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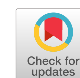
Phytochemical screening and evaluation of neuroprotective, anti-mutagenic and anti-genotoxic effects of Turkish endemic *Glaucium acutidentatum*



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ABSTRACT

Glaucium species (*Papaveraceae*) are the medicinal plant that has been used traditionally to have been used for centuries to treat memory impairment. The *aim* of this *study* is to investigate the *G. acutidentatum* methanol and water extracts' phytochemicals and neuroprotective, anti-inflammatory, anti-mutagenic and anti-genotoxic potentials.
Chemical composition was screened by using capillary gas chromatography–mass spectrometry (GC–MS). Neuroprotective effect was analyzed on nerve growth factor (*NGF*) differentiated-PC12 (dPC12) cells from neuroinflammation and neurodegeneration. Also, these extracts were screened for the mutagenic and anti-mutagenic activity by the *Salmonella*/microsome test system. In addition, genotoxic profiles and anti-genotoxic effects of these extracts were also analyzed by Comet technique.
Extracts of *G. acutidentatum* had strong neuroprotective effects against hydrogen peroxide (H_2O_2)-induced damage. Also, neurite length was dose-dependently increased in extracts exposed groups compared with the H_2O_2-treated group. The anti-inflammatory effect of these extracts was parallel to neuroprotective effect. However, these extracts showed strong anti-mutagenicity (72.9–75.0%) and anti-genotoxic properties. Any genotoxic effect was observed of these extracts in lymphocyte cells, analyzed by the Comet assay.
These results suggest that methanol and water extracts of *G. acutidentatum* had neuroprotective and anti-mutagenic effects and contained protective substances that decreasing damage to genetic material.
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1. Introduction

Neurodegenerative diseases (ND), defined as chronic and progressive diseases, are characterized by loss of neurons and axons in the central nervous system (CNS), sensory or cognitive systems. Diseases with different clinical phenotypes and genetic etiologies such as Alzheimer's disease (AD), Parkinson's disease (PD), Huntington's disease (HD), schizophrenia, and amyotrophic lateral sclerosis (ALS) are all formed by the neurodegeneration process. It is known that approximately 30 million people worldwide are affected by ND and it is estimated that by 2040, casualties caused by ND will be only surpassed by cancer (Stansley et al., 2012). Therefore, early diagnosis and treatment of NH are of crucial importance. Some drugs, approved by U.S. Food and Drug Administration (FDA), are used for treatment and prevention of ND (Lleo et al., 2006). The existing drugs against these diseases only delay the disease progression by alleviating the symptoms; however, they do not provide a cure and has failed to prevent the underlying degeneration of neurons. There is a desire to develop new alternative strategies which are capable of preventing the progressive neuronal loss caused by neuroinflammation and neurodegeneration.

Some plants have traditionally been used for centuries to treat memory impairment. One of this genus is *Glaucium*, belonging to the family of *Papaveraceae* (Orhan et al., 2004; Ahmed et al., 2013). There are some studies that demonstrate that various species of the *Glaucium* genus' secondary metabolites have anti-microbial, anti-bacterial, anti-inflammatory, anti-tumoral and analgesic activity and potency to cure age-related brain disorders (Orhan et al., 2004; Bournine et al., 2013; Soureshjan and Heidari, 2014; Hakemi-Vala et al., 2017). However, the neuroprotective, anti-inflammatory, anti-mutagenic and anti-genotoxic potentials of methanol and water extracts of *G. acutidentatum* Hausskn. et Bornm. is endemic to Turkey and have not been reported yet.

Abbreviations: AD, Alzheimer's disease; ALS, amyotrophic lateral sclerosis; CNS, central nervous system; D.W, distilled water; DMEM, Dulbecco's modified Eagle's medium; DMSO, dimethyl sulfoxide; dPC12, differentiated-PC12; FDA, U.S. Food and Drug Administration; GC–MS, gas chromatography–mass spectrometry; H_2O_2, hydrogen peroxide; HD, Huntington's disease; IL-10, interleukin 10; IL-6, interleukin 6; ND, Neurodegenerative diseases; *NGF*, nerve growth factor; PBS, phosphate buffered saline; PD, Parkinson's disease; *UNAM*, *Bilkent University Institute* of Materials Science and Nanotechnology.

