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LEVISTICUM OFFICINALE AND ITS EFFECTS ON BOVINE SPERMATOZOA ACTIVITY

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ABSTRACT

Lovage (*Levisticum officinale*) is a versatile medical, aromatic and spice plant of the family *Apiaceae*. Its extract has the ability to decrease blood pressure, to support digestion and bile secretion, to regulate irregular menstrual cycle and to act as a gentle aphrodisiac. The aim of this study was to identify major chemical components of the lovage extract followed by a comprehensive evaluation of its *in vitro* effects on selected quality parameters of bovine spermatozoa. Lovage extracts were subjected to high performance liquid chromatography (HPLC) which identified rutin, kaempferol, chlorogenic acid and neochlorogenic acid among the most dominant chemical components of the plant material. For the *in vitro* experiments, 10 ejaculates from sexually mature bulls were exposed to solutions with a gradually increasing concentration (300; 150; 75 and 37.5 µg/mL) of the extract. All analyses were performed following 0, 2, and 24 hours of *in vitro* culture. The motility evaluation was done using the computer-assisted sperm analysis (CASA) method, which revealed that higher concentrations of lovage extract (especially 300 µg/mL) had an immediate negative effect when compared to the control group ($P<0.01$), while 75 and 37.5 µg/mL had an immediate stimulating effect. The sample supplemented with 75 µg/mL extract showed a satisfactory motility even after 24 hours of incubation when compared to the rest of the samples. Cell viability is closely related to the motility, which were evaluated using the mitochondrial MTT test. Low concentrations of lovage stimulated the viability of the sperm cells while higher concentrations had an inhibitory effect ($P<0.05$; $P<0.001$). The NBT test was used to assess the superoxide radical production. The experimental data confirm the potential antioxidant effect of lower doses of lovage ($P<0.05$) and prooxidant properties of higher concentrations. The results generally suggest the beneficial effects of lower concentrations of the lovage extract and toxic effects of higher doses, providing a solid foundation for further research on the effects of lovage on male reproduction.

Keywords: lovage, male gametes, motility, mitochondrial activity, bull, free radicals

INTRODUCTION

Herbs have been used since ancient times for their medicinal or aromatic properties. An increased interest in using natural preservatives as alternatives to chemicals has brought a renewed attention towards aromatic plants (**Semeniuc *et al.*, 2017**). Culinary aromatic herbs are a good source of essential nutrients such as vitamins and minerals, as well as antioxidant compounds. Numerous studies have shown that high intake of plants, which are the source of antioxidants, could be associated with a lower incidence of cardiovascular diseases, cancer and other chronic pathologies. Bioactive compounds that are the key natural aromatic and fragrant ingredients of different spices and other aromatic plants, play an important role in human and animal health (**Nour *et al.*, 2017**). Over the past 20 years 61% of new chemically active substances derived from natural products have been discovered, leading to the re-introduction of medicinal plants into high-scale cultivation, breeding and their processing into foodstuffs and health products. Plant extracts, which are nowadays a popular option to increase the use of medical plants in medicine, biology, nutrition and agriculture, are usually a mixture of chemically diverse substances (**Sertel *et al.*, 2011**). Currently, their bioactive compounds and essential oils are used in active packing preparations for preservation purposes. Essential oils can be extracted from different parts of herbs by several techniques, such as distillation with water or steam, extraction using a suitable solvent, pressure or water extraction. The main constituents of essential oils are usually mono- and sesquiterpene together with carbohydrates, phenols, alcohols, ethers, aldehydes and ketones which are responsible for the biological activity of aromatic and medicinal plants as well as their aroma (**Semeniuc *et al.*, 2017**).

Several medicinal plants are empirically used to treat different aspects of male reproductive dysfunction, such as sexual asthenia, libido, erectile and ejaculatory disorders, as well as sperm abnormalities (azoospermia, oligospermia). Biological activities of many of these plants have been confirmed by in vitro, and/or in vivo animal studies as well in humans. At the same time, such natural additives may considerably improve the semen quality insemination by protecting the sperm function and fertilization ability, and in a more cost-effective way.

Lovage (*Levisticum officinale*) is a lasting, aromatic herb. Originally from Asia, the plant is also found in South Asia, Southern and Central Europe, where it has been cultured since the 12th century. All parts of this herb are strongly aromatic, the lute is grown for its seeds, leaves, roots and for its ethereal oils, which are used in perfumery, food, beverage and tobacco industries (**Santos *et al.*, 2005**).

