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Original Article

A two-generational reproductive study to assess the effects of *Juglans regia* on reproductive developments in the male and female rats

Estudo reprodutivo de duas gerações para avaliar os efeitos de *Juglans regia* no desenvolvimento reprodutivo de ratos machos e fêmeas

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Abstract

Environmental pollutants and lifestyle severely threaten human and animal health, leading to disturbances of various functions, including infertility. So, exploring a safe treatment that could effectively reverse infertility remains a challenge. The current study was intended to explore the fertility-enhancing effect of *Juglans Regia* oil in two successive generations of rats; F_0 and F_1. *J. Regia* oil was initially tested for in vitro antioxidant assay via ROS and DPPH, followed by in vivo toxicity testing. In the fertility assessment, eighteen pairs of male and female rats (n=36, 1:1, F_0 generation) were divided into three groups and dosed with 1 mL/kg and 2 mL/kg daily of *J. Regia* oil and saline, respectively, up to pre-cohabitation, cohabitation, gestation and lactation periods. The reproductive performance, including body weight, live birth index, fertility index, and litter size, was assessed. Hormonal and antioxidant markers of F_1 generations were assessed with the histopathological evaluation of male and female organs. The oil of *J. Regia* showed great antioxidant potential ($P < 0.05$) in DPPH (1,1-diphenyl-2-picrylhydrazyl) and ROS (Reactive Oxygen Species) methods ($P<0.05$). The continued exposure of the F_0 and F_1 generations to *J. Regia* oil did not affect body weight, fertility index, litter size, and survival index. We have found pronounced fertility outcomes in both genders of F_0 and F_1 generations with *J. Regia* 2 mL/kg/day in comparison to the control. Results showed that *J. Regia* significantly increased ($P < 0.05$) luteinizing hormone (LH), plasma testosterone, follicular stimulating hormone (FSH), glutathione peroxidase (GPx) and superoxide dismutase (SOD) activities in both generations. Histology of both generations reveals improved spermatogenesis and folliculogenesis with enhanced architecture. Altogether, the present results suggest that *J. Regia* improved fertility in both male and female rats by improving hormonal activities and oxidative stress.

Keywords: fertility, sex hormones, walnut, two-generation study, oxidative stress.

Resumo

Os poluentes ambientais e o estilo de vida ameaçam gravemente a saúde humana e animal, levando a distúrbios de diversas funções, incluindo a infertilidade. Assim, explorar um tratamento seguro que possa reverter eficazmente a infertilidade continua sendo um desafio. O presente estudo teve como objetivo explorar o efeito do aumento da fertilidade do óleo de *Juglans regia* em duas gerações sucessivas de ratos: F0 e F1. O óleo de *J. regia* foi inicialmente testado para ensaio antioxidante in vitro via ERO e DPPH, seguido por testes de toxicidade in vivo. Na avaliação da fertilidade, 18 pares de ratos machos e fêmeas (n = 36; 1:1; geração F0) foram divididos em três grupos e dosados com 1 mL/kg e 2 mL/kg diariamente de óleo e solução salina de *J. regia*, respectivamente, até os períodos de pré-coabitação, coabitação, gestação e lactação. Foi avaliado o desempenho reprodutivo, incluindo peso corporal, índice de nascidos vivos, índice de fertilidade e tamanho da ninhada. Marcadores hormonais e antioxidantes das gerações F1 foram mensurados por meio da avaliação histopatológica de órgãos masculinos e femininos. O óleo de *J. regia* apresentou grande potencial antioxidante ($P < 0,05$) nos métodos DPPH (1,1-difenil-2-picril-hidrazil) e ERO (Espécies Reativas de Oxigênio) ($P < 0,05$). A exposição contínua das gerações F0 e F1 ao óleo de *J. regia* não afetou o peso corporal, o índice de fertilidade, o tamanho da ninhada e o índice de sobrevivência. Encontramos resultados relevantes de fertilidade em ambos os sexos das gerações F0 e F1 com 2 mL/kg/dia de *J. regia* em comparação com o controle. Os resultados mostraram que *J. regia* aumentou significativamente ($P < 0,05$) as atividades do hormônio luteinizante (LH), testosterona plasmática, hormônio foliculoestimulante (FSH), glutationa peroxidase (GPx) e superóxido dismutase (SOD) em ambas as gerações. A histologia de ambas as gerações revela espermatogênese e foliculogênese melhoradas com arquitetura aprimorada. De modo geral, os presentes resultados sugerem que *J. regia* melhorou a fertilidade em ratos machos e fêmeas, as atividades hormonais e o estresse oxidativo.

Palavras-chave: fertilidade, hormônios sexuais, nozes, estudo de duas gerações, estresse oxidativo.

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1. Introduction

Infertility can be defined as the failure to accomplish a pregnancy clinically after 12 or more months of regular unsafe sexual intercourse (Szamatowicz and Szamatowicz, 2020). It has been estimated that around 15% of couples are infertile globally (Sun et al., 2019). Within all infertility issues, human sterility attributed to male fertility issues reports for 45-50% (Kumar and Singh, 2015), and female accounts for 46.6% of polycystic ovarian syndrome cases (PCOS), constituting the principal cause (Deshpande and Gupta, 2019). To date, factors underlying the dysfunction of the reproductive system are partly understood. The use of recent drugs to improve fertility dysfunction is insignificant in achieving higher pregnancy outcomes (Ye et al., 2020). So, the future focus will be on formulating more viable treatment choices to prevent or treat dysfunctions of the reproductive system to improve fertility.

