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Paper

Determination of possible contraceptive potential of methanolic leaf extract of *Mentha longifolia* L. in adult male rats: a biochemical and histological study

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Mentha longifolia L (ML) is locally used as an herbal contraceptive. We aim to assess the toxicity profile and validate the antifertility effects of its leaves in male rats. Adult male Sprague Dawley rats ($n = 10$ animals/group) were divided into four groups. The Control group received .9% saline, while groups II, III, and IV received 50, 75, and 100 mg/kg/day ML doses for 28 days. ML induced a dose-dependent decrease in percent fertility as well as the number of pups born in ML preexposed pairs, with maximum change observed in the ML3 group (60%, 3.75 ± 1.25) as compared to control (100%, 8.75 ± .48) group. A significant ($P < .01$) decrease in the rate of daily sperm production was observed in the ML3 group (.86 ± .16) in contrast to the control (2.93 ± .05). The levels of catalase and superoxide dismutase declined significantly in treatment groups (ML1-$P < .001$, ML2-$P < .05$, and ML3-$P < .01$). A significant increase in the production of reactive oxygen species ($P < .001$) was found in all treatment groups. The concentrations of LH ($P < .001$), FSH ($P < .001$), and testosterone ($P < .001$) were significantly reduced in a dose-dependent manner among ML-treated groups. Decreases in spermatogonial populations, mature spermatids, seminiferous tubule diameter, lumen diameter, and epithelial height were noticed at higher doses. The reversibility study depicted a reduction in both pregnancy outcomes and litter size. Besides contraceptive effects, *M. longifolia* L usage is associated with oxidative stress; therefore, it is important to consume this herb appropriately and its excessive usage should be prohibited.

Key words: *Mentha longifolia*; *herbal contraceptive*; *hormonal analysis*; *sperm motility*; *tissue histology*.

Background

Population explosion is one of the major contributors to environmental degradation and human suffering throughout the world.[1] The current statistics have shown that the world's population has increased to 7 billion. Every year, it is further growing by 80 million and is believed to reach 9–10 billion by the year 2050. One of the major contributing factors responsible for overpopulation is unattended and unplanned pregnancies. According to different epidemiological studies, 41% of worldwide pregnancies are undesired, 20% of which usually result in abortions and 47 000 maternal deaths, about unsafe abortions, emphasizing the need for awareness and practicing better contraceptive measures.[2,3] The word contraception refers to the 'intentional prevention of conception employing various sources such as devices, drugs, and surgical procedures'. The use of contraceptive practices is known to have many societal benefits such as reduction of maternal and infant mortality, reduced abortion rates, improvement in infant and maternal health, and more sustainable use of resources due to reduced population growth.[4–6] Multiple family planning methods include hormonal, physical barrier methods, sterilization, and behavioral methods. Almost 90% of the contraceptive users are women using either contraceptive patches, implants, injectables, contraceptive vaginal rings, or intrauterine methods.[7,8] However, the progress in the field of male contraceptives is still slow and limited.[9–11] Other chemical agents and sterility having the potential of non-hormonal male contraceptives prevent spermatogenesis and sperm motility, though these chemical or hormonal approaches have side effects ranging from obesity to carcinomas.[12–14] Due to the least toxic effects associated with the use of natural medicinal plants, recently, plants and their products have caught the attention of many scientists as a primary source of naturally occurring fertility regulating agents.[15]

Since olden times, plants have been rendered useful curatives against numerous diseases due to their pharmacological properties.[16] Several plant products possess antifertility activities and may be developed into contraceptives for both males and

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females.[8,17] Pakistan has numerous topographical and ecological zones as well as distinct biodiversity of plants, that traditionally also serves as medicine, food, and shelter to local communities.[18,19] Some of the most common plants used as male contraceptives in Pakistan include *Asplenium dalhousiae* Hook.,[15] *Chenopodium ambrosoides* Hook,[20] *Hedra nepalensis*[21], *Jatropha variegate*[22], *Adiantum venustum* D. Don, *Rumex hastatus* D. Don,[23] *Jatropha circas* Linn.,[24] *Chenopodium album* L,[25] *Mentha arvensis L.*[26], *M. longifolia* (L.) L.[23], and many more. For many centuries, plant preparation has been used to prevent conception.[27] These herbal plants possessing antifertility attributes such as estrogenic, abortifacient, anti-implantation, ecbolic, and spermicidal actions can be developed into both male and female contraceptives.[8] Such local plants are rich in phytoconstituents such as flavonoids, terpenes, tannins, diterpenoid lactones, and quinones that impair male fertility by mechanisms such as interfering with spermatogenesis progression, testosterone biosynthesis, or by apoptosis of spermatogenic cells in different animal models (dogs, rats, mice, monkey, and human), eliciting the search for an orally active and safe plant preparation for fertility regulation.[8,28–31]

