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Original Article

Efficacy of Some Antibiotics and Essential Oils Against *Acinetobacter baumannii*: An *in Vitro* Study

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Abstract

Background and Objective: *Acinetobacter baumannii* is considered as a main opportunistic pathogen in hospitals and exhibit high resistance against most antibiotic groups. The aim of this study was to evaluate the efficacy of some antibiotics and essential oils against this bacterium, *in vitro*.

Materials and Methods: Two hundred and one clinical samples were collected from the Children's Hospital of Damascus. The polymerase chain reaction was conducted to identify the genus and type of bacteria. Finally, the minimum inhibitory concentrations of several antibiotics and essential oils, including *Thymus syriacus, Origanum syriacum, Citrus aurantium, Cinnamomum verum, Syzygium aromaticum, Cupressus macrocarpa, Myristica fragrans, Biota orientalis*, and *Zingiber officinale*, were investigated on Luria-Bertani broth agar.

Results: Fifty-nine isolates of *A. baumannii* were identified and the results showed that the DNA fragments of 16S rRNA and the $bla_{OXA\text{-}51\text{-}like}$ gene were approximately equal to 280 bp and 350 bp, respectively. In addition, most effective antibiotics against 50% of bacteria in each isolate of *A. baumannii* were rifampicin, linezolid, and levofloxacin whereas most effective essential oils included *Cupressus macrocarpa, Citrus aurantium, Myristica fragrans,* and *Biota orientalis*.

Keywords: *Acinetobacter baumannii,* Essential oils, Fluoroquinolones, Drug therapy, Drug resistance

Background

Acinetobacter baumannii (*A. baumannii)* is a bacterium that is classified as opportunistic pathogens in hospitals, especially in immunocompromised patients and children. Regarding their role as pathogens, some studies in the last two decades have shown their involvement in many human infections, especially in intensive care units (1,2). *A. baumannii* colonizes the skin and upper respiratory tracts. It is isolated from urine, sputum, blood, and feces. In addition, it is usually found in hospitals on different surfaces. In other words, they are isolated from different locations within the hospitals (e.g., air, water faucets, bedsides, gloves, and catheters). Historically, this type of infection is associated with war-wounded due to the direct contamination of wounds in the surrounding environment. For example, it was the most isolated Gram-negative bacterium from the wounds of those wounded in the Vietnam War, as well as the case with those wounded in the US war in Iraq. Recent reports indicate an increased incidence of septicemia in military hospital patients (2). *A. baumannii* exhibits high resistance against most antibiotic groups because it owns genes that encode inhibitory enzymes (3). For example, carbapenem-resistant *A. baumannii* strains show high resistance to most antibiotics, particularly beta-lactamase groups (4), because these strains own $bla_{\text{OXA-51-like}}$ and $bla_{\text{OXA-23-like}}$ genes which encode enzymes that inhibit the action of these antibiotics (5). Further, other studies demonstrated that most clinical isolates of *A. baumannii* were resistant to most cephalosporins (6), and this bacterium was also completely resistant to aztreonam, cefotaxime in addition to amoxicillin-clavulanic acid combination (7).

Essential oils and plant extracts are used as new sources of antibacterial and antimicrobial agents in many fields (8), which include food preservation (9), pharmaceuticals, alternative medicine, and natural treatments (10-11).

Furthermore, many plant extracts and oils are commonly used as medicinal plants in Syria for several purposes, especially for respiratory and gastrointestinal disorders. Considering the above-mentioned explanations, this study aimed to conduct a survey on the antibacterial activity of several antibiotics and essential oils, *in vitro*, against the isolates of *A. baumannii* obtained from children.

Materials and Methods

Identification of Bacteria

Two hundred and one samples were collected from Children's Hospital of Damascus, Damascus, Syria from different sources (e.g., skin abscesses, bronchial secretions, urine, pharyngeal smears, and blood) during

May-December 2016.

First, the samples were cultured on peptone water for 3 hours at 37 °C. Then, 10 µL of the primary culture were taken and added to 5 mL of Luria-Bertani (LB) broth culture medium for bacterial multiplication, followed by incubating the bacterial fluid at 37 °C for 18 hours. Subsequently, according to (12), a swab of the bacterial fluid was transplanted onto the solid culture medium LB agar or/and selective media (Herellae Agar and Leeds Acinetobacter Agar). All used media were purchased from Himedia, India.

