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Inhibitory Effects of Several Spices on Inflammation Caused by Advanced Glycation Endproducts

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ABSTRACT

Advanced glycation endproducts (AGEs) is implicated in the pathogenesis of diabetic complications. Inhibiting the formation of AGEs and interfering with AGEs-mediated inflammation are two practicable strategies for developing a dietary adjuvant against diabetic complications. This study evaluated the protective capacities against diabetic complications of several spices based on their inhibition of the formation of AGEs in an *in vitro* BSA/glucose system and on the AGEs-induced production of proinflammatory cytokine in RAW 264.7 macrophages. Among the tested spices, cinnamon exhibited most strongly inhibited both the formation of AGEs and the AGEs-induced production of nitric oxide, interleukin-6 and tumor necrosis factor-α. Additionally, correlative results revealed that the capacity of spices to inhibit the formation of AGEs is attributable to phenolic compounds and, in contrast, the capacity to inhibit AGEs-induced inflammation is attributable to condensed tannin. This investigation demonstrates the potential of cinnamon to serve as a dietary adjuvant against diabetic complications.

Keywords: Advanced Glycation End-Products; Diabetic Complication; Inflammation; Spices

1. Introduction

During recent decades, growing evidence has shown that advanced glycation end-products (AGEs) participate importantly the pathogenesis of diabetic complications [1,2]. AGEs are a group of deleterious heterogeneous end products of nonenzymatic glycation. Covalent modification of protein and induction of oxidative stress and inflammatory response are two well-known deleterious effects of AGEs. Proteins, particularly those long-lived extracellular matrix molecules, when covalently crosslinked with AGEs result in undergo a structural change and exhibit functional abnormality [1]. Secondly, AGEs can disrupt cellular function by binding to specific cell surface receptors. One of the most extensively studied receptors is the receptor for AGEs (RAGE). The engagement of AGEs with RAGE triggers NADPH-oxidase, increasing intracellular oxidative stress, evoke signaling pathways such as protein kinase C, mitogen-activated protein kinase and the extracellular signal regulated kinase; activate transcription factor NF-κB and AP-1, and subsequently induce thrombogenic, fibrogenic, adhesive, chemoattractive and proinflammatory gene expression [3]. This AGEs-induced oxidative stress, fibrosis and inflammation accelerate the progress of diabetic vascular complications [4]. Accordingly, the inhibition of

AGE formation (anti-glycation), the blocking of AGE-RAGE interactions, and the suppression of RAGE expression or its downstream pathways have potential for use in therapeutic interventions against diabetic vascular complications. Indeed, agents that block the formation of AGEs, including aminoguanidine, thiamine and pyridoxine, and those that suppress AGEs-induced inflammation such as secreted form of RAGE (sRAGE), which blocks RAGE, have yielded promising results in animal models [5].

Spices that are rich in phytochemicals with anti-oxidant, anti-inflammatory, anti-carcinogenic, anti-microbial, hypolipidemic and other beneficial physiological properties, are extensively used in folk medicines for treating numerous chronic diseases and diabetes [6]. Although the beneficial effects of spices on diabetes have been studied for a long time, most researchers have focused on their hypoglycemic effect. The possible protective effects of spices against diabetic complications, especially regarding AGEs-induced inflammation, are few. It has been showed that AGEs can activate monocytes/macrophages to secret many chemoattractive and proinflammatory cytokines such as IL-1, IL-6, TNF-α and the inducible nitric oxide synthase (iNOS) by activation of transcription factor, NF-κB [7,8]. To this end, in this investigation, the protective properties of several culinary spices against

diabetic complications were determined from studying their capacity to inhibit the formation of AGEs *in vitro* and to inhibit AGEs-induced production of proinflammatory mediators such as nitric oxide (NO), interleukin-6 (IL-6) and tumor necrosis factor-α (TNF-α) in RAW 264.7 macrophages.

