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REPORT

Antibacterial and antibiofilm activities of *Scorzonera mackmeliana*

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Abstract: *Scorzonera* have been confirmed to have potent bioactivity. *Scorzonera mackmeliana* (Asteraceae), the endemic plant to Lebanon, has not yet been investigated. In the present study, we assessed the antibacterial activity of *S. mackmeliana* extracts against referenced bacterial strains. Extracts from different parts of the plant were evaluated against *Staphylococcus*, *Enterococcus*, *Escherichia* and *Pseudomonas* species. Phytochemical screening was done by standard biochemical tests and minimal inhibitory concentration (MIC), minimal bactericidal concentration (MBC) and minimal biofilm eradication concentration (MBEC) were determined by micro dilution method. The extracts possessed mainly alkaloids, phenols, flavonoids and coumarins. Gram-negative bacteria were most sensitive, whose MICs ranged between 48.98 and 341.85 mg/ml. Water stems extract, rich in phenols, was the most active with an MIC of 48.98 mg/ml. MBC was only recorded for water flowers extract, rich in resins, against *P. aeruginosa* and ethanolic roots extract, rich in terpenoids, against *S. epidermidis* with values of 160.85 mg/ml and 284.35 mg/ml, respectively. Furthermore, antibiofilm activity showed that the lowest MBEC was 0.1 mg/ml for water stems extract with an eradication ability of 91% ($p < 0.0001$). Hence, this study suggests *S. mackmeliana* as a promising candidate for future investigations to elucidate the major bioactive compound behind the antibacterial and antibiofilm effect.

Keywords: *Scorzonera mackmeliana,* phytochemical screening, antibiofilm activity, MIC, MBC, MBEC.

INTRODUCTION

The discovery of penicillin in 1928 by Alexander Fleming was a pillar in the history of modern medicine. Afterwards, the emergence of new antibiotics brought major advances in the control of bacterial infections. However, it was soon evident that bacterial pathogens became rapidly resistant to many of the discovered antibiotics (Barbour *et al*., 2004). As antimicrobials increased, so did the level and complexity of the resistance mechanisms exhibited by bacterial pathogens (Tenover, 2006). Such resistance involved all classes of antibiotics (Alanis, 2005). One major element enhancing bacterial resistance is biofilm formation.

Biofilms are communities of microorganisms attached to a solid surface and encased in a polysaccharide matrix. Biofilms have an enormous impact on medicine (Mah and O'Toole, 2001). The NIH revealed that among all microbial and chronic infections, 65% and 80%, respectively, are associated with biofilm formation (M. Jamal, 2017). Biofilms can form on medical devices like catheters, implants and contact lenses. These infections can only be treated by removal of the implant, thus increasing the trauma of the patient and the cost of treatment (Mah and O'Toole, 2001).

Biofilm grown cells express physiological properties distinct from planktonic cells. Those properties confer an increased resistance to antimicrobial agents (Mah and O'Toole, 2001). The most important resistance mechanisms conferred by biofilms are lesions in the mismatch repair system or in the DNA oxidative repair system, emergence of persistent bacterial cells and exclusion of biocides by the biofilm matrix (Li *et al*., 2017, Coutinho *et al*., 2009). Consequently, when cells exist in a biofilm, they become 10-1000 times more resistant to antimicrobials (Kanaan *et al*., 2017).

The World Health Organization (WHO) estimates that about 80% of the population from developing countries depend majorly on traditional medicines for their primary health care. For the remaining 20% as well as for those in industrialized countries, plant products play an important role in the health care. Despite the remarkable progress in

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synthetic organic chemistry, over 25% of the prescribed drugs in industrialized countries are derived directly or indirectly from plants which form a valuable source of medicines (Matias *et al*., 2013). Plants constitute a great source of bioactive compounds known for their antimicrobial potential. With increasing resistance against antimicrobials, plant extracts can be interesting alternatives (Coutinho *et al*., 2009). They demonstrated a low risk for resistance development due to their complex structural composition (Edinardo F.F. Matias, 2013).

