

ANTIOXIDANT ACTIVITY OF *TRIBULUS TERRESTRIS* – A NATURAL PRODUCT IN INFERTILITY THERAPY

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ABSTRACT

Antioxidant potential of *Tribulus terrestris* L. herbal preparations was evaluated using 1,1-diphenyl-2-picrylhydrazyl (DPPH), 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) di-ammonium salt (ABTS) free radicals, ferric reducing antioxidant power (FRAP) and inhibition of lipid peroxidation by ferric thiocyanate method (FTC). In addition, the quantification of total polyphenols and flavonoids was determinate using Folin-Chiocalteu reagent and AlCl₃, respectively. BHT was used as positive control. The obtained results demonstrated that *T. terrestris* preparations possess a significant antioxidant activity. IC₅₀ for DPPH and ABTS activity varied from 2.84 mg mL⁻¹ to 4.56 mg mL⁻¹ and from 0.28 mg mL⁻¹ to 0.31 mg mL⁻¹, respectively. All tested samples demonstrated stronger FRAP activity compared to BHT (2.29 µg TE ± 0.04 µg TE) and inhibit lipid peroxidation in linoleic acid system. The amount of total polyphenols ranged from 2.73 % ± 0.007 % to 3.17 % ± 0.008 %, whereas quantity of total flavonoids varied from 0.36 % ± 0.004 % to 0.58 % ± 0.01 %. All tested products demonstrated high antioxidant activity and inhibited lipid peroxidation. Therefore this investigation is useful for clarifying of pharmacological activity of *T. terrestris* and present new benefit of this plant in infertility therapy.

Keywords: *Tribulus terrestris* L., Antioxidant activity, Radical scavenging, Lipid peroxidation inhibition, Polyphenols

INTRODUCTION

Tribulus terrestris L. (Zygophyllaceae) (TT) popularly known as puncture vine is a perennial creeping herb with a worldwide distribution. Since ancient times it is regarded as an aphrodisiac in addition to its beneficial claims on various ailments such as urinary infections, inflammations, oedema and ascites¹. *Tribulus terrestris* growing in Bulgaria is a source for the industrial production of the original preparation "Tribestan™" produced by Sopharma AD, Bulgaria. Tribestan™ consists of the n-BuOH extract of the aerial parts of the same plant and is successfully applied for treatment of sexual deficiency². The active components of Tribestan™ are steroid saponins of furostanol type²⁻⁴. The dominating furostanol biglycosides have been identified as protodioscin and protogracillin⁴. An intensive screening on qualitative and quantitative composition of raw materials from TT and variety of preparations from different origin demonstrated that Bulgarian preparation Tribestan™ contains the highest amount of protodioscin and protogracillin⁵.

The aphrodisiac property of TT extract was explored in castrated rats⁶. Administration of TT to humans and animals improves libido and spermatogenesis⁷. Protodioscin is also found to increase the levels of testosterone, leutinizing hormone⁷, dehydroepiandrosterone⁸, dihydrotestosterone and dehydroepiandrosterone sulphate⁹. Clinical studies showed TT improved reproductive function, including increased concentration of hormones such as estradiol, with testosterone being very slightly influenced, thereby improving reproductive function, libido and ovulation^{4,10}.

Free radicals are a group of highly reactive chemical molecules with one or more unpaired electrons that can oxidatively modify biomolecules they encounter. Reacting almost immediately with any substance in their surrounding area, they begin a chain reaction leading to cellular damage¹¹. Oxidative damage, caused by reactive oxygen species (ROS), has been frequently associated with the pathogenesis of various conditions such as arthritis, cancer, inflammation, heart diseases¹² and contributes to defective spermatogenesis leading to male factor infertility¹³. Several clinical trials have examined the potential of antioxidant such as carnitines, vitamin E, vitamin C, selenium, carotenoids etc. supplementation to treat oxidativestress-induced male factor infertility¹³⁻¹⁴.

