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Structural Analysis of Saponin Isolate from the Soapbark Tree Extract

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ABSTRACT

The outbreaks of pandemics and epidemics have reenacted interest in high molecular weight bioactive molecules. Saponin is one of these molecules because of its associated health and commercial benefits. In this perspective, saponins from the aqueous extract of the soapbark (Q. saponoria) tree are attracting a rejuvenated interest among scholars. Therefore, this study was to provide further structural clarity to one of the identified saponin constituents of the soapbark tree extract. Adopting standard procedures for gas-liquid chromatography and in hyphenation with mass spectrometry, the alditol acetates and the associated methylated derivatives of the saccharide linkages of the saponin were evaluated. The space tandem mass spectrometric analyses and the NMR evaluation of the saponin fraction were also carried out. Obtained data showed the presence of a carbonyl proton peak at C-23. The finding suggests a deviation from previous reports for similar saponins with phytolaccinic acid aglycone. And may explain the possible bioactivity benefit of the molecule.

Keywords: Chromatography, Extract, Phytolaccinic, Saponin, Soapbark, Spectrometry.

Introduction

Recent findings have ignited a rejuvenated interest in phyto-principles and herbal therapy. This paradigm shift is not farfetched with the deploring trend of epidemics and pandemics, despite the vigorous techniques that are now, easily applicable to drug discovery. Unfortunately, the deteriorating economies particularly, of the low and middle-income countries (LMICs) have left the globe one step behind in addressing the emerging health issues.[1, 2, 3, 4] The COVID-19 (coronavirus disease 2019), a recent pandemic led to global travel restrictions and minimal economic activities.[5] Herbal therapy and nutraceuticals are now a big market in many countries because of their cost and accessibility.[6, 7] Some of the phytochemical constituents of the beneficial herbs have been isolated and elucidated. Among these constituents are the saponin molecules found in the soapbark tree (*Q. saponoria*) of the family Quillajaeceae.[8, 9, 10, 11] The soapbark tree has been reported for its beneficial application in the pharmaceutical, health and agricultural sectors.[12, 13] The aqueous extract of the bark of this plant has been demonstrated to be bioactive. The bioactive has been associated with the possession of saponin in the inner layer of the bark.[14] Some of the reported saponins from this herb which are collectively known as quillaja saponins include QS-21, QS-17 and QS-7.[14] Although some of these saponins have been associated with known beneficial bioactivities, others are still lacking in the available data that may provide their structural clarity.[14, 15]

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Such clarity could provide an understandable guide to the mechanism of their bioactivity and application(s). Therefore, this study was aimed at providing further lucidity to one of the previously identified saponin constituents of the aqueous extract of the soapbark tree, using gas-liquid chromatography (GLC), mass spectrometry (MS) and nuclear magnetic resonance (NMR) techniques.

Materials and Methods

Materials

All solvents and reagents used were of analytical grade obtained from the British Drug Houses (BDH) Chemical Company. Reference standards for glucose, fructose, rhamnose, glucuronic acid, galactose, xylose, arabinose, and lactose were obtained from the open market; the pure saponin fraction sample from the commercial extract product of *Q. saponoria*: Quil-A was obtained by semi-preparative RP-HPLC procedure and identified as described in our prior study.[8, 16] Model 3920 Gas Liquid Chromatography (GLC) (Perkin-Elmer, Germany); Model 5985 B GC/MS/DS GLC-Mass Spectrometer (MS) (Hewlett-Packard. USA); Macromass VG Quattro II MS/MS (Quadrupole-Hexapole-Quadrupole) (Macromass, UK); and INOVA 400 MHz Nuclear Magnetic Resonance (NMR) spectrometer (Varian, USA); and ultra-low temperature freezer MDF-150C118 (Antech, Germany) was applied in the structural elucidation.

Methods

Alditol acetate derivatization

Acid hydrolysis: Ten milligrams of the dried saponin sample were dissolved in 2 mL of 2 M trifluoroacetic acid (TFA), mixed using the vortex and kept at 100° C. The mixture was evaporated to dryness on a Rotary Evaporator RE-1100 (Bibby Scientific, UK) after 12 hours; and the residue recovered.[17-21]

To the above hydrolysis residue, 2 mL saturated aqueous solution of sodium borohydride ($NaBH_4$) was introduced. Two drops of glacial acetic acid and 2 mL of acetic anhydride in pyridine at a 1:1 ratio, were added to the mixture. The mixture was then kept at 90 to 100°C for 8 hours before evaporating to dryness on the rotary evaporator.[17, 19] The

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obtained residue (3.45 mg) was kept in the ultra-low temperature freezer at -70º C for subsequent use.

