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Full Length Research Paper

Antioxidant and *a*-glucosidase inhibitory compounds in *Lysimachia clethroides*

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Nine compounds were isolated from ethyl acetate and *n*-butanol extracts of *Lysimachia clethroides* and their structures were assigned from ^{1}H- and ^{13}C-NMR spectra and mass spectra as *β*-daucosterol (1), kaempferol (2), *β*-sitosterol (3), quercetin (4), stigmasterol (5), *β*-amyrin (6), betulinic acid (7), 3, 5,7,4'-tetrahydroxyflavanol (8) and *α*-amyrin (9). Compounds 7 and 8 were isolated from the plant for the first time, and also isolated from *Lysimachia* genus for the first time. Ethyl acetate and *n*-butanol extracts showed antioxidant activity, petroleum ether and *n*-butanol extracts showed *a*-glucosidase inhibitory activity. Compounds 2 and 4 had antioxidant activity with IC_{50} of 14.78±0.07 and 6.94±0.03 μg/ml, respectively, and *α*-glucosidase inhibitory activity with IC_{50} of 73.69±0.07 and 8.86±0.04 μg/ml, respectively. These results indicated that kaempferol and quercetin are the main active compounds in *L. clethroides.*

Key words: *Lysimachia clethroides*, chemical composition, antioxidant activity, *α*-glucosidase.

INTRODUCTION

Lysimachia clethroides Duby. belongs to Primulaceae family. The roots or whole plant of *L. clethroides* (LC) (Zhen-Zhu-Cai in Chinese) are used as a traditional Chinese herbal medicine for the treatment of edema, jaundice, rheumatism, amenorrhea, dysentery and fractures (Hu et al., 1999). Phytochemical research showed flavonoids, phenolic, saponins and organic acids to be the main compounds in *L. clethroides* (Yue et al., 2011; Zhou and Tu, 2009; You et al., 2007; Zhou and Tu, 2004; Ding et al., 2001; Ren et al., 2001; Yasukawa and Takido, 1986; Kitagawa et al., 1967; Wei et al., 2012a). Pharmacological investigations showed that L. clethroides have anti-tumor effect, hepatoprotective effect, and antibacterial activity (Xu et al., 2003; Tang et al., 2007; Wang et al., 2007; Wu et al., 2011; Wei et al, 2012b). Antioxidant activity of petroleum ether, ethyl acetate and *n*-butanol extracts of *L. clethroides* was reported in our research, and to the best of our knowledge, no studies have been reported on the *α*-glucosidase inhibitory activity of *L. clethroides*. So, the objective of this study was to carry out the phytochemical research to determine the active constituents of *L. clethroides*.

MATERIALS AND METHODS

Plant and extract preparation

The aerial parts of *L. clethroides* were collected from Guiyang, China, in October 2010, and identified by Professor Deyuan Chen (Guiyang College of Traditional Chinese Medicine). The specimen was deposited in Institute of Chinese Materia Medica, Henan University (20101006).

The air dried *L. clethroides* (6.75 kg) were extracted three times with 75% alcohol for 7 days at room temperature. After evaporation of the solvent in a vacuum pump, the concentrated extract (480 g) was suspended in water and extracted with petroleum ether, EtOAC (ethyl acetate) and *n*-BuOH, respectively. The solution was concentrated under reduced pressure to yield petroleum ether extract (LCPE) 16.1 g, EtOAC extract (LCEA) 80 g and *n*-BuOHextract (LCBU) 330 g, respectively.

General experimental procedures

The ^{1}H- and ^{13}C-NMR were recorded on Burker spectrometer (Burker, Switzerland) operating at 400 and 100 MHz for ^{1}H-NMR

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and ^{13}C-NMR, respectively. Mass spectra were recorded on Agilent 6890-5975 GC-MS (Agilent, America). The chemical shift values are reported in ppm (δ) unit and the coupling constants (J) are in Hz. The column chromatography was carried out on various adsorbents including silica gel 200 to 300 mesh, silica gel H and sephadex LH-20 (E. Merck, Darmstadt, Germany) and thin layer chromatography (TLC) were performed on precoated silica gel F_{254} plates (Qingdao Marine Ltd. Co., China). The detection was done at 254 nm by spraying with sulfuric acid reagent.

