Sjournals
Scientific Journals
Scientific Journal of Microbiology (2013) 2(2) 23-30
ISSN 2322-2948

Contents lists available at Sjournals
Scientific Journal of Microbiology
Journal homepage: www.Sjournals.com

Original article

Antimicrobial properties of plant extracts of *Thymus vulgaris L.*, *Ziziphora tenuior L.* and *Mentha Spicata L.*, against important foodborne pathogens *in vitro*

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ARTICLE INFO

Article history:
Received 07 January 2013
Accepted 25 January 2013
Available online 28 February 2013

Keywords:
Thymus vulgaris L.
Ziziphora tenuior L.
Mentha Spicata L.
Diffusion method Antimicrobial effects

ABSTRACT

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.*, *Ziziphora tenuior L.* and *Mentha Spicata L.* extracted with methanol 96°and the antimicrobial effects of extracts were evaluated on *Escherichia coli* PTCC 1330 and *Staphylococcus aureus* PTCC 1337 by "using the method of Collins" and "disk agar diffusion method". The results show that methanolic extracts were quite effective in 2000 µg/ml concentration on *Escherichia coli* PTCC 1330 and *Staphylococcus aureus* PTCC 1337 and were prevented from growth them on medium. In "disk agar diffusion method", 10, 20, 30 and 40 % methanolic extract concentrations, was deterrented effect on *Escherichia coli* PTCC 1330 and *Staphylococcus aureus* PTCC 1337. The *Thymus vulgaris L.*, *Ziziphora tenuior L.* and *Mentha Spicata L.* extracts presented the more effective impact on the growth of *Staphylococcus aureus* PTCC 1337than *Escherichia coli* PTCC 1330 ($p<0.05$). Among the bacteria tested, *E. coli* was the resistant bacterium against Thymus vulgaris L., Ziziphora tenuior L. and Mentha Spicata L. extracts E. coli is known to possess a high level of intrinsic resistance to most of the antimicrobial agents due to a very restrictive outer membrane barrier. As a result alcoholic extracts of *Thymus vulgaris L.*, *Ziziphora tenuior L.* and *Mentha Spicata L.*, have been strong antimicrobial activity against many food pathogen bacteria. Results showed

Thymus vulgaris L., *Ziziphora tenuior L*. and *Mentha Spicata L*. extracts can be used as natural antimicrobial in food products.

1. Introduction

Plants have been a potential source of medicine; though in a crude form, have been used from time immemorial to heal various ailments. A variety of bioactive compounds that are present in different parts of a plant has spurred a renewed interest in developing an alternate therapy. The traditional herbal medical system has been practiced globally from ancient times; consequently, a great volume of literature is available on the antimicrobial activity of a variety of plant species (Cowan, 1999). 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 (Ríos and Recio 2005; Fisher and Phillips 2009). 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. In recent years, it has been related that some essentials oils are capable of inhibiting bacteria of food origin and prolonging the shelf life of processed foods (Kim etal., 1995). Lamiaceae family consists of more than 4000 species in 200 genera. Many species within this family are medicinal plants that apply in human disease therapy as well as food in row and cooked forms. Many species of lamiaceae contain essential oils that showed biological activity on many fungal and bacteria plant pathogens.

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) (Ahmad etal., 2010; Moghtader., 2012).

One of them is *Mentha Spicata L*., interesting plant by its EOs, growing in humid places: on mountains at an altitude about 700 m and on plains. It is a perennial plant that has always green leaves. The flowers, with a corolla typically gamopetalous bilabiate, are grouped into small bunches. The fruit is a small hard tetra-achene. The secretary system is located in the leaves and in the stems. *Mentha Spicata L*. dried and fresh leaves are largely used in traditional medicine. Infusions and decoctions of aerial parts are used as carminative, digestive, antispasmodic, anti-inflammatory, expectorant and for the treatment of colds, head aches, hepatic injuries and asthma (Lemordant etal., 1977). The genus *Ziziphora* L. belongs to Labiatae family comprising 15 species and subspecies in Iran. *Ziziphora tenuior* L. is one of this genus member which widely distributed in the country. Ziziphora species were used as culinary herb in Iran. In Iranian folk medicine *Z. tenuior* (named as Kakoti in Persian) were used in treatment of fever, dysentery and uterus infection (Talebi etal., 2012).

