Original Article

Inhibitory effect of clonal oregano extracts against porcine pancreatic amylase *in vitro*

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Oregano (*Origanum vulgare*) is a rich source of natural phenolic antioxidants and has potential to be a source of nutritional ingredients for functional foods. Herbs such as oregano have long been used in food preservation and in traditional medicine in the treatment of common ailments and have potential for positive modulation of oxidation-linked diseases such as diabetes. One of the potentially important components of anti-diabetic activity by oregano extract is mild amylase inhibition by phenolic antioxidants to help contribute towards management of hyperglycemia. Previously, we reported the ability of rosmarinic acid, one of the principal phenolic components of oregano, to inhibit porcine pancreatic amylase (PPA) activity. Here, we investigated the effect of 50% ethanol extracts of eleven phenolic antioxidant-rich oregano clonal lines on the activity of PPA *in vitro*. To this end, we analyzed extract total soluble phenolic content by the Folin-Ciocalteu reagent method, rosmarinic acid (RA), protochatechuic acid (PA), quercetin, and *p*-coumaric acid (pCA) contents by HPLC, antioxidant activity as 1,1-diphenyl-2-picryl-hydrazyl (DPPH) radical scavenging, and PPA-inhibitory activity by incubation of the enzyme with clonal oregano extracts and characterization of the activity of the phenolic-bound enzyme. Clonal oregano extracts inhibited the activity of PPA *in vitro* by 9-57%. Amylase inhibition by oregano extract was associated with extract total phenolic content and RA, quercetin, PA, and pCA content, as well as extract antioxidant activity and protein content. Our finding that clonal oregano extracts can inhibit PPA supports a potential new functionality for oregano as a n anti-hyperglycemic agent. This provides an opportunity for a food-based strategy for modulation of starch breakdown to glucose, which could contribute to the management of hyperglycemia and diabetes complications in the long term.

Keywords: amylase inhibitors, oregano, herbal extracts, rosmarinic acid, hyperglycemia, diabetes mellitus, obesity

Introduction

Hyperglycemia is one of the major problematic symptoms associated with Type 2 diabetes mellitus, as well as pre-diabetes impaired glucose tolerance.[1] Elevated postprandial glucose levels and persistent hyperglycemia can lead to cellular damage and is associated with the development of retinal, renal, neurological, and cardiovascular disease.[2, 3]

As oral anti-diabetic therapies that act on the liver risk hepatic dysfunction, much recent attention has been placed on the development of agents to combat hyperglycemia as an anti-diabetic therapy.[4,5] Many anti-hyperglycemia agents currently in use, such as acarbose or metformin, act to inhibit or retard various reactions of glucose metabolism, are synthetic, and may have negative side-effects at high doses.[2,6] A current goal in anti-diabetic research is to identify anti-hyperglycemic agents that are safe and that lack any negative side-effects.

Traditional medicines from various cultures have given us potential prospects to explore the benefits of natural dietary remedies of symptoms associated with Type 2 diabetes mellitus, such as hyperglycemia. Traditional Indian and Chinese medicines have long used plant and herbal extracts as anti-diabetic agents.[7,8] These plants are typically rich in phenolic compounds. Herbs used in traditional Indian medicine, such as Holy Basil (*Ocimum sanctum*) and oregano (*Origanum vulgare*), are high in rosmarinic acid (RA) content.[9]

Recently, we reported the strong inhibitory activity of RA against porcine pancreatic amylase, the enzyme responsible for the breakdown of starch into glucose.[10] In light of this finding, we hypothesized that part of the anti-diabetic effect of RA-containing herb extracts may be due to the anti-amylase activity of RA. In the current study, we screened extracts of 11 clonal lines of oregano for anti-amylase activity. These clonal lines are of single seed origin and were isolated using tissue culture techniques from individual heterozygous seed.[11] Further, the results were compared to measurements of oregano extract, total phenolic and RA content, as well as anti-oxidant (free-radical scavenging) activity.

Methods

Plant materials

Shoots from specific clonal lines of oregano (*Origanum vulgare*) were generated previously from individual heterozygous seedlings following germination of a heterogeneous seed population.[11, 12]

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Shoot culture of oregano

Under aseptic conditions in a laminar flow hood, each existing clonal line was subcultured at room temperature on Petri plates containing Murashige & Skoog basal salt medium supplemented with 1 mg/ mL BAP, 3% sucrose, 1X Nitsch & Nitsch vitamins, and 0.3% gellan gum.[13]

Extraction procedure

One gram of fresh shoot tissue (per clone) was submerged in 5mL of 50% EtOH and incubated for 3d at -20˚C. Next, each sample was diluted with 5mL of 50% EtOH, homogenized, and centrifuged 20 min at 10,000 rpm at 4˚C. The supernatant was used as the crude oregano extract. Extractions using 95% EtOH were performed for HPLC analyses of RA content in each clonal line.