Nowadays, there is a growing interest in understanding the biological and pharmacological properties of medicinal plants. Modest attention has been paid to the investigation of the Lebanese endemic flora as well as to its applications. In this work, we were interested in studying the antibacterial and antibiofilm effects of *Scorzonera mackmeliana,* an endemic plant of Lebanon. To the best of our knowledge, it is the first time that the phytochemical components of ethanolic and water extracts from *Scorzonera mackmeliana* were screened. Thereafter, these extracts were evaluated for their antibacterial and antibiofilm activities against 5 bacterial strains comprising the two Gram types.

MATERIALS AND METHODS

S. mackmeliana was collected from Yammouneh in Bekaa (34.116046, 36.037830) during its flowering period in April (2013). It was identified in accordance with the two well-known guides of Lebanon's flora (G. Tohmé, 2007, Mouterde, 1966).

Extraction

The plant was rinsed with tap water and divided into several fractions: whole plant, flowers, stems, leaves and roots. Maceration method has been used for extraction. Each fraction was cut into small pieces and soaked in pure ethanol and in water successively and the containers were covered to preserve light-sensitive compounds. All the containers were placed on an agitator, first at room temperature, then at 37°C, for 8-12 hours for each of the specified temperatures. Following this, extracts were recovered by filtration. Aqueous and ethanol filtrates were then concentrated using a rotary evaporator (Heidolph, Germany) under reduced pressure at 60°C and 40°C respectively. Extracts were frozen at -80°C then freeze-dried to powdered form using lyophilizer (Christ Alpha 1-4 LD plus, Martin Christ, Germany)(Nostro *et al*., 2000).

Phytochemical screening

Aliquots from the ethanolic and aqueous extracts of each fraction of the plant were prepared and evaluated by qualitative reactions to screen the presence of phytochemical constituents: tannins, resins, coumarins, saponins, alkaloids, phenols, terpenoids, volatile oils, and flavonoids (Farhan H, 2012, Rawat V., 2013). The presence of each of those secondary metabolites in the extract was evaluated according to the extract response to the reaction, mainly by a color change and/or precipitate formation.

Bacterial strains, media and reagents

Three Gram-positive bacterial strains (*Staphylococcus epidermidis* (CIP 444), *S. aureus* (ATCC 25923) and *Enterococcus faecalis* (ATCC 29212)) and two Gram-negative ones (*Escherichia coli* (ATCC 35218) and *Pseudomonas aeruginosa* (ATCC 27853)) were used in this study. Bacterial Strains were stored in glycerol stocks at -80˚C as required. CIP 444 is a clinical strain isolated from an infected implanted device of a patient being hospitalized in the Mignot Hospital of Versailles, France (Kogan *et al*., 2006). Chokr A. has identified and characterized this strain for many of its properties. It was deposited and enclosed within the collection of microorganisms of Pasteur Institute in 2007 (Chokr *et al*., 2007, Kogan *et al*., 2006, Sadovskaya *et al*., 2006). The other strains are ATCC. Mueller-Hinton broth (MHB), Brain heart infusion (BHI), Brain heart agar (BHA), and Tryptic Soy Broth (TSB) were purchased from HIMEDIA (Mumbai, India), prepared and then autoclaved as indicated by the manufacturer.

Minimal Inhibitory concentration (MIC) and minimal bactericidal concentration (MBC) assays

MIC and MBC were determined by micro dilution method as recommended by the Clinical and Laboratory Standards Institute (CLSI). Briefly, serial two-fold dilution of water and ethanol extracts in Mueller-Hinton Broth were prepared in a 96 wells plate (100 µL per well) (Corning® Costar® 3598, Corning, NY 14831, USA). Wells with no extract were considered as positive controls. 100 µL of bacterial suspension are added to each well to have a final concentration of 5×10^5 CFU/mL. Wells without bacteria were considered as negative controls. Plates are incubated for 24 hours at 37°C. MIC is defined as the lowest extract concentration that yielded no visible growth. Contents of wells showing no visible growth were plated on Brain Heart Agar and incubated overnight at 37°C. Then, the number of colonies was counted, and MBC determined. MBC is defined as the lowest concentration that killed 99.9% or more of the initial inoculum (<5 CFU on the petri dish).