Fresh and dried lovage gooseberries are used in households as a substitute for "Maggi" spices for soups, sauces, pasta and meat. Fruits can also be used as root vinegar infusions for vegetables and mushrooms. The fruits may be whole or milled, added to bread, pastries, liqueurs and spirits.

The bioactive compounds found in lovage primarily exhibit diuretic and carminative effects. Lovage silicas are therapeutically used to help with urinary tract diseases, general weakness, chloride retention, and in case of elevated values of uric acid in the blood. Silica components support the formation of gastric juices and digestive enzymes, therefore the drug is used to treat dyspepsia associated with inadequate activity of the digestive organs. Lovage silica may be also used in perfumery, liqueur industry and as a bath additive. It is used for aromatization of some tobacco mixtures (**Ekiert, 2000**). In the pharmaceutical industry lovage is used for the preparation of phytopharmaceuticals (galenic and diuretic-acting preparations) (**Santos *et al.*, 2005**).

Based on a convincing evidence on a wide array of beneficial effects of lovage on the biological system, the aim of this study was a comprehensive evaluation of the major components of the *Levisticum officinale* plant extract, followed by the assessment of the *in vitro* effects of medical lovage on the motion parameters, mitochondrial integrity, and superoxide radical formation of bovine spermatozoa.

MATERIAL AND METHODS

Plant material collection and processing

Lovage leaves were harvested at the Botanical Garden of the Slovak University of Agriculture in Nitra, Slovakia at the end of July 2016. After drying, the leaves were crushed, weighed and soaked in ethanol (96 %, Centralchem, Bratislava, Slovakia) during two weeks at room temperature in the dark in order to prevent the degradation of active biomolecules. The ethanolic extracts were subjected to evaporation under reduced pressure at 40 °C (Stuart RE300DB rotary evaporator, Bibby Scientific Limited, UK, and vacuum pump KNF N838.1.2KT.45.18, KNF, Germany) to remove any residual ethanol. Crude extracts were dissolved in DMSO (Dimethyl sulfoxide; Sigma-Aldrich, St. Louis, USA) and adjusted to 1000 mg/mL as stock solution (**Tvrdá *et al*., 2016**).
For the chemical analysis, the leaves were freeze dried and milled. Methanol extracts were produced by adding 25 mL 80 % aqueous methanol (HPLC grade; Sigma-Aldrich) to 1 g of each sample. The mixtures were shaken on a horizontal shaker (250 rpm) at room temperature for 8 h. The samples were then filtered through filter paper (84 g/m^2; Munktell, Germany) and kept at 5 °C for further analysis.

HPLC-DAD analysis

Standards, methanol (HPLC grade), acetonitrile (gradient HPLC grade) and phosphoric acid (ACS grade) were purchased from Sigma-Aldrich. Double deionized water (ddH_2O) was treated (0.054 mS/cm^1) in a Simplicity 185 purification system (Millipore SAS, Molsheim, France). Standard solutions were prepared by dissolving 0.5 mg each of them with methanol in 10 mL. Following homogenization the lyophilized samples (2 g) were extracted with 20 mL of 80% methanol at laboratory temperature for 8 h by horizontal shaker (Unimax 2010; Heidolph Instrument GmbH, Germany). The extract was filtered through Munktell No 390 paper (Munktell & Filtrac, Germany) and stored in closed 20 mL vial tubes. Prior to injection the standard solutions and extracts were filtered through the Q-Max syringe filter (0.22 mm, 25 mm; Frisenette ApS, Knebel, Denmark) (**Bajčan *et al*., 2016**).
Chemical composition of the lovage extract was determined using the Agilent 1260 Infinity high performance liquid chromatograph (Agilent Technologies, Waldbronn, Germany) with quaternary solvent manager coupled with degasser (G1311B), sample manager (G1329B), column manager (G1316A) and DAD detector (G1315C). All HPLC analyses were performed on a Purosphere reverse phase C18 column (4 mm x 250 mm x 5 mm) (Merck, KGaA, Darmstadt, Germany). The mobile phase consisted of acetonitrile (gradient) (A) and 0.1% phosphoric acid in ddH_2O (B). The gradient elution was as follows: 0-1 min isocratic elution (20% A and 80% B), 1-5 min linear gradient elution (25% A and 75% B), 5-15 min (30% A and 70% B) and 20-25 min (40% A and 60% B). The initial flow rate was 1 mL/min1 and the injection volume was 10 mL. Column oven temperature was set up to 30 °C and the samples were kept at 4 °C in the sample manager. The data were collected and processed using the Agilent OpenLab ChemStation software for LC 3D Systems (**Luksic *et al*., 2016**).

