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Research Article

Evaluation of a *Trifolium Repens* L. Extract as a Potential Source of Antioxidants

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Abstract

The aim of this study was to perform *in vitro* evaluation of the antioxidant activity of an ethanol extract of *Trifolium repens* L. (TREE). The antioxidant properties of TREE were demonstrated using several established *in vitro* systems: 2, 2'-Azinobis-3-ethylbenzthiazoline-6-sulphonic acid (ABTS), 1, 1-Diphenyl-2-picrylhydrazyl (DPPH) and superoxide radical scavenging assays, ferrothiocyanate (FTC) and Thiobarbituric Acid (TBA) assays. The results showed that TREE had strong scavenging activities for DPPH, ABTS cation radical and superoxide anion radical. The radical scavenging IC_{50} values of TREE was 276 ± 14 μg/mL and 54 ± 2 μg/mL for DPPH and ABTS, respectively. The antioxidant activity was enhanced as the TREE concentration increased. The *Kb* value ($\times 10^{-4}$) of the TREE scavenging superoxide anion radical was 6.96 ± 0.42. The TREE also demonstrated good anti-lipid peroxidation capacities in FTC and TBA assays. The ethanol extract from *T. repens* showed potent antioxidant properties, and might be a valuable natural source of antioxidants that could be used in both the medical and food industries.

Keywords: *Trifolium repens* L.; Antioxidant activity; Radical scavenging; Lipid peroxidation; *In vitro*

Abbreviations

ABTS: 2,2'-Azinobis-3-ethylbenzthiazoline-6-sulphonic acid; BHT: Butylated Hydroxytoluene; DPPH: 1, 1-Diphenyl-2-picrylhydrazyl; FTC: Ferrothiocyanate; TBA: Thiobarbituric acid; TREE = Ethanol Extracts of *T. Repens*;

Introduction

Abundant evidence now suggests that reactive oxygen species (ROS) and free radicals may be a major cause of oxidative damage to biological molecules in the human body (e.g. DNA, proteins and membrane lipids) [1-5]. Increasing evidence also highlights that oxidative stress contributes to various disorders and diseases such as cancer, atherosclerosis, diabetes, arthritis and Alzheimer's [6-10]. Many synthetic antioxidants, such as Butylated Hydroxyanisole (BHA), Butylated Hydroxytoluene (BHT) and Tert-Butylhydroquinone (TBHQ), are widely adopted in the food, pharmaceutical and nutraceutical industries, but their potential health risks have led to restriction of the applications of these synthetic antioxidants. Consequently, natural antioxidants from plant extracts are now receiving much attention [11]. The antioxidant capacity from many extracts of medicinal herbs and their by-products are currently under active study [12,13].

Trifolium repens L. (Leguminosae) is widely distributed in the world [14]. As the dominant pasture legume in a number of temperate pasture ecosystem communities, *T. repens* performs vital functions due to its high feed value and as the major nitrogen-fixing plant species [15]. The whole plant has also been used in traditional Chinese medicine for treatment of epilepsy syndromes and bleeding for a long time [16]. A series of flavonoids have been separated from *T. repens* by Ursula et al., who reported the presence of quercetin, myricerin and kaempferol [17]. Foo et al. also separated myricetin-3-O-β-D-galactopyanoside, quercetin-3-O-β-D-galactopyanoside and kaempferol-3-O-β-D-galactopyanoside from this plant [18].

However, to the best of our knowledge, the antioxidant effects of *T. repens* have not been documented. The aim of this work was to evaluate the potential *in vitro* antioxidant effectiveness of TREE by 2, 2-diphenyl-1-picrylhydrazyl (DPPH), superoxide anion and 2, 2'-Azinobis-3-ethylbenzthiazoline-6-sulphonic acid (ABTS) radical scavenging activity tests and lipid peroxidation tests using ferrothiocyanate (FTC) and Thiobarbituric Acid (TBA).

Materials and Methods

Chemicals

1, 1-Diphenyl-2-picrylhydrazyl (DPPH) and Folin-Ciocalteau's reagent were purchased from Sigma-Aldrich (St.Louis, MO). Gallic acid, rutin, and Butylated Hydroxytoluene (BHT) were purchased from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China).