2. Materials and Methods

2.1. Chemicals Used

Folin-Ciocalteu phenol reagent, catechin, gallic acid, dimethyl sulfoxide (DMSO), bovine serum albumin (BSA), D-glucose, aminoguanidine, sulphanilamide, naphthylethylenediamine, 3-(4,5-dimethyldiazol-2-yl)-2,5 diphenyl tetrazolium bromide (MTT) and nitro blue tetrazolium chloride/5-bromo-4-chloro-3-indolyl phosphate p-toluidine salt (NBT/BCIP) tablets were purchased from Sigma Chemical Co. (St. Louis, MO, USA). All chemicals were analytical grade.

2.2. Preparation of Spice Extract

All of the tested dried spices were commercial products of Tomax Co. (Taiwan) and purchased from a local supermarket (Hsinchu, Taiwan) in the winter of 2009. Extraction was conducted by mixing 10 g of the dried spice powder with 300 ml methanol in a glass flask and then shaking violently (100 rpm) at 37°C for 20 h. After the suspension had been filtered through No. 2 filters, the filtrate was evaporated under a vacuum at a temperature of lower than 50°C to remove the methanol. Finally, the extracted solids were weighed and dissolved in DMSO.

2.3. Determination of Phenolic Compound, Flavonoid and Condensed Tannin

The Folin-Ciocalteu colorimetric approach and the aluminium chloride colorimetric method were utilized to measure the concentration of phenolic compounds and flavonoid in the spice extracts, respectively [9]. The content of condensed tannin was determined by using vanillin method according to a previous literature [10].

2.4. Evaluation of Antiglycating Capacity of Spice Extracts

An *in vitro* BSA/glucose glycation system of Vinson & Howard was adopted [11]. Briefly, spice extracts or aminoguanidine were incubated with 20 mg/ml BSA, 500 mM glucose and 0.02% (w/v) sodium azide in phosphate buffer (100 mM, pH 7.4) at 37°C for 3 weeks. The fluorescent intensity was measured using a fluorescent spectrometer with exciting and emission wavelengths of 330 and 410 nm, respectively. The antiglycating capacity (%) of spice extract was calculated as the difference be-tween the fluorescent intensity of the spice glycation solution with glucose and that without glucose as a proportion of the difference between the fluorescent intensity of DMSO glycation solution with glucose and that without glucose.

2.5. Preparation of AGEs-Modified Albumins Used in Cell Culture

The AGEs-modified albumins that were used in cell culture experiments were prepared following the method of Berbaum *et al.* [7].

2.6. Cell Culture Experiment

RAW 264.7 macrophages were obtained from Bioresource and BioResearch Center (Hsinchu, Taiwan) and cultured in DMEM medium that was supplemented with 10% fetal bovine serum (Invitrogen, Carlsbad, CA, USA). 1×10^5 cells were seeded into each well of a 96-well culture plate and cultured overnight. After washing with PBS, medium with spice extract and AGEs-modified albumins were added and the system was incubated for 24 h. The negative control presents the cells without any treatment. The cells which only treated with AGEs-modified albumins were assigned as positive control. The conditioned medium was collected, and the cell viability was evaluated by the MTT method.

2.7. Measurement of Amounts of Secreted NO, IL-6 and TNF-α

Nitrite concentration as an indicator of NO production was measured using the Griess reagent. The concentrations of IL-6 and TNF-α in the conditioned medium were measured using commercial ELISA kits (eBioscience, San Diego, CA, USA) following the manufacturer's instructions.

2.8. Measurement of Protein Levels of Inducible Nitric Oxide Synthase (iNOS)

After incubation with spice extract and AGEs-modified albumins for 12 h, the adherent cells were washed with PBS, collected, suspended in the lysis buffer (50 mM Tris, pH 7.6, 0.01% ethylenediaminetetraacetic acid (EDTA), 1% Triton X-100, 1 mM phenylmethanesulfonyl fluoride, and 1 µg/ml leupeptin) and centrifuged at 12,000 × g for 20 min at 4°C. The protein level of cellular iNOS was determined by western blotting and normalized to β-actin as described previously [12].

