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Antioxidant Activity of Eight Hydrosols from Morocco

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Abstract: Essential oils from aromatic and medicinal plants have received particular attention as potential natural agents for food preservation and medicine. Nevertheless, much less attention has been given to the components which remain dissolved in the distillation water. To this fraction which is quite fragrant and strongly flavoured with values of pH ranging from 4.5-5.5 is called hydrosol. The antioxidant activity of hydrosols produced by an enterprise from Morocco from eight aromatic species (*Pelargonium graveolens*, *Artemisia herba-alba*, *Cupressus atlantica*, *Ocimum basilicum*, *Juniperus phoenicea*, *Pinus pinaster*, *Marrubium vulgare* and *Hyssopus officinalis*) was evaluated. Several methods were used: Thiobarbituric acid Reactive Species (TBARS), Trolox Equivalent Antioxidant Capacity (TEAC), hydroxyl and superoxide scavenging abilities. The results showed that in TBARS and superoxide scavenging ability, *H. officinalis* hydrosol revealed to be the most effective ($IC_{50} = 136 \text{ mg mL}^{-1}$ and $IC_{50} = 2 \text{ mg mL}^{-1}$, respectively). In the TEAC assay, *M. vulgare* was the most effective ($IC_{50} = 72 \text{ mg mL}^{-1}$), although *H. officinalis* also presented a good activity ($IC_{50} = 87 \text{ mg mL}^{-1}$). *A. herba-alba* hydrosol presented the best capacity for scavenging hydroxyl free radical ($IC_{50} = 95 \text{ mg mL}^{-1}$).

Key words: Hydrosol, Morocco, antioxidant

INTRODUCTION

Essential oils and hydrosols were already used in Egypt over 6,000 years ago for medicinal, pharmacological and cosmetic purposes (Schorr, 2004).

An essential oil is defined internationally as the product obtained by hydrodistillation, steam distillation or dry distillation or by a suitable mechanical process without heating (*Citrus* fruits) of a plant or some parts of it. They are aromatic oily liquids, volatile, characterized by a strong odour, rarely coloured and generally with a lower density than that of water (Miguel, 2010).

When aromatic plants are distilled, the volatile fraction is released (essential oils) being collected on the water surface into a receiver or a separation funnel. Another substance also occurs during this process-the hydrosol. This is the water from the steam or hydrodistillation that comes into the receiver or separation funnel (Schorr, 2004). Therefore, hydrosol is obtained by steam distillation of aromatic plants along with essential oils and is used mainly in the cosmetic field and less frequently in the medical field (Inouye *et al.*,

2009). Hydrosols are also known as floral water, distillate water or aromatic water. They are considered co-products or the by-products of hydro and steam distillation of plant material. The chemical composition of hydrosols includes traces of essential oils and water-soluble components (Rao *et al.*, 2002; Baydar and Kineci, 2009; Tornuk *et al.*, 2011).

Generally the hydrosol is discarded which may bring considerable economic losses, since hydrosols are easy and inexpensive to produce (they are obtained at the same time as the essential oil) and they have the advantage to be practically innocuous for the human being (Lis-Balchin *et al.*, 2003; Tornuk *et al.*, 2011).

By comparison to essential oils, much less studies regarding the chemical composition (Babu and Kaul, 2005; Paolini *et al.*, 2008; Baydar and Kineci, 2009; Edris, 2009) and biological activities (Sagdic, 2003; Ozcan, 2005; Boyraz and Ozcan, 2006; Al-Turki, 2007) of hydrosols can be found. Recently, the use of some plant hydrosols as antimicrobial agents for preventing the bacterial deterioration of stored foods has also been a target of studies (Ozcan *et al.*, 2008; Vatansever *et al.*, 2008).

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Concerning the antioxidant properties of the hydrosols, information is scarce or even null. The present study aimed to assess the ability of eight hydrosols (*Pelargonium graveolens*, *Artemisia herba-alba*, *Cupressus atlantica*, *Ocimum basilicum*, *Juniperus phoenicea*, *Pinus pinaster*, *Marrubium vulgare* and *Hyssopus officinalis*) for preventing the oxidation.