2, 2'-Azinobis-3-ethylbenzthiazoline-6-sulphonic acid (ABTS) was purchased from Fluka (Menlo Park, CA). All other chemicals used for analysis were AnalaR grade and obtained from China Medicine (Group) Shanghai Chemical Reagent Corporation (Shanghai, China).

Plant materials

T. repens (whole plant) was collected in May 2013 from Wuhan, Hubei province, PR China, and authenticated by the corresponding author. A voucher specimen (No. 101) was deposited in the Department of Pharmacology, Medical College of Wuhan University of Science and Technology, China.

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Preparation of extracts

Air dried, powdered (less than 0.25 mm) *T. repens* (50 g) was extracted three time in 75% ethanol (600 mL) for 120 min at 80 °C, and the extracts were pooled. The solvent was evaporated under reduced pressure at 35°C and dried to obtain the ethanol extract of *T. repens* (TREE) as a powder.

Determinations of total phenolic and total flavonoid contents

Total phenolic content in the extracts was determined using Folin–phenol reagent as described by Harish et al. [19] with slight modification. Briefly, 1 mL of the extract was added to 10 mL deionized water and 2 mL of Folin–phenol reagent. The mixture was then allowed to stand for 5 min and 2 mL Na_2CO_3 sodium carbonate (7.5% w/v) was added. The absorbance was measured at 765 nm in a spectrophotometer. Phenolic content was calculated using gallic acid as the standard and expressed as gallic acid equivalents per mg/g dry extract.

Total flavonoid content was determined using the aluminium chloride colorimetric method as described by Jia et al. [20] with slight modification. Briefly, 1 mL of extract was placed in a 10 mL volumetric flask, the volume was made up to 5 mL with distilled water, and 0.3 mL $NaNO_2$ (5% w/v) was added. Five minutes later, 3 mL $AlCl_3$ (10% w/v) was added. After 6 min, 2 mL 1 M NaOH was added. Distilled water was again added to make a 10 mL volume. The solution was mixed thoroughly and the absorbance was measured against a blank at 510 nm. Flavonoid content was calculated using rutin as the standard and expressed as rutin equivalents per mg/g dry extract.

DPPH radical scavenging assay

The DPPH radical scavenging activity was performed according to the method of Zhou et al. [21]. Briefly, 2.7 mL of DPPH solution (0.2 mM, in ethanol) was mixed with 0.3 mL TREE at a range of concentrations. The reaction mixture was shaken and incubated in the dark at room temperature for 1h before the absorbance was measured at 517 nm. BHT at the same concentration was used as a positive control. The inhibition of the DPPH radical scavenging activity by the sample was calculated according to the following formula:

DPPH radical scavenging activity (%) =

$$\frac{Abs.of\ control - Abs.of\ sample}{Abs.of\ control} \times 100\%$$

ABTS radical scavenging assay

The ABTS radical scavenging assay was performed according to He et al. [22] with slight modifications. A solution of ABTS was prepared by (7 mM) mixing with potassium persulphate (2.45 mM) at room temperature in the dark for 16 h. The working solution was prepared by diluting this solution with 80% ethanol until the absorbance at 734 nm was 0.70±0.005. The TREE solution at a range of concentrations (0.3 mL) was mixed with ABTS solution (2.7 mL), and absorbance was read at 734 nm. BHT at the same concentration was used as a positive control. The inhibition of the ABTS radical scavenging activity by the sample was calculated according to the following formula:

ABTS radical scavenging activity (%) =

$$\frac{Abs.of\ control - Abs.of\ sample}{Abs.of\ control} \times 100\%$$

Superoxide anion scavenging activity assay

The superoxide anion scavenging activity of the TREE was conducted according to the method of Yao et al. [23] and Huang et al. [24] with slight modifications. Up to 0.5 mL of TREE (0.05mg/mL) was added to 4.5 mL of 50 mM Tris–HCl buffer (pH 8.2). The mixture was incubated at 25 °C for 20 min, and then 10 µL of 45 mM pyrogallol (25 °C) was added. The absorbance of the reaction mixture was measured at 325 nm every 30 s until the reaction time reached 6 min. The auto-oxidation rate constant (K_p) of pyrogallol was calculated from the curve of $A_{325\ nm}$ vs. time. The control did not contain test extracts and a concentration of ascorbic acid (Vc) identical to the sample was used as a reference. The inhibitory actions of test extract on the auto-oxidation rate of pyrogallol correlated with their ability to scavenge superoxide.

