

Original Article

UPLC/Q-TOF-MS profiling of phenolics from *Canarium pimela* leaves and its vasorelaxant and antioxidant activities



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ABSTRACT

Canarium pimela K.D. Koenig, Burseraceae, have a long history of use in the Chinese traditional medicine treatment of various ailments including hypertension, and our research team has reported the anti-hypertensive activity and delineated the mechanism involved in the action. The following research aims to evaluate the vasorelaxant and antioxidant activities of ethanol extract from *C. pimela* leaves and to analyze its chemical composition by ultra-performance liquid chromatography-quadrupole time-of-flight mass spectrometry (UPLC/Q-TOF-MS) that may correlate with their pharmacological activities. The results showed that pre-incubation of aortic rings with the extract (0.3, 1, 3, 10, 30 and 100 mg/l) significantly inhibited the contractile response of the rings to norepinephrine-induced contraction ($p < 0.01$ or $p < 0.05$). Crude ethanol extract and refined ethanol extract showed a highest inhibitory effect against 2,2-diphenyl-1-picrylhydrazyl hydrate scavenging activity (IC_{50} of crude ethanol extract = $15.42 \pm 0.14 \mu\text{g/ml}$ and IC_{50} of refined ethanol extract = $5.72 \pm 0.31 \mu\text{g/ml}$) and 2,2'-azinobis (3-ethyl-benzothiazoline-6-sulphonic acid ammonium salt) (ABTS) (IC_{50} of crude ethanol extract = $3.24 \pm 0.18 \mu\text{g/ml}$ and IC_{50} of refined ethanol extract = $1.88 \pm 0.07 \mu\text{g/ml}$) scavenging activity, which was considerably higher than that reported for butylated hydroxytoluene and lower of that measured for ascorbic acid. Moreover, its chemical composition was analyzed by UPLC/Q-TOF-MS. Sixteen compounds including nine flavonoids, four tannins, two phenolic acids and one dianthrone were identified for the first time as constituents of this species. And of this, six major phenolic components were simultaneous quantitative analysis by HPLC-UV, chlorogenic acid is the major compounds in *C. pimela* leaves. These results indicate that the phenolic-rich extract of *C. pimela* leaves is a promising natural pharmaceutical for combating hypertension and oxidative stress.

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Introduction

In 2012 and 2013 in the United States (Lindsley, 2015), the most dispensed prescriptions were anti-hypertensive drugs in all diseases (698 million). From 2003 to 2013, the death rate attributable to high blood pressure increased 8.2%, and the actual number of deaths rose 34.7% (Mozaffarian et al., 2016), and that this problem may increase in the coming decade. However, the pathogenesis and pathophysiology of hypertension is a complex heterogeneous disorder, of which is a positive and direct correlation with other cardiovascular and metabolic abnormalities. Oxidative stresses or inefficient antioxidant defenses as one of the factors appear to

be involved in the development and progression of hypertension (Wilcox, 2005; Elks et al., 2009).

In recent years, traditional and natural medicine have been extensively used to prevent and heal various cardiovascular problems including hypertension (Ho and Jie, 2007) and the anti-hypertensive activity of plant extract has been correlated with its antioxidant properties (Wilcox, 2005; Elks et al., 2009) and flavonoid contents (Hugel et al., 2016). As antioxidants, these compounds may prevent the progressive impairment of vascular endothelial cells due to oxidative stress to improve endothelial function. In addition, there is growing evidence that flavonoid-rich diets have been linked to improvements in endothelial function and blood pressure (Hugel et al., 2016).

Canarium pimela K.D. Koenig (CPL) belong to the family Burseraceae, locally called as “Wu lan” and “Chinese black olive” or “Mu Wei zi” is the characteristic cash crop of Guangdong Province (China) (Huang and Wang, 2009) because of its medicinal food

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homology (Jiangsu New Medical College, 1986). It is indigenous to the southeast area of China and has been introduced to other Asian tropical and semi-tropical regions. This plant, which was made into decoction, was used in the folklore medicine to treat anti-bacterium, anti-virus, anti-inflammation and detoxification in China (Ding, 1999). In recent years, the study of CPL mainly concentrated on cardiovascular. The CPL leaves extract has anti-hypertensive activity (Li et al., 1997). Its extract can help to improve myocardial blood perfusion and protect the myocardium. The liquid extracts from CPL leaves have a quick effect of the negative inotropic action on isolated frog hearts and anti-positive inotropic action of CaCl_2 , and the action can be blocked by atropine (Liang et al., 2006). The extracts from leaves of CPL have a bi-directional effect on heart function. After transient inhibition, myocardial contractility would transform to intensification with the descending heart rate (Cen et al., 2012). The use of isolated rat heart perfusion Langendorff model showed that CPL leaves liquid extraction may be beneficial to reduce cardiac oxygen consumption, maintain heart function, play a role in protecting the myocardium (Cen et al., 2013, 2014). The water extracts of fruit, leaves, and bark of CPL all lowered the rats blood pressure quickly, and the effect of extracts of fruit and leaves in lowering blood pressure was stronger than that of the bark extracts (Dong et al., 2006), and it was found that there was no relationship between the depressurization effect of CPL extract and the basal blood in stressed hypertension rats (Dong et al., 2007). In the isolated rat thoracic aortic annulus perfusion test, found that the leaves, fruit, and bark all have the vasodilatation effect, and the leaves were the most obvious (Liang et al., 2011). The leaves extracts can relax rat thoracic rings without endothelium, which mechanism is associated with activating β -receptor, opening of Ca^{2+} channel by the receptor dependent pathway as well as inhibition of Ca^{2+} influx in the vascular smooth muscle cell. The extracts also increase aorta rings tension in pre-contraction with KCl but are not associated with α receptor (Dong et al., 2008). All the above studies are pointed out the benefits of this plant in effect of hypertension activities. However, only the volatile oil was reported (Li et al., 2015), the chemical composition of phenolics of this plant that may correlate with its pharmacological activities is still a problem.

