DETERMINING SCIENTIFIC NAME USING *RBCL* GENE SEQUENCE METHOD AND ISOLATION OF SOME COMPOUNDS FROM *POLYSCIAS SCUTELLARIA*

Mai Do Van[1*], Thu Do Le Anh[1]

[1]**Faculty of Pharmacy, Nam Can Tho University, Vietnam.*

Corresponding Author: Mai Do Van

ABSTRACT

The objectives of the study were to analyze the morphological characteristics, sequence the RBCL gene and determine the scientific name of *Polyscias scutellaria*. *Polyscias scutellaria* samples were collected in Tan Loc Ward, Thot Not District, Can Tho City, Vietnam. Sequencing results of the RBCL gene segment showed that there is 100% similarity to the sequence of the rbcl gene segment of the species *Polyscias scutellaria* (Burm.f.) Fosberg, which has been published on the data bank. From the leaves of *Polyscias scutellaria* (Burm.f.) Fosberg (Araliaceae), Extracted and fractionated by exhaust extraction and liquid-liquid extraction techniques, obtaining fractionated extracts. The fractions were further isolated by chromatography and three compounds were obtained including stigmasterol from the diethyl ether fraction, kaempferol-3-O-rhamnoside from the ethyl acetate fraction and acid 3-O-β-D-glucopyranosyl-(1→4)-β-D-glucopyranosyl-(1→2)-β-D-(6-O-methyl) glucuronopyranosyl oleanolic (Polyscioside K) from the *n*-buthanol fraction - were characterized and isolated by various chromatographic methods. Their structures were elucidated by NMR (1D and 2D-NMR) in reference to the literature.

Keywords: *Polyscias scutellaria, Araliaceae, RBCL.*

INTRODUCTION

Polyscias is the second largest genus in the family Araliaceae. The Araliaceae family includes 55 genera and more than 1500 species, distributed mostly in tropical and subtropical regions, of which many species are used as oriental medicine and ornamental plants [9]. The genus *Polyscias* is derived from two Greek words: 'poly' meaning many and 'skia' meaning shade indicating thick foliage, which is characteristic of the genus *Polyscias*. Species of the *Polyscias* genus are usually perennial shrubs, rarely flowering, and are usually small or medium-sized trees with slender shapes and beautiful foliage. Pinnately compound leaves or simple leaves with pinnate lobes or pinnately compound leaves with leaflets of variable shape; Stipules absent or fused at the base into a small appendage. Native to Southeast Asia and Polynesian islands in the Pacific, tropical island areas. In addition, some species also grow and are cultivated in Indonesia, Malaysia, Lao and Vietnam [2].

Currently, in the world according to the plant monograph page of "The Plant list" [3], there are a total of 655 names, of which 176 species are accepted, there are 473 species. meaning, 6 unresolved species. Meanwhile, the plant classification system according to "Catalogue of Life" [8] has 174 species, 4 subspecies and 6 subspecies of the genus *Polyscias*. Previous studies only paid much attention to the species *Polyscias fruticosa* (L.) Harms. As for *Polyscias scutellaria*, it is rarely studied, *Polyscias scutellaria*, the shield aralia or plum aralia, is a tropical shrub or small tree reaching 2–6 meters in height. A native of the Southwest Pacific islands, it is commonly grown in gardens [8]. So the project was carried out to determine the scientific name of a tree species, possibly through analyzing morphological characteristics, analyzing the rbcl gene segment and comparing it with the gene segment. original rbcl in Genbank. To confirm the exact scientific name of the round-leaved Polyscias fruticosa plant collected in Thot Not District, Can Tho City, Vietnam we help survey the chemical composition with the hope that it will contribute to clarifying the chemical composition of the *Polyscias scutellaria* species. This round-leaved lentil aims to contribute scientific evidence to Vietnam's precious medicinal herb warehouse, thereby contributing to the exploitation and rational use of plant resources.

MATERIALS AND METHODS

Materials

Leaves of *Polyscias scutellaria* were collected in Tan Loc ward, Thot Not district, Can Tho city, Vietnam to extract, isolate and analyze DNA for gene sequencing, compared with the gene sequence samples of species of the genus *Polyscias*.

