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***In Vitro* Assays of Anti-Diabetic and Anti-Hypertensive Potential of Some Traditional Edible Plants of Qatar**

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ABSTRACT

Aizoon canariense, *Cynomorium coccineum*, *Glossonema edule*, and *Malva parviflora*, edible desert plants from Qatar, were selected to determine levels of phenolic bioactives and potential health benefits for managing early stages of type 2 diabetes and hypertension. Aqueous extracts of *C. coccineum*, contained soluble phenolics and had relatively high levels of antioxidant activity associated with α -glucosidase, α -amylase, and angiotensin-converting enzyme (ACE). *G. edule* and *M. parviflora* had moderate levels of anti-oxidant potential, soluble phenolics, and ACE inhibitory activity. The medicinal properties associated with *C. coccineum* suggest the plant may have potential as a diet-based solution for combating, preventing, and managing the early stage of type 2 diabetes when coupled with an overall healthy life style and pharmacological management strategies.

INTRODUCTION

Type 2 diabetes mellitus is a systemic, multi-layered chronic disease with worldwide impact, reaching epidemic proportions and projected to be the 7th leading cause of death by 2030 (WHO, 2013). Qatar is predicted to have the highest number of diabetic patients in the near future, with a reported increase of 130% by the year 2030 (Mushlin et al., 2012). Due to genetic susceptibility, children that have one or two diabetic parents are two to four times

higher risk for being diagnosed with type 2 diabetes (Harrison et al., 2003). Consanguinity, the incidence of which is the highest in the State of Qatar (51%), is frequently the cause of the prevalence of many genetic disorders and other inherited adult diseases, such as diabetes (Bener et al., 2013). In recent years, many edible food, medicinal, and spice plants have been investigated for potential benefits in managing type 2 diabetes (Kaur et al., 2015; Mirmiran et al., 2014).

Among the most important plant-based antioxidants are the dietary phenolics that are thought to be more effective as antioxidants than vitamin E or C *in vitro* and *in vivo* (Rice-Evans et al., 1997) because they potentially have direct enzyme inhibitory benefits as targets for hyperglycemia and managing carbohydrate metabolism. Specifically, apart from the antioxidant properties, phenolic compounds are natural inhibitors of α -glucosidase and α -amylase enzymes applicable for the management of early stages of hyperglycaemia associated with type 2 diabetes.

A method available for the management of type 2 diabetes is to impede the breakdown and intestinal absorption of glucose through the inhibition of pancreatic α -amylase and intestinal α -glucosidase enzymes (Pinto et al., 2009). The presence of the phenolics that act as α -glucosidase and α -amylase inhibitors in fruits, vegetables and spices could be a good approach to control post-prandial hyper-

glycemia and provide effective benefits without side effects. Further, many plants are also reported to be anti-hypertensive mainly for their potential to inhibit angiotensin converting enzyme (ACE) (Castro et al., 2000), which can be targeted for managing macrovascular complications of type 2 diabetes.

In the current study, phenolic bioactives from four traditional edible plants from Qatar were analysed for their potential in inhibiting α -glucosidase, α -amylase and ACE using *in vitro* assay models. In addition, the relevant total phenolic content, antioxidant potential and the different types of phenolic compounds and their content were analyzed using high performance liquid chromatography (HPLC). The studied food plants were once a part of the diet of the local Qatari population, but have been largely replaced by imported provisions. Establishing and incorporating the anti-diabetic potential of local food into diets could help in manage this disease.

MATERIALS AND METHOD

Plant samples. Indigenous plants, *Cynomorium coccineum* (local name: Trathuth), *Aizoon canariense* (local name: Jafna), *Glossonema edule* (local name: Yerawa), and *Malva parviflora* (local name: Khobeza) were collected locally and identified by a faculty member in the Department of Biological and Environmental Sciences, Qatar University, Doha. Porcine pancreatic α -amylase (EC 3.2.1.1), rat intestinal α -glucosidase (EC3.2.1.20), hippuric acid, rabbit lung ACE (angiotensin I-converting enzyme) (EC 3.4.15.1), 2,2-diphenyl-1-picrylhydrazyl (DPPH), acarbose, and ascorbic acid were purchased from Sigma Chemical Co. Standards for gallic acid were also purchased from Sigma Chemical Co.

