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Extract of the seed coat of *Tamarindus indica* inhibits nitric oxide production by murine macrophages in vitro and in vivo

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Abstract

The seed coat extract of *Tamarindus indica*, a polyphenolic flavonoid, has been shown to have antioxidant properties. The present studies investigated the inhibitory effect of the seed coat extract of *T. indica* on nitric oxide production in vitro using a murine macrophage-like cell line, RAW 264.7, and in vitro and in vivo using freshly isolated B6C3F1 mouse peritoneal macrophages. In vitro exposure of RAW 264.7 cells or peritoneal macrophages to 0.2–200 μg/mL of *T. indica* extract significantly attenuated (as much as 68%) nitric oxide production induced by lipopolysaccharide (LPS) and interferon gamma (IFN-γ) in a concentration-dependent manner. In vivo administration of *T. indica* extract (100–500 mg/kg) to B6C3F1 mice dose-dependently suppressed TPA, LPS and/or IFN-γ induced production of nitric oxide in isolated mouse peritoneal macrophages in the absence of any effect on body weight. Exposure to *T. indica* extract had no effect on cell viability as assessed by the MTT assay. In B6C3F1 mice, preliminary safety studies demonstrated a decrease in body weight at only the highest dose tested (1000 mg/kg) without alterations in hematology, serum chemistry or selected organ weights or effects on NK cell activity. A significant decrease in body weight was observed in BALB/c mice exposed to concentrations of extract of 250 mg/kg or higher. Oral exposure of BALB/c mice to *T. indica* extract did not modulate the development of T cell-mediated sensitization to DNFB or HCA as measured by the local lymph node assay, or dermal irritation to nonanoic acid or DNFB. These studies suggest that in mice, *T. indica* extract at concentrations up to 500 mg/kg may modulate nitric oxide production in the absence of overt acute toxicity.

Keywords: *Tamarindus indicia*; Nitric oxide; Macrophage

1. Introduction

Plant materials have long been used as traditional medicines for the treatment of a wide variety of ailments and diseases. Components of *Tamarindus indica*, a tree indigenous to India and South East Asia, have been used as a spice, food component, and snack. According to Thai traditional medicine, the fruit of *T. indica* is regarded as a digestive, carminative, laxative, expectorant and blood tonic. In addition, the seeds of *T. indica* are used as an anthelmintic, antidiarrheal, and an emetic, and the seed coat is used to treat burns and aid in wound healing as well as an antidysenteric (Farnsworth and Bunyapraphatsara, 1992).

Recently, Pumthong (1999) demonstrated the antioxidant activity of the seed coat extract of *T. indica*. The extract is composed of flavonoids including tannins, polyphenols, anthocyanidin, and oligomeric proanthocyanidins. Many of these flavonoids are also components of Pycnogenol®, a nutritional supplement which has been shown to have vasorelaxant activity, increase capillary permeability and participate in the cellular antioxidant network as indicated by its ability to regenerate the ascorbyl radical and to protect endogenous vitamin E and glutathione from oxidative stress (Packer et al., 1999; Rohdewald, 2002). Flavonoids found in various medicinal plants are natural antioxidants with free radical scavenging activity and they have also been

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shown to prevent free radical formation via inhibition of oxido-reductases (Middleton et al., 1986; Chen et al., 1993; Krol et al., 1995; Carlo et al., 1999).

Based on the reported antioxidant activity of the seed coat extract of *T. indica* which contains flavonoids among its major constituents, and the limited toxicological data available, the purpose of the present studies was to assess the anti-inflammatory potential of this extract and begin to access its safety. In vitro studies using *T. indica* seed coat extract were conducted to evaluate the modulation of nitric oxide (NO) production by RAW 264.7 macrophages using LPS and IFN-γ as stimulants. Confirmation of the effect in vivo was tested by orally exposing B6C3F1 mice to *T. indica* extract for 14 days and evaluating NO production by freshly isolated peritoneal macrophages following stimulation in vitro with lipopolysaccharide (LPS) and/or interferon gamma (IFN-γ), and in vivo or in vitro with 12-O-tetradecanoylphorbol-13-acetate (TPA). A 14-day toxicity study and studies to evaluate the effect of *T. indica* seed coat extract on components of innate and cellular immunity were performed to begin to assess safety.

2. Material and methods

2.1. Chemicals

The seed coat extract of *T. indica* was kindly provided by Dr. Maitree Suttajit (Chiang Mai University). Tamarind seeds were obtained from ripened tamarind fruits after removing the edible parts. The seeds were heated in a hot air oven at 140 °C, for 45 min, cooled and cracked to separate their outside brown layer. Only brown-red seed coats were collected and these were then ground into fine powder. In a separating funnel, 10 ml of 70% ethanol was added to 0.5 g of the ground tamarind seed coat. After vigorous shaking for 10 min the solution was passed through filter paper. This procedure was repeated until the filtrate became colorless. Five millilitres of the filtrate was further fractionated by mixing with 5 ml of chloroform. The mixture was shaken well and allowed to stand until the 2 layers were separated. Two millilitres of the upper aqueous layer was added to 5 ml of ethyl acetate. The mixture was shaken and left to separate into 2 layers. The lower layer containing a large amount of ethyl acetate was removed and the remaining layer was dissolved in 5 ml of methanol. Thin layer chromatography of the extract following acid hydrolysis demonstrated 6 distinct components each with different Rf values. The UV absorption spectra and IR spectra of the *T. indica* extract was found to be comparable with that of OPC extracted from grape seed and pine bark (Pumthong, 1999). Proximate anaylsis (conducted by Midwest Laboratories, Omaha, NE) revealed that the dried extract consisted of 89.87% carbohydrates, 0.32% protein and 0.38% fat. Using the Folin-Cicalteau method (Taga et al., 1984) the extract was shown to contain 247.25 mg/g of phenolic compounds.

The extract was suspended in 10% dimethylsulfoxide (DMSO) in sterile complete DMEM (Gibco, Grand Island, NY) for in vitro exposure or in phosphate buffered saline (PBS) for in vivo studies. IFN-γ was obtained from Research Diagnostics (Flanders, NJ). Dye solution 3(4,5-dimethyl thiazol-2-yl) 2,5-diphenyltetrazolium Bromide (MTT) and the Solubilization/Stop Solution in CellTiter 96 were obtained from Promega Corporation (Madison, WI). LPS (*Escherichia coli* serotype 0111:B4), vitamin C (ascorbic acid), vitamin E (α-tocopherol), β-carotene, TPA, 2,4-dinitrofluorobenzene (DNFB; purity ≥99%), acetone, α-hexylcinnamaldehyde (HCA; 85% purity), nonanoic acid (pelargonic acid; purity ≥97%), anti-asialo GM1, and all other reagents were obtained from Sigma Chemical Company (St. Louis, MO).

