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Research Article

In Vitro Evaluation of Contraceptive Efficacy of *Asplenium dalhousiae* Hook. and *Mentha longifolia* L. on Testicular Tissues of Adult Male Mice

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Abstract

Purpose: The present study aimed to investigate the antifertility potential of *Asplenium dalhousiae* Hook. and *Mentha longifolia* L. methanolic leaf extract using *in vitro* techniques in adult albino male mice.

Main method: Mice were dissected, testicular tissues collected and sliced into nine parts. Four different concentrations (1, 10, 100 and 1000µg/mL) of *A. dalhousiae* Hook. and *M. longifolia* L. were tested for 2 hours in 5% CO_2 incubator at 37°C. Sample with distilled water served as a control.

Results: No significant alteration in testosterone was seen among treatment groups as compared to control. However, a significant reduction in antioxidant enzymes was observed in treated groups, leading to physiological changes on mice testes. These effects are thought to be mediated by oxidative stress induced by both extracts.

Conclusion: the study concluded that *Asplenium dalhousiae* Hook. is found to have more profound antifertility effects as compared to *Mentha longifolia* L. on male reproduction by targeting process of steroidogenesis.

Keywords: *Asplenium Dalhousiae*; *Mentha Longifolia*; *In Vitro* Studies; Contraception

Introduction

Today, amongst the three means of mitigating against population growth are abortion, sterilization and contraception. The contraceptive mode of birth control is the most popular approach and different categories of contraceptive devices are being used. These are physiological devices, mechanical devices and surgical procedures. While contraceptives used for males have mainly focused towards prevention of sperm maturation. The chemical approaches possess numerous side effects including cholelithiasis, obesity, gastric trouble and carcinoma of cervix and breast, thromboembolism and asthma that has lost its effectiveness and popularity among women [1]. Researchers have dedicated their investigations on three general categories of male contraceptives physically blocking the vas deferens, use of heat for induction of temporary sterility and medication to halt the process of spermatogenesis [2]. With hazardous effects of each method of birth control, scientists are now focused on finding an oral herbal contraceptive that would be effective, reversible and more acceptable among the males.

One of the most inspiring quests in the realm of pharmacological and therapeutic sciences is the use of plant products as a key source of naturally occurring fertility regulating agents because of their minimal side effects [3]. Ethno botanical knowledge considering old therapeutic systems such as folklore, shamanism and herbalism is necessary to understand the use of plants having therapeutic potential [4]. Page et al. have reported high efficacy rates for plant based male contraceptives [5]. Different plants constituents like quinines, flavonoids, tannins, terpenes have been described to induce male infertility [6-8]. Variety of plants have been reported to hinder the different stages of spermatogenesis in many animals like mice, rats, monkey, dogs and humans [9,10]. *In vitro* evaluation of various plant extracts [11-14], have shown detrimental effects on various stages of spermatogenesis, testosterone production, sperm quality and anti-oxidant enzyme levels [15].

Therefore, this study was a preliminary design to evaluate the contraceptive efficacy of *A. dalhousiae* and *M. longifolia* leaf extract using *in vitro* technique to target the process of androgenesis and oxidative stress if any, in adult male mice.

Materials and Methods

The present study was conducted in the Reproductive Physiology laboratory, department of Animal Sciences, Quaid-i-Azam University, Islamabad. Animals were handled according to rules and regulation approved by local ethical committee of department of Animal Sciences which are research specific in animal handling.

Animals

Adult male Mus musculus mice with average weight of 25±5g were taken from the primate facility of Animal Sciences Department, Quaid-i-Azam University. Animals were randomly kept in stainless steel cages in well ventilated zone at room temperature of 20-26°C and 12 hours dark/light cycle, provided with pelleted food and water *ad libitum*. Animals were dissected, and testes were obtained. Testes were divided into nine equal parts.

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Plant material

***Asplenium dalhousiae* Hook.**

A. dalhousiae Hook. (the plant name has been verified from http://www.theplantlist.org) is a herb and is found in forest area, its local name is Gutti, and English name is Dalhousie spleenwort. The roots are of most medicinal importance when used as decoction. Its ethnomedicinal use in infants is as Ghutti [16,17]. Previous studies have shown that treatment with *A. dalhousiae* plant extract produced dose dependent damage to the DNA and also showed anti-proliferative and apoptotic activity in sperm [18].

