



Chemical composition and antifertility effect of a South African herbal mixture in female Sprague-Dawley rats

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ABSTRACT

Herbal contraceptives are often considered as an affordable and accessible option for birth control practice. This study evaluated the antifertility activity of a South African herbal mixture made from three plants (*Helichrysum caespititium* (DC.) Sond. ex Harv, *Bulbine frutescens* (L.) Willd and *Teucrium trifidum* Retz; ratio 1:1:1) in female Sprague-Dawley rats. Phytochemical profile of the herbal extract was analysed using ultra-high performance liquid chromatography coupled to quadrupole time-of-flight mass spectrometry (UPLC-QTOF/MS). To determine the effects of the herbal mixture on the oestrus cycle, 20 female Sprague-Dawley rats were divided into four groups (A, B, C, and D) of 5 rats per group. Group A served as the control and received distilled water for 21 days. Group B, C and D received the herbal mixture extract at a dosage of 100, 300, and 500 mg/kg, respectively, for 21 days, and vaginal smears were monitored daily. To evaluate the effects of the extract on the reproductive outcome, 21 female rats were divided into 3 groups (Groups E, F, and G), consisting of 7 rats per group. Group E served as the control and received distilled water. Group F received 300 mg/kg of the extract pre-coital for 5 days, while Group G received 300 mg/kg of the extract prior to mating with continuous administration for 14 days. The phytochemical analysis of the herbal extract revealed the presence of 19 compounds, notably verbascoside, which is known to exert either oestrogenic or anti-oestrogenic activities. The results showed that 300 and 500 mg/kg dose extended ($p < 0.05$) the duration of the metestrus phase when compared to the control, confirming the disruption of the oestrous cycle. The prolonged metestrus phase of the oestrus cycle by the herbal extract could be indicative of low reproductive hormones, decreasing the probability of conception. However, the herbal extract at 300 mg/kg had no antifertility activity in female Sprague-Dawley rats based on the number of litters in the treatment group relative to the control. It will be pertinent to assess other parameters such as anti-implantation activity, estrogenic/anti-estrogenic activity and hormonal analysis in future studies.

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1. Introduction

According to the United Nations, the world population will reach 9.7 billion in 2050 and 11.2 billion in 2100 from the current 8.7 billion as of November 2022 (United Nations, 2022). This expanding

population, especially in developing nations, is a social, economic, and environmental concern in terms of providing food, shelter, and overall quality of life. Conventional hormonal contraceptives have been primarily used to address the challenge of the ever-increasing population (Bhatt and Deshpande, 2021). While this method is reliable to a certain extent, research shows that this method has several side effects such as nausea, headaches, abdominal cramping, breast tenderness, and increased vaginal discharge or decreased libido (Britton et al., 2020).

In traditional medicine, many plants are currently being used for contraception and are generally referred to as “herbal contraceptives” (Kaur et al., 2011; Verma and Yadav, 2021). Herbal contraceptives provide alternatives for women who are uncomfortable with or do not have access to conventional contraceptive options. This is notable for women living in remote regions of developing countries with

Abbreviations: AnimCareREC, Animal Care, Health, and Safety Research Ethics Committee; GC–MS, Gas chromatography coupled to mass spectrometry; HEPA, High efficiency particulate air; HPLC, High performance liquid chromatography; HR-MS, High-resolution mass spectrometry; m/z , Mass to charge; MS, Mass spectrometry; PTFE, Poly-tetrafluoroethylene; RCF, Relative centrifugal field; UPLC-PDA, Ultra-high performance liquid chromatography coupled to photodiode array; UPLC-QTOF/MS, Ultra-high performance liquid chromatography coupled to quadrupole time-of-flight mass spectrometry; UPLC-QTOF/MS, Ultra-high performance liquid chromatography coupled to mass spectrometry; V, Voltage

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large populations such as India, China and Nigeria (Pradhan et al., 2013). Additionally, herbal contraceptives may be beneficial to women who are experiencing health issues as a result of the adverse effects of modern contraceptives (Moroole et al., 2019). Some of the plants used for contraception have been scientifically researched and have been confirmed to exert antifertility effects. One of such medicinal plants is *Asparagus africanus* (Tafesse et al., 2006; Pradhan et al., 2013; El-Ishaq et al., 2019). Evidence revealed that *Asparagus africanus* Lam. root water extract had anti-implantation activity effects in female Sprague Dawley rats.

