

Full Length Research Paper

Compounds with elastase inhibition and free radical scavenging activities from *Callistemon lanceolatus*

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Bioassay-guided investigation of the stems of *Callistemon lanceolatus* led to the isolation of five compounds such as betulinic acid (1), pyracrenic acid (2), arjunolic acid (3), catechin (4), and piceatannol (5). Their structures were elucidated on the basis of spectral studies as well as by comparison with literature data. Elastase inhibition and DPPH radical scavenging activities were investigated for the isolated compounds. Among these, pyracrenic acid (2) was identified as a potent elastase inhibitor with an IC₅₀ value of 1.5 µg/mL, more active than the control compound, oleanolic acid (IC₅₀ = 3.0 µg/mL). With regard to antioxidative studies, strong to moderate DPPH radical scavenging activities were observed with pyracrenic acid (2), catechin (4) and piceatannol (5).

Key words: *Callistemon lanceolatus*, elastase inhibition, radical scavenging, triterpenoids.

INTRODUCTION

Elastin is an extracellular matrix protein providing elasticity to the connective tissues (Daamen et al., 2007). It forms elastic fiber in the skin dermis, and has an influence on skin elasticity. Damage to the elastin fibers leads to the decreased skin elasticity. Elastase is the proteinase enzyme capable of degrading elastin (Nar et al., 2001). Therefore, inhibition of the elastase activity could be used as a method to protect against skin aging (Kim et al., 2007).

Exposure of skin to the ultraviolet (UV) light results in the activation of reactive oxygen species (ROS) system. ROS include activated and/or free radical oxygen compounds such as singlet oxygen, superoxide anion radical, hydroxyl radical and hydrogen peroxide. These species can attack the tissues in the dermis or epidermis to cause skin aging. Therefore, free radical scavenging compounds could also be used as a cosmetic ingredient to relieve the skin aging (Takeda et al., 2003).

It becomes of great interest to search new skin-care cosmetic ingredients from natural sources (Wang et al., 2008). Jeju Island is the largest island located in the

Southern most part of Korea. The island has diversity of a plant community with more than 1,800 plant species. We are continuously searching plants in Jeju to find natural products applicable in cosmetic preparations (Kim et al., 2007; Kim et al., 2008; Ko et al., 2009; Sultana and Lee, 2007).

Callistemon lanceolatus (Myrtaceae) is an evergreen shrub native to Australia, and is being cultivated in Jeju Island as an ornamental tree. Previous phytochemical studies on the leaves and flowers of this plant have yielded triterpenoids (Younes, 1975; Jeong et al., 2009), flavonoids (Mahmoud et al., 2002), ellagic acid derivatives and tannins (Marzouk, 2008).

In this study, the ethanol extract of *C. lanceolatus* was investigated because it showed strong elastase inhibition and DPPH radical scavenging activities. The bioactivity-guided fractionation of the extract led to the isolation of the active compounds; betulinic acid (1), pyracrenic acid (2), arjunolic acid (3), catechin (4), and piceatannol (5).

MATERIALS AND METHODS

Reagents and equipments

All solvents were of analytical grade. Oleanolic acid, N-Succ-(Ala)₃-*p*-nitroanilide (SANA) and Procine pancreatic elastase (PPE) were

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purchased from Sigma. UV spectra were obtained in methanol on a Biochrom Libra S22 UV-visible spectrophotometer. ^1H (400 MHz) and ^{13}C (100.60 MHz) NMR spectra were recorded on a JNM-LA 400 (JEOL) instrument, with chemical shifts in ppm relative to the solvent used. Vacuum liquid chromatography (VLC) and column chromatography (CC) were performed using silica gel 60H (15 μm , Merck) and silica gel (0.063–0.2 mm, Merck), respectively. Silica gel 60 F₂₅₄ coated on aluminum plates for thin layer chromatography (TLC) was supplied by Merck. Gel filtration chromatography (GFC) was performed on Sephadex LH-20 (25 - 100 μm , Fluka). Preparative HPLC experiment was carried out using Waters Alliance 2695 equipped with Sunfire™ 10 x 250 mm column.

Plant material

The stems of *C. lanceolatus* were collected in Jeju in July, 2007. A voucher specimen (No. 263) is deposited at the Natural Product Chemistry Lab, Department of Chemistry, Cheju National University, Korea.

Extraction and isolation

Air-dried and powdered stems of *C. lanceolatus* (1.5 kg) were extracted with 70% ethanol (30.0 L) using magnetic stirrer at room temperature for 24 h. The extract was filtered and concentrated using a rotary evaporator at a temperature not exceeding 45°C. This procedure was repeated two times. The combined extract (67.2 g) was suspended in water and successively partitioned into *n*-hexane (Hex), ethyl acetate (EtOAc) and *n*-butanol.

