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ORIGINAL ARTICLE

Chemical Composition and Neurotherapeutic Potential of *Glaucium corniculatum* Extracts

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ABSTRACT

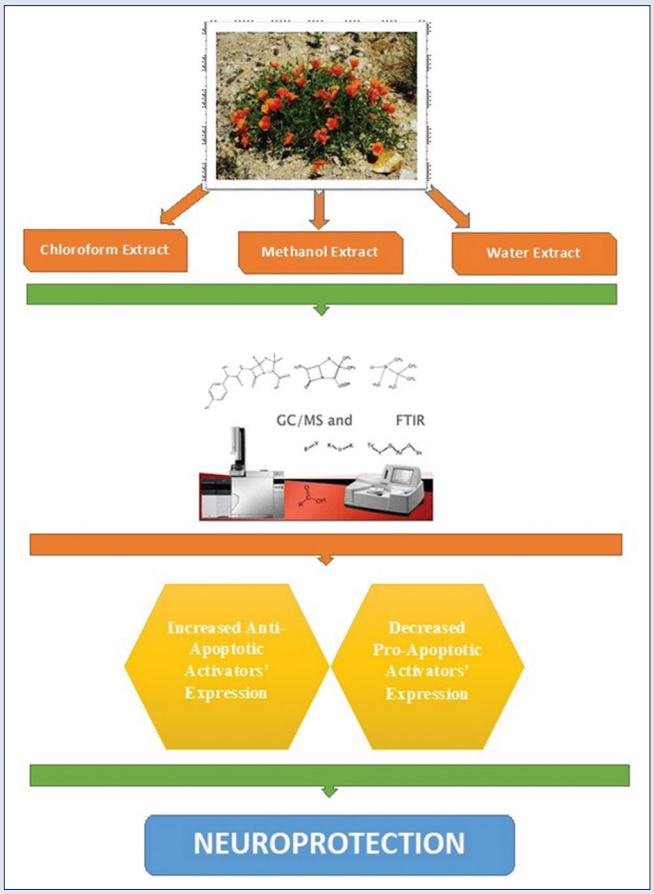
Background: The discovery of natural agents that suppress neural apoptosis is of strategic importance for the treatment of neurodegenerative diseases. *Glaucium corniculatum* has been used as a traditional medicine because of its memory-enhancing and neuroprotective properties. However, there has not been a study about this plant's apoptosis suppressive properties until now. **Objectives:** The present study examines the possible suppressive effects on apoptosis of three different *G. corniculatum* extracts (chloroform, methanol, and water) in neuronal-differentiated PC12 (dPC12) cells induced by hydrogen peroxide (H_2O_2) and presents the first association of compound content differences in extracts with the suppressive property. **Materials and Methods:** The chemical composition of these extracts was analyzed by Fourier transform infrared spectroscopy (FTIR) and gas chromatography-mass spectroscopy. The changes caused by the extracts in apoptotic markers were examined by real-time quantitative polymerase chain reaction and Western blot. **Results:** Extracts contained alkaloid, fatty acid, alcohol, alkane hydrocarbon, ester, ether, alkane, alkene, aliphatic, aromatic, and phenolic compounds with different relative proportions. In all the three extracts, the major compound was allocriptopine. Oleamide and 2-monopalmitin were the major fatty acids, respectively, identified in water and methanol extracts. Extracts were suppressed H_2O_2-induced apoptosis via decreasing the expression of Bax, Caspase-3 and -9, while increasing Bcl-2, in a concentration-dependent manner. The water extract showed the highest apoptosis suppression while methanol was the second. **Conclusion:** *G. corniculatum* extracts suppress apoptosis in dPC12 cells and allocriptopine, oleamide, and 2-monopalmitin may be responsible of apoptosis suppressive effect.

Key words: Alkaloids, apoptosis, chemical composition, fatty acids, *Glaucium corniculatum*, PC12 cell

SUMMARY

- The chemical composition differences in chloroform, methanol, and water extracts of *Glaucium corniculatum*, and the apoptosis-suppressing effects of these extracts on *in vitro* neurodegenerative model were reported for the first time. Chemical content in extracts was analyzed using Fourier transform infrared spectroscopy and gas chromatography-mass spectroscopy. Changes in apoptotic markers caused by extracts were examined by real-time quantitative polymerase chain reaction and Western blot. The extracts were found to increase Bcl-2 in a concentration-dependent manner, while reducing the expression of Bax, Caspase-3 and-9, suppressing hydrogen peroxide-induced apoptosis. It was found that the apoptosis-suppressing effect of the extracts can be exerted by allocriptopin, oleamide, 2-monopalmitine.

Abbreviations used: AChE: Acetylcholine esterase; AD: Alzheimer's disease; ALS: Amyotrophic Lateral Sclerosis; BSA: Bovine serum albümin; DMEM Dulbecco's Modified Eagle's Medium; DMSO: Dimethyl sulfoxide; dPC12: Neuronal-differentiated PC12; FABAL: Ege University Pharmaceutical Sciences Research Laboratory; FTIR: Fourier Transform Infrared Spectrophotometer; GC-MS: Gas chromatography mass spectroscopy; H_2O_2: Hydrogen peroxide; HD: Huntington's disease; $MgCl_2$: Magnesium chloride; NaCl: Sodium chloride; NGF: Nerve growth factor; PD: Parkinson's disease; ROS: Reactive oxygen species; TBS: Tris-buffered saline; UNAM: Bilkent University Institute of Materials Science and Nanotechnology.



