Suppression of *β*-Cell Apoptosis from H_2O_2-Induced Oxidative Stress in MIN6 cells using Methyl Gallate

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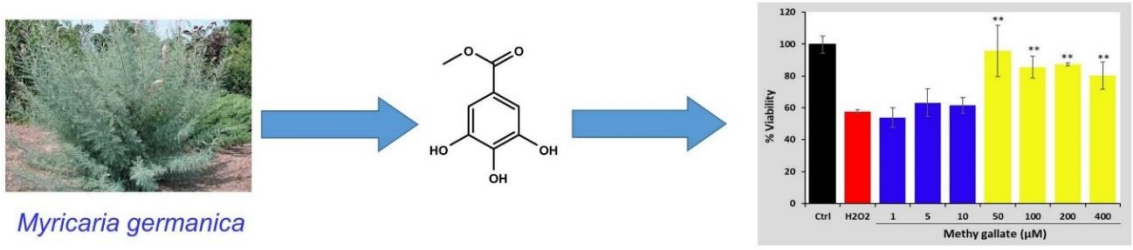
Summary: A major parameter for diabetic relevant diseases and hyperglycemia is the β-cell apoptosis. Anti diabetic drugs used widely these days chiefly target to lower hyperglycemia along with prevention of β-cells from apoptosis. In this study three natural products methyl gallate, syringic acid, and butanedioic acid from *Myricaria germanica* were analyzed for β-cell protection. Methyl gallate provided significant β-cell protection from H_2O_2-induced oxidative stress mediated apoptosis in MIN6 cells at 50 µM (95.5% ± 16.0 vs 57.6% ± 1.1) and at 100 µM (85.5% ± 7.0 vs 57.6% ± 1.1) concentrations.



Keywords: *Myricaria germanica*, Methyl gallate, β-cell protection, Oxidative stress, MIN6 cells.

Introduction

Natural products are attractive candidates for their potential use in the treatment and prevention of human ailments including diabetes. The major parameter for diabetic relevant diseases and hyperglycemia is the β-cell apoptosis. Anti diabetic drugs used widely these days chiefly target to lower hyperglycemia along with β-cell protection from apoptosis. Considering the global increase of diabetes and the literature record that antioxidants present in natural products protect cells by reducing H_2O_2-induced oxidative stress, the present study was planned on the isolation and anti-diabetic activity of the constituents of the plant *Myricaria germanica* as it contains a major array of polyphenols and flavonoids with extensive pharmacological profile. The plant also known as German Tamarisk or German false tamarisk among *Myricaria* species is usually found in China, growing to central Asia and towards Europe and extended to the temperate regions in the Mediterranean area. Genus Myricaria belong to family Tamaricaceae, it has only 4 genera and 110 species [1, 2]. It is a traditional remedial plant of Tibet used as analgesic [2] and for treating jaundice [3]. The leaves extract has been identified as active against microbes and employed as analgesic, to cure chronic bronchitis [2, 4]and joint pains [4, 5]. A number of flavonoids and phenolic compounds are reported from *Myricaria germanica* methyl gallate being one of the important and active compounds [6]. It is one of the highly specific and potent inhibitors against herpes simplex virus and investigated as an antioxidant, antimicrobial [7], free-radical scavenging activity [8, 9] and for inhibitory activity in lipid per oxidation [10]. Three pure compounds methyl gallate, syringic acid, and butanedioic acid were isolated from *Myricaria germanica* and analyzed for β-cell protection.

Experimental

General Experimental Procedures

The research exertion was conducted at HEJ Research Institute of Chemistry, I.C.C.B.S, University of Karachi. Stuff used for isolation and derivatization i.e. chemicals and analytical grade solvents were purchased from Sigma Aldrich (Munich, Germany), Scharlau (Barcelona, Spain). Distilled solvents were used for the purification of compounds through chromatographic technique i.e. column and thin layer chromatography. Yanaco (MP-S3) was used to determine the melting points of pure isolated compounds, in glass capillaries. The ultraviolet (UV) spectra of pure compounds were recorded using Hitachi (3200) Spectrophotometer. For

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obtaining the infrared spectra of the isolated pure compounds JASCO (302-A) IR spectrometer was used.^{1}H-NMR, spectra were recorded on 500 and 600 MHz instruments Bruker AM-400 and AMX-500 spectrometers. Coupling constants (*J*-value) were measured in Hertz, chemical shifts (δ) were recorded in ppm. The EI-MS spectra were recorded on JEOL (JMS 600H-1) mass spectrometer using flame ionization detector (FID).

Plant Material

Myricaria germanica (L.) Desv. leaves (5.5 kg) were collected from Bulchi Bagrote valley (Gilgit) Pakistan in the mid of May-2016. Sher Wali Khan, Head of the Department of Environmental Sciences, Karakuram International University (KIU), Gilgit-Baltistan identified the plant material. A Voucher specimen has been deposited in the herbarium of KIU (Voucher specimen No: 22).

