

The Aqueous Extract of A Mixture of *Eremomastax speciosa* and *Cyathula prostrata* (ESCP) Leaves Regularises an Overcrowding-Blocked Estrous Cycle, Enhances Implantation and Improves on Litter Size in Female Rats

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ABSTRACT

The people of the Bayang Tribe of Manyu Division of the South-West Region of Cameroon use the leave-aqueous macerations of both *Eremomastax speciosa* and *Cyathula prostrata*, either combined or separately, to regularize blocked or altered menstrual cycles in non-menopause women and resolve pregnancy-related complications. The present study was aimed at evaluating the effects of the aqueous extract of a mixture of both plant-leaves (ESCP) on an overcrowding-induced anestrus cycle, implantation and litter size in female albino rats. In part I of the experiment, a total of 32 female rats with blocked estrous cycle as a result of overcrowding, were partitioned into 4 groups of 8 rats each and treated with distilled water (10 ml/kg bw), 36, 72 and 144 mg/kg of the extract, respectively, for a period of 21 days during which vaginal smears were examined daily to assess any change in the estrous cycle. In part II of the experiment, thirty-two 32 normal-cycle female rats were crossed over night with males of proven fertility, treated in the same way as those in part I for 7 days, underwent a laparotomy, then allowed to complete the gestation. The extract unblocked the estrous cycle and induced a return to normal cyclicity. Percentage regulation increased significantly ($p < 0,001$) from 0% in week 1 of treatment to 62.5% in week 2 in animals receiving both the 72 mg and 144 mg/kg body weight doses. By the 21st day of treatment, animals treated with the 72 mg and 144 mg/kg body weight doses registered 75% regulation, compared to a 12.5% regulation recorded in the control group. The aqueous extract of a mixture of *Eremomastax speciosa* and *Cyathula prostrata* (ESCP) possesses a regulatory effect on the estrous cycle of anestrus rats provoked by overcrowding, though the mixture might have anti-implantation properties at higher doses.

Keywords

Overcrowding, *Eremomastax speciosa* and *Cyathula prostrata*, Estrous cycle, Implantation, Anestrus.

Abbreviations

AED: animal equivalent dose, C: *Cyathula*, E: *Eremomastax*, ESCP: *Eremomastax speciosa* and *Cyathula prostrata*, FSH: Follicle Stimulating Hormone, H&E: Hematoxylin & Eosin, hMG:

human menopausal gonadotropin, HPA: hypothalamo-pituitary-adrenal, LH: Luteinizing Hormone, POS: polycystic ovarian syndrome, PMS: premenstrual syndrome, P. O.: Post office, PR: percentage regulation.

Introduction

Environment, especially the habitat, interferes with the estrous cycle. Female rats whose areas are not sufficiently spacious are generally in anestrus. When female rats are exposed to overcrowding, they show a decrease in mood, lack of motivation and express fear. Also, chronic exposure to stressful conditions has negative effects on brain structures related to learning and memory [1]. This social effect, also called "the overcrowding effect" is a stressful effect. Under this condition, the hypothalamo-pituitary-adrenal (HPA) axis is stimulated to secrete cortisol, a primary steroid hormone responsible for controlling the body's stress response. This explains why cortisol is often associated with "fight-or-flight." At optimal levels, this hormone helps to regulate mood, fear and motivation. This natural response allows the body to focus its resources on overcoming the stress and once the body senses that the environment is "safe" again, functions are restored. However, when the individual is exposed to chronic stress, the body can't process the negative feedback loop responsible for triggering a necessary decrease in cortisol levels. This leaves the body thinking it is in a constant state of fight-or-flight, so it imposes the same limitations on non-essential functions as it would during a real threat. If all the cortisol receptors are responding appropriately, this will cause cortisol levels to spike and temporarily shut down non-essential functions like digestion and reproduction during stressful events. The main modification affecting the sexual cycle is anestrus, characterized by an absence of heat. The increase in glucocorticoids causes a stop in Luteinizing Hormone (LH) secretion, which in turn leads to anovulation. Generally, in women, elevated blood levels of cortisol are responsible for changes in menstruation, reduced sexual desire, decreased fertility or difficulties conceiving, heightened premenstrual syndrome (PMS) symptoms, worsening of symptoms related to polycystic ovarian syndrome (POS), herpes or other reproductive diseases. This may impair the reproductive function and result in infertility.

Infertility is a disease of the male or female reproductive system defined by the failure to establish a clinical pregnancy after 18 months or more of regular and unprotected sexual intercourse [2] to a point of a live birth [3]. Infertility can be primary or secondary. Primary infertility is when pregnancy has never been achieved by a person and secondary infertility is when pregnancy has occurred at least once and a failure to achieve subsequent pregnancy or pregnancies [3]. Furthermore, infertility is a medical condition that can cause psychological, physical, mental, spiritual and medical detriments to the patient. A unique quality of this medical condition involves affecting both the patient and the patient's partner within a couple [4].

