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Chemical composition and antioxidant activity of essential oil, various organic extracts of *Cistus ladanifer* and *Cistus libanotis* growing in Eastern Morocco

H. Zidane[1*], M. Elmiz[1], F. Aouinti[1], A. Tahani[1], J. Wathelet[2], M. Sindic[2] and A. Elbachiri[1]

[1]Laboratory of Physical Chemistry of the Natural Resources and Environment, University Mohammed Premier, BP 717, 60000 Oujda, Morocco.

[2]Unity of General and Organic Chemistry, Faculty of the Agronomic Sciences of Gembloux, Passage of the Prisoners 2, B-5030 Gembloux, Belgium.

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In the present work, we studied the chemical composition of the essential oil of *Cistus ladanifer* and *Cistus libanotis* growing in Eastern Morocco. The essential oils were obtained by hydrodistillation and their chemical composition was analysed using gas chromatography- mass spectrometry (GC–MS). Camphene, borneol, cyclohexanol-2, 2, 6 tremethyl, terpineol-4 and α-pinene were the main constituents of the essential oil of *C. ladanifer*, while in the essential oil of *C. libanotis* we obtained terpineol-4, γ-terpinene, camphene, sabinene, α-terpinene and α-pinene. The antioxidant potential of various extracts (water, ethanol, ethanol: water (50:50), methanol, methanol: water (50:50), acetonitrile) and essential oils of *C. ladanifer* and *C. libanotis* were carried out by the method of 1,1-diphenyl-1-picrylhydrazyl-hydrate (DPPH) free radical scavenging. Total phenolic and flavonoid contents were determined. The result show that *C. ladanifer* of the leaves of methanol: water (50:50) extract had the highest value of total phenolic content and the lowest was present in ethanol: water (50:50) extract of the stem and acetonitrile extract of the flowers of *C. libanotis.* From our experimental results, the extract of flowers, fruit, stem and leaves of those plants showed highest potential as free radical scavengers.

Key words: Antioxidant, phenolics, flavonoids, essential oil, extracts, gas chromatography- mass spectrometry (GC–MS).

INTRODUCTION

Cistaceae comprises eight genera and about 180 species. They are a medium-sized family typically consisting of heliophyte shrubs, subshrubs and herbs occurring in open areas on poor soils (Guzma´n and Vargas, 2009). They are pyrophylic (Alonso et al., 1992) and they are adapted to fires in Mediterranean forests.

The leaves of *Cistus ladanifer* produce a fragrant oleo-resin used in perfumes especially as a fixative. It is used in folk medicine for treatment of various skin diseases, as an anti-diarrheal, and as anti-inflammatory agents (Attaguile et al., 2000). In Eastern Morocco, *Cistus libanotis* and *C. ladanifer* are named by the local population as boubala and touzala, respectively. They are used as an anti-diarrheic, antiacid and antispasmodic by simple decoction of their leaves. In addition, anti-diarrheal effect of aqueous extract of *C. ladanifer* has been proved (Aziz

*Corresponding author. E-mail: zidane-hanane@live.fr. Tel: (00212)649853662. Fax: (212) (0)36500603.

et al., 2011). Furthermore, it produces a number of compounds with pharmacological properties: antihypertensive effect (Belmokhtar et al., 2009), antimicrobial (Guvenc et al., 2005; Greche et al., 2009), antioxidant (Amensour et al., 2010), antifungal (Mrabet et al., 1999), and other more.

The chemical composition of *C. ladanifer* essential oil was characterized by high amount of sesquiterpene (viridiflorol and ledol), and monoterpenoid (bornyl acetate and pinocarveol) (Oller-Lopez et al., 2005), whereas *C. libanotis* oil was dominated by hydrocarbons and oxygenated monoterpenes, monoterpene hydrocarbons, diterpenes, hydrocarbons and oxygenated sesquiterpenes (Ben Jemia et al., 2013).

