Available online at www.sciencedirect.com
ScienceDirect
Food Chemistry 104 (2007) 1539–1549
Food Chemistry
www.elsevier.com/locate/foodchem

Antioxidative effects of leaves from *Azadirachta* species of different provenience

Pongtip Sithisarn [a], Charlotte U. Carlsen [b], Mogens L. Andersen [b], Wandee Gritsanapan [a], Leif H. Skibsted [b,*]

[a] *Department of Pharmacognosy, Faculty of Pharmacy, Mahidol University, 447 Sri-Ayuthaya Road, Ratchatewi, Bangkok 10400, Thailand*
[b] *Food Chemistry, Department of Food Science, The Royal Veterinary and Agricultural University, Rolighedsvej 30, DK-1958 Frederiksberg C, Denmark*

Received 2 October 2006; received in revised form 26 November 2006; accepted 21 February 2007

Abstract

Twenty extracts of leaves from *Azadirachta* plants (Siamese neem tree of different provenience, neem tree and marrango tree), a traditional medicine, and frequently eaten in large amounts as parts of meals in Thailand, showed proportionality between total phenolics and radical-scavenging as measured, as reduction of Fremy's salt by ESR-spectroscopy. Scavenging effect of extracts from the neem tree was significantly lower than those of the Siamese neem tree and the marrango tree. The ability of the extracts to trap carbon-centred 1-hydroxyethyl radicals indicates prooxidative activity for a very high concentration of extract. The extracts were found to be efficient scavengers of compounds known to initiate lipid oxidation, as demonstrated by rate constants (25 °C) for reaction with ferrylmyoglobin: $k = (1.7 \pm 0.3) \times 10^3\ M^{-1}\ s^{-1}$ at pH 5.5 and $k = (7.7 \pm 1.4) \times 10^2\ M^{-1}\ s^{-1}$ at pH 7.4, respectively, and with riboflavin triplet-state: $k = (1.30 \pm 0.02) \times 10^9\ M^{-1}\ s^{-1}$ at pH 7.4. The rate constants for deactivation of ferrylmyoglobin by the extracts were larger than the rate constant for deactivation by the pure phenolic compounds present in the extract, demonstrating synergistic effects. All extracts showed high efficiencies as chain-breaking antioxidants. This was indicated by lowering of oxygen consumption rates in a peroxidising lipid emulsion, suggesting a role as dietary antioxidants. Siamese neem tree leaf extracts were found to interact with α-tocopherol in peroxidising liposomes, resulting in synergistic effects.

Keywords: *Azadirachta*; Siamese neem tree; Total phenolic; Radical scavenging; Antioxidant synergism; Protection against hypervalent iron

1. Introduction

Siamese neem tree, neem and marrango tree (*Azadirachta indica* A. Juss. var. *siamensis* Valeton, *Azadirachta indica* A. Juss. and *Azadirachta excelsa* (Jack) Jacobs, respectively) are large evergreen trees in the Meliaceae family, found throughout Asia, America and Africa (Sombatsiri, Ermel, & Schmutterer, 1995). Some parts of these plants are traditionally used as medicinal preparations for their antipyretic, antimalarial and anti-inflammatory effects, and for the treatment of skin diseases. Moreover, in Thailand, the young leaves and flowers of these plants are consumed as bitter tonic vegetables with fish and sweet sauce in order to promote good health (Clayton, Soralump, Chaukul, & Temsiririrkkul, 1996). In a previous study, the extracts of Siamese neem tree and the extracts of leaves, flowers and stem bark, were demonstrated to have strong antioxidant potential, as demonstrated by *in vitro* free radical-scavenging and by effects on lipid peroxidation in bronchogenic cancer cells (Sithisarn, Supabphol, & Gritsanapan, 2005). While the phytochemicals of Siamese neem tree and marrango tree are not characterized in any detail, there are many studies of constituents of the neem tree, including the limonoids, azadirachtin and nimbolide, and

* Corresponding author. Address: Food Chemistry, Department of Food Science, The Royal Veterinary and Agricultural University, Rolighedsvej 30, DK-1958 Frederiksberg C, Denmark. Tel.: +45 35283221; fax: +45 35283344.
E-mail address: ls@kvl.dk (L.H. Skibsted).

0308-8146/$ - see front matter © 2007 Elsevier Ltd. All rights reserved.
doi:10.1016/j.foodchem.2007.02.033

several triterpenes. Gallic acid, catechin, quercetin and rutin and other phenolic and flavonoids have also been isolated from the neem tree (Basak & Chakraborty, 1968; Nair, Gopal, & Issac, 1997; Nakov, Labode, & Akahtaedzhiev, 1982; Schroeder & Nakanishi, 1987; Siddiqui, Ghiasuddin, Faizi, & Siddiqui, 1992; Van der Nat et al., 1991).

Aging and many lifestyle diseases, such as cardiovascular disease, atherosclerosis, inflammation and cancer, are considered to relate to excess production of free radicals (Cross, 1987). A balanced diet, which includes fruit and vegetable with every meal, seems to be important in order to control oxidative stress in the dietary tract and also for control of oxidative damage to blood lipids and proteins. Leaf of the Siamese neem tree is one of the vegetables commonly used in Thailand and which appears to have a very high antioxidant content and accordingly appears to be of importance to public health (Sithisarn et al., 2005; Suttajit, Khansuwan, & Suttajit, 2002). This plant was therefore considered to be interesting for a more detailed study, in order to quantify the antioxidant capacity and to characterize the antioxidant activity of the leaf extracts. The characterization included a quantitative analysis of possible active compounds, and determination of radical-scavenging capacity and antioxidant activity for various types of oxidative stress in selected model systems. The close botanical relationships between the Siamese neem tree, the neem and marrango tree, prompted us to compare the antioxidant activity of the extracts from the leaves of these three plants. Accordingly, extracts of leaves of Siamese neem tree, neem and marrango tree, collected at different locations in Thailand, were studied for their radical-scavenging capacity, using both stable and transient radicals, by electron spin resonance spectroscopy for quantification, and laser flash photolysis and stopped flow spectroscopy for kinetic studies. An important aspect of these studies was to detect whether the activity of the extracts correlated with the total phenolic content as determined by the Folin–Ciocalteu method or whether synergistic effects between the different components in the extracts could be demonstrated.

2. Materials and methods

2.1. Chemicals

(±)-α-Tocopherol (all-rac-α-tocopherol) was purchased from Fluka (Switzerland). 2,2′-Azobis(2-amidinopropane)hydrochloride (AAPH) was purchased from Wako Chemicals Inc. (Richmond, VA,USA). Horse heart metmyoglobin (MMb, type III), methyl linoleate, polyoxyethylenesorbitan monolaurate (Tween-20), and quercetin were obtained from Sigma (St. Louis, MO, USA). Riboflavin, rutin, 4-pyridyl-1-oxide-*N*-*tert*-butylnitrone (POBN), phosphatidyl choline (PC) from soybean (99%) and ethanol were purchased from Sigma–Aldrich Co, Steinheim, Germany. Fremy's salt (potassium nitrosodisulfonate) was from Aldrich Chemical Co. (Milwaukee, WI, USA). Folin–Ciocalteu-reagent, disodium hydrogen phosphate, sodium dihydrogen phosphate, potassium dihydrogen phosphate, potassium phthalate, sodium chloride and sodium carbonate, of analytical grade, were obtained from Merck (Damstadt, Germany). Analytical grade hydrogen peroxide and analytical grade iron(II) sulphate heptahydrate were from Riedel-de-Haen (Selze, Germany). Solvents were all of HPLC grade and were supplied by Labscan Ltd. (Dublin, Ireland). Water was purified through a Millipore Q-plus purification train (Millipore Corp., Bedford, MA, USA). Rynchosin-glucoside was separated and identified as previously described (Sithisarn, Gritsanapan, Supabphol, & Chavasiri, 2003).