Treatment of α-amylase with clonal oregano extracts

Treatment of α-amylase was performed as previously described, with some modifications.[10] Fifty mg of powdered porcine pancreatic α -amylase (Sigma) was added to 27 mL of dH_2O and adjusted to pH 6.9. For each oregano extract, a volume equivalent to 400 µg total phenolic content was diluted to 3 mL with 50% EtOH, added to the above solution, and readjusted to pH 6.9. Amylase-oregano extract mixtures were incubated 24h at 4˚C with stirring. For comparison studies, 400 µg of synthetic antioxidants BHT and Trolox were in 50% EtOH were used. The control was 50% EtOH.

Characterization of α-amylase activity

-Amylase activity was determined by the method of McCue and Shetty[10], using starch as a substrate in a colorimetric reaction using 3,5-dinitrosalicylic acid. A standard curve was generated for the splitting products (reducing groups) using D-(+)-maltose monohydrate.[10] Activity was calculated as units/ mg protein, where 1 unit was defined as the amount of enzyme required to liberate 1 µmol of maltose under assay conditions. Protein content was determined using the Bio-Rad protein assay kit. Data was reported as amylase inhibition (AI) index values, defined herein as the ratio of the amylase activity of the control (enzyme alone) to that of the enzyme/ clonal extract mixture.[14] Values greater than 1 indicate α-amylase inhibition.

Total soluble phenolic content assay

The total soluble phenolics content in each extract was determined using a previously described method.[13] A phenolic standard curve was established at 725nm with (+)-catechin (25-200 µg/ mL) of in 95% EtOH.

Phenolic content determination by HPLC

One mL of each 95% EtOH clonal oregano extract adjusted to 200 µg total phenolic content per mL was passed through a 0.45 µm filter. HPLC analysis was performed using an Agilent 1100 series system equipped with an autosampler, a variable wavelength diode array detector (set at 333nm for RA; 306nm for all other phenolics), and a Zorbax SB-C18 column. The injection volume was 5 µL and flow rate 1mL/min. The solvents used were 10 mM phosphoric acid, pH 2.5, and MeOH. The MeOH concentration was increased to 60% for 8 min, to 100% for 7 min, then to 0% for 3 min, and maintained for another 7 min. Pure RA, PA, quercetin, and pCA was used for a standard curve and for sample spiking.

Antioxidant activity

Antioxidant activity was determined as percent scavenging of 1,1-diphenyl-2-picryl-hydrazyl (DPPH) radicals.[15] A volume equivalent to 200 µg total phenolic content for each extract was diluted to 1mL with 50% EtOH and mixed with 1mL of 0.1 mM ethanolic DPPH solution. After 30 min at RT, absorbance at λ = 517nm was measured.

Protein concentration

The protein concentration of each 50% ethanol extract of clonal oregano was determined using the Bio-Rad protein assay kit. Bovine serum albumin (BSA) was used for the standard.

