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Evaluation of NO-suppressing activity of several Mediterranean culinary spices

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Abstract

Excess nitric oxide (NO) produced by inducible nitric oxide synthase (iNOS) is implicated in the development of a number of diseases. Due to the absence of any natural specific enzymatic defense system *in vivo*, the consumption of certain foods which exhibit selective suppressive ability as regards NO overproduction might boost the host's protective effects against NO-mediated toxicity. Spices, rich in phenolics, are speculated conceivably to act as potential NO-scavengers or iNOS suppressors. The relative NO-suppressing activity of methanol extracts deriving from nine Mediterranean culinary spices was determined by measuring their inhibitory effect upon NO production for lipopolysaccharide (LPS)-activated RAW 264.7 macrophages. In addition, the specifics of the suppressing mechanism were further explored. All of the spices tested, with the exception of clove, displayed a rather linear dose-dependent NO-suppressing effect without there appearing to exist any effect upon cell viability. Furthermore, the NO-suppressing capacity of certain spices was able to be ranked based upon their IC_{50} (the concentration of spice extracts is required to cause 50% inhibition of NO production by LPS-activated RAW 264.7 cells), the ranking appearing as: rosemary (0.031%) > tarragon (0.052%) > cinnamon (0.059%) > oregano (0.106%) > basil (0.162%) > marjoram (0.236%) > allspice (0.269%) > and thyme (0.270%). Only cinnamon displayed excellent NO-scavenging ability, whereas all of the other spices demonstrated moderate to poor activities in this regard. Moreover, the inhibitory effect of tested spices upon the iNOS protein level was almost equivalent to their suppressive effect upon NO production. It would appear that inhibition of iNOS expression was the primary mechanism of action of spices as regards their exerting NO-suppressing activity.

Keywords: Nitric oxide; Reactive nitrogen species; Antioxidant; Phenolics; Spices

1. Introduction

Initially, nitric oxide was determined to act as a potent vasodilator, although its multiple functions and diverse roles have been progressively revealed sequentially with the passing of time (Brannan et al., 2001). In particular, nitric oxide (NO) and reactive nitrogen species (RNS), as deriving from the interaction of nitric oxide (NO) with oxygen or reactive oxygen species, both have been reported to participate in the development of oxidative tissue/cellular damage which has progressively become an established mechanism for tissue damage, and thereby received intense human-health interest (Burney et al., 1999). NO is a gaseous free radical that can be synthesized from arginine by a family of enzymes called nitric oxide synthases (NOS) in a biological system. To the best of our knowledge, at time of writing, four separate NOS isoforms have been identified. A new NOS, mitochondrial NOS, has been elucidated although little information would currently appear to be known about this entity (Ghafourifar and Richter, 1997). Constitutive expression of neural NOS

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and endothelial NOS typically produce a low basal level of NO, a compound which is an important mediator in the processes of vasodilation and neurotransmission. Inducible NOS (iNOS) is induced quantitatively by inflammatory stimuli in various cells such as macrophages, fibroblasts, smooth-muscle cells and hepatocytes, iNOS participating in the host immune defence system directed specifically against exogenous pathogens (Mayer and Hemmens, 1997). Once excess NO production by iNOS occurs, however, this will accelerate RNS formation, damage cellular macromolecules such as proteins, DNA, and lipids, and trigger numerous detrimental cellular responses (Radi et al., 1991a,b; Yermilov et al., 1995). In fact, a number of conditions including sepsis, cancer, diabetes, renal disease and atherosclerosis are characterized by abnormally high iNOS expression and high NO production. (Yu et al., 1994; Beckman and Koppenol, 1996; Cooke and Dzau, 1997). Elimination of NO both by NO-scavengers or iNOS inactivators aids to ameliorate such injury (Matheis et al., 1992; Hooper et al., 1997; Menezes et al., 1999). Therefore, it would appear likely that the scavenging of NO or the suppressing of iNOS by certain food items would serve as a promising indicator for the beneficial "health effects" of such food.

