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Phenolic contents and *in vitro* free radical scavenging activity of alcoholic extract of the fruits of *Tribulus terrestris* L.

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Abstract
Three flavonoids were isolated and identified using different spectroscopic procedures from the fruits of *Tribulus terrestris* L. viz. Querctin-3-O-α-L-rhamnopyranosyl (1```-6``)–O-β-D-glucopyranosoide, Quercetin and Isorhamentin-3-O-α-L-rhamnopyranosyl (1```-6``)–O-β-D- glucopyranosoide. The last compound is reported for the first time in the fruits under investigation.
Qualitative and quantitative determination of flavonoids and phenolic acids of the total alcoholic extract, different solvent extractives and isolated compounds in addition to their antioxidant activity adopting the DPPH method are presented. The results revealed that the total alcoholic, n –butanol and ethyl acetate fractions as well as rutin and quercetin have potent free radical scavenging activity (IC_{50} = 64.0 ± 2.45**,** 240.4 ± 5.61, 280.9 ± 4.28, 12.1± 1.13 and 11.2 ± 0.81 µg/ml respectively). Moreover, the antimicrobial and antifungal activities of the total alcoholic extract, its subfractions and isolated compounds showed that they possessed non-significant antifungal activity. However, the butanol and ethyl acetate fractions in addition to the quercetin exerted potent antimicrobial activity against all investigated strains.

Keywords: *Tribulus terrestris* L, flavonoids, antioxidant, DPPH, antimicrobial activity.

Introduction
Antioxidant research is an important topic in the medical field as well as in the food industry. The oxidation induced by ROS can result in cell membrane disintegration, membrane protein damage and DNA mutation, which can further initiate or propagate the development of many diseases, such as cancer, liver injury and cardiovascular disease [1]. Although the body possesses such defense mechanisms, as enzymes and antioxidant nutrients, which arrest the damaging properties of ROS [2, 3], continuous exposure to chemicals and contaminants may lead to an increase in the amount of free radicals in the body beyond its capacity to control them, and cause irreversible oxidative damage [4]. Therefore, antioxidants with free radical scavenging activities may have great relevance in the prevention of diseases in which oxidants or free radicals are implicated [5].
The massive increase in prevalence of multiple drug resistance has slowed down the development of new synthetic antimicrobial drugs and it necessitated the search for new antimicrobial from alternative sources. Natural compounds have become a source of numerous therapeutic agents [6]. phytochemical from medicinal plants showing antimicrobial activities have the potential of filling this need, because their structures are different from those of the more studied microbial sources, and therefore their mode of action are also very likely to differ [7 ,8]. Screening the active compounds from the plants has led to discovery of new medicinal drugs which have efficient protection and treatment role against various diseases. In this respect, polyphenolic compounds, like flavonoids and phenolic acids, commonly found in plants have been reported to have multiple biological effects, including antioxidant activity [9, 10, 11, 12]. Thus interest in natural antioxidant, especially of plant origin, has greatly increased in the world. *Tribulus terrestris L*. is a well-known and widely distributed species of the genus *Tribulus.* It is known with several common names: puncture vine, caltrop, goat head, bull's head, ground burr nut, devil's thorn [13] and Arabic names: Al-Gutub, Qutiba, Hasak or Ders El-Agouz [14].
The fruits are commonly used in traditional medicine as a diuretic and in treatment of hypertension, and hypercholesterolemia [14, 15, 16]. It is popularly claimed to improve sexual function possibly through increase in the free serum testosterone [17] and augmentation of nicotinamide adenine dinucleotide phosphate–diaphorase activity and androgen receptor expression [18]. Anthelmintic [19], antifungal [20, 21] and antibacterial [22] activities have also been

reported in T. terrestris extracts. Steroidal saponins from T. terrestris have been reported to possess anticancer activity [20, 23] and to protect hepatocytes from cell death [24].

As a part of our ongoing investigations on natural antimicrobial and antioxidants, the present study was designed to investigate the total content and HPLC profiles of phenolic and flavonoids of the T. terrestris fruits .As well as, studying the antioxidant and antimicrobial activities of different fruit extracts and isolated pure compounds.

2. Material and methods

2.1. General experiment procedures:

UV measurements were obtained on Shimadzu UV-1800 UV/ VIS spectrophotometer. ^{1}D NMR (^{1}H,^{13}C, DEPT-135) spectra was recorded in CD3OD using the residual solvents as an internal standard at 400 MHz for ^{1}H and 100 MHZ ^{13}C Bruker 400 instrument at 25 ^{0}C. Analytical TLC was performed on Merk Kieselgel 60 F_{254} plates with 0.25 mm layer thickness. Spots were visualized by UV light then after spraying with anisaldehyde/H_2SO_4 and aluminum chloride reagents.

Column chromatography (CC) was performed on Merck Kieselgel 60 (0.063-0.20 mm). Sephadex - LH 20 (Pharmacia, Uppsala, Sweden) and on Polyamide powder S for CC (Riedel –De Haen AG, Seezle –Hannover, Germany.

Analytical grade solvents used in this work; hexane (60-80 ^{0}C), chloroform, ethyl acetate, n-butanol, methanol, ethanol, 2, 2 –Diphenyl-1-picryl hydrazyl (DPPH) and ascorbic acid purchased from sigma - Aldrich (St. Louis, Mo, USA).

2.2. Plant material

T. terrestris fruits were obtained from commercial stores of medicinal plants (Harraz), Cairo, Egypt. The fruits were botanically authenticated by Dr. Abdelhalim Mohamed, Flora and Phytotaxonomy Department, Agricultural Research Center, Giza, Egypt. Herbarium specimen was deposited at the Department of Pharmacognosy, Faculty of Pharmacy, Beni-Suef University, Egypt.

