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Anticoagulation and Anticancer Constituents from *Eugenia caryophyllata* Thunb

Kyung Min Han*, Dong Hyun Kim*, Eun Mi Ahn, Youn Hyung Lee***, In Sik Chung*, Dae Keun Kim****, Byoung Mog Kwon*****, Sung Hoon Kim******, and Nam In Baek*†**

*Graduate School of Biotechnology & Plant Metabolism Research Center, Kyung Hee University, Yongin 449-701, Korea.
**Department of Herbal Food Science, Daegu Haany University, Gyeongsan 712-715, Korea.
***Major in Horticultural Biotechnology, Kyung Hee University, Yongin 449-701, Korea.
****Department of Pharmacy, Woosuk University, Jeonju 565-701, Korea.
*****Korea Research Institute of Bioscience and Biotechnology, Daejeon 305-333, Korea.
******Graduate School of East-West Medical Science, Kyung Hee University, Yongin 449-701, Korea.

ABSTRACT : From the EtOAc fraction of *Eugenia caryophyllata*, four compounds were isolated through activity-guided silica gel column chromatography. From the result of spectroscopic data including NMR, MS and IR, the chemical structures of the compounds were determined as 1-allyl-4-hydroxy-3-methoxybezene acetate (eugenol acetate, 1), 1-allyl-4-hydroxy-3-methoxybezene (eugenol, 2), 3β-hydroxyolean-12-en-28-oic acid (oleanolic acid, 3) and 2α,3β-dihydroxyolean-12-en-28-oic acid (maslinic acid, 4). Compounds 3 and 4 were isolated for the first time from this plant. Also, compounds 1, 2 and 3 exhibited relatively high platelet aggregation inhibitory activity with the IC_{50} values of 0.24, 0.09 and 0.07 mM, respectively. Compound 2 significantly prolonged activated partial thromboplastin time (aPTT) with the value of 124 ± 11.2 seconds as compared to the control with the value of 37.5 ± 2.2 seconds at the concentration of 50 μg/mℓ. Compounds 1 and 3 revealed inhibitory activity on farnesyl protein transferase (FPTase) with the IC_{50} values of 0.49 and 0.24 mM and compounds 1 and 2 highly inhibited the growth of rat-H-ras cells with the GI_{50} values of 6.63 and 5.70 μM, respectively.

Key words **:** *Eugenia caryophyllata*, PLT aggregation, aPTT, FPTase, rat-H-ras, eugenol, eugenol acetate, oleanolic acid, maslinic acid

INTRODUCTION

The flower buds of *Eugenia caryophyllata* Thunb (cloves) have been used as an herbal drug to treat several diseases such as digestive system disorders, bacterial and fungal infection (Zhang and Chen, 1997), toothache, headache, and joint pain (Shelef, 1983; Soto and Burhanuddin, 1995). This essential oil is also used as a local anesthetic and analgesic for sterilizing purposes. It was also reported to have anti-fungal, anticonvulsant (Harbone and Baxter, 1993), anticarcinogenic (Zheng *et al.*, 1992), antimutagenic (Miyazawa and Hisama, 2001) and antiplatelet activities (Cho *et al.*, 2004). Phytochemical analysis of clove oil has shown the presence of eugenol, acetyl eugenol, β-caryophyllene, methyl-*n*-pentyl ketone, salicylic acid methyl ester, humulene, benzaldehyde, chavicol, etc. (Jung and Shin, 1990). Its active ingredient, eugenol, comprises between 70% and 90% of the clove oil weight (Keene *et al.*, 1998). It reportedly participates in photochemical reaction (Mihara and Shibamoto, 1982) and possesses antioxidant activity (Ogata *et al.*, 2000), insecticidal activity (Park *et al.*, 2000), antiplatelet activity (Srivastava, 1993) and photocytotoxicity (Atsumi *et al.*, 2001). However the activities of other compounds have not been extensively studied so far. Especially, the methanol extracts of the plant showed significant inhibitory activity on FPTase and platelet (PLT) aggregation activity. So, in this paper the authors reported the isolation of some compounds from the flower buds of *E. caryophyllata* Thunb and their inhibitory activity not only on PLT aggregation and activated partial thromboplastin time (aPTT) delay activity related to anticoagulation activity but also on FPTase and rat-H-ras related to anticancer activity.

