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Evaluation of Ursolic Acid as the Main Component Isolated from *Catharanthus Roseus* against Hyperglycemia

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ABSTRACT. Ursolic acid with large amount (0.67% of dried plant weight) along with 7 compounds, namely as spatozoate (**1**), kaurenoic acid (**2**), ursonic acid (**3**), 3-hydroxy-11-ursen-28,13-olide (**4**), ursolic acid (**5**), vindoline (**6**) and mixture of β-sitosterol and stigmasterol were isolated from dichloromethane and ethyl acetate extracts which have shown anti-glucosidase activity of the whole plant of C.roseus. Some isolated compounds and their derivatives were also tested for anti-glucosidase and cytotoxicity.Ursolic acid was examined for hypoglycemic activity in *alloxan-induced diabetic mice* with dose of 200 and 300 mg/kg/day, respectively. The results have shown that the blood glucose level were reduced by 45.75% and 51.31% to compare with the control group. This study has confirmed that the main component of Vietnamese C. roseus has had significant anti-hyperglycemia activity.

1. INTRODUCTION

Catharanthus roseus (L.) is an important prerennial tropical plant belonging the Apocynaceae family, comsists of eight species, seven endemic to Madagasca (*C.coriaceus*, *C. lanceus, C.longifolius, C.ovalis, C.roseus, C. scitulus, C.trichophyllus*) and one, *C.pusillus* from India. Among them, *C. roseus* is widely used to treat with the numerous fatal human diseases because of its producing a wide spectrum of secondary metabolites, include monoterpenoids, glucosides, steroids, phenolics, flavanoids, anthocyanins and more than 130 different terpenoid indole alkaloids, which showed strong and important pharmacological activities [3, 22].

Much worldwide research attention has been paid in *C.roseus* as a model for medicinal plant improvement. Traditionally, *C. roseus* is being used for relieving muscle pain, depression of the central nerous system and wasps stings. It is also used to treat many cases of nose bleed, bleeding gums, mouth ulcer, sore throats, internal loss of memory, hypertention, cystitis, gastritis, enteritis, diarrhoea and the rised blood sugar levels. In Vietnam, *C. roseus* is used in the folk medicine for the treatment of fever, dialysis, skin diseases, and diabetes (Ho 2000). Hypoglycemia is one of the most noticeable activities of *C. roseus*. It has been also used for people with diabetes in South Africa, Australia, Philippine, South Vietnam and the UK. There have been some reports on the hypoglycemic activity of different parts of this plant [4, 12, 14].

Rapidly economic growth in the developed and developing countries has caused some severe human diseases, especially in diabetes. Currently, there are over 150 million diabetic patients worldwide and expected to increase to 300 million or more by the year 2015 [12]. In the big cities of Vietnam, approximately about 4% of the population have suffered from diabetes which have been equivelent with 3.3 million cases of diabetes in Vietnam in 2014 [5]. Its complications can cause of many diseases as cardiovascular disease, stroke, chronic kidney failure, foot ulcers, and damage to the eyes. Some chemical constituents of this species have been shown high

hypoglycemic activity such as vindoline, tetrahydroalstonine, catharanthine, lochericine, leurosine, vindolinine, vindogentianine, ursolic acid [9, 21].

In the present study, we have reported that some constituents were isolated from the whole *C. roseus* which have exhibited anti-glucosidase activity, cytotoxicity. This study has also affirmed that ursolic acid is the main hypoglycemic component of *C. roseus* growing in Vietnam.

2. MATERIALS AND METHODS

Plant material

The whole plant of *C.roseus* was collected in Hoai Nhon, Binh Dinh Province, in December 2012, and was identified by Mr. Nguyen The Anh, Institute of Chemistry, Vietnam Academy of Science and Technology. A voucher specimen No.005 has been kept at 6ºC in freezer in laboratory of Department of Organic Synthesis, Institute of Chemistry, Vietnam Academy of Science and Technology (VAST).

3. METHODS

General

ESI-MS: UPLC/MS/MS Xevo-TQMS. NMR: Bruker Avance 500 MHz. Column chromatography (CC): silica gel (70 – 230 and 230 – 400 mesh, Merck). Thin layer chromatography (TLC): DC-Alufolien 60 F_{254} (Merck). Yeast α glucosidase, p-nitrophenyl-α-D-glucopyranoside (pNPG) and alloxan monohydrate were purchased from Sigma.

