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Antibacterial and antibiofilm activity of *Ammi majus* seed against Gram-positive bacteria

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Abstract

Background and objective: *Ammi majus* belongs to family Apiaceae is an important plant used in a different country traditionally for treatment bacterial infection. This study aimed to investigate in vitro antibacterial and antibiofilm activity of *Ammi majus* seed extracts against six isolated Gram-positive bacteria.

Methods: The antibacterial activity of seed extracts were screened by disk diffusion and agar overlay bioautography method and their activities were further determined by minimum inhibitory and minimum bactericidal concentration. Biofilm formation was evaluated by the microtiter plate crystal violet assay. The quantity of bound bacteria was determined by measuring absorbance at (OD630 nm) using microtiter plate reader.

Results: Ethanol 80% and ethyl acetate extracts showed variable activity against all isolated bacteria while petroleum ether extract revealed resistance against studied bacteria. *Streptococcus mitis* showed more pronounced sensitivity against both extracts by disk diffusion (12 mm and 10 mm) and bioautography method with minimum inhibitory concentration 7.8125 mg/ml of ethanol 80% and 15.625 mg/ml for ethyl acetate extracts. Ethanol 80% and ethyl acetate extract significantly reduced biofilm formation as compared to control, showed antibiofilm activity at 0.4883-62.5 mg/ml and exhibited strongest antibiofim activity against *Staphylococcus aureus*.

Conclusion: *Ammi majus* seed extracts revealed highest antibacterial activity against Streptococcus species and strongest antibiofilm activity on Staphylococcus species.

Keywords: *Ammi majus*; Disk diffusion; Bioautography; minimum inhibitory concentration; Antibiofilm.

Introduction

Plants and their metabolites have a long history of use in folk medicine for the prevention and treatment of diseases and infection.[1] Treatment of infectious diseases lead to development excessive use of antibacterial agents which resulted in the antibacterial resistant which increase the chance of treatment failure. Antibacterial resistance due to accepting of resistance genes by horizontal gene transfer, target alteration of drugs, low permeability systems and grow in a specific growth state like biofilm are important mechanisms involved in bacterial resistance.[2] Bacterial biofilm is a sessile life form defined as community of microorganisms attached to a biotic or abiotic substrate surface and submerged into extracellular slimy matrix.[3] Bacterial biofilms have been reported to have useful effects on food chains, sewage treatment plants, on other hand have harmful effects serves to encourage bacteria persistence by resisting antibacterial treatment and host immune responses.[4] Biofilm causes numerous infections such as chronic otitis media, chronic prostatitis, chronic pneumonia in patients with cystic fibrosis, infections of orthopedic devices.[5,6] The factors that are contributed to resistance in biofilm forming bacteria includes the slow growth rate, decreased diffusion of antimicrobials and accumulation of enzymes that are involved in the resistance.[2] Approximately 60% of human infections are reported to be

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a result of biofilm formation on the human mucosa. As a result, lead to increased interest in the search to identify the alternatives therapy for microbial control[7] and almost exclusively focused on the effects of these against planktonic biofilm forms that are more resistant to antimicrobial agents and therefore more difficult to control.[8] Some plants have been reported to be able to prevent the formation of biofilm in some bacteria such as *Staphylococcus epidermidis, Staphylococcus aureus, Staphylococcus auricularis, Streptococcus mitis, Streptococcus salivarius, Streptococcus pneumoniae*.[9-11] Among plants of the *Apiaceae*, family *Ammi majus* native to northern Africa, southern Europe, western Asia and India. The fruit laterally compressed, oblong, mericarps of the cremocarp separated by a carpophore. Seed small, pendulous, albuminous.[12] The seed contains furanocoumarins, flavonoids, phenolic acid and other constituents.[13,14] The plant is reported to possess antimicrobial, antischistosomal, anti-inflammatory, anti-oxidant and antitumor activities.[14-16] To our knowledge and from searching literature, there are only a few studies on antibacterial activity and no study on antibiofilm activity of *Ammi majus* seed. This study aimed to investigate in vitro antibacterial activity and for the first time the antibiofilm activity of *Ammi majus* seed extracts against six isolated Gram-positive bacteria.

