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RESEARCH ARTICLE

Chemical characterization and *in vitro* evaluation of the antioxidant and antibacterial activity of *Pulicaria incisa* (Lam.) DC. essential oil

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Abstract

In the present study, *in vitro* antioxidant and antimicrobial activities of the essential oil of *Pulicaria incisa* (Lam.) DC., harvested from Oulad M'barek (Beni Mellal-Khenifra-Morocco) were evaluated. The essential oil obtained by hydrodistillation from the dried aerial parts was analysed by GC-MS. The major components were characterized as β-gurjunene (13.1%), β-bourbonene (11.5%), camphor (8.8%), γ-muurolene (7.9%), 1,8-cineole (eucalyptol) (6.1%), respectively. The *P. incisa* essential oil showed in the DPPH assay a value of IC_{50} 105.99 ± 4.25 µg/mL and in the FRAP assay a value of EC_{50} 87.21 ± 3.1 µg/mL, respectively. The antimicrobial activity of the oil was evaluated against five microorganisms using the *in vitro* paper disk diffusion for the determination of MIC and MBC values. The results obtained revealed different levels of sensitivity of the bacterial strains tested. The *Allorhizobium vitis* (S4) and *Agrobacterium tumefaciens (C58*) strains were the most sensitive to the investigated oil, with inhibition diameters >20 mm and a MIC and MBC of 0.25 (v/v) and 0.5 (v/v), respectively. Our results suggest that the essential oil of *P. incisa* contains bioactive compounds with antioxidant and antimicrobial properties supporting its traditional therapeutic use. This essential oil merits further study as a potential product for the management of phytopathogenic bacteria.

Keywords: *Pulicaria incisa* (Lam.) DC., Antioxidant activity, Antibacterial, Essential oil, Sensitivity.

Introduction

The genus *Pulicaria* belonging to the Asteraceae family, is represented by 80 species distributed in Europe, North Africa and Asia (Williams et al. 2003). *Pulicaria* species have been used in folk medicine as insect repellents, galactagogues, antiepileptics, and tonics (Ghazanfar, 1994), as well as for the treatment of colds, cough, colic, excessive sweating and as carminative (Ibn Sina, 1971). *Pulicaria incisa* (Lam.) DC. is a desert plant that has a pleasant aromatic odor known in Morocco under the vernacular name "*Aatitisa*". The decoction of the herb is used by the natives of some upper Egyptian areas, sweetened with sugar, as a substitute for tea (Nabiel 2003; Amer et al. 2007). The plant is also used as a traditional medicine for treating heart diseases and as a hypoglycemic agent (Mansour et al. 1990; Shabana *et* al. 1990; Nabiel, 2003; Saleh, 2003). *Pulicaria incisa* has been shown to contain large amounts of unsaturated fatty acids (Abd El-Gleel and Hassanien, 2012), to lower total lipid, total cholesterol and triglyceride levels and has been proposed as a potential cholesterol-lowering agent (Amer et al. 2007).

Today, such plant products receive significant attention from researchers and the pharmaceutical industry to replace current chemotherapy. However, despite all the studies mentioned concerning the use of this genus in medicine, no study has been focused on the antimicrobial effect of this essential oil against

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phytopathogenic bacteria. The objective of this study was to report the chemical composition of essential oil *Pulicaria incisa* (Lam.) DC. growing in Morocco and to investigate its antioxidant and antimicrobial properties.

Materials and Methods

Plant material and essential oil extraction

Aerial parts of *pulicaria incisa* (Pi) were collected in Morocco (June 2015) from Oulad M'barek (region of Beni Mellal-Khenifra). The plant was identified at the Biodiversity and Natural Substances Laboratory of the Ibn Tofail University of Kenitra-Morocco, on the basis of the document "Practical Flora of Morocco Volume 1, 2 and 3". The plant material was prepared and then dried in the shade at room temperature for four weeks.