In this context, and following our interest on the study of the chemical composition and valorization of CPL leaves, the aim of this study is to evaluate the potential of CPL as a cash crop for Guangdong Province in China, determining their vasorelaxant active parts by thoracic aorta rings of rat, their antioxidant properties determined by the 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging and 2,2'-azinobis (3-ethyl-benzothiazoline-6-sulphonic acid ammonium salt) (ABTS) radical scavenging, analyzing their ethanol: water extracts by UPLC/Q-TOF-MS, and also quantitative analyzed six phenolic compounds (chlorogenic acid, vitexin, isovitexin, hyperin, rutin, quercetin) by HPLC-UV.

Materials and methods

Chemicals

Methanol was of HPLC grade and purchased from Labscience, Inc. (Nevada, USA). Distilled water was supplied by Watson (Guangdong, China). Acetic acid and phosphoric acid of analytical grade were purchased from Zhiyuan Chemicals Ltd. (Tianjin, China). Rutin and quercetin (Batch No. 100080-201409 and No. 100081-201408) were obtained from the National Institutes for Food and Drug Control (Beijing, China). Other reference standards (purity $\geq 98\%$) including chlorogenic acid (No. 150511), vitexin (No. 151102), isovitexin (No. 151204) and hyperin (No. 150615) were purchased from Chengdu Purechem-standard Co., Ltd. (Sichuan, China). Norepinephrine (No. 20150414) were pur-

chased from Grand Pharmaceutical (China) Co., Ltd. Acetylcholine chloride (No. 60-31-1) and DPPH (No. C10088334) were purchased from Shanghai Macklin Biochemical Co., Ltd. (Shanghai, China). Isoprenaline hydrochloride (No. 20150301) was purchased from Shanghai Harvest Pharmaceutical Co., Ltd. (Shanghai, China). ABTS (No. F1719012) was purchased from Aladdin Industrial Corporation (Shanghai, China).

Preparation of plant extract

The fresh leaves of *Canarium pimela* K.D. Koenig, Burseraceae, were collected during the mid-August 2015 from the herbal garden of Zhongshan city, Guangdong province, China. The plant was taxonomically identified by Professor Jizhu Liu and a voucher specimen (No. 20150825) was deposited at School of Traditional Chinese Medicine, Guangdong Pharmaceutical University.

Dried and powdered leaf (20 g) of CPL was transferred to a round-bottom flask and was extracted with ethanol:water (50:50, v/v) with a solid-liquid ratio 1:50 (m/v). The flask was heated ($70 \pm 1^\circ\text{C}$) in an electric jacket under reflux for 80 min. The suspensions were filtered and the extract was vacuum-filtered, concentrated in a rotary vacuum evaporator at $50\text{--}60^\circ\text{C}$ (Jin et al., 2010) and freeze-dried to yield the crude ethanol extraction (CEE) (4.19 g). CEE was then purified by AB-8 resin and eluted as follows: water 2-bed volume (BV), 30% 2BV, 50% 3BV and 95% ethanol 2BV. When the eluent was clarified, beginning to collection till the end. Sugars were eluted first and the others were combined and dried as above to afford a brown phenolic extract – refined ethanol extraction (REE) (2.68 g).

Evaluation of CEE and REE on vascular tension in rats

Adult male and female Sprague Dawley (SD) rats (weighing 280–320 g) obtained from Medical Laboratory Animal Center of Guangdong Province (No. SCXK (粤) 2013-0002) were housed under conventional laboratory conditions throughout the period of experimentation. Animal experiments were carried out following the guide of Experimental Animal Ethics Committee of Guangdong Pharmaceutical University (No. gdp2016500) for the care and use of Laboratory animals. They were fed with standard pellets and water ad libitum and maintained at 22°C temperature, 85% relative humidity, and a 12 h day and night cycle.

The preparation of isolated rat thoracic aortic rings was based on the procedures described by Qin et al. (2014). Contraction of aorta rings was induced with norepinephrine (NE, 0.1 mM), and induced the relaxation with acetylcholine (ACh, 1 μM). The tension was measured using force-displacement transducer (Beijing, China) coupled with a MedLabs biological signal acquisition system (Nanjing, China).

Relaxation, a measure of inhibition of contraction in aortic ring pre-contracted with phenylephrine was measured in percentage and calculated as Eq. (1):

$$\% \text{ Relaxation} = \frac{T_c - T_t}{T_c} * 100 \quad (1)$$

where T_c stands for change in tension after contraction with norepinephrine, while T_t stands for change in tension after adding extract. Values were expressed as mean \pm standard deviation (SD).

The functional endothelial was assessed by add ACh (0.1 μM) to induce more than 60% relaxation of aortic rings pre-contracted with NE (0.1 mM). More than 60% relaxation of aortic rings can be used for experiments. The aortic rings were pre-contracted with 0.1 mM NE for 10 min, and then extracts was cumulatively added at different concentrations (0.3, 1.0, 3.0, 10.0, 30.0 and 100 mg/l). The vasorelaxant effect of the extract was compared with control group (saline).

