POLISH JOURNAL OF FOOD AND NUTRITION SCIENCES

www.pan.olsztyn.pl/journal/
e-mail: joan@pan.olsztyn.pl

Pol. J. Food Nutr. Sci.
2008, Vol. 58, No. 2, pp. 229-233

ANTIOXIDANT ACTIVITY OF PHENOLIC FRACTIONS OF *LITSEA MONOPETALA* (PERSIMON-LEAVED LITSEA) BARK EXTRACT

Muhammad Arfan[1], Hazrat Amin[1], Agnieszka Kosińska[2], Magdalena Karamać[2], Ryszard Amarowicz[2]

[1]Institute of Chemical Sciences, University of Peshawar, Peshawar, Pakistan; [2]Division of Food Science, Institute of Animal Reproduction and Food Research of the Polish Academy of Sciences, Olsztyn, Poland

Key words: *Litsea monopetala*, bark extract, phenolic compounds, antioxidant activity, column chromatography, HPLC

Phenolic compounds were extracted from *Litsea monopetala* bark using methanol. Four fractions (I-IV) were separated from extract on a Sephadex LH-20 column using methanol as the mobile phase evaluated for their total antioxidant activity, antiradical activity against DPPH•, (2,2-diphenyl-1-picrylhydrazyl radical), and reducing power. The total phenolics and tannin contents of the fractions were determined. The highest relative content in the crude extract was found for fraction I. The content of total phenolics in fractions ranged from 169 mg/g (fraction I) to 753 mg/g (fraction II). Condensed tannins were detected in fractions II-IV. The fractions exhibited the total antioxidant activity from 1.90 mmol Trolox/g (fraction I) to 7.06 mmol Trolox/g (fraction IV). Fraction III was characterised by the highest antiradical activity against DPPH• (EC_{50} = 0.011 mg/assay). Fractions II and III showed the strongest reducing power which was app. four times higher than that of fractions I. The presence of three main phenolic compounds in fraction I was confirmed using RP-HPLC.

INTRODUCTION

Anatomical parts of plants belonging to the genus *Litsea* are a rich source of biologically-active compounds, such as flavonoids (leaves of *Litsea coreana* and *Litsea japonica*) [Lee *et al*., 2005], butanolides (leaves of *Litsea acutivena*) [Cheng *et al*., 2001], sesquiterpenese (leaves and twigs of *Litsea verticillata*) [Zhang *et al*., 2003], 1,3-diarylpropan-2-ol (bark of *Litsea rotundifolia*) [Zhao *et al*., 2003], butanolide, cumarin, and syringaldehyde (bark of *Litsea akoensis*) [Tsai *et al*., 2000], and essential oils (leaves of *Litsea cubeba*, fruits, flowers and bark of *Litsea monopetala*, fruits of *Litsea glutinosa*) [Wang *et al*., 1999; Amer & Mehlhorn, 2006; Choudhury *et al*., 1996, 1997].

Biological activity of the genus *Litsea* extracts or individual chemical constituents isolated from these extracts has already been reported. The anti-diarrheal activity of methanol extract of dried bark and aerial parts of *Litsea polyantha* has been evaluated in mice using different models by Poonia *et al*. [2007]. The adulticidal activity of methanol extracts from *Litsea elliptica* Blume tree against adult of *Aedes aegypti* (L.) was reported by Hidayatulfathi *et al*. [2004]. The inhibitory potencies of flavonoids isolated from *Litsea japonica* leaves were described by Lee *et al*. [2005]. Sesquiterpenes and butenolides isolated from the leaves and twigs of *Litsea verticillata* were found to inhibit HIV-1 replication [Zhang *et al*., 2005]. Phytopesticidal and repellent efficacy of *Litsea salicifolia* against *Aedes aegypti* and *Culex quinquefasciatus* were reported by Phukan & Kalita [2005]. Citral refined from *Litsea cubeba* oil has been found to have a strong influence on fungi, especially *Aspergillus flavus* [Luo *et al*., 2004]. Both the methanolic extract and its fractions (0.01 mg/mL) from *Litsea cubeba* bark were found to inhibit nitric oxide and prostaglandin E_2 production in LPS-activated macrophages without significant cytotoxicity at less than 0.01 mg/mL concentration [Choi & Hwang, 2004]. Methanol extracts of *Litsea elliptica* leaves inhibited the growth of *Helicobacter pylori* strains with minimum inhibitory concentration of 100 μg/mL [Bhamarapravati *et al*., 2003]. The extract of *Litsea petiolata* exhibited significant antimutagenicity [Nakahara *et al*., 2002]. Lignans isolated from *Litsea verticillata* showed moderate anti-HIV activity [Hoang *et al*., 2002]. The methanol extract showed antibacterial activity, comparable to chloramphenicol, against 16 tested microorganisms [Mandal *et al*., 2000]. The bark of *Litsea monopetala* locally called *Medea sak* is used as nerves and bone tonic, stomachache, stimulant, analgesic and antiseptic in folk as well as established herbal in Pakistan and India. The leaves of this tree are used by the local people as fodder for cattle since they are claimed to enhance milk production. *Litsea monopetala* is mainly distributed in Western outer Himalayas, India, West Malesia, Thailand, and Burma.

