



Isolation, characterization & anti-fertility activity of the active moiety from the seeds of *Ensete superbum* Cheesm (Banakadali)

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Abstract

The ethanolic extract of the seeds of *Ensete superbum* Cheesm (Family Musaceae) furnished a compound with structure established as 4-(4-hydroxy-3-methyl-hex-5-enyl)-chroman-2, 7-diol on the basis of physical and spectral basis. The anti-implantation activity of the ethanolic extract at doses of 100, 125 and 250 mg/kg body weight when administered on days 1-7 were 86.9, 94.4, and 98.8% respectively, as compared to the isolated fraction at doses of 2.5 and 5 mg/kg body weight was 87.5 and 96.0% respectively as compared to their Control group. The isolated fraction was found to exhibit significant anti-estrogenic activity in immature female rats at the dose of 5 mg/kg, and the ethanolic extract exhibited very significant activity at the dose of 250 mg/kg-body weight. Histopathological studies revealed significant anti-ovulatory activity in immature female rats at the dose 5 mg/kg. The oral LD₅₀ value of the ethanolic extract of *Ensete superbum* Cheesm was found to be 3235.9 mg/kg.

Keywords: *Ensete superbum* Cheesm, NMR, chroman-2, 7-diol, anti-implantation, anti-estrogenic and anti-ovulatory activity.

1. Introduction

According to the provisional results of Census of India 2001, the Indian Population as at 00.00 hours of 1st March 2001 was 1,027,015,247, of which 531,277,078 were males and 495,738,169 were females, which is another subject of concern. The crude birth rate which was 49 per thousand population during 1901-11 has declined to about 31.3 per thousand population in 1991, and 25 per thousand population in 2002 [1]. India is the second country in the world after China to cross the one billion mark. It is now estimated

that by 2050, India will most likely overtake China to become the most populous country on the earth with 17.2 per cent population living. [2]. The complicated drug regimen, sexual dissatisfaction, failure rate, thrombosis, cardiovascular problems, nausea, vomiting, weight gain etc., make the use of hormonal contraceptives less acceptable. Therefore, our interest was stimulated in the development of new potent anti-fertility agent of natural origin with significant activity.

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The need of the hour is to develop an ideal *post-coital* non steroidal contraceptive especially from herbal sources, which would be safe, effective, acceptable, less expensive, simple to administer, long acting to obviate the frequent administration requires no medical supervision and 100% effective. WHO and ICMR provide systemic guidelines for the evaluation of anti-fertility plants to generate reproducible results, i.e., proper authentication and systemic screening methods [3]. So far no single plant is available which can be used on humans as a potent anti-fertility agent. Hence, the search needs to be continued.

With this background *Ensete superbum* Cheesm was studied for anti-fertility studies. The fraction (VIDR-2GD) isolated from its seeds has been reported to possess anti-implantation activity [4], but no work regarding its chemical characterization has been reported. The structural elucidation of the active isolated fraction of the drug and its anti-fertility were undertaken as the targets of our studies.

The crude ethanolic extract and the isolated compound from the seeds were screened as a post-coital anti-fertility agent in female rats on different days of gestation period i.e., day 4 and days 1-7. Studies with regard to its estrogenic anti-estrogenic and anti-ovulatory activities in immature female rats, the effect of the drug on estrous cycle and mating behavior and reversibility of action of the drug were also undertaken.

2. Material and Methods

2.1 Plant material

Ensete superbum Cheesm is reported as occurring naturally only in India mainly in Western Ghats of India from Bombay to Travancore Hills, Ravine Slopes, Assam, Jhadol and Ogna Forest Ranges [Udaipur Distr., Rajasthan], Deccan, Nasik, Khandesh and Poona

districts, Anamalei Mts (North of Cochin and Madura), Matheran Ram Ghat, Khandala, Dingidul, Waynarde Forest Area in Kerala [5].

2.1.1 Collection and Identification

Seeds of *Ensete superbum* Cheesm were collected from the forest area of Waynarde district in Kerala during the month of May-June. Seeds were dried in the shade at the temperature of $28 \pm 2^\circ\text{C}$ and were authenticated at NISCAIR (National Institute of Science Communication and Information Resources), CSIR, New Delhi (Ref. No.NISCAIR/RHM/F-3/2005 Consnt. No.544/19).