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INTRODUCTION

Neurodegenerative diseases, as a group of disorders, are characterized by slowly progressive irreversible neuronal losses. The oxidative stress caused by the increased reactive oxygen species (ROS) has been suggested as a common cause of various neurodegenerative diseases. ROS such as hydrogen peroxide (H_2O_2) are mediators of oxidative stress and cause lipid peroxidation and DNA damage.[1] These damage to the nerve cells causes neural apoptosis and eventually neurodegeneration.[2] Apoptosis is an active, morphologically distinct form of programmed cell death. Apoptosis is defined as the main mechanism responsible for neuronal loss during physiological aging, which controls the number of neurons and glial cells in the central and peripheral nervous system in their developmental stage. Also, it is a critical mechanism that regulates neuronal cell count and neurite density in the developing nervous system. Changes in the expression of Bcl-2 family members lead to an increase of pro-apoptotic proteins (Bax, Bak,…) and a decrease of anti-apoptotic (Bcl-2, Bcl-xL,…) proteins. However, caspase is one of the most important key executioners of apoptosis. The heterodimerization of pro-apoptotic members stimulates Caspase-9 and -3 action. This can lead to damage to the homeostasis of the membrane, nucleus, and cell skeleton, resulting in apoptosis.[3] Neuronal apoptosis may occur in response to stimuli associated with the pathogenesis of many neurodegenerative diseases. The most important of these is oxidative stress. Oxidative stress in the nerve cells acts on apoptosis by various mechanisms such as receptor activation, Caspase activation, Bcl-2 family proteins, and mitochondrial dysfunction and causes neuronal cell death, one of the main and common causes of neurodegenerative diseases such as Alzheimer's disease, Parkinson's disease, Huntington's disease, and amyotrophic lateral sclerosis.[4] In this context, the suppression of pro-oxidant-induced damage in neuronal cells can be a useful way to treat neurodegenerative diseases and diseases associated with aging-related cognitive decline. It is known that plant-based compounds have antioxidant and apoptosis-suppressing properties.[5-7] Therefore, plants containing these bioactive components may exhibit potential protective/therapeutic properties in neurodegenerative processes.

G. corniculatum is a traditional herbal agent, which has been widely used to brain-associated diseases such as forgetfulness.[8] Previous studies suggested that *G. corniculatum* extracts are rich in alkaloids, flavonoids, and fatty acids and have neuroprotective effects such as anti-inflammatory and anti-acetylcholinesterase (AChE).[9-13] Because these processes are common mechanisms of neurodegenerative events, it can be said that *G. corniculatum* extracts have a preventive/suppressive effect on neurodegeneration. However, *G. corniculatum* extracts' inhibiting effects on apoptosis caused by ROS and the neurotherapeutic effects have not been studied. In order to determine the source of these effects, it is very important to identify the active ingredients in the extracts. Therefore, in this study, the suppressive effects of three different extracts of *G. corniculatum* prepared with chloroform, methanol, and water solvent on H_2O_2-induced neuronal-differentiated PC12 (dPC12) cells were investigated at both gene and protein levels and the relationship between these effects and the different component contents of the extracts was tried to put forward.

MATERIALS AND METHODS

Cell lines, chemicals, and biochemicals

Pheochromocytoma PC12 cells were obtained from the Bilkent University Institute of Materials Science and Nanotechnology. High glucose Dulbecco's Modified Eagle's Medium (DMEM), horse serum, foetal bovine serum, L-glutamine, penicillin-streptomycin, bovine serum albumin, dimethyl sulfoxide, sodium bicarbonate, Tris-HCl, H_2O_2, sodium chloride (NaCl), magnesium chloride ($MgCl_2$), 1% Triton X-100, and Bradford reagent were purchased from Sigma-Aldrich (St. Louis, MO). Nerve growth factor (NGF) was purchased from Millipore (Billerica, MA, USA). Collagen type I from rat tail was purchased from Millipore Sigma (Burlington, MA, USA). Unless stated otherwise, all other reagents were from Sigma.

Plant material

G. corniculatum (L.) *RUD.* subsp. *refractum* (NAB.) CULLEN plants were collected from Beypazari district, northwest of Ankara, on July 27, 2015, and verified by Prof. Dr. Zeki AYTAÇ, Gazi University, Turkey. These samples were deposited in Gazi University, Herbarium of Faculty of Science. The herbarium plant number is ZA10700.

Preparation of plant extracts

Above-ground tissues of the plant samples were dried under shade milled to a powder using a grinder. Plant powder (10 g) was macerated with 150 mL of chloroform, methanol, and water, at 60°C–100°C for 4 h with a soxhlet device with heat regulator (LabHeat, Wärmetechnik GmbH, Mörlenbach, Germany). The extracts were filtered by using a 0.22-µm filter and evaporated to dryness (40°C) under reduced pressure by a rotary evaporator (Heidolph Laborota 4000, Schwabach, Germany) and stored in a refrigerator at 4°C until the time of use. The % yield was expressed as the mass of dried extract/mass of 1 g dried powder. The yields of extracts, per 1 g of dried powder, were 0.13%, 0.49%, and 0.39% in chloroform, methanol, and water extract, respectively.

Cell culture

The PC12 cells differentiate into neurite-like cells by NGF treatment, therefore, widely used as an *in vitro* model in neuroscience research, including studies of neurobiology, neuronal differentiation, neuroprotective, and neurotherapeutic events.[14] In this study, H_2O_2-induced dPC12 cells were used as a classical injury model to investigate the apoptosis suppression effect.

PC12 cells were maintained in DMEM supplemented with 10% fetal bovine serum, 10% horse serum, penicillin (100 U/mL), and streptomycin sulfate (100 µg/mL) in a humidified atmosphere of 5% CO_2. The medium was sterilized by using a 0.22-µm filter. PC12 cells were seeded onto T-75 cm^2 flasks coated with 50 ng/mL rat tail collagen, to achieve 70% confluence. The cells were subcultured every 2–3 days. 100 ng/mL of NGF was added to the media for 4 days to convert PC12 cells into neurite-differentiated cells.

