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Phenolic antioxidants from clonal oregano (*Origanum vulgare*) with antimicrobial activity against *Helicobacter pylori*

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Abstract

Phenolic phytochemicals consumed via our diet are good sources of natural antioxidants. These phenolic metabolites have beneficial effects on human health, including inhibition of mutagenesis and carcinogenesis. In addition some phenolic phytochemicals have also been shown to have antimicrobial and antifungal activity. Oregano (*Origanum vulgare* L.) is an important Mediterranean herb rich in phenolic compounds with antioxidant and antimicrobial activity. Using tissue culture techniques several high phenolics and rosmarinic acid-containing oregano clonal lines have been isolated with high antioxidant activity with antimicrobial potential. In this investigation the antioxidant activity of phenolic-enriched clonal oregano extracts, and their antimicrobial activity against ulcer-associated *Helicobacter pylori* have been evaluated. In all cases clonal extracts were compared to commercial oregano from heterogeneous sources. Total phenolics were the highest in 60% ethanol extracts of clonal oregano. Antioxidant activity based on 1,1-diphenyl-2-picrylhydrazyl-2-radical (DPPH) scavenging activity depended on the physiochemical nature of phenolic phytochemical and percentage inhibition activity based on 3-ethyl benzthiazoline-6-sulphonic acid ($ABTS^+$) assay correlated to the amount of total phenolics. Thiobarbutyric acid reactive substances (TBARS) and antioxidant protection factor (APF) method using β-carotene linoleic acid system were correlated to lipophilic phenolic phytochemicals. Antimicrobial activity against *H. pylori* was tested by the standard agar diffusion method. Phenolic profiles in oregano extracts were analyzed using HPLC. The differences in physico-chemical properties of phenolic acids consisting of C_6-C_1-COOH, and C_6-C_3-COOH structures were hypothesized to play a role in growth inhibition of *H. pylori*.

Keywords: Clonal oregano extracts; Antimicrobial activity; Antioxidant activity; Phenolic phytochemicals; Phenolic acids; *Helicobacter pylori*

1. Introduction

Antioxidants can be defined as compounds that can delay or prevent the oxidation of lipids or other molecules by inhibiting the initiation or propagation of an oxidizing chain reaction [1]. These dietary phenolic antioxidants have been shown to play important roles in delaying the development of chronic diseases such as cardiovascular diseases (CVD), cancer, inflammatory bowel syndrome and Alzheimer's diseases [2,13]. Phenolic antioxidants are products of secondary metabolism in plants and are good sources of natural antioxidants in human diets. Due to the carcinogenic potential of synthetic forms natural phenolic antioxidants are also being targeted as alternatives to minimize or retard oxidative deterioration in food and to improve the health-related functional value of the food [3,13]. Aromatic plants such as herbs and spices are especially rich in their phenolic content, and have been widely used to extend the shelf life of foods [3,4] and in traditional medicine as treatment for many diseases [6,13]. The antioxidant activity of phenolic compounds in plants is mainly due to their redox properties and chemical structure, which can play an important role in neutralizing free radicals, chelating transitional metals and quenching singlet and triplet oxygen, by delocalization or decomposing peroxides [1,5]. These properties are linked to beneficial health functionality of phenolic antioxidants due to their inhibitory effects against development of many oxidative-stress related diseases such as CVD, cancer and diabetes [6]. In addition some plant phenolics have shown antimicrobial and antifungal effects [6] and therefore current research is now focused on identifying antimicrobial activity against gastric ulcers, urinary tract infection and dental

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caries causing microorganisms using diets rich in phenolic phytochemicals.

Due to their naturally high phenolic antioxidant content, the use of extracts from the Lamiaceae family of herbs that includes herbs such as oregano, rosemary, thyme and spearmint are relevant for antioxidant applications. Among these oregano is an important plant widely used in South European cuisine and is a very popular herb in the Mediterranean countries. The leaves, dried herbs as well as the volatile oil of this aromatic perennial herb have been used medicinally for centuries. The positive effects of oregano on human health have now been attributed to its antioxidant activity both in the essential oil and soluble phenolic fractions [7,9,10]. Kikuzaki and Nakatani [11] isolated five different phenolic compounds from the methanol extract of leaves of oregano and among these rosmarinic acid was found to be present in highest concentrations. Rosmarinic acid is a caffeoyl ester and has now been shown to be an important antioxidant and anti-inflammatory compound [9,10].

