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Alaa Al Moubarak

*MS Student, Faculty of Science, Beirut Arab University, Beirut, Lebanon, moubarak1alaa@gmail.com*

Manal El Joumaa

*MS Student, Faculty of Science, Beirut Arab University, Beirut, Lebanon, manal.eljoumaa@gmail.com*

Jamilah Borjac

*Associate Professor, Faculty of Science, Beirut Arab University, Beirut, Lebanon, j.borjac@bau.edu.lb*

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## POTENTIAL MALE CONTRACEPTIVE EFFECT OF ERIBOTRYA JAPONICA LEAVES EXTRACT

### Abstract

Nowadays, the stability of population growth is becoming one of the common global social and health problems that could be solved via contraception. Current research in the field of male contraceptives is focusing on the use of oral herbs. *Eriobotrya japonica* (Loquat) is a medicinal plant that has been commonly valued for its anti-inflammatory, antioxidant, antidiabetic, hypocholesteremic, and antitumor properties. The present study aimed to investigate the effects of the oral administration of loquat aqueous leaves extract on the fertility of male Balb/c mice. For this aim, six-eight-weeks old male Balb/c mice (~25g) were orally administered (100 mg/Kg/day) loquat leaves extract for 10 and 30 days. The effects of this extract on semen parameters, seminiferous tubules architecture, levels of male sex hormones, glucose and cholesterol, and mRNA levels of key enzymes involved in steroidogenesis were determined. These parameters were also measured following 20 days post-treatment withdrawal. Our results showed that loquat extract administered for 10 and 30 days exerted antifertility effects including a significant decrease in sperm motility, viability and count, and in testosterone and progesterone levels concomitant with serious arrest in spermatogenesis. The levels of prolactin significantly increased with loquat treatment for 10 and 30 days. This extract inactivated the steroidogenesis pathway. All these alterations were significantly restored to normal in all the treated animals after 20 days of treatment withdrawal. No changes in the levels of glucose and cholesterol were noticed. Loquat extract is an effective and potential reversible male contraceptive herb.

### Keywords

Contraception, *Eriobotrya japonica*, semen parameters, spermatogenesis, steroidogenesis

## 1. INTRODUCTION

The world population is 7.7 billion and this number is expected to increase to 9.8 billion by the year 2050 (Worldometers, 2019). This rapid growth in population is becoming a worldwide health concern that will create serious pressure on resources in both rich and poor countries. Therefore, it requires implanting more reasonable family planning alternatives (Adebisi & Bello, 2011; Gill, Pande, & Malhotra, 2007).

Globally, only 60% of pregnancies are intended, while the rest unintended pregnancies represent ~200 millions of cases (Frohwrith, Blades, Moore, & Wurtz, 2016). In turn, undesired pregnancies contribute to 20 million unsafe cases of abortion and around 13% of maternal deaths annually (World Health Organization, 2016). Using effective methods of contraception decreased the ratio of unintended pregnancies by 70%, thus reducing about 25 million cases of abortions and subsequently the number of maternal deaths (Tsui, McDonald-Mosley, & Burke, 2010).

The currently used contraceptive methods for men and women do not sufficiently satisfy the variable personal requirements of couples through their reproductive life. Although increasing the availability of various choices to either partner can guarantee the spread of safe and effective methods that regulate fertility, the shortage of accessible male contraceptive means is a main obstacle toward involving men in family planning (Besculides & Laraque, 2004). Current research in the field of male contraceptives is greatly increasing the equitability of family planning between men and women (Lampiao, 2011).

Male contraceptive options involve non-hormonal or hormonal options. Non-hormonal options include vasectomy and condoms; however, their disadvantages are that vasectomy is mostly irreversible, and condom usage has poor long-term compliance and high failure rate (Amory, 2016; D'Anna et al., 2012). Hormonal-based methods rely on the suppression of the hypothalamic–pituitary–gonadal axis and spermatogenesis through the use exogenous testosterone, progestogens or gonadotropin-releasing hormone antagonists (Amory, Bremner, & Page, 2008; Chao, Page, & Anderson, 2014; Roth, Page, & Bremner, 2016). On the other hand, these methods have adverse side effects such as less libido, erectile dysfunction, mood and behavioral fluctuations, metabolic disturbances, venous thrombosis, migraine, myocardial infarction, high hemoglobin concentrations, and low high-density lipoprotein (HDL) cholesterol levels (Sabatini, Cagiano, & Rabe, 2011). Therefore, due to the wide range of side effects associated with these methods, the use of medicinal herbs is becoming more frequent among couples as a potent family-planning alternative (Ekor, 2014). Developing oral herbal contraceptives has many advantages including the familiarity of people with medicinal herbs, their few side effects, availability, affordable prices, well-documented effectiveness, and protection of privacy (Pradhan et al., 2013). The literature includes numerous research studies on plants with antifertility properties in male animal models. Medicinal plants that have shown antifertility activities include *Balanites roxburghii* (Rao, Shah, & Rajani, 1997a), *Phyllanthus amarus* (Rao, Shah, & Rajani, 1997b), *Carica papaya* seeds (Chinoy, D'Souza, & Padman, 1995), *Gossypium barbadense* (D'Anna et al., 2012), *Hibiscus rosa-sinensis*, *Embelia ribes*, *Daucus carota*, *Butea monosperma*, *Sapindus trifoliatis*, *Lonicera japonica*, *Lotus corniculatus*, and *Mentha arvensis* (Lampiao, 2011).