In addition to providing additional taste and flavor to foods, a wide spectrum of beneficial actions of spices, in particular, their antioxidant function, has been documented in several previous *in vivo* and *in vitro* studies (Madsen and Bertelsen, 1995; Lampe, 2003; Srinivasan, 2005). Recently, Dragland et al. used a ferric reducing/antioxidant power assay (FRAP) to evaluate systemically the antioxidant activity of certain food items, these authors further speculating that culinary herbs contribute significantly to the total intake of antioxidants in a normal diet and even more effectively so than a number of other food groups such as fruits, cereals and vegetables (Dragland et al., 2003). Thus it may be claimed that adopting a cuisine rich in spices may boost the chemoprotective capacity of fruits and vegetables in one's diet. In spite of the fact that the antioxidant activities of spices regarding, in particular, lipid oxidation and ROS scavenging have been extensively investigated (Martinez-Tome et al., 2001; Murcia et al., 2004), the NO-scavenging and NO-suppressing activities of spices, to the best of our knowledge, would still appear to be somewhat obscure.

In this study, nine commonly used culinary spices including rosemary, tarragon, cinnamon, oregano, basil, marjoram, allspice, thyme and clove were selected for investigation, and their direct NO-scavenging and NO-suppressing activities assessed. Additionally, the phenolic content and the antioxidant activity of each spice was also determined, respectively, by means of a Folin–Ciocalteu colorimetric method and a 2,2-diphenyl-1-picryl-hydrazyl (DPPH) radical scavenging assay, in order to attempt to elucidate any relationships that may exist between the phenolic content, and the NO-scavenging, NO-suppression and antioxidant activity of each selected spice.

2. Materials and methods

2.1. Materials

RAW 264.7 cells, a murine macrophage cell line, obtained from the Food Industry Research and Development Institute (Hsinchu, Taiwan), were cultured in DMEM medium supplemented with 10% heat-inactivated fetal bovine serum (Gibco BRL Life Technologies Inc., Grand Island, NY, USA). Lipopolysaccharide (LPS), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT), sulphanilamide, naphthylethylenediamine, Folin–Ciocalteu reagent and BCIP/NBT liquid substrate system were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Sodium nitroprusside was purchased from Roche Co. (Mannheim, Germany). All other chemicals were of analytical-grade purity.

2.2. Sample preparation

Nine commonly used Mediterranean culinary dried spices including rosemary, tarragon, cinnamon, oregano, basil, marjoram, allspice, thyme and cloves were purchased from a local supermarket (Taipei, Taiwan). The dried spices were ground and then extracted with 90% methanol following the procedures described by Halvorsen et al. (2002). Briefly, 3 mL of distilled water was added into a 50 mL tube containing 3 g of weighed dried spice. Methanol (27 mL) was then added to this homogenate and further extracted under violent shaking for a period of two hours. Following centrifuging at 5000*g* for 10 min, the supernatant was collected, aliquoted into small volume and vacuum-dried. Vacuum-dried solids from a 1 mL aliquot were re-dissolved with 0.2 mL of dimethyl sulfoxide (DMSO) for subsequent experimentation. The control experiments were carried out by adding the same amount of DMSO without spice extract.

2.3. Determination of suppressing effect on NO production by LPS-activated cells

In order to determine the effect of a spice extract on NO production, 1×10^5 RAW 264.7 cells were seeded into 96-well culture plates. Following incubation for 24 h, the adherent cells were washed three times with phosphate buffered saline (PBS). The cells were then incubated in prepared DMEM medium containing extracts from various spices, either with or without LPS (100 ng/mL). Following incubation for 24 h, the medium was collected for nitrite assay, at which time cell viability was evaluated using the MTT method (Mosmann, 1983). Finally, medium nitrite concentration was measured as an indicator of NO production by use of the Griess reaction (Kim et al., 1995). The NO-suppressing effect of spice extracts was expressed as the IC_{50} which denotes the concentration of spice extracts causing 50% inhibition of NO production by LPS-activated RAW 264.7 cells.