Oregano is a genetically heterogeneous species due to natural cross pollination. This heterogeneity results in large variation in phenolic content [12], which limits its use as an ingredient in functional foods. Tissue culture-based clonal propagation to select elite phenolic metabolite-producing phenotypes is a rapid and efficient alternative strategy. The genetic uniformity obtained from clonal selection is essential for maintaining ingredient quality, quantity [13] and maintain phytochmical profile consistency. Therefore, tissue culture-generated high phenolic clonal lines have been targeted after field cultivation for evaluation of antioxidant and anti-*Helicobacter pylori* activity. In this paper we report comparisons of ethanol and water soluble phenolics of clonal oregano and heterogeneous oregano from commercial sources. We have also characterized total phenolics, antioxidant activity and phenolic profiles using HPLC. Further we analyzed the antimicrobial activity of same extracts against gastric ulcer-associated bacterium *H. pylori*.

2. Materials and methods

2.1. Oregano

Dried powder of the O-1 clonal line developed at the University of Massachusetts and a commercial heterogenous powder mixture purchased from Bread and Circus (Hadley, MA) were used in this study.

2.2. Oregano extracts

Dry oregano powder was extracted with varying concentrations (0–95%) of ethanol. Among various extracts the highest phenolic containing extracts were chosen for further analysis and antimicrobial activity.

2.3. Water extracts

One gram of dry oregano powder was added to 200 ml of distilled water and boiled until the volume was reduced to 100 ml. The extract was then stirred at room temperature for 24 h. Water soluble extracts were obtained following centrifugation at $10,000 \times g$ for 14 min.

2.4. Ethanol extracts (10–95%)

One gram of dry oregano powder were stirred each in 100 ml of various concentrations of ethanol at room temperature for 24 h. The ethanol soluble extracts were obtained after centrifugation at $10,000 \times g$ for 14 min.

2.5. Total phenolic assay

Total phenolics were determined according to the modified method adapted by Shetty et al. [14]. One milliliters of oregano clonal line extracts and heterogeneous extracts were added into test tubes and mixed with 1 ml 95% ethanol, 5 ml water and than 0.5 ml 1N Folin–Ciocalteu reagent was added. After 5 min, 1 ml 5% Na_2CO_3 was added and the reaction mixture was allowed to stand for 60 min before the absorbance at 725 nm was measured. A standard curve was established for each assay using 50–500 μg of gallic acid in 95% ethanol.

2.6. Antioxidant assay with 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical

DPPH is a free radical which when dissolved in ethanol has a blue-violet colour. The loss of colour indicates radical scavenging activity and the objective is to inhibit the loss of colour with oregano extracts. DPPH was measured by modified method of Cervato et al. [8]. Three milliliters 60 μM DPPH in ethanol was added to 1 ml oregano water extracts or 60% ethanol extracts and then incubated at room temperature for 15 min. Absorbance was read at 517 nm using a Genesys 5 spectrophotometer (Spectronic Instruments, Rochester, NY).

The antioxidant activity was calculated as inhibition (%) of DPPH radical formation:

$$\text{inhibition (\%)} = \left(\frac{A_{517}^{\text{control}} - A_{517}^{\text{extract}}}{A_{517}^{\text{control}}} \right) \times 100$$

2.7. Antioxidant protection factor (PF)

Antioxidant protection factor was evaluated using an assay described by Andarwulan and Shetty [15]. One milliliter 200 μg/ml of β-carotene in chloroform was transferred into a round-bottomed flask and chloroform evaporated using a rotary evaporator under vacuum at 40 °C for 5 min. The β-carotene adhered to the sides of the flask was scraped off and dissolved with 20 μl purified linoleic acid and 184 μl

