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Research Article

Phytochemical Screening and Effect of Three Plants Collected from the Region of Khenchela, Algeria against Multi-drug Resistant Pathogenic Bacteria

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ABSTRACT

This work aims to perform phytochemical analysis of hydroethanolic extracts of three medicinal plants commonly used in folk medicine (*Juniperus oxycedrus, Eucalyptus globulus,* and *Matricaria chamomilla*) and to evaluate their antibacterial potential against multi-drug resistant bacteria. Disc diffusion method has been used to determine the antibacterial activities and minimum inhibitory concentrations (MIC) of different plant extracts against four Gram negative bacteria (*Escherichia coli, Klebsiella pneumoniae, Pseudomonas aeruginosa* and *Enterobacter aerogenes*) and two Gram positive bacteria (*Staphylococcus aureus* and *Bacillus subtilis*). Antibiotic sensitivity test was performed in Mueller Hinton agar with three commonly used antibiotics (tetracycline, vancomycin and oxacillin). Phytochemical analysis revealed the presence of tannins and free quinones in all the extracts, whereas alkaloids were absent. The results of antibiogram revealed that all strains were resistant to tested antibiotics with exception of *Staphylococcus aureus* that was found to be susceptible to oxacillin. Also, obtained results showed that hydroethanolic extracts exhibited dose-dependent growth inhibitory effects against *Staphylococcus aureus* and *Bacillus subtilis*, while no inhibitory effect on Gram negative bacteria was observed. In conclusion, the present work provides useful baseline information for the potential use of the studied plants in the fight against MDR strains.

INTRODUCTION

Antibiotic treatment is one of the main approaches of modern medicine which is used to combat infections [1]. However, overuse of antibiotics has become the major factor for the emergence and dissemination of multi-drug resistant strains of several groups of microorganisms [2]. Antibiotics that show low efficacy in treating human and animal diseases through antibiotic resistance must be replaced with new drugs to combat the burden of these pathogens. Hence, Plant-derived products are expected to be the best source of modern drugs [3, 4].

Medicinal plants have been a vital source of both curative and preventive medical therapy preparations for human beings, which also has been used for the extraction of important bioactive compounds [5-7]. These bioactive substances could include tannins, alkaloids, terpenoids, steroids and flavonoids [8].

The aim of the present study was to investigate the antibacterial activity of hydroethanolic extracts of three medicinal plants commonly used in folk medicine (*Juniperus oxycedrus, Eucalyptus globulus,* and *Matricaria chamomilla*) (Table1) against multi-drug resistant strains.

MATERIAL AND METHODS

Plant Collection

Plant materials were collected from the region of Khenchela (Eastern Algeria) (Fig. 1) in April 2021. Three medicinal plants (*Juniperus oxycedrus, Eucalyptus globulus,* and *Matricaria chamomilla*) (Fig. 2) were selected based on their potential against infectious diseases as determined by traditional use as folk medicine.

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Table 1: Scientific name, family, parts used and traditional use of selected plants

Plant	Family	Plant Part	Traditional Use
Juniperus oxycedrus	*Cupressaceae*	Leaf	Infectious diseases, chronic eczema and other several skin diseases, hyperglicemia, obesity, tuberculosis, bronchitis, and pneumonia [9-11].
Eucalyptus globulus	*Myrtaceae*	Leaf	Cardiac tonic, anthelmintic, stimulant, diuretic, antiseptic [12-14]; Cephalagia, tuberculosis, chronic cough, asthma, pneumonitis, burns, dyspepsia, flatulence, cardiac debility, pharyngodynia, skin diseases, chronic and intermittent fever, abcess, arthritis, burns, diabetes, cancer, diarrhea, dysentery, diphtheria, inflammatory conditions such as bronchitis, laryngitis, rhinitis, encephalitis [15, 16].
Matricaria chamomilla	*Asteraceae*	Flower	Digestive disorders, conjunctivitis, dysmenorrhea, cold, cough, kidney stones, eye infection, headaches, and insomnia [17]; colic and nervousness [18]; sinusitis, burns, wounds, ulcers, and stomachache [19]; flatulence, infections, and pharyngitis [20]; and hypertension, intestinal worms, anxiety, and hair loss [21].

