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ORIGINAL ARTICLE

Anti-Advanced Glycation End-product and Free Radical Scavenging Activity of Plants from the Yucatecan Flora

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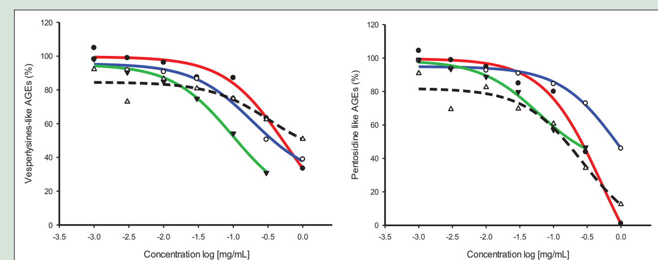
ABSTRACT

Background: Formation and accumulation of advanced glycation end-products (AGE) is recognized as a major pathogenic process in diabetic complications, atherosclerosis and cardiovascular diseases. In addition, reactive oxygen species and free radicals have also been reported to participate in AGE formation and in cell damage. Natural products with antioxidant and antiAGE activity have great therapeutic potential in the treatment of diabetes, hypertension and related complications. **Objective:** to test ethanolic extracts and aqueous-traditional preparations of plants used to treat diabetes, hypertension and obesity in Yucatecan traditional medicine for their anti-AGE and free radical scavenging activities. **Materials and Methods:** ethanolic extracts of leaves, stems and roots of nine medicinal plants, together with their traditional preparations, were prepared and tested for their anti-AGE and antioxidant activities using the inhibition of advanced glycation end products and DPPH radical scavenging assays, respectively. **Results:** the root extract of *C. fistula* (IC_{50}= 0.1 mg/mL) and the leaf extract of *P. auritum* (IC_{50}= 0.35 mg/mL) presented significant activity against vesperlysine and pentosidine-like AGE. Although none of the aqueous traditional preparations showed significant activity in the anti-AGE assay, both the traditional preparations and the ethanolic extracts of *E. tinifolia*, *M. zapota*, *O. campechianum* and *P. auritum* showed significant activity in the DPPH reduction assay. **Conclusions:** the results suggest that the metabolites responsible for the detected radical-scavenging activity are different to those involved in inhibiting AGE formation; however, the extracts with antioxidant activity may contain other metabolites which are able to prevent AGE formation through a different mechanism.

Key words: Antioxidant activity, diabetes, glycation-end products, traditional medicine

SUMMARY

- Ethanolic extracts from nine plants used to treat diabetes, hypertension and obesity in Yucatecan traditional medicine were tested for their anti-AGE and free radical scavenging activities.
- Significant activity against vesperlysine and pentosidine-like AGE was detected in the root extract of *Cassia fistula* and the leaf extract of *Piper auritum*.
- Traditional preparations and the ethanolic extracts of *Ehretia tinifolia, Manilkara zapota, Ocimum campechianum* and *Piper auritum* showed significant activity in the DPPH reduction assay.
- Results suggest that the metabolites responsible for the detected radical-scavenging activity are different to those involved in inhibiting AGE formation.



Abbreviations Used: AGE: Advanced glycation end-product; DPPH: 2,2-Diphenyl-1-picrylhydrazyl; DM: Diabetes mellitus; ROS: Reactive oxygen species; BSA: Bovine serum albumin; EtOH: Ethanol; EtOAc: Ethyl acetate; ANOVA: Analysis of variance; BA: *Brosimum alicastrum*; BS: *Bunchosia swartziana*; CF: *Cassia fistula*; CN: *Cocos nucifera*; ET: *Ehretia tinifolia*; MZ: *Manilkara zapota*; OC: *Ocimum campechianum*; PA: *Piper auritum*; RM: *Rhizophora mangle*; L: Leaves; S: Stems; R: Roots; T: traditional preparation; I: Inflorescences; W: Water

