The Relationship Between Neuroprotective Activity and Antigenotoxic and Acetylcholinesterase Inhibitory Effects of *Glaucium corniculatum* Extracts

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The purpose of this study was to investigate the relationship between the acetylcholinesterase (AChE) inhibitory and antigenotoxic effect with the neuroprotective activity of *Glaucium corniculatum* methanol and water extracts rich in rutin and quercetin flavonoids. Neuroprotective activity in terms of cell survival and development against oxidative damage was measured by MTT assay and microscopic analysis in H_2O_2-induced NGF-differentiated PC12 (dPC12) cells. QRT-PCR and western blot hybridization method was employed for the determination of AChE inhibition of the extracts in the same cell model, and the genotoxic and antigenotoxic effects were identified with Comet assay with human lymphocytes. H_2O_2-induced vitality loss in dPC12 cells was inhibited in pre-treated cells with these plant extracts. Moreover, extracts stimulated neurite formation and prevented the oxidative stress-induced reduction in neurite growth. In general, it was determined that *G. corniculatum* methanol extract containing higher amounts of rutin and quercetin was more effective than water extract in terms of AChE inhibitory, antigenotoxic and also neuroprotective effect. In this study, it was shown for the first time that both AChE inhibitory and antigenotoxic effects of *G. corniculatum* may be effective in neuroprotection and it's protective and therapeutic effects against neurodegeneration may be related to the flavonoid content.

Keywords: *Glaucium corniculatum*. Neuroprotection. Antigenotoxicity. AChE inhibition. Flavonoid.

INTRODUCTION

Neurodegenerative diseases are chronic and progressive group of diseases characterized by loss of function, damage and death of neurons, which are the main components of the nervous system and the main factor of ensuring neuronal communication (Jeong, 2017). In many neurodegenerative diseases, synaptic connections and neuronal extensions are known to be damaged due to increased oxidative stress in the neurons. In individuals with these diseases, the number of neurites decreases and neurite length shortens compared to non-patients (Subramanian, Crabtree, Acharya, 2008). Therefore, the discovery of agents that support neurite growth against oxidative damage is a key element in providing neuroprotection.

In recent years, the number of studies that are aimed at clarifying the underlying mechanisms of neurodegeneration has increased. One of the mechanisms underlying neurodegeneration is the DNA instability associated with neuronal dysfunction. It has been reported that genotoxic effects cause DNA damage and lead to neurodegeneration (Castillo *et al.*, 2016). It has been determined that neurons began to shrink in neurodegeneration and there is a statistically significant decrease in the number and intensity of spine in both apical and basal dendritic columns and

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this is related to neurodegenerative disease pathology (Duan *et al*., 2003). Therefore, it has been reported that modulation of neurodegeneration by substances with antigenotoxic properties may be a new way of developing interventions that can increase the neural defense response to neurodegeneration (Castillo *et al*., 2016). Modulation of neurodegeneration with antigenotoxic agents may emerge as a new way to develop interventions that may increase the response of neural defense to neurodegeneration.

Another underlying mechanism of neurodegeneration is an increase of concentration of AChE in various brain regions, which is an important neuromotor of the central nervous system. AChE associated signaling is increasingly recognized as playing an important role in different processes such as neurite outgrowth, synaptic transmission, control and synthesis of neurotrophic factors, and neuroprotection (Belluardo *et al*., 2005). However, studies show that current AChE inhibitor drugs are not fully successful in the treatment of neurodegenerative diseases (Birks, 2006). Therefore, the discovery of new AChE inhibitors has gained importance for the prevention of neurodegeneration. Recently, several preclinical studies have shown that some of the AChE inhibitors slow down neurodegeneration by presenting neuroprotective and antigenotoxic properties (Castillo *et al*., 2016). These studies demonstrated the importance of both antigenotoxic and AChE-inhibiting mechanisms for neuroprotection and increased the interest in the discovery of drugs that could act in either way.

Flavonoids, which are secondary metabolites, can inhibit oxidative damage and AChE, and suppress neurodegeneration. For this reason, flavonoids are considered as potential molecules in studies to develop a new group of drugs that are capable of resisting neurodegenerative diseases (Magalingam, Radhakrishnan, Haleagrahara, 2015). *Glaucium corniculatum* (Family: *Papaveraceae*), known as a red horned poppy, is one of the plants that contain flavonoids. It is a traditional herb that is used as memory enhancer. Although the effect on the AChE gene and protein level has not been previously determined, it is known to have *in vitro* and cellular AChE inhibitory properties (Orhan *et al*., 2004; Kocanci, Hamamcioglu,

Aslim, 2017a). It is one of the plants that may have a potential for curative and/or protective effect on neurodegenerative diseases because of both secondary metabolites it contains and the presence of preliminary data about the AChE inhibitor property (Orhan *et al*., 2004).

