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Study of Antioxidant Activity of Essential Oil of *Thymus serpyllum*

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This work is dedicated in memory of Professor Dr. Mushtaq Ahmad (Late).

ABSTRACT
The antioxidant activity of essential oil of *Thymus serpyllum* was determined by using DMPD/ferric chloride assay. The purple colored cation was formed by the oxidation of *N,N-dimethyl-p-phenylenediamine* (DMPD) with 0.05 M solution of ferric chloride. The purple colored radical cation obtained was decolorized by the antioxidants present in *Thymus serpyllum* oil. The antioxidant value of *Thymus serpyllum* oil was then measured by its ability to inhibit the color intensity of DMPD radical cation. It was also observed that the antioxidant value of *Thymus serpyllum* oil decreased with the passage of time.

Keywords: *Thymus serpyllum*; Antioxidant activity; DMPD/ferric chloride assay; DMPD radical cation

1. INTRODUCTION

Pure essential oils are a natural product and contain no preservatives or additives. A few of them can also be quite dangerous if not handled correctly. Some basic procedures should therefore be observed in order to ensure safety but also the quality of the oils in storage. Pure essential oils are extracted from an array of plant sources, petals, leaves, seeds, nut kernels, bark, stalks, flower heads, gums, resins of trees and contain the active ingredient of a plant in a highly concentrated and potent form. *Thymus serpyllum* belonging to genus *thymus* (include 300-400 species) has been extensively used for environmental, medicinal and edible uses[1-3]. *Thymus serpyllum* having many common names (wild thyme, creeping thyme, Breckland thyme, Ban Ajwain, Mother of thyme) belongs to family *Lamiaceae* (subfamily *Nepetoideae*, Tribe *Mentheae*) possessing interesting medicinal properties. Mostly plants contain abundant volatile aromatic oils, such as thymol (*Thymus*), Lavender oil (Lavendla), Rosemary oil (Rosemarinus) etc. All are used in perfumery[4]. *Thymus serpyllum* oil is a yellow liquid. The content of essential oil varies drastically with climate, time of harvest and storage conditions; extreme values are 0.75 % and 6.5 %. Antioxidants are substances or nutrients in our foods, which can prevent or slow the oxidative damage to our body. When our body cells use oxygen, they naturally produce free radicals (by-products), which can cause damage. Antioxidants act as "free radical scavengers" and hence prevent and stop damage done by these free radicals. Health problems such as heart disease, diabetics, cancer etc. are all contributed by oxidative damage. Antioxidants may also enhance immune defense and therefore low the risk of cancer and infection[5]. Evidences from biomedical and animal models suggest that nutritional antioxidant protects against the development of chronic diseases, including coronary heart disease, atherosclerosis and certain forms of cancer[6]. Antioxidant is a substance that when present in low concentrations relative to the oxidizable substrate significantly delays or reduces oxidation of the substrate. These are the substances that protect other substances from damaging oxidation reactions by reacting with free radicals and other reactive oxygen species within these substances. During this reaction the antioxidant is oxidized. Antioxidant plays an important role to detoxify such reactive oxygen intermediates in the oxidizable substances. Antioxidants act as chemical inhibitors to auto-oxidation damages. Examples of antioxidants include β-carotene lycopene, vitamins C, E and A, and other substances. Many antioxidants are highly substituted phenols, aromatic amines, amino acids and sulphur compounds.

Antioxidant action arises from a combination of several distinct chemical events, which include metal chelation, hydrogen donation from phenolic groups, oxidation to a non-propagating radical, redox potential, enzyme inhibition etc., interruption of auto-oxidation chain by an antioxidant taking place at the peroxy radical stage of the chain reaction. Antioxidants such as poly-phenols or aromatic amines act as chain breakers in the auto-oxidative process and usually not invariably oxidized themselves in doing so. Other act as synergists and enhance the activity of phenolic antioxidants, some other function by preventive mechanism and from inactive complexes with traces of pro-oxidant metals such copper, manganese or iron. The effectiveness of a particular antioxidant may be quite different in the presence and absence of enzymic, metallic pro-oxidant or of light[7].

The antioxidant activity of essential oils of *Thymus serpyllum* was determined by DMPD radical cation method and compared with that of standard antioxidant i.e. alpha-tocopherol and a relationship between the antioxidant activity and concentration of essential oil was determined[8].

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2. EXPERIMENTAL

2.1 *Materials and methods*

2.1.1 *DMPD/ferric chloride assay*

2.1.2 *Preparation of reagents*

All reagents and stock solutions required for the spectrophotometric studies were prepared in doubly distilled water, deionized water and ethyl alcohol, which were of analytical grade.

2.1.3 *DMPD solution (100 mM)*

209 mg of DMPD were dissolved in 10 ml of deionized water.

2.1.4 *Acetate buffer (0.1 M)*

2.2 g of Sodium acetate were dissolved in 1.5 ml of acetic acid and volume was made up to 250 ml with distilled water.

2.1.5 *Ferric chloride solution (0.05 M)*

0.807 g of Ferric chloride was dissolved in 100 ml of distilled water.

