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Origanum vulgare as a Possible Modulator of Testicular Endocrine Function in Mice

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***Abstract*—**This study was designed to assess the *in vitro* effects of *Origanum vulgare* L. (oregano) extract on the testicular steroidogenesis. We focused on identifying major biomolecules present in the oregano extract, as well as to investigate its *in vitro* impact on the secretion of cholesterol, testosterone, dehydroepiandrosterone and androstenedione by murine testicular fragments. The extract was subjected to high performance liquid chromatography (HPLC) which identified cyranosid, daidzein, thymol, rosmarinic and trans-caffeic acid among the predominant biochemical components of oregano. For the *in vitro* experiments, testicular fragments from 20 sexually mature Institute of Cancer Research (ICR) mice were incubated in the absence (control group) or presence of the oregano extract at selected concentrations (10, 100 and 1000 μg/mL) for 24 h. Cholesterol levels were quantified using photometry and the hormones were assessed by ELISA (Enzyme-Linked Immunosorbent Assay). Our data revealed that the release of cholesterol and androstenedione (but not dehydroepiandrosterone and testosterone) by the testicular fragments was significantly impacted by the oregano extract in a dose-dependent fashion. Supplementation of the extract resulted in a significant decline of cholesterol ($P < 0.05$ in case of 100 μg/mL; $P < 0.01$ with respect 100 μg/mL extract), as well as androstenedione ($P < 0.01$ with respect to 100 and 1000 μg/mL extract). Our results suggest that the biomolecules present in *Origanum vulgare* L. could exhibit a dose-dependent impact on the secretion of male steroids, playing a role in the regulation of testicular steroidogenesis.

***Keywords*—**Mice, *Origanum vulgare* L., steroidogenesis, testes.

I. Introduction

THE use of medicinal herbs in the management of health complications has been recorded for centuries now, and plant-based therapy has played a significant role in the development of a variety of contemporary pharmaceutical products. At the same time, as much as 60 % of the world's population takes advantage of bioactive ingredients originated from plants for medical reasons [1]. The World Health Organization (WHO) is a significant supporter of the use of medicinal plants and persuades scientists to devote more attention to a rational use of ethnopharmacological herbs and their products as a potential source of new medicaments [2].

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Throughout history, numerous plants have been traditionally used to counteract male reproductive dysfunction based on their beneficial effects on the endocrine as well as spermatogenic function of the testes. Malaysian ginseng (*Eurycoma longifolia*), a native Southeast Asian plant, has been shown to increase libido and stimulate the production of androgen hormones, such as testosterone hence to improve male sexual health [3]. Puncturevine (*Tribulus terrestris*), a tropical plant used in traditional folk medicine, is touted as a testosterone booster and remedy for impaired erectile function. At the same time, it is used to support normal testosterone production in men, especially once andropause starts [4]. Ashwagandha (*Withania somnifera*), a traditional Indian medical plant, is used in form of teas, extracts, and capsules to increase the sperm concentration and motility, as well as to enhance testosterone synthesis among infertile men [5]. Finally, Yohimbe (*Pausinystalia yohimbe*) may benefit people with low testosterone and associated symptoms. A study found that this herb may be as effective as sildenafil (Viagra) for erectile dysfunction in rats. Both drugs exhibit comparable effects on the central nervous system, such as an increased sexual arousal [6].

Oregano (*Origanum vulgare* L.) is a widespread plant rich in phenolic compounds with a variety of therapeutic effects. Belonging to the terpenes, oregano extracts and essential oils are predominantly constituted by monoterpenes and sesquiterpenes with antimicrobial, antifungal, anti-inflammatory and antioxidant properties [7]-[9]. Oregano leaves are a part of both traditional as well as official medicine. The silica should be composed of at least 60% of carvacrol and thymol, and it is used to treat nervousness, headache, migraines and anxiety. Moreover, side effects were not observed even in case of long-term administration of oregano extracts and oils. However, *Origanum* should be avoided during pregnancy, due to it enhancing effects on uterine contractions, leading to possible premature birth or miscarriage [10].

