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Research Article
Phytochemical and Biological Studies of *Tribulus terrestris* L. Growing in Egypt

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Abstract
Background and Objective: The genus *Tribulus* of the Zygophyllaceae family comprises several species which grow as shrubs or herbs in subtropical areas around the world. Phytochemical and biological studies of the different successive extracts of the aerial parts of *Tribulus terrestris* L. (Family Zygophyllaceae), growing in Egypt as well as the isolation and identification of the biologically active natural fractions or compounds were carried out. **Methodology:** The volatile constituents were prepared and analyzed by GC/MS. The lipoidal matter was prepared and analyzed by GLC. The carbohydrate and the amino acid contents were prepared and analyzed by HPLC. Saponin fraction was prepared. The activities of polar, non-polar extracts and saponin fraction as aphrodisiac, antimicrobial, cytotoxic and antioxidant were evaluated. **Results:** GC/MS of volatile constituents revealed the presence of thirty two compounds. The major compound was methyl linolenate (18.56%). The major fatty acid in the lipoidal matter is heptadecanoic acid (33.56%). HPLC analysis of carbohydrate and amino acid revealed the presence of inulin (5.61%) and glutamic acid (2.85%), respectively. Saponin yield was 7.38%. The results of the biological investigation of the polar and non-polar extracts revealed their aphrodisiac, antimicrobial, cytotoxic and antioxidant activities with different percentages. Phytochemical examination of the aqueous methanolic biologically active extract by HPLC revealed the presence of phenolic and flavonoid contents (11.16 and 6.076%) respectively as well as saponin. Isolation and identification of rutin, quercetin and diosgenin were carried out using several chromatographic and spectroscopic analysis. **Conclusion:** The herb under investigation revealed potent and remarkable biological activities due to the presence of different active constituents.

Key words: *Tribulus terrestris*, aphrodisiac effect, antioxidant activity, cytotoxicity activity

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Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

The genus *Tribulus* of the Zygophyllaceae family comprises several species which grow as shrubs or herbs in subtropical areas around the world[1]. From which *Tribulus terrestris* L. is very common in Egypt. *T. terrestris* L. is very common in the Nile delta including Cairo but not further South, Nile valley from Cairo to Wadi Halfa, the Mediterranean coastal strip from El-Sallum to Rafah, Sinai proper i.e. South of El-Tih desert (especially in waste places)[2]. This plant can be found in arid climate regions around the world, for example, in Southern USA, Mexico, Spain, Bulgaria, India and China[3]. On the other hand *T. terrestris* L. is used in folk medicine against various diseases. The fruits and seeds are of great importance in folk medicine, they are used as an aphrodisiac, diuretic, anthelmintic and to treat cough and kidney failure[4]. *T. terrestris* L. has the reputation of having anabolic effect. It is used in many countries as a dietary supplement against sexual impotency, oedema, abdominal distention and cardiovascular diseases[5]. Indeed, *T. terrestris* L. preparations are mainly used to improve performance in sports and for treatment of impotency[6]. More recently, it has been reported that an aqueous extract of *T. terrestris* L. appeared to possess aphrodisiac properties probably due to androgen increasing properties[1]. Earlier investigation performed on *T. terrestris* L. resulted in the isolation of steroidal saponins, flavonoids, carbohydrates and alkaloids[7].

Our aim of work is phytochemical and biological studies of the successive extracts of *Tribulus terrestris* L. (Family Zygophyllaceae), growing in Egypt, as well as the isolation and identification of the biologically active natural fractions or compounds which can be useful in drug industry as curative or complementary agent.

MATERIALS AND METHODS

Phytochemical part

Chemicals: All chemicals used in the present study are fine chemicals and solvents used are HPLC grade.

General experimental procedures: Modified Likens and Nikerson apparatus was used for preparation of the volatile constituents. GC/MS: Aglient 6890 GC equipped with an Aglient MS detector, with a direct capillary interface and fused silica capillary column HP-5-MS (Hewlett Packard, USA) for analysis of volatile constituents. GLC: Agilent Technologies 6890 N Network GC system (made in USA) for analysis of USM and FAME. HPLC: Qualitative and quantitative estimation of diosgenin content in the bioactive aqueous methanolic extract of the aerial parts of *T. terrestris* L. (Saponin hydrolysate, 50 mg) was carried out using High Performance Liquid Chromatography (HPLC). Diosgenin was analyzed by using HPLC modified method of Rani *et al.*[8]. The HPLC analysis was performed on clarity chromatography data system, the HPLC system consisted of two pressure pumps (Sykam S1122 delivery system), the injection port with a 2 mL loop (Sykam S5111 Injector valve bracket), a UV detector (Jasko-UV-2070 Plus, Intelkigent UV/Visible detector, Japan). For chromatographic separation, C-18 column (Thermo Hypersil Keystone, 5 μm, 250×4.6 mm) was used. Acetonitrile/water (15:85, v/v) was used for detection of diosgenin. Flow rate was adjusted to 0.8 mL min^{-1}. Sample volume (20 μL) was injected with the help of a micro syringe, the run time was adjusted to 15 min and UV absorbance was determined at 203 nm. AutoChrom 3000 software was the data acquisition system. Calibration curve for diosgenin determination was made using different concentrations of the authentic samples. The results obtained from HPLC analysis of the sample were calculated using the above mentioned calibration curve. The HPLC analysis of flavonoids and phenolic compounds was performed on HPLC Hewlett Packard (series 1050) equipped with autosampling injector, solvent degasser, UV detector and quarter HP pump (series 1050). The HPLC analysis of sugars was performed on a model HP 1050 HPLC equipped with refractive index detector. Amino acid analyzer: (LC 300 amino acid analyzer, Eppendorf, Germany). UV-visible spectrophotometer: UV-VIS double beam UVD-3500 spectrophotometer, Labomed, Inc. was used for recording UV spectra and measuring the absorbance in UV and visible range. NMR: Bruker AMX-500, Varian Inova-500, unity plus 300 NMR spectrometer apparatus using DMSO-d6 and CD3OD-d4 as solvents and TMS as internal standard. The data is expressed in δ-values in ppm and J-values in Hz. Portable ultraviolet lamp: (254, 365 nm, vl, 6LC, Marine Lavalee-Cedex, France) for localization of spots on thin layer chromatogram.

