World Journal of Agricultural Sciences 2 (3): 311-318, 2006
ISSN 1817-3047

The Genetic Relationship and Antimicrobial Activity of *Plantago* Species Against Pathogenic Bacteria

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Abstract: The antimicrobial effect of *Plantago lanceolata* and *Plantago Major* that collected from Jordan and *Plantago lanceolata* that introduced from France was investigated. Also, the genetic variation among the two *Plantago* sp. was determined. The *Plantago* sp. showed different antimicrobial activity in different extract amounts against five bacteria. *Plantago major* extract inhibited the growth of *S. aureus* at 200 mg ml^{-1} only. *Plantago lanceolata* extract inhibited the growth of all tested bacteria except *Klebsiella* sp. The collected plants of *Plantago lanceolata* from Jordan and France showed significant differences in their antimicrobial activity at different amounts of extracts. Based on variation of RAPD patterns, the similarity matrix between the two *Plantago* species was 35%. This indicated that the two species are not the same in their components, morphology and their activity.

Key words: Medicinal plants · antimicrobial activity · *Plantago* sp.

INTRODUCTION

In Jordan, about 485 species belonging to 330 general and 99 families are recorded as medicinal plants. Many of them are under collection pressure and often used for many medicinal purposes [1].

The antimicrobial agent may be defined as a chemical substance derived from a living source (plants, animals, or microbes) that in dilute solutions has the capacity to inhibit or destroy microorganism's growth [2].

Because of the side effects and the resistance that pathogenic microorganisms build against the antibiotics, much recent attention has been paid to extract biological active compounds from plant species that used in herbal medicine. In many parts of the world, medicinal plants are used for their antibacterial, antifungal and antiviral activities. These plant extracts were used as a source of medicinal agents to cure urinary tract infections, cervicitis, vaginitis, gastrointestinal disorders [3] and skin infections such as herpes simplex virus type I [4].

Plantain is the general name for several small herbs used medicinally because of their mucilaginous properties; *Plantago psyillium*, *Plantago ovata*, *Plantago major* and *Plantago lanceolata* are representatives of the species. The plant has been traditionally used as a remedy against insect bites, toothaches, fevers, ulcers and wounds. Other medicinal applications of the plantain species have included use as an astringent, demulcent and diuretic. Extracts of common plantain have been reported to exhibit antibacterial activity [5] and can suppress the humoral immune responses, especially in primary immune response [6]. A study conducted by Holetz *et al.* [7] indicated that *Plantago major* had some activity on *Staphylococcus aureus* (ATCC 25923). Another study by Hassawi and Kharma [8] showed no activity for *Plantago lanceolata* against *Candida albicans*.

A new technique called polymerase chain reaction (PCR) has opened a new era of biotechnology by which DNA molecules can be amplified and detected very rapidly [9]. This technology gives the chance to researchers to amplify specific regions of DNA in order to get millions of copies of DNA region flanked by two primers [10].

PCR- based genetic markers have been successfully applied in a wide range of plant and microbial species to discriminate between individuals [11, 12]. Several DNA molecular marker techniques based on PCR technology have been established [9]. One of these techniques is called random amplified polymorphic DNA (RAPD) which is simple, fast and provides an accurate assessment of the genetic variation of species and cultivars [10, 13].

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The aims of this study were to test the activity of the plant extracts of *Plantago lanceolata* and *P. major* that collected from Jordan and *P. lanceolata* that introduced from France on inhibiting the growth of five pathogenic bacteria (*Staphylococcus aureus*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Klebsiella* sp. and *Enterobacter* sp.) and also study the genetic variations among these plants.

MATERIALS AND METHODS

Plant material: The plant materials (*Plantago lanceolata* and *Plantago major*) were collected from Jordan. Also, *Plantago lanceolata* seeds were introduced from France and grown in the experimental station of Faculty of Agricultural Technology. Scientific, family and english names and common medicinal uses for these plants are summarized in Table 1.

Preparation of extracts: Plant materials were dried in shade at room temperature and ground by using a blender. Two hundred and fifty gram of plant powder was soaked in 1.25-1.5 l of 95% ethanol for 5 days at room temperature. The mixture was mixed daily for regular infusion. After a five-day period, the extract was filtered by using Whatman filter paper No. 1. The filtrate was dried by using a rotary evaporator at 60°C. The dried extract was stored in sterile glass bottles at -20°C until using [14].

