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A Pure Isolated from Fruit Seeds of Kolowe (*Chydenanthus excelsus*) The Identified as Single Peak By HPLC Analytic Column They Are Two Saponin Compounds of Isomered

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ABSTRACT

Analytical HPLC is a compounds separation tool that have better performances. The ability of the separation was not able to separate isomered saponins with physicochemical properties are very similar. It has been found two isomered saponins of an amorphous crystalline pure seed Kolowe (C. excelsus). The saponin separation using preparative HPLC column, and the results of analytical HPLC analytical column against the amorphous crystal showed a peak which indicates that it is a pure crystalline amorphous. However, the results of spectroscopic analysis with NMR-2D in a variety of techniques, the pure amorphous crystal is two saponins isomered. Two saponin are 3-O-[β-D-Xylopyranocyl(1→3)-β-D-glucopyranocyl(1→2)-β-D-glucuronopyranocyloxy]–28-O-(2-methylbuthyroyloxy)-oleana-12-en-15α, 16α, 22α-trihydroxy and 3-O-[β-D-Xylopyranocyl (1→3)-β-D-glucopyranocyl(1→2)-β-D-glucuronopyranocyloxy]-28-O-(3-methylbuthyroyloxy)-oleana-12-en-15α, 16α, 22α-trihydroxy.

Key words : Isomered saponins, *Chydenanthus excelsus*, Analytical HPLC

INTRODUCTION

HPLC analytical column is a High Performance Liquid Chromatography can separate chemical compounds that have the physicochemical properties are very similar. HPLC capability has been developed extensively for various needs of the separation of chemical compounds. The development will include a preparative HPLC column, a high performance liquid chromatography is used to separate compounds with properties very similar physicochemical and compound separation results obtained directly in the form of compound. HPLC analytical column only serves to analyze the chemical compounds in a material based on the principle of the separation, without obtaining the compound in the form of being. However, an analytical HPLC has the ability detection and separation of compounds are better than the preparative HPLC column. HPLC-related development needs of chemical analysis of the compounds having physicochemical properties are very similar, especially organic compounds. Organic compounds exist as isomers and among theisomered compound have slightly different chemical structures so that the compound has the physicochemical properties are very similar and consequently the difficulty of separation.

HPLC usage is not only the compounds that have been known, but also to detect unknown compounds, especially natural organic compounds. Natural organic compounds generally have physicochemical properties are very similar and also each of the classes of compounds are always found in a biological sources. Saponin for instance is an

extremely diverse natural compounds in any plant material, even within each of the fractions in the extracts of the parts (organs) of plants. Saponins in the extract fraction of one part of the plant is generally a compound isomers [4]. The molecular structure of saponins are formed regularly with the pattern of the aglycone triterpene and steroid and glycon form of D-glucose, D-xylose, D-galactose, L-arabinose, L-fucose and L-rhamnose. The regularity makes saponins very much the diversity of species with physicochemical properties that are very similar [4]. Similarities of physicochemical properties are also the cause of the difficulty of separating the compounds of saponin. Additionally, saponin belonged naturally very polar compounds are extracted from n-butanol to the aqueous solvent. Polar saponins trigger difficulties for the stationary phase chromatographic separation is used mostly made of polar compounds, so it can only separate the non-polar compounds. Materials for the separation of natural compounds of which use the principle of filtering based on molecular weight compounds such as sephadex, but for the saponins in general are isomeric compounds. Therefore, saponin is a natural compound that is very difficult to separate because it has physicochemical properties are very similar to one another, the diversity of compounds very much, and a class of natural compounds are very polar.

Since 1980s, HPLC are showing better improvement, including HPLC preparative column. Chromatography is used to separate compounds separately and they can be obtained according to his form. Development of preparative 1980s HPLC up to 2010 only on an HPLC column that can be used for the purposes of analysis and isolation of the compound of interest. Development of preparative 2010HPLC over the better that is HPLC specifically designed for the isolation of compounds. The development of preparative HPLC, providing an opportunity search compounds known saponin difficult to separate, and the compound has the potential in the pharmaceutical field. In addition, each research saponin likely find new compounds as a result of the scarcity of research on saponin. HPLC analytical ability to separate compounds are supporters for both preparative HPLC eluent and indicators determining the purity of the compound. If the HPLC spectrum of a compound showed a single peak, the compound is a pure compound. Therefore the role of the analytical HPLC in the isolation of compounds is still very necessary. However, there are several events in the separation of saponins associated with the use of HPLC for the purpose of ascertaining the purity of the compound. It has been found two saponins from Kolowe seeds (*C. excelsus*) identified spectroscopically of an amorphous solid [7], and also from the leaves of *Pittosporum undulatum* [2], [3]. This shows that the amorphous solid pure saponins expressed by analytical HPLC, still allowing a mixture of isomered saponins, and the spectroscopy progress especially NMR> 300 MHz that can detect a mixture of compounds that have the physico-chemical properties are very similar.

MATERIALS AND METHODS

Plant Material

Kolowe seeds taken from Kamaru, Buton islands, Indonesia. Kolowe fruit fresh, peeled and obtained kolowe seeds that are ready to be extracted.

Extraction

Fresh seeds are blended in methanol. Kolowe seeds juice was macerated for 24 hours successively. Total of 3.8 kg of fresh seed extract obtained 1.24 kg or approximately 32.63% of the fresh powder. Before the fractionation is done, 500 g of the methanol extract kolowe seeds mixed with 500 g of silica GF60, then put in a Buchner as a chromatography column, and then eluted successively with diethylether, n-hexane, and 96% methanol. Taken 100 g extract the results for fractionated elution gradient with a liquid-liquid technique. Solvent fractionation successively with n-hexane-water; ethylacetate-water; and n-butanol-water. The extract fractions obtained was 0.8 g fraction of n-hexane; 10.8 g ethyl acetate extract and 34.20 g n-butanol fraction, and the remaining fraction of the water. Extract fraction of ethyl acetate, n-butanol and water fraction screening of secondary metabolites namely flavonoids, alkaloids, tannins, triterpenes, steroids and saponins. N-butanol fraction and water proofed only contains saponins, and contains no other metabolites. Saponins are suspected in n-butanol fraction of the extract was further purified. Extracts from the n-butanol fraction was dissolved in methanol to dissolve, then added with diethylether solvent and then shaken aided by a simple centrifugal until precipitation. The precipitate formed is separated by filtration and obtained a precipitate that is suspected as a pure saponin extract. The screening saponin extract and other metabolites and proved only contain saponin.

Saponins Isolation from Kolowe Seeds

Saponin isolation conducted at the Laboratory of Chemistry, School of Chemistry, University of Sydney, Australia. Saponin isolation using preparative HPLC column is preceded by analytical HPLC analytical column. A total of 50 mg of saponin extract dissolved in methanol: water: acetic acid (75: 25: 05), then 12.0 mL injected to the analytical HPLC. Analytical HPLC system used is a Waters 510 pump; Waters U6K injector, Waters 490E UV detector wavelength 254 nm and Waters R410 RI, C18-86 Alltima Alltech column reversed phase, column size 25 cm x 4.6 mm, particle size adsorbent or stationary phase 5 μm. Eluent found was methanol: water: acetic acid (75: 25: 0.5), a

flow rate of 0.8 mL / min. At Waters 490E UV detector detected 4peak, while the R410 RI detector obtained 25 peak.

