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Phytochemical analysis, Antifungal, antibacterial and antioxidant properties of *Pulicaria mauritanica* from south-east of Morocco

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Abstract

The essential oil, methanol and petroleum ether extracts of *P. mauritanica* (Asteraceae) were assayed for their chemical composition, antimicrobial and antioxidant properties. The essential oils were obtained using a Clevenger apparatus while a soxhlet apparatus method was used to obtain methanol (polar) and petroleum ether (nonpolar) extracts. . GC-MS of essential oil revealed that the major compound is carvotanacetone representing 87.58% of the essential oil of *P. mauritanica.* Essential oil showed a strong inhibition of *Penicillium digitatum* mycelial growth (100% at 900ppm); ether extract gave 46.212 ± 1.543% of inhibition at 30,000 ppm, while methanol extract exhibited no activity against *P. digitatum*. Test of antibacterial activity of *P. mauritanica* essential oil against *listeria innocua*, *pseudomonas aeruginosa* and *staphylococcus aureus* revealed a strong antibacterial activity. Methanol extract showed a strong reduction of DPPH (IC_{50} = 0.027 ± 0.004 mg/mL), while essential oils and ether extract exhibited median reduction of DPPH, IC_{50} are successively 0.080 ± 0.005 mg/mL and 0.123 ± 0.0006 mg/mL.. Phytochemical screening of studied organic extracts showed that polyphenolic compounds are the major constituants. Linear correlation between total polyphenols and total flavonoids contents and antioxidant capacity was obtained; the correlation was positive.

1. Introduction

The oasis of Tata is situated in the southeast of Morocco. Geographically the area corresponds to the southern side of the mountains of the Anti-Atlas and occupies an area of 26,274 sq/km. The area is characterized by a hyperaridity marked by low rainfall of around 100 mm, of stormy character, and large fluctuations in the daily and yearly temperatures. Its population is mainly rural (70%) [1].

The antifungal activity against postharvest fungi, antibacterial activity against pathogenic bacteria and antioxidant activity of medicinal and aromatic species has been evaluated in previous work [2-9] in order to find alternatives to the use of chemicals as antifungals, anbacterials and antioxydants in agriculture, food and health industries. The use of natural plant products especially plants is an interesting alternative or a complementary control method because of their antimicrobial activity, nonphytotoxicity, systemicity and biodegradability [10-13]. *P. mauritanica* is an aromatic, endemic plant growing wild in southeast Morocco. It bears several vernacular names “Bamghar” and “Ifenzi oudaden”, among the Saharan population of Morocco. Decoction and maceration of its leaves and flowers are used to treat digestive and circulatory troubles [14].

The aim of this study is the contribution of the valorization *P. mauritan*ica through studying the chemical composition of essential oils (EOs) and organic extracts and evaluating the antifungal, antibacterial and antioxidant properties of different extracts.

2. Material and methods

2.1 Collection and preparation of plant

The aerial parts of *P. mauritanica* were randomly in the oasis of Tata in the southern-east of Morocco during May-July 2015. Plant samples were air dried in the shade then the leaves were grounded to a fine powder using a laboratory grinding mill (Polymix PX-MFC 90D, Switzerland) and stored in the dark at 4 °C until use for the extraction by petroleum ether and methanol. For the extraction of EOs the leaves of the plant were stored without grinding.

2.2 Extraction using organic solvents

Preparation of the plant samples for the *in vitro* antifungal screening against *P. digitatum* and antioxidant activity was conducted using soxhlet apparatus method. Twenty grams of plant powder were weighed in a thimble and subjected to 4 hours soxhlet extraction with 100 mL of petroleum ether as solvent in order to obtain apolar fraction. The solvent of extraction was removed by evaporation to dryness under reduced pressure at 40°C until the sample weight was constant. The remains of the plant material were extracted with methanol in a similar manner to obtain polar fraction and the yield (w/w) was determined for each extraction.

2.3 Isolation of EOs

One hundred grams of air dried leaves of *P. mauritanica* were submitted to hydro-distillation with a Clevenger-type apparatus according to the European Pharmacopoeia [15] and extracted with one litter of distilled water for 5 hours (until no more EOs were obtained). The EOs obtained were dried under anhydrous sodium sulphate and stored at 4 °C until used. The yield (w/w) was determined.

