THE POTENTIAL EFFECTS OF TRIBULUS TERRESTRIS L. ON CELLULAR PARAMETERS AND STERIOGENESIS IN VITRO

Lucia Zuščiková*a,1, Hana Grešfová1, Nikola Knížatová1, Eva Tvrda1, Anton Kovačík1, Norbert Lukáč1, Tomáš Jambor1

Address(es): Ing. Lucia Zuščiková,
1 Slovak University of Agriculture in Nitra, Faculty of Biotechnology and Food Sciences Institute of Applied Biology, Tr. A. Hlinku 2, 94976 Nitra, Slovak Republic.
2 Slovak University of Agriculture in Nitra, Faculty of Biotechnology and Food Sciences Institute of Applied Biology, Tr. A. Hlinku 2, 94976 Nitra, Slovak Republic.

*Corresponding author: xzuscikoval@uniag.sk

ARTICLE INFO
Received 16. 8. 2022
Revised 8. 11. 2022
Accepted 14. 11. 2022
Published xxx.xxxx.x

ABSTRACT

Tribulus terrestris L. is an herbaceous plant native to India, widely used as a natural sexual stimulant by traditional medicine in China, India, and Greece, and also for the treatment of various diseases such as low blood pressure, cardiovascular diseases, kidney, and skin diseases, etc. Tribulus extracts are rich in numerous biologically active compounds, such as steroidal saponins, alkaloids, lignan amides, and flavonoids, which are useful for the effective treatment of sexual performance, hormonal and sexual problems. The main goal of our in vitro study was to demonstrate the potential consequences of Tribulus terrestris L. extract on TM3 cell parameters such as cell viability, membrane integrity, lysosomal activity, and steroid hormone secretion. The extract was applied to mice Leydig cells at various concentrations (ranging from 37.5 µg/mL to 600 µg/mL) for 24 h. Based on the obtained data, we may claim statistically significant inhibition of cell viability at 300 µg/mL (P < 0.0001) and 600 µg/mL (P < 0.0001). At the same concentrations, there was a statistically significant decline in both cell membrane integrity (P < 0.05; P < 0.0001) and lysosomal activity (P > 0.05; P < 0.0001). Hormone secretion was significantly increased at 200 µg/mL (P < 0.05). To better understand how Tribulus terrestris L. affects the reproductive systems of animals or humans, further research must be done.

Keywords: Tribulus terrestris, Leydig cells, cell viability, hormones

INTRODUCTION

Reproduction is a crucial process that ensures species continuity, boosts productivity, and keeps animal species alive (Sorelle et al., 2019). The main problem in reproduction is an occurrence of infertility. Male fertility can be affected by a variety of factors, including physiological ones, pathological, psychological, lifestyle, and environmental depending ones (Dutta et al., 2021). Low sperm numbers, abnormal sperm morphology, and insufficient sperm motility are the main causes of infertility and subfertility in most males (Haghmorad et al., 2019). Since the last 2000 years, numerous medicinal herbs have been used to successfully treat infertility (Kumar et al., 2021). Due to the beneficial effects of herbal preparations as medicines, the usage of spices and herbs has gradually expanded in developing countries in recent years (Kowalczyk et al., 2022). Also, herb medicines are mostly affordable, safe, and widely available, and about 60% of the world’s population prefers more natural products before medical treatments (Jaradat and Zaïd, 2019; Rates, 2001). It has been proven that male reproductive health may be improved using several herbal drugs, which were quite effective in treating oligozoospermia, erectile dysfunction, reproductive endocrinological dysfunctions, and other issues (Sengupta et al., 2021). There are numerous useful herbs from phytomedicine, including extracts from Tribulus terrestris L. (Ramgir et al., 2022). This annual plant is a part of the Zygophyllaceae family (Sin et al., 2022) and it has a long history of use as a well-known traditional medicine in China (Zhu et al., 2017) and India since ancient times (Neychev & Mitev, 2016). In both Asia and Europe Tribulus terrestris plant has been used to cure sexual dysfunctions and for various medicinal purposes, including cardiac protection, anti-urothelial, antiabdometic, anti-inflammatory, and antioxidant benefits (Khaleghi et al., 2017), since its various parts contain several biologically active substances, including vitamins, alkaloids, saponins, flavonoids, steroids, tannins, flavonol glycosides, unsaturated fatty acids, etc. (Aladdadou et al., 2022; Tkachenko et al., 2020). Based on the Tribulus composition it has been reported higher improvements in several sexual parameters such as epithelial thickness, an increase of Leydig, spermatogonia, and Sertoli cell numbers. Besides that, Tribulus extracts improved sperm parameters including sperm concentration and motility (Aladdadou et al., 2022). Khaleghi et al., 2017). Thus, in our study, we focused on the effect of Tribulus terrestris L. plant extract on TM3 cell models of the Leydig cell line in vitro. Monitoring the impact on individual parameters could help in the possible cure or prevention of sexual and other problems.

