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Teucrium polium hexane extract downregulated androgen receptor in testis and decreased fertility index in rats

AAA Al-Tikriti[1], E Al-Khateeb[2] and MA Abbas[2]

Abstract

Teucrium polium L. (Lamiaceae) is a commonly used medicinal plant in folk medicine. Among several uses, *T. polium* is used to treat male fertility problems. This research was conducted to study the effect of *T. polium* on spermatogenesis, testosterone level, androgen receptor expression, and fertility in male rats. Administration of hexane extract for 6 days to aging rats increased testosterone level. When administered for 60 days, *T. polium* hexane extract downregulated androgen receptors, decreased sperm count without producing histological changes in different stages of spermatogenesis. Also, fertility index decreased without making teratogenic effects when treated males were mated with untreated females on the 55th day of extract administration. Therefore, the downregulation of androgen receptor could be due to the continued elevation in testosterone level over time. In conclusion, this study suggests that the prolonged use of *T. polium* in folk medicine may negatively affect male fertility.

Keywords

Teucrium polium, fertility index, androgen receptor, testosterone

Introduction

Teucrium polium (Lamiaceae) is commonly known as felty germander. It has been used for more than 2000 years in folk medicine.[1] In Jordan, it is known as Jaa'deh and it is one of the most prescribed plants by herbalists for diabetes, gastric spasms, and stomachache.[2] In addition, it is used for urinary tract inflammations, flatulence, indigestion, hypertension, obesity,[3] to treat kidney stones,[4] antinociceptive,[5] and for fertility problems.[6]

Phytochemical studies have revealed that *T. polium* contains several types of flavonoids such as salvigenin, cirsiliol,[7] luteolin, diosmetin, apigenin,[8] rutin,[9] cirsimaritin, and eupatorin.[10] In addition, steroidal compounds such as clerosterol, β-sitosterol, stigmasterol, brassicasterol, and campesterol[7,11] were isolated from different parts of *T. polium*. The oil of Jordanian *T. polium* contains 39 compounds. The main components were 8-cedren-13-ol (24.8%), β-caryophllene (8.7%), germacrene D (6.8%), and sabinene (5.2%).[12]

Most of the traditional uses of *T. polium* were investigated in vitro and in vivo using different animal models of disease such as its hypoglycemic and insulinotropic,[13] anti-inflammatory,[14] antinociceptive,[5] and antiulcer activites.[15] Furthermore, *T. polium* was reported to have potent antioxidant activity in vivo[16] as well as anticancer,[17] hypolipidemic[18] and anticonvulsant activities.[19]

In a previous study, Khleifat et al. reported a normal morphology of spermatozoa in rats treated with *T. polium* ethanolic extract collected from Jordan.[20] In fact, *T. polium* is not used only as herbal tea but its leaves are used also in cooking.[1] Up to our best knowledge, the effect of *T. polium* essential oil and nonpolar extracts on male fertility was not investigated previously. So, this work was designed to study

[1]Faculty of Pharmacy, Al-Ahliyya Amman University, Amman, Jordan
[2]Faculty of Pharmacy, Al-Ahliyya Amman University, Amman, Jordan

Corresponding author:
MA Abbas, Faculty of Pharmacy, Al-Ahliyya Amman University, Amman 19328, Jordan.
Email: manabbas@yahoo.com

the effect of both polar (alcoholic) extract and nonpolar (hexane) extract on male fertility and teratogenicity in rats. This study may highlight if the traditional use of this plant represents a real risk for the population or not.

Materials and methods

Plant material

T. polium aerial parts were collected from Deer Aala during April 2014. The plant was authenticated by Prof. Dr. Dawud Al-Eisawi (The University of Jordan, Faculty of Science/Department of Biological Sciences) and a voucher specimen was deposited at Al-Ahliyya Amman University-Laboratory of Graduate Studies (Abbas #Lam 4-2014). The aerial parts were dried at room temperature away from direct sunlight. Twenty-five grams of the ground aerial parts of *T. polium* were extracted in a soxhlet extractor (hot extraction) using 200 mL of 96% methanol and 200 mL of n-hexane sequentially. The extraction time was 4 h for each solvent. The resulting extracts were evaporated to dryness under reduced pressure using rotary evaporator and then stored at −20°C until used. Methanol and hexane were used to extract different phytochemicals (both hydrophilic and hydrophobic components) from this plant.

