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Original Article

Phytochemical analysis and antifertility potential of *Cynodon dactylon* in female Wistar rats: A herbal approach towards contraception

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ABSTRACT

Objective: The objective of the present study was to evaluate the antifertility activity of ether (ErCD), chloroform (CeCD) and ethyl alcohol (EyCD) extracts of the whole plant of *Cynodon dactylon* in female Wistar albino rats.

Methods: Acute oral toxicity and an antifertility study were performed in female Wistar rats with two dose levels (200 and 400 mg/kg, orally) of EyCD. The estrogenic and progestogenic effects of EyCD were further observed by administering it to immature Wistar rats by investigations of vaginal cornification, hormonal level, uterus weight, biochemical parameters, histopathology of the uterus and deciduoma formation, respectively. Isolation of EyCD was carried out by Flash Chromatography and isolated fraction was estimated by HPLC.

Results: No toxicity with any of the extract was found up to the dose of 2000 mg/kg body weight. EyCD treated rats exhibited maximum reduction in pregnancy (83.33%). Estimation of EyCD on vaginal cornification, estrogen-induced uterotrophic assay and deciduoma model demonstrated vaginal cornification, significant ($P < 0.01$) increase in uterine weight and uterine proliferation in histopathology and reduced deciduoma formation respectively. Hormonal and biochemical parameters confirmed the above findings indicating estrogenic potential and antiprogestogenic potential of EyCD that might be attributed to the presence of phytoestrogen (apigenin) in EyCD.

Conclusion: The results suggested that extracts of *C. dactylon* possess significant antifertility activity, which is consistent with the literature reported in folk medicine of this plant in fertility regulation.

1. Introduction

As the natural resources are limited, the development of country depends on population growth. Therefore, control of population growth is very important in developing countries like India. Oral contraceptives are the preferred method of contraception which act by inhibiting ovulation and inhibiting hypothalamus- pituitary mechanisms. In addition, these preparations increase the viscosity of cervical fluid rendering it hostile for sperm transport. There is stromal deciduation of the uterus towards the end of the cycle rendering changes in the motility and secretions of the fallopian tubes which affects fertilization. However, the associated adverse effects and high failure rate of available anti-fertility drugs limit their use (Back et al., 1988). Hence, there is an unmet need to identify and scientifically validate potent and effective herbs. Fertility regulation with plants or plant preparations and medicaments has been mentioned in the ancient texts of indigenous systems of medicine of many countries (Bhaduri, Ghose, Bose, Moza, & Basu, 1968; Desta, 1994; Kumar et al., 2017). In different parts of the world, herbal substances have been used in the abortion for unwanted pregnancy conditions (Gul, Rubab, Ahmad, & Iqbal, 2015).

Medicinal plants in India have been used since ancient time and screened for anti-implantation, abortifacient, contraceptive potential and hence anti-fertility effects. Herbal drugs are used since ancient times for fertility control and hence, needs pharmacological evaluation. Considering these herbal drugs are easily available and safe, the present study was performed. There is much evidence that shows some medicinal plants exhibit antifertility potential due to presence of numerous phytoconstituents including flavonoids, terpenoids, alkaloids and steroidal saponins that might be responsible for abrupt pregnancy by inhibiting implantation or inducing abortion (Sharma, Rani, Malhotra, Deswal, & Singh, 2015). The substances such as coumarin, flavonoid and sitosterol are proved to disrupt pregnancy that is of interest in human fertility control (Namulindwa, David, & Oloro, 2015).

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In recent years, many researchers' reports have substantiated the folkloric claims of abortifacient plants used by the people (Basak, Banerjee, & Manna, 2016). Abortifacient agents obtained from indigenous medicinal plants would be of immense benefit, especially to inhabitants of developing countries, since, the cost of these drugs would likely be within their reach as well as they might exhibit safety over risk factors compared to synthetic drugs (Gul et al., 2015).

Cynodon dactylon (L.) Pers. (Poaceae), commonly known as "durba" (Hindi), Bermuda grass (English), Karki (Kannada) is called perennial grass in India, known to be a tackler in Indian mythology and is offered to Lord Ganesha. It was found in warm climates all over the world between 450 south and north altitude. It was also found everywhere even on wasteland, roadside, dry places, and spreads vigorously on cultivated ground. *C. dactylon* is used as a folk remedy for cough and bronchitis, dropsy, hemorrhage, calculus, urogenital disorders, miscarriage, headache, convulsions, sores, cancer, cramps, cystitis, dysentery, epilepsy, leucoderma, hypertension, infections such as measles, rubella, fevers, and menorrhagia (Sen, Chakraborty, De, & Devanna, 2011; Suresh, Deepa, Harisaranraj, & Vaira, 2008). It is reported as abortifacient in ancient literature (Vaidyaratnam, 1995). *C. dactylon* is an important ingredient in various Ayurvedic preparations (Bagewadi, Siddanagouda, & Baligar, 2014). Despite these traditional claims, no in-depth scientific study has been performed regarding its antifertility effect especially in entire plant on female reproduction.

Present studies were designed to explore the activity of three different extracts of the whole plant of *C. dactylon* on fertility in female Wistar albino rats.

2. Materials and methods

2.1. Phytochemical studies

2.1.1. Plant collection and authentication of plant

Whole plant of *C. dactylon* were collected from local areas of Gulbarga, Karnataka and authenticated by Head, Department of Botany, Govt. Degree College, Gulbarga where voucher specimens are preserved. The voucher specimen number is HGCG NO-600.

2.1.2. Preparation of extracts

The whole plant *C. dactylon* was shade dried and pulverized to particle size (# 40). The coarsely powdered plant of *C. dactylon* (200 g) was successively extracted with the solvents of increasing polarity, viz.- petroleum ether (ErCD), chloroform (CeCD) and ethyl alcohol (EyCD) by a hot percolation method using Soxhlet apparatus at 40 °C for 48 h. The obtained extracts were concentrated under vacuum using Rota flash evaporator and dried. The percentage yield was calculated and reported (Mukherjee, 2002).

