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ANTIBACTERIAL EFFECT AND PHYTOCHEMICAL ANALYSIS OF THE SHOOT SYSTEM OF RUBUS CANESCENS DC. GROWING IN LEBANON

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Abstract

The misuse of antibiotics followed by improved fitness of resistant strains of infectious microorganisms hindered the efficacy of many known antimicrobial agents, and fueled research for the discovery of novel remedies. The current study aims at assessing the antimicrobial activity and understanding the mechanism of action of Rubus canescens DC. growing wild in Lebanon, as well as qualitatively determining its phytochemical profile. The antibacterial activity, MIC, and MBC of the extracts were evaluated by two-fold dilution. Time-kill curves were plotted to assess the bactericidal activity of the R. canescens DC. extracts against the growth of microorganisms, and TEM images were collected to confirm such effect. Overall, the extracts exhibited good antibacterial activity against MRSA and E. coli but not against S. pneumoniae and K. pneumoniae as determined by measuring the inhibition zones in plate-diffusion assays. TEM images of treated microorganisms revealed that the R. canescens DC. extracts induced irreversible deformations and damage to the cell membranes of the microorganisms leading to the leakage of cytoplasmic components and eventual cell death. Analysis of Time-Kill curves indicated that the extracts induced 100% killing of the test microorganisms within 10-18 h at the respective MBC. Finally, qualitative phytochemical analysis was conducted to decipher the active ingredients in the plant extracts.The current study reports the first data on the antimicrobial activity of different parts of R. canescens DC. Such promising data opens new avenues for broader assessment of the pharmacological profile of the scarcely investigated R. canescens DC.

Keywords

Rubus canescens DC., antibacterial activity, TEM, phytochemical analysis, Lebanon

1. INTRODUCTION

The use of medicinal natural resources for the treatment of a wide spectrum of diseases is an old aged practice. The first written records that document the utility of the healing power of plants date back to 2600 BC in Mesopotamia where the uses of approximately 1000 plant-derived substances have been described [Newman & Cragg, 2012]. Currently, medicinal plants continue to constitute a haven for home remedies in the developed and underdeveloped countries [Ahmad *et a*l., 2006]. According to the World Health Organization (WHO), about 80% of developing countries population depends on traditional medicines, while 25% of all modern medicines including both western and traditional medicine are derived from plants [Ong *et al*., 2005; Calixto, 2005]. Despite the emergence of high throughput screening for the discovery of lead molecules that can eventually be developed into drugs, the outcome from such approach did not meet expectations and drugs resulting from this approach are scarce [David *et al*., 2015]. This led scientists to go back to nature in attempt to find cures for diseases. The popularity of herbal medicine is increasing all over the world. The global herbal medicine market is expected to reach USD 117.02 billion per year by 2024, with Europe constituting the largest market in 2016 [Atanasov *et al*., 2015].

Plants of the genus Rubus (over 750 species) are known for its broad spectrum of biological activities such as antimicrobial [Thiem,& Goślińska, 2004; Escuredo *et al*., 2012; Panizzi *et al*., 2002], antioxidant [Fu *et al*., 2015; Gouveia-Figueira & Castilho, 2015], anticancer [Hsieh et al., 2013; George *et al*., 2013; Kim *et al*., 2005], anti-inflammatory [Fazio *et al*., 2013], and wound healing [Akkol *et al*., 2015]. In Lebanon, *Rubus canescens* DC. (*R. canescens* DC.) can grow throughout the four seasons, and is widespread at different altitudes. Typically, the plant annually grows to 28 in (70 cm) and has deep green leaves (3-5 leaves per leaflets), greenish flowers, and small dark seeds. While many species of the Rubus family are extensively studied for various biological applications, no studies have described the antimicrobial effects of the different parts of *R. canescens* DC. and its phytochemical profile. Therefore, we set out to assess the antimicrobial activity and understand the mechanism of action of the fruit juice, methanolic and aqueous extracts of leaves and stems of *R. canescens* DC. against multiple Gram-positive and Gram-negative bacteria, and to determine qualitatively its phytochemical profile.