2.4. Phytochemical analysis

2.4.1. Analysis by gaz chromatography-mass spectrometry (GC-MS) of EOs.

GC-MS analysis was carried out with a 5973N Agilent apparatus, equipped with a capillary column (95 dimethylpolysiloxane-5% diphenyl), Agilent HP-5MS UI (30 m long and 0.25 mm i.d. with 0.25 µm film thickness). The column temperature program was 50 °C during 2 min, with 4 °C/min increases to 180 °C, then 20 °C/min increases to 280 °C, which was maintained for 10 min. The carrier gas was helium at a flow-rate of 1 mL/min. Split mode injection (ratio 1:30) was employed. Mass spectra were taken over the m/z 30–500 range with an ionizing voltage of 70 eV. The individual compounds were identified by MS and their identity was confirmed by comparison of their retention indexes relative to C_8-C_{32} n-alkanes and mass spectra with those of authentic samples or with data already available in the NIST 2005 Mass Spectral Library and in the literature.

2.4.2. Total polyphenols content

Total polyphenols of methanol and petroleum ether extracts of *P. Mauritanica* were determined by Folin-Ciocalteu method [16]. 50 µL of mother solutions brought to appropriate dilutions was mixed with 1.25 mL of 10 fold diluted Folin-Ciocalteu phenol reagent. After 2 min of incubation at room temperature, 1 mL of 7.5% sodium carbonate was added and the mixture was incubated for 15 min at 50 °C. A calibration curve of gallic acid was established and the measurement was done at 760 nm. Total polyphenols content was expressed as milligram Gallic acid equivalent per gram of dried plant material (mg GAE/g dried plant).

2.4.3. Total flavonoids content

Quantitative determination of total flavonoids content of methanol and petroleum ether extracts of *P. Mauritanica* was carried out using a spectrometric assay using 2-aminoethyl-diphenylborate (NEU) (1% in methanol). 1 mL of dissolved extract in methanol extract brought to the suitable dilution was mixed with 50 µL of the NEU reagent. The absorption was determined at 409 nm by visible UV spectrophotometer and compared with quercetol standard (0.05 mg/mL) treated with the same reagent and under the same conditions as the extracts.

The percentage of total flavonoids quercetol equivalent per gram of dried extract (mg QE/g) was calculated using the following equation:

$$F\% = (A_{ext} \times 0.05 \times 100)/A_Q \times C_{ext} \quad (1)$$

Where A_{ext} is the absorption of the studied extract, A_Q is the absorption of quercetol and C_{ext} is the concentration of the extract in mg/mL. The yield of organic extract was used to obtain the percentage of total flavonoids quercetol equivalent per gram of dried plant material (mg QE/g dried plant).

2.4.4. Phytochemicals screening of alkaloids, terpenes, tannins and saponins

Tests of absence and presence of alkaloids, terpenes, tannins and saponins in methanol and petroleum ether extracts of *P. Mauritanica* were carried out according to different protocols. Methanol and petroleum ether extracts were submitted to thin-layer chromatography (TLC) Plates (GF 254 60; Merck). Plates were developed separately with Ethyl acetat/Methanol/Water (100/13.5/10, v/v/v) for alkaloids and with benzene for terpenes. The components were visualized under visible and UV light (365 nm) and sprayed with the following reagents in order to reveal spots of different groups: Dragendorff's and iodoplatinate's reagents for alkaloids and antimony chloride for terpenes. For the detection of tannins; to 2 mL of methanol and petroleum ether extracts are added a few drops of an aqueous solution of $FeCl_3$. To detect the saponins the calculation of the index of foam was used.

