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Genetic Relationship among *Salvia* Species and Antimicrobial Activity of their Crude Extract Against Pathogenic Bacteria

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Abstract: The effects of three *Salvia* species (*S. officinalis*, *S. dominica* and *S. spinosa*) on five bacteria (*Staphylococcus aureus, Pseudomonas aeruginosa, Klebsiella* sp. *Escherichia coli* and *Enterobacter* sp.) were investigated. Also, the genetic variation among the *Salvia* sp. was determined. The three *Salvia* sp. showed different antimicrobial activity in different extract amounts against the five bacteria. *Salvia officinalis* and *Salvia dominica* inhibited the growth of *Staphylococcus aureus* at all used extract amounts (200, 150, 100 and 50 mg mL^{-1}). *S. spinosa* showed no antimicrobial activity. Based on variation of RAPD patterns obtained with four primers (B-7, B-10, B-12 and B-17), the similarity among *Salvia* sp. was determined. The UPGMA-based (unweighted pair group method of arithmetic means) dendogram was constructed between the three species by using the similarity matrix results.

Key words: Medicinal plants, antimicrobial activity, *Salvia* sp.

INTRODUCTION

Medicinal plants are a valuable natural resource and regarded as potentially safe drugs. They have been playing an important role in alleviating human sufferings by contributing herbal medicines in the primary health care systems of rural and remote hilly areas where more than 70% of population depends on folklore and traditional system of medicines.

Jordan has not less than 2000 wild plant species belonging to about 700 genera. Among these plants, as many as 485 species from approximately 99 plant families are categorized as medicinal plants[1-4].

Medicinal plants are distributed all over the country with a wide range from the Eastern desert to other parts of the country. Those plants are massively used by Bedouins or local people in folk medicine as hot or cold drinks, or chewed raw materials as fresh or dry. Many medicinal plants are under collection pressure and often used for many medicinal purposes, some examples are: *Artemisia, Achillea, Salvia, Anthemis* and many others[5].

Rovinsky and Cizadlo[6] found that the antimicrobial effects of the acetone extraction of *Salvia divinorum* inhibited the growth of the gram negative organisms *Citrobacter freundi, Escherichia coli* and *Pseudomonas aeruginosa* and one gram positive organism, *Bacillus subtilis*. However, the extract had no effect on the growth of *Alcaligenes faecalis* and *Pseudomonas fluorescens* (gram negative organisms) and *Micrococcus luteus, Staphylococcus aureus, Staphylococcus epidermidis* and *Streptococcus lactis* (gram positive organism).

Nascimento *et al.*[7] found that the extracts from *Salvia officinalis* and *Achillea millefolium* did not inhibit the growth of *Staphylococcus aureus*, *Salmonella choleraesuis*, *Pseudomonas aeruginosa*, *Bacillus subtilis*, *Candida albicans*, *Proteus* sp. *Klebsiella pneumoniae* and *Enterobacter aerogenes*.

Tepe *et al.*[8] found that *Candida albicans*, *Candida krusei*, *Mycobacterium smegmatis*, *Acinetobacter lwoffii*, *Streptococcus pneumoniae* and *Clostridium perfringens* were the most sensitive microorganisms to the *Salvia cryptantha* essential oil, whereas *Salvia multicaulis* essential oil had pronounced activity against *Streptococcus pneumoniae*, *Candida krusei*, *Clostridium perfringens*, *Mycobacterium smegmatis*, *Candida albicans* and *Staphylococcus aureus*.

The RAPD technique was first employed by Williams *et al.*[9] to examine human DNA samples. This technique employs 10 base pair random primers to locate random segments of genomic DNA to reveal polymorphisms. These primers adhere to a specific nucleotide segment of the genomic DNA. RAPD analysis can provide a simple and reliable method for measuring genomic variation. Because it is a relatively straight-

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forward technique to apply and the number of loci that can be examined is unlimited, RAPD analysis is considered as having a number of advantages over RFLP's and other techniques.

The RAPD technique has further advantages over other systems of genetic documentation because it has a universal set of primers, no preliminary work such as probe isolation, filter preparation, or nucleotide sequencing is necessary. The ease and simplicity of the RAPD technique make it ideal for genetic mapping and DNA fingerprinting. In many instance, only a small number of primers are necessary to identify polymorphism within species[9].

