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STUDY OF THE ANTI-PROLIFERATIVE ACTIVITY OF STEROIDAL SAPONIN, DIOSCIN ISOLATED FROM THE RHIZOME OF COSTUS SPECIOSUS (INSULIN PLANT)

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ABSTRACT
Costus speciosus (family: *Costaceae*) is an Indian medicinal plant used in Ayurved as folklore medicine to cure various kinds of diseases, including diabetes, cancer and kidney problems. Chemical profiling of its methanol extract revealed the presence of a steroidal saponin in the form of glycoside as major constituent in its rhizome. Structural determination of this steroidal saponinglycoside has completedby chemical, spectral, and spectrometric methods. Biological evaluation of this compound has been conducted on different human cancer cell linesby using SRB assayin comparison with paclitaxel as control. Bioassay result exhibited significant activity against different cancer cell lines such as urinary bladder (HT-1376), pancreatic (HPAC-1376), prostate (DU-145), colon (HT-29) and thyroid (MDA-T32) cancer cells with IC_{50} values 9.47±2.0, 8.32±1.3, 13.85±1.3, 6.10±0.6, 6.86±0.6µg/mL respectively.

KEYWORD: *Costus speciosus*; rhizome; isolation & characterization; steroidal saponin; anti-proliferative activity; SRB assay.

INTRODUCTION
C. speciosus is a well-known medicinal plant, widely distributed all over India. All parts of the plant are very much useful as remedial agent for various kind of ailments.[1] In Hindi, it is named as Keu kand and in English language, it is called as spiral ginger or wild ginger or crepe ginger.[2] In the forest areas of Central India, especially in Bastara region of Chhattisgarh State, people of Gauli and Dhangar Communities are using the rhizome of this medicinal plant as nephroprotective agent, to cure various type of kidney related problems such as urolithiasis, urethritis, urinal disorder, and as diuretic.[3]

Now a days, two major health issues in our society are diabetes and cancer. These are very severe life threatening diseases to human beings. Of course, in the modern pharmacy, synthetic drugs are being used to provide relief and control of them. These drugs are very much expensive, not affordable to common people, habit forming andpossesses a lot of side effect due to prolonged use.[3, 4] In this connection, we are looking for suitable botanical medicines from natural resources as the replacement of expensive and harmful synthetic drugs for therapeutic uses for treatment of these diseases. In order to control, prevent, and treat these diseases, many medicinal plants and herbs are used as folklore remedial agents, either in processed form or in crude extract.[5] The product obtained by processing of herbs and medicinal plants are well known as commercial herbal products available in the market in various trade names. Medicinal plant and herb extracts are now generally considered as effective and reliable medicines in modern pharmacy as remedies to cure many acute and chronic diseases due to its minimum side effects, long history of use, affordability, and easy availability. Majority of world population relies therapeutically on medicinal plants and herbs for their primary healthcare and health promoting purposes.[6] Although botanical medicines have been used as remedial agents to cure various ailments from antiquity, there is no concrete exploration of scientific information concerning plants, crude plant extracts and various secondary metabolites from plants as remedial agents and functionary of plants crude products in human physiology to cure disease still remains unclear. Large numbers of plants and herbs are claiming various medicinal uses in the form of crude products, enriched or processed products as curative agents against many acute and chronic diseases for which there may not be proper medicine so far in modern pharmacy.[7] Many phytochemical investigations are going on all over the world in various aspects in order to

develop drug based on the ancient concept available in Ayurved. In this regard, we have selected a plant named as *C.* speciosus belonging to *Costaceae* family for its various medicinal properties reported in literature and evidence-based knowledge of local tribal people in some part of India.[8] All parts of the plant are very useful as folklore botanical supplement to cure various kinds of diseases including diabetes, kidney related problems, its mal functions and cancer reported in Ayurved.[5, 9, 10] Detailed phytochemical investigation has been carried out to find out the bio-active ingredients present into it followed by their structural characterization and anti-proliferative activity against five different human cancer lines.

RESULTS AND DISCUSSIONS

Literature survey shows that dry powder of the rhizome of this plant is used as folklore botanical remedy directly or as a poly-herbal formulation to cure specially diabetes and kidney related problems. From previous experience of local people, it would be considered as a natural remedy for healthier, safer and multi-potent botanical medicine for replacement of harmful synthetic drugs prescribed to control diabetes. The dry powder of rhizome of *C. specious* possesses excellent performance against hyperglycaemic effect. It is also called as insulin plant or anti-diabetic plant due to its possession of α-amylase and α-glycosidase inhibitory activity. Many poly-herbal formulations containing different parts of this plant are also available commercially in market in various form under different trade names. The major therapeutic credentials of different parts of the plant *C. specious* are central nervous depressant activity,[11] anti-stress,[12-14] anti-anxiety,[12] anti-oxidant activity,[14-20] anti-inflammatory activity,[17, 21] anti-analgesic activity,[21] anti-pyretic activity,[21] anti-diabetic,[24-29] anti-bacterial,[17, 30] anti-fungal activity,[17, 31] anti-proliferative & anti-cancer activity,[20, 22, 33] insulin secretary activity,[23, 35] genotoxicity & cytotoxicity,[36] α-amylase & α-glycosidase inhibitory activity,[36, 37] hypoglycaemic activity,[35, 38] biochemical & haematological effect,[39] anti-viral activity,[40] reproductive & aphrodisiac activity,[41,42] diuretic activity,[43,44] anti-cholinesterase,[45] micro-propagation activity,[46] larvicidal activity,[47] anti-nociceptive efficacy,[48] anti-hyper-glycaemic,[17,38,50,51] and hypolipidemic effects,[17, 50, 51] anti-convulsing activity,[52] etc. It has been reported that the *Costus* plant species contains flavonoids,[53, 54] sterol glycoside,[55] steroids,[57] etc.

But detailed phytochemical investigation of methanol extract of the rhizome of this plant has revealed a steroidal saponin present in the form of glycoside. It was present as major chemical constituent in methanol extract, about 2% of its dry weight. In addition, other minor chemical constituents belonging to sesquiterpenoids named as costunolide, erimanthin, santamarine were also present as minor constituents along with trace amount triterpenoid and steroid identified as lupeol, β-sitosterol, respectively. These were isolated by column chromatograph over silica gel with gradient solvent elution followed by preparative thin layer chromatography (PTLC).

The structural determination of the major chemical constituent diosgenin glycoside, also known as dioscin has been done by spectrometric, and spectroscopic methods on comparison with published values of ^{1}H NMR and ^{13}C NMR, MS spectral data reported in literature.[17]

The chemical structure of the active major ingredient is depicted below in figure-1.

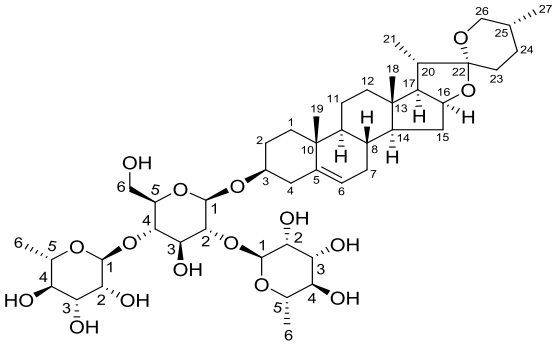


Figure-1: Chemical structure of dioscin isolated from dried rhizome of *C. speciosus.*

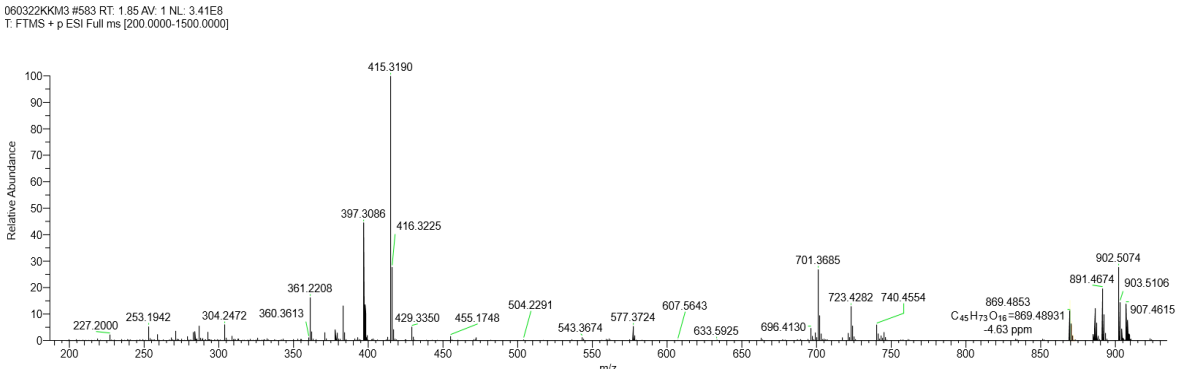


Figure 2: HRESI mass spectrum of dioscin (glycoside of diosgenin).

It is reported in literature that diosgenin is attached with a sugar moiety to form respective glycoside (dioscin). This sugar moiety in dioscin is a combined product of one glucose, attached to diosgenin (aglycone part) and two rhamnose moieties connected to glucose. These two rhamnose moieties are connected to the glucose moiety via1-2 linkage and 1-4 linkage. It has been confirmed by acid hydrolysis of glycoside (dioscin) isolated from methanol extract of *Costus speciosus* by us, the sugar moiety attached to diosgenin is a combined product of one glucose and two rhamnose moieties in ratio of 1: 2, evidenced by NMR and HRESI mass analysis. The interpretation of HRESI mass spectrum as depicted below in figure 3a and figure 3b.

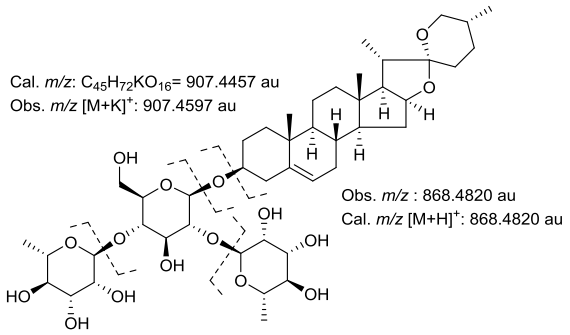


Figure 3a: Interpretation of HRESI mass spectrum of dioscin.

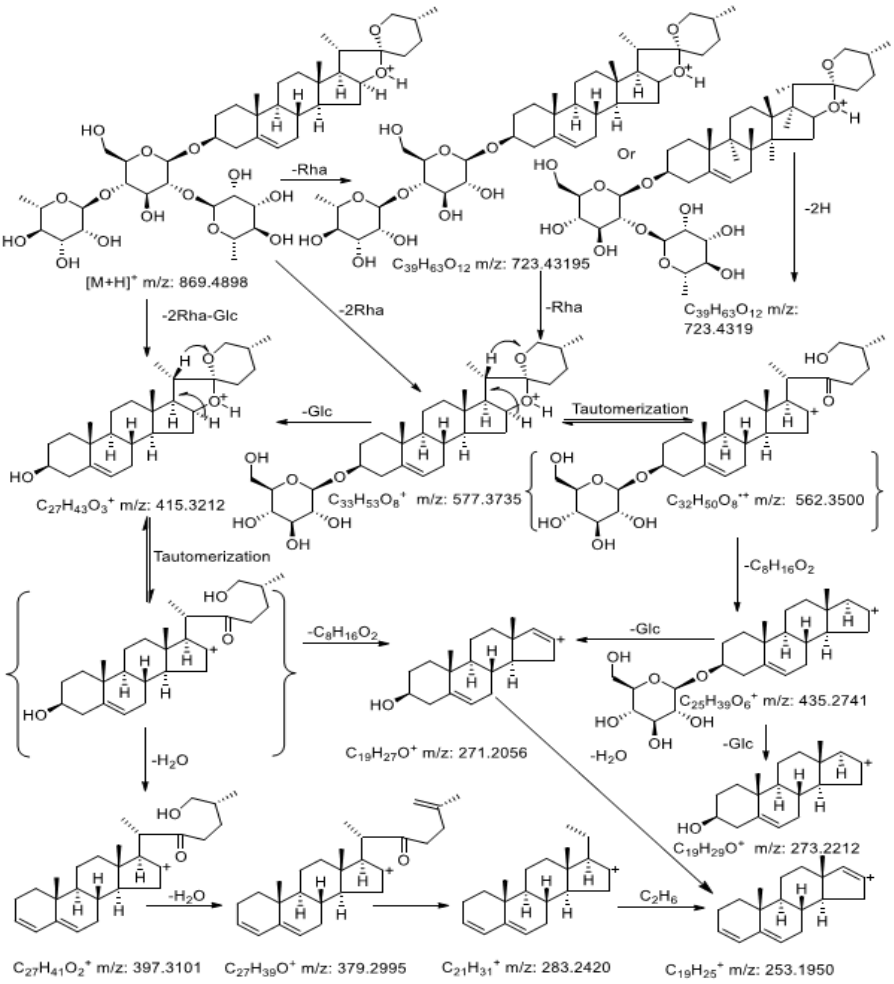


Figure 3b: Interpretation of HRESI mass spectrum of dioscin.

In HRESI mass spectrum, it demonstrated that the appearance of sharp peak at m/z 415.3190 au was responsible for formation of diosgenin ion as base peak with elimination of all sugar moieties from glycoside (dioscin). The appearance of the fragmentation peak at m/z 723.4319 au was occurred due to elimination of one rhamnose moiety from dioscin. The peak cited at m/z 869.4853 au was due to the formation of molecular ion peak, $[M+H]^+$ of glycoside whereas the peak at m/z 907.3615 au was involved for the formation of adduct of molecular ion with a potassium atom.

SUMMARY OF THE WORK

C. speciosus belonging to *Costaceae* family for its various medicinal properties reported in literature and evidence-based knowledge of local tribal people in some part of India. All parts of the plant are very useful as folklore botanical supplement or poly herbal preparation to cure various kinds of diseases including diabetes, kidney related problems, its mal-functions and cancer

reported in Ayurved. The present work describes the isolation of the major constituent along with very trace amount of lupeol and β-sitosterol from its methanol extract, structure elucidation of major constituent has been conducted by spectroscopic, spectroscopic methods, and evaluation of its anti-proliferative activity on five different human cancer cell lines by using sulfo-rhodamine B assay (SRB) assay in comparison with paclitaxel as control.

The study of mass spectrum of the dioscin (glycoside) revealed that peak appeared at m/z 869.4898 au was $[M+H]^+$. The peaks appeared at m/z 723.4319, 577.3735, 562.3500 415.3213 au by eliminating one rhamnose, two rhamnose or all of sugar moieties from glycoside sequentially or simultaneously. The intense peak cited in mass spectrum at m/z 415.3190 was due to formation of diosgenin ion on elimination of all sugar moieties giving rise to base peak.

MATERIALS AND METHODS

General experimental procedures

Melting points were determined using a Buchi melting point apparatus (Model number M560). Specific rotations were obtained using a JASCO DIP 1000 digital polarimeter. UV spectra were measured on Shimadzu UV-2100 UV-Vis spectrophotometer. NMR spectra were recorded in DMSO-d6 (manufactured by Euro-Isotope, UK; CAS number 2206-27-1) on a Bruker Avance 200 and a Varian 500 AR spectrometer using residual HDO as an internal standard. Chemical shifts are given in ppm (δ_H & δ_C), relative to residual HDO/partially deuterated methylated in DMSO-d6 at 2.49/3.30 & 29.00 ppm. UHPLC/MS analysis of the samples was performed using a Vanquish UHPLC system coupled to a Q-Exactive quadrupole orbitrap mass spectrometer (Thermo Fisher Scientific, USA) at the Ohio State University, College of Pharmacy, Instrumentation Facility. Silica gel 60 (230-400 mesh, Merck, Germany) was used for analytical TLC. Silica gel 60 (70-230 mesh, Merck, Germany) was used for column chromatography. All compounds were visualized by TLC using 10% aqueous H_2SO_4, vanillin-perchloric acid-EtOH followed by heating at 110° C for 5 min, DNP in EtOH and neutral $FeCl_3$ in MeOH.

Plant material

Rhizome of *Costus speciosus* (in local dialect Keu kand) was collected from Bejapur forest located in Bastar region of Chhattisgarh, India in March, 2019 with the help of local eminent naturopath Mr. Vijay Bharat and his associates. The material was authenticated by Dr. Hussain Barbhuiya, Landscape and Cosmetic Maintenance Section, A & SED Division, Bhabha Atomic Research Centre, Trombay, Mumbai. A voucher specimen was deposited in the Herbarium of the Landscape & Cosmetic Maintenance Division, BARC, Mumbai-400085, India.

Extraction and Isolation

Fresh quality rhizome (100 g; weight of fresh tendered material) of *Costus speciosus* were dried on shade at room temperature and ground to a powder (10.0 g; weight of shade dried material, 10% of fresh tendered weight) which was extracted with diethyl ether followed by methanol (50 mL) three times at room temperature. Removal of solvents afforded a brown viscous residue about 134 mg from ether and approximately 1.0 g sticky substancefrom methanol. This extract obtained from methanol was fractionated over silica gel (50 gm, 230-400 mesh, Aldrich, USA) and eluted with a step gradient of chloroform followed by a binary mixture of methanol in chloroform to furnish seven fractions, volume of each aliquot 50-100 mL. In some cases, the volume of aliquot collected was more than 100 mL. Fractions were monitored by TLC to examine the chemical composition. The fractions having similar chemical profiles were combined, and in some cases, further purified by using repetitive column chromatograph on open column chromatography over silica gel, gel permeation chromatography (GPC) with solvent gradual elution followed by preparative thin layer chromatography (PTLC).

Fractions 1 and 2 eluted with chloroform and a mixture of chloroform-methanol (0-5% and 5-10%, respectively) consisted mainly of trace amount of lipids.

Fractions 3 eluted with chloroform-methanol (10-15%) consisted mainly of trace amount of lipids along with a colourless solid substance as major constituent was obtained on removal of solvents. The major constituent was subjected to thin-layer chromatography (TLC) analysis. 10% aqueous H_2SO_4 solution was used as the spray reagent, and the TLC plate was subsequently heated at 120 ºC for five minutes. The analysis revealed the development of a pink color, suggesting the presence of a steroidal compound.

Fractions 4 eluted with methanol 20% in chloroform consisted mainly colourless solid substance as major constituent was obtained on removal of solvents designated as A. This was further chromatographed sequentially over silica gel and then over SephadexLH-20 column eluted with 10-15% methanol in chloroform to yield sub-fractions A_1-A_{10}. Sub-fractions A_5-A_7 were monitored by TLC and performed by using different spray reagents on micro TLC plate and visualization the colour changes on heating at 120 ºC for span of 5 min. It was observed that sub-fractions A_5-A_7 exhibited similar chemical profiles. They were combined (~750 mg) and separated on a silica gel open column to afford compound **1** (~700 mg) designated as dioscin. Preliminary investigation reveal that this compound is a steroidal saponin glycoside by performing Lieberman Bur-chard test (LB Test: trace amount sample dissolved in acetic anhydride in test tube and a drop of H_2SO_4 was added into it on side wall of the test tube; a pink colour was developed, and shake with water generated huge

lather indicated it was steroidal saponin), spraying with 10% aqueous H_2SO_4 followed by heating at 120 ºC for 5 min developed a pink colour and executing positive Molisch's test for glycoside.

Fraction 5 eluted with chloroform-methanol 25% contained fatty acids along with a phenolic compound. On evaporation, it yielded a trace residue B (~15.0 mg). The analysis of this compound was not conducted in the lieu of non-availability of enough amount of sample.

Fraction 6 eluted by a mixture of chloroform-methanol (30-35%) yielded sub-fractions that were combined and evaporated to furnish trace residue C (~5.0 mg). The analysis of it was not proceeded for insufficient quantity. Fraction 7 was also eluted by a mixture of chloroform-methanol (40-50%) yielding fractions that were combined and evaporated to furnish trace residue D (~5.0 mg). The analysis of it was not carried out due to inadequate of sample amount.

