Effect of *Viscum schimperi* on advanced glycation endproducts formation

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Abstract: Diabetes is a disease characterized by elevated uncontrolled glucose level. Hyperglycemia results in diabetic complication due to a reaction between sugar and amino acid of proteins to form advanced glycation endproducts (AGEs) in different tissues. Medicinal plants are considered as a great source of bioactive compounds that affect many ailments. In this regard; AGEs formation could be inhibited by many bioactive compounds isolated from medicinal plants. *Viscum schimperi* Engl. is a plant belongs to Loranthaceae and known for its antidiabetic activity. In this study; total methanol extract of *V. schimperi* (VT) was prepared, suspended in water and subjected to fractionation with chloroform followed by *n*-butanol to give (VC) and (VB) fractions respectively. The aqueous mother liquor was evaporated to form (VA) fraction. The inhibitory effect of all prepared fraction on the formation of advanced glycation endproducts (AGEs) was studied. The results revealed that *V. schimperi* extract and its different fractions inhibited protein glycation and oxidation of BSA induced by ribose together with decrease of protein aggregation. In conclusion; *V. schimperi* will be useful in management of diabetic complications based on its inhibition of advanced glycation endproduct formation.

Keywords: *Viscum schimperi,* advanced glycation endproducts, diabetic complications, anti-diabetic, Saudi plants.

INTRODUCTION

Diabetes is a chronic disease characterized by hyperglycemia due to lack of insulin or insulin resistance. The long term elevation of glucose level lead to development of a reaction between glucose and amino acid of protein to form Schiff's base which converted through cross linking to Advanced glycation end products (AGEs) (Singh *et al*., 2014). AGEs production can damage different tissues leading to the development of chronic diabetic complications including nephropathy, atherosclerosis, retinopathy, neuropathy, age-related diseases, and Alzheimer's disease (Ahmed, 2005; Brownlee, 1995; Schmidt and Stern, 2000; Singh *et al*., 2014; Vitek *et al*., 1994).

Herbs have been used for treatment of different ailments since beginning of human life. Herbal remedies are brought into focus with the objective of emphasizing already known indications, as well as, exploring others not previously practiced. In this regard, many plants are reported to be useful in treatment of diabetes (Pandeya *et al*., 2013). Moreover; some plant extracts have been evaluated for their effects on the formation of AGEs (Magadula *et al*., 2014; Okada and Okada, 2015; Sompong and Adisakwattana, 2015; Wu *et al*., 2015).

Viscum schimperi Engl. is a type of mistletoes belongs to family Loranthaceae (Kafaru, 1993).

The plant is widely growing in Al-taif governorate, Saudi Arabia and used as antidiabetic (Abdallah *et al*., 2015). *V. album* L. is the famous species growing in Europe and used to control blood pressure. Moreover it is used as immunostimulant, anti-cancer, antispasmodic, cytotoxic and anti-ulcer (Bown, 1995). *V. album* is known for its hypoglycemic effect and it ability to overcome symptoms of diabetes (Swanston-Flatt *et al*., 1989) due to its ability to increase insulin secretion (Gray and Flatt, 1999). Chemical investigation of *Viscum* genus revealed identification of different classes of compounds including lectins, viscotoxins, triterpenes, phenolics, polysaccharides, oligosaccharides and alkaloids (Abdallah *et al*., 2015; Zee-Cheng, 1997).

In a continuation of our work to understand the mechanism of antidiabetic activity of *V. schimperi*, (Abdallah *et al*., 2015; Abdel-Sattar *et al*., 2011); this study was undertaken to study the inhibitory effect of *V. schimperi* on advanced glycation end products formation.

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MATERIALS AND METHODS

Chemicals

All chemicals used in this study were of the highest available analytical grade. Bovine serum 1-diphenyl-2-picryl-hydrazil (DPPH), rutin, gallic acid, albumin (BSA), D-ribose, phosphate buffer saline, sodium azide, dimethyl sulfoxide (DMSO), (N-(caboxymethyl) lysine, thiol group ,5,5`-dithiobis (2-nitrobenzoic acid) (DTNB), thioflavin and amyloid cross β structure and HEPES buffer**.**

Plant materials

The aerial parts (leaves, stem, flower and fruits) of *Viscum schimperi* were collected from Al-Taif governorate, Saudi Arabia, in May 2014 and was kindly identified by Dr. Emad Alsherif Faculty of Science, King Abdulaziz University, Jeddah, Saudi Arabia. A specimen was deposited in the herbarium of the faculty of Pharmacy, King Abdulaziz University, Jeddah, Saudi Arabia (no. VS1167A).

Extraction of plant material for biological study

Powdered plant material 1Kg) of *V. schimperi* was extracted on cold with MeOH using Ultra-Turrax homogenizer (IKA, Staufen, Germany) till exhaustion. The total methanol extract was evaporated under reduced pressure to give dried residue (VT, 120g). Methanol extract was dissolved in the lease amount of water and fractionated with $CHCl_3$, *n*-butanol (saturated with water) to give (VF, 31g), and *n*-butanol (VB, 34g) fractions respectively. Mother liquor was also evaporated to give 32gm of (VA).

Standardization of methanol extract of V. schimperi

Metabolite profiling and standardization of total methanol extract were performed previously using UPLC-equipped with PDA detector and high resolution mass (Abdallah *et al*., 2015).

