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Chemical profile of xanthine oxidase inhibitor fraction of *Persicaria hydropiper*

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Abstract

Persicaria hydropiper, locally known as kesum, is an herb belongs to the family Polygonaceae. It has been used widely in many countries as food flavoring and possesses a wide range of medicinal values. The total phenolic content and xanthine oxidase inhibitory activity of the methanolic extract of *P. hydropiper* and fractions were determined spectrophotometrically. The butanol fraction was found to contain high phenolic content and was able to inhibit xanthine oxidase activity. Online profiling using liquid chromatography coupled with electrospray ionisation spectrometry (LC-ESIMS/MS) has revealed ten constituents in this active fraction. The major components were flavonoid derivatives and flavonoid sulphates, which were confirmed by comparison with an authentic standards as well as their MS/MS fragmentation patterns and UV spectra.

Introduction

Persicaria hydropiper (syn *Polygonum minus* or *Polygonum hydropiper*) or locally known as 'kesum' is a perennial herbaceous plant, belongs to Polygonaceae family. It can be found mostly in many parts of the world and grows wild in wet areas such as waterside and marsh (Miyazawa and Tamura, 2007a). Considering its peppery taste, *P. hydropiper* is used as a kind of spice to enhance the flavor of the dishes (Peng *et al*., 2003). The Japanese consume the sprout of *P. hydropiper* for dishes of 'sashimi' (Miyazawa and Tamura, 2007b). The juice from the leaves is used to treat headache, toothache, gastric ulcer, loss of appetite and dysentery (Rahman *et al*., 2002). The consumption of the leaves of *P. hydropiper* has been associated with a beneficial health effect, especially antioxidant and anti-inflammatory activities (Furuta *et al.*, 1986; Peng *et al.*, 2003, Yang *et al.*, 2013, Orbán-Gyapai *et al.*, 2015). Moreover, this plant comprises range of compounds, such as flavonoids, sesquiterpenoids, coumarins and phenylpropanoid glycosides (Furuta *et al*., 1986; Fukuyama *et al*.,1985; Brown *et al*.,1998; Peng *et al*., 2003).

Flavonoids and polyphenols have been reported to show potent xanthine oxidase (XO) inhibitors (Owen and Johns, 1999). The XO is the enzyme that catalyzes the metabolism of hypoxanthine and xanthine into uric acid, which plays an essential role in gout (Filha *et al*., 2006). Gout happens due to the deposition of uric acid in the joints leading to inflammation, which is also can be linked to hyperuricemia (Huo *et al*., 2015). Hence, the use of XO inhibitors that block the production of uric acid is one of the approaches to treat gout. The XO inhibitors have been reported in a wide range of plants used in traditional herbal medicines for the treatment of gout (Owen and Johns, 1999; Filha *et al*., 2006). The mechanism of XO revealed that it is an important biological source of superoxide radicals (Cos *et al*., 1998).

The identification of components present in the active fraction could be achieved using high performance liquid chromatography coupled with a photodiode-array detector and a mass spectrometry (HPLC-DAD/MS). This technique may provide powerful and economical tool for analysis in crude plant extracts. The HPLC-DAD could also provide extensive information on the structures while HPLC–MS provides information about the molecular weight and the molecular structure from its fragmentation data (Queiroz *et al.*, 2002). This hyphenated technique gives a precise idea of plant constituents and has been widely and successfully used in the identification of compounds in the plant extracts (Wolfender *et al*.,

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1998; Lee *et al.*, 2005; McNab *et al*., 2009; Hashim *et al.*, 2012).

It was reported that the crude methanolic extract of *P. hydropiper* exhibited strong antioxidant activity (Abas *et al*., 2006). In view of its popularity among the locals, it is important that the chemical profiles of the *P. hydropiper* can be established as the marker compounds. In this paper, the methanolic extract of *P. hydropiper* was fractionated into hexane, ethyl acetate, butanol and aqueous, and the XO activity of the resulting fractions was measured. The butanol fraction exhibited the strongest activity in the assays performed. In addition, the butanol fraction was profiled using LC-DAD-ESIMS/MS to obtain a better insight into the chemical constituents that could be contributing to the activity.