Methylation of the alditol acetate derivatives
Two milligrams of the alditol acetate residue obtained above, dissolved in 2 mL of dimethyl sulfoxide were mixed with 2 mL of methylsulfonyl carbanion in a small flask using a magnetic stirrer. The mixture was stirred under a nitrogen stream for 10 minutes at room temperature.[19] An excess of methyl iodide was added and further stirred for 20 minutes. The reaction mixture was diluted to twice its volume with water, and extracted twice with the same volume of chloroform. The extract was washed with water severally and evaporated in a vacuum using the freeze dryer. Obtained residue was dissolved in a 2 ml one-to-one mixture of ether-petroleum, and washed with water to remove traces of dimethyl sulfoxide.[17] The solution was then evaporated under streams of nitrogen, leaving 0.078 mg of the methylated residue that was preserved at -70º C in an ultra-low temperature freezer for further use.

Preparation of synthetic Alditol acetate derivatives from reference monosaccharides
Adopting the procedures as described above, ten milligrams of each sugar (reference standard) was used to derive their respective alditol acetate residue. These derivatives were used as the respective internal standards for the GLC evaluation.[17, 19]

GLC and GC-MS analyses
Instrumentation: The preconditioned fused silica capillary GLC column of dimensions 0.32 mm id x 25 m containing 3% OV-17 (Chompack) and Perkin- Elmer model gas chromatograph with a hydrogen flame detector was used for the GLC experiments. The GLC-packed column of 1.5 % silar 7CP on Gas Chrom Q (specifications: 100-120 mesh; 2 mm id x 183 cm) obtained from Chrompack was used for the GC-MS analyses. Sample preparation: One milligram of the alditol acetate derivative (of saponin sample or reference monosaccharides) was dissolved in 1 mL of hexane and 1 mg of the methylated residue was dissolved in 1 mL of chloroform for their respective GLC and GC-MS analyses.

Chromatogram and spectra development: Applying the stationary phase column as stated above, the GC was developed using the Perkin-Elmer Model 3920 GLC operated isothermally and conditioned at temperatures varied at a rate of 4º C/min from 190 to 220º C with the mobile phase flow rate maintained at 40 mL/min.[19] The hexane mobile phase was used for the GLC analysis of the alditol derivatives and data were interpreted for each monosaccharide unit using the chromatogram peaks obtained for the respective internal (reference) standards. The assigned monosaccharide units were further supported by the respective retention time of the alditol acetate derivatives of the reference monosaccharides.

Using chloroform mobile phase for the GC-MS analyses of the methylated derivatives, the GC-MS stationary phase column (cited above) was conditioned at a temperature program ranging from 170° C (delay 2 min) to 250° C.[17] All the GC-MS spectra for the permethylated alditol acetates were obtained using the Hewlett-Packard Model 5985 B GC/MS/DS. The MSD (mass spectra detector) is equipped with a dual electron impact-chemical ionization (EI-CI) source that has a membrane separator set at a source temperature of 160º C and an ionizing voltage of 70 eV. CI spectra were recorded at a source pressure of 120 Pa, using methane as reagent and carrier gas, at a source temperature of 150° C, and ionization voltage of 230 eV. Data were interpreted online using the National Institute of Standards and Technology (NIST) MS data library.

Mass spectroscopy (MS) of saponin fraction
Instrumentation: The Macromass VG Quattro II MS/MS (Quadrupole-Hexapole-Quadrupole) instrument fitted with electrospray ionization (ESI) source was used for the MS and space tandem mass spectrometric (MS/MS) analyses of the saponin fraction. In consistence with previous reports, the ionization temperature and voltage were conditioned at 200º C and 70 eV.[17] Sample preparation: A sufficient amount of the saponin fraction was dissolved with 10mM ammonium acetate 10 % solution to obtain a 1 mg/mL solution. The solution was ultra-sonicated, filtered through a sintered glass filter funnel, and used for spectra development.

