NATURAL DRUGS

INVESTIGATION OF THE POTENTIAL ANTI-FERTILITY ACTIVITY OF *ARBUTUS PAVARII* IN MALE MICE

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Abstract: Plants rich in flavonoids are widely studied as fertility regulators. Thus, the aim of the study was to examine the impact of flavonoid-rich extract from the leaves of *Arbutus pavarii* (*A. pavarii*) plant on the reproductive system of adult male Swiss albino mice. Twenty mice (20 ± 3 g) were included in this study; they were later grouped into four groups ($n = 5/\text{group}$) and were orally administered with three doses, that is, 250, 500 and 1000 mg/kg body weight (bw) of extract and vehicle for 28 days, while the remaining group served as the control. On the 29th day, the mice were sacrificed; collected were blood and testis samples, and these were utilized for biochemical, histopathological, sperm parameters, oxidative stress status, and morphometric analysis. The tested extracts at doses 250 and 500 mg/kg bw were observed to induce a significant decline in the sperm count, motility, and testicular level of total antioxidant status as well an increase in the testicular oxidative stress biomarkers, while the group treated with 1000 mg/kg bw showed a less pronounced effect. Histological analysis exhibited the disorganization of seminiferous tubule and a reduced spermatocyte number. Thus, our findings showed that extracts from the *A. pavarii* plant may induce spermicidal activity and interfere with testicular structures, ultimately leading to the spermatogenic arrest.

Keywords: *Arbutus pavarii*, flavonoids, anti-fertility, oxidative stress, histological analysis, mice

Medicinal plant extracts have been widely tested in animals to determine anti-fertility activities (1, 2). Recent research identified 48 out of 72 medicinal plants traditionally employed for fertility control as having a dose-dependent anti-fertility potential (1), which may be developed into novel orally active non-steroidal fertility regulating agents and contraceptives (2, 3).

Several studies have already reported the effect of secondary bioactive metabolites from medicinal plants have on the regulation of reproductive system function (4-6). For instance, bioactive metabolites are found to inhibit or induce ovarian folliculogenesis, which is often characterized by an increase in the weight of the ovary and uterus and opening and cornification of the vagina in females; meanwhile,

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in males, sperm abnormalities as a result of a cessation of spermatogenesis, degeneration in seminiferous tubules and regression of Leydig cells were also observed (7). Additionally, plant extracts may lead to teratogenic effects due to their oxidative activity, which in turn can lead to DNA fragmentation in somatic cells (8, 9).

Arbutus pavarii. Pampan (Ericaceae) has been identified as a plant commonly used in traditional folk medicine, as it is rich in bioactive metabolites such as polyphenols, flavonoids and alkaloids. The fruit of the plant is found to be rich in anti-septic, diuretic and laxative properties and has been suggested to improve blood pressure, while the leaves and flowers are found to have anti-microbial (10-12), anti-oxidant (13), anti-proliferative (14), and anti-cancer properties (15).

Most studies have suggested that *A. pavarii* is a good source of bioactive compounds including flavonoids, which are determined to have anti-microbial, anti-inflammatory, anti-mutagenic, anti-hepatotoxic, anti-cancer, and antioxidant properties (15-18) (Table 1). To date, research that focused on isolated flavonoids and their potential as anti-fertility agents remain to be limited (19, 20). Thus, this present study aims to evaluate and characterize potential anti-fertility properties of flavonoids extracted from the leaves of *A. pavarii* plant.

MATERIALS AND METHODS

Plant materials

Fresh leaves and flowers of *A. pavarii* were collected in January 2015 all the way from Al Marj, Libya. The plant was verified and authenticated by the Botany Department, Faculty of Sciences, University of Tripoli, Tripoli, Libya.

Microwave-assisted extraction of flavonoid

The microwave-assisted extraction method (32) using a domestic microwave oven system was used in extracting flavonoids (Black & Decker, Model No. MZ3000 PG, SL13YD, England). In brief, dried samples were mixed with methanol (80%). An intermittent microwave irradiation method was used in order to keep the temperature of the extraction mixtures below 80°C. With the power to 600 W, the suspension was radiated under a microwave oven in pre-setting procedures (30-sec radiation and 30 sec off) for three times, and the temperature of the extraction mixtures were kept below 80°C (32). After the extraction, the samples were filtered through Whatman No. 1 filter paper, and the filtrate was subjected to rotary evaporation

evaporator (Heidolph, LaboRota 4000, Germany). The residue was stored at -20°C for further analysis.

