The effect of *Apium graveolens* L., *Levisticum officinale* and *Calendula officinalis* L. on cell viability, membrane integrity, steroidogenesis, and intercellular communication in mice Leydig cells *in vitro.*

Tomas Jambor[1*], Julius Arvay[2], Eva Tvrda[3], Anton Kovacik[3], Hana Greifova[3], Norbert Lukac[3]

[1]BioFood Centre, Faculty of Biotechnology and Food Sciences, Slovak University of Agriculture in Nitra, Tr. A. Hlinku 2, 949 76 Nitra, Slovak Republic

[2]Department of Chemistry, Faculty of Biotechnology and Food Sciences, Slovak University of Agriculture in Nitra, Tr. A. Hlinku 2, 949 76 Nitra, Slovak Republic

[3]Department of Animal Physiology, Faculty of Biotechnology and Food Sciences, Slovak University of Agriculture in Nitra, Tr. A. Hlinku 2, 949 76 Nitra, Slovak Republic

*Address correspondence to MSc. Tomas Jambor, Ph.D., BioFood centre, Faculty of Biotechnology and Food Sciences, Slovak University of Agriculture in Nitra, Tr. A. Hlinku 2, 949 76 Nitra, Slovak Republic. tel. +42191516 635, tomasjambor1@gmail.com

Running head: Higher doses of extracts are cytotoxic for mice Leydig cells

Summary

Several plants have the potential to protect essential reproductive processes such as spermatogenesis or steroidogenesis, however, effective concentrations and main mechanisms of action are still unknown. This *in vitro* study was aimed to assess the effects of *Apium graveolens* L., *Levisticum officinale*, and *Calendula officinalis* L. extracts on the structural integrity, functional activity and gap junctional intercellular communication (GJIC) in mice Leydig cells. TM3 cells were grown in the presence of experimental extracts (37.5; 75; 150 and 300 µg/ml) for 24 h. For the present study, high-performance liquid chromatography analysis was used to quantify flavonoids or phenolic acids. Subsequently, Leydig cell viability was assessed by alamarBlue assay, while the cell membrane integrity was detected by 5-carboxyfluorescein diacetate-acetoxymethyl ester. The level of steroid hormones production was determined by enzyme-linked immunosorbent assay. Additionally, GJIC was assessed by scalpel loading/dye transfer assay. According to our results, *Apium graveolens* L. significantly increased the viability and cell membrane integrity at 75 µg/ml (109.0±4.3%) followed by a decline at 300 µg/ml (89.4±2.3%). In case of *Levisticum officinale* and *Calendula officinalis* L. was observed significant decrease at 150 µg/ml (88.8±11.66%; 87.4±6.0%) and 300 µg/ml (86.2±9.3%; 84.1±4.6%). Furthermore, *Apium graveolens* L. significantly increased the progesterone and testosterone production (75 and 150 µg/ml) however, *Levisticum officinale* and *Calendula officinalis* L. significantly reduced steroid hormones synthesis at 150 and 300 µg/ml. Finally, the disturbance of GJIC was significantly affected at 300 µg/ml of *Levisticum officinale* (82.5±7.7%) and *Calendula officinalis* L. (79.8±7.0%). The balanced concentration ratio may support the Leydig cell function, steroidogenesis as well as all essential parameters that may significantly improve reproductive functions.

Key words: Leydig cells, viability, membrane integrity, steroidogenesis, GJIC,

Introduction

Reproduction is an essential part of our common life, and the factors affecting it have always been a focus of extensive and continuous research. Nowadays, we recognize plenty of exogenous factors, which may interact with human and wildlife reproductive health, including heavy metals, endocrine disruptors, and other xenobiotics (Sedeh *et al.* 2012; Jambor *et al.* 2019). The majority of their negative effects, such as decreased testis weights, prostate cancer, poor semen quality, and insufficient production of steroid hormones, are frequently linked to damage of essential cellular organelles or disruptions to the processes responsible for normal reproductive functions (Smith 2007). In general, most of the mentioned problems could be solved by standard medical methods, especially surgical procedures, hormone therapy, or assisted reproductive technology methods. Inversely, an alternative therapy mediated by medicinal herbs may be another effective way to protect the reproductive system. Several studies have confirmed the higher compatibility of these plants with the human body and weak side effects in comparison to chemical drugs (Kooti *et al.* 2016). The most beneficial effect of medicinal herbs is related to the content of biologically active substances that are able to improve spermatogenesis, steroidogenesis, increase sperm count and motility, and in some cases, reverse the overall subfertility. However, properly balanced doses determine the potential effects of individual herbs. In many cases, the significant positive and protective effect was confirmed in the lower doses of medicinal plants, while the higher doses and long-term exposition could be hazardous for normal reproductive functions in males (Liu *et al.* 2004; Nantia *et al.* 2009).

Apium graveolens L. (Apiaceae) is one of the most confronted herbs with a high level of bioactive components such as limonene, sedanolide, alpha-pinene, or coumarin. *Apium* has a broad spectrum of effects such as anti-cancer, anti-microbial anti-inflammatory, and analgesic (Subhadradevi *et al.* 2011). *Levisticum officinale* from the same family as *A. graveolens L.*

contains a variety of bioactive molecules, and many previous studies confirmed anti-cancer, anti-bacterial, or spasmolytic effects. Extracts from *Levisticum* are also commonly used to treat rheumatism and urethritis (Ekiert 2000). *Calendula officinalis* L. (*Asteraceae*) is mainly known for its antitumor activity and cytotoxic effects on tumor cell lines. Besides, flowers from *Calendula* are traditionally used for their anti-inflammatory and antioxidant properties. They are also rich in pharmacologically active components, including coumarins, quercetin, beta-amyrin or narcissin (Preethi *et al*. 2010). Lower experimental concentrations of all plants mentioned above have been reported to have a significant impact on libido, spermatozoa quality, sexual hormone production or testis weight, and pituitary-gonadal axis (Halo *et al.* 2019; Saha *et al.* 2019; Tvrdá *et al.* 2019; Jambor *et al.* 2020). Nevertheless, current knowledge about the consequences of their higher concentrations on the reproductive functions is poor and extremely limited. Simultaneously, specific molecular mechanisms of action by which medicinal plants could modulate the reproductive processes and parameters are not sufficiently understood.