Methods

Materials

Petroleum ether, ethyl acetate, and ethanol from Scharlab S.L. (Spain), Muller Hinton agar, Blood agar, Nutrient agar, Nutrient broth (Merck Co. Germany). 0.1 % (v/w) Crystal Violet (Sigma-Aldrich, Germany), Dimethylthiazolyl diphenyl tetrazolium bromide (MTT) (Taizhou xi Anju pharma.co., China).

Sample collection and extraction

The fresh seed of *Ammi majus* was collected during July 2014 from Kurdistan region, Iraq, washed with distilled water and shade dried for 15 days, coarsely powdered and stored in moisture proof and light proof bottles at 6 ^{o}C until used. The identity of the plant was confirmed by the Department of Pharmacognosy, College of Pharmacy, Hawler Medical University. The 100 gm powdered seed was extracted with petroleum ether using ultrasonic (LUC-405, Korea) assisted extractor for 1h at 40 °C [17] then filtered. The residual plant materials were dried and re-extracted with ethanol 80 % for 1 h after filtration that yielded an extract which was after drying half of it dissolved in 10 ml (5N) HCl and refluxed for 1 hour. Liquid-liquid fractionation using ethyl acetate (10 x 3 ml) resulted in an ethyl acetate fraction on drying in vacuum the three fraction used for evaluation of the antibacterial and antibiofilm activity.

Microorganisms and inoculums preparation

The antibacterial activity of seed extracts was assessed against 6 Gram-positive bacteria which include *Staphylococcus epidermidis, Staphylococcus aureus, Staphylococcus auricularis, Streptococcus mitis, Streptococcus salivarius, Streptococcus pneumoniae.* The isolated pathogenic bacteria from urine were used in this work identified and confirmed by morphological studies and by using biochemical tests in the Microbiological Laboratory of the Biological Department, College of Education, Salahadin University. All bacteria were grown on Muller Hinton and Blood agar at 37 ^{o}C for 24 h, then stored at 4 ^{o}C until used. Individual pure colonies from each isolated plate were transferred to 10 ml Nutrient broth media. The study approved by the ethics committee of College of Pharmacy, Hawler Medical University, Erbil, Iraq.

Determination of antibacterial activity

Agar disc diffusion method

The agar disc diffusion method[18] was employed to determine the antibacterial activities of petroleum ether, ethanol 80 % and ethyl acetate extracts of *Ammi majus* seed against 6 Gram-positive bacteria. The bacterial cultures were first grown on

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Nutrient and blood agar plates at 37 °C for 18 to 24 h. One or several colonies of the respective bacteria were transferred into normal saline and adjusted to 0.5 McFarland turbidity standards. The inocula of the respective bacteria were streaked on Muller Hinton agar plates using a sterile swab and were then dried at 37 °C during 15 min. A sterile filter disc having 6 mm of diameter were soaked in different concentration 250, 125 and 62.5 mg/ml of each extract separately was placed at the surface of Muller-Hinton agar plates. The plates were incubated for 24 h at 37^{o}C, following incubation the plates were observed. The antibacterial activity was evaluated by measuring the clear zone surrounding the Whatman paper. Standard discs of the antibiotic azithromycin were applied as positive antibacterial controls.