In a previous study, we found that *G. corniculatum* methanol extract contained rutin (45 μg/mL) and quercetin (12 μg/mL), while the water extract contained rutin (41 μg/mL), quercetin (10 μg/mL) and luteolin (0.23 μg/mL) flavonoids (Kocanci, Hamamcioglu, Aslim, 2017b). The rutin and quercetin flavonoids are polyphenolic compounds found in fruits and vegetables. Some studies have focused on the neuroprotective, antigenotoxic and AChE inhibitory effects of these flavonoids (Ademosun *et al*., 2016; Barcelos *et al*., 2011). However, there is no study in the literature on the neuroprotective, antigenotoxic and AChE inhibitory effects of rutin and quercetin-rich *G. corniculatum* methanol and water extracts.

In this study, neuroprotective effects of rutin and quercetin-rich *G. corniculatum* methanol and water extracts on the viability and morphological development of neurites were investigated. In addition, the relationship otective effects of these extracts and antigenotoxic and AChE inhibitory effects were investigated.

MATERIAL AND METHODS

Plant Material

Crude plants were collected and verified by Prof. Dr. Zeki Aytaç (Gazi University, Faculty of Science, Department of Biology). The voucher specimen was kept in Herbarium of Gazi University, Faculty of Science, Department of Biology. *G. corniculatum (L.) RUD*. subsp. *refractum* (NAB.) CULLEN was collected from Beypazari district to the northwest of Ankara on 01.07.2014 and 27.07.2015.

Preparation of Plant Extracts

The aerial parts of the plant were dried in shade (protected from direct sunlight) at room temperature and then turned into powder using a blender. The powder was

extracted using the Soxhlet method described by Kocanci and colleagues (2017b).

PC12 Cell Culture, Differentiation and Treatment of Cells with Plant Extracts

In this study, PC12 cells were used as a neuron model. PC12 rat pheochromocytoma cells were obtained from Bilkent University Institute of Materials Science and Nanotechnology. PC12 cells were maintained in DMEM supplemented with 10% horse serum, 10% fetal bovine serum, 1% penicillin/streptomycin antibiotics and 1% (2 mM) L-Glutamine at 37°C in humidified atmosphere of 5% CO_2. Differentiation of the PC12 cells was induced by adding 100 ng/mL NGF in cell medium. The cells were maintained for 4 days in the NGF-containing medium before use. The medium was changed every other day.

We have previously reported that 12 and 24 h of extract incubation does not exert cytotoxicity to dPC12 cells with IC50 values of 1150±8-1650±5 μg/mL (Kocanci, Hamamcioglu, Aslim, 2017b)**.** Therefore, in the present study, 100-1000 μg/mL extract concentrations were tested for 24 h on the dPC12 cell line. Cells were treated with the reactive oxygen source H_2O_2 to produce oxidative damage to the cells. Cells in the control group were developed in normal culture conditions without treatment with plant extract or H_2O_2.

Neuroprotective Effect

The neuroprotective effect on cell viability of G. corniculatum extracts

To evaluate the neuroprotective effects of *G. corniculatum*, PC12 cells were developed in a collagen-coated 96-well plate and differentiated with NGF. dPC12 cells were preincubated with plant extracts (100, 250 and 500 μg/mL) for 24 h prior to H_2O_2 (final concentration: 200 μM) and incubated for 24 h. The activity of the treated dPC12 cells was determined using the MTT method. The cells were treated with 20 μL of MTT solution (5 mg/mL) (M5655-Sigma-Aldrich, USA) for 4 h at 37°C. The dark blue formazan crystals formed in viable

cells were solubilized with 200 μL of DMSO (K44917943 346-Millipore, USA) for 30 min. The absorbance was measured at 570 nm with a microplate reader (Epoch, BioTek). The results were expressed as the percentage of MTT reduction in relation to the absorbance of control cells at 100%.