A portion of EtOAc-soluble fraction (3 g) was subjected to VLC over a silica gel using gradient solvents (Hex-EtOAc-MeOH) to afford 21 fractions (fr. 01 - 21). Fraction 04 (250 mg) was applied to silica gel column chromatography with chloroform/methanol (12/1) to give four subfractions (fr. 04-1 to 04-4). The subsequent silica gel CC of fraction 04 - 2 (114 mg) with Hex/EtOAc (2/1) provided the compound 1 (25 mg). Similarly, the fraction 07 (714 mg) was also applied to column chromatography with chloroform/methanol (15/1) to give five fractions (fr. 07 - 1 to 07 - 5). The second fraction (07 - 2, 407 mg) was subjected to GFC with chloroform/methanol (10/1) to provide seven subfractions (fr. 07 - 2 - 1 to 07 - 2 - 7), among which the fifth fraction (07-2-5, 67 mg) was further purified by HPLC with acetonitrile/water (80/20) as a mobile phase to afford the compound 2 (7.4 mg). Another portion of EtOAc-soluble fraction (2.0 g) was again subjected to VLC over a silica gel using gradient Hex-EtOAc-MeOH solvents to afford 14 fractions (fr. 01' - FR 14'). Fraction 05' (150 mg) was silica gel column chromatographed with chloroform/methanol (4/1) to give seven subfractions (fr. 05' - 1 to 05' - 7). The fourth fraction (05' - 4, 14.4 mg) was identified as the compound 5, whereas the sixth fraction was identified as the compound 4 (7.3 mg). Fraction 05'-6 (48 mg) was subjected to GFC with chloroform/methanol (10/1) to provide five subfractions (fr. 05' - 6 - 1 to 05' - 6 - 5), among which the second fraction (05' - 6 - 2, 8.3 mg) was identified as the compound 3.

Betulinic acid (1) ^1H -NMR (400 MHz, CD₃OD) δ : 4.53 (1H, s, H-29), 4.39 (1H, s, H-29), 2.96 (1H, t, J = 7.8 Hz, H-3), 2.82 (1H, m), 2.06 (2H, m), 1.75 (2H, m), 1.52-0.70 (m, 20H), 1.49 (3H, s, H-30), 0.78 (3H, s), 0.76 (3H, s), 0.75 (3H, s), 0.63 (3H, s), 0.55 (3H, s); ^{13}C -NMR (100 MHz, CD₃OD) δ : 178.9 (C-28), 150.4 (C-20), 109.0 (C-29), 78.3 (C-3), 55.9 (C-17), 55.0 (C-5), 50.2 (C-9), 48.8 (C-19), 46.7 (C-18), 42.1 (C-14), 40.3 (C-8), 38.4 (C-1), 38.4 (C-4), 37.9 (C-13), 36.8 (C-10), 36.8 (C-22), 34.0 (C-7), 31.9 (C-16), 30.2 (C-15), 29.3 (C-21), 29.2 (C-21), 27.4 (C-23), 26.5 (C-2), 25.1 (C-12), 20.5 (C-11), 18.8 (C-30), 17.9 (C-6), 15.6 (C-25), 15.4 (C-26), 14.9 (C-24), 14.2 (C-27).

Pyracrenic acid (2) ^1H -NMR (400 MHz, CD₃OD) δ : 7.52 (1H, d, J = 15.8 Hz, H-7'), 7.03 (1H, d, J = 2.0 Hz, H-1'), 6.94 (1H, dd, J =

2.0, 8.0 Hz, H-5'), 6.78 (1H, d, J = 8.0 Hz, H-4'), 6.24 (1H, d, 15.8 Hz, H-8'), 4.71 (1H, d, J = 1.7 Hz, H-29), 4.60 (1H, d, J = 1.7 Hz, H-29), 3.03 (1H, m, H-3), 2.32 (1H, m), 2.24 (1H, m), 1.93 (2H, m), 1.80-0.70 (m, 21H), 1.70 (3H, s, H-30), 1.03 (3H, s), 0.99 (3H, s), 0.95 (3H, s), 0.92 (3H, s), 0.89 (3H, s); ^{13}C -NMR (100 MHz, CD₃OD) δ : 180.2 (C-28), 169.2 (C-9'), 152.0 (C-20), 149.4 (C-3'), 146.8 (C-7'), 146.6 (C-2'), 127.7 (C-6'), 122.9 (C-5'), 116.5 (C-8'), 115.6 (C-1'), 115.1 (C-4'), 110.2 (C-29), 82.3 (C-3), 57.5 (C-17), 56.9 (C-5), 51.9 (C-9), 50.4 (C-18), 48.4 (C-19), 43.6 (C-14), 41.9 (C-8), 39.6 (C-1), 39.6 (C-13), 39.1 (C-4), 38.3 (C-22), 38.1 (C-10), 35.5 (C-7), 33.3 (C-16), 31.7 (C-21), 30.8 (C-15), 28.5 (C-23), 26.8 (C-12), 24.8 (C-2), 22.1 (C-11), 19.5 (C-30), 19.3 (C-6), 17.1 (C-25), 16.7 (C-26), 16.6 (C-24), 15.1 (C-27).