2.1.6 *Alpha-tocopherol solution*

The stock solution of alpha-tocopherol was prepared by dissolving 300 mg of alpha-tocopherol acetate in 100 ml of ethanol, heated in a water bath for three hours at 80 ^{o}C and cooled. The volume was made again 100 ml with ethanol. The strength of the solution will be 300 mg / 100 ml. This was used as stock solution. Different concentrations of alpha-tocopherol solution were prepared as follows.

- **1 *µg*:** 0.1 ml into 10 ml of ethanol.
- **2.5 *µg*:** 0.25 ml into 10 ml of ethanol.
- **5 *µg*:** 0.5 ml into 10 ml of ethanol.
- **7.5 *µg*:** 0.75 ml into 10 ml of ethanol.
- **10 *µg*:** 1 ml into 10 ml of ethanol.

2.2 *Procedure*

The standard protocol as developed by Fogliano *et al.*[9] was followed. 100 mM DMPD solution was prepared by dissolving 209 mg of DMPD in 10 ml of deionized water, 1 ml of this solution was added to 100 ml of 0.1 M acetate buffer, pH 5.25, and the colored radical cation ($DMPD^{*+}$) was obtained by adding 0.2 ml solution of 0.05 M ferric chloride. One milliliter of this solution was directly placed in 1 ml plastic cuvette and its absorbance at 505 nm was measured. An optical density of 0.900 $\pm$ 0.100 unit of absorbance was obtained and it represents the uninhibited signal.

Fifty microliters of standard antioxidant i.e. alpha-tocopherol or of oil samples were added in the spectrophotometric cuvette and after 10 minutes at 25 ^{o}C under continuous stirring, the absorbance at 505 nm was measured. The buffered solution was placed in the reference cuvette. A curve was derived for α-tocopherol by plotting the absorbance at 505 nm as percentage of the absorbance of the uninhibited radical cation solution (blank) according to the equation:

$$\text{Inhibition of } A_{505}\ (\%) = (1 - A_f / A_o) \times 100$$

Where A_o is the absorbance or uninhibited radical cation and A_f is the absorbance measured 10 min after the addition of antioxidant sample.

2.3 *Chemical reaction*

$$DMPD_{(uncolored)} + \text{oxidant } (Fe^{+3}) + H \longrightarrow DMPD^{*+}_{(purple)}$$

$$DMPD^{*+}_{(purple)} + AOH \longrightarrow DMPD^{+}_{(uncolored)} + AO$$

α-tocopherol was used as standard antioxidant and standard curve was drawn by taking inhibition curve along Y-axis and conc. of α-tocopherol along X-axis[9].

3. RESULTS AND DISCUSSION

This study was brought about to evaluate antioxidant activity of *Thymus serpyllum* using DMPD radical cation. Now a day, antioxidants are widely being used in a number of medicinal products including drugs for heart diseases, diabetes, and cancer etc. The reason to choose *Thymus serpyllum* for this study is because of its prominent

antioxidant activity, and furthermore its wide occurrence in Pakistan. The aim of this study is to highlight the medicinal importance of *Thymus serpyllum* to use it as a raw material in the local production of antioxidant drugs. Several assays have been introduced for the measurement of antioxidant activity of single compounds and /or a complex mixture. It is accepted that the effects exerted by different compounds in various environments require different methods to be evaluated.

A different approach based on the chromatic properties of stable radical cation was first envisaged by Blois (1958) who used the radical of *α,α-diphenyl-β-pycryl-hydrazyl* (DPPH) to measure the antioxidant activity of several natural compounds. More recent Miller (1993), using the color solution of the ABTS radical cation, were able to set up a very simple and efficient method to measure the antioxidant status of human plasma. The same author used ABTS also to assess the efficiency of antioxidant compounds from different vegetables developing a decolorization assay.

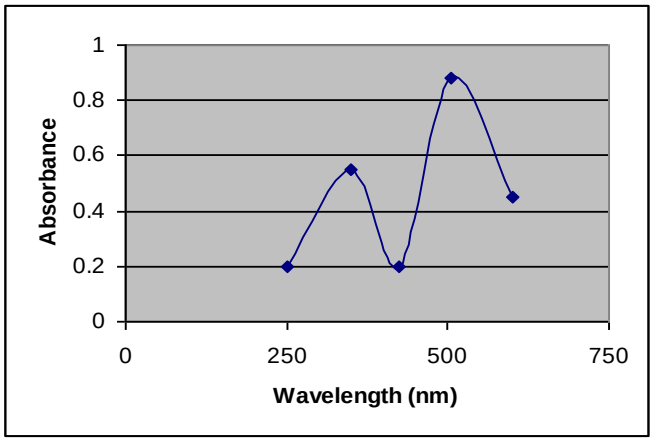

Fig-1: UV-Visible spectrum of the DMPD radical cation ($DMPD^{*+}$) that shows maximum absorbance at 505 nm.

