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# *Tribulus terrestris* fruit preparations impact HPLC chemical profiles and antioxidant, lipoxygenase and α-glucosidase inhibitory activities

**Preparações de frutas *Tribulus terrestris* afetam os perfis químicos de HPLC e as atividades antioxidantes, lipoxigenase e inibidoras de α-glucosidase**

**Las preparaciones de *Tribulus terrestris* afectan los perfiles químicos de HPLC y las actividades inhibidoras de antioxidantes, lipoxigenasa y α-glucosidasa**

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**Abstract**

*Tribulus terrestris* fruit has been used as a traditional and popular medicine for the prevention and treatment of several diseases, including sexual dysfunction, atherosclerosis and hypertension. The aim of this study was to evaluate the antioxidant, lipoxygenase and α-glucosidase inhibitory activities of an ethanol extract from Brazilian *T. terrestris* and its fractions sequentially partitioned into *n*-hexane, dichloromethane, ethyl acetate and *n*-butanol. The antioxidant capacities were determined by DPPH and ABTS scavenging free radicals, chelating metal ions, reducing power and total antioxidant activity by using phosphomolybdenum. High-performance liquid chromatography with diode array detection (HPLC-DAD) fingerprint analysis and quantitation of total phenolics were performed on the samples. The dichloromethane fraction showed the most complex HPLC-DAD chemical profile. The ethyl acetate and butanol fractions revealed the best phenolic compound and flavonoid recovery from *T. terrestris*. Concerning antioxidant activity, the ethyl acetate fraction presented better capacity for scavenging DPPH, ABTS and hydroxyl radicals, reductive power, total antioxidant capacity (TAC) and α-glucosidase inhibitory activity than the other fractions. These results correlated closely with the

levels of phenolic compounds and flavonoids. The hexane fraction showed the best metal chelating power and lipoxygenase inhibitory activity. The anti-diabetic and anti-inflammatory potential of Brazilian *Tribulus terrestris* depend on the method of preparation.
**Keywords:** Zygophyllaceae; Herbal extracts; Total phenolic contents; HPLC.

**Resumo**
O fruto do *Tribulus terrestris* tem sido utilizado como medicamento tradicional e popular para a prevenção e tratamento de diversas doenças, incluindo disfunção sexual, aterosclerose e hipertensão. O objetivo deste estudo foi avaliar as atividades antioxidante, inibitória da lipoxigenase e da α-glicosidase de um extrato etanólico de *T. terrestris* brasileiro e suas frações sequencialmente particionadas em *n*-hexano, diclorometano, acetato de etila e *n*-butanol. As capacidades antioxidantes foram determinadas por DPPH e ABTS sequestrando radicais livres, quelando íons metálicos, reduzindo o poder e a atividade antioxidante total usando fosfomolibdênio. Análise de impressão digital por Cromatografia Líquida de Alta Eficiência - Detector de arranjo de Diodos (CLAE-DAD) e quantificação de compostos fenólicos totais foram realizadas nas amostras. A fração diclorometano apresentou o perfil químico HPLC-DAD mais complexo. As frações acetato de etila e butanol revelaram a melhor recuperação de compostos fenólicos e flavonoides de *T. terrestris*. Com relação à atividade antioxidante, a fração acetato de etila apresentou melhor capacidade de sequestro de DPPH, ABTS e radicais hidroxila, poder redutor, capacidade antioxidante total (TAC) e atividade inibitória da α-glicosidase do que as outras frações. Esses resultados correlacionaram-se estreitamente com os níveis de compostos fenólicos e flavonóides. A fração hexânica apresentou o melhor poder quelante de metais e atividade inibitória da lipoxigenase. O potencial antidiabético e antiinflamatório do *T. terrestris* brasileiro depende do modo de preparo.
**Palavras-chave:** Zygophyllaceae; Extratos vegetais; Teores de fenólicos totais; CLAE.

**Resumen**
El fruto de *Tribulus terrestris* ha sido utilizado como medicina tradicional y popular para la prevención y tratamiento de diversas enfermedades, entre ellas la disfunción sexual, la aterosclerosis y la hipertensión. El objetivo de este estudio fue evaluar las actividades inhibidoras de antioxidantes, lipoxigenasas y α-glucosidasas de un extracto etanólico de *T. terrestris* brasileño y sus fracciones divididas secuencialmente en *n*-hexano, diclorometano, acetato de etilo y *n*-butanol. Las capacidades antioxidantes se determinaron mediante DPPH y ABTS eliminando radicales libres, quelando iones metálicos, reduciendo el poder y la actividad antioxidante total utilizando fosfomolibdeno. Se realizaron análisis de huellas dactilares por Cromatografía líquida de alta resolución - detector de matriz de diodos (HPLC-DAD) y cuantificación de compuestos fenólicos totales en las muestras. La fracción de diclorometano mostró el perfil químico HPLC-DAD más complejo. Las fracciones de acetato de etilo y butanol revelaron la mejor recuperación de compuestos fenólicos y flavonoides de *T. terrestris*. En cuanto a la actividad antioxidante, la fracción de acetato de etilo mostró mejor capacidad de captación de radicales DPPH, ABTS e hidroxilo, poder reductor, capacidad antioxidante total (TAC) y actividad inhibitoria de la α-glucosidasa que las demás fracciones. Estos resultados se correlacionaron estrechamente con los niveles de compuestos fenólicos y flavonoides. La fracción de hexano mostró el mejor poder quelante de metales y actividad inhibidora de la lipoxigenasa. El potencial antidiabético y antiinflamatorio de la *T. terrestris* brasileña depende de cómo se prepare.
**Palabras clave:** Zygophyllaceae; Extractos de plantas; Contenido fenólico total; HPLC.

## 1. Introduction

*Tribulus terrestris* L. (Zygophyllaceae) is native to India and is already found in various regions around the world because it is suited to warm regions (Ghanbari *et al.*, 2016; Khairwal & Kumar, 2013). Its fruit has been used in traditional medicine in China and India (Ayurveda) for hundreds of years in the treatment of infertility, impotence, erectile dysfunction, and low libido. In Western countries such as Brazil, the use of this species began in the 1970s. However, in recent years, *T. terrestris* has aroused even greater interest among athletes and physical exercise practitioners due to its invigorating and aphrodisiac characteristics and its possible role in increase the levels of male sex hormones such as testosterone (Dakshayini & Mahaboob Basha, 2018).

*T. terrestris* has also been reported as an enzyme inhibitor and antioxidant agent (Ercan & El, 2016; Lokhande *et al.*, 2014; Song *et al.*, 2016; Zheleva-Dimitrova *et al.*, 2012). However, the biological activity of an herbal preparation depends on the content and qualitative chemical profile, which can change according to the origin of the plant material, plant organ, and extractive methods, among other parameters. Several studies have shown the impact on the biological functions of *T. terrestris* as a result of chemical profile variations in samples from different geographic regions (Abdali-Mashhadi *et al.*, 2016; Dinchev *et al.*, 2008; Ivanova *et al.*, 2010; Zheng *et al.*, 2017).

The effects of *T. terrestris* on health are associated with the presence of diverse biologically active compounds, such as phenolic compounds (Hong *et al.*, 2013; Kim *et al.*, 2018; Lee *et al.*, 2017; Song *et al.*, 2016), flavonoids (Semerdjieva & Zheljazkov, 2019), saponins

(Kostova *et al.*, 2002; Zhu *et al.*, 2017), and alkaloids (Basaiyye *et al.*, 2018; Wu *et al.*, 1999). However, steroidal saponins and phenolic compounds are considered to be the most important bioactive metabolites (Ernawati *et al.*, 2017; Kim *et al.*, 2018; Lee *et al.*, 2017; Zhu *et al.*, 2017). Protodioscin is the most abundant saponin found in the aerial parts and fruit of *T. terrestris* (Dinchev *et al.*, 2008).