MATERIALS AND METHODS

Plant materials

The cloves, flower buds of *Eugenia caryophyllata*, were purchased from Boeun Medical Herb Shop, Kyungdong Market, Seoul, Korea, and identified by Prof. Dae-Keun Kim,

† Corresponding author: (Phone) +82-31-201-2661 (E-mail) nibaek@khu.ac.kr
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Woosuk University, Jeonju, Korea. A voucher specimen (KHU01011) was reserved at the Laboratory of Natural Products Chemistry, Kyung Hee University, Suwon, Korea.

Instrumentation

Melting points were determined on a Fisher-John apparatus (Fisher Scientific, USA) and uncorrected. Optical rotations were measured on a JASCO P-1010 digital polarimeter (JASCO, Japan). EI-MS was recorded on a JEOL JMSAX 505-WA (JEOL, Japan). IR spectra were run on a Perkin Elmer Spectrum One FT-IR spectrometer (Perkin Elmer, USA). ^{1}H-NMR (400 MHz) and ^{13}C-NMR (100 MHz) spectra were taken on a Varian Unity Inova AS 400 FT-NMR spectrometer (Varian, USA). The platelet count was carried out in a dual channel whole blood Lumi-ionized Calcium Aggregometer (Chrono-Log Corp, Ltd, USA). The plasma clotting times, activated partial thromboplastin time (aPTT) was counted with Automated Coagulation Laboratory 100 Instrument (Instrumentation Laboratory Company, Italy). Radioactivities were measured in a Wallac 1450 microbeta TRILUX liquid scintillation counter (Perkin Elmer, USA).

Isolation of compounds from the flower buds of *Eugenia caryophyllata.*

The 5.4 kg of cloves, flower buds of *E. caryophyllata*, were finely powdered, extracted with 15 ℓ of 80% aqueous methanol twice at room temperature overnight and filtered. The combined filtrate was concentrated under vacuum at 40 ℃ and the concentrate was sequentially partitioned into ethyl acetate (2 ℓ × 2) and water (2 ℓ) layers. The organic layer was concentrated to dryness by rotary evaporation at 35 ℃ to yield the active fraction, EtOAc extracts (ECE 390 g).

The EtOAc extract (200 g) was chromatographed on the silica gel column (Merck 70-230 mesh, 300 g, 7 ㎝ i.d. × 25 ㎝) and eluted with $CHCl_3$/MeOH (25 : 1 and 10 : 1, 5000 mℓ each) to afford five fractions (ECE1~ECE5). Each fraction was analyzed by TLC (silica gel 60 F_{254}), and fractions with similar patterns on the TLC plates were collected. The second fraction {ECE2, Ve/Vt (elution volume/total volume, $CHCl_3$-MeOH = 10 : 1) 0.05-0.10, 40 g} was successively chromatographed on the silica gel column (5.5 ㎝ i.d. × 20 ㎝) using *n*-hexane : $CHCl_3$: MeOH (20 : 3 : 1, 5 : 3 : 1, 7000 mℓ each) as eluting solution to yield six fractions (ECE2-1~ECE2-6), containing compound **2** {ECE2-3, Ve/Vt (*n*-hexane : $CHCl_3$: MeOH = 20 : 3 : 1) 0.85-0.90, Rf value on silica gel TLC in $CHCl_3$-MeOH (30 : 1) = 0.5, 14 g}. ECE2-2 (Ve/Vt (*n*-hexane : $CHCl_3$: MeOH = 20 : 3 : 1) 0.55-0.80, 19 g) was applied to silica gel column chromatography (4 ㎝ i.d. × 25 ㎝) and eluted with *n*-hexane : EtOAc (5 : 1, 3000 mℓ) to produce compound **1** {ECE2-2-2, Ve/Vt 0.65-0.80, Rf value on the silica gel TLC in $CHCl_3$-MeOH (30 : 1) = 0.6, 1 g}. ECE3 {Ve/Vt ($CHCl_3$-MeOH = 10 : 1) 0.10-0.45, 2 g) was chromatographed on the silica gel column (4.5 ㎝ i.d. × 14 ㎝) with $CHCl_3$: MeOH (30 : 1, 2500 mℓ), collecting four fractions (ECE3-1 to ECE3-4). ECE3-2 (Ve/Vt 0.25-0.40, 0.6 g) was applied to the silica gel c.c. (4.5 × 10 ㎝) and eluted with $CHCl_3$: MeOH (25 : 1, 1500 mℓ) to produce compound **3** {ECE-3-2-2, Ve/Vt 0.65-0.75, Rf value on silica gel TLC in $CHCl_3$-MeOH (12 : 1) = 0.5, 0.4 g}. ECE3-3 (Ve/Vt 0.40-0.65, 0.5 g) was subjected to the silica gel c.c. (4.5 × 9 ㎝) and eluted with $CHCl_3$: MeOH (10 : 1, 1500 mℓ) to produce compound **4** {ECE-3-3-2, Ve/Vt 0.60-0.75, Rf value on silica gel TLC in $CHCl_3$-MeOH (3 : 1) = 0.5, 0.25 g}.