The genus *Mentha* of the family *Lamiaceae* is a large plant genus, comprising approximately 25–30 reported species, having a sub-cosmopolitan distribution.[32] Plants of this genus are fast-growing, perennial, rarely annual herbs of aromatic fragrance with many hybrids and special selections most of which are found in Asia, Africa, Europe, Australia, and North America.[33] *M. longifolia* is a fast-growing herb with a peppermint-scented aroma.[34] Diverse biological functions have been reported for *M. longifolia* species such as antimycotic, antibacterial, anthelmintic, and insecticidal potencies.[35–39] The plant has been reported to provide a remedy against gastrointestinal infections, respiratory ailments, headaches, diarrhea, dysentery, cardiac diseases, high blood pressure, and pain in muscles and joints.[40,41] The essential oils of *M. longifolia* mainly contain luteolin-7-*O*-glycoside, luteolin-7, 3′-*O*-di-glycoside, quercetin-3-O-glycoside, apigenin, and kaempferol-3-*O*-glycoside all of which are a bio-flavonoids.[42] Flavonoids have been identified for their antifertility effect by various researchers.[43,44] Flavonoids possess anti-androgenic properties, producing toxic effects on the male reproductive organs in rats and dogs.[45,46] Previously, Zaman and colleagues reported that the whole plant of *M. longifolia* is used by the local people of Pakistan for antifertility purposes in men.[23] The interview with local community members further suggested that the whole plant is dried and plant powdered is used at night time by male partners for getting antifertility effects. A single report indicated that petroleum ether extract of the leaves of *M. arvensis* L., at the doses of 10 and 20 mg/mouse per day for 20, 40, and 60 days induced a reduction in the number of offspring of the treated male mated with normal females.[26] An earlier study from Pakistan has shown that the administration of *M. longifolia* leaves induces antifertility actions in women by disturbing the menstrual cycle, while no actions on the male reproductive system were reported.[47] Previously, in vitro studies performed by Abbas et al. (2019) showed that leaf extract of *M. longifolia* is known to possess antifertility effects by inducing oxidative stress.[48] However, besides that, not a single report is found regarding the antifertility effect of *M. longifolia* preparation in the male rat in vivo. Hence, the present study was designed to validate the possible antifertility effects of methanolic leaf extract of *M. longifolia* on the reproductive system of male rats by assessing sperm parameters, histological, and biochemical analysis.

Material and Methods

Plant material

The leaf samples of *M. longifolia*, commonly known as "wild mint" or "horse mint," were used for the present study. The name of the plant, *M. longifolia,* has been confirmed with The Plant List (http://www.theplantlist.org) which can be accessed at Royal Botanic Gardens Kew Science (http://apps.kew.org/wcsp/namedetail.do?name_id=124916). The samples were obtained from agricultural fields of Shangla, which is located at 34° 31′ to 33° 08′ North and 72° 33′ to 73° 01′ East on the globe. The samples were scientifically identified by Dr Mushtaq Ahmad, Professor, Department of Plant Sciences, QAU, and a sample was also preserved in the herbarium of Plant Sciences department, QAU, Islamabad under the accession number 69602.

Plant extract preparation

Leaves of *M. longifolia* were separated, air-dried, weighed, and ground in a Waring blender and then sieved. The methanol extract was prepared by keeping the leaf powder in methanol having a % w/w of 10.00. The extract was filtered through Whatman™ Qualitative Filter Paper Standard Grade, a circle whose pore size was 90 mm (GE Healthcare 1001090 Whatman™ Qualitative Filter Paper: Grade 1 Circles, Thermo Fisher Scientific) and subsequently concentrated in a rotary evaporator (Model: Hei-VAP Heidolph, Germany).[49]

Animals

Forty male Sprague Dawley rats *(Rattus norvegicus)* of pubertal ages with an average body weight of 200 ± 10 g were obtained from the primate facility of the Department of Zoology, Quaid-i-Azam University and maintained following the recommendations of the guidelines for the care and use of laboratory animals (NIH publications no. 80–23; 1996). The animals were housed in separate stainless steel cages in a well-ventilated room under standard conditions of 12/12 hour dark/light cycle and 25 °C room temperature during the experiment. All animals were fed with standard laboratory food and water ad libitum. Animal handling and experimental protocols were assessed and approved (BAS255) by the Department of Zoology ethical committee. The study is reported following ARRIVE guidelines.

Acute toxicity study

An acute oral toxicity study was performed according to OECD protocol 423 (acute toxicity class method). Fifteen adult Sprague Dawley rats (80–90 days old) were selected randomly and distributed into five groups each having three animals. The control group was given 1% methanol. While the animals of the first group were administered with a single dose of 5 mg/kg body weight of plant extract via oral gavage and animals were kept under observation for 14 days. A similar procedure was performed with higher doses of 75, 100, 300, and 2000 mg/kg of plant extract. If none of the animals showed mortality, then the dose was assigned as nontoxic. Behavioral changes and signs of mortality and morbidity were noted till the completion of the experiment on day 14.

Experimental design

Animals were randomly assigned into four groups (n = 10 /group). The control group was treated with .9% saline.

ML1 group received *M. longifolia* leaf extract 50 mg/kg b.w orally for 4 weeks.

ML2 group received *M. longifolia* leaf extract 75 mg/kg b.w orally for 4 weeks.
ML3 group received *M. longifolia* leaf extract 100 mg/kg b.w orally for 4 weeks.
The doses of the plant extract were selected after pilot studies.

Animal dissection and tissue collection

Following 28 days of treatment, animals were given anesthesia, provided via intraperitoneal injection of a ketamine/xylazine mixture (75/2.5 mg/kg, respectively).[50] Blood samples were obtained from each animal through the cardiac puncture, kept in heparinized tubes, and then centrifuged at 3000 rpm for 15 min. The separated plasma was stored at −20 °C for hormonal analysis. Both testes and epididymis were removed from each animal. One of the testes was washed in ice-cold saline and preserved in liquid nitrogen (−70 °C) for the daily sperm production analysis and antioxidant assay. A part of epididymis was washed, weighed, and minced for the processing of other sperm parameters such as sperm motility and viability. Whereas other testicular tissues and part of epididymis were fixed in 10% formalin for histological studies.

