

## Antioxidant activity of thymol: protective role in AAPH-induced hemolysis in diabetic erythrocytes

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**ABSTRACT:** In the aerobic environment, reactive oxygen species are the most lethal by-products of metabolism that mediate many diseases including cancer, diabetes, immune deficiency diseases and ageing. Present study is aimed to assess the antioxidant activity of Thymol and its implication in diabetes in vitro. Thymol was evaluated by ferric reducing antioxidant power (FRAP) assay and measurement of its total reducing power, total phenolic content and 1,1-diphenyl 2-picrylhydrazyl (DPPH) scavenging activity. Furthermore, the extract in vitro was also checked if it protects red blood cells (RBCs) from 2,2-azo-bis(2-amidinopropane) dihydrochloride (AAPH)-induced hemolysis in the samples of diabetic patients and healthy subjects.

Thymol demonstrated good ferric reducing ability and high reducing power that can be attributed to its higher amount of phenolic contents. Moreover, it scavenges upto a great extent. Thymol was able to inhibit RBC hemolysis by  $86.69 \pm 2.34\%$  and  $72.04 \pm 2.1\%$  in normal and diabetic patients respectively.

Our findings reveal that thymol possess antioxidant properties and could serve as a free radical scavenger, and this justifies its uses in folkloric medicines.

**Keywords:** Thymol, Diabetes, Antioxidants and ROS.

### I. INTRODUCTION

In the aerobic environment, reactive oxygen species are the most lethal byproducts of metabolism that mediate many diseases including cancer, diabetes, immunodeficiency diseases and ageing. 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 application have been restricted [1].

Many studies have demonstrated a correlation between the oxidative stress defense and the antioxidant properties of phytoconstituents [2,3]. 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 [4,5,6,7,8].

Thyme has been commonly used in foods mainly for the flavor, aroma and preservation and also in folk medicine since the ancient Greeks, Egyptians and Romans. Thymol, carvacrol and terpinene are major constituents of the oils of thyme [9,10]. Thymol can be used for the treatment of oral infectious diseases because of their inhibitory activity on oral bacteria [11,12].

In this study, we investigated the antioxidant properties of thymol and its effect on AAPH-induced hemolysis in erythrocytes obtained from diabetic patients and healthy subjects.

### II. MATERIALS AND METHODS

#### 2.1. Materials

Pure thymol, granule and white solid substance, from Sigma. 2,2- diphenyl-1-Picrylhydrazyl (DPPH) and 2,2-azobis (2-methyl propion-amidine) dihydrochloride (AAPH) from Aldrich.

#### 2.2. Methods

##### 2.2.1. Determination of Total Phenolic Content

Total phenolic content was determined by the method of [13] with slight modification. In each analysis, 1.58 ml of distilled water was pipetted into test tubes, followed by addition of 20  $\mu$ l of standard solution, sample solution, or water. Vortex all tubes. Then 100  $\mu$ l of Folin-Ciocalteu's (FC) reagent was added to each test tube, and the solutions were mixed again. After 30s and before 8 minute, 300 $\mu$ l of 20% sodium carbonate solution was added. The solutions were left at room temperature for 2 hour. Then the absorbance of the developed blue colour was determined at 765 nm. Gallic acid was used as a standard for the calibration curve and reported as gallic acid equivalents (mg) using the following equation based on the calibration curve:

$$A = 0.0013x + 0.00409 \quad R^2 = 0.99765$$

Where “A” is the absorbance and “x” is the gallic acid equivalents (mg).

### 2.2.2. 2,2-Diphenyl-2-picrylhydrazyl (DPPH) radical scavenging Activity

The free radical scavenging activity of thymol and standard reference compound *i.e.*, Gallic acid was analyzed by the DPPH assay as described by [14] with minor modification. In this assay, 1 ml of varying concentrations of thymol (0.25-5.0 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 ( $\mu\text{g/ml}$ ) has been used as a standard. The DPPH radical scavenging activity was calculated from the absorption value by the following equation:

$$\text{Radical scavenging activity (\%)} = \frac{\text{OD Control} - \text{OD Sample}}{\text{OD control}} \times 100$$

### 2.2.3. Reducing Power

Total reducing power was determined as described by [15] with some modifications. Thymol (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 [ $\text{K}_3\text{Fe}(\text{CN})_6$ ]; the mixture was then incubated at 50°C 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  $\text{FeCl}_3$  (0.1%), and the absorbance was measured at 700 nm.