Studies using RAPD have provided information regarding the taxonomic and the genetic relationships between *Brassica* sp.[10,11]. Genotype fingerprinting is a powerful and highly accurate tool for distinguishing cultivars of chrysanthemum, tomato and wheat[12-14] and the cultivars and landraces of collard[15].

The aims of this study were to test the activity of three *Salvia* sp. (*S. officinalis*, *S. dominica* and *S. spinosa)* that grown in the Jordanian habitats 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 *Salvia* sp.

MATERIALS AND METHODS

Plant material: Three *Salvia* sp. (*S. officinalis*, *S. dominica* and *S. spinosa*) were collected from different area in Jordan. Some information like scientific 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[16].

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.

Table 1: The scientific and english names and the common medicinal uses of *Saliva* species.

Scientific name	English name	Common medicinal uses	R*
Salvia dominica	Sage	Cold, stomach pain and indigestion	[19]
Salvia officinalis	White sage (common sage)	Stomach pain, gargle, antiseptic, astringent, antispasmodic and anti-inflammation	[18,21]
Salvia spinosa	Spiny-calyxed sage	Anti-stomach disturbances, anti-inflammatory gargle, antiseptic, anti-tussive, anti-hemorrhoids pain, anti-rheumatic, astringent, carminative and hypotensive	[19]

* References number

Screening of antimicrobial activities: Nutrient agar medium (NA) 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 min. After cooling to about 65°C, 25 mL of the medium poured in petri dish (90 mmx15 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 separated 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[17]. Young leaves from the collected plants were rinsed twice with a 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 hundred fifty microliter of DNA extraction buffer [2XCTAB (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 4 min. 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. DNA was precipitated by adding equal volume of isopropanol. Each tube were mixed and centrifuged for 5 min 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[18]. Deionized water was then added to adjust the final volume.

The PCR program was as following: 4 min 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.

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[19]:

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

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 extract concentrations of *Salvia* sp. (*S. officinalis*, *S. dominica* and *S. spinosa*) showed different antimicrobial activity against the five bacterial species (Table 2). The effect of bacteria, plants, extract amounts and their interaction were highly significant (Table 3).

Table 2: Antimicrobial activity of *Salvia* sp. at four extracts concentrations against five bacterial species

Bacteria	Concentration (mg mL^{-1})	Averages of inhibition zone (mm)		
		S. officinalis	*S. dominica*	*S. spinosa*
S. aureus	200	16.2	10.7	0
	150	15	10	0
	100	13	9	0
	50	14.2	10	0
P. aeruginosa	200	0	0	0
	150	0	0	0
	100	0	0	0
	50	0	0	0
Klebsiella sp.	200	8	0	0
	150	0	0	0
	100	0	0	0
	50	0	0	0
E. coli	200	0	0	0
	150	0	0	0
	100	0	0	0
	50	0	0	0
Enterobacter sp.	200	0	0	0
	150	0	0	0
	100	0	0	0
	50	0	0	0

Table 3: Source of variation and degree of freedom of the three *Salvia* sp., four extract concentrations and five bacterial species

Source of variation	df	Mean Square
Bacteria (B)	4	159.975**
Plant (P)	2	81.204**
Concentration (C)	3	5.538**
B * P	8	55.631**
B * C	12	1.392**
P * C	6	3.588**
B * P * C	24	0.931**
Error	180	0.029
Total	239	

