

Antimicrobial Activity of Isolated Bacterial Endophytes from *Cichorium intybus L*, *Pelargonium hortorum*, and *Portulaca oleracea* Against Human Nosocomial Bacterial Pathogens

Sharareh Lotfalian,¹ Azizollah Ebrahimi,^{1*} and Mohamad Reza Mahzoonieh¹

¹Department of Pathobiology, School of Veterinary Science, Shahrekord University, Shahrekord, IR Iran

*Corresponding author: Azizollah Ebrahimi, Department of Pathobiology, School of Veterinary Science, Shahrekord University, Shahrekord, IR Iran. Tel: +98-3814424427, Fax: +98-3814424427, E-mail: A_kahrizsangi@yahoo.com

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Abstract

Background: Bacterial endophytes are colonizers of the inner plant tissues in which they do not normally cause any substantial morphological changes or disease symptoms. Endophytic bacteria are safe microorganisms that reside within the plant hosts and are known to enhance the growth and development of host plants, probably by secreting growth hormones. These bacteria are known to enhance growth and products of plants by fixing atmospheric nitrogen, solubilization of phosphate, production of phytohormones and siderophores, and possession of antagonistic activity, as well as reducing the level of stress ethylene in host plants.

Objectives: In this descriptive study, we focused on the isolation of bacterial endophytes from three medicinal plants *Cichorium intybus L*, *Pelargonium hortorum*, and *Portulaca oleracea* and screened them for activities against nosocomial isolates of *Staphylococcus aureus*, *Enterococcus faecalis*, *Pseudomonas aeruginosa*, and *Acinetobacter baumannii*.

Methods: Random samples from asymptomatic leaves and branches of three medicinal plants (*Cichorium intybus L*, *Pelargonium hortorum*, and *Portulaca oleracea*) were collected. To isolate the endophytic bacteria, the disinfected portions of the plants were distributed onto the isolation media. To examine endophytic bacterial contents, bioassays were conducted using growing colonies in PA and YEA, inactivating them by chloroform. To test the antibacterial activity of the endophytic bacterial culture broth, filter-sterilized supernatants were poured into cylinders on each bacterial plate.

Results: A total of 24 phenotypically distinguishable bacterial endophytes were isolated in pure form from three medicinal plants. In part of the chloroform-inactivated colonies of all 24 isolated endophytes, the most effective herb was *C. intybus L*, followed by *Po. oleracea*, and in part of the supernatant culture broth, the most effective herb was *Po. oleracea*, followed by *C. intybus L*.

Conclusions: Endophytic microorganisms residing in *Cichorium intybus L*, *Portulaca oleracea*, and *Pelargonium hortorum* are a very promising source for production of bioactive compounds. In general, most isolated endophytes had an acceptable effect against indicator bacterial pathogens.

Keywords: Medicinal Plants, Endophytes, Antibacterial Activity, Iran

1. Background

The term “medicinal plants” includes various types of plants used in herbalism. Some of these plants demonstrate medicinal activity. These medicinal plants are considered rich sources of ingredients which can be used in drug development and synthesis.

Herbal medicine has been used for the treatment of various ailments in many countries. Antioxidants, such as vitamin C, polyphenols, carotenoids, tocopherols, and flavonoids may be responsible for the antibacterial activity of medicinal plants (1). In addition, these plants play a critical role in the development of human cultures around the world. Some plants are considered important sources of nutrition and, as a result, these plants are recommended for their therapeutic value (2).

Bacterial endophytes are colonizers of the inner plant

tissues in which they do not normally cause any substantial morphological changes or disease symptoms. They also are known to enhance the growth and development of host plants. The scientific community has become interested in bioprospecting these microorganisms for their potentially important secondary metabolite production, in particular for application in the pharmaceutical and food industries (3). The question is whether these substances are produced by the plant itself or as a consequence of a mutual relationship with beneficial organisms in their tissue. Much research has shown that in a microbe-plant relationship, endophytes contribute substances that possess various types of bioactivity, such as antibacterial and antifungal (4, 5).

In Iran, extracts from many types of local plants are traditionally used for the treatment of various ailments.

In one of our earlier works, regarding antimicrobial activity of endophytes originating from medicinal plants, we examined endophytes from native medicinal plants of Chaharmahal province, in west central Iran, and screened them for antimicrobial activity (6). However, little information is available on the occurrence or the potential significance of bacterial endophytes from medicinal plants *Cichorium intybus L*, *Pelargonium hortorum*, and *Portulaca oleracea*, which seem to be promising medicinal herbs.

