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

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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 for example 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 ($P < 0.05$; $P < 0.001$). 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 medications are mostly affordable, safe, and widely available, and about 60% of the world´s population prefers more natural products before medical treatments (Jaradat and Zaid, 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 disruptions, 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 (Sun *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-urolithic, antidiabetic, 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. (Aldaddou *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* extract improved sperm parameters including sperm concentration and motility (Aldaddou *et al*., 2022; Khaleggi *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 – Arborétum 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 (Ivanisova *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 (Luksic *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; Bibby 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 (Tvrda *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-difenyl-1-picrylhydrazyl (DPPH) method (Sanchéz-Moréno *et al*., 1998). Four hundred mL of sample was added to 3.6 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, Leicestershire, 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 acetonitrile (HPLC grade), and phosphoric acid (ACS grade) were purchased from Sigma-Aldrich (St. Louis, USA), while ddH_2O (double-deionized water) was prepared in a purification system Simplicity 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 ddH_2O) were included in the mobile phase. 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 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 grown 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% CO_2 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 cm^2 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 20™ (Bio-Rad Laboratories, California, USA). The final concentration of 4 x 10^3 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 in viable cells (Kamiloglu *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 Dulbeccos's phosphate-buffered saline (DPBS; Sigma-Aldrich, St. Louis, MO, USA) and incubated (95% atmospheric humidity; 37 °C; and 5% CO_2) 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, acetoxymethyl 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 precultured 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% CO_2; 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 x 10^3 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% (*w/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 & Shieh, 2006). Leydig cells were cultured in 96-well plates at a density of 4 x 10^3 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. Analyzes were accomplished according to the manufacturer's specifications (progesterone Cat. #K00225, testosterone Cat. #K00234, Dialab, Wiener Neudorf, Austria). The absorbance was measured at 450 nm wavelength by an ELISA microplate reader (Multiscan FC, ThermoFisher Scientific, Vantaa, Finland). Each sensitiveness 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 polyphenols 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 cynaroside 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	11.28 (±2.01)
Cynaroside	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 (±1.90)
Neochlorogenic acid	178.04 (±20.22)
Protocatechuic acid	69.84 (±6.72)
trans-Coumaric acid	28.03 (±1.99)
trans-Cinapic 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.

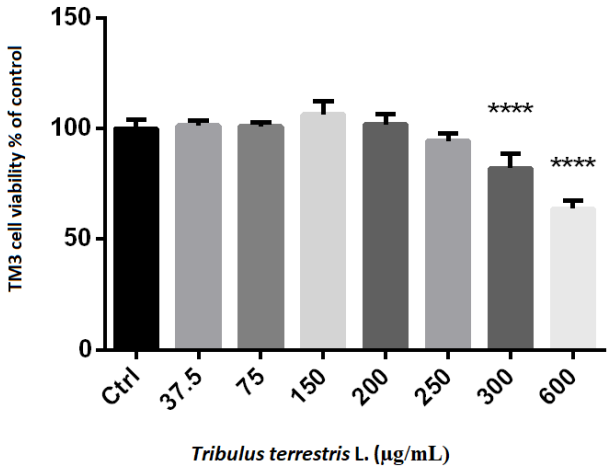


Figure 1 The effect of *Tribulus terrestris* L. exposure on TM3 Leydig cell viability *in vitro* after 24 h. Abbreviations: Ctrl (control group), each column is presented as the mean (±SEM) viability % of control (untreated) and experimental (treated) groups. Data were acquired from six independent experiments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

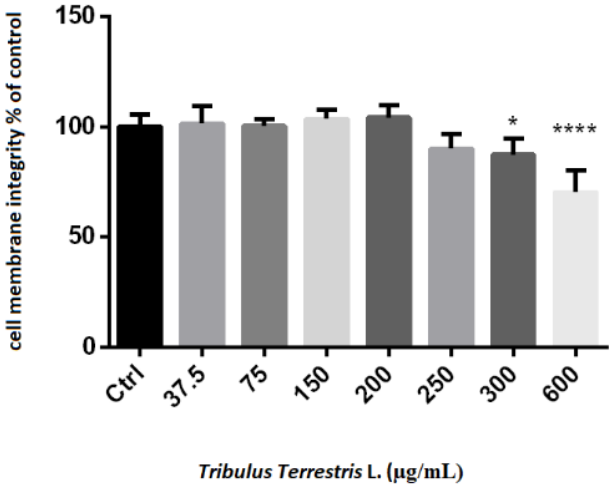


Figure 2 Membrane integrity of TM3 Leydig cell line treated with various doses of *Tribulus terrestris* L. for 24 h cultivation *in vitro*. Abbreviations: Ctrl (control group), each column is presented as the mean (±SEM) membrane integrity % of control (untreated) and experimental (treated) groups. Data 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%).

