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Protective capacities of certain spices against peroxynitrite-mediated biomolecular damage

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Abstract

Peroxynitrite, a potent cytotoxic agent, can damage a variety of biomolecules such as proteins, lipids, and DNA, and is considered as one of the major pathological causes of several diseases. Therefore, it would appear likely that interception of peroxynitrite by certain dietary compounds may represent one mechanism by which such foods may exert their beneficial action *in vivo*. A number of researchers have speculated that certain spices, rich in phenolics, may, conceivably, act as potential protectors against the actions of peroxynitrite. Eight culinary spices including cardamom, cinnamon, cloves, cumin, nutmeg, paprika, rosemary and turmeric were selected for study purposes. Further, the protective effects of methanol extracts of such spices against peroxynitrite-mediated damage to proteins, lipids and DNA were evaluated as determined by these extracts' ability to attenuate the formation of, respectively, nitrotyrosine in albumin, thiobarbiturate acid-reactive substances (TBARS) in liposome and strand breakages for plasmid DNA. All of the tested spices exerted some level of protective ability against peroxynitrite-mediated biomolecular damage. Amongst them, cloves deserve special attention due to their outstanding protective abilities against two of three forms of peroxynitrite-mediated biomolecular damage. Additionally, the phenolic content of certain spices appears to correlate well with such spices' protective effect against peroxynitrite-mediated tyrosine nitration and lipid peroxidation. Such an observation indicates that phenolics present in the spices contributed to such spice-elicited protection against peroxynitrite toxicity.

Keywords: Peroxynitrite; Reactive nitrogen species; Antioxidant; Phenolics; Spices

1. Introduction

In response to certain states of inflammation, perfusion injury and a variety of environmental toxins, the human body has been reported to produce, simultaneously, superoxide and nitric oxide, both of which entities further react with each other, exhibiting a nearly diffusion-limited rate constant to form peroxynitrite (Szabo, 2003). Peroxynitrite is more cytotoxic than its parent molecules, this arising via various oxidizing and nitrating mechanisms (Arteel et al., 1999). Such cytotoxicity can damage a large variety of biomolecules such as proteins, lipids, and DNA, a process which has been implicated in the development of several different human diseases (Beckman, 1996; Sadeghi-Hashjin et al., 1998; Digerness et al., 1999). Through the oxidation of certain sulfhydryl groups and thioethers, as well as via the hydroxylation and nitration of aromatic amino residues, peroxynitrite leads protein to dysfunction, and interferes with intracellular signal transduction (Kong et al., 1996; MacMillan-Crow et al., 1998; Go et al., 1999). Protein tyrosine residues are especially susceptible to peroxynitrite-dependent nitration, and the stable end product of such nitration, 3-nitrotyrosine, is considered to represent a biomarker for peroxynitrite-specific protein damage.

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Further, 3-nitrotyrosine has been detected in injured tissues for various coronary–artery disorders, neurological disorders and certain renal diseases (Beckman et al., 1996; Ischiropoulos, 1998; Pennathur et al., 2004). Certain lipids are also highly prone to peroxynitrite-mediated damage resulting in lipid peroxidation, an outcome that has been proposed to play a key role in the development of atherosclerosis (Radi et al., 1991; Graham et al., 1993; Rubbo and O'Donnell, 2005). Additionally, peroxynitrite has been reported to damage DNA via a number of mechanisms including base modification and mutation, as well as strand breakage (Salgo et al., 1995; Douki et al., 1996; Szabo and Ohshima, 1997). Induction of DNA strand breakage will activate poly (ADP-ribose) polymerase, leading cells to subsequently undergo apoptosis (Szabo, 2003). Undoubtedly, inherent defence against peroxynitrite actions *in vivo* is important for the human body in order to eliminate the detrimental consequences of peroxynitrite activity. Due to the human metabolism lacking a specific enzyme to decompose peroxynitrite, however, the burden of defence against sinister peroxynitrite activity *in vivo* relies solely upon the actions of certain nonenzymatic compounds (Klotz and Sies, 2003). Therefore, in addition to the actions of certain endogenous protective molecules such as glutathione, ascorbate and albumin, the consumption of foods which possess an abundance of peroxynitrite-scavenging components would appear to serve as a promising stratagem to boost a human being's inherent protection against the *in vivo* actions of peroxynitrite.