The present study was aimed at investigating the potential antioxidant effects *in vitro* of the phenolic fractions separated from *Litsea monopetala* bark crude extract using a Sephadex LH-20 column chromatography.

Author's address for correspondence: Dr. Ryszard Amarowicz, Institute of Animal Reproduction and Food Research of the Polish Academy of Sciences, ul. Tuwima 10, P.O. Box 55, 10-747 Olsztyn, Poland, tel.: (48 89) 523 46 27; fax: (48 89) 524 01 24; e-mail: amaro@pan.olsztyn.pl

MATERIALS AND METHODS

Chemicals

All solvents used for chromatography were of HPLC grade, unless otherwise specified. Ferric chloride, potassium ferricyanide, potassium persulfate, trifluoroacetic acid (TFA) and trichloroacetic acid (TCA) were acquired from the P.O.Ch. Company (Gliwice, Poland). Gallic acid, Folin and Ciocalteau's phenol reagent, 2,2-diphenyl-1-picrylhydrazyl radical (DPPH•), 2,5,7,8-tetramethyl-chroman-2-carboxylic acid (Trolox), and 2,2'-azino-bis-(3-ethylbenzothiazoline--6-sulfonic acid) (ABTS) were purchased from Sigma Ltd. (Poznań, Poland).

Extraction and fractionation

Litsea monopetala bark was collected from trees growing wild in Buner area of the North West Frontier Province (N-W.F.P), Pakistan. Phenolic compounds were extracted from the ground bark using methanol (4 L for 12 h; at room temperature). The extraction was repeated twice more, supernatants were combined and methanol was evaporated under vacuum at 45°C using a rotary evaporator. The prepared crude extract (48 g) was stored at –20°C until used.

A 0.5 g portion of the extract so obtained was dissolved in 8 mL of methanol and applied to a chromatographic column (2 × 60 cm) packed with Sephadex LH-20 and eluted with methanol. Fractions containing low-molecular weight phenolic compounds (4 mL) were collected using a fraction collector and their absorbance was measured at 280 nm. Eluates were then pooled into major fractions. Organic solvents were evaporated at 45°C using a rotary evaporator.

Total phenolics

The content of total phenolics in the fractions dissolved in methanol was determined according to the procedure described by Amarowicz *et al*. [2004a] using the Folin-Ciocalteau's phenol reagent. Gallic acid was used as a standard in this work.

Condensed tannins

The content of condensed tannins in the fractions dissolved in methanol was determined according to a modified vanillin method described by Price *et al*. [1978] and expressed as absorbance units at 500 nm per mg of extract (A_{500}/mg).

Total antioxidant activity

The total antioxidant activity (TAA) of the fractions dissolved in methanol was determined according to the Trolox equivalent antioxidant activity (TEAC) assay described by Re *et al*. [1999]. The total antioxidant activity was expressed as mmol Trolox/g of fraction.

Radical-scavenging activity

The capacity of the fractions to scavenge the "stable" free radical 2,2-diphenyl-1-picrylhydrazyl (DPPH•) was monitored according the procedure described by Amarowicz *et al*. [2002]. Briefly, 0.1 mL of methanolic solution of the fractions was mixed with 2 mL of methanol and then 0.25 mL of DPPH radical methanolic solution (1 mmol/L) was added. The mixture was vortexed thoroughly for 1 min. Finally, the absorbance of the mixture after standing at ambient temperature for 20 min was read at 517 nm.