MS Spectra development: Using the direct inlet (DI) technique, 1 mL of the sample was introduced into the source unit of the mass spectrophotometer with the aid of the sample cup and DI probe (which was heated once inserted into the instrument). The source unit was kept at 300º C. Ten microliters of the sample were injected into the ESI. A cone voltage-induced dissociation (CVID) was applied, increasing from 50 to 200 KV. Spectra scans were obtained from 300 to 3000 m/z. Collision-induced dissociation (space) MS/MS (CID-MS/MS): Low-energy collision-induced dissociation tandem space mass spectrometry (CID-MS/MS) experiment was performed using the same technique as described for the MS spectra development. However, argon was used as the collision gas in the space tandem collision cell. The cone voltage was set at 25kV and the collision energy at 180 eV.

NMR spectrometric analysis
Instrumentation: The Varian INOVA 400 MHz NMR which has a console equipped with one high-band (1H/19F) channel and two broadband-band channels was pre-conditioned for the experiment. The instrument was configured and optimized for ^{1}H sensitivity.

Sample preparation: A sufficient quantity of the saponin fraction was dissolved in deuterated methanol (CD_3OD) to approximately 0.4%$^{w}/_{v}$ solution at room temperature. The solution was vortexed for 1 minute at 400 rpm using the Basic Vortex Mixer 3067-71 (Thermo Scientific©, India). Aided by a teat, the solution was filtered using a glass cotton wool-fitted Pasteur pipette (previously washed with CD_3OD) into a clean 5 mm outer diameter NMR sample tube.
NMR Spectra development: The sample containing tube, closed with a Teflon tape-wrapped cap and held in the spinner, was placed in the sample depth gauge. Analysis was carried out with the 5 mm triple resonance inverse probe, and the NMR spectra (proton frequency 400 MHz) were recorded. The chemical shifts were reported in ppm referenced against the solvent peaks and the coupling constants (J_{H-H}) in Hetz (Hz). Also, the Heteronuclear Multiple Bond Correlation (HMBC) experiment, at delay times between 50 and 70 ms, was also performed.

Results and Discussion

Prior data have been reported on the isolation of saponins from the soapbark tree using RP-Chromatography with mobile systems of increasing polarity. The available data in this study are substantial contributions to the elucidation of the chemical structure of one of the saponins that were identified.[8]

GLC and GC-MS
GC-MS experiments are used to provide data on the glycone substituents. The obtained peaks for the alditol acetate and permethylated derivatives of the saponin glycone units provide supporting evidence(s) on the number, types and configuration of the constituting saccharide units, as well as the type of saponin.[24, 25] In such experiments, it is typical to hydrolyze the glycosidic linkages in other to release the constituting saccharide units of the glycone moiety. The obtained alditol acetates of the saccharide units, as in this study, present the monosaccharide derivatives in the open chain Fischer (projection) conformation which lacks the expression of epimerism common with simple sugars, due to the possession of chiral center(s).[24]

Alditol acetate derivatization
Acetylation of the alditols eliminates the anomeric centers and chirality typical of the ring forms of simple sugars. Thus, this presented the monosaccharides in the open-chain form that ultimately simplifies the GLC-chromatogram to a single peak for each sugar unit derivative.[25] In this form, they were stable, quantifiable and storable. The gas

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chromatogram of the alditol acetate was used to identify the sugar residues (Figure 1). The peaks and data interpretation using the internal standards indicated the presence of rhamnose (Rha), fucose (Fuc), galactose (Gal), glucuronic acid (GluA), and glucose (Glu) in the order of their retention times, as the monosaccharides that make up the glycon moieties. This was further corroborated by the GLC-chromatogram obtained for the respective standards. In all instances, the pattern of the peaks identified for the sugar units was consistent with earlier reports for the derivatives: rhamnitol pentaacetate, fucitol pentaacetate galactitol hexaacetate, tetraacetylgluconic acid, and glucitol hexaacetate respectively.[18, 22, 24] Similar monosaccharide constituents were reported for the quillaja saponins however, the absence of xylose in this study clearly suggests a departure from the norm associated with quillaja saponins that possess quillaic acid aglycone.[26] The internal standards were essentially included in this study to resolve identification difficulties inherent with the non-reproducibility of the correction factor of peak areas obtained by the ionization detectors, for alditol acetate derivatives from different sugars especially, when capillary columns are used. This is common, but may not be restricted to the flame-ionization detector alone.[25, 27, 28]