In the current study, our objective was to evaluate the chemical composition of the essential oil of *C. ladanifer* and *C. libanotis* and to examine the antioxidant potential of oils and various solvent extracts of plant parts (fruits, flowers, stems and leaves) by 1,1-diphenyl-2-picrylhydrazyl-hydrate (DPPH) free radical scavenging assay. Additionally, total flavonoid and phenolic contents were also determined.

MATERIALS AND METHODS

Plant material

C. ladanifer and *C. libanotis* were collected from Tafoughalt (Eastern of Morocco) and they were identified by Dr Haloui of the Department of Biology (University Mohamed Premier Oujda). Collected plants (5 May, 2012) were dried in the laboratory under normal air at room temperature.

Preparation of extract and isolation of essential oil

A weighed portion (2 g) of each dried and powdered sample (fruits, stems, flowers and leaves) was extracted with 100 ml of different solvent (water, ethanol, ethanol: water (50:50), methanol, methanol: water (50:50), acetonitrile) for 8 days at room temperature. The extracts were filtered and evaporated to dryness in a rotary evaporator. The extracts were dissolved in ethanol at 25 mg ml^{-1} concentration and stored until used. The essential oil from leaves of *C. ladanifer* and *C. libanotis* was obtained by hydrodistillation in a Clevenger-type apparatus during 5 and 4 h, respectively. Both oils were dried under anhydrous sulphate and stored at 4°C until used.

Gas chromatography–mass spectrometry (GC–MS) analysis

Analysis of essential oils was carried out by GC–MS using a Hewlett Packard 6890 gas chromatography equipped with a Hewlett Packard 6890 mass selective detector and a HP-5 MS capillary column 30 m x 0.25 mm (cross-linked Phynel-Methyl Siloxane) and film thickness of 0.25 µm. The column was temperature programmed as follows: 40°C for 5 min, and then the temperature was increased to 280°C at a rate of 10°C/min. Helium was used as a carrier gas and the oils (0.1 µl) were injected without any dilution in the splitless mode.

Mass analyzed conditions were ion source of 230°C, ionization energy of 70 eV, electron current of 2 A, and resolution of 1000. Mass unit was monitored from 30 to 450 m/z. Identification of components in the oil was based on retention indices relatives with n-alkanes and computer matching with the WILLEY 275.L library, as well as by comparison of the fragmentation patterns of mass spectra with those reported in the literature (Adams, 1995). The relative proportions (%) of each volatile compound as the percentage ration of the peak area of each compound to the total peak area of all identified compounds were calculated.

DPPH radical scavenging effect

Antioxidant activity was evaluated according to the method cited by Xiao-Juan et al. (2012). 100 µl of different concentration of extract or essential oils or standard (1 to 15 mg ml^{-1}) was added in 1, 4 ml of ethanol and 1 ml of solution of DPPH (0.004 %). The absorbance of the discoloration of the solution was measured at 517 nm. The percentage of inhibition was calculated by:

$$\text{Inhibition (\%)} = [(A_{DPPH} - A_{sample})/A_{DPPH}] \times 100$$

A_{DPPH} is the absorbance of DPPH solution and A_{sample} is the absorbance of the test sample. All tests were performed in triplicate. The lower absorbance of the reaction mixture indicates higher free radical scavenging activity.

Determination of total phenolic content

The concentration of total phenolics in different organic extracts was determined by using Folin Ciocalteu reagent (Singleton and Rossi, 1965); briefly, 20 µl of sample extract (1 mg ml^{-1}) was added to 100 µl of Folin Ciocalteu reagent and 300 µl of Na_2CO_3. 2.5 of distilled water was added after 5 min. The mixture was left 2 h and the supernatant was measured at 576 nm against a blank. The calibration curve was prepared by Ferulic acid at different concentration (0.2 to 2 mg ml^{-1}; Y= 0.0042X + 0.0014; R^2= 0.9991). A test was carried out in triplicate.

