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# INVESTIGATING THE ANTIBACTERIAL EFFECTS OF AQUEOUS AND ALCOHOLIC EXTRACTS OF THYME ON *BACILLUS SUBTILIS* BACTERIAS

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**ABSTRACT**

Thyme has been used as flavoring in many Iranian foods and its anti-microbial and anti-oxidant effects have been proven. For extraction for the aqueous extract 10 grams of leaves were initially added into 100 ml of distilled water and for the alcoholic extract, 10 grams of thyme leaves were added in 100 ml of ethanol 96%. In this study the standard *B.Subtilis* strain was used. For MIC and MBC study medium was Muller-Hinton Broth medium in which $5\times10^5$ active bacteria has been added per milliliter of liquid medium. Tubes were incubated for 48 h at 37 ° C and then results were monitored. In the MIC test of alcoholic extract of thyme, bacterial growth was observed within dilutions higher than 1.56%. MBC dilution was also within 1.56%. In the performed testing, MIC and MBC were both within 12.5% dilution.

**Keywords: Extract, Thyme, Bacillus Subtilis, MBC, MIC**

## INTRODUCTION

Considering the specific climatic conditions, Iran is one of the richest sources of medicinal plants [1]. Thyme is one of the most important a species of Lamiaceae family which includes approximately 215 genuses of perennial herbaceous and small shrubs in the world and 14 species of these genuses grow in different parts of Iran [1]. Thyme has been used as flavoring in many Iranian foods and its anti-microbial and anti-oxidant effects have been proven [2]. Antimicrobial activity of different species of thyme is related to phenolic compounds such as thymol, carvacrol [2, 3] and gamma terpinen [3]. The sensitivity of bacteria and fungi against thyme is different. Gram-negative bacteria are the most resistant and yeasts are the most sensitive microorganisms to the antimicrobial activity of thyme and Gram-positive bacteria show a moderate sensitivity [4]. The existing Carvacrol and thymol in thyme constitute a major component of the extract [5]. The essential oil of flower and leaves of thyme, has anti-spasmodic, carminative, anti-rheumatic and anti sciatica properties and is a powerful disinfectant [5]. Thyme oil has anti-bacterial and anti-fungal properties and stimulates the immune system. This plant is also used to treat rheumatism, muscle sprains, insect bites, disinfect wounds, pulmonary disease, and chronic cough, sore throat, cold stomach, intestines and stomach irritation [6]. The oil of this plant is also used for methods such as aromatherapy, fatigue, depression and irritation of skin and scalp [5, 6]. So many studies have been performed on chemical compounds and effects of extract and essential oils of thyme [7, 8, 9, 10]. But since the weather conditions and probably chemotype of the plant has large impact on the chemical composition of the extracts and essential oils and also no study has ever performed on the effects of thyme on bacillus subtilis, hence the present study was performed. The present study aims to investigate the effect of alcoholic and aqueous extracts of the *Zataria multiflora* Boiss plant on *Bacillus subtilis* bacterium. *Bacillus subtilis* is a saprophytic bacterium that can be easily separated from soil, air and water. This bacterium has a high competition with other microorganism. *Bacillus subtilis* produces lipopeptides that have antibiotic properties. The lipopeptides that are produced by *B.Subtilis* include ITURINS. *B.Subtilis* bacteria have many applications such as control of plant diseases in agriculture. *B.Subtilis* has not ever been raised as a human pathogen but this bacteria produce certain enzymes causing the allergy and hypersensitivity reactions.

## MATERIALS AND METHODS

### Extraction

The used thyme in study has been taken from research garden of Tehran Agricultural Jihad. The plant has been dried at laboratory temperature, away from sunlight, and its leaves were separated. Detached leaves were chopped into small sizes. For extraction for the aqueous extract 10 grams of leaves were initially added into 100 ml of distilled water and for the alcoholic extract, 10 grams of thyme leaves were added in 100 ml of ethanol 96%. Then it was placed on the rotator for 72 hours. After this period mixing plants and extracts were separated using filtration to achieve the initial extract. This process was done separately for both alcoholic and aqueous extracts. After the initial extraction, using rotator, aqueous and alcoholic solvents were separated from initial extract and concentrated extract was obtained.