Extraction and Isolation

The air dried leaves (5.5 kg) of *Myricaria germanica* (L.) Desv. were chopped and soaked in methanol for three days at room temperature (25 °C). The process was repeated thrice. The extracts were combined and solvent evaporated under reduced pressure. The resulting extract (0.685 kg) was phase separated between ethyl acetate and water. The ethyl acetate phase was dried (Na_2SO_4), charcoaled, and freed of the solvent. The residue was divided into petroleum ether soluble (100 g) and insoluble (25g) fractions. The petroleum ether insoluble fraction was subjected to column chromatography on silica gel and eluted with hexane-ethyl acetate (100:0 to 0:100) and then DCM-MeOH (100:0 to 0:100) to obtained 195 sub-fractions. Based on TLC, sub fractions were combined to obtain 10 fractions (Fr-1.0 to Fr-10.0). From fractions, Fr-1.0 and Fr-2.0 two pure compounds were obtained on washing with cold pet ether and identified as methyl gallate (**1**) and syringic acid (**2**) respectively. Another pure compound separated out on washing Fr-3.0 with cold dichloromethane and identified as dibutanoicacid (**3**) (Fig. 1).

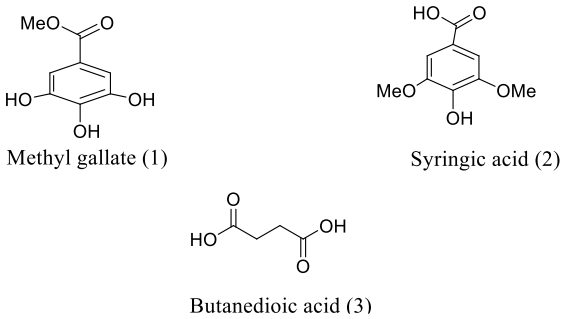
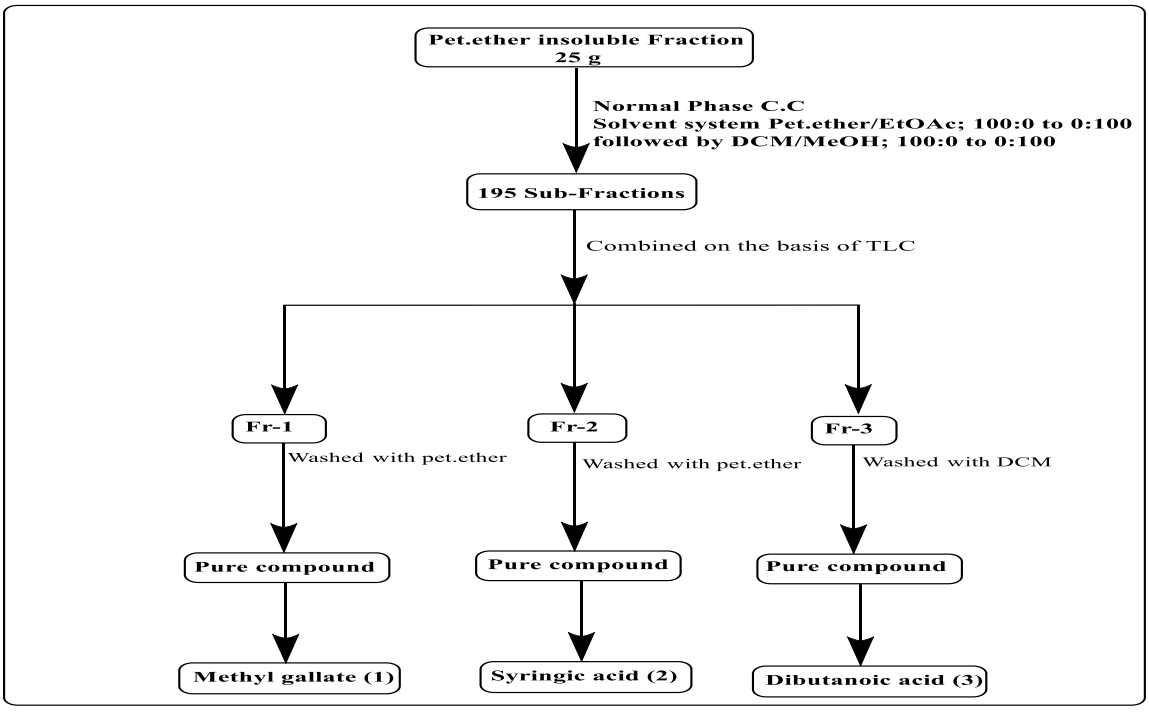


Fig. 1: Structures of the isolated compounds.



Scheme-1: Isolation Scheme.

