

Antioxidant Activity of Phenolic Fractions of *Mallotus philippinensis* Bark Extract

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Abstract

ARFAN M., AMIN H., KARAMAĆ M., KOSIŃSKA A., WICZKOWSKI W., AMAROWICZ R. (2009): **Antioxidant activity of phenolic fractions of *Mallotus philippinensis* bark extract.** Czech J. Food Sci., 27: 109–117.

Phenolic compounds were extracted from *Mallotus philippinensis* bark using methanol. Six fractions (I–VI) were separated from the extract on a Sephadex LH-20 column using ethanol and acetone-water as the mobile phases and were 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 in the fractions were determined. The content of total phenolics in the fractions ranged from 54 mg/g (fraction I) to 927 mg/g (fraction VI). Condensed tannins were detected in fractions II–VI. Total antioxidant activity (TAA) of phenolic fractions of *Mallotus philippinensis* bark extract ranged from 0.58 mmol Trolox/g (fraction I) to 6.82 mmol Trolox/g (fraction IV). Fraction IV also showed the strongest antiradical activity against DPPH• and reducing power. Several phenolic constituents in the fractions were detected by RP-HPLC using a gradient solvent system with UV-DAD detection.

Keywords: *Mallotus philippinensis*; bark; extract; natural antioxidants; phenolic compounds; tannins; antioxidant activity; antiradical activity

The plants of the genus *Mallotus* are a rich source of biologically active compounds such as phloroglucinols, tannins, terpenoids, coumarins, benzopyrans, and chalcones (AMAKURA & YOSHIDA 1996; TANAKA *et al.* 1998; CHENG & CHEN 1999; HUANG *et al.* 1999; AN *et al.* 2001, 2003; CHUNG *et al.* 2002; VAN KIEM *et al.* 2004; SUPUDOMPOL *et al.* 2004; WEI *et al.* 2004; MA *et al.* 2004; LIKHITWITAYAWUID & SUPUDOMPOL 2005). Biological activities of the extracts of the plants belonging to the genus *Mallotus*, or of individual chemical constituents isolated from these extracts, have been reported by several authors. The aque-

ous extract of *Mallotus philippinensis* showed a strong antipyretic effect similar to that of paracetamol (CHATTOPADHYAY *et al.* 2002). ISHII *et al.* (2003) reported that phloroglucinol derivatives of *Mallotus japonicus* had been shown to inhibit pro-inflammatory cytokine production. They exhibited the pharmacological ability to suppress prostaglandin E-2 production by activated macrophages (ISHII *et al.* 2002), and reduced nitric oxide production by a murine macrophage-like cell line (ISHII *et al.* 2001). Bergenin, which is the major constituent of *Mallotus japonicus* (Figure 1) was reported as hepatoprotective against hepatotoxicity in rats

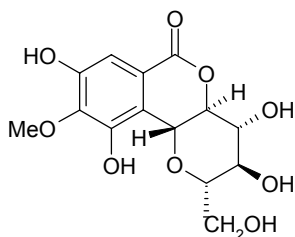


Figure 1. Chemical structure of bergenin

induced carbon tetrachloride (KIM *et al.* 2000). Lipophilic acetylbergenin isolated from *Mallotus japonicus* showed to possess more hepatoprotective activity than the much less lipophilic bergenin (LIM *et al.* 2000, 2001a, b). *Mallotus philippinensis* Muell. (*Euphorbiaceae*), also known as kamala or kamopollaka, was used as a source of a yellow dye (kamala dye), and as an antioxidant for ghee and vegetable oils (RAO & SESHADRI 1947)

In our previous study (ARFAN *et al.* 2007), several extracts of *Mallotus philippinensis* fruits and bark were prepared and evaluated for their total antioxidant activity, antiradical activity against DPPH• (2,2-diphenyl-1-picrylhydrazyl radical), and reducing power. The extracts prepared from the bark offered the strongest antioxidant activity.

At this moment, the application of botanicals and botanical preparations in the European Union in foods is prohibited (European Food Safety Authority 2004). On the other hand, in Asian countries such as Pakistan and India, the food legislation is not so restricted as in Europe and, therefore, *Mallotus philippinensis* bark extract can find applications as a natural antioxidant for food, an ingredient of functional foods, and nutraceutical.

The present study was aimed at the investigation of the possible *in vitro* antioxidant effects of the phenolic fractions isolated from the bark of *Mallotus philippinensis* using column chromatography.