Determination of total flavonoid content

Total flavonoid was determined using the method described by Pourmorad et al. (2006); briefly 0.5 ml of each extract (1 mg ml^{-1}) was added to 1.5 ml of ethanol, 0.1 ml of 10% aluminum chloride, 0.1 ml of 1 M potassium acetate and 2.8 ml of distilled water. The resulting solution was left at room temperature for 30 min. The absorbance was measured at 415 nm. The calibration curve was prepared by different concentration of quercetin solutions in ethanol (0.01 to 0.08 mg ml^{-1}; Y= 0.0048X - 0.0128; R^2= 0.9978). A test was carried out in triplicate.

Statistical data

All extractions were conducted in triplicates. Phenolic and flavonoid data are expressed as mean ± standard deviation. The means were compared by using the one-way and multivariate analysis of variance (ANOVA) followed by TuKey's using the SPSS 11.5 for Windows. The differences between individual means were deemed to be significant at $p < 0.05$.

RESULTS AND DISCUSSION

Chemical composition of the essential oils

The essential oil yields in leaf of *C. ladanifer* and *C. libanotis* were respectively 0.14 and 0.13% on dry weight basis (w/w). The essential oil volatile compounds of

Table 1. Chemical composition of the essential oil of *C. ladanifer* and *C. libanotis* .

Compound	Identification	Kovalts Indices	*C. libanotis* (%)	*C. ladanifer* (%)
Tricyclene	IK, MS	923	1.2	2.7
α-Thujene	IK, MS	930	–	1.0
α-Pinene	IK, MS	936	6.9	4.2
Camphene	IK, MS	952	12.2	15.5
Sabinene	IK, MS	977	11.2	0.8
β-Pinene	IK, MS	979	–	0.7
α-Phellandrene	IK, MS	1002	6.2	–
α -Terpinene	IK, MS	1019	7.3	1.8
p-Cymene	IK, MS	1028	4.7	2.3
Cyclohexanone, 2,2,6-trimethyl	IK, MS	1038	–	7.3
γ-Terpinene	IK, MS	1063	12.4	3.8
Terpinolene	IK, MS	1091	3.3	1.3
Sabinaketone	IK, MS	1154	0.8	–
α -Campholene aldehyde	IK, MS	1131	–	1.3
Camphore	IK, MS	1152	–	1.5
Exo-Methyl-Camphenilol	IK, MS	-	–	0.9
Borneol	MS	-	–	11.1
Terpineol-4	IK, MS	1183	17.4	6.3
P-Cymen-8-ol	IK, MS	1190	–	0.6
α-terpineol	IK, MS	1193	0.0	1.2
1-Methyladamantane	MS	1190	0.7	–
Verbenone	IK, MS	1217	–	0.8
l-Bornyl acetate	IK, MS	1293	–	0.9
p-Mentha-1,4-dien-7-ol	IK, MS	1336	–	0.8
(+)-2-Carene	IK, MS	1001	1.1	–
α-Cubebene	IK, MS	1360	–	2.2
6-Amyl-Apha-Pyrone	MS	–	1.0	–
Cyclosativen	IK, MS	1380	–	0.6
1S, Cis-Calamenene	MS	1528	0.7	–
δ-Cadinene	IK, MS	1490	–	6.4
Hexadecanoic acid	MS	1959	0.7	–
Diethyl phthalate	IK, MS	1602	2.2	2.9
Viridiflorol	IK, MS	1610	–	2.8
Cyercene	MS	1619	–	0.5
Bis (2-ethylhexyl) phthalate	MS	2560	–	0.2

%, Relative percentage area; ND, compound not identified in this sample; KI, Kovalts indices calculated using an apolar column (HP-5).

plants, their kovalts indices and their percentage are presented in Table 1.