For several years, locally grown plants in place of supplements have been used globally to vitalize, energize, and enhance male sexual functions. Several plants extract have now been identified for improving fertility and other sexual functions, including *Parkia Biglobosa* (Obeten et al., 2022), *Eugenia Uniflora* (Nkpurukwe et al., 2022), *Bryonia Laciniosa* (Sud and Sud, 2017), *Terminalia Catappa* (Oyeniran et al., 2021), *Tribulus Terrestris* (Zheleva-Dimitrova et al., 2012) and *Lepidium Meyenii* (Tafuri et al., 2021). The active constituents of these plants, including terpenoids, phenols, alkaloids, and saponins, may have a role in restoring fertility (Ogidi et al., 2019). Polyphenols, especially flavonoids, are recognized to have estrogenic (Akbaribazm et al., 2021) or androgenic capabilities (Abaho et al., 2022) that have a crucial role in maintaining fertility. Such findings pointed out that flavonoids can be crucial in recovering spermatozoa and spermatogenesis (Ye et al., 2020).

One of the flavonoid-rich fruit is walnut *(Juglans Regia* L.*)* of family Juglandaceae. It contains many bioactive compounds like serotonin and melatonin, also rich in magnesium and other minerals and polyunsaturated fatty acid (Tapia et al., 2013). A study conducted on essential oils taken from the leaves of *J. Regia* were analyzed by both GC-FID and GC-MS methods and exhibited a total of 38 compounds. High antioxidant activity was found in essential oil like polyunsaturated omega three and omega six fatty acids (Rather et al., 2012). Antioxidant potential and phenolic content were assessed in oils, kernels and bagasse pellets of various *J. Regia* cultivars.

Adelakun et al. (2019b) studied the reproductive capacity of *J. Regia* by conducting an experiment on rats. The findings of this research proposed that male rats showed increased reproductive capacity as a result of taking oil by increasing sperm count. Ikwuka et al. (2021) also support this finding that the frequent use of *J. Regia* boosts male fertility in Wistar rats. This improvement in sperm count and motility may be due to the presence of glycosides, saponins, PUFs and alkaloids that have antioxidant potential. Moreover, saponins may also improve sperm parameters, as reported earlier. Similarly, Masterson et al. (2020) found that *J. Regia* improved the semen quality and sperm motility.

Reproductive hormones are essential in the development and regular functions of the reproductive system. FSH and Testosterone are important for the fulfillment of reproductive abilities in males (Walker and Cheng, 2005). Studies showed that sex hormones like FSH, LH, and testosterone levels are associated with spermatogenesis (Khaki et al., 2009). Similarly, in females, the causes of infertility have been linked to disturbances in hormones, including FSH, LH, Estradiol, etc. LH plays a crucial role in the menstrual cycle and functions in conjunction with FSH. The FSH/LH production is governed by estrogen level. The increase in estrogen level makes the pituitary gland to start making additional LH and to halt FSH production. The LH shift makes the egg release from the ovary by a process named as ovulation (Ali and Hisham, 2016). Sudha and Reddy reported that infertility/ anovulation may be caused by hormonal imbalance, as balance in hormones is necessary to produce adequate follicles required for ovule development (Sudha and Reddy, 2013). Plant-derived polyphenols that influence endocrine/hormonal activities in humans have received great attraction for researchers because of their possible beneficial effects with no adverse effect.

J. Regia presented its exclusive actions by augmenting fertility and decreasing fertility-related problems, including hormonal imbalance. Assessment of body weight and blood parameters provides information regarding an animal's health and reproductive effects (Aly et al., 2009). However, no research has been executed to explore the beneficial role of *J. Regia* on reproduction in two generations. Therefore, a two-generational study was established to explore the beneficial and protective use of *J. Regia* oil on fertility and reproductive hormones in experimental animals.

2. Materials and Methods

2.1. Chemicals

All chemicals used in this study were of analytical grades including Ferric Chloride, Petroleum ether, Ethanol (96%), Formalin, Potassium hydroxide, Glacial acetic acid, Sulphuric acid, Sodium Hydroxide, Mayer's and Dragendoff's reagents, HCl, Tetramethylbenzidine (TMB), HRP (Horseradish peroxidase), FBS (Fetal bovine serum), Etoposide, DPPH, DMEM (Dulbecco's Modified Eagle Medium), Gallic acid and Folin-Ciocalteu reagent.

2.2. Identification and extraction of plant materials

The walnuts were purchased from a local market in Quetta (Baluchistan), Pakistan, along with their plants, and were identified at the KU Herbarium and Botanical Garden, University of Karachi. The specimens were identified as *Juglans Regia* L. of Juglandaceae, G.H. No. 95591. The fruits were then manually cracked, shelled, and subjected to expeller pressing to obtain oils. This method for acquiring oil from oleaginous seeds is the simplest and oldest method with no use of chemicals. The superior quality oil is extracted, and it utilizes low operation costs than solvent extraction.

2.3. Preliminary phytochemical screening

A small quantity of samples was taken for the phytochemical tests by methods in accordance with little modifications (Abdillah et al., 2015; Alshammaa, 2016; Esienanwan et al., 2020).

2.4. Antioxidant assays

2.4.1. Antioxidant assay by ROS

Primary cortical cells were used for the determination of Reactive Oxygen Species (ROS) generation. The method was adopted with some modifications (Kamiloglu et al., 2020; Vargas et al., 2014).