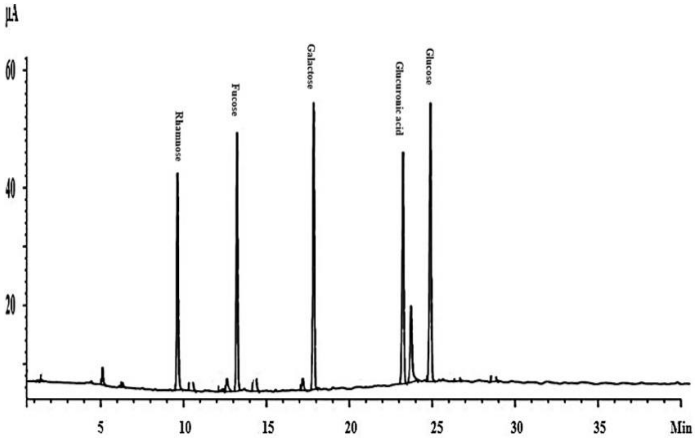

Figure 1: The identification of the monosaccharide residues of the pure fraction using the GC peaks of the alditol acetate derivatives.

Methylation of the alditol acetate derivatives

The sensitivity of sugar analysis is increased by the formation of permethylates of the alditol acetates. This is because the active hydrogen in the acetate is replaced by an alkylsilyl unit like the trimethylsilyl. In effect, the replacement reduces the propensity to form hydrogen bonding and increase volatility though, the thermal stability is maintained.[29] As such, the signal is increased with the silylation of the alditol acetates.[25, 29]

GC-MS peaks of the permethylated alditol acetates indicate the presence and linkage of glycone moieties. This was a suitable approach to resolve the absolute configuration of the monosaccharides in this study, as it proffers the position of the glycosidic linkages between sugar residues.[30] The absolute configuration of the silyated derivatives as identified by the primary m/z spectra analysis using the NIST-library is indicated in Table 1. The obtained data confirms the absence of xylose. Further interpretation of data in combination with previous reports suggests the presence of a bisdesmosidic glycosidic structure with a disaccharide at C-3 and trisaccharide at C-28 of the aglycone nucleus. Three types of aglycones have been associated with the quillaja saponins: quillaic acid, gypsogenin and phytolaccinic acid. However, reports on the structural elucidation of the saponins have shown that the absence of xylose may potentially indicate the possession of a phytolaccinic (sometimes called phytolaccagenic) acid aglycone nucleus.[31, 32]

Mass Spectrometry

In recent times, the generation of parent and daughter ions in mass spectrometry has formed a major part in providing structural clarity or identification of molecules. Molecular ions (parent ions) have been shown to be closely related to their parent molecule with regard to the m/z ratio. Dissociation of the energetically unstable molecular ions into charged fragments (daughter ions) can occur in a pattern that is consistent with specific bond characteristics, typical of a particular molecule. As such, the application of the MS and MS-space-tandem techniques in this study was geared to provide clarity to the molecular structure of the saponin fraction. CVID-MS (Figure 2) and the CID-MS/MS (Figure 3) data indicated typical peaks and fragmentation patterns which suggests that the daughter ions are probable products of the parent ion from the pure saponin fraction.

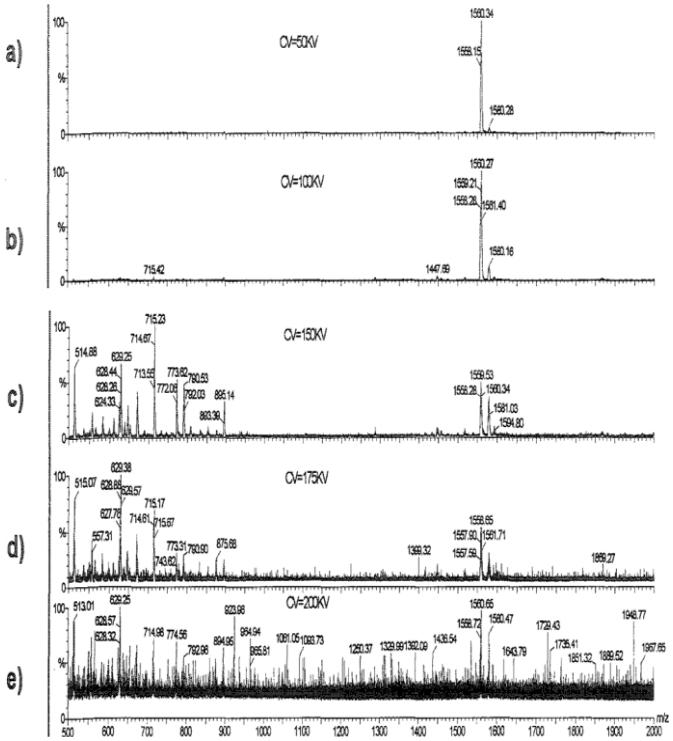

Figure 2: CVID of the pure fraction which indicates the increasing number of daughter ions and the progressive decrease of the size of the peak appearing at 1560 m/z with increasing CV from 50 KV at "a" through b, c, d to e, at 200 KV.