Biofilm formation

Assay of biofilm formation in polystyrene was performed essentially according to a standard procedure with some modifications (Kanaan *et al*., 2017). S. *epidermidis* grown in trypticase soy broth medium overnight at 37°C. Then the overnight cultured S. *epidermidis* CIP 444 suspension with defined volume at concentration of 4.16×10^5 CFU/mL (confirmed by viable count) was added to a trypticase soy broth medium supplemented with 0.25%

glucose. A total of 120µL of this bacterial suspension was inoculated into each well of a sterile 96-well flat-bottom polystyrene tissue culture-treated microtiter plate except for column 12 which was used as a negative control and filled only with the sterile medium, and then the plates were incubated for 24h at 37°C. The next step included discarding the biomass and washing the microtiter plates with saline water (0.9% NaCl) to remove any non-adherent bacteria, then drying the plates at room temperature for several minutes. After that, the remaining biofilm attached to the wall and the bottom of the wells was fixed by heating at 50°C for 50min, thus the plates were ready for treatment with the plant extracts.

Biofilm eradication test

After the fixation of the formed biofilm as previously described, each well of the microtiter plate was filled with 120µL of sterile physiologic water to be used as a diluent for the serial dilution of our plant extracts. A serial ½ dilution was then made with equal volume of the extract in the saline water in the wells except for column 11 which was used a positive untreated control, then the microtiter plates were incubated at 37°C for 18h. Tests were performed in quadruple. The wells were then washed 2 times by saline water, filled with 100µL 0.1% crystal violet and left at room temperature for 10min. The stain was then discarded and the wells were washed with saline water for 3 times. They were finally filled with 100µL of physiologic water and the OD_{490nm} was measured. The results were expressed as mean ± SEM.

STATISTICAL ANAYLSIS

Statistical analysis was done using Prism 5.0, GraphPad, San Diego, California USA, www.graphpad.com. Statistical differences were determined using One-way ANOVA followed by Dunnett post-test in order to compare results versus positive control. A p-value of ≤0.05 was considered statistically significant.

RESULTS

Phytochemical profile of the plants extracts

Each of the five plant fractions (whole plant, flower, leaves, stems, and roots) was extracted in water and in ethanol, giving 10 extracts in total. The phytochemical components were determined in each of the 10 extracts. Most ubiquitous phytochemical was coumarin, it has been found in all 10 extracts. Alkaloids were present in 8 fractions: all except flower water extracts and leaves water extracts. In the 3rd place, comes flavonoids and Phenols present in 7 extracts. Other secondary metabolites as resins, terpenoids and volatile oils were present in 5 from 10 extracts at variable amounts. Tannins and Saponins were completely absent from all extracts. Portions of each extract are represented in table 1.

Antibacterial activity

Different inhibitory potentials were displayed by the extracts toward the tested bacterial strains. For some extracts, no MIC and/or MBC were obtained at the used concentrations and the symbol (>) was used to indicate that a higher concentration might be needed to achieve an effect.

Gram negative strains were more sensitive to the extracts' activities than Gram positive ones. Among Gram negative bacteria, *P. aeruginosa* was the most sensitive where its growth was inhibited by 5 of the 10 extracts (Flowers' water and ethanol, stems' water, Roots' ethanol and whole plants' water extracts). In the second place comes *E. coli*, considering its sensitivity to 4 extracts (table 2).

As for Gram positive strains, *S. epidermidis* was more sensitive than *S. aureus and E. faecalis*. The first one's growth was inhibited by 4 extracts, the second one by 3 extracts, and *E.faecalis* was only sensitive to Leaves's water extracts (table 2).

The most active extract was Water Stem which was active on 4 of the 5 tested bacterial strains. It had the lowest MIC (48.98 mg/ml) on 3 bacterial strains: *P. aeruginosa*, *S. epidermidis* and *S. aureus* and an MIC of 97.95 mg/ml on *E.coli*. The second active compound is whole plant water extract, with an MIC of 122.25 mg/ml on 3 bacterial strains.

MBC was only obtained for Roots' ethanol extracts on *S. epidermidis*, (MBC value of 284.35 mg/ml), and for water flowers' extracts on *P. aeruginosa* (MBC value of 160.85 mg/ml).

Biofilm Eradication Assay

Biofilm eradication essay was performed on*S. epidermidis* pre-formed biofilm. Different biofilm eradication capabilities were displayed by each extract. A surprising pattern was registered; as the extract concentration decreased, the biofilm eradication increased. Some fluctuations were shown (fig. 1 and 2), but the general pattern is conserved.