To isolate the compound saponin used preparative HPLC techniques, Eluent was used by analytical HPLC eluent that is methanol-water-Astetic acid (75: 25: 0.5). Preparative HPLC system used was Waters 510 EF pump, injector Rheodyne 7125, 841 UV detector wavelength 250 nm and Waters R403 RI. Eluent flow rate of 11.25 mL/min at Alltech column Alltima reversed phase C18, column size 30 cm x 2.2 cm with stationary phase particle size of 10 lm. A total of 550 mg of n-butanol fraction of the extract is diluted with eluent has been provided, and then divided into three parts for injection purposes. The third solution is injected on a preparative HPLC and collected in 11 fractions. From the peak with a retention time of 23.06 minutes in analytical HPLC Waters R410 RI detector found an amorphous solid. Peak retention time were separated by preparative HPLC columns obtained two fractions with fractions 10 and 11. Fraction 11 formed an amorphous solid white as much as 11 mg without reset by preparative HPLC separation. The white amorphous solid with RL-4 which describes all four compounds were found by previous research on saponin separation of n-butanol fraction kolowe seed. The results of analytical HPLC analysis of the amorphous solid that showed a single peak so defined is a pure compound. Analytical HPLC analysis results in an amorphous solid RL-4 is shown in Figure 3

Spectroscopy Spectrum Analysis of RL-4
An amorphous solid, white colour from isolated fraction of n-butanol extract from kolowe seeds, then performed with FTIR, EIMS, and 400 MHz NMR.

a. NMR spectrum
NMR spectrometer Bruker Avance 400 used frequency of 400 MHz for ^{1}H NMR and 100 MHz for ^{13}C NMR. The techniques used proton spectrum recording, carbon, HH-COSY, 2D and ROE ROESY-1D, DEPT, HMQC, HSQC, TOCSY-1D, and HMBC. Snapshot dissolved in deuterated methanol (CD_3OD) in the NMR tube with an internal standard TMS (tetramethylsilane) for ^{1}H NMR and CD_3OD at delta 49.0 ppm for ^{13}C NMR. Snapshot taken spectra with a variety of techniques have been described previously.

b. Infrared spectrum (IR) and Mass spectra (MS)
Spectrometer used was Shimadzu FTIR 8400S using nujol techniques. Determination of the molecular mass of isolated compounds using techniques electrospray Ionization Mass Spectrometry (EIMS). Spectrometers are used Finningan MAT900 XL and Finningan LCQMS. Snapshot dissolved in methanol and mixed with sodium to facilitate ionization.

RESULTS AND DISCUSSION

Results of chemical screening against RL-4 indicates saponin and the basis for determining the instrument to determine its molecular structure. Object saponin molecule structure determination consists of three focus is the molecular structure of the aglycone, glycoside and other substituents. The main instrument of NMR structure determination using a variety of techniques, whereas the FTIR and EIMS as supporting data.

FTIR
Result from FTIR($_{Nujol}$).V_{max}.(cm^{-1}**);** IR Spectra Wavelength are3385; 3184, 2725, 2671, 1734, 1684, 1653, 1558, 1306, 1155, 1078, 721.

Mass Spectra
EIMS Result (HRMS);$(M+Na)^+$ m/z (% rel.): 1067 (100); Molecular mass RL-4 isolates were determined with EIMS technique by means of positive ionization. Isolates added sodium to facilitate ionization.Ion molecular mass (m / z) is 1067.5389 with an abundance of 100%. These ions are M + Na + thus isolates the molecular mass is reduced by atomic weight of sodium (23) obtained 1044.5389 or 1044. Molecular mass is consistent with the molecular formula $C_{52}H_{84}O_{21}$, was indicated as saponins.

NMR
The results of NMR analysis consisting of H and C NMR in various techniques. Chemical Shift analysis results NRM are:

a. **^{13}C NMR** (CD_3OD, 100 MHz) δ (ppm)are**:** 40,1; 27,1; 92, 4; 40,4; 56,6; 19,5; 37,1; 42,2; 48,1; 37,9; 24,7; 126,3; 144,4; 48,9; 68,4; 74,0; 45,3; 42,8; 47,6; 32,3; 46,0; 69,80; 69,75; 28,3; 16,9; 16,3; 17,9; 21,0; 66,4; 66,2; 33,6; 25,2; 105,6; 78,3; 86,8; 71,5; 78,4; 104,9; 75,2; 71,0; 67,1; 103,2; 76,1; 72,5;; 63,6; 177,9; 42,9; 28,0; 12,1; 17,3; 44,5; 22,8.

b. **^{1}H NMR** (CD_3OD, 400 MHz) δ (ppm) are : 1,65 (*m*); 1,01 (*m*); 1,89 (*m*); 1,75 (*m*); 3,22 (*dd, J* = 11,6 and 4 Hz); 0,79 (*d, J* = 11,6 Hz); 1,55 (*m*); 1,42 (*m*); 1,73 (*m*); 1,58 (*m*); 5,33 (*t, J* = 3,6 Hz); 3,85 (*d, J* = 7 Hz); 3,90 (*d, J* = 7 Hz); 2,40 (*dd, J* = 14 and 4 Hz); 2,41 (*t, J* = 12 Hz); 1,03 (*d, J* = 12 Hz); 2,09 (*t, J* = 12 Hz); 1,48 (*ddd*, J = 12; 5,8 and 1 Hz); 4,03 (*dd, J* = 12,3 and 5,7 Hz); 1,08 (*s*); 0,87 (*s*); 0,98 (*s*); 1,02 (*s*); 1,37 (*s*); 3,92; 3,82 (AB*q, J* = 11 Hz); 0,91 (*s*); 1,00 (*s*); 4,55 (*d, J* = 7,6 Hz); 3,81 (*m*); 3,75 (*m*); 3,60 (*m*); 3,78 (*m*); 4,61 (*d, J* = 7,6 Hz); 3,27 (*t, J* = 8 Hz); 3,32 (*t, J* = 8 Hz); 3,52 (*ddd, J* = 12; 8; and 5,2 Hz); 3,92 (*dd, J* = 12 and 5,2 Hz); 3,24 (*t, J* = 12 Hz); 4,96 (*d, J* = 8 Hz); 3,14 (*dd, J* = 9 and 8 Hz); 3,34 (*t, J* = 9 Hz); 3,08 (*dd, J* = 9 and 8 Hz); 3,29 (*m*); 3,83 (*dd, J* = 12 and 2 Hz); 3,55 (*dd, J* = 12 and 7 Hz); 2,39 (*sext, J* = 6,8 Hz); 1,70 (*ddd, J* = 14; 7; and 7,2 Hz); 1,49 (*ddd, J* = 14; 7; and 7,2 Hz); 0,90 (*t, J* = 7,2 Hz); 1,14 (*d, J* = 7 Hz); 2,23 (*dd, J* = 14; 4; and 7,2 Hz); 2,20 (*dd, J* = 14; 4; and 7,2 Hz); 2,07(*m*); 0,96 (*d, J* = 6,5 Hz).

Structure Elucidation of RL-4

^{13}C NMR spectrum There are 52 carbon signals that are correlated with proton signals. The number of signals in accordance with the results of the analysis of molecular mass in the mass spectrum (MS) is 1044. The results of spectral analysis DEPT DEPT 90 and 135 against the carbon signals are shown in Table 1.

