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LC–DAD–ESIMS/MS characterization of antioxidant and anticholinesterase constituents present in the active fraction from *Persicaria hydropiper*

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ABSTRACT

Persicaria hydropiper or 'kesum' is a herb used extensively as flavoring agent in food. The antioxidant activity was evaluated by measuring the hydroperoxide production resulting from linoleic acid oxidation using ferric thiocyanate (FTC) as well as the 1,1-diphenyl-2-picrylhydrazyl (DPPH) free radical scavenging ability. Moreover, inhibition activity against cholinesterase, an enzyme that responsible in several physio-pathological processes, was also determined. Identification of phytochemical constituents in the bioactive fraction of *P. hydropiper* was carried out by LC–DAD–ESIMS/MS technique. Fifteen compounds were identified including flavonoids, flavonoid glycosides and phenylpropanoid glycosides. Six of the compounds were isolated and their structures were elucidated in order to confirm their identities. The antioxidant and antiacetylcholinesterase activities of the isolated compounds were also evaluated.

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1. Introduction

Persicaria hydropiper (syn. *Polygonum minus* or *P. hydropiper*), locally known as 'kesum', is a herbaceous plant of the family Polygonaceae (Aronson, 2006). It is well known spice in Southeast Asian foods, especially by the Malays as flavor enhancer in some of the traditional dishes such as Laksa, due to its strong peppery taste (Peng et al., 2003). In Japan, China, and Europe, *P. hydropiper* has long been used as a hot-tasting spice. The sprouts of *P. hydropiper* is a Japanese traditional vegetables and is used mostly in 'sashimi' (Miyazawa & Tamura, 2007). Apart from that, *P. hydropiper* is also known for its medicinal uses. In traditional medicine, the juice of the leaves is used for headache, pain, toothache, gastric ulcer, dysentery, loss of appetite and dismenorrhoea (Cotelle et al., 1996; Rahman, Goni, Rahman, & Ahmed, 2002). Previous studies on *Persicaria* species reported a diverse array of compounds including flavonoids, chalcones, anthraquinones, napthoquinones, sesquiterpenoids, lignans, coumarins and stilbene glycosides (Calis, Demirezer, Sticher, Ganci, & Ruedi, 1999; Datta, Datta, Rashid, Nash, & Sarker, 2000; Datta, Datta, Rashid, & Sarker, 2002; Fukuyama, Sato, Miura, & Asakawa, 1985; Furuta, Fukuyama, & Asakawa, 1986; Manoharan, Benny, & Yang, 2005; Peng et al., 2003; Sun, Zimmermann, Campagne, & Sneden, 2000; Takasaki, Konoshima, Kuroki, Tokuda, & Nishino, 2001; Xiao, Xuan, Xu, & Bai, 2000).

Many health promoting effects have been ascribed to the action of antioxidant compounds (Cai, Luo, Sun, & Corke, 2004; Heim, Tagliaferro, & Bobilya, 2002; Jang et al., 2007; Sharma & Bhat, 2009). Free radicals or reactive oxygen species have been shown to be the causative agent in aging and several degenerative diseases such as cancer, atherogenesis, heart and neurodegenerative diseases (Schinella et al., 2010). Although the production of free radicals is an integral part of a normal metabolism, it still needs to be adequately controlled so as not to damage the structures and function of cells. For this purpose, humans rely on antioxidants produced by the body and those obtained from their diet. Apart from the well known antioxidants such as vitamins A, C, E and carotenoids, plant polyphenols (flavonoids, catechins, phenolic acids, coumarins, anthocyanins and tannins) have also been suggested to play an important role in the defense mechanism (Larson, 1988). These compounds are present in abundant in various plant foods (vegetables, cereals and legumes) and beverages such as herbal teas, coffee and cocoa (Moure et al., 2001; Schinella et al., 2010; Zhou et al., 2011). On the other hand, antioxidant activity has also been suggested to have significant role in the treatment of Alzheimer's disease (Chauhan & Chauhan, 2006; Resende et al., 2008). Hyphenated techniques such as LC–MS and LC–NMR,

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have now become the method of choice in metabolite profiling as well as increasing the throughput of biological screening process, in search of bioactive compounds in complex plant extracts (Wolfender, Rodriguez, & Hostettmann, 1998). The technique yields information about the molecular weight as well as the structure of the analytes using MS/MS or MS^n capabilities of the mass analyzer. Tandem mass spectrometry (MS^n) is one of the most sensitive methods for molecular analysis and very useful for distinguishing compounds with identical molecular weights. It also offers better selectivity due to its high mass-separation power. In the absence of standard reference compounds, the technique still allows for tentative identification of the analyte by making use of the UV spectrum of the analyte (Maillard, Wolfender, & Hostettmann, 1993). The usefulness of LC–DAD–MS/MS has been reported in numerous applications (Abas et al., 2010; Breksa Iii, Hidalgo, & Yuen, 2009; Nuengchamnong & Ingkaninan, 2009).

Previously we reported the crude methanolic extract of *P. hydropiper* exhibited strong antioxidant activity (Abas, Lajis, Israf, Khozirah, & Umi Kalsom, 2006). Considering the traditional uses and popularity among the locals, we further report the chemical profile of the active fraction using negative ion LC–DAD–$ESIMS^n$. In addition to antioxidant activity of fractions, the *in vitro* anticholinesterase activity of the fractions is described for the first time, providing further knowledge on this species chemistry and biological potential. The antioxidant and antiacetylcholinesterase activities of the compounds were also evaluated.