Oxidative damage in pancreatic islet tissues causes diabetes. Consumption of antioxidants through diet and supplements is believed to remove free radicals from living systems and provide health benefits (Altemimi *et al.*, 2017; Egnell *et al.*, 2017; Sarma *et al.*, 2010). The use of α-glucosidase inhibitors has been one of the treatments for the control of glycaemic levels since this enzyme, located in enterocyte villi, plays an important role in the final stage of carbohydrate digestion (Pappachan *et al.*, 2019). Another important enzyme involved in diabetic inflammation is lipoxidase. It is involved in diabetes complications, such as vein inflammation, retinopathy and nephropathy (Dobrian *et al.*, 2019; Domingueti *et al.*, 2016). There is some evidence suggesting the antioxidant (Dakshayini & Mahaboob Basha, 2018; Hammoda *et al.*, 2013; Khatri & Chhillar, 2015), antidiabetic (El-Shaibany *et al.*, 2015; Samani *et al.*, 2016) and anti-inflammatory (Kang *et al.*, 2017; Lee *et al.*, 2017) activities of *T. terrestris* in the literature.

Therefore, to further explore the potential of this plant, the aim of this study was to compare different methods of preparing *T. terrestris* fruit and their total contents of phenolic compounds, HPLC profiles and *in vitro* antioxidants, and lipoxygenase and α-glucosidase inhibitory activities.

## 2. Methodology

### 2.1 Chemicals and General Procedures

The chemicals used in this study were ethanol 92%; hexane; dichloromethane; ethyl acetate; *n*-butanol; methanol; Folin-Ciocalteu's solution; sodium carbonate ($Na_2CO_3$); gallic acid ($C_7H_6O_5$); aluminium chloride ($AlCl_3$); quercetin; 2,2-diphenyl-1-picrylhydrazyl (DPPH); 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid (ABTS); potassium persulfate ($K_2S_2O_8$); ascorbic acid ($C_6H_8O_6$); sulfuric acid ($H_2SO_4$); monosodium phosphate ($NaH_2PO_4$); disodium phosphate ($Na_2HPO_4$); ammonium molybdate ($(NH_4)_6Mo_7O_{24}$); butylated hydroxytoluene (BHT); ferrous sulfate ($FeSO_4$); ferrozine ($C_{20}H_{13}N_4NaO_6S_2$); ethylene diamine tetraacetic acid (EDTA); potassium ferricyanide ($K_3Fe(CN)_6$); trichloroacetic acid (TCA); ferric chloride ($FeCl_3$); 2-deoxyribose; thiobarbituric acid (TBA); hydrogen peroxide ($H_2O_2$), salicylic acid ($C_7H_6O_3$), α-glucosidase of *Bacillus stearothermophilus* EC 232-604-7, *p*-nitrophenyl-β-D-glucopyranoside (PNPG), potassium phosphate monobasic ($KH_2PO_4$), potassium phosphate dibasic ($K_2HPO_4$), lipoxygenase from *Glycine max* (soybean) EC 232-853-1, linoleic acid, boric acid, sodium hydroxide (NaOH), Tween 80, eugenol, acarbose, and protodioscin. They were purchased from Sigma–Aldrich®. All reagents were of analytical grade.

Except for those of the lipoxygenase inhibition assay, the spectrophotometric readings were recorded in a TECAN Infinity M200 PRO microplate reader operated by I-control software version 3.37. The analyses were performed in triplicate.

### 2.2 Sample Preparations

Dried fruits of *T. terrestris* were purchased from the Pharmaceutical Industry Catedral, Vespasiano, Minas Gerais, Brazil. The extracts and fractions were prepared according to the procedure by Oliveira *et al.* (2015). In this work, dried crude ethanolic extract (CE) and hexane (HEX), dichloromethane (DCM), ethyl acetate (EtOAc), *n*-butanol (BuOH) and methanol/water soluble (MWS) fractions were used.

### 2.3 HPLC Profiles

High-performance liquid chromatography with diode array detection (HPLC-DAD) profiles were performed on an Agilent 1200 series HPLC-DAD spectrometer (Agilent Technologies, Waldbronn, Germany) equipped with a quaternary pump (G1311A) with a degasser system (G13222A), ALS autosampler (G1322A) and TCC heater assembly (G1316A). A variable-wavelength ultraviolet detector (G1315D) was used to obtain chromatograms at 210 nm. The equipment was controlled by OpenLAB software version A07.04, build 04.07.28. For sample preparation, an aliquot of 10 mg of CE, 5 mg of the HEX, DCM, EtOAc, BuOH, AQSol fractions, and 1 mg of protodioscin (Sigma–

Aldrich®, Brazil) were dissolved in 1.0 mL MeOH at room temperature in an ultrasonication bath for 20 min. After centrifugation at 8400 g for 10 min, the supernatant (10 µL) was automatically injected onto the HPLC system. The corresponding protodioscin peak was characterized by retention time, UV spectroscopy and co-injection of the reference compound. Separations were performed on a C18 reversed-phase analytical column (Eclipse XDB-C18, Agilent Technologies, USA) with silica-based packing (5 µm × 150 mm × 4.6 mm I.D.). A security guard cartridge (Zorbax ODS 4-Pack, Agilent Technologies, USA) was used as a precolumn (5 µm × 12.5 mm × 4.6 mm I.D.) at a temperature of 40 °C. Water (A) and acetonitrile (B) were used as eluents, both containing 0.01% (v/v) phosphoric acid, with a linear gradient at a flow rate of 1.0 mL/min as follows: 0 min 95% A, 5% B; 60 min 5% A, 95% B, followed by 5 min of isocratic elution and then a return to initial conditions.

### 2.4 Spectrophotometric Quantification of Phenolic Compounds

The total phenolic contents of the *T. terrestris* extract and fractions were determined by applying Folin-Ciocalteu's colorimetric assay as described by Singleton and Rossi (1965) with modifications. Twenty microlitres of sample was added to 100 µL of Folin-Ciocalteu solution (10%) and 125 µL of $Na_2CO_3$ solution (7%). The absorbance was measured at 760 nm after 120 min of incubation at room temperature (24 ± 2 °C). The results were expressed as milligrams of gallic acid equivalents (GAE) per gram of dry extract (mg GAE/g).

The quantification of flavonoids (flavones/flavonols) was determined according to Chang *et al.* (2002) with minor modifications. Briefly, 100 µL of *T. terrestris* preparation was added to 100 µL of 10% $AlCl_3$-ethanol solution. After 40 min at room temperature (24 ± 2 °C), the absorbance was measured at 420 nm on a microplate reader. The results were expressed as milligrams of quercetin equivalents (QE) per gram of dry extract (mg QE/g).

### 2.5 Antioxidant Activities

#### 2.5.1 DPPH and ABTS Free Radical Scavenging Assays

The scavenging activity of DPPH (2,2-diphenyl-2-picrylhydrazyl) was determined by the method proposed by Brand-Williams *et al.* (1995) with modifications. Fifty microlitres of different concentrations of *T. terrestris* extract and fractions (0.625-20 mg/mL) were added to a solution of DPPH (250 µL). The absorbance measurements were performed at 517 nm after 60 min of incubation at room temperature (24 ± 2 °C). Butylated hydroxytoluene (BHT) was used as a positive control. The DPPH percentage inhibition was determined using the formula: % = $[(A_0 - A_1/A_0) \times 100]$, where A0 is the absorbance of the negative control and A1 is the absorbance of the samples. A solution containing the same amount of ethanol and DPPH solution comprised the negative control ($A_0$). The DPPH percentage inhibition was plotted against sample or standard content, and the $IC_{50}$ was determined (concentration of dried extract and fractions able to scavenge 50% of DPPH free radicals). The analyses were performed in triplicate.

The ABTS [2,2-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)] radical cation decolorization assay was carried out using the method described by Re *et al.* (1999) with modifications. The ABTS radical was prepared by reaction of the ABTS aqueous solution (7 mM) with $K_2S_2O_8$ (2.45 mM). This solution was stored in the dark for 16 h at room temperature (24 ± 2 °C). *T. terrestris* extract and fractions (30 µL) were added to 270 µL of ABTS solution, and the absorbance at 734 nm was read after 6 min. BHT was used as a positive control, and the ABTS percentage was calculated and expressed in the same way as the DPPH radical scavenging activity.