Materials and Methods

Chemicals

All chemicals were of analytical grade or HPLC grade and purchased from Merck (Darmstadt, Germany). Formic acid (Fisher, Loughborough, UK) was used as buffer. Water was purified by a MilliQ system (Millipore, Bedford, MA, USA). Xanthine oxidase (EC 1.1.3.22) from cow's milk (1.3 U/mL), xanthine and allopurinol were obtained from Sigma Chemical Co. (St. Louis, MO, USA). The buffer used was 1/15 M potassium phosphate-sodium phosphate buffer, pH 7.5. The substrate solution, 0.15 mM xanthine in water, and the enzyme solution; 0.28 U/mL in 1/5 M phosphate buffer (pH 7.5) were prepared immediately before use.

Plant materials and sample preparation

Persicaria hydropiper was obtained from the medicinal plant nursery of the Laboratory of Natural Products, Universiti Putra Malaysia and was identified by Dr. Shamsul Khamis. A voucher specimen (SK154/02) was deposited at the herbarium of the Laboratory of Natural Products. Air dried leaf powder (800 g) of *P. hydropiper* was extracted with methanol for three times, each time by soaking in 1.5 L of solvent for overnight. Then solvents were removed under reduced pressure to give 220 g of methanolic extract (yield 27.5%, w/w of dry plant material). A portion of the extract (78 g) was again dissolved in 200 mL of water: MeOH (3:1, v/v) mixture and fractionated with hexane, dichloromethane, ethyl acetate and butanol (3x 200 mL each fraction). The fractionation afforded five different fractions (hexane, dichloromethane, ethyl acetate, butanol and aqueous) which were then subjected to total phenolic content and XO assays. The bioactive fraction was further analyzed by HPLC-DAD-ESI-MS/MS. Samples for the analysis were prepared by dissolving 5 mg of the bioactive fraction in 1 mL MeOH and then filtered through a 0.45 µm filter (Waters, Milford, USA). The sample injection volume was 20 µL.

Instrumentation

Analyses were performed with a Thermo Finnigan model LCQDECA (Thermo Fisher Scientific, San Jose, CA, USA) 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 Xcalibur 1.2 (Thermo Fisher Scientific, San Jose, CA, USA) and Mass Frontier 5.0 (Thermo Fisher Scientific, San Jose, CA, USA) software. Analyte separation was carried out on a Hypersil GOLD C18 column (3 µm, 150 mm x 2.1 mm, Thermo Hypersil-Keystone Inc, Bellefonte, Pennsylvania, USA) with a flow rate of 250 µL/min. The sample was eluted using two mobile phases; acetonitrile containing 0.1% formic acid (solvent A) and water containing 0.1% formic acid (solvent B). The gradient program commenced from 10% to 50% A (30 min) followed by 50% to 100% A (50 min). The total analysis time was 80 min. The system operated in the negative mode at 4.8kV. The detection spectra were acquired from 100-1000 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%. The Spectramax Plus (Molecular devices, LLC, Sunnyvale, CA, USA) UV/VIS was employed as a microplate reader for measuring total phenolic content and XO inhibitory activity.

Determination of total phenolic content

Total phenolic content (TPC) was determined using the Folin Ciocalteu method (Singleton *et al*., 1999). To 0.1 mL of extract (1 mg/mL), 0.1 mL of Folin–Ciocalteu's reagent (50%, v/v) was added and then neutralized with 10 mL of sodium carbonate (7%, v/v; in distilled water). The blanks were prepared by replacing Folin–Ciocalteu's reagent with a solvent of extraction. After 60 min of incubation, the absorbance of the solution was measured at 725 nm using SpectraMax Spectrophotometer (Molecular devices, LLC, Sunnyvale, CA, USA). A standard curve of Gallic acid was constructed (0-100 ppm) and TPC content was expressed as mg gallic

Table 1. Total phenolic content and Xanthine oxidase inhibitory effects of Persicaria hydropiper fractions. Value expressed as IC_{50} (µg/mL). Each experiment was performed at least twice in triplicate