Tween 40 emulsifier. To this, 50 ml 50 mM H_2O_2 was added and shaken vigorously until a uniform emulsion was obtained. Aliquots (5 ml) of this emulsion were transferred to test tubes containing 100 μl phenolic extract from the oregano clonal line or the commercial heterogeneous mixture. The samples were vortexed for 1 min and incubated at 50 °C for 30 min. Subsequently, absorbance readings were recorded at 470 nm and compared to a control which had 100 μl ethanol in place of the extract. The antioxidant activity was expressed as protection factor and was calculated as follows:

$$\text{antioxidant protection factor (APF)} = \left(\frac{A_{470}^{\text{sample}}}{A_{470}^{\text{control}}}\right)$$

2.8. ABTS cation radical and antioxidant activity

The total antioxidant activity of dry oregano powder extract was measured by the $ABTS^+$ radical cation-decolorization assay involving preformed $ABTS^+$ radical cation [16]. $ABTS^+$ was prepared by reacting 5 ml of 7 mM ABTS (Sigma Chemical Co.) water solution with 88 μl of 140 mM potassium persulphate (ratio 1:0.35) and the mixture allowed to stand in the dark at room temperature for 12–16 h before use. Prior to assay $ABTS^+$ stock solution was diluted with ethanol (ratio 1:88) to give an absorbance at 734 nm of 0.70 ± 0.02 and was equilibrated to 30 °C. One milliliter ABTS was added to glass test tubes containing 50 μl of each extract and tubes were mixed by vortex mixer for 30 s. Tubes were incubated for 2.5 min and then read at 734 nm. The Trolox reference standard for relative antioxidant activities was prepared with 5 mM stock solution of Trolox in ethanol for introduction into the assay system at concentrations within the activity range of the assay (0–20 μM final concentration) for preparing a standard curve to which all data were referred. The percentage inhibition was calculated by:

$$\text{inhibition (\%)} = \left(\frac{A_{734}^{\text{control}} - A_{734}^{\text{extract}}}{A_{734}^{\text{control}}}\right) \times 100$$

2.9. Measurement of TBARS

Thiobarbutyric acid reactive substances (TBARS) were determined using a method adapted from McDonald and Hultin [17]. Emulsions were prepared by homogenizing 1% linoleic acid and 1% Tween 40 in 100 ml of distilled water. 0.8 ml of emulsions was added to glass tubes containing 0.2 ml of each extracts, and tubes were incubated at 50 °C for 10 h. One milliliter of mixtures was added to 2.0 ml TBA reagent (100 ml of Stock TCA–TBA–HCl solution mixed with 15% TCA, 0.375% TBA and 0.25 M HCl + 3 ml of 2% BHT in ethanol), vortexed and heated in a boiling water bath for 15 min. After cooling with tap water for 10 min, the solution was centrifuged for 15 min at 2000 × *g*. After 10 min, the absorbance was measured at 532 nm. TBARS was calculated from a standard curve prepared using 1,1,3,3-tetraethoxypropane.

2.10. HPLC analysis of phenolics [18,19]

Two milliliters oregano extracts was filtered through a 0.2 μm filter. Five microliters sample was injected using Agilent ALS 1100 autosampler into an Agilent 1100 series HPLC system equipped with DAD 1100 Photodiode array detector. The solvents used for gradient elution were [A] 10 mM phosphoric acid (pH 2.5) and [B] methanol. The methanol concentration was increased to 60% for the first 8 min and to 100% for 7 min, then decreased to 0% for 3 min and maintained for the next 7 min (total run time, 25 min). The analytical column used was Agilent Zorbax SB-C18, 250 mm × 4.6 mm i.d., with packing material of 5 μm particle size at a flow rate of 1 ml/min at ambient temperature. During each run the chromatogram was recorded at 306 and 333 nm and integrated using an Agilent Chemstation enhanced intergrator. Pure protocatechuic acid, caffeic acid, coumaric acid, rosmarinic acid and quercetin in 100% methanol were used to calibrate the standard curve and retention times.