Estimation of total flavonoids

The total content of flavonoids was estimated using aluminum chloride colorimetric assay according to Zou et al. (33). The results were expressed as mg rutin equivalents (RE) per gram of dry weight.

Estimation of total flavonols

Total flavonol content was determined by following the procedure described by Kumaran and Karunakaran (34). The results were expressed as mg RE per gram of dry weight.

Estimation of total anthocyanidin contents

The total anthocyanidin content was determined using the vanillin-HCl colorimetric method (35). Then, total results were expressed as mg catechin equivalent (CE) per gram of dry weight.

Estimation of total proanthocyanidins

The total content of proanthocyanidins was determined following the method of Li et al. (36). The results were expressed as mg CE per gram of dry weight.

Animals

All experimental procedures were performed in accordance with the European Union Directive 2010/63/EU for animal experiments. The ethics committee of the Biotechnology Research Center (Tripoli, Libya) approved the experimental protocol of the study (BEC-BTRC 02-2016). In total, 45 mature male Swiss albino mice, aged 8.5 old weeks and weighing between 18 and 20 g, were used in this study. The animals were bred and housed in the animal house at the Faculty of Sciences, University of Tripoli. The animals were placed in cages with free access to food and water, and they were allowed to acclimate to animal room conditions at 20°C-22°C, relative humidity 50-60% and 12-h light and dark cycle for 1 week prior to the commencement of the experiment.

Extract preparation

The dried extract was dissolved in ethanol (30%) of known concentrations and was later used for experimental studies. The doses were daily administered orally to mice via gavage throughout the study.

Sub-acute toxicity

A sub-acute toxicity test was performed as per the Organization for Economic Co-operation and

Table 1. Flavonoids with anti-fertility effects.

Flavonoid class	Flavonoid subclass	Natural sources	Other biological activities	References
Flavonol	Quercetin, myricetin, kaempferol, morin, rutin, isorhamnetin	Teas, onion, kale, scallions, broccoli, Atomato, currant, apples, berries	Anti-oxidant, anti-cancer, anti-microbial, anti-spermatogenic, hepatoprotective	(17, 21, 22)
Flavone	Apigenin, luteolin, chrysin, baicalein, tangeretin	Parsley, celery, red peppers, chamomile, thyme, peppermint	Anti-oxidant, anti-fertility, anti-cancer	(19, 23, 24)
Flavanone/dihydroflavone	Naringin, naringenin taxifolin, eriodictyol, hesperetin, hesperidin	Lemons, oranges, grapefruits, tomatoes, certain aromatic plants such as mint	Anti-oxidant, anti-inflammatory, anti-obesity, hepatoprotective	(25, 26)
Isoflavone/phytoestrogen	Genistin, genistein, glycitein, biochanin, daidzein, coumestrol	Legumes, soybean	Anti-oxidant, anti-fertility, anti-microbial, anti-inflammatory	(24, 27)
Anthocyanidin/anthocyanin	Cyanidin, delphinidin, malvidin, pelargonidin, petunidin, peonidin	Grapes, cherries, berries, black rice	Anti-oxidant, anti-cancer anti-inflammatory, anti-obesity, anti-diabetes	(28, 29)
Flavanol/flavan-3-ol	i. Monomers: (+)-catechin, (-)-epi-catechin, (+)-gallocatechin, (-)- epigallocatechin and their gallate derivatives ii. Dimers, oligomers and polymers (both are derived tannins): theaflavins, proanthocyanidins	Black and red grape, grape seeds, apples, apricots, cherries, berries, strawberry, teas, cocoa, chocolate	Anti-oxidant, anti-microbial, anti-cancer, anti-inflammatory, anti-diabetes, photo-protection, increasing fertility activity	(30, 31)

Development Guideline-423 (OECD-423 guidelines) (37). Mice were divided into four groups of five. Four doses of flavonoid extracts: 250, 500, 1000 and 2000 mg/kg were administered orally to mice via gavage for 28 days. Clinical observations were made daily for abnormal clinical signs or behavior of toxicity according to the specifications of the OECD (2001). Any death was also recorded, and independent observers are the ones who confirm the cause of death to exclude an exposure non-related mortality. Hematology, biochemistry and body weight (bw) were also assessed.