2. Objectives

In this descriptive study, we focused on the isolation of bacterial endophytes from three medicinal plants (*Cichorium intybus L*, *Pelargonium hortorum*, and *Portulaca oleracea*) and screened them for activity against the human nosocomial bacterial pathogens *Staphylococcus aureus*, *Acinetobacter baumannii*, *Enterococcus faecalis*, and *Pseudomonas aeruginosa*.

3. Methods

3.1. Collection of Plant Samples

Samples of three medicinal plants (*Cichorium intybus L*, *Pelargonium hortorum*, and *Portulaca oleracea*) were collected in spring 2013 and, after transferring them to Shahrekord University, they were taxonomically identified at the Botany Department. Asymptomatic leaves and branches of the three medicinal plants were thoroughly washed in running tap water, after which they were surface sterilized by submerging them in 75% ethanol for two minutes. The portions were further sterilized sequentially in 5.3% sodium hypochlorite solution for five minutes and 75% ethanol for 0.5 minutes. After being washed with distilled water and dried, each leaf was divided into segments. For isolation of endophytic bacteria, the disinfected portions were distributed onto the isolation media yeast extract agar (Merck, 64271, Germany) (yeast extract 5gr/L, glucose 10gr/L, agar 16gr/L) (YEA) and peptone agar (15g/L peptone water (Difco, 1807-17-4) and 15g/L agar (Quelab. 42-0223)) (PA) and incubated at room temperature for 3 - 7 days (7). Preliminary bacterial identification was done using Gram staining, catalase and oxidase activity, and biochemical tests on demand.

3.2. Bacterial Strains

Isolates of *S. aureus*, *A. baumannii*, *E. faecalis*, and *Ps. aeruginosa* were received from Isfahan and Shahrekord hospitals. Early morphological and biochemical tests were done to confirm the genera and species of the received isolates. Biochemical examinations, including Lysine Iron

Agar (LIA) (Quelab. 65-2097), Urease (Merck, 8483, Germany), Oxidation-Fermentation (OF), (Merck, 10282, Germany), and Triple Sugar Iron Agar (TSI) (Quelab. 39-4906) tests, in addition to Gram staining, growth in MacConkey agar (Merck, 64271, Germany), and cetrimide media (Biomark, 411-011, India), as well as oxidase (Merck, 3067, Germany) and catalase examinations followed, for confirmation of the isolates. The methods for isolation and identification of all isolates were based on the Quinn et al. guidelines (8).

3.3. Endophytic Bacterial Contents

To isolate the endophytic bacteria, selected colonies were diluted in peptone water (0.1%) and displayed as drops (Pasteur pipette) in PA and YEA media. Petri dishes were incubated at room temperature at 37°C for 24 - 48 hours, simultaneously. The bioassays were conducted using growing colonies in PA and YEA, and the bacteria were inactivated by chloroform for 15 minutes.

Plates were opened for 30 minutes to evaporate the substance. At the same time, 40 field isolates of *S. aureus*, *A. baumannii*, *E. faecalis*, and *Ps. aeruginosa* (10 isolates each) (BHI broth 24 hours at 37°C, (Himedia, 400-086)) were reactivated. A volume of 200µL of each culture, properly reactivated, was transferred to 10ml of semisolid BHI medium and shaken. This mixture was deposited onto the surface of plates (YEA) containing chloroform- (Merck, 64271, Germany) inactivated bacterial colonies. The plates were incubated at 37°C for 24 hours for the observation of inhibition halos (7) (Figure 1).

3.4. Endophytic Bacterial Broth Culture

To test the antibacterial activity of the endophytic bacterial culture broth, 200µl of each field isolate (109 CFU mL) was added into 15ml of YEA at 50°C, mixed thoroughly, and poured into a 9 cm-diameter Petri dish. After solidification, two to three sterilized stainless cylinders (5 mm internal diameter and 10mm high) were placed, open end up, on each plate. The culture broth of endophytic bacterial isolates grown in LB broth (Merck, 110285, Germany) (18 - 24 hours incubation at 37°C) was centrifuged (Sigma, serial no. 103286,) at 10,000 rpm for 15 minutes, and filter-sterilized supernatants (100µl of each) were poured into cylinders on each bacterial plate (9) (Figure 2).