**Strain Tested Bacteria**

In this study the standard *B.Subtilis* strain was used. PTCC1254 *Bacillus subtilis* strain was prepared from The Center for Veterinary Medicine of Tabriz Islamic Azad University. After the preparation, Bacteria strain was then identified by biochemical tests for definitive identification.

**Quantitative Analysis of the Sensitivity of Dilution Serials**

In this study in order to determine the minimum concentration that inhibits the growth of bacteria (MIC) and the minimum concentration that causes the death of bacteria (MBC), dilutions of 100%, 50%, 25%, 12.5%, 6.25%, 3.12%, 1.56%, 0.78% and 0.39% were obtained from the aqueous and alcoholic extracts of thyme. For each serial dilution, a control group of bacteria, a medium control group and an extract control group were determined. The medium used in this study is Muller-Hinton Broth medium in which $5\times10^5$ active bacteria has been added per milliliter of liquid medium. Tubes were incubated for 48 h at 37 ° C and then results were monitored.

For each dilutions of aqueous and alcoholic extracts, the last dilution where there was no turbidity (no growth) was considered as the MIC. And from all tubes without turbidity on Muller-Hinton Agar was cultured. The last dilution that was able to kill 99.9% of primary living bacteria was considered as MBC.

**RESULTS**

The results of tests conducted MIC and MBC on B.*subtilis* bacteria by aqueous and alcoholic extracts (96% ethanol) of thyme is presented in Table 1. In the MIC test of alcoholic extract of thyme, bacterial growth was observed within dilutions higher than 1.56%. MBC dilution was also within 1.56%. In the performed testing, MIC and MBC were both within 12.5% dilution. Both positive and negative control groups showed no unusual changes.

**Table 1: Test results of MIC and MBC of aqueous and alcoholic extracts of thyme on B.S bacteria**

| Extracts | Num | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Percent (%) | 100.0 | 50.00 | 25.00 | 12.50 | 6.25 | 3.12 | 1.56 | 0.78 | 0.39 | Bacteria control | Media control | Extract control |
| Alcoholic extract | MIC | - | - | - | - | - | - | + | + | + | + | - | - |
| | MBC | - | - | - | - | - | - | + | + | + | + | - | - |
| Aqueous extract | MIC | - | - | - | + | + | + | + | + | + | + | - | - |
| | MBC | - | - | - | + | + | + | + | + | + | + | - | - |