Reducing power

The reducing power of the fractions was determined by the method of Oyaizu [1986]. Briefly, the assay medium contained 0.1–0.5 mg of fraction I and 0.02-0.10 mg of fractions II-IV suspended in 2.5 mL of 0.2 mol/L phosphate buffer (pH 6.6) and 2.5 mL of 1% (w/v) potassium ferricyanide. After incubation at 50°C for 20 min, 2.5 mL of 10% (w/v) trichloroacetic acid was added to the mixture followed by centrifugation at 1750×*g* for 10 min. The supernatant (2.5 mL) was mixed with 2.5 mL distilled water and 0.5 mL of 0.1% (w/v) ferric chloride, and the absorbance of the resultant solution was read at 700 nm.

HPLC analysis

Phenolic constituents of fraction I were analysed using a Shimadzu HPLC system (Shimadzu Corp., Kyoto, Japan) consisting of two LC-10AD pumps, SCL 10A system controller and SPD-M 10A photodiode array detector. The chromatography was carried out using a pre-packed LiChrospher 100 RP-18 column (4 × 250 mm, 5 μm; Merck, Darmstad, Germany). Elution was employed for 50 min in a gradient system of 5-40% acetonitrile in water adjusted to pH 2.5 with TFA. Detector was set at 280 nm; injection volume was 20 μL and the flow rate was 1 mL/min.

RESULTS AND DISCUSSION

Four phenolic fractions (I-IV) were separated from the methanolic extract of *Litsea monopetala* bark using Sephadex LH-20 column chromatography (Figure 1). The content of total phenolics in fractions ranged from 169 mg/g (fraction I) to 753 mg/g (fraction II), (Table 1). Condensed tannins were detected in fractions II-IV. The highest content of condensed tannins determined using the vanillin/HCl method was found in fraction IV (Table 1). The content of total phenolics in the

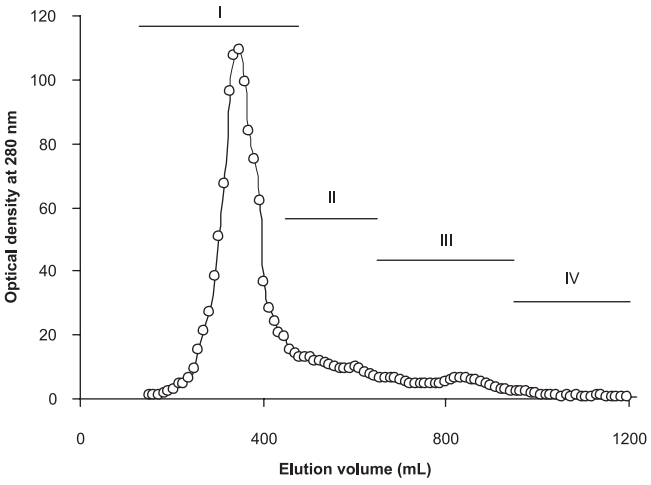

FIGURE 1. Separation of phenolic compounds from *Litsea monopetala* bark extract on a Sephadex LH-20 column with methanol as the mobile phase.

TABLE 1. Content of total phenolics, condensed tannins and total antioxidant activity in phenolic fractions of *Litsea* bark extract.

Fraction	Total phenolics[a] (mg/g)	Condensed tannins (A_{500}/mg)	Total antioxidant activity (mmol Trolox/g)
I	169 ± 5	-	1.90 ± 0.05
II	753 ± 26	0.021 ± 0.002	6.27 ± 0.12
III	695± 23	0.972 ± 0.018	6.16 ± 0.10
IV	698 ± 24	1.250 ± 0.027	7.06 ± 0.15

[a] reported as gallic acid equivalents

fractions separated from the *Litsea monopetala* bark crude extract was high. The results were several times higher then those reported previously for the tannin-rich extracts from leguminous and oil seeds, almonds, as well as seeds hulls [Amarowicz *et al*., 2000a, b, 2004b, 2005]. In our previous study, the content of total phenolics in fractions of *Mallotus philipinensis* bark extract ranged from 54 mg/g to 927 mg/g [Arfan *et al*., 2007]. Similar content of condensed tannins (from 0.021 to 1.321 absorbance units at 500 nm per mg of fraction) was noted previously by us in the fractions separated from *Mallotus philipinensis* bark extract using the same chromatographic method [Arfan *et al*., 2008].

