https://doi.org/10.31925/farmacia.2021.5.13 **ORIGINAL ARTICLE**

INVESTIGATION OF CLASS 1 INTEGRONS WITH ANTIBIOTIC RESISTANCE GENES IN MULTIDRUG-RESISTANT *ACINETOBACTER BAUMANNII* STRAINS AND DETERMINATION OF PLANT EXTRACT EFFECTS ON MULTIDRUG-RESISTANT ISOLATES

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Abstract

This study aimed to investigate the presence of resistance genes in multidrug-resistant *A. baumannii* isolates as well as to determine the antibacterial activity of selected plant extracts against isolates. 41 strains were isolated from various clinical samples. PCR tests were performed using the primers. Methanol was used as solvent for the preparation of the plant extracts. MIC values of the plant extracts were determined by the broth microdilution method. The $bla_{\text{OXA-23}}$, $bla_{\text{CTX-M-1}}$, $bla_{\text{CTX-M-2}}$, bla_{GES} genes and Class 1 integrons were detected in five isolated strains. The lowest MIC value (2.25 mg/mL) was determined for the *Echinacea purpurea* extract, while the highest MIC value (50 mg/mL) was determined for the *Morus alba* extract. Determination of the antibacterial effect of plants extracts used in the study against *A. baumannii* isolates shows the importance of screening the antibacterial activity of plants in the fight against antibiotic resistance.

Rezumat

Studiul a avut ca scop investigarea genelor de rezistență ale izolatelor de *A. baumannii* multirezistente, precum și determinarea activității antibacteriene a unor extracte vegetale supra a 41 specii microbiene izolate din diferite probe clinice. Metanolul a fost folosit ca solvent de extracție. Valorile concentrației minime inhibitorii ale extractelor obținute au fost determinate prin metoda microdiluției. Genele $bla_{\text{OXA-23}}$, $bla_{\text{CTX-M-1}}$, $bla_{\text{CTX-M2}}$ și bla_{GES}, alături de integroni din clasa 1 au fost decelate pentru 5 specii izolate. Valoarea MIC cea mai scăzută (2,25 mg/mL) a fost obținută pentru extractul din *Echinaceea purpurea*, în timp ce cea mai mare valoare MIC (50 mg/mL) a fost determinată pentru extractul de *Morus alba*. Determinarea efectului antibacterian ale extractelor vegetale împotriva izolatelor de *A. baumannii* arată importanța screening-ului activității antibacteriene a plantelor în lupta împotriva rezistenței la antibiotice.

Keywords: MIC, plant extract, resistance

Introduction

A. baumannii, a Gram-negative, nosocomial pathogen resistant to most current antibiotics, has become a major challenge for modern healthcare systems [1, 2]. *A. baumannii*, as free-living planktonic cells or biofilms, can live at a wide range of temperature, pH and humidity levels. It also uses various carbon and energy sources to survive. These characteristics contribute to the persistence and spread of *A. baumannii* in the hospital setting [1, 3, 4]. Recently, isolates with resistance to all known antibiotics have emerged and are therefore considered to be one of the most dangerous hospital pathogens worldwide [5, 6]. *A. baumannii* often causes pneumonia, bacteraemia, meningitis, and wound and urinary tract infections [7].

A. baumannii isolates resistant to more than two antibiotic groups (ampicillin-sulbactam, fluoroquinolones, aminoglycosides, cephalosporins and antipseudomonal carbapenems) are defined as MDR (multidrug-resistant) [5, 6]. The antimicrobial resistance mechanism in *A. baumannii* is divided into three categories: (i) enzymatic inactivation of antimicrobials; (ii) modification of antimicrobial targets; and (iii) reduced access to antimicrobial targets due to membrane modifications [8]. Enzymatic degradation by the β-lactamases in *A. baumannii* is the most common mechanism of β-lactam resistance [9]. The β-lactamases are divided into four groups as class A, class B (metallo β-lactamases), class C and class D (oxacillinases) [9]. Class A (TEM, SHV, CTX-M1, CTX-M2, VEB), class B (VIM, IMP), class C (AmpC) and class D (OXA-23, OXA-24,

OXA-51, OXA-58) β-lactamases have been reported worldwide in *A. baumannii* isolates [9]. The naturally occurring class D (OXA) carbapenemases in *A. baumannii* are the leading cause of carbapenem resistance in this microorganism [9].