2.4.2. Antioxidant assay by DPPH

An upgraded DPPH assay (1,1-diphenyl-2-picrylhydrazyl) was selected for the evaluation of tested compounds. The DPPH radicals diminish at 517 nm of absorbance in the presence of antioxidant potential. The test compounds at different concentrations (10, 20, 100, 200 and 300 µg/mL) were added to 0.1 mM DPPH (3 mL) and allowed to react. The mixture was swirled and incubated at room temperature for 30 min. A microplate reader (SkanIt Software 5.0 of RE, ver. 5.0.0.42) was utilized to assess the absorbance at 517 nm. The test compound reducing power was calculated with the absorbance of ascorbic acid, which served as control (Arif et al., 2022).

Radical Scavenging Activity was equated in percentage via Equation 1.

$$\% \; RSA = \left\{ \left(A_{control} - A_{sample} \right) / \; A_{control} \right\} \times 100 \qquad (1)$$

2.5. Brine shrimp toxicity testing

Toxicity of *A. Cepa* was evaluated to rule out % Mortality with the help of the Brine shrimp method (Artemia Saline). Etoposide served as a positive control in this method (Meyer et al., 1982).

2.6. Acute oral toxicity

The study on the acute oral toxicity of *J. Regia* was executed according to the OECD guidelines (OECD, 1987). Rats of both genders were used and were supplied with distilled water and feed *adlibitum*. Twenty rats were randomly grouped into four groups (n=5). A single oral dose was given; *J. Regia* was administered to rats (n=5) at a dose of (100,1000 and 2000 mg/kg bw) in comparison to the control. The rats were examined for toxicity signs like behavioral changes and mortality for 48 hours (Oyewusi et al., 2015; Saidu et al., 2007).

2.7. Animal ethical approval

The study was performed under the approval of the Board for Advanced Studies and Research dated 28-03-2019 (reference No 05071/pharm), University of Karachi and Pharmacology Departmental Research Committee, the for use of animals according to the National Institute of Health guidelines (NIH guidelines Islamabad, Pakistan) (NRC, 2011).

2.8. Experimental animals

Healthy and sexually mature Wistar rats of both sexes were equally used for the experimental fertility study. The animals were procured from the animal house, H.E.J, University of Karachi, and were kept in plastic cages having proper ventilation under standard temperature conditions (25 to 30 °C) with 12 h light and dark cycle. The experimental animals were placed in an animal house Department of Pharmacology, University of Karachi. Male rats aged 12 weeks (140-160 g) and female rats aged 14 weeks (160 g to 180 g) were used and given an acclimatization period for a week. The experimental protocol fulfills the guidelines on the appropriate use and care of laboratory animals (NRC, 2011), and the experimental protocol and design was permitted by the Board of Advanced Studies and Research (BASR), University of Karachi, ASRB/No./05071/Pharm {Resolution No. 10(P)07}.

2.9. Experimental protocol

The study was conducted for a total duration of seven months. A modified method of Vohra and Khera (2016) was used. Eighteen pairs of rats (n=36, F_0 generation) were grouped into six experimental groups (n=6) randomly; three males and three females in each group, and were accordingly treated. Group I served as control and administered distilled water daily; Group II animals were given a low dose of *J. Regia* oil, 1 mL/Kg/day; Group III animals were given *J. Regia* oil at a high dose of 2 mL/Kg/day.

All animals were dosed 30 days by gavage before mating then male animals in each group were singly paired with female animals for seven days. Female rats were subjected to the soiled bedding of an adult male rat to align their estrus cycle (female animals were fertile and receptive during this period) (Ain et al., 2022). The vaginal plug was noticed to confirm the pregnancy of each female rat. Body weight was weekly recorded before mating, during mating, and in gestational periods. All F_0 and F_1 female parental animals were monitored twice daily for any toxicity signs. F_0 and F_1 animals were treated with *J. Regia* oil throughout the pre-cohabitation (30 days), cohabitation (21 days), gestation (21 days), and lactation periods.

The expected female rats were permitted to carry the pregnancy to term. Afterwards fertility indices (fertility index, the number of pups, survival index) were estimated. Offspring relating to both generations were counted and observed for any abnormality on postnatal days 0, 4,7, 14 and 21 days.

The same doses of the *J. Regia* oil (as of the F_0 generation) were given to F_1 off-springs during their growth, adulthood, and up to breeding. Pregnant female animals of the F_1 generation were continuously given the *J. Regia* oil during their pre-cohabitation, cohabitation period, gestation and lactation until the F_2 generation pups were weaned. Afterwards, when pups were delivered, all F_1 animals were sacrificed and their blood samples and reproductive organs were taken for biochemical and histopathological assessment. The same protocol for the F_0 generation was followed till the birth of the F_2 generation (Vohra and Khera, 2016).