Chemicals and equipment for DNA analysis: CTAB Buffer (2% CTAB, 100 mM Tris pH 8.0, 20 mMEDTA pH8 0,1.4M NaCl), *β*-mercaptoethanol, chloroform: Isoamylalcohol (24:1), enzyme RNase, Isopropanol, ethanol (70%). All chemicals used in this experiment were sourced from Merck, Germany. PCRMix (NEXpro, Korea), PCR 2X MasterMix, purified agarose, GelRed dye, TAE 1X, Loading dye 6x, Ladder 1 kb plus (Thermo Scientific, USA), TE, purified water (2 times distilled and purified water) pasteurized at 121^{o}C for 20 minutes). ATTO CORPORATION AE 7344 electrophoresis, polyacrylamide ATTA Compact PAGE-Twin gel electrophoresis machine (ATTA, Japan), GeneAmp PCR System 2700 PCR machine (Amplied Biosystems – Malaysia), UV gel reader (BioBlockScientific, France).

Chemicals and equipment for isolation: Methanol, diethyl ether, ethyl acetate, *n*-butanol (Merck, Germany)….Nuclear magnetic resonance spectra: ^{1}H-NMR, ^{13}C-NMR, DEPT, COSY, HSQC, HMBC recorded on a BRUCKER AVANCE (500 MHz) chemical shift in δ (ppm), interaction constant (J) in Hz. Mass spectra were measured on an AGILENT TECHNOLOGIES 6120 (Quadrupole LC/MS). Thin layer chromatography (TLC) was performed on a pre-coated Merck-GF60 F254 aluminum silica gel plate, size 20 × 20 cm, adsorbent layer thickness 0.2 mm of Merck, Germany. Medium-pressure column chromatography using silica gel 60, Merck, particle diameter 0.040-0.063 mm; diaion HP-20; reverse phase silica gel RP - 18 (particle size 30 - 50 m).

Methods

Identify the scientific name

* Morphological description method

Observation and description of the external morphology of Dinguncle. Based on the improved method of [12], the parts described include: Stems, leaves...

* Total extraction and purification

Whole DNA was isolated from fresh leaves according to an improved CTAB extraction procedure [6].

First, 100 mg of leaf samples were weighed in a mortar and finely ground in 1 mL of CTAB 2X solution incubated at 65 ^{o}C for 15 min. Place the sample that has been ground in CTAB into the tube and add the CTAB, titrate to the 1.5 mL mark. Mix well and centrifuge at 13000 rpm for 10 min. After centrifugation, withdraw 1000 µL of the supernatant from each tube in turn and place in a new tube. Then add 10 µL of β-mercaptoethanol/tube. Carry out incubation at 65 ^{o}C for 60 minutes (every 10 minutes mix the samples well). Next, add 500 µL of chloroform to each tube, mix well and centrifuge at 13000 rpm for 10 minutes. Pipette 750 µL of the above solution into a new tube, then continue to add 500 µL of chloroform, mix well and centrifuge at 13000 rpm for 10 minutes. Transfer 550 µL of the above solution to a new tube, then add 500 µL of chloroform to each tube and centrifuge at 13000 rpm for 10 min. Withdraw 350 µL of the supernatant into a new tube, then add 5 µL of RNase to each tube, shake well and incubate the sample at 37 °C for 2 h. After 2 h of incubation, add 300 µL CTAB 2X and 500 µL chloroform to each tube. The sample was centrifuged at 13000 rpm for 10 min. Next, withdraw each tube 400 µL of the supernatant and put it in a new tube, and at the same time add 400 µL isopropanol (1:1 ratio), mix well and incubate at -20 ^{o}C for 30 minutes. The sample is centrifuged at 13000 rpm for min, carefully discarding the upper solution, leaving the precipitate deposited below. Add 500 µL 70% ethanol to each tube and centrifuge at 13000 rpm for 5 min to rinse the sample, then discard the alcohol and leave the precipitate. Add 500 µL of 70% ethanol further to each tube to rinse the sample a second time and centrifuge at 13000 rpm for 5 min. Then discard the alcohol and leave the precipitate. Use a micropipette to suck up the remaining alcohol in

each tube and let the sample dry (under a ceiling fan) for 1 hour. Finally, 30 µL TE were added to each tube (pH = 8.0) to dissolve the DNA and refrigerated at -20 °C.