2.1.3. Thin layer chromatography identification of bioactive components

One gram of each extract (ether, chloroform and ethyl alcohol) was dissolved in respective solvent and a few drops of distilled water were added for complete solubility, then each extract was subjected to different phytochemical tests (Khandelwal, 2008). All the three extracts (ErCD, CeCD and EyCD) were analyzed qualitatively by thin layer chromatography (TLC) to detect the bioactive components (steroid, alkaloid and flavonoid respectively). The Silica gel GS $_{254}$ percolated aluminum plates were used as TLC plates. The solvent system used for steroid was petroleum ether and acetone at a ratio of 7:3. The spots obtained were detected using anisaldehyde-sulfuric acid spraying reagent. The solvent system used for alkaloid was toluene, ethyl acetate and diethylamine at a ratio of 7:2:1. The spots obtained were detected using Wagner's spraying reagent. The solvent system used for flavonoid was ethyl acetate, formic acid, glacial acetic acid and water at a ratio of 10:1.1:1.1:2.6. The fluorescent spots obtained were detected using UV at 365 nm.

2.2. Pharmacological studies

2.2.1. Experimental animals

Wistar rats (180–220 g) of either sex, Swiss albino mice (20–25 g) and immature female Wistar rats (50–60 g) were obtained from Sainath Agencies, Hyderabad, Telangana, India (282/PO/Bt/S/2000/CPCSEA). They were maintained in HKES MTRIPS animal house at a temperature of (25 ± 1) °C and relative humidity of 45% to 55% under 12 h light: 12 h dark cycle. The animals had free access to food pellets and water was available *ad libitum*. Prior permission of IAEC was obtained for the conduction of experiments (IAEC approval No.- HKES/ MTRIPS/IAEC/91/2017–19).

2.2.2. Acute oral toxicity study

Acute oral toxicity studies were conducted as per OECD (Organization for Economic Cooperation and Development) guideline 425 (Ministry of Social Justice and Empowerment, 2008). Healthy adult Swiss mice of either sex weighing between 20 and 25 g were used for the study. The food, but not water, was withheld for 4 h before the extract was administered orally. All the extracts were given in progressive dose manner initiating from dose of 175 mg/kg., p.o. When no abnormality or death was observed, the next doses of 550, 1750 and 2000 mg/kg were chosen. At the dose of 2000 mg/kg, additional four mice were dosed. All the animals were observed for initially 30 m then 24 h for behavioral, neurological and autonomic profiles and for any lethality or death over the next 48 h.

2.2.3. Preparation of plant extracts

Dose of the different extracts (ErCD, CeCD and EyCD) obtained previously from whole plant of *C. dactylon* were selected based on acute toxicity studies on Swiss mice and administered at 200 and 400 mg/kg doses for all extracts in the present study. Each dried plant extracts were freshly suspended in gum acacia 2% just before oral administration to the animals during the experimental period.

2.2.4. Evaluation of ErCD, CeCD and EyCD on fertility outcome

Female Wistar albino rats (180–220 g) with regular oestrous cycles were allowed to mate with mature male rats (250–300 g) of proven fertility to induce pregnancy. The rats, in the ratio of 2:1, were left undisturbed overnight starting from the evening of proestrus and examined next morning for the evidence of copulation. Vaginal smear of each female rat was taken daily between 9:00 a.m. to 10:00 a.m. The rats exhibiting the copulation plug or thick clumps of spermatozoa in their vaginal smears were separated and that day was designated as day one of pregnancy. These rats were divided into seven groups each containing six animals. Group I (control) animals received vehicle (Gum acacia, 2%). Group II and III received ErCD, group IV and V received CeCD, group VI and VII received EyCD at the dose levels of 200 and 400 mg/kg respectively. All the treatments were given orally for seven days to pregnant rats. On 10th day of pregnancy, the animals were laparotomized under anesthesia. The number of implants present in both the uterine horns were determined. The abdominal wounds were sutured layer by layer using surgical suture (Orion Sutures India PVT ltd), and the animals were allowed to go term. After delivery, the numbers of pups born were noted. The pups born were observed for one month for any evidence of gross teratogenicity (Sharma et al., 2015). The parameters considered were the reduction in pregnancies and pregnancy index. The reduction in pregnancies was calculated as per the formula:

$$100 - \left(\frac{No.\ of\ rats\ showing\ implantation\ on\ day\ 10}{No.\ of\ rats\ showing\ presence\ of\ spermatozoa}\right) \times 100$$

Blood serum was further processed for the estimation of biochemical parameters such as LH and FSH.

Blood was centrifuged to separate the serum. The serum LH and FSH were analyzed by Clia (Chemiluminescence immunoassay): immulite, fully automated immunoassay analyzer (Mini vidas), Biomerieux, USA (Sharma et al., 2015).

2.2.5. Evaluation of EyCD for estrogenic activity: Vaginal cornification and uterotropic assay in immature ovariectomized female rats

Bilateral ovariectomy was done in immature female Wistar rats (55–65 g) under 5% Xylazine (10 mg/kg, i.p.) and Ketamine hydrochloride (80 mg/kg, i.p.) under sterile conditions. After one week, the animals were divided into six groups containing six animals each. Group I (control) was administered with vehicle i.e. gum acacia (2%). Group II was administered β-estradiol (1 μg/rat/day) suspended in olive oil, subcutaneously, which served as positive control. Animals of group III and IV received EyCD at the doses of 200 and 400 mg/kg p.o., respectively. All the doses and vehicle were administered orally for seven consecutive days. On the 8th day, the final body weight, vaginal opening and cornification of all the animals were observed. On the last day of treatment, the blood was withdrawn from *retro*-orbital plexus. Then, the animals were sacrificed with high dose of anesthesia and the uteri were dissected out, surrounding tissues were removed, then blotted on filter paper and weighed quickly on electronic balance. Blood serum was further processed for the estimation of biochemical parameters such as estrogen level, alkaline phosphatase and total cholesterol. The uterine horns from the control and treated animals were stored for 24 h in 10% formalin and histological examination was carried out (Sharma et al., 2015).

