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Chemical Constituents from *Lobelia chinensis* and Their Anti-virus and Anti-inflammatory Bioactivities

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In total, forty six compounds, including the novel compound lobechine (**1**), were characterized from the methanol extracts of *Lobelia chinensis*. The chemical structures of known metabolites were identified by comparing their spectroscopic and physical data with compounds reported in the literature. The structure of lobechine (**1**) was comprehensively established with the aid of 1D and 2D NMR spectroscopic analyses. In addition, selected isolates were screened for their inhibition of HSV-1 replication, superoxide anion generation, and elastase release. Among the tested compounds, scoparone (**10**) exhibited significant inhibition of superoxide anion generation with IC_{50} of 6.14 ± 1.97 μM and lobechine (**1**) exhibited moderate inhibition of elastase release with IC_{50} of 25.01 ± 6.95 μM, respectively.

Key words: *Lobelia chinensis*, Lobechine, Superoxide anion generation, Elastase release

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INTRODUCTION

Lobelia chinensis Lour. (Campanulaceae), distributed widely in East Asia in countries including Taiwan, China, Japan, and Korea, has been used as a diuretic, antidote, and hemostat in traditional Chinese medicine (Shibano et al., 2001). The major constituents of the genus *Lobelia* are reported to contain piperidine alkaloids (Felpin and Lebreton, 2004). Lobeline (Felpin and Lebreton, 2004), and radicamines A and B (Shibano et al., 2001) were reported to exhibit various biological activities. In our preliminary studies, the crude methanol extract of *L. chinensis* exhibited 19.8% inhibition of Herpes Simplex Virus Type I (HSV-1) replication at 100 μg/mL, and 22.6% and 29.9% inhibition of superoxide anion generation and elastase release, respectively, in FMLP/CB-activated human neutrophils at 10 μg/mL. For the purposes of discovering new antivirus and anti-inflammatory agents from natural sources, *L. chinensis* was selected as a target for investigating phytochemical diversity, as well as possible antivirus and anti-inflammatory candidates. In the present study, the chemical structures of forty-six constituents were established by spectroscopic methods and selected isolates were examined for their inhibition of HSV-1 replication, superoxide anion generation, and elastase release bioactivities.

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MATERIALS AND METHODS

General experimental procedures

Melting points were determined using a Fisher Scientific melting point measuring apparatus without corrections. The UV spectra were obtained on a Hitachi UV-3010 UV-Vis spectrophotometer. The IR spectra were obtained, as KBr discs, on a Jasco FT-IR-410 FT-IR spectrometer. Optical rotations were measured with a Jasco P-1010 polarimeter. Mass and high-resolution mass spectra were obtained on a Finnigan TSQ 700 mass spectrometer and a VG 70-250S mass spectrometer. ^{1}H- and ^{13}C-NMR, COSY, NOESY, HMQC, and HMBC spectra were recorded on a Bruker AVANCE-400 NMR spectrometer, using tetramethylsilane (TMS) as the internal standard. Standard pulse sequences and parameters were used

for the NMR experiments and all chemical shifts were reported in parts per million (ppm, δ). Column chromatography (CC) was performed on a silica gel (70-230 mesh and 230-400 mesh, ASTM, Macherey-Nagel), Diaion HP-20 (Mitsubishi), Diaion CHP-20P (Mitsubishi), and Sephadex LH-20 (Fluka). Thin layer chromatography (TLC) was performed with pre-coated Kieselgel 60 F 254 plates (E. Merck).

Plant material

Lobelia chinensis Lour. (Campanulaceae) was purchased in Chinese medicine shops in Taipei in July 2005 and the plant materials were identified and authenticated by Prof. C. S. Kuoh (Department of Bioscience, National Cheng Kung University, Tainan, Taiwan). A voucher specimen (CGU-LC) was deposited in the herbarium of Graduate Institute of Natural Products, Chang Gung University, Taoyuan, Taiwan.

