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Research Article

Total phenolics, flavonoids contents and antioxidant activity of essential oil and aqueous extracts of *Salvia aucheri* Boiss var. *mesatlantica*

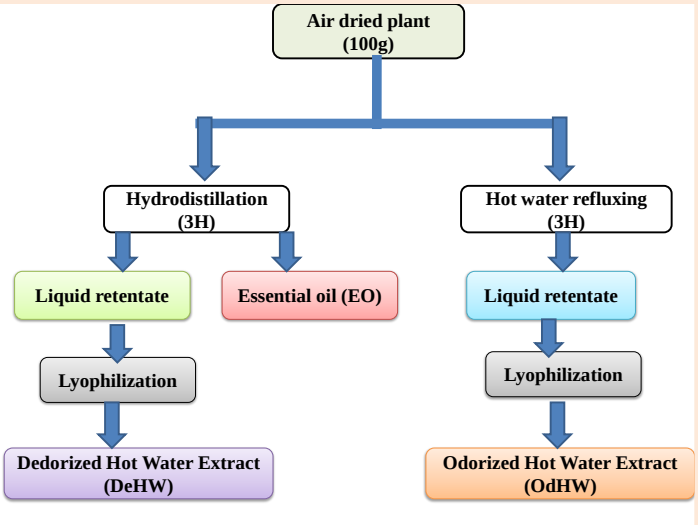
Mohamed Znini, Amal Laghchimi, Abdeslam Ansari, Mounir Manssouri, Lhou Majidi*

Université My Ismail, Laboratoire des Substances Naturelles & Synthèse et Dynamique Moléculaire, Faculté des Sciences et Techniques, BP 509, 52003, Errachidia, Morocco

Abstract

This study was designed to examine the total phenolic and flavonoid contents and antioxidant activity of essential oil (EO) and Odorized Hot Water Extract (OdHW) and Dedorized Hot Water Extract (DeHW)) of *Salvia aucheri* var. *mesatlantica* were using 2,2-diphenyl-1-picrylhydrazyl radical (DPPH•) assay and the β-carotene bleaching (BCB) test. The total phenolic contents of the different extracts as caffeic acid equivalents were found to be highest in OdHW (429.3 µg/mg) followed by DeHW (337.25 µg/mg) and the phenolic contents was lower EO (0.71 µg/mg). The inhibition on DPPH• and prevented the BCB of all extracts as ascorbic acid and BHT standards, respectively were in the order of OdHW extract > DeHW extract > EO. The findings show that a positive correlation was observed between the antioxidant activity and total phenolic levels of extracts. Arial parts of *S aucheri mesatlantica* being rich in phenolics may provide a good source of antioxidant.

Keywords: Total phenolics, Flavonoids, Antioxidant activity, Extracts, *S aucheri mesatlantica*



***Correspondence**
Author: Lhou Majidi
Email: lmajidi@yahoo.fr

Introduction

Free radicals are the molecules with unpaired electrons and commonly called reactive oxygen species (ROS). They are generated during the process of cellular oxidation, some examples includes superoxide anion, hydrogen peroxide, hydroxyl and nitric oxide radical [1] Free radicals react with nucleic acids, mitochondria, proteins and enzymes and resulted in their damage in the human health by causing severe diseases, such as cancer and cardiovascular diseases by cell degeneration [2]. Therefore much attention has been focused on the use of antioxidants to protect from damage due to free radicals. There are two basic categories of antioxidants, namely, synthetics and naturals. Synthetic antioxidants such as butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT) have been used as antioxidants since the beginning of this century. However, restrictions on the use of these compounds are being imposed because of their carcinogenicity and other toxic properties [3]. Thus, the interest in natural antioxidants has increased considerably. These compounds exhibit their antioxidant activity by various mechanisms including chain breaking by donation of hydrogen atoms or electrons that convert free radicals into more stable species and decomposing lipid peroxides into stable final products [4]. Among natural products, essential oils and plant extracts have been of great interest for their potential uses as alternative remedies for the treatment of many infectious diseases and the preservation of the foods from the toxic effects of the oxidants [5].