CVID-MS of the Saponin Fraction

Spectra data (Figure 2a) of the saponin fraction obtained at a cone voltage of 50 KV was clean with little or no noise at approximately m/z 1560. At the induction of cone voltage dissociation (CVID-MS) by increasing the cone voltage (CV) step wisely, different sets of typical daughter ions were observed at the CV of 100 (Figure 2b), 150 (Figure 2c), 175 (Figure 2d) and 200 KVs (Figure 2e). Following the increasing number of peaks obtained as the CV was increased, it was obvious that most of the proceeding signals (daughter ions) emanated from the molecular ion observed at m/z 1560. Interpreting the obtained spectra peaks in Figure 2c, d, e in collaboration with prior reported studies suggests the presence of aglycone ions (A-ion) at m/z 853; 791; and 513 which are typical of the phytolaccinic acid and sugar units at C-3; phytolaccinic acid and C-3 sugar unit with the absence of *O*-acetyl unit at C-23, and loss of carbon dioxide with H_2O from the glucuronic acid residue; and phytolaccinic acid with the absence of *O*-acetyl unit at C-23 respectively.[33,34, 35] The signal at m/z 629 which can also occur at 651 in sodiated positive mode, and 715 which is typical of galactose, have been reported for S13, a quillaja saponin that possesses a phytolaccinic acid aglycone.[33, 34, 35, 36]

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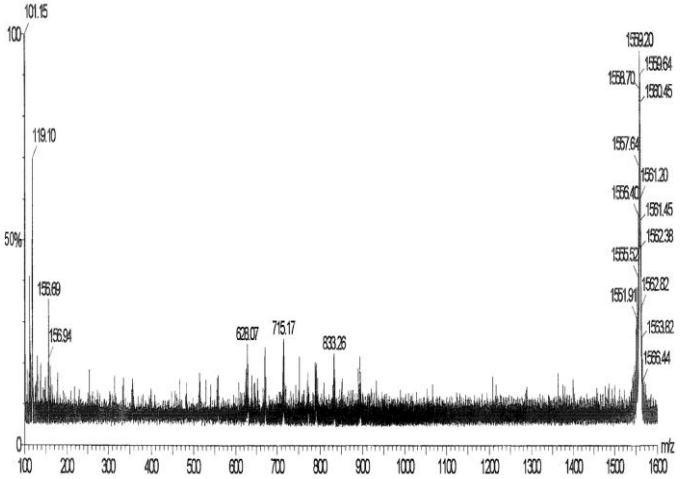

Figure 3: Low-energy collision-induced dissociation tandem space mass spectrometry (CID-MS/MS) of the pure fraction, indicating a series of daughter ions which are probable fragmentation products of the parent ion occurring at 1560 m/z.

CID-MS/MS space tandem of the Saponin Fraction

The CID-MS/MS Spectra signals at m/z 628.07 and 715.17 in Figure 3 further reinforced the suggestion of a phytolaccinic acid aglycone due to its archetypal form. Previous reports have shown that monohexoses exhibit base peaks due to deprotonated molecular ions [M – H]¯ at approximately m/z 179. [33] Under high-pH conditions as applied in the isolation of the saponin fraction used in this study,[8] the monosaccharides may undergo retro-aldolization reactions, yielding stable α-dicarbonyl compounds. [37, 38, 39, 40] Thus, the peak signals in the low m/z range of Figure 3 are likely attributable to deprotonated by-products from monohexose.[41] Usually, the m/z 101.15 is a deprotonated radical typical of fructose with the 1, 3 linkage that may also appear at m/z 100. However, similar signals for glucose have been shown to overlap those observed for fructose with minor differences only in their relative intensities.[41, 42] The peak at m/z 157 has been previously reported for the glucuronyl unit with the loss of H_2O at the negative ion mode.[41, 43]