Animals

Animals were kept under standard animal house conditions with 12 h light/12 h dark cycle. Room temperature was kept at 23 ± 2°C. Food and tap water were provided *ad-libitum*. Animal handling and care was in accordance with the National Institutes of Health guide for the care and use of laboratory animals and approved by the animal welfare committee at the faculty of pharmacy/Al-Ahliyya Amman University.

Determination of the median lethal dose (LD_{50})

BALB/c male mice 6 weeks old weighing (22–25 g) were obtained from the animal house of Applied Science University, Jordan. The intraperitoneal (i.p.) LD_{50} for methanolic and hexane extracts was determined according to the method of Alawi and Jeryes.[21] After i.p. injection of plant extract, mice were observed continuously for 2 h and then occasionally during the next 22 h. Percentage mortality was recorded after 24 h.

Effect of T. polium extracts on spermatogenesis, sperm count, and testosterone level

Forty eight male Wistar rats, 8 weeks old, weighing (160–180 g) were used. Rats were randomly divided into six groups. Group I received 0.1 mL of 1/10 LD_{50} of the methanolic extract of *T. polium* intraperitoneally daily (methanol extract-high dose) while Group II was given 1/20 LD_{50} of the methanolic extract (methanol extract-low dose). Group III served as the control group for the methanolic extract and received its vehicle (normal saline). 1/10 LD_{50} of the hexane extract of *T. polium* was administered for Group IV (hexane extract-high dose). Group V rats received 1/20 LD_{50} of the hexane extract (hexane extract-low dose). Group VI (control group for hexane extract) was given 0.1 mL dimethyl sulfoxide (DMSO), the vehicle of the hexane extract. Treatment period lasted for 60 days.

After 60 days of treatment, 1 mL of blood was collected from retro-orbital plexus under diethyl ether anesthesia, centrifuged at 2500 rpm for 10 min and stored at −20°C until testosterone level was determined. Total testosterone level was measured at the Specialty Hospital (Amman, Jordan) using Chemiluminescent Microparticle Immunoassay technology (ARCHITECT Testosterone assay, Abbott). Animals were killed by cervical dislocation under ether anesthesia. Body and organ weights of rats were recorded. The cauda epididymis was opened by a blade in 4 mL Hank's balanced salts solution to exude epididymal contents and sperms were counted immediately using a hemocytometer. One testis was fixed with formalin for immunohistochemical staining. The other testis was fixed with Bouin's fluid to be used later for histopathological examination. Paraffin sections (5 μm thick) were prepared. Sections were examined by two consultant pathologists at MedLabs Consultancy Group, Amman, Jordan.

Immunohistochemical staining of androgen receptor

Formalin-fixed, paraffin-embedded sections of the testis were mounted onto coated slides (Biocare Medical, USA), placed in oven at 70°C for 20 min, and deparaffinized in xylene. Later, tissue was hydrated using descending concentrations of ethanol and finally washed with distilled water. For antigen retrieval, sections were then heated for 40 min in 0.01M citrate buffer, pH 6 in a water bath at 95°C according

to the method of Shi et al.[22] Streptavidin–biotin-peroxidase staining was performed using cell and tissue staining kit HRP-DAB System (R&D System, USA). Sections were incubated with anti-androgen receptor monoclonal mouse IgG antibody (R&D System, USA; 10 μg mL^{-1}) diluted in phosphate buffered saline, pH 7.4, at 4°C overnight in a humidity chamber. Then, sections were then incubated with biotinylated secondary antibody and the primary/secondary antibody reaction was detected using high sensitivity streptavidin conjugated to horseradish peroxidase and revealed by 3,3' diaminobenzidine chromogen. The sections were mounted in glycerol jelly medium (10 g gelatin powder, 60 mL distilled water dissolved by warming and then 70 mL glycerol and one drop of saturated aqueous solution of phenol was added). Sections were then examined under Nikon light microscope.