Plant material: Samples of the aerial parts of *Tribulus terrestris* L. (Family: Zygophyllaceae) were collected from Cairo-Suez desert road (2014), Egypt and were kindly authenticated by Dr. Abdelhaleem Abdelmotagaly, Department of flora, the Agricultural Museum, Dokki, Giza, Egypt. The samples were air-dried, powdered and reduced to mesh no. 36 and kept in tightly closed containers.

Phytochemical methods

Preparation of successive extracts with selective organic solvents: Five hundred gram of the powdered dried aerial parts of *Tribulus terrestris* L. were extracted in continuous

extraction apparatus (Soxhlet) successively and exhaustively using solvents of increasing polarity in the following order: Petroleum ether (40-60°C), ether, chloroform, methanol and 50% aqueous methanol. These extracts were evaporated to dryness under vacuum at 40°C.

Investigation of volatile constituents

Preparation of volatile fraction: Fresh flowering aerial parts of *Tribulus terrestris* L. (500 g) were separately covered with sufficient water in a round bottom flask and subjected to hydrodistillation in a modified Likens and Nickerson apparatus[9] which allowed the distillation and simultaneous extraction of the volatile components in an organic solvent (n-pentane). The n-pentane layer was collected and cautiously evaporated, dehydrated over anhydrous sodium sulfate and stored in dark tightly closed container at 4°C to be analyzed by GC/MS.

GC/MS analysis of volatile constituents: GC/MS analysis of the volatile constituents of *Tribulus terrestris* L. was carried out on a gas chromatograph directly coupled to mass spectrophotometer (Agilent 6890) using Capillary column of fused silica, 30 m length, 0.32 mm ID and 0.25 mm thickness. Stationary phase HP-5MS, carrier gas Helium at flow rate of 1 mL min^{-1} and pressure 13 psi, temperature programming 60-280°C at a rate of 8°C/min, ion source temperature 230°C and ionization voltage 70 e.V. and injection volume 1 μL.

Investigation of the lipoidal matter content[10]: The residue of the petroleum ether extract was saponified according to the reported method[9]. Both the unsaponifiable and saponifiable fractions were analyzed using GC/MS adopting the methods of Tsuda *et al.*[11] and Finar[12], respectively.

Investigation of carbohydrates

Investigation of free sugars: The preparation was carried out according to Gertz[13]. The analysis was performed on a model HP1050 HPLC equipped with refractive index detector.

Investigation of polysaccharides: Preparation was achieved adapting the method of Fischer *et al.*[14]. The precipitated polysaccharide was hydrolyzed[15] and analyzed using HPLC equipped with refractive index indicator.

Investigation of protein and amino acid contents: This was carried out by Kjeldahl method using boric acid modification as described by Ma and Zuazaga[16].

Quantitative estimation of total flavonoid content: Total flavonoid content was estimated in the aqueous methanolic extract as rutin equivalent according to Rolim *et al.*[17] and assayed spectrophotometrically at 362.8 nm.

Quantitative estimation of total phenolic content as gallic acid equivalent: Total phenolic content was determined in the aqueous methanolic extract according to the method of Kujala *et al.*[18] and Singleton and Rossi[19] and then assayed spectrophotometrically at 745 nm.

Qualitative and quantitative estimation of flavonoid and phenolic compounds by HPLC: Flavonoid compounds were determined by HPLC according to the method of Mattila *et al.*[20], while phenolic compounds were determined by HPLC according to the method of Goupy *et al.*[21]. Analysis was carried out using HPLC equipped with UV detector set at 330 nm for flavonoid compounds and at 280 nm for phenolic compounds, respectively. Quarter HP pump (series1050). The column temperature was maintained at 35°C. Gradient separation was carried out with methanol and acetonitrile as a mobile phase at flow rate of 1 mL min^{-1}. Flavonoid standards from sigma Co. were dissolved in a mobile phase and injected into HPLC. Retention time and peak area were used for calculation of flavonoid concentration by the data analysis of Hewlett Packard software.

Isolation of the flavonoid compounds in the bioactive aqueous methanolic extract: 5 g of the dried aqueous successive methanol extract was chromatographed on polyamide column, gradient elution was carried out using water: Methanol (90:10), (80:20), etc. Fractions (50 mL) were collected then chromatographed on TLC of silica gel using (Ethyl acetate: Methanol: Water: Formic acid) (100:16:11:3) (v/v/v/v) as developing system and UV light before and after exposure to ammonia for detection. Fractions with similar pattern were pooled together and purified using preparative flash column RP C18 (PURIFLASH 4100) and gradient elution was applied with (Water: Acetonitrile) (99:1), (98:2) etc. as solvent system. Two flavonoid compounds were isolated which were further purified by rechromatography over a column of Sephadex LH-20 to afford compound 1 (15 mg) and compound 2 (25 mg). The isolated compounds were hydrolyzed[22] and identified by spectral analysis (UV, NMR) and comparison with reported literature[23,24].

