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Research Article

ANTIBACTERIAL EVALUATION AND PHYTOCHEMICAL SCREENING OF *EUCALYPTUS GOMPHOCEPHALA DC* AGAINST *PSEUDOMONAS AERUGINOSA*

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ABSTRACT

Objective: The aim was to evaluate the *in vitro* antibacterial activities of essential oil and extracts from *Eucalyptus gomphocephala* leaf on the growth of six strains of *Pseudomonas aeruginosa.*

Methods: Aqueous, ethanol, hexane, petroleum ether extracts and essential oil of *E. gomphocephala* leaf were investigated on six strains of *P. aeruginosa*. The antibacterial activity was determined by agar-well diffusion method and expressed as the average diameter of the zone of inhibition of bacterial growth around the wells. The minimum inhibitory concentration (MIC) of active extracts was determined by microdilution assay. The preliminary phytochemical screening of the leaf was conducted using various standard methods.

Results: The present study reveals that the essential oil does not show any activity against all strains. Extracts showed varying degrees of inhibition on the tested microorganisms. The petroleum ether and ethanol had a lower activity against strains. However, aqueous and hexane leaf extracts, of *E. gomphocephala* presented the highest anti *P. aeruginosa* activity against all strains tested. The MIC and minimum bactericidal concentration of the aqueous and hexane extracts ranged between 6.25 and 50 mg/ml. Qualitative analysis of *E. gomphocephala* revealed the presence of biological active compounds such as tannins, flavonoids, saponins and steroids/triterpenes.

Conclusion: To conclude, extracts of *E. gomphocephala* leaf may be pursued as an antibacterial agent for the treatment of infections caused by *P. aeruginosa.*

Keywords: *Eucalyptus gomphocephala,* Extracts, Antibacterial activity, *Pseudomonas aeruginosa*, Phytochemical screening.

INTRODUCTION

Infectious diseases remain a serious public health problem both in developing countries, where they are the main cause of the high mortality rates recorded, and in industrialized countries where there is an alarming incidence of antibiotic resistance. There is thus an increasing need for new compounds that can act by a direct antimicrobial effect or by an indirect effect, inhibiting resistance mechanisms of microorganisms [1]. Natural resources, particularly medicinal plants traditionally used against many infectious diseases, represent a high potential for discovering such compounds [2]. In this context, *Eucalyptus gomphocephala* (Murtaceae), a medicinal plant from which leaves are traditionally used against infectious diseases in Morocco, was investigated for biological activities and phytochemical composition.

Eucalyptus is native to Australia, and the genus *Eucalyptus* contains about 600 species. The Aborigines (native Australians) have traditionally used *Eucalyptus* leaves to heal wounds and fungal infections [3]. Leaf extracts of *Eucalyptus* have been approved as food additives [4]. Recently, attention has been focused on the medicinal properties of these extracts. Research data has demonstrated that the extracts exhibited various biological effects, such as antiseptics, against infections of the upper respiratory tract [5,6], antibacterial, antihyperglycemic [7], insecticidal activities [8,9], antioxidant and natural cytotoxic [10]. The genus *Eucalyptus* is known to be a rich source of bioactive natural products including terpenoids, tannins, flavonoids, and phloroglucinol derivatives [11]. *E. gomphocephala* (Myrtaceae) is a tree up to 40 meters in height [12]. Gallic acid, quercetin, myricetin, chlorogenic acid, gentisic acid and ellagic acid have been reported as components of this species [13,14]. Previous studies of the essential oil constituents of *E. gomphocephala* revealed the presence of α-pinene, β-pinene, limonene, myrcene, 1.8-cineole, allo-aromadendrene and globulol [12], trans-pinocarveol, pinocarvone, borneol, α-terpineol, globulol [15,16].

In the present investigation, the aqueous, ethanol, hexane, petroleum ether leaf extracts and essential oil of *E. gomphocephala* were evaluated for antimicrobial activity against six strains of *Pseudomonas aeruginosa* and subsequently phytochemical analysis of the leaf was carried out to determine the active phytochemical constituents responsible for antimicrobial activity. This work represents the first study that test the antibacterial effect of extracts of *E. gomphocephala.*

METHODS

Plant material

The plant was collected during two different seasons in July, and December 2012 in Zerhoun (central Morocco), and identified at National School of Forestry Engineering (Salé).

Preparation of extracts

Aqueous extract

Dried powder (100 g) of leaves of *E. gomphocephala DC* was prepared by extracting with distilled water under reflux for 2 hrs.

Organic extracts

Air-dried powdered leaves of *E. gomphocephala* (100 g) were macerated at a room temperature in petroleum ether, hexane and ethanol respectively for 24 hrs.

