2011 and authenticated (Voucher. AUS/ 2504) by Dr. Debojyoti Bhattacharya, Assam University Herbarium, Department of Life Science and Bioinformatics, Assam University, Silchar. The collected stem barks were then shade dried, pulverized and the powder (100g) obtained was soaked in petroleum ether to remove fatty and waxy materials. The remainder was then subject to successive Soxhlet extraction with methanol and water as solvents (1:4 w/v). Prepared methanolic and aqueous extracts were reduced in a vacuum evaporator (Buchi Rotavapor® R-210) at 40±1ºC and finally dehydrated in desiccators to get thick brown methanol (MEOI) and aqueous (AEOI) extracts. The extracts were dried in vacuum and the percentage yield of each was determined as 8.24% (MEOI) and 10.61% (AEOI), respectively using the formula: % yield = weight of the extract (mg)/weight of the whole tissue powder (mg) × 100

Extracts were then stored in a refrigerator at 8 ± 2°C for use in the *in vitro* and *in vivo* studies[7]. The AEOI and MEOI were dissolved in normal saline (pH 7.4) to obtain various concentrations (10, 20, 40, 80 and 100 µg/mL) of the extracts, which was used to perform the *in vitro* studies.

Evaluation of spermicidal potential (*in vitro*)

Semen sample collection and bioassay protocol

The human sperm used for this bioassay according to the procedures of the institutional ethical committee of National Institute of Health and Family Welfare (NIHFW), New Delhi, India. Human ejaculates have been obtained from male partners of patients those were referred to Infertility clinic of Department of Reproductive Biomedicine (RBM), NIHFW, New Delhi, India. Human sperm samples have been obtained from the male subject those had sexually abstinence for 72 to 96 h. At least 30 min before used all samples were liquefied at 37°C. Samples those shown a sperm count of more than 50×10^6 mL^{-1} and motility more than 60% were included for further study. Routine semen investigation such as concentration, motility, and morphology of sperms was done as per the Laboratory Manual for the Examination of Human Semen and Sperm-Cervical Mucus Interaction[8]. The motile sperms were counted using fresh unstained semen sample until assesses 200 spermatozoa. Experiment was carried out in triplicate for improving counting accuracy, procedures. The semen sample of same donor's was aliquot equally for further *in vitro* experimentations.

Evaluation of sperm immobilization properties (*in vitro*)

Different concentrations of the extracts (AEOI and MEOI) were mixed thoroughly with human ejaculate (100-150 million spermatozoa/mL) in 1:1 (*v/v*) ratio as reported by Waller *et al.*[9], 1980. A drop (10 µL) of each mixture was immediately taken on a glass slide and 5 fields were viewed under microscope with magnification of 400X for investigation of sperm motility. The semen sample mixed with test samples were incubated at 37ºC for 30 min. The above said method was repeated for next set of experimenations.

Hypo-osmotic swelling test (HOS)

This test was done by using non-commercial patented (Indian Patent, 2991/DEL, Nov. 2009) HOS test kit[9]. The test was carried out in two sets: Control (sperm in normal saline) and Treatment groups. In treatment groups four different concentrations (10µg/mL, 20µg/mL, 50µg/mL and 100µg/mL) of MEOI and AEOI were used. 5mL of sperm preparation was added to 50mL of HOS solution, mixed gently and incubated for 5 min at room temperature. 5mL of the stock solution was added to the two sets immediately after completion of the incubation time. Diversify sperm's tail coiling in random 100 sperm was indicator for swelling, that observed under a phase contrast microscope[10].

***In Vivo* studies**

Animals

In present study, wistar albino rats (150-200 g) and albino mice (20-25 g) of either used for *in vivo* studies. Animals were procured from the Regional Institute of Medical Sciences (RIMS), Imphal. The animals were housed in polypropylene cages; standard 12 h dark/light cycle and optimum temperature (27°C±2°C) was maintained. The standard chack food was fed to all animals under study and water *ad libitum*. Ensured hygienic housing and optimum comfort for animals by cleaning litter in the cages on daily basis. Institutional Animals Ethical Committee (IAEC), IBSD, Imphal (approval No.-IBSD/IAEC/ Trainee/Ph.cology/9) approval was taken for handling the animals before beginning of the experimentations.