MATERIAL AND METHODS

Plant material collection and processing
The leaves from Tribulus terrestris L. were harvested at the Institute of Forest Ecology – Arborérum Mlyňany (Slovak republic). For the quantification of the total phenolic content and antioxidant activity assessment, 1 g of the leaves were freeze-dried, mechanically comminuted, and extracted with 10 mL of 80% (v/v) ethanol (EtOH; Centralchem, Bratislava, Slovak republic) for 12 h during constant shaking at room temperature. After centrifugation (9000 rpm, 20 min), the supernatant was collected, filtered (PVDF syringe filter – 0.45 µm), and used for further experiments (Ivanova et al., 2020). In the case of the high-performance liquid chromatography (HPLC) analysis, 2 g of freeze-dried leaves were milled and extracted in 20 mL of 80% (v/v) aqueous methanol (HPLC grade, Sigma-Aldrich, St. Louis, USA). The mixture was shaken on a horizontal shaker (250 rpm) at laboratory temperature for 24 h. Afterward, the prepared extract was filtered (Whatman filter paper, Maidstone, United Kingdom) and kept at 4 °C until HPLC analyses (Lukšic et al., 2016). For the in vitro experiments, the leaves of Tribulus terrestris L. were freeze-dried, crushed, and extracted in 80% (v/v) EtOH for 12 h in the dark at room temperature. To remove any residual EtOH, the extract was subjected to evaporation under reduced pressure (Stuart RE300DB; Bibrary Scientific Limited, United Kingdom) at 40 °C. Finally, the crude extract was dissolved in a dimethyl sulfoxide (DMSO; Sigma-Aldrich, St. Louis, USA) and adjusted to 4000 µg/mL which served as a stock solution (Tvrdá et al., 2016; Jambor et al., 2020).

Quantification of total phenolic content
The total polyphenols amount of the Tribulus extract was quantified by the Folin-Ciocalteu method according to previous studies (Singleton and Rossi, 1965; Shymanska et al., 2018). One hundred µL of the experimental extract was mixed with one hundred µL of the Folin-Ciocalteu reagent, 20% (w/v) sodium carbonate (Sigma-Aldrich, St. Louis, USA), and 8.8 mL of ultrapure water. After 0.3 h in darkness, the absorbance was quantified at 700 nm wavelength using the Jenway 6405 UV/VIS spectrophotometer (Fisher Scientific, Leicestershire, United Kingdom). Gallic acid (GAE) was used as the standard, and the total phenolic content was calculated using the standard curve. Results were expressed as mg of GAE equivalents per kg of dry weight.
Measurement of antioxidant activity
The free-radical scavenging activity of the Tribulus extract was evaluated using the 2,2-diphenyl-1-picrylhydrazyl (DPPH) method (Sanchez-Moreno et al., 1998). Four hundred µL of sample was added to 3.0 mL of DPPH solution (25 mg DPPH in 100 mL EtOH, Sigma-Aldrich, St. Louis, USA). The absorbance of the mixture was quantified using the Jenway 6405 UV/VIS spectrophotometer (Fisher Scientific, Leicester, United Kingdom) with setup wavelength at 515 nm. Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid; Sigma-Aldrich, St. Louis, USA) was used as the standard, and the total antioxidant activity was expressed as mg of Trolox equivalents (TEAC) per g of dry weight.

High-performance liquid chromatography (HPLC-DAD) analysis
For the quantification of polyphenols and phenolic acids of Tribulus terrestris extract, the Agilent 1260 Infinity high-performance liquid chromatography (Agilent Technologies, Waldbronn, Germany) with a quaternary solvent manager coupled with a diode array detector (G1315C), degasser (G1311B), sample (G1329B), and column (G1316A) manager were used. In addition, selected standards acetone (HPLC grade), and phosphoric acid (ACS grade) were purchased from Sigma-Aldrich (St. Louis, USA), while ddH2O (double-deionized water) was prepared in a purification system Simplex 185 (Milipore SAS, Molsheim, France). HPLC measurements were performed on a Purosphere reverse phase C18 column (Merck, Darmstadt, Germany). The mobile phase D (acetonitrile) and the mobile phase C (0.1% phosphoric acid in ddH2O) were included in the mobile phase. The gradient elution (90% C and 10% D), 1 min elution (85% C and 15% D), 6–12 min linear gradient elution (85% C and 15% D), 1–6 min linear gradient elution (85% C and 15% D), 12–20 min (30% C and 70% D), and 20–25 min (30% C and 70% D). The initial flow rate was 1 mL/min, and the injection volume was 5 µL. The column thermostat was set at 30 °C and the samples were kept at 4 °C by the sample manager. All obtained data were collected and processed using the Agilent OpenLab ChemStation software for LC 3D Systems (Luksic et al., 2016).

Cell culture and in vitro setups
The mice TM3 line of Leydig cells (provided by American Type Culture Collection, ATCC #CRL-1714TM; Manassas, VA, USA) were used in Dulbecco’s Modified Eagle’s Medium/Nutrient Mixture (Ham’s) F12 along with HEPES with phenol red (DMEM/F12; Sigma-Aldrich, St. Louis, MO, USA). The medium was enhanced with 2.5% fetal bovine serum (FBS; BiochromAG, Berlin, Germany), 5% horse serum (HS; Gibco-Life Technologies, Auckland, New Zealand), 2.5 mM L-glutamine (Sigma-Aldrich, St. Louis, MO, USA), 1% penicillin and streptomycin formula (Sigma-Aldrich, St. Louis, MO, USA) and maintained at 37°C, 5% CO2 under the 95% humidified atmosphere. TM3 cells were routinely screened for contamination. The passage process was initiated after achieving 85–90% of cell confluent monolayer in 75 cm² flasks (TPP, Trasadingen, Switzerland). The cells were treated with Trypsin-EDTA (Sigma-Aldrich, St. Louis, MO, USA) and subcultured into 96 well plates. The Leydig cell density was estimated using the automated cell counter TC 20iE (Bio-Rad Laboratories, California, USA). The final concentration of 4 × 104 cells per well was established by adding a culture medium. The day before cell seeding, the well plates were coated in gelatin (0.1% w/v in physiologically buffered saline). Once the cells were grown in well plates, the medium was replaced to contain various concentrations of experimental extracts. Treated groups were compared to non-treated groups (control groups) after 24 h. After that, cell viability, membrane integrity, lysosomal activity, and hormone secretion were analyzed.

