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Antioxidant Activity of Essential Oils from Various Mexican Oregano Ecotypes and Oil Fractions Obtained by Distillation

Ramon Silva-Vazquez[1-3], Lorenzo Antonio Duran-Melendez[4], Gerardo Mendez-Zamora[5], Eduardo Santellano Estrada[4], Meizhen Xie[1,2], Nurhan Turgut Dunford[1,2]*, and Carla Goad[1,6]

[1]Department of Biosystems and Agricultural Engineering, Oklahoma State University, USA
[2]Robert M. Kerr Food & Agricultural Products Center, USA
[3]Research Center for Natural Resources, Mexico
[4]Department of Zootechnics and Ecology, Autonomous University of Chihuahua, Mexico
[5]Department of Agronomy, Autonomous University of Nuevo Leon, Mexico
[6]Department of Statistics, Oklahoma State University, USA

*Corresponding author
Nurhan Turgut Dunford, Department of Biosystems and Agricultural Engineering & Robert M. Kerr Food & Agricultural Products Center, FAPC Room 103, Stillwater, OK 74078-6055, USA, Tel: 405-744-7062; Fax: 405-744-6313; Email: Nurhan.Dunford@okstate.edu

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Abstract

Essential oil of oregano is known for its antimicrobial and antioxidant properties. The aim of this study is to investigate the antioxidant capacity of essential oils obtained from 5 different Mexican oregano ecotypes and the fractional distillation of these oils. Total phenolic content of the oil samples was analyzed. Antioxidant activity of the samples were examined by the following assays; Oxygen Radical Absorbance Capacity, 2,2-Diphenyl-1-Picrylhydrazyl, and Oxidative Stability Index (OSI). Chemical composition of the samples was determined by gas chromatography.

Thymol and carvacrol were the main components in all samples examined in this study. There were significant differences in chemical composition of oils obtained from various Mexican oregano ecotypes. Oregano essential oil fractions with antioxidant capacity similar to or higher than that of the synthetic antioxidant butylated hydroxytoluene were obtained by fractional distillation. This study demonstrated that oregano oil and its fractions can be viable alternatives to the synthetic antioxidants widely used in foods and animal feed.

INTRODUCTION

Food oxidation produces a rancid flavor and decreases the sensory and nutritional quality of the products making them unacceptable to consumers [1]. Antioxidants have been widely used for preservation of foods and feed. Synthetic antioxidants such as butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), tert-butylhydroquinone (TBHQ), and propyl gallate (PG) have been commonly used as antioxidants in various products. The consumer concerns about the potential adverse health effects of synthetic antioxidants [2,3] and increasing demand and willingness to pay premiums for "all natural" foods have motivated the industry to seek natural solutions to oxidation and extend the shelf life of food products.

There are numerous studies examining the use of essential oils as natural ingredients because of their preservative properties such as antioxidants and antimicrobials [4-6]. Oregano, also referred to as *origanum*, includes a wide range of species and subspecies that are distributed over about 60 families. *Alliaceae*, *Apiaceae*, *Asteraceae*, *Lamiaceae*, *Myrtaceae*, *Poaceae* and *Rutaceae* families are well known for their ability to produce essential oils of medicinal and industrial value [7, 8]. About 70 species, subspecies, varieties and hybrids characterized by a large morphological and chemical diversity produce valuable essential oils [9,10]. Greek [*Origanum vulgare* L. ssp, *hirtum*], Turkish (*Origanum onites* L.), Spanish (*Thymus capitatus* L.) and Mexican oregano (*Lippia graveolens* HBK or *Lippia berlandieri* Schauer; *Lippia palmeri* Watson, *Poliominthaa longiflora* Gray) are important essential oil producing species.

Oregano grows wild in 24 states in Mexico. *Lippia berlandieri* Schauer which is an herbaceous plant native to Mexico and belongs to the *Lamiaceae* family is the most economically important species. The state of Chihuahua is among the major producers of this oregano species in Mexico. The antioxidant properties of *Lippia berlandieri* Schauer have been reported [11,12]. Phenolic compounds, including flavonoids and phenolic acids, present in essential oils contribute to their antioxidant activities and retard oxidation reactions [13-15].