Determination of acute toxicity (ALD_{50})

In the present study, AEOI and MEOI was assessed for acute toxicity on albino mice (as per approval of IAEC, IBSD, Imphal), kept under prescribed conditions. The animals were kept for fasting for 4 h before starting experiment. OCED Guideline

423- Acute Oral Toxicity- Acute Toxic Class Method was adopted for this study. The AEOI and MEOI extracts orally administrated as a suspension (purified water used as a solvent at 20% w/v concentration). Behavioral abnormalities and mortality were recorded for 48 h after ingestion a single dose of 2000 mg/kg by the respective group of animals[11].

Evaluation of antifertility potential of AEOI and MEOI on male rats (*In vivo*)

Male antifertility potential of test products was evaluated as per WHO Protocol MB-50, 1983[5] with innovative modifications. According to the protocol, male rats divided into the following three groups with 12 rats each after confirmed their fertility: Gr. I, Control group (1 mL normal saline); and Gr. II & III, Treatment group (200 mg/kg of AEOI and MEOI, respectively) p.o. for 14 days.About 50% animals from group I, II & III (n=6) were made as Recovery groups (animals selected randomly and kept to recover from the possible effect of the respective test samples after the withdrawal of treatment on 14th day of experiment/treatment for further 14 days under normal food and water).

On the 14th day of treatment, male rats from groups I, II & III (n=6) were introduced to female rats in a ratio of 1:2 (male: female) for a single night. Successful mating of male and female animal was assured in following morning by detecting spermatozoa in the vaginal smear using a microscope and vaginal plug of females. The impregnated female rats were separated and allowed to deliver at time; counted numbers of pups delivered by each female rat[7]. On the 15th day after confirmation of mating, the weight of the male rats was noted and 50% of animals (treatment groups, n-6) from the Gr. I, II and II were sacrificed. The accessory sex organs were weighed, caudal epididymal sperm was counted (growing spermatozoa with head & tail), motility and morphology were studied as per the reported method. The changes in morphology of animal sperms under treatment in *in vivo* system, also compared with the changes of human sperm morphology under treatment with same extracts in *in vitro* condition[8,12]. The motile and immotile sperms in per unit area was calculated for determining percentage motility[8]. On 15th day of experiment, blood samples were collected from all sacrificed animals and allowed to coagulate. The serum was separated from coagulated blood by centrifugation at 2000 RPM for 10 min. The sera were aliquoted and stored at –20ºC until used for biochemical estimation (Day 1).

Male rats of the recovery groups (50% of animals from the group I, II and II, n=6) were also introduced to female rats in a ratio of 1:2 (male: female) for a single night on the 14th day of recovery (28th day of experiment) to study the reversable effect of male antifertility products under study. Successful mating of male and female animal was confirmed in next morning by observing spermatozoa in the vaginal smear using a microscope and vaginal plug of female rats. The impregnated female rats were separated and allowed to deliver at time; the number of pups delivered by each female rat was noted[5]. Again, on the 30th day of the experiment (Day 14 of recovery) the recovery group animals were sacrificed, serum was separated for biochemical estimation (Day 14).

Serum collected from Gr. I, II and III were analysed for Testosterone, Dihydrotestosterone (DHT) by the methods of Enzyme-linked Immunosorbent Assay and Prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$), Prostaglandin E_2 (PGE_2) by the method of Enzyme Immuno Assay (EIA) using commercial analytical rat kits (LSBio, USA) and estimated SGOT, SGPT, BIT, Triglyceride, Urea and Uric acid using commercial kit (Erba Mannheim, India).

Estimation of cholesterol in seminal plasma

Cauda epididymal fluid samples were obtained from a proximal and distal part of the cauda of animals according to the micro-puncture technique[13]. Each part was then flushed with 2 mL of normal saline. The samples were then centrifuged at 380 g for 10 min and the supernatants stored at –20°C. The total cholesterol content was estimated by using the technique of Eliasson, 1966 with slight modification[14]. In a test tube 2.5 mL of test reagent containing a mixture of 4.0 g of toluene-p-sulphonic acid, 60 mL of acetic anhydride and 40 mL of acetic acid, was gently mixed with 0.5 mL of concentrated sulphuric acid. Then the test tube was allowed to cool at room temperature (25º±2ºC). To that aliquot (0.1 mL) of seminal plasma was added, mixed well and the colour was allowed to develop in dark condition for 30 min. The green colour formed was then measured at 570 nm (Thermo Multiscan Spectrum) against water as a blank. A standard curve was made using standard cholesterol and total cholesterol content was calculated from that curve[14].

