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**Original article**

## Antioxidant and antimicrobial activity of *thymus vulgaris* L. on some pathogenic bacteria “*in vitro*”

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### ABSTRACT

Thyme (*Thymus vulgaris*) belongs to the Lamiaceae family, and has been used since antiquity in traditional medicine. It is recognized by its therapeutic virtues. The ancient-Iranians were familiar with many medicinal herbs and were aware of their usefulness in treatment of various diseases. In this study *Thymus vulgaris* L. to evaluate the antimicrobial activity, Disc Diffusion Test with Kirby-Bauer method was used. The Minimum Inhibitory Concentrations (MIC) and Minimum Bactericidal Concentration (MBC) were determined by using the dilution method. Methanolic extracts of *Thymus vulgaris* L. were investigated for their antioxidant activity by DPPH and FRAP assay. Data was analyzed by SPSS18 software and one-way ANOVA test analysis and Tukey test.  $p < 0.05$  was considered significant. The results show that methanolic extract were quite effective in 2000  $\mu\text{g/ml}$  concentration on *Pseudomonas aeruginosa* PTCC 1310 and *Streptococcus pyogenes* PTCC 1447 and were prevented from growth them on medium. In “disk agar diffusion method”, 10, 20, 30 and 40 % methanolic extract concentrations, was detertered effect on *Pseudomonas aeruginosa* PTCC 1310 and *Streptococcus pyogenes* PTCC 1447. The *Thymus vulgaris* L. extract presented the more effective impact on the growth of *Streptococcus pyogenes* PTCC 1447 than *Pseudomonas aeruginosa* PTCC 1310

( $p < 0.05$ ). IC<sub>50</sub> for antioxidant activity for *Thymus vulgaris* L. 1426.52  $\mu\text{g/ml}$  (by DPPH) and inhibition percent *Thymus vulgaris* L. 69 (by FRAP) and total phenolic content *Thymus vulgaris* L. 44.92 mg gallic acid equivalent/g of extract. Results showed *Thymus vulgaris* L. extracts can be used as natural antimicrobial in food products.

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## 1. Introduction

Iran has important potentialities in aromatic and medicinal plants because of the spontaneous flora; this is mainly connected to variety of its climate and nature of its grounds. The aromatic and medicinal plants are more and more used for several applications in pharmacy, medicine and food-processing therefore the necessity of their preservation. Wide spread of antibiotic resistance remains a serious clinical problem, which stimulates studies for search of new methods for coping with drug resistance or renews interest in traditionally used and forgotten methods, such as treatment with antibacterial plant extracts and essential oils (EOs). Combined therapy is traditionally used to increase antimicrobial activity and reduce toxic effects of agents (Alizadeh Behbahani et al, 2014; Ríos and Recio, 2005; Phillips et al, 2009).

The genus *Thymus* (thyme) contains about 350 species of aromatic perennial herbaceous plants and sub shrubs 40 cm tall in the family Lamiaceae, native to temperate regions in Europe, North Africa and Asia. Stems tend to be narrow or even wiry; leaves are evergreen in most species, arranged in opposite pairs, oval, entire, and small, 4 to 20 mm long, and usually aromatic. Thyme flowers are in dense terminal heads, with an uneven calyx, with the upper lip three-lobed, yellow, white or purple. Several members of the genus are cultivated as culinary herbs or ornamentals, when they are also called thyme after its best-known species, *T. vulgaris* or Thyme Green. *T. vulgaris* L. or common thyme is a low growing herbaceous plant, sometimes becoming somewhat woody. It is an evergreen shrub growing to 0.2 m (0 ft 8 in) by 0.3 m (1 ft) (Tabatabaei Yazdi et al 2013; Ahmad et al., 2010; Moghtader., 2012).

