# Dose-dependent Medicinal Effects of *Thymus haussknechtii* Velen Grown Wild in Turkey

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**Abstract**: In this study, it was aimed to determine dose-dependent interactions between phenolic contents and antioxidant, antibacterial and antifungal effect mechanisms of the infusions of *Thymus haussknechtii* Velen, naturally grown in the Eastern Anatolia region of Turkey. Therefore, the infusions of *Thymus haussknechtii* were tested and the interactions between phenolic contents and antioxidant, antibacterial and antifungal effect mechanisms were determined by way of different antioxidant, antibacterial and antioxidant test systems. The concentrations of *Thymus haussknechtii* showed strong hydrogen peroxide scavenging activity and free radical scavenging activity [1,1-diphenyl-2-picryl-hydrazil (DPPH) % inhibition]. Also, it was seen that *Thymus haussknechtii* infusions possessed strong antibacterial and antifungal activity against different gram negative and positive bacteria and fungi. In this study, positive correlations between antioxidant, antibacterial, antifungal potency and the total phenolic content of *Thymus haussknechtii* were found. When the concentration differences were examined, it was seen that concentrations of 4% had the most strong antioxidant, antibacterial and antifungal activity. As a result, *Thymus haussknechtii* can be reliable antioxidant, antibacterial antifungal substance at concentrations of 4% when it is used as a supplement to therapeutic regimens and for medicinal purposes.

**Keywords**: *Thymus haussknechtii* Velen, antioxidant, antibacterial, antifungal, phenolic content.

## INTRODUCTION

Plants have been used especially in rural areas for traditional medicine to cure many diseases. There is an increased interest to use natural antimicrobial compounds, like plant extracts of medicinal plants possess a characteristic flavour and colour (Aladağ *et al.,* 2009). Herbal medicine including plant extracts represents one of the most important fields of traditional medicine in Asia, Latin America and Africa countries (Lis-Balchin and Deans, 1997; Ozturk and Ercisli, 2006). To promote the proper use of herbal medicine and to determine their potential as sources for new drugs, it is essential to study medicinal plants, which have folklore reputation in a more intensified way (Mothana and Lindequist, 2004).

According to World Health Organisation, 65-80% of the world populations rely on traditional medicine to treat various diseases because the majority of humans living in Asia, Latin America and Africa countries where traditional medicine is dominant (Gurinder and Daljit, 2009; Chew *et al*., 2011).

Lamiaceae (formerly Labiatae) is one of the most important plant families. In this family genus *Thymus* has special position and they have about 215 species found throughout the world (Zaidi and Crow, 2005). *Thymus* species are well known as medicinal plants because of their biological and pharmacological properties. Recent studies have confirmed that *Thymus* species have strong antibacterial, antifungal, antiviral, antiparasitic, spasmolytic and antioxidant activities (Stahl-Biskup and Saez, 2002). It is believed that flavonoids are responsible for these activities. Also this compound is known to be synthesized by plants in response to microbial infection (Karou *et al*., 2005; Gokturk *et al*., 2004).

The genus *Thymus* is represented in Turkey by 38 species, and the ratio of endemism in the genus is 53% (Tumen *et al*., 1998). Several *Thymus* species are locally known as "kekik" or "tas kekik" and their dried herbal parts are used in herbal tea, condiment and folk medicine. The essential oils of some *Thymus* species are identified with the presence of high concentration of the isomeric phenolic monoterpenes thymol and carvacrol (Azaza *et al*., 2004). Recent studies have showed that *Thymus* species have strong antibacterial, antifungal, antiviral, antiparasitic and antioxidant activities (Karaman *et al*., 2001; Rasooli and Mirmostafa, 2002; Vardar-Unlu *et al*., 2003).

*Thymus haussknechtii* is one of the endemic species of *Thymus* naturally grown in the Eastern Anatolia region (especially, Erzincan -Sivas) of Turkey and locally known as "kekik" or "kum anığı". Available literature indicates antibacterial, antifungal and antioxidant properties of *Thymus haussknechtii* (Selma *et al*., 2012). On the other hand, as far as we know in the best way there have not been reporting the dose-dependent antifungal, antibacterial and antioxidant properties of *Thymus haussknechtii*. Therefore, the aim in the present study was

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to determine the dose-dependent possibble antifungal, antibacterial and antioxidant properties of *Thymus haussknechtii*.