A number of *in vivo* studies have suggested that oregano extracts could have the ability to stimulate male reproductive capacity at the levels of spermatogenesis as well as steroidogenesis [11], [12]. *In vitro* studies on cell cultures and tissues are nevertheless useful tools to thoroughly assess the behavior of biomolecules because of their uniform functional features and increased sensitivity. As the steroid hormones play a crucial role in the development and function of male sex, it is necessary to systemically assess the effect of plant extracts on their synthesis. We are unaware of any reports examining a direct in vitro effect of the *Origanum* extract on

the endocrine processes involved in male fertility. Hence, the aim of this study was to assess the impact of selected concentrations of the oregano extract on the secretion of cholesterol, testosterone, dehydroepiandrosterone and androstenedione by mouse testicular fragments. At the same time, we determined the predominant biomolecules of the *Origanum* extract and associated their presence with their potential to modulate testicular steroidogenesis.

II. Material and Methods

A. Plant Material Collection and Extract Preparation

Origanum leaves were provided during the summer of 2017. The plant material was dried, crusAhed, weighed and soaked in ethanol (96 %, Centralchem, Slovakia). The maceration took two weeks (20-22 °C, dark conditions). The resulting ethanolic extract was subjected to evaporation under reduced pressure at 40 °C (Stuart RE300DB rotary evaporator, Bibby Scientific Limited, UK) to dispose of the residual ethanol. The crude extract was dissolved in DMSO (dimethyl sulfoxide; Sigma-Aldrich, USA) and adjusted to 1000 mg/mL (stock solution) [13].

For the chemical assessment, the fresh oregano leaves were freeze dried and pulverized. Methanol extract for the HPLC analysis was prepared by blending 1 g of the plant material with 25 mL 80 % methanol (HPLC grade; Sigma-Aldrich), followed by shaking the mixture on a horizontal shaker (250 rpm, 20-22 °C, 8 h). Finally, the extract was filtered through filter paper (84 g/m^2; Munktell, Germany) and kept at 5 °C for further analysis.

B. HPLC-DAD Analysis

HPLC grade standards, acetonitrile (gradient HPLC grade), methanol (HPLC grade) and phosphoric acid (ACS grade) were obtained from Sigma-Aldrich. Double deionized water (ddH_2O) was prepared using a Simplicity 185 purification system (Millipore SAS, France). Standard solutions were obtained by dissolving 0.5 mg each in 10 mL methanol.

Origanum extract prepared beforehand and the standard solutions were filtered through Munktell No 390 paper (Munktell & Filtrac, Germany) and stored in closed 20 mL tubes. Prior to injection all liquids were filtered through the Q-Max syringe filter (0.22 mm, 25 mm; Frisenette ApS, Denmark).

Chemical assessment of the oregano extract was carried out using the Agilent 1260 Infinity HPLC (Agilent Technologies, Germany) with a Purosphere reverse phase C18 column (4 mm x 250 mm x 5 mm) (Merck, KGaA, Germany). The mobile phase carried acetonitrile (A) and 0.1% phosphoric acid in ddH_2O (B). The gradient elution consisted of: 0-1 min isocratic elution (20% A + 80% B), 1-5 min linear gradient elution (25% A + 75% B), 5-15 min (30% A + 70% B) and 20-25 min (40% A + 60% B). The initial flow rate was set at 1 mL/min with 10 mL injection volume. The column oven temperature was adjusted to 30 °C while the samples were kept at 4 °C in the sample manager. All data were collected and processed using the Agilent OpenLab ChemStation software for LC 3D Systems [14].

C. Testicular Tissue Collection and Cultivation

Male ICR mice (n = 20, 9-10 weeks old) were purchased and subsequently housed in plastic cages at 24±1 °C with a 12 h light/12 h dark photoperiod. The animals were provided with a standard pellet diet and water *ad libitum*. Institutional and national guidelines for the use of animals were followed, and all experiments were approved.

