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Cultivated Hypericum perforatum *Hypericin Extracts'* antibacterial effect against Susceptible and Methicillin-Resistant *Staphylococcus aureus*

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ABSTRACT

Staphylococcus aureus is important in many infections acquired in hospitals and communities. Infections caused by methicillin-resistant *S. aureus* predominantly originate from hospitals and are increasing in number in many countries. Therefore, many researchers have tried to find new compounds to act as substitutes for ineffective antibiotics. Hypericin is the active substance of *Hypericum perforatum* and material from the group of flavonoids with high antiviral and antibacterial properties. Due to the high demand and the limited number of *H. perforatum* pastures, *H. perforatum* is cultivated under different fertilizer treatments and it has been shown that, by increasing the amount of fertilizer, the amount of nitrogen and potash hypericin will rise in the fourth level of fertilizers (180 kg K_2O). The extent of the antibacterial effect was conducted using the sodden of plants in different dilution and disk diffusion methods. Each test was repeated three times, the mean diameter of the inhibition zone was measured on Mueller Hinton culture medium. Bacteria in various dilutions had significant effects, though the greatest impact occurred using a pure dilution and the lowest occurred in a dilution of 0.125 mg. The activity percentage effective decoctions of herbs against the MRSA, MSSA, and total strains were measured and the most being observed in MRSA strains.

1. Introduction

The increased prevalence of disease that, due to the consumption of foods infected with pathogenic bacteria or toxins, has been considered a critical public health issue for many years. Bacterial pathogens are more prevalent than any other pathogens transmitted via food and have caused several outbreaks of the disease. In this regard, bacteria such as *Salmonella*, *Listeria monocytogenes*, and *Escherichia coli* causes the highest illness and death rates (Mashhadian *et al.*, 2005). Moreover, considerable amounts of money have been spent on the treatment of diseases resulting from contaminated food. In Canada, for example, this amount is US $ 500 million (Todd, 1989). Among the most common of these bacteria is *E. coli* 0157, an enterohemorrhagic strain (Cornu and *et al*., 1999). This strain was first identified during an epidemiological investigation of disease outbreaks as a hemorrhagic colitis in the North America in 1982 (Riley *et al*., 1983). Since 1980, this strain of *E. coli* has been observed in large quantities of hemorrhagic colitis disorders and hemolytic uremic

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syndrome. This strain can be transmitted to humans in many ways including the consumption of water and food, most notably raw or under-cooked beef. In addition, this strain can be transmitted directly by animal-to-human or human-to-human contact (Armstrong *et al.*, 1996). Nowadays, antimicrobial agents, including protective additives and organic acids are used to prevent food contamination by pathogens and to increase the durability of foods (Kim *et al.*, 1995). Conversely, the spread of antibiotic-resistant strains of *Staphylococcus aureus* feature among the current problems faced by physicians and the number of available antibiotics used to treat these infections is reduced (Tiemersma *et al.*, 2004). Increasing antibiotic resistance and the subsequent identification of the adverse effects of chemical drugs has led to changes in the developmental approach of using medicinal plants, and many antibacterial substances originating from plants have subsequently been produced and marketed (Wuo, 1978; Azizi et al., 2002). In the meantime, it is known that *Hypericum perforatum* L., with a historical therapeutic use of over 2000 years, has a high-level of antimicrobial properties and, as an antimicrobial agent, can be applied against food pathogens. This herb has woody stems and is perennial, belonging to Hypericaceae that grows in different parts of Iran and temperate regions of Europe and Asia. Clinical and pharmacological evidence has shown that this herb has high antibacterial and antiviral affects (Biriskin *et al.*, 2001; Azizi *et al.*, 2002). Such properties are attributed to a special blend called hypericin. The accumulation of hypericin occurs in dark glands on the petals, sepals, anthers, and mutual leaves of the plant (Cellarova *et al.*, 1995).