2.2. Animals and maintenance

Female B6C3F1 mice were used for in vivo toxicity and NK studies and as a source of splenocytes for in vitro studies. Female BALB/c mice were used for local lymph node and irritancy studies. Mice were obtained from Taconic Farms (Germantown, NY) or Charles Rivers Laboratories in Wilmington, MA. Mice were maintained in the NIOSH animal facility (Morgantown, WV) under conditions specified within NIH guidelines (NIH, 1996) and experiments were conducted under an approved NIOSH Animal Care and Use Committee protocol. Mice were housed in polycarbonate "shoebox" cages with hardwood bedding and were provided tap water, and Agway Prolab Animal Diet (5% fat) *ad libitum*. Animals were weighed, individually identified by tail mark and assigned into homogeneous weight groups. Animal rooms were maintained between 18–26 °C and 40–70% relative humidity with light/dark cycles of 12 h intervals.

2.3. Cell culture

The murine macrophage cell line RAW 264.7 gamma NO (−) (ATCC CRL-2278) used in these studies was kindly provided by Dr. Gabriel K. Harris (National Institute for Occupational Safety and Health, Morgantown, WV). Cells were cultured and maintained in DMEM containing 10% heat-inactivated fetal calf serum (Hyclone, Utah), 100 U/ml penicillin and 100 μg/ml streptomycin (Gibco, Grand Island, NY) and grown at 37 °C with 5% CO_2 in humidified air. Exponentially growing cells were used for experiments when they reached 80% confluency.

2.4. In vivo exposure

B6C3F1 mice (5 animals/group) were administered increasing concentrations of *T. indica* seed coat extract (50, 250, 500 or 1000 mg/kg) or vitamin C (100 mg/kg) by oral gavage once a day for 14 days. The dose of vitamin C was chosen based on the previous studies by Bagchi et al. (1998). Twenty-four hours following the final exposure, animals were sacrificed by CO_2 inhalation and peritoneal macrophages were collected. In studies where peritoneal macrophages were stimulated in vivo with TPA, animals were injected intraperitoneally with 0.1 μg TPA diluted in 1 ml of PBS 2 h prior to sacrifice.

2.5. Collection of peritoneal macrophages

Murine peritoneal cells were removed by peritoneal lavage with 7 ml of ice cold PBS with 10% fetal calf serum. Differentials of the peritoneal cells indicated an average of 70% macrophages. Cells were kept cold on ice until transfer to a complete medium consisting of RPMI-1640 with 10% FCS, and penicillin (0.05 U/ml)-streptomycin (0.05 mg/ml). Cells were counted and 2×10^5 cells were plated in Costar 96-well flat-bottom plates (Corning, Corning, NY) and incubated for 2 h at 37 °C. Non-adherent cells were removed by washing with pre-warmed (37 °C) culture medium.

2.6. In vitro exposure

For in vitro studies, peritoneal macrophages or RAW cells, were plated at a concentration of 2×10^5 cells per well and incubated with test article or comparative controls at 37 °C for 24 h in the presence or absence of NO stimulants. The following stimulants and concentrations were selected based on the references cited; TPA (0.1 μg/ml, Bagchi et al., 1998), LPS (5 μg/ml, Lin and Lin, 1997), and IFN-γ (10 ng/ml, Virgili et al., 1997). The following comparative controls and concentrations were selected based on the references cited: 500 μM, vitamin C (Sandoval, 1977), 141.3 μM vitamin E (Bagchi, 1997) and 2.5 μM β-carotene, (Murakami et al., 2000). The concentrations of *T. indica* extract tested ranged from 0.2 to 200 μg/ml.

2.7. Measurement of nitrite and cell viability

Nitrite concentration was determined using the Griess reagent as described by Padgett and Pruett (1992). Following 24 h incubation with the stimulant and test article, 100 μl of cell-free supernatant from each well was transferred to another 96-well flat-bottom plate. Greiss reagent (100 μl, 1% sulfanilamide in 30% glacial acetic acid and 0.1% napthylethylenediamine dihydrochloride in 60% glacial acetic acid) was added. The absorbance of samples was measured at 545 nm with a spectrophotometric microplate reader (Spectra MAX 340). Nitrite in the samples was quantitated by comparison with a standard curve. A nitrite multi-point linear standard curve was generated using known concentrations of $NaNO_2$ ranging from 1.56 μM/ml to 200 μM/ml added to the complete culture media and analyzed with Softmax 3.3.3 ELISA software (Molecular Devices, Sunnyvale, CA).

Once 100 μl cell-free supernatant was removed from each well to determine nitrite concentration, cell viability was evaluated in the original plates by the addition of 15 μl of Dye Solution-MTT for 4 h. After washing 2 times with DMEM, the supernatant was removed and the formed insoluble formazan product was dissolved with 100 μl of Solubilization/Stop Solution. The optical densities of the culture wells were measured using a spectrophotometric microplate reader (Spectra MAX 340) at reference wavelengths of 570 and 730 nm. The optical density of formazan formed in control (untreated) cells was taken as 100%. Percent viability of test samples were determined as: % Viability = (average OD for test group/average OD for control group)×100.

2.8. Fourteen day toxicity study

Animals were weighed and assigned into homogenous weight groups. Animals were dosed with 50, 100, 500, or 1000 mg/kg of *T. indica* seed coat extract in 0.2 ml PBS per 20 g of body weight via oral gavage daily for 14 consecutive days. Animals were weighed daily and observed for signs of morbidity and mortality. On the final day of study, 24 h after the last dose, animals were weighed and sacrificed by CO_2 inhalation. Blood was collected for hematology (white blood cell count and differential, red blood cell count, hematocrit, hemoglobin, mean corpuscular hemoglobin, mean corpuscular volume, platelet count and mean platelet volume) and serum chemistry analysis (alkaline phosphatase, alanine transferase, aspartic transferase, blood urea nitrogen, uric acid, bilirubin, and creatine), and selected organ weights were taken including liver, brain, heart, spleen, kidneys, lungs, and thymus.

2.9. Irritancy assay

An irritancy study was performed to investigate the effects of oral exposure to the seed coat extract of *T. indica* on the development of dermal irritation to the moderate irritant nonanoic acid (NA) and the potent irritant DNFB. BALB/c mice were administered increasing concentrations of the seed coat extract of *T. indica* (50, 100 or 200 mg/kg) by oral gavage once a day for 14 days. On days 12, 13, and 14 separate groups of animals were dosed dermally with 50 μl of 60% nonanoic acid or 0.15% DNFB in acetone on the dorsal surface of each

ear (25 μl/ear). Ear thickness was measured prior to the first dermal exposure (day 11), and 24 h after the final exposure (day 15) to evaluate the irritant response as previously described by Woolhiser et al. (1998).

