Volume : 2 | Issue : 4 | Apr 2013 • ISSN No 2277 - 8179

Research Paper

Antioxidant Activity of Thymoquinone and its Protective Effect Against Oxidative Hemolysis

Medical Science

KEYWORDS : AAPH, Diabetes, free radical (FR),Thymoquinone (TQ).

Shazia Aman	Research Scholar, Department of Biochemistry, J.N.M.C.H., Aligarh Muslim University, Aligarh, U.P., India-202002
Shagufta Moin	Associate Professor, Department of Biochemistry, J.N.M.C.H., Aligarh Muslim University, Aligarh, U.P., India-202002

ABSTRACT

Reactive oxygen species (ROS) and Reactive nitrogen species (RNS) play major role in various diseases like diabetes, cancer, neurodegenerative and immunodeficiency diseases etc. Detoxification of such reactive oxygen intermediates is an essential target, yet naturally occurring antioxidants are warranted for better management of diseases. In this study, 2,2-Diphenyl-1-picrylhydrazyl (DPPH) radical scavenging assay, reducing power and Ferric Reducing Antioxidant Power assay were used for evaluating the antioxidant activity. It was also tested that whether TQ in-vitro protects red blood cells from (AAPH)-induced hemolysis in diabetic and healthy subjects. It scavenges upto 78.13%. TQ demonstrated significant ferric reducing ability at very low concentrations in healthy and diabetic plasma samples. Furthermore, TQ was able to inhibit RBC hemolysis by 86.22% and 71.04% in normal and diabetic patients respectively. Our findings reveal that thymoquinone possess excellent antioxidant properties and could serve as a free radical scavenger, and this justifies its uses in alternative medicines.

1.INTRODUCTION

ROS are formed and degraded by all aerobic organisms, leading to either physiological concentrations required for normal cell function, or excessive quantities, the state called oxidative stress. Intracellular production of those oxygen intermediates threatens the integrity of various biomolecules including proteins [1], lipids as well as lipoproteins and DNA. ROS are the most lethal byproducts of metabolism that mediate many diseases including cancer, diabetes, immunodeficiency diseases and ageing [2,3]. There should be a balance between antioxidants and prooxidants to maintain the body 's optimal physiological conditions. Synthetic antioxidants, such as BHT and BHA have recently been suspected to cause negative health effects and therefore their applications have been restricted [4].

Many studies have demonstrated a correlation between the oxidative stress defense and the antioxidant properties of phytoconstituents [5,6]. Phenolic phytochemicals found in significant quantities in vegetables, fruits, spices and seeds. They have been regarded as possible antioxidants. Their roles in food industry and in chemoprevention of diseases resulting due to oxidative stress have become an area of active research [7,8].

TQ (2-isopropyl-5-methyl-1,4-benzoquinone), is the main bioactive component of the volatile oil of the black seed or black cumin (Nigella sativa, Ranunculaceae family). It has been used as antioxidant, anti-inflammatory and antineoplastic medicines for more than 2000 years [9,10]. Generally Nigella sativa seeds contain more than 30% fixed oil and 0.40% to 0.45% volatile oil [11]. TQ represents 18.4 to 24% of the N. sativa volatile oil [12]. The pharmacological investigations of the seed extracts reveal a broad spectrum of activities including antidiabetic [13], neuroprotective [14], antiasthmatic [15], anti-inflammatory [16] and antimicrobial [17]. In this study, we investigated the antioxidant properties of TQ and its effect on AAPH-induced hemolysis in erythrocytes obtained from diabetic patients and healthy subjects.

1.MATERIALS AND METHODS

Thymoquinone (TQ), 1,1-diphenyl-2-picrylhydrazyl (DPPH), 2,2-Azobis(2-amidinopropane) dihydrochloride (AAPH) were purchased from Sigma-Aldrich, USA. Rests of the general chemicals used were of analytical grade bought from Sisco (India), HiMedia (India) and Qualigens (India).

