Assessing the antioxidant characteristics and polyphenol content of *Tribulus terrestris* microwave-assisted fruit extract

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ABSTRACT

Tribulus terrestris have been traditionally used as folk medicine owing to its rich source of polyphenolic compounds. The aim of the present study was to assess the antioxidant potential by performing quantification of polyphenolic compounds present in *Tribulus terrestris* fruit through HPLC analysis. Various extracts of *Tribulus terrestris* fruit were prepared through microwave-assisted extraction (MAE) carried out at three different time intervals of 1, 3, and 5 minutes respectively. The extracts were then subjected to antioxidant characterization. The results showed maximum values of TPC, TFC, DPPH, FRAP, and ABTS assay for the extract prepared at 3 minutes as 54.86±0.51mg GA/g, 544.33±4.50mg QE/g, 87.67±0.57%, 1051.67±3.21mM Fe^{+2}/g, and 86.34±2.08% respectively. However, the minimum values of TPC, TFC, DPPH, FRAP, and ABTS were observed for the extract prepared at 1 minute heating time as 39.27±0.36mg GA/g, 282.66±3.51mg QE/g, 60.33±1.15%, 756.34±4.50mM Fe^{+2}/g, and 66.33±1.52% respectively. Quantification of major polyphenolic compounds in microwave-assisted *Tribulus Terrestris* fruit extract was performed through HPLC analysis. The results of the analysis detected twenty-four polyphenolic compounds. The maximum concentration was detected for naringin i.e., 4694.18 mg/100g of dried extract among flavonoids, while the maximum phenolic acid was found to be salicylic acid i.e., 728.41 mg/100g of dried extract. The present study concluded that *Tribulus terrestris fruit* is a good source of natural antioxidants and various polyphenols.

Keywords: *Tribulus terrestris*, natural antioxidants, microwave-assisted extraction.

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1. Introduction

Reactive oxygen species (ROS) are originated as a natural byproduct of ongoing biological processes by normal oxygen metabolism in the body. However, the levels of ROS increase dramatically during stress resulting in a situation known as "oxidative stress", which can lead to cell damage. The pathogenesis of various diseases such as ischemic heart disease, diabetes, atherosclerosis, liver injury, immunosuppression, cancer, inflammation, and other neurodegenerative diseases is stimulated by ROS. ROS-induced oxidation results in the disintegration of cell membranes & proteins, further mutations in DNA have also been reported that may be the reason for the development and progression of numerous ailments [1]. In biological systems, antioxidants provide protective effects against oxidative stress by the termination of ROS chain reactions through the removal of free radical intermediates and inhibition of other oxidation reactions by getting themselves oxidized. During the last three decades, many antioxidant-based drugs and formulations have been used for the prevention as well as treatment of various complex diseases such as cancer and Alzheimer's disease [2].

Numerous therapeutic agents have been isolated from natural sources [3]. Plants contain a wide variety of free radical scavenging molecules including various phenolic compounds (e.g. phenols, flavonoids, tannins, quinones, lignans, coumarins, stilbenes), terpenoids (e.g. carotenoids) nitrogen compounds (e.g. amines, alkaloids, betalains), vitamins and a few other endogenous metabolites having a strong antioxidant activity [4]. Identification of these bioactive compounds from plants has led to the discovery of many plant-based medicinal drugs which exhibit greater efficiency, and protection against many diseases. Moreover, the interest in plant-derived antioxidants has increased dramatically all around the globe because of their non-toxic effect on synthetic antioxidants such as butyl hydroxyanisole (BHA) and butylhydroxytoluene (BHT) and various other potential health benefits [5].

