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Antioxidant and nitric oxide inhibition activities of selected Malay traditional vegetables

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Abstract

The antioxidant properties and the effect on nitric oxide (NO) production, in lipopolysaccharide-activated macrophages, of 12 traditional vegetables of the Malaysian Malays, including *Pithecellobium confertum*, *Averrhoa bilimbi*, *Portulaca oleracea*, *Solanum torvum*, *Solanum nigrum*, *Persicaria tenella*, *Cosmos caudatus*, *Pandanus amaryllifolius*, *Curcuma mangga*, *Ocimum basilicum*, *Anacardium occidentale* and *Melicope ptelefolia*, were investigated. Antioxidant activity of the methanolic extracts was evaluated by measuring the production of hydroperoxide and its degradation product (malonaldehyde) resulting from linoleic acid oxidation using ferric thiocyanate and thiobarbituric acid methods, respectively. Radical-scavenging potential was also evaluated using the 1,1-diphenyl-2-picrylhydrazyl radical. Griess assay was used to assess NO-inhibitory activity of the extracts. All species, except *P. confertum*, *S. torvum* and *P. amaryllifolius*, showed antioxidant activity. *M. ptelefolia*, *P. oleracea* and *P. tenella* showed in vitro activity on NO inhibition in murine peritoneal macrophages, whereas other plants showed no significant activity.

Keywords: Antioxidant activity; Traditional vegetables; Ferric thiocyanate; Thiobarbituric acid; 1,1-Diphenyl-2-picrylhydrazyl radical; Nitric oxide inhibition

1. Introduction

Traditional vegetables of the Malays in Malaysia (locally called 'ulam') comprise more than 120 species representing various families, from shrubs to large trees (Mansor, 1988). The leaves, shoots or rhizomes of the vegetables are eaten fresh as salad or cooked (Norhanom, Ashril, & Mustafa, 1999). They are consumed because of their taste, which adds variety and flavour to the diet, as well as for their health benefits. Nutritional studies have indicated that many of these vegetables are rich in carbohydrates, proteins, minerals and vitamins (Ismail, 2000). Some of the vegetables are also claimed to have medicinal properties, such as blood cleansing, induction of uterine contractions and prevention or cure of ailments such as diabetes, high blood pressure, cardiovascular disease, arthritis, fever and coughs. In addition, it is also believed that vegetables play a vital role in lowering the incidence of cancer, as well as the control of ageing and age-related diseases. The plants, including *Pithecellobium confertum*, *Averrhoa bilimbi*, *Portulaca oleracea*, *Solanum torvum*, *Solanum nigrum*, *Persicaria tenella*, *Cosmos caudatus*, *Pandanus amaryllifolius*, *Curcuma mangga*, *Ocimum basilicum*, *Anacardium occidentale* and *Melicope ptelefolia*, were selected for investigation because of the limited information on antioxidant and NO-inhibitory activities.

The leaves of *P. confertum* (=*Albizia splendens*) are used in traditional medicine for treatment of diarrhoea (Burkill, 1966) although the use of the seeds is not well