All extracts were subsequently filtered. The filtrates were concentrated using a rotary evaporator at 50°C, weighed and reconstituted in dimethyl sulfoxide (DMSO) to a concentration of 0.1 g/ml. The residues obtained were stored in a freezer at 4°C until use.

Essential oil

The extraction of essential oil was made on dried powder (100 g) of leaf of the plant by a Clevenger-type apparatus for 3 hrs. The obtained oil was dried over anhydrous sodium sulfate, filtered and stored at 4°C. The yield of each extract was calculated.

Test microorganisms

Six strains of *P. aeruginosa* were used in this study: Four clinical strains (P, P3, P65, and P381) coming from the Laboratory of Microbiology of Mohammed V Hospital of Meknes, two strains coming from sewerage (P2, P5). All bacterial strains were isolated and identified. P2, P5 and P381 are the strains pigmented (synthesize pigment of pyocyanine), P, P3 and P65 are non-pigmented.

Antimicrobial activity

The agar well diffusion method was employed for the determination of antimicrobial activities of the essential oil and extracts [17]. Suspension containing 10^7 CFU/ml of bacteria was spread on Mueller-Hinton agar. Well were loaded with different concentration (5, 10, 20 ul) of the oil and 10 ul of extracts at different concentration. The plates were incubated at 37°C for 24 h. The diameters of the inhibition zones (IZs) were measured in millimeters. Negative control was prepared using DMSO. Gentamicin (15 ug/disc) was used as a positive control with the tested bacteria. All the tests were performed in triplicate.

Parameters antibacterial (minimum inhibitory concentration [MIC] and minimum bactericidal concentration [MBC])

The MICs of active extracts were determined by macrodilution according to the recommendations of the Oussou *et al.* (2004) [18]. The MIC was calculated as the highest dilution showing complete inhibition of the tested strain. All experiments were conducted in duplicate. Referring to the results of the MIC assay, the test tube showing complete absence of growth were identified, and 5 µl of each was plotted onto agar plates, and incubated at previously-mentioned times and temperatures. The MBC was the concentration at which there was no microbial growth.

Phytochemical screening

Phytochemical properties of leaf of plant were tested using the following chemicals and reagents according to the method of Bruneton (2009) [19] and Sofowora (1982) [20] Alkaloids with Mayer and Dragendoff's reagents, tannin (FeCl3), saponins (foaming test), flavonoids (chip of magnesium and HCl), sterols and triterpens (ethylic, sulfuric acid and anhydride acetic).

Statistical analysis

The triplicate data were subjected to an analysis of variance and comparison of means was analyzed using SPSS package program version 20 (IBM, SPSS Statistics 20), differences were considered significant when $p<0.05$.

RESULTS AND DISCUSSION

Quantification and phytochemical analysis of extracts

The percentage quantities of *E. gomphocephala* extracts with different solvents was 7.26% for water, 10% for hexane, 2.67% for ethanol, 0.34% for petroleum ether and essential oil 0.88%. This shows that the extraction solvent affected the percentage yield of extracts. The yield (%) of hexane and aqueous extracts has the highest, but ethanol and petroleum ether extracts have the lowest yield.

A strong correlation (R=1) that is significant at the 0.01 was found between the results of yield and antibacterial activity.

The phytochemical screenings of *E. gomphocephala* leaves (Table 1) were reported to contain tannins (gallic tannins), flavonoids, saponins, steroids/triterpene and mucilage. These compounds have been reported to possess the antibacterial activity [19,21-23]. The presence of antibacterial activity in this plant may be due to the presence of one or more bioactive compounds. The phytochemicals (gallic acid, ellagic acid, myricetin and quercetin) found in *E. gomphocephala* [13,14] investigated show significant inhibitory effect against *P. aeruginosa* [24,25].

Table 1: Phytochemical components of the leaf of *E. gomphocephala*

S. no	Name of the compounds	Name of the test	Result
1	Alcaloïdes	Mayer	-
		Dragendorff	-
2	Tannins	Ferric chloride	+
	Catechic tannins	HCL	-
	Gallic tannins	Stiasny reaction	+
3	Flavonoids: Anthocyans	H_2SO_4/NH_4OH	-
	Flavones and genins	Cyanidine reaction	+
	Leucoanthocyans	Cyanidine reaction (without Mg)	-
4	Sterol and triterpenes	Chloroform/acetic acid/H_2SO_4	+
5	Mucilage	Alcoholic precipitation	+
6	Carbohydrates	Fehling's	-
7	Oses and holosides	H_2SO_4/ethanol/thymol	+
8	Cyanogenetics glycosides	Toluène	-
9	Free anthraquinone	Chloroform/NH_4OH	-
10	Combined anthraquinone		
	O-heterosids	HCl/NH_4OH	-
	C-heterosids	$FeCl_3/NH_4OH$	-
11	Saponins	Foam test	142.85

E. gomphocephala: Eucalyptus gomphocephala

Antibacterial test

The leaves plant were extracted using polar solvents (water, ethanol) and a non-polar solvent (hexane, petroleum ether), during two different seasons (July and September). The aqueous, ethanol, hexane, petroleum ether extracts and essential oil of *E. gomphocephala* were tested against *P. aeruginosa*. The antibacterial activity of the extracts was quantitatively assessed by either the presence or the absence of IZ and by measuring the diameter of the IZ around the wells. The results of antimicrobial activity of the plant extracts are presented in Table 2.