The animals were anesthetized by cervical dislocation, the testes were immediately collected and deposited into a sterile Petri dish with fresh, ice-cold PBS (Dulbecco's phosphate buffered saline, Sigma-Aldrich). Using tweezers and a blade knife, the testes were dissected into four approximately equal fractions. The obtained testicular fragments were washed twice with PBS and cultured in 0.5 mL DMEM (Dulbecco's Modified Eagle Medium, Sigma Aldrich) supplemented with 10 % fetal bovine serum (Sigma Aldrich) and 1 % antibiotic-antimycotic solution (Sigma Aldrich) and in the absence (control group) or presence of the *Origanum* extract supplemented at various concentrations (10, 100, 1000 µg/mL) for 24 h. The incubation was performed in 48-well plates at 37 °C, 95 % air and 5 % CO_2.

D. Biochemical Analyses

Once the *in vitro* culture had ended, the medium was removed from the plates and centrifuged (5000 RPM, 4 °C, 10 min). The supernatant was used for the quantification of cholesterol and hormones.

Cholesterol levels were quantified using the DiaSys commercial kit (Diagnostic Systems, Germany) and the Rx Monza semiautomatic photometer (Randox Laboratories, UK). The concentration of dehydroepiandrosterone (DHEA-S), testosterone and androstenedione in the media was determined by ELISA. The ELISA kits were purchased from Dialab (Austria). The absorbance was measured at 450 nm using Glomax Multi+ plate spectrophotometer (Promega, USA). Quantification of hormones was performed in duplicate.

E. Biochemical Analyses

Statistical assessment was carried out with GraphPad Prism 3.02 (GraphPad Software Incorporated, USA). One-way analysis of variance (ANOVA) and the Dunnett's test were used for statistical evaluations. The results are expressed as mean ± standard error of the mean (SEM). The level of significance was set at ***($P<0.001$) **($P<0.01$) and *($P<0.05$).

III. Results and Discussion

Knowledge obtained from the use of medicinal plants and their products traditionally used in ayurvedic medicine may be sorted taking into account a hierarchy of significance. Logically, data collected from complex *in vivo* studies come along with the highest value to the comprehension of the potential beneficial or adverse effects plant products may exhibit on the living organism. Nonetheless, a wide array of properties of plants and their products may be studied in depth

in vitro before being considered for any *in vivo* investigation. While such reports may reveal the possible shortcomings of *in vitro* research, suitably executed *in vitro* experiments have the potential to provide valuable information, and to serve as a sensitive predictor of clinical performance. In case of our study, *in vitro* assessments may provide more insight into the contradictory or controversial information gathered from previous investigations focused on the impact of plant extracts and/or oils on a biological system [15].

In this report, we assessed the biochemical composition of the *Origanum vulgare* L. extract and subsequently investigated its *in vitro* effects on the endocrine activity of mouse testicular tissue.

A. HPLC Analysis

Dominant biomolecules present in the oregano extract were identified and quantified by HPLC. The concentrations of the major chemical components identified are provided in Table I. The main biomolecule detected in the extract was cyranosid, followed by daidzein and thymol. Among the analyzed phenolic acids, rosmarinic, trans-caffeic and neochlorogenic acid were the most abundant.

TABLE I
MAJOR CHEMICAL COMPOUNDS IDENTIFIED AND QUANTIFIED [MG/KG] IN THE OREGANO EXTRACT

Cynarosid	280.32±8.55
Apigenin	5.56±0.66
beta-pinene	8.99±1.03
Carvacrol	20.22±1.55
Daidzein	77.09±6.44
Eucalyptol	4.78±0.92
gamma-terpinene	16.77±3.05
Chlorogenic acid	7.88±0.90
Kaempferol	16.88±3.43
Neochlorogenic acid	49.03±3.55
p-cymene	3.88±0.82
Rosmarinic acid	207.77±8.21
Rutin	38.82±3.09
Sinapinic acid	16.77±3.05
Thymol	48.03±3.98
trans-Caffeic acid	52.08±4.08
trans-Ferulic acid	8.66±1.56
trans-p-Coumaric acid	5.60±1.01
Vitexin	33.09±3.99