Tribulus terrestris L. is an annual or biennial, prostate herb. Its common names include Gokhru in Hindi, Khar-e-khasak in Urdu, and puncture vine, land (or small) caltrops in English. *Tribulus terrestris*is native to warm temperatures and is grown throughout the year. It is widely distributed in tropical and subtropical countries in Asia, North Australia, Africa, South Europe, China, and Japan [6,7]. It grows over a vast variety of soil types however, the growth of this plant is better in light textured soils. Usually, it is found in cultivated crops, roadsides, lawns, overgrazed pastures and neglected areas [8]. *Tribulus terrestris*is common throughout Pakistan from sea level to 3500m in sandy soils of barren lands and cultivated fields as a weed. *Tribulus terrestris*is well-known for its potential health benefits. It is used in various forms including direct usage as herb, the main component in the production of different medicines and food supplements, for example, physical rejuvenation, and therapy required for various conditions affecting the cardiovascular system, liver, kidney and immune system. The fruit is regarded as an aphrodisiac, diuretic and tonic. *Tribulus terrestris* has been reported to have various antioxidant compounds including phenols and flavonoids, which provide a wide range of benefits to human health. Antioxidant compounds and steroidal saponins isolated from *Tribulus terrestris* fruit possess cytotoxic and antitumor effects [9]. Other reported benefits include hypolipidemic, anti-diabetic, hepatoprotective, cardio-protective, diuretic, antiurolithic, anti-inflammatory, anti-bacterial, anti-fungal, aphrodisiac, antispasmodic, and immune-modulatory effects [10,11].

Keeping in view of the numerous health benefits of natural antioxidants, the present study was designed to explore the antioxidant capacity and polyphenolic content of *Tribulus terrestris* fruit

by performing various antioxidant tests including TPC (Total Phenolic Content), TFC (Total Flavonoid Content), DPPH (2,2-diphenyl-1-picrylhydrazyl), FRAP (Ferric Reducing Antioxidant Power), ABTS (2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) assay and HPLC analysis of the sample.

2. Materials and Method

2.1. Procurement of raw material:

Whole fruits of *Tribulus terrestris* were procured from the local market of Lahore. They were washed and air-dried at room temperature. In order to minimize the moisture content, they were dried in a hot air oven at 50^0C temperature. After the removal of moisture content, they were grinded to reduce particle size in order to facilitate the process of extraction.

2.2. Sample Preparation:

Microwave Assisted Extraction (MAE) technique was used to prepare the sample. The sample of *Tribulus terrestris* fruit was prepared in an adapted commercial kitchen microwave oven. The maximum output of the microwave oven was 700 W. For the preparation of microwave-assisted extract (MAE), 25g aliquot of *Tribulus terrestris* whole fruit powder was placed in a 250ml round bottom flask, 25ml distilled water was added in order to moisturize it for 30 minutes. The flask was connected to a Clevenger apparatus and heated at a power of 150W for different extraction times i.e., 1, 3, and 5 minutes respectively. N-Hexane was used to elute the volatile distillate and then dried by using anhydrous sodium sulfate. The n-hexane was then removed under vacuum conditions and extracts were refrigerated prior to the analysis [12].

2.3. Assessment of antioxidant characteristics

The microwave-assisted extracts collected after MAE at three various time intervals of *Tribulus terrestris* fruit were analyzed for their antioxidant potential by performing different tests including total phenolic content (TPC), total flavonoid contents (TFC), DPPH (1,1-diphenyl-2-picrylhydrazyl) assay, FRAP (Ferric Reducing Antioxidant Power) ABTS (2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) assay as discussed below:

2.3.1. Determination of Total Phenolic Contents (TPC):

Total phenolic contents of whole fruit of *Tribulus terrestris*microwave assisted extracts were calculated by using Folin-Ciocalteu reagent. The sample was weighed 50mg and then mixed with 0.5ml reagent and 7.5ml distilled water. The solution was left for few minutes and then mixed with 20% Na_2CO_3. The solution was heated at 40^oC in a water bath and then cooled down. Spectrophotometer was used to observe the level of absorbance at 765nm. Gallic acid curve was used to measure total phenolic contents present in the whole fruit of *Tribulus terrestris* and expressed as mg GAE/g of dry material [13].

2.3.2. Determination of Total Flavonoid Content (TFC):

Microwave-assisted extracts of whole fruit of *Tribulus terrestris* were analyzed for their flavonoid content. 10mg extract was taken into 250ml volumetric flask and 5ml of the ionized water was filled along 5% of 0.3ml sodium nitrite. $AlCl_3$ was added to it for a few minutes. 10ml ionized

water along with 1M NaOH was added into the solution. Spectrophotometer was then used to observe the level of absorbance at 510nm. The quercetin curve was used to measure total phenolic contents and expressed as mg QE/g of dry matter [14].