Determination of total phenolic content

Folin-Ciocalteu method was used for determination of phenolic contents in total extract and fractions as described before (Ainsworth and Gillespie, 2007). The results expressed in mg of gallic acid equivalents (GAE)/g dry weight

Determination of total flavonoid content

Aluminum chloride colorimetric assay as was used for determination of total flavonoid content in total extract and fractions as described before (Abdallah *et al*., 2014). The total flavonoid content was determined from the calibration curve, and expressed as mg rutin equivalents (RE)/g of dried extract.

Determination of antioxidant activity

1,1-diphenyl-2-picryl-hydrazil (DPPH) free radical scavenging assay was used to determine the antioxidant activity total methanolic extract and prepared fractions as described before (Abdallah *et al*., 2014; Sharma and Bhat, 2009). Extract concentration providing 50% inhibition (IC_{50} value) was calculated from the graph of inhibition percentage against extract concentration. Tests were carried out in triplicate. Ascorbic acid (1mM) was used as standard.

In vitro AGEs formation

Glycation of BSA in vitro at 37^{o}C was performed using: 10mg/ml BSA incubated with 100mM ribose in 100mM PBS (PH7.4) plus 0.02% sodium azide to prevent bacterial contamination followed by solublization of extracts with (DMSO) then dilution of each extract 10 times and at the end of this experiment the effect of each extract in different concentration was tested for its ability to form advanced glycation end products (Abdallah *et al*., 2016; Ghareib *et al*., 2015).

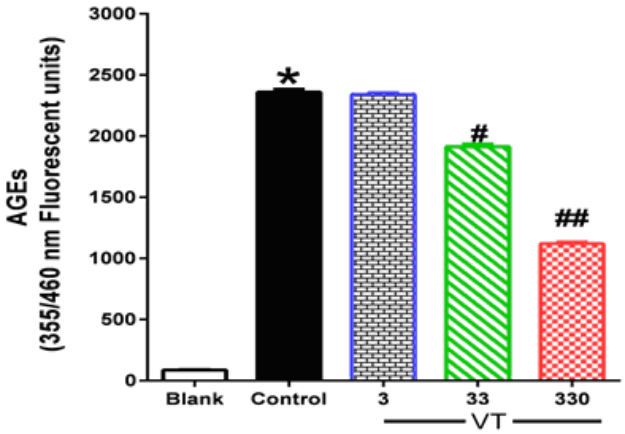

Fig. 1: Effect of VT (total extract, 3, 33 and 330 mg/ml) on advanced glycation end products formation when BSA (10%) incubated with ribose (100mM) for one week. Blank is a reaction mixture including BSA and ribose kept at -20^{o}C while control is the same reaction mixture but incubated at 37^{o}C. Results are expressed as mean ± SEM (n=3). * $p<0.05$ when compared to Blank, # $p<0.05$ when compared to Control, ## $p<0.01$ when compared to Control.

Determination of protein aggregation

Protein aggregation was determined using thioflavin T as a marker for this aggregation. Glycated BSA was incubated with thioflavin T (32µM) in 100mM phosphate buffer saline (PH 7.4), RT for 1 hour after that the fluorescence was measured (excitation 435nm/ emission 485nm).

RESULTS

The effect of total methanol extract of V. schimperi and its fractions on AGEs

The addition of VT in different concentrations (3, 33, 330 mg/ml) into the reaction media significantly decreased the AGEs formation at the two high concentrations (33, 330 mg/ml), meanwhile the lower concentration (3mg/ml)

showed weak inhibition on AGEs formation (fig. 1). Furthermore, testing the effect of different fractions of *V. schimperi* on AGEs formation in different concentrations (3, 33, 330mg/ml) revealed that VA significantly decreased the AGEs only at concentration (330 mg/ml) (fig. 2). Moreover, VC decreased the AGEs formation at all tested concentration (fig. 3). Finally, VB significantly decreased the AGEs formation only at the two high concentrations (330 and 33mg/ml) (fig. 4).

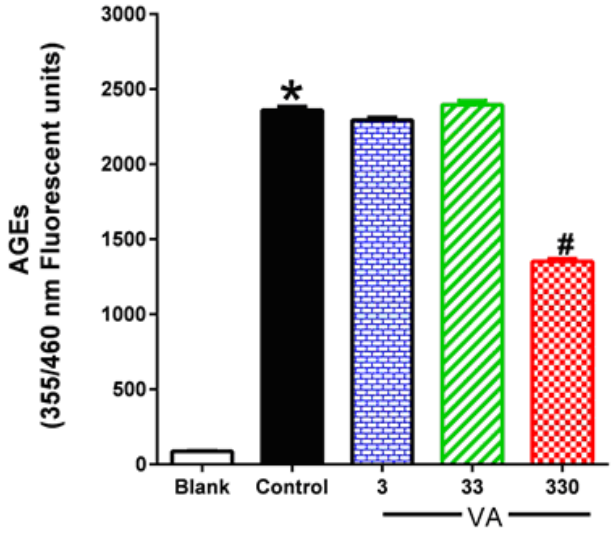

Fig 2. Effect of VA (Fraction A, 3, 33 and 330 mg/ml) on advanced glycation endproducts formation when BSA (10%) incubated with ribose (100 mM) for one week. Blank is a reaction mixture including BSA and ribose kept at -20°C while control is the same reaction mixture but incubated at 37°C. Results are expressed as mean ± SEM (n = 3). *p<0.05 when compared to Blank, # p<0.05 when compared to Control.