Materials in experiments *in vitro*

Gallic acid propyl (PG), butyl-p-hydroxyanisole (BHA) and butylated hydroxytoluene (BHT) were obtained from Sigma. 1,1-Diphenyl-2-picrylhydrazyl (DPPH) from Tokyo Chemical Industry Co. 6-hydroloxy-2,5,7,8-tetramethyl-chroman-2-carboxylic acid (trolox) from Aldrich Chemical Co. 2,2´-azino-bis(3-ethylbenzo-thiazoline-6-sulfonicacid) (ABTS) were obtained from Fluka. *α*-Glucosidase (EC 3.2.1.20), 4-Nitrophenyl- *α*-D-glucopyranoside (PNPG, 026K1516), 4-Nitrophenol (PNP, 10116387), acarbose (Lot 16869) and dimethyl sulfoxide (DMSO) were obtained from Sigma. Multiskan MK3 microplate reader (Thermo Instrument Co., Ltd. USA); UV-2000 spectrophotometer (Unico Instrument Co., Ltd, Shanghai); DELTA 320 PH-meter (Mettler-Toledo, USA), phosphate buffer (pH=6.8).

Antioxidant activity using DPPH assay

DPPH radical scavenging activity was assayed according to the method of Kang et al., (2008). 0.1 ml of different extracts of *L. clethroides* in methanol was mixed with 3.5 ml of DPPH methanol solution (0.06 mmol/L). The solution was measured at 515 nm after 30 min at room temperature with propyl gallate (PG), butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT) as positive control. The antioxidant activity was expressed as an IC_{50} value, that is, the concentration in μg/ml that inhibits DPPH absorption by 50%, and was calculated from the concentration-effect linear regression curve. All reactions were carried out with three replications.

Antioxidant activity using ABTS assay

Scavenging activity on ABTS radical of the extracts of *L. clethroides* was evaluated in accordance with literature (Kang et al., 2010). The different extracts of *L. clethroides* (0.15 ml) were mixed with ABTS radical stock solution (2.85 ml) and incubated at 37°C. The absorbance was observed at 734 nm after 10 min with PG, BHA and BHT as positive control. The percentage inhibition of $ABTS^{+}$ was calculated using the formula:

$$\text{Inhibition rate (\%)} = [(A_{control} - A_{sample})/A_{control}] \times 100,$$

where $A_{control}$ was the absorbance of the control and A_{sample} was the absorbance of the sample and the standard compound. All reactions were carried out with three replications.

α-Glucosidase inhibitory activity assay

The compounds were dissolved in DMSO, and stored at 4°C in refrigerator. The α-glucosidase inhibitory activity of the compounds was assessed according to the 96 microplate screening method reported by Kang et al. (2009, 2011). Absorbance (A) was detected at 405 nm. Enzymatic inhibition data were expressed as IC_{50} values (concentration of inhibitor required for 50% inhibition against *α*-glucosidase). The inhibitory rates (%) were calculated according to the formula: $[1-(OD_{test}-OD_{blank})/(\text{control } OD_{test}-\text{control } OD_{blank})] \times 100\%$. All reactions were carried out with three replications. Acarbose was used as positive control.

RESULTS

EtOAC extract (80 g) was subjected to column chromatography over silica gel (200 to 300 mesh) developing with $CHCl_3$-MeOH (100:1 to 8:2) to yield 5 fractions. Fraction 1 (7.830 g) was separated on a silica gel H with petroleum ether-acetone (5:1), and further chromatographed on sephadex-LH-20 to yield compound 3 (1.6 mg), 5 (10.5 mg) and 6 (7.7 mg). Fraction 2 (23.0 g) was separated on a silica gel H with petroleum ether--acetone (5:1), and further chromatographed on sephadex-LH-20 to yield compound 2 (37 mg). Fraction 3 (48 mg) was chromatographed on sephadex-LH-20 to yield compound 1 (23.4 mg). Fraction 4 was separated on a silica gel H with petroleum ether-acetone (3:1), and further chromatographed on sephadex-LH-20 to yield compound 4 (23.2 mg). Fraction 5 (1.7582 g) was separated on a silica gel H with petroleum ether-acetone (7:3), and further chromatographed on sephadex-LH-20 to yield compound 8 (7.0 mg).

