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ANTIBACTERIAL ACTIVITY OF *RHEUM RHAPONTICUM*, *OLEA EUROPAEA*, AND *VIOLA ODORATA* ON ESBL PRODUCING CLINICAL ISOLATES OF *ESCHERICHIA COLI* AND *KLEBSIELLA PNEUMONIAE*

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ABSTRACT

The aim of this study was to determine the antimicrobial activity of three selected Lebanese plants (*Rheum rhaponticum*, *Olea europaea*, and *Viola Odorata)* 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 2.5 and 80 µg/µl. The majority of these microorganisms were inhibited by 80 and 40 µg/µl of the crude extracts. The dichloromethane fraction of *Olea europea* exerted a significant inhibitory effect on 90% of the tested strains. Ethyl acetate extracts of all selected plants presented antibacterial activity with high potency. Aqueous extracts of *Rheum rhaponticum* and *Olea europaea* exerted antimicrobial activity against the majority of the tested strains while *Viola Odorata's* aqueous extract showed less activity. This study constitutes a good example for the screening of antimicrobial activities of plants on highly resistant organisms of clinical importance; however, toxicity of these extracts needs more investigation.

INTRODUCTION: Pathogenic bacteria constitute a major cause of morbidity and mortality in humans. The emergence and spread of bacterial resistance has made the treatment of infectious diseases more problematic. In this context, resistance in Gram negative bacteria presents a major challenge for the antimicrobial therapy and significantly narrows the treatment options of human infections [1]. Extended Spectrum β-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, 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. Plants have been used as remedies and treatments of diseases; there is evidence that they were used in ethnomedicine 60,000 years ago in Iraq [4, 5].

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 [5]. Plant derived antimicrobials represent a vast untapped source for medicines and further exploration of plant antimicrobials needs to occur [5]. In consequence, plants are starting to be considered as the base of modern medicine and antibiotic production [6].

The antimicrobial activity of a plant is highly related to secondary substances that are synthesized and produced by these plants [7]. Secondary metabolites are substances of low molecular weight, which were not products of the primary metabolic pathway of the producing organism and at first thought to be with no advantage to the plant. Nowadays it is believed that they have vital functions. They may act as messenger molecules under specific circumstances (e.g. against the aggression) or natural pressures in order to protect the producer organism. They also give plants their pigment and odors [7].

Rheum rhaponticum, *Olea europaea*, and *Viola Odorata* have been known to have antimicrobial, antitumor, anti-hypertensive, vasodilator, and anti-arrhythmic properties [8-11].

The aim of this study was to determine the antimicrobial activity of selected indigenous Lebanese plants (*Rheum rhaponticum*, *Olea europaea*, and *Viola Odorata)* against microorganisms with high level of acquired resistance to traditional antibiotics (*Escherichia coli* and *Klebsiella pneumoniae* producing Extended Spectrum Beta Lactamases) and to identify the specific fraction/s responsible for the antimicrobial activity.

MATERIALS AND METHODS:

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.

Selected Plants: The herbal sample consisted of 3 different indigenous Lebanese plants: *Rheum rhaponticum (roots)*, *Olea europaea (*leaves and stems), and *Viola Odorata (leaves).* They were collected from different Lebanese areas (Broumana, Ghazir, Jbeil, and Becharreh) and directly from nature. They were identified and characterized by a taxonomist. The name of the plant, time, place and date of collection were recorded.

Antimicrobial activity, ESBL and AmpC Detection: The Antimicrobial Susceptibility Testing was performed as recommended by the Clinical and Laboratory Standards Institute [12, 13]. The production of ESBL was detected phenotypically using the double disk synergy method described previously [14]. 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.

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 SFM guidelines were adopted for this antibiotic as alternative (Diameter<19 mm for Resistance). Although resistance to cephamycins cannot be a confirmatory test for AmpC production and might be conferred sometimes by ESBLs, resistance to cephamycin was looked at as an indicator for AmpC production since this is true in the majority of the cases.

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

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 sub-fractionate 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 sub-fractionation method without plant extract (solvent control).