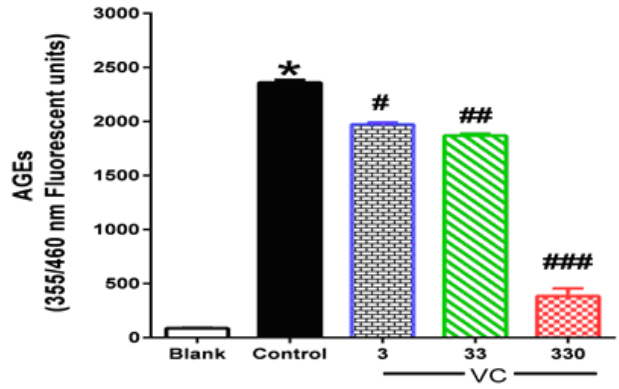

Fig 3. Effect of VC (fraction C, 3, 33 and 330 mg/ml) on advanced glycation end products formation when BSA (10%) incubated with ribose (100mM) for one week. Blank is a reaction mixture including BSA and ribose kept at -20°C while control is the same reaction mixture but incubated at 37°C. Results are expressed as mean ± SEM (n=3). *p<0.05 when compared to Blank, # p<0.05 when compared to Control, ## p<0.01 when compared to Control, ### p<0.001 when compared to Control.

The Effect of total methanol extract of V. schimperi and its different fractions on aggregation of the protein

The addition of VT in different concentration (3, 33, 330 mg/ml) into the reaction mixture significantly decreased the protein aggregation (fig. 5). VA significantly decreased the protein aggregation at concentration (330 mg/ml) only (fig. 6), meanwhile, VC and VB significantly decreased the protein aggregation at all tested concentration (fig. 7 and 8).

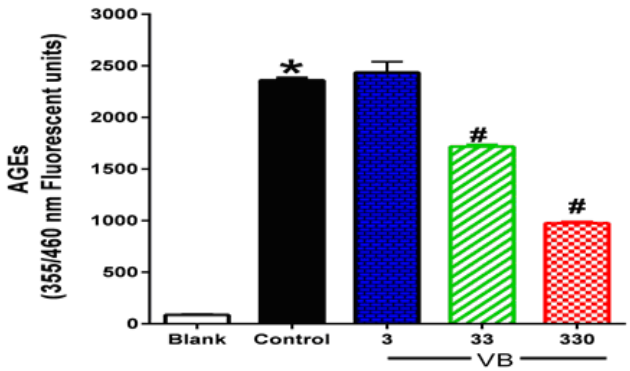

Fig. 4: Effect of VB (Fraction B, 3, 33 and 330 mg/ml) on advanced glycation endproducts formation when BSA (10%) incubated with ribose (100 mM) for one week. Blank is a reaction mixture including BSA and ribose kept at -20°C while control is the same reaction mixture but incubated at 37°C. Results are expressed as mean ± SEM (n = 3). *p<0.05 when compared to Blank, # p<0.05 when compared to Control

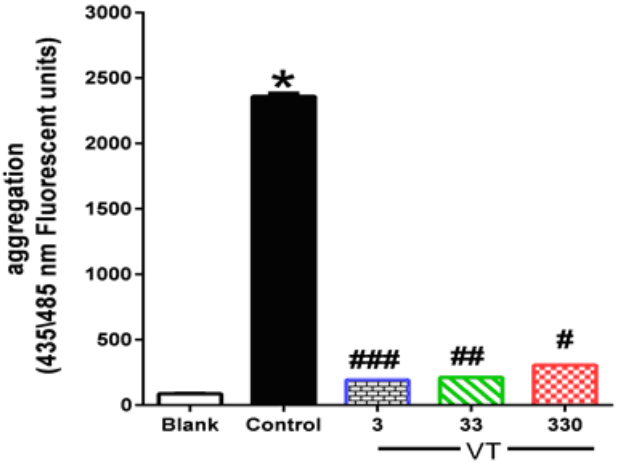

Fig. 5: Effect of VT (total extract, 3, 33 and 330mg/ml) on protein aggregation when BSA (10%) incubated with ribose (100mM) for one week. Blank is a reaction mixture including BSA and ribose kept at -20°C while control is the same reaction mixture but incubated at 37°C. Results are expressed as mean ± SEM (n=3). *p<0.05 when compared to Blank, # p<0.05 when compared to Control, ## p<0.01 when compared to Control, ### p<0.001 when compared to Control.

Standardization of methanol extract of V. schimperi

Standardization and metabolite profiling of total methanol extract of *V. schimperi* were performed previously (Abdallah *et al*., 2015) (please revise the supplementary figures) and results revealed the presence of 3-*O*-caffeoylquinic acid, 5-*O*-caffeoylquinic acid, feruloyl quinic acid, sinapoylquinic acid, eriodictyol, naringenin, eriodictyol hexoside and isorhamnetin-3-*O*-glucoside in concentrations (54±22µg/g), (4401±705µg/g), (327±117 µg/g), (163±59µg/g), (56±5.3µg/g), (45±14.3µg/g), (74±11µg/g) and (142±35.4µg/g) respectively.

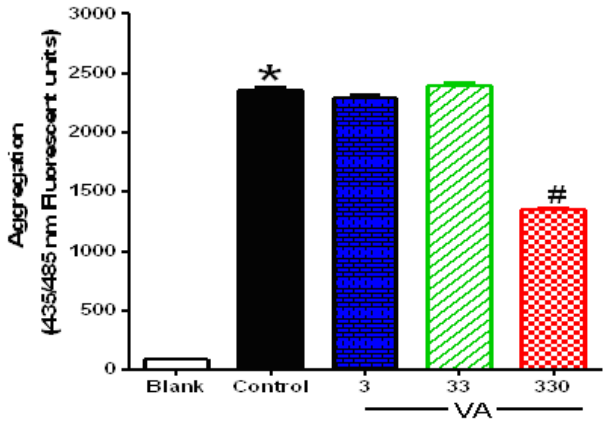

Fig. 6: Effect of VA (Fraction A, 3, 33 and 330 mg/ml) on protein aggregation when BSA (10%) incubated with ribose (100mM) for one week. Blank is a reaction mixture including BSA and ribose kept at -20°C while control is the same reaction mixture but incubated at 37°C. Results are expressed as mean ± SEM (n=3). * p<0.05 when compared to Blank, # p<0.05 when compared to Control.

