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ORIGINAL ARTICLE

Study of Antimicrobial Activity of Secondary Metabolites Extracted Fromspontaneous Plants from the Area of Laghouat, Algeria

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ABSTRACT

In the present study, we attempted to evaluate the antibacterial and antifungal potential of plant secondary metabolites: phenolic compounds, alkaloids and essential oils. These metabolites were extracted from eight spontaneous plants collected in the area of Laghouat, in the north of the Algerian desert. *Material and methods*The investigated plants are: *Datura stramonium*, *Peganum harmala*, *Ricinus communis*, *Nerium oleander*, *Citrullus colocynthis, Cleome arabica, Pistacia atlantica* and*Pistacia lentiscus*.The total phenolic compounds were extracted and quantified by UV-Visible Spectrophotometry. The essential oils of *Pistacia atlantica* and *Pistacia lentiscus* were obtained by hydrodistillation and analysed by GS/MS.The alkaloids were extracted from *Datura stramonium*, *Peganum harmala*, *Ricinus communis*, *Nerium oleander*, *Citrullus colocynthis and Cleome arabica*. The concentrations of the alkaloidic extracts were evaluated by UV-Visible. The antimicrobial activity of the extracts was assessed by the agar disc diffusion method on three bacteria and three fungi strains. The MIC evaluation of the active extracts was performed by the broth dilution method. *Results and conclusion:* The phenolic compounds obtained from the investigated plants did not exhibit any antimicrobial activity against the tested strains. The essential oils of *Pistacia atlantica* and *Pistacia lentiscus* showed an inhibiting activity against *Escherichia coli* ATTC 25922, *Staphylococcus aureus* ATTC 43300 MRSA+, *Pseudomonas aeruginosa* ATTC 27853 and *Fusarium oxysporum f. sp. albedinis* and *F. oxysporum f. sp. lycopersici.* The MIC of *Pistacia lentiscus* essential oil on bacteria was 0.25% (v/v). The alkaloidic extract of *Ricinus communis* was effective against *E. coli* ATTC 25922 and the MIC value determined is 0.02 mg/ml.

Key words: plant, phenolic compounds, essential oils, alkaloid, antibacterial activity, antifungal activity, agar disc diffusion method, broth dilution method

Introduction

During the twentieth century, when exploring the natural environment, man has made great discoveries that have enabled him to use a considerable number of natural resources.

Phytotherapy, the use of plants to medical purposes, is one of the oldest practices in the world. The traditional practice, based on empirical data, is considered as folk medicine and the approach based on scientific studies aims to extract and study active components from plants.

The toxic organic substances of plants present a various chemical composition (tannins, flavonoids, coumarins, alkaloids, terpenoids, lactones …etc). Furthermore, a significant number of microorganisms are pathogenic to Man, animal or plant. The damage caused by this microbial

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activity can lead to socio-economic and human losses.

As proved with the antibacterial effect of essential oils extracted from several species of thyme [8,10], many researches devoted to substances extracted from plants have shown an antimicrobial activity. Thus, it was established that essential oils, extracted from plants such as laurel, sage and rosemary, inhibited the growth of bacteria (*Staphylococcus aureus, Klebsiella pneumoniae*, *Enterococcus feacalis, Escherichia coli, Staphylococcus epidermidis* ... etc.) and fungi (*Candida albicans, Aspergillus Niger* and *Fusarium sp*.)[2,14,7].

The area of Laghouat (Algeria) has pre-Saharan characteristics. It offers, despite its geographical location and climate, a considerably rich flora. Some of these species are known for their aromatic and/or medicinal properties [5].

In the present work, we tested the antimicrobial activity of extracts of eight spontaneous plants, widely distributed in the region of Laghouat (Algeria), on pathogens of humans, animals and plants.

Our study was divided into two main steps:

Initially, we performed a chemical extraction and quantification of secondary metabolites (phenolic compounds, alkaloids and essential oils) from the studied plants;

Then, we tested the antimicrobial activity of crude plant extracts on pathogenic microorganisms.