A similar approach was followed by using *N,N-dimethyl-p-phenylenediamine* (DMPD). In the presence of suitable oxidant solution, a colored DMPD radical cation is formed. Antioxidant compounds which are able to transfer a hydrogen atom to $DMPD^{*+}$, cause a decolorization of the solution. $DMPD^{*+}$ radical cation was prepared by suitable oxidant solution at an acidic pH and λ_{max} was noted by using UV-Visible spectrophotometer. Spectrum of $DMPD^{*+}$ is reported in fig-1 that shows a maximum absorbance at 505 nm. The λ_{max} is also reported in the previous work[10,11]. Dependence of color formation on DMPD concentration is also noted. Absorbance at 505 nm was recorded 10 min after ferric chloride addition. The value of optical density remains stable for several hours, fig-2. Different oxidants were used previously for measuring antioxidant activity but the best results were obtained with ferric chloride which gives up to a final concentration of 0.1 mM, stable colored solution. DMPD solution, acetate buffer and ferric chloride solution were already prepared. A colored radical cation of DMPD was developed by mixing DMPD solution (1 ml), acetate buffer (250 ml) and ferric chloride solution and its absorbance was measured at 505 nm. Standard (α-tocopherol) and samples were separately added into cationic solution and absorbance was measured again at 505 nm. Activity of inhibition of standard (α-tocopherol) as well as sample was calculated through the valid equation and curves were drawn accordingly. Antioxidant activity was determined through these curves.

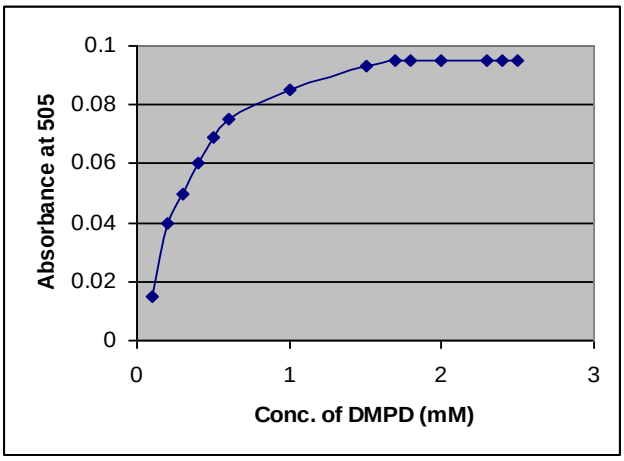

Fig-2: Dependence of color formation on DMPD concentration also noted. Absorbance at 505 nm was recorded 10 min after ferric chloride addition. The value of optical density remains stable for several hours.

The standard stock solution of α-tocopherol was prepared and then different concentrations from this stock solution were prepared. The absorbance and inhibition of standard α-tocopherol were given in the table-1, the standard curve for the absorbance was drawn from the observation shown in fig-3.

Table-1: Inhibition of DMPD with α-tocopherol.

Conc. of α-tocopherol (µg)	Absorbance (nm)	$(1-A_f / A_o) \times 100$ (%)
0.1	0.766	5.4
2.5	0.627	22.5
5.0	0.486	40
7.5	0.344	57.5
10	0.206	74.5

'A_f' is the absorbance measured 10 minutes after the addition of antioxidant
'A_o' is the absorbance of uninhibited radical cation
Absorbance of DMPD at 505 nm was 0.810

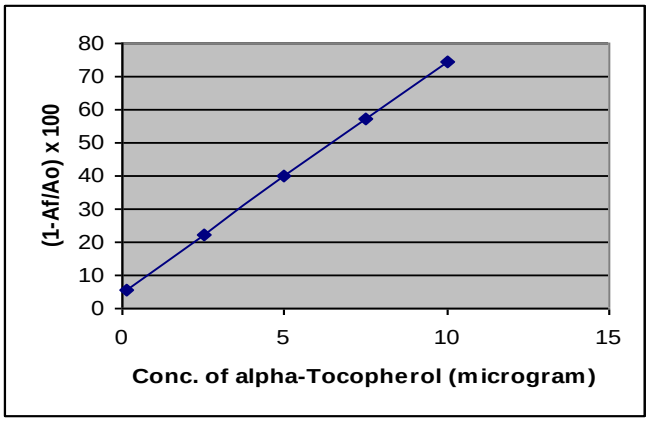

Fig-3: Calibration curve for the determination of antioxidant activity of *Thymus serpyllum* oil.

In our study, we took three samples. The antioxidant activity was measured of all three samples by taking the *Thymus serpyllum* extract in three different ways. The absorbance and inhibition for samples is shown in table-2. After that the concentration of antioxidant in the samples was calculated by comparing the standard α-tocopherol inhibition values.

Table-2: Concentration of antioxidant sample.

Type	Absorbance (nm)	$(1-A_f / A_o) \times 100$ (%)	Conc. with respect to α-tocopherol
Oil and α-tocopherol	0.416	41.40	5.23
Oil, α-tocopherol (acidic medium)	0.425	40.14	5.08
Oil, α-tocopherol (basic medium)	0.441	37.88	4.80

4. ACKNOWLEDGMENTS

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