Cell treatments

The dPC12 cells (1×10^6) were challenged with the oxidant H_2O_2 (100 µM for 24 h), in the presence or absence of different concentrations (100, 500, and 1000 µg/mL) of plant extracts for 24 h. The following five groups were formed:

- Group I: Control (untreated)
- Group II: H_2O_2 treated
- Group III: Pretreatment with H_2O_2 before *G. corniculatum* chloroform extracts
- Group IV: Pretreatment with H_2O_2 before *G. corniculatum* methanol extracts
- Group V: Pretreatment with H_2O_2 before *G. corniculatum* water extracts.

RNA isolation and gene expression study by real-time quantitative polymerase chain reaction

After treatment with H_2O_2 and extracts, the cells were harvested and washed with phosphate-buffered saline (PBS). Total RNA was isolated

using the RNeasy Mini Kit (74104-Qiagen, USD) following manufacturer's protocol. cDNA was prepared with 1 µg RNA as starting material using QuantiTect Rev. Transcription Kit (205311-Qiagen, USA) following the manufacturer' protocol. The reverse transcription reaction was performed sequentially for 15 min. at 42°C and for 15 min. at 95°C. Melt-curve analysis was used to determine the specificity of the amplified products. Applied Biosystems® 7500 Fast Real-Time polymerase chain reaction (PCR) System and SensiFAST™ SYBR Low-ROX Kit (BIO94005-BIOLINE, UK) were used for the PCR analysis. The gene-specific primers are shown in Table 1.

Western blot analysis

Total protein extracts from untreated/extracts-treated cells were subjected to Western blot analysis.[20] dPC12 cell treatment groups were washed with PBS. The cells were lysed in a buffer containing 1 M Tris buffer (pH 7.2), 2 M NaCl, 1 M $MgCl_2$, and 1% Triton X-100. The scraped cells were collected in sterile tubes. The tubes were vortexed at 4°C for 30 min. and clarified at 10,000 rpm for 20 min. at 4°C. The protein content was estimated using a Bradford assay and ultraviolet spectrophotometer. The cell lysates were loaded onto a 4%–12% gel and separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The bands of protein were transferred to polyvinylidene difluoride membranes using Trans-Blot Turbo RTA Transfer Kit, Mini, PVDF, for 40 Blots kit (1704272-Bio-Rad, USA). A solution of 2% nonfat dried milk in Tris-buffered saline (TBS) containing 0.1% Tween-20 was used to block non-specific binding for 30 min at room temperature before incubating with antibodies for 1 h. The membrane was treated for 1 h with specific antibodies dissolved in the block solution. The expression patterns of antibodies were detected using GAPDH was used as loading control after three washes with TBST for 10 min. Each membrane was incubated with a secondary antibody (Goat anti-rabbit IgG [H + L], AP conjugated secondary antibody) for 1 h at room temperature. After three washes with TBST, the blots were detected using an AP conjugate substrate kit (1706432-Bio-Rad, USA) and were used according to the manufacturer's instructions and analyzed using a Western blot detection system (ChemiDoc™ XRS + System-Bio-Rad, USA).

Fourier transform infrared spectroscopy analysis

Dried extract powder (10 mg) of each plant were encapsulated in 10 mg of KBr pellet, in order to prepare translucent sample discs. The powder of each plant sample was loaded in Fourier transform infrared spectroscopy (FTIR) spectroscopy (Shimadzu, IR Affinity1, Japan), with a scanning range from 400 to 4000 cm^{-1} with a resolution of cm^{-1}.

Gas chromatography-mass spectroscopy analysis

The phytochemical investigation of methanolic extract was performed at Ege University Pharmaceutical Sciences Research Laboratory on a gas chromatography-mass spectroscopy (GC-MS) equipment (Thermo Scientific Co.) Thermo GC-TRACE ultra ver.: 2.0, Thermo MS DSQ II. Experimental conditions of GC-MS system were as follows: HP-5 MS column, dimension: 30 m, ID: 0.25 mm, Film thickness: 0.25 µm. Flow rate of mobile phase (carrier gas: He) was set at 0.8 mL/min. In the gas chromatography part, temperature programme (oven temperature) was 100°C raised to 180°C at 15°C per min. It was increased from 180°C to 300°C with an increase of 5°C per min. The temperature was held at 300°C for 10 min. The injector temperature was 250°C. Samples dissolved in methanol and the results were compared by using Wiley Spectral library search program.

Statistical analysis

All experiments were conducted in triplicate. Statistical analysis was performed by SPSS 16 (SPSS Inc., Chicago, IL, USA). Statistical differences between control and experiment groups were examined using paired Student's *t*-test. For Western blot analysis, *t*-test (Excel 2007) was performed. Values of $P < 0.05$ were considered statistically significant. The results were reported as means ± standard deviation unless otherwise indicated.

RESULTS

Findings of content analysis in the extracts

The data on the peak values and the probable functional groups (obtained by FTIR analysis) present in the extracts (prepared in chloroform, methanol, and water solvents) of *G. corniculatum* are presented in Tables 2-4. The FTIR spectrums of *G. corniculatum* extracts are presented in Figure 1. The results of FTIR analysis confirmed the presence of alcohol, ester, ether, alkane, alkene, aliphatic, aromatic, and phenolic compounds.

Various bioactive compounds such as alkaloid, fatty acid, alkane hydrocarbon, and alcohol were found in extracts by GC-MS. In each of three extracts, the major compound was alkaloid and the water extract contained the most alkaloid. In all three extracts, the major alkaloid was allocriptopine (35.7% in chloroform extract, 51.6% in methanol extract, and 48.4% in water extract). Moreover, in all extracts, 4H-berberine N-oxide was second and tetrahydropalmatine was the third order common alkaloid. 9.8% fatty acid and 80.5% alkaloid were detected in chloroform extract; 15.8% fatty acid, 4.4% alkane hydrocarbon and 79.8% alkaloid were determined in methanol extract; and 4.8% alcohol, 7.5% fatty acid, and 87.7% alkaloid were detected in the water extract. The major fatty acid in chloroform extract was linoleic acid (3.7%).

