

Phytochemical analysis and beneficial effects of *Artemisia absinthium* L. essential oil in rat pseudopregnancy model

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Research Article

Keywords: *Artemisia absinthium*, Asteraceae, essential oil, in vivo, pseudopregnancy

Posted Date: June 10th, 2022

DOI: <https://doi.org/10.21203/rs.3.rs-1711199/v1>

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Abstract

Pseudopregnancy is a luteal phase syndrome in which steroids, prolactin inhibitor agents or ovariectomy/ovariohysterectomy are used in the treatment. New medical strategies should be developed because of the serious side effects of current treatment regimens. Several plants and natural compounds are investigated for their modulatory effect on hormonal balance in the treatment of pseudopregnancy. *Artemisia absinthium* L. (wormwood) is a medicinal plant that belongs to the Asteraceae family, used as an emmenagogue and to induce abortus traditionally. This plant has a regulatory effect on the dopaminergic system. In this study, the therapeutic potential of the *A. absinthium* essential oil was examined in an experimentally-induced pseudopregnancy model in rats. The pseudopregnancy model was induced by injection of a pregnant mare's serum gonadotropin and human chorionic gonadotropin to female rats. Essential oil of *A. absinthium* was orally administered to the rats at 12.5 mg/kg, 25 mg/kg and 50 mg/kg doses once daily for 10 days. Bromocriptine (3 mg/kg/per os) was administered to the reference group animals. Gas Chromatography analysis was conducted on the essential oil to reveal its phytochemical profile. *A. absinthium* essential oil at 25 mg/kg dose displayed beneficial effects in the pseudopregnancy model in rats. *Cis*-chrysanthenyl acetate (17.8), sabinyl acetate (11.6%), terpinen-4-ol (6.2%), caryophyllene oxide (5.5%) and (*E*)-nuciferol (5.5%) were found as the major components in the oil. *A. absinthium* essential oil rich in *cis*-chrysanthenyl acetate, sabinyl acetate, terpinen-4-ol, caryophyllene oxide, and (*E*)-nuciferol should be displayed therapeutic activity against pseudopregnancy.

Introduction

Pseudopregnancy, also known as pseudocyesis, is a luteal phase syndrome that shows clinical signs typical of late pregnancy and/or early postpartum periods, despite not being pregnant. This syndrome is characterized by physiological and behavioral effects (Campos and Link 2016; Chestnut 2019; Gobello et al. 2021). Clinically, enlarged mammary glands and lactation due to increased prolactin levels, weight gain, vomiting and loss of appetite can be observed. In addition to these findings, maternal behavior occurs during the pseudopregnancy period (Braunstein 2011; Chestnut 2019; Gobello et al. 2021). Diagnosis of pseudopregnancy syndrome is performed by clinical and ultrasonographic examinations. This syndrome is confirmed by the absence of the fetus/fetuses in ultrasonographic examination. In cases where the false pregnancy is not treated, it can progress and cause mastitis or a breast tumor. Treatment procedures such as steroids (estrogens, progestins), prolactin inhibitor agents (dopamine agonists) or ovariectomy/ovariohysterectomy aim to eliminate the clinical and behavioral outcomes (Tarín et al. 2013; Root et al. 2018; Papich 2021). Due to the serious side effects of the current medical treatment regimens in pseudopregnancy, new medical strategies should be developed. Natural resources and medicinal plants are of great importance in human civilization in treating various diseases. In the literature, *Pulsatilla* Mill. (Ranunculaceae), *Ferula* L. (Apiaceae), *Urtica* L. (Urticaceae), *Thuja* L. (Cupressaceae) species and *Matricaria chamomilla* L. (Asteraceae) used for the treatment of pseudopregnancy (Aslan et al. 2004; Madrewar and Glencross 2014; Özyurtlu and Alaçam 2005).

Artemisia absinthium L. is an important aromatic and medicinal plant that belongs to Asteraceae family, used as an emmenagogue and to induce abortus in traditional medicine as food for digestive properties (Baytop 1999; Judžentienė 2016). Besides the special use of wormwood in the spirit of absinthe, it is also used as a flavoring agent for other alcoholic beverages. Essential oil of wormwood had high concentrations of terpenes (Nguyen and Németh 2016) and wide a range of biological activities and used as antimicrobial, antiseptic, anthelmintic, anti-inflammatory, antidepressant, anti cold, carminative, choleric, digestive, stimulant (Goud and Swamy 2015; Nguyen and Németh 2016; Watson and Preedy 2008). Several studies have shown that *A. absinthium* has a regulatory effect on the dopaminergic system (Basiri et al. 2017; Sansar and Gamrani 2013; Zeraati et al. 2014). We investigated the therapeutic effects of petroleum ether, dichloromethane and methanol extracts of *A. absinthium* on pseudopregnancy model in our previous study (Demirel et al. 2018). Among tested extracts, petroleum ether extract exhibited a positive effect when compared to the control group. Therefore, this study aims to evaluate the potential activity of *A. absinthium* essential oil which is rich in nonpolar and volatile compounds for the treatment of pseudopregnancy.

Materials And Methods

Plant material

Artemisia absinthium L. aerial were collected in the city of Konya (between Taskent and Ermenek, Turkey), in the 2019 summer season. The plant was identified by Prof. Dr. Evren Yildiztugay (Faculty of Science, Selcuk University, Konya, Turkey) and one voucher specimen was deposited in the Department of Biology, Selcuk University (Voucher No: GZ-1928). The aerial parts of the plant samples were shade dried for 10 days at room temperature. The plant samples were powdered by using a laboratory mill and the powdered plant samples were stored in dark at room temperature.

Essential oil extraction and analysis

The essential oil was obtained by using hydro-distillation technique. 100 g dried plant samples were distilled in a Clevenger-type apparatus for 5 h. The essential oil was dried over sodium sulfate (anhydrous) and then the obtained essential oil was stored in an amber vial at + 4°C until analysis. The essential oil composition was characterized by gas chromatography-flame ionization detector (GC-FID) and gas chromatography-mass spectrophotometry (GC-MS) techniques. GC-MS analysis was performed by using an Agilent 5975 GC-MS system coupled to an Agilent 7890 A GC. To separate chemical components, the HP-Innowax column (60 m x 0.25 mm, 0.25 µm film thicknesses) was used. Other analytical parameters were reported in our earlier paper (Ak et al. 2021). The retention index (RI) calculated by co-injection with reference to a homologous series of *n*-alkanes (C8-C30) under the identical experimental circumstances was used to identify the components. In order to make more accurate identifications, RI values and mass spectra of the compounds were compared with those of literature and NIST 05 and Wiley 8th edition, respectively.

Animals

Thirty-six immature, healthy, 29-days-old female (70–75 g) Sprague Dawley rats were provided from Experimental Animal Center, Gazi University (Ankara, Turkey). The rats were quarantined for one week and housed in polysulfone cages with aspen shavings for bedding at 21–24°C and 45–55% humidity and with light-controlled (12 h light/12 h dark) conditions at the Laboratory Animals Breeding and Experimental Research Unit of the Faculty of Pharmacy, Gazi University. All animals were fed with a standard pellet diet and water *ad libitum* during the experimental period. The rats were maintained in accordance with the directions of the Guide for the Care and Use of Laboratory Animals. The experimental procedures of the present study were approved by the Experimental Animal Ethics Committee of Gazi University (G.U.ET- 17.015).