2. Materials and methods

2.1. Chemicals and reagents

All chemicals used were of analytical grade; solvents were of HPLC grade and purchased from Merck (Darmstadt, Germany). Ethanol was purchased from Scharlau Chemie S.A (Sentmenat, Spain). Redistilled reagent grade hexane, dichloromethane, ethyl acetate and methanol were used for extraction and separation of compounds. Water was purified by a Milli-Q system (Millipore, Bedford, MA, USA). Formic acid (Fisher, Loughborough, UK) was used as buffer. Linoleic acid, ascorbic acid, α-tocopherol, DPPH, ammonium thiocyanate, ferrous chloride, tacrine, acetylcholinesterase from electric eel (EC 3.1.1.7; Sigma C 2888), horse serum butyrylcholinesterase (EC 3.1.1.8; Sigma), DTNB (Dithio-bis-(2-nitrobenzoic acid)), acetylthiocholine iodide, butyrylthiocholine chloride, NaH_2PO_4 and Na_2HPO_4 were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Standard compounds such as quercetin and kaempferol were purchased from Extrasynthese (Genay, France). Column chromatography utilized silica gel 60 (7734 and 9385, Merck, Darmstadt, Germany).

2.2. Plant materials and sample preparation

P. hydropiper was obtained from the medicinal plant nursery of the Laboratory of Natural Products, Universiti Putra Malaysia. A voucher specimen (SK154/02) was deposited at the herbarium of the Laboratory of Natural Products. The leaves (800 g) were cut into small pieces and air-dried under the shade. The samples were then ground into fine powder and extracted for three times with methanol, each time by soaking in 1.5 l of solvent for overnight. The extracts were evaporated under reduced pressure to give 220 g of methanolic extract. A portion of the extract (78 g) was redissolved in 200 ml of water:MeOH (3:1, v/v) mixture and fractionated with hexane, dichloromethane, ethyl acetate and butanol (3 × 200 ml each fraction). The fractionation afforded five different fractions (hexane, dichloromethane, ethyl acetate, butanol and aqueous) which were then subjected to bioassays. The bioactive fraction was further analyzed by HPLC–DAD–ESIMS/MS. Samples for the analysis were prepared by dissolving 10 mg of the active fraction in 10 ml MeOH and then filtered with a C_{18}, Sep-Pak catridges (Waters, Milford, USA). The sample size for analysis was 20 μl.

2.3. Instrumentation

Mass spectra were acquired using Thermo Finnigan model LCQ^{DECA} (San Jose, CA) ion-trap mass spectrometer equipped with an ESI source. The instrument was coupled to a Surveyor HPLC binary pump, Surveyor diode array detector (DAD) (200–600 nm range; 5 nm bandwidth) and Surveyor autosampler. The hyphenated system was supported with an Xcalibur 1.2 and Mass Frontier 5.0 software. Analyte separation was carried out on a Hypersil GOLD C_{18} column (3 μm, 150 mm × 2.1 mm) with a gradient mobile phase comprising acetonitrile (solvent A) and water (solvent B), each containing 0.1% formic acid. The gradient program commenced from 10:90 (v/v) until 100:0 (v/v) of A:B over 65 min with a flow rate of 250 μl/min. The negative ion mass spectra were obtained from the LCQ^{DECA} ESI/MS detector on full ion scan mode (50–1000 amu) at a scan rate of 0.5 Hz and the capillary temperature was set to 275 °C. A data-dependent program was used in the liquid chromatography-tandem mass spectrometry analysis so that the most abundant ions in each scan were selected and subjected to MS/MS analysis. The collision-induced dissociation (CID) energy was adjusted to 35%. DPPH radical-scavenging activity and anticholinesterase activity were measured using a Spectramax Plus (Molecular Devices) UV/Vis Spectrophotometer. HPLC–DAD quantitative analyses were performed using a same Surveyor LC pump coupled with Surveyor DAD (Thermo Finnigan San Jose, CA). The experimental conditions (solvent gradient, DAD channel and columns) were the same as described above. The injection volume was 20 μl. Quantitative determination was carried using calibration curves of standards. Quercetin and quercetin-3-*O*-rhamnoside were selected as the external standards of calibration for flavonoids. Standard calibration curves were prepared in a concentration range 0.01–0.06 mg/ml with six different concentration levels. Triplicate injections were made for each level, and weight linear regression was generated. The amount of the compound was finally expressed in mg/100 g of extract.

2.4. Antioxidant activity

The ferric thiocyanate (FTC) lipid peroxidation inhibition and 1,1-diphenyl-2-picrylhydrazyl (DPPH) free radical-scavenging assays

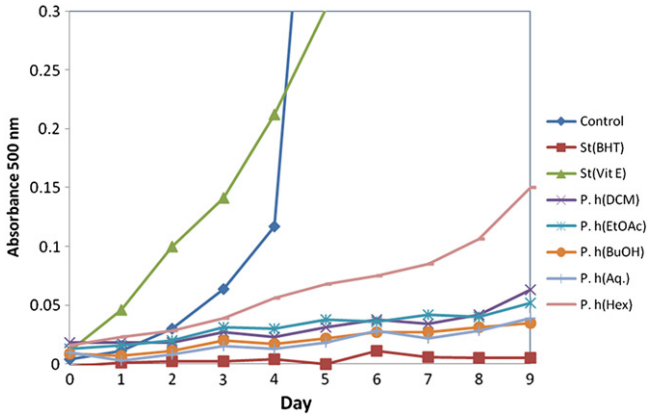


Fig. 1. Lipid oxidation using FTC method. Low absorbance value indicated strong antioxidant activity, (each sample, final concentration 0.2 mg/ml). Each experiment was performed at least twice in triplicates.

Table 1
Free radical-scavenging and anticholinesterase activities of methanolic extract and fractions of *Persicaria hydropiper*.