MTT Assay

With seeding density of $4x10^4$MIN6 cells/well, cells were seeded in 96 well plate and incubated. Then treated with different doses of methyl gallate, after 3 hours H_2O_2 were added and incubated for 24 hours and further incubated for 3 more hours to observe cell viability. Morphology of cells were examined by phase contrast microscopy. Readings were taken at 550 nm wavelength.

Cell viability assay was done with methyl thiazolyl tetrazolium (MTT) colorimetric assay. Pre-incubate cells with compounds and then with H_2O_2at O_2.CO_2 at 95.5 (%). After removing media, MTT was added. After 24 hours, DMSO was added to solubilize the formalin crystals and absorbance were taken at 550 nm.

Results and Discussion

Compound **1** was obtained as white solid. Its EI-MS spectrum showed molecular ion peak at *m/z* 184.0. IR absorption bands appeared at 3400 (OH), 1710.6 (C=O), 3055.5 (ar. C-H) and 2870 (asym-C-H) cm^{-1}. The ultraviolet absorption spectrum showed λ_{max}at 272 nm (Table-1). In the proton NMR spectrum, one singlet appeared at δ 7.02, for two aromatic protons and a three-proton singlet for methoxy protons at δ 3.80. In the ^{13}C-NMR spectrum, C-7 appeared at δ 169.0; both C-3 and C-5at δ 146.47, C-2 and C-6 at δ 110.03; C-4 and C-1 resonated at δ 139.74 and 121.44, respectively. Methoxy carbon was noted at δ 52.24 (Table-2).Based on the above data **1** was identified as methyl gallate.

Table-1: Physical properties of compound **1.**

	Compound-1
Color	**White**
Physical State	**Solid**
MP	**261.50 °C**
UV	**272**
IR (KBr)	**3400.0 (OH), 1735.0 (C=O), 3055.0 (ar. CH), 2870.0 (asym-CH).**
EI-MS [M$^+$] *m/z*	**184**

Table-2: ^{13}CNMR &^{1}HNMR spectroscopic data of compound **1.**

Position	**Chemical Shifts (δ)**		
	Compound-1		
	δ_C		**δ_H**
1	**119.33**	**C**	
2	**108.51**	**CH**	**7.02 s**
3	**145.75**	**C**	
4	**137.40**	**C**	
5	**145.75**	**C**	
6	**108.51**	**CH**	**7.02 s**
7	**166.43**	**C**	
8	**52.32**	**CH_3**	**3.80 s**

Compound **2** was isolated as snow white solid. The EI-MS spectrum exhibited [M$^+$] at *m/z* 198.0. IR absorption bands were noted at 3400 (OH), 1715 (C=O), 3058 (ar. C-H) and 2860 (asym. C-H) cm^{-1}.The absorption spectrum of UV had maxima at 274 nm (Table-3). In the ^{1}H-NMR spectrum, a singlet of two protons appeared at δ 7.15 for aromatic protons and protons of two aromatic methyl groups showed a singlet at δ 3.84.In the ^{13}C-NMR spectrum, C-7 appeared at δ 168.84, C-3 and C-5 at δ 149.12, C-4 at δ 140.61, C-1 at δ 120.6, C-2 and C-6 at δ 106.08, and C-8 and C-9 at δ 56.66 (Table-4).Based on the above data **2** was identified as syringic acid.

Table-3: Physical properties of compound **2.**

	Compound-2
Color	**White**
Physical State	**Solid**
MP	**205-209 °C**
UV	**274**
IR (KBr)	**2800-3400 (COOH), 3400.0 (OH), 1715.0 (C=O), 3058.0 (ar. C-H), 2860.0 (asym-CH).**
EI-MS [M$^+$]*m/z*	**198**

Table-4: ^{13}CNMR &^{1}HNMR spectroscopic data of compound **2.**

Position	**Chemical Shifts (δ)**		
	Compound-2		
	δ_C		**δ_H**
1	**120.64**	**C**	
2	**106.90**	**CH**	**7.15 s**
3	**147.33**	**C**	
4	**137.52**	**C**	
5	**147.33**	**C**	
6	**106.90**	**CH**	**7.15 s**
7	**167.91**	**C**	
8	**54.62**	**CH_3**	**3.86 s**
9	**54.62**	**CH_3**	**3.86 s**

Compound **3** was isolated as snow-white powder. The EI-MS spectrum showed molecular ion peak at *m/z* 118.0. IR absorption bands appeared at 3400 (OH), 1710 (C=O), 2850 (asymmetric-C-H) and UV absorbance spectrum showed maxima at 210 nm (Table-5). In the ^{1}H-NMR spectrum, a singlet appeared at δ 2.55 for H-2 and H-3. In the^{13}C-NMR spectrum, C-1 and C-4 appeared at δ 173.90, C2 and C-3 appeared at δ 28.90 (Table-6). Based on the above data **3** was identified as butanedioic acid (succinic acid).