2.4. Determination of NO-scavenging activity

The NO-scavenging activity of sample extract was determined according to the method of Sreejayan and Rao (1997) with some modification. Briefly, 60 μL of serial diluted sample extract was pipetted into a 96-well flat-bottomed plate. Following this, 60 μL of 10 mM sodium nitroprusside dissolved in PBS was added into each well and the plate was then incubated under light at room temperature for 150 min. Finally, an equal volume of Greiss reagent was added into each well in order to measure the nitrite content. The IC_{50} denotes the concentration of test spices required to quench 50% of the NO released by sodium nitroprusside.

2.5. Evaluation of inhibitory effect on iNOS protein levels

The RAW 264.7 cells were seeded at a density of 5×10^6 cells per 6-cm culture dish in a total volume of 5 mL and incubated for a period of 24 h. Following three washes of PBS, the adherent cells were incubated for 12 h in the presence or absence of LPS (100 ng/mL) and spice extract. Adherent

cells were then washed with PBS, collected, suspended in 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. Additionally, iNOS protein levels were determined by Immunoblot analysis. Briefly, supernatants of cell lysates containing an equal protein content were loaded into, and separated on, an 8% SDS-polyacrylamide gel and then transferred to polyvinylidene fluoride filters. Following this, filters were blocked and probed with anti-iNOS antibodies (BD Transduction Laboratories, Josesan, CA, USA). The filters were then incubated with secondary antibody conjugated to alkaline phosphatase and detected using NBT/BCIP solution. Finally, the band intensities were quantified with a software-supported photoimager (ImageMaster VDS; Amersham Pharmacia Biotech Co., Piscataway, NJ, USA).

2.6. Determination of total phenolic compounds present

The quantity of total phenolics in the spice extracts 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 alkaline solution. The absorbance at 765 nm was measured using a spectrophotometer. The total phenolic content for each spice extract was then calculated by means of the application of a standard curve prepared with gallic acid and expressed in terms of milligrams of gallic acid equivalents per milliliter of spice extract.

2.7. Determination of DPPH radical scavenging capacity

The DPPH radical scavenging capacity of spice extracts was determined based upon the method of Katsube et al. (2004). Briefly, 20 μL of serial diluted sample extract was pipetted into a 96-well flat-bottomed plate. Following this, 200 μL of DPPH solution was added into each well and the plate was shaken with plate shaker for 5 min. The change of absorption at 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 was expressed as the IC_{50}, denoting the concentration of test spices required to quench 50% of the DPPH radical present.

2.8. Statistical analysis

All results are presented as mean ± SEM for at least three independent tests. The significance of the differences at each sample concentration was analyzed by ANOVA and Duncan's multiple range test. The paired Student's *t*-test was used to evaluate the differences between the treatments and the controls. The correlation between two variants was analyzed by the Pearson test. All of the statistical analyses were performed by means of SPSS software with the level of significant difference set at $p < 0.05$ (SPSS for Windows, ver. 10.0; SPSS Inc., Chicago, IL, USA).