Agar overlay bioautography method

A thin layer chromatography (TLC) was used to separate bioactive constituents of ethanol 80 % and ethyl acetate extracts. 200 µL both ex-tracts were applied on pre-coated silica gel GF254 TLC plate (Merck), the best solvent system was applied for bioautography was Toluene: ethyl acetate: formic acid: water (10: 80: 2.5: 2.5) and dried for complete removal of solvents, then overlaid by Muller Hinton agar seed-ed with an overnight culture of isolated bacteria. The plate was incubated at 37 ^{o}C for 24 hours after that sprayed with an aqueous solution of 2 mg/ml dimethyl thiazolyl diphenyl tetrazolium bromide (MTT). The areas of inhibition were pale or yellow on a purple colored background.[19]

Determination of minimal inhibitory and minimal bactericidal concentration

The minimal inhibitory concentration (MIC) of *Ammi majus* extracts against each isolated bacteria was determined using broth microdilution assay as modified briefly, two-fold serial dilutions of ethanol 80 % and ethyl acetate extracts were prepared with Nutrient broth at a total volume of 100 µl per well in the 96-well plates. They started from the lowest concentrations of each extract 62.5 mg/ml. The microtiter plate wells were inoculated with 10 µl of 0.5 McFarland for each bacteria per well. After overnight incubation at 37 °C appropriate conditions, absorbance was measured at 490 nm using a microtiter plate reader (ELX800 Biotech USA) to assess the cell growth. The negative control consisted of Nutrient broth and bacterial cell suspension without the extract. Blank control contained only the medium. The MIC endpoint was defined as the lowest concentration of the test agent that completely inhibited growth or produced at least 90% reduction of absorbance in comparison to the negative control. All experiments were performed in triplicate and the average values were reported as MIC. The minimal bactericidal concentration (MBC) was defined as the lowest concentration of wells that did not allow visible growth when 10 µl of the well contents was placed on agar and grown 24 hours at 37 °C in appropriate conditions.[20]

Determination of antibiofilm activity

The effect of *Ammi majus* seed extracts on biofilm formation of each isolated pathogen *Staphylococcus epidermidis, Staphylococcus aureus, Staphylococcus auricularis, Streptococcus mitis, Streptococcus salivarius, Streptococcus pneumoniae* was examined using the modified crystal violet assay method.[21] Twofold serial dilutions of plant extract were made in sterile 96 flat wells microtiter plates containing 100 µl of Nutrient broth per well. The tested concentration ranged from (0.4883-62.5) mg/ml for ethanol 80 % and ethyl acetate fraction. A 10 µl of fresh bacterial suspension adjusted with (0.5 McFarland) was added to each well. Positive control (bacterial suspension in broth) and negative control (extract in broth). Following incubation at 37 °C for 24 hours, the content of each well was gently removed and unbound bacterial cells were removed from all wells by washing three times with 200 µl of sterile distilled water, stained with 0.1% crystal violet and incubated at the room temperature for

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30 minutes. Excess stain was removed thorough washing with distilled water and plates were fixed with 200 µl of ethanol 70 % for recording absorbance at 630 nm using an ELISA microplate reader.

The inhibition percentage of biofilm was calculated by the formula[21]

Percentage of biofilm inhibition = (Control OD630 nm–Test OD630 nm) / Control OD630 nm) x 100

Statistical analysis

Anti-biofilm and anti-bacterial activity data were obtained in triplicate and the results analyzed statistically by ANOVA using the statistical package for the social sciences (version 17.0). A *P* value of ≤0.05 was considered to indicate statistical significance.

Results

Antibacterial activity of seed extracts

In present work petroleum ether, ethanol 80% and ethyl acetate extracts of *Ammi majus* seed evaluated for their antibacterial activity by disc diffusion assay are presented in Table 1. All isolated Gram-positive pathogen revealed completely resistance to petroleum ether extract while susceptible to ethanol 80 % and ethyl acetate extracts of *Ammi majus* seed with variable degrees of inhibition zones. The diameter inhibition zone of ethanol 80% and ethyl acetate extract at 62.5 mg/ml ranged from (5-12 mm) and (3-10 mm) with the highest activity against *Streptococcus mitis*, along with MIC and MBC value for both extracts ranged from (3.9063- 15.625 mg/ml) and (7.8125-31.25 mg/ml) respectively showed in Table 2.

Table 1: Diameter inhibition zone of *Ammi majus* seed extracts against Gram positive bacteria (Mean±SD).