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documented. A common plant in tropical Asia, *A. bilimbi*, has been widely used in traditional medicine for cough, cold, itches, boils, rheumatism, syphilis, diabetes, whooping cough and hypertension (Goh, Chuah, Mok, & Soepadmo, 1995). A popular local vegetable, *P. oleracea*, has been used as folk medicine in different countries to treat different ailments of humans (Chan et al., 2000). A recent report indicated that an extract of *P. oleracea* accelerates wound healing and treatment of indomethacine and phenylbutazone-induced ulcers (Rashed, Afifi, & Disi, 2003). In the treatment of stomach pain and skin infections, local folk have recommended the use of *S. torvum* (Siemonsma & Piluek, 1994). Recently, the methanol extract of the fruits of *S. torvum* was found to exhibit interesting growth-inhibiting activity against bacteria commonly associated with pyogenic infections (Chah, Muko, & Oboegbulem, 2000). In Egypt, *S. nigrum* has traditionally been used for treating burns and infections (Mahmoud, Nawar, Amani, El-Mousallamy, & Barakat, 1989). Inhibition of free radical-mediated DNA damage (Sultana, Perwaiz, Iqbal, & Athar, 1995) and inhibition of cell growth in MCF-7 cells (Son et al., 2003) by the ethanol extracts of *S. nigrum* have also been reported. A well-known medicinal plant, *P. tenella* (=*Polygonum hydropiper*), has been reported to possess antifeedant, antimicrobial, cytotoxic, piscicidal and anticomplement activities (Furuta, Fukuyama, & Asakawa, 1986). More recently, it has been reported that the ethyl acetate fraction of this plant possessed antinociceptive (Rahman, Goni, Rahman, & Ahmed, 2002) and antioxidant activities (Peng et al., 2003). The sweet basil (*O. basilicum*) has been used for medicinal purposes, as a culinary herb and as an insect-controlling agent (Reenee et al., 1996). The ethanol extracts of *O. basilicum* were tested for antioxidant activity using trolox equivalent antioxidant capacity (TEAC), and showed a linear positive relationship with the total phenolic contents (Javanmardi, Stushnoff, Locke, & Vivanco, 2003). *C. caudatus* is believed to promote the formation of healthy bones and is said to be useful in 'cleansing the blood' (Burkill, 1966; Ismail, 2000). The methanol extracts of *C. caudatus* have been reported to show moderate antioxidant activity when tested using the xanthine–xanthine oxidase enzymatic assay (Norhanom et al., 1999). Recently, antioxidative and radical-scavenging activities of compounds isolated from this plant have been reported (Faridah, Shaari, Lajis, Israf, & Umi Kalsom, 2003). The leaves of *P. amaryllifolius* are used for colouring and to impart fragrance to foods while the whole plant is pounded for poulticing joints (Siemonsma & Piluek, 1994). The presence of volatile compounds in the leaves of *P. amaryllifolius*, particularly 2-acetyl-l-pyrroline (Apintanapong & Noomhorm, 2003; Laksanalamai & Ilangantileke, 1993; Wongpornchai, Sriseadka, & Choonvisase, 2003) plays an important role as an aroma. The rhizomes of *C. mangga* are traditionally used as a stomachic for the treatment of chest pains, fever and general debility, as well as to aid womb healing. It has been reported that the ethanolic extract of *C. mangga* showed antifungal activity against three plant pathogens (Suhaila, Suzana, Saleh, Ali, & Sepiah, 1996). The biological activity of cashew nut *(A. occidentale)* extracts has been quite well studied, but the antioxidant properties are less known. The leaves of *A. occidentale* have antibacterial activity against the gram-negative bacteria *Escherichia coli* (Kudi, Umoh, Eduvie, & Gefu, 1999) while the stem barks have been reported to inhibit prostaglandin production from cells (Ibewuike, Ogundaini, Bohlin, & Ogungbamila, 1997). Recently, the methanol extract of the stem bark of *A. occidentale* was reported to possess anti-inflammatory effects in an in vivo model (Olumayokun, Mutallib, Aduragbenro, & Janet, 2004). The antibacterial activity of *M. ptelefolia* has been reported previously (Rasadah & Zakaria, 1988) but data regarding its antioxidant activity were not found in the literature.

In view of the lack of investigation of antioxidant properties of these selected plants, we have investigated the activities of these plant extracts in inhibiting lipid peroxidation, scavenging DPPH radical and inhibiting nitric oxide production. Physiologically, antioxidants play a major role in preventing the formation of free radicals, which are responsible for many oxidative processes. Antioxidants may be synthetics, such as butylated hydroxyanisole (BHA), propyl gallate (PG) and butylated hydroxytoluene (BHT), or of natural origin, such as α-tocopherol, phenolic compounds as well as polyphenolics (Hall & Cuppett, 1997). Antioxidants isolated from plants also showed antibacterial, anticarcinogenic, anti-inflammatory, antiviral, antiallergic, estrogenic and immune-stimulating effects (Larson, 1988).

2. Materials and methods

2.1. Plant material

Plant samples were collected from the medicinal garden of the Institute of Bioscience, Universiti Putra Malaysia and were identified by Mr. Shamsul Khamis. Voucher specimens were deposited at the mini herbarium of the Laboratory of Natural Products at the Institute (Table 1).

2.2. Chemicals

Chemicals were purchased from Sigma Chemical Co. (USA), with the exception of ethanol, which was purchased from Scharlau (Barcelona, Spain). All chemicals and reagents were of analytical grade.