* DNA amplification by PCR reaction

The DNA sequence was amplified using primers rbcLa-F: 5'-ATGTCACCACAAACAGAGACTAAAGC-3' and rbcLa-R: 5'-GTAAAATCAAGTCCACCRCG-3' [10].

Thermal cycling for a CPR reaction: Performed in 35 heating cycles, including 5 min at 95 ^{o}C, 30 s at 95 ^{o}C, 30 s at 60 ^{o}C, 30 s at 72 ^{o}C, stretching the series for 5 min at 72 °C and the product was stored at 10 °C for 20 min.

* DNA electrophoresis on agarose gel

DNA after being extracted and purified will be checked by electrophoresis on 1% agarose gel. After electrophoresis, the gel was stained with redsafe dye (Biobasic, UK), and the results were recorded

* PCR product electrophoresis and sequencing

PCR products were electrophoresed and purified using the Wizard SV Gel kit and PCR Clean-up System (Promega). Based on the Sanger method [14]. Each dideoxynucleotide is labeled with a different colored fluorescent agent. Thus, all oligonucleotides terminating at the same dideoxynucleotide will have the same color. DNA sequences were sequenced by Phu Sa Biochem company (Vinh Long city) on an automatic sequence reader.

* Analyze data and compare DNA sequences

Molecular weight was calculated using Gel Analyzer software. Sequencing results were stored in FASTA format and analyzed using the latest BioEdit software version 7.0.5 [7]. Then by BLAST method on the NCBI gene bank system (National Center for Biotechnology Information) used for species identification.

Extraction and isolation

From 1500 g of *Polyscias scutellaria* leaves were extracted with 96% ethanol to obtain the extract, then vacuum evaporated to obtain 200 g of total extract. This high amount was dissolved in a minimum amount of water, then the resulting solution was shaken with the following solvents: Diethyl ether, ethyl acetate, *n*-buthanol. The obtained fractions were concentrated under reduced pressure to yield 30 g diethyl ether, 10 g ethyl acetate, 20 g *n*-buthanol.

Pure compounds have been isolated from 10 g of diethyl ether extract, 10 g of ethyl acetate extract and 10 g of *n*-buthanol extract in *Polyscias scutellaria* leaves by classical column chromatography through stationary phases of diaion HP - 20, forward phase silica gel and reverse phase silica gel monitor the fractions by thin layer chromatography. Then crystallize and purify many times to obtain the pure compound 15 mg PS01 from diethyl ether extract, 10 mg PS02 from ethyl acetate extract and 20 mg PS03 from *n*-buthanol extract.

Determination of the structure of the isolated and purified compounds was based on spectroscopic methods tests such as 1H-NMR, 13C-NMR, COSY, HSQC and HMBC. At the same time, it is based on references previously published by scientists.

RESULTS AND DISCUSSION

Determination of scientific name by *rbc*L gene sequencing

* Morphological characteristics of plant *Polyscias scutellaria*

Small tree 1 - 2 m tall, dark brown trunk with many white spots, very characteristic of the genus *Polyscias*. The leaves are simple, the leaf blade is tapered and concave like a disc or hemisphere, green or striped, hairless. Heart-shaped leaf base, serrated leaf blade.

Figure 1. Morphology of *Polyscias scutellaria*

Samples of fresh leaves of *Polyscias scutellaria* were extracted, DNA separated, gene sequenced, compared with the published gene sequence samples of species of the genus *Polyscias*, giving the following results:

* Extract the total DNA and perform the PCR reaction:

The total DNA after separation was electrophoresis on 1% agarose gel for clear DNA lines, clean electrophoresis tape, no RNA mixed. The total DNA after performing the gene multiplication reaction with the rbcL fragment was electrophoresed and compared with the standard 1000 bp ladder, showing that the obtained sequence size was about 600 bp. The product line on the electrophoresis tape is bold, clear, intact, and unbroken, so it is eligible for further purification to perform the sequencing reaction.