n-BuOH extract (330 g) was subjected to column chromatography over silica gel (200 to 300 mesh) developing with $CHCl_3$-MeOH (80:1 to 7:3) to yield 2 fractions. Fraction 1 (73.8 mg) was separated on a silica gel H with petroleum ether-acetone (60:1), and further chromatographed on sephadex-LH-20 to yield compound 9 (2.6 mg). Fraction 2 (452.1 mg) was separated on a silica gel H and further chromatographed on sephadex-LH-20 to yield compound 7 (5.0 mg).

According to the results of ^{1}H- and ^{13}C-NMR spectra and mass spectra, compounds 1 to 9 were assigned as *β*-daucosterol (1), kaempferol (2), *β*-sitosterol (3), quercetin (4), stigmasterol (5), *β*-amyrin (6), betulinic acid (7), 3, 5,7,4'-tetrahydroxyflavanol (8) and *α*-amyrin (9).

Antioxidant and *α*-glucosidase inhibitory activity *in vitro*

The antioxidant and *α*-glucosidase inhibitory activity of extracts and compounds from *L. clethroides* are shown in Tables 1, 2, 3 and 4.

Table 1 showed that in DPPH assay, the antioxidant activity of LCBU (IC_{50} = 9.86 μg/ml) was higher than that of BHT (IC_{50} = 18.71 μg/ml) as positive control. In ABTS assay, the antioxidant activity of LCBU (IC_{50} =7.43 μg/ml) was higher than that of BHT (IC_{50} = 7.72 μg/ml). The LCEA (IC_{50} = 9.02 μg/ml) was slightly lower than that of BHT. The results showed that the antioxidant activity of LCBU was higher than that of LCPE and LCEA; LCBU had the highest antioxidant activity *in vitro*.

Table 2 showed that the scavenging DPPH free radical activity of kaempferol and quercetin (IC_{50}=14.78 and 6.94

Table 1. Antioxidant activity of the extracts of *L. clethroides*

Sample	DPPH radical scavenging capacity IC_{50} (μg/ml)	ABTS radical scavenging capacity IC_{50} (μg/ml)
LCPE	NT	NT
LCEA	11.42±0.07	9.02±0.11
LCBU	9.86±0.13	7.43±0.06
BHA	3.2±0.03	1.88±0.02
BHT	18.71±0.18	7.72±0.04
PG	0.89±0.09	0.81±0.01

NT indicated not available because of low activity. BHA, BHT and PG were used as positive control.

Table 2. DPPH scavenging activity of compounds isolated from *L. clethroides*.

Compound	Concentration (μg/ml)	Inhibition (%)	IC_{50} (μg/ml)
Kaempferol	54.05	90.1±0.09	14.78±0.07
Quercetin	54.01	90.1±0.03	6.94±0.03
PG	10.81	88.96±0.01	3.96±0.01
BHA	64.86	90.00±0.01	10.27±0.04
BHT	108.11	80.53±0.02	40.8±0.05

PG, BHA and BHT were used as positive control.

Table 3. *α*-glucosidase inhibitory activity of the different extracts of *L. clethroides*.

Sample	Concentration (μg/ml)	*α*-glucosidase inhibitory activity	
		Inhibition (%)	IC_{50} (μg/ml)
LCPE	1500	98.78	190.81±3.21
LCBU	1500	96.37	10.95±0.99
Acarbose*	1500	57.26	1103.01±2.15

*Acarbose was used as positive control.

Table 4. *α*-glucosidase inhibitory activity of compounds isolated from *L. clethroides*.

Compound	Concentration (μg/ml)	*α*-glucosidase inhibitory activity	
		Inhibition (%)	IC_{50} (μg/ml)
Kaempferol	50	51.47±0.04	73.69±0.07
Quercetin	1500	98.76±0.06	8.86±0.04
Acarbose	1500	68.43±0.03	1081.27±0.04

Acarbose was used as positive control.

