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Antioxidant And Free Radical Scavenging Activity of *Tribulus terrestris. L*

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Abstract

In the present study, the ability of scavenging free radicals of the Methanol, Chloroform and Ethyl acetate of *Tribulus terrestris (Tt)* seed. *Tribulus terrestris* was determined by using 1, 1-diphenyl-2-picrylhydrazyl (DPPH), 2, 2'-Azinobis-(3-Ethylbenzothiazoline-6-Sulfonic Acid) (ABTS+), ferric reducing antioxidant power (FRAP), nitric oxide scavenging assay (NO), superoxide anion radical scavenging (SOD), hydroxyl radical scavenging assay (HRSA), Hydrogen peroxide radical assay (HPRA). The results showed that the Methanol seed extract of (Tt) has a significant antioxidant activity. Thus, the study suggests that (Tt) has a better source of natural antioxidants, which might be helpful in preventing the progress of oxidative stress.

Keywords

Tribulus terrestris L, DPPH, HRSA, HPRA, oxidative stress

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INTRODUCTION:

Nature has served as a rich repository of medicinal plants for thousands of years and an impressive number of modern drugs have been isolated from natural sources, notably of plant origin (1). Free radicals are created when cells use oxygen to generate energy. These by-products are generally reactive oxygen species (ROS) such as super oxide anion, hydroxyl radical and hydrogen peroxide that result from the cellular redox process. At low or moderate concentrations, ROS exert beneficial effects on cellular responses and immune function but at high levels, free radicals and oxidants generate oxidative stress, a deleterious process that can damage cell structures, including lipids, proteins, and DNA (2). Oxidative stress plays a major part in the development of chronic and degenerative ailments such as cancer, autoimmune disorders, rheumatoid arthritis, cataract, aging, cardiovascular and neurodegenerative diseases (3). Many herbs and spices are the subject of ongoing scientific investigations related to antioxidant properties and health (4). Nowadays, there is a growing interest in bioprospecting and the analysis of novel natural antioxidants for use in foods. Namely, some of synthetic antioxidants, such as butylated hydroxytoluene (BHT) and butylated hydroxyanisole (BHA), which have been widely used in foods and beverages, showed potential health hazards, because of the formation of possible toxic or carcinogenic components during their degradation (5,6).

Zygophyllaceae (Caltrop family) is a family of approximately 25 genera and 240 species (7) adapted to semidesert and Mediterranean climates. *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 (7) and Arabic names: Al-Gutub, Qutiba, Hasak or Ders El-Agouz (8). *Tribulus terrestris* has been used in folk medicine throughout history for conditions such as impotence, rheumatism, edema, hypertension, and kidney stones (9, 10). Literature showed that *Tribulus*

terrestris contains phenolic compounds (11), saponins (12), sterols (13) and alkaloids (14). In this study, we examined the antioxidant activity of *Tribulus terrestris* employing seven in vitro assay systems, i.e., DPPH, $ABTS^+$, FRAP, NO, SOD, HRSA and HPRA in order to understand the usefulness of this plant as a foodstuff as well as in medicine.

MATERIAL AND METHOD:

Chemicals

1, 1- diphenyl-2-picryl hydrazyl (DPPH), potassium ferricyanide were purchased from Sigma Chemicals Co. USA). Butylated hydroxytoluene (BHT) sulfanilamide, N-(1-naphthyl) ethylenediamindihydrochloride, EDTA and ferric chloride were purchased from Merck (Germany). All other chemicals were of analytical grade or purer.

Plant Material

Tribulus terrestris seed were collected in and around Chidambaram, Cuddalore, District in the month of January-February. The herbarium of the plant was identified and authenticated by the botanist Dr.V.Venkatesalu and the voucher specimen was deposited to the Department of Botany, Annamalai University, Tamil Nadu, India.