Sample	IC_{50} value (μg/ml)		
	AChE	BChE	DPPH
Methanol extract	278.87 ± 1.15[a]	839.56 ± 2.07[a]	36.20 ± 0.04[a]
Hexane fraction	362.6 ± 3.24[b]	544.64 ± 0.63[b]	31.25 ± 0.03[b]
Dichloromethane fraction	276.1 ± 1.01[a]	NA	36.45 ± 0.10[a]
Ethyl acetate fraction	231.93 ± 1.38[c]	268.97 ± 2.61[c]	13.30 ± 0.02[c]
Butanol fraction	234.1 ± 1.12[c]	496.24 ± 3.16[d]	17.93 ± 0.08[c]
Aqueous fraction	405.7 ± 0.50[d]	NA	93.75 ± 0.02[d]
Tacrine	9.73 ± 1.10[e]	18 ± 2.41[e]	–
Vitamin C	–	–	5.9 ± 0.04[e]
Quercetin	–	–	4.8 ± 0.01[e]

'–': not tested; NA: not available.
Values with the same lowercase within each column are not significantly different ($p > 0.05$) ($n = 3 \pm$ SD).

were carried out according to previously described protocols (Kikuzaki & Nakatani, 1993). Results are reported as mean ± SEM values. All *in vitro* experiments were conducted thrice, each time with three or more independent observations.

2.5. Anticholinesterase activity

Acetyl- (AChE) and butyrylcholinesterase (BChE) inhibitory activities were assessed, by slightly modifying the spectrophotometric method developed by Ellman, Courtney, Andres, and Featherstone (1961). Electric eel AChE and horse serum BChE were used, while acetylthiocholine iodide and butyrylthiocholine chloride were employed as substrates of the reaction. Briefly, 210 μl of DTNB (0.15 mmol/l in 0.1 mol/l phosphate buffer pH 7.4), 20 μl of AChE (0.5 U/ml in buffer) or BChE (0.04 U/ml in buffer) and 20 μl of test solution (samples were dissolved in DMSO) were mixed and incubated for 10 min at 25 °C. For controls, test solutions were replaced by the corresponding volume of DMSO or buffer. The reaction was then initiated by the addition of 20 μl of acetylthiocholine iodide (0.25 mmol/l) or butyrylthiocholine chloride (0.25 mmol/l). The final volume was 270 μl. The hydrolyzes of these substrates were monitored spectrophotometrically by the formation of yellow 5-thio-2-nitrobenzoate anion, as the result of enzyme-catalyzed reaction of DTNB with thiocholine, released by the enzymatic hydrolysis of acetylthiocholine iodide or butyrylthiocholine chloride, respectively, using a 96-well microplate reader (SpectraMax Plus, Molecular Devices, USA) at a wavelength of 412 nm. The rates of reaction were obtained over 180 s, with a 20 s interval. The measurements and calculations were evaluated by using Softmax PRO 5.2 software. Percentage of inhibition of AChE or BChE was determined by comparison of reaction rates of samples relative to blank sample (DMSO in phosphate buffer, pH 7.4) using the formula $(E - S)/E \times 100$, where E is the activity of enzyme without test sample, and S is the activity of enzyme with test sample. The experiments were carried out in triplicate. Tacrine was used as reference compound.

2.6. Isolation of compounds from the ethyl acetate fraction

The EtOAc fraction (4.73 g) was fractionated on a normal phase column chromatography (3 cm × 30 cm) with gradual elution of