Fig.1 Flowering *R. canescens* DC. plant.

2. MATERIALS AND METHODS

2.1 Chemicals

All reagents used in the present study were used as received from the supplier with no further purification. The culture media was purchased from Oxoid Ltd, United Kingdom. The culture media was prepared according to the manufacturer guidelines (Oxoid Ltd., UK) with distilled water and sterilized by autoclaving for 20 min at pressure of 15 lb/inch^2 to raise the temperature to 121°C. All the media's composition is given in g/L unless otherwise indicated.

Antimicrobial discs (Ceftriaxone (CRO-30 µg), Vancomycin (VA-30 µg), Cefoxitin (FOX-30 µg), Oxacillin (OX-1 µg), Azotreonam (ATM-30 µg), Cefotaxime (CTX-30 µg), Ofloxacin (OFX-5 µg), Gentamicin (CN-10 µg), Tetracyclin (TE-30 µg) and Nystatin (NS-100 µg) were obtained from acknowledged chemical suppliers (Medilic, Beirut).

2.2 Plant Collection and Extracts Preparation

The plant was identified by Prof. Nelly Arnold from Holy Spirit University of Kaslik (USEK), Lebanon. Leaves and stems from the collected *R. canescens* DC. were isolated, air-dried at room temperature in the dark, and grinded using a commercial blender. The juice was collected by squeezing and filtering the fruit. The leaves or stem powder (3 g) were extracted either with 350 ml of distilled water (water extract) or methanol (methanolic extract) by heating overnight at 60ºC. After cooling to room temperature, the mixture was filtered using gravity filtration technique, and the liquid phase was collected. The solution was concentrated under reduced pressure at 40ºC using rotary evaporator. The obtained oil residue was weighed and re-dissolved in sterile 25 mL of respective solvent (distilled water and methanol) to reach a concentration expressed in µL-sample/mL. All test solutions were kept in sterile dark bottles in the refrigerator and filtered through Sartorius Stedim Minisart syringe filter (0.2 µm pore-size) before use to ensure sterility [Sasidharan *et al*., 2011]. Methanol controls were tested, and no inhibition of bacterial growth was observed.

2.3 Microorganisms

Four different bacterial isolates were used throughout the current study, namely two Gram-positive bacteria: Methicillin-resistant Staphylococcus aureus (MRSA) and Streptococcus pneumoniae (S. pneumoniae), and two Gram-negative Extended Spectrum β-Lactamase (ESBL) producing bacteria: Escherichia coli (E. coli) and Klebsiella pneumoniae (K. pneumoniae). All isolates were kindly provided by the Microbiology Research Lab-Faculty of Science at Beirut Arab University and identified phenotypically as described by Bergey's Manual of Determinative Bacteriology [Holt & Krieg, 1994]. The bacterial isolates used throughout the present work were maintained on nutrient agar slants and stored at 4°C with regular transfer at monthly intervals. For long preservation, 25 % glycerol was added to slants and stored at 4°C.

2.4 Inoculum Preparation and Standardization

Inocula were prepared from a freshly prepared culture of each isolate, by suspending 4-5 colonies grown overnight on agar plate in sterile saline (0.85 %). Slowly, with agitation, 9.9 mL of 1 % chemically pure sulfuric acid were mixed with 0.1 mL of 1.175% aqueous solution of barium chloride (BaCl2.H2O) to make a total 10 mL per tube. The suspended barium sulfate precipitate corresponded approximately to 1.5×108 CFU/mL [Madigan et al., 2006]. The 0.5 McFarland standard provided an optical density comparable to the density of a bacterial suspension of 1.5×108 CFU/mL. The bacterial suspension was prepared by emulsifying 4 to 5 colonies from a freshly prepared culture of each isolate (16 to 24 h old nutrient agar slants) in 5 mL of 0.9% saline solution to achieve a turbid suspension. Matching turbidity was achieved using unaided eye by holding the bacterial suspension and McFarland tubes side by side and viewing them against a black background. If the bacterial suspension initially did not match with the standard's turbidity, the suspension may be diluted, or supplemented with more cells [Mahon et al., 1998].