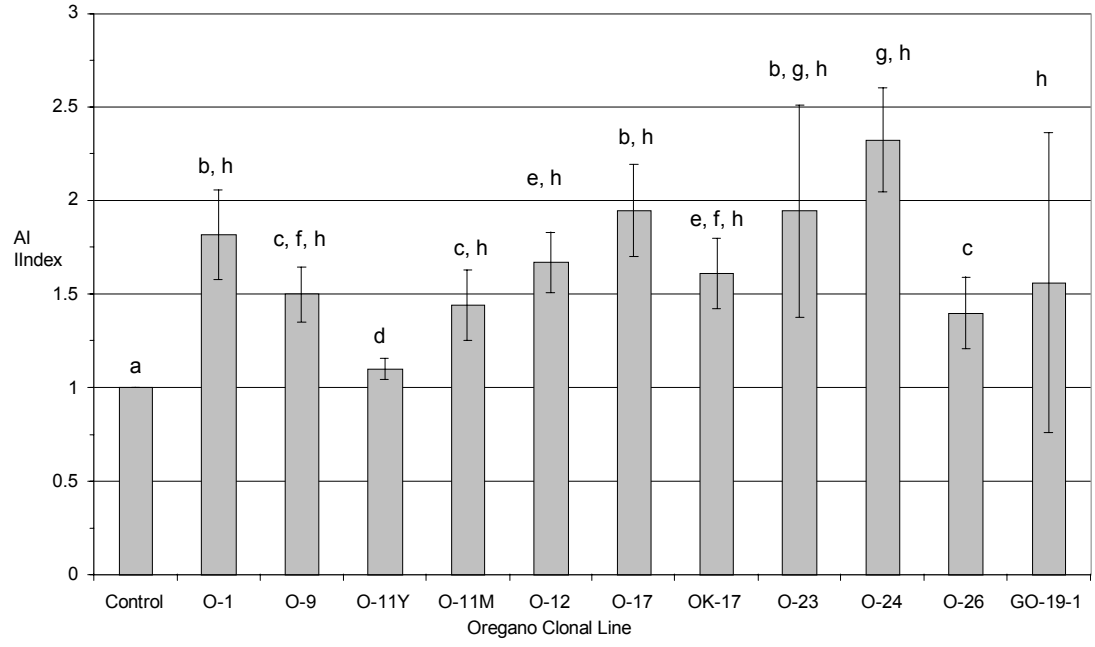

Figure 1. Inhibition of porcine pancreatic amylase activity *in vitro* by extracts of clonal oregano. Amylase inhibition (AI) index values above 1 indicate inhibitory activity. Data with different letters are significantly different by ANOVA at $P < 0.05$.

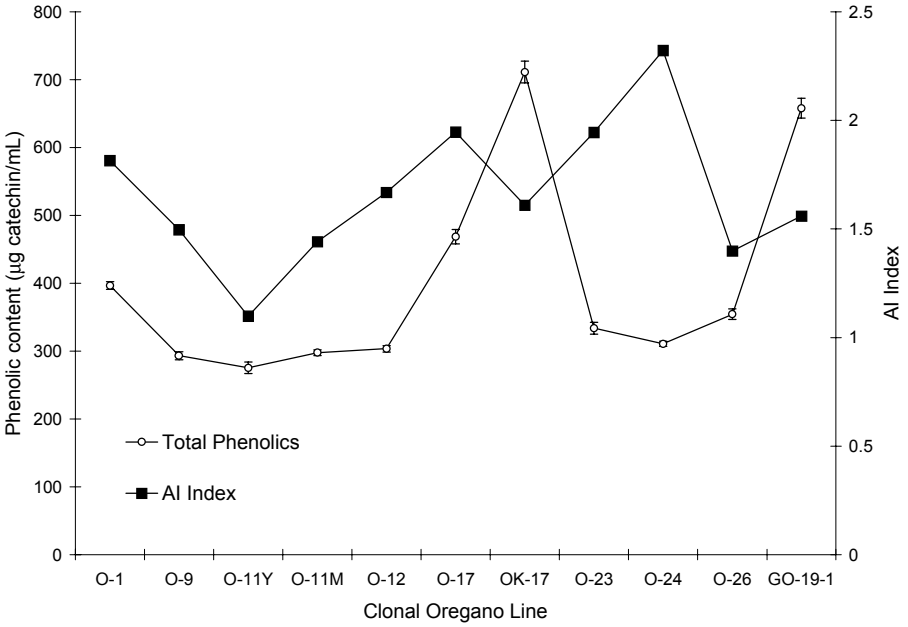

Figure 2. Comparison of clonal extract anti-amylase activity to total phenolic content and AI, amylase inhibition.

Descriptive statistics

Correlation coefficients were determined (using Microsoft Excel XP) for activities/ compounds that were suspected of possibly possessing synergistic action.

Protein structure analysis

RasMol (Windows Version 2.7.2.1; http://openrasmol.org/) Molecular Graphics Visualization Tool was used to visualize the published structure for porcine pancreatic amylase (Protein Data Bank code: 1DHK).[16-18]

Results and Discussion

Effect of clonal oregano extracts on α-amylase activity

In this study, extracts of clonal oregano lines were found to have strong inhibitory activity against PPA *in vitro* (Fig.1). The strongest anti-amylase activity was observed for extract O-24 which had an AI index value of 2.32 ± 0.28 and corresponded to 57% inhibition of enzyme activity. Eight of the 11 clonal oregano extracts tested had AI index values greater than or equal to 1.5. For these experiments, an AI index value of 1.5 corresponded to approximately 33% α-amylase enzyme inhibition.