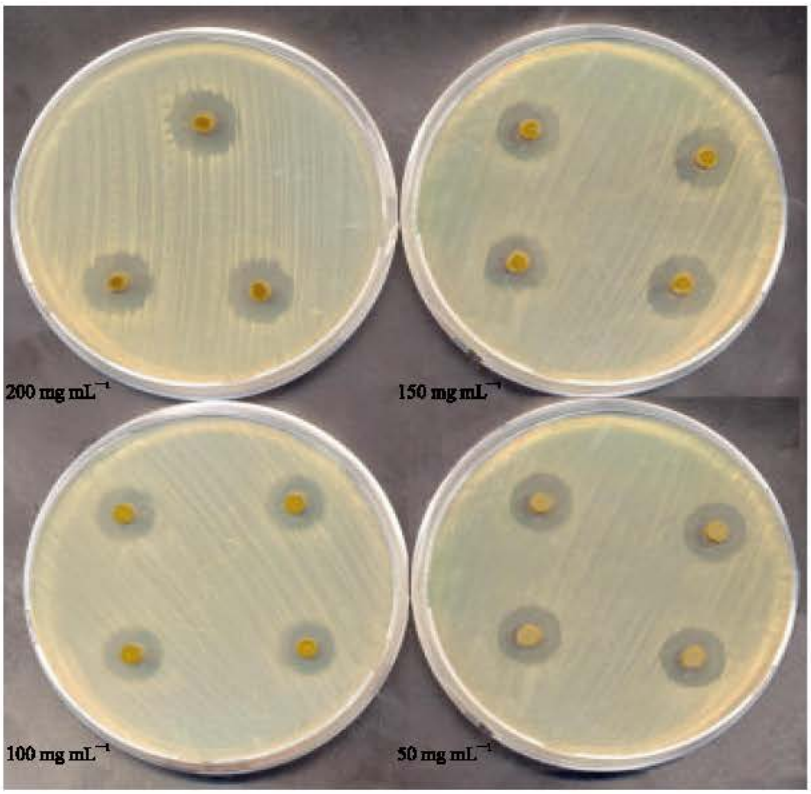

Fig. 1: Antimicrobial activity of *Salvia officinalis* against *Staphylococcus aureus* at four extracts amounts

The extract of *S. officinalis* revealed antibacterial activity against *S. aureus* and *Klebsiella* sp. It inhibited the growth of *S. aureus* (16.2, 15, 13 and 14.2 mm) at all used extract concentrations (200, 150, 100 and 50 mg mL^{-1}), respectively (Fig. 1), while *Klebsiella* sp. growth was inhibited (8 mm) at 200 mg mL^{-1} only.

S. dominica extract inhibited only the growth of *S. aureus* (10.7, 10, 9 and 10 mm) at the four concentrations (200, 150, 100 and 50 mg mL^{-1}), respectively. The extract of *S. spinosa* showed no antimicrobial activity against any of the tested bacteria.

The significant interaction between bacteria, plants and concentrations stated that the inhibition zones showed different results by using different plant species and different concentrations against all used bacteria. *S. officinalis* at concentration 200 mg mL^{-1} gave the best antimicrobial activity against *S. aureus*.

The total numbers of amplified bands were 50 (Fig. 2). Twenty-three bands produced from DNA amplification of *S. officinalis*, 13 bands from *S. dominica* and 14 bands from *S. spinosa*. PCR amplification of *S. officinalis* and *S. dominica* DNA were produced by using primers B-1, B-7, B-8, B-10, B-12, B-15 B-17 and B-18 while primers B-4, B-7, B-10, B-12, B-15 and B-17 produced amplified bands with *S. spinosa* DNA.

Band (677 bp) that amplified by primer B-7 was common in all *Salvia* sp. Band (778 bp) that amplified by primer B-10 was common in *S. officinalis* and *S. spinosa*. Band (700 bp) that amplified by primer B-12 was common in *S. dominica* and *S. spinosa*. Band (435 bp) that amplified by primer B-17 was common between *S. dominica* and *S. spinosa*. All the mentioned bands are marked in Fig. 2.

Similarity matrixes of *Salvia* species were calculated based on RAPD analysis resulted from the four primers (B-7, B-10, B-12 and B-17). The similarity between *S. officinalis* and *S. dominica* was 5%, similarity between *S. officinalis* and *S. spinosa* was 14% and similarity between *S. dominica* and *S. spinosa* was 44%. A dendrogram tree was drawn in Fig. 3 based on the results of the similarity matrix.