Figure 2. Total DNA electrophoresis

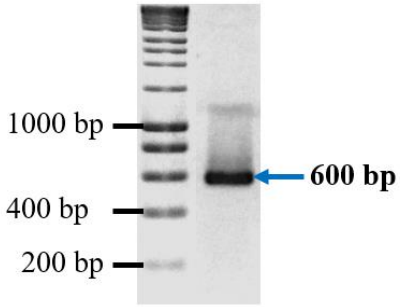

Figure 3. PCR product electrophoresis

* *rbc*L gene sequence

The obtained DNA sequence after sequencing consists of 544 bp, of which 544 bp is evident, and is included to compare with the published sequence, in which the G-C ratio is 42 %, the A-T ratio is 58 %. The NCBI/Blast tool was used to compare with the sequence published on the world gene bank (gene bank code: MN117993.1) [11], showing that the obtained gene sequence is similar to the *Polyscias scutellaria* (Burm.f.) Fosberg published with 544/544 homologous nucleotides (corresponding to 100% similarity rate).

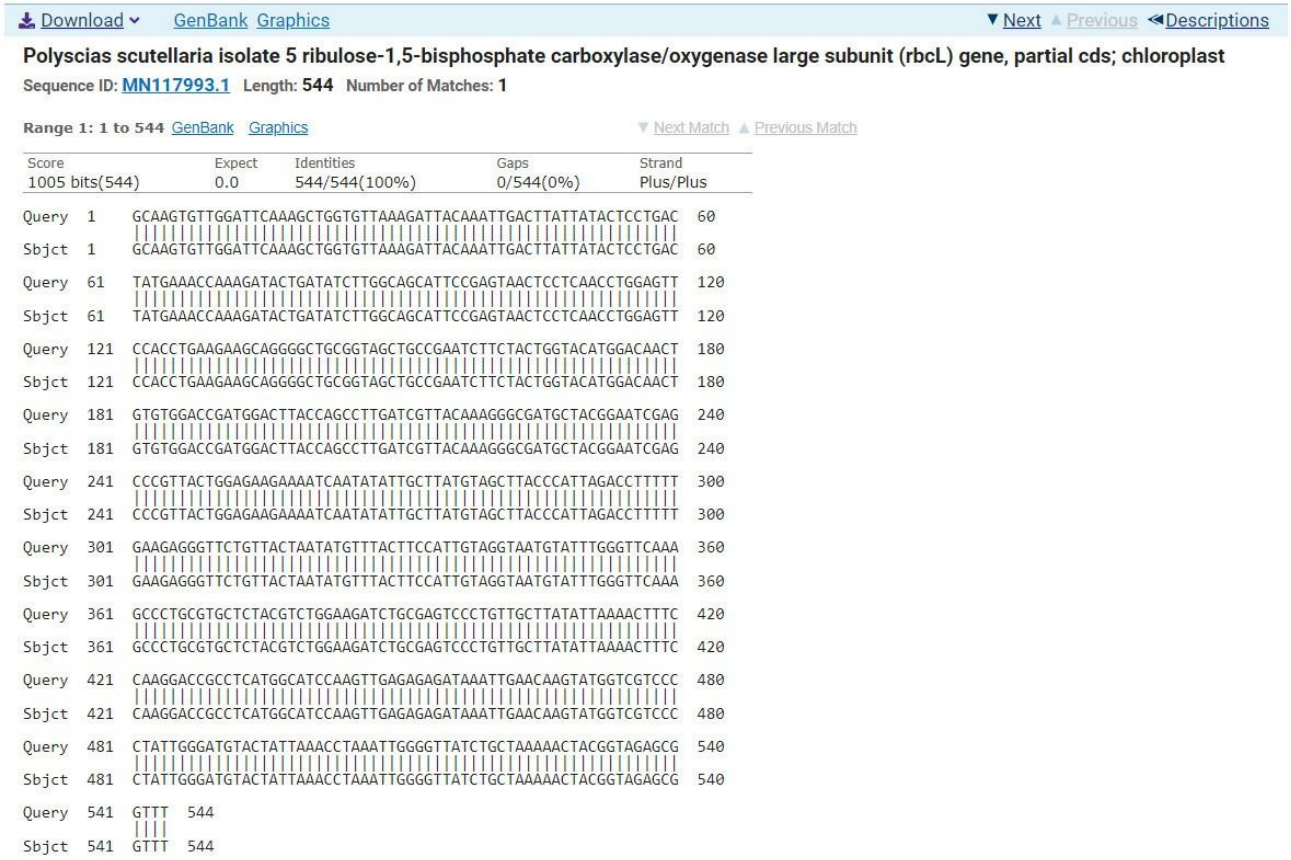

Figure 4. Comparison of the *rbc*L gene sequence of the study sample with the species sequence published in the world

In which: Query is the sequence of the research sample and Sbjct is the sequence of the species *Polyscias scutellaria* (Burm.f.) Fosberg published on the world gene bank (gene bank code: MN117993.1) [11]. The results of gene sequencing and comparison of the gene sequence of *Polyscias scutellaria* with the gene sequence of *Polyscias scutellaria* is a reliable basis for confirming the scientific name of the round-leaf clover, collected in Tan Loc ward, Thot Not district, Can Tho city, Vietnam which is *Polyscias scutellaria* belongs to the Ginseng family (Araliaceae).

Determine the structure of 3 isolated compounds

Pure compounds have been isolated from 10 g of diethyl ether extract, 10 g of ethyl acetate extract and 10 g of *n*-buthanol extract in *Polyscias scutellaria* leaves by classical column chromatography through stationary phases of diaion HP - 20, forward phase silica gel and reverse phase silica gel monitor the fractions by thin layer chromatography. Then crystallize and purify many times to obtain the pure compound 15 mg PS01 from diethyl ether extract, 10 mg PS02 from ethyl acetate extract and 20 mg PS03 from *n*-buthanol extract. All in powder form, color white, readily soluble in methanol.