There is significant evidence that gap junctional intercellular communication (GJIC) is essential for normal reproductive development. GJIC is made up of transmembrane proteins called connexins (Cx) and, they considered as major molecular regulators of male fertility. Namely, the most abundant expressed gap junction protein connexin 43 (Cx43) it necessary for spermatogenesis, steroidogenesis and healthy reproductive functions. Thus, testicular GJIC dysregulation caused by different stressors could affect the etiopathology of subfertility correlated with various reproductive abnormalities (Gilleron, 2015). Undoubtedly, there is a critical need to elucidate cellular interactions and clearly define effective doses of medicinal herbs for the reproductive system's proper functioning (Abbas 2017).

The present *in vitro* study aims to investigate the impact of ethanolic extract from *Apium graveolens* L., *Levisticum officinale*, and *Calendula officinalis* L. on mice TM3 Leydig cells

during 24 h cultivation. The experiments had in view to determine whether the use of the selected medicinal herbs of known composition exhibits any positive or negative effects on the mitochondrial activity or membrane integrity, sexual hormones release, as well as intercellular communication in mice Leydig cells.

Material and Methods

Preparation of the herbal extracts

The leaves from *Apium graveolens* L.*, Levisticum officinale,* and flowers from *Calendula officinalis L.* were collected at the local university´s field in Nitra (Slovak Republic). Plant material was dried in the shade, mechanically comminuted, weighed, and subsequently extracted with 96% ethanol (CentralChem, Bratislava, Slovak republic) for 2 weeks. After that, the ethanol was evaporated (Stuart RE300DB rotary evaporator, Bibby Scientific Limited, United Kingdom and vacuum pump KNF N838.1.2KT.45.18) under reduced pressure (0.5 bar/g) and elevated temperature 40 °C in order to remove any residual ethanol. The crude extract was dissolved in a standard organic solvent dimethylsulfoxide (DMSO; Sigma-Aldrich, St. Louis, USA) and adjusted to 100 mg/ml as a starting solution (Tvrdá *et al.* 2016).

HPLC-DAD analysis of phenolic compounds

In the case of quantitative analysis of the phenolic compounds, the aliquots of plant materials were subjected to the high-performance liquid chromatography (HPLC-DAD). One g of lyophilized leaves and flowers were dissolved in methanol (10 ml; 80%; Sigma-Aldrich, St. Louis, USA). Afterward, the mixture was shaken on a horizontal shaker (25 °C, during 8 h, at 250 rpm) and filtered through 84 g/m^2 filter paper (Munktell, Germany). The samples were subsequently extracted in 20 ml of 80% (v/v) methanol by shaking horizontally (Unimax 2010; Heidolph Instrument, GmbH, Germany). The high-performance liquid chromatograph (Agilent

126 1260 Infinity HPLC Technologies; Waldbronn, Germany) with quaternary solvent manager
127 coupled with degasser, sampler manager, Diode Array Detector, and column manager were
128 used to analyse phenolic content in the harvested leaves of *Apium graveolens* L., *Levisticum*
129 *officinale* and from flowers of *Calendula officinalis* L. HPLC measurements were performed
130 on a Purosphere reverse phase C18 column (Darmstadt, Germany). The mobile phase consisted
131 of acetonitrile and 0.1% phosphoric acid in double-deionized water (ddH_2O). The gradient
132 elution was as follows: 0-1 min isocratic elution (90% C and 10% D), 1-6 min linear gradient
133 elution (85% C and 15% D), 6-12 min (80% C and 20% D), 12-20 min (30% C and 70% D)
134 and 20-25 min (30% C and 70% D). The column thermostat was heated up to 30 °C, while the
135 samples were kept at 6 °C in the sampler manager. The collected data were processed using the
136 Agilent OpenLab ChemStation software for LC 3D Systems (Lukšič *et al.* 2016).
137
138 *TM3 Leydig cell culture*
139 The TM3 mouse Leydig cell line derived from the testis strain BALB/c nu/+ was obtained from
140 the American Type Culture Collection (ATCC; CRL-1714; Manassas, USA). As a non-
141 tumorigenic line, TM3 Leydig cells are commonly used for a short-term *in vitro* cultivation to
142 reflect variance in steroid hormone secretion. The cell culture medium consisted of DMEM/F12
143 (Dulbecco´s Modified Eagle´s Medium/Nutrient Mixture (Ham´s) F12; Sigma-Aldrich, St.
144 Louis, USA) supplemented with 5% HS (horse serum; Gibco-Life Technologies, New
145 Zealand), 2.5% FBS (fetal bovine serum; BiochromAG, Berlin, Germany) together with 2.5
146 $mmol^{-1}$ L-glutamine (Sigma-Aldrich, St. Louis, USA) and 1% penicillin/streptomycin solution
147 (Sigma-Aldrich, St. Louis, USA). Leydig cells were cultured at 37 °C with 5% CO_2 and 95%
148 saturated atmospheric humidity. Cells were regularly screened for contamination. The Leydig
149 cells density was determined using automated cell counter TC 20^{TM} (Bio-Rad Laboratories,
150 California, USA) and adjust with culture medium to a final concentration of 4 x 10^3 cells per

well. The cells were grown in a 96-well plate followed by pre-cultivation of the cells for 24 h until a monolayer was formed. Afterward, the medium was replaced to include varying concentrations of experimental extracts *Apium graveolens* L.*, Levisticum officinale,* and *Calendula officinalis* L. at 37.5; 75; 150 and 300 µg/ml. All treated groups were compared to the non-treated (control) Leydig cells cultured in cell-culture media. The applied concentration range was selected according to the results of our pilot range-finding experiments. The TM3 Leydig cells remained in culture for 24 h. The time of exposition has been chosen regarding to previous pilot study with bovine spermatozoa (Benko *et al.* 2019; Tvrdá *et al.* 2019). After the set time, cell viability, cell membrane integrity, steroid hormone production, and intercellular communication were evaluated.