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^{o}C for further use. The concentration of the original solution of the plant extract/fraction corresponds to the concentration obtained after re-suspension of the dried plant extracts. This was used as the stock solution and the most concentrated one from which the MIC series were prepared.

MIC and MBC Determination: The Microdilution Broth Method was used for the determination of the MIC of plant extracts as recommended by the Clinical and Laboratory Standards Institute [12, 13]. Broth (100 µl) was 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 10^6 CFU/ml. Within 15 minutes, the wells were inoculated with 100µl of this inoculum resulting in a 1:2 concentration of the content of the well in plant extract and of the bacterial suspension ($5x10^5$ CFU/ml). A routine bacterial count was performed in duplicates to verify the bacterial concentration. Positive and negative control wells were used. The negative control well consisted of 200 µl of 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 MBC was determined by sub-culturing samples from the tubes with concentrations above the MIC on new plates of 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 MIC_{90} 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 MIC_{50}.

RESULTS:

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:

- AmpC negative Quinolone resistant ESBL producers
- AmpC negative Quinolone susceptible ESBL producers
- AmpC positive Quinolone resistant ESBL producers
- AmpC positive Quinolone susceptible ESBL producers

TABLE 1: PHENOTYPIC PROFILES OF SUSCEPTIBILITY OF *ESCHERICHIA COLI* AND *KLEBSIELLA PNEUMONIAE* STRAINS

Strain	AM	AMC	PIP	TZP	CF	CXM	FOX	CTX	CRO	CAZ	CEF	IMP	GN	AN	SXT	OF	CIP	TGC	Group
Ec001SGH	R	R	R	S	R	R	S	R	R	R	R	S	S	S	S	S	S	S	ESBL+ QS
Ec002SGH	R	R	R	S	R	R	S	R	R	R	R	S	S	S	S	S	S	S	ESBL+ QS
Ec003SGH	R	R	R	S	R	R	R	R	R	R	R	S	R	S	R	R	R	S	ESBL+ QR AmpC +
Ec004SGH	R	R	R	S	R	R	R	R	R	R	R	S	R	S	R	R	R	S	ESBL+ QR AmpC +
Ec007SGH	R	R	R	S	R	R	S	R	R	R	R	S	S	S	R	R	R	S	ESBL+ QR
Ec010SGH	R	R	R	S	R	R	S	R	R	R	R	S	S	S	S	R	R	S	ESBL+ QR
Ec011SGH	R	R	R	S	R	R	S	R	R	R	R	S	R	S	R	R	R	S	ESBL+ QR
Ec012SGH	R	R	R	S	R	R	S	R	R	R	R	S	R	S	R	R	R	S	ESBL+ QR
Ec013SGH	R	R	R	S	R	R	S	R	R	R	R	S	R	S	R	S	S	S	ESBL+ QS
Ec016SGH	R	R	R	R	R	R	R	R	R	R	R	S	R	S	R	R	R	S	ESBL+ QR AmpC +
Ec017SGH	R	R	R	S	R	R	R	R	R	R	R	S	R	R	S	S	S	S	ESBL+ QS AmpC +
Ec018SGH	R	R	R	S	R	R	R	R	R	R	R	S	R	S	R	R	R	S	ESBL+ QR AmpC +
Ec019SGH	R	R	R	S	R	R	S	R	R	R	R	S	R	S	R	R	R	S	ESBL+ QR
EC020SGH	R	R	R	S	R	R	S	R	R	R	R	S	S	S	R	R	R	S	ESBL+ QR
EC021SGH	R	R	R	S	R	R	S	R	R	R	R	S	S	S	R	R	R	S	ESBL+ QR
Ec023SGH	R	R	R	S	R	R	S	R	R	R	R	S	S	S	R	R	R	S	ESBL+ QR
Ec026SGH	R	R	R	S	R	R	S	R	R	R	R	S	R	S	R	S	S	S	ESBL+ QS
Ec030SGH	R	R	R	S	R	R	S	R	R	R	R	S	R	S	R	R	R	S	ESBL+ QR
EC031SGH	R	R	R	S	R	R	S	R	R	R	R	S	R	S	R	R	R	S	ESBL+ QR
Ec032SGH	R	R	R	S	R	R	S	R	R	R	R	S	S	S	S	S	S	S	ESBL+ QS
Kp001SGH	R	R	R	S	R	R	S	R	R	R	R	S	R	S	R	S	S	S	ESBL+ QS
Kp002SGH	R	R	R	S	R	R	S	R	R	R	R	S	R	S	R	S	S	S	ESBL+ QS
Kp005SGH	R	R	R	S	R	R	S	R	R	R	R	S	R	S	R	S	S	S	ESBL+ QS
Kp006SGH	R	R	R	S	R	R	S	R	R	R	R	S	R	S	R	S	S	S	ESBL+ QS
Kp007SGH	R	R	R	R	R	R	S	R	R	R	R	S	R	S	R	R	R	I	ESBL+ QR
Kp008SGH	R	R	R	S	R	R	S	R	R	R	R	S	R	S	R	R	R	S	ESBL+ QR
Kp009SGH	R	R	R	R	R	R	S	R	R	R	R	S	R	S	R	R	R	S	ESBL+ QR
Kp010SGH	R	R	R	S	R	R	S	R	R	R	R	S	S	S	R	R	R	S	ESBL+ QR
Kp013SGH	R	R	R	S	R	R	S	R	R	R	R	S	S	S	R	S	S	S	ESBL+ QS
Kp016SGH	R	R	R	S	R	R	S	R	R	R	R	S	R	S	S	R	R	S	ESBL+ QR