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 (WHO (2002-2005)). The gram positive bacterium such as *Staphylococcus aureus* is mainly responsible for post operative wound infections, toxic shock syndrome, endocarditis,osteomyelitis and food poisoning. Some serotypes of *E. coli* can cause serious food poisoning in humans, and are occasionally responsible for product recalls due to food contamination (Benayache etal., 2001). The aim of this study was evaluated of antimicrobial effects of *Thymus vulgaris L*., *Ziziphora tenuior L*. and *Mentha Spicata L*. against *E. coli* and *Staphylococcus aureus* of the important food pathogen.

2. Materials and methods

2.1. Plant materials and extraction

Indigenously grown plants were collected from local village markets, in Mashhad, Iran. The species were identifying in the herbarium of Ferdowsi Mashhad University. 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. The powder was extracted with methanol according to the maceration method and the extract was filtered by Whatman no.1 filter paper. The filtrate was concentrated in a rotary evaporator at 40°C. The concentrated extract was oven dried at 40°C for 3 days and freeze dried for 48 h. The freeze dried extracts was stored at -20°C until use. (Ahmad and Beg, 2001).

2.2. Determination dry weight of alcoholic Thymus vulgaris L., Ziziphora tenuior L. and Mentha Spicata L. extracts

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 (Sattari et al, 2005).

2.3. Source of microorganisms

Two strains were chosen for investigation of which one was reference bacteria: Gram positive *Staphylococcus aureus* PTCC 1337 and Gram-negative *Escherichia coli* PTCC 1330 all organisms were stored at –70°C in glycerol Mueller-Hinton broth. Fresh subcultures were used for each experiment.

2.4. Preparation of Microbial suspension

To preparing microbial suspensions, requires 24-hour culture from each microorganism. So, 24 hours before experiments, microorganisms were inoculated from storage medium to nutrient agar medium slope. After 24 hours, the cultures were washed by Ringer solution and microbial suspensions were prepared. Then some of the bacterial suspension was poured in sterile tubes containing Ringer solution and its turbidity, was measured by spectrophotometer at 530 nm wavelength. It was diluted by Ringer solution until the solution turbidity equalizes with 5% McFarland standard solution. Suspension should have contains 1.5×10^8 CFU / ml (Valero and Salmeron., 2003).

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°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. Screening for antibacterial activity

Adding extracts to the culture medium "according of the method of Collins *et al.* (1995)" were done and to evaluated the antimicrobial effects of alcoholic *Thymus vulgaris L*., *Ziziphora tenuior L*. and *Mentha Spicata L*. extracts. Then 0.2 gram of methanol extract, were added to 5 ml of sterile distilled water. Then it was stirred with vortex system to assist being steady. Then 1 ml of this solution was added to sterile plates. The final concentration of the extract was 2000 µg / ml (Babayi et al., 2004). In the next step, Mueller Hinton agar (Merck-Germany) medium were sterile and used for bacteria, were added to the plates, and placed at room temperature, so the medium was prepared. One loop of each standard strain culture media was cultured inoculums on these medium. The plates were incubated for 24 hours at 37 ° C. The culture with extract and without bacteria was used as control (Babayi et al., 2004; Collins et al., 1998).

2.6. Statistical analysis

All the assays were carried out in triplicates. The experimental results were expressed as mean ± standard deviation. The data were analysed using one way analysis of variance (ANOVA) using SPSS version 17.

3. Results

The results of the antimicrobial effects of alcoholic extracts, by "using the method of Collins *et al.* (1995)" were show on in Tables 1 and 2). The results showed 2000 µg/ml concentration of both alcoholic extracts, were quite effective on reduce of growth *E.coli* and *S. aureus* and were had prevent growth over the medium.

The results of the antimicrobial effects of alcoholic *Thymus vulgaris L*., *Ziziphora tenuior L*. and *Mentha Spicata L*. extracts, by "the agar diffusion method" are presented in (Tables 3, 4 and 5).

Table 1
Antimicrobial effects of 2000µg/ml alcoholic *Thymus vulgaris L*., *Ziziphora tenuior L*. and *Mentha Spicata L*. extract concentrations, on *E.coli* (using the method of Collins et al. (1995)).