MATERIALS AND METHODS

Material. All solvents used were of analytical grade. Methanol, acetone, ethanol, acetonitrile, potassium ferricyanide, and trichloroacetic acid were acquired from the P.O.Ch. Company (Gliwice, Poland), (+)catechin, vanillin, Folin & Ciocalteu's phenol reagent, Sephadex LH-20, 2,2'-diphenyl-1-picrylhydrazyl radical (DPPH•), 2,2'-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS),

6-hydroxy-2,5,7,8-tetramethychroman-2-carboxylic acid (Trolox), and trifluoroacetic acid (TFA) were obtained from Sigma Ltd. (Poznań, Poland). *Mallotus philippinensis* bark was collected from the Buner area in N-W.F.P, Pakistan.

Extraction. *Mallotus philippinensis* bark was ground in a coffee mill. Phenolic compounds were extracted from the powdered material using methanol maceration (4 l for 12 h; at room temperature). The extraction was repeated twice more, the supernatants were combined, and methanol was evaporated under vacuum at 45°C using a rotary evaporator. The crude extract prepared (48 g) was stored at –20°C until used.

Column chromatography. A 0.5 g portion of the crude extract was dissolved in 8 ml of ethanol and applied to a chromatographic column (2 × 60 cm) packed with Sephadex LH-20, and subsequently eluted with 95% (v/v) ethanol. The fractions containing low-molecular weight phenolic compounds (5 ml) were collected using a fraction collector and their absorbance was measured at 280 nm. The tannin fraction was eluted from the column with acetone-water (1:1; v/v) (STRUMEYER & MALIN 1975). The eluates were then pooled into major fractions. Organic solvents were evaporated at 45°C using rotary evaporator and the water solution of the tannin fraction was lyophilised.

Total phenolics. The content of total phenolics in the fractions was determined according to the procedure described by AMAROWICZ *et al.* (2004b) using the Folin-Ciocalteu's reagent. (+)Catechin was used as the standard in this work.

Condensed tannins. The content of condensed tannins in the fractions 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 fraction (A_{500}/mg).

The condensed tannins present in the Sephadex LH-20 fractions were also hydrolysed according to the method described by PORTER *et al.* (1986). Briefly, into a 10 ml screw cap tube 6 ml of the acid butanol reagent (950 ml of *n*-butanol with 50 ml concentrated HCl), 1.0 aliquot of the extract, and 0.2 ml of the iron reagent (2% ferric ammonium sulfate in 2M HCl) were added and vortexed. The tube was capped loosely and put in a boiling water bath for 50 minutes. The tube was then cooled and the solution was transferred to a volumetric flask and made up to 25 ml with acid butanol. The absorbance at 550 nm was recorded using a Beckman DU 7500 diode array spectrophotometer

Table 1. Contents of total phenolics and condensed tannins in phenolic fractions of *Mallotus philippinensis* bark extract

Fraction	Total phenolics ^a (mg/g)	Condensed tannins ^b (A ₅₀₀ /mg)	Condensed tannins ^c (A ₅₅₀ /mg)
I	54 ± 1	–	–
II	357 ± 8	0.021 ± 0.001	0.047 ± 0.002
III	719 ± 17	0.972 ± 0.022	0.379 ± 0.002
IV	856 ± 20	1.25 ± 0.027	0.685 ± 0.007
V	794 ± 19	1.113 ± 0.023	1.220 ± 0.014
VI	927 ± 22	1.321 ± 0.030	1.745 ± 0.018

^areported as (+)-catechin equivalents; ^bdetermined using vanillin method; ^cdetermined after *n*-butanol/HCl hydrolysis

(Beckman Instruments, Fullerton, CA). The results were expressed as absorbance units at 550 nm per 1 mg of fraction (A₅₅₀/mg).

Total antioxidant activity. The total antioxidant activity (TAA) in the extracts 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 to the procedure described by AMAROWICZ *et al.* (2002). Briefly, 0.1 ml of methanolic solution containing different amounts of the fractions was mixed with 2 ml of methanol and then 0.25 ml of 1mM methanolic solution of DPPH radical was added. The mixture was vortexed thoroughly for 1 minute. Finally, the absorbance of the mixture was read at 517 nm after standing at ambient temperature for 30 minutes.

