

CHEMICAL STUDY OF A WATER EXTRACT OF ARGENTINE COMMERCIAL ORIGANUM

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Abstract

A chemical study was done of a water extract of Origanum x applii, "by product" of its essential oil hydrodistillation, in order to evaluate the existence and abundance of natural antioxidants as they have been reported in Lamiaceae plants.

Hydroquinone and its β -glucoside, arbutin were identified as the major non acidic phenolic compounds, while in an acidic fraction, rosmarinic acid was isolated in, at least, 0.8 % yield (in dry leaf basis) of this extract in the separation conditions, together with 3,4-dihydroxybenzoic acid, caffeic acid and acetoacetic acid among other minor compounds.

This results along with the estimation of a good antioxidant activity suggest that the extract can be used as source of natural antioxidants and therefore the economical value of this local origanum cropping could be improved and diversified in Argentina.

Resumen

Se realizó el estudio químico del extracto acuoso residual de la hidrodestilación de aceites esenciales del orégano comercial argentino Origanum x applii con la finalidad de evaluar la existencia y abundancia de antioxidantes naturales como han sido reportados en otras Labiadas.

Los principales compuestos fenólicos no ácidos aislados fueron identificados como hidroquinona y su β -glucósido, arbutina, mientras que en la fracción ácida se aisló ácido rosmarínico con un rendimiento de, al menos, 0,8 % (en base a hoja seca) de este extracto en las condiciones de la separación, acompañado por ácido 3,4-dihidroxibenzoico, ácido cafeico y ácido acetoacético entre otros compuestos minoritarios.

Estos resultados, junto con la estimación de un buen poder antioxidante, sugieren que este extracto puede ser usado como fuente de antioxidantes naturales, mejorando y diversificando de esta forma el valor económico del cultivo del orégano local en Argentina.

Introduction

Origanum x applii, an hybrid of O. vulgare and O. mejorana, is the major type of origanum cropped in Argentine, with two varieties named in Castellan language as "criolla" and "mendoza". Cooking use as dry leaf spice and sell abroad are the only purposes of its cultivation here. Origanum essential oil production is irrelevant in Argentine, though in other countries it is recognized as antimicrobial [1], antifungal [2] and antioxidant [3] among other activities.

In addition, an important antioxidant activity due to non volatile compounds has been reported in Lamiaceae plants including *Origanum* spp. [4-6].

Natural antioxidants are appreciated today for “natural” foods and furthermore, for any food, as synthetic antioxidants like BHT or BHA (2,6-diterbutyl, 4 hydroxitoluene or 3-terbutyl, 4 hydroxyanisol) are suspected of being carcinogens [4,7]. For example, rosmarinic acid, a phenylpropanoid first isolated from rosemary, has proved to have a potent antioxidative power [6,8]. In several countries, natural extracts from lamiaceae plants [8], mainly rosemary, sage, thymus and *origanum* spp., are being used as antioxidants in foods and they have got GRAS category from the US FDA.

Previous investigation on *Origanum* spp. has reported the presence of several phenolic compounds, glycosidated or not [9-12], among others [13].

To date chemical studies on *Origanum x applii* have referred only to its essential oil composition [14]. The known genetic heterogeneity in Lamiaceae [4,15] enhances the importance of chemical studies on non volatile compounds of this hybrid.

As a local commercial spice, the objective is to investigate antioxidative compound presence in the water extract of *Origanum x applii* var. *criolla*, a by product of essential oil hydrodistillation of its leaves. This water extract is normally seen as waste residue, but polar non volatile compounds of *origanum* have also been extracted.

The work goal is to describe some properties of this water extract and to identify the major compounds in order to evaluate its potential economical value. The *Origanum x applii* production could eventually be improved and diversify by obtaining its essential oil and as a source of natural antioxidants.

Materials and Methods

Plant material was dry leaves of *Origanum x applii* var. “*criolla*” from Fragarome S.A. (Province of Bs. As.). Essential oil extraction was carried out on a Clevenger type extractor by hydrodistillation of 40 g of *Origanum x applii* var. *criolla* dry leaf and 600 ml water during five hours. Vegetal material was filtered off and the remainder water extract was always taken to 500 ml with distilled water.

