Investigation of the properties and effects of *Salvia officinalis* L. on the viability, steroidogenesis and reactive oxygen species (ROS) production in TM3 Leydig cells *in vitro*

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Running head: *Salvia* affect reproductive functions in dose- and time dependency

Summary

The aim of our study was to reveal the *in vitro* effects of *Salvia officinalis* L. (37.5; 75; 150; 200; 250; 300 and 600 µg/mL) extract on the TM3 Leydig cell viability, membrane integrity, steroidogenesis and reactive oxygen species production after 24 h and 48 h cultivation. For the present study, the extract prepared from *Salvia officinalis* L. leaves was analysed by high performance liquid chromatography (HPLC) for selected flavonoids and phenolic acids followed by a determination of its free radicals scavenging activity (DPPH). Furthermore, Leydig cell viability was assessed by the mitochondrial toxicity assay (MTT), while the membrane integrity was evaluated by 5-carboxyfluorescein diacetate-acetoxymethyl ester (5-CFDA-AM). The level of steroid hormones was performed by enzyme-linked immunosorbent assay (ELISA) from the culture media, while the superoxide radical generation was measured by the nitroblue tetrazolium chloride (NBT) assay. The results show that experimental concentrations did not damage the cell membrane integrity and viability when present at below 300 µg/mL; it was only at 600 µg/mL that a significant (P<0.05) cell viability decline was observed after a 48 h cultivation. A significant (P<0.05) stimulation of testosterone secretion was recorded at 250 µg/mL for 24 h, while the prolonged cultivation time significantly (P<0.05) increased the testosterone and progesterone production at 150; 200; 250 and 300 µg/mL. Furthermore, none of the selected doses exhibited significant ROS-promoting effects however, the highest dose of *Salvia* initiated the free radical scavenging activity in cultured mice Leydig cells.

Keywords: *Salvia officinalis* L.; Leydig cells; Viability; Steroidogenesis; Reactive oxygen species

Introduction

There is overwhelming evidence about the potential ability of heavy metals, endocrine disruptors and other environmental pollutants to affect the health status of an individual. In addition, inadequate nutrition and current lifestyle are crucial factors responsible for reproductive disturbance (Jambor *et al*., 2018; Kovacik *et al*., 2018; Saha *et al*., 2019). Steroidogenesis running in Leydig cells converts cholesterol into various steroid hormones through the steroidogenic enzymes (P450scc, 3β-HSD, 17β-HSD) and the steroidogenic acute regulatory protein (StAR) under the control of the luteinizing hormone (LH) (Wang *et al*., 2017). The levels of steroid hormones depend on the number of cells and the steroid enzyme capacity to convert cholesterol. An exceptionally negative impact from the current environment may irreversibly inhibit the metabolic activity and steroidogenic functions of Leydig cells as well as decrease the semen quality or epididymal weight, increase the incidence of testicular cancer and subsequently affect the sexual behaviour (Sultan *et al*., 2001; Singh and Lin, 2012; Halo *et al*., 2019). Therefore, we attempt to point out natural sources which are able to effectively reverse this negative worldwide situation. Currently, medicinal plants are recognized as an alternative source of efficient biologically active compounds as opposed to synthetic medications and are used as primary health care remedies. Likewise, a great variety of them has been used for the enhancement of male fertility (Tohamy *et al*., 2012; Fattahi and Vaseghi, 2015). *Salvia officinalis* L. commonly known as sage is extremely rich in polyphenolic compounds, which are thought to be responsible for an abundant antioxidative potential. *Salvia* consists of a complex mixture of monoterpenes, diterpene, sesquiterpenes, flavonoids and phenolic acids. Together with another phytoconstituents such as cineol, borneol, rosmarinic acid, chlorogenic acid, salvianolic acid or vitamin C and E, sage represents the best way to protect individuals against different types of detrimental effects caused by xenobiotics (Kosar *et al*., 2010; Rahte *et al*., 2013). It has

been confirmed that *Salvia* products exhibit beneficial anti-inflammatory, antihyperglycemic, spasmolytic, antiseptic or hepatoprotective activities. *S. officinalis* L. is useful for the treatment of profuse perspiration, depression, anxiety as well as menopausal and sterility complications. Furthermore, some experimental studies indicate that molecules contained in *Salvia* scavenge free radical thereby minimize the damaging effects of oxidative insults (Esmaeili *et al*., 2009; Rahte *et al*., 2013). Overleaf, there is very limited information about the potential *in vitro* effects of *Salvia* on the Leydig cells function. There exists a strong conviction that bioactive phytoconstituents in *Salvia* may affect the production of steroid hormones, metabolic activity and reactive oxygen species generation (ROS), and further initiate changes in the reproductive health (Fattahi and Vaseghi, 2015; Tvrdá *et al*., 2017). The present *in vitro* study aims to evaluate the potential effects of the ethanolic extract from *S. officinalis* L. (37.5 – 600 µg/mL) on the TM3 Leydig cell line during different time periods. The experiments had in view to determine whether the use of the *Salvia* extract of known composition exhibits any positive effects on the mitochondrial activity or membrane integrity, as well as sexual hormone release and reactive oxygen species production in mice Leydig cells.

Material and methods

Plant material and extract preparation

The leaves from *Salvia officinalis* L. were harvested in the Botanical Garden at the Slovak University of Agriculture in Nitra. After freeze-dried (-80 °C), the plant material was crushed and weighed. An aliquot of the plant material was soaked in 96% ethanol (p.a. CentralChem, Bratislava, Slovak republic) for two weeks. In order to avoid the degradation of bioactive substances, the extraction was realized in the dark and at laboratory temperature. Subsequently, the ethanolic plant extract was subjected to evaporation (Stuart RE300DB

rotary evaporator, Bibby Scientific Limited, United Kingdom) under reduced pressure (vacuum pump KNF N838.1.2KT.45.18, KNF, Germany) and elevated temperature 40 °C in order to remove any residual ethanol. The crude plant extract was dissolved in a standard organic solvent DMSO (Dimethyl sulfoxide; Sigma Aldrich, St. Louis, USA) to equal 100.4 mg/mL as a stock solution.