Cell viability assay (alamarBlue)

To determine the effect of experimental concentrations (37.5 – 300 µg/ml) of the herbal extracts on the TM3 Leydig cell viability after 24 h exposure, alamarBlue™ assay was exploited. AlamarBlue™ cell viability reagent (AB; ThermoFisher Scientific, Invitrogen, Vantaa, Finland) is a sensitive oxidation-reduction indicator that fluoresces and changes the blue colour of resazurin to a pink reduced form - resorufin upon reduction by living cells mediated by mitochondrial enzymes (Hamid et al. 2004). Following respective exposure, the culture medium was removed, the treated cells were washed with PBS (phosphate-buffer saline; 7.2 pH) and cultured with serum-free DMEM/F12 containing 5% (v/v) alamarBlue solution at 37 °C under a humidified atmosphere of 95% air and 5% CO_2. After 30 min incubation, the fluorescence was measured at 530 nm against 590 nm (excitation/emission) wavelengths by a microplate reader (GloMax®-Multi$^+$; Promega Corporation, Madison, USA). The results are expressed as a percentage of the control (non-treated) group.

Cell membrane integrity assay (CFDA-AM)

To examine the impact of experimental concentrations (37.5 – 300 µg/ml) of the herbal extracts on TM3 cells membrane integrity after 24 h incubation, 5-carboxyfluorescein diacetate, acetoxymethyl ester (CFDA-AM; ThermoFisher Scientific, Invitrogen, Vantaa, Finland) was used according to the previous study (Schreer et al., 2005). In essence, culture media supplemented with herbal extracts was replaced with fresh cultured media together with 4 µM CFDA-AM. Subsequently, the TM3 cells were incubated for 30 min in the dark at 37 °C with 5% CO_2, and 95% saturated atmospheric humidity. The concentrations of the fluorescent metabolites of CFDA-AM were measured at wavelength 485 – 530 nm (excitation/emission) in a microplate reader (GloMax®-Multi$^+$; Promega Corporation, Madison, USA). The results are expressed as a percentage of the control (non-treated) group.

Enzyme-linked immunosorbent assay (ELISA)

To evaluate the progesterone and testosterone production, TM3 Leydig cells were incubated together with experimental concentrations (37.5 – 300 µg/ml) of the herbal extracts. After a 24 h *in vitro* cultivation period, the cell culture media was aspirated from each well and stored in Eppendorf tubes at -80 °C until assay. To investigate the level of steroid hormone, a commercially available ELISA kits (Dialab; progesterone Cat. #K00225 and testosterone Cat. #K00234, Austria) was used. The ELISA assay was carried out according to the manufacturer´s specifications. The optical density was measured by an ELISA microplate reader (Multiscan FC, ThermoFisher Scientific, Vantaa, Finland) at 450 nm wavelength. Cell culture media was collected from four independent (n=4) experiments. The results are expressed as a percentage of the control (non-treated) group.

Gap junctional intercellular communication assay (GJIC)

TM3 Leydig cells were cultured for 24 h exposure with selected concentrations (37.5 – 300 µg/ml) of the herbal extracts. After respective treatment, the scalpel loading/dye transfer (SL/DT) method was done as published previously Upham *et al*. (2016) whit slight modification. A gap junction permeable tracer lucifer yellow (1 mg/ml; Sigma-Aldrich, St. Louis, USA) was added to the cells and introduced into them by three parallel cuts made by a scalpel blade. After 6 min of incubation, the cells were washed three times with CaMg-PBS and fixed with a 4% formaldehyde solution. The images were captured by fluorescent microscope DMI 6000B (Leica Microsystems; Wetzlar, Germany) with DCF 345 FX camera. The area of cells stained with lucifer yellow was evaluated using ImageJ software (Schneider *et al.,* 2012). The results are expressed as a percentage of the control (non-treated) group.

Statistics

The obtained data were statistically analysed using 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. Statistical differences were expressed at a significance of $P<0.05$.

Results

Bioactive compounds prevalence in herbal extracts

We identified bioactive substances based on the retention time and the UV spectra chromatogram pattern. Detected levels of all flavonoids are summarized in Table 1 and phenolic acids in Table 2. The most prevalent flavonoids in *Apium graveolens* L. were vitexin (160.18±20.33 mg/kg) and cynaroside (49.57±5.45 mg/kg) followed by kaempferol, diaidzein,

or kaempferol. On the other hand, ferulic acid (523.04±42.12 mg/kg) and trans-p-coumaric acid (140.69±11.32 mg/kg) were identified as the predominant phenolic acids in the leaves of *A. graveolens* L. extract. Similarly, *Levisticum officinale* contained the highest amount of cynaroside (440.35±10.21 mg/kg) together with kaempferol (44.47±5.00 mg/kg) and rutin (40.32±3.77 mg/kg). The most prevalent phenolic acids were identified as chlorogenic acid (523.67±15.55 mg/kg) and neo-chlorogenic acid (365.90±3.09 mg/kg). From analysed flavonoids of *Calendula officinalis* L. rutin (34.36±2.87 mg/kg), kaempferol (22.77±2.01 mg/kg), and apigenin (22.01±2.09 mg/kg) were the most prevalent. From the phenolic acids were identified as rosmarinic acid (207.52±17.98 mg/kg) and chlorogenic acid (196.64±12.21 mg/kg).

Effects of the herbal extract on cell viability

As shown in Figure 1, experimental concentrations of *Apium graveolens* L. had a concentration-dependent effect on the cell viability of exposed cells compared to the control (100.0±6.7%). The results showed that 75 µg/ml (109.0±4.3%) caused a significant (*P<0.05)* increase in mitochondrial activity followed by a significant (*P<0.01*) decrease at the highest tested concentration (300 µg/ml; 89.4±2.3%). On the other hand, the same experimental concentrations of *Levisticum officinale* and *Calendula officinalis* L. had no significant effect up to 75 µg/ml on the presented parameter. However, higher concentrations of *Levisticum* initiated a significant (*P<0.05; P<0.01*) decline in the cell viability (88.8±11.66%; 86.2±9.3%) together with *Calendula* (*P<0.0001;* 87.4±6.0%; 84.1±4.6%) after 24 h cultivation comparing to the control (100.0±9.7% and 8.8%).