The extract pH and its titration curve were determined potentiometrically. Melting point were measured with a Büchi 511 Melting point apparatus. UV spectra and absorptions were recorded on a Shimadzu PR-1 spectrophotometer using 96° ethanol as reference and dilution solvent. FT-IR spectra were obtained on a Spectrum One FT-IR Perkin-Elmer spectrophotometer. The spectra were measured by using KBr pellets. ¹H-NMR and ¹³C-NMR spectra were recorded on a Bruker AC 300 spectrometer. Gas chromatography-mass spectra were obtained on a Shimadzu 50-50 CG-MS instrument, with a programmed temperature run (100-280°C) in an Ultra 2 column.

Isolation of major compounds of these extracts was performed by repeatedly SiO₂ column chromatography and recrystallization. Thin layer chromatography was performed on 0.25 mm thickness silicagel plates (Merck HF 254) with anisaldehyde / sulfuric acid and ferric chloride 5% in ethanol as revelators. Compound identification was established by spectroscopic methods and by comparison with literature references.

Total phenols were quantified by the Folin-Denis method [16] using gallic acid as standard. Results are expressed in gallic acid ppm and are the average of three determinations.

Table 1

		Mass g/l extract	Yield % dry leaf gallic acid	Total phenols ppm dilution	AAI each 1/10	Compounds
	Total Extract	21.0	26.0	5,200	1.5	(1) (2) (3) (4) (5) (6)
Fractionation scheme 1	Ethyl acetate extract pH 8.3	0.6	0.8	500	0.3	(1)
	n-butanol extract pH 8.3	0.8	1.0	200	0.15	(6)
	Ethyl acetate extract pH 3	3.2	3.8	1,800	1.2	(2) (3) (4) (5)
	n-butanol extract pH 3	1.4	1.7	800	0.2	(6)
	Residual water	-	-	700	0.5	
Fractionation scheme 2	Whole ethyl acetate extract pH 3	6.0	7.5	2,700	1.4	(1) (2) (3) (4) (5) (6)
	Whole n-butanol extract pH 3	4.0	5.0	1,300	0.5	(6)
	“pectin fraction”	5.0	6.3	200	0.2	
	Residual water	-	-	550	0.4	

The antioxidant activity index was evaluated by the β -carotene bleaching test (diffusion in agar method) [17], using a β -carotene / soybean oil model system and monitoring bleaching at room temperature under indirect solar light. The ratio between the time needed for β -carotene bleaching for a given fraction and the time for β -carotene bleaching in presence of BHT 0.02 % solution in identical conditions is reported. Results are the average of three determinations. Thin layer chromatography of the fractions followed by spraying with β -carotene/soybean oil in chloroform and monitoring of plate bleaching [17] was used to recognize antioxidative compounds as the spot colors where long-lasting.

In order to isolate compounds, extract fractionation was carried out by solvent extractions at different pHs. It results in four extracts: ethyl acetate and n-butanol extracts at pH 8.3 and ethyl acetate and n-butanol extracts at pH 3 (Scheme 1). Yields and parameters of these fractions are shown in Table 1. Compound (1) was isolated from the ethyl acetate extract at pH 8.3, compounds (2) to (5) from the ethyl acetate extract at pH 3, while compound (6) was identified in butanolic extracts at both pH (Figure 1).

Hidroquinone (1): colorless needles (acetone-benzene). mp: 170 °C. UV (λ max) 290 nm. IR (cm^{-1} , KBr pellet) 3250, 1520, 1470, 1240, 1222, 1200, 1080, 830, 760 super imposable with those in literature [20], MS: 110 (M^+ $\text{C}_6\text{H}_6\text{O}_2$). ^1H - NMR (ppm, d_6 -acetone): 9.0 (2H, s, phenolic OH); 6.67 (4H, s, aromatic protons).

