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Evaluation of antimicrobial and antioxidant activities of solvent extracts of *Anacyclus pyrethrum* L., from Algeria

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Abstract: In the present study, solvent extracts from aerial parts of *Anacyclus pyrethrum* L. were assessed for their total phenol content, antimicrobial and antioxidant (1,1-diphenyl-2-picrylhydrazyl free radical scavenging and ferric-ion reducing power) activities. The amounts of total phenolics and flavonoids in the solvent extracts were determined spectrometrically. (310.78 mg GA/g extract) and antioxidant activity (IC_{50} = 0.056 mg/mL). Increasing the concentration of the extracts resulted in increased ferric reducing antioxidant power for both extracts tested.

The methanolic extract exhibited the best antimicrobial activity against three gram-positive bacterium (*Listeria monocytogenes*: 100%, *Bacillus. cereus*: 69% and *Staphylococcus aureus*: 66%), as well as against Candida albicans (81%).

Finally, a relationship was observed between the biological activities potential and total phenolic and flavonoid levels of the extract. The results of this study provided an alternative of utilising *Anacyclus pyrethrum* aerial parts as readily accessible source of natural antioxidant in food cosmetic and pharmaceutical industry.

Keywords: *Anacyclus pyrethrum* L.; Solvent extracts; Antioxidant and antimicrobial activities; DPPH, Reducing power.

Introduction

Many oxidative stress related diseases are as a result of accumulation of free radicals in the body. A lot of researches are going on worldwide directed towards finding natural antioxidants of plants origins. The phenolic and flavonoid compounds are commonly found in plants and have been reported to have several biological activities including antioxidant and antimicrobial properties. The medicinal plants have become more and more important in primary health care, because of their secondary metabolites which may play numerous biological activities, against cancer and infectious diseases. Many pharmacological investigations are carried out to identify new drugs for the treatment of these diseases. Other hand, the synthetic antioxidants have disadvantages due to their possible toxicity and injurious properties to human health[1]. In addition, most consumers prefer additive free foods or a safer approach like the utilization of more effective antioxidant and antimicrobial agents from natural origins.

Accordingly, plant extracts and their derived secondary metabolites, such as phenolic components, offer the opportunity in this regard[2]. Among secondary metabolites, the

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polyphenol compounds play a wide range of biological effects including antibacterial, anti-inflammatory, antiallergic, hepatoprotective, antithrombotic, antiviral, anticarcinogenic and cardioprotective and vasodilatory effects[3]. The genus *Anacyclus* comprises about 13 annual and perennial species mainly centred in NW Africa but also found in other Mediterranean countries[4]. *A. pyrethrum* is a perennial herb of the Asteraceae family.

The rhizome of *A. pyrethrum* is used for treat problems associated with the toothache and sore throat. It is harvested in autumn and is dried. Its smell reminds licorice root. On the palate, it is felt that it contains essential oils with a tingling sensation on the tongue, hence its other name "Salivary[5].

Traditional medicine in Algeria has for centuries used the roots of *A. pyrethrum* for the treatment of respiratory infections and in the treatment of liver disease[6]. Everywhere in Algeria, the root is used as sternutatory, sialagogue and diaphoretic. It is regarded as a tonic to the nervous system. It is also used in respiratory infections and in the treatment of liver disease[6,7].

In some other countries, the roots are also considered aphrodisiac and sexual stimulant. In Indian medicine, the plant is widely recognized as tonic and rejuvenator[8]. It has been reported that the *A. pyrethrum* root has antibacterial and anti-inflammatory activities and is known for its insecticidal properties [6]. The chemical analysis of the roots show that they contain three fatty acids, one sterol and ten unsaturated amides, more specifically: pellitorine, anacycline, phenylethylamide, enetriyne alcohol, inulin, polyacetylenic amides I-IV, and sesamin. The plant contains also tannins, gum and essential volatile oil. Pyrethrine, an alkaloid, yielding pyrethric acid, is stated to be one of the active principles[9].