Extraction and isolation

The whole plants of *L. chinensis* (2.9 kg) were powdered and extracted with methanol at 70°C for 8 h (12 L × 7), and the combined extracts were concentrated under reduced pressure to generate a dark brown syrup (660 g). The crude extract was partitioned with chloroform and *n*-butanol, successively, to yield chloroform (93 g), *n*-butanol (87 g), and water solubles (480 g), respectively. The chloroform solubles were subjected to chromatography over a silica gel and eluted with a gradient of chloroform and methanol to generate 9 fractions. Fraction 3 was subjected to chromatography on a silica gel, and eluted with *n*-hexane and ethyl acetate (20 : 1) to give β-amyrin palmitate (**2**) (105.9 mg) (Chaturvedula and Schilling, 2002). Fraction 4 was subjected to column chromatography over a silica gel and eluted with a mixture of *n*-hexane and chloroform (50 : 1) to generate cycloeucalenol acetate (**3**) (298.5 mg) (Lee and Chang, 2000) and β-amyrin (**4**) (20.5 mg) (Wang et al, 2002). Fraction 5 was subjected to column chromatography over a slica gel with the solvent mixture of *n*-hexane and ethyl acetate (50 : 1) to yield cycloeucalenol (**5**) (40.0 mg) (Lee and Chang, 2000). Silica gel column chromatography of fraction 6 with chloroform followed by TLC generated citropten (**6**) (8.2 mg) (Kawaii et al., 1999), β-sitosterone (**7**) (1.0 mg) (Lee and Chang, 2000), and a mixture of β-sitosterol (**8**) and stigmasterol (**9**) (1120.8 mg) (Chen et al., 1997). Fraction 7 was purified by silica gel column chromatography with chloroform-ethyl acetate (20 : 1) to give scoparone (**10**) (5.1 mg) (Jang et al., 2005). Separation of fraction 8 by repeated column chromatography over silica gel using *n*-hexane-ethyl acetate gradients followed by purification with preparative TLC on silica gel yielded diosmetin (**11**) (3.8 mg) (Sahu et al., 1998), chrysoeriol (**12**) (2.5 mg) (Choi et al., 2005), aurantiamide acetate (**13**) (30.2 mg) (Park et al., 2006), methyl-(21*R*)-pheophorbide-a (**14**) (5.4 mg) (Lai et al., 2010), mixture of β-sitosteryl glucoside (**15**) and stigmasteryl glucoside (**16**) (1081.1 mg) (Chen et al., 1999), and 2,6-dimethoxy-*p*-benzoquinone (1.0 mg) (**17**) (Nishina et al., 1991).

The *n*-butanol solubles were subjected to chromatography over reversed-phase Diaion HP-20 gel using water and methanol gradients and generated 6 fractions. Purification of combined fractions 1 and 2 was performed by silica gel chromatography eluted with chloroform and methanol (9:1) to give adenine (**18**) (1.3 mg) (Wu et al., 2000), L-tyrosine (**19**) (37.1 mg) (Nord et al., 2004), uridine (**20**) (2.0 mg) (Chen et al., 1999), gallic acid (**21**) (0.6 mg) (Wu et al., 1995), D-glucose (**22**) (337.9 mg) (Shibano et al., 2004), D-fructose (**23**) (11.5 g) (Shibano et al., 2004), *meso*-erythritol (**24**) (1.45 g) (Monrad and Madsen, 2007), glycerol (**25**) (6.03 g) (Kiuchi et al., 1998), *myo*-inositol (**26**) (250.0 mg) (Takahashi et al., 2001), and succinic acid (**27**) (22.8 mg) (Nord et al., 2004), successively. Silica gel column chromatography of fraction 3 with a mixture of chloroform and methanol (5:1) generated 5-hydroxymethyl-pyrrole-2-carbaldehyde (**28**) (14.4 mg) (Hiermann et al., 2002), thymidine (**29**) (1.4 mg) (Ouwerkerk et al., 2002), and protocatechuic acid (**30**) (1.2 mg) (Wu et al., 1995). Chromatography of fraction 5 on a Sephadex LH-20 column eluted with water and step gradient with methanol followed by preparative TLC purification on silica gel led to the isolation of lobetyolinin (**31**) (3.2 mg) (Ishimaru et al., 1992), vanillic acid (**32**) (1.4 mg) (Chen et al., 1999), *p*-coumaric acid (**33**) (0.5 mg) (Chiang et al., 2003), methyl vanillate (**34**) (2.1 mg) (Chen et al., 1999), and caffeic acid (**35**) (13.2 mg) (Hu et al., 2007). Sephadex LH-20 column chromatography of fraction 6 eluted with a solvent pair of water and methanol and further purification with preparative silica gel TLC yielded linarin (**36**) (60.8 mg) (Quintin and Lewin, 2004), wogonoside (**37**) (8.5 mg) (Kang et al., 2008), apigenin (**38**) (2.5 mg) (Nagao et al., 2002), lobechine (**1**) (7.3 mg), lobetyol (**39**) (12.3 mg) (Ishimaru et al., 1991), methyl coumarate (**40**) (0.6 mg) (Chen et al., 1999), and methyl ferulate (**41**) (1.3 mg) (Lee et al., 2005).