Loquat (*Eriobotrya japonica*) is an evergreen tree that belongs to the Rosaceae family and has been widely used in traditional medicine. It was first grown in subtropical regions of China and later introduced to other countries, including Australia, Turkey, Lebanon, Japan, India, Spain, and Brazil (Baljinder, Seena, Dharmendra, Vikas, & Bansal, 2010). Loquat leaves have been shown to considerably contain phenolic compounds, flavonoids, triterpenoid constituents (oleanolic acid, corosolic acid, ursolic acid, amygdalin and maslinic acid), triterpenic acids, amygdalins, carotenoids, and sesquiterpene glycosides (Fu et al., 2012; Zhang et al., 2015). Loquat leaves extract is reported to possess anti-inflammatory (Kim et al., 2012), antioxidant (Polat et al., 2010), hypoglycemic (Lü et al., 2009; Tanaka et al., 2008), hypocholesteremic (Kim et al., 2012), hepatoprotective (Yoshioka et al., 2010), nephroprotective (Bae et al., 2010), as well as chemoprotective properties against breast (Kim et al., 2011), cervical, lung (Kang et al., 2006), leukemia (Kikuchi et al., 2011), and salivary gland cancers (Alshaker et al., 2011).

In the light of these facts, the present study was undertaken to investigate the effect of *Eriobotrya japonica* (loquat) aqueous leaves extract on fertility and spermatogenesis. Sperm parameters, morphology of the seminiferous tubules, levels of major male sex hormones, mRNA levels of key enzymes involved in spermatogenesis as well as on glucose and cholesterol levels in Balb/c male mice were evaluated.

## 2. MATERIALS AND METHODS

### 2.1 Extract Preparation

The leaves of *Eriobotrya japonica* (loquat) were collected in May 2016 from Hasbaya (South Lebanon, Lebanon). This plant was identified and authenticated by Dr. Salwa Mahmoud Abdul Rahman, Department of Biological Science, Faculty of Science at Beirut Arab University, Lebanon. After collection, 5% aqueous solution was prepared with a sample to solvent ratio of 1:20 (w/v). Five grams of dried loquat leaves were soaked in 100 mL of boiled distilled water (100°C) and left to simmer at room temperature for 30 minutes. The mixture was then filtered, aliquoted, and stored at -20°C to be used on a weekly basis.

### 2.2 Experimental Animals

Healthy male albino mice Balb/c mice (6-8 weeks old) with approximate weight ranging between 25-30 g were obtained from Beirut Arab University animal facility. Groups of six mice were placed per cage in an air conditioned room with temperature of 22-25 °C and photoperiod of 12Light:12Dark. The mice were left to acclimate for one week before initiating the experiments. All animals were carefully maintained under standard animal house conditions with free access to standard pellet diet and water. Experimental procedures were carried out in accordance with the guidelines of the Institutional Review Board at Beirut Arab University with approval code number 2019A-0037-S-R-0296.

### 2.3 Animal's Treatment

Animals were randomly divided into 6 experimental groups of 6 mice each.

Group A: Control mice received water for 10 days. Group B: mice received 100 mg/Kg aqueous loquat leaf extract per day for 10 days. Group C: Control for 30 days, mice received water for 30 days. Group D: mice receiving 100 mg/Kg aqueous loquat leaf extract for 30 days. Group E: Control mice receiving water for 50 days. Group F: mice receiving 100 mg/Kg aqueous loquat leaf extract for 30 days and left for 20 days after treatment.

Animals received loquat extract orally by gavage (gavage needle: Popper®, 7F 05785 G). The selected dose of loquat extract was chosen on the basis of previously published reports (Jian et al., 2018; Liu, Zhang, Xu, & Li, 2016; Wu et al., 2018). Mice were sacrificed after treatment. Blood samples were withdrawn in Vacurate® Vacuum blood collection tubes and centrifuged at 2800 x g for 10 minutes. Serum was aliquoted and stored at -20°C. Testis and epididymis were removed; one testis was placed in formalin solution for histological analysis, while the second testis was placed in liquid nitrogen before being stored at -80°C for RT-PCR. Epididymis was removed for measuring sperm parameters.

### 2.4 Sperm Suspension Preparation for Assessing Motility, Viability & Count

The right cauda epididymis was removed and placed in 1 mL of phosphate buffered saline at 37°C. The tissue was gently agitated and used to check sperm motility, viability, and sperm count using a haemocytometer. A drop of the sperm suspension (10 µL) was transferred on a clean glass slide, and sperm motility was assessed via counting progressive and immotile spermatozoa. Another 10 µL of the suspension was mixed well with 7 µL trypan blue; dead and alive sperms were counted using light microscope. Dead sperms were stained blue. For sperm count, 0.5 mL of the suspension was mixed with 0.5 mL 10% formalin. Then 10 µL of the mixture was transferred to a haemocytometer and the sperm count was evaluated.

## 2.5 Histological Analysis

Testis samples placed in 10% formalin were sent to Specialized Medical Laboratories (Beirut, Lebanon) for histological analysis. Briefly, the tissues were first dehydrated in 70% to 90% graded ethanol, cleared in xylene and then embedded in paraffin wax. Five to six  $\mu\text{m}$  sections were cut using a microtome. The sections were then de-paraffinized in xylene, passed through 70% to 90% alcohol, and then stained with hematoxylin and eosin (H&E) for examination. The prepared slides were observed under light microscope (400X).