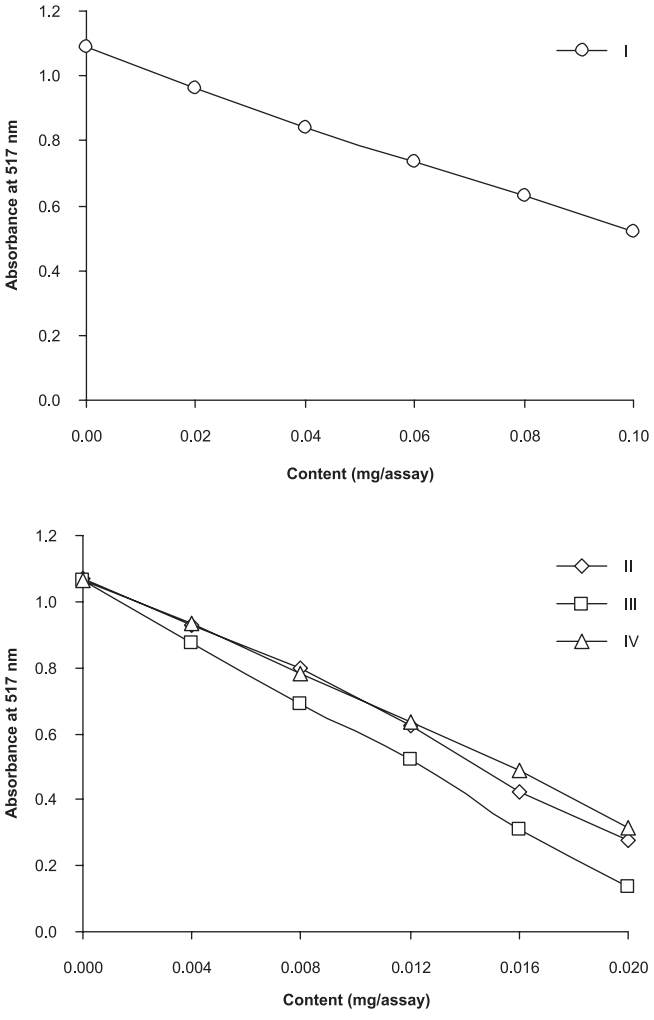

FIGURE 2. Scavenging effect of phenolic fractions of *Litsea monopetala* bark extract on the 2,2-diphenyl-1-picrylhydrazyl radical (DPPH•), as measured by changes in absorbance at 517 nm.