Numerous studies have been undertaken regarding the antibacterial effect of hypericin. Three types of antibacterial ointment with *H. perforatum* oil have been investigated, and the results indicated that the topical use of the substance was appropriate for the skin and vagina against six bacterial strains including *Streptococcus pyogenes*, two strains of Viridans *Streptococcus*, *Micrococcus luteus* ATCC 9341, *Lactobacillus acidophilus*, and *Moraxella catarrhalis* (Saddiqe *et al.*, 2010). Furthermore, the effect of the extract against standard samples and clinical *Helicobacter pylori* was evaluated, and it was determined that concentrations of 95.1–250 µg/ml of the extract has antibacterial properties (Yeşilada *et al.*, 1995). Therefore, the search and demand for medicinal plants has also increased medicinal plant cultivation. Herbs with wild origins are gathered; however, due to their lower production-rates, they are less responsive to the consumer market and action should be taken regarding their cultivation (Franz, 1983; Alizadeh *et al.*, 2012). However, in the cultivation of medicinal plants, the product is the chemical fruit, which can be affected by different variables, particularly nutrients, it is necessary to investigate the properties of their secondary metabolites.

2. Materials and Methods

2.1. Sample collection

Cuttings taken from the middle height Kalardasht (1000-1500 m) that based on the analysis of variance accounted for the largest amount of hypericin was transferred to growing bag (Rahnavard *et al.* 2012). Samples planting was Tonekabon with geographic coordinates 39 S 0483774 ‹UTM 4074954 and were selected with 25 meters above the sea level. After determining of hypericin in the genotypes of Kelardasht cuttings transferred to the field and planted inside of the growing bag with a depth of 50 cm, and total area of 5.0 square meters, and nitrogen (urea 46% N) was applied at four levels (0, 40, 80 and 120 kg N) and potassium (potassium 50% potash) in four levels (0, 60, 120 and 180 kg K_2O) with three replications. This project was carried out at factorial design in randomized complete block design as cutting length varied between 5 and 8 cm. Before planting the soil of samples were analyzed (Table 1). Analysis of factorial design was done using SPSS version 19.

Table 1. Soil analysis of cultivated samples

Soil Texture: loam													
Fe ppm	Mn ppm	Cu ppm	Zn ppm	Depth Cm	EC ds/m	pH	Mean N %	OM %	Mean p ppm	Mean k ppm	Sand %	Silt %	Clay %
27	6.28	1.04	0.6	0-30	0.21	7.18	6	33.3	42.17	121	56.5	32.3	11.2

2.2. Extraction of hypericin

In order to prepare the methanol extract, the samples were powdered dried flowering branch with the highest amount of hypericin. For extraction, 13% methanol was used with the method of cold maceration (Hajipour *et al.*, 2009). After relevant time the extracts were cleared and the solvent removed from the extracts prior to analysis with HPLC and were kept at 4^{o}C.

2.3. HPLC Conditions

HPLC was used of model Agilent 1200 Series, detector model UV-Agilent and was set with a wavelength of 503 nm. ZORBAX XDB C18 column with the dimension of 150×4.6 mm was used and 5 µm particle size mobile phases of sodium dihydrogen phosphate (15.6 g/lit): ethyl acetate: methanol (41: 39: 160) with isocratic washing (speed 1ml/min) was injected with 23 µl of sample. 1 mg standard hypericin was purchased from Sigma-Aldrich company with catalog number of 123 K56178 (Malgorzata *et al.*, 2010)**.**

2.4. Determination of hypericin in the samples

In order to determine the amount of hypericin was used of hypericin standard with concentration of 133 ppm and calculated by HPLC instrument software. Then the extract was injected with the amount of 20 µl. Hypericin concentration was calculated by comparing hypericin peak of curve area with a standard curve.

2.5. Preparation of decoction of herbs

In order to prepare a decoction of about 30 grams of dried plant with the most hypericin, samples were poured into the container and 200 to 300 ml of distilled water was added. The mixture was boiled for 30 minutes and then the extract was separated by the filter paper.