Extract	PE (mg/ml)			E 80% (mg/ml)			E a (mg/ml)			Azi
Bacteria	250	120	62.5	250	125	62.5	250	125	62.5	
Staphylococcus epidermidis	R	R	R	12±0.015	10±0.27	7±0.980	8±0.034	5±0.118	3±0.211	32±0.209
Staphylococcus aureus	R	R	R	10±0.067	9±0.300	7±1.33	10±0.012	7±0.044	5±0.580	35±1.03
Staphylococcus auricularis	R	R	R	14±0.146	10±0.01	8±0.980	15±0.150	9±0.203	7±0.123	46±0.315
Streptococcus mitis	R	R	R	20±0.122	15±018	12±0.13	16±0.220	14±0.01	10±0.01	30±0.231
Streptococcus salivarius	R	R	R	10±0.201	7±0.267	5±0.014	12±0.032	7±0.201	5±0.380	12±0.197
Streptococcus pneumoniae	R	R	R	14±0.176	8±0.139	7±0.056	10±0.071	6±0.030	4±0.087	30±0.200

PE: Petroleum ether; E80 %: Ethanol 80 %; Ea: Ethyl acetate extract ; R: Resistance; Azi: Azithromycin

Table 2: Minimum inhibitory and minimum bactericidal concentration of *Ammi majus* seed extracts against Gram-positive bacteria.

Bacteria	MIC (mg/ml)		MBC (mg/ml)	
	E80%	E a	E80%	E a
Staphylococcus epidermidis	7.8125	7.8125	15.625	15.625
Staphylococcus aureus	15.625	15.625	31.25	31.25
Staphylococcus auricularis	7.8125	3.9063	15.625	15.625
Streptococcus mitis	7.8125	15.625	15.625	31.25
Streptococcus salivarius	3.9063	15.625	7.8125	31.25
Streptococcus pneumoniae	7.8125	15.625	15.625	31.25