2.5 Antibiotic Susceptibility Testing

Antibacterial activities of commonly used antimicrobial agents were assessed using the disc-diffusion method according to the CLSI guidelines [CLSI, 2012]. Petri dishes were filled with 20 mL of sterile Müller Hinton agar. The test culture was swabbed on the top of the solidified media and allowed to dry. The loaded antibacterial discs namely: CRO-30 µg, VA-30 µg, FOX-30 µg, OX-1 µg, ATM-30 µg, CTX-30 µg, OFX-5 µg, CN-10 µg, TE-30 µg and

NS-100 µg, were placed on the surface of the medium and left for 30 min at room temperature for compound diffusion. The plates were incubated for 24 h at 37°C. Zones of inhibition were recorded in millimeters in table 2 and the experiments were repeated three times.

The antibacterial activity of crude *R. canescens* DC. extracts against the selected bacterial strains was assessed by the plate-well diffusion assay as described by Kudi *et al*. [Kudi *et al*., 1999]. A sterile cork-borer (8 mm diameter) was used to make wells in the set agar. An inoculum 1.5×10^8 CFU/mL equivalent to 0.5 McFarland was prepared, and 25 µL were swabbed over the surface of Müller-Hinton agar plate. 200 µL of the prepared plant extract was added to each well and the plates were incubated for 24 h at 37°C. Zones of inhibition were then measured in millimeters. The experiment was done in triplicate.

2.6 Determining MIC and MBC

The MIC was measured by following the CLSI M7-A10 and CLSI M27-A2 standard microdilution method in a sterile 96-well microtiter plates [CLSI, 2006; CLSI, 2009]. The wells were filled with 100 µL of sterile water, and 100 µL of plant extract solution were added to the wells by serial two-fold dilution. Each well was inoculated with 100 µL of McFarland standardized microbial suspensions diluted in Müller Hinton broth so that each well got 1.5 x 10^8 CFU/mL of bacteria. The 96-well microtiter plates were covered and incubated at 37°C for 24 h. The MIC is defined as the lowest concentration of plant extract that exhibited total inhibition of visible microbial growth. MBC was determined by inoculating the dilutions (20 µL) that showed no visible growth from the MIC into Müller-Hinton agar plates and incubating the plates at 37°C for 24 h. The lowest concentration that was lethal to the microorganism was considered as MBC. Experiments were done in triplicates.

2.7 Time-Kill Curve Assay

A time-kill curve was plotted to investigate the time needed by the extract under test to kill the bacteria. The selected extract that showed a bactericidal effect against the most promising bacterium under test was used. A 16 h old culture was harvested, the suspension was adjusted, and serially diluted to 1 x 105 CFU/mL spectrophotometrically and using the McFarland standard. Plant extracts for the selected bacteria were added to aliquots of 1 mL Müller Hinton broth followed by the addition of 1 mL of the inoculum to attain bactericidal concentration. Spread plate technique was used for counting the bacteria present in each sample at different time intervals [Yin et al., 2002].

2.8 Transmission Electron Microscopy Images

MRSA and ESBL E. coli were treated with the plant extracts at concentrations equivalent to MBC. Freshly taken samples were fixed using a universal electron microscope fixative. Series of dehydration steps followed using ethyl alcohol and propylene oxide. The samples were then embedded in labeled beam capsules and polymerized. Thin sections of cells exposed to extracts were cut using LKB 2209-180 ultra-microtome and stained with a saturated solution of uranyl acetate for 30 min and lead acetate for 2 min. Electron micrographs were taken using a Transmission Electron Microscope (JEM-100 CX Joel) at the Electron Microscope Unit, Faculty of Science, Alexandria University, Egypt.

3. RESULTS AND DISCUSSION

Screening experiments were conducted to evaluate the antibacterial effect of different *R. canescens* DC. extracts against two Gram-positive bacteria namely MRSA and *S. pneumoniae*, and two Gram-negative ESBL-producing bacteria, namely *E. coli* and *K. pneumoniae*, using the well-diffusion method. Zones of growth inhibition were recorded and summarized in table 1. The aqueous and methanolic extracts of leaves and stems, as well as fruit juice exhibited no effect against *K. pneumoniae* and *S. pneumoniae*.