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The aim of the study was i) to analyze of *G.acutidentatum* methanol and water extracts' phytochemicals ii) to investigate the ability of extracts to protect *d*PC12 cells from neuroinflammation and neurodegeneration when exposed to neurotoxic insult induced by H_2O_2 as an in vitro model, by analyzing the effect of on interleukin 6 (IL-6) and interleukin 10 (IL-10) production and neurite outgrowth iii) to identify the mutagenic and anti-mutagenic effects of the extracts by *Salmonella typhimurium* TA100 and iiii) to determine the genotoxicity profile and the anti-genotoxic properties of the extracts in human peripheral lymphocyte cells by single-cell gel electrophoresis (Comet) technique.

2. Materials and methods

2.1. Plant material

Crude plants were collected and verified by Prof. Dr. Zeki Aytaç (Gazi University, Faculty of Science, Department of Biology). The voucher specimen is kept in Herbarium of Gazi University, Faculty of Science, Department of Biology. *G. acutidentatum* HAUSSKN. ET BORNM was collected from southwest of Durulmus, Sivas on 21.06.2013.

2.2. Preparation of plant extracts

The plant's aerial parts were dried for 7–14 days in the shade (protected from direct sunlight) at environmental temperatures. The dried plant parts were then ground using a mixer. The powder was stored in a closed container. Powdered plants (30 g) were extracted with 300 mL methanol (65 °C for 4 h) and distilled water (100 °C for 4 h). Methanol and water extracts were filtered with Whatman no.1 filter paper and then concentrated by a rotary evaporator. For cell treatment, extracts were solved in cell culture medium (Alamdary et al., 2012).

2.3. Gas chromatography-mass spectrometer (GC–MS) analysis

Compound analyses were performed by using capillary gas chromatography–mass spectrometry (GC–MS) using Thermo brand Trace GC Ultra gas chromatography coupled with ISQ Single Quadrupole MS (Thermo Fisher Scientific, San Jose, CA, USA) instrument at *Akdeniz University Food* Safety and *Agricultural* Research Centre. 2 mg of the extract was prepared by dissolving in 100 μL of methanol, diluted 1:20.

The temperature conditions followed the program: The initial temperature was 100 °C. It was increased from 100 °C to 180 C with an increase of 15 °C per minute. It was increased from 180 °C to 300 °C with an increase of 5 °C per minute. Then the temperature was held at 300 °C for 10 min. The injector temperature was 250 °C. Helium was used as the carrier gas at a flow rate of 0.8 mL/min. HP-5 MS column (30 m × 0.25 mm × 0.25 μm) was used.

The spectra of chromatographic peaks were investigated using Xcalibur (version 2.07, Thermo Fisher Scientific, San Jose, CA, USA). Compounds were defined by comparing the mass spectral fragmentation with the standard reference spectra of the Wiley 7 N library database.

2.4. Cell culture and plant extract treatment

PC12 cells are derived from a pheochromocytoma of the rat *adrenal* medulla and that can be transformed into sympathetic like *neurons* by addition the *NGF* into the culture medium (Liu et al., 2010). Therefore, dPC12 is a well-defined model for studies on cellular biology and morphology of neurons.

PC12 cells were obtained from *Bilkent University Institute* of Materials Science and Nanotechnology (*UNAM*) collection. Cells were grown in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum, 10% horse serum, 1% penicillin/streptomycin and 1% (2 mM) L-Glutamine. Cells incubated at 37 °C under humidified conditions with 5% CO_2. Cells were split when they reached 70% confluence (every 3–4 days) (Chen et al., 2014). Cells were differentiated for 4 days using 100 ng/mL NGF (Haq et al., 2007). To examine the effects of plant extract on dPC12, cells were plated in 96-well plate and preincubated with at different concentrations for 24 h, then H_2O_2 was added to the medium for 24 h.

