

Effect of *Combretum hereroense* Root Extract on Estrous Cyclicity and Vaginal Cytology in Female Albino Rats with Experimentally Induced Reproductive Dysfunction

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Abstract

Infertility and reproductive dysfunction are still significant reproductive health issues, especially in environments with restricted access to traditional reproductive health care. Traditional medicines could also be a source of compounds with reproductive activities, but need to be tested for reproductive efficacy and safety. In parts of Kenya (in Baringo County) *Combretum hereroense* Schinz is traditionally used for reproductive health issues. However, information on its effects on estrous cyclicity is limited. This study examined the effect of *C. hereroense* root extract on estrous cycle characteristics and vaginal cytology in female albino rats with experimentally induced reproductive dysfunction. Thirty-six female albino rats aged 8–10 weeks were randomly allocated to six groups of six animals. The groups consisted of a normal control, negative control, positive control treated with Gynaecosid®, and three extract-treated groups receiving 400, 800 and 1200 mg/kg body weight of *C. hereroense* root extract. Reproductive dysfunction was induced after synchronization of the estrous cycle. Treatments were administered orally once daily for 14 days. Daily vaginal smears were used to determine estrous cycle length, cycle regularity and the proportion of days spent in proestrus, estrus, metestrus and diestrus. Vaginal cytology was assessed by recording leukocytes, cornified epithelial cells and nucleated epithelial cells. Differences in continuous variables were tested using one-way analysis of variance followed by Tukey's HSD test, while chi-square tests were used for categorical variables. Significant differences were observed among the six groups in leukocyte count ($F(5,30) = 79.42, p < 0.001$), cornified epithelial cell count ($F_{0.05(5,30)} = 68.37, p < 0.001$), nucleated epithelial cell count ($F_{0.05(5,30)} = 31.84, p < 0.001$) and estrous cycle length ($F_{0.05(5,30)} = 52.63, p < 0.001$). The negative control group had the longest cycle (8.7 ± 0.8 days) and the highest number of leukocytes (68.5 ± 6.3 cells/field), the lowest numbers of nucleated epithelial cells (10.5 ± 2.1 cells/field) and cornified epithelial cells (12.3 ± 3.6 cells/field) in this group. Mean cycle length decreased with increasing extract dose, from 6.9 ± 0.6 days at 400 mg/kg to 5.3 ± 0.5

days at 800 mg/kg and 4.6 ± 0.4 days at 1200 mg/kg. At 1200 mg/kg, the three vaginal cytological parameters were comparable to those recorded in the Gynaecosid® group. Cycle regularity and the distribution of days across the four estrous stages did not differ significantly between groups ($p > 0.05$). Overall, administration of *C. hereroense* root extract was associated with shorter estrous cycles and changes in vaginal cell composition in rats with experimentally induced reproductive dysfunction, with the effects being more pronounced at the higher doses. The 1200 mg/kg dose produced values close to those observed in the Gynaecosid® group. Further studies are needed to clarify the hormonal and cellular mechanisms underlying these effects and to establish the effective and safe dose range of the extract.

Keywords: *Combretum hereroense*, estrous cycle, vaginal cytology, reproductive dysfunction, female albino rats, medicinal plants

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Introduction

In addition to difficulty conceiving, infertility can also have social, psychological, and financial implications, especially when diagnosis and treatment is not available. Using a systematic review and meta-analysis, a global estimate of 17.5% of the prevalence of infertility was estimated, which equates to approximately one in six people who experience infertility throughout their reproductive years (Cox et al., 2022). Recent studies also suggest that infertility is still prevalent among individuals of reproductive age, and that there is considerable regional variation in its

prevalence and treatment (Liang et al., 2025). Infertility is particularly a significant burden in LMICs, where reproductive health services are generally under-resourced. Despite its multiple social and personal consequences, the issue of infertility in sub-Saharan Africa has not yet been given much policy attention compared to other reproductive health issues (Afferri et al., 2022). Several African contexts have documented that many women with a perceived difficulties to conceive do not access biomedical interventions, and that socioeconomic factors have a significant impact on access to services for infertility (Whittaker et al.,

2024). But the issue is not fertility alone: many of those who are affected are not able to get a proper diagnosis or treatment.

Another big hurdle is the price of the infertility treatment. Depending on the underlying cause, treatment may involve diagnostic investigations, medicines, surgery or assisted reproductive technologies. Patients are faced with paying most of the costs associated with these services, which can be costly. A systematic review of ART in LMICs revealed that treatment expenses were frequently higher than the annual income or GDP per capita. The average direct cost of one treatment cycle in the African studies in the review was around 227.7% of GDP/capita (Njagi et al., 2023). The high costs of treatment may therefore make fertility treatment inaccessible to many patients, especially when public financing and insurance support is limited (Afferri et al., 2022). Limited availability of specialized services adds to these challenges. Many African countries have an insufficient number of trained people, poor resources for infertility care, and few policy initiatives for care, with a small number of facilities providing all of these services (Afferri et al., 2022). Sub-Saharan Africa also provides recent evidence that many individuals seeking fertility problems do not go to biomedical care as their initial care, and that traditional and other care also remains important (Whittaker et al., 2024). Given these situations, the need for more local and easily accessible, affordable, and also compatible approaches to healthcare has also grown.

In many African communities medicinal plants are still playing a significant role in the health care system; however, over the past few years there has been a growing interest in the scientific community regarding the use of these plants as a source of biologically

active compounds. Experimental investigations have revealed the role of medicinal plants in a number of aspects of female reproduction, such as follicular development and ovarian steroid production (Mbemba et al., 2017). In addition, a wider investigation of herbal medicines in female infertility revealed an effect on pathways related to folliculogenesis, steroidogenesis, and other reproductive pathways (Akbaribazm et al., 2021). These results are consistent with the need for the continued study of medicinal plants, but do not constitute proof of efficacy or safety. Medicinal plants produce various secondary metabolites which could be related to their reproductive effects. Various medicinal plants have been identified to contain compounds including flavonoids, phenolics, tannins, alkaloids and terpenoids that have been studied for various biological activities (Akbaribazm et al., 2021). Female reproductive function is also relevant, as there is a link between oxidative stress and other events related to reproduction and ovarian activity (Lu et al., 2018). The biological effects observed in some plants may be due to the antioxidant activity of some of the compounds extracted from them, but this does not imply that antioxidant activity in a plant extract is proof of a reproductive effect.