Reducing power. The reducing power of the fractions was determined by the method of OYAIU (1986). Briefly, the assay medium contained 2.5 ml of the respective fraction in 0.2M 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 minutes. The supernatant (2.5 ml) was mixed with 2.5 ml of 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 fractions I–VI were analysed using a Shimadzu HPLC system (Shimadzu Corp., Kyoto, Japan) consisting of two LC-10AD pumps, SCL 10A system control-

ler, and SPD-M 10A photodiode array detector. Chromatography was carried out using a pre-packed LiChrospher 100 RP-18 column (4.6 × 250 mm, 5 µm; Merck, Darmstadt, Germany). The elution for 50 min in a gradient system of 5–40% acetonitrile in water adjusted to pH 2.5 with TFA was employed. The detector was set at 280 nm; the injection volume was 20 µl and the flow rate was 1 ml/minute.

Semi-preparative HPLC. The main phenolic compound from fraction II was separated using a semi-preparative Hibar pre-packed RT column (10 × 250 mm) with Lichrosorb RP-18 (7 µm) (Merck, Darmstadt, Germany). The mobile phase water-acetonitrile-acetic acid (88:10:2; v/v/v) (AMAROWICZ & WEIDNER 2001) was delivered at a rate of 4 ml/min using an injection volume of 500 µl. The detection was monitored at 280 nm.

ESI-MS. The pure compound separated from the bark extract was dissolved in 80% methanol (v/v) and applied on a column of Shimadzu liquid chromatograph mass spectrometer LC MS–QP 8000 system (Kyoto, Japan). The conditions of analysis were as follows: Luna 3 µ C (2) (2 × 150 mm) (Phenomenex; St. Torrance, CA, USA) column, mobile phase of 80% methanol (v/v), flow rate 0.2 ml/min; injection loop 10 µl; CDL temperature: 230°C; CDL voltage: +85 V; probe voltage: +4.5 kV, defragmentation voltage: +45 V; nebuliser gas flow 4.5 ml/minute.

RESULTS AND DISCUSSION

Six fractions (I–VI) containing phenolic compounds were obtained from the methanol extract of *Mallotus philippinensis* bark using Sephadex LH-20 column chromatography (Figure 2). Five fractions

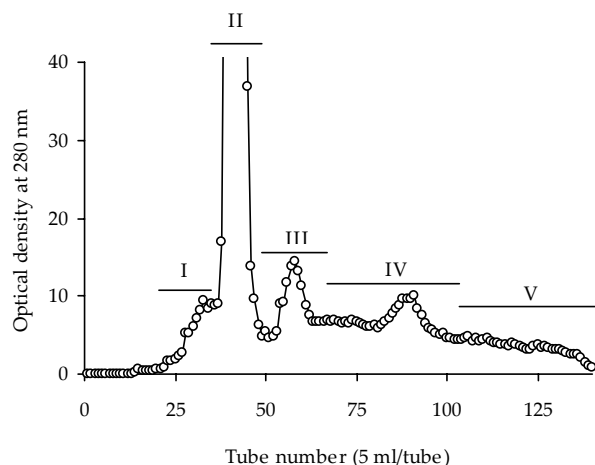


Figure 2. Separation of phenolic compounds from *Mallotus philippinensis* bark extract on a Sephadex LH-20 column with ethanol as the mobile phase

(I–V) were obtained with ethanol as the mobile phase. Fraction VI was eluted from the column with acetone-water (1:1, v/v). The content of total phenolics in individual fractions (Table 1) ranged from 54 mg/g (fraction I) to 927 mg/g (fraction VI). The content of total phenolics in the fractions separated from the *Mallotus philippinensis* bark crude extract was high. The results were several times higher than those reported previously for the tannin-rich extracts from leguminous and oil seeds, as well as seed hulls (AMAROWICZ *et al.* 2000a, b, 2004a). Condensed tannins were detected in fractions II–VI. Fraction VI was characterised by the highest content of condensed tannins. In fraction II, only trace quantities of condensed tannins were detected. The positive results of the vanillin reaction and colorimetric assay after *n*-butanol/HCl hydrolysis in fractions III–V confirm the presence of catechins or dimers/trimers of this compound, which is in accordance with the findings obtained by Sephadex LH-20 column chromatography with ethanol and acetone-water as the mobile phases (STRUMEYER & MALIN 1975; AMAROWICZ & SHAHIDI 1995). The high values found in the two assays used for the detection of condensed tannins are typical for this kind of column chromatography. The polarity of 50% acetone is sufficient to elute from the column condensed tannins with a high degree of polymerisation. The contents of condensed tannins in the fractions separated from the *Mallotus philippinensis* bark crude extract were high. Much lower values were obtained when the extracts of tannin-rich plant materials such as leguminous seeds, canola hulls,