DNA Isolation and Amplification by Polymerase Chain Reaction (PCR)

The isolation of DNA was carried out using the method of cetyltrimethylammonium bromide (13). The final extracted DNA was re-suspended in the Tris-EDTA (TE) buffer and the concentration was read using 2 µL of the sample in the nanodrop machine by the TE buffer as blank. Then, the concentration was made up to 100 ng/mL in each sample and stored at -20 °C until use.

Next, specific primers were used to amplify the 16S rRNA with a pair of primers including the 17 nucleotide forward primer of F16S (5'- TTTAAGCGAGGAGGAGG-'3) and the 18-nucleotide reverse primer of R16S (5'-ATTCTACCATCCTCTCCC-'3) (14). The primers of 16S rRNA yielded PCR products equal to 280 bp. According to (15), the $bla_{OXA\text{-}51^{-}like}$ gene was amplified with a pair of specific primers encompassing the 20-nucleotide forward primer of Fbla (5'-TAATGCTTTGATCGGCCTTG-'3) and the 20- nucleotide reverse primer of Rbla: (5'-TGGATTGCACTTCATCTTGG-'3). The primers of the $bla_{OXA\text{-}51^{-}like}$ gene gave a PCR product equal to 350 bp. The following reaction mixture contained 200 ng of bacterial DNA, 10 µmol of each forward and reverse primers, and the PCR mixture (3 mM of magnesium sulfate, 1X binding buffer, 0.2 mM dNTPs, and 1UTaq polymerase) taking into account the mixing of the reaction tube content well after the addition of each one of these substances. Finally, distilled water was supplemented until reaching a final volume of 25 µL. The essential steps of PCR are scheduled in Table 1.

PCR products were loaded on the 1.5% agarose gel and a 100 bp molecular weight DNA ladder was used for the validation of the length of the amplified products (Bio-Rad, USA; UV Tec GmbH, Germany).

Essential Oil Extraction

The samples of 100 g of wild thyme (*Thymus syriacus), Origanum syriacum, Citrus aurantium,* cinnamon (*Cinnamomum verum)*, *Syzygium aromaticum*, *Cupressus macrocarpa, Myristica fragrans*, *Biota orientalis*, and ginger (*Zingiber officinale)* were collected during the flowering season from different regions in Syria or purchased from local markets (Table 2). Then, they were air-dried (hydro-steam distillation) away from sunlight, and finally, grinded by an electrical mill. Moreover, the essential oils were extracted using a Clevenger-type apparatus according to the European pharmaceutical instruction method. The Clevenger-type apparatus was connected to a condenser and a cold water recycling device. Next, distilled water was added by the 1/10 volume to volume, and each sample was distilled for 2 hours. The floating essential oil was filtered through anhydrous sodium sulphate to dry the yielded essential oils, which were approximately about 1.6% for *T. syriacus, O. syriacum, C. Macrocarpa*, and *B. orientalis*, and about 0.6% for *C. aurantium, C. verum,*

Table 1. Polymerase Chain Reaction (PCR) Steps

PCR Step		Temperature	Duration
Initial denaturation		95 °C	5 minutes
37 cycles of amplification	DNA denaturation	95 °C	30 seconds
	DNA annealing	55 °C	30 seconds
	DNA extension	72 °C	60 seconds
Final extension		72 °C	10 minutes

Note. DNA: Deoxyribonucleic acid.

Table 2. Plants, Along With Their Families and Collection Sites

Scientific Name	Plant Family	Collection Site	Altitude (m)	Extracted Part
Thymus syriacusBoiss.	Lamiaceae	Alsoja mountain	840	Aerial parts
Citrus aurantium L.	Rutaceae	Latakia	300	Peels
Cinnamomum verum L.	Lauraceae	Market		Barks
Origanum syriacum L.	Lamiaceae	Alsoja mountain	840	Aerial parts
Cupressus macrocarpa L.	Cupressaceae	Market		Leaves
Syzygium aromaticum L.	Myrtaceae	Market		Leaves
Myristica fragransHaultt.	Myristicaceae	Market		Leaves
Biota orientalis L.	Cupressaceae	Market		Seeds
Zingiber officinaleRosc.	Zingiberaceae	Market		Rhizomes

S. aromaticum, M. fragrans, and *Z. officinale*. Eventually, the essential oils were collected in sealed dark glass bottles and kept in the fridge until use (16). For the antimicrobial activity test, several dilutions of the oils were done using dimethyl sulfoxide.