^{1}H NMR and ^{13}C NMR anomeric proton signal present on 4,55 ppm (*d, J* = 7,6 Hz); 4,61 ppm (*d, J* = 7,6 Hz); and 4,96 ppm (*d,J* = 8 Hz), and the anomeric carbon signals 105,6 ppm; 104,9 ppm; 103,2 ppm. This indicates RL-4 isolates are glycosides compounds. Aglycone triterpene indicate because there are six quaternary carbon signals at chemical shift 30 – 50 ppm. Carbon signals at δ 40,4 ppm; 42,2 ppm; 37,9 ppm; 48,9 ppm; 45,3 ppm; and 32,3 ppm. This signal usually shown a quaternary triterpen C-4, C-8, C-10, C-14, C-17, and C-20 for Oleana triterpen. Triterpenoids as aglycone glycoside compound in RL-4 reinforced by the presence of seven singlet methyl proton signals in chemical shift 0,5 – 2 ppm, which protons in δ 1,08 ppm (*s*), 0,87 ppm (*s*), 0,98 ppm (*s*), 1,02 ppm (*s*), 1,37 ppm (*s*), 0,91 ppm (*s*), and 1,00 ppm (*s*). Protons are usually attached to methyl carbon C-23, C-24, C-25, C-26, C-27, C-29, and C-30. Methyl carbon which usually becomes main clue to identify triterpen can not be used in RL-4 isolates because there are more than seven methyl carbon signals with chemical shift between 10 – 35 ppm, however seven existing methyl carbon signal characteristic as triterpen. Thus, the aglycone glycosides RL-4 has sufficient evidence as triterpen of Oleana and a saponin. Thus the determination of the structure RL-4 isolates compound carried out in three main stagesare the determination of the structure of the aglycone, part sugar, and other substituents structures.

a. Aglycon structure elucidation

Olefinic carbon signal at δ126,3 ppm and 144,4 ppm indicates that the saponin aglycone is olean-12-en triterpene. This was supported by the IR absorption characteristics C=C are cyclic in wave numbers 1653 cm^{-1}. The early prediction of the key considerations in determining the position of the aglycone carbon and proton. Determination of the position of the aglycone carbon and proton spectrum is done through observation HMBC, HMQC, HSQC, H-H COSY, TOCSY-1D, whereas configuration and geometry of the ring substituents conducted with the ROE-1D and ROESY-2D experiment.

Position of the carbon in the structure of RL-4 aglycone are shown in Table 2. On C-22 and C28 each provide two signals (Table 3). C-28 Atom are shifting paramagnetic at 66,4 ppm and 66,2 ppm which indicates a substituent which is more electronegative than hydroxyl groups, whereas the C-22 atom has shifted are diamagnetic at 69,80 ppm and 69,75 ppm which indicates a substituent which is more electropositive or influence of C-28 substituents. According basic framework of the saponin aglycone RL-4 is olean-12-en triterpen, which is shown in Figure 4. Chemical shift of C-28 provide two signals 66,4 ppm and66,2 ppm indicating there are two substituents are not simultaneously bound to the atom C-28, while the two signals on C-22 69,80 ppm and 69,75 ppm was the effect of two substituents from C-28. Chemical shift of C-28 to the low field indicate the esterification in C-28, while chemical shift of C-22 still within the range of influence of the hydroxyl as if without the substituent (CH_2) around 35 ppm [1], [6]. Accordingly the atom C-28 esterification, but not bonded together. Allegations of two substituents at C-28 bound not simultaneously indicate that the RL-4 is still the two compounds are mixed, because if the two substituents are bound simultaneously then not in accordance with the molecular mass of the analytical results are EIMS 1044. Determining configuration and geometry of ring substituents conducted with technique ROE-1 D and ROESY-2D. Results spectrum decision ROE-1 D and ROESY-2D shown on Table 3.

Geometry of olean-12-en triterpen are ring A/B, B/C,and C/D was trans while ring D/E can be trans or cis form. Ring geometry of A/B, B/C, and C/D RL-4 aglycon proved to be trans by ROE-1D and ROESY-2D experiment (Table 5), while positioning of D/E conducted based on location H-28 and H-18 because C-28 bonded to C-17 in β position. H-18 irradiation and the results affect the H-28 signal indicating that the location H-18 and H-28 in the same field which means the geometry of the ring D/E is cis. ROE 1D Spectrum, H-5 irradiation result influnce to H-3 and proton signal at δ 1,08 ppm (s) known as H-23. This shown H-3 are in same field with H-5 with α

configuration, then the position of the glycoside bond C-3 was β-anomer. It is also supported by ROE-1D experiments with H-23 dan H-24 irradiation, and H-1' anomerik coupling constants taht is ^3J = 7,6 Hz.Futhermore, C-15 and C-16 hydroxyl substituted proven with chemical shift C-15 68,4 ppm and C-16 74,0 ppm. Carbonyl signal in that area for the characteristics of the carbon ring hydroxy triterpenoids [2], [3], [5].

The position of hydroxyl bound to C-15 determined based on the location of the 27-Me proton (methyl). Irradiation is carried out on proton H-27 and the result does not give effect to the chemical shift of H-15. This shows that the proton H-27 not in same field with H-15 proton, which means the position of hydroxyl at C-15 atom is α. For the position of hydroxyl at C-16 is determined based on the location of the H-16 against HAB-28. Irradiation is carried out on H-22 in order to determine the position of the H-21. ROE-1D dan ROESY-2D H-22 irradiation spectrum give effect to the chemical shift H-16, H-18, and H-30. This indicates that the position of hydroxyl at C-16 is α and H-18 is β. Futhermore, C-21 are bounds two protons that forms a AB signal. ROE 1D experiments, H-22 irradiations give effect to H-21 on δ 1,48 ppm (*ddd, J* = 12; 5,8; 1 Hz) which means that the proton signal is Hβ-21,whereas in δ 2,09 ppm (*t, J* = 12 Hz) is Hα-21. Thus the geometry and position of the aglycone substituent of RL-4 is shown in Figure 5.

Subsequent observations focused on the results of the molecular structure substituents attached to the C-28 atom.The existence of two carbon signals at C-22 and C-28 indicates there are two different substituents attached to either carbon or one of them. Based on ROE-1D and ROESY-2D experiment to the C-22 atom allegedly bound to hydroxyl, while the C-28 atom bound to an acyl substituent. To ensure more of their substituents at C-22 and C-28 were identified by HMBC through correlation techniques H-22 and H-28 to carbon substituents signal, whereas the determination of the acyl structure is determined by the HMBC, HMQC, HSQC, and HH-COSY technique, Results of identification with these techniques are shown in Tables 4 and 5.

^{13}C NMR spectrum from isolat RL-4 isolate there are signal characteristics as carbonyl at δ 177,9 ppm. The carbonyl signal supported by the IR absorption (Table 1). Proton signal H_{AB}-28 (δ 3,92 ppm; 3,82 ppm; *q,J* = 11 Hz long distance correlated to the carbonyl signal, Futhermore, H-22 (4,03 ppm, *dd, J* = 12,2; 5,7 Hz) , did not show a long distance correlation with the carbonyl signal. This indicates that the acyl substituent attached to the C-28 is not on the C-22.Spektrum HMBC Spectrum there is signal on carbon with δ 175,0 ppm correlated with H-28 proton(δ 3,92 ppm; 3,82 ppm; *q,J* = 11 Hz), and the carbon signal characteristic as carbonyl. This carbonyl signal also long distance correlated with proton signals at (δ 2,23 and 2,20 ppm; 2 x *dd, J* = 14,4; 7,2 Hz) indicated as the protons in the molecule substituents. This indicated that there is two substituents attached to the C-28, but not bound together in accordance with the results of the analysis of molecular mass. Thus saponin from RL-4 are two isomered compound with a carboxylic ester substituents. Mixtures of two saponins in one isolate has also been found Higuchi *et al.* (1983) isolated from leaves of *Pittosporum undulatum* [2], [3].

b. Structure elucidation of Acyl subtituent

The parameters observed were signals associated with carbon signals on δ 177,9 ppm and 175,0 ppm. HMBC Spectrum carbonyl signal δ 177,9 ppm correlated with proton signal δ 2,39 ppm (*sext, J* = 6,8 Hz); 1,14 ppm (*d, J* = 7 Hz); sinyal H_{AB}-28 on δ 3,92 ppm and 3,82 ppm (*q,J* = 11 Hz); sinyal AB on δ 1,70 and 1,49 ppm (2 x *ddd, J* = 14; 7; 7,2 Hz). Carbon signal on δ 177,9 ppm is carbonyl signal which correlates long distance with H-28, which indicates that the carbon signal at 177.9 ppm is C-1'''' acyl subtituent. Futhermore, that the C-1 ''''results correlate long distance with four proton signal acyl groups, two proton signals attached to the same carbon signal that protons in δ 1,70 ppm (*ddd*; *J* = 14; 7; 7,2 Hz) and δ 1,49 ppm (*ddd*; *J* = 14; 7; 7,2 Hz) bound to carbon on δ 28,0 ppm. It is supported with HH-COSY spectrum (Table 6) which shows the proton is geminal. The geminal protons are correlated with protons in δ 2,39 ppm(*sext; J* = 6,8 Hz) and also with protons in δ 0,90 ppm (*t, J* = 7,2 Hz). Furthermore protons in δ 2,39 ppm(*sext, J* = 6,8) correlated with the proton signals δ 1,14 ppm (*d,J* = 7 Hz), and δ 1,14 ppm signal (*d,J* = 7 Hz) that uncorrelated with other proton signals. Thus, the carbon that binds protons in δ 1,14 ppm (*d,J* = 7 Hz) bound to the carbon that binds protons in δ 2,39 ppm(*sext; J* = 6,8). Thus the structure of the acyl-related carbon signal at δ 177,9 ppm an be determined, as shown in Figure 6.