2.11. Antimicrobial assay for Helicobacter pylori

H. pylori was cultured using the method of Stevenson et al. [20]. Isolates of *H. pylori* (strains ATCC 43504, originated from human gastric samples) were obtained from the American Type Culture Collection (Rockville, MD). *H. pylori* standard plating agar (HPSPA) was prepared using 10 g special peptone (Oxoid Ltd., Basingstoke, England), 15 g granulated agar (Difco Laboratories, Detroit, MI), 5 g sodium chloride (EM Science, Gibbstown, NJ), 5 g yeast extract (Difco Laboratories, Detroit, MI), and 5 g beef extract (Becton Dickinson and Co., St. Louis, MO) per litre to water. Antimicrobial activity against *H. pylori* was tested by the standard agar diffusion method. Broth media were prepared by using 10 g special peptone (Oxoid Ltd., Basingstoke, England), 5 g sodium chloride (EM Science, Gibbstown, NJ) per litre, 5 g yeast extract (Difco Laboratories, Detroit, MI), 5 g beef extract (Becton Dickinson and Co., St. Louis, MO) per litre of water. Hundred microliters stock *H. pylori* was added into test tubes contain 10 ml of broth media and incubated at 37 °C for 2–3 days. Sterile filter paper disks (5.5 mm), each containing 50, 100, 150 and 200 μg total phenolic content of oregano extracts were applied to the surface of HPSPA plates which were previously seeded with the test *H. pylori* from a 2–3 day culture. The plates were incubated at 37 °C for 3 days in Gas Pak jars (BBL Microbiology systems, Cockeysville, MD) with catalyst and Campy Paks (BBL) [21], and the net zone of inhibition was determined.

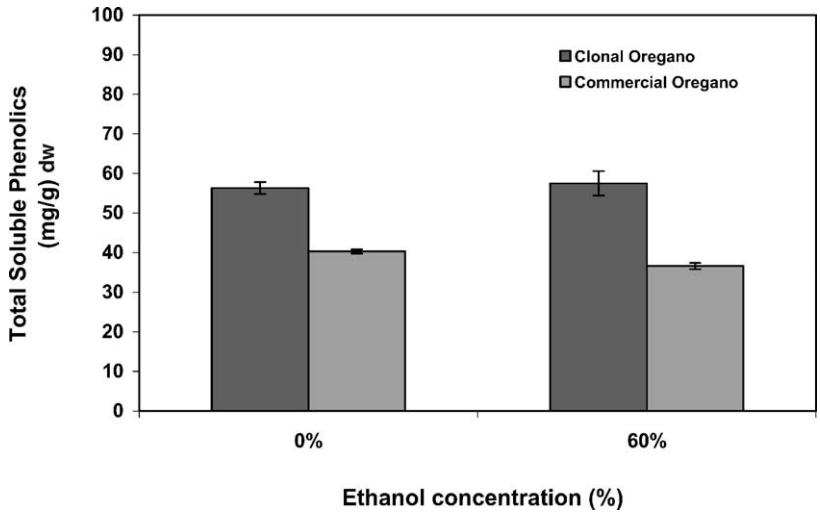

Fig. 1. Total soluble phenolics in water and ethanol extracts of clonal and commercial oregano samples.

3. Results and Discussion

3.1. Total phenolics

The total phenolic content in water extracts and all the ethanol extracts at different ethanol concentration was analyzed by the Folin–Ciocalteu method. The highest phenolic contents were found in water and 60% ethanol extracts of both clonal and commercial oregano. Therefore, these extracts were used for further antioxidant and antimicrobial studies. The results of clonal oregano were compared with the commercial heterogeneous oregano. In water extracts of the clonal oregano the total phenolic content was 52.8 mg/g dry weight (dw) compared to 39.4 mg/g dw in the commercial sample (Fig. 1). The ethanol extracts of clonal oregano had the slightly higher total phenolic content (55.35 mg/g dw) compared to the water extracts. For the commercial oregano sample, the total phenolic content in the ethanol extract was 35.43 mg/g dw which was lower than the water extract (Fig. 1). For both types of extractions the total phenolic content of the oregano clonal line was significantly higher than the commercial sample. The extraction process in this investigation used higher temperatures for extractions and therefore resulted in higher phenolic content for oregano clonal line than previously reported [22].