Ec: *Escherichia coli*, Kp: *Klebsiella pneumoniae,* 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/sulfamethoxazol, OF: ofloxacin, CIP: ciprofloxacin, TGC: tigecycline QR: quinolone Resistant, QS: quinolone sensitive S: Sensitive, I: Intermediate, R: Resistant; AmpC +: naturally occurring Cephalsporinase; ESBL+: ESBL producer

Inhibitory and Bactericidal Activities of the Plant Fractions:

Antimicrobial activity of *Rheum rhaponticum*: Both inhibitory and bactericidal effects of *Rheum rhaponticum* on *Escherichia coli* and *Klebsiella pneumoniae* were mainly observed with the crude extract of the plant, the ethyl acetate and the aqueous fractions. The best inhibitory activity, represented by the lowest MIC_{90} for both *E. coli* and *K. pneumoniae* (22µg/µl), was observed with the ethyl acetate fraction

(**Table 2**). The lowest MIC was recorded for the crude extract and ethyl acetate fraction with *Escherichia coli* at 5 µg/µl. The inhibitory and bactericidal effects of the dichloromethane fraction were not detected except for two strains of *Escherichia coli* and one *Klebsiella pneumoniae* (Table 2) with an of MIC 20 µg/µl. The Petroleum ether fraction did not show any detectable inhibitory effect within the tested concentrations.

It is worthy to note that the aqueous fraction that showed good antibacterial activity against *Escherichia coli* 2 exhibited very low activity on *Klebsiella pneumoniae* (Table 2). The MIC and MBC effects were detected within 1 dilution, which indicates simultaneous inhibitory and bactericidal activities.

