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Full Length Research Paper

Variation in antibacterial activity and phenolic content of *Hypericum scabrum* L. populations

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Hypericum scabrum **L. is one of the herbal and perennial medicinal plants belonging to Hypericaceae which all of its floral on aerial part has extensive use in preparing traditional medicines. The major constitutes of *H. scabrum* are α-pinen, thymol and carvacrol. The study was conducted to determine variations of antibacterial activity, thymol and carvacrol content in different populations of *H. scabrum* flowers. The antibacterial activity was tested by agar disc diffusion and serial dilution assays against four pathogens (*Staphylococcus areus*, *Pseudomonas aeroginosa, Basilluc cerus and Esherishia coli)*. The extract was characterized using high-performance liquid chromatography (HPLC). The contents of thymol and carvacrol, varied 0.93 to 1.30 and 0.25 to 0.39 mg/g extract, respectively. The highest content of thymol and carvacrol in all investigated samples was obtained from Sheyda and Lordegan population, respectively. The inhibition zones and MIC values for bacterial strains, which were sensitive to the extract of *H. scabrum* L., were in the range of 8 to 19 mm and 0.019 to 5000 mg/ml, respectively. The populations of Sheyda and Lordegan showed the strongest antibacterial activity.**

Key words: *Hypericum scabrum* L., population, antibacterial activity, thymol, carvacrol.

INTRODUCTION

Hypericum (Guttiferae or Hypericaceae) is a large genus of herbs or shrubs, which has more than 400 species and is accommodated in about 30 sections (Robson, 1990). The genus *Hypericum* grows widely at temperate regions of the earth and used as traditional medicinal plants in various parts of the world (Yazaki and Okada, 1994). Different species of *Hypericum* have been traditionally used for the treatment of wounds, eczema and burns. Also, they have been used as medicinal plants for centuries in the treatment of trauma, rheumatism, neuralgia, gastroenteritis, ulcers, hysteria, bedwetting and depression (Miller, 1998). It has also been used for its sedative, anti-inflammatory and antiseptic effects (Baytop, 1984; Ozturk et al., 2002; Mukherjee et al., 2000). During the last few years, antimicrobial, antifungal and antioxidant properties have been reported by many experimental studies (Rocha et al., 1995; Rabanal et al., 2002; Decosterd et al., 1991; Conforti et al., 2002; Butterweck et al., 2002). Thus, phytochemical investigations have led to the isolation of antimicrobial (Jayasuriya et al., 1989), anticancer (Agostinis et al., 2002), antidepressant (Butterweck et al., 2002), antiviral (Meruelo et al., 1988), antioxidant (Cakir et al., 2003), cytotoxic (Jayasuriya et al., 1989) and antifungal (Cakir et al., 2004, 2005).

The essential oils of *Hypericum* species grown in different region of the world have been extensively examined by GC and GC-MS (Cakir et al., 2004; Santos et al., 1999; Erken et al., 2001; Gudzic et al., 2001, Sajjadi et al., 2001; Baser et al., 2002; Schwob et al., 2002; Bertoli et al., 2003). *Hypericum* species contains the variety of compounds such as flavonoids (Chung et al., 1997; Dias et al., 1998; Ishiguro et al., 1991), xanthones (Gunatilaka et al., 1979; Rath et al., 1996; Wu et al., 1998a), chromenyl ketones (Ishiguro et al., 1990; Wu et al., 1998b), hyperforin derivatives (Decosterd et al., 1989; Maisenbacher and Kovar, 1992; Trifunovic et al., 1998), phloroglucinols (Decosterd et al., 1991; Ishiguro et al., 1994, 1998; n-alkanes (Brondz et al., 1983), napthodianthrones (Kitanov, 2001) and essential oils (Cakir et al., 1997). Chemical investigations showed that some benzoylphloroglucinol derivatives were present

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Figure 1. Areal plant and flowers of *Hypericum scabrum* L., Sab-z-ekoh, Chaharmahal va Bakhtiari, Iran.

in *Hypericum scabrum* L. (Matsuhisa et al., 2002). Alpha-pinene, spathulenol, para-cymene, acetophenone and carvacrol were the main essential oil constituents isolated from *H. scabrum* (Cakir et al., 1997).

It has been reported that essential oil yield and their components in plants is related to genetic and environment (Pourohit and Vyas, 2004; Rahimmalek et al., 2009). Chemical polymorphisms have been reported for many medicinal plants (Mockute et al., 2001; Russell and Southwell, 2003; Curado et al., 2006). Recent findings showed that some of the medicinal plant characteristics can be affected by genetic and ecological factors such as precipitation, temperature, plant competition and nitrogen content in the soil (Letchamo et al., 1995). However, there are no reports in assessment of major compounds (carvacrol and thymol) of *H. scabrum* growing in different climatic regions of the country.

