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Comparative analysis of extraction techniques and biological activities of the root of *Tribulus terrestris* L. grown in Northern Iraq

Türkiye ve Kuzey Irak'ın bazı bölgelerinde yetişen Tribulus terrestris L.'nin biyolojik aktivitelerinin araştırılması

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ABSTRACT

Tribulus terrestris root extracts were used in this study to find the yield percentage, total condensed tannins concentration, anti-microbial and antioxidant activities, as well as to identify and quantify phenolics components. A variety of extraction techniques such as microwave extraction (ME), convection extraction (CE), and accelerated solvent extraction (ASE) were employed, with methanol, ethanol and water used as solvents. The total condensed tannin concentration and antioxidant capacity were evaluated using UV-visible spectroscopy at 580 nm and 517 nm, respectively. Antimicrobial activity was determined by disc diffusion method. The LC-MS/MS was used to identify and quantify phenolic compounds. The ASE technique yielded the highest extraction efficiency (12.06%) when using methanol, while the conventional extraction technique yielded the lowest extraction efficiency (6.60%). The average total condensed tannin concentration in the TT root, measured by triplicate, was 10.83 mg/L. The methanol extract obtained using the ME technique exhibited the largest inhibitory zone (19.33 mm) against *Micrococcus luteus* LA2971. The ASE technique produced the highest radical scavenging activity (DPPH) with the methanol extract, while the CE technique showed the lowest DPPH scavenging activity in the ethanol extract. The ethanol extract had a greater capacity to scavenge DPPH than BHT. The highest and the lowest amounts of phenolic compounds were identified by using LC-MS/MS as Vanillin (125 µg/g) and chlorogenic acid (1.46 µg/g), respectively. Furthermore, the results demonstrated that hesperidin (10.79 µg/g), and quercetin (0.16 µg/g) had the highest and lowest quantities of flavonoids, respectively.

Key Words: *Tribulus terrestris*, LC-MS/MS, Antimicrobial activity, Antioxidant activity

ÖZ

Bu çalışmada, *Tribulus terrestris* kök ekstraktları verim yüzdesini, toplam yoğunlaştırılmış tanen konsantrasyonunu, antimikrobiyal ve antioksidan aktivitelerini bulmak ve ayrıca fenolik bileşenlerinin tanımlanması ve miktarlarının belirlenmesi için kullanılmıştır. Metanol, etanol ve suyun çözücü olarak kullanıldığı mikrodalga ekstraksiyonu (ME), konveksiyon ekstraksiyonu (CE) ve hızlandırılmış çözücü ekstraksiyonu (ASE) gibi çeşitli ekstraksiyon teknikleri kullanılmıştır. Toplam yoğunlaştırılmış tanen konsantrasyonu ve antioksidan kapasitesi, 580 nm ve 517 nm

'de UV-görünür spektroskopisi kullanılarak değerlendirilmiştir. Antimikrobiyal aktiviteyi belirlemek için disk difüzyon yöntemi kullanılmıştır. Fenolik bileşikleri tanımlamak ve miktarlarını belirlemek için LC-MS/MS kullanılmıştır. ASE tekniği, metanol kullanıldığında en yüksek ekstraksiyon verimliliğini (% 12.06) verirken, geleneksel ekstraksiyon tekniği en düşük ekstraksiyon verimliliğini (%6.60) sağlamıştır. TT kökündeki toplam yoğunlaştırılmış tanen konsantrasyonunun üçlü olarak ölçülmesiyle ortalama değeri 10.83 mg/L olarak bulunmuştur. ME tekniği kullanılarak elde edilen metanol özütü *Micrococcus luteus* LA2971'e karşı en büyük inhibitör zonu (19.33 mm) gösterdi. ASE tekniği metanol özütü ile en yüksek radikal temizleme aktivitesini (DPPH) üretirken, CE tekniği etanol özütünde en düşük DPPH temizleme aktivitesini gösterdi. Etanol özütü BHT'den daha fazla DPPH temizleme kapasitesine sahiptir. En yüksek ve en düşük fenolik bileşik miktarları LC-MS/MS kullanılarak sırasıyla Vanilin (125 µg/g) ve klorojenik asit (1.46 µg/g) olarak belirlendi. Ayrıca, sonuçlar hesperidin'in (10.79 µg/g) ve kuersetinin (0.16 µg/g) sırasıyla en yüksek ve en düşük flavonoid miktarlarına sahip olduğunu gösterdi.