Treatment protocol

Based on acute toxicity results, doses of 250, 500 and 1000 mg/kg were selected in evaluating the anti-fertility potential of flavonoid extracts (38). Mice were randomly divided into four groups (five mice per group). Each group received daily gavage doses of 250, 500 and 1000 mg/kg flavonoid extracts mg/kg bw for 28 days, while the control group was given ethanol at dose 0.003 mg/kg. On the 29th day, following the last treatment, the mice were anesthetized with chloroform and were then sacrificed; blood samples were then collected for biochemical analysis. The testes were dissected, cleared from surrounding tissues, blotted dry, and immediately weighed. The tested tissues were used in both biochemical and histopathological examinations.

Estimation of blood hematological and biochemical parameters

Blood samples were analyzed for routine hematological and biochemical parameters. Blood cell counts were performed with blood smears. Serum levels of alkaline phosphatase (ALP), aspartate aminotransferase (AST), and creatinine and urea were estimated. All analyses were performed using commercially available kits from Biomagreb, Tunisia.

Preparation of testicular homogenates

The testes were weighed and homogenized in saline (10% w/v) using a motor-driven Teflon pestle. Samples were centrifuged at 4500 rpm for 15 min at 4°C, and the supernatant was used for the estimation of testicular biomarkers.

Determination of testicular biochemical parameters

Total protein, high-density lipoprotein (HDL) (mg/dL), and low-density lipoprotein (LDL) (mg/dL), and cholesterol were measured using standard kits obtained from Biomagreb, Tunisia.

Determination of total antioxidant capacity

The total antioxidant capacity (TAC) was determined using a commercial kit according to manufacturer protocol (Anti-oxidant Assay Kit, cat. No. CS0790; Sigma-Aldrich, St. Louis, MO, USA).

Determination of lipid peroxidation

Lipid peroxidation ((MDA) level in testes homogenates were determined spectrophotometrically (39). Sample (1.0 mL) was then mixed with 2.0 mL of TCA-TBA-HCL reagent (0.37% thiobarbituric acid, 0.24 N hydrochloric acid and 15% trichloroacetic acid). The mixture was then incubated in a boiling water bath for 20 min. After cooling samples were centrifuged (Sigma 2K15, Germany) at 3000 rpm for 15 min, the supernatants were removed, and the absorbance of the supernatant was recorded at 532 nm. The amount of MDA was then expressed as nmol/mg protein.

Determination of protein carbonyl content

Protein carbonyl content in the testes homogenates was measured according to Lushchak et al. (40). The samples were incubated with 10 mM 2,4-dinitrophenylhydrazine (DNPH) in 2.5 M HCl for 1 h at room temperature in a dark place. The protein was precipitated with 20% trichloroacetic acid, followed by a washing step with ethanol-ethyl acetate (1 : 1), and dissolved in 6 M guanidine hydrochloride. Dinitrophenyl (DNP) hydrazone derivative absorbance was measured at 370 nm. Bovine serum albumin was used as a standard. The results were expressed as nmol/mg protein.

Determination of nitric oxide (NO) level

Nitric oxide in the testicular homogenates was determined according to Malyshev and Shnyra (41). 1.0 mL of the homogenate was added to an equal volume of Griess reagent, which contains 1% sulphanilamide and 0.1% N-(1-naphthyl)ethylenediamine dihydrochloride in 5% phosphoric acid. The mixture was then allowed to stand at room temperature for 30 min. The absorbance of the mixture was

read at 546 nm. A sodium nitrate solution was utilized to obtain a standard curve. The amount of nitrite was expressed as nmol/mg protein

Determination of sperm viability and motility after flavonoid treatment

Cauda epididymal sperm motility and testicular sperm count (maturing spermatozoa with head and tail) were made according to the procedure of Mortimer (42). The cauda epididymis was minced using a fine razor blade and suspended in 1 mL of PBS; then, it was shaken gently at 37°C for 15 min. The suspension was sieved through a nylon filter to separate the tissue from the released sperm. Total cauda sperm count, morphological defects, and motility were examined using low- and high-power objectives under light microscopy (Leica, Germany). The percentage of sperm motility was determined by counting both motile and immotile spermatozoa per unit area using a hemocytometer (Neubauer's chamber, Germany) (43). Counts were presented as million/mL of suspension.