2.2. Plant materials

The leaves of Siamese neem tree (*Azadirachta indica* A. Juss. var. *siamensis* Valeton), neem (*Azadirachta indica* A. Juss.) and marrango tree (*Azadirachta excelsa* (Jack) Jacobs) were collected from different locations in Thailand during April and May, 2004. The samples were identified by Mrs. Vachalee Prachasaisoradej, an Agricultural Scientist, Plant Variety Protection Division, Research Unit of Princess Sirinhorn Plant Herbarium, Bangkok Thailand, at which institution voucher specimens are deposited (BK63511, BK63512 and BK63513 for neem, Siamese neem tree and marrango tree, respectively). Samples were cleaned, dried in a hot air oven (55 °C) and powdered. Siamese neem tree leaves were labelled as S1–S12 for samples from Ratchaburi, Roi Et, Nakon Pathom, Mahasarakham, Ang Thong, Chanthaburi, Lampang, Lop Buri, Prachinburi, Nakhon Si Thammarat, Songkhla and Petchabun provinces, respectively. Marrango tree leaves samples were from Pak Panang, Meung districts, Nakhon Si Thammarat and from Pattalung provinces and identified as E1–E3, respectively. The Samples of neem leaves, from Nakhon Pathom, Chaing Rai, Chon Buri, Mahasarakham and Ratchaburi provinces, were identified as I1–I5, respectively.

2.3. Preparation of plant extracts

The aqueous extracts of leaves from Siamese neem tree, neem and marrango tree were obtained by the following procedure: dried powders of leaf samples were separately boiled with distilled water for 6–8 h (plant/water ratio 1:20, w/v) and subsequently filtered. The filtrate was evaporated to dryness in a rotary evaporator to yield dried leaf aqueous extracts. Determination of total phenolic content was determined by reduction of the phosphomolybdate ion (Folin–Ciocalteu reagent) by phenolates present in the extracts (Naithani, Nair, & Kakkar, 2006). In brief, 0.5 ml of plant extract (in duplicate), 30 ml of double-distilled water and 2.5 ml Folin–Ciocalteu reagent were mixed. After an interval of 8 min, 7.5 ml of 20% sodium carbonate solution were added and the volume adjusted to 50 ml. The mixture was allowed to stand at 20 °C for

2 h. The absorbance of the mixture was measured, using a UV–VIS spectrophotometer HP8453 (Hewlett Packard, USA) at 765 nm. Total phenolic contents were expressed as quercetin equivalents (QE) in milligrammes per gramme of sample. Different quercetin standards (0, 50, 100, 150, 250 and 500 mg/l) were used in order to obtain a standard curve.

2.4. Total flavonoid content and quercetin determination

Total flavonoid content was determined using a known method (Meda, Lamien, Romito, Millogo, & Nacoulma, 2005). Five millilitres of 2% aluminium chloride ($AlCl_3$) in methanol were mixed with the same volume of the sample solution. Absorption readings at 415 nm were taken after 10 min against a blank sample consisting of a 5 ml sample solution and 5 ml of methanol without $AlCl_3$. The total flavonoid content was determined, using a standard curve of quercetin (10–100 μg/ml) as a standard. The mean of three readings was used and expressed as mg of quercetin equivalents (QE)/100 g of sample (mg% QE). Quercetin content was determined by a validated method (Sithisarn & Gritsanapan, 2007).

2.5. Radical scavenging by reduction of Fremy's salt

According to the method of Rødtjer, Skibsted, and Andersen (2006), the extracts or solutions of the phenolic compounds (quercetin, rutin or rhynchosin-glucoside) were diluted with water (for extracts) and 25% methanol (for phenolic compounds). Three ml of diluted solution were mixed with 200 μl of Fremy's salt (820 μM) dissolved in a 25% saturated sodium carbonate solution. The concentration of the Fremy's salt was adjusted, based on spectrophotometric measurement ($\varepsilon_{270} = 933\ M^{-1}\ cm^{-1}$). The ESR spectra were recorded with a Miniscope MS200 ESR spectrometer (Magnettech, Berlin, Germany) 5 min after mixing. The measurements were carried out at room temperature with a microwave, power of 4 mW, and a modulation width of 0.25 mT. The intensity of the ESR signal was measured as the height of the central line relative to the height of a Mn(II)-marker attached to the cavity of the spectrometer. The radical-scavenging capacity was calculated on the basis of linear regression of results from experiments with four to five different concentrations of the sample or solution of the phenolic compound. The antioxidant activity was expressed as millimoles of Fremy's radicals reduced by 1 g of extract or phenolic compounds.

2.6. Radical-scavenging based on ESR detection of POBN spin adducts

As described by Rødtjer et al. (2006), 4 ml of 3.2 mM POBN dissolved in 1.0 M aqueous ethanol were mixed with 20 μl of 22 mM $FeSO_4$ and 50 μl of either a sample or water as a reference. Finally, 80 μl of 24 mM H_2O_2 were added. An aliquot of the reaction mixture was withdrawn directly into an ESR quartz capillary tube with an interior diameter of 0.75 mm (Wilmad, Buena, NJ, USA), and the ESR spectrum was recorded 2 min after the addition of the H_2O_2 solution on a Miniscope MS200 ESR spectrometer (Magnettech, Berlin, Germany). The measurements were carried out at room temperature with a microwave power of 4 mW, and a modulation width of 0.1 mT. Measurements were carried out with varying concentrations of plant extracts or pure phenolics (quercetin, rutin or rhynchosin-glucoside), and the concentration of spin adducts (%) was calculated from the height of the central peak of the ESR signal of the spin adduct by the following formula:

$$\text{Spin adducts } (\%) = \text{Peak height}_{\text{sample}} \times 100\%/\text{Peak height}_{\text{reference}}$$

2.7. Formation of hypervalent myoglobin in the presence of Siamese neem tree leaf extract

The rate of oxidation of metmyoglobin at 25.0 °C in an aqueous solution of pH 5.5 or 7.4 (ionic strength of 0.16) by hydrogen peroxide (4.0 or 8.0 mM), with or without plant extract (S1) present, was calculated from the slope of the linear regression between time and absorbance at 580 nm. A photodiode array spectrophotometer HP8453 was used.

2.8. Kinetics of reduction of ferrylmyoglobin by Siamese neem tree leaf extract

MbFe(III), dissolved in 5.0 mM phosphate buffer [ionic strength 0.16 adjusted with NaCl (pH 7.4)], was purified on a Sephadex G-25 column (Pharmacia Biotech, Uppsala, Sweden). The eluted MbFe(III) was diluted with phosphate buffer to yield a ~32 μM solution, based on $\varepsilon_{525} = 7700\ M^{-1}\ cm^{-1}$. After reaction of MbFe(III) with a 1.3 times molar excess of H_2O_2 for 3 min, the solution of MbFe(IV) = O was immediately used.

pH was measured relative to concentration standards (0.0100 and 0.00100 M HCl, ionic strength 0.16, adjusted with NaCl), employing the definition, $pH = -\log [H^+]$. pH was measured with Metrohm 6.0224.100 combination glass electrode connected to a Metrohm 713 pH meter. Solutions of Siamese neem tree (S1) extract were freshly prepared before use by dissolving the extract in a NaCl-containing phthalate and phosphate buffer with pH of 5.5 or 7.4, respectively. MbFe(IV) = O and Siamese neem tree solutions were placed in each syringe of a DX-17MW stopped-flow spectrofluorometer (Applied Photophysics, London, UK) at various temperatures (35, 25, 15 and 10 °C). The reactions were followed by absorbance measurement at 580 nm. Pseudo-first order rate constants for the reaction were calculated by nonlinear regression analysis. In the reaction mixture, the concentration of Siamese neem tree solution, calculated as total phenolic content (quercetin equivalents), was in excess, relative to

MbFe(IV) = O, by a factor of 10, and the buffer concentration was 40 mM; ionic strength was 0.16 ± 0.01, adjusted with NaCl. For each combination, pH was measured in thermostatted 1:1 mixtures of FeMb(IV) = O and Siamese neem tree solution. The myoglobin reaction product was identified by recording absorption spectra in the region 450–700 nm during the reaction between MbFe(IV) = O and Siamese neem tree leaf extract using an HP8453 UV–VIS diode array spectrophotometer .