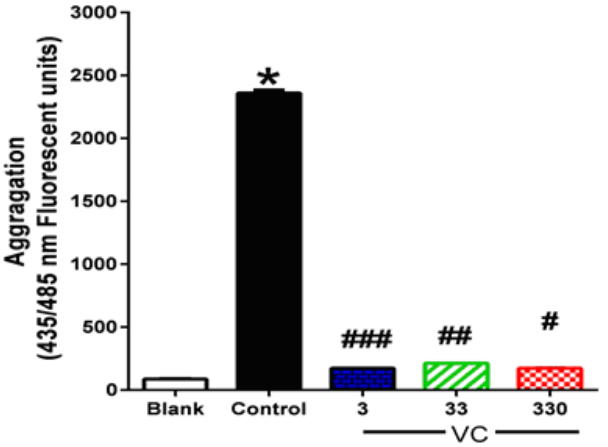

Fig. 7: Effect of VC (Fraction C, 3, 33 and 330 mg/ml) on protein aggregation when BSA (10%) incubated with ribose (100 mM) for one week. Blank is a reaction mixture including BSA and ribose kept at -20°C while control is the same reaction mixture but incubated at 37°C. Results are expressed as mean ± SEM (n=3). *p<0.05 when compared to Blank, #p<0.05 when compared to Control, ## p<0.01 when compared to Control, ### p<0.001 when compared to Control.

Moreover, determination of total flavonoids and phenolic contents in the studied extract and fractions showed results that are in agreement with that obtained from metabolite profiling. Among the tested fractions, the flavonoid content, expressed as rutin equivalents (RE), was highest in the VB (4.22 mg RE/g) followed by VT (3 mg RE/g). Meanwhile, highest phenolic content, determined as gallic acid equivalents (GAE), was found in VB (13 mg GAE/g) followed by VT (7mg GAE/g). Finally, only VT and VB were able to reduce the stable radical DPPH to the yellow colored diphenylpicrylhydrazine with IC_{50} 22 and 10µg/ml respectively.

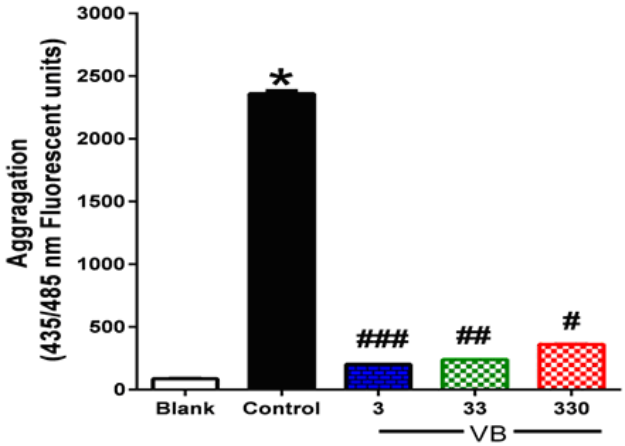

Fig. 8: Effect of VB (Fraction B, 3, 33 and 330 mg/ml) on protein aggregation when BSA (10%) incubated with ribose (100mM) for one week. Blank is a reaction mixture including BSA and ribose kept at -20°C while control is the same reaction mixture but incubated at 37°C. Results are expressed as mean ± SEM (n=3). *p<0.05 when compared to Blank, # p<0.05 when compared to Control, ## p<0.01 when compared to Control, ### p<0.001 when compared to Control.

DISCUSSION

The traditional antidiabetic drugs are very expensive despite many adverse effect and high rate of secondary failure. One of our considerations is to search about antidiabetic drug which is safer and affordable. The existing investigation reports the AGEs inhibitory effect of the methanolic extract of *V. schimperi* and its fractions. *V. schimperi* extract markedly inhibited protein glycation and oxidation of BSA induced by ribose together with decrease of protein aggregation and preventing loss of protein thiol group. In our work this results could be potentially beneficial in the stopping of AGE induced pathogenesis of the diabetic complications.

The maximum effect on AGEs formation was performed by n-butanol and chloroform fractions at concentration 330mg/ml. It was observed that increasing the concentration of tested fraction caused significant

decrease in the fluorescent intensity relative to the control. It was noticed that all fractions of *V. schamperi* were working better if they added on protein aggregation steps before AGEs formation. Moreover, results showed also that also n-butanol and chloroform fractions at different concentration markedly decrease protein aggregation.

Standardization and metabolite profiling of VT was performed by UPLC and detection was performed by PDA and high resolution MS detectors (Abdallah *et al*., 2015). The analysis revealed the presence of high percentage of triterpene of oleanolic acid nucleus in VC fraction.

Oleanolic acid was reported to reduce hyperglycemia due to inhibition of α-amylase and α-glucosidase activities. Moreover, it inhibits protein-tyrosine phosphatases leading to increase glucose uptake and insulin sensitivity. In addition; oleanolic acid previously showed anti-glycative effect in kidney of diabetic mice (Jang *et al*., 2010; Wang *et al*., 2010).