Table 1
List of plant species and yield of extracts

Scientific name	Part tested	Yield (w/w) (%)[a]	Voucher no.
Averrhoa bilimbi[b] Linn	Fruits	0.26	SK19/01
Anacardium occidentale Linn	Leaves	0.12	SK150/02
Cosmos caudatus Kunth	Leaves	0.23	SK148/02
Curcuma mangga Val	Rhizomes	0.06	SK149/02
Melicope ptelefolia (Champ. ex Benth) Hartley	Leaves	0.14	SK153/02
Ocimum basilicum L	Leaves	0.22	SK157/02
Pandanus amaryllifolius Ridl	Leaves	0.19	SK152/02
Pithcellobium confertum[c] Benth	Seeds	0.05	SK151/02
Persicaria tenella var. kawagoene	Leaves	0.27	SK154/02
Portulaca oleraceae L	Leaves	0.16	SK155/02
Solanum nigrum L	Leaves	0.09	SK156/02
Solanum torvum L	Leaves/fruit	0.13	SK26/01

[a] Weight (g) of crude methanolic extracts per 100 g of dried plant material.
[b] Plant sample extracted with water.
[c] Plant sample extracted with acetone.

2.3. Sample preparation and extraction

Leafy or whole vegetable samples were cut into small pieces, air-dried in the shade and ground into fine powder before being extracted with methanol. The seeds of *P. confertum* were blended and extracted with acetone and *A. bilimbi* fruits were blended and extracted with water. Extracts of each solvent were evaporated to dryness under reduced pressure before being subjected to the bioassays.

2.4. Antioxidant activity

2.4.1. Ferric thiocyanate method

The ferric thiocyanate (FTC) assay was carried out as described by Kikuzaki and Nakatani (1993) with slight modification. A mixture of 4 mg of samples (final concentration 0.02% w/v) in 4 ml of 99.5% ethanol, 4.1 ml of 2.51% linoleic acid in 99.5% ethanol, 8.0 ml of 0.02 M phosphate buffer (pH 7.0) and 3.9 ml of distilled water, contained in a screw-cap vial (∅38 × 75 mm) was placed in an oven at 40 °C in the dark. To measure the extent of antioxidant activity, 0.1 ml of the reaction mixture was transferred to a test tube (∅13 × 150 mm) and, to it, 9.7 ml of 75% (v/v) aqueous ethanol, followed by 0.1 ml of 30% aqueous ammonium thiocyanate and 0.1 ml of 0.02 M ferrous chloride in 3.5% hydrochloric acid were added. Three min after the addition of ferrous chloride to the reaction mixture, the absorbance was measured at 500 nm. The measurement was taken every 24 h until one day after absorbance of the control reached its maximum value. α-Tocopherol and BHT are used as standard antioxidants.

2.4.2. Thiobarbituric acid method

The test was conducted according to method of Kikuzaki and Nakatani (1993). The same samples as prepared for the FTC method were used. To 2 ml of the sample solution, was added 1 ml 20% aqueous trichloroacetic acid and 2 ml 0.67% aqueous thiobarbituric acid solution. The mixture was placed in a boiling water bath for 10 min. After cooling, it was centrifuged at 3000 rpm for 20 min. Antioxidant activity was recorded, based on the absorbance of the supernatant at 532 nm on the final day of the FTC assay.

2.4.3. Free radical-scavenging activity (1,1-diphenyl-2-picrylhydrazyl)

The potential antioxidant activity of plant extracts was assessed on the basis of the scavenging activity of the stable 1,1-diphenyl-2-picrylhydrazyl (DPPH) free radical according to the previous described procedures (Cottelle et al., 1996). Different concentrations of test samples were prepared in 96 microtiter plates. The reaction mixtures,consisting of 100 μl of test sample (various extracts dissolved in methanol) and 5 μl of DPPH in methanol (300 μM) were incubated for 30 min, and absorbance was measured at 515 nm. Percent inhibition by sample treatment was determined by comparison with the methanol-treated control group. The IC_{50} values denote the concentration of each sample required to give 50% of the optical density shown by the control. All test analysis were run in triplicate and averaged. Quercetin and ascorbic acid were used as positive controls.