2.6. Diluted plant extracts and prepared discs containing the extract

After extraction and preparation of decoction of herbs, the extracts were diluted with distilled water. For preparation of diskettes containing extracts the blank discs was used (6.4 mm, Padtan Teb, Iran).

Therefore the blank discs were placed in the tubes containing the dilutions of the extract and after 5-10 min they were taken and the disks were placed at 37 ° C for drying.

2.7. Bacteria Strains

The *Staphylococcus aureus* strains (sensitive and methicillin-resistant) was isolated from clinical samples from patients in Tonekabon city.

The disc-diffusion method was used for determining the susceptibility of bacteria to methicillin (Robetrs *et al.*, 2002). So that the 200 µl of turbidity of Equivalent 0.5 Mc Farland was prepared from any strains on Mueller-Hinton agar containing 4% NaCl was inoculated. After uniform culture, put it on oxacillin disc and after 24 hours were placed at 37°C, diameter of inhibition zone around each disk was measured and compared with the standard value (Baron *et al,*. 1990).

In this research the standard strains ATCC 25923 (sensitive to methicillin) and 1413 PTCC (methicillin- resistant) was used.

2.8. Investigation of the antimicrobial activity of extracts: The disc diffusion method

Each bacterial strain was incubated in nutrient broth at 37°C overnight (14 h), and the test bacterial solutions were prepared with the same broth to give a concentration of 1.5×10^{8} cfu/mg. Suspensions of microorganisms were transferred onto the surface of Muller-Hinton Agar media and spread evenly over the entire surface of the plates. Blank discs impregnated with 20 µl of a serial 20- fold dilution of extract compounds (pure, 0.5, 0.25, and 0.125 $mg.ml^{-1}$) were prepared using 50% DMSO. Also was used for the vancomycin disc as a control with contains 30 mg and for ensuring the experiment is repeated three times for each bacterial strain and the mean diameter of absence of growth recorded as the final diameter (Androw *et al,*. 2001).

3. Results

3.1. Culture results of Hypericum perforatum

Results of analysis of variance (Table 2) showed that the treatments had a significant effect on the amount hypericin. In this study the results from field studies showed that cuttings taken of middle height Kelardasht had high yield capability to produce hypericin in different fertilizer treatments. One way to increase secondary metabolites are the addition of plant dry weight, but with the addition of St.Johns, the flowers of the most important parts contain hypericin, but due to fact that in (*Hypericum perforatum*), flowers are the most important organs containing hypericin, and also there is no report on the effects of macronutrients on flavonoid metabolism. So increase the ratio in storage organs and manufacturer hypericin (Flowers) is the most important ways of increasing and improving the quality of this product (Martonfi *et al.*, 1994).

Table 2. Mean of square hypericin in the field under different fertilizer treatments

Mean of squares		
Hypericin	df	Sources of Variation
**3.179	3	N
**1.153	3	K
**0.057	9	Interaction Effect
0.0035	30	Error

** Significance at 1%

In this study we showed that by addition of N, hypericin was increased in plants. In 90 kg of N fertilizer, the most hypericin (2.836%) was observed and the lowest value (1.671%) was achieved in zero N fertilizer (Control treatment). The greatest amount of hypericin (2.765%) on the third level of potassium (P) and lowest in the control treatment was measured (Figure 2). Interaction of N and P fertilizers on the amount of hypericin were significant at the fourth level of N (90 kg/ha) and third level of P (60 kg/ha) (Figure 3).

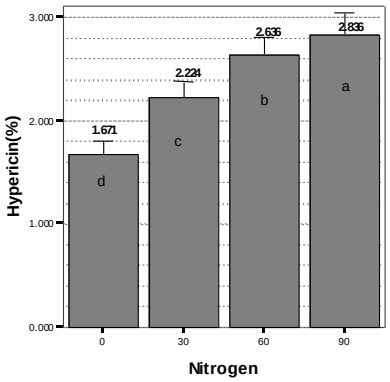

Figure 1. The effect of different amounts of nitrogen (N) on hypericin produced *H.perforatum L.*