Antibiotic Susceptibility Determination by Disk Diffusion

The isolates were grown in LB medium at 37 °C for 22 hours. Final inoculum bacterial numbers were adjusted to $1.5*10^8$ CFU/mL. A total of 0.1 mL of bacterial suspension was poured on each plate containing LB agar. Then, the lawn culture was prepared by sterile cotton swab and allowed to remain in contact for 1 minute. Additionally, the sterile antibiotic disks were placed on lawn cultures, followed by incubating Petri dishes at 37 °C for 24 hours and measuring the inhibition zone around each disk. All antibiotic disks were purchased from Himedia, India and contained imipenem (10 µg), meropenem (10 µg), cefprozil (30 µg), cefotaxime (30 µg), ceftazidime (30 µg), ciprofloxacin (5 µg), ofloxacin (5 µg), levofloxacin (5 µg), doxycycline (30 µg), amoxicillin + clavulanic acid (30 µg), tobramycin (10 µg), oxacillin (1 µg), rifampicin (5 µg), linezolid (30 µg), and azithromycin (15 µg).

Antibiotic Minimum Inhibitory Concentration (MIC) Determination

Antibiotics susceptibility was estimated by using 96-well plates (TPP, Switzerland) according to the well broth microdilution method. The two-fold dilution of each antibiotic was executed in LB broth® (Acumedia, Michigan, USA). Next, plate wells were inoculated with 1×10^6 CFU of bacteria (0.2 mL final volume) and incubated at 37 °C for 24 hours. In addition, MIC_{50} and MIC_{90} were interpreted as the lowest concentration that inhibited 50% or 90% of the visual growth of bacteria, respectively. Further, the MIC testing was performed according to the recommendations of the Clinical and Laboratory Standards Institute (17). The absorbance was determined at 590 nm (Thermo-Lab Systems Reader, Finland). Investigated antibiotics were imipenem (Sigma, St. Louis, USA), meropenem (Supelco, Merck, Germany), cefprozil (Bristol-Myers Squibb, New-York, USA), cefotaxime (Sigma), ceftazidime (Sigma), ciprofloxacin (Bayer, Istambul, Turkey), ofloxacin (Sigma), levofloxacin (Sigma), and doxycycline (Sigma, St. Louis, USA). The other antibiotics included amoxicillin + clavulanic acid (Sigma, St. Louis, USA), tobramycin (Sigma, St. Louis, USA), oxacillin (Thermo Scientific, Oxoid Germany), rifampicin (Sigma), linezolid (Thermo Scientific, Oxoid Germany), azithromycin (Thermo Scientific, Oxoid, Germany), cefoperazone (Sigma, St. Louis, USA), sulbactam (Supelco, Merck, Germany), and amikacin (Sigma).

Essential Oil MIC Determination

The microdilution broth susceptibility assay was used in this regard (18). Furthermore, the serial dilutions of each essential oil were prepared in the LB broth medium in 96-well microtiter plates using a range of concentrations for each essential oil from 0.02 to 5.26 µL/mL. Next, 100 µL of freshly grown bacteria, standardized until achieving a bacterial number of 1×10^6 CFU/mL in the LB broth, was added to each well, followed by doing positive and negative controls. Then, the plate was incubated for 24 hours at 37 °C by continuing the process of shaking. Finally, the lowest concentration inhibiting 50% or 90% of visual growth was recorded and interpreted as MIC_{50} and MIC_{90}, respectively.

Results

Identification of Bacteria

The results showed that 67 isolates (out of 201 samples) were positive when cultured on selective media (42%).

PCR Results

DNA isolation, PCR, and electrophoresis on the agarose gel were carried out and the results revealed that 59 (88%) isolates were identified as *Acinetobacter* genus (280 bp) and *baumannii* type (350 bp). Figure 1 shows the results of DNA fragment migration on the agarose gel.

Antibiotic Disk Diffusion Susceptibility Test

Based on the data in Figure 2, almost all used antibiotics had moderate to low effects against 50 tested isolates and the most effective antibiotics were rifampicin, levofloxacin, linezolid, and imipenem.

Antibiotic Minimum Inhibitory Concentration (MIC) Test

The results (Table 3) showed that most effective antibiotics against 50 isolates of A. baumannii were rifampicin (MIC50 = 4 µL/mL), linezolid (MIC50 = 8 µL/mL), and levofloxacin (MIC50 = 16 µL/mL) and the best antibiotic combination was cefoperazone/sulbactam + rifampicin (MIC50 = 4 µL/mL).