Fraction 8 was the result of column washing by using a mixture of chloroform-methanol 60-100%) to yield sub-fractions that were combined and evaporated to furnish trace residue E (~5.0 mg). The analysis of it was not done due to insufficient quantity.

Analysis of major chemical constituent isolated from methanol extract of *C. speciosus*

Compound dioscin: colourless solid crystalline substance; mp ~200-202 ºC. It showed molecular formula $C_{45}H_{72}O_{16}$ and mass 869.4853 au; HRESI mass $[M+K]^+$ (observed mass) 907.45972 au; calculated mass $[M+K]^+$ = 907.4457 au; UV (DMSO), λ_{max} (log ε): 265 nm; IR (neat), ν_{max} (cm^{-1}): 3354, 2931, 2114, 1644, 1452, 1374, 1129, 1036, 906, 812 and 613.

^{1}H NMR Data (500 MHz, DMSO-d6): δ_H 5.30 (brs, 1H, -OH), 5.06 (t, 1H, *J* = 2.5 Hz), 5.00 (s, 1H), 4.92 (d, 1H, anomeric proton, *J* = 5.0 Hz), 4.80 (brs, 1H, 1x-OH), 4.70 (brs, 2H, 2x-OH), 4.66 (3H, 1x CH_2-OH & 1x-OH), 4.49 (brs, 1H , 1x-OH), 4.40-4.32 (m, 1H, >CH-OH), 4.33 (d, 1H, >CH-OH, *J* = 5.0 Hz), 4.27 (d, 1H, anomeric H, *J* = 5.0 Hz), 4.26-4.23 (dd, 1H, >CH-OH, *J* = 5 Hz each), 4.06 (d, 1H, *J* = 10 Hz, >CH-OH), 3.99-3.3.95 (m, 1H), 3.85-3.80 (m, 1H), 3.72 (brs, 2H, 2x-OH), 3.50-3.47 (m, 2H), 3.27-3.20 (m, 2H), 3.18-3.13 (m, 6H, 2x-CH_2-OH), 3.12-3.06 (m, 1H, >CH-OH), 3.04-3.01 (t, 2H, -CH_2-OH), 2.93-2.90 (t, 1H, *J* = 5.0 Hz, >CH-OH), 2.39-2.37 (m, 1H), 2.16-2.11 (m, 1H), 1.93-1.86 (m, 2H -CH_2-), 1.81-1.76 (-CH_2-), (1.68-1.63 (m, 1H, -CH_2-), 1.62-1.58 (m, 1H, -CH_2-), 1.56-1.50 (m, 3H, -CH_2-), 1.47-1.41 (m, 3H, -CH_2-), 1.38-1.27 (m, 4H, -CH_2-), 1.21 (brs, 1H, 1x-OH), 1.16-1.11 (m, 2H, -CH_2-), 1.08 (s, 3H. -CH_3), 1.07 (s, 3H, -CH_3), 1.06 (s, 3H, -CH_3), 0.93 (s, 3H, -CH_3), 0.88 (s, 3H, -CH_3), 0.83 (d, 3H, -CH_3), 0.82 (d, 3H, -CH_3), 0.71 (d, 3H, -CH_3).

^{13}C NMR data (125 MHz, DMSO-d6): δ_C 140.32, 120.91, 109.62, 108.45, 102.93, 100.55, 98.17, 80.22, 79.77, 76.98, 76.84, 76.81, 76.65,76.15, 75.27, 73.88, 73.49, 72.57, 71.94, 70.75, 70.62, 70.46, 70.10, 68.67, 67.98, 65.93, 63.35, 63.09, 62.46, 61.80, 61.09, 60.02, 55.77, 49.60, 41.12, 40.10, 39.94, 39.81, 39.78, 39.60 (merged with DMSO), 39.44, 37.60, 36.84, 36.42, 33.10, 31.54, 31.50, 31.00, 30.99, 30.95, 29.83, 29.05, 28.50, 27.34, 20.40, 18.99, 17.84, 17.79, 17.11, 17.10, 16.11, 16.06, 15.80, 14.70.

DEPT NMR data at 135° (125 MHz, DMSO-d6) referring positive signals for CH and CH_3: δ_C 73.62, 65.68, 63.09, 60.84, 38.80, 37.35, 31.28, 28.79, 27.09, 20.15.

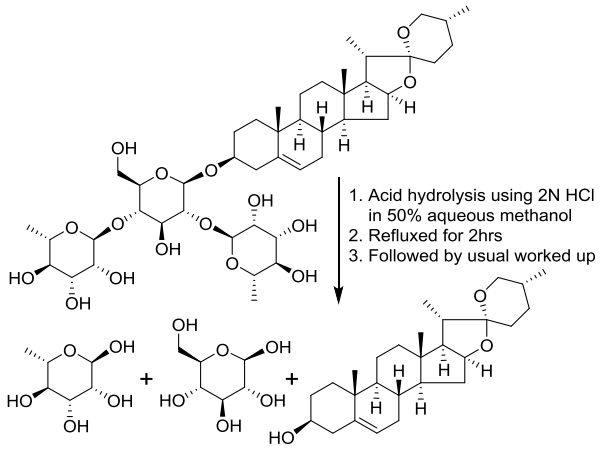
DEPT NMR data at 135° (125 MHz, DMSO-d6) referring negative signals for CH_2: δ_C 109.62, 108.45, 102.93, 100.55, 98.17, 18.75, 17.59, 17.54, 16.86, 16.84, 15.80, 14.44, 40.88, 38.84, 32.85, 30.73, 29.56, 62.23, 55.50, 49.36, 49.32, 79.98, 79.50, 76,74, 76.70, 76.61, 76.54, 76.04, 75.96, 75.91, 75.87, 75, 01, 73.23, 72.31, 71.66, 70.49, 70.38, 70.36, 70.21, 69.84, 68.40, 67.71.

HRESI mass analysis of compound dioscin

The mass of the compound has been determined as 869.4853 au $[M]^+$ (obs. mass) by HRESI mass spectrometry; Calculated mass $[M+K]^+$ 907.4457 au. The molecular formula of dioscin has been established as $C_{45}H_{72}O_{16}$.[58]

Acid hydrolysis of dioscin (1) / glycoside

A mixture of 11.0 mg of glycoside, 3.0 ml 5% aqueous HCl and 3.0 ml methanol was put in a 10 ml round bottom flask fitted with a small condenser. This mixture was refluxed for 2 hrs and cooled it to room temperature. On removal of solvents, a stick mass was obtained. About 5 ml of water and 5 ml ether was added into it to segregate aglycone and sugar by solvent partition. It was worked up by usual method. The aglycone part was extracted in ether and sugar was present in aqueous part. Solvents were removed to yield sugar and aglycone. Aglycone has been identified as diosgenin and sugar was identified as a mixture of glucose and rhamnose in ratio 1: 2 by ^{1}H NMR, HPLC and GC analysis with respect to authentic glucose and rhamnose by converting them to their respective acetate derivatives and circular paper chromatography with by using BAW (Butanol: Acetic acid: Water) as solvent system.



Scheme 1: Acid hydrolysis of dioscin, isolated from methanol extract *C. speciosus.*

Spectral analysis of respective aglycone upon acid hydrolysis of dioscin

Diosgenin (aglycone) was obtained upon the acid hydrolysis of dioscin (**1**, glycoside) followed by usual worked up of the reaction products. Analysis of its spectral data was mentioned below.

^{1}H NMR Data (500 MHz, DMSO-d6) of diosgenin (**2**): δ_H 5.34 (d, 1H, *J* = 5.0 Hz, -olefinic-H), 4.43-4.39 (m, 1H, >CH-OH), 3.55-3.46 (m, 2H, >CH-OH), 3.40-3.35 (dd/t, 1H), 2.29-2.23 (m, 3H, -CH_2-), 2.01-1.97 (m, 3H, -CH_2-),1.87-1.82 (m, 4H -CH_2-), 1.79-1.72 (m, 3H, -CH_2-), 1.65-1.46 (complex multiplet, 18H, -CH_2-), 1.31-1.27 (m, 2H, -CH_2-), 1.25 (s, 3H, 1x-CH_3), 1.19-1.17 (m, 3H, -CH_2-), 1.11-1.07 (m, 3H, -CH_2-), 1.02 (s, 3H, -CH_3), 0.79 (s, 3H, -CH_3), 0.79 (s, 3H, -CH_3), 0.78 (d, 3H, -CH_3).

^{13}C NMR data ($CDCl_3$, 125 MHz) of diosgenin (**2**): δ_C 134.46 (C-5), 122.53 (C-6), 109.42 (C-22), 80.99 (C-26), 71.92 (C-3), 68.63 (C-16), 67.02 (C-13), 65.53 (C-26), 63.00 (C-17), 62.30 (C-9), 41.79 (C-4), 40.44 (C-10), 37.38 (C-1), 36.81 (C-10), 32.22 (C-14), 32.01 (C-7),31.57 (C-23), 30.47 (C-15), 29.86 (C-25), 28.98 (C-24), 21.05 (C-11), 19.57 (C-19), 17.27 (C-27), 14.66 (C-21 & C-18).

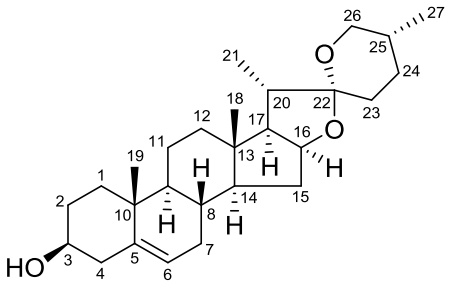


Figure 4: Structure and numbering of diosgenin.

Spectral analysis of respective sugars

Two type of sugars namely D-glucose and rhamnose was obtained upon acid hydrolysis of dioscin followed by usual worked up the reaction products. Analysis of their spectral data was given below.

^{1}H NMR Data (500 MHz, D_2O) of glucose (for β-rotamer): δ_H 4.62 (d, 1H, H-1), 3.88 (d, 1H, H-6a), 3.70 (d, 1H, H-6b), 3.48 (d, 1H, H-3), 3.46 (d, 1H, H-5), 3.39 (d, 1H, H-4, *J* = 5.0 Hz), 3.22 (d, 1H, H-2, *J* = 5.0 Hz).

^{1}H NMR Data (500 MHz, D_2O) of L-rhamnose: δ_H 5.10 (d, 1H, anomeric proton, H-1, *J* = 5.0 Hz), 4.85 (d, 1H, H-2, >CH-OH, *J* = 5.0 Hz), 3.92-3.91 (dd, 1H, H-3, >CH-OH), 3.88-3.85 (dd, 1H, H-4, >CH-OH, *J* = 5.0 Hz), 3.80-3.77 (dd, 1H, H-5, *J* = 5.0 Hz, >CH-OH), 3.60-3.57 (dd, 1H, >CH-OH, *J* = 5.0 Hz), 3.44-3.40 (dd, 1H, >CH-OH, *J* = 5.0 Hz), 3.39-3.33 (q, 1H, H-5, >CH-OH), 1.27 (d, 3H, *J* = 5.0 Hz, H-6, -CH_3).

ANTI-PROLIFERATIVE BIOASSAY

Anti-proliferative activity of dioscin against different cancer cell lines has been evaluated by using sulfo-rhodamine B (SRB) assay and was performed at The College of Pharmacy, The Ohio State University, Ohio, Columbus, USA.[59, 60]

Sulfo-rhodamine B (SRB) cell cytotoxicity assay is one of the most widely used method to detect cell viability. This assay is independent of cell metabolic activity. The incorporated dye released from stained cells after washing is directly proportional to the cell biomass and can be measured at 515 nm. Five different types of cancer cell lines such as human urinary bladder cancer cell line (HT-1376), pancreatic cell line (HPAC), prostate cancer cell line (DU-145), human colon cell line (HT-29) and human thyroid cell line (MDA-T32) were used to carry out bioassay for cell cytotoxicity study (Table-1).

Sample preparation

Test samples and controls (paclitaxel) were dissolved in 100% DMSO to prepare stock solutions of 10 mg/ml. Dilutions were first prepared using 10% DMSO in water and then 100% water.

Cell culture

Cancer cells [urinary bladder (HT-1376), pancreatic (HPAC), prostate (DU-145), colon (HT-29) and thyroid (MDA-T32)] were obtained from American Type Culture Collection, Manassas, VA, USA. Monolayer cells were cultured using T75 tissue culture flasks in Roswell Park Memorial Institute medium (RPMI) or Dulbecco's Modified Eagle Medium (DMEM), containing 10% fetal bovine serum and 1% antibiotic-anti-mycotic from Gibco. Also, HPAC used DMEM: F12 with insulin, transferrin, hydrocortisone, epidermal growth factor and MDAT-32 used RPMI-1640 with 1% NEAA (Non-essential amino acid). Cells were kept at 37 °C and in an atmosphere with 5% of CO_2.

Anti-proliferative assays

Bioassays were performed in triplicate in two independent experiments.

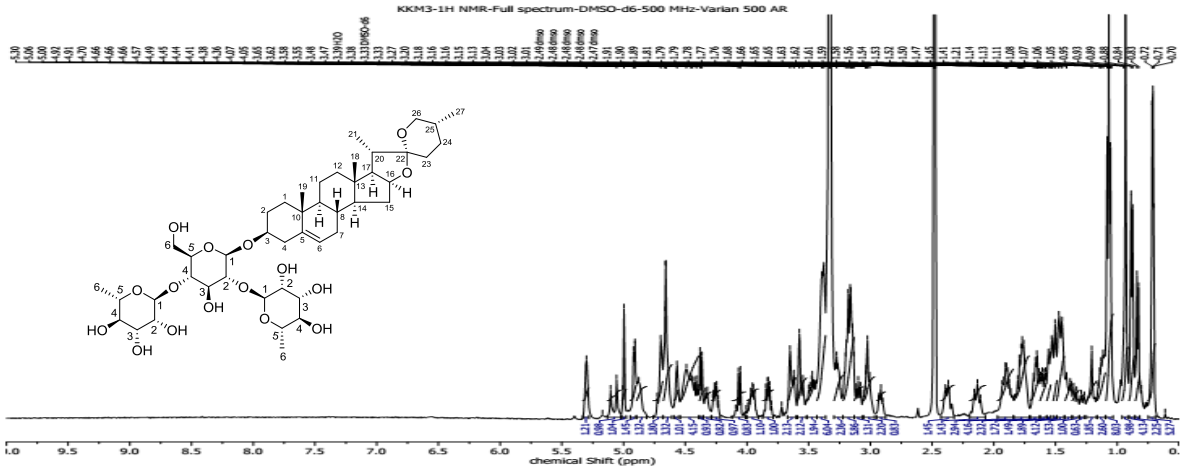


Figure S1: ^{1}H NMR spectrum (DMSO-d_6, 500 MHz,) of dioscin (1).

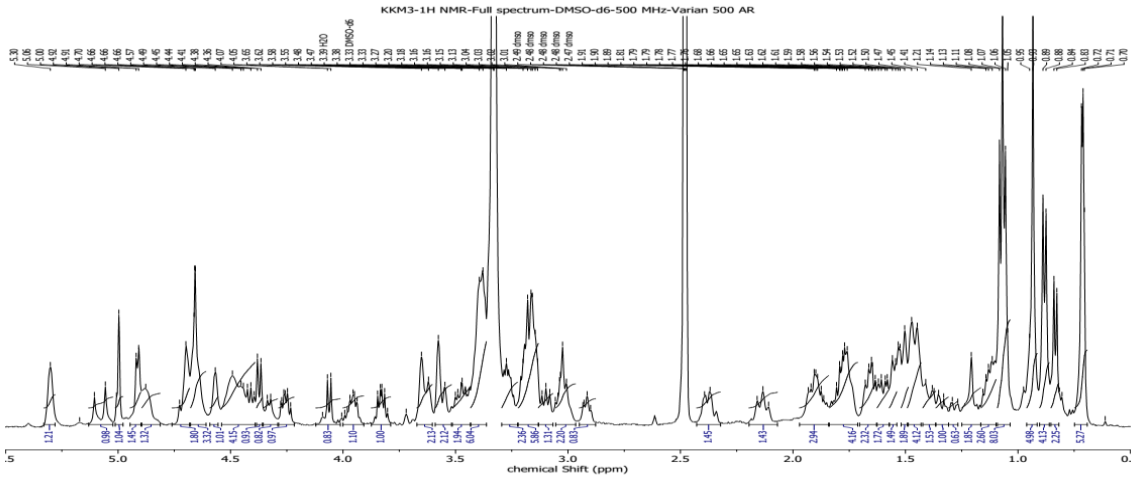


Figure S2: Expansion of ^{1}H NMR spectrum (DMSO-d_6, 500 MHz) of dioscin (1)

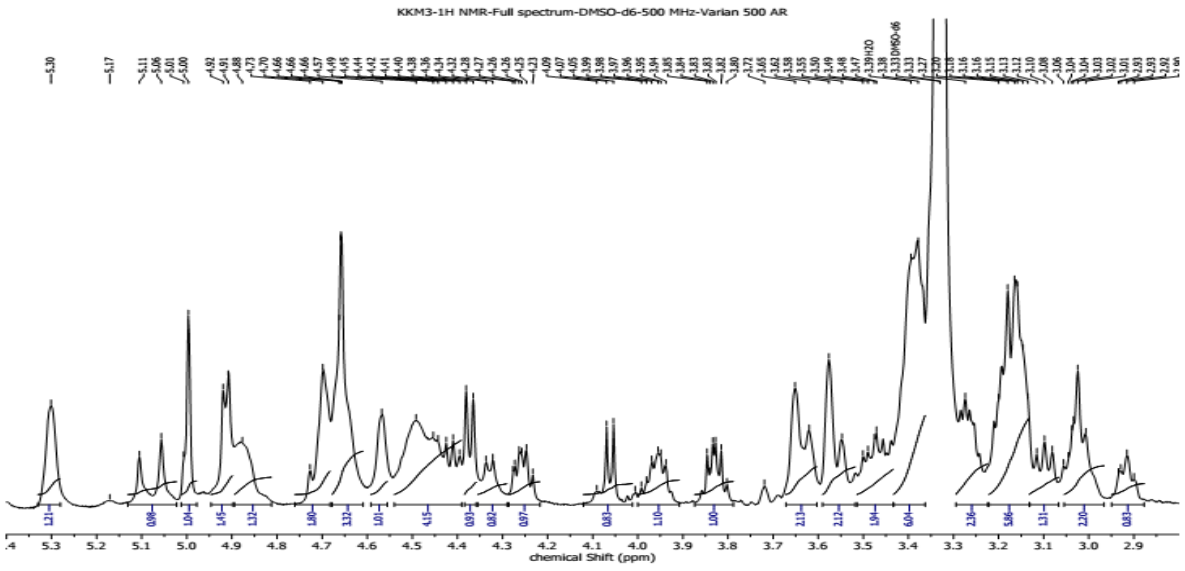


Figure S3: Expansion of ^{1}H NMR spectrum (DMSO-d_6, 500 MHz) of dioscin (1)