2.2.5.1. Histological assessment of uterus. The uterine horn was stored in 10% formalin and two bits from each cornua of the uterus were taken and subjected to fixation (10%formalin). The specimen was dehydrated by placing it three times in xylene (1 h each) and later in alcohol 70%, 90% and 100% strength, respectively, each for 2 h. The infiltration and impregnation were carried out by treating with paraffin wax twice, each time for 1 h. Blocks were made using "L" block field with molten wax. Sections were cut, 3–5 μm thick with microtome and slides were made by mounting the sections on glass slides coated with egg albumin/glycerin. The sections were stained with hematoxylin and eosin stain and subjected to microscopic examination (Aswar, Bodhankar, Mohan, & Thakurdesai, 2010).

2.2.5.2. Serum estrogen estimation. Blood was centrifuged to separate the serum. The serum estrogen was analyzed by Clia (Chemiluminescence immunoassay): immulite, fully automated immunoassay analyzer (Mini vidas), Biomerieux, USA (Aswar et al., 2010).

2.2.5.3. Serum alkaline phosphatase and cholesterol estimation. The serum alkaline phosphatase was measured by an Autoanalyzer Hitachi, model No-912 using kits (Roche, USA) [by pnpp (*P*-Nitrophenylphosphate)-kinetic method]. The serum cholesterol was measured by NX-500 Faji Dry chemistry fully automatic analyser (Sharma et al., 2015).

2.2.6. Evaluation of EyCD for progestogenic activity: histamine-induced deciduoma formation model

Adult female Wistar rats weighing 60 to 70 g were ovariectomized. They were allowed to recover for two days. Animals were divided into six groups containing six animals each. Group I-vehicle (distilled water, 10 mL/kg), Group II-EyCD (200 mg/kg p.o.) and Group III-EyCD (400 mg/kg p.o.), Group IV-hydroxyprogesterone caproate (0.04 mg/animal, s.c.). The treatment period was for 14 d. All these groups treated with estradiol (0.5 μg/ animal, once daily s.c.) for 4 days, followed by 9 days of vehicle or EyCD (200 and 400 mg/kg p.o.) or hydroxyprogesterone (0.04 mg/animal s.c.) administration .On 5th day of progesterone or vehicle or EyCD treatment uterine horns were exposed and 1.0 mg histamine dihydrochloride which was injected into lumen of one horn while in other horn (control), vehicle (distilled water) was injected. The animals were sacrificed on day 14th after the last treatment. Both uterine horns were removed and weighed. The degree of deciduoma formation was evaluated by the percent increase in the weight of the histamine-injected uterine horn as compared with the control horn. Prior to animal sacrifice, blood of each animal was withdrawn by retro orbital puncture (ROP) for estimation of progesterone. Histopathology of the ducidual uterine horn of all the animals was done to observe any secretory changes in horn and was compared with control and standard (Zanwar, Aswar, Hegde, & Bodhankar, 2010).

Blood was centrifuged to separate the serum. The serum progesterone was analyzed by Clia (Chemiluminescence immunoassay): immulite, fully automated immunoassay analyzer (Mini vidas), Biomerieux, USA (Zanwar et al., 2010).

2.2.7. Statistical analysis

Data was expressed as Mean ± S.E.M. and statistical analysis was carried out by One-way ANOVA followed by Dunnett's test using Graph Pad Prism version 5.00 for Windows Vista TM BASIC, Graph Pad Software, San Diego California USA. P value < 0.05 was considered as significant.

2.3. Preparation and standardization of apigenin

The Column, 12 g Normal Phase Column (High Resolution) and mobile phase, Dichloromethane (DCM): Methanol (Gradient) was used.

EyCD (200 mg) was dissolved in DCM and adsorbed in 2 g of silica, dried and loaded on the column, and eluted in a gradient manner with dichloromethane (DCM) and methanol mixture, by flash chromatography and the fractions were collected. The eluant was characterized by comparing with various standard flavones such as apigenin, luteolin using High Performance Liquid Chromatography (HPLC). Among the standards, the peak of the eluant matched with peak of apigenin.

HPLC Details: Jasco HPLC system Series 2000 comprising of PU2080 pump, Rheodyne injector with 20 μL loop, UV 2075 plus UV/Vis detector, Borwin software version 1.5 was used.

Apiagenin was estimated from ethanolic extract (EyCD) and fractions by RP- HPLC using Kromasil 100-5C_{18}column (250 × 4.6 mm, 5 μm). Mobile phase comprised of 25 mmol/L sodium acetate pH at 3 adjusted by using glacial acetic acid and acetonitrile (60:40, volumeratio) run at flow rate of 0.6 mL/min. Detector: UV (352 nm) (Shanmuga, Vazhayil, Varghese, & Nanjaian, 2017).

3. Results

3.1. Preliminary phytochemical screening

The percentage yield of ErCD, CeCD, EyCD extracts were 2.5%, 2.7% and 8.3%, respectively. Preliminary phytochemical assessment showed that ErCD contained only steroids, while CeCD and EyCD contained flavonoids, carbohydrates, alkaloids and glycosides (Table 1). Two steroidal compounds were detected by TLC of ErCD at R_f values of 0.6 and 0.46. Alkaloidal compounds of CeCD and

Table 1
Phytochemical constituents present in various extracts of *C. dactylon.*

Phytoconstituent types	Test carried out	ErCD	CeCD	EyCD
Carbohydrates	Fehling's test	–	+	+
Flavonoids	Lead acetate test	–	–	+
Steroids	Salkowski test	+	–	–
Tannins	Ferric chloride test	–	–	–
Alkaloids	Wagner's test	–	+	+
Proteins	Biuret test	–	–	–
Glycosides	General test	–	+	+

EyCD were detected by TLC at R_f value of 0.7 and 0.8, respectively and a flavonoidal compound of EyCD was detected by TLC at R_f value of 0.8.

