Phytochemical screening, quantitative analysis and antioxidant activity of Asteriscus imbricatus and Pulicaria mauritanica organic extracts

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Article history

Received: 20 September 2016 Received in revised form: 6 October 2016 Accepted: 8 October 2016 Abstract

Keywords

Asteriscus imbricatus Pulicaria mauritanica Phytochemicals Antioxidant activity Polyphenols Asteriscus imbricatus and Pulicaria mauritanica plants (Asteraceae) are well known for their medicinal value in Souss-Massa region near Agadir city in Morocco. However, several studies are needed to explain scientifically their efficacy. In this regard, different experiments are conducted on various organic extracts of these plants. The solvents used for the extraction are petroleum ether, chloroform, ethyl acetate and methanol. We performed a phytochemical characterization of flavonoids, terpenes and alkaloids compounds. We have quantified the total phenolic, total flavonoids and condensed tannins. We have also evaluated the antioxidant activity of these extracts. This study revealed the presence of flavonoids in the four extracts while the terpenes are present in petroleum ether and chloroform extracts for both plants species. Alkaloids are present only in A. imbricatus chloroform and ethyl acetate extracts. P. mauritanica is rich in flavonoids and polyphenols: P. mauritanica ethyl acetate extract contain 72.88±0.21 mg GE/g DWE of total polyphenols and 38.95±0.75 mg QuE/g of DWE of flavonoids. However, A. imbricatus ethyl acetate extract contains 25.36 mg GE/g DWE of total polyphenols and 3.68 mg QuE/g DWE of flavonoids. The condensed tannins are present in small quantity, 0.07 and 0.13 mg Cat/g of DW in ethyl acetate and metanolic extracts of A. imbricatus respectively. P. mauritanica contains also 0.03 and 0.05 mg mg Cat/g of DW of condensed tannins in ethyl acetate and metanolic extracts respectively. The results of the antioxidant activity showed that the ethyl acetate extract had the highest inhibition percentage of free radical DPPH with 48.02% and 69.68% for A. imbricatus and P. mauritanica respectively at the concentration of 1 mg/ml.

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Introduction

Medicinal plants are a precious heritage for humanity; our ancestors used these plants to ensure their health and transmitted their knowledge and their experiences generation to generation. About 80% of the world population uses this mode of therapy (Azaizeh *et al.*, 2003) especially in undeveloped countries when the modern medical system was absent (Tabuti *et al.*, 2003). Due to its geographical location and its climate, Morocco offers rich and varied flora with a marked endemism. Morocco is one of the Mediterranean countries that have a long medical tradition knowledge based on medicinal plants (Scherrer *et al.*, 2005). Many botanicals from medicinal plants are also investigated or used in agricultural field to develop bio-pesticides. These biopesticides do not develop resistance (Al-Adhroey et al., 2010) in contrast to the synthetic fungicides that result in several problems namely: the widespread development of resistance (Elad, 1992), the toxicity to non-target organisms and other environmental problems. The secondary metabolism of the plant is characterized by a structural diversity, which confers very important chemical and biological properties. Medicinal plant extracts were assessed for their antifungal potential against Botrytis cinerea fungi (Chebli et al., 2003a; Chebli et al., 2003b; Senhaji et al., 2014). Among the plants tested, the organic extracts of Asteriscus imbricatus and Pulicaria mauritanica plants have shown toxic effect against B. cinerea (Senhaji et al., 2014). For this reason, the organic extracts of those two plants were selected for a phytochemical study. Therefore, the present work



aims to study the phytochemical composition of the organic extracts by the characterization of flavonoids, terpenes and alkaloids compounds and by the quantification of total phenolic, total flavonoids and condensed tannins using calorimetric assay methods. In addition, antioxidant activity was carried out by means to inhibition of DPPH radical.

Materials and Methods

Preparation of plant organic extracts

The aerial part of *Asteriscus imbricatus* and *Pulicaria mauritanica* plant samples were collected respectively in Cap Ghir (geographic coordonates 30°37'48"N, 9°52'48"W) and Imozzer Idaoutanane (geographic coordonates 30°40'21"N, 9°28'50.90"W) areas near Agadir city in April. Herbarium voucher samples are deposited in the Mechanic, Process, Energy and Environment Laboratory, National School of Applied Sciences, University Ibn Zohr, Agadir, Morocco.