In addition to providing additional taste and flavor to foods, certain spices have been used as remedies in traditional medicine for centuries (Lampe, 2003; Srinivasan, 2005). Several healthy benefits of the consumption of a number of such spices include a digestion-stimulating action, a hypolipidemic effect, antidiabetic influence, presence of antilithogenic properties, antioxidant potential, anti-inflammatory properties, and antimutagenic and/or anticarcinogenic potential, benefits which appear to have been well documented previously (Lampe, 2003; Rajamani et al., 2005; Srinivasan, 2005). Recently, Dragland et al. (2003) speculated that the daily intake of 1 g of various potent antioxidant spices makes a relevant contribution to the total intake of antioxidants in a normal diet. In fact, in America, the *per capita* consumption of spices continues to grow, for example from 2.6 g/person per day in 1980 to approximately 4 g/person per day in 2000, and the importation of spices appears to be still growing at an average rate of 8.5% per year (Furth and Cox, 2004). It would appear that as a consequence of such changes to some peoples' dietary intakes, spices offer more potential than ever to serve as an important dietary antioxidant source. As best we are aware, previous research pertaining to the relative health benefits of spice intake appears to have centred around their defence ability against the presence, in the body, of reactive oxygen species such as superoxide, hydrogen peroxide and the hydroxyl radical (Martinez-Tome et al., 2001; Murcia et al., 2004; Suhaj, 2006). To the best of our knowledge, however, only limited information is available as regards the ability of certain dietary spices to contribute to/provide some form of medical defence against biologically relevant reactive nitrogen species, especially peroxynitrite (Chericoni et al., 2005). Preventing the formation of peroxynitrite, directly intercepting the presence of peroxynitrite, and repairing any peroxynitrite-damaged biomolecules have been previously recognized as constituting three significant and important defence levels against peroxynitrite *in vivo* (Arteel et al., 1999). In several of our previous studies, spices have been documented to exhibit potent preventative actions as regards the formation of peroxynitrite including direct scavenging the NO radical and eliminating NO production by suppressing iNOS expression (Tsai et al., 2005, 2007). Having said this, however, it would appear that comprehensive and comparative information regarding the peroxynitrite-intercepting ability of various spices is still somewhat scant. In this study, eight commonly used spices including cardamom, cinnamon, cloves, cumin, nutmeg, paprika, rosemary and turmeric were selected for investigation, and these spices' peroxynitrite-intercepting abilities were assessed based upon their inhibitory effect upon the generation of peroxynitrite-mediated protein and certain lipids, and also spices' inhibitory effect upon DNA damage. Additionally, the phenolics and flavonoids content, the antioxidant capacity including DPPH radical-scavenging activity, the Trolox equivalent antioxidant capacity (TEAC) and the oxygen-radical absorbance capacity (ORAC) of selected spices were also determined in order to elucidate any possible relationships that existed between spices' phenolics content, flavonoids content and peroxynitrite-intercepting ability.

2. Materials and methods

2.1. Chemicals used for investigation

Peroxynitrite was purchased from Upstate Co. (Lake Placid, NY, USA). Fluorescein, anti-3-nitrotyrosine antibodies, bovine serum albumin (BSA), dimethyl sulfoxide (DMSO), thiobarbituric acid (TBA), Folin–Ciocalteu reagent and BCIP/NBT liquid-substrate systems were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Plasmid DNA (PGEM-4Z vector), malondialdehyde tetrabutylammonium and Gybr gold were purchased from Promega (Madison, WI, USA), Fluka (Buchs, Switzerland) and Invitrogen Co. (St. Louis, MO, USA) respectively. All other chemicals used were of analytical-grade purity.

2.2. Sample preparation

Eight culinary dried spices including cardamom, cinnamon, cloves, cumin, nutmeg, paprika, rosemary and turmeric were purchased from a local supermarket (Hsinchu, Taiwan) in winter of 2005. All of them are the products of TOMAX ENTERPRISE CO., LTD (Taichung, Taiwan). The manufacturer would not divulge the origin and harvested period of the spices, as this was regarded as a commercial secret. The dried spices were ground and then extracted twice with a 10 times volume of methanol. Briefly, an aliquot of 30 mL of methanol was added into a 50 mL tube containing 3 g of weighed dried spice. The solution was mixed, and was then further extracted under violent shaking for a period of 12 h. Following centrifuging at 5000*g* for a period of 10 min, the supernatant was collected and vacuum-dried. The extraction yields for such supernatants are listed in Table 1. For the following experiments, the weighed extract

Table 1
Extraction yield, total phenolics and flavonoids content of various spices[1,2]

Common name	Botanical name	Yield of extraction (%)	Total phenolic content (mg GAE/g extract solids)	Flavonoid content (mg CE/ g extract solids)
Cardamom	*Elettaria cardamomum*	7.1	40 ± 2^{a}	226.8 ± 29.8^{d}
Cinnamon	*Cinnamomum cassia* Presl	10.4	269 ± 3^{f}	28.2 ± 0.5^{b}
Cloves	*Syzygium aromaticum*	21.7	527 ± 8^{h}	163.3 ± 12.0^{c}
Cumin	*Cuminum cyminum* L.	11.5	75 ± 1^{c}	243.1 ± 0.0^{d}
Nutmeg	*Myristica fragrans*	11.9	153 ± 1^{e}	$13.2 \pm 0.4^{a\ b}$
Paprika	Capsicum annuum L.	19.3	52 ± 1^{b}	ND[a]
Rosemary	Rosmarinus officinalis L.	17.4	127 ± 3^{d}	$20.1 \pm 1.3^{a\ b}$
Turmeric	*Curcuma longa*	7.1	349 ± 4^{g}	39.3 ± 0.4^{b}

[1] ND: undetectable.
[2] Each value represents the means ± SEM of triplicate tests. Means not sharing a common letter were significantly different ($p < 0.05$) when analyzed by ANOVA and Duncan's multiple range test.

solid was dissolved in DMSO to a final concentration of 10 mg/mL as a stock and then diluted with phosphate buffered saline (PBS) to the indicated concentration. The control experiments were carried out by adding the same amount of DMSO but without the addition of any spice extract.

