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Evaluation of reversible contraceptive potential of *Cordia dichotoma* leaves extract

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Article

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Abstract: Considering the safety-risk ratio of steroidal contraceptives, the present work was carried out to evaluate ethno-contraceptive use of *Cordia dichotoma* G. Forst., Boraginaceae, leaves (LCD). Preliminary pharmacological screening was performed on post-coital female albino rats. The leaves extract (LD50 5.50 g/kg bw) showed 100% anti-implantation activity (n=10) at 800 mg/kg dose level. (2-hydroxypropyl)-β-cyclodextrin (BCD) was used as bioavailability enhancer to form LCD-BCD complex, characterized by DLS, SEM and XRD analyses. The LCD-BCD complex (1:1, w/w) exhibited 100% pregnancy interception (n=20) at the dose level of 250 mg/kg and also showed strong estrogenic potential with a luteal phase defect. Qualitative and quantitative phytochemical analyses were carried out. The LCD extract was standardized by a validated HPTLC method and two contraceptive phytoconstituents, apigenin and luteolin were isolated. A detailed pharmacological analyses followed by chronic toxicity study were performed to predict the reversible nature of the developed phytopharmaceutical. The histological and biochemical estimations detected the reversible contraceptive potential after withdrawal. The observations suggested that the developed phyto-pharmaceutical has potential antifertility activity with safety aspects.

Introduction

Several adverse effects of steroidal contraceptives are observed, especially metabolic disorders and sex-characteristics after menopause (Tanis et al., 2001; Unny et al., 2003). In response to increasing drug development costs due to candidate safety and efficacy, one of the most challenging pursuits in the realm of pharmaceutical and medical sciences is the search for newer and more potent drugs with least toxic profile, self-administrable, less expensive and completely reversible (FDA, 2004). Plant drugs are generally considered as safe and cheap medicines, and interest in the pharmacological role of bioactive compound(s) present in plant is increased in last decade (Ulrich-Merzenich et al., 2009). The various reviews reported on medicinal plants and their active principles for fertility regulation (Unny et al., 2003).

Systemic standardization of ethno-medicinal herbal formulation into modern phytopharmaceutical provided the chance of increased efficacy and safety profile of traditional medicine. Additionally for achieving target bioavailability a certain degree of lipophilicity is prerequisite for a drug candidate. Inclusion with a carrier increases the absorption rate of the active pharmaceutical ingredient, which in turn can lead to substantial increase in oral bioavailability (Kesisoglou et al., 2007). Application of (2-hydroxypropyl)-β-cyclodextrin (BCD) in oral drug delivery is presently incorporated in various pharmacopoeias and national formularies (Loftsson & Brewster, 1996; Challa et al., 2005). The effects of BCD on p-glycoprotein and cytochrome P450 facilitated oral absorption (Ishikawa et al., 2005; Fenyvesi et al., 2008).

In the present work, a detailed post-coital female contraceptive assessment in rats was carried out after standardizing the leave extract of *Cordia dichotoma* G. Forst., Boraginaceae (LCD). Usage of the herb for its antifertility potency is documented (Anjaria et al., 2002; Bhattacharya, 2006). The anti-implantation activity of the bark of *C. dichotoma* is reported (Katolkar et al., 2012). The leaves of *C. curassavica* and *C. spinescens* were also used to cure menstrual pain and relieve postpartum pain respectively (Lans, 2007). Presence of diverse type of phyto-constituents, mono and poly saccharides, β-sitosterol, allantoin and flavonol glycosides of taxifolin, distylin and apigenin had been reported in these species (Samant & Pant, 2006; Ganjare et al., 2011).

The complex of standardized extract preparation and bioavailability enhancer was characterized, and the reversibility and toxicity profile of the developed preparation were analysed by chronic toxicity studies.

Material and Methods

Extract preparation

Leaves of *Cordia dichotoma* G. Forst., Boraginaceae, were collected from Acharya Jagadish Chandra Bose Indian Botanic Garden, West Bengal, in the month of March and identified by the Central National Herbarium, Botanical Survey of India. A voucher specimen was stored in department for future reference. The cleaned leaves were shade dried and subsequently powdered. The powder material was extracted with 40% ethanol (1:8 w/v) by continuous hot percolation in a Soxhlet extractor. The extract solution was washed with *n*-hexane, filtered and subsequently vacuum dried. The material had an extractive value of 15.8% (w/w). The powders were dispersed in distilled water prior to administration.

