Effects of ethanol extract of Dissotis rutundifolia on the histology of the ovary, uterus and Gonadotropins of adult female Whistar rats

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Effects of ethanol extract of *Dissotis rutundifolia* on the histology of the ovary, uterus and Gonadotropins of adult female Whistar rats.

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ABSTRACTS

This study was aimed at evaluating the effects of ethanol extract of Dissotis rutundifolia on the histology of the ovary and uterus of Adult whistar rats. A total of twenty (20) wister rats weighing 150- 180g were used in this experiment and were divided into 5 groups (1-5) of 4 rats per group. Group 1(control) were administered 2ml distill water, group 2 (low dose) received 25mg/kgbw daily, group 3 (low dose + withdrawal) 25mg/kgbw, group 4(hgh dose) received 100mg/kgbw while group 5 received (high dose + withdrawal received) 100mg/kgbw. the extracts was administered by gavage once daily for 14 days in all treated groups while in group 3 and 5 there was withdrawal for seven days. At the end of treatment the rats were sacrificed and the Ovary and Uterus harvested and histologically processed and stained using H&E stains while blood samples was obtained intraoccularly for hormonal assay all groups. The histological results showed a loss in follicles production and an increase in atretic follicles in ovaries in ovaries with loss of endometrial and glandular cells in the uterus. There was a significant decrease in serum levels of follicle stimulating hormone (FSH) and Luteinizing hormones (LH) with prolonged dioestrous stage as compared to the normal control group. In the withdrawal groups normal ostrous cycle was observed with production of ovarian primary follicles, restoration of uterine endometrium and glandular cells. The level of FSH and LH was also restored.

INTRODUCTION

Medicinal plants are commonly used in treating and preventing specific ailments and diseases, and are generally considered to play a beneficial role in healthcare. They are already important to the global economy. Demand is steadily increasing not only in developing countries but also in the industrialized nations [1]. World Health Organisation (WHO) estimates that approximately 80% of the developing world's population meets their Primary Healthcare needs through traditional medicine [2]. About 25% of prescription drugs dispensed in the United States contain at least one active ingredient derived from plant material. Some are made from plant extracts while others are synthesized to mimic a natural plant compound [3]. It has been estimated that one out of every three people in the United States had tried at least one form of alternative medicine [4]. A follow up study reported that the number of respondents using alternative therapies increased from 33.8% in 1990 to 42.7% in 1997 [5]. Within the last few decades, many plants have been screened for their biological and pharmacological properties by researchers. These efforts are continually being taken to examine the merits of traditional medicine in the light of modern science with a view aimed at adopting effectively beneficial medical practice and discouraging harmful ones [6].

D. rotundifolia Triana, a native of tropical West Africa belongs to the Melastomataceae family [7] and common names include Pinklady (English), Awede or Ajagunmorasin (Yoruba) and Ebafo (Bini) [8].

D. rotundifolia is a versatile perennial slender creeping herb with prostate or ascending stems up to 40cm high, rooting at the nodes and producing from seeds and stolon. The leaves are ovate to ovate-lanceolate, 1.5-7.0 cm long, 0.8-4.0 cm wide, 3-nerved, both surfaces sparsely to densely pilose, margins ciliate and somewhat crenuate, apex acute, base truncate to short-attenuate, petioles 0.5-2.5 cm long [9].

In Nigeria, D. rotundifolia is used mainly for the treatment of rheumatism and painful swellings. The leaves decoction is used to relieve stomach ache, diarrhea, dysentery, cough, stop abortion, conjunctivitis, circulatory problems and venereal diseases [8]. It is used in East Africa for the treatment of bilharzias in Cameroun [10]; the leaves are used for dysentery [11]. In tropical Africa, the whole plant is used as a remedy for rheumatism and yaws and as an antihelmintic and in Liberia for diarrhea [12]. Hot water extract of *D. rotundifolia* given orally is used for hookworm infestations [11]. Basically, when the plant is to be used in a concoction it is usually boiled or crushed and the applied or eaten. Other uses include; composting, manuring; ornamental, treatment for arthritis, rheumatism, dropsy, swellings, oedema, gout; febrifuges, as genital stimulants/depressants, treatments for naso-pharyngeal affections; infertility, antiaborifacients; pulmonary troubles; vermifuges, eye infections; malnutrition, debility; stomach troubles,etc. it has also been said to act as a painkiller [13]. All these practices and medicine span across West Africa. The use of it in the “cure” of infertility is not its most popular use but it has been used in that manner in folk tales [14]. In Cameroon it has also been used to treat fibromyoma [15]. Also it has been recorded that this plant has been used to treat bilhalzia and expulsion of placenta.

Phytochemical screening of the leaves of *D. rotundifolia* revealed the presence of alkaloids, tannins, saponins and cardiac glycosides [14]. It has also been found to contain trepenes, tannins, carbohydrate and flavonoids [16].

The female reproductive organ is a system which comprises of the primary sex organs which include a pair of ovaries and the accessory organs which include the fallopian tubes, uterus, cervix and vagina. These structures work together to carry out part of the process of procreation in females. Certain chemicals like drugs, plant extracts and metals acting on one or more of these structures could cause alterations in their histology, structure and function [17].

Dissotis rotundifolia has shown help in treating most uterine epithelium diseases and fibromyoma [18]. While treating these diseases the effects on the ovaries could lead to infertility considering the chemical constituents in the plant. This study is aimed at investigating the changes that occur to the oestrus cycle and the histological changes that occur on treating the adult female rats with the leaves extract. The objectives of the study was to investigate the effect of the methanol extract of *D. rotundifolia* on the histology of the ovaries and uterus, study the oestrus cycle and levels of Gonadotropins (FSH and LH) of female adult Whistar rats.

MATERIALS AND METHODS

Materials

Albino rats (Ratus novergus), syringes, 4 cages, 1 cage stand , hormone kit, 5 feeding troughs, 10% formosaline, 5 water bottles, 2 bags of vita chicken feed (Vital feeds grand cereals Ltd, Jos), saw duct, tissue processor, *Dissotus rotundifolia* (*melastomaceae*) (Pinklady), paraffin wax, distilled water, markers, saline water, dissecting set, refridgerator (Thermocool), EDTA bottles, organ bottles, weighing balance (Ohaus Explorer), 5ml syringes (Elsalmat pharmaceuticals), capillary tube, metal oral cannula, pins, disinfectant, hand gloves, measuring cylinder, glass slides, digital camera (Sony), Olympus microscope .

Animal source and handling

Twenty (20) Adult female rats weighing between 150 – 180g procured from the animal house of the Nigeria Institute for Trypanosomiasis and Onchocerciasis, Kaduna State, were used for this experiment. The rats were kept in the animal house of Bingham University, Karu and acclimatized for two weeks before the experiment commenced. The rats were fed on standard diet (Vital Feeds and Grand Cereals Ltd, Jos, Plateau State); water was given (ad libitum) and maintained under standard conditions. The animal house was well ventilated with a temperature range of 25-27 ˚C under day/night 12-12h photoperiodicity. The weight of the rats was taken prior to the commencement of treatment and 24 hours after the last day of administration before the animal were sacrificed.

