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Research Article

Phytochemistry

Isolation and Quantification of Antidiabetic and Antioxidant Components from Saturated Fraction of *Marrubium vulgare*. L Leaves.

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Abstract: *Marrubium vulgare L.* (Lamiaceae), aerial parts has been traditionally used to cure a variety of diseases including diabetes. A good class of secondary metabolites is present in the herb; however, the potential antidiabetic agent consistently stays a secret.. The present study aimed to identify the antidiabetic agents present in *M. vulgare* leaf extract. The isolation of compounds from the saturated fraction was done by column chromatography and their structures were elucidated using NMR, HRMS and MS spectroscopic methods. . The antidiabetic profiling of Methonoloc Extract (MeOH) extract (500 mg/kg b.w) and its chloroform fraction (250 mg/kg b.w) was investigated in STZ- induced diabetic rats. Four bioactive compounds namely marrubiin (MV-17), marrubinone B (MV-27), ladanein (MV-19) and apigenin 7-O-β-D-(6"- p- coumaroyl) glucoside (MV-25), were isolated from chloroform fraction, oleic acid(MV-20) and Palmitic acid (MV-21) from hexane fraction of *M. vulgare* leaf. Among these known compounds we first time report oleic and palmitic acid in *M. vulgare*. Both MeOH extract (500 mg/kg b.w) and its $CHCl_3$ fraction (250 mg/kg b.w) significantly (p?0.001) lowered the blood glucose level. Quantitative estimation by HPLC showed the highest concentration of MV-17, MV-20 and MV-27 in the $CHCl_3$ fraction. Moreover this fraction also showed significant antioxidant potential measured by DPPH assay. These results confirmed that the hypoglycemic potential of chloroform fraction (IIIM-2) may be largely due to compounds MV-17, 27 and 20.

Keywords: Oleic Acid, Marrubiin, Streptozotocin,, Ladanein, Antidiabetic.

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1. INTRODUCTION

Diabetes mellitus is a chronic metabolic disorder characterized by hyperglycemia together with impairment in glucose metabolism and other energy sources, such as lipids and proteins due to deficiency in insulin secretion, resistance to insulin action, or both.[1] There are about 463 million people with diabetes worldwide in the age group of 20-79 years, of whom 79% reside in developing countries. [2] In addition, the International Diabetes Federation [2] also estimated that the prevalence of this figure would rise to 700 million in 2045. In 2017 reports by WHO, it was estimated that 1.6 million deaths were due to diabetes alone which accounts for 9.9% of the global all-cause mortality among people.[3] As far as India is concerned, studies highlighted that the prevalence of diabetes is high and also there is a rapid increase in the urban population. It was estimated that about 134 million adults will be affected with diabetes in the year 2045.[4] Diabetes is controlled and managed by a combination of diet restriction, weight reduction programs and oral hypoglycemic synthetic drugs. Though these antidiabetic drugs play a key role in treatment of diabetes, yet are unable to accomplish ideal outcomes. Their association with a number of side effects and significant expenses demanded for an alternative drug with least or no side effects. Researchers found that such a demand could be fulfilled by medicines originated from plants, so all over the world, attempts are being made either to prepare herbal formulation or to isolate phytoconstituents from plants which act as natural antidiabetic agents.[5] Numerous traditional plants exist for treatments of diabetes, however about 800 plants are reported to possess antidiabetic potential with minor toxicities compared to synthetic drugs. [6,7] Researchers reported that several plants possessing antidiabetic potential contains a number of natural compounds like glycosides, alkaloids, terpenoids, flavonoids, carotenoids, however phytoconstituents responsible for their antidiabetic activity have not been fully identified. However, the search to identify such natural compounds is going on with great peace throughout the world. *Marrubium vulgare* which contains a good class of secondary metabolites like phenols, flavonoids, diterpenoids [8] is also evaluated for various biological activities including diabetes.[9] However previous studies of the herb related to its antidiabetic activity was restricted to crude extracts only [10-12] which contains almost all classes of compounds (phenols, flavonoids, diterpenoids tannins, essential oils, sugars), so to identify the active agent remains always a mystery. In the present study, we isolated six known compounds and evaluated the saturated fraction containing three major compounds for its antidiabetic potential.

2. MATERIALS AND METHODS

2.1 Plant material

Marrubium vulgare L. whole plant was collected at an altitude of 1743 m from Nowhatta, Srinagar (J&K) in the month of May 2018. The plant was authenticated by Dr. Anzar Ahmad Kharoo (Centre for Biodiversity & Taxonomy, Department of Botany, University of Kashmir) and sample of the plant material was deposited in the herbarium of the Department of Taxonomy, University of Kashmir under voucher specimen number, *Marrubium vulgare*- 2678 KASH [Ref No: F (voucher-specimen CBT/KU/18)]. Each part (leaf, flower, stem and root) of the herb was separated and washed thoroughly under running tap water and then rinsed in distilled water; they were allowed to dry for 10 min. These plant parts were then separately shade dried without any contamination for about 3 to 4 weeks. The dried samples were powdered (coarse) using a grinder and extraction of leaves (500 g) was done while rest samples were stored till further use.

2.2 Chemicals and drugs

Standard drug, Metmorfin hydrochloride was from Cipla Pharmaceutical Company, India; Streptozotocin (STZ) was from Sigma Chemicals (St. Louis, MO USA). Ebra Glucose Kit for estimation of glucose was of Transasia Bio-Medicals Ltd. 1,1-Diphenyl-2-picrylhydrazyl(DPPH) was of TCI company. All solvents used in the experiment (methanol, chloroform, DMSO) were of analytical grade.

2.3 Extraction and isolation of compounds [13,14]

Air dried leaves (500 g) were subjected to soxhletion using methanol (10 L) as solvent. After three siphoning the infusion was filtered through Whatman filter paper No. 1 and evaporated to dryness under vacuum at 40 °C using Rotary evaporator to get 100 g (20%) methanol crude extract. Out of 100 g extract, 80 g was dissolved in water and sequentially fractionated into n-hexane, chloroform and aqueous to obtain n-hexane (22.5 g), chloroform (45.5 g) and aqueous (12.0 g) fractions. $CHCl_3$ fraction (45.5 g) was chromatography over silica gel (100-200 mesh) and eluted using hexane:EtOAc gradient (0: 100% EtOAc) to give 105 fractions, based on TLC 22-26, 27-30, 64-72 and 100-105 fractions were pooled. Fraction 22-26 (30 gm) was further subjected to the silica gel column chromatography and elution of hexane: EtOAc (20:25) gave impure compound 1 (coded as MV-17) containing chlorophyll. Similarly from fraction 27-30 (5 gm) over silica gel column chromatography and at a polarity of 30% (EtoAc/hexane) also gave an impure compound 2 (MV-27) containing chlorophyll. Chlorophyll from both the fractions containing compound 1 and 2 was removed by rechromatographing them on HP-20 and eluted by increasing the gradient of methanol in water to yield pure compounds **1** (400 mg) and **2** (250 mg). Compound **3** (MV-19, 100 mg) was obtained from fraction 64-72 (5 g) on its subjection to silica gel column with mesh size of 100-200 and at polarity of 35% (EtoAc:hexane). Similarly fraction 100-105 (5 gm) was also rechromatographed on silica gel (100–200 mesh) columns and eluted with 90% EtoAc in hexane yielded the compound **4** (MV-25, 60 mg). Sub fraction 7-12 (5 g) of hexane fraction (22.5 g) yielded compound **5** (MV- 20, 200 mg) and 6 (MV-21, 100 mg) on subjection to silica gel (100-200 mesh) column chromatography with an elution gradient of 80:20 and 85:15 hexanes/EtOAc respectively.

2.4 Quantitative estimation of isolated compounds by HPLC

2.4.1 HPLC sample preparation[15,16]

Methanolic extracts of leaf and its hexane, chloroform and aqueous fractions were prepared with a concentration of 20 mg/mL in methanol (HPLC grade) and standards (isolated compounds) by dissolving 1 mg of each compound in 1 ml of methanol. All samples were first filtered through the Millipore filter (0.2µm). HPLC analysis was performed on a Shimadzu UFLC, composed of quaternary pump: LC-20AD, prominence degassing unit, Auto sampler: SIL-20A HT,

Column oven (CTO-10AS vp) and Photodiode Array Detector (PDA): SPD-M20A. Chromatographic separation for compound **1** (MV-17) and **6** (MV-21) was obtained with reverse phase column utilizing Merck RP-18 column (250 mm×4 mm, 5µm) with an isocratic system at 35°C. The Photodiode array detector (DAD) was set at wavelength of 205 nm and peak areas were integrated automatically by using software Lab Solutions® (shimadzu). Elution was performed at a flow rate of 0.8 ml/min and the injection volume was 10 µl. The mobile phase for quantitative analysis consisted of 65% ACN and 35% water with 0.1% formic acid. However for compound **5** (MV-20) mobile phase consisted of 95% ACN and 5% water with 0.1% formic acid and at a column temperature of 45 ^{0}C with flow rate of 1 mL/min. The total chromatographic analysis time was 30 min per sample for Compounds **1** and **6,** and 20 min for compound **5** eluting at retention times of 23.202, 10.082 and 3.440 min, respectively. For separation of compound **2**, **3** and **4**, same System (Shimadzu UFLC) was used under gradient program condition consisting of ACN: water (0.1% formic acid) solvent system with a flow rate of 0.75 mL/min at column temperature of 35 ^{0}C and an injection volume of 10 µl. The gradient program for RP-HPLC condition was performed as follows: initial 0.01 min, A–B (10:90, v/v), then 0.01–15 min, linear change from A–B (10:90, v/v) to A–B (40:60), 15–25 min, there is also linear change from A–B (40:60) to A–B (70:30) and the linear gradient elution is from A–B (70:30) to A–B (95:05) with the range of 35–40 min, then in next 40-45 min, the linear gradient is from A-B (95-05) to A-B (10:90). This was followed by A–B (10:90) from 45 min to 50 min. NMR spectra in chloroform-d ($CDCl_3$) and methanol-d_4 (CD_3OD) were recorded on Bruker Avance 400 Dgital NMR spectrometer operating at 400 MHz for ^{1}H and 100 MHz for ^{13}C . Chemical shifts are expressed in parts per million (δppm) and coupling constant (*J*) given in Hertz (Hz). ESI-HRMS were recorded on Aligent QTOF 6540 A instrument and MS analysis was carried out on LCMS-800 (Shimadzu).

2.5 Antioxidant activity

The 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging capacity assay of the crude methanolic extract and its fractions (Hx fr , Chf fr and Aq fr) were determined using the method described by Thida et al., 2018 [17] with slight modifications. In a 96 well plate a volume of 100 µL of 4 samples (M ex, Hx fr, Ch fr and Aq fr) at various concentration (1000- 15.62 µg/ml) were added to 100 µL of a methanol solution of DPPH (0.1 mM) in each well. The reaction mixture was incubated for 30 min.at room temperature in dark and the absorbance was measured at 517 nm using micro-plate reader of Tecan Infinite M Nano Elisa plate Reader (Austria).Ascorbic acid was used as a positive standard and methanol as positive control. All tests were performed in triplicates and percentage (%) of scavenging of the DPPH free radical was measured by using the following equation:

$$\text{Percentage Scavenging} = [(A_{\text{positive control}} - A_{\text{sample}})/(A_{\text{positive control}})] \times 100.$$

Where $A_{\text{positive control}}$ is the absorbance of the control reaction (containing all reagents except the test extract or standard), and A_{sample} is the absorbance of the test extract or standard.

Concentration of samples (extracts and standard) resulting in 50% inhibition on DPPH (IC_{50} value) were calculated using GraphPAD Prism Software Version 5.0**.**

2.6 Determination of anti-diabetic activity[Ref??18]

2.6.1 Animals

Thirty six male Albino rats (180-200 g) were obtained from the animal house of the CSIR IIIM, Jammu, India and maintained under standard controlled animal care facility having 12 hrs. of light/dark cycle, 24±2°C. The animals used in this study were cared for in accordance with the guidelines of Indian Institute of Integrative Medicine, Jammu, India. The study was approved by the Institutional Animal Ethics Committee (IAEC), study no. 1782/75/8/19. Throughout the experimental period, all six groups were fed normal chow standard pellet diet and water *ad libitum.*

2.6.2 Induction of diabetes[Ref??19]

The animals were allowed to fast for 12 hrs. and diabetes was induced by intraperitoneal injection (i.p) of freshly prepared Streptozotocin (STZ) in an ice cold citrate buffer at the concentration of 45 mg/ kg of body weight. The STZ treated animals were allowed to drink normal saline solution overnight to overcome drug-induced hypoglycemic shock. Rats having persistent glycosuria and hyperglycemia with a fasting blood glucose >250 mg/dL on the fifth day after the STZ injection were considered as diabetic and used for further experimentation.

2.7 Experimental design

In the experiment, a total of 36 male Wistar rats were divided into the following six groups for the oral administration of extract/fraction/drugs or vehicle.

I. Normal Control: Normal control: received normal feed and water p.o
II. Citrate buffer: Citrate buffer received citrate buffer (PH 4.5) i.p
III. DM Control: Diabetic control rats were receiving 0.1% sodium CMC solution orally
IV. Metformin: Diabetic rats were given metformin (500 mg/kg) for 14 days orally ([20,21]).
V. IIIM-1: Diabetic rats were administered orally with methanol extract of *M.vulgare* (500 mg/kg) for 14 days.
VI. IIIM-2: Diabetic rats were administered orally with chloroform fraction of *M.vulgare* (250 mg/kg) for 14 days.