Microorganisms: Five bacterial species were used in this study; they included one gram positive (*Staphylococcus aureus*) and four gram negative *(Escherichia coli*, *Pseudomonas aeruginosa*, *Klebsiella* sp., *Enterobacter* sp.). These microorganisms were obtained from the hospital of the University of Jordan.

Screening of antimicrobial activities: Nutrient agar medium (N.A.) was prepared by dissolving 28 g of N.A. in one liter of distilled water. The medium autoclaved at 121°C under 15 psi pressures for 30 minutes. After cooling to about 65°C, 25 ml of the medium poured in Petri-dish (90 mm x 15 mm (diameter x height)). The plates kept at room temperature for solidification and stored at 4°C until using.

Inoculums containing 10^6 bacterial cells /ml were spreaded on nutrient agar medium. Antimicrobial activity test was then carried out by using the hole-plate diffusion method. Holes were made on the media by using 6 mm cork borer. The dried plant extracts were dissolved in dimethylsulfoxide (DMSO) to final extract amounts of 200, 150, 100 and 50 mg ml^{-1}. Each hole (diameter 6 mm) in each plate was filled with 50 µl of plant extract. The inoculated agar plates were incubated at 37°C for 24 h. After the incubation period, the diameter of inhibition zone to each hole was measured in millimeter. The inhibition zone is the area surrounding the hole and there is no growth of the inoculated microorganism. DMSO used as negative control and showed no antimicrobial activity against any of the tested bacteria.

Genomic DNA isolation: DNA extraction was performed according to Doyle and Doyle [15]. Young leaves from the collected plants were rinsed twice with sterile distilled water and then blotted between two filter papers for 10 min. One hundred milligrams of leaves tissue were placed in 1.5 ml microfuge tubes and grounded in the presence of liquid nitrogen.

Four hundreds fifty microliter of DNA extraction buffer [2X CTAB (Hexadecyltrimethylammonium bromide), 1.4 M NaCl (Sodium chloride), 100 mM Tris-HCl (pH 8), 20 mM EDTA (Ethylenediaminetetraacetic Acid), 0.2% 2-mercaptoethanol, 1 mM 1,10-O-phenanthroline] were added to the grounded tissues. Samples were incubated at 65°C for 15-30 min in a water bath with continuous mixing. After cooling to room temperature, 600 µl of chloroform/isoamyl alcohol (24:1; v: v) were added to each tube and vortexed for few seconds. The tubes were centrifuged at 14000 rpm for four minutes. The aqueous phase of each sample was transferred into a new tube. One microliter of 10 mg ml^{-1} RNase was added to each tube and incubated for 60-70 min at 37°C. Chloroform/isoamyl alcohol extraction was repeated and the top phase was placed in new sterile tubes.

Table 1: The scientific, family and english names and the common medicinal uses of plant the plants species used in this study

Scientific and family name	English name	Common medicinal uses	Reference
Plantago lanceolata (Plantaginaceae)	Ribwort plantain	For tooth pain, bronchitis, purgatives, wound healing, cough.	7, 16
Plantago major (Plantaginaceae)	Greater plantain	For stomach upset, stomach ache, stomach and intestine inflammation, abscesses, cold, pimples, wounds, dysentery, burns, angina, asthma, fever, tuberculosis, whooping cough, chronic renal inflammation, dermal diseases, bronchitis, purgative.	7, 9, 16

DNA was precipitated by adding equal volume of isopropanol. Each tube were mixed and centrifuged for five minutes at 14000 rpm. Pellets were washed with 1 ml of 70% ethanol and left for air drying. Pellets were then dissolved in 50 µl sterile deionized water and stored at -20°C until using for PCR reactions. DNA concentrations where determined by using spectrophotometer.

RAPD reactions and PCR program: The final volume of the RAPD reaction was 25 µl, which contains 2.5 µl of 10X PCR buffer [50 mM KCl (Potassium chloride), 10 mM Tris-Cl, 5 mM $MgCl_2$ (Magnesium chloride), 0.1% Triton X-100)], 0.25 µl of 2-deoxyribonucleic acids (dNTPs; 10 mM each of dATP, dTTP, dGTP and dCTP), 10 pmole of Kit B primers (Operon DNA Technologies, Alameda, USA), 0.2 µl Taq DNA polymerase (5 units/µl) and 0.5-1 µl (about 20-100 ng) of genomic DNA template as suggested by Hoelzel [10]. Deionized water was then added to adjust the final volume.