Evaluation of fertility

For the assessment of fertility potential, the remaining four males from each group were withdrawn from treatment and caged separately with untreated fertile females (ratio 1:2) to breed for the next 60 days. Mating success was assessed by the presence of spermatozoa in the vaginal smear collected for three consecutive days. Hematoxylin staining was performed for spermatozoa assessment through microscopic examination (Nikon, 187842, Japan). The mated female rats were observed as pregnant and allowed to litter. The number of females conceived and their litter size was recorded using the following formula

$$\text{Fertility (\%)} = \{(\text{number of pups in the male's litters})/(\text{total number of pups}) * 100\}$$

Effect on sperm motility[51,52]

For the assessment of sperm motility, a small portion of cauda epididymis was cut and crushed in 1 ml of 37 °C normal saline solution to make a homogeneous mixture. About, 10 μl of homogenate sample was placed on a pre-warmed slide, a minimum of 10 fields were observed and 100 spermatozoa were calculated via a high-power microscope at 40× magnification.[53]

Evaluation of sperm viability

The Eosin-nigrosin staining test was used for the assessment of sperm viability. Semen samples were mixed with 25 μl of dye (eosin-nigrosin). The 15 μl droplets of this mixture were placed on a glass slide for smear preparation and air-dried at room temperature, and later examined under a light microscope at 40× resolution. The alive spermatozoa remained unstained (white) whereas dead cells were stained red. The percentage of dead and alive spermatozoa was calculated by counting at least 100 sperm cells.[54]

Daily sperm production

All frozen testicular samples were thawed at room temperature before homogenization. Tunica albuginea was removed; parenchyma was weighed and homogenized in 3 ml of .9% sodium chloride (NaCl) solution containing .5% Triton X-100 for 30 s.[55] This homogenate was diluted up to 5-folds. About, 20 μl of this homogenate sample was deposited on the Neubauer chamber to count late spermatids under the microscope at 40× magnification as previously given {David, 2019 #32}. Three readings were taken for each sample to calculate the average number of spermatids. The total number of all the spermatids per testis was assessed and divided by testicular weight to find the number of spermatids per gram testis i.e. the capability of sperm production. In the homogenate, spermatids were defiant to homogenization (nineteenth stage of spermiogenesis). The number of spermatids that were resistant to homogenization was divided by 6.3, which represents the number of days these spermatids reside in seminiferous epithelium calculated by using the formula:

$$Y = \text{no. of sperms } (N) \times 25 \times 1000 \times 5 \times 25 = Y/6.3$$

Biochemical analysis

Earlier frozen testicular tissues (90 mg) were thawed and homogenized in 3 ml of phosphate buffer (pH 7.4). This homogenate was then centrifuged at 12 000 rpm for 30 minutes at 4 °C. The supernatant has collected the determination of the antioxidant status of testicular tissue. The activity of antioxidant enzymes including catalase, superoxide dismutase, peroxidase, as well as stress biomarkers such as reactive oxygen species and thiobarbituric acid reactive species was estimated in testicular using Bio-Rad SmartSpec™ Plus Spectrophotometer following published protocols.[53,56]

Hormonal analysis

Plasma luteinizing hormone (LH) and follicle-stimulating hormone (FSH) concentrations were measured quantitatively by enzyme-linked immunosorbent assay kit (Reddot biotech, INC) with a microplate reader (Plato's R 496; AMP Diagnostics). Plasma testosterone concentrations were quantified by using enzyme immunoassay (EIA) kits (Biocheck Inc., USA). Results were expressed as ng/ml.

Tissue histology

Formalin (10%)-fixed testicular and epididymal tissues were dehydrated in ascending grades of alcohol, followed by embedding in paraffin. The embedded tissues were mounted on wooden blocks and 7 μm sections were cut using a microtome (Thermo, Shandon finesse 325, UK). The long ribbons containing tissues were stretched and fixed in previously prepared clean albumenized glass slides on Fischer to slide warmer at 60 °C. The slides were placed in an incubator for the completion of stretching overnight. On the following day, slides were stained and paramount with DPX.

Light microscopic study

Tissue sections were examined under the light microscope (Nikon, 187842, Japan) at 40× magnification. For morphometric analysis, the seminiferous tubule diameter and seminiferous tubule epithelial height of testicular tissue were measured by using Image J2X software (National Institute of Health, Bethesda, MD, USA).[50] Celebrated image in micrometer was used for setting scale and conversion of values from pixels to micrometers. The area of the seminiferous tubule and interstitial space was noted

Table 1. Effect of *M. longifolia* leaf extract on mean ± SEM body and reproductive organs weight (g) of control and treated adult male rats.

	Treatments			
Parameters	**Control**	**ML1 (50 mg/kg)**	**ML2 (75 mg/kg)**	**ML3 (100 mg/kg)**
Body weight gain (g)	239.0 ± 18.41	231.0 ± 5.33	263.0 ± 7.23	243.8 ± 18.66
Right testicular weight (g)	1.32 ± .02	1.06 ± .00	1.24 ± .04	1.60 ± .13^{b***c*}
Left testicular weight (g)	1.30 ± .07	1.12 ± .01	1.64 ± .07^{a*,d**}	1.54 ± .11^{b**}
Epididymis weight (g)	0.58 ± .03	0.69 ± .04	0.78 ± .02^{a*}	1.08 ± .06^{ab***c**}
Seminal vesicle weight (g)	1.24 ± .11	1.16 ± .15	1.12 ± .19	0.92 ± .10
Prostate weight (g)	0.65 ± .04	0.54 ± .01	0.69 ± .05	0.61 ± .07

Note: values are expressed as mean ± SEM. *$P < .05$, **$P < .01$, ***$P < .001$ (ANOVA followed by Dunnet's multiple comparison test) [a]Value versus ML1 [b]Value versus ML2 [c]Value versus control

by planimetry, using Image J2X software. The area in μm^2 was computed using the method of Jensen and colleagues.[51] Briefly, 25 pictures/animal (40×) of the known area was selected and the area of seminiferous tubules and interstitial space was determined by the free selection tool of the software. The area percentage (%) was calculated by the formula:

$$\%\ \mathbf{AS} = AS \times 100/T$$

where AS is the area covered by the seminiferous tubule, T is the total area of the field, and the percentage of the mean area was analyzed for comparison between the treated and control groups.