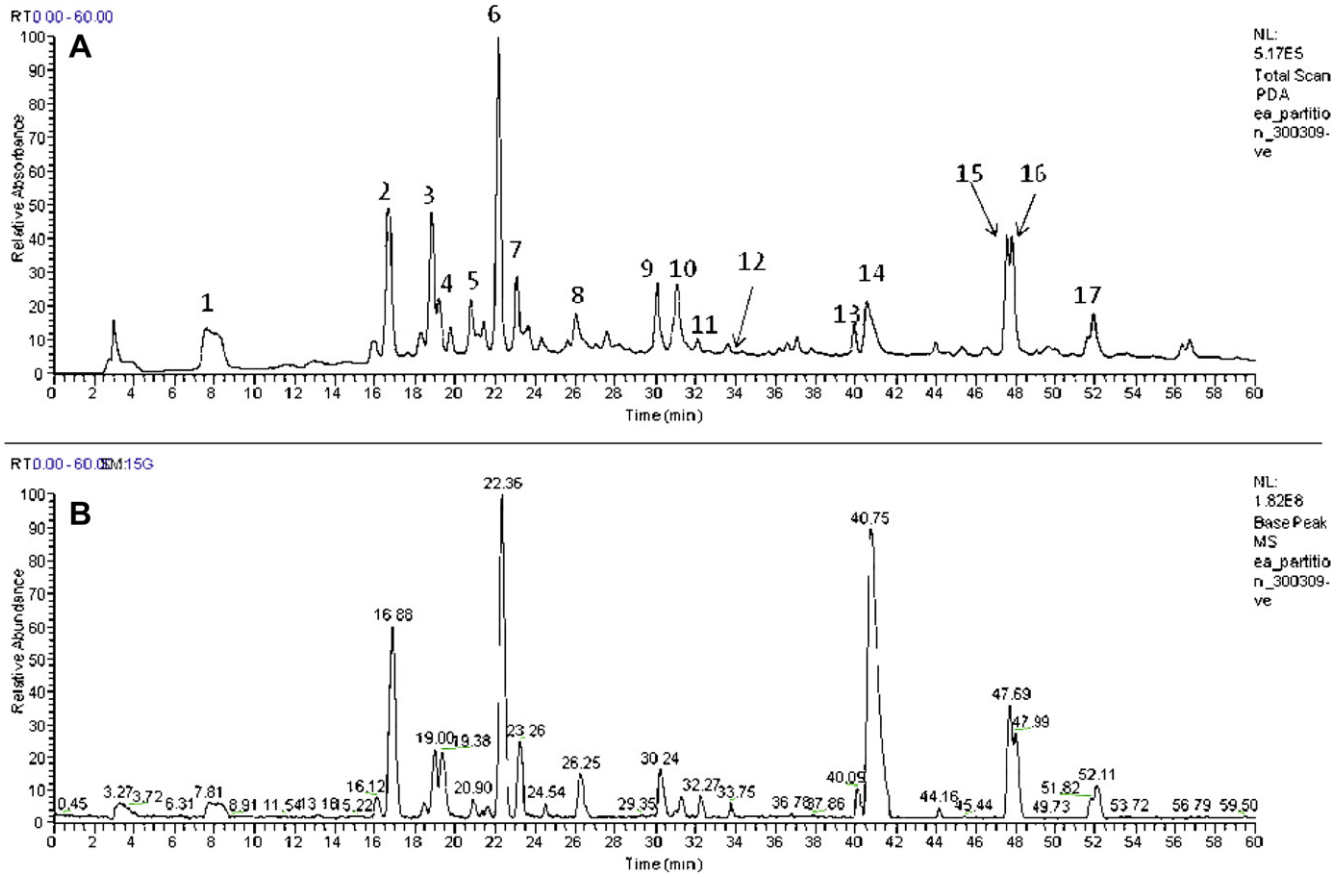


Fig. 2. (A) HPLC–DAD; (B) total ion chromatogram (TIC) profiles of ethyl acetate fraction of *Persicaria hydropiper*. For peak assignments, see Table 2. HPLC conditions are described in the text.

chloroform followed by the addition of methanol up to 100%. The eluent was collected by 100 ml fractions which were combined according to their TLC profiles to produce 9 pooled fractions (A–I). Fraction A (87.6 mg) was rechromatographed using sephadex LH-20, eluted with 100% MeOH to produce five fractions (1–5). Further fractionation of fraction A (4) using normal phase column chromatography (1.3 cm × 30 cm) with 100% chloroform as eluent yielded galloyl quercetin-3-*O*-glucoside (**2**; 7.3 mg). Repeated

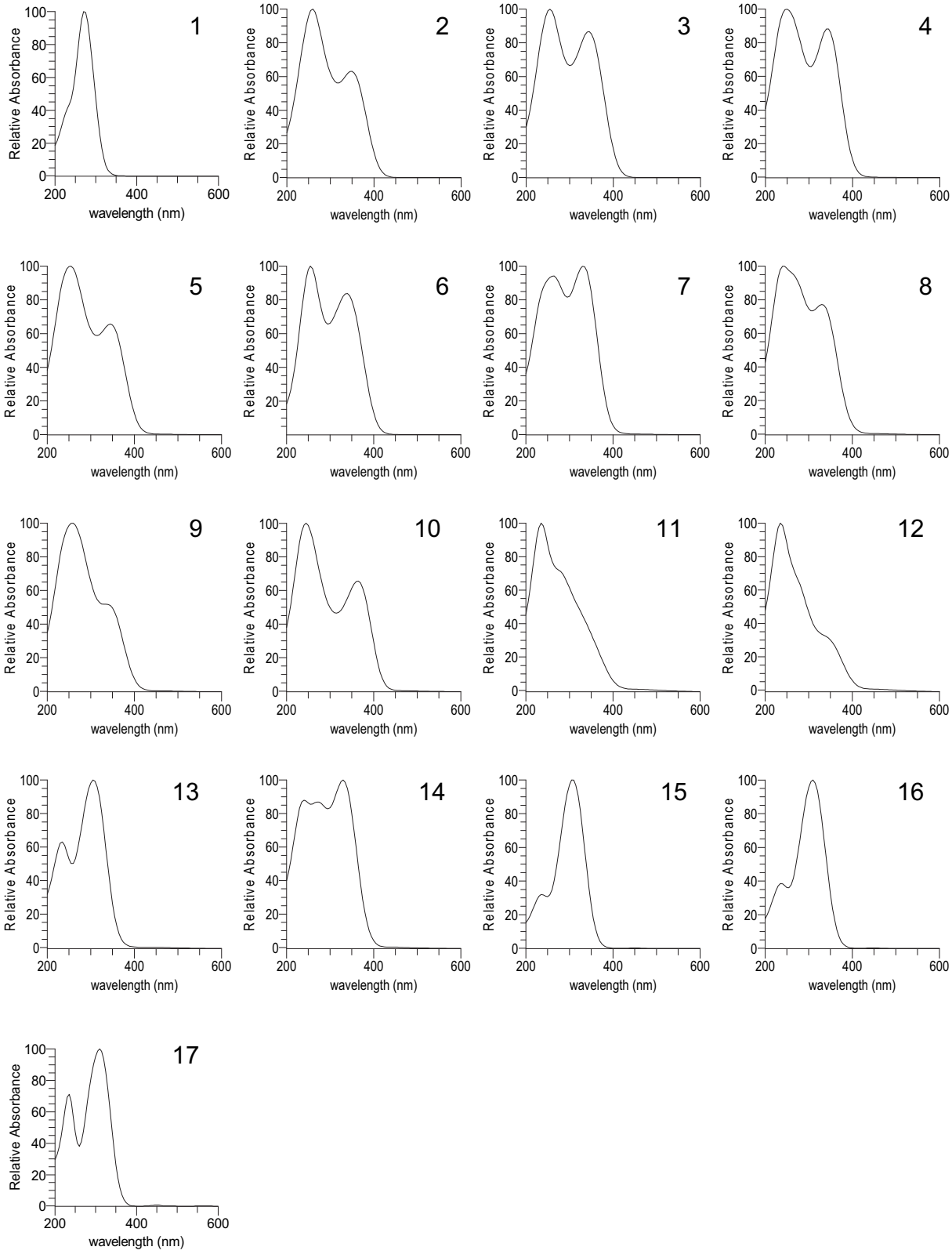


Fig. 3. UV spectrum for peaks **1**–**17** marked in Fig. 2A.