3. Results

3.1. Effects of spices extract on the NO production in LPS-activated RAW 264.7 macrophages

In order to avoid a possible cytotoxic effect of spices upon NO production, cell viability of the suspension was initially determined using an MTT method. Only clove at a concentration exceeding 0.4% caused cytotoxicity, whereas other spice extracts at the treated concentrations in this study did not influence cell viability (>90% of the control group). Briefly, the cell viabilities of clove, thyme, allspice, marjoram, and basil groups under the highest treated concentration (0.4%) were 94.4%, 102.4%, 98.6%, 106.2% and 107.9% of that of the control group, respectively. Viability of oregano and cinnamon treated cells at the highest concentration (0.2%) were 98.9% and 94.5% of that of the control group, respectively. Tarragon and rosemary treated cells were also almost alive, presenting 101.0% and 99.7%, respectively, of the survival rate of the control group at the highest treated concentration (0.1%). Analysis of NO production by measuring the nitrite level with the Griess reaction revealed that placing un-stimulated RAW 264.7 cells in culture medium for 24 h produced a basal quantity of nitrite (Fig. 1). When the cells were incubated with various individual spice extracts in the absence of LPS, medium nitrite concentration remained at a background level similar to that of the un-stimulated control (data not shown). After treatment with LPS for 24 h, the medium concentration of nitrite was observed to increase markedly compared with the control group (Fig. 1). Significant concentration-dependent inhibition of NO production was observed when cells were co-treated with LPS and various concentrations of the various spice extracts (Fig. 1). Due to the rather notable cytotoxic effect of clove, for the highest testing concentration for this spice (0.4%) we observed that such a level elicited only a 52% inhibition on NO production for LPS-activated RAW 264.7 cells. Therefore, the IC_{50} value for clove as regards NO production was not able to be calculated, and clove was excluded from involvement with the following cell experiments. The NO-suppressing activity of other spices was able to be ranked based upon their IC_{50}, and appeared as: rosemary (0.031%) > tarragon (0.052%) > cinnamon (0.059%) > oregano (0.106%) > basil (0.162%) > marjoram (0.236%) > allspice (0.269%) > and thyme (0.270%). The difference in NO-suppressing capacity between the most- and the least-effective spices in this regard was more than eight fold. Furthermore, in order to explore the NO-suppressing mechanism of test spices, we performed the following experiment.

3.2. Direct NO-scavenging activities of spices

The results for the NO-scavenging effect of test spices are depicted in Fig. 2. All of the tested spices revealed some level of NO-scavenging activity. At a concentration of 1%, the NO-scavenging ability (percentage) of spices was able to be ranked as follows: cinnamon (85%) and clove (83%) > allspices (70%), marjoram (68%) and rosemary (62%) > oregano (48%), tarragon (46%) and thyme (37%) > basil (24%) (Fig. 2). The corresponding IC_{50} values for NO-scavenging ability are listed in Table 1. Cinnamon and clove both exhibited an IC_{50} value lower than 0.1%, and both proved to be good NO-scavengers, whereas allspices, marjoram and rosemary revealed an IC_{50} value of between 0.25% and 0.5% revealing that they were only moderate NO-scavengers, whilst oregano, tarragon, thyme and basil, all of which featured an undetectable IC_{50}, were classified as rather poorly effective at NO-scavenging.