MATERIALS AND METHODS

Chemicals: 2-Thiobarbituric acid, nitrotetrazolium blue chloride, trichloroacetic acid, 2-deoxyribose, Trolox, 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid (ABTS), Butylated Hydroxytoluene (BHT), ascorbic acid, mannitol and fluorescein were purchased from Sigma-Aldrich Chemie, Steinheim, Germany. Phenazine Methosulfate (PMS), nicotinamide adenine dinucleotide disodium salt hydrate (NADH), 2,2'-azobis-2-methylpropanimidamide, dihydrochloride (AAPH) and ethylenediaminetetraacetic acid disodium salt dihydrate (EDTA) were purchased from Acros organics, New Jersey, USA. Di-potassium hydrogen phosphate anhydrous (K_2HPO_4) and hydrogen peroxide (H_2O_2) were purchased from Panreac Quimica, Montcada i Reixac, Barcelona, Spain. Potassium dihydrogen phosphate (KH_2PO_4) was purchased from Riedel-de-Haen Laboratory Chemicals, Germany. Potassium chloride (KCl) was purchased from Analar Normapur, Geldenaaksebaan, Leuven, Belgium. Butan-1-ol was purchased from Fisher Scientific UK Ltd, Loughborough, UK.

Preparation of hydrosols: According to the enterprise, samples (aerial parts) of each plant (1 kg) were alembic for steam distillation. Hydrosols of each plant (2 L) were obtained after 2 h. Then, the oil was removed by separation funnels. Hydrosols were kept in sterile and dark bottles under refrigerated conditions ($T = 4^\circ C$) until use. Samples of hydrosols were diluted in distilled water for further analysis.

Antioxidant activities

Thiobarbituric acid reactive species (TBARS): The ability of the hydrosols to inhibit malondialdehyde formation and therefore lipid peroxidation, was determined by using a modified Thiobarbituric Acid Reactive Species (TBARS) assay. Egg yolk homogenates were used as a lipid-rich medium obtained as described elsewhere (Dorman *et al.*, 1995). Briefly, 0.5 mL of 10% (w/v) homogenate and 0.1 mL of samples or control substance (BHT) with diverse concentrations were added to a test tube and made up to 1 mL with distilled water. Then, 1.5 mL of 20% acetic acid (pH 3.5) and 1.5 mL of 0.8%

(w/v) TBA in 1.1% (w/v) Sodium Dodecyl Sulphate (SDS) were added. The resulting mixture was vortexed and heated at $95^\circ C$ for 60 min. After cooling, at room temperature, 5 mL of butan-1-ol was added to each tube; the contents of the tubes were stirred and centrifuged at 3000 rpm for 10 min. The absorbance of the organic upper layer was measured at 532 nm using a Shimadzu 160-UV spectrophotometer. All of the values were based on the percentage antioxidant index (AI%), whereby the control was completely peroxidized and each oil demonstrated a degree of change; the percentage inhibition was calculated using the formula $(1 - T/C) \times 100$, where, C is the absorbance value of the fully oxidized control and T is the absorbance of the test sample. The antioxidant capacity was determined from three replicates. The percentage antioxidant index was plotted against the concentrations of samples and IC_{50} values were determined (concentration of hydrosol to prevent 50% of lipid oxidation).

Hydroxyl radical scavenging activity: The assay of OH^\cdot -scavenging activity was developed according to Chung *et al.* (1997) with small modifications. Briefly, the reaction mixture was prepared with 10 mM $FeSO_4 \cdot 7H_2O$, 10 mM EDTA, 10 mM 2-deoxyribose, 0.1 M phosphate buffer and sample or control substance (mannitol) in a test tube to give a total volume of 1.8 mL. Finally, 200 μL H_2O_2 was added to the mixture which was incubated at $37^\circ C$ for 4 h. After that, 1 mL trichloroacetic acid (2.8%) and 1 mL thiobarbituric acid (1%) were added to the test tube which was boiled for 10 min. After cooling, its absorbance was measured at 520 nm in a Shimadzu 160-UV spectrophotometer. The OH^\cdot -scavenging activity (%) was calculated using the following equation:

$$\text{Inhibition} = [(A_0 - A_1) / A_0] \times 100 (\%)$$

where, A_0 is the absorbance of the control (without sample) and A_1 is the absorbance in the presence of the sample. Tests were carried out in triplicate. The sample concentration providing 50% inhibition (IC_{50}) was obtained by plotting the inhibition percentage against hydrosol concentrations.