Plant samples were air-dried in the shade, at room temperature then grounded to a fine powder. The plants powders were undergone to a hot extraction in the Soxhlet apparatus, using four solvents in the order of increasing polarity: petroleum ether, chloroform, ethyl acetate and methanol. Dry residue of each extract was obtained after concentration and removal of the solvent by rotary evaporation. Each dry residue was dissolved in methanol to prepare a solution of 1 mg/ml. This solution was used to realize all the phytochemical tests (Harborne, 1998).

Characterization of flavonoids using thin layer chromatography (TLC)

The extracts were subjected to Thin Layer Chromatography examination (TLC) using commercial silica plates for the determination of the secondary metabolites. We tried different solvent systems to achieve better migration and components separation of the studied extracts. For each sample and analysis the same volume 20 μ l was used. Regarding flavonoids, the best separation of the constituents of studied extracts was obtained using the solvent system: toluene/acetic acid/water (125/72/3) (Markham, 1982). The revelation of different fluorescence was carried out under UV light at 365 nm after spraying with the NEU reagent (2 amino ethyl diphenylboric). Frontal ratio (Rf) was calculated as: the distance line-compound deposition on the line distance of solvent.

Analysis of flavonoids by HPLC

The HPLC analysis was performed using the

method described by Engida et al. (2013) to identify the aglycon flavonoids if they exist in the studied extracts. The stationary phase consists of a column type bonded silica reversed phase Nucleosil Symmetry C18 (150 x 4.6 mm, 5 μ m). The compounds were eluted with a gradient elution of mobile phases A and B. Solvent A consisted of deionized water and 1% acetic acid and solvent B consisted of methanol (HPLC grade) and 1% acetic acid. Acetic acid (1%) was added to reduce peak tailing. Gradient elution program was set as follows: 10% B-17.2% B (18 min), 17.2% B-23% B (12 min), 23% B isocratic (10 min), 23%-31.3% B (13 min), 31.3% B-46% B (12 min), 46% B–55% B (5 min), 55% B–100% B (5 min), 100% B isocratic (8 min), 10% B (2 min) and 10% B isocratic (5 min). The injection volume for all samples was 20 µl. Flavonoids were monitored at 280 nm at a flow rate of 1 ml/min. All determinations were run in triplicate. We first injected standard solutions containig quercetin, luteolin and myricetin at 0.01 mg/ml before injecting our extracts at 1mg/ ml.

Characterization of terpenes and alkaloids

For terpenes, the benzene was used as a migration solvent. After migration, the silica gel plate was sprayed with antimony chloride, and then placed in an oven at 110°C for 10 min. Any fluorescence detected, under UV at 365 nm, after this treatment proves that the tested material contains terpenes (Randerth, 1971). For alkaloids we used the solvent system consisting of (AcEt/MeOH/NH4OH) (9/1/1). The detection was carried out by spraying the Dragendorff reagent. The presence of alkaloids was shown by a bright orange color on the chromatogram (Randerth, 1971).

Total phenolic contents determination

About 200 µl of each methanolic extract was mixed with 1 ml of Folin-Ciocalteu reagent diluted ten times and 2 mL of H₂O. The mixture incubated at room temperature for 4 minutes after addition of 0.8 ml of sodium bicarbonate 7.5%. The total polyphenols were determined after 2 hours of incubation at room temperature. The absorbance of the blue color was measured at $\lambda_{max} = 765$ nm with a spectrophotometer Mapada UV-31. Quantification was done using a standard curve of gallic acid. The results were expressed in milligram gallic acid equivalents per g weight extract (mg GE/g of DW) according to Wong *et al.* (2006) method.

Quantification of flavonoids

The flavonoids were quantified by mixing

2mL of each methanolic extract with 100 μ l of reagent (2-aminoethyl diphenylboric (NEU) at 1% in methanol). The absorption of the extract was recorded at 409 nm and compared to that of quercetin (0.05 mg / ml) treated with the same reagent in the same conditions. The total flavonoids are calculated according to the formula described by Hariri *et al.* (1991):

 A_{ext} .: Absorption of the extract studied. A_{a} .: Absorption of quercetin.

 C_{ext}^{q} : Concentration of the extract in mg / ml.

Quantification of flavonoids was expressed in milligrams quercetin equivalents per one gram of dry weight extract (mg QuE/g of DW).