The aim of the present study was to investigate free radical scavenging and antioxidant activity of TT pharmaceutical

preparations using DPPH, ABTS, FRAP and FTC methods. In addition, the quantification of total polyphenols and flavonoids were determinate using Folin-Chiocalteu reagent and AlCl₃, respectively. The obtained results of antioxidant activity were also compared with the values of reference synthetic antioxidants.

MATERIALS AND METHODS

Tribulus terrestris products

Tribestan™ standardized dry extract (250 mg TT dry extract contains more than 112.5 mg furostanol saponins) (LOT № 21211) (Product 1) and Tribestan™ film-coated tablets (LOT № 40312) (Product 2) were purchased from Sopharma AD, Bulgaria; Tribulus™ (250 mg TT dry extract) (LOT № L 002.05.2010) was purchased from Ecofruct, Bulgaria (Product 3).

1,1-Diphenyl-2-picrylhydrazyl (DPPH), linoleic acid, ferrous chloride, 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS), 2,4,6-tri(2-pyridyl)-s-triazine (TPTZ), butylated hydroxytoluene (BHT), potassium persulphate, Folin-Chiocalteu reagent and 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox™) were from Sigma-Aldrich USA. All the other chemicals used including the solvents, were of analytical grade. All solvents were of HPLC grade and were purchased from Merck (Darmstadt, Germany) or Sigma-Aldrich (St. Louis, MO).

Measurement of antioxidant activity

DPPH radical scavenging activity

Free radical scavenging activity was measured by using DPPH method¹⁵. Different concentrations (0.2 mL) of products in MeOH were added to 2.5 mL methanol solution of 2,2'-diphenyl-1-picrylhydrazyl (DPPH) (0.05 mM). The absorbance was measured spectrophotometrically at 517 nm after 30 min. Results were evaluated as percentage scavenging of radical:

$$(\%) = \frac{Abs_{control} - Abs_{sample}}{Abs_{control}} \times 100,$$

DPPH radical scavenging activity

Where Abs_{control} is the absorbance of DPPH radical in MeOH, Abs_{sample} is the absorbance of DPPH radical solution mixed with sample. IC₅₀

value (concentration of sample where absorbance of DPPH decreases 50 % with respect to absorbance of blank) of the sample was determined. All determinations were performed in triplicate (n=3).

ABTS radical scavenging assay

For ABTS assay, the procedure followed the method of Arnao *et al.*, 2001 with some modifications¹⁶. The stock solutions included 7 mM ABTS solution and 2.4 mM potassium persulphate solution. The working solution was then prepared by mixing the two stock solutions in equal quantities and allowing them to react for 14 h at room temperature in the dark. The solution was then diluted by mixing 1 mL ABTS solution with 30 mL methanol to obtain an absorbance of 0.70 ± 0.01 units at 734 nm using a spectrophotometer. A fresh ABTS solution was prepared for each assay. Different concentrations (0.2 mL) of preparations were allowed to react with 2.5 mL of the ABTS solution and the absorbance was taken spectrophotometrically at 734 nm after 7 min. The ABTS scavenging capacity of the compound was calculated as

$$(\%) = \frac{\text{Abs}_{\text{control}} - \text{Abs}_{\text{sample}}}{\text{Abs}_{\text{control}}} \times 100$$

ABTS radical scavenging activity, where $\text{Abs}_{\text{control}}$ is the absorbance of ABTS radical in methanol; $\text{Abs}_{\text{sample}}$ is the absorbance of an ABTS radical solution mixed with sample. IC_{50} value (concentration of sample where absorbance of ABTS decreases 50 % with respect to absorbance of blank) of the sample was determined. All determinations were performed in triplicate (n=3).