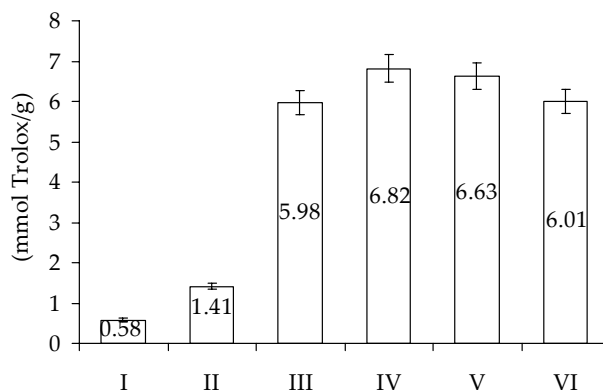


Figure 3. Total antioxidant activity (TAA) of phenolic fractions of *Mallotus philippinensis* bark extract (mmol Trolox/g)

and primrose seeds were separated using the same method (AMAROWICZ *et al.* 2000a, 2004a).

Total antioxidant activity (TAA) of phenolic fractions of *Mallotus philippinensis* bark extract ranged from 0.58 mmol Trolox/g (fraction I) to 6.82 mmol Trolox/g (fraction IV) (Figure 3). The values of TAA in fractions III–VI were several times higher than those reported in the extracts of phenolic compounds or their fractions separated from tannin-rich plant materials such as leguminous seeds, almonds, and hazelnuts (AMAROWICZ *et al.* 2004a, 2005; ALASALVAR *et al.* 2006). In our previous study (ARFAN *et al.* 2007), the extract obtained from the *Mallotus philippinensis* fruit using acetone exhibited the total antioxidant capacity of 1.54 mmol Trolox/g.

The fractions separated from the *Mallotus philippinensis* bark crude extract were investigated for the antiradical activity and reducing power. A freshly prepared DPPH• solution exhibits a deep purple colour with an absorption maximum at 517 nm. This colour disappears when an antioxidant is added to the assayed solution. For the reducing power assay, the presence of reductants (i.e. antioxidants) in the extracts causes reduction of the Fe³⁺/ferricyanide complex to the Fe²⁺ state. Therefore, it can be monitored by measuring the formation of Perl's Prussian blue coloration having a maximum absorbance at 700 nm.

Figure 4 depicts the concentration-dependent response curves of the fractions of *Mallotus philippinensis* bark extracts for the antiradical activity against DPPH•. The results are expressed as the decrease in the absorbance of the DPPH• solution at 517 nm. The antiradical activities of fractions III–VI were several times higher than