2.5. Antifungal activity

The antifungal activity of the EOs of *P. mauritanica* against *P. digitatum* was undertaken using the poisoned food method [17]. In poisoned food method, the pure EOs were dispersed firstly at distilled water and surfactant 2% Tween 80 to prepare a stock solution. Conveyable volumes of EOs from the stock solution were added to sterile molten PDA to obtain final concentrations ranging from 300 to 1,100 ppm. The control sets were prepared similarly using equal sterile water in place of the EOs. After solidification, the prepared plates were inoculated in center under aseptic conditions with a mycelial disc (5 mm diameter) from 7 days old cultures and incubated for 7 days at 25 ± 2 °C. Antifungal activity of organic extracts: methanol and petroleum ether of *P. mauritanica* against *P. digitatum* was tested *in vitro* using the same method as EOs test. Tree concentrations were tested 10,000; 20,000 and 30,000 ppm for each extract. After 7 days of incubation at 25 ± 2°C the fungitoxicity of EOs and organic extracts was expressed in terms of percentage of mycelial growth inhibition I (%) and calculated using equation:

$$I\ (\%) = (D_t - D_i)/D_i \times 100 \qquad (2)$$

With D_t is the average diameter of fungal colony in control and D_i is the average diameter of fungal colony in treatment.

To distinguish between the fungistatic and the fungicidal effects of the EOs and organic extracts on the target organism, a transfer experiment was done. Discs of fungi that had been 100% inhibited were transferred to fresh PDA to assess their viability after exposure to the EOs and the organic extracts.

2.6. Antibacterial activity

The 3 bacteria used as tested organisms are: *Staphylococcus aureus; Listeria innocua; Pseudomonas aeruginosa.*

The micro-organisms were transferred aseptically from stocks slopes into 10 mL of Mueller Hinton Broth (MHB) and incubated at 37°C then diluted to 106 UFC/mL. A dilution succession of *P. mauritanica* EOs ranging from 0.2, to 30 µg/mL in MHB supplemented with tween 80 (final concentration of 0.5 % (v/v)) was assessed to obtain 990 µl as final volume. 10 µL of every bacterial culture at 106 UFC/mL were added to prepared dilutions. Growth cultures containing EOs are incubated under 37 °C during 12 hours and are accompanied by the negative control where the EO are replaced by distilled water. Hemolysis tubes were incubated aerobically at 37 °C for 14 hours before the MICs and MBCs were determined. Turbidity of the cultures prevented visual determination of the MICs. To determine MBCs 10 µL broth was removed from each hemolysis tube and inoculated onto Mueller Hinton agar. The number of surviving organisms was determined after overnight aerobic incubation at 37 °C. The lowest concentration that maintained or reduced inoculum viability was the MIC, whereas the MBC was the concentration where less than 0.1 % of the initial inoculum survived. All tests were performed in triplicates.

2.7. Antioxidant activity

The antioxidant activity *in vitro* of EOs, methanol and petroleum ether extracts from *P. Mauritanica* was assessed by measuring the scavenging power of free radical 2, 2-diphenyl-1-picryhydrazyl (DPPH) according to literature [18-19] with some modifications. Methanol solution (500 µL) of EOs, methanol and petroleum ether extracts of *P. mauritanica* prepared at different concentrations (0.1 mg/mL, 0.05 mg/mL, 0.025 mg/mL, 0.0125 mg/mL) were mixed with 500 µL of methanol solution of DPPH (0.004 %). After an incubation period of 30 min in the dark at ambient temperature, the absorbance is read at 517 nm wavelength. The inhibition of free radical DPPH by butylhydroxytoluene (BHT) and Covi-ox T50 were also analyzed with the same concentrations and the same conditions for comparison. The inhibition of free radical DPPH percentage I (%) is calculated using equation:

$$I\ (\%) = 100 \times (A_{control} - A_{test})/A_{control} \quad (3)$$

Where $A_{control}$ is the absorbance of the control (containing all reagents without the test product) and A_{test} is the absorbance of the test compound (containing all reagents and the test product). The value IC_{50} is calculated from the graph of the DPPH scavenging effect percentage against the sample concentration and it is used to characterize the antioxidant activity of different examined extracts. All tests were performed in triplicate for each concentration.

2.8. Statistical analysis

All data were subjected to statistical analysis of variance using STATISTICA software, version 6, Stat-Soft, 2001, France. Newman & Keuls tests were used to segregate treatments which were significantly different ($p < 0.05$).

3. Results and discussion

2.1. Phytochemical analysis

The yield of EOs from *P. mauritanica* leaves and stems harvested in the oasis of Tata was high 0,861±0.013% compared with the yield obtained by Znini et al. [20] which was 0.45%.