The *n*-butanol fraction (VB) showed the highest phenolic and flavonoid content in all tested fraction and displayed a high antioxidant activity due to its constituents. Moreover, it was rich in phenolic compounds and *O*-phenolic quinic acid derivatives such as 3-*O*-caffeoyl quinic acid (OCQA) as a major constituent which was reported as AGEs inhibitor (Cui *et al*., 2009). Furthermore, OCQA is also reported to stimulate glucose transporter type 4 (GLUT4) in skeletal muscles, increases the activity of hexokinase and decreases the expression of glucose 6-phosphatase.

Further studies will be performed to isolate the bioactive compounds and study their effects as AGEs inhibitor.

CONCLUSION

Viscum schimperi has inhibitory effect against protein glycation with glucose and oxidative damage of BSA. Using *in vitro* models, *V. schimperi* markedly inhibited AGEs and protein aggregation.

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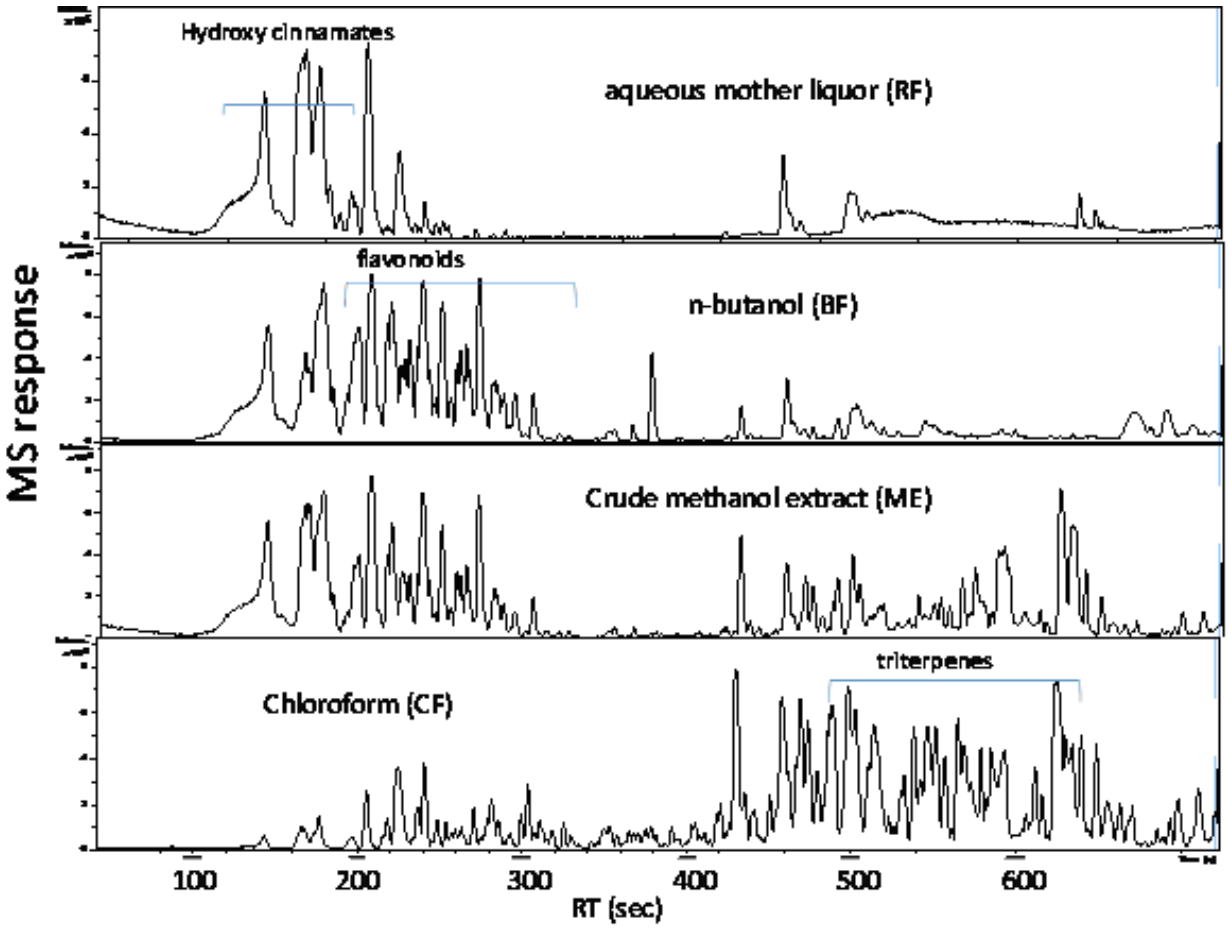
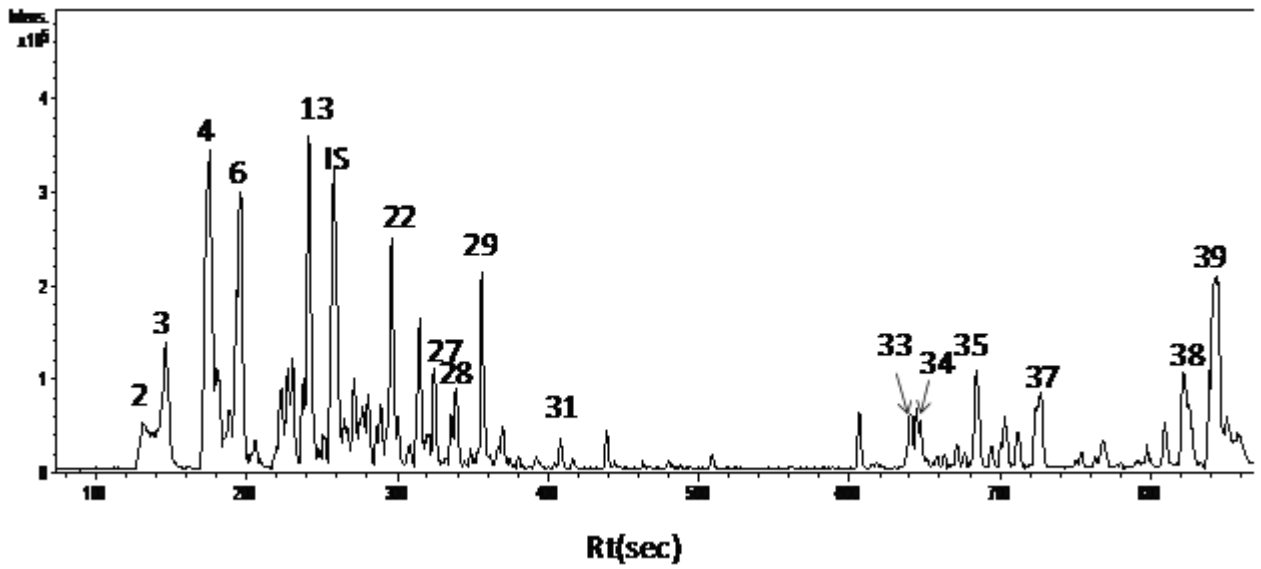
Appendix A. Supplementary data