Quantification of condensed tannins

The principle of this assay was based on attaching the aldehyde group of vanillin on 6 carbon of catechin ring to form a red chromophore complex that absorbs at 500 nm (Schofield *et al.*, 2001). The quantification of condensed tannins was performed according to the Heimler *et al.* (2006) method: Approximately 400µl of each extract was added to 3 ml of vanillin (4%) dissolved in methanol and 1.5 ml of concentrated hydrochloric acid. After 15min of incubation, the absorbance was measured at 500 nm. The concentration of condensed tannins was deduced from the calibration range established with catechin (0-30 µg/ml) and was expressed in mg catechin equivalents per one gram of dry weight extract (mg Cat/g of DW).

Antioxidant activity

The antioxidant activity was evaluated in vitro by measuring the trapping power of free radical DPPH (1.1-Diphenyl-2-picryhydrazyl) of different extracts of A. imbricatus and P. mauritanica. About 500 μ l of each extract at various concentrations (1 mg / ml, 0.5 mg / ml, 0.25 mg / ml, 0.125 mg/ml), was mixed with 500 μ l of a methanol solution of DPPH (0.004%). After 30 minutes of incubation in the dark and at laboratory temperature, the absorbance was read at 517 nm. The inhibition of free radical DPPH by BHT6 and the Coviox7 was also analysed with the same concentrations and the same conditions for comparison.

The inhibition of free radical DPPH in percentage (I%) was calculated as follows:

A blank: Absorbance of the blank (containing all

reagents except the test compound).

Sample A: Absorbance of the test compound.

The extracts were compared with those of two synthetic antioxidants BHT and COVIOX (Leitão *et al.*, 2002). All tests were performed in triplicate for each concentration.

Statistical analysis

Statistical analyses were conducted using SPSS (Statistical program for Social Sciences) version 16.0. All data were subjected to analysis of variance (ANOVA). Significant differences among means from triplicate analyses at (p<0.05) were determined by Newman and Keuls test.

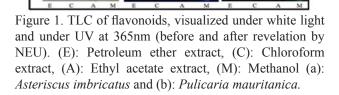
Results and Discussion

Flavonoids characterization using TLC and HPLC

The results of flavonoids characterization of A. imbricatus and P. mauritanica organic extracts are showed in the Figure 1. The analysis of TLC under UV light showed similar aspects between the two plants: Firstly the fluorescence appears in the four organic extracts, showing that they all contain flavonoids but in various proportions. The ethyl acetate extract has more bands with intense fluorescence compared to other extracts. Secondly this fluorescence is intensified when the polarity of the solvent increases, from petroleum ether, chloroform and ethyl acetate extract, but it decrease for the methanol. TLC analysis shows also that there is a difference concerning the number and color of the bands:

For *A. imbricatus* plant extracts (Figure 1a), petroleum ether extract revealed the presence of two bands essentially, B1 (light orange low intensity, Rf: 0.28) and the band B3 (orange, Rf: 0.21). While in the chloroform and ethyl acetate we found four bands: The B1 and B3 and also two other bands B2 (yellow, Rf: 0.17) and B4 (light yellow low intensity, Rf: 0.28). These four bands are found in ethyl acetate with high intensity of fluorescence. All the fluorescence that appeared on the chromatogram is yellow or orange color which means they represent the group of flavonols and flavons according to Bohm (1998).

For P. mauritanica plant extracts (Figure 1b), petroleum ether extract revealed the presence of two bands essentially, B1 (green, Rf: 0.625) and the band B2 (green, Rf: 0.56). While in the chloroform and ethyl acetate we found six bands, four new bands in addition to B and B2: The B3 (green, Rf: 0.45), B4 (orange-yellow, Rf: 0.39), B5 (orange-yellow, Rf:



0.26) and B6 (orange-yellow, Rf: 0.15). According to Bohm (1998) green, yellow and orange fluorescence represent the flavons and flavonols.

The HPLC chromatograms of three flavonoid standards: luteolin, myrcetin and quercetin and the petroleum ether and chloroform extracts of A. imbricatus are showen in Figure 2. The retention times of the standards flavonoids are: luteolin (78.58 min), myrcetin (79.664 min) and quercetin (82.562 min) (Figure 2a). The petroleum ether extract of A. imbricatus presents five peaks: 1(72.448), 2(76.915), 3(78.701), 4(83.091), 5(89.077) (Figure 2b). While, A. imbricatus chloroform extract displays three peaks with the following retention times: 1(78.216 min), 2(80.578 min), 3(86.878 min) (Figure 2c). Comparing the retention times of these peaks extracts to the retention times of flavonoids standards, we find that the third peak of petroleum ether extract and the first peak of chloroform extract of A. imbricatus correspond to the retention time of luteolin. The presence of luteolin which is an aglycon in both petroleum ether and chloroform extracts is due to their low polarity. Indeed according to Bruneton (2009) the aglycons are mostly soluble in no polar solvents. While the retention times recorded to the other extracts of A. imbricatus and to the P. mauritanica extracts, do not match any of the tested standards.