## 2.6 Hormonal and Biochemical Parameters Quantification

Testosterone and progesterone levels in plasma were quantitatively measured using ELISA kits (cat# AR E-8000 and AR E-8700 respectively, Labor Diagnostika Nord GmbH & Co.KG®, Germany) according to manufacturer's instructions. The level of prolactin was measured using ELISA kit and (cat# ab100736, Abcam®, USA) according to manufacturer's instructions. Blood glucose and cholesterol levels in plasma were quantitated using colorimetric assay kits (cat# 1001191 and 1001092, Spinreact®, Spain) according to manufacturer's recommendations. Experiments were carried out in triplicate.

## 2.7 Molecular Assays

### 2.7.1 RNA extraction and cDNA synthesis

Total RNA was purified from testis homogenate using Aurum™ total RNA mini kit (catalog # 732-6820, BIORAD®, USA) according to manufacturer's recommendation. RNA integrity was checked by agarose gel electrophoresis and ethidium bromide staining where the respective ribosomal RNAs appeared as two sharp bands corresponding to the 28S rRNA and 18 S rRNA. RNA was quantified spectrophotometrically at 260 nm. Reverse transcription was performed using QuantiTect® Reverse Transcription Kit (catalog # 205311, QIAGEN®, USA) according to manufacturer's recommendations. One microgram of RNA was reverse transcribed into cDNA using 20  $\mu\text{L}$  master mix consisting of 1  $\mu\text{L}$  Quantiscript Reverse Transcriptase (RT), 4  $\mu\text{L}$  Quantiscript RT Buffer, and 1  $\mu\text{L}$  RT Primer Mix at 42°C for 25 min, followed by enzymatic inactivation at 95°C for 3 min.

### 2.7.2 Real time polymerase chain reaction analysis

The expression levels of 3 $\beta$ -HSD, 17 $\beta$ -HSD, StAR, and CYP11A1 genes were quantified by quantitative real time polymerase chain reaction using QuantiFast® SYBR® Green PCR Kit (catalog # 204045, QIAGEN®, USA) according to manufacturer's instructions. The protocol involved two-step cycling. First, 10  $\mu\text{L}$  of 2x QuantiFast SYBR Green PCR Master Mix, 2  $\mu\text{L}$  of template cDNA, 2  $\mu\text{L}$  of each primer (1  $\mu\text{M}$ ), and 6  $\mu\text{L}$  RNase-free water were mixed in a final volume of 20  $\mu\text{L}$  PCR reaction mixture. Cycling involved a denaturation step at 95°C for 5 min, followed by 45 cycles each consisting of a denaturation at 95 °C for 10 s and annealing/extension at 57 °C for 30s. Primer sets for the PCR amplification were designed with the Primer3- Blast software (NCBI, USA), and were all purchased from BIO-RAD® (USA).

Sequences of primers were as follows: Glyceraldehyde-3 phosphate dehydrogenase (GAPDH) 5'-TGGTGCTCAGTG TAGCCCAG-3' (forward) and 5'-GGACCTGACCTGCCGTCTAG-3'(reverse) (Basma et al., 2016), Steroidogenic acute regulatory protein (StAR) 5'-ATCATTGTGCCGACTTCCCTAC-3'(forward) and 5'-ACCAGGTTAGCCTCAGTATTAGA-3'(reverse) (Hu et al., 2013), 3 $\beta$ -Hydroxysteroid dehydrogenase (3 $\beta$ -HSD)5'-AGCATCCAGACACTCTCATC-3' (forward) and 5'-GGAGCTGGTATGATATAGGGTA-3' (reverse), 17 $\beta$ -HSD 5'-TGCTCATTACCGTTGCTCC-3'(forward) and 5'-ATTGCGGGACCTGAAGGCA-3' (reverse) (Enzy Jinn et al., 2015), and Cholesterol side-chain cleavage enzyme (CYP11A1) 5'-AGGATGCGTCGATACTCTTCTCA-3' (forward) and 5'-CCATCAGATGCAGAGTTTCAA-3' (reverse) (Yang et al., 2018).

Experiments were carried out in triplicate. Negative controls containing RNase-free water instead of template cDNA for each primer sets primer were run to ensure that the absence of non-specific PCR products. The gene expression levels were measured by normalizing each gene's threshold cycle (Ct) to the Ct of glyceraldehyde-3 phosphate dehydrogenase (GAPDH) gene.  $\Delta$ Ct value was determined as the difference between the Ct of the gene of interest and the Ct of GAPDH. Relative gene expression was calculated using the  $2^{-\Delta\Delta CT}$  gene dosage ratio formula (GDR) where:

$$\Delta\Delta Ct = (\text{mCt gene of interest} - \text{mCt GAPDH}) \text{ test sample} - (\text{mCt gene of interest} - \text{mCt GAPDH}) \text{ control sample.}$$

## 2.8 Statistical Analysis

All statistical analyses were performed using Microsoft Excel, presented as mean with standard deviations. Statistical significance was tested using t-test and One-way ANOVA. Graphs were drawn using GraphPad prism software (Version 6). Statistical significance was reported as \* for *P*-value between 0.05 and 0.01, \*\* for *P*-value between 0.01 and 0.001, \*\*\* for *P*-value between 0.001 and 0.0001, and \*\*\*\* for *P*-value less than 0.0001.

## 3. RESULTS

### 3.1 Semen Analysis: Sperm Motility, Viability and Count

As illustrated in Fig. 1A, mice receiving loquat extract for 10 and 30 days (Groups B & D) showed a significant decrease in sperm motility by 44.5% and 68.4% respectively ( $P < 0.0001$ ) compared to their controls (Groups A & C). Besides, upon treating mice with loquat extract for 30 days then leaving them untreated for 20 days (Group E), a significant decrease of 10% ( $P < 0.05$ ) in sperm motility was observed, compared to the control (Group F). Moreover, after 20 days of loquat treatment withdrawal (Group F), there was a significant restoration in the motility of sperms by 65% ( $P < 0.0001$ ), compared to loquat treatment for 30 days (Group D).