Subsequent observations conducted on NMR signals associated with a carbon signal δ 175,0 ppm. The signals that are correlated with that carbon signals are tabulated in Table 6 and Table 7.

Total carbon signal associated with the carbonyl signal δ 175,0 ppm are four signal, while associated with the carbonyl signal at 177.9 ppm is five. This indicated that the acyl group associated with the carbonyl at δ 175,0 ppm there are carbon equivalent signal.

In Tables 7 and 8 shows the signal AB is at δ 2,23 ppm and 2,20 ppm (2 x *ddd, J* = 14,4; 7,2 Hz). AB signal is correlated with the signal-carbon single bond (δ 28,0 ppm), and correlated with proton signal at δ 2,09 ppm (*m*).

Proton signal at δ 2,09 ppm (*m*) that cross-correlated with the signal of protons in δ 0,96 ppm (*d, J* = 6,5 Hz). Thus, the carbon which is correlated with the signal AB is C-2’’’’’; carbon correlated with the signal on one bond at δ 2,09 ppm (*m*) there is C-3’’’’’, while C-4’’’’’ and C-5’’’’’ is equivalent were correlated with the proton signals δ 0,96 ppm (*d*; *J* = 6,5 Hz). Thus the structure of the acyl-related carbon signal at δ 175,0 ppm can be determined as shown in Figure 7. Detailed position of a second carbon and proton acyl groups substituted on RL-4 saponin aglycone are shown in Table 8.

Based on these descriptions concluded that the atom C-28 RL-4 substituted aglycone two types of acyl is 2-methylbuthyroyloxy and 3-methylbuthyroyloxy. Acyl associated with the carbonyl signal δ 177,9 ppm is 2-methylbuthyroyloxy with ester-1 symbol, while related to the carbonyl signal δ 175,0 ppm is 3- methylbuthyroyloxy with ester-1 symbol. Thus the presumtion RL-4 isolates is a mixture of isomered two compounds have proven.

c. Saccharide structure elucidation.

NMR Spectrum of RL-4 There are three anomeric proton signal is on δ 4,55 ppm (*d, J* = 7,6 Hz); 4,61 ppm (*d,J* = 7,6 Hz); 4,96 ppm (*d,J* = 8 Hz) and three anomeric carbon signals at δ 105,6 ppm; 104,9 ppm; 103,2 ppm. This indicates that part of the saponins sugar of RL-4 containing three monosaccharide. The position of carbon atoms and protons every monosaccharides identified by HH-COSY, HMBC, HMQC, HSQC, TOCSY-1D experiment; while the type of monosaccharides identified with ROE-1D and ROESY-2D experiment. Tabulation of data from such experiments are shown in Tables 9 and 10.

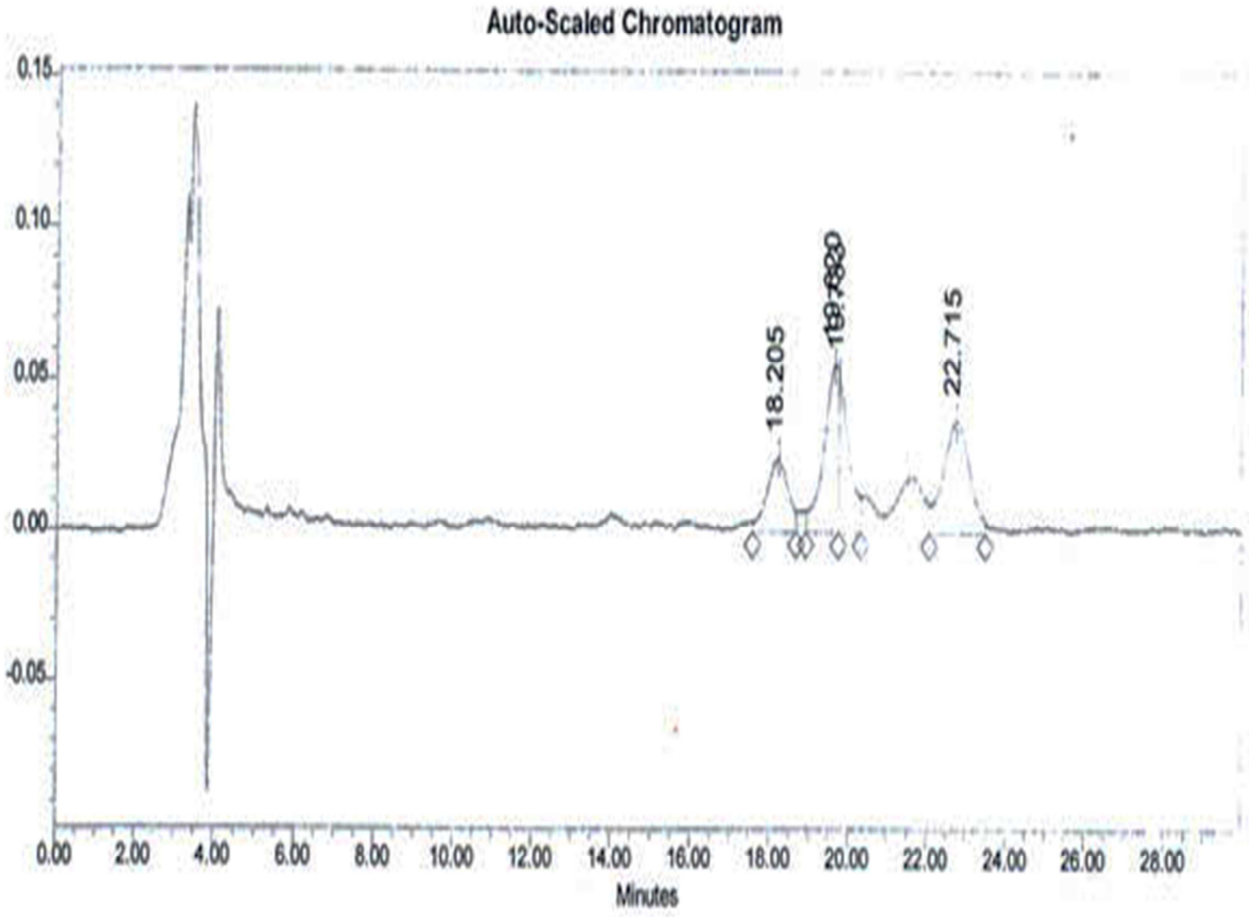
Analysis of the molecular structure of monosaccharides was performed three stages. The first stage of analysis of monosaccharides that is attached directly to the aglycone symbolized by M-1; monosaccharides that binds to M-1 symbolized by M-2, and monosaccharides are bound by M-2 is symbolized by M-3. Based on the experimental results, the structure of the monosaccharide M-1, glucuronic acid (Figure 8); M-2 monosaccharides, glucose (Figure 9) and monosaccharides are xylose M-3 (Figure 10).