Extraction and isolation

The dried and powdered *C. roseus* of whole plant (2.2 kg) were extracted with solvents of increasing polarity: *n*-hexane, dichloromethane, ethyl acetate and methanol, successively. The solvent was concentrated under vacuum to give 35.7g *n*-hexane-, 23.5g dichloromethane, 10.8g ethyl acetate and 72.2 g methanol extracts. The dichloromethane extract was chromatographed on silica gel column, eluting with increasing amount of MeOH (0-20%) in CH_2Cl_2 to yield 10 fractions, which were combined according to TLC monitoring. The fraction 2 was re-chromatographed over flash silica gel column eluting with *n*-hexane: EtOAc (9.5: 0.5) afforded 8mg spatozoate (**1).** 10 mg kaurenoic acid (**2)** was obtained by re-purification of the fraction 3 (82mg) on silica gel column (*n*-hexane: EtOAc = 9:1). The fraction 5 (315mg) was further purified on the silica gel column eluted with *n*-hexane: EtOAc (9:1) to give 12mg ursonic acid (**3)** and a mixture of *β*-sitosterol and stigmasterol (ratio of 2:1). The NMR spectral data of *β*-sitosterol and stigmasterol are in good agreement with those in reported literature (Suparb and Amorn 1991), and the ratio was determined based on signal integration of H-6, H-22 and H-23 on their ^{1}H NMR spectra. The NMR spectral data are in good agreement with those in the previous report (Suparb and Amorn 1991). The fraction 6-9 were rechromatographed over flash silica gel column eluting with CH_2Cl_2 : MeOH (93:7) to give 15 mg 3-hydroxy-11-ursen-28,13-olide (**4**) and 6.8g ursolic acid (**5**). 10.8g ethyl acetate extract was purified on silica gel column eluted with CH_2Cl_2:MeOH (93:7) to provide 102mg vindoline (**6**) and 8g ursolic acid.

Assay of cytotoxic activities using the cell lines: HepG2, KB, LU-1, MCF7, SK-Mel2, HL60, SW626, LNCaP and HT29

Cell culture

The monolayer cancer cell lines (HepG2, KB, LU-1, MCF7, SK-Mel2, HL-60, SW626, LNCaP and HT29) were grown in Dulbeco's modified Eagle medium (DMEM) with 2 mM L-glutamine, 10 mM HEPES, 1.0 mM sodium pyruvate, and 10% fetal bovine serum – FBS (GIBCO). The cells were subcultured every 3-5 days with the ratio of (1:3) and incubated at 37 $^{\circ}$C under humidified atmosphere of 5% CO_2.

Cytotoxic assay

The above mentioned human cancer cell lines are supplied by Prof. Dr. J. M. Pezzuto, Hawaii University, US and Prof. Jeanette Maier, Milan University, Italy in 2013. The five isolates were evaluated for their cytotoxic activities against seven (except HL60) human cancer cell lines according to the method described by Monk et al [10] and against HL60 cell line according to the method MTT [8]. The cytotoxic potential was assessed by determining the amount of sulforhodamine B (SRB) bound to proteins and was performed in a microtiter plate. The test samples were examined over a concentration range of 0.8 – 100 µg/mL. DMSO (10%) was used as the negative control. Ellipticine (Sigma) served as the positive control and examined at the concentration of 10, 2, 0.4 and 0.08 µg/mL, respectively. Experimental cultures were plated in microtiter plates (Costar, USA) containing 20µL of each test sample and 180µL of growth medium (10% FBS) per well at density of 6000 cells/well. The duration assay was adopted as 3 days. Test plates were incubated in a humidified atmosphere of 5 % CO_2, 37 ^{0}C for 72h, while the 0-day control was incubated for 1h. After incubation, cells were fixed for 30 min. to the plastic substratum by the addition of 100 µL of cold 20% aqueous trichloroacetic acid (TCA) for at least 1h at 4^{0}C. Fixed cells were then stained with 0.4 % SRB (w/v) dissolved in 1% acetic acid and washed four times with 1% acetic acid. The bound dye was then solubilized by the addition of 10mmol unbuffered Tris base (Sigma), absorption was measured at 515nm with a microplate reader (BioRad). All the experiments were performed three times with the mean absorbance values calculated.