This is of particular interest for the genus *Combretum*. African *Combretum* species are known to have diverse secondary metabolites like phenolic compounds, flavonoids, tannins, terpenoids and triterpenes, and some species have been explored for their medicinal potential (Silén et al., 2023). Phytochemical composition may vary significantly, however, in different species and parts of the plant. The results from one *Combretum* species cannot thus be readily extrapolated to the other species. *Combretum hereroense* Schinz is a species

to be explored further due to its traditional use in reproductive health. *C. hereroense* was reported amongst the plants traditionally used for reproductive-related conditions in Kenya in a systematic review of medicinal plants used for infertility, sexual dysfunction and reproductive related conditions in the East African Community (Kyarimpa et al., 2023). The ethnobotanical data offers a foundation for experimental testing of the plant – especially its roots that have been traditionally used in Baringo County.

There is still not much research done on the species. Kiptisia and Nandwa (2023) studied the root extracts of *C. hereroense* which are traditionally used to treat female infertility in Baringo County, Kenya. Their research found multiple classes of phytochemicals in the extracts of the roots and gave them preliminary data on acute oral toxicity. The results are relevant to setting up a scientific basis for further studies but do not provide information on the ability of the extract to change the reproductive function of women who have reproductive dysfunction. The estrous cycle is one useful method of analysing the reproductive function of female laboratory animals. Normally, the cycle lasts 4-5 days in rats and has four phases: proestrus, estrus, metestrus and diestrus. Vaginal smears have characteristic changes that occur in the cells that are shed into the vagina and can be used to determine the stage of the cycle. (Cora et al., 2015) Therefore, changes in cycle length and in the vaginal cytology may be valuable indicators of changes in vaginal reproductive cycling. Changes in reproductive cycling can occur in response to disruption of ovarian or endocrine function due to its close association with hypothalamic-pituitary-ovarian axis function (Estienne et al., 2021).

Although *C. hereroense* has been traditionally employed in reproductive disorders and some preliminary information is available on the phytochemical composition of the plant, there are few experimental data on its activity on reproductive cyclicity. In particular, it is unknown whether the root extract will enhance the extended or modified cycling that occurs in the experimental reproductive dysfunction. When used in conjunction with a vaginal cytology, the study of the length of the cycle may be used to make an initial evaluation without assuming any hormone profile and knowing that the extract is associated with changes in the reproductive cycle.

The present study was thus aimed at evaluating the effect of *C. hereroense* root extract on estrous cyclicity in female albino rats that have been made estrous dysfunction by experimental exposure. The estrous cycle length and smear cytology of the vagina were studied after being treated with graded doses of the extract and a conventional treatment group was included for comparison. Especially, this study assessed the changes in leukocytes, cornified epithelial cells and nucleated epithelial cells as evidence of various stages of estrous cycle. The null hypothesis was that the extract of root of *C. hereroense* will not significantly affect the estrous cyclicity and vaginal cytological characteristics of female albino rats made reproductively dysfunction.

Materials and Methods

Study design and study setting

The effect of *Combretum hereroense* root extract on reproductive dysfunction induced female albino rats, *Rattus norvegicus* on estrous cyclicity and vaginal cytology was investigated using controlled laboratory experimental

design. The study was carried out in Uasin Gishu county, University of Eldoret, Kenya. The experimental animals were kept and observed in the Faculty of Science laboratories in controlled environment.

The University of Eldoret lies at a latitude $0^{\circ}31'N$, $35^{\circ}17'E$, and an altitude of about 2100m a.s.l. (Figure 1). The immediate environment is part of the highland plateau of the Rift Valley, and has a moderate climate with temperatures

ranging generally between $12-24^{\circ}C$. The rain falls in two distinct periods, the long rains from mainly March-May and the short rains from October-December with an annual average of 900-1200mm. The landscape surrounding the area is mostly agricultural characterized by maize, wheat and horticultural cultivation, and areas of natural and semi-natural vegetation. The animal housing facilities provided controlled conditions in the laboratory, treatment and monitoring.