Superoxide anion scavenging activity: Measurements of superoxide anion scavenging activity of samples were based on the Soares method. Superoxide anions were generated in a non-enzymatic phenazine methosulfate-nicotinamide adenine dinucleotide (PMS-NADH) system by oxidation of NADH and assayed by reduction of Nitroblue Tetrazolium (NBT). The superoxide anion was generated in 3 mL of phosphate buffer (19 mM, pH 7.4),

containing NBT (43 μM) solution, NADH (166 μM) solution and different concentrations of hydrosols or control substance (ascorbic acid). The reaction was started with the addition of PMS solution (2.7 μM) to the mixture. The reaction mixture was incubated at 20°C for 7 min and the absorbance reading was performed at 560 nm in a UV/visible spectrophotometer, Ultrospec 1100 pro. The percentage of inhibition was calculated using the following equation:

$$\text{Inhibition} = [(A_0 - A_1)/A_0] \times 100 (\%)$$

where, A_0 is the absorbance of the control (without sample) and A_1 is the absorbance in the presence of the sample. Tests were carried out in triplicate. The sample concentration providing 50% inhibition (IC_{50}) was obtained by plotting the inhibition percentage against hydrosol concentrations.

ABTS free radical-scavenging activity or TEAC method:

The determination of ABTS radical scavenging was carried out as reported by Re *et al.* (1999). Briefly, the ABTS radical was generated by the reaction of (7 mM) ABTS aqueous solution with $\text{K}_2\text{S}_2\text{O}_8$ (2.45 mM) in the dark for 16 h and adjusting the absorbance at 734 nm to 0.7 at room temperature. Samples (10 mL) were added to 1490 mL ABTS, the absorbance at 734 nm was read at time 0 (A_0) and after 6 min (A_1). The percentage of inhibition was calculated using the following equation:

$$\text{Inhibition} = [(A_0 - A_1)/A_0] \times 100 (\%),$$

where A_0 is the absorbance of the control (without sample) and A_1 is the absorbance in the presence of the sample after 6 min. Tests were carried out in triplicate. The sample concentration providing 50% inhibition (IC_{50}) was obtained by plotting the inhibition percentage against hydrosol concentrations.

Chemical analysis of the essential oils

Chemical analysis of the essential oils gas chromatography (GC): Gas chromatographic analyses were performed using a Autosystem XL (Perkin Elmer, Shelton, CT, USA) gas chromatograph equipped with two Flame Ionization Detectors (FIDs), a data-handling system and a vapourizing injector port, into which two columns of different polarities were installed: a DB-1 fused-silica column (30×0.25 mm internal diameter (i.d.), film thickness 0.25 μm ; J and W Scientific, Rancho Cordova, CA, USA) and a DB-17HT fused-silica column (30×0.25 mm i.d., film thickness 0.15 μm ; J and W Scientific). Oven temperature was programmed from 45-175°C at 3°C min^{-1} , then at 15°C min^{-1} to 300°C, then held isothermal for 10 min; injector and detector temperatures, 280 and 300°C,

respectively; carrier gas, hydrogen, adjusted to a linear velocity of 30 cm sec^{-1} . The samples were injected using the split sampling technique, ratio 1:50.

Gas chromatography-mass spectrometry (GC-MS):

The GC-MS unit consisted of an Autosystem XL (Perkin-Elmer) gas chromatograph, equipped with a DB-1 fused-silica column (30×0.25 mm i.d., film thickness 0.25 μm ; J and W Scientific) and interfaced with a TurboMass mass spectrometer (software v. 4.1, Perkin-Elmer). Injector and oven temperatures were as above; transfer line temperature, 280°C; ion trap temperature, 220°C; carrier gas, helium, adjusted to a linear velocity of 30 cm sec^{-1} ; split ratio, 1:40; ionization energy, 70 eV; ionization current, 60 μA ; scan range, 40-300 u; scan time, 1 sec. The identity of the components was assigned by comparison of their retention indices, relative to C_9 - C_{15} n-alkane indices and GC-MS spectra from a home-made library, constructed based on analyses of reference oils, laboratory-synthesized components and commercially available standards.

Only the main components of which concentrations were superior to 10% were considered in the present study.

Statistical analysis: Statistical comparisons were made with one-way ANOVA followed by Tukey's multiple comparison test. The level of significance was set at $p < 0.05$. Statistical calculation was performed using SPSS 15.0 software.

RESULTS AND DISCUSSION

Figure 1 depicts the percentage of inhibition of some hydrosols for preventing lipid oxidation measured through the thiobarbituric acid reactive substances.