Detection of toxic metal elements

An atomic absorption spectroscopic study of LCD extract was carried out for detection of toxic metals. LCD powder was burnt into flame for 30 min. The resultant ash was poured in hydrochloric and nitric acid mixture (3:1), sonicated and filtered. Standard samples of arsenic, cadmium and lead were used as markers in concentration ranging 5 to 200 ppb diluted in HPLC water, and measurements were performed (Mitra et al., 2002).

Animals

Female Swiss albino mice (20 g) and albino Wister female rats (180-200 g) were used for acute and chronic toxicities screening respectively. Colony-bred adult male and female (140-220 g) rats were used for antifertility testing, white immature female (40-60 g) rats used for the estrogenic assessment. All the biological including animal experiments were performed after approval and according to the protocol of the Institutional Animal Ethics Committee (Reg. No.: 506/01/a/CPC SEA).

Acute toxicity studies and LD50 assessment

For acute toxicity study, LCD extract was administered orally at single doses of 0.5, 1, 2, 3, 3.5, 4, 4.5, 5, 5.5 and 6 g/kg bw to individual group. General behaviors were monitored with control group. The toxicological effect was assessed on the basis of mortality after 24 h. The percentage of mortality was converted to probits and the values are plotted against log dose. The LD50 was the dose intersected by probit 5 (Veerappan et al., 2007).

Screening of female rats for antifertility testing

The estrus cycle of rats was monitored for 14 days. Acyclic rats and rats with prolonged cycles were screened and eliminated. The process includes examination of vaginal smear to observe different phases and duration of estrus cycle (Marcondes et al., 2002).

Selection of dose

In contraceptive applications, Indian traditional documentation reported that 2-3 standard sized leaves of *C. dichotoma* are fried with rice and taken for 2-3 days per week (Bhattacharya, 2006). Four dose levels (*i.e.* 1000, 800, 400, and 200 mg/kg/day) were selected for contraceptive profile screening. Drugs were orally administered in post-coital female rats from day 1-7 of pregnancy.

Evaluation of post-coital pregnancy interceptive activity

The pharmacological assessment for post-coital contraceptive profile has been performed (Pattanayak & Mazumder, 2009). The animals were laparotomised under light ether anesthesia and aseptic conditions on day 10 of pregnancy. Both horns of the uterus were observed for the number and status of implants. The ovary was carefully observed and the number of corpora lutea was counted. The rats were allowed to recover and deliver after full term. The litters were allowed to grow to check their postnatal growth and monitor any congenital abnormalities up to 7 days post partum period. Rats that did not deliver had laparotomised on day 25 and their uteri were observed to reveal any evidence of implantation. LCD extract showed prominent action in pharmacological screening. Different dose levels of LCD-BCD complex were administered in post-coital female albino rats. The dose level exhibiting best activity profiles was subjected for detailed pharmacological assessments.

Characterization of developed formulation

(2-Hydroxypropyl)-β-cyclodextrin (BCD) was used as bioavailability enhancer. Inclusion complex of LCD-BCD (1:1, w/w) formulation was prepared by co-evaporation technique (Shahgaldian et al., 2003). BCD solution in water was mixed with LCD in 40% ethanol at room temperature. Excess solvent was evacuated from the complex using rotary evaporator at 70 ^{o}C. The semi-dried product was lyophilized, powdered and stored in a desiccator at room temperature. Powders were dispersed in distilled water prior to administration. Particle size distribution of the powder in water was measured by dynamic light scattering (DLS). The mean hydrodynamic (peak) and size distribution (width) of the preparation were

assessed by Zetasizer 2000 (Malvern, UK). The surface structure of the particles was visualized by scanning electron microscopy (SEM) (Hitachi VP-SEM S-3400N). Samples were gold sputtered in a sputter coater (Hitachi, E-1010) for 40 s and scanning was performed at 15 kV with 40,000 magnifications. X-ray diffraction (XRD) of BCD, LCD and LCD-BCD were recorded with a XPERT-PRO X-ray diffractometer (Model: PW 3050/60, PANalytical, Almelo, Netherlands). The 2θ range is 5-600 and the scan rate is 1^0/min with Cu K_α radiation (40 kV, 30 mA).