Evaluation of antioxidant effects of CEE and REE

DPPH radical scavenging assay

The antioxidant activity of the extract of CPL leaves evaluated by 2,2-diphenyl-1-picrylhydrazyl hydrate (DPPH) radical scavenging. The extract was dissolved in ethanol to prepare stock solutions over the concentration range 0.005–0.1 mg/ml, which was mixed with 2 ml of DPPH solution. Then, the mixture was incubated at room temperature for 30 min in the dark. The absorbance of the solution was measured at 517 nm. The antioxidant activity was also expressed in mg of ascorbic acid equivalents/g of extract (mg AAE/g of extract). The scavenging rate was calculated using Eq. (2):

$$\text{Radical scavenging rate (\%)} = \left(\frac{1 - A_{\text{sample}}}{A_{\text{control}}} \right) \times 100 \quad (2)$$

ABTS radical cation decolourization assay

The antioxidant activity of the extract of CPL leaves was also evaluated by ABTS radical cation decolourization assay, which were carried out as reported by Šliumpaitė et al. (2013) and with a slightly modified. Stock solution (7 mM) of 2,2'-azinobis (3-ethyl-benzothiazoline-6-sulphonic acid ammonium salt) (ABTS) was prepared by dissolving it in 2.45 mM potassium persulfate solution and storing the mixture in the dark at room temperature for 15–16 h before use. The ABTS^{•+} stock solution was diluted with phosphate buffer (PBS, 10 mM, pH7.4) before measurements to obtain the absorption of 0.800 ± 0.030 at 734 nm. The extract was dissolved in PBS and diluted to a series of concentrations (0.05, 0.10, 0.20, 0.23, 0.26, 0.30 mg/ml). A 4 ml of ABTS^{•+} solution was mixed with 40 μ l of the extract solution in the dark, the absorption was read after 10 min. All measurements were performed in triplicate. And its ability to scavenge ABTS^{•+} is compared with ascorbic acid.

Determination of total phenolics and flavonoids contents

The total phenolic contents of the extracts were determined by Folin–Ciocalteu method based on the procedures described by Siger et al. (2008). Gallic acid was used to compute the standard calibration curve (2, 4, 6, 8, 10 μ g/ml). All of the results were expressed as mg of gallic acid equivalents per gram extract (mg GAE/g of extract). The analyses were carried out in triplicate and the average value was calculated in each case.

Total flavonoids were carried out as reported by Ibrahim et al. (2015). Rutin was used as standard and the equivalents (w/w) were determined from a standard concentration curve (0.05, 0.10, 0.20, 0.30, 0.40, 0.50 mg/ml). Determinations were carried out in triplicates; results were represented as the mean values \pm standard deviations and expressed as mg rutin equivalents per gram extracts (mg RE/g of extract).

UPLC-Q-TOF/MS analysis

The leaf powder was dissolved in aqueous methanol (50% (v/v)) in an ultrasonic bath (Shumei KQ-300DA ultrasonic instrument, Kunshan, China) for 30 min and the mixture was centrifuged thrice with $9055.8 \times g$ for 10 min. The clear supernatant was used for UPLC-Q-TOF/MS analysis.

Acquity UPLC system (Waters Co., Massachusetts, USA) coupled with Micro-mass Q-TOF micro (Waters Co., Massachusetts, USA). UPLC was used as a separation means equipped with a binary solvent delivery system, an auto-sampler, and an Acquity UPLC™ BEH C18 column (4.6 \times 150 mm, 5 μ m, Waters Co., USA). A gradient elution was achieved using two mobile phases: 0.1% (v/v) aqueous acetic acid (solvent A) and methanol (B) at a flow rate of 0.21 ml/min with the following gradient conditions: 0 min, 22% B; 3.6 min, 29% B; 4.0 min, 33% B; 12.6 min, 35% B; 15.6 min, 70% B; 17.6 min, 70%

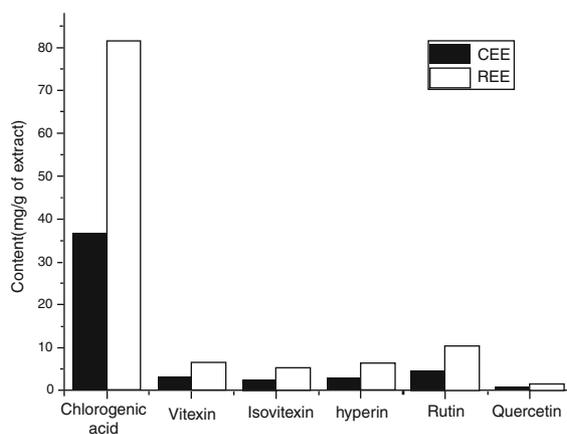


Fig. 1. The differences in the contents of the six components in CEE and REE were determined by HPLC-UV.

B; 22 min, 22% B. The sample injection volume was 5 μ l and the column temperature was maintained at 30 °C.

Mass spectrometric analyses were performed on Micro-mass Q-TOF Micro System equipped with an electrospray ionization (ESI) source in negative ion mode. The optimized parameters for the mass spectrometric analysis were as follows: capillary voltage, 3000 V; sample cone voltage, 30 V; extraction cone voltage, 2.0 V; desolvation gas flow rate, 600 l/h; desolvation gas temp, 300 °C; collision energies, 30–35 eV. All mass data acquisition were performed on MassLynx software (Version 4.1, Waters, Cicero, USA) using an independent reference spray via a Lock Spray™ interface to ensure accuracy, locking the $[M-H]^-$ of leucine enkephalin at m/z 554.2615 and the Q-TOF acquisition rate was set as 0.1 s and the inter-scan delay as 0.02 (Wu et al., 2015).