Essential Oil MIC Test

Based on the results (Table 4), all the applied essential oils were able, within the range of used concentrations, to inhibit 50% of the bacteria in each isolate. However, only some of these oils, especially those of *Cupressus macrocarpa, Citrus aurantium, Myristica fragrans*, and *Biota orientalis* were able to inhibit 90% of the bacteria in each isolate.

Discussion

Diseases caused by *A. baumannii* demonstrate a real threat to human health in developing countries, especially in children and in places of armed conflicts. The unacceptably high rates of infection or a significant increase in the

Table 3. Minimum Inhibitory Concentration (MIC) of Different Antibiotics Against *A. baumannii*

Antibiotic	Number of Resistant Isolates (%)	MIC_{50}	MIC_{90}
Imipenem (IP)	46 (92%)	64	128
Meropenem	46 (92%)	64	128
Ceftezole	50 (100%)	>256	>256
Cefprozil	50 (100%)	>256	>256
Cefotaxime	50 (100%)	>256	>256
Ceftazidime	46 (92%)	64	128
Ciprofloxacin	45 (90%)	32	128
Ofloxacin	45 (90%)	32	128
Levofloxacin	40 (80%)	16	32
Doxycycline	44 (88%)	32	128
AC	50 (100%)	>256	>256
Tobramycin	46 (92%)	32	128
Oxacillin	50 (100%)	>256	>256
Rifampicin	2 (4%)	4	8
Linezolid	2 (4%)	8	16
Azithromycin	43 (86%)	32	64
CPS	43 (86%)	32	64
CPS + rifampicin	2 (4%)	4	8
CPS + Ciprofloxacin	44 (88%)	32	32
CPS + Amikacin	46 (92%)	64	128
CPS + Tobramycin	45 (90%)	32	64
CPS + Ceftazidime	44 (88%)	32	64
IP + Rifampicin	44 (88%)	32	64
IP + Ciprofloxacin	45 (90%)	16	64
IP + Amikacin	46 (92%)	64	128
IP + Tobramycin	45 (90%)	32	64
IP + Ceftazidime	44 (88%)	32	64

Note. AC: Amoxicillin + Clavulanic acid; CPS: Cefoperazone + Sulbactam combination.

Table 4. Minimum Inhibitory Concentration (MIC) of Different Essential Oils Against *A. baumannii*

Essential Oil	$MIC_{50\ (\mu L/mL)}$	$MIC_{90\ (\mu L/mL)}$
Cinnamomum verum	0.08	1.28
Syzygium aromaticum	0.04	1.28
Cupressus macrocarpa	0.08	0.32
Origanum syriacum	0.08	1.28
Citrus aurantium	0.08	0.64
Thymus syriacus	0.04	-
Zingiber officinale	0.64	-
Myristica fragrans	0.01	0.64
Biota orientalis	0.16	0.64

number of cases of any epidemic disease strongly led to greater efforts to prevent it, either by following health guidelines or conducting vaccination campaigns. The previous research has long focused on the importance of infectious disease occurrence data (19). Nonetheless, standards adopted in the diagnosis of infectious diseases in developing countries still largely depend on clinical symptoms and signs more than laboratory analysis or pathological anatomy (20-21). Thus, the determination of the incidence rate of any disease, in which clinical symptoms are not specific, represents a major challenge in the chase of effective control and infectious disease prevention in developing countries. Despite the significant development in recent years, developing countries are still suffering from many problems in terms of infrastructure, funding sources, and trained personnel that are capable of executing a rapid detection of such diseases (22-23).

The antimicrobial resistance of *A. baumannii* against some antibiotics is related to the secretion of genes encoding enzymes which have the ability to inhibit the activity of several antibiotics (4). This property is mainly responsible for the emergence of resistance to several types of drugs such as tobramycin and fluoroquinolones (i.e., ciprofloxacin, levofloxacin, and ofloxacin). In this study, the resistance rates against these antibiotics were elevated and several ratios were recorded (i.e., 92%, 90%, 90%, and 80%, respectively) by using the minimum inhibitory concentration (MIC) method. Othman et al (24) revealed the presence of moderate resistance against tobramycin and ciprofloxacin in isolates from Tunisian hospitals (45% and 36%, respectively). On the other hand, the MIC of ciprofloxacin and levofloxacin in this study was significantly higher compared to the other studies (25-26).

