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- Note -

Isolation and Identification of Secondary Metabolites from the Ovary of *Nelumbo nucifera*

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The ovary parts of *Nelumbo nucifera* were extracted in 80% methanol (MeOH), and the concentrated extract was then partitioned using *n*-hexane, ethyl acetate (EtOAc), *n*-butanol (*n*-BuOH), and H_2O, successively. Using an octadecyl silica gel (ODS) column, silica gel (SiO_2) column chromatography, and a HPLC purification system, five compounds were isolated from the *n*-hexane fraction obtained from the extract of *N. nucifera* ovary. The chemical structures of the metabolites were determined using several spectroscopic methods, including NMR and GC/MS and MS of 1-eicosanol (**1**), cycloartenol (**2**), trans-squalene (**3**), pentadecanoic acid (**4**), and β-sitosterol (**5**). This study is a first attempt to isolate and identify secondary metabolites from the ovary of *N. nucifera*. The results indicated that the extract of *N. nucifera* ovary has biological effects, such as antibacterial and -tumor activity. Therefore, it could decrease the risk of HIV transmission through breastfeeding.

Key words : Column chromatography, GC/MS, *Nelumbo nucifera,* NMR

Introduction

Nelumbo nucifera Gaertn (Lotus), an annual aquatic herb with short rhizomes is grown and distributed throughout Asia including china and Egypt [13]. Almost all the tissues of Lotus, including flower stalks, flower petals, flower stamens, flower pistils, leaves, leaf stalks, seeds and rhizomes are widely used, and the leaves and embryos have been evaluated as important Chinese herbal drugs. In addition, seed kernels and rhizomes are usually used as a healthful cooked food, and they are often considered as human health immunomodulators. Stamens and petals containing flavonols and natural pigment are made into healthy tea and functional food additions, and they also have ornamental value [3, 14, 15].

Nelumbo nucifera leaves and embryos have been extensively studied about their antioxidant, antibacterial, anti-HIV and anti-obesity functions [3, 8]. The rhizomes have been reported to exhibit antibacterial, antidiarrhoeal, diuretic antihypertensive, and memory enhancing activities [4]. The seeds of *Nelumbo nucifera* have been studies effects of hepatoprotective [19], antioxidant [11], and antifertility [12]. Furthermore, in the traditional medicinal, its ovary has been reported to have the benefit for treating menorrhagia, uterine bleeding, hemorrhoids, diabetes, etc. It was previously reported that diverse compounds such as phenolics [15], triterpenes [7], alkaloids [10], and flavonoids [2, 6] were isolated from the leaves, embryo, stems, rhizomes and stamens of Lotus. However, it has not been studied yet to analyze the constituents from the ovary of *Nelumbo nucifera*. Hence, in this study, we performed the isolation and the structural elucidation of the metabolites in the ovary of *Nelumbo nucifera*.

Material and Methods

Plant materials

The ovary of *N. nucifera* was collected in July 2014 from Hamyang-gun Agricultural Development & Technology

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Center and identity was confirmed by Prof. Nam-In Baek, Department of Oriental Medicinal Materials & Processing, Kyung Hee University, Yongin, Korea. Voucher specimen (CMP1041M) was deposited at the herbarium of the Department of Herbal Crop Research, National Institute of Horticultural and Herbal Science, RDA, Eumseong, Korea.

Reagents and chemicals

Column chromatography was conducted using silica gel 60 (70-230 mesh, Merck, Darmstadt, Germany), RP-18 Lichroprep (40-63 mm, Merck, Germany), Thin layer chromatography (TLC) was conducted using a silica gel 60 F_{254}, RP-18 F_{254S} and detection reagent using 10% H_2SO_4. Extraction and column chromatography were conducted using *n*-hexane, ethyl acetate (EtOAc), methyl-chloride (CH_2Cl_2), and Methanol (MeOH), which were purchased from solvent (DAEJUNG Chemicals, Korea).

General experimental procedures

Nuclear magnetic resonance (NMR) spectrum was determined using a 400 MHz FT-NMR spectrometer (Varian Inova AS 400, Palo Alto, CA, USA) at 400 MHz for ^1H and 100 MHz for ^{13}C. The chemical shifts were referenced to residual solvent peaks ($CDCl_3$). The EI-MS data were collected on a gas chromatography (GC)-MS-QP 2010 PLUS spectrometer (Shimadzu, Kyoto, Japan). Electronic spray ionization (ESI)/MS spectrum was recorded on a 3200 Q-TRAP spectrometer (AB SCIEX, USA). Purification was performed using an HPLC system consisting of a 321 pump (Gilson, France), Ri detector (Shodex RI-101, Japan).