Statistical analysis

Data were expressed as mean ± SEM. One-way analysis of variance (ANOVA) followed by post hoc Tukey's test was performed for analysis of different groups using Graph pad prism 5 Software version 5.00 for Windows, GraphPad Software, San Diego CA, USA, (www.graphpad.com). Differences were measured to be statistically significant when $P < .05$.

Results

Acute toxicity

Acute toxicity of methanolic leaf extract of *M. longifolia* in rats after a single oral dose ranging from 5 to 2000 mg/kg body weight after 14 days showed no symptoms of mortality or morbidity and value expressed as LD0. There was no obvious difference in rat behavior or eating and drinking habit.

Effects on fertility potential

A decreased number of sperms was observed in the vaginal smears collected for three consecutive days after the treatment withdrawal period. A decrease in the percentage of conceived females and the number of live pups was observed after being impregnated with treated groups in comparison with control (100%) (Tables 1 and 2). The number of pups born per female also decreased dose dependently in all treated groups in contrast to controls. Significant reduction ($P < .05$) in the number of pups born to females mated with ML1 group males (5.25 ± .85) was observed when compared to control (8.75 ± .48). Similarly, the number of pups born to females mated with the ML3 group (3.75 ± 1.25) was significantly low ($P < .01$) than in the control. No critical change in the mean number of pups born per female was seen within treatment groups. A significant decrease ($P < .05$) in the number of pups was seen in ML3 (3.75 ± 1.25) group in contrast to ML1 (5.25 ± .85).

Effect on body weight and reproductive organs weight

There was an observed decrease in body weight of ML1 (231.0 ± 5.33), while an increase in ML2 (263.0 ± 7.23) and ML3 (243.8 ± 18.66) groups was evident as compared to the control (239.0 ± 18.41); however, the change was not significant as shown in Table 2. Similarly, a non-significant decrease in testicular weight in ML1 and ML2 groups was seen, while a significant ($P < .001$) decrease in the ML3 group as compared to the control group was noticed. A comparison of ML3 with ML2 showed a significant increase ($P < .01$). Furthermore, a dose-dependent increase in epididymis weight was observed in all the extract-treated groups ML1 (.69 ± .04), ML2 (.78 ± .02, $P < .05$), and ML3 (1.08 ± .06) as compared to the control group (.58 ± .03, $P < .001$). A significant change was witnessed in ML3 group when a comparison was made with ML1 ($P < .001$) and ML2 ($P < .01$). A decrease in weight of seminal vesicle occurred in a dose-dependent manner as compared to control; however, change was not significant. Similarly, a decrease in prostate gland weight was seen in ML1 and ML3 groups, while an increase in the ML2 group as compared with the control group was noted.

Effect on sperm motility, viability, and daily sperm production

A significant dose-dependent reduction in the percentage of sperm motility was seen in ML1 group (37.5 ± 8.53, $P < .05$), ML2 group (31.00 ± 5.81, $P < .01$), and ML3 group (22.5 ± 10.31, $P < .001$), in comparison with the control group (73.6 ± 2.58). A significant decrease in the percentage of viable sperm was also observed in ML2 group (47.5 ± 4.78, $P < .05$) and ML3 groups (42.5 ± 4.78, $P < .01$) and in contrast to the control (77.6 ± 3.42) as depicted in Table 3.

A decrease in daily sperm production was noticed in ML1 and ML2 groups in comparison with the control (2.93 ± .05) group; however, the change was not significant, while a significant decrease ($P < .01$) in sperm production was observed in ML3 group (.86 ± .16). (Table 3).

Effect of *M. longifolia* on oxidant/antioxidant activity and levels of hormones

A decrease in catalase levels in ML1 (5.17 ± .31), ML2 group (3.49 ± .58), and ML3 (2.87 ± .41) groups were noticed as compared to the control (5.88 ± .47) in a dose-dependent manner as shown in Table 4. A comparison of ML2 with ML1 showed a significant decrease ($P < .01$) in CAT levels. Furthermore, a decrease in SOD levels was seen in a dose-dependent manner among all the extract

Table 2. Comparative effects of *M. longifolia* leaf extract at on the percentage of females that conceived and the mean ± SEM number of pups born per female after 60 days of treatment withdrawal

	Treatments			
Parameters	**Control**	**ML1 (50 mg/kg)**	**ML2 (75 mg/kg)**	**ML3 (100 mg/kg)**
Percent fertility	100	77	95	60
Number of pups born/female	8.75 ± .48	5.25 ± .85^{a*}	7.25 ± .48	3.75 ± 1.25^{a***c*}

Note: values are expressed as mean ± SEM. *P < .05, **P < .01, ***P < .001 (ANOVA followed by Dunnet's multiple comparison test) [a]Value versus control [b]Value versus ML2 [c]Value versus ML1

Table 3. Mean ± SEM of per cent rate sperm motility, viability and DSP of experimental groups of rats, following 28 days of *M. longifolia* leaf extract treatment

	Treatments			
Parameters	**Control**	**ML1 (50 mg/kg)**	**ML2 (75 mg/kg)**	**ML3 (100 mg/kg)**
Motility %	73.6 ± 2.58	37.5 ± 8.53^{a*}	31.00 ± 5.81^{a**}	22.5 ± 10.31^{a***}
Viability %	77.6 ± 3.42	62.5 ± 11.09	47.5 ± 4.78^{a*}	42.5 ± 4.78^{a**}
Daily sperm production $\times 10^6$ / testis	2.93 ± .05	1.86 ± .23	1.86 ± .54	0.86 ± .16^{a**}

Note: values are expressed as mean ± SEM. *P < .05, **P < .01, ***P < .001 (ANOVA followed by Dunnet's multiple comparison test) [a]Value versus control [b]Value versus ML1 [c]Value versus ML2