The water solubles were subjected to chromatography over reversed-phase Diaion HP-20 gel using water and a step gradient of methanol, yielding 7 fractions. Fractions 4-6 were subjected to chromatography again on a Diaion CHP-20P gel, and eluted with water and a step gradient of methanol, leading to the isolation of adenosine (**42**) (1.1 mg) (Chen et al., 1997) and uracil

(**43**) (145.9 mg) (Huang and Liu, 2002); apigenin-7-*O*-[β-D-glucuronopyranosyl (1→2)-*O*-β-D-glucuronopyranoside) (**44**) (90.0 mg) (Stochmal et al., 2001); and chrysoeriol-7-*O*-[β-D-glucuronopyranosyl-(1→2)-*O*-β-D-glucuronopyranoside] (**45**) (341.8 mg) (Kowalska et al., 2007). Silica gel column chromatography of fraction 7 with a mixture of chloroform-methanol-water (7 : 1 : 0.05) yielded benzoic acid (**46**) (0.73 mg) (Chen et al., 2008).

Lobechine (1)

Yellow oil; UV λ_{max} (log ε): 294 (4.1), 257 (sh, 3.0) nm; IR ν_{max}: 3434, 2959, 2866, 1730, 1716, 1662 cm^{-1}; ^{1}H-NMR (Acetone-d_6, 400 MHz): δ 9.55 (1H, s, H-1'), 6.97 (1H, d, J = 3.6 Hz, H-3), 6.27 (1H, d, J = 3.6 Hz, H-4), 4.57 (2H, s, H-1'''), 4.41 (2H, t, J = 7.2 Hz, H-1''), 3.51 (2H, t, J = 7.2 Hz, H-1''''), 2.37 (2H, t, J = 7.2 Hz, H-3''), 2.04 (2H, quint, J = 7.2 Hz, H-2''), 1.56 (2H, quint, J = 7.2 Hz, H-2''''), 1.33 (2H, sext, J = 7.2 Hz, H-3''''), 0.90 (3H, t, J = 7.2 Hz, H-4''''); ^{13}C-NMR (Acetone-d_6, 100 MHz): δ 179.4 (C-1'), 173.6 (C-4''), 139.9 (C-5), 133.0 (C-2), 123.9 (C-3), 111.4 (C-4), 70.2 (C-1''''), 63.9 (C-1'''), 45.0 (C-1''), 32.0 (C-2''''), 30.8 (C-3''), 28.8 (C-2''), 19.5 (C-3''''), 13.6 (C-4''''); EI-MS m/z (%): 267 ($[M]^+$, 92), 238 (37), 194 (37), 180 (6), 167 (4), 80 (17); HR-EI-MS m/z 267.1472 $[M]^+$ (calculated for $C_{14}H_{21}O_4N$, 267.1471).

Cell culture and viruses

HSV-1 (KOS strain, VR-1493, ATCC) stocks was prepared and titrated in HeLa cells as previously described (Kuo et al., 2006). The virus stocks were stored at −80°C until further use.