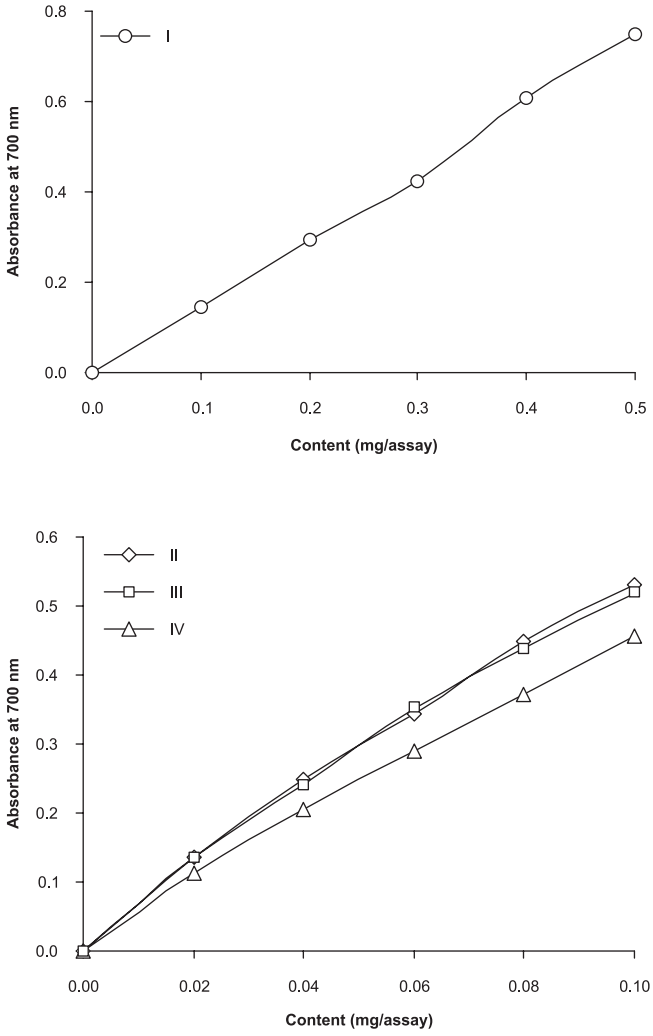

FIGURE 3. Reducing power of phenolic fractions of *Litsea monopetala* bark extract, as measured by changes in absorbance at 700 nm.

Figure 2 depicts the concentration-dependent antiradical activity response curves for the fractions of *Litsea monopetala* bark extracts. The results are expressed as the decrease in ab-

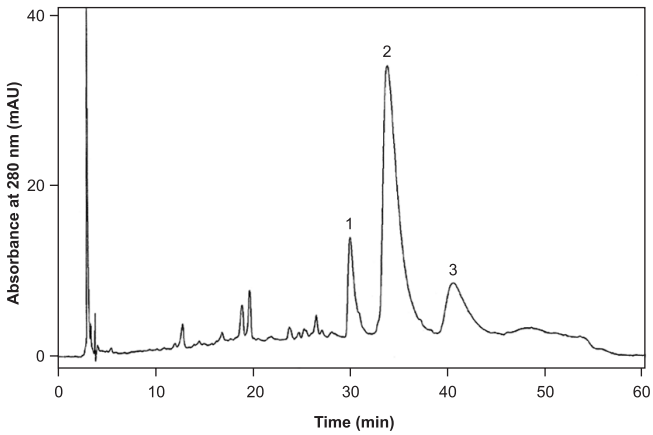

FIGURE 4. HPLC chromatograms of phenolic fraction I of *Litsea monopetala* bark extract.