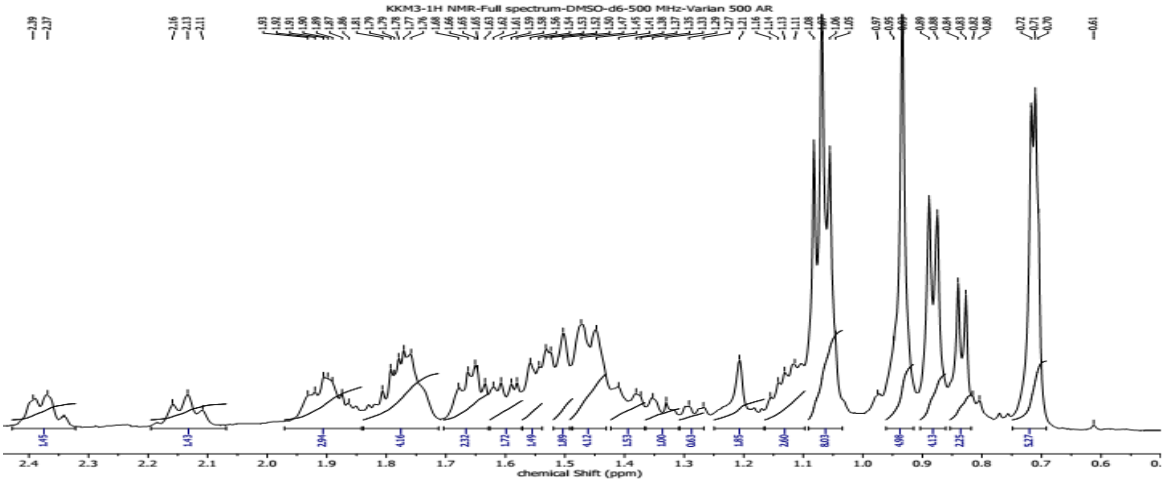


Figure S4: Expansion of ^{1}H NMR spectrum (DMSO-d_6, 500 MHz) of dioscin (1)

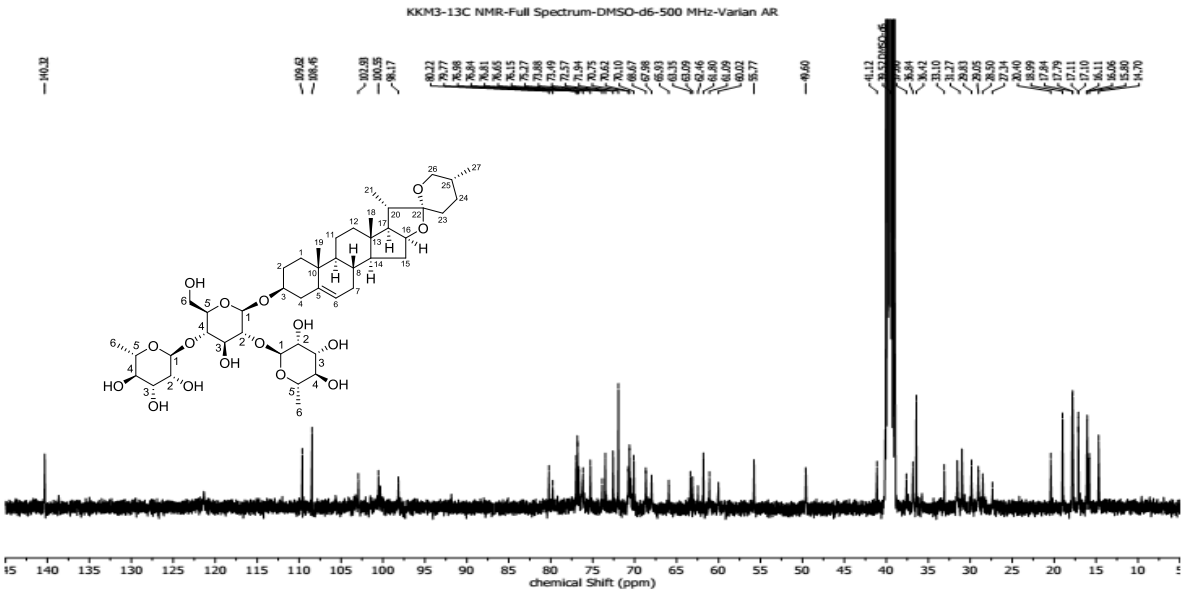


Figure S5: ^{13}C NMR spectrum (DMSO-d_6, 125 MHz) of dioscin (1)

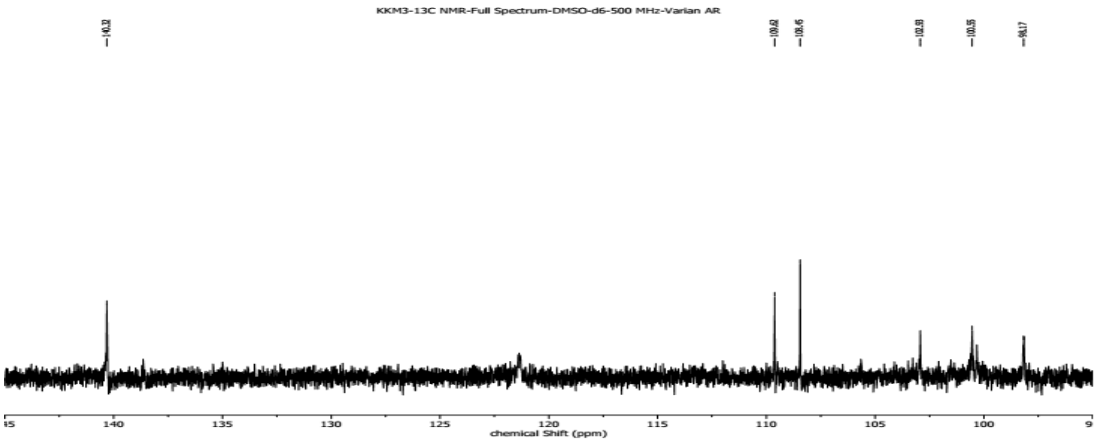


Figure S6: Expansion of ^{13}C NMR spectrum (DMSO-d_6, 125 MHz) of dioscin (1)

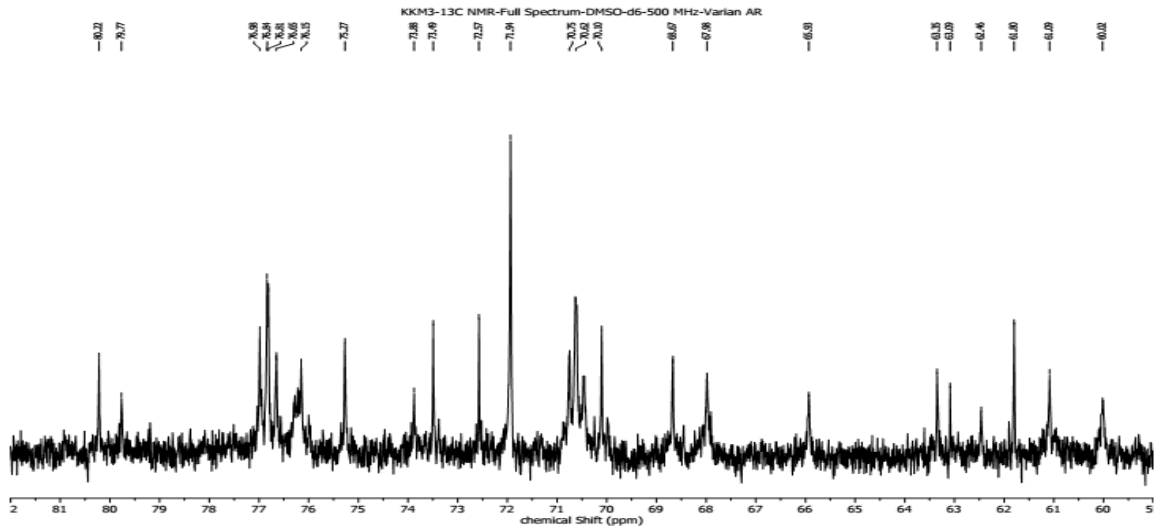


Figure S7: Expansion of ^{13}C NMR spectrum (DMSO-d_6, 125 MHz) of dioscin (1)

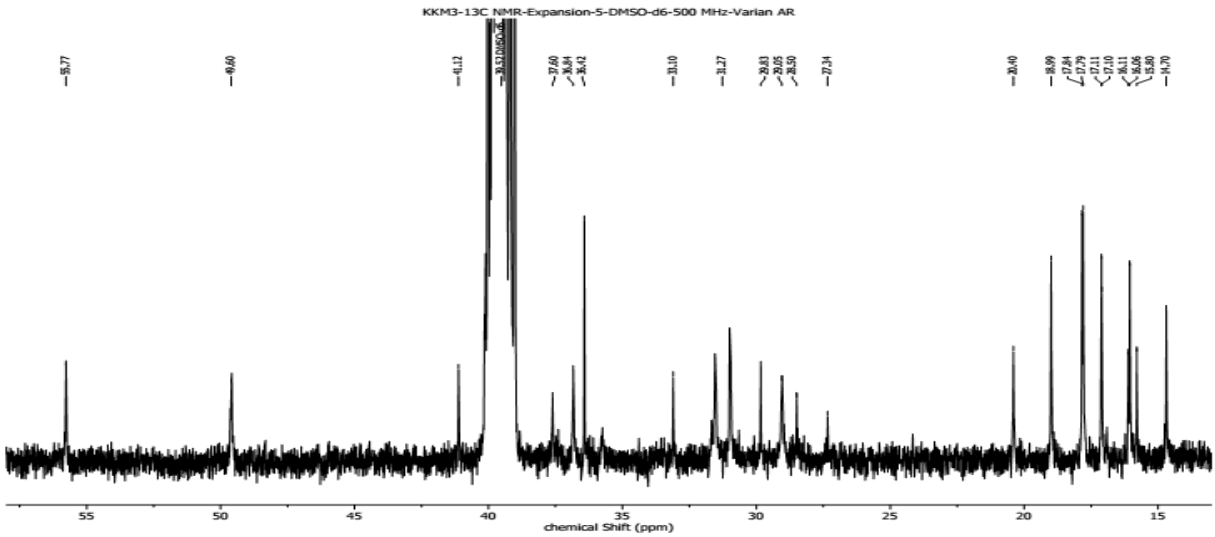


Figure S8: Expansion of ^{13}C NMR spectrum (DMSO-d_6, 125 MHz) of dioscin (1)

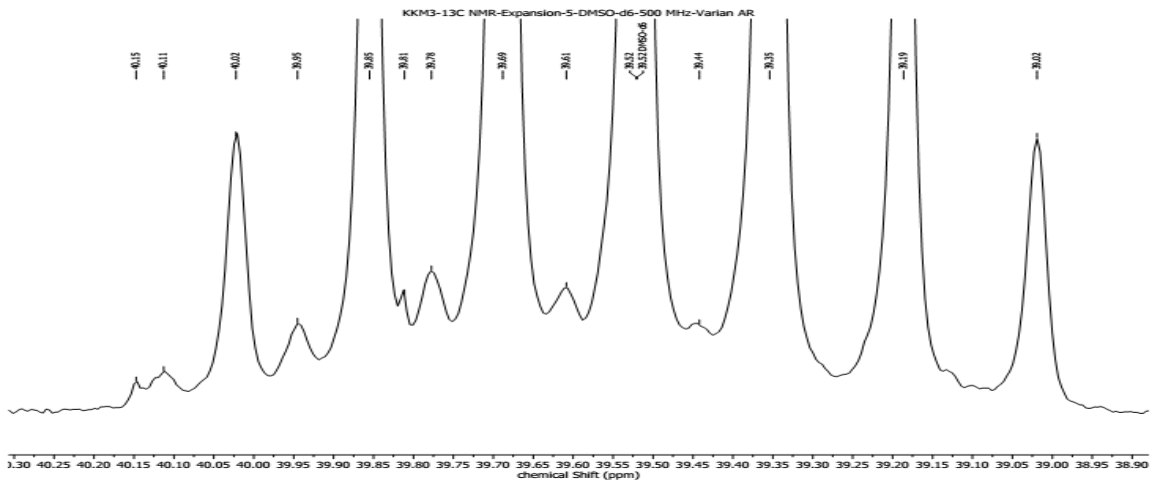


Figure S9: Expansion of ^{13}C NMR spectrum (DMSO-d_6, 125 MHz) of dioscin (1).

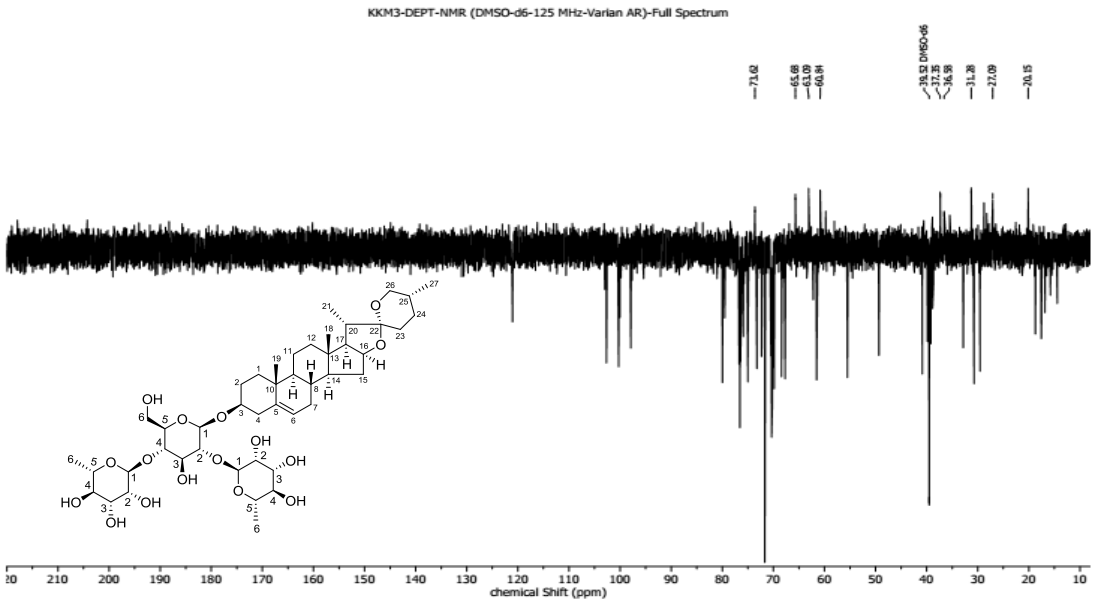


Figure S10: DEPT-135^{13}C NMR spectrum (DMSO-d_6, 125 MHz) of dioscin (1).

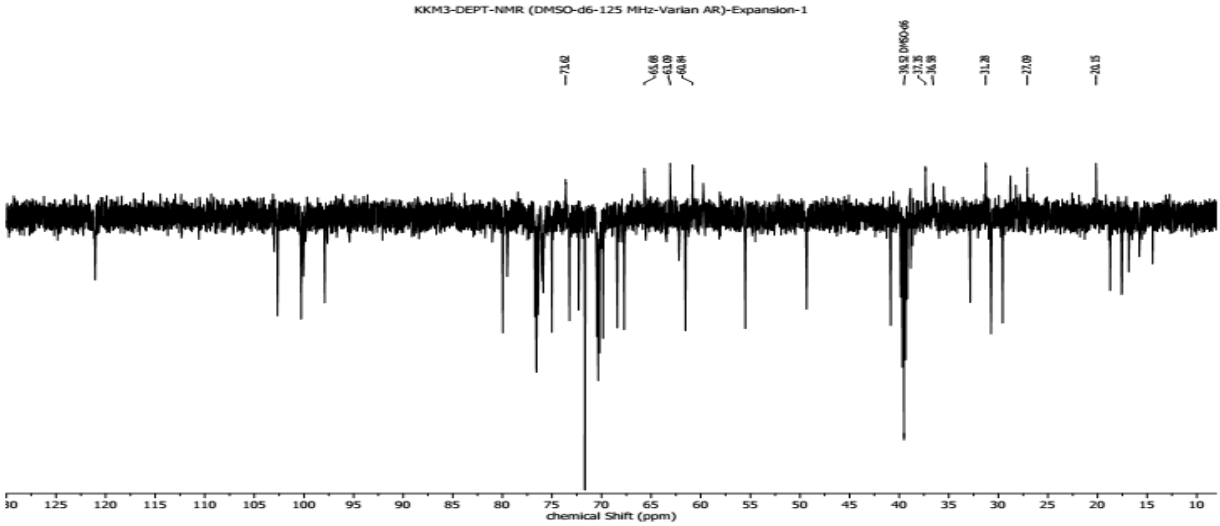


Figure S11: Expansion of DEPT-135°spectrum (DMSO-d_6, 125 MHz) of dioscin (1)

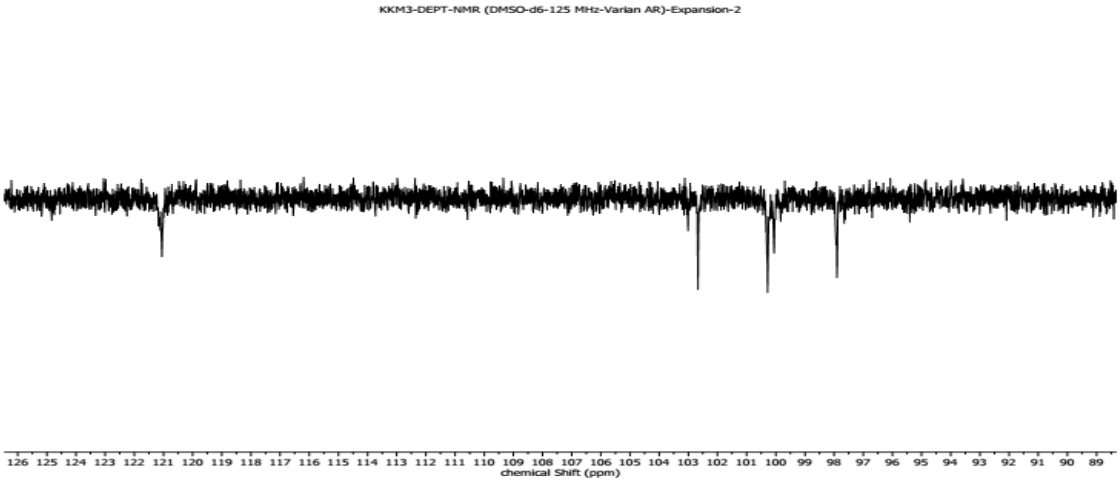


Figure S12: Expansion of DEPT-135° spectrum (DMSO-d_6, 125 MHz) of dioscin (1)

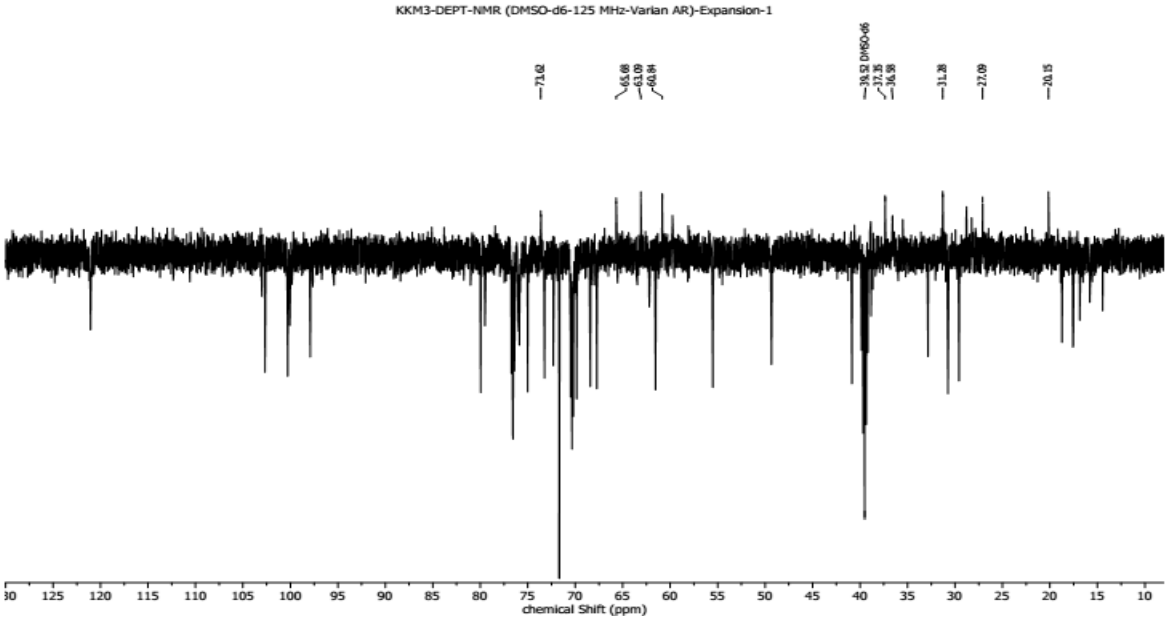


Figure S13: Expansion of DEPT-135° spectrum (DMSO-d_6, 125 MHz) of dioscin (1)