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INTRODUCTION

Advanced glycation end-products (AGEs) are a result of nonenzymatic reactions called Maillard reaction.[1] These reactions are initiated by a nucleophilic addition between the free amino group of a protein and a carbonyl group from a reducing sugar to produce, in a matter of hours, a reversible Schiff's base that can rearrange within a few days to a more stable and irreversible ketoamine or Amadori product.[2] This rearrangement is followed by a series of cascade reactions that include dehydration, oxidation, condensation, and cyclization to form cross-linked fluorescent and nonfluorescent AGEs.[3-5] Since the formation of AGEs depends on the presence of a sugar and a protein, hyperglycemic conditions, such as those in diabetes, favor AGE formation, causing alterations in the physicochemical and physiological functions of proteins, and ultimately resulting in cell damage.[6,7] The important role played by AGEs in the pathogenesis of microvascular damage that includes nephropathy, retinopathy, neuropathy, and cataracts in diabetes mellitus (DM) is well recognized;

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similarly, the high levels of AGEs and their receptors have been detected in obese individuals.[8] AGEs are also known to be involved in the development of Alzheimer's and other neurological diseases,[9] as well as in cardiovascular diseases, including atherosclerosis,[10] endothelial dysfunction, and vasorelaxation.[11]

Since the formation of AGEs is characteristic of individuals with DM, where their formation and accumulation develop at an accelerated rate and correlate with the severity of the complications, and are related to cell damage due to an increase in the production of reactive oxygen species involved in the auto-oxidation of glucose and reactions of the nonenzymatic protein glycosylation pathway,[12-15] antioxidants capable of radical scavenging or metal chelation,[16,17] as well as metabolites able to trap dicarbonyl species or break AGEs, have the potential to limit or reduce the amount of AGEs and their related complications.[12]

It has been reported that metabolites with anti-AGE and/or antioxidant activity can effectively protect against glucose-induced cytotoxicity.[18] However, although numerous AGE inhibitors, including the well-known aminoguanidine, ameliorated diabetic complications in an animal model, they also caused severe side effects including gastrointestinal disturbance, anemia, and flu-like symptoms.[19] Natural products represent an important option for the discovery and development of new anti-AGE pharmaceuticals; these can include metabolites with antioxidant activity since oxidative stress is involved and accelerates the formation of AGEs.[20] Plants, because of the complexity of their secondary metabolism that results in a great chemical diversity of natural products, are recognized as an important source of bioactive metabolites.[21] In addition, since many plants are traditionally used to treat diseases such as diabetes and others disorders associated with AGE formation, these particular species constitute a potentially important source for the search of novel secondary metabolites with anti-AGE activity.[22] We wish to report herein on the results of evaluating the anti-AGEs and radical scavenging activity of the organic extracts and aqueous traditional preparations of nine plants used in Yucatecan traditional medicine to treat diabetes, hypertension, and obesity.

MATERIALS AND METHODS

Collection of plant material

Plant material was collected in October–November 2013 and identified by field specialist Paulino Simá-Polanco; a voucher specimen of each species was deposited in the Herbarium of Unidad de Recursos Naturales CICY under the appropriate collection number: *Brosimum alicastrum* (BAL) Swartz (P. Simá 3153), *Bunchosia swartziana* Griseb (P. Simá 3155), *Ehretia tinifolia* (L.) (P. Simá 3152), and *Manilkara zapota* (L.) P. Royen (P. Simá 3154) were collected in Mopila, a community of Yaxcabá, Yucatán, México. Plants of C*assia fistula* (L.) (P. Simá 3158) were collected on the road from Dzitya to Chablekal, Yucatán, México, 200 m from the entrance of "Universidad de Chapingo"; fruits and inflorescences of *Cocos nucifera* (L.) (P. Simá 3166) were collected from a study site in San Crisanto, Yucatán, México; *Ocimum campechianum* Willdenow (P. Simá 3157) was collected on the road from Sierra Papacal to Chuburná Puerto, Yucatán, México, 100 m from the entrance to "Parque Científico y Tecnológico de Yucatán"; *Piper auritum* Kunth (P. Simá 3159) was collected in a private home in Baca, Yucatán, México; and *Rhizophora mangle* (L.) (P. Simá 3156) was collected in Chuburná Puerto, Yucatán, México.