Induction of pseudopregnancy model

A flow chart of the experimental procedure is given in Fig. 1. All the rats were randomly allocated into six groups consisting of six rats in each group, as follows: Sham (S) Group, Control (C) Group, Reference (R) Group, and three different Treatment Groups [T-1 (12.5 mg/kg), T-2 (25 mg/kg) and T-3 (50 mg/kg)] of *A. absinthium* essential oil. The pseudopregnancy model was not applied to the Sham Group. The rats of Sham Group were subcutaneously given NaCl 0.9%. To induce the pseudopregnancy model, initially, the 29 days-old rats received a single dose of injection of 50 IU/s.c. Pregnant Serum Gonadotropin (PMSG; Chronogest®, Intervet, Turkey). Three days after PMSG injection, the 32 days-old rats were administered a single dose of 20 IU/s.c. hCG (Choragon®, Erkim, Turkey) (Bar-Ami et al. 2006). The hCG injection day was considered as the day 0 of the pseudopregnancy.

The estrus stage of the rats was determined during the experimental procedure. On the 0, 5th, and 10th day of hCG administration, vaginal smear samples were taken from all the rats. The stages of the estrous cycle were classified as proestrus (oval nucleated epithelial cells), estrus (irregular-shaped cornified epithelial cells), metestrus (fragmented, cornified epithelial cells and smaller darker stained leukocytes), and diestrus (nucleated epithelial, predominate leukocytes) (Cora et al. 2015).

The treatment procedure

The application of the test materials were started immediately after hCG injection and continued for 10 days. The essential oil of *A. absinthium* was prepared in 2% Tween 80 aqueous solution (Abdollahi et al. 2003; Abidi et al. 2018). Tween 80 aqueous solution (2%; 2 mL/*per os*) was given to the Sham and Control Groups. Bromocriptine (Parlodel®, Meda Pharma, Turkey) was administered to the Reference Group (3 mg/kg/*per os*) animals. Treatment Groups received 12.5 mg/kg, 25 mg/kg and 50 mg/kg doses of *A. absinthium* essential oil in 2% Tween 80 aqueous solution, respectively.

Termination of the experimental procedure

On the 10th day of the treatment procedure, all the rats were sacrificed by exsanguination under general anesthesia (10 mg/kg/i.p. xylazine hydrochloride and 80 mg/kg/i.p. ketamine hydrochloride). The length and width of uterine tissue were measured by using a micrometer and its volume was calculated. The

uterine and ovarian tissues were removed and weighed on a precision scale. The mammary chains were bilaterally dissected and weighed.

The histopathological evaluation of mammary, uterine and ovarian tissues under a light microscope

The mammary, uterine, and ovarian tissues were immediately transferred into 10% neutral formaldehyde solution for 48 h. The samples were grossed by a pathologist to obtain proper slices from the tissues and rinsed with 0.9% saline solution. The tissue samples were put into the cassettes and processed using an automated tissue processor. The samples were embedded in paraffin, cut into 5- μ m-thick sections. The slides were stained with Haematoxylin and Eosin (H&E) and examined blindly under a light microscope. Mammary alveolar development, uterine and ovarian tissues were microscopically evaluated. The endometrial glands were counted. The thickness of the myometrium was measured. Furthermore, the stage of the estrus cycle was determined according to the histological appearance of uterine tissue (Dixon et al. 2014). The numbers of corpus luteum, regressed corpus luteum and tertiary follicles were counted in ovarian tissues to assess the ovarian follicular activity. Mammary alveolar development was scored at x 10 magnification as follows: -: No alveoli; +: 5–10 alveoli; ++: 10–50 alveoli; +++: more than 50 alveoli (Demirel et al. 2018).

Statistical analysis

Statistical analyses were performed using Graphpad Prism 6.0. The data are shown as the mean $\pm$ standard error of the mean (SEM). The analysis of variance (ANOVA) test was used to determine the significance of differences between groups. The results were considered statistically significant at $p < 0.05$.

Results

Chemical composition of the essential oil

GC-MS analysis showed that 60 compounds were identified in the tested essential oil. The compounds are listed in Table 1. The identified compounds accounted for 90.5% of the total compounds. *cis*-Chrysanthenyl acetate was the main compound with a percentage of 17.8. Other main compounds were sabinyl acetate (11.6%), terpinen-4-ol (6.2%), caryophyllene oxide (5.5%) and (*E*)-nuciferol (5.5%).

Table 1
Chemical constituents of essential oil from *Artemisia
absinthium* L. (%)

Compounds	RRI ^a	(%)
Sabinene	1124	0.1
Myrcene	1165	0.1
α -Phellandrene	1168	tr ^b
α -Terpinene	1183	tr
1,8-Cineole	1211	0.1
2-Hexanol	1225	tr
γ -Terpinene	1249	0.2
<i>p</i> -Cymene	1276	2.7
Terpinolene	1286	0.1
Perillen	1426	tr
<i>trans</i> -Linalool oxide (furanoid)	1451	0.8
β -Thujone	1454	0.1
<i>trans</i> -Sabinene hydrate	1469	0.1
(Z)- β -Ocimene oxide	1473	3.7
<i>cis</i> -Linalool oxide (furanoid)	1479	0.6
Camphor	1535	0.1
Linalool	1548	4.5
<i>cis</i> -Sabinene hydrate	1554	0.1
<i>trans-p</i> -Menth-2-ene-1-ol	1570	0.5
<i>cis</i> -Chrysanthenyl acetate	1581	17.8
Bornyl acetate	1593	0.2
β -Elemene	1601	0.6
Terpinen-4-ol	1612	6.2
<i>cis-p</i> -Menth-2ene-1-ol	1634	0.3
^a Relative retention indices calculated against n-alkanes.		
^b Trace (< 0.1%).		

Compounds	RRI ^a	(%)
α -Thujenal	1645	0.2
Sabina ketone	1655	0.6
Sabinyl acetate	1663	11.6
Lavandulol	1681	0.5
<i>trans</i> -Verbenol	1690	0.4
Carvotan acetone	1700	0.1
α -Terpineol	1706	1.2
<i>trans</i> -Sabinol	1711	0.5
β -Selinene	1743	0.4
Phellandral	1745	0.2
Naphthalene	1769	1.4
Neryl isobutyrate	1783	1.4
p-Methyl acetophenone	1800	1.0
Cumin aldehyde	1807	2.0
Perilla aldehyde	1810	0.4
Geraniol	1852	0.1
<i>p</i> -Cymen-8-ol	1861	1.7
Neryl isovalerate	1866	1.8
Geranyl isovalerate	1886	1.4
<i>cis</i> -Jasmone	1968	0.3
(<i>E</i>)-12-Norcaryophyll-5-ene-2-one	1996	0.6
Caryophyllene oxide	2017	5.5
(<i>E</i>)-Nerolidol	2051	0.3
Humulene epoxide II	2074	0.5
Heneicosane	2100	0.1

^a Relative retention indices calculated against n-alkanes.

^b Trace (< 0.1%).

Compounds	RRI ^a	(%)
Cumin alcohol	2121	0.8
Hexahydrofarnesyl acetone	2134	0.7
Spathulenol	2147	3.1
Nonanoic acid	2168	0.5
Thymol	2196	0.9
Selin-11-en-4 α -ol	2263	4.6
Decanoic acid	2274	0.3
(<i>E</i>)-Nuciferol	2355	5.5
Chamazulene	2436	0.2
Tetradecanoic acid	2698	0.3
<i>n</i> -Hexadecanoic acid	2912	0.6
Total identified		90.5
^a Relative retention indices calculated against n-alkanes.		
^b Trace (< 0.1%).		

