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ORIGINAL PAPER

Extracts of Mexican Oregano (*Lippia berlandieri* Schauer) with Antioxidant and Antimicrobial Activity

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Abstract Antimicrobial activity of fractions obtained from Mexican oregano (*Lippia berlandieri* Schauer) chloroform extract was tested by growth inhibition against *Escherichia coli*, *Staphylococcus aureus* and *Bacillus cereus*, and antioxidant capacity was tested by inhibition of linoleic acid oxidation. Fractions were obtained by differences in polarity or structure (phenolic and non-phenolic fraction). Gram-positive organisms were more susceptible to Mexican oregano extracts. Fraction 3 (by polarity) and phenolic fractions I, II, III, IV and V were the extracts with higher antimicrobial activity. The non-phenolic fraction had effect against *B. cereus*. Polarity fraction 5 and phenolic Fraction II had a high antioxidant capacity; a 0.08% concentration of fraction 5 had a similar effect as butylated hydroxytoluene at 0.01% concentration. Fractions of Mexican oregano with different polarity and functional groups had antioxidant and antimicrobial activity and can be used in a variety of applications.

Keywords Natural antimicrobials · Natural antioxidants · Mexican oregano

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Introduction

Advances in biological and chemical analytical methods have raised questions regarding the safety of chemical food additives. Because of new data relating chemical additives with toxicological problems (Kaur and Kapoor 2001), there is a tendency by the informed consumer to use natural food products. Many research groups are examining the chemical nature and activity of natural antimicrobials and antioxidants in fruits, vegetables, grains, herbs and other foods (Atoui et al. 2005). Interest in the role of antioxidants in human health has encouraged research in food science to determine how their content and activity can be maintained or even improved through cultivar development and processing technologies (Alasalvar et al. 2005). Therefore, efforts have concentrated on the use of natural extracts, such as those obtained from spices.

Recently, there has been an increase in the use of herbal spices; as in the past, their main use is as flavour enhancers, but there are also important medical and pharmaceutical applications. Spices have also been used in traditional culinary recipes for thousands of years in countries with warm weather, where they exhibit antimicrobial activity (Sherman and Flaxman 2001; Kalemba and Kunicka 2003). In particular, the antimicrobial activity of plant oils and extracts has formed the basis of many uses, including raw and processed food preservation, pharmaceuticals, alternative medicine and natural therapies (Hammer et al. 1999).

One of the spices with proven antimicrobial effects is oregano, which has been demonstrated to inhibit the growth of several foodborne pathogenic bacteria (Lambert et al. 2001; Chorianopoulos and Kalpoutzakis 2004; Burt et al. 2005). This spice has also been demonstrated inhibitory effect against foodborne fungi (Salmeron et al. 1990; Araujo et al. 2003; Portillo-Ruiz et al. 2005) not only on fungal growth

but also on the production of aflatoxins and spores. The antimicrobial activity of oregano has been attributed mainly to the presence of volatile compounds found in its essential oil, especially carvacrol and thymol, which have been demonstrated to be responsible for the antimicrobial effect of oregano as individual compounds (Kim et al. 1995; Skandamis and Nychas 2000). There are also reports on the antioxidant capacity of oregano, which is recognised as one of the most effective among common spices (Abdala and Roozen 2001). There are also reports on antioxidant compounds from oregano, different from thymol and carvacrol, including those with phenolic and glucosidic structures (Nakatani and Kikuzaki 1987; Kikuzaki and Nakatani 1989).