Growth, expressed as a percentage of the negative control, was calculated with the equation (OD: Optical Density):

$$\% \text{ growth} = \frac{\text{OD (test substance) - OD (day 0) x 100}}{\text{OD (negative control) - OD (day 0)}}$$

% inhibition = 100% - % growth

MTT method

The HL60 cell growth inhibition was determined by MTT assay. After incubation for 72h, the media was removed and the cells were incubated with 20μL MTT (5 mg/mL). After incubation for 4 h at 37 $^{\circ}$C in an atmosphere of 5 % CO_2 incubator, the formazan crystal formed were dissolved by adding 100 μL/well of DMSO. The optical density was measured at 515 nm wave length with ELISA Plate Reader (Bio-Rad) equipment. The number of viable cells was proportional to the extent of formazan production. Growth, expressed as a percentage of the negative control, was calculated with the equation:

$$\% \text{ growth} = \frac{\text{OD (test sample) x 100}}{\text{OD (negative control)}}$$

$$\% \text{ inhibition} = 100 \% - \% \text{ growth}$$

α-Glucosidase inhibitory activity *in vitro*

α-Glucosidase inhibitory activity was performed according to the method of Afrapoli-Moradi et al (2012). α-Glucosidase inhibitory activity was determined spectrophotometrically in a 96-well plate based on p-nitrophenyl-α-D-glucopyranoside (pNPG) as the substrate. The tested samples were dissolved in DMSO and added phosphate buffer 10 mM (pH 6.8). The mixture was putted in a 96-well plate at various concentrations as 1000µg/ml, 500µg/ml; 100µg/ml; 20µg/ml; 4 µg/ml. 20µl α- glucosidase (0,5U/ml) and 120 µl phosphate buffer 100 mM (pH 6.8) were added each well, mixed and incubated at 37°C for 15 min. The reaction was processed at 37°C for 60min and stopped by adding 80µL of 0.2M sodium carbonate solution. Absorbance of the wells was measured with a microplate reader at 405nm. The reaction system without plant extracts was used as control (mixture of DMSO 10%, phosphate buffer, enzyme and pNPG were used as the control). The

system without α-glucosidase was used as blank (mixture of tested sample, phosphate buffer and pNPG were used as blank). Acarbose was used as positive control. Each experiment was conducted in triplicate. The enzyme inhibitory rates of samples were calculated as follows:

$$\text{Inhibition (\%)} = (1 - A_{\text{Test sample}} / A_{\text{Control}}) * 100$$

$$\text{In which: } A_{\text{Control}} = OD_{\text{Control}} - OD_{\text{blank}}$$

$$A_{\text{Test sample}} = OD_{\text{Test sample}} - OD_{\text{blank mauthu}}$$

α-Glucosidase inhibitory activity was recorded spectrophotometrically in a 96-well plate based on pNPG as substrate (Li et al. 2011). The assay mixture (160μL) contained 8μl of a sample in DMSO (or DMSO itself as control), 112 μl phosphate buffer (pH 6.8) and 20μL enzyme solution (0.2 U/ml α-glucosidase in phosphate buffer), mixed and incubated at 37°C for 15 min, and then, 20μL substrate solution (2.5 mM pNPG prepared in the same buffer) added. The reaction was processed at 37°C for 15min and stopped by adding 80 μL of 0.2M Na2CO3 solution. Amount of the p-nitrophenol released from PNP-glycoside was quantified on a 96 microplate reader at 405 nm. The inhibitory rates (%) were calculated according to the formula: [1-(sample ODtest- sample ODblank)/ (control ODtest- control ODblank)] ×100%. Sample ODtest stand for solution of sample + enzyme + substrate. Sample ODblank stand for solution of sample + buffer. Control ODtest stand for solution of buffer + enzyme + substrate. Control ODblank stand for solution of buffer. All reactions were carried out with three replications. Acarbose was used as positive control.

Hypoglycemic effect assay in vivo

Experimental animal

Healthy BALB/c mice were housed in cages under laboratory conditions at standard conditions of temperature, light (12h light/dark cycle, 25°C and humidity 45-65%) in Institute of Biotechnology, VAST. The animals were fed with standard rodent diet and water ad libitum.

Experimental design

Hyperglycemia for 32 mice weighed from 27-32g by injecting alloxan monohydrate according to the method of Yanarday and Colak [24]. The fasting blood glucose was determined after 72h injected alloxan monohydrate solution and fasted 12h before completion. The blood glucose level greater than or equal to 8 mmol/L is considered diabetes mellitus [15]. Twenty four out of 32 induced diabetic mice were divided into four experimental groups so that the average blood glucose values in each group is equal. Group 1 diabetic control (DC). Group 2 diabetic rats given acarbose (50 mg/kg/day). Group 3 diabetic rats given ursolic acid (200 mg/kg/day). Group 4 diabetic rats given ursolic acid (300 mg/kg/day). The animals were treated for 9 days. To analyze the level of postprandial blood glucose, blood samples were collected from the eyes and mice were fasted for 6 h before the collection of blood samples.

% blood glucose level increased or decreased compared before experimental.