Plaque reduction assay

The plaque reduction assay followed procedures that were described previously (Kuo et al., 2001). Acyclovir was used as a positive control. HeLa cells (3.5×10^5/dish) were incubated with 100 PFU of HSV-1, and the test extracts or acyclovir was added to the cells at various concentrations. The viruses were adsorbed for 1 h at 37°C, and 1% methylcellulose was added to each well. After 5 days, the virus plaques that had formed in HeLa cells were counted by crystal violet staining. The activities of test extracts and acyclovir for inhibition of plaque formation were calculated.

Determination of cell viability

Approximately 3.5×10^5 HeLa cells were cultured in a 25 cm^2 flask and incubated with 0.1% DMSO or 25 μM Sam B for 5 days. Total, viable, and nonviable cells were counted three times under the microscope with the help of a hemocytometer following staining by Trypan blue, and the percentage of viable cells was calculated. Cell viability was also evaluated as lactate dehydrogenase (LDH) release using a LDH release assay (Roche). LDH activity was determined in milliunits per milliliter, where 1 mU is the amount of enzyme required to transform 0.0167 nM NAD per min (Kuo et al., 2008).

Preparation of human neutrophils

Blood was taken from healthy human donors (20-30 years old) by venipuncture, using a protocol approved by the institutional review board at Chang Gung Memorial Hospital. Neutrophils were isolated with a standard method of dextran sedimentation prior to centrifugation in a Ficoll Hypaque gradient and hypotonic lysis of erythrocytes. Purified neutrophils that contained > 98% viable cells, as determined by the Trypan blue exclusion method, were resuspended in a calcium (Ca^{2+})-free Hank's balanced salt solution (HBSS) buffer at pH 7.4, and maintained at 4°C prior to use.

Measurement of superoxide anion generation

The assay for the generation of superoxide anion was based on the SOD-inhibited reduction of ferricytochrome c (Hwang et al., 2009). In brief, after supplementation with 0.5 mg/mL ferricytochrome c and 1 mM Ca^{2+}, neutrophils (6×10^5 cells/mL) were equilibrated at 37°C for 2 min and incubated with drugs or an equal volume of vehicle (0.1% DMSO) for 5 min. Cells were activated with 100 nM FMLP during the pre-incubation of 1 μg/mL cytochalasin B (FMLP/CB) for 3 min. Changes in the absorbance with reduction in ferricytochrome c at 550 nm were continuously monitored in a double-beam, six-cell positioner spectrophotometer with constant stirring (Hitachi U-3010). Calculations were based on differences in reactions with and without SOD (100 U/mL) divided by the extinction coefficient for the reduction of ferricytochrome c (ε = 21.1/mM/10 mm).

Measurement of elastase release

Degranulation of azurophilic granules was determined by elastase release as described previously (Hwang et al., 2009). Experiments were performed using MeO-Suc-Ala-Ala-Pro-Val-*p*-nitroanilide as the elastase substrate. Briefly, after supplementation with MeO-Suc-Ala-Ala- Pro-Val-*p*-nitroanilide (100 μM), neutrophils (6×10^5/mL) were equilibrated at 37°C for 2 min and incubated with drugs or an equal volume of vehicle (0.1% DMSO) for 5 min. Cells were activated by 100 nM FMLP and 0.5 μg/mL cytochalasin B, and changes in absorbance at 405 nm were continuously monitored to assay elastase release. The results were expressed

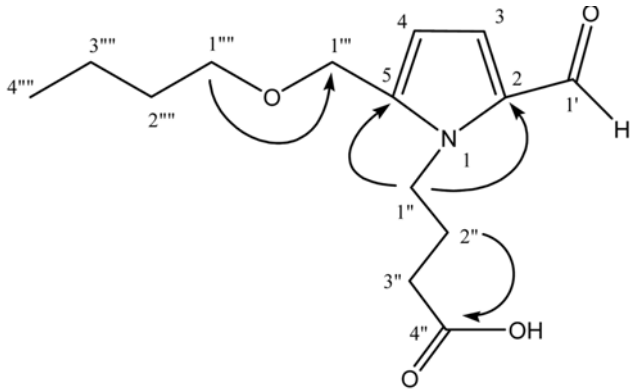
as the percentage of elastase release in the FMLP/CB-activated, drug-free control system.