Quantitative high-performance liquid chromatography (HPLC-DAD) analysis of the extract

All analytical standards such as methanol (HPLC grade), acetonitrile (gradient HPLC grade) and phosphoric acid (ACS grade) were purchased from Sigma Aldrich (St. Louis, USA). Before to HPLC analysis, the plant extract was filtered through a syringe filters Q-Max (0.22 μm, 25 mm, PVDF - Frisenette ApS, Knebel, Denmark) into the HPLC vials with PTFE septum. Standard solutions were dissolved in 10 mL of methanol and homogenised. Subsequently, the samples were extracted using 20 mL of 80% (v/v) methanol any horizontal shaker (Unimax 2010; Heidolph Instrument, GmbH, Germany). After filtration (Munktell No 390; Munktell & Filtrac, Germany), the samples were stored in vial tubes. The extracts and standard solutions were filtered through the Q-Max syringe filter and then injected. The high-performance liquid chromatograph (Agilent 1260 Infinity HPLC Technologies; GmbH, Waldbronn, Germany) with quaternary solvent manager coupled with degasser, sampler manager, Diode Array Detector and column manager were used to analysis of phenolic acids and flavonoids in the leaves extract of *Salvia officinalis* L. HPLC analyses were performed on a Purosphere reverse phase C18 column (4 mm x 250 mm x 5 mm; Merck, KGaD, Darmstadt, Germany). The gradient system with a mobile phase of 0.1% phosphoric acid in deionised water and gradient grade acetonitrile at a flow rate of 0.60 mL/min and the injection volume was 3 μL. The gradient elution was as follows: 0-1 min isocratic elution (90% C and 10% D), 1-6 min linear gradient elution (85% C and 15% D), 6-12 min (80% C and 20% D), 12-20

126 min (30% C and 70% D) and 20-25 min (30% C and 70% D). The post-run was set at 3 min.
127 Column thermostat was set to 30 °C and the sample was kept at 6 °C in the sampler manager.
128 Data were collected and processed using Agilent Open Lab Chem Station software for LC 3D
129 systems (Lukšič *et al*., 2016).
130
131 *DPPH radical scavenging assay*
132 Free radical scavenging activity of the *Salvia officinalis* L. was measured using the
133 2,2-difenyl-1-picrylhydrazyl (DPPH) method described by Sanchéz-Moréno *et al.* (1998). An
134 amount of 0.4 mL *Salvia* extract was added to 3.6 mL of DPPH solution (0.025 g DPPH in
135 100 mL ethanol; Sigma Aldrich, St. Louis, USA). The absorbance of the reaction mixture was
136 determined using the Jenway 6405 UV/VIS spectrophotometer (Fisher Scientific,
137 Leicestershire, United Kingdom) at wavelength 515 nm. The free radical scavenging activity
138 was expressed as percentage of DPPH inhibition by the following formula:
139
140 $$\% \text{ of inhibition} = [(Ac - As) / (Ac)] \times 100$$
141 where: Ac is the absorbance of DPPH alone and As is the absorbance of DPPH along with
142 sample.
143
144 *Estimation of total phenolic content*
145 Total polyphenolic content of *Salvia officinalis* L. extract was evaluated according to
146 Singleton and Rossi (1965) with slight modifications and described as the Folin-Ciocalteu
147 method. One hundred µL of sample were mixed with the same volume of the Folin-Ciocalteu
148 reagent (Sigma Aldrich, St. Louis, USA), 1000 µL of 20% (w/v) sodium carbonate (Sigma
149 Aldrich, St. Louis, USA), and 8800 µL of distilled water. After 30 min in darkness the
150 absorbance was measured at 700 nm wavelength using the Jenway 6405 UV/VIS

spectrophotometer (Fisher Scientific, Leicestershire, United Kingdom). The total concentration of phenols was calculated using the standard curve, gallic acid was used as the standard and the results were expressed as mg of gallic acid equivalents (GAE) per kg of dry matter.

Cell culture

The TM3 Leydig cell line, non-tumorogenic cells derived from mouse testis strain BALB/c nu/+ were purchased from the American type Culture Collection (ATCC #CRL-1714; Manassas, VA, USA) as a suitable model for our *in vitro* study. Cells were cultured in Dulbecco´s Modified Eagle´s Medium/Nutrient Mixture (Ham´s) F12 with HEPEs and NaHCO3 (DMEM/F12; Sigma Aldrich, St. Louis, USA) supplemented with 5% horse serum (HS; Gibco-Life Technologies, New Zealand), 2.5% fetal bovine serum (FBS; BiochromAG, Berlin, Germany), 2.5 mM L-glutamine (Sigma Aldrich, St. Louis, USA) and 1% penicillin/streptomycin solution (Sigma Aldrich, St. Louis, USA). TM3 cells were maintained at 37 °C under a humidified atmosphere of 95% air and 5% CO_2. The Leydig cells density was determined using a haemocytometer, adjusted with culture medium to a final concentration of 2 - 4 x 10^3 cells/well. The cells were seeded into 96-well plate and pre-cultured for 24 h. Afterwards, the medium was changed to include different experimental concentrations of ethanolic extract *Salvia officinalis* L. at 37.5; 75; 150; 200; 250; 300 and 600 µg/mL. The TM3 cells remained in culture during 24 h and 48 h respectively (Figure 1).