Most of the reports on oregano are related to *Origanum vulgare*, which is found mainly on the Mediterranean coast. However, more than 40 different kinds of herbal plants are known with the name of oregano, which share the characteristic odour and flavour given by the combined presence of thymol and carvacrol. Mexican oregano (*Lippia berlandieri* Schauer or *Lippia graveolens* HBK) is among those species and is characterised by a stronger flavour and a higher yield of essential oil (Huerta 1997). Mexican oregano has higher essential oil content than European oregano; carvarol content is higher and, therefore, has a stronger odour and slightly different biological activities (Arcila-Lozano et al. 2004). However, it has been demonstrated that yield and composition of oregano depend on growth conditions and extraction process (Dunford and Silva-Vazquez 2005). Most of the reports on oregano have been done on its essential oil, and there are few reports on the antimicrobial activity of other non-volatile compounds present in oregano. Lin et al. (2007) have recently described more than 20 flavonoids in Mexican oregano extract, using LC-DAD-ESI/MS analysis. There are also recent reports of antioxidant activity of extracts obtained from steams of Mexican oregano (Gonzalez-Güereca et al. 2007), as well as antimutagenic and antioxidant activities of oil-free extracts (Martínez-Rocha et al. 2008).

The search for compounds with antimicrobial and/or antioxidant activity, but without the strong odour characteristic of oregano, can lead to products with a wider range of applications. Therefore, the aim of this work was to obtain different extracts and fractions from Mexican oregano (*L. berlandieri* Schauer) and to evaluate their antimicrobial and antioxidant properties.

Materials and Methods

Biological Material and Chemicals

Mexican Oregano (*L. berlandieri*) was bought at a local market in Chihuahua, Mexico, which was previously collected from wild plants from the region. Bacterial strains used were *Escerichia coli* ATCC 25922, *Staphylococcus aureus* ATCC 6538 and vegetative cells of *Bacillus cereus* ATCC 11778 and were obtained from the culture collection at the School of Chemical Sciences, University of Chihuahua, Chihuahua, México. Stains were maintained on Brain Heart Infusion medium (BIOXON, Mexico City, Mexico) and for fresh inoculum preparation, the microorganisms were grown to exponential phase in nutrient broth (BIOXON, Mexico City, Mexico), at 35 °C for 18 h. All chemical reagents and solvents used were purchased from J.T. Baker (Mexico City, Mexico), unless otherwise described.

Preparation of Mexican Oregano Fractions

For solid–liquid extraction, 500 g of dry and pulverised oregano were placed in a closed flask with chloroform and after 24 h, filtered and the solvent evaporated until a paste-like extract was obtained (Kikuzaki and Nakatani 1989). For further separation, 2 g of extract were placed in a chromatography column packed with silica gel 70–230 mesh (Merck, Mexico City, Mexico) and successively eluted with chloroform, chloroform–acetone (70:30, *v*/*v*), chloroform–acetone (30:70, *v*/*v*), acetone–methanol (70:30, *v*/*v*) and acetone–methanol (30:70, *v*/*v*), collecting fractions of 50 mL each. Fractions were concentrated in a rotary evaporator (Büchi Mod 461, Flawil, Switzerland). Initial characterisation of the fractions was carried out by obtaining the spectra with an infrared spectrophotometer (Spectrum GX. Perkin Elmer, Waltham, MA, USA), reading absorbance in attenuated reflectance, using a Zinc Selenite crystal to place the sample, and with the following specifications: range of lecture of 4,000 to 600 cm^{-1}, with a 1-cm^{-1} interval, and a 4-cm^{-1} resolution.

In order to separate the phenolic from the non-phenolic fraction of the chloroform extract, a liquid–liquid extraction was done. In a separatory funnel, 2 g of the extract were diluted in 40 mL of chloroform and washed three times with 120 mL of 0.1 N sodium hydroxide. The chloroform phase was separated, and once the solvent was evaporated, the crude non-phenolic fraction was obtained. To further purify this fraction, 0.3 g of it were diluted in ethanol and centrifuged at 3,600×*g* at 10 °C for 15 min. Ethanol was evaporated from the supernatant to concentrate the non-phenolic fraction. The basic aqueous phase was acidified with 6N HCl to pH 3.0, and 40 mL of chloroform were added to extract the phenolic fraction. The phenolic fraction was dissolved in chloroform and separated by preparative thin layer chromatography (TLC) on silica (Merck, Mexico City, Mexico) eluting with benzene–methanol 95:5. The phenolic fractions were localised with ultraviolet light and extracted from the silica gel by Soxhlet, using the same solvent as in the TLC.