Quantification

Quantitative analyses were performed on an Agilent 1260 Infinity series equipped with a UV detector. The chromatographic separation was carried out on an Agilent ZORBAX SB-C18 column (250 mm \times 4.6 mm, 5 μ m, Agilent Co., USA) with the mobile phase consisting of 0.5% (v/v) aqueous phosphoric acid and methanol (B). The gradient elution program was as follows: 0 min, 23% B; 8 min, 32% B; 30 min, 35% B; 50 min, 70% B; 55 min, 23% B. The flow rate was at 1 ml/min, the UV detection wavelength was set at 360 nm, the injection volume was 20 μ l and the column temperature was maintained at 30 °C.

Calibration curves were obtained by HPLC injection of chlorogenic acid, vitexin, isovitexin, hyperin, rutin, quercetin standard solutions in 50% (v/v) aqueous methanol with the concentration of 571.87, 25.70, 34.96, 25.06, 55.14 and 9.91 μ g/ml, respectively. The data relevant for obtaining the calibration curves are shown in Table 1. Quantification of individual compounds (Table 2, Fig. 1) was obtained using the calibration data of the commercial standard. Compounds concentrations were calculated in triplicate and the mean value calculated in each case.

Statistical analysis

Results are expressed as means \pm standard deviation (SD). Statistical analyses were performed by paired *t* test or the Bonferroni test after one-way analysis of variance (one-way ANOVA). The correlation was established by regression analysis computed using origin 8.0. All statistical tests with $p < 0.05$ were considered significant. Significance was established when the probability level.

Table 1
Regression equation, correlation coefficients, linearity ranges, LOD and LOQ for six analytes.

Compounds	Linearity ranges (mg/l)	Calibration curve ^a	(r ²)	LOD ^b (mg/l)	LOQ ^c (mg/l)
Chlorogenic acid	57–570	$y = 0.2402x + 0.3208$	0.9998	0.13	0.48
Vitexin	2.5–25	$y = 0.3108x - 0.1698$	0.9996	0.52	1.89
Isovitexin	3.5–35	$y = 0.6288x - 0.3924$	0.9994	0.44	1.12
Hyperin	2.5–25	$y = 0.642x - 0.3703$	0.9995	0.41	1.02
Rutin	5.5–55	$y = 0.5521x - 0.3689$	0.9995	0.97	2.53
Quercetin	1–10	$y = 0.9747x - 0.4955$	0.9978	0.21	0.65

^a y = peak area, x = concentration in mg/l of extract.

^b LOD, limit of detection.

^c LOQ, limit of quantification.

Table 2
HPLC quantification of six components identified in CPL leaves extracts.

	150612		150820		150930	
	Means \pm SD (mg/g)	RSD (%)	Means \pm SD (mg/g)	RSD (%)	Means \pm SD (mg/g)	RSD (%)
Chlorogenic acid	13.39 \pm 0.09	0.70	6.71 \pm 0.09	1.39	13.42 \pm 0.20	1.51
Vitexin	1.63 \pm 0.03	1.80	0.56 \pm 0.01	1.43	1.05 \pm 0.01	1.06
Isovitexin	0.93 \pm 0.01	0.66	0.47 \pm 0.01	0.92	0.86 \pm 0.02	1.76
Hyperin	1.04 \pm 0.01	0.86	0.51 \pm 0.01	1.35	0.88 \pm 0.11	0.89
Rutin	2.04 \pm 0.04	1.88	0.85 \pm 0.02	1.91	1.62 \pm 0.02	1.00
Quercetin	0.35 \pm 0.01	1.49	0.16 \pm 0.01	1.37	0.37 \pm 0.01	0.65
Total (mg/g of leaf)	19.38 \pm 2.28		9.26 \pm 0.025		18.65 \pm 0.06	

All values are expressed as mean \pm standard deviation ($n = 3$).

Results and discussion

Total phenols and flavonoids contents

Total phenols and flavonoids contents of CEE and REE of *C. pimela* leaves were determined in this study (Table 3). The value of the total phenolic content of CEE and REE were 259.1 ± 55 (mg GAE/g of extract) and 415.6 ± 2.37 (mg GAE/g of extract), respectively. And that of the total flavonoids of CEE and REE were 373.9 ± 6.56 (mg RE/g of extract) and 824.1 ± 5.86 (mg RE/g of extract), respectively. The result revealed that the total phenolic (approximately 1.6-fold) and flavonoids (approximately 2.2-fold) content of REE were increased compared with CEE, and the differences between CEE and REE were significant ($p < 0.01$ or $p < 0.001$). That was likely due to the proteins, carbohydrates or other small polar hydrophilic molecules removal.

Effect of CEE and REE on vascular tension in rats

Having a relaxing effect on blood vessels is the most direct effect to antihypertension. Studies have reported that the effect of relaxing the aortic ring showed a non-endothelium-dependent vasodilation by Dong et al. (2008). So the relaxation effect of extracts on endothelium-intact aortic rings pre-contracted with 0.1 mM NE was evaluated at the addition of cumulative concentrations (0.3, 1.0, 3.0, 10.0, 30.0 and 100 mg/ml) in the present study.