Lippia essential oil is a mixture of several components, mainly phenolic monoterpenes including thymol, carvacrol and their precursor *p*-cymene [10,16-18]. There are reports that oregano essential oils also contain a glucoside, 4-(3,4-dihydroxybenzoyloxymethyl) phenyl-β-

D-glucopyranoside, exhibiting antioxidant activity [19,20]. Variations in chemical composition of essential oils are shown to affect their antioxidant properties significantly [21]. When phenols were concentrated in essential oils by molecular distillation, antioxidant capacity of the oils increased [22]. The chemical composition of essential oils of the same species may vary depending on the harvesting season and geographical location of plant growth (Burt 2004). Furthermore, Mexican oregano growth experiments carried out under controlled conditions in a greenhouse showed that plant maturity level had a significant effect on the essential oil content of the leaves and young plants had the highest essential oil content [23].

The main objective of this study was to investigate the effect of essential oil chemical composition on its antioxidant properties. The variations in chemical composition of essential oils from cultivated and wild Mexican oregano species were examined in this study. Oil fractions obtained by distillation of the essential oils extracted from the leaves and the flowers of the plants were also examined for their chemical composition and antioxidant capacity.

MATERIALS AND METHODS

Plant material

Samples collected from cultivated and wild *Lippia berlandieri* Schauer and *Poliomintha longiflora* Gray Mexican oregano grown in Coahuila, Durango, and the southern region of Chihuahua State in Mexico, were used in the experiments. The collected samples were herborized according to the methodology described by Lot and Chiang [24] and vouchers were deposited in the CIIDIR Instituto Politecnico Nacional Herbarium, located in Durango, Mexico. The morphological analyses of the plants were based on the taxonomic characteristics excluding those influenced by environmental variables. The samples were collected during the flowering growth stage of the plants. Only dried flowers and leaves were used in steam distillation. Essential oil samples were obtained using a distillation unit (1500 kg feedstock holding capacity) built in-house at the Natural Solutions Company, Jimenez, Chihuahua, Mexico. Five Mexican oregano ecotypes (the same species grown in different environments and might have a different phenotypic expression due to the interaction of genes with the environment) were investigated in this study: Cultivated (CUL), Jimenez (JIM), Zaragoza (ZAR), Salaices (SAL) belonging to *Lippia* genus and *Poliomintha longiflora* (POL). a summarizes the plant growth location and harvest dates.

Oil fractionation

Essential oil samples were fractionated by using a glass distillation unit (Vigreux distilling column, Kimble Chase Life Science and Research Products LLC). Four fractions, F1, F2, F3 and F4 were collected from oils obtained from each oregano ecotypes. F1, F2 and F3 fractions were collected by heating each oil sample separately at 120, 140 and 180 °C, respectively, until no condensate was received in the collection flask at the set temperature, followed by a temperature increase and collection of the next fraction in a separate flask. F4 was the residual oil in the flask after collecting F3. The volatile components coming off the oil passed through a glass condenser which was cooled with water at 22 °C to facilitate condensation. Thymol crystals were obtained by heating the SAL oil at 180°C until a solid residue was obtained.

Chemical composition of oil samples

Oil samples were analyzed by a HP 6890 Plus gas chromatography system equipped with a flame ionization detector (FID) (HP Company, Wilmington, DE). A Perkin Elmer PE-1 capillary column (30 m x 0.25 mm, 0.25 μm film thickness) was used for separation of the oil components. The chemical standards used for identification of the peaks, carvacrol, thymol, *p*-cymene, gamma-terpinene, menthol, cineole, borneol, camphene, linalool, limonene, myrcene, trans-caryophyllene and eugenol were purchased from Sigma (Sigma-Aldrich, St. Louis, MO). The helium carrier gas flow rate was 26 cm/s. The injector temperature was maintained at 250°C. A temperature program with total run time of 25 min was used. The column temperature, after an initial isothermal period of 1 min at 55°C, was increased to 95°C at a rate of 3°C/min, and maintained at this temperature for 1 min. Then, the column temperature was further increased to 220°C at a ramp rate of 20°C/min and maintained at this temperature for 3.4 min. The detector conditions were as follows: temperature 250°C and H_2, air and make-up gas (He) flow rates were 40 mL/min, 400 mL/min and 30 mL/min, respectively. Oil samples (1μL) were injected by an auto sampler (HP 7683, HP Company, Wilmington, DE). Peak areas were calculated and data collection was managed using an HP Chemstation (A.09.01, Agilent Technologies, Palo Alto, CA).

