeylanicum.*

In situ immunofluorescence of ER-β in uterus and ovary

In ovary intact cyclic mice uterus, ER-β expression has been found in a cell-specific manner. The cell-specific immunolocalization of ER-β has been established by immunofluorescence localization of ER-β. The expression of ER-β in the uterine section of cyclic ovary intact vehicle treated control mice revealed moderate (++) receptor expression in the stromal cells (Fig. 2 C, D). Luminal epithelium exhibits very low (+) expression of the receptor. Endometrial glands embedded in the stromal region showed similar pattern of ER-β receptor expression intensity as the surrounding stroma. Myometrium and perimetrium cells are devoid of ER-β expression. Oral administration of CRBE of *Cynoglossum zeylanicum* to the ovary intact cyclic female mice showed strong (+++) expression of ER-β in the luminal epithelium and uterine gland. The endometrial stromal cells exhibit intense (++++) expression of ER-β in extract treated mice uterine section with moderate expression in myometrium and perimetrium as presented in the Fig. 3 C, D.

The immunofluorescence of ER-β in the ovary of control and CRBE treated females showed expression of ER-β in cell specific manner. The expression has been found in the follicles of different developmental stages (Fig 5 and 6). The immunofluorescence of ER-β in the cyclic control group of females showed moderate intensity (++) in ovarian stromal cells. All follicular cells in the control group, including primary (1°), secondary (2°) and tertiary (preantral) (3°) follicles, failed to exhibit ER-β expression (Fig. 5 C, D). The thecal cells and Granulosa cells of developing follicles at any stage of their proliferation have not shown the presence of-ERβ expression. However, in the mature antral follicle (Graafian follicle) cells surrounding the oocyte showed moderate signals of expression of estrogen receptor-β. The granulosa cells, cumulus cells, of the matured Graafian follicle express moderate expression of ER-β, while thecal cells express moderate to strong expression as shown in Fig. 5 E. Administration of CRBE to cyclic ovary intact females accelerated the expression of ER-β in stromal cells of the ovary at a higher level (strong +++) of signals than that of the control females. The cumulus cells and granulosa cells of growing follicle surrounding the developing oocyte failed to express ER-β signals (Fig. 6 C &D). However, the peripheral theca cells surrounding the oocyte of a mature Graafian follicle showed intense (++++) signal of the receptor as depicted in the Fig. 7 B. Mature oocyte of the, Graafian follicle failed to show the ER-β immunoreactivity (Fig. 7 B). The growing follicular cells of secondary preantral follicles as well as the mature Graafian follicles expresses moderate to high expression of ER-β in the multilayer granulosa cells as seen in the Fig. 7 C.

The ovariectomized (OVX)- vehicle-treated control female mice uterus showed absence of ER-β expression signal in its all types of cells, i.e., in the luminal epithelium, stromal cells or other areas of the endometrial region (Fig. 8 C, D). The OVX- CRBE treated mice showed moderate

expression (++) of ER-β in the luminal epithelium. The stromal cells express strong (+++) signal as shown in Fig. 9. In the uterine gland, perimetrium, and myometrium, there is a moderate expression (++) of ER-β in the OVX-CRBE-treated females (Fig. 9 C, D). The quantitative analysis of the signals of CRBE treated females showed significantly higher signals than that of the control samples of uterus and ovary as presented in Table 1 and Table 2 respectively.

proliferation under the influence of estrogen (O'Brien *et al.*, 2006). In the present study, the CRBE of *Cynoglossum zeylanicum* treated mice uterus, endometrial epithelium showed more proliferation ($P < 0.5$) with finger-like projections. The thickness of and luminal epithelial was higher in treated females in comparison to that of the control. Increased cellular proliferation is an evidential support of presence of estrogenic phytocompounds in the CRBE, which targets the endometrial epithelium with native steroid's agonistic effects.

Table 1. Quantification of Immunofluorescence of ER-β signals in various cell types in uterus of control and treated groups. Intensity values are expressed as means of observations.

Uterus	Luminal epithelium	Stroma	Endometrial gland	Myometrium	Perimetrium
Control	+	++	++	–	–
CRBE treated	+++	++++	+++	++	++
OVX- Control	+	–	–	–	–
OVX - CRBE treated	++	+++	++	++	++

Table 2. Quantification of Immunofluorescence of ER-β signals in various cell types in ovary of control and CRBE treated females. Intensity values are expressed as means of observations.

Ovary	Stromal tissue	Follicle grade			Graafian Follicle		
		1°(Primary)	2° (Secondary)	3° (Preantral)	Theca cells	Cumulus cells	Granulosa cells
Control	++	–	–	–	++	++	++
CRBE treated	+++	++	+++	+++	++++	–	–

+ <20% (Low); ++ 20–40% (moderate); +++ (40–80%) Strong ;++++ (>80%) Intense.