Semen sample collection and processing

Semen samples (n = 20) were obtained from four adult Holstein Friesian breeding bulls (Slovak Biological Services, Nitra, Slovak Republic). One ejaculate was collected from each bull on a regular collection schedule (once a week for five consecutive weeks) using an artificial vagina. Following collection, sperm concentration and motility were assessed using phase-contrast microscopy (200 x). Only ejaculates with the required quality (minimum 70 % motility and concentration of 1×10^9 sperm/mL) were used for the subsequent experiments. Institutional and national guidelines for the care and use of animals were followed, and all experimental procedures were approved by the State Veterinary and Food Institute of Slovak Republic (no. 3398/11-221/3) and Ethics Committee.
Each sample was diluted in PBS (Dulbecco's Phosphate Buffer Saline without calcium chloride and magnesium chloride; Sigma-Aldrich) containing various concentrations of the lovage extract (300; 150; 75; 37.5 µg/mL) using a dilution ratio of 1:40. The specific concentrations were selected upon similar studies on the *in vitro* effects of plant extracts on male reproductive cells, which have suggested an optimal concentration range oscillating between 300 and 20 µg/mL for a complex and meaningful short- as well as long-term *in vitro* analysis of sperm vitality parameters (**Tvrdá *et al*., 2016;2018**). The samples were cultured at laboratory temperature (22-25°C). After culture periods of 0, 2 and 24h, spermatozoa motility, mitochondrial activity and superoxide production were assessed in each group.

Spermatozoa motility analysis

Spermatozoa motion characteristics were assessed using the computer-aided sperm analysis (CASA; Version 14.0 TOX IVOS II.; Hamilton-Thorne Biosciences, Beverly, MA, USA). The system was set up as follows: frame rate - 60 Hz; minimum contrast - 20; static head size - 0.25-5.00; static head intensity - 0.40-2.00; static elongation - 20-100; default cell size - 4 pixels; default cell intensity - 40. Ten µL of each sample were placed into the Makler counting chamber (depth 10µm, 37 °C; Sefi Medical Instruments, Haifa, Israel) and spermatozoa motility (MOT; percentage of motile spermatozoa; motility >5 m/s; %) was evaluated immediately. Ten microscopic fields were subjected to each analysis to include at least 300 cells (**Tvrdá *et al*., 2018**).

Mitochondrial activity (MTT test)

Spermatozoa mitochondrial activity was evaluated using the colorimetric metabolic activity (MTT) test, which is based on the conversion of a yellow tetrazolium salt (3-(4,5-dimetylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; MTT) to blue formazan particles by mitochondrial succinate dehydrogenase of intact mitochondria within living cells. The tetrazolium salt (Sigma-Aldrich) was dissolved in PBS (Dulbecco's Phosphate Buffer Saline without calcium chloride and magnesium chloride; Sigma-Aldrich) at 5 mg/mL. Ten µL of the tetrazolium solution was added to each sperm suspension. After a 2 h incubation (shaker, 37 °C, 95 % air atmosphere, 5 % CO2), the formazan crystals were dissolved in 80 µL of acidified (0.08 mol/L HCl; Centralchem) isopropanol (Centralchem). Optical density was determined at a wavelength of 570 nm against 620 nm as reference using a Multiskan FC microplate photometer (Thermo Fisher Scientific Inc., Waltham, MA, USA). Data are expressed as percentage of the control set to 100 % (**Knazicka *et al.*, 2012**).