2.9. Measurement of mRNA Levels of iNOS

After they had been incubated with 200 µg/ml spice extract and 1 mg/ml AGEs-modified albumins for 6 h, ad-

herent cells were collected and the total RNA was isolated using Trizol reagent (Invitrogen, Carlsbad, CA, USA). The mRNA level of iNOS was determined using the reverse transcription polymerase chain reaction and performed as described previously [12].

2.10. Statistical Analysis

All experimental results are expressed as mean ±SD from three independent tests. The significance of the differences between treatments was evaluated by ANOVA, and then Duncan's multiple range test was performed to make multiple comparisons. The condition for significance of the difference between datasets was $p < 0.05$. The correlation between two variants was analyzed by performing the Pearson test. All statistical analyses were conducted using SPSS software (SPSS 12.0 for Windows; SPSS Inc., Chicago, IL, USA).

3. Results

3.1. Concentration of Phenolic Compound, Flavonoid, and Condensed Tannin

As listed in **Table 1**, the amounts of phenolic compounds in different spice extracts varied considerably. Cinnamon extract contained the most abundant phenolic compounds, whereas cumin and parsley extracts contained the least phenolic compounds. The amounts of flavonoid and condensed tannin varied significantly. Thyme contained the most flavonoid. Cumin contained the least—only 1.1 mg CEs/g, or roughly 1.8% of that in thyme. Cinnamon, cumin and rosemary contained more than 10 mg CEs/g, and were rich in condensed tannin. The concentration of condensed tannin in cardamom extract was lower than the detection limit.

3.2. Anti-Glycating Capacities of Spice Extracts

As listed in **Table 2**, aminoguanidine, a well-known in-

hibitor of the formation of AGEs, exhibited complete inhibition at concentrations over 100 μg/ml and exhibited 74.4% inhibition of the formation of fluorescent AGEs at a concentration of 50 μg/ml. All spice extracts suppressed the glucose-mediated formation of fluorescent AGEs in a dose-dependent manner at concentrations of 50 - 200 μg/ml. For ease of comparison, the IC50, which is the concentration required to inhibit the formation of fluorescent AGEs by 50%, was calculated. The anti-glycating capacities of the spice extracts, given by IC50 declined in the order; cinnamon, thyme, turmeric, rosemary, basil, parsley, cumin and cardamom.

3.3. Effects of Spice Extracts on AGEs-Stimulated Secretion of NO, IL-6 and TNF-α

The viabilities of all cells that were treated with any of the spices exceeded 95% of that of the DMSO control group (data not shown). This result indicated that the spice extracts at the concentrations (50 - 200 μg/ml) used herein were not cytotoxic. **Figure 1** presents the effect of the spice extracts on the AGEs-induced secretion of NO. As described elsewhere [7,8], treating RAW 264.7 macrophages with AGEs-modified albumins (1 mg/ml) induced substantial NO secretion. Additionally, co-treatment of the cells with AGEs-modified albumins and various concentrations of the spice extracts significantly inhibited NO production in a concentration-dependent manner. Based on the results obtained at a concentration of 200 μg/ml, the capacities of the different spice extracts to inhibit the AGEs-induced secretion of NO declined in the order; cinnamon, rosemary, cumin, thyme, basil, turmeric, cardamom and parsley. The inhibition by spice extracts of IL-6 and TNF-α secretion was weak at treatment concentrations of less than 200 μg/ml, so only the results of treatment at concentration of 200 μg/ml are presented in **Figure 2**. All of the tested spice extracts

