Research Article

Antibacterial Activity of the Extracts Obtained from *Rosmarinus officinalis*, *Origanum majorana*, and *Trigonella foenum-graecum* on Highly Drug-Resistant Gram Negative Bacilli

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Our aim was to determine the antimicrobial activity of three selected plants (*Rosmarinus officinalis*, *Origanum majorana*, and *Trigonella foenum-graecum*) against Extended Spectrum Beta Lactamase (ESBL)—producing *Escherichia coli* and *Klebsiella pneumoniae*— and to identify the specific plant fraction responsible for the antimicrobial activity. The plants were extracted with ethanol to yield the crude extract which was further subfractionated by different solvents to obtain the petroleum ether, the dichloromethane, the ethyl acetate, and the aqueous fractions. The Minimum Inhibitory Concentrations (MIC) and Minimum Bactericidal Concentrations (MBC) were determined using broth microdilution. The MICs ranged between 1.25 and 80 $\mu\text{g}/\mu\text{l}$. The majority of these microorganisms were inhibited by 80 and 40 $\mu\text{g}/\mu\text{l}$ of the crude extracts. The petroleum ether fraction of *Origanum majorana* significantly inhibited 94% of the tested strains. Ethyl acetate extracts of all selected plants exhibited relatively low MICs and could be therefore described as strong antibacterial.

1. Introduction

Disease causing bacteria have always been considered a major cause of morbidity and mortality in humans. The appearance of resistant microorganisms paved the way to the occurrence of infections that are only treated by a limited number of antimicrobial agents. The emergence of resistant Gram negative bacteria presents a major challenge for the antimicrobial therapy of infectious diseases and increases the incidence of mortality and morbidity. Bacterial resistance to antimicrobial agents is a medical problem with public health, socioeconomic, and even political implications [1]. Extended Spectrum Beta Lactamase (ESBL) producing bacteria are spread worldwide. Their prevalence in Lebanon is increasing through the years and their incidence depends on the region and environment [2, 3]. In view of the increase in ESBL resistance crisis and the negligible development of antibiotics

in the past few years, there is an urgent need for new antibacterial compounds in order to fight the emergence of these new resistant pathogens.

For centuries, plants have been used as remedies and treatments of diseases. The Middle Eastern Mediterranean region is rich in plant species; there are about 2,600 species of which many are considered to have medicinal effects. However, there is relatively limited research on medicinal plants in this region [4]. Plant-derived antimicrobials represent a vast untapped source for medicines, and further exploration of plant antimicrobials needs to occur [4]. In consequence, plants are starting to be considered as the base of modern medicine and antibiotic production [5].

Many studies have investigated the antimicrobial activity of different plant species in various geographical regions in search for new antibiotics. The use of plant derivatives as antimicrobials has not been extensively addressed until

TABLE 1: Phenotypic profiles of susceptibility of *Escherichia coli* and *Klebsiella pneumoniae* strains.

	Number of strains	AM	AMC	PIP	TZP	CF	CXM	FOX	CTX	CRO	CAZ	CEF	IMP	GN	AN	SXT	OF	CIP	TGC	Resistance profile
<i>E. coli</i>	5	R	R	R	S	R	R	S	R	R	R	R	S	S	S	S	S	S	S	ESBL+ QS
	5	R	R	R	S	R	R	R	R	R	R	R	S	R	S	R	R	R	S	ESBL+ QR AmpC +
	10	R	R	R	S	R	R	S	R	R	R	R	S	S	S	R	R	R	S	ESBL+ QR
<i>K. pneumoniae</i>	5	R	R	R	S	R	R	S	R	R	R	R	S	R	S	R	S	S	S	ESBL+ QS
	5	R	R	R	R	R	R	S	R	R	R	R	S	R	S	R	R	R	I	ESBL+ QR