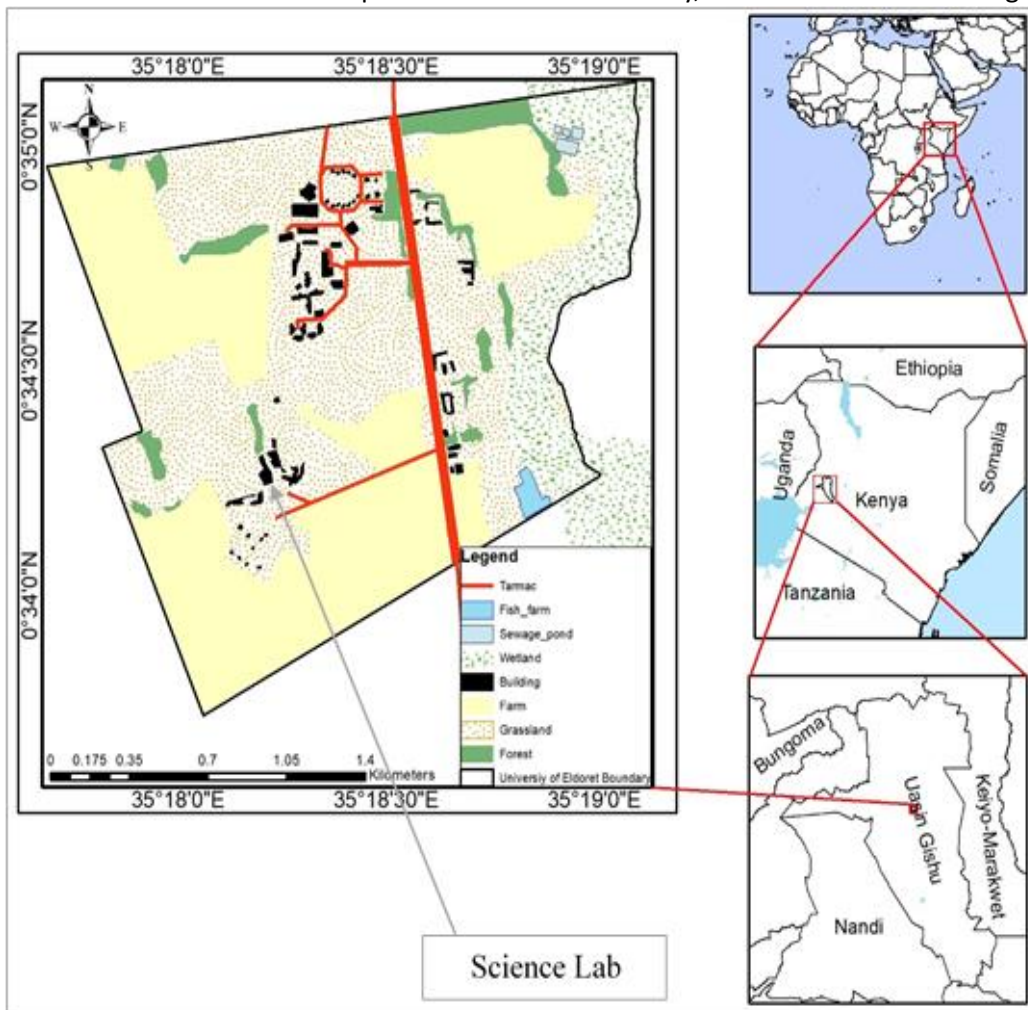


Figure 1: Map of University of Eldoret, Uasin Gishu County, Kenya

Experimental animals and husbandry

Thirty-six female albino rats (*Rattus norvegicus*), 8-10 weeks old, weighing 180-220 g were used. Three to four animals were kept in each cage (the cage was made of polycarbonate and has

an approximate size of $40 \times 25 \times 20$ cm) and obtained from an accredited laboratory animal supplier. Animals were kept in a light/dark room with 12 hrs of light and 12 hrs of darkness, the relative humidity in the room was 50–60%, and

the temperature was maintained at $22 \pm 2^\circ\text{C}$. Standard commercial rodent feed and clean drinking water was provided freely to the rats. Cages were cleaned frequently and appropriate bedding and environmental enrichment were given. For two weeks the animals were given time to become acclimatized before experimental procedures were started. The general health, activity and behaviour were observed every day during the study period. The housing conditions have been adopted from the original experiments.

Collection and authentication of plant material

Roots of *C. hereroense* were harvested from the natural stands in Baringo County, Kenya in the dry season. The plant material was identified and authenticated by a botanist from University of Eldoret with a voucher specimen kept in the University of Eldoret herbarium for future reference. During the collection, care was taken to not cause unnecessary damage to the source plants. The collected root materials were washed with the running tap water to get rid of soil and adhering materials, then cut into smaller pieces and dried in the shade at room temperature for about 14 days. After drying the roots were finely ground in a mechanical grinder and kept in clean and airtight containers until extraction.

Preparation of the root extract

The root extract was prepared as per the method described by Ahmed et al. (2014). The extraction of the powdered root material was done through maceration. In brief, 100 g of the powdered material was extracted using the extraction solvent (500 mL) and stirred intermittently at room temperature for 24 h. The mixture was filtered on Whatman No. 1 filter paper and the filtrate was concentrated by rotary evaporator at reduced pressure.

The crude extract obtained was transferred to amber bottles and stored in the refrigerator at 4°C for future use. The crude extract was made up in distilled water before it was administered. Three different doses (400, 800 and 1200 mg/kg b.wt) were prepared. Dosages were given based on the weights of the rats and new solutions were prepared each day during the treatment. The dose range selected was similar to the experimental protocol and toxicity data are available on the root extract. The use of root extract for experimental studies is supported by the previous studies carried out by Kiptisia and Nandwa (2023) on *C. hereroense* root extract from Baringo County which reported phytochemical constituents and no mortality or major abnormalities during acute oral exposure.

Synchronization of estrous cycles and induction of reproductive dysfunction

Before induction of reproductive dysfunction, the estrous cycles of the female rats were synchronized. Synchronization was carried out using Steron® tablets. The 5-mg tablet was dissolved in 10 mL of distilled water and rats were given 2, 1-mg doses per day for 2 days oral administration. After synchronization, reproductive dysfunction was achieved by giving medroxyprogesterone acetate (MPA) orally at 5 mg/kg body weight for 7 days in a row. The induction period was monitored for changes in estrous cyclicity by using vaginal smears. Induced animals that did not exhibit normal or acceptable estrous cycles were kept for the treatment phase.

Experimental grouping and treatment

The experimental grouping and treatment were as follows: The experimental grouping and treatment were:

Table 1: Experimental grouping and treatment

Group	Description	Treatment
C1	Normal control	Distilled water, 1 mL/rat/day
C2	Negative control	Distilled water, 1 mL/rat/day
C3	Positive control	Gynaecosid®, 5.3 mg/kg body weight
C4	Low-dose extract	<i>C. hereroense</i> root extract, 400 mg/kg
C5	Medium-dose extract	<i>C. hereroense</i> root extract, 800 mg/kg
C6	High-dose extract	<i>C. hereroense</i> root extract, 1200 mg/kg

Normal control was treated as untreated animals and negative control was treated as animals suffering from induced reproductive dysfunction and given distilled water. Gynaecosid® was used as positive control. The three graded doses of *C. hereroense* root extract were given to the extract groups. All treatment were given orally for 14 days continuously. Body weight was measured during treatment and was adjusted for individual dose. Treatment preparations were freshly prepared each day. The changes in behaviour, activity, feeding and other clinical signs were watched for throughout the experiment. The six-group allocation and treatment protocol was the same as the original experimental protocol.

Monitoring of estrous cyclicity

Vaginal cytology was used to monitor estrous cyclicity daily during the synchronization, induction and treatment periods. Vaginal samples were taken with sterile cotton buds that were briefly moistened with 0.9% normal saline every morning. The rats were gently handled, and the cotton bud was slowly inserted into the vagina and spun to pick up vaginal exfoliates. The material was then immediately placed on a clean microscope slide and spread out into a thin smear. The smears were then air-dried and stained with hematoxylin and eosin. Leukocytes, nucleated epithelial cells and cornified epithelial cells were examined under a light microscope and the relative abundance and appearance

of these cells were used to identify the estrous cycle stage. The four phases were known as the proestrus, estrus, metestrus and diestrus. This method is based on the method used to stage the rat estrous cycle based on vaginal smears (Cora et al., 2015). Daily observations were recorded for each animal. The cytological features of the various phases of the cycle were documented by making representative microscopic fields pictures. The original study protocol also included the collection of daily vaginal smears, H&E staining and microscopic examination of the three major cell types.