Extract	***E.coli***
Thymus vulgaris L.	++
Ziziphora tenuior L	+
Mentha Spicata L.	+

(++) 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 *Ziziphora tenuior L*. and *Mentha Spicata L*.

Table 2
Antimicrobial effects of 2000µg/ml alcoholic *Thymus vulgaris L*., *Ziziphora tenuior L*. and *Mentha Spicata L*. extract concentrations, on *S. aureus* (using the method of Collins et al. (1995)).

Extract	***S. aureus***
Thymus vulgaris L.	++
Ziziphora tenuior L	++
Mentha Spicata L.	++

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

Table 3
Average diameter (mm) of microbial free zone area of by alcoholic *Thymus vulgaris L*. extract, on *E. coli* and *S. aureus* (disk agar diffusion method).

Microorganism		***E. coli***		
Thymus vulgaris L.concentration	10	20	30	40
Average diameter (mm) of microbial free zone area	9.2±0/3224	11.6±0/2887	14.3.1±0/5774	18 ±0/2887
Microorganism		***S. aureus***		
Thymus vulgaris L.concentration	10	20	30	40
Average diameter (mm) of microbial free zone area	12.3±0/3224	15.7±0/5221	18.3 ±0/5221	21.9±0/2887

Tables 4
Average diameter (mm) of microbial free zone area of by alcoholic Mentha Spicata L. extract, on *E. coli* and *S. aureus* (disk agar diffusion method).

Microorganism		***E. coli***		
Mentha Spicata L. concentration	10	20	30	40
Average diameter (mm) of microbial free zone area	7.1±0/5224	9.6±0/2887	11.1±0/5774	14 ±0/5
Microorganism		***S. aureus***		
Mentha Spicata L. concentration	10	20	30	40
Average diameter (mm) of microbial free zone area	10.2±0/3224	12.9±0/5221	15.3±0/5774	18.2±0/2887

Table 5
Average diameter (mm) of microbial free zone area of by alcoholic *Ziziphora tenuior L*. extract, on ***E. coli*** and *S. aureus* (disk agar diffusion method).

Microorganism		***E. coli***		
Ziziphora tenuior L. concentration	10	20	30	40
Average diameter (mm) of microbial free zone area	6.6±0/5224	7.9±0/2887	11±0/5	12.7 ±0/5
Microorganism		***S. aureus***		
Ziziphora tenuior L. concentration	10	20	30	40
Average diameter (mm) of microbial free zone area	9.1±0/3224	11.7±0/5774	13.2±0/5332	16.4±0/2887

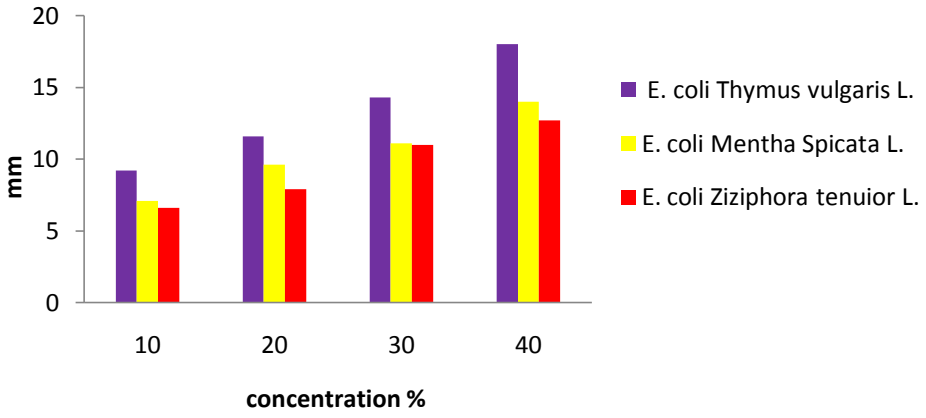

Fig. 1. Antimicrobial activity of alcoholic *Thymus vulgaris L*., *Ziziphora tenuior L*. and *Mentha Spicata L*. extracts, on *E. coli*.

