Effects of heavy metals on the antibacterial properties of *Verbascum speciosum* Schard

Efectos de los metales pesados sobre las propiedades antibacteriales de *Verbascum speciosum* Schard

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ABSTRACT

The effects of heavy metals, from Hame-Kasi Mine in Western Iran, on the *in vitro* antibacterial effects of the aqueous extracts of polluted *Verbascum speciosum* Schard. (Scrophulariaceae) flowers in different concentrations were investigated. Determination of heavy metals (Cu, Fe, Mn, and Zn) in collected soils and plants from the control and mine area was performed using Flame Atomic Absorption Spectrophotometry.The control sites were from 5 km distance of the mine. The antibacterial activity of the extracts was examined against 10 bacterial strains (three Gram-positive and seven Gram-negative bacteria). Results showed high Fe, Mn and Zn concentration in polluted soil, and high Fe concentration in polluted plant in compared to control samples. The control and polluted aqueous extract appeared to be highly effective on *Salmonella paratyphi* in different concentrations, but non effective on six other bacteria strains.

Keywords: Heavy metals, *Verbascum speciosum*, antibacterial activity, Hame-Kasi Mine, Iran.

RESUMEN

Se investigó los efectos de los metales pesados provenientes de la mina Hame-Kasi en el oeste de Irán sobre los efectos antibacteriales *in vitro* de los extractos acuosos florales en diferentes concentraciones del control y contaminado de *Verbascumspeciosum* Schard. (Scrophulariaceae). La determinación de los metales pesados (Cu, Fe, Mn y Zn) en suelos y plantas colectados del área de control y de la mina se realizó mediante la espectrofotometría de absorción atómica de llama. Los controles estuvieron a 5 km de la mina. La actividad antibacteriana de los extractos se evaluó contra 10 cepas de bacterias (tres Gram-positivas y siete bacterias Gram-negativas). Los resultados mostraron mayores concentraciones de Fe, Mn y Zn en los suelos contaminados y mayor concentración de Fe en las plantas contaminadas en comparación con las muestras del control. El extracto acuoso del control y del contaminado pareció ser altamente efectivo sobre *Salmonella paratiphi* en diferentes concentraciones, pero no efectiva en las otras seis cepas bacterianas.

Palabras clave: Metales pesados, *Verbascum speciosum*, actividad antibacteriana, Mina Hame-Kasi, Irán.

INTRODUCTION

It is known that environmental stresses, such as heavy metals, industrial chemicals and pollutants may restrict plant growth and photosynthetic rates. Such conditions lead to the synthesis of compounds deriving from secondary metabolites (Noori *et al.*, 2009; 2010).

The incidence of microbial infections has increased dramatically in the past 20 years. Accordingly, increases in the rates of morbidity and mortality because of microbial infections have been regarded as a major problem (Tatli and Akdemir, 2005). For the treatment of bacterial disease antibiotics are sometimes used. The misuse of antibacterial agents increases the incidence of bacteria resistant strains and make the treatment of infection difficult (Diab, 2002). One of the solutions to solve antibiotic resistant incident problem among pathogenic bacteria is to develop new drug from natural sources such as plants. Herbal therapy is used in the management of disease before the advent of orthodox medicines (Diab 2002). In addition, extract or components of plants were used to treat infections in age-old practice in a large part of the world, especially in developing countries, where there is dependence on traditional medicine for a variety of diseases. Some plants remain a common source of antimicrobial agents which is reported to have minimal side effects. The medical and antimicrobial properties of aqueous plants extracts have been known since ancient times. The extracts of several wild and medicinal plants have been tested against some bacterial and fungal growth and for antimicrobial properties (Fareed *et al.,* 2008). While species used in traditional medicines continue to be the most reliable sources for the discovery of useful compounds, the screening of plant growing under various stresses has provided yet another source for compounds with useful activities against microbes (Rajakaruna *et al.,* 2002).

Over the centuries, plants have been served man as a source of drugs for the treatment of microbial infections (Momoh and Adikwu, 2008) and many efforts have been made to discover new antimicrobial compounds from various kinds of sources such as micro-organisms, animals and plants (Kavitha *et al*., 2012).