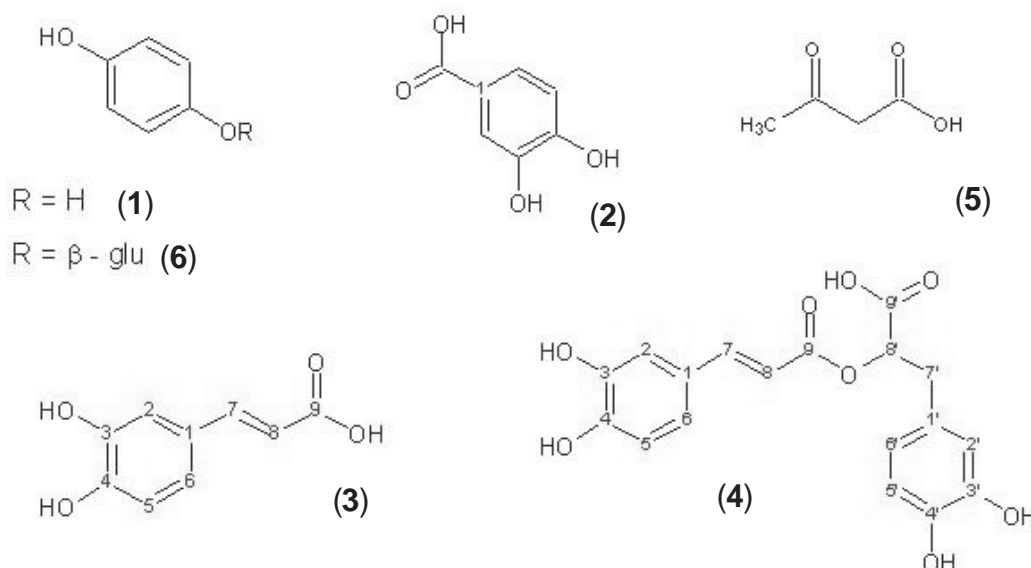


Figure 1

3,4-dihydroxybenzoic acid (protocachucic acid) (2): white needles (ethyl acetate). Mp: 200 °C; UV (λ_{max}) 293 nm. IR (cm^{-1} , KBr pellet): 3290, 2650-2500, 1670, 1600, 1295, 1255, 943, 765 super imposable with that of literature [20], ^1H -NMR (ppm, d_6 -acetone): 7.52 (1H, d ($J=2$ Hz), 2-H); 7.47 (1H, dd ($J=9$ and 2 Hz), 6-H); 6.89 (1H, d ($J=9$ Hz), 5-H); ^{13}C -NMR (d_6 -acetone): 167.5 (7-C), 150.6 (4-C), 145.4 (3-C), 123.5 (6-C), 122.5 (1-C), 117.5 (2-C)*, 115.6 (5-C)* (*assignments could be interchanged) identical with literature data [20]

Caffeic acid (3): colorless needles (ethyl acetate-benzene) UV (λ max): 292 nm, 323 nm; IR (cm^{-1} , KBr pellet): 3230, 1645, 1620, 1453, 1289, 1280, 980, 820; ^1H -NMR (ppm, d_6 -acetone): 7.50 [1H, d ($J=16$ Hz) H-7]; 7.16 (1H, d ($J=2$ Hz), H-2); 7.03 [1H, dd ($J=9$ and 2 Hz), H-6], 6.80 [1H, d ($J=9$ Hz), H-5]; 6.25 [1H, d ($J=16$ Hz), H-8] identical with literature data [20].

Rosmarinic acid (4): amorphous solid, UV λ max (log ϵ): 289 nm (3.7), 326 nm (3.8); IR (cm^{-1} , KBr pellet): 3400, 2800, 1710, 1690, 1605, 1520, 1450, 1363, 1290, 1205, 1170, 1120, 1085, 989, 820; MS: 194 (0% !), 163 (100%); $^1\text{H-NMR}$ (ppm, d6-acetone): 7.56 [1H, d (J=16 Hz) 7-H]; 7.17 [1H, d (J=2 Hz), 2-H]; 7.05 [1H dd (J= 9 and 2 Hz) 6-H]; 6.85 [1H, d (J= 9 Hz) 5-H]; 6.84 [1H, d (J=2 Hz), 2'-H]; 6.75 [1H, d (J= 8.5 Hz), 5'-H]; 6.66 [1H, dd (J=8.5 and 2 Hz) 6'-H]; 6.30 [(1H, d (J= 16 Hz) 8-H)]; 5.20 [(1H, m, 8'-H)], 3.07 [(2H, dq, 7'-H)]; $^{13}\text{C-NMR}$ (ppm, d6-acetone): 171.2 (9'-C), 166.8 (9-C), 148.8 (4-C), 146.5 (3-C), 146.1 (7-C), 145.6 (3'-C), 144.7 (4'-C), 129.1 (1'-C), 127.4 (1-C), 122.7 (6-C), 121.5 (6'-C), 117.2 (2'-C), 116.3 (5-C), 115.8 (5'-C), 115.1 (2-C), 114.8 (8-C), 73.7 (8'-C), 37.4 (7'-C) identical with literature data [5,12].