2.3. Samples preparation

2.3.1. Extraction

The fruits of *T. terrestris* (5 kg) were finely powdered and extracted by cold maceration with 70% ethanol till exhaustion (7 × 2 L). The ethanol extract was evaporated under reduced pressure at 40 ^{0}C till dryness to yield semisolid residue (500 g). About 450 g of the residue were suspended in 400 ml distilled water and partitioned successively with n-hexane (6 × 500 ml), chloroform (3 × 500 ml), ethyl acetate (5 × 500 ml) and n-butanol saturated with water (8 × 500 ml). Solvent of different extractives were evaporated separately under reduced pressure to yield 40, 10, 15 and 40 g residue for n-hexane, $CHCL_3$, ethyl acetate and butanol extracts, respectively.

2.3.2. For spectrophotometric analysis

The powdered of the fruits of *T. terrestris* (5g) was deffated with 15ml petroleum ether, dried and extracted by stirring at 130rpm with 100ml of ethanol, at room temperature for 24h, then filtered on cellulose paper [25] (soares *et al.* 2009). The filtrate was dried, reconstituted with ethanol and stored at 4 ^{0}C until use.

2.3.3. For HPLC –DAD analysis

The total ethanolic extract (0.02g) was dissolved in 3ml methanol and filtered through a 0.2 µm millipore membrane filter before injection.

2.4. Determination of total phenolics

The method adopted was based on measuring the intensity of the color developed through complexing of total alcoholic extract with Folin-Ciocalteu's phenol reagent and the concentration was calculated as gallic acid equivalent with reference to a pre-established standard calibration curve [26].

2.5. *Determination of total flavonoids*

The method adopted was based on measuring the intensity of the color developed using UV-visible spectrophotometer, when flavonoids were complexed with aluminium chloride and the percentage was calculated as rutin with reference to pre-established standard calibration curves [27].

2.6. HPLC-DAD analysis for flavonoids and phenolic acids

The analysis for phenolic compounds was performed on the Agilent 1200 series rapid resolution liquid chromatograph (Agilent Technologies, Santa Clara, CA) consisting of a vacuum degasser, anauto-sampler, a binary pump and diode-array detection (DAD) sys-tem. Data analysis was performed with Agilent HPLC Chem-Station software. This instrument was equipped with a Phenomenex Prodigy 5 ODS-2 column (4.60 mm × 250 mm, 5 micron, CA, USA). Samples were centrifuged at 2655 × g for 10 min prior to HPLC injection. Simultaneous monitoring was performed for determination at 254, 280, 300, 520, and 640 nm; and spectral data were collected from 200 to 700 nm. The column temperature was setat 30 °C, and the injection volume was 20 µL, with a flow rate of 0.5 mL/min. Acidified water (6% acetic acid, v/v) and acetonitrile were used as mobile phases A and B, respectively [28]. The gradient elution was programmed as follows: 0–40 min, 0–25% B; 40–80 min, 25–85% B; 80–90 min, 85–100% B; 90–95 min, 100% B. All identified phenolic compounds (phenolic acids and flavonoids) were quantified with external standards, using calibration curves. The retention time and peak area were identified and used to calculate the phenolics and flavonoids concentrations by the data analysis of Agilent 1200 series software.

2.7. Free radical scavenging activity (DPPH assay)

The scavenging activity of *T. terrestris* total alcoholic fruit extract, different solvent extractives and isolated compounds against 2, 2-diphenyl-1-picrylhydrazyl radical (DPPH) were investigated spectrophotometrically as described by [29]. Briefly, 500 µL of total alcoholic extract, different fractions, α-Tocopherol and 25 µL of isolated compounds and ascorbic acid were separately mixed with 2 ml of DPPH solution (0.02 mg/ml). The mixtures were kept in the dark for 15 min at room temperature and then the absorbance was measured at λ517 nm. The percentage of absorbance inhibition at λ 517 nm was calculated using the following equation:

$$A\% = 100 - [(A_{sample} - A_{blank}) \times 100) / A_{control}]$$

All tests were performed in triplicate and the graph was plotted with the average of the three determinations ± SE. IC_{50}, concentration of the tested sample in µg/ml that scavenged 50% of free radicals, was calculated from the concentration response curve**.**

2.8. *In vitro* antimicrobial activity

2.8.1. Test microorganisms:

Eight bacterial strains and two fungal strains were used in the present study. The bacteria used were *Staphylococcus aureus* (RCMB 010027), *Staphylococcus epidermidis* (RCMB 010024), *Streptococcus pyogenes* (RCMB 010015) as Gram-

positive and Gram-negative *Neisseria gonorrhoeae* (RCMB 010076), *Proteous vulgaris* (RCMB 010085), *Klebsiella pneumoniae* (RCMB 0010093), *Pseudomonas aeruginosa* (RCMB 010043) and *Escherichia coli* (RCMB 010056). The fungal strains used were *Aspergillus fumigates* (RCMB 02564) and *Candida albicans* (RCMB 05035). All of micro-organisms were obtained from the Regional Center of Mycology and Biotechnology Antimicrobial Unite (RCMB), Cairo, Egypt.

2.8.2. Culture media and antibiotics for antimicrobial assay

Brain Heart Infusion as liquid and solid media (HiMedia), Mueller- Hinton agar (HiMedia) and Potato Dextrose Agar medium (HiMedia) were used. Ampicillin (Oxoid, UK), Gentamycin (Oxoid, UK) were used as standard antibacterial agents and Amphotericin B (Sigma Chemical Co., St. Louis, Mo.) was used as a standard antifungal agent.