Concerning sperm viability results which are shown in Fig. 1B, loquat treatment for 10 and 30 days (Groups B & D) caused significant decreases in sperm viability by 28.4% and 67.2% ( $P < 0.0001$ ) respectively, compared to their controls (Groups A & C). Additionally, mice treated with loquat extract for 30 days and left 20 days untreated (Group F) showed a significant decrease in their sperm viability by 14.2% ( $P < 0.05$ ) compared to their control (Group E). More importantly, a withdrawal period of 20 days (Group F), showed a significant restoration in the viability of sperms by 63% ( $P < 0.0001$ ), compared to loquat treatment for 30 days (Group D).

As for sperm count results which are shown in Fig. 1C, mice receiving loquat extract for 10 and 30 days (Groups B & D) showed a significant decrease in their sperm count by  $6.6 \pm 0.54$  and  $8.15 \pm 0.5$  corresponding to 66% and 86.7% decrease ( $P < 0.0001$ ) respectively compared to their controls (Groups A & C). On the other hand, after 20 days of loquat treatment withdrawal (Group F), the sperm count was restored by 85% ( $P < 0.0001$ ), compared to loquat treatment for 30 days (Group D).

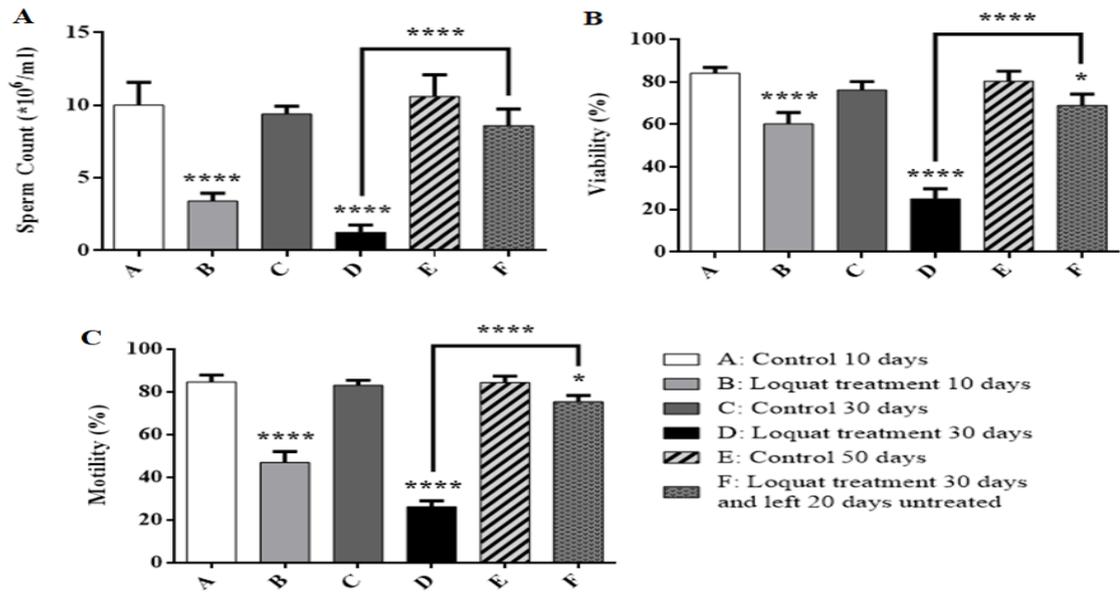


Fig.1 Effect of loquat leaves extract on sperm count (A), viability (B) and motility (C) in Balb/c mice. All bars of sperm counts are represented as means of different mice  $\pm$  SD (n = 6). Asterisks on bars represent significance relative to the control and those drawn up wards represent inter-categorical statistical significance (each category with the preceding one). (\*) and (\*\*\*) correspond to  $P < 0.05$  and  $P < 0.0001$ , respectively.

### 3.2 Histopathology

As shown in Fig. 2, cross sections of seminiferous tubules from mice treated with loquat leaves extract for 10 day (panel B) and 30 days (panel C) showed arrest in spermatogenesis, decrease in the number of spermatozoa and in the diameter of seminiferous tubules, and destruction of germinal epithelium, compared to sections from normal control mice (panel A). These histopathological changes were more pronounced at 30 days than those at 10 days. However, tubular sections from mice treated with loquat leaves and left 20 days without any treatment showed a restoration in the number of spermatozoa and germinal epithelium (panel D).