Table 1: Antibacterial activity of *R. canescens* DC. extracts.

Extract	Microorganism	Inhibition zone (mm)	MIC (µl-extract/L)	MBC (µl-extract/L)	MBC/MIC ratio
Leaves/MeOH	MRSA	34 ± 1.7	7.81	7.81	1
Stems/MeOH	MRSA	29 ± 2.1	62.5	62.5	1
Fruit juice	MRSA	32 ± 2.1	125	125	1
Leaves/H_2O	MRSA	46 ± 2.5	31.3	31.3	1
Stems/ H_2O	MRSA	32 ± 2.0	15.6	15.6	1
Fruit juice	ESBL *E. coli*	18 ± 3.1	15.6	15.6	1

On the other hand, the various extracts exhibited antibacterial activity against MRSA with inhibition zones ranging between 32-46 mm. Interestingly, the growth of ESBL *E.coli* was only inhibited by fruit juice with an average inhibition zone of 18 mm. These results are supported by Panizzi *et al*. who reported inhibition zones between 20-25 mm for *S. aureus* and *E. coli* when tested against a crude methanolic extract of *Rubus ulmifolius*. On ther hand, the water extracts of the same plant were less effective against the same microorganisms with inhibition zones in the range of 8-15 mm [9]. Saklani *et al.* evaluated the antimicrobial activity of different extracts of wild edible fruits of *Rubus ellipticus*. At 10 mg/ml, the ethanol extract, but not the aqueous one, inhibited the growth of *S. aureus* and *E. coli* [Saklani *et al*., 2012]. Velićanski *et al*. demonstrated the antibacterial potential of a *Rubus idaeus L*. (raspberry) fruit and pomace extracts against selected Gram-positive and Gram-negative organisms [Velićanski *et al*., 2012]. Raspberry fruit extracts showed strong antibacterial activity against several microbial strains including *S. aureus*; however, *E. coli* was found to be the most resistant. In our study, the fruit juice exhibited bactericidal effect on both MRSA and *E. coli*, thus demonstrating the beneficial effects of the Lebanese *R. canescens* DC. berries, which can be consumed as a source rich in antibacterial bioactive ingredients. The resistance of MRSA and ESBL *E. coli* to commercial antibiotics was assessed using the antibiotic susceptibility assay as described in CLSI guidelines [CLSI, 2012], and results are summarized in table 2.

Table 2: Antimicrobial susceptibility of the tested microorganisms to common antimicrobial agents. S: susceptible; I: intermediate; R: resistant.

Extract	Inhibition zone (mm)		Zone diameter interpretative standards		
	MRSA	**ESBL *E. coli***	**S**	**I**	**R**
Ceftriaxone (30 µg)	12.00 ± 0.60	10.00 ± 0.50	≥23	20-22	≤19
Vancomycin (30 µg)	17.00 ± 1.00	10.00 ± 0.8	≥17	15-16	≤14
Cefoxitin (30 µg)	17.00 ± 1.00	23.00 ± 1.00	≥18	15-17	≤14
Oxacillin (1 µg)	10.00 ± 0.58	12.00 ± 0.60	≥13	11-12	≤10
Azotreonam (30 µg)	13.00 ± 0.80	15.00 ± 0.56	≥21	18-20	≤17
Cefotaxime (30 µg)	12.00 ± 0.70	8.00 ± 0.80	≥26	23-25	≤22
Ofloxacin (5 µg)	31.00 ± 0.40	11.00 ± 0.58	≥16	13-15	≤12

Based on the sensitivity assay data, MIC values for the plant extracts that showed the most promising inhibitory effect against the growth of some bacterial strains were determined and summarized in table 1. MIC values against MRSA ranged between 7.81 µl-extract/L for the methanolic leaf extract, and 125 µl-extract/L for the fruit juice. On the other hand, the MIC value for the fruit juice against ESBL *E.coli* was 15.6 µl-extract/L.