Assessment of estrous cycle length and regularity

The length of the estrous cycle was calculated based on the order of daily vaginal-cytology examinations. A complete cycle was considered to be the period of time between two consecutive estrus events. The number of days to cycle was noted for each animal and means were calculated for each experimental group. To determine cycle regularity, the sequence of the identified estrous stages of the period of observation was used. Animals with the anticipated four stages, but that had been prolonged or interrupted several times in the same stage, were considered to have irregular cycles, while those animals that progressed normally through the four stages without any such disruption were considered to have regular cycles. The experimental groups were compared in

terms of the percentage of the animals showing regular and irregular cycles.

Quantification of vaginal cytology

The three different cell types found in the vagina and used in the estrous cycle assessment were leukocytes, cornified epithelial cells and nucleated epithelial cells and were evaluated under the microscope. The number of cells was counted from representative microscopic fields, all at the same magnification, and at the same assessment technique for each experimental group. The leukocytes were recognized by their small size and distinct nuclei, while the nucleated epithelial cells were recognized by their distinct epithelial morphology and distinct nuclei. Cornified epithelial cells were characterized by their flattened, irregular shape and lack or poor definition of their nuclei. Both the relative abundance of these cell populations and the classification of the vaginal cytology were used to aid in the assignment of each smear to an estrous stage. These cell characteristics have been well used for staging the rat estrous cycle (Cora et al., 2015).

Body-weight monitoring

Body weights were measured for each individual during the experimental period. General condition determined by body weight measurements and amount of extract or control treatment for each animal calculated accordingly. Doses were modified to achieve the desired mg/kg of body weight if needed.

Data analysis

The data obtained by daily vaginal-cytology observations was tabulated and verified prior to analysis. Continuous data, such as estrous cycle length, and vaginal cell counts were presented as mean $\pm$ SD. One way analysis

of variance (ANOVA) was used to compare the six experimental groups. Tukey's honestly significant difference (HSD) test was applied in the case of an overall ANOVA indicating a significant difference between means for pairwise comparisons. Proportions of animals in regular or irregular cycles and the distribution of observations on the four estrous stages were evaluated by using chi-square test, if the expected frequencies were greater than 5. The data were analysed statistically using Statgraphics Centurion software. P values less than 0.05 were considered to be statistically significant.

Ethical considerations

The study was carried out under the institutional requirement in the care and use of laboratory animals. Attention was paid to minimising pain, distress and unnecessary manipulation when handling animals and conducting experimental procedures. The principles of Replacement, Reduction and Refinement were observed and animal numbers were kept to a minimum based on experimental design. Animals were observed during the study to detect any signs of trouble, suffering or reactions to the treatment. Animals were humanely handled in relation to procedures that required them to be restrained or anaesthetised and were humanely euthanised at the end of experimental work according to institutional procedures. The original protocol similarly specified application of the 3Rs, daily monitoring and humane treatment of the experimental animals.

Results

Effect of *Combretum hereroense* Root Extract on the Estrous Cycle of Female Albino Rats with Experimentally Induced Reproductive Dysfunction

Cycle Length

The estrous cycle of female albino rats was monitored over two consecutive cycles using vaginal smear histology. Leukocytes, epithelial and cornified cells were used to identify the stages of the cycle: Proestrus, Estrus, Metestrus and Diestrus. Cycle length and stage distribution were analyzed to determine the effect of *C. hereroense* root extract. The infertility/Extract group (C2)

exhibited the longest mean cycle length of 5.17 ± 0.29 days, which was significantly higher than all other groups. Groups C4 and C5 each recorded a mean of 4.33 ± 0.47 days, C1 had a mean of 4.17 ± 0.29 days and the normal control group (C3) had the shortest mean cycle length of 4.0 ± 0.0 days. One-way ANOVA revealed a statistically significant difference in mean cycle length among groups ($F_{(5,10)} 0.05 = 10.08, p = 0.002$). Post-hoc Tukey HSD analysis showed that the mean cycle length of the C2 group was significantly greater than the other groups (C1, C3, C4 and C5) ($p < 0.05$). The other groups were not significantly different. The mean cycle length of each group is shown in Figure 2.

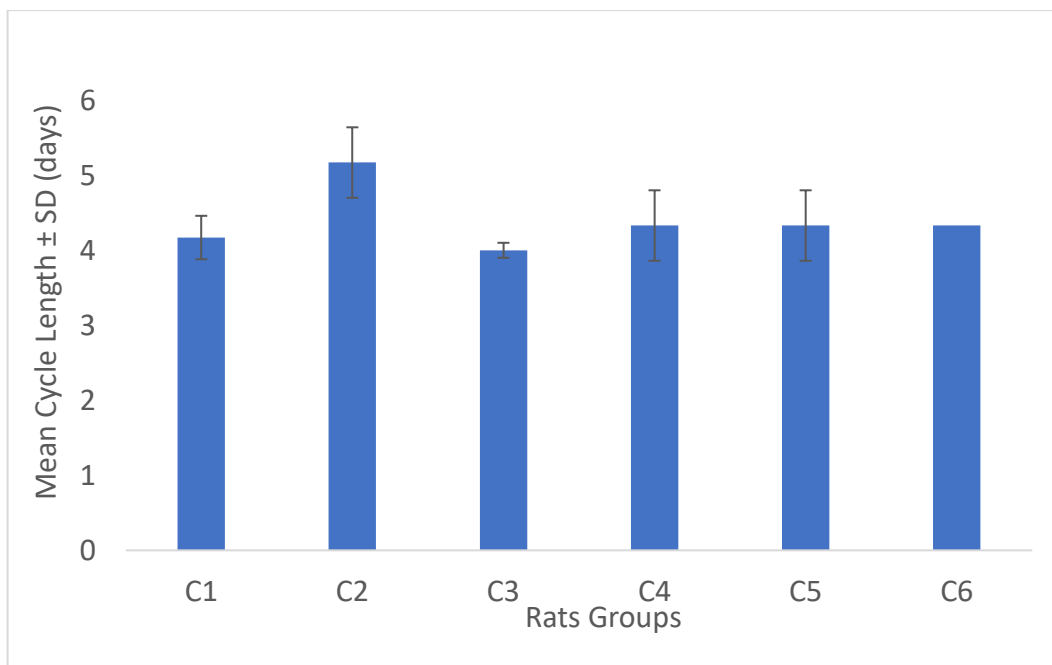


Figure 2: Mean estrous cycle length (days) of female albino rats following treatment with different doses of *Combretum hereroense* root extract

Cycle Regularity

The regularity of the cycles was assessed by comparing to the normal estrous cycle of 4-5 days (Table 2). The results showed that all rats in C1, C3, C4, C5 and C6 had normal cycles while one rat

in C2 had an irregular cycle. There was no significant difference in cycle regularity between groups ($\chi^2 = 1.0, df = 5, p = 0.91$) when analysed by chi-square.

