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Effect of drought stress on antibacterial activity of *Thymus daenensis* subsp*. daenensis* Celak.

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***Thymus daenensis* subsp*. daenensis* is an endemic aromatic and medicinal plant of Iran. An experiment was conducted to study the effect of drought stress on growth and on antibacterial activity of *T. daenensis* subsp. *daenensis* Celak in pot experimental of field, Shahrekord, Southwest Iran. Plants were exposed to three drought treatments at before flowering (75 days after sowing): (1) well-watered at 100% field capacity (control), (2) severe drought stress at 20% field capacity, (3) mild drought stress at 40% field capacity, and (4) hard drought stress at 60% field capacity, until complete flowering (120 days after sowing). The antibacterial activity of the ethanol extract was assayed against four pathogens (*Staphylococcus aureus, Bacillus cereus, Salmonella typhimurium* and *Streptococcus agalactiae*) by agar disc diffusion method. The results of showed that the ethanol extracts from the different treatments studied showed antibacterial activities, with the diameters of the inhibition zone ranging from 8 to 31 mm. There were no significant differences in the antibacterial activity of the extracts from the different treatments in almost concentrations against four pathogens. But, the highest level of antibacterial activities against *S. aureus, B. cereus, S. typhimurium* and *S. agalactiae* were demonstrated by the extract (1000 µg/ml) of drought stress at 60% field capacity (IZ = 26 mm), drought stress at 20% field capacity (IZ = 18.7 mm), drought stress at 20% field capacity (IZ = 20.2 mm) and drought stress at 20% field capacity (IZ = 23.7 mm), respectively. Increased drought levels did not significant reduce antibacterial activity as compared with controls.**

Key words: *Thymus daenensis* subsp*. daenensis*, drought stress, antibacterial activity, ethanol extract.

INTRODUCTION

The genus *Thymus* L. belongs to the mint family (Lamiaceae), and consists of about 215 species of herbaceous perennials and small shrubs in the world. 14 species of *Thymus* has been reported in Flora Iranica (Jalas, 1982; Stahl-Biskup and Saez, 2002), four of which, *Thymus carmanicus* Jalas, *Thymus daenensis* Celak subsp. *daenensis* Celak, *T. daenensis* Celak subsp. *lancifolius* (Celak.) Jalas, *Thymus persicus* (Roniger ex Reach. F.) and *Thymus trautvetteri* Klokov and Desj.-Shost have been known to be endemic (Rechinger, 1982). *T. daenensis* subsp. *daenensis* is an endemic subspecies of Iran. This subspecies generally grows in high altitudes in Zagros mountains range (Ghasemi Pirbalouti et al., 2011).

The areal parts and volatile constituents of thyme, a perennial dwarf shrub, are used as medicinal herbs. *Thymus* species are commonly used for herbal tea, flavoring agents (condiment and spice) and medicinal purposes (Stahl-Biskup and Saez, 2002). Infusion and decoction of aerial parts of *Thymus* species are used to carminative, digestive, antispasmodic, anti-inflammatory, and expectorant and for the treatment of colds in Iranian

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traditional medicine (Zargari, 1990; Nickavar et al., 2005; Ghasemi Pirbalouti, 2009). Recent studies have shown that *Thymus* species have strong antibacterial, antifungal, spasmolytic and antioxidant activates (Rahimmalek et al., 2009; Jordan et al., 2009). Previous studies showed that essential oil and extract of *T. daenensis* exhibited anti-microbial activities against *Candida albicans* (Ghasemi Pirbalouti et al., 2009a), *Listeria monocytogenes* (Ghasemi Pirbalouti et al., 2009b), *Campylobacter jejuni* and *Campylobacter coli* (Ghasemi Pirbalouti et al., 2010a), *Staphylococcus aureus, Escherichia coli, Pseudomonas aeruginosa* and *Klebsiella pneumoniae* (Ghasemi Pirbalouti et al., 2010b), *Escherichia coli O157:H7* (Ghasemi Pirbalouti et al., 2010c) and *Saprolegnia parasitica* (Ghasemi Pirbalouti et al., 2009c).