Fonseca et al (5) showed that the emergence of carbapenem-resistant isolates is responsible for the emergence of carbapenem- (imipenem and meropenem)

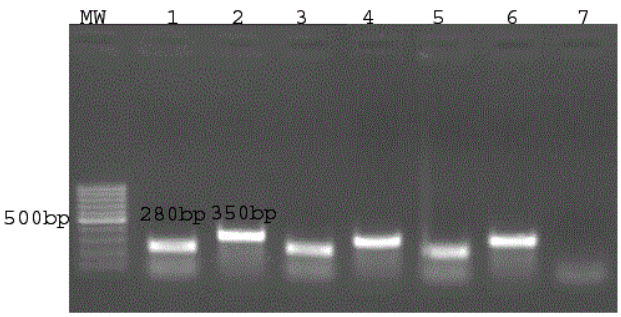

Figure 1. DNA Fragment Electrophoresis on Agarose Gel
Note. MW: 100 bp molecular weight; DNA ladder bands 1 and 2 display positive control (for genus and type). In addition, bands 3-6 represent two selective isolates, and band 7 indicates the negative control.

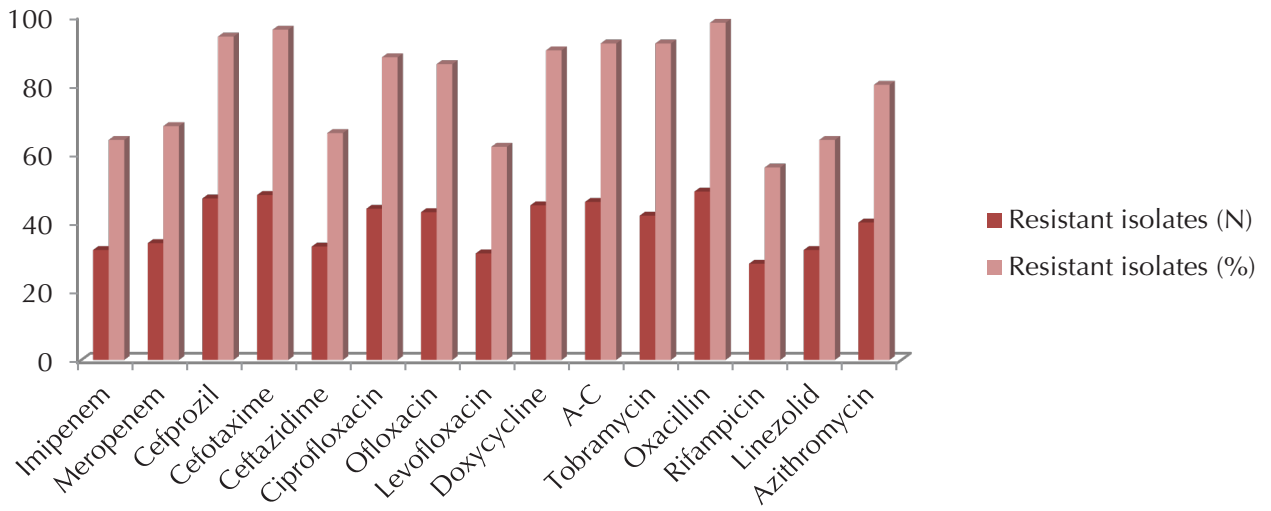

Figure 2. Disk Diffusion Susceptibility of Different Antibiotics Against A. baumannii. A-C = Amoxicillin + Clavulanic Acid

and rifampicin-resistant bacteria. In our study, the percentage of rifampicin-resistant isolates was very low (4%) by using the MIC method with the MIC_{90} of less than 8 µg/mL. On the contrary, the percentage of imipenem- and meropenem-resistant isolates was 92% against both antibiotics. Considering international publications, in this study, the MIC of rifampicin was higher compared with the study of Aranda et al (25). However, a relatively lower MIC of imipenem was reported in several previous studies as 2 µg/mL (27), 8 µg/mL (28), and 16 µg/mL (29) compared to this study.

Beta-lactamase enzymes produced by the *A. baumannii* strains are responsible for the emergence of penicillin and cephalosporin resistance. In our study, it was clear that almost all studied isolates were resistant to these two groups. Accordingly, significant resistance was observed to all cephalosporin used in this work with a value of MIC_{90} exceeding 128 µg/mL, which is consistent with the results of previous studies using cefuroxime (27, 29) while it contradicts the values revealed by other studies using cefazolin (27-28).