TABLE 2: MICS AND MBCS OF THE DIFFERENT FRACTIONS OF *RHEUM RHAPONTICUM ON ESCHERICHIA COLI* AND *KLEBSIELLA PNEUMONIAE*

Bacterial strain	Crude (µg/µl)		Petroleum ether (µg/µl)		Dichloromethane (µg/µl)		Ethyl acetate (µg/µl)		Aqueous (µg/µl)	
	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC
Ec001SGH	40	40	ND	ND	ND	ND	10	10	80	80
Ec002SGH	40	40	ND	ND	ND	ND	5	10	80	ND
Ec003SGH	20	20	ND	ND	ND	ND	10	10	40	40
Ec004SGH	20	40	ND	ND	ND	ND	10	10	40	80
Ec007SGH	40	80	ND	ND	ND	ND	40	80	80	ND
Ec010SGH	40	40	ND	ND	ND	ND	10	20	80	80
Ec011SGH	40	40	ND	ND	ND	ND	20	40	80	ND
Ec012SGH	40	40	ND	ND	ND	ND	10	10	80	80
Ec013SGH	40	40	ND	ND	ND	ND	20	20	40	80
Ec016SGH	40	40	ND	ND	20	ND	10	10	40	40
Ec017SGH	5	5	ND	ND	ND	ND	5	10	10	10
Ec018SGH	20	20	ND	ND	ND	ND	20	20	ND	ND
Ec019SGH	20	20	ND	ND	ND	ND	5	5	40	40
Ec020SGH	40	40	ND	ND	20	ND	20	20	ND	ND
Ec021SGH	20	20	ND	ND	ND	ND	10	10	40	40
Ec023SGH	40	40	ND	ND	ND	ND	10	20	40	80
Ec026SGH	40	40	ND	ND	ND	ND	10	20	80	80
Ec030SGH	80	80	ND	ND	ND	ND	10	10	80	ND
Ec031SGH	20	20	ND	ND	ND	ND	20	40	80	80
Ec032SGH	40	40	ND	ND	ND	ND	40	40	80	ND
Control	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Kp001SGH	80	80	ND	ND	ND	ND	40	40	ND	ND
Kp002SGH	40	40	ND	ND	20	ND	10	20	ND	ND
Kp005SGH	80	80	ND	ND	ND	ND	20	40	ND	ND
Kp006SGH	20	20	ND	ND	ND	ND	20	20	ND	ND
Kp007SGH	80	80	ND	ND	ND	ND	20	20	ND	ND
Kp008SGH	80	80	ND	ND	ND	ND	10	20	80	ND
Kp009SGH	80	ND	ND	ND	ND	ND	20	20	80	80
Kp010SGH	80	ND	ND	ND	ND	ND	10	20	ND	ND
Kp013SGH	80	80	ND	ND	ND	ND	20	20	ND	ND
Kp016SGH	80	ND	ND	ND	ND	ND	20	40	80	ND
Control	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
MIC_{90}	80		N/A		N/A		22		80	

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

Antimicrobial Activity of *Olea europaea*: As **Table 3** shows, *Olea europaea* extracts exhibited inhibitory and bactericidal effects on *Escherichia coli* and *Klebsiella pneumoniae.* The only fraction that did not show this effect was the petroleum ether where only 2 strains were inhibited and killed at 80 µg/µl. The lowest MIC_{90} was observed with dichloromethane and ethyl acetate fractions at 40 µg/µl for *Escherichia coli* and with ethyl acetate fraction at 44 µg/µl for *Klebsiella pneumoniae* (Table 3). The lowest MIC was recorded for the dichloromethane and ethyl acetate fractions with *Escherichia coli* at 5 µg/µl (Table 3).

TABLE 3: MICS AND MBCS OF THE DIFFERENT FRACTIONS OF *OLEA EUROPA ON ESCHERICHIA COLI* AND *KLEBSIELLA PNEUMONIAE*

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

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

The crude extract and aqueous fraction that showed good antibacterial activity with *Escherichia coli* exhibited very low activity on *Klebsiella pneumoniae.* The dichloromethane and ethyl acetate fractions exerted antibacterial effect even when the crude extract, petroleum ether and aqueous fractions did not show any antibacterial activity such was the case with Ec018SGH, Kp006SGH, Kp007SGH and Kp016SGH.

For the majority of the tested strains, bactericidal activity was observed at 80 µg/µl for the crude, the aqueous and the petroleum ether fractions and at 40 µg/µl for the dichloromethane and the ethyl acetate fractions. Crude extract, petroleum ether, dichloromethane, ethyl acetate and aqueous fractions showed, respectively, bactericidal effect against 53%, 10.5%, 87%, 100% and 43% of the tested strains. The MIC and MBC effects were detected within 1 dilution. As for *Klebsiella pneumoniae*, bactericidal effect was observed with 80µg/µl of the crude and aqueous fractions against Kp002SGH and Kp005SGH, respectively.