The literature indicates that there is no scientific evidence to support the anti-diabetic effect of *A. pyrethrum* in spite of its ethnobotanical usage. The aim of this work is to evaluate the antimicrobial and antioxidant properties of the extracts of *A. pyrethrum*. Additionally, the total phenolic and flavonoid contents of chloroform, methanolic and water extracts have been determined.

Results and Discussion

Total phenolics and flavonoids contents

The phenolic and flavonoid compounds are commonly found in plants and have been reported to have several biological activities including antioxidant and antimicrobial properties[10,11]. The total phenolic and flavonoid contents of *A. pyrethrum* extracts from different solvents were examined and are presented in Table 1. These compounds showed differences in their total contents depending on solvents polarities.

The highest content of total phenolic was found in methanolic extract (310.78 mg GAE/g) followed by aqueous extract (183.82 mg GAE/g), whereas the contents obtained with chloroform extract (91.8 mg GAE/g) was much smaller. These compounds are known to act as antioxidants not only because of their ability to donate hydrogen or electrons but also to because they are stable radical intermediates.

Literature survey reveals that the most antioxidant activities from plant sources are correlated with phenolic compounds[12,13]. Total flavonoids varied from 24.20 to 92.50 mg QE/g. Methanolic and water extracts had the highest levels with 92.50 and 72.50 mg QE/g respectively, while chloroform extract had the lowest amounts (24.20 mg QE/g). Flavonoids

as one of the most diverse and widespread group of natural compounds are probably the most important natural phenolics.

Table 1. Total phenol content of *A. pyrethrum* crude extracts

Extracts	Total polyphenol content [(a)]	Total flavonoid content [(b)]
Methanol	310.78 ± 5.2	92.50 ± 4.2
Water	183.82 ± 3.1	72.50 ± 2.1
Chloroform	91.8 ± 1.7	24.20 ± 1.2

[(a)] µg gallic acid equivalent per mg of extract (mg GA/g); [(b)] mg quercetin equivalent per g of extract (mg QE/g); Values expressed are means ± SD of three parallel measurements

Antioxidant properties

Radical scavenging activity

The antioxidant activity of the extracts was determined by the 1,1-diphenyl-2-picrylhydrazyl (DPPH) test system. Table 2 demonstrates DPPH scavenging activity, expressed in percentage, caused by different concentrations of solvent extracts from *A. pyrethrum*. The weakest radical scavenging activity (77.29 %) was exhibited by the chloroform extract of 0.50 mg/mL, whereas the strongest activity (89.58 %) was exhibited by the methanolic extract at a concentration of 0.16 mg/mL. The next highest activity (78.19 %) was for the water extract at a concentration of 0.2 mg/mL. As shown in Table 2, the antioxidant activity of extracts increased with an increase in their concentrations. At higher concentrations, the antioxidant activity of extracts was closer to the scavenging effect of ascorbic acid.

Therefore, DPPH scavenging activity is usually presented by the IC_{50} value. Concentrations of the antioxidant providing 50% inhibition of DPPH in the test solution (IC_{50}) were calculated and presented in Table 2.

Table 2. DPPH radical-scavenging of the crudes extracts from *A. pyrethrum* at different concentrations

Sample	Antioxidant activities					
	Extract concentration (mg/mL)	0.055	0.071	0.10	0.16	
Methanol	Scavenging effect on DPPH (%)	44.79	61.22	79.70	89.58	
	DPPH IC_{50} (mg/mL)					0.056
	Extract concentration (mg/mL)	0.055	0.086	0.12	0.2	
Water	Scavenging effect on DPPH (%)	19.84	41.33	62.32	78.19	
	DPPH IC_{50} (µg/mL)					0.114
	Extract concentration (mg/mL)	0.055	0.10	0.16	0.50	
Chloroform	Scavenging effect on DPPH (%)	12.33	30.73	51.68	77.29	
	DPPH IC_{50} (mg/mL)					0.154
	Extract concentration (mg/mL)	0.04	0.05	0.06	0.08	
Ascorbic acid	Scavenging effect on DPPH (%)	39.40	51.03	68.57	97.84	
	DPPH IC_{50} (mg/mL)					0.048