Based on these descriptions of the part structure of the sugar saponin RL-4 isolates can be determined as shown in Figure 11.

d. Saponin Structure elucidation from RL-4 isolate

Based on these descriptions concluded that the RL-4 isolates from kolowe seed (*C. excelsus*) is a mixture of two saponins isomered. The second difference lies in the structure that saponin acyl substituent attached to the C-28 atom. Acyl substituent is 2- methylbuthyroyloxy first and second substituents 3- methylbuthyroyloxy. Thus the RL-4 isolates obtained two saponin symbolized by saponin 2 and saponin 3. Name saponin 2 is: 3-*O*-[β-D-Xylopyranocyl (1→3)-β-D-glucopyranocyl (1→2)-β-D-glucuronopyranocyloxy]-28-*O*-(**2-**methylbuthyroyloxy)-olean-12-en-15α, 16α, 22α-trihydroxy, whereas saponin 3 is: 3-*O*-[β-D-Xylopyranocyl (1→3)-β-D-glucopyranocyl(1→2)-β-D-glucuronopyranocyloxy]-28-*O*-(**3-**methylbuthyroyloxy)-olean-12-en-15α, 16α, 22α-trihydroxy The structure of the two saponins is shown in Figure 12.

Figure 1. HPLC analytic result of the saponin extract Kolowe seedby Waters 490E UV detector.



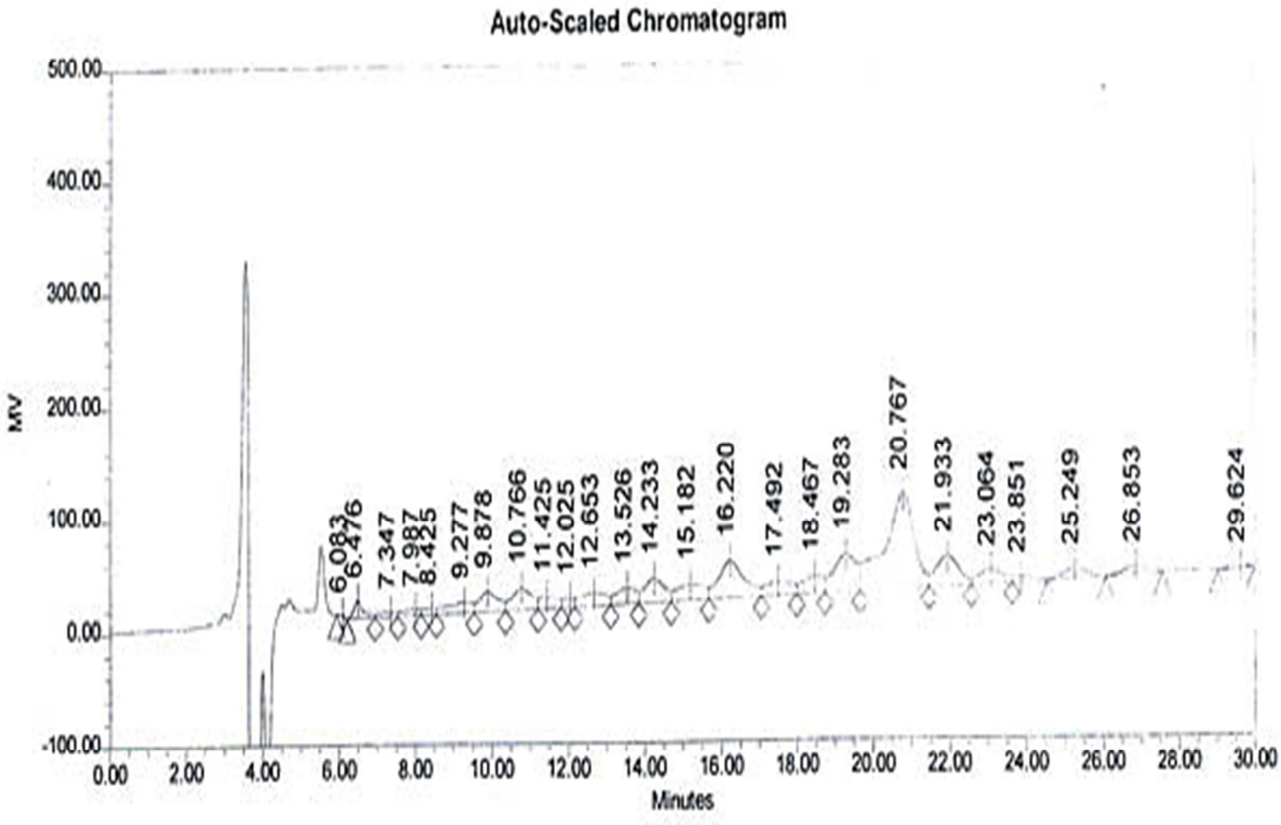


Figure 2. . HPLC analytic result of the saponin extract Kolowe seedby Waters 490E UV detector

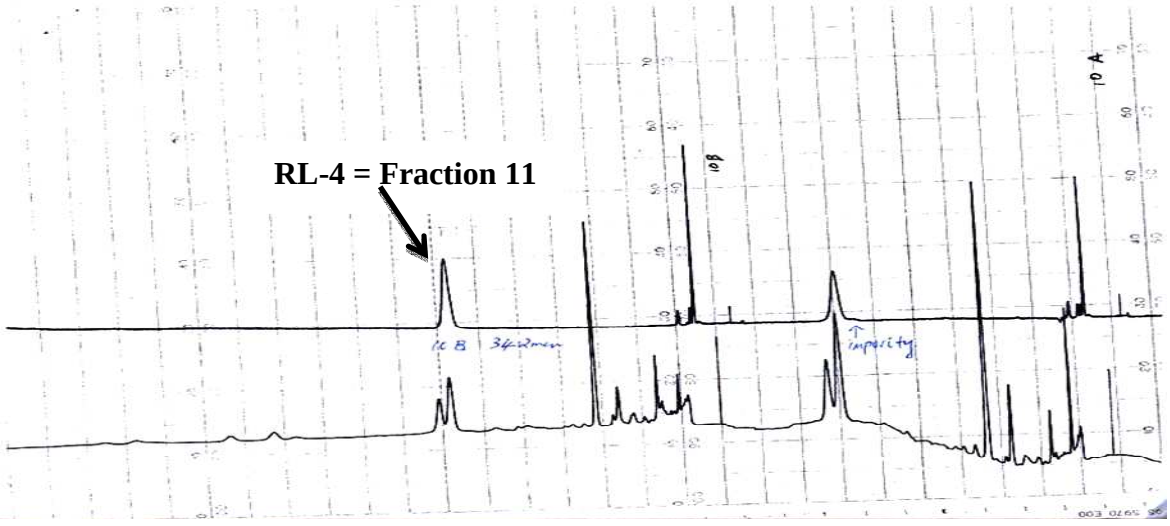


Figure 3. The results of analytical HPLC analysis with RI detector on amorphous solids separation results by preparative HPLC peak with a retention time of 23.06 on analytical HPLC chromatogram of n-butanol fraction extract kolowe seeds with RI detector