The result revealed that CEE ($E_{max} = 92.34 \pm 9.78\%$) and REE ($E_{max} = 100.18 \pm 8.08\%$) of CPL leaves extracts both have a relaxing effect on thoracic aorta rings pre-contracted with NE compared to the normal control group (saline group), and showing a concentration-dependent relax effects (Fig. 2). Isoproterenol (ISO), a β receptor agonist, which can expansion of periphery blood vessel as a positive control group. The vasorelaxant effect of CEE group (10, 30 and 100 mg/ml), REE group (3, 10, 30 and 100 mg/ml) and ISO group (3, 10, 30 and 100 mg/ml) shows a large significance compared to the normal control group ($p < 0.05$ or $p < 0.01$) (Table 4). In addition, both CEE group and REE group have a similar effect to ISO group ($EC_{50} = 0.73 \pm 0.00064$ mg/l) (Fig. 2). At the same concentration, the vasorelaxant effect of REE group ($EC_{50} = 5.2 \pm 0.002$ mg/l)

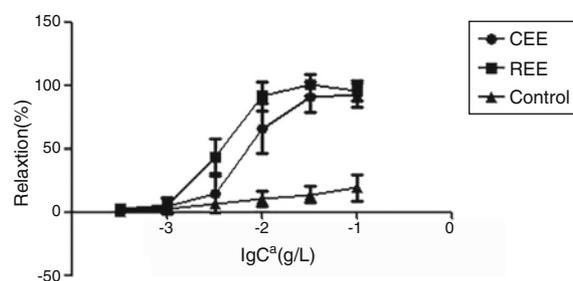


Fig. 2. Effects of CEE and REE of CPL leaf on vasorelaxant activity in rat aortic rings. All values are expressed as mean \pm standard deviation ($n = 10$). ^algC, cumulative concentration.

was stronger than CEE group ($EC_{50} = 10 \pm 0.007$ mg/l). Based on the results of the total phenolic and flavonoids content of the extracts, it is speculated that the active compounds of vasodilation may be related to its phenolic and flavonoids components.

Antioxidant activity

Oxidative stress-induced hypertension and hypertensive-induced kidney injury have fostered an increased interest in the therapeutic potential of antioxidants. Antioxidants can be used as a supplement to treat various diseases caused by oxidative stress including hypertension. However, the majority of studies have focused on the use of synthetic antioxidants to prevent or attenuate the detrimental effects of oxidative stress both *in vivo* and *in vitro* (Elks et al., 2011). Recently, many pharmacologists have noticed that looking for antioxidants from natural products (Newman and Cragg, 2007). There are many ways to evaluate the anti-oxidation, DPPH[•] and ABTS^{•+} scavenging assays are the most common method.

Table 5 presents the results of the antioxidant activity obtained for the studied extracts, expressed in terms of the amount of extract needed to decrease the DPPH[•] and ABTS^{•+} concentration by 50% (IC_{50}), as well as in terms of the ascorbic acid equivalents (AAE) on a leaves basis (μ g AAE/ml of extracts). The IC_{50} values for ascorbic acid and for BHT were also obtained and reported in Table 5 for comparative purposes. It may be

Table 3
Extraction yields, total phenolic content and flavonoids content of CEE and REE of CPL leaf extracts.

Extracts	Extraction yield (%)	Total phenolic content (mg GAE ^a /g of extract)	Flavonoids content (mg RE ^d /g of extract)
CEE ^b	20.95	259.1 ± 5.5	373.9 ± 6.56
REE ^c	13.4	415.6 ± 2.37**	824.1 ± 5.86 ^{□□□}

Compare with the total phenolic content of CEE, ** $p < 0.01$.

Compare with the flavonoids content of CEE, ^{□□□} $p < 0.001$.

Values of total phenolic content are expressed as mean ± standard deviation ($n = 3$).

^a GAE, gallic acid equivalents.

^b CEE, crude ethanol extract.

^c REE, refined ethanol extract.

^d RE, rutin equivalents.

Table 4
Vasorelaxation effects of CEE and REE on rat aortic rings.

Number	Blank group Relaxation (%)	Isoproterenol group (ISO)		CPL leaf extract group		
		Final concentration IgC (g/l)	Relaxation (%)	CEE Final concentration IgC (g/l)	REE Relaxation (%)	REE Relaxation (%)
1	0.63 ± 1.47	−5.5	0.9 ± 2.03	−3.5	2.16 ± 2.81	2.98 ± 3.51
2	3.05 ± 2.33	−5.0	8.64 ± 7.45	−3.0	4.68 ± 5.61	5.89 ± 5.65
3	6.81 ± 3.70	−4.5	18.76 ± 9.1**	−2.5	14.25 ± 14.69	44.00 ± 13.54** [□]
4	10.57 ± 6.16	−4.0	34.72 ± 16.7**	−2.0	65.77 ± 19.49**	91.25 ± 11.91** ^{□□}
5	13.84 ± 6.42	−3.5	50.62 ± 14.18**	−1.5	90.38 ± 11.9**	100.18 ± 8.08** [□]
6	19.23 ± 10.38	−3.0	54.16 ± 11.05**	−1.0	92.34 ± 9.78**	95.97 ± 7.88**

Compare with blank groups, ** $p < 0.01$; compared with CFE, [□] $p < 0.05$, ^{□□} $p < 0.01$.

All values are expressed as mean ± standard deviation ($n = 10$).

Table 5
Antioxidant activity.