2.5. Nitric oxide inhibitory activity

2.5.1. Cell culture

The RAW264.7 cells were grown in plastic culture flasks in Dulbecco's Modified Eagle's Medium (DMEM) with phenol red containing HEPES, L-glutamine supplemented with 10% foetal bovine serum (FBS) and 1% antibiotic/antimycotic solution (Gibco/BRL) under 5%

CO_2 at 37 °C. After 4–5 days, the cells were removed from the culture flask by scraping and were then centrifuged for 10 min at 3000 rpm and at 4 °C. The medium was then removed and the cells were suspended with fresh DMEM without phenol red containing HEPES, and L-glutamine with the same supplements. Cell counts and viability were measured using a standard trypan blue cell counting technique. The cell concentration was adjusted to 1×10^6 cells/ml in the same medium. Apart from the normal cell controls, all cells were cultured in the above media (50 μl) which also contained triggering agents such as 200 U/ml of recombinant murine IFN-γ (Pharmingen) and 10 μg/ml lipopolysaccharide seeded into the wells of 96-well tissue culture plates. Then 50 μl of serially diluted plant extracts in media containing dimethylsulphoxide (DMSO) were dispensed into the wells of the cell plates to yield a final concentration of DMSO at 0.1% per well. Extracts were used in triplicate and the cells were incubated for 24 h at 37 °C, 5% CO_2, in a fully humidified incubator. Controls included media only, cells in media containing triggering agents and 0.1% DMSO.

2.5.2. Measurement of nitrite

To determine the nitric oxide concentration, the stable nitric oxide conversion product, nitrite (NO_2^-) was measured using the Griess reagent (Chi, Cheon, & Kim, 2001). After 24 h of incubation, 50 μl aliquots were removed from supernatants of cultured cells and incubated with an equal volume of the Griess reagent (1% sulfanilamide, 0.1% *N*-(l-naphthyl)-ethylene diamine dihydrochloride, 2.5% H_3PO_4) at room temperature for 10 min. The absorbance at 550 nm was determined in a Spectramax Plus (Molecular Devices) UV/Vis microplate reader. The concentrations of nitrite were derived by regression analysis using serial dilutions of sodium nitrite as a standard. Percentage inhibition was calculated, based on the ability of extracts to inhibit nitrite below the levels produced by cells cultured in the presence of triggering agents and DMSO, which was considered as 0% inhibition.

2.5.3. Measurement of cell viability

To determine that the observed nitric oxide inhibition was not false positive due to cytotoxic effects, a cytotoxicity assay (Heras et al., 2001) was also performed following culture. After removal of media, the cells were topped up with 100 μl of complete DMEM. To each well, 20 μl of a solution of 5 mg/ml of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) in PBS 7.2 were added. The cells were incubated at 37 °C with 5% CO_2 for 4 h. The medium was then carefully discarded and the formazan salt formed was dissolved in DMSO. The absorbance was read at 570 nm. The absorbance of formazan in control (untreated cells) was taken as 100% viability.

2.6. Statistical analysis

Results are given as means ± SEM values. All the experiments in vitro were conducted at least three times, each time with three or more independent observations. Significance analysis was performed by ANOVA ($*P < 0.1$, $**P < 0.05$, $***P < 0.001$).

3. Results and discussion

3.1. Antioxidant activity

There are many different antioxidant components in plants, and it is relatively difficult to measure each antioxidant component separately. Therefore, several different methods have been developed to evaluate the antioxidant activity of biological samples (Chang, Luu, & Cheng, 1983; Lingnert, Vallentin, & Eriksson, 1979; Lopez, Martinez, Del Valle, Ferrit, & Luque, 2003).

