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Bioactive Compounds from Culinary Herbs Inhibit a Molecular Target for Type 2 Diabetes Management, Dipeptidyl Peptidase IV

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ABSTRACT: Greek oregano (*Origanum vulgare*), marjoram (*Origanum majorana*), rosemary (*Rosmarinus officinalis*), and Mexican oregano (*Lippia graveolens*) are concentrated sources of bioactive compounds. The aims were to characterize and examine extracts from greenhouse-grown or commercially purchased herbs for their ability to inhibit dipeptidyl peptidase IV (DPP-IV) and protein tyrosine phosphatase 1B (PTP1B), enzymes that play a role in insulin secretion and insulin signaling, respectively. Greenhouse herbs contained more polyphenols (302.7−430.1 μg of gallic acid equivalents/mg of dry weight of extract (DWE)) and flavonoids (370.1−661.4 μg of rutin equivalents/mg of DWE) compared to the equivalent commercial herbs. Greenhouse rosemary, Mexican oregano, and marjoram extracts were the best inhibitors of DPP-IV (IC_{50} = 16, 29, and 59 μM, respectively). Commercial rosemary, Mexican oregano, and marjoram were the best inhibitors of PTP1B (32.4−40.9% at 500 μM). The phytochemicals eriodictyol, naringenin, hispidulin, cirsimaritin, and carnosol were identified by LC−ESI-MS as being present in greenhouse-grown Mexican oregano and rosemary. Computational modeling indicated that hispidulin, carnosol, and eriodictyol would have the best binding affinities for DPP-IV. Biochemically, the best inhibitors of DPP-IV were cirsimaritin (IC_{50} = 0.43 ± 0.07 μM), hispidulin (IC_{50} = 0.49 ± 0.06 μM), and naringenin (IC_{50} = 2.5 ± 0.29 μM). Overall, herbs contain several flavonoids that inhibit DPP-IV and should be investigated further regarding their potential in diabetes management.

KEYWORDS: *hispidulin, marjoram, oregano, rosemary, type 2 diabetes mellitus, DPP-IV, PTP1B*

INTRODUCTION

Type 2 diabetes mellitus (T2DM) affects 8.3% of Americans and cost the United States $175 billion in 2012.[1] Currently, the only effective therapies for the management of T2DM are lifestyle intervention and treatment with pharmaceuticals; however, these approaches have poor adherence[2] and can be expensive to maintain.[1] Thus, there is a need to identify natural compounds that can aid in the management of this disease.

Plants produce bioactive compounds that inhibit the pharmaceutical targets related to T2DM, dipeptidyl peptidase IV (DPP-IV) and protein tyrosine phosphatase 1B (PTP1B).[3,4] DPP-IV is a serine protease responsible for the degradation of the insulinotropic incretin glucagon-like peptide 1 (GLP-1). Pharmaceutical inhibitors of this enzyme have successfully reduced hyperglycemia and hemoglobin A1C levels as a monotherapy and in combination with other antidiabetic agents.[5] PTP1B is a tyrosine phosphatase responsible for the reversal of insulin receptor autophosphorylation. Reducing the activity of this enzyme is hypothesized to prolong the insulin signaling cascade and thus increase insulin sensitivity.[6] A synthetic inhibitor of PTP1B reduced enzyme activity and plasma insulin levels in high-fat-diet-induced obese mice.[7]

A review of recent scientific publications on culinary herbs indicates that herbs from a traditional Mediterranean diet alleviate inflammation, hypertension, hyperlipidemia, and hyperglycemia in vivo.[8] Investigation of the hypoglycemic mechanisms showed that oregano (*Origanum vulgare*), marjoram (*Origanum majorana*), and rosemary (*Rosmarinus officinalis*) can inhibit α-glucosidase[9−11] and act as peroxisome proliferator-activated receptor γ (PPAR-γ) agonists in vitro;[12,13] however, there has been no investigation into the effect of these herbs on DPP-IV or PTP1B.

Mexican oregano (*Lippia graveolens*) is an herb from Latin cuisine that has traditional medicinal uses. In a comprehensive review of the traditional uses of 28 varieties of *Lippia* from around the world, only the use of *L. graveolens* had been reported as a therapy for diabetes.[14] Mexican oregano is also of specific interest in diabetes research because it contains one of the highest concentrations of flavonoids of all foods listed in the U.S. Deparment of Agriculture (USDA) database. Remarkably, there have been no studies examining its potential in diabetes management.

It is well-known that the concentration of phytochemicals such as polyphenols in plants can be impacted by growth conditions[15,16] and drying methods;[17] therefore, it is important to consider origin when examining the therapeutic potential of these herbs. Thus, the first aim of this study was to examine the differences in polyphenol concentration and antioxidant capacity of greenhouse-grown and commercial herbs and the ability of herb extracts from different sources to inhibit DPP-IV and PTP1B. Our second aim was to identify and quantify specific compounds responsible for DPP-IV inhibition in culinary herbs using bioassay-guided fractionation, LS−ESI-

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MS, and HPLC. Our third aim was to examine these compounds for the ability to inhibit DPP-IV independently. To complement this work, computational modeling was used to provide insight into the theoretical enzyme−ligand interactions which identifies molecular targets for inhibition within the enzyme. Through these aims, we have systematically identified compounds that inhibit DPP-IV, an enzyme that plays a role in insulin secretion.

MATERIALS AND METHODS

Materials. Seeds for *O. majorana* and *O. vulgare* ssp. *hirtum* were purchased from Richter's Herbs (Goodwood, Ontario, Canada). Propagated cuttings of *L. graveolens* and *R. officinalis* were purchased from Companion Plants (Athens, OH). Commercial dry herbs were purchased at local grocery stores in the area of Urbana, IL. Fluorescein (S71283), *p*-nitrophenyl phosphate (≥98%, BP2534-1), and all solvents used in extraction, chromatography, and mass spectrometry were of HPLC grade and were purchased from Fisher Scientific (Pittsburgh, PA). Sitagliptin phosphate monohydrate (≥98%, S8576), sodium orthovanadate (≥90%, S6508), Trolox (97%, 238813), gallic acid (≥97.5%, G7384), rutin (≥94%, R5143), hispidulin (≥98%, SML0582), cirsimaritin (≥90%, SMB00174), naringenin (≥95%, N5893), rosmarinic acid (96%, 536954), porcine DPP-IV (D7052, EC 3.4.14.5), AAPH (97%, 440914), 2-aminoethyl diphenylborinate (D9754), and Folin−Ciocalteu phenol reagent (F9252) were purchased from Sigma Chemical Co. (St. Louis, MO). Carnosol (≥96%, 89800) was purchased from Cayman Chemical Co. (Ann Arbor, MI). Eriodictyol (95%, 20056) was purchased from Indofine Chemical Co. (Hillsburough, NJ). Human recombinant PTP1B (BML-SE332-0050, EC 3.1.3.48) was purchased from Enzo Life Sciences (Farmingdale, NY).

Greenhouse Herb and Extract Preparation. Fresh herbs were grown in the University of Illinois Plant Care Facility under greenhouse conditions (average temperature 24.0 ± 0.3 °C) between the months of March and June 2013 and were not exposed to any pesticides or fertilizers. After three months, leaves were removed from the plants, washed, frozen at −80 °C, and freeze-dried within 48 h using a Labconco Freezone 6L (Kansas City, MO) freeze-dryer with a collector temperature of −50 °C. The dried leaves were ground using a commercial coffee grinder and pulverized using a mortar and pestle until a fine powder developed. The powder was sifted through a 20 mesh sieve to remove any remaining large pieces of material. The herb powder was then extracted in an excess of 100% methanol using a Soxhlet exhaustive extraction apparatus for at least 24 h. The methanolic extract was filtered using no. 4 Whatman filter paper by gravity flow and concentrated by evaporation under a fume hood at room temperature overnight. An aliquot was removed and evaporated to near dryness (∼3 mL) using a Buchi rotoevaporator. After rotoevaporation, water was added, and the aliquot was freeze-dried to remove any residual water and methanol. The remaining methanolic extract was fractionated by flash chromatography.