Sample extraction. The edible portions of the selected plants were extracted with water to determine the chemical constituency. For *A. canariense* and *M. parviflora*, the entire plants were macerated before extraction. For the *C. coccineum*, the inner flesh of the stem was removed and then extracted. The fruit of *G. edule* were cut into small pieces with a cheese grater and then uniformly mixed before extraction. All samples (2.5 g) were extracted in 100 mL of distilled water under reflux at 95°C for 30 min. Each of the samples was subsequently centrifuged at

11,000 g for 10 min to separate cellular debris from the extract.

Soluble phenolics. Total soluble phenolics in each of the test samples were determined using a previously established assay (Kwon et al., 2006). In brief, a 0.5 mL of sample extract was weighed and added to a test tube. Each of the samples in the test tubes was subsequently mixed with 1 mL of 95% ethanol, 5.5 mL of distilled water, and 0.5 mL of 50% Folin-Ciocalteu reagent. After five minutes, 1 mL of 5% sodium carbonate was added to stop the reaction and the absorbance of the mixture was determined at 725 nm using a UV-visible light spectrophotometer (Agilent Model 8453) after a 1 h dark incubation. Gallic acid was used as a standard, and the results were expressed as μg gallic acid/mL of sample fresh weight.

Antioxidant activity. The antioxidant activity was determined using a DPPH radical scavenging method modified from Kwon et al. (2006). A 250 μL aliquot of the sample extract was mixed with 1.25 mL of DPPH (60 μM in ethanol) and then centrifuged at 5000 g for one minute. The absorbance was measured at 517 nm using the previously described Agilent UV-visible light spectrophotometer. The absorbance readings were compared with a control containing 95% ethanol in place of the sample extract. The inhibition was calculated as:

$$\% \text{ Inhibition} = \frac{(\text{Absorbance}_{\text{control}} - \text{Absorbance}_{\text{extract}})}{\text{Absorbance}_{\text{control}}} \times 100$$

α -Amylase inhibition. The α -amylase inhibitory activity was determined using a modified assay of that described in the Worthington Enzyme Manual (Worthington, 1993; Kwon et al., 2006). A total of 500 μL of 0.02 M sodium phosphate buffer (pH 6.9 with 0.006 M NaCl) containing 0.5 mg/mL of α -amylase were pre-incubated at 25°C for 10 min. After the pre-incubation, 500 μL of a 1% starch solution in 0.02 M sodium phosphate buffer (pH 6.9 with 0.006 M NaCl) was added to each tube at timed intervals. The reaction was stopped using 1.0 mL of dinitrosalicylic (DNS) acid color reagent. The test tubes were incubated in a boiling water bath for 5 min and then cooled to room temperature. The

reaction mixture was diluted by adding 5 to 15 mL of distilled water, and the absorbance was measured at 540 nm using the previously described UV-Visible light spectrophotometer. The absorbance readings were compared with the controls that contained buffer instead of sample extract. The percentage α -amylase inhibitory activity was calculated using the same equation used for percentage inhibition in the DPPH radical inhibition assay.

α -Glucosidase. Inhibitory activity of α -glucosidase was measured following a modified procedure described by McCue et al. (2005). The α -glucosidase was assayed using 50 μ L of sample extracts and 100 μ L of 0.1 M phosphate buffer (pH 6.9) containing α -glucosidase solution (1 U/mL), which was then incubated in 96-well plates at 25°C for 10 min. After the pre-incubation period, 50 μ L of 5 mM *p*-nitrophenyl- α -*D*-glucopyranoside solution in 0.1 M phosphate buffer (pH 6.9) was added to each well at timed intervals. The reaction mixtures were incubated at 25°C for 5 min.

Before and after incubation, absorbance readings of the samples were recorded at 405 nm using a microplate reader (Semi-Automated ELISA System, TECAN SUNRISE Reader, Model Sunrise, Austria) and compared with a control that had 50 μ L of buffer solution in place of the extract. The α -glucosidase inhibitory activity was expressed as percent inhibition and was calculated using the same equation used for determining the percent inhibition in the DPPH radical inhibition assay. Dose dependency was tested using 0.5 μ L to 25 μ L following the same protocol.