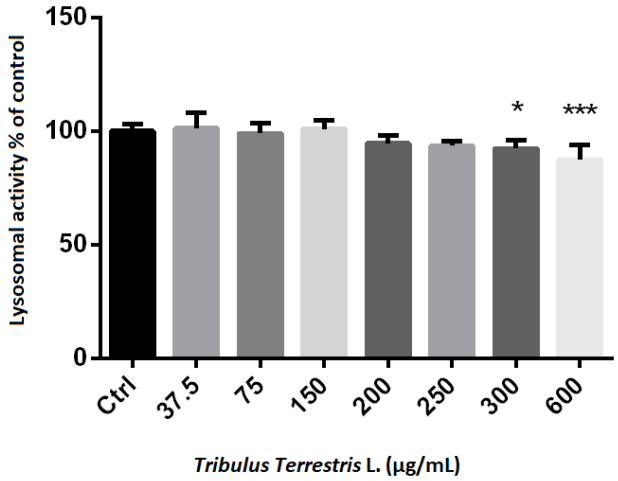
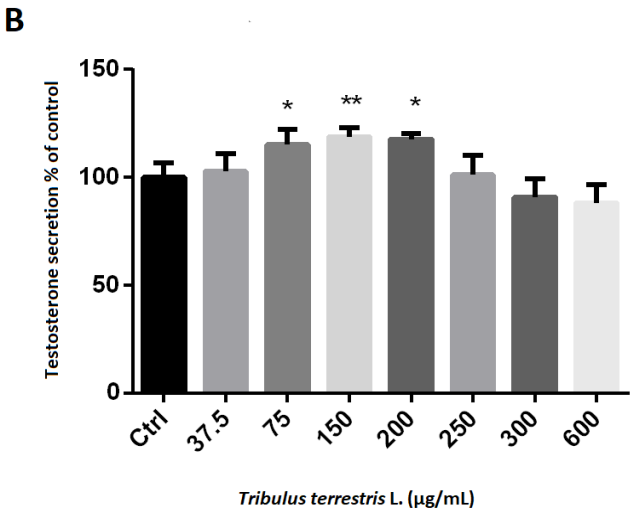


Figure 3 The effect of *Tribulus terrestris* L. exposure on TM3 Leydig cell lysosomal activity *in vitro* after 24 h. Abbreviations: Ctrl (control group), each column is presented as the mean (±SEM) lysosomal activity % of control (untreated) and experimental (treated) groups. Data were acquired from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

Progesterone and testosterone secretion

To further verify the effect of *Tribulus terrestris* L. on steroid hormone secretion *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 (115.5 ± 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.



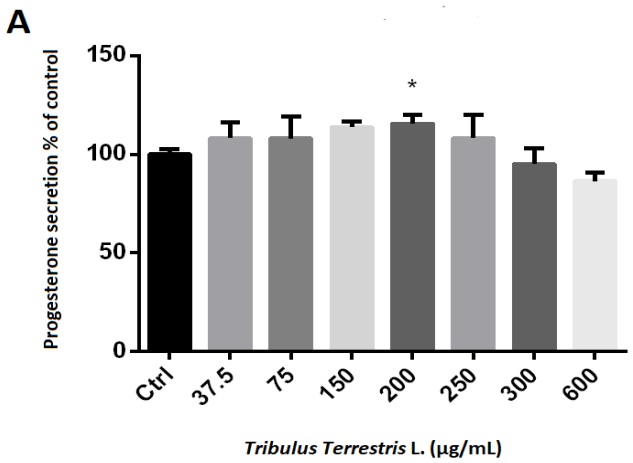


Figure 4 The impact of *Tribulus terrestris* L doses on progesterone (A) and testosterone (B) secretion in TM3 Leydig cells *in vitro*. Abbreviations: Ctrl (control group), each column is presented as the mean (±SEM) hormone secretion % of control (untreated) and experimental (treated) groups. Data were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

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, Folin-Ciocalteau reagent indicates 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, were occurred 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-

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 terres*tris (TT) (14 days) extract in mice reproductive systems damaged by cyclophosphamide (CP). It was proven, that *Tribulus* extract improved sperm characteristics, specifically, 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, Salahshoor *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, testosterone 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 results were in Mal + TT dose (10 mg/kg) compared to the Mal group. The same trend was observed in other parameters, where there was a significant improvement in all concentrations (2.5; 5; 10 mg/kg) of group Mal + TT 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 ng/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 (from 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 herb extract. The same tendency was in a study conducted with rats who had polycystic ovary syndrome (PCOS), testosterone showed an anti-androgenic 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 (Sandeep *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 (250-600 µg/mL), led to decreasing viability parameters (cell viability, membrane activity, and lysosomal activity), along with hormone production in the Leydig cell line significantly.