Investigation of saponin

Extraction and preparation of saponin: 1 kg of dried powdered aerial parts of *T. terrestris* L. was defatted by refluxing with petroleum ether (40-60°C) then extracted by refluxing with 85% aqueous methanol The dried aqueous methanol extract was dissolved in water and purified by liquid-liquid extraction using chloroform and ethyl acetate, respectively. The aqueous layer was evaporated under reduced pressure at constant temperature till completely dried. The residue of was dissolved in the least amount of methanol (5 mL) then was poured in 3 L of acetone in order to precipitate the crude saponin. The saponin was separated by centrifugation. The process was repeated twice.

Isolation of diosgenin: 50 g of the prepared saponin was chromatographed on column of silica gel G, gradient elution was carried out with (dichloromethane: methanol) as solvent system (80:20), (60:40), etc. Fractions of 50 mL were collected and chromatographed on TLC of silica gel using (Chloroform: Methanol:Water) (61:32:7) (v/v/v) as solvent system and detected with Khagi-Mischner reagent with heating at 110°C for 10 min. Similar fractions were pooled together giving subfractions T1, T2 and T3. 0.5 gm of T3 was subjected to hydrolysis according to the method of Cary[25]. The hydrolysate was chromatographed by TLC of silica gel using (Petroleum Ether: Benzene: Ethyl acetate: Acetic acid) (10:20:6:0.5) (v/v/v/v) and (Chloroform: Methanol) (95: 5) (v/v) as mobile phases and visualized by spraying with Khagi-Mischner reagent followed by heating at 110°C for 10 min.

Calibration curve for diosgenin determination was made using different concentrations of the authentic samples. The results obtained from HPLC analysis of the sample were calculated using the above mentioned calibration curve. Qualitative and quantitative estimation of diosgenin in the bioactive aqueous methanolic extract of the aerial parts of *T. terrestris* L. was carried out using High Performance Liquid Chromatography (HPLC) as previously reported in details.

Biological part

Materials

Animals: Male albino rats, of 217±7.2 g as (Mean±SD) were used. Animals were obtained from the animal house of National Research Centre, Cairo, Egypt. The animals were kept individually in stainless steel cages at room temperature. Water and food were given *ad libitum*. Adult normal male and female albino mice of 21-25 g body weight were used in an acute toxicity test.

Microorganisms for antimicrobial activity: The following bacteria and yeast were used: *Bacillus subtilis* ATCC 9139, *Staphylococcus aureus* ATCC 6538 and *Lactobacillus* species local isolate as gram positive bacteria, *Escherichia coli* ATCC 11229, Pseudomonas aeruginosa ATCC 15442 and *Salmonella typhimurium* ATCC 14028 as gram negative bacteria while *Candida albicans* ATCC 10231 was used as yeast. Bacteria and yeast strains were kindly obtained from culture collection of Bacteriological lab, Water Pollution Research Department, NRC, Egypt.

Media: Mueller-Hinton agar (MHA) and tryptone soya broth (TBS) were used in the microbiological evaluation.

Cancer cell lines: HepG2 (hepatocellular carcinoma cell line). These were obtained from ATCC, USA.

Chemicals used in the cytotoxic activity: 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT), (Sigma Aldrich, MO). DMSO (Lab Scan, Ireland).

DPPH radical for antioxidant *in vitro* evaluation were purchased from Sigma Co

Methods: Acute lethal toxicity test of extracts (aqueous methanol, petroleum ether and saponin) was carried out according to Goodman *et al.*[26]. The 24 h mortality counts among equal sized groups of mice (8 animals/group) receiving progressively increasing oral dose of the extracts were recorded.

Preparation of dosage form: Petroleum ether and aqueous methanolic successive extracts as well as saponin of *Tribulus terrestris* L. were dispersed separately in water using gum acacia to be given orally to rats. For the control, the vehicle was prepared through dissolving the same amount of gum acacia in water.

Aphrodisiac effect

Castration of rats: Castration was induced in rats as described by Van Coppenolle *et al.*[27]. The animals were anesthetized by intraperitoneal injections of ketamine (0.05 mL kg^{-1}) and xylazine (0.05 mL kg^{-1}). Castration was performed through the scrotum sack, cutting and opening the scrotum and then taking out the testicles.

Design of the experimental study: In this experiment forty-two male rats were divided into seven groups.

Group one (6 rats) was served as normal healthy control. Group two were normal rats and injected subcutaneous by testosterone propionate (10 mg kg^{-1} rat b.wt.). The remaining thirty rats were castrated and served as aphrodisiac rat model. Group three, four and five were orally administered by polar aqueous methanol extract of *Tribulus terrestris* (5 mg kg^{-1} rat b.wt.), non-polar petroleum ether extract of *Tribulus terrestris* (5 mg kg^{-1} rat body weight) and saponin fraction of *Tribulus terrestris* (5 mg kg^{-1} rat b.wt.). Group six were injected subcutaneous by testosterone propionate (10 mg kg^{-1} rat b.wt.), while group seven was castrated rats and given no medication, it served as castrated control. At the end of the experiment (2 months) blood samples were collected from all rats after an overnight fast for the determination of plasma testosterone using ELISA technique[28] and plasma luteinizing hormone (LH)[29]. The plasma levels of creatinine[30] and urea[31] were determined as indicator of kidney function, while the activity of aspartate transaminase (AST) and alanine transaminase (ALT) were determined as indicator of liver function according to the method of Reitman and Frankel[32]. The entire prostate was removed from all animals and weight as indicator to the aphrodisiac properties of *Tribulus terrestris*. Final body weight of all rats was recorded at the end of the experiment. Also body weight gain was calculated. Animal procedures were performed in accordance with the Ethics Committee of the National Research Centre, Cairo, Egypt.