Crude/Fraction	*Total phenolic content (mg GAE/100g dry extract)*	*Xanthine oxidase inhibitory (IC_{50} value, µg/mL)*
Crude methanol	419.86 ±1.34^a	29.27±5.43^a
Hexane	4.34 ±0.90^b	ND
Dichloromethane	8.74 ±1.53^c	ND
Ethyl acetate	68.95 ±3.93^d	165.25±2.97^b
Butanol	224.38 ±3.38^e	28.72±7.61^a
Aqueous	6.39 ±1.50^c	ND
Allopurinol	ND	6.76±0.11^c

Values with same lowercase in the same column are not significantly different ($p > 0.05$) according to Duncan Test, SPSS Version 16 (n=3±SD). ND = Not determined.

acid equivalents (GAE) per 100 gram of dry extract.

Inhibition of xanthine oxidase activity

The XO activities with xanthine as the substrate were measured spectrophotometrically, based on the previous procedure with slight modification (Filha *et al.*, 2006). The assay mixture consisted of 0.036 mL of extract solution, 0.055 mL of 1/15 M phosphate buffer (pH 7.5) and 0.014 mL of enzyme (0.2 U/mL) solution, which was prepared immediately before use. After preincubation of the mixture at 25°C for 2 min, the reaction was initiated by adding 0.107 mL of substrate solution (0.6 mM in water). The assay mixture was incubated at 25°C for 20 min and the absorbance was measured at 295 nm every 6 s for 10 min, using a SpectraMax Spectrophotometer (Molecular devices, LLC, Sunnyvale, CA, USA). All assays were conducted in triplicate. Thus, inhibition percentages are the mean of triplicate observations. A negative control (blank; 0% XO inhibition activity) was prepared containing methanol solution (1%, v/v/ 0.1%, w/v in assay mixture) without extract solution. Allopurinol, a known inhibitor of XO, was used as a positive control at a final concentration of 10 µg/mL in the assay mixture. The average values from three determinations were used to calculate XO inhibition activity using the equation below:

$$\% \text{ Inhibition} = (1-(\text{test inclination}/\text{blank inclination})) \times 100$$

where test inclination is the linear change in absorbance per minute of test material, and blank inclination is the linear change in absorbance per minute of blank.

Statistical analysis

Data were analyzed using Statistical Package for Social Science (SPSS™) software for Windows, Version 16.0 (SPSS Inc., Chicago, IL). One-way analysis of variance (ANOVA) was used to determine significant differences among the means at $p < 0.05$. All results are expressed as mean ± standard deviation of three experiments. Pearson's correlation was performed to analyze the relationship between XO inhibitory activity and TPC. For the correlation purpose, IC_{50} values of XO inhibitory activity were converted to $1/IC_{50}$ values to denote the same trend of activity and value.

Results and discussion

Total phenolic content and inhibition of xanthine oxidase activity

Phenolic compounds play an important role in plant constituents due to its ability to inhibit lipid peroxidation (Owen and Johns, 1999). The Folin-Ciocalteu method is widely used to determine the total phenolic content, even though different phenolic compounds gave different responses (Wong *et al.*, 2006; Djordjevic *et al*., 2011). The phenolic contents (mg/100 g) in the extract and fractions, determined from regression equation of calibration curve ($y = 0.006x + 0.0198$, $R^2 = 0.9943$) and expressed in gallic acid equivalent (GAE) varied between 4.34 and 419.86 (Table 1). For the fractions assayed, it was evident that butanol fraction had considerably high contents of phenolic substances (224.38 ± 3.38 mg GAE/ 100 g dry extract). Previous reports on different extracts of *P. hydropiper* confirmed that this plant had high total phenolic content (Wong *et al*., 2006; Huda-Faujan *et al*., 2009).