***Mentha longifolia* L.**

Its common name is safaid podina. The main constituents in *Mentha longifolia* (the plant name has been verified from http://www.theplantlist.org) are terpenoids and flavonoids [19]. In addition, Mentha species have been used as a traditional remedy for treatment of bronchitis, flatulence, nausea, cuts, wounds, bruises [20], ulcerative colitis, anorexia, and liver complaints due to its ant inflammatory, carminative, diaphoretic, antiemetic, antispasmodic, stimulant, analgesic, against gastric problems, emmenagogue, and anti-inflammatory activities [21-23]. Recently, we published the data demonstrating contraceptive activity of *A. dalhousiae* by inducing and monitoring steroidogenesis and spermatogenesis in adult male rats (*In vivo*) [16].

The leaf sample of *Asplenium dalhousiae* Hook. and *Mentha longifolia* L. was obtained from agricultural fields of District Shangla and its nearby villages Alpuria, Shahpur, Lilowni, Chakesar, Hayatabad, Ajaori and Kass, Pakistan. All the plants were identified by Dr. Mushtaq Ahmad at Plant Systematics and Biodiversity laboratory and Herbarium of Pakistan, Quaid-i-Azam University Islamabad. Both the plant samples were deposited in the herbarium of Plant Sciences department, QAU, under accession number 105632 (*Asplenium dalhousiae* Hook.) and 69602 (*Mentha longifolia* L.).

Plant extract preparation

Leaves of *A. dalhousiae* and *M. longifolia* were separated and methanolic extract was prepared according to the method described by Ain et al. The leaves were air-dried for about 2 weeks, ground in Waring blender and then sieved through filter paper. The dried powder was extracted with methanol (leaf to solvent ratio 1:10) followed by filtration using Whatman™ Qualitative Filter Paper Standard Grade, circle whose pore size was 90mm (GE Healthcare 1001090 Whatman™ Qualitative Filter Paper: Grade 1 Circles, Thermo Fisher Scientific) and then concentrated on a rotary evaporator (Model: Hei-VAP Heidolph, Germany; Gulfraz, Qadir, Nosheen, & Parveen, 2007).

Experimental design

Six adult male mice (Mus musculus) were used in this experiment. Experimental design was planned in reference to Moundipa et al. study with slight modifications [24]. The four doses 1µg/mL, 10µg/mL, 100µg/mL, 1000µg/mL were selected for each plant according to *in vitro* studies using plant extracts as suggested by Srivastav et al. in their respective study [12]. Adult mice testes slices were used to incubate intact Leydig cells with their environment in media containing different doses of plants extract. Animal testes treated with distilled water only served as control. Asplenium treated groups having different concentrations of plant extracts were named as Asp 1 (1µg/mL), Asp 2 (10µg/mL), Asp 3 (100µg/mL) and Asp 4 (1000µg/mL) while Mentha treated groups as Ment 1 (1µg/mL), Ment 2 (10µg/mL), Ment 3 (100µg/mL) and Ment 4 (1000µg/mL). Stock solution of plant extracts were prepared in methanol and was diluted with cell culture media. Methanol concentration in the media was kept less than 0.5%.

Biochemical analysis

Following centrifugation of testicular tissues supernatant was isolated and used for assessment of antioxidant status.

Estimation of ROS: Estimation of Reactive Oxygen Species (ROS) was carried out in testicular tissue of control and treated animals. Hayashi et al. protocol was followed for ROS detection [25]. 0.1M sodium acetate buffer was prepared by dissolving 4.1g of sodium acetate in 500ml of distilled water. The pH was maintained at 4.8. Then 10mg of N, N-Diethyl-p-phenylenediamine sulphate salt (DEPPD) in 100ml of sodium acetate buffer was dissolved and a second solution was prepared by adding 50mg of ferrous sulphate ($FeSO_4$) in 10ml of sodium acetate buffer. Both the solutions were mixed in a ratio of 1:25 and incubated in dark for 20min at room temperature. Then 20µl was taken from the solutions mixture, 1.2ml of buffer and 20µl of homogenate were taken in a cuvette and absorbance was checked at 505nm by using Smart Spec TM plus Spectrophotometer. Three readings were taken for each tissue sample after every 15 seconds.