Vaginal cytology

The cytological evaluation was performed according to the classification specified in the materials and methods section. The vaginal smear samples taken before the induction of the pseudopregnancy model showed that the mean duration of the estrous cycle was 4–5 days in all rats. This regular cycle in the S Group continued during the experimental period. However, in the other groups, it was found that all the animals were in the luteal (metestrus-diestrus) phase of the estrus cycle after hormone administrations. The cells (nucleated epithelial cells) of the luteal stage continued to be observed in all the rats of the C Group during the experimental procedure. In the treatment groups, irregular-shaped cornified squamous epithelial cells began to be observed at the end of the procedure. Therefore, it was demonstrated that the estrous cycle started in these rats. These alterations were similar in all treatment groups (Fig. 2).

Mammary, ovarian and uterine tissue weights and uterine volume

The macroscopic views of the uterine horns and ovaries are given in Fig. 3. There was no pathological alteration in the macroscopic views of the uterine horns and ovaries of the S group. Both uterine horns were enlarged in the C group compared to the other groups. *A. artemisia* essential oil treatment provided better healing macroscopically when compared to C group. The mean value of the uterine volume of the

C group animals ($267.90 \pm 7.19 \text{ mm}^3$) was higher than those of the treatment groups (T-1: 251.20 ± 12.82 ; T-2: 251.20 ± 0.00 and T-3: $259.10 \pm 7.85 \text{ mm}^3$) (Table 2).

Table 2

The comparison according to groups of the weight of mammary gland, ovarian and uterine tissue and uterine volume

Groups	Mean $\pm$ S.E.M.			
	Mammary gland weight (g)	Ovarian weight (g)	Uterine weight (g)	Uterine volume (mm^3)
S	1.30 ± 0.09	$0.07 \pm 0.00^{**}$	0.22 ± 0.02	251.2 ± 3.97
C	1.70 ± 0.12	0.22 ± 0.04	0.25 ± 0.01	267.90 ± 7.19
R	1.48 ± 0.20	0.13 ± 0.02	0.22 ± 0.02	257.40 ± 6.31
T-1	1.37 ± 0.02	0.16 ± 0.02	0.17 ± 0.00	251.20 ± 12.82
T-2	1.29 ± 0.07	$0.12 \pm 0.01^*$	0.16 ± 0.02	251.20 ± 0.00
T-3	1.48 ± 0.15	0.13 ± 0.01	0.17 ± 0.01	259.10 ± 7.85
The experimental groups were compared with the control group (*: $p < 0.05$; **: $p < 0.01$); S.E.M. Standard error of the mean; S: Sham Group; C: Control Group; R: Reference Group; T-1: Treatment Group-1 (12.5 mg/kg); T-2: Treatment Group-2 (25 mg/kg); T-3: Treatment Group-3 (50 mg/kg)				

Ovarian weights were significantly different in S ($0.07 \pm 0.00 \text{ g}$, $p < 0.001$) and T-2 ($0.12 \pm 0.01 \text{ g}$, $p < 0.05$) groups when compared to the C group ($0.22 \pm 0.04 \text{ g}$). The greatest decrease in ovarian weight was detected in the T-2 group among the treatment groups. Although the uterine weights reduced in the treatment groups, no significant difference was found (Table 2).

Due to pseudopregnancy induction, the mammary glands enlarged in the C group. The mammary glands' development in the treatment groups, especially in the T-2 group, was lower than that of the C group (Fig. 4). There was no statistically significant difference between the groups in terms of mammary gland weight. However these parameters were found to be the highest in the control and the lowest in the T-2 group (Table 2).

Histopathological analysis

Microscopic findings of ovarian, uterine and mammary tissues are presented in Table 3. Significant difference was determined in terms of total corpus luteum (CL) number ($p \leq 0.05$). The number of total CL was found to be the lowest in the S (4.67 ± 1.09) and T-3 (9.33 ± 2.01) Groups and the highest in the C group (24.17 ± 6.27). Although there was no difference among the groups in terms of regressed CL numbers, it was observed the highest in the R (3.33 ± 1.12) and T-1 (3.50 ± 0.76) Groups and lowest in the S (1.00 ± 0.26) group ($p \leq 0.05$). Mucosal plica in epithelium of endometrium were noticeable in the C and T-1 Groups. The epithelium of endometrium had a hyperplastic appearance and the endometrial glands were observed to be quite enlarged in these groups. All rats of the S Group were observed in the follicular

phase. Although luteal phase was detected in all animals of the C Group, both follicular and luteal phases were observed in the treatment and reference groups. No significant difference was observed in terms of the number of endometrial glands. The least alveolar development was determined in R and T-2 Groups. Histopathological views of ovarian, uterine and mammary tissues were given in Fig. 5, 6, and 7, respectively.

Table 3

The histopathological parameters of ovarian and uterine tissues, and mammary glands in all groups

Parameters	S	C	R	T-1	T-2	T-3
Total corpus luteum	4.67 ± 1.09 ^c	24.17 ± 6.27 ^a	15.33 ± 4.09 ^{abc}	19.67 ± 4.52 ^{ab}	15.00 ± 4.40 ^{abc}	9.33 ± 2.01 ^{bc}
Regressed corpus luteum	1.00 ± 0.26	2.83 ± 1.08	3.33 ± 1.12	3.50 ± 0.76	1.67 ± 0.56	1.83 ± 0.60
Tertiary follicle	5.00 ± 1.03	2.67 ± 0.76	5.00 ± 1.03	3.33 ± 0.67	2.67 ± 0.72	2.50 ± 0.67
The number of endometrial glands	7.67 ± 1.12	6.83 ± 1.01	8.00 ± 1.26	7.50 ± 0.76	8.83 ± 1.70	9.00 ± 1.29
Thickness of myometrium	238.33 ± 24.28	248.33 ± 19.56	255.00 ± 29.07	263.33 ± 24.59	265.00 ± 22.17	256.67 ± 17.26
Mammary alveolar development	2.00 ± 0.26	1.83 ± 0.31	1.50 ± 0.22	2.00 ± 0.26	1.50 ± 0.55	1.83 ± 0.31
a,b,c Means with different superscripts within a row are different (p < 0.01); S: Sham Group; C: Control Group; R: Reference Group; T-1: Treatment Group-1 (12.5 mg/kg); T-2: Treatment Group-2 (25 mg/kg); T-3: Treatment Group-3 (50 mg/kg)						

Discussion

Various pharmacological agents have been used to treat pseudopregnancy, including anti-prolactins, progestogens, serotonin agonists, and dopamine agonists. Progestins are not fully effective in the treatment of pseudopregnancy and cause recurrence of the syndrome. Furthermore, they have a wide range of serious side effects. Therefore, progestogens have not been used for the treatment of pseudopregnancy. In recent years, medical therapy used to treat pseudopregnancy include dopamine agonists and serotonin antagonists. Dopamine agonists are preferred more frequently in the treatment of pseudopregnancy because they cause the least side effects and longer duration of action. Prolactin secretion is regulated by multiple neuro-transmitters and hormones. The major control mechanism of prolactin secretion is the activation of prolactin-inhibiting dopaminergic neurons in the hypothalamus. Ergot derivatives such as bromocriptine or cabergoline reduce prolactin secretion since they have a strong dopamine D2-receptor agonist activity. The serotonin antagonists indirectly inhibit prolactin secretion by stimulating the secretion of endogenous dopamine. Although dopamine agonist drugs that are selective prolactin inhibitors have the ability to suppress prolactin, they are expensive drugs (Gobello et al. 2001;

Gobello 2006; Ramsey 2017; Root et al. 2018). Therefore, novel drug candidates have been investigated for the treatment of pseudopregnancy, among them natural products are promising ones. According to the previous studies, *Pulsatilla alpina* subsp. *apiifolia* (Scop.) Nyman (Martin et al. 1988); *Ferula asafoetida* H.Karst. (Bharath Kumar et al. 2017); *Urtica dioica* L. (Bisht et al. 2017); *Thuja orientalis* L. (Park et al. 2014) and *Matricaria chamomilla* L. (Kabiri et al. 2019) used for the treatment of pseudopregnancy due to their dopaminergic effect. The dopaminergic effect of *Artemisia absinthium* L. has been revealed by previous studies (Kharoubi et al. 2010; Zeraati et al. 2014). Therefore, in our previous study, we investigated the therapeutic effects of the different extracts of *A. absinthium* on pseudopregnant rats (Demirel et al. 2018). The petroleum ether, dichloromethane and methanol extracts were administered to the rats at 100 mg/kg dose (per os) for ten days. According to the results, petroleum ether extract of the plant exhibited modulatory effect on hormonal and histological changes against pseudopregnancy. This active extract was rich in nonpolar and volatile compounds and over 70% of the compounds were found to be hydrocarbons. In the present study, we aimed to assess the potential activity of *A. absinthium* essential oil, which also contains nonpolar ingredients, in the treatment of pseudopregnancy in rats.