## MATERIALS AND METHODS

*Thymus haussknechtii* was collected from its wild habitat in Çayırlı, Erzincan province; specimen was deposited in the Erzincan University Herbarium, Turkey. *Thymus haussknechtii* used in this study was collected and identified by the second author. Aerial part of plant was air-dried in the dark room and then ground to powders using a mechanical grinder. Infusion was made by pouring 100ml of boiling water on 8 g of plant material. The mixture was left to stand for 20min and then filtered and diluted to the concentrations (1, 2, 4, 8% g/100ml). All bacterial strains used in this study, were obtained from the Refik Saydam Hygiene Center Presidency (RSHCP, Ankara, Turkey).

### *Total phenolic content*

Total phenol content was determined by the method adapted from Vinson *et al*. (Vinson *et al*., 1995), using the Folin-Ciocalteu reagent according to method of Singleton and Rossi (Singleton and Rossi, 1965). Briefly, 1.5ml of *Thymus* infusions, 1ml of HCl and 5ml of methanol were added to each tube. The tubes were capped, mixed thoroughly and heated at 90$^{o}$C for 2 hours. After 20min, the solution was completed to 10ml with distilled water. The solution was filtered and Folin-Ciocalteu reagent was added to the solution. The tubes were capped, mixed thoroughly and the blue coloration was read at 750nm against a blank standard. Results were expressed in milligrams of catechin/l of plant material extract.

### *Antioxidant activity tests*

$H_2O_2$ scavenging activity of *Thymus* infusions was measured by the method of Ruch *et al*. (Ruch *et al.*, 1989). A solution of hydrogen peroxide (40mM) was prepared in phosphate buffer (pH 7.4). Hydrogen peroxide concentration was determined as spectrophotometric by measuring the absorption at 230 nm using a spectrophotometer (Beckman DU 520). The infusions of *Thymus* were added to a hydrogen peroxide solution (4ml *Thymus* extracts +0.6ml $H_2O_2$). Absorbance of mixtures at 230nm was determined after 10 min against a blank solution containing phosphate buffer without hydrogen peroxide.

Free radical scavenging activity [1,1-diphenyl-2-picrylhydrazil (DPPH) % inhibition] was determined by the method of Yamaguchi *et al*. (Yamaguchi *et al*., 1998). 500µM solution of DPPH in absolute ethanol was prepared. Then, 1ml of the solution was added to 0.2ml of *Thymus* infusions. Also, 0.8ml of Tris-HCl was taken and 100mM Tris-HCl buffer (pH 7.4) and added to the solutions. The mixtures were shaken vigorously and allowed to stand in darkness and at room temperature for 20min. Then the absorbance was measured at 517nm using a spectrophotometer (Beckman DU 520).

### *Preparation of discs*

What man 1 filter paper was cut 6mm in diameter and discs were autoclaved at 121$^{o}$C for 15min. (Trans Trst MT-25-30A) made sterile. Discs were prepared and 20µl infusions were absorbed for each disc.

### *Antimicrobial activity tests*

Bacterial and fungal suspensions were prepared with the recommended by Clinical Laboratory Standards Institute (CLSI, 2002). Briefly, bacterial suspension was prepared with direct colony suspension method from 24-hour culture of bacteria in 0.9% normal saline (NaCl) to be equal turbidity of 0.5 McFarland ($1\times10^{8}$ CFU/ml). Densitometer device (Den-1, Latvia) was used for measuring the concentration of cell. Suspensions were inoculated on Mueller-Hinton agar (Oxoid CM337) with the help of swab. After the medium surface was dried, the prepared extraction discs and control of antibiotic discs (Penicillin10U, Erythromycin 15µg, Chloramphenicol 30µg, Ampicillin 10µg and Nystatin 10µg) were placed on medium surface. Antimicrobial and antifungal activities were determined by measurement of zone of inhibition around each paper disc. The discs zone diameters on the Mueller-Hinton agar surface were measured at 35$^{o}$C after 24 hours incubation for the bacteria and at 30$^{o}$C after 24 hours incubation for *Candida albicans*.

## STATISTICAL ANALYSIS

Data are presented as mean standard deviation (means ± S.D.) of at least three independent experiments. One-way ANOVA followed by Scheffe's test were performed to determine statistical differences between groups with the aid of SPSS soft ware version 11.0 (SPSS, Chicago, IL, USA). Differences were considered significant at $P<0.05$. Experiments were repeated three times.

## RESULTS

Antioxidant effect mechanisms of different *Thymus* infusions were determined according to a direct interaction with total phenolic content of *Thymus* infusions. Remarkable increasing of total phenol content dependent increasing concentrations of *Thymus* infusions was determined. The total phenol content of *Thymus* infusions is presented in (table 1).