1-Allyl-4-hydroxy-3-methoxybezene acetate (eugenol acetate, 1)

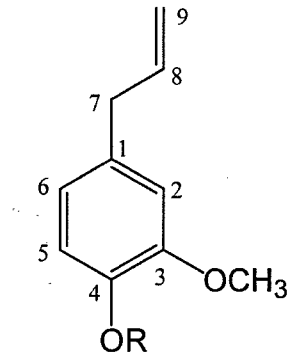
Pale brown oil ($CHCl_3$); EI/MS *m/z*: 206 $[M]^+$, 164, 149, 131, 43; IR_δ ($CHCl_3$, cm^{-1}) 2850, 1765, 1605, 1270, 1200, 1150, 990, 910, 825; ^{1}H-NMR (400 MHz, $CDCl_3$, δ) 6.95 (1H, d, *J* = 7.8, H-5), 6.78 (1H, d, *J* = 1.6 Hz, H-2), 6.76 (1H, dd, *J* = 7.8, 1.6 Hz, H-6), 5.97 (1H, ddt, *J* = 17.0, 9.6, 7.2 Hz, H-8), 5.09 (1H, dd, *J* = 17.0, 1.6 Hz, H-9 trans), 5.05 (1H, dd, *J* = 9.6, 1.6 Hz, H-9 cis), 3.79 (3H, s, $-OCH_3$), 3.36 (2H, d, *J* = 7.2 Hz, H-7), 2.28 (3H, s, acetate-CH_3), ^{13}C-NMR (100 MHz, $CDCl_3$, δ) 169.0 (acetate-carbonyl), 150.6 (C-3), 138.8 (C-4) 137.7 (C-1), 136.8 (C-8), 122.3 (C-5), 120.5 (C-6), 116.0 (C-9), 112.5 (C-2), 55.7 ($-OCH_3$), 40.0 (C-7), 20.6 (acetyl-Me).

1-Allyl-4-hydroxy-3-methoxybezene (eugenol, 2)

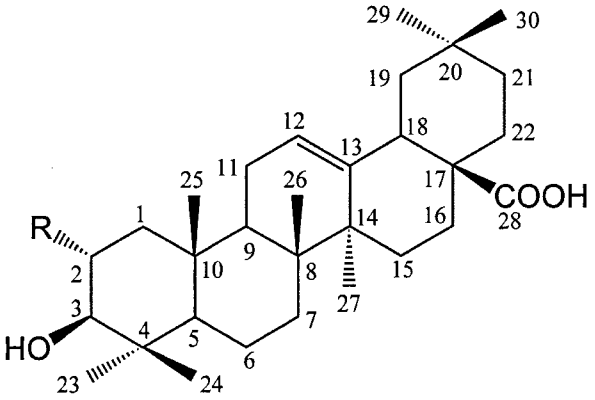
Pale brown oil ($CHCl_3$); EI/MS *m/z*: 164 $[M]^+$, 149, 131, 121, 103, 91, 77, 65, 55; IR_υ ($CHCl_3$, cm^{-1}) 3500, 3080, 1630, 1605, 1507, 1260, 1230, 990, 910; ^{1}H-NMR (400 MHz, $CDCl_3$, δ) 6.88 (1H, d, *J* = 8.7 Hz, H-5), 6.71 (1H, d, *J* = 2.4 Hz, H-2), 6.70 (1H, dd, *J* = 8.7, 2.4 Hz, H-6), 5.97 (1H, ddt, J = 17.0, 9.6, 7.2 Hz, H-8), 5.09 (1H, dd, *J* = 17.0, 1.6 Hz, H-9 trans) 5.07 (1H, dd, *J* = 9.6, 1.6 Hz, H-9 cis), 3.86 (3H, s, $-OCH_3$), 3.34 (2H, d, *J* = 7.2 Hz, H-7), ^{13}C-NMR (100 MHz, $CDCl_3$, δ) 146.3 (C-3), 143.7 (C-4), 137.8 (C-1) 131.9 (C-8), 121.1 (C-6), 115.5 (C-9), 114.1 (C-2), 111.0 (C-5), 55.8 ($-OCH_3$), 39.9 (C-7).