Total phenolic content

The total phenolic content (TPC) of the oil samples was determined by the Folin-Ciocalteu method [25]. In summary, a mixture of 0.1 ml of oil, 500 μL of Folin-Ciocalteu reagent (FCR) (Sigma-Aldrich), 1.5 mL of 20% sodium carbonate, and 7.9 mL deionized water was allowed to react for 2 h at ambient temperature. Absorbance of the solution was measured at 765 nm on a DU520 UV-Vis spectrophotometer (Beckman, Brea, CA) at time 2 h. Gallic acid (Sigma-Aldrich) was used to prepare an external standard curve for quantification. TPC of the samples was reported as Gallic acid Equivalents (GE) per gram of sample used.

DPPH Radical Scavenging Capacity

2,2-Diphenyl-1-picrylhydrazyl (DPPH) tests were carried out on oil samples [26]. Prior to absorbance measurements, 2 mL of 60 μM DPPH solution (Sigma-Aldrich, St. Louis, MO) was added to 50 μL of oil. Absorbance of the sample was recorded on a DU520 model UV–Vis spectrophotometer (Beckman, Brea, CA) at 517 nm for 60 min at 30 s intervals. The percent radical inhibition at 60 min is determined using the following equation [26]:

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

Where;

A_{sample} absorbance of the sample at 60 min

$A_{control}$ the absorbance of assay solution at time 0 min

Oxygen radical absorbance capacity

The oxygen radical absorbance capacity (ORAC) assay was

performed by using a HTS-7000 Microplate (Perkin-Elmer, Covina, CA) reader at excitation and emission wavelengths of 485 and 535 nm, respectively. All the reagents were prepared in pH 7.0 phosphate buffer. Oil, 20 µL, was mixed with 160 µL of fluorescein (TCI, Portland, OR, USA) (0.2 µg/mL) and 20 µL 2,2-azobis(2-methylpropionamidine) dihydrochloride (AAPH; Sigma-Aldrich; 33.2 mg/ml) in a 48-well clear plate and reacted at 37°C for 45 min. The same procedures were used to prepare blank and standard which were 20 µL of phosphate buffer (pH 7.0) and 20 µL of 10 µM Trolox (Sigma-Aldrich), respectively. The AAPH solution is always made fresh daily and the oregano oil dilution ratio was 1:1 000 000. ORAC values were calculated by using the area under the kinetic curve (AUC) and the dilution factor (DF) as:

$[(AUC_{sample} - AUC_{blank}) = (AUC_{Trolox} - AUC_{blank})] \times DF \times$ (µmol Trolox/g sample)

Where; AUCsample, AUCblank and AUCTrolox were the area under the curve for the sample, blank and Trolox, respectively.

ORAC value was expressed as µmol Trolox/g sample.

Oxidative Stability Index (OSI)

Oxidative Stability Index (OSI) is an automated method that determines oil stability measuring the conductance produced when evolving volatile organic acids are collected in deionized water. Stable secondary oxidation products (volatile organic acids) are produced while oil is oxidized by a stream of air is bubbled through the oil usually at 110°C. The OSI tests were carried out using a 743 Rancimat instrument (Metrohm, Herisau, Switzerland) according to the AOCS Air Oxidation Method (AOM-AOCS Cd 12b-92) [27].

DATA ANALYSIS

All experiments were carried out at least in duplicates. Means were compared by Tukey's HSD test at a 95% confidence interval. Statistical analysis of the experimental data was performed using SAS (ver. 9.4, SAS Institute, Inc., Cary, N.C.).