On day 5th post STZ treatment blood glucose estimation was done and rats with high blood glucose (> 250 mg/dL) were selected and grouped as given above. The different doses of extract, fraction and metformin were prepared in 0.1% sodium CMC (carboxymethyl cellulose) and then administered every day till the completion of the experiment (i.e., 14 days), whereas untreated and diabetic control groups

were treated with saline and 0.1% sodium CMC respectively every day orally. At the end of the experiment, the blood samples were collected for biochemical studies. The serum was separated by centrifugation and subjected for assay immediately or stored at −20°C.

2.7.1 Estimation of blood glucose level

Non - fasted blood samples were collected through retro orbital plexus puncture method [22,23] in sample tube (1 mL) on 5th, 8th and 15th day of the study. The blood samples were centrifuged at 5000 rpm for 10 minutes to separate serum by using an Ultra-Centrifuge Machine (THERMO SCIENTIFIC FRESCO 21) and estimation of glucose level was done by glucose oxidase-peroxidase (GOD-POD) method, using ERBA GLUCOSE kits whereas standard was taken as 100 mg/dl.

3. STATISTICAL ANALYSIS

All data are presented as mean ± standard error of mean (S.E.M). Statistical analysis was performed by analysis of variance (ANOVA) followed by Bonferroni's post hoc test. P-values less than 0.05 were considered to be significant.

4. RESULTS

In this study, four compounds viz marrubiin, marrubinone B, ladanein and apigenin 7-O-β-D (6"-p-coumaroyl) glucoside were isolated from chloroform and two (oleic acid and palmitic acid) from hexane fraction (Fig. 1) of *M. vulagare* and their structures were established by using various spectroscopic techniques (^{1}H, ^{13}C NMR values, HRMS and MS) and direct comparison with literature [24-29] [Supplementary File 1 Fig S1- S24]. The isolated compounds were as follows:

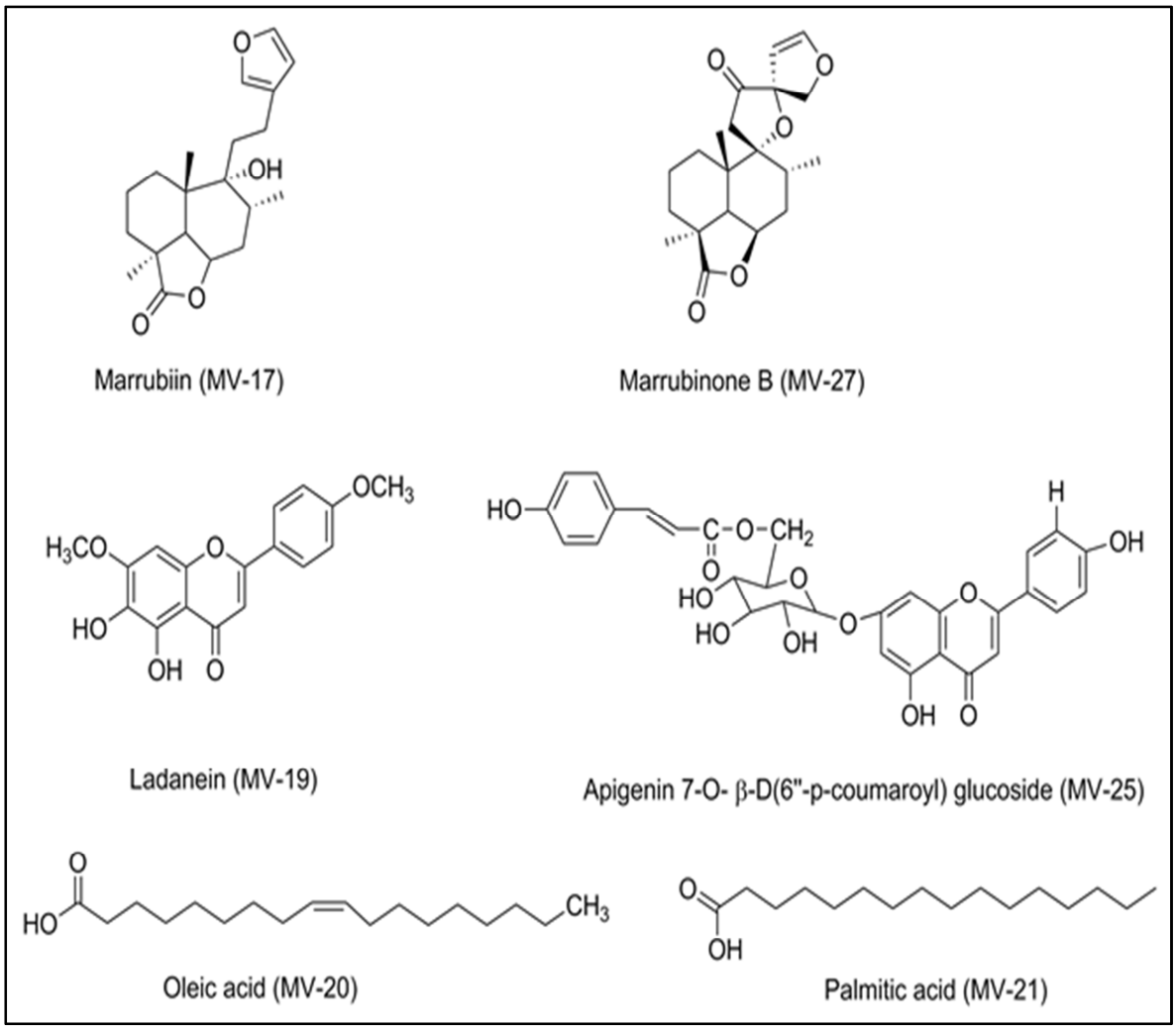

Fig 1: Chemical structure of the known compounds isolated from *M. vulgare* leaf

Compound 1: Marrubiin

White Powder, ^{1}H NMR (400 MHz $CDCl_3$): δ_H 7.37 (s,1H), 7.24 (s,1H), 6.28 (s, 1H), 4.75 (dd,*J*=8.0,2.8Hz), 2.58-2.50 (m,2H), 2.24 (d,*J*=4.6Hz,1H), 2.0-2.09 (m,3H), 1.93-1.87 (ddd,*J*=7.2,10.1,14.4Hz,1H), 1.78-1.71 (m,4H), 1.51- 1.44 (m,2H), 1.30 (s,3H), 1.28 (s,1H), 1.14 (s,1H), 1.07 (s,3H), 0.97 (d,*J*=6.4Hz,3H); ^{13}C NMR (100 MHz $CDCl_3$): δ_C 21.15 (C-12), 35.29 (C-11), 28.51 (C-3), 75.95 (C-9), 125.19 (C-13), 143.26 (C-15), 110.86 (C-14), 45.01 (C-5), 43.94 (C-4), 76.33 (C-6), 39.89 (C-10), 18.32 (C-2), 32.51 (C-8), 31.67 (C-7), 22.41 (C-18), 138.74 (C-16), 183.96 (C-19), 16.74 (C-17), 21.15(C-20), 28.79 (C-1). ESI-MS m/z 333.2.$[M+H]^+$(cal. for $C_{20}H_{28}O_4$, m/z 332.44).

Compound 2: Marrubinone B

White crystal form, ^{1}H NMR (400 MHz $CDCl_3$): δ_H 1.70 (1H, H-1α), 1.61 (1H, H-2α), 1.81. (1H, H-2β), 1.51 (1H, m, H-3α), 2.14 (1H, H-3β), 2.27 (1H, d, *J*=4.3Hz, H-5α), 4.74 (1H,

t-like, 6β), 2.19 (1H, dd, *J*= 5.8,13.7,H-7β), 2.17 (1H, H-8β), 2.78 (1H, d, *J*=18.5Hz, H-11 pro R), 2.36 (1H, d, *J*=18.5Hz, H-11 pro S), 4.98 (1H, d, *J*= 2.4, H-14), 6.61 (1H, d, *J*=2.3,H-15), 4.31(1H, d, *J*=10.7Hz, H-16 pro R), 4.46 (1H, d, *J*=10.7Hz, H-16 pro S), 0.71(3H, d, *J*=6.3Hz, H-17), 1.31 (3H, S, H-18), 1.06(3H ,S, H-20)); ^{13}C NMR (100 MHz $CDCl_3$): δ_C 28.58 (C-1), 17.99 (C-2), 28.27 (C-3), 44.22 (C-4), 44.64 (C-5), 75.88 (C-6), 31.44 (C-7), 32.20 (C-8), 84.84 (C-9), 38.83 (C-10), 40.32 (C-11), 212.76 (C-12), 92.96 (C-13), 102.92 (C-14), 151.57 (C-15), 78.18 (C-16), 15.90 (C-17), 22.76 (C-18), 183.57(C-19), 23.96 (C-20); ESI-MS m/z 345.2$[M-H]^-$ (cal. for $C_{20}H_{26}O_5$, m/z 346.42).

Compound 3: Ladanein

yellow powder, ^{1}H NMR (400 MHz DMSO): δ_H 3.94 (3H, s, OMe-7), 3.87 (3H, s, OMe-4′), 6.95 (1H, s, H-8), 6.89 (1H,s, H-3), 7.13 (2H,d, *J*= 8.9, H-3′, H-5′), 8.07 (2H, *J*= 8.9, H-2′, H-6′), 12.60(1H,s, OH); ^{13}C NMR (100 MHz DMSO): δ_C 55.63 (OMe-7), 56.38 (OMe-4′), 91.26 (C-8), 103.19 (C-3), 105.17 (C-10), 114.65 (C-5′ and C-3′), 123.04 (C-1′), 128.30 (C-2′ and C-6′), 130.06 (C-6), 146.24 (C-9), 149.76 (C-5), 154.50 (C-7), 162.35 (C-4′), 163.43 (C-2), 182.29 (C-4). ESI-MS m/z 315.10 $[M+H]^+$, m/z 313$[M-H]^-$ (cal. for $C_{17}H_{14}O_6$, m/z 314.29).

Compound 4: Apigenin 7-O-β-D (6''- p- coumaroyl) glucoside

White powder, 1H NMR (400 MHz CDCl3,CD_3OD): δ_H 3.83 (3H,m ,H-2'',3'',4''), 4.16 (H, dd, *J*= 4Hz and 12Hz, H-6a''), 4.46 (1H, d, *J*= 12Hz, H-6b''), 5.15 (1H, d, *J*=8Hz, H-1''), 5.26 (1H, brs, 2''-OH), 5.38 (1H, brs,3''-OH), 5.51 (1H, brs,4''-OH), 6.32 (1H, d, *J*=16Hz, H-2'''), 6.47 (1H, d, *J*=1.9Hz, H-8), 6.66 (2H, d, *J*=8Hz, H-6'''and8'''), 6.81 (1H, s , H-3), 6.92 (1H, d, *J*=8Hz, H-3' and 5'), 7.35 (1H, d, *J*=8Hz, H-5'' and 9'''), 7.48 (1H,d, *J*=16Hz, H-3'''), 7.93 (2H, d, *J*=8Hz, H-2' and 6'), 12.95 (1H, s, 5-OH) ^{13}C NMR (100 MHz $CDCL_3$, CD_3OD): δ_C 162.72 (C-2), 103.04 (C-3), 181.98 (C-4), 156.92 (C-5), 99.52 (C-6), 164.30 (C-7), 94.75 (C-8), 161.38 (C-9), 106.40 (C-10), 121.01 (C-1'), 128.55 (C-2'), 116.02 (C-3'), 161.16 (C-4'), 116.02 (C-5'), 128.55 (C-6'), 99.52 (C-1''), 73.86 (C-2''), 76.26 (C-3''), 70.06 (C-4''), 73.86 (C-5''), 63.45 (C-6''), 166.46 (C-1'''), 113.76 (C-2'''), 144.93 (C-3'''), 124.92 (C-4'''), 130.08 (C-5''' and-9'''), 115.69 (C-6'''and-8'''), 159.79 (C-7''') ESI-MS m/z 579.19 $[M+H]^+$, (cal. for $C_{30}H_{26}O_{12}$, m/z 578.53).

Compound 5: Oleic acid

White powder, ^{1}H NMR (400 MHz $CDCl_3$): δ_H 5.35 (2H, m), 2.34 (2H, t, J=7.4), 2.02 (4H, m), 1.63 (2H, m), 1.26-1.32 (20H, m), 0.88 (3H, t, *J*=6.4); ^{13}C NMR (100 MHz $CDCl_3$): δ_C 179.87 (C-1), 130.13 (C-9), 129.83 (C-10), 34.13 (C-2), 32.02 (C-16), 29.87 (C-7), 29.78 (C-12), 29.68 (C-15), 29.62 (C-5), 29.53 (C-14), 29.33 (C-4), 29.23 (C-6), 29.16 (C-13), 27.32 (C-8), 27.26 (C-11), 24.78 (C-3), 22.78 (C-17), 14.18 (C-18); ESI-MS m/z 281$[M-H]^-$ (cal. for $C_{18}H_{34}O_2$, m/z 282.47).

Compound 6: Palmitic acid

White powder, ^{1}H NMR (400 MHz CDCl3): δ_H 9.00 (OH), 2.33 (2H, d, *J*=7.5Hz), 1.61 (2H, m), 1.24 (24 H, m), 0.86 (3H,t, *J*=6.6 Hz); ^{13}C NMR (100 MHz CDCl3): δ_C 178.91 (C-1), 34.06 (C-2), 32.15 (C-3), 29.92 (C-4), 29.91 (C-5), 29.89 (C-6), 29.87 (C-7), 29.81 (C-8), 29.72 (C-9), 29.66 (C-10), 29.58 (C-11), 29.46 (C-12), 29.30 (C-13), 24.78 (C-14), 22.91 (C-15), 14.31 (C-16); ESI-MS m/z 279.40$[M+K]^+$ (cal. for $C_{16}H_{32}O_2$, m/z 256.42).