	IC ₅₀ of DPPH [*] (μg/ml)	IC ₅₀ of ABTS ⁺⁺ (μg/ml)	mg AAE ^a /g of extracts
Ascorbic acid	4.23 ± 0.23	1.78 ± 0.01	–
BHT	20.87 ± 0.40	–	–
CEE	15.42 ± 0.14	3.24 ± 0.18	274.39 ± 2.54
REE	5.72 ± 0.31***	1.88 ± 0.07 ^{□□□}	740.52 ± 40.65

Compare with CEE, *** $p < 0.001$, ^{□□□} $p < 0.001$.

All values are expressed as mean ± standard deviation ($n = 3$).

^a AAE, ascorbic acid equivalents.

observed that the ascorbic acid (IC₅₀ = 4.23 ± 0.23 μg/ml) was only slightly more effective DPPH^{*} scavenger compared to REE (IC₅₀ = 5.72 ± 0.31 μg/ml of extracts), and REE was more effective DPPH^{*} scavenger compared to CEE (IC₅₀ = 15.42 ± 0.14 μg/ml of extracts) and BHT (IC₅₀ = 20.87 ± 0.40 μg/ml), in addition, the differences were significant ($p < 0.001$).

ABTS⁺⁺ decolorization assay gave quite similar results compared to those obtained by the DPPH^{*} assay (Table 5). Statistical analysis proved that there were significant differences between CEE (IC₅₀ = 3.24 ± 0.18 μg/ml) and REE (IC₅₀ = 1.88 ± 0.07 μg/ml) in ABTS radical cation decolorization assay ($p < 0.001$), and the ascorbic acid (IC₅₀ = 1.78 ± 0.01 μg/ml) was only slightly more effective scavenger compared to REE.

The antioxidant activity results revealed that leaves extracts have an antioxidant activity considerably higher than that reported for BHT and lower of that measured for ascorbic acid, and the antioxidant activity of REE is better than CEE. So the extracts maybe could protect the blood vessels from the detrimental effects produced by oxidative stress and play a role in relaxing blood vessels and lowering blood pressure (Hugel et al., 2016).

Analysis phenolic compounds in the leaf extract of CPL

The identification of the components was carried out by HPLC-UV, UPLC-MS/MS. A total 16 compounds have been identified and tentative identified by comparing their spectral data with reference compounds, when available, or corroborated with the literature.

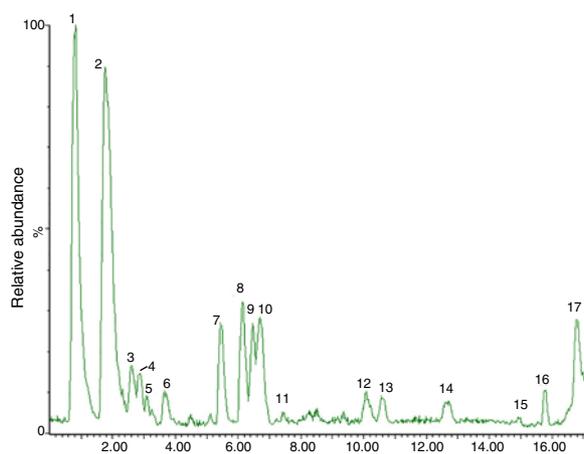


Fig. 3. Base peak chromatograms data detected in *Canarium pimela* L. leaves.

The base-peak chromatography of the methanol extract from *C. pimela* leaves was displayed in Fig. 3. UPLC-MS data for the identified compounds, namely their retention time, the molecular ion [M–H][–] and the main product ions obtained by UPLC-MS/MS were provided in Table 6. Identified phenolic compounds belonged to various classes including nine flavonoids, four tannins, two phenolic acids and one dianthrone.

Two phenolic acids compounds (1 and 2) were identified according to the fragmentation. Peak 1 was identified as the quinic acid in the negative mode, revealing the diagnostic precursor ion of m/z 191 at 0.800 min and product ion spectra with m/z 85 and 93, consisting of data from the literature. Peak 2 was assigned to chlorogenic acid (3-*O*-caffeoylquinic acid) due to the presence of characteristic product ions at m/z 191 [quinic acid–H][–], 179 [caffeic acid–H][–] and 161 [caffeic acid–H₂O–H][–]. It was further confirmed by reference as well.

Four tannins compounds (3, 12, 14 and 16) were identified as tannins. The MS/MS spectra of hydrolysable tannins usually show diagnostic product ions at m/z 633, 463, 301 and 169, which corresponded to the moiety of HHDP-gall Oyl hexoside, ellagic acid

Table 6
Phytochemical constituents identified in *Canarium pimela* leaves extracts.