Table 4. Effect of methanol leaf extract of *M. longifolia* on biochemical parameters of rat testis

	Treatments			
Parameters/hormones	**Control**	**ML1 (50 mg/kg)**	**ML2 (75 mg/kg)**	**ML3 (100 mg/kg)**
CAT (U/mg)	5.88 ± .47	5.17 ± .31	3.49 ± .58^{b**}	2.87 ± .41^{c**}
SOD (U/mg)	7.9 ± .58	5.16 ± .44^{a**}	4.99 ± .67^{a***}	1.27 ± .39^{a***c**}
POD (nmole)	4.17 ± .56	5.56 ± .50	6.56 ± 1.35	6.93 ± 1.50
ROS (μmol/min)	1.26 ± .03	4.23 ± .64^{a**}	4.63 ± .35^{a***}	4.90 ± .57^{a***}
TBARS (nM/mg)	0.79 ± .47	1.87 ± .21	2.51 ± .32	3.77 ± .40^{a-e}
LH (ng/ml)	0.89 ± .02	0.47 ± .06^{aa***}	0.11 ± .04^{ab***}	0.20 ± .06^{a***b**}
FSH (ng/ml)	2.07 ± .20	0.57 ± .19^{a***}	0.40 ± .16^{a***}	0.50 ± .12^{a***}
Testosterone (ng/ml)	11.89 ± .23	1.21 ± .19^{a*}	8.02 ± .38^{a***b**}	7.94 ± .50^{a***b**}

Note: values are expressed as mean ± SEM. *P < .05, **P < .01, ***P < .001 (ANOVA followed by Dunnet's multiple comparison test) [a]Value versus ML1 [b]Value versus ML2 [c]Value versus control

treatment groups i.e. ML1 ($P < .001$), ML2 ($P < .001$), ML3 ($P < .001$) as compared to the control group, while the comparison of ML3 with ML2 showed a significant decrease in SOD concentration in ML3 group ($P < .01$). The biochemical analysis of peroxidase levels showed a significant increase in the ML1 (5.56 ± .50) group as compared to the control. A non-significant increase in POD levels was seen in ML2 (6.56 ± 1.35) and ML3 (6.93 ± 1.50) groups as well in contrast to the control group (4.1 ± .56).

There was observed a significant increase in the number of reactive oxygen species among ML1 ($P < .01$), ML2 ($P < .001$), and ML3 ($P < .001$) as compared to control (Table 3). An increase in the number of TBARs was observed in ML1 and ML2 groups, however, the change was not significant. A highly significant increase ($P < .001$) in TBARs among ML3 was observed as compared to the control and ML2 group.

Plasma FSH and LH concentrations were significantly ($P < .001$) reduced in the ML1, ML2 and ML3 treated group as compared to the control (Table 3). The comparison of ML1 with increasing doses showed a highly significant decrease in LH concentrations in ML2 ($P < .001$) and ML3 ($P < .01$) groups. A dose-dependent decrease in testosterone levels was seen in all the experimental groups (ML1–1.21 ± .19, ML2–8.02 ± .38, ML3–7.94 ± .50) when contrasted with the control group (11.89 ± .23).

Effect of *M. longifolia* on histomorphology of reproductive tissues

Data regarding the histological analysis of testis and epididymis-caput and cauda epididymis for all the experimental groups are presented in Table 4.

Effects on testis

A critical diminishment ($P < .001$) in mean seminiferous tubule diameter was found in all the treatment groups (ML1, ML2, and ML3) when contrasted with control (Table 4). A highly significant reduction was noticed in the diameter of seminiferous tubule among ML2 ($P < .001$) and ML3 ($P < .05$) treated groups as compared to the ML1 group, while the comparison of ML3 with that of ML2 showed a significant ($P < .001$) decrease in diameter. Similarly, mean tubular lumen diameter in ML1 ($P < .01$) and ML3 ($P < .01$) treated groups were significantly reduced in comparison with control groups. Unequal arrangement of seminiferous tubules with interstitial spaces and a low number of spermatozoa in the epithelium was observed in higher dose treatments as shown in Fig. 1. A highly significant reduction in mean epithelial thickness ($P < .001$) was observed in all the plant extract treated groups as compared to control. Most prominently in ML3 (100 mg/kg treated) group when its comparison was made with ML1 and ML2 groups. A prominent decrease ($P < .001$) in height of epithelium

Table 5. Assessment of mean ± SEM of seminiferous tubule diameter, tubular lumen diameter, seminiferous tubule epithelial height, tunica albuginea height, and number of different cell types in seminiferous tubules in the testis of control and treated groups

	Treatments			
Morphometric parameters	**Control**	**ML1 (50 mg/kg)**	**ML2 (75 mg/kg)**	**ML3 (100 mg/kg)**
Seminiferous tubule diameter (μm)	1729.05 ± 59.47	1578.52 ± 53.32^{a***}	132.94 ± 51.03^{ab***}	987.52 ± 26.93^{abc***}
Tubular lumen diameter (μm)	522.18 ± 23.84	425.51 ± 11.91^{a**}	518.72 ± 23.71^{b**}	416.85 ± 21.22^{ac**}
Epithelial height(μm)	593.03 ± 27.79	429.60 ± 12.18^{a***}	431.51 ± 22.36^{a***}	193.99 ± 5.94^{abc***}
Tunica albuginea height (μm)	210.07 ± 6.77	165.35 ± 9.00^{a***}	113.06 ± 5.15^{ab***}	58.92 ± 1.65^{abc***}
Number of cell types				
Spermatogonia	86.60 ± 4.46	76.42 ± 1.99^{a*}	73.33 ± 2.02^{a**}	69.58 ± 1.13^{a***}
Primary spermatocytes	68.17 ± .75	65.75 ± 1.96	63.25 ± 1.99	58.25 ± 1.624^{ab*}
Secondary spermatocytes	64.00 ± 1.23	6.67 ± 2.09	61.92 ± 2.18	61.58 ± 1.82
Spermatids	108.60 ± 5.37	92.58 ± 1.23^{a***}	91.17 ± 1.65^{a***}	90.92 ± 1.38^{a***}