AM: ampicillin, AMC: amoxicillin/clavulanic acid, PIP: piperacillin, TZP: piperacillin/tazobactam, CF: cephalotin, CXM: cefuroxime, FOX: cefoxitin, CTX: cefotaxime, CRO: ceftriaxone, CAZ: ceftazidime, CEF: cefepime, IMP: imipenem, GN: gentamicin, AN: amikacin, SXT: trimethoprim/sulfamethoxazole, OF: ofloxacin, CIP: ciprofloxacin, TGC: tigecycline QR: quinolone Resistant, QS: quinolone sensitive S: Sensitive, I: Intermediate, R: Resistant, AmpC +: Overproduction of AmpC, ESBL+: ESBL producer.

recently since most antibiotics were derived from bacterial or fungal origin. With the increase in resistance and the realization that the effective life span of any antibiotic is limited, new sources especially plant sources are currently being heavily investigated. Thousands of phytochemicals with antimicrobial activity have already been identified but they should be subjected to animal and human studies to study their toxicity and their effectiveness in whole organism systems. Several phytochemicals are already being studied in humans [6].

Rosmarinus officinalis (Rosemary), *Origanum majorana* (Marjoram), and *Trigonella foenum-graecum* (Fenugreek) have been known to have antimicrobial, antioxidant, antidiabetic, and antitumorigenic activities [7–10]. In addition, some of them are known to affect the cell's adhesive properties, self-aggregation, and protein secretion; and this might help in the treatment of cardiovascular diseases and thrombosis [10].

The aim of this study was to determine the antimicrobial activity of selected indigenous Lebanese plants (*Rosmarinus officinalis*, *Origanum majorana*, and *Trigonella foenum-graecum*) against microorganisms with high level of acquired resistance to traditional antibiotics (*Escherichia coli* and *Klebsiella pneumoniae* producing ESBL) and to identify the specific fraction/s responsible for the antimicrobial activity.

2. Materials and Methods

2.1. Bacterial Strains. Twenty strains of *Escherichia coli* and ten strains of *Klebsiella pneumoniae* were isolated at the clinical microbiology laboratory of the Saint George Hospital-University Medical Center, between December 2007 and May 2009. In addition to being ESBL producers, these isolates exhibited different profiles of resistance. In addition, four control strains were used throughout the experiments: these strains were *E. coli* 25922 (as beta lactamase negative strain) and *E. coli* ATCC 35218 (as beta lactamase positive strain), and 2 clinical isolates of *E. coli* and *Klebsiella pneumoniae* producers of CTXM-15 previously identified by our laboratory using molecular techniques were also included as positive controls for ESBL production.

2.2. Selected Plants. The herbal sample consisted of three different Lebanese plants: leaves of *Rosmarinus officinalis* (Rosemary), leaves of *Origanum majorana* (Marjoram), and seeds of *Trigonella foenum-graecum* (Fenugreek). They were collected directly from nature, identified, and characterized by a taxonomist. The name of the plant, time, place, and date of collection were recorded.

2.3. Antimicrobial Activity, ESBL, and AmpC Detection. The Antimicrobial Susceptibility Testing was performed as recommended by the Clinical and Laboratory Standards Institute (CLSI) [11]. The production of ESBL was detected phenotypically using the double disk synergy method described by Jarlier and Marty [12]. The strain showing a key hole effect between one or more of the third cephalosporin disks and the amoxicillin/clavulanic acid disk or showing a boost of the inhibition zone of one of the third generation cephalosporin disks toward the amoxicillin/clavulanic acid disk was considered as an ESBL producer. Another phenotypic method was used for the detection of overproduction of a class C beta-lactamase AmpC. This method relied on the resistance to cefoxitin being a major difference between ESBL producers (susceptible to cefoxitin) and AmpC overproducers (resistant to cefoxitin). The antimicrobial agents that were tested were ampicillin, piperacillin, imipenem, amoxicillin/clavulanic acid, piperacillin/tazobactam, cephalotin, cefoxitin, cefuroxime, ceftriaxone, ceftazidime, cefepime, gentamicin, ciprofloxacin, ofloxacin, tigecycline, and trimethoprim/sulfamethoxazole. The breakpoints for the different antibacterial agents recommended by the CLSI were used. Since tigecycline has no CLSI breakpoints, the (Comité de l'Antibiogramme-Société Française de Microbiologie) CA-SFM guidelines [13] were adopted for this antibiotic as an alternative (Diameter < 19 mm for Resistance). Although resistance to cephamycins cannot be a confirmatory test for AmpC overproduction and might be conferred sometimes by ESBLs, resistance to cephamycin was looked at as an indicator for AmpC overproduction since this is true in the majority of the cases.