3β-Hydroxyolean-12-en-28-oic acid (oleanolic acid, 3)

White powder ($CHCl_3$); m.p. 296-298 ℃; $[\alpha]_D = +70.0^\circ$ (*c* = 0.4, $CHCl_3$); EI/MS *m/z*: 456 $[M]^+$, 438, 248, 207, 203, 189; IR_υ ($CHCl_3$, cm^{-1}) 3420, 2930, 1680; ^{1}H-NMR (400 MHz, pyridine-d_5, δ) 5.49 (1H, br. s, H-12), 3.35 (1H, dd, *J* = 14.0, 4.0 Hz, H-3), 1.32 (3H, s, H-23), 1.28 (3H, s, H-27),



1 (eugenol acetate) : R = acetyl
2 (eugenol) : R = H



3 (oleanolic acid) : R = H
4 (maslinic acid) : R = OH

Fig. 1. Chemical structures of compounds isolated from the flower buds of *Eugenia caryophyllata*.

1.07 (3H, s, H-24), 1.05 (3H, s, H-30), 1.04 (3H, s, H-25), 0.99 (3H, s, H-26), 0.95 (3H, s, H-29), ^{13}C-NMR (100 MHz, pyridine-d_5, δ) 179.4 (C-28), 144.3 (C-13), 122.2 (C-12) 77.8 (C-3), 55.6 (C-5), 47.8 (C-9), 46.5 (C-19), 46.1 (C-17), 41.8 (C-14), 41.6 (C-18), 39.7 (C-8), 39.5 (C-4), 38.5 (C-1), 36.9 (C-10), 34.6 (C-29), 33.8 (C-21), 32.9 (C-22), 32.7 (C-7), 31.8 (C-20), 28.3 (C-23), 27.9 (C-15), 27.7 (C-2), 25.6 (C-27), 23.3 (C-16), 23.3 (C-30), 23.3 (C-11), 18.2 (C-6), 17.1 (C-26), 16.1 (C-24), 15.1 (C-25).

2α, 3β-Dihydroxyolean-12-en-28-oic acid (maslinic acid, 4)

White powder ($CHCl_3$-MeOH); m.p. 246-251 ℃; $[\alpha]_D$= 59.4° (*c* = 1.2, MeOH); EI/MS *m/z*: 472 $[M]^+$, 426, 408, 248, 223, 203; IR_{υ} ($CHCl_3$, cm^{-1}) 3400, 2910, 1675; ^{1}H-NMR (400 MHz, pyridine-d_5, δ) 5.46 (1H, br. s, H-12), 4.09 (1H, ddd, *J* = 11.4, 9.0, 4.4 Hz, H-2), 3.39 (1H, d, *J* = 9.0 Hz, H-3), 1.26 (3H, s, H-23), 1.25 (3H, s, H-27), 1.07 (3H, s, H-24), 1.01 (3H, s, H-30), 0.99 (3H, s, H-25), 0.98 (3H, s, H-26), 0.93 (3H, s, H-29), ^{13}C-NMR (100 MHz, pyridine-d_5, δ) 179.0 (C-28), 144.4 (C-13), 121.7 (C-12) 83.2 (C-3), 68.0 (C-2), 55.4 (C-5), 47.7 (C-9), 47.3 (C-1), 46.2 (C-17), 46.0 (C-19), 41.7 (C-18), 41.5 (C-14), 39.4 (C-8), 39.3 (C-4), 38.0 (C-10), 33.8 (C-21), 32.8 (C-29), 32.8 (C-7), 32.7 (C-22), 30.5 (C-20), 28.9 (C-23), 27.8 (C-15), 25.7 (C-27), 23.5 (C-11), 23.3 (C-30), 23.3 (C-16), 18.4 (C-6), 17.2 (C-26), 17.1 (C-25), 16.4 (C-24).

