

Original article

COMPARATIVE *IN VITRO* ACTIVITY OF MEDICINAL PLANTS *Arnebia densiflora* and *Ecballium elaterium* AGAINST ISOLATED STRAINS of *Klebsiella pneumoniae*

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Abstract

In vitro activity of the extracts of medicinal plants *Ecballium elaterium* and *Arnebia densiflora*, which is endemic to Turkey, was screened against isolated strains of *Klebsiella pneumoniae* that were resistant in disc diffusion test (trimethoprim-sulfamethoxazol, sulbactam-ampicillin, clavulonate-amoxicillin, ceftriaxone, cefepime, imipenem, ceftazidime, tobramycin, gentamicin, ofloxacin, ciprofloxacin). Broth microdilution susceptibility testing was performed according to the Clinical and Laboratory Standards Institute. The extracts of *A. densiflora* root bark and *E. elaterium* leaf, stem and fruit samples were analyzed against the strains of *K. pneumoniae* at the concentrations range from 128 to 0.0312 µg/mL. This is the first report shows that the extracts of *A. densiflora* and *E. elaterium* are effective as much as the antibiotics amoxicillin and ofloxacin against some of the isolated strains of *K. pneumoniae* (K₂, K₃, K₅, K₆ and K₁₀) at the concentration of 32 µg/mL. The extracts were active at the concentration of 64 µg/mL onto rest of the strains, which are close to the effective dose of controls as well. However, the activities of all the extracts against standard control strain of *K. pneumoniae* (RSKK 574) were comparatively better (8 µg/mL) than that of the tested isolated strains of *K. pneumoniae*, in which the activities varied between 32 to 64 µg/mL.

Key words: *Arnebia densiflora*, Activity, ESβL, *Ecballium elaterium*

İzole *Klebsiella pneumoniae* Suşlarına Karşı Tıbbi Bitkilerden *Arnebia densiflora* ve *Ecballium elaterium*'un Karşılaştırmalı *in vitro* Aktivitesi

Ecballium elaterium ve *Arnebia densiflora* (Nordm.) Ledeb, ekstreleri, disk difüzyon testinde (trimetoprim-sulfametoksazol, sulbaktam-ampisilin, klavulonate-amoksisilin, seftriakson, sefepim, imipenem, seftazidim, tobramisin, gentamisin, ofloksasin, siprofloksasin) dirençli izole suş *Klebsiella pneumoniae*'e karşı tarandı. Duyarlılık testi olarak Klinik ve Laboratuvar Standartları Enstitüsü önerileri doğrultusunda sıvı mikrodilüsyon testi uygulandı. *A. densiflora*'nın kök kabuğu ve *E. elaterium* yaprak, gövde ve meyva ekstreleri izole *K. pneumoniae* suşuna ve kontrole (ampisilin and ofloksasin) karşı 128 ve 0.0312 µg/mL aralığında değişen etki gösterdi. Bu çalışma *A. densiflora* ve *E. elaterium* ekstrelerinin izole *K. pneumoniae* (K₂, K₃, K₅, K₆ ve K₁₀) suşlarına karşı 32 µg/mL konsantrasyonunda amoksisilin ve ofloksasin antibiyotikleri kadar etkili olduğunu gösteren ilk rapordur. Ekstreler, geri kalan suşlarda 64 µg/mL konsantrasyonunda aktiftirler, bu da kontrolün etkili dozuna yakındır. Bununla birlikte tüm ekstreler standart kontrol suş *K. pneumoniae* (RSKK 574)'ye karşı (8 µg/L) izole suş *K. pneumoniae*'ye 32 ve 64 µg/mL arasında değişen etkiden karşılaştırmalı olarak daha iyidir.

