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Antimicrobial activity of xerophytic plant (*Cotula cinerea* Delile, 1831) extracts against some pathogenic bacteria and fungi

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ABSTRACT

Objective: To investigate the antimicrobial activities of sequentially different solvent extracts of an Algerian commonly available plant namely *Cotula cinerea*, against five different human pathogenic microbes namely, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *Escherichia coli* and *Candida albicans*. **Methods:** The antimicrobial activity was evaluated using agar disc diffusion method. Aerial parts of *C. cinerea* were extracted using solvents of different polarity (70% ethanol, *n*-butanol, ethyl acetate and petroleum ether). **Results:** Petroleum ether and *n*-butanol extracts have the most effective antimicrobial activity while Gram-negative *K. pneumoniae* was most sensitive. Linear regression analysis was performed to find correlations between extract concentrations and inhibition activity. Results showed a significant increase in mean diameter of inhibition zone with increasing extract concentrations of all solvent except *n*-butanol. Two-way ANOVA test was used to compare the effect of *Brocchia cinerea* extracts on the antimicrobial properties. All plant extracts have shown significant differences in their actions as antimicrobial agents. **Conclusion:** The inhibition of microbial growth at low concentration as 25% of 1mg mL⁻¹ indicated a potent antimicrobial activity of *B. cinerea* extracts.

1. Introduction

Medicinal plants, which form the backbone of traditional medicine, have been in the last few decades the subject of very intense pharmacological studies. This has been brought by the acknowledgement of the value of medicinal plants as potential sources of new therapeutic compounds and drug development[1]. According to the World Health Organization, about 80% of the world's population living in developing countries relies essentially on plants for primary health care[2]. In recent years, pathogenic microorganisms have developed a multiple drug resistance through the indiscriminate use of commercial antimicrobial drugs commonly used in the treatment of infectious diseases [3]. Therefore, there is a need to develop alternative antimicrobial drugs from various sources such as medicinal

plants[4].

Plants with antimicrobial activities have become more interesting because many people are aware of problems associated with the over-prescription and misuse of traditional antibiotics. However, only about 20% of the plants found in the world have been subjected to a pharmacological or biological testing[5]. Plants in the environment are exposed to a range of abiotic stresses like osmotic, salinity and temperature, which affect their growth and other metabolism process such as a wide variety of secondary metabolites production, like polyphenols, tannins, terpenoids, alkaloids etc, which may have antimicrobial properties[6, 7].

The Algerian flora plays a key role in supporting traditional medicine, which is widely practiced in the country. This flora holds a rich diversity of medicinal and endemic plants[8]. Many of these plants used in Algerian traditional medicine have the potential to provide pharmacologically active natural products[9, 10]. Ethnopharmacological interest in the sources of these compounds has increased nationally and worldwide, particularly in the search for drugs to counter multi-

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Table 1

Resistance pattern of target organisms to antibiotics.

Strain	Phenotype of resistance	Phenotype of sensibility
<i>Staphylococcus aureus</i>	AMC, AMX, CE, CF, OX, PEN	CHL, CIP, GEN, PEF
<i>Escherichia coli</i>	AMC, AMX, CIP, PEF, SXT	CE, CF, CEF, C, GEN, IMI, STR
<i>Pseudomonas aeruginosa</i>	AMC, AMX, ATM, CF, CIP, PEF, SXT	GEN, IMI, PEF, PIP
<i>Klebsiella pneumoniae</i>	IMI	C, SXT
<i>Candida albicans</i>	ND	ND

(AMC: amoxicillin + acid clavulanic, AMX: amoxicillin, ATM: aztreonam, C: chloramphenicol, CE: cefatxin, CF: cefalotin, CEF: cefazolin, CIP: ciprofloxacin, GEN: gentamicin, IMI: imipenem, ND: not determined, OX: oxacillin, PEF: pefloxacin, PEN: penicillin, PIP: piperacillin, STR: streptomycin, SXT: trimethoprim/sulfamethoxazol).

resistant microorganisms.