Cytotoxicity tests
Cell viability
To determine the effect of herbal extracts on TM3 Leydig cell viability, alamarBlue™ assay was applied by using alamarBlue reagent (ThermoFisher Scientific, Invitrogen, Vantaa, Finland). AlamarBlue fluorometric assay relies on the irreversible, non-specific, enzymatic degradation of the chemical by living cells, where blue resazurin is reduced into pink resorufin, referring to mitochondrial dehydrogenase activity and viable cells (Kanniloga et al., 2020). Before the extract’s exposure, Leydig cells were pre-cultured in advance. Following that, the present culture medium was replaced by experimental doses (37.5 – 600 µg/mL) of Tribulus terrestris L. for 24 h. Treated cells were washed with Dulbecco’s phosphate-buffered saline (DPBS; Sigma-Aldrich, St. Louis, MO, USA) and incubated (95% atmospheric humidity; 37 °C; and 5% CO2 with DMEM/F12 (serum-free) containing a specific concentration of alamarBlue reagent 5% (v/v)). Fluorescence was measured after 30 minutes at 530/590 nm wavelengths (excitation/emission) by using a plate reader GlomaxMulti+ (Promega Corporation, Madison, WI, USA).

Cell membrane integrity
The integrity of the membrane was evaluated with a test using 5-carboxyfluorescein diacetate, acetoxyethyl ester (CFDA-AM; Thermo Fisher Scientific, Waltham, Massachusetts, USA). When 5-CFDA-AM enters the cell, non-specific intracellular esterases cleave it to produce fluorescent carboxyfluorescein, which is effectively retained by living cells with intact plasma membranes (Gorokhova et al., 2012). In brief, TM3 cells were seeded and preincubated before treatment with herbal extracts. Afterward, the supplemented culture medium was washed with DPBS and changed to a fresh medium (DMEM/F12) involving 4 µM CFDA-AM, and cells were incubated for 30 minutes at 37 °C; 5% CO2 and 95% atmospheric humidity. Final fluorescent metabolites were measured using a fluorescence plate reader GlomaxMulti+, using 485 and 530 nm wavelengths (excitation/emission).

Lysosomal activity
The ability of TM3 Leydig cells to integrate and accumulate the slightly cationic supravital red dye, which is binding to anionic and phosphate groups of lysosomes, is the basis of the neutral red uptake (NRU; Thermo Fisher Scientific, Waltham, MA, USA) assay (Ivanova & Uhlig, 2008). Shortly, the TM3 cell line was cultured for 24 h before herbal doses exposure at a specific density (4 × 104 cells per well) in 96- well plates with gelatin. Subsequently, the cell culture medium was removed and replaced with experimental concentrations of Tribulus terrestris L, starting from 37.5 µg/mL to 600 µg/mL for 24 h. After treatment, cells were washed with DPBS and covered with NR diluted in DMEM/F12 at a determined concentration of 0.005% (v/v) and incubated for 2 h. The destaining process was performed using 1% (v/v) acetic acid in 50% (v/v) ethanol for 20 min. Finally, the absorbance of the dissolved NR was measured at 525/660-720 nm wavelength by using a spectro-fluoro-luminometer GlomaxMulti+.

| Table 1 Inter-Assay, Intra-assay variability, and sensitivities of steroid hormones |
|-----------------|----------|----------|----------|
| Hormone         | Inter-Assay Variability (%) | Intra-Assay Variability (%) | Sensitivity |
| Progesterone    | ≤9.3     | ≤4.0     | 0.05 ng/mL |
| Testosterone    | ≤8.3     | ≤7.0     | 0.10 ng/mL |

Determination of steroid hormones (ELISA)
Steroid hormones (progesterone and testosterone) were evaluated by an enzyme-linked immunosorbent assay (ELISA). The fundamental principle of ELISA is to utilize an enzyme to detect the binding of antigen (Ag) antibody (Ab) (Ma & Shihe, 2006). Leydig cells were cultured in 96-well plates at a density of 4 × 104 cells per well for 24 h. Afterward, the cells were exposed to 37.5-600 µg/mL of herbal extract for 24 h. The medium from each well was removed and centrifuged at 3000 rpm; 4 °C; 10 min. Separated supernatant was preserved at ~ 80 °C in Eppendorf tubes until assay. To determine testosterone and progesterone level, commercially available ELISA kits were used. Analyses were accomplished according to the manufacturer’s specifications (progesterone Cat. #K00225, testosterone Cat. #K00218, Wiesbaden, Germany). The absorbance was measured at 450 nm wavelength by an ELISA microplate reader (Multispec FC, ThermoFisher Scientific, Vantaa, Finland). Each sensiveness and variability of analyzed hormones are shown in Tab 1.