2.1.2 Extraction and Isolation

The kernels of the dried seeds of *Ensete superbum* Cheesm, Banakadli (1 kg) were initially crushed in mortar pestle, and ground to fine powder by the electric grinder at room temperature ($25 \pm 2^\circ\text{C}$) and then extracted with distilled water. The extract was filtered and the filtrate dried under reduced pressure (25%). Separately the powdered drug (2.5 kg) was then extracted with 95% (v/v) ethyl alcohol in Soxhlet apparatus [4]. The extract was filtered, the filtrate was distilled under reduced pressure to give red colored solid (48 gm). The ethanolic extract was further dissolved in 250 ml of water and heated with a concentrated solution of basic lead acetate until the precipitation was complete. The precipitate was treated with sodium sulphate and calcium carbonate to form a slug and dried at $47 \pm 2^\circ\text{C}$. It was then treated with absolute ethyl alcohol and refluxed. The alcohol was removed under reduced pressure to yield a yellow solid and recrystallized with methanol, to a yellowish white solid (isolated fraction, 2.5gm) [4]. The extracts and the isolated fraction were taken for the desired phytochemical and pharmacological studies. Isolated fraction was also characterized for its possible structure.

2.2 Phytochemical and chemical screening

Sample of the dried powder of seeds was subjected to quantitative standards like moisture content, total ash value, acid insoluble ash value, and water soluble ash value [6]. The alcoholic extracts and the isolated compound of the seeds were subjected to various qualitative chemical tests for the presence of different constituents like carbohydrate, glycoside, alkaloid, steroids, saponins, flavonoids, tannins etc. whereas the isolated compound was further characterized using colour, DSC, solubility, chemical tests, TLC/HPTLC (Camag), FTIR spectra, ¹H NMR (Advanced Dry 400 MHz - Bruker Spectrospin), ¹³C NMR (400 MHz - Bruker Spectrospin), GC-MS (model-5975 Algenet, Technologies).

The extract and its isolated compound was subjected to TLC/HPTLC using solvent system (toluene: ethyl acetate: formic acid 5:4.5:0.5) [7] [8].

2.3 Pharmacological screening

2.3.1 Animals

After approval of Institutional Ethics Committee (DIPSAR) all the animal experiments were conducted on in-bred female *wistar* rats (150-275 gm body weight), male *wistar* rats (200-300 gm body weight) and immature female *wistar* rats (30-40 gm body weight). Albino mice (30-50 gm. body weight) were used for the acute toxicity study. Rats were selected as experimental animals for studying the anti-fertility effects because of their short gestation period (i.e. 20-22 days), they are easily available at economic costs. Further, they are easy to handle, and only a small amount of drug is required since the weight and the size of the animal is small.

All the animals were kept in the Institutional animal house (DIPSAR) under identical

conditions (12:12 dark: light cycle). The room temperature was maintained at 25±3°C throughout the studies. They were housed in plastic cages with proper bedding (rice husk) and were fed with "standard laboratory feed pellets" diet and water *ad libitum*.

2.3.2 Preliminary screening for the anti-implantation activity

Four groups of matured female albino rats (6 rats per group) were selected for ethanolic extract, group I served as control and received 2% gum acacia (1 ml each) while, groups II, III and IV received the drug at the doses of 100, 125 and 250 mg/kg body weight respectively, orally, on day 4 and days 1-7 *post-coital* respectively. Three groups of matured female albino rats (6 rats per group) were selected for the isolated compound Group I served as control and received 2% gum acacia (1 ml each) while Groups II and III received the drug at the doses of 5 and 2.5 mg/kg body weight respectively, orally, on days 1-7 and day 4 *post-coital* respectively. On day ten of pregnancy, the animals were laparotomized and the numbers of implants present in both the uterine horns as well as the number of corpora lutea on each ovary were counted. The animals were allowed to complete the gestation period and the number of litters delivered if any, were counted [9].