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REFERENCES

Abadjieva D., Grigorova S., Gjorgovska N., Kistanova E. (2019). Dose-dependent effect of *Tribulus Terrestris* dry extract on reproductive organs of growing male rabbits. *Macedonian Journal of Animal Science, 9* (1), 19-23. https://repository.ukim.mk/bitstream/20.500.12188/15585/1/MJAS%209-1%20Dessislava.pdf.

Abbas M. W., Hussain M., Akhtar S., Ismail T., Qamar M., Shafiq Z., Esatbeyoglu T. (2022). Bioactive Compounds, Antioxidant, Anti-Inflammatory, Anti-Cancer, and Toxicity 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 potential of *Tribulus terrestris in vitro*. *Pharmaceutical Biology, 53* (4), 469-476. https://doi.org/10.3109/13880209.2014.924019.

Agric., 76 (2), 270-276. https://doi.org/10.1002/(SICI)1097-0010(199802)76:2<270::AID-JSFA945>3.0.CO;2-9.

Aldaddou W. A., Aljohani A. S. M., Ahmed I. A., Al-Wabel N. A., El-Ashmawy I. M. (2022). 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-Bana M., Hussein J. (2018). Mitigation of Alpha-Cypermethrin-Induced Hepatotoxicity in Rats by *Tribulus terrestris* Rich in Antioxidant Compounds. *Jordan Journal of Biological Sciences, 11* (5), 517-525. https://jjbs.hu.edu.jo/files/vol11n5/Paper%20Number%207.pdf

Arafa M. H., Amin D. M., Samir 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 doi:10.1007/s11101-005-2833-x

Durgawale P., Datkhile K. (2017). Study of Polyphenol Content and Anti-Oxidative Potential of *Tribulus terrestris* Dry Fruit Extract. *International Journal of Pharmacognosy and Phytochemical Research, 9* (5), 716-721. https://doi.org/10.25258/phyto.v9i5.8154

Dutta S., Sengupta P., Slama P., Roychoudhury S. (2021). Oxidative Stress, Testicular Inflammatory Pathways, and Male Reproduction. *International Journal of Molecular Sciences, 22* (18), 10043. https://doi.org/10.3390/ijms221810043

Gorokhova E., Mattsson L., Sundström A. M. (2012). A comparison of TO-PRO-1 iodide and 5-CFDA-AM staining methods for assessing viability of planktonic algae with epifluorescence microscopy. *Journal of Microbiological Methods, 89*(3), 216-221. https://doi.org/10.1016/j.mimet.2012.03.005

Haghmorad D., Mahmoudi M. B., Haghighi P., Alidadiani P., Shahvazian E., Tavasolian P., Hosseini M., Mahmoudi M. (2019). Improvement of fertility parameters with *Tribulus Terrestris* and Anacyclus Pyrethrum treatment in male rats. *International Brazilian Journal of Urology, 45* (5), 1043-1054. https://doi.org/10.1590/s1677-5538.ibju.2018.0843

Ivanisova E., Menhartova K., Terentjeva M., Harangozo L., Kantor A., Kacaniova M. (2020). The evaluation of 844 chemical, antioxidant, antimicrobial and sensory properties of kombucha tea beevrage. *J Food Sci Technol, 57* (5), 1840-1846. https://doi.org/10.1007/s13197-019-04217-3

Ivanova L., Uhlig S. (2008). A bioassay for the simultaneous measurement of metabolic activity, membrane integrity, and lysosomal activity in cell cultures. *Analytical Biochemistry, 379*(1), 16-19. https://doi.org/10.1016/j.ab.2008.04.035.

Jambor T., Arvay J., Ivanisova E., Tvrda E., Kovacik A., Greifova H., Lukac N. (2020). 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. *Physiological research, 69* (4), 661-673. https://doi.org/10.33549/physiolres.934457

Jaradat N., Zaid A. N. (2019). Herbal remedies used for the treatment of infertility in males and females by traditional healers in the rural areas of the West Bank/Palestine. *BMC Complementary and Alternative Medicine, 19* (194), 1-2. https://bmccomplementmedtherapies.biomedcentral.com/articles/10.1186/s12906-019-2617-2.