2.8.3. Determination of the antimicrobial activity

The antimicrobial activity of total alcoholic extract, different fractions and isolated compounds of *T. terrestris* fruits was screened by agar disc diffusion method with slight modification [30, 31]. The bacterial cultures were grown in Brain Heart Infusion broth at 37 ^{0}C. After 6 h of growth, 100 µl of each microorganism at concentration of 1 x 10^6 cells/ml was inoculated on the surface of Muller–Hinton agar plates for bacteria and Potato Dextrose agar plates for fungi. DMSO with concentration up to 2% was used to dissolve the samples. Filter paper discs (6 mm in diameter) saturated with 20 µl of the tested samples were placed on the surface of the inoculated plates. The plates were incubated at 37 ^{0}C for 24 h. The diameter of the inhibition zone was measured in millimeter, and was recorded as mean ± SD of a triplicate experiment. Ampicillin (10 µg), gentamicin (10 µg) discs for bacteria, and amphotericin B (5 µg) for fungi were used as positive standards. All the results are shown in table (6 and 7).

2.9. Isolation of pure compounds

Phytochemical screening revealed that butanol and ethyl acetate soluble fractions are rich in flavonoids. The butanol soluble fraction (40 g) was chromatographed over 250 g polyamide column (150 x 5 cm). Gradient elution was carried out with 100% water then with water containing 5% methanol till 100% methanol. Fractions 250 ml were collected and TLC monitored using solvent system chloroform: methanol: water (15:6:1). The spots were visualized by UV light, $AlCL_3$ and after spraying with anisaldehyde/ H_2SO_4 spray reagents. Similar fractions were pooled together, evaporated to dryness under vacuum at 40 ^{0}C, whereby 13 fractions (F1-F13) were obtained. Flavonoids were mainly eluted in fraction 9 (1.3g) eluted with 50% methanol. This fraction was rechromatographed on 100 g VLCRP C_{18}-column (100 x 3) using Methanol: Water (60:40) as a mobile phase for elution. Fractions of 5 ml were collected, analyzed by TLC using solvent system chloroform: methanol: water (15:6:1) and similarly visualized Similar fractions were polled together to give 15 fractions. Fraction 7 (40 mg) was subjected to sephadex column C (SPLH 20) using methanol: water (8:2) as a mobile phase and 1 ml fractions were collected to give 50 fractions. After TLC monitoring as mentioned above. Similar fractions (16-25) and (34-40) were collected to give compound A1 (15 mg) and compound A2 (8 mg), respectively.

Ethyl acetate fraction (15 g) was chromatographed on 250 g polyamide column (150 x 5 cm). Gradient elution was performed starting with water with gradual increase of MeOH (5% methanol till 100%). Fractions of 200 ml were collected and monitored by TLC using different solvent systems, similar fractions were pooled together to yield 8 fractions (F1-F8), evaporated under reduced pressure, weighed and kept in desiccator for further chromatographic separation to isolate the individual compounds. Chemical screening revealed that fraction 5 (0.5 g) eluted by 70% methanol was rich in flavonoids. It was chromatographed on 50 g silica gel column (50 x 2 cm) using methylene chloride with gradual increase of methanol (1% methanol till 20%). Fractions of 10 ml were collected, analyzed by TLC using solvent system chloroform: methanol (9:1, 8:2 and 7:3) visualized as previously mentioned. Similar fractions were pooled together to give 9 fractions. Fraction 3 eluted by methylene chloride: methanol (97:3) showed one major spot, on concentration, pure compound was precipitated A3 (10mg).

3. Results and discussion

Three flavonoids were isolated from the fruit of *T. terrestris* fig (1). The qualitative chemical tests on A_1, A_2 and A_3 not only proved their phenolic nature as evident upon spraying with $FeCl_3$ spray reagent. Upon acid hydrolysis of A_1 and A_2 followed by comparison with authentic sugar samples indicated the presence of glucose and rhamnose.

3.1. Compounds characterization

3.1.1. Querctin-3-O-α-L-rhamnopyranosyl (1```-6``)–O-β-D-glucopyranosoide (A_1). Yellow amorphous powder (15 mg). Melting point (m.p); 182-185 ^{O}C. UV (λ max nm) MeOH: 258, 266 sh, 299 sh, 358. HRESI/MS $[M+H]^+$ in positive ionization mode 611.1627 calculated for $C_{27}H_{30}O_{16}$. ^{1}H-NMR (400 MHZ, CD3OD δ PPM) 6.22 (1H,d, *J = 2.01* Hz, H-6), 6.41 (1H, d, *J = 2.01* Hz, H-8), 7.69 (1H, d, *J = 2.0* Hz, H-2`) 7.63 (1H, *dd, J = 8.0 Hz, H-6*`) 6.88 (1H, *d, J = 8.0,H-5`)* 5.12 (glc.H-1``, 1H, *d, J = 7.5* Hz) 4.55 (Rha.H-1```, 1H, *d, J = 1.5* Hz) 3.07-3.66 (rha-glc, 10 H, m), 1.13 (rha, CH3, *3H, d, J = 6.3 Hz).*

^{13}C-NMR (100 MHz, CD3OD, ppm) 157.09 (C2), 134.21 (C3), 178.0 (C4), 157.92 (C5), 98.53 (C6), 164.61(C7), 93.45 (C8), 161.57 (C9), 104.20 (C10), 121.69 (C1`), 114.63 (C2`), 144.0 (C3), 148.40 (C4`), 116.27 (C5`), 122.14 (C6`). Glu, 101.1 (C_1``) 74.31 (C2``), 76.67 (C3``), 72.51 (C4``), 75.59 (C5``), 67.13 (C6``). Rha, 103.31 (C1```), 69.97 (C2```), 70.69 (C3```), 70.81 (C4```), 68.30 (C5```), 16.48 (C6```) (CH3) all data presented in table **(1).** From these results, compound A_1 was assumed to be Quercetin-3-O-rutinoside **(**Rutin**)** and it was confirmed by comparison of its spectral data with literature values [32, 33].