Determination of estrogenic/antiestrogenic property

Ovariectomy was performed in immature female albino rats (Zarrow et al., 1964). After one week of the operation, animals were divided into four groups. The first group served as control and received the vehicle only (aqueous BCD solution). The second group received a suspension of estradiol valerate in distilled water containing dimethyl sulphoxide (10%, v/v) at a dose of 1 mg/kg body weight. The third group received the test dose (LCD-BCD) and the fourth group received a combination of test (LCD-BCD) and standard doses. All the treatments were given orally for seven days. On 24 h after the last dose, the animals were weighed, vaginal openings and vaginal smears were observed and sacrificed by cervical dislocation under light ether anesthesia. The uterine horns were removed, trimmed, opened longitudinally, blotted between cold and wet tissue papers and weighed immediately. The uterus was homogenized with cold normal saline solution containing 10 mg of tissue/mL. The homogenate was cold centrifuged at 900 x *g* for 15 min and the supernatant was used for the estimation of glucose, cholesterol and alkaline phosphatase (Pattanayak & Mazumder, 2009).

Determining variations in estrous cycles, blood biochemical parameters and histology of ovary and uterus

LCD-BCD was administered on 1-7 d of pregnancy. Before and after treatment, 2 mL of blood was drawn from orbital plexus. Serum gonadal hormones (estradiol, progesterone, DHEA-S, testosterone and cortisol), gonadotrophic hormones (FSH and LH), lipid-carbohydrate profiles (cholesterol (TC), HDL-C, triacylglyceride, VLDL-C, LDL-C and glucose), marker of lipid peroxidation (malondialdehyde) and serum antioxidant levels (glutathione and nitric oxide) were estimated.

Test of reversibility of anti-fertility effect and chronic toxicity studies

Adult female rats were acclimatized for a period of one month. During the period normal behavioral parameters like food intake, body weight gain and stool quality were checked. Normal cyclic rats (n=24) were divided into two groups (n=12) and were treated with LCD-BCD complex or vehicle for 21 days, and kept in normal condition for further 21 days.

The animals of each group were further divided into two groups. For the first group of each (n=6), the estrus cycles were monitored and animals in pro-estrus phase were allowed to mate with male. After completion of one gestation period, number of litters, average wt of pups on day 7 and viability index were checked.
The animals of other group were sacrificed, blood collected via cardiac puncture, and the liver, kidneys and lungs were taken for histological analysis. Serum biochemical parameters, SGPT and SGOT, ALP, creatinine and uric acid, MDA, GSH and NO were estimated.

Isolation of bioactive constituents and standardization of the extract

LCD was screened for presence of phenolic contents, flavonoids, tannins, essential oils, alkaloids, tannins, saponins, glycosides, steroids and terpenes by different qualitative chemical analyses (Harborne, 1998). LCD (10 g) was treated with 2N HCl for 1 h at 100 ^oC. The cooled solution was saturated with sodium chloride, partitioned with ethyl acetate and dried under nitrogen atmosphere. The dried residue was dissolved in ethanol and separation was performed in preparative TLC method developed in our laboratory. Silica gel (0.3 mm thickness) was used as stationary phase and toluene-ethyl acetate-methanol mixture (5:4.5:0.5) as mobile phase. After development of chromatogram, the separated spots were visualized by UV light at 254 nm and the major components are recovered by treating each portion of adsorbent with methanol. After separation, 10 mg of compound A and 23 mg of compound B were isolated. Preliminary identification of these two compounds was carried out by Forestal chromatogram and melting point analysis. The spectral analyses (mass and nuclear magnetic resonance spectroscopy) of compounds A and B were carried out for identification.

Quantification of A and B in LCD was carried out by HPTLC analysis. Definite volumes of ethyl acetate fractions were applied on TLC plate. Densitometric scanning was performed at 254 nm. The linearity, precision, repeatability, accuracy, robustness, limit of detection (LOD) and limit of quantification (LOQ) were estimated for validation of the developed analytical method. Instrumental precision and repeatability of the method were checked by repeated scanning (n=6) of the same spots, expressed as relative standard deviation (% RSD). Variability of the method was studied by analyzing aliquots of standard solution on same day (intra-day precision) and other days (inter-day precision), and the results were expressed as % RSD. LOD and LOQ were determined at signal/noise

ratio of 3:1 and 10:1 respectively. The accuracy of the method was assessed by performing recovery study by addition of 50 and 100% of standard and expressed as average percent recovery. Robustness of the developed method was assessed after deliberate alterations of any one of experimental parameter. The plates were prewashed by methanol and activated at 110 °C for 2, 5, and 7 min respectively, prior to chromatography. Time from spotting to chromatography and from chromatography to scanning was varied by ±10 min. Development distance was altered from 7 to 6 cm and 8 cm. Time of hydrolysis was altered from 1 h to 50 and 70 min. Robustness of the method was carried out at the concentration level of 200 ng/spot in triplicate.