Anahtar Kelimeler: *Tribulus terrestris,* LC-MS/MS, Antimikrobiyal aktivite, Antioksidant aktivite

Introduction

Traditional medicine has been extensively used for several years to treat diseases and provide healthcare in many parts of the world. The information showed that there is a strong association between the using of medicinal plants and laboratory testing (Singh et al., 2008). Phytotherapy involves the use of biologically active plant components (Alanis et al., 2007). Growth inhibition or decrease in harmful pathogens is the most intriguing area of use for therapeutic plant extracts (Kotzekidou et al., 2008). Chronic inflammation affects more than a billion people worldwide and has been associated with several serious medical conditions, including respiratory difficulties, skin problems, gastrointestinal problems, and nervous system abnormalities. In addition, corticosteroids and nonsteroidal anti-inflammatory medications help reduce the discomfort caused by these symptoms (Rodriguez & Davoudian, 2016). One of the most prevalent illnesses that can be fatal, and the biggest reason of death globally is cancer. It is projected that there would be around 19.3 million new cancer diagnoses globally in 2020 and an estimated 10 million cancer-related deaths, according to the International Agency for Research on Cancer (Sung et al., 2021). Numerous biologically active metabolites found in fruits and vegetables can be used as alternatives to synthetic medications (Stankovi´c et al., 2016). *Tribulus terrestris* L. plants are related to Zygophyllaceae kin and broadly grown subtropics and Mediterranean, including Mexico, Spain, Bulgaria, India, Pakistan, and China (Chhatre Saurabh et al., 2014). Majeed & Mahmood (1988) and Saad Aldein (1986) indicated that traditional medicine used Tribulus plants as tonics, sexual stimulants, analgesics, astringents, stomachics, diuretics, lithontriptics, and renal anti-infectives in Iraq. *Tribulus terrestris* plant is generally used to cure the illness in cuta-neous pruritus, eyes, chest tightness or pain, hypoimmunity, cerebral disease and cancer. Moreover, it exhibits significant pharmacological, antioxidant, antimicrobial, and antiinflammatory activities, enhancement of hormone and gonadotropin levels, alleviation of muscle damage, improvement of mitochondrial dysfunction, genetic and cytological effects of cultured human lymphocytes, and regulation of enzyme activities (Tian et al., 2019). The aim of this study was to determine the best optimization conditions and to study the biological activity of the *T. terrestris* plant root using different extractions and solvents. Therefore, this study will contribute to previous and future studies.

Materials and Methods

Plant Materials

Tribulus terrestris roots were collected from Koysinjaq region Iraq. The plant was recognized by botanist Dr. Abdulghani Omer Ismaeel Sarmamy, University of Salahaddin Department of Biology/Erbil-Iraq. The roots were air-dried under standard room conditions and ground by using an electric stainless-steel blender. The powder was sieved through various mesh sizes and kept at

room temperature until evaluation. The trials were carried out in the Faculty of Forestry Laboratory, Kahramanmaraş Sütçü İmam University.

Extraction Methods

Microwave extraction

Ten grams of the extract were combined with 100 mL each of ethanol (95%), methanol (95%), and water in a glass container. The mixture was stirred, and the extraction was carried out for 30 minutes at various temperatures. After extraction, the liquid was filtered using Whatman No. 1 filter paper. A rotary vacuum evaporator was used to collect and evaporate the liquid extract (Laghari et al., 2011).

Conventional extraction method

Ten grams of powdered extract were mixed with 100 mL of methanol and heated at 40°C for 2 hours. The liquid and solid phases were separated using Whatman No. 1 filter paper. A rotary vacuum evaporator was then employed to obtain purified extract. Water and ethanol were used in the same manner at this stage (Crozier et al., 1997).

Accelerated solvent extraction

Ten grams of the dried extract were weighed and placed in a 10 mm specific fiber filter glass. Automatic extraction was performed using methanol and ethanol at 60 °C under pressure for 40 minutes. After extraction, the liquid form was evaporated using rotary vacuum to obtain the pure form (Comlekcioglu et al., 2013).

Determination of yield extract

The 0.5 grams of root extract were subjected to extraction using various methods, with 50 mL each of ethanol, methanol, and water. The extraction yield of the roots was determined using the formula given below (Anokwuru et al., 2011):

$$Percentage\ yield = \frac{W2-W1}{W0} \times 100$$

Where,

$W2\ is\ the\ extract\ dry\ weight\ with\ container\ (gr)$

$W1\ is\ the\ weight\ of\ container\ alone\ (gr)$

$W0\ is\ the\ oven\ dry\ weight\ of\ sample\ (gr)$

Total condensed tanins

A UV-Vis spectrophotometer was used for the experiment. The solution was prepared by dissolving 0.05 g of Fe_2SO_4 in a mixture of 5 mL of HCl (35%) and 95 mL of N-butanol. To calculate condensed tanin, 0.01 gr extract and mimosa tannin were added separately to a testing tube, which was then filled with 10 mL extract solution and incubated in water bath for one hour. The wavelength selected for measuring the absorbance was 580 nm (Makkar et al., 1995; Rasul et al., 2024).