3.1.2.Isorhamentin-3-O-α-L-rhamnopyranosyl(1```-6``)–O-β-D-glucopyranosoide *(A2).* Yellow amorphous powder, 10 mg, melting point (m.p); 182-184 ^{o}C. UV (λ max nm) MeOH: 258, 349 nm. HRESI/MS $[M+H]^+$ in positive ionization mode 625.1786 calculated for $C_{28}H_{32}O_{16}$.

^{1}H-NMR (400 MHZ, CD3OD, δ PPM): **6.21**(1H, *d, J = 2.01* Hz*, H-6*), 6.40 (1H, *d, J = 2.01* Hz*, H-8)*, 6.92 (1H, *d*, *J* = 8 Hz, H-5`), 7.65 (1H, *dd*, *J*1 = 8 Hz, *J*2 = 2 Hz, H-6`), 7.94 (1H, d, *J* = 2 Hz, H-2`), 1.11 (3H, *d*, *J* = 6 Hz, Me-6```) 3.20–3.90 (~14H, *m*, sugar protons), 3.96 (3H, s, OCH3 at C-3`), 4.55 (1H, *d*, *J* = 1.5 Hz, H-1```), 5.22 (1H, *d*, *J* = 8 Hz, H-1``);

^{13}C-NMR (100MHz, CD3OD), δ [ppm]: 157.58 (C-2), 134.03 (C-3), 177.84 (C-4), 161.58 (C-5), 98.75 (C-6), 165.24

(C-7), 93.64 (C-8), 157.12 (C-9), 104.12 (C-10), 121.57 (C-1`), 114.69 (C-2`),149.46 (C-3`), 146.40 (C-4`), 113.11 (C-5`), 122.56 (C-6`), 55.34(OCH3 at C-3`), 101.11 (C-1``), 74.50 (C-2``), 76.67 (C-3``), 72.42 (C-4``), 75.94 (C-5``), 67.11(C-6``), 103.06 (C-1```), 70.20 (C-2```), 70.66 (C-3```), 70.85 (C-4```), 68.88 (C-5```), 16.48 (C-6```). All data presented in table (2). From these results, compound A_2 was assumed to be isorhamentin -3-o-rutinoside and it was confirmed by the comparison of its spectral data with the literature values [34, 33].

3.1.3 Quercetin (A3). Yellow microcrystals, 8mg, melting point (m.p):314-316°C. UV (λ max nm) MeOH; 256, 301 sh., 370. EI/MS $[M-H]^-$ in negative ionization mode 301.2 calculated for $C_{15}H_{10}O_6$. ^{1}H-NMR (400 MHZ, CD3OD δ PPM) 6.19 (1H, *d, J = 2.0 Hz*, H-6), 6.40 (1H, *d, J = 2.0 Hz*, H-8), 7.75 (1H, *d, J = 2.0* Hz, H-2`), 6.89 (1H, *d, J = 8.0 Hz*, H-5`), 7.64 (1H, *dd, J = 8.0 & 2.0* Hz, H-6`). $^{13}$C-NMR (100 MHz, CD3OD, δ ppm) 147.9 (C2), 136.8 (C3), 177.0 (C4), 158.3 (C5), 99.1 (C6), 164.8 (C7), 93.9 (C8), 162.0 (C9), 104.0 (C10), 124.5 (C1`), 116.0 (C2`), 146.5 (C3`), 149.8 (C4`), 116.4 (C5`), 121.0 (C6`), and all data presented in table (3). Finally the spectral data ^{1}H and ^{13}C-NMR showed basically agreement with the literature values [35, 36, 31].

Table 1: ^{1}H and ^{13} C-NMR spectral data of compound A1 (400 and 100 MHz, CD_3OD).

C-no	Multiplicity (DEPT)	^{13}C-NMR (δ)	^{1}H-NMR (δ)	J_{HH} (HZ)
2	C	157.09	-	
3	C	134.21	-	
4	C	178.00	-	
5	C	157.92	-	
6	CH	98.53	6.19 (d)	*J* = 2.0
7	C	164.61	-	
8	CH	93.45	6.38 (d)	*J* = 2.0
9	C	161.57	-	
10	C	104.20	-	
1`	C	121.69	-	
2`	CH	114.36	7.66 (d)	*J* = 2.0
3`	C	144.43	-	
4`	C	148.40	-	
5`	CH	116.27	6.88 (d)	*J* = 8.0
6`	CH	122.14	7.62 (dd)	*J* = 8.0, 2.0
Glucose				
1``	CH	101.01	5.12 (d)	*J* = 8.0
2``	CH	74.31	3.51 (m)	
3``	CH	76.67	3.51 (m)	
4``	CH	72.51	3.63 (m)	
5``	CH	75.79	3.51 (m)	
6``	CH_2	67.13	3.79 (d)	*J* = 10.5
Rhamnose				
1```	CH	103.31	4.53 (d)	*J* = 2.0
2```	CH	69.97	3.42 (m)	
3```	CH	70.69	3.53 (m)	
4```	CH	70.81	3.81 (m)	
5```	CH	68.30	3.42 (m)	
6```	CH_3	16.48	1.12 (d)	*J* = 6.5

Table 2: ^{1}H and ^{13} C-NMR spectral data of compound A2 (400 and 100 MHz, CD_3OD).