Infertility is estimated to impact about 10-25% (estimated range from 48-180 millions) of couples of reproductive age. Based on a report from the 2006-2010 National Survey of Family Growth,

about 6% of married couples in the United States are infertile and 12% have impaired fecundity. By contrast, in China the prevalence of infertility was found to be at about 25%. The average infertility in Africa is 10.1% of couples, with a high percentage of 32% in some countries and certain tribes have higher infertility rates. While primary infertility is higher in other regions of the world, the secondary type is more common in Africa. The infertility rate is very high in Cameroon and it ranges from 15% to 30% depending on the age and the socioeconomic level of couples [5]. Female infertility occurs in about 37% of all infertile couples and ovulatory disorders account for more than half of the cause of the infertility [6].

Infertility treatments depend on the type and its possible cause(s). Some treatment options include pro-fertility drugs like: clomiphene citrate, taken by mouth, enhances ovulation by causing the pituitary gland to release more FSH and LH, which stimulate the growth of an ovarian follicle containing an egg; Gonadotropins, such as human menopausal gonadotropin or hMG (Menopur) and FSH (Gonal-F, Follistim AQ, Bravelle), injectable, stimulate the ovary to produce multiple eggs; Metformin (Fortamet), used in insulin resistance conditions and helps improve insulin resistance, which can improve the likelihood of ovulation; Letrozole (Femara) belongs to a class of drugs known as aromatase inhibitors and works in a similar fashion to clomiphene; utrogestan, which can be taken through the oral route; Bromocriptine (Cycloset, Parlodel), a dopamine agonist, might be used when ovulation problems are caused by excess production of prolactin (hyperprolactinemia) by the pituitary gland. Infertility can also be remedied through surgery including laparoscopic or hysteroscopic surgery and tubal surgeries as well as through assisted conception (intrauterine insemination, assisted reproductive technology and *in vitro* fertilisation). These drugs have numerous side effects like bloating, headache, gastric-upset, mood swings, developing ovarian hyperstimulation syndrome. Their use is further hindered by the fact that these drugs tend to be expensive/unavailable, while surgery procedures are sometimes uncomfortable and some remote areas may lack the facilities to carry out these surgeries; hence, many women especially in the developing countries suffering from infertility lack the means and finance of acquiring these treatments. These above shortcomings make individuals to think of alternative ways to solve their problems, which make them rely on medicinal plants.

Medicinal plants have been used from ancient times for their therapeutic values as well as to impart flavor to food. Plants have been used in the management of illnesses for decades and have continuously grown over time as complementary medicine since they are readily and cheaply available healthcare alternatives [7]. Nowadays, there is a growing interest in the use of crude extracts and dry powder samples of medicinal and aromatic plants for the development and preparation of alternative traditional medicine and food additives. Drugs derived from medicinal plants may have possible therapeutic relevance in the treatment of several diseases like infertility [8]. Although there are other options

to treat infertility with pro-fertility drugs as mentioned above, medicinal plants still remain the best option to treat infertility as they are relatively cheap and easily accessed with no or minimal side effects.

Eremomastax speciosa and *Cyathula prostrata* are medicinal plants used in Tropical Africa, Asia and Australia to treat different ailments. Data exists on the actions of these plants in different experimental designs [9-13]. The people of the Bayang Tribe of Manyu Division of the South-West Region of Cameroon use the leave-aqueous macerations of both plants, either combined or separately, to regularize blocked or altered menstrual cycles in non-menopause women, enhance implantation, prevent miscarriages or save risky pregnancies and induce multiple conceptions (twins, triplets, quadruplets, etc). In a previous study, we reported the aphrodisiac activity of the aqueous extract of *E. speciosa* in normal male rats [14]. However, until our study, no studies have investigated these claims. The present study was aimed at evaluating the effects of the aqueous extract of a mixture of *Eremomastax speciosa* and *Cyathula prostrata* leaves on an overcrowding-induced anestrus cycle, implantation and litter size in female albino rats.

Materials

Plant material

Collection, identification and preparation of the aqueous extract of a mixture of *Eremomastax speciosa* and *Cyathula prostrata* leaves

Eremomastax speciosa and *Cyathula prostrata* leaves were harvested from Mamfe, Manyu Division of the South-West Region of Cameroon in the month of March 2022. Both plants were identified at the National Herbarium Yaounde where a specimen of each is placed at voucher numbers N° 6112 /6344 HNC and N° 7002/7724 HNC, respectively.