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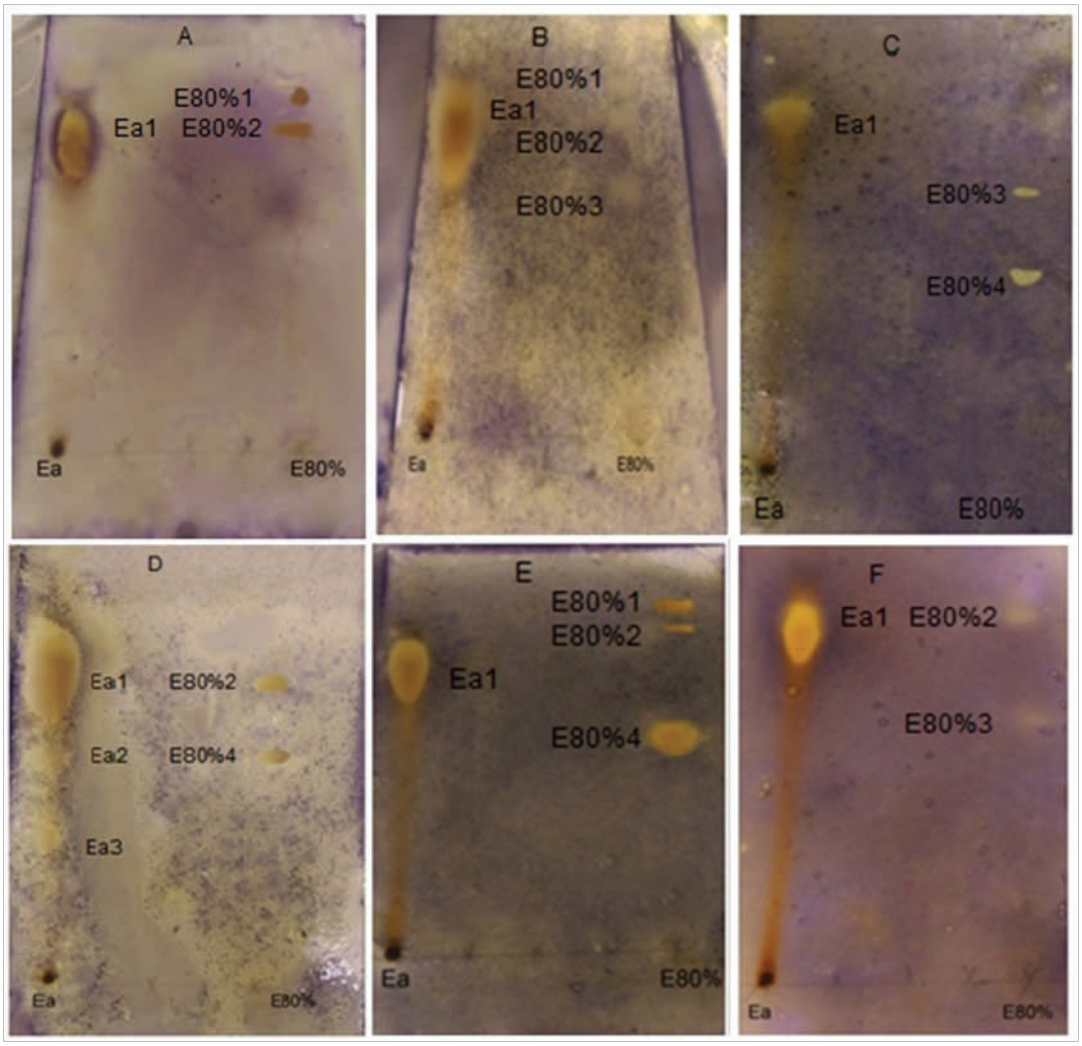
The results of agar overlay bioautography showed the presence of one or more active constituents in seed extracts responsible for antibacterial activity (Figure 1). In ethanol 80 % extract four constituents were found to be active ethanol 80 % extract (E80%) 1-4 with retardation factor values 0.9, 0.8, 0.6 and 0.5, respectively. Constituent E80% 1 showed activity against *Staphylococcus epidermidis, Staphylococcus aureus, and Streptococcus salivarius*. Constituent E80% 2 showed activity against all isolated bacteria except *Staphylococcus auricularis*. Constituent E80% 3 showed activity against *Staphylococcus aureus, Staphylococcus auricularis and Streptococcus pneumoniae.* Constituent E80% 4 showed activity against *Staphylococcus auricularis*, *Streptococcus mitis*, *Streptococcus salivarius.* In ethyl acetate extract three constituents ethyl acetate extract (Ea) 1, Ea 2 and Ea 3 at retardation factor values 0.8, 0.6 and 0.35 respectively were found to be active. Constituent Ea 1 showed a zone of inhibition against all isolated bacteria, while Ea 2 and Ea 3 were found only active against *Streptococcus mitis.*



E80%: Ethanol 80%; Ea: Ethyl acetate extracts

Figure 1: Bioautogram plates of ethanol 80% and ethyl acetate extracts against A. *Staphylococcus epidermidis* B. *Staphylococcus aureus* C. *Staphylococcus auricularis* D. *Streptococcus mitis* E. *Streptococcus salivarius* F. *Streptococcus pneumonia.*

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Anti-biofilm activity of seed extracts

The antibiofilm activity of ethanol 80% and ethyl acetate seed extracts showed a significant reduction in the biofilm at different concentration ranged from 0.4883-62.5 mg/ml against six isolated bacteria were monitored by assaying crystal violet dye and the reduction in cell attachment was measured by a microplate reader. The inhibition of biofilm formation increased with increasing concentration (Table 3 and 4).

Table 3: Antibiofilm activity of ethanol 80% extract against Gram-positive bacteria.