Note: values are expressed as mean ± SEM. $^{*}P < .05$, $^{**}P < .01$, $^{***}P < .001$ (ANOVA followed by Dunnet's multiple comparison test) ^{a}Value versus control ^{b}Value versus ML1 ^{c}Value versus ML2

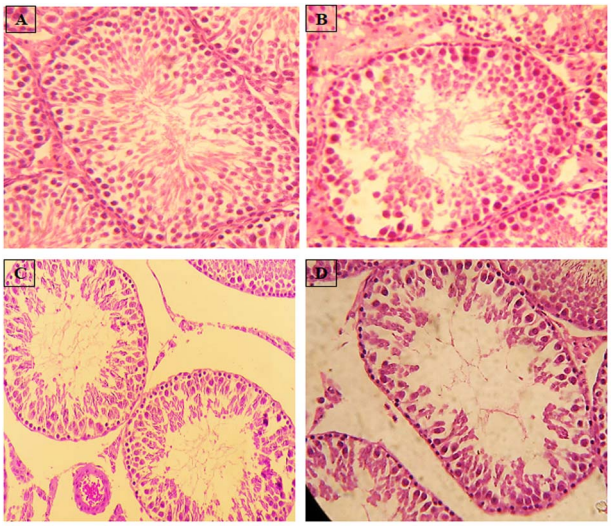

Fig. 1. Photomicrograph of seminiferous tubules. (**A**) Control: showing dense tubules, lumen engorged with spermatids and normal germ cells. (**B**) ML1: showing tubules with degenerated epithelium. (**C**) ML2: showing further degenerations increased interstitial space and empty lumen with less number of spermatogenic cells. (**D**) ML3: showing maximum damage to epithelial layer with increased interstitial space, tubules with accelerated formation of empty lumen. Magnification 40×. Spermatogonia (SP), Elongated spermatids (ES), Interstitial space (IS), Epithelium (E).

was noticed in all the treatment groups as compared to the control group; however, a significant change was witnessed in ML3 when its comparison was made with ML1 ($P < .001$) and ML2 ($P < .001$) (Tables 5 and 6).

Effects on epididymis-caput epididymis and cauda epididymis

A highly significant reduction ($P < .001$) in the ductular diameter was observed in ML2 and ML3 groups when compared with ML1 and the control group. Mean luminal diameter significantly decreased in ML2 ($P < .01$) and ML3 ($P < .001$) groups as compared to the control group, but not in ML1 group, as depicted in Fig. 2. Mean epithelial cell height also showed a significant decrease in ML2 ($P < .05$) and ML3 ($P < .01$) treated groups as compared to the control group.

No significant difference was observed in the ductular diameter of cauda epididymis among the control and treated groups. However, a highly significant reduction ($P < .001$) in mean lumen diameter was observed in all treated groups when compared to the control group, predominantly in ML3 group ($P < .001$). In the case of mean epithelial cell height, no significant difference was observed among control and treated groups as well as when a comparison was made within groups.

Discussion

Family planning programs have been considered an essential instrument in accelerating global fertility decline. Shortcomings of some currently available male contraceptive methods are a major obstacle to the involvement of men in family planning.[57] On a global scale, many local plants have been tested and identified for their antifertility effect in males.[58,59] This is a pioneer study to examine the antifertility effects of *M. longifolia* leaf extract in adult male rats. The possible outcome of *M. longifolia* leaf extract on the percentage of females that conceived after being impregnated with treated male rats and reversibility of antifertility effect after treatment withdrawal was also evaluated. Our investigation indicated that *M. longifolia* leaf extract decreases fertility in male rats through a reduction in sperm motility and viability rate, altered antioxidant and hormonal levels, and impaired spermatogenesis at higher doses.

Sperm motility and viability are some of the most important parameters to determine the capability of mature sperms for normal fertilization.[21] The results of the present study showed a significant reduction in the percent rate of sperm motility and viability among all the extract-treated groups against the control group. This reduction in sperm motility and viability could be attributed to the reduced levels of serum testosterone and enhanced production of reactive oxygen species as previously suggested as well.[20] Several other studies focusing on the use of antifertility plants in males have also shown that contraceptive actions of plants are mediated through a reduction in sperm motility rate and sperm number.[60] Following the plant-based contraceptive research reported by,[61] our results showed a marked reduction in daily sperm production in a dose-dependent manner against control, with the highest reduction seen in 100 mg/kg treated animals. This reduction might be attributed to multiple possible mechanisms, one of which includes the potential of the plant extract to cross the testicular barrier and disturb normal spermatogenesis or sperm movement, affecting the motility of mature spermatozoa.[62] Thus, it is suggested that reduced daily sperm production after exposure of rats to plant extract might

Table 6. Mean ± SEM of ductular diameter (μm), luminal diameter (μm) and epididymis cell height (μm) of epididymis in control and treated groups after 28 days of *M. longifolia* extract treatment

		Treatments			
Parameters		**Control**	**ML1 (50 mg/kg)**	**ML2 (75 mg/kg)**	**ML3 (100 mg/kg)**
Ductular diameter (μm)	Caput	753.45 ± 17.60	74.79 ± 64.88	518.65 ± 19.53^{ab***}	457.06 ± 13.91^{ab***}
	Cauda	526.96 ± 22.72	427.50 ± 34.93	469.90 ± 36.06	431.28 ± 3.79
Lumen diameter (μm)	Caput	593.68 ± 14.92	516.73 ± 16.30	443.23 ± 39.53^{a**}	436.65 ± 31.39^{a***}
	Cauda	487.34 ± 18.84	173.07 ± 7.05^{a***}	268.46 ± 18.61^{a***b**}	312.36 ± 29.38^{ab***}
Epithelial height (μm)	Caput	92.01 ± 3.60	79.59 ± 4.40	76.68 ± 5.17^{a*}	73.90 ± 2.31^{a**}
	Cauda	63.69 ± 4.83	54.91 ± 4.86	51.92 ± 3.20	62.83 ± 4.34