Commercial Herb and Extract Preparation. Commercial dry herbs were ground into a fine powder using a commercial coffee grinder, pulverized with a mortar and pestle, and sifted. The herbs were then extracted in methanol at room temperature for 4 h, after which the extract was filtered using no. 4 Whatman filter paper by gravity flow, the methanol was reserved, and new methanol was added to the herbs for a second extract overnight at room temperature. After the extract was filtered once more, methanol from the 4 h extraction and that from the overnight extraction were pooled. Methanolic extract was evaporated to near dryness (∼3 mL) using a Buchi rotoevaporator (Newcastle, DE). After rotoevaporation, water was added, and the extracts were freeze-dried to remove any residual water and methanol.

Flash Chromatography and Fraction Preparation. Fractionation was performed using a Büchi Sepacore flash chromatography system (Newcastle, DE) with dual C-605 pump modules, a C-615 pump manager, a C-660 fraction collector, and a C-635 UV photometer, with SepacoreRecord chromatography software. The column used was a 40 × 150 mm flash column with approximately 90 g of preparative C18 reversed-phase bulk packing material (125 Å, 55−105 μm, Waters Corp., Milford, MA). The column was equilibrated with 20% methanol and 0.5% acetic acid in water for 10 min at a flow rate of 30 mL/min. After injection of the samples (20 mL), the column was developed with a binary gradient to 100% methanol over 45 min. The effluent was monitored at 280 nm, and fractions based on absorbance were collected in the fraction collector by the software program. Fractions containing each absorbance peak were then pooled. This procedure was repeated until all the extract was used. Fraction designations correspond to the following elution times (min): (Greek oregano) OA, 4−5; OB, 6−10; OC, 11−19; OD, 20−23; OE, 24−28; OF, 29−45; (Mexican oregano) LA, 4−5; LB, 6−10; LC, 11−13; LD, 14−17; LE, 18−20; LF, 21−25; LG, 26−28; LH, 29−31; LI, 32−45; (rosemary) RA, 4−6; RB, 7−15; RC, 16−22; RD, 23−26; RE, 27−30; RF, 31−32; RG, 33−35; RH, 36−39; RI, 40−45; (marjoram) MA, 4−6; MB, 7−13; MC, 14−19; MD, 20−21; ME, 22−23; MF, 24−25; MG, 26−27; MH, 28−45. The fraction pools were then concentrated by evaporation in the hood at room temperature, rotoevaporation, and then freeze-drying. The fraction extracts were then weighed and stored at −20 °C for future use.

Total Polyphenols. The total phenolic concentration was quantified on the basis of the principles introduced by Singleton and Rossi[18] adapted to a microassay. Samples (100 μg of extract/mL) or gallic acid standard and 1 N Folin−Ciocalteu phenol reagent were added to a 96-well flat-bottom plate and allowed to stand for 5 min before the addition of 20% Na_2CO_3. The mixture was allowed to stand for 10 min before the absorbance was read at 690 nm using a Synergy 2 multiwell plate reader (Biotek Instruments, Winooski, VT). The results are expressed as micrograms of gallic acid equivalents (GAE) per milligram of dry weight of extract (DWE) using a gallic acid standard curve: $y = 0.0195x + 0.0054$, $R^2 = 0.9997$.

Total Flavonoids. The total flavonoid concentration was quantified using an adaptation of the method used by Oohmah et al.[19] Briefly, 20 μL of sample (100 μg/mL) or rutin standard was added to a flat-bottom 96-well plate followed by the addition of 180 μL of distilled water. A solution of 2-aminoethyl diphenylborinate (10 mg/mL, 20 μL) was then added to each well, and the absorbance was read at 380 nm using a Synergy 2 multiwell plate reader (Biotek Instruments). The results are expressed as micrograms of rutin equivalents (RE) per milligram of DWE using a rutin standard curve: $y = 0.0028x + 0.0487$, $R^2 = 0.99$.

Oxygen Radical Absorbance Capacity. Antioxidant capacity was measured by the oxygen radical absorbance capacity (ORAC) assay previously described.[20] Trolox standard, blanks, or samples in assay buffer (75 mM phosphate buffer, pH 7.4) were added to the wells of a 96-well black-walled plate. Fluorescein (116.9 nM) was added to each well, and the plate was incubated at 37 °C for 15 min. AAPH (40 mM) was added to each well, and fluorescence was read at 485 and 582 nm every 2 min at sensitivity 60 at 37 °C using a Synergy2 multiwell plate reader (Biotek Instruments). The results are expressed as millimoles of Trolox equivalents (TE) per milligram of DWE.

DPP-IV Inhibition. DPP-IV inhibition was measured using the DPP-IV Glo protease assay (G8351, Promega, Madison, WI) following the manufacture's protocol. Briefly, 50 μL of DPP-IV Glo reagent was added to a white-walled 96-well plate containing 50 μL of blank, 40 μL of enzyme control, 40 μL of sample, 40 μL of positive control sitagliptin, or 40 μL of pure compounds. The samples were prepared in an assay buffer (100 mM Tris, 200 mM NaCl, 1 mM EDTA, pH 8.0) at a concentration of 100, 10, 1, or 0.1 μg of GAE/mL. A stock solution of sitagliptin was prepared by adding 10.48 mg of sitagliptin phosphate monohydrate to 10 mL of assay buffer, making a 2 mM solution which was then diluted to 10, 1, 0.1, and 0.01 μM. Pure compounds were prepared in assay buffer at a concentration of or 100, 10, 1, and 0.1 μM. The blank contained only assay buffer, while the enzyme control contained assay buffer and 10 μL of purified porcine DPP-IV enzyme (88% homology to the human enzyme)[21] at 0.075 U/nL. DPP-IV enzyme was also added to each sample. Luminescence was then measured after gentle mixing and incubation at 26 °C for 30 min