Table 2: Cycle Regularity of Female Rats

Group	Normal Cycles (4–5 days)	Irregular Cycles (<4 or >5 days)	Total Rats
C1	3	0	3
C2	2	1	3
C3	3	0	3
C4	3	0	3
C5	3	0	3
C6	3	0	3

Percentage of Days Spent in Each Estrous Stage

Female albino rats were determined to be in one of the stages of the estrous cycle by examining vaginal smears using a combination of leukocytes, nucleated epithelial cells and cornified epithelial cells. Four stages were seen as Proestrus, Estrus, Metestrus and Diestrus. The percentage of days spent in each stage over two consecutive cycles was calculated to evaluate the effect of *C. hereroense* root extract on stage distribution. The infertility/Extract group (C2) recorded the highest proportion of days in the Estrus stage (30%), compared to 25% in C1 and C3 and 26% in C4 and C5. In the Proestrus stage, C1 and C3 each recorded 25%, while C4 and C5 recorded

24% and C2 showed the lowest proportion at 22%. The percentages of metestrus were the same in all groups (25%). There were no significant differences in diestrus between the different groups, with 23% in C2 and 25% in the other groups. The normal control group (C3) exhibited an equal distribution across all four stages (25% each) (Figure 3). No significant association was found between the treatment group and distribution of estrous stage ($\chi^2 = 2.24$, d.f=12, $p = 0.998$) by statistical analysis with Chi-square test. C2 had a marginal increase in the number of days spent in Estrus, and a marginally decreased number of days spent in Proestrus and Diestrus, but none of these were statistically significant.

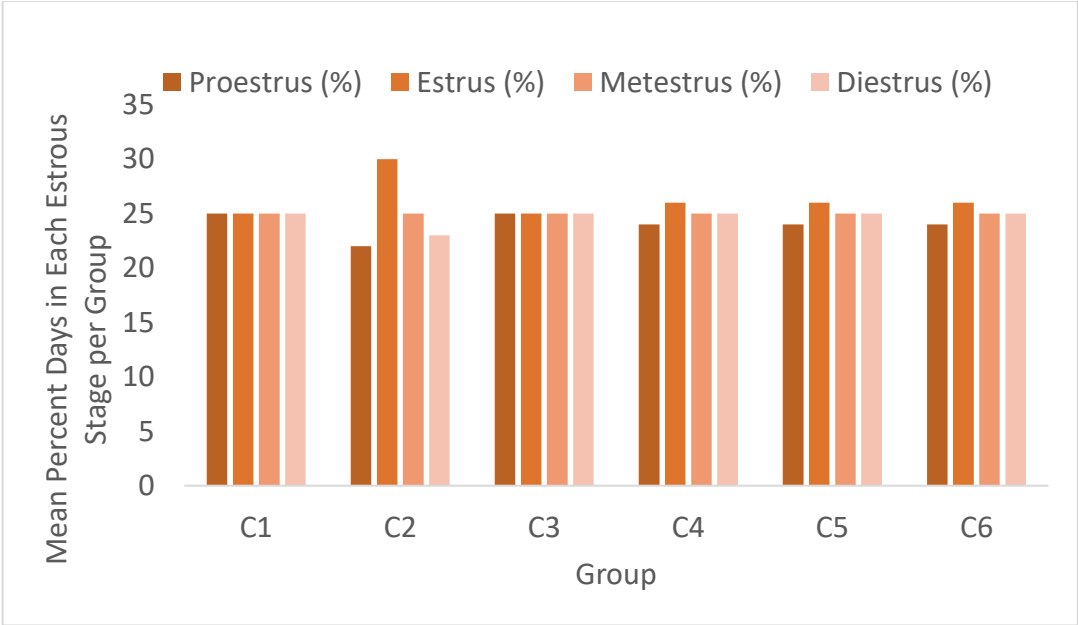


Figure 43: Distribution of estrous cycle stages (%) among female albino rats following treatment with different doses of *Combretum hereroense* root extract.

Effect of *Combretum hereroense* Root Extract on Vaginal Cytological Characteristics

The effect of *Combretum hereroense* root extract on vaginal histology and estrous cyclicity is presented in Table 3. Following treatment, differences were observed among the experimental groups in leukocyte count, cornified epithelial cell count, nucleated epithelial cell count and estrous cycle length. The negative control group recorded the highest mean leukocyte count (68.5 ± 6.3 cells/field), while the normal control group recorded the lowest (8.6 ± 2.1 cells/field). Mean leukocyte counts declined with increasing doses of *C. hereroense* root extract, measuring 45.6 ± 5.7 , 28.2 ± 4.3 and 18.9 ± 3.1 cells/field in the low-, medium- and high-dose groups, respectively. The positive control recorded a mean leukocyte count of 16.8 ± 3.4 cells/field. These differences were statistically significant (one-way ANOVA, $F_{0.05(5,10)} =$

79.42 , $p < 0.001$). Tukey's HSD test showed that the high-dose extract group did not differ significantly from the positive control, but both groups had significantly lower leukocyte counts than the negative control ($p < 0.05$).

The mean number of cornified epithelial cells ranged from 12.3 ± 3.6 cells/field in the negative control group to 72.4 ± 5.8 cells/field in the normal control group. Rats treated with the low, medium and high doses of the extract recorded mean counts of 28.8 ± 4.9 , 46.5 ± 5.4 and 61.5 ± 5.7 cells/field, respectively, while the positive control group recorded 63.7 ± 6.2 cells/field. The one-way ANOVA showed that there was a significant difference among the treatments ($F(0.05(5, 10) = 68.37$, $p < 0.001$). A post hoc analysis also indicated that the extract used in the high dosage group was similar to the positive control and significantly different from the negative control ($p < 0.05$) (Table 3).