Arjunolic acid (3) ^1H -NMR (400 MHz, CD₃OD) δ : 5.23 (1H, m), 3.68 (1H, m), 3.50 (1H, d, J = 5.8 Hz), 2.84 (1H, dd, J = 3.7, 10.0 Hz), 1.14 (3H, s), 1.00 (3H, s), 0.92 (3H, s), 0.88 (3H, s), 0.80 (3H, s), 0.71 (3H, s); ^{13}C -NMR (100 MHz, CD₃OD) δ : 181.8 (C-28), 144.1 (C-13), 121.4 (C-12), 78.5 (C-3), 68.0 (C-2), 67.6 (C-23), 47.8 (C-5), 47.3 (C-9), 46.3 (C-17), 45.8 (C-1), 45.7 (C-19), 42.3 (C-4), 41.5 (C-14), 41.1 (C-18), 38.9 (C-8), 37.6 (C-10), 33.6 (C-21), 32.7 (C-29), 32.5 (C-7), 31.8 (C-22), 30.5 (C-20), 29.3 (C-15), 27.3 (C-16), 25.5 (C-27), 23.1 (C-30), 22.7 (C-11), 17.8 (C-6), 16.5 (C-25), 16.4 (C-26), 12.5 (C-24).

Catechin (4) ^1H -NMR (400 MHz, CD₃OD) δ : 6.84 (1H, d, J = 2.0 Hz, H-2'), 6.77 (1H, dd, J = 8.1 Hz, H-5'), 6.71 (1H, dd, J = 2.0, 8.1 Hz, H-6'), 5.92 (1H, d, J = 2.4 Hz, H-8), 5.85 (1H, d, J = 2.4 Hz, H-6), 4.56 (1H, d, J = 8.0 Hz, H-2), 3.97 (1H, ddd, J = 8.0, 8.0, 4.8 Hz, H-3), 2.85 (1H, dd, J = 4.8, 16.0 Hz, H-4), 2.50 (1H, dd, J = 8.0, 16.0 Hz, H-4); ^{13}C -NMR (100 MHz, CD₃OD) δ : 158.0 (C-9), 157.7 (C-5), 157.1 (C-7), 146.4 (C-3'), 146.4 (C-4'), 132.4 (C-1'), 120.2 (C-6'), 116.2 (C-5'), 115.4 (C-2'), 101.0 (C-10), 96.4 (C-6), 95.7 (C-8), 83.0 (C-2), 69.0 (C-3), 28.7 (C-4).

Piceatannol (5) ^1H -NMR (400 MHz, CD₃OD) δ : 6.97 (1H, d, J = 2.0 Hz, H-2'), 6.89 (1H, d, J = 16.0 Hz, H-8), 6.83 (1H, dd, J = 2.0, 8.0 Hz, H-6'), 6.76 (1H, d, J = 16.0 Hz, H-7), 6.75 (1H, d, J = 8.0 Hz, H-5'), 6.43 (2H, d, J = 2.0 Hz, H-2 & H-6), 6.16 (1H, t, J = 2.0 Hz, H-4); ^{13}C -NMR (100 MHz, CD₃OD) δ : 159.6 (C-3 & C-5), 146.5 (C-3'), 146.5 (C-4'), 141.3 (C-1), 131.1 (C-1'), 129.7 (C-8), 127.0 (C-7), 120.2 (C-6), 116.4 (C-2'), 113.8 (C-5'), 105.8 (C-2 & C-6), 102.6 (C-4).

Elastase inhibition activity assay

Procine pancreatic elastase (PPE) was assayed using spectrophotometric method (Kraunsoe et al., 1996) with N-Succ-(Ala)₃-*p*-nitroanilide (SANA) as the substrate. The release of *p*-nitroaniline for 15 min at 25°C was monitored by measuring the absorbance at 410 nm. In the reaction mixture were contained 0.2 M Tris-HCl buffer (pH 8.0), 1 $\mu\text{g}/\text{mL}$ elastase, 0.8 mM SANA (ESIV; elastase substrate IV, Calbiochem) as substrate and different inhibitors (dissolved in 70% ethanol or DMSO). Each inhibitor was pre-incubated for 15 min at 25°C and the reaction was started by adding substrate. Blanks contained all the components except the enzyme. The oleonic acid was used as a positive control. Each treatment was replicated thrice.