The results have shown that *A. absinthium* essential oil at 25 mg/kg dose displayed beneficial effects in the pseudopregnancy model in rats by considering the reduction in volume of uterine and the weights of uterine, ovary and mammary tissues when compared to the control group. Moreover, *A. absinthium* essential oil showed histological changes of ovarian and mammary tissues in the treatment groups.

The normal estrous cycle in rats is 4 to 5 days. The estrous cycle consists of 4 stages: proestrus (12 h), estrus (12 h), metestrus (21 h), and diestrus (57 h) (Paccola et al. 2013; Cora et al. 2015). In pseudopregnancy, after ovulation, the diestrus period is prolonged by 13–18 days, the corpora lutea become permanent and progesterone continues to be secreted (Anderson and Musah 2013; Abd-Elkareem 2017). In the first days of pseudopregnancy, the progestative process is similar to the gestational period and the structure of the CL is regulated by the production of prolactin. The nocturnal surge of prolactin stimulates the neuroendocrine pathway in pseudopregnant rats (Terkel 1988). These changes in the estrous cycle can be revealed by vaginal cytology (Cora et al. 2015). Irregular estrous cycle is observed in pseudopregnant rats. The nucleated epithelial cells of the diestrus stage continue to be seen in the extended period. In the present study, vaginal cytology was assessed on the 0, 5th, and 10th day of treatment protocol in pseudopregnant rats. The nucleated epithelial cells of the diestrus stage were seen in all the rats of the C Group during the experimental procedure. In the treatment groups, irregular-shaped cornified squamous epithelial cells were found at the end of the experiment. It was thought that the estrous cycle started in these rats. These findings in treatment groups were similar. Thus, it was concluded that vaginal cytology was not a determinative factor on the effect of *A. absinthium* essential oil on pseudopregnancy.

Although there is no embryo implantation in the uterus, in pseudopregnancy cases, the histomorphological changes are detected in the tissue (Abd-Elkareem 2017). These changes are nonspecific trauma-related decidual cell differentiation and hyperemic and swelling featured uterine

tissue which are observed at the middle stage of pseudopregnancy (Peel et al. 1979; Anderson and Musah 2013). Owing to the enhancement of plasma progesterone level, mucosal plica, glandular formation, epithelial proliferation and thickening of the uterine wall are seen in this syndrome. Epithelial proliferation, crypt formation, increased mucosal plica and glands are characteristic findings of the last stage of pseudopregnancy (Albers et al. 2015; Abd-Elkareem 2017). The numbers of CL were significantly increased in the C group when compared to the treatment groups. Thus, similar to other studies, increased mucosal plica and glandular hypertrophy in endometrium were distinct in C and T-1 groups. Mucosal plica was determined in some cases in the other groups. The previous studies have shown that the mammary epithelial cells are increased at the stage of metestrus as seen in pseudopregnant females (Hvid et al. 2012). In the current study, while mammary gland, alveolar and ductal developments were found to be much enhanced in C and T-1 groups, the mammary tissue was recovered in T-2 and R groups. In overall assessment, 25 mg/kg of *A. absinthium* essential oil was determined as the effective dose on uterine and mammary tissue in pseudopregnant rats.

In our previous study, GC-MS analysis revealed that, among the volatile compounds, *cis*-chrysanthenyl acetate and β -thujone were the major ones in the most active petroleum ether extract (Demirel et al. 2018). According to the phytochemical findings of the present study, *cis*-chrysanthenyl acetate was the main compound with a percentage of 17.8. Moreover, sabinyl acetate, terpinen-4-ol, caryophyllene oxide and (*E*)-nuciferol were determined as other main compounds. These findings were in accordance with the outcome of the previous studies in which *cis*-epoxyocimene, *cis*-chrysanthenyl acetate, bornyl acetate, eucalyptol, myrcene, linalool, neryl butanoate, sabinene, *trans*-sabinyl acetate, *trans*-thujone, α - and β -thujone were detected in the essential oil of the plant (Bailen et al. 2013; Batiha et al. 2020; Dhen et al. 2014; Jiang et al. 2021; Judzentiene and Budiene 2010; Julio et al. 2015; Orav et al. 2006; Pino et al. 1997; Rezaeinodehi and Khangholi 2008). In agreement with our results, *cis*-chrysanthenyl acetate was reported as the major component in the essential oil of *A. absinthium*. Sharopov et al. (2012) investigated three *A. absinthium* samples from two different localities and *cis*-chrysanthenyl acetate (17.9%) was the main component in one of the samples. In addition, high concentration of *cis*-chrysanthenyl acetate was reported by Bailen et al. (2013) in the essential oils obtained from *A. absinthium* grown under various environmental conditions. Similar results were also reported by Judzentiene and Budiene (2010), Julio et al. (2015) and Obistioiu et al. (2014). However, chamazulene (Msaada et al. 2015), bornyl acetate (Pino et al. 1997), neryl butanoate (Orav et al. 2006), sabinene (Orav et al. 2006) were found to be the main compounds in the essential oils of *A. absinthium* from different countries. The observed differences could be explained by climatic (rainfall, temperature, sunlight etc.), geographical conditions (altitude, soil structure etc.) and harvest time.

Thujone, a psychoactive compound, causes hallucinations and enhances the activity of dopamine. Medicinal plants that have thujone have been reported to be used as an abortifacient, contraceptive and uterotonic and for the treatment of amenorrhea and uterine carcinomas (Dhiman et al. 2012). However, long-term use of *A. absinthium* essential oil can be toxic and cause mental disorders such as insomnia, convulsions, and hallucinations. Judzentiene et al. (2012) investigated wormwood essential oil toxicity depending on oil composition. The most toxic essential oils were found to be those containing significant

amounts of trans-sabinyl acetate and thujon, while other samples containing an equivalent amount of sabinyl acetate but not thujon were found to be significantly less toxic. α - and β -thujone were listed as potentially dangerous compounds in *A. absinthium* by The European Food Safety Authority (EFSA). However, α - and β -thujone content in the oil between 0% and 70.6% does not cause any side effects (Szopa et al. 2020). In our study, β -thujone content was determined as 0.1% in the oil.

Conclusion

Natural sources are crucial due to their therapeutic potential against several diseases. Especially, medicinal plants and their secondary metabolites lead to discovery of novel drugs. Hence, studying the bioactivity of natural products is essential for evaluating promising drugs. Herein, we aimed to evaluate the potential effect of *A. absinthium* essential oil in a pseudopregnancy model in rats. The outcome of the study has shown that *A. absinthium* essential oil rich in *cis*-chrysanthenyl acetate, sabinyl acetate, terpinen-4-ol, caryophyllene oxide, and (*E*)-nuciferol displayed therapeutic activity in terms of improving mammary development.