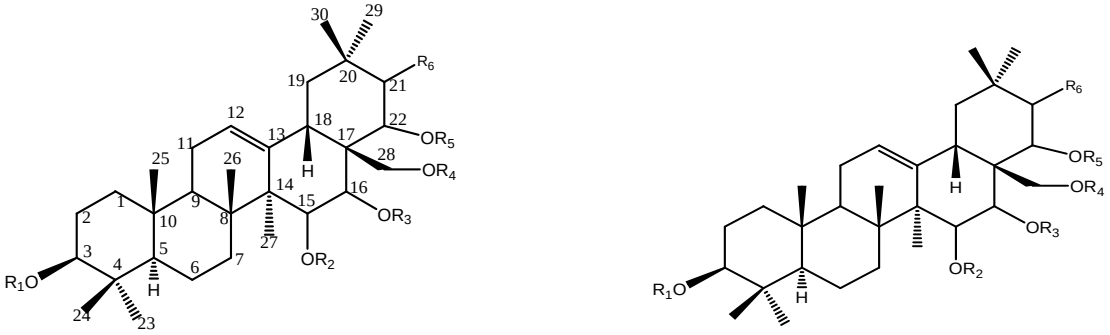


Figure 4. Hypothetical structure of saponin aglycone RL-4 Isolate

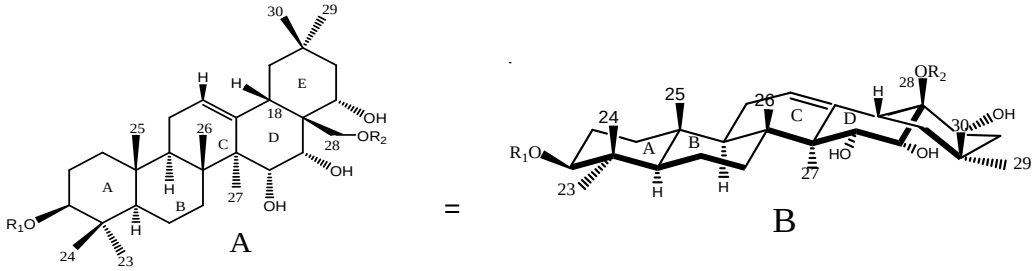


Figure 5. Saponin aglycon structure from RL-4 (A) = normal; (B) = conformation

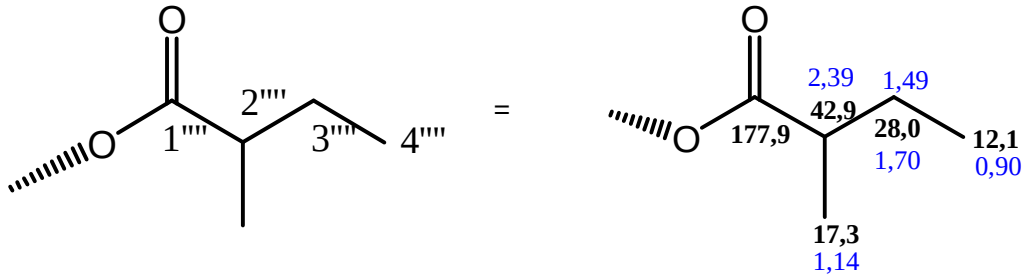


Figure 6. Acyl structure (ester) saponin from RL-4 related to carbonyl signal at 177,9 ppm

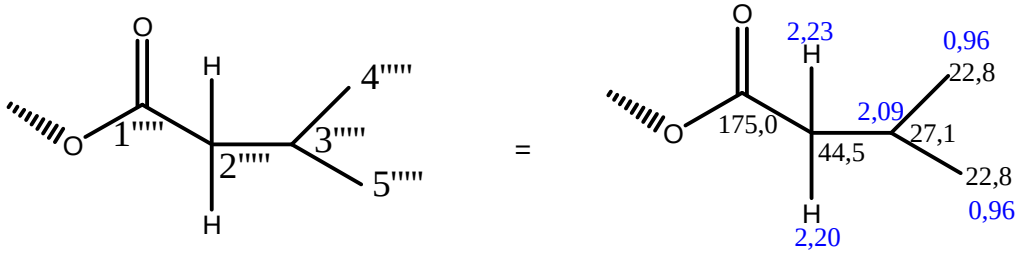


Figure 7. Acyl structure in saponin from RL-4 associated with the carbonyl signal 175,0 ppm

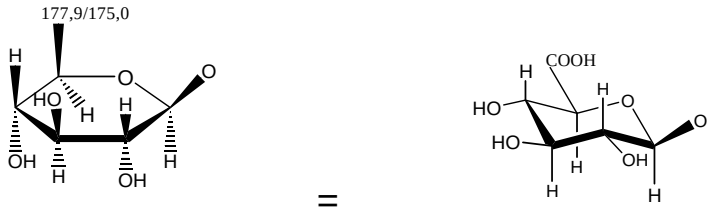


Figure 8. M1 structure monosaccharides (glucuronic acid) saponin sugar part of the RL-4

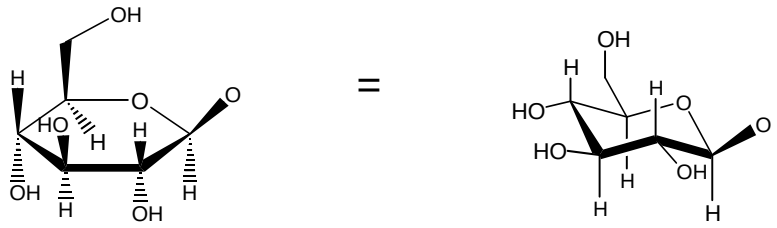
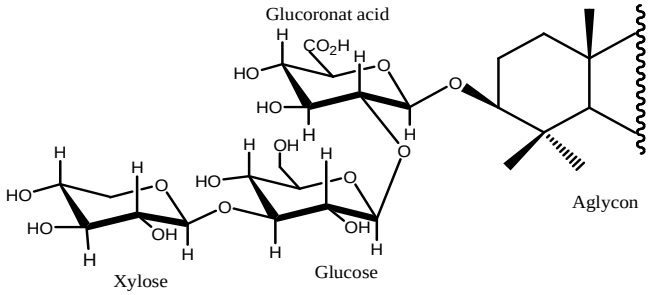


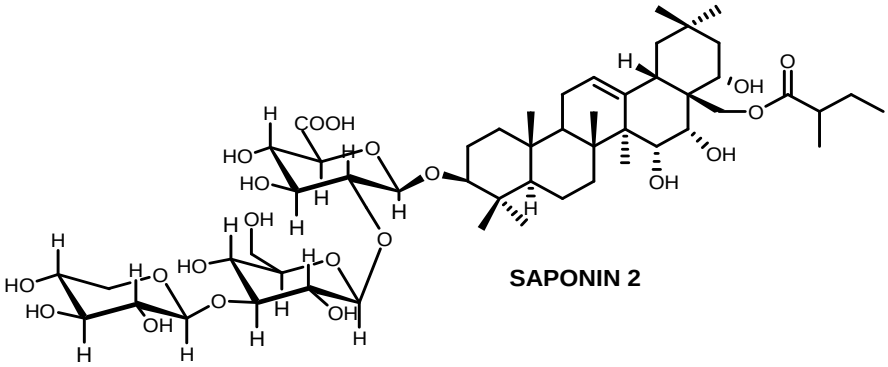
Figure 9. M2 structure monosaccharides (glucose) sugar saponin part of isolates RL-4

Figure 10. M3 Structure of monosaccharides (xylose) saponin sugar part of the isolates RL-4

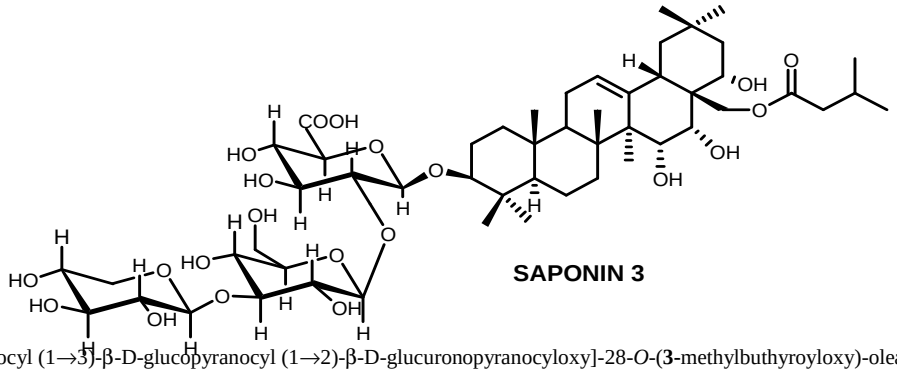


3-*O*-[β-D- Xylopyranocyl (1→3)-β-D- glucopyranocyl (1→2)-β-D- glucuronopyranocyloxy]

Figure 11. The structure of the sugar saponin RL-4 isolates from seed kolowe (C. excelsus)



3-*O*-[β-D-Xylopyranocyl (1→3)-β-D-glucopyranocyl (1→2)-β-D-glucuronopyranocyloxy]-28-*O*-(**2**-methylbuthyroyloxy)-olean-12-en-15α, 16α, 22α-trihydroxy.