The genus *Salvia* (sage) is one of the largest and the most important aromatic and medicinal genera of the *Lamiaceae* family which contains 900 different species widespread throughout Mediterranean region, South-East

Asia and Central America. About 14 species are also found in Morocco, where, 5 of these species are endemic [6] *Salvia* is a rich source of phytochemicals including phenolic acids, polyphenols, flavonoid glycosides, and essential oils [7-10].

Salvia aucheri var. *mesatlantica* is a spontaneous shrub and endemic to the middle and high Atlas of Morocco (**Figure 1**). The leaves and stems of this plant, known locally as ‘‘Tagoltamte”, are used in decoction by the local population as herbal tea and against stomach ailments, rheumatism and digestive disorder [10].

Figure 1 *Salvia aucheri* var. *mesatlantica* in its native habitat in south-eastern of Morocco.

Recently, we have reported the first studied of the chemical composition of essential oil of *S. aucheri mesatlantica* and its application as a green inhibitor for the corrosion of steel in 0.5 M H_2SO_4 [10]. We reported also, the antifungal activities of liquid and vapour-phase of the essential oil against fungi commonly causing deterioration of apple [11]. As far as our literature survey could ascertain, antioxidant activities of *S. aucheri mesatlantica* have not previously been published. Therefore, the objective of this study was to determine the antioxidant activities of its essential oil, as well as the two various extracts obtained by water, using two different methods: 2,2-diphenyl-1-picrylhydrazyl (DPPH•) radical activity assay and the β-carotene bleaching (BCB) test. Total phenolics and flavonoids content of plant extracts are also reported.

Experimental

Plant material

The aerial parts of *S. aucheri mesatlantica* were harvested in May 2009 in the wild in the mountain Assoul located at the south-east of Errachidia (Morocco) at an altitude of 2000 m. Identification of the species was confirmed by biology unity and voucher specimens were deposited in the herbarium of Faculty of Sciences and Technology of Errachidia.

Preparation of the Extracts

Essential oil isolation (EO)

The EO used in this study was the same we used in our previous study [10]. It was prepared by hydrodistillation for 3h using a Clevenger type apparatus and analyzed by gas chromatography (GC) and gas chromatography/mass spectroscopy (GC/MS). The EO yield was approx 1.2% and a total of 38 components, accounting for 95.4% of the total oil, were identified (**Table 1**). It contained camphor as the main component with 49.80%, followed by 1,8-cineol (9.50%), the viridiflorol (8.8%) and camphene (7.80%).

Table 1 Chemical composition of essential oil from *S. aucheri mesatlnatica* from Morocco [10].

N°[a]	Components	RI a[b]	RI p[c]	%[d]
1	Tricyclene	921	995	0.3
2	α-Pinene	930	1007	2.9
3	Camphene	943	1046	7.8
4	β-Pinene	967	1088	1.2
5	Myrcene	976	1132	0.2
6	p-Cymene	1007	1229	1.5
7	1,8-Cineol*	1016	1183	9.5
8	Limonene*	1016	1167	1.9
9	Camphenilone	1051	1407	0.2
10	Linalool	1078	1498	0.2
11	α-Campholenal	1096	1436	0.1
12	Camphor*	1119	1467	49.8
13	*trans*-Pinocarveol*	1119	1599	1.0
14	*cis*-Verbenol	1122	1626	0.4
15	Pinocarvone	1132	1511	0.4
16	Borneol	1143	1646	1.7
17	p-Cymen-8-ol	1154	1789	0.5
18	Terpinen-4-ol	1155	1551	0.4
19	Myrtenal	1163	1570	0.5
20	α-Terpineol	1166	1643	0.4
21	Myrtenol	1174	1734	0.3
22	trans-Carveol	1192	1777	0.3
23	Carvone	1210	1673	0.2
24	Bornyl acetate	1265	1529	1.0
25	Carvacrol	1275	2135	0.5
26	α-Terpinyl acetate	1329	1643	0.4
27	Geranyl acetate	1358	1706	0.1
28	γ-Cadinene	1506	1706	0.3
29	*trans*-Calamenene	1509	1777	0.1
30	Caryophyllene oxyde	1569	1919	0.3
31	Globulol	1575	1994	0.2
32	Viridiflorol	1584	2021	8.8
33	Epoxyde d'Humulene II	1594	1973	0.3
34	Caryophylla-4(14),8(15)-dien-5α-ol	1621	2220	0.3
35	τ-Cadinol	1627	2102	0.5
36	β-Eudesmol	1636	2157	0.3
37	α-Cadinol	1640	2161	0.3
38	Cadalene	1657	2140	0.3
	Total identified			**95.40**
	Monoterpene Hydrocarbons			15.8
	Oxygenated Monoterpenes			67.9
	Sesquiterpene Hydrocarbons			0.7
	Oxygenated Sesquiterpenes			11.0