C-no	Multiplicity (DEPT)	^{13}C-NMR (δ)	^{1}H-NMR (δ)	J_{HH} (HZ)
2	C	157.09	-	
3	C	134.21	-	
4	C	178.00	-	
5	C	157.92	-	
6	CH	98.53	6.19 (d)	*J* = 2.0
7	C	164.61	-	
8	CH	93.45	6.38 (d)	*J* = 2.0
9	C	161.57	-	
10	C	104.20	-	
1`	C	121.69	-	
2`	CH	114.36	7.66 (d)	*J* = 2.0
3`	C	144.43	-	
4`	C	148.40	-	
5`	CH	116.27	6.88 (d)	*J* = 8.0
6`	CH	122.14	7.62 (dd)	*J* = 8.0, 2.0
Glucose				
1``	CH	101.01	5.12 (d)	*J* = 8.0
2``	CH	74.31	3.51 (m)	
3``	CH	76.67	3.51 (m)	
4``	CH	72.51	3.63 (m)	
5``	CH	75.79	3.51 (m)	
6``	CH_2	67.13	3.79 (d)	*J* = 10.5
Rhamnose				
1```	CH	103.31	4.53 (d)	*J* = 2.0
2```	CH	69.97	3.42 (m)	
3```	CH	70.69	3.53 (m)	
4```	CH	70.81	3.81 (m)	
5```	CH	68.30	3.42 (m)	
6```	CH_3	16.48	1.12 (d)	*J* = 6.5

Table 3: ^{1}H and ^{13} C-NMR spectral data of compound A3 (400 and 100 MHz, CD_3OD).

C-no	^{13}C-NMR(δ)	^{1}H-NMR(δ)	J_{HH} (HZ)
2	147.9	-	
3	136.8	-	
4	177.0	-	
5	158.3	-	
6	99.1	6.19	d, *J* = 2.0
7	164.8	-	
8	93.9	6.40	d, *J* = 2.0
9	162.0	-	
10	104.0	-	
1`	124.5	-	
2`	116.0	7.75	d, *J* = 1.8
3`	146.5	-	
4`	149.8	-	
5`	116.4	6.89	d, *J* = 7.8
6`	121.0	7.64	dd, *J* = 7.8,1.8