2.3.3. 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay:

The radical scavenging activity of microwave-assisted extracts from the whole fruit of *Tribulus terrestris* were assessed by using a standard DPPH solution prepared in n-hexane. 2ml solution of 25 µg/ml n-hexane was taken in a cuvette along with the addition of antioxidant solution. The spectrophotometer was used to calculate the absorbance of the control and extract sample at 517nm. The process was repeated thrice and the final value was evaluated by following the formula [15].

$$\% \text{ Inhibition} = [(A(c) – A(s)/A(c)] \times 100$$

Where,

Ac = absorbance of control;

As = absorbance of the sample

2.3.4. Ferric Reducing Antioxidant Power Assay (FRAP):

In order to analyze the reducing antioxidant activity, FRAP assay was used. 40mmol/L of Hydrochloric acid (HCL), 20mmol/L of 2.5ml ferric chloride, and 0.3ml/L of acetate buffer were added in the FRAP reagent having 10mmol/L of 2.5ml 2,4,6-Tripyridyl-S-triazine (TPTZ). The FRAP reagent was heated at 37^{o}C and mixed with 50µL of the prepared solution in tubes. The standard solution which was used in the process was ferric sulfate. The spectrophotometer was used to observe the level of absorbance at 593nm. The value of FRAP was expressed as equivalent to 1mmol/L of ferric sulfate [16].

2.3.5. ABTS (2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)) assay:

ABTS assay was carried out by following the methods described by the reported method [17]. ABTS was dissolved in double distilled water up to a concentration of 7.4mM, then potassium persulphate was added up to 2.6mM concentration into it. The working solution was prepared through the process of mixing equal quantities of the two stock solutions. These solutions were then allowed to react at room temperature in the dark for 12-16 hours. Afterward, the solution was diluted by 1ml of ABTS solution along with 60ml methanol. 150µL of *Tribulus terrestris* whole fruit extracts were left to react for two hours at room temperature with 2850µL freshly prepared solution of ABTS in the dark. The level of absorbance was recorded at 734nm through a spectrophotometer. ABTS scavenging activity (%) was calculated by following the formula [18].

$$\text{ABTS scavenging activity (\%)} = [(A_0\text{-}A_1)/A_0] \times 100$$

Where,

A_0 = ABTS absorbance of the control;

A_1 = ABTS absorbance in the presence of the sample.

2.4. HPLC analysis

2.4.1. Sample for HPLC analysis

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

2.4.2.HPLC-DAD analysis of the polyphenol compounds

For the determination of phenolic components in *Tribulus terrestris* fruit, a High-performance liquid chromatograph (Agilent-1260) attached with a DAD (Diode-array detection) system was used in this study. This HPLC-DAD was equipped with an auto-sampler, a pump, and a vacuum degasser. The samples were analyzed on reverse phase CortecsC18-column (2.7µm, 4.6× 150 mm). Before injection into the HPLC system, all the samples were centrifuged for 10 minutes at 2655×g. The spectral data was determined at 200-700 nm. The chromatographic conditions set were as: temperature of the column: 30^{o}C, flow rate: 0.5 mL min-1, and sample volume: 5 µL. Two mobiles phases were used *i.e.* A: H_3PO_4in double distilled water (0.1% v/v) and B: acetonitrile. For this experimentation, gradient elution was performed having details as: isocratic for 0–2 min; A (90%) & B (10%), gradient elution for 1-5 min; A (85%) & B(15%), 5-10 min; A (80%) & B (20%), hold for 10 minutes, 10-15 min; A (30%) & B (70%).Peak area and retention time were used for the identification and determination of flavonoids and phenolic components present in the sample [19].

2.5. Statistical Analysis

Data was analyzed statistically using Statistics (9.0) software. Results were expressed as mean ± S.D. ANOVA (analysis of variance) for completely randomized design (CRD) was performed and to assess the significance ($P \leq 0.05$) differences among means Tukey's test was applied.

3. RESULTS AND DISCUSSION

Antioxidant Characterization

The medicinal properties of *Tribulus terrestris* are mainly attributed to its bioactive components such as phenolic compounds, flavonoids and saponins content etc [20]. Phenolic compounds and flavonoids are the major active components of medicinal herbs and plants which play a major role in the determination of their antioxidant potential. The antioxidant characterization of *Tribulus Terrestris fruit* has been carried out in the present study and the results are presented in Table 1. The results of total phenolic contents (TPC) and total flavonoid content (TFC) obtained in the present study are presented as mg of gallic acid per gram (mg GA/g) and mg of quercetin per gram (mg QE/g) respectively.