Results and discussion

AAS analysis of heavy metal in LCD

The minerals responsible for irreversible toxicities of reproductive organs were found in different parts of herbs grow in polluted soil due to industrial and anthropogenic activities (Wang et al., 1996). The contents of arsenic (Chattopadhyay et al., 1999), cadmium (Johnson et al., 2003) and lead (WHO, 1995) in LCD preparation were found to be lower than the permissible limits (<5 ppb) mentioned in US-FDA and AYUSH (Sahoo et al., 2010).

Acute toxicity study

Except reduced locomotion (hypoactivity) in the dose equal to or lower than 3.5 g/kg bw, no signs of toxicity were observed in rats treated with a single dose of LCD extract. At higher doses, a regular dose-dependent increase in mortality was observed. The oral LD50 of LCD was found to be 5.50 g/kg (data not shown).

Phytochemical characterization

Qualitative chemical examinations of LCD extract exhibited prominent presence of flavonoids, tannins, terpenoids and glycosides, with traces of alkaloids and saponins. Two flavonoid aglycones, A and B were isolated and characterized.

Compound A: Pale yellow powder; mp 340-343 °C; bluish black coloration with aqueous ferric chloride; TLC on cellulose plate, R_f 0.8 (pale brown spot on Forestal chromatogram, black in ammonia vapor); UV (MeOH) λ_{max} 268, 306, 327 nm; ^{1}H NMR (CD_3OD and DMSO-d_6 (4:1, v/v), 500 MHz) δ 12.95 (1H, s, OH-5), 10.82 (1H, s, OH-7), 10.34 (1H, s, OH-4'), 7.92 (2H, d, *J*=8.77 Hz, H-2', H-6'), 6.91 (2H, d, *J*=8.77 Hz, H-3', H-5'), 6.77 (1H, s, H-3), 6.47 (1H, d, *J*=2.00 Hz, H-8), 6.18 (1H, d, *J*=1.99 Hz, H-6); ^{13}C NMR (DMSO-d_6, 125 MHz) δ 181.8 (C-4), 164.2 (C-2), 163.6 (C-7), 161.1 (C-5 and C-4′), 157.1 (C-9), 128.2 (C-2′ and C-6′), 120.4 (C-1′), 115.9 (C-3′ and C-5′), 103.8 (C-10), 102.4 (C-3), 99.1 (C-6), 94.3 (C-8); HREIMS *m/z* 271.0602 (calcd for $C_{15}H_{10}O_5$, 271.0625), confirmed apigenin (AP) (Harborne, 1998).

Compound B: Yellow powder; mp 326-328 °C; bluish black coloration with aqueous ferric chloride; TLC on cellulose plate, R_f 0.65 (pale brown spot on Forestal chromatogram, black in ammonia vapor); UV (MeOH) λ_{max} 253, 268, 306, 339 nm; ^{1}H NMR (CD_3OD and DMSO-d_6 (4:1, v/v), 500 MHz) δ 12.96 (1H, s, OH-5), 7.38-7.41 (2H, m, H-2', H-6'), 6.88 (1H, d, *J*=8.30 Hz, H-5'), 6.66 (1H, s, H-3'), 6.43 (1H, d, *J*=2.07 Hz, H-8), 6.18 (1H, d, J=2.06 Hz, H-6); ^{13}C NMR (DMSO-d_6, 125 MHz) δ 182.4 (C-4), 164.2 (C-2), 163.8 (C-7), 160.2 (C-5), 158.6 (C-9), 148.6 (C-4′), 146.2 (C-3′), 122.4 (C-1′), 119.2 (C-6′), 114.3 (C-5′), 112.3 (C-2′), 103.4 (C-10), 102.3 (C-3), 98.3 (C-6), 94.2 (C-8); HREIMS *m/z* 309.0375 (calcd for $C_{15}H_{10}O_6Na$, 309.0375) indicated presence of luteolin (LT) (Harborne, 1998).

Standardization of LCD extract

HPTLC method validation parameters, inter and intra-day precisions and recovery studies were performed (Table 1). Different sample solutions and solvent systems were tried in order to resolve the marker compounds. The developed TLC densitometric method was validated in terms of precision, repeatability and accuracy. The linearity range for AP and LT was found to be 50-200 ng/spot, with correlation coefficient >0.99 in both the cases. The method was found to be precised with % RSD (n=3) for intra-day in the range of 0.98-1.15 and 0.91-0.96, and for inter-day in the range of 1.13-1.28 and 1.01-1.22 for 50 and 200 ng of AP and LT respectively, indicating the proposed method was accurate and reproducible. The LOD and LOQ values for both were found to be 10 and 40 ng respectively. The average recoveries at two different levels of AP and LT were found to be 99.24 and 99.87% respectively. Robustness of the method was checked after deliberate alterations of the analytical parameters, showed that areas of peaks of interest remained unaffected by small changes of the operational parameters. Less than 2% of RSD along with appreciable recovery are indicative of the robustness of the method. AP and LT were quantified in LCD sample and are found to be 0.14 and 0.32% (w/w) with R_f of 0.70 and 0.65 respectively.