Iran has a rich flora (Ghahreman and Attar, 1999) which more than 500 plant species are widely used in folkloric medicine due to their antimicrobial and anticarcinogenic properties (Ayenechi, 1991; Zargari, 1993). Traditional medicines have been used for a wide variety of purposes for many thousands of years in Iran and all over the world. There are increasing interests in medicinal plants as a natural alternative to synthetic drugs (Fabio *et al.,* 2007), particularly against microbial agents. There are over a hundred chemical substances that have been derived from plants for drugs and medicines (Ashafa *et al.,* 2008).

The genus *Verbascum* (Scrophulariaceae) is represented by 49 species in Iran (Rechinger, 1963-1988). Some species of the genus have widely been used throughout centuries to treat internal and external infections. According to Meurer Grimes *et al.* (1996), many other internal and external uses of the leaves and flowers of several *Verbascum* species have been documented in many societies in Europe, Asia, Africa and northern America. *Verbascum* species contain a wide range of compounds, such as phenolic compounds, terpenes, and, alkaloids (Tatli and Akdemir, 2004). *V. thapsus* L. has been used for the treatment of different diseases including asthma, spasmodic cough, migraine and earache (Turker and Camper, 2002). *V. thapsus* L., *V. fruticulosum* L. and *V. undulatum* Lam., have been investigated for their antibacterial, antifungal, antiviral, and antimalarial activities through *in vitro* and *in vivo* tests (Magiatis *et al.,* 2001; Turker *et al.,* 2001). Based on McCutcheon *et al* (1995) report, extracts of *V. thapsus* revealed antiviral activity against Herpes virus type 1. Furthermore, aqueous extracts of *V. fruticulosum* demonstrated stronge growth inhibition on the malaria parasite (Sathiyamoorthy *et al.,* 1999). Flowers of *V. phlomoides* L., *V. densiflorum* Bertol. and *V. thapsus* L. species have been used for their ethnopharmacological effects among common people in Turkey. Their flowers prepared as drug have diuretic and expectorant effects (Dülger *et al.,* 2002).

Heavy metals are defined as that group of elements that have specific weights higher than about 5 g/cm^3. A number of them (Co, Fe, Mn, Mo, Ni, Zn, Cu) are essential micronutrients and are required for normal growth and take part in redox reactions, electron transfers and other important metabolic processes in plants. Metals which are considered nonessential (Pb, Cd, Cr, Hg etc.) are potentially highly toxic for plants (Rama Devi and Prasad, 1998; Sebastiani *et al.*, 2004; Rai *et al*., 2005). Large areas of land are contaminated with heavy metals resulting from mines, industries, urban activities and agricultural practices (Khan *et al.,* 2000; Clemens, 2001). Excessive concentrations of trace elements (Cd, Co, Cr, Hg, Mn, Ni, Pb and Zn) are toxic and lead to growth inhibition, decrease in biomass and death of the plant (Zenk, 1996). Heavy metals inhibit physiological processes such as respiration, photosynthesis, cell elongation, plant-water relationship, N-metabolism and mineral nutrition (Zornoza *et al*., 2002).

This paper reports the demonstration of *in vitro* activity by the aqueous extract of polluted *Verbascum speciosum* flowers against pathogenic bacteria (*Bacillus cereus*, *B. megaterium*, *B. subtilis*, *Citrobacter amalonaticus*, *Enterobacter aerogenes*, *Klebsiella pneumoniae*, *Proteus vulgaris*, *Pseudomonas aeruginosa*, *Salmonella parathyphi* and *Serratia marcescens*).

MATERIALS AND METHODS

Study site

The Hame-Kasi Mine is situated on the western region of Hamadan, Iran (lat. 34º 57´ N, long.

48º 8´ E) with 10 km^2 area. The mean temperature varies between +40ºC and -19ºC (Golestani, 2001; Barati, 2002).