Chemical composition of Tribulus terrestris root determined by LC-MS/MS

Phenolic substances detection and measurement using the previously published LC-MS/MS method was used. The analytical method's applicability as well as the standard compounds' qualitative and quantitative determination have already been confirmed. Table 1. Linearity ranges and quotes for rectilinear regression for the standard chemicals were presented. Correlation coefficients were found to be higher than 0.99. The limit of detection (LOD) and the limit of quantitation (LOQ) of the reported analytical method are shown in Table 1. For the analyzed compounds, LOD varied between 0.05 and 25.8 µg/L, while LOQ ranged from 0.17 to 85.9 µg/L. Additionally, the recovery rates of the phenolic compounds were between 96.9% and 106.2%. The conclusion was calculated using the following equation (Ertas et al., 2015).

Quantification of compound (µg/g)= $\frac{RxU^f}{100}$

Where,

R = (LC–MS/MS result (µg)),

U^f= (percent relative uncertainty at 95%

confidence level (%))

Antimicrobial Activity

Preparation microorganism medium

Mueller-Hinton and Sabouraud-dextrose agar were used for the cultivation of bacteria and fungi, respectively. Subsequently, plates were inoculated with 10 µL of Gram-positive and Gram-negative bacteria and 20 µL of fungi. The microorganisms were supplied by Department of Microbiology, Kahramanmaraş Sütçü University, Türkiye. Detailed information regarding the microorganisms is provided in Table 2. (Rasul et al., 2024).

Table 2. Information relating to microorganisms used in the study

Microorganisms	***Type***
Pseudomonas aeruginosa (*Pa*) DSM50071	Gram (-)
Escherichia coli (*Ec*) DM	Gram (-)
Klebsiella pneumoniae (*Kp*) FMC5	Gram (-)
Staphylococcus aureus (*Sa*) Cowan1	Gram (+)
Bacillus megaterium (*Bm*) DSM32	Gram (+)
Bacillus subtilis (*Bs*) IMG22	Gram (+)
Micrococcus luteus (*Ml*) LA2971	Gram (+)
Enterococcus faecium (*Ef*) Clinical isolate	Gram (+)
Candida utilis (*Cu*) NRRL-Y-900	Fungi
Candida albicans (*Ca*) ATCC1023	Fungi
Yarrowia lipolytica (*Yl*) NCIM3589	Fungi
Saccharomyces cerevisiae (*Sc*) WET136	Fungi

Preparation of disc diffusion agar

Root extracts were tested for antimicrobial activity against eight bacteria and four fungi, as shown in Table 2. A volume of 150 µl of each root extract (20 mg/mL) was immersed into 10 mm in diameter a sterile disc. Following the inoculation process, plant extract-prepared discs were positioned on the inoculation medium and let the diffuse at room temperature. The negative (methanol-ethanol-water) and positive (10 µg/mL gentamicin and ampicillin) were used like controls groups. Furthermore, 150 µl Itracnazol and flucozanol were created as antifungal antibiotics and impregnated into discs. Except for fungi, which were kept at 27 °C for 24 to 48 hours, at 37 °C for 18 to 24 hours, all Petri dishes were incubated. The inhibitory zones were calculated with a ruler, and the three repeats' mean diameter was recorded (Shankar et al., 2010; Rasul et al., 2024).

Root Extracts DPPH Antioxidant Activity

To evaluate the antioxidant activity of the root extracts in ethanol and methanol, Blois's method was used to test the stable 1,1-diphenyl-2-picrylhydrazyl (DPPH) free radical scavenging capacity. Sample solutions (0.1, 0.2, and 0.3 mL) were mixed with ethanol and methanol in separate test tubes. Subsequently, 1 mL of DPPH solution was added to each mixture. The solutions were thoroughly shaken and left to stand at room temperature in the dark for 30 minutes. Absorbance changes were measured at 517 nm using a UV-Visible spectrophotometer. The referance standart was used as Butylated hydroxytoluene (BHT). The following formula was used to determine the optical density and percentage of DPPH radical scavenging capacity (Rasul et al., 2024; Göçeri et al., 2022).

DPPH Radical Scavenging Power (%):

$$\frac{\mathbf{Abs_{control}} - \mathbf{Abs_{sample}}}{\mathbf{Abs_{control}}} \times \mathbf{100}$$

Where,

$Abs_{control}$ (DPPH absorbance)

Abs_{sample} (Sample and BHT absorbance)

Statistical analyses

Descriptive statistical methods, including mean and standard deviation were applied to each result. Statistical analysis was performed using

analysis of variance in SPSS software version 18 (SPSS Inc., Chicago, USA). The mean differences were assesed either non-significant (P>0.05) or significant (P<0.05).