column chromatography (1.3 cm × 30 cm) of fraction B (441.3 mg) using $CHCl_3$:MeOH (9:1, v/v) afforded quercetin-3-*O*-rhamnoside (**6**; 10.8 mg). Fraction C (100 mg) was further rechromatographed on sephadex LH-20 (1.3 cm × 20 cm), eluted with 100% MeOH to give rhamnetin (**12**; 5.3 mg). The same work flow was applied for fraction D (47.3 mg) yielded 3,5-dihydroxy-4-methoxybenzoic acid (**1**; 5.4 mg). Due to the polarity of some compounds present in this fraction, the EtOAc fraction was also subjected to reversed phase column chromatography (5 cm × 10 cm) packed with 200 g reversed phase silica gel in 100% MeOH and recondition using MeOH:H_2O (2:8, v/v). The EtOAc fraction (5 g), dissolved in a minimum volume of MeOH:H_2O (2:8, v/v) was loaded onto the glass column (1.3 cm × 30 cm) and eluted with MeOH:H_2O (2:8, v/v) with 20% increment of MeOH. Collected fractions were pooled into 8 major sub-fractions (A_{RP}–H_{RP}) based on their TLC profiles. Fraction E_{RP} (49.4 mg) was rechromatographed using 100% EtOAc to give quercetin (**10**; 8.3 mg). Fraction B_{RP} (210.5 mg) was subjected onto sequential reversed phase column using MeOH:H_2O (2:8, v/v) as eluent. Further recrystallization of fraction $B_{RP\text{-}FC}$ has yielded quercetin-3-*O*-glucoside (**3**; 5.3 mg).

2.7. Statistical analysis

Data were analyzed using Statistical Package for Social Science (SPSS™) software for Windows, Version 16.0 (SPSS Inc., Chicago, IL). Differences in means were determined using ANOVA. Results are expressed as a mean of three determinations ±SD. Significance level was set as $p < 0.05$.

3. Results and discussion

3.1. Evaluation of antioxidant (FTC and DPPH) and anticholinesterase activities

The various solvent fractions of *P. hydropiper* exhibited higher antioxidant activity in comparison to α-tocopherol. As shown in Fig. 1, a low absorbance value represents a high level of antioxidant activity. Except for the hexane fraction, the antioxidant activity of all the solvent fractions was comparable to butylated hydroxytoluene (BHT), a synthetic antioxidant. *P. hydropiper* also showed significant free radical-scavenging activity, as reflected in the IC_{50} values of the fractions (Table 1). The ethyl acetate fraction in particular, strongly inhibited free radicals with an IC_{50} value of 13.3 μg/ml.

Anticholinesterase activity was carried out using two enzymes, namely acetyl- (AChE) and butyrylcholinesterase (BChE). Among the fractions, the ethyl acetate fraction demonstrated the highest activity for both AChE and BChE with IC_{50} values of 231.9 μg/ml and 268.9 μg/ml, respectively (Table 1). A moderate correlation between the DPPH radical-scavenging activity and AChE activity ($r^2 = 0.700$) and weak correlation against BChE activity ($r^2 = 0.214$) of the fractions were observed. Following this, the ethyl acetate fraction was further characterized using LC–MS/MS in order to get a better idea on the constituents that could be contributing to both activities.

3.2. HPLC–DAD–ESIMS/MS analysis of ethyl acetate fraction

Fig. 2 shows the LC–DAD and total ion chromatogram (TIC) of the ethyl acetate fraction of *P. hydropiper*. Seventeen peaks were discernable from the LC–DAD profile, fifteen of which were identifiable based on the LC–MS/MS data and subsequent confirmation by comparison with literature data and/or co-chromatography with authentic standards. The compounds in this bioactive fraction consisted mainly of flavonol glycosides and phenylpropanoids. The classes of compounds were recognizable from their characteristic UV spectra (Fig. 3). The chromatographic and spectroscopic data are summarized in Table 2 and an overview of the identified compounds is given in Fig. 4. Six compounds including 3,5-dihydroxy-4-methoxybenzoic acid (**1**), galloyl quercetin-3-*O*-glucoside (**2**), quercetin-3-*O*-glucoside (**3**), quercetin-3-*O*-rhamnoside (**6**), quercetin (**10**), and rhamnetin (**12**) were successfully isolated and were unambiguously identified by spectroscopic methods including MS, UV, IR and NMR spectrometry. The detailed NMR data will be provided upon request.

Peak **1** had the UV λ_{max} at 270 nm, with the retention time at 7.62 min. The parent ion at m/z 183 $[M\text{-}H]^-$ was further fragmented to fragment ions at m/z 168 and m/z 124, corresponds to the loss of methyl group (15 amu) and carboxylic radical (44 amu). Based on the spectroscopic data of the isolated compound, peak **1** was identified to be that of 3,5-dihydroxy-4-methoxybenzoic acid.

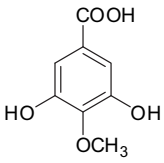
Table 2
Retention times, MS, MS/MS and UV values of the main constituents present in the ethyl acetate fraction of *Persicaria hydropiper*.