2.9. Laser flash photolysis kinetic experiment

Riboflavin and Siamese neem tree leaf extract (S1) were dissolved in aqueous phosphate buffer ($I = 0.16$, 5 mM, pH 7.4) to yield concentrations of 407 μM riboflavin and 0, 300, 600 and 900 μM for the extracts (calculated as total phenolic content of quercetin equivalents). The samples were purged for 20 min with high purity argon in cuvettes closed by rubber septa. All solutions were protected from light prior to the experiments. Laser flash photolysis experiments were carried out with a LKS.50 spectrometer from Applied Photophysics Ltd. (Leatherhead, UK) as previously described (Becker, Cardoso, & Skibsted, 2005). The third harmonic at 355 nm of a pulse Q-switched Nd-YAG laser was used to pump a dye laser Spectron Laser System (Rugby, UK) using coumarin 120 which has an emission peak at 440 nm. The intensity of the laser pulse was approximately 2.7 mJ cm^{-2}. A R928 photomultiplier tube from Hamamatsu (Hamamatsu, Japan) was used to detect the transient absorption (300–800 nm). Appropriate UV cut-off filters were used to minimize the sample degradation by the monitoring light. The samples were excited in 1.0 × 1.0 cm fluorescence cuvettes from Hellma (Mulheim, Germany). All samples were prepared using fresh solutions thermostatted at 25 ± 0.5 °C and purged with argon before experiment.

2.10. Oxygen consumption assay

As previously described (Carlsen & Skibsted, 2004), 250 μl of 28 mM methyl linoleate (dissolved in methanol) were mixed with 62.5 μl of 0.04 g/ml of Tween-20 in methanol. Methanol was removed using a gentle nitrogen flow, followed by adding 2.5 ml of 50 mM thermostatted (25 °C) air-saturated phosphate buffer (pH 6.8) and 10 μl of sample extracts or pure compound (quercetin and rhynchosin-glucoside) at different concentrations. 25 μl of 0.2 mM metmyoglobin aqueous solution were subsequently added in order to initiate oxidation. Immediately after metmyoglobin addition, measurements of the oxygen consumption were started by transfer of the emulsion to the thermostatted cell equipped with a microelectrode OX MQCH-4134 (Unisense, Denmark), measuring the oxygen concentration every 1 s by a Unisense Picoameter PA2000 oxygen analyzer (Unisense, Aarhus, Denmark). A two-point calibration of air-saturated water (25 °C) and mixture of 0.1 M sodium ascorbate and 0.1 M sodium hydroxide was used to calibrate the electrode and the oxygen analyzer before use. The initial oxygen consumption rate $v(O_2)$ in μmol l^{-1} s^{-1} was calculated from:

$$v(O_2) = -\text{slope } [O_2]_{\text{initial}} \times 10^6/100$$

The slope (percent O_2 per second) was calculated from the oxygen consumption in the 80–40% interval in relation to the initial 100% oxygen concentration corresponding to water saturated with air; $[O_2]_{\text{initial}} = 2.6 \times 10^{-4}$ mol l^{-1} at 25 °C. The influence of each of the extracts on the initial rate of oxygen consumption was expressed as an antioxidative index relative to the rate in the absence of extract:

$$I_{\text{oxygen}} = v(O_2) \text{ with extract present}/v(O_2) \text{ without extract present}$$

In order to compare the effect of total phenolic content in each extract on oxygen consumption rate, I_{oxygen} was divided by total phenolic content (mg quercetin equivalents/1 g extract).

2.11. Peroxidation of liposomes

The method of Roberts and Gordon (2003) was used with minor modifications. Two millilitres of a solution containing 1.5 μmol soybean phosphatidyl choline dissolved in chloroform was mixed with 1 ml of hexane. The solvent was subsequently removed under reduced pressure (approximately 150 mbar) on a rotary evaporator with the water bath set at 30 °C. Nitrogen was introduced to re-establish atmospheric pressure in the evaporation flask after the complete evaporation of solvents. The lipid residue was rehydrated with 10 ml of 0.01 M phosphate buffer (pH 7.4 and 6.0) and vortexed for 10 min following 30 s of sonication, yielding a white homogeneous suspension of multilamellar liposomes. The liposomes were stored in the rotary evaporation flask protected from light with aluminium foil and kept under nitrogen at all times. Unilamellar liposomes were prepared from the suspension of multilamellar liposomes using an Avestin Liposofast Basic small volume (500 μl) extrusion device (Avestin Europe GmbH, Mannheim, Germany). The suspension was passed 21 times through a double layer of polycarbonate membranes with a pore size of 100 nm (Roberts & Gordon, 2003). α-Tocopherol was incorporated by using hexane containing 0.50 mol% of α-tocopherol. Siamese neem tree leaf extract (S1, 0.50 mol%, calculated as quercetin equivalents) were added to the liposome system with the phosphate buffer.

Unilamellar liposome suspensions (2.5 ml) were pipetted into quartz cuvettes and incubated for 10 min at 37 °C in the water-jacket cell holder of a HP8453 UV–VIS diode array spectrophotometer with automatic cell changer. Lipid peroxidation was initiated by introducing 20 μl of 75 mM AAPH dissolved in the phosphate buffer. Up to six samples were measured in each run with phosphate buffer as blank and liposome suspension without antioxidant as control. The absorbance was measured at 234 nm (maximum absorption of conjugated dienes) every 10 min for

1080 min in total. The lag phase before onset of oxidation was measured in minutes corresponding to the intercept between the tangent of the propagation phase and the tangent to the lag phase (Roberts & Gordon, 2003).

3. Results

3.1. General

The potential of various aqueous *Azadirachta* leaf extracts as antioxidants was, in agreement with the evaluation protocols previously described (Becker, Nissen, & Skibsted, 2004), evaluated by comparing (i) total content of phenolics, (ii) the capacity and activity as radical-scavengers, and (iii) the capacity to inhibit lipid oxidation in model systems of increasing organisation.

3.2. Total phenolic content

Phenolic compounds play an important role as antioxidants, and a good correlation between the concentration of plant phenolics and the total antioxidant capacity has been reported (Madsen, Nielsen, Bertelsen, & Skibsted, 1996). There is no previous report of the phenolic content in various *Azadirachta* plants, and the concentration of total phenolics was determined using Folin–Ciocalteu assay and the results are found in Table 1. As is evident from the table, *Azadirachta* leaf extracts have a phenolic content ranging from 12 to 79 mg/g quercetin equivalents (QE). The average total phenolic contents of Siamese neem tree, marrango tree and neem were 32.6 ± 16.1 ($n = 12$), 53.0 ± 24.8 ($n = 3$) and 40.9 ± 5.5 ($n = 5$) mg QE/g extract, respectively, with the lowest phenolic content in the Siamese neem tree (S5) and six times as high phenolic content in marrango tree (E3).

3.3. Radical-scavenging

3.3.1. Scavenging of long lived radicals

The scavenging activities of leaf aqueous extracts from *Azadirachta* plants toward stable radicals were studied, using the reduction of Fremy's salt as detected by ESR spectroscopy. Antioxidants in the extracts react with Fremy's salt, which is a stable radical, and produce diamagnetic reduction products, and the intensity of the ESR signal is decreased (Rødtjer et al., 2006). Linearity was observed between the amount of the extracts added in the assay and the decrease of the ESR signal of Fremy's salt. The amounts of Fremy's salt scavenged by one gramme of extracts were calculated on the basis of the linear regression and may be found in Table 1. The extracts with the higher contents of phenols showed the best scavenging of Fremy's salt radicals. Marrango tree leaf extract scavenged with 0.76 ± 0.50 mmol per gramme of extract ($n = 3$), the highest average amount of Fremy's salt radical. Siamese neem tree and neem tree showed lower capacities with 0.58 ± 0.43 ($n = 12$) and 0.33 ± 0.04 ($n = 5$) mmol Fremy's salt per gramme of extract, respectively. The leaf extract of Siamese neem tree from Ratchaburi province (S1) showed the strongest activity among all extracts. Flavonoids in Siamese neem tree leaf (quercetin, rutin and rhynchosin-glucoside) exhibit high efficiency with respect to quenching of Fremy's salt radicals (Table 1).