The aims of this study were: (1) to evaluate the antibacterial properties of the extract of five localities of *H. scabrum* grown in various geographical regions of Iran, (2) to determine the variation of thymol and carvacrol contents of different populations, and (3) to assess the relationships between variations of thymol and carvacrol contents and the environmental factors involved in different geo-ecological regions.

MATERIALS AND METHODS

Plant material

The flowers of wild populations of *H. scabrum* L. were collected from Isfahan and Chaharmahal va Bakhtiari provinces, central of Iran (Figure 1). Each sample was labeled and its location was recorded using a global positioning system (GPS, Vista Garmin) receiver. The samples of the plants were identified by regional floras and authors with floristic and taxonomic references (Rechinger, 1982), and voucher specimens were deposited at the Herbarium of IAU, Shahrekord Branch, Shahrekord, Iran. The accessions of plants were transferred from natural habitats at the early flowering stage on May, 2010.

Soil physical and chemical characteristics such as pH, electrical conductivity, texture, organic carbon, nitrogen (N), phosphorus (P), and potassium (K) contents were obtained through soil-sampling and analysis. The slope and elevation information were obtained from the digital elevation model (DEM) using two well-known GIS software packages ILWIS (3.0 Academic). Climatic conditions of natural habitats were determined using the nearest meteorology station.

Sample preparation

Harvested flowers were dried at room temperature for one week. The extracts were obtained by stirring 100 mg of ground samples with 30 ml of pure ethanol (analytical grade; Merk, Germany) for 30 min. Samples were filtered by a Whatman no 4. filter paper.

Reagents and chemicals

Methanol (HPLC grade), ethanol (analytical grade), acetonitrile (analytical grade) and water (HPLC grade) were purchased from Merck Co (Darmstadt, Germany). The standard of carvacrol acid was purchased from ROTH (Karlsruhe, Germany).

Preparation of standard solution

Stock standard solutions were prepared by accurately weighing 31.35 mg thymol reference standard and 7.5 mg carvacrol into separate 50 ml volumetric flasks and dissolving in acetonitrile/ water

(50:50, v/v).

Working standard solutions (1, 2.5 and 5 ml) were prepared by dilution from the stock standard solution. The mixture was stirred carefully and refluxed in a water bath at 90°C for 1 h.

Identification of phenolic compounds using HPLC

The isolation and analysis method for carvacrol and thymol were conducted according to previously published protocols (Krause and Ternes, 1999; Hajimehdipoor et al., 2010; Shekarchi et al., 2010). The obtained mixture was injected to HPLC system (Kanauer, Germany). An HP 1000 series liquid chromatography system comprising vacuum degasser, quaternary pump, auto-sampler, thermostatted column compartment and diode array detector was used. Column Machery-NAGEL, Nucleosin-100-5 C18, Loop 20 µl was maintained at 30°C.

Solvents used for separation were water (eluent A) and acetonitrile (eluent B). The gradient program was as follows: 70% A/30% B, 0 to 5 min; 42% A/58% B, 5 to 18 min; 70% A/30% B, 18 to 30 min. The calibration curves (correlation coefficient) for thymol and carvacrol were Y = 89322x-382440 (r^2 = 0.998) and Y = 74919x-247838 (r^2 = 0.994), respectively. Samples were filtered through a 0.45 µm membrane filter before injection.

The flow rate was kept 1 ml min^{-1}. The injection volume was 20 µl and peaks were monitored at 330 nm. The chromatographic peaks of carvacrol were confirmed by comparing their retention times and UV spectra with that of their reference standard. Working standard solutions were injected into the HPLC and peak area responses were obtained. Standard graphs were prepared by plotting concentration versus area. Quantification was carried out from integrated peak areas of the samples using the corresponding standard graph.

Bacterial strain

Two Gram-positive (*Staphylococcus aureus* and *Bacillus cereus*) and two Gram-negative (*Escherichia coli* and *Pseudomonas aeruginosa*) bacterial strains were all clinical isolates obtained from Food Microbiology Laboratory, Veterinary Medicine Faculty, Islamic Azad University-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. An initial bacterial suspension containing 10^7 CFU/ml was made from the flask broth culture. Subsequent dilutions were made from the previous suspension, which were then used in tests.