2.4. Preparation of Crude Extract. Fresh plants were dried in the shade at room temperature and ground in a coffee

TABLE 2: MICs and MBCs of the different fractions of *Rosmarinus officinalis* on *Escherichia coli* and *Klebsiella pneumoniae*.

Bacterial strain	Crude ($\mu\text{g}/\mu\text{l}$)		Petroleum ether ($\mu\text{g}/\mu\text{l}$)		Dichloromethane ($\mu\text{g}/\mu\text{l}$)		Ethyl acetate ($\mu\text{g}/\mu\text{l}$)		Aqueous ($\mu\text{g}/\mu\text{l}$)	
	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC
Ec001SGH	80	80	X	X	ND	ND	10	10	80	80
Ec002SGH	20	20	X	X	ND	ND	2.5	5	40	80
Ec003SGH	10	10	X	X	ND	ND	2.5	5	20	20
Ec004SGH	40	40	X	X	ND	ND	5	10	40	80
Ec007SGH	80	80	X	X	ND	ND	10	10	80	ND
Ec010SGH	40	40	X	X	ND	ND	5	5	80	80
Ec011SGH	40	40	X	X	ND	ND	5	5	40	80
Ec012SGH	40	40	X	X	ND	ND	5	10	40	40
Ec013SGH	20	20	X	X	ND	ND	10	10	40	80
Ec016SGH	10	20	X	X	ND	ND	2.5	2.5	20	20
Ec017SGH	40	40	X	X	ND	ND	2.5	2.5	5	5
Ec018SGH	20	20	X	X	ND	ND	5	10	80	80
Ec019SGH	10	20	X	X	ND	ND	2.5	2.5	40	40
Ec020SGH	20	20	X	X	ND	ND	2.5	5	40	80
Ec021SGH	20	20	X	X	ND	ND	2.5	2.5	40	40
Ec023SGH	40	40	X	X	ND	ND	5	10	40	80
Ec026SGH	20	40	X	X	ND	ND	2.5	5	80	80
Ec030SGH	40	40	X	X	ND	ND	5	10	40	80
Ec031SGH	20	20	X	X	ND	ND	5	5	40	80
Ec032SGH	10	20	X	X	ND	ND	2.5	5	40	80
Control	ND	ND	X	X	ND	ND	ND	ND	ND	ND
MIC ₉₀	44		X		N/A		10		80	
Kp001SGH	20	20	X	X	ND	ND	2.5	2.5	40	80
Kp002SGH	20	20	X	X	ND	ND	5	5	80	80
Kp001SGH	20	20	X	X	ND	ND	2.5	2.5	40	80
Kp002SGH	20	20	X	X	ND	ND	5	5	80	80
Kp005SGH	10	20	X	X	ND	ND	5	5	20	20
Kp006SGH	20	20	X	X	ND	ND	10	10	80	ND
Kp007SGH	20	20	X	X	ND	ND	5	5	80	80
Kp008SGH	40	40	X	X	ND	ND	5	5	40	80
Kp009SGH	40	40	X	X	ND	ND	5	5	80	80
Kp010SGH	40	40	X	X	ND	ND	10	10	80	80
Kp013SGH	40	40	X	X	ND	ND	20	20	80	80
Kp016SGH	40	80	X	X	ND	ND	2.5	2.5	80	80
Control	ND	ND			ND	ND	ND	ND	ND	ND
MIC ₉₀	40		X		N/A		10		80	