μg/ml, respectively) was lower than that of PG (IC_{50}=3.96 μg/ml), but higher than that of BHT (IC_{50}=40.8 μg/ml). The scavenging DPPH free radical activity of kaempferol was lower than that of BHA (IC_{50}=10.27 μg/ml), but quercetin was higher than that of BHA. The antioxidant activity sequence was PG>quercetin>BHA>kaempferol>BHT. So, kaempferol and quercetin isolated from LCEA had high antioxidant activity, and were the active antioxidant component in LCEA.

Table 3 showed that the *α*-glucosidase inhibitory activity of LCBU (IC_{50}= 10.95 μg/ml) was higher than that of LCPE (IC_{50}= 190.81 μg/ml), which were all higher than that of acarbose (IC_{50}=1103.01 μg/ml).

Table 4 showed that kaempferol (IC_{50}=73.69 μg/ml) and quercetin (IC_{50}=8.86 μg/ml) had good *α*-glucosidase inhibitory activity, which were obviously higher than that

of acarbose (IC_{50}=1081.27 μg/ml) as positive control. The α-glucosidase inhibitory activity sequence was quercetin> kaempferol>acarbose. Kaempferol and quercetin were typical flavonoids isolated from LCEA. So, kaempferol and quercetin were the active components in *L. clethroides* against *α*-glucosidase.

DISCUSSION

Phytochemical research showed the main chemical compositions in *L. clethroides* to be flavonoids, saponins and organic acids. Nine compounds were isolated from ethyl acetate and *n*-butanol extracts of *L. clethroides,* and their structures were assigned from ^{1}H- and ^{13}C-NMR spectra and mass spectra. Betulinic acid (7) and 3, 5,7,4'-tetrahydroxyflavanol (8) were isolated from the plant for the first time, and also isolated from *Lysimachia* genus for the first time.

Results showed that the antioxidant activity of LCBU was higher than that of LCPE and LCEA, but scavenging DPPH activity of LCBU was different from scavenging ABTS activity. DPPH and ABTS methods are based on the spectrophotometric determination to determine antioxidant activity of extracts, which are widely used for determination of total antioxidant activity *in vitro* from different angles. The nature of the ABTS method does not directly reflect the activity of the tested substance; it is only used to characterize the ability of reaction of the test samples and oxidation of ABTS, rather than blocking the oxidation process. Although, the antioxidant capacity was evaluated based on scavenging free radicals, the rate of free radical scavenging was different, the rate of scavenging ABTS was faster than that of DPPH. The antioxidant result of the methods was different, because of the different reaction mechanisms and reaction conditions. Therefore, the combination of the two methods can more fully reflect the antioxidant activity.

α-Glucosidase inhibitors can be competitive to inhibit *α*-glucosidase activity in the small intestine, and can slow down or inhibit the absorption of glucose in the intestine. Thus, it can effectively reduce the peak of post-prandial glycemia, adjust blood sugar levels, reduce the stimulation of high glucose on the pancreas, improve insulin sensitivity, protect the pancreas function and effectively prevent and improve the occurrence and development of diabetic complications. Results showed that LCBU and LCPE had *α*-glucosidase inhibitory activity by establishing α-glucosidase inhibitory model *in vitro*.

Currently, there are many antioxidant functional factors that can eliminate reactive oxygen species in the plant, for example, flavonoids, tannins, alkaloid and catechins from plants have the effect of antioxidant and scavenging oxygen free radical (Wang et al., 2011; Guo et al., 2009). At the same time, the *α*-glucosidase inhibitor structure types are also diversity, flavonoids and their glycosides, polyphenols, triterpenoid glycosides, alkaloids, peptides, lipids, and acids have inhibition activity. Flavonoids, saponins and organic acids are the main compounds from *L. clethroides;* these compounds may be the active compounds.

By further separation and purification, kaempferol and quercetin were typical flavonoids isolated from LCEA. The results showed that kaempferol and quercetin had antioxidant and *α*-glucosidase inhibitory activity, they were one of active compounds in *L. clethroides.*

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