Estimation of lipid peroxidation: The TBARS method given by Wright et al., was used to estimate lipid peroxidation [26]. Reaction solution consisted of 0.02mM ferric chloride ($FeCl_3$) of 100mM, 0.2ml ascorbic acid of 100mM, 0.2ml of testicular sample and 0.58ml phosphate buffer (0.1M) with pH value of 7.4 made the total volume 1ml. In water bath at 37°C, final mixture was incubated for 1 hour and 1ml of 10% trichloroacetic acid was used to end the reaction. Then 1ml of 0.67% thiobarbituric acid was added and in water bath of boiling water, all the tubes were kept for 20 minutes and finally moved to crushed ice-bath. After that, centrifuged for 15 minutes at 25000 rpm and at 535nm readings were noted from spectrophotometer. Results were explained as µmol of TBARS/min/mg tissue at 37°C with coefficient of molar extinction of $1.56\times105\ M^{-1}\ cm^{-1}$.

Catalase (CAT) activity: With small modification, Chance and Maehly, method was used to find out the activity of CAT [27]. To measure CAT levels in testis, 0.1ml homogenate, 2.5ml of 50mM phosphate buffer (pH 5.0) and 0.4ml of 5.9mM H_2O_2 were added in a cuvette. After one minute at wavelength of 240nm variations in absorbance of solution were noted. Absorbance change of 0.01 units in one minute was called one unit of CAT activity.

Sodium dismutase (SOD) activity: By using the protocol of Kakkar et al, activity of SOD was determined [28]. For this purpose, 0.3ml of testicular sample, 1.2ml of sodium pyrophosphate buffer (0.052mM; pH 7.0) and 0.1ml of phenazine methosulphate (186µM) were mixed and reaction was started by the addition of 0.2ml of NADH (780µM). Finally, after 1 minute by the addition of 1ml of glacial acetic acid reaction was ended and at 560nm readings were noted and results were explained as units/mg of protein.

POD activity: Guaiacol Peroxidase (POD) activity was determined by using method as recommended by Chance and Maehly

Table 1: *In vitro* effect of *A. dalhousiae* and *M. longifolia* leaf extract on antioxidant/oxidant enzymes in mice testes after 2 hours of incubation.

Groups	SOD (U/mg protein)	POD (mU/mg protein)	CAT (mU/mg protein)	ROS (U/mg tissue)	TBARS (pM/mg/min)
Control	311.34±24.78	0.91±0.02	7.40±0.66	2.12±0.17	0.98±0.31
Asp 1 (1µg/mL)	262.60±21.66	0.70±0.05	2.05±0.58***	2.33±0.03***	3.18±0.47***
Asp 2 (10µg/mL)	160.50±17.06***	0.38±0.01***	1.07±0.18***	2.57±0.08**	3.63±0.32***
Asp 3 (100µg/mL)	103.12± 2.60***	0.80±0.08	2.15±0.33***	2.49±0.07*	1.06±0.25
Asp 4 (1000µg/mL)	60.89±16.05***	0.54±0.03**	3.39±0.66***	2.46±0.07	1.11±0.32
Ment 1 (1µg/mL)	242.00±28.38	0.71±0.05	2.32±0.52***	2.36±0.07	2.47±0.33*
Ment 2 (10µg/mL)	159.72±17.57***	0.59±0.11*	3.16±0.10***	2.35±0.05	2.62±0.46*
Ment 3 (100µg/mL)	203.52±10.78**	0.72±0.05	5.28±0.40	2.64±0.03***	0.30±0.05
Ment 4 (1000µg/mL)	169.00±16.40***	0.67±0.09	6.39±0.72	2.40±0.11*	2.12±0.40

Values are expressed as mean ± SEM. *p< 0.05, **p< 0.01, ***p< 0.001

[27]. Reaction was carried out by adding 0.3ml of 40Mm H_2O_2, 2.5ml of 50mM phosphate buffer (pH= 5.0) and 0.1ml of 20mM guaiacol into 0.1ml of homogenate. After one-minute changes in absorbance were noted at 470nm. Absorbance change of 0.01 unit in one minute was called as one unit of POD activity.

Protein estimation-principle of the assay: In order to evaluate total protein content in testicular tissue, 20µL of standard and supernatant was added to eppendorf containing 1mL of Reagent (R) and vortex to mix properly. After 10 minutes of incubation at 37°C optical density was measured against a reagent blank at 546nm using chemistry analyzer (AMP diagnostics, Austria).

Hormonal analysis

Testosterone levels were determined in testicular tissue homogenate through ELISA kits (Amgenix, USA) according to the manufacturer instructions. All the samples were measured in a single assay and results were expressed as ng/ml.

Statistical analysis

All the results are expressed as mean ± SEM. One-way ANOVA followed by Dunnett's test was done using Graph pad Prism software version 5.00 for windows, Graph Pad software, San Diego CA, USA (www.graphpad.com).