Extraction and isolation of the metabolites obtained from the ovary of *Nelumbo nucifera*

The ovary of *Nelumbo nucifera* (9.9 kg) was extracted with 80% MeOH at room temperature. The extracts were filtered through a filter paper and evaporated under reduced pressure at 40℃ to yield 40 g of extract. The extract was poured into H_2O (1 l) and then extracted with *n*-hexane (1 l × 3), successively. *n*-Hexane layer was concentrated under reduced pressure to obtain the *n*-hexane faction of ovary from *Nelumbo nucifera* extract (16 g). The *n*-hexane fraction (16 g) was used for the isolation of compounds. *n*-hexane fraction (16 g) was applied on a silica gel column (∅ 7.5 × 20 cm), and eluted with *n*-hexane : EtOAc (30 : 1 → 10 : 1 → 6 : 1) and CH_2Cl_2 : MeOH (30 : 1) to obtain 12 fractions (NNE1 to NNE12). Sub-fraction NNE9 (167 mg) was recrystallized from $CHCl_3$ to obtain NNE9W [compound **1**, 18.9 mg, TLC R_f = 0.25(silica gel 60 F_{254}, *n*-hexane : EtOAc = 10 : 1)]. A precipitate of fraction 9 (NNE91, 128 mg) was purified using an HPLC system consisting of a 321 pump (Gilson, France), Ri detector (Shodex RI-101, Japan) and a Phenomenex column [(250×10 cm, 10 um, Luna 10 u silica, USA)] in *n*-hexane : EtOAc (20 : 1) to obtain 4 fractions (NNE91-1 to NNE91-4) including NNE91-3 [compound **2**, 21.9 mg, TLC R_f = 0.4(silica gel 60 F_{254}, CH_2Cl_2 : MeOH = 90 : 1), R_f = 0.2(RP-18 F_{254S}, MeOH : H_2O = 40 : 1)]. Sub-fraction NNE1 (400 mg) was applied on a silica gel column (∅ 3×20 cm) and eluted with *n*-hexane : EtOAc (50 : 1) to obtain 4 fractions (NNE1-1 to NNE1-4) including NNE1-1 [compound **3**, 9.5 mg, TLC R_f = 0.7(silica gel 60 F_{254}, *n*-hexane : EtOAc = 50 : 1)]. Sub-fraction NNE5 (5 g) was applied on a silica gel column (∅ 5×27 cm) and eluted with *n*-hexane : EtOAc (10 : 1 -> 3 : 1 -> 1 : 1) and CH_2Cl_2 : MeOH (15 : 1 -> 10 : 1) to obtain 16 fractions (NNE5-1 to NNE5-16) including NNE5-10 [compound **4**, 35.2 mg, TLC R_f = 0.5(silica gel 60 F254, CH_2Cl_2 : MeOH = 15 : 1)]. NNE5-7 was recrystallized from $CHCl_3$ to obtain NNEW5-7W [compound **5**, 30 mg, TLC R_f = 0.4(silica gel 60 F254, *n*-hexane : EtOAc = 5 : 1), R_f = 0.2(RP-18 F_{254S}, MeOH : H_2O = 40 : 1)]. Compound **1** (1-Eicosanol): white powder; EI/MS: 298.32 $[M]^+$; ^1H-NMR (400 MHz, $CDCl_3$): 3.61(4H, m, OCH_2), 1.60-1.18(CH_2), 0.85(3H, t, *J*=6.4 Hz, CH_3).

Compound **2** (cycloartenol): white powder; negative ESI/MS: 425 $[M-H]^-$ for $C_{30}H_{50}O$; ^1H-NMR (400 MHz, $CDCl_3$): δH 5.07(1H, m, H-24), 3.05(1H, dd, *J*=9.6, 5.4 Hz, H-3), 1.66(3H, s, H-18), 1.57(3H, s, H-26), 0.86(1H, s, H-28), 0.78(1H, s, H-30), 0.52(1H, d, *J*=0.79, H-19), 0.30(1H, d, *J*=0.81, H-19). ^{13}C NMR (100 MHz, $CDCl_3$): δc 130.8(C-25), 125.2(C-24) 78.8(C-3), 52.6(C-17), 48.7(C-14), 47.9(C-8), 47(C-5), 45.2(C-14), 40.4(C-4), 36.3(C-12), 35.8(C-22), 35.5(C-20), 31.9(C-15), 30.3(C-1), 29.6(C-2), 29.6(C-19), 28.12,(C-7), 26.4,(C-16), 26(C-11), 25.9(C-27), 25.7(C-10), 25.4(C-30), 24.9(C-23), 21.1(C-6), 19.9(C-9), 19.2(C-28), 18.2(C-21), 18.0(C-18), 17.6(C-26), 14.3(C-29).