#### 2.5.2 Hydroxyl Radical Scavenging Assay

The effect of *T. terrestris* preparations on hydroxyl radicals was determined by using the deoxyribose method as Kunchandy and Rao (1990) with modifications. The reaction mixture contained 1375 µL of sodium phosphate buffer (0.1 M; pH 7.4), 200 µL of 2-deoxyribose (10 mM), 200 µL of $FeSO_4$ + EDTA (10 mM), 200 µL of $H_2O_2$ (10 mM), and 25 µL of different concentrations of *T. terrestris* extract or fractions (0.625-20 mg/mL). The reaction was initiated by the addition of $H_2O_2$ and incubated at 37 °C for 4 h followed by the addition of 1 mL of TCA

(2.8%) and 1 mL of TBA (1%). The resulting solution was boiled in a water bath for 10 minutes and then cooled to room temperature (24 ± 2 °C). The absorbance was measured at 532 nm. The results were expressed in $IC_{50}$ (mg/mL).

**2.5.3 Total Antioxidant Capacity (TAC)**

The total antioxidant capacity (TAC) was determined as described by Prieto *et al*. (1999) with modifications. The method of ammonium molybdate reduction was measured with 20 µL of *T. terrestris* extract and fractions, and they were mixed with 1500 µL of the reagent solution (0.6 M sulfuric acid, 28 mM monosodium phosphate, and 4 mM ammonium molybdate) for 90 minutes at 95 °C. The absorbance was measured at 695 nm. The results were expressed in milligrams of ascorbic acid equivalent per gram of dry preparation (AAE mg/g).

**2.5.4 Chelating Metal Ions**

The capacity of chelating metal ions by *T. terrestris* extract and fractions was evaluated by the method described by Wang *et al*. (2004) with modifications. An aliquot of 50 µL of sample was incubated with 40 µL of $FeSO_4$ (2 mM) and 80 µL of ferrozine (5 mM) for 10 minutes. The absorbances were measured at 572 nm. EDTA was used as positive control. The percentage of chelating ability was determined according to the following formula: $(A_0-A_1)/A_0*100$, where $A_0$ is the absorbance of the control and $A_1$ is the absorbance of *T. terrestris* preparations.

**2.5.5 Reductive Potential**

The reductive potential of the samples was determined according to the method reported by Oyaizu (1986) with minor modifications and described by Aazza *et al*. (2011). Fifty microlitres of different concentrations of *T. terrestris* extract or fraction was mixed with 500 µL of phosphate buffer (200 mM, pH 6.0) and 500 µL of potassium ferricyanide $[K_3Fe(CN)_6]$ (1%). The mixture was incubated at 50 °C for 20 minutes. An aliquot of 500 µL of trichloroacetic acid (10%) was added to the mixture, which was then centrifuged for 10 minutes at 3,000 rpm. The upper layer of solution (500 µL) was mixed with 500 µL of distilled water and 100 µL of $FeCl_3$ (0.1%). The absorbance was measured at 700 nm.

**2.6 Enzyme Inhibition Assays**

**2.6.1 Lipoxygenase Inhibition Activity**

The capacity of *T. terrestris* to inhibit lipoxygenase was determined according to the method of Frum and Viljoen (2006) with small modifications. It was evaluated by reaction of 10 µL of lipoxygenase solution (0.054 g in 1 mL of 0.005% borate buffer, Tween 80 0.1 M, pH 9) to 450 µL of borate buffer, 10 µL of samples, and 25 µL of linoleic acid (0.001 M). Eugenol was used as standard. The absorbance was measured at 234 nm every 20 s for 5 min on a spectrophotometer (U-5100 HITACHI). The percentage inhibition of the enzyme was calculated, and the $IC_{50}$ values were compared.

**2.6.2 α-Glucosidase Inhibition Activity**

α-Glucosidase inhibition was based on El-Guendouz *et al*. (2016), with some modifications. Initially, a mixture of 25 µL of *T. terrestris* extract or fractions, 50 µL of α-glucosidase, and 200 µL of phosphate buffer (100 mM, pH 6.8) was incubated at room temperature for 10 min. After preincubation, 150 µL of p-nitrophenyl-β-D-glucopyranoside (PNPG) (0.5 mM) solution in the phosphate buffer was added to each well. The reaction mixtures were incubated at 37 °C for 20 minutes, and then sodium carbonate solution (0.4 mM) was added to stop the reaction. The control had 25 µL of 92% ethanol or methanol instead of test samples. Acarbose was used as a standard. The absorbance was measured at 405 nm on a microplate reader. The inhibition percentage of the enzyme was calculated, and the $IC_{50}$ values were compared.

### 2.7 Statistical Analysis

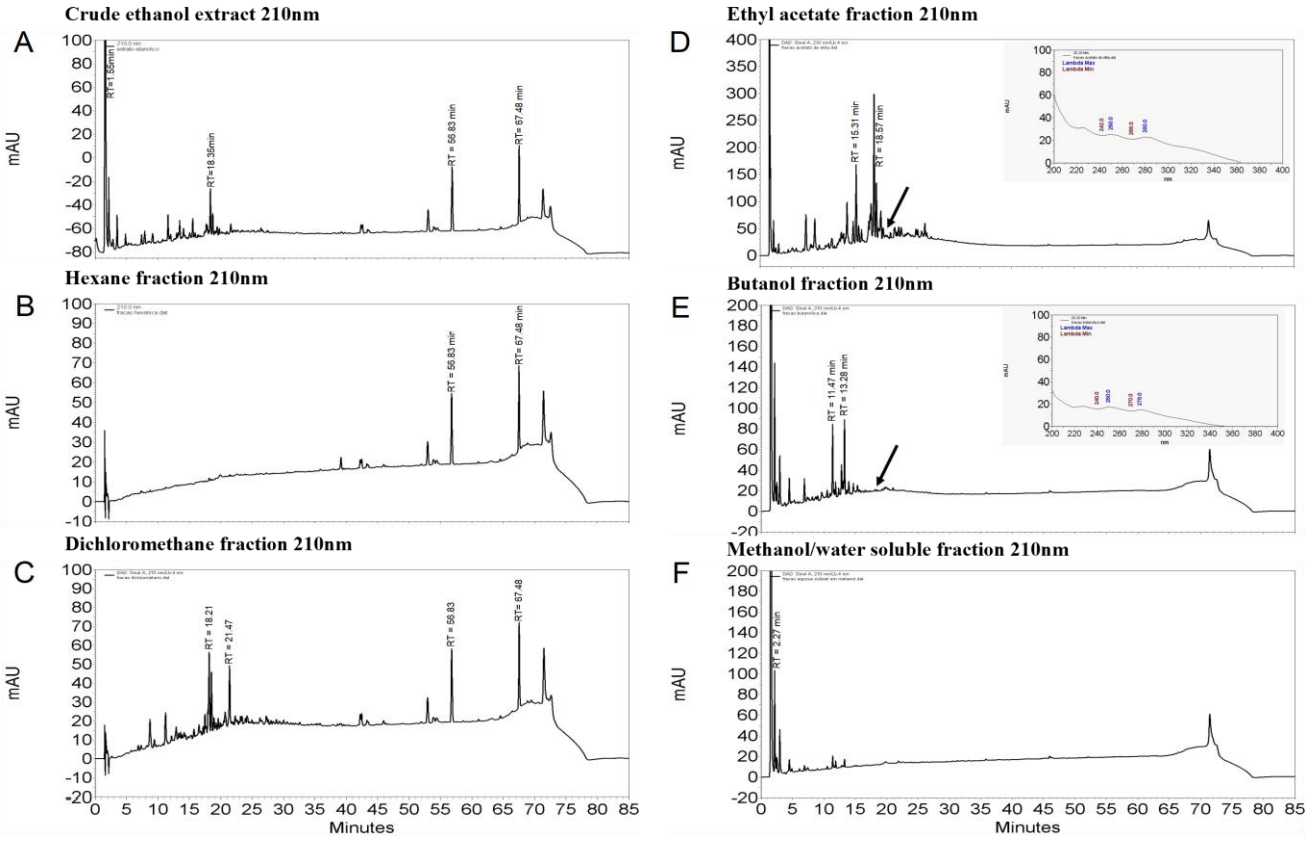
All analyses were performed in triplicate. Five values for each sample were averaged (n = 5). The results were expressed as the means ± SD. The significant difference for more than two samples was determined by using one-way ANOVA and the Tukey comparison test. The value of $p<0.05$ was considered to be significant. Principal component analysis (PCA) was used to identify the relationships between the extract and fractions of *T. terrestris* and the phenol contents and antioxidant and enzyme inhibitory activities. All experiments were performed in triplicate using R software 3.5.2 (Vienna, Austria).