Water stress has considerable adverse impact on productivity of agricultural plants. Drought stress can cause quantity and quality yield losses in both crop species and medicinal plants (Rout and Shaw, 2001). Moisture deficiency induces various physiological and metabolic responses like stomatal closure and decline in growth rate and photosynthesis (Flexas and Medrano, 2002). The results of Baher et al. (2002) showed that greater soil water stress decreased plant height and total fresh and dry weight of *Satureja hortensis*. Razmjoo et al. (2008) also showed that water stress caused a significant reduction in plant height, the number of branches and flowers, peduncle length, head diameter, fresh and dry flower weight and essential oil content of Chamomile (*Matricaria chamomila*). Koocheki et al. (2008) reported that drought stress increased chlorophyll content in *Ziziphora clinopodioides*, specific leaf weight in *Zataria multiflora* and *Z. clinopodioides*, and temperature in *Z. multiflora* and *Teucrium polium* as compared with controls. Another study (Simon et al., 1992) showed that mild and moderate plant water stress increased sweet basil leaf essential oil content and altered oil composition. They reported that after 21 days of plant water deficit, the oil content of leaves increased from 3.1 to 6.2 µl/leaf dry weight.

T. daenensis has adaptability to high altitude and semiarid of climates. Its cultivation may be an alternative option in areas with drought problems. However, the performance of this plant in water stress environments, and the effect of this stress on growth and antibacterial activity have not been studied well. The objective of this research was to evaluate the effect of deficit water on growth characteristics and antibacterial activity of *T. daenensis* subsp. *daenensis* Celak.

MATERIALS AND METHODS

Treatments

Plastic white pots with a top diameter of 20 cm and a depth of 25 cm were filled with clay loam soil. Ten seeds of *T. daenensis* collected from natural rangelands of Iran were sown in each pot. This experiment was conducted using a randomized complete design with three replications under natural conditions in Shahre-kord (latitude 32° 20´ N, longitude 50° 51´ E, altitude 2061 m asl), South-west Iran. Type of study area climate by Emberger's climatology method (Emberger, 1953) is cold and semiarid. Plants were exposed to three drought treatments before flowering (about 75 days after sowing): (1) well-watered at 100% field capacity (control), (2) severe drought stress at 20% field capacity, (3) mild drought stress at 40% field capacity and (4) hard drought stress at 60% field capacity, until complete flowering (about 120 days after sowing).

Extract preparation

Air-dried and powdered leaves and flowers of *T. daenensis* were macerated at room temperature with 500 ml of ethanol: water (80:20) for 48 h. The extractions continued two times and then were concentrated in a rotary evaporator under reduced pressure (Model Zirbus 302®, Italy). The extract samples were stored in universal bottles and refrigerated at 4°C prior to use.

Bacterial strain

The extracts were screened for antimicrobial activity using the agar diffusion technique (Rota et al., 2004) against four microorganisms of significant importance. The bacterial strains were used to assess the antimicrobial properties of the test samples, three Gram-negative strains: *S. agalactiae, S. aureus* and *B. cereus* and one Gram-negative strain: *Salmonella typhimurium*. All clinical isolates were obtained from Food Microbiology Laboratory, Veterinary Medicine Faculty, IAU, Shahrekord Branch, Iran, and identified using conventional morphological as well as biochemical tests. Stock cultures of bacteria were kept in 20% glycerol PBS (phosphate buffered saline) at -70°C. Active cultures were generated by inoculating 100 µl of the thawed microbial stock suspensions into 5 ml nutrient broth (Merck, Germany) followed by overnight incubation at 37°C. The density of bacteria culture required for the test was adjusted to 0.5 McFarland standards (1.0×10^7 CFU/ml), and were measured using the spectrophotometer (Eppendorf, AG, Germany). Absorbance at 600 nm of known bacterial densities was determined to obtain a standard calibration curve. Subsequent dilutions were made from the aforementioned suspension, which were then used in tests.

Antimicrobial test

BHI agar (Merck, Germany) was used to prepare the culture medium, which was then autoclaved at 121°C for 15 min. Plates were prepared with 10 ml agar inoculated with 1 ml of each bacterial suspension. Filter paper disks (Whatman No. 1, 6 mm diameter) were impregnated with 60 µl of extract, and incubated at 35°C for 18 h. The extracts (serial dilutions 2 to 1000 µg/disc) were dissolved in dimethyl sulfoxide (DMSO, 15 µl) before the test for antimicrobial activity. Discs (6 mm diameter) of ampicillin, ciprofloxacin, flumequine and penicillin (10 µg) were used as positive controls. The scale of measurement was the following (disk diameter included): P ≥ 20 mm zone of inhibition is strongly inhibitory; <20 to 12 mm zone of inhibition is moderately/mildly inhibitory; and <12 mm is no inhibitory. All the data collected for each assay are the averages of three determinations.

Analysis of data

The differences between experimental groups were compared using one-way ANOVA, and comparison of the means of the treatments evaluated zby Duncan's multiple range test at $p < 0.05$

Table 1. The antibacterial activity of the ethanol extracts from the different treatments against four pathogens.