[a] Order of elution are given on apolar column (Rtx-1)
[b] RI a = retention indices on the apolar column (Rtx-1)
[c] RI p = retention indices on the polar column (Rtx-Wax)
[d] % = relative percentages of components are given on the apolar column except for components with an asterisk (*) (percentages are given on the polar column)

Preparation of the Deodorized Hot Water Extract (DeHW)

After completion of hydrodistillation, the liquid retentate was collected, filtered and centrifuged at 5000 rpm for 30 min. The supernatant was also filtered to eliminate any residues and lyophilized to give finally DeHW in a yield of 14.78% (w/w) [12].

Preparation of the Odorized Hot Water Extract (OdHW)

A portion (100 g) of dried plant material was extracted with 1L of water under refluxing for 3 h. The liquid retentate was collected, filtered and centrifuged at 5000 rpm for 30 min. The supernatant was also filtered to eliminate any residues and lyophilized to give finally OdHW in a yield of 17.6% (w/w).

Assay for total phenolics contents (TPC)

Total phenolics constituent of the extracts were determined by using the Folin-Ciocalteu reagent according to the method described by Sarikurkcu et al. (2010) with slight modifications[13]. 0.1 ml of extract solution, containing 1000 µg extract, was added to a volumetric flask. Then, 45 ml distilled water and 1 ml Folin–Ciocalteu reagent was added and flask was shaken vigorously. After 3 min, a 3 ml of Na_2CO_3 (2%) solution was added and the mixture was allowed to stand for 2h by intermittent shaking. Absorbance was measured at 760 nm. TPC were expressed as caffeic acid equivalents (CAEs), using a calibration curve of a freshly prepared caffeic acid solution used as standard agent. For the caffeic acid, the curve absorbance versus concentration is described by the equation:

$$\text{Absorbance} = 0.0653\ \text{CAEs}\ (\mu g) - 0.001 \qquad (R^2 : 0.9799).$$

Assay for total flavonoids contents (TFC)

TFC was determined by using the method of Sarikurkcu et al. (2010) with slight modifications [13]. Briefly, 1 ml of 2% aluminium trichloride ($AlCl_3$) in methanol was mixed with the same volume of the extract solution (1000 µg). Absorbance values of the samples were determined at 430 nm after 15 min duration against a blank. TFC were expressed on a dry weight basis as quercetin equivalents (QEs), using a calibration curve of a freshly prepared quercetin solution used as standard agent. For quercetin, the curve absorbance versus concentration is described by the equation:

$$\text{Absorbance} = 0.1195\ \text{QEs}\ (\mu g) + 0.0319 \qquad (R^2 : 0.9996)$$

Antioxidant activity

DPPH• assay

The antioxidant activity of *S. aucheri mesatlnatica* essential oil and two water extracts extracts were assessed by measuring their scavenging abilities to 2.2'-diphenyl-1-picrylhydrazyl stable radicals. The DPPH• assay was performed as described [14]. In succinct terms, the aliquots (50 µl) of various concentrations of the test compound were added to 5 ml of a 0.004% methanol solution of DPPH•. After a 30 min incubation period at room temperature the absorbance was read against a blank at 517 nm. Under the same operating conditions, natural antioxidant reagent Ascorbic acid (3-7 µg/ml) was used as the positive control and all tests were carried out in triplicate. Inhibition free radical DPPH• in percent (I%) was calculated in following way: :

$$I\% = (1 - A_{sample}/A_{blank}) \times 100$$

where A_{blank} is the absorbance of the control reaction (containing all reagents except the test compound), and A_{sample} is the absorbance of the test compound. The antiradical activity was finally expressed as IC_{50} (µg/ml), the extract concentration required to cause a 50% inhibition.

