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Original Article

Proanthocyanidins from the stem bark of *Rhus tripartita* ameliorate methylglyoxal-induced endothelial cell apoptosis



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ABSTRACT

In traditional Arabian medicine, the *Rhus tripartita* plant (family Anacardiaceae) has been used to treat inflammatory conditions. Although *Rhus* extracts have been reported for their cardioprotective effects, information regarding their active principle compounds remains insufficient. The present investigation was aimed at determining the antioxidant chemical constituents of the methanolic extract of *R. tripartita* stem bark and evaluating their ability to ameliorate methylglyoxal-induced endothelial cell apoptosis. Ten flavonoid compounds (1–10) were isolated and identified using DPPH radical scavenging bioassay-guided chromatographic separation. A new proanthocyanidin (rhuspartin) (1) was isolated and identified as 3,5,13,14-flavantetrol-(4 β →8)-catechin, using extensive spectroscopic data and high resolution-mass spectrometry. Among the compounds (1, 5, 7–10) tested for toxicity toward cultured endothelial cells (HUVECs), the non-cytotoxic compounds 1 and 7 evinced cytoprotective potential that reversed the methylglyoxal-induced apoptosis (by 62% and 64%, respectively) through downregulation of caspase 3/7.

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1. Introduction

The family Anacardiaceae, which consists of 73 genera and approximately 600 species [1,2], is comprised mainly of trees, shrubs, and/or woody vines. The majority of these belong to the genus *Rhus* [3,4], including over 250 species that bear the common name sumac [5]. It is widely distributed in the tropics, subtropics, and various global temperate zones and is known to be rich in flavonoids, bioflavonoids, and proanthocyanidins [6–10]. In particular, *Rhus tripartita* (Ucria) Grande is distributed primarily in North Africa and in northeastern part of Saudi Arabia [5]. In Arabian traditional medicine, this plant has been used for the treatment of cardiovascular and gastrointestinal disorders along with inflammatory conditions [5,11]; its other reported biological activities also include antioxidant, antidiarrheal, and antiulcer effects [12].

Methylglyoxal (MGO), a highly reactive dicarbonyl compound, is generated endogenously as well as in several food-stuffs and beverages during processing, cooking, or storage as a by-product of glycolysis [13,14]. High level of plasma MGO is associated with type 2 diabetes mellitus and considered as a causative factor in atherosclerosis and macrovascular diseases [15,16]. Notably, in cultured endothelial cells (ECs), MGO rapidly causes a hyperglycemic state that damages ECs function and becomes a prominent factor in most diabetic complications [17,18]. Furthermore, ECs play an important role in modulating vascular function and homeostasis; thus, their dysfunction as result of inflammation and apoptosis can be an initiating event in atherogenesis [19].

In our previous work, we demonstrated the therapeutic nature of *R. tripartita* stem bark including its cardiovascular, antioxidant, and anti-inflammatory effects [5,8]. The aim of the present study was to isolate and identify the antioxidant compounds, in particular those of phenolic nature, from the stem bark of *R. tripartita*. In addition, we assessed their activity toward reversing the endothelial apoptosis mediated by MGO to highlight the potential use of this plant as a source of safe and effective drugs against diabetic cardiovascular complications.

2. Materials and methods

2.1. General methods

Optical rotations were measured on a Perkin–Elmer Model 343 polarimeter (Waltham, MA, USA). CD spectra were recorded on a Jasco J-815 spectrophotometer in 1-cm cuvettes at room temperature. Nuclear magnetic resonance (NMR) spectra were recorded in deuterated dimethylsulfoxide (DMSO- d_6) on an UltraShield Plus system (Bruker Biospin GmbH, Rheinstetten, Germany) operating at 700 MHz for ^1H and at 175 MHz for ^{13}C .

HPLC was carried out using a Phenomenex Jupiter Proteo column (Jupiter Proteo 90 Å, 250 × 10 mm, 4 μm; Torrance, CA, USA) on a Shimadzu HPLC-LC-20 AD series binary gradient pump and a Shimadzu SPD-M20A detector (Tokyo, Japan). Column chromatography (CC) was carried out on Sephadex LH-20 (Pharmacia, Uppsala, Sweden) using ethanol as the eluent.