4. Results

Segments of surface-sterilized leaves and stems of *Cichorium intybus L*, *Pelargonium hortorum*, and *Portulaca oleracea* incubated in yeast extract agar, peptone water, and

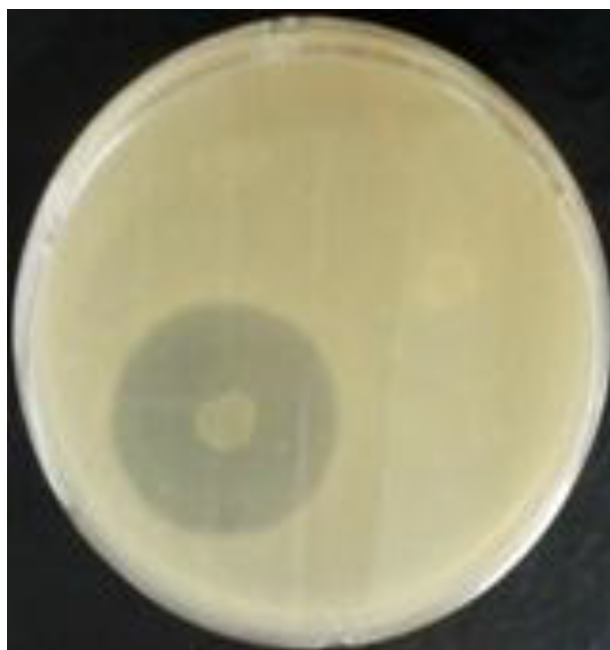


Figure 1. Chloroform-inactivated colonies of isolated endophytes showed antimicrobial activity against *S. aureus*.

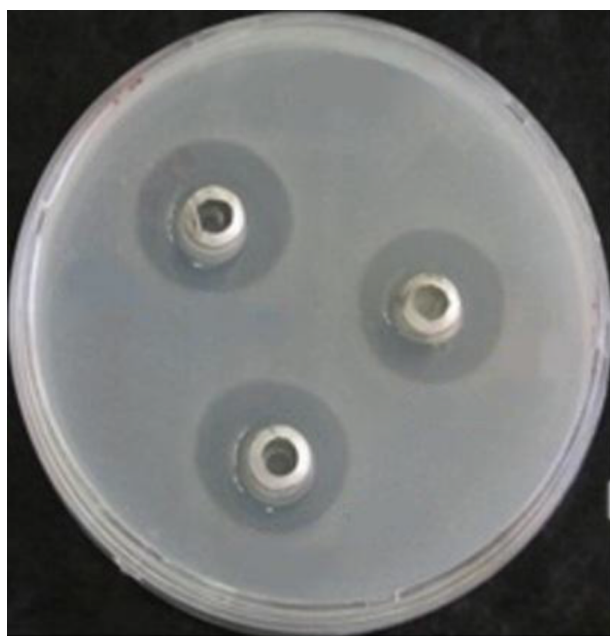


Figure 2. Supernatant culture broth of the isolated endophytes showed antimicrobial activity against *S. aureus*.

brain heart infusion agar plates showed growth of morphologically distinguishable bacterial colonies surround-

ing the segments after 24 - 48 hours. A total of 24 phenotypically distinguishable bacterial endophytes were isolated in pure form from three medicinal plants. Of the herbs of these 24 isolates, 7 were from *Cichorium intybus* L (6 leaves, 1 branch), 10 from *Pelargonium hortorum* (only branch), and 7 were from *Portulaca oleracea* (3 leaves, 4 branches). The bacterial endophytes were characterized based on micromorphological, Gram staining, and catalase examinations. Of the 24 isolated bacterial endophytes, 5 were Gram-positive (2 cocci and 3 *Bacillus* spp) and 19 were Gram-negative (7 Bacilli and 12 Coccobacilli). Filamentous forms were not detected in any of the plant samples.

Antimicrobial activity of all bacterial endophytes was assessed against 40 bacterial isolates of *S. aureus*, *A. baumannii*, *E. faecalis*, and *Ps. aeruginosa* (10 isolates each). The isolate which inhibited growth of any of the test isolate(s) was considered to have antibacterial activity, and the length of the inhibition zone was measured (Tables 1 and 2). Of the 24 isolated endophytes screened, chloroform-inactivated colonies of four endophytes from the leaves and one from the branches of *C. intybus* L, as well as two endophytes from the leaves and three endophytes from the branches of *P. oleracea* showed an average inhibition zone of more than 9.5 mm against *Staphylococcus aureus* and *E. faecalis* isolates (Table 1), while supernatant culture broth from all 24 endophytes from three plants showed an average inhibition zone of more than 21 mm against *S. aureus* isolates (Table 2).