2.10. Reproductive performance of F_0 and F_1 rats

The reproductive parameters studied were mentioned in the form of ratios, weights, and indices that analyze all stages starting from conception till weaning. These fertility parameters were computed as follows (Equations 2 to 6):

$$Fertility\ index(\%) = \left(\frac{Females\ count\ giving\ birth\ /\ females\ count\ lived\ together}{}\right) \times 100$$ (Chiang and Flaws, 2019) (2)

$$Litter\ size = count\ of\ pups\ /\ count\ of\ gestated\ females$$ (Vohra and Khera, 2016) (3)

$$Live\ birth\ index(\%) = \left(\frac{count\ of\ pups\ alive\ at\ day\ 0\ /\ count\ of\ pups\ born}{}\right) \times 100$$ (4)

$$Survival\ index - 4\ days(\%) = \left(\frac{count\ of\ pups\ alive\ on\ day\ 4\ /\ day\ 0\ alive\ pups\ count}{}\right) \times 100$$ (5)

$$Survival\ index - 21\ days(\%) = \left(\frac{count\ of\ alive\ pups\ on\ day\ 21\ /\ day\ 4\ alive\ pups\ count}{}\right) \times 100$$ (Narayana et al., 2005) (6)

2.11. Blood parameter assessment

All F_1 generation male and female animals were sacrificed and their blood samples were collected by heart puncture and kept in B-Ject gel clot activator vacuum tubes under aseptic conditions. Samples were centrifuged in Eppendorf machine at 4000 rpm for 10 min. The separated serum was kept at -80 °C for hormonal and biochemical testing. All samples were analyzed for hormonal estimation (FSH, LH, testosterone, and estradiol (Alam, 2019; Sultana et al., 2018) via ELISA Roche Diagnostics, Basil). SOD and Glutathione (GPx) were estimated by the kit acquired from Glory Science Co., Ltd. (Glory Science Co.2022#45).

2.12. Histopathological evaluation

The rats were sacrificed by cutting off the diaphragm. Ovaries and testes of the selected rats of F_1 generation were identified with the help of a histopathologist; ovaries were dissected out from the surrounding fats, and measured weight. The separation of the horns of the uterus from the vagina was done by cutting the uppermost point of the cervix than preserved in 10% formalin, and later treated through usual processes and embedded in paraffin. Similarly, testes were removed from the scrotum, dissected from adherent tissues, weighed then fixed in 10% formalin, and later treated through usual processes and embedded in paraffin. Blocks of paraffin were cut serially and longitudinally at 4 µm thickness, followed by Hematoxylin-Eosin staining. Stained slides were analyzed and compared in groups (Ellenburg et al., 2020).

2.13. Statistical analysis

Statistical analysis was implemented using the SPSS software (version 20.0). Multiple groups were compared by one-way analysis of variance applying the Bonferroni post-hoc test. The results were regarded to be statistically significant if the value lies between $P < 0.05$ and highly significant when $P < 0.01$.

3. Results

3.1. % yield of J. Regia oil

The percentage yield of *J. Regia* oil was 47.62% from 8 Kg of fruits.

3.2. Preliminary phytochemical screening

The presence of *J. Regia* constituents was mentioned in Table 1. The qualitative phytochemical analysis reveals that *Juglans Regia* possesses flavonoids, saponins and phenols.

3.3. Antioxidant assays

3.3.1. Antioxidant assay of J. Regia by ROS

The DCF-DA method was employed to determine the ROS production and % Viability of *J. Regia* oil on primary cortical neuronal cells and was found nontoxic and significantly prevented H_2O_2-induced oxidative damage. % Cell viability in neuronal cells after incubation using indicated concentrations of *J. Regia* is shown in Figure 1, which showed protective efficacy against H_2O_2-induced toxicity on neuronal cells and had no cytotoxic effect in neuronal cells at concentrations up to a dose of 100 (µg/mL).

Table 1. Phytochemical Analysis of *Juglans Regia*.

Phytochemicals	Juglans Regia
Phlobatannins	-
Flavonoids	+
Glycosides	-
Alkaloids	-
Saponins	+
Phenols	+
Terpenoid	-
Tannin	-

The presence of phytochemicals is represented as (+), while absence is denoted as (-).

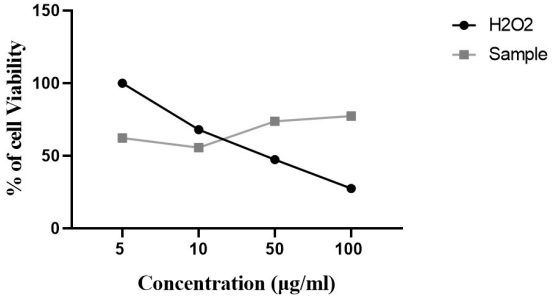

Figure 1. Percentage Viability of *J. Regia* in comparison to H_2O_2.

3.3.2. Antioxidant assay of J. Regia by DPPH

The % inhibition of *J. Regia* oil at different concentrations is shown in Table 2. The oil was observed with radical scavenging potential by DPPH utilizing Ascorbic Acid as a reference standard. Among various concentrations, *J. Regia* exhibited the highest activity at 100 µg/mL and 300 µg/mL i.e., 74.65 ± 1.24% and 74.24 ± 4.94%, respectively; while Ascorbic acid exhibited the strongest scavenging activity of 88.14 ± 1.28% at 300 µg/mL.

Table 2. DPPH-radical scavenging potential of *Juglans Regia* oil at different concentrations: Ascorbic Acid as positive control.

Samples	Concentration (µg/mL)	% Inhibition (Mean ± SD)
Juglans Regia Oil		
	10	64.81 ± 5.75
	100	74.65 ± 1.24
	200	68.67 ± 5.16
	300	74.24 ± 4.94
Ascorbic Acid (Standard)	10	75.96 ± 0.92
	20	77.94 ± 2.02
	100	81.8 ± 1.47
	200	85.49 ± 0.45
	300	88.14 ± 1.28

3.4. Brine shrimp lethality testing

The *J. Regia* oil was assessed for brine shrimp lethality studies, and the results were mentioned in Table 3. No significant toxicity to brine shrimp Artemia salina was found with *J. Regia* oil at different concentrations, and significant results represented the oil is biologically safe. The % mortality of *J. Regia* at 1000 µg/mL concentration is 20% compared to standard Etoposide, which shows 100% mortality at 1000 µg/mL, indicating oil is significant and safe for use.