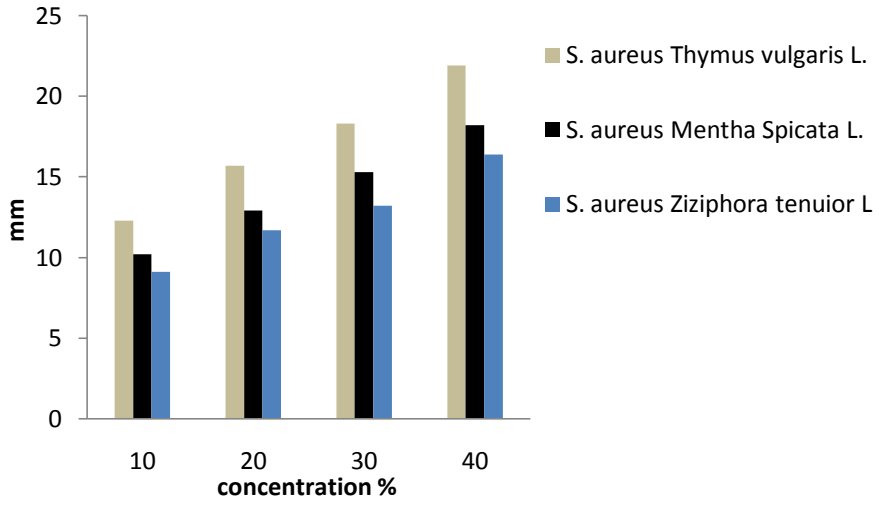

Fig. 2. Antimicrobial activity of alcoholic *Thymus vulgaris L.*, *Ziziphora tenuior L.* and *Mentha Spicata L.* extracts, on S. aureus.

4. Discussion

Based on the results methanolic extract of *Thymus vulgaris L.*, *Ziziphora tenuior L.* and *Mentha Spicata L.* in this study have significant antimicrobial activity on the studied microorganisms. The results show that alcoholic *Thymus vulgaris L.*, *Ziziphora tenuior L.* and *Mentha Spicata L.*alcoholic extracted at all concentrations (10, 20, 30 and 40%) had the inhibitory effect on *S. aureus* and *E. coli*. The results show that *Thymus vulgaris L* extracted at all concentrations had the inhibitory strong effect on *E. coli*. Thymol is one of main compound of *T.spicata*, *Satureja thymbra*, *Salvia fruticosa*, *Laurus nobilis*, *Mentha pulegium*, *Inula viscosa*, *Pimpinella anisum*, *Eucalyptus camaldulensis*, and *Origanum minitiflorum* plants growing wild in southern Turkey. (Muller-Riebau et al., 1995). Thymol has microbial activity because of its phenolic structure. There is evidence supporting the belief that thymol, when applied two to three time's 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.

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 etal., 2008). Nedorostova et al. (2009) tested the essential oils of 27 plants species on *L. monocytogenes* ATCC 7644, *S. aur*eus ATCC 25923, *E. coli* ATCC 25922, *P. aeruginosa* ATCC 27853, and *S. enterica Enteritidis* ATCC 13076. Of these, 13 were active, and only the essential oils of *Allium sativum* and *Armoracia rusticana* were capable of inhibiting all of the bacteria. *Staphylococcus aureus* was inhibited by all active oils followed by *E. coli* (8), *L. monocytogenes* (7), *S. enterica* Enteritidis (6), and *P. aeruginosa* (2).

Antimicrobial effect of the extracts was different, depending on the type of microorganisms, thus, the gram-positive bacterium *Staphylococcus aureus*, was higher sensitivity compared to gram-negative bacteria *E. coli* (Table

3, 4 and 5) and showed inhibitory effects at lower concentrations of *Thymus vulgaris L*., *Ziziphora tenuior L*. and *Mentha Spicata L*. extracts. Alcoholic extract of *Thymus vulgaris L*., extract was more effective *Ziziphora tenuior L*. and *Mentha Spicata L.* (Figure 2, 3).

Therefore, using *Thymus vulgaris L*., *Ziziphora tenuior L*. and *Mentha Spicata L*. as a natural antimicrobial compounds in vitro requires further research on mechanism of the pharmacy plant on the microorganisms.

5. Conclusion

In conclusion, it can suggest that *Thymus vulgaris L*., *Ziziphora tenuior L*. and *Mentha Spicata 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.

Acknowledgment

The authors wish to express their profound gratitude and thank sincerely to Research's Deputy of Ferdowsi University of Mashhad for providing the cost of this project and help with implementation of this project with code 16135.

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