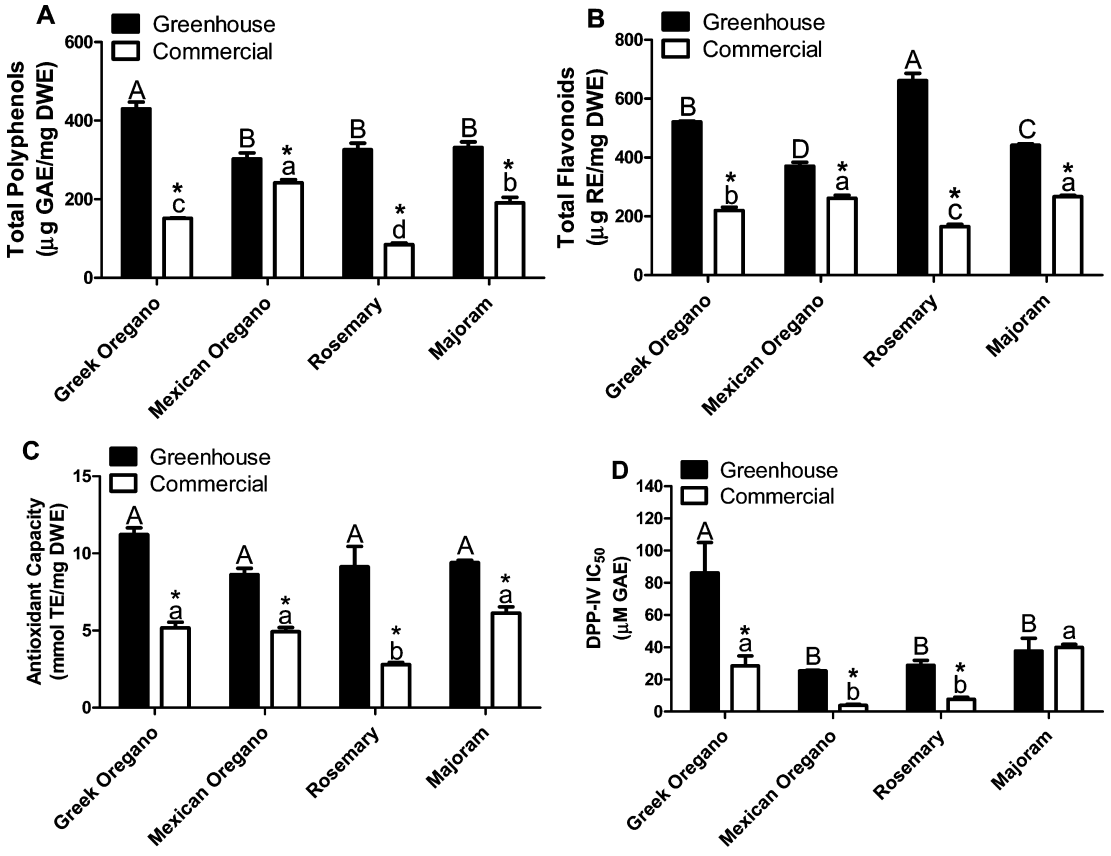

Figure 1. (A) Concentration of polyphenols, (B) concentration of flavonoids, (C) antioxidant capacity, and (D) DPP-IV IC_{50} for greenhouse and commercial herbs. The data represent the mean ± SEM from at least three independent studies done in triplicate. Capital letters indicate significant difference among greenhouse herbs. Lowercase letters indicate significant difference among commercial herbs. Asterisks indicate significant difference between greenhouse and commercial herbs. Significance is reported at $p < 0.05$. GAE = gallic acid equivalents, RE = rutin equivalents, and TE = Trolox equivalents.

using a Synergy2 multiwell plate reader (Biotek Instruments). Percent inhibition was calculated from the blank and enzyme control for each sample. IC_{50} was calculated from the quadratic formula generated from log(concentration) and percent inhibition, and the units were converted to micromolar.

PTP1B Inhibition. PTP1B inhibition was determined by analysis of *p*-nitrophenyl phosphate (*p*-NPP) degradation adapted from the methods of previous researchers.[22−24] Briefly, 50 μL of blank, 40 μL of enzyme control, 40 μL of sample, or 40 μL of positive control sodium orthovanadate was added to a flat-bottomed clear 96-well plate followed by the addition of 50 μL of *p*-NPP (10 mM). The samples and sodium orthovanadate were prepared in assay buffer (50 mm HEPES, 100 mm NaCl, 0.1% BSA, 1 mm DTT, pH 7.3) and contained 100, 10, 1, or 0.1 μg of GAE/mL on the basis of data obtained from the polyphenol analysis for the samples or 100, 10, 1, or 0.1 μM for sodium orthovanadate. The blank contained only assay buffer, while the enzyme control contained assay buffer and 10 μL of purified PTP1B enzyme (10 μg/mL). Purified PTP1B enzyme was also added to each sample. The plates were incubated at room temperature for 20 min, the reaction was terminated with the addition of 50 μL of 1.5 M NaOH, and the absorbance was read at 405 nm using a Synergy2 multiwell plate reader (Biotek Instruments). Each sample at each concentration was also run without enzyme in the same experiment to control for the inherent color present in each sample. Percent inhibition was calculated from the blank and enzyme control for each sample. IC_{50} was calculated from the quadratic formula generated from log(concentration) and percent inhibition, and the units were converted to micromolar.

LC−ESI-MS and HPLC Analysis. A turbo ion spray electrospray source and an Agilent 100 series HPLC system (G1379A degasser, G1357A binary capillary pump, G1389A autosampler, G1315B photodiode array detector, and G1316A column oven) running under Applied Biosystems Analyst 2.0 (build 1446) LC−MS software were utilized. The mass spectrometer was calibrated at least daily with a standard calibration mixture recommended by Applied Biosystems, and the signal detection was optimized as needed. The data were acquired in the MOF MS mode, negative. The MS parameters were as follows: accumulation time, 1 s; mass range, 200−1000 Da; source gas 1, 50 units; source gas 2, 35 units; curtain gas, 25 units; ion spray voltage, 4500; source heater, 400 °C; declustering potential 1, 80; focusing potential, 265; declustering potential 2, 15; ion release delay, 6; ion release width, 5. The column was a 3 mm × 150 mm Inertsil reversed-phase C-18, ODS-3, 3 μm column (Metachem, Torrance, CA). The initial solvent system was 20% methanol and 80% water with 0.25% formic acid at a flow rate of 0.25 mL/min. After injection (15 μL) the column was developed with a linear gradient to 100% methanol over 50 min. The column effluent was monitored at 280 nm in the PDA detector. The software package was set to collect mass data between 150 and 1000 amu.

Phenolic Compound Quantification by HPLC. HPLC analysis was conducted on a Shimadzu LC-20 HPLC system (LC-20AT quaternary pump, DGU-20A5 degasser, SIL-20A HT autosampler, and SPD M20A photodiode array detector) running under Shimadzu LCSolutions version 1.22 chromatography software (Columbia, MD). The column used was a 4.6 mm × 250 mm Inertsil ODS-3 reversed-phase C-18, 5 μm column from Varian. The initial conditions were 20% methanol and 80% water with 0.05 M phosphoric acid at a flow rate of 1 mL/min. The effluent was monitored at 280 nm. After injection of 25 μL, the column was held at the initial conditions for 2 min and then developed to 100% methanol in a linear gradient over 55 min. A standard curve based on nanomoles injected was prepared from rosmarinic acid, eriodictyol, naringenin, hispidulin, cirsimaritin, and

carnosol prepared at concentrations of 0.1, 0.2, 1, and 2 nmol/μL. The molar extinction coefficients for each standard were 1.702×10^{-3}, 1.375×10^{-3}, 1.686×10^{-3}, 1.254×10^{-3}, and 10.087×10^{-3} abs/nmol, respectively, and were used to quantify the concentrations of the compounds of interest.

Computational Modeling. Molecular files for the compounds tested were acquired from Chemical Book (www.chemicalbook.com). Structural hydrogens were added to the molecular files, and the CHARMm force field was applied using Accelrys Discovery Studio 3.5 Vizualizer (Discovery Studio 3.5, Accelrys Software Inc., San Diego, CA). The structure of DPP-IV was acquired from the Protein Data Bank (PDB ID 1X70, www.rcsb.org). Because DPP-IV exists as a homodimer in the crystal structure with sitagliptin and stabilizing water molecules, the ligands from the crystal structure, water molecules, and a monomer unit of DPP-IV were removed from PDB file 1X70 using Discovery Studio 3.5. Rotational bonds for each compound were assigned, and the amino acid TYR547 was made flexible in the DPP-IV structure using AutoDock Tools (version 1.5.6).[25] AutoDock Vina[26] search box parameters were determined using a rigid form of DPP-IV. The orientation of the search box was predetermined by AutoDock Tools; however, the dimensions in angstroms were changed to encapsulate DPP-IV as a whole (center $X = -8.425$, center $Y = 54.618$, center $Z = 33.543$, size $X = 68$, size $Y = 64$, size $Z = 78$). In a physiological system a ligand may have access to other regions of the enzyme beyond the catalytic site. Thus, the enzyme was encapsulated as a whole to model any potentially novel interactions between the ligands of interest and DPP-IV outside of its active site. A text configuration file was created detailing the search parameters, and the exhaustiveness of the search was set to 150. The exhaustiveness is proportional to the number of runs per search and was increased from the default setting of 8 to 150 due to the large search space used in this study, as such a low value may have given inconsistent results. Additionally, exhaustiveness was increased to 150 on the basis of the consistency of preliminary research modeling sitagliptin. The best binding conformation was determined by the lowest binding affinity (kcal/mol) of each computational docking for each compound tested. Interactions between the amino acids of DPP-IV and each compound were observed using Discovery Studio 3.5. AutoDock Vina provides the results based on the best docking conformation, which is given a root-mean-square deviation of zero. Since only the best docking conformation for each ligand was chosen, the root-mean-square deviations could not be compared.

Statistics. Data are expressed as the mean ± SEM of at least three replications. Statistical analysis was conducted using SAS Enterprise Guide (version 6.1, SAS Institute Inc., Cary, NC) and GraphPad Prism (version 5.02, GraphPad Software, Inc., San Diego, CA). One-way analysis of variance (ANOVA) with Tukey's post-test was used to evaluate the differences between group means. Regression analysis was used to evaluate associations between variables. Analyses were considered statistically significant at an α-level of $p < 0.05$.

