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The Antimicrobial Activity and the Genetic Relationship of *Achillea* species

Abeer Kharma and Dhia Hassawi
Department of Biotechnology, Faculty of Agricultural Technology,
Al-Balqa Applied University, Al-Salt, 19117, Jordan

Abstract: The antimicrobial effect of three *Achillea* sp. (*A. fragrantissima, A. biebersteinni* and *A. santolina*) on five bacteria (*Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Klebsiella* sp. *Escherichia coli* and *Enterobacter* sp.) was investigated. Also, the genetic variation among the *Achillea* sp. was determined. The three *Achillea* sp. showed different antimicrobial activity in different extract amounts (50, 100, 150 and 200 mg mL^{-1}) against the five bacteria. *A. fragrantissima* inhibited the growth of *S. aureus* at all used extract amounts. *A. biebersteinni* inhibited the growth of all tested bacteria except *P. aeruginosa.* The extract of *A. santolina* inhibited the growth of *S. aureus* at 150 and 200 mg mL^{-1}, the growth of *Klebsiella* sp. at extract amounts 150 and 200 mg mL^{-1} and the growth of *Enterobacter* sp. at the four extract amounts. The three *Achillea* sp. revealed no activity against *P. aeruginosa.* Based on variation of RAPD patterns obtained with four primers (B-1, B-10, B-15 and B-18), the similarity between *A. fragrantissima* and *A. santolina* was 35%. The UPGMA-based (unweighted pair group method of arithmetic means) dendogram was constructed between the two species by using the similarity matrix results.

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

INTRODUCTION

Due to the widespread and often indiscriminate use of antimicrobial drugs, many microorganisms have acquired resistance to specific antibiotic treatments and these strains are particularly evident in the hospital environment (Evan's, 1999). This has created immense clinical problems in the treatment of infectious diseases (Davis, 1994). In addition to this problem, antibiotics are sometimes associated with adverse effects on host which include hypersensitivity, depletion of beneficial gut and mucosal microorganisms, immunosuppression and allergic reactions (Idose *et al*., 1968). Research on medicinal plants has increased and their antimicrobial activity has been screened in number of studies (Mahasneh, 1986; Nadir *et al*., 1986; Desta, 1993; Brantner and Grein, 1994; Alkofahi *et al*., 1997; Mahasneh *et al*., 1999; Afifi and Abu-Irmaileh, 2000).

Medicinal plants have been tested for biological, antimicrobial and hypoglycemic activity (Atta and Alkofahi, 1998; Glombitza *et al*., 1994; Vats *et al*., 2002). They have also tested for antiulcerogenic, antihelminthic, hepatoprotective, analgesic, antipyretic, antileishmania and insecticidal activities (Sultana *et al*., 1995; Abreu *et al*., 1999; Karim and Quraan, 1986; Dafni and Yanive, 1994; Chariandy *et al*., 1999).

Herbalists praise *Achillea* for its anti-inflammatory, antipyretic, carminative, astringent, antispasmodic, stomachic and bactericidal action. Traditional use of *Achillea* against serious illnesses in ancient Persia attracted the attention of researchers interested in studying this plant's therapeutic effects on the immune system.

Random amplified polymorphic DNA (RAPD) is one of the molecular marker techniques which based on the amplification of random sequences of the genomic DNA using arbitrary oligonucleotide primers without requiring prior sequence information (Ko *et al*., 1994; Vazquez *et al*., 1996).

RAPD is rapidly growing as it is often highly polymorphic, gives reproducible fingerprints of any complex genome, its identification does not require radioactivity and only very small quantities of plant material are required for this type of analysis. RAPD analysis is simple, fast and provides an accurate assessment of the genetic variation of species and cultivars (Hancock and Callow, 1994; Hoelzel, 1992).

The objectives of this study were to test the activity of the plant extracts of three *Achillea* species (*A. fragrantissima*, *A. biebersteinii* and *A. santolina*) on inhibiting the growth of five pathogenic bacteria (*Staphylococcus aureus*, *Escherichia coli*, *Pseudomonas*

Corresponding Author: Dhia Hassawi, Department of Biotechnology, Faculty of Agricultural Technology, Al-Balqa Applied University, Al-Salt, 19117, Jordan Tel: 00962-5-3530469

aeruginosa, *Klebsiella* sp. and *Enterobacter* sp.) and also study the genetic variations among these medicinal plants.