Table 3: Effect of *Combretum hereroense* Root Extract on Vaginal histology and Estrous Cyclicity

Treatment group	Leukocytes (cells/field)	Cornified epithelial cells (cells/field)	Nucleated epithelial cells (cells/field)	Estrous cycle length (days)
Normal control	8.6 ± 2.1^a	72.4 ± 5.8^a	38.6 ± 4.2^a	4.2 ± 0.3^a
Negative control	68.5 ± 6.3^d	12.3 ± 3.6^d	10.5 ± 2.1^c	8.7 ± 0.8^c
Positive control (Gynaecosid)	16.8 ± 3.4^b	63.7 ± 6.2^{ab}	34.1 ± 3.5^a	4.5 ± 0.4^a
<i>C. hereroense</i> Low dose	45.6 ± 5.7^c	28.8 ± 4.9^c	18.6 ± 2.8^b	6.9 ± 0.6^b
<i>C. hereroense</i> Medium dose	28.2 ± 4.3^b	46.5 ± 5.4^b	27.4 ± 3.2^{ab}	5.3 ± 0.5^{ab}
<i>C. hereroense</i> High dose	18.9 ± 3.1^b	61.5 ± 5.7^{ab}	35.8 ± 3.8^a	4.6 ± 0.4^a

Values are presented as mean $\pm$ standard deviation (SD). Means within a column sharing different superscript letters differ significantly at $p < 0.05$ according to Tukey's Honestly Significant Difference (HSD) post hoc test following one-way ANOVA.

For nucleated epithelial cells, the lowest mean count was observed in the

negative control group (10.5 ± 2.1 cells/field), whereas the normal control

group had the highest count (38.6 ± 4.2 cells/field). Mean counts increased from 18.6 ± 2.8 cells/field in the low-dose group to 27.4 ± 3.2 and 35.8 ± 3.8 cells/field in the medium- and high-dose groups, respectively. The positive control recorded a mean of 34.1 ± 3.5 cells/field. The variation among groups was significant ($F_{0.05(5, 10)} = 31.84, p < 0.001$) with the high-dose extract group showing no significant difference from the positive control ($p > 0.05$).

The mean estrous cycle length was longest in the negative control group (8.7 ± 0.8 days) and shortest in the normal control group (4.2 ± 0.3 days). Mean cycle lengths of 6.9 ± 0.6 , 5.3 ± 0.5 and 4.6 ± 0.4 days were recorded in the low-, medium- and high-dose groups, respectively, compared with 4.5 ± 0.4 days in the positive control. One-way ANOVA showed a significant treatment effect on estrous cycle length ($F_{0.05(5, 10)} = 52.63, p < 0.001$). Tukey's HSD test indicated that the estrous cycle length in the high-dose group was not significantly different from that of the positive control ($p > 0.05$).

Discussion

Effect of *Combretum hereroense* Root Extract on the Estrous Cycle of Infertile Female Albino Rats

Cycle Length

The estrous cycle results showed that there was a change in normal reproductive cyclicity of female albino rats induced to be infertile. The untreated infertile animals reversed to a normal cycle pattern, while animals treated with *Combretum hereroense* root extract exhibited a cycle pattern closer to the healthy animals. This implies recovery of reproductive function after induction of infertility with the extract improving the physiological processes associated with maintaining normal ovarian cyclicity.

The estrous cycle reflects the activity of the ovary and is dependent on the integrated activity of the hypothalamic–pituitary–ovarian axis. To ensure normal passage through the various stages of the cycle, the growth of the follicles, ovulation and development of the luteal tissues must be regulated in a very specific fashion by the changing levels of gonadotropins and ovarian hormones. Disruptions in this pathway could cause a delay in follicular development, changes in ovulation or extended periods of reproductive activity. In experimental rodents, ovarian dysfunction often is associated with alterations in the vaginal histology and the length of the estrous cycle, which provides a valuable clue to the extent of reproductive dysfunction (Goldman et al., 2007; Hubscher et al., 2005; Byers et al., 2012). A prolonged cycle in untreated non-pregnant females, which is observed during the cycle of an infertile female, can thus be considered as a result of poor ovarian activity and poor hormonal coordination after induction of infertility. Others have noted reproductive issues that were linked to decreased follicular development, decreased steroid hormone production and altered ovarian responsiveness in animal models of experimental infertility (Dumesic et al., 2015; Gougeon, 2010). The role of estrogen in the regulation of early events in the estrous cycle has been discussed and insufficient estrogen production or delayed development of the follicles can prolong the cycle (Couse & Korach, 1999). This group's reproductive cycle alteration is in agreement with previous reports that ovarian dysfunction is associated with reproductive cycle irregularity.

The shorter and regular cycle period after treatment was observed with *C. hereroense* may suggest that the extract helped in improving ovarian functions. The mechanism is not known,

but it is a possibility that it is due to the phytochemical compounds present in the Combretum species. Flavonoids, phenolic compounds, tannins and terpenoids have been previously found in Combretum plants, and some of the phytochemicals found in the plants have anti-oxidant and protective properties (Eloff et al., 2008; Cock, 2015). These properties are significant in the female reproductive function, as oxidative imbalance has been shown to affect follicular development, oocyte quality and ovarian steroidogenesis (Agarwal et al., 2012; Ruder et al., 2008).

Oxidative stress is one of the factors that has gained a new recognition as being involved in the reproductive dysfunctions. Too many ROS can injure the cells of the ovary, and cause problems with the production of hormones, which in turn can interfere with the growth of the follicles and the normal reproductive cycle. In fact, several studies demonstrated that antioxidant-rich plant extracts provided the benefits of anti-aging effect on ovarian tissue and consequently improved reproductive outcomes by minimizing ovarian tissue oxidative damage (Alahmar, 2019). Thus, the increase in cyclicity after the administration of *C. hereroense* could be a result of the bioactive compounds that the plant contains, creating a more favourable environment for the recovery of the ovary.

The findings of the present study are also similar to those of previous studies on medicinal plants which have been used in reproductive studies. Flavonoids and other phenolic compounds found in plants have been reported to affect female reproductive function by their antioxidant properties, by affecting pathways involved in estrogen, and by improving ovarian structure and function (Patisaul & Jefferson, 2010). The occurrence of these

observations lends support to the suggestion that the bioactive compounds of *C. hereroense* might have a similar mode of action to restore reproductive cyclicity.