Quantification of the superoxide production (NBT test)

The nitroblue-tetrazolium (NBT) test was used to quantify the intracellular formation of the superoxide radical, by assessing blue NBT formazan deposits, generated by the reduction of the membrane permeable, yellow-colored, nitroblue tetrazolium chloride (2,20-bis(4-Nitrophenyl)-5,50-diphenyl-3,30-(3,30-dimethoxy-4,40-diphenylene) ditetrazolium chloride; Sigma-Aldrich) by the superoxide radical. The NBT salt was dissolved in PBS containing 1.5 % DMSO (dimethyl sulfoxide, Sigma-Aldrich) to a final concentration of 1 mg/mL and added to the cells (100 µL per well). After a 1 h incubation (shaker, 37 °C, 95 % air atmosphere, 5 % CO2), the cells were washed twice with PBS and centrifuged at 300 x g for 10 min. Lastly, the cells and formazan crystals were dissolved in 2 mol/L KOH (potassium hydroxide; Centralchem) in DMSO. Optical density was determined at a wavelength of 620 nm against 570 nm as reference by a Multiskan FC microplate photometer (Thermo Fisher Scientific Inc.). Data are expressed in percentage of the control set to 100 % (**Tvrdá *et al*., 2016**).

Statistical analysis

Statistical analysis was carried out using the GraphPad Prism program (version 3.02 for Windows; GraphPad Software, La Jolla, CA, USA, http://www.graphpad.com). Descriptive statistical characteristics (mean, standard error) were evaluated at first. One-way ANOVA was used for specific statistical evaluations. Dunnett's test was applied as a follow-up test to ANOVA, based on a comparison of every mean to a control mean, and computing a confidence interval for the difference between the two means. The level of significance was set at *** - $P < 0.001$, ** - $P < 0.01$, * - $P < 0.05$.

RESULTS AND DISCUSSION

High performance liquid chromatography (HPLC) was used for the identification and quantification of major chemical compounds present in the lovage extract. The quantitative determination was performed by the external standard method and the concentrations of the identified compounds are showed in Table 1. The main compound detected in the extract was rutin (4049±5.09 mg/kg). From the analyzed phenolic acids, chlorogenic acid, neochlorogenic acid, trans-p-coumaric acid, trans-caffeic acid and trans-ferulic acid were quantified with the first being the most abundant (515±6.69 mg/kg). Four flavonoid glycosides, cynarosid, rutin, apigenin and kaempferol were found in the extract (Table 1).

Table 1 Major chemical compounds identified and quantified [mg/kg] in the Lovage extract

Cynarosid	43.57±4.04
Rutin	4049±5
Apigenin	31.51±3.66
Kaempferol	42.17±2.22
Chlorogenic acid	515±6.69
Neochlorogenic acid	315±1.98
trans-p-Coumaric acid	9.04±0.67
Trans-Caffeic acid	27.87±1.99
Trans-Ferulic acid	18.77±1.30

(Mean ± SEM; n=3) ND – not detected.

In the second part of the experiment the *in vitro* effects of *Levisticum officinale* on three quality parameters of bovine sperm were assessed. All evaluations we

performed at 0, 2, and 24 hours using working solutions with 300; 150; 75; 37.5 µg/mL plant extract.

The first parameter was the sperm motility evaluated using the CASA method. It was revealed that the highest extract concentration had an immediate negative effect on sperm motion when compared to the control group. On the other hand, the lowest concentrations showed an immediate stimulatory effect on the motility. Despite the stimulus no statistically significant differences were observed between the samples. The highest concentration of the lovage extract exhibited negative effects during the second hour of the analysis, while an increased motility was detected following the addition of 75 µg/mL extract. In the residual samples, the motility was significantly decreased. Following 24 h, 300 µg/mL extract was almost destructive to the sperm motion when compared to the control group ($P < 0.01$). The second highest the concentration (150 µg/mL) also exhibited adverse effects on the germ cells, while a positive effect was observed at concentrations of 75 µg/mL and 37.5 µg/mL. The sample containing 75 µg/mL lovage extract exhibited the highest motility. It may be suggested that higher concentrations of medical lovage may have a toxic effect on the sperm motility under *in vitro* conditions. On the contrary, media containing 75 µg/mL and 37.5 µg/mL extract exhibited a stimulating effect on the sperm motion behavior (Table 2).