Effect of the herbal extract on cell membrane integrity

The results present in Figure 2. have revealed that almost all applied concentrations of *Apium graveolens* L. positively affect this parameter with significant ($P<0.05$) impact at 75 µg/ml (109.6±7.9%). Significant reduction ($P<0.01$) was recorded at 300 µg/ml (85.9±2.9%). In respect to remaining extracts, 150 µg/ml (88.8±11.6%) and 300 µg/ml (86.2±9.3%) of *Levisticum officinale* significantly ($P<0.05$; $P<0.01$) reduced presented parameters. In addition, a significant ($P<0.0001$) cytotoxic effect was confirmed at the same concentrations of *Calendula officinalis* L. Reduced cell membrane integrity fluctuated between 87% (±6.2%) and 84% (±6.8%). Experimental groups were compared to the control (100.0 ±3.9%; 9.2 and 7.4%).

Effect of the herbal extract on hormone production

As seen in Figure 3A applied doses (75 and 150 µg/ml) of *Apium* significantly enhanced progesterone production (116.0±3.1% and 114.4±8.5%) followed by decline at 300 µg/ml. On the other hand, higher experimental concentrations of *Levisticum* decreased progesterone release at 300 µg/ml (90.9±8.5%), while the same dose of *Calendula* reduced steroid production significantly (86.1±7.5%). All experimental groups were compared to the control group (100.0±4.9%;6.7% and 1.6%). Figure 3B indicated the strongest stimulating potential of *Apium graveolens* L. with a significant increase at 150 µg/ml (114.4±2.1%), while the highest concentration (300 µg/ml) caused a non-significant decline. Overleaf, a weak stimulating effect was observed after *Levisticum* and *Calendula* treatment. Higher concentrations (150 and 300 µg/ml) initiate a gradual decline in testosterone production, but only *Calendula* caused a significant decrease ($P<0.05$; $P<0.001$). The level of testosterone was defined at 87.9±4.9% and 77.5±6.8% comparing to the control (100.0±3.2% and 4.9%).

Effect of the herbal extract on intercellular communication

As seen in Figure 4A, exposure to none of the treatments by *Apium* (37.5-300 µg/ml) caused significant changes in intercellular communication. Overleaf, this biomarker was significantly ($P<0.05$) inhibited at 300 µg/ml of *Levisticum officinale* (82.5±7.7%) and *Calendula officinalis* L. (79.8±7.0%). All treated groups were compared to the control group (100.0±4.6%; 4.6% and 4.4%). The representative images of GJIC activity are shown in Figure 4B.

Discussion

Numerous studies have shown that medicinal herbs, which are a rich source of different phytoconstituents, could be associated with many health benefits. Bioactive compounds appear to play an important protective role in cardiovascular diseases, hepatic diseases, reproductive problems, the onset of cancer, and other chronic pathologies (Nour *et al.* 2017). The results of our *in vitro* study indicate a significant dose-dependent effect of medicinal herbs extracts on TM3 Leydig cells. Lower applied doses of positively affect selected cellular parameters, while the highest concentrations (150 and 300 µg/ml) of *Calendula* and *Levisticum* progressively reduced cell viability and cell membrane integrity, decreased progesterone, and testosterone secretion as well as inhibited intercellular communication.

The quantitative evaluation of experimental extract performed by HPLC-DAD analysis confirmed a wide range and variegated ratio of polyphenols and phenolic acids (Table 1 and 2). Many of them are capable to positively affect the reproductive functions in males. A high proportion of bioactive molecules was confirmed by Yao *et al*. (2010). Their study identified major phenolic acids in different cultivars of *Apium graveolens* such as p-coumaric acid (105 mg/kg) ferulic acid (99.3 mg/kg), followed by flavonoids apigenin (92.1 mg/kg), luteolin (90.5 mg/kg) or kaempferol (94.6 mg/kg). Similar to our results, Złotek *et al.* (2019) identified ferulic acid, ellagic acids, p-coumaric acid, caffeic acid, kaempferol, rutin, apterin, and quercetin-3-O-

deoxhexoside-O-hexodside as the most abundant in *Levisticum officinale* L. Frum (2017) has monitored the level of polyphenols in *Calendula officinalis* L. where the highest concentrations of rutin, syringic acid, and gallic acid were recorded. The lower amounts of cinnamic acid, resveratrol, and ferulic acid were also detected. All presented studies above confirmed similar levels of bioactive substances in our experimental medicinal herbs. We are convinced that their detailed identification and monitoring is definitely required for a better understanding of the physiological mechanism as well as to help understand the potential changes in the male reproductive system.

Mutual comparison of individual cellular models confirmed different reactions to presented medicinal herbs extracts. The vast majority of *in vitro* studies are focused on tumorogenic cell lines where the increasing concentrations of herbal extract inhibit cancer proliferation. In contrast, the result of our *in vitro* study confirmed that lower experimental concentrations might positively affect essential parameters of non-tumorigenic cells, especially the cell viability and cell membrane integrity, but with increasing doses start at 150 to 300 µg/ml are able to significantly damage these parameters. Comparable consequences have previously been reported by Subhadradevi *et al.* (2011). Mouse lung fibroblast L929 cells were exposed to *Apium graveolens* at concentrations ranging from 2 to 20 µg/ml during 48 h and the number of viable cells was determined by the MTT assay. The herbal extract statistically inhibited this parameter in a concentration-dependent manner. Sertel *et al*. (2011) evaluated the impact of *Levisticum officinale* extract on the head and neck squamous carcinoma cells (HNSCC) using XTT cytotoxicity assay. The biological model was cultured together with experimental concentrations (0.0001 to 10 mg/ml) of extract for 72 h *in vitro*. The concentration-response curve showed a steady rise in the viability up to 0.1 mg/ml with a subsequent rapid decrease in cell viability to 4.7% (1 and 10 mg/ml) when compared to the untreated control cells. The beneficial effects of *Calendula officinalis* L. were confirmed by