Compound **3** (trans-squalene): yellow oil; EI/MS: 410.71 $[M]^+$; ^1H-NMR (400 MHz, $CDCl_3$): 5.10(6H, overlapped), 1.98~2.07(overlapped, CH_2), 1.58-1.66(24H, overlapped, CH_3).

Compound **4** (pentadecanoic acid): brown oil; EI/MS: 242.39 $[M]^+$; ^1H-NMR (400 MHz, $CDCl_3$): 2.31(2H, m, H-2), 1.59~1.22(overlapped, CH_2), 0.86(3H, t, terminal-CH_3).

Compound **5** (β-sitosterol): white powder; positive

ESI/MS: 415 $[M+H]^+$; ^{1}H-NMR (400 MHz, $CDCl_3$): 5.33(1H, d, J=4.8 Hz, H-6), 3.50(1H, J=9.4, 5.2, H-3), 0.99(3H, s, H-19), 0.90(3H, d, J=6.4 Hz, H-21), 0.82(3H, t, J=7.6 Hz, H-29), 0.81(3H, d, J=7.6 Hz, H-26), 0.79(3H, d, J=6.8 Hz, H-27), 0.65(3H, s, H-18). ^{13}C NMR (100 MHz, $CDCl_3$): δc 140.74(C-5), 121.69(C-6), 71.79(C-3), 56.75(C-14), 56.05(C-17), 50.12(C-9), 45.83(C-24), 42.31(C-4), 42.29(C-13), 39.76(C-12), 37.24(C-1), 36.49(C-10), 36.13(C-20), 33.93(C-22), 31.9(C-2), 31.9(C-8), 31.65(C-7), 29.14(C-25), 28.23(C-16), 26.07(C-23), 24.29(C-25), 23.06(C-28), 21.07(C-11), 19.8(C-26), 19.38(C-19), 19.02(C-27), 18.76(C-21), 11.97(C-18), 11.84(C-29).

GC/MS analysis

A DB-5MS column (30 m x 0.25 μm ID x 0.25 mm) was used for the GC/MS experiment. The oven temperature was programmed as follows: 200℃ for 2 min, increased to 290℃ at a rate of 5℃/min and held for 20 min. The injector and detector temperatures were set at 280℃ and 250℃, respectively. Identification was performed by comparing their mass spectra with those of a library (Wiley Library, version 2012).

Results and Discussion

The 80 % methanol extract was fractionated into *n*-hexane layer, EtOAc layer, *n*-BuOH layer and H_2O layer through solvent fractionation. The repeated silica gel, ODS column chromatographies and prep-HPLC of *n*-hexane fractions supplied compounds 1-5(Fig. 1). Structural identifications of these compounds were carried out by interpretation of extensive spectroscopic data and comparison with the data described in the literature.

1-Eicosanol

Compound **1** yielded a pale purple color on the silica gel TLC after being sprayed with 10% aq. H_2SO_4 and heated. The molecular ion was detected at *m/z* 298.32 $[M]^+$ in the EI-MS spectrum. The ^{1}H-NMR spectrum showed signals such as an oxygenated methylene proton (δ_H 3.61), several methylene protons (δ_H 1.60~1.18), and methyl proton (δ_H 0.85), indicating that compound **1** was a fatty alcohol.

The type and composition formula of the fatty alcohol included in the compound was determined using GC/MS analyses for the non-methylated fatty alcohol, which were obtained through direct analysis of compound **1**. Qualitative analysis and composition formula determination of the fatty acids was conducted by comparing retention time (11′ 18″) and the peak area of each peak with authentic chemicals in the GC/MS experiment. This information was further confirmed by comparing the molecular ion peak (*m/z* 298 $[M]^+$) and fragmentation ion peaks with those of the Wiley Library in the GC/MS experiment. Compound **1** was identified as a 1-eicosanol.