Table 1: Forward and reverse primer polymerase chain reaction sequences for real-time polymerase chain reaction[15-19]

Primers	Sequence of nucleotides (nt) 5'-3'	Size (nt)	PCR product length (bp)
Bax	F CTGCAGAGGATGATTGCTGA	20	207
	R GAGGAAGTCCAGTGTCCAGC	20	
Caspase-3	F TGGCCCTGAAATACGAAG	18	210
	R GGCAGTAGTCGCCTCTGA	18	
Caspase-9	F GGATCTGGACTGCGTCTCATCAA	23	190
	R CCGAGACCTTGGAACACAGAGAA	23	
Bcl-2	F ATCGCTCTGTGGATGACTGAGTAC	24	134
	R AGAGACAGCCAGGAGAAATCAAAC	24	
GAPDH	F CAACTCCCTCAAGATTGTCAGCAA	24	118
	R GGCATGGACTGTGGTCATGA	20	

All qRT-PCR tests were performed in triplicate, repeated at least 2 times. An equal amount of that cDNA was taken to do PCRs for several apoptotic genes with GAPDH as an internal control. PCR: Polymerase chain reaction; qRT-PCR: Real-time quantitative PCR; GAPDH: Glyceraldehyde-3-phosphate dehydrogenase

Table 2: Fourier transform ınfrared spectroscopy analysis of *Glaucium corniculatum* chloroform extract

n	Wave number/cm	Functional group assignment	Compounds
1	3320.82	O–H stretching of carboxylic acid groups	Carbonyl compound
2	3006.48	O–H stretching band	Hydroxy group
3	2913.91-2846.42	C–H stretch	Aliphatic compound
4	1731.76	C=O stretching	Ester
5	1652.7	C=O amide stretching vibrations	Ketone compound
6	1600.63-1542.77-1498.42	Aromatic C=C stretching	Aromatic compound
7	1461.78	C–H, aliphatic asymmetric bending	Aliphatic compound
8	1376.93-1 -1340.28	CH3, –CH2, CH aliphatic symmetric bending	Aliphatic compound
9	1272.79	R–CO–O–R', C–O–C asymmetric stretch	Aliphatic ester
10	1236.15	C–N stretching vibrations	Alkene compound
11	1162.87	C–O–C, symmetric stretch	Ether
12	1120.44	Aromatic C–H deformation	Alkane
13	1081.87	C–O stretching	Alcohol
14	1033.66	C–O stretching	Alcohol
15	985.447	C–H bending vibration	Alkane
16	933.378	C–H bending vibration	Alkane
17	862.025	C–H out of plane bending	Aromatic compound
18	802.242	A trisubstituted double bond	Alkene compound
19	717.39	Mono-substituted benzene ring	Phenolic compound
20	647.965	C–Br stretch, aliphatic	Aliphatic bromo compound

Table 3: Fourier transform ınfrared spectroscopy analysis of *Glaucium corniculatum* methanol extract

Number	Wave number/cm	Functional group assignment	Compounds
1	3342.03	O–H stretch, polymeric OH	Hydroxy compound
2	2921.63-2854.13	C–H stretch, aliphatic	Alkane compound
3	1733.69	C=O stretching	Ester
4	1643.05-1604.48-1500.35	Aromatic C=C stretching	Aromatic compound
5	1461.78	C–H, aliphatic asymmetric bending	Aliphatic compound
6	1365.35-1346.07	CH3, –CH2, CH aliphatic symmetric bending	Aliphatic compound
7	1280.5-1236.15	R–CO–O–R', C–O–C asymmetric stretch	Aliphatic ester
8	1160.94	C–O–C, symmetric stretch	Ether
9	1116.58	Aromatic C–H deformation	Alkane compound
10	1078.01	C–O stretching	Alcohol
11	1033.66	C–O stretching	Alcohol
12	985.447	C–H bending vibration	Alkene compound
13	927.593	C–H bending vibration	Alkene compound
14	865.882	C–H out of plane bending	Aromatic compound
15	823.455	A trisubstituted double bond	Alkene compound

Table 4: Fourier transform ınfrared spectroscopy analysis of *Glaucium corniculatum* water extract

Number	Wave number/cm	Functional group assignment	Compounds
1	3251.4	O–H stretch, polymeric OH	Hydroxy compound
2	2937.06	C–H stretch, aliphatic	Alkane compound
3	1610.27-1571.7-1538.92	C=C stretching	Aromatic compound
4	1461.78	C–H, Aliphatic asymmetric bending	Aliphatic compound
5	1378.85	CH3, –CH2, CH aliphatic symmetric bending	Aliphatic compound
6	1274.72-1236.15	R–CO–O–R', C–O–C asymmetric stretch	Aliphatic ester
7	1193.72	Not found	
8	1135.87	C–O–C, symmetric stretch	Ether
9	1122.37	Aromatic C–H deformation	Alkane compound
10	1081.87	C–O stretching	Alcohol
11	1037.52	C–O stretching	Alcohol
12	985.447	C–H bending vibration	Alkene compound
13	871.667	C–H out of plane bending	Aromatic compound
14	821.527	A trisubstituted double bond	Alkene compound
15	655.679	C–Br stretch, aliphatic	Aliphatic bromo compound

2-Monopalmitin (9.4%) was the major fatty acid that is specific to methanol extract, while oleamide (7.5%) was the major fatty acid that is specific to water extract. Palmitic acid, ethyl linoleate, and 1,2-Dipalmitin were found only in chloroform; 2-Monopalmitin, AC1NSYIY, and 1-Monopalmitin were found only in the methanol extract; and oleamide was found only in water extract [Table 5].