2.2. Plant material

R. tripartita stem bark was collected from Hail in the north-western region of Saudi Arabia in April 2013. The plant was identified and authenticated by an expert taxonomist at the Herbarium Unit, College of Pharmacy, King Saud University. The voucher specimen has been deposited (SY 202/2013) at the herbarium of the Faculty of Pharmacy, King Saud University, Riyadh, Saudi Arabia.

2.3. Extraction and isolation

Using 4000 ml of 80% aqueous methanol, 1100 g of air-dried powdered stem barks was extracted by maceration three times. The alcoholic extract was filtered and concentrated under reduced pressure at 40 °C to yield a dry extract of 231 g (21%). Part of the dry extract (100 g) was partitioned in 400 ml of distilled water and subjected successively to solvent fractionation with dichloromethane (CH_2Cl_2), ethyl acetate (EtOAc), and *n*-butanol (*n*-BuOH) (3 × 400 ml) until complete exhaustion in each fractionation step to yield a dichloromethane fraction (RTSM1) (7.3 g), ethyl acetate (RTSM2) (20 g), *n*-butanol (RTSM3) (8.7 g), and an aqueous fraction (RTSM4) (13.8 g). The EtOAc fraction (RTSM2) was subjected to gel filtration chromatography by using a Sephadex LH-20 column (Pharmacia) (90 × 4 cm) and EtOH as the mobile phase. The fractions (50 ml each) were collected and examined by thin-layer chromatography (TLC; Silica gel 60 F254 plate, Merck, Kenilworth, NJ, USA) using EtOAc-HOAc-HCOOH- H_2O (30/0.8/1.2/8) as the solvent system. The TLC plate was examined under UV (254 and 366 nm) before and after spraying with vanillin H_2SO_4 (reagent A) and Neu's spray reagent (reagent B). A total of 86 fractions were collected, and similar fractions were combined to yield seven groups: RTSM2-I, fr. 1–15; RTSM2-II, fr. 16–20; RTSM2-III, fr. 21–35; RTSM2-IV, fr. 36–45; RTSM2-V, fr. 46–60; RTSM2-VI, fr. 61–74; and RTSM2-VII, fr. 75–86. The sub-fractions was subjected to reversed-phase HPLC (Jupiter Proteo 90 Å, 250 × 10 mm, 4 μm) using a 5–70% CH_3CN – H_2O gradient over 40 min, and the following compounds were obtained: (2), 16.1 mg yellow amorphous powder; (4), 7.9 mg yellow amorphous powder, and (5), 11.6 mg yellow amorphous powder from RTSM2-II; (3) and (6), 10.2 and 3.8 mg yellow amorphous powders, respectively, from RTSM2-III; (7) and (1), 14.1 and 11.3 mg yellow amorphous powders, respectively, from RTSM2-IV; (8), (9) and (10), 9.1, 12.6 and 5.5 mg yellow amorphous powders, respectively, from RTSM2-V.

2.4. 1,1-Diphenyl-2-picrylhydrazyl (DPPH) radical-scavenging assay

The radical-scavenging activity of the isolated compounds against DPPH^{*} was assessed with a rapid TLC screening method using 0.2% DPPH in MeOH. At 30 min after spraying, the active compounds appeared as yellow spots against a purple background [20,21].

2.5. Cell culture and compounds preparation

Human umbilical vein endothelial cells (HUVEC 16549) were maintained in Dulbecco's modified Eagle medium-GlutaMax (DMEM-GlutaMax) medium (Gibco, Gaithersburg, MD, USA), supplemented with 10% bovine serum (Gibco) and 1 × penicillin-streptomycin mix (Invitrogen, Carlsbad, CA, USA) at 37 °C with 5% CO₂ in a humid chamber. Stocks of compounds (1, 5, 7–10; 1 mg, each) were prepared by first dissolving in 50 µl DMSO (Sigma, Munich, Germany) and then in complete medium (1 mg/ml; DMSO <0.1%, final), followed by reconstitution in six working concentration (25, 12.5, 6.25, 3.12 and 1.56 µg/ml) in complete medium. The DMSO (<0.1%) acted as an untreated or negative control. The standard pro-apoptotic agent MGO [22] and anti-apoptotic drug aminoguanidine (AG) [23] were also prepared in DMSO and culture medium.