Cell viability (MTT) assay

To assess the effect of the *Salvia officinalis* L. experimental doses (37.5 – 600 µg/mL) on the TM3 Leydig cells viability after 24 h and 48 h exposure, mitochondrial toxicity assay (MTT; 3-4,5-dietyltiazol-2-yl)-2,5-diphenyltetratzolium bromide) was exploited. This

colorimetric assay measures the reduction of soluble yellow tetrazolium salt (Sigma Aldrich, St. Louis, USA) to water-insoluble blue formazan crystals by mitochondrial succinate dehydrogenase activity in living cells (Mosmann, 1983). Following respective exposure, the culture medium was removed, and the cells were treated with 1 mg/mL of tetrazolium salt during 3 h at 37 °C under a humidified atmosphere of 95% air and 5% CO_2 (Figure 2). Afterward, the formed formazan crystals were dissolved by DMSO (Sigma Aldrich, St. Louis, USA), gently shaking for 20 min and read by an ELISA reader (Multiscan FC, ThermoFisher Scientific, Vantaa, Finland) at 570 nm against 620 nm wavelengths. Cells from four independent experiments were analysed for each treatment. All data were expressed in percentage of control (non-treated cells) group.

Cell membrane integrity (CFDA-AM) assay

The loosing of cell membrane integrity was assessed directly in the cell culture wells using the fluorescent probe 5-carboxyfluorescein diacetate, acetoxymethyl ester (CFDA-AM; ThermoFisher Scientific, Invitrogen, Vantaa, Finland) described by Schreer *et al*. (2005) with a slight modification. In brief, cell culture media supplemented with *Salvia officinalis* L. (37.5 – 600 µg/mL) were removed from the plates after 24 h and 48 h exposure. Subsequently, confluent monolayers of TM3 cells were cultured in the presence of 4 µM CFDA-AM for 30 min under a humidified atmosphere of 95% air and 5% CO_2. The concentrations of the fluorescent metabolites of CFDA-AM were measured using the wavelength of 485 – 530 nm (excitation/emission) respectively. Fluorescence was measured as arbitrary units and reading for the wells without cells were subtracted from those for the experimental wells to account for background fluorescence. The experimental data were expressed as percentage of non-treated cells (control group).

Enzyme-linked immunosorbent assay (ELISA) for detecting steroid hormones

To examine the progesterone and testosterone secretion after 24 h and 48 h exposition, TM3 cells were cultured in the presence of experimental doses of *Salvia officinalis* L. mentioned above. After *in vitro* incubation, the cell culture media was removed from each experimental well and stored in Eppendorf tubes at -20°C until assay. The production of steroid hormones was determined by enzyme-linked immunosorbent assay (ELISA). The ELISA kits were purchased from Dialab (testosterone, Cat. #K00234; progesterone, Cat. #K00225, Austria) and the procedure was carried out according to the manufacture´s instructions. The sensitivities for steroid hormones are presented in Table 1. The absorbance was measured at 450 nm wavelength by an ELISA reader (Multiscan FC, ThermoFisher Scientific, Vantaa, Finland). The results are expressed as percentage of the control (non-treated) group.

Nitroblue-tetrazolium reduction (NBT) assay

The superoxide radicals produced in TM3 Leydig cell line were evaluated by the nitroblue-tetrazolium (NBT) assay after respective treatment by *Salvia officinalis* L. (37.5 – 600 µg/mL) for 24 h and 48 h. This colorimetric assay is conducted by evaluating cells containing blue formazan deposits. They are formed by reduction of the membrane permeable yellow coloured nitroblue-tetrazolium chloride (2,2´bis(4-nitro-phenyl)-5,5´-diphenyl-3,3´-dimethoxy-4,4´-diphenylene) diterazolium chloride (Sigma Aldrich, St. Louis, USA) and superoxide radicals (Choi *et al*., 2006). After *in vitro* cultivation, the culture media were removed and TM3 Leydig cells were further incubated in the presence of 100 µL/well NBT working reagent (1 mg NBT per 1 mL culture media dissolved in DMSO) under a humidified atmosphere of 95% air and 5% CO_2 for 3 h. Afterwards, the formazan products were solubilized in 100 µL/well of 2M KOH (potassium hydroxide; p.a. CentralChem, Bratislava,

Slovak Republic). The resulting colour reaction was measured spectrophotometrically at wavelength of 620 nm against 570 nm as reference by an ELISA reader (Multiscan FC, ThermoFisher Scientific, Vantaa, Finland). All experimental data were expressed in percentage of control (optical density of formazan from cells not exposed to tested extract).

Statistical analysis

The obtained data were statistically analysed using the GraphPad Prism 5.0 (GraphPad Software Incorporated, San Diego California, USA). One-way analysis of variance (ANOVA) followed by Dunnett´s multiple comparison test was used for statistical evaluations. Results were expressed as the mean ±standard deviation (S.D). All experiments were repeated at least three times. Each experimental group was represented by six culture wells of the cells. Statistical differences were expressed at a significance of $P<0.05$.