Ferric reducing/antioxidant power (FRAP)

The FRAP assay was done according to the method described by Benzie and Strain, 1996 with some modifications¹⁷. The stock solutions included 300 mM acetate buffer, pH 3.6, 10 mM TPTZ solution in 40 mM HCl, and 20 mM $\text{FeCl}_3 \times 6\text{H}_2\text{O}$ solution. The fresh working solution was prepared by mixing 25 mL acetate buffer, 2.5 mL TPTZ solution, and 2.5 mL $\text{FeCl}_3 \times 6\text{H}_2\text{O}$ solution and then warmed at 37 °C before using. 0.2 mL of 5 mg mL⁻¹ extract in MeOH was allowed to react with 2.8 mL of the FRAP solution for 30 min in the dark condition. Readings of the colored product (ferrous tripyridyltriazine complex) were then taken spectrophotometrically at 593 nm. Results are expressed in mg Trolox equivalent (TE). All determinations were performed in triplicate (n=3).

Determination of inhibition of lipid peroxidation in linoleic acid system by FTC method

The antioxidant activity of studied compounds against lipid peroxidation was measured through ammonium thiocyanate assay, as described by Takao *et al.*, with some modifications¹⁸. The reaction solution, containing 0.2 ml 5 mg mL⁻¹ extract in MeOH, 0.2 ml of linoleic acid emulsions (25 mg/ml in 99% ethanol) and 0.4 ml of 50

mM phosphate buffer (pH 7.4), was incubated in the dark at 40 °C. A 0.1 ml aliquot of the reaction solution was then added to 3 ml of 70 % (v/v) ethanol and 0.1 ml of 30% (w/v) ammonium thiocyanate. Precisely 3 min after the addition of 0.1 ml of 20 mM ferrous chloride in 3.5% (v/v) hydrochloric acid to the reaction mixture, the absorbance of the resulting red color was measured spectrophotometrically at 500 nm. Aliquots were assayed every 24 h until the day after the absorbance of the control solution (without compound) reached maximum value. BHT (5 mg mL⁻¹ in MeOH) was used as positive control. All determinations were performed in triplicate (n=3).

Determination of total polyphenols

The determination of total polyphenols was performed according to the European Pharmacopoeia¹⁹ involving Folin-Chiocalteu reagent and pyrogallol as standard. The analyses were carried out at 760 nm. The measurements were carried out using a Shimadzu UV-VIS spectrophotometer (Germany). The content of total polyphenols was calculated as % pyrogallol equivalent (PE). All determinations were performed in triplicate (n = 3).

Determination of total flavonoids content

The content of the flavonoids was spectrophotometrically determined at 430 nm by creating a complex with AlCl_3 according to the European Pharmacopoeia²⁰. The content of flavonoids was calculated as % hyperoside equivalent (HE). The measurements were carried out using a Shimadzu UV-VIS spectrophotometer (Germany). All determinations were performed in triplicate (n = 3).

RESULTS

DPPH, ABTS and FRAP activity

The radical scavenging activity of TT extracts was compared with those of BHT and expressed as IC_{50} (concentration of sample where absorbance of DPPH or ABTS decreases 50 % with respect to absorbance of blank) (Table 1). Product 3 possessed the highest DPPH activity (IC_{50} 2.84 mg mL⁻¹), although it is lower than BHT (IC_{50} 0.60 mg mL⁻¹). All samples demonstrate moderate ABTS activity compared to the control BHT (IC_{50} 0.02 mg mL⁻¹) with IC_{50} ranged from 0.28 mg mL⁻¹ (Product 1) to 0.30 mg mL⁻¹ (Product 3). In FRAP assay reduction of ferric tripyridyl triazine (Fe III TPTZ) complex to ferrous form (which has an intense blue color) at low pH can be monitored by measuring the change in absorption at 593 nm. The FRAP activity of TT extracts was compared with those of BHT and expressed as mg Trolox equivalent (TE). The studied TT preparations manifested stronger FRAP activity compare to the control BHT (2.29 ± 0.04 mg TE), whereas Product 3 was found to be the most potent (3.33 ± 0.02 mg TE).