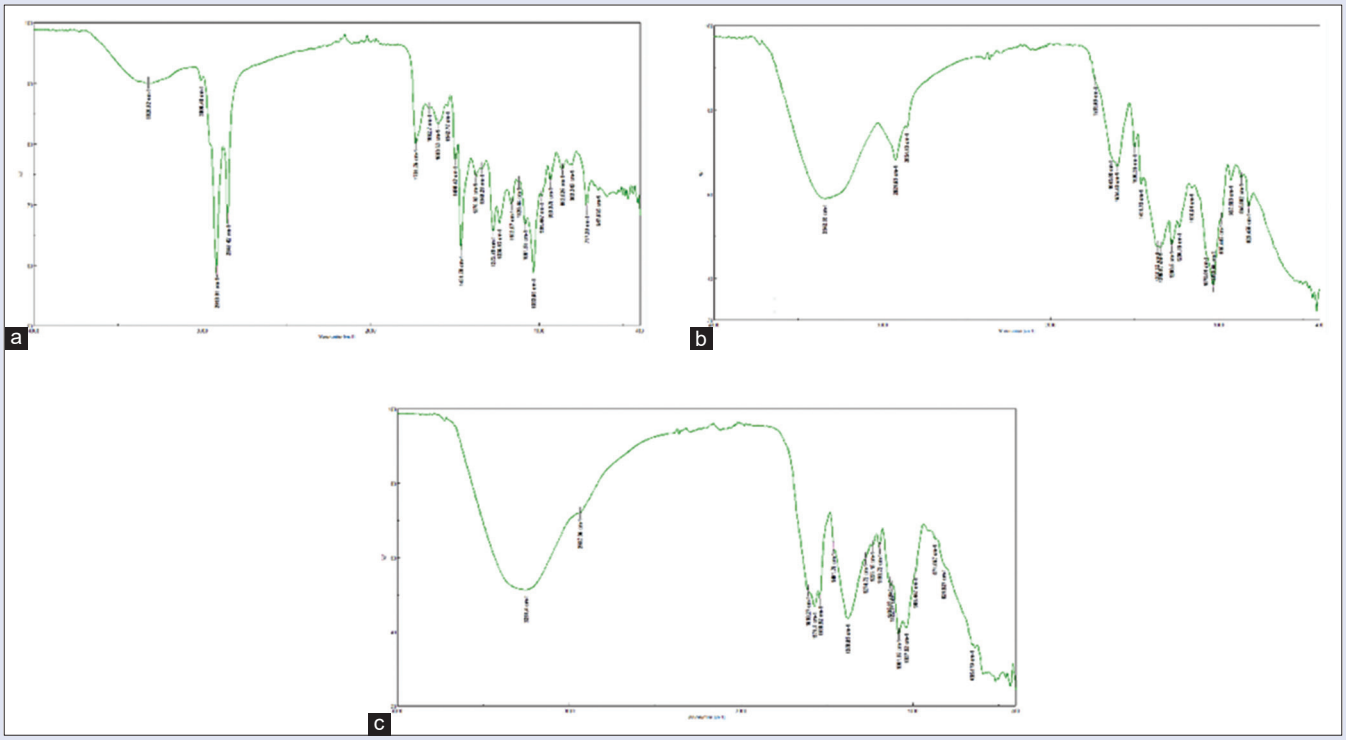

Figure 1: Fourier transform infrared spectroscopy spectrums wave numbers of *Glaucium corniculatum* extracts (a) chloroform extract, (b) methanol extract, (c) water extract

Table 5: Phytocomponents identified in the chloroform, methanol, and water extracts of *Glaucium corniculatum* by gas chromatography-mass spectroscopy

Compound	Synonym	Compound quantities in extracts (%)		
		Chloroform	Methanol	Water
Alkaloids				
6H-Dibenzo[a, g] quinolizine, 5,8,13,13a-tetrahydro-2,3,9,10-tetramethoxy-, (ñ)-	Tetrahydropalmatine	18.7	13.0	13.1
Tetrahydroberberine N-oxide	4H-Berberine N-oxide	26.1	15.2	26.2
[1,3]Benzodioxolo[5,6-e][2]benzazecin-14 (6H)-one, 5,7,8,15-tetrahydro-3,4-dimethoxy-6-methyl	Allocryptopine	35.7	51.6	48.4
Fatty acids				
n-Hexadecanoic acid	Palmitic acid	1.8	–	–
9,12-Octadecadienoic acid (Z, Z)-	Linoleic acid	3.7	2.0	–
Linoleic acid ethyl ester	Ethyl linoleate	1.9	–	–
Hexadecanoic acid, 1-(hydroxymethyl)-1,2-ethanediyl ester	1,2-Dipalmitin	2.4	–	–
Hexadecanoic acid, 2,3-dihydroxypropyl ester (CAS)	1-Monopalmitin	–	1.6	–
8,11-Octadecadienoic acid, methyl ester	AC1NSYIY	–	2.8	–
Hexadecanoic acid, 2-hydroxy-1-(hydroxymethyl) ethyl ester	2-Monopalmitin	–	9.4	–
9-Octadecenamide, (Z)-	Oleamide	–	–	7.5
Alkane hydrocarbon				
1H-Indene, 1-hexadecyl-2,3-dihydro- (CAS)	1-n-Hexadecylindan	–	4.4	–
Alcohol				
Phenol, 2,6-bis (1,1-dimethylethyl)-4-methyl- [CAS])	Butylated hydroxytoluene	–	–	4.8

CAS: Caspase

Glaucium corniculatum extracts modulated the expression of apoptosis-related mRNAs expression Induced by hydrogen peroxide