Antimicrobial activity: *Bacillus subtilis* ATCC 9139, *Staphylococcus aureus* ATCC 6538, *Lactobacillus* species local isolate, *Escherichia coli* ATCC 11229, *Pseudomonas aeruginosa* ATCC 15442, *Salmonella typhi* ATCC 14028 and *Candida albicans* ATCC 10231 (24 h aged each) inoculated in Tryptone Soya Broth (TSB) and incubated at 37°C for 17-24 h. One milliliter from each culture was transferred into 9 mL (0.9% NaCl solution) and diluted to 10^5 (colony forming unit) CFU/1 mL. The antimicrobial activity of the ether, chloroform, methanol and 50% aqueous methanol successive extracts were evaluated by the agar well diffusion method using Mueller Hinton Agar No. 2 (MHA) (Thermo Scientific). The plant extracts were sterilized by filtration through 0.45 µ membrane before testing. Briefly, bacteria were grown on MHA at 37°C overnight, a loop full of growth was then inoculated into Mueller Hinton broth (Thermo Scientific) and incubated at 37°C on a rotary shaker until the turbidity of the growth was equivalent to the density of 0.5 McFarland standard. The microorganism was then spread (0.1 mL) on the surface of MHA (spreading technique). Wells of uniform diameter (6 mm) were made on the solidified agar. About 25 mg mL^{-1} of plant extracts and the negative control (solvent without plant extract) were placed separately in each well. Plates were then left at room temperature for 1 h to allow the solutions diffusion into the MHA and then incubated at 37°C overnight. Finally, the inhibition zone measured from the base of the plate resting 5-7 cm above black flat[33,34].

Cytotoxic activity: The successive extracts (petroleum ether, diethyl ether, chloroform, methanol and 50% aqueous methanol extracts) were tested using the cell line technique according to Thabrew *et al.*[35]. Cell viability was assessed by the mitochondrial dependent reduction of yellow MTT [3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide] to purple formazan[36]. Cells batch cultured for 10 days then seeded in concentration of 10×10^3 cells/well in fresh complete growth medium in 96 multiwell micro titer plastic plates (10^4 cells/well) at 37°C for 24 h under 5% CO_2 for 24 h before treatment to be attached to the wall of plate using water jacketed carbon oxide incubator (Sheldon, TC2323, Cornelius, OR, USA). Media was aspirated, fresh medium (without serum) was added and cells were incubated either (100, 50, 25, 12.5, 6.25, 3.125, 1.56 and 0.78 mg mL^{-1}). Cells were suspended in RPMI 1640 medium, 1% antibiotic-antimycotic mixture (10,000 U mL^{-1} potassium penicillin, 10,000 µg mL^{-1} Streptomycin Sulfate and 25 µg mL^{-1} Amphotericin B) and 1% L-glutamine in 96 well flat bottom micro plate at 37°C under 5% CO_2. After 48 h of incubation, medium was aspirated, 40 µL MTT salt (2.5 µg mL^{-1}) were added to each well and incubated for further 4 h at 37°C under 5% CO_2. To stop the reaction and dissolving of formed crystals, 200 µL of 10% Sodium Dodecyl Sulfate (SDS) in unionized water was added to each well and incubated over night at 37°C. A positive control which composed of 100 µg mL^{-1} of *Annona Cherimola* extract was used as known as cytotoxic natural agent which gives 100% lethality under same conditions. The absorbance was then measured using micro plate multi-well reader (Bio-Rad Laboratories Inc., model 3350, Hercules, California, USA) at 595 nm and reference wavelength 620 nm. A statistical significance was tested between samples and negative control (cell with vehicle) using independent t-test by SPSS 11 program. DMSO is the vehicle used for dissolution of plant extracts and its final concentration on the cells was less than 0.2%. A probit analysis was carried for IC_{50} and IC_{90} determination using SPSS 11 program. IC_{50} (concentration which reduces survival of the exposed cancer cells to 50%) and IC_{90}

(concentration which reduces survival of the exposed cancer cells to 90%) was obtained from the curves.

Determination of the *in vitro* antioxidant activity: The free radical scavenging activity of successive extracts (petroleum ether, diethyl ether, chloroform, methanol and 50% aqueous methanol extracts) was measured by 1,1-diphenyl-2-picryl-hydrazil (DPPH$^{\cdot}$) using the method of Shimada *et al.*[37]. All plant extracts were screened at 100 µg mL^{-1} while the most potent active extracts (gave more 90%) were assayed at 25-75 µg mL^{-1}. 0.1 mM solution of DPPH in methanol was prepared. Then, 1 mL of this solution was added to 3 mL of extract solution at different conc. (25-75 µg mL^{-1}). The mixture was shaken vigorously and allowed to stand at room temperature for 30 min. Then the absorbance was measured at 517 nm in Asys microplate reader. Lower absorbance of the reaction mixture indicated higher free radical scavenging activity. DPPH scavenging effect % was calculated according to the following Eq:

$$100-\frac{A_0-A_1}{A_0}\times 100$$

where, A_0 was the absorbance of the control reaction and A_1 was the absorbance in the presence of the sample[38].