### 2.2.4. Ferric Reducing Antioxidant Power (FRAP) assay

FRAP assay was carried out by the method of [16] with slight modification. The method is based on the reduction of a ferric 2,4,6-tripyridyl-s-triazine complex ( $\text{Fe}^{3+}$ -TPTZ) to its ferrous ( $\text{Fe}^{2+}$ -TPTZ), intensive blue colored form in the presence of antioxidant. 300 mM acetate buffer, pH 3.6, 10 mM TPTZ and 20 mM  $\text{FeCl}_3$  were mixed in a ratio of 10:1:1 to be a working FRAP reagent. 100  $\mu\text{l}$  of thymol was mixed with 3ml of FRAP reagent and incubated at 37°C 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  $\mu\text{mol/l}$ ).

### 2.2.5. AAPH-induced RBC hemolysis assay

Blood was obtained from healthy human and diabetic patients separately 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 [17]. The packed RBC was suspended in four volumes of PBS solution after the last wash. AAPH, a peroxy radical initiator, was used for RBC hemolysis [17]. 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 thymol. 0.5 ml of 200 mM AAPH was added. The reaction mixture was shaken gently while being incubated at 37°C 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:

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

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

## 2.3. Statistical analysis:

All the data have been expressed as mean  $\pm$  Standard deviation (SD) for n=3. Statistical significance of the data was determined by student's t- test. The probability of occurrence was selected at p-value  $\leq$  0.05.

## III. RESULTS

### 3.1. Total phenolic content

The total amount of phenol was determined through Folin-Ciocalteu and has been expressed as milligrams equivalent to Gallic acid per milligram of phytochemical (Standard curve equation:  $Y = 0.0013x + 0.00409$ ;  $r^2 = 0.9953$ ). The ethanolic solution of thymol was found to have a good amount of phenolic compounds. It was found to have  $0.495 \pm 0.061$  mg GAE/mg of thymol.

### 3.2. DPPH assay

Thymol was found to have good radical scavenging activity. And it was found to be concentration dependent. As shown in fig.3, TL scavenges upto 82.26%. In a similar assay, gallic acid as standard reference compound scavenges upto 94.67%, having IC<sub>50</sub> value of 2.867µg/ml. IC<sub>50</sub> value is the concentration at which antioxidants show 50% inhibition of free radicals generated. IC<sub>50</sub> value of TL is 393.3±11.54 µg/ml (Fig.1).