Collection of soil and plant material and preparation

Ten samples of the soil (depth 10-15 cm) were taken and sieved through a 1 cm sieve. Ten samples of mature fresh plants of *Verbascum speciosum* (Scrophulariaceae) were collected from Hame-Kasi Mine area during 2007. Control samples were also collected at the 5 km distance from the mine based on Barati (2002) by the reason control and polluted area are similar in all of ecological data with the exception of mine grains. Specimens of each plant sample were prepared for reference as herbarium vouchers. Soil and dissected flower samples were air dried under shadow for heavy metal content as well as extraction and examination of antibacterial effects.

Determination of heavy metals

Determination of heavy metals (Cu, Fe, Mn, Zn) in control and polluted soil and plant samples was carried out using Shimadzu Atomic Absorption/ Flam Emmission Spectrophotometer (Model AA- 670) in Azad University of Hamadan.

Bacteria

A total of ten bacteria were used in the screening. Three Gram-positive bacteria strains *viz*.; [*Bacillis cereus* (PTC 1247), *B. megaterium* (PTC 1017), *B. subtilis* (PTC 1365)] and seven Gram-negative bacteria strains viz.; [*Citrobacter amalonaticus* (PTC 1499), *Enterobacter aerogenes* (PTC 1221), *Klebsiella pneumoniae* (PTC 1053), *Proteus vulgaris* (PTC 1079), *Pseudomonas aeruginosa* (PTC 1430), *Salmonella parathyphi* (PTC 1230) and *Serratia marcescences* (PTC 111)] used in this study were all reference isolates. They were obtained from the Pad-Tan Teb Company, Iran. The bacteria species were cultured in the sterile Mueller-Hinton Agar plates. All cultures were incubated at 37ºC for 18-24 h.

Extraction of the plant material

Five grams of coarsely powdered flowers were successively extracted with EtOH 70% and distilled water for 72 h by using Soxhlet. The extracts were filtered through Whatman No. 1 filter paper (Germany), evaporated under vacuum. The residues were separately dissolved in the same extracting solvent with 0.08, 0.4, 2, 10 and 50 ppm dilutions and kept in refrigerator till use.

Antibacterial testing

in vitro antibacterial activities of the control and polluted extracts in different dilutions were determined by paper disc method described by Dülger *et al.*, (2002). Ampiciline (10 µg/disk), nalidixic acid (30 µg/disk), peniciline (10 µg/disk) and tetracycline (µg/disk) were used as standards to determine the sensitivity for each microbial species tested. All plates were then covered and incubated for 18-24 at 37ºC. The inhibition zones on the each medium were calculated in mm (including the diameter of the disc) from the lower surface of the Petri dishes (Lopez-Garcia *et al.*, 1992). The control is consisted of paper disc soaked with appropriate solvent and evaporated to dryness. All the assays were carried out in triplicate. On each plate an appropriate reference antibiotic disc was applied depending on the antibiogram test bacterial strains table from the Pad-Tan Teb Company, Iran for comparing.

RESULTS

Bioaccumulation and heavy metals in control and polluted soils and plants

Table 1 and Figure 1 showed bioaccumulation coefficient (BAC) based on Kabata-Pendias and Pendias (2001) and heavy metal means (in ppm) in control and polluted soils and *Verbascum speciosum* using atomic absorption/ flam emission specterophotometeric methods. Results showed that polluted soil had the highest Zn concentration (25915 ppm) and polluted *Verbascum speciosum* had the highest Fe amount (15390 ppm). Mn concentration in both polluted (5855 ppm) and control (3218 ppm) soils were also high in comparison to the rest samples.

Antibacterial effects of control and polluted plant extracts

The results of the effects of control and polluted *Verbascum speciosum* extracts on tested bacterial strains using antibiogram tests table (Table 2) were shown in Table 3 and Figure 2. The means of the diameters of the inhibition zones were reported in table and figure using EXCEL. As shown in Table 3 and Figure 2, antibacterial activity were observed in four of examined taxa (*Bacillus subtilis*, *Enterobacter aerogenes*, *Proteus vulgaris* and *Salmonella parathyphi*) and not observed in the rest. Therefore, samples without any antibacterial activity were deleted in these Table 3 and Figure 2.

Bacillus subtilis

Polluted *Verbascum speciosum* extracts in 10 and 50 ppm concentrations showed antibacterial effects on *Bacillus subtilis*, while other concentrations were not effective.