Ethanol 80 % extract	Percentage biofilm inhibition											
	St.epidermidis		*St. aureus*		*St. auricularis*		*S. mitis*		*S.salivarius*		*S. pneumoniae*	
	%	*P* value	%	*P* value	%	*P* value	%	*P* value	%	*P* value	%	*P* value
Control	0		0		0		0		0		0	
0.4883	14.9	0.041	50.7	0.019	1.8	0.049	2.9	0.033	7	0.043	3	0.02
0.9766	17.7	0.035	55.5	0.046	24.5	0.026	7.5	0.025	37.7	0.032	12.6	0.036
1.9531	21.5	0.029	61.9	0.039	28.1	0.037	21.3	0.045	46.5	0.048	16.6	0.015
3.9063	29.9	0.036	65	0.047	31.8	0.017	31.1	0.028	48.2	0.018	31.3	0.04
7.8125	41.1	<0.001	80.9	<0.001	66.3	<0.001	33.4	<0.001	51.7	<0.001	35.3	<0.001
15.625	48.5	<0.001	87.3	<0.001	75.4	<0.001	35.4	<0.001	57	<0.001	41.4	<0.001
31.25	59.8	<0.001	92	<0.001	78.1	<0.001	44.9	<0.001	60.5	<0.001	75.2	<0.001
62.5	71.9	<0.001	95.2	<0.001	81.8	<0.001	55.4	<0.001	70.1	<0.001	89.8	<0.001

P value represents the comparisons between control and different concentrations

Table 4: Antibiofilm activity of ethyl acetate extract against Gram-positive bacteria.

Ethyl acetate extract	Percentage biofilm inhibition											
	St.epidermidis		*St. aureus*		*St. auricularis*		*S. mitis*		*S.salivarius*		*S. pneumoniae*	
	%	*P* value	%	*P* value	%	*P* value	%	*P* value	%	*P* value	%	*P* value
Control	0		0		0		0		0		0	
0.4883	7.4	0.04	4.9	0.019	32.7	0.032	11.5	0.029	12.6	0.031	6.8	0.021
0.9766	16.8	0.017	10.8	0.044	50	0.02	14.4	0.03	17.4	0.04	11.8	0.033
1.9531	20.5	0.027	22.8	0.03	50.9	0.04	18.4	0.01	23.3	0.025	18	0.03
3.9063	23.3	0.020	71	0.027	51.8	0.023	23.3	0.048	33	0.037	29.2	0.019
7.8125	31.7	0.016	72.2	0.046	55.4	0.039	27.9	0.027	39.8	0.02	44	0.049
15.625	52.3	<0.001	78.3	<0.001	68.1	<0.001	38.4	<0.001	55.3	<0.001	57.7	<0.001
31.25	63.5	<0.001	90.3	<0.001	87.2	<0.001	44.3	<0.001	71.8	<0.001	62.7	<0.001
62.5	73.8	<0.001	93.9	<0.001	90	<0.001	59.3	<0.001	87.3	<0.001	85.7	<0.001