Preparation of plant extracts

Leaves, stems, and roots of each species (inflorescences only in the case of *C. nucifera*) were first dried for 3 days at room temperature and then for 3 days in an oven at 50°C. The plant material was ground and extracted by maceration (three times) with ethanol (400 ml/20 g of plant material), at room temperature, for 72 h. Only water from *C. nucifera* was extracted by liquid-liquid extraction with ethyl acetate in the ratio of 2:1 (v/v, three times). The plant material was eliminated by successive filtration through cheesecloth and filter paper (Whatman No. 1), and the ethanol filtrates were eliminated under reduced pressure to produce the corresponding crude extracts. For the traditional preparations, an infusion of the dry plant material commonly used in traditional medicine was prepared by adding it to boiling water (20 g/L) and allowing it to stand for 20 min. The plant material was eliminated by filtration and the resulting aqueous extract was frozen and lyophilized; in the case of *C. nucifera*, since water is traditionally used as a remedy, water from a coconut was filtered, frozen, and lyophilized to produce the corresponding crude extract of the aqueous traditional preparation. The yields of the extracts are listed in Table 1.

Inhibition of anti-advanced glycation end products

The anti-AGE assay was carried out following the methodology previously reported by Séro *et al.*[5] Briefly, the samples (1 μg to 1 mg) were incubated with D-ribose (0.5 M) and bovine serum albumin (BSA, 10 mg/ml) in a Na-phosphate buffer (50 mM pH 7.4); the solutions were incubated in black microtiter plates (96 wells) at 37°C for 24 h in a closed system before AGE fluorescence measurement. Both vesperlysines-like (λ_{exc} 370 nm; λ_{em} 440 nm) and pentosidine-like (λ_{exc} 335 nm; λ_{em} 385 nm) AGE fluorescence were measured using a microplate spectrofluorometer Infinite M200 (Tecan, Lyon, France).

To avoid quenching phenomena, the fluorescence resulting from the incubation in the same conditions of BSA (10 mg/ml) and the tested extract (1 μg to 1 mg) was subtracted from each measurement. The negative control, i.e. 100% inhibition of AGEs formation, consisted of wells with only BSA. A control, i.e. no inhibition of AGEs formation, consisted of wells with BSA (10 mg/ml) and D-ribose (0.5 M). The final volume assay was 100 μL. The percentage of AGE formation was calculated as follows for each extract concentration: AGEs (%) = (fluorescence intensity [sample] – fluorescence [blank of sample]/fluorescence intensity [control] – fluorescence [blank of control]) ×100.

Dose–effect curves were best fit with a sigmoidal dose–response equation using Sigma Plot 12.5 software (Systat Software GmbH: Erkrath, Germany), which enabled calculation of the IC_{50} values. Results were compared with those of reference products, aminoguanidine and quercetin.

Antioxidant activity by the 2, 2-Diphenyl-1-picrylhydrazyl radical scavenging method

The assay was carried out following the method reported by Brand-Williams *et al.*,[23] with modifications and scaled to a 96-well microplate. For the quantitative analysis, different test solutions (1×10^{-1}, 1×10^{-2}, 1×10^{-3}, and 1×10^{-4} mg/mL) of the various crude extracts were prepared and 20 μL of the test solution was combined with 180 μL of a 0.1 mM solution of 2, 2-Diphenyl-1-picrylhydrazyl (DPPH) in EtOH. The 96-well microplate was left to stand for 20 min in dark, and the absorbance was determined by reading at 540 nm in a microplate reader (ELx800-Biotek). Vitamin C (ascorbic acid) solutions were similarly prepared and used as positive control. A mixture of EtOH (20 μL) and DPPH solution (20 μL) was used as blank. The DPPH radical reduction percentages were calculated using the formula: DPPH radical reduction (%) =1 – ([absorbance of sample/absorbance DPPH] ×100).