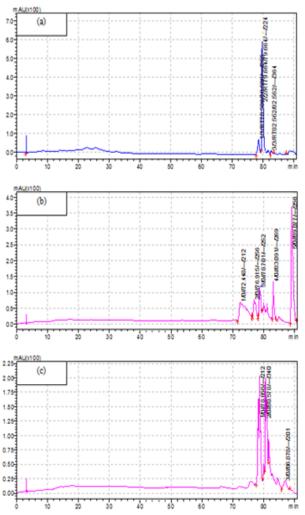


Figure 2. HPLC chromatogram of three flavonoid standards: luteolin (78.58), myrcetin (79.664), quercetin (82.562) (a). HPLC chromatogram of *Asteriscus imbricatus* petroleum ether extract (b), showing five peaks: 1(72.448), 2(76.915), 3(78.701), 4(83.091), 5(89.077) and (c): HPLC chromatogram of *Asteriscus imbricatus* chloroform extract, showing four peak: 1(78.216 min), 2(80.578 min) and 3(86.878 min) obtained at 280 nm.

Terpenes and alkaloids characterization

For these two plants, a blue fluorescence is observed in both petroleum ether extract and chloroform extract which means that these extracts contain terpenes while the two other extracts do not contain terpens. In the other hand the blue fluorescence spots that appear on the *A. imbricatus* chromatogram have a condensed color compared to the spots of *P. mauritanica* chromatogram, which allows us to say that *A. imbricatus* plant is rich in terpenes than *P. mauritanica*. The presence of alkaloids is demonstrated by spraying Dragendorff reagent on the chromatogram.

For *A. imbricatus,* we notice the appearance of orange spot in chloroform and ethyl acetate extracts, which indicate that these extracts contain the alkaloids. While the petroleum ether and methanol

Table 1. Secondary metabolites groups existing in Asteriscus imbricatus and Pulicaria mauritanica organic extracts

	Flavonoids		Terpenes		Alkaloids		Condensed	
							Tannins	
Organic	AI	PM	А	PM	А	PM	А	PM
extracts								
Petroleum	+	+	+	+	-	-	-	-
ether								
Chloroform	++	++	+	+	+	-	-	-
Ethyl	+++	+++	-	-	+	-	+	+
acetate								
Methanol	+	+	-	-	-	-	+	+

+: Presence; -: Absence

AI: Asteriscus imbricatus

PM: Pulicaria mauritanica

extracts showed no stain. While the absence of oranges spots in *P. mauritanica* extracts after spraying with Dragendorff reagent shows that this plant does not contain alkaloids.

The results of phytochemical study are summarized in Table 1. The flavonoids are present in the four extracts, terpenes are present in the two extracts petroleum ether and chloroform extracts for the two plants, while the alkaloids are present only in chloroform and ethyl acetate extracts of A. imbricatus. We found that the secondary metabolites content of the 2 plants depends on the extracts. This change may be related to the polarity and capability to extract substances to be dissolved in the used solvent. The petroleum ether and the chloroform (no polar) were the most powerful regarding the terpenes extraction. Ethyl acetate (moderately polar) was the strongest in extracting more flavonoids. The alkaloids were extracted by the medium polar solvents chloroform and ethyl acetate, whereas the condensed tannins were more extracted by methanol which is polar solvent (Cowan, 1999). These results are in agreement with previous studies which were conducted on plants bellowing to Asteraceae family: Koffi et al. (2009) have detected the presence of sterols, polyterpenes, polyphenols, flavonoids, alkaloids, tannins and saponins in the leaves of Chromolaena odorata (Asteraceae) and have shown that the leaves of the plant Ageratum conyzoides L (Asteraceae) contain sterols, polyterpenes, polyphenols, flavonoids, alkaloids and saponins. Djellouli et al. (2013) reported that Anvillea Radiata, Cotula cinerea and Matricarea pubescens (Asteraceae) contain the saponines, the terpenoïdes, the alkaloids, the tannins,