2.10. Local lymph node assay (LLNA)

The LLNA was conducted in female BALB/c mice following oral exposure to *T. indica* extract to evaluate the modulatory effect of *T. indica* seed coat extract on the development of a T cell-mediated contact hypersensitivity response. The assay was conducted as previously described by Woolhiser et al. (1998). Five mice per treatment group received vehicle or increasing concentrations of *T. indica* extract by oral gavage for 14 consecutive days. On days 10, 11 and 12, separate groups of mice were dosed with 50 μl of either 30% HCA or 0.15% DNFB on the dorsal surface of both ears (25 μl on each ear). On day 15, the mice were injected intraveneously via the lateral tail vein with 0.2 ml (20 μCi) of ^{3}H-thymidine (specific activity of 0.2 μCi/ml). Five h later the mice were sacrificed by CO_2 inhalation. The left and right draining cervical lymph nodes of each animal, located at the bifurcation of the jugular vein, were excised and placed into 4 ml cold phosphate-buffered saline (PBS). A single cell suspension was generated by dissociating the lymph nodes between the frosted ends of two microscope slides. Cells were washed twice with 10 ml PBS and precipitated in 3 ml 5% trichloroacetic acid (TCA) overnight at 4 °C. Following resuspension in 1 ml TCA, the cells were transferred to 5 ml of scintillation cocktail. ^{3}H-thymidine incorporation was determined with a Packard, Tri-carb 2500TR Beta Counter. Samples were counted for 5 min each and mean DPM-background was calculated for each dose group. The stimulation index for each test group was calculated by dividing the group mean by the mean for the vehicle control group.

When evaluating the potential immunomodulatory effect of *T. indica* extract following DNFB exposure, the LLNA and irritancy assays were combined to reduce animal usage (Woolhiser, 1998). Prior to the first topical DNFB exposure in the LLNA, animals' ears were pre-measured and then post-exposure measurements were taken 24 h following the final exposure and prior to injection with 3H-thymidine.

2.11. Natural killer cell (NK) activity

Following 14 days of oral exposure to *T. indica* extract or VH ($n=5$), an in vitro cytotoxicity assay as described previously (Holsapple et al., 1988; Duke et al., 1985) was used to evaluate NK cell function. Positive control animals were administered 0.2 ml of a 1:10 dilution of anti-asialo GM1 by i.p. injection 24 h prior to assay. All animals were sacrificed and spleens were collected 24 h following the final exposure. Single cell suspensions were made and splenocytes were adjusted to 2×10^{7} cells per ml in complete media (RPMI, 10% fetal calf serum, 50 IU penicillin and 50 μg streptomycin). Splenocyte and ^{51}Cr-labeled Yac-1 cells (ATCC, TIB 160, Manassas, VA) were prepared in ratios of 200:1, 100:1, 50:1, 25:1, 12.5:1, 6.25:1 and replicate wells plated for each concentration. After a 4-h incubation at 37 °C and 5% CO_2, 100 μl of supernatant was transferred to tubes and counted on a gamma counter (Packard Cobra II Auto-Gamma). Maximum release was determined by lysing ^{51}Cr-labeled Yac-1 cells with 0.1% Triton X in complete media. Spontaneous release was determined by incubating Yac-1 tumor cells in complete media. The results were expressed in percent lysis for each of the effector to target ratios.

2.12. Statistical analysis

Statistical analysis was performed using Graph Pad Prism version 3.0 (Graph Pad Software Inc., San Diego, CA). For multiple comparisons, data were analyzed by one-way ANOVA followed by Dunnett's test when significant differences were detected ($P\leqslant0.05$). Comparison between two groups was made using a student *t*-test. Linear regression was used to determine dose-responsiveness. Data were considered statistically significant at $P\leqslant0.05$.

3. Results

3.1. Modulation of NO production by LPS & IFN-γ stimulated RAW 264.7 cells following in vitro exposure to seed coat extract of T. indica

To investigate the effect of the seed coat extract of *T. indica* on NO production, the accumulation of nitrite, the stable metabolite of NO, was measured in the culture media of RAW 264.7 cells using Greiss reagent. Resting RAW 264.7 cells were stimulated with LPS (5 μg/ml) and/or IFN-γ (10 ng/ml) to stimulate NO production. Cells were simultaneously treated with increasing concentrations of the seed coat extract of *T. indica* or with vitamin C (500 μM), vitamin E (141.3 μM), or beta-carotene (2.5 μM) for 24 h. As shown in Fig. 1a, in vitro exposure to seed coat extract of *T.indica* at concentrations ranging from 0.2 to 200 μg/ml inhibited nitrite production in a concentration-dependent manner reaching a 68% suppression at the high dose. An approximate 54% suppression in NO production was observed in cells treated with the comparative controls, vitamin C (500 μM), vitamin E (141.3 μM), or beta-carotene (2.5 μM). Based on the MTT assay, the observed decrease in NO production by mice exposed to *T. indica* extract, vitamin E or β-carotene occurred in the absence

of any effect on cell viability (Fig. 1b). An approximate 10% reduction in cell viability was seen following exposure to vitamin C.

3.2. Fourteen day oral toxicity study

Prior to accessing the modulatory effects of *in vivo T. indica* extract exposure on NO production, a 14-day toxicity study was conducted to select the doses to be administered. No deaths or abnormal clinical signs were observed in any of the mice treated with the seed coat extract of *T. indica* throughout the 14 days of the study. Exposure of B6C3F1 mice to 1000 mg/kg of the extract resulted in a decrease in body weight, reaching a maximum of 14% weight loss on day 11 (Fig. 2a). Treatment with the seed coat extract of *T. indica* at concentrations up to 1000 mg/kg had no significant effects on hematological parameters or serum chemistries (data not shown). Furthermore, organ weights including, spleen, lungs, thymus, brain, kidney and liver, were not altered by treatment with the seed coat extract of *T. indica* (data not shown). Based on this data, 500 mg/kg was chosen as the highest dose to be used in the subsequent in vivo studies using B6C3F1 mice. Given that weight loss was observed in BALB/c mice exposed to 500 μg/kg for 14 days (Fig. 2b), 200