% decrease compared to before experimental = $100 \times (G1 - G2)/G1$

% increase compared to after experimental = $100 \times (G2 - G1)/G2$

In which: G1: The blood glucose level before experimental

G2: The blood glucose level after experimental

Statistical Analyses

All the treatments were calculated in a completely randomized design with at least thrice. Data were analyzed using program Microsoft Excel 2013. The IC_{50} (50% inhibitory concentration) was determined by plotting concentrations against % growth using nonlinear regression analysis from Table Curve software

4. RESULTS AND DISCUSSION

Chemical structures of the isolated compounds

From dichloromethane and ethyl acetate extracts of the whole of *C. roseus* having anti-glucosidased, seven compounds: spatozoate (**1**), kaurenoic acid (**2**), ursonic acid (**3**), 3*β*-hydroxy-11-ursen-28,13-olide (**4**), ursolic acid (**5**), vindoline (**6**) along with a mixture of *β*-sitosterol (**7**) and stigmasterol (**8**) with ratio of 2:1 were isolated and determined (Fig 1). This is the first time reported on isolation of compounds **1**, **2**, **3** and **4** from *C. roseus* in Vietnam.

*Spatozoate (**1**)*

ESI-MS *m/z*: 335.02 $[M+Na]^+$. ^{1}H-NMR (500 MHz, $CDCl_3$): δ 7.75 – 7.74 (1H, m, H-6), 7.72-7.70 (1H, m, H-3), 7.53-7.51 (2H, m, H-4 & H-5), 7.42 (2H, br d, *J* = 7.5 Hz, H-2'/H-6'), 7.39-7.33 (3H, m, H-3'- H-4'), 5.34 (2H, s, H-8), 4.19 (2H, t, *J* = 6.5 Hz, H-2''), 1.65-1.60 (2H, m, H-3''), 1.41-1.36 (2H, m, H-4''), 0.93 (3H, t, *J* = 7.5 Hz, H-5''). ^{13}C-NMR (125 MHz, $CDCl_3$): δ 167.7 (C-7), 167.4 (C-1''), 135.6 (C-2), 132.5 (C-1), 131.9 (C-1'), 131.1 (C-5), 130.9 (C-4), 129.0 (C-6), 128.9 (C-3), 128.6 (C-2'/C-6'), 128.4 (C-3'/C-5'), 128.37 (C-4'), 67.5 (C-8), 65.6 (C-2''), 30.5 (C-3''), 19.2 (C-4''), 13.7 (C-5'').

*Kaurenoic acid (**2**)*

ESI-MS *m/z*: 301.17 $[M-H]^-$. ^{1}H-NMR (500 MHz, $CDCl_3$): δ 4.80 (1H, s, H-17a), 4.74 (1H, s, H-17b), 2.64 (1H, br s, H-13), 1.24 (3H, s, H-18), 0.95 (3H, s, H-20). ^{13}C-NMR (125 MHz, $CDCl_3$): δ 183.8 (C-19), 155.9 (C-16), 103.0 (C-17), 57.1 (C-9), 55.1 (C-5), 49.0 (C-15), 44.3 (C-8), 43.9 (C-13), 43.7 (C-4), 41.3 (C-1), 40.7 (C-7), 39.7 (C-3), 39.7 (C-10), 37.9 (C-14), 33.1 (C-12), 29.0 (C-18), 21.9 (C-6), 19.1 (C-2), 18.5 (C-11), 15.6 (C-20).

*Ursonic acid (**3**)*

ESI-MS *m/z*: : 477.22 $[M+Na]^+$ (positive ion), 453.26 $[M-H]^-$ (negative ion). ^{1}H-NMR (500 MHz, $CDCl_3$): δ 5.26 (1H, *t*-like, H-12), 2.58-2.51 (1H, m, H-2a), 2.41- 2.36 (1H, m, H-2b), 2.20 (1H, d, *J* = 11.5 Hz, H-18), 1.09 (3H, s, CH_3), 1.08 (3H, s, CH_3), 1.06 (3H, s, CH_3), 1.03 (3H, s, CH_3), 0.96 (3H, d, *J* = 6.5 Hz, CH_3), 0.86 (3H, d, *J* = 6.5 Hz, CH_3), 0.83 (3H, s, CH_3). ^{13}C-NMR (125 MHz, $CDCl_3$): δ 217.8 (C-3), 183.7 (C-28), 138.1 (C-13), 125.6 (C-12), 55.3, 52.6, 48.0, 47.4, 46.8, 42.1, 39.5, 39.3, 39.1, 38.8, 36.7, 36.6, 34.2, 32.5, 30.6, 28.0, 26.6, 24.1, 23.5, 23.4, 21.4, 21.2, 19.6, 17.0, 16.9, 15.2.