MATERIALS AND METHODS

Plant material: The plant materials of three *Achillea* species (*A. fragrantissima*, *A. biebersteinii* and *A. santolina*) were collected from Jordan environment. 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 5 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 (Kandil *et al*., 1994)

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 (NA) was prepared by dissolving 28 g of NA in one liter of distilled water. The medium was 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 (90×15 mm (diameter×height)). The plates kept at room temperature for solidification and stored at 4°C until using.

Inoculums containing 10^6 bacterial cells mL^{-1} 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 50, 100, 150 and 200 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 (1990). 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 [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 microlitre 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 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-HCl, 5 mM $MgCl_2$ (Magnesium chloride), 0.1% Triton

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

Scientific and family name	English name	Common medicinal uses	Reference
Achillea biebersteinii (Composite)	Yarrow	Carminative, insect repellant	Alkofahi (1996)
Achillea fragrantissima (Composite)	Lavender cotton	Carminative, depurative, anti-spasmodic, anti-diabetic, anti-diuretic, used for stomachic, internal hemorrhage, tumors, severe cough, infections, fever, rheumatic pain	Farnham (1996), Navaroo *et al.* (1996), Oran and Al-Eisawi (1998), Scott *et al.* (1996), Weising *et al.* (1995)
Achillea santolina(Composite)	Santolina milfoils	Anti-colic, kidney stones, anti-diabetic, tooth pain, dysentery, carminative, insect repellant	Alkofahi *et al.* (1996), (1997), Karim and Quraan (1986), Oran and Al-Eisawi (1998)

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, U.S.A.), 0.2 μL Taq DNA polymerase (5 units μL^{-1}) and 0.5-1 μL (about 20-100 ng) of genomic DNA template as suggested by Hoelzel (1992). 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 hours 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 (Lynch, 1990):

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

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 significant (Table 2). Three *Achillea*

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

Source of variation	df.	Mean square
Bacteria (B)	4	315.962**
Plant (P)	2	20.679**
Extract amounts (E)	3	72.411**
B * P	8	77.278**
B * E	12	13.710**
P * E	6	0.924**
B * P * E	24	2.675**
Error	180	0.083
Total	239	

** Significant at $p<0.01$

Table 3: Antimicrobial activities of the three *Achillea* sp. at four extract amounts against five bacterial species

		Averages of inhibition zone (mm)		
Bacteria	Extract amount (mg mL^{-1})	*A. fragrantissima*	*A. biebersteinii*	*A. santolina*
S. aureus	200	19.5	17.2	11.2
	150	16.2	14.2	8.2
	100	12.5	13.5	0
	50	12.2	11.7	0
P. aeruginosa	200	0	0	0
	150	0	0	0
	100	0	0	0
	50	0	0	0
Klebsiella sp.	200	10.2	8.5	9
	150	0	8	8
	100	0	7.5	0
	50	0	0	0
E. coli	200	0	8.5	0
	150	0	7.7	0
	100	0	7	0
	50	0	0	0
Enterobacter sp.	200	0	9	12
	150	0	8.2	10.7
	100	0	0	9.7
	50	0	0	8