Relationship between clonal extract phenolic content and α -amylase inhibition

Previous research has noted the ability of various phenolic compounds to inhibit α-amylase activity.[19, 20] Therefore, to further define the nature of the amylase inhibition mechanism in response to clonal oregano extracts, the extract anti-amylase activities were compared to extract total soluble phenolic contents (Fig.2). Anti-amylase activity appeared to be related to phenolic content in some extracts but not in others. In Figure 2, anti-amylase activity of extracts O-1 to O-17 was strongly linked to total soluble phenolic content (correlation coefficient = 0.85). However, anti-amylase activity of the remaining extracts (OK-17 to GO-19-1) was largely unrelated to total soluble phenolic content (correlation coefficient = -0.51). As some of the extracts in this latter group still possess strong anti-amylase activity, it is possible that other factors, such as phenolic synergies or synergies to other extract components (i.e. proteins), may be involved.

Relationships between α- amylase inhibition and specific phenolic contents in clonal oregano extracts

Oregano is known to be a rich source of RA, a biphenolic compound previously reported to possess strong anti-amylase activity.[10] To explore potential phenolic compounds involved in the anti-amylase activity of clonal oregano extracts, we determined the concentration of selected phenolic compounds known to occur in oregano and related herb species. Table 1 shows the contents of

Table 1. Concentration[a] of select phenolic compounds in extracts[b] of clonal oregano by HPLC

Oregano Clone	RA	PA	Quercetin	pCA
0-1	64.5 ± 0.5	-[c]	12.0 ± 0.1	
0-9	42.6 ± 0.4	-	8.8 ± 0.04	0.15 ± 0.01
0-11Y	114.0 ± 1.4	-	9.9 ± 2.3	0.53 ± 0.09
0-11M	116 ± 1.4	-	9.5 ± 0.1	-
0-12	101 ± 0.2	-	16.5 ± 0.2	1.10 ± 0.02
0-17	184 ± 2.3	-	12.6 ± 0.1	0.99 ± 0.02
OK-17	136 ± 1.4	-	18.4 ± 0.4	0.61 ± 0.04
0-23	76.0 ± 1.1	-	15.3 ± 1.3	-
0-24	53.1 ± 0.1	-	5.5 ± 0.3	-
0-26	31.5 ± 0.3	-	8.9 ± 0.4	0.24 ± 0.01
GO-19-1	72.6 ± 0.2	8.5 ± 0.1	15.2 ± 0.01	-

[a] Data are µg phenolic per 200µg of total phenolic content per extract. [b] Data are mean ± SD of three replicates.
[c] '_' symbol = not detected, or of negligible amount. RA, rosmarinic acid; PA, protocatechuic acid; pCA, para-Coumaric acid.

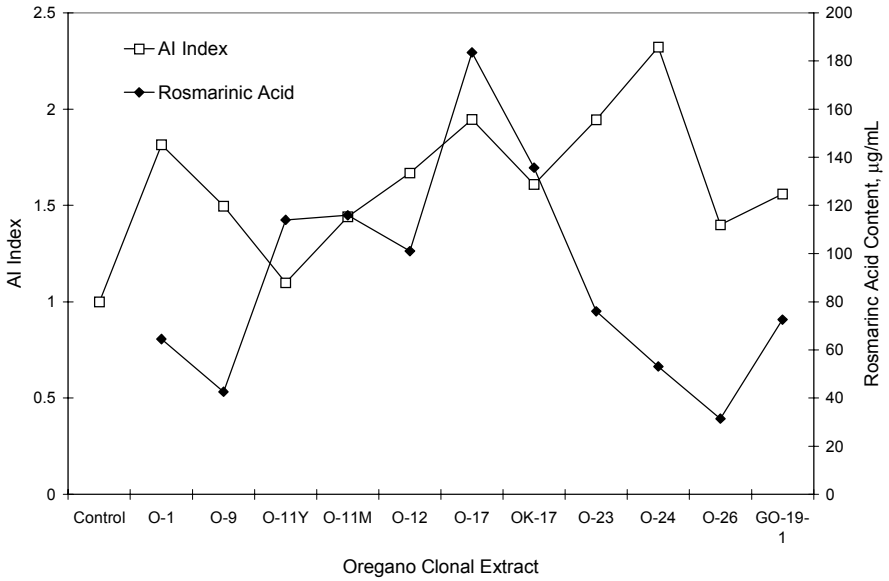

Figure 3. Comparison of clonal oregano extract anti-amylase activity to rosmarinic acid content and AI amylase inhibition.