Preparation of Plant Extract

Tribulus terrestris seed were shade dried at room temperature (32 ± 2ºC) and the dried seed was ground into fine powder using a pulveriser. The powder was sieved and kept in deep freezer until use. 100 g of dry fine seed- powder was taken and mixed with 300 ml of three different organic solvents (methanol, chloroform, and ethyl acetate,) and magnetically stirred in a container over night at room temperature. The extract was filtered using a muslin cloth and concentrated at 40 ± 5ºC.

DPPH radical scavenging activity

The antiradical efficiency was assessed using the DPPH ˙ method as described by Blios (13). In this method commercially available methanol soluble stable free radical DPPH was used. In its radical form, DPPH has an absorption band at 515 nm, which disappears upon reduction by an antioxidant compound or a radical species. For the photometric assay, different volumes of the extracts were taken in different test tubes. The volume was adjusted to 100 µL with methanol. 5.0 mL of 0.1 MM methanolic solution of DPPH˙ was added to these tubes and shaken vigorously. The tubes were allowed to stand for 20 min at 27ºC. The control was prepared as above but without the test extract and methanol was used for the baseline correction. Changes in the absorbance of the samples were monitored at 517 nm. Results were compared with the activity of BHT. The percentage of DPPH˙ discolouration of the samples was calculated using the following formula:

DPPH radical scavenging activity (%) = [(A517 of control – A517 of sample) /A517 of control] × 100.

2, 2'-Azinobis-(3-Ethylbenzothiazoline-6-Sulfonic Acid) ($ABTS^+$) Assay

The total antioxidant activity of the samples was measured by [2, 2'-azino-bis (3-ethylbenzthiazoline-6-sulphonic acid)] $ABTS^{\bullet+}$ radical cation decolorization assay according to the method of Re *et al.* (14). $ABTS^{\bullet+}$ was produced by reacting 7mM $ABTS^+$ aqueous solution with 2.4mM potassium persulfate in the dark for 12-16 hours at room temperature. The radical was stable in this form for more than two days when stored in the dark at room temperature. Then, 2ml of diluted $ABTS^{\bullet+}$ solution was added to the sample varying concentrations of *Tribulus terrestris* seed extracts. The blank contained water in place of *Tribulus terrestris* seed extract. After 30 minutes of incubation at room temperature, the absorbance was recorded at 734nm and compared with standard BHT. Percentage of inhibition was calculated.

$$\% \text{ Scavenging} = \frac{\text{Control OD} - \text{Test OD}}{\text{Control OD}} \times 100$$

Ferric Reducing Antioxidant Potential (FRAP) Assay

Ferric reducing ability was evaluated using different concentrations of crude extract. The FRAP reagent contained 10mM of TPTZ solution in 40mm. HCl 20m M $FeCl_3$,$6HO_2$, and acetate buffer (300mM, pH 3.6) (1:1:10, v/v/v). A 100µL 50% aqueous ethyl acetate of the test compounds was added to 3mL of the FRAP reagent, and the absorbance was measured at 593nm after incubation at room temperature for 6min, using the FRAP reagent as a blank (15).

Nitric oxide scavenging activity

Nitric oxide scavenging activity was determined according to the method suggested by Sreejayan and Rao (16). Nitric oxide generated from sodium nitroprusside in aqueous solution at physiological pH interacted with oxygen to produce nitrite ions, which can be estimated using the Griess reagent. Scavengers of nitric-oxide act against oxygen, leading to reduced production of nitrite ions. In brief, 3.0 mL of 10 mM sodium nitroprusside in phosphate

buffered saline was mixed with different concentrations of the extract and incubated at 25°C for 150 min. 0.5 mL of the incubated solution was removed and diluted with 0.5 mL of Griess reagent (1% sulphanilamide, 2% orthophosphoric acid and 0.1% N-1-naphthylethylenediamine dihydrochloride). The absorbance of the chromophore formed during diazotization of the nitrite with sulphanilamide and subsequent coupling with N-1-naphthylethylenediamine dihydrochloride was measured at 546 nm and percentage of scavenging activity was measured with reference to standards.