For each extract, we defined the minimal biofilm eradication concentration (MBEC). It corresponds to the lowest concentration exhibiting highest biofilm eradication. For each MBEC, the percentage of eradication (eradication potential) was also determined. It is calculated as the following:

$$\%\ \text{eradication} = \left[\frac{\text{O.D.}(\text{positive contol}) - \text{O.D.}(\text{treated well})}{\text{O.D.}(\text{positive contol}) - \text{O.D.}(\text{negative control})}\right] \times 100$$

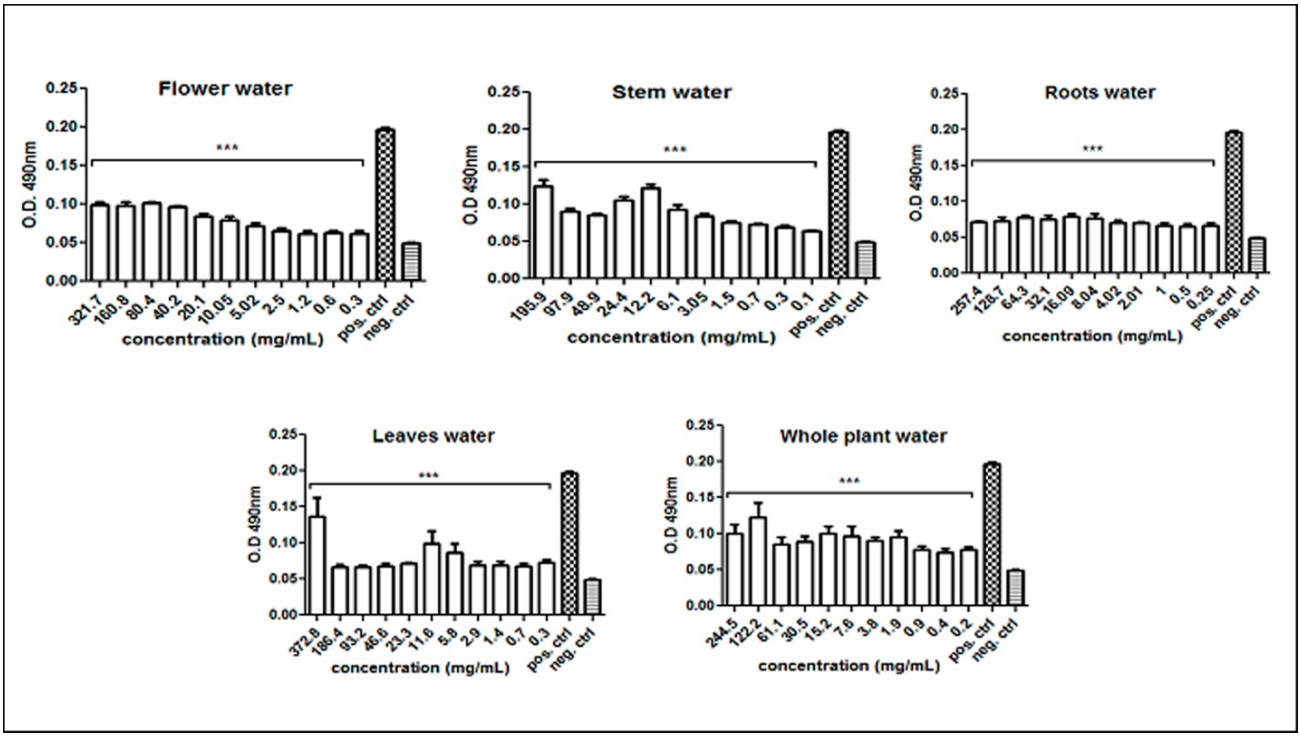

Fig. 1: Eradication effect of water extracts on *S. epidermidis*. Graphs of the different water extracts showing crystal violet optical density (O.D.) as function of the extract concentration; and reflecting the variation of the biofilm concentration by the different extract concentrations. ▭ :treated wells, ▩ :positive control or untreated wells, ▤: negative control. There was significant difference for the treated compared to the untreated wells; ***: P<0.0001.