Determination of total testosterone in aging rats

Eight aging rats (14 months old) weighing 330–350 g were used. One milliliter blood was obtained from retro-orbital plexus before plant extract administration. Hexane-extract of *T. polium* was administered by i.p. injection for 6 consecutive days and again 1 mL blood was obtained from retro-orbital plexus at the end of treatment period for total testosterone determination.

Fertility study

Thirty-six virgin, nontreated Wistar female rats (7–8 weeks old) weighing (160–180 g) were used for mating the treated males. On the day 55th of treatment, each male rat was caged separately with one untreated adult female for 5 days. Mated females were housed individually. After birth, the number and weight of viable newborns were recorded. Fertility index was calculated by dividing the number of impreganted females by the total number of mated females. The pups were examined thoroughly for signs of physical deformity.

Qualitative analysis of phytochemical constituents

Test for flavonoids: *T. polium* extract was heated with 10 mL ethyl acetate for 3 min and filtered. The appearance of yellow color after the addition of 1 mL diluted ammonia solution to the filtrate indicates the presence of flavonoids.[23]

Test for tannins: About 0.1% ferric chloride reagent was added to plant extract, a blue-black coloration indicates the presence of tannins.[23]

Test for alkaloids: After heating *T. polium* extract with HCl for 20 min on a boiling water bath, the solution was filtered. One milliliter of the filtrate was treated with Mayer's reagent and then with Dragendroff's reagent. Appearance of turbidity indicates the presence of alkaloids.[23]

Test for terpenoids: *T. polium* extract was mixed with chloroform. Concentrated was gently added to the mixture. An interface with a reddish-brown coloration appears if terpenoids are present.[23]

Liebermann–Burchard test for the detection of phytosterols: *T. polium* extract was dissolved in chloroform and heated with glacial acetic acid and acetic anhydride. After cooling, few drops of concentrated H_2SO_4 were added along the side of the test tube. The appearance of a reddish violet color at the junction of the two layers and a bluish green color in the acetic acid layer indicates the presence of phytosterols.[23]

Statistical analysis

Statistical analysis was made with SPSS, 15th version. Relative testis weight, sperm count, number of newborns, weight of newborns and total testosterone after 60 days treatment were analyzed using one-way analysis of variance followed by least significant difference analysis. Testosterone level in aging rats was analyzed using *t*-test. All data were presented as mean $\pm$ standard error of the mean. In all statistical analysis tests differences were considered significant when $p < 0.05$.

Results

Determination of the median lethal dose (LD_{50})

The LD_{50} of methanolic extract was found to be 827.9 mg kg^{-1} and that of hexane extract was 812.8 mg kg^{-1}. Decrease in locomotor activity was observed in animals treated with both extracts compared with their control groups.

Effect of T. polium extracts on spermatogenesis, sperm count, and testosterone level

Treatment of male rats with *T. polium* extracts for 60 days resulted in a decrease in the relative testis weight of both animal groups treated with high and low doses of hexane extract ($p \leq 0.05$) in comparison to vehicle

Table 1. Relative testis weight, total serum testosterone, and epididymal sperm count after 60 days of treatment. Values are represented as mean ± SEM.

Treated groups	Relative testis weight (%)	Total serum testosterone (ng mL^{-1})	Epididymal sperm count (million cells mL^{-1})
Methanol extract (high dose)	0.50 ± 0.01	5.73 ± 1.69	47.6 ± 0.24[a]
Methanol extract (low dose)	0.49 ± 0.02	6.75 ± 2.32	48.2 ± 0.58[a]
Control group (0.9%NaCl)	0.48 ± 0.03	3.44 ± 1.04	57.6 ± 0.74
Hexane extract (high dose)	0.39 ± 0.03[a]	4.04 ± 0.92	22.4 ± 4.86[a]
Hexane extract (low dose)	0.39 ± 0.02[a]	3.02 ± 1.04	27.0 ± 1.94[a]
Control group (DMSO)	0.50 ± 0.02	3.13 ± 0.60	47.8 ± 1.24

DMSO: dimethyl sulfoxide.
[a]Statistically significant difference, $p \leq 0.05$.

(DMSO)-treated group. No such change was observed in animals treated with methanolic group (Table 1).