Drugs such as sulbactam, colistin, imipenem, and rifampicin have been used in the treatment of *A. baumannii* (30-32). The combination of several drug groups such as colistin-rifampicin, colistin-imipenem, imipenem-rifampicin, and cefoperazone/sulbactam-imipenem showed better efficacy against these bacteria (33-35). Thus, it is clear that the combination of several types of antibiotic groups may be the appropriate alternative treatment for these bacteria. In this context, Tunyapanit et al (36) found that the proportion of isolates resistant to cefoperazone/sulbactam combination did not exceed 3% with the MIC_{90} of less than 2 µg/mL, which is absolutely inconsistent with the results of our study where the proportion of isolates resistant to this combination was 86% with the MIC_{90} of more than 64 µg/mL. Conversely, our finding concurs with that of this study which proved that cefoperazone/sulbactam-rifampicin combination is the best pharmacological combination in terms of both sensitivity and the MIC value. Finally, although linezolid is normally used against Gram-positive bacteria, it showed a good synergistic effect against *A. baumannii* in combination with colistin (37-38). The present study evaluated the effect of linezolid against *A. baumannii* and revealed good results in this regard.

Recently, plant extracts have been developed and used in several fields such as natural antioxidants or antimicrobial agents (39-40). The antibacterial mechanisms of natural compounds found in herbs and spices were discussed as well (41).

Most applied plants in this study are used in traditional medicine in all Syrian regions in order to treat many disorders, especially respiratory and gastrointestinal diseases. Therefore, it was possible to investigate the efficacy of these plants against *A. baumannii*. All studied essential oils were able to inhibit 50% of the bacteria in each isolate. However, only some of these essential oils had the ability to inhibit 90% of bacteria, especially the essential oils of *Cupressus macrocarpa, Citrus aurantium, Myristica fragrans*, and *Biota orientalis* and the MIC_{90} values for these essential oils were 0.32, 0.64, 0.64, and 0.64 µg/mL, respectively.

There are only a few studies concerning the role of essential oils and plant extracts in the treatment of antibiotic-resistant *A. baumannii*. For instance, Miyasaki et al (42) showed that most of the de-tanninized parts of the *Scutellaria baicalensis* have a good effect against these bacteria (MIC_{90} = 128 µg/mL). Karaman et al (43) also demonstrated that the methanolic extract of *Juniperus oxycedrus L.* has a good effect against *A. Baumannii* and

many other bacteria. In addition, Intorasoot et al (44) found that the extracts of *Cinnamomum verum*, *Syzygium aromaticum*, and *Ocimumbasilicum* Linn. volatile oils had an effective minimum bactericidal concentration against *A. baumannii* (MBC_{90} = 0.5, 1, and 2 µg/mL, respectively). Furthermore, Kumar et al (45) reported the essential oils of *Citrus maxima* and *Citrus aurantifolia* showed potential antimicrobial properties against *A. baumannii*. However, KumariPushpa et al (45) concluded that the secondary metabolites of *Myristica fragrans* Houtt can be an indispensable source of antimicrobial compounds against *A. baumannii*. In addition, *Ehretia microphylla* and *Piper betle L* leaf extracts showed a good inhibitory effect against *A. baumannii* (47-48). On the other hand, according to Duraipandiyan et al (49), the ethyl acetate extract of *Toddaliaasiatica* represented high efficacy against these bacteria (MIC = 125 µg/mL). Finally, Saghi et al (50) found good efficacy of *Origanum syriacum, Thymus syriacus,* and *Satureja* extracts against *A. baumannii* (MIC = 2.6, 0.44, and 0.3 µg/mL, respectively).

Conclusion

In general, *A. baumannii* strains were resistant to most antibiotic groups and alternative therapies. Rifampicin, linezolid, and levofloxacin were the most effective antibiotics against these bacteria using the minimum inhibitory concentration method whereas cefoperazone/sulbactam-rifampicin was the best antibiotic combination. Eventually, all essential oils used in this study were able to inhibit 50% of these bacteria while only some of these oils showed the ability to inhibit 90% of these bacteria.

Conflict of Interest

The authors declare no conflict of interests.

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Ethical Statement

Ethical permission was obtained from the Ethical Committee of Damascus Children Hospital.

Authors' Contribution

We confirm that the manuscript, as well as the order of authors listed in the manuscript, has been contributed, reviewed and approved by all named authors.

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Informed Consent

The verbally informed consent was obtained from the children's parents.

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