## DISCUSSION

Herbs have long been used around the world in terms of their properties. In the last decade because of drug resistance special attention has been paid to these resources as natural reservoirs [11]. So far, the effects of aqueous and alcoholic extracts of thyme have not been studied on *B.subtilis* bacteria and antimicrobial properties of these extracts on *B.subtilis* with other bacterial species and genera will be done. In 2002 Singh et al. showed that thyme essential oil on Enterohaemorrhagic Escherichia Coli (E.Coli) has an inhibitory effect and among the tested compounds, thymol and carvacrol were cited as the effective factors. They have expressed that these factors have an effect on gram-negative and gram-positive bacteria [12]. In another study by Goudarzi on the aqueous and alcoholic extracts of thyme on Enterohaemorrhagic Escherichia coli, its antimicrobial effect has been proven [11]. In this study the lowest dilution of MIC was 0.78% and the last dilution that caused a 99.9% death of bacteria was 0.78% [11]. In another study conducted by Nouri et al in 2012, antimicrobial effect of thyme essential oil on E.COLI on minced beef was evaluated during storage at refrigerator temperature to replace chemical preservatives and consumers' health care. In this study and at concentration of 0.03%, strong antibacterial effects were reported and stated that during storage period, bacterial growth reduces while increasing essential oil concentration and statistically, essential oil has a significant effect on bacterial growth [13]. In 2003, Burt and colleagues found that at low concentration such as 0.12% and 0.25%, thyme essential oil has bacteriostatic and bactericidal effect on E.COLI respectively [14]. In 2002, Shahnazi and colleagues reported that thyme has Bactericidal and bacteriostatic effects on different bacteria [15]. In another study conducted in 2004 by Sokmen et al, the chemical composition of essential oil of Thymus spathulifukus was analyzed using GC and GC / MC. 28 compound was obtained in which the major components include thymol (36.5%), carvacrol (29.8%) Parasymn (10%) and Gamatrpynn (6.3%) [16]. In another study conducted by Karaman et al (2001), different concentrations of essential oil from Thymus revolutus which is a native of Turkey, was tested against 11 bacterial and 4 fungal and these results show that there are significant anti-bacterial and anti-fungal effects [17]. In a study by sokmen and et al in 2004, antimicrobial and antioxidant properties of essential oil and methanol extracts of thymus spa was assessed in vitro situation. Test results of antimicrobial and antioxidant activity of essential oil showed that strong inhibitory effect on the growth of micro-organisms studied, apart from the four species had fungal but the methanol extract had moderate antibacterial effect and there was no antifungal effect [16]. In another study conducted in 2005 by Hersch et al. 189 species of gram-negative and 135 species of gram-positive bacteria which were locally common (Escherichia coli, Salmonella enteritidis, Shigella sonnei, Pseudomonas aeruginosa, Klebsiella pneumonia, Streptococcus pneumonia, staphylococcal aureus cocos, Staphylococci) were tested in vitro situation with essential oil of 11 species of plants taken from heavily infected patients. All species showed relative resistance to selected antibiotics but among the 11 selected species, Thymus vulgaris and Cinnamomumverum, Drigannumvulagare showed severe antibacterial effects [18]. In a survey by Osman in 2003, essential oils of (hydrosol) two species of Thymus vulgaris and Thymus sepyllum and three species of Oregano named *O.majorana*, *O.omtes* and *O.vulgar* that have great applications in food products and beverages were tested to investigate the amount of their inhibitory effects on growth of E.coli bacteria, *Staphylococcus aureus* and *Yersinia enterocolitica*. To do this, disk diffusion method was applied and serial dilution method was used to determine the extent of bactericidal property. In the disk diffusion method, the entire essential oil test was positive on growth inhibition and the method of serial dilutions, fatality of the bacteria was observed. The most sensitive bacteria to these essential oils have been reported as *staphylococcus aureus* [19]. In another study antibacterial properties of volatile oils of two species of Thymus pubescens and Thymus serpyllum within pre- and post-flowering period were evaluated on the E.coli bacteria, Staphylococcus aureus, Bacillus subtilis and

Pseudomonas aeruginosa and their antibacterial effects have been confirmed by Rasouli et al 2002. These essential oils, even in high concentrations, except of *Pseudomonas aeruginosa* had strong fatality effect on the rest of the bacteria [20]. The study of Rasouli et al and our study on *B.Subtilis* bacteria have high compliance. In general these studies have documents that considering the in vitro evaluation of the bacteria prove the antimicrobial effect of thyme. The study of this research proved the antimicrobial effect of thyme and determines MIC and MBC values at different dilutions. In General, our study is consistent with all previous studies and confirms the antimicrobial effect of alcoholic and aqueous extracts of thyme. Nowadays with the increasing bacterial resistance to antibiotics and also the threats of food shortages and spoilage as a serious threat to the health of living organisms and humans, need to find cheap natural and effective antimicrobials is a particular need. Hence, medicinal plants can be the best alternative to chemical drugs. In this study it is recommended to investigate the effects of *Thymus vulgaris* extract on other bacteria and pathogenic and non-pathogenic fungi. As an antimicrobial composition, thyme requires extensive research in all fields of biology.

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