Results and Discussion

Root extracts yield percentage

Table 3 shows the yield calculations for the *T. terrestris* root extract in methanol, ethanol, and water extracts.

Table 3. Yield percentage of Tribulus terrestris root extracts

Method	*Solvents*	*Yield*- (%)*
Microwave (ME)	Methanol	9.23 ± 0.15
	Ethanol	8.30 ± 0.15
	Water	7.30 ± 0.15
Conventional	Methanol	6.65 ± 0.06
	Ethanol	6.70 ± 0.06
	Water	6.60 ± 0.15
Accelerated solvent (ASE)	Methanol	12.06 ± 0.15
	Ethanol	10.03 ± 0.15
	Water	NT

Note: ethanol (E), methanol (M), water (W), *The values aredisplayed as the three measures' mean ± SD, NT: not tested

The extraction method and solvent significantly influenced the yield. The highest yield was obtained with methanol (12.06%) using the accelerated solvent extraction (ASE) technique, whereas the lowest yield was recorded with water (6.60%) using the conventional extraction method. Variations in extract yields with different solvents can be explained by the polarity of both the extracted compounds and the solvents used (Hsu et al., 2006; Shabir et al., 2011). Methanol provided the highest yield in the ASE process, consistent with findings of Chatha et al. (2006), which reported methanol as yielding the highest rice bran extract.

Total condensed tannin in root extracts

Root extracts were investigated with standart mimosa calibration which is given at Table 4. The regression equality (y=66.35x+0.411, R^2=0.999).

Table 4. Total condensed tannin concentration in root extracts

Test	*Condensed Tannin Concentration (mg/L)*	*Average mg/L*	*Standart Deviation*	*Variation*
1	9.90	10.83	±1.39	12.79
2	10.17			
3	12.42			

Higher plant species contain polymeric flavonoid molecules known as condensed tannins. These secondary metabolites are particularly beneficial in crops used as feed sources. Their presence in pulses is considered a favorable agronomic trait, provided they constitute 3–4% of the dry weight (Robbins et al., 1998). The roots had an average total condensed tannin concentration of 10.83 mg/L, consistent with findings from Sebata et al. (2005), who reported 0.039 mg/g condensed tannin levels in *T. terrestris*. Additionally, Mohammed et al. (2013) observed low tannin concentrations in ethanol extracts of *T. terrestris* fruits.

Chemical compounds in root extracts determined by LC-MS/MS

The qualitative investigation of phenolics and flavonoids components was done by using LC-MS/MS. Many characterization studies have been conducted on *T. terrestris*. A total of 27 phenolics, non-phenolics, and flavonoids components of *T. terrestris* were examined in the methanol extract using a triple-quadruple analyzer-equipped mass spectrometer. A negative ionization mode was used to investigate phenolics and flavonoids substances. Table 1 shows the outcomes of the methanol extracts of the roots.

Table 1. Identification of phenological compounds of root extract

Type	*Compounds*	*RT[a]*	*Parent ion(m/z)[b]*	*LOD/LOQ (µg/L)[e]*	*U[f]*	*Quantification (µg/g)[g]*
Phenolic acids	Gallic acid	4.29	169.05	4.8/15.9	5.1	10.10±0.51
	Vanillin	8.77	151.05	10.1/33.7	4.9	125±6.12
	Tannic acid	6.46	182.95	10.2/34.2	5.1	98.02±5.00
	p-Coumaric acid	9.53	162.95	15.2/50.8	5.1	2.91±0.14
	Chlorogenic acid	5.43	353	7.3/24.3	4.9	1.46±0.07
	tr-caffeic acid	7.37	178.95	4.4/14.7	5.2	2.08±0.10
	Protocatechuic acid	5.63	152.95	25.8/85.9	5.1	4.04±0.20
	Rosmarinic acid	9.57	358.9	10.4/34.8	4.9	Nd
	Salicylic acid	11.72	136.95	4/13.3	5.0	37.43±1.87
	4-OH Benzoicacid	11.72	136.95	3.0/10.0	5.2	34.52±1.79
Non-Phenolic	Quinic acid	3.32	190.95	22.3/74.5	4.8	12.04±0.57
	Malic acid	3.54	133.05	19.2/64.1	5.3	78.15±4.14
	tr-Aconitic acid	4.13	172.85	15.6/52	4.9	6.63±0.32
Flavonoids	Quercetin	14.48	300.9	2.0/6.8	7.1	0.16±0.01
	Myricetin	11.94	317	9.9/32.9	5.9	0.31±0.01
	Fisetin	12.61	284.95	10.7/35.6	5.5	0.72±0.04
	Coumarin	12.52	146.95	9.1/30.4	4.9	1.07±0.05
	Rutin	10.18	609.1	17.0/56.6	5.0	1.52±0.07
	Naringenin	14.66	270.95	2.6/8.8	5.5	2.23±0.12
	Kaempferol	15.43	284.95	2.0/6.6	5.2	0.46±0.02
	Hyperoside	10.43	463.1	12.4/41.4	4.9	7.47±0.36
	Apigenin	17.31	268.95	0.1/0.3	5.3	0.47±0.02
	Hesperetin	15.29	300.95	3.3/11.0	5.3	0.22±0.01
	Hesperidin	9.69	611.1	21.6/71.9	4.9	10.79±0.53
	Rhamnetin	18.94	314.95	0.2/0.7	6.1	0.52±0.03
	Luteolin	15.43	284.95	5.8/19.4	6.9	Nd
	Chrysin	21.18	253	0.05/0.17	5.3	Nd