flavonoids, and steroids. Edeoga et al. (2005) found that both of Emilia coccinea and Tridax procumbens (Asteraceae) contain alkaloids, tannins, saponins, and flavonoids. Our results are also in agreement with previous studies conducted on plants bellowing of the same genus: The phytochemical screening of Asteriscus graveolens, revealed the presence of alkaloids coumarins, condensed tannins, terpenes and cyanogenic compounds (Alilou et al., 2014). This study showed also that Asteriscus graveolens contain the luteolin that we have also identified in Asteriscus imbricatus. For the Pulicaria genus: the phytochemical screening of Pulicaria crispa revealed the presence of the following metabolites: triterpenoids, alkaloids and flavonoids (Elshiekh et al., 2015). Indeed the essential oil composition of A. imbricatus and P. mauritanica were described respectively by Alilou et al. (2008) and Cristofari et al. (2011).

Quantification of total phenolic, total flavonoids and condensed tannins

As the Table 2 shows, the total polyphenols and flavonoids contents according to the organic extracts investigated varied in the same way for the two plants studied and from extract to extract. The ethyl acetate is the richest extract in polyphenols and flavonoids followed by chloroform extract, the methanolic extract and finally the petroleum ether extract. P. mauritanica contain more flavonoids and total polyphenols compared to A. imbricatus. Indeed P. mauritanica ethyl acetate extract contain 72.88±0.21 mgGE/g DWE of total polyphenols and 38.95±0.75 mgQuE/g of DWE of flavonoids. However A. imbricatus ethyl acetate extract contain 25.36 mgGE/g DWE of total polyphenols and 3.68 mgQuE/g DWE of flavonoids. There are no condensed tannins in the two plants studied in both extracts petroleum ether and chloroform. While ethyl acetate and methanol extracts contain a very low quantity of condensed tannins. The total phenol and flavonoids content of plants belonging to the Asteraceae family were described by several previous works: methanolic extract of Atractylis gummifera contain 17 mgGE/g DWE of total phenolic and 7 mgQuE/g of DWE of flavonoids (Khadhri et al., 2013). Chrysanthemum trifurcatum contain 07.23 g/100g DWE and Asteriscus maritimus contain 21.24 g/100g DWE of total polyphenols (Khadhri et al., 2013). While Artemisia herba alba contains only 13.06 mg/g DWE of total polyphenols (Djeridane et al., 2006). In other studies they found that ethyl acetate extract of Pulicaria jaubertii contain 322.98 mgGE/g DWE of total phenolic compounds and

Table 2. Total phenolic, total flavonoids and condensed tannins contents of *Asteriscus imbricatus* and *Pulicaria mauritanica* organic extracts.

	Total pl	nenolics	Total fla	avonoids	Condensed Tannins		
Organic	(mg G E/	g DWE) ¹	(mg QuE	g of DW)²	(mg Cat E/g of DW) [®]		
	AI	PM	AI	PM	AI	PM	
extracts							
Petroleum	7.63ª±0.16	20.08°±0.22	0.37ª±0.05	10.63°±1.38	0°*	0°	
ether							
Chloroform	21.14°±0.22	58.41°±0.1	2.21°±0.3	30.5°±0.72	0 ^b	0°	
Ethyl	25.36°±0.07	72.88°±0.21	3.68°±0.01	38.95°±0.75	0.07°±0.001	0.03°±0.002	
acetate							
Methanol	13.59°±0.16	39.37°±0.28	1.7 ^c ±0.04	25.79 ^c ±0.49	0.13 ^ª ±0.002	0.05°±0.003	

*Each value represents the mean of three replicates \pm SD. Means followed by different letters in each column are significantly different at P < 0.05 according to Newman and Keuls test.

¹: milligrams gallic acid equivalents/gram dry weight of extract.

²: milligrams quercetin equivalents/ gram dry weight of extract.

³: milligrams catechin equivalents/ gram weight of extract.

AI: Asteriscus imbricatus and PM: Pulicaria mauritanica

159.8± 22.11 mg QuE/ g of DWE of flavonoids (Algabr *et al.*, 2010) and methanolic extract of *Pulicaria inuloides* contain 91.2± 0.95 mg G E / g DWE of total phenolic compounds and 75± 85.7 mg QuE/ g of DWE of flavonoids (AL-HAJJ *et al.*, 2014). Moreover, ethyl acetate extract of *Pulicaria dysenterica* contain 118.29µg mg G E / g DWE of total phenolic compounds and 55.76 ±0.83 µgQuE/ g of DWE of flavonoids (Boga *et al.*, 2014). If we compare our results with these studies, we can say that *P. mauritanica* can be considered as a very rich plant in total polyphenols and flavonoids while *A. imbricatus* is moderately rich in these compounds.