2.3.3 Preliminary screening for the estrogenic/anti-estrogenic activity

Immature female rats (30-40 gms), were divided into four groups of 6 animals each. Group I served as control and received 2% gum acacia and 0.1 ml 0.5% Carboxy Methyl Cellulose, while group II received the drug (ethanolic extract / isolated compound) at the dose of 125 mg/kg/5 mg/kg respectively. Group III received 0.05 µg oestradiol benzoate (s.c) in 0.5% Carboxy Methyl Cellulose while group IV

received the drug (ethanolic extract/isolated compound) at the dose of 125mg/kg/5mg/kg body wt. (orally) respectively and 0.05 µg oestradiol benzoate (s.c). The treatment was continued for 4 days, 24 hrs after the last dose (i.e., on 5th day, the animals were then sacrificed and their ovaries and uteri dissected and weighed [10].

2.3.4 Preliminary screening for the anti-ovulatory studies.

Immature rats were divided into four groups of six rats each. Group I served as control and received 2% gum acacia and 0.1 ml 0.5% Carboxy Methyl Cellulose, while group II received the drug (ethanolic extract / isolated compound) at the dose of 125 mg/kg / 5 mg/kg respectively. Group III received eCG (10 I.U. s.c.) and hCG (10 I.U. s.c.) while group IV received the drug (ethanolic extract / isolated compound) at the dose of 125mg/kg / 5mg/kg body wt. (orally) respectively and eCG (10 I.U. s.c.) and hCG (10 I.U. s.c.). The animals were sacrificed 24 h after the hCG administration. Ovaries and uteri were dissected. The histopathological analysis of the ovaries and uteri of different groups was done [11].

2.4 Determination of oral LD₅₀

Acute toxicity was determined by oral route in 3 adult albino mice of either sex (35 -50 g). The alcoholic extract was suspended in 2% gum acacia in distilled water. The initial dose was at a level of 300 mg/kg body weight going upto 5000 mg/kg. Mortality during the next 24 hr was recorded and approximate LD₅₀ value determined [12].

2.5 Statistical analysis

The data was analyzed statistically to determine whether there was any significant difference among different groups using ANOVA single factor test, using XL STAT 2006 software [12].

3.0 Results

3.1 Phytochemical and chemical screening

The moisture content, total ash, water soluble ash, acid insoluble ash, alcohol soluble extractive value and water soluble extractive values were found to be 0.8, 2.8, 1.49, 0.55, 5.8, 22.92% respectively.

The absorption maximum of 1% methanolic solution of isolated compound was observed at 254 nm. The FTIR as neat film in NaCl : showed the presence of the following functional groups in the isolated fraction, FTIR ν_{max} /cm⁻¹ 1014.5 (C---O stretching); 1415.7 (aliphatic C=C stretching); 1554. (aromatic C=C stretching); 2873.7 (C-H stretching); 2966.3 (CH₂ stretching in heterocyclic ring system and acyclic system); 3417 (hydrogen bonding -OH stretching). ¹H NMR in CDCl₃ at 400 MHz exhibited various chemical shifts (ppm) observed as δ 7.26 (singlet), 3H (Ar-H); δ 6.58 (doublet), 3H (Unsaturated H); δ 5.05(singlet), 1H (-OH of the Hetero nucleus) ; δ 4.65 (singlet), 2H (-OH in conjugation); δ 2.31, 1H (CH-OH); δ 1.30 – 2.05 (multiplet) (CH₃ of the cyclic and acyclic system); δ 0.98 (multiplet) (CH₃). The isolated compound was further studied for carbons using ¹³C NMR in CDCl₃ at 400 MHz which exhibited various chemical shifts (ppm) observed at δ 24.22—39.10 (Sp3 hybridized carbon, CH₃ and CH₂ of acyclic system); δ 48.37 - 49.63 (Sp3 hybridized carbon, CH₂ of cyclic system); δ 64.00 (C—OH cyclic) ; δ 64.26 (C—OH acyclic); δ 74.47 (Sp2 hybridized carbon, C=C alkenes); δ 130.83 (aromatic carbons). On subjecting the compound to GC-MS, a sharp single peak was observed at 47.365 and which on Electron impact fragmentation showed molecular ion peak at m/z 279, (M+H); m/z 253, (loss of 26 CH₂=CH₂); m/z 221 (M-57); m/z 207 (M-71), m/z 193, m/z 167, (76%) (C₉H₁₀O₃); m/z 149 100% Base peak (167 –18 (H₂O) m/z 132 [(base peak- 31(CH₂ OH)] m/z 113, (M-base peak); m/z 93 (113-18(H₂O) (Fig. 1).