In the FTC method, the amount of peroxide in the initial stages of lipid oxidation was measured every 24 h, over a period of 7 days (the absorbance of the positive control reached a maximum on the sixth day). As shown in Fig. 1, a low absorbance value represents a high level of antioxidant activity. *O. basilicum*, *P. oleracea*, *S. nigrum*, *C. caudatus*, *A. occidentale*, *M. ptelefolia* and *P. tenella* exhibited higher activities than α-tocopherol. However, none of the extracts were better than BHT (Fig. 1, inset). *A. bilimbi* and *C. mangga* showed moderate antioxidant activities while *P. confertum*, *P. amaryllifolius* and *S. torvum* seemed to be inactive. During the oxidation process, peroxide is gradually decomposed to lower molecular weight malondialdehyde, the amount of which is measured by the thiobarbituric acid (TBA) assay. The absorbance values of all samples at 532 nm, measured on the last day of the experiment (one day after the control reached maximum), are shown in Fig. 2. The results were in agreement with the results obtained from the FTC method. The absorbance values for *O. basilicum*, *P. oleracea*, *S. nigrum*, *C. caudatus*, *A. occidentale*, *M. ptelefolia* and *P. tenella* (0.048, 0.068, 0.052, 0.066, 0.096, 0.089 and 0.107, respectively), were lower than that of α-tocopherol (0.32), indicating stronger antioxidant activity.

C. caudatus, *A. occidentale* and *P. tenella*, in addition to exhibiting strong antioxidant activity in both FTC and TBA assays, also showed strong activity in the free radical-scavenging assay. From these results, we could therefore suggest that the consumption of these vegetables could possibly offer some dietary benefits since they contain constituents, which are able to protect against lipid peroxidation and to scavenge free radicals. The moderate antioxidant activity of *C. mangga*, indicated by the FTC and TBA assays, is possibly due to their essential oil constituents (Jitoe et al., 1992).

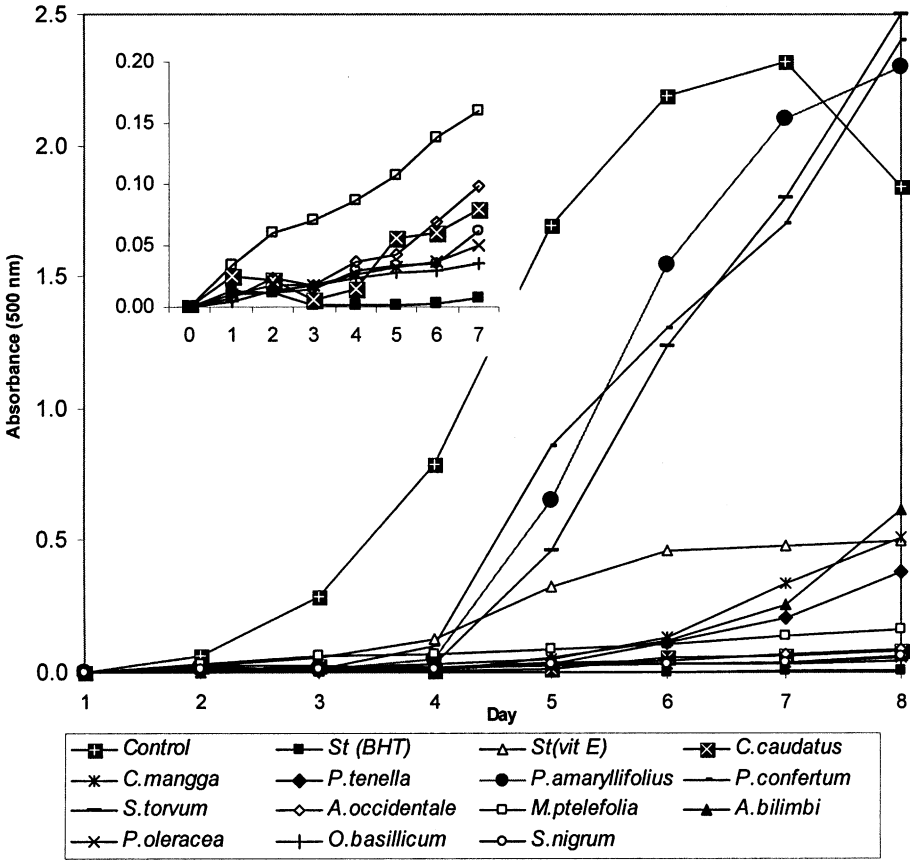

Fig. 1. Lipid oxidation was monitored daily using FTC method. Low absorbance value indicated strong antioxidant activity. Inset shows the expanded plots of the five most active extracts and BHT. The final concentration of each sample was 0.02% w/v. Each experiment was carried out in triplicates and repeated twice.