Phenolic compounds found in plants have been well known for their ability of scavenging free radicals, which is referred to as antioxidant activity (Young and Woodside 2001). Several assays have been frequently used to estimate antioxidant capacities for clinical studies including 2,2- azinobis (3-ethyl-benzothiazoline-6-sulfonic acid) (ABTS) (Leong and Shui, 2002; Miller and Rice, 1997), 2,2- diphenyl-1-picrylhydrazyl (DPPH) (Gil et al., 2002), ferric reducing antioxidant power (FRAP) (Guo et al., 2003; Jimenez et al., 2001), and the oxygen radical absorption capacity (ORAC) (Prior et al., 2003). The DPPH method can only be dissolved in organic media, especially in ethanol, this being an important limitation when interpreting the role of hydrophilic antioxidants. Both radicals show similar bi-phase kinetic reactions with many antioxidants. However, the ferric reducing antioxidant power (FRAP) method is based on the reduction of a ferroin analogue, the  $\text{Fe}^{3+}$  complex of tripyridyltriazine  $\text{Fe}(\text{TPTZ})^{3+}$  to the intensely blue-coloured  $\text{Fe}^{2+}$  complex  $\text{Fe}(\text{TPTZ})^{2+}$  by antioxidants in acidic medium.

Bacteria related food poisoning is the most common, but fewer than 20 different bacteria actually are the culprits. More than 90 percent of the cases of food poisoning each year are caused by *Staphylococcus aureus*, *Salmonella*, *Clostridium perfringens*, *Campylobacter*, *Listeria monocytogenes*, *Vibrio parahaemolyticus*, *Bacillus cereus*, and entero-pathogenic *Escherichia coli*. The antimicrobial properties of medicinal plant essential oils have been stirring interest from the perspective of their making up an alternative to the use of chemical additives in foods.

About 80 percent of the population of relies on traditional medicine because western-trained medical personnel are limited or not really accepted by the community, and traditional healers are easily consulted, living in the same community. That points to the demand for Traditional Medicine Practitioners (TMPs) for medicinal plants and the fact that the majority of the people, rural and urban alike, depend largely on herbal medicines for treating a variety of diseases. This reliance is mainly due to the high cost of conventional medicine and inaccessibility of modern health care facilities in most areas.

The aim of this research was to compare the total phenolics content and efficiency of DPPH and FRAP assays to estimate antioxidant activities and evaluated of antimicrobial effects of in *Thymus vulgaris* L. of the important food pathogen.

## 2. Materials and methods

### 2.1. Preparation of *Thymus vulgaris* L.

Indigenously grown plants were collected from local village markets, in Tehran, Iran. The plant material was thoroughly washed with clean water to remove soil and other dirt. Then the leaves were separated, air dried for complete drying. The dried plant material was powdered using a heavy duty blender (Tabatabaei Yazdi et al 2013).

### 2.2. Extract preparation

Maceration method was used to prepare extracts. The amount 50 gram of *Thymus vulgaris* L. powder was added to 250 ml methanol 96 degree. The alcoholic extract mixture was preserved at laboratory temperature for 24 hours and was stirred every few hours with a glass rod. The collecting supernatant was centrifuged by 3000 rpm for 10 min. The resulting extract (supernatant) volume has reached to the original with ethanol, and then samples were stored into the dark container at refrigerator temperature after filtering by 0.45  $\mu$  Whatman filter paper (Alizadeh behbahani et al, 2013).

### 2.3. Determination dry weight of alcoholic *Thymus vulgaris* L. extract

At first the weight of a tube were measured, and then 1ml of alcoholic extracts were poured in it. The contents of the tube were dried at room temperature. After drying the extract, the tubes were weighed again. Weight differences are equivalent weight of 1ml alcohol extract. Average of three replicates, was calculated as the dry weight of the extract (Alizadeh behbahani et al, 2014).

### 2.4. Source of microorganisms

Two strains were chosen for investigation of which one was reference bacteria, Gram positive *Streptococcus pyogenes* PTCC 1447 and Gram-negative. *Pseudomonas aeruginosa* PTCC 1310 all organisms were stored at  $-70^{\circ}\text{C}$  in glycerol Mueller-Hinton broth. Fresh subcultures were used for each experiment.