Acetoacetic acid (5) identified as rosmarinic acid impurity by spectroscopic assignments [19] and GC-MS analysis: $^1\text{H-NMR}$ (ppm, d6-acetone): 2.18 (s, $\text{CH}_3\text{CO-}$ moiety), 3.80 (s, $-\text{CH}_2-$ moiety); MS: 102 (M^+), 84, 60, 43 (BP)

Arbutin (6): colorless needles (ethyl acetate) mp 199 °C; UV (λ max) 290 nm; IR (cm^{-1} KBr pellet) 3390, 2910, 1651, 1513, 1220, 1066, 1014, 832, 778 super imposable with those in literature [20]; $^1\text{H-NMR}$ (ppm, d6-DMSO): 9.05 (1H, s, phenolic OH); 6.86 (2H, dd (J= 2.3 – 6.6, H-2,-6)); 6.66 (2H, dd, J=2.3-6.6, H-5,-3); 5.27 (1H, d J = 4.85, 2'-OH), 5.08 (1H,d, J=3.8 3'-OH); 5.01 (1H, d, J=4.8, 4'-OH); 4.62 (1H, d, J=7.4, 1'- βH Glu); 4.58 (1H, t, 6'-OH); 3.68 (1H, dd, (J=3.8 J=11.25, 6' βH), 3.46 (1H, m, 6' αH); 3.4-3.2 (4H, m, 2',3',4', 5'H Glu).

A second and simpler fractionation scheme (Scheme 2) was done to reduce losses and follow the distribution of phenols and antioxidant activity among the fractions (Table 1). It consists of direct ethyl acetate and n-butanol extractions at pH 3 of the water extract, followed by concentration of the remainder water solution, then a “pectin fraction” precipitation with 2.5 parts in volume of ethanol and filtration [18].

Results and discussion

Origanum x applii water extract obtained as mentioned above has acidic reaction (pH=5.6). Its titration curve with NaOH showed two steps: the first one corresponds to organic acids (0.04 equiv./liter extract between pH 7 and 3) and the second accounts for weaker acids probably phenols, as Ferric chloride and Folin test were positive. However, Shinoda test for flavonoids and gelatin test for tannins resulted negative.

The whole extract showed strong UV absorptions at 285 and 325 nm, even on 1/100 and 1/200 dilution that supported the idea of phenols with extended conjugation [19] in good concentration. Folin-Denis test showed 5,200 ppm of phenols expressed as gallic acid.

The parameters of the fractions obtained after the extraction Scheme 1 are shown in Table 1. Chromatographic separation was focused on the fractions that demonstrated antioxidant activity in a screening with the TLC β -carotene bleaching test [17] and led to the isolation of the major compounds in the fractions ((1)-(6)) whose structures are shown in Figure 1.

Ethyl acetate extract at pH 3 gave the best mass yield, phenol concentration and antioxidative activity among the fractions. As it is shown in Table 1, phenolic compound concentration of this

fraction, in spite of being high, is only around 1/3 of the initial one, however, it represents more than 3/4 parts of total antioxidant activity of the original water extract. The natural antioxidative compound rosmarinic acid (**4**) was isolated here (at least with 0.8 % in dry leaf basis) among other phenolic acids that also have antioxidant activity by themselves and reinforce the effect in different ways.

Besides, it is remarkably that extracted masses are low and the sum of phenols in all fractions does not account for total phenols in the whole extract. Emulsions and dark precipitates that appeared during the extraction procedures and were filtered off, may explained the differences. Besides, turbidity on acidification and an abundant brownish-white precipitate that separated when ethanol was added to the water extract, led us to suspect of a pectin-like behaviour.

To establish how phenols distribute among the fractions and to reduce their losses while fractioning, a simpler scheme that considered pectin precipitation was carried out as described in Scheme 2. "Pectin fraction" is to be studied, but preliminary colour test with toluidine O blue (green colour), Folin test and UV spectra have shown the presence of phenols in it, but no significant antioxidant activity appeared in this fraction tests.

Again, the highest amount of phenols and antioxidative behaviour was in the ethyl acetate extracts. However, there are some more water soluble phenolic compounds only extracted with n-butanol and others that remained in the aqueous solution and in the "pectin fraction", that indicate different degrees of antioxidant activity and will be the subject of further studies.

Conclusions

Origanum x apalii has got non-volatile antioxidative compounds like both *O. vulgare* and *O. majorana*. Rosmarinic acid is in good concentration in this water extract considered a waste residue, along with other phenolic compounds that undoubtedly enhance the observed activity by different cooperative mechanisms.

The valorization of residues may be the key to the success of some kind of developments. In this sense, this work supports the possibility of using water residues of essential oil hydrodistillation of argentine commercial *origanum* as sources of natural antioxidants for food and health purposes.

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