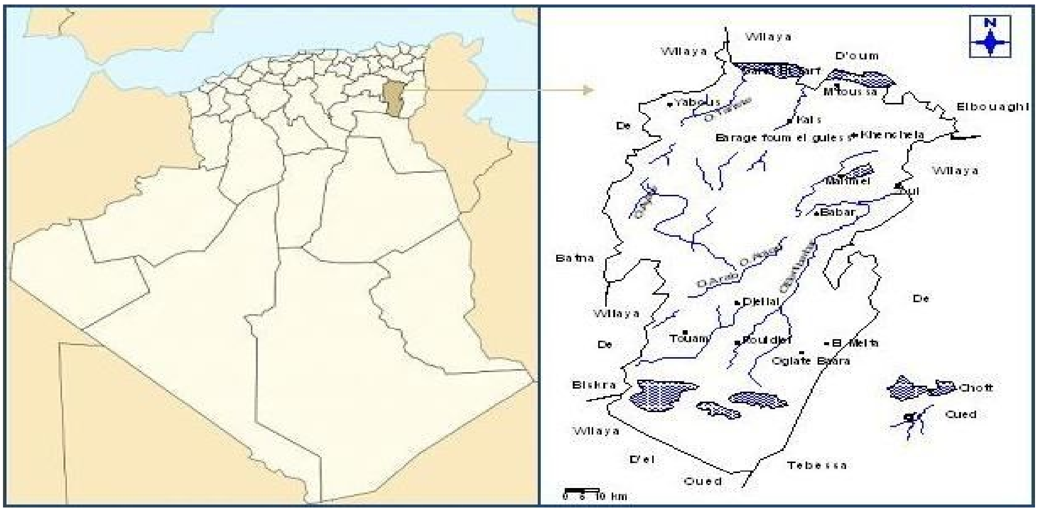

Figure1: Map of the study region: Khenchela district in Eastern Algeria

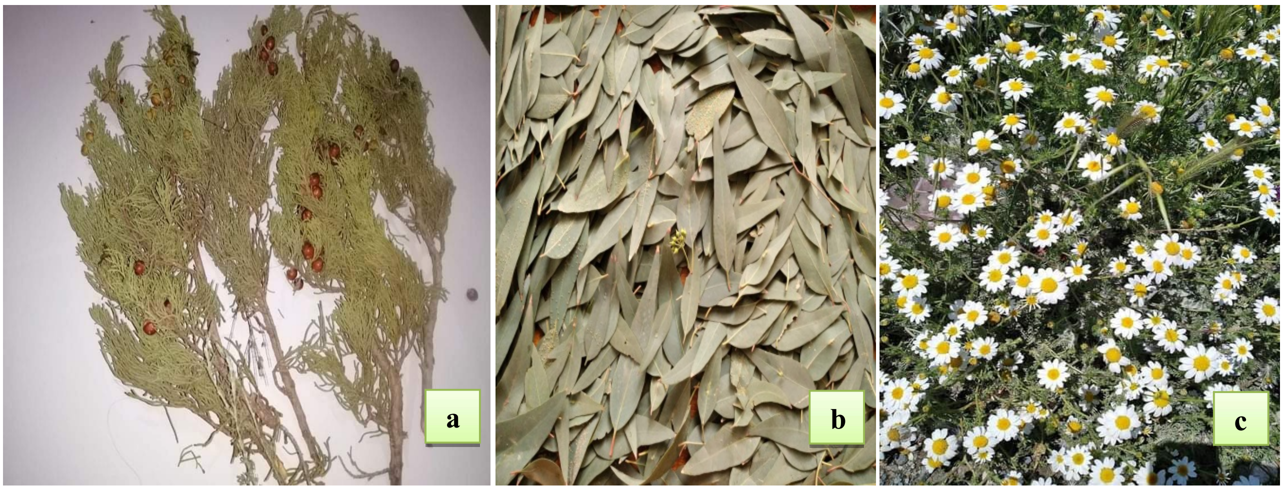

Figure2: (a) *Juniperus oxycedrus;* **(b)** *Eucalyptus globulus;* **(c)** *Matricaria chamomilla*

The collected plants were washed and dried in shade. The dried materials were then finely pulverized by grinding using aluminium collection blender and the powders obtained were weighted.