(Mean ± SEM; n=3)

Previous data are available with respect to the biochemical composition and biological properties of oregano and its products. A comprehensive evaluation of biomolecules present in *Origanum vulgare* extracts and oils was provided by Teixeira et al. [8] and Vazirian et al. [9]. Similarly to our results, thymol, gama-terpinene, carvacrol, eucalyptol and p-cymene were the predominant components identified in previous reports. In another study, focusing on the variations in the chemical composition of the essential oil of *Origanum vulgare* collected in Lithuania in flowering and seeding stages, linalyl acetate, gamma-terpinene, beta-pinene and carvacrol were the main components of the flowering stage, while carvacrol, alpha-pinene, beta-pinene and trans-caryophyllene were the main compounds of the seeding stage of the plant [16]. According to Teixeira et al. [8], hot water extracts prepared from oregano had the strongest antioxidant properties and the highest phenolic content. On the other hand, the essential oil proved to have the highest antibacterial activity, inhibiting the growth of all tested pathogenic bacteria and causing greater reductions on *Listeria*. Vazirian et al. [9] furthermore state that the plant could be a good natural antioxidant preservative.

B. Biochemical Analyses

In the second part of this study we assessed the *in vitro* impact of *Origanum vulgare* extract on the steroidogenic activity of mouse testicular fragments.

As a building block of sex hormones cholesterol plays a crucial role in male steroidogenesis. As shown in Fig. 1, the cholesterol levels were significantly different between the control (0.51±0.16 μg/mL) and experimental groups (0.31±0.06 μg/mL in case of 10 μg/mL extract; 0.27±0.08 μg/mL with respect to 100 μg/mL extract; 0.12±0.02 μg/mL in relation to 1000 μg/mL extract). We detected a significant decrease of the cholesterol quantity, depending directly on the dose of extract used ($P<0.05$ in case of 100 μg/mL; $P<0.01$ with respect to 1000 μg/mL extract).

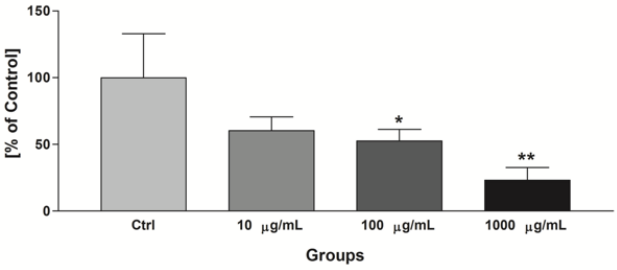

Fig. 1 The *in vitro* impact of the oregano extract on the cholesterol levels in mouse testicular fragments; Each bar represents mean (± SEM) values expressed as % of the control and experimental groups. The results were collected from four separate experiments **($P<0.01$); *($P<0.05$).

Similarly to cholesterol, androstenedione secretion was affected significantly, particularly in case of 100 and 1000 μg/mL extract ($P<0.01$, Fig. 2). The levels of androstenedione decreased depending on the concentration of the extract used in the experiment (57.21±5.14 ng/mL in case of 10 μg/mL extract; 29.23±1.71 μg/mL with respect to 100 μg/mL extract; 25.61±2.25 μg/mL in relation to 1000 μg/mL extract) when compared to the control (88.50±8.25 ng/mL).

As shown in Fig. 3 the dehydroepiandrosterone levels were not significantly different between the control (9.79±0.10 μg/mL) and experimental groups (9.39±0.90 μg/mL with respect to 10 μg/mL extract; 8.83±1.00 in case of 100 μg/mL extract; 9.97±0.10 μg/mL extract; $P>0.05$). The repeated analysis showed that the *Origanum vulgare* extract does not affect the quantity of dehydroepiandrosterone in the medium.