Results

Chemical constituents and antioxidant properties

HPLC-DAD analysis was chosen as a suitable method for the investigation of the quality and quantity of flavonoids and phenolic acids. We identified bioactive substances on the basis of the retention time and the UV spectra chromatogram pattern. Cynaroside (41.9 ± 7.4 mg/kg) prevailed amongst the analysed flavonoids, followed by rutin (39.1 ± 5.2 mg/kg), kaempferol (38.0 ± 7.0 mg/kg) and apigenin (28.3 ± 4.0 mg/kg). Except these, daidzein (17.3 ± 1.9 mg/kg), quercetin (14.6 ± 3.0 mg/kg), vitexin (4.4 ± 0.9 mg/kg) and resveratrol (2.2 ± 0.2 mg/kg) were identified as well. Overleaf, rosmarinic acid (257.8 ± 30.0 mg/kg) was identified as the predominant phenolic acid in the leaves of *Salvia officinalis* L. extract. We also detected *trans*-caffeic acid (58.6 ±7.2 mg/kg), neo-chlorogenic acid (55.8 ± 5.0 mg/kg), *trans*-coumaric acid (54.5 ± 6.2 mg/kg) as well as chlorogenic acid (53.3 ± 4.9 mg/kg). The

total content of phenolic acids was predominantly represented by *trans*-sinapic acid (12.4 ± 1.0 mg/kg) and ferulic acid (11.2 ± 0.9 mg/kg). In the present *in vitro* study, we showed that the free radical scavenging activity of *Salvia officinalis* L. extract from leaves determined by the DPPH method was at 90.07 ± 4.01 % inhibition of DPPH level. The total phenolic content determined by the Folin-Ciocalteu method was set at 7855.68 ± 59.92 mg of gallic acid per kg.

Effects of Salvia officinalis L. on TM3 cell viability

Exposure of TM3 mice Leydig cells to various doses of *Salvia officinalis* L. (from 37.5 to 600 µg/mL) after 24 h and 48 h cultivation was evaluated with respect to cell viability. The results have revealed that the viability of the treated cells was not significantly (P>0.05) affected during 24 h exposure. A moderate increase was recorded up to 200 µg/mL (110.5 ± 9.1%) with a subsequent decrease at 600 µg/mL (91.3 ± 5.2%) when compared to the control group. As seen in Figure 3a. none of the selected concentrations had a cytotoxic effect. To further explore the cytotoxicity, the mitochondrial toxicity assay was carried out for the 48 h cultivation. The viability of TM3 Leydig cells progressively increased with a significant (P<0.05) level at 200 µg/mL (117.4 ± 5.3 %). In case of higher doses of *Salvia officinalis* L. a gradual decline was recorded at 250 and 300 µg/mL with significant (P<0.05) effects at 600 µg/mL (Figure 3b.). All experimental doses were compared to the control, without treatment (100 ± 5.9 %). The presented data suggest that higher concentrations of *Salvia officinalis* L. may decrease the cell viability in a dose- and time-dependent manner.

Effects of Salvia officinalis L. TM3 cell membrane integrity

Mice Leydig cell line TM3 was treated by different concentrations of *Salvia officinalis* L. during 24 h and 48 h cultivation *in vitro.* As presented in Figure 4a., the cell membrane

integrity was not significantly (P>0.05) affected in the whole applied range of concentrations (37.5 – 600 µg/mL) of the extract after 24 h treatment when compared to the control group (100 ± 7.0%). An extended time of cultivation with higher experimental concentrations (300 and 600 µg/mL) caused a moderate decline in the membrane integrity of Leydig cells. The results shown in Figure 4b. illustrate that the highest dose of *Salvia officinalis* L. (600 µg/mL) immediately decreased (88.1 ± 7.3 %) this parameter without significant changes (P>0.05). All experimental groups were compared to the control group (100 ± 4.3%).

Assessment of steroid hormone production

TM3 Leydig cells were cultured in media supplemented with increasing doses of *Salvia officinalis* L. (37.5 - 600 µg/mL) for 24 h and 48 h. A slight increase of the progesterone production was observed in case of all tested concentrations of *Salvia* after a 24 h treatment. The highest stimulating effect, but no significant (P>0.05) was confirmed at 300 µg/mL (108.9 ± 5.0%) when the mean values of the hormone secretion fluctuated between 100.0 ± 4.1% in the control group (3.38 ng/mL) and 108.9 ± 5.0% in the experimental group exposed to 300 µg/mL (3.68 ng/mL) (Figure 5a.). Figure 5b. presents a dose-dependent growth in the progesterone secretion after a 48 h cultivation. According to our results both of experimental concentrations (150 and 200 µg/mL) significantly (P<0.05) increased the steroid hormone production (120.5 ± 10.3%; 117.5 ± 13.0%). The mean value of progesterone secretion was 2.05 and 2.00 ng/mL, whereas in untreated (control) group 1.70 ng/mL progesterone was recorded. In the remaining doses, a gradual decrease without significant changes was observed. As seen in Figure 6a., the tested extract caused a progressive increase in the testosterone production up to 200 µg/mL, followed by a significant (P<0.05) stimulation at 250 µg/mL (115.8 ± 8.4% respectively 2.44 ng/mL) after 24 h treatment. A similar tendency was confirmed after 48 h incubation. We found that the concentrations of

Salvia officinalis L. (200, 250 and 300 μg/mL) significantly (P<0.05) stimulated the testosterone secretion (119.9 ±9.2% 123.0 ± 7.3% and 121.0 ± 5.4%). The mean values varied from 1.90 ng/mL to 1.97 ng/mL. All applied concentrations were compared with the control cells (1.42 ng/mL) (Figure 6b.). According to the obtained results, we may suppose that the higher applied doses positively affected the steroidogenic process essential for normal reproductive functions.

Measurement of reactive oxygen species (ROS)

It turned out that the previous fundamental parameters were not markedly damaged. Therefore, we wanted to study if either *Salvia officinalis* L. stimulates or inhibits the intracellular production of ROS after 24 h and 48 h cultivation. According to the nitroblue-tetrazolium assay, none of the applied doses (37.5 – 600 μg/mL) significantly affected the superoxide radicals released after 24 h incubation. However, treatment with the highest concentration (600 μg/mL) led to a progressive decrease (86.5 ± 5.0%) in the ROS production without significant changes (P>0.05) in comparison to the control group (100.0 ± 4.8%) (Figure 7a.). The extended time of cultivation showed similar results. As shown in Figure 7b. a reduced generation of superoxide radicals (89.1 ± 7.3%) was recorded only in case of the highest dose of the tested extract. All data were compared to the untreated cells (100.0 ± 4.7%). The experimental data suggest that the chosen doses of *Salvia officinalis* L. had a weak antioxidative potential and did not negatively affect all presented parameters.