3.5. Acute oral toxicity

Regarding the safety of the *J. Regia* plant, acute toxicity showed that it is safe at low concentrations and lethal at higher doses (2000 mg/kg). Moreover, some dose-dependent behavioral changes were also observed in high-dose treated groups, including depression, clustering, folding and sleeping, unsteady gait, and loss of appetite. The results regarding the acute toxicity (LD_{50}) of *J. Regia* by using the Karber Method are mentioned below (as shown in Table 4). The arithmetic method of Karber was utilized for calculation.

3.6. Fertility assessment

The two-generation fecundity study was conducted for seven months that showed no congenital abnormalities in any of the pups of all the groups of F_0 and F_1 generations. The body weights of F_0 and F_1 animals are mentioned in Table 5, representing insignificant differences ($P>0.05$) among both generations and control.

Table 3. Percentage Mortality of *Juglans Regia* oil as compared to Control and Etoposide (Standard).

Samples	Concentration (µg/mL)	No. of Shrimps	No. of Survivors	% Mortality
Control Distilled Water	10	30	30	0
	100	30	30	0
	1000	30	30	0
Standard (Etoposide)	10	30	5	83.33
	100	30	1	96.6
	1000	30	0	100
Juglans Regia Oil	10	30	30	0
	100	30	25	16.66
	1000	30	24	20

Table 4. Determination of acute toxicity (LD_{50}) in rats using Karber Method.

Dose	Dose Difference	No. of dead Animals	Mean Mortality	Dose Difference - Mean Mortality
100	0	0	0	0
1000	900	0	0	0
2000	1000	2	1	1000
Control	0	0	0	0

LD_{50} = Highest Dose – Σ (Mean Mortality × Dose Difference) /n; LD_{50} = 2000 – (0 + 0 + 1000) / 5; LD_{50} of *J. Regia* Oil = 1800 mg/kg/BW.

The number of pups was counted in each generation and noticed for congenital abnormalities. In all groups, no congenital abnormality was observed. The number of pups increased significantly in the first and second generations compared to the control group (df 5, 30; F 3.37; P < 0.05).

Post Hoc analysis through the Bonferroni test reveals that high-dose administration of *Juglans Regia* produced statistically significant differences in the number of pups in both generations as compared to low-dose group and control group rats, as shown in Table 6.

3.7. Reproductive performance

The reproductive performance was assessed by observing several parameters, including live birth index, fertility index, and litter size. Insignificant changes were found in all treated groups' live birth, fertility, and survival indexes (P > 0.05). All these parameters were improved in all treated groups, just like the control group. However, a significant increase in litter size was noticed in the treated group in both generations (P < 0.05) when compared to the control group as shown in Table 7.

3.8. Hormonal parameters

FSH, LH, Estradiol and Testosterone levels were tested in the plasma of both control and treated male and female rats. In higher doses, statistically significant raised values of FSH and LH were found in female rats (df 5,30; F 32.38; P < 0.05) (df 5,30; F 105.2; P < 0.05), respectively, when compared to the control. Similarly, statistically significant differences in estradiol (df 5,30; F 101.8; P < 0.05) and testosterone (df 5,30; F 81.49; P < 0.05) were observed in male rats after administration of high doses of *J. Regia* as compared to control. A significant decrease was found in Estradiol in female animals (df 5,30; F 60.05; P < 0.05) as compared to the control (as shown in Figure 2).

3.9. Oxidative parameters

SOD levels in treated rats were found to be statistically highly significant in male rats (df 5,30; F 25.02; P < 0.01) and female rats (df 5,30; F 6.28; P < 0.01) with high dose treatment as compared to control. Additionally, statistically significant results in male (df 5,30; F 27.4; P < 0.05) and female (df 5,30; F 13.13; P < 0.05) were also observed on GPx levels with high dose treatment as compared to control (as shown in Figure 3).

Table 5. Effect of *J. Regia* oil on weekly body weight of F_0 and F_1 generation female rats as compared to control.

Weeks	F_o			F_1		
	Control	T1 (1 mL/ kg)	T2 (2 mL/kg)	Control	T1 (1 mL/kg)	T2 (2 mL/kg)
I	164 ± 0.51	170 ± 2.25	167.5 ± 1.60	170.5 ± 2.95	166.3 ± 1.22	166.3 ± 1.76
II	166.1 ± 0.94	168.3 ± 0.88	167.1 ± 0.94	171.5 ± 3.14	165.8 ± 0.70	165.5 ± 0.76
III	170.6 ± 1.17	168.1 ± 0.70	165.3 ± 0.71	172.5 ± 3.19	165.1 ± 0.94	165.3 ± 0.80
IV	172.3 ± 2.29	169.1 ± 0.47	166.8 ± 0.47	174.3 ± 3.54	164.8 ± 0.30	165 ± 1.0
V	174 ± 1.65	170.3 ± 0.88	169.5 ± 0.56	175 ± 3.09	166.8 ± 0.40	167.5 ± 1.11

n = 6, Mean ± SEM. F_0 presents Parent Generation, while F_1 present 1st Generation, T1 shows low dose group while T2 shows high dose group

Table 6. Effect of *J. Regia* on the number of pups/ litter of F_1 and F_2 generation rats.