They were washed under running tap water, air-dried for about 1 month then ground into a powdered form separately using an electric blender. One hundred grams of each powder were combined and macerated into 1800ml of distilled water and kept for 72 hours accompanied by mechanical agitation. This was followed by filtration using the Whatman filter paper No 2. The filtrate obtained was evaporated in an oven (TITANOX S.r.l) at 40°C for 4 days at the end of which a 26.5g black residue was obtained giving an 11.3 %. This residue was conserved at -20°C until used. Meanwhile, this protocol of extraction was repeated separately for each plant and the residue obtained was submitted to the Organic Chemistry Unit of the Department of Chemistry, Faculty of Science, and University of Buea for phytochemical analyses.

Determination of Administrative Doses

Administrative doses of the extract were determined based on the folk use. *Eremomastax speciosa* and *Cyathula prostrata* can be used separately or as a combination to remedy menstrual cycle related infertility in women. Traditionally, when used as a combination, about 15 leaves from each plant are jointly macerated

into 150-200mL of tap water and administered to an adult female of reproductive age suffering from an irregular menstrual cycle or miscarriages. Based on these and other screening tests, the therapeutic dose used in treating these difficulties was determined. This stood at 5.80 mg/kg from which the animal equivalent dose (AED) following the method of Nair and Jacob (2016) was calculated and stood at 36 mg/kg. A stock solution of 3.6 mg/mL concentration was then prepared by dissolving 360 mg of the mixture into 100 ml of distilled water. From this solution, 1 mL was administered to each rat of 100 g body weight, equivalent to 36 mg/kg or 3.6 mg/100g body weight dose. The effects of the mixture at 72 mg/kg and 144 mg/kg doses were examined. Stock solutions of 7.2 mg/ mL and 14.4 mg/ mL were prepared by dissolving 720 mg and 1440 mg, respectively into 100 ml of distilled water and like with the therapeutic dose, 1 mL from either solution was administered to the respective animals.

Solutions and reagents

Three milligrams (3 mg) of methylene blue powder (Sigma chemicals, USA) were introduced into a 1000 ml measuring cylinder containing 300 ml of distilled water. The cylinder and its contents were homogenized by shaking then filled to the 1000 ml mark with distilled water, which gave a 0.3% methylene blue solution used to color vaginal smears.

Nine milligrams (9 mg) of sodium chloride (NaCl) crystals (Sigma Chemicals, USA) were weighed and introduced into a 1000 ml measuring cylinder containing 300 ml of distilled water. The cylinder and its contents were homogenized by shaking then filled to the 1000 ml mark with distilled water which gave a 0.9% NaCl solution used to collect vaginal fluid.

One (1) milliliter of Eosin Y (CARLO ERBA Reagents, Milano, Italy) and 1 mg of Hematoxylin (CARLO ERBA Reagents, Milano, Italy) were measured separately and added together in a 100 ml measuring cylinder containing 30 ml distilled water. The cylinder and its contents were homogenized by shaking then filled to the 100 ml mark with distilled water, which gave a 1% Hematoxylin & Eosin (H&E) 1:1 solution used to stain vaginal smears. Penicillin-G was purchased from a local Pharmacy and preparation of its solution consisted in dissolving 600 mg of powdered Penicillin-G 1000000IU into 2 ml of distilled water. It was properly shaken and then used on the laparotomized females to prevent post-surgical infection.

Animals

Breeding of animals

Animals used in this experiment were rats of the Wistar strain of either sex, aged 12 weeks and weighing between 150 g and 200 g each. They were raised in the Animal Facility of the Department of Animal Biology and Conservation of the Faculty of Science, University of Buea under standard conditions of temperature (25 ± 1°C) and humidity (50–80%) with a 12/12 h light/dark cycle and in standard cages with each cage containing 5 animals. They had free access to food and water.

Screening of females

A total of seventy (70) female rats obtained from the Animal Facility of the Department of Animal Biology and Conservation of the Faculty of Science, University of Buea, Cameroon and aged 3 months were recruited into this experiment. Each female was monitored for 4 consecutive cycles (20days) during which vaginal fluid was collected, smears prepared and viewed under the light microscope (UNICO 380, USA) at X100 for the presence of different white blood cell types. Vaginal fluid for each female was taken between 07:00am and 08:00am local time and this collection time was respected throughout the experiment. The following procedure was used to collect the vaginal fluid: the female was held in a supine position with one hand and using the other hand, a dropper containing about 0.5ml of 0.9% NaCl solution was introduced into the vagina of the animal, while ensuring that the fluid introduced into the vagina returned with pressure. The fluid was sent in and withdrawn about 4 times ensuring the color of the 0.9% NaCl in the dropper had changed from colorless to milky before the dropper was withdrawn [15].

The smear was prepared by placing a drop of the fluid on a clean microscope slide and spreading with the help of the dropper. It was allowed to dry for about 30 minutes following which it was fixed with methanol for 15 minutes then stained with 0.3% methylene blue for 10 minutes. The smear was then examined under the light microscope (UNICO 380, USA) at magnification X100 to view and identify the phase of the cycle based on the leukocytes present. Photomicrographs of the smears were taken and recorded. This exercise was aimed at ascertaining the regularity of their estrous cycle before the experiment [16]. Only females with regular cycles comprised chronologically of pro-estrus, estrus, metaestrus and diestrus were retained and used in the next stage of the experiment.