Materials and Methods

Plant Material:

Eight plants were selected for this study: *Cleome arabica*, *Citrullus colocynthis*, *Datura stramonium*, *Nerium oleander, Peganum harmala* and *Pistacia atlantica* were collected in the area of Laghouat (400 km south of Algiers); *Ricinus communis* was obtained from Beni Isguen (Ghardaia, 600 km south of Algiers); and *Pistacia lentiscus* was collected from Berouaguia (Media, 88 km south of Algiers).

Only the leaves of the plants were used, after being collected and dried, to perform the extraction of the secondary metabolites.

Plant Extracts:
Extraction of Phenolic Compounds:

The total phenolic compounds were extracted from the eight plants according to the method proposed by Djeridane *et al*. [9]. The concentrations of the methanolic extracts were evaluated following the adapted method proposed by Singleton *et al.* [19], using Folin-Ciocalteu's method. The quantification was performed by UV-Visible Spectrophotometry.

Extraction of the Essential Oils:

The essential oils of *Pistacia lentiscus* and *Pistacia atlantica* were extracted by hydrodistillation (Buchbauer, 2000 *in* Lahlou, [21] then analysed by GC/SM.

Extraction of Alkaloids:

The alkaloids were extracted from only six plants. The extraction protocol was according to the adapted method proposed by Ross and Rain [15].

The quantitative analysis of the ethanolic alkaloidic extracts was performed following the adapted method proposed by Singh *et al*. [24]. The quantification was conducted by UV-Visible Spectrophotometry.

The Microbial Strains:

Six microbial strains were analyzed. We have chosen three bacterial species, and a fungus as pathogens of Human and animals: *Escherichia coli* ATTC 25922, *Staphylococcus aureus* ATTC 43300 MRSA+, *Pseudomonas aeruginosa* ATTC 27853; and *Aspergillus fumigatus*.

Two phytopathogenic fingi were studied: *Fusarium oxysporum f. sp. albedinis* (*Foa*) and *F. oxysporum f. sp. lycopersici* (*Fol*).

The bacterial strains were kindly provided by the laboratory of Microbiology, Faculty of Biological Sciences, USTHB, Algeria.

A. fumigatus has been provided by the Laboratory of Mycology, Institute Pasteur Dely Brahim, Algeria.

Isolates of *Fusarium oxysporum* were a gift of the laboratory of Phytopathology, National Institute of Agricultural sciences, El-Harrach, Algiers.

Growth Conditions:

The bacterial strains were maintained on nutrient agar; and the fungal strains on PDA medium.

Overnight cultures of the bacterial strains were prepared in nutrient broth, and then incubated for 24 h at 37°C as proposed by Hammer *et al.*, [14] and Faleiro *et al.*, [12]. The fungal strains were incubated on PDA 5 to 7 days at room temperature before performing the susceptibility tests.

Susceptibility Tests:
Agar Disc Diffusion Method:

In preliminary tests of antimicrobial activity, we adopted the agar disc diffusion method standardized by the WHO report (1977 *in* [22]. The bacterial strains were incubated on Mueller-Hinton medium, and the fungal strains on PDA medium.

Overnight cultures of bacteria were used to prepare dilutions in saline solution (0.9%, w/v) to a turbidity of 0.5 Mac Farland standards (10^8 CFU/ml) [18]. The dilutions of fungi were prepared in saline solution from the cultures on PDA (5-7 days), corresponding to a concentration of 10^6 cells/ml [26].

Once the agar on Petri dishes (90 mm) seeded, disks of Whatman No. 6 paper (6 mm in diameter) [11], were soaked, each, of a fixed volume of 20 µl of plant extract. Discs were deposited at equal distances on the surface of the agar. We used as a negative control disc impregnated with solvent without extract.

The Petri dishes were left at room temperature for 20 to 30 min before incubation to ensure a good diffusion of the extracts in the agar [11]. Cultures were then incubated at 37°C for 24 h for bacteria and at room temperature for 48 h for fungi [4].