Cotula cinerea L., syn. *Brocchia cinerea* Del. (Asteraceae), a xerophytic plant widely distributed in sandy and desert grounds^[11, 12]. This medicinal plant popularly known as (Gartoufa or Chouhiya), is commonly used in Algerian folk medicine, as well in the rest of the Maghreb region, like an anti-inflammatory, analgesic, antipyretic, antiseptic and for treatment of various diseases, including digestive problems (constipation, colics), rheumatism, urinary and pulmonary infections. It is much appreciated in green tea or mixed with food to enhance the flavour^[9, 10]. Several compounds have been isolated from *B. cinerea*, including flavonoids, sesquiterpene lactones, sesquiterpene coumarins and tannins^[13, 14]. The objective of this work is to evaluate the antimicrobial activity of *B. cinerea* extracts, brought by deferent solvents, against some human pathogenic bacteria and fungi species.

2. MATERIALS AND METHODS

2.1. Plant material

Brocchia cinerea samples were collected from its natural range of distribution in El-Oued (Algerian Sahara Desert) (about 4km Southeast of El-Oued city; 33° 20'N to 33° 19'N, 6° 52'E to 6° 53'E) in March 2011.

2.2. Extraction protocol

2.2.1. Extracting solvents

The extraction was carried out by using solvents of different polarity: (i) petroleum ether (non-polar), (ii) 70% ethanol, (iii) *n*-butanol, (iv) ethyl acetate (all three moderately polar).

2.2.2. Preparation of plant

The freshly picked aerial parts of the plant used in the screening were air-dried at room temperature for 2 weeks, with no direct sunlight. Once dried, plant was ground into fine powder and stored at 4 °C until extraction.

2.2.3. Preparation of the extracts

The powdered plant material (20 g) was macerated for 24 h (3–24h) in a mixture of ethanol/water (70:30; v/v) with frequent agitation at room temperature (25±1 °C). Then the mixture was filtered using filter paper (Whatman No. 1) under the vacuum of a water pump and the ethanol was evaporated under low pressure using a rotary evaporator at 50 °C. The residue was taken as the hydro alcohol extract^[15]. The remaining aqueous extract was partitioned with petroleum

ether, ethyl acetate and *n*-butanol (3×100 ml for each solvent). These extracts were dried under reduced pressure using a rotatory evaporator at 40 °C. The residues were taken as the petroleum ether, ethyl acetate and *n*-butanol extracts of the plant^[16].

2.3. Antimicrobial activity

2.3.1. Microbial strains and growth conditions

Five clinical isolates of microorganisms were used to assess the plant-antimicrobial properties, including the Gram-positive *Staphylococcus aureus*, the Gram-negative *Escherichia coli*, *Pseudomonas aeruginosa* and *Klebsiella pneumoniae*, while the yeast-like fungus was *Candida albicans* (see Table 1 for strains and their resistance phenotypes). All strains were obtained from the Microbiology Laboratory of Hospital Benamor Djilani (El-Oued, Algeria) and were maintained at 4 °C on slants of Nutrient Agar (NA) for bacteria and Sabouraud Dextrose Agar (SDA) for the yeast. Active cultures were prepared by transferring a loop of cells from the agar slant to a test tube containing 5 ml of Nutrient Broth for bacteria and Sabouraud Dextrose broth for the yeast. They were then incubated overnight to the logarithmic phase of growth; about 6–10 hours at 37 °C for bacteria and 12–16 h at 30 °C for the yeast.

2.3.2. Antimicrobial assay (Disk diffusion assay)

The disc-diffusion assay^[17] was used to determine growth inhibition caused by plant extracts. Inoculum, containing 10⁶–10⁸ CFU per milliliter, was spread on Mueller-Hinton (MH) agar plates for bacteria and 10⁴–10⁵ CFU per milliliter was poured over the base plates forming a homogenous top layer on SDA for fungus strain. Using a sterile forceps, Whatman's filter discs (Ø = 5 mm), impregnated with different dilutions of extracts [25, 50, 75 and 100%] from the initial concentration of 1 mg ml⁻¹, were deposited on inoculated plates and left at 4 °C for 2 h before their incubation to allow the diffusion of the extract. Saturated discs with solvents (ethanol, petroleum ether, ethyl acetate, and *n*-butanol) were air-dried then used as negative controls. The plates were evaluated after incubation at 37 °C for 24 h for bacterial and 48 h at 30 °C for fungus, after which, inhibition zones around each disc were measured (disk diameter included).