F_1				F_2			
Control	T_1 (1 mL/kg)	T_2 (2 mL/kg)	P Value	Control	T_1 (1 mL/kg)	T_2 (2 mL/kg)	P Value
4.17 ± 0.47	4.67 ± 0.42	6.50 ± 0.76*	P<0.05	4.17 ± 0.30	4.67 ± 0.42	6 ± 0.68*	P<0.05

n = 6, Mean ± SEM. *P < 0.05 significant. F_1 presents 1st Generation Pups, while F_2 presents 2nd Generation Pups, T1 shows low dose group, while T2 shows high dose group.

Table 7. Developmental Findings in F_1 and F_2 Rat Pups.

Parameters	F_0 Parents/F_1 Pups			F_1 Parents/F_2 Pups			P-Value
	Control	T1 (1 mL/kg)	T2 (2 mL/kg)	Control	T1 (1 mL/kg)	T2 (2 mL/kg)	
No. of Pregnant Rats	6	6	6	5	6	6	P > 0.05
Litter Size	4.5	4.33	6.5*	5	4.66	6*	P < 0.05
Fertility Index (%) of F_0 female	100	100	100	83.3	100	100	P > 0.05
Live Birth Index (%)	100	100	97.61	100	100	100	P > 0.05
Survival index at day 4 (%)	100	100	100	100	100	100	P > 0.05
Survival index at day 21 (%)	100	100	100	100	100	100	P > 0.05

F_0 presents Parent Generation, while F_1 presents 1st Generation, F_2 presents 2nd Generation, T_1 shows low dose group while T_2 shows high dose group. n = 6, Mean ± SEM. *P < 0.05 significant.

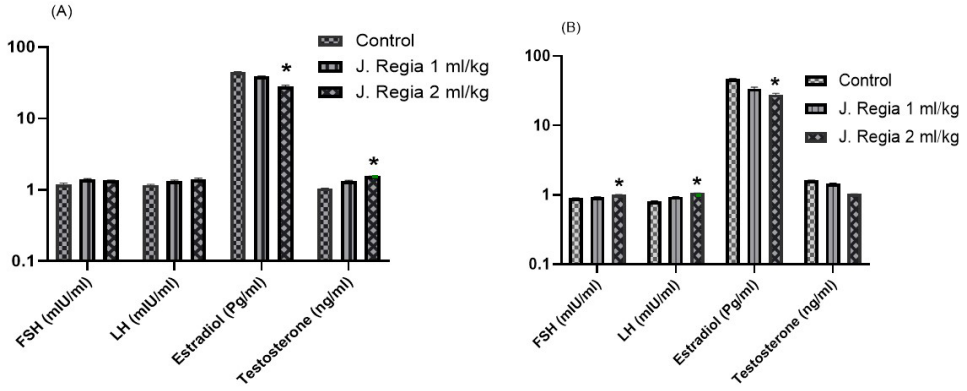

Figure 2. Effect of *J. Regia* oil on Hormonal Parameters as compared to control: Male (A); Female (B). n = 6, Mean ± SEM; *P < 0.05 significant, as compared to control.

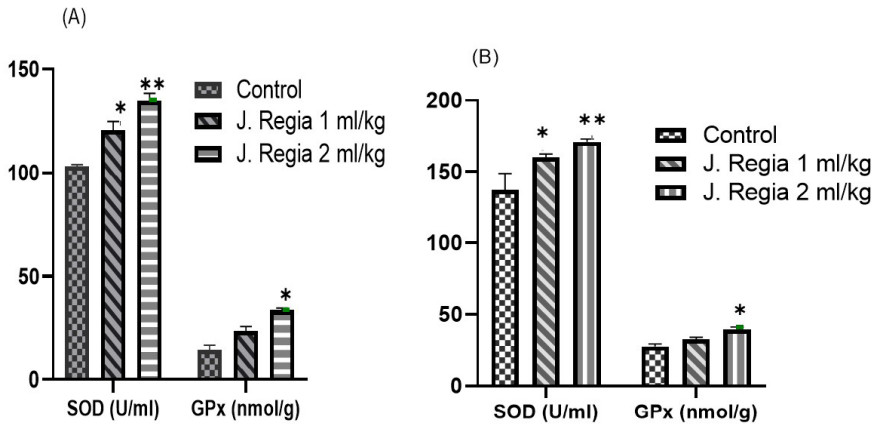

Figure 3. Effect of *J. Regia* oil on Oxidative Parameters as compared to control: Male (A); Female (B). n = 6, Mean ± SEM; *P < 0.05 significant; **P < 0.01 highly significant as compared to control.

3.10. Histopathological evaluation

Figure 4 displays the effect of *J. Regia* on histopathological parameters. Control ovaries (a) showed the external cortex surrounded by an intact capsule having a profuse stroma. A lot of blood vessels parted by unattached connective tissues in the medulla were also observed. The histological ovarian architecture of treated low-dose (b) animals was similar to controls. Similarly, high-dose *J. Regia* treatment slide (c) showed Graafian follicle and zona pellucida along with preantral, small antral, and large antral follicles and corpus luteum of different stages were observed.