Based on existing evidence, the antifertility properties of several medicinal plants are linked to the presence of a variety of phytoconstituents such as flavonoids, terpenoids, alkaloids, and steroidal saponins (El-Ishaq et al., 2019; Malpani et al., 2020; Alhaj et al., 2022; Moroole et al., 2022). For example, alkaloids are one of the bioactive class of phytochemicals that alter the reproductive system in humans and animals (Bala et al., 2014). *Areca catechu* L. contains alkaloids which alter the reproductive system in female rats (Shrestha et al., 2010; Bala et al., 2014). Phytochemicals such as terpenoids, alkaloids, saponins, glycosides and tannins impair ovarian functioning and oestrous cyclicity through the interaction of ovarian and extra ovarian hormones (Bhatt and Deshpande, 2021).

Historically, traditional medicines consist of single plant species (Seleteng-Kose, 2022). However, traditional health practitioners realised through the accumulation of therapeutic experience that combining different plant species to form a herbal mixture could significantly improve therapeutic effects (Seleteng-Kose, 2022), as this could lead to possible synergistic or antagonistic pharmacological effects (Ndhlala et al., 2011). Herbal mixtures are combinations of individual plants or plant parts used to treat various medical conditions. The preparation of the mixtures could be simple or complex depending on the complexity of the illness to be treated (Ndhlala et al., 2011).

In South Africa, herbal mixtures are known to be used for contraception (Moroole et al., 2019). These mixtures are often composed of various parts of individual plant species which are combined at defined ratio and administered to patients by traditional health practitioners. However, scientific evidence to substantiate the efficacy of these medicinal plants as herbal contraceptives is limited, thereby justifying the need for more research. For instance, Moroole et al. (2022) reported the safety and contraceptive efficacy of the herbal mixture consisting of *Helichrysum caespitium* (DC.) Sond. ex Harv, *Bulbine frutescens* (L.) Willd and *Teucrium trifidum* Retz. However, the efficacy results were not conclusive. Additionally, the study design for the contraceptive study had the limitation of a short drug administration period (3 days). Furthermore, the phytochemical analysis was conducted using GC–MS which revealed a few compounds. This suggests the need for a high throughput technique for the determination of the phytochemicals present in the herbal mixture. Therefore, this study was aimed at profiling the compounds and evaluating the antifertility activity of a herbal mixture consisting of *Helichrysum caespitium* (DC.) Sond. ex Harv, *Bulbine frutescens* (L.) Willd, and *Teucrium trifidum* Retz. in female Sprague-Dawley rats. This investigation is expected to provide evidence on the potential contraceptive efficacy of the locally sourced herbal mixture.

2. Materials and methods

2.1. Materials

High Performance Liquid Chromatography (HPLC) grade methanol and HPLC grade acetonitrile were purchased from Thembane Chemicals (Johannesburg, South Africa), poly-tetrafluoroethylene (PTFE) syringe filter was purchased from Sigma Aldrich, (Johannesburg, South Africa), saline was purchased from Dischem (Potchefstroom, South Africa), crystal violet powder was purchased from Merck Pty

Ltd (Johannesburg, South Africa) and cotton-tipped swab was purchased from CDR Laboratories (Johannesburg, South Africa).

2.2. Plant collection and extraction

2.2.1. Plant collection

Fresh leaves of *Helichrysum caespitium* and *Teucrium trifidum* were collected with the assistance of a traditional health practitioner in Ngaka Modiri Molema District Municipality, Mafikeng, North West Province, South Africa. Fresh roots of *Bulbine frutescens* were collected at the North-West University, Potchefstroom, South Africa. Voucher specimen for *Helichrysum caespitium* and *Teucrium trifidum* were deposited at the SD Phalatshe Herbarium of the North-West University, Mafikeng campus, South Africa with the voucher number Moroole M.A 2 and Moroole M.A 4. Voucher specimen (voucher number RM001) for *Bulbine frutescens* was deposited at AP Goossens Herbarium of the North-West University, Potchefstroom, South Africa. Plant collection permit was obtained prior to collection.

2.2.2. Aqueous extraction of herbal plant mixture

The plant materials were washed under running tap water and washed with distilled water to remove any dirt. The root parts of *Bulbine frutescens* were shade-dried at room temperature for two weeks, while the leaves of *Teucrium trifidum* and *Helichrysum caespitium* were air-dried in an oven at 40 °C for 24 h. The dried parts of each plant material were pulverised into fine powder using an electric blender (Kenwood Liquidiser, South Africa) and solid particles were removed by using a kitchen sieve with 1 mm pores.