P value represents the comparisons between control and different concentrations

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Discussion

This study included evaluation three *Ammi majus* seed extracts against 6 Gram-positive bacteria to determine their antibacterial and antibiofilm activities. Results of the antibacterial activity study demonstrated that ethanol 80% extract showed higher activity than ethyl acetate extract against bacterial species. In a comparison of microbial sensitivity to both extracts by disk diffusion assay, Streptococcus species presented more pronounced sensitivity to ethanol 80% and ethyl acetate extracts than Staphylococcus species. Petroleum ether extract did not show activity against 6 Gram-positive bacteria even at maximum concentration 250 mg/ml, while ethanol 80% and ethyl acetate extract exhibited activity even at minimum concentration 62.5 mg/ml and more pronounced inhibition against *Streptococcus mitis* while the lowest inhibition for ethanol 80% extract was 5 mm against *Streptococcus salivarius* and for ethyl acetate extract was 3 mm against *Staphylococcus epidermidis* (Table 1). This may be because the bacterial inhibition can vary with plant extracts, the solvent used for extraction and the organism to be tested.[22] The antibacterial activity of ethanol 80% and ethyl acetate extract against *Staphylococcus epidermidis* and *Staphylococcus aureus* in agreement with the result recorded by other works,[23] while in contrast with the same study about petroleum ether extract activity against same bacteria. The antibacterial activity of ethanol 80% and ethyl acetate extract against *Staphylococcus auricularis, Streptococcus mitis, Streptococcus salivarius, Streptococcus pneumoniae* has not been previously described. In this study, bioautography is an important and easy way for determination bioactive constituents responsible for antibacterial activity even in a complex matrix which is found in seed extracts. The bioautography assay was performed for extracts showed antibacterial activity by disk diffusion method, applied to 6 Gram-positive bacteria and microbial growth inhibition appeared as pale or yellow zones around constituents with antibacterial activity against a violet background. In ethanol 80% extract constituent E80% 2 showed activity against five isolated bacteria while E80% 1, 3 and 4 which revealed activity only against three bacteria. All isolated bacteria showed sensitivity to constituent Ea 1 of ethyl acetate extract but *Staphylococcus aureus* and *Streptococcus mitis* showed more pronounced sensitivity in comparison to other bacteria (Figure 1). The MIC value of the extracts were assessed visually and by microtiter plate reader methods in order to minimize error and the lowest MIC value was 3.9063 for ethanol 80 % extract with MBC 7.8125 mg/ml against *Streptococcus salivarius,* also for ethyl acetate extract against *Staphylococcus auricularis* with MBC 15.625 mg/ml (Table 2). Surprisingly, ethanol 80% and ethyl acetate extract have larger inhibition zones against *Streptococcus mitis* and lowest MIC value against *Streptococcus salivarius and Staphylococcus auricularis*; this may be due to the presence of bioactive constituents in extract which is difficult to spread on the agar surface, but when they are diluted in broth showed a high antibacterial activity. Most of the plant have been studied for their activity against bacterial infection for the development of new antimicrobial agents but few plant extract has been investigated for their antibiofilm activity and antibiofilm activity of *Ammi majus* seed has not been reported previously. The study was only conducted on the extracts demonstrating activity to the isolated bacteria. Results indicated that both extracts showed significant inhibition of biofilm $P<0.05$ at concentration 0.4883 mg/ml against isolated bacteria whereas after 3.9063 mg/ml showed significant reduction of biofilm formation with $P<0.001$ for ethanol 80% extract. Seed extracts exhibited stronger antibiofilm activity against Staphylococcus species than Streptococcus species. Staphylococci

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have the ability to form biofilms on an implanted medical device or damaged tissues and these biofilms are difficult to eradicate.[21] The highest antibiofilm activity documented for ethanol 80% extract 95.2% and ethyl acetate extract 93.9% against *Staphylococcus aureus*, while the lowest documented for ethanol 80 % extract 55.4 % and ethyl acetate extract 59.3% against *Streptococcus mitis* (Table 3 and 4). However, none of the extracts was able to inhibit biofilm formation completely this may be due to solubility and diffusion of phytochemical constituents in agar media. The antibiofilm activity of the seed extracts against Streptococcal and Staphylococcal species may be an important tool for decrease microbial colonization on the surfaces and the epithelial mucosa which is main causes of infections by interrupting the release of the adhesion compound lipoteichoic acid from the cell surface,[24] or alteration of bacterial gene expression could be mediated the anti-biofilm action.[25] As a result concluded that the antibacterial and antibiofilm activity of *Ammi majus* seed due to their bioactive constituent present in it because phenolic acid, terpenes, tannin and flavonoids [10,26-29] were found to exhibit marked antibacterial and antibiofilm activity.

Conclusion

As a result concluded that during use of a different solvent for extraction of active constituents ethanol 80% extract showed higher activity than ethyl acetate extract against bacterial species, *Ammi majus* seed extracts revealed highest antibacterial activity against *Streptococcus species* and strongest antibiofilm activity on *Staphylococcus species*. A number of constituents (Ea 1-3 and E80 % 1-4) revealed their activity by agar overlay method and future works were suggested for isolation and identification each of these constituents responsible for antibacterial activity by using a different instrument like TLC, HPLC, IR, NMR, etc.

Conflicts of interest

The authors report no conflicts of interest.

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