Declarations

Author contribution MAD and IS designed the study. GZ realized phytochemical analysis. MAD and IS performed the *in vivo* study. AOC performed the histopathological analysis. MAD, IS, AOC, and KTA analyzed the data. All authors wrote the manuscript.

Funding This research received no specific grant from any funding agency in the public, commercial, or not-profit sectors.

Data availability The data that support the findings of the present study are available from the corresponding author (MAD) upon request.

Ethics approval The study was approved by Experimental Animal Ethics Committee of Gazi University (G.U.ET- 17.015).

Consent to participate This is an experimental animal study.

Consent for publication This is an experimental animal study.

Conflict of interest The authors declare no competing interests.

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Figures

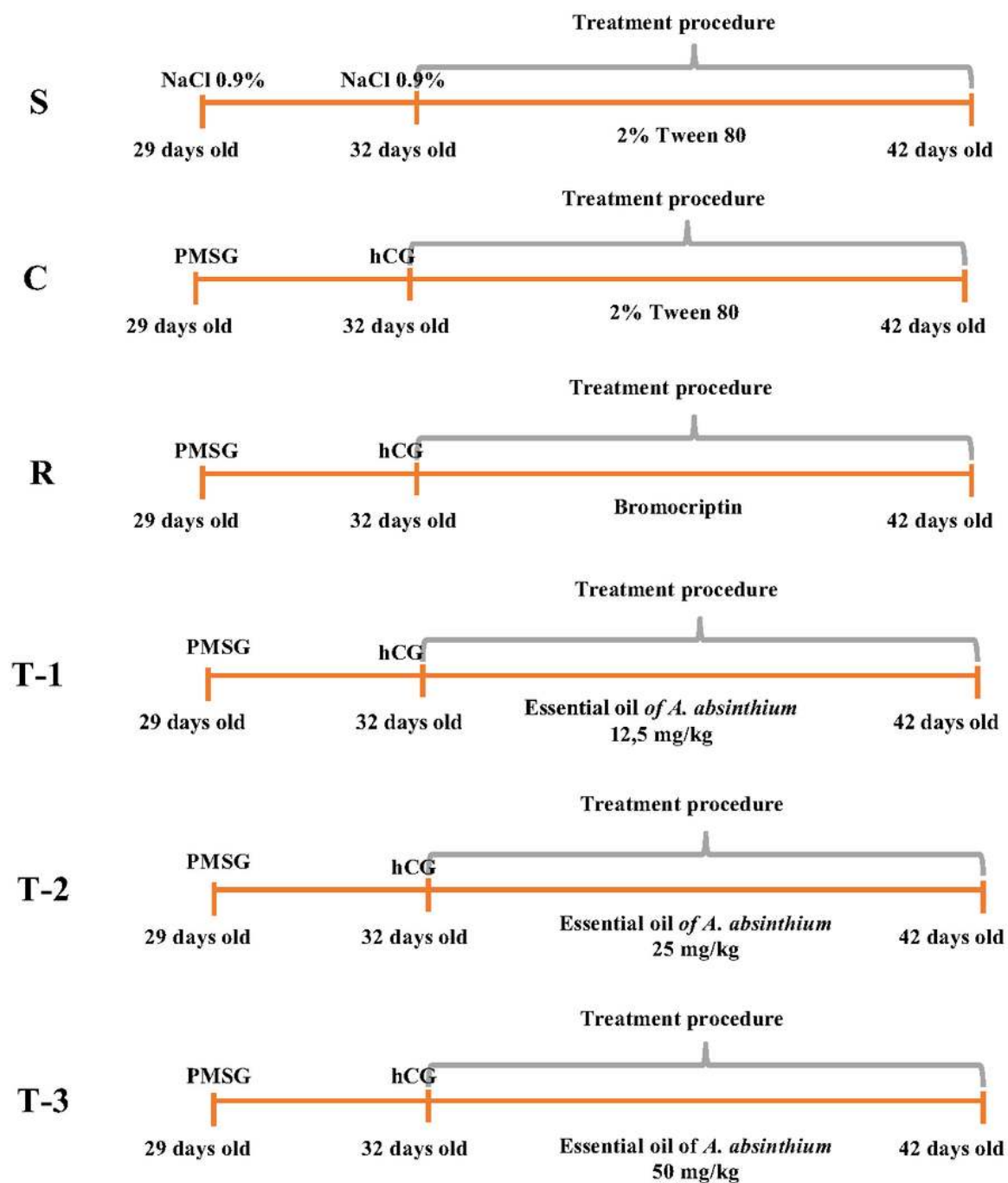
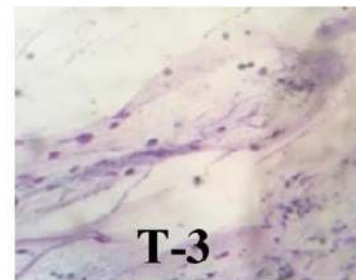
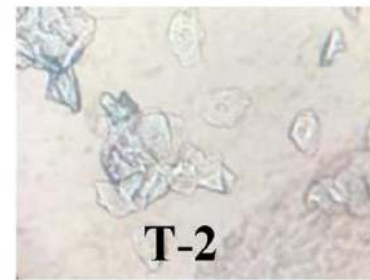
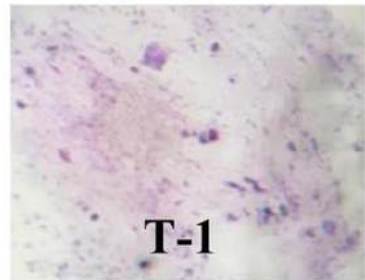
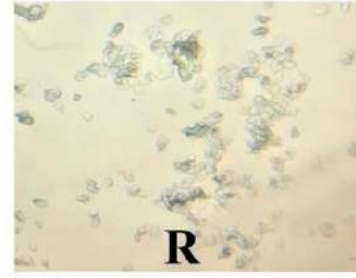
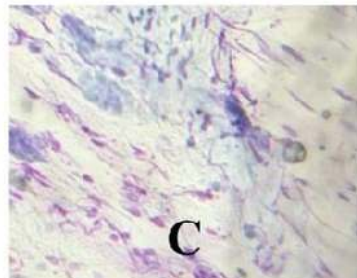
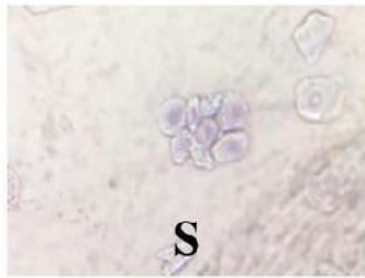