Pharmacological screening

It was observed that LCD revealed anti-implantation potential in a dose-dependent manner (Table 2). The test substance, LCD when administered at 1000 mg/kg (T4), no implantation sites were observed but caused behavioral changes. The observable symptoms

Table 1. Validation of TLC densitometric method for quantification of apigenin (AP) and luteolin (LT).

	Marker	Present (ng)	Added (ng)	Recover (ng)	Recovery (%)[a]	Average recovery (%)
Recovery study	AP	70	35	105.11±3.24	100.10±3.08	99.24
		70	70	137.72±0.84	98.37±0.60	
	LT	160	80	236.49±2.13	98.54±0.89	99.87
		160	160	323.85±2.30	101.20±0.72	
	Marker	Concentration (ng)		Intra-day precisiona		Inter-day precisiona
Intra-day and inter-day precision	AP	50		0.98		1.13
		200		1.15		1.28
	LT	50		0.96		1.01
		200		0.91		1.22
Instrumental precision (AP and LT)[b]				1.18, 1.24		
Repeatability (AP and LT)[b]				0.88, 1.02		
LOD (ng)				10		
LOQ (ng)				40		
Specificity				Specific		
Linearity (correlation coefficient) for AP and LT				0.99999, 0.99976		
Range (ng spot^{-1})				50-200		
SD (%) for AP and LT				0.30, 1.90		

[a]% RSD, n=3; [b]% RSD, n=6.

included reduced food intake and general weakness that revealed through slow and crippled movements. The 800 mg/kg dose of LCD (T3) also resulted in 100% pregnancy interception without any behavioral changes (n=10). It executed the contraceptive potency in a relatively higher dose level. Thus incorporation of bioavailability enhancer in the extract formulation was considered.

Characterization and pharmacological evaluation of developed formulation

The mean particle size of the LCD-BCD formulation, analyzed by DLS, was found to be 174.7 nm with a poly-dispersity index of 0.281 (Figure 1A). Spherical non-adherent particles were observed upto one month of preparation indicated stability of the formulation (Figure 1B). The interaction of drug with polymer was clearly visible in XRD patterns (Figure 1C). In the product (Figure 1C, b), the intensity of BCD at 18.77^0 contrast to the intensity at 16.86^0 was thirteen times amplified, implied some change in BCD matrix mixture. The diffraction peak of LCD powder revealed several sharp peaks at diffraction angles (2θ) of 19.35^0, 21.42^0, 22.36^0, 25.83^0, 28.37^0 and 40.51^0. These peaks were mostly absent in LCD-BCD complex indicates formation of amorphous state and complexation. Reduction in crystalline nature obviously pretends a role for better oral bioavailability.

LCD-BCD formulation exhibited 100% anti-implantation activity in all 800, 500 and 250 mg/kg dose levels (Table 2). LCD-BCD at 250 mg/kg dose (T7) was found to be selective among all the treatments for post-coital antifertility testing (n=20), hence considered for detailed pharmacological investigations.

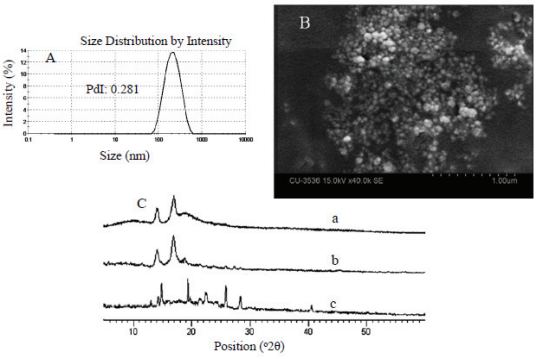

Figure 1. Characterization of LCD-BCD complex. A. Size distribution in DLS; B. Surface structure by SEM and C. Drug-polymer interaction observed in XRD pattern, [a]BCD, [b]LCD-BCD complex, [c]LCD.

Pharmacological findings

Effect in ovariectomised rats (OVX)

T7 caused a significant increase in uterine weight (193.8%) in OVX as compared to control (Table 3). The

Table 2. Contraceptive property assessment.