Compound PS01

Compound PS01 is in the form of colorless, needle-shaped crystals, soluble in chloroform. The IR spectrum (KBr) has characteristic signals at 3421.6 cm-1 (O-H), 2936.87 (C-H), 1049 (C-O). ^{1}H-NMR spectrum shows the presence of 6 methyl groups, including 2 single nasal signals at δ_H 0.70 (H-18), δ_H 1.01 (H-19) and 3 single nasal signals doublet at δ_H 0.97 (H-21), δ_H 0.81 (H-26), δ_H 0.79 (H-27) and 1 triple methyl at δ_H 0.83 (H-29) indicate that this is 1 branch is outside the main frame. In addition, the ^{1}H-NMR spectrum also shows the presence of three olefin proton signals at δ_H 5.35 (H-6), δ_H 5.15 (H-22), δ_H 5.02 (H-23). Analysis of the coupling constants of these two double-tip olefin protons shows that they are trans-coupled to each other by coupling constant J=15.0; 8.5 Hz, showing that there are 2 pairs of C=C double bonds. At the same time, a methine signal appears

bound to the OH group at δ_H 3.52 (H-3). The ^{13}C-NMR spectrum combined with the DEPT spectrum shows the presence of 29 carbons, including 6 methyl groups, 9 methylene groups, 11 methine groups and 3 quaternary carbon atoms. In addition, there is also 1 carbon signal. hydroxymethine at δ_C 71.9 ppm (C-3), indicating the existence of a hydroxyl group. Spectral data also indicate the presence of two double bonds in the molecule confirmed by two carbon pairs at δc 140.8 (C-5) and 121.7 (C-6); δ_C 138.3 (C-22) and 129.3 (C-23) of the -HC=CH- double bond on the open carbon chain. These are characteristic signals of the stigmasta-5,22-diene framework.