β-sitosterol:stigmasterol (2:1)

^{1}H-NMR (500 MHz, $CDCl_3$): δ 5.36 - 5.34 (m, 1H), 5.18 - 5.13 (m, 0.36H), 5.04 - 5.02 (m, 0.32H), 3.56 - 3.49 (m, 1H).

β-sitosterol : ^{13}C-NMR (125 MHz, $CDCl_3$): δ 140.8 (C-5), 121.7 (C-6), 71.8 (C-3), 56.8, 56.1, 50.2, 45.9, 42.3, 42.3, 39.8, 37.3, 36.5, 36.2, 34.0, 31.9, 31.7, 29.2, 28.3, 26.1, 24.3, 23.1, 21.1 , 19.8, 19.4, 19.1, 18.8, 11.8.

Stigmasterol: ^{13}C-NMR (125 MHz, $CDCl_3$): δ 140.8 (C-5), 138.3 (C-22), 129.3 (C-23), 121.7 (C-6), 71.8 (C-3), 56.9 (C-14), 56.0 (C-17), 51.2, 50.2, 42.2, 40.5, 39.7, 37.3, 36.2, 35.8, 33.7, 32.4, 31.9, 31.5, 28.9, 25.4, 24.4, 21.2, 21.1, 19.4, 12.2, 12.0.

*3-hydroxy-11-ursen-28,13-olide (**4**)*

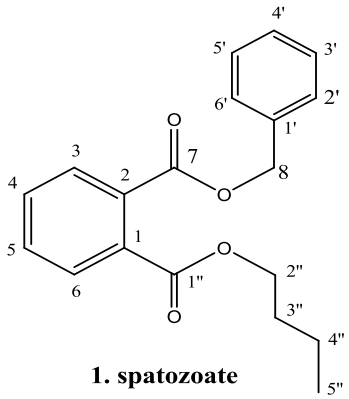
HR-ESI-MS *m/z*: 477.3339 $[M+Na]^+$. ^{1}H-NMR (500 MHz, $CDCl_3$): δ 5.96 (1H, dd, *J* = 10.5, 10.0, H-11), 5.53 (1H, dd, *J* = 10.5, 3.5 Hz, H-12), 3.22 (1H, dd, *J* =11.0, 4.5 Hz, H-3α), 2.13 (1H, dt, *J* = 13.0, 6.0 Hz, H-16 α), 1.95 (1H, br s, H-9), 1.16 (3H, s), 1.05 (3H, s), 1.00 (3H, d, *J* = 6.5 Hz), 0.99 (3H, s), 0.94 (3H, d, *J* = 6.5 Hz), 0.91 (3H, s), 0.79 (3H, s). ^{13}C-NMR (125 MHz, $CDCl_3$): δ 179.9 (C-28), 133.4 (C-12), 128.8 (C-11), 89.7 (C-13), 78.8 (C-3), 60.6, 54.8, 53.0, 45.1, 42.0, 41.7, 40.3, 38.9, 38.3, 38.1, 36.4, 31.3, 31.2, 30.8, 29.7, 27.8, 27.0, 25.6, 22.8, 19.2, 18.9, 17.9, 17.8, 17.7, 16.1, 14.9.

*Ursolic acid (**5**)*

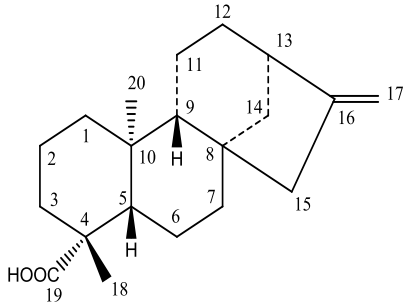
ESI-MS *m/z*: 479.23 $[M+Na]^+$ (positive ion), 455.27 $[M-H]^-$ (negative ion). ^{1}H-NMR (500 MHz, DMSO-d_6): δ 5.12 (br s, H-12), 4.30 (1H, d, *J* = 4.5 Hz), 3.01- 2.99 (1H, m, H-3), 2.10 (1H, d, *J* = 11.5 Hz, H-18), 1.04 (3H, s, CH3-27), 0.90 (3H, d, *J* = 9.5 Hz, H-30), 0.89 (3H, s, H-23),0.86 (3H, s, H-25), 0.81 (3H, d, *J* = 6.5 Hz, H-29), 0.75 (3H, s, H-26), 0.67 (3H, s, H-24). ^{13}C-NMR (125 MHz, $CDCl_3$): δ 178.3 (C-28), 138.2 (C-13), 124.6 (C-12), 76.9 (C-3), 54.8, 52.4, 47.0, 46.8, 41.6, 39.1, 38.5, 38.4, 38.3, 38.2, 36.5, 36.3, 32.7, 30.2, 28.3, 27.5, 27.0, 23.8, 23.3, 22.9, 21.1, 18.0, 17.0, 16.9, 16.1, 15.2.