Cycloartenol

Compound 2 yielded a dark brown color on the silica gel TLC after being sprayed with 10% aq. H_2SO_4 and heated. The molecular ion was detected at *m/z* 425 $[M-H]^-$ in the ESI negative MS spectrum. The ^{1}H-NMR spectrum (400 MHz, $CDCl_3$) demonstrated an olefine methine (δ_H 5.07, m, H-24) and an oxygenated methine (δ_H 3.45, dd, *J*=9.6, 5.4, H-3), and the chemical shift and coupling constants of the latter were in accordance with those of a 3β-OH substitution pattern. Additionally, in the high magnet field, seven singlet methyl signals δ_H 1.66(3H, s, H-18), 1.57(3H, s, H-26), 0.86(1H, s, H-28), 0.78(1H, s, H-30), 0.52(1H, d, *J*=0.79, H-19), 0.30(1H, d, *J*=0.81, H-19), and two doublet methylene signals

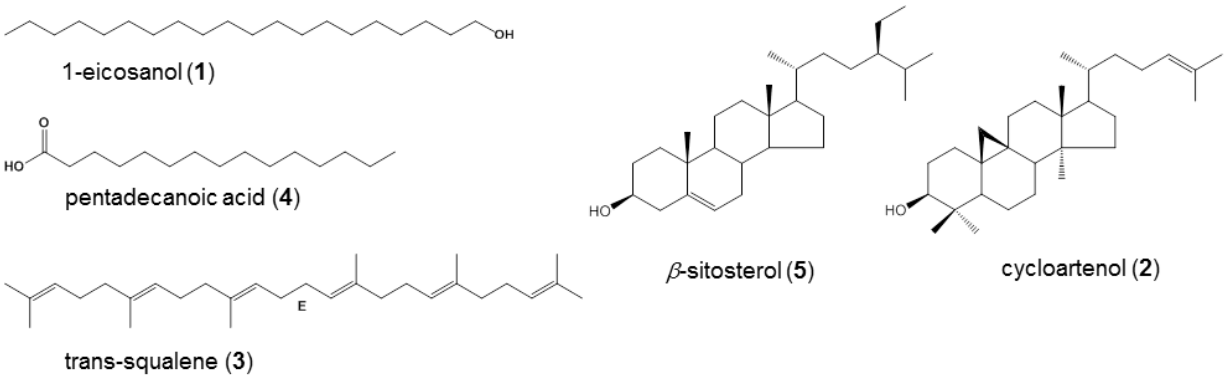

Fig. 1. Chemical structures of compounds (1-5) isolated from the ovary of *Nelumbo nucifera*.

δ_H 0.52(1H, d, *J*=0.79, H-19), δ_H 0.30(1H, d, *J*=0.81, H-19) were observed. These indicate that compound **2** could be a cycloartane-type triterpenoid.

The ^{13}C-NMR spectrum (100 MHz, $CDCl_3$) exhibited the presence of 30 carbon signals, consisting of seven methyl signals δ_C 29.6(C-19), 25.9(C-27), 25.4(C-30), 19.2(C-28), 18.2(C-21), 17.6(C-26), 14.3(C-29), two olefine carbon signals δC 130.8(C-25), 125.2(C-24), and an oxygenated methine carbon signal 78.8(C-3). The multiplicity of each carbon was determined using a DEPT experiment. The doublets at δ_C 0.52 [*J*=0.79 Hz, H-18a] and 0.30 [*J*=0.79 Hz, H-18b] indicated a cyclopropyl group. All the above resonances are characteristics of a cycloartane-type triterpenoid. ^{13}C-NMR has shown recognizable signals δ_C 130.8 and 125.2 ppm, which are assigned C_{25} and C_{24} double bonds respectively. The value at δC 18.0 corresponded to methylene signal indicated a cyclopropyl group. As a result, compound **2** was determined to be cycloartenol which were confirmed by comparison to that reported in the literature [16].

Trans-squalene

Compound **3** yielded a pink color on the silica gel TLC after being sprayed with 10% aq. H_2SO_4 and heated. The molecular ion was detected at *m/z* 410.71 $[M]^+$ in the EI-MS spectrum. The ^{1}H-NMR spectrum showed signals such as six olefine mehtine protons (6H, δ_H 5.10, overlappted), several methylene protons (δ_H 1.98~2.07), and eight methyl protons (24H, δ_H 1.58-1.66, overlappted), indicating that compound **3** was a compound.

The composition formula and mass were determined using GC/MS analyses for the non-methylated which were obtained through direct analysis of compound **3**. Qualitative analysis and composition formula determination of the fatty acids was conducted by comparing retention time (15′ 52″) and the peak area of each peak with authentic chemicals in the GC/MS experiment. This information was further confirmed by comparing the molecular ion peak (*m/z* 410 $[M]^+$) and fragmentation ion peaks with those of the Wiley Library in the GC/MS experiment. As a result, compound **3** was identified as trans-squalene.