Anahtar kelimeler: *Arnebia densiflora*, Aktivite, GSβL, *Ecballium elaterium*

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INTRODUCTION

Traditional healers have long used plants to prevent or to cure infectious diseases. Many of these plants have been investigated scientifically by researchers for their antimicrobial activity, consequently, a large number of plant products have been shown to inhibit the growth of pathogenic microorganisms (1-5). Different plant extracts have been screened to observe their *in vitro* antimicrobial activities against virus, gram-negative, gram-positive bacteria and fungi as yeast or filamentous (4). Considerable results showed that it is worth to study plant extracts and phytochemicals for their biological activity against microorganisms. *Arnebia densiflora* is one of the endemic medicinal plants, which is known for its wound healing effects in folk medicine in Turkey (6). Its major phytochemicals shikonin and derivatives belong to the class of naphthaquinones, which is composed of mostly colored compounds (7, 8). Specifically, fruit juice of the (*Ecballium elaterium*) has been used in folk medicine for sinus relief in Anatolia (9). The active compound was determined as triterpenoid cucurbitacin B, which has mainly antiinflammatory effects (10, 11). Studies also showed that traditional medicine *E. elaterium* has inhibitory effects on inflammatory cytokines; Interleukin-1 α , Interleukin-1 β and tumor necrosis factor α (11).

Klebsiella pneumoniae is a gram-negative rod that causes bacterial pneumonia (about 3%) and nosocomial infections. Multi-drug resistance is highly common in *K. pneumoniae* and is under the control of transmissible plasmids. Hence, increasing resistance against antimicrobial agents is a major concern of medical microbiology in recent years (12). Plasmid-mediated extended-spectrum β -lactamases (ES β Ls), produced by organisms such as *K. pneumoniae*, confer clinically significant resistance to broad-spectrum of penicillins, monobactams, cephalosporins and a variety of other antibiotic classes that have been found to be associated with subsequent infections due to ESBLs-producing organisms in Enterobacteriaceae. The first ESBLs report was on *K. pneumoniae* in 1983 (13). Published research on ESBLs has now originated from more than 30 different countries, reflecting the truly worldwide distribution of ESBLs producing organisms. ESBLs have been classified based on their amino acid sequences (i.e., SHV, TEM, CTX-M, BES, GES, OXA, VEB). CTX-M type showed dramatical increase since 1995 in the most parts of the world, including North and South America, Asia and Europe. ESBL-producing isolates of *K. pneumoniae* were mostly recovered from hospitalized patients and long term care centers (12). Those isolates were often associated with outbreaks of infection specifically in high-risk regions, such as neonatal and intensive care units. Since the enormous increase in the number of ESBL-producing isolates, the situation will create significant therapeutic problems in the future.

The purpose of this study is to find out natural-novel compounds that are active against the isolates of *K. pneumoniae*. In this study the activity of the extracts of selected medicinal plants *A. densiflora* and *E. elaterium* were evaluated against isolated strains of *K. pneumoniae*.

EXPERIMENTAL

Plant extracts

A. densiflora was collected from hill slopes of Sivrihisar, Eskişehir-Turkey in May 2007. A specimen (GUE-2601) was placed in the Herbarium of Faculty of Pharmacy, Gazi University, Ankara, Turkey. Reddish-purple color root barks of the plant were air dried, weighed as 0.5 g and each weighed sample was extracted at room temperature with different solvents such as petroleum ether (*Ad-PE*), hexan (*Ad-HX*), chloroform (*Ad-CHL*), ethyl acetate (*Ad-ETOAC*), *n*-butanol (*Ad-BUT*) and ethanol (*Ad-ETOH*) separately. Each extraction was carried out 2 times with 25 ml solvent in each time. Six different solvents were applied; all the extracts were evaporated *in vacuo* at 48^oC. Plant materials of *E. elaterium* were collected from Imrahor region of Ankara-Turkey. Air dried leaves, stems and fruits of the plants were grounded to powder.