Induction of Anestrous

At the end of this screening period, 56 females with regular estrous cycle were then grouped randomly into 4 cages of 14 rats each. Each cage was 30cmx20cmx18cm in dimensions with the floor covered with clean, contaminant-free wood-shaves. They were left together for 50 days during which vaginal smears were prepared and viewed as explained earlier at 5 day intervals. The main objective of such grouping was to induce the blockade of the sexual cycle using the overcrowding-stress factor. In these conditions, the sexual cycle blockade was confirmed by the nonappearance of the standard characteristic phases of the sexual cycle notably the pro-estrus-estrous-metestrus-diestrus, in this chronology.

Animal grouping and treatment

"Evaluating the effects of the aqueous extract of a mixture of *Eremomastax speciosa* and *Cyathula prostrata* (ESCP) leaves on anestrous"

To evaluate the correcting effect of the aqueous extract of a mixture of *Eremomastax speciosa* and *Cyathula prostrata* leaves on anestrous, 32 female rats with blocked estrous cycle and

obtained from the previous phase of the experiment were randomly partitioned into 4 groups of 8 rats each and treated as follow:

- Group 1: Rats receiving distilled water (DW, 10 ml/kg bw) and considered as the control
- Group 2: Rats receiving the 36 mg/kg bw (ESCP, 36 mg/kg) dose of the plant mixture;
- Group 3: Rats receiving the 72 mg/kg bw (ESCP, 72 mg/kg) dose of the plant mixture;
- Group 4: Rats receiving the 144 mg/kg bw (ESCP, 144 mg/kg) dose of the plant mixture.

Both distilled water and the plant extract were administered orally using the endogastric gavage cannula once daily for a period of 21 days consecutively. Vaginal smears were prepared and examined daily in order to assess any change in the estrous cycle. At the end of the 21 days treatment, the number of females whose cycles had returned to normal were identified and the percentage of success called percentage regulation (PR) per group calculated from the formula:

Percentage regulation (PR) = [Number of females with regulated cycles /Initial Number] X 100

On day 22 following commencement of treatment, animals were terminated through cervical dislocation. Blood was collected in anticoagulant-free test-tubes. Selected organs including the ovaries, uterus, liver and kidneys were isolated, freed from all connective tissue, rinsed with distilled water, blotted and weighed using an electronic balance (NVT 1601/1, OHAUS, USA). A 15% homogenate of the ovaries was prepared. Following centrifugation at 3000rpm for 15 minutes, the supernatant and the serum were collected, kept at -20°C and used to assay total proteins and total cholesterol.

Evaluating the effects of the aqueous extract of a mixture of *Eremomastax speciosa* and *Cyathula prostrata* (ESCP) leaves on implantation and litter size

Thirty-two (32) ovary-intact normal-cycle female rats were crossed over night with males of proven fertility. Vaginal fluid was collected the next day following the procedure described earlier. A drop of the fluid was placed on a clean microscope slide, stained with hematoxylin & eosin (H&E) to determine females with smears having spermatozoa, considered positive smears. The presence of spermatozoa in the vaginal smears was an indication of effective copulation and fertilization. Consequently, this day was considered day 1 of gestation. At the end of the microscopic observations, all the females were partitioned into 4 groups of 8 rats each and treated as in the previous experiment. However, treatment here lasted 7 days according to the instructions of the traditional healer.

On day 10 following commencement of treatment, the females underwent a laparotomy. They were given intraperitoneal injections of diazepam (10mg/kg bw) followed by that of ketamine (50 mg/kg bw) five minutes after the first injection. Following loss of reflex movements by the animal, the fur along the abdominal region

were shaved and the exposed skin prepared for aseptic surgery (97% alcohol wipe). All surgical instruments and stitching thread were conserved in a 90% ethanol when not in use. An incision of about 4cm long was made on the skin along the median line of the abdominal region and a second made on the peritoneum. The uterine horns were withdrawn using forceps, implantation sites counted and recorded, then incisions closed by stitching with the thread. The peritoneum and skin were then stitched, after which an intramuscular injection of penicilline G (2000 UI/kg bw/day) was given for 3 days to prevent any post-surgical infection [17,18]. At the end of the laparotomy, the animals were followed-up right up to delivery from which the following parameters were recorded: number of pregnant females, number of implantation sites and litter size.

Statistical Analyses

Values were expressed as Mean±SEM. Mean values were calculated for each animal and quantitative comparisons between groups established from those means. Analysis of Variance (ANOVA) followed by Duncan test was used in the SPSS for windows version 20.0 software. Significant levels were tested at $p < 0.05$.

Results

Results of the phytochemical tests

Phytochemical analyses of each plant revealed the presence of phytoconstituents of pharmacological importance. The results are presented in Table 1.