Table 1. Anti-oxidant characterization of microwave-assisted extracts from *Tribulus terrestris* whole fruit powder obtained at various time intervals

Anti-oxidant characterization	**1 minute**	**3 minutes**	**5 minutes**
TPC (mg GA/g)	39.27±0.36C	54.86±0.51A	48.17±0.68B
TFC (mg QE/g)	282.66±3.51C	544.33±4.50A	406.67±3.05B
DPPH (% inhibition)	60.33±1.15C	87.67±0.57A	74.66±1.52B
FRAP (mM Fe^{+2}/g)	756.34±4.50C	1051.67±3.21A	884.66±3.51B
ABTS (% inhibition)	66.33±1.52C	86.34±2.08A	72.66±1.53B

The results presented in Table 1 clearly indicated that microwave-assisted extract of *Tribulus Terrestris fruit* elucidated at 3 minutes exhibited the maximum antioxidant activity among all of the three extracts. The data were expressed as mean ± standard deviation. The values of TPC, TFC, DPPH, FRAP and ABTS for extract prepared at 3 minutes were 54.86±0.51mg GA/g, 544.33±4.50mg QE/g, 87.67±0.57%, 1051.67±3.21mM Fe^{+2}/g and 86.34±2.08% respectively. However, the minimum values of TPC, TFC, DPPH, FRAP, and ABTS were observed for the extract prepared at 1 minute heating time as 39.27±0.36mg GA/g, 282.66±3.51mg QE/g, 60.33±1.15%, 756.34±4.50mM Fe^{+2}/g and 66.33±1.52% respectively. The results of the present study indicated that heating time had a significant effect on the antioxidant potential of the extract.

Earlier studies also provide evidence in accordance with the results of the current study. The values of TPC and DPPH for this study are comparable to the results reported [21]. They showed the values of TPC and DPPH from *Tribulus terrestris* collected from various regions in order to observe the effects of geographical and climate conditions on the chemical composition, antioxidant potential, and growth of the plant [22]. also reported the values of total phenolic compound (TPC) found in Tribulus terrestris, and the results of their research study are less as compared to the present study. They reported the value of TPC in *Tribulus terrestris* as 19.1±1.6 mg GA/g of water extract which is less than the reported result of the present study. Stefanescu et al. (2020) also reported a lesser value of TPC for the herbal drug made with *Tribulus terrestris* as compared to the results of the present study [23]. Ali et al. reported the value for total phenolic compounds (TPC) as 14.48 ± 0.16 mg GA/g of *Tribulus terrestris* extract [18]. Jain et al carried out a research study in order to observe the total phenolic content and antioxidant potential of various medicinal herbs including Tribulus terrestris. The results of their study showed less value for TPC (10.2±0.53mg GA/g)) and FRAP assay (560±2.80 mM Fe^{+2}) as compared to the present study however, the results of DPPH % inhibition (82.64±0.98) reported by the study are comparable to the values of the present study [24].

Patil et al. (2012) determined the total flavonoid content (TFC) of *Tribulus terrestris* extract in both ethanol and acetone [25]. The flavonoids of both the extracts were found to be in the range of 601.3 to 452.7 mg quercetin equivalent/g of dry sample by using the standard plot of quercetin. The results showed that the content of flavonoids in ethanol extract of *Tribulus terrestris*is higher

than that of the acetone extract. These values are comparable to the results of the present study which showed the maximum value of TFC as 544.33±4.50 mg QE/g and the minimum value as 282.66±3.51 mg QE/g for the extracts obtained at 3 minutes and 1 minute respectively. A comparative study was out by [26], in which they compared the antioxidant potential of three different plants including Tribulus terrestris, *Tribulus macropterus* and *Tribulus arabicus*. They used different parameters for the determination of antioxidant potential of the plants including DPPH, ABTS assay and β-carotene bleaching. The results of their study showed less value for DPPH % inhibition (40%) of Tribulus terrestris, however the values of ABTS assay

(79.2%) was comparable to the values presented in the present study. The difference between findings of the present study and the earlier researches may exist due to the use of different extraction techniques or methods used by various researchers.