Antimicrobial Assay

Oregano extracts and their fractions were suspended in ethanol to a concentration of 20,000 or 30,000 ppm; from this stock, they were further diluted in nutrient broth (BIOXON, Mexico City, Mexico) to obtain concentrations of 200 ppm. In the case of the non-phenolic fraction, concentrations of 200 and 300 ppm were used to evaluate how effective the fraction was to inhibit the microorganisms tested. Growth curves were done in nephelometric flasks with 100 mL nutrient broth added with 200 ppm of each fraction and inoculated with a 1% *v*/*v* of microbial fresh culture. Control growth curves contained the equivalent amount of ethanol that was added with the oregano fractions and the inoculum of each microorganisms and were done at the same time as the experimental growth curves. Flasks were incubated in an aerobic chamber (New Brunswick Scientific Mod. Innova 4300, Edison, NJ, USA) with agitation (100 rpm) at 37 °C, and optical density (OD) at 510 nm was measured in a microplate reader (BioRad Mod 550, Tokyo, Japan) every 2 h. Growth curves of the microorganisms in the presence of phenolic fractions were done in microplates, and growth was measured using a microplate reader for turbidity measurements (BioRad Mod. 550, Tokyo, Japan; Kim et al. 1995). All experiments were done by duplicate.

Antioxidant Assay

The assay was done by a modification of the method detailed by Terao and Matsushita (1981). Fractions were suspended in acetone to a final concentration of 0.1% *v*/*v* in 10 mL. To the reaction tubes, 80–83 mg of linoleic acid was added to obtain a final concentration of 0.008% (*v*/*v*). The tubes were incubated at 60 °C for 4 h, taking a sample every 30 min. After incubation, 1 mL ethanol, 1 mL of thiobarbituric acid (TBA) solution (0.04 M TBA in acetic acid, chloroform and 0.3 M sodium sulfite solution in a 12:8:1 *v*/*v* proportion) and 2 mL of trichloroacetic acid (0.28 M in distilled water) were added and tubes were incubated at 96 °C for 30 min. After incubation, 0.5 mL of the reaction tube was diluted with 1 mL acetone, and the OD of the coloured reaction product was measured at 532 nm. As negative control, no fraction was added to the reaction tube. As a positive control, a 0.01% (*w*/*v*) concentration of commercial butylated hydroxytoluene (BHT) was used. For each curve, the slope of the lineal part was calculated, and lower slope values were considered to posses more antioxidant capacity.

Statistical Analysis

Antimicrobial and antioxidant activity were evaluated by analysing the slope values of the lineal portion of the growth or oxidation curve using analysis of variance (ANOVA). Significant values were subjected to mean analysis by least significant difference, using a significance level of 95%. Statistical analyses were performed on Statistica version 5.0 Statsoft, Inc. (2000).

Results and Discussion

Fractions of Mexican Oregano Obtained

The aim of this study was to obtain an extract that contained many polar and non-polar compounds; thus, chloroform was used to obtain the Mexican oregano extract. The initial chloroform extract yielded 8.27% on a dry matter basis. From this initial extract, six different fractions were collected by column chromatography and are identified by Arabic numbers. Fraction 1 was the less polar fraction, and the polarity was increased up to fraction 6 that had the highest polarity. The initial characterisation of all fractions demonstrated that all contained a proportion of phenolic compounds. In order to evaluate if the phenolic compounds were responsible for the biological activity of all column chromatographic (CC) fractions, a separation based on functional structure was done, as described above. Two fractions were initially separated, a phenolic and a non-phenolic fraction. From the phenolic fraction, a further separation was carried by TLC, and eight different fractions were obtained, numbered by consecutive Roman numbers. All fractions were evaluated for antimicrobial and antioxidant properties.