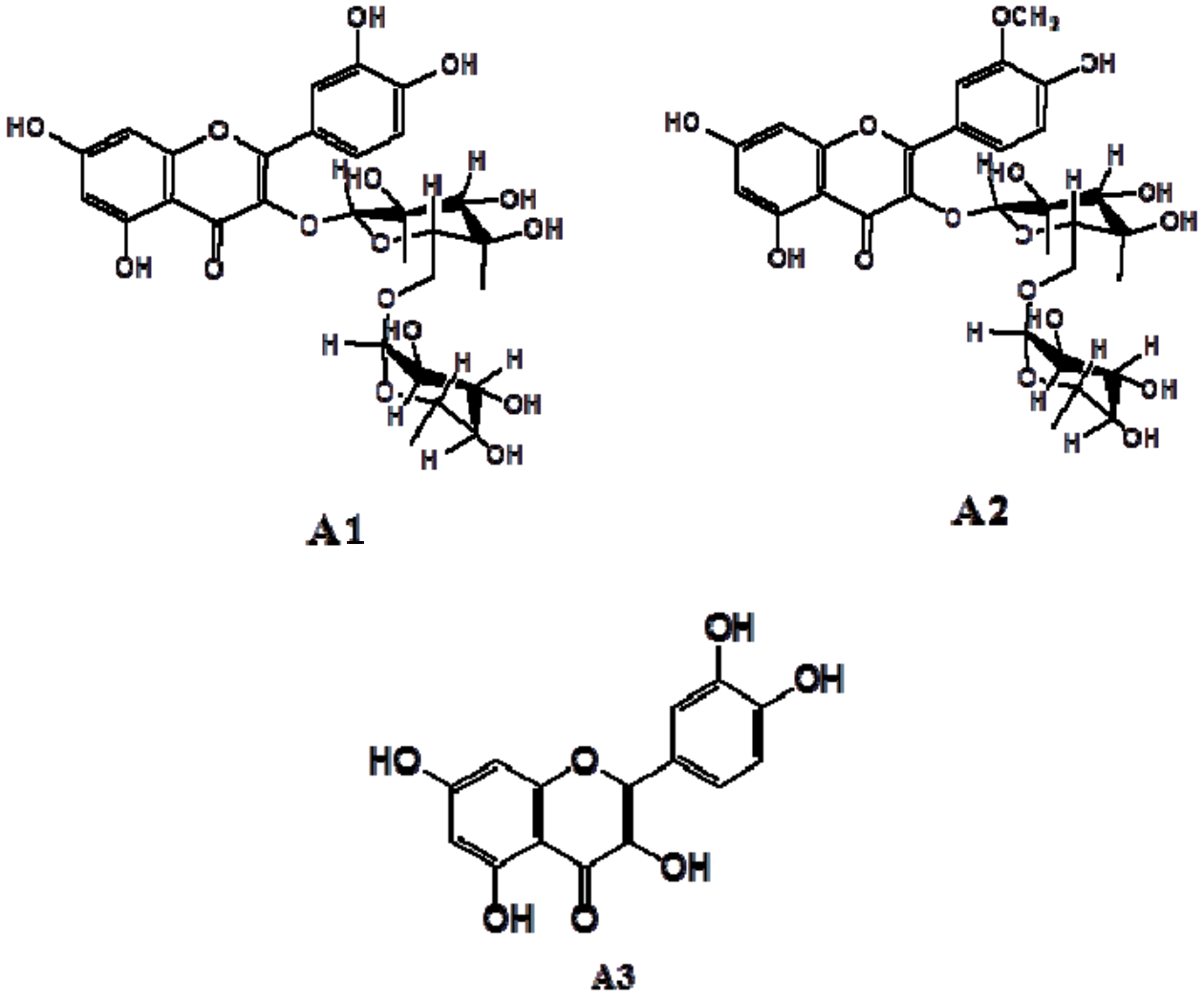

Fig 1: Chemical structures of compounds 1-3

3.2. Quantitative determination of total flavonoids and phenolic content of the fruit of T. terrestris using colorimetric assay.

Phenolic and flavonoid compounds are the major active ingredients in plants. Both of these classes exhibited good antioxidant potential. In this study, the total phenolic and flavonoid contents of the fruits of *T. terrestris* were determined using spectrophotometric assay. Further analysis of the studied alcoholic extract using RP-HPLC, demonstrated the presence of different phenolics and flavonoids which are known for their antioxidant potential [37].

The data showed that *T. terrestris* alcoholic extract has high phenolic and flavonoid contents which attributed to their antioxidant and antimicrobial activities. Moreover, the amount of total flavonoids estimated as rutin equivalent was 140 ± 0.576 mg/g of the dried powdered fruit. The content of total phenolic was estimated in the fruits as gallic acid was 250 ± 1.17 mg/g of the dried powdered fruit. The analysis of the correlation between the total phenolic and flavonoids compounds and antioxidant activities confirmed the importance of flavonoids content on the rate of antioxidant activity.

3.3. Qualitative and quantitative determination of flavonoids and phenolic acids of the fruit of T. terrestris L using HPLC –DAD analysis

According to RP-HPLC analysis of phenolics and flavonoids profiles of *T. terrestris* fruits. Thirty one phenolic compounds were identified and their relative % and concentration in the fruits were presented in tables (4 and 5), involved 9 flavonoids (hesperidin, rutin, quercitrin, quercetin, naringin, naringenin, hesperitin, apigenin and kaempferol) and 22 phenolic acids (pyrogallic, gallic, p-hydroxy benzoic, protocatechuic, catechol, caffeic, vanillic, syringic, chlorogenic, ferulic, p-coumaric, ellagic, coumarin salicylic, cinnamic, 3,4,5 methoxy cinnamic, 4-amino benzoic acid, catechin, epicatechin, iso-ferulic, resveratrol and coumaric). On the other hand, the percentage of total identified flavonoids was 11.629 mg/100 gm of dried ethanol extract. Naringin and hesperidin were the major flavonoids identified with concentration; 4663.88 and 2113.61 mg/100g respectively, of the dried ethanol extract. The percentage of total identified phenolic acids was 5.641 mg/100 gm of dried ethanol extract. Syringic acid and Salicylic acid were the major phenolic acids detected in concentration 656.443 and 656.443 mg /100 g of dried ethanol extract.

Table 4: Results of determination of flavonoids in the fruit of *T. terrestris L* using HLPC- DAD analysis.

No	Flavonoid	Relative %	Concentration of flavonoids (mg/100 gm dried extract)
1	Naringin	7.7462	4663.88
2	Narenginin	2.0337	1470.40
3	Rutin	3.4256	1090.25
4	Hesperidin	2.5322	2113.61
5	Hespertin	0.2174	206.48
6	Quercetrin	5.5840	1177.71
7	Quercetin	0.3347	255.34
8	kaempferol	0.4885	384.87
9	Apigenin	0.5624	247.19
Total identified flavonoids 11629.73 Percentage of total identified flavonoids 11.629			

Table 5: Results of determination of phenolic acids in the fruit of *Tribulus terrestris L.* using HPLC-DAD analysis.

No	Phenolic compounds	Relative %	Concentration of phenolic compounds (mg/100 g dried extract)
1	Syringic acid	0.2333	656.443
2	Gallic	0.1996	130.552
3	Pyrogallol	0.0831	203.625
4	4-amino-benzoic acid	0.6035	66.41
5	Protcatechuic acid	0. 4043	101.897
6	Catechin	0.1850	101.155
7	Chlorogenic	0.06343	118.615
8	Catechol	1.2940	443.72
9	Epicatechin	0.8526	246.16
10	P-hydroxy benzoic acid	0.9284	205.33
11	Caffeic	0.2310	24.93
12	Vanillic	1.1189	25.76
13	Ferulic	1.2077	86.97
14	Iso-ferulic	3.9815	367.19
15	Resveratrol	1.5799	37.63
16	Ellagic	0.7609	128.13
17	Coumaric	3.7176	83.89
18	Salicylic	2.1091	625.71
19	3,4,5 methoxy cinnamic	0.2957	14.02
20	Coumarin	0.2684	25.99
21	P-coumaric acid	0.5197	62.88
22	Cinnamic	0.5789	32.17
Total identified phenolics 5641.35 Percentage of total identifiedphenolics 5.641			

3.4. In vitro DPPH scavenging activity

In traditional societies nutrition and health care are strongly interconnected and many plants have been consumed both as food and for medicinal purposes. The consumption of wild medicinal plants play a central role in the diet, but very few ethnopharmacological and phytopharmacological studies have dealt exhaustively with the potential health benefits of such diets.