Table 1
Total phenolic content of leaf aqueous extracts in mg quercetin equivalents of *Azadirachta* plants determined by Folin–Ciocalteu assay ($n = 2$), Antioxidative index from oxygen depletion rates in methyl linoleate emulsion (pH 6.8, 25 °C) in the presence of *Azadirachta* leaves extracts (200 μg/ml) ($n = 2$) or flavonoids, and radical-scavenging capacities of *Azadirachta* leaf extracts determined by electron spin resonance spectroscopy using Fremy's salt ($n = 2$)

	Total phenolic content (mg QE/g extract)	$I_{oxygen} \times 100$	mmol Fremy's salt/g extract
S1 (Ratchaburi, Central)	69.9	0.039 ± 0.74	1.48
S2 (Roi Et, Northeast)	17.3	2.41 ± 0.00	0.23
S3 (Nakhon Pathom, Central)	18.7	0.78 ± 0.61	0.18
S4 (Mahasarakham, Northeast)	30.7	0.61 ± 0.47	0.51
S5 (Ang Thong, Central)	12.8	2.76 ± 0.08	0.07
S6 (Chanthaburi, East)	37.0	0.45 ± 0.35	0.81
S7 (Lampang, North)	21.8	1.23 ± 0.11	0.40
S8 (Lopburi, Central)	26.5	2.38 ± 0.47	0.24
S9 (Prachinburi, Central)	27.9	1.05 ± 0.00	0.42
S10 (Nakhon Si Thammarat, South)	33.8	0.43 ± 0.20	0.59
S11 (Songkhla, South)	49.6	0.25 ± 0.00	1.09
S12 (Petchabun, North)	45.6	0.19 ± 0.71	1.00
E1 (Pak Panang, Nakhon Si Thammarat, South)	30.3	1.10 ± 0.18	0.20
E2 (Meung, Nakhon Si Thammarat, South)	49.2	0.38 ± 0.38	0.92
E3 (Pattalung, South)	79.4	0.13 ± 0.28	1.17
I1 (Nakhon Pathom, Central)	48.1	0.48 ± 0.13	0.38
I2 (Chaing Rai, North)	35.7	0.87 ± 0.09	0.32
I3 (Chon Buri, East)	42.9	0.65 ± 0.19	0.37
I4 (Mahasarakham, Northeast)	35.0	0.59 ± 0.28	0.28
I5 (Ratchaburi, Central)	42.6	0.51 ± 0.28	0.32
S1 (100 μg/ml)		0.045 ± 0.09	
S1 (400 μg/ml)		0.030 ± 0.00	
Quercetin (50 μg/ml)		37.5 ± 0.16	4.14
Quercetin (250 μg/ml)		14.6 ± 0.61	
Rutin			10.7
Rhynchosin-glucoside (250 μg/ml)		31.2 ± 0.09	9.88

3.3.2. Scavenging of short-lived radicals

The scavenging effects of *Azadirachta* leaf extracts toward short-lived radicals were examined using the Fenton reaction. In the Fenton reaction, hydroxyl radicals are formed by oxidation of Fe^{2+} by hydrogen peroxide, reaction (1). The hydroxyl radicals react immediately with ethanol present in the assay, forming 1-hydroxyethyl radicals, reaction (2).

$$Fe^{2+} + H_2O_2 + H^+ \rightarrow Fe^{3+} + {}^{\cdot}OH + H_2O \quad (1)$$

$$CH_3CH_2OH + {}^{\cdot}OH \rightarrow CH_3{}^{\cdot}CHOH + H_2O \quad (2)$$

The 1-hydroxyethyl radicals are then either trapped by the spin trap 4-pyridyl-1-oxide-*N*-*tert*-butylnitrone (POBN) or react with the antioxidant compounds in plant extracts to generate ethanol and a stable phenoxyl radical reactions (3) and (4).

$$CH_3{}^{\cdot}CHOH + POBN \rightarrow POBN/\ CH_3{}^{\cdot}CHOH \quad (3)$$

$$CH_3{}^{\cdot}CHOH + ArOH \rightarrow CH_3CH_2OH + ArO^{\cdot} \quad (4)$$

The spin adduct, POBN/$CH_3{}^{\cdot}CHOH$ is detected by ESR, while the phenoxyl radical is transformed to non-radical compounds. Radical-scavenging capacity of the extracts will result in a decrease in the spin adduct signals, while a prooxidative activity will result in an increase. The addition of low concentration of the extracts of the leaves of *Azadirachta* plants in the Fenton reaction based assay resulted in a decrease in the level of spin adducts demonstrating radical-scavenging effects. Antioxidative effects were observed with phenolic compound concentrations up to 100 μg quercetin equivalents per ml (Fig. 1), while a higher concentration (1000 μg QE/ml) exhibited prooxidative effects as the relative spin adduct concentration is higher than 100%, as shown in Table 2.

3.4. Quenching of triplet riboflavin

Riboflavin is a photosensitizer as the triplet diradical formed by absorption of visible light is a strong oxidant. The effect of Siamese neem tree leaf extract as a quencher of triplet state was evaluated as previously by real time kinetic analysis by laser flash photolysis (Becker et al., 2005). Following excitation with a 440 nm light pulse of 8 ns, an aqueous solution of riboflavin was found to form the diradical triplet state of riboflavin, as evidenced by the transient absorption spectra recorded after 1 μs of the laser-pulse in the 300–800 nm spectral region. Repeating the experiments with Siamese neem tree leaf extract, together with riboflavin, the decay rate constant was calculated using exponential fitting to the absorption time traces. The decay of triplet riboflavin in each experiment was fully described by single exponential decay, as tested statistically, and the pseudo-first order rate constant is plotted in Fig. 2 as a function of varying extract concentration. From Fig. 2, it is further seen that the pseudo-first order rate constant for triple riboflavin deactivation is linearly dependent on the extract concentration for excess of

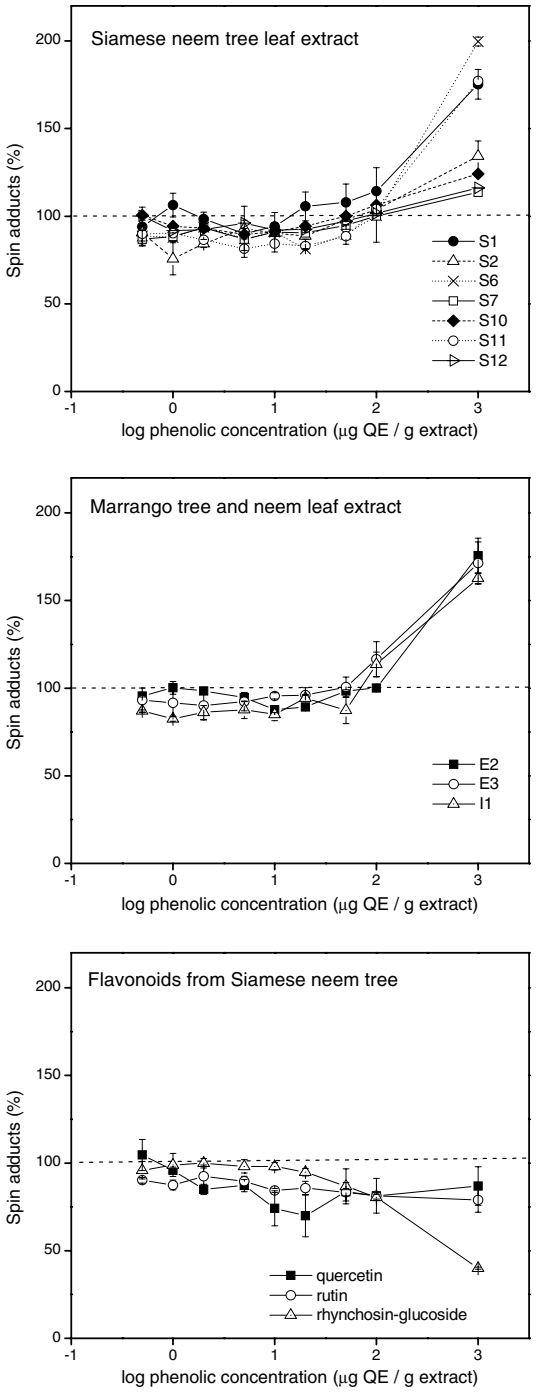

Fig. 1. The effect of *Azadirachta* leaf extracts and flavonoids from Siamese neem tree on the formation of spin adducts in the Fenton assay ($n = 2$). The level of spin adducts formed in a control experiment without addition of extracts is equal to 100% and the extracts may be identified in Table 1.