2.6. Cytotoxicity assay

The cytotoxic effect, if any, of compounds (1, 5, 7–10) was tested on HUVECs using the cell proliferation assay (TACS cell proliferation MTT assay Cell Proliferation Assay Kit, Trevigen, Gaithersburg, MD, USA). Briefly, HUVECs (0.5×10^5 /well) were seeded in a 96-well flat-bottom plate (Becton–Dickinson Labware, Bedford, MA, USA) and incubated overnight. The cells were treated with the different doses (in triplicate) of the test compounds, including untreated (DMSO) and positive (MGO) controls. At day 3 post-treatment, cells were treated with MTT reagent (10 µl/well) and incubated at room-temperature for 4 h in the dark. Immediately upon the appearance of purple color, detergent solution (100 µl/well) was added and the cells were further incubated for 1.5 h at 37 °C. The optical density (OD; $\lambda = 570$) was measured (Microplate reader ELx800; BioTek, Winooski, VT, USA). Non-linear regression analysis was performed (Excel 2010; Microsoft Corp., Redmond, WA, USA) to determine the cell proliferation fraction using the following equation:

$$\text{Cell proliferation fraction} = \frac{\text{ODs} - \text{ODb}}{\text{ODc} - \text{ODb}}$$

where ODs, ODb, and ODc are the absorbance of sample, blank, and negative control, respectively. Data were subjected to non-linear regression analysis (Excel) and presented as % cell survival in relation to the untreated control.

2.7. Anti-apoptotic/cytoprotective assay

The two non-cytotoxic compounds 1 and 7 were further tested for their cytoprotective/anti-apoptotic potential on HUVECs. In brief, HUVECs (0.5×10^5 /well) were seeded in a 96-well flat-bottom plate (Becton–Dickinson Labware) and incubated

overnight. The pro-apoptotic agent MGO (0.5 mM) was added to the cells followed by co-treatment with different doses (25, 12.5, 6.25 and 3.12 µg/ml) of 1 and 7 as well as the standard anti-apoptotic drug AG (0.05 mM) in triplicate. At day 3 post-incubation, MTT assay was performed as in section 2.6 and OD were measured. Data were subjected to non-linear regression analysis and presented as % increase in cell proliferation/survival in relation to the standard control.

2.8. Apoptotic signaling (caspase-3/7) assay

To assess *in vitro* caspase-3/7 activation, HUVECs (0.5×10^5 /well) were treated with the anti-apoptotic compounds 1 and 7 (25 µg/ml, each), AG (0.05 mM), and MGO (0.5 mM), and were assayed on day 3 (Apo-ONE-cas3/7 Assay Kit; Promega, Madison, WI, USA) as per the supplied manual. Briefly, caspase-3/7 reagent was added (100 µl/well) and cells were incubated in the dark at room temperature for 5 h. The OD was measured, and data were subjected to non-linear regression analysis and presented as % inhibition of caspase-3/7 activity in relation to the standard control.

3. Results and discussion

3.1. Identification of isolated compound

A radical-scavenging guided phytochemical study on the stem bark of *R. tripartita* led to the isolation and identification of 10 flavonoids (Fig. 1). The structures of these compounds were elucidated by extensive 1D and 2D NMR analyses, accurate mass measurements, and comparison with reported data. The flavonoids were identified as catechin (2) [24], gallicocatechin (3) [24], taxifolin (4) [25], epicatechin (5) [24], epigallocatechin (6) [24], epicatechin-3-O-rhamnoside (7) [26], mesuaferone-A (8) [27], myricetin-3-O-glucopyranoside (9) [28], and 2'',3''-dihydrohinokiflavone (10) [29]. All physical and spectral data of these compounds were in agreement with the respective published data.

Rhuspartin (1) was obtained as a yellow amorphous solid ($[\alpha]_D^{25} 144.7^\circ$ (c 0.3, MeOH)). High-resolution electrospray ionization mass spectrometry (HRESIMS) showed a pseudomolecular ion peak at m/z 561.1400 $[M-H]^-$ (calcd 561.1402) consistent with a molecular weight of 562 amu. The molecular formula was established as C₃₀H₂₆O₁₁, implying 18 degrees of unsaturation. The UV spectrum in MeOH showed absorption bands at λ_{max} 215 and 282 nm, while the IR spectrum showed strong absorption bands at 3387 (OH), 1620 (C=C) and 1165 (C-O, 2° alcohol) cm⁻¹.