3.2. Acute oral toxicity

In acute oral toxicity studies, no changes in the behavior and autonomic profiles as well no mortality were observed in all the mice treated up to the dose of 2000 mg/kg in all the extracts of *C. dactylon.* The extracts ErCD, CeCD and EyCD were safe up to a dose level of 2000 mg/kg of body weight.

3.3. Effect on fertility outcome

On the 10th day of pregnancy, laparotomization was carried out in female rats and anti-implantation effect was expressed as percentage of animals showing absence of implantation in uteri. A dose-dependent anti-implantation effect was observed in treated pregnant rats as shown in (Table 2). The extracts significantly reduced the pregnancy index, the mean number of implants and the number of litters born ($P < 0.0001$) as compared to control group rats. The reduction in pregnancy was found to be 0%, 16.67%, 66.7% and 83.33% in control, ErCD, CeCD and EyCD ($P < 0.0001$) respectively (Table 2). No mortality of pregnant rats was observed during the study period.

The effect of ErCD, CeCD and EyCD on biochemical parameters has been presented in (Table 3). The EyCD at both doses showed a significant ($P < 0.0001$) decrease in serum LH and FSH level compared to control.

3.4. Effect on vaginal cornification, uterotrophic, estrogenic activity and other biochemical parameters

EyCD (200 and 400 mg/kg) treatment in immature rats exhibited vaginal opening and cornification. The number of cornified cells in vaginal smear was considerably less than the group treated with ethinyl estradiol (+++) but notably higher (from + to ++) than the control group. Oral administration of EyCD at doses of 200 and 400 mg/kg body weight caused a significant ($P < 0.01$) increase in body weight as well as uterine weight of ovariectomized rats when compared to control group. The high dose of EyCD (400 mg/kg) caused a significant increase in body weight compared to the standard. Similar changes were observed on morphometrical indices of uterus. EyCD at both the doses produced significant ($P < 0.01$) increase in the diameter of the uterus, diameter of endometrium, its thickness and height of endometrium of ovariectomized rats compared to control. EyCD at the dose of 400 mg/kg body weight produced significant increase in thickness of endometrium when compared with the group treated with ethinyl estradiol ($P < 0.001$), which showed dose-dependent estrogenic effect of the extract (Table 4).

3.4.1. Effect on other biochemical parameters

The effect of extract on biochemical parameters has been presented in (Table 5). The EyCD showed marked ($P < 0.001$) estrogenic potential as observed by significant rise in serum estrogen level in immature ovariectomized rats as compared to control in both the doses. Further, the extract showed nonsignificant decrease in the serum cholesterol compared to control. Estrogenic activity of the extract was further confirmed by significant ($P < 0.001$) decrease in level of serum alkaline phosphate at the dose of 400 mg/kg.

3.4.2. Effect on histopathology of uterus

The histological analysis of the uteri was summarized in Fig. 1. Uterus of the control group was small and inactive and showed a slit-like lumen. Also, compaction of stromal cells was seen. Some glands with small diameter were observed in the stroma. Perimetrium layer and circular and longitudinal muscles layers of the myometrium were thin. Treatment with estradiol showed marked proliferation showing an increase in number of endometrial glands, the uterus lumen had multiple folds, increased endometrial and myometrial thickness, the cells were normal and no inflammation was observed. Both the doses of EyCD showed sparse proliferation of glands, multiple folds of lumen, endometrial and myometrial hyperplasia, normal cellular arrangement and no inflammation.

Table 2
Antifertility activity of extracts of *C. dactylon* in female Wistar rats (mean ± SEM, $n = 6$).

Doses/ ($mg \cdot kg^{-1}$p. o.)	No. of rats showing presence of spermatozoa	No. of rats showing implantation on day 10	Mean No. of implants on day 10	Mean No. of pups delivered	Reduction in pregnancies /%	Pregnancy index	No. of death from day 1 to 22 of pregnancy
Control (Gum acacia)	6	6	9.00 ± 0.68	9.16 ± 0.70	0	100.0 ± 0.0	0
ErCD (200 $mg \cdot kg^{-1}$)	6	5	3.50 ± 1.47**	2.33 ± 0.98***	16.66	40.43 ± 13.45***	0
ErCD (400 $mg \cdot kg^{-1}$)	6	5	3.16 ± 1.51**	1.50 ± 0.76***	16.66	29.67 ± 8.47***	0
CeCD (200 $mg \cdot kg^{-1}$)	6	2	2.83 ± 1.90**	0.50 ± 0.34***	66.7	1.81 ± 1.81***	0
CeCD (400 $mg \cdot kg^{-1}$)	6	2	0.50 ± 0.34***	0.16 ± 0.16***	66.7	0.0 ± 0.0***	0
EyCD (200 $mg \cdot kg^{-1}$)	6	1	0.66 ± 0.66***	0.0 ± 0.0***	83.34	0.0 ± 0.0***	0
EyCD (400 $mg \cdot kg^{-1}$)	6	1	0.50 ± 0.50***	0.0 ± 0.0***	83.34	0.0 ± 0.0***	0

Note: **$P < 0.001$, ***$P < 0.0001$ *vs* control group.

Table 3
Effect of EyCD on biochemical parameters (mean ± SEM, n = 6).