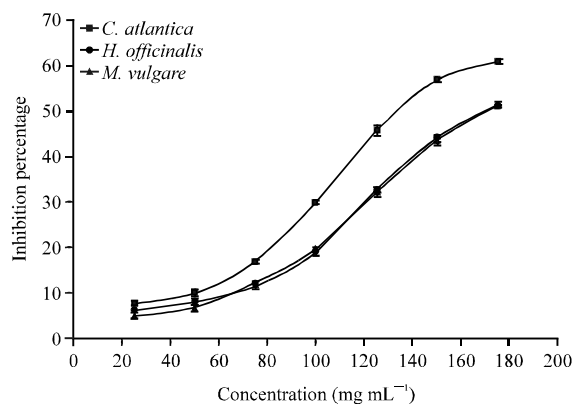


Fig. 1: Percentage of inhibition of malondialdehyde formation, measured by the TBARS method, of hydrosols

Table 1: Antioxidant activity of hydrosols expressed as IC₅₀ (mg mL⁻¹) for TBARS, ABTS, superoxide and hydroxyl assays

Plants	TBARS	ABTS	Superoxide	Hydroxyl
<i>A. herba-alba</i>	-	259±0.001 ^b	107±0.001 ^b	95±0.001 ^c
<i>C. atlantica</i>	170±0.001 ^a	278±0.001 ^a	76±0.001 ^c	135±0.001 ^a
<i>J. phoenicea</i>	-	-	68±0.001 ^d	-
<i>M. vulgare</i>	170±0.001 ^a	72±0.001 ^d	162±0.001 ^a	-
<i>H. officinalis</i>	136±0.001 ^b	87±0.001 ^c	2±0.001 ^e	106±0.001 ^b
<i>P. graveolens</i>	-	-	-	-
<i>O. basilicum</i>	-	-	-	-
<i>P. pinaster</i>	-	-	-	-

-: Not possible to be evaluated due to the low antioxidant ability which did not allow determining IC₅₀ values, values in the same column followed by the same letter are not significantly, different by Tukey multiple comparison test (p<0.05)

Hyssopus officinalis hydrosol was the best antioxidant. *Cupressus atlantica* and *Marrubium vulgare* presented similar activity. In all cases, the activity was dose-dependent. The remaining hydrosols did not present any antioxidant activity measured by this method. Regarding Table 1, the values IC₅₀ values also proved the best activity of *H. officinalis*. Nevertheless, such value was significantly higher than the control (BHT). The IC₅₀ found for this compound was 0.1 mg mL⁻¹.

For the lowest concentrations of *Marrubium vulgare* and *H. officinalis* hydrosols their ability for scavenging ABTS radicals was similar, nevertheless higher concentrations of *M. vulgare* hydrosol was more effective as antioxidant than *H. officinalis*. The capacity for scavenging ABTS radicals of *Artemisia herba-alba* and *C. atlantica* hydrosols were significantly inferior to those of *Marrubium vulgare* and *H. officinalis* (Fig. 2, Table 1). In any case, the activities were dose-dependent although for higher concentration saturation was achieved, more evident for *C. atlantica*, *A. herba-alba* and *H. officinalis* hydrosols (Fig. 2).

The best activities found for *Marrubium vulgare* and *H. officinalis* in terms of scavenging ABTS radicals did not coincide to those for preventing the lipid oxidation for which *C. atlantica* was the most active. Such results reveal the diverse ways by which samples may prevent oxidation although the capacity for scavenging some type of radicals (e.g., ABTS) has been subject to some criticism because they are foreign to biological systems (Awika *et al.*, 2003). However, the operational simplicity, reproducibility and flexible usage for determining both hydrophilic and lipophilic antioxidant ability of food extracts and physiological fluids explain its frequent usage (Apak *et al.*, 2007). BHT was the control substance used in this assay and the IC₅₀ value found for it was 0.004 mg mL⁻¹. This antioxidant had an activity 18,000 fold higher than the hydrosol of *M. vulgare* which was the best antioxidant among the hydrosols assayed.