The ¹H, ¹³C, and DEPT-135 NMR spectroscopy (Table 1) in combination with 2D ¹H-¹³C heteronuclear single quantum correlation (HSQC) analysis of (1) in DMSO-*d*₆ (supplementary data, S2–S7) revealed the presence of one *sp*³ methylene group H-4' [δ_H 2.64 (1H, dd, *J* = 5.0, 16.1 Hz) and δ_H 2.44 (1H, dd, *J* = 7.0, 16.1 Hz); δ_C 27.9]; one *sp*³ methine H-4 [δ_H 4.49 (1H, d, *J* = 2.0 Hz); δ_C 30.8]; four oxygen-bearing *sp*³ methines H-2 [δ_H 5.21 (1H, brs); δ_C 79.9], H-3 [δ_H 4.35 (1H, m); δ_C 70.7], H-2' [δ_H 4.43 (1H, d, *J* = 7.1 Hz); δ_C 80.5], and H-3' [δ_H 3.74 (1H, m); δ_C 66.4]; ten *sp*² aromatic methines grouped by the ¹H-¹H COSY experiment into four spin systems of one 1,2,3-trisubstituted aromatic spin

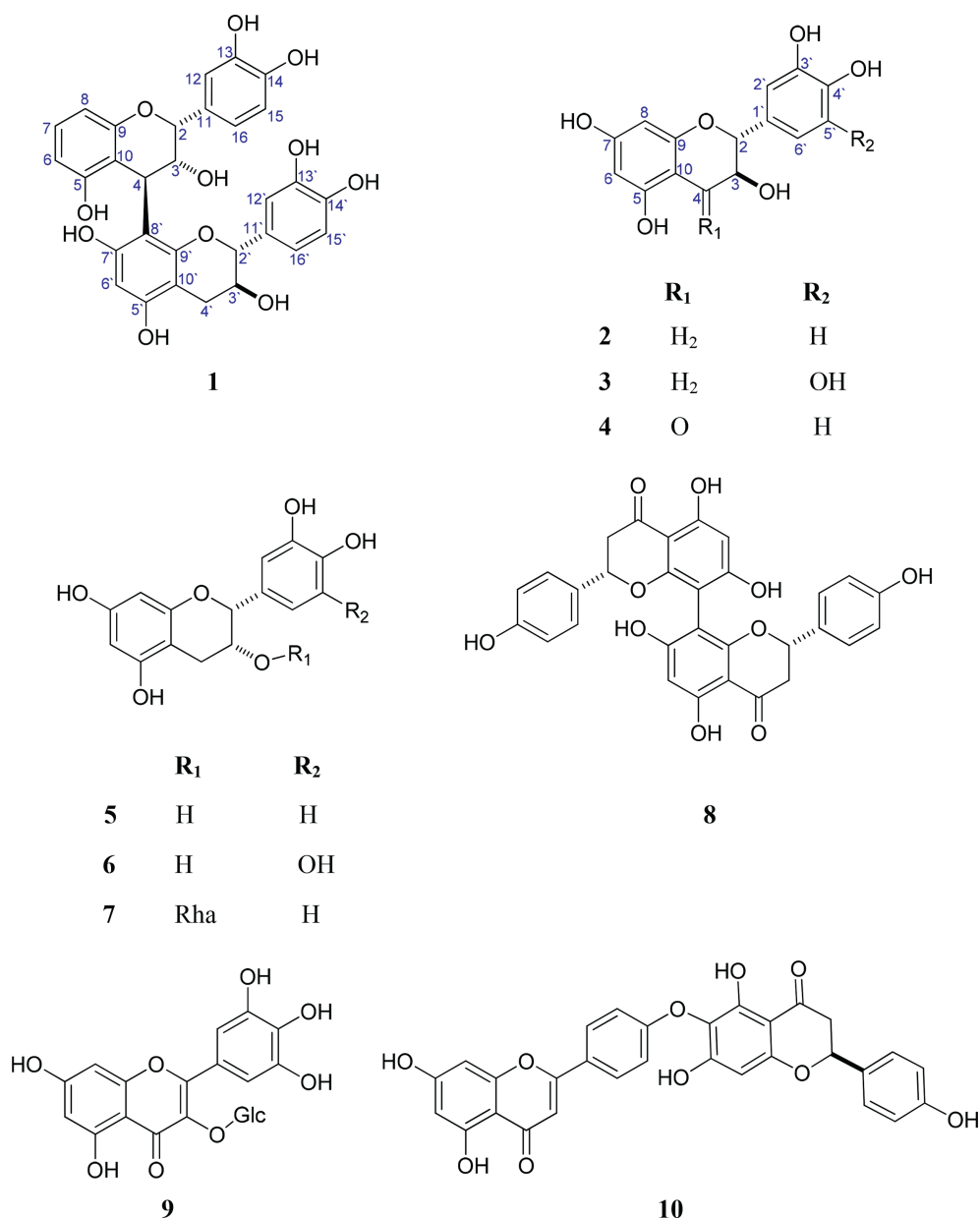


Fig. 1 – Chemical structures of the isolated compounds (1–10); Glc: glucose, Rha: rhamnose.

system H-6 [δ_{H} 6.15 (1H, d, $J = 8.0$ Hz), δ_{C} 107.8], H-7 [δ_{H} 6.34 (1H, m), δ_{C} 128.8], and H-8 [δ_{H} 6.33 (1H, d, $J = 8.0$ Hz), δ_{C} 102.3] of ring A upper unit; an isolated aromatic proton H-6' [δ_{H} 5.91 (1H, s), δ_{C} 97.0] of ring A lower unit; and two ABX aromatic systems of H-12 [δ_{H} 6.69 (1H, s), δ_{C} 113.0], H-15 [δ_{H} 6.50 (1H, d, $J = 8.0$ Hz), δ_{C} 115.7], and H-16 [δ_{H} 6.48 (1H, d, $J = 8.0$ Hz), δ_{C} 115.8] of ring B upper unit; and H-12' [δ_{H} 6.64 (1H, s), δ_{C} 114.4], H-15' [δ_{H} 6.71 (1H, d, $J = 8.0$ Hz), δ_{C} 115.5], and H-16' [δ_{H} 6.28 (1H, d, $J = 8.0$ Hz), δ_{C} 