Both *T. polium* methanol and hexane extracts caused a reduction in sperm count (Table 1) compared to the control groups. The decline in epididymal sperm count was more pronounced in rats treated with hexane extract. On the other hand, administration of *T. polium* extracts for 60 days resulted in no statistically significant difference in total testosterone level (Table 1).

Histological study and immunohistochemical staining of androgen receptor

Testes sections showed regular seminiferous tubule with normal germinal epithelium morphology. Spermatozoa were present in the lumen in all treated groups. No granulomas, necrosis nor inflammation were seen in all treated and control groups (Figure 1). A clear reduction in androgen receptor immunostainig was observed in testis sections of rats treated with hexane extract compared to vehicle (DMSO)-treated group (Figure 2).

Determination of total testosterone level in aging rats that received hexane extract of T. polium for 6 days

Administration of *T. polium* hexane extract for 6 days to aging rats resulted in a significant increase in the serum total testosterone level. The mean total testosterone level was 0.40 ± 0.17 ng mL^{-1} before treatment and 2.11 ± 0.84 ng mL^{-1} 6 days after treatment.

Effect of T. polium extracts on fertility of male rats

Fertility index was 80% for the methanol high dosed animals while it was 100% for both controlled and low dosed animals (Table 2). On the other hand, both high and low hexane doses caused marked dropping in pregnancy rate compared to their control group injected with DMSO. Number and weight of newborns were not statistically different in animals received hexane and methanol extract compared with their control groups. No abnormalities in newborns were detected in any group.

Qualitative analysis of phytochemical constituents of T. polium

Methanolic extracts showed positive tests for the presence of flavonoids, tannins, alkaloids but not terpenoids. Hexane extract, on the other hand, gave positive results for the presence of alkaloids, terpenoids, and phytosterols but not flavonoids.

Discussion

In the present work, the effect of *T. polium* polar (methanol) extract and nonpolar (hexane) extract on the testis was investigated. Several authors have reported the effect of polar extracts of *T. polium* on testis but none have investigated the effect of nonpolar extracts. Relative testis weight, spermatogenesis, and total testosterone level of rats treated with *T. polium* methanol extract did not change significantly in the present study compared to their control group. Administration of ethanol extract of *T. polium*, collected from Saudi Arabia, caused a significant reduction in testis weight in mice treated for 90 days.[24] On the other hand, Mohammed reported an increase in testicular weight, Leydig cell, spermatogonia, spermatocytes, spermatozoa, and testosterone level after 8 weeks of oral treatment in mice.[6] The difference in results between the present study and the previous