Discussion

In our study, we investigated the *in vitro* effect of *Salvia officinalis* L. extract on selected essential cellular processes running in TM3 Leydig cells. We confirmed, that some of the tested concentrations may positively affect steroidogenesis without negative consequences

on the cell viability and membrane integrity together with ROS scavenging activity in a dose- and time- dependent nature. *Salvia officinalis* L. is characterized by the presence of numerous bioactive phytoconstituents such as flavonoids, phenolic acids, tannins, terpenoids, and other secondary metabolites. The results of our HPLC-DAD analysis confirmed higher amounts of cynaroside (41.9 mg/kg), rutin (39.1 mg/kg) and kaempferol (38.0 mg/kg), followed by apigenin, daidzein, quercetin or resveratrol. Furthermore, total phenolics content of the tested extract was found to be 7855.68 ± 59.92 mg/kg when compared with the gallic acid equivalent. The estimation of the radical scavenging effect performed by DPPH assay demonstrates 90.07 ± 4.01 % inhibition of the stable DPPH free radical. Adbelkader *et al*. (2014) studied the extract from leaves of *Salvia officinalis* L. by simple chemical tests and showed the presence of flavonoids (quercetin, caffeic acid), triterpenoids and steroids (*β*-sitosterol, *β*-amirin) as well as cinnamic derivatives (chlorgenic acid). Results from the colorimetric analysis focused on the total phenolic content (31.15 ± 1.05 mg/g GAE) and DPPH radical scavenging activity (130.56 ± 0.86%) confirmed a strong antioxidant activity of this plant extract. Kosar *et al*. (2010) determined the quantitative and qualitative content of phytochemicals in the *Salvia officinalis* leaves by HPLC analysis. A number of components could not be identified however, their chemical class was tentatively determined. The major component was rosmarinic acid, followed by luteolin-7-*O*-glycoside, luteolin and caffeic acid. The authors also confirmed a significant scavenging DPPH property in a concentration dependency. They were convinced that abietane diterpenes and rosmarinic acid were responsible for the potent scavenging activity of the *Salvia* taxa.

Many experimental studies confirmed antimicrobial, anti-inflammatory or anti-carcinogenic properties of *Salvia* species. Furthermore, *Salvia* species have been reported to improve male reproductive functions, however current information about the exact mechanism and specific effects are limited. According to our results, a prolonged time of

cultivation significantly increased the viability of TM3 Leydig cells at 200 µg/mL. Overleaf, at the highest concentrations (600 µg/mL) a progressive decline was observed. In addition, during the evaluation of the cell membrane integrity, essential for the sufficient activity of steroidogenesis, we did not confirm any significant damage of the membrane continuity after 24 h or 48 h. A recent study determined the protective *in vivo* effect of *Salvia officinalis* L. extract on the rat's testis. Experimental animals were exposed to diazinon (200 mg/kg) and daily treated by *Salvia officinalis* L. (100 mg/kg) for four weeks. The results confirmed, that rats with diazinon exhibited a decreased number of spermatogonia, spermatocytes, Leydig and Sertoli cells. On the other hand, data obtained from rats treated by diazinon together with the tested extract confirmed the protective effect of *Salvia* in possible tissue damage. The amount of Leydig cells, sperm cells, Sertoli cells as well as diameter of seminiferous tubules were positively affected (Fattahi and Vaseghi, 2015). The irreversible damage in the cell viability *in vitro* induced by *Salvia officinalis* L. were investigated by Lima *et al*. (2004). Hepatocytes isolated from rat liver were cultured in the presence of an essential oil from *Salvia* (2 – 2000 nl/mL) for 30 min. The results showed that the tested oil was not toxic when present at concentrations below 200 nl/mL. Progressive changes were recorded at 2000 nl/mL, where a significant LDH leakage and GSH decrease were observed indicating cell damage. Cell viability assay was performed on FL83B mouse hepatocytes by Chen *et al*. (2019) *in vitro.* Experimental cells were treated with 200, 500 and 1000 µg/mL of tea extract from *Salvia officinalis* L. leaves for 24 h. The tested extract at 200 and 500 µg/mL had no significant effects on the growth of mouse hepatocytes. However, treatment with the highest dose significantly reduced the cell viability. The improving effects of *Salvia* on male reproductive system may come from the effect of *Salvia* phytoconstituents, specifically rosmarinic acid, quercetin, kaempferol, rutin, thujone, rosmanol and many others. The potential impact of rutin on Leydig cell viability was evaluated by Sun *et al.* (2017). TM3 cells were treated by rutin at

10, 20 and 40 µmol/L during 12 h, 24 and 48 h. As the results showed, the cell viability did not significantly differ among the groups, whit different concentrations and time courses. Chen *et al.* (2007) monitored whether quercetin and resveratrol may affect tumor Leydig cell proliferation in different time periods *in vitro.* The results of the study did not confirm any cytotoxic effect. The experimental concentrations (0.5 – 50 µM) had no significant effects after a five-day exposure. Although the *in vitro* effects of *Salvia officinalis* L. on Leydig cells have not been entirely documented, many reports investigated a significant impact on the spermatogenesis, testosterone production, and erectile functions (Jasem *et al.,* 2010; Alzweiri *et al.,* 2011; Ismail *et al.,* 2013).