3.2 Pharmacological screening

3.2.1 Preliminary screening for the anti-implantation activity

The calculated mean percent anti-implantation activity of the ethanolic extract at the doses of 100, 125 and 250 mgs/kg body weight compared to the mean percent anti-implantation activity of its isolated compound at the doses of 5 and 2.5 mg/kg bodyweight on day 4 and days 1-7 compared to their Control group is as given in Table I. The percent anti-implantation loss for treated groups was analyzed statistically with respect to Control and was found to be significant in all cases ($p<0.05$).

3.2.2 Preliminary screening for the estrogenic/anti-estrogenic activity

Estrogenic/anti-estrogenic activity of the ethanolic extract and its isolated compound at the dose of 125mg/kg and 5mg/kg respectively, was calculated on the basis of the weights of ovaries and uteri administered orally to immature female albino rats as shown in (Table 2).

3.2.3 Preliminary screening of the ethanolic extract of *Ensete superbum* seeds for the anti-ovulatory studies

Anti-ovulatory activity of the ethanolic extract at the dose of 5mg/kg-body weight was studied on immature female albino rats. The

Table 1. Comparative anti-implantation activity of the ethanolic extract and the isolated compound of *Ensete superbum* Cheesm seeds

Treatment	Percentage anti-implantation	
	Day 4 (post-coital)	Days 1-7 (post-coital)
Ethanolic extract Control	28.9	32.8
100 mg	53.65	86.9
125 mg	65.36	94.4
250 mg	82.29	98.77
Isolated compound Control	27.6	25.95
2.5 mg	65.9	87.5
5.0 mg	72.6	96

Table 2. Comparative anti-estrogenic activity of the ethanolic extract and the isolated compound of *Ensete superbum* Cheesm seeds

Treatment	Mean Weight of Ovaries ± S.D. (mg)	Mean Wet Weight of Uteri ± S.D. (mg)
Ethanolic extract		
Control	27.02 ± 0.38	78 ± 1.32
Drug 125mg/kg (oral)	25.03 ± 0.59	65.03 ± 0.80
Oestradiol Benzoate (0.05 µg) s.c	36.17 ± 1.05	154.53 ± 1.94
Oestradiol Benzoate (0.05 µg) s.c. + Drug (125 mg/kg) oral	30.87 ± 0.66	107.5 ± 0.70
Isolated compound		
Control	26.45 ± 0.82	35.94 ± 1.05
Drug 5 mg/kg (oral)	25.16 ± 0.73	34.93 ± 0.98
Oestradiol Benzoate (0.05 µg) s.c	41.9 ± 1.57	115.74 ± 4.76
Oestradiol Benzoate (0.05 µg) s.c. + Drug (5 mg/kg) oral	35.91 ± 0.99	77.64 ± 4.24