Testicular histology

One (right) of the two testes of each animal was removed and placed in 10% neutral buffered formalin, dehydrated in graded ethanol, cleared in xylene and embedded in paraffin wax. Sections were cut at 5 µm thicknesses, stained with hematoxylin and eosin, and observed under a light microscope (44). Morphometric analyses of the height and diameter of 10 randomly selected seminiferous tubules from each testis were assessed using the ImageJ software.

Statistical analysis

Statistical analysis was performed via the GraphPad Prism software version 6.0, 2013 (San Diego, California, USA). The data were expressed as mean ± standard deviation. Statistical significance was assessed using the one-way analysis of variance, followed by Tukey's multiple comparisons test with a significance level at $p = 0.05$.

Table 2. Flavonol, anthocyanidin and proanthocyanidin contents of flavonoids extracted from leaves.

Treatment	Content (mg/g DW) ^a
Flavonoids ^b	409.05 ± 3.25
Flavonols ^b	187.97 ± 0.79
Anthocyanidin ^c	906.85 ± 0.43
Proanthocyanidin ^c	1250.02 ± 0.76

^aData presented as mean of three replicates. ^bmg rutin equivalents g⁻¹ DW. ^cmg catechin equivalents g⁻¹ DW

Table 3. Effects of the extract on biochemical and hematological parameters in male mice after 28 days of treatment.

Parameters	Control	250 mg/kg	500 mg/kg	1000 mg/kg
RBCs ($10^{12}/L$)	6.49 ± 0.68	6.71 ± 0.59	6.05 ± 1.18	5.28 ± 0.80
WBCs ($10^9/L$)	6.20 ± 1.34	6.73 ± 0.68	6.83 ± 0.37	6.10 ± 0.95
LY (%)	58.00 ± 4.17	60.66 ± 14.15	61.30 ± 9.97	57.76 ± 3.01
PLT ($10^9/L$)	265.0 ± 1.00	259.66 ± 1.15	263.67 ± 48.29	260.0 ± 1.00
HGB (g/dL)	11.26 ± 0.30	11.56 ± 1.10	10.86 ± 0.80	10.67 ± 1.51
HCT (%)	34.10 ± 0.90	32.80 ± 1.56	38.76 ± 1.89	36.66 ± 0.92
MCV (fL)	87.66 ± 0.66	84.67 ± 0.67	76.13 ± 3.77	66.46 ± 6.31
MCH (pg)	27.03 ± 1.66	26.0 ± 0.00	27.66 ± 0.66	23.70 ± 0.62
MCHC (g/L)	35.40 ± 0.36	34.26 ± 0.30	34.73 ± 0.89	31.50 ± 1.50
ALP (U/L)	123.33 ± 8.50	119.47 ± 2.73	118.33 ± 1.53	122.33 ± 5.51
AST (U/L)	56.66 ± 21.38	57.73 ± 4.15	55.33 ± 12.70	58.3 ± 2.65
Creatinine (mg/dL)	0.52 ± 0.03	0.67 ± 0.11	0.69 ± 0.01	0.63 ± 0.03
Urea (mg/dL)	2.55 ± 0.48	3.00 ± 0.2	2.50 ± 0.62	2.6 ± 0.30

Data are presented as mean $\pm$ standard deviation (SD) (n = 5); RBCs: red blood cell count; WBCs: white blood cell count; LY: lymphocyte ratio; PLT: platelet count; HGB: hemoglobin; HCT: hematocrit; MCV: mean corpuscular volume; MCH: mean corpuscular hemoglobin; MCHC: mean corpuscular hemoglobin concentration; ALP: alkaline phosphatase; AST: aspartate aminotransferase.

Figure 1. Effects of oral administration of flavonoid-rich extract for 28 days on the body and testicular weights in male rats treated with two different doses of the extract. Data are expressed as mean $\pm$ SD, n = 5. Significant ($p < 0.05$) compared with the control group.

RESULTS

Contents of flavonoids, flavonols, anthocyanidin, and proanthocyanidin in *A. pavarii* leaf extract

Both the contents of flavonoids and flavonols (mg/g dry weight (DW)) in alcoholic *A. pavarii* leaf extract were determined and expressed as rutin, 409.05 ± 3.25 and 187.97 ± 0.79 mg/g DW, respectively (Table 2). Meanwhile, the contents of anthocyanidin and proanthocyanidin (mg/g DW) in the extract were determined and expressed as catechin equivalents, 906.85 ± 0.43 and 1250.02 ± 0.76 mg/g DW, respectively (Table 2).