Groups Parameters	Control	ErCD (200 mg·kg^{-1})	ErCD (400 mg·kg^{-1})	CeCD (200 mg·kg^{-1})	CeCD (400 mg·kg^{-1})	EyCD (200 mg·kg^{-1})	EyCD (400 mg·kg^{-1})
LH /(ng·mL^{-1})	36.88 ± 0.64	35.00 ± 0.81	33.83 ± 0.94	33.33 ± 0.61	32.17 ± 0.70*	29.97 ± 1.07***	26.52 ± 1.92***
FSH /(ng·mL^{-1})	125.5 ± 0.54	123 ± 0.73	122.8 ± 0.87	121.3 ± 0.80	120.2 ± 0.60*	115.7 ± 2.08***	111.1 ± 2.21***

Note: *P < 0.01, ***P < 0.0001 *vs* control group.

Table 4
Effect of EyCD on female rat body weight gain, uterine weight, morphometrical indices of uterus and biochemical parameters (mean ± SEM, n = 6).

Parameters	Control	β-estradiol (1 μg)	EyCD (200 mg·kg^{-1})	EyCD (400 mg·kg^{-1})
Body weight gain /g	15 ± 1.58	27 ± 3.39*	24 ± 3.31*	32 ± 2.55#
Uterine weight/mg	71.50 ± 17.29	197.5 ± 19.31*	187.5 ± 34.73*	190.0 ± 27.39*
Vaginal cornification	Nil	+++	+	++
Morphometrical indices of uterus				
Diameter of uterus/mm	1.40 ± 0.24	2.54 ± 0.14*	2.36 ± 0.31*	2.38 ± 0.27*
Diameter of endometrium /mm	1.06 ± 0.22	2.18 ± 0.15*	2.0 ± 0.28*	2.12 ± 0.28*
Thickness of endometrium /mm	0.30 ± 0.06	0.66 ± 0.08*	0.64 ± 0.10*	0.80 ± 0.09* #
Height of endometrium /mm	23.20 ± 1.27	31.00 ± 2.51*	30.80 ± 1.79*	30.10 ± 1.45*
Biochemical Parameters				
Serum estradiol/ (pg·mL^{-1})	54.38 ± 1.99	154.6 ± 7.85***	93.10 ± 1.91**	150.3 ± 9.10***
Cholesterol/ (mg·dL^{-1})	78.80 ± 2.81	86.43 ± 2.10	75.46 ± 2.29	68.50 ± 4.66
Alkaline phosphatase/ (U·L^{-1})	178.2 ± 8.04	123.6 ± 11.32**	158.0 ± 10.14	132.7 ± 7.41**

Note: *P < 0.01; **P < 0.001, ***P < 0.0001 *vs* control group. # Significant in relation to standard: P < 0.01.

Table 5
Effect of EyCD on female rat decidual weight and biochemical parameters (mean ± SEM, n = 6).

Parameters	Control	Hydroxyprogesterone caproate (0.04 mg/animal, s.c.)	EyCD (200 mg·kg^{-1})	EyCD (400 mg·kg^{-1})
Decidual weight/mg	107.4 ± 8.24	140.8 ± 3.61*	94.95 ± 6.38	73.18 ± 9.98*
Biochemical Parameters				
Serum progesterone /(ng·mL^{-1})	11.98 ± 1.99	39.22 ± 0.95***	2.13 ± 0.42***	2.47 ± 0.70***

Note: *P < 0.01, ***P < 0.0001 *vs* control group.

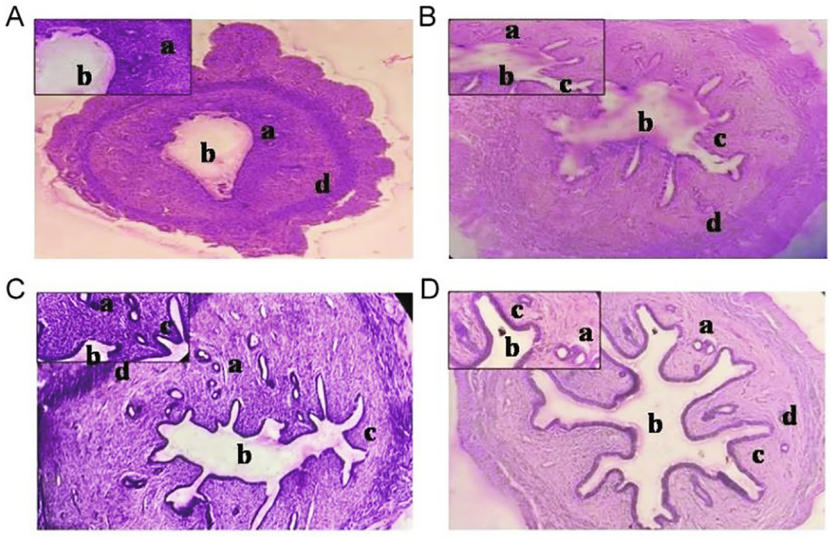

Fig. 1. Histo-architecture of uterus of estrogen (1 μg/rat/day) and EyCD-treated (200 and 400 mg/kg) female rats (H&E, 5-Am sections) in control group (A), β-estradiol group (B), EyCD (200 mg/kg) (C) and EyCD (400 mg/kg) group (D). There is an increased proliferation of endometrial glands, folds of lumen and hyperplasia of endometrium and myometrium (400 ×). (a. endometrial glands; b. lumen of the uterus; c. folds of lumen; d. myometrium thickness.)