Extract Preparation

Dissotis rotundifolia (Melastomaceae) was obtained in the month of April, 2013 from Itoku market, Abeokuta, Ogun state, Nigeria. It was authenticated by Dr (Mrs) Olorunmaiye P.M; Weed Scientist of Plant Physiology and Crop Production Department, Federal University of Agriculture Abeokuta, Ogun State. The leaves of the plant were removed and air dried under room temperature. The dried leaves were grounded into fine powder and 250g of the powder was soaked in 2.0 liters of methanol. The solution was filtered after 24hours while the filtrate was concentrated to a semi solid form using the rotary evaporator. 20g of the concentrate was obtained by maceration method and dissolved in distilled water for dosage preparation. The LD_{50} of the extract had been predetermined as 500mg/kg body weight [14]. The stock solution was prepared using 3g of the extract dissolved in 75ml of distilled water. This led to a stock solution with a concentration of 40mg/ml of solution.

Experimental protocol

A total of twenty (20) wister rats were used in this experiment and were divided into 5 groups (1-5), each cage containing rats of weight range 150g to 180g.

Table 1 Treatment protocol

Group name	Number in group	Treatment
Group1 (Control Group)	4	Receive distilled water (2ml)
Group2 (Low dose)	4	25mg/body weight
Group3 (Low dose + withdrawal)	4	25mg/body weight
Group 4 (High dose)	4	100mg/body weight
Group 5 (High dose + withdrawal)	4	100mg/body weight

All of these were dispensed between 0900 hours and 0930 hours for 14 days. Administration was by gavage using metal oral canulas. After 14 days of administration, 2 from Group 1 and all of Groups2 and Group4 were sacrificed. The others (the remaining 2 from control and Groups 3 and 5) were left for 7 days as a withdrawal experiment and then sacrificed on the 8th day. The body weights of the rats where then measured just before administration, after 7 days of administration, after 14 days of administration and for the remaining left after 21 days.

Animal Sacrifice

Animals were sacrificed 24hours after last administration. Blood was taken intraoccularly from all groups for hormonal assay for FSH and LH and the animals were demobilized by cervical dislocation. The ovary and uterus were harvested after abdominal incision. The ovaries and uterus were then fixed in 10% formal saline and stained using Haematoxylin and Eosin stains.

The blood was used for hormonal assay for FSH and LH.

Histological Analysis

The harvested organs were carefully dissected out, trimmed of fat and connective tissue. The tissues were processed.

Statistical analysis

All the data were statistically evaluated using One-way Anova (Analysis of variance) on SPSS/17.0 software (SPSS Inc, Chicago, USA) and the data were presented as Means ± Standard Error of Mean (SEM). Differences were considered to be of statistical significance at the error probability of less than 0.05 ($P<0.05$).

RESULTS

Table 2 showed the phytochemical analysis of *D. rotundifolia.*

The results obtained showed that there was a significant decrease in weight of the treated Whister rats groups as compared to the normal control group and the withdrawal treated groups. From the results obtained it was observed that the methanolic extract of *Dissotis rotundifolia* induced a dose dependent weight loss when compared to the control group, while there was a significant weight gain in the withdrawal groups for 7 days.

Table 2 Phytochemical Analysis Results

Phytochemical	Presence
Tannins	+
Saponins	+
Alkaloids	+
Flavonoids	-
Phenols	+
Glycosides	-
Terpenes	-
Resins	+
Carbohydrate	+

+ = Present - = Absent

Weight studies

Table3. Effect of *Dissotis rotundifolia* on Body Weight of Female Wister Rats

Body weights	**(Control)**	**(Low Dose)**	**(High dose)**
	(Mean ±SEM)	(Mean ±SEM)	(Mean ±SEM)
Initial Weight	150±5.00	149.5±4.39	145±6.48
After 7 days	156±3.75	134±6.73*	132.5±4.58*
After 14 days	162±2.00	123±2.03*	118±4.25*
Withdrawal (7dayspost administration)	164±4.50	142±4.78*	140.8±3.04*

** = P < 0.05 level of significance (Anova).*

Microscopic findings of the effects of ethanol extract of *Dissotis rutundifolia* on normal and treated ovaries and uterus of adult Whistar rats.

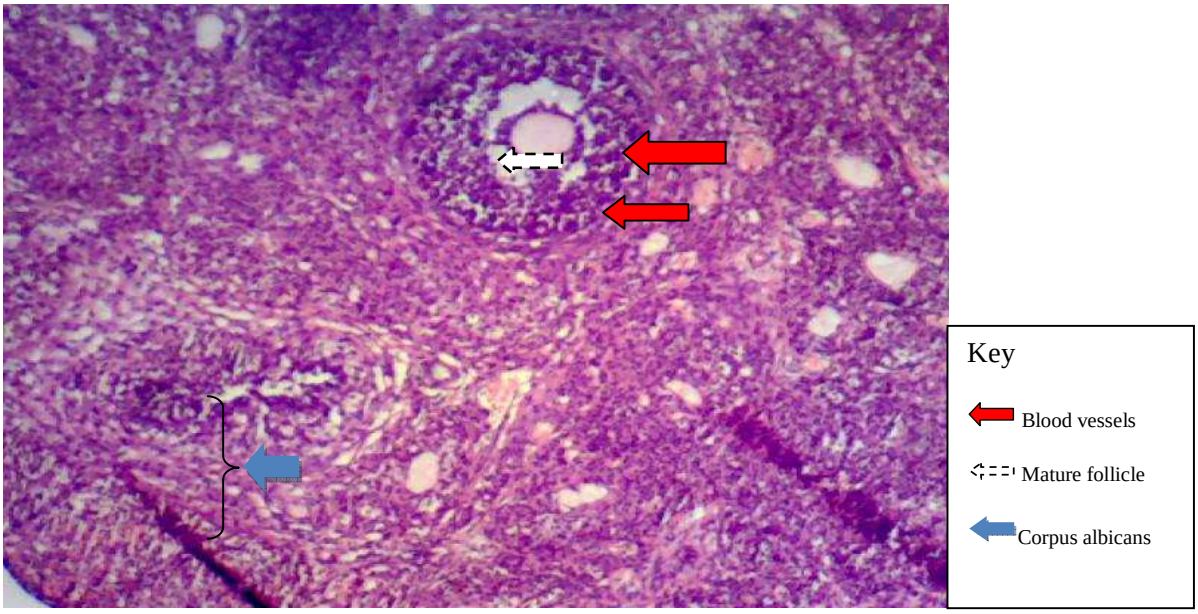

Plate 1: Micrograph of Control ovary showing ovum, mature follicle, corpus albicans and blood vessels in the stroma with numerous follicular and stroma cells (H & E stain x400).