Superoxide anion scavenging activity assay

The superoxide anion radicals are produced in 2 ml of phosphate buffer (100 mM, pH 7.4) with 78M - nicotinamide adenine dinucleotide (NADH), 50M nitro blue tetrazolium chloride (NBT) and test samples at different concentrations. The reaction mixture is kept for incubation at room temperature for 5min. It is then added with 10 M of 5-methylphenazinium methosulphate (PMS) to initiate the reaction and incubated for 5min at room temperature. The color reaction between superoxide anion radical and NBT is read at 560nm. The reaction mixture without test sample is used as control and without PMS is used as blank (17). The scavenging activity is calculated as follows, % Scavenging activity = [(Absc– Abss)/Absc] × 100

Hydroxyl radical scavenging activity

The scavenging activity for the sample extracts on hydroxyl radical was measured according to the method of Klein *et al.,* (18). 20 µg concentration of the extract was added with 1.0 mL of iron – EDTA solution (0.13% ferrous ammonium sulfate and 0.26% EDTA), 0.5 mL of EDTA solution (0.018%), and 1.0 mL of DMSO (0.85% v/v in 0.1 M phosphate buffer, pH 7.4) sequentially. The reaction was initiated by adding 0.5 mL of ascorbic acid (0.22%) and incubated at 80-90ºC for 15min in a water bath. The reaction was terminated by the addition of 1.0 mL of ice – cold TCA (17.5% w/v). Then, 3.0 mL of Nash reagent (75.0 g of ammonium acetate, 3.0 mL of glacial acetic acid, and 2.0 mL of acetyl acetone were mixed and raised to 1L with distilled water) was added and left at laboratory temperature for 15 min. The reaction mixture without sample was used as control. The intensity of the colour formed was measured spectrophotometrically at 412nm against reagent blank. The percentage of hydroxyl radical scavenging activity (HRSA) was calculated using the following formula:

HRSA (%) = [A control– A sample / A control] × 100.

Scavenging activity against hydrogen peroxide

The scavenging capacity of extracts on hydrogen peroxide was determined according to the method of Nebavi *et al.,* (19). Test tubes were prepared with 2.0 ml of various extracts (50- 400 ppm) and a solution of H_2O_2 (1.2 ml, 40 mM) in phosphate buffer (pH 7.4). A blank solution was prepared in the same way but without H_2O_2. After incubation of the mixture during 10 min, the absorbance was recorded at 230 nm. BHT was used as reference standard. The scavenging activity was calculated using the following Formula:

% Scavenging activity = [(Ac – At)/ Ac] 100,

Where, Ac absorbance of the control, At absorbance of the extract

RESULTS AND DISCUSSION

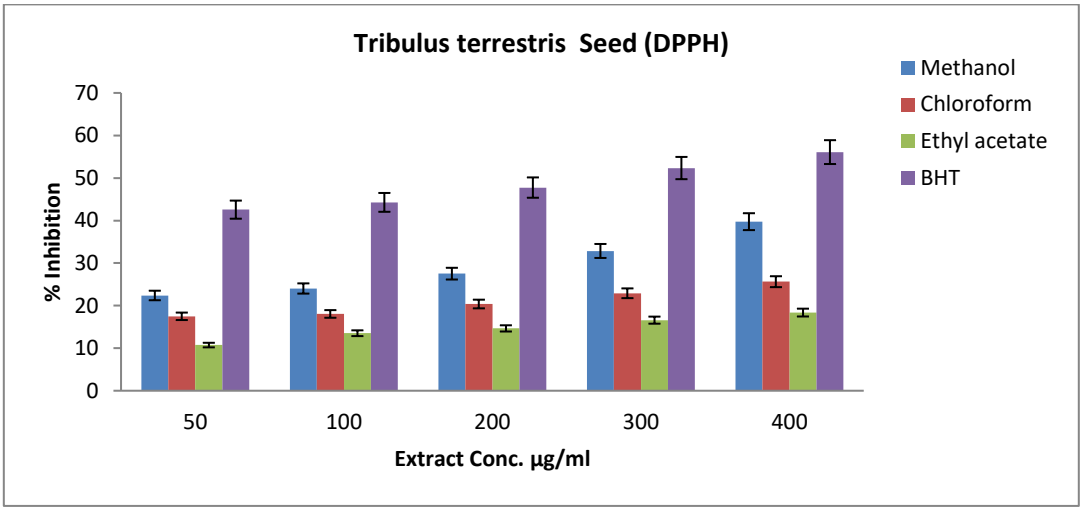
The antioxidant activities were determined by using 1, 1diphenyl-2- picryhydrazyl (DPPH) as a free radical. The DPPH scavenging activity of seed extracts of *Tribulus terrestris* was determined by its absorbance at 570nm, which is due to the presence of antioxidants. The percentage of DPPH radical scavenging activity of methanol, chloroform and ethyl acetate extract of *Tribulus terrestris* seed were expressed in Fig 1. This shows the maximum DPPH scavenging activity of methanol extracts of *Tribulus terrestris* seed was 39.74% whereas BHT was found to be at 56.09% at 400 µg/ml. DPPH radical is considered to be a model of lipophilic radical. In this mode, scavenging activity is attributed to hydrogen donating ability of antioxidants (20). Although ethyl acetate extract of *Tribulus terrestris* seed possess good DPPH scavenging activity, it was evident that the extract could serve as free radical inhibitors or scavengers.

The percentage of $ABTS^+$ radical scavenging activity of methanol, chloroform and ethyl acetate of *Tribulus terrestris* were expressed in Fig 2. Methanol of *Tribulus terrestris* were displayed a maximum $ABTS^+$ scavenging activity of 53.90% at 400 µg/ml whereas for BHT was found to be 56.09% at 400 µg/ml. ABTS assay is an excellent tool for determining the antioxidant activity of hydrogen ion donating antioxidants and of chain-breaking antioxidants (21).

The ferric reducing ability of the extracts revealed that all of them gave well FRAP activity. The reduction capacity of FRAP radicals was determined by the decrease in its absorbance at 593 nm, which is

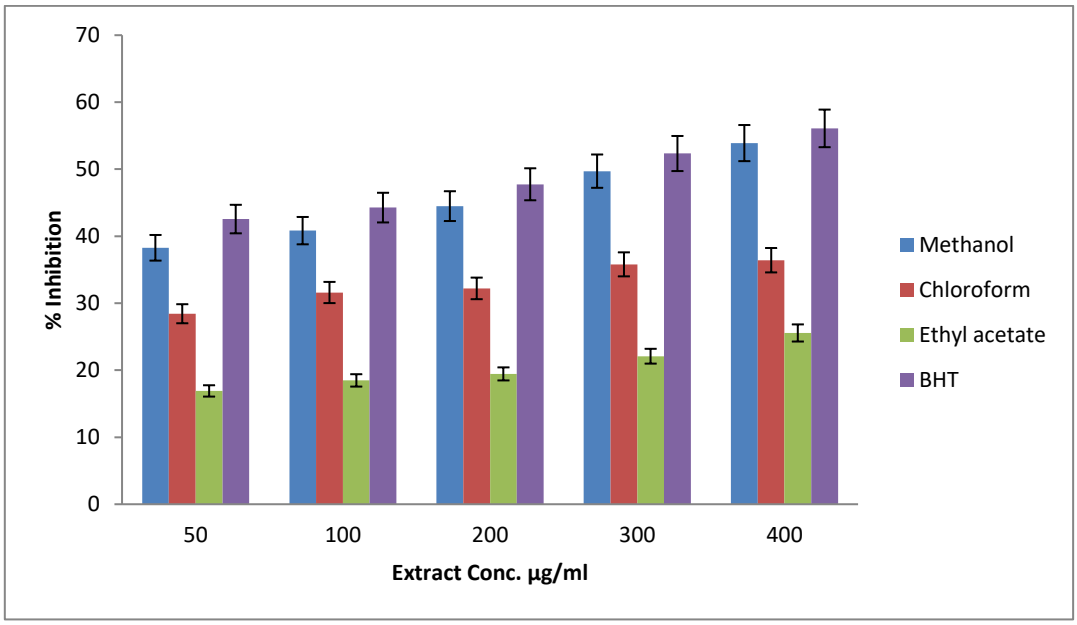
induced by antioxidants. The percentage of FRAP radical scavenging activity of methanol, chloroform, and ethyl acetate extract of *Tribulus terrestris* seed were revealed in Fig 3. Methanol of *Tribulus terrestris* were exhibited a maximum FRAP scavenging activity of 47.35% at 400 µg/ml whereas BHT was found to be 56.09% at 400 µg/ml. In FRAP assay, non-enzymatic antioxidants react with prooxidants and inactive them. In a redox reaction, antioxidants act as 'reductants'. In this context, the antioxidant power can be referred to as 'reducing ability'. In this FRAP assay, an easily reducible oxidant, Fe III is used in excess. Thus, there is a reduction of Fe III-TPTZ complex by antioxidant (22). The decrease in the concentration of FRAP is a measure of the antioxidant activity of *Tribulus terrestris*.