116.9] of ring B lower unit. In addition, 14 quaternary carbon atoms comprising nine oxygenated aromatic carbons C-5, C-9, C-13, and C-14 (δ_{C} 156.6, 153.9, 145.1, and 144.5 ppm, respectively) of the upper unit and C-5', C-7', C-9', C-13', and C-14' (δ_{C} 155.1, 155.3, 153.6, 144.5, and 144.4 ppm, respectively) of the lower unit; and five non-oxygenated aromatic carbons C-10, C-11, C-8', C-10', and C-11' (δ_{C} 112.7, 130.9, 104.4, 99.5, and 130.4 ppm, respectively) were observed. Nine hydrogen

resonances lacked correlations in the HSQC spectrum of 1 and were therefore recognized as being located on hetero-atoms that were identified subsequently as hydroxyl protons for OH-3 [δ_{H} 6.84 (1H, brs)] and OH-3' [δ_{H} 4.92 (1H, d, $J = 4.2$ Hz)], in addition to seven hydroxyls on aromatic systems OH-5, OH-13, OH-14, OH-5', OH-7', OH-13', and OH-14' [δ_{H} (8.63–9.24)].

The NMR data indicated the structure of dimeric proanthocyanidins with two flavan-3-ol moieties. The presence of two flavanyl units was determined from the ^1H and ^{13}C NMR data, with the occurrence of two sets of distinct key signals C-2, C-3, and C-4 for the upper unit, and C-2', C-3', and C-4' for the lower unit [30,31]. The resonance changes of sets indicated that conformational isomerism may exist, which caused by the steric interaction (restricted rotation) between the upper and lower units. This kind of isomerism is well known and observable in many cases of procyanidin oligomers.