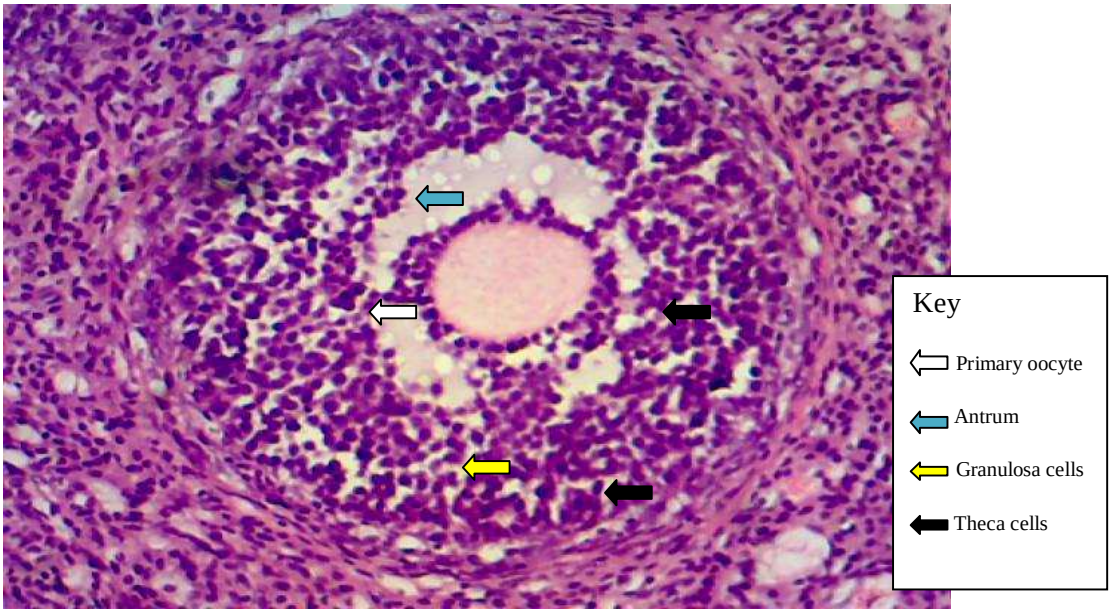

Plate 2: micrograph of Control ovary showing normal mature follicle with primary oocyte, antrum, granulose cells and theca cells (H & E stain x1000).

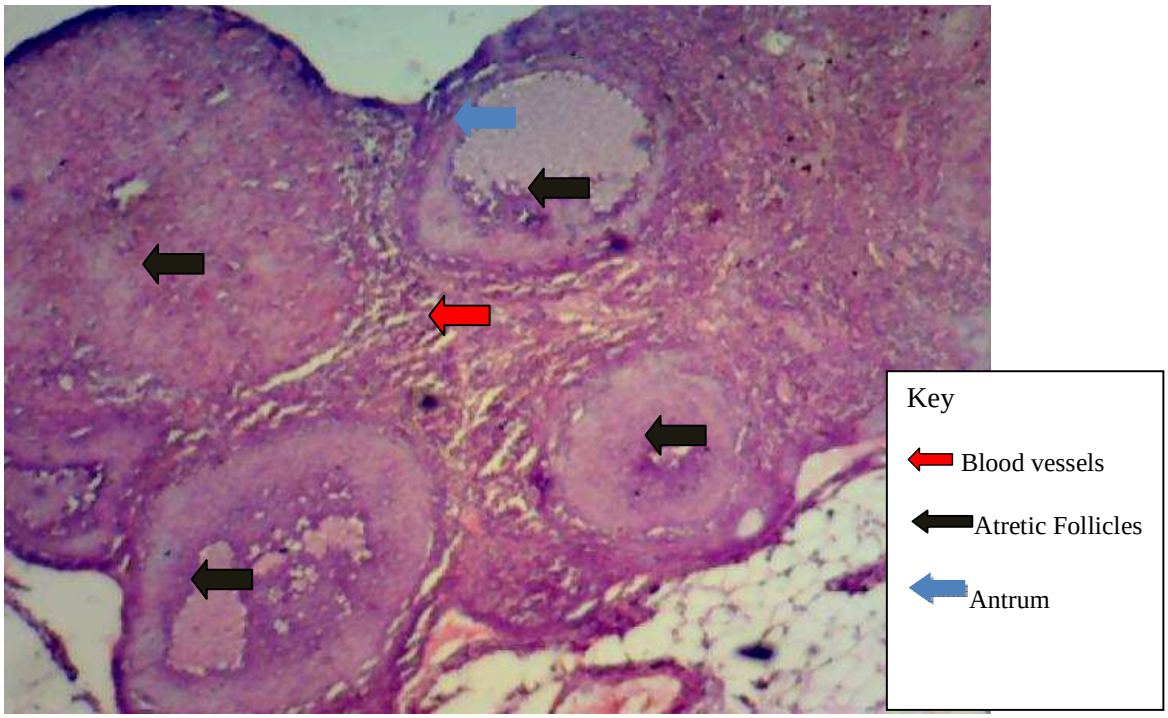

Plate 3: Micrograph of Low dose ovary showing numerous atretic follicles (eventually leading to corpus luteum) and secondary follicles with no new follicles being formed amongst few and abnormal spaces in the stroma. (Mass degeneration of follicular cells) (H & E stain x400).

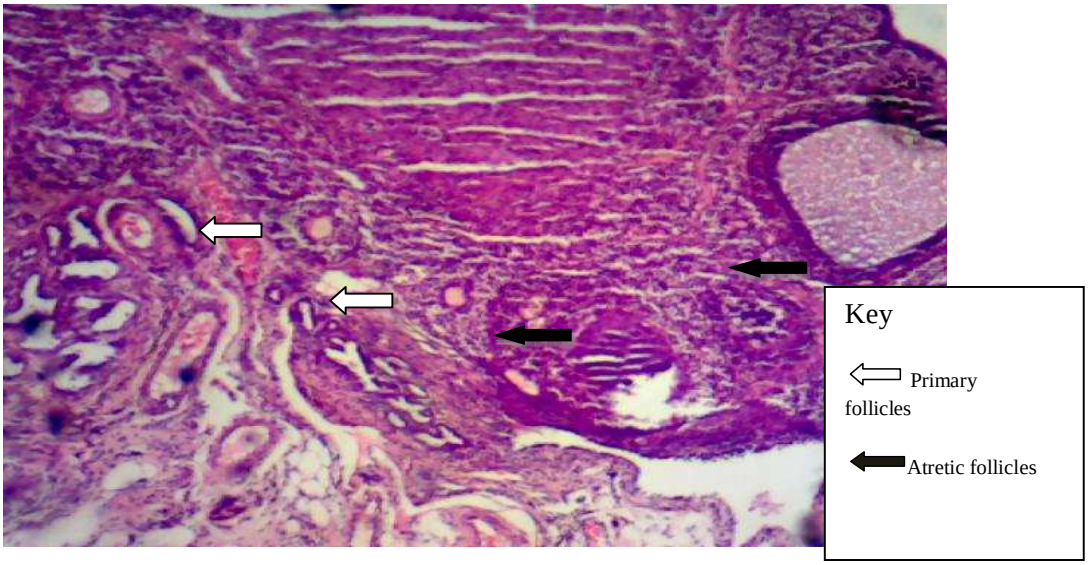

Plate 4: Micrograph of Low dose + withdrawal ovary showing the primary follicles and empty atretic follicles without mature follicles present in them and abnormal spaces within the stroma (regeneration in progress) (H & E stain x400).