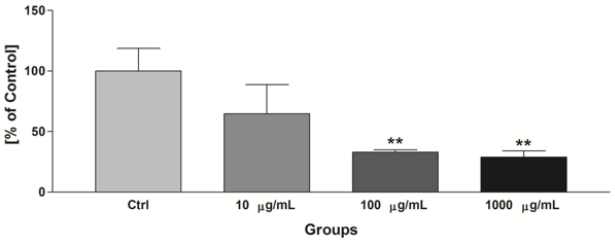

Fig. 2 The *in vitro* impact of the oregano extract on the androstenedione production in mouse testicular fragments; Each bar represents mean (± SEM) values expressed as % of the control and experimental groups. The results were collected from four separate experiments **(P<0.01); *(P<0.05).

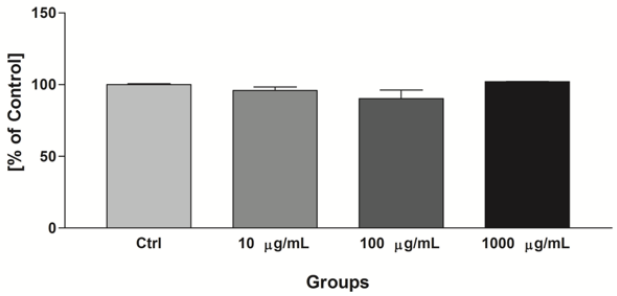

Fig. 3 The *in vitro* impact of the oregano extract on the dehydroepiandrosterone levels in mouse testicular fragments; Each bar represents mean (± SEM) values expressed as % of the control and experimental groups. The results were collected from four separate experiments **(P<0.01); *(P<0.05).

Similarly, the testosterone concentrations were not significantly affected by the exposure to the *Origanum* extract (P>0.05). A minor decrease of testosterone production was recorded at all experimental groups (18.50±0.91 ng/mL in case of 10 μg/mL extract; 16.42±1.73 ng/mL with respect to 100 μg/mL extract; 18.00±1.78 ng/mL in relation to 1000 μg/mL extract), without significant changes (P>0.05) in comparison with the control (18.87±1.14 ng/mL). The analysis showed that the oregano extract does not affect the quantity of testosterone in the medium (Fig. 4).

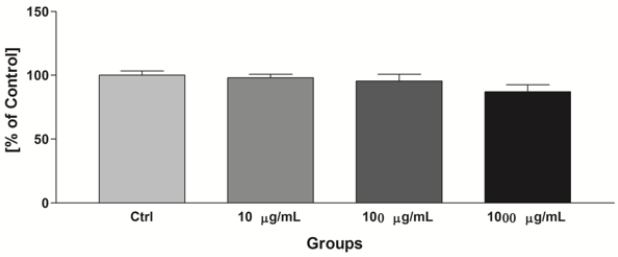

Fig. 4 The *in vitro* impact of the oregano extract on the testosterone levels in mouse testicular fragments; Each bar represents mean (± SEM) values expressed as % of the control and experimental groups. The results were collected from four separate experiments **(P<0.01); *(P<0.05).

As far as we are concerned, no study exists on the assessment of testicular steroidogenesis *in vitro* following exposure to the *Origanum* extract. A number of *in vivo* reports have studied the effects of oregano extracts or essential oils on the male reproductive system in rats. Hollenbach et al. [17] observed no difference in weight gain between the experimental groups treated with oregano essential oil and the control, supporting the hypothesis of no systemic toxicity of *Origanum*. Positive effects of thymol and carvacrol on male reproduction were also stated by Guvenc et al. [11], revealing that the administration of both major chemical components isolated from oregano had no negative effects on the testicular histopathology. Furthermore, exposure to both biomolecules *in vivo* decreased the oxidative damage to spermatozoa and improved their quality parameters. What is more, Elsawy et al. [12] state that *Origanum vulgare* treatment (20 mg/kg) prevented as well as reversed toxic changes to male reproductive tissues and cells of rats exposed to ethylene glycol. Nevertheless, our *in vitro* study shows that the *Origanum* extract does exhibit significant modulatory effects on the endocrine activity by decreasing the levels of cholesterol as well as androstendione by rat testicular fragments. A similar observation was noted by Hollenbach et al. [17]. In their report, the *Origanum* essential oil acted directly on sexual organs, reducing their weight and causing tissue injury of the testes. At the highest dose (27% V/V), these changes could be associated with metabolic disorders, as testosterone levels were decreased as well. The direct action of oregano could have been exhibited on the Leydig cells, affecting testosterone production as suggested by Chen et al. [18]. Changes in Leydig cells as well as the testicular vascular system could be considered as indirect effects resulting in subfertility [19].