Table 1: Results of anti-advanced glycation end-products and 2, 2-Diphenyl-1-picrylhydrazyl radical scavenging activity for organic extracts and traditional aqueous preparations

Scientific name	Medicinal use	Code[a]	Extract type[b]	Yield (%)	Anti-AGEs IC_{50} (mg/mL)		DPPH EC_{50} (μg/mL)
					Vesperlysine-like AGEs	Pentosidine-like AGEs	
Moraceae	Diabetes, obesity	BAL	EtOH	10.0	0.5	0.6	>300
Brosimum alicastrum L.		BAS	EtOH	4.4	>1	>1	>300
		BAR	EtOH	6.4	0.4	1.0	>300
		BAT	H_2O	17.2	>1	>1	>300
Malpighiaceae	Hypertension	BSL	EtOH	7.4	1	>1	>300
Bunchosia swartziana G.		BSS	EtOH	4.8	>1	>1	>300
		BSR	EtOH	7.7	>1	>1	290±1
		BST	H_2O	18.0	>1	>1	>300
Fabaceae	Diabetes	CFL	EtOH	21.0	0.5	0.2	287.5±3
Cassia fistula L.		CFS	EtOH	17.0	0.2	0.9	216.6±15
		CFR	EtOH	26.0	0.1	0.1	250±17
		CFT	H_2O	28.0	>1	>1	>300
Arecaceae	Diabetes, obesity	CNI	EtOH	18.0	>1	>1	220±14
Cocos nucifera L.		CNW	AcOEt	12.0	>1	>1	>300
		CNT	H_2O	7.8	>1	>1	>300
Boraginaceae	Hypertension	ETL	EtOH	12.0	0.7	>1	>300
Ehretia tinifolia L.		ETS	EtOH	5.0	1	0.3	100±0.9*
		ETR	EtOH	4.0	>1	>1	>300
		ETT	H_2O	14.0	>1	>1	139.5±0.7*
Sapotaceae	Diabetes, Hypertension	MZL	EtOH	8.0	0.4	0.8	98.5±10*
Manilkara zapota V.		MZS	EtOH	12.0	>1	>1	190±14
		MZR	EtOH	17.0	0.4	0.7	107.5±0.7*
		MZT	H_2O	22.0	0.5	1	165±7*
Lamiaceae	Diabetes, hypertension	OCL	EtOH	24.0	>1	>1	235±7
Ocimum campechianum W.		OCS	EtOH	11.0	>1	>1	>300
		OCR	EtOH	8.0	>1	>1	>300
		OCT	H_2O	23.0	>1	>1	150±28*
Piperaceae	Diabetes	PAL	EtOH	10.0	0.35	0.35	135±7*
Piper auritum K.		PAS	EtOH	12.0	>1	>1	>300
		PAR	EtOH	7.0	>1	>1	155±7*
		PAT	H_2O	22.0	>1	>1	279.5±13
Rhizophoraceae	Diabetes	RML	EtOH	30.0	0.3	>1	200±14
Rhizophora mangle L.		RMS	EtOH	11.0	0.45	>1	210±35
		RMR	EtOH	15.0	>1	>1	245±1.4
		RMT	H_2O	12.0	>1	>1	>300
Aminoguanidine	Anti-AGEs	-	Control		1.0	0.17	-
Ascorbic acid	Antioxidant	-	Control		-	-	51±1.4

[a]First two letters refer to the genus and species of each plant; the third letter corresponds to - L: Leaf; R: Root; S: Stem; I: Inflorescence; W: Water; T: Traditional preparation; [b]EtOAc: Ethyl acetate; EtOH: Ethanol; H_2O: Aqueous; *P<0.05; AGEs: Advanced glycation end-products; DPPH: 2, 2-Diphenyl-1-picrylhydrazyl

The different concentrations were plotted against the decoloration percentages, and a lineal regression statistical analysis (Excel) of the resulting curve allowed the expression of the antioxidant activity of each sample in terms of its EC_{50} value. All samples were analyzed in triplicate in three different days.

Data analysis

To determine the most active samples in the antioxidant assay, statistical treatment of the data was carried out using the Sigma Plot 10.0 software, with a statistical significance between the mean differences of $P < 0.05$ by analysis of variance with Tukey's *post hoc* test.