4.1 HPLC quantification of isolated compounds

With isocratic and gradient methods, identification of the isolated compounds in methanolic extracts as well as their fractions (Hx fr, Chf and Aq fr) was done. Confirmation of the presence of all the six compounds (Figure 1) was achieved by having the same retention times as that of standard marrubiin (Ret. Time=23.202), marrubinone B (Ret Time= 31.771), ladenein (Ret Time= 29.560), Apigenin 7-O-β-D (6''- p- coumaroyl) glucoside (Ret. Time=23.155), oleic acid (Ret. Time= 3.440) and Palmitic acid (Ret. Time =9.927). Quantitative estimation of isolated compounds present in chloroform fraction (Fig 2) through HPLC is given in table 1. (Concentration of isolated compounds in Mex, Hex fr and Aq fr is provided as supplementary file 2 Fig S2 (A-F))

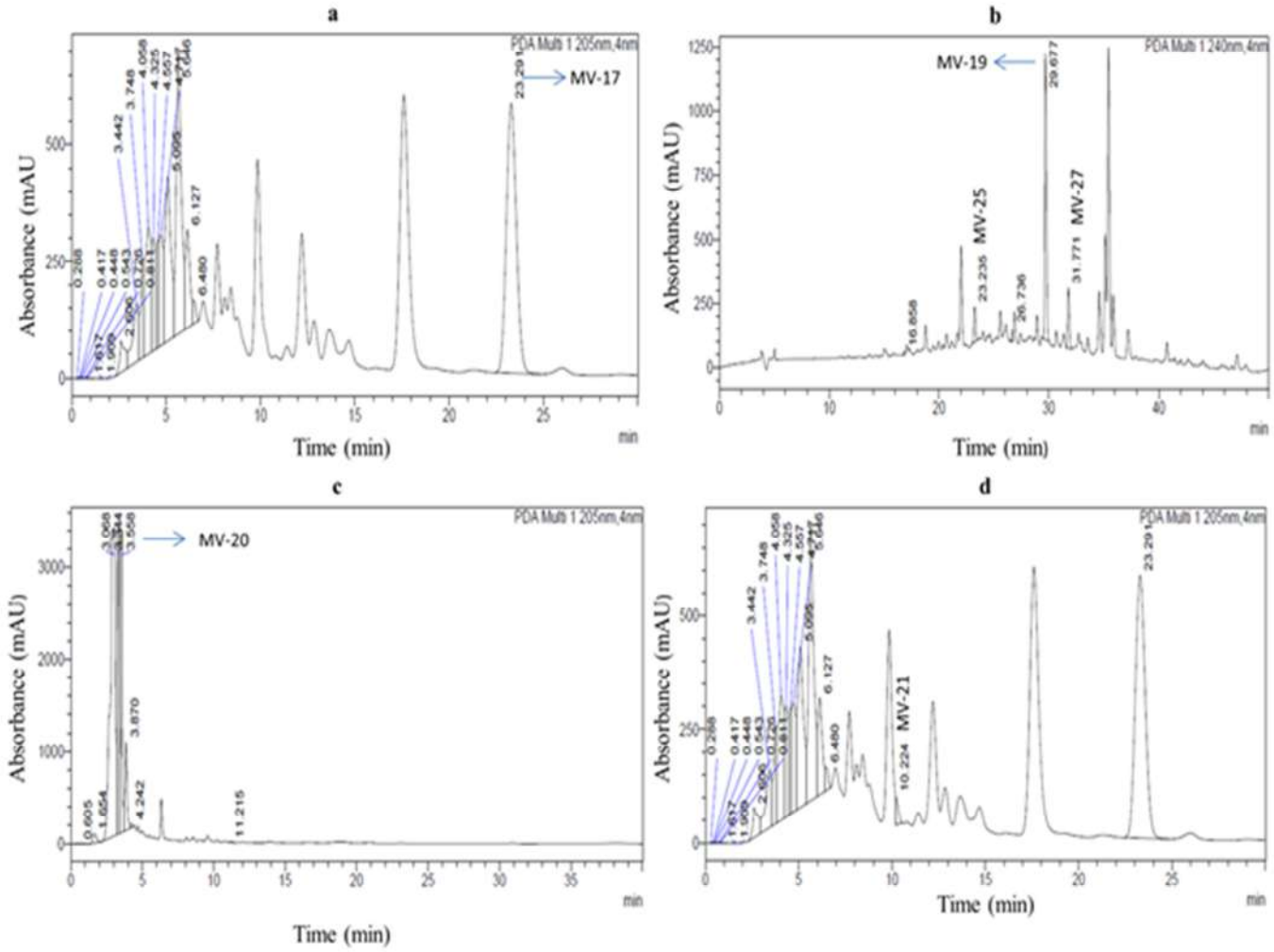

Fig 2: UFLC-DAD chromatogram showing presence of (a) Marrubiin (MV-17), (b) Marrubinone B (MV-27), Ladanein (MV-19) and Apigenin 7-O-β-D (6"- p- coumaroyl) glucoside (MV-25), (c) Oleic acid (MV-20), (d) Palmitic acid (MV-21) in Chloroform fraction (IIIM-2) of *M. vulgare* leaf.

Table 1: *Quantitative estimation (HPLC) of isolated compounds in M. vulgare leaf*

		Concentration in mg/g			
Code	**Compound**	**Methanol**	**Hexane**	**Chloroform**	**Aqueous**
MV-17	Marrubiin	0.08	0.75	80.53	0
MV-27	Marrubinone B	8.88	19.6	33.48	0.08
MV-19	Ladanein	5.77	5.92	42.13	0.05
MV-25	Apigenin 7-O-β-D (6"- p- coumaroyl) glucoside	11.94	2.9	5.99	4.51
MV-20	Oleic acid	40.6	12.28	71.89	0
MV-21	Palmitic acid	0.16	0.21	1.92	1.24

Amount of each compound in mg/g of extract was calculated from concentration in 10 µl injection volume determined by HPLC

4.2 Antioxidant activity

DPPH (1,1-diphenyl-2-picrylhydrazyl) radical scavenging activity of *M. vulgae* leaf extract and its fractions being represented in fig. 3 showed highest % of inhibition capacity exhibited by chloroform fraction (85.12 %). All the samples showed concentration-dependent increase in radical scavenging capacity with highest IC_{50} inhibition was recorded in chloroform fraction (308.20 µg/mL). The IC_{50} values of methanol extract and its fractions compared with standard ascorbic acid is presented in table 2.

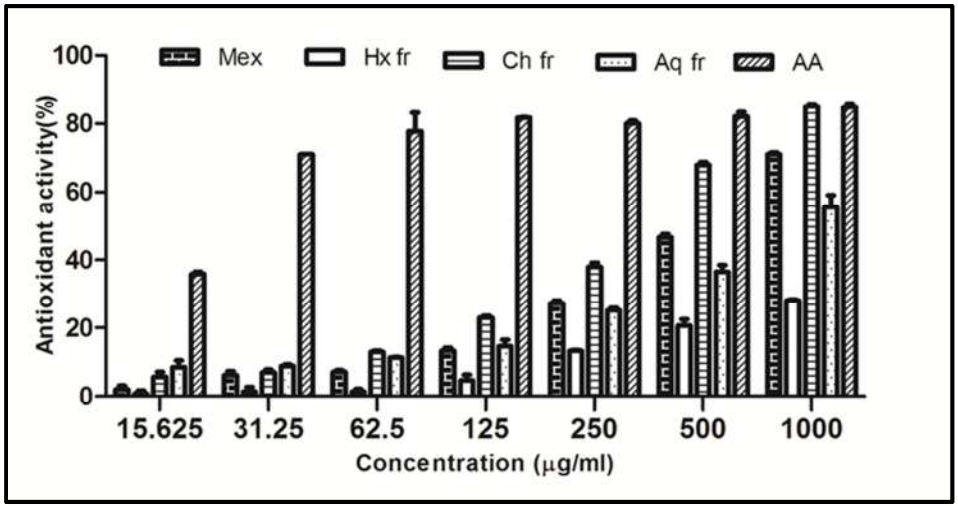

Fig 3: DPPH radical-scavenging activity of the root extract and its fractions. Data was analyzed using a two-way ANOVA (analysis of variance). Significant difference ($p<0.05$) was observed.

Table 2: ***Inhibitory concentration 50 (IC_{50} in µg/mL) of M. vulgare leaf samples and standard***

Extract/fraction	IC_{50} (in µg/mL)
Methanol	529.03
Hexane	>1000
Chloroform	308.20
Aqueous	890.90
Ascorbic acid[a]	15.85

[a]Ascorbic acid was used as standard

4.3 Antidiabetic Activity

The effect of *M. vulgare* Methanol extract (500 mg/kg) its chloroform fraction (250 mg/kg) and standard metformin (500 mg/kg) on variations in blood glucose in normal control group, citrate buffer, diabetic control, Metformin and plant extracts (IIIM-1 and IIM-2) treatment groups are shown in Fig. 4. The blood glucose level (Table **3)** significantly increases ($p<0.001$) in diabetic rats compared to normal rats. After oral administration of metformin and extracts (IIIM-1 and 2) for 15 days there was significant reduction in the blood glucose level compared with diabetic control rats (DM control). Both IIIM-1 (500mg/kg) and IIIM-2 (250 mg/kg) caused a significant (***$p<0.001$) decrease in blood glucose level compared to diabetic control at the end of 15thday. It is evident from the table that diabetic control rats had elevated blood glucose level and methanol extract (IIIM-1) and chloroform fraction (IIIM-2) were able to improve the metabolism significantly by comparing with the untreated rats see Fig. 4**.**

Table 3: ***Effect of Methanolic leaf extract (IIIM-1) and Chloroform fraction (IIIM-2) of M. vulgare on blood glucose level of STZ-induced diabetic rats after 15 days treatment***

Group	Day5 post STZ	Day 8	Day15
Normal Control	99.5±4.0	149.6±7.3	92.1±6.8
Citrate buffer	106.0±3.4	134.4±7.4	94.0±7.5
DM Control (Vehicle)	535.9±59.1#	685.8±47.2#	614.8±13.8#
Metformin	383.6±16.1	623.8±58.7	250.8±67.6 ***
IIIM-2	461.4±79.2	451.8±98.0 ***	83.6±14.5 ***
IIIM-1	556.6±40.4	495.0±65.0 *	83.7±16.5 ***

All the values are expressed as mean ±SEM n=6 for each group Data analyzed by ONE WAY ANOVA followed by Bonferroni's post hoc test #P<0.001 : significant difference from diabetic control from normal control. ***P<0.001. Very highly significant; **p<0.01 Highly significant; *p<0.05 Significant compared with respective diabetic control

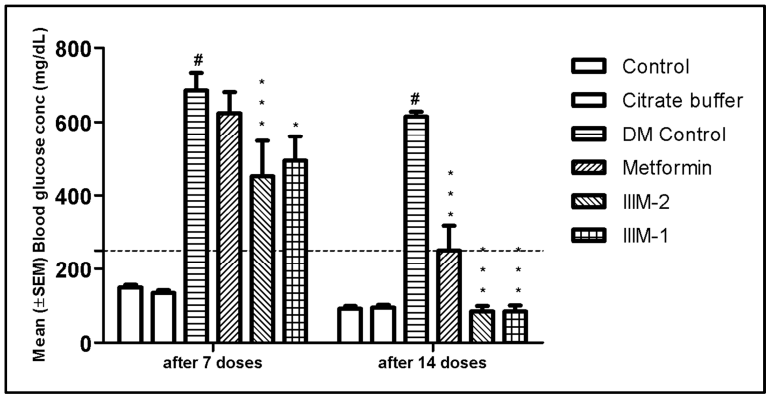

Fig 4: Blood glucose levels of diabetic rats treated with *M.vulgare* extracts (IIIM-1, IIIM-2) or vehicle or metformin. # P<0.001 compared to normal, ***P<0.001, *P<0.05, compared to DM control

4.4 Quantitative estimation of isolated compounds in M. vulgare leaf samples

<< PDA >>
ID#1 Compound Name: MV17

Title	Sample Name	Ret. Time	Area	Height	Conc.	Injection Volume
28-01-21AMIT026.lcd	M1	22.721	19263	635	0.015	10.000000
28-01-21AMIT027.lcd	M2	23.342	197988	5898	0.150	10.000000
28-01-21AMIT028.lcd	M3	23.291	21324525	578662	16.106	10.000000
28-01-21AMIT029.lcd	M4	0.000	0	0	0.000	10.000000
28-01-21AMIT030.lcd	M5	0.000	0	0	0.000	10.000000
28-01-21AMIT031.lcd	M6	0.000	0	0	0.000	10.000000
28-01-21AMIT032.lcd	M7	0.000	0	0	0.000	10.000000
28-01-21AMIT033.lcd	M8	0.000	0	0	0.000	10.000000
28-01-21AMIT034.lcd	M9	0.000	0	0	0.000	10.000000
28-01-21AMIT035.lcd	M10	0.000	0	0	0.000	10.000000
28-01-21AMIT036.lcd	M11	0.000	0	0	0.000	10.000000
28-01-21AMIT037.lcd	M12	0.000	0	0	0.000	10.000000
28-01-21AMIT038.lcd	M13	0.000	0	0	0.000	10.000000
Average		23.118	7180592	195065	5.423	
%RSD		1.490	170.589	170.310	170.589	
Maximum		23.342	21324525	578662	16.106	
Minimum		22.721	19263	635	0.015	
Standard Deviation		0.345	12249331	332215	9.251	

(A)