Table 2 Spermatozoa motility [%] in the absence (Control) or presence of the lovage extract during different time periods

Motility [%]	0 h	2 h	24 h
Control	82.00±4.74	45.75±4.90	17.75±1.53
300 µg/mL	76.25±3.56	40.50±4.54	1.00±0.44**
150 µg/mL	84.00±5.06	42.25±2.12	2.00±0.22**
75 µg/mL	85.25±2.17	53.75±2.78	19.25±3.28
37.5 µg/mL	83.00±3.67	46.75±5.05	18.25±2.45

**P<0,01. X±S.D.

Mitochondria provide the sperm cells with the energy they need to support important functions. The activity of mitochondria is closely related to motility, as mitochondria also provide fuel for the sperm movement. To determine the viability of the germ cells following the addition of the lovage extract, an optical method based on the measurement optical density taking advantage of an ELISA reader was used. MTT test results are shown in Figure 1. After an immediate measurement of the viability, no statistically significant changes in the experimental groups were detected when compared with the control group. After a two-hour incubation, a significantly decreased mitochondrial activity was recorded, especially in case of higher concentrations of the extract. The most significant detrimental concentration was 300 µg/mL extract when compared to the control group ($P<0.05$). In contrast, samples containing 75 µg/mL and 37.5 µg/mL lovage extract showed a stimulating effect similar to that of the motility. A rapid decline of viability at 300 µg/mL and 150 µg/mL extract were observed after 24 hours as well. Samples containing a higher extract content, exhibited a significantly decreased viability ($P < 0.001$), while the mitochondrial activity was higher in samples containing 75 µg/mL and 37.5 µg/mL extract. As such, it may be hypothesized that lower concentrations of *Levisticum* have a stimulating *in vitro* effect on the viability of bull spermatozoa.

The NBT test was used to evaluate if spermatozoa were producing the superoxide radical and, if so, what was its quantity. After the first analysis no increase of the radical production on a statistically significant level was observed, with the exception of the sample with the highest concentration of the extract (300 µg/mL) where the superoxide concentration was slightly increased. After two hours the differences between the samples were negligible as well. The final analysis confirmed antioxidant effects of 75 µg/mL and 37.5 µg/mL extract when compared to the control ($P<0.05$) and its prooxidant properties at higher concentrations. Upon completion of the experiments, it may be may concluded that lower concentrations of lovage caused a significant decrease of the superoxide production, while higher concentrations may induce the production of dangerous free radicals. The results are graphically depicted in Figure 2.

Preliminary, although limited data are available with respect to the chemical composition and biological activity of lovage and its extracts on reproduction. **Kemzūraitė *et al.* (2014)** state that silica from lovage leaves and seeds are used in the food, beverage, perfume and tobacco industries, while roots have been known for centuries as a medicine that has carminative and spasmolytic properties.

The isolation of the lovage components by hydrodylation and solvent extraction has shown that the chemical composition of the extracts depends on the geographical location, ripeness, time of collection, anatomical part of the plant and the insulation method. A comprehensive description of bioactive substances present in the lovage silica was reported by **Sertel *et al.* (2011)**. Essential oil obtained from lovage leaves contained 9 constituents (73.74% of the total oil content). The largest fraction consisted of monoterpenes, particularly α-terpinyl acetate (48.15%). A surprising and unexpected, but repeatedly observed effect was that subtoxic etheric oil concentrations of *Levisticum* stimulated the cell proliferation and viability. Dose-dependent cytotoxic effects were found at higher concentrations. Effects comparable to those obtained in this study have been previously reported in combination with standard anticancer agents such as doxorubicin. The proliferation - stimulating effect of other cytotoxic compounds may be interpreted as rescue mechanism.

Paumgartten *et al.* (1998) investigated the effect of b-myrcene, one of the main components of lovage bitter essential oil on rat reproduction. No signs of maternal toxicity and no increase of external visible malformations were observed at none of the dose levels. Only at the highest tested dose (500 mg/kg) b-myrcene caused an increased rate of resorption and a higher frequency of anomalies of the fetal skeleton. No adverse effects of b-myrcene were recorded with respect to the postnatal weight gain, but the release of primary fur, teeth growth and eye opening were slightly delayed in the exposed offspring.

Affonso *et al.* (2012) examined the effect of *Schinus terebinthifolius* (Brazilian spice) with properties similar to lovage, on the reproduction of rats. The main components of the plant extract were a-fenein (20.75%), ß-pinene (10.11%), ß-myrcene (9.30%), a-fallandene (14.94%), limonene (20.81%) and isosylvestrene (13.87%). The authors reported that a 60-day treatment of rats with essential oil from the *S. terebinthifolius* did not significantly change the weight of the reproductive organs, and no contraceptive activity of the oil was detected.