DISCUSSION

The extract of *Salvia dominica* inhibited the growth of *S. aureus* only (Table 2). *S. officinalis* inhibited the growth of two bacteria (*S. aureus* and *Klebsiella* sp.); the higher activity was against *S. aureus*. Essawi and Srour[20] showed that *S. officinalis* inhibited the growth of *S. aureus* (ATCC 25923) and *P. aeruginosa* (ATCC 27853)

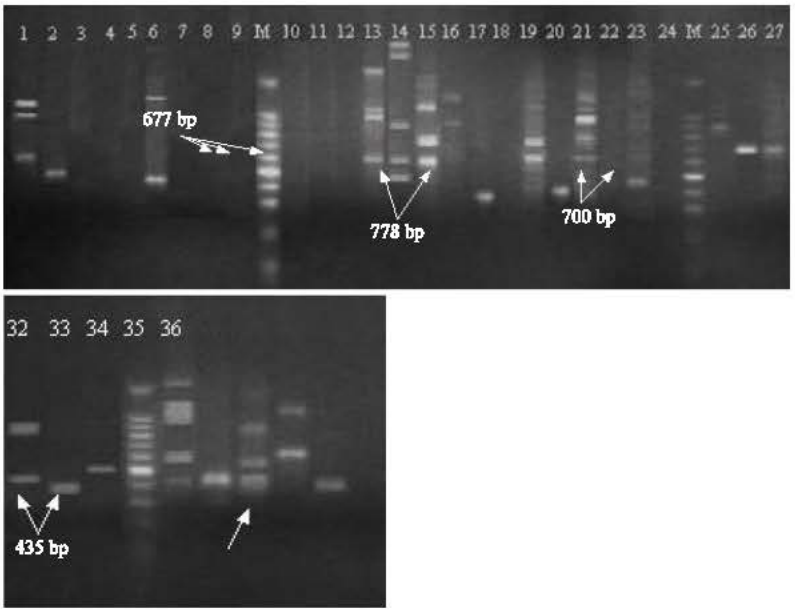

Fig. 2: DNA from *Salvia officinalis, S. dominica* and *S. spinosa* that amplified with different primers. The first number of lanes for each primer represents *S. officinalis,* the second number represents *S. dominica* and the third number represents *S. spinosa*

Lanes	Primers	Lanes	Primers	Lanes	Primers
1, 2, 3	B-1	13, 14, 15	B-7	25, 26, 27	B-12
4, 5, 6	B-4	16, 17, 18	B-8	28, 29, 30	B-15
7, 8, 9	B-5	19, 20, 21	B-10	31, 32, 33	B-17
10, 11, 12	B-6	22, 23, 24	B-11	34, 35, 36	B-18

M: DNA marker

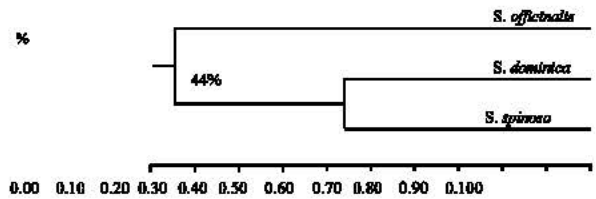

Fig. 3: Dendrogram of three *Salvia* species generated by UPGMA cluster analysis of RAPD data obtained with four primers

by using hole-plate diffusion method at extract concentration 150 mg/hole; *P. aeruginosa* could be inhibited due to the using of high extract concentration (150 mg/hole) or due to the difference in the sensitivity between the two *P. aeruginosa* strains that used in their study.

S. aureus was sensitive to the extracts of *S. dominica* and *S. officinalis* while other tested bacteria (*P. aeruginosa*, *Klebsiella* sp., *E. coli* and *Enterobacter* sp.) were resistant for the used concentrations.

In general the gram positive bacteria (*S. aureus*) was more sensitive to all plant extracts than gram negative bacteria (*Klebsiella* sp., *P. aeruginosa*, *E. coli* and *Enterobacter* sp.). This may be attributed to the fact that cell wall in gram positive bacteria consist of a single layer, while gram negative cell wall is a multilayered structure and quite complex.

S. spinosa showed no antibacterial activity against the tested bacteria at used concentrations. This species may not have antimicrobial agent or need to be used in higher concentrations.

S. officinalis and *S. dominica* showed antibacterial activity. Although the similarity matrix was 5% which indicated that high differences are existing between the two species, they could have similar gene which responsible for producing the antibacterial agent. The similar band between these two species with *S. spinosa* (677 bp) and the similar bands between *S. dominica* and *S. spinosa* (435 and 700 bp) indicated that these bands are not responsible for producing antibacterial agent because *S. spinosa* showed no antimicrobial activity against any

of the tested bacteria. On the other hand, similarity matrix between *S. dominica* and *S. spinosa* was 44%. This similarity could be due to similarity in some components or morphology.

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