Table 1: Metabolites identified in methanol extract of *V. schimperi* using UPLC/UV/MS

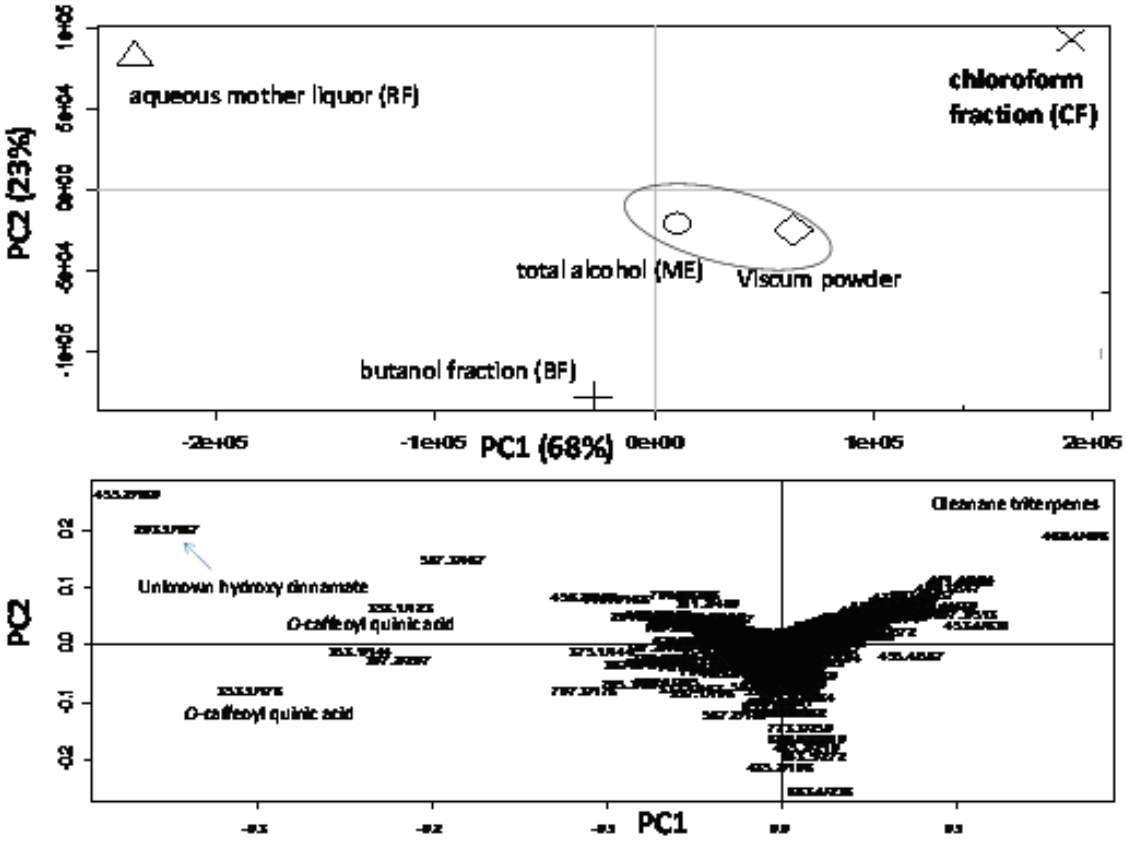
Peak	rt (sec)	UV	Formula	Identification	$[M-H]^-$ (*m/z*)	Error (ppm)	MS^n product ions (%)
1	35	nd	$C_7H_{11}O_6$	Quinic acid	191.0546	1.5	
2	134	295 shd, 325	$C_{16}H_{17}O_9$	1-*O*-caffeoylquinic acid	353.0853	3.1	191(47)
3	147	295 shd, 325	$C_{16}H_{17}O_9$	4-*O*-caffeoylquinic acid	353.0854	6.7	191(37)
4	176	308	$C_{25}H_{27}O_8$	Unknown	455.1672	8.6	293(8), 128(10)
5	189	295 shd, 325	$C_{16}H_{17}O_9$	3-*O*-caffeoylquinic acid	353.0866	3.5	191(100)
6	195	295 shd, 325	$C_{16}H_{17}O_9$	5-*O*-caffeoylquinic acid	353.0879	0.3	179(22), 73(30)
7	206	325	$C_{18}H_{21}O_{10}$	Sinapoylquinic acid	397.1142	0.5	223(82)
8	220		$C_{20}H_{22}O_9$	Viscutin III	405.0796	7.6	273(30)
9	223	283 shd, 312	$C_{18}H_{15}O_8$	Rosmarinic acid	359.0748	6.8	173(60)
10	227	277, 325 shd	$C_{21}H_{23}O_{10}$	Tetrahydroxyflavan-*O*-β-D-hexoside	435.1306	2.0	273(17)
11	231	280, 322 shd	$C_{26}H_{31}O_{14}$	Luteoliflavan-*O*-[pentosyl-hexoside]	567.1743	4.2	435(30), 273(10)
12	237	289 shd, 325	$C_{17}H_{19}O_9$	Feruloyl quinic acid	367.1035	0.2	193(20), 73(65)
13	242	328	$C_{18}H_{21}O_{10}$	Feruloyl quinic acid-methyl ether	397.1151	2.8	367(9), 191(60)
15	251	281, 330	$C_{34}H_{45}O_{18}$	Unknown	741.263	2.4	579(100), 463(20)
16	260		$C_{21}H_{23}O_9$	Unknown	419.1351	0.7	257(30)
17	265	260, 340	$C_{21}H_{21}O_{11}$	Eriodictyol hexoside	449.1128	8.5	287(23)
18	270	nd	$C_{15}H_{11}O_6$	Eriodictyol	287.0562	0.3	
19	271	275, 330 shd	$C_{13}H_{14}NO_4$	Unknown	248.0923	2.0	164(17)
20	278	nd	$C_{31}H_{29}O_{12}$	Isorhamnetin-*O*-pentosyl-*O*-α-L-rhamnopyranoside	593.1635	4.9	447(32), 315(6)
21	283	nd	$C_{23}H_{25}O_{11}$	Isorhamnetin -3-*O*-glucoside	477.1418	3.2	315