RESULTS AND DISCUSSION

The anti-AGE assay comprised the simultaneous quantification of both vesperlysines-like (λ_{exc} 370 nm; λ_{em} 440 nm) and pentosidine-like (λ_{exc} 335 nm; λ_{em} 385 nm) AGEs. It has been reported that on evaluating the anti-AGE activity of extracts, semipurified fractions, or pure metabolites, samples must be tested for their anti-AGE activity against both vesperlysines and pentosidine-like AGEs, since interference between AGEs and the samples can occur.[5] In this investigation, extracts were considered to be active when a decrease in the fluorescence of at least one wavelength was observed and when the IC_{50} was below than that of the natural product quercetin. Evaluation of the anti-AGE activity of the ethanol extracts and aqueous traditional preparations of nine Yucatecan medicinal plants traditionally used to treat diabetes, hypertension, and obesity showed that the root extract of *C. fistula* (CFR) presented the highest inhibition activity against vesperlysine and pentosidine-like AGEs (IC_{50} = 0.1 mg/mL for both), higher even than the control aminoguanidine (IC_{50} = 1.0 and 0.17 mg/mL) [Table 1]; the organic extracts from the leaves and stems of *C. fistula* (CFS), together with those of the leaves of *B. alicastrum* (BAL), stems of *E. tinifolia* (ETS), leaves of *P. auritum* (PAL), and leaves of *R. mangle* (RML) showed anti-AGEs activity, but only against vesperlysine-like AGEs (IC_{50} = 0.50, 0.20; 0.5, 0.2; 0.35, and 0.3 mg/mL, respectively) [Table 1].

Alternatively, testing of the same group of samples for their antioxidant activity using the DPPH radical scavenging test showed

a significant level of activity in the organic extracts from the stem of *E. tinifolia* (ETS) (EC_{50} = 100 ± 9 μg/mL), the leaves of *M. zapota* (MZL) and roots of *M.zapota* (MZR) (EC_{50} = 98 ± 10 and 107 ± 7 μg/mL, respectively), the leaves (PAL) and roots (PAR) of *P. auritum* (EC_{50} = 135 ± 7 and 155 ± 7 μg/mL, respectively), and the aqueous traditional preparations of *E. tinifolia* (ETT), *M. zapota* (MZT), and *O. campechianum* (OCT) (EC_{50} = 139 ± 0.7, 165 ± 7, and 150 ± 28 μg/mL, respectively) [Table 1].

It has been reported that the antioxidant effect of plant products is mainly due to the radical scavenging activity of phenolic metabolites such as flavonoids, tannins, and phenolic terpenes,[24] and it has been reported that crude extracts and pure metabolites with antioxidant properties often show anti-AGE activity.[17,25] However, the unexpected results obtained in this study, where these two biological activities are not necessarily associated with each other, suggest that there might be other mechanisms (e.g. trapping of dicarbonyl species or AGE-breaking) involved in the anti-AGE activity of the antioxidant extracts. The root extract of *C. fistula* (CFR) exhibited the best anti-AGE activity, with results similar to those reported in the literature for bioactive plant crude extracts.[5] Even though to date, there are no reports on the anti-AGE activity of *C. fistula,* a high anti-AGE activity has been reported for the flower extract of *C. auriculata*.[26] In addition, anthraquinones isolated from the seeds of *Cassia tora* also exhibited a strong inhibitory activity against AGE formation,[27] while phytochemical studies carried out on the roots of *C. fistula* showed the presence of rhamnetin 3-O-gentibioside,[28] and the secondary metabolite composition of different parts of the plant is recognized as being phenolic antioxidants such as anthraquinones, flavonoids, and flavan-3-ol derivatives.[29]

The anti-AGE and antioxidant activity detected in the organic extract from the leaf extract of *P. auritum* (PAL) coincides with reports on the literature that the strong anti-AGE activity in the hexane leaf extract of the plant, when tested in both *in vitro* and *in vivo* models, is related to its antioxidant activity.[30] However, the radical scavenging activity of the leaf extract of *P. auritum* is not in agreement with previous reports in the literature describing low levels of antioxidant activity in the essential oil of *P. auritum*, which was found to contain metabolites such as safrole, myristicin, *D*-germacrene and *β*-caryophyllene,[31] or the lack of antioxidant activity of the methanol and aqueous extracts from *P. auritum*.[32]

CONCLUSIONS

The results of this investigation contribute to the knowledge about the biological activity in species that are commonly used to treat diabetes, hypertension, and obesity in the Yucatan peninsula. Additional studies are necessary to identify the metabolites responsible for the biological activity detected in the root extract of *C. fistula* (CFR).

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Conflicts of interest

There are no conflicts of interest.

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