Statistical analysis: The results of animal experiments were expressed as Mean±SE and they were analyzed statistically using the one-way analysis of variance ANOVA followed by Duncan's t-test.

RESULTS

Phytochemical part

Preliminary phytochemical screening: Preliminary phytochemical screening revealed that the powdered dried aerial parts of *Tribulus terrestris* L. contains: Volatiles, sterols and/or terpenes, carbohydrates and/or glycosides, flavonoids (free and combined), saponins, coumarins, traces of alkaloids and/or nitrogenous compounds.

Volatile constituents: The GC/MS analysis of volatile constituents of *T. terrestris* L. revealed thirty two identified compounds. Oxygenated components were found to be the main constituents (53.13%) while the percentage of hydrocarbons reached (46.87%). Oxygenated monoterpenes were mainly dill ether (0.79%) and oxygenated sesquiterpenes were mainly butylated hydroxytoluene (9.28%) and dihydro agarofuran (0.51%). Oxygenated diterpenes were mainly phytol (0.65%). Diterpene hydrocarbons were found mainly neophytadiene (2.53%) and eicosane (0.69%). Fatty acids and esters exhibited (37.07%) of the volatile constituents.

Lipoidal matter: The yield of lipoidal matter in the aerial parts of *T. terrestris* L. was (2.78%). The percentage of the USM fraction was (48%), the percentage of total fatty acid fraction was (46%). n-Nonadecane C19 (8.8%) is the main hydrocarbon identified in the USM followed by n-Tricosane C23 (4.96%), n-Heneicosane C21 (4.33%) and n-Tetracosane C24 (4.31%). The sterols identified in the USM comprise: Cholesterol (3.56%), campesterol (1.71%) and stigmasterol (1.08%). The triterpenoid compound α- amyrine (5.65%) was identified in the USM. The percentage of identified unsaturated fatty acids was (28.68%). linolenic acid was the major unsaturated fatty acid (17.6%). The percentage of identified saturated fatty acids was (53.7%). Heptadecanoic acid was the major saturated fatty acid (33.56%).

Carbohydrate content: The HPLC analysis showed that the major free sugars in *T. terrestris* L. were raffinose (0.7%), arabinose (0.62%), galacturonic acid (0.59%) and stachyose (0.41%). The main polysaccharide hydrolysate was inulin and arabinose (5.61 and 2.38%), respectively.

Protein and amino acid contents: The percentage of total protein content of the aerial parts of *T. terrestris* L. was found to be 16.63%.

The analysis of the amino acid content of the aerial parts of *T. terrestris* revealed the presence of phenylalanine, threonine, valine, leucine and lysine as essential amino acids with different proportions. Aspartic acid, serine, glutamic acid, glycine, alanine, tyrosine and arginine are also present as non-essential amino acids in the plant with different proportions. The major essential amino acid in *T. terrestris* L. was leucine (0.5%) while the major non-essential amino acid was glutamic acid (2.85%).

Flavonoid and phenolic contents in the bioactive aqueous methanolic extract: The percentage of flavonoid content was 6.076% while that of phenolic content was 11.16%. The results of HPLC analysis (Table 1 and 2) of flavonoids revealed the presence of nine identified flavonoids which are naringin, rutin, hyperoside, quercetrin, naringenin, quercetin, hesperetin, kampferol and apigenin. The major compounds are: Hyperoside, naringin and hesperetin which represent

0.053, 0.040 and 0.025%, respectively. The results of HPLC analysis of phenolic compounds revealed that the total number of identified phenolic compounds is fourteen compounds which are pyrogallol, gallic acid, protocatechuic acid, catechin, catechol, chlorogenic acid, p-hydroxybenzoic acid, caffeic acid, vanillic acid, ferulic acid, salicylic acid, ellagic acid, coumaric acid and cinnamic acid. The major compounds were: Salicylic, ellagic and pyrogallol which represent 0.045, 0.026 and 0.014%, respectively.

Flavonoid compounds in the bioactive aqueous methanolic extract: Two flavonoid compounds were isolated using different chromatographic techniques. Identification of the isolated compounds was achieved using physical, chemical and spectral analysis including acid hydrolysis, chromatographic analysis, UV, NMR. The two compounds were identified as rutin (15 mg) and quercetin (25 mg).

Investigation of saponin content in the bioactive methanolic extract: The total yield of the isolated saponin was 73.76 g in *T. terrestris* L. which represent 7.38% of the dried powdered aerial parts of *T. terrestris*. Quantitative estimation of diosgenin using HPLC analysis revealed that its percentage was 0.51% in the isolated saponin.

Biological part

Acute toxicity: The results of acute toxicity showed no mortality/or toxicity of the 24 h of oral administration of the successive extracts of *T. terrestris* L. up to a dose of 1 g kg^{-1} mice body weight 10 mg kg^{-1} mice body weight of the prepared saponins.

Aphrodisiac effect: Table 3 showed nutritional parameters and prostate weight of the studied experimental groups. Final body weight and body weight of castrated rats were reduced non-significantly compared with normal rats. Prostate weight of castrated rats given no medication or given oral administration of petroleum ether extract of *Tribulus terrestris* showed significant reduction compared with normal and other castrated rats. Oral administration of aqueous methanol

Table 1: Identified flavonoids in the methanolic extract of *T. terrestris* L.