Table-5: Physical properties of compound **3.**

	Compound-3
Color	**White**
Physical State	**Solid**
MP	**188.0 °C**
UV	**210**
IR (KBr)	**2800-3400.0 (COOH), 1710.0 (C=O), 2850.0 (asym-C-H).**
EI-MS [M$^+$] *m/z*	**118**

Table-6: ^{13}CNMR &^{1}HNMR spectroscopic data of compound **3.**

Position	**Chemical Shifts (δ)**		
	Compound-3		
	δ_C		**δ_H**
1	**173.92**	**C**	
2	**28.90**	**CH_2**	**2.55 s**
3	**28.90**	**CH_2**	**2.55 s**
4	**173.92**	**C**	

B-Cell protection activity of methyl gallate from H_2O_2-induced oxidative stress mediated apoptosis in MIN6 cells. Methyl gallate showed significant β-cell protection activity from H_2O_2-induced oxidative stress mediated apoptosis in MIN6 cells at different concentrations shown in Fig 2.

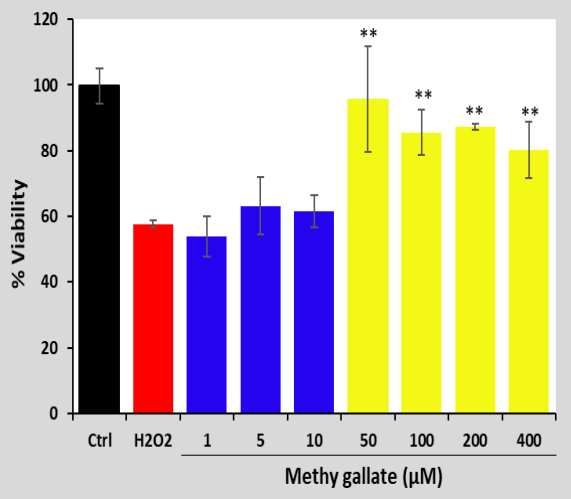


Fig. 2: *In vitro* β-cell protective activity for methyl gallate having different doses.

Table-7 shows values for cell viability mean percentages with standard deviation for the different concentrations of methyl gallate in MTT assay. Significant β-cell protection activity from H_2O_2-induced oxidative stress 95.5 ± 16.0, 85.5 ± 7.0, 87.2 ± 0.9, 80.2 ± 8.5% vs 57.6 ± 1.1% mediated apoptosis in MIN6 cells at 50, 100, 200 and 400µM concentration. Methyl gallate with 50 and 100µM indicated potent in vitro β-cell protective activity of 95.5% and 85.5 % respectively.

Table-7: Cell viability mean percentages with standard deviation for different concentration of methyl gallate in MTT assay.

Methyl gallate	Cell viability mean (%)	Standard Deviation (%)
Control (-ve)	99.6	5.3
H_2O_2 (400 µM)	57.6	1.1
1µM	53.8	6.1
5 µM	63.1	8.7
10 µM	61.5	4.8
50 µM	95.5	16.0
100 µM	85.5	7.0
200 µM	87.2	0.9
400 µM	80.2	8.5
Control (+ ve)	93.2	3.1

There is a global increase of chronic diseases including diabetes which need effective drugs. Plants containing a major array of polyphenols and flavonoids with extensive pharmacological profile offer potential source especially considering that out of 250,000 terrestrial plants, not more than 1% has been screened pharmacologically, and only a fraction of them have been used for T2DM studies. Anti diabetic drugs used widely these days chiefly target to lower hyperglycemia along with β-cell protection from apoptosis since the major parameter for diabetic relevant diseases and hyperglycemia is the β-cell apoptosis. The literature record further shows that antioxidants present in natural products protect cells by reducing H_2O_2-induced oxidative stress. Hence, the present study was undertaken on the isolation of constituents of plant *Myricaria germanica* and determination of the β-cell protection activity of the constituents.

Conclusion

Phytochemical-based therapies offer effective pharmacological approaches for the treatment of diabetes. Hencethis study was conducted on *Myricaria germanica.* Three compounds methyl gallate, syringic acid, and butanedioic acid were isolated and analyzed for β-cell protection. Methyl gallate provided significant β-cell protection from H_2O_2-induced oxidative stress mediated apoptosis in MIN6 cells at 50 µM (95.5% ± 16.0 vs 57.6% ± 1.1) and at 100 µM (85.5% ± 7.0 vs 57.6% ± 1.1) concentrations. It may prove useful as a potential agent in the treatment of disorders associated with oxidative stress–induced cell damage. Further studies are needed to elucidate the mechanism of its action as anti-diabetic agent.

Disclosure statement

There is no potential conflict of interest.

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