The antioxidant capacity was measured by using methods of Ruch *et al*. (Ruch and others 1989) and Yamaguchi *et al*. (Yamaguchi *et al*., 1998). A statistical significant difference ($p<0.05$) was found between the control and *Tymus haussknechtii* infusions in $H_20_2$ scavenging activity potential (table 2). The free radical scavenging capacity of

**Table 1**: Total phenol content of *Thymus haussknechtii* infusions

| Sample | *Thymus haussknechtii* Infusions | | | |
|---|---|---|---|---|
| | 1 % | 2 % | 4 % | 8 % |
| Total Phenol Content (mg/l catechin equivalent) | 87±5.36a | 186±6.42b | 315.7± 4.34c | 612±3.28d |

**Table 2**: Effects of *Thymus haussknechtii* infusions on antioxidant mechanisms

| Parameters | Control | *Thymus haussknechtii* Infusions | | | |
|---|---|---|---|---|---|
| | | 1 % | 2 % | 4 % | 8 % |
| $H_20_2$ Scavenging Activity (mM $H_2O_2$) | 3.984± 0.2082a | 1,721± 0.112b | 0.145± 0.021c | 0.058± 0.002d | 0.186± 0.026e |
| Free Radical Scavenging Activity (% DPPH inhibition) | 0.00a | 18.914± 2.172b | 44.72± 1.374c | 83.39± 1.210d | 77.41± 1.432e |

Values were expressed as means ± S.D., n=3. Different letters on the same line were considered to be statistically significant when $p<0.05$.

**Table 3**: Antibacterial activities of the various infusions of *Thymus haussknechtii* using agar disc diffusion method

| Bacterium Name and Numbers | | *Thymus* Sensitivity | | | | Antibacterial Substances Sensitivity | | | |
|---|---|---|---|---|---|---|---|---|---|
| Name | Number | 1% | 2% | 4% | 8% | Penicillin 10 U | Erythromycin 15 µg | Chloramphenicol 30µg | Ampicillin 10µg |
| *Bacillus cereus* | ATCC 10876 | 6mm | 16mm | 24mm | 22mm | 10mm | 28mm | 30mm | 18mm |
| *Bacillus sphaericus* | ATCC 4525 | - | 13mm | 18mm | 18mm | 18mm | - | 22mm | 17mm |
| *Bacillus subtilis* | ATCC 6633 | 7mm | 15mm | 22mm | 22mm | 25mm | 30mm | 30mm | 18mm |
| *Bordetella bronchiseptica* | ATCC 10580 | - | 7mm | 11mm | 11mm | - | 16mm | 25mm | 12mm |
| *Enterococcus faecalis* | ATCC 29212 | 5mm | 12mm | 26mm | 26mm | 22mm | 25mm | 32mm | 30mm |
| *Escherichia coli* | ATCC 25922 | - | 7mm | 11mm | 12mm | - | 11mm | 25mm | 22mm |
| *Escherichia coli O: 157 H: 7* | RHFS 232 | - | 8mm | 16mm | 16mm | - | 12mm | 22mm | 23mm |
| *Klebsiella pneumoniae* | ATCC 13883 | - | 12mm | 20mm | 19mm | - | 13mm | 28mm | 20mm |
| *Listeria monocytogenes* | ATCC 7644 | - | 9mm | 19mm | 18mm | - | 9mm | 20mm | 24mm |
| *Micrococcus luteus* | ATCC 9341 | 11mm | 17mm | 34mm | 32mm | 20mm | 23mm | 28mm | 20mm |
| *Proteus mirabilis* | ATCC 7002 | 7mm | 12mm | 20mm | 18mm | 15mm | - | 20mm | 23mm |
| *Pseudomonas aeruginosa* | ATCC 27853 | - | 7mm | 13mm | 13mm | - | - | - | 14mm |
| *Salmonella enteritidis* | ATCC 13076 | - | 10mm | 21mm | 20mm | - | 11mm | 34mm | 25mm |
| *Salmonella typhimurium* | ATCC 14028 | - | 9mm | 18mm | 19mm | - | 12mm | 33mm | 22mm |
| *Staphylococcus aureus* | ATCC 25923 | - | 10mm | 25mm | 26mm | 20mm | 21mm | 23mm | 29mm |
| *Staphylococcus aureus* | ATCC 16217 | - | 12mm | 25mm | 25mm | - | 19mm | 22mm | 20mm |
| *Streptococcus epidermidis* | ATCC 12228 | - | 13mm | 40mm | 38mm | 16mm | 20mm | 22mm | 22mm |
| *Streptococcus pyogenes* | ATCC 21599 | - | 7mm | 24mm | 23mm | 11mm | - | 19mm | 20mm |

Key: - → no measurable zone

the *Tymus haussknechtii* infusions against common free radicals (DPPH) *in vitro* were further determined. Table 2 shows the DPPH scavenging activities of the *Tymus haussknechtii* infusions.