RESULTS AND DISCUSSION

Characterization and Comparison of Greenhouse and Commercial Herbs. Figure 1A–C represents the total polyphenol concentration, total flavonoid concentration, and antioxidant capacity measured in greenhouse-grown and commercially available Greek oregano, Mexican oregano, rosemary, and marjoram. Greenhouse-grown herbs had significantly higher polyphenol concentrations (range 302.7–430.1 μg of GAE/mg of DWE), flavonoid concentrations (range 370.1–661.4 μg of RE/mg of DWE), and antioxidant capacity (range 8.62–11.22 mmol of TE/mg of DWE) compared to the commercially available products.

Of all greenhouse-grown herbs, extracts from Greek oregano contained the highest concentration of polyphenols (430.1 ± 17.1 μg of GAE/mg of DWE) and rosemary extract contained the highest concentration of flavonoids (661.4 ± 24.6 μg of RE/mg of DWE), while there was no statistical difference in antioxidant capacity among the extracts from the greenhouse grown herbs. Of all commercial herbs, Mexican oregano extract contained the highest concentration of polyphenols (241.5 ± 8.0 μg of GAE/mg of DWE) and Mexican oregano and marjoram extracts contained the highest concentration of flavonoids (260.9 ± 11.0 and 266.9 ± 3.8 μg of RE/mg of DWE). Rosemary extract had the lowest antioxidant capacity (2.8 ± 0.1 mmol of TE/mg of DWE) of the commercial herbs. There was a positive correlation ($R^2 = 0.70$, $P < 0.001$) between total polyphenol and total flavonoid concentrations, total polyphenol concentration and antioxidant capacity ($R^2 = 0.92$, $P < 0.001$), and total flavonoid and antioxidant capacity ($R^2 = 0.74$, $P < 0.01$) of the extracts.

Extraction in polar solvents results in a yield of approximately 15%.[27−30] Adjusting for extract yield, the total polyphenol concentration for the dry herbs ranged from 1266 mg of GAE/100 g of dry weight (DW) (commercial rosemary) to 6452 mg of GAE/100 g of DW (greenhouse Greek oregano). These values are consistent with those reported in the literature for culinary herbs.[31] The total flavonoid concentration ranged from 2474 mg/100 g of DW (commercial rosemary) to 9921 mg/100 g of DW (greenhouse rosemary), and the antioxidant capacity ranged from 41.9 mmol of TE/100 g of DW (commercial rosemary) to 168.3 mmol of TE/100 g of DW (greenhouse Greek oregano). These values are consistent with the reported antioxidant capacity of methanol extracts from culinary herbs.[32]

Previous reports indicated that field-grown herbs have significantly lower total polyphenol concentration and antioxidant capacity[16] than greenhouse-grown herbs. Drying temperatures can also negatively impact the total polyphenol concentration and antioxidant capacity.[17] Furthermore, the cultivar or plant origin can play an important role in dictating the concentrations of antioxidants.[32] All of the aforementioned factors may have contributed to the differences observed between greenhouse and commercial herbs.

DPP-IV and PTP1B Inhibition by Greenhouse and Commercial Herbs. Figure 1D indicates the concentration required to inhibit DPP-IV by 50%. Lower concentrations indicate a fraction is a more potent inhibitor. Among all greenhouse-grown herbs, rosemary (IC_{50} = 28.7 ± 3.1 μM), Mexican oregano (25.3 ± 0.3 μM), and marjoram (37.7 ± 7.9 μM) extracts were the most potent inhibitors of DPP-IV. For all commercially produced herbs, rosemary (6.5 ± 0.4 μM) and Mexican oregano (IC_{50} = 3.9 ± 0.6 μM) extracts were the most potent inhibitors of DPP-IV.

Commercial Greek oregano extract (28.4 ± 6.3 μM) was a better inhibitor of DPP-IV than greenhouse-grown Greek oregano extract (86.2 ± 18.8 μM), commercial Mexican oregano extract (3.9 ± 0.6 μM) was a better inhibitor of DPP-IV than greenhouse-grown Mexican oregano extract (25.3 ± 0.3 μM), and commercial rosemary extract (6.5 ± 0.4 μM) was a better inhibitor than greenhouse rosemary extract (28.7 ± 3.1 μM). There was no difference in DPP-IV inhibition between the extracts of commercially produced and greenhouse-grown sources for marjoram. The positive control sitagliptin had an IC_{50} of 0.05 ± 0.01 μM.

Percent inhibition of PTP1B by 500 μM herb extracts was measured from each greenhouse and commercial herb. Extracts from greenhouse herbs did not inhibit PTP1B; however, commercial Mexican oregano (37.3% ± 6.8%), rosemary (40.9% ± 7.2%), and marjoram (32.4% ± 17.5%) extracts inhibited PTP1B at this concentration (data not shown). The

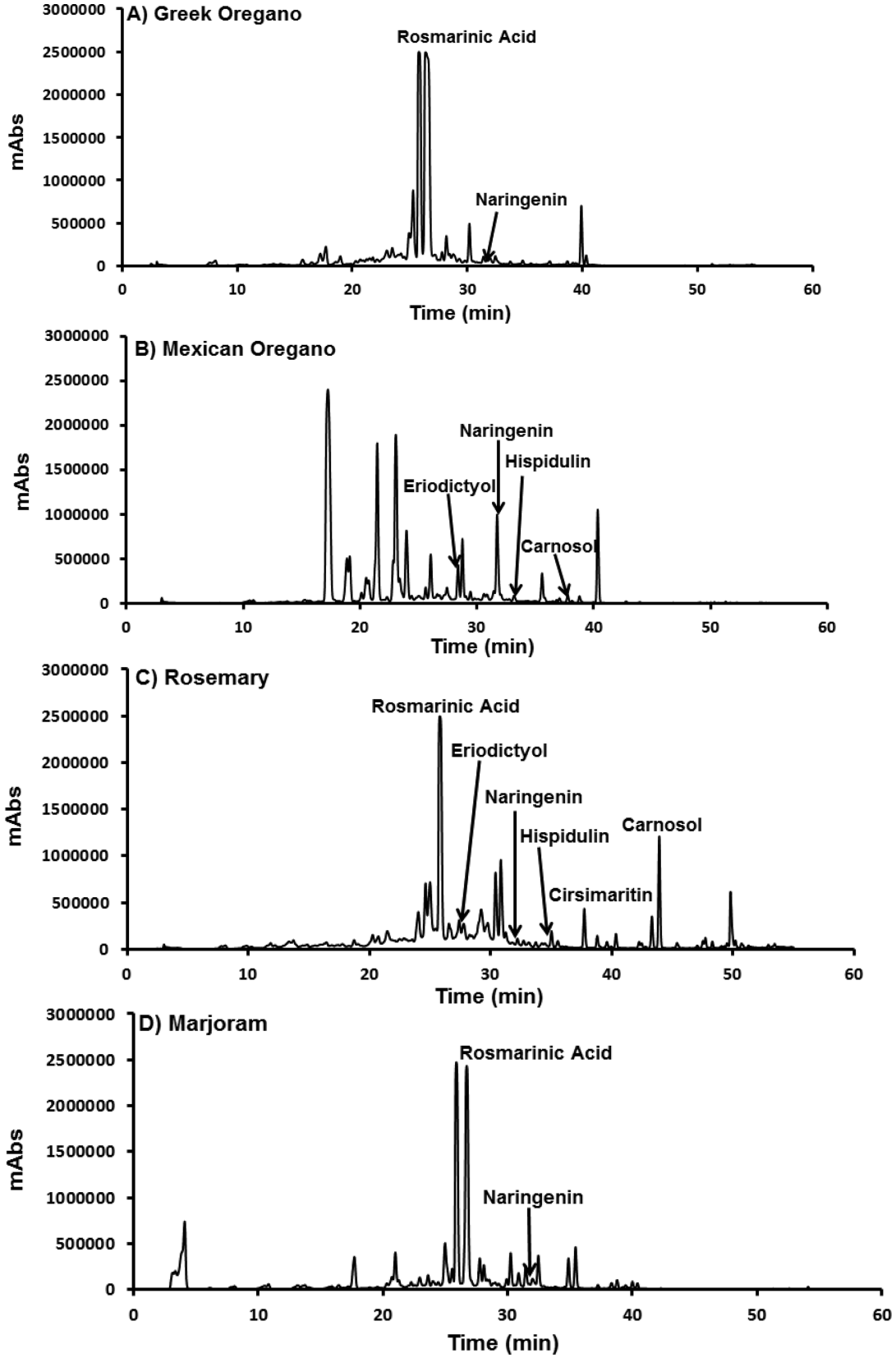

Figure 2. Representative HPLC chromatograms of the greenhouse-grown herbs. Positively identified compounds are indicated.

positive control sodium orthovanadate inhibited PTP1B by 100% at 500 μM and by 50% at 5.7 $\pm$ 2.7 μM. Comparison of the LC−ESI-MS chromatograms between commercial and greenhouse Greek oregano reveals many differences between the two in peak retention times and intensities (data not shown). We are currently performing further analysis to better understand the chemical differences in the composition of the commercial and greenhouse herb extracts.