ACE inhibition. ACE inhibition was assayed by a method modified by Kwon et al. (2006), using hippuryl-histidyl-leucine (HHL) as a substrate and the ACE-I enzyme from rabbit lung (EC 3.4.15.1). A volume of 50 μ L of sample extracts was incubated with 100 μ L of 1 M NaCl-borate buffer (pH 8.3) containing 2 mU of ACE-I solution at 37°C for 10 min. After pre-incubation, 100 μ L of a 5 mU substrate (HHL) solution was added to the reaction mixture. Test solutions were incubated at 37°C for 1 h. The reaction was stopped with the addition of 150 μ L of 0.5 N HCl to the reaction.

A 5 μ L sample of the stopped reaction mixture was injected into a high-performance liquid chromatography (HPLC) apparatus (Agilent 1100 with UV-Detector 1100, Autosampler 1200 & Column compartment 1200, Agilent Technologies, Palo Alto, CA). The solvents used for the gradient were 10 mM phosphoric acid (pH 2.5) and 100% methanol. The methanol concentration was 60% for the first 8 min and then increased to 100% for 5 min followed by a decrease to 0% for the next 5 min (total run time of 18 min). The analytical column was an Agilent Nucleosil 100-5C18, 250 mm x 4.6 mm inside diameter, filled with packing material of 5 μ m particle size, and used a flow rate of 1 mL/min at ambient temperature. During each run, the absorbance was recorded at 228 nm for the detection of liberated hippuric acid and retention time by comparison with a calibration standard of hippuric acid. The percentage inhibition was calculated using a formula similar to that used for determining the inhibition of for antioxidant activity of α -amylase.

$$\% \text{Inhibition} = \frac{(A_{\text{control}} - A_{\text{extract}})}{(A_{\text{control}} - A_{\text{blank}})} \times 100$$

HPLC analysis of phenolics. For analysis of phenolics by HPLC, a 2 mL samples of the extracts were filtered (pore size, 0.2 μ m), and 5 μ L was injected into the HPLC apparatus (Agilent 1100 with UV-Detector 1100, Auto-sampler 1200 and Column compartment 1200, Agilent Technologies, Palo Alto, CA). For gradient elution, the solvents were: 10 mM phosphoric acid (pH 2.5) and 100% methanol. The methanol concentration was increased from 0 to 60% for the first 8 min and to 100% over the next 7 min, then decreased to 0% for the next 3 min and maintained for the next 7 min (total run time of 25 min).

The analytical column, an Agilent Zorbax SB-C18, 250 mm x 4.6 mm i.d. packed with 5 μ m particle size, used a flow rate of 1 mL/min at ambient temperature. Absorbance was recorded at 306 nm. Calibration standards were cinnamic acid, rosmarinic acid, protocatechuic acid, *p*-coumaric acid, chlorogenic acid, caffeic acid, ferulic acid, gallic acid and quercetin. Extract constituents were identified by comparison of retention times and diode

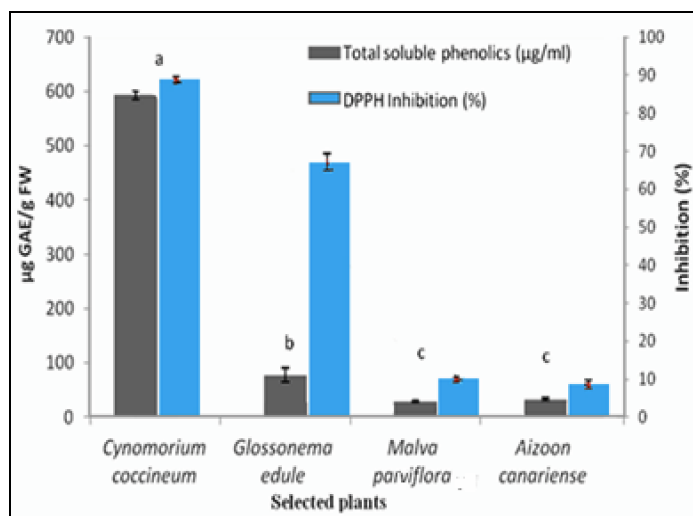
array spectral characteristics with the standards. The results were expressed as $\mu\text{L/g}$ of sample fresh weight.