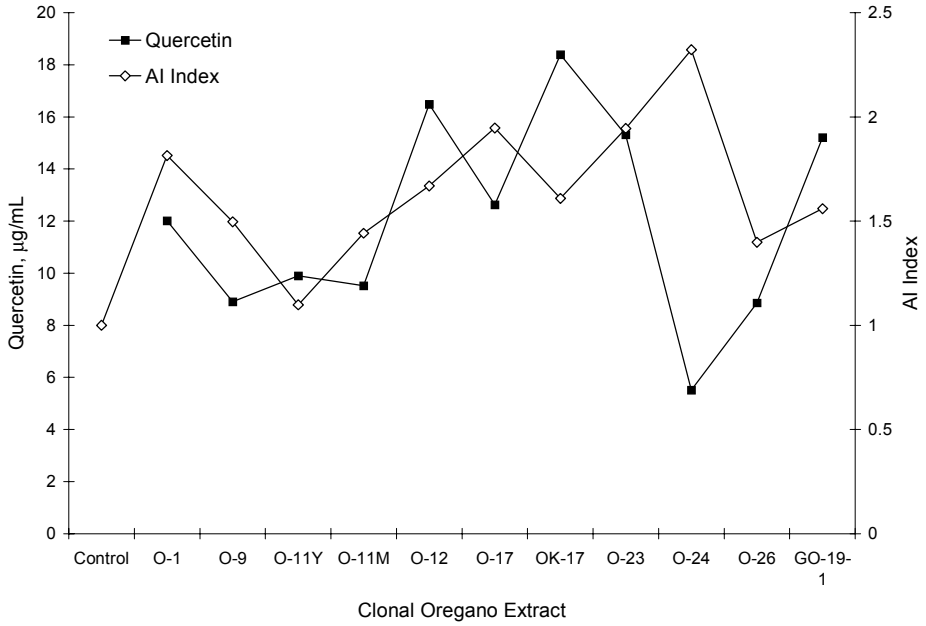

Figure 4. Comparison of clonal oregano extract anti-amylase activity to quercetin content and AI amylase inhibition.

RA, PA, quercetin, and pCA that were detected by HPLC in samples (200µg total phenolics) of each oregano clonal extract. The major phenolic detected in all of the samples was RA. The second highest phenolic detected was quercetin. pCA was detected in 6 of the 11 extracts, while PA was detected in only one (GO-19-1).

The anti-amylase activities of the clonal oregano extracts were compared to the contents of RA, quercetin, and pCA, both individually and in sum with PA. Overall, anti-amylase activity of the clonal oregano extracts did not correlate well with RA content (coefficient = - 0.01), as shown in Figure 3. However, for 4 of the extracts (O-1, O-9, O-17, and OK-17) anti-amylase activity was strongly correlated to RA content (coefficient = 0.62). There was a strong correlation (coefficient = 0.75) between anti-amylase activity and quercetin content for all but two (OK-17 and O-24) of the clonal oregano extracts (Fig. 4). Anti-amylase activity in clonal oregano extracts O-17 and OK-17 were positively correlated to pCA content (coefficient = 1.0), however, none of the other extracts were associated with pCA content (Fig. 5). For 7 of the 11 clonal oregano extracts (O-1, O-9, O-12, O-17, OK-17, O-26, and GO-19-1), anti-amylase activity was strongly correlated (coefficient = 0.81) to the sum total phenolic content of RA, quercetin, pCA, and PA, as detected by HPLC (Fig. 6). As the correlations between clonal extract anti-amylase activity and phenolic contents are not absolute, we are led to believe that other extract components may be involved.

Relationship between clonal extract antioxidant activity and α-amylase inhibition

Three of the best known mammalian α-amylase inhibitors are acarbose, a carbohydrate inhibitor, Tendamistat, a proteinaceous inhibitor from *Streptomyces*, and α-amylase inhibitor 1, a lectin-like proteinaceous inhibitor from the common bean *Phaseolus vulgaris*.[18] Structural analysis indicates that the proteinaceous inhibitors interact with