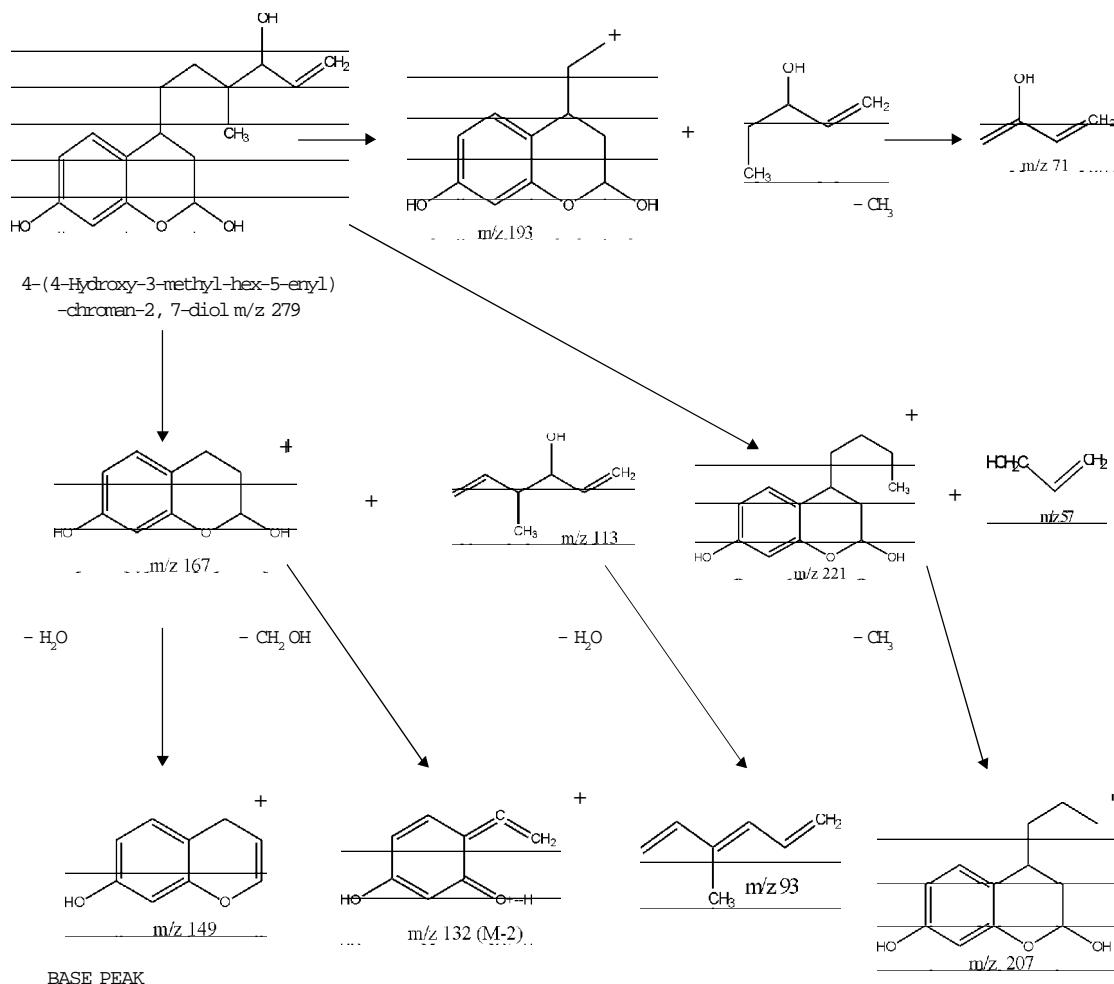


Fig.1: Fragmentation patterns of the isolated compound of the seeds of *Ensete superbum* using EIMS

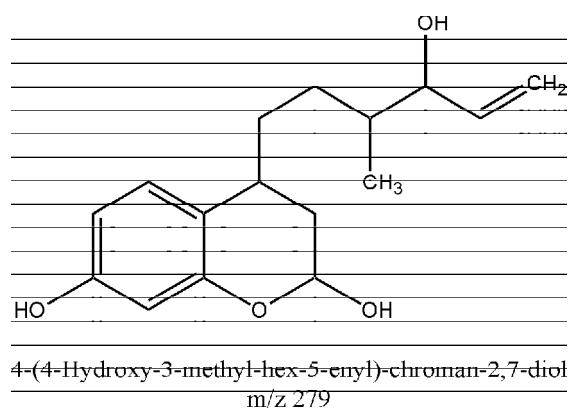


Fig.2: Possible structure of the isolated compound of *Ensete superbum* Cheesm seeds

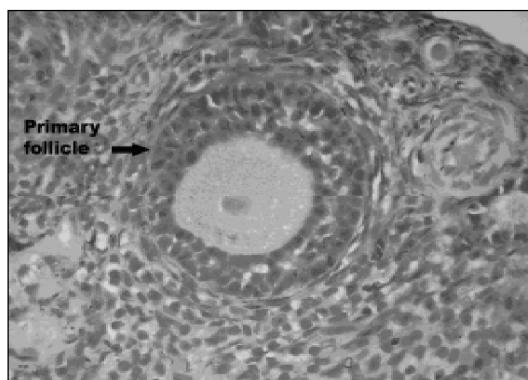


Photo I
GROUP I (Control, 2% Gum Acacia) Immature ovary with numerous primary follicles, no corpus luteum