The herbal mixture was prepared by weighing 50 g of each three powdered plants into a clean beaker and homogeneously mixed to form a uniform mixture. After being thoroughly mixed, 120 g of the herbal mixture was weighed and added into 2 L of distilled water in a glass beaker. The beaker containing the herbal mixture was placed onto a heater stirrer to heat up until boiling point. The mixture was covered with aluminium foil to prevent contamination and allowed to cool overnight. The cooled plant extract was transferred into a new beaker in preparation for centrifugation. The plant extract was centrifuged at 3000 RCF for 2 min and filtered using a Whatman filter paper and a vacuum pump. Shortly after filtration, the plant extracts were frozen at –20 °C for 24 h in preparation for freeze drying. The resultant extract from the herbal mixture extract was freeze-dried using a ViTis AdVantage 2.0 2849 BenchTop Freeze Dryer (United Scientific, USA) at –42 °C for 5 days. Thereafter, 18.56 % yield was obtained and the extract was stored at –80 °C until needed for further experimental analysis.

2.3. Phytochemical fingerprinting using ultra-high performance liquid chromatography mass spectrometry (UPLC-MS)

2.3.1. Sample preparation

A total of 10 mg of the herbal mixture extract was weighed and reconstituted in 1 mL of HPLC grade methanol. The methanol mixture was filtered using poly-tetrafluoroethylene (PTFE) syringe filter with 0.2 µm pore size to remove any undissolved particles.

2.3.2. UHPLC-Q-TOF/MS analysis of the herbal mixture aqueous extract

The liquid chromatography separation was performed using Waters Acquity Ultra Performance Liquid Chromatographic (UPLC) system coupled to a PDA Detector. The system comprises of a sampler manager, solvent manager, column oven and binary pump. An Acquity UPLC BEH C₁₈ column (150 mm × 2.1 mm, 1.7 µm, Waters) was used for this analysis. Mobile phase “B” consisted of 5 % (100 % Acetonitrile) and mobile phase “A” consisted of 95 % of water (0.1 % formic acid in 1000 mL H₂O). The flow rate was maintained at 0.300 mL/min and column compartment was maintained at 40 °C. The following gradient elution was applied: 95 % A: 5 % B to 15 % A: 85 % B in

16 min, changed to 5 % A: 95 % B in 0.5 min, back to initial ratio in 0.5 min and equilibrating the system for 1 min. A 2.0 μ L injection volume was used, and the total run time was 18 min. For the UPLC-MS analysis, the UPLC system was interfaced with a Xevo G2QToF MS (Waters, USA). The same elution gradient, flow rate and column were used. The mass spectrometer was operated in negative dual AJS electrospray ionisation mode. Nitrogen was utilized as the desolvation gas at a flow rate of 600 L/H, while the desolvation temperature was maintained at 400 °C. The capillary and cone voltages were set to 2450 V and 46 V, respectively, and the source temperature was 100 °C. The MS scan rate was at 1 spectra/s and the spectra were recorded by scanning the mass range from m/z 100 to 1200. Data were centroided during acquisition using independent reference lock-mass ions via the LockSpray™ interface to ensure mass accuracy and reproducibility. Data was also collected and processed using chromatographic software Masslynx 4.1.

2.4. In vivo antifertility study

The animal study was conducted at the DSI/NWU Preclinical Drug Development Platform (PCDDP) vivarium, and research on animals was approved by the North-West University Animal Care, Health, and Safety Research Ethics Committee (NWU-AnimCareREC) with the approval number NWU-00438-21-A5.

2.4.1. Oestrus cycle analysis

2.4.1.1. Animal husbandry. Twenty laboratory bred female Sprague-Dawley rats, aged between 80 and 100 days, were acclimatised for 7 days while housed in individual ventilated cages. Two female rats were housed per cage, with relative humidity 45–55 %, under room temperature of 22 ± 2 °C and a 12 h light/dark cycle. The cages were equipped with rack isolator systems with input and exhaust fan filter units providing HEPA filtered inlet and outlet air. The animals were provided with environmental enrichment. The rats were fed daily with standard rodent maintenance chow (NutritionHUB (Pty) Ltd) and water *ad libitum*. Good hygiene was maintained through constant cleaning of the cages by removal of faeces and changing of beddings. All rats were ear clipped for identification and given a unique reference number.

2.4.1.2. Herbal mixture administration. Twenty female Sprague-Dawley rats were randomly divided into 4 groups consisting of 5 animals each (Fig. S1). Group A received distilled water and served as the control. Group B, C & D received the aqueous extract of the herbal mixture (*Helichrysum caespitium*, *Bulbine frutescens* and *Teucrium trifidum*) at doses of 100 mg/kg, 300 mg/kg and 500 mg/kg respectively, daily for 21 days. After daily administration of the test substance via oral gavage, vaginal smears were collected from each rat for oestrus cycle analysis. After the completion of 21 days treatment, the extract was withdrawn, and oestrus cycle was studied for another 21 days. The three doses selected were lower than the lethal dose (LD_{50}) of 2500 mg/kg determined from the acute oral toxicity study (Moroole et al. 2022).