The percentage of inhibition was calculated as:

$$\text{Inhibition (\%)} = (1 - B/A) \times 100$$

Where A is the enzyme activity without inhibitor, and B is the activity in the presence of inhibitor.

DPPH free radical scavenging activity test

The free radical scavenging activity was assayed spectrophotometrically (Blois, 1958) using deep blue 2,2-Diphenyl-1-picrylhydrazyl

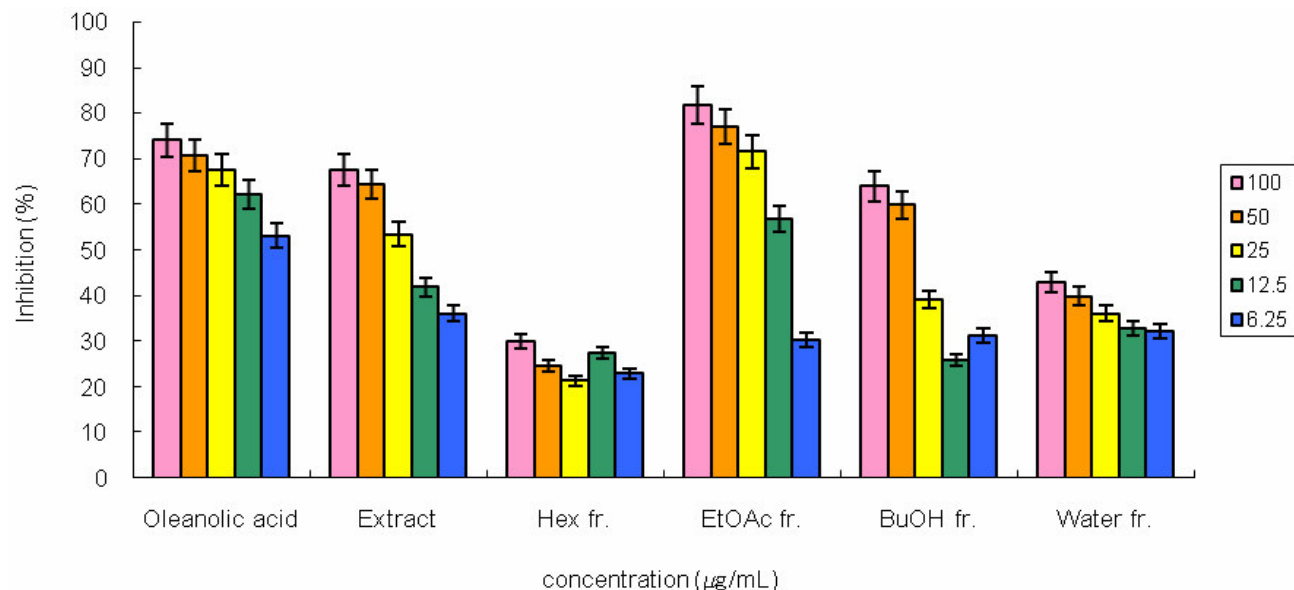


Figure 1. Elastase inhibition activities of the extracts from *C. lanceolatus*.

(DPPH) radical. The radical scavenging activity can be followed by a loss of absorbance at 525 nm. Sample stock solutions (1mg/mL) were diluted to final concentrations of 50, 25, 12.5 and 6.25 µg/mL in 70% ethanol or DMSO. DPPH solution was prepared using 1.2 mL DPPH (0.2 mM in ethanol), 3 mL ethanol and 0.5 mL DMSO. DPPH solution (0.45 mL) was added to 0.05 mL of sample solutions of different concentrations, shaken well by vortex, and allowed to react at room temperature. The absorbance values were measured after 10 min at 525 nm by UV/Vis spectrophotometer. The free radical scavenging activity of samples was calculated according to the formula:

$$\text{DPPH radical scavenging activity (\%)} = \frac{[1 - (\text{Abs}_{\text{sample}} - \text{Abs}_{\text{blank}})]}{\text{Abs}_{\text{control}}} \times 100$$

Where $\text{Abs}_{\text{sample}}$ is the absorbance of the experimental sample, $\text{Abs}_{\text{blank}}$ is the absorbance of the blank; $\text{Abs}_{\text{control}}$ is the absorbance of the control. The ascorbic acid (vitamin C) was used as a positive control. Each treatment was replicated thrice.