Table 1. Anticoagulation and anticancer activities of the compounds from the flower buds of *Eugenia caryophyllata*

	PLT aggregation[a] IC_{50}[†] (mM)	aPTT[b] (50 μg/mℓ)	FPTase[c] IC_{50} (mM)	rat-H-ras[d] GI_{50}[§] (μM)
Compound 1	0.24	37.5 ± 3.4 s	0.49	6.63
Compound 2	0.09	124 ± 11.2 s	1.42	5.70
Compound 3	0.07	29.4 ± 2.5 s	0.24	ND[¶]
Compound 4	0.57	36.9 ± 3.5 s	0.82	ND
control	0.16 [A]	37.5 ± 2.2 s [B]	0.18 [C]	29.6 [D]

[†]IC_{50} value is the concentration at which 50% of the activity is inhibited. [‡]The data are presented as mean ± standard deviation of three replications. [§]GI_{50} is the concentration at which 50% of the cell growth is inhibited. [¶]No data; 50% inhibition was not achieved. [a]Inhibitory activity on platelet aggregation; [b]Activated partial thromboplastin time; [c]Inhibitory activity on Farnesyl protein transferase; [d]Inhibitory activity on the growth of rat-H-ras cell lines. Control: [A]aspirin; [B]heparin (negative control); [C]2-hydroxy-cinnamaldehyde; [D] dehydrotrametenolic acid.

PLT aggregation assay

The stock solutions of compounds were prepared by dissolving them in dimethyl sulfoxide (DMSO) and further diluted in 0.9% saline when in use. Blood samples from healthy volunteers who had not taken any drugs for at least 15 days were collected by venopuncture into a plastic flask containing 3.15% sodium citrate (1 : 9, v/v). Platelet rich plasma (PRP) was obtained by centrifugation of the citrated blood at room temperature at 120 × g for 10 min. The platelet count was adjusted to 3.0×10^8 platelets per mℓ and platelet aggregation was monitored in a dual channel whole blood Lumi-ionized Calcium Aggregometer (Chrono-Log Corp, Ltd, USA). Human PRP (400 μℓ) was incubated at 37℃ for 2 min in the aggregometer with stirring at 1000 rpm. A fixed amount (50 μℓ) of compounds as samples or aspirin as a positive control drug was added and incubated at 37 ℃ for 3 min. After incubation, platelet aggregation was induced by the addition of collagen (50 μg). Changes in light transmission were recorded for 7 min after stimulation with collagen. The aggregation was expressed as % inhibition (*X*) using the following equation. X (%) = $A - B/A \times 100$; *A*, maximal aggregation of the control; *B*, maximal aggregation of sample-treated PRP. The doses of

agonists, which exhibited maximal aggregation in the control, were determined in preliminary experiments. IC_{50} values were calculated to determine the concentration at which 50% of the activity was inhibited.

aPTT assay

Anticoagulation activity of compounds was evaluated by measuring plasma clotting times as described earlier (Yuk *et al.*, 2000). The plasma clotting times such as activated partial thromboplastin time (aPTT) were counted with Automated Coagulation Laboratory 100 Instrument (Instrumentation Laboratory Company, Italy) using platelet poor plasma that was obtained by centrifugation of the human platelet rich plasma at $850 \times g$ for 15 min. The plasma was incubated with compounds or heparin for 7 min at 37℃. Then, 100 ㎕ of the incubated plasma was mixed with 50 ㎕ of cephaline in the process plate, and the coagulation was started by the addition of 1mM $CaCl_2$, 100 ㎕ of thromboplastin and bovine thrombin. Anticoagulation activity was evaluated by assaying prolongation of the plasma clotting time from at least three separate experiments