Chloroform-inactivated colonies of four endophytes from the leaves of *C. intybus* L, of two endophytes from the leaves of *P. oleracea*, and five from the branches of *Pe. hortorum* showed an average inhibition zone of more than 10.5 mm against *S. aureus* and *E. faecalis* isolates (Table 1), while the supernatant culture broth of three endophytes from the leaves and one from the branches of *C. intybus* L, as well as three from the leaves and four from the branches of *Po. oleracea* showed an average inhibition zone of more than 9.5mm against *E. faecalis* isolates. Chloroform-inactivated colonies of five endophytes of *Po. oleracea* showed an average inhibition zone of more than 10.5mm against *E. faecalis* isolates (Table 1), while the supernatant culture broth of 24 isolated endophytes of three herbs all showed inhibition zones of less than 4mm against *Pseudomonas aeruginosa* and *A. baumannii* isolates (Table 2). The chloroform-inactivated culture broth of 24 isolated endophytes of three herbs all showed inhibition zones of less than 1.5 mm against *A. baumannii* isolates (Table 1).

5. Discussion

We screened only the stem and leaves of *Cichorium intybus* L, *Pelargonium hortorum*, and *Portulaca oleracea*, al-

Table 1. Antibacterial activity of chloroform-inactivated bacterial colonies isolated from *C. intybus* L, *P. hortorum*, and *P. oleracea* against 40 field isolates (10 each) of human bacterial pathogens^{a,b}

Herb	Endo.	Average Inhibition Zone (mm)				
		Morph.	<i>E. faecalis</i>	<i>A. baumannii</i>	<i>Ps. aeruginosa</i>	<i>S. aureus</i>
<i>C. intybus</i> L	1L	Bacillus spp	6.8 (2.53)	0 (0)	2 (1.8)	0 (0)
	2L	G- Bacilli	19.4 (5.45)	0 (0)	1.5 (1.4)	10.6 (3)
	3L	Bacillus spp	23 (5.46)	0 (0)	5.3 (3.5)	14.9 (4.2)
	4L	G+ Cocci	12.4 (4.01)	0 (0)	4.6 (2.25)	0 (0)
	5L	G- Bacilli	22.9 (4.54)	0 (0)	3.2 (2.03)	6.9 (2.27)
	6L	G- Bacilli	5.4 (3.75)	0 (0)	3.7 (2.3)	0 (0)
<i>P. oleracea</i>	1B	G- Cocco bacill	6.2 (3.48)	0 (0)	1.6 (1.5)	9.8 (3.8)
	1L	G- Cocco bacill	2 (0)	0 (0)	1.6 (1.5)	0 (0)
	2L	G- Cocco bacill	12 (1.9)	0 (0)	1.5 (1.4)	0 (0)
	3L	G- Cocco bacill	11 (1)	0 (0)	0 (0)	0 (0)
	1B	G- Cocco bacill	0 (0)	0 (0)	2.5 (2.3)	1.2 (1.13)
	2B	G- Cocco bacill	17.6 (6.03)	0 (0)	1.7 (1.6)	9.8 (3.8)
	3B	G- Cocco bacill	19.6 (5.4)	0 (0)	3.9 (2.4)	2.8 (1)
	4B	Bacillus spp	14.6 (4.97)	0 (0)	2.7 (2.5)	6.8 (2.3)
	1B	G- Bacilli	13 (4.3)	0 (0)	7 (3.5)	1 (0)
	2B	G- Cocco bacill	20.8 (4.85)	0 (0)	1.7 (1.6)	10 (2)
<i>P. hortorum</i>	3B	G- Bacilli	3 (0)	1.5 (0)	1.5 (0)	17.8 (3.7)
	4B	G- Bacilli	4.7 (2.3)	0 (0)	1.2 (1.3)	1.5 (1)
	5B	G- Cocco bacill	2 (1.8)	0 (0)	0 (0)	1.3 (1.2)
	6B	G- Cocco bacill	0 (0)	0 (0)	1 (0)	1.5 (1)
	7B	G- Bacilli	0 (0)	0 (0)	3.2 (2.03)	0 (0)
	8B	G+ Cocci	1.2 (1.1)	0 (0)	0 (0)	0 (0)
	9B	G- Cocco bacill	16 (4.47)	0 (0)	1.2 (1.13)	8 (3)
	10B	G- Cocco bacill	17.2 (4.7)	0 (0)	1 (0.9)	1.5 (1)

^aEndo stands for endophytes, morph for morphology, L for leaf, and B for branch.