MIC: Minimum Inhibitory Concentration, MBC: Minimum Bactericidal Concentration, ND: Not Detected, X: missing extract, μg : microgram, μl : microliter, N/A: Not Applicable.

bean grinder. The dried plant material was weighed and then soaked in 80% ethanol for 7 days with continuous shaking in a shaker at room temperature. At day seven, the plant material was filtered and the filtrate collected. This was repeated and the filtrates were combined and concentrated in a rotary evaporator to obtain the crude extract (fraction 1).

2.5. Fractionation Method. The crude extract of each plant was further partitioned by extraction with different solvents in a 1:1 (v/v) ratio in order to subfractionate the plant

components according to their polarity: petroleum ether (fraction 2), dichloromethane (fraction 3), and ethyl acetate (fraction 4). Extractions were repeated three times and fractions were combined. The remaining aqueous layer was collected as fraction number 5. Fractions 1 and 5 were dried using a freeze dryer, but fractions 2, 3, and 4 were dried under the hood to dryness due to the inconvenience of introducing vapor solvent into the freeze dryer. Controls were prepared for each fraction by drying the same amount of solvent and following the same subfractionation method without plant extract (solvent control).

TABLE 3: MICs and MBCs of the different fractions of *Origanum majorana* on *Escherichia coli* and *Klebsiella pneumoniae*.

Bacterial strain	Crude ($\mu\text{g}/\mu\text{l}$)		Petroleum ether ($\mu\text{g}/\mu\text{l}$)		Dichloromethane ($\mu\text{g}/\mu\text{l}$)		Ethyl acetate ($\mu\text{g}/\mu\text{l}$)		Aqueous ($\mu\text{g}/\mu\text{l}$)	
	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC
Ec001SGH	80	80	X	X	X	X	2.5	5	80	80
Ec002SGH	80	ND	5	5	10	10	5	5	80	80
Ec003SGH	80	80	5	5	5	10	5	5	80	ND
Ec004SGH	80	80	X	X	5	5	2.5	5	80	ND
Ec007SGH	ND	ND	10	10	20	20	20	20	ND	ND
Ec010SGH	40	40	2.5	2.5	5	10	5	5	80	80
Ec011SGH	80	80	X	X	2.5	5	2.5	5	80	ND
Ec012SGH	80	ND	X	X	5	5	5	5	80	80
Ec013SGH	40	80	X	X	10	10	5	5	80	ND
Ec016SGH	80	80	1.25	1.25	5	5	2.5	2.5	40	40
Ec017SGH	20	40	10	10	10	10	2.5	2.5	20	20
Ec018SGH	40	40	2.5	2.5	10	10	5	5	ND	ND
Ec019SGH	ND	ND	ND	ND	ND	ND	2.5	5	80	80
Ec020SGH	80	80	5	5	ND	ND	5	5	80	80
Ec021SGH	40	80	X	X	ND	ND	5	5	80	80
Ec023SGH	ND	ND	X	X	ND	ND	5	5	80	80
Ec026SGH	40	80	2.5	2.5	5	10	5	5	80	ND
Ec030SGH	80	80	X	X	X	X	5	5	80	ND
Ec031SGH	40	80	2.5	5	10	10	5	5	80	80
Ec032SGH	80	80	1.25	1.25	5	10	2.5	5	80	ND
Control	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
MIC ₉₀	80		N/A		N/A		5		80	
Kp001SGH	ND	ND	2.5	5	10	10	5	10	ND	ND
Kp002SGH	80	80	2.5	2.5	5	10	5	5	80	ND
Kp005SGH	ND	ND	1.25	2.5	10	20	5	5	80	ND
Kp006SGH	40	80	10	10	10	10	20	20	ND	ND
Kp007SGH	80	80	1.25	1.25	20	20	5	10	ND	ND
Kp008SGH	ND	ND	X	X	ND	ND	5	10	80	ND
Kp009SGH	ND	ND	5	10	10	20	5	5	80	ND
Kp010SGH	80	80	X	X	X	X	5	10	80	ND
Kp013SGH	ND	ND	X	X	X	X	2.5	2.5	80	ND
Kp016SGH	ND	ND	5	10	20	ND	10	10	80	80
Control	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
MIC ₉₀	80		N/A		N/A		11		80	