2.4.1.3. Vaginal cytology. The oestrus cycle was studied by stained preparation of vaginal smear of the animals. Vaginal cytology was performed daily at approximately the same time from 9:00 to 10:00 am. A cotton-tipped swab wetted with ambient temperature physiological saline was introduced into the vagina of the restrained rat. The cotton swab (approximately 5 mm wide and 12 mm long) was inserted into the vaginal tract of the rat (to a depth of approximately 1 cm), gently turned and rolled against the vaginal wall (at an estimated angle of 45°) then removed. Afterwards, the cells were transferred to a dry glass slide by moving the swab across the slide. The slides were air-dried and stained using 0.1 % crystal violet stain (0.1 g

of crystal violet powder in 100 mL of double distilled water). Each slide was placed in a beaker containing 0.1 % crystal violet solution for 1 min and then into a beaker of distilled water for 1 min. The wash process with distilled water was repeated for another 1 min. Excess water was removed from the edge of the slides with tissue paper while avoiding contact with the stained smear. After staining, each slide was examined under a light microscope with a magnification range between 5 $\times$, 10 $\times$ and 40 $\times$. The proportion and type of cells (leukocytes, cornified epithelial and nucleated epithelial cells) found in the vaginal smears were used to identify and classify the four stages of the oestrous cycle.

2.4.2. Antifertility activity - Reproductive outcome in rats

2.4.2.1. Animal husbandry. A total of 42 laboratory bred rats, consisting of 21 male and 21 female Sprague-Dawley rats, aged between 80 and 100 days were acclimatised for 7 days while housed in individual ventilated cages. Two animals of the same gender were housed per cage, under the conditions described in Section 2.4.1.1.

2.4.2.2. Herbal mixture administration and experimentation. The antifertility activity study was evaluated following existing protocols (Ganguly et al. 2007; Sewani-Rusike 2013; Moroole et al. 2022). To determine the antifertility activity of the herbal mixture, the most effective dose (300 mg/kg) that disrupted the oestrus cycle was selected. Twenty-one female Sprague-Dawley rats were randomly divided into 3 groups (Group E, F & G) consisting of 7 animals each (Fig. S2). Male rat beddings were placed in female rat cages during acclimatisation for synchronization of the oestrous cycle. Vaginal smears were collected from each female rat and examined under the microscope to confirm the stage of oestrus cycle. Group E received distilled water and served as the control. Group F received 300 mg/kg of the aqueous extract of the herbal mixture pre-coital for 5 days, by oral gavage followed by pairing with male rats at ratio of 1:1 (one male rat and one female rat in a cage) for 9 days. Group G received 300 mg/kg of the aqueous extract of the herbal mixture pre-coital for 5 days, followed by pairing with male rats at ratio of 1:1 for 9 days during which oral administration of the extracts continued daily during the 9-day mating period. After mating, the female animals were separated from the males and observed for 19 days for pregnancy. If any sign of pregnancy was observed, the animals were allowed to complete one gestation period. After the completion of one gestation period, the number of litters was determined for all experimental groups. The litters were allowed to mature for two weeks and the growth of litters from the treated group were compared to that of the control group. This was done by checking for any signs of physical abnormalities (Appearance of fur, eyes, behaviour, tumours, and genitalia).

2.5. Data analysis

The data from the oestrous cycle analysis and reproductive outcome were expressed as mean $\pm$ standard deviation. Statistical analysis of the variance between experimental and control values was carried out using one-way ANOVA and followed by a Bonferroni post-hoc test. The statistical significant difference was considered when $p < 0.05$.

3. Results and discussion

3.1. Phytochemical characterisation of the aqueous herbal mixture extract

The phytochemical analysis of the aqueous extract of the herbal mixture using UPLC-QTOF/MS revealed the presence of 19 compounds and one unidentified compound (Table 1). Major peaks

Table 1
Tentative phytochemical compounds identified in the aqueous extract of the herbal mixture using ultra-high performance liquid chromatography coupled to quadrupole time-of-flight mass spectrometry (UPLC-QTOF/MS).