The results revealed that all extracts showed varying degrees of inhibition on the tested microorganisms at a concentration of 100 mg/mL. The petroleum ether and ethanol extracts showed no activity on P2, P5 strains and had a lower activity against other strains during the two seasons studied. There is no statistically significant difference between seasons for these extracts ($p>0.05$). Aqueous and hexane extracts, there are a high sensitivity of all strains but different depending on the season ($p=0.00<<0.05$). Hexane extract shows a max zone of inhibition of 31 mm against strain P2. Compared to the results obtained with the control (gentamicin), the IZs obtained with hexane extract were highly interesting but no significant difference ($p\leq0.051$). For the negative control (DMSO), we have recorded no growth inhibition for all bacterial strains. Based on these results, we can suggest that there is not a general method for preparing an extract because depending on the solvent used, the activities may be different for the same plant drugs. Indeed, each species has its own personality, it is expressed differently depending on the preparation technique.

According to Paris and Hurabielle (1981) [26], the value of herbal drugs depends in part in caring for their harvest, among other things, the time of harvest. The chemical composition of herbal drugs varies with the vegetative cycle of the plant: Variations may be qualitative: The appearance of an active ingredient and disappearance of another may

be quantitative: The content of active ingredients can pass through a maximum and decreases then quickly. From the results, we can suggest that the active principles responsible for the antibacterial activity are concentrated during the summer (Fig. 1).

The essential oil of the plant revealed no antibacterial activity at the different concentrations and seasons. Study of Elaissi *et al.* (2012) [16] also shows that the active ingredient 1,8 cineol, met in large quantities in the genus *Eucalyptus* [15] and considered its antibacterial activity had no effect on *P. aeruginosa.* Another aspect is also to be considered: In fact, the pharmacological response can vary depending not only on the nature of the extract, but also on the dose. Moreover, the same drug from which two extracts were made may have the same activity but at different doses. This is the case of our plant (July) (Table 3).

The highest inhibitory activity on the microorganisms was recorded at 100 mg/ml and as the concentration of the extract decreased, the zone of inhibition also decreased (Fig. 2).

For each extract, the sensitivity results in a proportional decrease in the concentration of the extract IZ, which clearly shows that the extracts were active in a dose-response relationship (R=0.992; p<0.05).

MIC and MBC

Extracts whose showed the best activity were tested to determine the MIC and MBC. The MIC and MBC of the hexane and aqueous extracts of the leaves ranged between 6.25 and 50 mg/mL (Table 4).

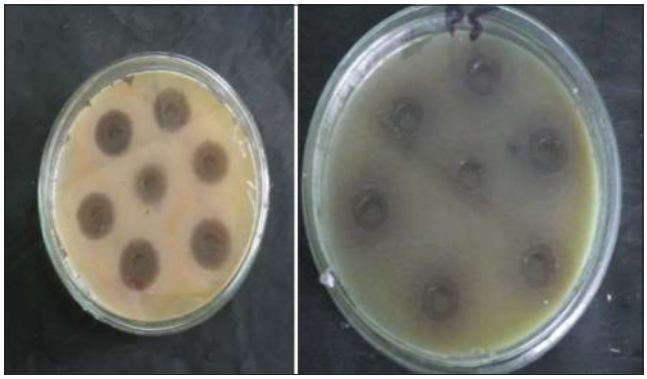

Fig. 1: Antibacterial activity of aqueous extract (100 mg/ml) in July (left) and December (right)

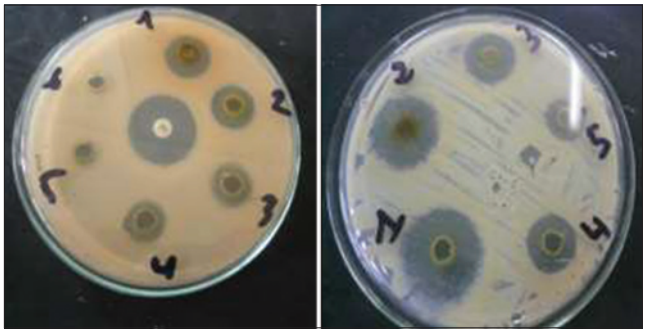

Fig. 2: Antibacterial activity of aqueous (left) and hexane (right) extracts at different concentration (1, 2, 3, 4, 5, 6 respectively 80 mg, 70 mg, 50 mg, 30 mg, 10 mg, 5 mg; CN: Gentamicin 15)