2.3. Determination of total phenolic compounds

The quantity of total phenolics in the spice extract was determined by means of a Folin–Ciocalteu colorimetric method (Singleton et al., 1999). Briefly, optimal diluted sample was reacted with Folin–Ciocalteu phenol reagent in an alkaline solution. The absorbance of the solution at a wavelength of 765 nm was measured using a spectrophotometer (Ultrospec 2100 pro, Amersham Pharmacia Biotech Co. Piscataway, NJ, USA). The total phenolic content of each spice extract was expressed in terms of milligrams of gallic-acid equivalents (GAE) per gram of spice extract.

2.4. Determination of total flavonoids

Total flavonoid content in the spice extract was determined according to the method of Zhishen et al. (1999). Briefly, 0.25 mL of optimal diluted sample was added into a tube containing 1 mL of double distilled water. Following this, 0.75 mL of 5% $NaNO_2$, 0.075 mL of 10% $AlCl_3$, and 0.5 mL of 1 M NaOH were added at zero, at 5 min and at 6 min, sequentially. Finally, the volume of reacting solution was adjusted to 2.5 mL with double distilled water. The absorbance of the solution at a wavelength of 510 nm was detected using a spectrophotometer. The flavonoid content in each spice extract was expressed in terms of milligrams of catechin equivalents (CE) per gram of spice extract.

2.5. Determination of peroxynitrite-induced fluorescein oxidation

Firstly, the protective effect of spice extracts against peroxynitrite action was assessed according to the extent of the spice extract's inhibitory activity upon the level of peroxynitrite-induced fluorescein oxidation. Briefly, 50 μL of various concentrations of spice extract and 150 μL of fluorescein solution dissolved in phosphate buffer were pipetted into wells of a 96-well flat-bottomed plate. Following this, peroxynitrite was added to a final concentration of 1 mM and the reaction solution was incubated for 30 min at 37 °C. The change in fluorescent intensities (FI) subsequent to the addition of peroxynitrite was measured by a microplate multimode detector with excitation and emission wavelengths of, respectively, 535 and 485 nm. The protective capacity of spice extract against peroxynitrite action was expressed as the percentage inhibition of peroxynitrite action, and calculated as follows:

$$\text{Percentage inhibition}(\%) = [1 - (\text{FI}_{\text{spice w/o peroxynitrite}} - \text{FI}_{\text{spice w/peroxynitrite}})/(\text{FI}_{\text{control w/o peroxynitrite}} - \text{FI}_{\text{control w/peroxynitrite}})] \times 100\%$$

2.6. Determination of peroxynitrite-induced nitrotyrosine formation

The nitration of tyrosyl residue in BSA was performed according to the method of Ippoushi et al. (2003). Briefly, various concentrations of spice extract dissolved in PBS were mixed with BSA solution. Following this, peroxynitrite was added and the reaction solution was incubated for a period of 30 min at 37 °C. Next, the 3-nitrotyrosine concentration of the solution was determined by Immunoblot analysis. Briefly, 10 μL of reaction solution was loaded into, and separated on, a 10% SDS–polyacrylamide gel and then transferred to polyvinylidene fluoride filters. Following this, filters were blocked and then probed with anti-3-nitrotyrosine antibodies. The filters were then incubated with secondary antibody conjugated to alkaline phosphatase and detected using NBT/BCIP solution. Finally, the relative intensities of the bands on the filters were quantified with a software-supported photoimager (ImageMaster VDS; Amersham Pharmacia Biotech Co. Piscataway, NJ, USA).

2.7. Determination of peroxynitrite induced lipid peroxidation

The work reported on herein, adopted the liposomes as a lipid bilayer model, with the extent of lipid peroxidation occurring as a consequence of peroxynitrite activity being determined colorimetrically, by determining the presence of TBA-reactive substances (TBARS). Lecithin was suspended in PBS and further formed liposomes by sonication with an ultrasonic homogenizer. In order to assess the protective effect of spice extracts against peroxynitrite-mediated lipid peroxidation, spice extract and peroxynitrite were added sequentially into the liposomes solution and then incubated at 37 °C for a period of 30 min. Finally, double the liposome solution's volume of TBA reagent (0.375% TBA, 15% TCA, 0.25 N HCl) was added, and the final solution heated to 100 °C and maintained as such for 10 min, and then centrifuged at 12,000*g* for a period of 10 min. The solution's supernatant was then removed and the solution's absorbance at an incident wavelength of 532 nm measured. The TBARS levels were calculated by means of the application of a standard curve prepared with malondialdehyde.

2.8. Determination of peroxynitrite-induced DNA strand breakage

Herein, the extent of DNA strand breakages was measured by the conversion of supercoiled (SC) plasmid DNA to open circular (OC) and linear (LN) forms of DNA according to the method of Ippoushi et al. (2003). Briefly, 0.24 μg of plasmid DNA was incubated, separately, with

the various spice extracts investigated herein, and then treated with peroxynitrite. Following incubation of the solution at 37 °C for a period of 30 min, samples were loaded into and electrophoresed in a 0.8% agarose gel. Following staining of the gel with Gybr gold, the gel was visualized under UV light and photographed. The relative intensity of each DNA band present in the gel was quantified using a software-supported photoimager (ImageMaster VDS; Amersham Pharmacia Biotech Co. Piscataway, NJ, USA).