RESULTS AND DISCUSSION

The ethanol extract of *C. lanceolatus* stem exhibited strong anti-elastase activity dose-dependently with 50% inhibition (IC_{50}) at a concentration of 20.2 µg/mL. Oleanolic acid (IC_{50} at 3.0 µg/mL), being used as the ingredient of anti-wrinkle cosmetic formulations in Korea, is employed as the positive control in this experiment (Liu, 1995). In order to identify the active components, solvent fractionation was performed that is, the extract was suspended in water and partitioned successively with *n*-hexane, ethyl acetate, and *n*-butanol. Among all the fractions, the ethyl acetate soluble fraction was found to have the most effective anti-elastase activity with IC_{50} at 11.7 µg/mL (Figure 1).

The ethanol extract also showed strong antioxidant activity with DPPH radical scavenging experiment. Compared to ascorbic acid displaying 50% scavenging activity (RS_{50}) at 10.4 µg/mL, the extract exhibited RS_{50} at 17.0 µg/mL on this test. Each of the solvent fractions above mentioned were also subjected to the DPPH experiment. In this study, the scavenging effect was observed in the order of with *n*-butanol (RS_{50} 8.1 µg/mL), water (RS_{50} 14.1 µg/mL) and ethyl acetate (RS_{50} 19.4 µg/mL) fractions (Figure 2).

In order to identify the components responsible to these activities, ethyl acetate fraction was chosen for further study. Repeated column chromatography of the ethyl acetate fraction over silica gel and Sephadex LH-20 or employment of prep HPLC, resulted in the isolation of five compounds 1 - 5 as the active components (Figure 3). The structures of the isolated compounds were identified using spectroscopic data and their comparison with the literature values.

The compound 1 was inferred to be a triterpene based on ^{13}C NMR spectrum with 30 carbon peaks and ^1H NMR spectrum with a broad range of aliphatic signals including six methyl singlets. Further investigation of ^{13}C NMR spectrum revealed the characteristic signals for carboxylic acid (δ_{C} 178.9), vinyl carbons (quaternary C at δ_{C} 150.4 and CH_2 at δ_{C} 109.0) as well as an oxygen-bearing methine (δ_{C} 78.3). These informations suggested that the compound 1 is betulinic acid, which was confirmed by comparing the data to those in the literature (Cichewicz and Kouzi, 2004). By the examination of NMR spectra, the compound 2 was suggested as a derivative of betulinic acid bearing an additional aromatic ester moiety at C-3. The aromatic ester was identified as caffeate by ^1H NMR displaying *trans* olefin signals at δ 6.24 (d, $J = 15.8$ Hz)

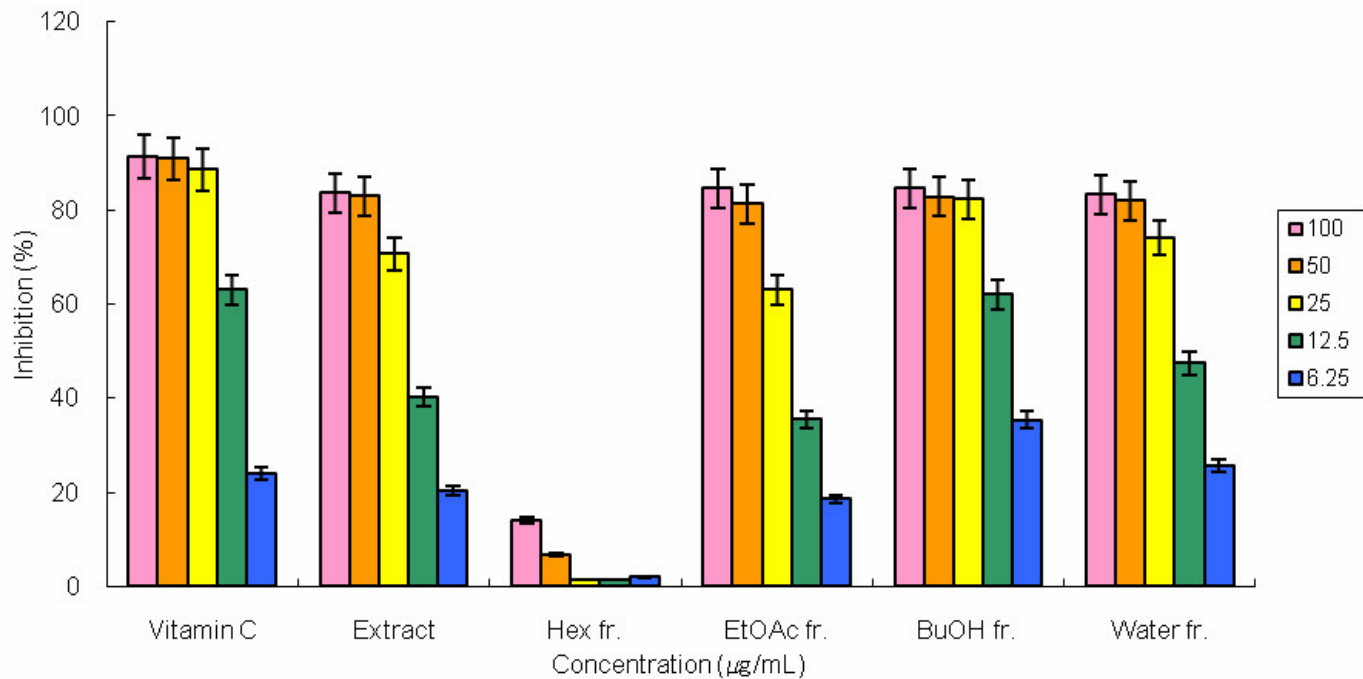
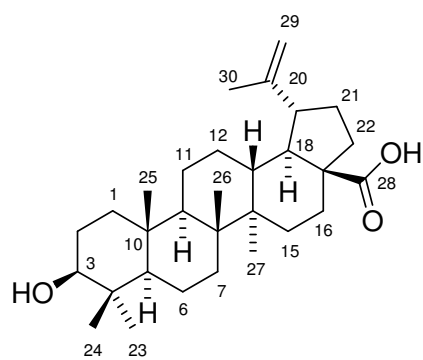
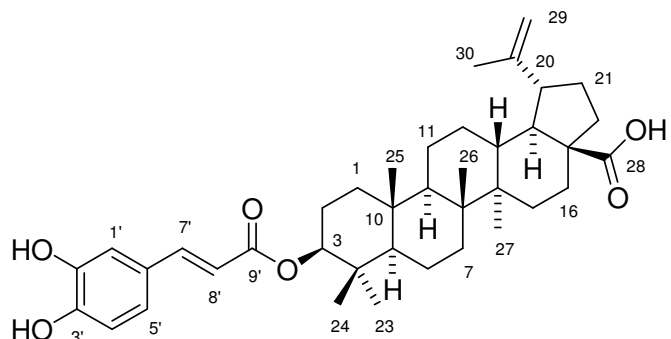