The investigation on the XO inhibitory activity of the crude methanol showed inhibition activity at 100 µg/mL. Based on the result of crude extract, the fractions of *P. hydropiper* were assayed. Among the fractions assayed, butanol and ethyl acetate fractions demonstrated XO activity which showed inhibitory

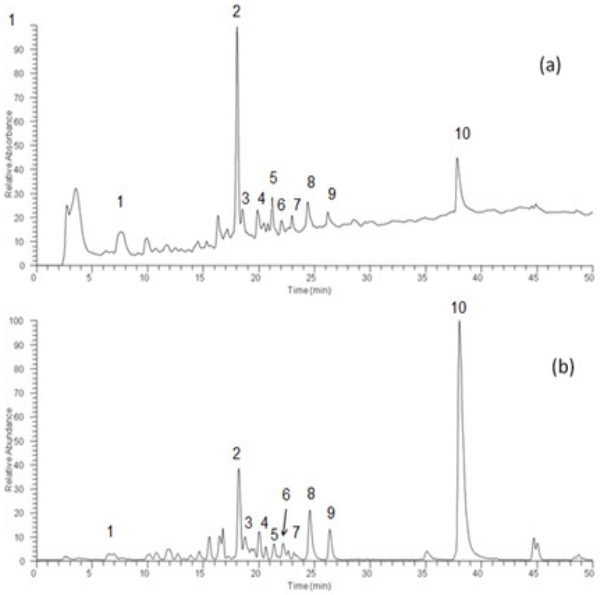

Figure 1. (a) LC-DAD and (b) total ion chromatogram (TIC) profiles of butanol fraction of *Persicaria hydropiper*. The peak numbers correspond to compounds identification presented in Table 2

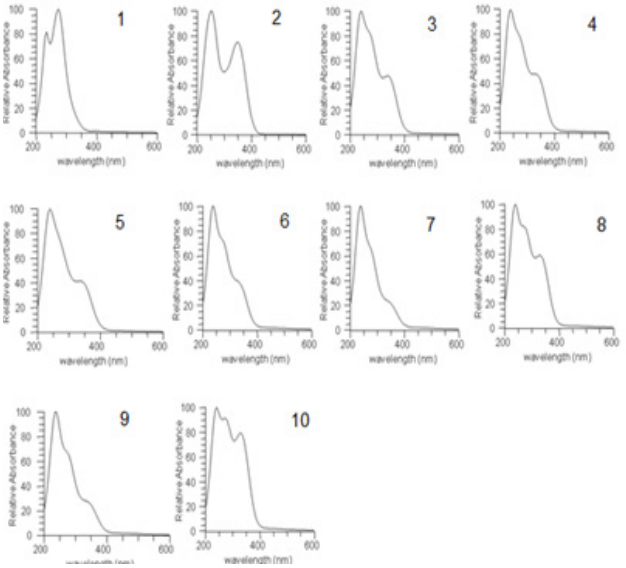

Figure 2. HPLC- UV profile of the butanol fraction of *Persicaria hydropiper*

effects greater than 50% with the IC_{50} values of ethyl acetate and butanol was 165.3 and 28.7 µg/mL, respectively (Table 1). There was a strong positive correlation between XO inhibitory activity and TPC of *P. hydropiper* fractions (R = 0.68). This suggests that most of the present phenolic compounds directly contribute to XO inhibitory activity.

For the positive control, allopurinol exhibited higher activity compared to butanol and ethyl acetate fractions. The other fractions considered to have minimal or no XO inhibitory activity. Due to our continued interest in identifying the constituents that could be contributing to the bioactivity, butanol fraction was further characterized using HPLC-DAD-ESIMS/MS.

Identification of flavonoid glycosides from butanol fraction

Liquid chromatography coupled to mass spectrometry (LC/MS) technique was applied to confirm the results obtained by LC-DAD analysis. Figure 1 shows the LC-DAD and total ion chromatogram (TIC) of the butanol fraction of *P. hydropiper*. Ten peaks were detected from the LC-DAD profile and were identifiable based on the LC-MS/MS data in negative ion mode and subsequent confirmation by comparison with literature data and/or co-chromatography with authentic standards. The negative ion mode was chosen because it appeared more selective and more sensitive for further LC-MS analysis of flavonoids in plants. The compounds in this bioactive fraction consisted mainly of flavonol glycosides and flavonol sulphates. The classes of compounds were recognizable from their characteristic UV spectra (Figure 2).