Statistical analysis. Statistical analysis was a one-way analysis of the variance (ANOVA). Post-hoc comparisons were done using LSD test or a planned comparison in SPSS version 16.0. ($p < 0.05$).

RESULTS AND DISCUSSION

Antioxidant activity, soluble phenolics, and HPLC analysis. The total antioxidant activity and total soluble phenolics of all four edible plants of Qatar showed significantly different results (Figure 1). *Cynomorium coccineum* had significantly higher antioxidant activity and total soluble phenolics as comparison with the other three plants. *Glossonema edule* had high antioxidant activity, but moderate total soluble phenolics, whereas *Malva parviflora* and *Aizoon canariense* had low antioxidant activity and low total soluble phenolic content.

Figure 1. Total soluble phenolics and DPPH inhibitory potential of plants¹.



¹Data are mean \pm SE (n=9). Columns with different letters indicate statistically significant differences among groups at $p < 0.5$. The Pearson's correlation coefficient between total soluble phenolics and DPPH inhibitory potential was 0.79.

Among the studied edible plants, *Cynomorium coccineum* is of particular interest in for the medicinal applications in ancient times. Zucca et al. (2013) evaluated both the aqueous and methanolic extracts of *C. coccineum* for total antioxidant capacity using selected ET-based methods, such as DPPH assay and

ABTS-based assay to measure the Trolox Equivalents Antioxidant Capacity (TEAC), and the FRAP method and ORAC assay based on pyrogallol red. Significant antioxidant capacities using DPPH assay and ORAC-PYR assays were observed. Gao (2011) has reported that the extract of another species of *Cynomorium*, *C. songaricum*, contains proanthocyanidins that improve DPPH free-radical scavenging activity.

Jin et al. (2014) has noted that the antioxidant potential of acetone extract of *C. songaricum*, which contains catechins, is significantly superior antioxidant activity compared with Vitamin E, but is inferior to ascorbic acid. Nuo et al. (2010) has reported high DPPH and ABTS free-radical scavenging activity by *C. songaricum* polysaccharides. Lu et al. (2004) investigated the antioxidant effects of aqueous extract *C. songaricum in vitro* on five types of edible oils, and reported that soybean oil possessed antioxidant capacity. Zhao et al. (2009) has described the antioxidant capacity of phenolic compounds isolated from *C. songaricum*, which has a stronger antioxidant effect at a concentration of 0.2596 mg/mL when compared with vitamin C.

In the current study, *Malva parviflora* had low DPPH inhibitory activity and low total soluble phenolics content. Although aqueous extracts were used, the results agreed with a report by Afolayan et al. (2008) indicating the methanolic extract of *M. parviflora* had 9.3% DPPH inhibition and the methanol extract inhibited 94.3% ABTS (2,2'-azinobis [3-ethylbenzothiazoline-6-sulfonic acid] diammonium salt) radical ion. Farhan, et al. (2012) reported aqueous and ethanolic extracts at 4 mg/mL of crude leaves and stems of *M. parviflora* grown in Lebanon have high DPPH inhibitory activity, enhancing the scavenging activity by 59% and 70%, respectively. The current study, however, does not support the findings of Farhan et al. (2012), but this could be due to differences in extraction methods, genetic variation in the edible plants, growing conditions, or assay methodology.

The investigation of hydroxycinnamic acids and other phenolic acids has not previously been investigated in the four species of plants studied in this report. Using HPLC chromatography, the current

study demonstrated the presence of various phenolic acids, such as gallic acid, chlorogenic acid, caffeic

acid, protocatechuic acid, and cinnamic acid, along with quercetin in all four edible Qatari plant samples

Table 1. Phenolic compounds in aqueous extracts of plants.