ID#4 Compound Name: MV 19

Title	Sample Name	Ret. Time	Area	Height	Conc.	Injection Volume
07-12-20AMIT069.lcd	M1	29.686	1789132	162684	1.154	10.000000
07-12-20AMIT070.lcd	M2	29.678	1833807	146178	1.183	10.000000
07-12-20AMIT071.lcd	M3	29.677	12852914	1116842	8.426	10.000000
07-12-20AMIT072.lcd	M4	29.687	46793	4433	0.009	10.000000
07-12-20AMIT073.lcd	M5	29.657	553075	41823	0.342	10.000000
07-12-20AMIT074.lcd	M6	29.699	220314	19111	0.123	10.000000
07-12-20AMIT075.lcd	M7	29.673	267374	28204	0.154	10.000000
07-12-20AMIT076.lcd	M8	29.672	11765	1306	-0.014	10.000000
07-12-20AMIT077.lcd	M9	29.415	217646	10681	0.121	10.000000
07-12-20AMIT078.lcd	M10	29.747	128198	7320	0.062	10.000000
07-12-20AMIT079.lcd	M11	0.000	0	0	0.000	10.000000
07-12-20AMIT080.lcd	M12	29.673	247444	26902	0.141	10.000000
07-12-20AMIT081.lcd	M13	0.000	0	0	0.000	10.000000
Average		29.660	1651678	142317	1.064	
%RSD		0.286	228.395	230.510	233.120	
Maximum		29.747	12852914	1116842	8.426	
Minimum		29.415	11765	1306	-0.014	
Standard Deviation		0.085	3772348	328055	2.479	

(B)

<< PDA >>
ID#1 Compound Name: MV20

Title	Sample Name	Ret. Time	Area	Height	Conc.	Injection Volume
29-01-21AMIT006.lcd	M1	3.310	13953713	1130665	8.120	10.000000
29-01-21AMIT007.lcd	M2	3.445	4219726	351902	2.455	10.000000
29-01-21AMIT008.lcd	M3	3.558	24706332	3289843	14.377	10.000000
29-01-21AMIT009.lcd	M4	0.000	0	0	0.000	10.000000
29-01-21AMIT010.lcd	M5	3.304	5832576	689794	3.394	10.000000
29-01-21AMIT011.lcd	M6	3.458	3604843	506489	2.098	10.000000
29-01-21AMIT012.lcd	M7	3.311	7422794	483501	4.319	10.000000
29-01-21AMIT013.lcd	M8	3.308	1564279	97113	0.910	10.000000
29-01-21AMIT014.lcd	M9	3.475	3271905	363177	1.904	10.000000
29-01-21AMIT015.lcd	M10	3.470	6216486	897505	3.617	10.000000
29-01-21AMIT016.lcd	M11	3.312	6499317	806205	3.782	10.000000
29-01-21AMIT017.lcd	M12	3.463	5834513	809373	3.395	10.000000
29-01-21AMIT018.lcd	M13	0.000	0	0	0.000	10.000000
Average		3.401	7556953	856870	4.397	
%RSD		2.729	86.260	100.167	86.260	
Maximum		3.558	24706332	3289843	14.377	
Minimum		3.304	1564279	97113	0.910	
Standard Deviation		0.093	6518616	858303	3.793	

(C)

<< PDA >>
ID#1 Compound Name: MV21

Title	Sample Name	Ret. Time	Area	Height	Conc.	Injection Volume
28-01-21AMIT026.lcd	M1	10.064	43037	80	0.031	10.000000
28-01-21AMIT027.lcd	M2	10.219	58427	3933	0.042	10.000000
28-01-21AMIT028.lcd	M3	10.224	537939	60301	0.384	10.000000
28-01-21AMIT029.lcd	M4	10.095	346600	8159	0.247	10.000000
28-01-21AMIT030.lcd	M5	10.217	1969	360	0.001	10.000000
28-01-21AMIT031.lcd	M6	10.197	301731	14019	0.215	10.000000
28-01-21AMIT032.lcd	M7	9.853	410851	20889	0.293	10.000000
28-01-21AMIT033.lcd	M8	0.000	0	0	0.000	10.000000
28-01-21AMIT034.lcd	M9	10.123	92530	4642	0.066	10.000000
28-01-21AMIT035.lcd	M10	10.104	167842	5674	0.120	10.000000
28-01-21AMIT036.lcd	M11	10.095	239502	14449	0.171	10.000000
28-01-21AMIT037.lcd	M12	10.110	744555	42234	0.531	10.000000
28-01-21AMIT038.lcd	M13	0.000	0	0	0.000	10.000000
Average		10.118	267726	15885	0.191	
%RSD		1.046	86.365	119.800	86.365	
Maximum		10.224	744555	60301	0.531	
Minimum		9.853	1969	80	0.001	
Standard Deviation		0.106	231220	19031	0.165	

(D)

ID#2 Compound Name: MV 25

Title	Sample Name	Ret. Time	Area	Height	Conc.	Injection Volume
07-12-20AMIT069.lcd	M1	23.243	3197696	303739	2.387	10.000000
07-12-20AMIT070.lcd	M2	23.238	771371	75904	0.580	10.000000
07-12-20AMIT071.lcd	M3	23.235	1599334	139724	1.197	10.000000
07-12-20AMIT072.lcd	M4	23.236	1204425	100740	0.902	10.000000
07-12-20AMIT073.lcd	M5	23.226	1029234	117118	0.772	10.000000
07-12-20AMIT074.lcd	M6	23.241	72478	5932	0.060	10.000000
07-12-20AMIT075.lcd	M7	23.238	2236642	240153	1.671	10.000000
07-12-20AMIT076.lcd	M8	23.241	122617	13494	0.097	10.000000
07-12-20AMIT077.lcd	M9	23.240	87926	10666	0.071	10.000000
07-12-20AMIT078.lcd	M10	23.236	27173	3372	0.026	10.000000
07-12-20AMIT079.lcd	M11	23.218	308575	34809	0.235	10.000000
07-12-20AMIT080.lcd	M12	23.182	1029900	121372	0.773	10.000000
07-12-20AMIT081.lcd	M13	0.000	0	0	0.000	10.000000
Average		23.231	973947	97252	0.731	
%RSD		0.074	101.229	98.953	100.458	
Maximum		23.243	3197696	303739	2.387	
Minimum		23.182	27173	3372	0.026	
Standard Deviation		0.017	985922	96234	0.734	

(E)

ID#5 Compound Name: MV 27

Title	Sample Name	Ret. Time	Area	Height	Conc.	Injection Volume
07-12-20AMIT069.lcd	M1	31.784	697004	69204	1.776	10.000000
07-12-20AMIT070.lcd	M2	31.775	1538008	146901	3.920	10.000000
07-12-20AMIT071.lcd	M3	31.771	2626547	237672	6.695	10.000000
07-12-20AMIT072.lcd	M4	31.819	6474	605	0.016	10.000000
07-12-20AMIT073.lcd	M5	31.767	231842	21299	0.590	10.000000
07-12-20AMIT074.lcd	M6	31.788	352137	33249	0.897	10.000000
07-12-20AMIT075.lcd	M7	31.773	173083	14530	0.440	10.000000
07-12-20AMIT076.lcd	M8	31.766	12025	1095	0.030	10.000000
07-12-20AMIT077.lcd	M9	31.786	31919	3125	0.080	10.000000
07-12-20AMIT078.lcd	M10	30.818	2119943	125490	5.403	10.000000
07-12-20AMIT079.lcd	M11	0.000	0	0	0.000	10.000000
07-12-20AMIT080.lcd	M12	31.617	976619	87579	2.489	10.000000
07-12-20AMIT081.lcd	M13	0.000	0	0	0.000	10.000000
Average		31.679	796873	67341	2.030	
%RSD		0.916	115.163	113.262	115.218	
Maximum		31.819	2626547	237672	6.695	
Minimum		30.818	6474	605	0.016	
Standard Deviation		0.290	917705	76272	2.339	

(F)

Fig. S2. HPLC chromatogram showing concentration of (A) Marrubiin, (B) Ladanein, (C) Oleic acid, (D) Palmitic acid, (E) Apigenin 7-O-β-D (6''- p- coumaroyl) glucoside, (F) Marrubinone B in Mex (M1), Hx fr (M2), Ch fr (M3) and Aq fr (M4) *0f M. vulgare* leaf.

4.5 NMR analysis of compound 1 (MV-17)

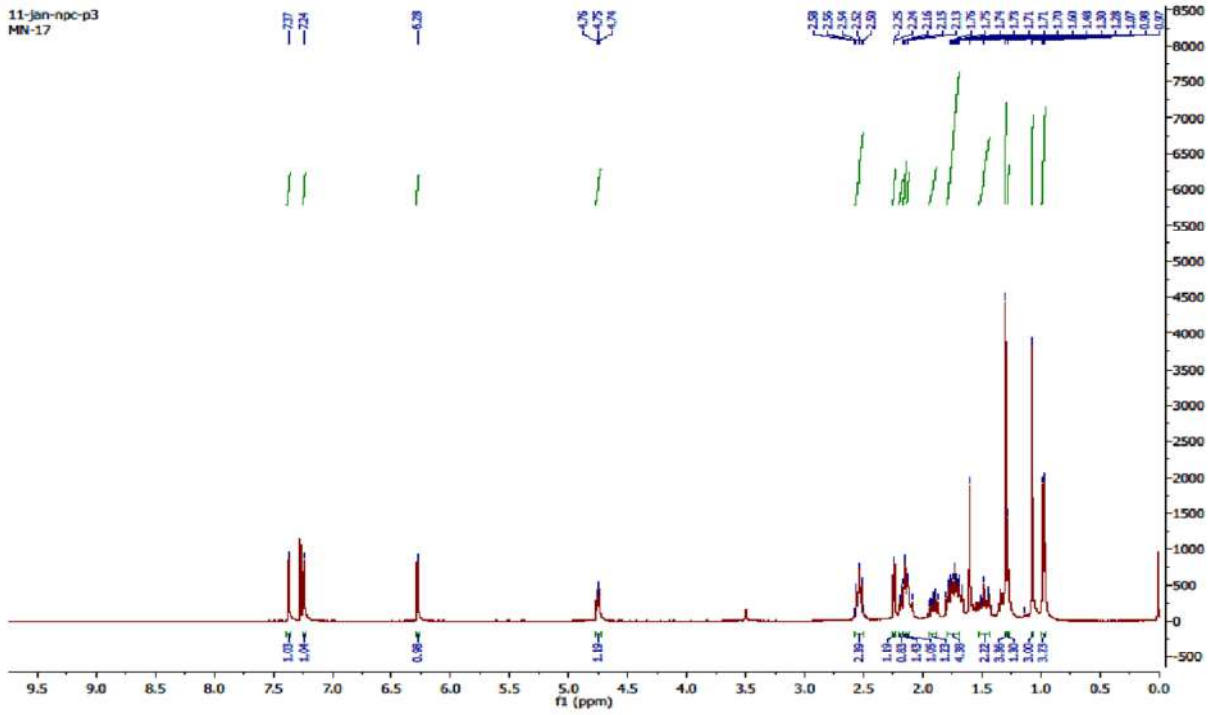

Fig. S1. ^{1}H spectrum of marrubiin (MV-17)

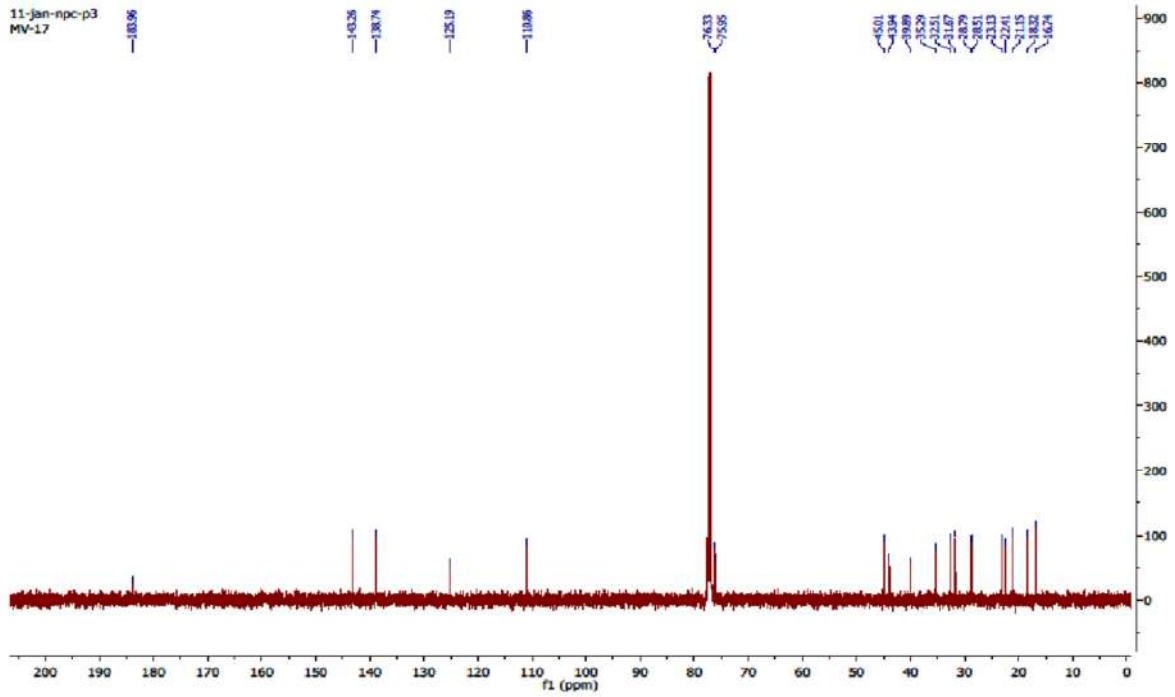

Fig. S2. ^{13}C spectrum of Marrubiin (MV-17)