Pentadecanoic acid

Compound **4** yielded a yellow color on the silica gel TLC after being sprayed with 10% aq. H_2SO_4 and heated. The molecular ion was detected at *m/z* 242.39 $[M]^+$ in the EI-MS spectrum. The ^{1}H-NMR spectrum showed signals such as a methylene proton (2H, δ_H 2.31) and several methylene protons (δ_H 1.59~1.22), and terminal methyl proton (3H, δ_H 0.86), indicating that compound **4** was a fatty acid compound. The composition formula and mass were determined using GC/MS analyses for the non-methylated which were obtained through direct analysis of compound **4**. Qualitative analysis and composition formula determination of the fatty acids was conducted by comparing retention time (4′ 72″) and the peak area of each peak with authentic chemicals in the GC/MS experiment. This information was further confirmed by comparing the molecular ion peak (*m/z* 242 $[M]^+$) and fragmentation ion peaks with those of the Wiley Library in the GC/MS experiment. Compound **4** was identified as a pentadecanoic acid.

β-Sitosterol

Compound **5** yielded a hot pink color on the silica gel TLC after being sprayed with 10% aq. H_2SO_4 and heated. The molecular ion was detected at *m/z* 415 $[M-H]^-$ in the ESI negative MS spectrum. The ^{1}H-NMR spectrum (400 MHz, $CDCl_3$) demonstrated an olefine methine (δ_H 5.33, m, H-6) and an oxygenated methine (δ_H 3.50, dd, J=9.4, 5.2, H-3), and the chemical shift and coupling constants of the latter were in accordance with those of a 3β-OH substitution pattern. Additionally, in the high magnet field, two singlet methyl signals δH 0.99(3H, s, H-18), 0.65(3H, s, H-19), and three doublet methyl signals δH 0.90(1H, d, *J*=6.4, H-21), and 0.79(1H, d, *J*=7.6, H-27), 0.81(1H, d, *J*=7.6, H-26), and one triplet methyl proton δ_H 0.82(3H, t, *J*=7.6 Hz, H-29) were observed. These indicate that compound 6 could be a stigmastane-type sterol. The ^{13}C-NMR spectrum (100 MHz, CDCl3) exhibited the presence of 29 carbon signals, consisting of six methyl signals δ_C 19.8(C-26), 19.38(C-19), 19.02(C-27), 18.76(C-21), 11.97(C-18), 11.84(C-29), two olefine carbon signals δ_C 140.74(C-5), 121.69(C-6), and an oxygenated methine carbon signal δ_C 71.79(C-3). The multiplicity of each carbon was determined using a DEPT experiment. All the above resonances are characteristics of a stigmastane-type sterol. As a result, the NMR spectra of compound **5** showed signals typical of stigmastane-type sterol, which led to its identification as β-sitosterol, a well-known sterol in all plant. β-sitosterol which were confirmed by comparison to that reported in the literature [5].

All the isolated compounds have been reported to exhibit various activities. For example, 1-eicosanol was examined as antibacterial activity [9]. Cycloartenol which is obtained

from *Cimicifuga simplex* has several biological effects [20]. Squalene was reported to be anti-tumor activity [18]. Pentadecanoic acid has also been found to be decrease the risk of HIV transmission through breastfeeding [20]. And β-sitosterol have been reported to high levels of *β*-sitosterol concentrations in blood have been correlated with increased severity of heart disease in men having previously suffered from heart attacks [1]. Finally, this study indicated that the ovary of *Nelumbo nucifera* includes five beneficial metabolites such as 1-eicosanol, cycloartenol, squalene, pentadecanoic acid, and *β*-sitosterol. Furthermore, the ovary of *Nelumbo nucifera* will be used as a good material for biological effects such as antibacterial activity, anti-tumor activity, and decrease the risk of HIV transmission through breastfeeding.

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연꽃의 자방으로부터 80% MeOH로 추출하고, 얻어진 추출물을 *n*-hexane, ethyl acetate, *n*-butanol 및 H_2O으로 용매 분획하였다. 이 중 *n*-hexane 분획물에 대해 silica gel과 octadecyl silica gel column chromatography 및 Prep-HPLC system을 반복 수행하여 5 종의 물질을 분리 하였다. 각 화합물의 화학구조는 NMR, GC/MS및 ESI/MS 등의 분광학적 스펙트럼을 측정하고, 해석하여 1-eicosanol (**1**), cycloartenol (**2**), trans-squalene (**3**), pentadecanoic acid (**4**) 및 β-sitosterol (**5**)으로 동정하였다. 이 화합물들은 연꽃 자방추출물에서 처음으로 분리하고 동정하였으며 앞으로 이 화합물들에 대한 다양한 생리적 및 약리적 활성을 검토함으로써 건강기능성 식품 또는 의약품의 소재로서의 충분한 가치가 있다고 여겨진다.