Antioxidant activity in linoleic acid system

In the present study, the inhibition of lipid peroxidation of TT extracts was determined using FTC method (Table 2). All studied samples demonstrated ability in this method.

Table 1: DPPH, ABTS-radical scavenging and FRAP-activity of studied *Tribulus terrestris* products

Sample	DPPH IC_{50} mg mL ⁻¹	ABTS IC_{50} mg mL ⁻¹	FRAP mg TE
Product 1	4.56	0.28	2.76 ± 0.24
Product 2	4.30	0.31	2.44 ± 0.05
Product 3	2.84	0.30	3.33 ± 0.02
BHT	0.60	0.02	2.29 ± 0.04

Table 2: Antioxidant activity of *Tribulus terrestris* products in linoleic acid system

Sample	Absorption at 500 nm				
	1 st day	2 nd day	3 rd day	4 th day	5 th day
Control	1.13 ± 0.03	1.42 ± 0.20	1.94 ± 0.14	2.24 ± 0.02	2.66 ± 0.06
Product 1	0.90 ± 0.09	0.95 ± 0.04	1.02 ± 0.01	1.04 ± 0.35	1.07 ± 0.01
Product 2	1.01 ± 0.07	1.02 ± 0.01	1.07 ± 0.01	1.05 ± 0.01	1.11 ± 0.02
Product 3	1.11 ± 0.06	0.83 ± 0.03	1.13 ± 0.03	1.09 ± 0.04	1.19 ± 0.07
BHT	1.02 ± 0.03	1.00 ± 0.01	1.02 ± 0.03	1.01 ± 0.01	1.01 ± 0.03

Total polyphenols and flavonoids content

The amount of total polyphenols was expressed as % pyrogallol equivalent in 100 g dry extract, the flavonoid content was expressed as g hyperoside equivalent in 100 g dry extract (Figure 1). The amount of

total polyphenols ranged from 2.73 % \pm 0.007 % to 3.17 % \pm 0.008 %. The highest content of total flavonoids was found in the supplement - *Tribulus* (Product 3) (0.58 % \pm 0.01 %). Results from the correlation between total phenolics, flavonoids and antioxidant activity of studied preparations were presented in Table 3.

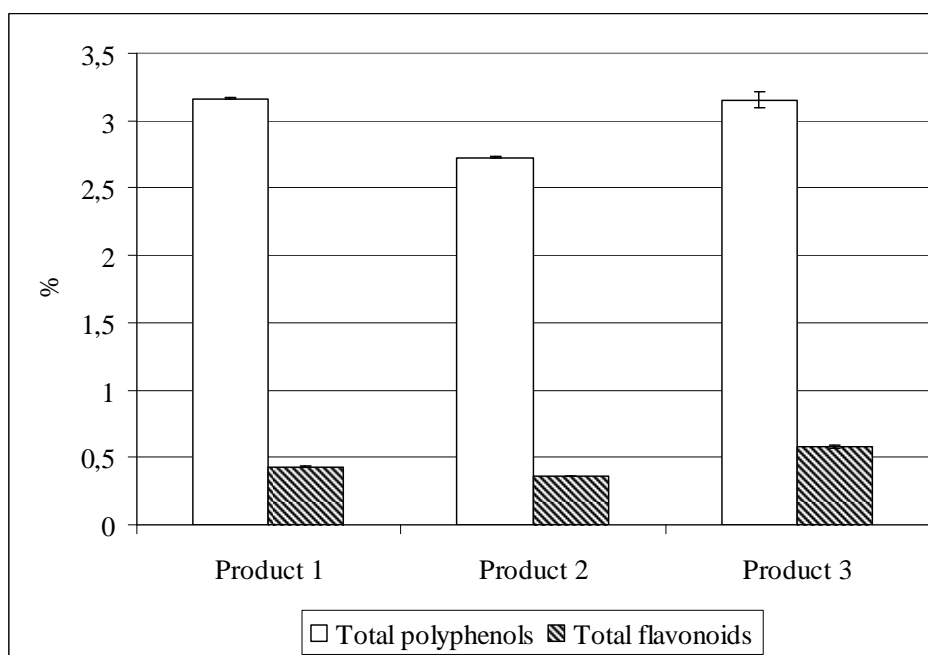


Fig. 1: Total polyphenols and total flavonoids content in studied *Tribulus terrestris* products