Antimicrobial Effect

Fractions from Mexican oregano obtained by CC were inhibitory to *S. aureus*, as observed in Fig. 1A. Fraction 6 allowed a small growth of the bacteria, but the rest of the fractions inhibited bacterial growth (ANOVA $F=60.71$ $p<0.001$). The effect of all CC fractions on *E. coli* (Fig. 1B) was minimal. Statistical analysis showed differences among all fractions and of all the fractions to the control growth curve (ANOVA $F=577.97$, $p<0.001$). Fractions 2 and 3 were the ones that showed a smaller slope and, therefore, had higher inhibitory capacity. For *B. cereus*, the effect of the CC fractions derived from Mexican oregano was also highly statistically significant (ANOVA $F=727$, $p<0.001$). The control curve was different from fractions; although all fractions inhibited bacterial growth, fractions 3, 5 and 6 showed the strongest antimicrobial activity (Fig. 1C). There was a differential effect of each fraction against the microorganisms tested; *S. aureus* and *B. cereus* were more susceptible than *E. coli,* in accordance with reports on the preferential effect of phenolics found in spices against

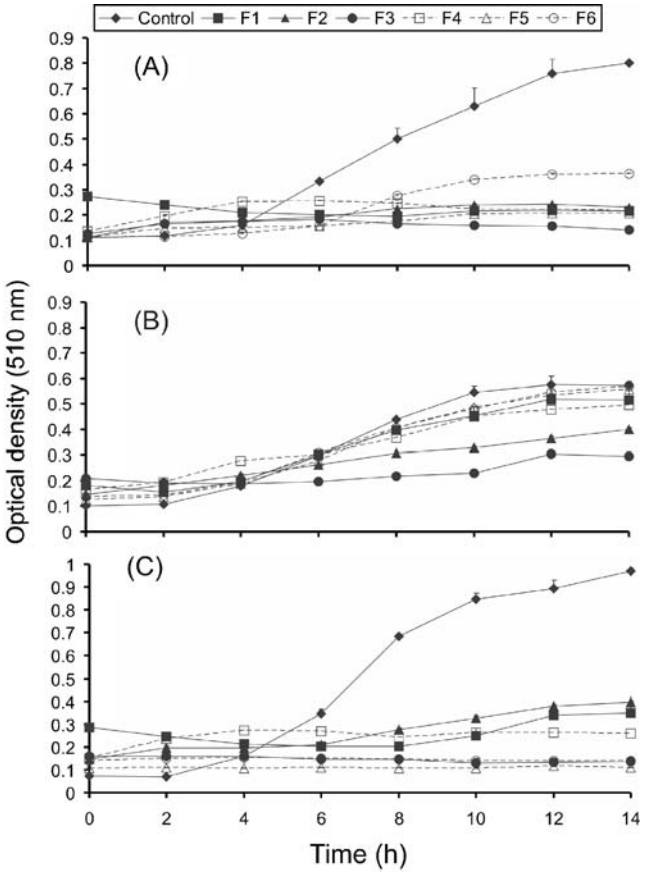

Fig. 1 Growth curves of (**A**) *S. aureus*, (**B**) *E. coli* and (**C**) *B. cereus* in the presence of Mexican oregano fractions obtained by column chromatography. Data are the average + std. of two replicates. Significance level used in statistical analysis was 0.05

Gram-positive bacteria (Mejlhom and Dalgaard 2002). Of all the fractions, fraction 3 showed the highest antimicrobial effect and had a characteristic oregano odour due to the presence of thymol and carvacrol. On the other hand, fraction 5 also showed an important antimicrobial effect but had no oregano odour. Lambert et al. (2001) found a limited application of oregano essential oil as food preservative, due to its strong odour; therefore, fraction 5 could be an alternative to oregano essential oil as a food additive.