MIC: Minimum Inhibitory Concentration, MBC: Minimum Bactericidal Concentration, ND: Not Detected, X: missing extract, μg : microgram, μl : microliter; N/A: Not Applicable.

2.6. Study of Antimicrobial Activity of the Plant Extracts.

The plant powders were weighed and dissolved in sterile distilled water. The solutions were filtered through 0.22 μm sterile filter membranes and stored at 4°C for further use. The concentration of the original solution of the plant extract/fraction corresponds to the concentration obtained after resuspension of the dried plant extracts. This was used as the stock solution and the most concentrated one from which the Minimum Inhibitory Concentration MIC series were prepared.

2.7. Determination of the Inhibitory and Bactericidal Concentrations. The Microdilution Broth Method was used for the determination of the MIC of plant extracts as recommended

by the Clinical and Laboratory Standards Institute [14]. Broth (100 μl) were dispensed in each well of a sterile microdilution tray. An appropriate volume of plant extract suspension was added to the first tube in each series (after removing the same volume of broth) in order to achieve the desired concentration after the addition of the bacterial inoculum. A standardized bacterial inoculum was prepared and adjusted to 0.5 McFarland and then diluted to 10⁶ CFU/ml. Within 15 minutes, the wells were inoculated with 100 μl of this inoculum resulting in a 1 : 2 concentration of the content of the well in plant extract and of the bacterial suspension (5 × 10⁵ CFU/ml). A routine bacterial count was performed in duplicates to verify the bacterial concentration. Positive and negative control wells were used. The negative

TABLE 4: MICs and MBCs of the different fractions of *Trigonella foenum-graecum* on *Escherichia coli* and *Klebsiella pneumoniae*.

Bacteria	Crude ($\mu\text{g}/\mu\text{l}$)		Petroleum ether ($\mu\text{g}/\mu\text{l}$)		Dichloromethane ($\mu\text{g}/\mu\text{l}$)		Ethyl acetate ($\mu\text{g}/\mu\text{l}$)		Aqueous ($\mu\text{g}/\mu\text{l}$)	
	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC
Ec001SGH	ND	ND	ND	ND	ND	ND	X	X	20	ND
Ec002SGH	ND	ND	ND	ND	ND	ND	X	X	ND	ND
Ec003SGH	ND	ND	ND	ND	ND	ND	X	X	20	ND
Ec004SGH	ND	ND	ND	ND	ND	ND	X	X	20	ND
Ec007SGH	ND	ND	ND	ND	ND	ND	X	X	20	ND
Ec010SGH	ND	ND	ND	ND	ND	ND	X	X	20	ND
Ec011SGH	ND	ND	ND	ND	ND	ND	X	X	20	ND
Ec012SGH	ND	ND	ND	ND	ND	ND	X	X	20	ND
Ec013SGH	ND	ND	ND	ND	ND	ND	X	X	20	ND
Ec016SGH	ND	ND	ND	ND	ND	ND	X	X	20	ND
Ec017SGH	ND	ND	ND	ND	ND	ND	X	X	ND	ND
Ec018SGH	ND	ND	ND	ND	ND	ND	X	X	ND	ND
Ec019SGH	ND	ND	ND	ND	ND	ND	X	X	20	ND
Ec020SGH	ND	ND	ND	ND	ND	ND	X	X	20	ND
Ec021SGH	ND	ND	ND	ND	ND	ND	X	X	10	10
Ec023SGH	ND	ND	ND	ND	ND	ND	X	X	ND	ND
Ec026SGH	ND	ND	ND	ND	ND	ND	X	X	20	ND
Ec030SGH	ND	ND	ND	ND	ND	ND	X	X	20	ND
Ec031SGH	ND	ND	ND	ND	ND	ND	X	X	20	
Ec032SGH	ND	ND	ND	ND	ND	ND	X	X	20	ND
Control	ND	ND	ND	ND	ND	ND	X	X	ND	ND
MIC ₉₀	N/A		N/A		N/A		X		20	
Kp001SGH	ND	ND	ND	ND	ND	ND	X	X	ND	ND
Kp002SGH	ND	ND	ND	ND	ND	ND	X	X	20	ND
Kp005SGH	ND	ND	ND	ND	ND	ND	X	X	20	ND
Kp006SGH	ND	ND	ND	ND	ND	ND	X	X	ND	ND
Kp007SGH	ND	ND	ND	ND	ND	ND	X	X	20	ND
Kp008SGH	ND	ND	ND	ND	ND	ND	X	X	20	ND
Kp009SGH	ND	ND	ND	ND	ND	ND	X	X	20	ND
Kp010SGH	ND	ND	ND	ND	ND	ND	X	X	20	ND
Kp013SGH	ND	ND	ND	ND	ND	ND	X	X	ND	ND
Kp016SGH	ND	ND	ND	ND	ND	ND	X	X	20	ND
Control	ND	ND	ND	ND	ND	ND	X	X	ND	ND
MIC ₉₀	N/A		N/A		N/A		X		N/A	