For the organic extracts methanol was the best solvent extracting a larger quantity of material (20.35±0.202%) compared with petroleum ether (2.762±0.151%) (Table 1).

Table 1: Yields of EOs, methanol and petroleum ether extracts from *P. Mauritanica*

	Methanol	**Petroleum ether**
Yield (%)	20,350±0.202[a]	2.762±0.151[b]

Each value is presented as mean ± standard deviation (n = 3). Different letters a, b are significantly different according to Newman & Keuls tests at $p < 0.05$.

The results of GC-MS analysis of *P. mauritanica* EOs harvested in the oasis of Tata revealed the identification of 16 constituents representing 94.45 % of the total EOs composition. The results in table 2 show that the major compound is the oxygenated monoterpene carvotanacetone representing 87.58 % of the EOs. The other two most abundant compounds were 4-methoxy-2, 3, 6-trimethylphenol (1.48 %) and 4-Carene (0.82 %). GC/MS analysis results revealed that the EOs of *P. mauritanica* has a commercial potential for the production of oxygenated monoterpene carvotanacetone.

Table 2: GC/MS of *P. mauritanica* EOs

N°	**RT**	**Name of compounds**	**Area %**
1	6.91	Alpha-pinene	0.3
2	7.87	O-cymene	0.14
3	15.54	4-Carene	0.82
4	15.8	Alpha-Terpineol	0.09
5	15.86	Trans-para-menthan-2-one	0.54
6	16.03	Tetrahydrocarvone	0.2
7	16.88	Carvomenthol	0.27
8	17.91	Carvotanacetone	87.58
9	19.25	Dihydroedulan	0.12
10	19.4	Thymol	0.57
11	19.7	3-Methyl-4-isopropylphenol	0.6
12	23.51	Vetylbois	0.81
13	25.51	3-tert-Butyl-4-hydroxyanisole	0.15
14	26.56	Delta-cadinene	0.11
15	28.29	Caryophyllene oxide	0.67
16	29.9	4-methoxy-2,3,6-trimethylphenol	1.48
Total identified			94.45

The presence of carvotanacetone as main component has been also recorded for EOs of *P. Mauritanica* collected in Mellab, Alnif, Zagora and Errachidia regions of Morocco (87.3%) [20]. same compound has been

also recorded in EOs of *Pulicaria undulate* from South Yemen (91.4%) and Sudan (55.87%) [21, 22], and *Pulicaria inuloides* from North Yemen (47.3%) [23].

Methanol extract of *P. mauritanica* showed a high content of total polyphenols and total flavonoids in comparison with petroleum ether extract. Saponins, alkaloids and hydrolysable tannins are present in methanol extract of *P. mauritanica* while terpenes and condensed tannins are absent in this organic extract. In the petroleum ether extract of *P. Mauritanica,* terpenes and alkaloids are found while saponins and tannins are absent (Table 3). Correlation between total polyphenols content and total flavonoids content reveals a positive correlation (Figure 1).

Table 3: Phytochemical investigation results of *P. mauritanica* extracts.

Phytochemical groups	Methanol extract	Petroleum ether extract
Total polyphenol content(mg GAEs/g dried plant)	31.321 ± 5.667^{a}	2.437 ± 0.027^{b}
Total flavonoid content (mg QEs/g dried plant)	4.207 ± 1.178^{b}	0.00^{b}
Terpenes	-	+++
Saponins	+++	-
Alkaloids	+	+++
Condensed tannins	-	-
Hydrolysable tannins	+++	-

-: Not detected; +++: Present in high; +: Present in low quantity

Each value is presented as mean ± standard deviation (n = 3). Different letters A, B and a, b are significantly different according to Newman & Keuls tests at $p < 0.05$.