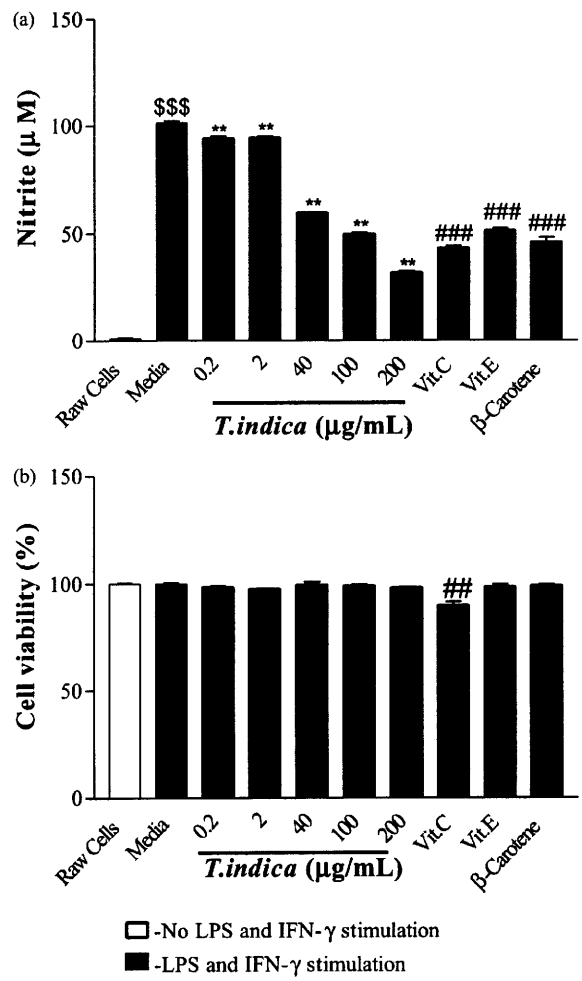

Fig. 1. Nitrite production (a) and percent cell viability (b) of RAW 264.7 cells treated with increasing concentrations of *T. indica* extract, Vit C (500 μM), Vit E (141.3 μM) or β-carotene (2.5 μM) and simultaneously stimulated with LPS (5 μg/ml) and IFN-γ (10 ng/ml) for 24 h. Results are expressed as mean±SE for groups of 5 mice. Nitrite production by LPS and IFN-γ stimulated RAW cells was compared to non-stimulated cells by student's *t*-test ($$$ represents $p < 0.0001$). For LPS and IFN-γ stimulated cells, data from *T. indica* extract exposed groups were analyzed by one-way ANOVA followed by Dunnett's test when significant differences were detected ($P \leqslant 0.05$), ** represents $P < 0.01$. Vit.C, Vit.E, and β-carotene exposed groups were individually compared to the media exposed RAW cells using *t*-test (## represents $P < 0.001$, ### represents $P < 0.0001$).

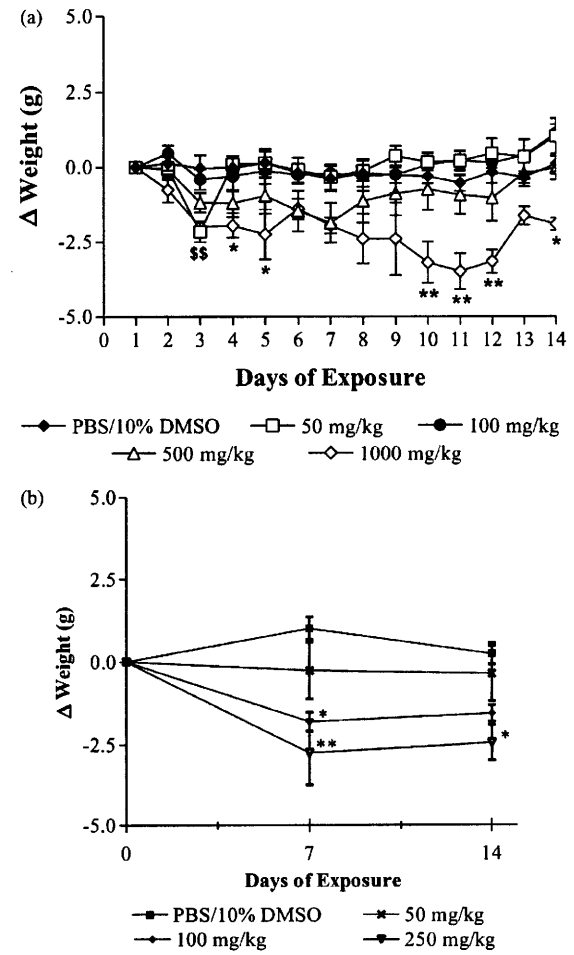

Fig. 2. Change in body weight (g) during the 14-day time course of oral exposure to VH (PBS/10% DMSO) or increasing concentrations of *T. indica* extract in B6C3F1 mice (a) or BALB/c mice (b). Results are expressed as mean±SE for groups of 5 mice. For each time point, data were analyzed by one-way ANOVA followed by Dunnett's test when significant differences were detected ($P \leqslant 0.05$). *represents $P < 0.05$, and **represents $P < 0.01$ as compared to VH control. $$ represents $P < 0.01$ in both the 50 and 1000 mg/kg groups as compared to VH control.