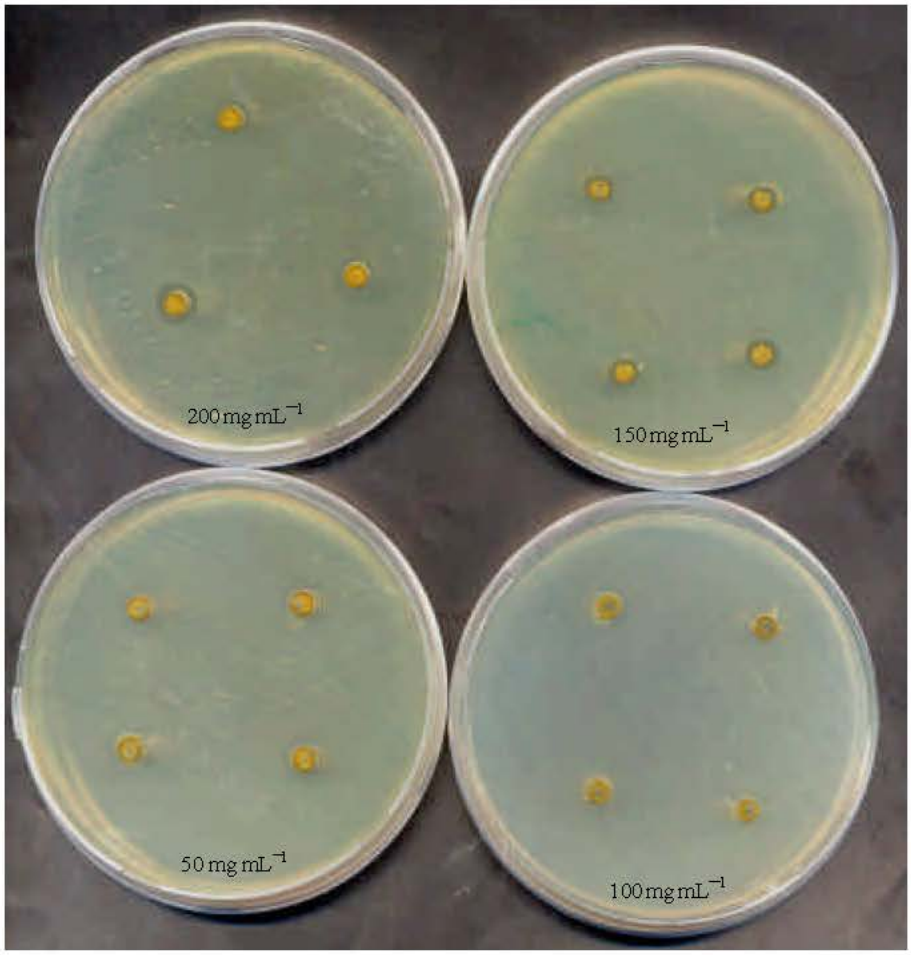

Fig. 1: Antimicrobial activities of *Achillea biebersteinii* against *Enterobacter* sp. at four extract amounts

sp. (*A. fragrantissima*, *A. biebersteinii* and *A. santolina*) showed different antimicrobial activity in different extract amounts against the five bacterial species (Table 3).

A. fragrantissima inhibited the growth of *S. aureus* at all used extract amounts. The inhibition zones were (19.5, 16.2, 12.5 and 12.2 mm) at extract amounts (200, 150, 100 and 50 mg mL^{-1}) respectively. The *A. fragrantissima* extract inhibited the growth of *Klebsiella* sp. at 200 mg mL^{-1} only. *A. biebersteinii* inhibited the growth of all tested bacteria except *P. aeruginosa*. It was highly active (17.2, 14.2, 13.5 and 11.7 mm) against *S. aureus* at extract amounts (200, 150, 100 and 50 mg mL^{-1}) respectively. Also, it was active against *Klebsiella* sp. and *E. coli* at three extract amounts (200, 150 and 100 mg mL^{-1}); it inhibited the growth of *Enterobacter* sp. (Fig. 1) at two extract amounts (200 and 150 mg mL^{-1}).

The extract of *A. santolina* inhibited the growth of *S. aureus* at amounts 200 and 150 mg mL^{-1}, the growth of *Klebsiella* sp. at extract amounts 200 and 150 mg mL^{-1} and the growth of *Enterobacter* sp. at the four extract amounts.

The three *Achillea* sp. revealed no activity against *P. aeruginosa*. The results also indicated that *A. biebersteinii* inhibited the growth of the four bacteria and the extract amount 200 mg mL^{-1} showed the highest inhibition zones. The significant interaction between bacteria, plants and extract amounts indicated that *Achillea* sp. showed different antimicrobial activity against the tested bacteria and the extracts amounts affected on some bacteria but revealed no effect on others.

For RAPD technique, 20 arbitrary decamer primers (Kit B QIAGEN Operon, USA) including B-1 to B-20 were