From the analysis of IR, ^{1}H, ^{13}C-NMR spectrum data, combined with DEPT, HMBC, HSQC spectra and comparison with documents [13], it shows that there are similarities. Therefore, the structure of compound PS01 was determined to be stigmasterol. This compound has been found in *Polyscias fruticosa* [5].

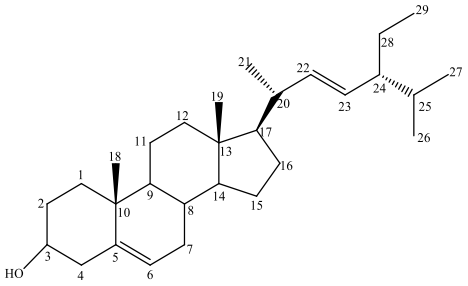

Figure 5. Structure of stigmasterol

Compound PS02

Compound PS02 was obtained as a pale yellow powder, easily soluble in acetone. The HR-ESI-MS spectrum gives the molecular ion tip $[M+Na]^+$ at m/z 455.0930 (theoretical 455.0954), allowing the molecular formula to be determined as $C_{21}H_{20}O_{10}$. ^{1}H-NMR spectrum of compound 3 at the weak magnetic field region, showing the presence of 1 -OH group at δ_H 12.71 (s, 1H) and 2 aromatic proton signals at δ_H 6.48 (d, 1H) , J = 2.0 Hz, H-8), δ_H 6.27 (d, 1H, J = 2.0 Hz, H-6) are meta-paired to characterize the aromatic nucleus A of the flavonoid framework. The ^{1}H–NMR spectrum also shows two aromatic proton signals at δ_H 7.86 (d, 2H, J = 8.5 Hz), δ_H 7.02 (d, 2H, J = 9.0 Hz) demonstrating the presence of an aromatic nucleus with 2 substituents at position 1,4 (nucleus B). The ^{1}H-NMR spectrum also shows signals of 5 oxymethin groups at δ_H 5.54 (brs, 1H), δ_H 4.23 (brs, 1H), δ_H 3.70 (dd, 1H, J = 9.0, 2 ,5 Hz), δ_H 3.33 (m, 1H), δ_H 3.30 (m, 1H) and 1 double-ended methyl group at δ_H 0.90 (d, 3H, J = 5.5 Hz), confirmed presence of sugar molecule α-L-rhamnopyranosyl in the structure of compound 3. ^{13}C-NMR spectrum combined with HSQC spectrum helps identify 21 carbon signals of compound 3, which includes 15 carbon signals of flavonoids (including 1 carbonyl group at δ_C 179.3; there are 6 sp^2 carbon signals linked directly with the oxygen atom at δ_C 165.0; 163.2; 160.9; 158.5; 158.0 and 135.6; there are 5 aromatic carbon signals at δ_C 131.7; 116.3; 105 ,8; 99.8; 94,3) and 6 signals of sugar molecule α-L-rhamnopyranosyl (5 methine signals at δ_C 102.8; 73.0; 72.2; 71.5; 71.4 and 1 signal of the methyl group at δ_C 17,7). Survey of HMBC spectral data confirmed that compound 3 is a flavonoid glycoside. Besides, the correlation between the proton anomer signal at δ_H 5.54 (H-1") and the carbon signal at δ_C 135.6 (C-3) demonstrates the association of the sugar molecule at C-3. of the aglycon fraction.