MIC: Minimum Inhibitory Concentration, MBC: Minimum Bactericidal Concentration, ND: Not Detected, X: missing extract, μg : microgram, μl : microliter; N/A: Not Applicable.

control well consisted of 200 μl of Nueller Hinton Broth (MHB); the positive well consisted of 200 μl MHB with a bacterial suspension but without a plant extract. The tray was incubated at 35°C for 18–24 hours after which the MIC was recorded as the highest dilution of each plant extract that still retained an inhibitory effect resulting in no visible growth or in other terms absence of turbidity observed with the naked eye. The Minimum Bactericidal Concentration (MBC) was determined by subculturing samples from the tubes with concentrations above the MIC on new plates of Mueller Hinton Agar (MHA). The MBC corresponded to the lowest concentration of the extract associated with no bacterial culture.

All experiments were performed three independent times in duplicate form. The MIC₉₀ is defined as the Minimum Inhibitory Concentration required to inhibit the growth of 90% of organisms; it was calculated as the percentile below which 90% of the individual MICs values fall. In view of the relatively small population of tested bacteria, it was not advantageous to calculate MIC₅₀.

3. Results

3.1. Resistance Phenotypes of the Tested Strains. As shown in Table 1, the patterns of resistance of the tested strains could

be divided into four categories:

- (i) ESBL positive, Quinolone resistant, with no overproduction of AmpC,
- (ii) ESBL positive, Quinolone susceptible, with no overproduction of AmpC,
- (iii) ESBL positive, Quinolone resistant, with overproduction of AmpC,
- (iv) ESBL positive, Quinolone susceptible, with overproduction of AmpC,