Statistics
The acquired data were statistically analyzed by GraphPad Prism 6.01 (GraphPad Software Incorporated, San Diego, CA, USA). Firstly, the descriptive attributes, such as minimum, maximum, mean, and standard error of the mean, etc., were investigated. One-way analysis of variance (ANOVA) followed by Dunnett’s multiple comparison test was used for statistical evaluations. Results were represented as the mean ± standard error of the mean (SEM). Each of the experiments was repeated at least four times (n = 4). The following levels of statistical significance (* P < 0.05; ** P < 0.01; *** P < 0.001; **** P < 0.0001) were used to evaluate whether there were significant differences between the control group and the experimental groups.

RESULTS AND DISCUSSION
Total polyphenols and antioxidant activity measurements
The collected data from the biochemical assessment of Tribulus terrestris are presented in Tab 2. According to the Folin-Ciocalteu method, the total polyphenols content of the experimental extract was 98.87 mg GAE/g dry weight. Besides that, DPPH method revealed, that the free-radical scavenging activity of Tribulus was 92.99 mg TEAC/g dry weight.
Table 2 Biochemical profile and antioxidant capacity of Tribulus terrestris L.

| Parameter                 | Value (± SEM) |
|---------------------------|---------------|
| The total phenol content  | 98.87 ± 10.22 mg GAE/g d.w. |
| DPPH assay                | 92.99 ± 2.99 mg TEAC/g d.w. |

Abbreviations: Data are presented as means (± SEM) from three independent measurements. d.w. – dry weight, GAE - Gallic acid equivalents, TEAC – Trolox equivalents.

Bioactive constituents (HPLC-DAD analysis)

The concentrations of phenolic molecules identified in the Tribulus terrestris L. extract are summarized in Table 3. The main detected polyphenols were rosmarinic acid (9271.61 ± 662.09 mg/kg d.w.) followed by daidzein (574.41 ± 60.72 mg/kg d.w.), and chlorogenic acid (273.33 ± 25.55 mg/kg d.w.). Two flavonoid glycosides such as cymaroside and rutin, as well as two flavonoid aglycones naringenin and quercetin, were also found in the experimental extract. From the analyzed phenolic acids, neochlorogenic acid (178.04 ± 20.22 mg/kg d.w.) was the most abundant.

Table 3 Content of biologically active compounds in Tribulus terrestris L.

| Phenolic Compounds         | Concentration (mg/kg d.w.) (± SEM) |
|----------------------------|-----------------------------------|
| Rutin                     | 74.54 (±8.89)                     |
| Vitexin                   | 11.25 (±2.01)                     |
| Cynarosside               | 166.88 (±13.99)                   |
| Resveratrol               | 3.75 (±0.28)                      |
| Apigenin                  | 9.25 (±0.92)                      |
| Kaempferol                | 19.26 (±2.37)                     |
| Quercetin                 | 25.03 (±3.09)                     |
| Daidzein                  | 574.41 (±60.72)                   |
| Naringenin                | 35.78 (±3.22)                     |
| Pyrogallol                | 20.23 (±1.82)                     |
| Catechin                  | 16.56 (±2.12)                     |
| Catechol                  | 24.98 (±1.99)                     |
| Neochlorogenic acid       | 178.04 (±20.22)                   |
| Protocatecholic acid      | 69.84 (±6.72)                     |
| trans-Coumaric acid       | 28.03 (±1.99)                     |
| trans-Cinnamic acid       | 16.94 (±1.02)                     |
| Ferulic acid              | 17.90 (±2.55)                     |
| Rosmarinic acid           | 9271.61 (±662.09)                 |
| Chlorogenic acid          | 273.33 (±25.55)                   |
| p-Coumaric acid           | 80.45 (±7.28)                     |
| Ellagic acid              | 19.40 (±2.00)                     |
| Cinnamic acid             | 28.34 (±1.73)                     |
| Vanillic acid             | 18.99 (±0.98)                     |
| Salicylic acid            | 3.23 (±0.55)                      |

Abbreviations: Data are presented as means (± SEM) from three independent measurements. d.w. – dry weight.

Effect of Tribulus terrestris L. on Leydig cell viability

Individual concentrations of experimental doses of Tribulus terrestris L. had a visible concentration-dependent impact on cell viability, compared to a control sample (100 ± 4.214%) after 24 h (Figure 1). Obtained data revealed that 300 µg/mL (82.01 ± 6.741%) of herbal extract induced a significant (P < 0.0001) decrease in cell viability. We observed a similar significant (P < 0.0001) trend with the highest extract concentration of 600 µg/mL (63.76 ± 3.734%). As for the other concentrations, exposed TM3 cells were not significantly affected (P > 0.05). On the other hand, we can notice a slight increase in the viability of treated cells, at 150 µg/mL (106.3 ± 6.069%), but without statistical significance. According to the presented data, higher concentrations of Tribulus terrestris L. may reduce cell viability.

Figure 1 The effect of Tribulus terrestris L. exposure on TM3 Leydig cell viability in vitro after 24 h. Abbreviations: Ctrl (control group), each column is presented as the mean (±SEM) viability % of control (untreated) and experimental (treated) groups. Data were acquired from six independent experiments (n = 6). The levels of significance were formed at **** (P < 0.0001).