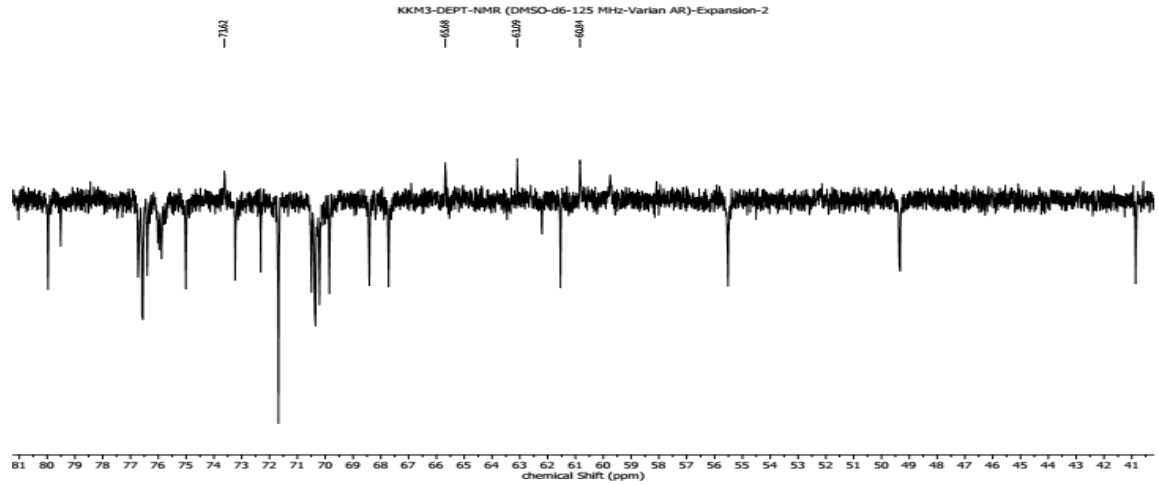


Figure S14: Expansion of DEPT-135^{13}C NMR spectrum (DMSO-d_6, 125 MHz) of dioscin 1)

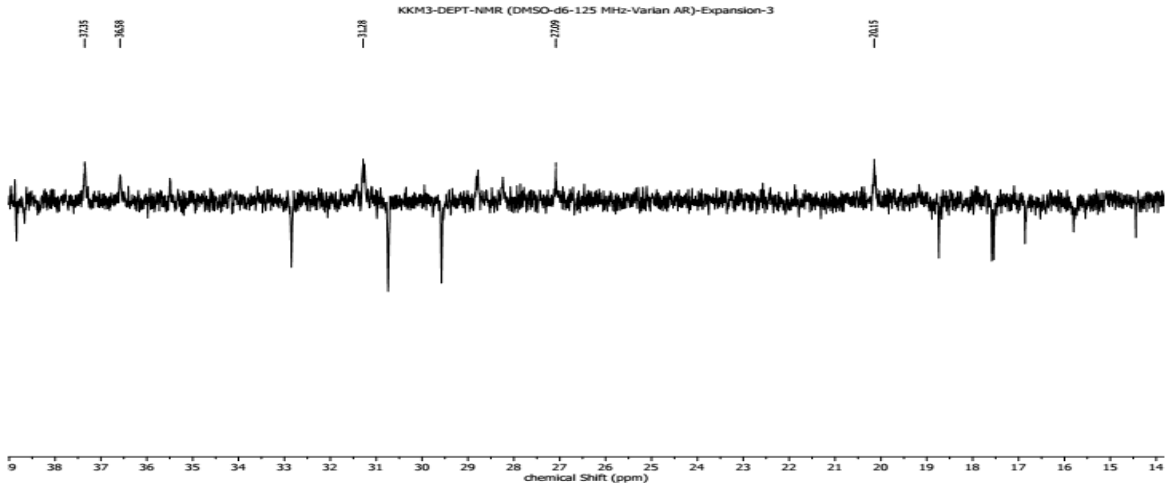


Figure S15: Expansion of DEPT-135°spectrum (DMSO-d_6, 125 MHz) of dioscin (1)

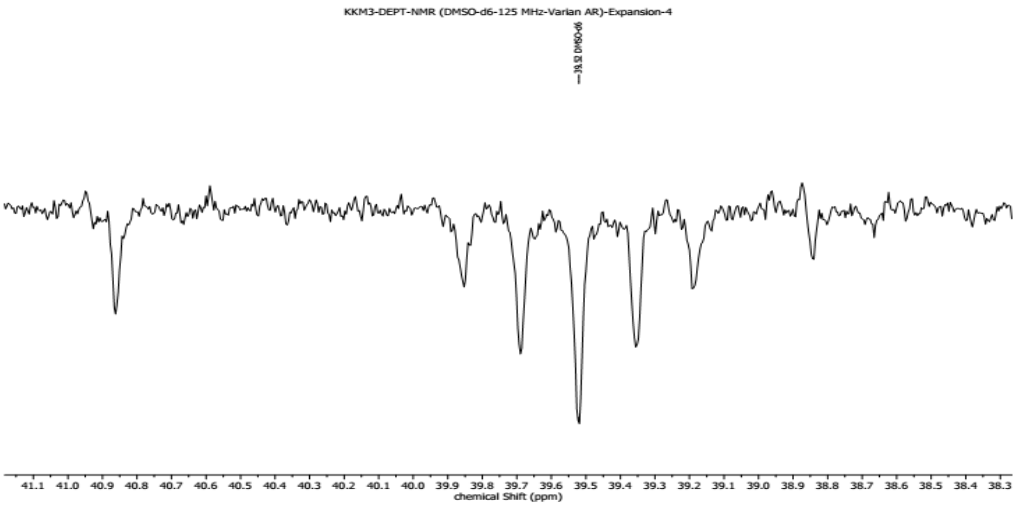


Figure S16: Expansion of DEPT-135°spectrum (DMSO-d_6, 125 MHz) of dioscin (1)

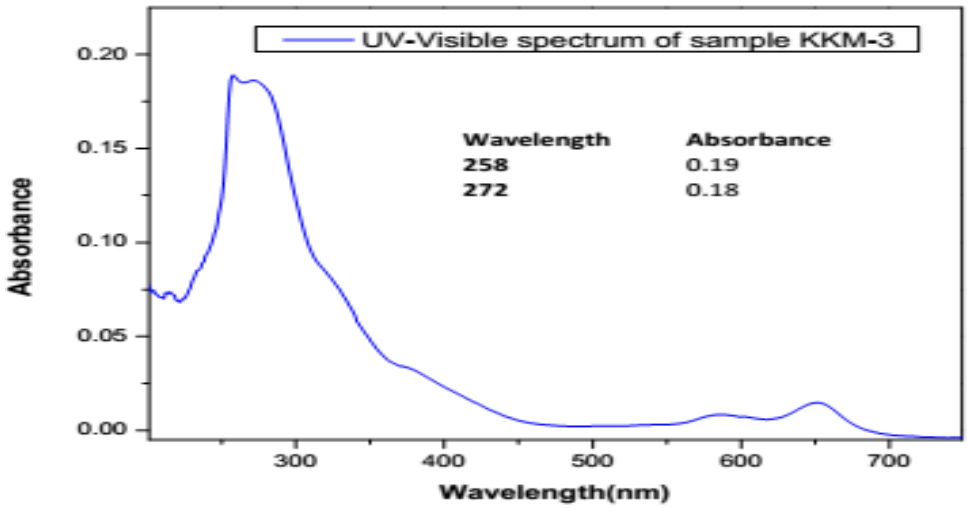


Figure S17: UVspectrum (DMSO)ofdioscin(1).

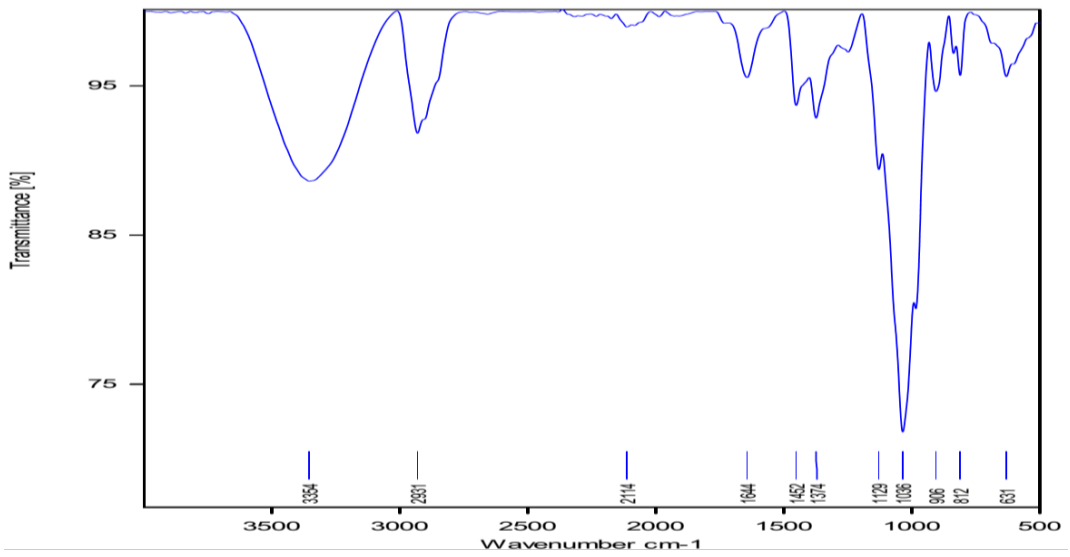


Figure S18: IR absorption spectrum of dioscin (1)

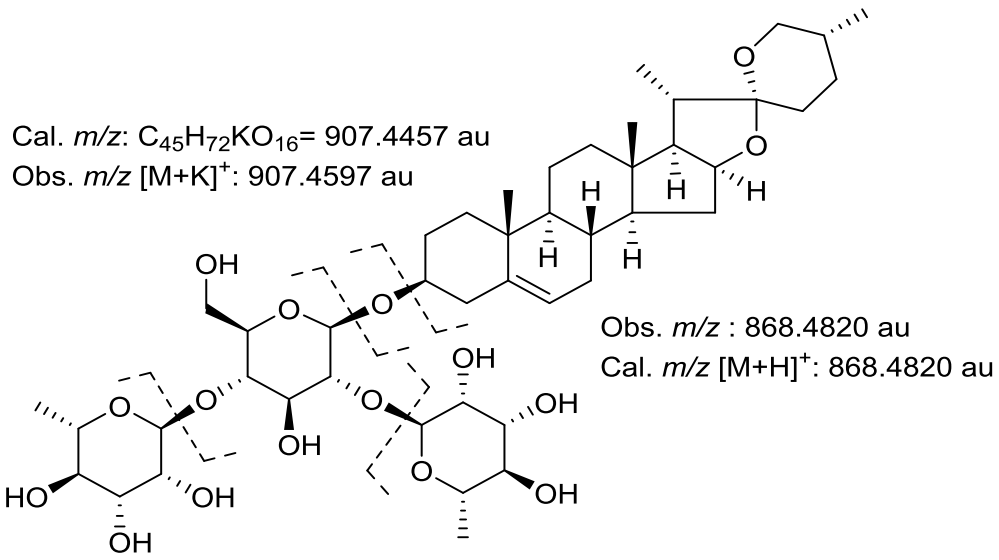


Figure S19: Interpretation of HRESI-MS of dioscin (1).

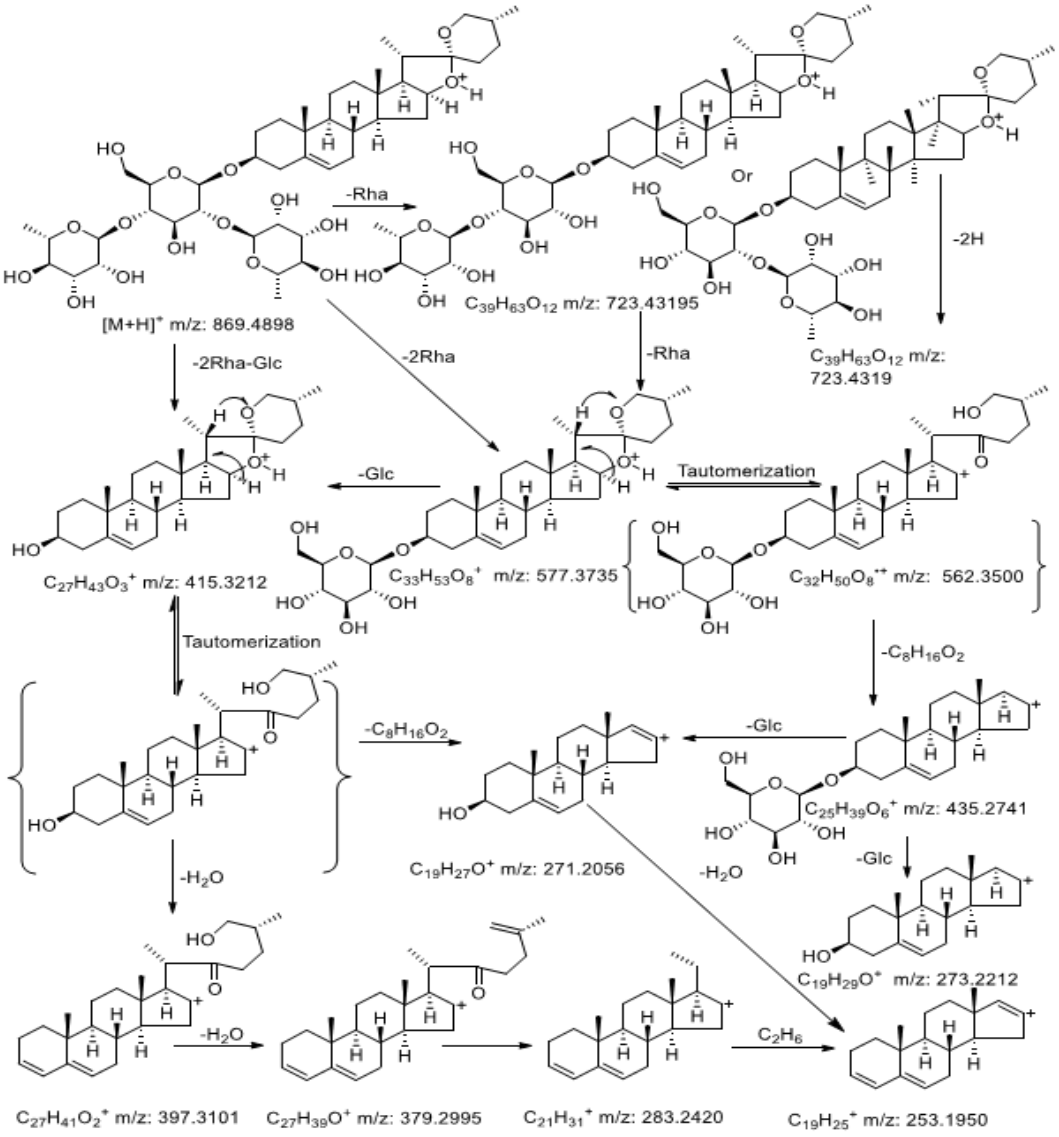


Figure S20: Interpretation of HRESI-MS of dioscin (1).

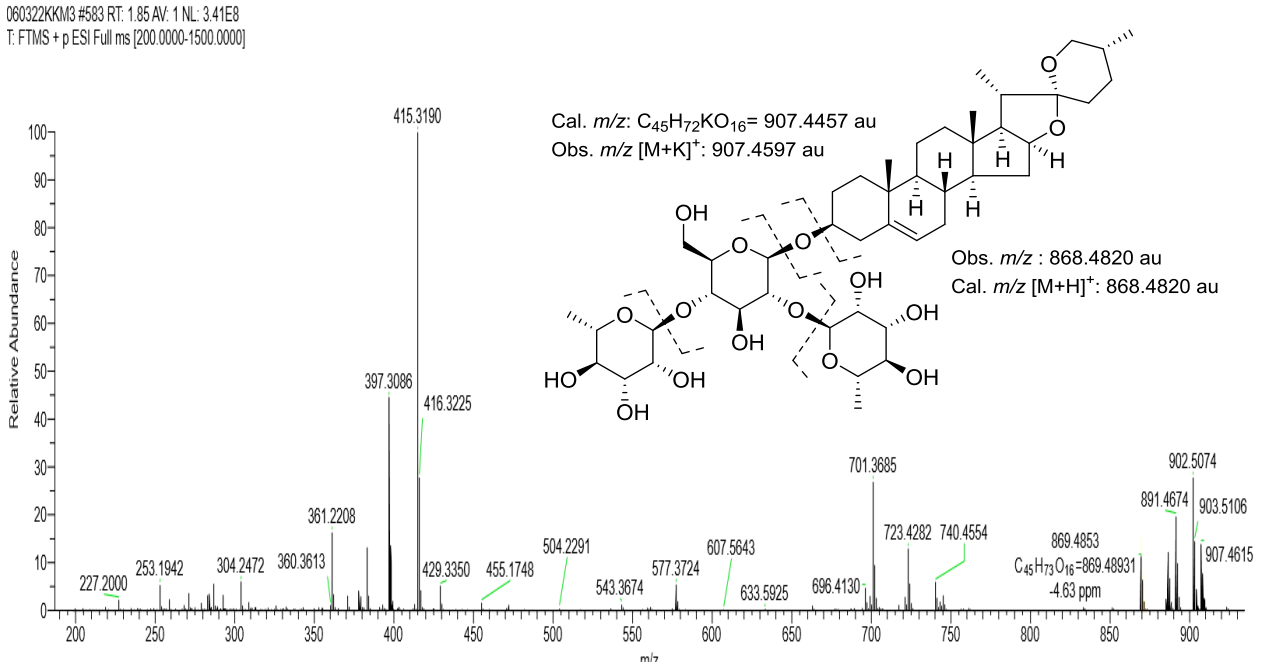


Figure S21: HRESI mass spectrum of dioscin (1).