3.2. Inhibitory and Bactericidal Activities of the Plant Fractions

3.2.1. Antimicrobial Activity of *Rosmarinus officinalis*. *Rosmarinus officinalis* exerted both inhibitory and bactericidal effects on *Escherichia coli* and *Klebsiella pneumoniae*. These effects were observed with the crude extract, the ethyl acetate, and the aqueous fractions. Dichloromethane fraction did not show any inhibitory effect within the tested concentrations. The best inhibitory activity represented by the lowest MIC₉₀ was observed with the ethyl acetate fraction at 10 µg/µl for *Escherichia coli* and *Klebsiella pneumoniae* (Table 2). The lowest MIC (2.5 µg/µl) was recorded for the ethyl acetate fraction with *Escherichia coli* and *Klebsiella pneumoniae* (Table 2). This concentration inhibited 40% of the tested bacteria. The ethyl acetate fraction exhibited as well a bactericidal activity on the majority of the strains at concentrations between 2.5 and 5 µg/µl. The crude and the aqueous fractions inhibited most of the strains at a concentration of 40 µg/µl, the crude extract exhibited a bactericidal effect on 16 strains at 20 µg/µl, and the aqueous fraction exerted a bactericidal effect on 23 strains at 80 µg/µl. The solvents' controls that were systematically run for all solvents did not exert any antibacterial activity. Bacterial growth was observed for the positive controls while no growth was observed for the negative controls.

3.2.2. Antimicrobial Activity of *Origanum majorana*. Both inhibitory and bactericidal effects of *Origanum majorana* on *Escherichia coli* and *Klebsiella pneumoniae* were observed with the crude extract of the plant, the petroleum ether, the dichloromethane, the ethyl acetate, and the aqueous fractions. MIC₉₀ was not always easy to calculate in view of the unavailability of enough extract or the very high concentration needed to achieve an inhibitory effect. The lowest MIC₉₀ (5 µg/µl) was observed with the ethyl acetate fraction for *Escherichia coli* and at 11 µg/µl for *Klebsiella pneumoniae* (Table 3). While the lowest MIC (1.25 µg/µl) was recorded for the petroleum ether fraction with *Escherichia coli* and *Klebsiella pneumoniae*, the MIC and MBC effects were detected within 1 dilution, which suggested simultaneous inhibitory and bactericidal activity and a bactericidal nature of the compound. The concentrations, at which most of the bacterial suspensions were cleared, were 80 µg/µl for the crude extract and the aqueous fraction, 5 µg/µl for the petroleum ether fraction, 10 µg/µl for the dichloromethane fraction, and 5 µg/µl for the ethyl acetate fraction. Bacterial

growth was observed for the positive controls while no growth was observed for the negative controls.

3.2.3. Antimicrobial Activity of *Trigonella foenum-graecum*. The inhibitory effects of *Trigonella foenum-graecum* on *Escherichia coli* and *Klebsiella pneumoniae* (Table 4) were only observed with the aqueous fraction. Crude extract, petroleum ether, and dichloromethane fractions did not show any inhibitory effect within the tested concentrations. The ethyl acetate fraction could not be tested since the amount obtained was not enough. The aqueous fraction inhibited 23 out of 30 strains at 20 µg/µl. However, the lowest MIC was recorded at 10 µg/µl with Ec021SGH. In addition, bactericidal activity was only observed with this strain at 10 µg/µl. The solvents' controls did not exert any antibacterial activity. Bacterial growth was observed for the positive controls while no growth was observed for the negative controls.

4. Discussion

Production of Extended Spectrum Beta Lactamase enzymes emerged in Gram negative bacteria and caused the infections to become more difficult to treat in view of their resistance to a wide range of antibiotics [14].

Rosmarinus officinalis [15], *Origanum majorana* [16], and *Trigonella foenum-graecum* [16] were traditionally used for the treatment of several illnesses such as urinary tract infections, rheumatoid cholecystitis, diarrhea, and hypertension. Their antimicrobial potential was tested against a variety of bacteria and was shown to exert variable activities [8, 17–19]. In view of the important spread of the ESBL producers in Lebanon [3, 20], it was important to investigate whether these indigenous plants have any antibacterial activity against ESBL producing *Escherichia coli* and *Klebsiella pneumoniae* [21].