Peak No:	Retention time (Min)	Molecular formula	[M–H] [–] (m/z)	Tentative Identified compound	Fragments	λ _{max} (nm) (PDA)	Intensity (mAU)	Class
1	2.83	C ₂₀ H ₃₀ O ₁₂	461.1652	Chlorogenic acid derivatives	375, 353, 315, 191, 135, 113	233.9, 324.9	176,000	Phenol
2	3.06	C ₁₇ H ₃₀ O ₁₂	425.1655	alpha-D-Glucopyranoside, alpha-D-glucopyranosyl, 3-(2-methylbutanoate)	407, 389, 245, 161, 143	238.9	579,000	Glycoside
3	3.35	C ₁₆ H ₁₈ O ₉	353.0873	Chlorogenic acid	191, 179, 161, 135	240.9, 321.9	138,000	Phenol
4	3.88	C ₉ H ₈ O ₄	179.0351	Caffeic acid	135	240.9, 321.9	268,000	Phenol
5	3.95	C ₁₇ H ₂₈ O ₁₁	407.1569	1-Glucosyl acylclidiydroelenic acid	389, 305, 233, 179, 161, 143		362,000	Secoiridoid
6	4.13	C ₂₉ H ₃₆ O ₁₆	639.1927	Phenylethanoid glycoside	621, 529, 459, 415, 201, 179	240.9, 328.9	256,000	Glycoside
7	4.17	C ₂₉ H ₃₆ O ₁₆	639.1923	Phenylethanoid glycoside	621, 529, 459, 415, 201, 161	240.9, 328.9	258,000	Glycoside
8	4.62	C ₂₃ H ₁₈ O ₁₂	485.0761	Dihydromyricetin-methyl-gallate	241, 139	241.9, 350.9	323,000	Flavonoid
9	4.79	C ₂₅ H ₂₄ O ₁₂	515.0858	Unidentified	245.9, 350.9	245.9, 350.9	127,000	Unknown
10	4.99	C ₂₉ H ₃₆ O ₁₅	623.1990	Verbascoside	461, 315, 179, 161	242.9, 328.9	1,230,000	Glycoside
11	5.18	C ₂₅ H ₂₄ O ₁₂	515.1201	Dicaffeoylquinic acid	353, 241, 191, 179, 161	243.9, 321.9	64,500	Phenol
12	5.27	C ₂₉ H ₃₆ O ₁₅	623.1990	Isoverbascoside	461, 263, 179, 161	243.9, 325.9	233,000	Glycoside
13	5.32	C ₂₉ H ₃₆ O ₁₅	623.1974	Forsythiaside	461, 353, 263, 191, 179, 135	244.9, 325.9	131,000	Glycoside
14	5.87	C ₄₁ H ₇₂ O ₁₀	723.5027	Glycoside derivative	677, 451, 161	244.9, 325.9	51,600	Glycoside
15	6.43	C ₂₂ H ₂₅ O ₁₂	481.1336	Caffeic acid derivative	329, 299, 241, 179, 139	238.9	73,100	Phenol
16	7.14	C ₃₁ H ₃₂ O ₁₅	643.1650	Glycoside derivative	551, 483, 461, 357, 323, 279	246.9, 303.9	65,200	Glycoside
17	7.46	C ₂₆ H ₃₂ O ₁₃	551.1770	Neosarininoside B/Arbortristoside C	337, 213, 183, 141	269.9	131,000	Glycoside
18	7.62	C ₄₅ H ₆₄ O ₁₇	875.4087	Triterpenoid glycoside derivative	857, 769, 727, 625, 147		189,000	Glycoside
19	8.79	C ₄₅ H ₈₆ O ₂₈	1073.5208	Triterpenoid glycoside derivative	927, 875, 769, 329	269.9, 325.9	168,000	Glycoside
20	9.85	C ₄₅ H ₇₅ O ₁₈	901.4237	Triterpenoid glycoside derivative	883, 795, 753, 651, 427, 319	248.9, 303.9	201,000	Glycoside

identified in the chromatogram were exposed to negative mode of ESI-QTOF-MS (Fig. 1). Additionally, for compound identification, the ion peaks and MS fragments of the compound were used and compared with compound databases such as PubChem, ChemSpider, MONA and literature. The compounds identified consisted of glycosides (63.16 %), phenols (26.32 %), secoiridoid (5.26 %) and flavonoids (5.26 %).