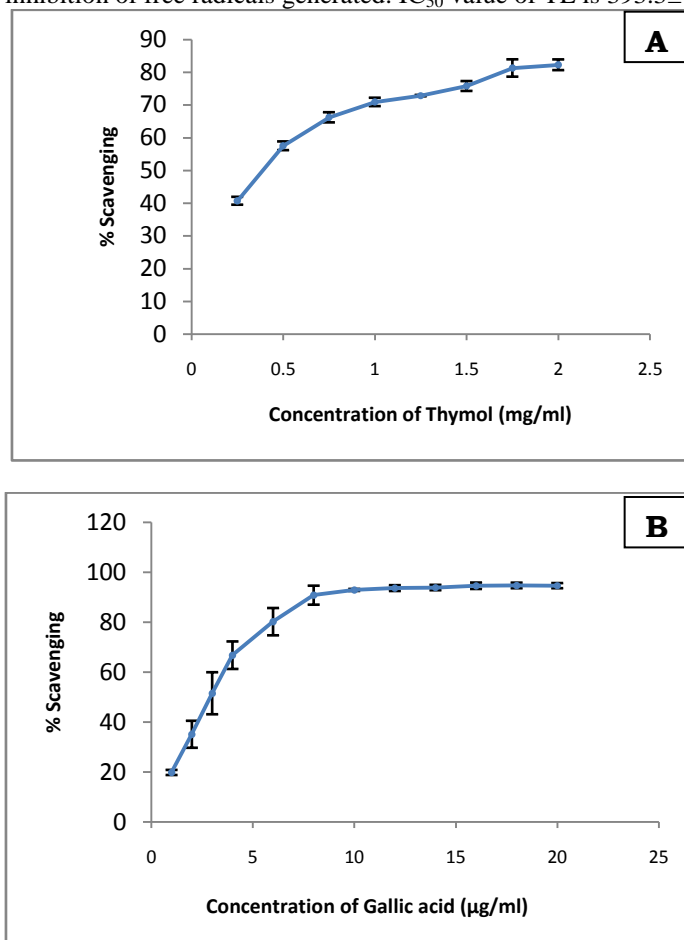
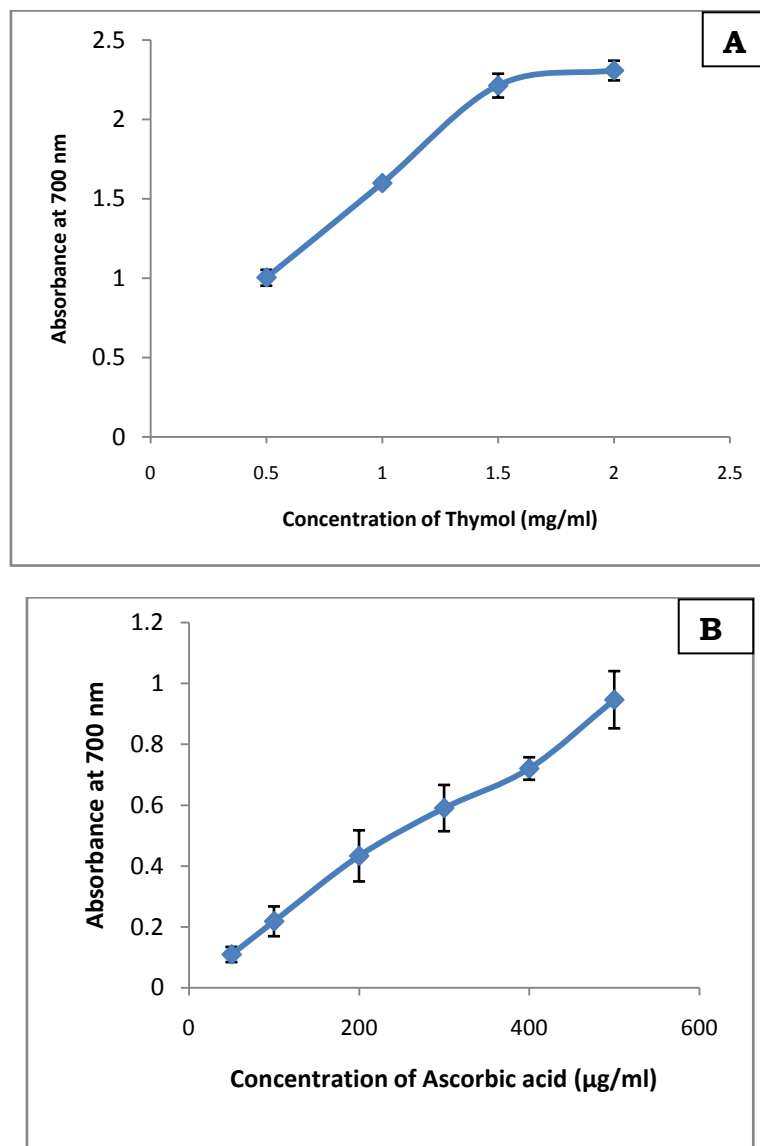


Fig.1: Percent DPPH scavenging activities of (a) TL and (b) GA. All the points represent Mean ± SD of triplicate samples.

### 3.3. Reducing Power

Total reducing power of TL was measured by Zhu *et.al.*, method [15] in which reduction of Fe<sup>3+</sup>(CN<sup>-</sup>)<sub>6</sub> to Fe<sup>2+</sup>(CN<sup>-</sup>)<sub>6</sub> was determined in presence of thymol 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 thymol increased with the increase in concentration (Fig. 2).