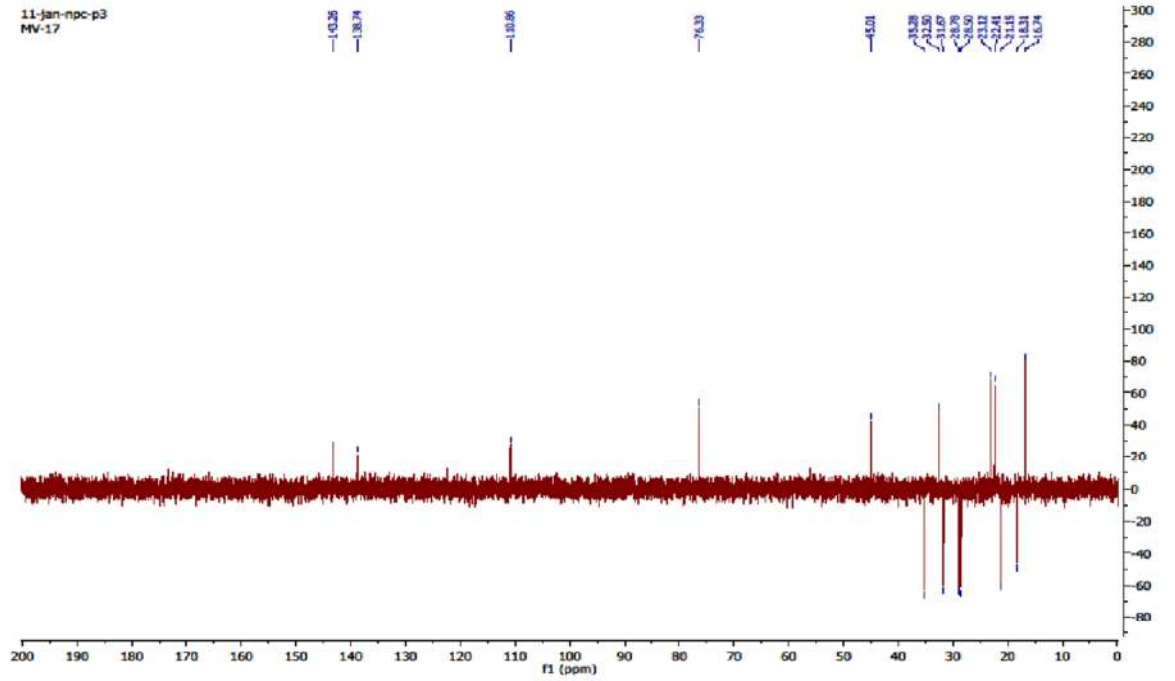

Fig. S3. DEPT of Marrubiin (MV-17)

4.6 HRMS of Marrubiin (MV-17)

Data File	MV-17.d	**Sample Name**	MV-17
Sample Type	Sample	**Position**	Vial 5
Instrument Name	Instrument 1	**User Name**	
Acq Method	vishal_12-01-13.m	**Acquired Time**	30-04-2019 PM 1:16:55
IRM Calibration Status	Success	**DA Method**	Default.m
Comment			

Sample Group	**Info.**
Acquisition SW Version	6200 series TOF/6500 series Q-TOF B.05.01 (B5125)

Compound Table

Compound Label	RT	Mass	Formula	MFG Formula	MFG Diff (ppm)	DB Formula
Cpd 6: C20 H28 O4	0.3	332.1987	C20 H28 O4	C20 H28 O4	0.31	C20 H28 O4

Compound Label	*m/z*	RT	Algorithm	Mass
Cpd 6: C20 H28 O4	333.2058	0.3	Find by Molecular Feature	332.1987

Fig. S4. HRMS of Marrubin (MV-17)

4.7 NMR analysis of compound MV-27

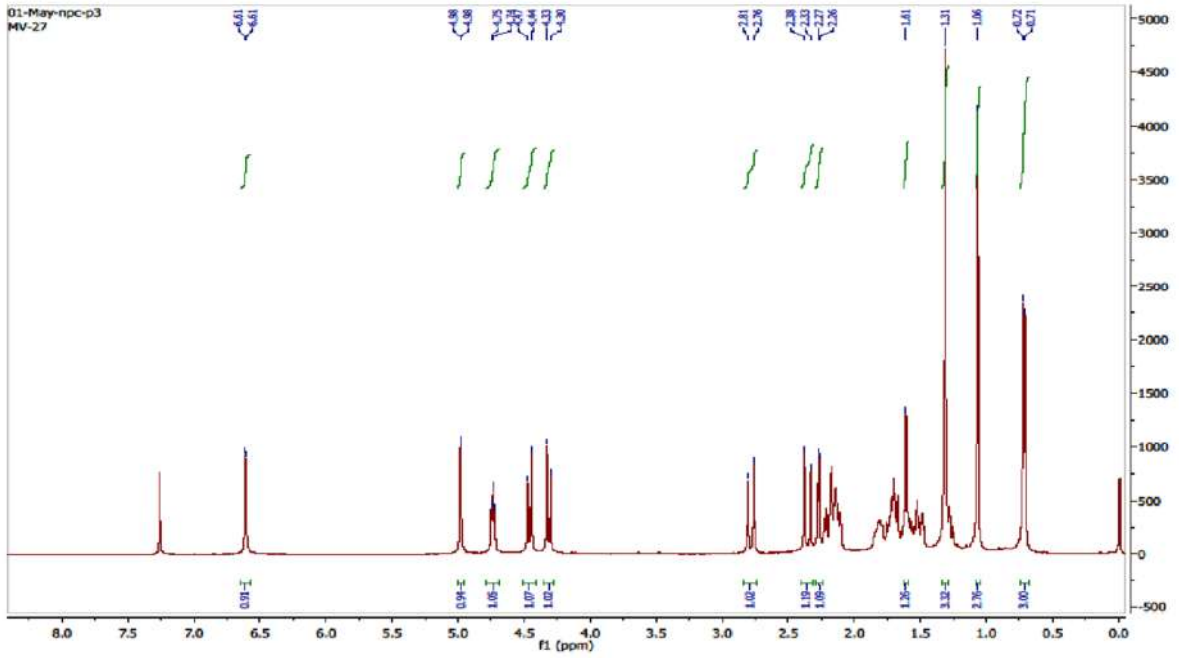

Fig. S5. ^{1}H spectrum of Marrubinone B (MV-27)

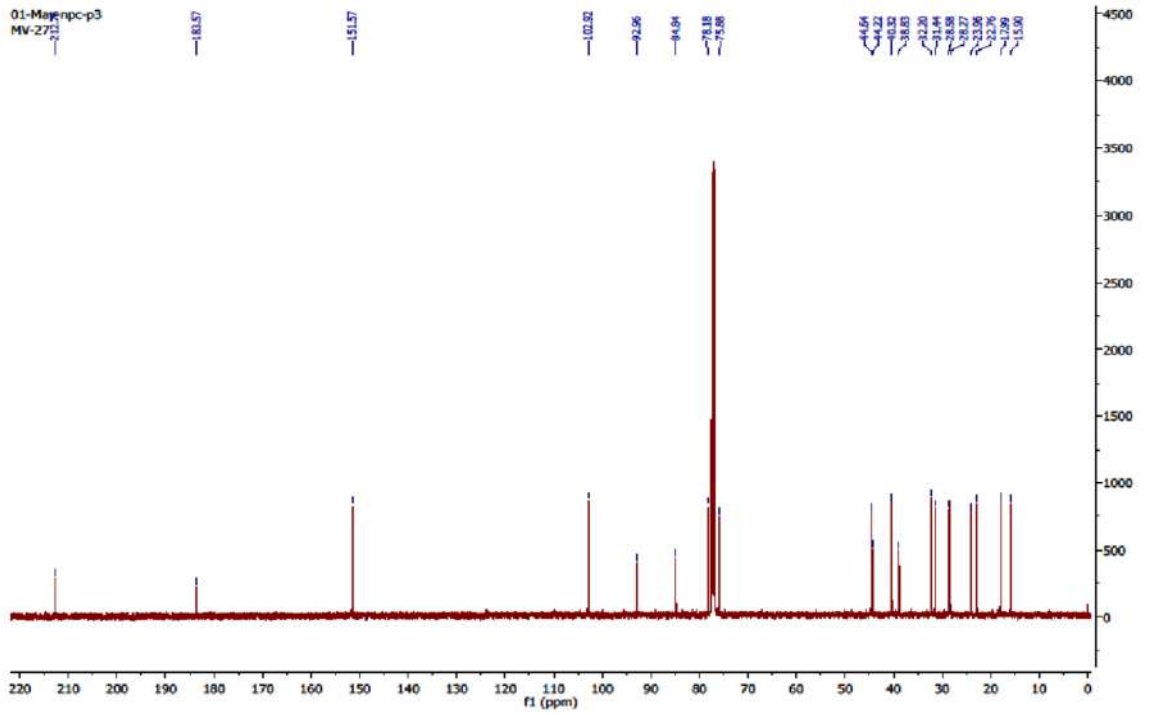

Fig. S6. ^{13}C spectrum of Marrubinone B (MV-27)

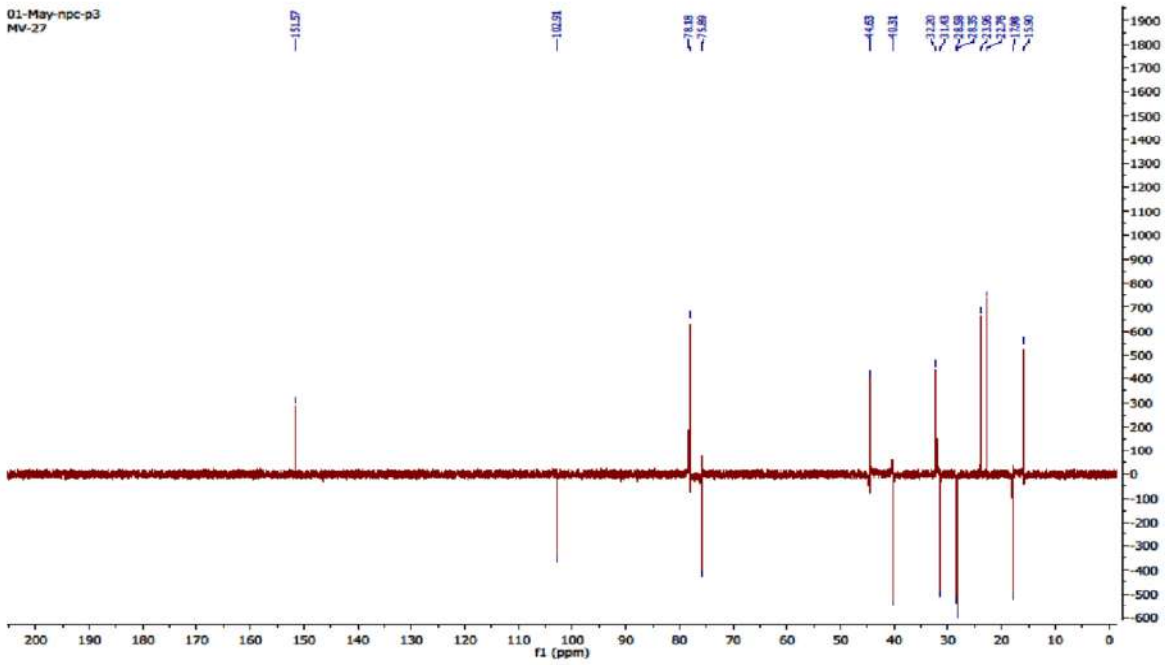

Fig. S7. DEPT of Marrubinone B (MV-27)

4.8 HRMS of Marrubinone B (MV-27)

Data File	MV-27.d	**Sample Name**	MV-27
Sample Type	Sample	**Position**	Vial 4
Instrument Name	Instrument 1	**User Name**	
Acq Method	vishal_12-01-13.m	**Acquired Time**	30-04-2019 PM 1:12:35
IRM Calibration Status	Success	**DA Method**	Default.m
Comment			

Sample Group	**Info.**
Acquisition SW Version	6200 series TOF/6500 series Q-TOF B.05.01 (B5125)

Compound Table

Compound Label	RT	Mass	Formula	MFG Formula	MFG Diff (ppm)	DB Formula
Cpd 8: C20 H26 O5	0.3	346.1774	C20 H26 O5	C20 H26 O5	1.67	C20 H26 O5

Compound Label	*m/z*	RT	Algorithm	Mass
Cpd 8: C20 H26 O5	347.1848	0.3	Find by Molecular Feature	346.1774

Fig. S8. HRMS of Marrubinone B (MV-27)

4.9 NMR analysis of compound MV-19

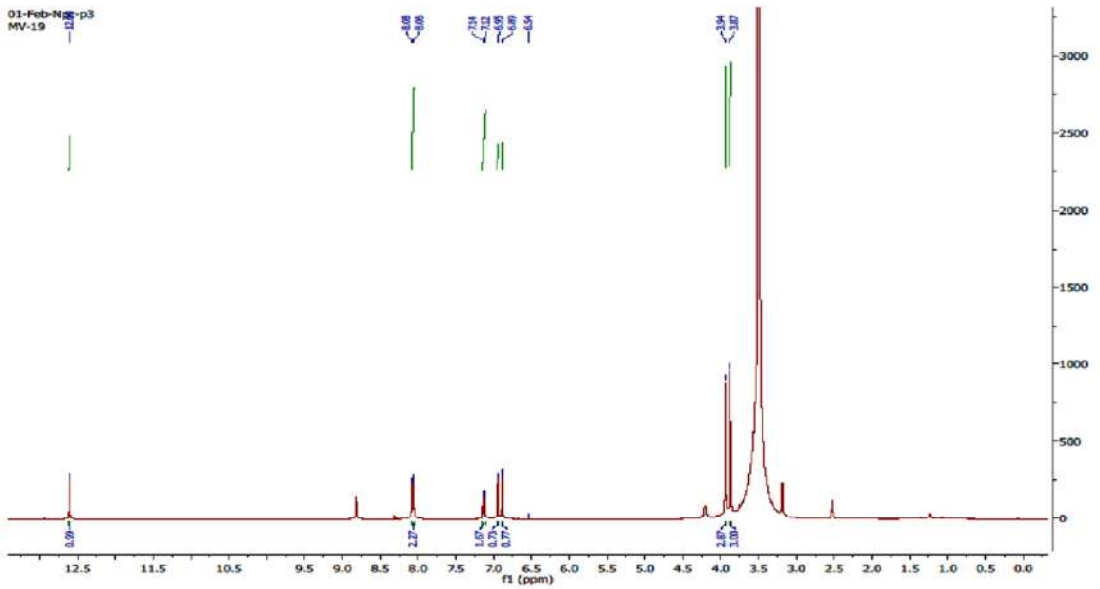

Fig. S9. ^{1}H spectrum of Ladanein (MV-19)