Twenty eight (28) and 18 compounds were identified in the essential oil of *C. ladanifer* and *C. libanotis*, respectively. As a result of GC-MS analyse, *C. ladanifer* contained, camphene (15.5%), borneol (11.1%), cyclohexanol-2, 2, 6 tremethyl (7.3 %), terpineol-4 (6.3 %) and α-pinene (4.2 %) as the major compounds. The main constituents of the oil of *C. libanotis* were terpineol-4 (17.4%), γ-terpinene (12.4%), camphene (12.2%), sabinene (11.2%), α-terpinene (7.3%), α-pinene (6.9%) and α-phellandrene (6.2%). The comparison of our result with literature shows important qualitative and quantitative differences in compositions. According to Mariotti et al. (1997) report, major compounds of the essential oil of *C. ladanifer* have been determined as α-pinene, viridiflorol, ledol and bornyl acetate.

As can be seen from Table 1, camphene is the major compounds of the *C. ladanifer* oil together with borneol and cyclohexanol-2, 2, 6 tremethyl in the present study. When compared with these compounds, bis (2-ethylhexyl) phthalate and cyercene were detected in a small quantity in the oil. Another study from Portugal has investigated the main constituents in the oil of *C.*

Table 2. Comparison of total phenolic and flavonoid contents of *C. ladanifer* and *C. libanotis*.

Plant		Water extract	Ethanol extract	Methanol extract	Ethanol: water extract (50:50)	Methanol: water extract (50:50)	Acetonitrile extract
Phenolic content (FA mg/g dry weight)							
C. ladanifer	Fl	66.48±0.14^{c}	53.25±0.24^{d}	139.84±0.56^{a}	158.60±0.32^{a}	182.41±0.09^{a}	75.38±0.24^{a}
	F	95.82±0.1^{a}	114.10±0.27^{a}	130.22±0.16^{b}	98.47±0.06^{b}	110.01±0.05^{b}	71.29±0.15^{b}
	S	28.23±0.03^{d}	82.83±0.18^{b}	61.19±0.13^{c}	36.89±0.07^{d}	33.77±0.08^{d}	70.33±0.04^{b}
	L	83.31±0.14^{b}	65.76±0.1^{c}	47.96±0.13^{d}	40.50±0.15^{c}	53.49±0.22^{c}	27.51±0.11^{c}
C. libanotis	Fl	26.07±0.15^{b}	30.88±0.15^{d}	35.93±0.08^{c}	69.36±0.03^{a}	40.98±0.02^{b}	24.14±0.03^{d}
	F	26.08±0.66^{b}	75.62±0.77^{a}	43.87±0.15^{a}	39.30±0.45^{b}	28.96±0.75^{c}	36.17±0.09^{a}
	S	38.58±0.14^{a}	41.70±0.19^{c}	40.50±0.11^{b}	24.14±0.1^{d}	24.39±0.09^{d}	27.51±0.24^{c}
	L	25.11±0.31^{b}	44.59±0.19^{b}	40.02±0.08^{b}	26.55±0.22^{c}	43.15±0.22^{a}	33.53±0.29^{b}
Flavonoid content (QE mg/g dry weight)							
C. ladanifer	Fl	78.13±0.09^{a}	59.64±0.3^{c}	62.76±0.36^{b}	48.43±0.19^{a}	46.14±0.1^{a}	23.71±0.21^{c}
	F	18.93±0.06^{b}	83.94±0.17^{a}	71.27±0.23^{a}	29.32±0.08^{b}	31.19±0.04^{b}	60.26±0.05^{a}
	S	5.85±0.14^{c}	18.10±0.73^{d}	20.59±0.41^{d}	7.93±0.15^{d}	3.98±0.16^{d}	3.56±0.3^{d}
	L	16.44±0.67^{d}	61.10±0.53^{b}	51.13±0.55^{c}	25.99±0.41^{c}	18.10±0.41^{c}	28.69±0.21^{b}
C. libanotis	Fl	10.21±0.19^{c}	63.59±0.15^{c}	35.96±0.23^{a}	41.78±0.17^{a}	32.85±0.17^{a}	22.05±0.11^{a}
	F	8.76±0.15^{d}	14.16±0.28^{d}	14.78±0.24^{d}	11.25±0.12^{d}	3.57±0.14^{d}	6.06±0.11^{d}
	S	16.23±0.09^{b}	31.81±0.14^{b}	26.62±0.14^{b}	12.70±0.11^{c}	14.99±0.04^{c}	11.25±0.07^{b}
	L	20.39±0.1^{a}	15.82±0.55^{c}	24.96±0.18^{c}	18.93±0.74^{b}	17.89±0.12^{b}	10.63±0.89^{c}