### 2.5. Determination of antibacterial activity

#### 2.5.1. Diffusion method

The disc diffusion method is used as a preliminary essay for antibacterial activity prior to more detailed studies. The determination of bacterial susceptibility was done by diffusion on Mueller-Hinton agar. The pH of Mueller-Hinton agar was adjusted at 7.2-7.4. Preparation of inoculums and inoculation by flooding method were realized according to the standard method described by Kirby and Bauer. The Mueller-Hinton agar was covered completely by bacterial suspension (2- 3 ml). Impregnated discs (6mm) with extract plant were applied to the bacterial surface. Then it was fixed on the media with a light little pressure. Inverted and incubated after 15 min at  $37^{\circ}\text{C}$  for 24 hours. After incubation time, the diameter of free zone was measured exactly by using a ruler in millimeters. All experiments were performed with 3 replicates (Bauer et al., 1996).

#### 2.5.2. Determination of minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC)

Dilutions of 400, 200, 100, 50, 25, 12.5 mg/l of ethanol extract of *Salvia* were prepared in sterile tubes. Then 100 microliters of each dilution by using a sampler to the higher concentrations less were added to 1 to 6 sterile U-shaped, Round-bottom 96-well micro plate then 100 microliter of 24 h culture of bacteria *Pseudomonas aeruginosa* PTCC 1310 and *Streptococcus pyogenes* PTCC 1447 with a turbidity equivalent to a standard 0.5 McFarland were separately added to the wells containing 100  $\mu\text{l}$  of the extract. In well No. 7, 100  $\mu\text{l}$  of bacterial suspension with a turbidity equivalent of the bacteria standard 0.5 McFarland Separately with 100  $\mu\text{l}$  MHB medium as a positive control and well No. 8, 200  $\mu\text{l}$  of sterile MHB medium was added as a negative control. Optical density of wells in micro plate ELISA reader at a wavelength of 630 nm was read. Then Micro plate was incubated for 24 h at  $37^{\circ}\text{C}$ . After 24 hours of re-absorption of light at a wavelength of 630 nm was determined by ELISA reader. Compared before and after incubation the optical density of each well and also check the wells, turbidity the lowest concentration of the test substance in the wells was related to no turbidity was observed in the concentration as the minimum inhibitory concentration (MIC) is considered. In order to determine the minimum bactericidal concentration (MBC) of the tested extracts, from the MIC concentration Wells and three wells of

further concentration which no detectable turbidity by sterile cotton swab was cultured on MHA medium and was incubated for 24 h at 37°C. After 24 hours of incubation, the plates were examined for bacteria growth. Concentration of tested extract on solid medium that no growth of tested bacteria was observed, as MBC was considered (Espinel-Ingroff et al. 2002). In order to confirm the results, experiments were repeated at 3times.

## 2.6. Determination of antioxidant using DPPH

The antioxidant activity of plant extract and the standard antioxidants were assessed on the basis of radical scavenging effect of the stable DPPH free radical. Gallic acid was used to prepare a standard solution. In a modified assay (Bruits et al., 2001), 200 µl of a 100 mM solution of DPPH radical in methanol was mixed with 20 µl of 12.5-3200 µg/ml extracts, gallic acid respectively and solutions were left at room temperature for 30 minutes. The DPPH radical inhibition was measured at 515 nm by using a micro-plate reader model Biotek ELx808. The IC 50 of each sample (concentration in µg/ml required to inhibit DPPH radical formation by 50%) was calculated by Matlab software. The extract methanolic solution without DPPH was used as a blank to be subtracted from all measurements. The antioxidant activity (AOA) is given by,

$$100 - [(A) \text{ sample} - (A) \text{ blank}] \times 100 / [(A) \text{ control}]$$

The IC50 value for each sample, defined as the concentration of the test sample leading to 50% reduction of the initial DPPH concentration, was calculated from the non linear regression curve of Log concentration of the test extract (µg/ml) against the mean percentage of the radical scavenging activity.