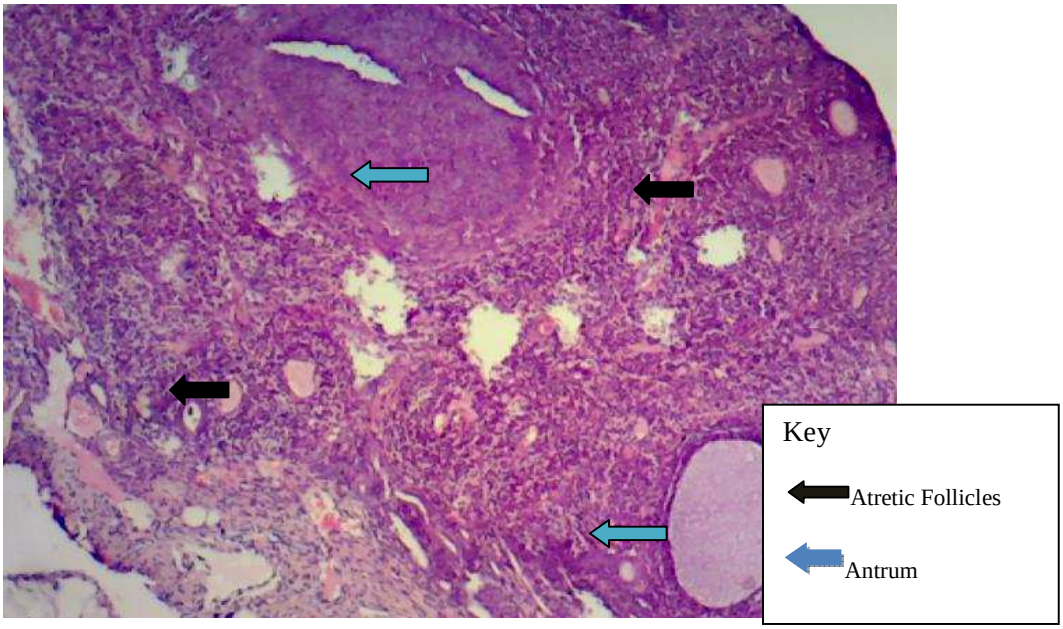

Plate 5: Micrograph of High dose ovary showing atretic follicles, antrums without primary oocytes within them and poorly developed blood vessels and abnormal spaces in the stroma (Massive degeneration of glandular cells) (H & E stain x400).

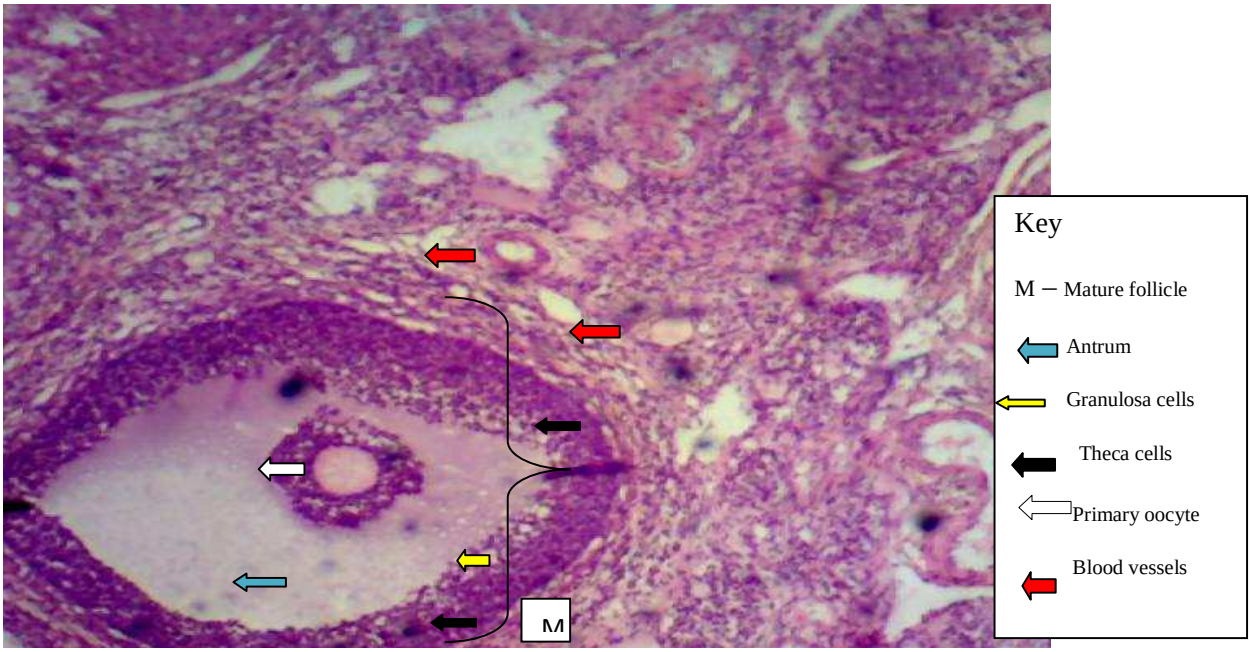

Plate 6.Micrograph of High Dose + Withdrawal ovary showing a mature follicle (H & E stain x1000).
Microscopic finding of the Uterus