Sub-acute toxicity evaluation of extract

Sub-acute toxicity evaluation has found no substantial changes in hematological and biochemi-

cal parameters as shown in Table 3. At 2000 mg/kg bw, no mortality or clinical signs of toxicity were observed. This indicates that extracts are under category 5 (45). Hence, an LD_{50} cut-off of 1000 mg/kg body weight was chosen, and doses of 250, 500 and 1000 mg/kg body weight were used to examine the anti-fertility properties of the flavonoid extract.

The effect of the extract on body weight and testes weight

The mean total body and testicular weights of mice were measured at baseline and at the completion of the experiment as presented in Figure 1. After 28 days, a small increase was noted in terms of body weight at doses of 250 and 500 mg/kg bw compared to the control group. However, these changes were deemed not significant. No significant

difference was observed for testes weight either (Fig. 1).

The effect of the extract on lipid profile parameters

No significant differences were observed in the levels of HDL compared with the control group (Table 4) ($p > 0.05$). An increase in the level of cholesterol was only observed at the lowest dose of 250 mg/kg bw compared with the control group ($p < 0.05$). The level of LDL was significantly higher in groups treated with 250 mg/kg bw and 500 mg/kg bw of the extract compared with the control group ($p < 0.05$) (Table 4). However, no significant differences were determined at 1000 mg/kg bw.

The effect of the extract on sperm parameters
Sperm physical properties

As shown in Figure 2, sperm analysis has shown that the administration of flavonoid extract at three doses has significantly decreased sperm count, abnormality, and motility compared to the control group. Specifically, the sperm count was observed to decrease by 15.8% ($p < 0.003$), 16.4%, ($p < 0.003$) and 15.2% ($p < 0.05$) for 250, 500 and 1000 mg/kg extract dose, respectively, while sperm motility decreased by 77.9% ($p < 0.05$), 77.1% ($p < 0.001$) and 30.9% ($p < 0.05$) in comparison to the control group (Figs. 3A and 2C). Sperm abnormalities, including head and tail defects (small or big head, coiled or bent tail), were significantly reduced

Figure 2. Effects of oral administration of flavonoid-rich extract for 28 days on the epididymal sperm count (A); abnormality (B) and motility (C) in male mice treated with three different doses of the extract. Data are expressed as mean $\pm$ SD, $n = 5$. A significant ($p < 0.05$) compared with the control group.

Table 4. Effect of the extracts on testicular levels of cholesterol (C), high-density lipoprotein (HDL) and low-density lipoprotein (LDL) contents in male mice treated with three different doses of extracts.

Treatment	C (mg/dL)	HDL (mg/dL)	LDL (mg/dL)
Control	78.80 $\pm$ 2.82	41.50 $\pm$ 2.12	15.80 $\pm$ 0.28a
250 mg/kg	86.50 $\pm$ 2.12*	40.50 $\pm$ 0.70	24.70 $\pm$ 1.08*
500 mg/kg	81.50 $\pm$ 3.53	39.00 $\pm$ 1.41	25.60 $\pm$ 4.80*
1000 mg/kg	80.50 $\pm$ 2.12	36.50 $\pm$ 2.12	16.60 $\pm$ 4.80

Data are presented as mean $\pm$ standard deviation (SD). * significant difference compared with control ($p \leq 0.05$).

Figure 3. Effects of oral administration of flavonoid-rich extract for 28 days on the diameter of seminiferous tubules (A) and germ epithelial height (B) in male mice treated with three different doses of the extract. Data are expressed as mean $\pm$ SD, $n = 5$. Significant ($p < 0.05$) compared with the control group.

Figure 4. Effect of oral administration of flavonoid-rich extract for 28 days on testicular protein carbonyl (A), lipid peroxidation (B), nitric oxide (C) and total anti-oxidants (D) in male mice treated with three different doses of the extract. Data are expressed as mean $\pm$ SD, $n = 5$. Significant ($p < 0.05$) compared with the control group.

in mice treated with 250 and 500 mg/kg ($p < 0.05$) compared to the control group (Fig. 2B).