Oxidative stress is a major predisposing risk factor for many chronic diseases as well as male infertility problem [38, 39]. In the past few years, there has been growing interest in the involvement of reactive oxygen species (ROS) in several pathological situations. ROS produced in vivo include superoxide radical (O_2.•-), hydrogen peroxide(H_2O_2) and hypochlorous acid (HOCl). H2O2 and O_2.•-can interact in the presence of certain transition metal ions to yield a highly-reactive oxidizing species, the hydroxyl radical (•OH) [40] Phenolic compounds and flavonoids have been reported to be associated with antioxidative action in biological systems, acting as scavengers of singlet oxygen and free radicals [41, 42].

To assess the potential antioxidant activity of *T. terrestris L* fruit, total ethanolic extract and its different solvent fractions of the plant were screened for their *in vitro* DPPH free radical scavenging activity. The obtained results (fig) revealed that the total alcoholic, n –butanol and ethyl acetate have Potent free radical scavenging (0.064 ± 0.002, 0.240 ± 0.056 and 0.280 ± 0.004 mg/ml), respectively with a higher extent than other fractions compared to α-Tochopherol as a reference standard (0.041 ± 0.003 mg/ml). Consequently, the DPPH free radical scavenging activity of flavonoidal compounds isolated from butanol and ethyl acetate fractions of *the* fruit was evaluated to identify the most active constituents responsible for the antioxidant activity. The obtained result revealed that compound A_1 and A_3 had the strongest free radical activity. Moreover, IC_{50} of compound A_1 (12.1 ± 1.13**)** and A_3 (11.2 ± 0.81) close to ascorbic acid (10.2 ± 0.93), proving their potent antioxidant effect. Finally, the antioxidant activity of *T. terrestris* could be attributed to its flavonoidal content. Flavonoids act as scavengers of various oxidizing species i.e. super oxide anion, hydroxyl radical or peroxy radicals, they also act as quenchers of singlet oxygen [43].

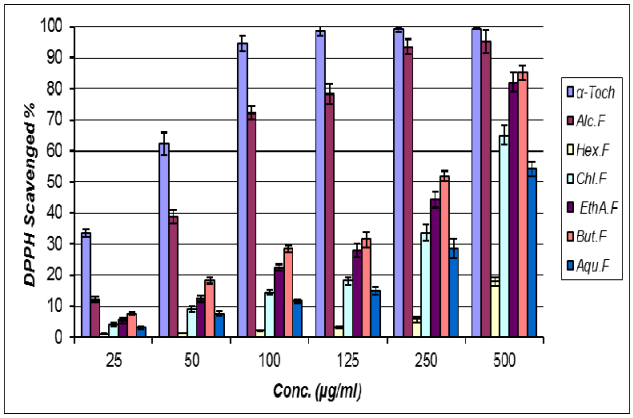

Fig (2): The effect of total alcoholic extract, other different fractions of *T. terrestris* fruit and α-tochopherol as a reference drug on DPPH scavenged %. α –Toch: Alpha tochopherol, MTT: Total alcoholic extract, Hex.fr: Hexane fraction, Chl.fr: Chloroform fraction, EtoAc.fr: Ethyl acetate fraction, But.fr: Butanol fraction, Aque.fr: Aqueous fraction.

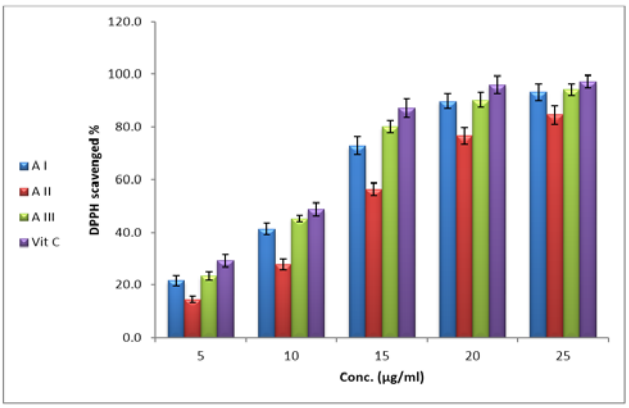

Fig 3: The effect of isolated compounds from *T. terrestris* fruit and vitamin C as reference drug on DPPH free radical scavenged %. A1: rutin, A2: Isorhamentin rutinoside and A3: Quercetin

3.5. *In-vitro* antimicrobial activity

The antimicrobial activity of total alcoholic extract, other different solvent fractions and isolated compounds were evaluated by measuring the diameter of inhibition zone for all samples at volume 20 µl per disc and the results are reported in table ( ).

There was a difference in the inhibition of growth among the compounds against different strains of micro-organisms. The isolated compounds, total ethanolic extract and different fractions showed antibacterial, but no antifungal activities. The butanol and ethyl acetate fractions showed the highest inhibitory effect against E.coli (16 ± 0.51 & 15 ± 0.21 mm) and K. pneumonia (17 ± 0.34 & 12 ± 0.53 mm), respectively.