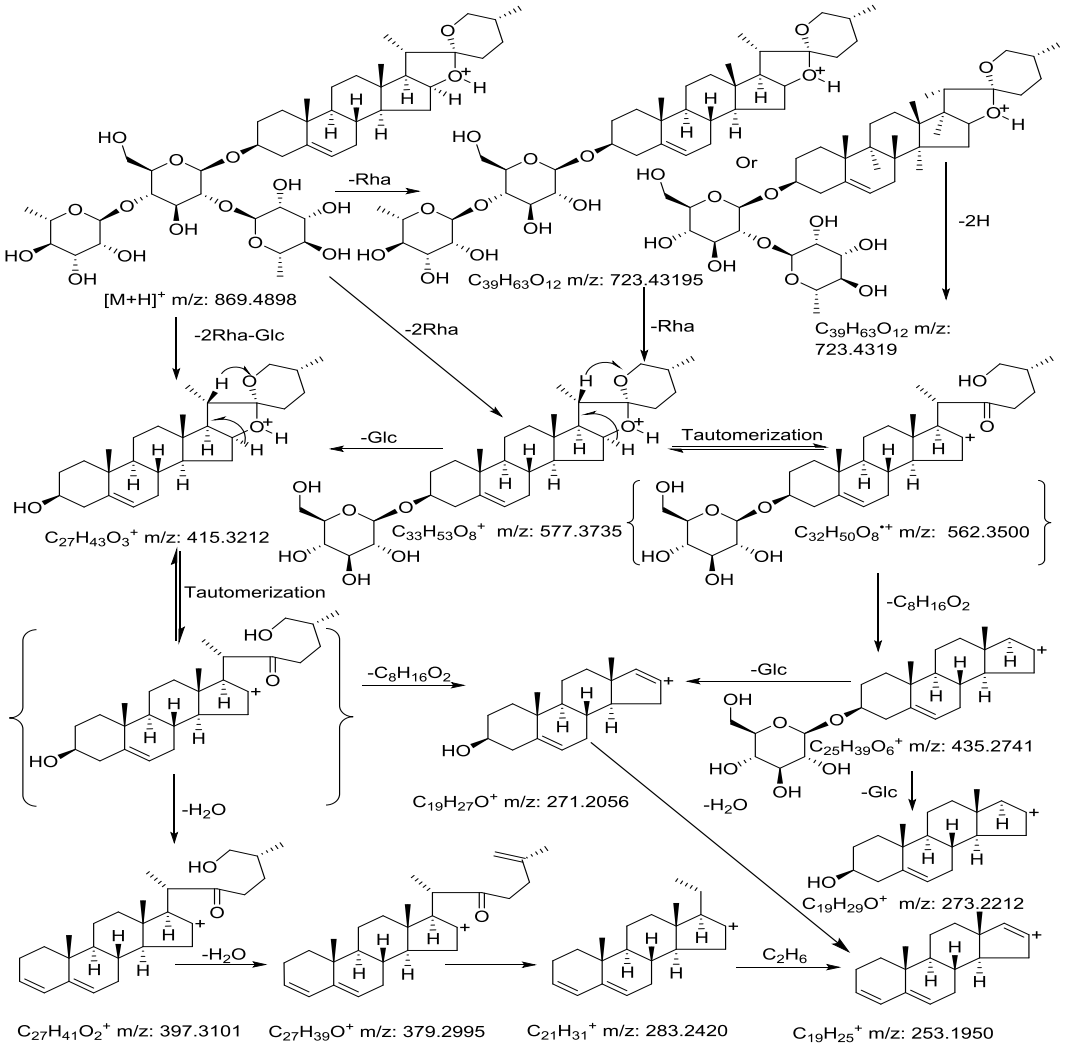


Figure S22: HRESI mass interpretation of dioscin.

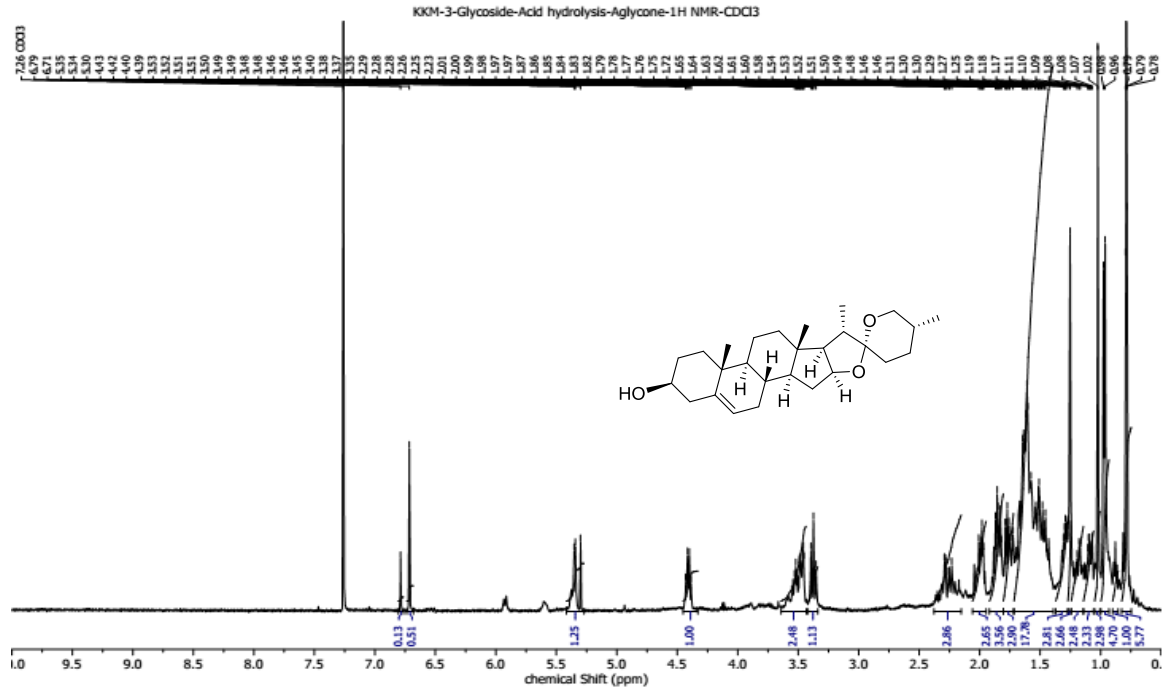


Figure S23: ^{1}HNMR spectrum ($CDCl_3$, 500 MHz) ofdiosgenin(2), aglyconeobtained from acid hydrolysis of glycoside, dioscin (1).

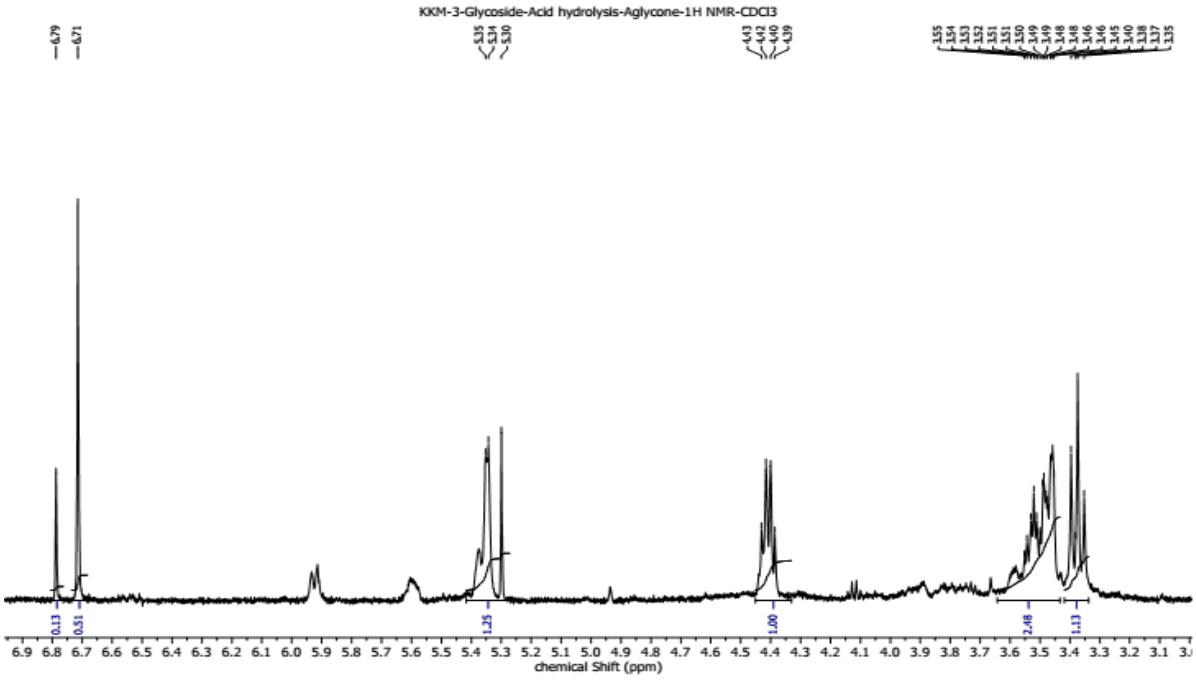


Figure S24: ^{1}HNMRspectrum ($CDCl_3$, 500 MHz) ofdiosgenin (2), aglyconeobtained from acid hydrolysis of glycoside, dioscin (1)

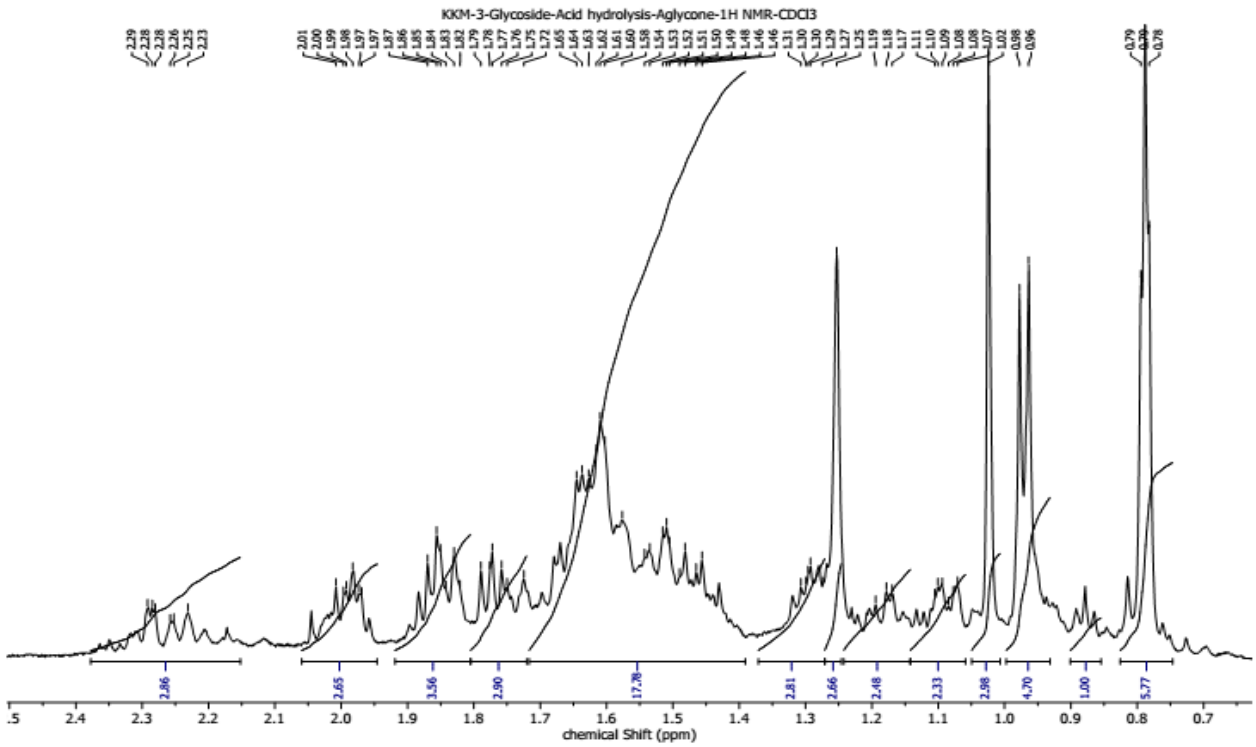


Figure S25: Expansion of ^{1}HNMRspectrum ($CDCl_3$, 500 MHz) of diosgenin (2), aglycone obtained from acid hydrolysis of glycoside, dioscin (1)

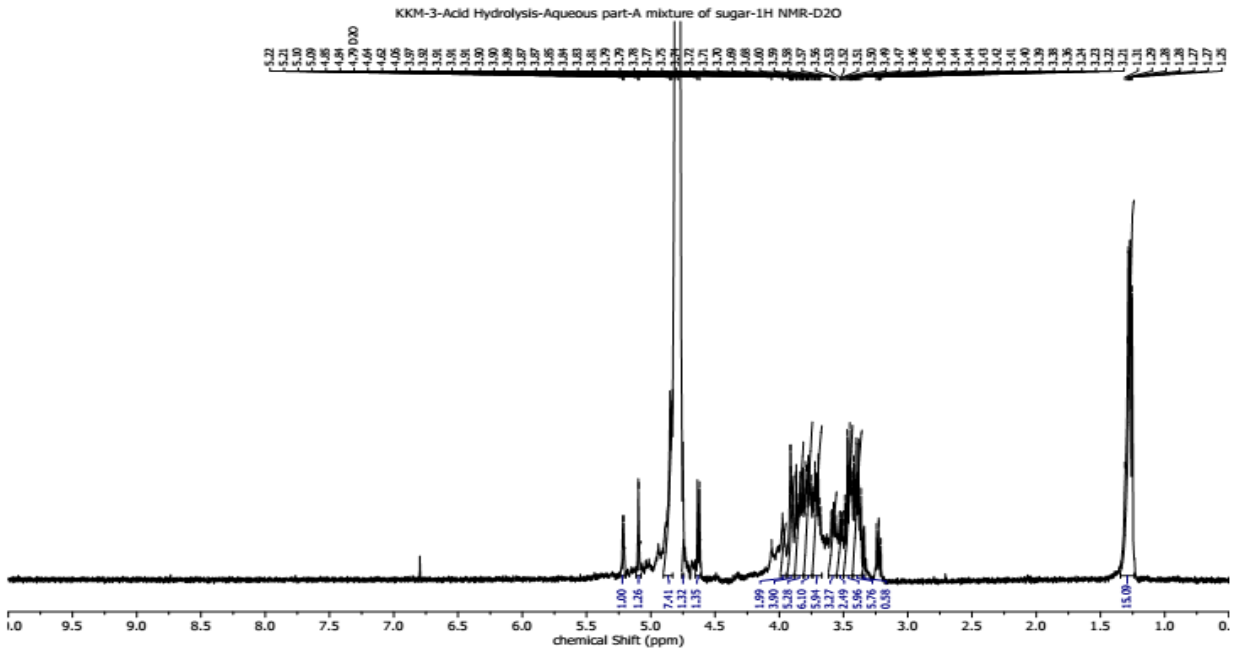


Figure S26: ^{1}HNMRspectrum (D_2O, 500 MHz) ofa mixture of sugars(3) obtained from acid hydrolysis of glycoside, dioscin (1)

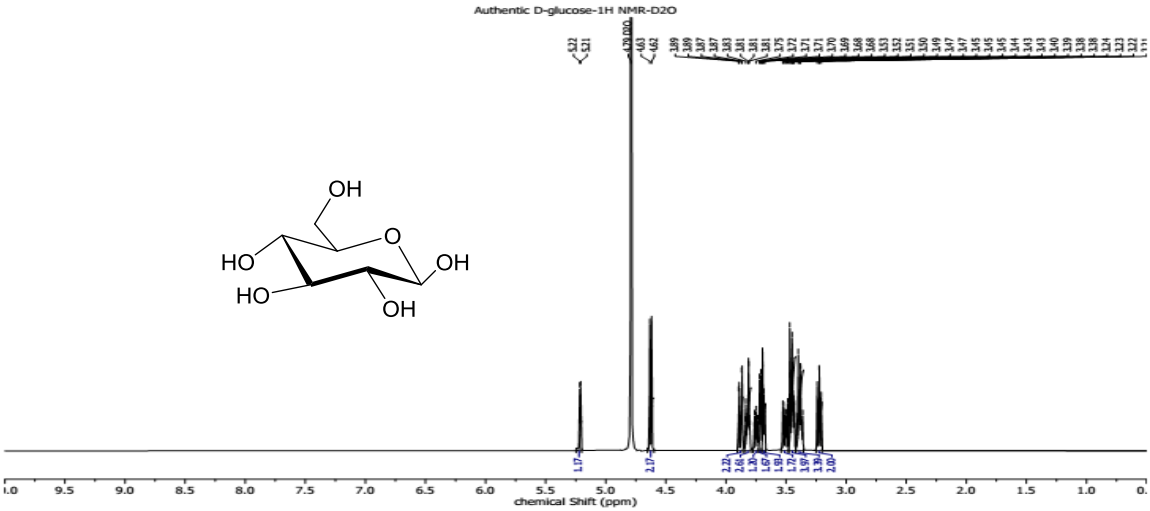


Figure S27: ^{1}HNMRspectrum (D_2O, 500 MHz) ofauthentic D-glucose (4).

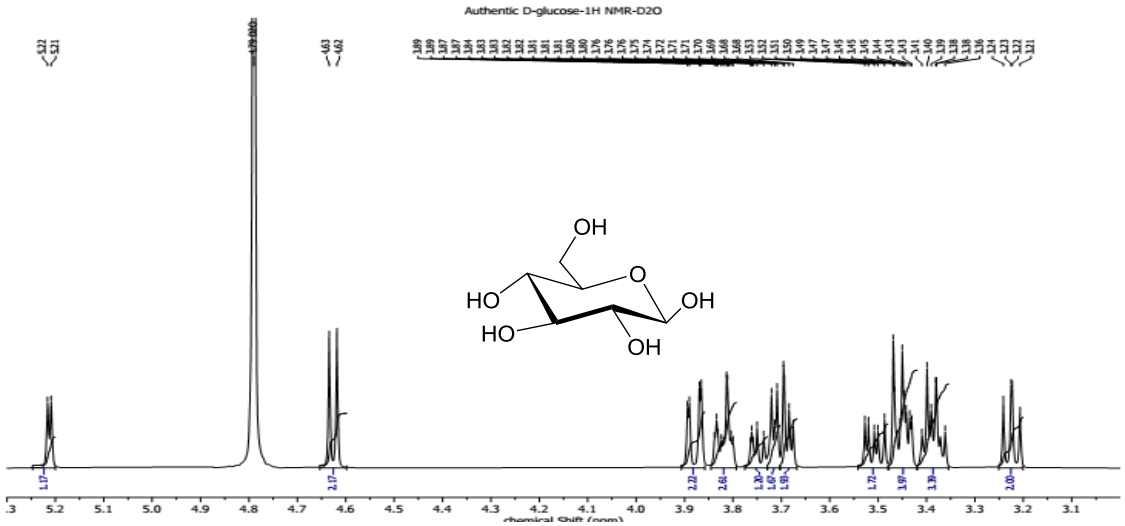


Figure S28: Expansion of ^{1}HNMRspectrum (D_2O, 500 MHz) ofauthentic D-glucose (4).