Antioxidant activity in a linoleic acid system using ferrothiocyanate (FTC) and thiobarbituric acid (TBA)

The FTC method was adapted from Chen et al. [25] with slight modification. A 100µg sample of TREE was dissolved in 1 mL of ethanol, and 1 mL linoleic acid (2.5%, prepared in ethanol, w/v) or distilled water and 2 mL of phosphate buffer (50 mM, pH 7.0) were added. The mixtures were capped and incubated at 40 °C in the dark. Aliquots (0.1 mL) were withdrawn and mixed with ferrous chloride (0.1 mL, 20 mM in 3.5% HCl), 30% ammonium thiocyanate (0.1 mL) and 75% ethanol (9.7 mL), and 3 min later, the absorbance was measured at 500 nm. Aliquots were withdrawn and assayed in an identical fashion at 24 h intervals until a constant maximum value was reached. BHT was used for comparative purposes in this test.

The method of Huang et al. [26] using TBA was also employed to determine the antioxidant activity of TREE with slight modification. A mixture of 20% trichloroacetic acid (2 mL) and aqueous TBA (2 mL) was mixed with 1 mL of extract solution prepared as described for the FTC method above. The mixture was incubated in a boiling water bath for 10 min, cooled, and the supernatant separated via centrifugation at 2000×g for 20 min. Absorbance of the resulting supernatant was measured at 532 nm. The inhibition rate was calculated according to the following formula:

TBA inhibition (%) =

$$\frac{Abs.of\ control - Abs.of\ sample}{Abs.of\ control} \times 100\%$$

Statistical Analysis

All experiments were done in triplicate, and the results were reported as mean ± SD. Data were analysed with one-way ANOVA.

Results & Discussion

Total phenolics and flavonoids have been reported to go hand-in-hand with antioxidant activity [27-29]. The TREE yield was 40.46% , the total phenolic content (34.67±0.30 mg/g) of the TREE was reported as gallic acid equivalents and total flavonoids (52.25±0.18

Table 1: Extract yields and content of total phenolics, flavonoids and antioxidants in an ethanol extract from *Trifolium repens* L.

Sample	Extract yield (%)	TP(mg GAE/g of extract)	TF(mg RUE/g of extract)	IC_{50} value [a](μg/ml)	
				ABTS	DPPH
TREE	40.46	34.67±0.30	52.25±0.18	54±2	276±14
BHT				6±0.09	166±2

TP: Total Phenolics; TF: Total Flavonoids; GAE: Gallic Acid Equivalents; RUE: Rutin Equivalents; TREE: Ethanol Extracts of *T. repens*; [a] IC_{50}: The Concentration Required to Scavenge 50% of Radical. Each value represents the mean ± SD of three replicate measurements.