Preparation of Plant Extracts

The hydroethanolic extracts were obtained by maceration of 25 g plant powder in 200 mL of ethanol-water mixture (v/v) for 48h at room temperature. The filtrates obtained on filter paper were then evaporated using a rotary evaporator. The resulting solutions were dried in an oven (50°C). The obtained residues were stored at 4°C until their use [22]. A yield for each extract was calculated using the following formula: Extract yield% = R/S × 100 (where R; weight of extracted plants residues and S; weight of plant raw sample).

Preliminary Phytochemical Screening

Phytochemical analysis for compounds which include flavonoids, tannins, quinones, saponins, terpenoids and alkaloids was done using standard methods described by Harborne (1998) and Bruneton (1999)with little modifications [23, 24].

Detection of Flavonoids

To 2-3 mL of each extract, zinc powder was added in a test tube, followed by drop wise addition of concentrate HCl. Formation of purple red or cherry color indicates the presence of flavonoids.

Detection of Tannins

To 2 ml of test solution, a few drops of 5% ferric chloride solution were added. Formation of blue color indicated the presence of hydrolysable tannins.

Detection of Free Quinones

The presence of free Quinones is confirmed by the addition of a few drops of NaOH when the aqueous phase turns yellow, red, or purple color.

Detection of Saponins

The extract was diluted with 20 mL of distilled water and it was shaken in a graduated cylinder for 15 m. A 1 cm. layer of foam indicated the presence of Saponins.

Detection of Terpenoids

5 mL of each plant extract was mixed in 2 mL of chloroform followed by the careful addition of 3 ml concentrated (H_2SO_4). A layer of the reddish brown coloration was formed at the interface thus indicating a positive result for the presence of terpenoids.

Detection of Alkaloids

To 1 ml of test solution or filtrate was added a drop or two of the Mayer's reagent along the sides of the test tube. A white or creamy precipitate confirmed the test as positive.

Tested Bacterial Strains

Clinical isolates of six pathogenic bacteria (*Escherichia coli, Klebsiella pneumoniae, Pseudomonas aeruginosa, Enterobacter aerogens, Staphylococcus aureus* and *Bacillus subtilis)*, obtained using selective media following standard protocols from private and public laboratories, Khenchela, were used. Isolates were identified by their ability to grow on selective media, by gram reaction, by their morphology and by various distinct biochemical reactions [25].

Inoculums Preparation

Bacterial strains were pre-cultured overnight at 37°C in nutrient agar plates. Afterward, each strain was suspended in sterile 0.85% NaCl solution and the turbidity was measured using spectrophotometry (optical density [O.D.] =0.08-0.1 at 625 nm) [26].

Antibiotic Sensitivity Test

The antibiogram was performed by disc diffusion method in Mueller Hinton agar with three commonly used antibiotics. To determine the antibiotics sensitivity profiles of bacteria, the antibiotics were used at the following concentrations: tetracycline (30µg), vancomycin (30µg), and oxacillin (5µg).

Antibacterial Activity

The disc diffusion method is used to evaluate antimicrobial activity of each plant extract. The dried plant extracts were dissolved in dimethyl-sulfoxide (DMSO) to obtain stock solutions of 100mg/ mL and 200 mg/mL and sterilized by 0.45 µm millipore filters. The discs were placed on the inoculated Mueller Hinton agar plates and impregnated with 10µl of extracts and their mixture. Antimicrobial activity was evaluated by measuring the zone of inhibition against the test organisms [27].

Determination of Minimum Inhibitory Concentrations (MIC's) of the Effective Plant Extracts

The minimum inhibitory concentrations (MIC) of the plant extracts were determined using disc diffusion method. Dilutions of each extract ranging from 200 to 0.195 mg/mL were prepared in DMSO. 10 µl of each dilution was poured into the discs which are placed in inoculated Mueller Hinton agar plates. The lowest concentration of each of the plant extracts that inhibits the microbial growth after 24 h was considered to represent the minimal inhibitory concentration (MIC) of that extract.