Peak[e]	Retention time[e] (RT)	Molecular ion peak $(M\text{-}H)^-$	MS/MS fragments ions (Intensity, %)	UV	Compounds	Relative amounts[d] (mg/100 g extract)
1	7.62	183	168 (100), 124	270	3,5-Dihydroxy-4-methoxybenzoic acid[a]	–
2	16.68	615	463 (100), 301, 179, 151	260, 350	Galloy Quercetin-3-*O*-glucoside[a]	0.836
3	18.84	463	301 (100), 179, 151	255, 345	Quercetin-3-*O*-glucoside[a]	0.23
4	19.21	447	285 (100)	250, 345	Kaempferol-3-*O*-glucoside[c]	0.009
5	20.82	599	447 (100), 285	255, 345	Galloyl kaempferol-3-*O*-glucoside[b]	0.11
6	22.19	447	301 (100), 179, 151	255, 340	Quercetin-3-*O*-rhamnoside[a]	1.49
7	23.10	431	269 (100)	265, 330	Apigenin-7-*O*-glucoside[b]	0.09
8	26.04	365	285 (100), 267	240, 330	*Unidentified*	–
9	30.10	599	447, 301 (100), 179, 151	260, 335	Galloyl quercetin-3-*O*-rhamnoside[b]	0.19
10	31.10	301	179 (100), 151	245, 365	Quercetin[a]	0.19
11	32.11	593	447, 285 (100)	235	Kaempferol rutinoside[c]	0.03
12	33.61	315	300 (100), 271, 255	235	Rhamnetin[a]	0.03
13	39.94	779	633 (100), 615, 453	235, 305	Hydropiperoside[b]	–
14	40.55	379	299 (100)	240, 270, 330	*Unidentified*	–
15	47.55	925	779 (100), 615, 453	235, 305	Vanicoside D[b]	–
16	47.81	955	809 (100), 663, 615, 487	235, 310	Vanicoside B[b]	–
17	51.94	997	851 (100), 705, 663, 487	235, 310	Vanicoside A[b]	–

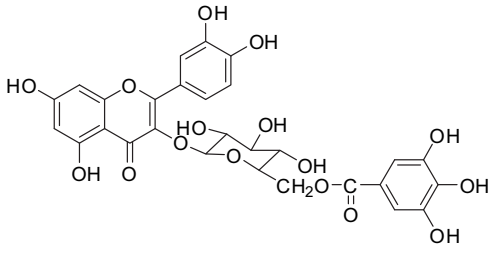
[a] Isolated and identified by spectroscopic data.
[b] Identified in the literature from the family Polygonaceae.
[c] Identified by comparison with standard compounds.
[d] value are means ($n = 3$).
[e] Peak numbers and retention time refer to Fig. 2.

The UV spectra of peaks **2**–**6** and **9**–**12** were typical of flavonoids (235–285 nm and 300–350 nm) (Markham, 1982). Mass fragmentation pattern revealed them to be mainly glycosides of the ubiquitous flavonoids, quercetin (peaks **2**, **3**, **6** and **9**) and kaempferol (peaks **4**, **5** and **11**). Peak **2** gave an $[M-H]^-$ ion at m/z 615, which fragmented further to give daughter ions at m/z 463, due to the loss of a galloyl moiety (−152 amu), followed by a subsequent loss of a glucosyl unit (−162 amu) to give the aglycone fragment ion at m/z 301. Further MS/MS^n yielded daughter ions at m/z 179 and m/z 151 confirming the aglycone for peak **2** as quercetin (Seeram, Lee, Scheuller, & Heber, 2006). Comparison with literature (Peng et al., 2003) and spectroscopic data on the isolated compound identified peak **2** to be that of galloyl quercetin-3-*O*-glucoside. The free quercetin (peak **10**) was also isolated and unequivocally identified by spectroscopic data. Peak **3** was due to quercetin-3-*O*-glucoside (Peng et al., 2003), confirmed by co-chromatography with the spectroscopic data on the isolated compound. The significantly ionized peak **6** showed an $[M-H]^-$ ion at m/z 447 which fragmented further to the aglycone (m/z 301) by losing a rhamnosyl unit (−146 amu). Its identification as quercetin-3-*O*-rhamnoside was confirmed by spectroscopic data of the isolated compound. Meanwhile peak **9** had similar fragment ions with peak **6** plus an additional galloyl moiety, based on the $[M-H]^-$ ion at m/z 599. Thus, peak **9** was identified to be that of galloyl quercetin-3-*O*-rhamnoside (Peng et al., 2003).

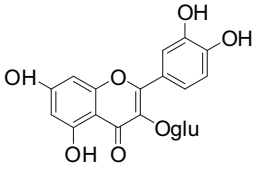
Peak **4** showed $[M-H]^-$ ion at m/z 447 which subsequently lost a glucosyl unit (−162 amu) to give the aglycone fragment ion at m/z 285. Comparison with an authentic standard confirmed its identity as kaempferol-3-*O*-glucoside. Meanwhile peak **5** also had similar aglycone fragment ions as that of peak **4**. However its $[M-H]^-$ ion



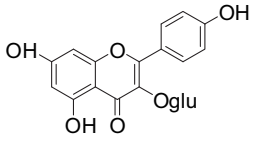
3,5-dihydroxy-4-methoxybenzoic acid **(1)**



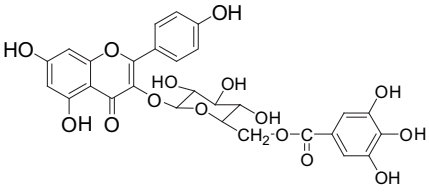
Galloyl quercetin-3-*O*-glucoside **(2)**