Table 1: Phytocomposition of *Eremomastax speciose* and *Cyathula prostrata* leaves-aqueous extracts.

Phytoconstituent or Phytocompound	Plant	
	<i>Eremomastax speciosa</i>	<i>Cyathula prostrata</i>
Phenols	+	+
Triterpenes	-	+
Sterols	+	-
Alkaloids	+	-
Sugars	+	+
Saponins	+	+

Table 2: Summary of the actions of the aqueous extract of a mixture of *Eremomastax speciosa* and *Cyathula prostrata* (ESCP) leaves on overcrowding-blocked estrous cycle of female Wistar rats.

Duration of treatment(days)	Treatment	Initial number of females	Number with regulated cycle	Percentage regulation
0-7	Distilled water (10ml/kg)	8	0	0%
	ESCP, 36mg/kg	8	0	0%
	ESCP, 72mg/kg	8	0	0%
	ESCP, 144mg/kg	8	0	0%
8-14	DW(10ml/kg)	8	0	0%
	ESCP, 36mg/kg	8	4	50%
	ESCP, 72mg/kg	8	5	62.5%
	ESCP, 144mg/kg	8	5	62.5%
15-21	DW (10ml/kg)	8	1	12.5%
	ESCP, 36mg/kg	8	5	62.5%
	ESCP, 72mg/kg	8	6	75%
	ESCP, 144mg/kg	8	6	75%

Values presented as M±SEM; DW: distilled water; ESCP: *Eremomastax speciosa* and *Cyathula prostrata*

Regulatory effects of the aqueous extract of a mixture of *Eremomastax speciosa* and *Cyathula prostrata* (ESCP) leaves on overcrowding-blocked estrous cycle.

Administration of the aqueous extract of a mixture of *Eremomastax speciosa* and *Cyathula prostrata* leaves at the 36, 72 and 144 mg/kg bw doses to anestrus females for a 21 day period witnessed a return to a normal regular cycle in the extract-treated animals, compared to the group treated with distilled water (Table 2).

During the first week of treatment, no female had the cycle regulated. However, from the second week of treatment, a return to normal cyclical events of the estrous cycle was noticed in all extract-treated females with 50% registered in the 36 mg/kg dose and 62.5% for the 72 mg/kg and 144 mg/kg doses. In the course of the third week, there was an increase in the percentage regulation, compared to the second week of treatment, with the groups receiving the extract at 72 and 144 mg/kg attaining 75% regulation; while the negative control group recorded a 12.5% regulation (Table 2).

Effects of the aqueous extract of a mixture of *Eremomastax speciosa* and *Cyathula prostrata* (ESCP) leaves on the relative weight of ovaries, uteri and liver of anestrus female rats.

Compared to the control group, treatment of anestrus female rats with ESCP at the doses 36,72 and 144 mg/kg uninterruptedly for 21 days did not induce any significant ($p > 0.05$) effect on the relative weight of some selected organs (Table 3).

Effects of the aqueous extract of a mixture of *Eremomastax speciosa* and *Cyathula prostrata* (ESCP) leaves on the serum, ovarian, uterine and liver protein concentration of anestrus female rats.

Table 4 summarizes data obtained in relation to the level of proteins in the serum, ovary, uterus and liver. According to the table, there was a significant ($p < 0,001$) increase in the concentration of uterine proteins in the group that received the 144mg/kg dose

(6.78 ± 0.33 µg/mg) and the 72 mg/kg dose (p < 0.05) (6.04 ± 0.39 µg/mg), compared to the control group (5.05 ± 0.38 µg/mg). Also, a significant (p < 0.05) increase in the level of liver proteins was recorded in animals that received the 36 mg/kg dose (92.77 ± 0.99 mg/g), compared to the control group that recorded a 76.22 ± 1.24 mg/g concentration in liver proteins. Furthermore, a significant (p < 0.05) increase in serum proteins was registered in the 72 mg/kg extract-treated group (56.28 ± 1.15 mg/ml), compared to the control group (44.51 ± 1.38 mg/ml).

Table 3: Effects of the aqueous extract of a mixture of *Eremomastax speciosa* and *Cyathula prostrata* (ESCP) leaves on the relative weight of ovaries, uteri and liver of anestrus female rats.

Treatment	Relative weight of organs (mg/100g bw)		
	Uteri	Ovaries	Liver
DW (10ml/kg)	240.28 ± 2.03	50.42 ± 0.93	3351.85 ± 4.08
ESCP, 36mg/kg	240.57 ± 1.80	45.71 ± 1.00	3177.71 ± 4.37
ESCP, 72mg/kg	222.28 ± 1.81	52.42 ± 1.17	3186.85 ± 4.29
ESCP, 144mg/kg	224.71 ± 2.10	44.43 ± 0.77	3193.71 ± 4.95

Values presented as M±SEM; DW: distilled water; ESCP: *Eremomastax speciosa* and *Cyathula prostrata*.