The gene expression results showed that H_2O_2 significantly increased Bax, Caspase-3 and-9 while decreased Bcl-2 gene expression relative to the control, non-treated dPC12 cells ($^*P < 0.05$ Significance level of the H_2O_2 treated group compared to the control group) [Figure 2]. However, a significant decrease in expression of Bax, Caspase-3 and-9 and a significant increase in the Bcl-2 gene were observed in dPC12 cells treated with *G. corniculatum* extracts (100–1000 µg/mL), relative to the H_2O_2-treated group. The effect of the 1000 µg/mL extract concentration on mRNA levels was statistically significant compared to the H_2O_2-treated group ($^{\#}P < 0.05$ The significance level of the extract applied groups compared to the H_2O_2 treated group), whereas the effect at concentrations of 100 and 500 µg/mL was variable. Although there was no obvious difference between extracts, the water extract concentration of 1000 µg/mL was the best effective application in all mRNA

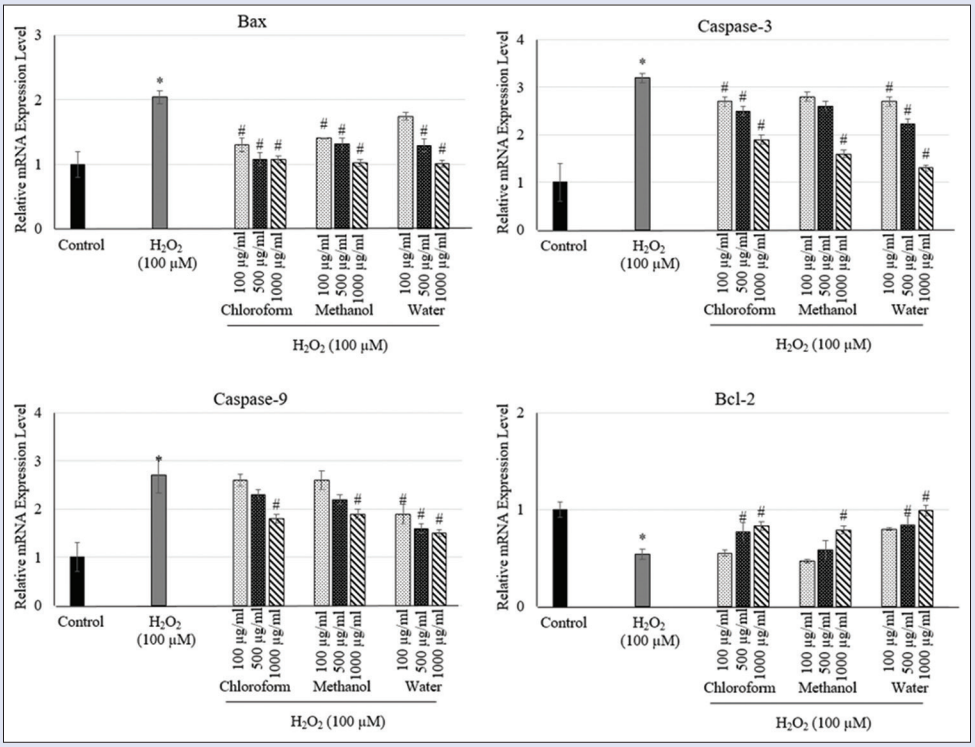

Figure 2: The effect of H_2O_2 and *Glaucium corniculatum* chloroform, methanol and water extracts applied after H_2O_2 on the expression levels of target genes in dPC12 cells .*$P < 0.05$ versus control, #$P < 0.05$ versus H_2O_2 alone. dPC12: Neuronal-differentiated PC12; H_2O_2: Hydrogen peroxide

expressions. The inhibition caused by the 1000 µg/mL concentration of water extract compared to H_2O_2-treated group was 50 ± 0.3% in Bax, 59 ± 0.3% in Caspase-3, and 44 ± 0.2% in Caspase-9 mRNA expressions. The induction caused by the water extract at a concentration of 1000 µg/mL compared to the H_2O_2 applied at the level of Bcl-2 mRNA was 100 ± 0.1%.

Glaucium corniculatum extracts modulated the expression of apoptosis-related proteins expression induced by hydrogen peroxide

After exposure to 100 µM H_2O_2, the Bax, Caspase-3 and-9 protein expressions increased and Bcl-2 decreased, according to the control, non-treated dPC12 cells (*$P < 0.05$). These trends were reversed by *G. corniculatum* extracts in a dose-dependent manner. Consistently, the upregulation of cleaved Bax, Caspase-3 and-9, which are pro-apoptotic markers, was significantly suppressed by posttreatment with *G. corniculatum* extracts. Also, the downregulation of Bcl-2 reversed by posttreatment with *G. corniculatum* extracts. Although there was no obvious difference between extracts, the water extract concentration of 1000 µg/mL was the best effective application in all the protein expressions (#$P < 0.05$). Protein expression results confirmed the gene expression data [Figure 3].

DISCUSSION

Recently, researchers have made considerable efforts to search for natural substances with neurotherapeutic potential, and particular attention has been paid to medicinal plants. *G. corniculatum* has been used as a memory-enhancing agent since ancient times.[8] There has been mounting evidence that the extracts of *G. corniculatum* possess significant neuroprotective activity with anti-inflammation and anti-AChE effects.[11-13] However, there have been no previous reports regarding the relationship between the active compounds in *G. corniculatum* and apoptosis suppression effect against H_2O_2-induced cell toxicity in dPC12 cells. This is the first study to report that *G. corniculatum* extracts have potential as an apoptosis suppressor agent. Also, this is the first report to demonstrate that these plant extracts inhibited caspase signaling triggered by the demodulation of Bcl-2 family members in H_2O_2-damaged dPC12 cells.

Changes in the chemical structure of the substances change the solubility properties in the solvents and thus different chemical profiles can be produced in the extractions prepared with different solvents. In studies conducted with plant metabolites, extracts prepared with different solvents are used to expand the metabolic content.[21] In this study, chloroform, methanol, and water extracts of *G. corniculatum* were prepared and experimental studies were performed by using different concentrations of these three extracts.