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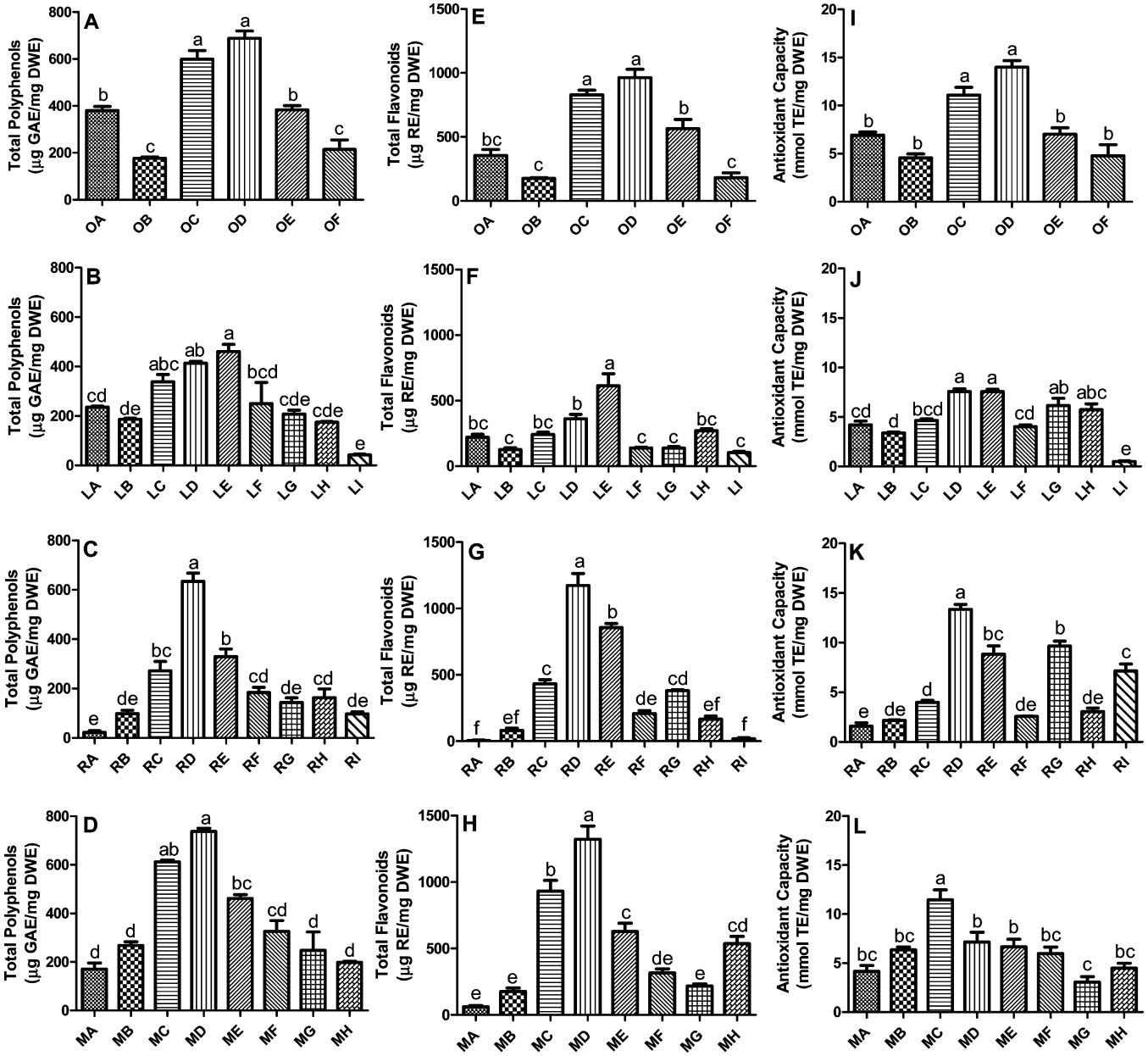

Figure 3. Total polyphenols (A–D), total flavonoids (E–H), and antioxidant capacity (I–L) for all fractions of greenhouse herbs: Greek oregano (A, E, I), Mexican oregano (B, F, J), rosemary (C, G, K), and marjoram (D, H, L). The data represent the mean ± SEM from at least three independent studies done in triplicate. Columns with differing letters are different with a significance of $p < 0.05$. GAE = gallic acid equivalents, RE = rutin equivalents, and TE = Trolox equivalents.

Jiang et al.[4] identified more than 200 natural compounds that were reported to inhibit PTP1B with IC_{50} < 100 μM; these included flavones, phenolic acids, and phenolic terpenoids. The natural compounds present in the herb extracts examined in this study did not inhibit PTP1B to the same extent. Jiang et al.[4] reported the values for pure compounds rather than whole extracts, and they reported natural compounds from a wide variety of sources such as plant roots, bark, and leaves as well as fungi and marine algae, and it is possible that the types of compounds that best inhibit PTP1B are not found in high concentrations in the leaves of culinary herbs.

Characterization of Fractions from Greenhouse Herbs. Figure 2 shows the comparative HPLC chromatograms of the greenhouse-grown herbs. Positively identified compounds are indicated. Figure 3 indicates the polyphenol concentration, flavonoid concentration, and antioxidant capacity of all fractions collected from each herb extract based on retention times using flash chromatography as indicated in the Materials and Methods. For Greek oregano (O), fractions C and D had the highest concentration of polyphenols and flavonoids and the highest antioxidant capacity. For Mexican oregano (L), fraction E had the highest of these three measurements, while, for rosemary (R), it was fraction D. For marjoram (M), fraction D had the highest concentration of polyphenols and flavonoids; however, fraction C had the highest antioxidant capacity. This could be due to the presence of nonphenolic antioxidants such as iridoid glycosides,[33] terpenoids, or saponins.[34]

DPP-IV and PTP1B Inhibition by Fractions from Greenhouse Herbs. Table 1 contains the IC_{50} values for

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Table 1. DPP-IV and PTP1B Inhibition for All Fractions of Greenhouse-Grown Herbs[a]