Results

Antioxidant enzymes

Superoxide dismutase (SOD): The activity of SOD was significantly decreased (p<0.001) in Asp 2, Asp 3, Asp 4 (10µg/mL, 100µg/mL, 1000µg/mL) and Ment 2, Ment 4 (10µg/mL and 1000µg/mL) groups when compared with control group. A significant change (p<0.01) was noted in Ment 3 (100µg/mL) treated groups when compared to control while no significant results were observed in Asp 1 (1µg/mL) treated group and Ment 1 (1µg/mL) treated groups. The specific activity of superoxide dismutase (U/mg protein) in testicular tissues of control and treated groups are presented in (Table 1).

Peroxidase (POD) activity: Peroxidase activity (POD) was found significantly decreased (p<0.001) in Asp 2 (10µg/mL) treated groups as compared to the control group. A significant reduction (p<0.01) in Asp 4 (1000µg/mL) and (p<0.05) in Ment 2 (10µg/mL) treated groups was observed when compared to control. Other treatments exhibited no significant change when compared with control group (Table 1).

Catalase (CAT): The CAT activity was significantly decreased (p<0.001) in Asp 1, Asp 2, Asp 3, Asp 4 (1µg/mL-1000µg/mL) treated groups and Ment 1, Ment 2 (1µg/mL, 10µg/mL) treated groups when compared to control group. While no significant change was observed in Ment 3, Ment 4 (100µg/mL and 1000µg/mL) treated groups when compared to control (Table 1).

Reactive Oxygen Species (ROS): In all the treated groups, increase in ROS was observed in comparison to control group and expressed in Table 1. Asp 1, Asp 2, Asp 3 (1 - 100µg/mL) treated groups showed significant increase (p<0.001, p<0.01 and p<0.05 respectively) in ROS in comparison to control. Similarly, significant increase (p<0.001 and p<0.05) was observed in Ment 3, Ment 4 (100µg/mL and 1000µg/mL) treated groups when compared to control. Other treatments of Asp 4 (1000 µg/mL) and Ment 1, Ment 2 (1-10 µg/mL) treated groups did not showed any significant change as compared to the control.

Estimation of lipid peroxidation: The value of lipid peroxidation was increased in all treated groups then control group as expressed in Table 1. Results showed a significant increase (p<0.001) in TBARS in Asp 1, Asp 2 (1µg/mL and 10µg/mL) treated groups when compared to control. A significant increase (p<0.05) in TBARS was also observed in Ment 1, Ment 2 (1µg/mL and 10µg/mL) treated groups in comparison to control. No significant change was noted in Asp 3, Asp 4 (100ug/ml, 1000ug/ml) and Ment 3, Ment 4 (100ug/ml and 1000ug/ml) treated groups when compared to control.

Hormonal analysis

The average intratesticular testosterone concentrations (µg/mL) were estimated from testicular homogenate after *in vitro* exposure to plant extracts. Treatment with different concentrations of plant extracts of *A. dalhousiae* Hook. and *M. longifolia* L. for two hours *in vitro* caused no significant alteration in testicular testosterone concentrations when compared to the control group (Figure 1 and 2).

Discussion

The study was conducted to investigate the antifertility potential of medicinal plants. A large number of plants are known to cause contraceptive effects in males by disturbing process of spermatogenesis, steroidogenesis, sperm maturation and sperm transport [29]. The present study purposed to evaluate the contraceptive efficacy of methanolic leaf extract of *Asplenium dalhousiae* Hook. and *Mentha longifolia* L. on the reproductive system of male mice.

From the literature surveyed, no study on contraceptive efficacy of *A. dalhousiae* and *M. longifolia* L. leaf extracts has yet been done *in vitro*, so present study is the preliminary contribution. *In vitro* exposure of testicular tissues to methanolic plant extracts caused minimal reduction in intratesticular testosterone levels. This non-statistical reduction in intratesticular testosterone levels might be dose and incubation time dependent. Significant reduction in intratesticular testosterone levels could be possible by increasing the dose regime and incubation time period. Intratesticular levels of testosterone are crucial for the proper functioning of accessory sex organs as well as maintenance of spermatogenesis [30]. As these two phenomena are androgen dependent, disturbances in the androgen production (specifically testosterone) may inhibit spermatogenesis. Reduced testosterone production is directly linked to reduced Leydig cell number, eventually leading to spermatogenic arrest and disruption in seminiferous epithelium. Aqueous extract of bulb of *Allium sativum* (Amaryl lidaceae) was used in rats at 5%, 10% and 15% in diet for a period of 30 days which showed significant reduction in plasma and intratesticular testosterone levels ultimately inducing spermatogenetic arrest [31]. *Chromolaena odorata* (Asteraceae) alkaloid leaves extract administered to rats for 60 days caused significant reduction in serum LH and FSH levels, as well as testicular and serum testosterone levels in a dose dependent manner [32].