The present study showed that different extracts/fractions exhibited antimicrobial activity against ESBL producing *Escherichia coli* and *Klebsiella pneumoniae*. The crude extracts, except those of *Trigonella foenum-graecum*, the ethyl acetate, and the aqueous fractions of all the plants exhibited an inhibitory effect. Contrary to *Rosmarinus officinalis* and *Trigonella foenum-graecum*, the petroleum ether fraction of *Origanum majorana* showed potent inhibitory effect against the tested strains. In most of the cases, inhibitory and bactericidal effects were detected by the same concentrations. The lowest MIC was recorded with petroleum ether fraction of *Origanum majorana* at 1.25 µg/µl although the petroleum ether fractions were not associated with high antibacterial activity through the study.

Our results show that all plant extracts, except *Rosmarinus officinalis*, exhibited more pronounced antibacterial activity on *Escherichia coli* than on *Klebsiella pneumoniae*. This phenomenon was also observed by Safary et al. [22] who showed that *Quercus brantii* 80% ethanol extract exhibited antibacterial activity against some tested Gram negative bacteria but not against *Klebsiella pneumoniae*. This difference may be correlated to the presence of a capsule

around *Klebsiella pneumoniae*. In the present study, the MIC results varied between 1.25 and 80 $\mu\text{g}/\mu\text{l}$. Some MICs of the same extracts varied against the different tested strains, although some of the tested strains had the same antimicrobial susceptibility patterns. In their investigation, Ahmad and Aqil [23] postulated that the presence of different intrinsic levels of tolerance to antimicrobials in the tested microorganisms caused the variation of the MIC values among the isolates with relatively similar antimicrobial susceptibility patterns.

The low activity of the crude extracts against the tested *Escherichia coli* and *Klebsiella pneumoniae* suggests either that the crude extracts held very low concentration of active antibacterial compounds or that the crude extract contained compounds that inhibited the antibacterial activity of the effective compounds. Petroleum ether fractions, except for, *Origanum majorana*, did not show significant inhibitory effect. This could be due to the fact that the plants did not contain enough secondary compounds with active antibacterial activity against these pathogens extractable with petroleum ether or that these compounds do not exhibit antibacterial activity.

All aqueous extracts exerted antimicrobial activity against the majority of the tested strains. This antimicrobial activity was moderately low, which may possibly be related to the fact that most of the secondary metabolites were extracted either by petroleum ether, dichloromethane, or ethyl acetate solvents.

Extraction of secondary metabolites highly depends on using extractory techniques that depend on the chemical properties of these compounds. Water-soluble compounds and proteins can be extracted in water or polar solvents whereas water insoluble compounds can be extracted with organic solvents [24].

The crude extract of each plant was partitioned by extraction with different solvents in order to subfractionate the plant components according to their polarity. Solvents were applied starting by the least polar to the more polar. These solvents were selected in order to extract compounds with different polarities. Petroleum ether is known to extract the nonpolar metabolites. Dichloromethane is known to extract compounds with medium polarity, and ethyl acetate is known to extract the polar compounds [25, 26].

Since most of the identified MICs, especially MICs of the crude extract and aqueous fraction, consisted of the highest concentrations tested, and since the MBC would normally be the concentrations of higher dilutions, some of the MBCs were not determined such as with *Trigonella foenum-graecum* fractions. Moreover, some MICs and MBCs were found to be in the same tube at the same concentration. One possible explanation is that the MIC might have matched with the concentration found between the two consecutive tubes showing bactericidal effect and turbidity concentration, respectively.

Our findings reported here show that different extracts of *Rosmarinus officinalis*, *Origanum majorana*, and *Trigonella foenum-graecum* inhibited the growth of ESBL producing *Escherichia coli* and *Klebsiella pneumoniae* at different rates. However, the toxic effects of plant extracts were not explored

or tested in this work. The selective toxicity of an antimicrobial agent on eukaryotic cells is crucial and would impact on the usefulness of this extract as a medicinal compound. Antibacterial extracts that are toxic on human cells may be useful as nonmedicinal antimicrobial agents, such as surface disinfectants. In addition, purification and identification of the bioactive components is needed to examine the mechanisms of action of these agents especially that these mechanisms probably differ from those of the commonly used antibiotics.

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