Differences between the treated groups suggest that the reproductive response to *C. hereroense* might be a function of the level of active compounds delivered. The dose-related effects of plant extracts can be observed due to the capacity of the phytochemicals to be absorbed, metabolized and interact with cellular targets. Moderate doses of plant-based treatments for reproductive disorders have been recorded to yield better effects as compared to lower or higher doses (Salehi et al., 2019; Venturella et al., 2021; Mohamed et al., 2026). With the reproductive use of *C. hereroense* in mind, it is likely that it will be essential to determine an effective therapeutic dose. Estrous cycle length restoration is an indicator of reproductive regulation, but is only one component of a fertility evaluation. Further study of the reproductive hormone profile, ovarian histology, and follicular development and oxidative stress markers would offer additional evidence of the mechanisms by which the reproduction of females is affected by *C. hereroense*.

Cycle Regularity

Regularity of the estrous cycle revealed that the majority of animals were cycling normally during the study period. Induction of infertility caused some changes in reproductive parameters, but the bulk of the groups involved in the experiment had a similar series of estrous cycles. The results showed there was no significant differences between the groups, thus suggesting that *C. hereroense* root extract had no effect on the normal progression of estrous cycle and was tolerated by the treated animals. A normal estrous cycle is

indicative of proper functioning of the hypothalamus, pituitary and ovary. This hormonal relationship regulates follicular development, and ovulation and formation and regression of corpus luteum. Disruptions in this pathway can lead to irregular cycling and/or extended reproductive phases or a lack of normal ovulatory events (Goldman et al., 2007; Hubscher et al., 2005). Regularity of the cycles in most of the groups treated indicates that the ovaries continued to produce sufficient hormones to maintain regularity in most of the infertile groups.

Some previous studies have demonstrated that even when reproductive impairment does occur, cycle regularity is not necessarily lost. However, in some individuals, there is a decrease in the quality of the follicles, the competence of the oocyte and/or the concentration of hormones, but the pattern of estrous cycling is relatively unchanged (Dumesic et al., 2015; Agarwal et al., 2012). This could be the reason why the present study found a change in cycle length, but not an increase in cycle irregularity. The maintenance of regular cycles in the animals fed with *C. hereroense* extract is also critical since some of the bioactive plant components can affect reproductive hormones and hence ovarian function. However, the present results indicated that the extract was not sufficient to stimulate or suppress hormones abnormally to disrupt the normal progression in the cycles. The same thing has been observed in studies on medicinal plants that contain remarkable amounts of antioxidants, in which treatment helped in reproductive functions without affecting physiological regulation.

This response may be partly due to the biological activity of *C. hereroense*. Some species of the genus *Combretum* have several secondary metabolites such as flavonoids, phenolic compounds and

tannins which have been correlated with antioxidant and protective effects against tissues (Eloff et al., 2008; Cock, 2015). Compounds with anti-oxidative effects are known to promote ovarian stability by reducing ovarian oxidative damage which includes impact on follicular development and production of hormones, therefore such compounds may be useful in preserving ovarian stability (Ruder et al., 2008; Alahmar, 2019).

In the infertile group, only one animal showed an irregular cycle and this could be due to variation between animals and not be a definite treatment effect. Cycle characteristics between experimental animals can vary based upon the sensitivity to the hormones, number and quality of the ovaries, and their reaction to induction of infertility (Byers et al., 2012; Cora et al., 2015). Overall, the results suggest that *C. hereroense* did not disrupt reproduction, but rather kept the reproductive cycle normal. All the results indicate that there was no negative effect on the regularity of the estrous cycle in female albino rats when treated with *C. hereroense* root extract. Rather, the extract seemed to be fit for normal reproductive cycling, and further investigations were warranted for further use in reproductive function.

Percent Days Spent in Each Estrous Stage

The distribution of days in estrous stages revealed that the root extract of *C. hereroense* was not causing significant differences in the progression of the proestrus, estrus, metestrus and diestrus phases. The overall distribution was similar in all groups with the normal control animals showing a usual distribution of cycle stages. There was no significant difference among treatments, indicating that treatment did not affect the normal estrous cycle sequence for the maintenance of normal cycle progression. All the stages of the estrous cycle are

separate physiological events associated with a shift in levels of ovarian hormones. Proestrus is the follicular growth phase and increased estrogen production, estrus the ovulation phase and highest estrogen activity, metestrus the transition to luteal development and diestrus the increased progesterone activity and preparation of the reproductive tract for possible implantation (Goldman et al., 2007; Cora et al., 2015). Variations in the ratios of the duration of these stages can thus be interpreted as changes in the endocrine function of the ovary.

The stage distribution of both treated and control animals is similar, suggesting that *C. hereroense* produced little effect on these underlying hormonal processes. This is important because some of these plant compounds can alter reproductive timing under high doses of the compounds (Patisaul & Jefferson, 2010). In the present study, however, the extract did not disrupt the normal pattern of cycle progression, indicating its effects were not due to disruptions in endocrine regulation.

The difference in the percentage of estrus days and proestrus days within the groups may be a natural variation and not a direct effect of the treatments. Estrous stage duration can vary naturally among laboratory rodents due to differences in age, physiological condition, environmental factors and individual hormonal responses (Byers et al., 2012; Cora et al., 2015). These differences were not significant at the statistical level and are not likely to be a significant change due to the extract.

Normal distribution of estrous stages may be linked to the phytochemical contents of *C. hereroense*. Medicinal plants contain flavonoids and other phenols that have been identified to have antioxidant activity in supporting reproductive tissues, but without any effect on normal hormone-mediated

processes (Agarwal et al., 2012). In a similar manner studies have been conducted on *Combretum* species that have shown antioxidant and anti-inflammatory properties which could be beneficial in protecting cellular functions during physiological stress (Eloff et al., 2008; Cock, 2015).

In similar studies using plant-based reproductive interventions, similar results were found, with extracts having a beneficial effect on reproductive performance without disrupting normal estrous cycling. These effects have been blamed on the protection of ovarian tissue and/or changes in antioxidant levels and/or maintenance of endocrine balance (Ruder et al., 2008; Alahmar, 2019), rather than the direct effect on hormones. The present results then indicate that *C. hereroense* can promote reproductive recovery, without altering the normal structure of the estrous cycle.