Note: values are expressed as mean ± SEM. *P < .05, **P < .01, ***P < .001 (ANOVA followed by Dunnet's multiple comparison test) [a]Value versus control [b]Value versus ML1 [c]Value versus ML2

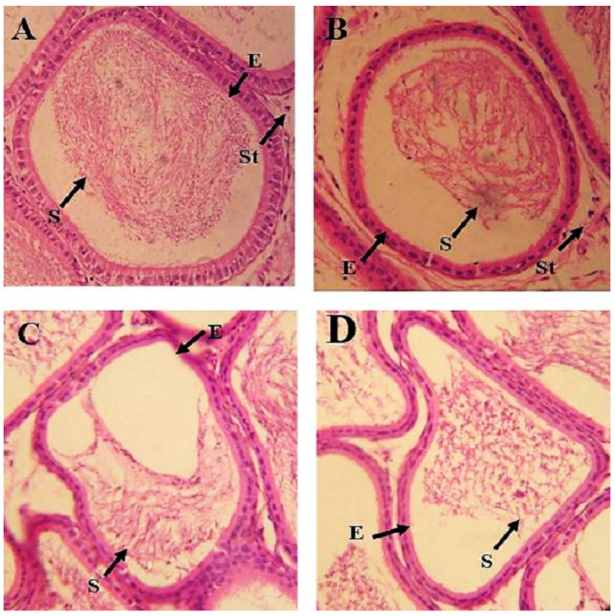

Fig. 2. Comparative photomicrograph of adult male rat caput epididymis (H&E, 40×) from: (**A**) Control group; showing normal morphology of caput epididymis with closely spaced tubules lined by thin pseudostratified epithelium and lumen filled with spermatozoa, (**B**) ML1; showing no much difference from control group, having widely spaced tubules, (**C**) ML2; showing tubules of smaller diameter, reduced pseudostratified epithelium and lumen with distorted spermatozoa, (**D**) ML3; showing further decrease in tubular diameter and pseudostratified epithelium. Stroma (St), Epithelium (E), Spermatozoa (S).

be responsible for the decrease in the number of germ cells, reduced spermatogenesis, and reduced production of androgen and pituitary gonadotropins.

Oxidative stress is associated with the imbalance between the antioxidant defense system and the generation of reactive oxygen species.[63] The SOD, CAT, and POD are key enzymes that scavenge free radicals in male reproductive organs.[64] The present study found a substantial decrease in SOD levels in all groups, with a significant decrease in the 100 mg/kg treated group ($P < .001$). Furthermore, the levels of CAT and POD were increased in all treated groups, significantly in the 50 mg/kg treated group ($P < .01$). These results differ from the findings of,[65] who reported a decrease in CAT after administration of 60 mg/kg *Terminalia chebula* extract to rats for 28 days. One possibility of a reduction in SOD levels might be the excessive production of anions in response to the *M. longifolia* administration, and this decreased SOD expression increased the expression of POD and CAT as compensatory adjustments in testicular tissues. It is also possible that an increased generation of ROS may inhibit the activity of SOD and cause increased oxidative stress. This would result in decreased sperm motility and viability and ultimately lead to male sterility. A significant and dose-dependent increase in ROS and TBARS was observed with maximum concentrations of TBARS seen in a 100 mg/kg treated group. Our study also revealed that increased production of TBARS in testicular tissue also elevates the ROS levels in each treated group, as proposed by.[65]

The testicular tissue histology showed dose-dependent degenerative changes in the histo-architecture of seminiferous tubules depicted by a decrease in the tubular and luminal diameter of seminiferous tubules, epithelial height and thickness, and the number of germ cells, with the most pronounced effects in the group receiving 100 mg/kg dosage. These observations are in line with those reported by Jahan et al. (2009) and Sewani-Rusike and Gundidza (2011) in their respective studies.[66,67] Moreover, empty lumen and enlarged interstitial spaces were seen, affecting the normal process of spermatogenesis. These changes can be attributed to reduced testosterone production observed in our study.[15,68] A decrease in the number of germ cells was evident in the present study among extract-treated groups as compared to the control. The germ cells rely on the Sertoli cells for their nourishment, and this interaction is responsible for normal spermatogenesis and any damage to these cells would result in the spermatogenic arrest.[69] Furthermore, it can be inferred that the extract component crossed the blood testes barrier (BTB) and directly exerted the aforementioned effects on seminiferous tubule architecture.

Our study showed a significant dose-dependent decline ($P < .001$) in serum levels of FSH, LH, and testosterone among all treated groups, favoring results of [70,71]. These results suggest that the mechanism of *M. longifolia* action on rat testis was the upstream suppression of LH and FSH. The absence of LH stimulation at the testicular level resulted in low Leydig cell count and Leydig cell dysfunction, thereby causing a subsequent decline in testosterone secretion, reducing sperm counts.[20] A similar decrease in Leydig cell count was seen in all treated groups of our study. The mechanism behind the anti-spermatogenic action of *M. longifolia* extract treatment is yet to be explored; however, it might be speculated that the non-availability of pituitary gonadotropins and testosterone hormone is the possible cause of failure of spermatogenesis.