Each test was repeated 3 times and the antimicrobial activities were evaluated by measuring inhibition zone diameters.

The concentrations of the tested solutions were prepared from decimal dilutions of crude extracts in the convenient solvent (methanol/ethanol).

MIC Determination by the Method of Broth Dilution:

MICs were determined using the nutrient broth for the bacterial strains, and GYP broth for fungi. Only active dilutions with concentrations less than or equal to 1 mg/ml were tested.

Constant volumes of liquid culture medium (5ml) were distributed in test tubes. Subsequently, we inoculated each tube with 1 ml of bacterial or fungal suspension (10^8 cells / ml for bacteria; and 10^6 cells/ml for fungi). Afterward, 500µl of each solution was added into each tube at increasing concentrations. A negative control tube was inoculated without extract. The test tubes were incubated for 24 hours at 37°C for bacteria and 48 to 25°C for fungi. During the incubation period, the tubes were submitted to a manual agitation every hour. After incubation, the MIC was recorded as the lowest concentration demonstrating no apparent growth, compared to the negative control.

Results and discussion

Quantitative Analysis of Plant Extracts:
Quantification of Total Phenolic Compounds:

The concentrations in total phenolic compounds of the extracts, equivalent of gallic acid, are shown in Table 1.

The quantitative analysis reveals that the *Pistacia atlantica* presents the highest concentration of total phenolic compounds from the eight plants with 6039.80mg/100g of dry matter. The concentrations of the other plants ranged from 206.25mg/100g to 1884.80mg/100g. The lowest levels of total phenolic compounds were recorded for *Cleome Arabica* with 191.20mg/100g, and for *Peganum harmala* with 34.80mg/100g of dry matter.

Essential Oils:

The yields of essential oils, expressed in ml/100g of dry matter, are presented in Table 2.

The levels of essential oils in the leaves of *Pistacia atlantica* and in *Pistacia lentiscus* are, respectively, 0.13% and 0.17%. The yields of the two plants are close, but low compared to other species. For example, *Mentha pulegium* (collected in Bulgaria), presents a yield of 1.54% in essential oil [25].

GC/MS analysis has revealed more than 100 compounds, of which the majority has been identified by the library of the mass spectrometer, in the essential oils of the tow studied species of *Pistacia*.

Thus, the major compounds of the essential oil of *Pistacia atlantica* cited in percentage in decreasing order are: Terpinen-4-ol (12.58%), *T*-Muurolol (5.22%), Viridiflorol (4.69%), Spathulenol (4.58%), *Cis*-3-hexenyl benzoate (4.38%), and δ-cadinen (3.53%).

In the study undertaken by Gourine *et al.* [13], the authors revealed that the major compounds of essential oil from leaves of *Pistacia atlantica,* collected in August from the same geographical area as our sample, were: β-Pinen (19.08%), α-Terpineol (12.82%), Bicyclogermacren (8.15%) and Spathulenol (9.45%). We notice the difference in the chemical composition of both oils. The reasons of this significant difference could be: the season of collection, experimental conditions of extraction and analysis.

Barrero *et al*. [3], studied the essential oil from leaves of *Pistacia atlantica* collected in Marrakesh, Morocco, GC/MS revealed that the

major compounds of this oil were: Terpinen-4-ol (21, 7%) and Elemol (20.0%), suggesting a chemical composition difference with our oil.

In the essential oil of *Pistacia lentiscus*, the major compounds, expressed as a percentage in decreasing order, are: β-Caryophyllen (17.09%), Myrcen (7.05%), δ-Cadinen (6.91%), *T*-Muurolol (6.71%), α-Terpineol (4.91%) and α-Humulen (4.04%).

A study conducted by Kivçak *et al*. [20] on the essential oil of *Pistacia lentiscus* leaves, collected in Turkey, has revealed that the major compounds of this extracts were: Terpinen-4-ol (29.2%), Caryophyllen (29.2%), and *p*-Cymene (7.1%).