2.4. Statistical analyses

The aim of linear regression analysis (LRA) was to find a statistically significant correlation between

different concentrations of each extracts and their overall antimicrobial activity assessed as diameter of inhibition disregarding tested strains. The analyzed data pertaining to antimicrobial activity of different *B. cinerea* extracts was also subjected to two-way ANOVA. The ANOVA was performed to test the effect of dilution levels of extracts and the tested species on the level of antimicrobial activities. Interaction between (tested microbe-species * extract concentration) was also included in the analysis for each plant extract. Besides the explanatory ability of LRA, it could be used, supported by ANOVA outputs, to detect potency "effectiveness" of the extract itself disregarding its concentration. Both ANOVA and LRA were considered statistically significant (*), highly significant (**), or very highly significant (***) probability-value $p < 0.05$, $p < 0.01$ and $p < 0.001$, respectively.

3.RESULTS

Hydro-alcohol extract of *B. cinerea* shown much higher extraction yield (w/w %) than other extracts, of which *n*-butanol extract had the lowest yield. Since extract yield increases with extracting solvent polarity. As a result, 70% ethanol, which was the most polar of all solvents and that was used for fractionation, has afforded the maximum yield (11.0) compared to petroleum ether (1.0), *n*-butanol (6.0) and ethyl acetate (1.2).

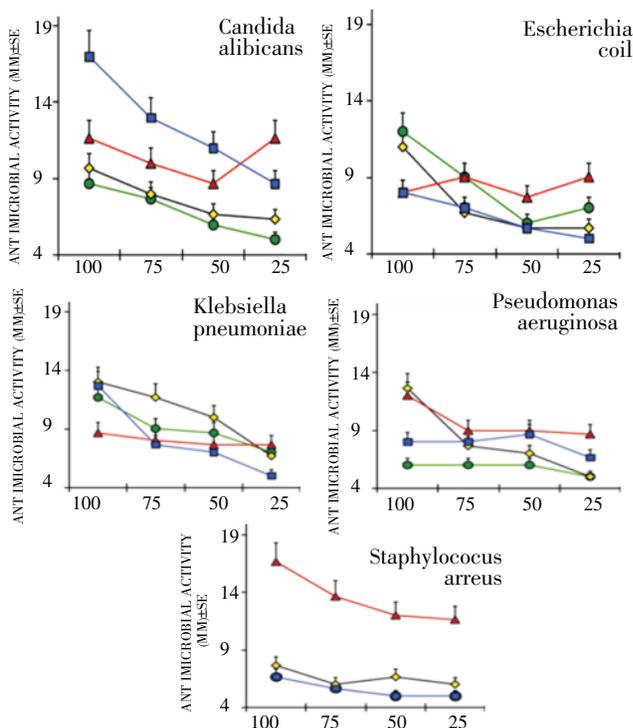


Figure 1. Antimicrobial activity (mm±SE) of *B. cinerea* extracts (Initial concentration of extract is 1 mg ml⁻¹, disc diameter = 5 mm).

The inhibition zone, referring to antimicrobial activity of *B. cinerea* extracts, was measured after incubation of the plates. Each of the extracts was tested three times and the average (\pm SD) of three values was determined (Figure 1). Generally, the results showed that the inhibitory effect of extracts increased with increasing of concentrations. Although the

antimicrobial activity assayed for *B. cinerea* extracts showed an overall inhibition effect against *K. pneumoniae* (16.67±5.77 mm) with *n*-butanol extract, and 17±1.73mm with petroleum ether extract. Little activity was observed of the tested strains (*E. coli*, *P. aeruginosa*, *S. aureus* and *C. albicans*) at a concentration of 0.25 mg mL⁻¹. The petroleum ether extract was twice as active against *P. aeruginosa*. High activity against *S. aureus* was found with *n*-butanol and ethyl acetate extracts where inhibition zones equal to 12±5.20 and 11.67±3.79 mm, respectively. Moreover, the hydro alcohol extract was found to be the most active extract against *E. coli* (Figure 1). Similarly, only the hydro alcohol extract of *B. cinerea* had antiyeast activity against *C. albicans*. Comparing the results of growth in inhibition zones for all four extracts, it is evident that petroleum ether extract possesses moderate antimicrobial properties as compared to the most active extract (*n*-butanol) and weakly active extracts (ethyl acetate and hydro alcohol).

In the latter case, a moderate activity was observed against *K. pneumoniae*, *P. aeruginosa* and *S. aureus* of *n*-butanol extract at 25% of concentration whereas *C. albicans*, *E. coli* and *P. aeruginosa* remained uninhibited at equal concentration of ethyl acetate extract. A moderate activity was also observed against all tested bacteria and fungi at 25% of hydro alcohol extract.