In our previous study, we determined that 200 µM and higher H_2O_2 concentrations cause more than 50% PC12 cell death in 24 h of application.[12] Therefore, in this study, 100 µM H_2O_2 (35%–40% inhibition) was used to examine the neurotherapeutic effects of *G. corniculatum* extracts in dPC12 cells. H_2O_2 is a well-known and widely used oxidative stress agent.[22] The chronic or high levels of oxidative stress in cells cause neuronal cell death.[23] We examined whether H_2O_2-induced oxidative stress resulted in apoptosis. The balance between the expression levels of Bax, Caspase-3,-9 and Bcl-2, the genes and proteins responsible for apoptosis, plays a critical role in determining the fate of cells, survival, or death. In this study, we confirmed that H_2O_2 treatment induced pro-apoptotic factors (Bax, Caspase-3,-9) and reduced anti-apoptotic factor (Bcl-2) at the gene and protein levels. We found that *G. corniculatum* extracts suppressed the increase of pro-apoptotic factors and the decrease of anti-apoptotic factor in H_2O_2-treated dPC12 cells. The gene and protein expression results demonstrated that the

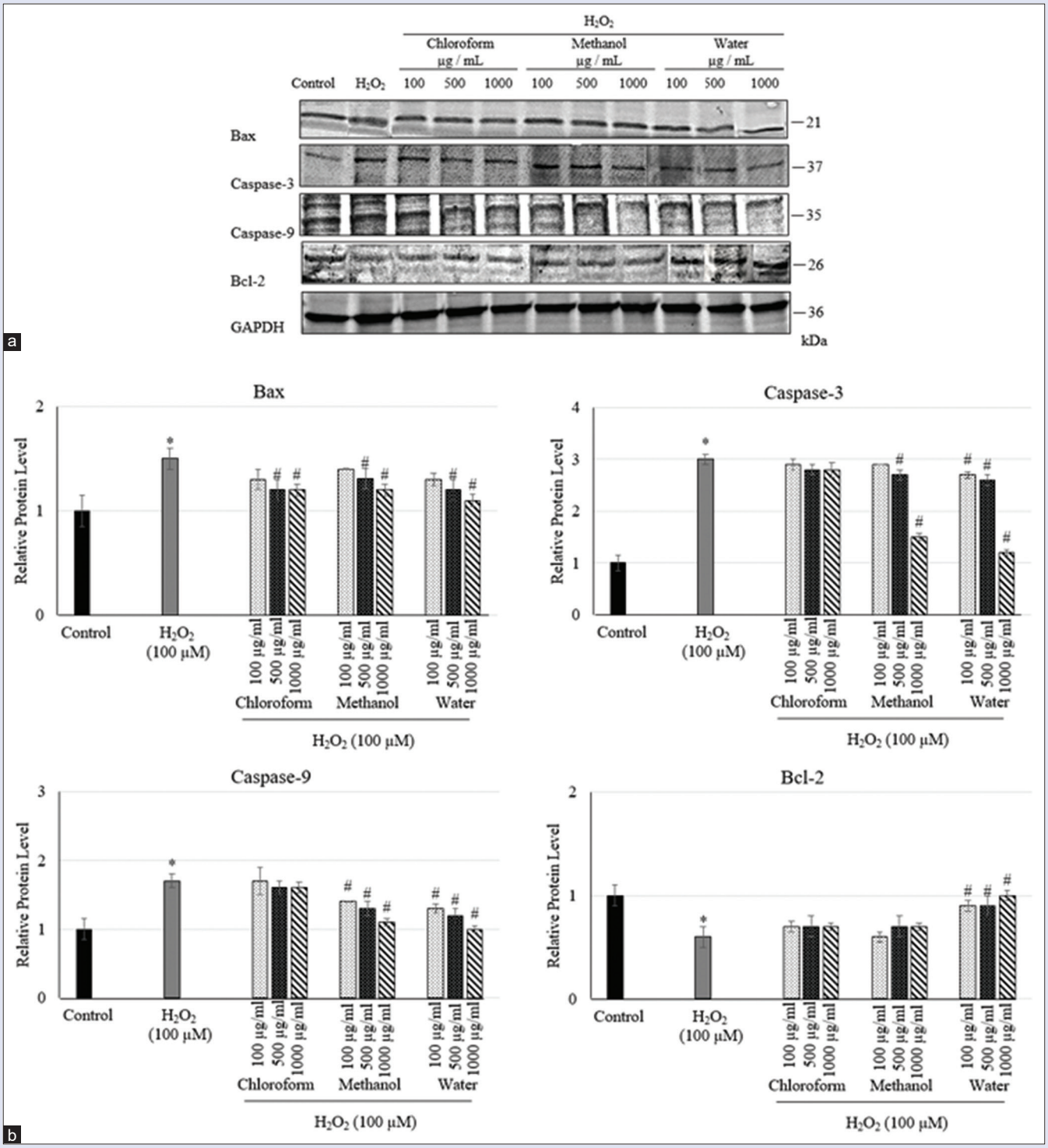

Figure 3: The effect of H_2O_2 and *Glaucium corniculatum* chloroform, methanol and water extracts applied after H_2O_2 on the expression levels of Bax, Caspase-3,-9 and Bcl-2 proteins in dPC12 cells. (a) Representative Western blot results. (b) The average relative quantitative expressions. *$P < 0.05$ versus control, #$P < 0.05$ versus H_2O_2 alone. dPC12: Neuronal differentiated PC12; H_2O_2: Hydrogen peroxide

ability to suppress apoptosis by *G. corniculatum* extracts associated with a downregulation of Bax, Caspase-3 and-9 and upregulation of Bcl-2. Previous studies have suggested that neuronal apoptosis could be suppressed by inhibition of Bax, Caspase-3 and-9 and induction of Bcl-2.[24,25] Hence, decreasing Bcl-2 family expression (Bax and Bcl-2 ratio) might indicate that the extracts of *G. corniculatum* suppress apoptosis in dPC12 cells and strongly correlates with the intrinsic mitochondrial apoptotic signaling pathway.