	DPP-IV IC_{50} (μM GAE)			
fraction	Greek oregano	Mexican oregano	rosemary	marjoram
A	>500	207.0 ± 52.3 ab	>500	>500
B	206.3 ± 47.2 a	130.3 ± 27.2 ab	>500	231.08 ± 52.0 a
C	>500	162.8 ± 37.0 a	>500	>500
D	317.4 ± 60.7 a	160.7 ± 9.3 a	>500	>500
E	**20.3 ± 3.9** b	56.5 ± 13.8 bc	65.3 ± 14.9 a	146.1 ± 49.3 ab
F	23.3 ± 1.9 b	19.0 ± 5.9 c	22.4 ± 11.9 bc	29.9 ± 1.1 b
G	NA	1.5 ± 0.7 c	**0.4 ± 0.1** c	47.3 ± 10.1 b
H	NA	1.3 ± 0.2 c	6.8 ± 1.5 bc	**1.9 ± 0.4** b
I	NA	**0.8 ± 0.3** c	30.1 ± 4.6 b	NA
	PTP1B inhibition (%) (500 μM)			
fraction	Greek oregano	Mexican oregano	rosemary	marjoram
A	7.0 ± 3.5 b	NI	NI	NI
B	13.3 ± 4.2 b	6.3 ± 3.3 c	NI	NI
C	1.3 ± 1.0 b	2.2 ± 2.1 c	NI	NI
D	NI	NI	NI	17.1 ± 10.4 b
E	32.1 ± 3.3 b	NI	NI	3.7 ± 3.1 b
F	**77.4 ± 18.4** a	NI	NI	4.7 ± 2.7 b
G	NA	12.1 ± 3.1 c	49.8 ± 20.6 a	NI
H	NA	**97.5 ± 1.0** a	NI	**77.7 ± 4.3** a
I	NA	78.9 ± 4.4 b	**87.2 ± 2.9** a	NA

[a]The data represent the mean ± SEM from at least three independent studies done in triplicate. Values within a column followed by different letters are significant at $p < 0.05$. GAE = gallic acid equivalents, and IC_{50} = concentration required to inhibit enzyme activity by 50%. NI = no inhibition at 500 μM. NA = not applicable. Bold numbers indicate the most potent fractions.

DPP-IV for the fractions of each herb extract. From Mexican oregano, fractions LF, LG, LH, and LI were the most potent inhibitors of DPP-IV (IC_{50} range 0.8−19.0 μM), from rosemary, fraction RG was the most potent inhibitor of DPP-IV (IC_{50} = 0.4 ± 0.1 μM), from marjoram, fraction MH was the most potent inhibitor of DPP-IV (IC_{50} = 1.9 ± 0.4 μM), and from Greek oregano, fractions OE and OF were the most potent inhibitors of DPP-IV (IC_{50} range 20.3−23.3 μM). Fractions from these herbs also inhibited PTP1B. At 500 μM, the most potent inhibitors of PTP1B were fractions LH (97.5% ± 1.0%), RI (87.2% ± 2.9%), MH (77.7% ± 4.3%), and OE (77.4% ± 18.4%).

LC−EIS-MS Composition Analysis. To identify specific compounds from these herbs that inhibit DPP-IV, the most potent fractions were analyzed by LC−ESI-MS. Figure 4 shows representative chromatograms and a mass spectrum for rosemary fraction RG. Table 2 indicates which compounds were identified in the most potent fractions for Mexican oregano and rosemary. Eriodictyol, naringenin, hispidulin, and cirsimaritin have been previously identified in Mexican oregano.[35] Other compounds previously found in Mexican oregano that were tentatively identified but not confirmed due to lack of an analytical standard were sakuranetin or scutellarein[36,37] and desmethoxycentaureidin.[38] Rosmarinic acid, hispidulin, cirsimaritin, carnosol, and 7-methylrosmanol have been previously reported in rosemary.[39−43] Rosmarinic acid glucoside[44] and rosmanol isomers[39−42] were tentatively identified in rosemary fractions on the basis of comparison to the literature.

Concentrations of Purified Compounds. The concentrations of compounds identified through bioassay-guided fractionation were quantified using HPLC. Table 3 indicates the concentrations of the compounds in each herb.

Naringenin was quantified in all four species of culinary herbs, ranging from 0.15 mg/g of DW in Greek oregano and marjoram to 4.49 mg/g of DW in Mexican oregano. On the basis of the literature, Mexican oregano (3.72 mg/g of DW)[35] and Greek oregano (2.20 mg/g of DW)[45] concentrations are comparable to these results. There have yet to be reports on the concentration of naringenin aglycon in rosemary or marjoram; however, the concentration of naringenin glycoside has been reported to be 7.16 mg/g of DW[46] in rosemary. In other species of the same family, thyme (*Thymus vulgaris*) contains 0.64 mg/g of DW[47] naringenin glycoside, while the aglycon has been identified in savory (*Satureja cuneifolia*) and thyme (*Thymbra spicata*) at 0.30 and 0.23 mg/g of DW, respectively.[48] Cirsimaritin was also identified in all four culinary herbs in concentrations ranging from 0.05 mg/g of DW in marjoram to 1.10 mg/g of DW in rosemary. The cirsimaritin concentration has previously been reported in rosemary and Mexican oregano to be 0.08 mg/g of DW[49] and 0.68 mg/g of DW,[35] respectively, agreeing with the results of this study.

Eriodictyol was quantified at concentrations ranging from 0.30 ± 0.02 mg/g in marjoram to 1.38 ± 0.02 mg/g in Mexican oregano. Lin et al.[35] measured the range of concentrations of eriodictyol in three samples of Mexican oregano at 0.72−0.91 mg/g, and eriodictyol has been identified as being present in Greek oregano at a concentration of 0.4 mg/g by Agiomyrgianaki et al.[45] There have been no reports on the concentration of eriodictyol in rosemary and marjoram; however, eriodictyol has been identified in thyme,[47] indicating its presence in other species of the Lamiaceae family.

Rosmarinic acid was present in Greek oregano, rosemary, and marjoram extracts at concentrations that ranged from 16.87 mg/g of DW in rosemary to 28.77 mg/g of DW in Greek oregano. Almela et al.[40] report that methanol extract from rosemary contains 32.6 mg/g of DW rosmarinic acid, while