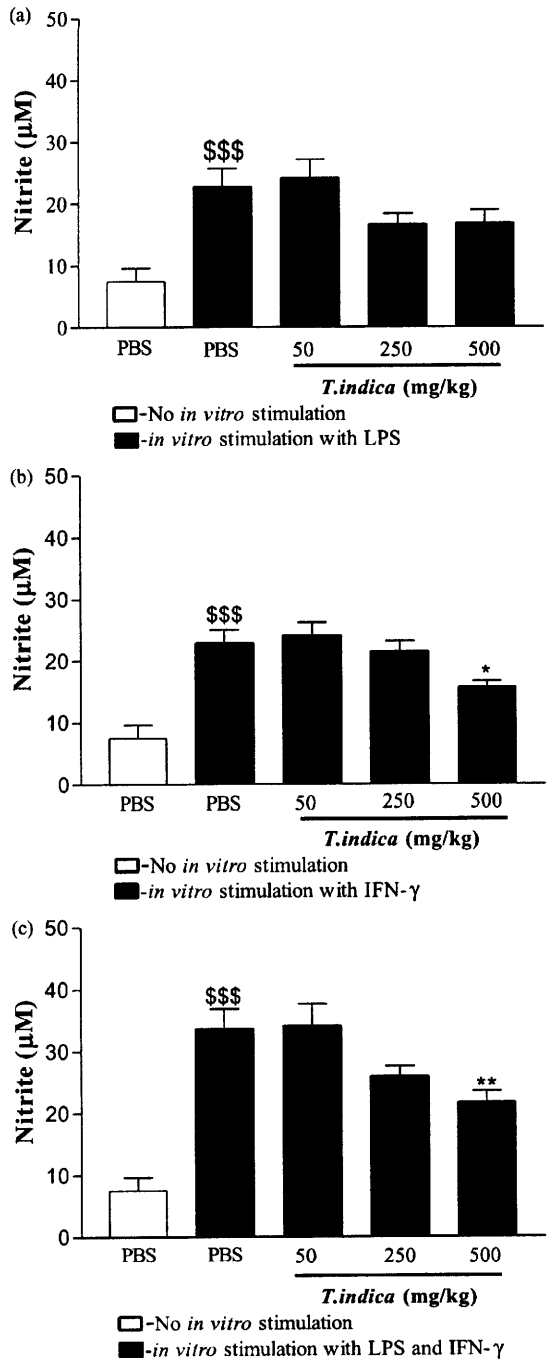

Fig. 3. Nitrite production by peritoneal macrophages from mice treated with PBS (PBS/10% DMSO) or increasing concentrations of *T. indica* extract and stimulated *in vitro* with LPS (a), IFN-γ (b), or LPS and IFN-γ (c). Results are expressed as mean±SE for groups of 5 mice. Levels of nitrite from IFN-γ and/or LPS stimulated macrophages from PBS control animals were compared with levels from non-stimulated macrophages using student's *t*-test ($$$ represents $P<0.0001$). Levels of nitrite from *T. indica* extract treatment groups and IFN-γ and/or LPS stimulated macrophages from PBS control data were compared by one-way ANOVA followed by Dunnett's test when significant differences were detected ($P\leqslant 0.05$). (*represents $P<0.05$, ***represents $P<0.0001$).

mg/kg was chosen as the highest dose in studies using BALB/c mice.

3.3. Modulation of NO production by peritoneal macrophages following in vivo exposure to seed coat extract of T. indica

To assess the potential for *T. indica* extract to modulate NO production in vivo, female B6C3F1 mice were treated with vehicle, 10% DMSO in PBS, or increasing concentrations of the extract (50, 250, 500 mg/kg) by oral gavage for 14 days. On day 15 animals were sacrificed and peritoneal macrophages were collected and treated with LPS (5 μg/ml), IFN-γ (10 ng/ml), or LPS and IFN-γ for 24 h. As shown in Fig. 3a, b, and c, treatment with LPS and/or IFN-γ induced a significant increase in nitrite production when compared with unstimulated peritoneal macrophages. Combined exposure to LPS & IFN-γ increased nitrite production greater than exposure to either LPS or IFN-γ alone ($P<0.05$). Exposure to *T. indica* extract dose-dependently inhibited NO production by IFN-γ and LPS and IFN-γ stimulated cells reaching statistical significance in the highest *T. indica* extract exposure group (500 mg/kg). No effect on the viability of peritoneal macrophages due to test article exposure was observed in this study (data not shown). Cell viability was greater than 90% for all groups.

Following oral exposure to *T. indica* extract, when TPA was used as the in vitro stimulant, NO production by peritoneal macrophages was significantly inhibited in a dose-dependent manner reaching 55% inhibition at the 500 mg/kg dose, a level comparable to that seen following exposure to 100 mg/kg Vit. C (Fig. 4A). As shown in Fig. 4B, nitrite production induced by intraperitioneal exposure to TPA was also inhibited dose-dependently by oral exposure to the seed coat extract of *T. indica*, reaching statistical significance at 250 mg/kg dose and showing 70% inhibition at the 500 mg/kg dose. Cell viability was not affected by exposure to either *T. indica* extract or TPA in either of these studies (data not shown).

3.4. Modulation of the dermal irritant response to nonanoic acid and DNFB following in vivo exposure to the seed coat extract of T. indica

Topical exposure to the moderate irritant nonanoic acid (60%) induced an approximate 28% increase in ear swelling in BALB/c mice. Fourteen days of oral exposure to the seed coat extract of *T. indica* (up to 200 mg/kg) did not have any modulatory effect on this response (Fig. 5). Likewise, no effect was seen following 14-day oral exposure to *T. indica* seed coat extract in a similar study using 0.15% DNFB as the irritant (data not shown).

3.5. Modulation of the induction of T-cell mediated sensitization and NK cell activity following in vivo exposure to seed coat extract of T. indica

Topical exposure to 0.15% DNFB, a potent sensitizer, induced lymphocyte proliferation in the draining lymph nodes of BALB/c mice with a stimulation index of 31. Fourteen days of oral exposure to the seed coat extract of *T. indica*, at concentrations up to 200 mg/kg, or Vit C (100 mg/kg) did not have any modulatory effect on this response (Fig. 6). A similar study was conducted using the more moderate sensitizer HCA at a concentration of 30% with no modulatory effect on lymph node cell proliferation observed following oral exposure to *T. indica* extract (data not shown).

NK activity in vehicle exposed B6C3F1 mice demonstrated a 20% lysis at the 200:1 effector to target ratio. A 72% suppression in NK activity was observed in animals exposed to the positive control, anti-asialo GM1. Fourteen day oral exposure to the seed coat extract of *T. indica* at doses up to 500 mg/kg or to the comparative control, Vit.C (100 mg/kg), had no effect on NK activity (Fig. 7).