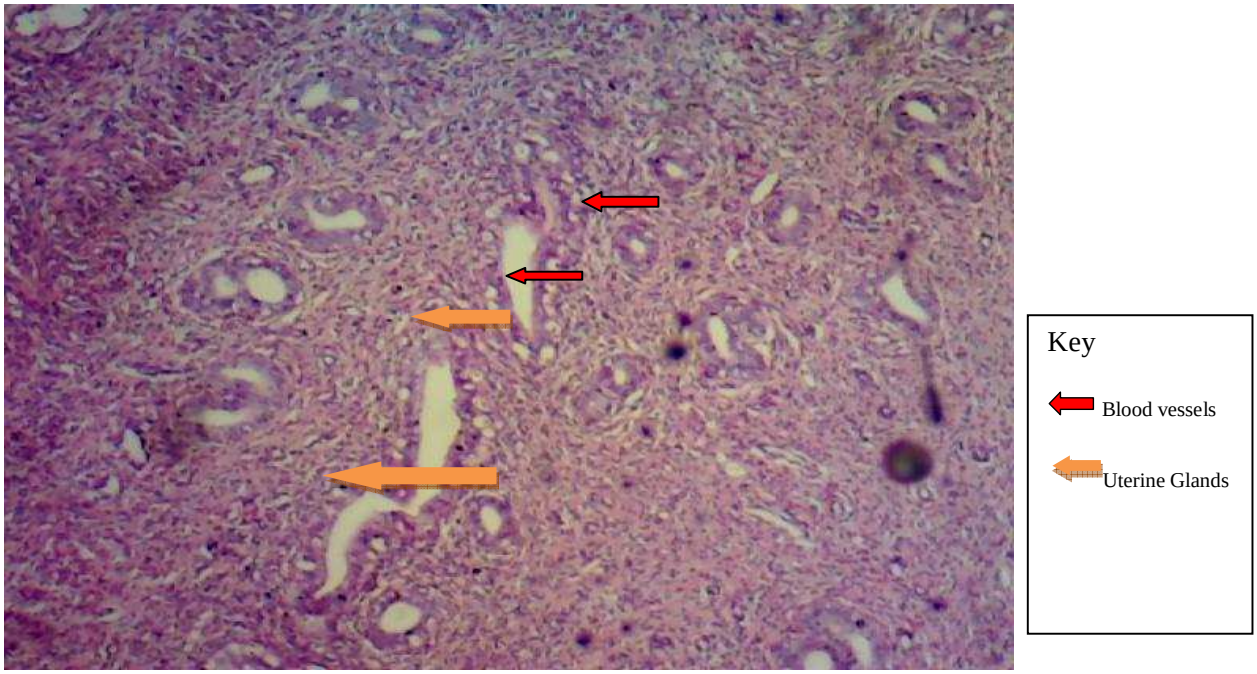

Plate 7: Micrograph of Control uterus magnification showing normal histo-architecture of endometrium with abundant blood supply and uterine glands (H & E stain x400).

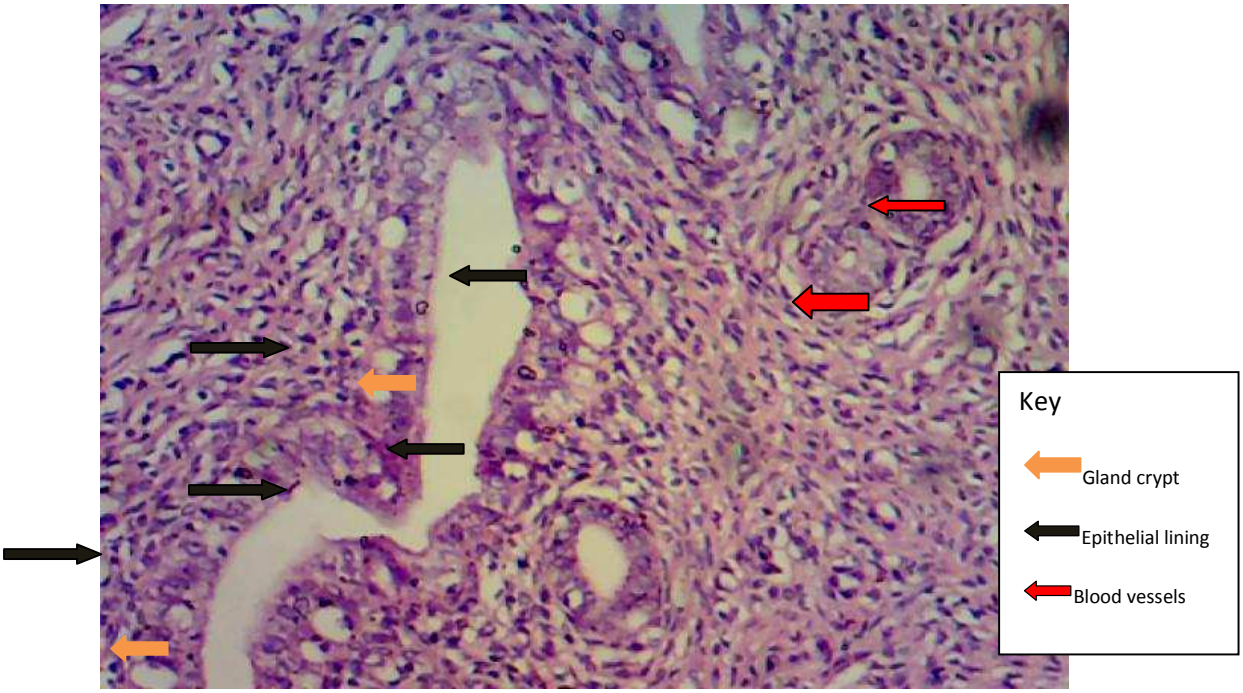

Plate 8: Micrograph of Control uterus showing uterine gland crypt, secretory epithelial lining and numerous blood vessels of the Uterine gland (H & E stain x1000).

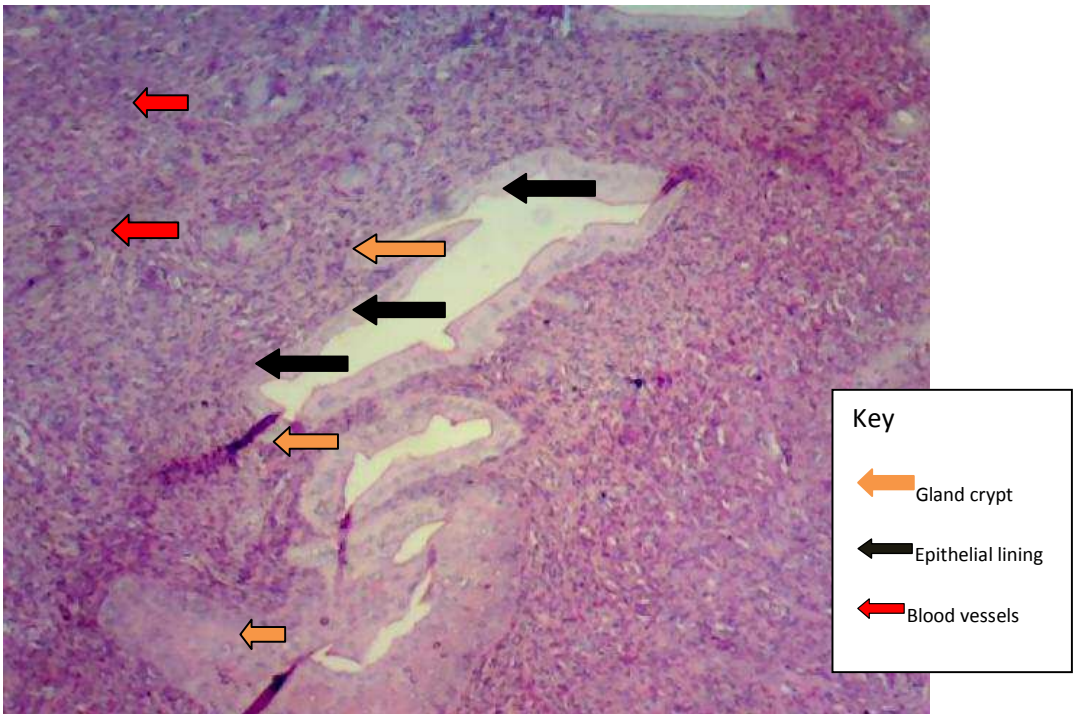

Plate 9: Micrograph of Low dose uterus showing uterine glands, loss of uterine lining (epithelium in glands) incapable of secretions and very little blood supply (degeneration of glandular cells) (H & E stain x400).