Values are mean ± SD (standard mean deviation) (n=3). In each column, different letters mean significant differences between plant parts ($P < 0.001$). Values followed by the same letter under the same column are not significant different ($P \geq 0.05$). FA, Ferulic acid equivalents; QE, quercetin equivalents; Fl, flowers; F, fruit; S, stem; L, leaves.

ladanifer, viridiflorol, glubulol, an unknown sesquiterpene alcohol and 15-nor-labdan-8-ol (Gomes et al., 2005). In some reports, main components have been determined as 1, 8-cineol and viridiflorol (Viuda-Martos et al., 2011). These changes in the essential oil compositions might arise from several factors such as geological, geographical, seasonal and climatic (Perry et al., 1999).

Contents of total phenols and flavonoids

Phenolic compounds are the main class of natural antioxidants and there is a close relationship and positive correlation between the phenolic content and antioxidant activity of plant tissues. Table 2 summarises the results from the quantitative determination of the phenol and flavonoid content of the different extracts. Total phenol content was determined as ferulic acid equivalents in milligrams per gram (FA mg/g dry weight) while total flavonoid contents were calculated as quercetin equivalents in milligrams per gram (mg QE mg/g dry weight). The total phenol contents varied between different plant parts: aqueous methanolic extract from *C. ladanifer* flowers had higher total phenol content (182.41 mg FA/g) than other extract. Significant differences were also found among different plant parts.

As for total flavonoid content, the ethanolic extract of *C. ladanifer* fruit had higher total flavonoid content than other extract, and concerning the total flavonoid content of *C. libanotis* , the highest value was observed in flower from the ethanolic extract. The lowest total flavonoid content was signalled in the acetonitrile extract of stem from *C. ladanifer* (3.56 QE mg/g dry weight) and in the aqueous methanolic extract of fruit from *C. libanotis* (3.57 QE mg/g dry weight). The amount of active compounds like phenolic and flavonoid deposited in each part of the plant is usually different. In similar study, the total phenolic content of ethanolic and acetone extract of *C. ladanifer* (leaf) were 255.19 and 334.46 mg GAE (Gallic acid equivalent)/g of plant extract respectively, which had highest values compared to our results (Andrade et al., 2009). Amensour et al. (2010) reported that total phenolics in methanolic and ethanolic extract of *C. ladanifer* were 18.43 and 11.87 mg GAE/g of extract, respectively; much lower compared to our values. Screening of the polyphenolic composition of aqueous extracts of those plants was studied, given as abundance compounds ellagitannins (Barrajón-catalán et al., 2011).

DPPH radical scavenging activities

Free radical scavenging properties of different extracts from different *C. ladanifer* and *C. libanotis* parts and