2.9. Measurement of DPPH radical-scavenging capacity

The DPPH radical-scavenging capacity of spice extracts was determined as has been described earlier (Tsai et al., 2007). Briefly, 20 μL of serially diluted sample extract was pipetted into wells of a 96-well flat-bottomed plate. Following this, 200 μL of 0.2 mM DPPH solution was added into each well and the plate was shaken with a plate shaker for 5 min. The change in level of absorption of incident radiation at a wavelength of 540 nm subsequent to the addition of DPPH was then measured by an ELISA reader (EL800, BIO-TEK Instruments Inc., Winooski, VT, USA). The scavenging capacity of spice extract was expressed as the IC_{50} which denotes the concentration of the tested spices required to quench 50% of the DPPH radical present.

2.10. Measurement of Trolox equivalent antioxidant capacity (TEAC)

The TEAC of each spice extract was determined by use of a commercial total antioxidant status detecting kit (Randox Laboratories Ltd., Antrim, UK) and expressed in millimoles of Trolox equivalents (TE) per gram of spice extract.

2.11. Measurement of oxygen radical absorbance capacity (ORAC)

The ORAC assay was performed as described by Huang et al. (2002), and was expressed in millimoles of Trolox equivalents per gram of spice extract. Briefly, 25 μL of optimal diluted sample extract were pipetted into wells of a 96-well flat-bottomed plate. Following this, 150 μL of fluorescein solution was added into each well and the plate was incubated at 37 °C for 30 min in the dark. Next, 25 μL of 2,2-azobis (2-amidinopropane) dihydrochloride (AAPH) solution was added to the wells as a source of peroxyl radical, following which, the solution's fluorescence readings were taken every 2 min for a total period of 120 min by means of a microplate multimode detector (Zenyth 3100, Anthos Labtec Instruments Inc, Lagerhausstr, Wals, Austria). Finally, the difference between the area under the fluorescence decay curve for each sample and the corresponding area for blank was determined in order to calculate the ORAC value, as has been described previously by Cao and Prior (1999).

2.12. Statistical analysis

All results presented herein are expressed as mean ± SEM for at least three independent tests for each spice extract. The significance of the differences between the treatments was analyzed by ANOVA, and followed by Duncan's multiple range test for multiple comparisons. The correlation between two variants was analyzed by application of the Pearson test. All of the statistical analyses were performed by means of SPSS software with the level of significant difference between compared data sets being set at $p < 0.05$ (SPSS for Windows, ver. 10.0; SPSS Inc., Chicago, IL, USA).

3. Results

3.1. Phenolics and flavonoids contents of spice extracts

A summary of the phenolics and flavonoids contents of spice extracts is revealed in Table 1. Quite clearly, there exists quite a large variation amongst the tested spices as regards phenolic content. Cloves contained 527 mg gallic-acid equivalent per gram of spice extract, which was the most-substantial value for this parameter amongst the tested spices. Contrasting this, cardamom, paprika and cumin revealed the lowest such levels, they only containing, respectively, 40, 52 and 75 mg gallic-acid equivalent per gram of spice extract. Similarly, the tested spices revealed widely ranging levels for flavonoids content. The relative abundance of flavonoids amongst the various tested spice extracts, however, differed quite markedly from the corresponding values for extract-contained phenolics. Cumin and cardamom featured the greatest levels of flavonoids, these two spices revealing, respectively, 243.1 and 226.8 mg catechin equivalents per gram of spice extract. By contrast, the flavonoids content of paprika was quite low, and on occasion, this level was less than the detection limit of our analytical method.

3.2. Protective capacities of spice extracts against peroxynitrite-mediated fluorescein oxidation

Table 2 lists a summary of the protective effect of all tested spice extracts against peroxynitrite-mediated fluorescein oxidation. All of the tested spices demonstrated a dose-dependent inhibitory effect upon the extent of peroxynitrite-mediated fluorescein oxidation. At a concentration of 100 μg/mL, turmeric inhibited 96.3% of peroxynitrite-mediated fluorescein oxidation, this being the most effective of the tested spices against peroxynitrite-mediated fluorescein oxidation. Cloves, nutmeg, cinnamon and rosemary all exhibited an analogous inhibitory effect of >50%, and proved to be quite good at preventing the oxidation of fluorescein by peroxynitrite. By contrast, cumin, paprika and cardamom only displayed an inhibitory effect of <50% upon peroxynitrite-mediated fluorescein oxidation, they being, thus, classified as relatively poor such protectors.

Table 2
Protective capacities of spice extracts against peroxynitrite-mediated fluorescein oxidation[1,2]

Treated conc. (μg/mL)	Percentage inhibition (%)		
	100	50	25
Cardamom	28.5 ± 2.3^{a}	16.2 ± 5.4^{a}	15.2 ± 8.6^{a}
Cinnamon	63.5 ± 1.9^{cd}	42.7 ± 0.3^{c}	21.1 ± 13.4^{a}
Cloves	74.5 ± 1.8^{e}	61.5 ± 1.5^{d}	62.4 ± 6.3^{c}
Cumin	41.2 ± 4.6^{b}	30.5 ± 3.7^{b}	18.9 ± 10.1^{a}
Nutmeg	71.0 ± 6.1^{de}	47.4 ± 2.3^{c}	34.4 ± 2.1^{ab}
Paprika	38.7 ± 5.6^{b}	38.0 ± 4.5^{bc}	21.0 ± 5.6^{a}
Rosemary	58.0 ± 3.3^{c}	48.0 ± 2.9^{c}	34.7 ± 4.6^{ab}
Turmeric	96.3 ± 1.6^{f}	70.0 ± 0.7^{d}	51.7 ± 3.3^{bc}

[1] Protective capacity was expressed as percentage inhibition of spice extract on the peroxynitrite-mediated fluorescein oxidation with reference to the control value.