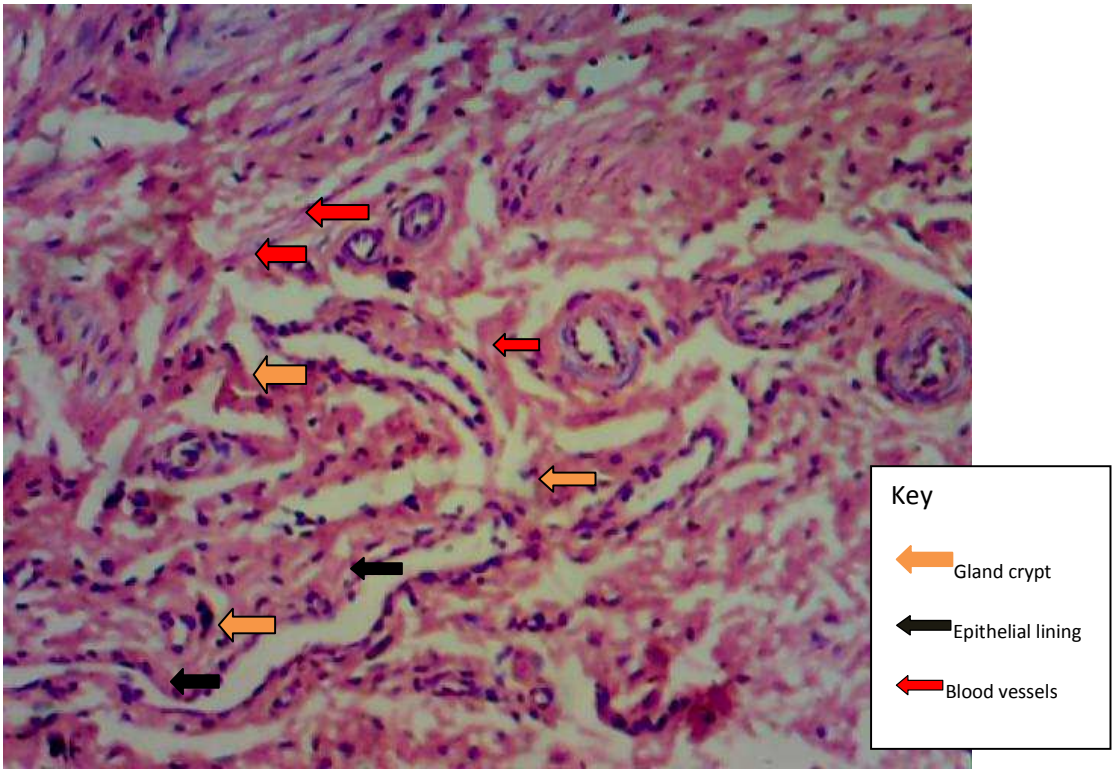

Plate 10: Micrograph of Low dose + withdrawal uterus showing numerous blood vessels, newly formed uterine glands with rejuvenated endometrium lining capable of secretions (early secretory phase i.e. regeneration) (H & E stain X1000).

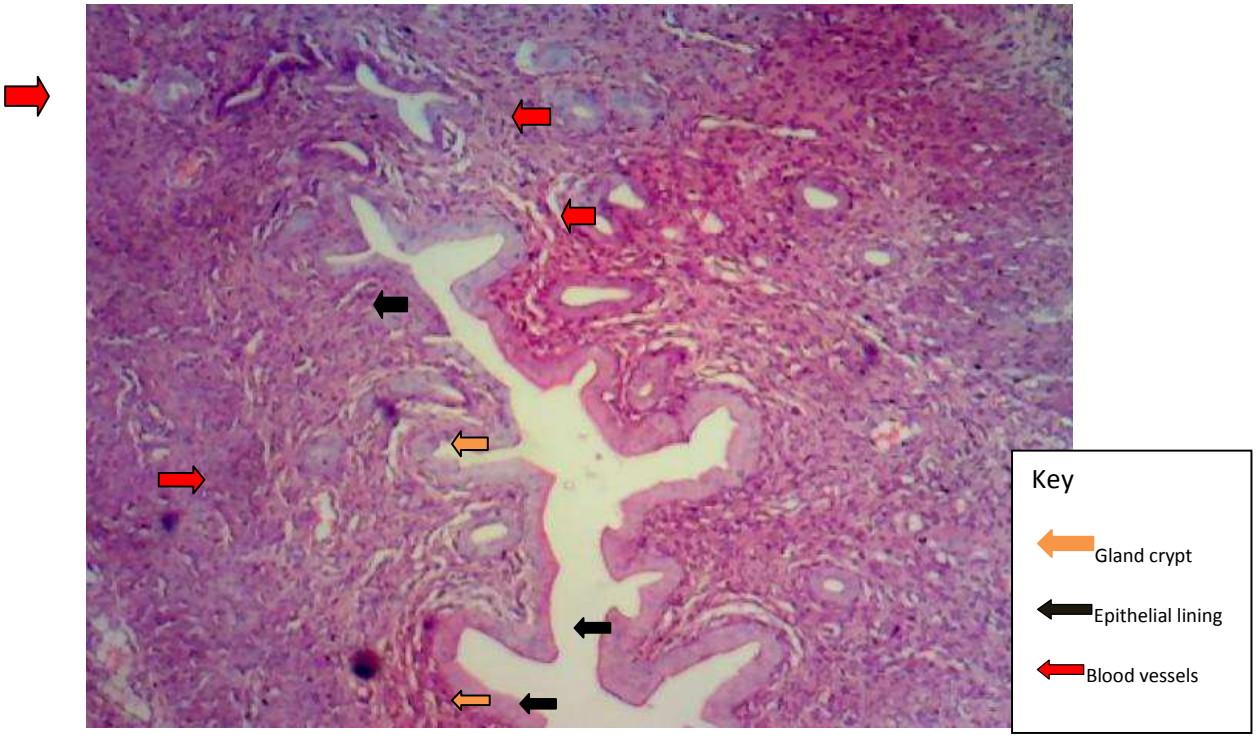

Plate 11: Micrograph of High dose uterus showing loss of the endometrium, numerous blood vessels with deep and tortuous uterine glands (Degenaration of endometrium and glandular cells) (H & E stain x400).