The susceptibility of microbial species to crud *B. cinerea* extracts was in the following decreasing order: For hydro alcohol extract, *K. pneumoniae* > *P. aeruginosa* > *E. coli* > *C. albicans* > *S. aureus*. For petroleum ether extract, *K. pneumoniae* > *P. aeruginosa* > *E. coli* > *S. aureus* > *C. albicans*. For *n*-butanol extract, *K. pneumoniae* > *S. aureus* > *P. aeruginosa* > *E. coli* > *C. albicans*. For ethyl acetate extract, *K. pneumoniae* > *S. aureus* > *E. coli* > *P. aeruginosa* > *C. albicans*.

Table 2.

Linear Regression analysis applied between extract dilutions and antimicrobial activity. (C*I: Concentrations*Inhibition, ***: very highly significant).

Extracts	Slope	Intercept	R ²	S(C*I)	SS(I)	F	P
Hydro alcohol	6.16	4.13	0.304	28.88	584.98	25.34	0.0000 ***
Petroleum ether	5.60	4.57	0.224	26.25	655.73	16.76	0.0001 ***
<i>n</i> -butanol	2.37	8.53	0.030	11.13	886.98	1.78	0.1874
Ethyl acetate	4.29	4.47	0.192	20.13	449.65	13.80	0.0004 ***

The linear regressions have slopes that were statistically significantly non-zero, i.e. one can assume that a relationship exists between inhibition activity and extract concentrations of hydro alcohol ($F = 25.34$; $p < 0.00001$), Petroleum ether ($F = 16.76$; $p = 0.0001$), and Ethyl acetate ($F = 13.80$; $p = 0.0004$). However, the linear regression slope was not statistically significantly different from zero ($F = 1.78$; $p = 0.1874$), indicating there was no significant trend between *n*-butanol extract concentrations and inhibition zone (Table 2). Additionally comparison of regressions showed that slopes were not significantly different ($F = 1.4$; $p = 0.2325$) while the intercepts were highly significantly different ($F = 9.5$; $p < 0.00001$).

Two-way ANOVA revealed that all plant extracts have shown significant differences in their actions as antimicrobial agents with either tested strains or extract concentration or even their interaction. ANOVA Fisher-

values in “Strain test” and “Extract dilutions” factors were all highly or very highly significant for the four extracts (except in dilutions of *n*-butanol). Thus antimicrobial activity of *B. cinerea* varied significantly between germs (in particularly within Petroleum ether extract $F = 39.2$, $p < 0.001$) and according to dilutions (especially within Petroleum ether extract $F = 23.8$, $p < 0.001$). In general, the interaction effect of the two factors (Strain test * Extract concentration) has no statistically significance on variation of antimicrobial activity in all extracts except Petroleum ether ($F = 3.3$; $p = 0.002$) (Table 3).

Table 3

Two-way analysis of variance (ANOVA). (**: highly significant, ***: very highly significant).

Plant extracts	Variation sources	SS	DF	MS	F	P	
Hydro alcohol	Strain test	97.6	4	24.4	4.1	0.007	**
	Extract dilutions	191.3	3	63.8	10.6	<0.001	***
	Interaction	56.2	12	4.7	0.8	0.667	
	Residual Error	240.0	40	6.0			
	Total	585.0	59	9.9			
Petroleum ether	Strain test	334.4	4	83.6	39.2	<0.001	***
	Extract dilutions	152.4	3	50.8	23.8	<0.001	***
	Interaction	83.6	12	7.0	3.3	0.002	**
	Residual Error	85.3	40	2.1			
	Total	655.7	59	11.1			
<i>n</i> -butanol	Strain test	229.4	4	57.4	4.1	0.007	**
	Extract dilutions	45.5	3	15.2	1.1	0.370	
	Interaction	48.7	12	4.1	0.3	0.988	
	Residual Error	563.3	40	14.1			
	Total	887.0	59	15.0			
Ethyl acetate	Strain test	120.9	4	30.2	6.0	<0.001	***
	Extract dilutions	90.2	3	30.1	6.0	0.002	**
	Interaction	38.6	12	3.2	0.6	0.793	
	Residual Error	200.0	40	5.0			
	Total	449.7	59	7.6			

4. DISCUSSION

Infectious diseases represent a serious public health problem and remain the major cause of death throughout the world. Alternative natural products of plants could be of high interest to mitigate the increasing incidence of resistance to antibiotics. Some plant extracts and phytochemicals are known to have antimicrobial properties, which could be of great importance in the therapeutic treatments. In the last years, various studies have been conducted in different countries, demonstrating the efficacy of this type of treatment [18, 19, 20]. Algeria recently also increased researches in traditional herbal medicines following scientific findings testifying of their effectiveness in healing several health issues.