**Table 1. Concentrations of phenolic compound, flavonoid and condensed tannin.**

Common name	Botanical name	Phenolic (mg GAEs/g extract)	Flavonoid (mg CEs/g extract)	Condensed tannin (mg CEs/g extract)
Basil	*Ocimum basilicum*	68.9 ± 1.0[d]	24.8 ± 0.3[d]	9.3 ± 1.9[bcd]
Cardamom	*Elettaria cardamomum*	69.7 ± 1.1[d]	11.6 ± 0.6[e]	ND[a]
Cinnamon	*Cinnamomum verum*	226.7 ± 7.0[a]	59.7 ± 2.2[b]	14.7 ± 7.2[d]
Cumin	*Cuminum cyminum*	39.4 ± 1.8[e]	1.1 ± 0.0[f]	12.5 ± 2.3[cd]
Parsley	*Petroselinum crispum*	45.1 ± 1.0[e]	2.5 ± 0.1[f]	6.5 ± 2.2[abc]
Rosemary	*Rosmarinus officinalis*	93.6 ± 0.3[c]	41.2 ± 1.7[c]	12.0 ± 1.0[cd]
Thyme	*Thymus vulgaris*	141.1 ± 0.7[b]	62.4 ± 1.3[a]	4.6 ± 2.0[ab]
Turmeric	*Curcuma longa*	135.7 ± 9.2[b]	40.4 ± 1.3[c]	1.7 ± 5.4[a]

Means not sharing a common letter in the same column were significantly different ($p < 0.05$) when analyzed by ANOVA and Duncan's multiple range test.

Table 2. Antiglycating capacities of spice extracts.

Treating conc.	Inhibitory capacity (%)			IC$_{50}$ (µg/mL)
	200 µg/mL	100 µg/mL	50 µg/mL	
Basil	80.7 ± 0.9^d	59.2 ± 1.1^d	37.5 ± 2.0^d	83.7
Cardamom	68.6 ± 0.9^f	45.7 ± 2.6^e	24.7 ± 1.5^f	129.5
Cinnamon	99.3 ± 1.4^a	88.7 ± 1.4^a	62.6 ± 1.1^a	26.0
Cumin	81.6 ± 0.9^d	48.9 ± 2.0^e	25.2 ± 1.1^f	111.6
Parsley	74.5 ± 1.5^e	57.7 ± 5.0^d	31.9 ± 1.4^e	99.1
Rosemary	91.1 ± 1.9^b	69.0 ± 0.4^c	46.0 ± 1.8^c	58.8
Thyme	94.9 ± 0.3^b	78.1 ± 1.3^b	52.9 ± 3.9^b	44.2
Turmeric	89.9 ± 0.6^c	72.4 ± 0.5^c	49.7 ± 0.3^b	50.6
	800 µM	400 µM	200 µM	
Aminoguanidine	102.9 ± 2.2	86.4 ± 1.7	60.3 ± 2.8	-