TM3 cell membrane integrity

Mice TM3 Leydig cell line was exposed to different concentrations of Tribulus terrestris L. during 24 h cultivation in vitro. The data presented in Figure 2 demonstrate that the cell membrane integrity was not significantly (P > 0.05) affected by lower extract concentrations (37.5-250 µg/mL) compared to the control group (100 ± 5.757%). However, higher extract doses of 300 µg/mL and 600 µg/mL negatively influenced cell membrane integrity (87.34 ± 7.271%; 70.41 ± 10.07%), which was significantly (P < 0.05; P < 0.0001) decreased in both concentrations. Based on the gained results, we can state a similar effect of the herbal extract on viability as well as on membrane integrity.

Figure 2 Membrane integrity of TM3 Leydig cell line treated with various doses of Tribulus terrestris L. for 24 h cultivation in vitro. Abbreviations: Ctrl (control group), each column is presented as the mean (±SEM) membrane integrity % of control (untreated) and experimental (treated) groups. Data was acquired from six independent experiments (n = 6). The levels of significance were formed at * (P < 0.05) and **** (P < 0.0001).

Lysosomal activity

Figure 3 shows the impact of Tribulus terrestris L. treatment on lysosomal activity in TM3 Leydig cells. Herbal extract at lower dosages (37.5-250 µg/mL) did not result in significant (P > 0.05) elevation of lysosomal activity compared to the control group (100 ± 3.021%). As with the previous specific parameters (viability, membrane integrity), we can see similarities in obtained results. Statistically significant (P < 0.05; P < 0.001) changes occurred in experimental samples supplemented with 300 µg/mL and 600 µg/mL of herbal extract. These experimental groups led to a significant reduction of cell lysosomal activity (92.40 ± 3.535%; 87.44 ± 6.595%).
To further verify the effect in vitro, their levels were measured in the mice TM3 cell line (Figure 4A, Figure 4B) for a period of 24 h. When compared to the control group (100 ± 2.685%), a dose of 200 µg/mL extract showed a significant (P < 0.05) increase in progesterone secretion (115.3 ± 4.399%) (Figure 4A). The lowest concentrations (37.5-150 µg/mL) also had a stimulating effect on secretion, but with a non-significant impact (P > 0.05). On the contrary, experimental groups with higher doses (200-600 µg/mL) led to a drop in hormone secretion without significance (P > 0.05). In the case of secreted testosterone (Figure 4B), there was not a significant change in the lowest herbal concentration (37.5 µg/mL). However, in the following experimental groups 75, 150, and 200 µg/mL of herbal dosage, there was a significant (P < 0.05; P < 0.01; P < 0.05) increase in testosterone secretion (115.3 ± 7.192%; 118.8 ± 4.121%; 117.8 ± 2.436%) compared to the control group. Overleaf, a visual decrease of hormone secretion was observed in the highest concentrations (250-600 µg/mL) of Tribulus terrestris L. Likewise in Figure 4A, but without significant impact.

Progestrone and testosterone secretion

Natural plant products and therapeutic plant extracts have aroused interest throughout history. The usage of natural products and products based on them has increased rapidly over the past few decades for their beneficial health effects, yet there is still a lack of research data in this area. In our experiment, we analyzed the effect of Tribulus terrestris L. extracts on selected cellular mechanisms in TM3 Leydig cells in vitro. HPLC-DAD analyses confirmed a wide range of biologically active compounds with the highest amount of rosmarinic and chlorogenic acid, followed by three flavonoid aglycones namely daidzein, naringenin, and quercetin. Previous studies performed by Zheng (2017) and Tian et al. (2020) identified more than twenty major constituents presented in the Tribulus terrestris L. extract. They confirmed 13 saponins, 6 flavonoids, and derivatives of caffeic acid. In contrast to our study, quercetin and kaempferol were the most abundant. The different levels of phenolic compounds could be caused by growing conditions, area of cultivation, as well as by plant processing methods. A varied representation of detected phenolic compounds confers certain characteristics to Tribulus. The specific content of our sample could be related to its antioxidant activity, which was discussed in a previous study (Abbas et al. 2022.) Our study evaluated antioxidant activity by DPPH scavenging method. Gained results indicate 92.99 ± 2.99 mg trolox equivalent per gram dry weight of the extract. The significant potential to scavenge free radicals was evaluated by Durgawale et al. (2017) previously. The DPPH and ABTS (2, 2'-Azino- bis (3-ethylbenzothiazoline-6-sulfonic acid) Diammonium salt) confirmed dose-dependent inhibition of free radicals’ production with the highest potential at 30 and 40 µg/mL of Tribulus terrestris L. extract. Antioxidant potential fluctuated between 84.6% to 90% respectively. In the case of total phenolic content quantification, indicating 98.87 ± 10.22 mg gallic acid equivalent per gram dry weight of the extract. Durgawale et al. (2017) study’s estimated the total phenolic content of the extract at 6.65 ± 0.64 mg GAE/g of dry weight. Similarly, lower phenolic content was declared by Ali et al. (2018), where the total amount was set at 14.48 ± 0.16 mg GAE/g dry weight. According to this study’s findings, Tribulus terrestris L. has a slightly positive impact on monitored parameters in lower dosages. On the contrary, higher extract concentrations indicated significant decreases in presented parameters. Many conducted studies have confirmed the positive effect of Tribulus terrestris L. extract on male or female reproductive systems. It was claimed that Tribulus extracts, and dietary supplements improved male and female libido disorders, impotence, infertility, and sperm mobility in addition to improving muscle tone and spermatogenesis (Kostova and Dinchev, 2005). Abadjieva et al. (2019) analyzed the effect of Tribulus on the reproductive organs of growing male rabbits during 42 days. With increasing dosage there were changes in testicular weight, also the extract had a positive impact on the development of gonadal tissue. Compared to the control group, the population of cells in the lumen of the seminiferous tubules increased. In the group with the concentration of 5 mg/kg body weight, there were observed changes in the epithelial layer of gonads and a reduction in the number of Leydig cells. Besides that, the Karimi Jashni et al. (2011) research evaluated the effect of Tribulus terrestris on spermatogenesis in rats (oral dose once daily for 8 weeks), where the population of primary spermatocytes significantly (P < 0.05) increased in extract dose of 10 mg/kg. In the case of viability, previously Khalegi et al. (2017) observed the effect of Tribulus extract on human sperm viability in vitro after 0 seconds, and once every 15, 30, 60, and 120 minutes of incubation, where doses of 40 and 50µg/mL of Tribulus terrestris extract considerably increased sperm viability, but non-