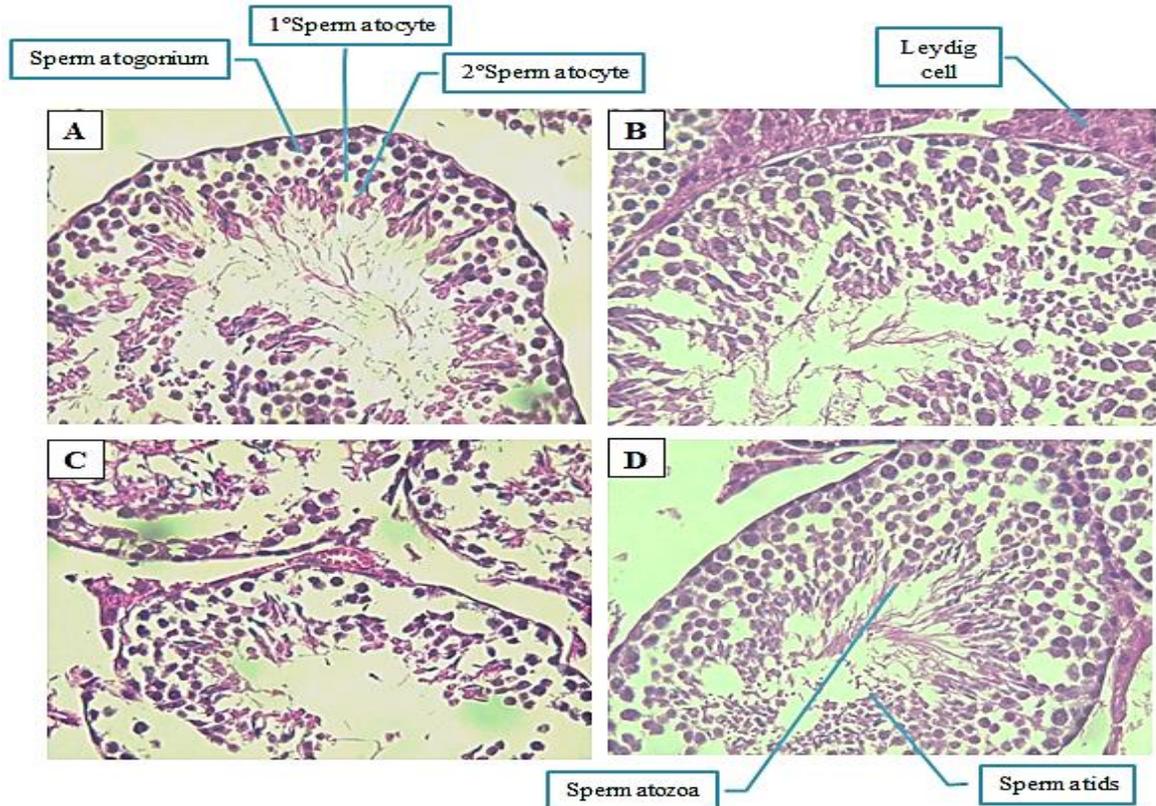


Fig.2: Histopathological changes in the seminiferous tubules of control and loquat extract treated mice groups (400X). (A) Cross sections of seminiferous tubules from normal mice. (B) Tubular section from mice treated with loquat extract for 10 days. (C) Tubular section from mice treated with loquat extract for 30 days. (D) Tubular section from mice treated with loquat extract for 30 days and then left untreated for 20 days.

### 3.3 Hormonal, Blood Glucose and Cholesterol Levels

Compared to the control groups (Group A & C), the level of testosterone was significantly reduced by 48.8% and 87.3% ( $P < 0.01$  and  $< 0.0001$ ) upon loquat treatment for 10 and 30 days respectively (Groups B & D) (Fig. 3A). More importantly, testosterone level was significantly restored by 79% upon 20 days of loquat treatment withdrawal (Group F) ( $P < 0.01$ ), compared to loquat treatment for 30 days (Group D).

As for progesterone level which is shown in figure 3B, loquat treatment extract for 30 days (Group F) induced a significant reduction in the level of progesterone by 88.3% ( $P < 0.0001$ ), compared to the normal control (Group E). Moreover, the level of progesterone was significantly restored by 82% upon 20 days of loquat treatment withdrawal (Group F) ( $P < 0.01$ ), compared to loquat treatment for 30 days (Group D).

Findings regarding prolactin level (Fig. 3C) showed that loquat treatment for 10 and 30 days (Groups B & D) led to a significant increase in prolactin level by 66.8% and 67% ( $P < 0.0001$ ), respectively compared to their controls (Groups A & C). Moreover, after 20 days of loquat treatment withdrawal (Group F), the level of prolactin was significantly restored by 47% ( $P < 0.0001$ ), compared to loquat treatment for 30 days (Group D).

Moreover, Fig. 3D & E showed that neither blood glucose nor cholesterol levels were affected by loquat extract treatment as no significant differences were observed upon comparing their levels among different experimental groups.