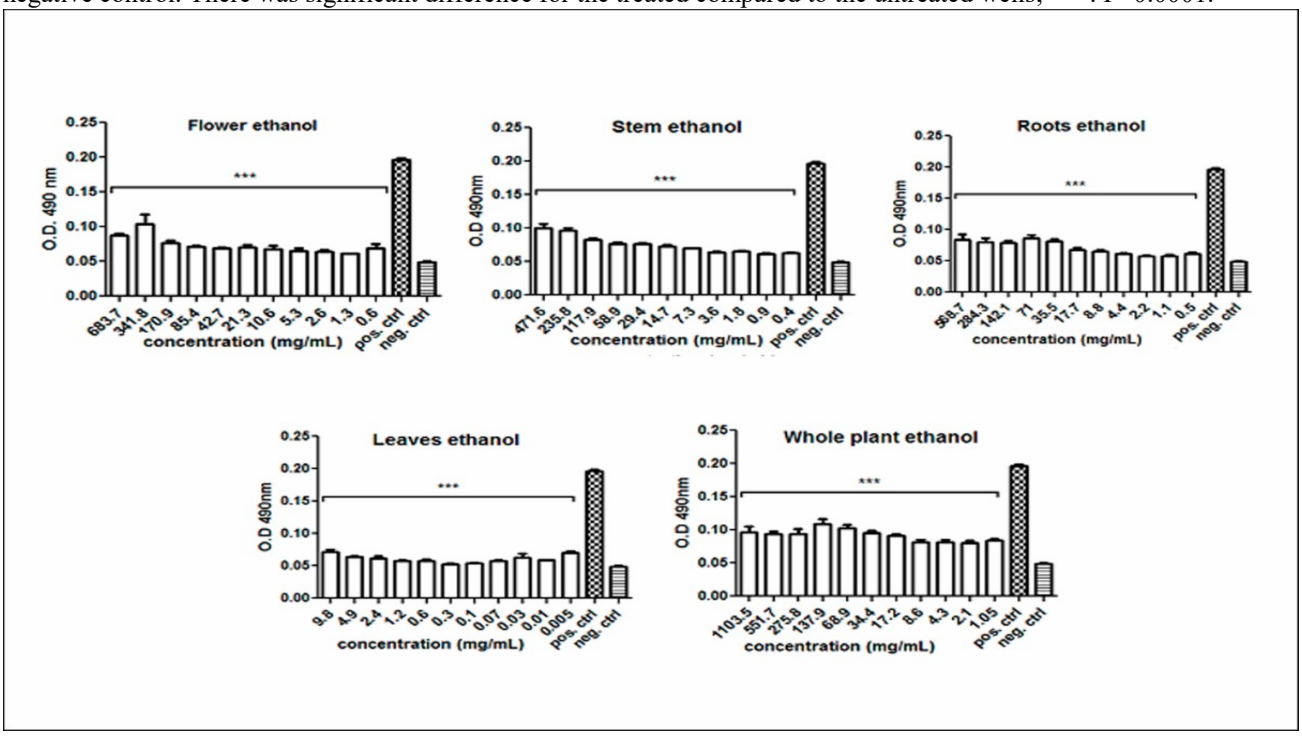

Fig. 2: Eradication effect of ethanol extracts on *S. epidermidis*. Graphs of the different ethanol extracts showing crystal violet optical density (O.D.) as function of the extract concentration; ▭ and reflecting the variation of the biofilm concentration by the different extract concentrations: treated wells,: ▩positive control or untreated wells: ▤ negative control. There was significant difference for the treated compared to the untreated wells; ***: P<0.0001.