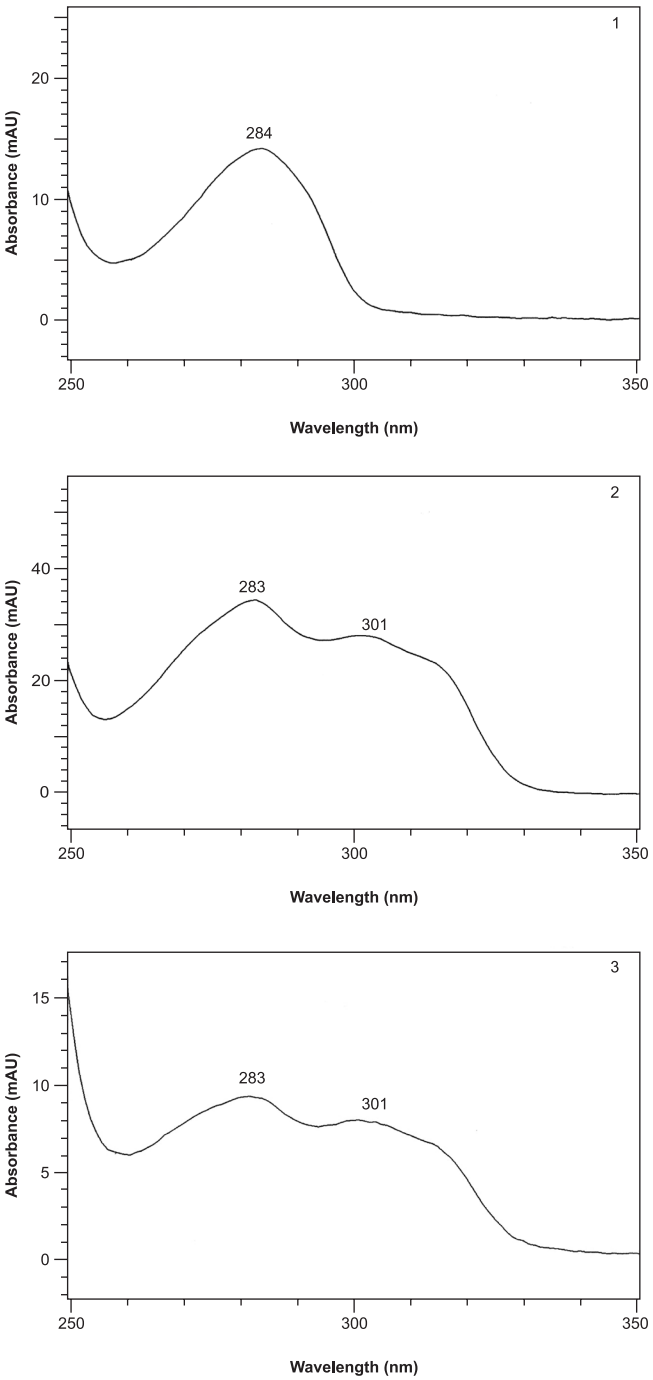

FIGURE 5. UV-DAD spectra of compounds separated from fraction I of the *Litsea monopetala* bark extract using HPLC method; 1-3 – numbers of peaks corresponding to Figure 4.

sorbance of the DPPH• solution at 517 nm. The antiradical activities of the fractions II-IV were several times higher than those of fractions I. The EC_{50} values calculated for fractions I, II, III, and IV were 0.098, 0.013, 0.011, and 0.014 mg/assay. It is worth noting that fraction IV, showing the highest TAA value (Table 1), was a weaker scavenger of DPPH• as compared to fractions II and III.

Fractions II and III showed the strongest reducing power (Figure 3) which was approximately four times higher than that of fraction I. In the assay used, the presence of reductants (*i.e.* antioxidants) in the fraction caused a reduction of the Fe^{3+}/ferricyanide complex to the Fe^{2+} state. Therefore, it can be monitored by measuring the formation of Perl's Prussian blue coloration having the maximum absorbance at 700 nm.

The strong antioxidant and antiradical properties of the fractions of *Litsea monopetala* bark extracts are in accordance with literature data. The antiradical activity of the pine bark procyanidins were reported by Virgili *et al*. [1998] and Packer *et al*. [1999]. The extracts of pine bark were also active in an emulsion model system [Cui *et al*., 2005]. The inhibition of lipid peroxidation in liposomes was caused by *Larus nobilis* bark extract [Simić *et al*., 2003]. The antiradical activity against DPPH• was noted for the bark extract obtained from *Betula platyphylla* var. *japonica* [Ju *et al*., 2004] and *Uncaria tomentosa* [Sandoval *et al*., 2000], and *Yucca periculosa* [Torres *et al*., 2003].

Fraction I was subjected to RP-HPLC analysis. The RP--HPLC chromatogram recorded at 280 nm was characterised by three dominant peaks with retention time of 29.45 min, 34.06 min, and 40.09 min (Figure 4). The main phenolic compound from the fraction I of *Litsea monopetala* crude extract was recorded with retention time of 34.06 min (Figure 4; peak 2). The UV spectrum of compound giving peak 1 was characterised by the maximum at 284 nm (Figure 5). Compounds 2 and 3 exhibited the same UV spectra with maxima at 282 nm and 301 nm. Interpretation of these results is difficult. Most certainly, in fraction I neither derivatives of hydroxycinnamic acid nor flavonols/flavans were present. The maximum of catechins UV spectra is close to 284 nm but the negative results of vanillin reaction (Table 1) confirm the absence of flavano-3-ols in fraction I. The same UV spectrum of compounds 2 and 3 can evidence to their similar structure. Therefore, it can be suggested that they can be positional isomers of one phenolic compound. Further work is in progress in our research laboratories to characterise these and other compounds from fractions I-IV.