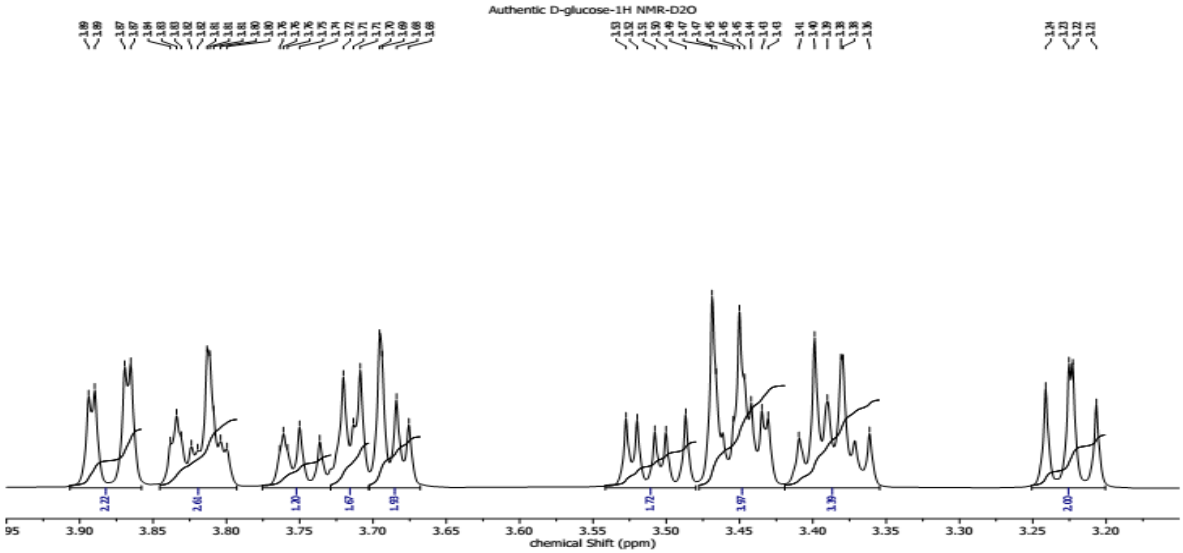


Figure S29: Expansion of ^{1}HNMRspectrum (D_2O, 500 MHz) ofauthentic D-glucose (4)

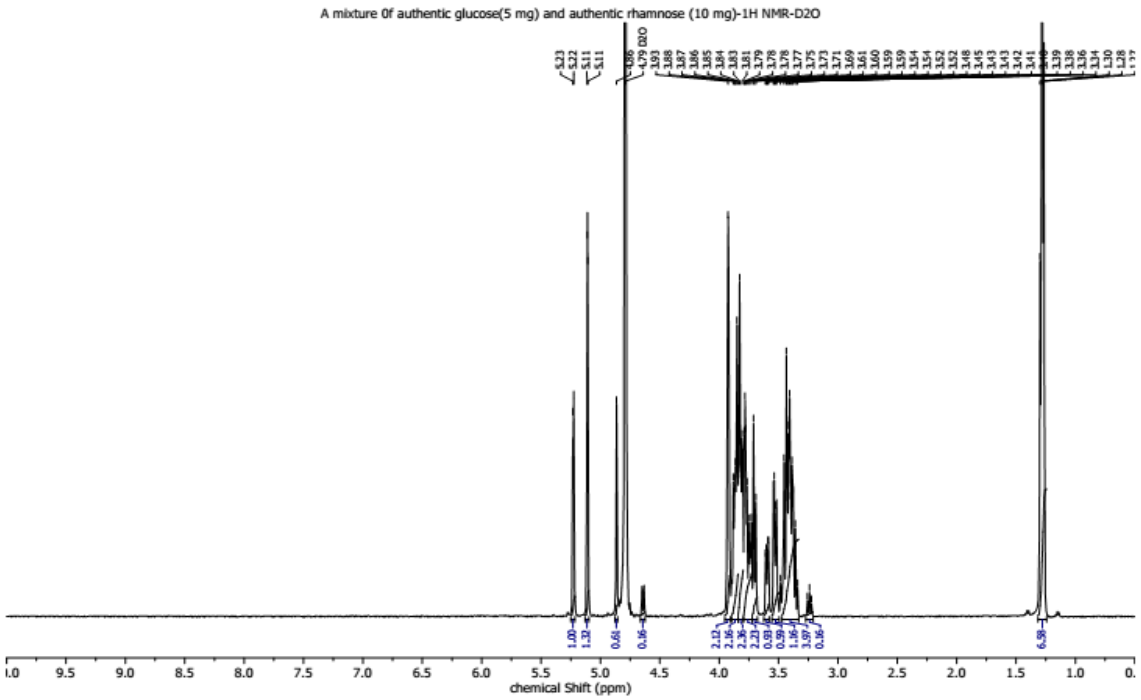


Figure S30: ^{1}HNMRspectrum (D_2O, 500 MHz) of a mixture of authentic D-glucose (5 mg) and authentic L-rhamnose (10 mg)/simulated sugars.

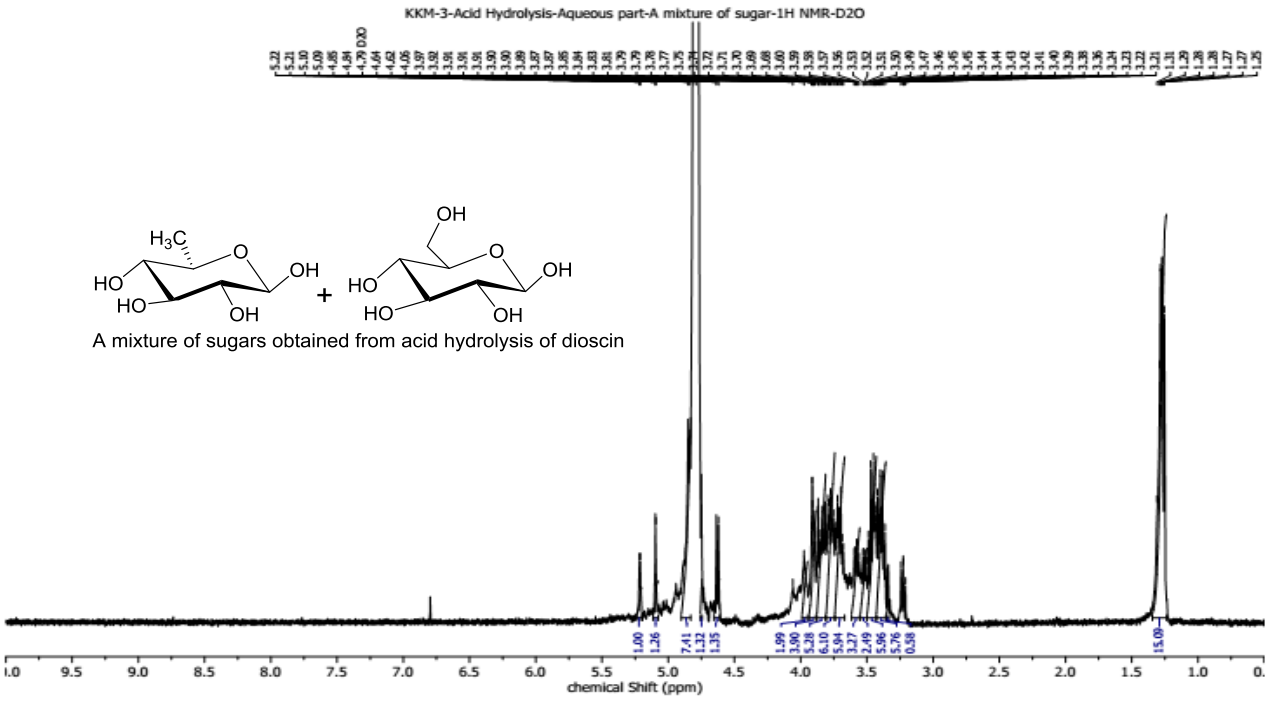


Figure S31: ^{1}HNMRspectrum (D_2O, 500 MHz) ofa mixture of sugars (3) generated from acid hydrolysis of glycoside, dioscin (1)

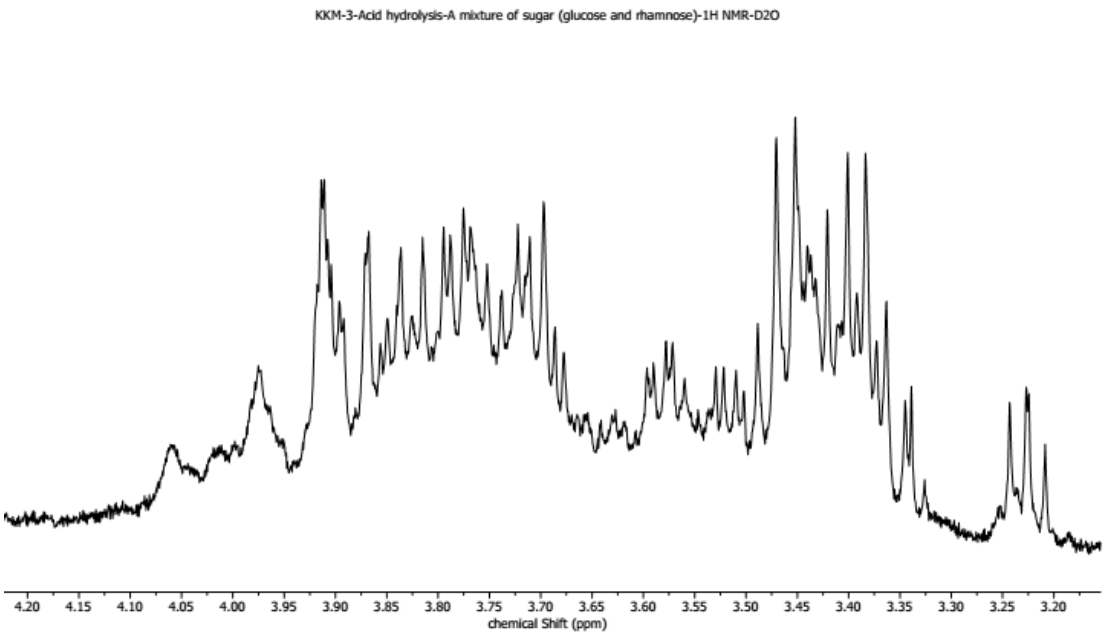


Figure S32: Expansion of ^{1}HNMR spectrum (D_2O, 500 MHz) of a mixture of sugars (3) generated from acid hydrolysis of glycoside, dioscin (1)

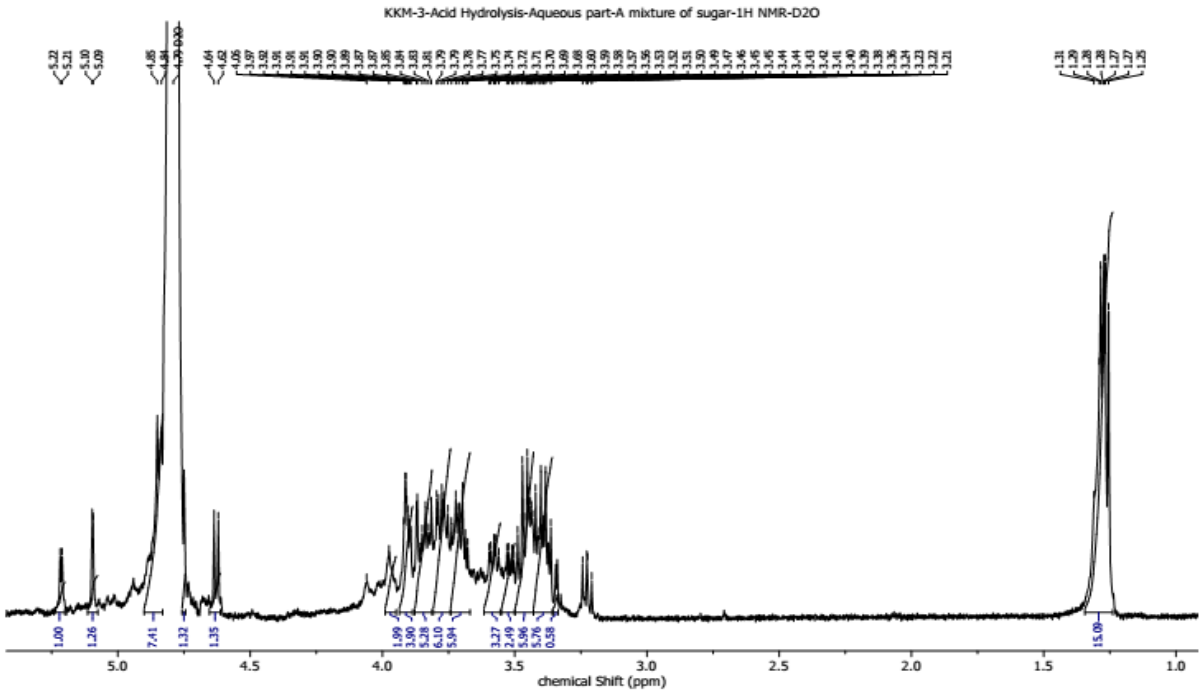


Figure S33: Expansion of ^{1}HNMRspectrum (D_2O, 500 MHz) ofa mixture of sugars (3) generated from acid hydrolysis of glycoside, dioscin (1)

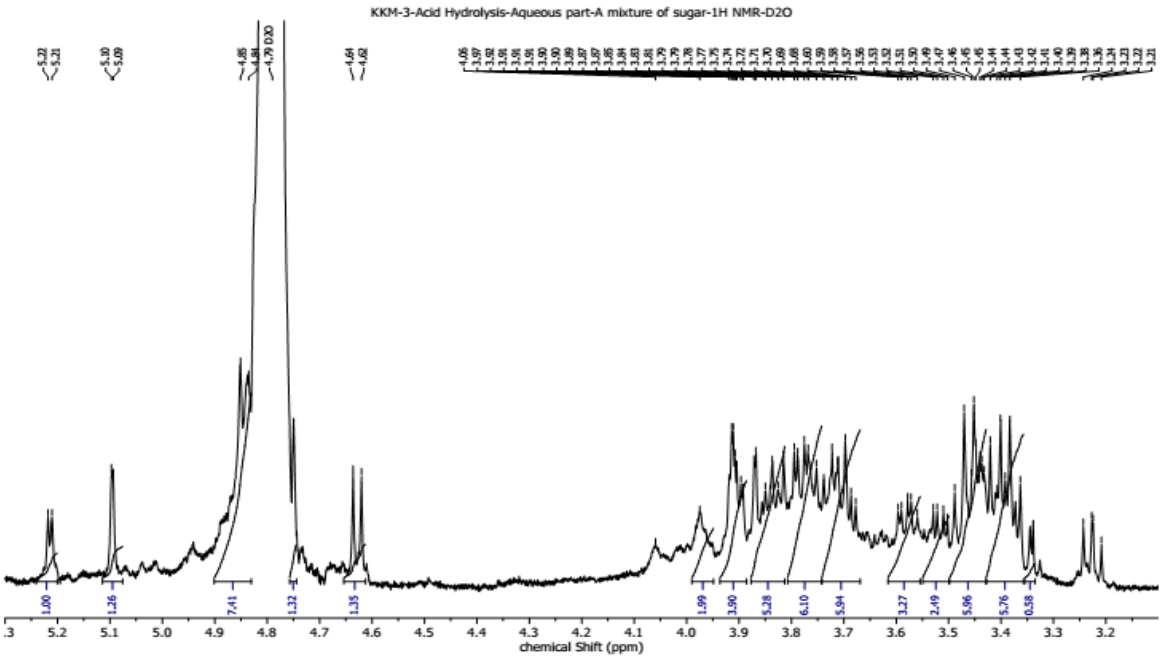


Figure S34: Expansion of ^{1}HNMRspectrum (D_2O, 500 MHz) of a mixture of sugars (3) generated from acid hydrolysis of glycoside, dioscin (1)

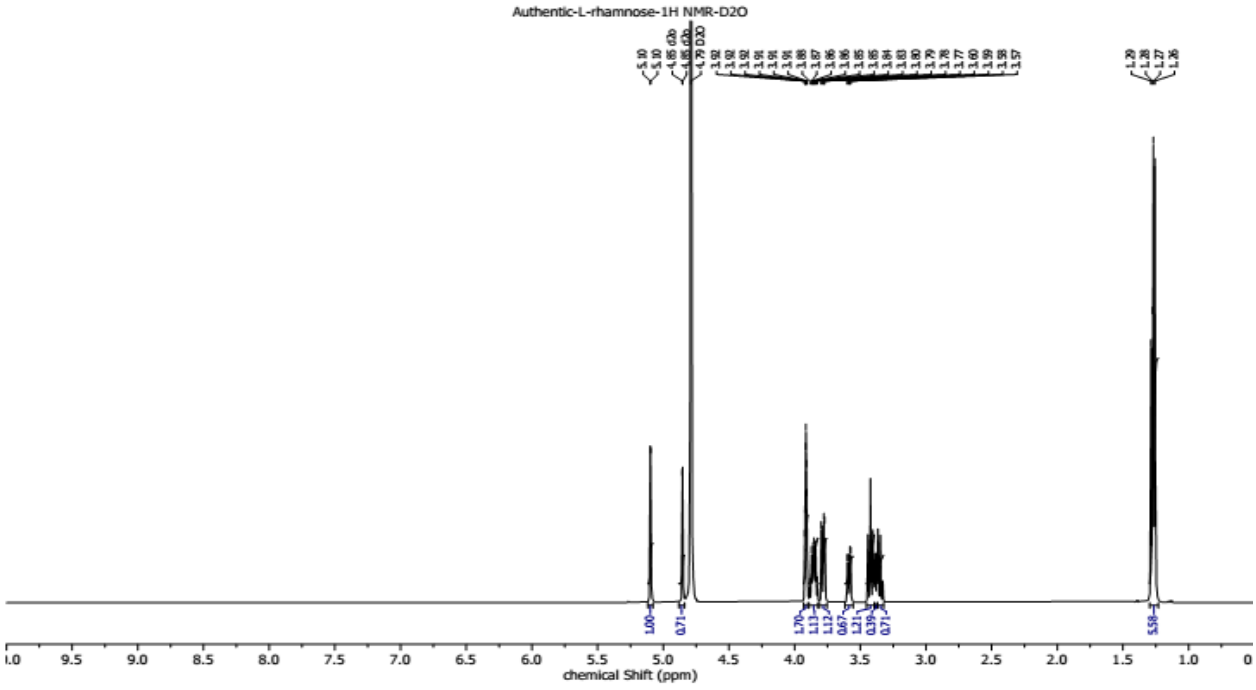


Figure S35: ^{1}H NMR spectrum (D_2O, 500 MHz) of authentic L-rhamnose (5).

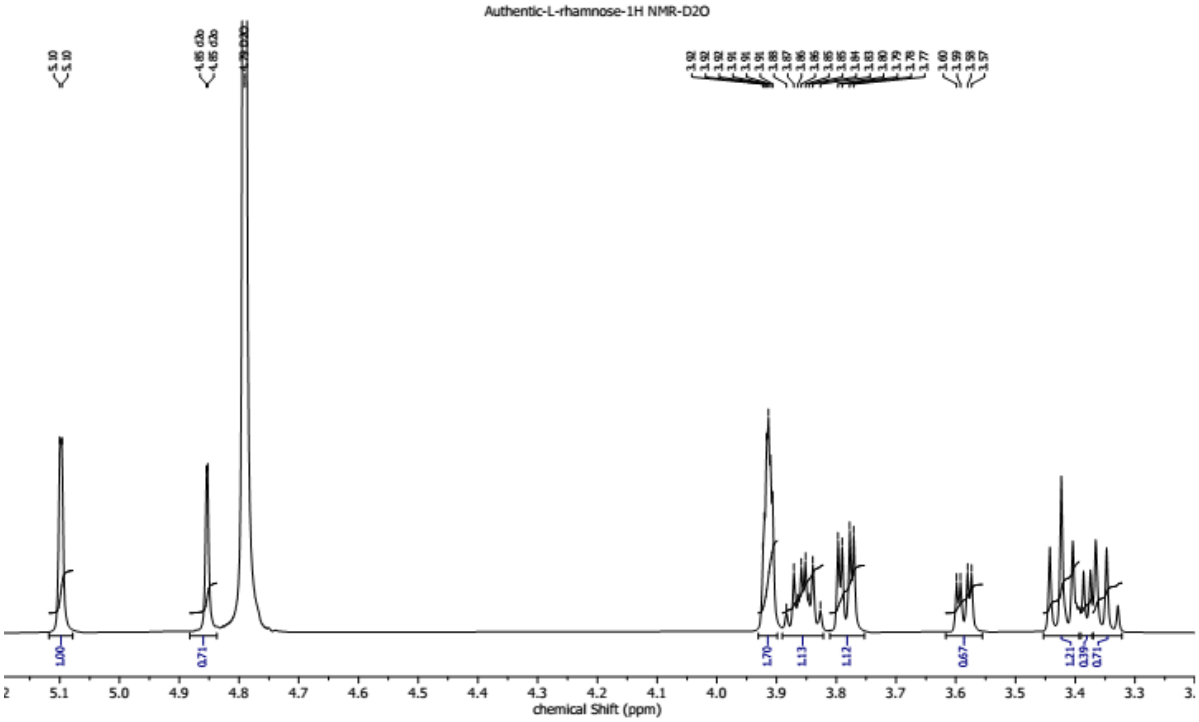


Figure S36: Expansion of ^{1}H NMR spectrum (D_2O, 500 MHz) of authentic sugar L-rhamnose (5)