Treatments	Concentration (µg/ml)									
	1000	500	250	125	62.5	31.2	15.6	7.8	3.9	1.9
Streptococcus agalactiae										
Control	15.67 ± 0.58	13.44 ± 0.48	8.64 ± 0.19	-	-	-	-	-	-	-
Serve drought stress	23.83 ± 4.33	19.34 ± 2.5	14 ± 0.39	6.65 ± 3.84	-	-	-	-	-	-
Mild drought stress	20.67 ± 1.73	15 ± 0.19	4.22 ± 4.22	3.56 ± 3.56	2.89 ± 2.89	-	-	-	-	-
Hard drought stress	17.89 ± 1.89	15 ± 0.19	11.56 ± 1.46	-	-	-	-	-	-	-
Staphylococcus aureus										
Control	17.45 ± 4.29	12.89 ± 2.45	7 ± 3.78	3.67 ± 3.67	-	-	-	-	-	-
Serve drought stress	19.33 ± 2.34	9 ± 4.5	6.44 ± 3.49	-	-	-	-	-	-	-
Mild drought stress	14.89 ± 0.95	12 ± 2.01	3.89 ± 3.89	3.22 ± 3.22	-	-	-	-	-	-
Hard drought stress	26 ± 3.91	17.11 ± 0.87	15.11 ± 0.29	8.89 ± 0.22	3.11 ± 3.11	-	-	-	-	-
Salmonella typhimurium										
Control	14.33 ± 0.51	11.01 ± 0.88	7.78 ± 0.29	-	-	-	-	-	-	-
Serve drought Stress	22.22 ± 0.79	13.89 ± 1.39	8.78 ± 4.62	4.89 ± 4.89	4.78 ± 4.78	4.67 ± 4.67	4.11 ± 4.11	-	-	-
Mild drought stress	17 ± 2.17	12.78 ± 1.49	3.67 ± 3.67	3.67 ± 3.67	3.67 ± 3.67	-	-	-	-	-
Hard drought stress	17.78 ± 1.68	14.78 ± 0.29	11.56 ± 1.49	3.78 ± 3.78	3.22 ± 3.22	-	-	-	-	-
Bacillus cereus										
Control	18.39 ± 2.64	14.81 ± 1.09	4.33 ± 4.33	3.67±3.67	-	-	-	-	-	-
Serve drought Stress	18.67 ± 2.41	13.33 ± 1.57	7.89 ± 4.52	4.78 ± 4.78	4.33 ± 4.33	-	-	-	-	-
Mild drought stress	17.11 ± 3.49	8.78 ± 4.42	3.22 ± 3.22	3.22 ± 3.22	2.78 ± 2.78	-	-	-	-	-
Hard drought stress	17.55 ± 0.95	15.51 ± 0.39	12.44 ± 1.97	10.89 ± 2.43	3.33 ± 3.33	-	-	-	-	-

a: The inhibition zone diameter (mm) ± S.E.

level. All data processing was performed with SPSS software Version 17.

RESULTS AND DISCUSSION

The results of showed that the ethanol extracts from the different treatments studied showed antibacterial activities, with the diameters of the inhibition zone ranging from 8 to 31 mm (Table 1). The results of ethanol extract effects of different treatments (control and drought stress treatments) against *B. cereus* and *S. aureus* in different dilutions (2 to 1000 µg/disc) showed that there were not significant differences in the antibacterial activity. In concentration of 1000 µg/ml, the ethanol extract effect of different treatments against *S. typhimurium* showed that it was significant difference ($p \leq 0.05$) in the antibacterial activity. But, other concentrations (dilutions 2 to 500 µg/ml) were not significant different in the antibacterial activity against *S. typhimurium*. The results showed only in concentration 500 µg/ml, significant antibacterial effect ($p \leq 0.05$) in the antibacterial activity. But, other concentrations (dilutions 2 to 500 µg/ml) were not significant different in the antibacterial activity against *S. typhimurium.*

The results showed only in concentration 500 µg/ml, significant antibacterial effect ($p \leq 0.05$) against *Streptococcus agalactiae*. However, in other concentrations (dilutions 2 to 250 µg/ml) there were no significant differences in the antibacterial activity against *S. agalactiae.* In high concentrations, the extracts showed strong activity (inhibition zone ≥ 20 mm) and moderate activity (inhibition zone <20 to 12 mm). The highest level of antibacterial activities against *S. aureus, B. cereus, S. typhimurium* and *S. agalactiae* were demonstrated by the extract (1000 µg/ml) of drought stress at 60% field capacity (IZ = 26 mm), drought stress at 20% field capacity (IZ = 18.7 mm), drought stress at 20% field capacity (IZ = 20.2 mm) and drought stress at 20% field capacity (IZ = 23.7 mm), respectively.