As a steroid hormone, testosterone originates from cholesterol, involving two possible pathways. The first step in male steroidogenesis involves cleavage of a side-chain of cholesterol catalyzed by P450scc (CYP11A1), leading to pregnenolone formation. Pregnenolone may be transformed to progesterone, and subsequently to androstenedione by 3β-hydroxysteroid dehydrogenase (3β-HSD). The final step involves a reduction of androstenedione to testosterone by 17β-hydroxysteroid dehydrogenase (17β-HSD) (Δ-4 pathway). The Δ-5 pathway involves the conversion of pregnenolone to DHEA-S by 17β-hydroxylase and 17,20-lyase. DHEA-S will be subsequently transformed to androstenediol followed by testosterone by 3β-HSD [20].

In this study, a significant dose-dependent decrease of cholesterol was recorded. In theory, its decline should lead to a subsequent decrease of all hormones observed; however, this did not occur. We may hypothesize that the declining levels of cholesterol had no effect on the Δ-5 pathway, which was translated into non-significant changes in the production of DHEA-S. As such, this intermediate may become the central source for testosterone secretion. On the contrary, the decline of cholesterol is well-represented by a significant decrease of androstenedione, the central intermediate of the Δ-4 pathway. Based on this observation, we may speculate that the biocomponents of the oregano extract could have impacted the

natural balance of the testicular steroidogenesis, leaning towards the Δ-5 pathway and resulting in lower levels of cholesterol available for androstenedione secretion. Cholesterol is a crucial molecule for the synthesis of all sex hormones, and it is a fact that its low availability contributes to poor steroidogenesis [21]. In theory, the decreasing concentrations of cholesterol should have had an impact on the dynamics of male steroidogenesis; however, the end product (testosterone) was not significantly affected in this study. We may argue that although relatively low, cholesterol levels were still within acceptable limits to provide substrate for subsequent steroidogenic transformations. Moreover, we may assume that the Δ-5 pathway was less affected by the biomolecules present in *Origanum*, and thus compensated for the imbalance caused by the exposure to the extract. A similar hypothesis was postulated by Jambor et al. [22]. Nevertheless, to verify this assumption, more intermediates of both pathways (progesterone and androstenediol) should be investigated. Even though a non-significant, but a decreasing trend was observed in case of both DHEA-S and testosterone, which could become more noticeable with increasing time of the *in vitro* culture. Thus, it may be feasible to prolong the culture of testicular fragments in the presence of the oregano extract in order to re-evaluate its impact on the steroidogenesis throughout a longer timeframe.

Interactions of biomolecules present in the oregano extract are another question to be answered in further studies. While it has been revealed that thymol, carvacrol and terpinen-4-ol present in the oregano extract may exhibit beneficial effects on the male reproductive system [12], [23], [24], it is important to assess their synergy or antagonism in a complex mixture such as a plant extract or essential oil. At the same time, future *in vitro* studies are highly encouraged in order to assess the impact of medicinal herbs on specialized cells and tissues which may help to predict their behavior in a living organism.

IV. Conclusion

Our preliminary results provide more specific evidence on the biological activity of the *Origanum vulgare* extract on male reproductive structures. The use of medicinal herbs with numerous biological properties in practical andrology is highly anticipated. Our report obviously cannot predict a definitive *in vivo* or *in vitro* outcome since a direct impact of the oregano and its products on male reproductive performance needs to be evaluated further. As such, assessments of the toxicity, pharmacokinetics and bioavailability of oregano and its bioactive components in relation to the functional activity of the male reproductive system are indispensable.

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