A description of all compounds and corresponding fragment ions obtained by tandem MS are summarized in Table 2 and an overview of the identified compounds is given in Figure 3. Peak 1 had a pseudomolecular ion at m/z 409 [M-H]-. Further fragmentation contribute to daughter ions at m/z 329 and 269, corresponds to the loss of sulphate (-80 amu) and subsequent loss of methoxy radical (30 amu). Thus, it was concluded that peak 1 is quercetin 7,3'-dimethylether-3-sulphate (rhamnazin-3-sulphate). This compound has been reported from the same plant previously (Barron *et al.*, 1988).

The UV spectra of peaks 2, 5 and 7 were typical flavonols (235-285 nm and 300-350 nm), demonstrating that the flavonols were substituted at 3-OH position (Markham, 1982; Tiberti *et al.*, 2007). Peak 2 had a pseudomolecular ion $[M–H]^-$ at m/z 477 and MS/MS produced a major fragment at m/z 301 $[M-H-176]^-$ due to the loss of glucuronyl group. Further MS/MS3 yielded daughter ions at m/z 179 and 151, which matches the fragmentation pattern of quercetin (Seeram *et al*., 2007). On the basis of the mass spectral data and chromatographic retention as well as standard compound, peak 2 is identified as a quercetin-3-*O*-glucuronide, the presence of which has previously been reported in *P. hydropiper* (Peng *et al*., 2003). Peak 5 showed the MS/MS spectrum at m/z 447 ion produces the fragment ions at m/z 301, representing the glycosidic cleavage by the loss of a rhamnose sugar (-146 amu) unit. Thus, peak 5 is tentatively identified as quercetin-3-*O*-rhamnoside.

Table 2. Summary of molecular ions, fragments and tentative compounds identified based on UV, MS/MS and comparison with literature data

Peak	RT (min)	$[M-H]^-$ *m/z*	MS/MS *m/z*	UV (λ_{max}) (nm)	Tentative compounds
1	6.77	409	329, 191	235, 275	Rhamnazin-3-*O*-sulphate[a,b]
2	18.02	477	301, 179, 151	255, 350	Quercetin-3-*O*-glucuronide[a]
3	18.52	447	285, 267, 241	240, 340	Luteolin-3-*O*-glucoside[c]
4	19.85	365	285, 257, 227, 185	235, 335	Kaempferol-3-*O*-sulphate[b]
5	21.18	447	301, 179, 151	240, 340	Quercetin-3-*O*-rhamnoside[a,c]
6	22.02	431	269, 225	235, 270	Apigenin-7-*O*-glucoside[b]
7	22.96	527	447, 301, 179, 153	235, 270	Quercitrin-7-sulphate[b]
8	24.37	365	285, 267, 241	235, 275, 330	Luteolin-7-*O*-sulphate[a]
9	26.19	395	315, 300	235, 270	Isorhamnetin-3-*O*-sulphate[b] (persicarin)
10	37.82	379	299, 284, 267, 241	240, 275, 330	Luteolin-3'-methyl ether 7-sulphate[a]

[a] Isolated and identified by spectroscopic data.
[b] Identified in the literature from the family Polygonaceae.
[c] Identified by comparison with standard compound

Its identification as quercetin-3-*O*-rhamnoside was confirmed by standard compound and comparison with literature (Peng *et al.*, 2003). Meanwhile, peak 7 had similar fragment ions with peak 5 with an additional sulphate moiety (80 amu), based on the $[M-H]^-$ ion at m/z 527. Thus, peak 7 was tentatively identified as quercitrin-7-sulphate.