Not: RT[a]; Retention time, Parent ion (m/z)[b]; Molecular ions of the standard compounds (mass to charge ratio), LOD/LOQ[e] (µg/L); Limit of detection/Limit of quantification, U[f] (%); Percent relative uncertainty at 95% confidence level (k=2), Nd: not detected.

A total of 24 compounds were identified, including phenolic, non-phenolic and flavonoids. Vanillin was the most plentiful phenolic compound (125 µg analyte/g), followed by tannic acid (98.02 µg analyte/g), salicylic acid (37.43 µg analyte/g), 4-OH benzoic acid (34.52 µg analyte/g), gallic acid (10.10 µg analyte/g), protocatechuic acid (4.04 µg analyte/g), *p-coumaric* acid (2.91 µg analyte/g), tr-caffeic acid (2.08 µg analyte/g), and chlorogenic acid (1.46 µg analyte/g). Among the three non-phenolic acids detected, malic acid was the most prevalent (78.15 µg analyte/g), followed by quinic acid (12.04 µg analyte/g) and trans-aconitic acid (6.63 µg analyte/g). In terms of flavonoids, extract contained notable amounts hesperidin (10.79 µg analyte/g), hyperoside (7.47 µg analyte/g), naringenin (2.23 µg analyte/g), rutin (1.52 µg analyte/g), coumarin (1.07 µg analyte/g), rhamnetin (0.52 µg analyte/g), apigenin (0.47 µg analyte/g), kaempferol (0.46 µg analyte/g), myricetin (0.31 µg analyte/g), hesperetin (0.22 µg analyte/g), quercetin (0.16 µg analyte/g) (Table 1). The concentrations of chlorogenic acid, caffeic acid, and 4-hydroxybenzoic acid differed from those reported by Reshma et al. (2015). Similarly, the quantity of gallic acid was lower compared to an earlier study by Mishra & Bisht (2012). Hammoda et al. (2013) confirmed the presence of quinic acid. Flavonoids, a chemically and biologically diverse group of secondary metabolites, are widely distributed and play a crucial role in the chemotaxonomic studies of plant genera and families (Mitra et al., 2012). The flavonoid analysis revealed high concentrations of hesperidin and low concentrations of quercetin, as shown in Table 1. Unlike earlier research by Mitra et al. (2012), which identified chrysin as the primary flavonoid in *T. terrestris* leaves, the

current study found hesperidin to be the dominant flavonoid. Furthermore, the lack of luteolin and chrysin in this investigation is consistent with Mitra et al. (2012) findings. However, the concentrations of rutin and coumarin differed from those reported by Ivanova et al. (2010) and Mohammed et al. (2013). Numerous studies have demonstrated that the primary constituents of *T. terrestris* include flavonoids, alkaloids, α-amyrin, and saponins (Abirami & Rajendran, 2011; Gomathi et al., 2012). Variations in the primary components of *T. terrestris* may be attributed to differences in the plant's geographic origin, extraction methodologies, and the types of solvents used.

Antimicrobial activity of root extract

Researchers worldwide have investigated the effects of plant extracts on fungi and bacteria. Through oral traditions, the specific plants used and their application methods for various diseases have been passed down. To validate any potential antibacterial activity, plants must be tested against diverse microorganisms (Duraipandiyan & Ignacimuthu, 2011). In this study, the antimicrobial activity of *T. terrestris* root extract (20 mg/mL) was assessed using the disc diffusion method against eight bacterial and four fungal strains. The antimicrobial and antibiotic inhibition zones are presented in Tables 5 and 6, respectively. According to the results, the root extracts significantly inhibited all tested microorganisms ($P < 0.05$), as shown in Table 3.4. The root extracts demonstrated substantial inhibition zones against *Y. lipolytica* NCIM35, *C. albicans* ATCC1023, and *M. luteus* LA2971 in the in vitro antimicrobial screening. Moderate inhibition zones were observed for *B. megaterium* DSM32, *S. aureus* Cowan1, *P. aeruginosa* DSM50071, and *B. subtilis* IMG22. In contrast, low inhibition zones were recorded for *S. cerevisiae* WET136, *E. coli* DM, *E. faecium*, and *K. pneumoniae* FMC5.