Means not sharing a common letter in the same column were significantly different ($p < 0.05$) when analyzed by ANOVA and Duncan's multiple range test.
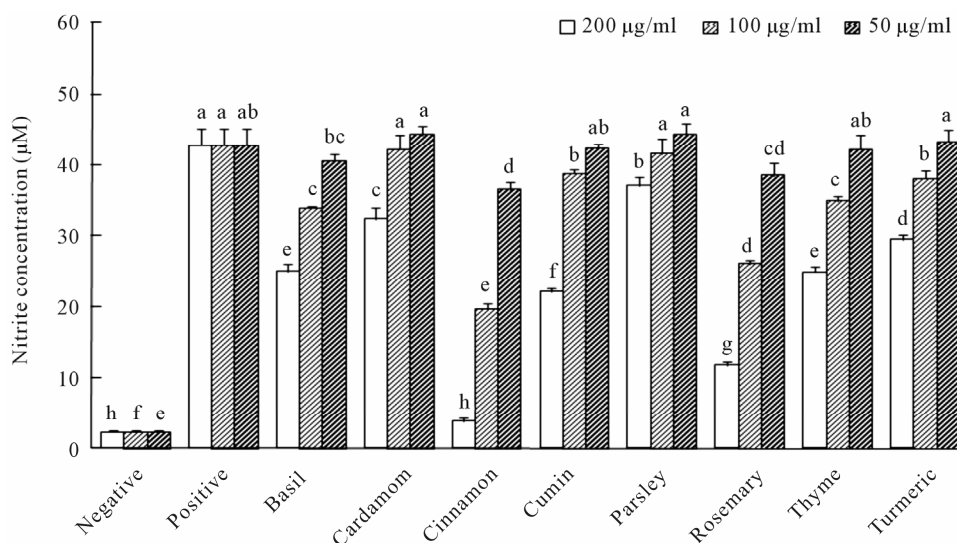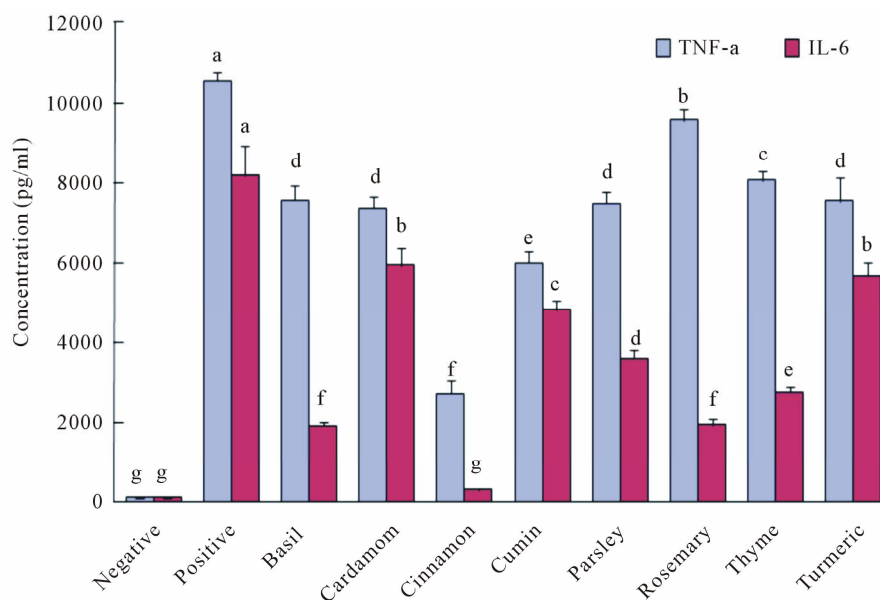

Figure 1. Effect of spice extracts on NO production in AGE-stimulated RAW264.7 cells.