Table 1 – ^1H (700 MHz) and ^{13}C NMR (175 MHz) data (in $\text{DMSO}-d_6$) for compound **1**.

no.	7	
	δ_{C} , mult.	δ_{H} , mult (J in Hz)
Upper Unit		
2	79.9, CH	5.21, brs
3	70.7, CH	4.35, m
4	30.8, CH	4.49, d (2.0)
5	156.6, C	
6	107.8, CH	6.15, d (8.0)
7	128.8, CH	6.34, m
8	102.3, CH	6.33, d (8.0)
9	153.9, C	
10	112.7, C	
11	130.9, C	
12	113.0, CH	6.69, s
13	145.1, C	
14	144.5, C	
15	115.7, CH	6.50, d (8.0)
16	115.8, CH	6.48, d (8.0)
OH-3		6.84, brs
OH-5		8.63–9.24, s
OH-13		8.63–9.24, s
OH-14		8.63–9.24, s
Lower Unit		
2'	80.5, CH	4.43, d (7.1)
3'	66.4, CH	3.74, m
4'	27.9, CH_2	a. 2.64, dd (5.0, 16.1) b. 2.44 dd (7.0, 16.1)
5'	155.1, C	
6'	97.0, CH	5.91, s
7'	155.3, C	
8'	104.4, C	
9'	153.6, C	
10'	99.5, C	
11'	130.4, C	
12'	114.4, CH	6.64, s
13'	144.5, C	
14'	144.4, C	
15'	115.5, CH	6.71, d (8.0)
16'	116.9, CH	6.28, d (8.0)
OH-3'		4.92, d (4.2)
OH-5'		8.63–9.24, s
OH-7'		8.63–9.24, s
OH-13		8.63–9.24, s
OH-14		8.63–9.24, s

An analysis of the vicinal coupling constants of the C-ring protons H-2 and H-3 revealed the nature of the units. A large value (7.1 Hz) for $J_{2,3}$ indicated a (+)-catechin unit (2,3-*trans*) for the lower unit and a broad singlet indicated an (–)-epicatechin unit (2,3-*cis*) or epicatechin derivatives (3,5,13,14-flavantetrol unit) [31]. Thus, the upper unit was identified as 3,5,13,14-flavantetrol, whereas the lower unit was identified as (+)-catechin.

The ^1H - ^{13}C heteronuclear multiple bond correlation (HMBC) analysis of **1** (Fig. 2A) showed the connectivity of the unit based on the observed correlation between H-4 and C-3, C-9, and C-8', indicating that the connection between the upper and lower units was established between C-4 and C-8'. Comparison of **1** with compounds **2** and **5** showed that

dimerization at C-8' led to a loss of proton H-8' and a downfield shift of C-8' from δ_{C} 94.5 in (**2**) to δ_{C} 104.4 in (**1**). Moreover, downfield shifts were also observed for C-4 from δ_{C} 28.3 in (**5**) to δ_{C} 30.8 in (**1**), for C-3 from δ_{C} 65.0 in (**5**) to δ_{C} 70.7 in (**1**), and for C-2 from δ_{C} 78.1 in (**5**) to δ_{C} 79.9 in (**1**). Furthermore, key HMBC correlations from H-7 (δ_{H} 6.34) to C-5 (δ_{C} 156.6) accompanied by the ^1H - ^1H COSY correlation between H-7/H-6, and H-7/H-8 indicated the loss of the hydroxyl group at C-7 and confirmed the 3,5,13,14-flavantetrol unit.

The absolute configuration at the chiral centre C4, and thus the interflavanoid linkage, was determined from CD spectroscopic data and suggested to be β -configuration (4R) owing to a positive Cotton effect at 220–240 nm (Fig. 2B) [31,32]. The systematic conformational analysis was performed by the MOE software package with the Merck Molecular Force Field MMFF94 [33]. The electronic circular dichroism (ECD) was determined for the stable conformers obtained and the geometry was optimized at the B3LYP/6-31G(d) level using the Gaussian09 program package [34]. The overall ECD spectra were then generated according to the Boltzmann weighting of each conformer. Comparison of the theoretical spectra with the experimental spectra revealed a good agreement between the calculated β -configuration (4R) and the measured ECD curves (Fig. 2B), whereas the diastereomer α -configuration (4S) showed the opposite results. Thus, based on the above evidence, compound **1** was determined to be 3,5,13,14-flavantetrol-(4 β →8)-catechin and named rhuspartin.