Antimicrobial test

The disc diffusion method of Iennette (1985) was used with some modification to determinate rate of inhibition growth of bacteria by extract. Brain heart infusion (BHI) agar (Merck, Germany) was used to prepare the culture medium and autoclaved at 121°C for 15 min. Plates (8 cm diameter) were briefly prepared with 10 ml agar inoculated with 1 ml of each bacterial suspension. Sterile paper discs (6 mm in diameter) were impregnated with 60 µl of dilutions with concentration of 100 microgram per disc and incubated at 35°C for 18 h. The extract was dissolved in dimethyl sulfoxide (DMSO, 15 µl) before the test for antimicrobial activity. Discs (6 mm diameter) of ampicillin, erythromycin and ciprofloxacin (10 µg) were used as positive controls. Bacterial growth inhibition was determined as the diameter of the inhibition zones around the discs (mm). The growth inhibition diameter was an average of three measurements, taken at three different directions. All tests were performed in triplicate.

The MICs were determined by serial dilution assay. All extracts were initially tested at 10 mg/ml and serially diluted to 0.019 mg/ml. Each tube was inoculated with 5 ml of bacterial suspension at a density of 10^7 CFU/ml, and incubated at 37°C for 24 h. The growth of microorganisms was observed as turbidity determined using the measure optical density at 600 nm, by spectrophotometer (Eppendorf, AG, Germany). Erythromycin was included as positive control in each assay. Extract-free solution was used as a negative control. Control tubes were incubated under the same condition. All assays were carried out in triplicate.

Analysis of data

The differences between experimental groups were compared using one-way analysis of variance (ANOVA). All data processing was performed with SPSS software Version 11.5.

RESULTS

The results showed that most of populations of *H. scabrum* were in high altitudes (2226 to 2822 m above sea level). The soil and climatic information of selected regions was summarized in Table 1.

The amounts of thymol and carvacrol of flowers (mean of four replicates) ranged from 0.93 to 1.30 mg/g and 0.25 to 0.039 mg/g extract, respectively (Table 2). In present study, comparison of the amounts of thymol and carvacrol of *H. scabrum* extract in different geographic conditions showed that there are some qualitative and quantitative differences between five localities in Isfahan and Chaharmahal va Bakhtiari provinces of Iran (Table 2).

The highest amount of thymol in all investigated samples was recorded in Sheyda population from Chaharmahal va Bakhtiari province (1.30 mg/g extract), while the lowest was observed in Sabz-e-kooh population from the same province (0.93 mg/g extract). Also, highest amount of carvacrol in all investigated samples was recorded in Lordegan population from Chaharmahal va Bakhtiari province (0.39 mg/g extract), while the lowest was observed in soork population from the same province (0.25 mg/g extract) (Table 2). The results showed that some of the geographic, climatology and edaphic factors had no significant effects on the thymol and carvacrol content.

Preliminary screening of the *in vitro* antibacterial activity of five extracts from different localities against four pathogens microorganisms was studied using the paper disc agar diffusion technique. The results showed significant variation in the antibacterial properties of extracts (Table 2). The extracts showed moderate activity (inhibition zone <20 to 12 mm) and low activity (inhibition zone <12 mm). Attending to this, the major effectiveness was achieved by the extracts from Sheyda and Lordegan population from Chaharmahal va Bakhtiari province.

Table 1. Geographical and climatic of natural habitats of populations *Hypericum scabrum*.

Region	Province †	Elevation (m asl^{-1})	Latitude	Longitude	P [a]	T [b]	pH	E.C. [d]	O.C. [e]	P [f]	N [g]	K [h]
Sheyda	Ch	2822	32° 36′ N	50° 32′ E	382	10	7.94	0.589	1.482	25.1	0.150	328
Lordegan	Ch	2479	31° 22′ N	51° 00′ E	593	15	7.72	0.511	1.404	20.8	0.143	800
Chadegan	Is	2327	32° 33′ N	50° 18′ E	348	11	7.64	0.414	1.287	35.1	0.103	532
Soork	Ch	2288	32° 04′ N	51° 03′ E	443	11	7.71	0.529	0.975	26.5	0.992	512
Sabz-e-kooh	Ch	2226	31° 49′ N	50° 51′ E	593	14	7.6	0.623	2.786	59.1	0.288	711

† Ch: Chaharmahal va Bakhtiari, Is: Isfahan. [a] annual precipitation (mm), [B] average temperature (°C), [D] electrical conductivity (dS/m), [e] organic carbon (%), [f] available phosphorous (mg/kg), [G] total nitrogen (%), [h] available potassium (mg/kg).

However, more precise data on the antimicrobial properties were obtained through determination of bacteriostatic concentrations. The minimum inhibitory concentration (MIC; µg extract /mL medium) (against four microorganisms) of five extract are shown in Table 2. The extract with the most bacteriostatic properties were: Sheyda and Lordegan populations with MIC≤19 to 625 µgml^{-1} against three strains tested.