Table 5. Antimicrobial activity of root extract

Organisms		***Inhibition Zones (mm)****							
		Extraction tecniques							
		Microwave			***Conventional***		***Accelerated solvent***		
		CH_3OH	C_2H_6O	H_2O	CH_3OH	C_2H_6O	H_2O	CH_3OH	C_2H_6O
Kp	Mean	11	11	–	12.67	–	–	11.67	11
	SD	±0.0	±0.0	–	±0.57	–	–	±0.57	±0.0
Pa	Mean	14.67	11.67	11	12.67	12.67	–	14.67	12.67
	SD	±0.57	±0.57	±0.0	±0.57	±0.57	–	±0.57	±0.57
Ec	Mean	11.67	12	11	11.67	11	–	12.67	11.67
	SD	±0.57	±0.0	±0.0	±0.57	±0.0	–	±0.57	±0.57
Sa	Mean	11	14.67	11.67	11	13.67	–	11.67	11
	SD	±0.0	±0.57	±0.57	±0.0	±0.57	–	±0.57	±0.0
Bm	Mean	12.67	11	12.67	13	12.67	–	13.67	12.67
	SD	±0.57	±0.0	±0.57	±0.0	±0.57	–	±0.57	±0.57
Ef	Mean	–	–	–	11.67	11	11	12.67	12
	SD	–	–	–	±0.57	±0.0	±0.0	±0.57	±0.0
Bs	Mean	11	11	11	12.67	11	–	13.33	13
	SD	±0.0	±0.0	±0.0	±0.57	±0.0	–	±0.57	±0.0
Ca	Mean	17.67	15.67	11.67	15.67	14.67	11	16.33	15.67
	SD	±0.57	±0.57	±0.57	±0.57	±0.57	±0.0	±0.57	±0.57
Ml	Mean	19.33	15.67	14.67	15	16	13.67	14.67	14.67
	SD	±0.57	±0.57	±0.57	±1.0	±1.0	0.57	±0.57	±0.57
Cu	Mean	–	–	–	–	–	–	–	–
	SD	–	–	–	–	–	–	–	–
Sc	Mean	–	11.67	–	–	12	11	–	12
	SD	–	±0.57	–	–	±0.0	±0.0	–	±0.0
Yl	Mean	15.67	16.67	13.67	11	11.67	15.67	11.67	11.67
	SD	±0.57	±0.57	±0.57	±0.0	±0.57	±0.57	±0.57	±0.57

Note: CH_3OH: methanol, C_2H_6O: ethanol, H_2O: water, ME: (microwave extraction), CE:(conventional extraction), ASE:

(accelerated solvent extraction), *Kp*: *K. pneumoniae*, *Pa*: *P. aeruginosa, Ec*: *E. coli*, *Sa*: *S. aureus* Cowan1, *Bm*: *B. megaterium*, *Ef*: *E. faecium* clinical isolate, *Bs*: *B. subtilis*, *Ca*: *C. Albicans, Ml*: *M. luteus*, *Cu*: *C. utilis*, *Sc*: *S. cerevisiae*, *Yl*: *Y. lipolytica*, (–) means no inhibition zone, 150 µL/disc (20 mg/mL) extracts were used. *The values are displayed as the three replications' mean ± SD. There were statistically significant mean differences (P<0.05). f-test value (5.184) and p-value (0.000).

Table 6. Antibiotic activity of root extract

	Inhibition zones (mm)*			
Organisms	***Gentamicin***	***Ampicillin***	***Fluconazol***	***Itraconazol***
K. pneumonie	43.33±1.15	–	-	-
E. coli	35.67±1.15	–	-	-
P. aeruginosa	33.67±0.57	7.00±0.0	-	-
B. megaterim	33.33±0.57	6.33±0.57	-	-
S. aureus	35.67±0.57	6.00±0.0	-	-
B. subtilis	40.00±1.0	6.67±0.57	-	-
E. faecium	31.00±1.0	–	-	-
M. luteus	34.33±0.57	8.66±0.57	-	-
C. albicans	-	-	12.67±0.57	13.00±1.0
C. utilis	-	-	16.33±0.57	18.33±0.57
S. cerevisiae	-	-	13.67±0.57	13.67±0.57
Y. lipolytica	-	-	12.67±0.57	12±0.0