*Vindoline (**6**)*

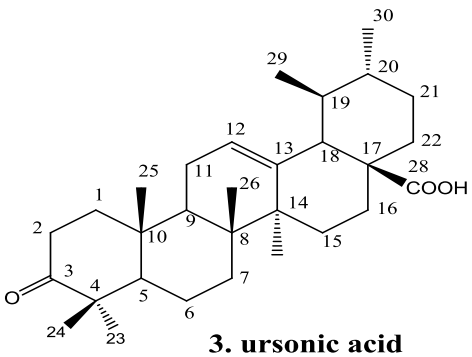
^{1}H-NMR (500 MHz, $CDCl_3$): δ 6.90 (1H, d, *J* = 8.0 Hz, H-14), 6.31 (1H, dd, *J* = 8.5, 2.5 Hz, H-15), 6.09 (1H, d, *J* = 2.5 Hz, H-17), 5.86 (1H, ddd, *J* = 10.0, 4.5, 1.5 Hz, H-7), 5.47 (1H, s, H-4), 5.24 (1H, d, *J* = 10.0, H-6), 3.80 (3H, s, $COOCH_3$), 3.79 (3H, s, OCH_3), 3.76 (1H, s, H-2), 3.53-3.49 (1H, m), 3.43 (1H, dt, *J* = 9.0, 4.0 Hz), 2.85-2.81 (1H, m), 2.69 (3H, s, NCH_3), 2.67 (3H, s, H-19), 2.56-2.51 (1H, m), 2.36-2.31 (2H, m), 2.08 (3H, s, $OCOCH_3$), 1.68-1.63 (1H, m), 1.17-1.13 (1H, m), 0.50 (3H, t, *J* = 7.5 Hz, H-21). ^{13}C-NMR (125 MHz, $CDCl_3$): δ 171.9 ($OCOCH_3$), 170.8 (-$COOCH_3$), 161.2 (C-16), 153.7 (C-18), 130.5 (C-6), 125.0 (C-13), 124.1 (C-7), 122.7 (C-14), 104.6 (C-15), 95.8 (C-17), 83.4 (C-2), 79.6 (C-3), 76.4 (C-4), 67.1 (C-19), 55.3 (Ar-OCH_3), 52.8 (C-12), 52.2 ($COOCH_3$), 51.9 (C-11), 51.1 (C-8), 44.0 (C-10), 42.9 (C-5), 38.2 (-NCH_3), 30.8 (C-20), 21.0 (-$OCOCH_3$), 7.6 (C-21).