Antimicrobial activities of Mexican oregano's phenolic fractions obtained by TLC against *S. aureus* are shown in Fig. 2A. At the end of the incubation time, fraction VII showed a poor antimicrobial effect. The rest of the fractions were antimicrobial, especially those with intermediate polarity (fractions III, IV and V) which had the highest antimicrobial effect (ANOVA $F=1157.655$ $p<0.001$). Figure 2B shows the effect of the phenolic fractions of Mexican oregano versus *E. coli*, and it can be observed that as polarity of the fractions increases (from fraction I to fraction VIII), the antimicrobial effect diminishes. Fractions VII and VIII did not even present a lag phase. Statistical analysis (ANOVA $F=423.85$ $p<0.001$) shows that fraction VI has no antimicrobial effect, while fractions I, II, III and IV have a moderate antimicrobial activity. The antimicrobial effect of the phenolic fractions against *B. cereus* (Fig. 2C) is significant, and as with *S. aureus*, it diminishes as the polarity increases (ANOVA $F=86.62$, $p<0.001$). Ultee et al. (2002) suggested a mechanism of action of phenolic compounds to *B. cereus* and proposed that for carvacrol, the hydroxyl group present, causes cellular damage in an irreversible way.

To determine if non-phenolic compounds might have antimicrobial effect, the non-phenolic fraction was evaluated, and in order to remove any possible interference from waxy material, it was cool-centrifuged (3,600×*g* at 10 °C for 15 min). At a concentration of 200 ppm, the non-phenolic fraction was slightly effective vs. *E. coli*, was not

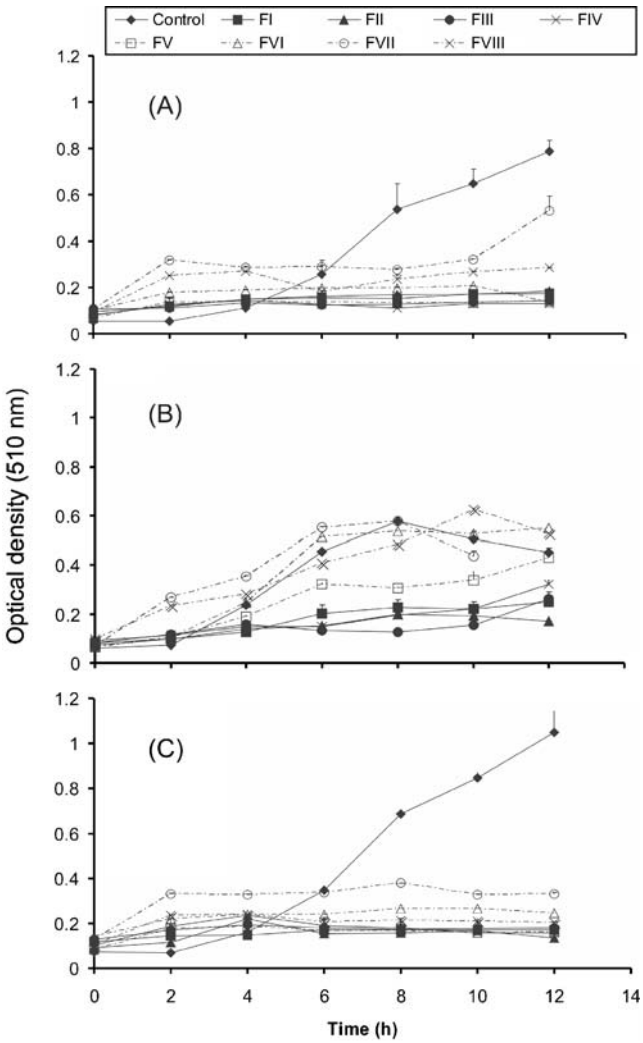

Fig. 2 Growth curves of (**A**) *S. aureus*, (**B**) *E. coli* and (**C**) *B. cereus* in the presence of Mexican oregano fractions obtained by TLC of the phenolic fraction. Data are the average + std. of two replicates. Significance level used in statistical analysis was 0.05