Note: *The values are displayed as the three replications' mean±SD, (–) means no inhibition zone

A range of inhibitory zones (11–19.33 mm) was observed against Gram-positive (*+*) bacteria, including *B. Megaterium*, *S. Aureus*, *B. Subtilis*, *E. Faecium*, and *M. Luteus* (Table 5). The highest inhibition zone was recorded against *M. Luteus* using methanol extracts obtained through microwave extraction. This finding aligns with Ali et al. (2015), who reported that one of the non-polar fractions of plant extracts inhibited *M. Luteus*. However, the inhibition zone reported in this study was smaller than the 27.1 mm zone reported by Upadhyay et al. (2010) using olive essential oil. The inhibition zones of *M. Luteus* against methanol extracts were smaller than gentamicin but larger than ampicillin, underscoring the plant's antimicrobial potential. The observed inhibition might be attributed to the phenolics, and flavonoids present in the methanol extract, for instance vanillin (Vaghasiya et al., 2004), flavonoids (Kasim et al., 2011), and quinic acid (Zhang et al., 2013). Antimicrobial tests against Gram-positive bacteria revealed the lowest inhibition zone (11 mm) from methanol (ME), ethanol (CE), and water extracts under various extraction techniques (Table 5). *B. Subtilis* showed inhibition zones ranging from 11–13.33 mm for all extracts except CE-water, which displayed no inhibition. These results are lower than those reported by Mohammed (2008), and Baburao et al. (2009), but consistent with Sanjeeva et al. (2011). For *S. Aureus*, all extracts at 20 mg/disc demonstrated inhibition zones of 11–14.67 mm, except CE-water, which showed no inhibition. These findings deviate from the results of Soleimanpour et al. (2015) and Zou et al. (2014) but are consistent with Sanjeeva et al. (2011) and Dastagir et al. (2012). The antimicrobial activity of *T. Terrestris* root extracts against *B. Megaterium* was less effective than the results reported by Dığrak et al. (2001). Against Gram-negative (−) bacteria, including *K. Pneumoniae*, *E. Coli*, and *P. Aeruginosa*, inhibition zones ranged from 11–14.67 mm (Table 5). Water-based extractions (microwave and conventional) did not inhibit *K. Pneumoniae*, while conventional extractions also failed to inhibit *P. Aeruginosa* and *E. Coli*. Methanol extracts obtained through ASE inhibited *E. Coli* with a zone of 12.67 mm, consistent with findings by Usman et al. (2007) and Jindal et al. (2012). For *P. Aeruginosa*, the methanol extract demonstrated a zone of 14.67 mm, similar to results by Mohammed et al. (2008) but lower than those of Kianbakht & Jahaniani, (2003). Generally, *T. Terrestris* root extracts demonstrated moderate antimicrobial activity against Gram-positive (+) bacteria (*S. Aureus, B. Megaterium*, *B. Subtilis*, *E. Faecium*, and *M.*

Luteus). The highest inhibition zone (19.33 mm) was recorded against *M. Luteus* using methanol extract via microwave extraction. Among Gram-negative (-) bacteria, the highest zone (14.67 mm) was observed for *P. Aeruginosa*. The antifungal activity of *T. Terrestris* roots showed no inhibition against *C. utilis*. However, the highest antifungal activity (17.67 mm) was observed against *C. albicans* using methanol extracts from microwave extraction (Table 5). This was followed by ethanol extracts, which demonstrated significant inhibition against *Y. Lipolytica* (16.67 mm). *S. Cerevisiae* exhibited the lowest inhibition zone (11 mm). The antifungal activity of methanol and ethanol extracts supports findings by Al-Bayati et al. (2008) and can be attributed to compounds, for instance vanillin (Fitzgerald et al., 2005) and saponins (Zhang et al., 2006). Compared to standard antibiotics, gentamicin demonstrated superior antimicrobial effects, while plant extracts outperformed ampicillin (Table 6). Importantly, *C. albicans* showed greater susceptibility to *T. Terrestris* root extracts than to standard antifungal drugs fluconazole and itraconazole. The antimicrobial activity of *T. Terrestris* roots against *S. Cerevisiae* and *Y. Lipolytica* was comparable to that of standard drugs. Organic solvent-based extracts (methanol and ethanol) generally exhibited stronger antimicrobial activity compared to water extracts, highlighting the role of solvent choice in extracting bioactive compounds. These findings confirm that methanol and ethanol are more effective than water for extracting antimicrobial agents from plants (Moniharapon & Hashinaga, 2004; Parekh et al., 2005). Overall, *T. Terrestris* root extracts demonstrated moderate antibacterial and anticandidal activities.