22	300	275, 323 shd	$C_{28}H_{35}O_{13}$	Unknown	579.2105	3.8	417(68)
23	303	nd	$C_{21}H_{21}O_{10}$	Naringenin	433.1158	4.0	271(100)
24	308	278,324	$C_{31}H_{37}O_{16}$	Unknown	665.2107	3.0	209(71), 187(100)
25	314	nd	$C_{28}H_{33}O_{14}$	Trihydroxyflavanone-Di-Me ether-O-[pentosyl-hexoside]	593.1899	3.6	461(17)
26	319	277, 330	$C_{27}H_{29}O_{12}$	Chrysin-O-di-α-rhamnopyranoside	545.1686	3.9	253(11)
27	324	277, 330	$C_{23}H_{25}O_{10}$	Trihydroxyflavanone-Di-Me ether-O-hexoside	461.1479	5.5	257(30)
28	338	nd	$C_{37}H_{41}O_{17}$	Unknown	757.2372	3.0	559(24), 397(13)
29	354	nd	$C_{40}H_{59}O_{19}$	Unknown	843.369	4.1	
30	367	nd	$C_{29}H_{31}O_{14}$	Unknown	603.175	5.0	397(25),
31	401	nd	$C_{15}H_{11}O_5$	Naringin	271.0604	2.8	175(87), 113(61)
32	608	nd	$C_{33}H_{55}O_{14}$	Unknown	675.3606	1.3	
33	638	nd	$C_{18}H_{29}O_3$	Hydroxy-octadecatrienoic acid	293.2109	4.5	235(10)
34	643	nd	$C_{30}H_{47}O_4$	Dihydroxy-oleana-dien-oic acid	471.3477	0.6	453(15), 293(30)
35	682	nd	$C_{18}H_{31}O_3$	Hydroxy-8,12-octadecadienoic acid	295.2266	4.2	277(10),
36	700	nd	$C_{30}H_{45}O_4$	Unknown triterpene	469.3331	1.7	297(81)
37	722	nd	$C_{18}H_{33}O_3$	10-Hydroxy-octadecenoic acid	297.2419	5.5	
38	821	nd	$C_{30}H_{45}O_3$	Betulinic acid	453.3387	3.7	
39	837	nd	$C_{30}H_{47}O_3$	Oleanolic acid	455.3542	2.5	345(8), 277(21)



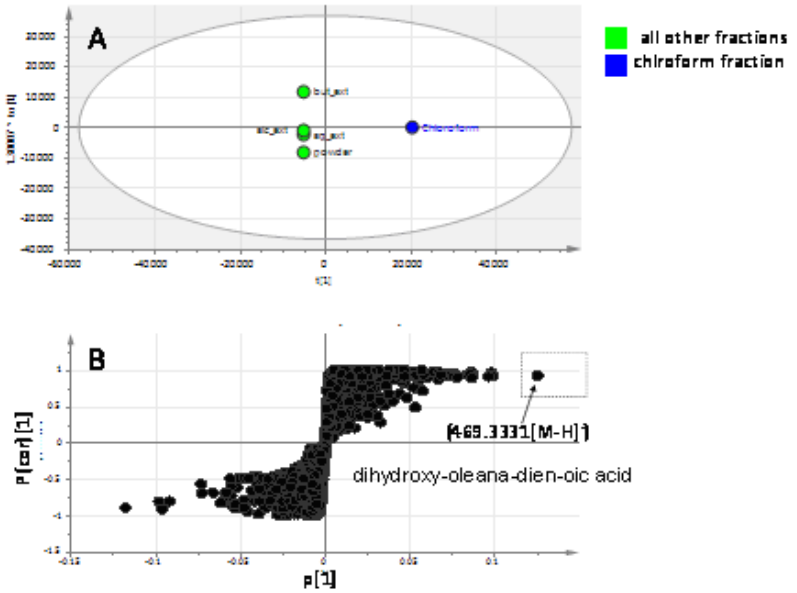
Supplementary Fig. 1: UPLC-qTOF-MS base peak chromatograms of of the methanol extracts of *Viscum schimperi* crude methanolic extract (ME) and its fractions, viz. chloroform (CF), n-butanol (BF), in addition to aqueous mother liquor (RF) showing abundance of metabolite classes in each respective extract. Chromatographic conditions are described under Materials and Methods.