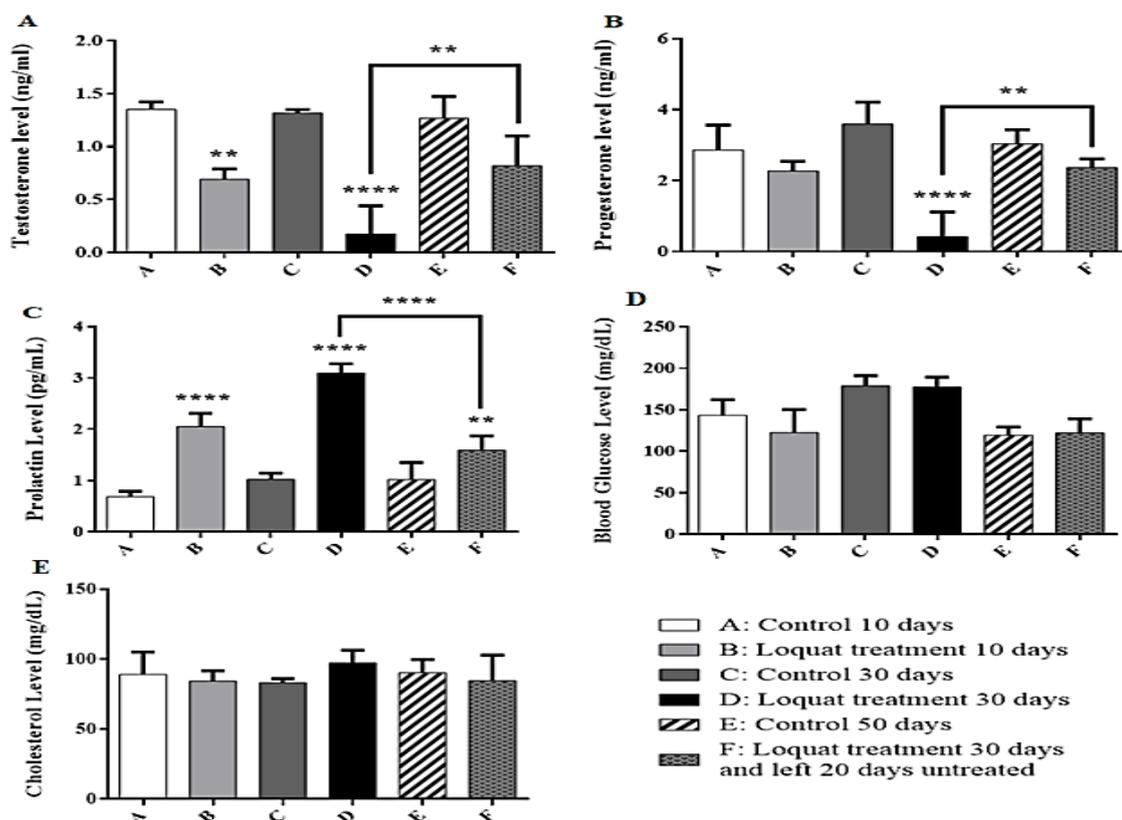


Fig.3: Effect of loquat leaves extract on testosterone (A), progesterone (B), prolactin (C), blood glucose (D), and cholesterol (E) levels in Balb/c mice. All bars are represented as means  $\pm$  SD (n = 6). Asterisks on bars represent significance relative to the control and those drawn up wards represent inter-categorical statistical significance (each category with the preceding one). (\*\*) and (\*\*\*\*) correspond to  $P < 0.01$  and  $P < 0.0001$ , respectively.

### 3.4 Gene Expression

As shown in Fig. 4A, the expression level of the StAR gene in mice receiving loquat extract for 10 and 30 days (Groups B & D) was significantly decreased by 2.3 and 9 folds ( $P < 0.0001$ ) respectively, compared to their normal control (Groups A & C). Furthermore, after 20 days of loquat treatment withdrawal (Group F), the expression level of StAR gene was restored by 8.7 folds (p-value  $< 0.0001$ ), compared to loquat treatment for 30 days (Group D).

The expression level of the CYP11A1 gene in mice receiving loquat extract for 10 and 30 days (Groups B & D) was significantly decreased by 2.6 and 4 folds ( $P < 0.0001$ ) respectively compared to their control (Groups A & C) (Fig. 4B). It was also shown that a withdrawal period of 20 days (Group F) was able to significantly restore the expression level of CYP11A1 gene by 3.7 folds ( $P < 0.0001$ ), compared to loquat treatment for 30 days (Group D).

As shown in Fig. 4C, the expression level of the  $3\beta$ HSD gene in mice receiving loquat extract for 30 days (Group D) was significantly decreased by 8.16 folds ( $P < 0.001$ ) compared to their normal control (Group C). It was also noted that loquat treatment withdrawal (Group F) was able to restore the level of  $3\beta$ HSD gene by 10.16 folds ( $P < 0.0001$ ) compared to its treatment for 30 days (Group D).

Regarding the expression level of  $17\beta$ HSD gene which is shown in Fig. 4D, loquat treatment for 10 and 30 days (Groups B & D) caused significant downregulations in the level of this gene by 3.15 and 5 folds respectively ( $P < 0.01$  and  $< 0.0001$ ), compared to their controls (Groups A & C). Additionally, a withdrawal period of 20 days (Group F) was able to significantly restore expression level of  $17\beta$ HSD gene by 5.5 folds (p-value  $< 0.0001$ ), compared to loquat treatment for 30 days (Group D).