Antioxidant activity of root extracts

When exposed to radical scavengers, the purple free radical 1,1-diphenyl-2-picrylhydrazyl (DPPH), which typically absorbs at 517 nm, is significantly reduced due to the donation of hydrogen atoms or electrons. The extract's absorbance at 517 nm reflects its maximum capacity to scavenge free radicals (Taslimi et al., 2017; Gulcin & Alwasel, 2023). As an antioxidant interacts with DPPH, it neutralizes its free radical properties by donating an electron or hydrogen atom. In the presence of a free radical scavenger, the electron pairs, causing the absorption to disappear, and the extent of decolorization corresponds to the number of electrons consumed (Banerjee and Bonde, 2011). Table 7 presents the radical scavenging activity of *T. terrestris* root's methanol and ethanol extracts in comparison to butylated hydroxytoluene (BHT).

Table 7. Antioxidant activity of root extracts

		DPPH radical scavenging activity (%)					
		Extract volume (mL)			***BHT volume (mL)[2]***		
Methods	***Solvents[1]***	***0.1***	***0.2***	***0.3***	***0.1***	***0.2***	***0.3***
Microwave[2] extraction	MeOH	79.68	80.47	83.01	91.0	92.0	90.5
Conventional[3] extraction	MeOH	79.52	82.93	84.52	91.0	92.0	90.5
ASE[4]	MeOH	83.73	82.38	85.23	91.0	92.0	90.5
Microweve[5] extraction	EtOH	77.11	79.44	80.18	68.0	66.4	78.0
Conventional[6] extraction	EtOH	76.0	76.18	76.93	68.0	66.4	78.0
ASE[7]	EtOH	78.51	78.32	79.81	68.0	66.4	78.0

Note: E: ethanol, M: methanol, ASE; Accelerated solvent extraction method, BHT: Butylated hydroxytoluene; [1]:53mg/L;[2]:0.0073 mg/mL;[3]:0.0101 mg/mL; [4]:0.0131 mg/mL; [5]:0.0073 mg/mL; [6]: 0.0091 mg/mL; [7]: 0.0109 mg/mL

According to the data, *T. Terrestris* root's extracts had the highest DPPH scavenging capacity (85.23%) when measured using the ASE method's methanol extract. On the other hand, ethanol extract using the traditional extraction method had the lowest DPPH scavenging

capacity (76.0%). A study showed that the ASE method has more effective scavenging capability than other methods (Pietrzak et al., 2014). In this investigation, the ethanol fraction's DPPH scavenging power outperformed BHT used as positive control. However, methanol extract's capacity to scavenge DPPH was inferior to that BHT (Table 7). In addition, comparison of the results with BHT is consistent with the results of Zheleva-Dimitrova et al. (2012). The ethanol extracts of TT roots exhibited strong antioxidant capacity. The presence of polyphenolic chemicals in the roots of TT may be the cause of this.

Conclusion

This study focused on the different extraction methods, solvents, antimicrobial and antioxidant activities, and chemical compositions of *T. terrestris* L. root extract. The highest extraction yield was achieved using the accelerated solvent extraction (ASE) technique with methanol. Additionally, total condensed tannins were present in the *T. terrestris* root extract. A total of 27 chemical compounds were identified in the methanol extract using LC-MS/MS, with vanillin being the most prevalent phenolic component. The ASE-derived methanol extract exhibited the highest DPPH scavenging capacity, while the ethanol extract obtained through traditional extraction methods demonstrated the lowest DPPH scavenging activity. Notably, all ethanol extract fractions exhibited higher DPPH scavenging power than the BHT reference. The antimicrobial activity of the extracts had been assessed using the disc diffusion method. The results demonstrate that, under the prescribed conditions, the various *T. terrestris* root extracts exhibited potent antimicrobial and antifungal properties. Among the extracts, the microwave-extracted methanol extract demonstrated the strongest antimicrobial inhibition zone against *M. luteus* and *C. albicans* (antifungal). These findings suggest that the microwave extraction technique could serve as an effective alternative to other extraction methods. The plant (*T. terrestris*) shows promise as a source of antioxidants and could potentially aid in the treatment of diseases related to free radical damage. Furthermore, the roots of this plant appear to be safe and effective for treating a range of infections caused by microorganisms. Thus, this study contributes to a deeper understanding of the pharmacological actions of *T. terrestris* as a biological therapy. Consequently, consuming *T. terrestris* could protect humans against pathogenic microbes and oxidative stress without adverse side effects. However, further in vivo research is needed to fully explore this plant's potential biological functions. Traditional healers may find that the various substances in *T. terrestris* roots are a promising source of naturally occurring bioactive compounds.

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Author's Contributions

The contribution of the authors is equal.

Statement of conflict of interest

Authors have declared no conflict of interest.

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