Peak 3 resulted in a pseudomolecular ion $[M–H]^-$ at m/z 447, which subsequently lost a glucosyl unit (-162 amu) to give the aglycone fragment ion at m/z 285 and 241. The fragmentation pattern showed the typical mass of the luteolin aglycone in the negative mode (Francisco *et al*., 2014). Thus, the fragmentation of peaks 3 was identified as luteolin-3-*O*-glucoside when compared to standard compounds. The MS/MS fragmentations of peaks 4 and 8 appear that the compounds were isomer due to $[M-H]^-$ ion at 365. The MS/MS^n spectrum of $[M-H]^-$ ion shows an ion at m/z 285, which indicates a loss of a 80 unit. Peak 4 exhibited the MS/ MS^n spectrum at m/z 285 followed by ion at m/z 257 and 227 were due to the loss of CO, sequentially. Further ion at m/z 185 indicates the loss of CO_2. The fragmentation spectrum in which the main ions match with the fragmentation pattern of kaempferol (March and Miao, 2004). Meanwhile, for peak 8, the MS/MSn spectrum at m/z 285 ion resulted in a fragmentation spectrum of m/z 267, due to the loss of H_2O. Other fragments were in which the ions match with the fragmentation pattern of luteolin. Peak 8 was tentatively identified as luteolin-7-*O*-sulphate.

Peak 6 exhibited the parent ion at m/z 431. This molecular ion was subsequently fragmented further to produce apigenin aglycone fragment ions at 269 and 225, due to the loss of glucosyl moiety (162 amu). This compound was tentatively identified as apigenin-7-*O*-glucoside (Ma *et al.*, 1997; Hughes *et al*., 2001). Peak 9 gave the molecular ion peak at m/z 395. The ion was then fragmented to m/z 315, due to the loss of sulphate $[M-H-SO_3]^-$. It showed the fragmentation of sulphated flavonoids

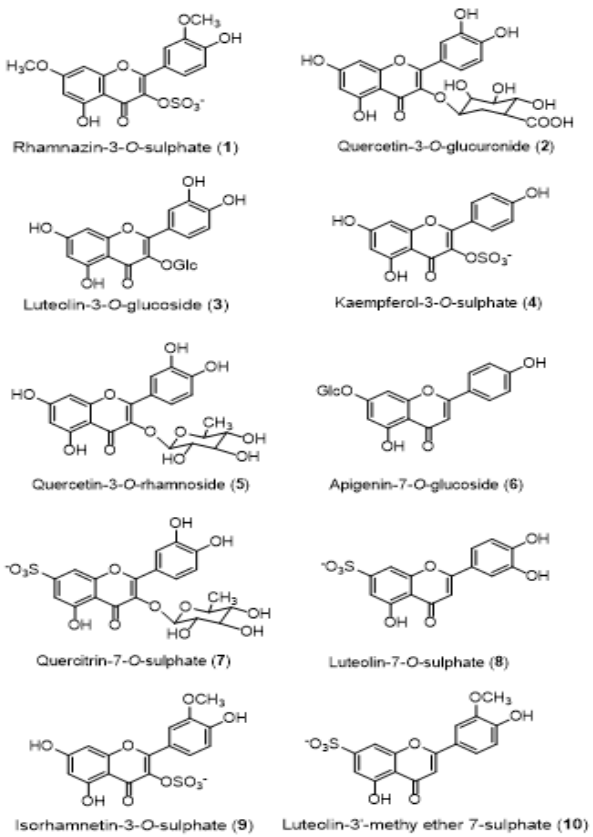

Figure 3. Structures of compounds identified using LC-DAD-MS/MS analysis

and identified as isorhamnetin-3-*O*-sulphate or persicarin. The methylated flavonoids, isorhamnetin, m/z 315 provided significant $[M-H-15]^-$ fragments corresponding to loss of CH_3 from the deprotonated molecular ion (Álvarez-Fernández *et al*., 2015). Isorhamnetin-3-*O*-sulphate has been reported in *P. hydropiper* (Harborne, 1975). The ESI mass spectrum of peak 10 exhibit molecular ion $[M–H]^-$ at m/z 379 and MS/MS produced a major fragment at m/z 299 and 284, which correspond to the loss of sulphate group and then one methyl unit. Peak 10 was characterized as luteolin-3'-methyl ether 7-sulphate, accounting for the loss of the sulphate residue and the methyl unit respectively.

Conclusion

The butanol fraction of *P. hydropiper* was significant as XO inhibitor. The compounds were identified as flavonol glycosides and flavonol sulphates. Characterization of the compounds was based on the fragmentation pattern of the MS spectra. Based on the profile, all the major peaks obtained were phenolic compounds. Thus, it can be concluded that phenolic compounds are responsible for inhibiting XO.

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