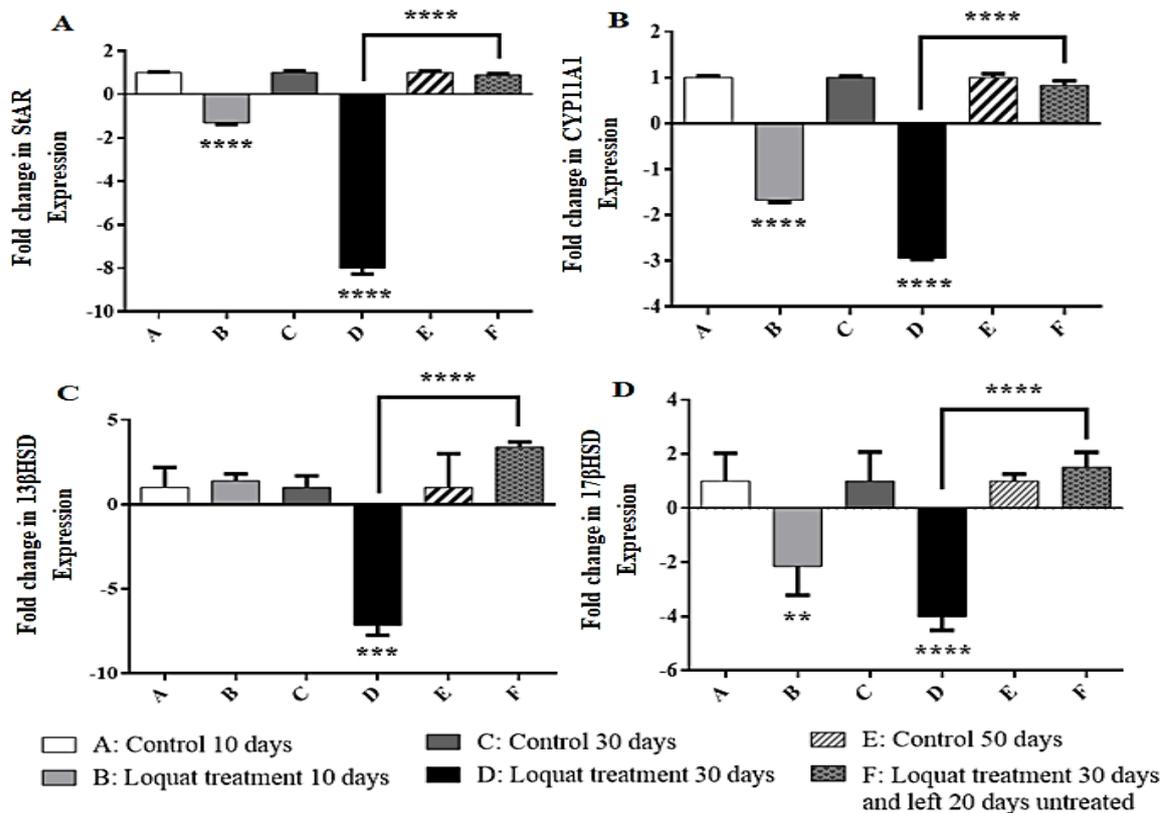


Fig.4: Effect of loquat extract on the expression of StAR (A), CYP11A1 (B), 3βHSD (C), and 17βHSD (D) genes. Expression levels of treated samples and controls were normalized to their respective GAPDH, and the fold change was determined relative to the control. All bars represent mean of three determinations  $\pm$  SEM. (\*\*), (\*\*\*) and (\*\*\*\*) on bars and on lines drawn upwards represent inter-categorical statistical significance corresponding to  $P < 0.05$ ,  $< 0.01$  and  $< 0.001$ .

#### 4. DISCUSSION

This study evaluates the anti-fertility activity of *Eriobotrya japonica* (loquat) aqueous leaves extract on the basis of its effect on the sperm parameters, histo-morphological examination of the seminiferous tubules, levels of major male sex hormones, mRNA levels of some enzymes involved in spermatogenesis as well as on glucose and cholesterol levels in male mice.

The present investigation showed that *Eriobotrya japonica* aqueous extract administered for 10 and 30 days exerted antifertility effects in male Balb/c mice through significant reductions in sperm motility, viability and count. However, a withdrawal period of 20 days restored these semen parameters indicating a reversible effect of loquat extract on sperm infertility. Sperm motility, viability and count are the most sensitive tests for spermatogenesis and they are highly correlated with fertility. For example, low sperm count and inactive spermatozoa have been widely responsible for male infertility (Braide, Agube, Essien, & Udoh, 2003; Joshi & Bansal, 2012; Udoh, Udoh, & Umon, 2005). Also, reduced sperm count in testes and epididymis might have resulted from either an inhibition of meiotic division of spermatocytes or a suppressive effect of the extract on spermatogenesis (Akbarsha et al., 2001; Obianime & Aprioku, 2009). On the other hand, altered sperm motility and viability are mainly attributed to disturbances in epididymal function (Gupta, 2012; Rajalakshmi, 1992).

Histopathologically, loquat extract treatments for 10 and 30 days caused a serious arrest in spermatogenesis which resulted from an evident decrease in the number of spermatozoa and diameter of seminiferous tubules. Also, a destruction of the testicular architecture was marked by distorted germinal epithelium and reduced spermatogenesis. Partial reversibility of such deteriorated histoarchitecture was noted after a withdrawal period of 20 days.

These histomorphological findings along with the semen analysis results confirm the ability of loquat aqueous extract to impair spermatogenesis in male mice.

Semen analysis and histopathological analysis revealed that loquat extract impairs the epididymal function which is commonly known to be dependent on the level of androgens (Bagaria et al., 2006). Besides, low sperm motility and counts suggest an undersupply of testosterone to the epididymis, thus leading to impaired epididymal function (Bhutia et al., 2008). Therefore, the level of testosterone was assessed to explain the exact mechanism exerted by loquat on epididymal impairment.

At the hormonal level, a significant decrease in testosterone level was observed at 10 and 30 days of loquat extract administration; however, there was a restoration in the level of testosterone after 20 days of withdrawal of treatment. Low testosterone levels are commonly known to impair spermatogenesis and thus lead to infertility (Visser & Weber-Schöndorfer, 2015). Similar results were observed in studies using *Quassia amara* (Raji & Bolarinwa, 1997), *Carica papaya* (Udoh & Kehinde, 1999; Udoh et al., 2005), *Mucuna urens* (Udoh & Ekiyepong, 2001), *Polygonum hydropiper*, *Citrus limonum*, *Piper nigrum*, and *Juniperis communis* (Daniyal & Akram, 2015).