Test flavonoids	Retention time	Area (%)	Test results of flavonoids (%)
Naringin	11.358	1.633	0.0398
Rutin	11.772	1.760	0.0071
Hyperoside	11.910	6.803	0.0532
Quercetrin	12.819	3.275	0.0094
Naringenin	13.819	2.733	0.0056
Quercetin	14.061	0.649	0.0026
Hesperetin	14.409	3.474	0.0248
Kampferol	14.632	2.102	0.0036
Apigenin	15.686	1.107	0.0064

Table 2: Identified phenolic compounds in the methanolic extract of *T. terrestris* L.

Test items	Retention time	Area (%)	Test results of phenolic compounds (%)
Pyrogallol	6.044	0.2940	0.01427
Gallic acid	6.403	0.3332	0.00106
Protocatechuic acid	7.596	0.4986	0.00694
Catechin	7.845	0.5106	0.00402
Catechol	7.973	0.3288	0.00320
Chlorogenic acid	8.470	1.0399	0.00916
P-hydroxybenzoic acid	8.803	0.4689	0.00429
Caffeic acid	9.283	1.3444	0.00351
Vanillic acid	10.185	1.1538	0.00376
Ferulic acid	11.021	1.6229	0.00524
Salicylic acid	12.527	1.4919	0.04541
Ellagic acid	12.663	3.1160	0.02625
Coumaric acid	13.054	1.2683	0.00324
Cinnamic acid	13.961	0.6186	0.00099

Table 3: Prostate weight and nutritional parameters of different studied groups

Groups	Initial body weight (g)	Final body weight (g)	Body weight gain (g)	Prostate weight (g)
Normal control	217.5 ± 3.82^{a}	302.7 ± 2.81^{a}	85.2 ± 4.1^{a}	0.967 ± 0.02^{a}
Normal testosterone	217.2 ± 2.902^{a}	301.2 ± 1.796^{a}	84.0 ± 1.57^{a}	1.17 ± 0.05^{c}
Castrated control	217.5 ± 3.343^{a}	298.7 ± 2.185^{a}	81.2 ± 2.372^{a}	0.555 ± 0.046^{b}
Castrated testosterone	217.7 ± 3.545^{a}	300.3 ± 4.301^{a}	82.7 ± 6.102^{a}	0.912 ± 0.032^{a}
Tribulus aqueous methanol extract	217.7 ± 2.498^{a}	302.5 ± 2.012^{a}	84.8 ± 4.252^{a}	1.02 ± 0.031^{a}
Tribulus PE. extract	217.5 ± 3.084^{a}	301.3 ± 2.185^{a}	83.8 ± 2.749^{a}	0.672 ± 0.025^{b}
Saponin fraction of *Tribulus*	217.2 ± 2.868^{a}	296.8 ± 2.072^{a}	79.7 ± 2.072^{a}	0.897 ± 0.023^{a}

In each column same letters means non-significant difference, different letter means the significance among the tested groups at 0.05 probability

Table 4: Biochemical parameters of the different studied groups

Groups	Testosterone (ng mL^{-1})	LH (ng mL^{-1})	Creatinine (mg dL^{-1})	Urea (mg dL^{-1})	AST (U mL^{-1})	ALT (U mL^{-1})
Normal control	1.47 ± 0.145^a	0.42 ± 0.022^a	0.603 ± 0.026^a	28.6 ± 0.737^a	62.7 ± 1.429^a	24.7 ± 1.69^a
Normal testosterone	1.683 ± 0.101^a	0.405 ± 0.031^a	0.628 ± 0.025^a	29.2 ± 0.672^a	64.8 ± 1.222^a	24.2 ± 1.376^a
Castrated control	0.535 ± 0.017^b	0.728 ± 0.035^b	0.645 ± 0.021^a	31.6 ± 1.437^a	64.3 ± 2.403^a	25.3 ± 0.882^a
Castrated testosterone	1.12 ± 0.048^a	0.682 ± 0.021^b	0.593 ± 0.019^a	29.4 ± 0.686^a	63.2 ± 1.108^a	25.5 ± 1.586^a
Tribulus aqueous methanol extract	1.22 ± 0.031^a	0.57 ± 0.017^c	0.58 ± 0.031^a	30.1 ± 1.002^a	63.5 ± 1.995^a	25.8 ± 1.558^a
Tribulus PE. extract	0.58 ± 0.011^b	0.753 ± 0.023^b	0.60 ± 0.018^a	29.9 ± 1.369^a	64.0 ± 2.379^a	25.3 ± 1.333^a
Saponin fraction of *Tribulus*	1.01 ± 0.042^c	0.555 ± 0.019^c	0.615 ± 0.023^a	29.7 ± 0.556^a	62.7 ± 1.605^a	26.2 ± 0.601^a

In each column same letters means non-significant difference, different letter means the significance among the tested groups at 0.05 probability

extract of *Tribulus terrestris* or its saponin fraction showed significant elevation in prostate weight compared with castrated rats given no medication.

Table 4 represented biochemical parameters of different experimental groups. Castrated rats showed significant reduction in plasma level of testosterone, while plasma level of luteinizing hormone (LH) elevate significantly. Oral administration of castrated rats with aqueous methanol extract of *Tribulus terrestris* or saponin for 2 months showed significant elevation in plasma level of testosterone compared with all castrated rats. Plasma level of LH reduced significantly in castrated rats given oral administration of aqueous methanol extract of *Tribulus terrestris* or saponin compared with all castrated rats groups. The present results revealed that castration leads to low androgenic status which appeared through reduction in testosterone plasma levels and reduction in prostate weight. Also castration elevates plasma levels of LH. Oral administration of aqueous methanol and saponin extracts of *Tribulus terrestris* showed significant aphrodisiac effect through significant elevation of plasma levels of testosterone and reduction of LH plasma levels, which accompanied with significant elevation of prostate weight to reach normal levels.