[2] Each value represents the mean ± SEM of triplicate tests. Means not sharing a common letter were significantly different ($p < 0.05$) when analyzed by ANOVA and Duncan's multiple range test.

3.3. Protective capacities of spice extracts against peroxynitrite-mediated nitrotyrosine formation

As shown in Fig. 1, subsequent to BSA exposure to peroxynitrite, a significant level of formation of nitrotyrosine was observed. Furthermore, the level of peroxynitrite-induced nitrotyrosine formation under such conditions was attenuated by the addition of various spice extracts. At a concentration of 100 μg/mL, all of the tested spices, apart from cardamom and paprika, almost completely inhibited the formation of nitrotyrosine following BSA exposure to peroxynitrite. Therefore, the protective capacities of spice extracts were further evaluated by using a lower concentration of test spice, namely 10 μg/mL, as a consequence of which, we were able to rank the spices as regards their ability to attenuate the formation of nitrotyrosine as follows: cloves (95.5%) > nutmeg (67.7%), rosemary (66.5%), turmeric (66.5%), cinnamon (57.8%) > paprika (36.0%) > cumin (26.4%) and >cardamom (9.5%).

3.4. Protective capacities of spice extracts against peroxynitrite-mediated lipid peroxidation

A summary of the results for the protective effects of spice extracts against peroxynitrite-induced lipid peroxidation is presented in Fig. 2. When liposomes were exposed to 1 mM of peroxynitrite, a significant level of formation of TBARS was detected. Furthermore, the formation of TBARS as induced by peroxynitrite's lipid-peroxidation activity was suppressed by the addition of spice extracts. The relative protective effect of spices in this regard was able to be ranked based upon the particular spice extract's proportional inhibition of peroxynitrite-induced TBARS formation, the results being as follows: cinnamon (63.9%) ≧ cloves (58.5%), rosemary (54.0%), turmeric (44.3%) ≧ nutmeg (35.5%) > cardamom (8.5%), cumin (6.1%) and paprika (1.6%).

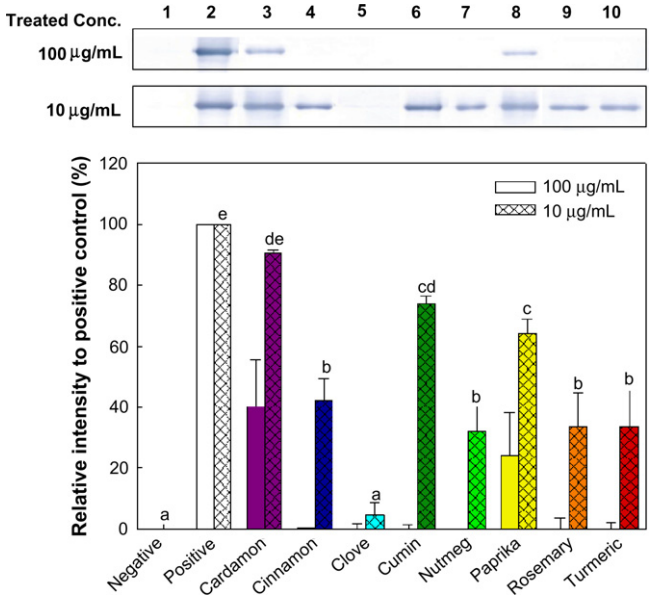

Fig. 1. Protective capacities of spice extracts against peroxynitrite-mediated nitrotyrosine formation in BSA. Lane 1 presents the result of the BSA without any treatment. The other lanes represent BSA preincubated without (2) or with extract from cardamom (3), cinnamon (4), cloves (5), cumin (6), nutmeg (7), paprika (8), rosemary (9) and turmeric (10), respectively, followed treatment with peroxynitrite (100 μM) at 37 °C for 30 min. The values are expressed as means ± SEM of triplicate tests. Means not sharing a common letter were significantly different ($p < 0.05$) when analyzed by ANOVA and Duncan's multiple range test.

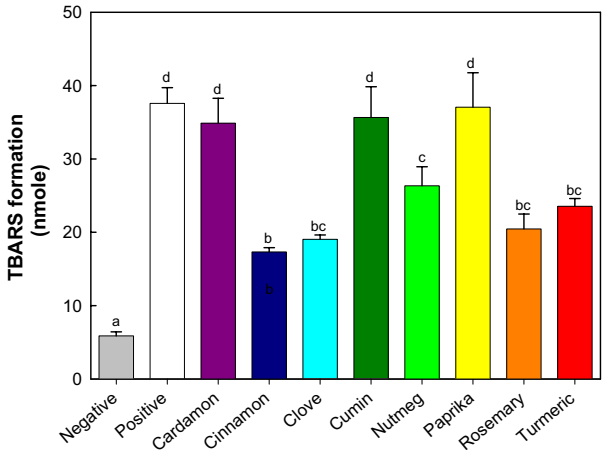

Fig. 2. Protective capacities of spice extracts against peroxynitrite-induced lipid peroxidation. Lane 1 presents the result of the liposomes without any treatment. The other lanes represent liposomes preincubated without (2) or with 100 μg/mL extract from cardamom (3), cinnamon (4), cloves (5), cumin (6), nutmeg (7), paprika (8), rosemary (9) and turmeric (10), respectively, followed treatment with 1 mM peroxynitrite at 37 °C for 30 min. The values are expressed as means ± SEM of triplicate tests. Means not sharing a common letter were significantly different ($p < 0.05$) when analyzed by ANOVA and Duncan's multiple range test.