Statistical analysis

Results were expressed as mean ± S.E. Computation of 50% inhibitory concentration (IC_{50}) was computer-assisted (PHARM/PCS v.4.2). Statistical comparisons were made between groups using the Student's *t* test. Values of $p < 0.05$ were considered to be statistically significant.

RESULTS AND DISCUSSION

Compound **1** was purified as a light yellow syrup, and the HR-EI-MS of **1** exhibited a molecular ion peak at *m/z* 267.1472, corresponding to the molecular formula of $C_{14}H_{21}O_4N$ (calculated 267.1471). The UV absorption maxima at 294 and 257 nm suggested the existence of a pyrrole ring (Koukoulitsa et al., 2006). The IR absorption bands at 1730, 1716 and 1662 cm^{-1} displayed the presence of carbonyl and carbon-carbon double bond groups, respectively. In the ^{1}H-NMR spectrum, a set of mutually coupled proton signals at δ 6.97 (1H, d, J = 3.6 Hz) and 6.27 (1H, d, J = 3.6 Hz) were characteristic of the H-3 and H-4 of a pyrrole ring. In addition, one formyl singlet at δ 9.55 (1H, s) and one oxymethylene singlet at δ 4.57 (2H, s), along with the HMBC analysis and comparison with the published data, suggested that compound **1** possessed the basic skeleton of 5-hydroxymethyl-pyrrole-2-carbaldehyde (Hiermann et al., 2002). In the upfield region of ^{1}H-NMR spectrum, two sets of mutually coupled proton signals at δ 4.41 (2H, t, J = 7.2 Hz, H-1''), 2.37 (2H, t, J = 7.2 Hz, H-3''), and 2.04 (2H, quint, J = 7.2 Hz, H-2''); and δ 3.51 (2H, t, J = 7.2 Hz, H-1''''), 1.56 (2H, quint, J = 7.2 Hz, H-2''''), 1.33 (2H, sext, J = 7.2 Hz, H-3''''), and 0.90 (3H, t, J = 7.2 Hz, H-4''''), were determined as $CH_2CH_2CH_2$ and butyl fragments, respectively, under the COSY experiment. In HMBC analysis, the methylene group at δ 3.51 (H-1'''') displayed ^3J correlation with the carbon at δ 63.9 (C-1'''), suggesting that the butyl moiety was connected with the hydroxymethyl group. Another methylene group at δ 4.41 (H-1'') exhibited long range ^3J correlations with the carbons at δ 133.0 (C-2) and 139.9 (C-5), which confirmed its attachment to the nitrogen atom. Moreover, ^3J correlation between the quintet at δ 2.04 (H-2'') and the carboxylic acidic carbon at δ 173.6 (C-4'') was observed, and this could further determine the presence of butyric acid at *N*-1 position of the pyrrole ring. Consequently, the chemical structure of **1** was established as shown in Fig. 1. It was reported from the natural source for the first time and thus trivially named lobechine. In addition to the new compound **1**, the known metabolites **2-46** were also characterized by comparison of their spectroscopic and physical data with compounds reported in the literature.



lobechine (**1**)

Fig. 1. Structure and significant HMBC correlations of lobechine (**1**).

The crude extract, chloroform soluble, *n*-butanol soluble, water soluble, and the pure compounds **1**, **4-6**, **11**, and **13** from the chloroform soluble were examined for their antiviral activity at 100 μg/mL or 100 μM. The percentages of inhibiting HSV-1 plaque formation for the tested compounds are displayed in Table I. Among the examined extract and solubles, the chloroform fraction demonstrated the most significant inhibition of HSV-1 replication with a percentage of 25.13 ± 2.69%. However, the purified principles from the chloroform soluble did not display marked effects on the inhibition of HSV-1 replication.

In order to investigate the effects of fractions and

Table I. Percentages of fractions and isolated compounds on the inhibition of HSV-1 replication

Analyte	Inhibition percentages[a]
Crude extracts	19.80 ± 4.49
Chloroform solubles	25.13 ± 2.69
n-butanol solubles	16.24 ± 2.15
Water solubles	12.27 ± 4.19
1	N.A.[b]
4	32.02 ± 1.86
5	15.50 ± 4.50
6	13.16 ± 8.64
11	2.63 ± 1.24
13	29.39 ± 3.10
Acyclovir	98.5 ± 1.3

[a]percentages at 100 μg/mL for fractions, 100 μM for pure compounds, and 10 μM for acyclovir.
[b]not active.