2,2-Diphenyl-2-picrylhydrazyl (DPPH) radical scavenging Activity

The free radical scavenging activity of TQ and standard reference compound i.e, Gallic acid was analyzed by the DPPH assay as described by Sanchez-Moreno et al. (1998)[18] with minor modification. In this assay, 1 ml of varying concentrations of TQ (0.25-2.0 mg/ml) dissolved in 1 ml of ethanol, were mixed with 1 ml of ethanol solution of DPPH (0.2 mM). The mixture was vortexed and incubated for 30 min. The optical density of the solution was measured at 517 nm using Hitachi 2010 spectrophotometer. Gallic acid (μg/ml) has been used as a standard. The DPPH radical scavenging activity was calculated from the absorption value by the following equation:

Radical scavenging activity (%) = [(OD control – OD sample)/ OD control] X 100

Reducing Power

Total reducing power was determined as described by Zhu et al., (2002)[19] with some modifications. TQ (0.5-2.0 mg/ml) in 1 ml of ethanol were mixed with 2.5ml of phosphate buffer (0.2M, pH 6.6) and 2.5 ml of 1% potassium ferricyanide [K3Fe(CN)6]; the mixture was then incubated at 50ºC for 30 minute. 2.5 ml of trichloroacetic acid (10%) was then added to the mixture, which was then centrifuged at 3000 rpm for 10 min. 2.5 ml of supernatant was mixed with 2.5 ml of distilled water and 0.5 ml FeCl3 (0.1%), and the absorbance was measured at 700 nm.

Ferric Reducing Antioxidant Power (FRAP) assay

FRAP asssay was carried out by the method of Benzie & Strain (1996)[20] with slight modification. The method is based on the reduction of a ferric 2,4,6-tripyridyl-s-triazine complex (Fe3+ -TPTZ) to its ferrous (Fe2+ -TPTZ), intensive blue colored form in the presence of antioxidant. 300 mM acetate buffer, pH 3.6, 10 mM TPTZ and 20 mM FeCl3 were mixed in a ratio of 10:1:1 to be a working FRAP reagent. 100 μl of TQ was mixed with 3ml of FRAP reagent and incubated at 37ºC for 30 min. The absorbance at 593 nm was monitored. All reagents were freshly prepared before used. Aqueous solution of known Fe (II) concentration was used for calibration (in a range of 100-1000 μmol/l).

AAPH-induced RBC hemolysis assay

Blood was obtained from healthy human donor and collected into heparinized tubes through the Blood Bank, J. N. Medical College, Aligarh Muslim University, Aligarh. Erythrocytes were separated from plasma and the buffy coat, and washed three times with 5 volumes of phosphate buffered saline (PBS), pH 7.4. During every wash, RBCs were centrifuged at 4000rpm for 10 min to obtain packed cell preparation [21]. The packed RBC was suspended in four volumes of PBS solution after the last wash. AAPH, a peroxyl radical initiator, was used for RBC hemolysis [21]. Addition of AAPH to the suspension of washed erythrocytes induces the oxidation of membrane lipids and proteins, resulting in hemolysis. 0.5 ml of the erythrocyte suspension was mixed with 0.5 ml of PBS solution containing varying amounts of TQ. 0.5 ml of 200 mM AAPH was added. The reaction mixture was shaken gently while being incubated at 37ºC for 3 hr. After incubation, reaction mixture was diluted with eight volumes of PBS and centrifuged at 4000 rpm for 5 min. The Absorbance (A) of the supernatant was recorded at 540 nm. Percent inhibition was calculated by the following equation:

$$\% \text{ Inhibition} = [A_{AAPH} - A_{Phytochemical}] / A_{AAPH}$$

Where A_{AAPH} is the absorbance of AAPH at 540 nm and $A_{Phytochemical}$ is the absorbance of thymoquinone at 540 nm.