Fig. 8 — HPLC chromatogram of methanol (MEOI) and aqueous (AEOI) extracts of *Oroxylum indicum* stem bark, Standard compounds Oroxylin-A (Sigma, USA) and Baicalin (Sigma, USA).

Table 1 — HPLC profile of Oroxylin-A (Retention Time-19.358) and Baicalin (Retention Time-8.285)

Concentration (µg)		Area	
Oroxylin-A	Baicalin	Oroxylin-A	Baicalin
0.0125	0.0125	3649646	59753
0.0250	0.0250	8082041	93225
0.0500	0.0500	17169355	179917
0.1000	0.1000	32090898	319935

The body weight was not changed significantly, weight of other vital organs and behaviour of the animals upon oral treatment with the extracts, which demonstrates the general well-being of the animals, but the weight of the testis along with other accessory organs such as the vas deferens, prostate gland and epididymis were significantly decreased in

Table 2 — Quantification of Oroxylin-A and Baicalin in methanol (MEOI) and Aqueous (AEOI) extracts of *Oroxylum indicum* stem barks

Bioactive compound	Retention time		Area		Concentration of bioactive compound in 10 µg sample (10 µL)		Content (%)	
	MEOI	AEOI	MEOI	AEOI	MEOI	AEOI	MEOI	AEOI
Oroxylin-A	19.343	19.645	2837803	2368235	0.859 µg	0.7144 µg	8.59	7.144
Baicalin	8.275	8.285	156422	382621	0.446 µg	1.21 µg	4.46	12.10

comparison to the control animals after 14 days of treatment. The epididymal sperm count, motility was decreased and morphological abnormalities such as globular shape of head, tail coiling and tail fusion of two or more sperm was also seen. Decrease in the weights of the sex organs may be attributed to the reduced levels of testosterone as observed in the treatment group animals as it is already known that the various sex organs are androgen dependent for the maintenance of structure and function. Any change in the level of this hormone could also influence sperm motility and viability within the epididymis thereby affecting the overall reproductive function. However, the serum levels of dihydrotestosterone remained unaltered which is associated with the maintenance of male libido[16].

Along with androgens, prostaglandins (PGs) are also involved in male reproduction. Higher concentration of PGs has been observed in the seminal fluid[17] that stimulate influx of calcium through a receptor associated mechanism which facilitates the acrosome reaction. Prostaglandin E_2 (PGE_2) has been reported to stimulate sperm motility while Prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) inhibits it[18,19]. In this work, both the test products shown an increase level of PGE2 and PGF2α, however the effect was more prominent with the methanol extract. It is interesting to note the elevation in the serum PGE2 and PGF2α levels. It is plausible that the increase in PGF2α could be responsible for the sterile effect of the rats during the treatment period since it is inversely correlated with sperm motility[20,21] while increased PGE2 could be responsible for facilitating the reversible action of *Oroxylum indicum* extract which corroborates with the findings in this study in which the male fertility was restored following two weeks of extract withdrawal (Fig 7). The reversible effect was witnessed through an increase in the pup numbers delivered by female rats inseminated by male rats of the recovery group in comparison to the pup number delivered by female rats impregnated by males of the treatment groups.

Cholesterol is an important component that acts as a precursor in the synthesis of androgens, therefore reduced levels would indicate an inadequate level of androgen synthesis in the testis. Our study showed that animals treated with the test extracts had significantly reduced cholesterol content in seminal plasma in which the MEOI showed higher activity.

Conclusion

Both the aqueous (AEOI) and methanol (MEOI) extracts of the stem bark of *Oroxylum indicum* significantly (**P <0.01) decreased the weight reproductive organ in male rats. The MEOI treated rats showed significant (**P <0.01) decrease in sperm motility and sperm counts. AEOI, MEOI treatment significantly (**P <0.01) reduced level of testosterone, but sharply raised dihydrotestosterone and prostaglandin in rats. The results support the traditional claim for use of *O. indicum* as a male contraceptive agent, where MEOI have shown reversible action on male reproductive system leading to contraception without destructively effecting the libido. Reversal of fertility was also seen upon withdrawal of the treatment indicating reversible contraception. However, further investigations are essential to established the mechanism of action and to identify the specific roles of individual components found in the extracts those are responsible for imparting the anti-fertility activity.

Conflict of interest

Authors declare no competing interests.

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