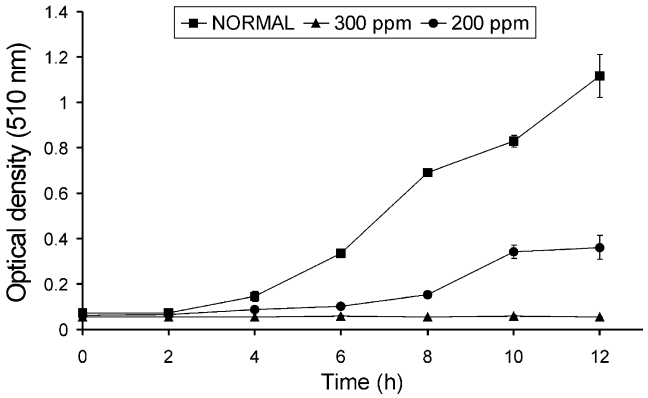

Fig. 3 Growth curves of *B. cereus* Control (normal) and with centrifuged non-phenolic fraction of Mexican oregano at 200 and 300 ppm. Data are the average + std of two replicates. Significance level used in statistical analysis was 0.05

inhibitory to *S. aureus* (data not shown) but had antimicrobial effect against *B. cereus*. Therefore, the cool-centrifuged non-phenolic fraction was further studied with *B. cereus* (Fig. 3). When added at 200 ppm, it allowed a slight growth of *B. cereus*, reaching a stationary phase after 10 h of incubation. When the fraction was added at 300 ppm, bacterial growth was inhibited (ANOVA F=420.30 $p<$ 0.001). The differential effect of the non-phenolic fraction of oregano against *B. cereus* deserves further studies in order to identify the compounds present in this fraction, as well as to identify their mechanisms of action. Some studies show the relative activity of plant oils and extracts by comparing results from different oils tested against the same organism. According to Hammer et al. (1999), some authors concentrate exclusively on one essential oil or one microorganism. While these data are useful, the reports are not directly comparable due to methodological differences such as choice of plant extract(s), test microorganism(s) and antimicrobial test method. In the present study, the antimicrobial effect of fractions with different polarity, as well as phenolic or non-phenolic fractions, was differentially inhibitory versus *E. coli, S. aureus* and *B. cereus.*

Table 1 Average slopes of oxidation curves of linoleic acid, in the presence of BHT or each of the fractions of Mexican oregano obtained by column chromatography

Fraction	Slope
Negative control	0.912±0.020[a]
F1	0.617±0.018[b]
F2	0.627±0.111[b]
F3	0.494±0.031[c]
F4	0.284±0.039[d]
F5	0.188±0.006[d]
F6	0.192±0.001[d]
BHT (positive control)	0.0003±0.004[e]

Values are the average of two replicates. Different letters represent different groups within the column. Significance level used in statistical analysis was 0.05

Table 2 Average slopes of oxidation curves of linoleic acid, in the presence of BHT or each of the phenolic fractions of Mexican oregano obtained by TLC

Fraction	Slope
Negative control	0.854±0.028[a]
FI	0.388±0.021[d]
FII	0.338±0.020[d]
FIII	0.527±0.085[b]
FIV	0.363±0.010[d]
FV	0.418±0.029[d]
FVI	0.448±0.013[cd]
FVII	0.493±0.014[bc]
FVIII	0.370±0.010[d]
BHT (positive control)	0.012±0.006[e]

Values are the average of two replicates. Different letters represents different groups within the column. Significance level used in statistical analysis was 0.05

Antioxidant Effect

When the antioxidant effect of the CC fractions was evaluated, it was demonstrated that as the polarity of the fraction