RESULTS AND DISCUSSION

Essential oil chemical composition

The essential oil yields obtained from the samples examined in this study were similar, about 4% based on the feedstock weight. However, there were significant differences in chemical composition of the oils from different ecotypes (Tables 1,2). Carvacrol (CAR), thymol (THY) and *p*-cymene (CYM) were the major components in all the essential oil samples (Table 2). Oil from JIM was rich in CAR (about 60%) while SAL, ZAR and POL had the highest THY (about 55%), cineole (CINE) (25%) and γ-terpene (TER) content (about 18%) contents, respectively. All the other components identified in the oils were at concentrations less than 5%.

Essential oil fractionation by distillation was very effective to enrich oils in desired components. Oil from SAL did not produce any condensate at 120 and 140°C (no F1 and F2 fractions). Yet, it was possible to obtain thymol crystals with 94% purity from the same ecotype, SAL. JIM did not produce F4 fraction, meaning that there was no residual oil in the flask after heating the oil at 180°C. Fractionation of CUL oil enhanced CYM concentration in F1, F2 and F3 fractions. Considering that CYM has a lower boiling point, 177 °C, and smaller molecular weight (MW), 132.2, than CAR (237.7 °C and MW of 150.2) and THY (232 °C and MW of 150.2) the latter result is expected. As a result, F4 from CUL oil was enriched in THY. The original oil extracted from SAL was rich in THY, 54.6%. F3 fraction from SAL oil was enriched in CYM, 35.4%, and concentration of TER increased 135% (from 0.1% to 13.5%) in the same fraction.

Total Phenolic Content

Oils from CUL and JIM had significantly higher TPC value than that of the oils from other ecotypes (Table 3). THY + CAR contents of these oils, 58.9% in CUL and 63.4% in JIM, were significantly higher than the oils from other ecotypes examined in this study ($p < 0.05$) (Table 2). Hence, in general TPC results were correlated with the phenolic content in the samples as determined by GC. Indeed, the contribution of CAR to the TPC was significantly higher than the other components present in the oil, $R^2 = 0.6388$ (Table 4). Although the concentrations of borneol (BOR) and myrcene (MYR) were very low in the samples, the statistical analysis of the data indicates that their contributions to the TPC were substantially. The contribution of THY to TPC was very low, $R^2 = 0.01403$.

Fractionation of the oil obtained from JIM almost doubled the TPC of the fraction JIMF3, which had the highest TPC, 87.9%, among all the oils examined in this study. Since JIMF3 contained the highest CAR concentration among the oils examined in this study, the latter result is in agreement with the findings discussed in the previous paragraph that CAR is a significant contributor to TPC. The change in the TPC of the fractions obtained from SAL and CUL was not as pronounced as that of JIMF3 because of the much lower CAR content in the samples (Table 2).

Antioxidant capacity

DPPH: The antioxidant capacity of the essential oil samples was also evaluated by examining DPPH of the samples. This assay measures the reducing capacity of antioxidants toward DPPH which is a stable organic nitrogen free radical. BHT was the reference antioxidant that is widely used in food systems. As expected DPPH inhibition increased with increasing BHT concentration used in the test (Table 5). About 96% DPPH inhibition could be achieved using BHT at 2% concentration (weight %). Table 4 shows the DDPH inhibition data for oregano essential oil concentration at 2.5% (volume basis, 5 µL oil in 2 mL DDPH solution) in the solution. DPPH inhibition of CUL, SAL and JIM at 2.5% (volume) was similar to that of BHT at 2% level. Although POL (64.5%) and ZAR (85.7%) demonstrated significantly lower DPPH inhibition capacity than the oils obtained from other ecotypes (94-95% DPPH inhibition) their radical scavenging activity was still higher than that of the BHT at 0.5% level.

The highest contributors to the DPPH activity were CYM ($R^2 = 0.72220$) and THY ($R^2 = 0.41090$) among the compounds constituting oregano essential oil (Table 4). Hence, the samples with relatively high CYM and THY exhibited high DPPH scavenging activity (Tables 2,4). Fractionation of the oils from CUL, SAL and JIM by distillation did not improve DPPH inhibition beyond that

Table 1: Oregano ecotypes, their growth locations and harvest date.