the extract. The activation of riboflavin by light and deactivation by plant phenolic compounds (ROH) can be expressed as:

$$Rib + h\nu \rightarrow {}^1Rib^* \quad (5)$$

$${}^1Rib^* \rightarrow {}^3Rib^* \quad (6)$$

$${}^3Rib^* + ROH \rightarrow {}^2Rib.^- + {}^2ROH.^+ \quad (7)$$

Table 2
The effects of *Azadirachta* leaf extracts and flavonoids from Siamese neem tree at the concentration of 1000 μg/ml (calculated as quercetin equivalents) on the formation of spin adducts in Fenton assay ($n = 2$)

	Spin adducts (%)
S1 (Ratchaburi, Central)	175 ± 8
S2 (Roi Et, Northeast)	134 ± 9
S6 (Chanthaburi, East)	200 ± 2
S7 (Lampang, North)	114 ± 12
S10 (Nakhon Si Thammarat, South)	124 ± 2
S11 (Songkhla, South)	177 ± 2
S12 (Petchabun, North)	116 ± 4
E2 (Meung, Nakhon Si Thammarat, South)	171 ± 12
E3 (Pattalung, South)	176 ± 10
I1 (Nakhon Pathom, Central)	163 ± 41
Quercetin	87 ± 5
Rutin	79 ± 7
Rhynchosin-glucoside	40 ± 0.6

The level of spin adducts formed in a control experiment, without addition of phenols, is equal to 100%.

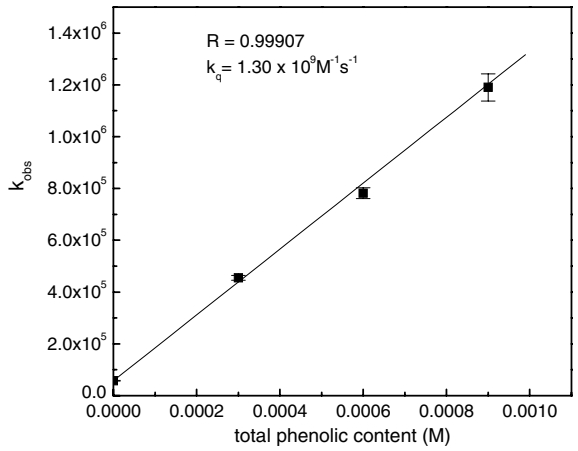

Fig. 2. Dependence on quencher concentration of pseudo-first order rate constant for quenching of triplet riboflavin excited state by aqueous extract of leaves from Siamese neem tree (S1) with pH 7.4 at 25 °C. The second order quenching constant, k_q, was determined by linear regression.

From the observed first-order rate constant, the second-order rate constant (k) for reaction (7) was found to be $1.30 \pm 0.02 \times 10^9$ l mol^{-1} s^{-1} by linear regression, which is nearly the same as the value obtained for (−)-epigallocatechin gallate (EGCG) and (+)-catechin and better than the one obtained for rutin, which were found to be $(1.70 \pm 0.02) \times 10^9$, $(1.44 \pm 0.04) \times 10^9$ and $(0.97 \pm 0.02) \times 10^9$ l mol^{-1} s^{-1}, respectively. This suggested that Siamese neem tree leaf extract is an efficient reactant towards triplet excited state riboflavin and comparable to other phenolic compounds investigated.

3.5. Kinetics of formation of hypervalent myoglobin in the presence of Siamese neem tree leaf extract

Ferrylmyoglobin is as a hypervalent iron compound and a strong prooxidant, which may be formed by reaction of myoglobin with peroxides. The oxidation of metmyoglobin to MbFe(IV) = O, with or without Siamese neem tree leaf extracts, was studied at two pH values (5.5 and 7.4), using concentrations of 4.0 and 8.0 mM hydrogen peroxide and a common pigment concentration of 32 μM. The results shown in Fig. 3, demonstrate the time profile of absorption for 580 nm at which wavelength MbFe(IV) = O has maximal absorption. It was found that Siamese neem tree leaf extract can slow down the oxidation of metmyoglobin to ferrylmyoglobin, as shown in the slower increase in absorbance of the hypervalent iron pigment. The activity was found to be better at acidic pH than at neutral pH, and at higher concentrations of hydrogen peroxide.

3.6. Kinetics of MbFe(IV) = O reduction by Siamese neem tree leaf extract

The observed kinetics for reduction of MbFe(IV) = O by excess Siamese neem tree leaf extract could be described by (pseudo) first-order reactions for all experimental conditions (Fig. 4). The reaction products formed from reduction of MbFe(IV) = O by Siamese neem tree were spectrally identified as MbFe(III) (results not shown). The temperature dependence of the reduction rate of MbFe(IV) = O by Siamese neem tree leaf extract was studied at temperatures of 10, 15, 25 and 35 °C at pH 5.5 and 7.4, and the observed pseudo-first order rate constants were converted to second order rate constants by division with the myoglobin concentration. The regression analysis of the second-order rate constants for the different temperatures, as a function of temperature according to the Arrhenius equation (Fig. 5) resulted in the values shown in Table 3 for activation enthalpies, $\Delta H^{\#}$, and the activation entropies, $\Delta S^{\#}$, at 25 °C for pH 5.5 and 7.4.

From the results, the rate constant for deactivation of ferrylmyoglobin by Siamese neem tree at pH 7.4 and 25 °C was found to be 771 ± 139 M^{-1} s^{-1} which is lower than the values obtained for green tea extract and (−)-EGCG which were reported as 2300 ± 77 and 1170 ± 83 M^{-1} s^{-1}, respectively. However, the deactivation of ferrylmyoglobin was more efficient than that observed for chlorogenate, rutin and apigenin, for which rate constants were reported to be 216 ± 50, 105 ± 1 and 125 ± 6 M^{-1} s^{-1}, respectively (Hu & Skibsted, 2002). The rate constant for deactivation of ferrylmyoglobin by Siamese neem tree leaf extract at lower pH (5.5) had the value 1696 ± 301 M^{-1} s^{-1}, suggesting that Siamese neem tree leaf extracts deactivated ferrylmyoglobin more efficiently in acidic solution than at neutral pH.

3.7. Effect on lipid oxidation

3.7.1. Lipid emulsions

Oxygen is consumed in the propagation phase of lipid oxidation, and the effect of the antioxidants can be directly measured as the change in the rate of depletion of oxygen. A comparison of the relative oxygen depletion rates, in absence and presence of extracts, for the methyl linoleate