Results of our *in vitro* study demonstrate, that some experimental concentrations of *Salvia officinalis* L. extract may progressively enhance the production of steroid hormones. Especially, a significant increase in the testosterone production was confirmed at 250 µg/mL after 24 h cultivation, followed by a significant rise in the testosterone release at 200, 250 and 300 µg/mL for 48 h. Progesterone secretion was significantly enhanced at 150 and 200 µg/mL after 48 h of exposition. Bahr and Ibrahim (2015) performed a study focused on the examination of hydroalcoholic leaves extract from *Salvia officinalis* L. on the testosterone levels and testicular tissue changes in male rats. In rats fed with 150 and 200 mg/kg a significant increase in the serum testosterone levels and seminiferous tubule diameter as well as number of spermatozoa was observed. Salah *et al*. (2016) monitored the effect of *Salvia officinalis* L. (300 mg/kg body weight) on the testosterone production in albino male rats after five weeks of treatment. Compared with the non-treated rats, the level of testosterone was significantly increased together with LH and follicle stimulating hormone (FSH). We have compared our data with a few *in vitro* studies focusing on the potential impact of bioactive constituents occurring in *Salvia officinalis* L. extracts on the steroid hormone production by Leydig cells. A recent study determined the effects of resveratrol and quercetin (1 – 50 µM)

on the steroidogenesis in MA-10 Leydig cells. Progesterone production was significantly decreased by resveratrol at 25 and 50 μM after 24 h cultivation. An opposite tendency was recorded after quercetin treatment. The same experimental doses progressively increased progesterone release, only the highest concertation stimulated hormone production significantly. Resveratrol inhibited progesterone secretion through down-regulation of StAR gene expression at the transcriptional and mRNA levels. Inversely, quercetin stimulated hormone secretion by up-regualtion of StAR promoter activity and mRNA expression (Chen *et al*., 2007). To further information, the effect of rutin on the testosterone release in mice Leydig cells was examined. The results confirmed an increased steroid hormone production in a dose-dependency. A significant growth was observed at 20 and 40 μmol/L of rutin after a 24 h incubation (Sun *et al*., 2017). A further *in vitro* study determined the effect of kaempferol from *Alcea rosea* (1 μg/mL) on isolated rats Leydig cells. Radioimmunological analysis of steroid hormone production revealed a significantly higher level of testosterone in the control group than in the experimental cells after a 24 h cultivation (Papiez *et al*., 2002). All of the presented results suggested that there are differential effects of phytoconstituents on Leydig cells functions. As such, there is a necessity to favor and evaluate parallel effects of a wide range of bioactive compounds collectively before focusing on individual actions. On the contrary, *in vitro* studies support our present finding, where lower doses have no significant impact on Leydig cell functions, while high concentrations may negatively affect the cell viability or steroidogenesis.

The evaluation of superoxide radical production in our study did not confirm any significant impact. Some of the tested concentrations slightly increased the superoxide production, however higher doses (250 – 600 μg/mL) gradually scavenged these radicals regardless of cultivation time. We assume, that a prolonged incubation with *Salvia* may support the protection against oxidative stress. Consistent with our study, Frei and Higdon

426 (2003) reported that *Salvia officinalis* L. effectively scavenged free radicals and modulated
427 the antioxidant pathways. This may be due to its phytoconstituent, namely, rosmarinic acid
428 which protects membrane lipids against oxidative insults. Fattahi and Vaseghi (2015) indicate
429 that the extract of *Salvia officinalis* L. inhibits the production of free radicals and repair
430 tissues thereby minimizing cell damage caused by excessive free radicals. Zupko *et al*. (2001)
431 showed that various *Salvia officinalis* species have an inhibitory effect on the lipid
432 peroxidation induced by Cu^{2+} and Fe^{2+} - containing compound that have free radical
433 scavenging activities. A recent study reported that *Salvia officinalis* L. tea consumption may
434 improve the lipid profile inducing a decrease of the highly atherogenic LDL-C particles,
435 which are rapidly oxidable. It follows that phytoconstituents of *Salvia* may protect essential
436 components of cellular membranes and prevent irreversible changes to the molecular
437 mechanisms as well as viability damage (Elida *et al.,* 2010). Sun *et al*. (2017) reported that
438 some experimental concentrations (10; 20 and 40 µmol/L) of rutin decreased ROS generation
439 and malondialdehyde (MDA) levels in TM3 Leydig cells after a 24 h cultivation.
440 Additionally, the activities of antioxidant enzymes, especially superoxide dismutase (SOD),
441 catalase (CAT) or peroxidase (POD) were remarkably increased by rutin at 20 and 40 µmol/L.
442 According to a previous *in vitro* study, the extract from *Salvia officinalis* L. may affect the
443 antioxidant status of Caco-2 cell line. This experimental model was treated by 60 µg/mL of *S.*
444 *officinalis* L. during 24 h. The cellular content of reduced glutathione (GSH) was significantly
445 increased when compared with the control group. In the contrary, no significant differences in
446 the SOD and CAT activity were observed. In addition, the same experimental concentration
447 has not induced single-strand DNA breaks investigated by the comet assay after a 24 h
448 exposition (Aherne *et al*., 2007). Apparently, the unique composition of *Salvia officinalis* L.
449 leads to the regulation of signal transduction pathways of cell growth and proliferation,
450 induction of apoptosis, modulation of enzymatic activity related to the secretion of steroid

hormones as well as to the regulation of hormone metabolism. Our data highly emphasize on the need to further evaluate the exact effects of phytoconstituents present in *Salvia officinalis* L. extract on the *in vitro* cellular parameters or processes running in Leydig cells.