The minimal bactericidal concentration (MBC) is a measure of the potency of the extract in killing bacterial strains. *R. canescens* DC. extracts were tested for their bactericidal effect against the selected bacteria strains. The lowest concentration that was lethal to the bacterium was considered as MBC. As depicted in table 1, MBC values against MRSA and ESBL *E. coli* were equal to the MIC values. This suggested that the extracts exhibited bactericidal effect against the tested microorganisms with MBC/MIC < 4 (Table 1; MBC/MIC=1). It is noteworthy mentioning that MBC/MIC ratio less than 4 suggested a bactericidal effect, while a bacteriostatic effect is suggested at a ratio higher than 4. These observations are in correlation with Thiem & Goślińska who reported MBC/MIC ratios between 1 and 2 for the leaves butanolic fraction of *Rubus chamaemorus* against MRSA and ESBL *E. coli* [Thiem,& Goślińska, 2004]. Similar bactericidal effect was also reported by the Cai *et al*. who tested the antibacterial activity of volatile oil extracted from *Rubus parvifolius* L. leaves against several Gram-positive and Gram-negative microorganisms. The authors reported MIC values of 5 mg/mL against *S. aureus*, *E. coli*, and *K. pneumonia* [Cai *et al*., 2012].

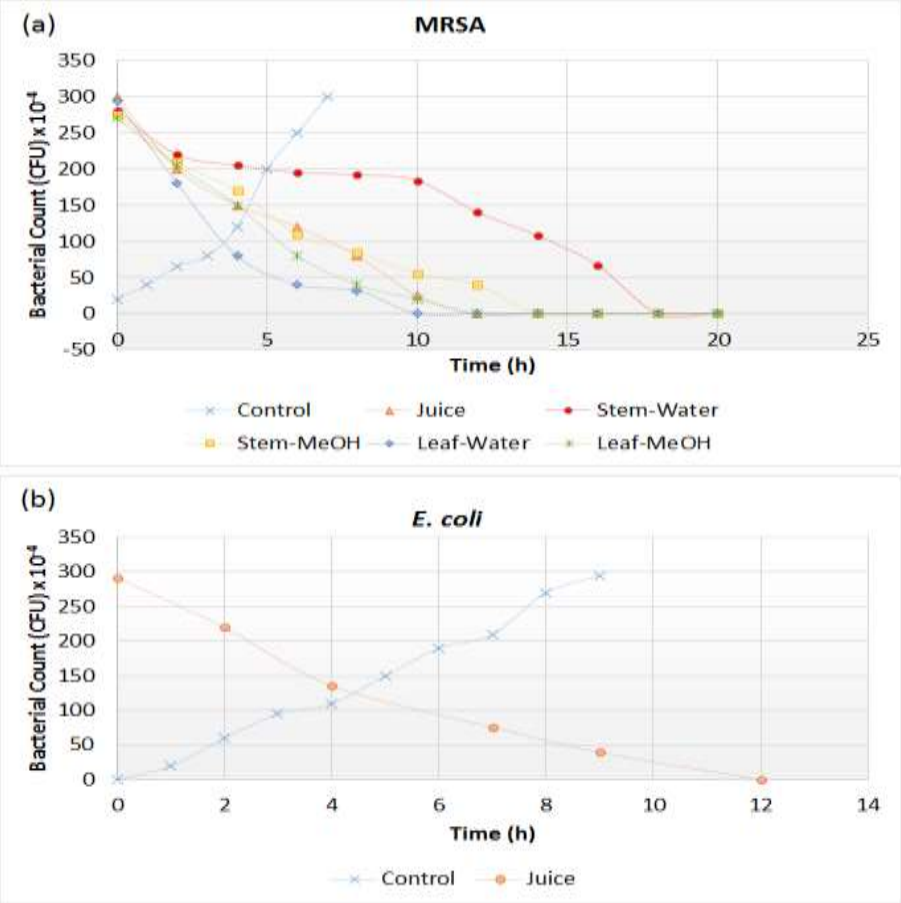

Fig.2: (a) Time kill-curve of MRSA treated with *R. canescens* DC. aqueous extracts, methanolic extracts, and fruit juice; (b) Time kill-curve of ESBL *E. coli* treated with juice. All assays were compared to a negative control.