In the herbal extract, the most abundant compound was verbascoside with peak intensity of 1,230,000 mAU. Verbascoside is a phenylethanoid glycosidic compound that has been found to exhibit *in vitro* estrogenic and anti-estrogenic activity (Papoutsis et al., 2006; Wu et al., 2021). Various studies revealed that plants that are exhibiting estrogenic and anti-estrogenic activity may have antifertility properties (Sharma and Bhinda, 2005; Bhaskar et al., 2009; Sandeep et al.,

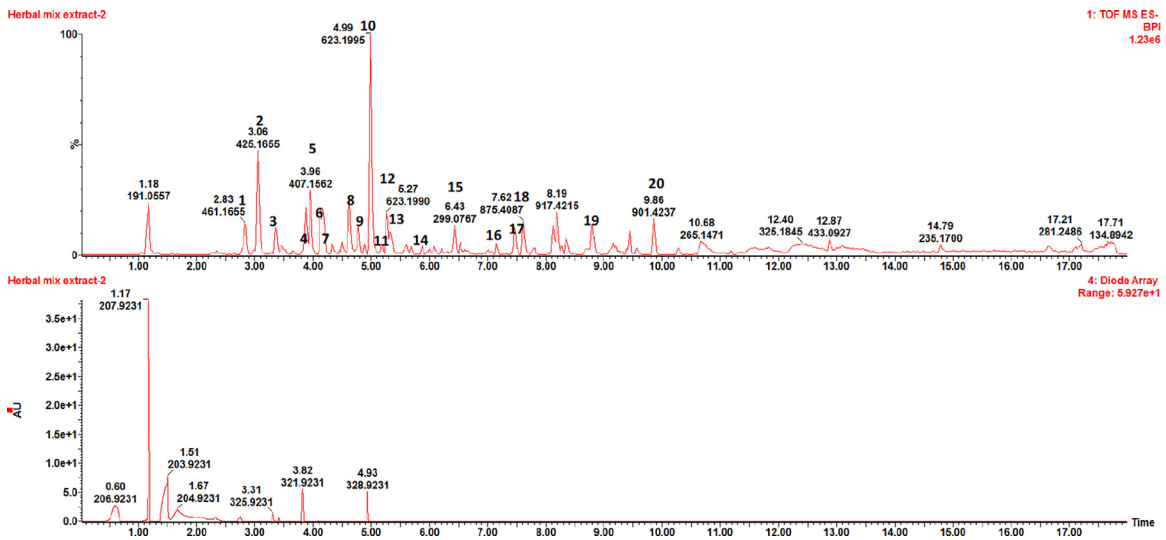


Fig. 1. Ultra-high performance liquid chromatography coupled to quadrupole time-of-flight mass spectrometry (UPLC-QTOF/MS) and Ultra-high performance liquid chromatography coupled to photodiode array detector (UPLC-PDA) chromatogram of the aqueous extract of herbal mixture.

2011; Patil and Patil, 2012). Additionally, preliminary screening of plants evaluated for antifertility activities have shown the presence of phytochemicals such as phenol, flavonoid and glycosides (Pattanayak and Mazumder, 2009; Kumar et al., 2017; Shah and Jhade, 2018; El-Ishaq et al., 2019). Therefore, the synergism produced by these phytochemicals could be responsible for the antifertility properties of the herbal mixture as prescribed by traditional health practitioners.

3.2. Oestrous cycle analysis

The female rodent reproductive cycle is referred to as the oestrous cycle, and it resembles the human reproductive cycle, also known as the menstrual cycle. The oestrous cycle is a periodic process that explains variations in reproductive hormone levels resulting from ovarian activity in the presence of pituitary hormones (Aritonang et al., 2017; Ajayi and Akhigbe, 2020). The oestrous cycle is characterized into four stages namely, proestrus, oestrus, metoestrus, and dioestrus. Evidently, the short length of the oestrous cycle of rats makes them an excellent model for the evaluation of reproductive system and the changes that occur during the reproductive cycle (Aritonang et al., 2017). To identify the various phases of the oestrus cycle, cells present in the vaginal smears were viewed under the microscope and classified into 4 phases (Fig. 2), with proestrus phase having nucleated epithelial cell, oestrus phase with predominantly epithelial cells, metestrus phase with mostly non-nucleated cells and small leucocytes and dioestrus phase having large number of leukocytes and small nucleated cells

A comparison of the oestrous cycle of the control and treated groups revealed a disruption of the oestrus cycle due to extension of the duration of the metestrus phase ($p < 0.05$) (Table 2). The metestrus phase is the third stage of the oestrus cycle and is marked by a regression in the uterus lining and a decrease in reproductive hormone levels, such as estrogen and progesterone (Westwood, 2008; Byers et al., 2012). This disruption of the oestrus cycle indicates that the extracts has some effects on the reproductive hormone levels. This could be due to the possible effect on the hypothalamic-pituitary-gonadal (HPG) axis which may have disrupted the feedback loop resulting in altering of hormone levels (Patil and Patil, 2013). The results obtained in this study are similar to that of Mishra et al. (2009), where the aqueous extract of *Bougainvillea spectabilis* Willd.