Peak no.	R_t (min)	$[M-H]^-$ (m/z)	Calculated (m/z)	Formula	Fragment ions (m/z)	Identified compounds	Ref/Std.
1	0.800	191.0576	191.0556	$C_7H_{12}O_6$	85,93,127	Quinic acid	^a Ref.
2	1.787	353.8078	353.1036	$C_{16}H_{18}O_9$	191,179,135	Chlorogenic acid	^b Std.
3	2.635	633.0703	633.0728	$C_{27}H_{22}O_{18}$	463,301	Corilagin	^a Ref.
4	2.839	401.1617	401.1423	$C_{25}H_{22}O_5$	269,161,101	Unkonwn	-
5	3.112	505.1626	505.1506	$C_{30}H_{18}O_8$	343,293,169,151	Protohypericin	^a Ref.
6	3.672	451.1029	451.1501	$C_{24}H_{20}O_9$	341,217,177	Cinchonain I	^a Ref.
7	5.420	431.0978	431.1065	$C_{21}H_{20}O_{10}$	311,283	Vitexin	^b Std.
8	6.148	431.0987	431.0992	$C_{21}H_{20}O_{10}$	341,311,283	Isovitexin	^b Std.
9	6.494	463.0876	463.1071	$C_{21}H_{20}O_{12}$	300	Hyperoside	^b Std.
10	6.721	609.1458	609.1792	$C_{27}H_{20}O_{16}$	300,179,151	Rutin	^b Std.
11	7.403	433.0771	433.0820	$C_{20}H_{18}O_{11}$	300,271,255	Quercetin-3-O-arabinoside	^a Ref.
12	10.132	461.0773	461.0720	$C_{21}H_{18}O_{12}$	315,300	4'-O-methylellagic acid-3-O- α -L-rhamnopyranoside	^a Ref.
13	10.635	451.1029	451.1019	$C_{24}H_{20}O_9$	341,217,189	Cinchonain I isomer	^a Ref.
14	12.685	475.0904	475.0877	$C_{22}H_{20}O_{12}$	460,328,313	3,3'-Di-O-methylellagic acid-4-O-rhamnopyranoside	^a Ref.
15	14.503	301.0331	301.0292	$C_{15}H_{10}O_7$	179,151,121	Quercetin	^b Std.
16	15.764	343.1539	343.1545	$C_{18}H_{16}O_7$	328,191,163	3,4,3'-Tri-O-methylellagic acid	^a Ref.
17	16.705	537.7937	537.1107	$C_{30}H_{18}O_{10}$	417,375	Amentoflavone	^a Ref.

^a Ref.: Identified by comparison with references.

^b Std.: Identified by comparison with a standard.

hexoside, ellagic acid and gallic acid respectively (Zhang et al., 2016). Peak 3 exhibited the precursor ion $[M-H]^-$ at m/z 633 and ion fragments were 463 $[M-galloyl-H_2O-H]^-$ and 301 $[ellagic\ acid-H]^-$, coincident with a gallic group and hexosyl residue, indicating that this was probably an HHDP-hexosyl-gallate, thus peak 3 was identified as corilagin (Da et al., 2010). The molecular ion of peak 12 was $[M-H]^-$, m/z 461. It yielded the major fragment at m/z 315 with a loss of a rhamnose residue (146 u), and a further loss of a methyl radical (15 u) present another characterized fragment at m/z 300, which was proposed as 4'-O-methyl ellagic acid-3-O- α -L-rhamnopyranose with the reference (Li et al., 2013). Peak 14 shared the similar fragmentation regularities with peak 12, which is the loss of rhamnose residue and methyl, with the exception that peak 14 lost two methyl radicals (15 u) during the process. Thus, peak 14 was tentatively identified as 3,3'-di-O-methyl ellagic acid-4-O-rhamnopyranose (Sirat et al., 2010). Peak 16 was assigned to be methyl ellagic acid due to its same fragment pattern with reference identified as 3,4,3'-tri-O-methyl ellagic acid with the molecular ion at m/z 343 and diagnostic fragments at m/z 328, 191 and 163 (Gao et al., 2014).

Furthermore, nine flavonoids (6, 7, 8, 9, 10, 11, 13, 15 and 17) were identified in the CPL leaves extract. Peak 6 and 13 were tentatively proposed as cinchonain I and cinchonain I isomer (Zhang et al., 2016) based on their same molecular ion $[M-H]^-$ at m/z 451 ($C_{24}H_{20}O_9$) and similar major fragment ions at 341 $[M-C_6H_6O_2-H]^-$ and 217 $[M-2C_6H_6O_2-CH_2-H]^-$, which revealed the presence of two dehydrobenzene rings and two methylene. Peak 7 and 8 exhibited the same precursor ion $[M-H]^-$ at m/z 431 in the negative mode of ESI/MS spectra. Two main fragment ions presented at m/z 341 ($[M-H-90]^-$), and 311 ($[M-H-120]^-$), which confirmed that these compounds are mono-C-glycoside flavonoids. It has been demonstrated that a slight difference to distinguish the position of the sugar residue is to observe the peak of the $[M-H-18]^-$ fragment ion. When this is present, it means the sugar substituent lies on the 6 position as in compound 8 (isovitexin). On the contrary, its absence reveals that the sugar is located in position 8 as compound (7 vitexin) (Gattuso et al., 2006). These fragmentations are consistent with the data from literature and online databases (Da et al., 2010). Both two compounds were further confirmed by the authentic standards as well. Peak 9, peak 10, peak 11, peak 15 and peak 17 presented the same fragment ion at m/z 301 with the formula $C_{15}H_9O_7$, which is corresponding to the aglycon flavonol quercetin. Peak 9, peak 10 and peak 15 were characterized as hyperoside (quercetin-3- β -D-galactoside), rutin (quercetin-3- β -D-rutinoside) and quercetin, and confirmed by the authentic standards and lit-

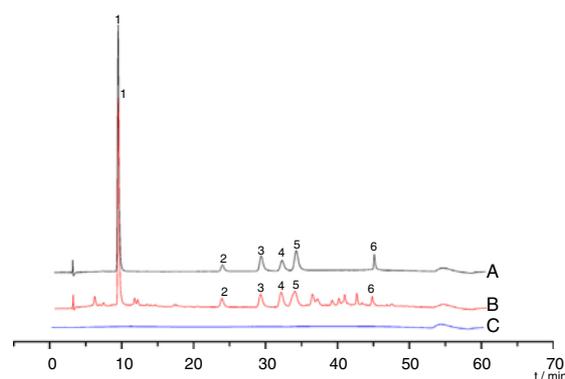


Fig. 4. HPLC chromatogram of mixed references solution (A), sample solution (B), and blank solution (C) at 360 nm. (1) Chlorogenic acid, (2) vitexin, (3) isovitexin, (4) hyperin, (5) rutin, (6) quercetin.