NMR Spectrometric Analysis

Evidence provided by the chemical shifts (Figures 4 and 5) and the configuration of the five anomeric protons (Table 2), as well as, prior literature data,[22, 23] provided the fundamental information for the proposed structure (Figure 6). The interpretation of the ^{1}H NMR spectra in Figure 4, supported by reported data in prior studies, showed that the saponin fraction is a bisdesmosidic compound that has two different saccharide linkages located at two terminals: disaccharide at C-3 indicated at a chemical shift of 3.86, and trisaccharide with no apparent proton signal at C-28. Also, indicated are five anomeric protons (Table 2) which resonated as doublets.[33, 44] These proton signals and configuration, as supported by their coupling distances, for the pyranose sugar residues are consistent with previous reports.[32] Proton peak resonates appearing as singlets were recorded at the chemical shifts of 5.6 and 9.5 ppm for the olefinic and aldehyde protons attached to the C-12 and C-23 of the aglycone, respectively. The substitution positions of the acyl groups were further elucidated with the three-bond heteronuclear connectivity observed as cross-peaks. Data indicated a carbonyl at 1.16 ppm in the HMBC spectrum corresponding to the proton peak at C-24 (Figure 5). Correlational observation of the ^{1}H-HMBC in a similar report confirmed the disaccharide *β*-D-Galp-(1→2)-*β*-D-GlcpA and trisaccharide *α*-L-Rhap-(1→2)-[*β*-D-Glcp- (1→3)]-*β*-D-Fucp residues are linked to C-3 and C-28, respectively. Comparing the chemical shifts and the pattern of the cross-peaks to previously reported quillaja saponins, data suggests the absence of the quillaic acid aglycone.[45, 46]

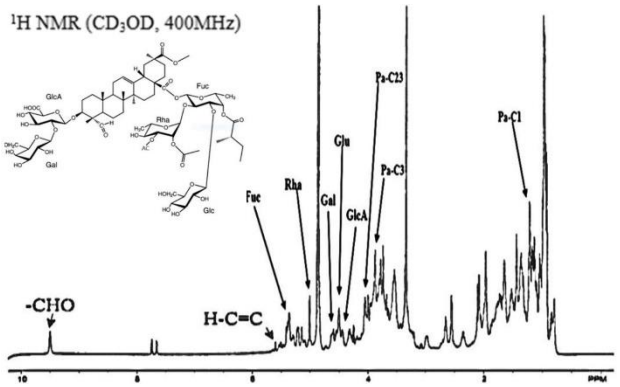

Figure 4: NMR spectra of the pure fraction from 1-10 ppm indicating the ^{1}H peaks due to the phytolaccinic acid (Pa), carbonyl, sugar anomeric and olefinic protons.

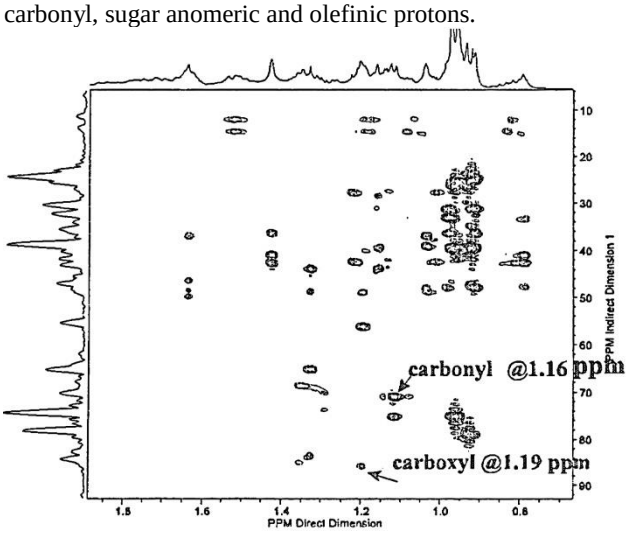

Figure 5: The Heteronuclear Multiple Bond Correlation (HMBC) spectra of the pure fraction. The peak at 1.16 ppm indicates correlational interaction of carbon 4 of the aglycone with the carbonyl (carbon at position 24) proton.