β-Carotene bleaching (BCB) test

In this assay antioxidant capacity is determined by indirectly measuring the inhibition of the volatile organic compounds and the conjugated diene hydroperoxides arising from linoleic acid oxidation. Antioxidant activity was

carried out according to the β-carotene bleaching method with minor modifications [15]. β-Carotene (2 mg) was dissolved in 10 ml chloroform. 1 ml of the chloroform solution was mixed with 20 µl linoleic acid and 200 mg Tween-20. The chloroform was evaporated under vacuum at 45°C, then 50 ml oxygenated water was added, and the mixture was vigorously shaken. The emulsion obtained was freshly prepared before each experiment. An aliquot (250 µl) of the β-carotene-linoleic acid emulsion was distributed in each of the tubes and 30 µl of various concentrations of each extract are added. The tubes were incubated at 45°C for 2h, and the absorbance was measured at 470 nm against a blank. Readings of all samples were performed immediately (t = 0 min) and after 2h of incubation (t = 120 min). Under the same operating conditions, BHT (0.5-5 µg/ml) was used as positive control and all tests were carried out in triplicate. The antioxidant activity of the extracts was evaluated in term of β-carotene bleaching inhibition in percent (I%) using this formula :

$$I\% = (A_{\beta\text{-carotene after 2h assay}}/A_{\text{initial }\beta\text{-carotene}}) \times 100$$

where $A_{\beta\text{-carotene after 2 h assay}}$ is the absorbance of β-carotene after 2 h assay remaining in the samples and $A_{\text{initial }\beta\text{-carotene}}$ is the absorbance of β-carotene at the beginning of the experiments. The results were expressed as IC_{50} values (µg/ml).

Statistical analysis

Experimental results were expressed as mean ± SD of three parallel measurements and analyzed by SPSS (SPSS 10 for Windows) statistical software. Differences between means were determined using Tukey multiple comparisons and least significant difference (LSD). Correlations were obtained by Pearson correlation coefficient in bivariate correlations. *P* values < 0.05 were regarded significant.

Results

Assays for total phenolics and flavonoids contents (TPC and TFC)

On the basis of the absorbance values of the various extract solutions, results of the colorimetric analysis of TPC and TFC are given in (**Figure 2**).

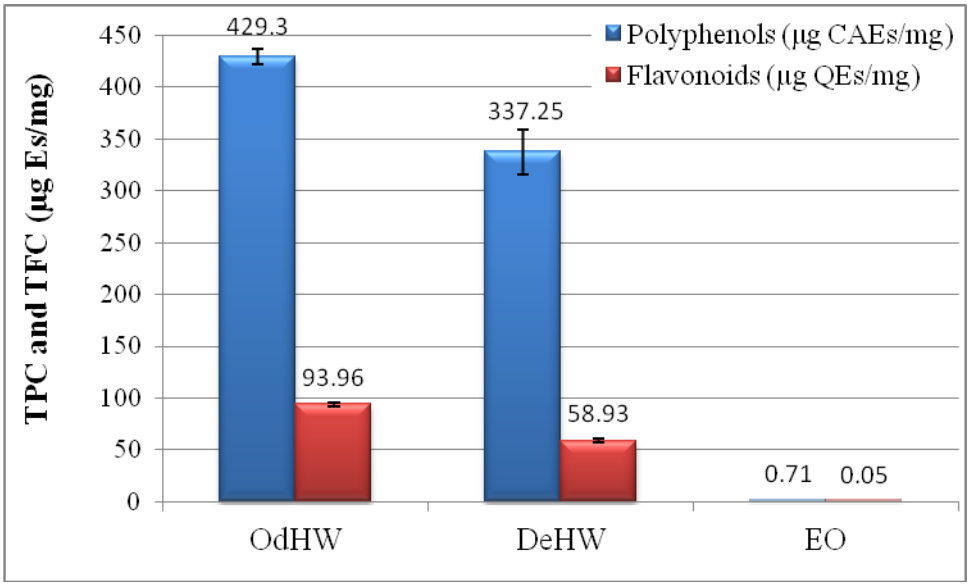


Figure 2 TPC and TFC (µg Es/mg extract) of all extracts from *S. aucheri mesatlnatica.*