Effect of *Combretum hereroense* Root Extract on Vaginal Cytological Characteristics

The present study indicated that the root extract of *C. hereroense* positively affected multiple reproductive parameters that were affected after inducing the female rats (albino rats) to be infertile. The extract had an effect on features of the vaginal smear and estrous cycle duration which suggested it affected the activity of the ovary and hormonal regulation. This effect was more pronounced in higher doses of extract where the reproductive characteristics were closer to those of fertile controls suggesting a dose dependent effect. Vaginal Cytological is a commonly employed simple and informative technique to determine the reproductive status, based on the changes in vaginal cell populations, which corresponds with the fluctuations in ovarian hormones during the estrous cycle. During proestrus

and estrus, there is proliferation and maturation of vaginal epithelial cells, which is associated with an increase in nucleated and cornified cells, while during proestrus and diestrus, there is increased infiltration of leukocytes into the vagina (Goldman et al., 2007; Cora et al., 2015). This is reflected in a change in the cell population profile from a predominant leukocyte-dominated profile in untreated animals with impaired fertility to higher percentage of epithelial cell maturation after extract treatment, indicating better hormonal coordination and restoration of normal cycle progression.

The increased number of leukocytes seen in the untreated rats may relate to reproductive stress and changes in tissue state due to infertility induction of infertile rats. Inflammation and oxidative imbalance are well established factors involved in female reproductive disorders, since they could affect ovarian steroid production, follicular development and quality of the reproductive environment (Agarwal et al., 2012; Ruder et al., 2008). The observed decrease in leukocytes following treatment by *C. hereroense* could be a result of modulation of inflammation response and/or improvement in the tissue conditions necessary for normal reproductive function.

Biological activity of *C. hereroense* can be associated to its phytochemical composition. It has been found in previous investigations that there are flavonoids, tannins, phenolics and terpenoids present in the leaves of Combretum species, many of the compounds identified have antioxidant and anti-inflammatory properties (Eloff et al., 2008, Cock, 2015). These compounds can prevent the damage of reproductive tissues by reducing oxidative damage and contributing to cell function. This is especially significant in infertility where

too much ROS can affect granulosa cell function, affect follicular development, and affect the production of hormones (Agarwal et al., 2012; Alahmar, 2019).

Administering the extract led to a greater number of cornified and nucleated epithelial cells, further indicating higher hormonal activity of the ovary. It is known that in normal physiology, estrogens stimulate proliferation and cornification of the vaginal epithelium, leading to predictable changes to the vaginal cell composition during the cycle (Goldman et al., 2007; Hubscher et al., 2005). The results of these analyses suggest that the high dose extract group was similar to fertile control animals, indicating that treatment may have helped to restore the impaired reproductive estrogen processes in the infertile animals. Reproductive response similar to this has been reported in studies on medicinal plants that have antioxidant and reproductive supportive properties. In experimental models of reproductive dysfunction flavonoids and phenolics rich extracts have been shown to improve ovarian function, reproductive hormone balance and normalize estrous patterns (Noh et al., 2020). Additionally, certain compounds found in plants that have estrogen-modulating activity have been reported to affect reproductive tissues, but not physiologically. Further, some plant-derived compounds are found to be estrogen-modulating without altering the normal physiological regulation (Setchell & Cassidy, 1999; Patisaul & Jefferson, 2010). These results imply the potential role of the phytochemicals found in *C. hereroense* in the improvement of the vaginal histology.

The reproductive regulating effect of the extract is further substantiated from the changes in estrous cycle length noticed. Disturbances in the development of the ovarian follicles and modified secretion of reproductive

hormones are commonly associated with infertility and can extend estrous cycle duration (Gougeon, 2010; Dumesic et al., 2015). As the dose of the extract increased, the cycle length shortened, indicating better synchrony of ovarian events and re-establishment of more normal reproductive timing. Antioxidant plant products have also been shown to improve reproductive cycling and recovery of tissues after recovering ovarian tissues and promoting improved endocrine function (Ruder et al., 2008). The greater the response at the higher extract dose indicates that the concentration of the active compounds might affect their reproductive improvement. Patterns of this kind have also been reported with medicinal plants in which elevated doses of the bioactive compounds may lead to optimized therapeutic effects until a threshold is reached (Patisaul & Jefferson, 2010; Salehi et al., 2019; Mohamed et al., 2026). But, the precise mechanism and specific compounds responsible for such effects will demand more phytochemical and molecular studies.

Conclusion

The results indicated that the root extract of *Combretum hereroense* had an impact on the estrous cycle characteristics and vaginal cytology in female albino rats with experimentally induced reproductive dysfunction. The negative control animals exhibited the most significant differences, characterized by extended estrous periods, elevated levels of leukocytes and a greater decrease in cornified and nucleated epithelial cells. The treatment with extract had less effect on these changes at the concentration of 800 and 1200 mg/kg. The three vaginal cytological parameters measured and the length of the estrous cycle were similar to the

Gynaecosid® group when the 1200 mg/kg dose was used.

There was no significant difference between the groups in the distribution of animals by their cycle regularity and in the percentage of days in each estrous stage. The combined results indicate that the root extract might reverse some of the changes in estrous cycles and vaginal cytology resulting from experimentally induced reproductive dysfunction. However, additional studies are required to elucidate the biological mechanisms that underlie these effects, and to define the safety and dose range of the extract.

Recommendations

The hormonal changes observed due to the response of *C. hereroense* root extract should be studied further. Measurement of oestrogen, progesterone, FSH and LH would help determine if the changes in estrous cycling are associated with the alterations in reproductive hormone levels. Further dose-response studies are also suggested especially at doses lower and higher than those used in the present study. This would help determine the dose at which the extract starts to have measurable effects and if higher doses are safe or not. Studies that follow after 14 days of treatment would also be helpful in determining if the changes observed are sustained with continued treatment.

The components which are known to be present in the root extract should be identified and studied separately or in combination. This would help explain the biological activity observed in the present study and give a better idea of the biological effect of the extract on reproductive function. There also should be a histological examination of the ovary and uterus performed in further investigation. Correlation of tissue changes with the vaginal cytology and

estrous cycle patterns would give more complete information on the reproductive effects of the extract. The conclusions drawn in the present work apply to rats only and thus further studies in other animal species and appropriate safety evaluation is required.

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