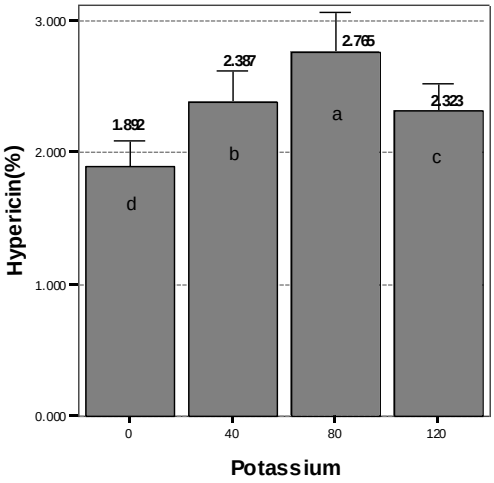

Figure 2. The effect of different amounts of potassium (P) on hypericin produced *H.perforatum L.*

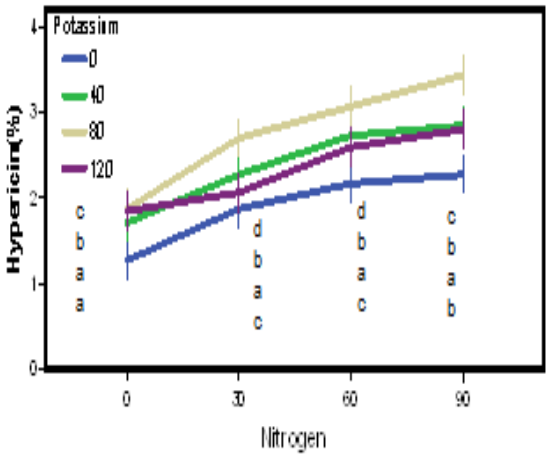

Figure 3. The Interaction of nitrogen and potassium on the hypericin produced *H.perforatum L.*

3.2. Bacterial Results

The various concentrations of hypericin had significant differences at the bacteria (table 2). The greatest impact on net dilution and the lowest in a dilution of 0.125 was obtained (Figure 4). Should be noted that significant differences were found between bacteria in terms of the zone diameter in different concentrations and more halo was formed at MRSA strains (Figure 5).

Activity percent of effective decoction of *H. perforatum* against strains, was obtained in the highest at MRSA and the lowest at MSSA strains (Figure 6).

Table 3. Analysis of variance of zone diameters bacteria in various concentrations

Sources of Variation	df	Mean of Square	F
Dilution	3	129.518	209.363**
Bacteria	1	5.320	8.600**
Interaction Effect	3	1.379	$2.230^{n.s}$
Error	14	.619	
Total	21		

* Significance at 1%

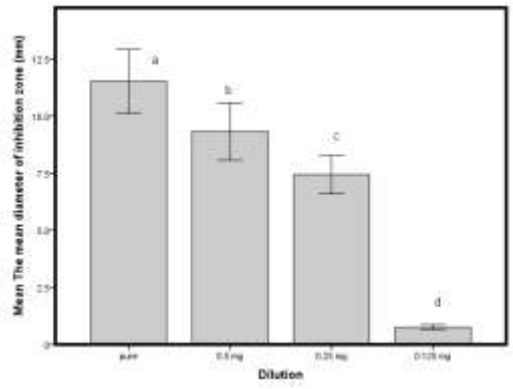

Figure 4. Effect of dilution in the formation of halos.

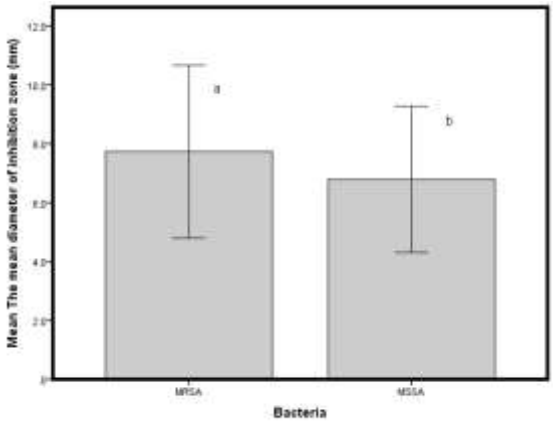

Figure 5. Effect of bacteria in formation of halos.