The essential oil of *Pistacia lentiscus* (700 km east of Algiers), extracted and studied by Benyoussef *et al*. [6] presents, as major compounds: Terpinen-4-ol (17.3 to 34.7%), α-Terpineol (10.4 to 11.0%) and δ-Germacrène (8.4 to 15.8%).

The differences noticed between our oils and the essentials oils studied by theses authors could be due to the nature of the soil, of the plant from which the sample was taken (male or female), climate and the season of the samples collection. The experimental protocols followed for oil extraction and analysis could also influence the chemical composition.

Alkaloids:

The concentrations of the alkaloidic extracts expressed in g/100 g dry matter, equivalent to caffeine, are presented in Table 3.

As shown in the table 3, the alkaloidic extracts of *Citrullus colocynthis* and *Peganum harmala* present the highest concentrations of alkaloids, approaching 20g/100g. The concentrations of the other extracts ranged between 8.140 and 11.990 g/100g. The lowest concentration was noticed in *Cleome arabica*, with 2.900g/100g.

However, it is to be noticed that the method of quantitative analysis we have adopted (UV-Visible spectrophotometry) is not very sensitive technique, where there can be interference of detection. This could explain the high values recorded for the tests to quantify the phenolic compounds and alkaloids.

Activity Tests:
Agar Diffusion Method:

After the incubation period required for microbial growth, we conducted the appreciation of results of the antimicrobial activity of each substance tested.

Activity of the Phenolic Compounds:

The phenolic compounds extracted from the eight plants showed no reproducible inhibitory effect against the studied strains of *E. coli* and *S. aureus*. Yet, the methanolic extracts of *Citrullus colocynthis* and *Ricinus communis* exhibited an antibacterial activity against *P. aeruginosa* ATTC 27853. However, the tested concentrations of these extracts exceeded 1 mg/ml (3.68 mg/ml and 5.08 mg/ml respectively); thus, these results were ignored since the studied concentrations were considered too pronounced.

No inhibitory activity of the phenolic extracts was observed against the fungal strains of *A. fumigatus* and *F. oxysporum f. sp. albedinis* (*Foa*). Only the crude methanolic extract of *Peganum harmala* demonstrated an antifungal activity against *F. oxysporum f. sp. lycopersici* (*Fol*). Nevertheless, the concentration of this extract exceeded 1mg/ml (1.74 mg/ml), and the dilution of the crude extract (1/10) showed no activity. Thereby, this result was ignored.

Activity of Essential Oils:

Tow dilutions of the essential oils (in ethanol) of *P. atlantica* and *P. lentiscus* were tested against the microbial strains: 2.5 % (v/v) and 1 % (v/v). Both the dilutions of the tow essential oils exhibited an antibacterial activity against the three bacterial strains. Thus, we tried to establish the MIC for these extracts.

The essential oils of the Pistachios exhibited no activity against *A. fumigatus*.

Unlike our extracts, many essential oils extracted from different plant species, have demonstrated their inhibitory effect against this fungal strain:

Inouye *et al*. [17] studied the inhibitory activity of seven essential oilson the apical growth of *A. fumigatus*. These authors showed a fungistatic effect with oilEssentials of lemon, lavender and tea tree. A fungicidal activity was detectedwith oils extracted from lemongrass and perilla. The activity the essential oilsextracted from the thyme and cinnamon bark varied according the tested concentrations. The differences in activity of oils from this study and ours could be, mainly, due to the chemical composition of the tested oils, the operating conditions of extraction and the employed method for the screening of activity.

Other authors revealed the antifungal activity of essential oils against different species of *Aspergillus*. This was the case with Shin [23]. This studyshowed a significant inhibitory activity with the essential oils extracted from*Styrax*

tonkinensis, *Lavandula angustifolia*, *Melaleuca alternifolia*, *Pelargoniumgraveolens* and *Rosmarinus officinalis* on *A. Niger* and *A. flavus*. The results obtained by this author, differ from ours, primarily because of the studied fungal strains, then, the diversity of chemical compositionof the tested oils.