Group	Test substance	Oral dose (LCD, mg/kg)	n	nP	PI	nCL	nI	nL	nLD*	mLW	VI(%)*
C	Vehicle	-	6	6	-	9.3±0.3	8.7±0.2	8.3±0.2	0.0±0.0	15.4±0.1	100±0.0
T1	LCD	200	6	5	16.7	5.7±0.2	3.8±0.8	3.6±0.2	0.6±0.4	15.1±0.1	80.0±13.3
T2	LCD	400	6	3	50	5.5±0.2	1.3±0.6	2.0±0.0	1.3±0.7	15.2±0.0	33.3±33.3
T3	LCD	800	10	0	100	4.8±0.3	0.0±0.0	-	-	-	-
T4	LCD	1000	6	0	100	5.2±0.3	0.0±0.0	-	-	-	-
C1	BCD	-	5	0	100	9.0±0.3	8.6±0.2	8.2±0.2	0.0±0.0	15.9±0.1	100±0.0
T5	LCD-BCD	800	6	0	100	4.3±0.2	0.0±0.0	-	-	-	-
T6	LCD-BCD	500	6	0	100	4.3±0.2	0.0±0.0	-	-	-	-
T7	LCD-BCD	250	20	0	100	4.8±0.2	0.0±0.0	-	-	-	-
T8	LCD-BCD	100	6	4	33.3	4.5±0.2	2.0±0.7	0.5±0.3	0.5±0.5	13.9±0.0	50.0±50.0

n, nP, PI, nCL, nI, nL, nLD, mLW and VI represent the number of animals in a group, number of pregnant animals, percentage pregnancy interception, number of corpora lutea, number of implantation sites, number of litters born on parturition, number of litters that died within 7 d of parturition, mean live litter weight on day 7 and viability index respectively. The results are presented as mean±S.E. One way analyses of variances, nCL: F=36.72 (df=9,67), nI: F=106.39 (df=9,67), nL: F=244.55 (df=4,18), nLD: F=2.88 (df=4,16), mLW: F=10.75 (df=4,13) and VI: F=3.34 (df=4,16). The values are significant at p<0.005 except *p>0.03.

Table 3. Evaluation of estrogenic and anti-estrogenic profiles.

Dose (mg/kg, bw)	NOV (%)	NC (%)	Uterine weight (mg/100g, bw)	Biochemical changes in uterus		
				Glucose (mg/100 g)	Cholesterol (mg/100 g)	ALP (IU/100 g)
Control	0	0	61.2±0.5 (n=16)	0.92±0.01 (n=10)	5.30±0.04 (n=10)	0.50±0.01 (n=10)
EV (1)	100	100	217.6±1.8 (n=17)	1.71±0.01 (n=11)	7.43±0.03 (n=11)	0.89±0.01 (n=11)
LCD-BCD (250)	47	35	179.8±1.5 (n=15)	1.42±0.01 (n=9)	6.39±0.03 (n=9)	0.65±0.01 (n=9)
EV (1) + LCD-BCD (250)	80	60	208.2±1.0 (n=10)	1.64±0.01 (n=8)	6.91±0.04 (n=8)	0.84±0.01 (n=8)

NOV is the number of animals with open vagina; NC represents number of animals with predominantly cornified cells like estrus stage. The results are presented as mean±S.E. One way analysis of variance, uterine weight: F=2746 (df=3,54); uterine glucose: F=2140 (df=3,34); uterine cholesterol: F=651 (df=3,34) and uterine ALP: F=961 (df=3,34). The values are significant at p<0.01.

uteri were fluid filled. T7 induced vaginal opening in OVX and the smear showed a condition of estrous or proestrous, while the control rats had closed vaginas. A significant increase of glucose (54.35%), cholesterol (20.57%) and alkaline phosphatase (30%) were observed in uterine tissue homogenates. Co-administration of EV with LCD–BCD formulation caused a significant increase in uterine weight (240.2%) in OVX, but the extent of uterotropic effect was considerably less than that produced by EV alone (Table 3). Thus LCD revealed estrogenic activity when administered alone, but exhibited impeded estrogenic activity when administered with estrogen.

Serum hormonal and biochemical profiles

Figure 2 depicts the results of biochemical assay. There was diminution in serum progesterone (13.90%), and increment in estradiol (14.51%) and DHEA-S (46.93%) contents. In case of gonadotrophin hormone, FSH was observed to be significantly decreased (106.67%) with a slight reduction in LH levels, i.e. LH-FSH ratio alters. Body weight had been found to be 3.14% elevated after seven days treatment period. It indicated that drug administration significantly influenced corpus luteum function by affecting steroid biosynthesis. Probably the remarkable increase in serum estradiol levels caused a reduction in the functional life-span of the corpus luteum (Williams & Lemke, 2002).