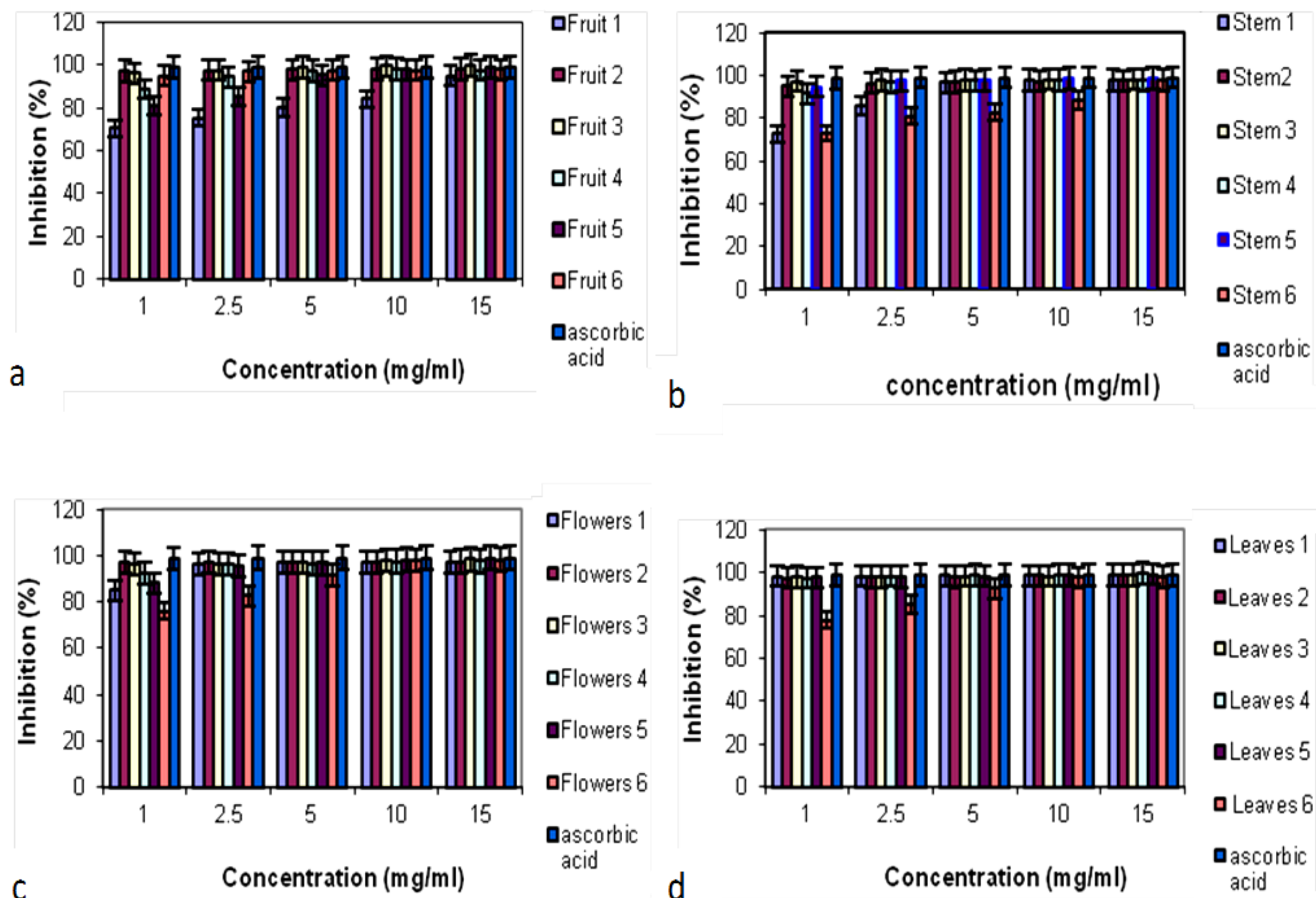

Figure 1. DPPH radical scavenging activities of fruit (a), stem (b), flowers(c) and leaves (d) from *C. ladanifer* of different solvent extracts at various concentrations (1, Water extract; 2, ethanol extract; 3, methanol extract; 4, water : ethanol (50:50) extract; 5, water: methanol (50:50) extract; 6, acetonitrile extract).

essential oils are presented in Figures 1, 2 and 3. Higher percentage inhibition value indicated higher antioxidant activity. All extracts of different plant parts showed higher scavenging ability of DPPH radicals when compared to those reported for essential oils of the leaf of *C. libanotis* and *C. ladanifer* (70.7 and 67.7%, respectively).