Considering the results from the (Figure 2), TPC was highest in the hot water extracts with a content of 429.30 ± 7.2 µg CAEs/mg (42.93± 0.72%) and 337.25 ± 21.87 µg CAEs /mg (33.73± 2.19%) for the OdHW and DeHW extracts, respectively. However, EO was the poorest extract in polyphenols with 0.71 ± 0.03 µg CAEs/mg (0.07 ± 0.003%). Similarly, the quantitative determination of flavonoids also found that OdHW extract was the richest in flavonoids content with 93.96 ± 1.7 µg QEs/mg (9.4 ± 0.17%) followed by DeHW extract with 58.93± 1.61 µg QEs/mg (5.89 ± 0.16%), while the content of the EO was very low (0,05 µg QEs/mg). The Statistic analysis of TPC

and TFC revealed a high significant difference ($p< 0.05$) between all extracts. In addition, the results showed that all the samples tested had higher concentrations of polyphenols than flavonoids.

Determination of antioxidant activities

DPPH• assay

DPPH• is a highly stable free radical with purple color. After reacting with an antioxidant it turned to a stable yellow color compound (diphenyl-picrythydrazine). Reduction in the color was measured by spectrophotometer (λ_{max} =517 nm). The results of antiradical activities of studied extracts and ascorbic acid (positive control) were presented in **Table 2**.

Table 2 Scavenging activity, expressed as (%) and IC_{50} values (µg/ml), on DPPH• test of *S. aucheri mesatlnatica* extracts and ascorbic acid*.

Sample	Concentration (µg/ml)	Scavenging effect on DPPH• (%)	IC_{50} (µg/ml)**
EO	500	22.90±4.89	1561.67 ± 230.94 [a]
	1000	40.53±3.69	
	1500	49.04±3.25	
	2000	61.28±0.85	
DeHW	12	40.24±2.78	14.73 ± 0.68 [b]
	14	47.28±1.21	
	16	55.49±1.47	
	18	58.04±1.02	
	20	63.31±2.31	
OdHW	2	15.75±3.56	6.63 ± 0.47 [c]
	4	27.28±3.03	
	6	43.81±2.19	
	8	59.20±1.43	
	10	68.37±1.51	
Ascorbic acid	1	14.97±0.47	3.01 ± 0.16 [d]
	2	28.75±1.95	
	3	49.99±2.29	
	4	64.84±2.78	
	5	79.25±1.88	

* Values expressed are means ± S.D. of three parallel measurements.
**Values followed by different letters were significantly different at $P < 0.05$ according to the Tukey multiple comparisons and least significant difference (LSD).

The free radical scavenging activity of the all samples showed a concentration-dependent activity profile. It increased with an increase in their concentrations. It can be seen that various extracts exhibited a significant difference to free radical scavenging activities ($p< 0.05$), which reached at higher value in the presence of the highest extract concentration.

According to the results obtained, the OdMW extract exhibited high DPPH• radical scavenging activity with an IC_{50}= 6.63 ± 0.47 µg/ml. This activity was followed by DeHW extract with an IC_{50}= 14.73 ± 0.68 µg/ml, whereas the EO showed the weakest activity potential with an IC_{50}= 1561.67 ± 230.94 µg/ml, which is 235 times less active than the OdMW extract. In comparison with the standard antioxidant (IC_{50} = 3.01 ± 0.16 µg/ml), all the extracts tested are less active. It thus appears that the OdMW and DeHW extracts are 1.35 times and 5 times less active than ascorbic acid, respectively. Thus, the DPPH• scavenging effect increased in the order of EO < DeHW < OdHW < Ascorbic acid.

β-Carotene bleaching (BCB) test

The potential of the plant to inhibit lipid peroxidation was evaluated using the BCB test. The results of *S. aucheri mesatlnatica* samples and standard (BHT) are presented in **Table 3**.