Enterobacter aerogenes

Fifty ppm concentration of control *Verbascum speciosum* extract showed the highest antibacterial effect (11.2 mm) on this bacterium and other extracts were not influenced.

Proteus vulgaris

Concentrations of 0.08 and 0.4 ppm of control *Verbascum speciosum* extract didn't show any inhibition zone, while others were effective.

Table 1. Comparison of bioaccumulation coefficient (BAC) and heavy metal means (ppm) in control and polluted soils and *Verbascum speciosum* using atomic absorption/ flam emission spectrophotometric methods at the Hame-Kasi Mine, Hamadan, Iran.

	Mean ± SD heavy metal (ppm)				Bioaccumulation coefficient (BAC)	
Sources	Soil		*Verbascum speciosum*		*Verbascum speciosum*	
Heavy metals	Control	Polluted	Control	Polluted	Control	Polluted
Cu	1.70 ± 0.35	2.30 ± 0.98	25.30 ± 0.85	40 ± 1.53	0.6	1.01
Fe	278 ± 0.68	822.50 ± 0.55	311.50 ± 0.86	15390 ± 1.05	0.9	4.89
Mn	3218 ± 1.36	5855 ± 0.77	21 ± 0.46	39 ± 0.76	0.004	0.08
Zn	112 ± 0.96	25915 ± 0.49	56 ± 0.63	129.50 ± 1.55	0.002	0.06

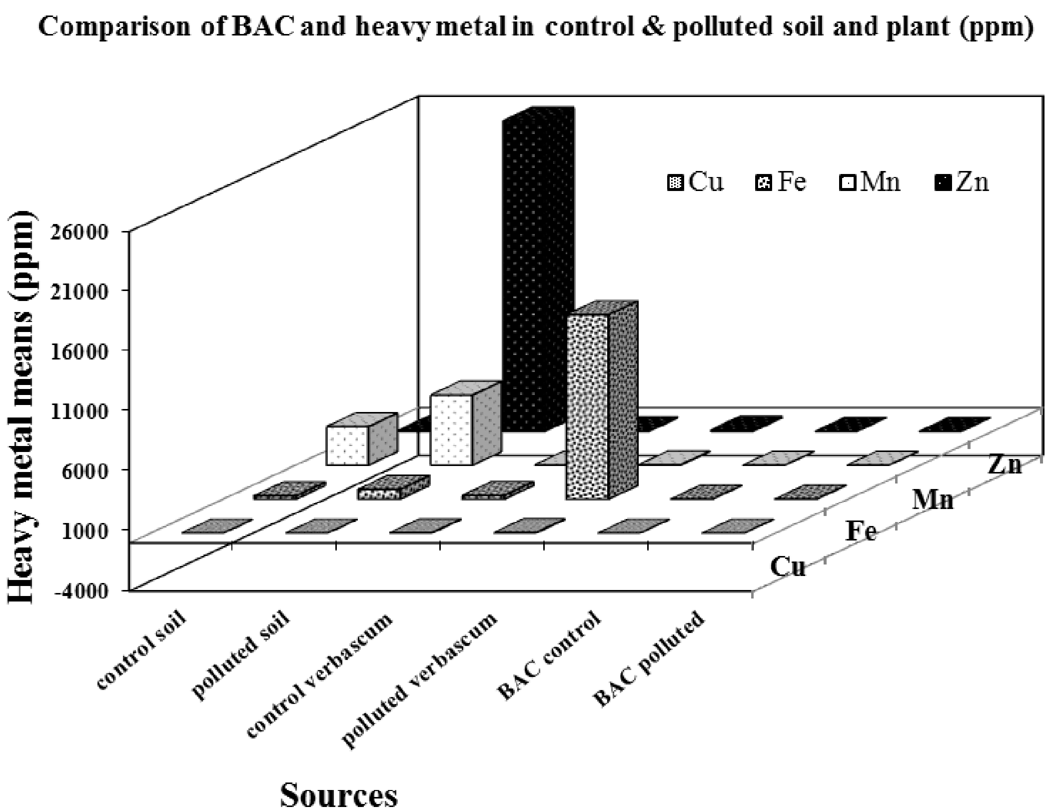

Figure 1. 3-D column with a cylindrical shape for comprising bioaccumulation coefficient (BAC) and heavy metal means (ppm) in control and polluted soils and *Verbascum speciosum* at the Hame-Kasi Mine, Hamadan, Iran.