FPTase inhibitory activity assay

The FPTase assay was done using a scintillation proximity assay (SPA) kit following the protocol described by the manufacturer (Amersharm Bioscience), except that a biotinylated substrate peptide containing the Ki-Ras carboxyl-terminal sequence was used. The C-terminal peptide of Ki-Ras (Peptron Inc, Korea) was used as a Biotin-KKKSKTKCVIM synthesized by solid-phase peptide synthesis. FPTase activity was determined by measuring the transfer of [^{3}H]-farnesyl from [^{3}H]-farnesyl pyrophosphate to Biotin-KKKSKTKCVIM. Typical reaction mixtures (100 ㎕ total) contained 50 mM HEPES, pH 7.5, 30 mM $MgCl_2$, 20 mM KCl, 5 mM DTT, 0.01% Triton X-100, 150-250 nM [^{3}H]-farnesyl pyrophosphate (60 μM, 1 Ci/㎕), 25-50 ng (approximately 2.5-5 nM) of recombinant rat FPTase or 10 ㎕ of partially purified Q-Sepharose-derived FPTase, the indicated concentration of 2-hydroxycinnamaldehyde or dimethyl sulfoxide (DMSO) vehicle control (10%, v/v, final), and 10-200 nM Biotin-KKKSKTKCVIM. After 45 min incubation in a water bath at 37℃, reactions were stopped by adding 150 ㎕ of STOP/bead reagent into each tube or well. The reaction mixtures were mixed with vortex and left to stand for 30 min at room temperature. Samples were measured in a Wallac 1450 microbeta TRILUX liquid scintillation counter. IC_{50} values were calculated to determine the concentration at which 50% of the activity was inhibited relative to the DMSO vehicle control (Reiss *et al.*, 1990).

Rat-H-ras inhibitory activity assay

H-ras (H-ras gene transformed NIH3T3 cell) solid tumor cell lines were cultured in 10% of bovine serum albumin or RPMI-1640 medium (containing L-glutamine and not containing sodium bicarbonate; Gibco-BRL) under 37℃ and 5% CO_2 condition. The subculture was carried out 2 times a week. The cells were separated from the wall using 0.5% of trypsin and 5.3 mM EDTA (ethylenediamine tetra acetic acid) dissolved in phosphate buffer solution (PBS). In each well of 96-well plate (Falcon), $3 \times 10^3 - 6 \times 10^3$ cells were added and cultured at 37 ℃ and 5% CO_2 condition for 24 hr. The compounds were dissolved in dimethyl sulfoxide (DMSO) and diluted to the concentration required for the test. The final concentration of DMSO was set to be lower than 0.5%. After 24 hr, cells were replenished with fresh complete medium containing compounds or 0.1% DMSO. After a further 48 hr of incubation, cell proliferation reagent WST-1 (Roche) was added to each well. Then the culture plate was placed for 3 hrs at 37℃ and 5% CO_2 condition. When the reaction was completed, light absorption at 450-690 ㎚ was measured by using an ELISA reader to identify the cancer cell growth inhibition effect. The GI_{50}, the concentration when the cancer cell growth is inhibited by 50%, was about 5.5 mu./㎖ for the NIH3T3 cell and about 1.2 mu./㎖ for the H-Ras cell.

RESULTS AND DISCUSSION

The methanol extract of *Eugenia caryophyllata* showed inhibitory activity on FPTase with the value of $67 \pm 4.6\%$ and on PLT aggregation of $76 \pm 2.9\%$ at the concentration of 100 ug/㎖. Therefore, the methanol extract was fractionated into EtOAc layer, *n*-BuOH layer and H_2O layer through solvent fractionation. The repeated silica gel column chromatographies of EtOAc fraction, which showed the inhibitory activity on FPTase with the value of $79 \pm 3.8\%$ and on PLT aggregation with $84 \pm 3.6\%$ at the same concentration as above, supplied two phenylpropanoids and two triterpenoids, compounds **1-4**.