RESULTS AND DISCUSSION

Extraction Yield

According to the results presented in Fig. 3, the highest yield was recorded for the hydroethanolic extract of *J. oxycedrus* leaves, which was 20.44%, followed in descending order by *E. globules* leaves extract and *M. chamomilla* flowers extract with percentages of 15.56 % and 15.4%, respectively.

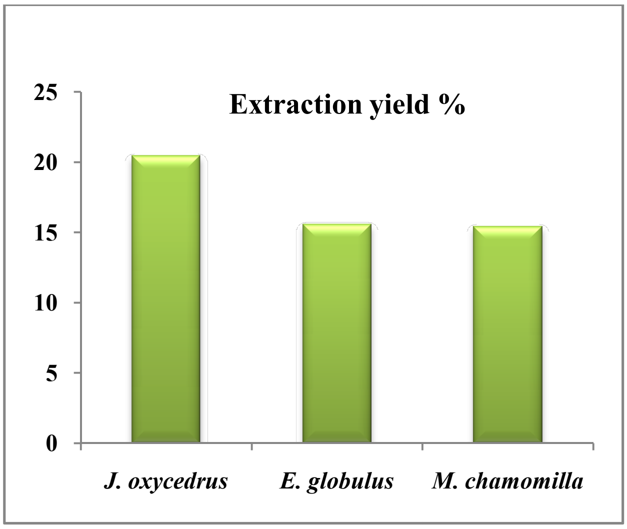

Figure 3: Percentage yield of plant extracts

Phytochemical Screening

The results of the preliminary phytochemical screening revealed the absence of flavonoids in *J. oxycedrus* and *M. chamomilla* extracts. However, these substances were found to be present in *E. globulus* extract. Saponins were absent in all extracts except of *J. oxycedrus* extract. Terpenoids were found to be present only in *E. globulus* extract. Alkaloids were absent in all the extracts, whereas tannins and free quinones were present in all the extracts as shown in Table 2.

Table 2: Preliminary Phytochemical Screening of selected plant extracts

Phytochemicals	Plant extracts		
	J. oxycedrus	*E. globulus*	*M. chamomilla*
Flavonoids	-	+	-
Tannins	+	+	+
Free quinones	+	+	+
Saponins	+	-	-
Terpenoids	-	+	-
Alkaloids	-	-	-

(+): Present; (-): Absent

The phytochemical compounds detected are known to have medicinal importance. For example, Tannins, according to research, are known to have antibacterial [28], antitumor and antiviral activities [29]. They work by precipitating microbial protein thus making nutritional protein unavailable for them [30]. Similarly, quinones are an important family of natural products. They have a variety of biological effects, such as anticancer and antimicrobial activities [31, 32]. These phytochemical compounds identified in hydroethanolic extracts may be responsible for the biological activities shown by *J. oxycedrus*, *E. globules* and *M. chamomilla* and the reason for their use as a traditional medicine.

Antibiotic Susceptibility Test

In this study, antimicrobial susceptibility was evaluated by disc diffusion method using antibiotics from three different classes: tetracyclines, glycopeptides and β-lactams against clinical isolates of Gram negative (*Escherichia coli, Klebsiella pneumoniae, Pseudomonas aeruginosa* and *Enterobacter aerogenes*) and Gram positive bacteria (*Staphylococcus aureus* and *Bacillus subtilis*). According to Table 3, all strains were resistant to tested antibiotics with exception of *Staphylococcus aureus* that was found to be susceptible to oxacillin. In literature, organisms expressing *in vitro* resistance to three or more antimicrobial classes are referred to as multidrug-resistant organisms [33].