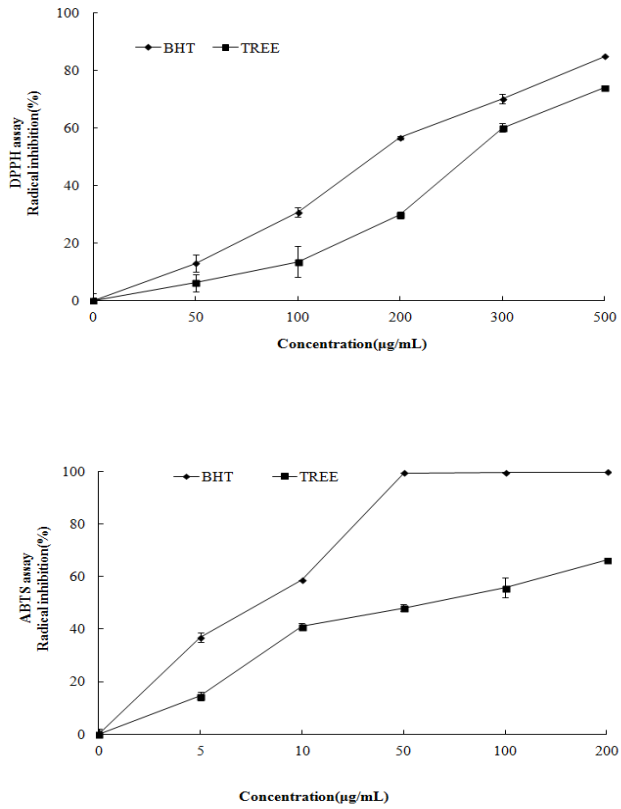

Figure 1: DPPH and ABTS radical scavenging activities of extracts from *Trifolium repens* L. TREE, ethanol extracts of *T. repens*; BHT, butylated hydroxytoluene. Y-error bars, standard deviation.

mg/g) was calculated as rutin equivalents (Table 1). The results show that *T. repens* has relatively high flavonoid content.

The DPPH and ABTS assays were used to measure the capacity of antioxidant compounds to scavenge these free radicals. These two free radicals are commercially available, stable, easy to handle, and the reaction with antioxidants can be followed easily by a conventional UV/visible absorption spectrophotometer. The scavenging activity of the extracts and the reference compound, BHT, is summarized in Figure 1 and the concentrations required to inhibit each radical by 50% (IC_{50}) are shown in Table 1. The TREE showed excellent scavenging of free radicals in both the DPPH (IC_{50} = 276±14μg/mL) and the ABTS (IC_{50} = 54±2μg/mL) scavenging assays.

Superoxide anion radical ($\cdot O_2^-$) is a relatively weak, but the most common, free radical generated *in vivo*. It acts as a precursor of singlet oxygen and hydroxyl radical, which have potential of reacting with biological macromolecules and thereby inducing tissue damage [30]. The rate of this auto-oxidation reaction is dependent on the $\cdot O_2^-$ concentration, which is generated by the auto-oxidation of pyrogallic acid under alkaline conditions. Excessive production of superoxide anion radical has been regarded as the beginning of ROS accumulation in cells, resulting in redox imbalance and related harmful physiological consequences [31]. The result shown in Table 2 indicate that the TREE can slow down the auto-oxidation reaction of pyrogallic acid; the *Kb* value ($\times 10^{-4}$) of the control, TREE and Vc samples were 8.61±0.64, 6.96±0.42 and 0.75±0.086, respectively. The results showed that TREE inhibited the superoxide anion radical to a certain extent but was not as strong as ascorbic acid, and also indicated that the TREE is capable of scavenging superoxide radicals, and could help prevent or ameliorate oxidative damage.

The amount of lipid peroxidation can be measured by the FTC method. The peroxides are formed by linoleic acid when it is oxidised and converts Fe^{2+} to Fe^{3+}, which can then be determined by absorbance at 500 nm as it can complex with SCN^- to form a thiocyanate [32]. The antioxidant activity exhibited by extracts according to the FTC methods is shown in Fig. 2A. The BHT showed a high capacity to inhibit linoleic acid peroxidation, with absorbance values always under 0.15 during the 9 days of testing. The absorbance of the control showed a steady increase and reached a peak on the 8th day. The absorbance of TREE containing samples increased slowly, which implied a strong antioxidant capability.

The peroxide in the FTC assay will gradually decompose to malondialdehyde, which can be measured by the TBA assay. The TBA binds malondialdehyde to form a red pigment at low pH and high temperature (100), which can be quantified by absorbance at 532 nm [25]. As shown in Figure 2B, the inhibition of peroxidation obtained for BHT and TREE were 64.99±2.25% and 46.54±4.25%, respectively. Thus the TREE also showed excellent antioxidant activity, in agreement with the results of the FTC assay.