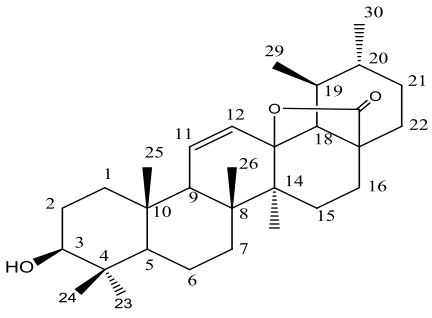
1. spatozoate



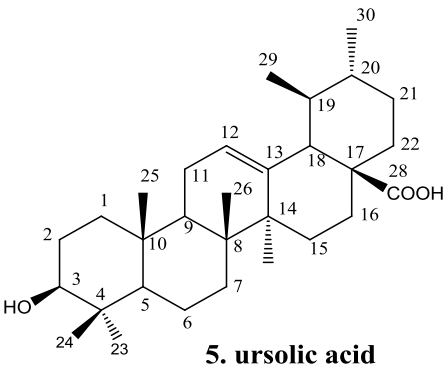
2. kaurenoic acid



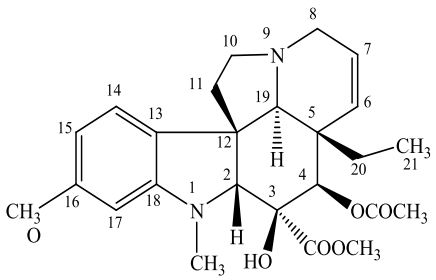
3. ursonic acid



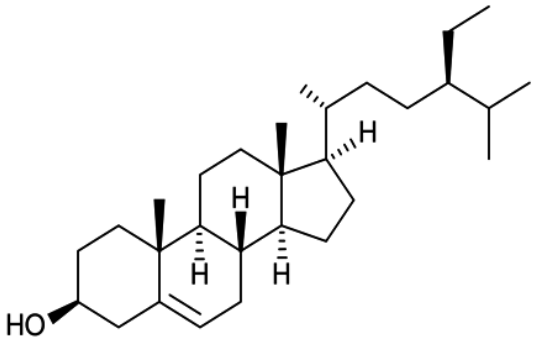
4. 3*β*-hydroxy-11-ursen-28,13-olide



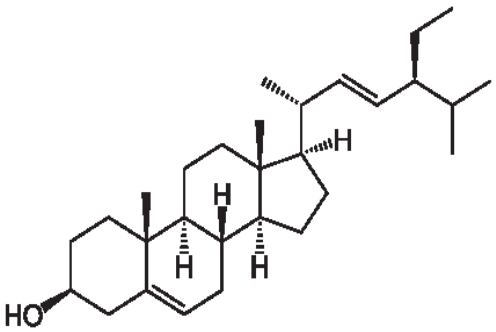
5. ursolic acid



6. vindoline



7. β-sitosterol



8. stigmasterol

Figure 1. Structures of the compounds isolated from *C. roseus*

Inhibitory activity against α-glucosidase *in vitro*

Some isolated compounds have been compounds were tested anti-glucosidase activity. The results have shown that almost the tested **compounds** have inhibitory activity against α-glucosidase, except for compound **2**. Three compounds **3**, **4** and **5** exhibited inhibitory α-glucosidase better than acarbose. Especially, ursolic acid (**5**) is the strongest activity with IC_{50} value as 3.83µg/mL (Table 1). This result is in very close agreement with the study of Kang et al [7] whom reported that ursolic acid isolated from EtOAC extract of *Osmanthus fragrans* disclosed great inhibitory activity of α-glucosidase with IC50 =3.38 µg/mL. These results are shown extra evidences to demonstrate for hyperglymecia activity of *C. roseus*.

Table 1. Inhibitory effect of some isolated compounds against α-glucosidase.

Compounds	IC_{50} (µg/mL)
1	282.42
2	>500
3	45.92
4	92.65
5	3.83
Acarbose	152.56

Cytotoxicity of isolated compounds

Compounds **1 – 4** were tested for cytotoxicity on nine human cancer cell lines: liver cancer (Hep-G2), mouth cancer (KB), lung cancer (LU-1), breast cancer (MCF-7), melanoma cancer (SK-Mel2), acute leukemia (HL60), ovarian cancer (SW626), androgen-sensitive human prostate adenocarcinoma (LNCaP) and colon adenocarcinoma (HT-29). Their IC50 values are given in the Table 3. Among them, compound **3** was found to be the most active one against all tested cancer cell lines with the IC_{50} values ranged from 4.36 to 30.15μg/mL. Compounds **2** was found to have moderate cytotoxicity against all nine cell lines with the IC_{50} in the range of 49.07 to 70.30 μg/mL. Compound **4** was inactive on all tested cell lines (IC_{50} > 100 μg/mL) (Table 2).

Table 2. Cytotoxic activity of compounds **2** - **4**, **7** and **8**

Compounds	*In vitro* cytotoxicity IC_{50} (μg/mL)								
	Hep G2	KB	LU-1	MCF7	SK-Mel2	HL60	SW626	LNCaP	HT-29
1	>100	>100	84.37	76.71	>100	71.56	>100	79.51	>100
2	58.37	70.30	61.77	52.07	49.07	52.06	50.78	39.55	56.27
3	4.36*	5.31*	19.79*	25.36*	24.23	14.80	33.78	12.19	30.15
4	>100	>100	>100	>100	>100	>100	>100	>100	>100
Ellipticine	0.42	0.49	0.44	0.42	0.42	0.43	0.37	0.44	0.34

*. IC_{50} of compound **3** on four cell lines Hep G2, KB, LU-1 and MCF7 were repored on by Thien et al [20].

Some previous works on evaluating of cytotoxic of *C. roseus* have been conducted. Admad et al [1] reported that crude aqueous extract of *C.roseus* exhibited differential effects of inhibiting the proliferation of the Jurkat cell line in time-and dose-dependent manner. It is possible that the chemical pathways between the major active compounds may act in concert to suppress Jurkat cells proliferation. The previous report of Siddiqui et al [17] revealed that chroroform fraction extract of *C. roseus* showed the highest cytotoxic activity against HCT-16 colorectal carcinoma cell line. Some compounds isolated from *C. roseus* such as catharanthine, vindoline showed high cytotoxic activity.