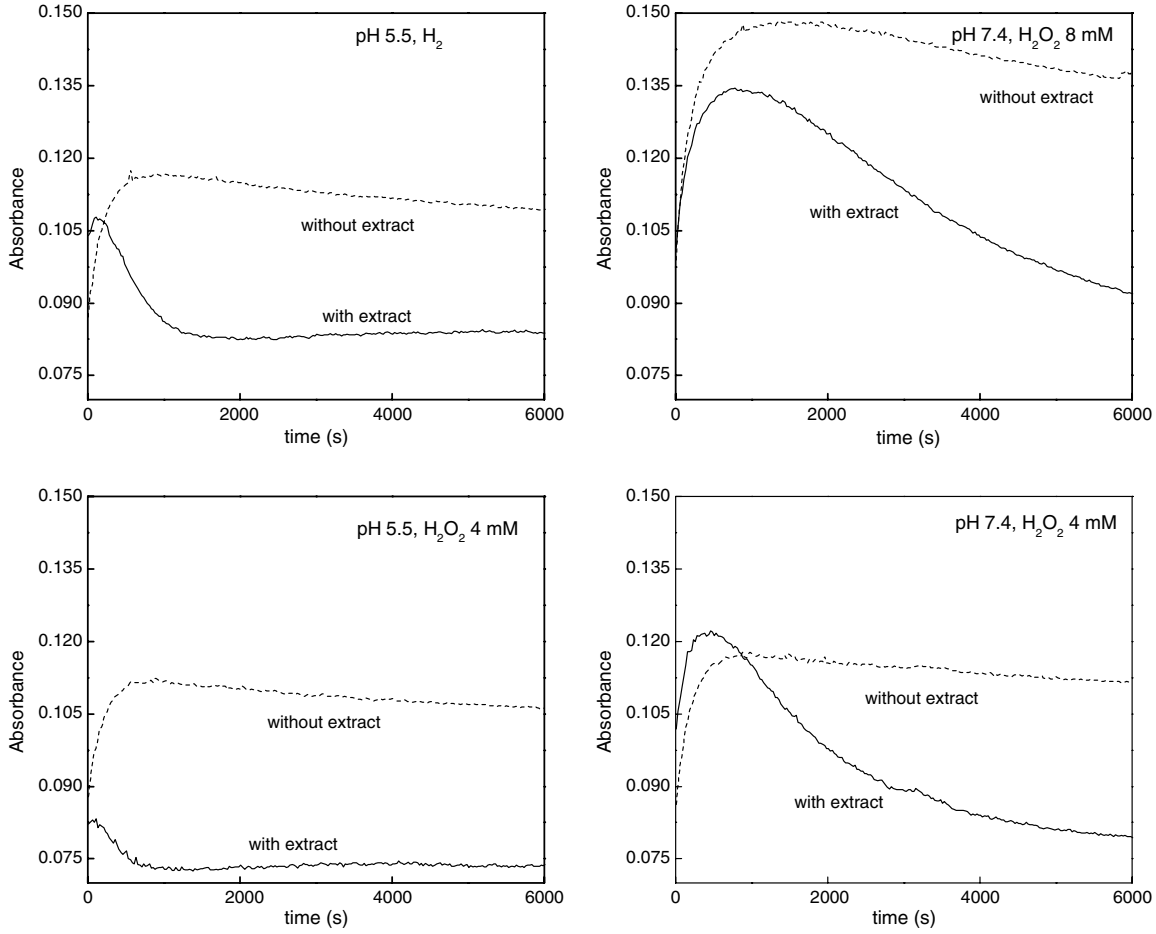

Fig. 3. Time profile of absorption at 580 nm of aqueous MeFe(IV) = O resulting from addition of 8 mM and 4 mM H_2O_2 to 32 μM MbFe(III), with and without 0.16 mM Siamese neem tree leaf extract (S1, calculated as quercetin equivalents) solution with pH 5.5 and 7.4 and ionic strength 0.16 (NaCl). Spectra are recorded with a 25 s interval following 5 s of mixing time in a 1 cm cuvette at 25 °C. Eight hours elapsed from initiation of the reaction to recording of the final spectrum.

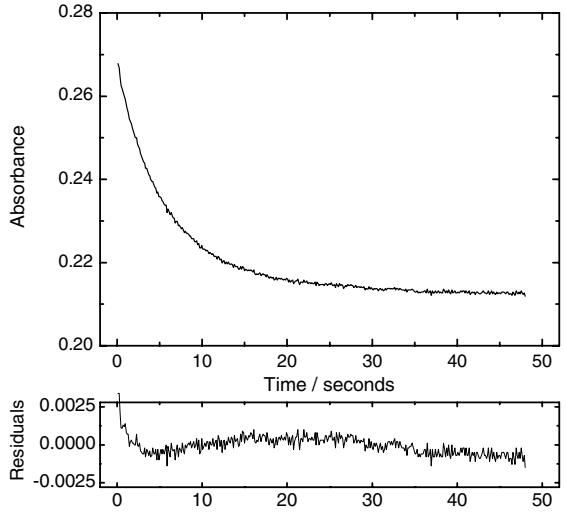

Fig. 4. Relative absorbance at 580 nm during reaction between 0.0320 mM MbFe(IV) = O and 0.16 mM Siamese neem tree leaf extract (S1, calculated as quercetin equivalents) at pH 7.4 (40 mM phosphate buffer) and 25 °C at ionic strength 0.16 (NaCl) using the stopped flow technique. Lower panel shows residuals from a non-linear regression analysis: $A(t) = a + b\ \exp(-k_{obs}t)$, from which a pseudo first order rate constant, $k_{obs} = 0.194 \pm 0.012\ s^{-1}$, was obtained.

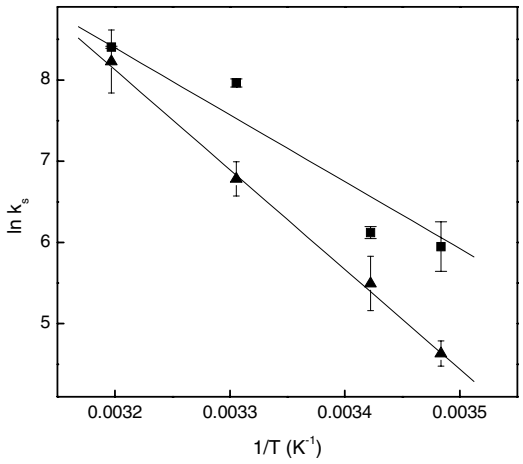

Fig. 5. Arrhenius plot for second order rate constants for reduction of MbFe(IV) = O by Siamese neem tree leaf extract (S1) with a phenol concentration of 0.16 mM at pH 5.5 ■ and 7.4 ▲ in an aqueous solution of ionic strength 0.16 (NaCl) from which the activation parameters of Table 3 were calculated using transition state theory.

Table 3
Rate constants and activation parameters for deactivation of MbFe(IV) = O by Siamese neem tree (S1) leaf extract at 25 °C in aqueous solution with pH 5.5 and 7.4

Sample	Rate constant (M^{-1} s^{-1}) at 25.0 °C	$\Delta H^{\#}$ (kJ mol^{-1})	$\Delta S^{\#}$ (J mol^{-1} K^{-1})
S1 pH 5.5	1696 ± 301	66 ± 9	35 ± 31
S1 pH 7.4	771 ± 139	80 ± 9	76 ± 32

emulsion used for the assay, may be found in Table 1. The *Azadirachta* leaf extracts explored in this study showed different effects on the oxygen depletion. All extracts were highly effective in inhibiting the oxygen consumption and comparable to the flavonoids, quercetin and rhynchosin-glucoside. Marrango tree had the largest effect on oxygen depletion rate with the average value of 0.54 ± 0.50 ($n = 3$) for ($I_{oxygen} \times 100$), while neem and Siamese neem tree showed a lower average effect, with 0.62 ± 0.15 ($n = 5$) and 1.05 ± 0.95 ($n = 12$), respectively. However, the most effective extract is Siamese neem tree (S1), which was found to have a clear concentration-dependent effect (Table 1). The quercetin contents of each aqueous extract are given in Table 4, together with the total flavonoid content. From a simple comparison, it is seem that there is no simple correlation between the flavonoid or quercetin content and the antioxidative index.

Table 4
Quercetin and total flavonoid content in the aqueous extracts of *Azadirachta* leaves evaluated for the antioxidant capacity and activity

Plant extract	Quercetin content in aqueous extract (mg %)[a] (by TLC densitometry)	Total flavonoid content in aqueous extract (mg% QE)[a]
S1 (Ratchaburi, Central)	13.2 ± 0.85	289 ± 5.31
S2 (Roi Et, Northeast)	6.19 ± 0.16	147 ± 3.71
S3 (Nakhon Pathom, Central)	14.2 ± 1.29	346 ± 12.6
S4 (Mahasarakham, Northeast)	22.3 ± 1.75	477 ± 5.83
S5 (Ang Thong, Central)	9.59 ± 0.40	158 ± 12.1
S6 (Chanthaburi, East)	8.61 ± 1.59	284 ± 16.3
S7 (Lampang, North)	9.37 ± 1.95	224 ± 14.5
S8 (Lopburi, Central)	24.4 ± 2.48	110 ± 15.9
S9 (Prachinburi, Central)	48.1 ± 1.50	414 ± 3.37
S10 (Nakhon Si Thammarat, South)	18.4 ± 1.72	294 ± 8.56
S11 (Songkhla, South)	27.8 ± 1.96	511 ± 6.85
S12 (Petchabun, North)	21.8 ± 0.45	493 ± 3.91
E1 (Pak Panang, Nakhon Si Thammarat, South)	22.2 ± 1.10	131 ± 4.69
E2 (Meung, Nakhon Si Thammarat, South)	36.2 ± 1.40	268 ± 4.35
E3 (Pattalung, South)	34.0 ± 2.45	398 ± 9.11
I1 (Nakhon Pathom, Central)	31.8 ± 0.18	145 ± 15.39
I2 (Chaing Rai, North)	14.6 ± 1.05	93.1 ± 3.37
I3 (Chon Buri, East)	33.6 ± 1.38	128 ± 8.56
I4 (Mahasarakham, Northeast)	16.8 ± 0.20	193 ± 6.85
I5 (Ratchaburi, Central)	26.7 ± 1.69	193 ± 3.19

[a] Expressed as mean ± SD ($n = 3$).