3.2. Antioxidant activity

The antioxidant activity reflected by the DPPH radical scavenging assay were similar in water and 60% ethanol extracts, even though total phenolic content in both the extracts was different (Fig. 2). The percent inhibition of DPPH was from 80 to 82%. This suggests that the physico-chemical nature of the individual phenolics in the extracts may be more important in contributing to the antioxidant activity than the total phenolics content measured by the Folin–Ciocalteu assay. Further, it could also indicate that a critical concentration of phenolics is sufficient to obtain the desired

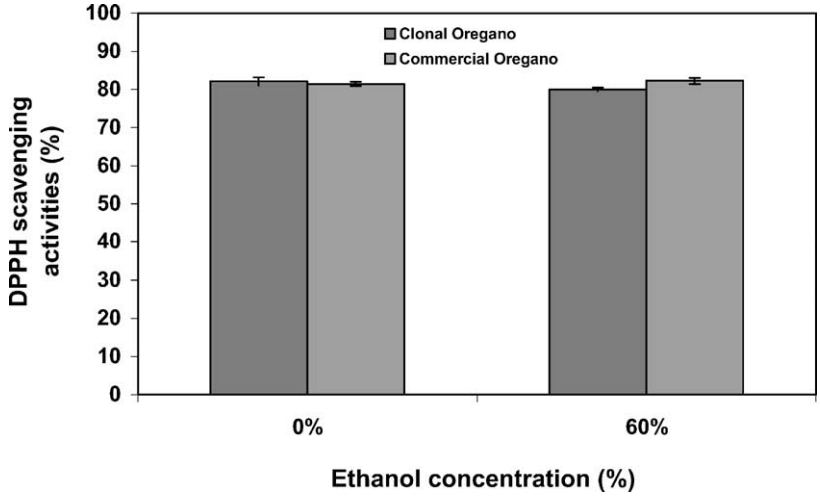

Fig. 2. DPPH radical inhibition activity of water and ethanol extracts of clonal and commercial oregano samples.

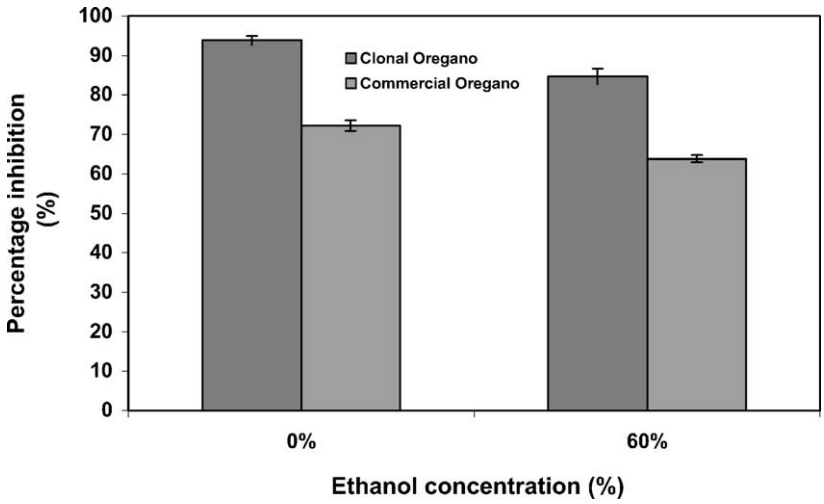

Fig. 3. ABTS radical inhibition by water and ethanol extracts of clonal and commercial oregano samples at 49.4 μg/ml of soluble phenolic content.