The neuroprotective effect on neurit development of G. corniculatum extracts

PC12 cells were seeded into collagen-coated 24 well microplates containing poly-D-lysine and laminin (10 μg/mL) coated glass coverslips ($5x10^3$ cell/cm²). The cells were differentiated with NGF. dPC12 cells were treated with methanol and water extracts of *G. corniculatum* (100-500 μg/mL) for 24 h prior to exposure to 200 μM H_2O_2 for 24 h. The cells were stained with 20% giemsa dye after fixation with 3.7% paraformaldehyde. The coverslips were examined under the light microscope (Leica ICC50 HD) using the LAS V4.3.0 software. The neurite density index (number of neurites per cell) and neurite length were analyzed in randomly selected 100 cells (Chen *et al.*, 2014).

COMET Assay

The Comet assay was performed using human blood lymphocytes. Biocoll separating solution was used for the isolation of the human lymphocytes. 50 μM H_2O_2 was chosen to induce a consistent level of DNA damage in human lymphocytes. The study was approved by Gazi University Clinical Research Ethics Committee (Date and Number: 14/08/2017, 362). The cells were exposed to the following treatments: (1) to evaluate the genotoxicity of the extracts, the cells were exposed to *G. corniculatum* in 100, 250 and 500 μg/mL concentrations for 1 h at 37°C; to evaluate antigenotoxic potential, (2) the cells were incubated with H_2O_2 (50 μM for 15 min.) prior to their exposure to the *G. corniculatum* extracts. For the test of genotoxicity, the cells were treated with PBS as a control and H_2O_2 as a positive control. Before starting the Comet assay, we determined cell viability by spectrophotometric analysis as a trypan

blue indicator. We found that the cell viability was higher than 85% in all groups. The slides were treated for 2 h at 4°C in freshly prepared, chilled lysis buffer solution (10M NaOH, 0.2 M EDTA, pH > 13), followed by incubation in electrophoresis (25 V and 300 mA) for 20 min. The slides were then neutralized with Tris buffer (0.4 M Tris, pH adjusted to 7.5) for 15 min. rinsed with distilled water and stained with ethidium bromide (20 µg/mL) for 5 min. under dark condition. The stained slides were examined at 546 nm excitation and 590 nm barrier filters at 40X objective under a fluorescent microscope. For each extract concentration, 100 cells were counted in the slides and examined with a computerized imaging system (Comet Assay III, Perceptive Instruments Ltd., UK). The results were evaluated in terms of tail intensity, tail moment and tail length. Tail DNA damage (%) was determined by the following formula:

Tail DNA damage% = 100 x (DNA tail density / (DNA head density + DNA tail density)) (Behravan *et al.*, 2011).

AChE Inhibitory Effect of *G.corniculatum* Extracts in H$_2$O$_2$-induced dPC12 Cells

1x10^5 dPC12 cells, placed on collagen-coated 6-well plates, were exposed to 100 µM H$_2$O$_2$ for 24 h at 37°C. Then the H$_2$O$_2$ was withdrawn and the cells were cultured in differentiation medium containing the 100, 500 and 1000 µg/mL of plant extracts for 24 h at 37°C (These concentrations were selected in order to clearly determine the effects on gene and protein levels). Cells without H$_2$O$_2$ and plant extract treatment were used as control. After the applications, the AChE gene and protein expression levels were determined in dPC12 cells.

RNA isolation and gene expression analysis by RT-PCR

Total cellular RNA was extracted from different experimental groups using the RNeasy Mini Kit (74104-Qiagen, USA) based on the manufacturer's instructions. RNA quantification was performed by Epo Take3 ELISA Reader using routine procedures.

RNA isolates with 260/280 nm absorption between 1.8-2.1 were subjected to study. 1 µg RNA was then reverse transcribed into cDNA using a QuantiTect Reverse Transcription Kit (205311-Qiagen, Germany) according to the manufacturer's instructions. The resulting cDNA was amplified by PCR with the following set of primers: AChE, forward 5′-CGCAGCAATACGTGAGCCTGAA -3′ and reverse 5′-TGTGGCGCTGAGCAATTTGGG-3′; GAPDH, forward 5′-CAACTCCCTCAAGATTGTCAGCAA-3′ and reverse 5′-GGCATGGACTGTGGTCATGA-3′. RT-PCR reactions were performed using SYBR Green PCR Kit (SensiFAST SYBR® Low-ROX Kit, BIO94005-Bioline, USA) in a *7500* Fast Real-Time PCR System (Applied Biosystems, Walthman, USA). 40 cycles were carried out, each consisting of denaturation at 95°C for 5 sec, annealing for 30 sec at 65°C. The specificity of the product of the amplification was determined by melting curve analysis for each primer pair. The mRNA expression of the target gene was normalized according to the GAPDH mRNA expression in a given sample. The data were analyzed by the comparative CT method and the fold change was calculated by $2^{-\Delta\Delta CT}$ method (Livak, Schmittgen, 2001).