In aerobic organisms, oxygen is essential for efficient energy production but paradoxically, produces chronic toxic stress in cells. Oxidative Stress (OS) results when production of ROS exceeds the capacity of cellular antioxidant defences to remove these toxic species [33]. Anti-lipid peroxidation and radical scavenging are the two main types of protective mechanisms that exist in the body to remove the toxic oxygen by-products. Sabudak et al. [34] reported the contents of fatty acids and other lipids from five plants belonging to *Trifolium* species. The whole plant hexane extracts of five plants contained eight fatty acids consisting of linolenic (31.1%) and palmitic (18.9%) acids as the most abundant unsaturated and saturated fatty acids, respectively. The total unsaturation for the oils of five *Trifolium* species was 30.6-42.2%. All the five *Trifolium* extracts showed more or less antioxidant activity in β-carotene-linoleic acid system however, inhibition percentages were very low in DPPH assay. In Poland, Kicel and Wolbiś [35] determined the flavonols and isoflavones in the *T. repens* flowers and leaves by a RP-HPLC method and test the antioxidant activity by DPPH assay. The results showed

Table 2: Inhibition of pyrogallic acid auto-oxidation by extracts from *Trifolium repens* L.

Sample	Control	TREE	Vc
K_b value ($\times 10^{-4}$)	8.61±0.64	6.96±0.42	0.75±0.086

TREE: Ethanol Extracts of *T. repens*; Vc: Ascorbic Acid; K_b: Auto-Oxidation Rate Constant. Each value represents the mean ± SD of three replicate measurements.

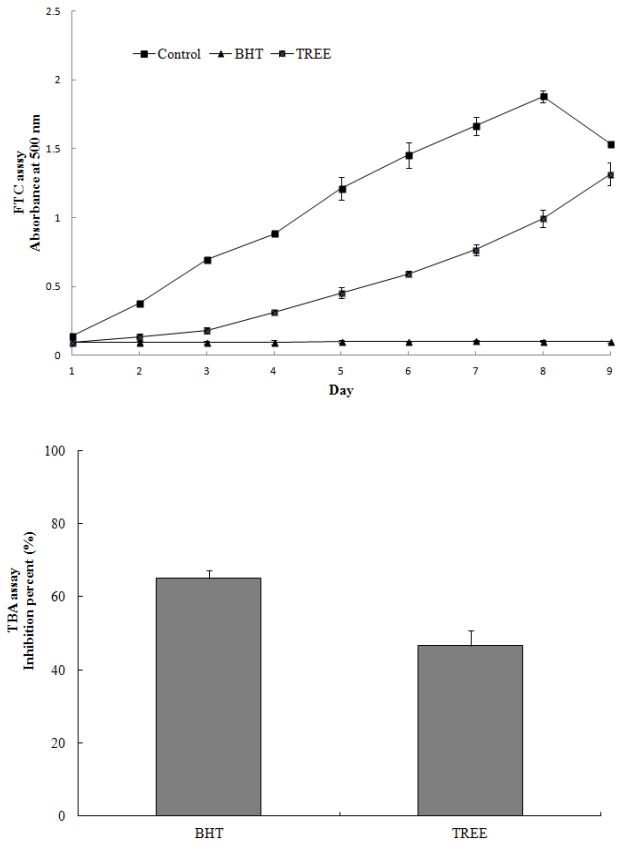

Figure 2: Antioxidant activity monitored daily using the FTC and TBA methods. Low absorbance indicates strong antioxidant activity. (A) FTC assay. (B) TBA assay. TREE, ethanol extracts of *T. repens*; BHT, butylated hydroxytoluene. Y-error bars, standard deviation.

that the flowers are richer source of phenolics ranging from 28.7 to 38.8 mg GAE/g, and flavonoids is up to 20 mg HP/g (calculated for hyperoside), but poor with isoflavones both in the flowers and leaves, the antioxidant activity towards DPPH with EC50 values ranging from 72.3 to 179.3 mg/mL. In our research, the total phenolic and total flavonoid contents of the whole plant were determined and the *in vitro* antioxidant tests revealed that TREE has not only good radical scavenging but anti-lipid peroxidation activities. This study might help in developing this promising antioxidant.

Conclusion

In the present study, the ethanolic extract of *T. repens* was evaluated for antioxidant activities *in vitro*. Our results showed that TREE had strong scavenging free radical and anti-lipid peroxidation capacities. The observed strong antioxidant activity of *T. repens* may be due to the presence of phenolic and flavonoid compounds. The *T. repens* showed potent antioxidant properties, and might be a valuable natural source of antioxidants that could be used in both the medical and food industries. However, further research on the pharmacological activities of this plant is still needed.

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