Both dilutions of our studied essential oils were active against *Fol,* and the MIC was to be determined.

Only the dilutions of the essential oil of *P. lentiscus* exhibited an antifungal activity against *Foa*. The essential oil of *P. atlantica* did not show a reproducible antifungal activity against this fungal strain.

Activity of Alkaloids:

No reproducible antibacterial activity was shown from the studied alkaloidic extracts against the studied strains of *S. aureus* and *P. aeruginosa*. However, an antibacterial activity against *E. coli* ATTC 25922 was recorded with the diluted ethanolic extract of *Ricinus communis*. The concentration of this dilution was below 1mg/ml (0.52mg/ml); thus, we considered interesting to determine the MIC.

The alkaloidic extracts of the studied plants did not exhibit a reproducible antifungal activity against *Fol*.

Only, the alkaloidic extract of *Datura stramonium* showed an antifungal activity against both *A. fumigatus* and *Foa*. The concentrations of the active dilutions were both below 1 mg/ml (0.04mg/ml and 0.4mg/ml, respectively), therefore, we tried to establish the MIC.

Karou *et al*. [19] studied the inhibitory effect of alkaloids extracted from aspontaneous plant collected in Burkina Faso, on Gram positive and Gram negative bacteria. These authors found, contrary to our study, a positive activity against strains of *S. aureus* and a small inhibitory effectagainst strains of *E. coli*.

Hufford *et al*. [16] tested two alkaloids extracted from *Liriodendron tulipifera*, on some microorganisms. These compoundsshowed, contrary to ours, an antimicrobial activity on *S. aureus* and *Aspergillus flavus*.

These differences in activity could be the result of the diversity of the chemical composition of the tested alkaloids, since they belong to different plant species.

Determination of the Mic by the Broth Dilution Method:

After the preliminary tests of activity, studied by the agar diffusion, method, we tried to establish the MIC of the active extracts. Only extracts with an inhibitory activity reproducible in the three trials, and presenting concentrations below the threshold fixed concentration (1mg/ml), were studied.

No growth for the three bacterial strains was observed with all the analyzed concentrations of the essential oil of *P. lentiscus*, ranging from 0.25% to 4% (v/v). We deduced that the MIC of this essential oil on the studied strains of *E. coli*, *S. aureus* and *P. aeruginosa* corresponds to 0.25% (v / v).

The inhibitory effect of the essential oil of *P. lentiscus*, observed in preliminary tests on *Fol* could not be completely reproduced has in MIC tests.

Moreover, we noticed a very slight growth in the tube containing a concentration of 0.25% of the essential oil, and a more significant growth with 0.5% and 1% of oil. With 2% and 4% of oil, the growth was more important.

These inconsistent results could be explained by the presence of athreshold concentration above which the substance loses its activity.

Table 1: Total phenolic compounds concentration of the investigated plants

Methanolic plant extract	Concentration (mg/100g)
Peganum harmala	34.80
Cleome arabica	191.20
Datura stramonium	206.25
Nerium oleander	345.00
Citrullus colocynthis	735.20
Ricinus communis	1016.40
Pistacia lentiscus	1884.80
Pistacia atlantica	6039.80

Table 2: The yields of essential oils in Pistacia atlantica and Pistacia lentiscus

Plant	Levels (v/m)% of dry matter
Pistacia atlantica	0.13
Pistacia lentiscus	0.17

Table 3: Total alkaloids concentration in studied plants

Plant	Yield (g/100g of dry plant material)
Cleome arabica	2.900
Datura stramoniom	8.140
Rricinus communis	10.410
Nerium oleander	11.990
Peganum harmala	19.400
Citrullus colocynthis	19.680

We observed an inhibition of the growth of *Foa* with the concentrations 0.25% and 0.5% of essential oil of *P. lentiscus*. A Slight inhibitory effect was observed with the concentrations equal to 1%, 2% and 4% of oil. These results lead us to suspect a possible degradation of the oil, or the existence of a gap of concentrations in which the substance could be active.