3.5. Protective capacities of spice extracts against peroxynitrite-induced DNA strand breakages

As shown in Fig. 3, when plasmid DNA was exposed to peroxynitrite, the native supercoiled (SC) DNA was converted to a relaxed open circular (OC) DNA with associated single-strand breakages, and also converted to linear (LN) DNA with associated double-strand breakages. Moreover, all spice extracts were capable of preventing peroxynitrite (1 mM)-mediated DNA strand breakages. At a level of 100 μg/mL, cardamom, cinnamon, cloves, cumin, nutmeg, paprika, rosemary and turmeric extract decreased DNA strand breakage by, respectively, 29%, 81%, 44%, 22%, 80%, 26%, 40%, and 49% of the corresponding value for the positive control (plasmid incubated only with peroxynitrite). The ranking of the tested spice extracts' protective ability against peroxynitrite-mediated DNA strand breakages differed quite significantly from the rankings for the corresponding spice-extracts'-protective ability against peroxynitrite-mediated tyrosine nitration and lipid peroxidation.

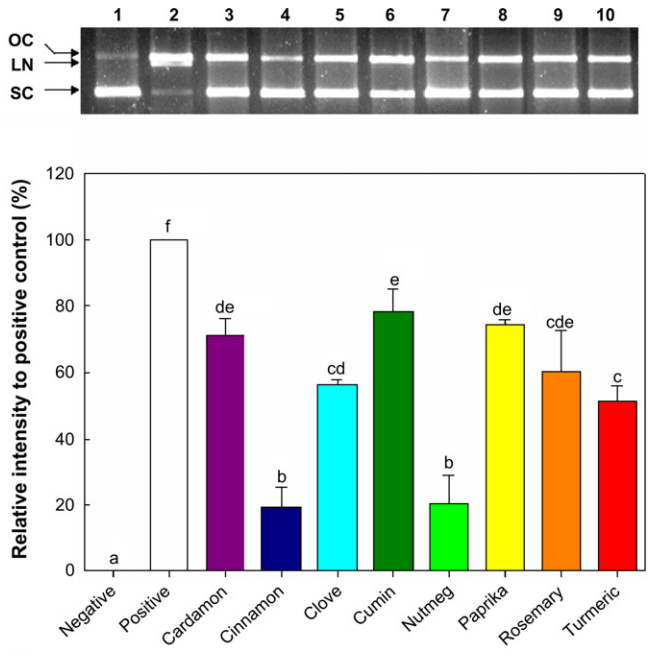

Fig. 3. Protective capacities of spice extract against peroxynitrite-induced DNA strand breakages. Lane 1 presents the result of the plasmid DNA without any treatment. The other lanes represent plasmid DNA preincubated without (2) or with 100 μg/mL extract from cardamom (3), cinnamon (4), cloves (5), cumin (6), nutmeg (7), paprika (8), rosemary (9) and turmeric (10), respectively, followed treatment with peroxynitrite (1 mM) at 37 °C for 30 min. The values are expressed as means ± SEM of triplicate tests. Means not sharing a common letter were significantly different ($p < 0.05$) when analyzed by ANOVA and Duncan's multiple range test.

3.6. Antioxidant activity of spice extracts

Many different methods have been established for evaluating the antioxidant capacity of certain biological samples, with such methods being classified, roughly, into one of two categories based upon the nature of the reaction that the method involved (Huang et al., 2005). The methods involving an electron-transfer reaction include the total phenolics assay using Folin–Ciocalteu reagent, the TEAC and the DPPH radical-scavenging assay. The ORAC testing assay used herein belongs to that group of methods for evaluating the antioxidant capacity which involve the hydrogen-atom transfer reaction (Huang et al., 2005). The antioxidant capacities of the test spice extracts as assessed by different assay methods are summarized in Table 3. When the TEAC assay was used to provide a raking order of antioxidant capacity for the test spices, the results, in decreasing order of antioxidant capacity, were as follows: cloves > rosemary, cinnamon, turmeric > nutmeg > cumin > paprika and cardamom. The DPPH scavenging capacity of spices was presented as IC_{50}, ie the concentration of spice extract needed to quench 50% of the DPPH radical. Of the eight spices tested, the most-active was cloves, this spice extract featuring an IC_{50} value of 62 μg/mL. In contrast, paprika and cardamom presented an IC_{50} value of >1000 μg/mL, and would thus appear to be rather poor DPPH radical scavengers. In this study, we adopted an ORAC assay method to assess the hydrogen-atom transfer capacity of the tested spices and found that the magnitude of the resultant ORAC value for the tested spices exhibited similar trending with corresponding TEAC values.