RESULTS

DPPH assay

TQ was found to have good radical scavenging activity. And it was found to be concentration dependent. As shown in fig.1, TQ scavenges upto 78.13%. In a similar assay, gallic acid as standard reference compound scavenges upto 94.67%, having IC50 value of 2.867±0.404 µg/ml. IC50 value is the concentration at which antioxidants show 50% inhibition of free radicals generated. IC50 value of TQ is 1.07±0.036 mg/ml.

Fig.1. About DPPH assay here:

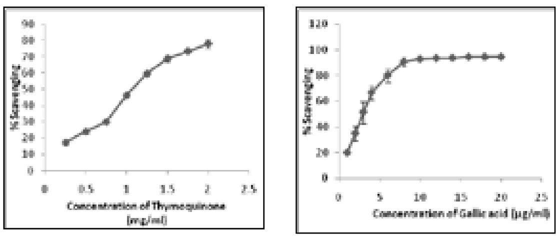

Fig.1: Percent DPPH scavenging activities of (a) TQ and (b) GA. All the points represent Mean ± SD of triplicate samples.

Reducing Power

Total reducing power of TQ was measured by Zhu et.al., method [19] in which reduction of $Fe3+(CN^-)6$ to $Fe2+(CN^-)6$ was determined in presence of TQ by measuring absorbance at 700nm resulting from the formation of Perl 's Prussian blue complex. Ascorbic acid was used as standard. In the present study, reducing power of TQ increased with the increase in concentration.

Fig. 2. About reducing power here:

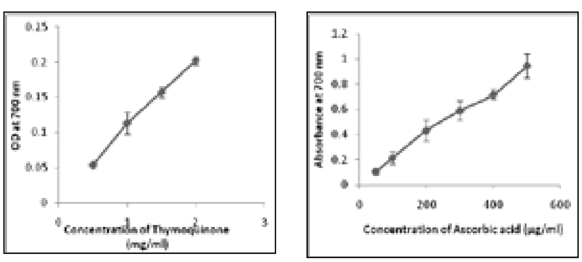

Fig.2: Total Reducing ability at various concentrations of (a) TQ and (b) Ascorbic acid. All the points represent Mean ± SD of triplicate samples.

FRAP assay

FRAP value was calculated from the standard graph of ferrous sulphate (Standard curve equation: Y= 0.71274x + 0.09092; r2= 0.999). FRAP value of TQ was found to be 2.731±0.592 mM Fe2+/mg of thymoquinone.

AAPH induced RBC hemolysis assay

The incubation of RBCs with different concentrations of thymoquinone (TQ) and ascorbic acid (AA) showed a potent protective effect against AAPH-induced RBC lysis. This protective effect also showed an increase in concentration dependent manner. 50% inhibition of RBC lysis was attained at a concentration of 40 and 7.5µg/ml in healthy subjects, when incubated with TQ and AA respectively. Similarly, IC50 of diabetic subjects was found to be 50 and 10 µg/ml when incubated with TQ and AA respectively.

Fig.3. About AAPH assay here:

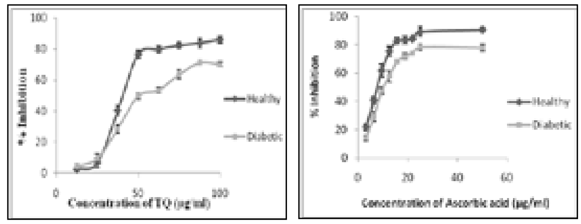

Fig.3: Percentage inhibition of RBC lysis in AAPH assay by increasing concentrations of (a)TQ and (b)AA in both healthy (♦) and diabetic (■) subjects. All the points represent Mean ± SD of triplicate samples.