Plants are capable of synthesizing secondary metabolites known as phytochemical compounds that act as plant defence mechanisms against macro and microorganisms [5, 10]. In many cultures in Asia, Africa and some parts of America, traditional medicine has been used for decades to protect human health and also to treat various diseases [11]. Alkaloids, flavonoids, phenolics and tannins are among the most important phyto-chemicals used in phytotherapy [5, 12]. In recent years, many researchers have pointed out the role of plant antibacterial activities in fighting antibiotic resistant bacteria [13]. The efficacy of plant-derived compounds in controlling various MDR pathogens, including *Acinetobacter sp.*, is being investigated [1].

Considering these points, the present study investigated the presence of antibiotic resistance genes and Class 1 integrons in multidrug-resistant *A. baumannii* isolates from intensive care unit patients, and the effects on these isolates of methanol extracts from nine selected plants (*Morus alba*, *Zingiber officinale*, *Vaccinium myrtillus*, *Rosa canina*, *Hypericum perforatum*, *Lycium barbarum*, *Aquilaria agallocha*, *Nigella sativa* and *Echinacea purpurea*) were determined.

Materials and Methods

Forty-one *A. baumannii* strains isolated from various clinical specimens (tracheal aspirate, blood, urine, broncho-alveolar lavage) of patients hospitalized in the Rize Training and Research Hospital Intensive Care Unit, Turkey, were identified and their antibiograms were performed *via* the Vitek 2 Compact automated system. Isolation of DNA from the bacteria was performed using the DNA boiling method [14].

PCR tests were performed using primers of bla_{VIM}, bla_{NDM}, bla_{IMP}, bla_{GES}, bla_{CTXM-1}, bla_{CTXM-2}, bla_{OXA-58}, bla_{OXA-23}, bla_{OXA-51} and Class 1 integrons [15]. The PCRs were performed in a final volume of 50 µL. The PCR component mix was as follows: 5 µL of genomic DNA, 2 µL of each primer (20 pmol/µL), 10 µL reaction buffer (Promega, Madison, WI, USA), 3 µL 25 mM $MgCl_2$, 4 µL of each dNTP (2.5 mM) and 1.5 U of GoTaq® Flexi Polymerase (Promega, Madison, WI, USA). All PCR results were run on 1% agarose gel containing 0.5 µg/mL ethidium bromide and then visualized under ultraviolet (UV) light.

When choosing the plants to be used in the study, those which are frequently studied in the literature and widely consumed and utilized by people for various purposes were preferred. *Morus alba*, *Zingiber officinale*, *Vaccinium myrtillus*, *Rosa canina*, *Hypericum perforatum*, *Lycium barbarum*, *Aquilaria agallocha*, *Nigella sativa* and *Echinacea purpurea* were purchased as dry and ground from a local herbal shop. To ensure that each of the samples was completely and uniformly dried, they were oven dried and weighed at intervals for seven days. Samples (10 g) of each of the powdered plants were then extracted with 100 mL of methanol for 2 h using a magnetic stirrer. At the end of the process, the plant extracts were first filtered through Blue Ribbon filter paper and in the second stage, through 0.25 µM filters. The clarified plant extracts were placed in glass flasks and their solvents were completely evaporated in a rotary evaporator. Their concentrations were determined by dissolving each in a specific volume of methanol. The extracts were then stored in a cool, dark environment until analysis.