Time-kill curves were plotted to assess the bactericidal activity of the *R. canescens* DC. extracts against the growth of MRSA and ESBL *E. coli*. Bacterial growth was monitored using the spread plate technique. The assay was done at concentration equivalent to 1xMBC for MRSA and ESBL *E. coli*. Untreated MRSA and ESBL *E. coli* were used as negative controls and showed an increase in absorbance with time (Figure 2). The antibacterial extracts tested in the present study were particularly effective with respect to the time needed to exert lethal effect on the microbial growth. Analysis of the Time-kill curves depicted in Figure 2a indicated that the active extracts needed 10-18 h to induce 100% killing of MRSA at the respective MBC. On the other hand, the Time-kill curve for the juice against ESBL *E. coli* (Figure 2b) showed that the extract needed 12 h to induce 100% killing of the microorganisms at the respective MBC.

Transmission electron microscopy (TEM) images were collected to test the effect of the *R. canescens* DC. extracts against MRSA and ESBL *E. coli*. Images of control (untreated) microorganisms and treated ones are depicted in Figures 2a-e (MRSA) and 2f-g (*E. coli*). The ultrastructure of treated bacterial cells displayed noticeable morphological changes. Figures 2b demonstrated that upon treatment of MRSA with methanolic leaf extract at the corresponding MIC, the cells exhibited morphological changes such as disintegration of protoplasm, vacuolization, deformation and shrinkage of cell wall, as well as condensation of DNA, all of which could have led to cell death. Ultrastructural analysis of MRSA treated with juice at the corresponding MIC (Figure 2c) revealed morphological alteration exhibited by the presence of ghost cells, cell wall rupture and shrinkage of cytoplasm leading to leakage of cellular content and eventual cell death. Moreover, loss of typical structural organization and structural integrity was observed.

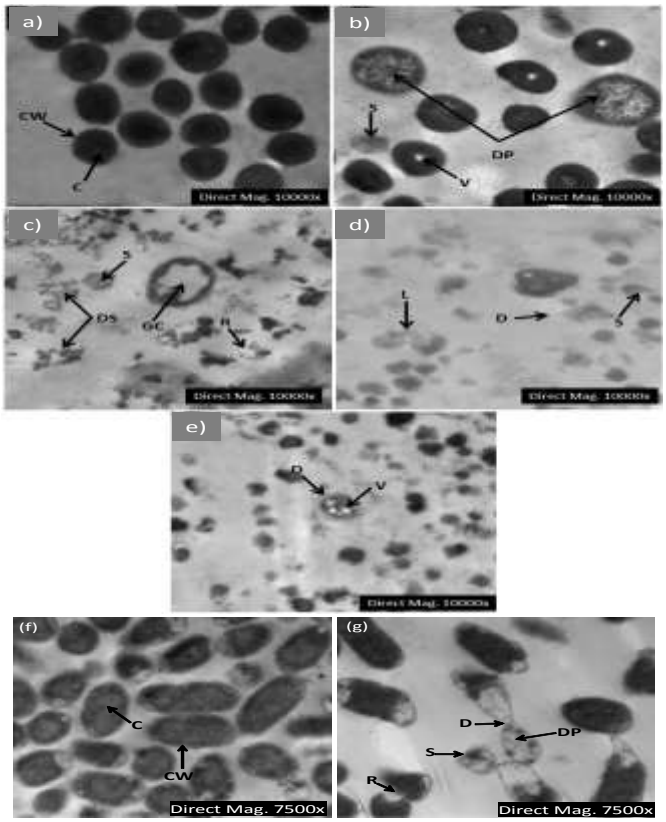

Fig.3: TEM of MRSA treated with methanolic leaf extract (b), juice (c), aqueous stem extract (d), and aqueous leaf extract (e), and compared to a control (a). (f-g) TEM of *E. coli* treated with juice (g) and compared to an untreated control (f). Scale: 100 nm. C: cytoplasm, CW: cell wall; D: damage; DP: disintegration of protoplasm; DS: disintegration of cells; GC: ghost cells; L: leakage of cellular content; R: rupture; S: shrinkage; V: vacuolization.