3.2. Cytotoxicity assay

Although compounds **5**, **8**–**10** showed high toxicity toward HUVECs, compounds **1** and **7** were non-toxic even at the highest dose (50 $\mu\text{g/ml}$). The determined 50% cytotoxicity concentration (CC_{50}) values of the tested compounds (in order) were **5** (2.75 $\mu\text{g/ml}$), **10** (8.52 $\mu\text{g/ml}$), **8** (16.47 $\mu\text{g/ml}$), **9** (18.35 $\mu\text{g/ml}$), **1** (215.65 $\mu\text{g/ml}$) and **7** (235.25 $\mu\text{g/ml}$).

3.3. Anti-apoptotic or cytoprotective potential of compounds **1** and **7**

The MTT assay showed the dose-dependent anti-apoptotic/cytoprotective activities of compound **1** (Fig. 3A) and compound **7** (Fig. 3B) against MGO-induced apoptosis in HUVECs. Among the four doses tested, the best activity was observed with the highest dose (25 $\mu\text{g/ml}$) for **7** (approximately 64%) and **1** (approximately 62%), nearing that of the AG (0.05 mM)-treated cells that exhibited approximately 71% reversal of cell apoptosis in relation to MGO toxicity. Tested safe doses above 25 $\mu\text{g/ml}$ did not show significant enhancement in their activities (data not shown).

3.4. Apoptotic signaling (caspase-3/7) assay

Further insight into the possible mechanism of HUVEC cytoprotecting or anti-apoptotic activity of compounds **1** and **7** (optimal dose 25 $\mu\text{g/ml}$) was revealed through downregulation of cellular caspase-3/7 by about 41% and 38%, respectively

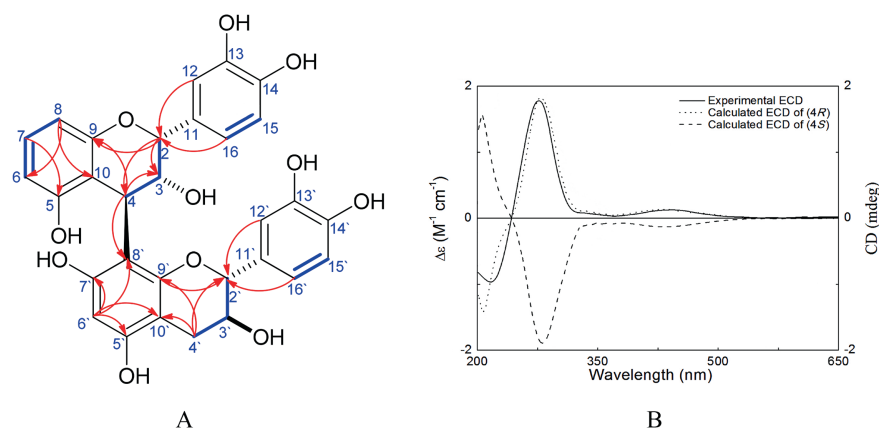


Fig. 2 – A) Selected COSY (—) and HMBC (H→C) correlations for compound 1. B) Attribution of the absolute configuration of compound (1) by comparing calculated CD spectra with experimental spectra.