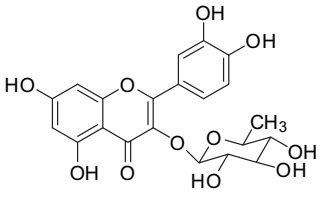
Quercetin-3-*O*-glucoside **(3)**



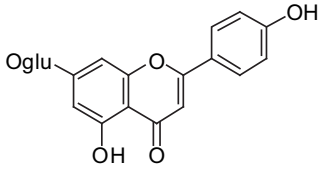
Kaempferol-3-*O*-glucoside **(4)**



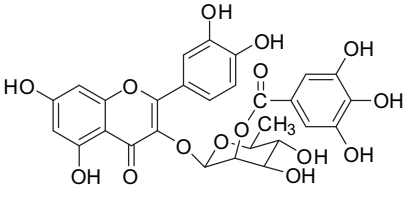
Galloyl kaempferol-3-*O*- glucoside **(5)**



Quercetin-3-*O*-rhamnoside **(6)**

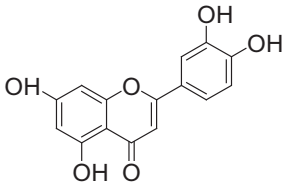


Apigenin-7-*O*-glucoside **(7)**

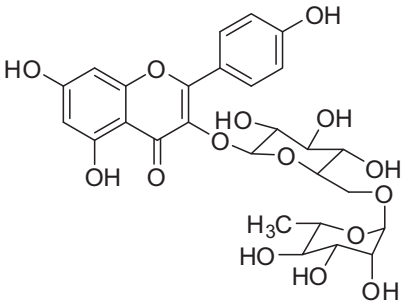


Galloyl quercetin-3-*O*-rhamnoside **(9)**

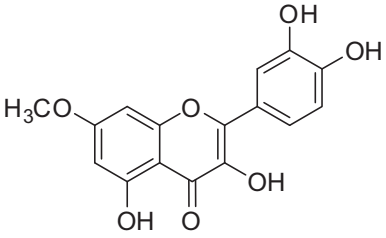
Fig. 4. Structures of compounds identified using HPLC–DAD–MS/MS analysis.



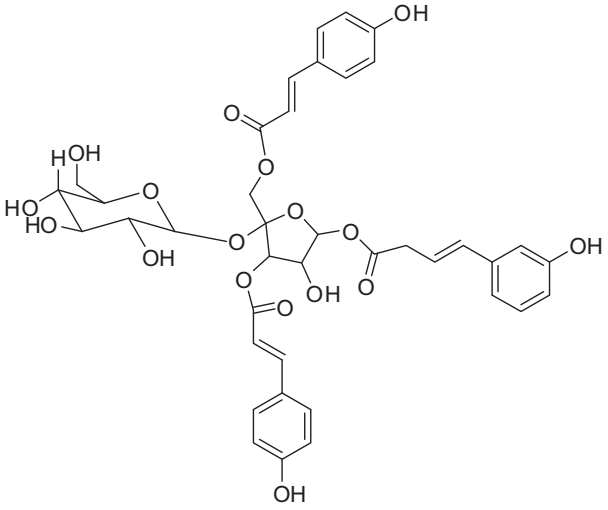
Quercetin **(10)**



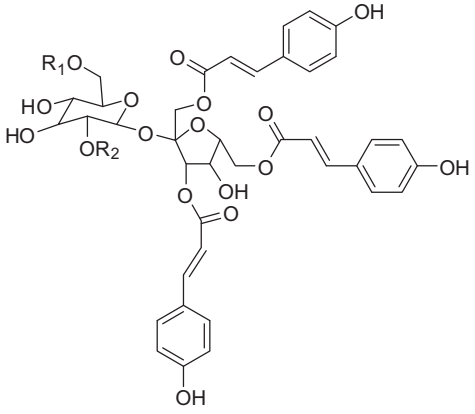
Kaempferol rutinoside **(11)**

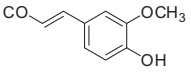


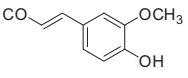
Rhamnetin **(12)**



Hydropiperoside **(13)**



Vanicoside A **(17)**; R_1=  ; R_2= $COCH_3$

Vanicoside B **(16);** R_1=  ; R_2 =H

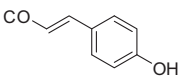
Vanicoside D **(15)** R_1=  ; R_2= H

Fig. 4. (*continued*).

was 152 amu larger than that of peak **4** suggesting that peak **5** was that of galloyl kaempferol-3-*O*-glucoside. Both compounds have been reported previously from the same species (Peng et al., 2003). The $[M-H]^-$ ion of peak **11** appeared at m/z 593. Again, loss of a rhamnosyl unit (−162 amu), gave the aglycone ion at m/z 285, typical of kaempferol. Its identification was further confirmed by co-chromatography with an authentic standard.

Apart from quercetin and kaempferol, two other flavonoids (peaks **7** and **12**) were also detected in the bioactive fraction. Peak **7** exhibited a $[M-H]^-$ ion at m/z 431 which upon MS/MS lost a glucosyl moiety to give a fragment ion at m/z 269. This compound was thus identified as apigenin-7-*O*-glucoside, which has been reported previously from the same species (Peng et al., 2003). Identification of peak **12** as that of rhamnetin was confirmed from spectroscopic data of the isolated compound. Peaks **8** and **14** with $[M-H]^-$ ions at m/z 365 and 379, respectively, were left identified at this stage since their UV and mass data were insufficient to provide positive identification.