Plants are important source of potentially useful structures for the development of new chemotherapeutic agents. The first step towards this goal is the *in vitro* antibacterial activity assay. Therefore, *in vitro* antibacterial activity of *Tymus haussknechtii* was evaluated by disc diffusion method using Mueller-Hinton Agar with determination of inhibition zones (IZ). Results (table 3) showed that except for 1% the infusions of *Thymus haussknechtii,* which showed antibacterial activity against only five bacteria. The other *Thymus haussknechtii* infusions have great potential of antibacterial activity with increasing concentrations against all of the eighteen bacteria tested. The IZ values for bacterial strains, which were sensitive to *Thymus haussknechtii*, were in the range of 5-40mm. Especially, 4% infusions of *Thymus haussknechtii* showed the highest inhibitory activity against *Streptococcus epidermidis* (40mm) and least activity observed in *Enterococcus faecalis* (5mm) at 1% infusions of *Thymus haussknechtii*. Also, 4% and 8% infusions of *Thymus haussknechtii* showed significant antifungal activity against *Candida albicans* when compared with Nystatin

**Table 4**: Antifungal activities of different infusions of *Thymus haussknechtii* using agar disc diffusion method

| Bacterium Name and Numbers | | *Thymus* Sensitivity | | | | Antifungal Substance Sensitivity |
|---|---|---|---|---|---|---|
| Name | Number | 1% | 2% | 4% | 8% | Nystatin 10 µg |
| *Candida albicans* | ATCC 10231 | - | 15mm | 21mm | 21mm | 22 mm |

NA → not applicable, Key: - → no measurable zone

(table 4). On the other hand, it was not seen any antifungal activity at the 1% infusions of *Thymus haussknechtii* against *Candida albicans*.

## DISCUSSION

It is well-known that phenolic compounds contribute to quality and nutritional value in terms of modifying color, taste, aroma, and flavor and also in providing health support effects. They also serve in plant defense mechanisms to counteract reactive oxygen species (ROS) in order to survive and prevent molecular damage and damage by microorganisms, insects, and herbivores (Vaya *et al*., 1997; Sengul *et al*., 2009). A number of studies showed that antioxidant and antimicrobial activity of plant extracts is correlated with total phenol contents rather than with any individual phenolic compound (Prior *et al*., 1998; Kilicgun and Altiner, 2010). As a matter of fact, current results showed that *Tymus haussknechtii* exhibited a potential free radical scavenging activity. The antioxidant activity of *Tymus haussknechtii* infusions may depend on the presence of polyphenols, which may act as a reducing agent. As a matter of fact in a study, it was found that the higher the phenolic content of the extract, the stronger the free radical scavenging activity (Chew *et al*., 2011). On the other hand, the same increasing for DPPH radical scavenging activity and $H_20_2$ scavenging activity could not be determined at higher concentrations (8%). Furthermore, the scavenging activity significantly decreased at higher concentration (table 2). This finding suggests that the same plant optimizing antioxidant capacity may indicate a concentration-dependent pro-oxidant activity. This result can be interacted with increasing phenolic content of the *Tymus haussknechtii* infusions at higher concentrations (8%). As a matter of fact, several studies also have reported that phenolics act as pro-oxidants, under certain conditions, such as high doses or the presence of metal ions (Raza and John, 2005; Watjen *et al*., 2005).

When the antibacterial and antifungal activity results of *Thymus haussknechtii* were examined, it was seen that the antibacterial and antifungal activities of *Thymus haussknechtii* increased with increasing concentration (from 2 to 8g/100ml). These effects may be attributed to polyphenol compounds of *Thymus haussknechtii*. Moreover, recent studies imply that *Thymus* species have strong antioxidant antifungal and antibacterial activities because of their potential sources of phenolic compounds (Mahesh and Satish, 2008; Proestos *et al*., 2005; Tepe *et al*., 2011). However, to the best of our knowledge, no detailed study concerning dose-dependent properties for the preventive effects on *in vitro* antioxidant antifungal and antibacterial activities of *Thymus haussknechtii*, has been made so far. This may be the first and promising study to provide data on this matter.

## CONCLUSION

Current findings confirm the antibacterial, antioxidant and antifungal activity of *Thymus haussknechtii.* These results have the using possibility of *Thymus haussknechtii* for drug development and human consumption for the treatment of infectious diseases, especially wound infections caused by resistant microbes. The effects of *Thymus haussknechtii* on more pathogenic organisms and toxicological investigations and further purification however, needs to be carried out. Also, However, to establish *Thymus haussknechtii* utility as especially a natural antimicrobial, antioxidant and antifungal agents in treatment of infectious diseases, further researches are needed to evaluate the effectiveness of *Thymus haussknechtii*.

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