Table 2

The effect of a herbal mixture extract consisting of *Helichrysum caespitium* (DC.) Harv, *Bulbine frutescens* (L.) Willd and *Teucrium trifidum* Retz on the oestrous cycle of female Sprague- Dawley rats.

21 Days Oestrus cycle analysis – Treatment				
Treatment (mg/kg)	Proestrus	Oestrus	Metestrus	Dioestrus
0 (Control)	3 ± 1.58	4.2 ± 3.27	6.6 ± 2.07	7.2 ± 3.83
100	1.2 ± 1.30	4.4 ± 3.65	11.8 ± 4.02	3.6 ± 5.13
300	1.6 ± 0.55	6.2 ± 1.30	11.6 ± 2.07*	1.6 ± 1.52
500	1.4 ± 1.14	4.4 ± 2.19	11.2 ± 1.92*	4 ± 4.06

Data are expressed as mean ± SD. $n = 5$. * $P < 0.05$.

leaves disrupted the oestrous cycle by prolonging the metestrus phase, with an increase of about 145.28 %. The study also revealed that the serum level of oestrogen was significantly reduced in female rats (Mishra et al., 2009).

The present study revealed that the effects of the herbal mixture on the oestrus cycle is dose-dependent. The mid-dose (300 mg/kg) and high-dose (500 mg/kg) were more effective in disrupting the oestrous cycle. In addition, the extension of the metestrus phase by the extracts could be indicative of the estrogenic/anti-estrogenic activity of the herbal mixture which could be due to the presence of verbascoside (Sharanabasappa et al., 2002; Wu et al., 2021). Furthermore, the disruption of the oestrous cycle and metestrus phase may suggest the antifertility properties of the herbal extract, as it delays the advent of other phases and reduces the frequency of ovulation and fertilisation (He et al., 2017). The study conducted by Kaingu et al. (2018) corroborates the present finding where the administration of aqueous extracts of *Croton menyharthii* Pax and *Uvariadendron kirkii* Verdc. in female rats disrupted the oestrus cycle and its metestrus phase.

The reversibility of a contraceptive agent is essential in the normal functioning of the female reproductive system, as such agents are not meant to cause infertility but antifertility. In the current study, the effect of the herbal extracts on the oestrus cycle was reversible, i.e., there was no significant difference between the control and treatment groups when extract was withdrawn for 21 days (Table 3). The reversibility of the herbal mixture may signify a relative short half-life of the extract in rats, indicating that its components could be rapidly eliminated once it is absent from the system leading to transient effects.

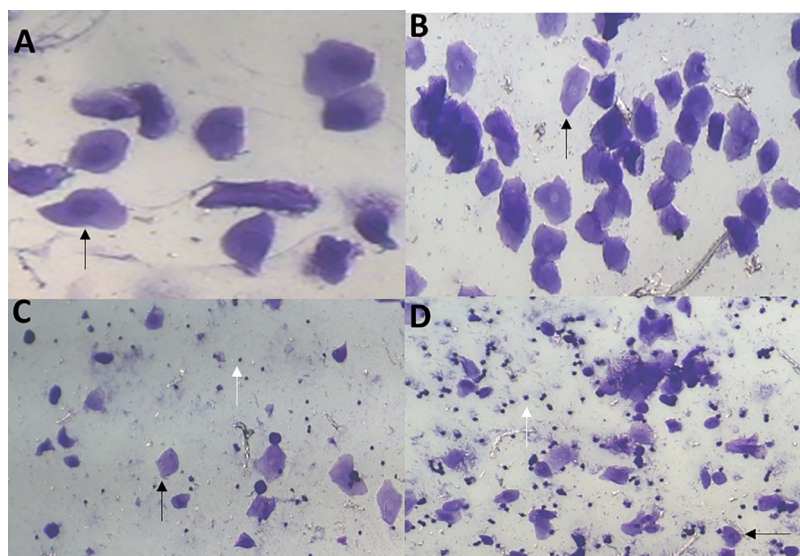


Fig. 2. Image showing the cytological assessment of the crystal violet-stained vaginal smears of female Sprague-Dawley rats at 40X magnification (A) Proestrus: predominantly small well-formed nucleated epithelial cells (B) Oestrus: Predominantly large cornified epithelial cells (C) Metestrus: leucocytes with smaller numbers of non-nucleated epithelial cells (D) Dioestrus: Predominantly leucocytes with small number of nucleated epithelial and cornified cells.