The process of spermatogenesis involves different stages of differentiation; including diploid spermatogonia, spermatocytes, round spermatids, and haploid mature spermatozoa. As this

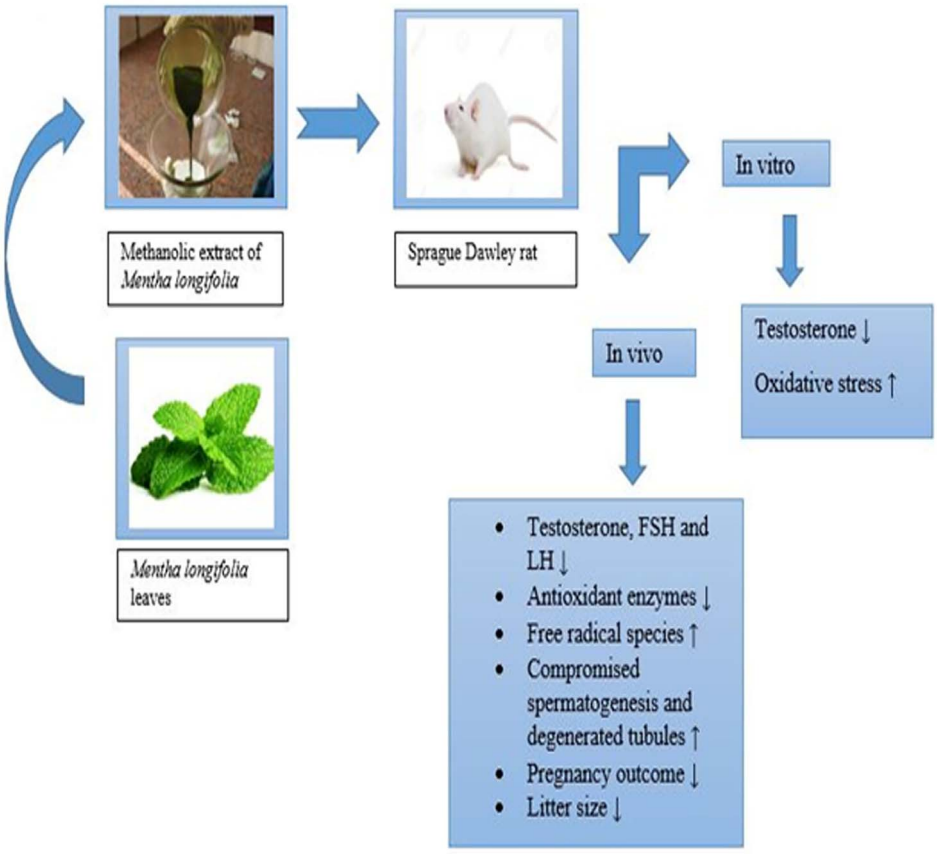

Fig. 3. Mechanism of antifertility potential of *M. longifolia* in adult male rats.

differentiation is controlled by testosterone and pituitary gonadotropins, both of which were found to be reduced in our study, degenerative changes in the seminiferous tubules were observed, failing normal spermatogenesis. The low count of spermatogonia and mature spermatids in the *M. longifolia*-treated rats appeared to be due to the suppressive effect of the treatment on spermatogenesis.

Furthermore, the fertility study showed a decrease in the number of sperms in the vaginal smear of mated females at the termination of the treatment schedule. However, withdrawal of treatment for eight weeks induced partial recovery of fertility, revealed by the attained pregnancy of untreated females when mated with treated males and their litter size. Both the percentage of pregnancy and litter size was reduced in all treated groups as previously reported by Mc et al. (2015) in his work on *Opuntia elatior* extract in male rats.[72] Partial recovery of male fertility could be attributed to the short period of 8 weeks of treatment withdrawal. As these findings are preliminary, additional studies are required to isolate the active principle component responsible for the antifertility effect of *M. longifolia* and to further elucidate their precise mode of action. Further long-term studies should also be directed in the future for the evaluation of complete reversible fertility with this extract (Fig. 3).

Conclusion

In conclusion, this is a critical scientific study to assess the plant's toxicity profile and validate its contraceptive potential. The administration of *M. longifolia* leaf extract at high doses has a potent ability to induce toxic effects in male rats by decreasing sperm motility and viability, reducing plasma levels of testosterone and pituitary gonadotropins, causing histopathological alteration of reproductive organs, and elevating free radical species that eventually results in a spermatogenic arrest. These effects are likely to bring alteration in factors controlling the pituitary-gonadal axis and oxidative stress induced in testicular tissues by plant extract. Thus, it can be suggested that because of the pronounced effects of the extract on certain physiological aspects of testes and its reversible fertility suppression efficacy, *M. longifolia* leaves might be utilized in the development of contraceptive drugs for men; however, the associated oxidative stress should also matter of concern. Therefore, in a normal routine, people who consume *M. longifolia* should be warned to consume this herb appro.

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Authors' contributions

Sarwat Jahan led the experimental design and approved the study. Mehwish David and Qasim Shah conceived the study, performed the experimental work, and analyzed the results. Qurat-ul-ain performed the initial part of the study and Umar Ijaz helped in compiling the results. The plant was collected, identified, and extracted by Mushtaq Ahmed. Mehwish David, Qasim Shah, Tayyaba Afsar, and Suhail Razak wrote the paper with input

from other authors. Huma Shafique, Tariq Nahar Alanezi, Ali Almajwal, and Umar Ijaz made substantial contributions to the interpretation of data and revising of the manuscript for intellectual content. All authors read and approved the final manuscript.

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Conflict of interest statement. None declared.

Competing interests

The authors declare that they have no competing interests.

Availability of data and materials

All the data are contained in the manuscript.

Ethical approval and consent to participate

The study protocol was approved by the ethics committee at Quaid I Azam University, Islamabad, Pakistan. Studies reported in the manuscript fully meet the criteria for animal studies specified in the ACS ethical Guidelines.

Consent for publication

Not applicable.

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