Each 0.5 g weighed plant parts were extracted with (*Ee*-PE), hexan (*Ee*-HX), chloroform (*Ee*-CHL), ethyl acetate (*Ee*-ETOAC), *n*-buthanol (*Ee*-BUT) and ethanol (*Ee*-ETOH) separately. The extractions were carried out at room temperature; solvents were applied to powdered plant material for 2 times as 25 ml solvent in each time. The extracts were evaporated *in vacuo* at 48°C. Precisely weighed 10 mg of each extract was employed for the *in vitro* activity testing.

Microbiological studies

Test materials

Dried plant extracts of *A. densiflora* and *E. elaterium* were dissolved in dimethylsulphoxide at a final concentration of 256 µg/mL and sterilized by filtration using 0.22 µm Millipore (MA 01730, USA) and in further; they were used as the stock solutions. Reference antibacterial agents, ampicillin (AMP; Faco), ofloxacin (OFX; Hoechst Marion Roussel) were obtained from their respective manufacturers and dissolved in phosphate buffer solution (ampicillin; pH: 8.0, 0.1 mol/L), and in water (ofloxacin). The stock solutions of these agents were prepared in medium according to CLSI (formerly; National Committee for Clinical Laboratory Standards) recommendations (14-16).

Microorganisms and Inoculums preparation

Isolated strains of 10 *K. pneumoniae* that are resistant to trimethoprim-sulphamethoxazole (Oxoid), sulbactam-ampicillin (Oxoid), clavulonate-amoxicillin (Oxoid), ceftriaxone (Oxoid), cefepime (Oxoid), imipenem (Oxoid), ceftazidime (Oxoid), tobramycin (Oxoid), gentamicin (Oxoid), ofloxacin (OFX), ciprofloxacin (Oxoid) by disc diffusion test were used for determination of antibacterial activity. *K. pneumoniae* RSKK 574 (Refik Saydam Central Hygiene Institute-Culture Collection, The Ministry of Health of Republic of Turkiye, Ankara) was used as the control strain. Mueller Hinton Broth (MHB; Oxoid) and Mueller Hinton Agar (MHA; Oxoid) were applied for growing and diluting of the bacterial suspensions. The microorganism suspensions used for inoculation were prepared at 10⁷cfu (colony forming unite)/ml by diluting fresh cultures at McFarland 0.5 density (10⁸cfu/mL). Suspensions of all bacteria (10µl) were added in each well of the diluted extracts density of 10⁵cfu/ml (14-17).

In vitro antibacterial activity test

In vitro broth microdilution test was performed in accordance with the guidelines of CLSI. Ampicillin and ofloxacin were used as reference agents. Moreover, standard strain of *K. pneumoniae* (RSKK 574) was used as a control. Media (100µL) were placed into each 96 wells of the microplates. Extract solutions at 256 µg/mL were added into first rows of microplates and two fold dilutions of the compounds (128-0.0312 µg/mL) were made by dispensing the solutions to the remaining wells. 10µL of bacterial suspensions were inoculated into all the wells. The sealed microplates were incubated at 35°C for 18h. The lowest concentration of the extracts that completely inhibits macroscopic growth was determined as minimum inhibitory concentration (MIC) (4, 14).

RESULTS AND DISCUSSION

Antimicrobial activity of the variable plant extracts of *A. densiflora* and *E. elaterium* were tested against the isolated strains of *K. pneumoniae* (K₁ to K₁₀) and compare with controls (ampicilline and ofloxacin) in the same condition. Broth microdilution testing was performed for determination of the MICs. The results of the microdilution test of plant extracts were shown in Table 1.