Morphometric results

Treatment with different flavonoid extract doses for 28 days led to an atrophy of the seminiferous tubules. The diameter of the seminiferous tubules was determined to be significantly smaller at 250 ($0.63 \pm 0.17 \mu\text{m}$), 500 ($0.67 \pm 0.25 \mu\text{m}$) and 1000 mg/kg ($0.72 \pm 0.22 \mu\text{m}$) ($p < 0.01$) compared with the control group ($2.25 \pm 0.39 \mu\text{m}$) (Fig. 3A). The germ cell height of seminiferous tubules signif-

icantly decreased compared to that of the control. Mean epithelial height was determined to be at 0.49 ± 0.10 , 0.54 ± 0.10 , 0.57 ± 0.13 and $0.62 \pm 0.17 \mu\text{m}$ in the groups treated with 250, 500 and 1000 mg/kg, respectively, compared with the control group ($0.62 \pm 0.17 \mu\text{m}$) ($p < 0.05$) (Fig. 3B).

The effect of the extract on testicular total anti-oxidant and oxidative biomarkers

The levels of lipid peroxidation (measured as MDA) and nitric oxide (NO) within the testicular tissues were significantly higher following treat-

ment with flavonoid extracts. In contrast to controls, as the flavonoid extract dose increased, the level of MDA and NO was observed to increase significantly ($p < 0.001$) (Fig. 4). Changes in protein carbonyl content within the testicular tissues were found to be lower at doses of 250 and 500 mg/kg bw compared to the highest dose (1000 mg/kg bw). The changes in the anti-oxidant levels within the testicular tissue were found to be dose-dependent (Fig. 4).

Histopathological findings

Histological analysis showed normal architecture of the seminiferous tubule, with a normal pattern of spermatogenesis in the testes of control mice (Fig. 5A). In contrast, histopathological changes were detected in the testes of flavonoid-treated mice (Figs. 5B, C and D).

The lowest dose, that is, 250 mg/kg of flavonoid extract has been determined to exert the more pronounced effect on testicular tissue compared to other applied doses, while the highest dose had the least pronounced effects (Fig. 5). Meanwhile, the doses of 250 and 500 mg/kg bw of flavonoid extracts caused decreased maturation of spermatogenic cells, resulting in spermatogenesis arrest (18% at 250 mg/kg and 15% at 500 mg/kg) and abnormal arrangement of germ cell layers (11% at 250 mg/kg and 8% at 500 mg/kg) (Fig. 5C and 5E, Table 5).

A loss of germ cell attachment that sloughed into seminiferous tubules lumen was also observed (Figs. 5, Table 5). Paternal germ cell exfoliation was also identified as another toxic effect of flavonoid

Figure 5. Photomicrograph of testicular histology of the (A) control rats showing normal seminiferous tubules and normal structure of germinal epithelium. (B) Mice treated with flavonoid extract at dose 250 mg/kg bw, showing exfoliation of germ cells in the center of seminiferous tubule and disarrangement of spermatids in the lumen. C&E: Mice treated with flavonoid extract at dose 250 mg/kg bw, exhibiting spermatogenesis arrest. D: Mice treated with flavonoid extract at dose 250 mg/kg bw, showing germ cell degeneration. F: Mice treated with flavonoid extract at dose 250 mg/kg bw, showing vacuoles. Magnification: 40x. Scale bar = 100 μ m.

Table 5. Mice testicular toxicological histopathology findings induced by flavonoid extract in male mice treated with three different doses.

Treatment	Control	250 mg/kg*	500 mg/kg*	1000 mg/kg*	P-value
Germ cell exfoliation	Non	+ (19%)	+ (15%)	+ (11%)	< 0.5
Germ cell degeneration	Non	+ (12%)	+ (9%)	+ (9%)	< 0.5
Germ cell disarrangement	Non	+ (11%)	+ (8%)	+ (10%)	< 0.5
Vacuoles	Non	+ (15%)	+ (10%)	+ (10%)	< 0.5
Spermatogenesis arrest	Non	+ (18%)	+ (15%)	+ (12%)	< 0.5

*Extract doses

exposure, which was found to be 19% in the 250 mg/kg treated group and 15% in the 500 mg/kg treated group (Fig. 5B, Table 5). Moreover, it was noted that an important percent of vacuoles within germ cell layers are due to loss of Sertoli cells and germ cells (15% and 10% at doses of 250 and 500 mg/kg, respectively) (Fig. 5F, Table 5).