Figure 1: Effect of Methanol, Chloroform and Ethyl acetate seed extracts of *Tribulus terrestris* on DPPH assay



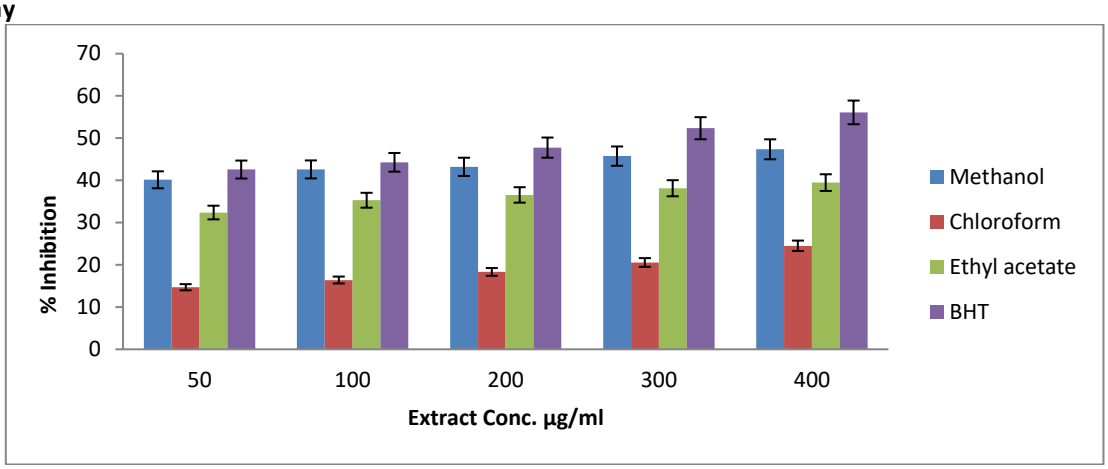
Each value is expressed as mean ± standard deviation (n=3).

Figure 2: Effect of Methanol, Chloroform and Ethyl acetate extract of *Tribulus terrestris* (Linn) seed on ABTS^{+} assay