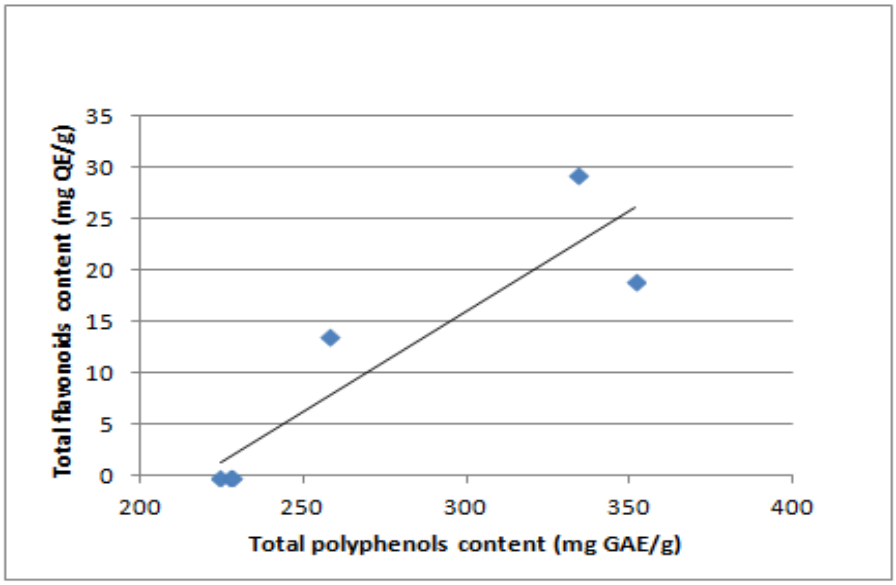


Figure 1: Correlation between total polyphenols content and total flavonoids content Correlation coefficient (R=0.906) of *P. Mauritanica.*

2.2. Antifungal activity

The effects of different concentrations of *P. mauritanica* EOs on mycelial growth of *P. digitatum* after an incubation period of 7 days at 25 ± 2°C are summarized in Table 4.

EOs of *P. mauritanica* were highly active against *P. digitatum*. Significant decrease in the mycelium growth with increase in the EOs concentration was observed. Complete inhibition was observed at 900 ppm. This result suggests that the EOs have a significant activity ($p<0.05$) and inhibited the mycelium growth of *P. digitatum*. The transfer of the mycelium disk totally inhibited to new plates containing fresh PDA (without EOs) showed that the mycelium of *P. digitatum* grew after incubation of 7 days, indicating a fungistatic effect of *P. mauritanica* EOs on *P. digitatum* (no fungicidal activity) at these concentrations.

The organic extracts of *P. mauritanica* were tested for tree concentrations 10,000; 20,000 and 30,000 ppm. Methanol extract representing the polar fraction favoured the mycelium growth of *P. digitatum* contrairy to the petroleum ether representing apolar extract which gave 46.21 ± 1.54 % of inhibition at 30,000 ppm (Table 5).

Table 4: Percent of inhibition of mycelial growth of *P. digitatum* measured after seven days of incubation with different concentrations of *P. mauritanica* EOs.

Concentrations (ppm)	Mycelial growth inhibition (%)
0	0.00 ± 0.00
100	1.135 ± 0.155
300	17.262 ± 7.539
400	30.952 ± 3.174
500	41.071 ± 1.190
600	51.785 ± 3.571
700	65.476 ± 8.730
800	83.333 ± 3.174
900	100.00 ± 0.000
1000	100.00 ± 0.000
1100	100.00±0.000

Each value is presented as mean ± standard deviation (n = 3).

Table 5: Inhibition of radial growth of *P. digitatum* on PDA medium with *P. mauritanica* organic extracts.

Type of extract	Inhibition rate (%)		
	Concentrations (ppm)		
	10,000	20,000	30,000
Methanol extract	-50.564 ± 13.536^{e}	-52.661 ± 7.462^{e}	-36.561 ± 12.6^{d}
Ether extract	5.044 ± 4.060^{c}	29.275 ± 5.130a^{b}	46.212 ± 1.543^{a}

Each value is presented as mean ± standard deviation (n = 3). Different letters of a, b, c, d, and e are significantly different according to Newman & Keuls tests at ($p < 0.05$).