3-*O*-[β-D-Xylopyranocyl (1→3)-β-D-glucopyranocyl (1→2)-β-D-glucuronopyranocyloxy]-28-*O*-(**3**-methylbuthyroyloxy)-olean-12-en-15α, 16α, 22α-trihydroxy.

Figure 12. Saponin structure 2 and 3 of the RL-4 isolated from the seeds kolowe (*C. excelsus*)

Table 1. Tabulation of carbon-type RL-4 isolateof the DEPT ^{13}C NMR spectrum

No	δ C (ppm)	DEPT	No	δ C (ppm)	DEPT	No	δ C (ppm)	DEPT
1	40,1	CH_2	19	47,6	CH_2	37	78,4	CH
2	27,1	CH_2	20	32,3	C	38	104,9	CH
3	92,4	CH	21	46,0	CH_2	39	75,2	CH
4	40,4	C	22	**69,80**	CH	40	71,0	CH
5	56,6	CH	23	**69,75**	CH	41	67,1	CH_2
6	19,5	CH_2	24	28,3	CH_3	42	103,2	CH
7	37,1	CH_2	25	16,9	CH_3	43	76,1	CH
8	42,2	C	26	16,3	CH_3	44	72,5	CH
9	48,1	CH	27	17,9	CH_3	45	63,6	CH_2
10	37,9	C	28	21,0	CH_3	46	177,9	C
11	24,7	CH_2	29	66,4	CH_2	47	42,9	CH
12	126,3	CH	30	66,2	CH_2	48	28,0	CH_2
13	144,4	C	31	33,6	CH_3	49	12,1	CH_3
14	48,9	C	32	25,2	CH_3	50	17,3	CH_3
15	68,4	CH	33	105,6	CH	51	44,5	CH
16	74,0	CH	34	78,3	CH	52	22,8	CH_3
17	45,3	C	35	86,8	CH			
18	42,8	CH	36	71,5	CH			

Table 2. Positions of carbon and proton in saponin aglycone of RL-4 isolates

carbon (C)		H Position and chemical shift	HMBC Correlation
Position	δ (ppm)	δ (ppm), mult, *J* (Hz)	
1	40,1	**H-1** (1,65; *m*; 1,01, *m*)	H-2; H-3; H-5; H-9; 25-Me;
2	27,1	**H-2** (1,89; *m;* 1,75, *m*)	H-1; H-3;
3	92,4	**H-3** (3,22; *dd;* 11,6; 4)	H-1; H-1; H-2; H-3; 23-Me; 24-Me
4	40,4	-	H-2; H-3; H-5; H-6; 23-Me; 24-Me
5	56,6	**H-5** (0,79; 11,6)	H-3; H-6; H-7; H-9; 23-Me; 24-Me; 25-Me
6	19,5	**H-6** (1,55; *m*; 1,42, *m*)	H-5; H-7
7	37,1	**H-7** (1,73; *m*)	H-5; H-6; 26-Me
8	42,2	-	H-9; H-11; H-15; 26-Me; 27-Me
9	48,1	**H-9** (1,58; *m*)	H-1; H-7; H-11; H-12; 25-Me; 26-Me
10	37,9	-	H-1; H-2; H-5; H-6; H-9; H-11; 25-Me
11	24,7	**H-11** (1,89; *m*)	H-9; H-12;
12	126,3	**H-12** (5,33; t; 3,6)	H-9; H-11; H-18;
13	144,4	-	H-11; H-12; H-15; H-18; H-19; 27-Me;
14	48,9	-	H-7; H-9; H-12; H-15; H-16; H-18; 26-Me; 27-Me
15	68,4	**H-15** (3,85; *d*, 7)	H-16; 27-Me
16	74,0	**H-16** (3,90; *d*, 7)	H-15; H-18; H-22; H_{AB}-28;
17	45,3	-	H-15; H-16; H-18; H-19; H-21; H-22; H_{AB}-28
18	42,8	**H-18** (2,40; *dd*; 14; 4)	H-12; H-19; H-22; H-16;
19	47,6	**H-19** (2,41; *t;* 12; 1,03; *d*; 12)	H-18; H-21; 29-Me; 30-Me;
20	32,3	-	H-18; H-19; H-21; H-22; 29-Me; 30-Me
21	46,0	**H-21** (2,09; *t;* 12; 1,48; *ddd*; 12; 5,8; 1)	H-19; H-22; 29-Me; 30-Me
22	69,80/69,75	**H-22** (4,03; *dd*; 12,2; 5,7)	H-16; H-18; H-21; H_{AB}-28
23	28,3	**H-23** (1,08, s)	H-3; H-5; 24-Me
24	16,9	**H-24** (0,87, s)	H-3; H-5; 23-Me
25	16,3	**H-25** (0,98, s)	H-1; H-5; H-9
26	17,9	**H-26** (1,02, s)	H-7; H-9;
27	21,0	**H-27** (1,37, s)	H-15
28	66,4/66,2	**H-28** (3,92; 3,82, AB*q*, 11)	H-16; H-18; H-22
29	33,6	**H-29** (0,91, s)	H-19; H-21; 30-Me
30	25,2	**H-30** (1,00, s)	H-19; H-21; 29-Me

Spectrum reffernces NMR: H-H COSY, HMBC, HMQC, HSQC, TOCSY-1D

Table 3. Experimental results ROE-1D and 2D-NOESY in determining the geometry of the ring substituents saponin aglycone and configuration of RL-4 isolates

Irradiation Proton	Influences proton
H-5	H-3. Hα-6 (1,55 ppm), Hα-7, H-9, H-23 (Me)
H-12	H-18, Hβ-19 (2,41 ppm), $(H-11)_2$,
H-18	H-12, Hβ-19, H-22, H_{AB}-28, H-30 (Me)
Hα-19	H-27 (Me)
H-22	H-16, H-18, Hβ-21 (1,48 ppm), H_{AB}-28, H-30 (Me)
H-23 (Me)	H-1', H-3, H-5, Hα-6
Hβ-19	H-18
H-24 (Me)	H-25 (Me)
H-25 (Me)	Hβ-1 (1,65 ppm), Hβ-2 (1,75 ppm), H-11, H-24 (Me)
H-26 (Me)	Hα-11, H-15, H-24 (Me)
H-27 (Me)	Hα-19, H-9
Hβ-6	H-24 (Me), H-25 (Me), H-26 (Me
H-29 (Me)	Hα-19, Hα-21 (2,09 ppm)
H-30 (Me)	H-18, Hβ-21, H-22

Refferences: ROE-1D and ROESY-2D

Table 4. HMBC correlations and HMBC / HMQC Tabulation signals acyl saponin RL-4 which relate to the carbonyl signal at δ 177,9 ppm