Salmonella parathyphi

All of control and polluted *Verbascum speciosum* extracts in different concentrations showed antibacterial effects. 0.4 ppm concentration of control *Verbascum speciosum* extract had also the highest inhibition zone (10 mm).

DISCUSSION

Heavy metals contamination of arable soil showed several problems, including phytotoxic effects of certain elements such as Cd, Pb, Zn, and Cu, which are well known as micronutrients and cause several phytotoxicity if critical endogenous levels are exceeded (Mengel and Kirkby, 2001; Susarla *et al.*, 2002, Chehregani *et al.*, 2005). In a related study (Herms and Mattson, 1992) showed nutrient availability which may affect plant investments in defense relative to investments in growth. Another and even a more serious problem is posed by the uptaking of potentially noxious elements through food or forage plant species and their being transferred to the food chain and, finally, to humans (Kloke, 1980). Large areas of land are contaminated with heavy metals resulting from mines, industries, urban activities and agricultural practices (Khan *et al.*, 2000; Clemens, 2001). Heavy metals inhibit physiological processes such as respiration, photosynthesis, cell elongation, plant-water relationship, N-metabolism and mineral nutrition (Zornoza *et al.*, 2002). Some external mechanisms that limit the uptake of metals by root can help plants tolerate a certain amount of toxic metal in soil. One of them is the formation of non-toxic metal-ligand chelates in rhizosphere involving organic acids and other substances exuded from roots. The tolerance of heavy metals is also enhanced by the action of mycorrhizae (Khan *et al.*, 2000; Baranowska-Morek, 2003).

There are many interactive effects of several concurrent stresses from environmental conditions. In many cases plants resistant to heavy metal stress have lower nutritional requirements and specific mineral and water economies to cope with this stress (Siedlecka *et al.*, 2001; Baranowska-Morek, 2003). Gunthardt-Goerg and Vollenweider (2007) reported

Table 2. Antibiogram test table and comprising *in vitro* antibacterial effects of standard antibiotic discs on four examined bacteria strains.

Sensitivity/Bacteria	Mean ± SD inhibition zone in diameter (in mm) around the disc						
	Sensitivity			Four examined bacteria			
Antibiotic	Resistant	Intermediate	Sensitive	*Bacillus subtilis*	*Entrobacter aerogenes*	*Proteus vulgaris*	*Salmonella paratyphi*
Ampiciline	< 13	14-18	> 16	-	-	-	18 mm
Nalidixic acid	< 13	14-16	> 18	-	-	-	22 mm
Peniciline	< 28	-	≥ 29	28 mm	-	-	-
Tetracycline	< 14	15-18	> 18	16 mm	14 mm	12 mm	-

Table 3. Comprising *in vitro* antibacterial effects of the extracts of control and polluted *Verbascum speciosum* flowers in different concentrations at the Hame-Kasi Mine, Hamadan, Iran..

			Mean ± SD inhibition zone in diameter (mm)									
Extract concentration (ppm)			0.08		0.4		2		10		50	
Bacteria	Strains	Gram +/-	C	P	C	P	C	P	C	P	C	P
Bacillus subtilis	PTC 1365	+	0	0	0	0	0	0	0	8.0± 0.82	0	7.8± 0.24
Entrobacter aerogenes	PTC 1221	-	0	0	0	0	0	0	0	0	11.2± 0.71	0
Proteus vulgaris	PTC 1079	-	0	8.00± 0.57	0	8.80± 0.50	8.07± 0.77	9.80± 1.09	9.20± 0.76	9.80± 0.85	9.80± 0.86	7.80± 0.24
Salmonella paratyphi	PTC 1230	-	8.10± 0.46	9.80± 1.25	10.00± 0.85	8.70± 0.24	9.10± 0.53	8.20± 0.92	8.10± 0.72	6.80± 0.24	7.20± 0.57	7.70± 0.62
Control plate	-	No one	0	0	0	0	0	0	0	0	0	0