3.7.2. Liposomes

The antioxidant activity of Siamese neem tree leaf extract was further investigated as the ability to inhibit lipid oxidation in liposomes made of soybean phosphatidyl choline when initiated by AAPH, a hydrophilic azo compound radical initiator. The extent of oxidation was monitored spectrophotometrically by following the formation of conjugated dienes as absorbance at 234. The formation of conjugated dienes began immediately upon addition of AAPH to the liposomes without extract, while a clear lag phase was observed when Siamese neem tree leaf extracts were added to the liposome solution at both pH 7.4 and 6.0, again demonstrating Siamese neem tree leaf extract as an efficient antioxidant, Figs. 6 and 7. Vitamin E, α-tocopherol, is a lipophilic natural antioxidant known to efficiently inhibit lipid peroxidation, and α-tocopherol also showed a clear lag phase prior to formation of conjugated dienes in the liposome assay. The observed lag phases for 0.5 mol% of α-tocopherol were 95 and 45 min at pH 7.4 and 6.0, respectively. For 0.75 μM (0.5 mol% calculated as quercetin equivalents) Siamese neem tree leaf extracts, the lag phases were even longer and 175 and 60 min at pH 7.4 and 6.0, respectively. In liposomes, with both the vitamin antioxidant and the extract added, the lag phases were found to be 275 and 100 min for 0.25 mol% α-tocopherol and 0.25 mol% Siamese neem tree leaf extract added at pH 7.4 and 6.0, respectively. Adding Siamese neem tree leaf extract together with α-tocopherol is seen to prolong the lag phase relative to the sum of the lag phases calculated for the two individual compounds at both pH 7.4 and 6.0 (Figs. 6 and 7). The combination of α-tocopherol and Siamese neem tree leaf extract thus gives synergistic effects for both neutral and slightly acidic pH.

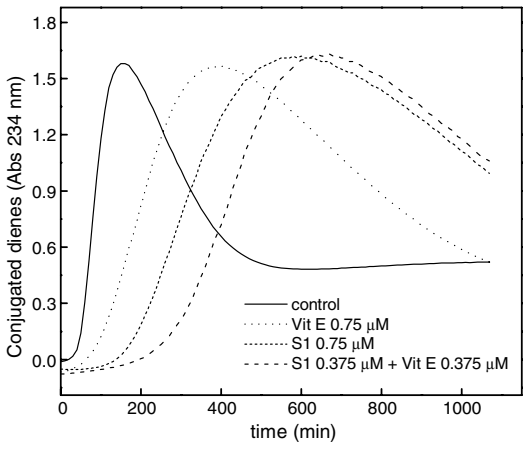

Fig. 6. Formation of conjugated dienes in peroxidising liposomes at 37 °C, pH 7.4, measured as absorbance at 234 nm. Values are means of duplicate experiments.

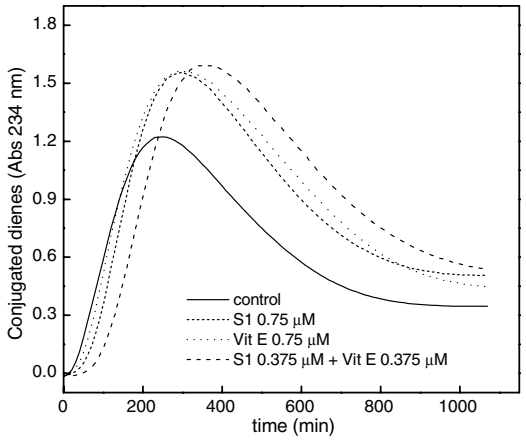

Fig. 7. Formation of conjugated dienes in peroxidising liposomes at 37 °C, pH 6.0, measured as absorbance at 234 nm. Values are means of duplicate determinations.

4. Discussion

Antioxidants are present in plant materials in complex mixtures and their interaction may result in synergistic effects in foods or in the body after ingestion (Becker et al., 2004). Such effects may be difficult to predict from analysis of total phenolic content of plant extracts or even from a more detailed analysis. The evaluation of the antioxidant properties of leaves from different species of *Azadiracta* extracts clearly illustrates the importance of using combinations of methods for natural mixtures, as found in plant material or plant extracts.

Siamese neem tree, neem tree and marango tree were all found to have high concentrations of phenols. However, large individual differences in phenol content between plants of different provenience were seen. The radical-scavenging capacity of the extracts, as determined using the semi-stable radical Fremy's salt, correlated linearly with the phenol content for each of the three series of plant material. However, it is clearly seen from Fig. 8 that the phenols present in Siamese neem tree more efficiently scavenge the stable radical than do the phenols present in marrango tree and especially the neem tree. A comparison with the total flavonoid content and the quercetin content confirms these patterns, and these observations deserve further attention in relation to the structure of individual and unusual phenols present in the plants (Sithisarn et al., 2005). The scavenging of short-lived radicals by the *Azadirachta* leaves, as tested by 1-hydroxylethyl radicals generated in the Fenton reaction, showed that all types of extracts were active up to a certain concentration of around 100 μg quercetin equivalents/l of extract. Above this level, prooxidative properties became dominant (Table 2), and most likely related to the reducing properties of the phenol, as was also seen for other types of plant material rich in phenols (Rødtjer et al., 2006). Notably, three flavonoids isolated from Siamese neem tree did not show a similar shift to become prooxidants, again indicating the importance of antioxidant interaction in complex mixtures (Fig. 1). In food systems, light may induce radical formation and one of the Siamese neem tree extracts (S1) was tested for its capacity to quench triplet riboflavin, the primary radical in photooxidation of many foods. Triplet riboflavin is formed following light absorption and the extract was shown, using real time kinetic methods, to protect as effectively or even better than did tea extracts or the purified plant phenols tested so far (Becker et al., 2005).

Purified plant phenols, e.g. quercetin, rutin and the rhynchosin-glucoside, all known to be important in *Azadirachta* species and plant extracts, may have radical-scavenging capacities which correlate with the number of phenol groups present, but they have different effects as antioxidants in various substrates. Potential antioxidants should accordingly always be tested in a relevant oxidation substrate. In emulsions, the extracts were found to be efficient antioxidants. Notably, no simple correlation between phenol content and capacity to inhibit oxygen consumption was seen, indicating an important interaction between different phenols and other compounds in