The essential oil was isolated from dry plant material by hydro-distillation, using a Clevenger type apparatus for 3 hours. The obtained oil is characterized by a very strong odour and yellowish colour. The yield relative to the dry matter measured from three samples is determined (3x 400 g). Finally, the essential oil was stored in the dark at 21°C for further experiments.

Gas chromatography-mass spectrometry analysis

The GC/MS unit consisted of a Shimadzu GC-2010 gas chromatograph, equipped with BP-5 capillary column (30 m x 0.25 mm i.d., film thickness 0.25 µm; SGE, Ltd.), and interfaced with a Shimadzu QP2010 Plus mass spectrometer (software version 2.50 SU1). Oven temperature was programmed, 60-200°C, at 3°C.min^{-1}, and then held isothermal for 5 min; transfer line temperature, 300°C; ion source temperature, 200 °C; carrier gas, helium, adjusted to a linear velocity of 36.5 cm.s^{-1}; split ratio, 1:40; ionization energy, 70 eV; scan range, 40-400 u; scan time, 1 s. Components identification was carried out by comparison of their retention indices relative to C_9-C_{20} *n*-alkanes on the BP-5 column confirmed by comparison of recorded mass spectra with those of a computer library (Shimadzu corporation library and NIST05 database/ Chem Station data system) and by comparing with authentic reference compounds whenever possible.

Bacterial strains tested

The panel of five pathogenic strains was used, included *Pseudomonas savastanoi* pv *savastanoi* (PSS 2066-1) (Bouaichi et al. 2015), *Allorhizobium vitis* (*A. vitis* S4) (Popoff et al. 1984), *Agrobacterium tumefaciens* (A. tumefaciens C58) (Kersters et al. 1973), *Clavibacter michiganensis* subsp. *michiganesis* (CMM 1616-3) and *Pectobacterium carotovorum* subsp. *carotovorum* (PCC 2657-1) from the pathogenic collection of the laboratory of "Plant Bacteriology and Biological control "RUPP CRRA-Meknes" (Morocco).

Antibacterial activity by disk diffusion method

The antibacterial activity of the essential oils was determined by using the paper disk diffusion technique (Popoff et al. 1984). From fresh colonies (18 to 24h old), a bacterial suspension was performed in sterile distilled water. The turbidity of this suspension is adjusted to 0.5 McFarland and then diluted to the serial dilution. Petri dishes containing YPGA medium (5g yeast extracts; 5 g bacto peptone; 10g glucose; 20g agar; distilled water to 1.0 L) were inoculated by Sterile disk (6 mm diameter) was soaked by 2 µL of essential oil and placed in the centre of the medium. The Petri dishes are first left for 1-hour under-flow laminar cabinet for diffusion before to be incubated at 26 °C in the oven during of 24h to 48h. The negative control was a sterile disk soaked with 2 µL of sterile distilled water. Streptomycin (20 µg/mL) was involved as a positive control to compare its effect with bio-agent. Antibacterial activity is determined by measuring the diameter of the inhibition zone around each disc. All tests were performed three repetitions.

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Determination of the minimal inhibitory concentration (MIC) and minimal bactericidal concentration (MBC)

The microdilution method was used to determine the minimum inhibitory concentration (MIC) of the oil (Ismaili et al. 2004). The redox dye resazurin was used to determine the MIC of the samples of the essential oil of *P. incisa* for a decreasing range of essential oils (0.5; 1; 2.5; 5; 10; 20 µL/mL), the use of a 0.2% (v/v) concentration of agar as a stabilizer overcame the solubilisation problem between the oil and the medium while avoiding chemical emulsifiers (Remmal et al. 1993). After incubation at 26 °C, observation of the range provides access to the Minimum Inhibitory Concentration (MIC), which corresponds to the lowest concentration of extract or oil capable of inhibiting bacterial growth. All tests were performed three repetitions.

Antioxidant Activity

DPPH• assay

The DPPH• test was carried out as described for previous studies (Cuendet et al. 1997; Kirby and Schmidt, 1997; Burits and Bucar, 2000 and Sahin et al. 2004). 50 µL of various dilutions of the essential oil were mixed with 2 mL of a 2.3% methanol solution of DPPH•. After an incubation period of 30 min, the absorbance of the samples was read at 517 nm using a spectrophotometer.