Conclusion

Herbal medicines derived from plant extracts are being increasingly utilized to treat a wide variety of human diseases. The current *in vitro* study highlights the potential beneficial effects of *Salvia officinalis* L. that may be linked to its antioxidant properties and efficiency to affect cellular functions of exposed TM3 Leydig cell line. Experimental doses of *Salvia* revealed a dose- and time- dependent stimulation of the cell viability and steroid hormone production. What is more, none of the experimental doses significantly damaged the membrane integrity, and the production of reactive oxygen species has not been significantly affected. In view of these *in vitro* observations, we assume that a balanced concertation ratio may support the Leydig cell function, steroidogenesis as well as viability, which may significantly improve reproductive performance in males.

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Declaration of interest

There is no conflict of interest.

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598 **TABLE**

599 Table 1. Intra-assay, inter-assay variability and sensitiveness for the selected steroid hormones

Hormone	Intra-assay variability (%)	Inter-assay variability (%)	Sensitivity
Progesterone	≤4.0	≤9.3	0.05 ng/mL
Testosterone	≤7.0	≤8.3	0.10 ng/mL

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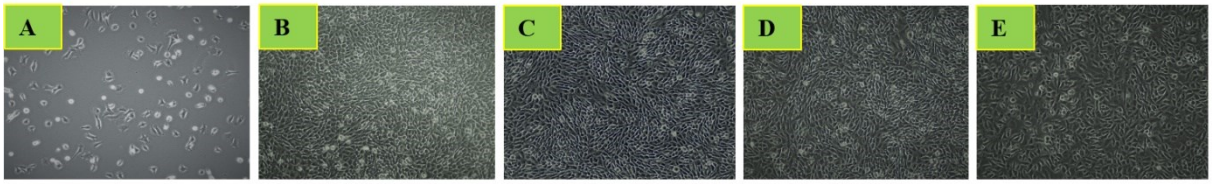
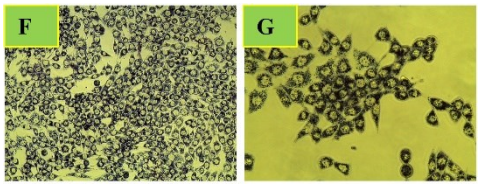
FIGURES

Figure 1. TM3 Leydig cells growing in different experimental concentrations of *Salvia officinalis* L. during 24 h *in vitro* cultivation.



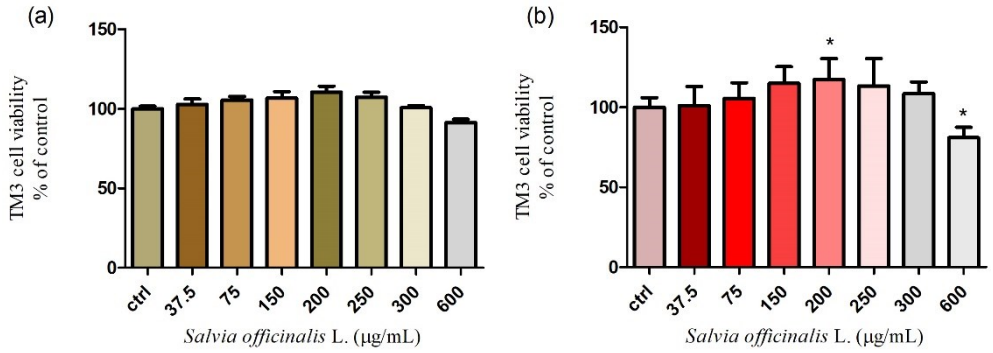
A – after subculturing; B – untreated (control) cells; C – 150 µg/mL; D – 300 µg/mL; E – 600 µg/mL. The cells were observed under a phase-contrast microscope (Leica Microsystems CMS GmbH; Wetzlar, Germany); magnification 200x. (colorless)

Figure 2. Formazan crystals formed after 3 h incubation with yellow tetrazolium salt.



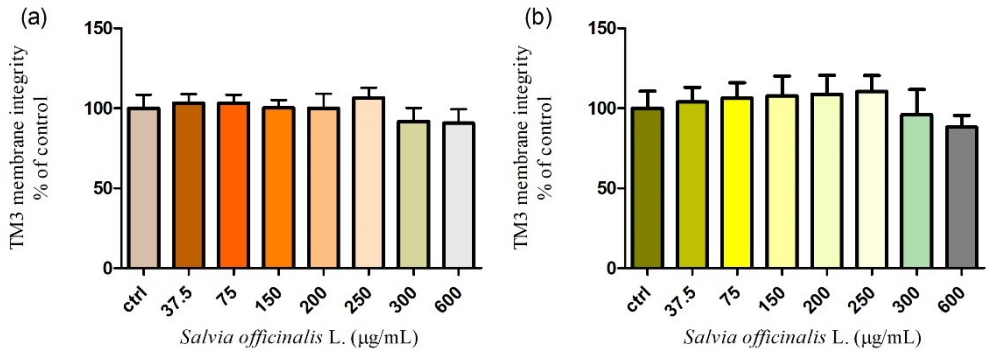
Formazan deposits indicate mitochondrial succinate dehydrogenase activity in living cells. The cells were observed under a phase-contrast microscope (Leica Microsystems CMS GmbH; Wetzlar, Germany)); magnification: F – 200x; G – 400x; (colorless)

Figure 3. Cell viability of TM3 Leydig cells treated with different concentrations of *Salvia officinalis* L. for (a) 24 h and (b) 48 h cultivation.