The PCR program was as following: four minutes at 95°C, 40 cycles of 30 sec at 94°C as denaturation step, 60 sec at 36°C as annealing step and 80 sec at 72°C as extension step; one cycle of 10 min at 72°C. Following amplification, samples were stored at 4°C until using for electrophoresis.

Amplified products were separated by electrophoresis in 1.2% agarose gel with 1X TBE buffer (0.089 M Tris, 0.089 M Boric acid and 0.002 M EDTA). Five microliter of 10 mg ml^{-1} ethidium bromide stain was added to the 100 ml agarose solution before pouring in the casting tray. The agarose gel was poured in the tray and allowed to solidify. A running buffer of 0.5X TBE was added to about 0.5 cm above the gel level. PCR products were loaded with 6X gel loading dye. The gel chamber was connected to the power supply in a way that DNA runs towards the anode (+ve pole). Electrophoresis was performed at 100 volts for about 2 h and bromophenol blue dye have migrated to about 7.5 cm. Amplification products were visualized under ultraviolet light at 254 nm and photographed using Biorad Gel Documentation System (BioRAD. Gel DOC 2000).

Statistical analysis: The experiment for the antimicrobial activity was conducted and analyzed as a factorial experiment in a Completely Randomized Design (CRD).

Estimation the size of amplified DNA fragment: The amplified fragment DNA size was estimated by using regression equation which explains the relation between the logarithm of the DNA marker size (bp) (Y, dependent variable) and the mobility distance of the marker (mm) (X, independent variables) on each gel. Correlation coefficient (R) was calculated to determine the strength relationship between the two factors of the regression equation.

Similarity matrix calculation: Similarity matrix was calculated by using the following equation [16]:

$$\text{Similarity matrix} = 2N_1/(N_1+N_2+N_3)$$

Where N_1 is the number of shared bands in species a and b, N_2 is the number of bands present in a but not in b and N_3 is the number of bands present in b but not in a.

RESULTS

The effect of bacteria, plants, extract amounts and their interaction were highly significant (Table 2). The two *Plantago* species showed significant differences against the tested bacteria (Table 3). *P. major* inhibited the growth (8.7 mm) of *S. aureus* at 200 mg ml^{-1} only. *P. lanceolata* extract inhibited the growth of all tested bacteria except *Klebsiella* sp. It affected the growth of *S. aureus* and *E. coli* (Fig. 1) at three extract amounts (200, 150 and 100 mg ml^{-1}) and affected the growth of *P. aeruginosa* and *Enterobacter* sp. at all used extract amounts (200, 150, 100 and 50 mg ml^{-1}). The largest inhibition zone (12.2 mm) produced by *P. lanceolata* at 200 mg ml^{-1} against *S. aureus*. The significant interaction indicated that although *P. lanceolata* affected on the bacteria at most extract amounts; *P. major* affected only on *S. aureus* at one extract amount.

For RAPD technique, 20 arbitrary decamer primers (Kit B QIAGEN Operon, USA) including B-1 to B-20 were used. The number of bands produced per primer by using the PCR varied from 0 to 7 with each species.

Table 2: Source of variation and degree of freedom (df) of the antimicrobial activity of two *Plantago* sp. at four extract amounts against five bacteria

Source of variation	df	Mean square
Bacteria (B)	4	20.422**
Plant (P)	1	146.306**
Extract amounts (E)	3	26.306**
B*P	4	12.103**
B*E	12	4.259**
P*E	3	16.039**
B*P*E	12	1.899**
Error	120	0.069
Total	159	