Table 1: The sugar residues of the pure fraction as identified by their permethylated alditol acetate derivatives and fragment $^m/_z$ peaks

S/N	Identified Monosaccharide	Permethylated alditol acetate derivatives (NIST)	Fragment ion peaks (m/z)
1	Fucose (Fucp)	1,5-di-*O*-acetyl-2,3,4-tri-*O* -methyl-fucitol	131.05
2	Rhamnose (Rhap)	1,5-di-*O*-acétyl-2,3,4-tri-*O* -méthyl-rhamnitol	175.12
3	Glucuronic acid (GlcAp)	1,5,6-tri-*O*-acetyl-2,3,4-tri-*O* -methyl-6-d 2-glucitol	192.01
4	Galactose (Galp)	1,5-di-*O*-acetyl-2,3,4-tri-*O*-methyl-galactitol	233.25
5	Glucose (Glcp)	1,5-di-*O*-acetyl-2,3,4-tri-*O*-methyl-glucitol	205.01

Table 2: Configuration and the coupling constants of the sugar anomeric protons at their assigned ^{1}H NMR chemical shift (*δ*)

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S/N	Residue	^{1}H NMR Chemical shift (δ) ppm	J_{H-H} Coupling Hz	Sugar Configuration
1.	GlcA-1	4.42	dJ = 6.24	β -Dp
2.	Gal-1	4.57	dJ = 6.10	β -Dp
3.	Fuc-1	5.34	dJ = 6.24	α –Lp
4.	Glu-1	4.50	dJ = 1.50	β -Dp
5.	Rha-1	4.99	dJ = 6.29	α –Lp

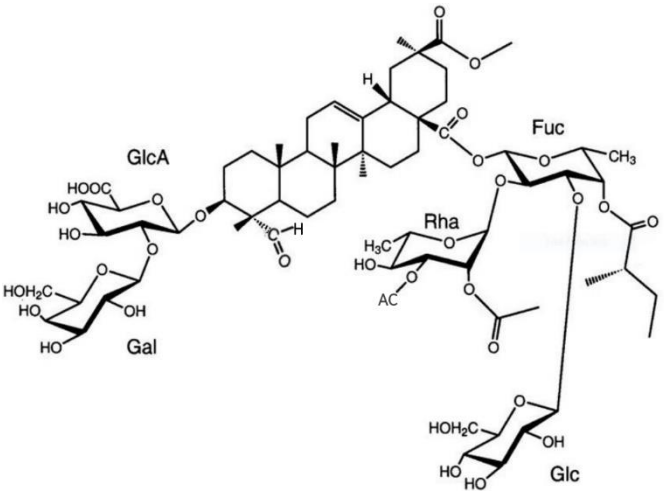

Figure 6: The proposed structure for the pure saponin fraction-3β, 23α-oxo-30-methoxy-30-oxoolean-12-en-28-oic acid

However, evidence shows consistency with a saponin which possesses a phytolaccinic acid aglycone as proposed by the structure in Figure 6.[31, 32, 47, 48, 49, 50] The obtained m/z spectra of the A-ions of the saponin fraction were consistent with ion fragments associated with 3β, 23α-Dihydroxy-30-methoxy-30-oxoolean-12-en-28-oic acid, also known as phytolaccinic or phytolaccagenic acid. The aglycone nucleus which is the basis for the proposed structure in this study has been reported for phytolaccagenin saponin in *Phytolacca americana*.[31, 51, 52, 53]

Prior investigation has associated the immunogenic and adjuvant activities of some quillaja saponins with the presence of the carbonyl unit (at C-23) that is linked to the C-4 as shown in this study.[54] This finding may explain the reported adjuvant activity of the saponin fraction in the earlier study.[16]

Conclusion

The structural complements of the ^{1}H NMR chemical shifts in this study support the concept that has been reported by previous studies for saponins. However, the presence of the carbonyl (aldehyde) proton peak at C-23 indicates a 23-oxo-phytolaccinic acid aglycone which is a deviation from the 23-*O*-acetylphytolaccinic acid aglycone as reported for such saponins containing non-quillaic acid aglycone. Previous reports on similar non-quillaic saponin isolated from *Q. saponoria* lack the carbonyl moiety as identified in this study. As such, provided evidence supports further studies to elaborate on the activity of the saponin fraction for academic benefit and also, the ultimate application of the herbal principle.

Conflict of Interest

The authors declare no conflict of interest.

Authors' Declaration

The authors hereby declare that the work presented in this article is original and any liability for claims relating to the content of this article will be borne by them.

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