Liver and kidney functions of all rats groups showed non-significant changes, which indicate the complete safety of the studied extracts.

Antimicrobial activity: The results revealed that the chloroform extract of *T. terrestris* L. is the most powerful extract, it has the highest percentage of bacterial removal against the five bacterial strains tested. Against *E. coli* the most powerful fractions were chloroform extract, methanol extract, 50% aqueous methanol and diethyl ether extract which have percentage of bacterial removal equal to 93.87, 91.25, 91.00 and 90.00% respectively. Against *Staphylococcus aureus* the most powerful fractions were chloroform extract, 50% aqueous methanol extract, diethyl ether extract which have percentage of bacterial removal of 88.51, 84.38 and 68.5, respectively. Against *Candida albicans* the most powerful extracts were chloroform and methanol extracts which have percentage of bacterial removal of 93.39 and 60.26%, respectively. Against *Bacillus subtilis* the most powerful extracts are diethyl ether extract, chloroform extract, methanol extract which have percentage of bacterial removal of 95.87, 80.22 and 77.78, respectively. Against *Salmonella typhimurium* the most powerful extracts were chloroform, methanol and diethyl ether extracts which have percentage of bacterial removal of 72.30, 67.66 and 67.31%, respectively.

Cytotoxic activity: The results revealed that methanol and petroleum ether extracts of *T. terrestris* L. were the most effective fractions against human hepatocellular carcinoma cell line ((HepG2) cell line) which have LC_{50} values equal to 16.1 and 21.5 μg mL^{-1}, respectively.

***In vitro* antioxidant activity:** DPPH scavenging activity of the successive extracts (petroleum ether, diethyl ether, chloroform, methanol and 50% aqueous methanol extracts) as well as saponin fraction of *T. terrestris* L. revealed that polar extract (methanol and 50% aqueous methanol) and saponin have the highest antioxidant activity, 26.5 and 18.5%, respectively.

DISCUSSION

Biological investigation of the aerial parts of *T. terrestris* revealed its aphrodisiac, microbiological, cytotoxic and antioxidant activities with different degrees.

Successive extracts of the aerial parts of *T. terrestris* L. as well as the prepared saponin were tested for the acute lethal toxicity. The results showed of the tested extracts were safe up to a dose of 1 g kg^{-1} mice body weight while saponin is safe up to 10 mg kg^{-1} mice body weight.

Concerning the aphrodisiac activity, the petroleum ether extract, aqueous methanolic extract and prepared saponin of *T. terrestris* L. were tested by oral administration at doses of 5 mg kg^{-1} body weight. It is well known that in males, LH acts on the interstitial cells (Leydig cells) in the testis, causing production and secretion of androgens and secondarily promotes spermatogenesis via androgen. The successful and

complete male germ cell development is dependent on the balanced endocrine interplay of hypothalamus, pituitary and the testis. Gonadotropin releasing hormone (Gnrh) secreted by the hypothalamus elicits the release of gonadotropins follicle stimulating hormone (FSH) and luteinizing hormone (LH) from the pituitary gland. FSH binds with receptors in the sertoli cells and stimulates spermatogenesis. LH stimulates the production of testosterone in Leydig cells, which in turn may act on the Sertoli and peritubular cells of the seminiferous tubules and stimulates spermatogenesis[39].

The failure of pituitary to secrete FSH and LH will result in disruption of testicular function leading to infertility. Testosterone and estradiol inhibit control the secretion of gonadotropins[40]. The increased FSH level in men with azoospermia or severe oligozoospermia (<5 million sperm mL^{-1}) indicate damaged seminiferous tubule[41]. Studies have shown that in infertile men with germinal epithelial injury, no stimulation of spermatogenesis occurs due to low or lack of production of androgen binding proteins. Gonadotropins (FSH, LH) and testosterone are the prime regulators of germ cell development. Abnormal spermatogenesis is often associated with altered serum gonadotropins and testosterone[42]. FSH, LH and testosterone evaluation is useful in the management of male infertility[43].

Infertility is a problem of global proportions, affecting on an average 8-12% of couples worldwide[44]. Based on the National Women's Health Information Center (NWHIC), the annual incidence of male infertility is at least two million cases. Recent studies have indicated that the prevalence of oligozoospermia is extremely high in the metropolis as well as in the smaller towns of India[45]. Infertility is defined as the inability to achieve pregnancy after 1 year of unprotected coitus[46]. Male infertility is considerably to be less complicated than female infertility, but can account for 30-40% of infertility[47]. Except for some physical defects, low sperm count (Oligozoospermia) and poor sperm quality are responsible for male infertility in more than 90% of the cases. Out of these, in about 30-40% the cause is unexplained and in the rest of the cases critical illness, malnutrition, genetic abnormalities, pollution, side effects of some medicines, hormones and chemicals plays a major role[48].

Tribulus is a well known pharmaceutical herb that has been used for a long time in the traditional Chinese and Indian systems of medicine for the treatment of various diseases. It has been found that the genus *Tribulus* is rich in biologically active furostane, cholestane and spirostane type steroidal saponins[49].