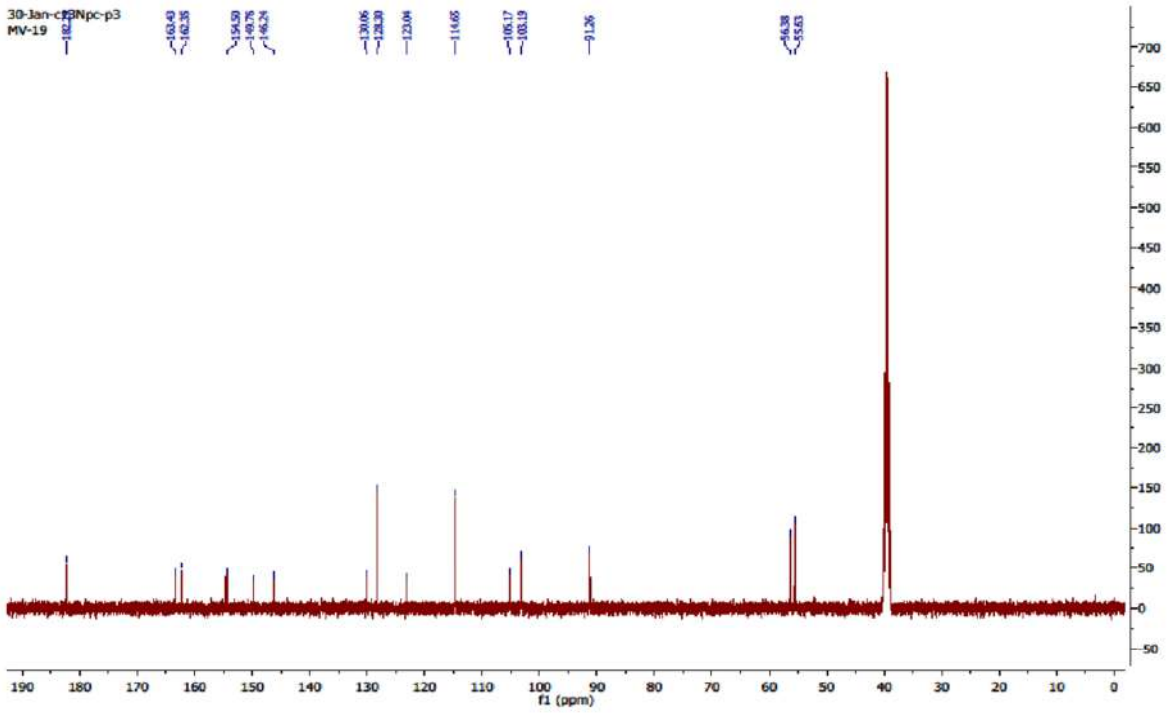

Fig. S10. ^{13}C spectrum of Ladanein (MV-19)

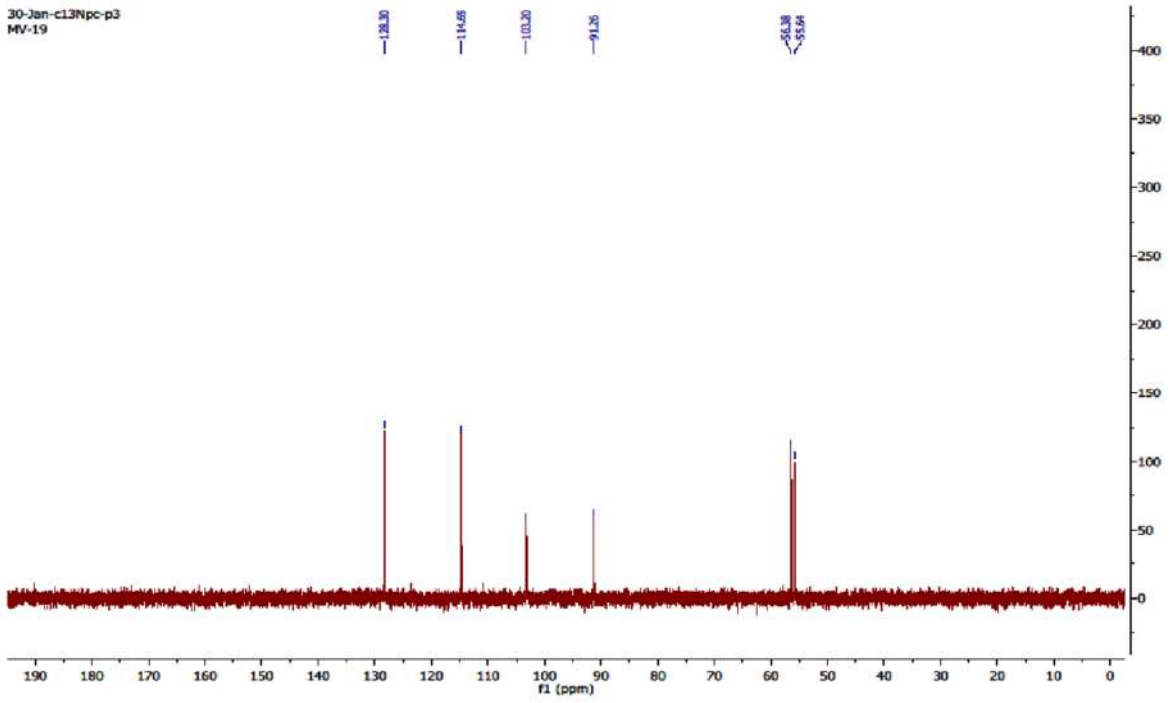

Fig. S11. DEPT of Ladanein (MV-19)

4.10 MS spectrum of Ladanein (MV-19)

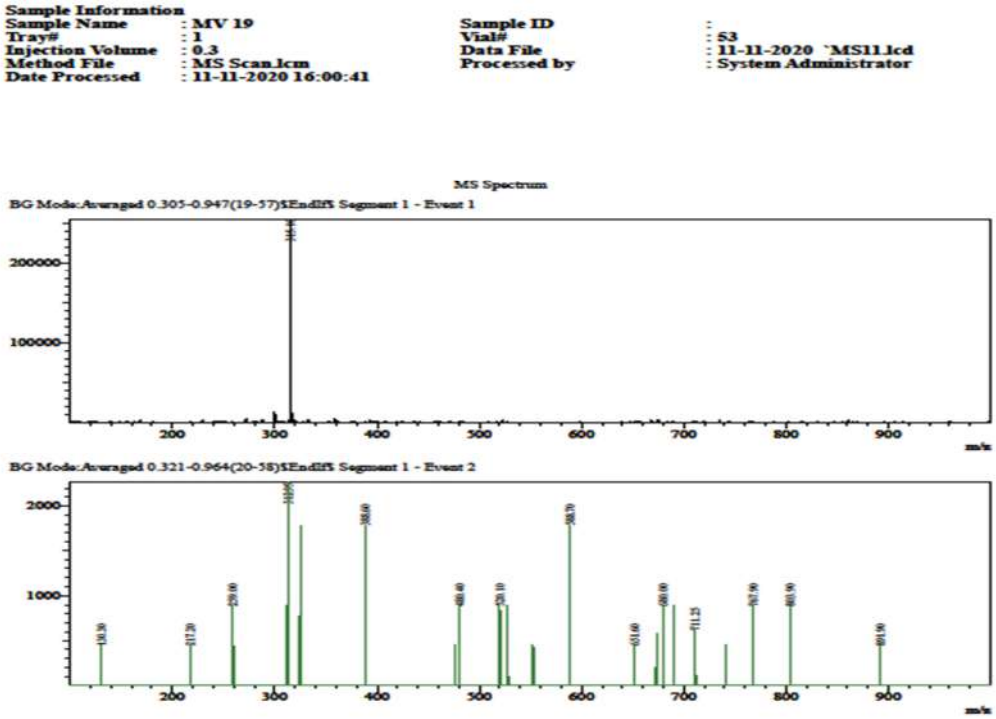

Fig. S12. ESI-MS m/z 315 $[M+H]^+$ m/z 313$[M-H]^-$ (cal. for $C_{17}H_{14}O_6$, m/z 314.29). spectrum of Ladanein (MV-19)

4.11 NMR analysis of compound MV-25

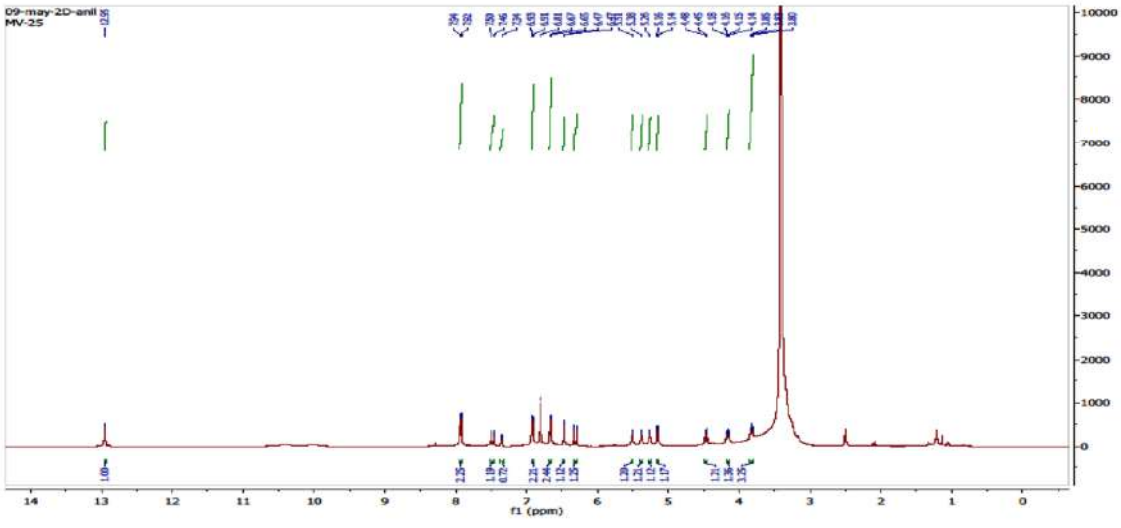

Fig. S13. ^{1}H spectrum of Apigenin 7-O-β-D(6''- p- coumaroyl) glucoside (MV-25)

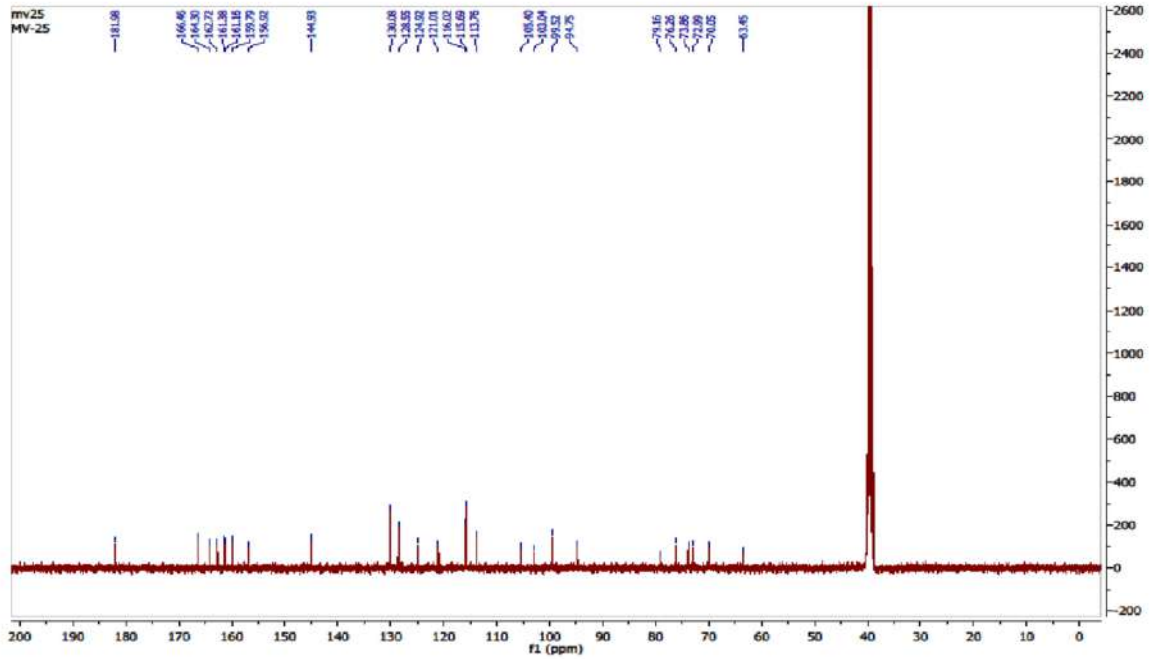

Fig. S14. ^{13}C spectrum of Apigenin 7-O-β-D (6"- p- coumaroyl) glucoside (MV-25)