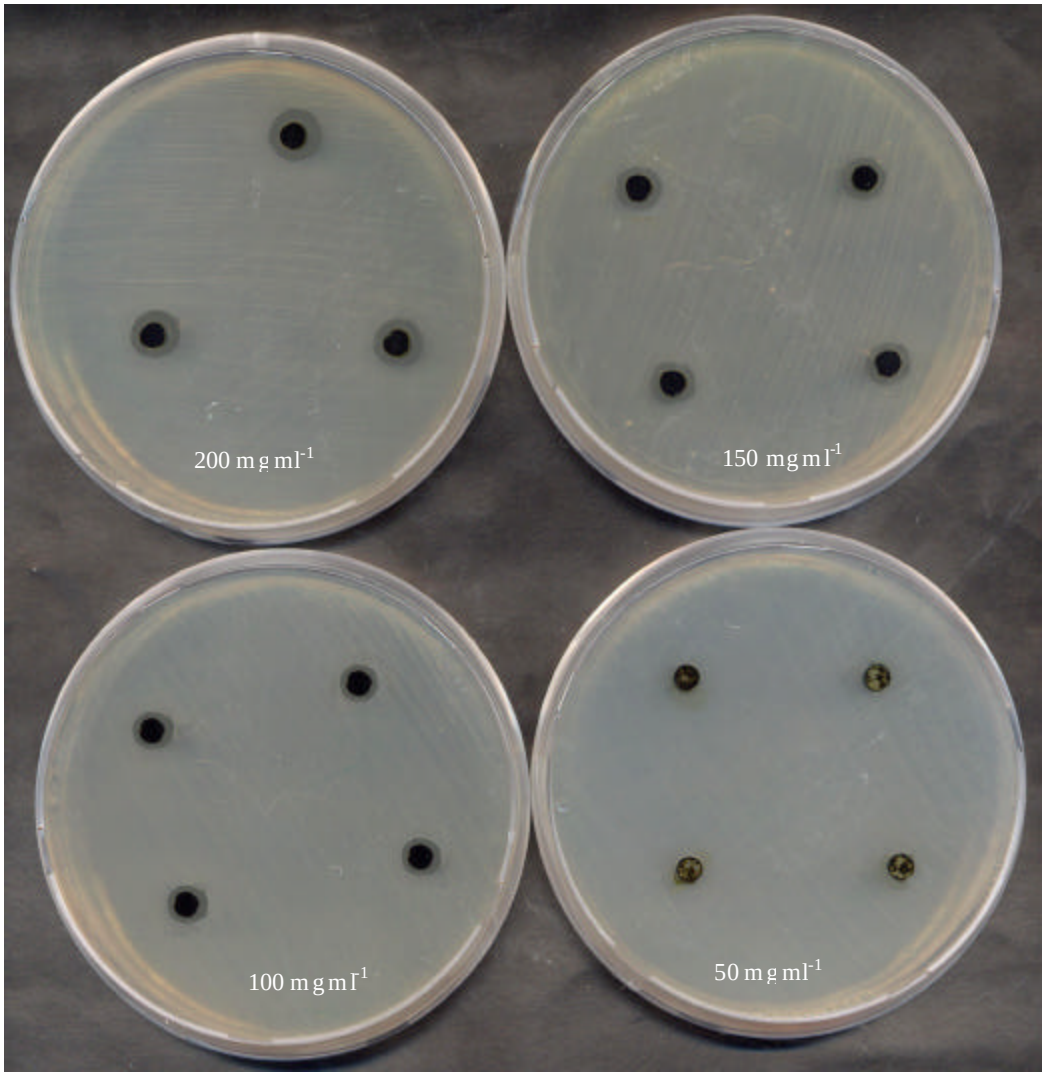

Fig. 1: Antimicrobial activity of *Plantago lanceolata* against *Escherichia coli* at four extracts amounts

Table 3: Antimicrobial activity of *Plantago* sp. at four extracts amounts against five bacterial species

Bacteria	Extract amount (mg ml^{-1})	Averages of inhibition zone (mm) *P. major*	*P. lanceolata*
S. aureus	200	8.7	12.2
	150	0.0	11.7
	100	0.0	8.2
	50	0.0	0.0
Klebsiella sp.	200	0.0	0.0
	150	0.0	0.0
	100	0.0	0.0
	50	0.0	0.0
P. aeruginosa	200	0.0	10.5
	150	0.0	10.0
	100	0.0	8.5
	50	0.0	7.2
E. coli	200	0.0	9.0
	150	0.0	8.0
	100	0.0	7.2
	50	0.0	0.0
Enterobacter sp.	200	0.0	9.5
	150	0.0	8.7
	100	0.0	8.2
	50	0.0	7.2

Table 4: Source of variation and degree of freedom of the antimicrobial activity of *Plantago lanceolata* at four extract amounts against five bacteria

Source of variation	df	Mean square
Bacteria (B)	4	91.053**
Plant (P)	1	8.556**
Extract amount (E)	3	96.540**
B*P	4	18.759**
B*E	12	15.586**
P*E	3	8.873**
B*P*E	12	2.326**
Error	120	0.089
Total	159	

Table 5: Comparing between antimicrobial activity of *Plantago lanceolata* which collected from Jordan and France at four extract amounts against five bacterial species