Antimicrobial Activity of *Viola odorata*: **Table 4** shows clearly that *Viola odorata'*s plant extract and fractions exhibited very low or absent antibacterial activity at the tested concentrations. *The* Ethyl acetate and aqueous fractions showed inhibitory and bactericidal effects on *Escherichia coli*. Only ethyl acetate fraction exerted inhibitory and bactericidal effects on *Klebsiella pneumoniae.* The MIC and MBC effects were detected within 1 dilution.

The concentrations at which most of the strains were inhibited were 5 µg/µl for ethyl acetate fraction and 80µg/µl for the aqueous fractions. The lowest MIC was recorded for the ethyl acetate fraction with *Escherichia coli* at 2.5 µg/µl. The MIC_{90} was determined with ethyl acetate fraction at 10 µg/µl for *Escherichia coli* and at 5.5 µg/µl *Klebsiella pneumoniae* (Table 4).

The concentration of ethyl acetate fraction at which bactericidal effect was observed for most of the strains was 10 µg/µl. Crude extract, petroleum ether and dichloromethane fractions did not show any inhibitory effect within the tested concentrations. Although the aqueous fraction showed antibacterial activities with *Escherichia coli,* it did not exhibit any activity on *Klebsiella pneumoniae.* Bacterial growth was observed for the positive controls while no growth was observed for the negative controls.

TABLE 4: MICS AND MBCS OF THE DIFFERENT FRACTIONS OF *VIOLA ODORATA ON ESCHERICHIA COLI* AND *KLEBSIELLA PNEUMONIAE*

Bacterial strain	Crude (µg/µl)		Petroleum ether (µg/µl)		Dichloromethane (µg/µl)		Ethyl acetate (µg/µl)		Aqueous (µg/µl)	
	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC
Ec001SGH	ND	ND	X	X	X	X	10	10	80	80
Ec002SGH	ND	ND	ND	ND	ND	ND	5	10	ND	ND
Ec003SGH	ND	ND	ND	ND	ND	ND	10	10	ND	ND
Ec004SGH	ND	ND	X	X	X	X	2.5	5	80	ND
Ec007SGH	ND	ND	ND	ND	ND	ND	10	10	ND	ND
Ec010SGH	ND	ND	ND	ND	ND	ND	5	10	ND	ND
Ec011SGH	ND	ND	X	X	X	X	5	10	80	ND
Ec012SGH	ND	ND	X	X	X	X	2.5	5	80	80
Ec013SGH	ND	ND	X	X	X	X	5	10	80	80
Ec016SGH	ND	ND	ND	ND	ND	ND	5	10	80	80
Ec017SGH	ND	ND	ND	ND	ND	ND	10	10	ND	ND
Ec018SGH	ND	ND	ND	ND	ND	ND	10	ND	ND	ND
Ec019SGH	ND	ND	ND	ND	ND	ND	5	10	80	80
Ec020SGH	ND	ND	ND	ND	ND	ND	5	10	ND	ND
Ec021SGH	ND	ND	X	X	X	X	2.5	2.5	40	40
Ec023SGH	ND	ND	X	X	X	X	5	10	80	ND
Ec026SGH	ND	ND	ND	ND	ND	ND	5	10	80	ND
Ec030SGH	ND	ND	X	X	X	X	5	10	80	ND
Ec031SGH	ND	ND	ND	ND	ND	ND	2.5	5	80	ND
Ec032SGH	ND	ND	ND	ND	ND	ND	5	10	ND	ND
Control	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND

Kp001SGH	ND	ND	ND	ND	ND	ND	5	10	ND	ND
Kp002SGH	ND	ND	ND	ND	ND	ND	5	10	ND	ND
Kp005SGH	ND	ND	ND	ND	ND	ND	5	10	ND	ND
Kp006SGH	ND	ND	ND	ND	ND	ND	10	ND	ND	ND
Kp007SGH	ND	ND	ND	ND	ND	ND	5	10	ND	ND
Kp008SGH	ND	ND	X	X	X	X	5	5	ND	ND
Kp009SGH	ND	ND	ND	ND	ND	ND	5	10	ND	ND
Kp010SGH	ND	ND	X	ND	X	ND	5	10	ND	ND
Kp013SGH	ND	ND	X	X	X	X	5	10	ND	ND
Kp016SGH	ND	ND	ND	ND	ND	ND	5	ND	ND	ND
Control	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
MIC_{90}	N/A		N/A		N/A		5.5		N/A	