The antiradical capacity of the studied essential oil was calculated using the following formula: $I\ (\%) = 100 * [(A_0 - A_1) / A_0]$ Where: A_0 is the absorbance of the control at 30 min, and A_1 is the absorbance of the sample at 30 min.

The DPPH assay was expressed as IC_{50}, which is the antiradical concentration required to cause 50% of inhibition (Harififar et al. 2007). The IC_{50} was calculated by plotting inhibition percentages against concentrations of the sample. Gallic acid and ascorbic acid were used as positive controls.

Reducing power (FRAP) determination

The reductive potential (Fe^{+3}) in essential oils is determined using the method described by Oyaizu (1986). One milliliter of sample solution at different concentrations was mixed with 2.5 mL of phosphate buffer (0.2 M, pH 6.6) and 2.5 mL of potassium ferricyanide [K_3 Fe $(CN)_6$] (1%). The mixture was incubated in a water bath at 50 °C for 20 min, then 2.5mL of TCA (trichloroacetic acid) 10% was added to the mixture to stop the reaction and the test tubes were centrifuged at 3000 rpm for 10 min. 2.5 mL of supernatant was mixed with 2.5 mL of distilled water and 0.5 mL of $FeCl_3$ (0.1%) and the absorbance was measured by a spectrophotometer at 700 nm. The sample concentration providing 0.5 of absorbance (EC_{50}) was calculated by plotting absorbance at 700 nm against the corresponding sample concentration. BHT and quercetin were used as reference compounds.

Results and Discussion

Chemical composition of EO

The EO from aerial parts of *P. incisa* was obtained by hydrodistillation and gave a yield of 0.2 % (w/w dry plant material). The yield obtained in the current study is different compared to those obtained by Chaib et al. who found 0.50 % (w/w) (Chaib et al. 2017). Our results are in agreement with other studies carried out on the same species, which reported that the genus *Pulicaria* yields relatively moderate quantities of essential oil (Shahat et al 2017, Mustafa et al. 2018).

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The chemical composition of *P. incisa* EO showed the presence of a total of 22 compounds (Table 01). The major components characterized were β-gurjunene (13.1%), β-bourbonene (11.5%), camphor (8.8%), γ-muurolene (7.9%), 1,8-cineole (eucalyptol) (6.1%), ledol (4.9%), α-terpineol (4.2%) and other compounds at low percentages. A similar study carried out on *P. incisa* leaf and flower oil, which showed a different chemotype to our studied species, were characterized by a high content of carvotanacetone with 66.0 and 50.9 and 13.3 and 24.3% of chrysanthenone, respectively (Shahat et al., 2017).

Table 1. GC-MS data for essential oil components identified in *P. incisa*.

RRI [a]	KI [b]	Compound	%
1035	1031	1,8-cineole (Eucalyptol)	6.1
1136	1133	α-Terpineol	4.2
1144	1141	Camphor	8.8
1170	1169	Borneol	3.8
1180	1177	Terpinen-4-ol	0.6
1197	1196	β-Cyclocitral	1.2
1239	1237	Pulegone	0.7
1353	1352	Thymol acetate	2.3
1359	-	γ-Elemene	0.6
1390	1388	β-Bourbonene	11.5
1411	1408	Caryophyllene	0.9
1436	1433	β-Gurjunene	13.1
1480	1479	γ-Muurolene	7.9
1514	1513	γ-Cadinene	3.3
1527	1522	δ-Cadinene	0.9
1583	1582	Caryophyllene oxide	1.6
1608	1602	Ledol	4.9
1652	1650	β-Eudesmol	0.5
1662	1658	Patchouli alcohol	0.6
1834	-	Hexahydrofarnesylacetone	4.5
1939	1938	Cembrene	1.2
1946	1943	Phytol	1.1

[a] RRI Relative retention indices calculated against n-alkanes on BP-5 column; [b] KI Kovats indices from literature (Adams, 2007) for DB-5 capillary column.