For *C. ladanifer*, methanolic extract were observed in this order: 97.8% (leaf) > 97.3% (stem) > 96.1% (flower) > 95.9% (fruit) whereas for *C. libanotis*, it was found as 96.9% (flower and stem) > 95.9% (fruit) > 93.4% (leaf). In addition, DPPH scavenging ability of the extracts of plant parts was similar to ascorbic acid, suggesting that plant extracts have a potent antioxidant activity mainly due to their richness on phenolic compounds. Oils was found less effective than the control. The use of water with alcoholic solvent presents a good advantage to extract polyphenols and can also modulate the polarity of alcohol solvent used (Mohammedi and Atik, 2011). These extract possess a remarkable potential in scavenging of free radicals since they present a number of hydroxyls acting as hydrogen donators.

Conclusion

There is a growing interest of industry to replace synthetic chemicals from natural products with bioactive properties from plant origin. The results indicate a significant qualitative difference found in terms of chemical composition in the oils. The most abundant compounds in *C. ladanifer* were camphene, borneol, cyclohexanol-2, 2, 6 tremethyl, terpineol-4 and α-pinene, while the essential oil of *C. libanotis* were terpineol-4, γ-terpinene, camphene, sabinene, α-terpinene and α-pinene. The present data suggest the importance of comparing and exploring the variety of essential oil and phenolic content from different *C. ladanifer* and *C. libanotis* parts, since this variability of repartition of bioactive substances between leaf, stem, flower and fruit entrained the variability of their potential antioxidant activities.

Figure 2. DPPH radical scavenging activities of fruit (a), stem (b), leaves (c) and flowers (d) from *Cistus libanotis* of different solvent extracts at various concentrations (1, Water extract; 2, ethanol extract; 3, methanol extract; 4, water: ethanol (50:50) extract; 5, water: methanol (50:50) extract and 6: acetonitrile extract).

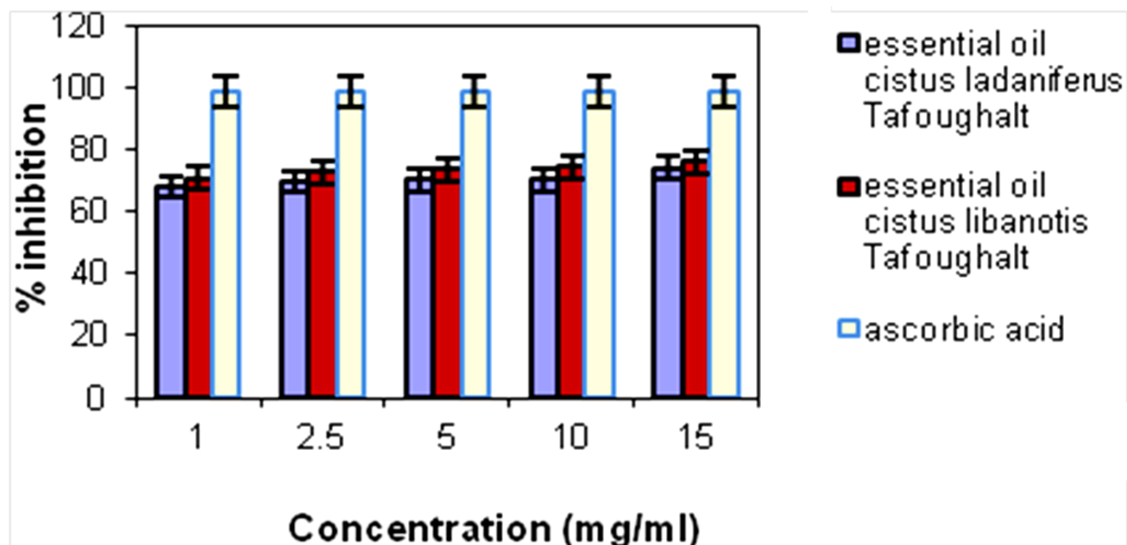

Figure 3. DPPH radical scavenging activities of essential oil of *C. ladanifer* and *C. libanotis*.

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