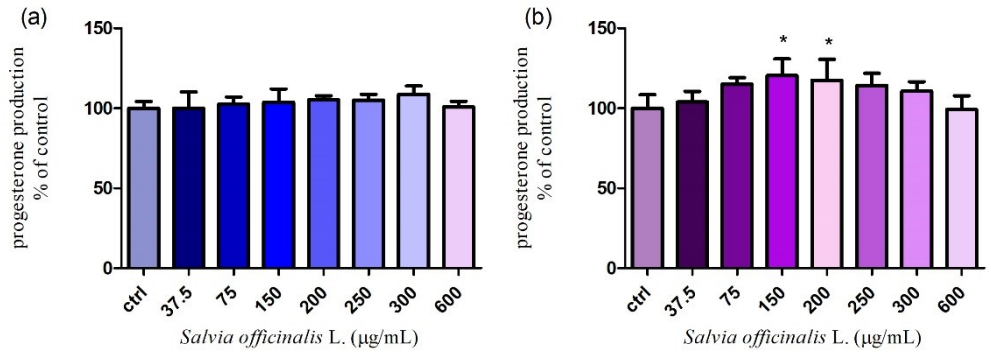


Abbreviations: ctrl – control group. Each bar represents the mean (±S.D.) viability % of control (untreated) and treated groups. Data were obtained from four (n=4) independent experiments. The level of significance was set at * (P<0.05). Statistical differences between the values of control experimental groups is indicated by an asterisk. (colorless)

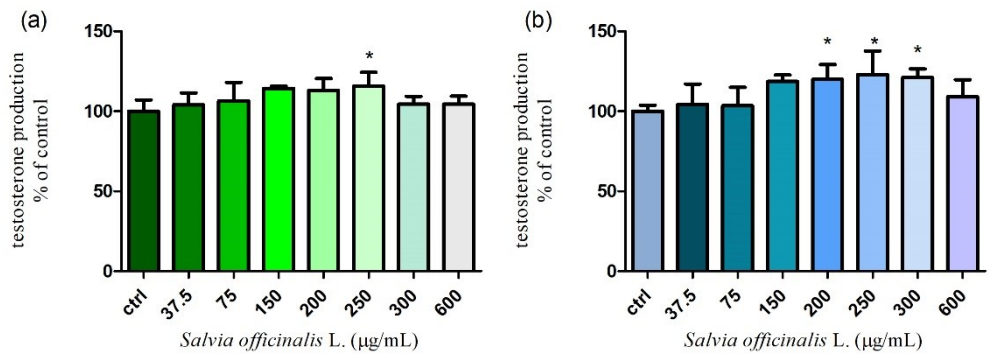
Figure 4. Membrane integrity of TM3 Leydig cells treated with different concentrations of *Salvia officinalis* L. for (a) 24 h and (b) 48 h cultivation.



659 Abbreviations: ctrl – control group. Each bar represents the mean (±S.D.) membrane integrity
660 % of control (untreated) and treated groups. Data were obtained from four (n=4) independent
661 experiments. The level of significance was set at * (P<0.05). Statistical differences between
662 the values of control experimental groups is indicated by an asterisk. (colorless)
663
664 Figure 5. Progesterone production in TM3 Leydig cells treated with different concentrations
665 of *Salvia officinalis* L. for (a) 24 h and (b) 48 h cultivation.



666
667
668 Abbreviations: ctrl – control group. Each bar represents the mean (±S.D.) progesterone % of
669 control (untreated) and treated groups. Data were obtained from four (n=4) independent
670 experiments. The level of significance was set at * (P<0.05). Statistical differences between
671 the values of control experimental groups is indicated by an asterisk. (colorless)
672
673 Figure 6. Testosterone production in TM3 Leydig cells treated with different concentrations
674 of *Salvia officinalis* L. for (a) 24 h and (b) 48 h cultivation.



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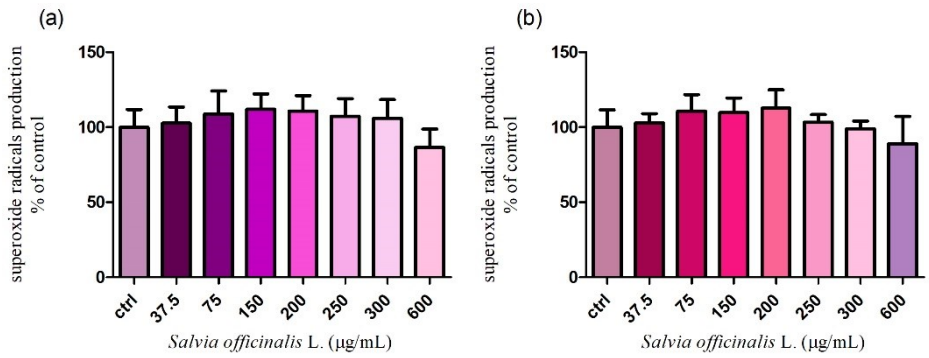
677 Abbreviations: ctrl – control group. Each bar represents the mean (±S.D.) testosterone % of
678 control (untreated) and treated groups. Data were obtained from four (n=4) independent
679 experiments. The level of significance was set at * (P<0.05). Statistical differences between
680 the values of control experimental groups is indicated by an asterisk. (colorless)

681

682 Figure 7. The intracellular formation of superoxide radicals in TM3 Leydig cells treated with
683 different concentrations of *Salvia officinalis* L. for (a) 24 h and (b) 48 h cultivation.



684

685 Abbreviations: ctrl – control group. Each bar represents the mean (±S.D.) free radical % of
686 control (untreated) and treated groups. Data were obtained from four (n=4) independent
687 experiments. The level of significance was set at * (P<0.05). Statistical differences between
688 the values of control experimental groups is indicated by an asterisk. (colorless)

689

690

691 **Highlights**

692 - Only the highest dose of Salvia officinalis L. decreased cell viability significantly

693 - Cell membrane integrity was not significantly affected by Salvia officinalis L

694 - 150 and 200 µg/mL of Salvia significantly increased progesterone production

695 - Significant stimulation of testosterone release was recorded at 200; 250 and 300µg/mL

696 - ROS scavenging activity of Salvia was observed without significant changes