## 3. Results and Discussion

### 3.1 HPLC Profiles

HPLC fingerprints allowed us to distinguish between the chemical profiles of the *T. terrestris* preparations (Figure 1). The most complex chemical profiles were seen in the CE extract and DCM fraction. These chromatograms showed great similarity in the chemical profile of the preparations, with the predominance of peaks in the retention time (RT) range of 0 to 30 min corresponding to substances of high and intermediate polarities.

**Figure 1 -** Chromatograms of *T. terrestris* fruit preparations at 210 nm. Legend: A = Crude ethanol extract, B = hexane fraction, C = dichloromethane fraction, D = ethyl acetate fraction, E = butanol fraction, F= methanol/water soluble fraction.




Source: Authors.

Compounds with low polarity, corresponding to the peaks with retention 50 min, were also detected in these preparations. The two low polarity peaks with the highest intensities (56.83 and 67.48 min) were also observed in the HEX fraction. However, the intensities of both peaks were different, indicating quantitative differences. The EtOAc and BuOH fractions presented a complex profile in the region of high polarity compounds (0-20 min), and the MWS fraction evidenced the presence of two intense peaks with RTs below 2,0 min. In general, diverse chemical profiles of the *T. terrestris* preparations were observed. The chemical composition of *T. terrestris* fruit is well established, and a wide

range of compounds have been reported to occur in this plant, including flavonoids, alkaloids, lignan amides, cinnamic acid amides, steroidal and triterpene saponins (Nebieridze *et al*., 2018; Semerdjieva & Zheljazkov, 2019; Song *et al*., 2016). Some studies have shown that steroidal saponin and cinnamic acid amide compounds are responsible for the antidiabetic, anti-inflammatory and antioxidant activities of *T. terrestris* extracts (Ernawati *et al*., 2017; Kim *et al*., 2018; Lee *et al*., 2017; Zhu *et al*., 2017). According to several studies, protodioscins are the main components of the aerial parts of *T. terrestris* (De Combarieu *et al*., 2003; Kostova *et al*., 2002; Shishovska *et al*., 2015). Although saponins show weak UV absorption, even in the short wavelength range, Shishovska *et al*. (2015) reported that the HPLC-DAD method is suitable for routine control of raw plant material and pharmaceuticals based on *T. terrestris*. To investigate the presence of protodioscin in the samples, the protodioscin peak from the sample chromatograms was identified by the retention time by comparison with the protodioscin standard chromatogram, co-injection with a reference compound and $UV_{210}$ spectrum matching. Protodioscin was detected in the EtOAc and BuOH fractions characterized as low-intensity peaks at 20.25 min.

### 3.2 Phenolics Spectrophotometric Quantification

The total phenolic content of the extract and fractions measured by the Folin-Ciocalteu method is shown in Table 1. There were significant differences ($p < 0.05$) between the total content of phenolic compounds in the *T. terrestris* preparations. As shown in Table 1, the EtOAc fraction (45.44 ± 0.00 mg GAE/g of dry extract) and BuOH fraction (31.88 ± 0.15 mg GAE/g of dry extract) were the highest. The HEX (8.01 ± 0.43 mg GAE/g of dry extract) and MWS fractions (9.82 ± 0.15 mg GAE/g of dry extract) had the lowest total phenolic contents.

**Table 1** - Phenol contents and antioxidant and enzyme inhibitory activities of *T. terrestris* fruit.

| Preparation | Total phenols content (mg GAE/g)[1] | Total flavonoids content (mg QE/g)[2] | DPPH[3] | ABTS[3] | Hydroxyl radical scavenging[3] | TAC (mg AAE/g)[4] | Chelating metal ions[3] | Lipoxygenase inhibition[3] | α-Glucosidase inhibition[3] |
|---|---|---|---|---|---|---|---|---|---|
| **EtOH** | 17.04±0.14$^{c}$ | 16.09±0.47$^{c}$ | 0.57±0.03$^{c}$ | 0.11±0.02$^{c}$ | 0.25±0.13$^{a}$ | 61.46 ±1.06$^{bc}$ | - | 0.119 ± 0.001$^{b}$ | - |
| **Hex** | 8.01±0.43$^{e}$ | 6.70±0.81$^{d}$ | 10.73±0.18$^{d}$ | 1.02±0.003$^{d}$ | - | 44.10±2.62$^{c}$ | 1.81±1.02$^{b}$ | 0.083 ± 0.013$^{c}$ | - |
| **DCM** | 16.28±0.40$^{c}$ | 15.01±0.68$^{c}$ | 0.59±0.01$^{a}$ | 0.15±0.02$^{c}$ | - | 77.78±5.72$^{bc}$ | - | 0.106 ± 0.013$^{bc}$ | - |
| **EtOAc** | 45.44±0.00$^{a}$ | 25.82±0.69$^{b}$ | 0.08±0.00$^{b}$ | 0.04±0.01$^{b}$ | 0.017±0.003$^{b}$ | 120.78±13.53$^{a}$ | - | 0.130 ± 0.011$^{b}$ | 0.044±0.0012$^{a}$ |
| **BuOH** | 31.88±0.15$^{b}$ | 29.18±0.00$^{a}$ | 0.48±0.03$^{a}$ | 0.12±0.01$^{c}$ | - | 80.16±2.71$^{ab}$ | - | 0.199 ± 0.012$^{a}$ | 0.966±0.25$^{b}$ |
| **MWS** | 9.82±0.15$^{d}$ | 7.75±0.35$^{d}$ | - | 0.72±0.01$^{a}$ | - | 72.70±0.87$^{bc}$ | - | - | - |
| **EDTA** | NA | NA | NA | NA | NA | NA | 0.033±0.001$^{a}$ | NA | NA |

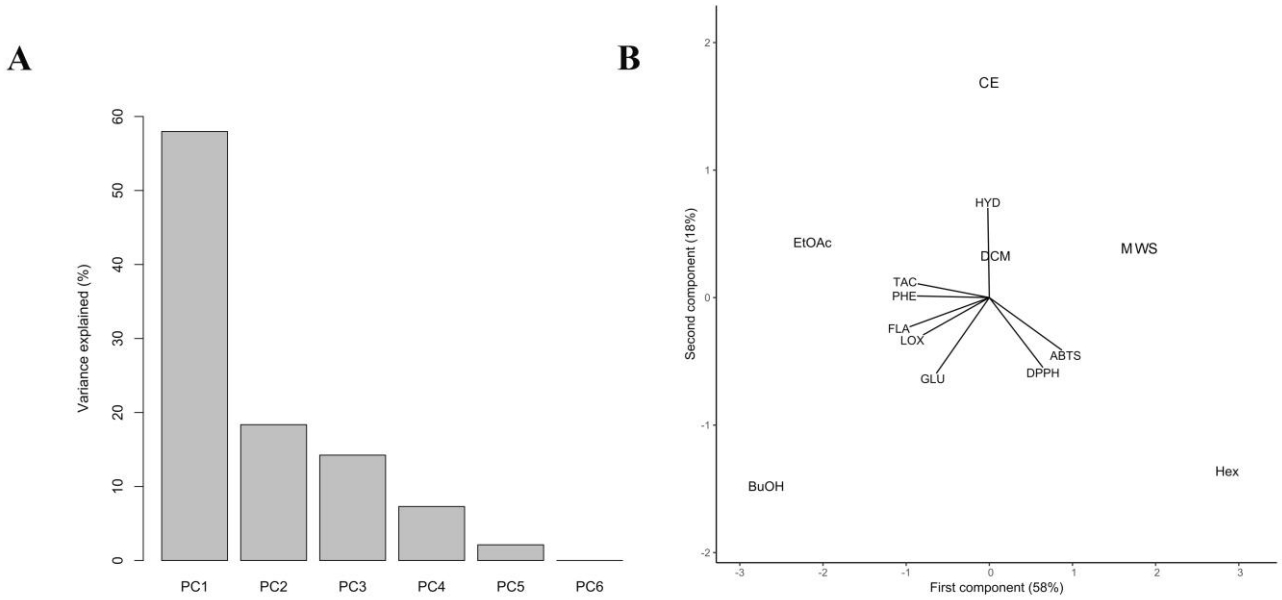
[1]**GAE**: gallic acid equivalent; [2]**QE**: quercetin equivalent; [3]The values are presented as $IC_{50}$=mg/mL; [4]**TAC**: Total antioxidant capacity expressed as mg of ascorbic acid equivalents/g of dry extract (mg AA/g); -: without activity or very poor activity; NA = not available; EtOH: ethanol extract; HEX: hexane fraction; DCM: dichloromethane fraction; EtOAc: ethyl acetate fraction; BuOH: butanol fraction; MWS: methanol/water soluble fraction. Samples with the same letter are not significantly different ($p < 0.05$) by Tukey's test. Source: Authors.