In the present study oral administration of aqueous methanol extract and saponin fraction of *T. terrestris* L. elevate testosterone and LH plasma levels. Studies have suggested that Gokshura (*Tribulus terrestris* L.) can enhance the hormone levels to within a normal range. It can increase the testosterone by increasing the luteinizing hormone and the gonadotropin releasing hormone[50]. This hormone is effective in building muscles as well as improving fertility and libido and it has also been proved to be active in stimulating spermatogenesis and sertoli cell activity in rats[51]. Protodioscin, a steroidal glycoside found in the *T.terrestris* L.[44], increased the levels of testosterone, dihydrotestosterone and dehydroepiandrosterone and thereby improved libido, erectile dysfunction[45] and low semiological indices[46]. Also diosgenin, a steroidal compound and one of the main sapogenins present in *T. terrestris* L., is a typical initial intermediate for synthesis of steroidal compounds, oral contraceptives and sex hormones. Diosgenin has very impressive pharmacological profile and could be used as a medicine for the treatment of different types of disorders in the future[52].

The results of antimicrobial activity of *T. terrestris* L. revealed that *T. terrestris* L. has potent antimicrobial activity as previously discussed in details.

The cytotoxic activity of the successive extracts of *T. terrestris* L. was studied on $HepG_2$ cell line. The most effective fractions were the methanol and petroleum ether extracts of *T. terrestris* L. which have LC_{50} values equal to 16.1 and 21.5 µg mL^{-1} respectively. Concerning the antioxidant effect the polar extracts as well as the prepared saponin of *T. terrestris* L. have the highest antioxidant activity which was 26.5, 18.5%, respectively. Phytochemical investigation of the most biologically active extracts revealed the presence of phenolic compounds as well as saponin. Investigation of the flavonoid compounds led to the isolation and identification of several flavonoid and phenolic compounds. It was reported that quercetin and rutin which were detected in the present study showed significant antiradical capacity by means of two different in vitro tests: Scavenging of the stable DPPH radical dot radical and of authentic peroxynitrite ($ONOO^-$). Quercetin showed maximum activity (IC_{50} (DPPH radical dot) 5.5 µM, IC_{50}($ONOO^-$) 48.8 µM). Potential antiradical interactive effects among the two compounds were also investigated and results indicated possible synergy between quercetin and rutin towards ($ONOO^-$)[53]. Much work has been carried out in recent years on the beneficial effect of phenolic compounds which act as natural antioxidants and help to neutralize free radicals. Quercetin is an anti-oxidative flavonoid widely distributed in the plant kingdom. Phenolic hydroxyl groups at the B-ring and the 3-position are responsible for its free radical scavenging activity. With respect to its relationship with molecular targets relevant to cancer prevention, quercetin aglycone has been

shown to interact with some receptors, particularly an aryl hydrocarbon receptor, which is involved in the development of cancers induced by certain chemicals. Quercetin aglycone has also been shown to modulate several signal transduction pathways which are associated with the processes of inflammation and carcinogenesis. Rodent studies have demonstrated that dietary administration of this flavonol prevents chemically induced carcinogenesis, especially in the colon, whilst epidemiological studies have indicated that an intake of quercetin may be associated with the prevention of lung cancer. Dietary quercetin is, therefore, a promising agent for cancer prevention[54]. Flavonoid compounds have been investigated for their protective action against oxidative mechanisms in different *in vivo* and *in vitro* models, which seems to be linked to their antioxidant properties. Naringenin which was detected in the biologically active extracts has intensive antibacterial activity against methicillin resistant *Staphylococcus aureus* (MRSA) and *streptococci*. An alteration of membrane fluidity in hydrophilic and hydrophobic regions may be attributed to this effect which suggests that this flavonoid might reduce the fluidity of outer and inner layers of membranes. The correlation between antibacterial activity and membrane interference supports the theory that flavonoids may demonstrate antibacterial activity by reducing membrane fluidity of bacterial cells. The 5,7-dihydroxylation of the A ring and 2, 4 or 2, 6-dihydroxylation of the B ring in the flavanone structure is important for anti-MRSA activity. A hydroxyl group at position 5 in flavanones and flavones are important for their activity against MRSA. Substitution with C8 and C10 chains may also enhance the antistaphylococcal activity of flavonoids belonging to the flavan-3-ol class. 5-hydroxyflavanones and 5-hydroxyisoflavanones with one, two, or three additional hydroxyl groups at the 7, 2 and 4 positions inhibited the growth of *Streptococcus mutans* and *Streptococcus sobrinus*[55].

It is important to clarify that the detected flavonoid compounds in the biologically active extracts are powerful antioxidants. They possess also many types of pharmacological activities, including anti-inflammatory, hepatoprotective, vasorelaxant, antiviral and anticarcinogenic effects. They also have important roles in plant growth, reproduction and pathogen and predator resistance[56].

It is important to note that the presence of steroidal saponin, diosgenin in *T. terrestris* exhibits anticarcinogenic activity via reducing peroxidation reaction and marker enzymes through enhancing the intrinsic antioxidant defense system[57]. It is also a typical initial intermediate for synthesis of steroidal compounds, oral contraceptives and sex hormones[52].

CONCLUSION

The herb under investigation revealed potent and remarkable biological activities due to the presence of different active constituents. All the obtained results confirm the advantage of its use as effective therapeutic agents from botanical origin. In the proper dosage from this can be realized after doing the necessary toxicological and pharmaceutical studies which are in progress.

SIGNIFICANT STATEMENTS

The present study discovers the aphrodisiac effect of *Tribulus terrestris* L., which growing in Egypt. All the obtained results confirm the advantage of its use as effective therapeutic agents from botanical origin. Thus, this study has been succeeds in finding a new natural source of aphrodisiac treatment and possibly using it instead of synthetic pharmaceutical preparations.

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