many experimental studies focused on cancer diseases in most cases. However, only a few studies provide information about the cytotoxic concentrations in non-carcinoma cells. Alnuqaydan *et al*. (2015) measured the cytotoxicity of the extract from *C. officinalis L.* at different concentrations for 4, 24, and 48 h on HaCaT cells *in vitro*. *Calendula* showed limited toxicity with a significant effect in the highest concentration. Only 4.4 and 4.2 mg/ml expressed as 2% (v/v) and 5% (v/v) showed a significant toxicity. The viability of HUVEC cells was monitored after 48 h *in vitro* cultivation with *C. officinalis* L. (0.5 – 500 µg/ml) by MTT assay. The results suggest a gradual decline up to 10 µg/ml, followed by a radical cytotoxic effect at 250 and 500 µg/ml (Preethi *et al*. 2010). According to the current knowledge, extract from selected medicinal herbs used in our study could protect sensitive cellular organelles and cell homeostasis in a concentration-dependent manner. It is caused by the mutual ratio of bioactive molecules whose high levels have been confirmed by the previous part of our analysis. Obtained results suggest that some experimental concentrations may negatively affect basal cellular parameters what could result from higher toxic potential of selected extracts. Furthermore, we can assume that the cellular membrane destruction or cell death could destroy steroidogenesis enzymes activity resulting in decreased hormone production. To resolve this issue, further investigations are required. At the same time, we are convinced that adequately applied dose settings could improve males' reproductive functions. The cell structure and mitochondrial activity are closely related to the steroidogenic process ongoing in Leydig cells responsible for steroid hormone production.

Our *in vitro* study's data suggest that the secretion of progesterone and testosterone could be positively affected by the lower doses (75 and 150 µg/ml) of *Apium graveolens* L. However, at the highest concentration of *Apium graveolens* L*., Levisticum officinale*., and *Calendula officinalis* L. has recorded a significant decrease in steroidogenic capacity resulting in a decline of progesterone and testosterone levels. The efficacy of hydro-alcoholic extracts of

A. graveolens L. on the serum levels of testosterone in male rats was investigated by Kooti *et al.* (2016). Male Wistar rats were orally administered to 200 and 300 mg/kg of *A. graveolens* L. for 20 days. The results showed a slight decrease in testosterone production at 300 mg/kg, but without significant changes. Similarly, Madkour (2014) administered orally male albino rats at 200 mg/kg per day of *A. graveolens* L. oil for 8 weeks. The radioimmunoassay revealed an increased concentration of testosterone when compared to the control group. Interestingly, Helal (2014) confirmed a slight decrease in testosterone secretion in male Wistar rats after 6 weeks of exposure to 50 µg/kg per body weight of *A. graveolens* L. Ghaedi *et al*. (2018) published an experimental study focused on the effect of *Levisticum officinale* extract on the testis histology and testosterone production in diabetic rats. Treatment of rats with 500 mg/kg significantly increased the testis weight and serum testosterone levels. The authors assumed that effective concentrations might reduce testicular tissue destructions. The effect of *Calendula* on the male reproductive functions of rats was evaluated by Kushwaha *et al*. (2007). Healthy male albino rats were orally administrated 200 mg/kg body weight of an extract from *C. officinalis* for 60 days. The results confirmed a significant decrease in sperm motility and density as well as a significant reduction in serum testosterone level.

Gap junctional intercellular communication control testis functions at multiple steps such as testis development, steroid hormone production or spermatogenesis. At the same time, GJIC is extremely sensitive to exogenous stressors, and in many cases could partly participate in subfertility. Similarly, to our results Gao *et al*. (2014) evaluated the effect of *Apium graveolens* L. seed extract on expression of gap junctional protein in human stomach cancer cell line – Hs746T *in vitro*. Semi-quantitative RT-PCR, and Western blot analysis revealed an increase in endogenous Cx43 mRNA and protein expression following by *Apium* treatment, especially at 100 µg/ml after 72 h. Nakamura *et al.* (2005) evaluated the effect of kaempferol, as an important molecule of *Calendula* and *Levisticum* on GJIC of MSU-2 human foreskin

fibroblasts (HCT116) and human colon cancer cells (KNC). GJIC was measured 7 days after addition of experimental doses (5 and 10 μM). Kaempferol was found to enhance the level of GJIC in KNC cells to 1.33 times (5 μM) and 1.29 times (10 μM) higher than control- untreated cells. On the other hand, no enhancement of GJIC was detected in HCT116 cells following kaempferol treatment.

We are convinced, that dysregulation of GJIC presented in our study could be an essential part of the toxic mechanism related to the action of experimental extracts. According to presenting data, the TM3 mice Leydig cells are susceptible to the highest doses of applied medicinal herbs extracts with a toxic impact on essential cellular organelles and functions. However, as we mentioned before, the exact determination of proper concentrations may definitely affect the activity of mice Leydig cells and ensure sufficient production of male steroid hormones. Nowadays, the majority of experimental studies provide a broad spectrum of information, which is not consistent. Therefore, systematic and detailed research is definitely required for an exact conclusion formulation.

Conclusion

Presented data revealed significant concentration-dependent effects of *Apium graveolens* L. *Levisticum officinale* and *Calendula officinalis* L. on cell viability, membrane integrity, steroidogenesis, and intercellular communication of TM3 Leydig cells after short time cultivation. It has been shown that although medically used plants have a strong potential to inhibit the onset of many pathological conditions as well as support reproductive abilities, higher applied doses can encourage toxic effects mediated through reduced viability, membrane integrity as well as GJIC inhibition. Given these *in vitro* observations, we assume that a balanced concentration ratio may support the Leydig cell function, steroidogenesis, and all essential parameters that may significantly improve reproductive capacity in males.

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Declaration of interest

There is no conflict of interest.