3.5. Effect on deciduoma formation and serum progesterone

Oral administration of EyCD at doses of 200 and 400 mg/kg body weight caused a significant (P < 0.01) decrease in uterine weight of ovariectomized rats when compared to control. Only hydroxyprogesterone caproate (0.04 mg/animal, s.c) caused a significant (P < 0.01) increase in weight of uterus as well as progesterone level compared to control. The EyCD had no effect on uterine weight as well as progesterone level (Table 5). The histological analysis of the uteri was summarized in Fig. 2. Uterus of

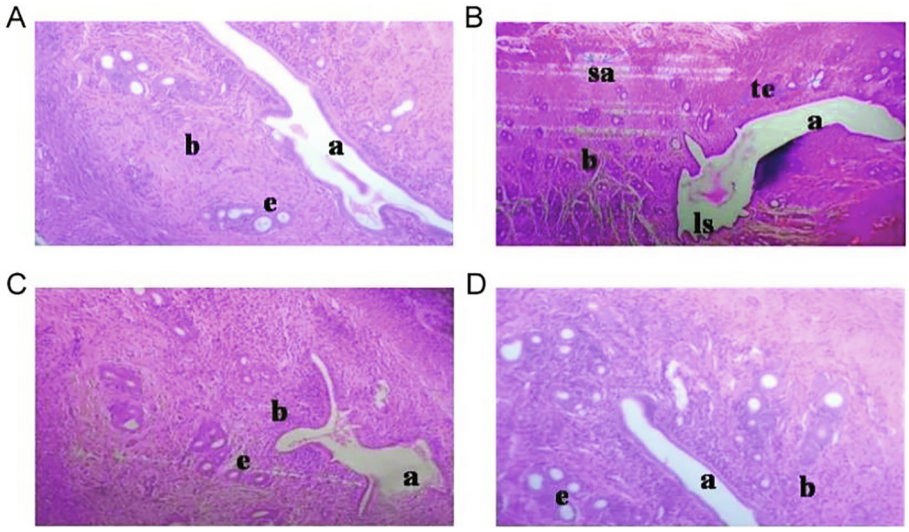

Fig. 2. Histo-architecture of the uterus of progesterone (0.04 mg/animal) treated rats in control group (A), progesterone (0.04 mg) group (B), EyCD (400 mg/kg) (C) and EyCD (200 mg/kg) group (D). It shows an increased no. of tortuous endometrial glands, luminal secretion and spinal arterioles. EyCD (200 and 400 mg/kg) treated female rat shows lumen without secretion, proliferative endometrim, round endometrial glands (400 ×). (a. lumen; b. endometrium; e. round endomerial glands; te. tortuous endometrial glands; ls. luminal secretion, sa. spinal arterioles.)

the control group was small and inactive and showed a slit-like lumen. Also, compaction of stromal cells was seen. Some round endometrial glands with small diameter were observed in the stroma. Treatment with progesterone showed marked secretory changes such as luminal secretion, edematous or decidual stroma, many tortuous endometrial glands, vacuolation in cells showing spinal arterioles. Both the doses of EyCD showed compact stroma, lumen without secretion, round endometrial cells and without spinal arterioles. The histology confirms the nonprogestogenic activity of EyCD.

3.6. HPLC results

The high-performance liquid chromatogram of EyCD (Fig. 3) and fraction (Fig. 4) shows the presence of apigenin (Fig. 5) as

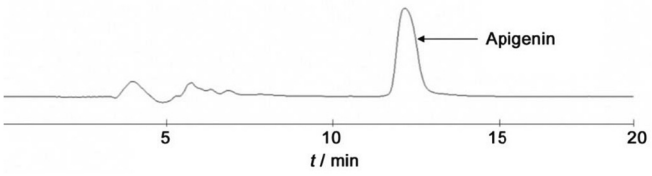

Fig. 3. RP-HPLC/UV chromatogram of EyCD (1000 μg/mL) (Retention time: 12.208 min).

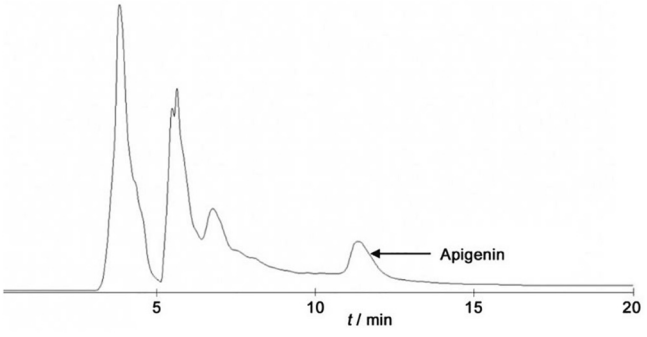

Fig. 4. RP-HPLC/UV chromatogram of fraction (1000 μg/mL) (Retention time: 12.342 min).

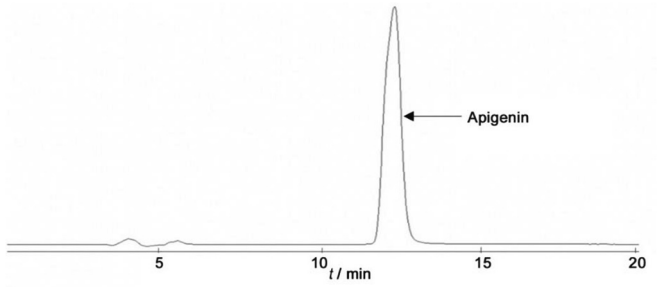

Fig. 5. RP-HPLC/UV chromatogram of 50 μg/mL apigenin (Retention time: 12.225 m).

0.3% and as 0.06% respectively as the peak eluted by extract and fraction at retention time of standard apigenin.