C = Control and P = Polluted. 0 = no zone of inhibition stress associated with a biotic (bacteria, fungi and insects) or abiotic (frost, drought, mineral deficiency, heavy metal pollution in the soil, acidic deposition, and ozone) helpful for the validation of symptoms in broadleaved and conifer trees. Remarkably resistant plants are involved in phytoremediation of metal polluted sites (Sebastiani *et al.*, 2004). Such plants are uncommon and according to Khan *et al*. (2000), about 400 hyperaccumulator species have been identified. As Table 1 and Figure 1 showed high concentration of Cu, Fe, Mn and Zn in polluted soil and plants compare to controls, it is believed that *Verbascum speciosum* is able to accumulate huge amounts of heavy metals in its tissues and it is a hyperaccumulator species.

In this study investigations showed that the ethanolic-aqueous plants extracts possesses antimicrobial activity against some of the tested organisms. Previous reports showed that the aesthetic, medical and antimicrobial properties of plants extracts have been known since ancient times (Janovska *et al.*, 2003). The extracts of several wild and medicinal plants have been tested against some bacterial and fungal growth and for antimicrobial properties (Fareed *et al.*, 2008). *Verbascum speciosum* has grown in a natural environment imposing extreme edaphic stress on plants. The soils consist of high concentrations of some heavy metals (Cu, Fe, Mn and Zn) (Table 1 and Figure 1). The screening of plant growing under various stresses has provided yet another source for compounds with useful activities against microbes (Rajakaruna *et al.*, 2002). So, plants growing in Hame-Kasi Mine may be good candidates for the study of plants with antimicrobial properties. Antimicrobial activity was most consistently detected in *Verbascum speciosum*. Notably, the flower of the species exhibit activity against *Entrobacter aerogenes* with 11.2 mm inhibition zone (Table 2 and Figure 2). Contrary Dülger *et al.* (2002) founds that *Verbascum* L. species have antimicrobial activity against the Gram-positive (Gr +) bacteria, but not have activity against the Gram-negative (Gr -) bacteria used in their study. Polluted *Verbascum speciosum* flower extracts in 10 and 50 ppm concentrations showed antibacterial effects on *Bacillus subtilis* (Gr +). In addition 50 ppm concentration of control *Verbascum speciosum* flower