## 2.7. Ferric ion reducing activity (FRAP)

The FRAP assay was performed as described previously (Benzie and Strain, 1996). Briefly, 180µl of solution of FRAP(10 parts of 300 mM sodium acetate buffer at pH 3.6, 1 part of 10 mM TPTZ solution and 1 part of 20 mM FeCl3. 6H2O solution) was mixed with 20 µl of 12.5-3200 µg/ml methanolic extracts microplate in oven at ~37°C. Absorbance was determined at 595 nm after 6 min of incubation at room temperature by micro-plate reader (Biotek ELx808).

$$\text{Inhibition (\%)} = [(A) \text{ blank} - (A) \text{ sample}] / [(A) \text{ blank}] \times 100$$

## 2.8. Determination of total phenol content (TPC)

Total phenolic compound contents were determined by the Folin-Ciocalteu method (Ebrahimzadeh et al., 2008). The extract samples (0.5 ml of different dilutions) were mixed with Folin Ciocalteu reagent (5 ml, 1,10 diluted with distilled water) for 5 min and aqueous Na2CO3 (4 ml, 1 M) were then added. The mixture was allowed to stand for 15 min and the phenols were determined by micro-plate reader (Biotek ELx808) at 765 nm. The standard curve was prepared by 0, 50, 100, 150, 200, and 250 mg ml<sup>-1</sup> solutions of gallic acid in methanol, water (50,50, v/v). Total phenol values are expressed in terms of gallic acid equivalent (mg/g of dry mass).

## 2.9. Statistical analysis,

All the assays were carried out in triplicates. Data was analyzed by SPSS18 software and one-way ANOVA test analysis and Tukey test. p <0.05 was considered significant.

## 3. Results

The results of the antimicrobial effects of alcoholic extract, by “using the method of Collins et al. (1995)” were show on in Tables 1).

**Table 1**

Antimicrobial effects of 2000µg/ml alcoholic *Thymus vulgaris* L., extract concentrations, on *E.coli* (using the method of Collins et al. (1995)).

Microorganism	<i>Thymus vulgaris</i> L.
<i>Streptococcus pyogenes</i>	++
<i>Pseudomonas aeruginosa</i>	+

(++) in Table showed no bacterial growth on culture and strong antibacterial activity of alcoholic *Thymus vulgaris* L.

(+) in Table showed no bacterial growth on culture and strong antibacterial activity of alcoholic *Thymus vulgaris* L.

The results showed 2000 µg/ml concentration of both alcoholic extracts, were quite effective on reduce of growth *Streptococcus pyogenes* and *Pseudomonas aeruginosa* and were had prevent growth over the medium.

The results of the antimicrobial effects of alcoholic *Thymus vulgaris* L. extract, by "Kirby-Bauer" are presented in (Tables 2).

**Table 2**

Average diameter (mm) of microbial free zone area of by alcoholic *Thymus vulgaris* L. extract, on *Streptococcus pyogenes* and *Pseudomonas aeruginosa* (Kirby-Bauer).

Microorganism	P. aeruginosa			
	10	20	30	40
Thymus vulgaris L.concentration	10	20	30	40
Average diameter (mm) of microbial free zone area	8.2±0/24a	11.8±0/87b	14.5±0/57c	17 ±0/87d
Microorganism	S. pyogenes			
	10	20	30	40
Thymus vulgaris L.concentration	10	20	30	40
Average diameter (mm) of microbial free zone area	11.6±0/24a	14.9±0/52b	17±0/52c	20.6±0/87d

The values of MIC and MBC of ethanol extract of sage have been presented in table 3 against the referred bacteria. MIC of methanolic extract for *Streptococcus pyogenes* and *Pseudomonas aeruginosa* was 15.3 and 31.33 mg/ml and their MBC of the was 24.6 and 45.6 mg/ml, respectively.

**Table 3**

MIC and MBC of sage ethanol extracts against tested bacteria (mg/ml).

Microorganism	MIC	MBC
<i>Streptococcus pyogenes</i>	15.3	24.6
<i>Pseudomonas aeruginosa</i>	31.33	45.6

The study showed IC50 for antioxidant activity for *Thymus vulgaris* L. 1122.21 µg/ml (by DPPH) and inhibition percent *Thymus vulgaris* L. 74 µg/ml (by FRAP) (Table 4). Total phenolic content *Thymus vulgaris* L. 32.72 mg gallic acid equivalent/g of extract.

**Table 4**

Comparison of antioxidant activity *Thymus vulgaris* L. by FRAP and DPPH assay.