Similar results were obtained by Borran *et al.* (2017), who studied the CE extract of *T. terrestris* fruit from Iran. The dried powder was extracted with an ethanol:water mixture (70:30) using the maceration method, and a quantity of 52.9 ± 2.3 mg GAE/g of dry extract was identified for the total phenolic contents. In addition to flavonoids, lignanamides and cinnamic acid amides have also been reported as bioactive phenolic compounds in *T. terrestris* (Hong *et al.*, 2013; Kim *et al.*, 2018; Song *et al.*, 2016).

The total flavonoid content (TFC) (Table 1) ranged from 6.70 ± 0.81 mg QE/g of dry extract of the HEX fraction to 29.18 ± 0.00 mg QE/g of dry extract of the BuOH fraction. As shown in Figure 2A, it is possible to observe that PCA efficiently reduced the dimensionality of the original data to a smaller number of variables. More than 75% of the original variation in the first two components was retained. Therefore, by an initial set of 8 variables, most of the variance could be explained with only two principal components. As shown in Figure 2B, the total content of phenolic compounds and flavonoids in the EtOAc fraction was positively correlated.

The values of phenols and flavonoids in the CE extract and fractions were within the range found for samples of *T. terrestris* from Bulgaria (Zheleva-Dimitrova *et al.*, 2012) and India (Dwivedi & Sengar, 2018; Rajendrabhai, 2017). The flavonoids of *T. terrestris* are mainly derivatives of heterosides of quercetin, kaempferol, and isorhamnetin (Zhu *et al.*, 2017).

**Figure 2 -** Principal component analysis based on data of response of extract and fractions of *T. terrestris* and phenol contents, antioxidant and enzyme inhibitory activities. Legend: A: Scree plot of explained variance of each principal component. The x-axis represents each of the principal components (PC), and the y-axis represents the percentage of variance explained by each of these components. B: Scatterplot of the extract and fractions of *T. terrestris* and measured parameters on the plane defined by the first two components of principal component analysis. EtOH = crude ethanol extract; Hex = hexane fraction; DCM = dichloromethane fraction; EtOAc = ethyl acetate fraction; BuOH = butanol fraction; MWS = methanol/water soluble fraction; PHE = phenol content; FLA = flavonoid content; DPPH = DPPH free radicals; ABTS = ABTS radical scavenging; HYD = hydroxyl radical scavenging activity; TAC = total antioxidant capacity; LOX = lipoxygenase inhibitory activity; GLU = α-glucosidase inhibitory activity.




Source: Authors.

### 3.3 Antioxidant Activity

Several *in vitro* assays have been carried out to estimate the antioxidant capacities of the *T. terrestris* fruit preparations, since several authors have stated that antioxidant activity should not be concluded based on a single method (Alam *et al.*, 2013; Dudonné *et al.*, 2009).

#### 3.3.1 DPPH and ABTS Free Radical Scavenging Assays

The DPPH assay is one of the most well-established methods for determining the antioxidant activity of natural products. Antioxidants that interact with DPPH either transfer electrons or hydrogen atoms to DPPH and neutralize its free radical character (Choi *et al.*, 2016). In the ABTS free radical method, antioxidants reduce $ABTS^{\cdot+}$ to ABTS and decolorize it (Alam *et al.*, 2013). The ABTS assay is particularly interesting for plant extracts because the absorption at 734 nm eliminates colour interference (Dudonné *et al.*, 2009).

The capacity for scavenging DPPH and ABTS free radicals is shown in Table 1. The best DPPH and ABTS antioxidant activities were obtained in the EtOAc fraction ($IC_{50}$=0.08 ± 0.00 mg/mL and $IC_{50}$ = 0.04 ± 0.01 mg/mL, respectively). The results of the PCA analyses were in accordance with the results obtained by analysis of variance (Figure 2B and Table 1). Since the results were expressed in $IC_{50}$, the lowest values represent the best results. In these cases, parameters that showed the lowest values had inverse factor coordinates and contributed to the highest correlation. The EtOAc fraction, probably due to its higher total concentration of phenols, was plotted on the opposite side of the ABTS and DPPH antioxidant activities, indicating highly correlated contributions.

The capacity for scavenging DPPH radicals appears to be better in fruits than in fresh leaves from India, as reported by Khatri and Chhillar (2015). The capacity of fruit from China to scavenge ABTS free radicals was higher than that of samples from Bulgaria (Zheleva-Dimitrova *et al.*, 2012).

**3.3.2 Hydroxyl Radical Scavenging Assay**

Hydroxyl radicals are potent reactive oxygen species in biological systems that react with polyunsaturated fatty acid moieties of cell membrane phospholipids and cause many degenerative diseases, cancer, and ageing (Alam *et al.*, 2013; Asikin *et al.*, 2016). In the hydroxyl radical scavenging assay, hydroxyl radicals are generated by the Fenton reaction, and the inhibition of deoxyribose degradation could be attributed to the inhibition of radicals (Sannigrahi *et al.*, 2010).

The results for the ethanolic extract and ethyl acetate fraction were $IC_{50}$=0.25±0.13 mg/mL and $IC_{50}$=0.017±0.003 mg/mL, respectively. The hydroxyl radical scavenging assay of *T. terrestris* can be attributed to its flavonoid content. Flavonoids act as scavengers of various oxidizing species, superoxide anions ($O_2$-•), hydroxyl radicals and peroxy radicals, and they also act as quenchers of singlet oxygen (Keshtmand *et al.*, 2015).

Therefore, the results presented in this study are in agreement with the fact that the total phenolic and flavonoid contents are major contributors to the antioxidant activity of *T. terrestris*, which is in accordance with the literature (Lokhande *et al.*, 2014; Zheleva-Dimitrova *et al.*, 2012).

**3.3.3 Total Antioxidant Capacity**

Total antioxidant activity (TAC) is a quantitative antioxidant method expressed in terms of the number of equivalents of ascorbic acid per gram of dry extract. The TAC of the extract and fractions of *T. terrestris* is shown in Table 1. There were significant differences ($p < 0.05$) between the TAC of the CE extract and fractions. EtOAc (120.78 ± 13.53 mg AAE/g of dry extract) and BuOH (80.16 ± 2.71 mg AAE/g of dry extract) fractions showed the highest TAC, while the HEX (44.10 ± 2.62 mg AAE/g of dry extract) fraction had the lowest TAC. The EtOAc and BuOH fractions of *T. terrestris* fruits are rich sources of polyphenols (Table 1). As expected, a positive correlation between the total phenolic compounds, flavonoids, and TAC was confirmed by PCA (Figure 2B). The TAC assay is based on the reduction of molybdenum (VI) to molybdenum(V) in the presence of a reducing agent (antioxidant), which formed a green phosphomolybdate(V) complex that can be evaluated spectrophotometrically at 765 nm. Many natural products, including phenols and flavonoids, can cause this reduction (Ahmed *et al.*, 2015). A wide range of phenolic phytochemicals have been associated with biologically active compounds in *T. terrestris* fruit (Hong *et al.*, 2013; Kim *et al.*, 2018; Kumari *et al.*, 2015; Semerdjieva & Zheljazkov, 2019; Song *et al.*, 2016). For example, Kumari *et al.* (2015) demonstrated that *T. terrestris* fruit extract restored spermatogenesis, and the authors attributed this effect to the presence of antioxidant flavonoids rather than to steroidal saponins. Asadmobini *et al.* (2017) also reported a close relationship between antioxidant activities and sperm mobility, one of the main uses of *T. terrestris*.