Discussion

The coordinated interaction of estrogen receptors with the cells of the uterus and ovary is a key regulatory process for endometrial receptivity of the uterus. The expression of the steroid receptor in certain cell types at a particular time is crucial for endometrial preparation for successful implantation. (Robertshaw *et al.*, 2016). In the present study, the agonistic effects of the phytocompounds present in the CRBE of *Cynoglossum zeylanicum* has been evidenced in the ovarian and uterine cells. A differential expression pattern of estrogen receptors in the cell types of ovary and uterus suggests the estrogenic property of the compounds and its effects on ER expression in these tissues. It has been well established that the uterine endometrial epithelium shows profound

The ER-β signals were found strong in stromal cells and glandular epithelium while moderate in luminal epithelium in the CRBE treated group. It has been observed that the ER-β expression shifted to stromal areas in the extract treated uterus when compared with the control, indicating the possibility of the extract triggering the receptors of stromal cells and epithelial cells of the stromal area rather than the lumen. In the present work, ER-β expressed in ovarian stromal cells, thecal cells and granulosa cells. The phytocompounds in the plant extract have been found to target the stromal cells of the endometrium, raising the possibility that the extract could aid in the decidual cell reaction (DCR). Decidual cell reaction is a phenomenon where the stromal cells change into decidual cells during the

peri-implantation period of mouse gestation. Studies have revealed that estrogen stimulates cell proliferation strictly in the epithelial cells of ovariectomized adult mouse uteri (Quarmby *et al.*, 1984). In the present study, the ovariectomized mice treated with plant extract showed uterine epithelial cell proliferation in the luminal epithelium implying the estrogenic nature of the compounds present in *Cynoglossum zeylanicum.* The compounds present in the extract may either activate the increased expression or initiate increased transcription of estrogen receptor.

As per the traditional knowledge, the traditional healers prescribed the root bark of *Cynoglossum zeylanicum* to women seeking a desired pregnancy but were unable to conceive under normal circumstances. During the first 5 days after menstrual flow, desired ladies should take a water suspension of the paste of the root bark orally as per the oral literature. The information hypothesize the influence of the plant extract on features of either uterine pre-receptivity status or ovarian follicular recruitment events, despite the fact that the reasons for the female patient's infertility and reproductive condition are unknown.

This finding revealed that different cell types in the uterus and ovary express the estrogen receptor in varying intensities in response to the CRBE administration. These findings demonstrate that active estrogenic compounds in the plant extract either enhance the function of endogenous estradiol or bind with ER with higher affinity than the native steroid. This function of CRBE aids uterine endometrium proliferation and modulate estrogen receptor expression. Evidence on the coexistence of ligand-binding sites and ER in the ovary suggests that the effect of estrogen in thecal cells and follicular cells are mediated by ER-α and ER-β respectively. ER-β is responsible for follicular growth and maturation and any disarray in the ER-β gene unable to prevent growth and maturation of follicles. A study on differential expression of ER-β in rat ovary revealed that ER-β was expressed only in nuclei of granulosa cells (Sar *et al.*, 1999). The ER-β is the most prevalent estrogen receptor in the ovary. It has been reported that thecal cells express estrogen receptor alpha (ER-β) and granulosa cells express estrogen receptor beta (ER-β) in the ovary (Lee *et al.*, 2021). In the present experiment, The ER-β has been expressed in all developing follicles (primary, secondary and preantral) in the following oral administration of CRBE to the cyclic ovary intact females. The receptor has been localized with high intensity in thecal cells than that of the granulosa cells in the mature Graafian follicle of the CRBE treated females. This finding suggests that the phytocompounds present in the plant extract induced the granulosa cell proliferation and functions to alter the ability of the thecal cell to express ER, which aids in follicular development. The uterus undergoes constant coordinated variations in proliferation in distinct cell types with different levels of circulating estrogen (Weihua *et al.*, 2000). During the proliferative phase of the ovary, follicular growth leads to an increase in estrogen levels, which leads to regeneration and growth of the endometrial glands, stroma, and endothelial cells of the uterus (Kodaman *et al.*, 2004).

One of our previous studies found phytoconstituents in the plant *Cynoglossum zeylanicum* that have a substantial binding affinity for the steroid receptors- ER (ER-α and ER-β) and PR, confirming the plant's steroidogenic capabilities (Gowala *et al.*, 2020). The present in vivo study on female reproductive tissue showed the existence of estrogenic compounds (phytoestrogens) in the CRBE of *Cynoglossum zeylanicum*. Phytoestrogens can interact with ER-α and ER-β, acting as agonists or antagonists depending on whether 17β-estradiol is present or not. (Shanle and Xu, 2011). The selective estrogen receptor modulators (SERMs) exhibit tissue-specific estrogen agonist or antagonist activity. Selected estrogen receptor modulators (SERMs) are phytoestrogens that have a higher affinity for ER-α than ER-β (Lorand *et al.*, 2010). This finding suggests that the CRBE may be able to modulate stromal cell alterations to decidual cells during early gestation in mice. The crude root bark extract of *Cynoglossum zeylanicum* specially induced the stromal cells of endometrium and thecal cells of ovary to express the ER-α. The ovarian follicular growth and maturation could be influenced by ER-α expression in thecal cells. It has been documented that about 15% of all breast cancers

lack progesterone receptors (PR) and estrogen receptors (ER). Since they lack ER and PR receptors, these cells are resistant to cytotoxic chemotherapy and targeted therapies (Anestis *et al*., 2019). Likewise, endometrial cancer those are negative for both ER and PR do not respond to endocrine therapy according to a meta-analysis of studies reporting ER/PR status (MacKay *et al*., 2020). According to the findings of this study, It has been observed that the extract (CRBE) of *Cynoglossum zeylanicum* stimulate the expression of estrogen receptors in stromal and thecal cells of uterus and ovary respectively. This discovery could open up new avenues in pharmaceutics for targeted endocrine therapy. These findings provide strong evidence and the first recorded report of *Cynoglossum zeylanicum* extract having estrogenic properties that target stromal cells. This discovery elucidates the role of the plant's estrogenic properties in female reproduction regulation.

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