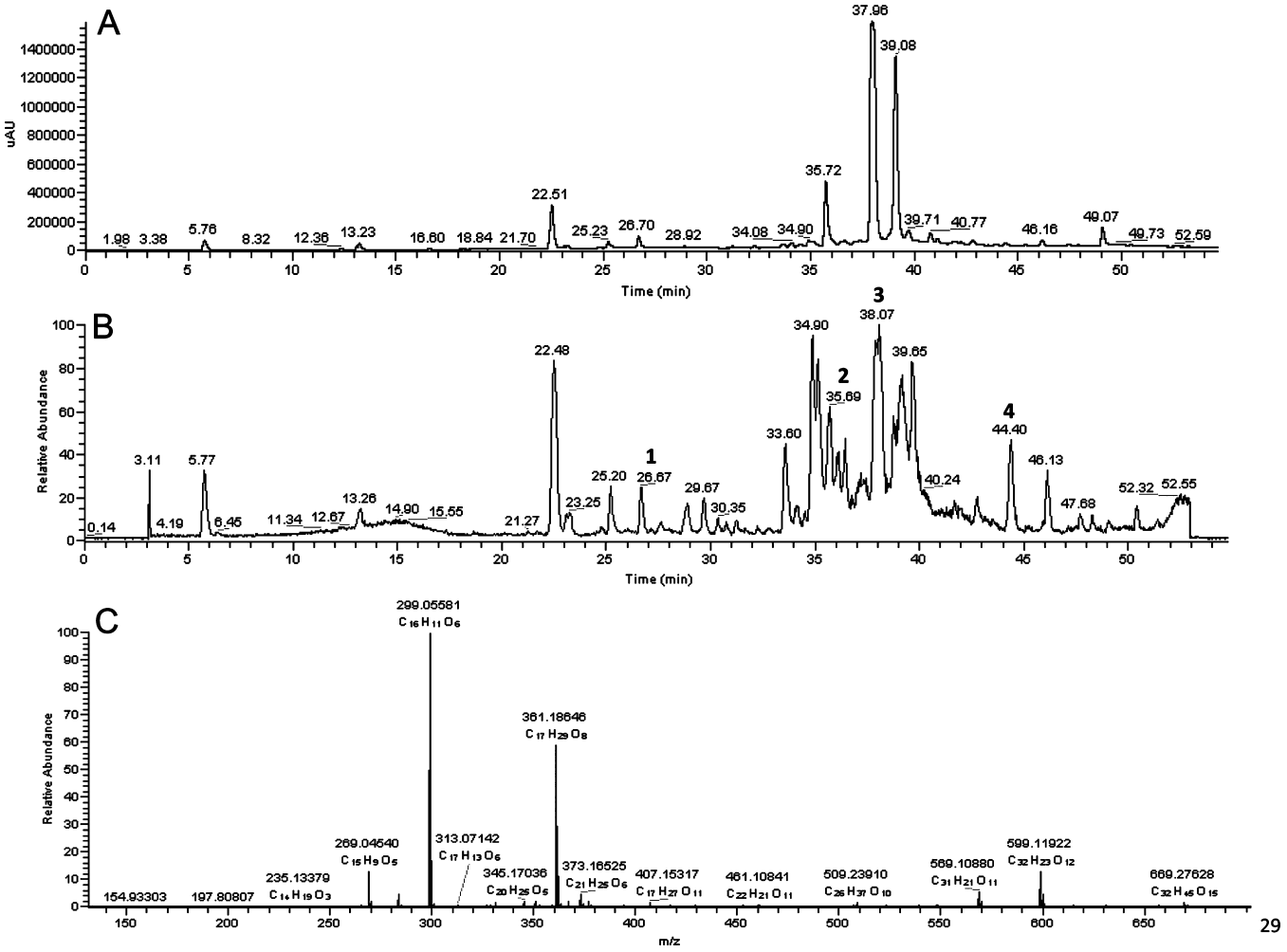

Figure 4. Representative chromatograms [(A) absorbance at 280 nm, (B) total ion current] and mass spectrum (C) from LC−ESI-MS for rosemary fraction RG: rosmarinic acid (**1**), hispidulin (**2**), cirsimaritin (**3**), carnosol (**4**).

Table 2. Composition As Determined by LC−ESI-MS of the Most Potent Fractions, Lowest DPP-IV IC_{50}, from Mexican Oregano and Rosemary

Mexican oregano				rosemary			
fraction	peak (t_R)	m/z	compound	fraction	peak (t_R)	m/z	compound
LF	28.48	287.06	eriodictyol	RF	32.64	345.17	rosmanol isomers[39−42]
	31.76	271.06	naringenin		38.63	520.14	rosmarinic acid 3-*O*-glucoside[44]
	35.72	299.06	hispidulin	RG	26.67	359.08	rosmarinic acid
LG	32.15	271.06	naringenin		33.6	345.17	rosmanol isomers[39−42]
	36.11	299.06	hispidulin		34.9	345.17	rosmanol isomers[39−42]
	38.26	313.07	cirsimaritin		35.69	299.06	hispidulin
	41.26	329.18	carnosol		38.07	313.07	cirsimaritin
LH	36.03	299.06	hispidulin		39.14	520.14	rosmarinic acid 3-*O*-glucoside[44]
	38.24	313.07	cirsimaritin		39.65	345.17	rosmanol isomers[39−42]
	39.28	285.08	sakuranetin,[36] scutellarein[36]		44.40	329.18	carnosol
	41.32	329.18	carnosol	RH	38.01	313.07	cirsimaritin
LI	40.24	329.03	desmethoxycentaureidin[38]		39.79	345.17	rosmanol isomers[39−42]
					44.51	329.18	carnosol
				RI	34.9	345.17	rosmanol isomers[39−42]
					44.40	329.18	carnosol

Shekarchi et al.[50] reported that ethanol extraction of Greek oregano yields a rosmarinic acid concentration of 25.0 mg/g of DW, concentrations which are comparable to those presented in this study.

Hispidulin was identified in Mexican oregano at 0.14 mg/g of DW and rosemary at 0.08 mg/g of DW. Previously, Mexican oregano has been reported to contain 1.00 mg/g of DW,[35] while rosemary has been reported to contain 0.02 mg/g of

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Table 3. Concentrations of Compounds (mg/g of DW) in Greek Oregano, Mexican Oregano, Rosemary, and Marjoram[a]

	rosmarinic acid	eriodictyol	naringenin	hispidulin	cirsimaritin	carnosol
Greek oregano	28.77 ± 0.42 a	0.38 ± 0.01 b	0.15 ± 0.03 b	ND	0.09 ± 0.01 c	0.04 ± 0.01 b
Mexican oregano	ND	1.38 ± 0.02 a	4.49 ± 0.29 a	0.14 ± 0.02 a	0.25 ± 0.00 b	0.29 ± 0.01 b
rosemary	16.92 ± 0.47 b	0.43 ± 0.02 b	0.36 ± 0.01 b	0.08 ± 0.00 b	1.10 ± 0.02 a	24.86 ± 1.27 a
marjoram	16.87 ± 0.69 b	0.30 ± 0.02 c	0.15 ± 0.01 b	ND	0.05 ± 0.00 c	0.04 ± 0.00 b

[a]The data represent the mean ± SEM from at least three independent extractions. Values within a column followed by different letters are significant at $p < 0.05$. ND = none detected. DW = dry weight.

Table 4. Binding Affinity and DPP-IV IC_{50} of the Compounds of Interest[a]

flavonoid	binding affinity (kcal/mol)	IC_{50} (μM)	functional group, location	fraction
hispidulin	−9.4	0.49 ± 0.1 b	OH, 4′, 5, 7; OCH_3, 6	LF, LG, LH, RG
eriodictyol	−8.9	10.9 ± 0.4 a	OH, 3′, 4′, 5, 7	LF
naringenin	−8.6	2.5 ± 0.3 b	OH, 4′, 5′, 7	LF, LG
cirsimaritin	−8.4	0.43 ± 0.07 b	OH, 4′, 5; OCH_3, 6, 7	LG, LH, RG, RH
other				
carnosol	−8.8	>100		LG, RG, RH, RI
rosmarinic acid	−7.8	14.1 ± 1.7 a		RG, MH
sitagliptin	−9.6	0.06 ± 0.03 b		control

[a]The data represent the mean ± SEM from at least three independent studies done in triplicate. Values within a column followed by different letters are significant at $p < 0.05$. Binding affinities were obtained through computational modeling. IC_{50} values were determined by biochemical assay.

DW[49] and 1.5 mg/g of DW[39] hispidulin. Carnosol was measured in all herbs; the highest concentration was 24.86 mg/g of DW in rosemary, which is similar to 21.5 mg/g of DW previously reported by Kontagianni et al.[39] While the values obtained in this study deviate some from the reported values, it is important to note that there is variation in the literature regarding the concentrations of secondary plant metabolites due to the differences in growth conditions, processing, and extraction.

DPP-IV Inhibition by Purified Compounds. Table 4 lists the IC_{50} of DPP-IV for the compounds of interest. Of the compounds identified, cirsimaritin (0.43 ± 0.07 μM), hispidulin (0.49 ± 0.1 μM), and naringenin (2.5 ± 0.3 μM) were the most potent inhibitors of DPP-IV. Eriodictyol (10.9 ± 0.4 μM) and rosmarinic acid (14.1 ± 1.7 μM) were also good inhibitors of DPP-IV. Carnosol was the least effective inhibitor, having an IC_{50} over 100 μM. These results are comparable to those published for flavonoids by Fan et al.,[3] where the IC_{50} values for luteolin, apigenin, and naringenin were 0.12, 0.14, and 0.24 μM, respectively.

Computational Modeling. Table 4 lists the calculated binding affinities between the compounds of interest and DPP-IV. Computational modeling indicates that hispidulin (−9.4 kcal/moL), eriodictyol (−8.9 kcal/mol), carnosol (−8.8 kcal/moL), and naringenin (−8.6 kcal/moL) have the most negative binding affinities for the DPP-IV enzyme, suggesting that they could easily bind to DPP-IV.

Figure 5A−E shows the theoretical ligand−amino acid interactions between DPP-IV and hispidulin, eriodictyol, naringenin, carnosol, and the pharmaceutical inhibitor sitagliptin. Hispidulin and eriodictyol interacted with amino acids in the catalytic region of DPP-IV.[51] The experimental conformation of sitagliptin was compared to the conformation of sitagliptin in the crystal structure of PDB file 1X70 through Discovery Studio 3.5 to validate the results of the computational model. Both conformations had 14 identical interactions and interacted with all three pockets of DPP-IV.