Oliaee *et al.* (2014) examined the effect of a similar plant, *Artemisia kopetdaghensis* on rat reproduction. Concentrations of the *A. kopetdaghensis* extract at 200 and 400 mg/kg did not have a significant effect on the duration of pregnancy. However, administration of 200 and 400 mg/kg extract resulted in a 30 to 44% abortion in the animals. None of the potential benefits of *A. kopetdaghensis* was related to a significant change in the number of newborns. Likewise, the extract practically had no significant effect on their weight.

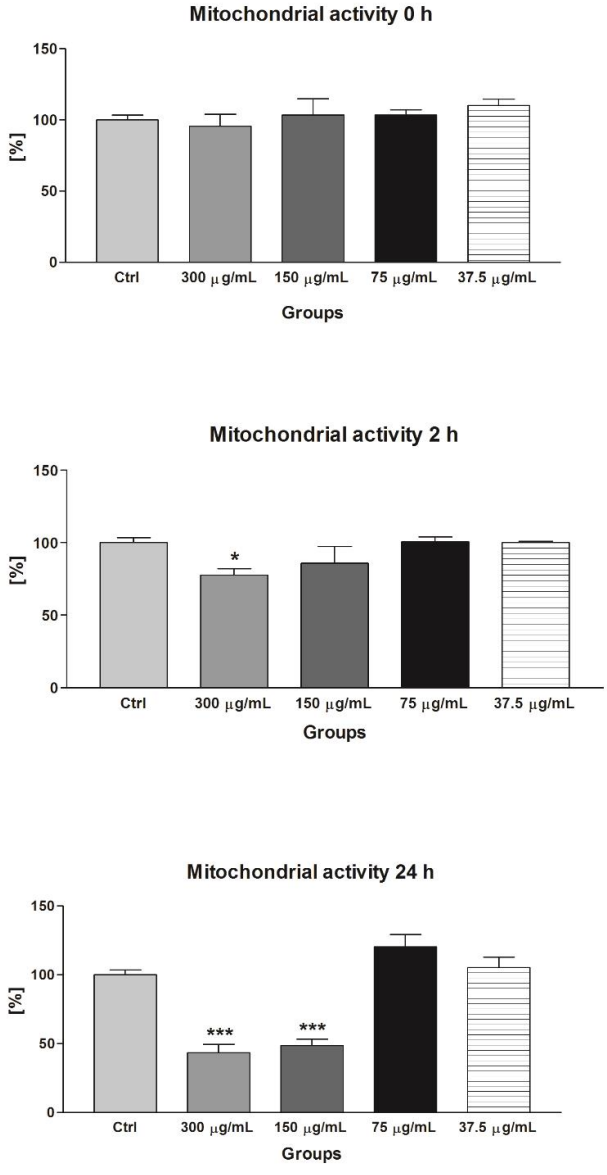

Figure 1 The effect of various concentrations of the lovage extract on the viability of bovine spermatozoa at 0h, 2h and 24h. Each bar represents mean (±SEM) optical density as the percentage of controls, which symbolize 100%. The data were obtained from five independent experiments. The level of significance was set at *** $P<0.001$; ** $P<0.01$; * $P<0.05$.