antioxidant activity after which there is a saturation effect and the presence of additional phenolics does not increase the antioxidant activity. The differences in antioxidant activity in a particular assay are largely a function of the ratio of hydrophilic and hydrophobic nature of phenolics. DPPH assay essentially measures the antioxidant activity of the water soluble phenolics and it is possible that the amount of hydrophilic phenolics having antioxidant activity in the DPPH assay using clonal oregano and commercial samples may be similar. In order to clarify these possibilities additional antioxidant assays were used to find the differences in phenolic profile-related antioxidant activity of these two oregano samples. In the $ABTS^{+}$ radical cation-decolorization assay [16,23] extracts containing 50 μg phenolics/ml were compared. The percentage inhibition of $ABTS^{+}$ for both the oregano samples was higher in water extracts than ethanol extracts (Fig. 3) suggesting the sensitivity of the assay towards water soluble antioxidants. When the clonal extracts were compared to the commercial extracts of oregano, both water and ethanol extracts of the clonal oregano had higher activity, indicating differences in the physio-chemical properties of the phenolics in the two samples (Fig. 3). In the β-carotene assay, though there was slightly higher activity in the clonal extracts than the commercial extracts (Fig. 4), for both the clonal and the commercial oregano samples the antioxidant protection factor of the water and ethanol extracts was similar (Fig. 4). Higher antioxidant protection activity of the clonal extracts in the β-carotene assay may suggest the possible higher levels of lipophilic phenolic antioxidants in them compared to the commercial samples. This was substantiated by the measurement of TBARS value. The TBARS value for clonal extracts were generally lower than commercial extracts (Fig. 5). Further ethanol extracts had lower TBARS value than water extracts. β-carotene assay and TBARS measure the ability of the antioxidant in preventing the oxidative deterioration of lipids and fatty acids. Therefore the higher antioxidant activity of the phenolic antioxidants from the clonal oregano in these

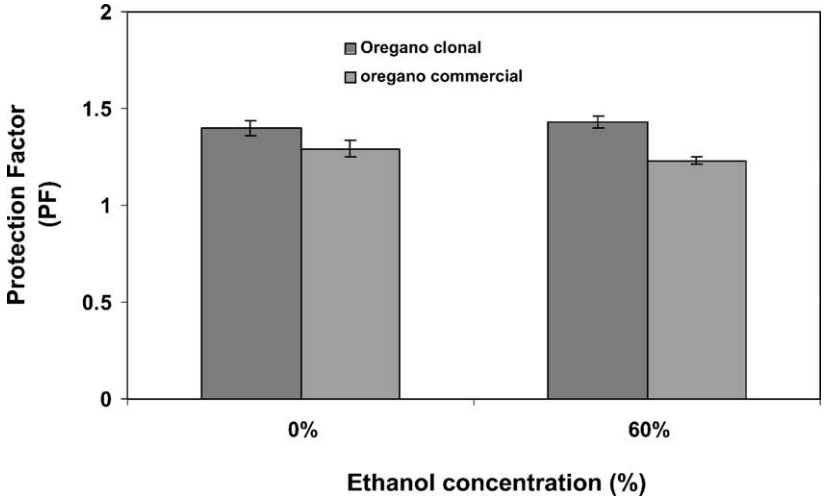

Fig. 4. Antioxidant protection factor of water and ethanol extracts of clonal and commercial oregano samples measured by β-carotene linoleic acid assay.

Table 1
Individual phenolic compounds analyzed by HPLC in water and 60% ethanol extracts of clonal and commercial oregano samples

Phenolics (mg/g dw)	Oregano			
	Colonal		Commercial	
	Water	60% ethanol	Water	60% ethanol
Protocatechuic acid	1.11 ± 0.79	0.25 ± 0.18	0.43 ± 0.35	N.D.
Caffeic acid	1.41 ± 0.16	1.51 ± 0.26	0.84 ± 0.03	0.66 ± 0.02
Coumaric acid	2.57 ± 0.06	3.91 ± 0.59	0.95 ± 0.06	0.96 ± 0.10
Rosmarinic acid	6.95 ± 0.26	8.90 ± 0.43	4.83 ± 0.14	5.92 ± 0.15
Quercetin	1.04 ± 0.02	3.24 ± 0.45	1.99 ± 0.87	1.11 ± 0.33
Total	13.08	17.81	9.04	8.65

N.D.: not detected.

two assays suggest a possible biological functionality in preventing the oxidative degradation of membrane lipids.