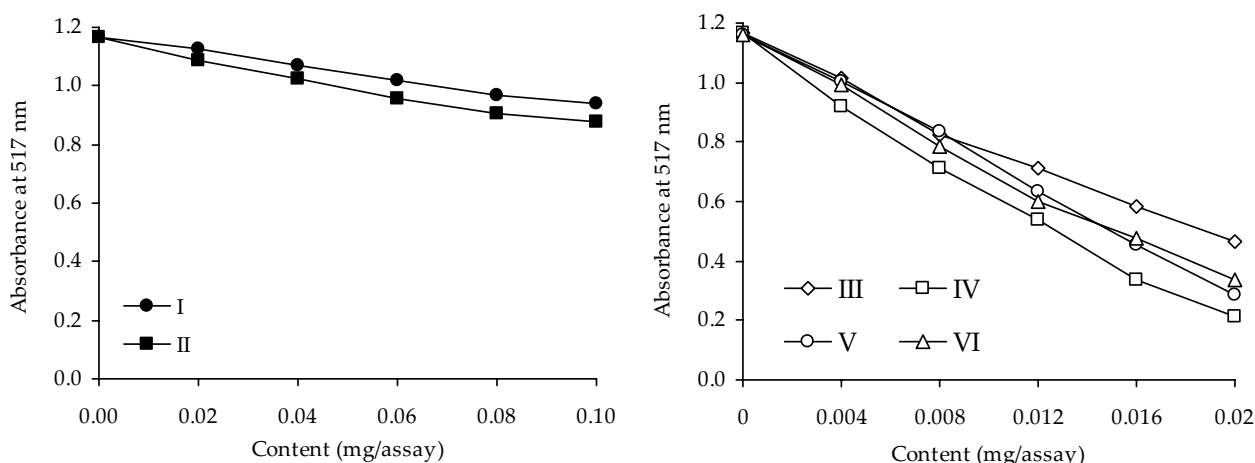


Figure 4. Scavenging effect of phenolic fractions of *Mallotus philippinensis* bark extract on the 2,2-diphenyl-1-picrylhydrazyl radical (DPPH[•]), as measured by changes in absorbance at 517 nm

those of fractions I and II. Like in the case of total antioxidant activity, fraction IV was found as the strongest scavenger of DPPH[•]. Fraction IV also exhibited the strongest reducing power that was ~10 times higher than those observed in fractions I and II (Figure 5).

Antiradical activity and reducing power in fractions III–VI were similar to those of the extract from the leaves of bearberry (*Arctostaphylos uva-ursi*), which is also a rich source of condensed tannins (AMAROWICZ *et al.* 2004b). A strong antiradical activity against DPPH[•] is typical for the tannins-rich plant extracts (MUIR 1995; CHEN & CHANG 1995; AMAROWICZ *et al.* 1999, 2000a, b; SHAHIDI *et al.* 1997, 2001).

The antioxidant and antiradical properties of plant bark have been reported in several studies. CUI *et al.* (2005) noted that the addition of pine (*Pinus*

massoniana) bark extract to a linoleic acid emulsion acted as a strong inhibitor of the peroxidation process. Procyanidins obtained from pine bark showed a strong antiradical activity (VIRGILI *et al.* 1998; PACKER *et al.* 1999). SIMIĆ *et al.* (2003) described the inhibition of lipid peroxidation in liposomes, induced by Fe²⁺/ascorbate system of *Larus nobilis* bark extract. The methanol extract from *Yucca periculosa* demonstrated scavenging properties towards DPPH[•] in TLC autographic and spectrophotometric assays (TORRES *et al.* 2003). The extract of cat's claw (*Uncaria tomentosa*) bark was an effective scavenger of DPPH[•] (SANDOVAL *et al.* 2000). JU *et al.* (2004) reported the extract from the dried bark of *Betula platyphylla* var. *japonica* as a strong DPPH[•] scavenger and lipid peroxidation inhibitor.

The RP-HPLC chromatograms of the phenolic fractions of *Mallotus philippinensis* bark extract

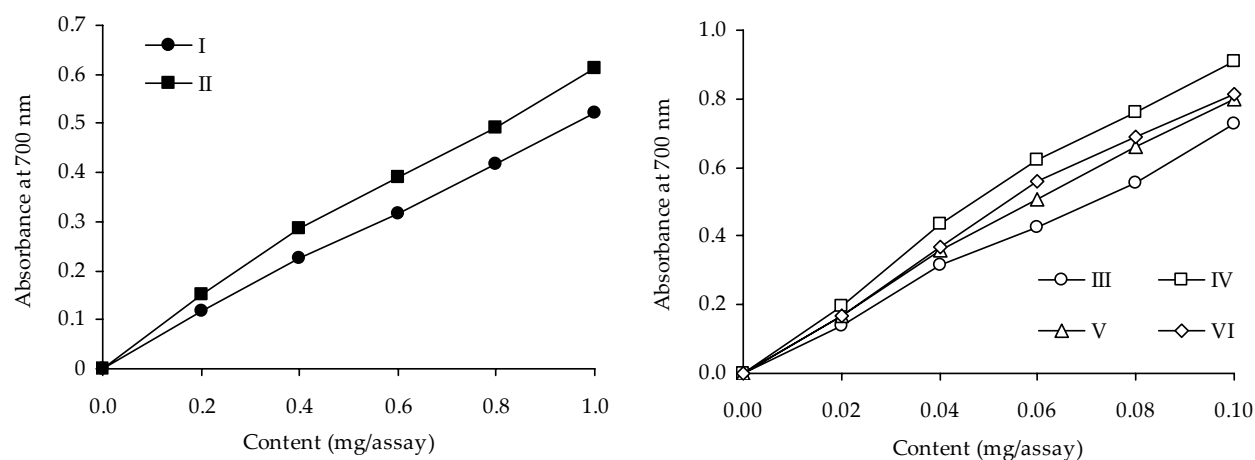


Figure 5. Reducing power of phenolic fraction of *Mallotus philippinensis* bark extracts, as measured by changes in absorbance at 700 nm