In present study, level of antioxidant enzymes in male reproductive organs was evaluated. As Ghosh et al. have described that treatment with aqueous ethanolic extract of T. *chebula* induced a substantial decrease in SOD and CAT levels in testicular tissues, the present findings also displayed similar results where all the plant extract treated groups showed highly significant reduction ($p<0.001$) in SOD and CAT activity as compared to control group [33]. These results are in contrast with findings of Khaki et al where increased level of SOD were seen when rats were administered with 400mg/kg/day of citrus extract for 30 consecutive days [34]. Another work was done on mice to check the effect of aqueous extract of *Choerospondias axillaris* fruit commonly used for the treatment of cardiovascular diseases in Mongolia [35]. In a study, aqueous extract of cloves of *Allium sativum* (Amaryl lidaceae) at the concentration of 500 and 1000mg/kg/day caused a significant reduction in percentage of morphologically normal spermatozoa and sperm concentration in male rats, a significant decrease in SOD activity, decline in testosterone concentration and alteration in normal spermatogenesis [36].

Reduction in POD levels prevents this conversion resulting in cell damage and death. In present study, decreased levels of POD activity was observed in all the plant extract treated groups (*A. dalhousiae* Hook. and *M. longifolia* L.) as compared to control.

The results of current experiment showed that exposure of testicular tissue to methanolic leaf extract of *A. dalhousiae Hook.* and *M. longifolia* L. impart hazardous and deleterious effects on male reproductive organs. These toxic effects are thought to be attributed by oxidative stress induced by plant components, not explored previously. Further research is required to illuminate the underlying mechanisms causing oxidative stress. Oxidative stress is a result of disturbance in oxidant/anti-oxidant system [34]. Under normal circumstances, a balance between reactive oxygen species and antioxidants is maintained. Oxidative stress is induced by high production of free radicals i.e. TBARs and ROS. A significant rise in levels of reactive oxygen species and in lipid peroxidation like TBARS was detected in all the treated groups when compared with control group. The highly significant increase ($p< 0.001$) in TBARs was seen in Asp 1 and Asp 2 (1µg/ml and 10µg/ml). Similar results have been reported in previous studies which suggested that increased production of free radicals such as TBARS elevates level of ROS in all the treated groups [16,33].

Due to higher content of polyunsaturated fatty acids in the plasma membrane of spermatozoa, they are highly susceptible to elevated levels of ROS [37-39]. This can lead to lipid peroxidation and thus disruption of lipid membranes, resulting in reduced production of testosterone and decreased androgenesis [34]. In our studies, antioxidant enzyme content was measured to evaluate oxidative stress induced by plant extract. Increased level of reactive oxygen species was observed in the plant extract treated groups as compared to control group. A significant increase ($p<0.001$) has been observed in reactive oxygen species levels of all the Asplenium treated groups (Asp 1, Asp 2 and Asp 3) while Ment 3 (100µg/mL) and Ment 4 (1000µg/mL) treated groups showed significant increase ($p<0.001$ and $p<0.05$) in ROS levels respectively as compared to control groups.

Conclusion

Results of the present study reveals that exposure to *A. dalhousiae* Hook. and *M. longifolia* L. leaf extract exert physiological effects on testes of adult male mice. The extract has shown to cause decrease in intratesticular testosterone levels and significant reduction in antioxidants levels mediated by oxidative stress. However, based on the more production of reactive oxygen species, *Asplenium dalhousiae* Hook. is found to have more profound antifertility effects as compared to *Mentha longifolia* L. on male reproduction. Future studies can be designed in order to evaluate the unresolved contraceptive potential of these plant extracts by changing dose regime and increasing incubation period and to isolate the active components responsible for contraceptive potential of *A. dalhousiae* Hook. and *M. longifolia* L. The detailed mechanisms involved in encouraging reproductive toxicity needs to be elucidated. With these supportive studies, both *A. dalhousiae* Hook. and *M. longifolia* L. plant can be utilized in the development of contraceptive drugs for men.

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