^bNumbers in parentheses show standard deviation of averages.

though endophytes could also occur in the roots, flower, and seeds. The leaves of *C. intybus* L were found to harbor more endophytes than the branch segments (Table 1), while for *P. hortorum*, the condition appeared to be the reverse. This higher species richness in one anatomical site may be attributed to micro-environmental peculiarities, as specific conditions in essential nutrients drive the survival of tissue-specific endophytes. Differences in the prevalence of endophytes in different parts of plants have also been reported by others (10, 11).

Antimicrobial activity of endophytic bacteria is not uncommon. Li et al. (12) have explored endophytic actinomycetes associated with pharmaceutical plants in the rain forest of Yunnan, China and detected endophytic *Strepto-*

myces displaying antimicrobial activity against *S. aureus*, *Ps. aeruginosa*, and *Candida albicans*. In one of our earlier works, we showed that most fungal and bacterial endophytes from four medicinal plants (*Stachys lavandulifolia*, *Rumex pulcher*, *Hypericum scabrum*, *Starja bacteriarica*, and *Achillea kellalensis*) displayed considerable activity against indicator fungal and bacterial strains (6).

In the present study, five bacterial endophytes from seven endophytes of *C. intybus* L that were chloroform-inactivated showed antibacterial activity (more than 9.5 mm inhibition zone) against *E. faecalis* and *S. aureus* isolates (Table 1). In the supernatant broth culture of bacterial endophytes of this herb, all endophytes from the leaves and branches showed antibacterial activity against *S. au-*

Table 2. Antibacterial activity of supernatant culture broth of endophytes isolated from *C. intybus*L, *P. hortorum*, and *P. oleracea* against 40 field isolates (10 each) of human bacterial pathogens^{a,b}

Herb	Endo.	Average inhibition zone (mm)				
		Morph.	<i>E. faecalis</i>	<i>A. baumannii</i>	<i>Ps. aeruginosa</i>	<i>S. aureus</i>
<i>C. intybus</i> L	1L	Bacillus spp	0 (0)	0 (0)	2 (1.2)	21.3 (0.5)
	2L	G- Bacilli	5.4 (2.3)	3.6 (1.8)	1.9 (1.2)	21.3 (0.6)
	3L	Bacillus spp	6 (2.3)	2 (1.2)	0 (0)	22.1 (0.96)
	4L	G+ Cocci	9.8 (2.1)	1.7 (1.09)	3 (1.5)	21.8 (0.9)
	5L	G- Bacilli	10.3 (2.2)	2.2 (1.3)	1.9 (1.2)	21.7 (0.67)
	6L	G- Bacilli	10.4 (2.2)	2.9 (1.8)	2.2 (1.4)	22 (0.9)
<i>P. oleracea</i>	1B	G- Cocco bacill	10.4 (2.2)	2 (1.2)	1.9 (1.2)	21.4 (0.63)
	1L	G- Cocco bacill	11 (2.5)	1.8 (1.1)	1.5 (1)	22.5 (0.9)
	2L	G- Cocco bacill	11.9 (2.04)	2.3 (1.5)	2 (1)	22 (1)
	3L	G- Cocco bacill	10.8 (2.3)	0 (0)	2 (1.2)	21.4 (0.9)
	1B	G- Cocco bacill	9.9 (2.19)	2.3 (1.5)	2.2 (1.4)	21.3 (0.6)
	2B	G- Cocco bacill	10.5 (2)	1.9 (1.2)	1.7 (1.09)	21 (0.9)
	3B	G- Cocco bacill	9.2 (2.03)	2.1 (1.3)	2.3 (1.4)	21.6 (0.9)
	4B	Bacillus spp	10.9 (2.2)	1.9 (1.2)	2.3 (1.4)	21.2 (0.7)
	1B	G- Bacilli	6.4 (2.5)	3.6 (1.7)	1.6 (1.5)	20.9 (1.03)
	2B	G- Cocco bacill	8.2 (2.6)	1.9 (1.2)	2.1 (1.4)	21.6 (1.7)
<i>P. hortorum</i>	3B	G- Bacilli	8 (2)	2.1 (1.3)	1 (0)	21.5 (1)
	4B	G- Bacilli	7.8 (2.1)	1.9 (1.2)	0.8 (0.7)	21.2 (1.1)
	5B	G- Cocco bacill	8.2 (2.3)	1.5 (1.4)	2.4 (1.5)	21 (0.8)
	6B	G- Cocco bacill	0 (0)	2.3 (1.5)	1.7 (1.09)	21.2 (0.7)
	7B	G- Bacilli	5.7 (2.2)	2.2 (1.4)	2.5 (1.6)	20.5 (0.7)
	8B	G+ Cocci	0.8 (0.7)	1.7 (1.09)	2 (1.2)	21.9 (0.88)
	9B	G- Cocco bacill	5 (2)	2 (1.2)	1.8 (1.1)	21.6 (0.55)
	10B	G- Cocco bacill	1.4 (1.3)	1.9 (1.2)	1.8 (1.1)	20.9 (0.64)