erature (Hsu et al., 2017; Zhou et al., 2014) according to the MS and MS/MS data. Peak 11 was considered to be quercetin-3-O- α -L-arabinofuranoside, yield the precursor ion $[M-H]^-$ at m/z 433 and major fragment ion at m/z 300 ($[M-H-133]^-$), a typical loss of arabinose residue, which is consistent with fragment ions reported in the literature (Li et al., 2009) and Massbank database. Peak 17 with the molecular ion $[M-H]^-$ at m/z 537 and major MS2 fragments at m/z 417 and 375, was assigned to amentoflavone due to its same fragment pattern with reference (Zhou et al., 2014).

Peak 5 present a molecular ion $[M-H]^-$ at m/z 505 ($C_{30}H_{18}O_8$), and MS² fragments at m/z 343, 293, 169 and 151 were tentatively identified as protohypericin according to reference (Holscher et al., 2009).

Specificity was conducted for the interference of diluent with the principal peaks of chlorogenic acid, vitexin, isovitexin, hyperoside, rutin, and quercetin. The target compounds were identified on HPLC chromatograms by comparing the retention time of the sample against the known standard. The chromatograms of mixed standard solution, sample solution, and blank solution were given in Fig. 4. And six compounds of *C. pimela* leaves from different batches including batch 150612, 150820 and 150930 was simultaneous quantitative analyzed. The contents of six analytes were shown in Table 2. Simultaneous quantitative analysis of some phenolics (mg/g powdered leaves) revealed the presence of chlorogenic acid (13.09 ± 0.09), vitexin (1.63 ± 0.03), isovitexin (0.93 ± 0.01), hyperin (1.04 ± 0.01), rutin (2.04 ± 0.04) and quercetin (0.35 ± 0.01) by using HPLC-UV in the extract. Results showed that chlorogenic

acid (69.1%, 72.5%, and 72.0%) possessed the highest share among the six components from the extract in three batches. And the total amounts of six compounds of CEE and REE (Fig. 1) identified by HPLC follows a trend similar to that of total phenolic content and flavonoids content, which was further validated that polyphenols may be active ingredients.

In the current study, 16 compounds of the extract were characterized by using UPLC/Q-TOF-MS technique, and all the 16 compounds are referenced for the first time as constituents of CPL leaves. UPLC/Q-TOF-MS analysis revealed that aqueous methanol extract contains a complex mixture of phenolic compounds including phenolic acids and flavonoids, of which chlorogenic acid is the major compounds in *C. pimela* leaves. Additionally, most of these were reported to have vasorelaxant and antioxidant effects. It was reported that chlorogenic acid can reduce oxidative stress and improves NO bioavailability, leading to the attenuation of endothelial dysfunction and hypertension (Suzuki et al., 2006). Quercetin was reported to possess anti-hypertensive action (Marunaka et al., 2017). Isovitexin and vitexin were reported to have a relaxation effect on agonist-induced vascular contraction regardless of endothelial function (Je et al., 2014). Rutin can improve kidney and heart structure and function (blood pressure, left ventricular stiffness, vascular responses, echocardiog., histol.) (Diwan et al., 2017). However, further separation and purification of bioactive compounds in the leaf extracts, as well as the elaboration of their molecular mechanisms are warranted in the next study.

Conclusion

In this work, we have evaluated the vasorelaxant and antioxidant activity of the ethanolic of CPL leaves, determined the phenolic as active ingredients, and analyzed the compounds by UPLC-MS. Sixteen compounds were identified or tentatively identified including two phenolic acids, quinic acid and chlorogenic acid, four tannins, corilagin, 4'-O-methyl ellagic acid-3-O- α -L-rhamnopyranose, 3,3'-di-O-methyl ellagic acid-4-O-rhamnopyranose and 3,4,3'-tri-O-methyl ellagic acid, and nine flavonoids, cinchonain I, cinchonain I isomer, vitexin, isovitexin, hyperin, rutin, quercetin-3-O-arabinoside, quercetin and amentoflavone, and an anthrone, protohypericin. All the components were determined for the first time as constituents of this species. Furthermore, six of these compounds, including chlorogenic acid, vitexin, isovitexin, hyperin, rutin and quercetin were simultaneously analyzed by HPLC-UV for the first time. The current results indicate that the phenolic-rich extract of CPL leaves is a promising natural pharmaceutical for combating hypertension and oxidative damages.

Ethical disclosures

Protection of human and animal subjects. The authors declare that the procedures followed were in accordance with the regulations of the relevant clinical research ethics committee and with those of the Code of Ethics of the World Medical Association (Declaration of Helsinki).

Confidentiality of data. The authors declare that they have followed the protocols of their work center on the publication of patient data.

Right to privacy and informed consent. The authors have obtained the written informed consent of the patients or subjects mentioned in the article. The corresponding author is in possession of this document.

Authors' contributions

YFD and YLL collected and identified the samples. JW and YY performed the experiments interpreted the results, and JW drafted the manuscript. XAF conducted the UPLC/Q-TOF-MS analysis and designed the study. QCX and YZC supervised the laboratory work. ZFL and JFB interpreted the results and contributed to critical reading of the manuscript. All the authors have read the final manuscript and approved the submission.

Conflicts of interest

The authors declare no conflicts of interest.

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