3.3. HPLC analysis of extracts

HPLC analysis was carried out to identify the phenolic profiles in clonal and commercial oregano extracts. Five major phenolic metabolites were found and these were; rosmarinic acid, caffeic acid, coumaric acid, protocatechuic acid and quercetin (Table 1). In general, clonal extracts had a higher content of these phenolics than commercial samples in both water and ethanol extracts. In both the clonal and the commercial oregano samples rosmarinic acid was the major phenolic. In the clonal oregano, rosmarinic acid content was 8.9 and 6.95 mg/g dw in 60% ethanol and water extracts respectively. In commercial extracts the rosmarinic acid content was 4.83 mg/g dw for the water extract and 5.92 mg/g dw for the ethanol extract. The phenolic metabolite found at next highest concentration was coumaric acid. The highest content of coumaric acid was observed in ethanol extracts of the clonal line at a level of 3.91 mg/g dw compared to 2.57 mg/g dw in water extracts. In commercial extracts coumaric acid was in the range of 0.95 mg/g dw for both water and ethanol extracts. Another major phenolic of significance that was observed in the oregano extracts was quercetin which was found at levels of 3.24 mg/g dw in clonal ethanol extracts. Other phenolics such as protocatechuic acid and caffeic acid were generally found in lower concentrations in the clonal extracts and commercial extracts.

3.4. Anti-Helicobacter pylori activity

H. pylori is associated with gastric ulcers in humans [24]. Current therapies using antibiotics are expensive and have the potential for the development of antibiotic resistance [25]. Therefore, dietary sources have the potential to help manage *H. pylori*. In this research the ability of high phenolic clonal oregano to inhibit the growth of *H. pylori* was investigated and compared to the commercial sample. It was observed that clonal extracts were more effective in inhibiting the growth of *H. pylori* than commercial extracts at the same phenolic concentration. When evaluating the water extracts of clonal oregano 50 μg of phenolic per disk showed highest antimicrobial activity. Increasing the concentration

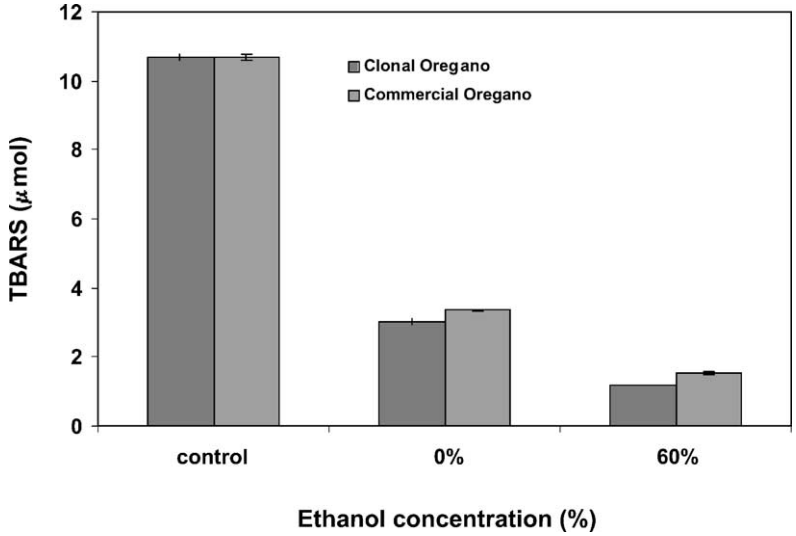

Fig. 5. Amount of TBARS produced in the presence of water and ethanol extracts of clonal and commercial oregano samples.