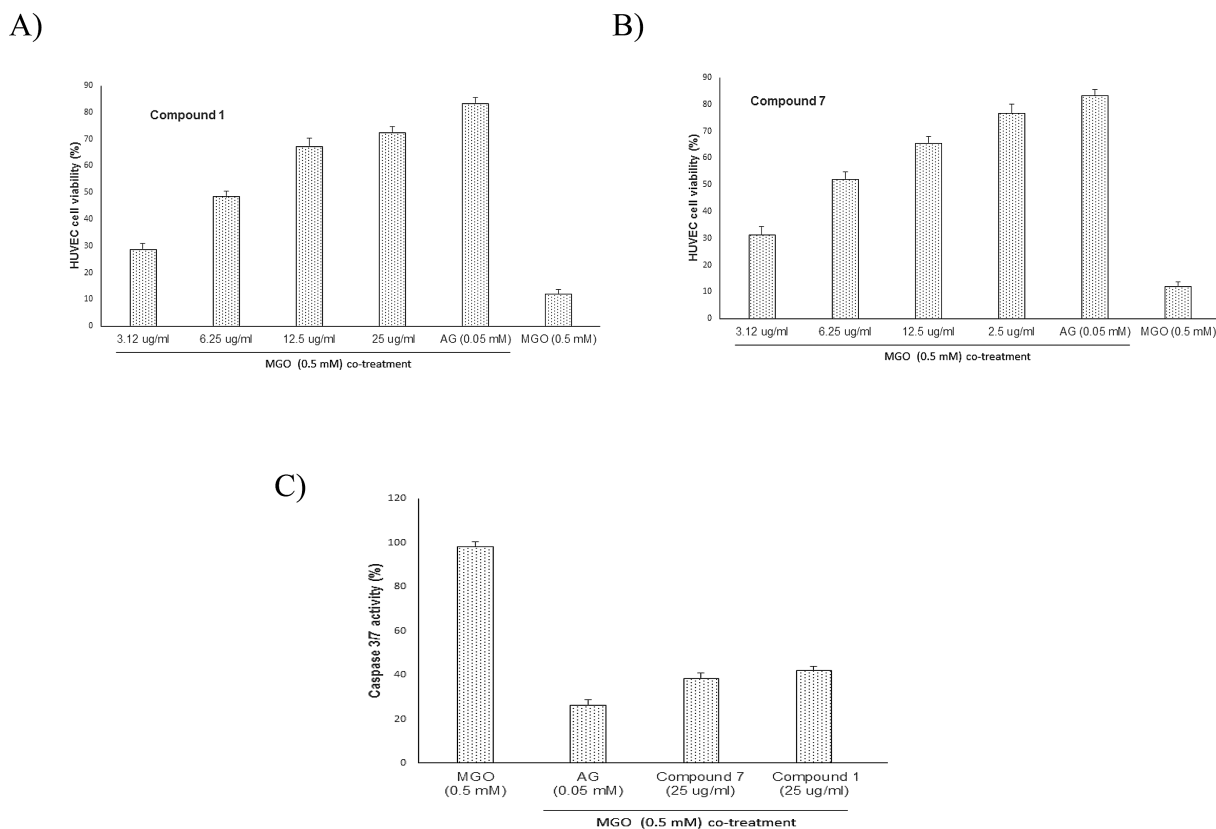


Fig. 3 – MTT assay showing the dose-dependent anti-apoptotic/cytoprotective activity of *R. tripartita* derived compounds 1 (A) and 7 (B) against MGO-toxicity in cultured HUVEC cells. (C) Apoptosis assay showing inhibition of MGO-induced cellular caspase-3/7 activation by *R. tripartita* derived compounds 1 and 7 in cultured HUVEC cells.

compared to AG (approximately 26%) in relation to MGO alone treated cells (Fig. 3C).

This is consistent with the multiple known mechanisms that mediate the damaging effect of MGO on the cells including oxidative stress and disruption of the functionality of cellular proteins, which has negative impact on cell signaling [35]. Similar therapeutic effects have been considered to underlie the effectiveness of a number of chemical compounds which previously shown to protect endothelial

cells against carbonyl stress-induced cellular impairments through both antioxidative mechanisms and the modulation of apoptotic gene expression [36].

4. Conclusions

Ten flavonoids, including a new proanthocyanidin (1), were isolated and identified from *R. tripartita* stem bark. The

structural determination was performed using extensive NMR and HRESIMS analysis. Of the isolated compounds evaluated for anti-apoptotic effects on cultured HUVECs, the new compound 1 as well as 7 showed cytoprotective potential that reversed the MGO-induced apoptosis through downregulation of caspase3/7.

These data contribute toward the exploration of the structural diversity and biological activity of flavonoids for the prevention and treatment of diabetic cardiovascular diseases. Furthermore, the identified new compound represents a potentially valuable addition to the growing number of proanthocyanidins being isolated from the genus *Rhus*.

Conflicts of interest

All authors declare no conflict of interest.

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List of abbreviations

AG	aminoguanidine
DMSO- d_6	deuterated dimethylsulfoxide
DPPH	1,1-diphenyl-2-picrylhydrazyl
ECs	endothelial cells
ECD	electronic circular dichroism
HMBC	heteronuclear multiple bond correlation
HRESIMS	high-resolution electrospray ionization mass spectrometry
HSQC	heteronuclear single quantum correlation
HUVEC	human umbilical vein endothelial cells
MGO	methylglyoxal
MTT	MTT3-[4,5-dimethylthiazole-2-yl]-2,5-diphenyltetrazolium) bromide
NMR	nuclear magnetic resonance
TLC	thin-layer chromatography

Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.jfda.2019.02.002>.

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