Figure 3 The effect of Tribulus terrestris L. exposure on TM3 Leydig cell lysosomal activity in vitro after 24 h. Abbreviations: Ctrl (control group), each column is presented as the mean (±SEM) lysosomal activity % of control (untreated) and experimental (treated) groups. Data were acquired from six independent experiments (n = 6). The levels of significance were formed at * (P < 0.05) and *** (P < 0.001).

Figure 4 The impact of Tribulus terrestris L doses on progesterone (A) and testosterone (B) secretion in TM3 Leydig cells in vitro. Abbreviations: Ctrl (control group), each column is presented as the mean (±SEM) hormone secretion % of control (untreated) and experimental (treated) groups. Data were acquired in both cases from four independent experiments (n = 4). The levels of significance were formed at * (P < 0.05) and ** (P < 0.01).
significantly. We discovered the same trend in our results, where the viability of Leydig cells elevated at the dose of 150 µg/mL of Tribulus terrestris L. (P < 0.05).

Pavin et al. (2018) indicated protective properties of Tribulus terrestris (TT) (14 days) extract in mice reproductive systems damaged by cyclophosphamide (CP). It was proven, the Tribulus terrestris improved sperm motility and viability, and there was a no-significant positive impact on sperm membrane integrity in the group of CP+TT (100 mg/kg + 11 mg/kg) compared to the control and CP groups.

Also, Salashhoor et al. (2020) investigated whether the extract of Tribulus terrestris L. could enhance the toxic effect of Malathion (Mal) on the male reproductive system in vivo. TT was administrated orally, and daily for 8 weeks. The determined parameters were progressive motility, sperm cell morphology, the number of sperm cells, testostereone level measurement, etc. In the case of progressive motility and sperm viability, there was a minor increase in the group with TT extract compared to the control, but without significance. Statistically significant increase in sperm motility along with sperm membrane integrity was observed in the TT-treated group compared to the Mal group.

In our study, we estimated Tribulus terrestris L.’s impact on steroid hormones secretion of TM3 Leydig cells in vitro after 24 h, since steroidogenesis is one of the main mechanisms of male fertility. Visible significance occurred in the case of testosterone secretion at concentrations 75, 150, and 200 µg/mL of Tribulus (P < 0.05; P < 0.01; P < 0.05). Progesterone secretion was significantly enhanced at the dose of 200 µg/mL. Authors in a recent study confirmed the protective effect of Tribulus terrestris extract on testis steroidogenesis in copper overload rats (Arafa et al., 2019). The results demonstrated backward restoration without significance in both testosterone and luteinizing hormone secretion with Tribulus terrestris extract (10 mg/kg) after 90 days. A similar influence was researched in Tarko et al. (2022) study. They elucidated the effect of Tribulus terrestris alone and in combination with xylene on basic bovine ovarian cell functions in vitro for 48 h. Testosterone release was positively stimulated at a concentration of 100 µg/mL Tribulus extract with xylene compared to the control group, with significance (P < 0.05).

Regardless of the previous data, our obtained results managed to show the cytotoxicity impact of Tribulus terrestris L. extract on cellular models after 24 h. In all monitored viability parameters was a significant reduction after Tribulus treatment. In the case of cell viability, there was a decline at concentrations 300 and 600 µg/mL (P < 0.0001). A similar pattern was observed by Neychev et al. (2007), where they explored different aspects of the effect of Tribulus under cellular conditions, using normal human skin fibroblasts as a cell model. Firstly, they analyzed cytostatic/cytotoxic effect of Tribulus, which had a cytostatic impact at lower concentrations (0.06 to 5.6 µg/mL) after 24 h. Nevertheless, increasing concentration (over 6.7 µg/mL) caused a statistically significant decline in cell viability. Additionally, Abudayyak et al. (2015) investigated the potential disrupting and cytotoxic activities of Tribulus terrestris on rat kidney proximal tubular epithelial cell model in vitro after 24 h. The results estimated a concentration-dependent effect, where the cytotoxicity was not observed upon water and chloroform Tribulus extracts. The methanol extracts of Tribulus at concentrations 62.5; 125; 250 and 500 µg/mL have recorded a decrease in cell viability (68.5; 62.6; 28.0 and 8.2% of control-100%). Consequently, we identified decreased testosterone and progesterone production at doses of 300 and 600 µg/mL of fresh herb. The same tendency was in a study conducted with rats who had poisoning 1% iodoide and 5 µg/mL of TT (0.01% TT) which caused an increase in cell death activity drop at a concentration of 50.9 µg/mL in vitro during 24 h. Tribulus was able to weaken the symptoms of PCOS, in a dose-dependent manner (Sandee et al., 2015).