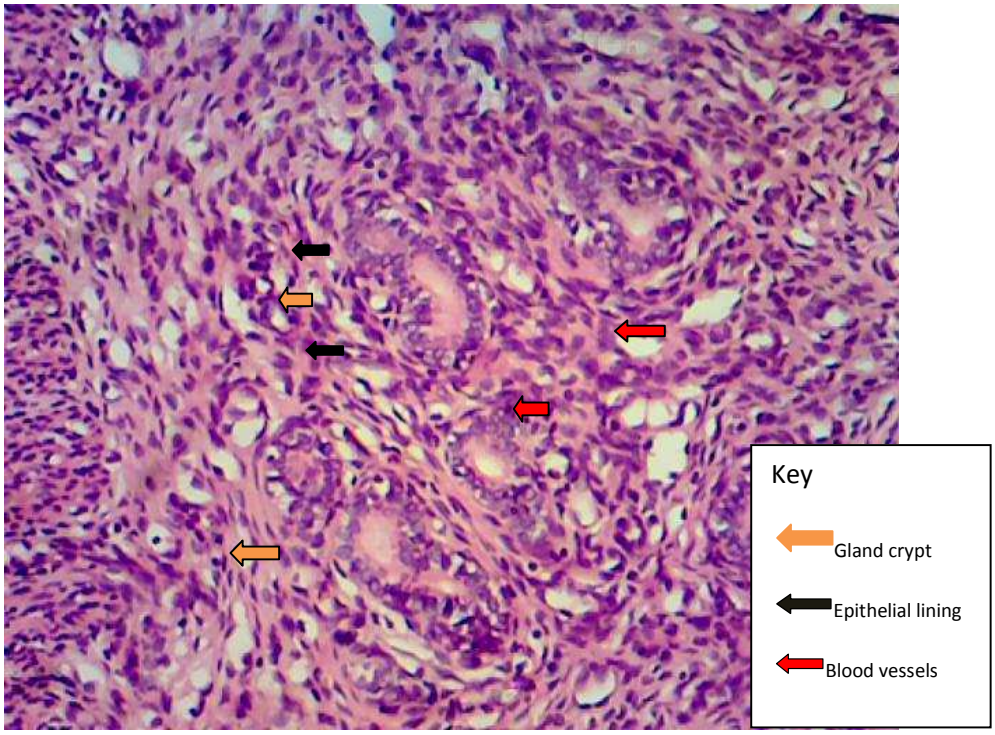

Plate 12: Micrograph of High dose + Withdrawal uterus showing endometrium with highly proliferated lining and secretions in the uterine glands (earliy secretory stage i.e. endometrial regeneration) (H & E stain x1000

Hormonal Assay

Table 4: Effect of Methanolic extract of *Dissotis rotundifolia* on hormones

Hormone	(Control)	(Low Dose)	(High Dose)
	(Mean±SEM)	(Mean±SEM)	(Mean±SEM)
LH	17.5±2.50	5.5±2.17*	3.9±0.31*
LH (After 7 days of Withdrawal)	15.5±2.50	17.5±2.10	10.8±0.47*
FSH	26±1.0	4.4±0.17*	4.6±0.50*
FSH (After 7 days of Withdrawal)	20.5±4.50	20.25±2.78	20±1.93

** = P < 0.05 level of significance (Anova).*

Serum level of both hormones showed reduction in treated rats. The reduction in LH level was dose dependent. The high dose results showed a more significant change compared to the control group. There was a return of hormone levels to higher levels after 7 days withdrawal except for withdrawal group for High dose.

The reduction in FSH did not look dose dependent even though there was significant decrease when compared to the control group. There was return of hormonal levels to higher levels after 7 days of withdrawal.

Analysis on Oestrus Cycle

These are images of the vaginal smear depicting oestrus cycle stages as observed in normal rats (Control Group).

Plate 13: Micrograph showing vaginal smears of rats in proestrus stage. Cells are rounded (intermediary cells) and sparse. (x100)

Plate 14: Micrograph showing vaginal smears of rats in Oestrus (ovulatory) stage. The cells are sparse, keratinized cells lying in groups or large flakes. (x100)

Plate 15: Micrograph showing vaginal smears of rats in Dioestrus stage of oestrus cycle. This mainly made up of cellular debris in high concentration. (x100)

Effects of *Dissotis rotundifolia* on Oestrus Cycle of Wister Rats

Table 5: Oestrus Cycle before Experiment (8 days).

Group	Proestrus (Days)	Oestrus (Days)	Dioestrus (Days)
Group1 (Control)	2	2	4
Group2 (Low dose)	2	2	4
Group3 (Low dose + withdrawal)	2	2	4
Group4 (High dose)	3	1	4
Group5 (High dose + withdrawal)	2	2	4

The oestrus cycle table showing the normal pattern of oestrus cycle phases within a course of 8 days (2 cycles) before beginning of the experiment. The table shows normal cycle with an average of 2 days of proestrus (1 day per cycle), 2 days of oestrus (1 day per cycle) and 4 days of dioestrus (2 days per cycle) for all the sample rats.

Table 6: **Oestrus Cycle during Administration (8 Days).**

Group	Proestrus (Days)	Oestrus (Days)	Dioestrus (Days)
Group1 (Control)	2	2	4
Group2 (Low dose)	2	0	6
Group3 (Low dose + withdrawal)	1	1	6
Group4 (High dose)	2	1	5
Group5 (High dose + withdrawal)	2	0	6

The table above shows oestrus cycle within a time of 8 days during administration. There is prolonged dioestrus stage (5 or 6 days out of 8 days) in the treatment groups with either 1 or 0 days of oestrus (ovulation) and 2 days in proestrus (1 day per cycle). These effects are not dose dependent.

Table 7: Oestrus Cycle during Withdrawal

Group	Proestrus(Days)	Oestrus (Days)	Dioestrus(Days)
Group1 (Control)	2	2	4
Group3 (Low dose + withdrawal)	2	2	4
Group5 (High dose + withdrawal)	3	1	4

Table showing oestrus cycle during withdrawal with the amount of days spent in each oestrus cycle stage through the duration of 8 days. This showed a return to normal oestrus cycle durations for the treatment groups with 2 - 3 days in proestrus (at most 2 days per cycle), 1-2 days in oestrus (at least 1 day per cycle) and 4 days in dioestrus stage (days per cycle). The recovery does not seem hindered by the dose initially administered.

DISCUSSION

Dissotis rotundifolia has been used across west and east Africa where it is abundant to cure illnesses such as stomach ache, diarrhea, dysentery, cough, stop abortion, conjunctivitis, dysentery, venereal diseases and hookworm infestations with some of these claims having being scientifically verified. [14]. Also it is used in West Africa in belief that it cures fibromyoma which has been scientifically verified. This is basically due to its anti-inflammatory and antimicrobial actions [14]. The plant has been used to treat these illnesses without study of the side effects.

This study demonstrates that methanolic extract of *Dissotis rotundifolia* has morphological, histological, biological and physiological effects on the ovary and uterus (major reproductive organs) of female wister rats.