Thymus vulgaris L.	DPPH assay (radical scavenging activity)		
FRAP assay	Linear equation	R-squared	IC50 (µg/ml)
74	$y = 0.022x + 16.16$	$R^2 = 0.900$	1122.21

Data are displayed with mean ± SD.

#### 4. Discussion

Based on the results methanolic extract of *Thymus vulgaris* L. in this study has significant antimicrobial activity on the studied microorganisms. The results show that methanolic *Thymus vulgaris* L. methanolic extracted at all concentrations (10, 20, 30 and 40%) had the inhibitory effect on *Streptococcus pyogenes* and *Pseudomonas aeruginosa*. The results show that *Thymus vulgaris* L extracted at all concentrations had the inhibitory strong effect on *Streptococcus pyogenes*. *Thymus vulgaris* are recurrent flavonoids, these metabolites are a group of pigments contained in plants and they are responsible for flower and fruit coloration. Flavonoids are present in dietary fruit and vegetables and responsible for many biological properties included antioxidant activities (Tripoli et al, 2007).

*Thymus vulgaris* is a wide bush that grows in all Mediterranean areas including Iraq, the essential oil of common thyme (*Thymus vulgaris*), contains 20-54% Thymol. Thymol, an antiseptic, is the main active ingredient in various mouthwashes such as Listerine. Before the advent of modern antibiotics, oil of thyme was used to medicate bandages. Thymol has also been shown to be effective against various fungi that commonly infect toenails. Thymol can also be found as the active ingredient in some all-natural, alcohol-free hand sanitizer.

Thymol has microbial activity because of its phenolic structure. There is evidence supporting the belief that thymol, when applied two to three times daily, can eliminate certain kinds of fungal infections that affect fingernails and toenails in humans. Regular application to the affected nail over periods of about three months has been shown to eliminate the affliction by effectively preventing further progress by simply cutting the nail as one normally would, all infected material is eventually eliminated.

An important factor to consider when analyzing the results is the use of various thyme essential oil lots because, as Nascimento et al., (2007) point out, lots can be affected by intraspecific genetic variation and differences in the conditions of cultivation of the plant or preparation methods, such as climate, sowing time, soil, use of pesticides, use of fertilizers, state of plant material (dry or fresh) and extraction technique. These variations can affect the chemical composition of the oil and the contents of the active substances significantly, thereby influencing their antimicrobial activity. Furthermore, adverse storage conditions can affect the stability of the oil components and reduce their activity. In the present study, two different lots were mixed.

The antimicrobial nature of thymol is caused by thymol's ability to alter the hyphal morphology and cause hyphal aggregates, resulting in reduced hyphal diameters and lyses of hyphal wall (Numpaque et al., 2011). Additionally, thymol is lipophilic, enabling it to interact with the cell membrane, altering cell membrane permeability by permitting the loss of macromolecules (Segvic et al., 2007). The mechanism of action of extract plant and essential oil and their components as antimicrobials has not been fully elucidated. This is complicated by the fact that there are a large number of chemical compounds present in extract and EOs and often they are all needed for antibacterial activity and the extract and EOs does not seem to have a specific cellular target. Thus the antimicrobial mechanism of extract and EOs may not be attributable to one specific mechanism, but rather there may be several targets in the cell. Most of the focus on antimicrobial mechanisms for extract and EOs has been on the cell membrane and targets interconnected with the membrane. For bioactivity, the extract and EOs pass through the cell wall and cytoplasmic membrane (Bakkali et al., 2008).

## 5. Conclusion

In conclusion, *T. vulgaris* is an important source of phenolic compounds. The result of the present study showed that the extract of this plant contains high amount of flavonoids, and exhibited a great antioxidant and antibacterial activity. In this context, thyme can be used as an easily accessible source of natural antioxidants and antibiotics in commercial food products and drugs. It can suggest that *Thymus vulgaris* L extract in In-vitro have considerable antimicrobial ability over the studied strains. In addition, more studies are needed in In-Situ be done, to identifying the effective dose of the extract on the microorganisms, and finally introduce the extract as a natural and novel antimicrobial compound.

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