In comparison with other species of the same genus, for instance the composition EO of *Pulicaria gnaphalodes* presented the main components namely: geraniol, 1,8-cineole (eucalyptol), α-pinene, chrysanthenone, α-terpineol and filifolone (Asghari et al., 2014). Al-Hajj et al., 2014 who examined the composition of *Pulicaria inuloides* which was reportedly rich in 5-isopropyl-2-methyl-2-cyclohexen-1-one (55.1%), methylbenzene (20.6%) and (*Z*)-citral (2.9%). Another study was conducted on the aerial parts of *Pulicaria undulata* (L.) from Iran, major components identified were 4-terpineole (20.12%), α-terpinene (4.02%), γ-terpinene (7.00%), *cis*-sabinene hydrate (8.29%), linalool (5.60%), *cis*-calamenene (13.37%) and Junipene (8.66%) (Ravandeh et al., 2011).

In contrast, these authors found different constituents and these variations in composition are due to many factors such as the species studied of the same genus, the time of harvesting, the method of extraction and the part of the plant to be analysed. According to Harkat-madouri et al., the quantitative and qualitative

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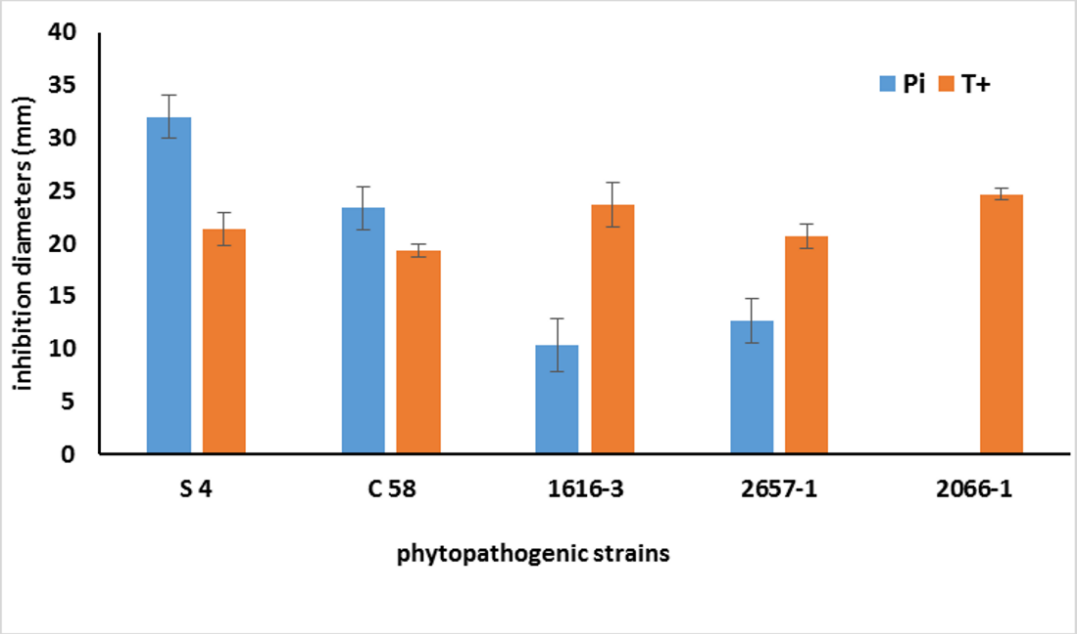
difference of the compounds of essential oils may depend on environmental, agronomic, age and geoclimatic factors and also on the used extraction techniques and the experimental conditions (Harkat-madouri et al., 2015).

Antimicrobial Activity

The obtained results showed a variation between the tested strains. Essential oil of *P. incisa* showed inhibition against all tested strains, apart from PSS 2066-1 (Figure 01).