Phytochemical	<i>Cynomorium coccineum</i>	<i>Malva parviflora</i>	<i>Aizoon canariense</i>	<i>Glossonema edule</i>
	----- (µL /L) ----- ^{1,2}			
Gallic acid	8.12 ± 0.29 ^a	1.61 ± 0.42 ^{bc}	2.30 ± 0.59 ^b	0.67 ± 0.23 ^c
Chlorogenic acid	5.56 ± 0.29 ^a	2,18 ± 0.14 ^b	2.47 ± 0.27 ^b	0.49 ± 0.12 ^c
Caffeic acid	0.94 ± 0.17 ^a	0.80 ± 0.10 ^a	2,23 ± 0.46 ^b	0.49 ± 0.11 ^a
Protocatechuic acid	3.51 ± 0.66 ^{ac}	2.50 ± 0.51 ^{ab}	5.42 ± 1.09 ^c	0.77 ± 0.29 ^b
Cinnamic acid	5.95 ± 0.95 ^a	3.54 ± 0.60 ^a	4.28 ± 0.71 ^a	0.01 ± 0.01 ^b
Quercetin	1.51 ± 0.42 ^a	4.13 ± 0.55 ^b	-----	2.08 ± 0.45 ^{ab}

¹Means ± standard error, n=9.

²Means with different letters within rows are significantly different, p<0.05..

Cynomorium coccineum, in general, had higher amounts of the phenolic phytochemicals. The gallic acid content (8.12 µL/L) was highest in *C. coccineum*, whereas quercetin content was highest in *Malva parviflora* (4.13 µL/L). Protocatechuic acid and cinnamic acid content were highest in *Aizoon canariense* at 5.42 µL/L and 5.95 µL/L, respectively. Zucca et al. (2013) also has reported the presence of gallic acid in the extracts of *C. coccineum*. Rizk et al. (1990) reported the presence of flavonoids, mainly apigenin, 6,8 di-C-glucoside, and luteolin 7-O-glucoside, in unripe fruit of *Glossonema edule*, whereas *A. canariense* has been reported to contain alkaloids, coumarins, saponins, tannins, flavonoids, steroids, and triterpenes. In a study by Farhan et al. (2012) the methanolic fractions of polyphenols from leaves and stems of *M. parviflora* contained different amounts of phenolic compounds, flavonoids, saponins, alkaloids, resins and tannins.

α-Glucosidase/amylase inhibition. Based on the *in vitro* assay model, only *Cynomorium coccineum* extracts inhibited α-glucosidase and α-amylase enzymes. Aqueous extract of *C. coccineum* demonstrated a relatively high α-glucosidase inhibitory activity (Table 2). In contrast, a moderate inhibition of α-amylase was observed in the aqueous extracts of *C. coccineum* where 300 µL of extract caused a 54.4% inhibition (Fig. 2). α-Amylase and α-glucosidase are key enzymes involved in starch breakdown and subsequent glucose release leading to rapid intestinal absorption. When inhibited by a high

carbohydrate diet, these enzymes are beneficial in maintaining the postprandial blood sugar level and managing hyperglycemia (Puls et al., 1977). A major drawback of currently used α-glucosidase and α-amylase inhibitors, such as pharmacologically beneficial acarbose, are side effects, such as abdominal distention, flatulence, tympanitis, and diarrhea. These side effects are possibly due to complete inhibition of α-amylase, leaving the carbohydrate to ferment in the colon (Bischoff et al., 1985; Bischoff, 1994).

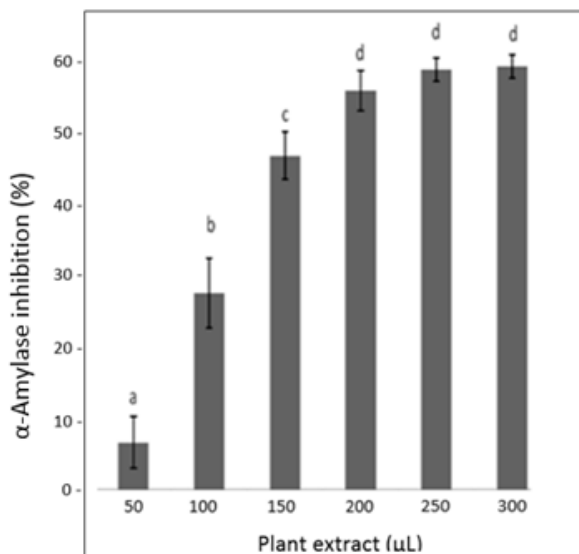
Table 2. α-Glucosidase inhibitory potential of *C. coccineum* aqueous extract.