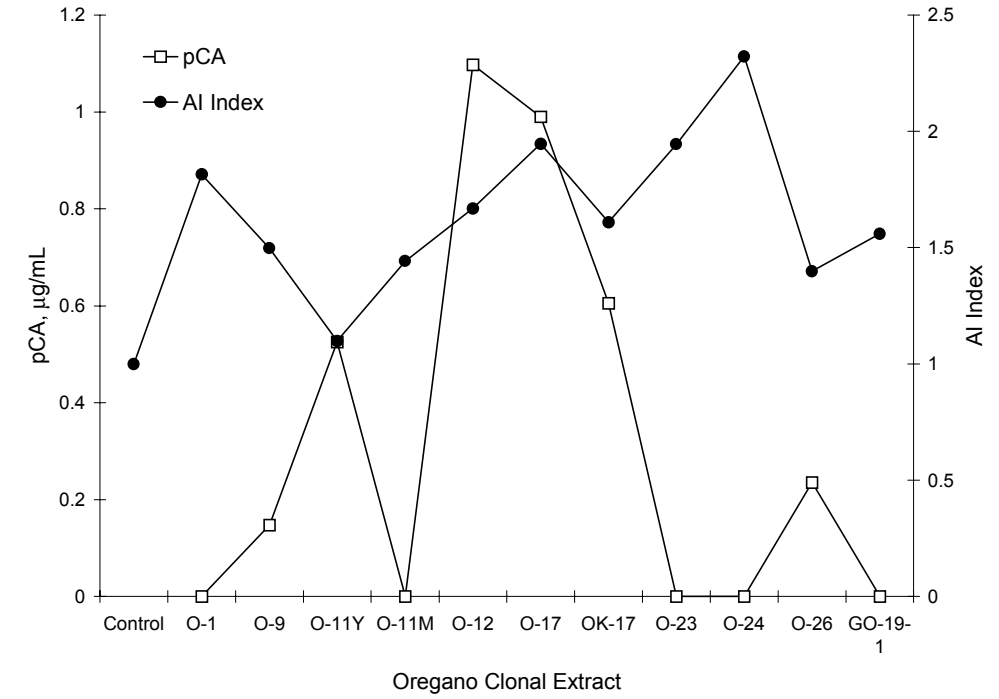

Figure 5. Comparison of clonal oregano extract anti-amylase activity to pCA content. AI, amylase inhibition; pCA, para-coumaric acid.

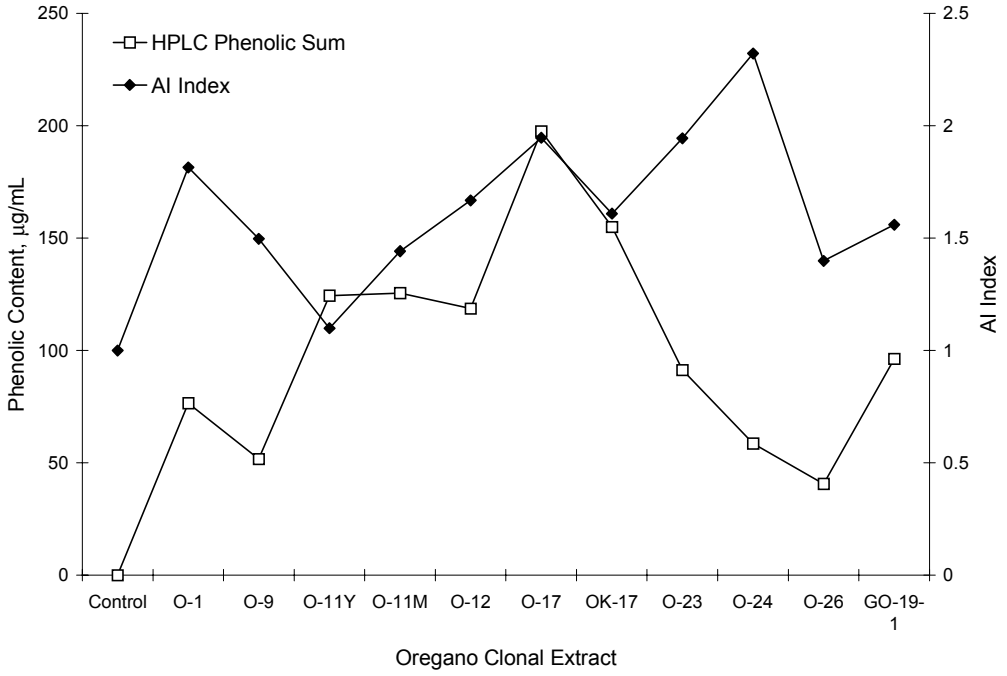

Figure 6. Comparison of clonal oregano extract anti-amylase activity to the sum total content of the phenolics rosmarinic acid, quercetin, para-coumaric acid, and protocatechuic acid, as detected by HPLC. AI, amylase inhibition.

with α-amylase and block access to the active site, while the carbohydrate inhibitor acarbose directly interacts with residues in the active site (Glu233, Asp300, and Asp197) to inhibit the enzyme.[18] In this work, analysis of the published structure of PPA (PDB code: 1DHK), revealed the occurrence of 5 sets of disulfide bridges that occur on the outer surface of the enzyme (Fig. 7). We hypothesize that reduction of these cysteine residues by antioxidants could cause structural alterations that may negatively affect the activity of the enzyme.