Ecotypes	Type	Growth location	Harvested
Cultivated (CUL)	Cultivated Variety (*Lippia berlandieri*)	Jimenez, Chihuahua	10-01-2014
JIM	Wild (*Lippia berlandieri*)	Jimenez, Chihuahua area	10-01-2014
ZAR	Wild (*Lippia berlandieri*)	Zaragoza, Chihuahua area	10-01-2014
SAL	Wild (*Lippia berlandieri*)	Salaices, Chihuahua area	10-01-2014
POL	Wild (*Poliomintha longiflora*)	Coahuila, Mexico area	10-01-2014

Table 2: Chemical composition of Mexican oregano essential oils (% of total oil collected) from various ecotypes and fractions obtained by distillation.

Compounds[1]/	CAR	THY	CYM	TER	MENT	CINE	BOR	CAM	LIN	LIM	MYR	TCAR	EUG
Sample type													
POL	13.9	28.5	5.5	17.9	0.8	0.6	-	-	-	-	-	0.07	0.6
ZAR	4.5	0.6	21.7	-	4.4	25.1	-	0.3	-	2.7	-	0.03	-
CUL	24.5	34.4	9.0	0.3	1.1	0.6	0.6	-	1.0	0.1	-	0.4	0.2
SAL	1.6	54.6	12.3	0.1	1.0	0.6	0.2	-	0.5	0.1	0.2	-	0.8
JIM	60.0	3.4	16.1	5.4	0.8	0.3	-	-	0.3	0.3	0.09	0.4	0.1
Thycryst	0.6	94.4	-	-	0.3	-	-	-	-	-	-	0.9	0.03
CUL F1	9.2	0.6	52.1	-	-	-	-	-	-	-	-	-	-
CULF2	1.7	8.7	42.8	9.9	0.5	2.6	-	-	-	-	-	-	-
CULF3	2.2	11.6	42.9	10.5	0.7	2.4	0.4	0.06	1.5	-	-	0.05	-
CULF4	12.5	53.5	6.5	-	1.5	0.3	0.2	-	-	-	-	0.2	0.08
SALF3	0.3	9.9	35.4	13.5	0.3	2.0	-	0.2	0.4	-	-	0.3	0.07
SALF4	2.1	63.7	8.1	3.4	1.1	1.6	0.2	0.08	0.5	0.4	-	0.05	1.0
JIMF1	9.2	0.6	52.1	14.5	0.2	0.9	-	-	0.2	0.1	-	0.04	-
JIMF2	11.4	0.8	56.0	16.5	0.3	0.9	-	-	0.3	-	-	0.05	-
JIMF3	73.4	4.4	6.4	1.7	-	-	0.9	-	0.3	0.2	0.3	0.09	0.3

Abbreviations: CUL: Cultivated; JIM: Jimenez (JIM); Zaragoza (ZAR); Salaices (SAL) belonging to *Lippia* Genus and *Poliomintha longiflora* (POL) and CAR= Carvacrol; THY= thymol, CYM= *p*-cymene; TER= Gamma-Terpinene; MENT= Menthol; CINE= Cineole; BOR= Borneol; CAM= Camphene; LIN= Linalool; LIM= Limonene; MIR= Myrcene; T-CAR= *Trans*-Caryophyllene and EUG= Eugenol
The columns with no data indicate values ≤0.001, under detection limit of the analytical technique used.

Table 3: TPC, ORAC and DPPH of the samples.