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FIGURES CAPTIONS

Figure 1 The effects of *Apium graveolens* L., *Levisticum officinale* and *Calendula officinalis* L. on TM3 Leydig cell viability *in vitro* after 24 h cultivation

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 and experimental groups are indicated as: [a]Significant difference from the control *P< 0.05*; [b]Significant difference from the control *P< 0.01;* [c]Significant difference from the control *P< 0.001;* [d]Significant difference from the control *P< 0.0001.*

Figure 2 The effects of *Apium graveolens* L., *Levisticum officinale* and *Calendula officinalis* L. on TM3 Leydig cell membrane integrity *in vitro* after 24 h cultivation

ctrl – control group. Each bar represents the mean (±S.D) cell membrane integrity % 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 and experimental groups are indicated as: [a]Significant difference from the control *P< 0.05*; [b]Significant difference from the control *P< 0.01;* [c]Significant difference from the control *P< 0.001;* [d]Significant difference from the control *P< 0.0001.*

Figure 3A Progesterone production in TM3 Leydig cells exposed to different concentrations of experimental extracts from *Apium graveolens* L., *Levisticum officinale* and *Calendula officinalis* L. *in vitro* after 24 h cultivation

ctrl – control group. Each bar represents the mean (±S.D) progesterone production % 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 and experimental groups are indicated as: [a]Significant difference from the control *P< 0.05*; [b]Significant difference from the control *P< 0.01;* [c]Significant difference from the control *P< 0.001;* [d]Significant difference from the control *P< 0.0001.*

Figure 3B Testosterone production in TM3 Leydig cells exposed to different concentrations of experimental extracts from *Apium graveolens* L., *Levisticum officinale* and *Calendula officinalis* L. *in vitro* after 24 h cultivation

ctrl – control group. Each bar represents the mean (±S.D) testosterone production % 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 and experimental groups are indicated as: [a]Significant difference from the control *P< 0.05*; [b]Significant difference from the control *P< 0.01;* [c]Significant difference from the control *P< 0.001;* [d]Significant difference from the control *P< 0.0001.*

Figure 4A Intercellular communication in TM3 Leydig cells exposed to different concentrations of experimental extracts from *Apium graveolens* L., *Levisticum officinale* and *Calendula officinalis* L. *in vitro* after 24 h cultivation

ctrl – control group. Each bar represents the mean (±S.D) GJIC % of control (untreated) and treated groups. Data were obtained from three (n=3) independent experiments. The level of significance was set at *(P<0.05).* Statistical differences between the values of control and experimental groups are indicated as: [a]Significant difference from the control *P< 0.05*; [b]Significant difference from the control *P< 0.01;* [c]Significant difference from the control *P< 0.001;* [d]Significant difference from the control *P< 0.0001.*

Figure 4B The representative images of GJIC activity in the control group, and after 24 h exposure to 300 µg/ml of *Apium*, *Levisticum* and *Calendula* followed by SL/DT technique. The lucifer yellow dye spreading into the Leydig TM3 cells is related to the GJIC extent.