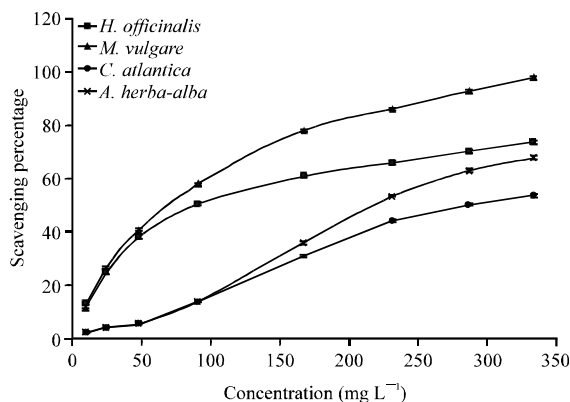


Fig. 2: Percentage of ABTS radical scavenging of hydrosols

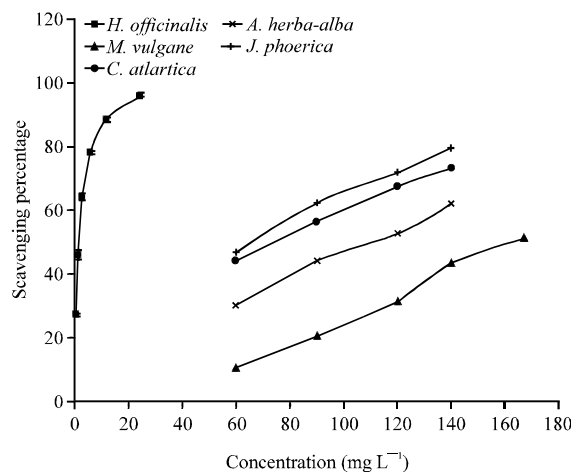


Fig. 3: Percentage of superoxide radical scavenging of hydrosols

An antioxidant may also act through the ability for scavenging superoxide anion radicals. Figure 3 depicts the scavenging percentage of these radicals by some hydrosols. *H. officinalis* is significantly the best hydrosol in contrast to the *M. vulgare* hydrosol. The activity of all hydrosols possessing such property was dose-dependent. Nevertheless *O. basilicum*, *P. graveolens* and *P. pinaster* hydrosols were unable to scavenge superoxide anion radicals. Ascorbic acid, the control substance checked in this assay, revealed to be much better as scavenger of superoxide anion radicals than the hydrosols, due to its significant low IC₅₀ value (0.02 mg mL⁻¹), that is, 100-fold better than the *H. officinalis* hydrosol, the best antioxidant among the samples assayed.

Concerning hydroxyl scavenging only three hydrosols were effective: *A. herba-alba*, *H. officinalis* and

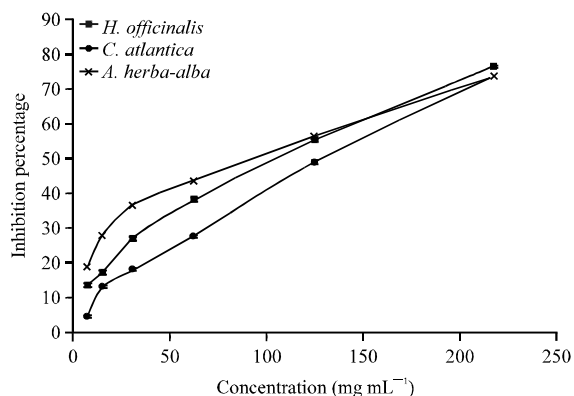


Fig. 4: Percentage of hydroxyl radical scavenging of hydrosols

C. atlantica. Although for higher concentrations, there was no difference among the samples, for lower concentrations, *A. herba-alba* hydrosol was the most active in contrast to *C. atlantica*, the activity of *H. officinalis* hydrosol was between those two ones (Fig. 4). The remaining hydrosols did not show any capacity for scavenging hydroxyl radicals. Mannitol ($IC_{50} = 0.007 \text{ mg mL}^{-1}$), the control substance used in this assay revealed to be significantly better than hydrosols. *H. officinalis* and *C. atlantica* were the sole hydrosol samples possessing activity in all antioxidant assays checked in the present work (Fig. 1-4, Table 1). On the opposite site, *Ocimum basilicum*, *Pelargonium graveolens* and *Pinus pinaster* can be reported as being completely ineffective as antioxidants. Even the best antioxidant activities found for *H. officinalis* and *C. atlantica* hydrosols were significantly worse than the control substances. Such may be expected since hydrosols include traces of essential oils and water-soluble components.

The chemical composition of *C. atlantica* and *H. officinalis* hydrosols revealed the presence of terpinen-4-ol (83.1%) and camphor (76.4%) in high percentages, respectively, in a total of 97.1 and 96.6% compounds identified. Such yields are unusual when compared to the essential oils of the same species (Barrero *et al.*, 2005; Kizil *et al.*, 2010; Arjouni *et al.*, 2011a, b; Moro *et al.*, 2011). In spite of that, the components cannot be considered as being responsible for the antioxidant activities found in the samples, because the same major components were also detected in *P. graveolens* and *P. pinaster* hydrosols and no activity was found for these samples.

The presence of those major compounds was unexpected and further work must be carried out to find out the reasons responsible for this high yield. In

addition, the activities found cannot be attributed to the major compounds present in the hydrosols. The presence of other compounds involved in the inhibition of oxidation needs to be identified.

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