Such decrease in testosterone level is possibly due to some common mechanisms that have been shown to be inhibited by plant extracts including the gonadotropin secretion, steroidogenesis, or decreased progesterone levels. Our findings indicate that loquat extract exerts inhibitory effects against progesterone, a hormone that has been identified to influence spermiogenesis, sperm capacitation, acrosome reaction as well as testosterone biosynthesis (Oettel & Mukhopadhyay, 2004). On the other hand, loquat treatment for 10 days and 30 days significantly increased the levels of prolactin. Similar to other parameters, the level of prolactin was restored to normal after 20 days of loquat treatment withdrawal. These findings indicate that loquat treatment induces hyperprolactinemia in male mice. Although the exact functions of prolactin in male reproduction are not well-established yet, this hormone has been associated with antifertility. High prolactin levels were shown to reduce testosterone synthesis either through increased secretion of adrenal corticoids or through inhibition of GnRH release from the hypothalamus (Gill-Sharma, 2009). Both high prolactin and low testosterone levels are clear evidences of the antifertility effects of loquat leaves extract.

As for steroidogenesis, the gene expression levels of StAR protein and three critical enzymes: CYP11A1, 3 $\beta$ HSD and 17 $\beta$ HSD, were assessed. Loquat extract treatment for 10 and 30 days significantly downregulated the expression level of the StAR and CYP11A1 genes; while after 20 days of its withdrawal, the expression level of these genes was restored to normal levels. These findings indicate that loquat extract exerts reversible inhibitory effects on steroidogenesis at two rate-limiting steps: (1) the transport of free cholesterol into the inner mitochondrial membrane to initiate steroid biosynthesis which is mediated by StAR, and (2) the conversion of cholesterol to pregnenolone in the mitochondria by CYP11A1 (Baljinder et al., 2010; Clark, 2016). Likewise, the mRNA level of the 3 $\beta$ HSD and 17 $\beta$ HSD were shown to be significantly downregulated upon loquat treatment of mice for 30 days. Since 3 $\beta$ HSD is involved in the synthesis of progesterone from pregnenolone, reductions in its level lead to low progesterone levels and infertility (Rasmussen, Ekstrand, & Zamaratskaia, 2013). Also, 17 $\beta$ HSD enzyme catalyzes the conversion of androstenedione into testosterone, thus its downregulation leads to reduced testosterone levels and subsequently impaired spermatogenesis (Asare et al., 2013). Our analysis of mRNA expression levels of selected genes involved in steroidogenesis showed major dysregulations in the gene levels of CYP11A1, 3 $\beta$ HSD and 17 $\beta$ HSD. In turn, these dysregulation could be responsible for the observed hormonal imbalance and diminished levels of testosterone and progesterone in male mice. Our molecular results suggest that loquat extract targets the steroidogenesis pathway.

To further investigate whether loquat extract exerts antispermatogenic effects indirectly through altering glucose and cholesterol levels, we determined their levels in the serum of treated mice. Several studies showed that some herbs exert antispermatogenic activities through inducing changes in cholesterol and glucose levels which are normally essential for steroidogenesis, maintenance of sperm motility, and fertilization ability of mature sperms and seminal plasma (Muroso, Derk, & Akgul, 2006; Vijaykumar, Sangamma, Sharanabasappa, & Patil, 2004).

In addition, our assumption was based on previously reported hypoglycemic and hypocholesteremic activities of loquat extract in animal models with induced diabetes (Tanaka et al., 2008) and hypercholesteremia (Kim et al., 2012). However, this was not the case with our study where loquat treatment did not induce any changes in the levels of glucose and cholesterol. This greatly proves the direct antifertility effects of loquat extract which involve distortions in the epididymal histoarchitecture, blockage of spermatogenesis and inhibition of steroidogenesis.

As revealed by our study, the antifertility activity of loquat extract is significant. This activity may be due to the presence of triterpenes and flavonoids in loquat leaves which are well-documented for their ability to suppress spermatogenesis (Chaudhary et al., 2007). The anti-fertility effects of triterpenes are attributed to their disrupting actions on the surface of sperm cells which reduce sperm motility and inhibitory actions against major enzymes involved in spermatogenesis such as hyaluronidase and acrosin (Farnsworth & Waller, 1982). A study by Obianime, Aprioku, and Esomonu (2010) showed that the antifertility effects of *Ocimum gratissimum* were attributed to the high content of these bioactive compounds. More importantly, several studies in the literature have indicated that oleanolic acid and ursolic acid – two major triterpenes found in loquat leaves – are potential antifertility agents (Ampofo-Yeboah et al., 2013; Chattopadhyay, Dungdung, Mandal, & Majumder, 2005; Rajasekaran et al., 1988). These triterpenes have exerted antifertility effects in several species whereby they suppress the attainment of puberty, gonad function and gamete production (Srinivasulu & Changamma, 2017).

## 5. CONCLUSIONS

The present study revealed that loquat is an effective male contraceptive herb. Its administration at a dose of 100 mg/kg body weight over 30 days was more potent than its administration for 10 days. Moreover, a withdrawal period of 20 days showed significant restorations of the normal structure and functions of epididymis. In fact, loquat extract mediates its antifertility effects through direct impairment of steroidogenesis and spermatogenesis. It can be considered as a promising ideal and reversible male contraceptive. Further research is needed to overcome the limitations of this study which include the use of a single dose of aqueous loquat leaf extract, and to assess the levels of hypothalamic and pituitary hormones. Additional fertility test can be performed where we can repeat this experiment but with additionally mating every male with a female partner in order to study the effect of this extract on pregnancy rate and fetuses. Moreover, the effect of this extract on female fertility can be examined. Further investigation can be done to evaluate the effects of singular constituents and bioactive compounds found in loquat leaves extract. Also, our data open up future work for the assessment of the effect of loquat extract on the genetic material and chromosomes of sperm in terms of structural abnormalities, DNA fragmentation, and single strand breakage.”

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