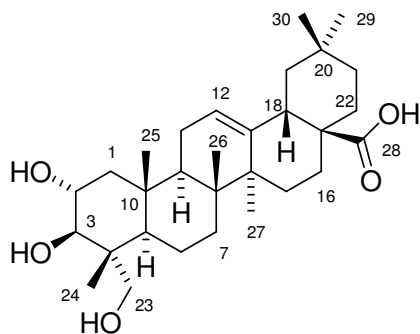
Figure 2. DPPH radical scavenging activities of the extracts from *C. lanceolatus*.



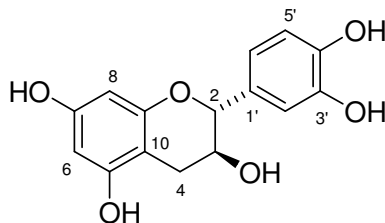
betulinic acid (1)



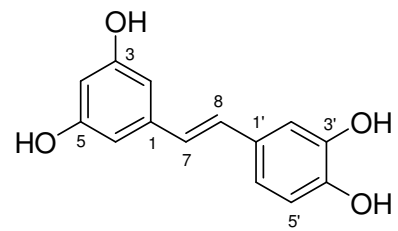
pyracrenic acid (2)



arjunolic acid (3)



catechin (4)



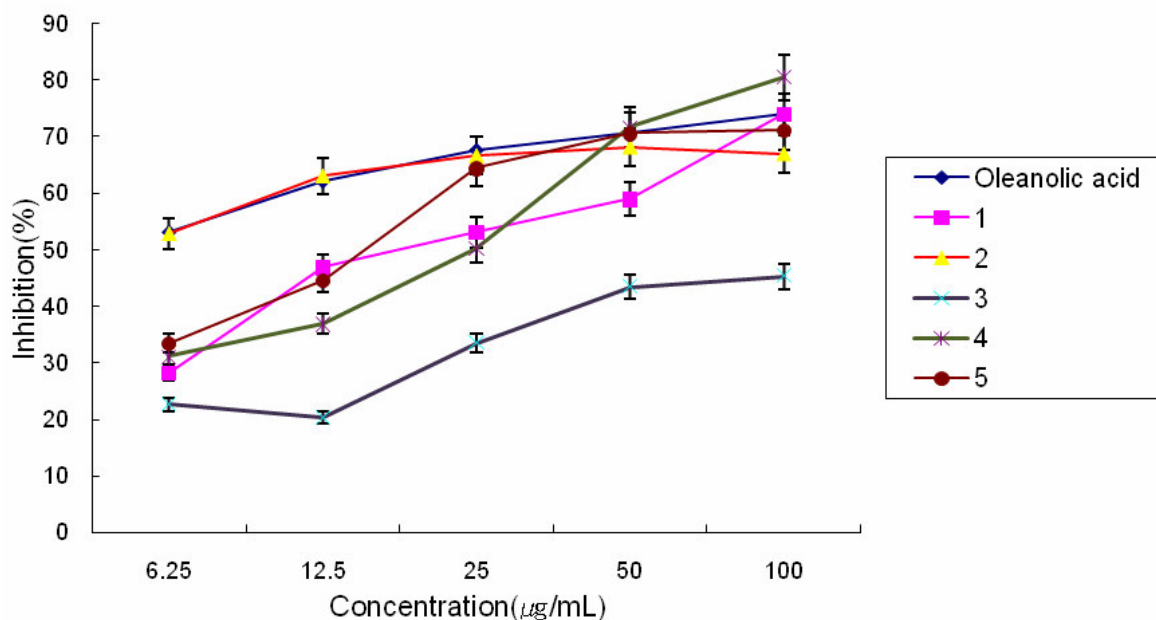
piceatannol (5)