A relation between ovarian estrogen production and adrenal androgen synthesis had also been evaluated. Elevated levels of estrogen could have a direct adrenal effect (Steinmetz et al., 1997). A stress condition might be generated upon treatment with LCD-BCD. Cortisol (15.18%) and malondialdehyde (MDA, 11.33%) levels were raised, while GSH level fall down (5.23%) with increased adrenal DHEA-S output (46.93%) (Figure 2).

Estrogen increased the cortisol-binding globulin and the free cortisol level elevated. Estrogen also decreased the ability of liver to metabolize cortisol and contributed to the elevation of unbound cortisol (Speroff et al., 1999). Paradoxically, a diminished level of testosterone (20.76%) was also found, due to binding with serum proteins that might be induced with use of contraceptives (Palatsi et al., 1984). Explanation can also be drawn from enhanced level of DHEA-S. Testosterone was produced from DHEA. The dosage form of LCD might be interrupting the pathway of conversion. Some antiandrogenic terpenoids were isolated from *Cordia multispicata* of Boraginaceae family (Kuroyanagi et al., 2001). Terpenoidal components present in LCD might be responsible for this kind of effects.

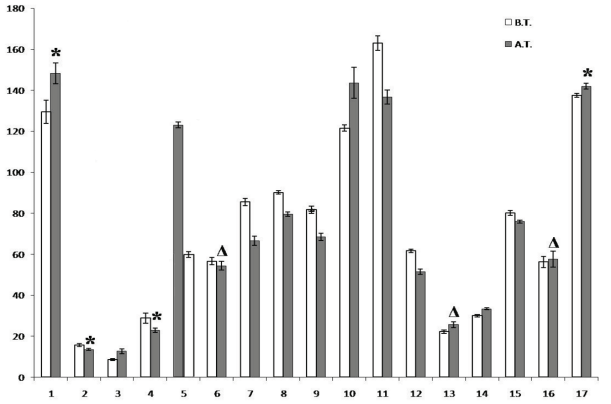

Figure 2. Effects of LCD-BCD formulation on biochemical profiles. B.T.: before treatment, A.T.: after treatment; [1]estradiol (pg/mL), [2]progesterone (ng/mL), [3]DHEA (ng/mL), [4]testosterone (ng/10 mL), [5]FSH (mIU/dL), [6]LH (mIU/dL), [7]glucose (mg/dL), [8]cholesterol (mg/dL), [9]triacylglyceride (mg/dL), [10]HDL-cholesterol (mg/L), [11]VLDL-cholesterol (mg/L), [12]LDL-cholesterol (mg/dL), [13]cortisol (ng/mL), [14]MDA (µg/10 mL), [15]GSH (µg/mL), [16]NO (µg/dL), [17]Body weight (g). The changes with respect to B.T. are significant at $p<0.01$, except *$p<0.05$ and $^{\Delta}p>0.1$.

From Figure 2, it can be ascertained that a significant decrease in blood glucose (22.12%), possibly disrupts oxidative energy metabolism in uterus during implantation, providing environment unreceptive for implantation (Keshri et al., 2004). Although LCD-BCD did not promise any beneficial serum antioxidant status, a significant decline in blood cholesterol (11.71%), VLDL-C (16.28%), triacylglyceride (16.26%) and LDL-C (16.26%), and increase in HDL-C (18.27%) confirmed that LCD has favorable effect on lipid profile and seems to be free of cardiovascular risk factors, unlike steroidal oral contraceptives (Wahl et al., 1983).

Relation with marker compounds

AP and LT, isolated from Boraginaceae family, have significant contraceptive property (Hiremath et al., 2000), individually at dose level of 25 mg/kg. In the developed dosage form, these two compounds were cumulatively administered at a level of approximately 1.25 mg/kg (0.5% of 250 mg/kg dose, T7) and exhibited 100% contraceptive potential (Table 2). These two components showed weak estrogenic actions, when administered alone (Hiremath et al., 2000). But LCD-BCD exerted strong estrogenic potential (Table 3). Herbal medicines consist of the active compound(s) along with other ingredients. The results of the activation of a signal cascade can be due to signal amplification, and is much greater than the summation of the single effects (Imming et al., 2006; Wagner & Ulrich-Merzenich, 2009). Both the compounds might have super-additive agonistic effect to each other that possibly imported estrogenic contraceptive property.