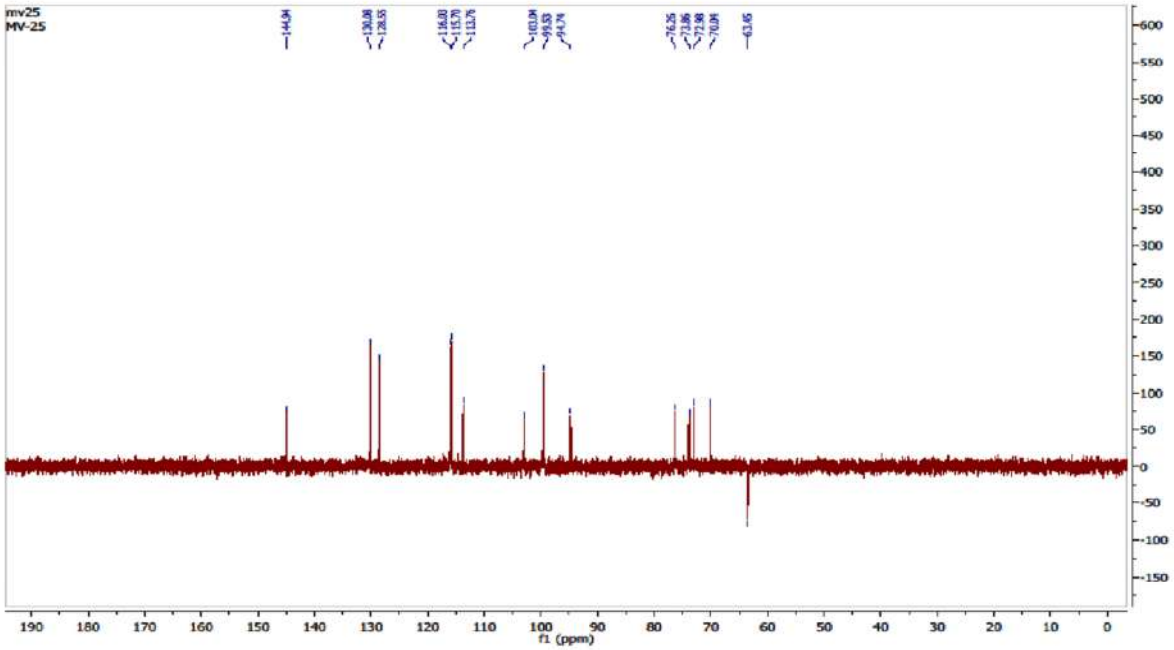

Fig. S15. DEPT of Apigenin 7-O-β-D (6"- p- coumaroyl) glucoside (MV-25)

4.12 HRMS of Apigenin 7-O-β-D (6"- p- coumaroyl) glucoside (MV-25)

Data File	MV-25.d	**Sample Name**	MV-25
Sample Type	Sample	**Position**	Vial 3
Instrument Name	Instrument 1	**User Name**	
Acq Method	vishal_12-01-13.m	**Acquired Time**	30-04-2019 PM 1:08:15
IRM Calibration Status	Success	**DA Method**	Default.m
Comment			

Sample Group	**Info.**
Acquisition SW Version	6200 series TOF/6500 series Q-TOF B.05.01 (B5125)

Compound Table

Compound Label	RT	Mass	Formula	MFG Formula	MFG Diff (ppm)	DB Formula
Cpd 2: C30 H26 O12	0.3	578.1426	C30 H26 O12	C30 H26 O12	-0.26	C30 H26 O12

Compound Label	*m/z*	RT	Algorithm	Mass
Cpd 2: C30 H26 O12	579.1498	0.3	Find by Molecular Feature	578.1426

Fig. S16. HRMS of Apigenin 7-O-β-D (6"- p- coumaroyl) glucoside (MV-25)

4.13 NMR analysis of compound MV-20

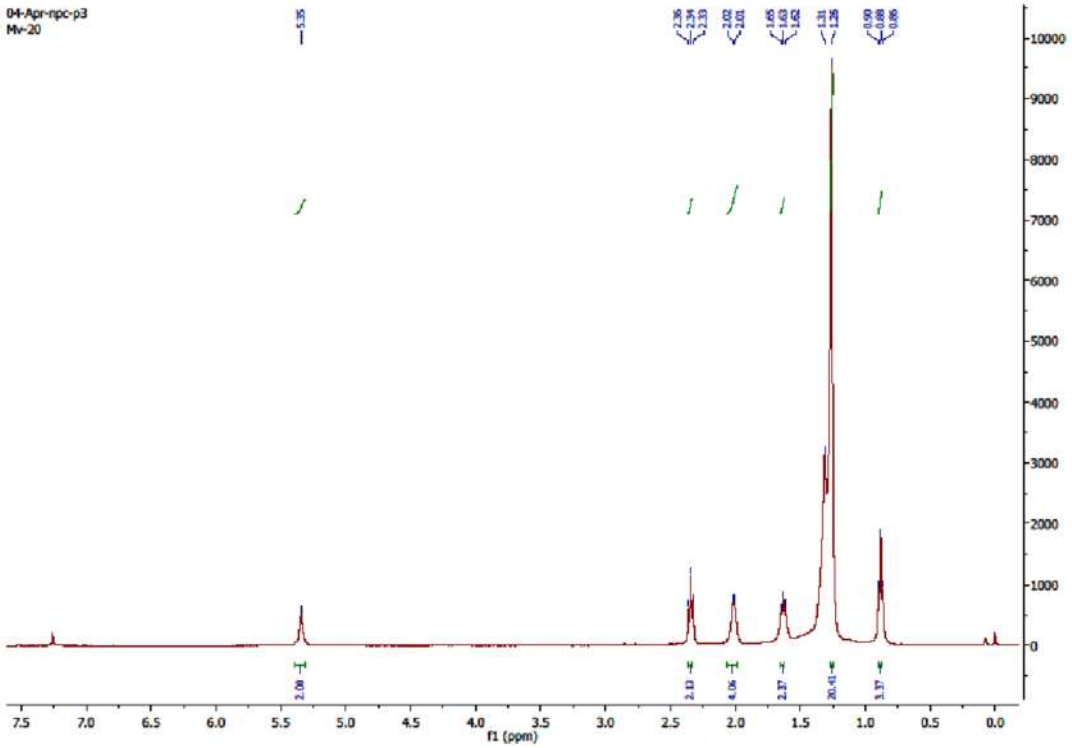

Fig. S17. ^{1}H spectrum of oleic acid

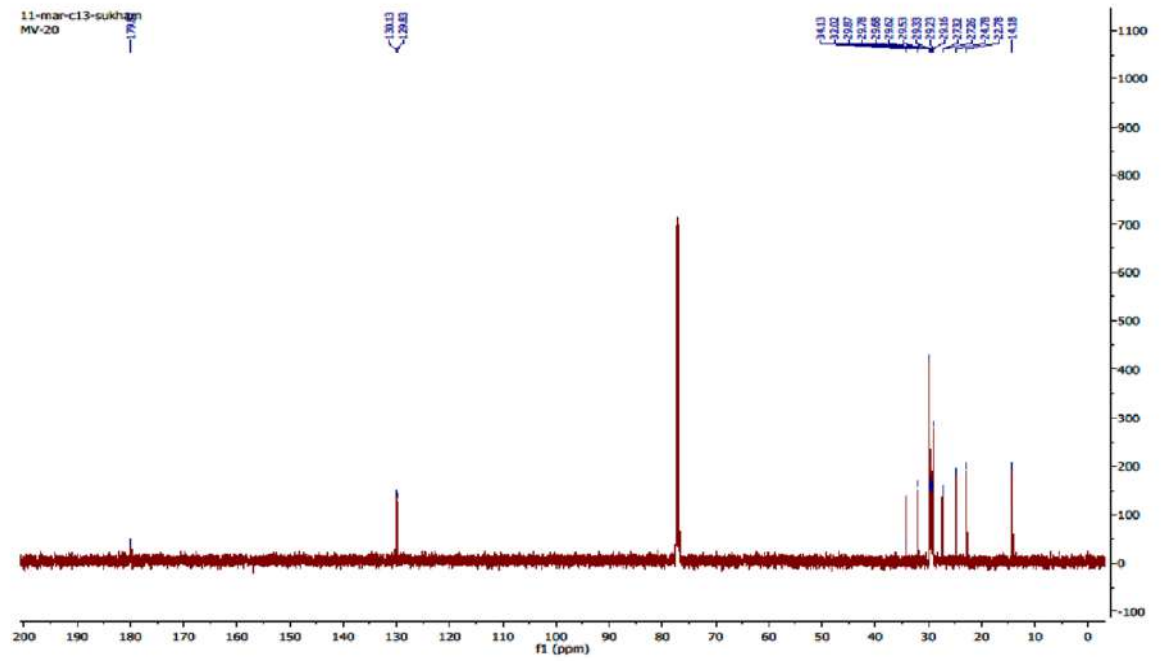

Fig. S18. ^{13}C spectrum of oleic acid

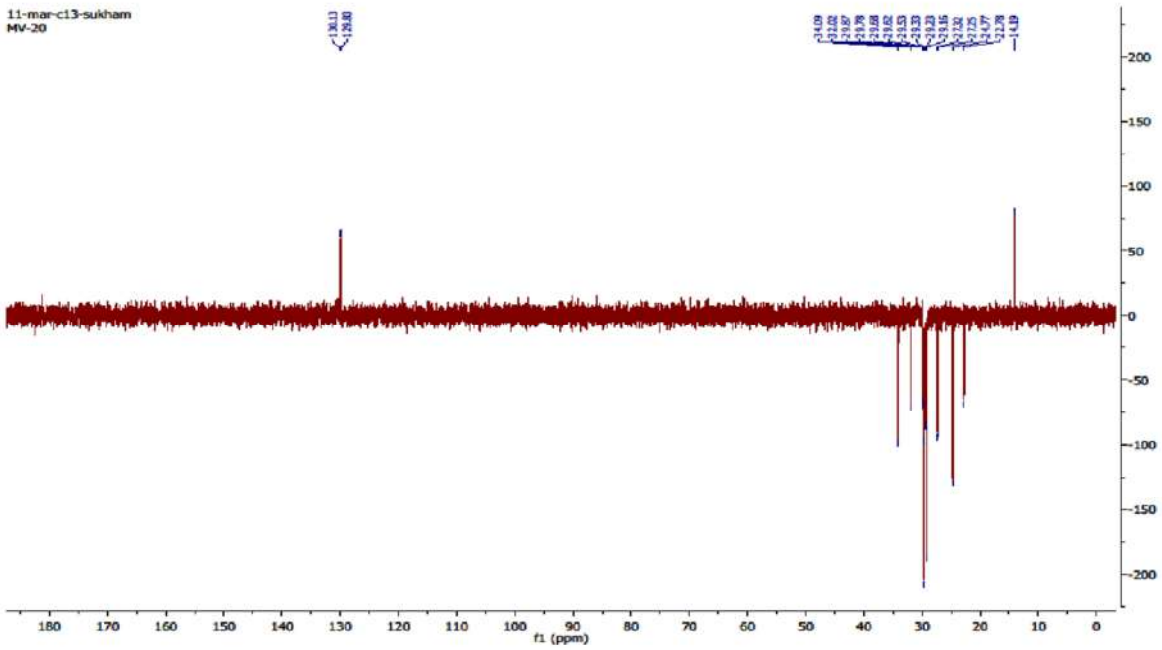

Fig. S19. DEPT of Oleic acid (MV-20)

4.14 HRMS of Oleic acid

Data File	MV-20.d	**Sample Name**	MV-20
Sample Type	Sample	**Position**	Vial 9
Instrument Name	Instrument 1	**User Name**	
Acq Method	vishal_12-01-13.m	**Acquired Time**	22-03-2019 PM 1:15:19
IRM Calibration Status	Success	**DA Method**	Default.m
Comment			

Sample Group	**Info.**
Acquisition SW Version	6200 series TOF/6500 series Q-TOF B.05.01 (B5125)

Compound Table

Compound Label	RT	Mass	Formula	MFG Formula	MFG Diff (ppm)	DB Formula
Cpd 4: C18 H34 O2	0.3	282.2576	C18 H34 O2	C18 H34 O2	-6.14	C18 H34 O2

Compound Label	*m/z*	RT	Algorithm	Mass
Cpd 4: C18 H34 O2	281.2504	0.3	Find by Molecular Feature	282.2576

Fig. S20. HRMS of Oleic acid (MV-20)