Microscopic examination of the control group testis figure (a) showed an architecture of tubular seminiferous epithelia normally present with occasional Sertoli cells and spermatogenic cells at the interstitial area and base of the tubule having negligible connective tissues at the tubular surroundings. Microscopic examination of the low-dose treated testis (b) showed normal seminiferous tubules similar to the control group. Similarly, testis (c) treated with a high dose of *J. Regia* showed normal architecture of tubular seminiferous epithelia along with Sertoli cells and spermatogenic cells having minimal connective tissues at the tubular surrounding.

4. Discussion

J. Regia's therapeutic benefits have been recognized since ancient civilizations. In traditional medicine, walnuts were used to treat diabetes, cancer, inflammation, diarrhea, prostate disorders and cardiovascular disorders (Zhang et al., 2021). Walnut fruit contains polyphenols, fatty acids, cardiac glycosides, carbohydrates, steroids, minerals, tannins, proteins, dietary fiber, melatonin, α-tocopherol, plant sterols, folate, vitamin E, vitamin C, and vitamin A family compounds (Rusu et al., 2020).

In the current study, *J. Regia* oil represented the presence of flavonoids, saponins and phenols. Phenolic compounds are usually associated with antiatherogenic, antioxidant, anti-inflammatory, and anticancer properties. Slatnar et al. (2015) reported high content of walnut phenolic content in the skin of *J. Regia* that covers the kernel. Recent studies suggest the presence of polyphenols, squalene and tocopherols strongly correlated to the antioxidant capacity of *J. Regia* oil (Gao et al., 2019; Jahanban-Esfahlan et al., 2019).

The antioxidant capacity of various oils can be determined accurately and rapidly using DPPH testing. In

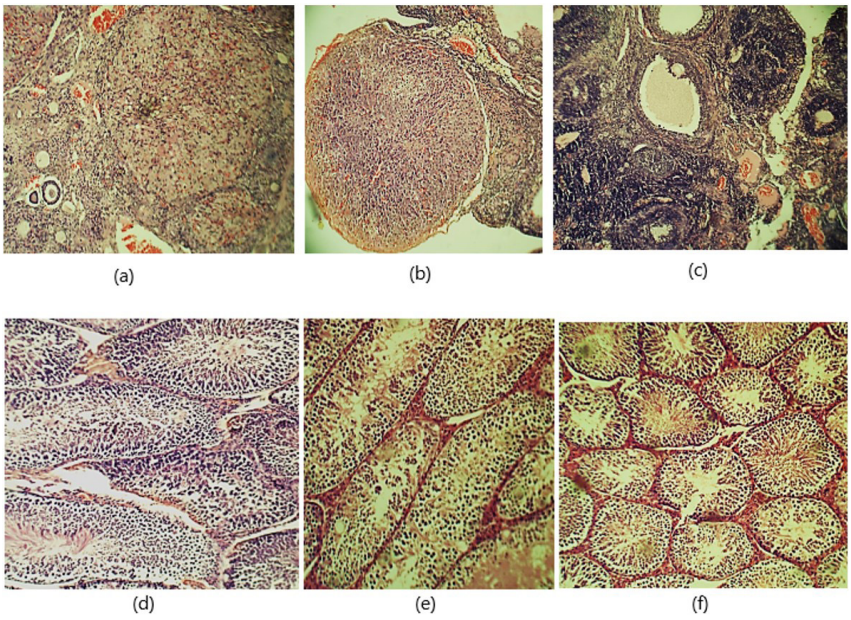

Figure 4. Effect of *J. Regia* and on Histopathological Parameters (a) Micrograph rat ovary of control (10x); (b) Micrograph rat ovary low dose *J. Regia* treated (10x); (c) Micrograph rat ovary high dose *J. Regia* treated (10x); (d) Micrograph rat testis of control (10x); (e) Micrograph rat testis low dose *J. Regia* treated (10x); (f) Micrograph rat testis high dose *J. Regia* treated (10x).

the current study, *J. Regia* showed 74.65% % inhibition at 100 µg/mL, that showed its highest free radical scavenging activity. Gao et al. (2022) documented the antioxidant role of *J. Regia* oil through DPPH and reported that it is due to the presence of δ-tocopherol and TPC in *J. Regia* oil. Our findings concluded that *J. Regia* oil exhibited greater antioxidant capacity, and this antioxidant potential is due to the occurrence of phenolic compounds, tocopherols and according to some recently reported data also, melatonin, an indoleamine that exhibits high antioxidant capacity (Arranz et al., 2008; Pycia et al., 2019).

The ROS production and % viability testing of *J. Regia* oil was tested by the DCF-DA method. It was found non-toxic in comparison to H_2O_2 and increased neuronal cell viability by 77.42% at 100 µg/mL. Laubertová et al. (2015) reported *J. Regia* oil can increase SOD activity, can enhance the antioxidant capacity of cells, decrease ROS activity and can prevent the release of cytokines depending on oil concentration. Based on our results, we supposed that *J. Regia* oil has the antioxidant ability to neutralize reactive oxygen intermediates and may contribute to progressing endogenous antioxidant defenses.

Toxicity studies are experimental screening procedures used to affirm the safety of any drug or herbal product with animal models. The critical criterion for toxicological evaluation is Mortality (Liu et al., 2019). In the Brine shrimp lethality test, the % mortality of *J. Regia* oil was found to be 20% at a maximum concentration that shows it is safe at low concentrations and lethal at higher doses. Similarly, the result of its oral acute toxicity test revealed that it has a high safety range when administered orally. The results attained in our study of acute oral toxicity revealed no mortality at the evaluated dose of 1000 mg/kg BW. Thus, it is proposed that the oral lethal dose (LD50) of *J. Regia* oil is up to 1000 mg/kg BW. The animals were noticed to be normal, having no changes within their eyes, fur, skin and mucous membranes. Neither any noticeable toxicity signs were observed, including tremors, coma, convulsions, nor any behavioral change was found.