These flavonoids formed H-bonds to the amino acids Thr565 and Arg560. Hispidulin and naringenin formed H-bonds with Lys512, while only hispidulin formed an H-bond with Asn562. Figure 5F shows the relative positions of these flavonoids in the catalytic region of DPP-IV. The arrow in Figure 5G shows the location of the catalytic region relative to the whole enzyme.

Carnosol had electrostatic interactions with amino acids in the S1 and S2 pockets of DPP-IV and van der Waals interactions with the S3 pocket. The S1 pocket consists of the key catalytic amino acids Ser630, Asn710, and His740, the S2 pocket consists of Glu205 and Glu206, and the S3 pocket consists of Ser209, Phe357, and Arg358.[52] Sitagliptin interacted with all three pockets of DPP-IV and had the lowest binding affinity of all compounds tested (−9.6 kcal/mol), but does not interact with the catalytic region. Given that the best binding conformations of naringenin, hispidulin, and eriodictyol did not show interactions with the pockets of DPP-IV, these compounds may have synergistic effects with sitagliptin in the inhibition of DPP-IV.

Computational modeling of cirsimaritin (diagram not shown) revealed that this flavonoid also binds within the catalytic region and has several amino acid interactions in common with hispidulin, naringenin, and eriodictyol. In contrast to the computational modeling, carnosol was not a good inhibitor of DPP-IV. This could be due to the relatively weak electrostatic interactions predicted between carnosol and DPP-IV compared to the several hydrogen bonds predicted for the flavonoids. This may also be the result of competition between the substrate and carnosol for the binding pockets and access to the catalytic amino acids.

Our model was validated on the basis of the comparison of sitagliptin interactions between the crystal structure and experimental conformation. The two arrangements had most interactions in common and bonded to the active site in a similar manner. The experimental conformation and the crystal structure both have sitagliptin having electrostatic interactions with all the residues of the S1 and S2 pockets of DPP-IV, and both have interactions with the S3 pocket. The two arrangements deviated where the crystal structure has sitagliptin interacting electrostatically with Arg358 of DPP-IV while the

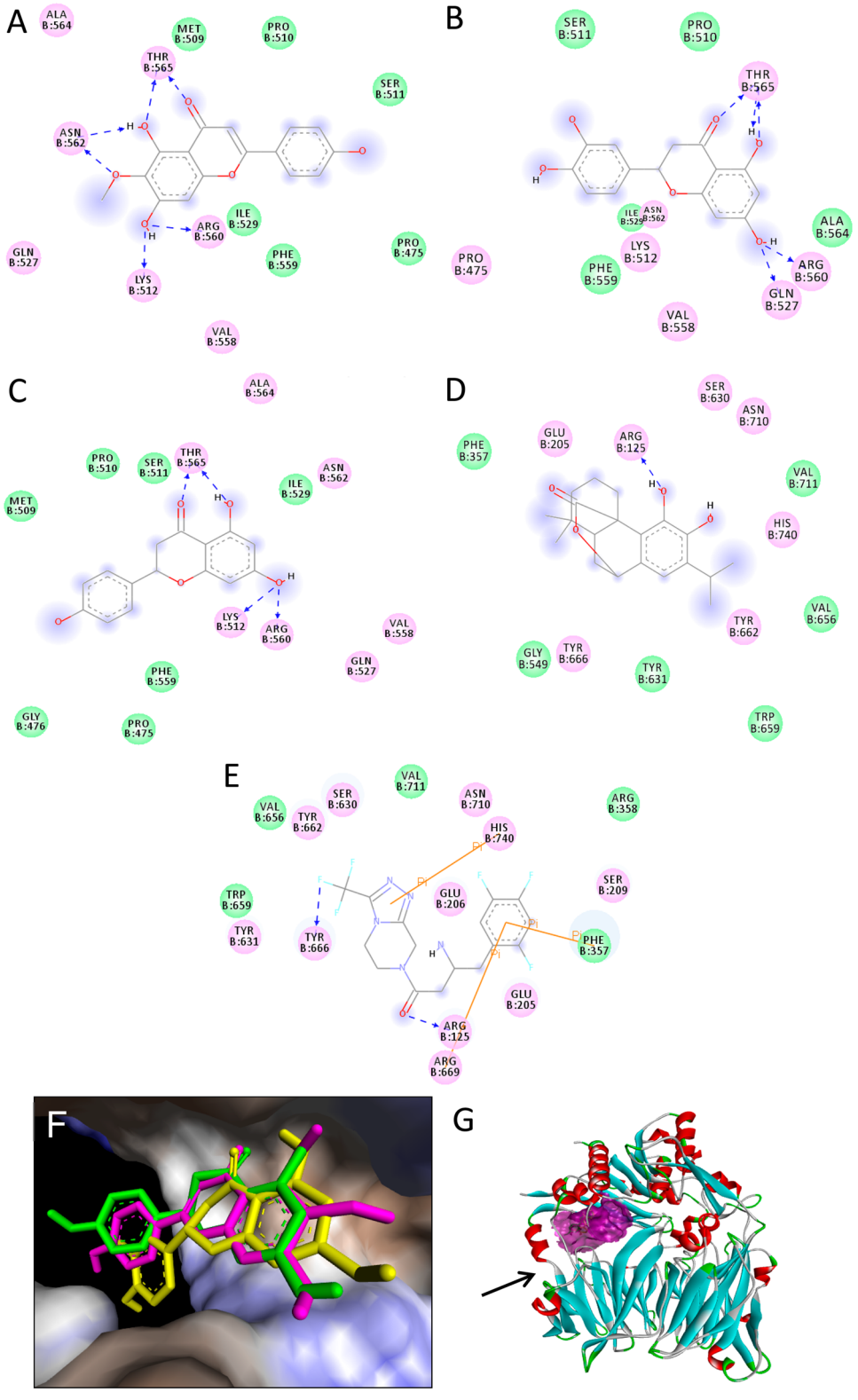

Figure 5. Computational amino acid–ligand interactions for (A) hispidulin, (B) eriodictyol, (C) naringenin, (D) carnosol, and (E) sitagliptin: electrostatic interactions (pink), van der Waals interactions (green), H-bonds (blue arrows), π-interactions (orange lines). (F) 3-D molecular docking of hispidulin (purple), eriodictyol (yellow), and naringenin (green) in DPP-IV. (G) Location of the catalytic pockets (arrow) relative to the region in which the flavonoids bind (purple).

experimental results have sitagliptin having only a van der Waals interaction with the same residue and the crystal structure has sitagliptin interacting with Val207 and Tyr547 while the experimental conformation does not. These deviations may be a result of the structure of sitagliptin being rotated in the final experimental conformation, rather than being planar as in the crystal structure. Furthermore, the experimental conformation for sitagliptin interactions with the

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S1, S2, and S3 subsites has been published by Nabeno et al.,[53] further validating this model.

Conclusion. In summary, extracts of greenhouse-grown herbs were a better source of flavonoids and polyphenols compared to commercially produced herbs; however, this did not impact the concentration required to inhibit DPP-IV. Commercially produced herbs inhibited PTP1B; however, this effect remained only in fractions of greenhouse-grown herbs. Extracts from rosemary and Mexican oregano were the most potent inhibitors of DPP-IV and contained the flavonoids cirsimaritin, hispidulin, and naringenin, which are potent inhibitors of DPP-IV independently. Future work in this area should focus on optimization of the growth and processing conditions for Mexican oregano and rosemary to increase their concentrations of these flavonoids. Overall, this research supports further investigation into the potential for rosemary and Mexican oregano and their flavonoids to be used in the management of T2DM.

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Notes

Mention of trade names or commercial products in this paper is solely for the purpose of providing specific information and does not imply recommendation or endorsement by the U.S. Department of Agriculture (USDA). The USDA is an equal opportunity provider and employer.

The authors declare no competing financial interest.

ABBREVIATIONS USED

DPP-IV, dipeptidyl peptidase IV; DW, dry weight; DWE, dry weight of extract; GAE, gallic acid equivalents; GLP-1, glucagon-like peptide 1; ORAC, oxygen radical absorbance capacity; PTP1B, protein tyrosine phosphatase 1B; RE, rutin equivalents; TE, Trolox equivalents; T2DM, type 2 diabetes mellitus

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