**3.3.4 Chelating Metal Ions**

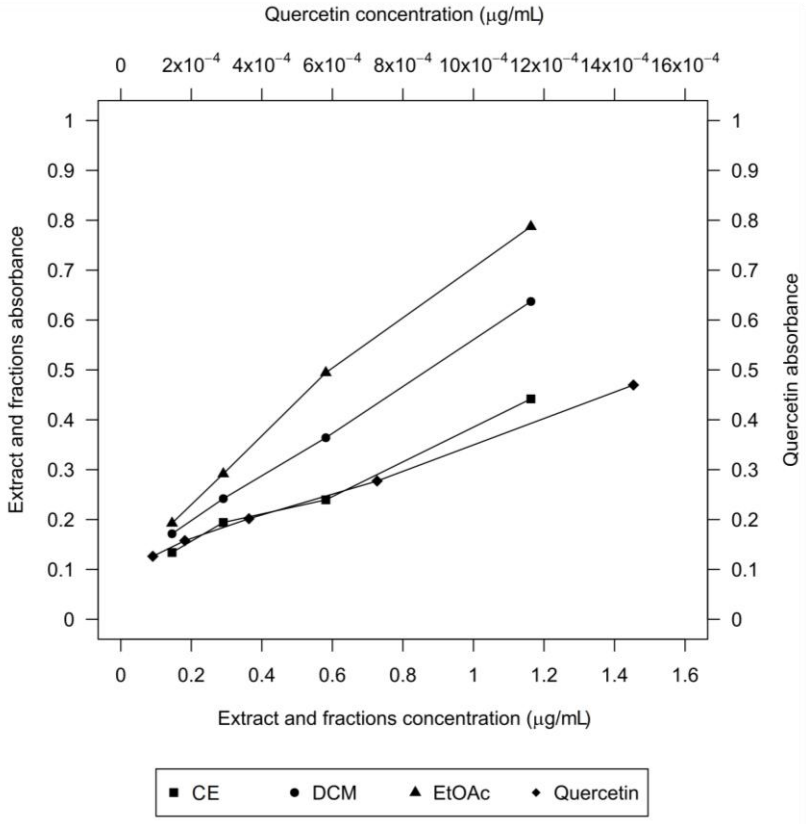
A previous study showed that the ethanolic extract of *T. terrestris* exhibited a remarkable protective effect against cadmium-induced testicular damage. The observed effect comprised significant recovery from testicular histological alterations, tissue peroxidation, and Cd accumulation. One of the explanations for the protective effect was attributed to inhibition of testicular tissue peroxidation by antioxidant and metal chelating (Rajendar *et al.*, 2011).

For this reason, we evaluated the *T. terrestris* preparation by using ferrous metal ion chelating capacity. This method is usually used because metals can catalyse free radical reactions and stimulate lipid peroxidation. In this study, even though the HEX fraction was a significantly less potent metal chelating agent than EDTA, this was the only prepared sample that demonstrated this activity (Table 1). This result indicates that not only phenolic compounds but also low polarity compounds are involved in *T. terrestris* fruit antioxidant activities.

### 3.3.5 Reductive Potential

The reducing power of the extract and fractions was also analysed. The results are shown in Figure 3. In this study, only the ethanolic extract and ethyl acetate and dichloromethane fractions presented the capacity for reducing power. The ethyl acetate fraction of *T. terrestris* presented high absorbance and concentration. This result may be related to the total content of phenolics.

**Figure 3 -** Reductive potential of the extract and fractions of *T. terrestris* fruit at 700 nm. Legend: EtOH: crude ethanol extract; DCM: dichloromethane fraction; EtOAc: ethyl acetate fraction; standard quercetin.




Source: Authors.

### 3.4 Enzyme Inhibition Assays

#### 3.4.1 Lipoxygenase Inhibition Activity

The species *T. terrestris* showed the ability to inhibit lipoxygenase. The best anti-inflammatory activity corresponding to the lowest $IC_{50}$ value was obtained for the hexane fraction ($IC_{50}$= 0.083 ± 0.013 mg/mL), and the worst anti-inflammatory activity was found in the butanol fraction ($IC_{50}$= 0.199 ± 0.012 mg/mL) (Table 1). The methanol/water soluble fraction did not inhibit the enzyme. Thus, the inhibition of lipoxygenase increased with decreasing polarity of the prepared samples.

In this study, lipoxygenase from *Glycine max* (soybean) was used. Soy lipoxygenase exhibits structural homology to human lipoxygenase. However, in plants, lipoxygenase metabolizes linoleic and linolenic acid, and in animals, lipoxygenase metabolizes arachidonic acid (Gardner, 1995). All lipoxygenases contain a single nonheme iron per molecule of enzyme. Iron is intimately involved in the dioxygenation reaction of polyunsaturated fatty acids, which yields hydroperoxides (Nelson & Seitz, 1994).

The hexane fraction also presented the best capacity for chelating metal ions. This result may be correlate with the ability of this fraction to inhibit lipoxygenase due to the presence of iron in the lipoxygenase structure. This plant showed *in vitro* lipoxygenase inhibition and can be suggested as a potential anti-inflammatory agent. Other authors also reported the anti-inflammatory activity of *T. terrestris* (Kang *et al.*, 2017; Lee *et al.*, 2017).

### 3.4.2 α-Glucosidase Inhibition Activity

The inhibition of the α-glucosidase activity of *T. terrestris* seems to be particularly associated with the presence of phenolic compounds and flavonoids. The ethyl acetate and butanol fractions showed the best $IC_{50}$ values of 0.044 ± 0.0012 mg/mL and 0.966 ± 0.25 mg/mL, respectively. Similar studies from India (Lamba *et al.*, 2011), Turkey (Ercan & El, 2016) and Korea (Song *et al.*, 2016) have reported *T. terrestris* α-glucosidase inhibitory activity. This species presents promising action as an α-glucosidase inhibitor and might have a beneficial effect on diabetes mellitus control.

## 4. Conclusions

Among all *T. terrestris* fractions, the ethyl acetate fraction showed the highest free radical scavenging activity in all the tested models compared to the ethanol extract and other fractions. The highest scavenging activities, reducing power, total antioxidant capacity and inhibition of α-glucosidase of the ethyl acetate fraction can be ascribed to the phenolic compounds and flavonoids of the plant. The capacity for scavenging DPPH, ABTS and hydroxyl radicals represents the fraction direct radical scavenging activity. The HEX fraction showed the best metal chelating power, and this chelating ability might contribute to its lipoxygenase inhibitory activity. Thus, the *in vitro* antioxidant, lipoxygenase and α-glucosidase inhibitory activities of *T. terrestris* were confirmed.

Polar samples of Brazilian *T. terrestris* fruits have *in vitro* antioxidant and α-glucosidase inhibitory activities. In contrast, the nonpolar samples have chelating power and lipoxygenase inhibitory activity. The antidiabetic and anti-inflammatory potential of Brazilian *T. terrestris* depends on the preparation method used.

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## References

Aazza, S., Lyoussi, B., & Miguel, M. G. (2011). Antioxidant and antiacetylcholinesterase activities of some commercial essential oils and their major compounds. *Molecules*, *16*(9), 7672-7690.

Abdali-Mashhadi, A.-R., Direkvand-Moghadam, F., jalali, M., Albobaji, M., Direkvand-Moghadam, A., & Delpisheh, A. (2016). The measurement of the quercetin of different parts of *Tribulus terrestris* by HPLC. *Future Natural Products*, *2*(1), 21-26.

Ahmed, D., Khan, M. M., & Saeed, R. (2015). Comparative analysis of phenolics, flavonoids, and antioxidant and antibacterial potential of methanolic, hexanic and aqueous extracts from *Adiantum caudatum* leaves. *Antioxidants*, *4*(2), 394-409.