Figure 1

Flow chart of the experimental procedure

5th day of treatment



10th day of treatment

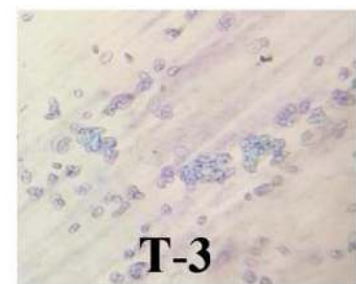
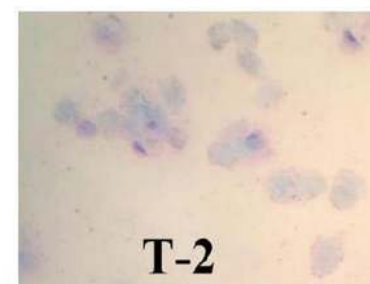
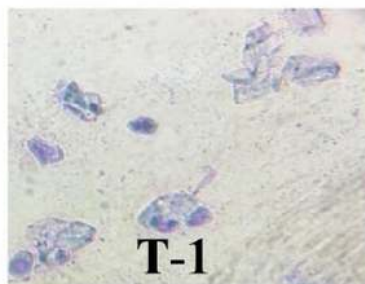
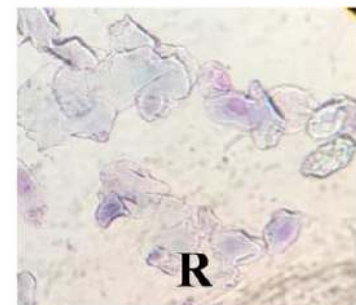
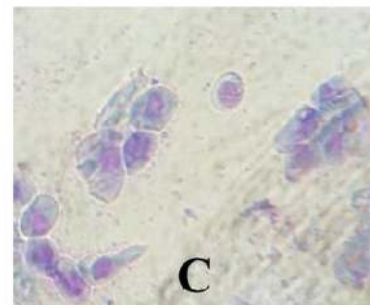
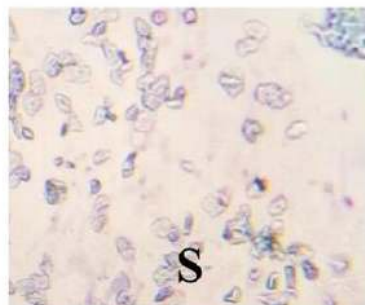


Figure 2

Vaginal cytology findings on 5th and 10th of treatment. S: Sham Group; C: Control Group; R: Reference Group; T-1: Treatment Group-1 (12.5 mg/kg); T-2: Treatment Group-2 (25 mg/kg); T-3: Treatment Group-3 (50 mg/kg)

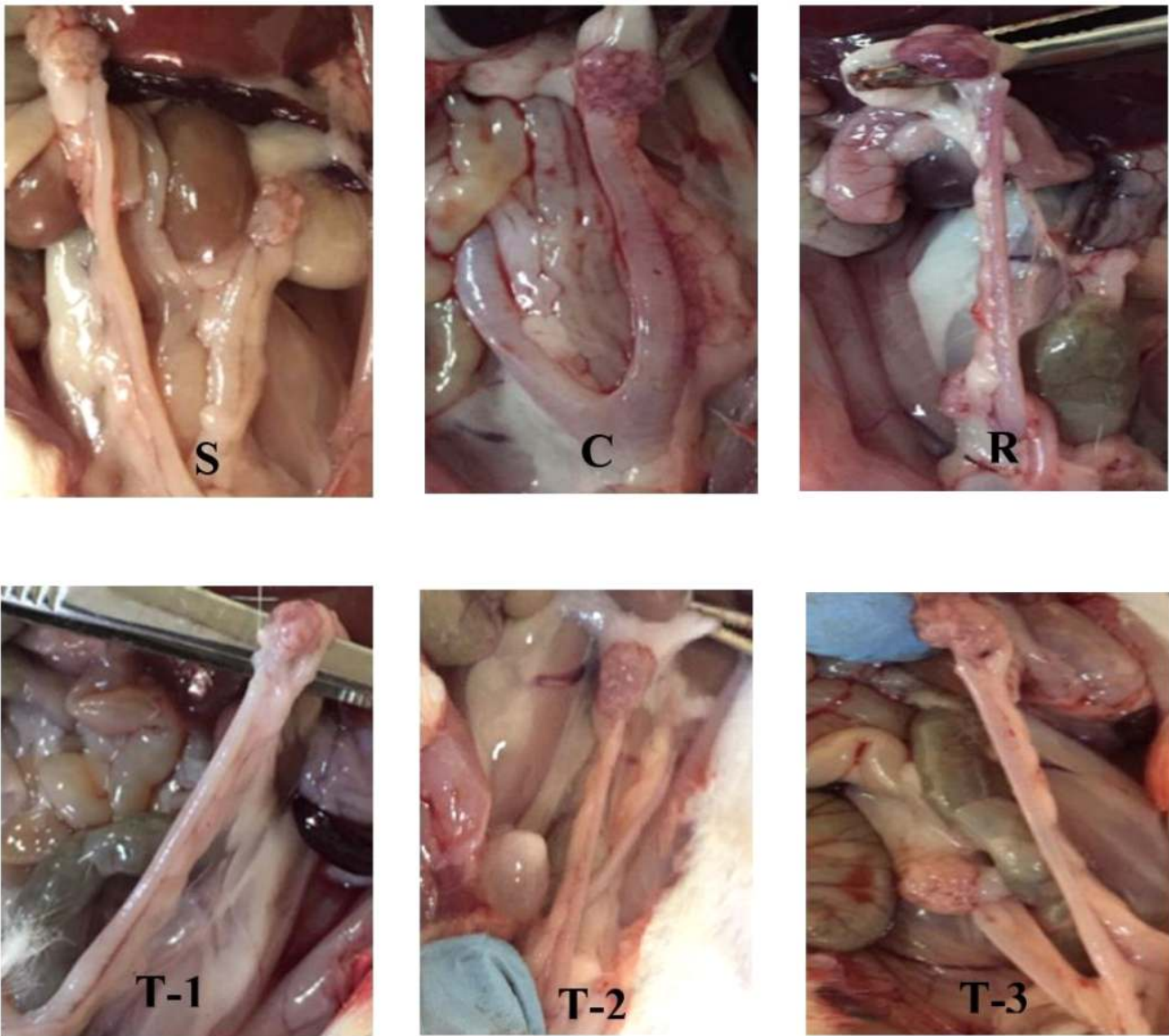


Figure 3

Macroscopic view of uterine tissues. *S: Sham Group; C: Control Group; R: Reference Group; T-1: Treatment Group-1 (12.5 mg/kg); T-2: Treatment Group-2 (25 mg/kg); T-3: Treatment Group-3 (50 mg/kg)*