Effect of *Dissotis rotundifolia* (Melastomaceae) methanol extracts on Histology of Ovary and Uterus

The histology of the ovary of rats from the control group showed numerous primordial cells and mature follicles indicating a normal microaarchitecture of the ovary. All rats in groups 2 and 3 which received 25mg/kg body weight of the extract while groups 4 and 5 received 100mg/kg body weight of methanolic extract of *Dissotis rotundifolia.* These showed fewer follicles when compared to the control group. Their ovarian follicles were not seen at different stages of maturation and mature follicles were completely absent unlike those from the control group. This should be due to the high amount of ascorbic acid found in the plant as reported by, [19]. Their work, which was carried out as attempt to verify and investigate the phytochemical constituents of the plant (*Dissotis rotundifolia*), showed presence of ascorbic acid in it. The effect of ascorbic acid on follicular cells has been noted by [20 shato]. Their experiments showed that the presence of ascorbic acid caused degeneration and destruction of follicular cells of ovary and loss of epithelial lining of the uterus. Thus due to presence of ascorbic acid there was degeneration of the follicular cells observed in the cortex of the ovaries belonging to treated rats.

The histology of the uterus from rats in control groups showed large lamina content, healthy endometrium with rounded uterine glands lined with pseudostratified columnar cells of the endometrium and columnar calls of the uterine glands. Uterus from rats in treatment groups showed shortened glandular epithelium. They also exhibited thin glandular linings of glands that were tortuous unlike the rounded glands in the uterus of the glandular cells. The changes did not look dose dependent. All of these changes may be due to the acidic content in *Dissotis rotundifolia* as reported by [19]. This claim is backed up by the works of Shattock and Solomon in 2004.

Effect of *Dissotis rotundifolia* (*Melastomaceae*) Extract on Oestrus Cycle

The extract of *Dissotis rotundifolia* has shown a decrease in duration of proestrus and oestrus stages of ovulation but an increase in duration of dioestrus phase of ovulation. The fact that dioestrus is especially prolonged significantly will reduce frequency of ovulation, [21]. The dioestrus, which is prolonged, is characterised by activities of corpus luteum that produce progesterone in the absence of pregnancy [21]. This should be due to the anti-inflammatory properties of the extract [14]. Studies have shown that the process of ovulation is comparable to an inflammatory process. Anti-inflammatory drugs have been used to arrest ovulation [22]. The anti-inflammatory property of the saponins present in the extract as shown by the phytochemical analysis has been shown to inhibit the action of cyclo-oxygenase enzy. It was revealed that traditional non-steroidal anti-inflammatory drug produce their effects by blocking COX-2. COX-2 deficient mice suffer from defect in reproductive fuctions such as ovulation and fertilization, since it is important in ovulation through its role as an enzyme for follicular rupture, .

Effect of *Dissotis rotundifolia* (Melastomaceae) on FSH and LH

Concerning reproductive hormonal changes in this study, a significant decrease in the serum levels of LH and FSH were recorded in groups treated with *Dissotis rotundifolia* compared to control group. The ovulatory process is initiated when follicular tissue is stimulated by a surge of pituitary gonadotropins (FSH/LH). This could lead to an increase in circulation of these hormones. FSH is best known for its role in stimulating follicular development and both are primary hormones for initiating ovulation [14]. These hormones significantly reduced in treated rats. For LH there was a significant decrease in the serum level which was dose dependent. For FSH there was also significant decrease though not dose dependent. The reduction in the levels of these hormones are implicated in the degeneration of the follicular cells observed in the histology as shown above and consequently leading to

anovulation, promoting infertility in the wister rats following treatment using the extract. LH is required for the proliferation of the endometrium for the reception of fertilized ovum and the decrease in the serum levels was also implicated in the lack of proliferation and thinning the endometrium of treated rats. All of these factors lead to infertility in the treatment rats. The reason for these could be due to the presence of high level of pytoestrogens like saponins as shown in the phytochemical analysis. Saponins have been found to reduce fertility in animals upon continuous administration. Phytoestrogenic plants have both estrogenic and antiestrogenic effects on mammalian systems. They prevent implantations and other estrogen-dependent activities in the reproductive system. They do this by causing hormonal imbalances in the systems of the subject concern. Some plants that possess oxytocic effects have been found to also have anti-fertility effects.

Effect of withdrawal from treatment with Dissotis rotundifolia on histology of Ovary and Uterus.
Concerning the effect of withdrawal (for a period of 7 days), there was obvious recovery of all the parameters used to analyse the effects of the extract on reproductive parameters of the female wister rats. This was performed only on Groups 3 and 5 as they were the withdrawal groups. The ovaries showed new follicles being formed even though not all of the atretic follicles had been disposed. There was also a presence of a mature follicle. The stroma of the ovaries also showed thickening unlike those of the treatment groups (2 and 4). The Histo-architecture is similar to the control groups.

The uterus of the withdrawal groups showed recovery after withdrawal for a period of 7 days. It showed development of new, rounded uterine glands with thick pseudostratified columnar epithelium of the endometrium and columnar cells lining the uterine glands. There were also little or no abnormal spaces within the endometrial layer. This result shows that there was recovery of the uterine tissue when compared to treatment groups (Groups 2 and 4) and looked more like those of the control uterus. The recovery was not dose dependent.

Effect of withdrawal from treatment with *Dissotis rotundifolia* on oestrus cycle
The oestrus cycle pattern stabilized back to normal (2 days of prooestrus, 2 days of oestrus and 4 days of dioestrus) in group 3 but (3 days of proestrus, 1 day of oestrus and 4 days of dioestrus) in group 5 all within an 8 day span. These figures are very similar with those of the control group's oestrus cycle showing recovery in the oestrus cycle.

Effect of withdrawal from treatment with *Dissotis rotundifolia* (Melastomaceae) on Serum levels of FSH and LH
The serum levels of LH and FSH showed significant recovery back to normal levels (except for the level of FSH level in the high dose withdrawal group; Group 5 which was significantly lower than normal levels but still higher than that of the treatment group), when compared to the control group where the hormonal levels were significantly higher than those of the treatment groups. The reduced levels in FSH in the high dose recovery group was reportedly due to high levels of saponins than the low dose withdrawal group (100mg/kg body weight compared to 25mg/kg body weight respectively)

CONCLUSION

The results of these studies have demonstrated that *Dissotis rotundifolia* extract partially or completely blocks ovulation by promoting degeneration of follicular cells and stroma hyperplasia in the ovarian cortex. It also shows that administration of this extract also caused destruction of the endometrial lining of the uterus preventing implantation of fertilized ovum if any. Administration of the extract also showed reduction in the serum levels of FSH and LH in treatment rats thus leading to unfavourable conditions for ovulation or pregnancy. There was significant recovery in all the above stated parameters showing a return to normalcy in the withdrawal group rats. *Dissotis rotundifolia* extract has an anti-fertility potential and considering recovery after withdrawal, shows potential for use as contraceptive in developing countries with less side effects and complications.

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