Protein expression analysis by Western blot

The cells from different experimental conditions were lysed with solubilisation buffer (pH 7.8) (10 mM Tris-HCl, pH 7.2, 1 M NaCl, 50 mM MgCl$_2$, 1% Triton X-100). The protein concentration was measured with a Bradford protein assay kit (B6916-Sigma-Aldrich, USA) based on the manufacturer's instructions. Proteins (30 µg per lane) were separated on 4-12% polyacrylamide gels, they were transferred to PVDF membrane, blocked with 2% milk in Tris-buffered saline (20 mm Tris, 150 mm NaCl, pH 7.6), and incubated with the primary antibodies for 2 h (PB9417-Boster, USA). After washing, the membranes were probed with a secondary antibody (SA00002-2- Proteintech, USA) for 1 h. Then, blots were developed using the AP Conjugate Substrate solution (1706432-Bio-Rad, USA) for a period of 30 min. to detect the target protein bands. The bands formed on the membrane were detected on a gel imaging system

(ChemiDoc ™ XRS + System-Bio-Rad, USA). The intensity of the bands was determined using the BIORAD ImageLab 5.21 software. These expression levels of AChE were normalized to the housekeeping protein GAPDH (10494-1AP-Proteintech, USA) (Gong *et al.*, 2012).

STATISTICAL ANALYSIS

The results were statistically evaluated using the SPSS 16.0 program. In all the experiments, the difference between the control groups and the differences between the inductor and plant extract applications were analyzed by t-Test. The differences between the means were determined by the One-Way ANOVA Post Hoc Tukey HSD test. The relation between the amount of flavonoid in the extracts and the AChE inhibitory effect was determined by Pearson correlation. Statistical significance was determined to be the 95% significance level. The results were presented as mean value ± standard deviation (5 parallels in 3 repetitions for the neuroprotection assay, 3 parallels in 3 repetitions for all other analyses).

RESULTS

Neuroprotective Effects of *G. corniculatum* Extracts

The neuroprotective effects against H_2O_2-induced cytotoxicity

To investigate the effects of *G. corniculatum* extracts on cell viability after H_2O_2 exposure, the dPC12 cells were treated with 200 µg/mL of H_2O_2 for 12 h. As shown in Figure 1, the exposure of dPC12 cells to 200 µM H_2O_2 alone sharply reduced the dPC12 cell viability (approximately 50% of the control group value) (* $p < 0.05$). However, H_2O_2-induced cytotoxicity in dPC12s cells was inhibited in pre-incubated cells with plant extracts (** $p < 0.05$). This protective effect is associated with increased concentration of the extracts and the relative cell viability reached its highest value at the concentration of 500 µg/mL of water extract (96±2%). The results demonstrated a protective effect of *G. corniculatum* extracts against H_2O_2-induced cytotoxicity. This observation suggests that *G. corniculatum* extracts were likely to promote cell survival or delay the oxidative stress-induced death of neurons.
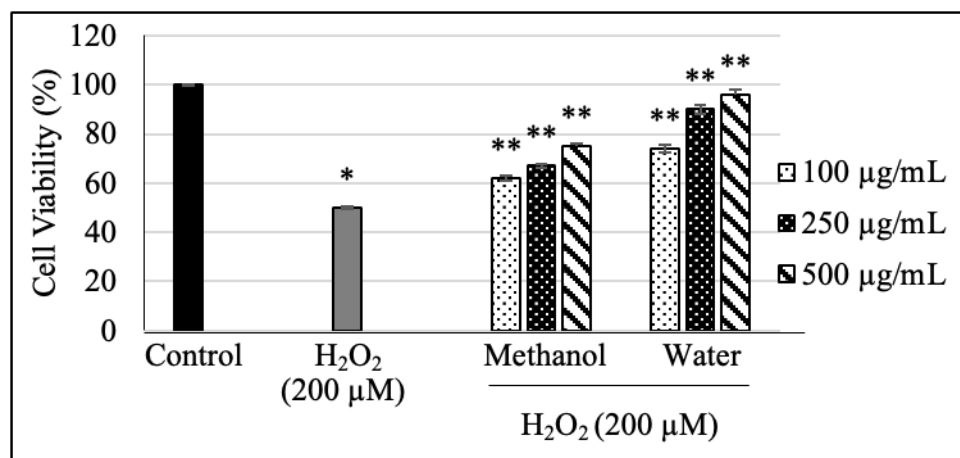
FIGURE 1 - Neuroprotective effects of *G. corniculatum* extracts on the viability of dPC12 cells against H_2O_2-induced cytotoxicity. Control: untreated dPC12 cells. * $p < 0.05$ vs.control group. ** $p < 0.05$ vs. H_2O_2-treated group.