Table 3
Antioxidant capacities of spices as determined by means of TEAC, DPPH scavenging and ORAC assays[1]

	TEAC (mmol TE/g extract solids)	IC_{50} of DPPH scavenging (μg extract solids/mL)	ORAC (mmol TE/g extract solids)
Cardamom	0.50 ± 0.01^{a}	>1000	0.81 ± 0.01^{a}
Cinnamon	1.53 ± 0.04^{d}	168	3.13 ± 0.03^{c}
Cloves	2.13 ± 0.00^{e}	62	6.15 ± 0.11^{g}
Cumin	0.62 ± 0.01^{a}	768	2.01 ± 0.09^{b}
Paprika	0.57 ± 0.00^{a}	>1000	0.92 ± 0.08^{a}
Nutmeg	1.34 ± 0.01^{c}	294	3.57 ± 0.11^{d}
Rosemary	1.18 ± 0.04^{b}	149	4.36 ± 0.24^{b}
Turmeric	1.42 ± 0.09^{cd}	233	5.44 ± 0.06^{f}

[1] Each value represents the mean ± SEM of triplicate tests. Means not sharing a common letter were significantly different ($p < 0.05$) when analyzed by ANOVA and Duncan's multiple range test.

3.7. Correlation between phenolics content, antioxidant capacity and protective activity against peroxynitrite

The correlation coefficients between the total phenolics content of spice extracts, antioxidant capacity and protective activity against peroxynitrite are presented in Table 4. As expected, the phenolics content correlated highly with the TEAC as well as with DPPH scavenging capacity, due to these three assays applied similar reaction principle to determine the antioxidant capacity. A significant correlation was also shown to exist between the phenolic content and the ORAC value for each spice. Additionally, we found, for the first time as best we are aware, that the phenolics content of a particular spice was highly correlated with its protective abilities against peroxynitrite-mediated fluorescein oxidation, tyrosine nitration and lipid peroxida-

Table 4
Correlation coefficients between the phenolics content of a particular spice extract and its protective ability against peroxynitrite-mediated protein, lipid and DNA damages

	Correlation coefficient (probability)
TEAC value	0.93 (0.001)
IC_{50} of DPPH	−0.76 (0.030)
$ORAC_{ROO\cdot}$ value	0.87 (0.005)
Peroxynitrite-mediated fluorescein oxidation	0.77 (0.025)
Peroxynitrite-mediated tyrosine nitration	0.83 (0.011)
Peroxynitrite-mediated lipid peroxidation	0.74 (0.036)
Peroxynitrite-mediated DNA strand breakage	0.36 (0.399)

tion, although the spice extract's phenolic content did not correlate well with the extract's protective ability against peroxynitrite-mediated DNA strand breakage.

4. Discussion

Peroxynitrite action can cause one or two electron-oxidation steps as well as encouraging the nitration of certain biomolecules (Arteel et al., 1999). Therefore, in this study, we evaluated the peroxynitrite-intercepting abilities of several different spices by measuring their relative protective effects against peroxynitrite-mediated tyrosine nitration, lipid peroxidation, and DNA strand breakage. Amongst the tested spices, cloves displayed the most-potent protection against peroxynitrite-mediated tyrosine nitration and lipid peroxidation, and cinnamon and nutmeg showed the best protection against peroxynitrite-mediated DNA strand breakage, whereas cumin, cardamom and paprika were poor protectors against peroxynitrite-mediated biomolecular damage. Eugenol is the most-abundant component of cloves, and is the component which has been previously identified as being the main contributor to the ability of essential oil of cinnamon to attenuate peroxynitrite-induced nitrotyrosine formation and lipid peroxidation (Chericoni et al., 2005). Thus, we believe it not unreasonable to postulate here that eugenol contributed greatly to the potent protection of clove extract against peroxynitrite activity. Additionally, despite the observation that paprika was a rather weak protector against peroxynitrite action amongst the tested spices, based upon our results, the potential for effective protection to be exerted by paprika *in vivo* cannot be completely ruled out, given that it has been documented previously that a daily intake of 30 g of fresh paprika can enhance the resistance of serum lipoprotein against autoxidation (Ahuja and Ball, 2006). Therefore, it appears clear that greater effort need be directed to the ongoing investigation of such foods in the context of supporting the application of such foods as a dietary protective booster against peroxynitrite action. Such further investigations should include: the identification of all of the active components of such spices, the determination of the relative bioavailability of such constituents, and the evaluation of their effective concentration *in vivo*.