^aEndo stands for endophytes, morph for morphology, L for leaf, and B for branch.

^bNumbers in parentheses show standard deviation of averages.

reus, and four endophytes showed antibacterial activity against *E. faecalis*. In each part, one bacterial endophyte showed broad spectrum antimicrobial activity, indicating possible biotechnological applications of endophytes living in the tissues of this herb. However, isolation, purification, and detection of active compound(s) are necessary for their further utilization.

Regarding *Pe. hortorum*, the supernatant culture broth of all isolated endophytes showed high antibacterial activity against *S. aureus* (Table 2), but in part of the chloroform-inactivated colonies, five out of ten endophytes of this herb were effective against *E. faecalis* (Table 1).

Pelargonium species are rich sources of monoterpenes, sesquiterpenes, coumarins, tannins, phenolic acids,

cinnamic acids, flavones, flavonoids, and flavonol derivatives (13). The antimicrobial activity of extracts of Pelargonium and their constituents has also been reported by others against *S. aureus* and some other bacteria (14).

For *Po. oleracea*, the supernatant culture broth of all isolated endophytes showed high antibacterial activity against *S. aureus* and *E. faecalis* (Table 2), but in part of the chloroform-inactivated colonies, only five of seven isolated endophytes were effective against *E. faecalis* (Table 1). Chan et al. (15) reported that two active ingredients, namely linoleic and oleic acids, were identified from *Po. oleracea* with synergistic antibacterial activity when combined with erythromycin against MRSA, indicating that they possibly act by inhibiting the efflux pumps

of the bacteria cells. The antibacterial activity of the extract of this herb is also reported elsewhere (16). In view of the ever-increasing demand for novel antimicrobial substances, the endophytes identified in examined medicinal plants could be new candidates for a potential source of new antibiotics (3).

In part of the supernatant culture broth, all seven endophytes from *C. intybus L* showed high antibacterial activity against *S. aureus*, while four endophytes were effective against *E. faecalis* (Table 2). In part of the chloroform-inactivated colonies, five endophytes of this herb were effective against *E. faecalis* and *S. aureus* (Table 1).

Nandagopal and Kumari reported antibacterial activity of the root extracts of *C. intybus L* against *S. aureus*, *Ps. aeruginosa*, and *E. coli* (17). Patkowska and Konopiński (18) showed antagonistic activity of selected bacteria of the soil environment of the root of *C. intybus L* towards fungi pathogenic towards this plant. In terms of each isolated endophyte, antibacterial activities varied considerably against all examined pathogenic bacteria, and also between the two methods (chloroform-inactivated and supernatant culture broth) of examination (Tables 1 and 2), suggesting that bacterial growth inhibition is mediated by a variety of antimicrobial metabolites.

In conclusion, endophytic microorganisms residing in *Cichorium intybus L*, *Portulaca oleracea*, and *Pelargonium hortorum* are a very promising source for production of bioactive compounds effective against some human nosocomial bacterial pathogens. Further research should be conducted to classify the endophytes residing in the studied herbs and exploit the substances produced by them.

Footnotes

Authors' Contribution: Sharareh Lotfalian performed the examinations; Azizollah Ebrahimi supervised the study and wrote the manuscript; Mohamad Reza Mahzoonieh supervised the study.

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