significantly inhibited IL-6 secretion. Cinnamon almost completely inhibited AGEs-induced IL-6 secretion and was the most potent of the tested spices. Spice extracts had less of a capacity to inhibit AGEs-stimulated TNF-α secretion in RAW264.7 macrophages than they did to inhibit the secretion of NO and IL-6. Similarly, cinnamon was the most effective inhibitor of AGEs-stimulated TNF-α secretion.
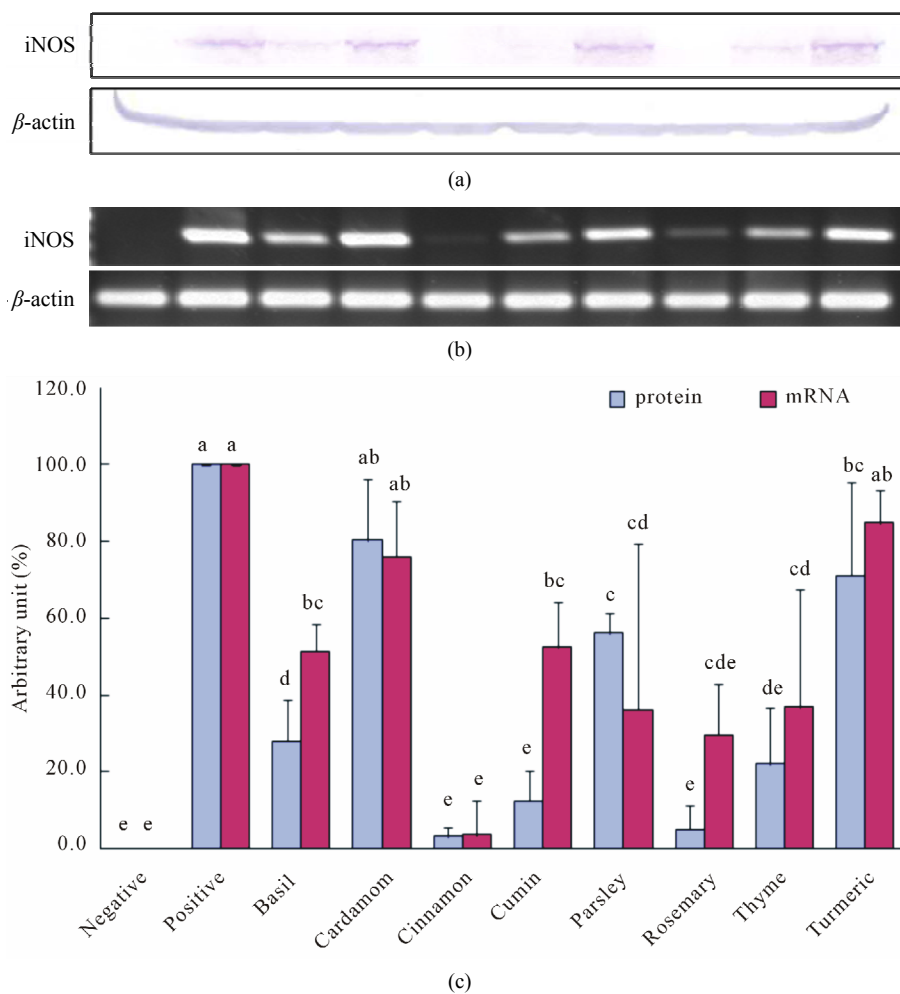
3.4. Effects of Spice Extracts on Protein and mRNA Levels of iNOS in AGEs-Stimulated RAW264.7 Cells

To investigate the mechanism of the inhibition by the spice extract on AGEs-induced NO secretion, the protein and mRNA levels of iNOS were evaluated. As shown in

Figure 3, AGEs-modified albumins induced significant expression of iNOS protein in RAW264.7 cells (positive) and except for cardamom, this expression was attenuated by co-incubation with spice extracts. At a concentration of 200 µg/ml, the capacities of the spice extracts to inhibit the expression of iNOS protein declined in the order cinnamon, rosemary, cumin, thyme, basil, parsley and turmeric. Similarly, AGEs-modified albumins induced iNOS mRNA expression, which was inhibited by spice extracts. As expected, the amounts of NO secreted were strongly correlated with iNOS protein levels (r = 0.849, p = 0.008) and the iNOS mRNA levels (r = 0.794, p = 0.019). These results indicated that spice extracts inhibited NO secretion mostly by down-regulating gene expression of iNOS.

Figure 2. Effect of spice extracts on TNF-*α* and IL-6 production in AGE-stimulated RAW264.7 cells.

Figure 3. Effect of spice extracts on protein and mRNA levels of iNOS in AGE-stimulated RAW264.7 cells. (a) Protein levels; (b) mRNA levels; (c) Quantitative results.

3.5. Correlative Analysis

The concentrations of both phenolic compound (r = 0.922, p = 0.001) and flavonoid (r = 0.943, p < 0.001) in a particular spice extract were significantly correlated with its antiglycating capacity. However, interestingly, the concentration of condensed tannin in a particular spice extract was correlated with its capacity to inhibit the AGEs-induced production of NO (r = 0.852, p = 0.007) and IL-6 (r = 0.736, p = 0.037).