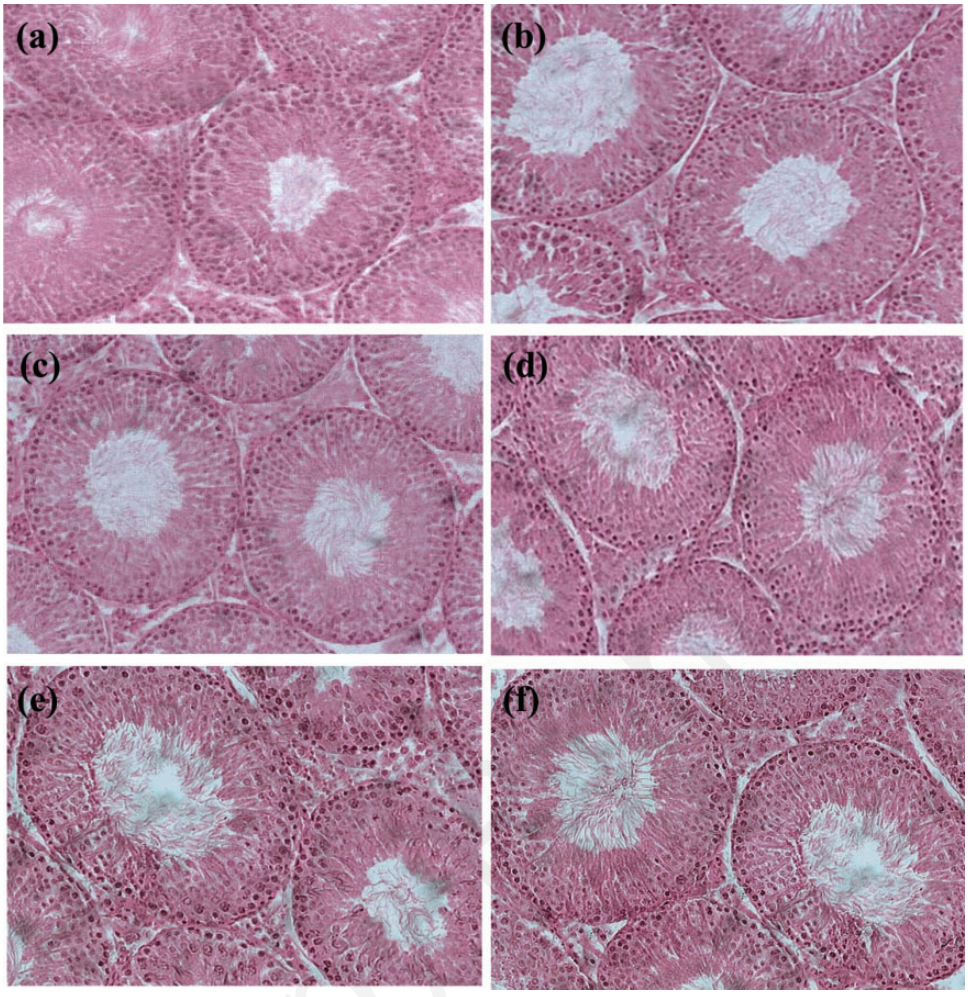

Figure 1. Testis cross sections. (a) Control (normal saline-treated group) showing normal interstitial cells and seminiferous tubules with lumen full of spermatozoa. (b) Methanol extract high dose. (c) Methanol extract low dose. In both (b) and (c) no signs of tissue damage were noted. (d) Control (DMSO) same as Figure1(a). (e) Hexane extract high dose and (f) hexane extract low dose. Normal germinal epithelium morphology is seen in (e) and (f). Spermatozoa are present in the lumen of seminiferous tubules and interstitial cells are intact (100×) (H&E stain). DMSO: dimethyl sulfoxide.

studies could be due to the use of different animal models, period of treatment, extraction method, route of administration, and/or variation in the phytochemical constituents of the plant due to different environmental and geographical factors.

Administration of *T. polium* hexane extract to male rats for 60 days resulted in a significant decrease in relative testis weight ($p \leq 0.05$) and sperm count compared to vehicle-treated group. The presence of phytosterols in hexane extract was confirmed using Liebermann–Burchard test. Several phytosterols were isolated from *T. polium* namely β-sitosterol, stigmasterol, campesterol, brassicasterol, and clerosterol.[11] According to Malini and Vanithakumari, β-sitosterol administration resulted in a reduction in the size of testes after 48-day treatment in male albino rats.[25] Therefore, the presence of phytosterols in hexane extract may explain, at least in part, the reduction in relative testis weight in hexane extract-treated animals recorded in this experiment.

In the present study, sperm count decreased in rats treated with methanol extract of *T. polium* and more noticeably in rats treated with hexane extract. In a previous study conducted in Jordan, Khleifat et al. injected rats intraperitoneally with ethanolic extract of *T. polium* for 6 weeks.[20] No change in sperm count was recorded. It is well known that the decrease in sperm count negatively affects semen quality. Therefore, male fertility will decline.[26] This explains the findings of the current study in which sperm count