We have noticed no bacterial growth in the tubes containing the different concentrations of the alkaloidic extract of *Ricinus communis*, tested on the studied strain of *E. coli*. These concentrations ranged from 0.02 to 0.64 mg/ml.

We established that the MIC, of this extract on this strain is 0.02mg / ml.

The inhibitory effect of the alkaloidic extract of *Datura stramonium*, observedon, both *Foa* and *A. fumigatus*, could not be reproduced by the broth dilution method. These results suggest that this extract has lost its inhibitory activity. This degradation could be the result of prolonged storage of the alkaloid extract.

Conclusion:

The results of the activity tests of phenolic compounds allow us to establishthat these metabolites, extracted from the investigated plants, show no antibacterial activity against *E. coli* ATCC 25922, *S. aureus* ATCC 43300 MRSA + and *P. aeruginosa* ATCC 27853.

We also found that these compounds showed no antifungal activityagainst *Aspergillus fumigatus*, *Fusarium oxysporum f. sp. lycopersici* and*Fusarium oxysporum f. sp. albedinis*.

An antibacterial activity of essential oil of *Pistacia lentiscus* was found against *E. coli* ATCC 25922, *S. aureus* ATCC 43300 MRSA + and *P. aeruginosa*ATCC 27853 The MIC of this oil on these bacteria is 0.25% (v / v).

The results of the agar diffusion method and broth dilution method of the essential oil of *Pistacia lentiscus* on *F. oxysporum sp. albedinis* suggest that this oil could have a certain antifungal activity against this fungus. This inhibitory activity is expressed at certain concentrations of oil, whose values are less than or equal to 0.5%(v/v).

The antifungal activity of the essential oil of *Pistacia lentiscus* noticed on *F. oxysporum f.sp. lycopersici* in the agar disc diffusion tests, could not be reproduced by the method of broth dilution. However, we observed a slower growth of this fungus with concentrations equal to 0.25%, 0.5% and 1% (v / v).

The alkaloidic extracts of *Cleome arabica*, *Citrullus colocynthis*, *Peganum harmala*, *Ricinus communis* and *Nerium Oleander* showed no antifungal effect against *A. fumigatus* and *F. oxysporum f. sp. albedinis*. Only the alkaloidic extract of *Datura stramonium* was found to be active against these fungi, when tested by the agar diffusion method. However, the MIC of this extract could not be determined, as a result of a loss of activity because of a prolonged conservation.

No antibacterial activity was observed on *E. coli* ATCC 25922 with alkaloidic extracts of *Cleome arabica*, *Citrullus colocynthis*, *Peganum harmala*, *Datura stramonium* and *Nerium Oleander*. Yet, the alkaloidic extract *Ricinus communis*, exhibited an inhibitory activity against this strain, with a MIC equal to 0.02 mg/ml.

The alkaloidic extracts of *Cleome arabica*, *Citrullus colocynthis*, *Peganum harmala*, *Datura stramonium*, *Nerium Oleander* and *Ricinus communis* did not exhibit any antibacterial activity against *S. aureus* MRSA +ATCC 43300 and *P. aeruginosa* ATTC 27853.

In the absence of essential oil of *Pistacia atlantica*, the MIC could not be determined for this oil proved active against *E. coli* ATCC 25922, *S. aureus* MRSA +ATCC 43300, *P. aeruginosa* ATTC 27853, and against *F. oxysporum f.sp. lycopersici.*

It would be interesting to repeat this study on more extanded number of samples of the studied plants, in time and space to:

Confirm the reproducibility of positive activities observed in this work;

Analyze the stability of toxicity in time;

Determine the minimum bactericidal and fungicidal concentrations;

To study with more depth the activity observed on the strains of *Fusarium oxysporum*;

Quantify components of tested substances according to the physiological state of theplants;

To characterize the molecule responsible for the activity observed with analyzed extracts and to study their molecular modes of action.

To study a possible synergistic activity, of the active compounds with other antimicrobial agents.

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