DISCUSSION

Previous findings on other plants have shown that the variation of their quantitative extract composition is attributed to the geographic direction (Kokkini et al., 1997; Karousou., 1998; Yavari et al., 2010).

Karousou et al. (2005) showed that high carvacrol content in two species (*Coridothymus capitatus* Reichenb. fil. and *Satureja thymbra* L.) is associated to the dry dwarf-shrub formations of the lowland, whereas a high thymol content is related to the more mesic timber or highland formations. Also, they reported that the relation between oil composition and the natural habitats of the collected plants suggests the use of natural habitat unit as a tool for the assessment and prediction of variation in essential oil in a single species (Karousou et al., 2005).

Yavari et al. (2010) reported that there were positive relationship between some essential oil characters of *Thymus migricus* and some environmental factors. The influence of environmental factors over p-cymene, gamma-terpinene, linalool and thymol concentration was evidently showed. Essential oil yield was fairly strongly related to the concentrations of Ca^{2+} and K^{+}, percentage of organic matter, altitude, temperature and soil texture (Yavari et al., 2010).

A number of studies in the phenol-rich *Lamiaceae* species: *Thymus vulgaris* L. (Gouyon et al., 1986), *Thymus 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 a total of 46 spice and herb extracts from different regions contained high levels of phenolics and exhibited antibacterial activity against foodborne pathogens. They suggested that there were highly positive relationships (R^2 = 0.73 to 0.93) between antibacterial 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 extract from different populations appears to be explainable by phenolic compound (thymol and carvacrol). These results agree with those reported by other researchers (Consentino et al., 1999; Davidson and Naidu, 2000; Skocibusic et al., 2006; Rota et al., 2008). These chemical differences can be most probably explained by the variability of the genetic factors as well as the existence of different chemotypes. Sheyda and Lordegan populations might be a potential thymol and carvacrol-rich source for mass-cultivation in order to improve commercial purposes.

The extract of populations of *H. scabrum* has a stronger antibacterial activity as compared to the positive antibacterial standards. The phenolic compounds, such as carvacrol, are widely reported to possess high levels of antimicrobial activity (Baydar et al., 2004; Nejad et al., 2008). Carvacrol, which is the main component of *H. scabrum* 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).

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The results showed that Gram-negative bacteria (*E. coli* and *P. aeruginosa*) were more sensitive than Gram-positive bacteria (*S. aureus and B. cereus*) (Table 2).

Table 2. The amounts of carvacrol and thymol (mg/g extract), zones of growth inhabitation (mm) and Minimum inhibitory concentration (MIC) (µg/ml) of different population of *Hypericum scabrum*.

Population	Thymol (mg/g extract)	Carvacrol (mg/g extract)	Zones of growth inhabitation (mm)				Minimum inhibitory concentration (µg/ml)			
			E. c [a]	*S. a*	*P. a*	*B. c*	*E. c*	*S. a*	*P. a*	*B. c*
Sheyda	1.3005	0.3839	18	16	19	16	312.5	312.5	78.5	625
Lordegan	1.2975	0.3927	15	19	17	19	625	<19	156	39
Chadegan	0.9490	0.2658	11	10	10	12	2500	2500	625	2500
Soork	0.9436	0.2567	11	9	15	11	2500	5000	625	5000
Sabz-e-kooh	0.9358	0.2585	10	13	8	10	2500	1250	625	625
*ANOVA	$p \leq 0.01$	$p \leq 0.05$	$p \leq 0.01$	$p \leq 0.01$	$p \leq 0.01$	$p \leq 0.01$	-	-	-	-

[a] *E. c: Escherichia coli; P. a: Pseudomonas aeruginosa; S. a: Staphylococcus aureus; B. c: Bacillus cerus*. N = 3. *One-way analysis of Variance (ANOVA).

Antibacterial activity of extracts varied related to the test organisms. Whiles, Cos et al. (2006) reported that Gram-negative bacteria are generally more resistant compared to the Gram-positive ones.

Conclusion

In the present study, we demonstrated the potent antibacterial activity of *H. scabrum* extract against pathogens strains, which justifies the large use of this plant in traditional medicine. We considered that it would be very useful to promote thymol and carvacrol chemotypes crop culture in order to guarantee the quality of products. In addition, the *H. scabrum* might be a potential thymol and carvacrol-rich source for commercial cultivation. However, further research is needed to evaluate the effectiveness of *H. scabrum* essential oils and extracts in food ecosystems to establish their utility as natural antimicrobial agents in food preservation and safety. It may be concluded that the best geographic localities for the large scale production of major constituents, for example, Sheyda and lordegan populations or ecological conditions similar to Sheyda might be a potential thymol and carvacrol-rich source for commercial cultivation.

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