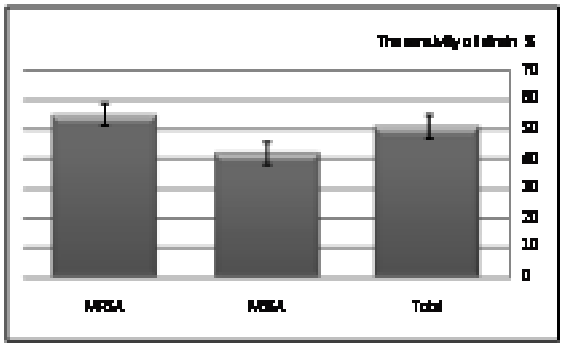

Figure 6. The sensitivity of the bacterial strains to boiled activities of *H.perforatum*.

4. Discussion

Environmental factors alter the biosynthesis of secondary metabolites in *H. perforatum L.* and an experiment in different media (sandy soil and medium culture) was also indicated that the production of these materials is controlled by culture conditions (Briskin *et al.*, 2000). Furthermore, different amounts of minerals caused several changes in the production of the aforementioned. In addition to macro elements, it was reported that micro-elements also has an effect not only upon naphthodianthrone, but they were also seen to be effective on hyperforin and proanthocyanidin activities (Gray *et al.*, 2000). Fertilizer tests performed on *H. perforatum* showed that fertilizer increased plant biomass and the amount of hypericin and hyperforin. The test also showed that yield, plant height, and number of branches rose per-flower (Briskin *et al.*, 2001). Medical properties of chemical compounds known as *Hypericum* are shown by Sadiqqe *et al.* (2010). According to the result of this study the combination hypericin of this plant has more effective antimicrobial effects on gram-positive bacteria. The effectiveness of *H. perforatum* extract against gram-positive bacteria over gram-negative bacteria has been mentioned in other reports (Reichling *et al.*, 2001; Avato *et al.*, 2004).

On the other hand, a glimpse at the results of this study shows good antibacterial effect of decoction of cultivated *Hypericum* against strains of MSSA and MRSA since the activity of effective compounds is associated with plants. Additionally, factors affecting the production of these metabolites have an important role in the extraction of active ingredients and their activities, as well as having superior antibacterial effect upon the decoction of herbs compared with other methods which indicates that the use of water as a solvent can help to achieve combinations of plant extracts. The antibacterial effect of the alcoholic extract of 20 species of medicinal plants from Golestan province against MRSA and MSSA strains showed that the best anti-staphylococcal effect of ethanol extracts came from eight species of medicinal plants such as barberry (Dadgar *et al.*, 2006). The results of anti-staphylococcal effect obtained from the decoction of the plant, do not match with the results of this research.

Thus, the solvent method and type of either plant (wild or cultivated plant planting's effect) is both effective and has antibacterial properties. To explain this process, water was chosen as a solvent when studying the anti-bacterial effect, and was compared with the effect of alcoholic extract which confirms that water is used in the extraction of most of the plant material. However, many plant compounds are complex organic compounds and their solubility in water is lower than alcohol. Furthermore, the valuation of the antibacterial effect of the decoction of herbs against MSSA and MRSA strains showed that the extract's dilution had a direct impact on the level of activity and effect of antibacterial properties.

Because a growing resistance of *S. aureus* to methicillin and other antibiotics was observed across different countries, the results of this study and especially considerable impact of high concentrations of decoction of *H. perforatum* is important on the strains of MSSA, MRSA, and particularly MRSA. This indicates that, in addition to the effective results of oil extraction, chloroform, and essential oils of these plants as proven in other studies, the effect of decoction is important and desired. However, the clinical application of these plants needs further and broader research before these plants can be successfully used and standardized to replace current ineffective antimicrobials.

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