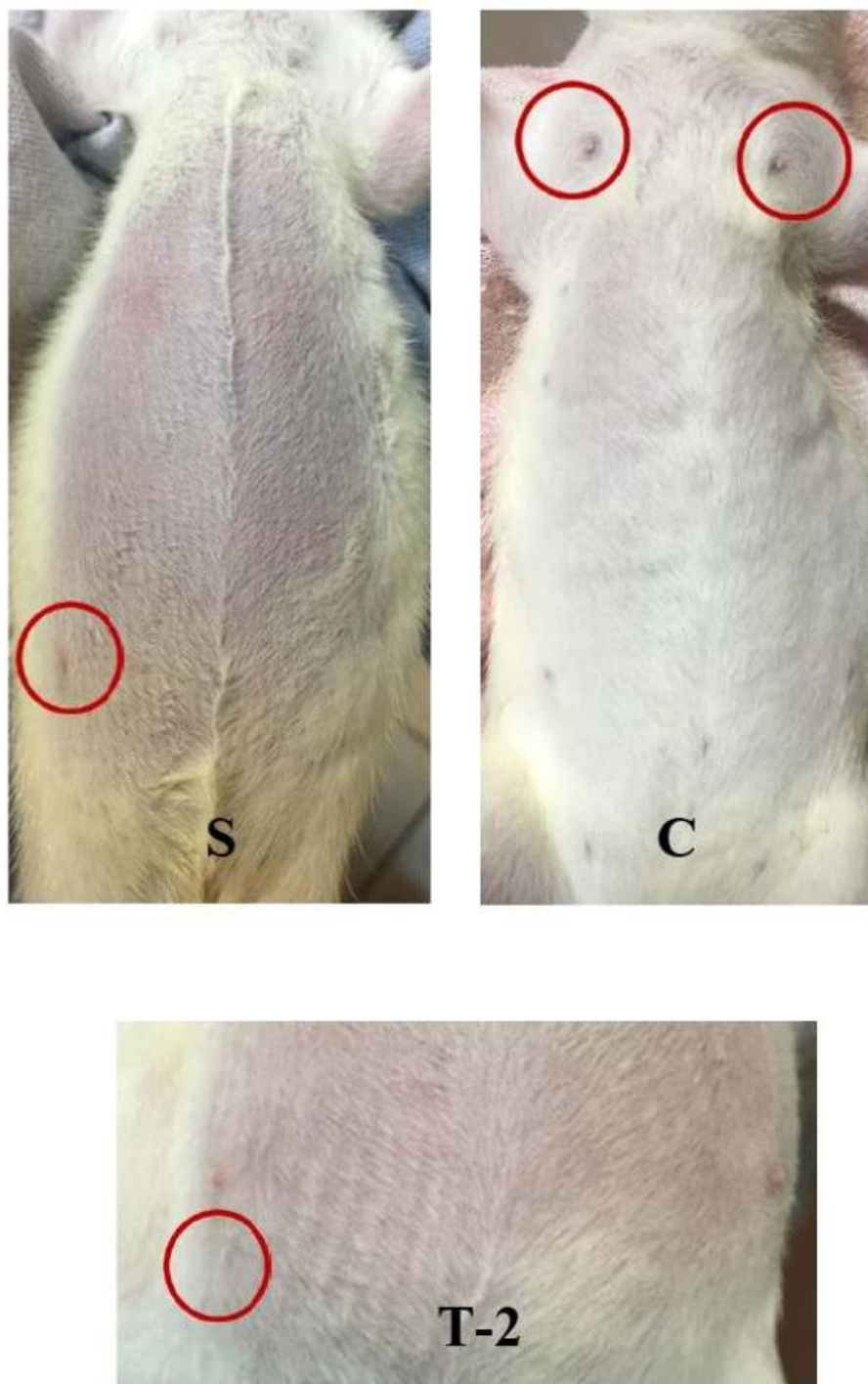


Figure 4

Macroscopic view of mammary glands. *S: Sham Group; C: Control Group; T-2: Treatment Group-2 (25 mg/kg)*

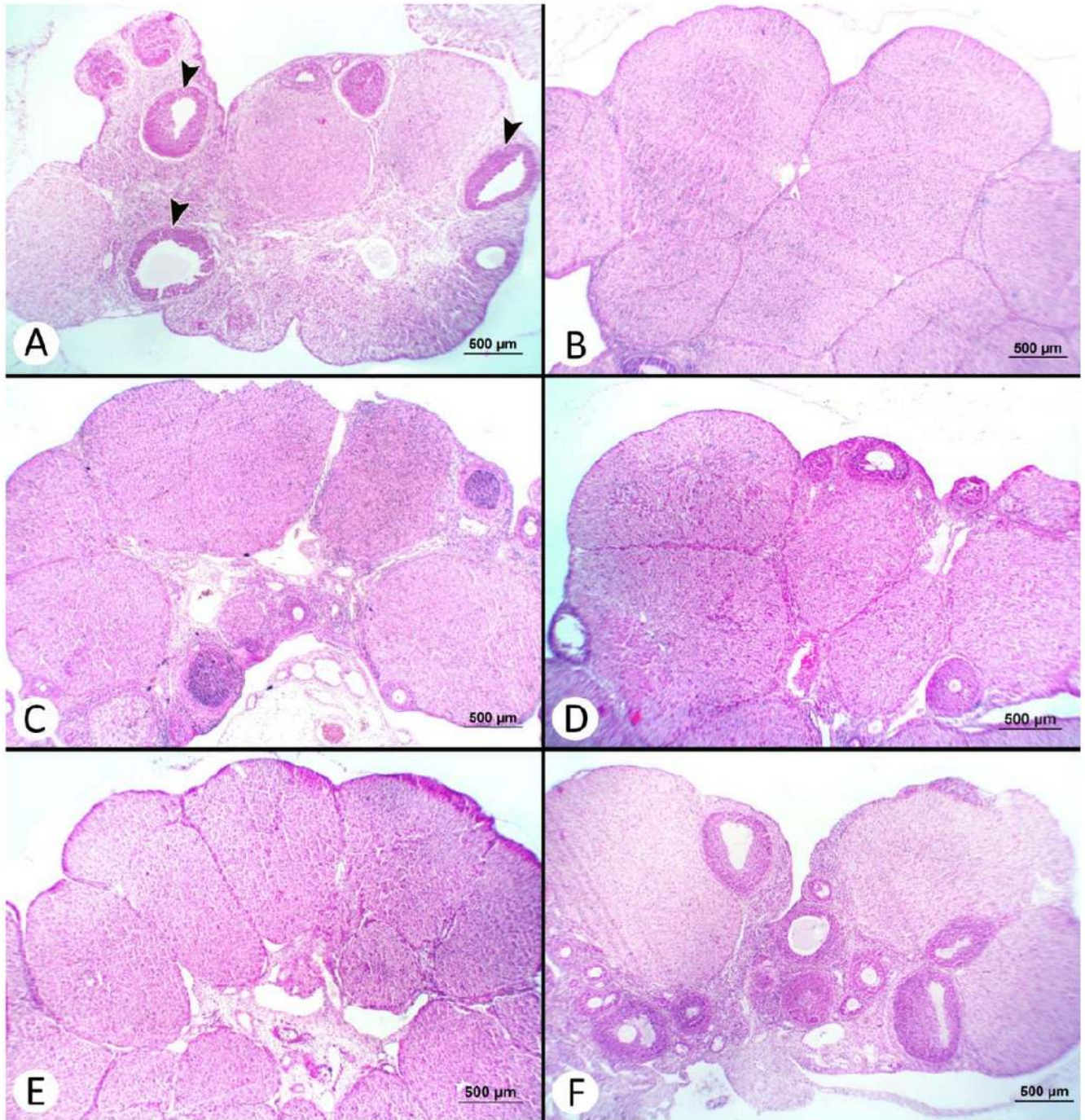


Figure 5

Histopathological view of ovarian tissues (H&E x 50). **A:** The appearance of multiple tertiary follicles (arrowheads) development in sham group. **B:** Microscopic view of the corpus luteum in control group. **C:** Microscopic view of section of ovary in reference group. **D:** Microscopic view of section of ovary in treatment group of 12.5 mg/kg. **E:** Multiple active and regressing corpus luteums in treatment group of 25 mg/kg. **F:** Follicular development in treatment group of 50 mg/kg.