SAMPLE	TPC	ORAC	DPPH
	(as mg Gallic acid/g oil)	(mmol Trolox/g oil)	(% Inhibition)
POL	13.2 ± 0.2	58.8 ± 4.4	85.7
ZAR	23.2 ± 1.7	47.9 ± 2.2	64.5
CUL	49.1 ± 0.5	53.0 ± 3.3	95.5
SAL	34.1 ± 8.6	53.7 ± 2.8	94.0
JIM	44.3 ± 1.2	33.5 ± 4.6	95.4
Thycry	30.1 ± 3.0	41.0 ± 4.8	95.8
CUL F1	11.5 ± 1.0	48.5 ± 7.8	68.6
CUL F2	13.8 ± 0.8	49.4 ± 2.3	59.1
CUL F3	14.0 ± 0.7	54.4 ± 8.0	63.8
CUL F4	44.5 ± 0.3	49.2 ± 2.9	95.2
SAL F3	13.9 ± 2.1	38.1 ± 8.9	54.1
SAL F4	33.2 ± 1.0	46.1 ± 3.2	94.0
JIM F1	23.1 ± 0.2	34.5 ± 3.7	58.0
JIM F2	22.4 ± 3.2	27.5 ± 2.9	68.3
JIM F3	87.9 ± 0.1	37.2 ± 0.2	95.2

Table 4: The coefficient of determination (R^2) was computed for each compound versus each of the three assays ORAC, DPPH, and TPC.

Compound	TPC	ORAC	DPPH
CAR	0.63880	0.12724	0.20458
BOR	0.62067	0.01512	0.17867
MYR	0.57269	0.02084	0.18695
PCYM	0.31740	0.10433	0.72220
GTER	0.23224	0.05375	0.30962
CAM	0.05176	0.00303	0.15552
EUG	0.03123	0.12614	0.28182
CINE	0.02789	0.01297	0.10357
TCAR	0.02647	0.05435	0.14385
THY	0.01403	0.08784	0.41090
MENT	0.00252	0.08359	0.00012
LIN	0.00120	0.05794	0.00023
LIM	0.00024	0.00327	0.02650

Table 5: DDPH inhibition capacity of BHT at different concentrations.

BHT concentration (%, weight basis)	Inhibition (%)
0.5	63.77
1.0	86.95
1.5	90.09
2.0	96.01

of the original oils, 94-95% inhibition (Table 4). However, a reduction in THY content in the fractions resulted in a significant decrease in DPPH inhibition.

ORAC: This method uses competitive kinetics to assess the ability of antioxidants to compete with fluorescein (FL), a molecular probe, to scavenge the peroxyl radicals generated by 2, 2′-Azodi (2-amidinopropane) dihydrochloride. Antioxidant reacts with the peroxyl radicals by donating a hydrogen atom to prevent the oxidation of the FL. Trolox, a vitamin E analogue, is used as a standard in this assay. A high ORAC value indicates a strong antioxidant capacity.

The highest ORAC value was measured for POL which exhibited the lowest TPC among the oils examined in this study (Table 4). This oil had the second lowest and the highest THY + CAR and TER contents, respectively, than the other oils. Although the statistical analysis of the data indicated that CAR was the highest contributor to the ORAC activity of the samples (Table 4), the correlation coefficients for ORAC values were extremely low, $R^2 = 0.127224$. The latter result indicates that synergistic effects of the various oil components and/or unidentified components in the oil samples had a significant effect on the ORAC value.

OSI: Effect of the essential oil concentration on canola oil stability was evaluated using the OSI method with selected oils, POL, JIM, CUL, SAL, JIMF3 and THYCRY (Figure 1). All the samples examined in the study significantly improved the oxidative stability of canola oil with increasing concentration of the added oregano oil except POL ($p = 0.5875$) and THYCRY ($p = 0.0520$). JIM ($p = 0.0013$) and JIMF3 ($p = 0.0003$) which had the highest CAR contents were the most effective oils in improving the OSI of the canola oil. Increase in OSI was less pronounced above 0.4% JIM addition while OSI continued increasing even after 0.7% JIM3 addition to canola oil.

CONCLUSIONS

Essential oil contents of the five Mexican oregano ecotypes examined in this study were similar. This study clearly demonstrated that chemical composition of the oils obtained from different ecotypes varied significantly. Distillation of the oils is a very effective method for enriching the oils in desired components, i.e. CAR or THY enrichment. Chemical composition of the oils had a significant effect on TPC, ORAC and DPPH activity of the oils. Contributions of each oil component to the antioxidant activity of the oils were determined. JIM and JIM3 which had very high CAR content improved the OSI of the refined canola oil significantly. In summary, data provided in this study is helpful for developing New Mexican oregano oil applications in food systems.

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