Table 1. Antimicrobial activity as MICs ($\mu\text{g/mL}$) of the extracts of *A. densiflora* (Ad), *E. elaterium*(Ee) and the references tested against isolated strains of *Klebsiella pneumoniae* ($K_{n=1-10}$)

Extracts	Microorganisms										
	K_1	K_2	K_3	K_4	K_5	K_6	K_7	K_8	K_9	K_{10}	
Ad-PE	R (SXT; AMC; SAM; CRO; IMP; TOB)	R (SXT; AMC; CRO; IMP; TOB)	R (AMC; SAM; CRO; CPM)	R (AMC; CRO; CAZ; CPM; TOB; GM)	R (AMC; CRO; CPM; GM)	R (AMC; SAM; CRO; CPM)	R (SXT; AMC; SAM; CRO; CPM; OFX)	R (SXT; AMC; SAM; CRO; CPM; OFX; CIP)	R (SXT; AMC; SAM; CRO; CPM; OFX; CIP)	R (AMC; SAM; CRO; OFX)	<i>K. pneumoniae</i> RSKK 574
Ad-HX	64	32	32	64	32	32	64	64	64	32	8
Ad-CHL	64	32	32	64	32	32	64	64	64	32	8
Ad-ETOAC	64	32	32	64	32	32	64	64	64	32	8
Ad-BUT	64	32	32	64	32	32	64	64	64	32	8
Ad-ETOH	64	32	32	64	32	32	64	64	64	32	8
Ee-PE	64	32	32	64	32	32	64	64	64	32	8
Ee-HX	64	32	32	64	32	32	64	64	64	32	8
Ee-CHL	64	32	32	64	32	32	64	64	64	32	8
Ee-ETOAC	64	32	32	64	32	32	64	64	64	32	8
Ee-BUT	64	32	32	64	32	32	64	64	64	32	8
Ee-ETOH	64	32	32	64	32	32	64	64	64	32	8
AMP	-	--	-	-	-	-	-	-	-	-	2
OFX	32	32	32	32	32	32	32	32	32	32	<0.12

R: Resistant, SXT: Trimethoprim-Sulfamethoxazol, SAM: Sulbactam-Ampicillin, AMC: Clavulonate-Amoxicillin, CRO: Ceftriaxone, CPM: Cefepime, IMP: Imipenem, CAZ: Ceftriaxone, TOB: Tobramycin, GM: Gentamicin, CIP: Ciprofloxacin, AMP: Ampicillin, OFX: Ofloxacin. Ad: (*A. densiflora*), Ee: (*E. elaterium*), PE: (Petroleum ether), HX: (Hexan), CHL: (Chloroform), ETOAC: (Ethyl acetate), BUT: (Butanol), ETOH: (Ethanol).

Since there is no complete study reported up to date on the active compounds of *A. densiflora*, six different extracts were prepared with the aim of capturing as much as phytochemicals in each of the extract. It is well known that according to their polarity, different phytochemical groups can be extracted in to different solvents. Root bark was extracted with the increasing polarity of the solvents such as petroleum ether (*Ad-PE*), hexan (*Ad-HX*), chloroform (*Ad-CHL*), ethyl acetate (*Ad-ETOAC*), *n*-butanol (*Ad-BUT*) and ethanol (*Ad-ETOH*) separately.

The extracts showed *in vitro* activity on the isolates of K_{-2, 3, 5, 6}, and K₁₀ similar to the control antibiotics ampicilline and ofloxacin at the concentration of 32 µg/mL as shown in Table 1. On the other hand, isolates K_{-1, 4, 7, 8}, and K₉ were inhibited at the concentration of 64 µg/mL by the plant extracts, the observed activities were two times the consantration of the control antibiotics. Even though the standard strain was more sensitive to the control antibiotics ampicillin (2 µg/mL) and ofloxacin (0.12 µg/mL), the extracts were highly effective on the standard strain of *K. pneumoniae* at the dose of 8 µg/mL. The activity of the extracts varied according to the isolated strains, but no difference was observed among the activity of the different extracts. All the extracts were active either at the concentration of 32 µg/mL or 64 µg/mL. As for *E. elaterium*, similar results were obtained with *A. densiflora*. The fruit juice (or sap) of *E. elaterium* has been used for sinus relief in Anatolia for ages (9). According to the clinical studies, effective compound found to be triterpenoid cucurbitacin B, which has anti-inflammatory effect demonstrated on animals (11). In our study, activity of the extracts of *E. elaterium* did not differ with the applied solvents, whereas it differed along with the isolates. In consistent with *A. densiflora*, the MICs values of the extracts of *E. elaterium* were comparable with the MICs values of control antibiotics ampicillin and ofloxacin on the isolates of K_{-2, 3, 5, 6}, and K₁₀ at the concentration of 32 µg/mL. Rests of the strains were two times less sensitive to the plant extracts compared to the above isolates at 64 µg/mL.