Figure 1

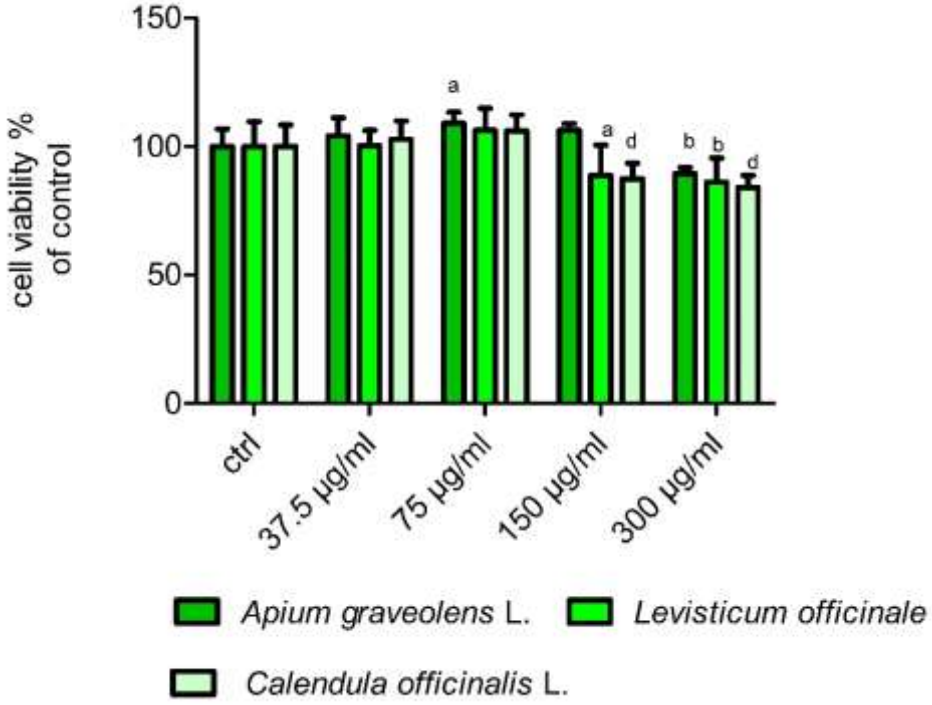


Figure 2

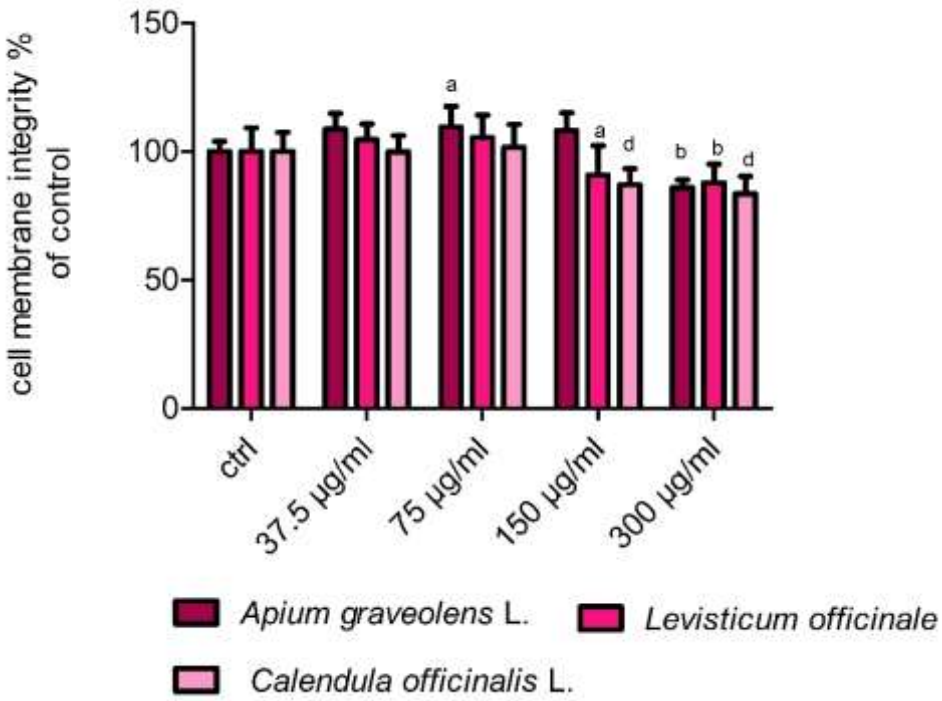


Figure 3A

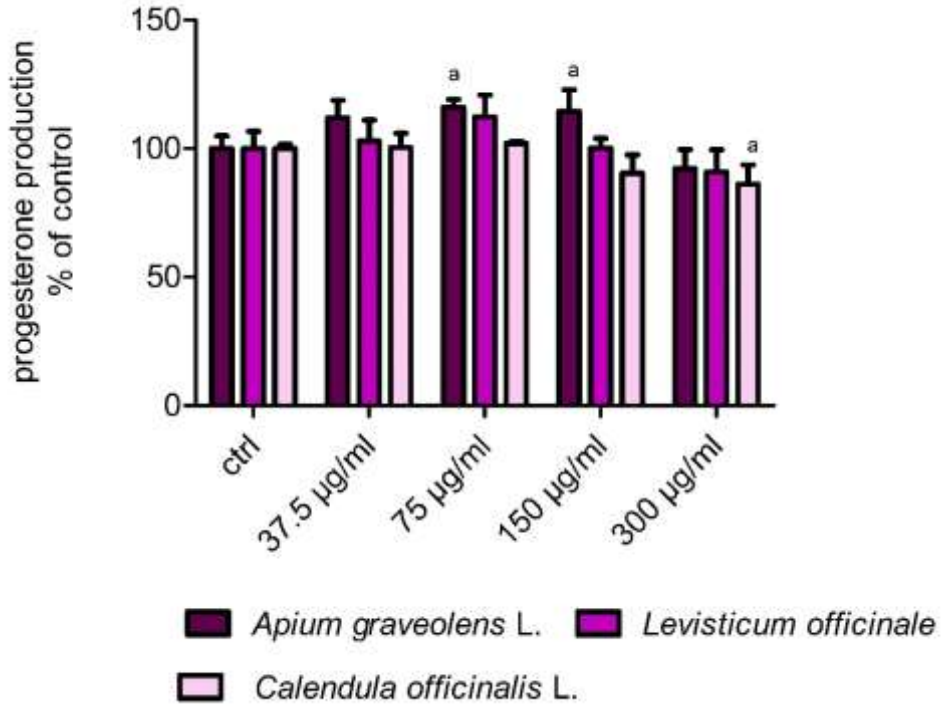


Figure 3B

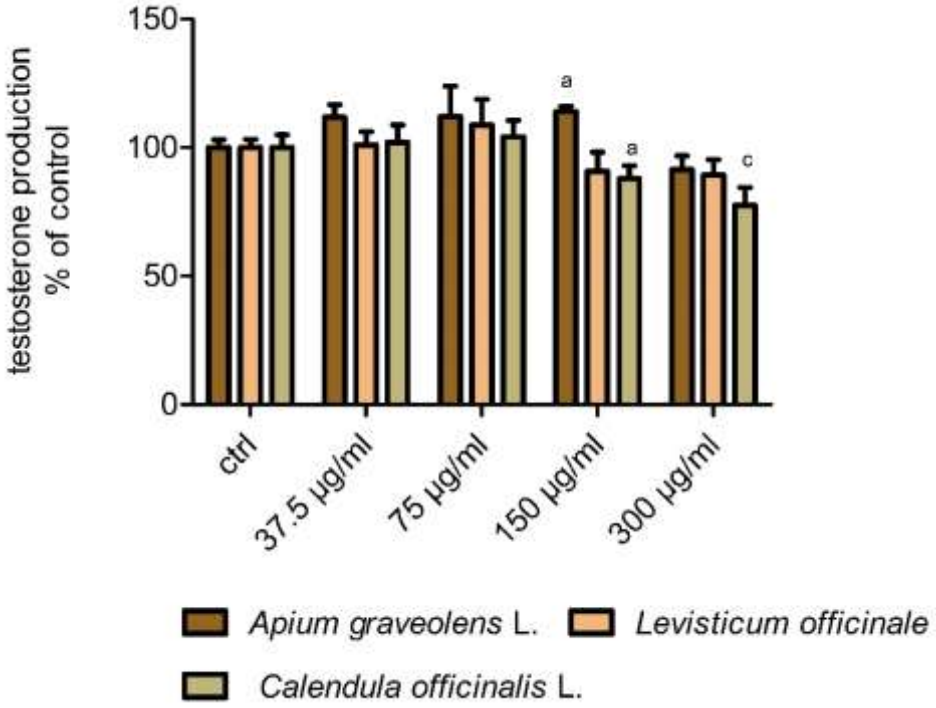
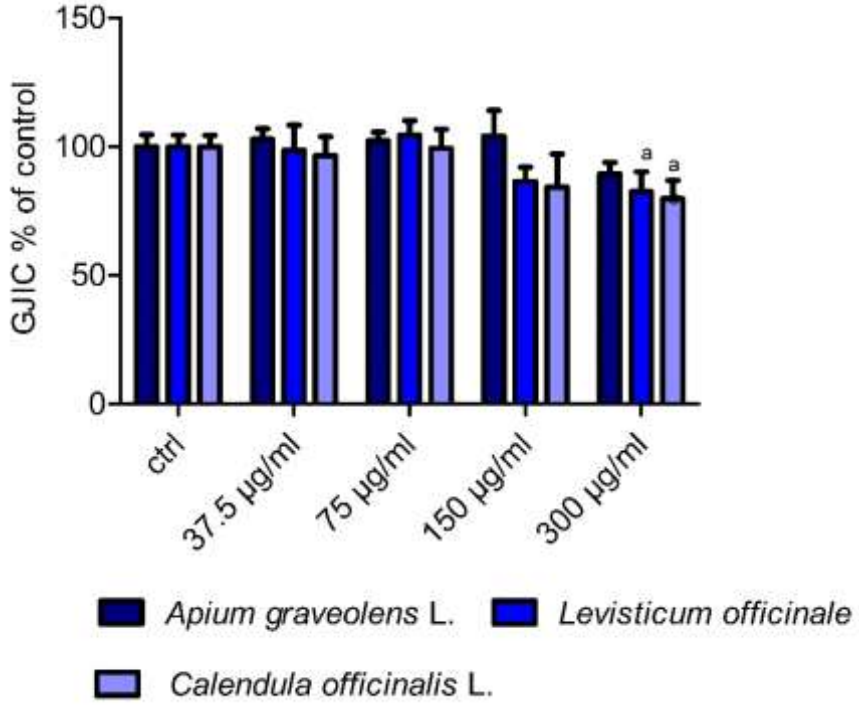