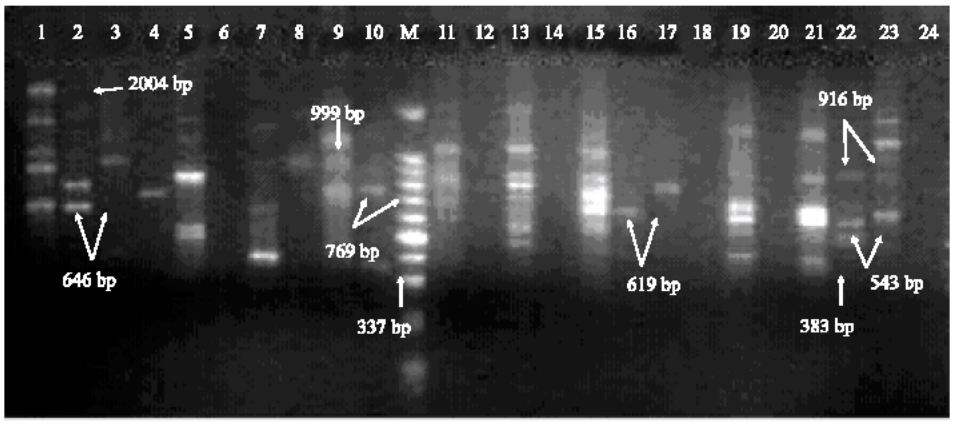

Fig. 2: DNA from *Achillea fragrantissima* and *Achillea santolina* that amplified with different primers. The odd numbers of lanes for *A. fragrantissima* and the even numbers for *A. santolina*

Lanes	Primers	Lanes	Primers	Lanes	Primers
1 and 2	B-1	9 and 10	B-10	17 and 18	B-16
3 and 4	B-5	11 and 12	B-11	19 and 20	B-17
5 and 6	B-7	13 and 14	B-12	21 and 22	B-18
7 and 8	B-8	15 and 16	B-15	23 and 24	B-20

M: DNA marker

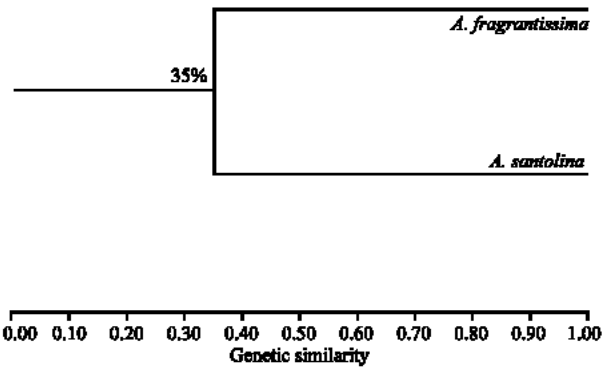

Fig. 3: Dendrogram of two *Achillea* sp. generated by UPGMA cluster analysis of RAPD data obtained with four primers

used. The number of bands produced per primer by using the PCR varied from 0 to 7 for each species.

Amplification of *A. fragrantissima* DNA produced 52 bands from 12 primers (B-1, B-5, B-7, B-8, B-10, B-11, B-12, B-15, B-16, B-17, B-18 and B-20). The largest band molecular weight was 2004 bp while the smallest was 383 bp (Fig. 2). However, 6 primers had amplified *A. santolina* DNA (B-1, B-5, B-8, B-10, B-15 and B-18) that produced 12 bands. The largest band molecular weight was 999 bp while the smallest was 337 bp (Fig. 2).

Five bands were found common in both species; these bands were produced by four primers: B-1 (646 bp), B-10 (769 bp), B-15 (619 bp) and B-18 (543 and 916 bp). Based on variation of RAPD patterns obtained with the four primers, the similarity of the two species was 35%. The UPGMA-based (unweighted pair group method of arithmetic means) dendrogram was constructed by using the similarity matrix result (Fig. 3).

DISCUSSION

Achillea fragrantissima was active against *S. aureus* and *Klebsiella* sp. (Table 3). *A. biebersteinii* extract (10 mg/hole) inhibited the growth of *S. aureus*, *Klebsiella* sp., *E. coli* and *Enterobacter* sp. This result did not agree with the result of Al-Kofahi *et al.* (1996) who found that *A. biebersteinii* extract inhibited the growth of *P. aeruginosa* but showed no activity against *E. coli* and *S. aureus*. Since *A. biebersteinii* was collected from Jordan in both studies and extracted by the same procedure, these differences could be due to the genetic variations (mutations) between the bacterial strains.

Among *Achillea* genera, the best results revealed by *A. biebersteinii* extract which showed activity against all tested bacteria except *P. aeruginosa*.

S. aureus was the most sensitive bacteria that inhibited by the three *Achillea* species extracts. On the other hand, *P. aeruginosa* was resistant to all *Achillea* species extracts.

The genetic variations among species were studied by using RAPD analysis. The three *Achillea* sp. showed different antimicrobial activity. This indicated the presence of antimicrobial agents in all of them. These agents could be the same in the three species but

produced in different extract amounts or each species produced different antibacterial compound that caused variation in their activity.

The similar PCR product bands of two *Achillea* sp. (*A. fragrantissima* and *A. santolina*) (Fig. 2) might suggest the presence of similar gene or genes that are responsible of producing antibiotic agents in the tested species.

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