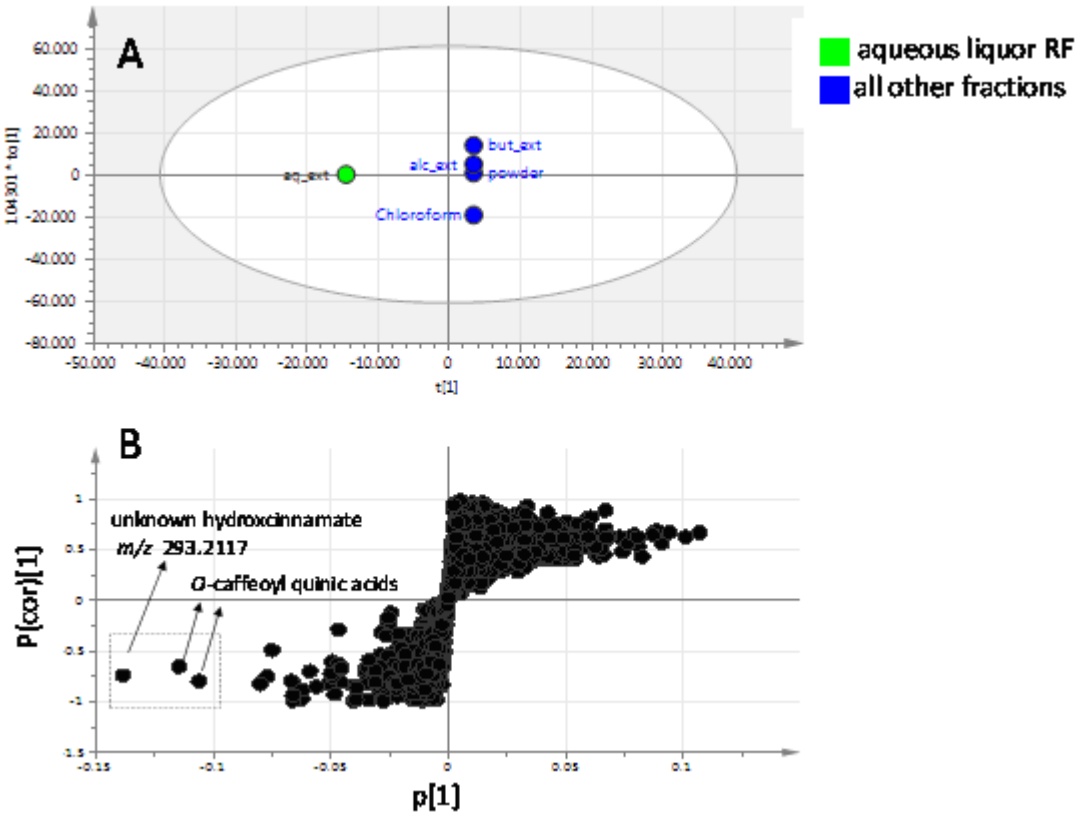
Supplementary Fig. 2: Representative UPLC-MS trace of *V. schimperi* methanol extract characterized by 2 main regions: (100-400 sec) with peaks principally due to flavonoids & phneolic acids and region (600-850) for triterpenes & fatty acids. Peak numbers follow those listed in (table 1) for metabolite identification using UPLC-PDA-MS. IS denotes for umbelliferone internal standard spiked at a concentration of 20µg.



Supplementary Fig. 3: UPLC-qTOF-MS (*m/z* 100-1000) principal component analyses of the different *V. schimperi* extracts. The metabolome clusters are located at the distinct positions described by two vectors of principal component 1 (PC1 = 57%) and principal component 2 (PC2 = 32%). (A) Score Plot of PC1 vs PC2 scores. (B) Loading plot for PC1 and PC2 with contributing mass peaks and their assignments, with each metabolite denoted by its mass/R_t (sec) pair. It should be noted that ellipses do not denote statistical significance, but are rather for better visibility of clusters as discussed.



Supplementary Fig. 4: (UPLC-qTOF-MS (m/z 100-1000) A) OPLS-DA score plot and (B) loading S-plots derived from CF sample modelled against all other extracts. The S-plot shows the covariance p[1] against the correlation p(cor)[1] of the variables of the discriminating component of the OPLS-DA model. Cut-off values of P<0.01 were used; variables selected are highlighted in the S-plot with m/z retention time in seconds and identifications are discussed in the text.



Supplementary Fig. 5: UPLC-qTOF-MS (m/z 100-1000) (A) OPLS-DA score plot and (B) loading S-plots derived from aqueous mother liquor (RF) sample modelled against all other extracts. The S-plot shows the covariance p[1] against the correlation p(cor)[1] of the variables of the discriminating component of the OPLS-DA model. Cut-off values of P<0.01 were used; variables selected are highlighted in the S-plot with m/z retention time in seconds and identifications are discussed in the text.