The methanolic extract of *A. pyrethrum* had the highest radical scavenging activity with the lowest IC_{50} value of 0.056 mg/mL. This was higher than the water extract with an IC_{50} value of 0.114 mg/mL and chloroform extract with an IC_{50} value of 0.154 mg/mL. These results demonstrate a positive correlation between free radical and the phenolic contents in the methanolic and water extracts.

This result was in favour of the implication of phenolic compounds in the antioxidant activity of *A. pyrethrum* extracts by hydrogen transferring reaction.

Reducing power

There are many methods to assay antioxidant capacity. But ferric reducing antioxidant power (FRAP) method we used in the present study is rather quick and simple to perform[14]. In this assay, Fe^{3+}/ferricyanide complex is reduced to the ferrous form by antioxidants and can be monitored by measuring the formation of navy blue colour at 700 nm[15].

In the present study, methanolic extract exhibited a stronger reducing power compared to other extracts as shown in Table 3. Indeed, the absorbance of methanolic extract at 0.10 mg/mL was 0.359, which were stronger than that of water and chloroform extracts (0.281 and 0.091, respectively).

Similarly, at 0.15 mg/mL the highest ferric reductive capacity resided with methanolic extract (0.569) compared with water and chloroform extracts (0.301 and 0.182, respectively). On the other hand, ascorbic acid has the strongest reducing power compared to all the extracts. At 0.15 mg/mL, the reducing power of ascorbic acid was 1.84.

Table 3.The total antioxidant capacities of the crudes extracts of *A. pyrethrum* and synthetic antioxidant determined as ferric reducing antioxidant power (FRAP).

Extract source	**FRAP**	
	0.1 (mg/mL)	**0.15 (mg/mL)**
Methanol	0.359 ± 0.14	0.569 ± 0.06
Water	0.281 ± 0.05	0.301 ± 0.03
Chloroform	0.091 ± 0.06	0.182 ± 0.04
Ascorbic acid	1.32 ± 0.04	1.84 ± 0.08

Antimicrobial activity

The in vitro antibacterial activities of solvent extracts of *A. pyrethrum* against the employed bacteria were qualitatively and quantitatively assessed by the presence or absence of inhibition zones. Methanolic extract showed the highest inhibition activity (*Listeria monocytogenes*: 100%, *Candida albicans* 81%, *Bacillus cereus*: 69% and *Staphylococcus aureus*: 66%) and water extract showed the second highest inhibition activity (*Listeria monocytogenes*: 71% and *Candida albicans*: 68%). Other hand, the inhibition activity for chloroform extract was the lowest with inhibition < 50% against all bacteria strains. Rest of the bacterial strains (*Pseudomonas aeruginosa* and *Escherichia coli*) showed no inhibition (Table 4).

The antibacterial activity appears to correlate well with the total phenolic values of *A. pyrethrum* specie. It is suggested that the phenolic compounds which are antioxidants are responsible for the antibacterial activity[16].

The purification of the antimicrobial compounds from the methanolic extracts, using bioactivity-directed fractionation and the characterization of the bioactive compounds need to be pursued. Therefore, *A. pyrethrum* showed significant antibacterial activity and could be considered as one of the sources of natural antibiotics for medicinal use.