REFERENCES

1. Arfan M., Amin H., Karamać M., Kosińska A., Amarowicz R., Shahidi F., Antioxidant activity of phenolic fraction of *Mallotus philipinensis* fruits and bark extract. J. Food Lipids, 2007, 14, 280–297.
2. Arfan M., Amin H., Karamać M., Kosińska A., Amarowicz R., Shahidi F., Antioxidant activity of phenolic fraction of *Mallotus philipinensis* bark extract. Czech J. Food Sci., 2008, submitted.
3. Amarowicz R., Naczk M., Shahidi F., Antioxidant activity of crude extracts of canola/rapeseed hulls. J, Am. Oil. Cem. Soc., 2000a, 77, 957–961.
4. Amarowicz R., Naczk M., Zadernowski R., Shahidi F., Antioxidant activity of condensed tannins of beach pea, canola hulls, evening primrose, and faba beans. J. Food Lipids, 2000b, 7, 195–205.
5. Amarowicz R., Karamać M., Weidner S., Abe S., Shahidi F., Antioxidant activity of wheat caryopses and embryos extracts. J. Food Lipids, 2002, 9, 201–210.
6. Amarowicz R., Pegg R.B., Rahimi-Moghaddam P., Barl B., Weil J.A., Free radical scavenging capacity and antioxidant activity of selected plant species from the Canadian prairies. Food Chem., 2004a, 84, 551–562.