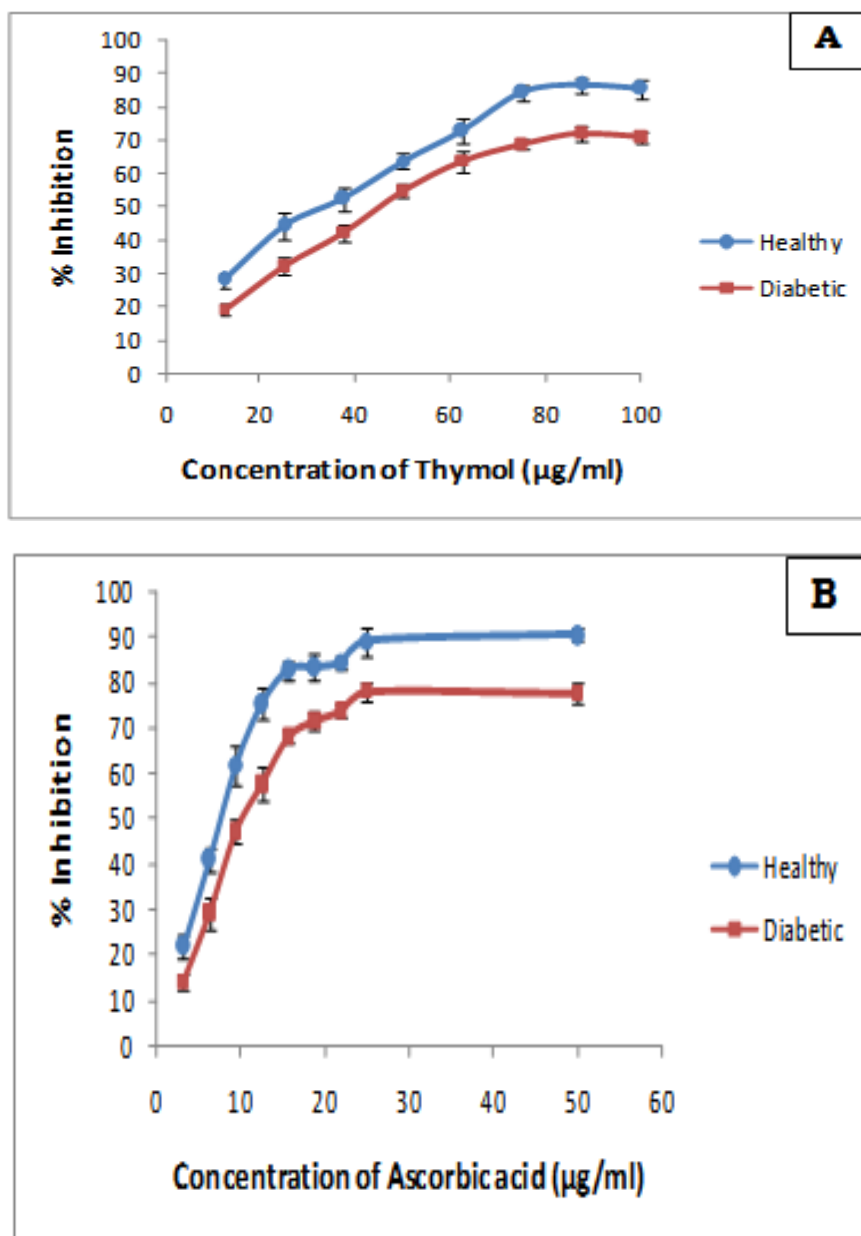
**Fig.2: Total Reducing ability at various concentrations of (a) TL and (b) Ascorbic acid. All the points represent Mean  $\pm$  SD of triplicate samples.**

### 3.4. FRAP assay

FRAP value was calculated from the standard graph of ferrous sulphate (Standard curve equation:  $Y = 0.71274x + 0.09092$ ;  $r^2 = 0.999$ ). FRAP value of TL was found to be  $25.315 \pm 7.693$  mM  $Fe^{2+}$ /mg of thymol.

### 3.5. AAPH induced RBC hemolysis assay

The incubation of RBCs with different concentrations of thymol 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 (Fig.3). 50% inhibition of RBC lysis was attained at a concentration of 32 and  $7.5 \mu\text{g/ml}$  in healthy subjects, when incubated with TL and AA respectively. Similarly,  $IC_{50}$  of diabetic subjects was found to be 45 and  $10 \mu\text{g/ml}$  when incubated with TL and AA respectively.



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

#### IV. DISCUSSION

Free radicals and oxidative stress are 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 [18]. These FRs induce damage to proteins, DNA, lipids and other biomolecules in oxidative stress-related diseases [19,20]. In the present study, thymol showed antioxidant activity and protected human RBC from free radical induced damage. Its significant antioxidant and free radical scavenging ability might be helpful in slowing the progress of various oxidative stress-related diseases.

The antioxidant activity of thymol was determined by DPPH radical scavenging ability. This method is based on the reduction of DPPH. It gives strong absorption maximum at 517nm due to the presence of odd electron of DPPH. As the odd electron of the radical becomes paired off in the presence of a hydrogen donor, which is a free radical scavenging antioxidant, the absorption strength decreases [21]. Decolorization in the presence of thymol is stoichiometric with respect to the number of electrons captured. Its excellent scavenging activity can be attributed to good amount of phenolic compounds. It is further supported by the reductive power of thymol which increases with the increase in concentration of thymol. Different studies have been indicated

that the reducing properties are generally associated with the presence of reductones, which have been shown to exert antioxidant action by breaking the FR chain through the donation of a hydrogen atom [22,23].

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

The biomembrane are mostly susceptible to FR attack due to its content of polyunsaturated fatty acids (PUFA). Lipid oxidation of human RBC membrane mediated by AAPH, induces membrane damage and subsequent hemolysis [17,15]. The present study revealed that thymol protects these PUFA from oxidation in the membrane of RBC incubated with AAPH in both diabetic and normal subjects significantly. Thymol protected RBC lysis upto  $86.69 \pm 2.34\%$  and  $72.04 \pm 2.1\%$  in healthy and diabetic blood respectively. Thymol showed protection against lipid peroxidation of RBC membrane, it can also be used in the prevention of cardiovascular diseases.

## V. CONCLUSION

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

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