CONCLUSION

In conclusion, we confirmed a significant antioxidant potential of Tribulus as well as a rich content of phenolic compounds. In addition, dose-dependent effect of Tribulus terrestris L. extract on TM3 Leydig cells parameters, such as cell viability, membrane integrity, lysosomal activity, and steroid hormone secretion was observed. The results showed a slight improvement in presented parameters, mostly at the lowest concentrations (37.5-200 µg/mL) of Tribulus dose. In the case of sex-steroid hormones secretion, lower doses up to 200 µg/mL significantly stimulated the steroidogenic process and increased progesterone and testosterone release. On the other hand, the highest doses of experimental extract (2500 µg/mL), led to decreasing viability parameter cell characteristics, sperm activity, and lysosomal activity, along with hormone production in the Leydig cell line significantly.

Acknowledgments: This work was supported by the Scientific Agency of the Republic of VEGA No. 1/008321 and the Slovak Research and Development Agency Grant No. APVV-21-0168 and APVV-20-0218. This publication was also supported by the Operational program Integrated Infrastructure within the project: Demand-driven research for the sustainable and innovative food, Drive4ISFood 313011V336, co-financed by the European Regional Development Fund.

REFERENCES

Abedjiva D., Grigorova S., Gjorgjova N., Kistanova E. (2019). Dose-dependent effect of Tribulus terrestris dry extract on reproductive organs of growing male rats. Biologia, 74(4), 295-301. https://doi.org/10.7414/Biologia/1299091176786766915

Abedjiva D., Grigorova S., Gjorgjova N., Kistanova E. (2020). Dose-dependent effect of Tribulus terrestris dry extract on reproductive organs of growing male rats. Biologia, 75(1), 16-23. https://www.researchgate.net/publication/336980735_Dose-dependent_effect_of_Tribulus_terrestris_dry_extract_on_reproductive_organs_of_growing_male_rats

Abbas M. W., Hussain M., Akhtar S., Ismaill T., Qamar M., Shafiq Z., Etatabeyolu T. (2022). Bioactive Compounds, Antioxidant, Anti-Inflammatory, Anti-Cancer, and Antibacterial Activity Assessment of Tribulus terrestris—In Vitro and In Vivo Studies. Antioxidants, 11 (6), 1160. https://doi.org/10.3390/antiox11061160

Abudayyak M., Jannuzzi A. T., Özhan G., Alpertunga B. (2015). Investigation on the toxic effect of Tribulus terrestris in vitro. Pharmaceutical Biology, 53 (4), 477-496. https://doi.org/10.1080/13546801.2014.924019

Agric. 76 (2), 270-276. https://doi.org/10.1002/SCIC.1190701100110089276.2-270-276.https://doi.org/10.1002/SCIC.1190701100110089276.2-270-276.https://doi.org/10.1002/SCIC.1190701100110089276.2-270-276.

Aladdouw A. W., Aljohani A. S. M., Ahmed I. A., Al-Wabel N. A., El Ashmawy I. M. (2019). Ameliorative effect of methanolic extract of Tribulus terrestris L. on nicotine and lead-induced degeneration of sperm quality in male rats. Journal of Ethnopharmacology, 295. https://doi.org/10.1016/j.jep.2022.115337

Ali S. I., Gaafar A. A., Abdallah A. A., El-Daly S. M., El-Banaa M., Hussein J. (2018). Mitigation of Alpha-Cypermethyl-Induced Hepatotoxicity in Rats by Tribulus terrestris Rich in Antioxidant Compounds. Journal of Biological Sciences, 18 (5), 517-525. https://papers.ssrn.com/sol3/papers.cfm?abstract_id=320073

Arafa M. H., Amin D. M., Samar G. M., Atteia H. H. (2019). Protective effects of tribulus terrestris extract and angiotensin blockers on testis steroidogenesis in copper overloaded rats. Ecotoxicology and Environmental Safety, 178, 113-122. https://doi.org/10.1016/j.ecoenv.2019.04.012

Arafa M. H., Amin D. M., Samar G. M., Atteia H. H. (2019). Protective effects of tribulus terrestris extract and angiotensin blockers on testis steroidogenesis in copper overloaded rats. Ecotoxicology and Environmental Safety, 178, 113-122. https://doi.org/10.1016/j.ecoenv.2019.04.012

Arafa M. H., Amin D. M., Samar G. M., Atteia H. H. (2019). Protective effects of tribulus terrestris extract and angiotensin blockers on testis steroidogenesis in copper overloaded rats. Ecotoxicology and Environmental Safety, 178, 113-122. https://doi.org/10.1016/j.ecoenv.2019.04.012

Durgawale P., Datkhile V., Shukla A., Mansi A. (2021). Bioactivity, Toxicity Assessment of Tribulus terrestris L. on the Reproductive System of Animals and Humans: Potential and Limitations. Evidence-Based Complementary and Alternative Medicine, https://doi.org/10.1155/2022/4766409.
Zhu W., Du Y., Meng H., Dong Y., Li L. (2017). A review of traditional pharmacological uses, phytochemistry, and pharmacological activities of Tribulus terrestris. Chemistry Central Journal, 11 (1), 60. https://doi.org/10.1186/s13059-017-0289-0