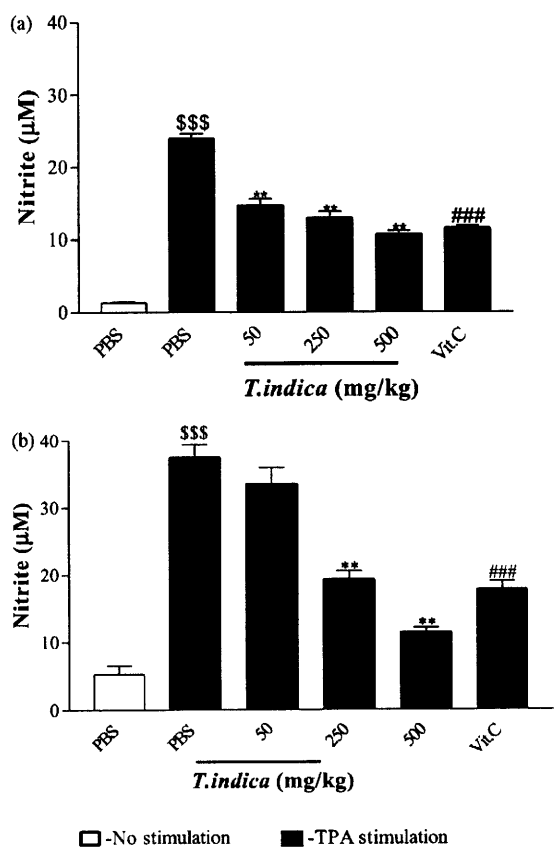

Fig. 4. Nitrite production by peritoneal macrophages from mice orally gavaged with PBS (PBS/10% DMSO), increasing concentrations of *T. indica* extract or Vit.C (100mg/kg) and stimulated in vitro (a) or in vivo (b) with 0.1 μg/ml of TPA. Results are expressed as mean±SE for groups of 5 mice. The PBS exposed (TPA stimulated and unstimulated) mice were compared using a student's *t*-test ($$$ represents $P<0.0001$). For TPA stimulated groups, *T. indica* extract exposed groups and the PBS exposed group were compared using ANOVA followed by Dunnett's post test when significant differences ($P<0.05$) occurred. **represents $P<0.01$. The Vit.C exposed group was compared with the PBS exposed group using a student's *t*-test (### represents $P<0.0001$).

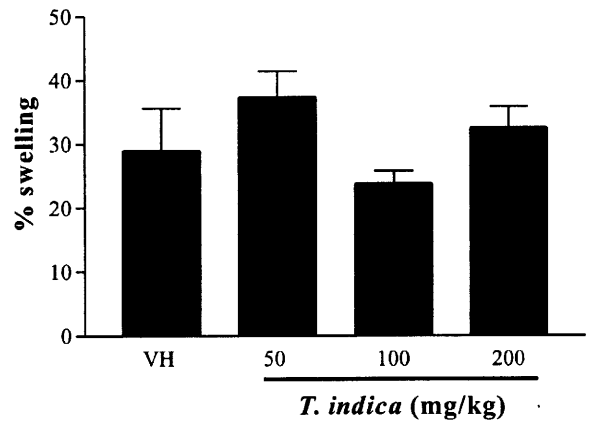

Fig. 5. BALB/c mice ($n=5$) were orally exposed to either VH (PBS/10% DMSO) or the concentration of the seed coat extract of *T. indica* shown for 14 days. Starting on the 12th day following initial oral exposure, animals were exposed topically with 60% nonanoic acid for 3 consecutive days. Ear thickness was measured pre-exposure and 24 hrs post-final exposure. Bars represent mean±SE of the calculated percent ear swelling.

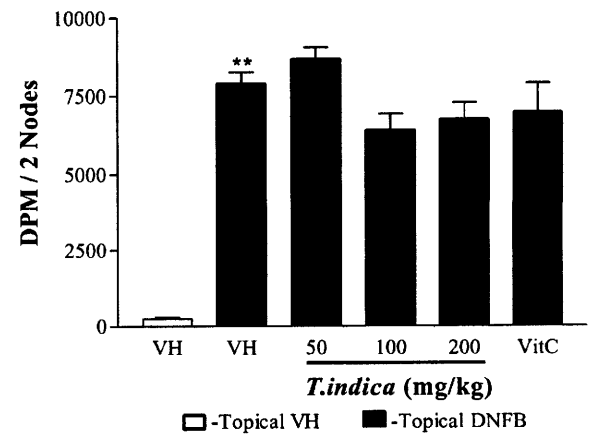

Fig. 6. BALB/c mice ($n=5$) were dosed orally with either vehicle (PBS/10% DMSO) or increasing concentrations of the seed coat extract of *T. indica* as shown. Open bar indicates topical exposure to vehicle (acetone), and closed bars represent topical exposure to 0.15% DNFB. Bars represent mean±SE. **represent $P<0.01$ as compared to the open bar by student's *t*-test.

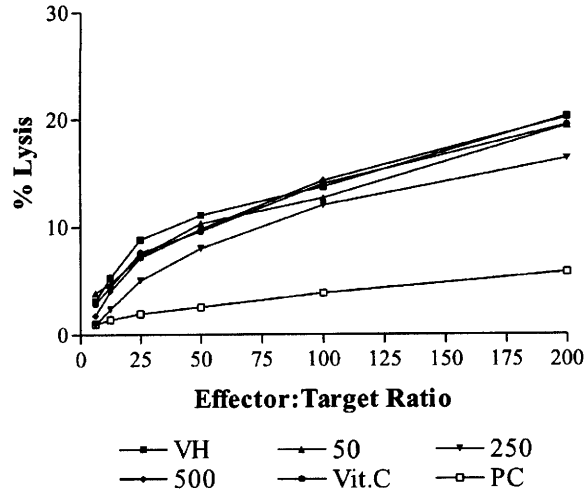

Fig. 7. Natural killer cell activity in female B6C3F1 mice ($n=5$) following a 14-day exposure to the seed coat extract of *T. indica* or vitamin C. Data are presented as mean±SE. Anti-asialo GM1 was used as a positive control (PC).

4. Discussion

Although individual components have not been identified, the seed coat extract of *T. indica* contains high amounts of polyphenolic flavonoids which are known to exhibit strong antioxidant scavenging activity against peroxyl radicals generated by ABTS/H_2O_2/peroxidase and ABTS/H_2O_2/myoglobin systems, hydroxyl radicals produced by ABTS/H_2O_2/$FeCl_3$ (Feton reaction) and superoxide anions generated by hypoxanthine-xanthine oxidase (neotetrazolium) system (Pumthong, 1999). Due to the multiple phenolic groups in their structure, flavonoids are potentially able to quench free radicals by forming more stable oxidized products. Various other naturally occurring polyphenolic flavonoids such as rutin, quercetin, apigenin, wogonin, luteolin, tectorigenin, galangin, morin, naringenin (van Acker et al., 1995; Kim et al., 1999 and Raso et al., 2001), epigallocatechin gallate from tea (Lin, 1997; Chan et al., 1997), Ginkgo biloba extract (EGb 761) (Kobuchi, et al., 1997), silymarin (Kang et al., 2002) and Pycnogenol (Virgili et al., 1998) have been reported to inhibit NO production. Therefore, the present studies were designed to investigate the potential for the seed coat extract of *T. indica* in modulation of NO production.