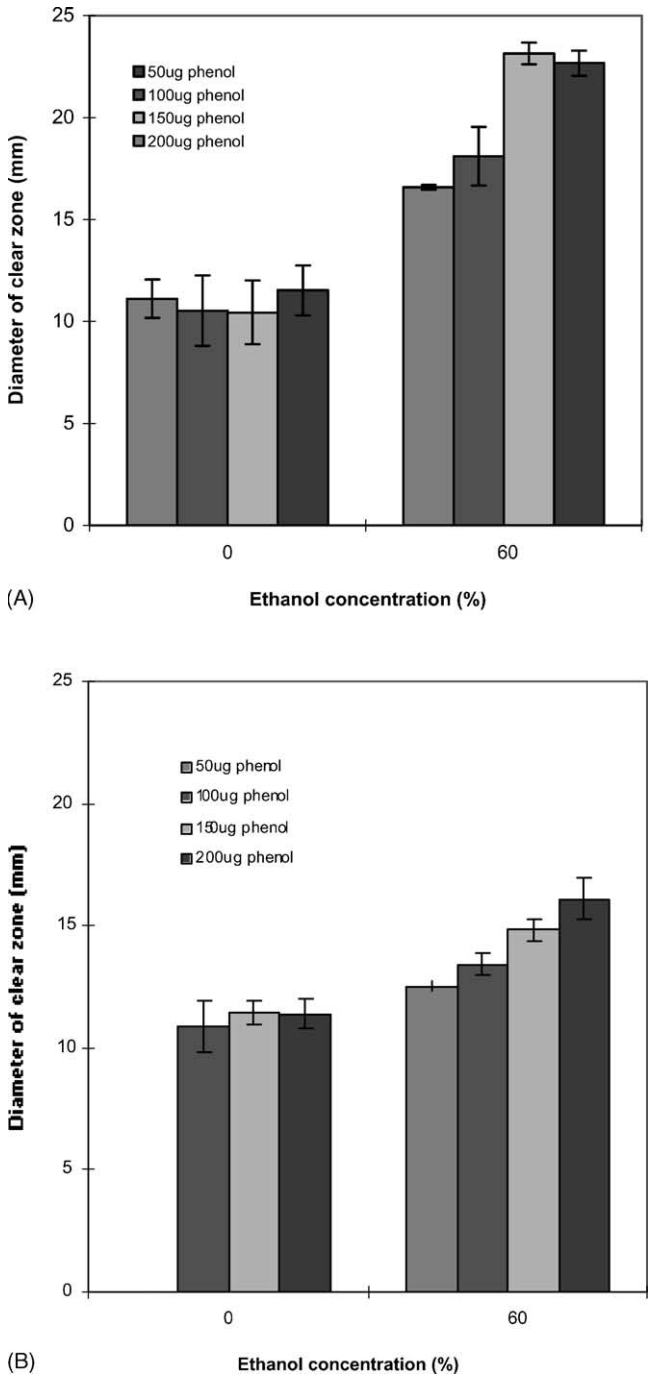

Fig. 6. Antimicrobial activities against *H. pylori* of water and ethanol extracts from (A) clonal oregano and (B) commercial oregano.

to 200 μg of phenolic per disk did not increase the antimicrobial activity. Similar antimicrobial activity in the water extracts was obtained in commercial samples when 100 μg of phenolic per disk or higher was used (Fig. 6). This suggests a lower minimal inhibitory concentration of phenolics from clonal oregano (50 μg of phenolic per disk) compared to the commercial sample (100 μg of phenolic per disk). The ethanol extracts of clonal and commercial oregano had higher activity against *H. pylori* and increased in a dose dependent manner (Fig. 6). The inhibitory activity was much higher for clonal oregano than commercial oregano. This result indicates that the ethanol soluble phenolics of clonal oregano had the most potent anti-*Helicobacter* activity. These differences could potentially be due to higher antioxidant activity of the clonal lines assayed by all the different antioxidant assays as well as the higher concentration of major phenolic phytochemicals assayed by HPLC. Higher antioxidant activity based on the β-carotene assay and TBARs indicates good ability to function at the lipid water interface and higher activity in the $ABTS^{+}$ and DPPH assays indicate the presence of higher water soluble phenolic antioxidants. These two types of antioxidants may act in synergy and inhibit the growth of *H. pylori* by various mechanisms such as cytosolic hyperacidity, disruption of electron transport chain, membrane destabilization, disruption of membrane transport, inhibition of H^{+}-ATPase, ion channels, and inhibit bacterial metabolism [26].

4. Conclusions

An elite clonal line of oregano with high phenolic content, antioxidant activity and consistent phenolic profile was identified and characterised. The high phenolic and antioxidant activity correlated well with high antimicrobial activity against ulcer-associated *H. pylori*. In general the clonal oregano had a much higher total phenolic, antioxidant activity and antimicrobial activity against *H. pylori* compared to the commercial heterogeneous oregano extracts. The identification of an elite phenolic clonal line will allow the development of dietary phenolic ingredients whose antioxidant potential could enhance protective host defence responses in humans (2) and at the same time inhibit prokaryotic pathogenic bacteria like *H. pylori* linked to gastric infections. This research offers innovative strategies to develop functional foods and supplements against chronic bacterial infections.

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