Table 4: Antibacterial activity of solvent extracts of *A. pyrethrum*

Microorganisms	**Zone of inhibition in mm (% inhibition)**				
	Methanolic extract	Water extract	Chloroform extract	Gen[a]	AmB[b]
Gram-positive bacterium					
L. monocytogenes	14 (100)	10(71)	8(57)	14	Nd
S. aureus	20(66)	16(53)	11(36)	30	Nd
B. cereus	16(69)	12(52)	10(43)	23	Nd
Gram-negative bacterium					
P. aeruginosa	6	6	6	16	Nd
E. coli	6	6	6	22	Nd
Yeast					
C. albicans	18(81)	15(68)	10(45)	Nd	22

Mean diameter of zone of inhibition in millimetres. The percentage of inhibition is related to the extracts compared to standards Gentamicin and Amphotericin; [a]GEN: Gentamicin; [b]AmB: Amphotericin B; nd: not determined; 6 mm is the disk diameter (no inhibition)

Conclusion

The results of this work show that *A. pyrethrum* methanolic extract exhibited promising antimicrobial and antioxidant activities. The biological activities of *A. pyrethrum* could be attributed to the phenolic compounds that are responsible for these activities. Therefore these results present a good basis for further investigation of the potential application of *A. pyrethrum* for its medicinal purposes as well as in the food industry.

Experimental Section

Plant Material

The stems/leaves of *A. pyrethrum* were harvested from the mounts of Tlemcen (north-west of Algeria), in April 2009. The plants collected were identified by Pr. Noury Benabadji, "Laboratory of Ecology and Ecosystem Management", University of Tlemcen (Algeria). A voucher specimen was deposited in this laboratory. Plant samples were dried in the share and conserved for future use.

Preparation of the solvent extracts

The air dried *A. pyrethrum* aerial parts (stems/leaves) were ground to fine powder using a grinder. Then the powdered plant material (50 g) was extracted twice (250 ml) using the following sequence of solvents with increasing polarity: chloroform, methanol and water at 30 ◦C for 24 h each. The sample was then filtered through filter paper in a Buchner funnel. The filtered solution was evaporated at reduced pressure at 40 ◦C and then dissolved in appropriate solvent.

Determination of total phenolic contents

Total phenolic constituent of the extracts were determined by employing the methods given in the literature[17,18]. 200 µL of the dilute extract solution containing 40 µg of the extract was added to 1 mL of Folin-Ciocalteu reagent (diluted in distillated water). After 4 min, 800 µL of Na_2CO_3 (75 mg/mL) solution was added and the mixture was allowed to stand for 45 min at room temperature. At the end of the incubation, the absorbance was measured at 760 nm with a spectrophotometer (Optizen POP spectrophotometer, Daejeon, South Korea).

The same procedure was also applied to the standard solutions of gallic acid, and a standard curve was obtained. The concentrations of phenolic compounds expressed as mg gallic acid equivalent per g (mg GAE/g) of extract were calculated according to the standard gallic acid graph. All experiments were carried out in triplicate, and gallic acid equivalent values were reported as X ± SD of triplicates.

Determination of total flavonoids contents

Total flavonoid contents of the extracts were determined using the Dowd method as adapted by Querttier *et al*., 2000[19]. One milliliter of 2% aluminium trichloride ($AlCl_3$) in methanol was mixed with the same volume of extracts (200 µg). The absorption at 430 nm was measured after 10 min against a blank sample consisting of 1 mL methanol without $AlCl_3$. The concentrations of flavonoid compounds expressed as mg quercetin equivalent per g (mg QE/g) of extract were calculated according to the standard quercetin graph. All experiments were carried out in triplicate, and quercetin equivalent values were reported as X ± SD of triplicates.