The effectiveness of EOs against fungi has been demonstrated [24-31]. Generally, the major components determine the biological properties of the EOs. The bioactivity of monoterpenes, which contain oxygen functionalities such as ketones, alcohols, and aldehydes, has been demonstrated against plant pathogens [32].
Seven Moroccan Labiatae oils, whose major components are carvacrol, linalyl acetate and thymol, completely inhibited the mycelial growth of *B. cinerea* [33].
Some mechanisms have been proposed to explain the inhibition of mycelium growth such as the possibility that the terpenes may increase the concentration of lipidic peroxides and result in cell death [34], or that they may act on the hyphae of the mycelium, affecting the permeability of cells membrane and inducing leakage of components from the cytoplasm, and therefore, the death of the mycelium [35-37]. Omidbeygi et al [38] suggests that components of the EOs cross the cell membrane, interacting with the enzymes and proteins of the membrane, so producing a flux of protons towards the cell exterior which induces changes in the cells and, ultimately, their death. Cristani et al.[39] reported that the antimicrobial activity is related to the ability of terpenes to affect not only permeability but also other functions of cell membranes, these compounds might cross the cell membranes, thus penetrating into the interior of the cell and interacting with critical intracellular sites. High antifungal activity of petroleum ether extract could be explained by the important content on terpenes present in the petroleum ether extract but absent in methanol extract. Mohamed et al. [40] found in previous work that the butanolic and ethereal extracts of *Verbascum eremobium* were the most effective in reducing fungi growth of different fungi species and he indicated in his study that the effect of plant extracts on fungal pathogen may be attributed to their content on secondary metabolites (example, alkaloids, phenolic, flavonoids and terpenoids compounds) with known antifungal activity.

2.3. Antibacterial activity

Table 6 shows MICs and MCBs values obtained under closed conditions. The EOs of *P. mauritanica* examined exhibited antibacterial activity which generally increased in the following order: *P. aeruginosa*< *S. aureus* < *L. innocua.*

Table 6: MICs and MCBs of tested bacteria

	Gram +/-	MIC (µg/mL)	MCB (µg/mL)
S. aureus	+	0.7	1.6
P. aeruginosa	-	0.8	3
L. innocua	+	0.6	0.9

In our tests Gram positive bacteria are more sensitive to the EOs of *P. mauritanica* than gram negative bacteria. It has been established in previous works that Gram-positive bacteria are much more sensitive to drug action than Gram negative bacteria [41].

Ali NA et al. [21] revealed in previous paper that *Pulicaria undulate* EOs whose major components was carvotanacetone showed a strong bactericidal activity against *S. aureus*.

3.4. Antioxydant activity

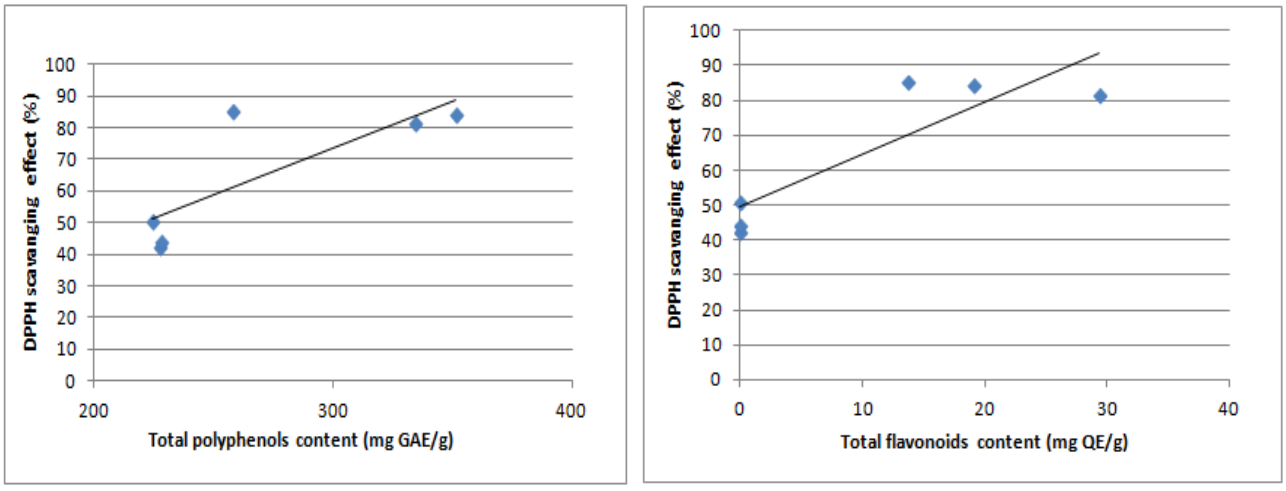
Antioxidant activity is most commonly evaluated by the DPPH scavenging activity test [42]. The values of IC_{50} of *P. mauritanica* EOs, methanol and petroleum ether extracts are presented in table 7.