The broth microdilution method was used to determine the MIC of the plant extracts against *A. baumannnii* isolates. Experiments were performed in triplicate using 96-well plates. Ampicillin was used as control at concentrations of 100 - 0.78125 mg/mL. All isolates were grown on LB (Luria Bertani) medium at 37°C. In the first 11 wells, 50 µL of LB broth was placed, while, as a sterility control, 100 µL of LB broth was added to the 12^{th} well and evaluated. In addition, the 11^{th} well was prepared as a growth control with 50 µL LB broth + 50 µL bacteria. Serial dilutions were made up to the 10^{th} well, starting with 50 µL of the first concentration of extracts placed into the first well. Plates were incubated at 37°C and MIC values were determined as the concentration of the plant extract in the first well where no growth had occurred [16].

Results and Discussion

Identification and antibiograms of 41 *A. baumannii* strains isolated from various clinical specimens (tracheal aspirate, blood, urine and bronchoalveolar lavage) of patients hospitalized in the Rize Training and Research Hospital Intensive Care Unit, Turkey, were studied using the Vitek 2 Compact automated system. Antibiotic resistance profiles of the 41 identified *A. baumannii* isolates are shown in Table I. The bla_{OXA-51} gene was used to identify *A. baumannii* isolates, and this gene was detected in all of the isolates included in this study. In 38 isolates, the bla_{OXA-23} (92.6%) class D β-lactamase gene was detected. Among the Class A β-lactamase genes, bla_{CTXM-1} was detected in 36 isolates (87.8%), bla_{CTXM-2} in 18 isolates (43%) and bla_{GES} in 1 isolate. The presence of the studied metallo β-lactamase genes (bla_{VIM}, bla_{NDM} and bla_{IMP}) and the bla_{OXA-58} gene encoding Class D β-lactamase was not observed in any isolate. Class 1 integrons were detected in 34 isolates.

Most of the *A. baumannii* pathogens were isolated from the respiratory tract of hospitalized patients [9]. The clinical effect of *Acinetobacter* increases morbidity or mortality and *A. baumannii* infections are responsible for the increase in patient deaths in critically ill patients.

Many studies have shown that *A. baumannii* has a high rate of multiple drug resistance (MDR) [9].

Table I

Antibiotic resistance rates of *A. baumannii* isolates

Antibiotic	S (n/%)	I (n/%)	R (n/%)
PIP	-	-	100% (41)
SXT	13.51% (6)	2.7% (2)	83.78% (35)
CAZ	-	2.7% (2)	97.3% (40)
IMP	2.7% (2)	-	97.3% (40)
MEM	2.7% (2)	-	97.3% (40)
GN	24.32% (10)	13.51% (6)	62.16% (25)
NET	27.03% (11)	10.81% (5)	62.16% (25)
TOB	32.43% (13)	-	67.57% (27)
TIG (N = 35)	20% (8)	80% (30)	-
TZP	-	2.7% (2)	97.3% (40)
COL	97.3% (40)	2.7% (2)	-
LEV	-	-	100.0% (41)

PIP: Piperacillin, SXT: Trimethoprim-sulfamethoxazole, CAZ: Ceftazidime, IMP: Imipenem, MEM: Meropenem, GN: Gentamicin, NET: Netilmicin, TOB: Tobramycin, TIG: Tigecycline, TZP: Piperacillin-tazobactam, COL: Colistin, LEV: Levofloxacin

Extended-spectrum β-lactamases (ESBLs) are plasmid-mediated and produced by Gram-negative bacilli that provide resistance to penicillin, cephalosporin and monobactams. These ESBLs are commonly found worldwide in *Enterobacteriaceae*, *Pseudomonas aeruginosa* and *A. baumannii*. Included in ESBL members are TEM-, SHV-, or CTX-M-type β-lactamases. The CTX-M-type β-lactamases are divided into five groups according to amino acid sequence: CTX-M-1, CTX-M-2, CTX-M-8, CTX-M-9 and CTX-M-25 [17]. The CTX-M β-lactamases produced by *A. baumannii* strains are plasmid-mediated and therefore find a wide distribution in hospitals. In this study, the PCR method was used to detect the presence of CTX-M-1 and CTX-M-2 β-lactamase encoding genes in the isolates. It was determined that 36 strains (87.8%) tested positive for $bla_{\text{CTX-M-1}}$ and 18 isolates (43%) tested positive for $bla_{\text{CTX-M-2}}$.