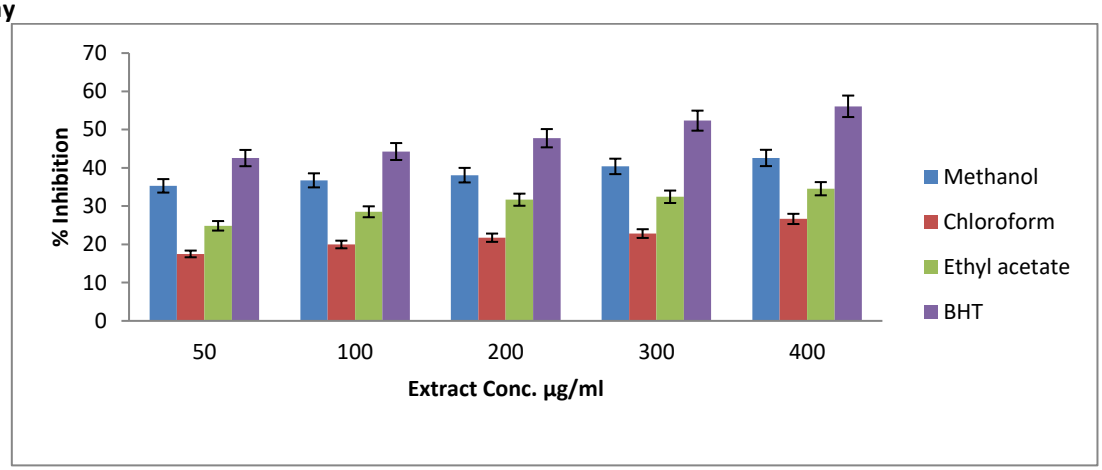
Each value is expressed as mean ± standard deviation (n=3).

Figure 3: Effect of Methanol, Chloroform and Ethyl acetate seed extract of *Tribulus terrestris* (Linn) on FRAP assay



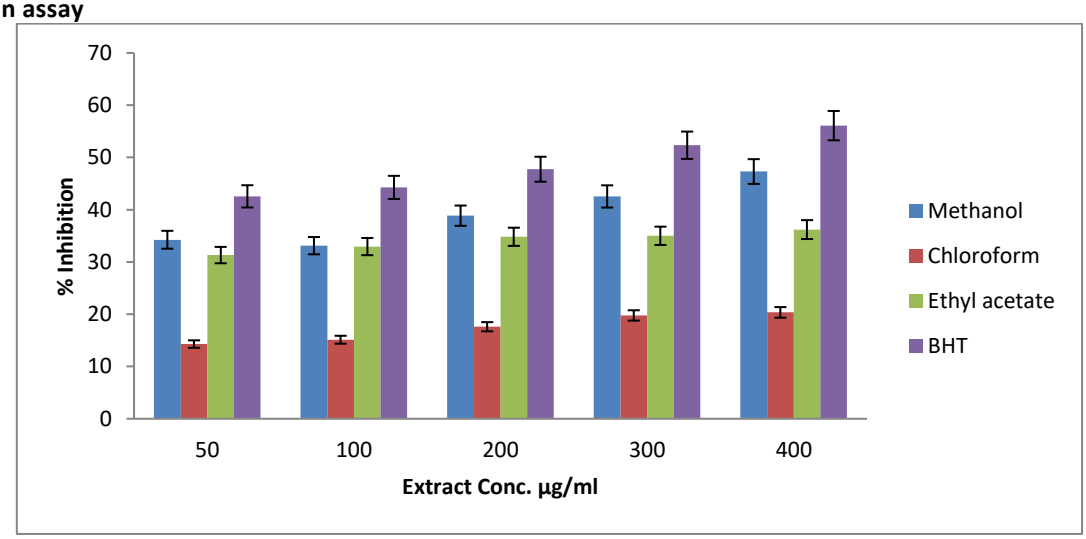
Each value is expressed as mean ± standard deviation (n=3).