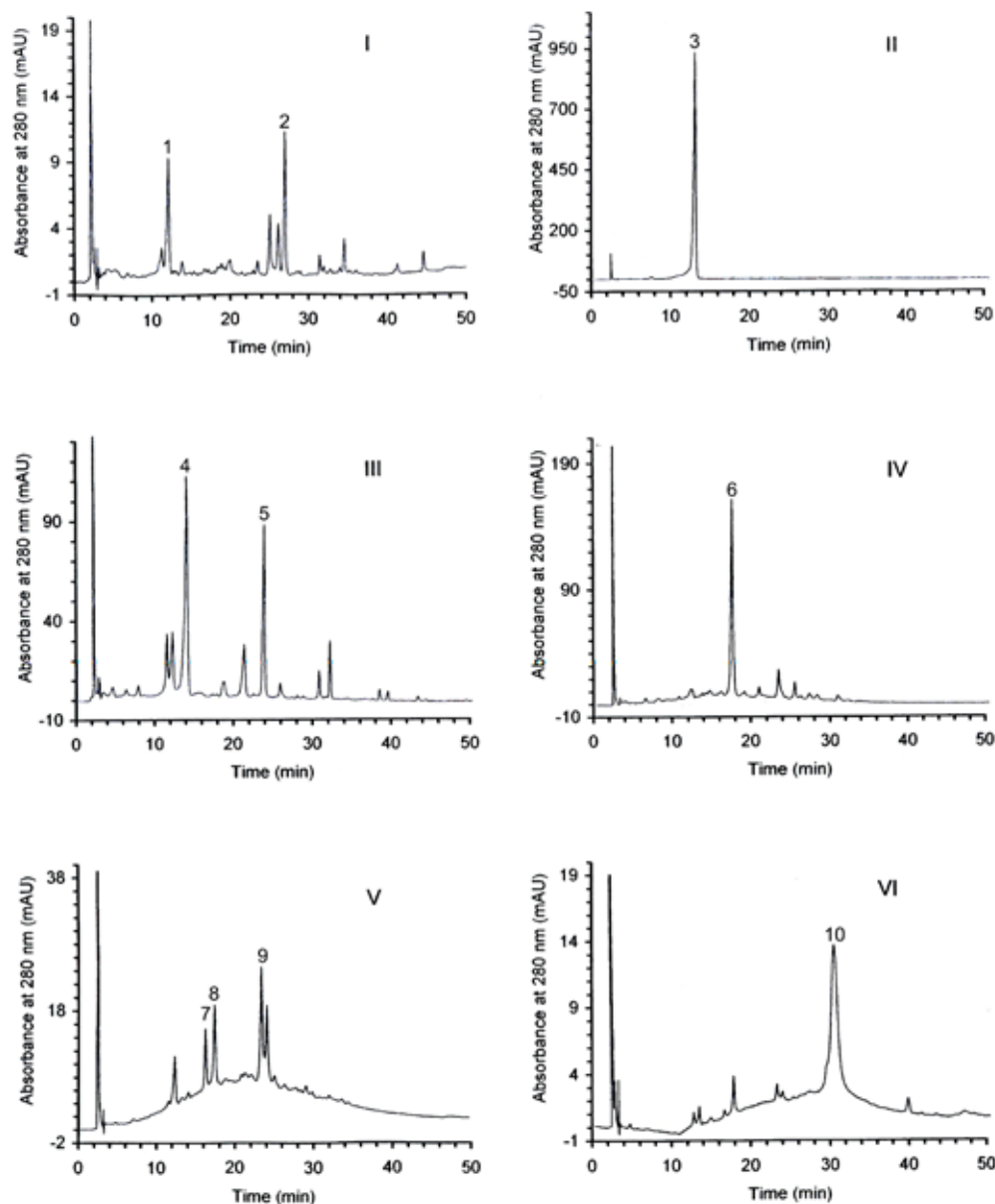


Figure 6. HPLC chromatograms of phenolic fractions of *Mallotus philippinensis* bark extract