In a multicentre study conducted in 2016 in Turkey, 443 isolates of *A. baumannii* were studied, and CTX-M-1 was identified in 63 isolates and CTX-M-2 in 42 isolates [18]. The presence of CTX-M-1 and CTX-M-2 β-lactamases in 96 *A. baumannii* isolates from Turkey was investigated and no positive results were obtained for the isolates [19]. In the same study, the presence of OXA-23 and OXA-51 class D β-lactamases was detected in all strains [19].

Similar results were obtained in this study. The presence of OXA-51 β-lactamase was detected in all 41 isolates and OXA-23 β-lactamase was found in 38 isolates. The $bla_{\text{OXA-51}}$ gene is naturally found on the *A. baumannii* chromosome. The $bla_{\text{OXA-23}}$ is plasmid in origin and is found in isolates in many countries around the world. The presence of the $bla_{\text{OXA-23}}$ gene in clinical isolates of *A.baumannii* from Turkey varies from 31 to 92% [19]. In our study, the presence of OXA-40 and OXA-58 class D β-lactamases was not observed in any of the isolates. Of the class A carbapenemases, the GES-type β-lactamase encoding bla_{GES} was detected in only one isolate, whereas none of the class-B β-lactamase encoding genes (bla_{VIM}, bla_{NDM}, bla_{IMP}) were found.

Integrons are genetic elements that recognize and capture mobile gene cassettes carrying antimicrobial drug resistance determinants. Class 1 integrons are associated with multidrug-resistant pathogens. Class 1 integrons are frequently found in *A. baumannii* [20]. In this study, Class 1 integrons were identified in 34 of the 41 multidrug-resistant *A. baumannii* isolates.

The MIC values of nine plant extracts against six *A. baumannii* isolates with different antibiotic resistance phenotypes and genes were determined. The codes of the isolates, the resistance genes they carry and the MIC values of the plant extracts against these isolates are given in Table II.

Table II

MIC values of plant extracts against multidrug-resistant isolates

		Plant Extracts Used in the Study								
Bacteria codes	**Resistance genes carried by isolates**	***Aquilaria agallocha***	***Nigella sativa***	***Lycium barbarum***	***Hypericum perforatum***	***Zingiber officinale***	***Morus alba***	***Rosa canina***	***Vaccinium myrtillus***	***Echinacea purpurea***
AB38	Class 1 integron, OXA-51, OXA-23	–	20 g/mL	35 mg/mL	10 mg/mL	30 mg/mL	12.5 mg/mL	35 mg/mL	–	2.75 mg/mL
AB43	Class 1 integron, OXA-51, OXA-23, CTX-M1, CTX-M2	–	20 g/mL	35 mg/mL	10 mg/mL	–	50 mg/mL	–	–	2.25 mg/mL
AB45	Class 1 integron, OXA-51, OXA-23, CTX-M1, CTX-M2	–	20 g/mL	17.5 mg/mL	10 mg/mL	30 mg/mL	25 mg/mL	–	–	2.75 mg/mL
AB46	OXA-51, OXA-23, CTX-M2	–	20 mg/mL	35 mg/mL	–	15 mg/mL	–	–	–	2.25 mg/mL
AB59	OXA-51, OXA-23, CTX-M1	–	20 g/mL	35 mg/mL	–	15 mg/mL	–	–	–	–
AB69	Class 1 integron, OXA-51, CTX-M1	–	20 mg/mL	17.5 mg/mL	–	–	–	–	–	–

Among the plant extracts, *Echinacea purpurea* was found to have the lowest MIC against the multidrug-resistant *A. baumannii* isolates. The MIC of *Echinacea purpurea* methanol extract was 2.75 mg/mL for AB38 and AB45, and 2.25 mg/mL for AB43 and AB46.