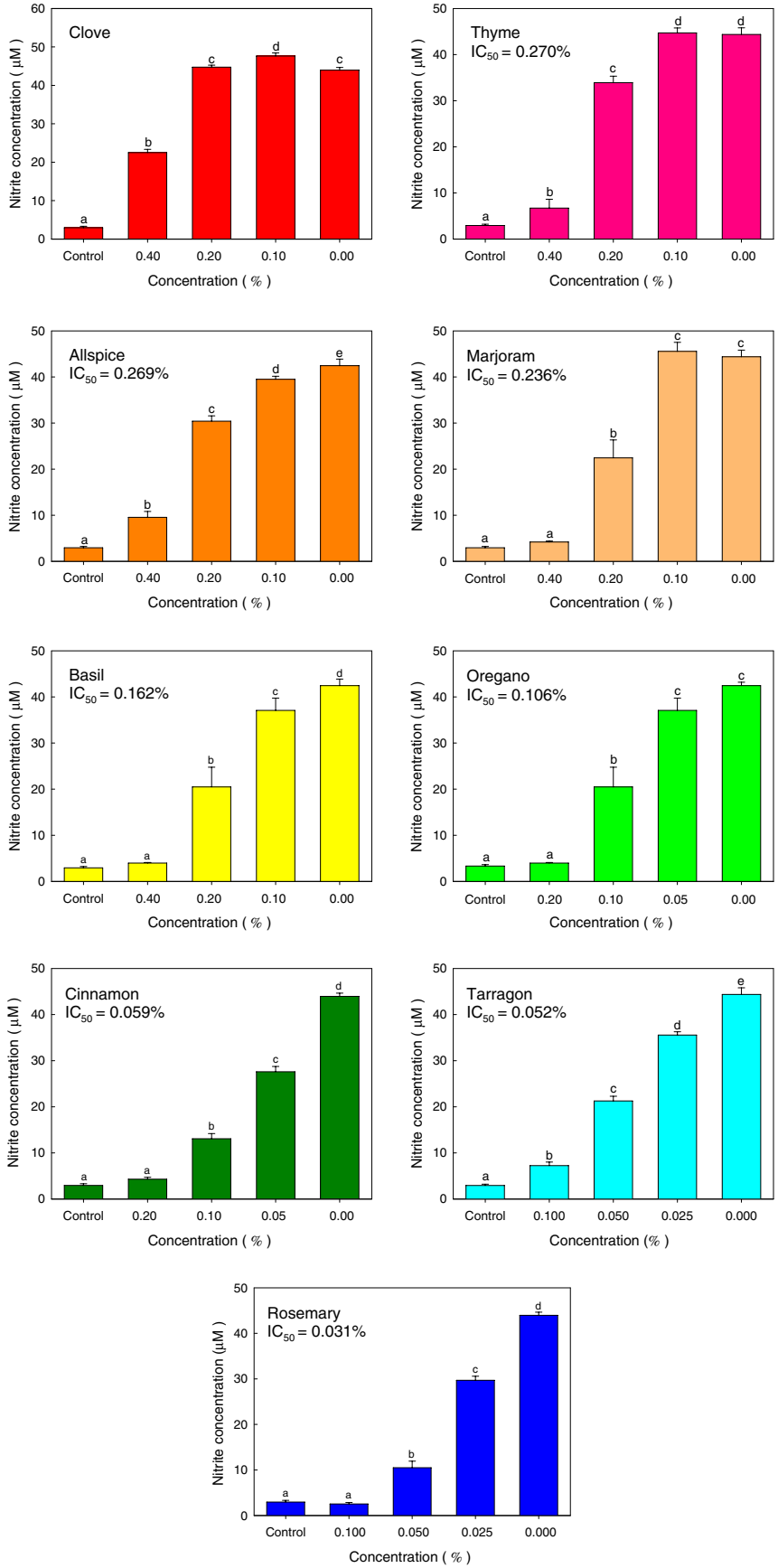

Fig. 1. Effect of various spice extracts on the NO production in LPS-activated RAW 264.7 cells. The values are expressed as means ± SEM of triplicate tests. Means not sharing a common letter were significantly different ($p < 0.05$) when analyzed by ANOVA and Duncan's multiple range test.