Table 4: Effects of the aqueous extract of a mixture of *Eremomastax speciosa* and *Cyathula prostrata* (ESCP) leaves on the serum, ovarian, uterine and liver protein concentration of anestrus female rats.

Treatment	Total Proteins			
	Serum (mg/ml)	Ovaries (µg/mg)	Uteri (µg/mg)	Liver (mg/g)
DW (10ml/kg)	44.51±1.38	16.18 ± 1.15	5.05 ± 0.38	76.22 ± 1.24
ESCP, 36mg/kg	53.76 ± 1.08	23.08 ± 0.93	4.95 ± 0.42	92.77 ± 0.99*
ESCP, 72mg/kg	56.28 ± 1.15	18.80 ± 0.73	6.04 ± 0.9**	84.80 ± 1.13
ESCP, 144mg/kg	45.93 ± 0.89	16.42 ± 0.82	6.78 ± 0.3***	84.77 ± 0.93

Values presented as M±SEM; DW: distilled water; ESCP: *Eremomastax speciosa* and *Cyathula prostrata*; *p<0.05 compared to the control; **p<0.01 compared to the control; ***p<0.001 compared to the control.

Effects of the aqueous extract of a mixture of *Eremomastax speciosa* and *Cyathula prostrata* (ESCP) leaves on the total cholesterol in anestrus female rats.

From Table 5, no significant (p > 0.05) difference in total serum and ovarian cholesterol was noticed between the extract-treated rats and the control group.

Table 5: Effects of the aqueous extract of a mixture of *Eremomastax speciosa* and *Cyathula prostrata* (ESCP) leaves on the total cholesterol in the serum and ovaries of anestrus female rats.

Treatment	Total cholesterol (mg/ml or µg/mg)	
	Serum (x10 ⁻²) (mg/ml)	Ovaries (µg/mg)
DW (10ml/kg)	38.17 ± 1.75	15.54 ± 0.92
ESCP, 36 mg/kg	34.37 ± 1.25	17.34 ± 0.98
ESCP, 72 mg/kg	38.52 ± 1.95	14.69 ± 0.71
ESCP, 144 mg/kg	40.63 ± 1.26	13.22 ± 0.69

Values presented as M±SEM; DW: distilled water; ESCP: *Eremomastax speciosa* and *Cyathula prostrata*.

Effects of the aqueous extract of a mixture of *Eremomastax speciosa* and *Cyathula prostrata* (ESCP) leaves on implantation and litter size.

Table 6 shows the influence of the aqueous extract of a mixture of ESCP on implantation. As it can be noticed, there was no significant (p > 0.05) difference in the number of pregnant females between the extract-treated and the control animals. Also, the animals treated with the 144mg/kg dose of the extract recorded a non-significant (p > 0.05) decrease in the number of implantation sites and litter size, compared to those treated with the extract at lower doses (36 mg/kg et 72 mg/kg).

Table 6: Summary of the effects of the aqueous extract of a mixture of *Eremomastax speciosa* and *Cyathula prostrata* (ESCP) leaves on implantation.

Treatment	Parameters			
	Number of females crossed	Number of females pregnant	Average implantation	Average Litter size
DW (10ml/kg)	8	8	5.14 ± 0.49	5.86 ± 0.43
ESCP, 36 mg/kg	8	8	7.00 ± 0.40	7.00 ± 0.40
ESCP, 72 mg/kg	8	8	7.28 ± 0.43	7.28 ± 0.43
ESCP, 144 mg/kg	8	8	6.71 ± 0.36	6.71 ± 0.36

Values presented as M±SEM; DW: distilled water; ESCP: *Eremomastax speciosa* and *Cyathula prostrata*.

Discussion

Our findings demonstrate that the aqueous extract of a mixture of *Eremomastax speciosa* and *Cyathula prostrata* (ESCP) leaves possess regulatory potentials on overcrowding-induced anestrus in female Wistar rats. In most female mammals, the sexual cycle is followed by an anestrus phase characterized by a temporal or permanent stop of ovarian activity [17]. Anestrus is a consequence of stress on the physiology of the ovary [18]. In our study, stress induced by overcrowding might have caused an activation of the hypothalamo-pituitary complex by raising the plasma concentration of cortisol. The increased level of cortisol is responsible for the inhibition of the secretion of Luteinizing Hormone (LH), whose pro-ovulatory effects have been demonstrated [19]. The regulation of the sexual cycle of overcrowding-induced anestrus cycle following administration of the aqueous extract of a mixture of ESCP leaves suggests that the mixture possesses the potentials to unblock a blocked estrous cycle. The potential to unblock the estrous cycle could be attributed to the action of some phytoconstituents found in the mixture.