2.5. Cell viability assay

Cell viability was assessed by MTT [3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazoliumbromide] assay. The cytotoxicity of the extracts was tested against dPC12 cells. Before the treatment, the cells were plated at an appropriate density (1×10^4 cells/200 μL) in a 96-well plate and incubated for 48 h at 37 °C. Then, cells were treated with the *G. acutidentatum* extracts (100, 250 and 500 μg/mL) for another 24 h. After 24 h of incubation, 20 μL of MTT (5 mg/mL) in phosphate buffered saline (PBS) was added to each well and incubated at 37 °C for 4 h. MTT medium was carefully aspirated from the wells and then the formed formazan crystals were solubilized in 200 μL of dimethyl sulfoxide (DMSO). Using microplate reader, the absorbance was measured at 570 nm (Epoch, BioTek) (Heo et al., 2001). The cell viability was determined using the following formula Cell Viability (%) = [Abs (sample) / Abs (control)] × 100.

2.6. Neuroprotective effect of extracts

To evaluate the neuroprotective effects of *G. acutidentatum*, dPC12 cells were pretreated with different concentrations of plant extracts (100, 250 and 500 μg/mL) for 24 h prior to exposure to 200 μM H_2O_2. After 24 h, the cell viability was calculated by MTT assay according to above formula (Park et al., 2012).

Table 1
Compounds present in the water and methanol extracts of *G. acutidentatum.*

Water extract					Methanol extract				
RT [min]	Compound	Formula	Structure	Peak area%	RT [min]	Compound	Formula	Structure	Peak area%
3.64	Cyclohexanone	$C_6H_{10}O$	Ketone	4.05	3.64	2-methoxy-4-vinyl phenol	$C_9H_{10}O_2$	Phenolic	0.94
4.64	Desulphosinigrin	$C_{10}H_{17}NO_6S$	Glycoside	1.54	6.86	Phenol, 4-(3-hydroxy-1-propenyl)-2-methoxy	$C_{10}H_{12}O_3$	Alcohol	0.84
5.13	Undetectable compound			2.14	7.50	Neophytadiene	$C_{20}H_{38}$	Terpene hydrocarbon	0.83
5.46	Undetectable compound			1.12	9.25	n-hexadecanoic acid	$C_{16}H_{32}O_2$	Fatty acid	3.38
7.02	Undetectable compound			2.17	11.84	9,12-octadecadienoic acid	$C_{18}H_{32}O_2$	Fatty acid	27.10
8.66	Hexadecanoic acid methyl ester	$C_{17}H_{34}O_2$	Fatty acid	0.98	21.98	Norglaucine	$C_{20}H_{23}NO_4$	Alkaloid	2.45
9.20	n-hexadecanoic acid methyl ester	$C_{17}H_{34}O_2$	Fatty acid	3.94	23.12	Glaucine	$C_{21}H_{25}NO_4$	Alkaloid	28.78
10.96	9-octadecenoic acid(Z) methyl ester	$C_{19}H_{36}O_2$	Fatty acid	9.26	25.14	1-hentetracontanol	$C_{41}H_{84}O$	Alcohol	17.15
22.99	Glaucine	$C_{21}H_{25}NO_4$	Alkaloid	50.79	25.26	Undetectable compound			8.43
25.13	Butane	$C_9H_{20}O_2$	Alkane	9.48	27.83	2(1H)-pyridone	C_5H_5NO	Alkaloid	10.10
27.73	2(1H)-pyridone	C_5H_5NO	Alkaloid	12.85					
29.85	Oxoberberine	$C_{20}H_{17}NO_5$	Alkaloid	1.70					

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