Figure 4: Effect of Methanol, Chloroform and Ethyl acetate seed extracts of *Tribulus terrestris* on Nitric oxide assay



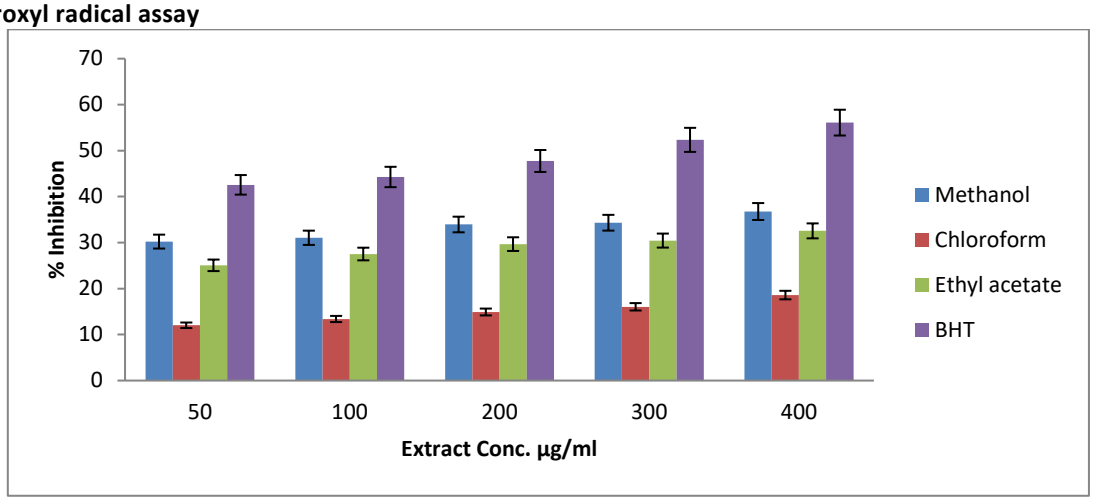
Each value is expressed as mean ± standard deviation (n=3).

Figure 5: Effect of Methanol, Chloroform and Ethyl acetate seed extract of *Tribulus terrestris* on Superoxide Anion assay



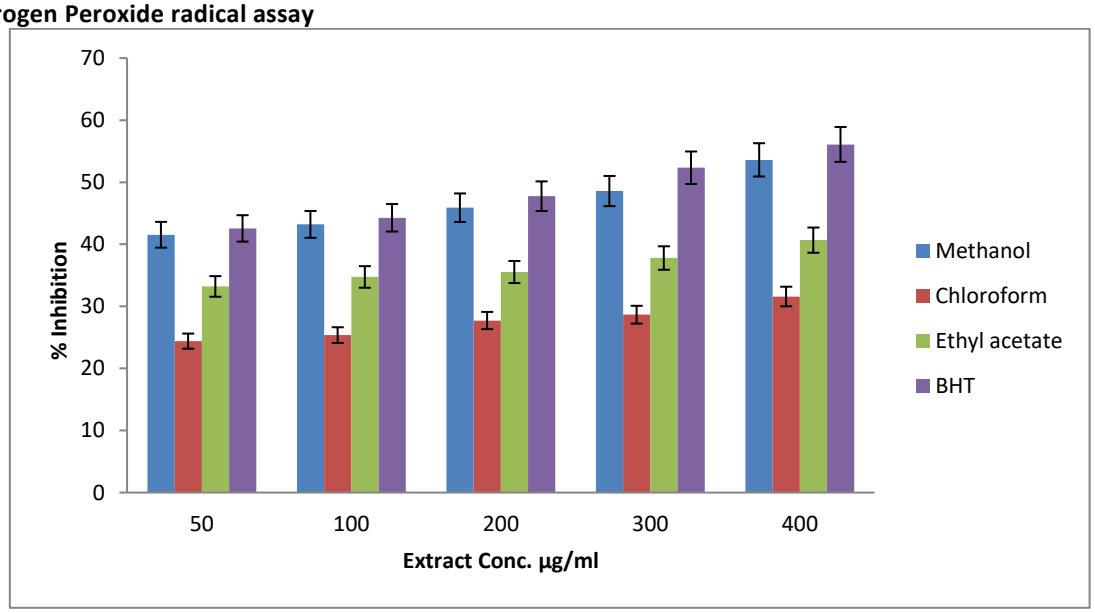
Each value is expressed as mean ± standard deviation (n=3).