Table 3: Antibiotic susceptibility of pathogenic strains

Bacterial strains	Antibiotics		
	Tetracycline	Vancomycin	Oxacillin
Escherichia coli	R	R	R
Klebsiella pneumoniae	R	R	R
Pseudomonas aeruginosa	R	R	R
Enterobacter aerogenes	R	R	R
Staphylococcus aureus	R	R	S
Bacillus subtilis	R	R	R

R: Resistant; S: Susceptible

Antibiotic-resistant bacterial infections are already widespread on the globe [34]. In February 2017, World Health Organization (WHO) published its first ever list of antibiotic-resistant 'priority pathogens' that pose the greatest threat to human health. These priority pathogens are resistant to multiple antibiotics and have in-built abilities to resist treatment and transfer along genetic material that leads other bacteria to become drug-resistant as well [35]. Therefore, new drugs are desperately needed for battling these rapidly evolving pathogens.

Antibacterial Activity

The antibacterial activity of plants extract was evaluated against six pathogenic bacterial strains at two doses (200 mg/mL and 100mg/mL) using disc diffusion assay. Both the zones of inhibition as well as MIC values of the extract were calculated. The results of the disc diffusion assay (zones of inhibition) are shown in Table 4, while the results of MIC are depicted in Table 5.

Table 4: Antibacterial activity of hydroethanolic extracts of selected plants

Bacterial strains	**Inhibition zone diameter (mm)**								
	J. oxycedrus		***E. globulus***		***M. chamomilla***		**Mixture**		**DMSO**
	100 (mg/mL)	200 (mg/mL)	100 (mg/mL)	200 (mg/mL)	100 (mg/mL)	200 (mg/mL)	100 (mg/mL)	200 (mg/mL)	
Escherichia coli	NI	NI	NI	NI	NI	NI	NI	NI	NI
Klebsiella pneumoniae	NI	NI	NI	NI	NI	NI	NI	NI	NI
Pseudomonas aeruginosa	NI	NI	NI	NI	NI	NI	NI	NI	NI
Enterobacter aerogenes	NI	NI	NI	NI	NI	NI	NI	NI	NI
Staphylococcus aureus	7.6	12.3	11.6	16	11.6	15	11	13	NI
Bacillus subtilis	10.3	13.3	14	15.3	8.3	8.6	ND	ND	NI

NI: No inhibition zone; ND: Not determined

Table 5: Minimum Inhibitory Concentration (MIC) of effective plant extracts

Bacterial Strains	**Minimum Inhibitory Concentration (mg/ml)**		
	J. oxycedrus	***E. globulus***	***M. chamomilla***
Staphylococcus aureus	6.25	6.25	6.25
Bacillus subtilis	6.25	6.25	12.5

All the extracts showed dose-dependent growth inhibitory effects against Gram positive bacteria, while no inhibitory effect on Gram negative bacteria was observed. Also, the control DMSO showed no inhibition.

Hydroethanolic extract of *E. globulus* had the maximum zone of inhibition against *S. aureus* (16 mm) at 200mg/mL, whereas hydroethanolic extract of *M. chamomilla*, *J. oxycedrus* and the mixture showed zone of inhibition (15 mm, 12.3mm and 13mm) against the same strain, respectively. *J. oxycedrus, E. globulus* and *M. chamomilla* at concentration 200mg/mL made an inhibition zone with diameters of 13.3 mm, 15.3 mm and 8.6 mm against *B. subtilis* respectively. The minimum inhibitory concentrations of all extracts were 6.25 mg/mL for *S. aureus* and the same MIC was recorded for *B. Subtilis* with exception of *M. chamomilla* extract which was 12.5 mg/mL.

Several studies have reported that *J. oxycedrus, E. globulus* and *M. chamomilla* exhibited different degrees of antibacterial activity against a wide spectrum of bacteria. These differences may be explained by susceptibility testing conditions, method of extraction, and even strain to strain differences [36]. According to the present study, these plants also can be alternatives to antibiotics against some multidrug-resistant bacteria.

CONCLUSION

Juniperus oxycedrus, Eucalyptus globulus, and *Matricaria chamomilla* have been used as herbal medications since ancient times, are still popular today, and probably will continue to be used in the future because they contain various bioactive phytochemicals that could provide therapeutic effects.

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