Figure 3. Structures of the isolated compounds 1 - 5 from *C. lanceolatus*.

Table 1. Elastase inhibition activities of the isolated compounds 1 - 5 from *C. lanceolatus*.

Compounds	Inhibition (%)					IC ₅₀ (μ/mL)
	100 μ/mL	50 μ/mL	25 μ/mL	12.5 μ/mL	6.25 μ/mL	
Oleanolic acid	74.1	70.7	67.6	62.1	53.1	3.0
1	73.9	58.9	53.0	46.8	28.2	21.6
2	66.9	68.1	66.6	63.0	52.8	1.5
3	45.3	43.4	33.5	20.6	22.6	
4	80.5	71.6	50.2	36.9	31.1	20.2
5	71.1	70.6	64.4	44.6	33.5	15.6

IC₅₀ : Concentration (μg/mL) at which the inhibition of elastase activity is 50%.

**Figure 4.** Elastase inhibition activities of the compounds 1 - 5 isolated from *C. lanceolatus*.

and 7.52 (d, $J = 15.8$ Hz) coupled with 1,3,4-trisubstituted benzene peaks at δ 6.78 (d, $J = 8.0$ Hz), 6.94 (dd, $J = 8.0, 2.0$ Hz) and 7.03 (d, $J = 2.0$ Hz). Therefore, the compound 2 was identified as betulinic acid 3 β -*O*-caffeate, pyracrenic acid, which was confirmed by comparing its data to literature values (Pan et al., 1994). Very recently, the compounds 1 and 2 have been isolated from the aerial part of *C. lanceolatus* (Jeong et al, 2009).

Based on ¹³C NMR spectrum showing 30 carbon peaks and ¹H NMR spectrum with some aliphatic signals including six methyl singlets, the compound 3 was also inferred as a triterpene. Spectrum of ¹³C NMR revealed the characteristic signals for carboxylic acid (δ_C 181.8), an olefin (quaternary C at δ_C 144.1 and CH at δ_C 121.4) as well as oxygen-bearing methines (δ_C 68.0, 78.5) and methylene (δ_C 67.6). Comparison of these data to the literature value (Higuchi and Kawasaki, 1976) confirmed that the compound 3 is arjunolic acid (2 $\alpha,23$ -dihydroxy-oleanolic acid). The compound 3 were identified as cate-