Peak **13** gave $[M-H]^-$ ion at m/z 779. After MS/MS^n the ion fragmented further to give daughter ions at m/z 633, 615 and 453, due to subsequent losses of coumaroyl, hydroxyl and hexose moieties, respectively. Based on comparison with literature, peak **13** was assigned as hydropiperoside, a phenylpropanoid glucoside, previously isolated from *P. hydropiper* (Fukuyama, Sato, Miura, Asakawa, & Takemoto, 1983). Hydropiperoside has also been isolated from *Polygonum lapathifolia* and *Polygonum pensylvanicum* (Brown, Larson, & Sneden, 1998; Takasaki et al., 2001).

Peaks **15**–**17** were also identified as phenylpropanoid glucosides. Peak **15** showed an $[M-H]^-$ ion at m/z 925 and has the similar fragment ions with hydropiperoside. This indicated that the compound had an additional coumaroyl moiety as supported by the fragment ion at m/z 779 $[M-H\text{-coumaroyl}]^-$. Other fragment ions were present at m/z 615 $[M-H\text{-dicoumaroyl-OH}]^-$ and 453 $[M-H\text{-dicoumaroyl-OH-hexose}]^-$. Thus, peak **15** was identified as 6′-*O*-*p*-coumarylhydropiperoside or vanicoside D (Brown et al., 1998).

The ESI mass of peak **16** exhibited peak at m/z 955. MS/MS^n yielded daughter ions at m/z 809 and at m/z 663, indicating the subsequent losses of 2 coumaroyl moieties, and at m/z 487 which indicated a further loss of feruloyl moiety. Peak **16** was therefore determined to be that of vanicoside B (Kiem et al., 2008). Meanwhile, peak **17**, with $[M-H]^-$ ion at m/z 997, also lost of two coumaroyl moieties upon MS/MS^n, subsequently giving ions at m/z 851 and m/z 705. Other fragment ions were also observed at m/z 663 for $[M-H-COCH_3]^-$ and at m/z 487 which indicated a loss of a feruloyl moiety. Peak **16** was therefore identified to be that of vanicoside A (Kiem et al., 2008). Peaks **15**–**17** were determined as phenylpropanoid glucosides derivatives. The vanicosides contain *p*-coumaroyl esters at the 1, 3 and 6 positions of sucrose and a feruloyl ester at the 6′ carbon of glucose.

In this investigation the six isolated compounds were also studied for their biological activity. Table 3 shows the free radical-scavenging and acetylcholinesterase activities of isolated compounds. In consistent from the previous report the free radical-scavenging activity of flavonoids decreased with glycosylation, when compared with the corresponding aglycones (Rice-Evans, Miller, & Paganga, 1997). The results showed that 3,5-dihydroxy-4-methoxybenzoic acid (**1**), quercetin (**10**) and quercetin-3-*O*-rhamnoside (**6**) exhibited significant antioxidant activity with their IC_{50} values of 8.08, 11.14 and 18.46 μg/ml respectively, whereas rhamnetin (**12**), quercetin-3-*O*-glucoside (**3**) and galloyl quercetin-3-*O*-glucoside (**2**) showed weak antioxidant activity. Based on the results, it can be concluded that the antioxidative activity of the ethyl acetate fraction was contributed by phenolics compounds. It was also observed that quercetin aglycone was a stronger radical scavenger compared to its glycosides, which in agreement with those reported by Fan, Terrier, Hay, Marston, and Hostettmann (2010). On the other hand, the quercetin aglycone showed the lowest enzyme inhibition than its glycosides (Table 3). The activity of enzyme inhibition was in the order of tacrine >**12** > **3** > **6** > **2** > **10** > **1**. Fan, Hay, Marston, and Hostettmann (2008) reported that sugar moiety attached to the flavonoids is necessary for inhibition of AChE.

Results obtained from quantitative analyses demonstrated that the flavonoids content in ethyl acetate fraction of *P. hydropiper* was 3.205 mg/100 g. The linear regression analytical data for the calibration plots showed a good linear relationship with $r^2 > 0.993$ within the test ranges. The relationship between peak areas (y) and concentrations (x) was $y = 216049x - 373789$. Quercetin-3-*O*-rhamnoside was the major constituent (1.49 mg), followed by galloyl quercetin-3-*O*-glucoside (0.836 mg). Based on the relative amounts of the external standard, it was observed that the flavonoids were the major contributors to the antioxidant and anticholinesterase activities of the fraction.

Table 3
Radical-scavenging activity and AChE of isolated compounds.

Compounds	DPPH (IC_{50} value, μmol/l)	AChE (% inhibition at 100 μmol/l)
Quercetin (**10**)	11.14 ± 0.01^a	9.30
Quercetin-3-*O*-glucoside (**3**)	45.9 ± 0.01^b	28.06
Galloyl quercetin-3-*O*-glucoside (**2**)	58.3 ± 0.03^c	15.95
Quercetin-3-*O*-rhamnoside (**6**)	18.46 ± 0.30^d	23.94
Rhamnetin (**12**)	31.2 ± 0.04^e	33.38
3,5-Dihydroxy-4-methoxybenzoic acid (**1**)	8.08 ± 0.15^f	6.22
Vitamin C	6.8 ± 0.11^f	–
Tacrine	–	99.52

'–': not tested.
Values with the same lowercase within each column are not significantly different ($p > 0.05$) ($n = 3 \pm$ SD).

4. Conclusion

This is the first report describing the chemical profile of antioxidant and anticholinesterase constituents present in the active fraction of *P. hydropiper* using HPLC–DAD–MS/MS. The results of the study showed the main constituents were flavonoids, flavonoid glycosides and phenylpropanoid glycosides. The strong antioxidant activity found in the ethyl acetate fraction of *P. hydropiper* could lead to the use of this species in the food industry to prolong the shelf life of food products. In addition, the consumption of *P. hydropiper* can be a cheap and practical approach to the prevention of disease especially those related to aging.

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