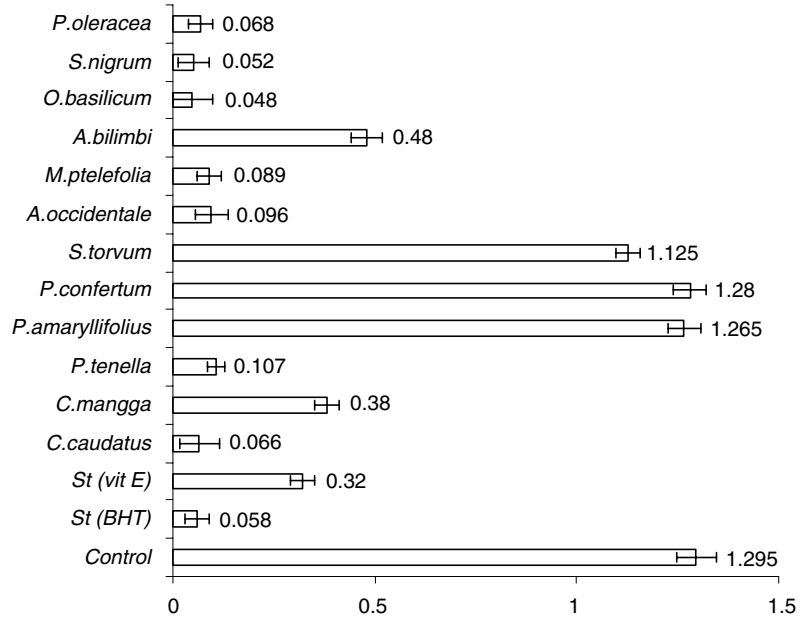

Fig. 2. Amount of malondialdehyde in lipid peroxidation, measured using TBA assay. The final concentration of each sample was 0.02% w/v. Each experiment was carried out in triplicate and performed twice.

Table 2
Radical-scavenging activities of the Malay traditional vegetables

Samples	DPPH radical-scavenging-activity IC_{50} (μg/ml)
Anacardium occidentale	15.3 ± 0.11
Averrhoa bilimbi	na
Cosmos caudatus	20.2 ± 0.05
Curcuma mangga	100 ± 0.1
Melicope ptelefolia	30.0 ± 0.02
Pandanus amaryllifolius	na
Pithcellobium confertum	na
Persicaria tenella	17.2 ± 0.07
Portulaca oleracea	70.4 ± 0.03
Solanum nigrum	250 ± 1.2
Solanum torvum	125 ± 0.5
Ocimum basilicum	190 ± 0.7
Vitamin C	5.9 ± 0.04
Quercetin	4.8 ± 0.01

na, inactive.

The radical-scavenging activities of the samples were determined from the reduction in the optical density (OD) of diphenyl-*p*-picrylhydrazyl (DPPH) free radical at 515 nm. The extracts of *P. tenella*, *A. occidentale* and *C. caudatus* were found to be strong free radical-scavengers, exhibiting IC_{50} values of less than 30 μg/ml. However, *M. ptelefolia*, *P. oleracea* and *C. mangga* showed moderate free radical-scavenging activities ($IC_{50} \leqslant 100$ μg/ml), as shown in Table 2.

Of all the plants examined, the MeOH extracts of *O. basilicum*, *S. nigrum*, *A. occidentale*, *M. ptelefolia*, *C. caudatus* and *P. tenella*, exhibited particularly strong antioxidant activities. Previous phytochemical studies on these species, except for *M. ptelefolia*, have indicated the presence of flavonoids (Faridah et al., 2003; Mahmoud et al., 1989; Peng et al., 2003; Ranjana, Vikas, & Ilyas, 1989; Reenee et al., 1996; Urones, Marcos, Perez, & Barcala, 1990). Since the flavonoids have also been known to have antioxidant properties, their presence in these species could therefore be the basis for the observed antioxidant activity (Cottelle et al., 1996; Saija et al., 1995). Although the presence of flavonoids in *M. ptelefolia* has not been known, the isolation of several derivatives of isoquinoline (alkaloid) (McCormick, McKee, Cardellina II, & Boyd, 1996) and benzopyran, including those with a phenolic moiety, have been reported (Kamperdick, Yan, Van Sung, & Adam, 1997). Since benzopyrans bear close structural resemblance to α-tocopherol, a well-known antioxidant, it is possible that the antioxidant activity of *M. ptelefolia* is caused by these compounds. The presence of phenolics, which are also known for their antioxidant property (Lien, Ren, Bui, & Wang, 1999), has also been reported in some species, including *O. basilicum* and *A. occidentale* (Jayasinghe, Jayasinghe, Goto, Aoki, & Wada, 2003; Javanmardi et al., 2003; Kogel & Zech, 1985; Subramaniam, Joseph, & Fair, 1969). Although previous studies have shown the presence of steroidal glucoside in *S. nigrum* (Hu et al., 1999), terpenoids in *P. tenella* and *O. basilicum* (Chang, Wang, Wu, Kuo, & Chao, 1999; Fukuyama, Sato, Asakawa, & Takemoto, 1982) their contribution to antioxidant activity in these species is not expected.