The MIC of *Nigella sativa* methanol extract was 20 mg/mL against all studied clinical isolates. It was found that the MIC of *Hypericum perforatum* methanol extract for AB38, AB43 and AB45 was 10 mg/mL, while this extract did not inhibit the growth of AB46, AB59 or AB69 at the tested concentrations. The MIC value of *Lycium barbarum* methanol extract against isolates AB38, AB43, AB46 and AB59 was 35 mg/mL and 17.5 mg/mL against AB45 and AB69.

The MIC of *Zingiber officinale* methanol extract was found to be 30 mg/mL for AB38 and AB45 and 15 mg/mL for AB46 and AB59, whereas it did not inhibit the growth of AB43 or AB69 isolates at the tested concentrations. The MIC values of the methanol extract of *Morus alba* against AB38, AB43 and AB45 isolates were determined as 12.5 mg/mL, 50 mg/mL and 25 mg/mL, respectively.

The *Rosa canina* methanol extract inhibited the growth of AB38 alone among the studied isolates. It was observed that the methanol extracts of *Aquilania agalloeha* and *Vaccinium myrtillus* exhibited no anti-microbial activity against the multidrug-resistant *A. baumannii* clinical isolates (AB38, AB43, AB45, AB46, AB59 and AB69) at the concentrations tested.

Since no new antibiotics have been developed against carbapenem-resistant *A. baumannii* isolates, the anti-microbial activity of plant-derived substances used in traditional medicine has been of particular interest [21]. Secondary metabolites are responsible for the antimicrobial activities of plants. Various plant active compounds with effective activity against *A. baumannii* have been identified worldwide [21].

In the present study, the MIC values of nine different plant methanol extracts were determined against MDR *A. baumannii* clinical isolates having six different resistance gene patterns. In one study, antimicrobial activity of methanol, acetone and chloroform extracts of *Zingiber officinale* against MDR *A. baumannii* was investigated [13]. According to the results, the chloroform plant extract had the highest activity against MDR *A. baumannii*, with a MIC value of 25 mg/mL. In our study, the methanol extract of *Zingiber officinale* was found to have a MIC of 15 mg/mL against MDR *A. baumannii*. In a study conducted by Miyasaki *et al.*, the MIC value of *Rosa rugosa* extract against *A. baumannii* isolates was determined as 50 µg/mL [21, 22]. In this study, the antimicrobial activity of *Rosa canina* methanol extract on *A. baumannii* isolates was investigated and the MIC value was found to be 35 mg/mL. Javadian *et al*. examined the antimicrobial activity of *Peganum harmala* and *Heracleum persicum* against *A. baumannii* and suggested that extracts obtained from both of these might be useful for treating bacterial infections [23]. The antibacterial activity of *Pinus pinaster* bark extract against MDR *A. baumannii* isolates was investigated in another study that found the extract could be useful in the fight against resistant bacteria [24]. In another study, the antibacterial activities of *Oliveria decumbens*, *Pelargonium graveolens*, *Eugenia caryophyllata*, *Ziziphora tenuir* and *Trachyspermum copticum* oils were evaluated against 32 clinical isolates of *A. baumannii*. Essential oils of *O. decumbens*, *E. caryophyllata* and *T. copticum* were found to have the best antibacterial activity against the clinical isolates of *A. baumannii* [25]. Zhang *et al.* investigated the antibacterial activity of *Mentha arvensis* ethanol extract on MDR *A. baumannii* isolates and found this extract to be an effective antibacterial against the isolates [26].

This study investigated the antibacterial effect of methanol extracts of nine different medicinal plants on MDR *A. baumannii* isolates. According to the results, the growth of the isolates harbouring different antibiotic resistance genes was inhibited by different plant extracts.

Conclusions

The emergence of resistant strains of bacterial pathogens has resulted in limited treatment options. Traditionally, plants play an important role in the treatment of diseases and have secondary metabolites that are reported to be potentially active against a variety of bacteria. Therefore, plants can be used as a resource in the search for alternative medicines to replace existing antibiotics.

Conflict of interest

The authors declare no conflict of interest.

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