δ C (ppm)	Correlation	
	HMQC/HSQC δ H (ppm), mult., *J* (Hz)	HMBC δ H (ppm), mult., *J* (Hz)
177,9	-	(2,39*; sext;* 6,8), (1,70; 1,49; 2 x *ddd*; 14; 7; 7,2), (1,14; *d*; 7), (H_{AB}-28)
42,9	2,39*; sext;* 6,8	(1,70; 1,49; 2 x *ddd*; 14; 7; 7,2), (0,90*; t*; 7,2), (1,14; *d*; 7)
28,0	1,70; 1,49; 2 x *ddd*; 14; 7; 7,2	(0,90*; t*; 7,2), (2,39*; sext;* 6,8), (1,14; *d*; 7)
12,1	0,90*; t*; 7,2	(2,39*; sext;* 6,8), (1,70; 1,49; 2 x *ddd*; 14; 7; 7,2)
17,3	1,14; *d*; 7	(2,39*; sext;* 6,8), (1,70; 1,49; 2 x *ddd*; 14; 7; 7,2)

Table 5. HH-COSY correlation tabulation acyl saponin from RL-4 isolates associated with the carbonyl signal at δ 177,9 ppm

δ H (ppm); mult.; *J* (Hz)	δ H (ppm); mult.; *J* (Hz)
(2,39*; sext;J* = 6,8 Hz)	(1,70; *ddd*; *J* = 14; 7; 7,2 Hz) (1,49; *ddd*; *J* = 14; 7; 7,2 Hz) (1,14; *d*; *J* = 7 Hz)
(1,70; *ddd*; *J* = 14; 7; 7,2 Hz)	(1,49; *ddd*; *J* = 14; 7; 7,2 Hz) (0,90*; t*; *J* = 7,2 Hz)
(1,49; *ddd*; *J* = 14; 7; 7,2 Hz)	(0,90*; t*; *J* = 7,2 Hz)

Table 6. HMBC correlations and HMBC NMR Tabulation signals in saponin RL acyl-4 which relate to the carbonyl signal at δ 175,0 ppm

δ C (ppm)	Correlation	
	HMQC/HSQC δ H (ppm), mult., *J* (Hz)	HMBC δ H (ppm), mult., *J* (Hz)
175,0	-	H_{AB}-28 (3,92; 3,82) (2,23; 2,20; 2 x *dd*, 14,4; 7,2)
44,5	2,23; 2,20; 2 x *dd;* 14,4; 7,2	(2,09; m); (0,96; *d*, 6,5)
27,1	2,09; *m*	(2,23; 2,20; 2 x *dd*, 14,4; 7,2); (0,96,; *d*, 6,5)
22,8	0,96; *d*, 6,5	(2,23; 2,20; 2 x *dd*, 14,4; 7,2); (2,09; *m*)

Table 7. Tabulation of correlation HH-COSY NMR signals in saponin RL acyl-4 signals that relate to the carbonyl δ 175,0 ppm

δ H (ppm), mult., *J* (Hz)	δ H (ppm), mult., *J* (Hz)
(2,23; *dd*; *J* = 14,4; 7,2 Hz)	(2,20; *dd*; *J* = 14,4; 7,2 Hz) 2,09; *m*
(2,20; *dd*, *J* = 14,4; 7,2 Hz)	2,09; *m*
2,09; *m*	(0,96; *d*, *J* = 6,5 Hz)

Table 8. Tabulation position proton and carbon acyl group substituted on the C-28 saponin aglycone RL-4

Carbon (C)		Proton (H)	HMBC Correlation
Position	**δ (ppm)**	**δ (ppm), mult, *J* (Hz)**	
Ester-1			
1''''	177,9		H-2''''; H_{AB}-3''''; H-5''''; H-22
2''''	42,9	**H-2''''** (2,39; sext., 6,8)	H_{AB}-3''''; H-4''''; H-5''''
3''''	28,0	**H-3''''** (1,70; 1,49; 2 x *ddd*; 14; 7; 7,2)	H-2''''; H-4''''; H-5''''
4''''	12,1	**H-4''''** (0,90; *t*; 7,2)	H-2''''; H_{AB}-3''''; H-5''''
5''''	17,3	**H-5''''** (1,14; *d*; 7)	H-2''''; H_{AB}-3''''
Ester-2			
1'''''	175,0	-	H_{AB}-2'''''; H-3'''''
2'''''	44,5	**H-2'''''** (2,23; 2,20, 2 x *ddd*; 14; 4; 7,2)	H-3'''''; H-4/H-5'''''
3'''''	27,1	**H-3'''''** (2,09; *m*)	H-2''''' H-4/H-5'''''
4'''''	22,8	**H-4'''''** (0,96, *d;* 6,5)	H-2'''''; H-3''''';
5'''''			

Refferences: HMBC, HMQC, HSQC, HH-COSY spectrum.

Table 9. Tabulation of of carbon and proton position saponin sugar part of the RL-4

Carbon (C)		Position and [δ H (ppm)	HMBC Correlation
Position	**δ (ppm)**	**mult; *J* (Hz)]**	
Monosaccharides-1			
1'	105,6	**H-1'** (4,55; *d*; 7,6)	H-2'; H-3; H-5';
2'	78,4	**H-2'** (3,82; *m*)	**H-1''**, H-1' H-3'; H-4'
3'	86,7	**H-3'** (3,75; *m*)	H-1'; H-2'**;** H-4' H-5'; **H-1''**
4'	71,5	**H-4'** (,60; *m*)	H-2'; H-3'; H-5'
5'	78,4	**H-5'** (3,81; *m*)	H-1'; H-3'; H-4'
6'	-	.	
Monosaccharides-2			
1''	103,2	**H-1''**(4,96; *d*; 8)	H-2''; H-3''; H-5'', **H-2'**
2''	76,1	**H-2**'' (3,14; *dd*; 9; 8)	H-1''; H-3''; H-4''
3''	78,4	**H-3''** (3,34; *t;* 9)	H-1''; H-2''; H-4''; H-5'', **H-1'''**
4''	72,5	**H-4''** (3,08; *dd*; 9; 8)	H-2''; H-3''; H-5''; H_{AB}-6''
5''	75,2	**H-5''** (3,29; *m*)	H-1''; H-3''; H-4''; H_{AB}-6''
6''	63,6	**H-6''** (3,83; *dd;* 12; 2) **H-6''** (3,55; *dd*; 12; 7)	H-4''; H-5''
Monosaccharides-3			
1'''	104,9	**H-1'''** (4,61;*d*, 7,6)	**H-3'''**; H-2'''; H-3'''; H_{AB}-5'''
2'''	75,2	**H-2'''** (3,27; *t;* 8)	H-1'''; H-3'''; H-4'''
3'''	78,3	**H-3'''** (3,32; *t*; 8)	H-1'''; H-2'''; H-4'''; H_{AB}-5'''
4'''	71,0	**H-4'''** (3,52; *ddd;* 12; 8; 5,2)	H-2'''; H-3'''; H_{AB}-5'''
5'''	67,1	**H-5'''** (3,92; *dd;* 12; 5,2) **H-5'''** (3,24; *t;* 12)	H-1''; H-3'''; H-4'''

Refferences: HMQC, HSQC, HH-COSY, HMBC, TOCSY-1D spectrum

Table 10. Experiments result from ROE-1D and ROESY-2D

Irradiation proton	Influences Proton
H-1'	H-3; H-3'; H-5' ; 23-Me
H-1''	H-3'; H-3'; H-3''; H-5''
H-1'''	H-3; H-3'''; Hα-5''' (3,24 ppm)

CONCLUSION

1. A compound isolated from natural products or synthesized expressed purely based on single peak from the analysis of HPLC analytical column was allowed as a mixture of compounds isomered with physico-chemical properties that are very similar
2. Mixtures of two isomered saponin compounds with physicochemical properties are very similar which cannot be separated by HPLC analytical column, its molecular structure can be determined by NMR 400 MHz with HH-COSY; HMBC; HMQC; HSQC; ROE-1D; ROESY-2D; TOCSY-1D, DEPT experiment and helped with MS and FTIR.
3. Two saponins isomered from Kolowe seed (*C. excelsus*) which cannot be separated by HPLC analytical column, it can be determined the structure by NMR 400 MHz

Acknowledment
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