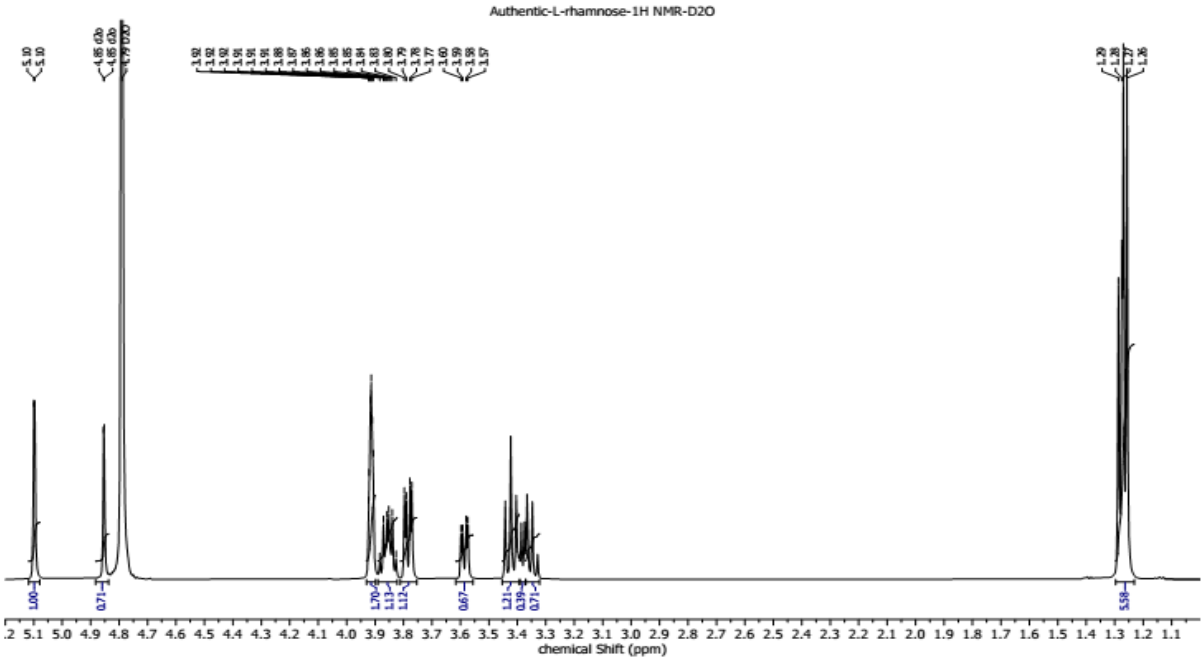


Figure S37: Expansion of ^{1}H NMR spectrum (D_2O, 500 MHz) of authentic sugar L-rhamnose (5)

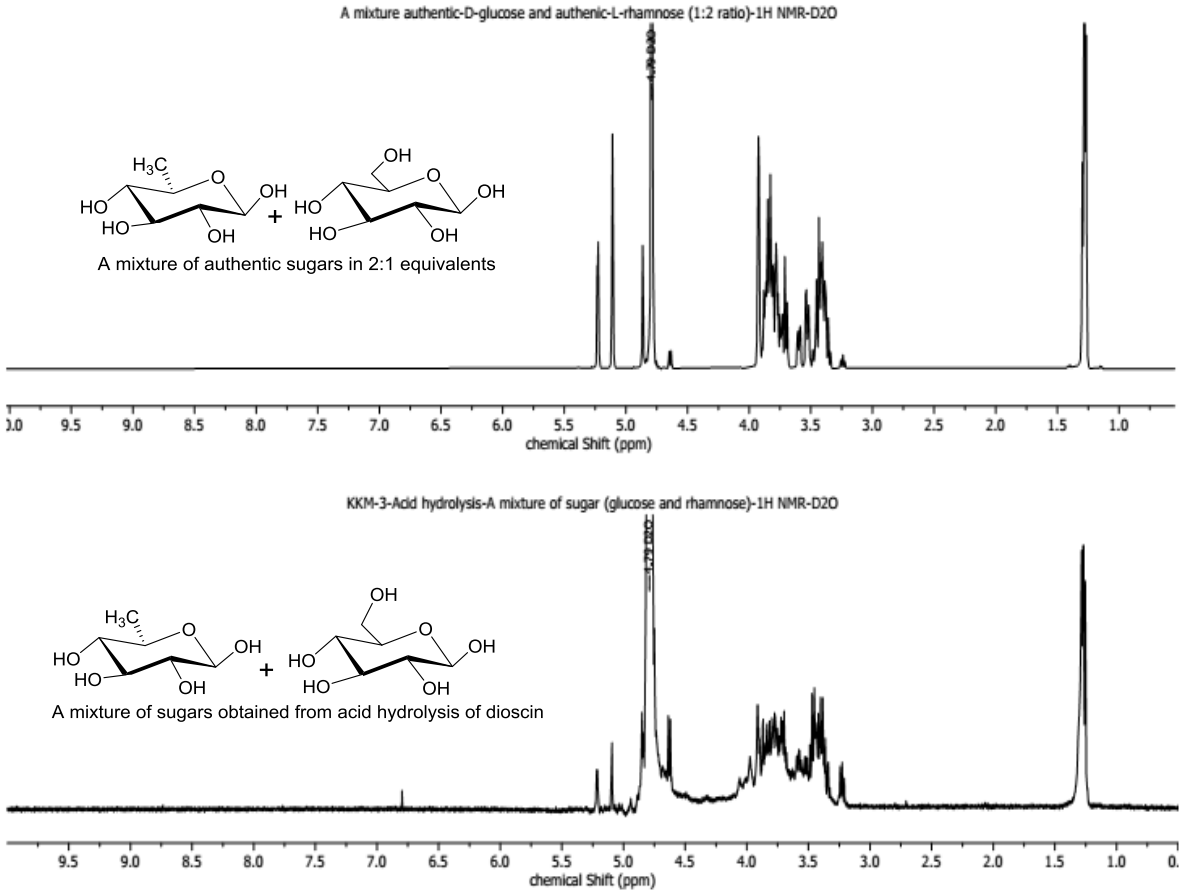


Figure S38: Stacking plot of ^{1}H NMR spectrum (D_2O, 500 MHz) of sugars (3)from acid hydrolysis of dioscin, and a mixture of authentic sugars D-glucose (4) and L-rhamnose (5) in 1:2 equivalents amount.

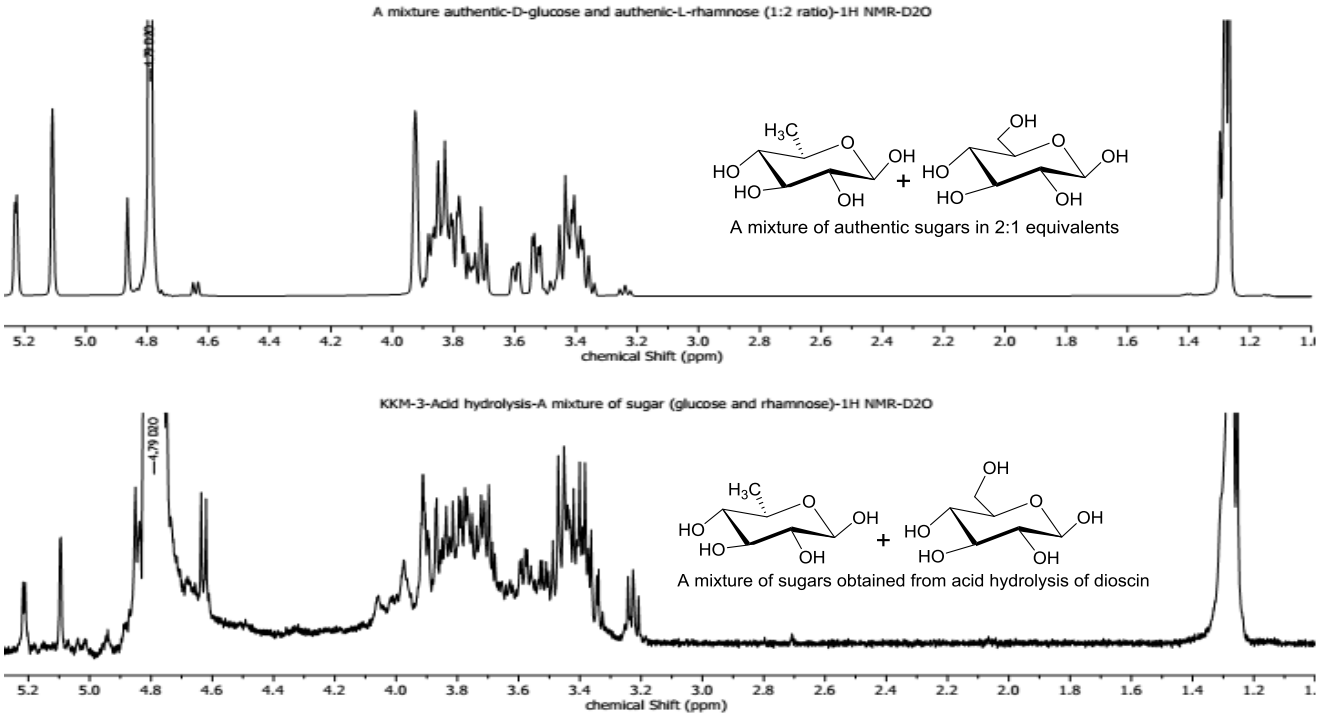


Figure S39: Expansion of staking plot of ^{1}HNMRspectrum (D_2O, 500 MHz) of a mixture of sugars (3) from acid hydrolysis of dioscin, and a mixture of authentic sugars D-glucose and L-rhamnose in 1:2 equivalent ratio.

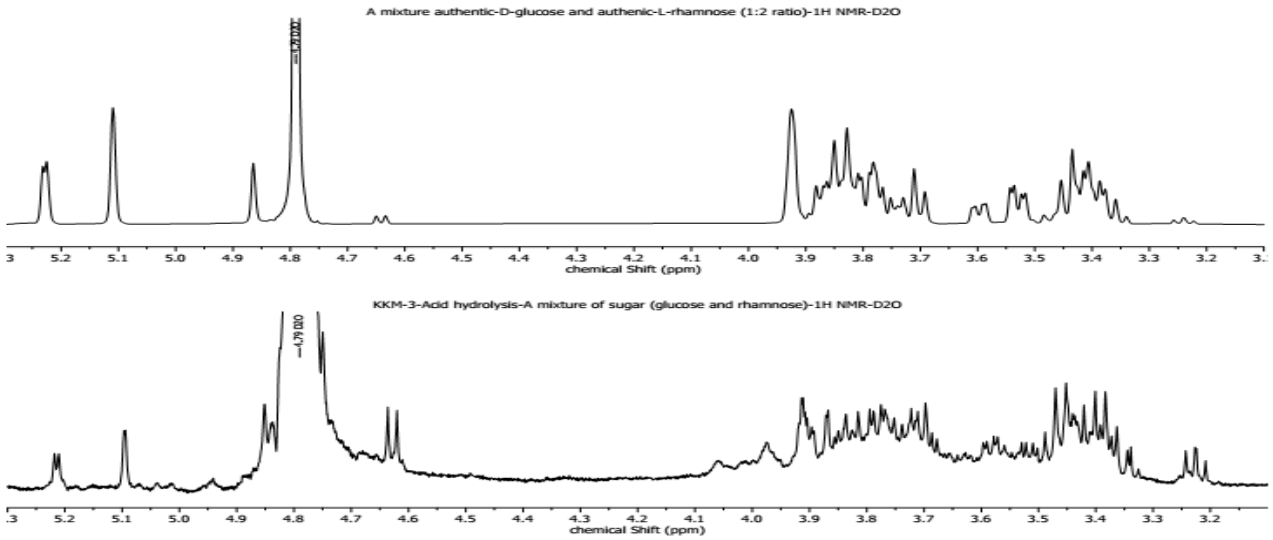


Figure S40: Expansion of stacking plot of ^{1}H NMR spectrum (D_2O, 500 MHz) of a mixture of sugars (3) from acid hydrolysis of dioscin, a mixture of authentic sugars D-glucose and L-rhamnose in 1:2 equivalent ratio.

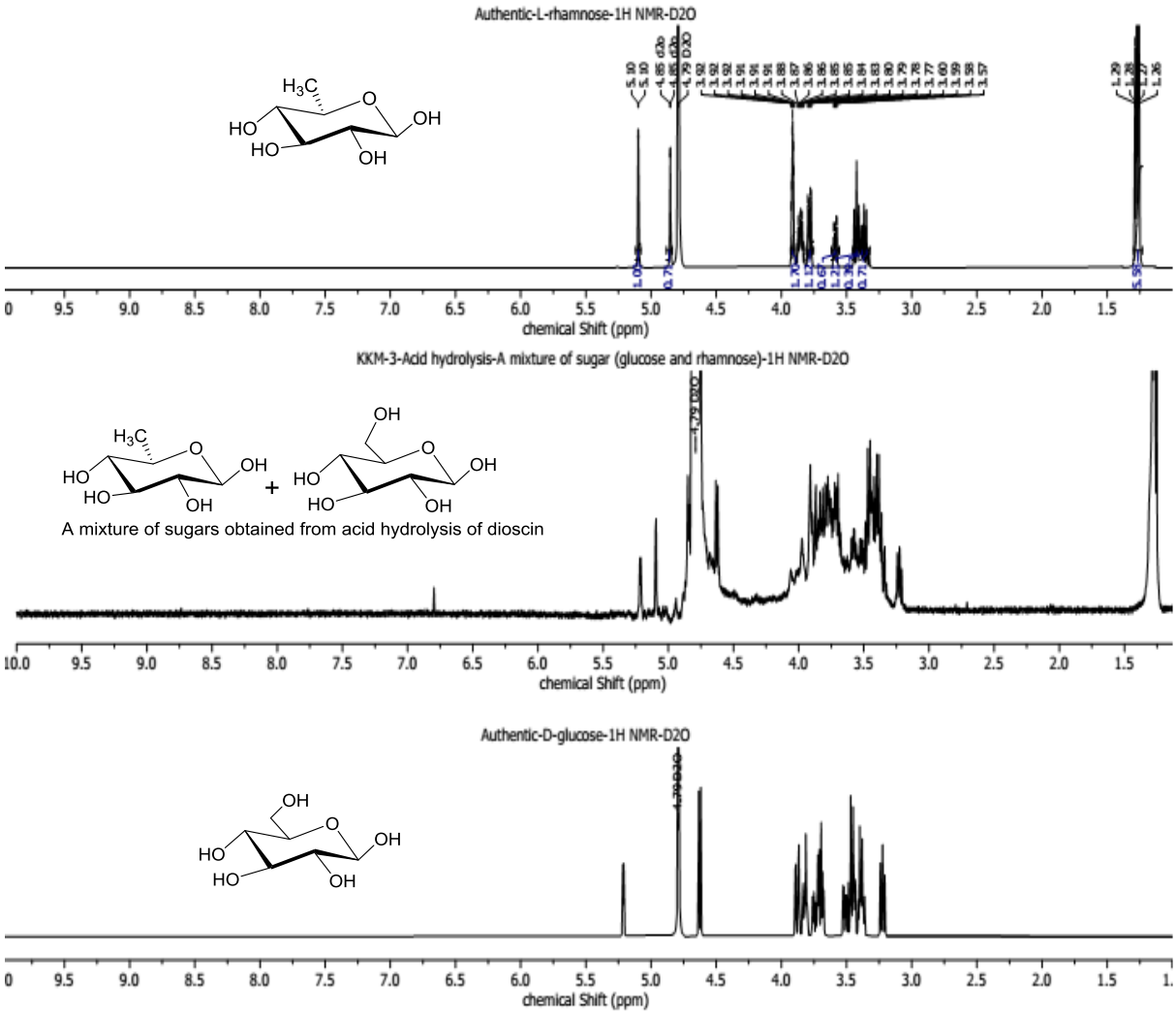


Figure S41: Stacking plot of ^{1}H NMR spectrum (D_2O, 500 MHz) of a mixture of sugars (3) from acid hydrolysis of dioscin and a mixture of authentic sugars D-glucose (4) and L-rhamnose (5) in 1:2 equivalent amount.

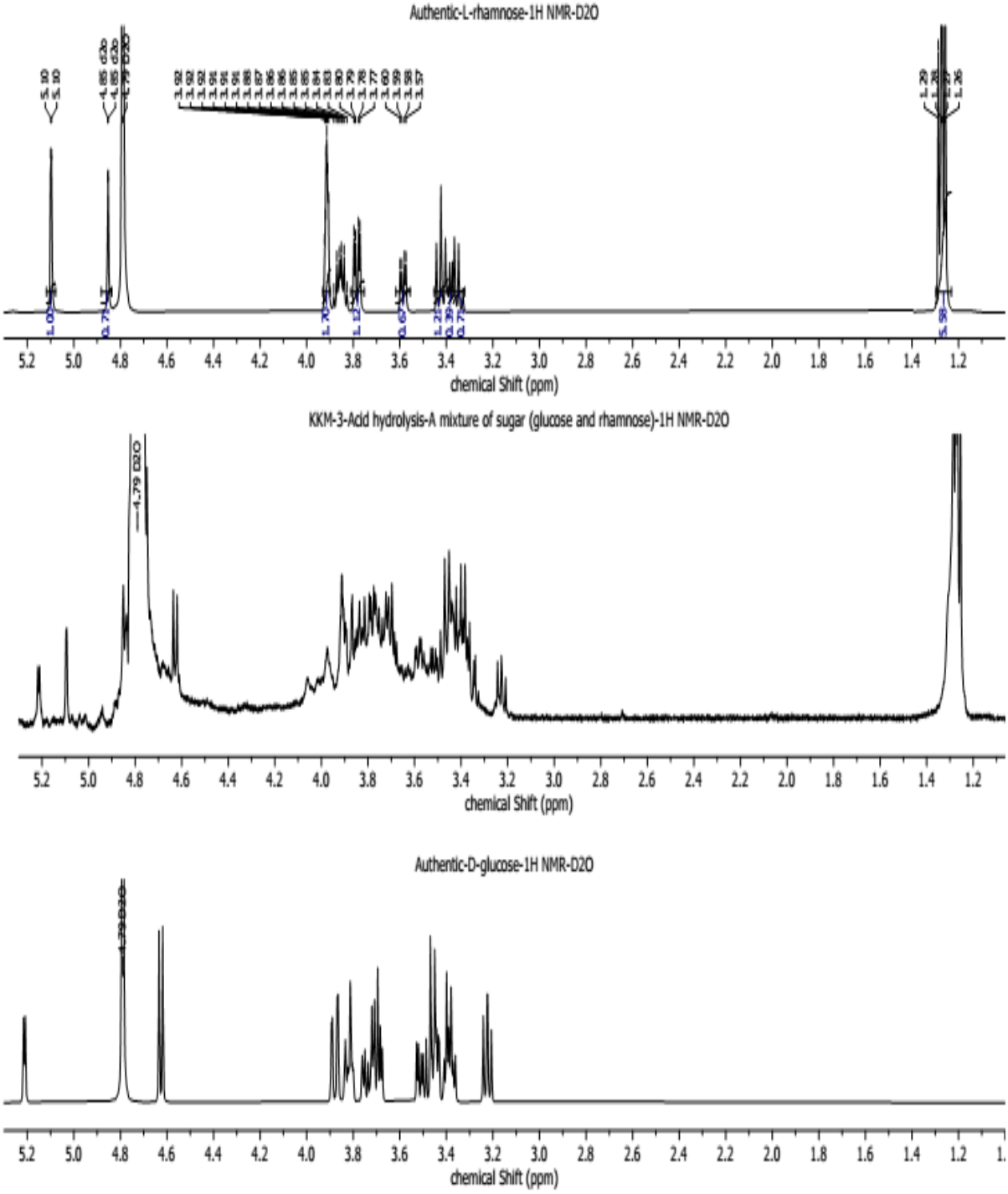


Figure S42: Expansion of staking plot of ^{1}HNMRspectrum (D_2O, 500 MHz) of L-rhamnose (5), a mixture of sugar (3) obtained from acid hydrolysis of dioscin, and authentic sugars D-glucose (4)

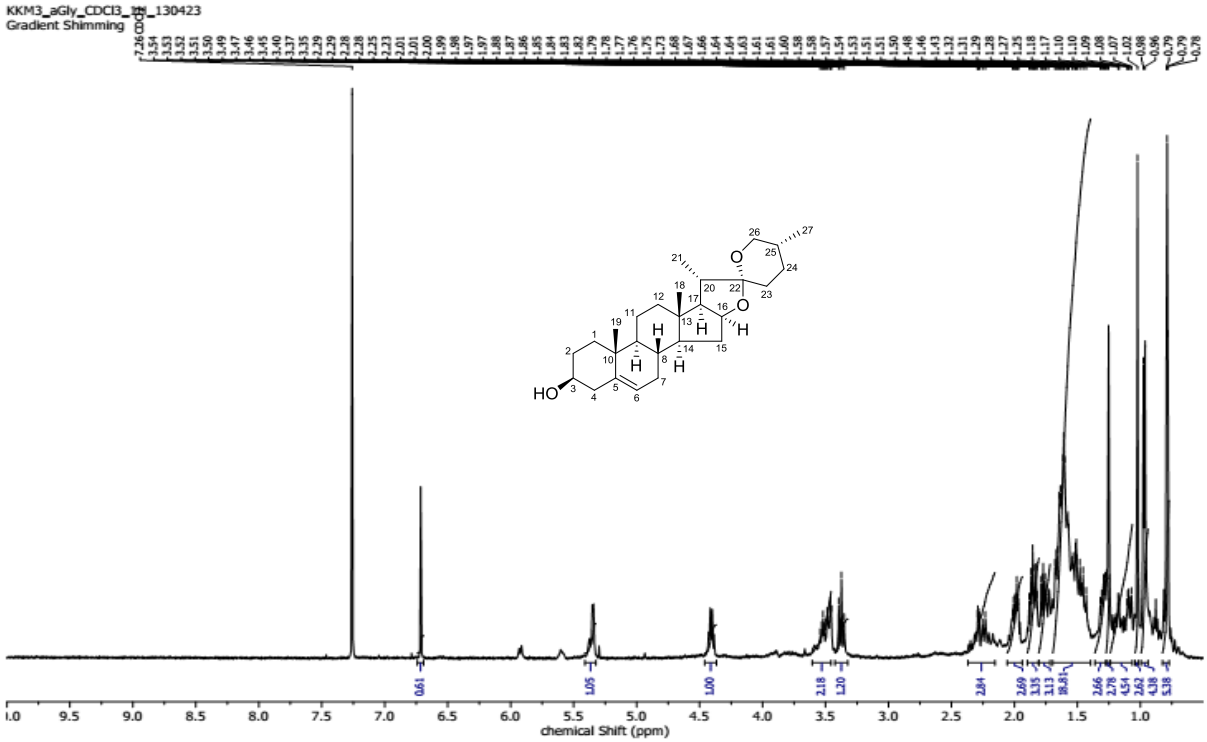


Figure S43: ^{1}H NMR spectrum ($CDCl_3$, 500 MHz) of diosgenin (2) obtained from acid hydrolysis of dioscin.