Microscopic observation revealed significant changes in the ultrastructure of MRSA treated with aqueous stem extract at the corresponding MIC (Figure 2d). The cells exhibited structural disorganization due to irreversible cell wall damage in addition to leakage of cytoplasm, severe ruptures as well as cellular shrinkage. When MRSA was treated aqueous leaf extract (Figure 2e), disruptions in cell shape, disintegration in protoplasm, and vacuolated cells were observed. Juice-induced morphological changes of *E. coli* were observed in figure 2g. The majority of cells displayed disintegration of protoplasm, cellular shrinkages and ruptures, and loss of integrity of nuclear material.

The phytochemical analysis presented in this study screened for saponins, terpenoids, flavonoids, anthraquinones, glycosides, steroids, triterpenoids, tanins, alkaloids, and total phenol. All extracts tested positive for flavonoids, terpenoids, anthraquinones, triterpenoids, and phenols. On the other hand, no extract tested positive for the presence of glycosides as assessed by the Keller-Killani test. Interestingly, the fruit juice did not test positive for tanins, alkaloids, saponins, and steroids. The methanolic and aqueous leaves and stem extracts tested positive for the chemicals under investigation except glycosides (Table 3). The detected components in *R. canescens* DC. are known to exhibit antimicrobial effects. For example, it has been reported that the presence of terpenes accounts for strong to moderate antibacterial activity against Gram-positive and Gram-negative bacteria [Oyedeji & Afolayan, 2005], while the presence of anthraquinones in all the tested extracts can add to the antimicrobial activity [Comini *et al*., 2010]. Flavonoids, which tested positive in all extracts, have demonstrated good antibacterial activity [Cushnie & Lamb, 2005], and tannins showed toxic effect against filamentous fungi, yeast, and bacteria [Sharma *et al*., 1999]. The total phenol content in the plant extracts was quantified spectrophotometrically using the Folin-Ciocalteu test. All extracts tested positive for phenols, thus contributing to the observed antibacterial activity [Lucchini *et al*., 1990]. Alkaloids, which can exert antibacterial activity through different mechanisms [Cushnie *et al*., 2014], was detected in all extracts except the fruit juice.

Table 3: Summary of the phytochemical analysis of *Rubus canescens* DC. extracts. (+) symbolizes the presence of constituent under test while (–) its absence.

Extract	Fruit juice	Leaves / H_2O	Stems / H_2O	Leaves / MeOH	Stems / MeOH
Flavonoids	+	+	+	+	+
Saponins	-	+	+	+	+
Terpenoids	+	+	+	+	+
Anthraquinones	+	+	+	+	+
Glycosides	-	-	-	-	-
Steroids	-	+	-	+	+
Triterpenoids	+	+	+	+	+
Tanins	-	+	+	+	+
Alkaloids	-	+	+	+	+
Phenols	+	+	+	+	+

4. CONCLUSIONS

The current study reports the first data on the antimicrobial activity of different parts of *R. canescens* DC., a thorny shrub widely distributed in Lebanon. The extracts were particularly bactericidal against MRSA and *E. coli*. The active extracts needed 10-18 h to induce 100% killing of the microorganisms under test. TEM images confirmed the formation of ghost cells in all test microorganisms. Such promising antibacterial activity opens new avenues for broader assessment of the pharmacological profile of the scarcely investigated *R. canescens* DC. For example, we recently reported the hepatoprotective and antioxidant activity of *R. canescens* DC. extracts [Mohammad *et al*., 2019].

HUMAN AND ANIMALS RIGHTS

No human or animal subjects were used in the experiments of the current study.

CONFLICT OF INTEREST

The authors declare no potential conflict of interest.

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