DISCUSSION

Biomolecules, including lipids, proteins and DNA, are biotargets of ROS-mediated oxidative injury. Oxidative damage to these molecules is involved in the pathogenic mechanisms of many human diseases including Alzheimer 's diseases, Parkinson 's disease, diabetes mellitus, atherosclerosis, ischemia-reperfusion injury and ageing processes [22,23]. In the present study, TQ showed significant antioxidant and free radical scavenging ability which might be helpful in slowing the progress of various oxidative stress-related diseases.

The antioxidant activity of TQ was determined by DPPH radical scavenging ability. Many of the pharmacological activities have been attributed to the quinine constituents of the seed [11]. It is further supported by the reductive power of TQ which increases with the increase in concentration of TQ. Different studies have been indicated that the reducing properties are generally associated with the presence of reductones, which have been shown to exert protective action of antioxidant is usually due to inhibition of FR-induced chain reaction through the donation of a hydrogen atom [24,25].

The ability of phytochemicals to reduce ferric ions was determined by FRAP assay. TQ showed good reducing ability of ferric tripyridyl triazine complex into ferrous-(TPTZ) complex. All these results confirmed the good antioxidant power of TQ.

Red blood cells are vulnerable to lipid peroxidation due to their high content of polyunsaturated fatty acids (PUFA). Lipid oxidation of human RBC membrane mediated by AAPH, induces membrane damage and subsequent hemolysis [21]. The present study revealed that TQ protects these PUFA from oxidation in the membrane of RBC incubated with AAPH in both diabetic and normal subjects significantly. TQ inhibit RBC lysis upto 86.22±2.15% and 71.04±1.39% in healthy and diabetic blood respectively. These results indicate the possibility of employing TQ as an antioxidant substance to ameliorate the oxidative damage.

CONCLUSION

The data reported in the present study demonstrates that TQ has free radical scavenging activity and good reducing power. It can also provide protection against RBC hemolysis induced by free radicals or oxidative stress. Therefore, herbal medicines based on TQ can be used for the prevention and treatment of oxidative stress related disorders, such as, diabetes, vascular diseases, cancer and rheumatism.

ACKNOWLEDGEMENTS

Authors are thankful to the Department of Biochemistry, Faculty of Medicine, Aligarh Muslim University, Aligarh for instruement facilities created with the financial help. We also acknowledged UGC for funding scholarship.