Razmjoo et al. (2008) showed that water stress caused a significant reduction in essential oil content of Chamomile (*M. chamomila*). The reduction in essential oil content may be due to disturbance in photosynthesis and carbohydrate production under stress condition and suppression of the plant growth (Flexas and Medrano, 2002). Reduction in oil content and compositional alterations in the essential oils as a consequence of drought has also been described in mints (Charles et al., 1990) and sweet basil (Simon et al., 1992). However, Holtzer et al. (1988) believed that depending upon the plant species and plant genotype, drought stress can increase, decrease or have no effect on the levels of metabolites. In this experiment, increased drought levels did not significantly reduce antibacterial activity as compared with control. This is similar to the report that there were no differences in the water-soluble mucilage content extracted from Boraginaceae from wet and dry habitats (Pollak and Albert, 1990), and two species of *Ziziphus* (Clifford et al., 2002). However, contrasts with early findings in *Brunella grandifolia* (Jeremias, 1966) in which mucilaginous substances increased during drought-stress. Shabih et al. (1999) reported that when moisture deficiency does not limit plant growth and survival, the production of secondary metabolites such as essential oil is even stimulated by limited stressful environments.

In a study (Bettaieb et al., 2012), the essential oil compositions and phenolic contents fruit of cumin (*Cuminum cyminum* L.) under different levels of stress drought were examined. Their results indicated that water deficit enhanced the palmitic acid percentage, antioxidant activity by four different test systems (DPPH, β-carotene/linoleic acid chelating and reducing power assays), and the essential oil yield (1.64%) and increased by 1.40 folds under moderate water stress. In addition, they reported that stress drought results on the modification of the essential oil chemotype from γ-terpinene/phenyl-1, 2 ethanediol in the control to γ-terpinene/cuminaldehyde in stressed ones. According to the results, they suggested that water deficit treatment may regulate the production of bioactive compounds in cumin seeds, influencing their nutritional and industrial values.

In the present study, comparison of the antibacterial activity of *T. daenensis* subsp*. daenensis* extract in different treatments may have been caused by drought stress effect on quantitative extract. A number of studies in the phenol-rich Lamiaceae species *Thymus vulgaris* L. (Gouyon et al., 1986), *T. piperella* L. (Boira and Blanquer, 1998) and *Origanum vulgare* L. (Vokou et al., 1993) have shown that the preponderance of carvacrol or thymol in their essential oils is associated to climatic conditions. Shan et al. (2007) reported that there were highly positive relationships (R^2 = 0.73 to 0.93) between antibacterial and antioxidant activities and phenolic content of the tested extracts against each bacterium. Some studies claim that the phenolic compounds present in spices and herbs might also play a major role in their antimicrobial effects (Hara-Kudo et al., 2004). However, no large scale systematic investigation of the relationship between bacterial inhibition and total phenolic content of spices and herbs has been reported. In this study, most of the antimicrobial activity in extracts from different treatments appears to be explainable by phenolic compounds (thymol and carvacrol). These results agree with those reported by other researchers (Consentino et al., 1999; Davidson and Naidu, 2000; Skocibusicet al., 2006; Rota et al., 2008). The phenolic compounds, such as thymol and carvacrol, are widely reported to possess high levels of antimicrobial activity (Baydar et al., 2004; Nejad et al., 2008). Thymol and carvacrol, which are the main components of *T. daenensis* essential oil and extract, have been considered as biocidal, resulting in bacterial membrane perturbations that lead to leakage of intracellular ATP and potassium ions and ultimately cell death (Helander et al., 1998; Juven et al., 1994; Ultee et al., 1999).

Conclusion

In the present study, we demonstrated that the potent antibacterial activity of *T. daenensis* subsp. *daenensis* extract against food borne pathogens strains, which justifies the large use of this plant in traditional medicine. *T. daenensis* was moderately tolerant to drought stress, because water deficiencies inhibited various growth parameters of this plant to various degrees, and the results of ethanol extract effects of different treatments against four pathogens in most dilutions (2 to 1000 µg/disc) showed that there were no significant differences in the antibacterial activity. Finally, the results of this research can be concluded that *T. daenensis* can be grown successfully on arid and semiarid climates.

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ABBREVIATIONS

DMSO, Dimethyl sulfoxide; **IZ,** inhibition zone.

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