DISCUSSION

In recent years, researchers have focused on identifying and developing cost-effective and safe contraceptives in an attempt to curb the growing population in certain countries, such as Egypt and India. Several studies found that plants rich in bioactive compounds including flavonoids that may act as fertility regulators (19, 20). However, the effects of flavonoids from plant sources on the reproductive system remain to be controversial and are not fully understood.

Fertility and implantation activity of plant extracts found to be related to oestrogenic activity in males, while in females resulted from their action as estrous cycle disruptors in an animal laboratory (20, 46-48). Prior studies suggest that flavonoids may positively affect fertility activity by improving spermatogenesis (49, 50). The lack of consistency in findings can be attributed primarily to the presence of many classes of flavonoid compounds (e.g. anthocyanin, proanthocyanidin, iso?avonoid, and flavonol), which may exhibit different effects on fertility (51). Additionally, since most studies have focused on investigating the female reproductive system, this present study aimed to focus on evaluating the effect of the flavonoid-rich extract on the reproductive system of male mice.

This current study showed that flavonoids that are orally administered did not affect the health condition of mice or demonstrate signs of general toxicity; however, they can interfere with male reproductive function (52, 53). In addition, no significant changes in body and testis weights were recorded in the animals after extract treatment, which was deemed in agreement with previous findings (6, 52). Moreover, the elevated level of cholesterol in the testes is attributed to decreased androgen production, which can often lead to the accumulation of cholesterol in the testes and impaired spermatogenesis (54).

In previous studies, male mice treated with flavonoid extract at low doses (=15 mg/mL) or high doses (250-500 mg/kg bw) showed a significant reduction in the sperm motility, abnormality, semi-

niferous tubules, and height of the germinal epithelium in testes, which was in concord with present results (7, 20, 53).

Similar to previous studies that reported an inverse relationship between sperm membrane integrity and oxidative stress biomarkers, such as malondialdehyde (MDA), protein carbonyl (PC) and nitric oxide (NO) (55, 56), this present study found that the testicular level of MDA, NO and PC has increased in treated groups compared with that of the control group ($p < 0.05$). Taking into account the histopathological observations, these findings suggest that flavonoids may act as pro-oxidants, as such the spermicidal activity observed on sperm membrane structures was due to oxidative damage (55, 57).

Oxidative stress is often a result of an imbalance between the production and the removal of reactive species and free radicals by anti-oxidants. Due to the high content of polyunsaturated fatty acids in the plasma membrane of spermatozoa, sperm is found to be vulnerable to reactive oxygen species (ROS) during the critical phases of spermatogenesis (58). Oxidative lipid damage within the sperm membrane may lead to an accumulation of lipid peroxidation products, including MDA, which consequently lower sperm motility and decrease spermatogenesis (7, 22, 56). Additionally, although NO was identified to play role in the physiologic cell signaling mechanisms, an increase in NO was found to damage many biological molecules, further reducing the fertility rate (59). Moreover, oxidation of proteins has been associated with male fertility (60) as demonstrated by an increase in the level of reactive species, oxidation adducts, and induction of spermatogenic arrest.

The level of seminal ROS and their toxic effects are restricted by seminal anti-oxidants, which have been found to pose beneficial effects on sperm parameters and developmental potential (61). Seminal anti-oxidants include enzymatic antioxidants such as catalase, superoxide dismutase, glutathione peroxidase, and non-enzymatic antioxidants such as glutathione, ascorbic acid and tocopherols.

Furthermore, similar to previous studies that have reported an inverse association between anti-oxidant status and infertility, this present study found that sperm motility was significantly lower among mice treated with flavonoid extracts, which also markedly reduced the amount of TAC (62, 63).

CONCLUSION

Our findings demonstrate that the daily administration of *A. pavarii* extract at doses of 250 and 500 mg/kg bw may lead to the suppression of spermatogenesis in male Swiss albino mice, subsequently reducing their fertility ability. However, the long-term effects need to be further investigated to better understand whether discontinuation of the treatment would reverse the anti-fertility effects of the flavonoid-rich plants.

Conflict of interest

The authors declare no conflict of interest.

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