Alam, M. N., Bristi, N. J., & Rafiquzzaman, M. (2013). Review on *in vivo* and *in vitro* methods evaluation of antioxidant activity. *Saudi Pharmaceutical Journal*, *21*(2), 143-152.

Altemimi, A., Lakhssassi, N., Baharlouei, A., Watson, D. G., & Lightfoot, D. A. (2017). Phytochemicals: extraction, isolation, and identification of bioactive compounds from plant extracts. *Plants*, *6*(4), 42.

Asadmobini, A., Bakhtiari, M., Khaleghi, S., Esmaeili, F., & Mostafaei, A. (2017). The effect of *Tribulus terrestris* extract on motility and viability of human sperms after cryopreservation. *Cryobiology*, *75*, 154-159.

Asikin, Y., Takahashi, M., Mizu, M., Takara, K., Oku, H., & Wada, K. (2016). DNA damage protection against free radicals of two antioxidant neolignan glucosides from sugarcane molasses. *Journal of the Science of Food and Agriculture*, *96*(4), 1209-1215.

Basaiyye, S. S., Naoghare, P. K., Kanojiya, S., Bafana, A., Arrigo, P., Krishnamurthi, K., & Sivanesan, S. (2018). Molecular mechanism of apoptosis induction in Jurkat E6-1 cells by *Tribulus terrestris* alkaloids extract. *Journal of Traditional and Complementary Medicine*, *8*(3), 410-419.

Borran, M., Minaiyan, M., Zolfaghari, B., & Mahzouni, P. (2017). Protective effect of *Tribulus terrestris* fruit extract on cerulein-induced acute pancreatitis in mice. *Avicenna Journal of Phytomedicine (AJP)*, *7*(3), 250-260.

Brand-Williams, W., Cuvelier, M. E., & Berset, C. (1995). Use of a free radical method to evaluate antioxidant activity. *LWT - Food Science and Technology*, *28*(1), 25-30.

Chang, C.-C., Yang, M.-H., Wen, H.-M., & Chern, J.-C. (2002). Estimation of total flavonoid content in propolis by two complementary colometric methods. *Journal of food and drug analysis*, *10*(3), 3.

Choi, C.-I., Eom, H. J., & Kim, K. H. (2016). Antioxidant and α-glucosidase inhibitory phenolic constituents of *Lactuca indica* L. *Russ. J. Bioorganic Chem.* , *42*(3), 310-315.

Dakshayini, P., & Mahaboob Basha, P. (2018). Tribulus terrestris fruit extract improves antioxidant defense in female reproductive tract: A comprehensive study in diabetic rats. *J. Innov. Pharm. Biol. Sci.*, *5*(2), 101-107.

De Combarieu, E., Fuzzati, N., Lovati, M., & Mercalli, E. (2003). Furostanol saponins from *Tribulus terrestris*. *Fitoterapia*, *74*(6), 583-591.

Dinchev, D., Janda, B., Evstatieva, L., Oleszek, W., Aslani, M. R., & Kostova, I. (2008). Distribution of steroidal saponins in *Tribulus terrestris* from different geographical regions. *Phytochemistry*, *69*(1), 176-186.

Dobrian, A. D., Morris, M. A., Taylor-Fishwick, D. A., Holman, T. R., Imai, Y., Mirmira, R. G., & Nadler, J. L. (2019). Role of the 12-lipoxygenase pathway in diabetes pathogenesis and complications. *Pharmacology & Therapeutics*, *195*, 100-110.

Domingueti, C. P., Dusse, L. M. S. A., Carvalho, M. d. G., de Sousa, L. P., Gomes, K. B., & Fernandes, A. P. (2016). Diabetes mellitus: the linkage between oxidative stress, inflammation, hypercoagulability and vascular complications. *Journal of Diabetes and its Complications*, *30*(4), 738-745.

Dudonné, S., Vitrac, X., Coutière, P., Woillez, M., & Mérillon, J.-M. (2009). Comparative study of antioxidant properties and total phenolic content of 30 plant extracts of industrial interest using DPPH, ABTS, FRAP, SOD, and ORAC Assays. *Journal of Agricultural and Food Chemistry*, *57*(5), 1768-1774.

Dwivedi, D., & Sengar, N. (2018). Investigation of phytochemical constituents from *Tribulus terrestris* roots, leaves and fruits. *Journal of Chemistry and Chemical Sciences*, *8*(1), 55-58.

Egnell, M., Fassier, P., Lécuyer, L., Gonzalez, R., Zelek, L., Vasson, M.-P., Hercberg, S., Latino-Martel, P., Galan, P., Druesne-Pecollo, N., Deschasaux, M., & Touvier, M. (2017). Antioxidant intake from diet and supplements and risk of digestive cancers in middle-aged adults: results from the prospective nutrinet-santé cohort. *British Journal of Nutrition*, *118*(7), 541-549.

El-Shaibany, A., Molham, A.-H., Al-Tahami, B., & Al-Massarani, S. (2015). Anti-hyperglycaemic activity of *Tribulus terrestris* L aerial part extract in glucose-loaded normal rabbits. *Trop. J. Pharm. Res.*, *14*(12), 2263-2268.

El-Guendouz, S., Aazza, S., Lyoussi, B., Antunes, M. D., Faleiro, M. L., & Miguel, M. G. (2016). Anti-acetylcholinesterase, antidiabetic, anti-inflammatory, antityrosinase and antixanthine oxidase activities of Moroccan propolis. *Int. J. Food Sci.*, *51*(8), 1762-1773.

Ercan, P., & El, S. N. (2016). Inhibitory effects of chickpea and *Tribulus terrestris* on lipase, α-amylase and α-glucosidase. *Food Chemistry*, *205*, 163-169.
Ernawati, T., Radji, M., Hanafi, M., Mun'im, A., & Yanuar, A. (2017). Cinnamic acid derivatives as α-glucosidase inhibitor agents [cinnamic acid derivative; α-glucosidase inhibitor; antidiabetic; synthesis; natural products]. *Indones. J. Chem.*, *17*(1), 10.

Frum, Y., & Viljoen, A. M. (2006). *In vitro* 5-lipoxygenase and anti-oxidant activities of South African medicinal plants commonly used topically for skin diseases. *Skin Pharmacol. Physiol.*, *19*(6), 329-335.

Gardner, H. W. (1995). Biological roles and biochemistry of the lipoxygenase pathway. *HortScience*, *30*(2), 197-205.

Ghanbari, A., Moradi, M., Raoofi, A., Falahi, M., & Seydi, S. (2016). *Tribulus terrestris* hydroalcoholic extract administration effects on reproductive parameters and serum level of glucose in diabetic male rats. *International Journal of Morphology*, *34*(2).

Hammoda, H. M., Ghazy, N. M., Harraz, F. M., Radwan, M. M., ElSohly, M. A., & Abdallah, I. I. (2013). Chemical constituents from *Tribulus terrestris* and screening of their antioxidant activity. *Phytochemistry*, *92*, 153-159.

Hong, S. S., Choi, Y.-H., Jeong, W., Kwon, J. G., Kim, J. K., Seo, C., Ahn, E.-K., Lee, H. H., Ko, H.-J., Seo, D.-W., & Oh, J. S. (2013). Two new furostanol glycosides from the fruits of *Tribulus terrestris*. *Tetrahedron Lett.*, *54*(30), 3967-3970.

Ivanova, A., Lazarova, I., Mechkarova, P., & Tchorbanov, B. (2010). HPLC method for screening of steroidal saponins and rutin as biologically active compounds in *Tribulus Terrestris* L. *Biotechnol. Biotechnol. Equip.*, *24*(sup1), 129-133.

Kang, S. Y., Jung, H. W., Nam, J. H., Kim, W. K., Kang, J. S., Kim, Y. H., Cho, C. W., Cho, C. W., Park, Y. K., & Bae, H. S. (2017). Effects of the fruit extract of *Tribulus terrestris* on skin inflammation in mice with oxazolone-induced atopic dermatitis through regulation of calcium channels, orai-1 and TRPV3, and mast cell activation. *Evid. Based Complementary Altern. Med.*, *2017*, 8312946.