Many phenolic compounds are known to possess antioxidant activity *in vitro* and *in vivo*.[21-23] Since oregano is a rich source of the phenolic antioxidants RA and quercetin, we sought to explore whether antioxidant activity of the clonal oregano extracts might be involved in the anti-amylase activity. Antioxidant activity of the clonal oregano extracts was determined as percentage DPPH free-radical scavenging. Additionally, two synthetic antioxidants, BHT and Trolox, were also tested for anti-amylase activity. Anti-amylase activity of 6 of the 11 clonal oregano extracts and the 2 synthetic antioxidants was found to strongly correlate (coefficient = 0.87) to

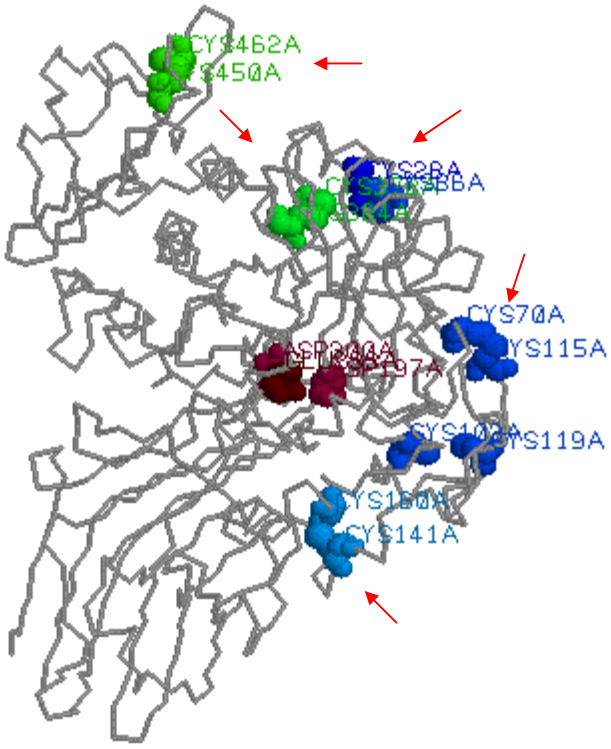

Figure 7. Structural representation of porcine pancreatic alpha-amylase. Cysteine residues are shown in green (chain B) and in blue (chain A). Three important residues of the active site (Glu233A, Asp300A, Asp197A) are shown in red (center of molecule). Arrows indicate each of the 5 disulfide bridges located on the outer surface of the enzyme.

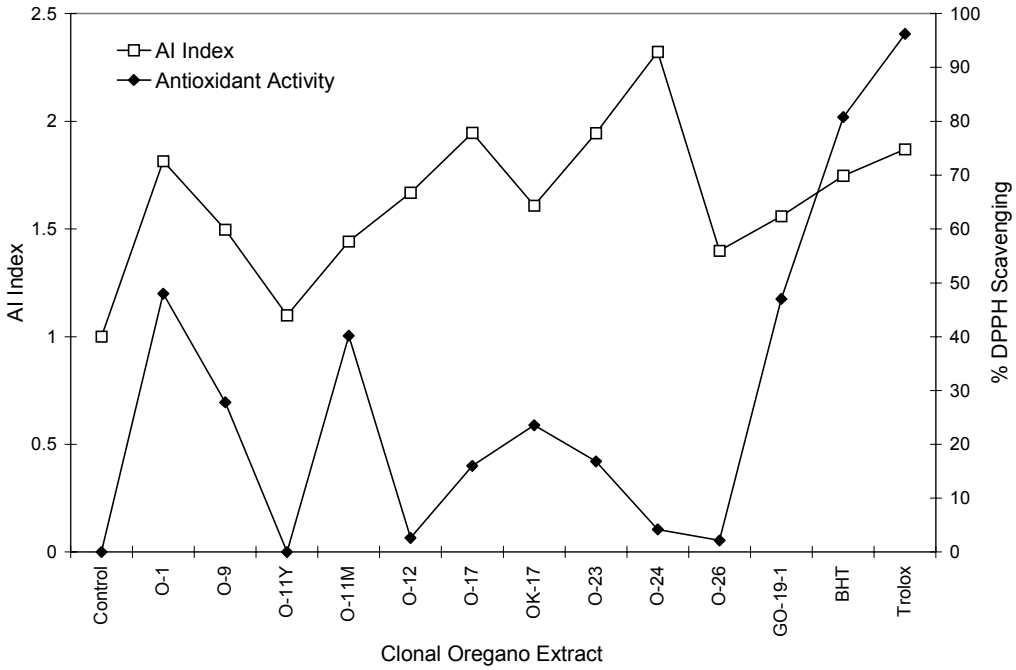

Figure 8. Comparison of clonal oregano extract anti-amylase activity to antioxidant activity given as percentage DPPH free-radical scavenging. AI, Amylase inhibition.

antioxidant activity (O-1 to O-11M, O-26, and GO-19-1; Fig. 8). However, as the activities of the remaining 5 extracts (O-12 to O-24; Fig. 8) did not correlate, other mechanisms including direct interactions remain possible.