7. Amarowicz R., Troszyńska A., Baryłko-Pikielna N., Shahidi F., Extracts of polyphenolics from legume seeds – correlation between their total antioxidant activity, total phenolics content, tannins content and astringency. J. Food Lipids, 2004b, 11, 278–286.
8. Amarowicz R., Troszyńska A., Shahidi F., Antioxidant activity of almond extract. J. Food Lipids, 2005, 12, 344–358.
9. Amer A., Mehlhorn H., Repellancy effect of forty-one essential oils against *Aedes*, *Anopheles*, and *Culex* mosquitoes. Parasitol. Res., 2006, 99, 478–490.
10. Bhamarapravati S., Pendland S.L., Mahady G.B., Extracts of spice and food plants from Thai traditional medicine inhibit the growth of the human carcinogen *Helicobacter pylori*. In Vivo, 2003, 17, 541–544.
11. Cheng H.-I., Lin W.-Y., Duh C.-Y., Lee K.-H., Tsai I.-L., Chen I.-S., New cytotoxic butanolides from *Litsea acutivena*. J. Nat. Prod., 2001, 64, 1502–1505.
12. Choi E.M., Hwang J.K., Effects of methanolic extract and fractions from *Litsea cubeba* bark on the production of inflammatory mediators in RAW264.7 cells. Fitoterapia, 2004, 75, 141–148.
13. Choudhury S.N., Singh R.S., Ghosh A.C., Leclercq P.A., *Litsea glutinosa* (Lour) C.B. Rob., a new source of essential oil from northeast India. J. Essential Oil Res., 1996, 8, 553–556.
14. Choudhury S.N., Ghosh A.C., Singh R.S., Choudhury M., Leclercq P.A., Essential oils of *Litsea monopetala* (Roxb.) Pers. A new report from India. J. Essential Oil Res., 1997, 9, 635–639.
15. Cui Y., Xie H., Wang J., Potential biomedical properties of *Pinus massoniana* bark extract. Phytother. Res., 2005,19, 34–38.
16. Hidayatulfathi O., Sallehuddin S., Ibrahim J., Adulticidal activity of some Malaysian plant extracts against *Aedes aegypti* Linnaeus. Trop. Biomed., 2004, 21, 61–67.
17. Hoang V.D., Tan G.T., Zhang H.J., Tamez P.A., Hung V.V., Cuong N.M., Socjarto D.D., Fong H.H., Pezzuto J.M., Natural anti-HIV agents-part I: (+)-demethoxyepiexcelsin and verticillatol from *Litsea verticillata*. Phytochem., 2002, 59, 325–329.
18. Ju E.M., Lee S.E., Hwang H.J. Kim J.H., Antioxidant and anticancer activity of extracts from *Betula plantyphylla* var. *japonica.* Life Sci., 2004, 74, 1013–1026.
19. Lee S.Y., Min B.S., Kim J.H., Lee J., Ki T.J., Kim C.S., Kim Y.H., Lee H.K., Flavonoids from the leaves of *Litsea japonica* and their anti-complement activity. Phytother. Res., 2005, 19, 273–276.
20. Luo M., Jiang L.K., Xiao M., Li B., Zou G.L., Effects of citral on *Aspergillus flavus* spores by quasi-elastic light scattering and multiplex microanalysis techniques. Acta Biochim. Biophys. Sin., 2004, 34, 277–283.
21. Mandal S.C., Kumar C.K., Majumader A., Majumader R., Maity B.C., Antibacterial activity of *Litsea glutinosa* bark. Fitoterapia, 2000, 71, 439–441.
22. Nakahara K., Trakoontivakorn G., Alzoreky N.S., Ono H., Onishi-Kameyama M., Yoshida M., Antimutagenicity of some edible Thai plants, and a bioactive carbazole alkaloid, mahanine, isolated from *Micromelum minutum*. J. Agric. Food Chem., 2002, 50, 4796–4802.
23. Oyaizu M., Studies on products of browning reaction: Antioxidative activities of products of browning reaction prepared from glucosamine. Jap. J. Nutr., 1986, 44, 307–315.
24. Packer L., Rimbach G., Virgili F., Antioxidant activity and biological properties of a procyanidin-rich extract from pine (*Pinus maritime*) bark, pycnogenol. Free Radic. Biol. Med., 1999, 27, 704–724.
25. Phukan S., Kalita M.C., Phytopesticidal and repellent efficacy of *Litsea salicifolia* (Lauraceae) against *Aedes aegypti* and *Culex quinquefasciatus*. Indian J. Exp. Biol., 2005, 43, 472–474.
26. Poonia B.S., Sasmal D., Mazumdar P.M., Anti-diarrheal activity of methanol extract of *Litsea polyantha* bark in mice. Fitoterapia, 2007, 78, 171–174.
27. Price M.L, Van Scoyoc S., Butler L.G., A critical evaluation of the vanillic reaction as an assay for tannin in sorghum grain. J. Agric. Food Chem., 1978, 26, 1214–1218.
28. Re R., Pellegrini N., Proteggente A., Pannala A., Yang M., Rice-Evans C., Antioxidant activity applying an improved ABTS radical cation decolorization assay. Free Rad. Biol. Med., 1999, 26, 1231–1237.
29. Sandoval M., Charbonnet R.M., Okuhama N.N., Roberts J., Krenova Z., Trentacosti A.M., Miller M.J.S., Cat's claw inhibits TNFα production and scavenges free radicals: role in cytoprotection. Free Radic. Biol. Medic., 2000, 29, 71–78.
30. Simić M., Kundaković T., Kovaèević N., Preliminary assay on the antioxidative activity of *Laurus nobilis* extracts. Fitoterapia, 2003, 7, 613–616.
31. Torres P., Avila J.G., de Vivar A.R., García A.M., Martín J.C., Aranda E. Céspedes C.L., Antioxidant and insect growth regulatory activities of stilbenes and extracts from *Yucca perculosa.* Phytochemistry, 2003, 64, 463–473.
32. Tsai I.-L., Yaun I.-L., Duh C.-Y., Jeng Y.-F., Chen I.-S., A new cytotoxic butanolide from the stem bark of *Litsea akoensis.* Chinese Pharm. J., 2000, 52, 235–239.
33. Virgili F., Kobuchi H., Packer L., Procyanidins extracted from *Pinus maritime* (Pycnogenol®): scavengers of free radical species and modulators of nitrogen monoxide metabolism in activated murine RAW 264.7 macrophages. Free Radic. Biol. Med., 1998, 24, 1120–1129.
34. Wang F., Yang D., Ren S., Zhang H., Li R., Chemical composition of essential oil from leaves of *Litsea cubeba* and its antifungal activities. J. Chinese Medic. Materials, 1999, 22, 400–402.
35. Zhang H.J., Tang G.T., Santarsiero B.D., Mesecar A.D., Hung N.V., Coung N.M., Soejarto D.D., Pezzuto J.M., Fong H.H., New sesquiterpenes from *Litsea verticillata*. J. Nat. Prod., 2003, 66, 609–615.
36. Zhang H.J., Nguyen V.H., Nguyen M.C., Soejarto D.D., Pezzuto J.M., Fong H.H., Tan G.T., Sesquiterpenes and butenolides, natural anti-HIV constituents from *Litsea verticillata*. Planta Med., 2005, 71, 452–457.
37. Zhao Y., Shong G.Q., Guo Y.W., Rotundifolinol, a new 1,3-diarylpropan-2-ol from bark of *Litsea rotundifolia* var. oblongifolia. J. Asian. Nat. Prod. Res., 2003, 5, 273–277.

Received January 2008. Revision received February and accepted March 2008.