The present investigation explored the use of one such plant, *B. cinerea* Del., endemic in North Africa, for treating infectious diseases. The antimicrobial activity assayed for *B. cinerea* extracts showed that the hydro alcohol extract was less active against the tested strains, and only the relatively polar fraction (*n*-butanol) was more active against the germs. The ANOVA confirm that *n*-butanol showed a

high significant variation in antimicrobial activity against tested strains, however there is no significant variation for its concentrations, which was also demonstrated by LRA. This result supported the fact that active compounds are concentrated more in this fraction. The other extracts (Hydro alcohol, Petroleum ether and Ethyl acetate), they may be more effective when they are used with high concentration. This statement was clearly revealed either by LRA or by ANOVA. The wide range of variation in antimicrobial activity shown by *B. cinerea* extracts might be reflecting the differences in chemical concentration and composition among the plant extract with each solvent. A successful extraction procedure of active botanical compounds from plant material is dependent on the type of solvent used in the extraction procedure [21, 22, 23].

In this report, an extraction procedure was performed to extract components of the plant-sample using a combination of several solvents. For the antibacterial activity, the plant extracts were active against both Gram-positive and Gram-negative bacteria, though they were more active against the later. Concerning the anticandidal property, the present study showed no or weak activity for plant extracts. In our study, the highest activity was recorded against the Gram-negative bacteria: *K. pneumoniae*, which was the most susceptible bacterium of all the tested strains. These results may be of great importance in healing procedures since *K. pneumoniae* can be commonly involved in urinary, intra-abdominal and respiratory infections [24, 25].

Markouk et al. [11] reported that acetate extract of *B. cinerea* collected from Zagora (Sothern Morocco) exhibited an antibacterial effect with MIC of 200 μ g/mL against all tested bacteria and *n*-butanol extract has been shown to be highly effective, especially against *Pseudomonas fluorescens* and *Bacillus* sp., with MIC of 12 μ g/mL. In the same study, ethyl ether extracts of *B. cinerea* were found to be inactive against all tested bacteria. These results are in concordance with ours, which confirm that bioactive components of any plant may differ in their solubility depending on: (i) the extraction solvents [21, 26], (ii) the nature of biologically active components such as alkaloids, saponins, tannins, phenols etc. [26, 27], and (iii) the geographical origin of plant material [28], because ecological conditions in general (including abiotic factors “edaphic, climatic, water stress...” or biological interactions “such as intra and/or interspecific competitions...”) may have large impact on growth and fitness of vegetation species [4, 27]; particularly by affecting their metabolism process within secondary metabolites production [5]. Ahmed et al. [14] reported that *B. cinerea* is particularly rich with flavonic compounds besides sesquiterpene-lactone and sesquiterpene coumarins, which have been also isolated from this plant.

The results reported here can be considered as the first information on the antimicrobial properties of *Brocchia cinerea*, an endemic species of the Algerian Saharan flora. This may also contribute to the knowledge of antimicrobial properties in *Brocchia* species reported elsewhere.

5. CONCLUSION

In the light of this study, *B. cinerea* is a prospective wild

plant for the isolation of new antimicrobial substances. Although further investigations are clearly necessary to clarify and identify the bioactive constituents, we believe that our results presented herein may be a contribution for other researchers, who would deeply investigate the antimicrobial activity of *B. cinerea*. Moreover, our results on antimicrobial assays has justified and partially supported the Algerian common usage of the plant. The screening of some medicinal plant crud extracts has shown that some of those were potentially rich sources of antimicrobial agents.

Finally, promoting human well being deserves to join efforts in considering and valorising Saharan natural patrimony, as well as carrying out more scientific research on plants living in drylands with considering both chemical, biological, toxicological and pharmacological investigations as well therapeutic aspects.

Conflict of interest statement

We declare that we have no conflict of interest.

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