4.15 NMR analysis of compound MV-21

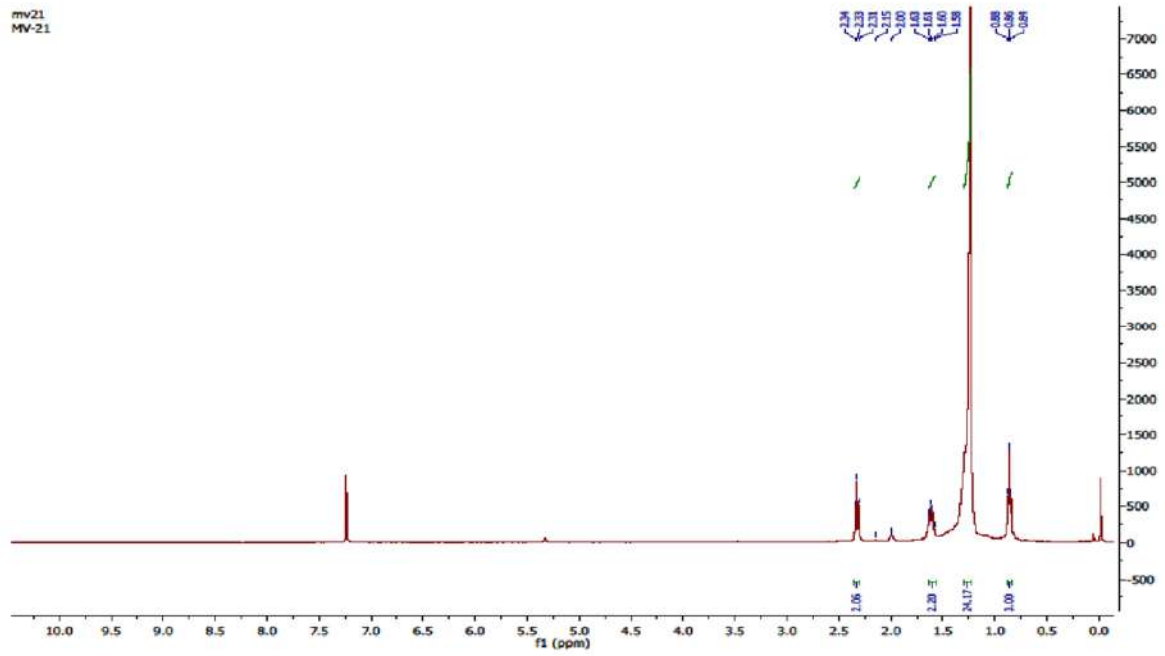

Fig. S21. ^{1}H spectrum of Palmitic acid (MV-21)

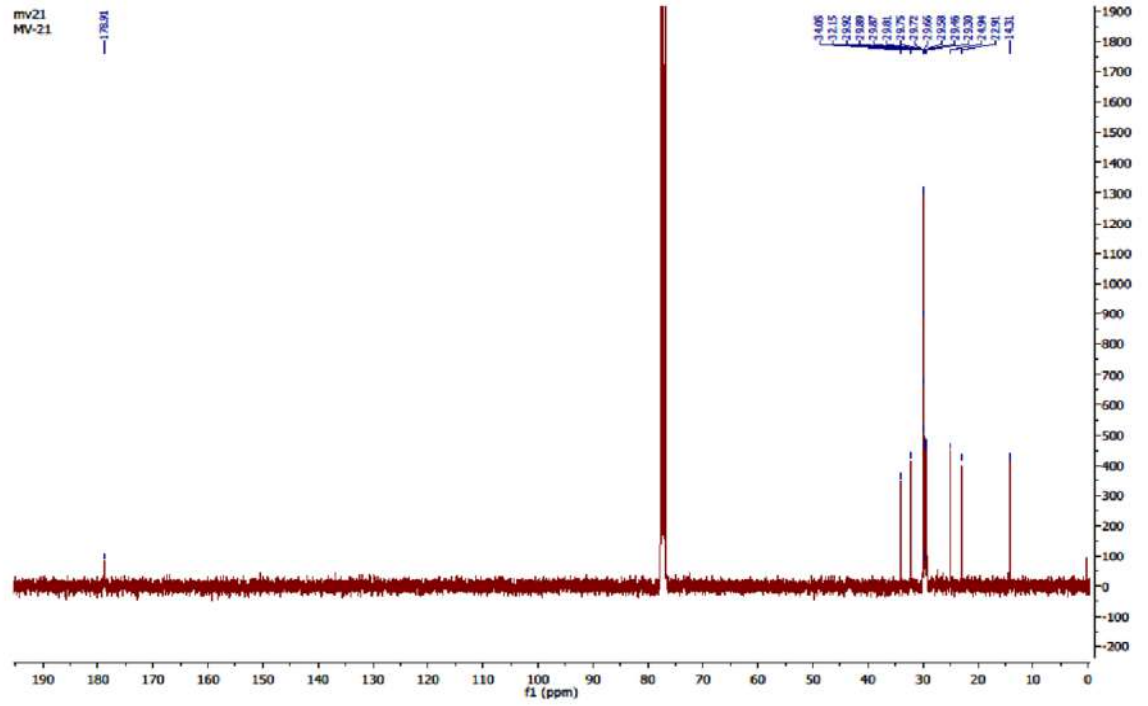

Fig. S22. ^{13}C spectrum of Palmitic acid (MV-21)

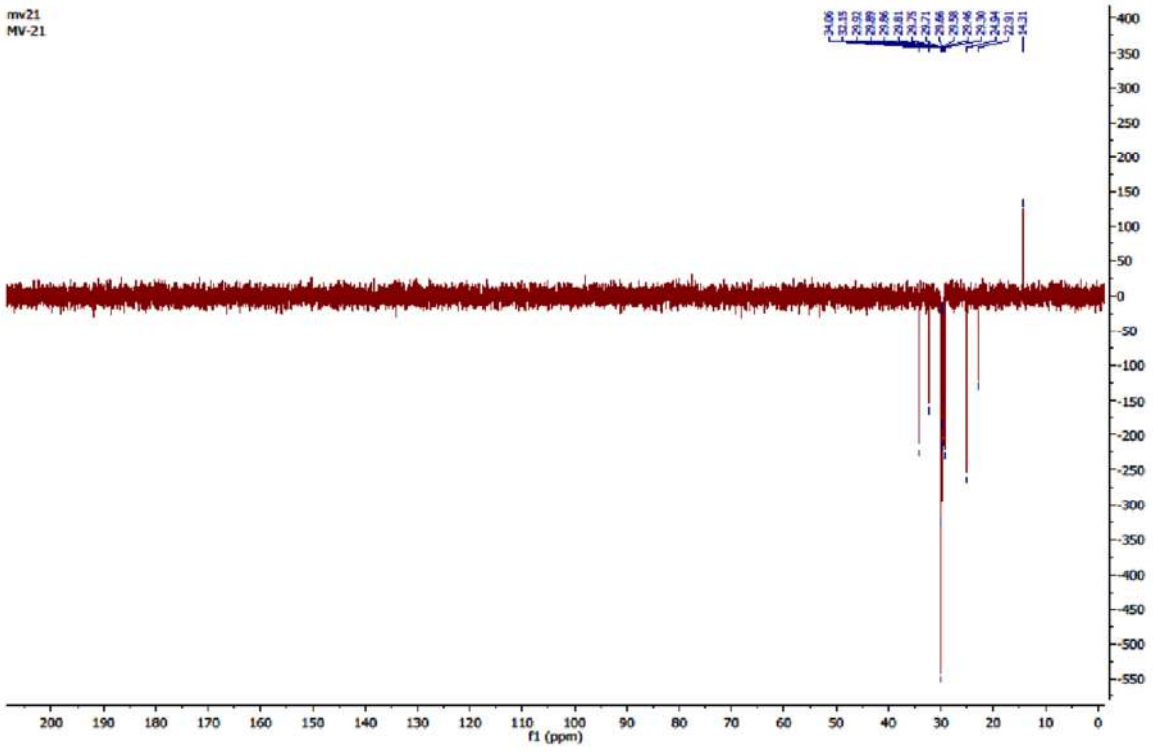

Fig S23. DEPT of Palmitic acid (MV-21)

4.16 MS spectrum of Palmitic acid (MV-21)

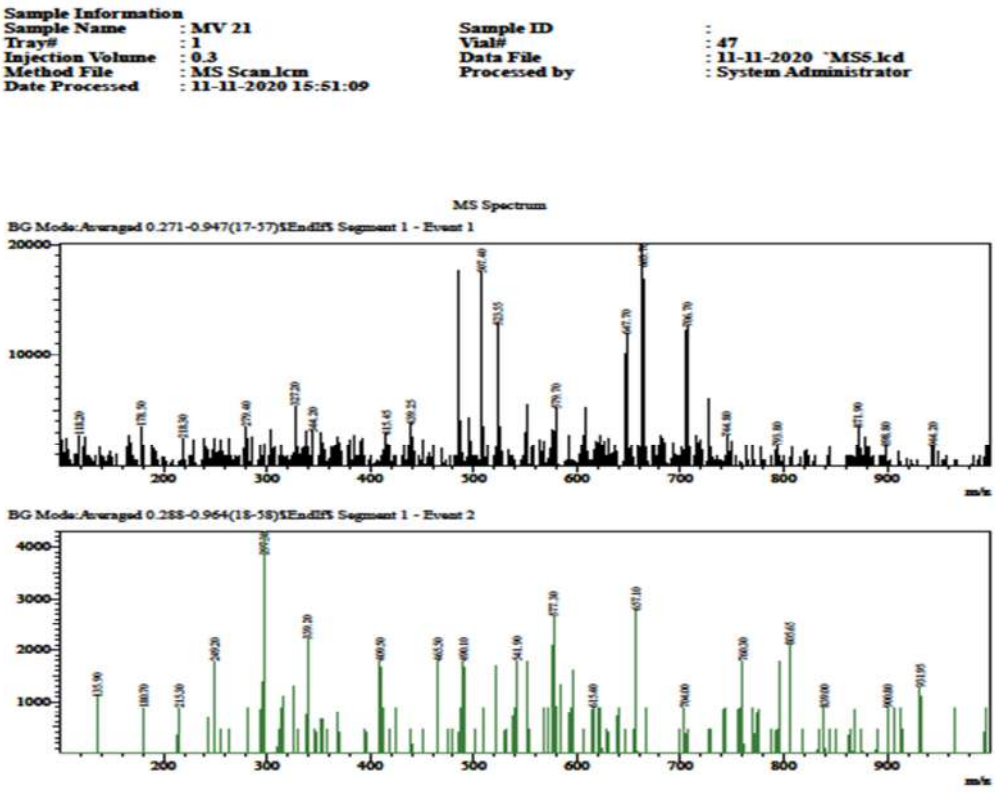

Fig. S24. ESI-MS m/z 279.30[M+K]$^+$ (cal. for $C_{16}H_{32}O_2$, m/z 256.42).

5. DISCUSSION

Diabetes which is characterized by hyperglycemia is associated with long term damage, dysfunction and failure of various organs mostly kidney, retina, heart and nervous system [30] .Though various synthetic antidiabetic drugs are available but there is still increasing demand for herbal treatment due to its easy availability, affordability and less side effects compared to synthetic ones [31],. Various reports implicated that presence of some classes of phytoconstituents (terpenoids, flavonoids, saponins, tannins and alkaloids) in herbal plants are responsible for antidiabetic activity. [32,33] So researchers turn no stone left to make formulations and isolate novel antidiabetic agents in order to curb this deadly metabolic disorder. In the present study our aim was to isolate secondary metabolites from *M. vulgare* leaf extract and comprehensively evaluate their antioxidant and antidiabetic activity. Earlier reports related to antidiabetic activity of *M. vulgare* was restricts to crude extracts [10,12,34], however in this study we first time report antioxidant and antidiabetic activity of bioactive compounds from saturated fraction (chf fr) of *M. vulgare* leaf. All fractions (Hex fr, Ch fr and Aq fr) including crude methanol extract showed potent radical scavenging activity in concentration dependent manner with highest % exhibited by Ch fr (IIIM-2), in which highest amount of diterpenoids (marrubiin= 80.53 mg/g , marrubinone B = 33.48 mg/g DW) and fatty acid (oleic acid= 71.89 mg/g DW) was found through HPLC analyses. Previous studies suggested that presence of flavonoids and verbascoside derivative in the aerial parts of *M. vulgare* could be responsible for antidiabetic activity [10,12,35], but to our best knowledge, none evaluated any saturated fraction containing one or two compounds for antidiabetic activity; however marrubiin are reported to possess analgesic, antiedematogenic, and gastroprotective activities [36-38] and marrubinone B acting as natural killer (NK) cell stimulator by its lytic activity against K_{562} leukemia cell line.[39] Various biological activites of oleic acid are repoted like improvement in insulin resistance, inhibition of glucose production and food intake, preventing various different type of disorders such as cardiovascular, autoimmune, skin injury and cancer.[40-42]In our present study we not only isolated marker compounds characterized them through different spectral analysis (MS, HRMS, ^{1}H NMR and ^{13}C NMR) but also evaluated saturated fraction (IIIM-2) containing three major compounds for its antidiabetic potential. We observed blood glucose level of diabetic animals starting decreasing from 5th day onwards of drug treatment that was continued to maintain till 14th day, which was comparable to metformin 500 mg/kg. There was an 86.4% decrease in blood glucose level with both MEex (IIIM-1) and Ch fr (IIIM-2) at a dose of 500 mg/kg and 250 mg/kg compared to metformin 500 mg/kg treatment (59.2%) respectively. The underlying mechanism as well as evaluation of lipid profile was not done in this study due to lack of funds or financial support. However previous studies [41,43,44] suggested that more than one mechanisms could be linked. One might due be inhibitory action of the major compounds of chloroform fraction against α–glucosidase by retarding the posprandial glucose and other may be extrapranceatic effect.

6. CONCLUSION

It is evident from the present work that diterpenoids isolated from the leaf of *M. vulgare* has a significant potential of antidiabetic effect and moderate antioxidant capacity The presence of other compounds in Methanol extract might be taking part to aid in the activity but their involvement is not so much as is evident from the results of chloroform fraction

containing considerably high amount of diterpenoids (marrubiin and marrubinone B) and oleic acid. Hence, further studies needed to elucidate the exact mechanism responsible for antidiabetic activity moreover pure marrubiin and marrubinone B should be evaluated for the activity.

7. ACKNOWLEDGMENT

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8. AUTHORS CONTRIBUTION STATEMENT

Shabir Ahmad Dar collected, extracted and isolated the compounds from plant sample, drafted and interpreted the research paper and data, Parveen Kumar in assistance with Shabir Ahmad Dar performed in vivo study, Prasoon Gupta characterized the isolated compounds, Sreedhar Madishetti designed the research project and analyzed the data, Manik Sharma revised and made necessary corrections in the paper, and Amit Kumar performed HPLC. All authors have read and approved the final manuscript.

9. CONFLICT OF INTEREST

Conflict of interest declared none.

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