From the analysis of HR-ESI-MS, ^{1}H-NMR, ^{13}C-NMR spectral data, combined with HMBC, HSQC spectra and comparison with the literature [5], there are similarities. Therefore, compound 3 was identified as kaempferol-3-O-rhamnoside. This is a flavonoid compound that has been shown to have antioxidant activity [1] with the following structure:

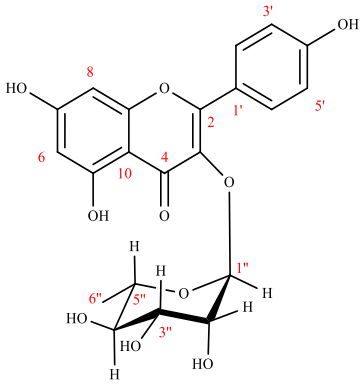

Figure 6. Structure of kaempferol-3-*O*-rhamnosid

Compound PS03

Compound PS03 is obtained as an amorphous, colorless powder, soluble in pyridine, giving a purple color with VS reagent. UV spectrum (MeOH) shows maximum absorption peak at 204 nm. The IR spectrum (MeOH) of compound PS03 shows vibrational signals (cm-1) in the region 3362 (O-H), 2942, 2915, 2831 (C-H), 1022 (C-O). HR-ESI-MS spectrum for molecular ion tip [M-H]- at m/z 969.5013 (theoretical 969.5059), allowing to determine the molecular formula of PS03 as $C_{49}H_{78}O_{19}$.

^{1}H-NMR and ^{13}C-NMR spectrum data show that compound PS03 has 49 carbon atoms, including the aglycon part is oleanolic acid and the glycoside part is the sugar β-6-O-methylglucuronate (6-O-Me-GlcA) and 2 β-glucose (Glc). Except, the downward field shift of carbon at δ_C 80.2 (C-2') of Me-GlcA compared to that at δ_C 176.5 (C-28), shows that Glc-2 of compound PS03 will bind into C-2'. Furthermore, the HMBC spectrum of compound PS03 shows a correlation between the three anomer protons at δ_H 4.88 (d, 7.5, H-1') of Me-GlcA, 4.90 (d, 8.0, H-1'') of Glc -1, 5.37 (d, 7.5, H-1''') of Glc-2 and carbon at δ_C 89.5 (C-3) of aglycon, 81.7 (C-4') of Me-GlcA, 80.2 (C-2') of Me-GlcA, respectively.

From analyzing UV, IR, HR-ESI-MS, ^{1}H, ^{13}C-NMR spectral data, combined with DEPT, HMBC, HSQC, COSY spectra and comparing with documents [15]. Therefore, the structure of compound PS03 was determined to be acid 3-O-*β*-D-glucopyranosyl-(1→4)-*β*-D-glucopyranosyl-(1→2)-*β*-D-(6-O -methyl) glucuronopyranosyl oleanolic (Polyscioside K). This is a compound that was previously found in the species *Polyscias fruticosa* [5].

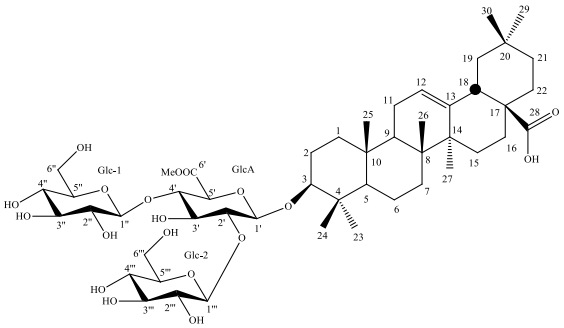

Figure 7. Structure of Polysciosid K

CONCLUSION

By method of barcoded DNA combined with morphological characteristics, *Polyscias scutellaria* was collected in Tan Loc ward, Thot Not district, Can Tho city, Vietnam with scientific name *Polyscias scutellaria* (Burm.f.) Fosberg belonging to the Ginseng family (Araliaceae). This result helps to accurately identify the scientific names of the research subjects by the rbcL gene sequencing method.

From the leaves of *Polyscias scutellaria* grown in Thot Not district, Can Tho City, Vietnam for the first time, we isolated and identified the structure of 3 compounds: *stigmasterol,* kaempferol-3-*O*-rhamnosid *and* acid 3-O-*β*-D-glucopyranosyl-(1→4)-*β*-D-glucopyranosyl-(1→2)-*β*-D-(6-O -methyl) glucuronopyranosyl oleanolic (Polyscioside K). These compounds help guide further studies in terms of biological effects as well as deeper chemistry.

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