Keshtmand, Z., Ghanbari, A., Khazaei, M., & Rabzia, A. (2015). Protective effect of *Tribulus terrestris* hydroalcoholic extract against cisplatin-induced apoptosis on testis in mice. *Int. J. Morphol.*, *33*(1).

Khairwal, V., & Kumar, M. (2013). Lead acetate induced oxidative stress and its possible reversal by *Tribulus terrestris* root extract in testes of *Swiss albino* mice. *J. Environ. Sci. Toxicol. Food Technol.*, *6*(3), 79-85.

Khatri, S., & Chhillar, A. K. (2015). Evaluation of *in vitro* free radical scavenging activity of *Tribulus terrestris*. *Int. J. Basic Appl. Biol.*

Kim, H. S., Lee, J. W., Jang, H., Le, T. P. L., Kim, J. G., Lee, M. S., Hong, J. T., Lee, M. K., & Hwang, B. Y. (2018). Phenolic amides from *Tribulus terrestris* and their inhibitory effects on nitric oxide production in RAW 264.7 cells. *Arch. Pharm. Res.*, *41*(2), 192-195.

Kostova, I., Dinchev, D., Rentsch, G. H., Dimitrov, V., & Ivanova, A. (2002). Two new sulfated furostanol saponins from *Tribulus terrestris*. *Z. Naturforsch. C. J. Biosci.*, *57*(1-2), 33-38.

Kumari, M., Kumar, P., & Singh, P. (2015). Safety evaluation of *Tribulus terrestris* on the male reproductive health of laboratory mouse. *Int. J. Pharm. Phytopharm. Research*, *4*(5), 281-287.

Kunchandy, E., & Rao, M. N. A. (1990). Oxygen radical scavenging activity of curcumin. *Int. J. Pharm.* , *58*(3), 237-240.

Lamba, H., Bhargava, C., Thakur, M., & Bhargava, S. (2011). α-glucosidase and aldose reductase inhibitory activity *in vitro* and anti-diabetic activity *in vivo* of *Tribulus terrestris* L. (Dunal). *Int. J. Pharm. Pharm.*, *3*, 270–272.

Lee, H. H., Ahn, E. K., Hong, S. S., & Oh, J. S. (2017). Anti-inflammatory effect of tribulusamide D isolated from *Tribulus terrestris* in lipopolysaccharide-stimulated RAW264.7 macrophages. *Mol. Med. Rep.*, *16*(4), 4421-4428.

Lokhande, K., Kulkarni, C., Shinkar, M., Jadhav, S., & Salunkhe, S. (2014). Evaluation of antioxidant potential of Indian wild leafy vegetable *Tribulus terrestris*. *Int. J. Adv. Pharm. Biol. Chem.*, *3*, 2277-4688.

Nebieridze, V. G., Skhirtladze, A. V., Kemertelidze, E. P., & Ganzera, M. (2018). Megastigmane glycosides from leaves of *Tribulus terrestris*. *Chem. Nat. Compd.*, *54*(1), 63-65.

Nelson, M. J., & Seitz, S. P. (1994). The structure and function of lipoxygenase. *Curr. Opin. Struct. Biol.*, *4*(6), 878-884.

Oliveira, N. N. P. M., Félix, M. A. R., Pereira, T. C. S., Rocha, L. G. P., Miranda, J. R., Zangeronimo, M. G., Pinto, J. E. B. P., Bertolucci, S. K. V., & Sousa, R. V. d. (2015). Sperm quality and testicular histomorphometry of wistar rats supplemented with extract and fractions of fruit of *Tribulus terrestris* L. *Braz. Arch. Biol. Technol.*, *58*, 891-897.

Oyaizu, M. (1986). Studies on products of browning reaction antioxidative activities of products of browning reaction prepared from glucosamine. *The Japanese journal of nutrition and dietetics*, *44*(6), 307-315.

Pappachan, J. M., Fernandez, C. J., & Chacko, E. C. (2019). Diabesity and antidiabetic drugs. *Molecular Aspects of Medicine*, *66*, 3-12.

Prieto, P., Pineda, M., & Aguilar, M. (1999). Spectrophotometric quantitation of antioxidant capacity through the formation of a phosphomolybdenum complex: Specific application to the determination of vitamin E. *Analytical Biochemistry*, *269*(2), 337-341.

Rajendar, B., Bharavi, K., Rao, G., Kishore, P., Kumar, P. R., Kumar, C. S., & Patel, T. P. (2011). Protective effect of an aphrodisiac herb *Tribulus terrestris* Linn on cadmium-induced testicular damage. *Indian journal of pharmacology*, *43*(5), 568.

Rajendrabhai, V. D. (2017). Detection of phytochemical and pharmacological properties of crude extracts of *Tribulus terrestris* collected from tribal regions of Baglan (MS), India. *Int J Pharmacognosy Phytochem Res*, *9*(4), 508-511.

Re, R., Pellegrini, N., Proteggente, A., Pannala, A., Yang, M., & Rice-Evans, C. (1999). Antioxidant activity applying an improved ABTS radical cation decolorization assay. *Free Radical Biology and Medicine*, *26*(9), 1231-1237.

Samani, N. B., Jokar, A., Soveid, M., Heydari, M., & Mosavat, S. H. (2016). Efficacy of the hydroalcoholic extract of *Tribulus terrestris* on the serum glucose and lipid Profile of women with diabetes mellitus: a double-blind randomized placebo-controlled clinical trial. *Journal of Evidence-Based Complementary & Alternative Medicine*, *21*(4), NP91-NP97.

Sannigrahi, S., Mazuder, U. K., Pal, D. K., Parida, S., & Jain, S. (2010). Antioxidant potential of crude extract and different fractions of *Enhydra fluctuans* Lour. *Iranian journal of pharmaceutical research: IJPR*, *9*(1), 75.

Sarma, A. D., Mallick, A. R., & Ghosh, A. (2010). Free radicals and their role in different clinical conditions: an overview. *International Journal of Pharma Sciences and Research*, *1*(3), 185-192.

Semerdjieva, I. B., & Zheljazkov, V. D. (2019). Chemical constituents, biological properties, and uses of *Tribulus terrestris*: a review. *Natural Product Communications*, *14*(8), 1934578X19868394.

Shishovska, M., Arsova-Sarafinovska, Z., & Memeti, S. (2015). A simple method for determination of protodioscin in *Tribulus terrestris* L. and pharmaceuticals by high-performance liquid chromatography using diode-array detection. *J. Chem. Eng. Res. Updates*, *2*, 12-21.
Singleton, V. L., & Rossi, J. A. (1965). Colorimetry of total phenolics with phosphomolybdic-phosphotungstic acid reagents. *Am. J. Enol. Vitic.*, *16*(3), 144-158.

Song, Y. H., Kim, D. W., Curtis-Long, M. J., Park, C., Son, M., Kim, J. Y., Yuk, H. J., Lee, K. W., & Park, K. H. (2016). Cinnamic acid amides from *Tribulus terrestris* displaying uncompetitive α-glucosidase inhibition. *Eur. J. Med. Chem.*, *114*, 201-208.

Wang, B.-J., Lien, Y.-H., & Yu, Z.-R. (2004). Supercritical fluid extractive fractionation – study of the antioxidant activities of propolis. *Food Chemistry*, *86*(2), 237-243.

Wu, T.-S., Shi, L.-S., & Kuo, S.-C. (1999). Alkaloids and other constituents from *Tribulus terrestris*. *Phytochemistry*, *50*(8), 1411-1415.

Zheleva-Dimitrova, D. Z., Obreshkova, D., & Nedialkov, P. T. (2012). Antioxidant activity of *Tribulus terrestris* - a natural product in infertility therapy.

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. *J. Am. Soc. Mass Spectrom.*, *28*(11), 2302-2318.

Zhu, W., Du, Y., Meng, H., Dong, Y., & Li, L. (2017). A review of traditional pharmacological uses, phytochemistry, and pharmacological activities of *Tribulus terrestris*. *Chem. Cent. J.*, *11*(1), 60.