4. Discussion

The present findings showed that the low and high doses of extracts ErCD, CeCD and EyCD (200 and 400 mg/kg) have shown 16.67%, 66.7% and 83.34% reduction in the pregnancies respectively when compared with the control group. All the extracts have shown a marked reduction in a number of implants as well as a number of litters born. Several plants have been studied and are found to avert implantation or causes detachment of the implanted embryo; They act as abortifacients or contraceptives. Some plants show disruption of the estrous cycle and inhibition of ovulation, thereby exhibiting a contraceptive effect (De Boer & Cotingting, 2014). Therefore, an attempt was made to study the effect of most potent extract, EyCD on estrogenic as well as progesterone activity. The results indicated that the extract-treated rats had cornified cells in vaginal smear pointing to the disruption of estrous cycle, with an increase in time span of rats to remain in estrous phase, confirming its contraceptive potential. This activity might be attributed to estrogenic potential of extract due to presence of various phytoestrogens. The estrogenic effect was further confirmed by estimating serum estrogn levels and morphometric study of endometrium. The morphometrical indices of uterus indicated a significant increase in the diameter of the uterus, increase in diam-

eter and thickness of endometrium as well as the height of endometrium in ovariectomized rats, at both the doses (200 and 400 mg/kg) compared to control group. Estrogen causes a rise in uterine weight, fluid retention thereby causing, ballooning of the uterus, vaginal opening, and cornification, thereby leading to non-receptive condition and altering the hormone level in the uterus (Burton & Wells, 2002). Phytoestrogens are also reported to relieve post-menopausal symptoms, reduction of osteoporosis, improvement in blood cholesterol levels and lowering the risk of certain hormone-related cancers, which was seen with Genistein (Arcoraci et al., 2017; Suthar, Banavalikar, & Biyani, 2001). Our study supports the above findings with reference to cholesterol and alkaline phosphatase. In the present study, significant antifertility activity, marked vaginal cornification, increase in uterine and body weight of bilaterally ovariectomized immature rats suggests phytoestrogenic activity of the EyCD. The extract (EyCD) showed presence of flavonoids that might be acting as phytoestrogen and is responsible for estrogenic and antifertility activity. Earlier researchers have also reported antifertility activity for flavonoids (Hiremath, Badami, Hunasagatta, & Patil, 2000; Moersch, Morrow, & Neuklis, 1967; Shah, Jhade, & Chouksey, 2016). *C. dactylon* is reported to contain flavones such as apigenin, luteolin, flavone glycosides-orientin (8-C-β-*D*-glycosyl luteolin), vitexin (8-C-β-*D*-glycosyl apigenin), isoorientin (6-C-β-*D*-glycosyl luteolin) and isovitexin which have been reported to exhibit phytoestrogenic nature (Kalita & Milligan, 2010). The present study shows the presence of apigenin in EyCD and fraction as 0.3% and 0.06% respectively as confirmed by HPLC.

In corollary to this hypothesis, the putative rise in serum estrogen by EyCD might be due to phytoestrogens that are present in EyCD getting converted into hormone estrogen in the body. The hormonal data of FSH and LH which was reduced in the EyCD group indicated that estrogen might inhibit the release of these hormones from adenohyposis by negative feedback mechanism. The diminished FSH is an indication of disturbance of estrus cycle and ovulation. For maintenance of pregnancy and corpora luteum, LH was required. The significant attenuation of serum LH concentration could be associated with the physiological process of luteolysis preceding parturition. The deciduoma model indicated that EyCD decreased the uterus weight. As against, progestogenic secretary changes, we observed no secretions and decidual stroma in EyCD group. There was significant reduction in progesterone level as compared to vehicle-treated animals. The reduced progesterone level might be attributed to low LH concentration. Hence, the antifertility activity observed in the present study is mainly due to its estrogenic potential and inhibition of LH, FSH and progesterone which could be due to presence of apigenin in the extract.

As mentioned earlier CD is also reported to be used as primarily for antidiabetic (Singh, Kesari, Gupta, Jaiswal, & Watal, 2007) as well as for other therapeutic effects (Rai, Jaiswal, Rai, Sharma, & Watal, 2010) at the doses (200 mg to 1 g/kg, p.o) which has shown antifertility activity in the present study. Hence, care must be taken while consuming the herb or its preparation by pregnant women.

5. Conclusion

From the present study, it can be concluded that all the extracts of *C. dactylon* have shown dose-dependent inhibition of pregnancy and possess significant antifertility effects. EyCD exhibited potent antifertility activity that can be attributed to its estrogenic activity and inhibitory effect on LH, FSH and progesterone which may be because of presence of apigenin in the extract. In conclusion, this study provides novel evidence in support of continuing action of the traditional use of *C. dactylon* in gynecologic disorders.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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References

Arcoraci, V., Atteritano, M., Squadrito, F., Anna, R. D., Marini, H., Santoro, D., et al. (2017). Antiosteoporotic activity of genistein aglycone in postmenopausal women: Evidence from a post-hoc analysis of a multicenter randomized controlled trial. *Nutrients, 9*(2), 179.

Aswar, U. M., Bodhankar, S. L., Mohan, V., & Thakurdesai, P. A. (2010). Effect of furostanol glycosides from Trigonella foenum-graecum on the reproductive system of male albino rats. *Phytotherapy Research, 24*(10), 1482–1488.

Back, D. J., Grimmer, S. F. M., Orme, M. L. E., Proudlove, C., Mann, R. D., & Breckenridge, A. M. (1988). Evaluation of Committee on Safety of Medicines yellow card reports on oral contraceptive-drug interactions with anticonvulsants and antibiotics. *British Journal of Clinical Pharmacology, 25*(5), 527–532.

Bagewadi, Z. K., Siddanagouda, R. S., & Baligar, P. G. (2014). Hytoconstituents investigation by lc-ms and evaluation of anti-microbial and anti-pyretic properties of *Cynodon dactylon*. *International Journal of Pharmaceutical Sciences and Research, 5*(7), 2874–2889.

Basak, S., Banerjee, A., & Manna, C. K. (2016). Role of some ethno medicines used by the Santal tribal people, of the district Bankura, WB, India, for abortifacient purposes. *Journal of Medicinal Plants Studies, 4*(2), 125–129.