Doses	Inhibition (%) ±SE
10 µl	96.02 ± 0.05
15 µl	96.92 ± 0.21
20 µl	95.75 ± 0.06
25 µl	96.13 ± 0.15
30 µl	95.89 ± 0.07
35 µl	95.81 ± 0.25
40 µl	95.46 ± 0.10
50 µl	95.53 ± 0.21

Thus, integrating natural inhibitors from dietary edible plants with lower inhibitory effects against α-amylase activity and a stronger inhibitory activity against α-glucosidase can be potentially targeted as a more complimentary and effective therapy for postprandial hyperglycemia control with minimal side effects (Kwon et al. 2006). The result of this *in*

in vitro study indicates a biochemical rationale for why *C. coccineum* has the potential to be highly effective in modulating sugar uptake with a potentially moderate effect on rapid starch break-down.

Figure 2. α -Amylase inhibitory potential of *C. coccineum* aqueous extracts.



Data are mean % \pm SE values (n=9). ^{a-d}Columns with different letters indicate statistically significant differences among groups at $p < 0.05$.

ACE inhibition assay. In an *in vitro* assay, the aqueous extracts from *Cynomorium coccineum*, *Glossonema edule*, and *Malva parviflora* inhibited ACE inhibitory activity, but the aqueous extracts of *Aizoon canariense* did not inhibit ACE. Of the three studied plants, an aqueous extract of *C. coccineum* had a higher ACE inhibitory action, $38.46 \pm 3.38\%$, than *Glossonema edule*, $27.81 \pm 0.96\%$ inhibition, and *Malva parviflora*, $11.12 \pm 6.06\%$ inhibition. The long-term, macrovascular complications of diabetes is hypertension. ACE is an important enzyme in maintaining vascular tension through the activation of and conversion of histidyl-leucine dipeptide to angiotensin II, a vasoconstrictor (Skeggs et al. 1956).

Angiotensin II leads to stimulated synthesis and release of aldosterone, increasing blood pressure by retaining sodium in the distal tubules (Lieberman 1975). Inhibiting ACE is considered a beneficial therapeutic approach in the treatment of high blood pressure in both diabetic and non-diabetic patients.

CONCLUSIONS

The current study indicates a strong possibility that *Cynomorium coccineum*, an edible spice and food plant used in Qatar, could help manage glucose and glucose-induced high levels of reactive oxygen species (ROS) linked to hyperglycemia and type 2 diabetes-associated hypertension. To our knowledge, studies on anti-diabetic potential of *Cynomorium coccineum*, *Aizoon canariense*, and *Glossonema edule* have not previously been studied.

Cynomorium coccineum which grows throughout the Mediterranean region, including North Africa, the Arab peninsula, and beyond, is an edible food and medicinal plant, commonly eaten during famines and prized for medicinal properties. The high antioxidant potential, indicated by the strong DPPH inhibitory potential, plus the total soluble phenolics, potent α -glucosidase inhibition, moderate α -amylase inhibition, and ACE inhibiting potential, indicate that *Cynomorium coccineum* can be used as an edible source for preventing and managing the early stages of type 2 diabetes. The α -glucoside inhibitory activity in the extract of *Cynomorium coccineum* suggest that further investigation of this activity will be worthwhile, including a comparative study with acarbose, the α -glucosidase inhibitory clinical drug.

The moderate antioxidant potential and total soluble phenolics and ACE inhibitory potential demonstrated by *Malva parviflora* and *Glossonema edule* may also be useful for potentially managing hypertension and oxidation-linked vascular complications. Indeed, additional studies may validate the biochemical rationale of traditional edible food plants and overall health benefits of plants that were historically an important part of the Qatari diet.

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