Figure 1. Growth inhibition of phytopathogenic strains (*A. vitis* S4), (*A. tumefaciens* C58), (CMM 1616-3), (PCC 2657-1), (PSS 2066-1) using of *P. incisa* essential oil.



(Pi = P*ulicaria incise essential oil. T+= Streptomycin (20 µg/mL).*

The analysis of variance of the treatment factor showed a highly significant difference (P = 0.05) between treatment with essential oil and the negative control (sterile distilled water). The antagonistic experience has shown that the essential oil exhibited a higher antibacterial activity than the positive control, represented by streptomycin (20 µg/mL) against the bacterial pathogens *A. vitis* (S4) and *A. tumefaciens* (C58) which were more sensitive with zones of inhibition greater than 20 mm, they have been considered sensitive to this essential oil. On the contrary, CMM 1616-3, PCC 2657-1 strains were less sensitive with inhibition diameters of 10.33 and 12.66 mm, respectively.

The essential oil of *P. incisa* was active with a minimum inhibitory concentration (MIC) of 0.25% (v/v) against *A. vitis (*S4), *A. tumefaciens* (C58) and CMM 1616-3 with a minimum bactericidal concentration (MBC) of 0.5% (v/v). However, the MIC and MBC of this oil against PCC 2657-1 was the same as 0.5%. A similar study showed that the antibacterial activity of the essential oil *Pulicaria incisa* against a common nosocomial pathogen, *Acinetobacter baumannii* ATCC 19606, with a MIC up to 19 µg/mL (Chaib et al. 2017).

Various species of *Pulicaria* have revealed their antibacterial potency, such as the antibacterial activity of *Pulicaria undulata* against *Staphylococcus aureus, Bacillus subtilis*, *Escherichia coli* and *Pseudomonas aeruginosa* (Mossa et al. 1987, Rustaiyan et al. 1991, Elegami et al. 1994; EL-Kamali et al. 1998). Another study on the essential oil of *Pulicaria odora* showed inhibitory activity against Gram-positive *Staphylococcus aureus* and *Streptococcus pyogenes*; the Gram-negative strain *Vibrio cholerae* and the yeast *Candida albicans* with a MIC ranging from 2 to 10 µL/mL (v/v) (Ezoubeiri et al. 2005).

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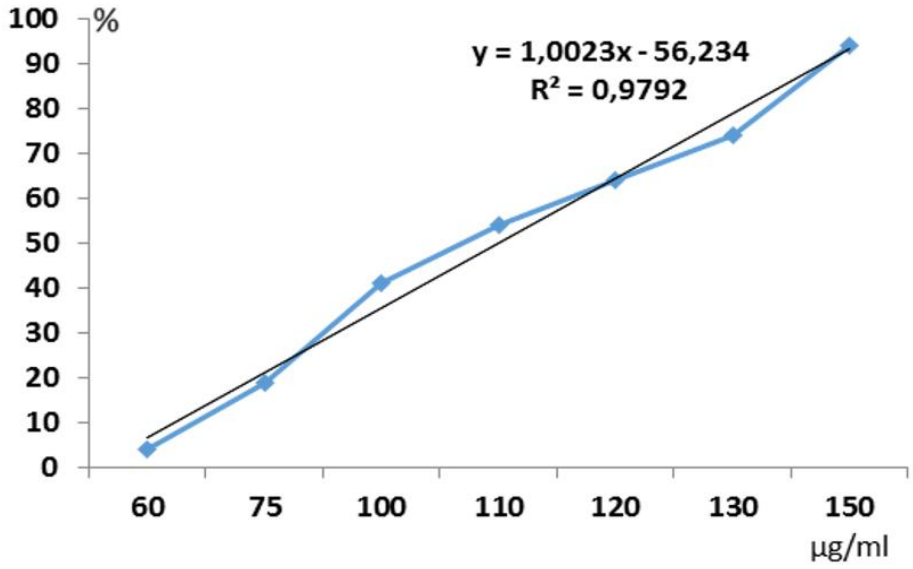
Antioxidant activity

DPPH Radical Scavenging Assay

The antioxidant activity of *P. incisa* EO and standard antioxidant (ascorbic acid and gallic acid) towards the DPPH radical was evaluated using a spectrophotometer following the reduction of this radical accompanied by its passage of the violet colour (DPPH•) to the yellow colour (DPPH-H) measurable at 517nm. From the values obtained, we calculated the percentages of inhibition using the formula given previously. The values obtained made it possible to draw the curve of figure 02, which represents the variation of the percentage of inhibition following the concentrations of the essential oil.