Results such as the relative abundance of phenolic compounds amongst the different spices tested herein, and the significant correlations that existed between spice-extract phenolic content and antioxidant capacity, as measured by TEAC, DPPH scavenging and ORAC methods, would appear to be highly consistent with corresponding results presented by previous researches (Teissedre and Waterhouse, 2000; Zheng and Wang, 2001; Dragland et al., 2003; Shan et al., 2005). Most importantly, the results that significant correlations existed between the phenolic content of a spice extract and the extract's protective ability against peroxynitrite-mediated fluorescein oxidation and tyrosine nitration, as well as against lipid peroxidation, indicated firstly, that phenolic compounds were also responsible for the protective abilities of certain spices against the actions of peroxynitrite *in vitro*. Plant phenolics generally possessing an aromatic ring bearing one or more hydroxy substituents are being regarded as those substances derived from the shikimate pathway and phenylpropanoids metabolism. Furthermore, phenolics can be classified into several groups by the number of constitutive carbon atoms in conjunction with the structure of the basic phenolic skeleton (Robards et al., 1999). As best we are aware, a number of workers have gone to considerable effort to explore the relationship between the structure of phenolic compounds and these compounds' peroxynitrite-intercepting activity as assessed by means of these compounds' ability to prevent peroxynitrite-mediated oxidation of fluorescent substrates (Haenen et al., 1997; Heijnen et al., 2001a,b). Both the catechol group in ring B and 3-hydroxyl group in ring C provide the greatest contribution to the peroxynitrite-intercepting ability of flavonoids (Haenen et al., 1997). Furthermore, in 2001, Heijnen et al. reported that 3-hydroxyl group in ring C was the peroxynitrite-intercepting reactive centre of flavonoids and its reactivity was positively affected by the electron-donating capacity of the substituted groups at 5 and 7 positions. In addition, the galloyl group enhances the peroxynitrite-intercepting ability of certain tannins (Chung et al., 1998). In fact, the structural features of flavonoids that are deemed to be necessary for high peroxynitrite-intercepting activity are also applicable to the intercepting abilities of other reactive oxygen/nitrogen species, such as superoxide anions and nitric oxide (Hu et al., 1995; Van Acker et al., 1995; Chen et al., 2002; Nakagawa and Yokozawa, 2002; Taubert et al., 2003). Therefore, it appears reasonable to speculate that the protective capacity of certain spice extracts against peroxynitrite activity was associated with the extracts' phenolic content as well as the extracts' antioxidant activity. Although flavonoids which have a C6–C3–C6 skeleton are the most widespread and diverse phenolics in most plants (Robards et al., 1999). However, phenolic volatile oils are found to be the main active antioxidants in most spices (Shan et al., 2005). Additionally, in our study, the flavonoids content of spices did not correlated with their protective capacities against peroxynitrite-mediated oxidative and nitrating damages. Therefore, we suggested that flavonoids played a minor role on the spices protective effect against peroxynitrite-mediated oxidative and nitrating damages.

Nitration of tyrosine by peroxynitrite requires a one-electron oxidation procedure as a primary step to form the tyrosyl radical, this being followed by the addition of the nitrogen-dioxide radical to the tyrosyl radical (Daiber et al., 1998). It thus seems likely that phenolics can attenuate the level of peroxynitrite-mediated tyrosine nitration by interacting with the tyrosyl radical, as well as with the nitrogen-dioxide radical. Due to the presence of O-semiquinone radicals in peroxynitrite-treated red wine sample, scavenging peroxynitrite-derived radicals have, for some

time, been considered as the protective mechanism underlying the activities of red wine's polyphenols against peroxynitrite-mediated tyrosine nitration (Ferroni et al., 2004). Two possible reaction mechanisms involving certain plant phenolics and peroxynitrite would appear to have been proposed: as preferential nitration steps for monophenolates and electron donation by catechlolates (Pannala et al., 1998). For this current study, although we were not able to specifically demonstrate the real mechanism underlying the protective effects against peroxynitrite activity offered by phenolic compounds, based upon our results, however, we believe that it is not unreasonable to propose that the total quantity of phenolic compounds present in a spice extract was a key determinant to the relative protective ability of that spice against peroxynitrite activity.

Somewhat incompatibly, variability in the tested spice extracts' protective effect upon peroxynitrite-mediated DNA strand breakage was not associated with the individual extract's phenolic content nor was it associated with extracts' antioxidant capacities as determined by DPPH scavenging, TEAC and ORAC assays. As best we are aware, the underlying mechanism of DNA strand breakage as induced by peroxynitrite is considerably complicated and not entirely clear. Peroxynitrite-induced DNA strand breakage has previously been suggested to arise both directly via sugar damage and indirectly via base damage, most of which effects are inflicted through oxidative rather than nitrating pathways (Burney et al., 1999). For neither peroxynitrite itself nor for the hydroxyl radical, is the hydroxy radical-like intermediate ($ONOOH^{\bullet}$) or peroxynitrous acid suggested to be responsible for the DNA strand-breaking damage arising from peroxynitrite activity (Szabo and Ohshima, 1997). Therefore, the results of peroxynitrite's activity as regards DNA damage might be partly attributable to differences in reaction sequence between phenolics with peroxynitrite or with $ONOOH^{\bullet}/ONOOH$, however, further study is clearly warranted in order to fully interpret the characteristics of spices' protection against peroxynitrite-induced DNA stand breakage.

The present study has provided, to the best of our knowledge, the first evidence that certain spices effectively protected certain biomolecules from peroxynitrite-mediated nitrating and oxidizing damage, and that the protective abilities of such spices depended, to a certain extent, upon their phenolic-compound content as well as their antioxidant capacity. Whether the reaction of the tested spice extracts with peroxynitrite represents a biologically efficient and important detoxification pathway *in vivo* still remains to be demonstrated. Regardless of such uncertainty, peroxynitrite-induced tissue discoloration, nutrient loss and lipid peroxidation have been previously reported to have been detected in post-mortem animal tissue (Brannan et al., 2001; Brannan and Decker, 2001; Connolly and Decker, 2004). Therefore, the application of such plant extracts to the conservation of muscle-derived foods would likely be expected.

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