Table 7: IC_{50} values (in [mg/ml] ± standard deviation) for the examined extracts of P. mauritanica in comparison with synthetic antioxidant references (BHT and Covi-ox T50).

Samples	IC_{50} (mg/mL)
Essential oils	0.080 ± 0.005^{c}
Methanol extract	0.027 ± 0.004^{b}
Petroleum ether extract	0.123 ± 0.0006^{d}
BHT	0.026 ± 0.002^{b}
Cov-iox T50	0.003 ± 0.0004^{a}

Each value is presented as mean ± standard deviation (n = 3). Different letters a, b, c, d, are significantly different according to Newman & Keels tests at ($p < 0.05$).

Methanol extract of *P. mauritanica* showed a strong scavenging activity of DPPH (IC_{50}=0.027mg/mL). Petroleum ether extract and EOs of *P. mauritanica* exhibited low scavenging effect (IC_{50}=0.123 mg/mL and IC50=0.080 mg/mL respectively). It appears that for the organic extracts the DPPH scavanging effect increased proportionally to the total polyphenols and to the total flavonoids content. A linear relationship between inhibition percentage of DPPH, the total polyphenols content and the total flavonoids content was established (Figure 2) and the coefficients of correlation exceed 0.8. Therefore, it can be concluded that the total polyphenol and total flavonoids compounds were the major components contributing to the antioxidant property of methanol extract of *P. mauritanica*. This has also been observed by many other authors for different others species [43-46]. Similar result obtained for the scavenging effect of DPPH by petroleum ether extract of *P. mauritanica* was found by Tian et al. [47] using *Galla chinensis* petroleum ether extract. This result can be explained by the low contents of total polyphenols content and the absence of flavonoids. Polyphenols are represented in majority by tannins and flavonoids [48], these two groups are considered as potent antioxidants. The antioxidant activity of phenolic compounds is mainly due to their redox properties, which play an important role in neutralizing free radicals, quenching singlet and triplet oxygen.



(A) (B)

Figure 2: Correlation between DPPH scavenging effect assay and total polyphenols content (A) and correlation between DPPH scavenging effect assay and total flavonoids content. Correlation coefficient R = 0.811 and R= 0.903, respectively, for total polyphenols content and total flavonoids content.

The ability of *P. Mauritanica* EOs to quench hydroxyl radicals, which are implicated in some diseases, revealed the presence of antioxidant compounds in this plant [49]. The scavenging activity observed for this EOs could be explained partially by the high amounts of oxygenated monoterpe carvotanacetone recorded for this EOs. Oxygenated monoterpenes have been recognized for their higher antioxidative capacity [50]. However, it's

difficult to attribute the antioxidant effect of a total EOs to one or few active compounds. Both minor and major compounds should make a significant contribution to the EO's activity [51].
EOs, Methanol and petroleum ether extract from *P. mauritanica* act as "radicals scavengers", therefore, could serve as safe natural alternative for synthetic antioxidants in food and pharmaceutical industries.

Conclusion

In conclusion, the EOs isolated from the aerial parts of *P. mauritanica* harvested in Tata oasis located in south-east of Morocco represent 0,861±0.013% of the dried plant material. *P. mauritanica* EOs exhibited a composition strongly dominated by carvotanacetone 87.58%. The observed antimicrobial activity against bacteria and *P. digitatum* fungus of this EOs can be attributed to its high content of carvotanacetone. The inhibition effects of petroleum ether extract of aerial parts of *P. mauritanica* against the mycelial growth of *P. digitatum* can be attributed to the presence of high content of terpens and alkaloids. The results obtained of the antioxidant activity show that methanol extract of this plant contains high levels of phenolics and flavonoids compounds.

Conflict of interest

None.

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