Figure 2. Percent inhibition of DPPH• as a function of the concentrations of *P. incisa* EO.



The IC_{50} values determined in µg/mL express the effective concentration of antioxidant extract required for entrapment and the 50% reduction in DPPH• (Table 2). The essential oil of *P incisa* could bring back the stable free radical (1,1-diphenyl-di-picrylhydrazyl) IC_{50} of 105.99 ± 4.25 µg/mL showing lower antioxidant activity than Ascorbic acid and Gallic acid. It appears from these results that the two acids are the most effective antioxidants with an IC50 of 12.081 ± 0.006 and 20.036 ± 0.002 µg/mL, respectively. In Egypt, Abd El-Gleel and Hassanien (2012) showed that methanolic extract of *P. incisa* with high anti-radical activity and high levels of antioxidant can act quickly (in the first 10 minutes) on free radicals. According to Elmann et al. the *P. incisa* infusion was found to be a very potent free-radical scavenger with an IC_{50} value of 45 µg/mL and 80% inhibition of DPPH absorbance at 517 nm Elmann et al. (2012). Other studies have shown the antioxidant effect of *pluricaria* species; Ragab and Raafat revealed the antioxidant and cytotoxic effect of the alcohol extract of *Pulicaria jaubertii* (Ragab and Raafat, 2016). In addition, Al-Hajj et al. showed the antioxidant activity of the methanolic extract of *Pulicaria inuloides* and standard ascorbic acid which recorded 81-43.55% and 70.91-50 and 50.44-10%, respectively (Al-Hajj et al. 2014).

Table 2. Antioxidant test by DPPH• expressing the effective concentration 50% in µg/mL.

	DPPH IC_{50} (µg /mL)
EO of *P. incisa*	105,99 ± 4,25
Ascorbic acid	12,081 ± 0,006
Gallic acid	20,036 ± 0, 002

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FRAP assay

The antioxidant activity of *P. incisa* EO was evaluated using the FRAP method. The presence of reducing agents in the essential oil of the plant leads to the reduction of Fe_3^+ / ferricyanide complex in ferrous form. The EC_{50} values are inversely proportional to the antioxidant capacity of the compound because it expresses the amount of antioxidant needed to decrease the concentration of the free radical (Fe_3^+) by 50%. The EC_{50} values for the essential oil of *P. incisa* and for ascorbic acid and gallic acid and BHT are shown in Table 3.

Table 3. Reducing power *P. incisa* EO by the method from FRAP.

Samples	**EC $_{50}$ (µg /mL)**
EO of *P. incisa*	87,21 ± 3,1
Ascorbic acid	17,50 ± 0,01
Gallic acid	23,21 ± 0,09
Butylated hydroxytoluene (BHT)	28,82 ± 0,20

The FRAP test revealed that the EO has a relatively low reducing power compared to ascorbic acid, gallic acid and BHT which showed a potent antiradical activity with EC_{50} values of about 17, 50 ± 0.01, 23.21 ± 0.09 and 28.82 ± 0.20 ug/mL, respectively. These values are lower than that recorded by the essential oil of *P. Incisa* whose value is of the order of 87, 21 ± 3.1 ug/mL.

The EO of the species of *P. incisa* has shown that it has an antimicrobial and also antioxidant effect suggests prospects for application in the agricultural, cosmetic and pharmaceutical sectors. The results of this study could contribute to the valorisation of this Moroccan aromatic and medicinal plant. Further research is needed to identify the minor constituents of this essential oil and to examine other potentials.

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