HPLC Analysis

According to RP-HPLC analysis of *Tribulus terrestris* fruit, twenty-four compounds were identified and their relative percentage along with a concentration (mg/100g of dried extract) is presented in table 2.

Table 2. Classification and Concentration (mg/100g dried extract) of Polyphenol Compounds Identified in *Tribulus terrestris* fruit

Classification of compounds	**Compounds Concentrations of polyphenolic compounds (mg/100g dried extract)**	
Flavonoids	Naringin	4694.18
	Hesperidin	2124.43
	Narenginin	1398.56
	Quercetrin	1192.86
	Rutin	1099.92
	Kaempferol	388.42
	Apigenin	252.93
	Quercetin	249.55
Phenolic acids	Salicylic acid	728.41
	Iso-ferulic acid	389.34
	p-hydroxy benzoic acid	219.77
	Chlorogenic aicd	138.64
	Gallic aicd	137.88
	Catechol	136.98
	Ellagic acid	124.13
	Catechin	114.79
	Ferulic acid	95.24
	Coumaric acid	92.81
	p-Coumaric acid	58.31
	Resveratrol	42.17
	Cinnamic acid	39.28
	Vanillic acid	22.80
	Coumarin	21.72
	Caffeic acid	19.46

Among the twenty-four identified compounds, 8 were flavonoids (naringin, rutin, naringenin, hesperidin, apigenin, quercetin, kaempferol, quercetin) while the remaining 16 compounds were phenols (iso-ferulic acid, coumaric acid, catechin, resveratrol, ferulic acid, catechol, vanillic acid, ellagic acid, cinnamic acid, salicylic acid, p-coumaric acid, p-hydroxybenzoic acid, coumarin, vaffeic acid, gallic acid, chlorogenic acid). The compound which was detected in maximum concentration was naringin (4694.18 mg/100g dried extract) while caffeic acid (19.46 mg/100g dried extract) was detected in the minimum concentration. The total number of identified compounds was found to be lower than that detected by [6]. They performed the RP-HPLC analysis of *Tribulus terrestris* fruit and identified similar concentrations of certain phenolic compounds. These authors reported total 31 compounds *i.e.* phenolic acids: 22 & flavonoids: 9. The concentrations were also expressed in mg/100g of the dried ethanol extract. The results showed similar concentrations of certain compounds identified in the present study with a little variation. The present showed a slight increase in the concentration of most of the compounds except a few which showed less concentration.

Kulig et al performed a research study to determine the antioxidant potential of certain herbs including *T.terrestris* fruit, by the qualitative as well as a quantitative screening of polyphenols through HPLC analysis [27]. The results showed that the fruit of *Tribulus terrestris* possess the highest polyphenols content among all the analyzed herbs. The number of identified compound are less than the present study. The results showed the detection of total 10 polyphenol compounds among which rutin was found to have the highest concentration. The presence of rutin in *Tribulus terrestris* was also confirmed by [28]. They performed HPLC analysis for the screening of steroidal saponins and rutin as biologically active compounds in *Tribulus terrestris* [29], also performed the HPLC analysis of *Tribulus terrestris*. The results of their study revealed the presence of thirty-two compounds among which nine were flavonoids including naringin, rutin, hyperoside, quercetin, naringenin, quercetin, hesperetin, kaempferol, and apigenin. The highest concentrations were recorded for hyperoside, naringin and hesperetin.

4. CONCLUSION

Tribulus Terrestris is a good source of natural antioxidants. The fruit of *Tribulus terrestris* exhibits strong antioxidant potential and significant amounts of phenols and flavonoids. The heating time of a microwave oven can affect the antioxidant potential of the extract. The therapeutic properties of *Tribulus Terrestris fruit* can be attributed to its antioxidant capacity. Due to the presence of strong antioxidant potential in *Tribulus Terrestris fruit* without any noticeable side-effect, the present study provides a baseline for food technologists and pharmacological industries to manufacture effective antioxidant drugs or formulations using *Tribulus Terrestris fruit* that can provide a wide range of health benefits.

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