Table 3 Inhibition of β-carotene discoloration, expressed as (%) and IC_{50} values (µg/ml), of *S. aucheri mesatlnatica* extracts and BHT*.

Sample	Concentration (µg/ml)	β-carotene discoloration (%)	IC_{50} (µg/ml)**
EO	300	26.33±10.34	934.5 ± 10.27 [a]
	500	28.86±10.83	
	1000	43.36±9.49	
	2000	65.31±9.96	
DeHW	5	6.32±1.17	35.19 ± 1.11 [b]
	10	17.90±2.01	
	25	46.90±1.32	
	50	60.91±1.11	
	100	69.65±1.43	
OdHW	5	21.56±2.65	17.55 ± 2.78 [c]
	10	42.46±3.04	
	25	64.14±2.53	
	50	71.20±2.94	
	100	74.24±1.97	
BHT	0.5	40.83±0.32	1.07 ± 0.19 [d]
	1	50.18±0.18	
	2	58.56±0.09	
	5	68.44±0.11	

* Values expressed are means ± S.D. of three parallel measurements.
**Values followed by different letters were significantly different at $P < 0.05$ according to the Tukey multiple comparisons and least significant difference (LSD).

Similar activity pattern was observed when compared to the results of DPPH• system, BCB test showed that all samples inhibits oxidation of the linoleic acid, in a dose-dependent and significant manner ($p< 0.5$). In this study, OdHW extract exhibited also high activity of oxidation of the linoleic with an IC_{50} value 17.55 ± 2.78 µg/ml. This activity was followed again by DeHW extract with IC_{50}= 33.54 ± 1.11 µg/ml), indicating that the OdMW extract was almost twice more active than DeHW extract. However, EO was less active with an IC_{50} = 1363 ± 10.27 µg/ml. As can be seen from Table 3, antioxidant activity of BHT (IC_{50} =1.07 ± 0.19 µg/ml) was found to be higher than that of all samples tested.

Discussion

Literature review shows the presence of different compounds such as phenolic acids, polyphenols, flavonoid and essential oils in the *Salvi*a family plants [7-10]. The presence of these compounds in the aqueous extracts of *S. aucheri mesatlantica* may also be the main cause of its high antiradical activities and high TPC. Indeed, OdHW and DeHW extracts are rich in polyphenol (429.30 ± 7.2 and 337.25 ± 21.87 µg CAEs/mg, respectively). These values are higher than that obtained in methanol and deodorized aqueous extracts of *S. tomentosa* from Turkey (200 and 149 µg GAEs/mg, respectively) [16]. It is also higher than that of methanol extracts of *S. verbenaca* collected in 10 different sites in Tunisia (55.03 to 136.33 µg GAEs/mg) [17] and aqueous extract of the *S. officinalis* leaves from Brazil (7.6 ± 1.2 µg GAEs/mg) [18]. The difference in levels of polyphenols in plants is probably due to the composition of phenolic extracts [19] and biotic (species, organ and physiological stage) or abiotic (salinity, luminosity, water deficit and edaphic factors) conditions [20]. On the other hand, OdHW extract exhibited also high antioxidant activity with its IC_{50} was lower than those of some species of *Salvia* reported in the literature. For instance, the methanol extracts of six *Salvia* species were screened for their possible antioxidant activities by Tepe et al [7] and it was determined that the most active plant was *S. euphratica* subsp. *Euphratica* with an IC50 value of 20.7 ± 1.22 µg/ml, followed by *S. sclarea* (IC50 = 23.4 ± 0.97 µg/ml) among the polar subfractions. Also, it was stated that the free radical scavenging activity of *S. tomentosa* aqueous methanol extract was superior to all other extracts prepared by using solvents of varying polarity (IC50 =18.7 µg/ml) [16]. Moein et al [21] reported that he polar subfractions C and F of ethyl acetate extract of *S. mirzayanii* possessed high scavenging activities against free radicals with IC50 = 37.9 ± 0.85 and 40.05 ±

1.4 µg/ml, respectively. Other results have shown that the various extracts of plants exhibited highest antioxidant property. For instance, the aqueous extract of *Xylopia. aethiopia* exhibited antioxidant activities DPPH assay with an IC50 of 280 µg/mL [22]. This value is lower than that of our sample. The antioxidant activities of the methanol extracts from seeds of Helianthus annuus were investigated. The result showed that these extract exhibited a significant inhibition of DPPH activity (85% at 100 µg/ml) [23].