4. Discussion

In this investigation, all eight of the tested spices inhibited the formation of AGEs *in vitro* to varying degrees, and their antiglycating capacities were correlated with the concentration of the phenolic compound. Consistent with earlier studies [13], the result supports the hypothesis that antioxidant phenolic compounds contribute to the antiglycation capacity of spices to an extent that depends strongly on their amount. Among the tested spices, cinnamon most strongly inhibited the formation of AGEs. The mechanisms by which cinnamon inhibits the formation of AGEs include antioxidation and the trapping of reactive carbonyl species [14]. Due to concern of safety, the evaluation and development of dietary inhibitors of the formation of AGEs has become an important trend in food research. Saraswat *et al.* [15] recently evaluated 17 dietary plants with strong antioxidant properties and found that three spices-ginger, cumin and cinnamon-exhibited marked antiglycation activity, and could reduce the formation of different AGEs *in vitro*. Additionally, the antiglycating property of spices has been demonstrated in vivo and suggested to be responsible for the slowing of the development of diabetic complications [16]. For example, cumin has been shown to inhibit glycation and maintain the chaperone activity of α-crystallin, and thereby, to delay the progress of cataract in diabetic rats [17].

More importantly, in this investigation, spices were found also to inhibit the AGEs-induced production of NO, IL-6 and TNF-α. Of the tested spices, cinnamon, rosemary and cumin were the most effective inhibitors thereof. The amounts of condensed tannin in the tested spices correlated significantly with the capacities of the spices to inhibit the AGEs-induced production of NO and IL-6. This result implies that the inhibition by spices of AGEs-induced inflammatory response primarily involved condensed tannin. Berbaum *et al.* [7] indicated that AGEs and lipopolysaccharide (LPS) follow similar or convergent downstream signaling pathways to induce the expression of proinflammatory genes in RAW264.7 macrophages. Indeed, in the authors' earlier investigation, spices were shown to reduce the production of NO and the expression of iNOS in LPS-activated RAW264.7

macrophages [18]. Additionally, based on the previous studies, the possible active components present in spices could be reasonably posited. Trans-cinnamaldehyde has been proven to be the active component that was responsible for the anti-inflammatory activity of cinnamon methanolic extract by considering the LPS-induced production of NO in RAW264.7 macrophages as an inflammatory model [19]. Based on the same model, carnosic acid and carnosol were identified as major contributors to the anti-inflammatory activity of rosemary [20]. Thymoquinone has been proposed to be the main anti-inflammatory component of cumin [21]. However, further work must be done to identify the components that are responsible for the inhibiting activity of spices toward the AGEs-induced inflammatory response. Based on our preliminary study, trans-cinnamaldehyde seems to be the most likely candidate component that is responsible for the potent inhibitory activity of cinnamon (data not shown).

In the past, owing to a lack of understanding of the biochemistry of AGEs and its relationship to diabetic complications, the protective effects of spices against diabetes have been studied mostly with reference to their hypoglycemic activity. Information regarding whether spices can protect against diabetic complications has heretofore been scant. Recently, cumin, ginger, turmeric and cinnamon have been reported to help treat cataract and nephropathy in diabetic rats [22-25]. Turmeric and its constituent, curcumin, can delay the development of a cataract but does not have any effect on blood glucose [25]. Clearly, as well as having a hypoglycemic capacity, spices can protect against diabetic complications in other ways.

5. Conclusion

In this investigation, very interestingly, cinnamon exhibited the strongest capacity not only to inhibit the formation AGEs but also to inhibit the AGEs-mediated inflammatory response. Although a definitive conclusion concerning the use of cinnamon as a hypoglycemic agent cannot yet be drawn, cinnamon has been extensively used as an anti-diabetic supplement since its hypoglycemic activity was reported in 2003 [26]. The results herein suggest the possible application of cinnamon in the primary and secondary prevention of diabetic complications, based on its potent inhibition of the formation of AGEs and of AGEs-mediated inflammation.

6. Acknowledgements

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