The female sexual cycle is controlled by the secretion of steroid hormones, particularly estrogens. Secretion of these hormones is not uniform, but varies with the stage or phase of the cycle. Follicle Stimulating Hormone (FSH) release stimulate the development and maturation of follicles bringing the production of estrogens, responsible for the manifestation of receptivity (heat). These estrogens exert a negative feedback control on secretion of LH. Simultaneously, a positive feedback control of estrogens stimulates the release of LH by the pituitary and it is the pic of LH that triggers ovulation.

The increase in uterine and ovarian proteins noted in animals treated with the higher doses of the extract further supports the hypothesis of pro-estrogenic actions of the plants studied. These effects could be attributed to some phytochemicals. In effect, phytochemical analyses of either plant revealed the presence of alkaloids in the *Eremomastax speciosa* aqueous leaf-extract. It is known that alkaloids are enzyme inhibitors having effects similar to those of endogenous hormones. Alkaloids attach on estrogenic receptors and regulate the activities of key enzymes and their metabolism [20]. These compounds act directly on estrogenic receptors, causing an increase in proteins. The regulatory effect of the mixture of ESCP on the blocked cycle noted in this study would be due to the presence of alkaloids in *Eremomastax speciosa*.

Considering the obligatory involvement of cholesterol in the synthesis of steroid hormones including estrogens, the no change in ovarian cholesterol level amongst the extract-treated and control animals is a further indication that the extract did not act through the biosynthesis of estrogens when unblocking the anestrus cycle induced by overcrowding. We could also hypothesize that the bioactive substances contained in both plants mimic actions of estrogens on target cells of these hormones with the consequence of increasing protein levels as discussed above.

In order to better evaluate the effects that the plant mixture could have on the ovarian activity of anestrus female rats, we assessed the effect of the extract on implantation. Implantation is a crucial phenomenon during which the fertilized egg attaches itself to the uterine mucosa. It generally follows a series of biochemical, biophysical and hormonal phenomena. Studies have shown that the morphology of the endometrium is important for the implantation of the blastocyst [21]. As such, estrogens intervene during the follicular phase favoring the thickening of the endometrium, which is necessary for implantation. However, once the blastocyst is fixed, an increase in estrogens will inhibit implantation. Progesterone favors implantation [22]. Compared to the control, the extract had no effect on the number of implantation sites. Nevertheless, a non-significant decrease in the number of implantation sites and litter size were recorded in animals treated with the largest dose of the extract.

Phytochemical screening also revealed the presence of saponins and sterols. According to Stranbury [23], these compounds possess estrogenic actions, which explain the pro-estrogenic activity that was observed. Earlier, Mosani et al. [24] showed that only a small amount of estrogen is sufficient to induce implantation. The effect of estrogens on implantation is associated to the ability to form catechol-estrogens which are active substances produced by the hydroxylation of carbon at positions 2 and 4 to the extent that estrogens with low binding potential cannot induce implantation [25]. Furthermore, Vasudeva and Sharma [26] then Ryan et al. [27] have proven that an exact balance between estrogens and progesterone is essential for implantation.

Conclusion

We can conclude that the aqueous extract of a mixture of *Eremomastax speciosa* and *Cyathula prostrata* possesses a

regulatory effect on the estrous cycle of anestrus rats provoked by overcrowding. However, the mixture has anti-implantation properties at higher doses.

References

1. Ezequiel Batista do Nascimento, Aline Lima Dierschnabel, André de Macêdo Medeiros, et al. Memory impairment induced by different types of prolonged stress is dependent on the phase of the estrous cycle in female rats. *Hormones and Behavior*. 2019; 10: 45-63
2. Mélodie Vander Borgh, Christine Wyns. Fertility and infertility: Definition and epidemiology. *Clinical Biochemistry*. 2018; 62: 2-10.
3. World Health Organisation (WHO). *Infertility. International Classification of Diseases, 11th Revision (ICD-11)* Geneva: WHO. 2020.
4. Seyede Batool Hasanpoor-Azghdy, Masoumeh Simbar, Abouali Vedadhir. The emotional-psychological consequences of infertility among infertile women seeking treatment: Results of a qualitative study. *Iranian Journal of Reproductive Medicine*. 2014; 12: 131-138.
5. Gwet-Bell Ernestine, Gwet Bea B, Akoung Nicole Pharm, et al. The 5 main challenges faced in infertility care in Cameroon. *International federation of fertility societies*. 2018; 3: 16.
6. Unuane D, Herman T, Brigitte V, et al. Endocrine disorders and female infertility. *Best Practical Research In Clinical Endocrinology and Metabolism*. 2011; 25: 861-873.
7. Hossain MA, AL-Raqmi KA, AL-Mijizy ZH, et al. Study of total phenol, flavonoids contents and phytochemical screening of various leaves crude extracts of locally grown *Thymus vulgaris*. *Asian Pacific Journal of Tropical Biomedicine*. 2013; 9: 705-710.
8. Oke B, Aslim C, Ozturk S, et al. Essential oil composition, antimicrobial and antioxidant activities of *Satureja cuneifolia* Ten. *Food Chemistry*. 2009; 112: 874-879.
9. Julius E. Oben, Sheila, E. Assi, Gabriel, A. Agbor, et al. EFFECT OF EREMOMASTAX SPECIOSA ON EXPERIMENTAL DIARRHOEA. *African Journal Traditional Complementary and Alternative Medicine*. 2006; 3: 95-100.
10. Bolanle Ibrahim, Abimbola Sowemimo, Anzel van Rooyen, et al. Antiinflammatory, analgesic and antioxidant activities of *Cyathula prostrata* (Linn.) Blume (Amaranthaceae). *Journal of Ethnopharmacology*. 2012; 141: 282-289.
11. Schnablegger GE, Venables L, Koekemoer TC, et al. *Cyathula prostrata* ethanol extract activates the extrinsic pathway of apoptosis in HeLa and U937 cell lines. *South African Journal of Botany*. 2013; 88: 380-387.
12. Samuel O. Onoja, Chinemerem Eke, Emmanuel Ejiofor, et al. Antioxidant, anti-inflammatory and anti-nociceptive properties of hydro-methanol extract of *Eremomastax speciosa* (Hochst.) Cufod leaf. *African Journal of Traditional, Complementary and Alternative Medicine*. 2017; 14: 56-63.