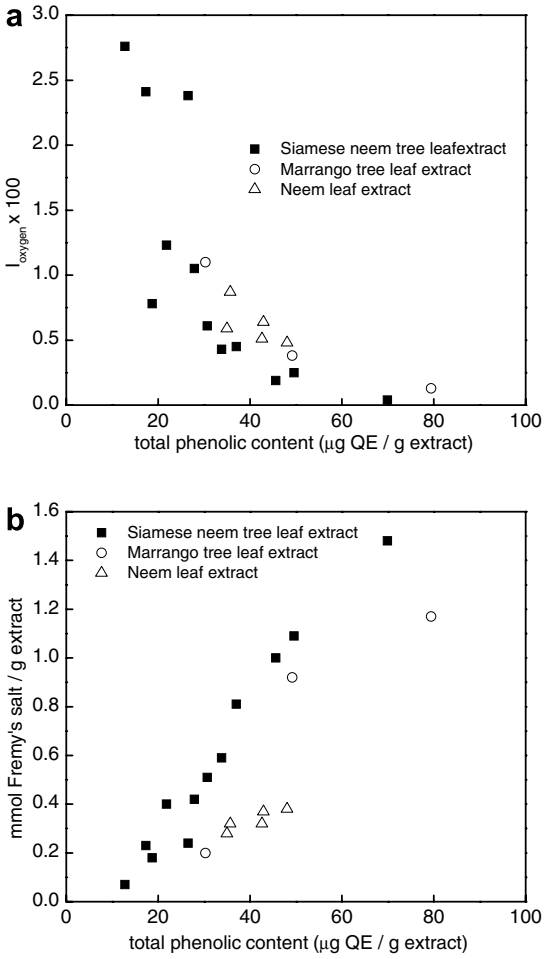

Fig. 8. Correlation of total phenolic content in *Azadirachta* leaves extracts and (a) antioxidative index from the oxygen depletion rate, $I_{oxygen} \times 100$, and (b) radical-scavenging capacity, expressed as mmol Fremy's salt/g extract. Total phenolic content, I_{oxygen} values, and mmol Fremy's salt values are the means of two determinations.

the individual extract. In order to describe such synergism in more detail, the interaction between vitamin E and one extract was studied in the more organized liposomes. In the liposomes, the extract (S1) was more efficient as an antioxidant than was vitamin E on a molar basis. The two different types of antioxidants, moreover, showed a significant synergistic interaction (Figs. 6 and 7). Such synergism may be part of the health-promoting properties often ascribed to Siamese neem tree leaves. These results strongly encourage human studies in which vitamin E status would be correlated with the intake of Siamese neem tree leaves as part of the diet.

Polyphenols are often poorly absorbed by humans and, moreover, they are excreted actively by the body. However, they may be active in the gastro-intestinal tract (Vulcain, Goupy, Caris-Veyrat, & Dangles, 2005). Vegetables are often eaten together with meat, and we have considered another mechanism for the health-promoting effect of Siamese neem tree leaves, as eaten in the Thai diet. The meat pigment myoglobin, and also hemoglobin, easily form hypervalent iron when exposed to hydroperoxides. In the present study, it was clearly demonstrated that Siamese neem tree leaf extracts were efficient in preventing the formation of hypervalent iron and, if formed, the extracts inactivate the hypervalent state rather efficiently. Hypervalent iron is an efficient prooxidant and may cleave peroxides, generating free radicals, and both prevention of formation and deactivation by the phenolic mixture in Siamese neem tree leaves accordingly protect the gastroinstetinal tract against free radicals. More detailed investigations, as reported in Carlsen, Kröger-Ohlsen, Bellio, and Skibsted (2000), also considering binding of myoglobin to the Siamese neem tree phenols, would be valuable for a deeper understanding.

References

Basak, S. P., & Chakraborty, D. P. (1968). Chemical investigation of *Azadirachta indica* leaf (*M. azadirachta*). *Journal of the Indian Chemical Society, 45*, 466–467.

Becker, E. M., Cardoso, D. R., & Skibsted, L. H. (2005). Deactivation of riboflavin triplet-excited state by phenolic antioxidant: mechanism behind protective effects in photooxidation of milk-based beverages. *European Food Research and Technology, 221*, 382–386.

Becker, E. M., Nissen, L. R., & Skibsted, L. H. (2004). Antioxidant evaluation protocols: Food quality or health effects. *European Food Research and Technology, 219*, 561–571.

Carlsen, C. U., Kröger-Ohlsen, M. V., Bellio, R., & Skibsted, L. H. (2000). Protein binding in deactivation of ferrylmyoglobin by chlorogenate and ascorbate. *Journal of Agricultural and Food Chemistry, 48*, 204–212.

Carlsen, C. U., & Skibsted, L. H. (2004). Myoglobin species with enhanced prooxidative activity is formed during mild proteolysis by pepsin. *Journal of Agricultural and Food Chemistry, 52*, 1675–1681.

Clayton, T., Soralump, P., Chaukul, W., & Temsiririrkkul, R. (1996). *Medicinal plants in Thailand* (vol. 1). Bangkok: Amarin Printing.

Cross, E. C. (1987). Oxygen radicals and human disease. *Annals of Internal Medicine, 107*, 526–545.

Hu, M., & Skibsted, L. H. (2002). Kinetics of reduction of ferrylmyoglobin by (−)-epigallocatechin gallate and green tea extract. *Journal of Agricultural and Food Chemistry, 50*, 2998–3003.

Madsen, H. L., Nielsen, B. R., Bertelsen, G., & Skibsted, L. H. (1996). Screening of antioxidative activity of spices. *Food Chemistry, 57*, 331–337.

Meda, A., Lamien, C. E., Romito, M., Millogo, J., & Nacoulma, O. G. (2005). Determination of the total phenolic, flavonoid and proline contents in Burkina Fasan honey, as well as their radical scavenging activity. *Food Chemistry, 91*, 571–577.

Nair, M. S., Gopal, S., & Issac, D. (1997). Optimised isolation procedure for biologically active compounds nimbolide and 28-deoxonimbolide from *Azadirachta indica* leaves. *Phytochemistry, 46*(7), 1177–1178.

Naithani, V., Nair, S., & Kakkar, P. (2006). Decline in antioxidant capacity of Indian herbal teas during storage and its relation to phenolic content. *Food Research International, 39*(2), 176–181.

Nakov, N., Labode, O., & Akahtaedzhiev, K. (1982). Study of the flavonoid composition of *Azadirachta indica*. *Farmatsiya (Sofia), 32*, 24–28.

Roberts, W. G., & Gordon, M. H. (2003). Determination of the total antioxidant activity of fruits and vegetables by a liposome assay. *Journal of Agricultural and Food Chemistry, 51*, 1486–1493.

Rødtjer, A., Skibsted, L. H., & Andersen, M. L. (2006). Antioxidative and prooxidative effects of extracts made from cherry liquer pomace. *Food Chemistry, 99*(1), 6–14.

Schroeder, D. R., & Nakanishi, K. (1987). A simplified isolation procedure for azadirachtin. *Journal of Natural Product, 50*(2), 241–244.

Siddiqui, B. S., Ghiasuddin Faizi, S., & Siddiqui, S. (1992). Triterpenoids from the fresh fruit coats of *Azadirachta indica*. *Phytochemistry, 31*(12), 4275–4278.

Sithisarn, P., Gritsanapan, W., (2007). Variability of quercetin content in Siamese neem tree leaves in Thailand by TLC-densitometry. In *Proceeding of international conference on medicinal and aromatic plants*. 15–18 January; Chiang Mai, Thailand.

Sithisarn, P., Gritsanapan, W., Supabphol, R., & Chavasiri, W. (2003). Antioxidative compound in the leaves of Siamese neem tree. *Thai Journal of Pharmaceutical Sciences, 27*, 73.

Sithisarn, P., Supabphol, R., & Gritsanapan, W. (2005). Antioxidant activity of Siamese neem tree. *Journal of Ethnopharmacology, 99*, 109–112.

Sombatsiri, K., Ermel, K., & Schmutterer, H. (1995). Other Meliaceous plants containing ingredients for integrated pest management and further purpose. In H. Schmutter (Ed.), *The neem tree Azadirachta indica* A. Juss. and other meliaceous plants. Germany: VCH.

Suttajit, S., Khansuwan, U., & Suttajit, M. (2002). Antioxidative activity of Thai medicinal herbs. *Thai Journal of Pharmaceutical Science, 26*, 32.

Van der Nat, J. M., Van der Sluis, W. G., Thart, L. A., Van dijk, H., De Silva, K. T. D., & Labadie, R. P. (1991). Activity-guided isolation and identification of *Azadirachta indica* bark extract constituents which specifically inhibit chemiluminescence production by activated human polymorphonuclear leukocytes. *Planta Medica, 57*(1), 65–68.

Vulcain, E., Goupy, P., Caris-Veyrat, C., & Dangles, O. (2005). Inhibition of the metmyoglobin-induced peroxidation of linoleic acid by dietary antioxidants: Action in the aqueous vs. lipid phase. *Free Radical Research, 39*, 547–563.