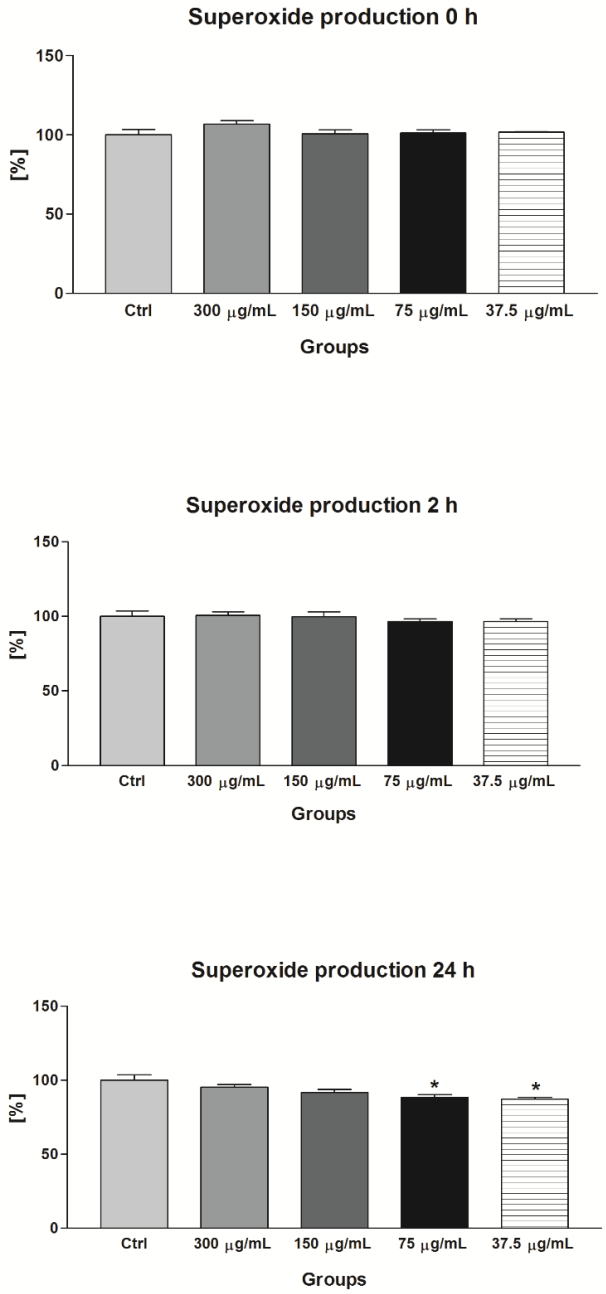

Figure 2 The effect of various concentrations of the lovage extract on the spermatozoa superoxide production at 0h, 2h and 24h. Each bar represents the mean (±SEM) optical density as the percentage of controls, which symbolize 100 %. The data were obtained from five independent experiments. The level of significance was set at *** P<0.001; ** P<0.01; * P<0.05.

Today, medicinal plants are widely used as an alternative to drugs. Although plant products have fewer side effects when compared with synthetic drugs, they are not completely free of side effects or toxicity. Adverse effects of medicinal plants may be the result of contamination with herbicides or pesticides, falsification of the active compounds, improperly prepared plant products, misidentification of plant components and the intrinsic toxicity of some herbs. As such, potential side effects of any medicinal plants must be determined before any research or clinical application. Unfortunately, unlike those synthetic drugs that could be toxic due to known side effects, there is still insufficient data on the undesirable consequences of the use of plant substances in vivo or in vitro (**Oliaee *et al.*, 2014**).

It may be assumed that the individual chemical components of the medical lovage could affect the examined bovine sperm parameters. Harmful effects have been confirmed at higher concentrations and a protective impact at lower. The above-described analyzes were mainly focused on the *in vitro* effect of various concentrations of lovage, providing more specific information on its individual effects. Lovage belongs to widely used spices and aromatic plants which is why more information on the broad spectrum of its effects on the general health and reproduction is highly welcome.

CONCLUSION

In this study the chemical composition of the medical plant *Levisticum officinale* and its *in vitro* effects on male reproduction were investigated. Furthermore, based on numerous health benefits of lovage on the living organism, this study aimed to find out what effect it has on the activity of male reproductive cells. To achieve the set goals, HPLC was applied to study the major chemical constituents of the lovage extract. At the same time, working solutions with a gradually increasing concentration of the lovage extract (300; 150; 75; 37.5 µg/mL) were prepared and studied for their impact on the motility, mitochondrial activity and superoxide production of bovine spermatozoa. After evaluation of the obtained results, it may be concluded that higher concentrations of lovage extract (300 and 150 µg/mL) act adversely on the germ cells when compared to the control, and increase the production of free radicals, which may cause cellular oxidative stress. On the other hand, concentrations of 75 and 37.5 µg/mL exhibited significant positive effects, translated into an increased motility and mitochondrial activity, followed by a decreased formation of free radicals. Finally, it may be assumed that some of the substances present in lovage could act as stimulants at lower concentrations, while their higher concentrations could become toxic to the cell survival. At the same time, further data validation using more complex sperm analyses and *in vivo* experiments are highly recommended.

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