were characterised by ten (1–10) dominant peaks (Figure 6). Three main peaks were recorded on the chromatogram as coming from fraction V, two peaks from fraction I, and two from fraction III. On the chromatograms of fractions II, IV, and VI, only one main peak was observed. The lack of a flat base line recorded on the chromatograms of fractions V and VI confirms the presence of condensed tannins in these fractions. The compounds giving peaks 1 and 3 in the chromatograms were characterised by an absorption maximum at 272 nm and a shoulder at 306 nm. At 272 nm, a maximum was recorded with the compound giving peak 8, and at 278 nm with peaks 5 and 7. The compounds attributed to peaks 4, 6, and 9 were characterised by maxima of UV spectra at 274, 278, and 276 nm,

respectively. The maxima of UV spectra of compounds 2 and 10 were shifted towards a longer wavelength and recorded at 282 nm (2), 282 and 302 nm (10). The UV spectra show the absence of hydroxycinnaminates, flavonols and flavanols in the fractions. UV spectra of those phenolic compounds are characterised by the maxima at wavelengths of 320–330 nm (phenolic acids) and 340–350 nm (flavonols and flavanols) (WEIDNER *et al.* 1999; MABRY *et al.* 1970). The strong absorption band at 278 and 282 nm (peak 2 and 5) can be attributed to catechins or procyanidins (AMAROWICZ & SHAHIDI 1995).

The ESI-MS spectrum in the positive mode of the compound separated from the *Mallotus philippinensis* bark using a semi-preparative HPLC

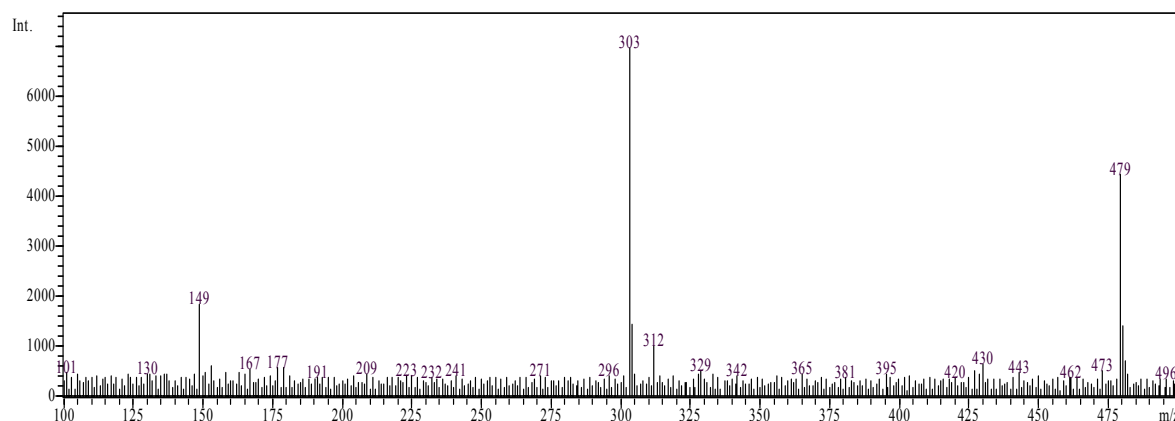


Figure 7. ESI-MS spectrum of the main compound separated from fraction II of the *Mallotus philippinensis* bark ex-

showed a $[M+H]^+$ at an m/z of 479 and dominant peaks of 303 and 304 where the glucuronic acid moiety was cleaved from the glycoside (Figure 7). Mass spectra indicated that the compound has a molecular weight of 478. Molecular weight of the aglycon was calculated to be 303. Most probably it was a derivative of bergenin whose MW is 328 (TAKAHASHI *et al.* 2003).

CONCLUSION

From the results achieved it can be concluded that the fractions separated from the *Mallotus philippinensis* possess strong antioxidant and antiradical properties. After toxicological studies on some eventual harmful compounds present in the extract or its fractions, we suggest to use this material as natural antioxidant for food, functional foods, or nutraceuticals. Because of the presence of condensed tannins in *Mallotus philippinensis* bark, sensory studies are needed before practical applications.

Acknowledgements. One of the authors (M. Arfan) acknowledges the research grant obtained from the University of Peshawar own resources for carrying out part of this research.

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Received for publication November 14, 2007

Accepted after corrections April 6, 2009

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