Although first recognized for its role as a vasodilator and in the regulation of blood flow and pressure, nitric oxide has been identified as an important intra- and intercellular regulatory molecule with functions as diverse as neural communication, the generation of memory and host defense (for review see, Garthwaite, 1991; Moncada and Higgs, 1993; Rand, 1992; Moncada et al., 1991; Ignarro, 2002). NO produced by the endothelium and/or platelets inhibits platelet aggregation and adhesion, inhibits leukocyte activation and modulates smooth muscle cell proliferation (Mocada and Higgs, 1993). In the peripheral nervous system, NO is released by a widespread network of nerves, previously recognized as nonadrenergic and noncholinergic. These nerves mediate some forms of neurogenic vasodilation and regulate certain gastrointestinal, respiratory and genitourinary functions (Rand, 1992). NO is also synthesized in neurons of the central nervous system, where it acts as a neuromediator with many physiological functions, including the formation of memory, coordination between neuronal activity and blood flow (Garthwaite, 1991; Snyder and Bredt, 1992; Prast and Philippu, 2001). In contrast to its role as an antioxidant and neuroprotectant, altered NO formation and function have been shown to play a role in the pathphysiology of neurological diseases including Alzheimer's Disease, Parkinson's Disease, cerebral ischemia and hepatic encephalopathy (Rao, 2002).

Low concentrations of NO from activated macrophages are beneficial as, along with other reactive nitrogen intermediates, they are responsible for cytostatic and cytotoxic activity against infectious organisms and tumor cells. In addition, NO plays a regulatory role in the function of natural killer cells and the expression of cytokines such as IFN-γ and transforming growth factor-β (Bogdan et al., 2000). However, overproduction of NO has been found to be associated with various diseases such as septic shock, autoimmune diseases, and chronic inflammation by increasing vascular permeability and the extravasations of fluid and proteins at the inflammatory site (Moncada, et al., 1991; Kolb and Kolb-Bachofen, 1992; Ialenti et al., 1992). Excess NO can react with DNA, causing mutations and eventually carcinogenicity (Ohshima and Bartsch, 1994; Tamir and Tannenbaum, 1996). Furthermore, NO can react with superoxide to form peroxynitrite ($ONOO^-$), a more potent oxidant and cytotoxic agent (Ischiropoulos et al., 1992). Therefore, inhibition of high-output NO production could be a useful strategy for treatment of various inflammatory diseases.

Several studies have investigated the therapeutic potential and safety of components of *T. indica*. A polysaccharide isolated and purified from the seeds of *T. indica* has been shown *in vitro* to have immunomodulatory activity such as phagocytic enhancement and inhibition of leukocyte migration and cell proliferation (Sreelekha et al., 1993). Tamarind seed polysaccharide has been tested in B6C3F1 mice with results demonstrating the absence of carcinogenicity in mice of either sex following long-term dietary exposure (Sano et al., 1996).

The in vivo and in vitro studies presented here demonstrated the suppressive effect of the seed coat extract of *T. indica* on NO production using both a murine macrophage cell line and freshly isolated peritoneal macrophages. This effect was dose-dependent and

occurred at concentrations below which cytotoxicity occurred *in vitro* and in the absence of systemic toxicity in vivo. Additionally, at the concentrations required to suppress NO production, no effect was seen on NK cell activity or the development of a T cell-mediated sensitization response. Results from these studies were comparable to those reported for more well known natural dietary supplements. Using a similar model, LPS and IFN-γ stimulated RAW 264.7 cells, NO production was demonstrated to be inhibited approximately 70% or 55% by Pycnogenol or *Ginkgo biloba* extract respectively (Virgili et al., 1998; Kobuchi et al., 1997).

Excess NO production has been associated with many diseases such as autoimmunity, rheumatoid arthritis, inflammatory bowel disease and septic shock (Moncada et al., 1991; Nathan, 1992). The results of these acute and in vitro studies which demonstrated suppression of NO production at concentrations of the crude seed coat extract of *T. indica* while producing no measured adverse effects suggests that further chemical analysis, identification and testing of the active components of the extract is warranted.

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References

Bagchi, D., Garg, A., Krohn, R.L., Bagchi, M., Tran, M.X., Stohs, S.J., 1997. Oxygen free radical scavenging abilities of vitamin C and E, and a grape seed proanthocyanidin extract *in vitro*. Research Communications in Molecular Pathology and Pharmacology 95, 179–189.

Bagchi, D., Garg, A., Krohn, R.L., Bagchi, M., Bagchi, D.J., Balmoori, J., Stohs, S.J., 1998. Protective effects of grape seed proanthocyanidins and selected antioxidants against TPA-induced hepatic and brain lipid peroxidation and DNA fragmentation, and peritoneal macrophage activation in mice. General Pharmacology 30, 771–776.

Bogdan, C., Rollinghoff, M., Diefenbach, A., 2000. The role of nitric oxide in innate immunity. Immunological Reviews 173, 17–26.

Carlo, G.D., Mascolo, N., Izzo, A.A., Capasso, F., 1999. Flavonoids: old and new aspects of a class of natural therapeutic drugs. Life Sciences 65, 337–353.

Chan, M.M., Fong, D., Ho, C.T., Huang, H.T., 1997. Inhibition of inducible nitric oxide synthase gene expression and enzyme activity by epigallocatechin gallate, a natural product from green tea. Biochemical Pharmacology 54, 1281–1286.

Chen, S., Hwang, J., Deng, P.S.K., 1993. Inhibition of NAD(P)H: Quinone acceptor oxidoreductase by flavones: a structure activity study. Archives of Biochemistry and Biophysics 302, 72–77.

Duke, S.S., Schook, L.B., Holsapple, M.P., 1985. Effects of N-nitrosodimethylamine on tumor susceptibility. Journal of Leukocyte Biology 37, 383–394.

Farnsworth, N.R., Bunyapraphatsara, N., 1992. Thai Medicinal Plants Recommended for Primary Health Care System. Prachachon, Thailand.

Garthwaite, J., 1991. Glutamate, nitric oxide and cell-cell signalling in the nervous system. Trends in Neurosciences 14, 60–67.

Holsapple, M.P., White Jr, K.L., McCay, J.A., Bradley, S.G., Munson, A.E., 1988. An immunotoxicological evaluation of 4,4′-thiobis-(6-t-butyl-m-cresol) in female B6C3F1 mice. 2. Humoral and cell-mediated immunity, macrophage function, and host resistance. Fundamental and Applied Toxicology 10, 701–716.

Ialenti, A., Ianaro, A., Moncada, S., Rosa, M.D., 1992. Modulation of acute inflammation by endogenous nitric oxide. European Journal of Pharmacology 211, 177–182.

Ignarro, L.J., 2002. Nitric oxide in the regulation of vascular function: an historical overview. J Card Surg 17, 301–306.

Ischiropoulos, H., Zhu, L., Chen, J., Tsai, M., Martin, J.C., Smith, C.D., Beckman, J.S., 1992. Peroxynitrite mediated tyrosine nitration catalyzed by superoxide dismutase. Archives of Biochemistry and Biophysics 298, 431–437.