The antimicrobial activity of the isolated flavonoids showed high degree of antimicrobial activity against gram positive and gram negative bacteria. The results obtained in table ( ) showed that quercetin, has the strongest antibacterial activity. It was found that quercetin has the strongest antimicrobial activity against *S. epidermidis*, *N. gonorrhoeae*, *P. vulgaris and E. coli* with a mean zone of inhibition diameter of 18 ± 0.19, 15.5 ± 0.51, 11.9 ± 0.33 and 20.2 ± 0.33 mm, respectively, while rutin and isorhamentin rutinoside showed no activity against all four previous mentioned organisms, but isorhamentin rutinoside has moderate antimicrobial activity against *K. pneumoniae and P. aeruginosa* with a mean zone of inhibition diameter 18.6 ± 0.33 and 16.5 ± 0.35, respectively.

Table 6: antibacterial and antifungal activity of total alcoholic extract and different fractions of *T. terrestris* fruit.

Tested microorganism	Total alcoholic	Hexane	Chloroform	Ethyl acetate	Butanol	standard
Inhibition zone diameter (mm)						
Fungi						Amphotericin B
A. fumigatus (RCMB 02564)	NA	NA	NA	NA	NA	28.6 ± 0.14
C. albicans (RCMB 05035)	NA	NA	NA	NA	NA	21.8 ± 0.12
Gram positive						Ampicillin
S. aureus (RCMB 010027)	16 ± 0.12	10 ± 0.35	11 ± 0.24	14 ± 0.12	14 ± 0.12	28.6 ± 0.14
S.epidermidis (RCMB 010024)	14 ± 0.16	10 ± 0.33	9 ± 0.62	12 ± 0.19	13 ± 0.42	25.2 ± 0.18
S. pyogenes (RCMB010015)	15 ± 0.17	9 ± 0.55	11 ± 0.28	13 ± 0.51	14 ± 0.12	26.4 ± 0.34
Gram negative						Gentamycin
N.gonorrhoeae (RCMB 010076)	17 ± 0.34	10 ± 0.22	13 ± 0.14	10 ± 0.20	14 ± 0.17	19.4 ± 0.18
P. vulgaris (RCMB 010085)	11.9 ± 0.33	NA	NA	9 ± 0.22	10 ± 0.42	23.4 ± 0.30
K.pneumonia (RCMB 0010093)	18 ± 0.32	NA	NA	12 ± 0.53	17 ± 0.34	26.2 ± 0.15
P. aeruginosa (RCMB 010034)	18 ± 0.33	10 ± 0.29	11 ± 0.42	14 ± 0.21	15 ± 0.21	17.3 ± 0.12
E. coli (*RCMB 010056)*	20 ± 0.16	8 ± 45	9 ± 0.17	15 ± 0.21	16 ± 0.51	25.3 ± 0.18

The test was done using the agar disc diffusion technique, disc diameter: 6.0 mm, RCMB: Regional Center for Mycology and Biotechnology Antimicrobial unit test organisms. NA: No activity, data are expressed in the form of mean ± SD.

Table 7: Antibacterial and antifungal activity of flavonoid compounds isolated from *T. terrestris* fruit.

Tested microoganisms	Rutin	Isorhamentin rutinoside	Quercetin	Standard
Inhibition zone diameter (mm)				
Fungi				Amphotericin B
A. fumigatus	NA	NA	NA	24.6± 0.10
C. albicans	NA	NA	NA	21.8 ± 0.12
Gram positive				Ampicillin
S. aureus	14.2 ± 0.72	17.4 ± 0.19	22 ± 0.21	28.6 ± 0.14
S. epidermidis	NA	NA	18 ± 0.19	25.2 ± 0.18
S. pyogenes	12.3 ± 0.58	16.2 ± 0.63	20 ± 0.53	26.4 ± 0.34
Gram negative				Gentamycin
N. gonorrhoeae	NA	NA	15.5 ± 0.51	19.4 ± 0.18
P. vulgaris	NA	NA	11.9 ± 0.33	23.4 ± 0.30
K. pneumoniae	12.6 ± 0.21	18.6 ± 0.33	19.4 ± 0.39	26.2 ± 0.15
P. aeruginosa	14.8 ± 0.42	16.5 ± 0.35	18.6 ± 0.58	17.3 ± 0.12
E. coli	NA	15.3 ± 0.39	20.2 ± 0.33	25.3 ± 0.18

The test was done using the agar disc diffusion technique, disc diameter: 6.0 mm, RCMB: Regional Center for Mycology and Biotechnology Antimicrobial unit test organisms. NA: No activity, data are expressed in the form of mean ± SD.

Finally, the total alcoholic extract, different fractions and isolated compounds have activity against most reference bacteria, but their antibacterial activity was much lower than ampicillin and gentamicin. Similar results were also observed by Kianbakht and Jahanianiian (2003) who reported that the methanolic extracts of different plant organs (fruits, stems, leaves and roots) of *T. terrestris* growing in Iran inhibited the growth of *S. aureus*, *E. faecalis*, *E. coli* and *P. aeruginosa* and plant showed considerable activity against all bacteria. *In addition to*, one of the uses of *T. terrestris* is in urinary infections. Since the present study showed activity against the most prevalent Gram positive bacteria in urinary infections namely *E. coli*, the use of the plant as a urinary anti-infective is validated.

4. Conclusion

All isolated compounds and different fractions of *T. terrestris* fruits exhibited free radical scavenging antioxidant activity and strongly inhibited many of pathogenic microorganism such as *E. coli*, *K. pneumoniae and P. aeruginosa*, so this plant may represent a new natural sources of antioxidant and antimicrobial drugs that could be useful in the therapy of

pathologies. Moreover, the protective and antioxidant effects of *T. terrestris* ethanolic extract in sodium valproate induce testicular toxicity in rats can be attributed to its contents of flavonoids and other phenolics (Hifnawy, *et al.* under publication).

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