Table 1: Results of the phytochemical screening

Plant Species	Plant part	Solvent	Quantity of phytochemicals*								
			Alkaloids	Tannins	Resins	Phenols	Saponins	Flavonoids	Terpenoids	Coumarins	Volatile oil
Scorzonera mackmeliana	Flowers	Ethanol	+	-	-	+	-	+++	-	+	+
		Water	-	-	+++	-	-	-	-	+	-
	Stems	Ethanol	+++	-	-	-	-	++	-	++	-
		Water	+	-	-	+++	-	+	+	+	-
	Leaves	Ethanol	+	-	-	+	-	+++	-	+	-
		Water	-	-	+	-	-	-	-	++	-
	Roots	Ethanol	++	-	-	++	-	-	+++	+	-
		Water	+	-	+	++	-	+	+++	+	-
	Whole plant	Ethanol	++	-	+++	+++	-	+++	-	++	+
		Water	++	-	+++	+++	-	++	++	++	-

: - : negative ; + : small amount ; ++ : average ; +++ : large

Table 1: Results of MIC and MBC of the plant extracts against the tested bacteria

Plant Species	Plant Part	Solvent	MIC (mg/mL)					MBC (mg/mL)				
			S. aureus	*E. faecalis*	*S. epidermidis*	*E. coli*	*P. aeruginosa*	*S. aureus*	*E. faecalis*	*S. epidermidis*	*E. coli*	*P. aeruginosa*
S. mackmeliana	Flowers	W	160.85	>160.85	>160.85	160.85	160.85	>160.85	>160.85	>160.85	>160.85	160.85
		E	>341.85	>341.85	341.85	>341.85	341.85	>341.85	>341.85	>341.85	>341.85	>341.85
	Stems	W	48.98	>97.95	48.98	97.95	48.98	>97.95	>97.95	>97.95	>97.95	>97.95
		E	>235.8	>235.8	>235.8	235.8	>235.8	>235.8	>235.8	>235.8	>235.8	>235.8
	Leaves	W	186.4	186.4	>186.4	>186.4	>186.4	>186.4	>186.4	>186.4	>186.4	>186.4
		E	>8.9	>8.9	>8.9	>8.9	>8.9	>8.9	>8.9	>8.9	>8.9	>8.9
	Roots	W	>128.7	>128.7	>128.7	>128.7	>128.7	>128.7	>128.7	>128.7	>128.7	>128.7
		E	>284.35	>284.35	142.18	>284.35	284.35	>284.35	>284.35	284.35	>284.35	>284.35
	Whole plant	W	>122.25	>122.25	122.25	122.25	122.25	>122.25	>122.25	>122.25	>122.25	>122.25
		E	>620.7	>620.7	>620.7	>620.7	>620.7	>620.7	>620.7	>620.7	>620.7	>620.7

* : W: Water; E: Ethanol

Table 3: Water extracts (A) and ethanol extracts (B) with their MBEC and percentages of eradication. Results are presented in decreasing order of biofilm eradication potential

A			B		
Water Extracts	MBEC (mg/ml)	% of Eradication	Ethanol Extracts	MBEC (mg/ml)	% of Eradication
Flower	1.2	92 %	Leaves	0.3	98 %
Stem	0.1	91 %	Roots	2.2	95 %
Roots	1	90 %	Whole Plant	2.1	95 %
Leaves	93.2	89 %	Flower	1.3	92 %
Whole Plant	0.4	84 %	Stem	0.9	91 %

As shown in figures 1 and 2 and table 3 (A&B), all extracts exhibited an eradicating activity. Biofilm eradication was observed for all the ranges of concentrations used for each extract. For most of the extracts, the highest effects observed corresponded to low extracts concentrations (between 2.2 mg/ml and 0.3 mg/ml). Both, water and ethanol extracts, showed high biofilm eradication potentials. Percentages of eradication were between 89% and 98% at extracts MBEC (table 3 A and B). The highest eradicating effect was attributed to leaves ethanol extracts with 98% of biofilm eradication. Roots ethanol and whole plant ethanol extracts showed

95% of biofilm eradication at their MBEC (fig. 2, table 3). The highest water extracts' effects were attributed to flower water with 92% of biofilm eradication, followed by stem water and roots water extracts with 91% and 90% of biofilm eradication, respectively, at their MBEC (fig. 1, table 3).