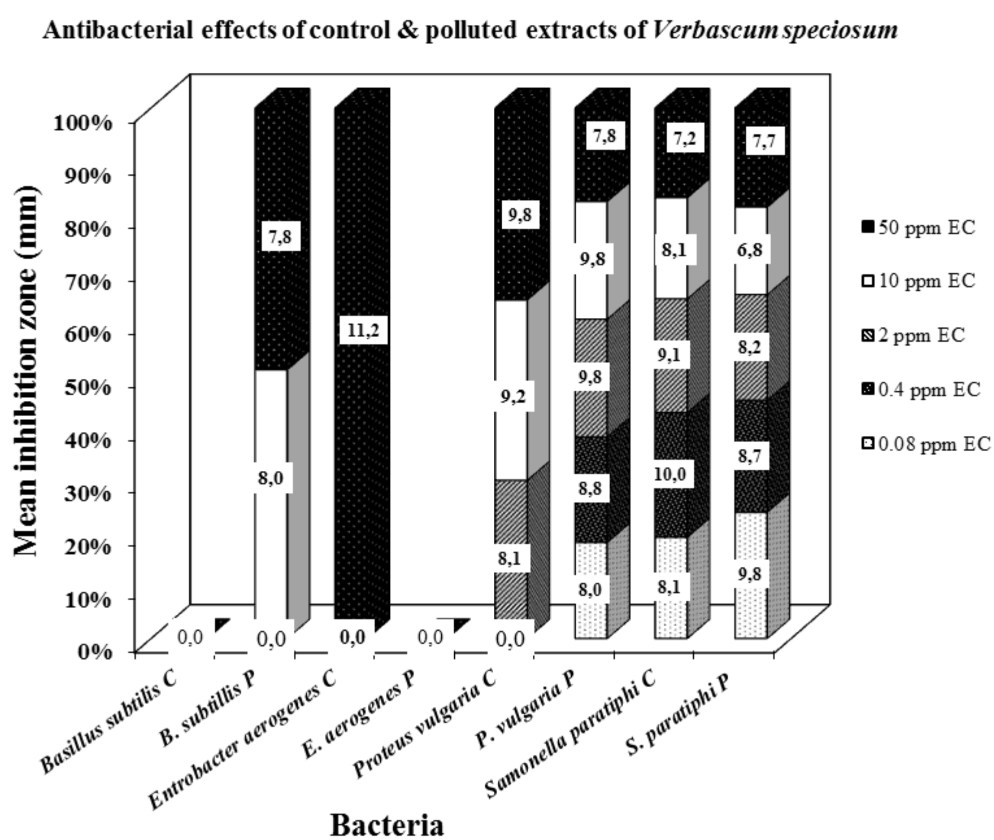

Figure 2. Stacked column with a visual effect histogram for comprising *in vitro* antibacterial effects of control (C) and polluted (P) *Verbascum speciosum* extracts in different concentrations. EC = extract mean concentration at the Hame-Kasi Mine, Hamadan, Iran.

extract showed the highest antibacterial effect on *Entrobacter aerogenes* (Gr -). Control and polluted *Verbascum speciosum* flower extracts in different concentrations also showed antibacterial effects on both *Proteus vulgaris* and *Salmonella paratyphi* (Gr-) bacteria (Table 2 and Figure 2).

Table 2 and Figure 2 showed a wide variation in *in vitro* antibacterial effects of control and polluted flower extracts of *Verbascum speciosum*. All the flower extracts exception polluted extract in 10 ppm and 50 ppm concentrations are not effective against *Bacillus subtilis*. Polluted flower extracts of the species in different concentrations were also effective against *Proteus vulgaris*. Rajakaruna *et al*., (2002) showed plants growing in serpentine soils, however are physiologically adapted to deal with the stresses of the environment. It is possible that various compounds that are induced by stress, either for tolerance or as a by-product of some physiological process, may in fact possess antimicrobial properties. But, further work is needed using high performance liquid chromatography (HPLC), dry chemistry (DC) or gas chromatography (GC) to evaluate and comparison of the nature of adaptation in all studied plants. Studies by other researchers (Tahara *et al.*, 1994; Hashem and Sahab, 1999) have also shown the induction of antimicrobial compounds by plants exposed to high Cu. Studies done on species growing in high metal environments such as serpentine habitats have shown that plants that accumulate heavy metals may have better defenses against herbivores and pathogens (Boyd, 1998). Boyd *et al.* (1994) have shown that the Ni hyperaccumulating plant, such as *Streptanthus polygaloides* (Brassicaceae) may effective against the some pathogenic fungi. Other studies (Weiersbye and Przybylowicz, 1999; Ghaderian *et al.,* 2000) further support the hypothesis that metal hyperaccumulation by plants is closely linked to enhanced protection against disease.

It is evident that the four examined bacteria did not show any sensitivity to the plant extracts compared to the standard antibiotic disks (Tables 2 and 3). Finally results showed that the control and polluted flower extracts of *Verbascum speciosum* did not have strong antimicrobial effects against four examined bacteria, as compared to the examined standard antibacterial antibiotics. Notably, the effect of extract depends on the type of solvent, plant species and part of plant used.

Finally it is believed that existence antimicrobial compounds in polluted plants may be responsible for environmental stresses and may have a protective defensive role against heavy metal high concentrations.

CONCLUSIONS

The present study investigated antimicrobial properties of *Verbascum speciosum* Schard. found in Hame-Kasi Mine in Western Iran, a natural environment imposing extreme edaphic stress on plants. The soils consist of high concentrations of some heavy metals. Plants growing in this area, however, are physiologically adapted to deal with the stresses of the environment. It is possible that various compounds that are induced by stress, either for tolerance or as a by-product of some physiological process, may in fact possess antimicrobial properties. Hence, plants growing in mine environment may be good candidates for the study of plants with antimicrobial properties.

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