Inhibitory activity against α-glucosidase *in vitro* of the derivatives of ursolic acid

Ursolic acid (UA) is a pentacyclic triterpenoid which are available in many herbs and fruits. In this study, ursolic acid was isolated from the whole of *C. roseus* with large amount (0.67% of dried plant weight). In previous research, we have successfully synthesized a series of ursolic acids which was listed in the Table 3 (Thien et al. 2013). In order to find out their derivatives as hypoglycemic agents, we have further tested anti-glucosidase activity of ursolic acid's derivatives

Table 3. Inhibitory effect of derivatives of ursolic acid against α-glucosidase

Compounds	IC_{50} (µg/mL)
3*β*-Acetoxy-urs-12-ene-28-oic acid	9.08
3*β* -Succinoyl-urs-12-en-28-oic acid	125.07
3*β* -phthaloyl-urs-12-en-28-oic acid	0.93
3-Acetoxyimino-urs-12-en-28-oic acid	24.16
N-(3*β*-Acetoxy-urs-12-en-28-oyl)-11-aminoundecanoic acid methyl ester	>500
3*β* -Acetoxy-urs-12-en-28-carboxamide	105.25
N-(3*β* -Hydroxy-urs-12-ene-28-oyl)-11-amino undecanoic acid	85.05
3*β* -hydroxy-urs-12-ene-28-carboxamide	27.96
3*β* -phthaloyl-urs-12-en-28-carboxamide	6.35
Ursolic acid	3.25
Acarbose	135.70

Almost derivatives of ursolic acid exhibited inhibitory against α-glucosidase better than acarbose, except compound N-(3*β*-Acetoxy-urs-12-en-28-oyl)-11-aminoundecanoic acid methyl ester. Especially, derivative 3*β* -phthaloyl-urs-12-en-28-oic acid is 3.5 times more active than its lead compound (Table 3). This result has been similar with the recent report of Wu et al [23].

Effects of ursolic acid on alloxan-induced diabetic mice *in vivo*

As shown in the Table 5, the blood glucose level on alloxan-induced diabetic mice after oral administration of graded doses of 200mg and 300mg of ursolic acid /kg/day. The results showed that alloxan-induced mice after oral administration of graded doses of ursolic acid with the dose as 200 and 300 mg/kg/day, the blood glucose level were reduced respectively 19.26% and 25.10 % to compare with not drinking ursolic acid (day 0). Meanwhile, the blood glucose concentration of the control group was increased 26.21% comparing with before experiment. This showed that after 9 days alloxan-induced mice oral ursolic acid with doses of 200mg and 300 mg/kg/day, the serum glucose level was reduced 45.75% and 51.31% compared with the control group (Table 4).

Table 4. Hypoglymecic effect of ursolic acid isolated from *C. roseus* on alloxan-induced diabetic mice.

Group	Blood glucose level			
	Day 0	Day 10th	% change compared with day 0	Percentage decrease compared with group 1
Group 1	16,70 ± 5,74	22,63 ± 4,03	↑26,21	-
Group 2	16,57 ± 3,88	14,73 ± 5,39*	↓11,07	37,28
Group 3	16,18 ± 3,27	13,02 ± 4,08*	↓19,54	45,75
Group 4	16,73 ± 2,05	12,53 ± 1,91*	↓25,10	51,31

* *$p<0.05$: Statistical significance versus diabetes control*

The earlier reports revealed that the oral administration of leaves juice of *C. roseus* in healthy and alloxan-induced diabetic rabbits showed a significant antidiabetic activity and it had a more prolonged effect at 1.0 mL/kg than the dlibencalmide dose at 40µg/kg in the period of 18-24h after treatment [11]. Another research by oral administrating of aquenous extract of *C. roseus* at a dose of 250mg, 350mg, and 450mg/kg body weight for 30 days to diabetic rats led to significant reduction in blood glucose, reduction in lipid profile and also prevented a decrease in body weight [12].Also, Qi et al [16] reported that ursolic acid is able to significantly relieve renal damage in mice with diabetic nephropathy induced by alloxan which may be involved in decreasing blood glucose level.

5. CONCLUSIONS

In conclusions, numberous pharmacological works and traditional usage have proved the high medicial properties of *C. roseus*. In this study, ursolic acid along with 7 compounds isolated from the whole plant of *C.roseus* have anti-glucosidase activity. Compounds **3**, **4** and **5** have shown inhibitory α-glucosidase better than acarbose. Especially, ursolic acid (**5**) is shown the highest activity against α-glucosidase *in vitro* with IC_{50} value by 3.83 µg/mL. While, the derivative 3*β* -phthaloyl-urs-12-en-28-oic acid has shown the greatest inhibition 3.5 times more than its lead compound. This study has confirmed that the main component with anti-hyperglycemia of *C. roseus* growing in Vietnam.

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