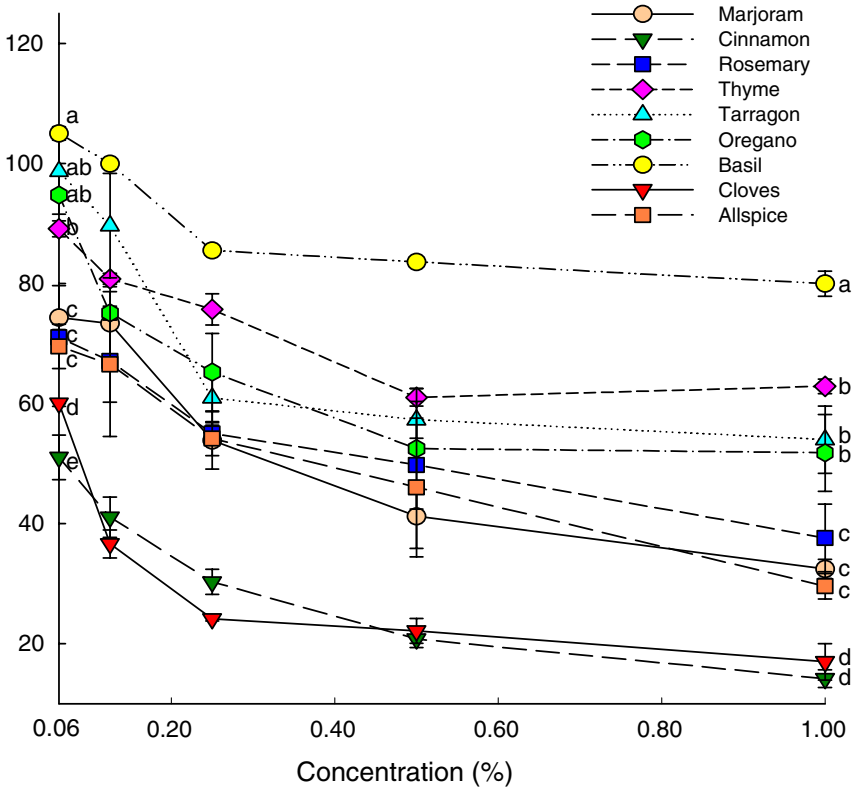

Fig. 2. Scavenging effect of various spice extracts on the NO radical released by sodium nitroprusside. The data are expressed as the means ± SEM of triplicate tests. Means not sharing a common letter were significantly different ($p < 0.05$) when analyzed by ANOVA and Duncan's multiple range test.

Table 1
Total phenolics content, NO and DPPH-scavenging activities of spice extracts

Spices	Botanical Name	IC_{50} of NO scavenging (%)	Phenolics content (mg GAE/mL extract)[b]	IC_{50} of DPPH-scavenging (%)
Rosemary	*Rosmarinus officinalis*	0.452	2.11 ± 0.07^{d}	0.105
Tarragon	*Artemisia dracunculus*	ND[a]	0.88 ± 0.04^{g}	0.234
Cinnamon	*Cinnamomum cassia*	0.068	6.90 ± 0.03^{b}	0.024
Oregano	*Origanum vulgare*	ND	2.45 ± 0.03^{c}	0.099
Basil	*Ocimum basilicum*	ND	0.92 ± 0.05^{g}	0.141
Marjoram	*Maiorana hortensi*	0.268	2.49 ± 0.08^{c}	0.119
Allspice	*Pimenta dioica*	0.332	1.79 ± 0.01^{e}	0.242
Thyme	*Thymus vulgaris*	ND	1.55 ± 0.07^{f}	0.130
Cloves	*Syzygium aromaticum*	0.080	15.66 ± 0.17^{a}	0.019

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

3.3. Effect of spice extracts on the iNOS protein expression in LPS-activated RAW 264.7 cells

For this work, rosemary and tarragon were used at a 0.1% treating concentration, cinnamon and oregano being used at 0.2%, whereas basil, marjoram, allspice and thyme were all used at 0.4% for the specific treating concentration which inhibited 90% of NO production by LPS-activated RAW 264.7 cells. This was undertaken so as to examine the effect of spice extract upon the iNOS protein expression. Fig. 3 reveals that RAW 264.7 cells did not express a detectable level of iNOS protein when incubated for 12 h in culture medium without LPS, whereas incubation of such cells in the presence of LPS markedly increased the iNOS protein content. All spices revealed a significant inhibitory effect upon the expression of iNOS (Fig. 3). Meanwhile, the inhibitory effect of spice extract upon the iNOS protein level was almost equivalent to the suppressing effect upon NO production.

3.4. Total phenolic content and DPPH radical scavenging activity of spice extracts

The phenolic content of spice extracts is revealed in Table 1. Clove, contains 15.66 mg gallic acid equivalent per milliliter of spice extract, which was the highest figure