J. Regia oil is a rich source of polyunsaturated fatty acids (PUFs) required for many physiological mechanisms and fertility (Panth et al., 2016). PUFs are used as an energy source during oocyte maturation and embryo development before implantation (Wathes et al., 2007). Robbin and colleagues reported that 75 g of walnuts per day added to a diet enhanced sperm motility, vitality, and morphology in a group of healthy young men as compared to the control group and considered improved semen quality and sperm motility associated with an increase in blood serum omega-6 and omega-3 PUFs (Robbins et al., 2012). Current data demonstrated an increased number of pups production in both F_1 and F_2 generations with both doses of *J. Regia* oil. This indicated that its oil presented beneficial actions by augmenting the fertility power of the female and male rats. *J. Regia* contains a significant amount of polyunsaturated fatty acids such as linoleic acid, oleic acid and linolenic acid (Arranz et al., 2008). Thus preventing the conversion of testosterone to dihydrotestosterone by inhibiting 5-α reductase and this way restores the plasma testosterone level in males and estrogen in postmenopausal women (Assi et al., 2022; Bostani et al., 2014). However, it seems that enhanced testosterone levels using *J. Regia* oil were because of its effect on Leydig cells, as well as its intrusion with the testosterone biosynthesis was probably due

to the prostaglandin synthesis stimulation (Assi et al., 2022). We observed that rats in both F_1 and F_2 generations represented fertility-enhancing effects by increasing litter size. Similarly, F_2 off-springs showed improved live birth index in the high dose group compared to the control group. No effects were observed on the survival index.

Reproductive hormones are essential in the development and regular functions of the reproductive system. FSH and Testosterone are important for fulfilling reproductive abilities in males (Walker and Cheng, 2005). Studies showed that sex hormones like FSH, LH, and testosterone are associated with spermatogenesis (Khaki et al., 2009). FSH, LH, testosterone, and Estradiol levels were estimated in both male and female animals. Significantly raised levels of LH and FSH were observed by *J. Regia* oil low and high doses in female rats. Similarly, Testosterone levels were found to be significantly raised in male rats, depicting its potential as a fertility-enhancing agent. These results are in agreement with previous results that explained that *J. Regia* significantly improves sex hormones, especially LH and FSH that enhance conception and testicular growth (Ghorbani et al., 2014). Additionally, Estradiol levels were significantly decreased and maintained as in the control group. Since FSH/LH surge is required for ovulation, that is triggered by LH releasing hormones and depends on estradiol levels (Jeje et al., 2021). Our results suggest that *J. Regia* oil in higher doses significantly improved FSH levels by balancing the estradiol and improving fertility, probably due to the increased pituitary sensitivity to gonadotropin-releasing hormone (Lienou et al., 2012). Our findings are in accordance with a study that explained that *J. Regia* successfully raised sex hormones in rats (Bostani et al., 2014). *J. Regia* contains α-linolenic acid, which is converted to arachidonic acid, to make prostaglandins that play an important role in ovulation and testicular steroidogenesis (Adelakun et al., 2019a). Robbins et al. (2012) explained that *J. Regia*, if added to the diet, improved sperm vitality, motility, and morphology.

Oxidative stress plays a crucial role in the pathogenesis of numerous inflammatory diseases and reproductive performances (Alahmar, 2019). Reactive oxygen species in physiological processes may behave as key signaling molecules, but excessive levels may also mediate pathological processes involving reproduction. Glutathione and Superoxide dismutase (SOD) are physiological antioxidants, the master detoxifiers and maestro of the immune system (Ozougwu, 2016). ROS affects multiple pathological and physiological processes in the ovaries, from oocyte maturation to fertilization leading to infertility (Lu et al., 2018). Glutathione and SOD shield eggs from damage caused by oxidative stress during folliculogenesis in females as well as they improve sperm count, motility and spermatozoa maturation in males (Mannucci et al., 2022). The current study represented improved glutathione and SOD levels after both low and high doses of *J. Regia* oil consumption in rats that confirmed the antioxidant potential of *J. Regia* oil. These results are in-accordance with Miao et al. (2020) that exhibited *J. Regia* oil significantly improved the antioxidant capacity by restoring glutathione and SOD levels and reducing the release of inflammatory factors from LPS-induced intestinal injury.

5. Conclusion

This study has revealed that *J. Regia* effectively promotes conception and fertility in both males and females by enhancing ovulation and spermatogenesis. Thus, the results of this study render access to a safer natural alternative to overcome fertility issues. Plant products for the treatment of infertility issues will be more acceptable for economic reasons and side effects than chemical agents.

5.1. Limitations and areas for future research

Two major limitations in the current study could be addressed in future research. First, the study focused mainly on the bi-generational study model and only the microscopic structure of the ovaries and testes of the experimental animals. If more resources had been available, we would have studied the correlation of the *Juglans Regia* oil on fertility by conducting infertility models, i.e., female and male infertility models. Furthermore, due to a limited budget, we restrict towards F_2 generation; in case of more facilities and financial resources, we must go for further F_3 and F_4 generation to scrutinize mirror images of study results. Secondly, further genomic markers will be evaluated for the exact molecular mechanisms.

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