REFERENCE

[1] Stadtman, E.R., Levine, R.L. (2000). Protein oxidation. Ann. N.Y. Acad. Sci. 899, 191-208. [2] Hassan, I., Chibber, S., Khan, A.A., Naseem, I. (2012). Riboflavin Ameliorates Cisplatin Induced Toxicities under Photoillumination. PLoS ONE 7(5): e36273. doi:10.1371/journal.pone.0036273. [3] Crujeiras, A.B., Díaz-Lagares, A., Carreira, M.C., Amil, M., Casanueva, F.F. (2013). Oxidative stress associated to dysfunctional adipose tissue: a potential link between obesity, type 2 diabetes mellitus and breast cancer. Free Radic. Res. 47(4), 243-56. [4]. Branen AL. (1975). Toxicology and biochemistry of butylated hydroxyanisole butylated hydroxytoluene. J. Am. Oil Chem. Soc. 52, 59-63. [5]. Aruoma OI. (2003). Methodological considerations for characterizing potential antioxidant actions of bioactive components in plant foods. Mutat Res; 523, 9-20. [6]. Amarowicz R, Pegg RB, Rahimi-Moghaddam P, Barl B, Weil JA. (2004). Free radical scavenging capacity and antioxidant activity of selected plant species from the Canadian prairies. Food Chem; 84, 551-562. [7]. Nakatani N. (2000). Phenolic antioxidants from herbs and spices. Biofactors 13, 141-146. [8]. Noguchi N, Niki E. (2000). Phenolic antioxidants: a rationale for design and evaluation of novel antioxidant drug for atherosclerosis. Free Radical Biol Med; 28, 1538-1546. [9] Trang NT, Wanner MJ, Phuong le VN, Koomen GJ, Dung NX. (1993). Thymoquinone from Eupatrium ayapana. Planta Med., 59; 99. [10] Hosseinzadeh H, Parvardeh S. (2004). Anticonvulsant effects of thymoquinone, the major constituent of Nigella sativa seeds, in mice. Phytomedicine, 11; 56-64. [11] Ali BH, Blunden G. (2003). Pharmacological and toxicological properties of Nigella sativa. Phytother. Res., 17, 299-305. [12] Arslan SO, Gelir E, Armutcu F, Coskun O, Grel A, Sayan H, Celik IL. (2005). The protective effect of thymoquinone on ethanol-induced acute gastric damage in the rat. Nutr. Res., 25, 673-680. [13] Arayne MS, Sultana N, Mirza AZ, Zuberi MH, Siddiqui FA. (2007). In vitro hypoglycemic activity of methanolic extract of some indigenous plants. Pak. J. Pharm. Sci., 20 (4), 261-268. [14] Ismail N, Ismail M, A. Latiff L, Mazian M, Mariod AA. (2008). Black cumin seed (nigella sativa Linn.) oil and its fractions protect against beta amyloid peptide-induced toxicity in primary cerebellar granule neurons. J. Food Lipids, 15, 519-533. [15] Boskabady MH, Mohsenpoor N, Takaloo L. (2010). Antiasthmatic effect of Nigella sativa in airways of asthmatic patients. Phytomed., 17 (10), 707-713. [16] Ragheb A, Attia A, Eldin WS, Elbarbry F, Gazarin S, Shoker A. (2009). The protective effect of thymoquinone, an anti-oxidant and anti-inflammatory agent, against renal injury: A review. Saudi J. Kidney Dis. Transpl., 20 (5), 741-752. [17] Allahghadri T, Rasooli I, Owalia P, Nadooshan MJ, Ghazanfari T, Taghizadeh M, Astaneh SDA. (2010). Antimicrobial property, antioxidant capacity, and cytotoxicity of essential oil from cumin produced in Iran. J. Food Sci., 75 (2), H54-H61. [18]. Sanchez-Moreno, Larrauri JA and Saura-Calixto F. (1998). A procedure to measure the anti-radical efficiency of polyphenols. J Sci Food Agric; 76, 270-276. [19]. Zhu JX, Wang Y, Kong LD, Yang C, Zhang X. (2004). Effects of Biota orientalis extract and its flavanoid constituents, quercetin and rutin on serum uric acid levels in oxonate-induced mice and xanthine dehydrgenase and xanthine oxidase activities in mouse liver. J Ethnopharmacol; 93, 133-140. [20]. Benzie IFF, Strain JJ. (1996). The ferric reducing ability of plasma (FRAP) as a measure of Antioxidant Power: the FRAP assay. Anal Biochem; 239, 70-76. [21]. Miki M, Tamai H, Mino M, Yamamoto Y, Niki E. (1987). Free radical chain oxidation of erythrocytes by molecular oxygen and its inhibition by α-tocopherol. Arch Biochem Biophys; 258, 373-380. [22] Hassan, I., Chibber, S., Naseem, I. (2010). Ameliorative effect of riboflavin on the cisplatin induced nephrotoxicity and hepatotoxicity under photoillumination. Food Chem. Toxicol. 48(8-9), 2052-8. [23]. Pham-Huy LA, He H, Pham-Huy C. (2008). Free radicals, antioxidants in disease and health. Int J Biomed Sci ; 4, 89-96. [24]. Shimada K, K Fujikawa K, Yahara and T Nakamura. (1992). Antioxidative properties of xanthan on the autooxidation of siyabean oil in cyclodextrin emulsion. J Agric Food Chem; 40, 945-948. [25]. Shon MY, TH Kim, NJ Sung. (2003). Antioxidants and free radical scavenging activity of Phelinus baumii extracts. Food Chem; 82, 593-597.