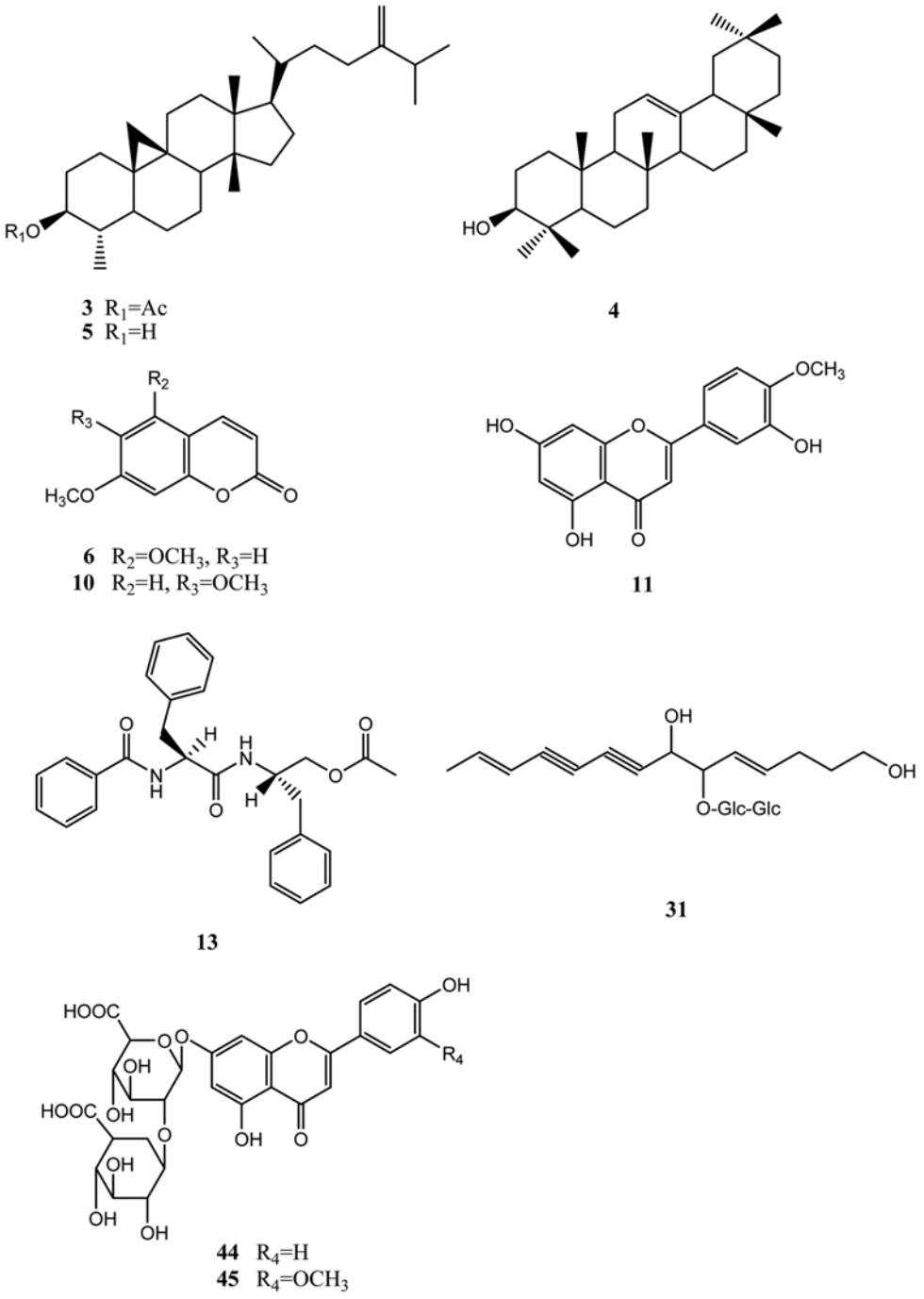


Fig. 2. Structures of the isolates for bioactivity examination.