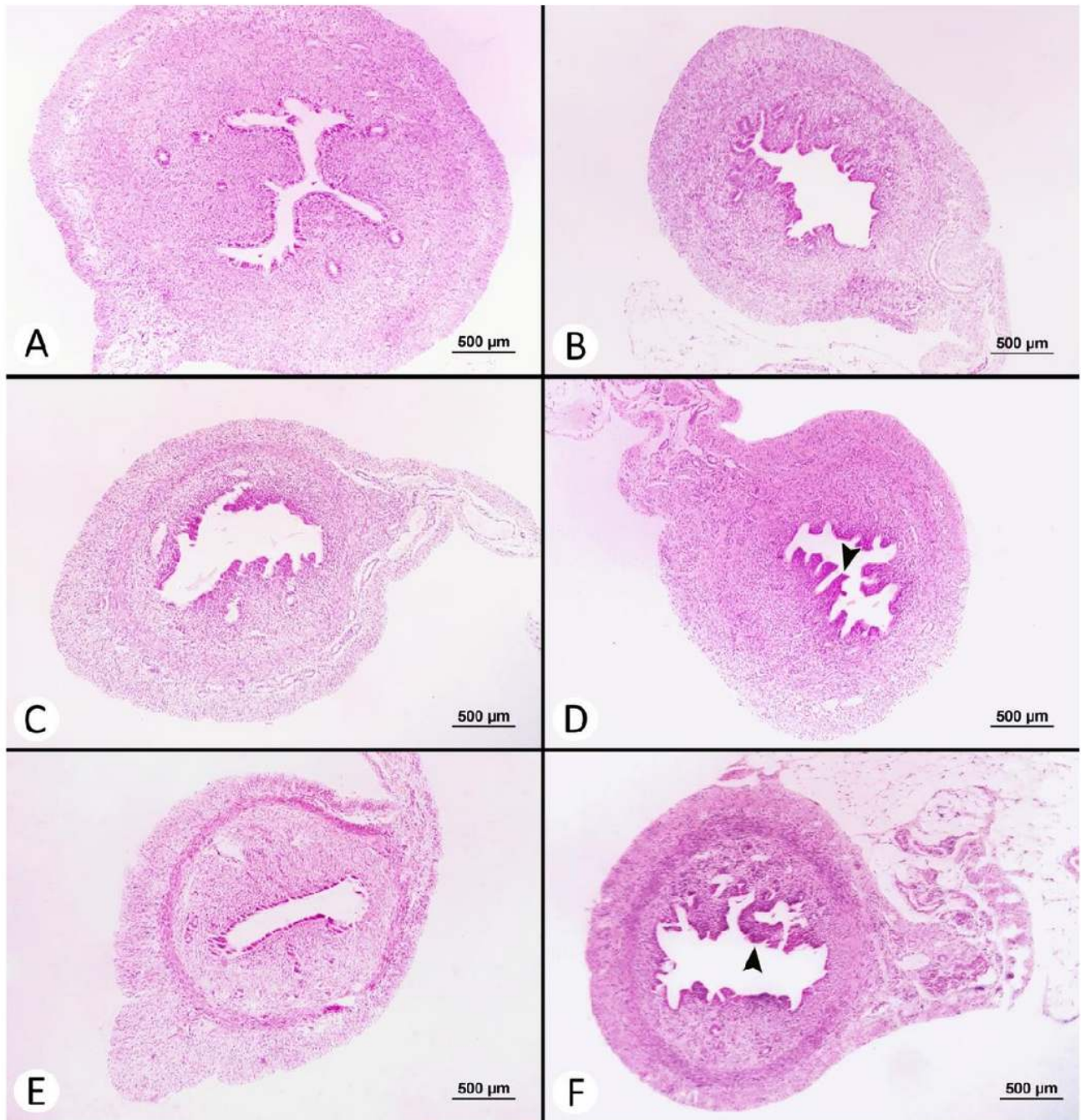


Figure 6

Microscopic view of uterine tissues (H&E x 50) **A:** Apoptosis of the endometrial epithelium and development of the endometrial glands in sham group. **B:** Histological view of the uterus in control group. **C:** Desquamation of the endometrial epithelium in reference group. **D:** Mucosal plica formation towards the luminal space due to hyperplasia of the endometrial epithelium (arrowhead) in treatment group of

12.5 mg/kg. **E:** Endometrial epithelium lined with columnar epithelium in treatment group of 25 mg/kg. **F:** Appearance of mucosal plica formation (arrowhead) in treatment group of 50 mg/kg.

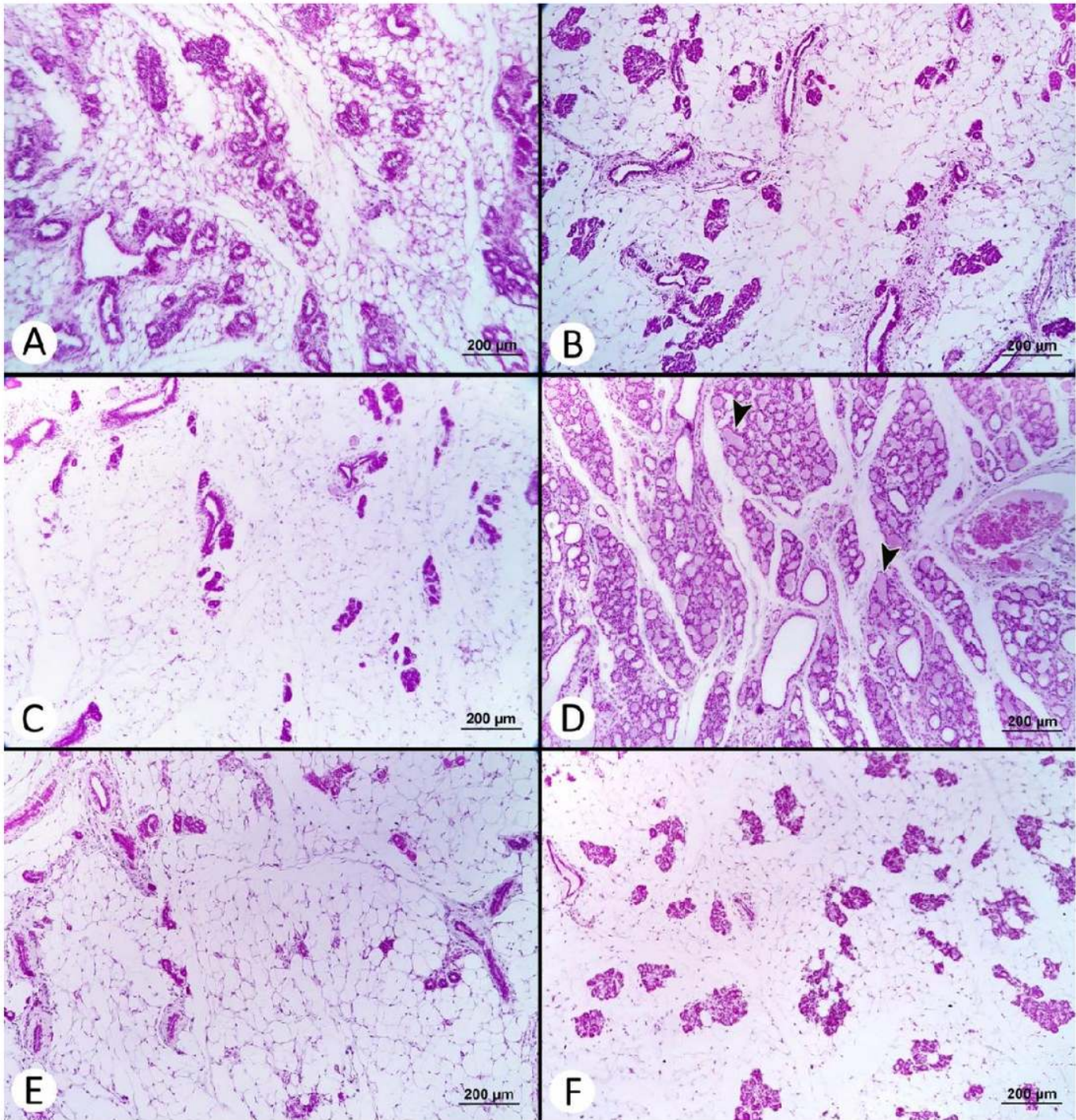


Figure 7

Microscopic view of mammary tissues (H&E x 50) **A:** Appearance of prominent mammary alveoli in sham group. **B:** Microscopic view of the mammary gland in control group. **C:** Appearance of inactive mammary glands in reference group. **D:** The appearance of abundant milk synthesis within the active acini in the mammary gland (arrowheads) in treatment group of 12.5 mg/kg. **E:** Appearance of atrophic mammary glands in treatment group of 25 mg/kg. **F:** Microscopic view of mammary gland in treatment group of 50 mg/kg.