Table 3: Correlation between total polyphenols and flavonoids content and antioxidant activity of studied *Tribulus terrestris* products

Activity	Total polyphenols	Total flavonoids
DPPH	-0.355	-0.891
ABTS	-0.769	-0.139
FRAP	0.762	0.999

DISCUSSION

In the present study the radical scavenging and inhibition of lipid peroxidation of *Tribulus terrestris* commercial preparations were investigated for the first time. The obtained results of antioxidant activity were also compared with the values of reference synthetic antioxidant. In addition the quantification of the total polyphenols and flavonoids was carried out.

Numerous studies revealed DPPH radical scavenging activity of extracts from different well known medicinal plants as *Hypericum* ²¹, *Acacia catechu*, *Citrus limon* ²², *Stevia rebaudiana* ²³, *Trigonella foenum-graecum* ²⁴ as well as *Tribulus terrestris* ethanol extract ²⁵. However, no detailed evaluation of antioxidant capacity with different in vitro methods on TT commercial preparations was undertaken so far.

The DPPH- radical scavenging activity of studied samples decreased in order: BHT (IC₅₀ 0.60 mg mL⁻¹) > Product 3 (IC₅₀ 2.84 mg mL⁻¹) > Product 2 (IC₅₀ 4.30 mg mL⁻¹) \approx Product 1 (IC₅₀ 4.56 μ g mL⁻¹). There are no significant differences between ABTS radical scavenging activities of the tree TT preparations.

Generally, it is known that total polyphenols (a wide class of components including phenolic acids, catechins, flavonols and anthocyanins), are highly correlated with antioxidant activity, and the bioavailability of polyphenols has been reported ²⁶⁻²⁷. Previous investigation demonstrated that the quantity of main flavonoids in TT was about 1.5 times that of main saponins ²⁸.

The analysis of the correlation between the total phenolic compounds and flavonoids and antioxidant activities showed

significant dependence in the case of DPPH - free radical neutralizing properties and total flavonoids content ($r = -0.891$), as well as FRAP activity and the total phenolic compounds amount (-0.999) (Table 2). This fact unambiguously confirmed the importance of flavonoid amount on the rate of antioxidant activity and explained the stronger effect of Product 3 in DPPH and FRAP assays.

During linoleic acid peroxidation, peroxides were formed and these compounds oxidized Fe²⁺ to Fe³⁺. The Fe³⁺ ion formed a complex with SCN⁻, which had a maximum absorbance at 500 nm. Thus, a high absorbance value was an indication of high peroxide formation during the emulsion incubation.

The results obtained demonstrated that the absorbance of the control at 500 nm increased to a maximal value of 2.66 \pm 0.06 after 96 h. However, the antioxidant activity of studied preparations was slightly less effective than that of BHT; all of them inhibited lipid peroxidation stronger than the control. These results indicate that extract from *Tribulus terrestris* significantly reduce the formation of hydroperoxide, thus implying that this species is powerful natural antioxidants.

CONCLUSION

All tested TT preparations exhibited radical scavenging, antioxidant activity and strongly inhibited lipid peroxidation compared to the control (BHT). This plant is a possible new powerful natural source of antioxidants and could be useful in therapy of free radical pathologies. Therefore this investigation is useful for clarifying of pharmacological activity of *T. terrestris* and present new benefit of this plant in infertility therapy.

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