Compound **1**, colorless oil, showed the absorbance bands due to the carbonyl (1765 ㎝$^{-1}$) and olefine (1605 ㎝$^{-1}$) in the IR spectrum ($CHCl_3$) and molecular ion peak ([M]$^+$) at *m/z* 206 in the EI/MS spectrum. In the ^{1}H-NMR spectrum (400 MHz, $CDCl_3$), olefinic methine (δ 6.95-δ 5.97), exomethylene (δ 5.08 and δ 5.05), methoxy (δ 3.79), sp^3-methylene (δ 3.36) and acetate-methyl (δ 2.28) signals were observed. In the ^{13}C-NMR spectrum (100 MHz, $CDCl_3$), twelve signals consisting of one acetate (δ_C 169.0 and 20.6), two olefinic oxygenated quaternary (δ_C 150.6 and δ_C 138.8), one olefinic quaternary (δ_C 137.7), four olefinic methine (δ_C 136.8, 122.3, 120.5 and 112.5), one exomethylene (δ_C 116.0), one methoxy (δ_C 55.7)

and one sp^3-methylene (δ_C 40.0) signals were observed. These findings led to the conclusion that compound **1** is a phenylpropanoid with one acetate and one methoxy. Compound **1** was finally identified as 1-allyl-4-hydroxy-3-methoxybezene acetate (eugenol acetate) through the comparison of several physical and spectral data with those of the literature (Kwon and Lee, 2001).

Compound **2**, colorless oil, showed the absorbance bands due to the hydroxy (3500 cm^{-1}) and olefine (1630 cm^{-1}) in the IR spectrum ($CHCl_3$) and molecular ion peak (M^+) at *m/z* 164 in the EI/MS spectrum. In the ^{1}H-NMR spectrum (400 MHz, $CDCl_3$), olefinic methine (δ 6.88-δ 5.97), exomethylene (δ 5.09-5.07), methoxy (δ 3.86) and sp^3-methylene (δ 3.34) signals were observed. In the ^{13}C-NMR spectrum (100 MHz, $CDCl_3$), ten signals consisting of two olefinic oxygenated quaternary (δ_C 146.3 and 143.7), one olefinic quaternary (δ_C 137.8) and three olefinic methine (δ_C 131.9, 121.1, 115.5 and 114.1), one exomethylene (δ_C 111.0), one methoxy (δ_C 55.8) and one sp^3-methylene (δ_C 39.9) signals were observed. From these results, it was concluded that compound **2** is a phenylpropanoid with one hydroxy and one methoxy. Compound **2** was finally identified as 1-allyl-4-hydroxy-3-methoxybezene (eugenol) through the comparison of several physical and spectral data with those of the literature (Kwon and Lee, 2001).

Compound **3**, white powder, showed the absorbance bands due to the hydroxy (3500 cm^{-1}) and olefine (1680 cm^{-1}) in the IR spectrum ($CHCl_3$) and molecular ion peak (M^+) at *m/z* 456 in the EI/MS spectrum. In the ^{1}H-NMR spectrum (400 MHz, $CDCl_3$), an olefinic methine (δ 5.49) and an oxygenated methine (δ 3.35) signals were observed. Also in the high magnet field region, several methine and methylene signals, and seven singlet methyl signals (δ 1.32, 1.28, 1.07, 1.05, 1.04, 0.99 and 0.95) were observed. In the ^{13}C-NMR spectrum (100 MHz, pyridine-d_5), thirty signals consisting of one carboxyl (δ_C 179.4), one olefinic quaternary (δ_C 144.3) and one olefinic methine (δ_C 122.2), one oxygenated methine (δ_C 77.8), five methane, nine methylene and seven methyl (34.6, 28.3, 25.6, 23.3, 17.1, 16.1 and 15.1) signals were observed. These findings led to the conclusion that compound **3** is a pentacyclic triterpenoid of oleanane type with seven singlet methyls. Compound **3** was finally identified as 3β-hydroxyolean-12-en-28-oic acid (oleanolic acid) through the comparison of physical and spectral data with those of literature (Ikuta *et al.*, 2002).