Table 2: Zone of inhibition the polar and non-polar extracts of *E. gomphocephala* in the month of July and September (100 mg/ml)

Strains	Aqueous extract		Ethanol extract		Hexane extract		Petroleum ether extract		Gentamicin
	Jul	Dec	Jul	Dec	Jul	Dec	Jul	Dec	
P	15±1.59	10±1	12.5±1.5	13.33±0.47	25.5±2.5	11.33±0.47	12.5±2.5	14.66±0.34	25.5±1.5
P3	15±2.12	10±1	13±00	13.33±0.47	25±00	16.33±0.47	11±01	12±01	24.5±1.5
P65	16.66±0.85	10±0	10±01	10.33±0.47	24±01	14.16±0.47	9±00	11.66±0.23	21.5±1.5
P381	15.75±2.74	9±0	9±01	10.33±0.47	28.5±1.5	14.33±0.47	11±00	11.33±0.47	22.5±0.5
P2	15.25±3.24	9±0	-	-	29±02	15±01	-	-	20±01
P5	15.66±0.94	9±0	-	-	25±00	14±1,41	-	-	21.5±0.5

E. gomphocephala: Eucalyptus gomphocephala, Jul: July; Dec: December

Table 3: The effect of the hexane and aqueous extracts (mm) in different concentrations (mg/ml)

Strains	Aqueous extract (mg/ml)						Hexane extract (mg/ml)					
	80	70	50	30	10	5	80	70	50	30	10	5
P	16.5±2.5	15±1.5	15±2	14±3	12.5±1.5	10±1	25.5±2.5	18.5±1.5	13±1	10±1	7±1	-
P3	16.5±1.58	15±2	13±2	11.5±2.5	10±1	9,5±0,5	21±4	20±4	15±0	13.5±0.5	9±0.5	7±0.5
P65	13±2	12.5±2.5	9.5±1.5	9±1	8.5±0.5	7±0	20±1	15±1	12±0	10±0	8±1	6±1
P381	13,5±1.5	11.5±0.5	11±1	10.5±0.5	8±1	6±1	21.5±0.35	12.5±0.35	10.5±0.5	9.5±0.5	9±0.5	8±0
P2	13.5±2.5	10.5±2.5	11±1	-	-	-	21±1	12±1	11±1	8±0	6±1	-
P5	15±1	14.5±0.5	12±1	11±1	-	-	20±1	13±0,5	11±1	7±0	-	-

Table 4: Antibacterial parameters (MIC and MBC) of the hexane and aqueous extracts

Strains	Aqueous extracts (mg/ml)			Hexane extracts (mg/ml)		
	MIC	MBC	MBC/MIC	MIC	MBC	MBC/MIC
P	12,5±00	18.75±6.25	1-2	9.37±3.12	12.5±00	1-2
P3	6,25±00	12.5±00	2	12.5±00	18.75±6.25	1-2
P65	12,5±00	12.5±00	1	18.75±6.25	50±00	1-4
P381	6,25±00	9.37±3.12	1-2	18.75±6.25	25±00	1-2
P2	9,37±3,12	9.37±3.12	1	50±00	50±00	1
P5	9,37±3,12	12.5±00	1-2	50±00	50±00	1

MIC: Minimum inhibitory concentration, MBC: Minimum bactericidal concentration

The MBC/MIC ratio sets the bacteriostatic or bactericidal nature of the extract. The MBC/MIC of hexane and aqueous extract of *E. gomphocephala* are equal to 1 or 2 reports. Extract studied seem to be bactericidal activity against *P. aeruginosa*. Highest MIC and MBC for hexane extract were those for pigmented strains; however it is the opposite for the aqueous extract. The hexane extract elucidate that MIC and MBC are higher than aqueous extract, but it is difficult to say that the difference is significant (p≤0.045). The correlation between MIC and IZs of hexane (p>0.005) and aqueous (p>0.05) extracts is low non-significant.

CONCLUSION

The present study confirmed antimicrobial properties of extracts from *E. gomphocephala* that showed significant growth inhibition for *P. aeruginosa* tested whose the problem relates to the emergence of strains that possess multiple resistances to a range of antibiotics, thereby making them difficult to treat. The phytochemical analysis showed the presence of effective biological compounds like glycosides, flavonoids, terpenoids, tannins and saponins. These derivatives could be potential control of clinical pathogenic bacteria [27].

The encouraging results indicate the *E. gomphocephala* might be exploited as natural antibiotic for the treatment of several infectious diseases caused by this germ, and could be useful in understanding the relations between traditional cures and current medicines.

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