Kamiloglu S., Sari G., Ozdal T., Capanoglu E. (2020). Guidelines for cell viability assays. *Food Frontiers, 1*(3), 332-349. https://doi.org/10.1002/fft2.44.

Karimi Jashni H., Malekzadeh Shiravani S., Hoshmand F. (2011). The effect of the Tribulus terrestris extract on spermatogenesis in the rat. *Journal of Jahrom University of Medical Sciences, 9* (4), 8-13. http://jmj.jums.ac.ir/article-1-64-en.html.

Khaleghi S., Bakhtiari M., Esmaeili F., Asadmobini A. (2017). *Tribulus terrestris* Extract Improves Human Sperm Parameters In Vitro. *Journal of Evidence-Based Integrative Medicine, 22* (3), 407-412. https://doi.org/10.1177/2156587216668110

Kostova I., Dinchev D. (2005). Saponins in *Tribulus terrestris* – chemistry and bioactivity. *Phytochemistry Reviews, 4* (2-3), 111-137.

Kowalczyk A.,Kupczyński R., Gałęska E., Araujo J. P., Czerniawska-Piątkowska E. (2022). Clinical Application of Bio extracts inn Supporting the Reproductive System of Animals and Humans: Potential and Limitations. *Evidence-Based Complementary and Alternative Medicine*. https://doi.org/10.1155/2022/4766409.

Luksic L., Arvay J., Vollmanova A., Toth T., Skrabanja v., Trcek 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.

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Neychev V. K., Nikolova E., Zhelev N., Mitev 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:10.3181/00379727-207-2320126.

Neychev V., Mitev 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., Izaguirry A. P., Soares M. B., Spiazzi C. Ch., Mendez A. S. L., Leivas F. G., dos Santos Brum D., Cibin F. W. S. (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.

Ramgir S. S., Renu K., Vellingiri B., George A., Tirupapuliyur D., Thiagarajan P., Gopalakrishnan 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.

Rates S. M. (2001). Plants as source of drugs. *Toxicon, 39* (5), 603-613. https://doi.org/10.1016/S0041-0101(00)00154-9.

Salahshoor M. R., Abdolmaleki A., Faramarzi A., Jalili C., Shiva R. (2020). Does *Tribulus terrestris* improve toxic effect of Malathion on male reproductive parameters*? Journal of pharmacy and bioallied sciences, 12* (2), 183-191. https://doi.org/10.4103/jpbs.jpbs_224_19

Sánchez-Moreno C., Larrauri J. A., Saura-Calixto F. (1998). A procedure to measure the antiradical efficiency of polyphenols. *J. Sci. Food*

Sandeep P. M., Bovee T. F. H., Sreejith K. (2015). Anti-Androgenic Activity of Nardostachys jatamansi DC and *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/B978-0-12-815565-3.00015-1.

Shymanska O., Vergun O., Rakhmetov D., Brindza J., 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.uniag.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?url=medicinal-plants-and-female-reproduction-disorders-due-to-oxidative-stress.

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., Harrath A. H., Aldahmash W., Grossman R., Sirotkin A. V. (2022). Potential protective effect of puncturevine (*Tribulus terrestris*, L.) against xylene toxicity on bovine ovarian cell functions. *Physiol Research, 71* (2), 249-258. https://doi.org/10.33549/physiolres.934871

Tian Ch., Chang y., Liu X., Zhang Z., Guo Y., Lan Z., Zhang P., Liu M. (2020). Anti-inflammatory activity in vitro, extractive process and HPLC-MS characterization of total saponins extract from *Tribulus terrestris* L. fruits. *Industrial Crops and Products*, 150, 1123-1143. https://doi.org/10.1016/j.indcrop.2020.112343

Tkachenko K., Frontasyeva M., Vasilev A., Avramov L., Shi L. (2020). Major and Trace Element Content of *Tribulus terrestris* L. Wildlife Plants. *Plants, 9* (12), 1764. https://doi.org/10.3390/plants9121764.

Tvrda E., Michalko J., Matusikova I., Lukac N. (2016). In vitro effects of the Chlamydomonas reinhardtii extract on bovine spermatozoa. *Journal of Microbiology, Biotechnology and Food Sciences, 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

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/s13065-017-0289-x