Photo II
GROUP II (Drug 5 mg/kg) Mature ovary. Numerous primary follicles with theca interna cells lining the follicular cavity

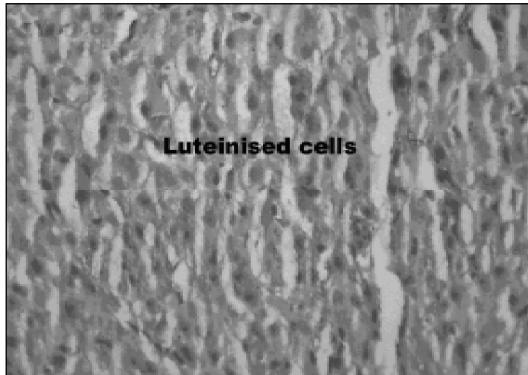


Photo III
GROUP III (eCG + HCG) Ovary shows corpus luteum along with its luteinised cells and a primary follicle

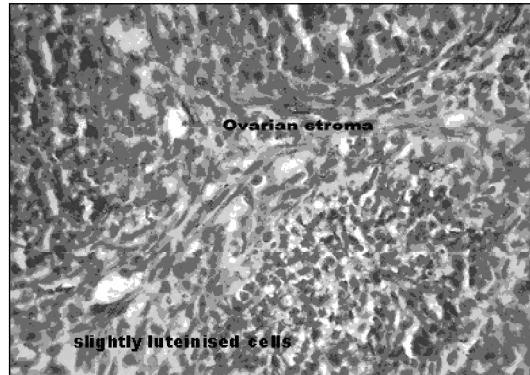


Photo IV
GROUP IV (Drug 5 mg/kg + eCG + HCG) Immature ovarian stromal tissue along with slightly luteinised cells

Fig. 3. Comparative Histological Photographs of the ovaries of different treatment groups (400X).

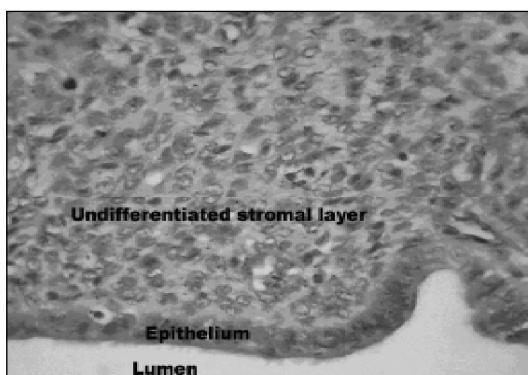


Photo V
GROUP I (Control, 2% Gum Acacia) Immature uterus with flat lining and undifferentiated endometrium, low epithelial layer and undifferentiated stromal layer

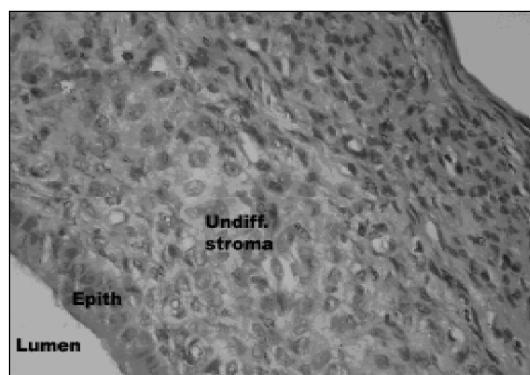


Photo VI
GROUP II (Drug 5 mg/kg) Proliferative phase showing epithelial lining and, undifferentiated stroma