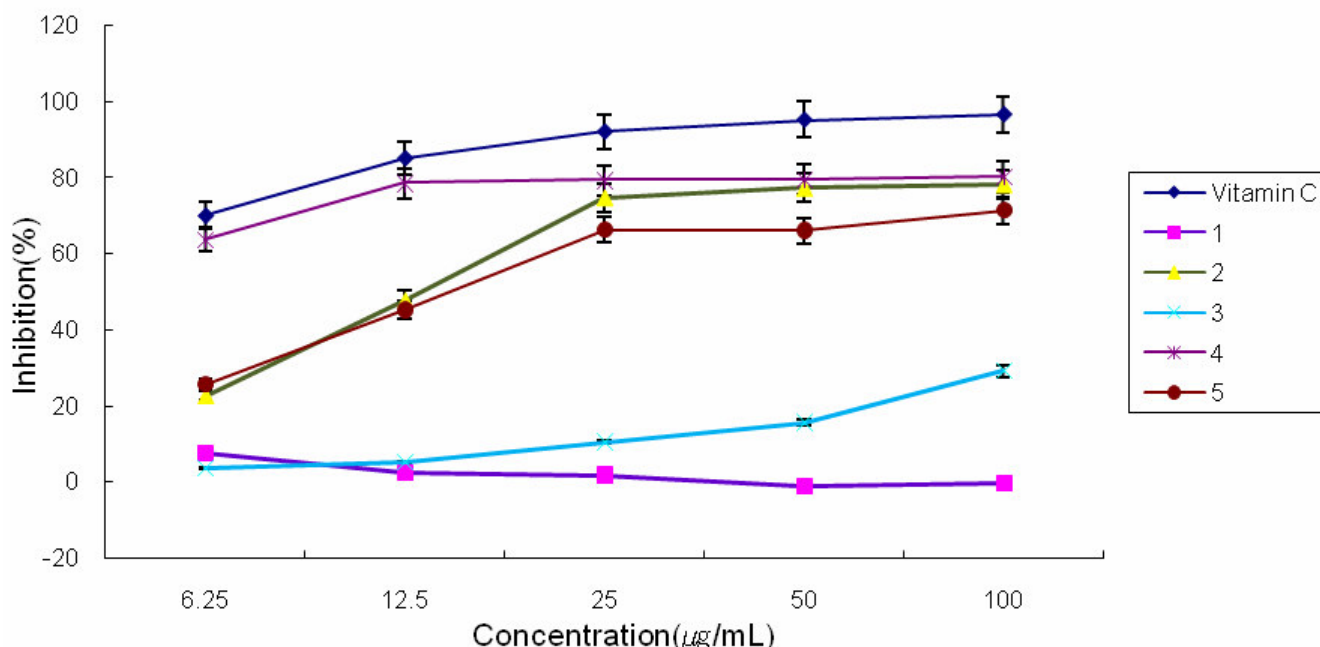
chin by comparing its data to those in the literature (Chung et al., 1996). The compound 5 has a structure of *trans*-stilbene skeleton, and identified as piceatanol, previously isolated from the fruit of *C. citrinus* (Park and Lee, 2008). Interestingly, the compounds 3-5 were isolated for the first time from the stems of *C. lanceolatus* in this study.

The isolated compounds 1-5 were investigated for their anti-elastase activities using N-Succ-(Ala)₃-*p*-nitroanilide as the substrate. The test solutions for each compound were prepared at varying concentrations (6.25 to 100 μg/mL) and examined for their inhibition activities by monitoring the absorbance at 410 nm due to the release of *p*-nitroaniline. The results are summarized in Table 1 and Figure 4. Among all the compounds tested, pyracrenic acid (2) showed strong elastase inhibition activity (IC₅₀ 1.5 μg/mL), which is two times more active than the oleanolic acid. Betulinic acid (1), catechin (4) and piceatanol (5) showed the moderate activities with IC₅₀ 21.6,

Table 2. DPPH radical scavenging activities of the compounds 1 - 5 from *C. lanceolatus*.

Compounds	Inhibition (%)					RC ₅₀ (μ /mL)
	100 μ /mL	50 μ /mL	25 μ /mL	12.5 μ /mL	6.25 μ /mL	
Ascorbic acid	96.5	95.1	92.0	85.0	69.9	4.2
1	-0.5	-1.3	1.6	2.1	7.3	-
2	78.1	77.2	74.7	47.7	22.6	13.1
3	29.0	15.5	10.4	5.1	3.6	-
4	80.1	79.4	79.2	78.4	63.6	4.4
5	71.2	65.9	66.2	45.1	25.6	14.5

RS₅₀ : Concentration (μg/mL) at which the radical scavenging activity is 50%.

**Figure 5.** DPPH radical scavenging activities for the compounds 1 - 5 from *C. lanceolatus*.

20.2 and 15.6 μg/mL, respectively.

DPPH radical scavenging activities were also investigated for the isolated compounds 1-5. The results are summarized in Table 2 and Figure 5. Compared to the ascorbic acid (RS₅₀ 4.2 μg/mL), catechin (4) exhibited the comparable activity with RS₅₀ 4.4 μg/mL. Pyracrenic acid (2) and piceatannol (5) showed moderate activities with RS₅₀ 13.1 μg/mL and 14.5 μg/mL, respectively.

Conclusion

Bioassay-guided investigation of the stems of *C. lanceolatus* led to the isolation of three triterpenoids (1 - 3), together with catechin (4) and piceatannol (5). We investigated their elastase inhibition and radical scavenging activities for the development of skin anti-wrinkle ingredients in cosmetic formulation. Among the isolated

compounds, pyracrenic acid (2) exhibited very strong elastase inhibition activity with an IC₅₀ value of 1.5 μg/mL, which is two times more active than oleanolic acid. On the other hand, catechin (4) displayed strong DPPH radical scavenging activity (RS₅₀ 4.4 μg/mL) comparable to that of ascorbic acid (RS₅₀ 4.2 μg/mL). The examined activities of the extracts as well as the isolated compounds from *C. lanceolatus* implied that this plant could be potential candidate for the development of novel cosmetic additives.

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