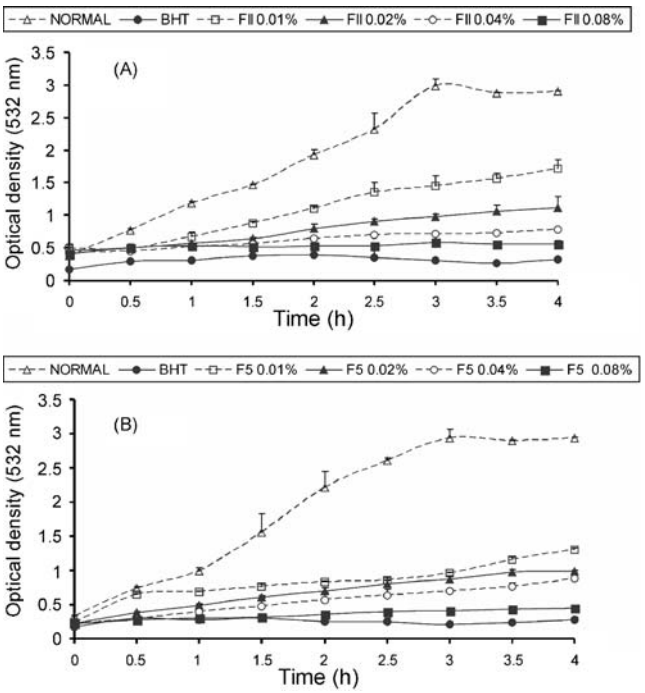

Fig. 4 Linoleic acid oxidation curves of fractions obtained from Mexican oregano at different concentrations, and comparison with a positive control (BHT 0.01%). (**A**) Fraction 5 obtained from a chloroform extract by column chromatography and (**B**) Fraction II obtained from a crude phenolic fraction by thin layer chromatography. Data are the average + std of two replicates. Significance level used in statistical analysis was 0.05

increased, so did the antioxidant capacity (Table 1). Fractions 4, 5 and 6 showed high antioxidant activity, although it was statistically lower than the positive control (BHT) (ANOVA $F=91.91$, $p<0.001$). The antioxidant activity of the TLC-separated phenolic fractions is shown in Table 2, and it can be observed that all fractions had a moderate antioxidant activity. Fractions I, II, IV, V, and VIII were similar in their antioxidant capacity (Table 2) but were all different from the positive control (BHT; ANOVA $F=82.60$, $p<0.001$). Of all fractions, FII was the one with smaller slope value and, therefore, the one with more antioxidant capacity.

In order to evaluate the effect of concentration on the antioxidant activity of those fractions that were the best candidates, increasing concentrations (0.01–0.08%) of CC fraction 5 (F5) and TLC phenolic fraction II (FII) were tested. Results are shown in Fig. 4 and demonstrate that as the concentration increased, the slope of the linoleic acid oxidation curves decreased, which demonstrates an increment in antioxidant activity. For F5, the higher concentration tested (0.08%) was less antioxidant than the positive control (BHT at 0.01%), so a larger concentration will be needed form this fraction to obtain the equivalent antioxidant activity of BHT at 0.01% (Fig. 4B). On the other hand, the 0.08% concentration of FII was not different from the positive control (BHT). Therefore, a 0.08% concentration of FII is equivalent to 0.01% concentration of BHT (Fig. 4A).

There are reports of the presence of antioxidant molecules different to carvacrol and thymol in oregano, such as the ones reported by Nakatani and Kikuzaki (1987) who obtained a glucoside from a water-soluble oregano extract with a similar activity as BHT. On the other hand, Kikuzaki and Nakatani (1989) obtained different phenolic compounds that demonstrated a similar activity as BHT (0.01%) at a 0.002% concentration. On the other hand, Viekari et al. (1993) isolated different phenolic fractions from an ethanolic extract of European oregano, with antioxidant activities similar to BHA and BHT at a 0.02% concentration. Singh et al. (2005) worked on the antioxidant capacity of essential oils as well as acetone extracts of several spices, and it was comparable to that of mustard oil. Martinez-Rocha et al. (2008) demonstrated the presence of rosmarinic acid in Mexican oregano, as well as other polar bioactive antioxidant compounds. These reports demonstrate the variety of antioxidant compounds present in oregano and the reason why it is considered one of the best antioxidant spices.

Conclusion

A number of extracts and fractions with different polarity and/or structural properties were obtained from Mexican oregano (*L. berlandieri* Schauer) and demonstrated to have antimicrobial and antioxidant capacity, including some with no strong oregano odour. The characterisation of antimicrobial and antioxidant compounds in Mexican oregano could lead to a wider use of the spice in the food industry as well as in other applications.

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