3.2. Nitric oxide inhibitory activity

The inhibitory effect on lipopolysaccharide (LPS)- and interferon-γ (IFN-γ)-induced NO production was shown by the extracts of *M. ptelefolia*, *P. oleracea* and *P. tenella*. Preincubation of macrophage cells with *M. ptelefolia*, *P. oleracea* and *P. tenella* extracts inhibited NO production significantly ($P < 0.001$) in a concentration-dependent manner. This inhibition is not due to their, cytotoxicity, as indicated by their cell viability values. Other samples did not show any significant effect on NO release (Table 3).

Table 3
Effect of the traditional vegetables on NO synthesis

Sample	IC_{50} value (μg/ml)	Percentage inhibition[a]	Percentage cell viability[a]
Anacardium occidentale	na	16.1 ± 5.28	98.5 ± 0.47
Averrhoa bilimbi	na	22.3 ± 4.01	103.68 ± 0.48
Cosmos caudatus	na	15.4 ± 1.54	110 ± 0.13
Curcuma mangga	na	19.2 ± 10.1	96.6 ± 0.01
Melicope ptelefolia	nd	95.0 ± 3.9***	96.7 ± 0.37
Pandanus amaryllifolius	na	34.1 ± 4.53	87.3 ± 0.39
Pithcellobium confertum	na	23.5 ± 3.48	75.6 ± 0.23
Persicaria tenella	8	87.8 ± 2.93***	192 ± 0.60
Portulaca oleracea	44	94.8 ± 3.57***	92.0 ± 0.60
Solanum nigrum	na	27.6 ± 4.99	108 ± 0.57
Solanum torvum	na	25.2 ± 5.26	78.2 ± 0.50
Ocimum basilicum	na	30.2 ± 3.87	70.6 ± 0.14

The data represent the means ±SD of triplicate cultures of three independent experiments. Significance analysis was performed by ANOVA (***$P < 0.001$).

na=activity more than 250 μg/ml, nd, activity below 4 μg/ml.

[a] Values expressed at concentration 250 μg/ml.

The role of the free radical (NO) in inflammatory processes is well known (Schinella, Tournier, Prieto, Mordujovich, & Rios, 2002). Free radicals liberated from phagocyte cells are important in inflammatory processes because they are implicated in the activation of nuclear factor κB (NF-κB), which induces the transcription of inflammatory cytokines and COX-2. Furthermore, antioxidants have been shown to be able to effectively block the activation of NF-κB through the stabilization of NF-κB/IκB-α complex (Huang et al., 2001). The results of this study showed that *M. ptelefolia*, *P. tenella* and *P. oleracea* also contain constituents that can inhibit the production of NO_2^-, as measured in the Griess assay. Since these plant species also exhibited radical-scavenging activities, it is possible that the same or similar compounds may be responsible for the activities in each of the species.

In conclusion, *O. basilicum*, *P. oleracea*, *S. nigrum*, *C. caudatus*, *A. occidental*, *M. ptelefolia* and *P. tenella* are good sources of compounds with antioxidant properties while *M. ptelefolia*, *P. oleracea* and *P. tenella* extracts exhibited strong NO-inhibitory activity. This report further suggests that a number of the local traditional vegetables might have beneficial chemopreventive effects in addition to providing potential new sources of natural antioxidants and NO production inhibitors.

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