Bhaduri, B., Ghose, C. R., Bose, A. W., Moza, B. K., & Basu, U. P. (1968). Antifertility activity of some medicinal plants. *Indian Journal of Experimental Biology, 6*, 252–253.

Burton, J., & Wells, M. (2002). The effect of phytoestrogens on the female genital tract. *Journal of Clinical Pathology, 55*(6), 401–407.

Committee for the purpose of control and supervision of Experimental Animals (CPCSEA), OECD Guidelines for the testing of Chemicals, revised draft guidelines 425(#26), Acute oral toxicity-Acute toxic class method, revised document, India: Ministry of Social Justice and Empowerment; 2008.

De Boer, H. J., & Cotingting, C. (2014). Medicinal plants for women's healthcare in southeast Asia: A meta-analysis of their traditional use, chemical constituents, and pharmacology. *Journal of Ethnopharmacology, 151*(2), 747–767.

Desta, B. (1994). Ethiopian traditional herbal drugs. Part III: Anti-fertility activity of 70 medicinal plants. *Journal of Ethnopharmacology, 44*(3), 199–209.

Gul, S., Rubab, B., Ahmad, N., & Iqbal, U. (2015). Herbal drugs for abortion may prove as better option in terms of safety, cost & privacy. *Journal of Scientific and Innovative Research, 4*(2), 105–108.

Hiremath, S. P., Badami, S., Hunasagatta, S. K., & Patil, S. B. (2000). Antifertility and hormonal properties of flavones of Striga orobanchioides. *European Journal of Pharmacology, 391*(1–2), 193–197.

Kalita, J. C., & Milligan, S. R. (2010). *In vitro* estrogenic potency of phytoestrogen-glycosides and some plant flavanoids. *Indian Journal of Science and Technology, 3*(12), 1142–1147.

Khandelwal, K. R. (2008). *Practical pharmacognosy techniques and experiments* (15th Ed., pp. 149–156). Nirali Prakashan, Pune: Pragati Books Pvt. Ltd.

Kumar, S., Dagar, S., Kumar, P., Singh, J., Kumar, S., & Kumar, D. (2017). Antifertility effect of hydroalcoholic extract of *Pandanus odoratissimus* L. leaves. *Porto Biomedical Journal, 2*(5), 167–169.

Moersch, G. W., Morrow, D. F., & Neuklis, W. A. (1967). The antifertility activity of isoflavones related to genistein. *Journal of Medicinal Chemistry, 10*(2), 154–158.

Mukherjee, P. K. (2002). *Quality control of herbal drugs* (1st Ed., pp. 399–407). Business Horizons: Pharmaceutical Publishers, New Delhi.

Namulindwa, A., David, N., & Oloro, J. (2015). Determination of the abortifacient activity of the aqueous extract of *Phytolacca dodecandra* (LHer) leaf in Wistar rats. *African Journal of Pharmacy and Pharmacology, 9*(3), 43–47.

Rai, P. K., Jaiswal, D., Rai, D. K., Sharma, B., & Watal, G. (2010). Antioxidant potential of oral feeding of *Cynodon dactylon* extract on diabetes-induced oxidative stress. *Journal of Food Biochemistry, 34*(1), 78–92.

Sen, S., Chakraborty, R., De, B., & Devanna, N. (2011). An ethnobotanical survey of medicinal plants used by ethnic people in West and South district of Tripura, India. *Journal of Forestry Research, 22*(3), 417–426.

Shah, S. K., Jhade, D., & Chouksey, R. (2016). Antifertility activity of ethanolic and aqueous extracts of *Aloe vera* Mill on female wistar rats: Rising approaches of herbal contraception. *Journal of Pharmaceutical Sciences and Research, 8*(9), 952–957.

Shanmuga, S. R., Vazhayil, B. K., Varghese, L., & Nanjaian, M. (2017). Development and validation of RP-HPLC method for simultaneous determination of apigenin and luteolin in ethanol extract of *Clerodendrum serratum* (Linn.) leaves. *Asian Journal of Applied Sciences, 5*(1), 52–60.

Sharma, P., Rani, S., Malhotra, H., Deswal, S., & Singh, S. (2015). Antifertility potential of hydroalcoholic extract of *Cordia dichotoma G* Forst. leaves: A folklore medicine used by Meena community in Rajasthan state in India. *Asian Pacific Journal of Reproduction, 4*(2), 100–105.

Singh, S. K., Kesari, A. N., Gupta, R. K., Jaiswal, D., & Watal, G. (2007). Assessment of antidiabetic potential of *Cynodon dactylon* extract in streptozotocin diabetic rats. *Journal of Ethnopharmacology, 114*(2), 174–179.

Suresh, K., Deepa, P., Harisaranraj, R., & Vaira, A. V. (2008). Antimicrobial and phytochemical investigation of the leaves of *Carica papaya* L., *Cynodon dactylon* (L.) Pers., *Euphorbia hirta* L., *Melia azedarach* L. and *Psidium guajava* L. *Ethnobotanical Leaflets, 1*, 1184–1191.

Suthar, A. C., Banavalikar, M. M., & Biyani, M. K. (2001). Pharmacological activities of Genistein, an isoflavone from soy (Glycine max): Part II—Anti-cholesterol activity, effects on osteoporosis & menopausal symptoms. *Indian Journal of Experimental Biology, 39*(6), 520–525.

Vaidyaratnam, P. S. (1995) Orient Longman Series. *Indian medicinal plants: a compendium of 500 species, Vol. 2*, 289–292.

Zanwar, A. A., Aswar, U. M., Hegde, M. V., & Bodhankar, S. L. (2010). Estrogenic and embryo-fetotoxic effects of ethanol extract of *Linum usitatissimum* in rats. *Journal of Complementary and Integrative Medicine, 7*(1), 1–15.