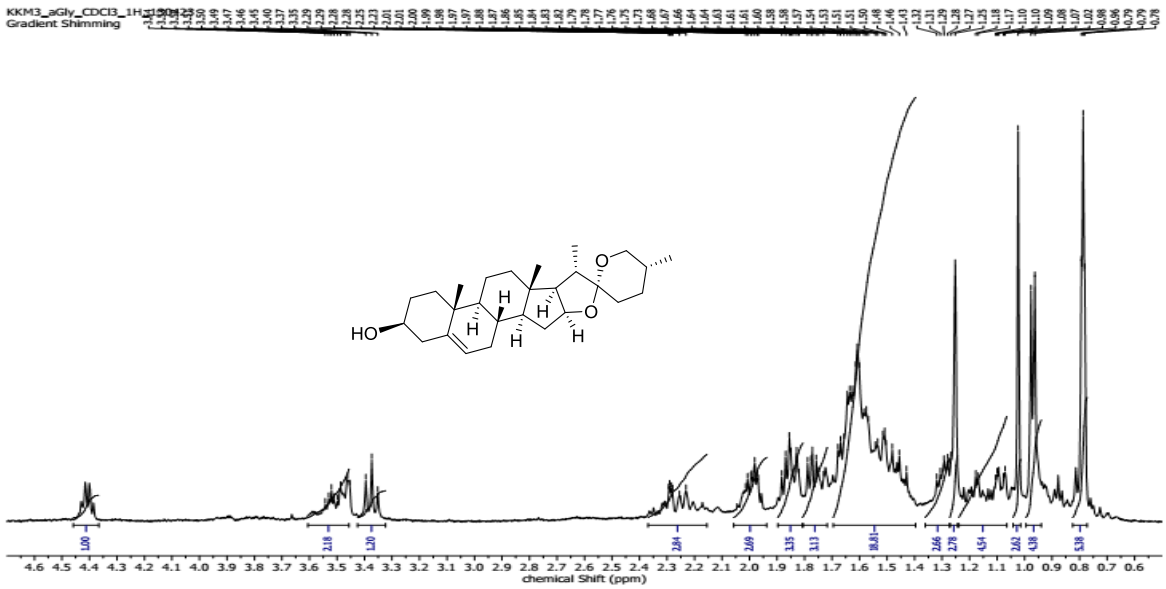


Figure S44: Expansion ^{1}H NMR spectrum ($CDCl_3$, 500 MHz) of diosgenin (2) obtained from acid hydrolysis of dioscin.

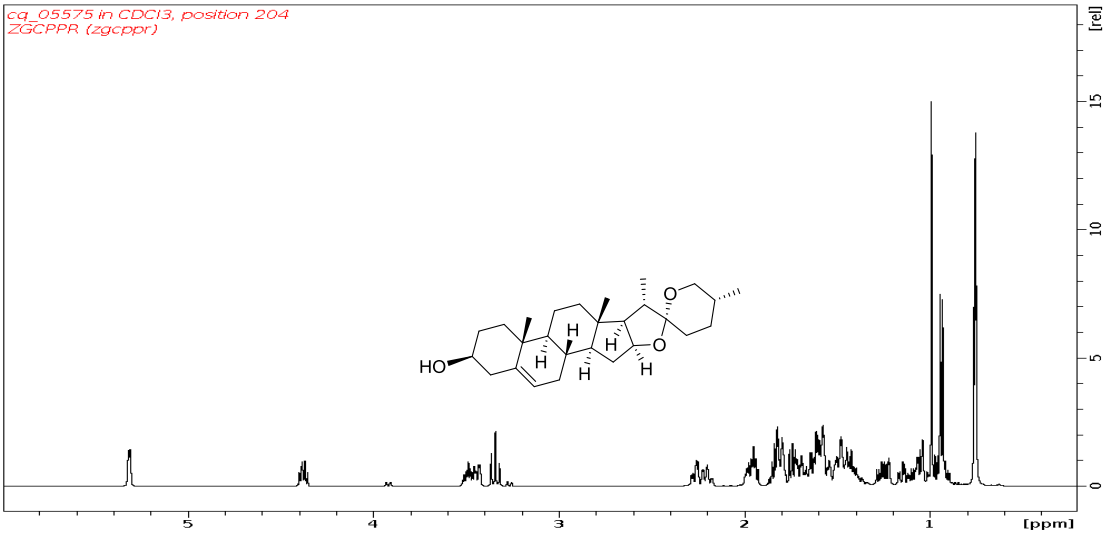


Figure S45: ^{1}H NMR spectrum ($CDCl_3$, 500 MHz) of standard diosgenin available in literature.

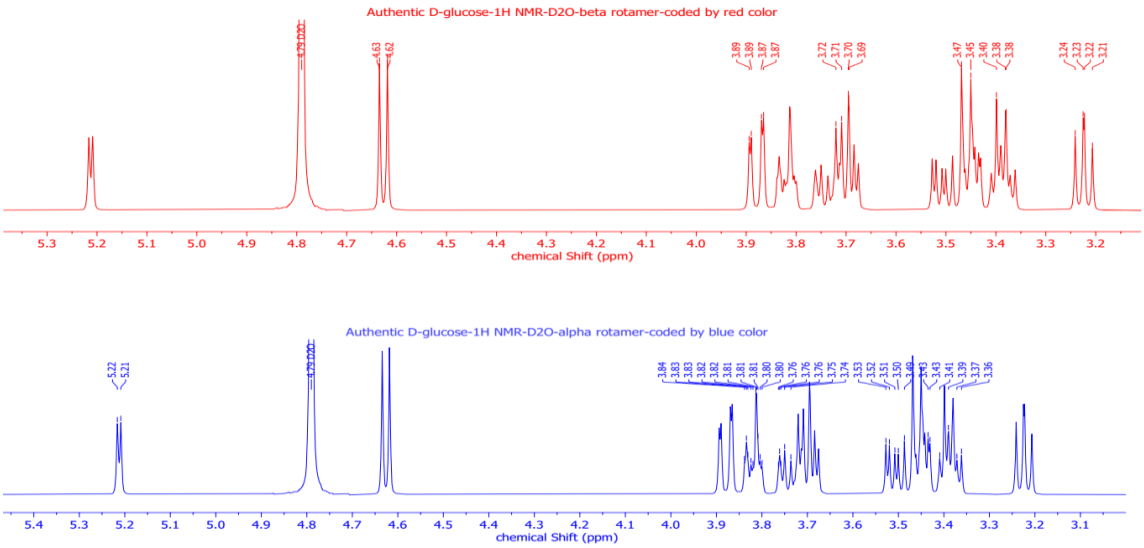


Figure S46: Interpretation of ^{1}H NMR spectrum ($CDCl_3$, 500 MHz) of rotamer of D-glucose.

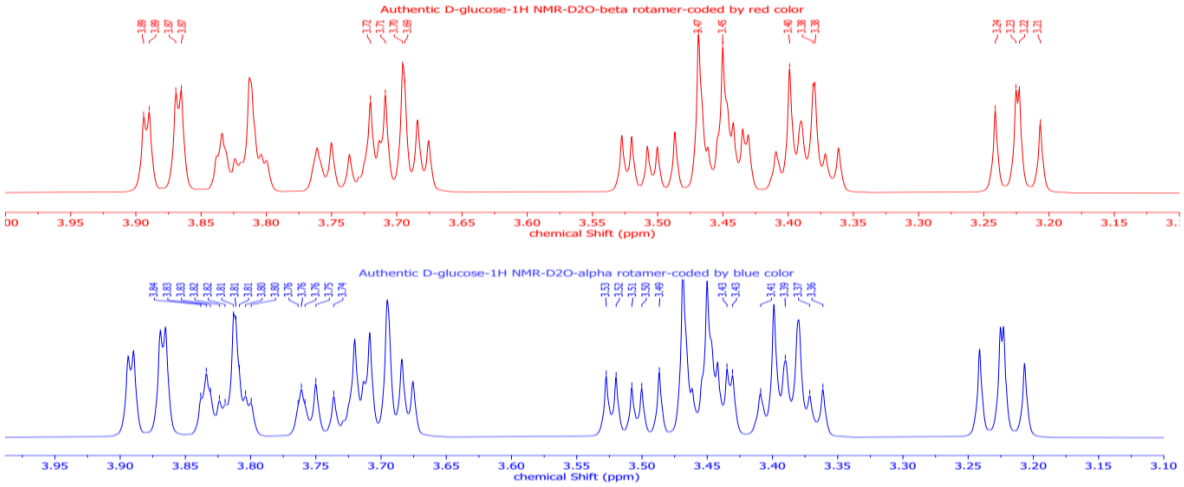


Figure S47: Extensive interpretation of ^{1}H NMR spectrum ($CDCl_3$, 500 MHz) of rotamer of D-glucose.

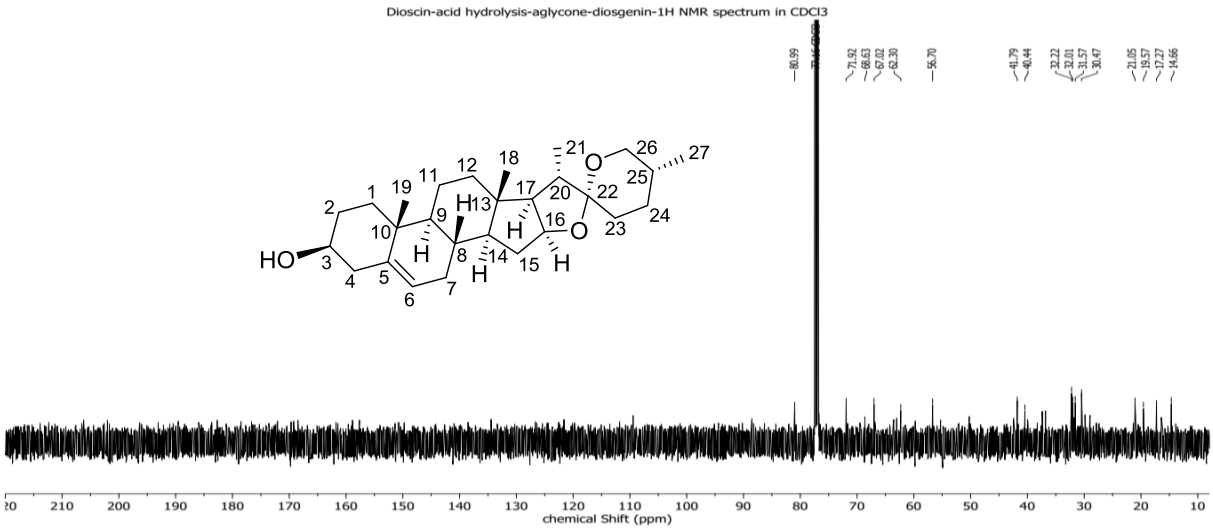


Figure S48: ^{1}H NMR spectrum ($CDCl_3$, 500 MHz) of diosgenin obtained from acid hydrolysis of glycoside dioscin.

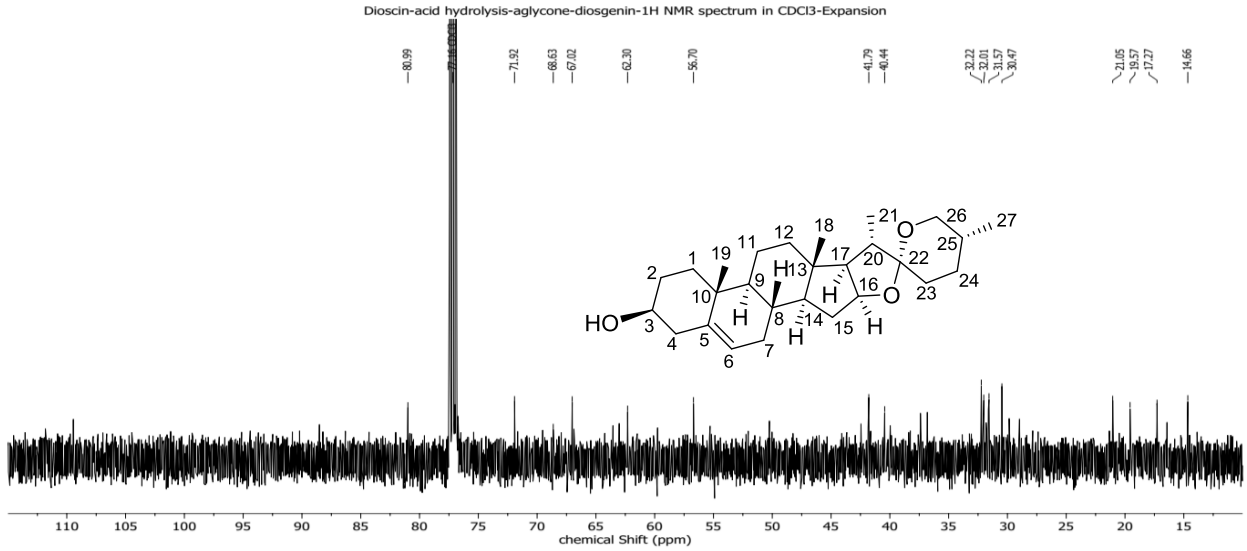


Figure S49: Expansion of ^{1}H NMR spectrum ($CDCl_3$, 125 MHz) of diosgenin obtained from acid hydrolysis of glycoside dioscin.

Table 1: Anti-proliferative activity of dioscin against different cancer cell lines, using sulfo-rhodamine B (SRB) assay.

Sample	Result of sulfo-rhodamine B (SRB) assay on different human cancer cell lines									
	HT-1376 (Urinary bladder)		HPAC (Pancreatic)		DU-145 (Prostate)		HT-29 (Colon)		MDA-T32 (Thyroid)	
	% Inhibition ±SE[a,b]	IC_{50} ±SE[c] µg/mL	% Inhibition ±SE[a,b]	IC_{50}±SE[c] µg/mL	% Inhibition ±SE[a,b]	IC_{50}±SE[c] µg/mL	% Inhibition ±SE[a,b]	IC_{50}±SE[c] µg/mL	% Inhibition ±SE[a,b]	IC_{50}±SE[c] µg/mL
Dioscin	62.43±0.3	9.47±2.0	80.298±0.6	8.32±1.3	66.08±0.5	13.85 ±1.3	70.06 ± 0.7	6.10 ± 0.6	74.77 ± 0.4	6.86 ± 0.6
Paclitaxel	-	13.17±0.5	-	0.92±3.3	-	0.011±0.0005	-	0.0028±0.0 0007	-	0.69±0.13

[a] Samples tested in triplicate and in two separate experiments at **20µg/mL**

[b] % Inhibition <50 deemed inactive

[c] IC_{50} ≥20 deemed inactive

ASSOCIATED CONTENT

Supporting Information (Spectroscopic data consisting IR, UV, ^{1}H NMR, ^{13}C NMR, DEFT, HRESIMS) of compound dioscin were enclosed in attached file (**Annexure-1**).

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CONFLECT OF INTEREST

The authors declare that there is no conflict of interest regarding the publication of this article.

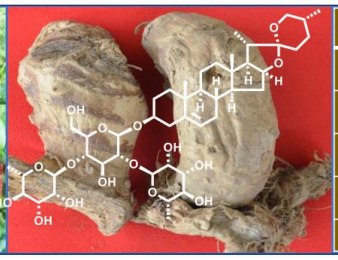
DATA AVAILABILITY

The data that support the findings of this study are available from corresponding author upon reasonable request.

AUTHOR CONTRIBUTION STATEMENT

AKB performed isolation, structural characterization, chemical transformation/reaction and drafted manuscript. IC and ESA contributed to the samples/reagents/materials/analysis tools and analysed anti-proliferative assay the data. EJCB supervised biological experimental, analysed result of anti-proliferative assay, contributed final revision of the manuscript, and managed to acquire HRESI-MS data with the help from IC and ESA.

GRAPHICAL ABSTRACT



Cell lines	IC_{50}-Dioscin	IC_{50}-Paclitaxel
HT-1376	9.47±2.0	13.17±0.5
HPAC-1376	8.32±1.3	0.92±3.3
DU-145	13.85 ±1.3	0.011±0.0005
HT-29	6.10 ± 0.6	0.0028±0.00007
MDA-T32	6.86 ± 0.6	0.69±0.13

CONCLUSION

Dry rhizome of *Costus speciosus* (Keu kand in local dialect and spiral ginger in English) was extracted with various solvents having different polarity at room temperature to afford respective crude extracts. Chemical profiling was carried out by column chromatography over silica gel with gradient elution by using a binary mixture of methanol and chloroform. It was found that a steroidal saponin glycoside was present as major constituent in the methanol extract of *C. speciosus*. The major chemical constituent was identified as steroidal saponin in the form of glycoside (performing positive LB test for steroid, Molisch test for glycoside and formation of lather/foam while shaking with water) and it was present in about 2% of dry weight of rhizome along with other minor constituents named as costunolide, erimanthin, santamarine, as well as the triterpenoid lupeol and the sterol β-sitosterol. The structural determination of the major ingredient was carried out by spectroscopic and spectrometric methods and identified as diosgenin glycoside. The anti-proliferative activity of it was conducted on different cancer cell lines such as urinary bladder (HT-1376), pancreatic (HPAC-1376), prostate (DU-145), colon (HT-29) and thyroid (MDA-T32) by SRB assay. It exhibited anti-proliferative activity at the micro molar (µM) level against the aforesaid cell lines (Table-1) in comparison to paclitaxel as control.

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