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

DISCUSSION: Due to the high level of resistance to third generation cephalosporins and monobactams, the choice of effective and safe antibiotic treatment is becoming limited. Alternative agents or extracts obtained from natural medicinal plants need to be introduced or combined with antibiotics for therapeutical use. In the present study, extracts from three different plants were tested for antimicrobial activity against ESBL producing *Escherichia coli* and *Klebsiella pneumoniae*.

Most studies [15-18] found more antimicrobial activity in plant extracts tested against Gram-positive than Gram-negative bacteria. This was attributed to the fact that Gram-negative bacteria possess an outer membrane that acts as a barrier to many environmental substances [19]. 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 *Viola odorata*, the ethyl acetate and the aqueous fractions of all the plants exhibited an inhibitory effect. Contrary to the dichloromethane fractions of *Rheum rhaponticum* and *Viola odorata,* the dichloromethane fractions of *Olea europaea* 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 ethyl acetate fraction of *Viola odorata* at 2.5 µg/µl although the remaining fractions of this plant were not associated with detectable antibacterial effect.

In Nigeria, ESBL producing *Escherichia coli* and *Klebsiella pneumoniae* were inhibited by aqueous extracts of *Thonningia sanguinea.* The results showed significant inhibition with MIC values between 3.125 and 6.250 mg/ml [20]. In the present study, the MIC_{90} of the different fractions varied between 5.5 and 80 µg/µl.

In addition, our results show that the crude extract (ethanol extract), the ethyl acetate and the aqueous fractions of the selected plants showed antimicrobial activity against almost all the tested organisms. *Viola odorata* crude extracts were the only extracts that did not show any antibacterial activity. These results are similar to the results obtained by Ahmad and Aqil [21] after testing the alcohol crude extract and subfractions from 15 traditionally used medicinal plants.

In the present study the individual MICs varied between 2.5 and 80 µg/µl. 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 [21] 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.

Petroleum ether fractions did not show significant inhibitory effects within the tested concentrations. 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 these compounds do not exhibit antibacterial activity. Consequently the obtained fraction contained a minimal amount of active agents, resulting in a concentration that is possibly too low to be active.

Dichloromethane fractions of *Olea europaea* exhibited their inhibitory activity against 90% of the tested strains. Their inhibitory activity was mostly exhibited at 20µg/µl and 40µg/µl. An antibacterial activity of dichloromethane extracts was shown by another study [22]. They found that the dichloromethane extract of *Ageratum conyzoides* was weakly active against *Escherichia coli* only with a zone size of 7 mm around a 6 mm diameter well.

Dichloromethane fractions of *Olea europaea* showed the strongest antibacterial inhibitory effect at 5 µg/µl. Moshi *et al.,* [23] showed that the dichloromethane extract of *Whitfieldia elongate* exhibited strong activity against *Vibrio cholerae*, *Staphylococcus aureus, Bacillus cereus,* and *Candida neoformans.* On the other hand, in the present study, dichloromethane fractions of *Viola odorata* did not show inhibitory activity.

All aqueous extracts exerted antimicrobial activity against the majority of the tested strains. This antimicrobial activity was exhibited with low potency 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. *Viola odorata* aqueous extract only showed inhibitory effect against 40% of the tested microorganisms. The low percentages of inhibited microorganisms may possibly be related to the intrinsic insensitivity of *Klebsiella pneumoniae*.

This study has shown that the highest antibacterial activity against ESBL producing *Escherichia coli* and *Klebsiella pneumoniae* was mainly manifested by *Rheum rhaponticum* and *Olea europaea*, while *Viola odorata* exhibited less antibacterial affect. 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 non-medicinal 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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