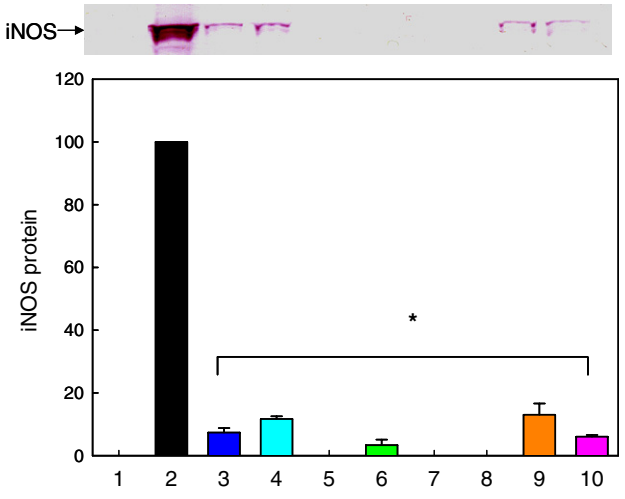

Fig. 3. Inhibitory effect of various spices on LPS-induced iNOS protein levels in RAW 264.7 cells. Lane 1 presents the results of the cells without any treatment. The other lanes represent cells treated without (lane 2) or with 0.1% extract from rosemary (lane 3), tarragon (lane 4), and 0.2% extract from cinnamon (lane 5) and oregano (lane 6), and 0.4% extract from basil (lane 7), marjoram (lane 8), allspice (lane 9) and thyme (lane 10), respectively, in the presence of LPS (100 ng/mL) for 12 h. The values are expressed as means ± SEM of triplicate tests. $^{*}p < 0.05$ as determined by paired Student's *t*-test as compared with the LPS-treated group (lane 2).

amongst the tested spices. Tarragon and basil were the lowest, they only containing, respectively, 0.88 and 0.92 mg gallic acid equivalent per milliliter of spice extract. Obviously, thus, there exists quite a large variation amongst the tested spices as regards phenolic content. The antioxidant activity of spices was evaluated by means of a DPPH radical scavenging assay and expressed as the IC_{50}. As shown in Table 1, the antioxidant activity of spices was able to be ranked based upon their IC_{50} value, the results revealing clove (0.019%) >cinnamon (0.024%) > oregano (0.099%) > rosemary (0.105%) > marjoram (0.119%) > thyme (0.130%) > basil (0.141%) > tarragon (0.234%) > allspice (0.242%). A significant correlation appeared to exist between the phenolic content of a particular spice and the IC_{50} of DPPH-scavenging ($r = -0.71$, $p < 0.05$), although the phenolic content of a particular spice neither correlated with the IC_{50} value for NO-suppressing nor with the spice's NO-scavenging ability.

4. Discussion

Foods and phytochemicals exert a NO-suppressing activity via three different pathways: (1) the blocking of iNOS expression, (2) the inactivation of iNOS catalytic function and (3) the scavenging of NO (Sheu and Yen, 2001). In this study, we selected nine commonly used spices to evaluate their NO-suppressing activity by LPS-activated macrophages and to investigate the actual suppressing mechanism involved. Amongst the nine study-tested spices, rosemary revealed the most-pronounced NO-suppressing activity, followed by tarragon, cinnamon, oregano, basil, marjoram, allspice and thyme, however, due to the cytotoxic effect of certain spices being more pronounced than the NO-suppressing activity, clove was not able to be used to determine its NO-suppressing action. When exploring the mechanism of NO suppression, it was found that cinnamon was an excellent NO scavenger, and marjoram, allspice and rosemary exhibited moderate NO-scavenging ability, whereas the other tested spices all performed poorly as regards NO-scavenging ability. Finally, a significant suppression of the iNOS protein by all spices was observed and almost equivalent to their suppressive effect upon NO production. Hence, according to the results of this current study, spices appear to be more likely to provide a NO-suppressing effect primarily through regulation of cellular iNOS expression.

Kim et al. (1998) screened 48 species of edible plants in Japan and found that families of *Araceas*, *Cruciferae*, *Labiatae* and *Liliaceae* exhibited strong suppressing activity upon NO production by LPS-activated RAW 264.7 cells. Indeed, the potent NO-suppressing activities of *Liliaceae* plants, such as garlic, green onion and leek, have also been documented in our, and others', previous studies (Tsai et al., 2005; Wang et al., 2005). All of the *Labiatae* plants tested in the present study, including rosemary, oregano, basil, marjoram, and thyme, revealed significant NO-suppressing activity for LPS-activated cells and thus, provided further convincing evidence to illustrate, at least partially, the relative benefits of the spices' anti-inflammation, anti-cancer or antioxidant effects.