Antioxidant activity

The results of the antioxidant activity of the studied plants are presented in the Figure 3. The tested extracts are all DPPH inhibitors but there is a significant difference between them. Indeed at a concentration of 1 mg / ml for the two plants A. imbricatus and *P. mauritanica* respectively the ethyl acetate extract had the highest inhibition percentage of free radical DPPH (48.02%) and (69.68%). Followed by chloroform extract (38.16%) and (54.52%), the methanol extract (31.72%) and (41.45%). While, the petroleum ether extract showed the lowest percentage (20.56%) and (36.19%).

The Figure 3 shows also that the inhibition percentage of DPPH for all extracts decreases with decreasing concentration. We note also that there is a correlation between the content of phenolic compounds in each extract and the scavenging activity. Indeed, the coefficient of correlation between

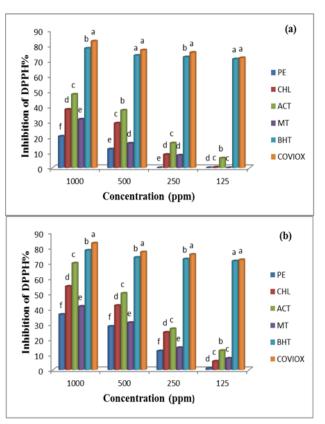


Figure 3. The inhibition percentage of free radical DPPH of the organic extracts. (PE): Petroleum ether, (CHL): Chloroform, (ACT): Ethyl acetate (MT): Methanol Significant differences (P < 0.05) between means were indicated by different letters above histogram bars according to Newman and Keuls test (a): *Asteriscus imbricatus* and (b): *Pulicaria mauritanica*.

antioxidant capacity and polyphenol content in the four organic extracts was $R^2 = 0.969$ and $R^2 = 0.965$

for A. imbricatus (Figure 3a) and P. mauritanica (Figure 3b) respectively. This correlation indicates that the antioxidant capacity of these extracts is due to their content in phenolic compounds. Other studies have also reported that there is a positive correlation between total phenolic content and antioxidant activity (Wong et al., 2006; Djeridane et al., 2006). We determined graphically the concentration corresponding to 50% inhibition of DPPH (IC₅₀) for the ethyl acetate extract which showed the highest percentage of inhibition, the value is $IC_{50} = 1.48 \text{ mg/}$ mL for A. *imbricatus* and $IC_{50} = 0.63$ mg/mL for P. mauritanica. We can compare our results with the studies that have investigated the antioxidant activity of other plants belonging to the Asteraceae family: Alilou et al. (2014) found that IC_{50DPPH} was 0.2498 mg/ml for the essential oil of Asteriscus graveolens. While, Echinops spinosus ethanol extract showed the highest ability to reduce DPPH radicals 90% and the $IC_{_{50DPPH}}$ value for this extract was 147µg/ mL (Khedher *et al.*, 2014). While the IC_{50DPPH} of Pulicaria gnaphalodes methanol extract was 9.1±0.5 µg/ml (Kamkar et al., 2013). The IC_{50DPPH} of Pulicaria jaubertii ethyl acetate extract was 1.24µg/ml (Algabr et al., 2010). In another study Pulicaria dysenterica methanol extract shows 22 % of DPPH inhibition (Boga et al., 2014). From this comparison we can say that Asteriscus imbricatus have a moderate antioxidant activity while Pulicaria mauritanica presents an interesting antioxidant activity compared with plants of the same family.

Conclusion

Phytochemical study of the four organic extracts of Asteriscus imbricatus and Pulicaria mauritanica was useful to provide information on the potential of these plants as a source of secondary metabolites. At the same time, it also provides data on the characteristics of the extraction method using solvents of varying polarities. In this study, it was prouved that the ethyl acetate extracts contain the high quantity of total phenolic and flavonoids compounds. There is a positive correlation between the content of total phenolic and flavonoids compounds in the extracts and the antioxidant activity. While petroleum ether and chloroform extracts of the two plants were rich in terpenes compounds. It was showed also that P. mauritanica plant is rich in phenolic compounds, while A. imbricatus plant is rich in terpenes and contains alkaloids. Finally, the extracts obtained using polar solvents were more rich in phenolic compounds whereas the extracts obtained using no polar solvents were rich in terpenes.

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