Relationship between clonal extract protein content and α-amylase inhibition

Phenolic compounds are known to bind proteins, including enzymes.[24] As the inhibition profile for clonal

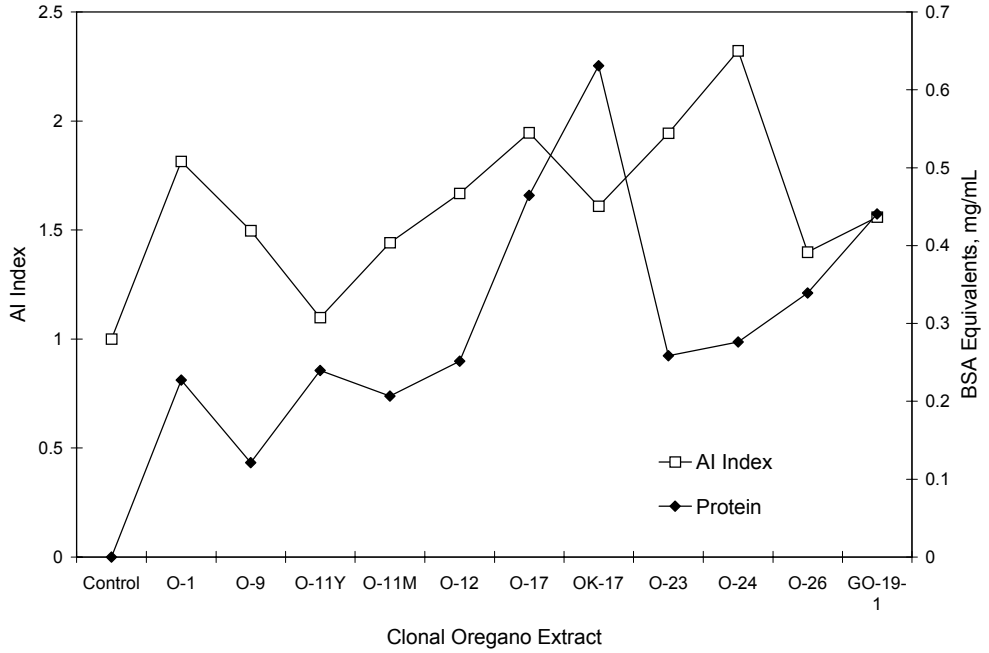

Figure 9. Comparison of clonal oregano extract anti-amylase activity to extract protein content. AI, Amylase inhibition; BSA, bovine serum albumin.

oregano extracts against PPA was not absolutely correlated to phenolic content, there remains a possibility for the involvement of protein-phenolic synergies in the observed PPA inhibition. Therefore, we compared the extract protein content to extract PPA inhibition (Fig. 9). Overall, there was a moderate correlation between extract protein content and PPA inhibition (coefficient = 0.36). For the first 6 extracts listed in Figure 9 (O-1 to O-17), the correlation was much stronger (coefficient = 0.74). When the data for 2 additional extracts (O-23, O-24) was added, the correlation decreased somewhat but remained strong (coefficient = 0.65). These results suggest that protein may be involved in the PPA inhibitory activity mediated by clonal oregano extracts, perhaps through protein-phenolic synergies.

Conclusion

In this study, extracts of clonal oregano lines were found to have strong inhibitory activity against PPA *in vitro*. PPA inhibition varied by extract and ranged from 9% (O-11Y) to 57% (O-24). Generally, PPA inhibition was related to extract total phenolic content and the sum content of the four major phenolics in oregano, RA, quercetin, PA, and pCA. We were unable to identify a specific phenolic as solely responsible for oregano extract PPA inhibition, as some extract anti-PPA activities correlated well with RA content, while others correlated better with quercetin content. PPA inhibition by oregano extract was generally related to antioxidant activity. The synthetic antioxidants BHT and trolox were found to inhibit PPA, although at intermediate efficacy compared to the oregano extracts. Finally, PPA inhibition by oregano extract was also found to correlate with extract protein content, suggesting a possible role for protein-phenolic synergies.

The finding that phenolic antioxidant-rich oregano extracts inhibit α-amylase activity may help to explain how these constituents of traditional Asian anti-diabetes medicines could confer their therapeutic benefits. Further, the results of our study suggest the potential for oregano as a part of functional foods for the dietary control of hyperglycemia and support its inclusion as a natural, safe, anti-diabetic therapy for modulation of Type 2 diabetes mellitus.

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