Figure 4A



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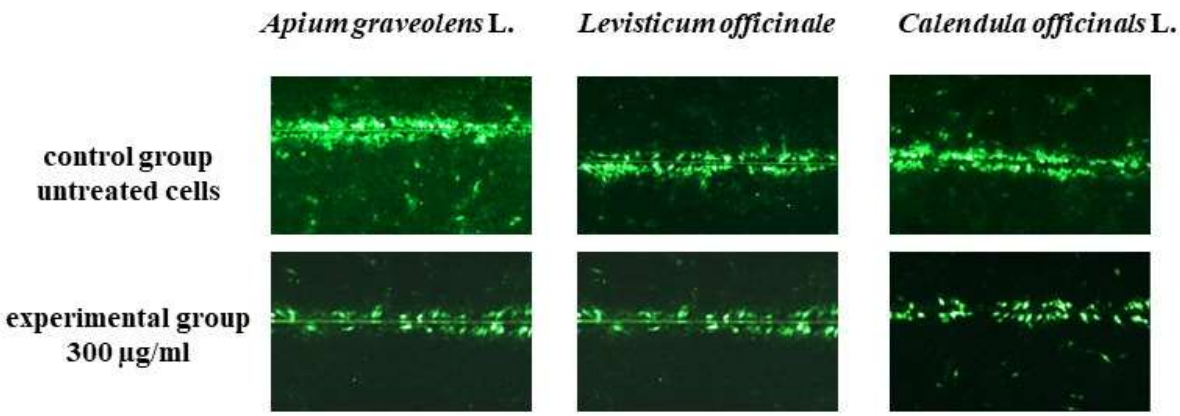
661 **Figure 4B**



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666 **TABLES CAPTIONS**

667 **Table 1** Major flavonoids identified and quantified (mg/kg) in *Apium graveolens* L., *Levisticum*
668 *officinale* and *Calendula officinalis* L.

669

670 **Table 2** Major phenolic acids identified and quantified (mg/kg) in *Apium graveolens* L.,
671 *Levisticum officinale* and *Calendula officinalis* L.

672

673 **Table 1**

Polyphenols	***Apium graveolens* L.**		***Levisticum officinale***		***Calendula officinalis* L.**	
	Mean [mg/kg]	S.D. [mg/kg]	Mean [mg/kg]	S.D. [mg/kg]	Mean [mg/kg]	S.D. [mg/kg]
Rutin	5.98	0.98	40.32	3.77	34.36	2.87
Vitexin	160.18	20.33	-	-	6.27	0.96
Cynaroside	49.57	5.45	440.35	10.21	12.99	1.11
Resveratrol	3.32	0.76	-	-	13.80	1.24
Apigenin	7.00	1.02	33.43	3.19	22.01	2.09
Kaempferol	7.88	1.14	44.47	5.00	22.77	2.01
Quercetin	4.95	0.78	-	-	17.42	1.55
Diaidzein	7.45	1.02	-	-	14.71	1.72
Catechin	-	-	-	-	12.22	0.98
Myricetin	-	-	-	-	11.16	1.02

674 S.D. – standard deviation

675

676 **Table 2**

Phenolic acids	*Apium graveolens* L.		*Levisticum officinale*		*Calendula officinalis* L.	
	Mean [mg/kg]	S.D. [mg/kg]	Mean [mg/kg]	S.D. [mg/kg]	Mean [mg/kg]	S.D. [mg/kg]
Neo-chlorogenic acid	8.79	1.54	365.90	3.09	36.55	2.55
Protocatechuic acid	130.78	12.78			-	-
trans-p-Coumaric acid	140.69	11.32	10.99	1.08	7.36	0.99
Sinapinic acid	-	-	5.30	1.04	55.30	4.01
trans-Sinapic acid	21.99	2.05			56.32	4.44
Ferulic acid	523.04	42.12	88.61	6.55	18.01	2.01
trans-ferulic acid	-	-	19.02	2.99	5.94	0.67
Rosmarinic acid	90.89	7.86			207.52	17.98
Chlorogenic acid	17.39	1.12	523.67	15.55	196.64	12.21
p-Coumaric acid	22.76	1.77			-	-
Caffeic acid	-	-	55.65	4.01	28.88	3.09
trans-Caffeic acid	-	-	22.33	2.69	57.97	3.63
Cinnamic acid	-	-			21.99	2.88
Gallic acid	-	-			6.99	0.78

677 S.D. – standard deviation

678