Determination of antioxidant activity

1,1-diphenyl-2-picrylhydrazyl (DPPH) radical-scavenging activity

The antioxidant activity of the samples was determined by the 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging[20]. In test tubes, 0.25 ml of DPPH 0.8 mM in MeOH was added to accurately weighed aliquots of the extracts dissolved in 3.75 mL of MeOH, corresponding to concentration ranges of extract between 0.01 to 0.2 mg/mL. After mixing, the samples were maintained in the dark, at room temperature for 30 min. The absorbance at 517 nm was measured using a UV/Vis V-530 spectrophotometer and compared with a control without extract. A blank was prepared for each sample using methanol instead of the DPPH solution. Ascorbic acid was used as reference compound. Antioxidant activity was expressed as a percent inhibition of DPPH radical, and calculated from the equation:

$$\text{Scavenging activity (\%)} = \mathbf{100\ [(Abs\ control - Abs\ sample)/Abs\ control]}$$

IC_{50} values were determined from the plotted graphs of scavenging activity against the concentration of the extracts. These values are defined as inhibitory concentration of the extract necessary to decrease the initial DPPH radical concentration by 50% and are expressed in mg/ml. Triplicate measurements were carried out.

Ferric reducing antioxidant power assay (FRAP)

The total antioxidant capacity of sample was determined using a FRAP assay of Oyaizu (1986) [21]. Different concentrations of extracts (0.10 and 0.15 mg/mL) in distilled water were mixed with 2.5 mL of phosphate buffer (200 mM, pH 6.6) and 2.5 mL of potassium

ferricyanide ($K_3Fe(CN)_6$) (1%). The mixture was incubated at 50 °C for 20 min. 2.5 mL of trichloroacetic acid (10%) was added to the mixture, which was then centrifuged at 3000 rpm for 10 min. The upper layer of the solution (5 mL) was mixed with 5 mL of distilled water and 1 mL of $FeCl_3$ (0.1%) and the absorbance was measured at 700 nm with a spectrophotometer (Optizen POP spectrophotometer, Daejeon, South Korea). Increased absorbance of the reaction mixture indicated increased reducing power. Ascorbic acid was used as the standard compound. Phosphate buffer (pH 6.6) was used as blank solution. The data presented are the average of three measurements given as mean ± standard deviation.

Antimicrobial activity

Bacterial and yeast

The bacterial strains used in this study, i.e. *Staphylococcus aureus*, *Listeria monocytogenes*, *bacillus cereus* (Gram positive) *Escherichia coli* and *Pseudomonas aeruginosa* (gram negative) were isolated at the Medical Reanimation Department of the Hospital University Center of Tlemcen in Algeria. The *C. albicans* fungal isolate was obtained from the dermatology department of the Hospital University Center of Tlemcen.

Preparation of inocula

Bacterial strains preserved in nutrient agar at 4°C, were revivified in nutrient solution and incubated at 37±1°C during 18 to 24 h. 0.1 mL of each culture was added to 10 mL BHIB (Brain Heart Infusion Broth, pronadisa Hispanalab). *C. Albicans* preserved at 4°C in the Sabouraud agar supplemented with chloramphenicol was revivified in nutrient solution and incubated at 30±1°C during 24 to 48 h. 0.1 mL of each culture was added to 10 mL sterile physiological water. For antimicrobial assay, bacterial strains were grown on Mueller-Hinton Agar (MHA, Pronadisa Hispanalab) while *C. albicans* was grown on Sabouraud Dextrose Agar + chloramphenicol (SDA, Merck).

Disc-diffusion method

Bacterial and yeast inocula reached microbial densities in the range 10^6 to 10^7cfu/mL. Antibacterial activities of samples of the stems/leaves were assessed using the paper disk agar diffusion method according to Rios et al., 1987[22]. All extracts of the whole aerial plant parts were dissolved in DMSO. Absorbent disks (Whatman disk 6-mm diameter) were impregnated with 20 µg of extracts and then placed on the surface of inoculated plates (90 mm) and incubated at 37°C for 24 h. Negative controls were prepared using a disk impregnated with the same solvent employed to dissolve the plant extracts. Gentamicin (10 µg / disc) and amphotericin B (25 µg / disc) were used as the standard antibiotics for bacteria and fungus, respectively. Antimicrobial activity was assessed by measuring the inhibition zone. All the tests were performed in triplicate.

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