13. Peter Kelechi Ajuogu, Richard Ere, Medubari B Nodu, et al. The influence of graded levels of *Cyathula prostrata* (Linn.) Blume on semen quality characteristics of adult New Zealand white bucks. *Translational Animal Science*. 2020; 4: 1134-1139.
14. Nchegang B, Mezui C, Longo F, et al. Effects of the Aqueous Extract of *Eremomastax speciosa* (Acanthaceae) on Sexual Behavior in Normal Male Rats. *BioMedical Research International*. 2016; 2016: 10.
15. Esther Ngadjui, Pierre Watcho, Telesphore Benoit Nguelefack, et al. Effects of *Ficus asperifolia* on normal rat estrus cyclicity. *Asian Pacific Journal of Tropical Biomedicine*. 2013; 3: 53-57.
16. Grace A Essiet, Godwin C Akuodor, Daniel OJ Aja, et al. Effects of *Salacia lehmbachii* ethanol root bark extract on estrous cycle and sex hormones of female albino rats. *Asian Pacific Journal of reproduction*. 2018; 7: 274-279.
17. Egbe B. Besong, Ateufack Gilbert, Albert Kamanyi. Validating sex-enhancing potentials of aqueous extract of *Pseudopanax arboreus* (Araliaceae) (L. F. Phillipson) on normal male rats. *International Journal of Multidisciplinary and current research*. 2017; 5: 1-10
18. Egbe B. Besong, G. Ateufack, Smith B. Babiaka, et al. Leaf-methanolic extract of *Pseudopanax arboreus* (Araliaceae) (L. F. Phillipson) reverses amitriptyline-induced sexual dysfunction in male rats. *Biochemistry Research International*. 2018; 14.
19. Paccola CC, Resende CG, Stumpp T, et al. The rat estrous cycle revisited: A quantitative and qualitative analysis. *Animal Reproduction*. 2013; 10: 677-683.
20. Spattini G, Borghi V, Thinoczy J, et al. Follicular development and plasma concentration of LH and prolactin in anoestrus female dogs treated with the dopamine agonist cabergoline. *Therigenology*. 2006; 68: 826-833.
21. Kei-Ichiro Maeda, Hiroko Tsukamura. The impact of stress on reproduction :Are glucocorticoids inhibitory or protective to gonadotropin secretion?. *Endocrinology*. 2006; 147: 1085-1086.
22. Whitten PL, Heather BP. Cross-species and inter-assay comparisons of phytoestrogen action. *Environmental Health Perspectives*. 2001; 109: 5-20.
23. Diedrich K, Fauser BCJM, Devroey P, et al. The role of the endometrium and embryo in human implantation. *Human Reproduction Update*. 2007; 13: 365-377.
24. Mosami Galvankar, Neha Singh, Deepak Modi. Estrogen is essential but not sufficient to induce endometriosis. *Journal of Bioscience*. 2017; 42: 251-263.
25. Stanbury J. *Botanical Medicine acting on the female reproduction system*. 2000.
26. Vasudeva N, Sharma SK. Estrogenic and pregnancy interceptory effects of *Achyranthes aspera*. *African Journal of Traditional Medicine*. 2007; 4: 7-11.
27. Ryan M. Marquardt, Tae Hoon Kim, Jung-Ho Shin, et al. Progesterone and Estrogen Signaling in the Endometrium: What Goes Wrong in Endometriosis. *International Journal of Molecular Science*. 2019; 20: 3822.