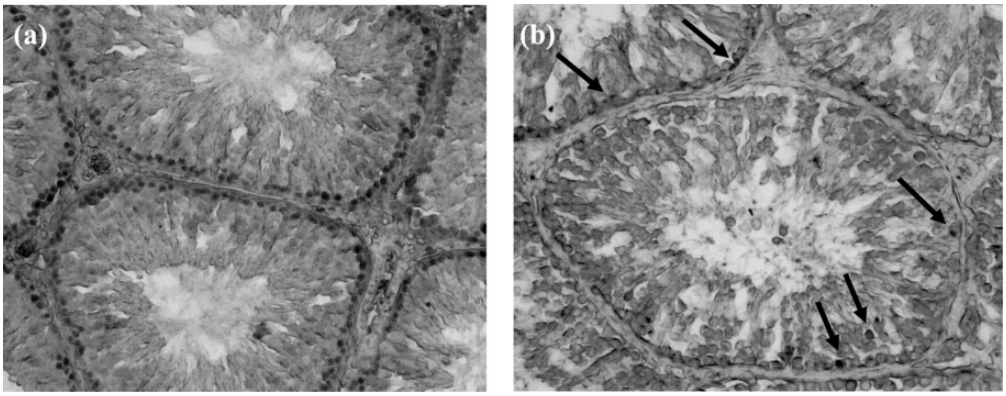

Figure 2. Immunohistochemical staining of androgen receptor in testis. (a) Testis of rat treated with vehicle (DMSO). Dark staining of cells indicates the presence of androgen receptor. (b) Testis of rat treated with hexane extract of *T. polium*. Note the weak staining in (b) which indicates the weak expression of androgen receptor (arrows). Magnification power (100×). DMSO: dimethyl sulfoxide.

Table 2. Results of fertility test.[a]

Treated groups	Fertility index (%)[b]	Average number of newborns (mean ± SEM)	Weight of newborns (g) (mean ± SEM)	Abnormalities in newborns
Methanol extract (high dose)	80	9.5 ± 0.86	6.3 ± 0.30	None
Methanol extract (low dose)	100	10.0 ± 0.89	5.87 ± 0.24	None
Control group (0.9%NaCl)	100	10.4 ± 0.87	5.82 ± 0.09	None
Hexane extract (high dose)	60	10.33 ± 1.76	6.43 ± 0.35	None
Hexane extract (low dose)	60	8.33 ± 2.90	6.35 ± 0.55	None
Control group (DMSO)	100	11.2 ± 0.66	6.4 ± 0.18	None

DMSO: dimethyl sulfoxide.
[a]No statistically significant difference was observed at $p \leq 0.05$.
[b]Fertility index (%) = (No. pregnant/No. copulated) × 100.

as well as fertility index decreased remarkably in hexane-treated rats.

Spermatogenesis was unaltered in rats treated with hexane and methanol extracts of *T. polium*. Therefore, other factors have affected sperm viability, for example, changes in epididymis. Future studies are needed to investigate the cause of this decrease in sperm count in *T. polium*-treated animals.

Al-Ashban et al. suggested that *T. polium* aqueous extract could have a mutagenic effect since it increased sperm abnormalities in male mice.[24] On the other hand, Khleifat et al. reported a normal morphology of sperms in rats treated with *T. polium* ethanolic extract.[20] In our study, methanolic and hexane extracts of *T. polium* were administered to males and those males were mated with untreated females. No gross malformations were detected in newborns.

Spermatogenesis depends on the presence of testosterone which exerts its action by the binding to androgen receptors in the testis.[27] In this study, androgen receptors were downregulated in testes of rats given *T. polium* hexane extract for 60 days. The downregulation of androgen receptors could be due to prolonged exposure to plant extract that elevated testosterone level over time. In conclusion, this study suggests that the prolonged use of *T. polium* in folk medicine may negatively affect male fertility.

In the present study, no histopathological changes in the liver and kidney were observed. This was in contrary to the findings of some other authors reporting hepatotoxic effects of this plant.[28]

T. polium extract increased testosterone level significantly in aging rats when administered for 6 consecutive days. Luteolin, apigenin,[8] and kaempferol[29] were reported to be present in *T. polium.* These active constituents possess aromatase (estrogen synthase) inhibitory activity and/or affected the expression of aromatase enzyme.[30,31] Testosterone is aromatized to

estrogens in male brain and the latter exerts a negative feedback on the secretion of gonadotropins.[32] If estrogen synthesis decreases, more gonadotropins are released. This will stimulate the testes to synthesize more testosterone. It is well documented that aromatase enzyme inhibition improves testosterone levels in human males.[31] Therefore, the effect of *T. polium* extracts on testosterone level could be due to aromatase (estrogen synthase) inhibition and/or the down-regulation of its gene expression. Further research is needed to explore the mechanism of action by which *T. polium* extract exerts its effect.

Declaration of Conflicting Interests

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