Luksic L., Arvay J., Vollmanova A., Toth T., Skrbanja v., Tcek J., Germ M., Kreft I. (2016). Hydrothermal treatment of Tartary buckwheat grain hinders the transformation of rutin to quercetin. Journal of cereal science, 72, 131-134. https://doi.org/10.1016/j.jcs.2016.10.009

Neychev V. K., Nikolova E., Zhelev N., Mitov V. I. (2007). Saponins from Tribulus terrestris L. Are Less Toxic for Normal Human Fibroblasts than for Many Cancer Lines: Influence on Apoptosis and Proliferation. Society for Experimental Biology and Medicine, 232 (1), 126-133. doi:10.3181/00379727-207-2329126

Neychev V., Mitov V. (2016). Pro-sexual and androgen enhancing effects of Tribulus terrestris L.: Fact or Fiction. Journal of Ethnopharmacology, 179, 345-355. https://doi.org/10.1016/j.jep.2015.12.055

Pavin N. F., Izaquiryn A. P., Soares M. B., Spiauzzi C. Ch., Mendez A. S. L., Leivas F. G. (2018). Tribulus terrestris Protects against Male Reproductive Damage Induced by Cyclophosphamide in Mice. Oxidative Medicine and Cellular Longevity, 2018, 9 p. https://doi.org/10.1155/2018/5758191

Ranger S. S., Renu K., Vellingin B., George A., Tirupapuliyur D., Thiagarajan P., Gopalakrishnan A. V. (2022). Phytochemical Therapeutics for Male Infertility: Critical Insights and Scientific Updates. Journal of Natural Medicine, 76, 546-573. https://doi.org/10.1007/s11418-022-01619-0

Rates S. M. (2001). Plants as source of drugs. Toxicin, 39 (5), 603-613. https://doi.org/10.1016/S0041-0101(00)01549-9

Salahshoor M. R., Abdolmaleki A., Faramarzi A., Jalili C., Shiva R. (2020). Major and Trace Element Content of Tribulus terrestris L. and Their Beneficial Effects on Polycystic Ovary Syndrome-Induced Rat Models. Metabolic syndrome and related disorders, 13 (6), 248-254. https://doi.org/10.1089/met.2014.0136

Sengupta, P., Durairajanayagam, D., Agarwal, A. (2021). Herbal medicine used to treat andrological problems: Asia and Indian subcontinent: Withania somnifera, Panax ginseng, Centella asiatica. Herbal Medicine in Andrology, 93-106. https://doi.org/10.1016/B978-0-815565-3.00015-1

Shymanska O., Vergun O., Rakhetmov D., Brindza I., Ivanisova E. (2018). A Total Content of Phenolic Compounds in the Ethanol Extracts of Galega officinalis L. and G. orientalis Lam. Agrobiodiversity for Improving Nutrition, Health and Life Quality, (2), 140-145. https://agrobiodiversity.uneag.sk/scientificpapers/article/view/163

Singleton V. L., Rossi J. A. (1965). Colorimetry of Total Phenolics with Phosphomolybdic-Phosphotungstic Acid Reagents. American Journal of Enology and Viticulture, 16 (3), 144-158. https://www.ajevonline.org/content/16/3/144.article-info

Sorelle D. N., Ferdinand N., Narcisse V. B., Tchoumboue. (2019). Medicinal Plants and Female Reproduction Disorders due to Oxidative Stress. Archives of Veterinary Science and Medicine, 2, 058-073. https://fortuneonline.org/articles/medicinal-plants-and-female-reproduction-disorders-due-to-oxidative-stress.html

Sun X.-Ch., Song X., Guo F., Yuan Y.-H., Wang S.-Y., Wang S., Liu K.-L., Lv X.-Y., Han B., Zhang Ch., Liu J.-T. (2022). Terrestrosin D, a spirostanol saponin from Tribulus terrestris L. with potential hepatorenal toxicity. Journal of Ethnopharmacology, 283. https://doi.org/10.1016/j.jep.2021.114716

Tarko A., Štochmaľová A., Hrabovszká S., Vachanová A., Továrová D., Thiagarajan P., Gopakrishnan A. V. (2022). Phytomedicinal Therapeutics for Male Infertility: Critical Insights and Scientific Updates. Journal of Natural Medicine, 76, 546-573. https://doi.org/10.1007/s11418-022-01619-0

Tsezos A., Iliodromiti A. G., Vougiouklakis G. G. (2019). Medicinal plants and female reproduction disorders due to oxidative stress. Archives of Veterinary Science and Medicine, 2, 058-073. https://fortuneonline.org/articles/medicinal-plants-and-female-reproduction-disorders-due-to-oxidative-stress.html

Tvrda E., Michalko J., Matusíková I., Lukác N. (2016). In vitro effects of the Chlamydomonas reinhardtii extract on bovine spermatozoa. Journal of Microbiology, Biotechnology and Food Science, 6 (3), 972-975. https://doi.org/10.15414/jmbfs.2016.17.6.3.972-975

Zheng W., Wang F., Zhao Y., Sun X., Kang L., Fan Z., Qiao L., Yan R., Liu S., Ma B. (2017). Rapid Characterization of Constituents in Tribulus terrestris from Different Habitats by UHPLC/Q-TOF MS. Journal of the American Society for Mass Spectrometry, 28 (11), 2302-2318. https://doi.org/10.1007/s13361-017-1761-5