The NMR spectrum of compound **4** was nearly the same as oleanolic acid (**3**) with the exception of the resonances of C-2 atom. C-2, a methylene in oleanolic acid, was revealed as an oxygenated methane and as the correlation between C-2 and H-3 and H-23 in the gHMBC spectrum. Also, through the observation of the coupling constant (J = 11.4, 9.0, 4.4 Hz) of H-2, the configuration of C-2 hydroxyl was determined to be á-bond. The ^{1}H- and ^{13}C-NMR data of compound **4** were assigned based on those of the literature (Kuang *et al.*, 1989). Consequently, compound **4** was determined to be 2α,3β-dihydroxyolean-12-en-28-oic acid (maslinic acid).

Each isolated compound was applied to some evaluation systems for the anticoagulation and anticancer activity.

Thrombosis, the formation of an unwanted clot within the blood vessels or heart, is the most common abnormality of hemostasis, and the venous thrombosis is triggered by blood stasis or inappropriate activation of the coagulation cascades, often as a result of a defect in the normal defense hemostatic mechanism (Mycek *et al.*, 1997). High molecular weight dextran was reported to induce blood stasis by platelet aggregation and the formation of microthrombin. It also induced intravascular red blood cell aggregation, decreased the number of platelet and fibrinogen, and significantly increased fibrin degradation products (FDP) (Zhen *et al.*, 1996; Mchedlishvili *et al.*, 1999). The above changes are the causes of myocardial injuries in rats with circulatory thrombi.

FPTase, a member of the prenyltransferase enzyme family, is a key post translational modification step for Ras protein and this is a mandatory process for retention of transforming ability. Therefore, inhibitors of Ras farnesylation are promising as effective antitumor agents. Many synthetic FPTase inhibitors have been reported including peptidomimetics in the past few years; however, only a few examples of natural product inhibitors have been reported (Anthony *et al.*, 1999; Reiss *et al.*, 1990; Williams *et al.*, 1999).

The H-ras proto-oncogene has been frequently activated in several animal and human tumors and is thought to play an important role in the emergence of these malignancies (Bos, 1989). Some specific H-ras alleles were found at a higher frequency in patients with breast tumor than in normal patients. In fact, most human breast carcinoma revealed elevated levels of H-ras RNA and protein (Hand *et al.*, 1987). Ras transfers signals in discrete intracellular pathways (Marshall, 1995), with the route via Raf-1, MEK, Erk, or PI-3-kinase/Akt pathway, and finally induces the expression of the immediate early genes to turn on a cascade of downstream genes in regulating vital cellular processes (Maculuso *et al.*, 2002). These results suggest a role of this oncogene in the initiation and/or progression of this malignancy (Chin *et al.*, 1999).

Compounds **1**, **2** and **3** exhibited an inhibitory activity on platelet aggregation with the IC_{50} values of 0.24, 0.09 and 0.07 mM, respectively, which were higher or similar activity in comparison to a well known positive control, aspirin (Srivastava, 1993), of the IC_{50} value of 0.16 mM. Compound **4**, which has an additional hydroxyl at C-2 of oleanolic acid (**3**),

showed relatively low activity with the IC_{50} value of 0.57 mM. In addition, compound **2** significantly prolonged aPTT with the value of 124 ± 11.2 seconds as compared to control of the value of 37.5 ± 2.2 seconds at the treatment concentration of 50 μg/mℓ.

Compounds **1** and **3** revealed relatively high inhibitory activity on FPTase with the values of 0.49 and 0.24 mM, respectively, which were slightly lower compared to a positive control, 2-hydroxycinnamaldehyde (Kwon *et al.*, 1996), of the IC_{50} value of 0.18 mM and other active compounds (Kang *et al*, 2004). However, compounds **2** and **4** exhibited no significant inhibitory activity on FPTase. Compounds **1** and **2** inhibited the growth of rat-H-ras cells with the GI_{50} values of 6.63 and 5.70 μM, respectively, which were much higher inhibitory activity than naturally occurred inhibitory compound, dehydrotrametenolic acid, a lanostane-type triterpene acid from *Poria cocos* (Kang *et al.*, 2006), with the GI_{50} value of 29.6 μM. Reports dealing with compounds showing inhibitory activity on the cell growth of H-ras-rat cells are very rare. As the result of these data, the authors could suggest that eugenol (**2**) may be a crucial agent in cancer treatment.

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