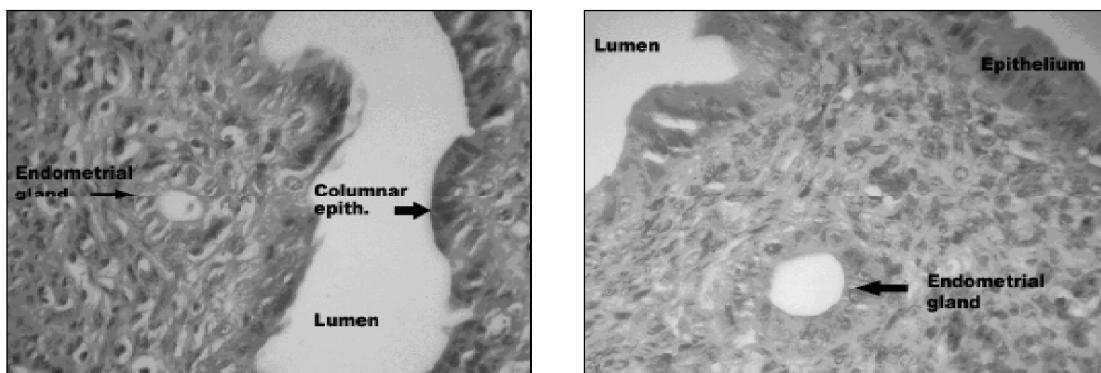


Photo VII
GROUP III (eCG + HCG) Hyperplastic lining thrown into folds, glandular differentiation in stroma and endometrium and columnar changes in the epithelial lining.

Photo VIII
GROUP IV (Drug 5 mg/kg + eCG + HCG) The uteri showed some features of differentiation both in the uterine lining and stroma

Fig. 4. Comparative Histological Photographs of the uteri of different treatment groups (400X)

histopathological analysis of the ovaries and uteri of different groups was observed as in Fig. 3 and 4.

3.3 Determination of oral LD_{50}

The oral LD_{50} of the ethanolic extract of the seeds was calculated to be 3235.9 mg/kg.

4. Discussion

The ethanolic extract and its isolated compound of the seeds of *Ensete superbum* cheesm was subjected to Phytochemical and Pharmacological screening. HPTLC profile of isolated fraction in solvent system (toluene: ethyl acetate: formic acid 5:4.5:0.5), showed a single spot with R_f of 0.69. FTIR frequency at 1456cm^{-1} ^1H NMR 7.26(s), 3H of aromatic hydrogen and ^{13}C NMR δ 130.83 confirms the presence of aromatic moiety in the compound, a prominent peak at 3417cm^{-1} by FTIR due to bonded –OH stretching and d 4.65 (s) by ^1H NMR confirms the presence of phenolic moiety. ^1H NMR δ 1.30 –2.05 and ^{13}C NMR δ 24.22–39.10 and δ 48.37–49.63 confirms the Sp3 hybridized carbon, CH_3 and CH_2 of acyclic system and Sp3 hybridized carbon, CH_2 of the

cyclic system respectively. On the basis of the fragments obtained in mass spectra, [fig I], the molecular ion peak $\text{C}_{16}\text{O}_4\text{H}_{22}$ with m/z 279 of the compound was then confirmed from database Chem Draw. From the spectral data, the structure of the compound appears to be a chroman derivative (Fig. 2).

The pharmacological study revealed that both the ethanolic extract and its isolated compound showed a significant anti-implantation activity as shown by the decrease in number of implants due to an imbalance in endogenous estrogen and progesterone levels and when given along with ethinyl estradiol exhibited anti-estrogenic activity. This shows that the extract and its isolated compound possibly acted as competitive antagonist to the much more potent ethinyl estradiol. The above results were also evidenced by the histopathological analysis.

The estrogenicity of the compound can be understood in view of the superficial similarity to Equol and estrogen, the similarity include an aromatic ring system with hydroxy group at the same position and another hydroxy group on the acyclic ring system attached to the

heterocyclic ring [13-14]. A strategically located phenolic hydroxyl group, with no alkyl substitution in the *ortho* position is necessary for high affinity binding to the receptor and potent estrogenic activity *in vivo*. Extending a side chain away from the binding site in a bis phenolic compound produces a binding ligand with anti-estrogenic activity *in vitro* [15]. Significant anti-estrogenic activity of the fraction suggests that it might either be interrupting the release of estrogen on day 4 (the day of Estrogen surge) or by antagonizing the action of estrogen. No teratogenic effects were seen and the drug

showed reversibility of action. Therefore, to conclude the isolated compound of molecular formula C₁₆O₄H₂₂, appears to be a chroman derivative 4-(4-hydroxy-3-methyl-hex-5-enyl)-chroman-2, 7-diol, possess significant anti-implantation activity, which could be attributed to its anti-estrogenic action.

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