Table 3

Post treatment effect of a herbal mixture extract consisting of *Helichrysum caespitium* (DC.) Harv, *Bulbine frutescens* (L.) Willd and *Teucrium trifidum* Retz on the oestrous cycle of female Sprague-Dawley rats.

21 Days Oestrus cycle analysis – Post-Treatment				
Treatment (mg/kg)	Proestrus	Oestrus	Metestrus	Dioestrus
0 (Control)	5.8 ± 1.30	5.8 ± 0.45	6 ± 1.41	3.4 ± 1.52
100	4.6 ± 1.14	7.6 ± 1.14	5.8 ± 0.84	3 ± 1.55
300	6.2 ± 0.84	5.6 ± 0.55	4.8 ± 1.10	4.4 ± 0.55
500	6.2 ± 1.64	5.8 ± 0.45	5.2 ± 1.64	3.8 ± 1.64

Data are expressed as mean ± SD. n = 5. * P < 0.05.

Table 4

The antifertility activity of a herbal mixture extract consisting of *Helichrysum caespitium* (DC.) Harv, *Bulbine frutescens* (L.) Willd and *Teucrium trifidum* Retz at dose of 300 mg/kg in female Sprague-Dawley rats.

Number of litters	
Control	9.57 ± 4.54
Pre coital administration (5 days)	8.43 ± 4.58
Pre continuous administration (14 days)	9.57 ± 4.79

Data are expressed as mean ± SD. n = 7. * P < 0.05.

3.3. Antifertility activity - Reproductive outcome

Herbal medicines have been used for centuries to treat varieties of conditions and are often regarded to be safe with limited side effects (Gurumani and Balamurugan, 2014). Many plants have been reported to have antifertility properties, but only few have been reported to be evaluated in laboratory animals (Gurumani and Balamurugan, 2014). The present study examined the antifertility activity of a herbal mixture used by traditional healer practitioners by evaluating the effects on reproductive outcome. The results revealed that all female rats in the treatment groups fell pregnant and one female rat out of the control group did not fall pregnant. This could be due to the male rat's inability to consummate or due to infertility.

In the current study, there was no significant reduction in the number of litters of the treatment groups compared to the control group (Table 4). This indicates that at the tested dose, the herbal mixture did not exhibit antifertility activity. The absence of antifertility could also be attributed to the removal of extracts following the prescribed treatment duration. As shown in Table 3, the oestrus cycle returned to its normal cycle when the extracts were withdrawn, indicating that the extracts might have temporarily influenced reproductive hormones and as soon as the extract was withdrawn, the hormone levels were restored, hence conception. In addition, Moroole et al. (2022) used the same dose of 300 mg/kg, which showed no efficacy. This could imply that the dosage administered may not be sufficient at inhibiting fertilization or implantation which is essential process involved in preventing conception.

4. Conclusion

In the present study, the phytochemical analysis revealed the presence of 19 compounds and one unidentified compounds in the tested herbal mixture. The results revealed that the herbal mixture used by traditional health practitioners in the North-West Province had effects on the oestrus cycle in female Sprague-Dawley rats. The effect was exerted by extending the duration of the metestrus phase of the oestrus cycle. The effects could be attributed to the presence of verbascoside which exerts both estrogenic and anti-estrogenic activity, which could

potentially influence reproductive hormones. However, the herbal mixture showed no antifertility activity at the tested dose. Further studies such as anti-implantation activity, estrogenic/anti-estrogenic activity and hormonal analysis remain pertinent to determine the effects of the herbal extract on reproductive hormones.

Declarations

Ethical statements (and/or informed consent in case of human study)

This research was approved by the North-West University Animal Care, Health, and Safety Research Ethics Committee (NWU-AnimCar-eREC) with the approval number NWU-00438-21-A5.

Availability of data and material

All data generated or analysed during this study are included in this published article.

Data availability

All data generated or analysed during this study are included in this published article.

Declaration of competing interest

We declare that no known competing financial interests or personal relationships that could have appeared to influence the work reported in this article.

CRediT authorship contribution statement

Opeyemi J. Fadeyi: Conceptualization, Formal analysis, Investigation, Writing – original draft, Data curation. **Makhotsa Lekhooa:** Conceptualization, Data curation, Formal analysis, Supervision. **Molelekwa A. Moroole:** Supervision. **Cor Bester:** Data curation. **Adeyemi O. Aremu:** Conceptualization, Data curation, Formal analysis, Supervision. **Rose Hayeshi:** Conceptualization, Data curation, Formal analysis, Supervision.

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Supplementary materials

Supplementary material associated with this article can be found in the online version at doi:10.1016/j.sajb.2024.05.025.

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