When the extracts were evaluated in terms of apoptosis suppressor properties at both gene and protein levels, all extracts were similar but effective. The highest apoptosis suppression effect in all genes and proteins expressions was determined in the water extract. However,

in the methanol extract at a concentration of 1000 mg/mL, significant suppression in Caspase-3 and-9 proteins was noted. Accordingly, it can be said that water extract is the most effective in suppressing apoptosis and methanol extract is effective in second order. It is estimated that the ability of all extracts studied here to suppress apoptosis in dPC12 cells could be related to their content.

In our study, the possible component contents of *G. corniculatum* extracts were analyzed by FTIR and GC-MS. The results showed that the extracts contained alkaloid, fatty acid, alcohol, alkane hydrocarbon, ester, ether, alkane, alkene, aliphatic, aromatic, and phenolic compounds. Several *in vivo* and *in vitro* studies have reported that bioactive components such as alkaloids and fatty acids are good sources of apoptosis suppressants.[26,27] This suggests that *G. corniculatum* may be a good source of apoptosis-suppressing compounds. Results showed the presence of alkaloids and fatty acids in all the three extracts. This result is similar to the literature in which the *Papaveraceae* family is found to be rich in fatty acids and alkaloids.[28,29] In all the three extracts, the ratio of alkaloids was higher than the other compounds and the major alkaloid was found to be allocriptopine. In a study by Novak *et al*., it was emphasized that allocriptopine was the major alkaloid in G. corniculatum extracts[9] and that allocriptopine was determined in *G. corniculatum* extracts.[30] Our results supported these studies. Other alkaloids in the extracts were 4H-Berberine N-oxide (tetrahydro berberine) and tetrahydropalmatine. Tetrahydroberberine is a berberine-derived alkaloid and has been demonstrated by several studies showing the anti-inflammatory, antioxidant and anti-apoptotic properties of berberine alkaloids.[31-33] Also, previous studies have shown that the destruction of allocriptopine and tetrahydro berberine are effective in apoptotic death processes.[34-36]

The alkaloid yield of the extracts was water > methanol > chloroform. Thus, water extract with the best suppressive effect of apoptosis was determined to be the richest extractor from these compounds. A positive correlation was found between the apoptosis-suppressing effect and the alkaloid ratio in the extracts. This suggests that there may be a relationship between the alkaloid ratio in the extracts and the apoptosis-suppressing effect.

In our study, it was determined that the fatty acid composition in the extracts showed differences. The amounts of fatty acids in the extracts are in the form of methanol > chloroform > water. This result is consistent with studies indicating that fatty acids are well soluble in organic solvents such as methanol and chloroform compared to water.[37,38] In the results of the fatty acid composition in the extracts, linoleic acid in chloroform extract, 2-Monopalmitin in methanol extract and oleamide in water extract were determined as major. These results are similar to the previous study in which *G. corniculatum* major fatty acids were determined.[28] In the chloroform extract, the major fatty acid was linoleic acid and the other fatty acids such as palmitic acid, ethyl linoleate and 1,2-Dipalmitin also prevent. 2-Monopalmitin, AC1NSYIY and 1-Monopalmitin fatty acids, which are found only in the methanol extract and 2-Monopalmitin was a major compound for methanol extract. Similarly, oleamide was the only fatty acid in the water extract while it was not found in chloroform and methanol extracts. The presence of the most effective apoptosis-suppressing effect in water and methanol extracts suggested that the major fatty acids (2-Monopalmitin and oleamide) in these extracts may be responsible for this effect. There are studies supporting this belief. The previous studies have shown that 2-Monopalmitin plays a role in the activation of many anti-apoptotic gene-activating systems.[39] In addition, oleamide has been shown to be effective in apoptotic death processes.[34-36]

Although there are some studies showing that alkaloids and fatty acids are effective on neurodegenerative diseases due to antioxidant and apoptosis-suppressing effects,[6,40,41] no studies have investigated the *G. corniculatum* extracts bioactive compound content's relationship with these mechanisms. Our findings suggest that *G. corniculatum* may serve as a powerful suppressor in the treatment of apoptosis-induced neurodegeneration. In this study, it was determined that *G. corniculatum* extracts' phytochemicals such as alkaloids and fatty acids may be related to apoptosis-suppressive effect. Furthermore, it was determined that the content of *G. corniculatum* extract varies with the extraction solvent and the apoptosis suppressing effect of each extract differs. Therefore, we suggest that *G. corniculatum* might enhance the cellular apoptotic condition through the synergistic effects of the combination of all bioactive components and this may be better than the use of the active compounds alone. We believe that these discoveries support the potential benefit of *G. corniculatum* extracts as a novel apoptosis suppressor agent which should be used in future clinical studies.

CONCLUSION

This study revealed for the first time that chloroform, methanol, and water extracts of *G. corniculatum* exert apoptosis-suppression activity by increasing the anti-apoptotic Bcl-2 and decreasing the activation of pro-apoptotic Bax, Caspase-3 and-9 in neurite-like cells. Hence, these extracts show the therapeutic potential for neurodegenerative diseases. The highest effect was determined in water extract and secondly in methanol extract and the level of their capability to induce apoptosis was concentration dependent. The chemical differences between the extracts that can explain the difference in effect seen between the extracts were determined by extensive chemical identification. It was shown that the different alkaloids and fatty acids in the extracts may be related to apoptosis suppression. The present study suggests that these extracts contain bioactive compounds and these may be neurotherapeutic agents that can be used to treat neurodegenerative diseases. However, future *in vitro* and *in vivo* studies can be highly helpful for a detailed assessment of these compounds.

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Conflicts of interest

There are no conflicts of interest.

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