Bacteria	Extract amount (mg ml^{-1})	Averages of inhibition zone (mm) *P. major* (Jordan)	*P. lanceolata* (France)
S. aureus	200	12.2	17.0
	150	11.7	12.7
	100	8.2	10.2
	50	0.0	0.0
Klebsiella sp.	200	0.0	0.0
	150	0.0	0.0
	100	0.0	0.0
	50	0.0	0.0
P. aeruginosa	200	10.5	10.0
	150	10.0	0.0
	100	8.5	0.0
	50	7.2	0.0
E. coli	200	9.0	8.5
	150	8.0	0.0
	100	7.2	0.0
	50	0.0	0.0
Enterobacter sp.	200	9.5	9.2
	150	8.7	0.0
	100	8.2	0.0
	50	7.2	0.0

Table 6: The PCR product of *Plantago* species by using B-4 and B-10 primers

Primer	DNA fragment size	*P. major**	*P. lanceolata**
B-4	545	+	+
	718	+	-
	847	-	+
B-10	381	-	+
	545	+	+
	847	+	-
	1317	+	-

* (+) indicated the presence of band and (-) indicated the absence of band

Fifteen bands resulted from the PCR amplification of *P. major* DNA and 16 bands of *P. lanceolata* DNA (Fig. 2). The largest band (1470 bp) was amplified from *P. major* DNA by using primer B-7. The amplification of *P. lanceolata* DNA by using primer B-10 produced the smallest band (381 bp). Two bands were similar in size (545 bp) and produced by primers B-4 and B-10 in both species.

The similarity matrix of the two *Plantago* sp. was 35% based on two primers (B-4 and B-10) used in RAPD analysis (Table 6). From the similarity matrix results, a dendrogram was drawn (Fig. 3).

The extract of the species that collected from Jordan and that introduced from France showed antimicrobial activity. The effect of bacteria, plants, extract amounts and their interaction were significant (Table 4). Both extracts inhibited the growth of all tested bacteria except *Klebsiella* sp. (Table 5). *P. lanceolata* from Jordan inhibited the growth of *P. aeruginosa* and *Enterobacter* sp. at all used extract amounts and inhibited *E. coli* at three extract amounts (200, 150 and 100 mg ml^{-1}). *P. lanceolata* from France inhibited the growth of *P. aeruginosa*, *Enterobacter* sp. and *E. coli* at extract amount (200 mg ml^{-1}) only. The growth of *S. aureus* was

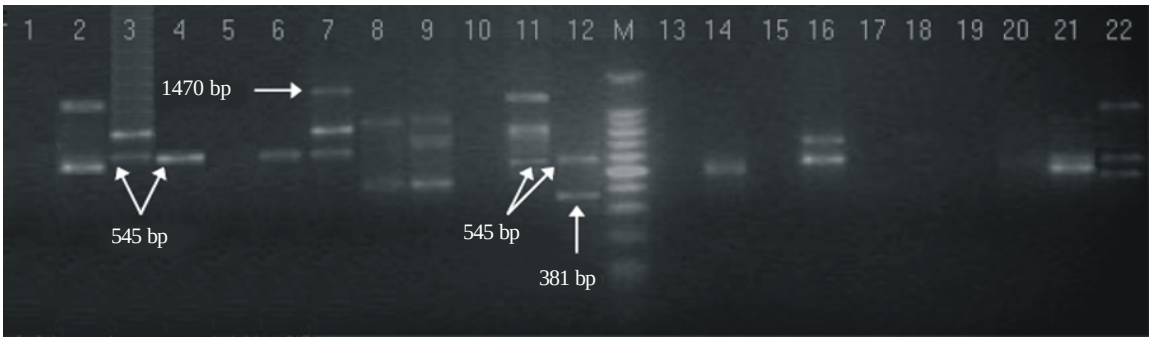

Fig. 2: DNA from *Plantago major* and *P. lanceolata* that amplified with different primers. The odd number of lanes for *P. major* and the even number for *P. lanceolata*

Lanes	Primers	Lanes	Primers
1, 2	B- 2 (TGATCCCTGG)	11, 12	B- 10 (CTGCTGGGAC)
3, 4	B- 4 (GGACTGGAGT)	13, 14	B- 11 (GTAGACCCGT)
5, 6	B- 6 (TGCTCTGCCC)	15, 16	B- 12 (CCTTGACGCA)
7, 8	B- 7 (GGTGACGCAG)	17, 18	B- 14 (TCCGCTCTGG)
9, 10	B- 8 (GTCCACACGG)	19, 20	B- 15 (GGAGGGTGTT)
		21, 22	B- 17 (AGGGAACGAG)