Chronic toxicity studies

The various parameters were assessed to estimate chronic toxicity of LCD-BCD. Comparable number of litters born with 100% viability index indicated reversible contraceptive nature of LCD. The morphological and histological examinations (Figure 3) showed that no toxic sign of organ damage in the test group, and results were comparable with control. The levels of liver marker enzymes, SGPT, SGOT and ALP were normal. Renal function parameters, marker of lipid peroxidation and antioxidant status were also unaltered and did not show any toxic pitfall (Table 4).

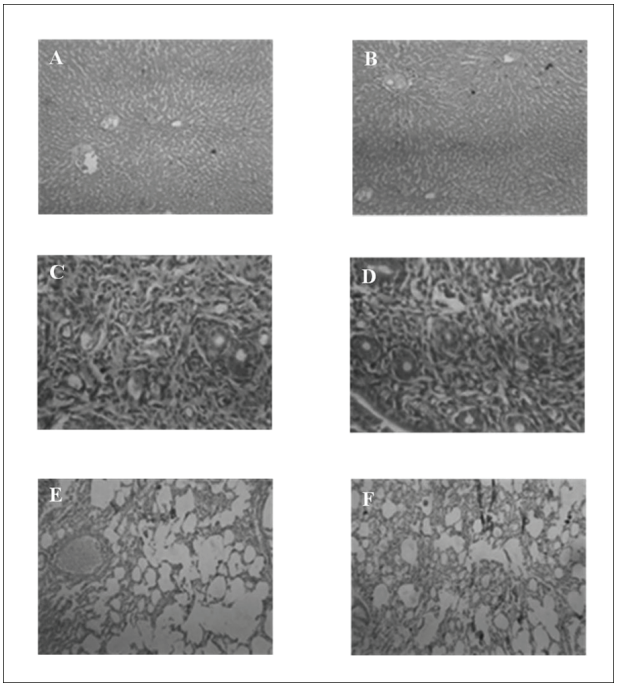

Figure 3. Comparative histological changes. Liver: A. Control; B. Treated. Kidney: C. Control; D. Treated. Lungs: E. Control; F. Treated.

Table 4. Reversible contraceptive property assessment and chronic toxicity studies.

Parameter	Unit	Control (n=6)	Test (n=6)	% changes	Statistical parameters	
					ANOVA (F)	Significance (*p*)
Number of litter born	-	8.33±0.42	8.83±0.31	6.00	0.92	0.36
Viability index	-	100.0±0.0	100.00±0.00	0.00	-	-
Mean litter weight	g	14.97±0.15	14.79±0.17	-1.20	0.63	0.45
Ovary	mg/100 g bw	25.48±0.46	25.88±0.88	1.57	0.38	0.55
Uterus	mg/100 g bw	81.61±0.46	82.80±0.37	1.46	4.08	0.07
Vagina	mg/100 g bw	33.11±0.33	34.35±0.15	3.75	12.02	0.01
Liver	g/100 g bw	3.92±0.47	3.95±0.30	0.77	0.19	0.68
Kidney	mg/100 g bw	391.61±2.41	393.20±3.64	0.41	2.24	0.40
Heart	mg/100 g bw	348.45±1.91	351.09±1.86	0.76	0.97	0.35
Adrenal	mg/100 g bw	15.40±0.36	14.98±0.18	-2.73	1.08	0.32
SGPT	IU/L	26.42±0.77	28.96±1.01	9.61	3.95	0.08
SGOT	IU/L	21.37±0.71	21.21±0.57	-0.75	0.03	0.86
ALP	IU/L	78.96±3.74	81.18±2.22	2.81	0.26	0.62
Uric acid	mg/dL	6.13±0.19	5.59±0.19	-8.81	4.00	0.07
Creatinine	mg/dL	0.31±0.01	0.34±0.02	9.68	1.63	0.23
MDA	µg/mL	2.89±0.10	2.67±0.10	-7.61	2.35	0.16
GSH	µg/mL	77.36±1.64	76.53±2.25	-1.07	0.09	0.77
NO	µg/mL	0.58±0.02	0.56±0.01	-3.45	0.31	0.59

Conclusion

The developed formulation of standardized *Cordia dichotoma* leaf extract showed potent contraceptive potential. It has strong estrogenic potential but lack of major cardiovascular risks associated with estrogens, even after long term of administration.

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Authors' contributions

PB (PhD student) contributed in collecting plant sample, confection of herbarium, performing the laboratory work, *i.e.* chromatographic analysis and animal experimentations, and drafted the paper. AS is supervised the overall project work. Authors have read the final manuscript and approved the submission.

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