Moreover, it is apparent that the high activity of aqueous extracts is due to their high TPC and TFC. Therefore, the correlation coefficients between the antioxidant capacities, TPC and TFC for all extracts were determined. **Table 4** lists the Pearson's coefficients between TPC, TFC and various antioxidant capacities.

Table 4 Pearson's coefficients between TPC, TFC and IC_{50} values (µg/ml) obtained by the DPPH$^{\bullet}$ assay and BCB test of all extracts of *S. aucheri mesatlantica*.

Test	TPC	TFC	DPPH$^{\bullet}$ assay (IC_{50})	BCB test (IC_{50})
TPC	1.000	0.970*	0.960*	0.962*
TFC		1.000	0.866**	0.871**
DPPH$^{\bullet}$ assay (IC_{50})			1.000	0.999*
BCB test (IC_{50})				1.000
** Correlation is significant at the 0.01 level				
* Correlation is significant at the 0.001 level				

Analysis of the results revealed that the TPC showed a high significant correlation with IC_{50} values obtained by DPPH$^{\bullet}$ and BCB tests (r^2= 0.960 and 0.962, respectively) (p< 0.001)). Similarly, a very significant correlation (p <0.01) was observed between the TFC and the IC_{50} values obtained by DPPH$^{\bullet}$ and BCB tests (r^2 = 0.866 and 0.871, respectively). In addition, a highly significant correlation (p < 0.001) was observed between the IC_{50} values calculated by the two different techniques (r^2 = 0.999). We noted also that the IC_{50} values of DPPH$^{\bullet}$ assay were lower than those of BCB test, except essential oil. This might be attributed that, the linoleic acid peroxidation inhibitory activity was mostly controlled by some non-polar metabolites present in extracts such as essential oils, while these hydrophilic antioxidants and antiradical activity were mainly attributed to polar secondary metabolites such as phenolics and flavonoids. This is in agreement with the results obtained by Frankel and Meyer in 2000 have suggested that antioxidants which exhibit apolar properties are most important because they are concentrated in the lipid-water interface, thereby preventing the formation of lipid radicals and β-carotene oxidation. In contrast, the polar antioxidants are diluted in the aqueous phase and thus are less effective in protecting of lipids [24]. It was reported that sample that inhibits or retards the bleaching β-carotene can be described as a scavenger of free radicals and as a primary antioxidant [25].

Many studies have conclusively shown close relationship between total phenolic contents and antioxidative activity of the fruits, plants and vegetables [26]. The key role of phenolic compounds as scavengers of free radicals is emphasized in several reports. Their antioxidant activity is mainly due to their redox properties which make them act as reducing agents, hydrogen donors, and singlet oxygen quenchers [27, 28]. Therefore, synergistic or additive actions of the different compounds present in the extracts cannot be ruled out. Indeed, in the present study, the results obtained by the two methods show that the OdHW has significant antioxidant activities than DeHW and EO. This behavior can be explained by the synergistic action between volatile and non-volatile compounds present in the OdHW compared to the DeHW that is devoid in volatile compounds lost during the preparation of EO by hydrodistillation.

Conclusion

In recent times, the essential oils and various extracts of plants have attracted attention as sources of natural products. They have been studied for their potential uses as alternative remedies for the treatment of many oxidative diseases as well as the preservation of foods from the toxic effects of oxidants. In this respect, studying with the endemic species may be of great interest since their bioactive properties and secrets could be lost forever without being tapped. So far we know this is the first report that envisages the antioxidant activities of essential oil and various extracts of *S. aucheri mesatlantica*. Indeed, the activity of these extracts was attributed to the higher phenolic content. Therefore, aerial parts of *S aucheri mesatlantica* may provide a good source of strongly antioxidant substances for use as a natural additive in food and pharmaceutical industries.

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