Kang, J.S., Jeon, Y.J., Kim, H.M., Han, S.H., Yang, K.H., 2002. Inhibition of inducible nitric-oxide synthase expression by silymarin in lipopolysaccharide-stimulated macrophages. Journal of Pharmacology and Experimental Therapeutics 302, 138–144.

Kim, H.K., Cheon, B.S., Kim, Y.H., Kim, S.Y., Kim, H.P., 1999. Effects of naturally occurring flavonoids on nitric oxide production in the macrophage cell line RAW 264.7 and their structure -activity relationships. Biochemical Pharmacology 58, 759–765.

Kobuchi, H., Droy-Lefaix, M.T., Christen, Y., Packer, L., 1997. *Ginkgo biloba* extract (Egb 761): Inhibitory effect on nitric oxide production in the macrophage cell line RAW 264.7. Biochemical Pharmacology 53, 897–903.

Kolb, H., Kolb-Bachofen, V., 1992. Nitric oxide: A pathogenic factor in autoimmunity. Immunological Today 13, 157–160.

Krol, W., Czuba, Z.P., Thereadgill, M.D., Cunningham, B.D.M., Pietsz, G., 1995. Inhibition of nitric oxide (NO) production in murine macrophages by flavones. Biochemical Pharmacology 50, 1031–1035.

Lin, Y.L., Lin, J.K., 1997. (−)-Epigallocatechin-3-gallate blocks the induction of nitric oxide synthesis by down-regulating lipopolysaccharide-induced activity of transcription factor nuclear factor-κB. Molecular Pharmacology 52, 464–472.

Middleton, E., Kandaswami, C., 1986. The impact of plant flavonoids on mammalian biology: Implications for immunity, inflammation and cancer. In: Harborne, J.B.. Chapman & Hall, London, pp. 619–652.

Moncada, S., Higgs, E.A., 1993. The L-arginine-nitric oxide pathway. The New England Journal of Medicine 329, 2002–2012.

Moncada, S., Palmer, R.M.J., Higgs, E.A., 1991. Nitric oxide: physiology, pathophysiology and pharmacology. Pharmacological Reviews 43, 109–142.

Murakami, A., Nakashima, M., Koshiba, T., Maoka Nishino, H., Yano, M., Sumida, T., Kim, O.K., Koichi, K., Ohigashi, H., 2000. Modifying effects of carotenoids on superoxide and nitric oxide generation from stimulated leukocytes. Cancer Letters 149, 115–123.

Nathan, C., 1992. Nitric oxide as a secretory product of mammalian cells. FASEB Journal 6, 3051–3064.

NIH, 1996. Guide for the Care and Use of Laborator Animals. National Academy Press, Washington, DC.

Ohshima, H., Bartsch, H., 1994. Chronic infections and inflammatory processes as cancer risk factors: possible role of nitric oxide in carcinogenesis. Mutation Research 305, 253–264.

Packer, L., Rimbach, G., Virgili, F., 1999. Antioxidant activity and biologic properties of a procyanidin-rich extract from pine (*Pinus maritima*) bark, Pycnogenol. Free Radical Biology & Medicine 27, 704–724.

Padgett, E.L., Pruett, S.B., 1992. Evaluation of nitrite production by human monocyte-derived macrophages. Biochemical Biophysical Research Communication 186, 775–781.

Prast, H., Philippu, A., 2001. Nitric oxide as modulator of neuronal function. Progress in Neurobiology 64, 51–68.

Pumthong, G., 1999. Antioxidative activity of polyphenolic compounds extracted from seed coat of *Tamarindus indica* Linn. Chiang Mai University, Thailand.

Rand, M.J., 1992. Nitrergic transmission: nitric oxide as a mediator of non-adrenergic, non-cholinergic neuro-effector transmission. Clinical and Experimental Pharmacology and Physiology 19, 147–169.

Rao, V.L., 2002. Nitric oxide in hepatic encephalopathy and hyperammonemia. Neurochemistry International 41, 161–170.

Raso, G.M., Meli, R., Carlo, G.D., Pacilio, M., Carlo, R.D., 2001. Inhibition of inducible nitric oxide synthase and cyclooxygenase-2 expression by flavonoids in macrophage J774A.1. Life Sciences 68, 921–931.

Rohdewald, P., 2002. A review of the French maritime pine bark extract (Pycnogenol®), a herbal medication with a diverse clinical pharmacology. International Journal of Clinical Pharmacology and Therapeutics 40, 158–168.

Sandoval, M., Zhang, X.J., Liu, X., Mannick, E.E., Clark, D.A., Miller, M.J.S., 1997. Peroxynitrite-induced apoptosis in T84 and RAW 264.7 cells: attenuation by L-ascorbic acid. Free Radical Biology & Medicine 22, 489–495.

Sano, M., Miyata, E., Tamano, S., Hagiwara, A., Ito, N., Shirai, T., 1996. Lack of carcinogenicity of tamarind seed polysaccharide in B6C3F1 mice. Food and Chemical Toxicology 34, 463–467.

Snyder, S.H., Bredt, D.S., 1992. Biological roles of nitric oxide. Scientific American 266 (5), 68–71,74-77.

Sreelekha, T.T., Vijayakumar, T., Ankanthil, R., Vijayan, K.K., Nair, M.K., 1993. Immunomodulatory effects of a polysaccharide from *Tamarindus indica*. Anti-Cancer Drugs 4, 209–212.

Taga, M.S., Miller, E.E., Pratt, D.E., 1984. Chia seeds as a source of natural lipid antioxidants. Journal of the American Oil Chemists Society 61, 928–931.

Tamir, S., Tannenbaum, S.R., 1996. The role of nitric oxide (NO) in the carcinogenic process. Biochimica et Biophysica acta 1288, F31–F36.

van Acker, S.A.B.E., Tromp, M.N.J.L., Haenen, G.R.M.M., van der Vijgh, W.J.F., Bast, A., 1995. Flavonoids as scavengers of nitric oxide radical. Biochemical and Biophysical Research Communications 214, 755–759.

Virgili, F., Kobuchi, H., Packer, L., 1998. Procyanidins extracted from *Pinus maritima* (Pycnogenol®) scavengers of free radical species and modulators of nitrogen monoxide metabolism in activated murine RAW 264.7 macrophages. Free Radical Biology and Medicine 24, 1120–1129.

Woolhiser, M.R., Hayes, B.B., Meade, B.J., 1998. A combined murine local lymph node and irritancy assay to predict sensitization and irritancy potential of chemicals. Toxicology Methods 8, 245–256.