Figure 6: Effect of Methanol, Chloroform and Ethyl acetate seed extracts of *Tribulus terrestris* (Linn) on Hydroxyl radical assay



Each value is expressed as mean ± standard deviation (n=3).

Figure 7: Effect of Methanol, Chloroform and Ethyl acetate seed extracts of *Tribulus terrestris* (Linn) on Hydrogen Peroxide radical assay



Each value is expressed as mean ± standard deviation (n=3).

The reduction capacity of Nitric oxide radicals was determined by the decrease in its absorbance at 593nm, which is induced by antioxidants. The percentage of nitric oxide radical scavenging activity of methanol, chloroform, and ethyl acetate extract of *Tribulus terrestris* seed was specified in Fig 4. Methanol of *Tribulus terrestris seed* were exhibited a maximum nitric oxide scavenging activity of 42.59% at 400µg/ml whereas BHT was found to be 56.09% at 400 µg/ml. Nitric oxide is a free radical produced in mammalian cells, involved in the regulation of various physiological process including neuro transmission, vascular homeostasis, antimicrobial and antitumor activities. However, excess production of NO is associated with several diseases. It would be interesting to develop potent and selective inhibitors of NO for potential therapeutic use (23). In the present study, *Tribulus terrestris* exhibited potent nitric oxide radical scavenging activity, which competes with oxygen to react with nitric oxide and thus inhibits the generation of nitrite.

Superoxide is a highly reactive molecule that reacts with various substances produced through metabolic processes. Percentage scavenging activity of superoxide anion examined at different concentrations of methanol, chloroform, and ethyl acetate extract of *Tribulus terrestris* seed was revealed in Fig 5. Methanol of *Tribulus terrestris* were exhibited a maximum superoxide anion scavenging

activity of 47.31% at 400 µg/ml whereas BHT was found to be 56.09% at 400 µg/ml. Superoxide anion radical is one of the strongest reactive oxygen species among the free radicals that are generated after oxygen is taken into living cells. Superoxide anion changes to other harmful ROS and free radicals such as hydrogen peroxide and hydroxyl radical, which induce oxidative damage (24) .

The percentage scavenging activity of hydroxyl radical examined at different concentrations of methanol, chloroform and ethyl acetate extract of *Tribulus terrestris* were revealed in Fig 6. Methanol of *Tribulus terrestris* were exhibited a maximum hydroxyl radical scavenging activity of 36.76% at 400 µg/ml whereas BHT was found to be 56.09% at 400 µg/ml. Hydroxyl radical are the major active oxygen causing lipid peroxidation in enormous biological damage. The highly reactive hydroxyl radical can cause oxidative damage to DNA, lipid and protein (25). In this study, was found to scavenge O_2 significantly and in dose dependent manner and may protect the DNA, protein, and lipid from damage.

The percentage of hydrogen peroxide radical scavenging activity of methanol, chloroform, and ethyl acetate of *Tribulus terrestris* seed extracts were expressed in Fig 7. Methanol of *Tribulus terrestris* were exposed a maximum hydrogen peroxide radical scavenging activity of 53.61% at 400 µg/ml whereas for BHT was found to be 56.09% at 400 µg/ml. Although hydrogen peroxide itself is not very reactive, it can sometimes cause cytotoxicity by giving rise to hydroxyl radicals in the cell. Thus, removing H_2O_2 is very important throughout food systems (19).

CONCLUSIONS

The present study demonstrates that different extract obtained from the seeds of *Tribulus terrestris* possessed antioxidant and radical-scavenging activities. The results indicated that the methanol extract of *Tribulus terrestris* have proton-donating ability, could serve as free radical inhibitors or scavengers, acting possibly as primary antioxidants. This study supports the contention that traditional medicines remain a valuable source in the potential discovery of natural product pharmaceuticals. Significant antioxidant activity showed by *Tribulus terrestris* provides a scientific validation for the traditional use of this plant.

CONFLICTS OF INTEREST

The authors declare that they no conflicts of interest.

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