Total phenolics content of a spice has been usually found to correlate highly with $ABTS^{+}$, DPPH, and O^{2-} scavenging activities of that spice, and therefore, phenolics were considered as the main contributors responsible for the free-radical scavenging activity of a spice (Parejo et al., 2002; Katsube et al., 2004; Tsai et al., 2005). In accord with the observations of these workers, it was observed herein that a significant correlation existed between the total phenolics content of a spice and its DPPH-scavenging ability. However, in this study, NO-scavenging activity did not correlate with total phenolics content, which suggested that some specific phenolic components rather than the total phenolics were the critical determinant for the NO-scavenging capacity of spices. The specific structural feature of phenolics needed for NO scavenging has been investigated previously, and it has been found that the presence of a catechol group in a flavonoid is essentially required for excellent NO-scavenging ability (De la Puerta et al., 2001) and that gallic acid linked to flavan-3-ol also plays an important role in the NO scavenging of catechins (Nakagawa and Yokozawa, 2002). Actually, such structure–activity relationships have also been applied for the scavenging of other reactive oxygen/nitrogen species, such as O^{2-} and peroxynitrite (Haenen et al., 1997; Chung et al., 1998; Heijnen et al., 2001; Taubert et al., 2003). Although the relative complexity and diversity of constituents in spices might be a causative factor for lacking relationship between phenolics

content and NO-scavenging activity, the specific detail of the structure–activity relationship is still needed to establish, in order to further establish and/or further illustrate the phenomenon that a spice's phenolic content correlates with DPPH-scavenging activity but not with its NO-scavenging ability.

According to the present results, the concentration that spices need to be present at in order to exert NO scavenging was somewhat greater than the concentration needed to suppress iNOS. Additionally, due to the presence of hemoglobin in the blood of human body, a compound which possesses 30 times the NO-scavenging activity of any of the flavonoids (Van Acker et al., 1995), it would seem rather unlikely that plant extracts and/or phenolics act significantly as NO-scavengers *in vivo* and thus, probably do not contribute significantly to a reduction in the level of *in vivo* NO-mediated cytotoxicity when compared to the action of hemoglobin. However, the application of such plant extracts and/or phenolics in the conservation of muscle-derived foods would likely be expected, given that NOS activity which is involved in peroxynitrite-induced lipid peroxidation and discoloration has often been detected in post-mortem tissues (Brannan et al., 2001; Brannan and Decker, 2002).

The NO-suppressing activity of certain Mediterranean spices in this study was mainly exerted through inhibition of iNOS expression. The rather striking NO-suppressing activity of rosemary has been previously documented according to one of its constituents, namely "carnosol", a compound which inhibits iNOS expression by blocking the activation of NF-κB (Chan et al., 1995; Lo et al., 2002). Cinnamic aldehyde, a compound found to be present in, specifically, the essential oil of cinnamon, as well as clove and allspice, has also been identified as a potent iNOS suppressor (Lee et al., 2002). In addition, several flavonoids, present ubiquitously in certain plants including apigenin, quercetin, kaempferol and chrysin (Liang et al., 1999, 2001) have been reported to decrease the transcriptional activity of iNOS gene. Although the real bioactive components of a number of these spices still warrant further exploration, all of the phytochemicals mentioned above did provide, at least to some extent, some potential illustration of the iNOS-suppressing activity of such spices.

In addition to exhibiting strong antioxidant activity, the examined spices demonstrated excellent suppressing activity as regards NO production by LPS-activated cells, this being facilitated mainly through the inhibition of iNOS expression. An obvious difference as regards NO-suppressing activity existed between the various spices tested herein, thus the results of this study might provide a potential evaluating standard for individuals to select spices which feature high NO-suppressive activity. Rosemary proved to be the most-pronounced NO suppressor amongst the nine tested spices, although this result did not take into consideration the absorption, distribution and metabolism of this spice, or any other, within the human body.

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