Despite numerous studies on the plant essential oils and extracts were screened with different method on bacteria and fungi species including *K. pneumonia*, in line with our knowledge neither *A. densiflora*, nor *E. elaterium* screened with gram-negative bacteria for their antibacterial activity.

A. densiflora, belongs to Boraginaceae family, in which plants known for their medicinal effects (18). Crude extracts prepared from colored roots of *Alkanna tinctoria*, *Lithospermum erythrorhizon* and *A. nobilis* showed antibacterial activity (18). It is reported that isolated two compounds, alkannin and shikonin, from the crude extracts showed activity against gram-positive bacteria but they were inactive against gram-negative bacteria. In our case, instead of individual compounds, extracts, which include all the phytochemicals of the plant, were applied and they demonstrated significant antimicrobial activity. This might be due to either synergistic effect of the compounds or individual effect of some other compounds that were not pronounced in that study. In a recently published study, a variety of root extracts of *Cordia gellatii* screened with total 10 strains of gram-positive and gram-negative bacteria including *K. pneumonia* by broth microdilution and agar diffusion tests. This study demonstrated that MICs varied between 125-1000 µg/mL for all tested microorganisms. In the mean time, all tested extracts except dichloromethane extract (MIC; 500 µg/mL) were shown antibacterial activity against *K. pneumonia* at a concentration of 1000 µg/mL (19). In another study the extracts of *Plagiochasma appendiculatum* were screened with disc diffusion test for their antibacterial effect and significant activity was obtained. In this study, the activity of the only one, ethanolic extract was same as the control standard antibiotics (gentamicin, tetracycline and streptomycin; 10 µg/disc) and the rest of the extracts demonstrated MICs ranges between 100 to 200 µg/disc against *K. pneumonia* (3). In our study the MICs of most of the extracts were the same with the standard antibiotics. Therefore the activity of the extracts that were used might be considered as noteworthy. Some plants from Northern Argentina were screened for their antimicrobial activity

with a microplate assay using alamar blue (2). The MIC values were recorded as 250-1000 µg/mL, whereas the MICs values of gentamicin and amoxicillin were 0.31 and 0.16, respectively. In an additional research report, Sudanese medicinal plant extracts were tested with micro-dilution assay and MICs values were between 100 to 3130 µg/mL for the different extracts of different plant parts (20). MIC value of antibiotic neomycin was 1.56 µg/mL. The literature shows that although different methods were applied to test antibacterial activity including *K. pneumonia*, there is no available data testing plant extracts or compounds on more than one strain of *K. pneumonia*. As a conclusion of the studies completed so far, in the majority of the reports MIC values of the extracts ranged between 10 to 3130 µg/mL, in which 0 to 500 µg/mL are considered as effective antimicrobials (2, 19-21). The extracts that were applied in this study demonstrated MICs values at 32 and 64 µg/mL, which were in similar magnitude with the currently used antimicrobial agents. Although MIC values for these already used antimicrobials are in resistant ranges, *E. elaterium* and *A. densiflora* extracts might be useful. Although, all the results on *E. elaterium* and *A. densiflora* extracts showed the first significant activity against isolated strains of *K. pneumonia* as control; further study for exploring their responsible compounds is under evaluation.

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