DISCUSSION

With the emergence of the increasing numbers of antibiotic-resistant bacteria, new natural therapeutic agents are needed. One of *Scorzonera* taxa, *S. mackmeliana*, which is an endemic plant mostly distributed through the Bekaa valley of Lebanon, was used in this study to be assayed for antimicrobial activity. To the best of our knowledge, this is the first time that extract (s) or product (s) from this specie were investigated for any bioactivity. It was demonstrated in many studies that such taxa have been used for the treatment of many diseases. Yang *et al.* isolated few compounds with anticancer effect from the roots of *Scorzonera divaricata Turcz* a traditional natural medicine used in european countries (Yang *et al*., 2016b). Similar to that, *Scorzonera hispanica,* a vegetable cultivated in Central and Southern Europe, was also suggested to have chemotherapeutic effect against colon cancer (Granica *et al*., 2015). Anti-inflammatory activity was identified from Turkish Scorzonera species, *S. latifolia*, *S. cana var*. *jacquiniana*, *S. tomentosa*, *S. mollis* ssp. *szowitsii*, *S. eriophora*, *S. incisa*, *S. cinerea*, and *S. parviflora* (Acikara *et al*., 2014). *Scorzonera latifolia*, mainly found in central and east anatolia, was also found to have antinociceptive activity (Acikara *et al*., 2014). In terms of antimicrobial effect, the extract of species, endemic to Turkey, was described by *Ugur et al.* to have inhibitory effect on the growth of multiresistant strains of *Stenotrophomonas maltophilia* (Ugur *et al*., 2010). Furthermore, *Scorzonera sandrasica*, plant from Turkey, was found to modulate quorum sensing-regulated behavior in *Chromobacterium violaceum* (Bosgelmez-Tinaz G *et al* 2007).

From Lebanese endemic plants, few were described to have bioactivities; *Arbutus andrachne* the strawberry tree, *Punica granatum* the pomegranate fruit and *Sideritis libanotica* were found to have antioxidant properties (Abidi *et al*., 2016, Kozik *et al*., 2015, Formisano *et al*., 2015). Also, essential oils of the Lebanese *Juniperus excelsa M.* have been identified to have antibacterial activity towards *Aggregatibacter actinomycetemcomitans* and *Streptococcus* (Azzimonti *et al*., 2015). *S. mackmeliana,* the endemic medicinal plant of Lebanon, is still to date unexplored.

In the present study, our results showed different MIC(s) for the different parts of *S. mackmeliana* plant and such pattern effect was described previously (Benli *et al*., 2008, Benli *et al*., 2009). Ethanol extracts showed lower antibacterial activities than water extracts. The best MIC was that of the stem water extract followed by whole plant water extract. The first one had antibacterial activity against four different bacterial strains, *S. aureus* (gram positive), *S. epidermidis* (gram positive), *P. aeruginosa* (gram negative) and *E. coli* (Gram negative); while the second showed an activity against three bacterial strains: *S. epidermidis*, *P. aeruginosa* and *E. coli*. The phytochemical screening of both extracts showed a major presence of phenol that is less represented in other parts of the plant; this active phytochemical constituent has most likely played a role in the beneficial antibacterial effect. High phenolic levels in extracts have been demonstrated to be responsible for antibacterial activities of many plant extracts, such as drumstick peel (*Moringa oleifera*) and leaves of *Pergularia daemia Forsk* (Sarkodie *et al*., 2016, Surendra *et al*., 2016).

Regarding biofilm eradication, flower water extracts yielded the highest eradication activity between water extracts. They are mainly rich in resins which might be at the origin of their eradication potential. Water extracts of stems also showed highly potent antibiofilm eradication properties. Such activity was also relevant for the other parts of the plant. The major ubiquitous constituent found in all the extracts was coumarin, which might be the potential lead molecule for the biofilm eradication. Such compound has been previously reported to have a potent antibiofilm activity (Yang *et al*., 2016a, Lee *et al*., 2014).

As for ethanol extracts, leaves, flowers and whole plant showed the strongest anti-biofilm eradication activity. This might be due to their high content in Flavonoids. Flavonoids form complexes with both extracellular and soluble proteins as well as with bacterial membranes (Savoia, 2012). Roots ethanol extracts are also highly active against biofilms. They are rich in terpenoids which might act on biofilm eradication by disrupting lipid structures and inhibiting quorum sensing (Negi, 2012).

CONCLUSION

Herein, we demonstrated substantial antibacterial and antibiofilm effect of *S. mackmeliana*; further investigations on the active compounds and the mechanism of action are needed. From this study, it can be concluded that extracts from *S. mackmeliana* alone or synergistically with antibiotics could be tested as novel effective antibacterial agents against resistant bacterial strains.

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