M: DNA marker

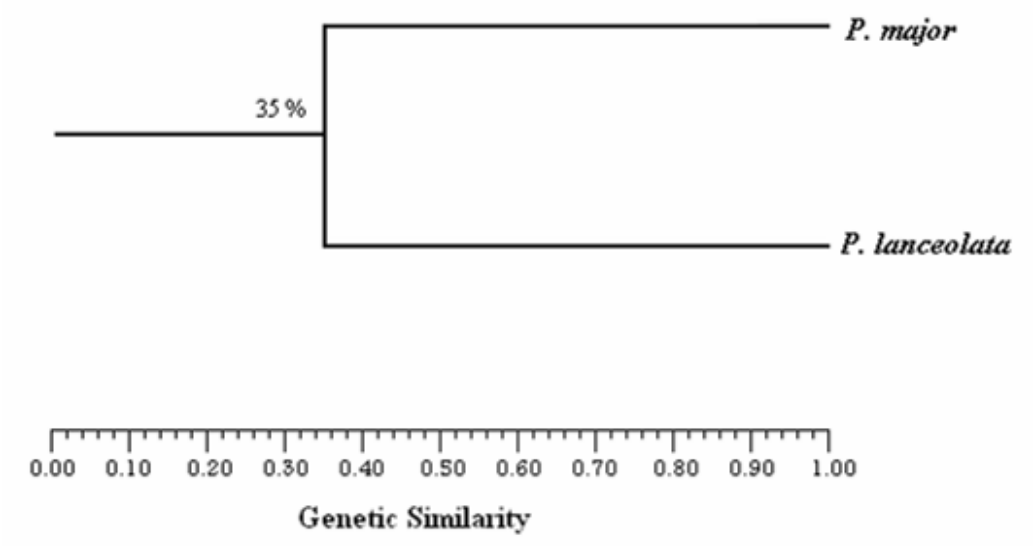

Fig. 3: Dendrogram of two *Plantago* sp. generated by UPGMA cluster analysis of RAPD data obtained with two primers (B- 4 and B- 10)

inhibited at three extract amounts (200, 150 and 100 mg ml^{-1}); the inhibition zones were (12.2, 11.7 and 8.2 mm) with the species from Jordan and (17, 12.7 and 10.2 mm) with the species from France, respectively. The significant interaction indicated that bacteria strains showed different response to species of Jordan and France at different amounts of extracts. The interaction showed, French *P. lanceolata* at extract amount 200 mg ml^{-1} gave the best antimicrobial activity (17.00 mm) against *S. aureus*.

DISCUSSION

Plantago lanceolata had antimicrobial activity against all used microorganism except *Klebsiella* sp. (Table 3). This result agree with Desta [17], who revealed that *P. lanceolata* collected from Ethiopia showed activity against *S. aureus* (ATCC 13709), *E. coli* (ATCC 9637) and *P. aeruginosa* (ATCC 27853) but no activity against *K. pneumoniae* (ATCC 10031). Our results indicated that *P. lanceolata* has an antimicrobial agent that could be effective against most bacteria.

The bacteria *Klebsiella* sp. was resistance to the extract of the two *Plantago* species; this could be due to cell wall components or higher concentration of extract need to be used.

P. major showed less antibacterial activity than *P. lanceolata*. One of the different bands in both species could be responsible for producing the antibacterial agents (if these agents are different); or the similar band (545 bp) could be responsible for producing the antibacterial agents (if these agents are same). Also, both *Plantago* species could have the same antibacterial agent but in different extract amounts that cause highly variation in their antibacterial activity. The similarity matrix between the two species was 35%. This indicated that the two species are not the same in their components, morphology and their activity.

The collected plants of *P. lanceolata* from Jordan and France showed significant differences in their antimicrobial activity. French *P. lanceolata* gave higher activity against *S. aureus* than Jordanian *P. lanceolata*; but Jordanian *P. lanceolata* showed higher activity against other bacteria (*P. aeruginosa*, *E. coli* and *Enterobacter* sp.) (Table 5). This variation in quality or composition of the same plant species could be due to differences in the environmental conditions and genetic variations. Skoula *et al.* [18] worked on *Salvia fruticosa* that collected from different geographic locations in the island of Crete. They found that the essential oils of these plants gave different response when all plants cultured under the same condition for three years. They suggested that this result was due to variation in quality and composition of plants and this might depends more on the genetic background of the plants and less on climate variation.

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