pure compounds on the reduction of respiratory burst by human neutrophils in response to FMLP/CB, the generation of superoxide anion was determined and the results were shown in Table II. The chloroform soluble potently inhibited superoxide anion generation in a concentration-dependent manner with an IC_{50} value of 4.75 ± 0.97 μg/mL. In addition, the isolates **1**, **3**, **6**, **10**, **31**, **44**, and **45** were examined for inhibition of the generation of superoxide anion. Among these, scoparone (**10**) significantly inhibited the generation of superoxide anion with the IC_{50} value of 6.14 ± 1.97 μM, compared with the reference compound LY294002, a phosphatidylinositol-3-kinase inhibitior (IC_{50} values of 1.40 ± 0.23 μM). It was shown that this compound is the active principle in the *L. chinensis* extracts.

Table II. Effects of fractions and isolated compounds on inhibition of superoxide anion generation by human neutrophils in response to FMLP/CB

Analyte	IC_{50}	Inhibition percentages[a]
Crude extracts	N.D.	22.63 ± 5.87*
Chloroform solubles	4.75 ± 0.97 μg/mL***	
n-butanol solubles	N.D.	45.51 ± 4.91***
Water solubles	N.D.	−0.12 ± 1.97
1	N.D.	34.96 ± 6.34***
3	N.D.	14.87 ± 10.92
6	N.D.	44.21 ± 6.13**
10	6.14 ± 1.97 μM***	
31	N.D.	4.30 ± 3.56
44	N.D.	42.07 ± 8.13**
45	N.D.	23.45 ± 8.13*
LY294002[b]	1.40 ± 0.23 μM***	

[a] percentages at 10 μg/mL for fractions and 10 μM for pure compounds. N.D.: not determined. All data were expressed as means ± S.E.M. of three separated experiments. ***P < 0.001; **P < 0.01; *P < 0.05 compared with the control value.
[b]LY294002, a phosphatidylinositol-3-kinase inhibitior, was used as a positive control for inhibition of superoxide anion generation.

Table III. Effects of fractions and isolated compounds on inhibition of elastase release by human neutrophils in response to FMLP/CB

Analyte	IC_{50}	Inhibition percentages[a]
Crude extracts	N.D.	29.94 ± 5.76**
Chloroform solubles	2.45 ± 0.46 μg/mL***	
n-butanol solubles	N.D.	24.05 ± 6.06*
Water solubles	N.D.	0.74 ± 1.58
1	25.01 ± 6.95 μM	
3	N.D.	−0.70 ± 2.31
6	N.D.	22.59 ± 0.64***
10	N.D.	19.52 ± 4.46*
31	N.D.	1.54 ± 2.38
44	N.D.	16.28 ± 6.59
45	N.D.	5.10 ± 3.55***
LY294002[b]	2.64 ± 0.29 μM***	

[a]percentages at 10 μg/mL for fractions and 10 μM for pure compounds. N.D.: not determined. All data were expressed as means ± S.E.M. of three separated experiments. ***P < 0.001; **P < 0.01; *P < 0.05 compared with the control value.
[b]LY294002 was used as a positive control for inhibition of elastase release.

Moreover, neutrophil degranulation was measured based on the extent of release of elastase, a primary granule-derived protease. The crude extract and fractions were determined for their effects on the inhibition of elastase release (Table III). Also the chloroform soluble displayed the most significant inhibition of elastase release with an IC_{50} value of 2.45 ± 0.46 μg/mL. Among the tested isolates **3**, **6**, **10**, **31**, **44**, and **45**, no significant inhibitions were found and the inhibition percentages ranged from −0.70 to 22.59%. However, lobechine (**1**) exhibited moderate inhibition of elastase release with IC_{50} of 25.01 ± 6.95 μM, compared to the reference compound LY294002, a phosphatidylinositol-3-kinase inhibitior (IC_{50} values of 2.64 ± 0.29 μM).

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