The neuroprotective effect on neurite development

Average neurite index and neurite length were evaluated to monitor neurite growth. Accordingly, the untreated dPC12 cells were with elongated and spreading morphology (Figure 2A, control). When the cells treated with plant extract alone were compared with control cells, it was seen that treatment with extracts significantly increased neuronal index/regeneration in dPC12 cells in a concentration-dependent manner (Table I). 100 and 250 µg/mL methanol extracts did not increase the neurite index significantly ($p > 0.05$). Whereas, other extract applications triggered an increase in the number of neurites per cell according to the control (* $p < 0.05$). It was determined that the extract applications increased the neurite index, although they decreased the neurite length slightly compared to the control. This indicates that the extracts support the development of neurites, helping create new neurites.

While in the H_2O_2 treated group the neurite lengths were decreased and the neurite branchings almost completely disappeared compared to the control (Figure 2B). In the cells treated with H_2O_2 compared to control, the number of neurites per cell (87%) and neurite length (38%) were significantly reduced (* $p < 0.05$) (Table I). This showed that H_2O_2 induced damage to the neurite development of dPC12 cells.

Both *G. corniculatum* extracts prevented H_2O_2-induced neurite damage in dPC12 cells (Figure 3B and 3C). Morphological changes in the dPC12 cells demonstrated that pretreatment of cells with the *G. corniculatum* extracts resulted in a significant increase in neurite length due to concentration whereas only 500 µg/mL of methanol extract resulted in a significant increase in neurite index (Table I) (** $p < 0.05$). There was no significant difference between the effect of water and methanol extracts on neurite length when applied plant extract concentrations were taken into consideration. These results showed that *G. corniculatum* extracts effectively precluded H_2O_2-induced neurite degeneration in dPC12 cells and plant extracts had a significant protective effect against H_2O_2-induced neurite length damage. These results obtained from morphological observations were in parallel with the results of the neuroprotective effect on cell viability determined by the MTT method.
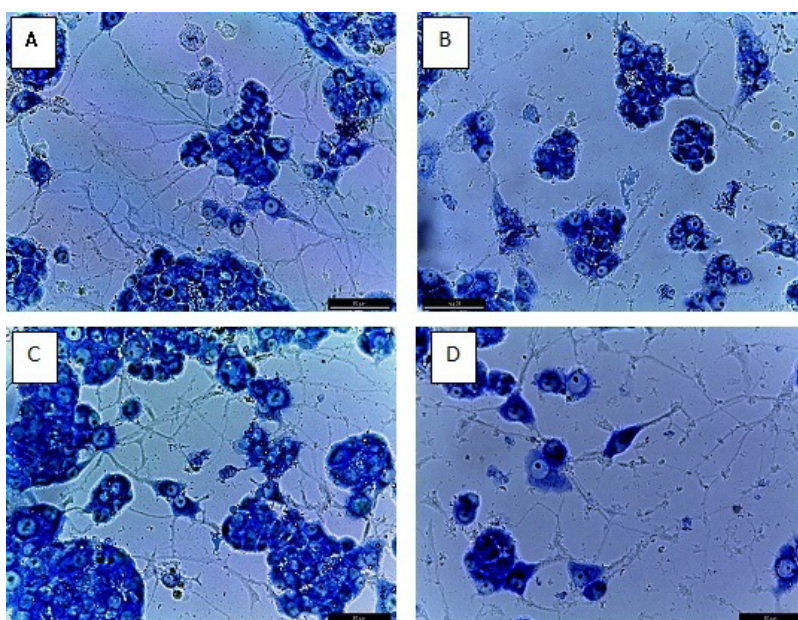
FIGURE 2 - Microscopic images of dPC12 cells with the *G. corniculatum* extracts and H_2O_2 treatments (40X magnification) A: Control (untreated dPC12 cells), B: H_2O_2 treated dPC12 cells, C: 500 µg/mL methanol extract per-treated cells D: 500 µg/mL water extract pre-treated cells.
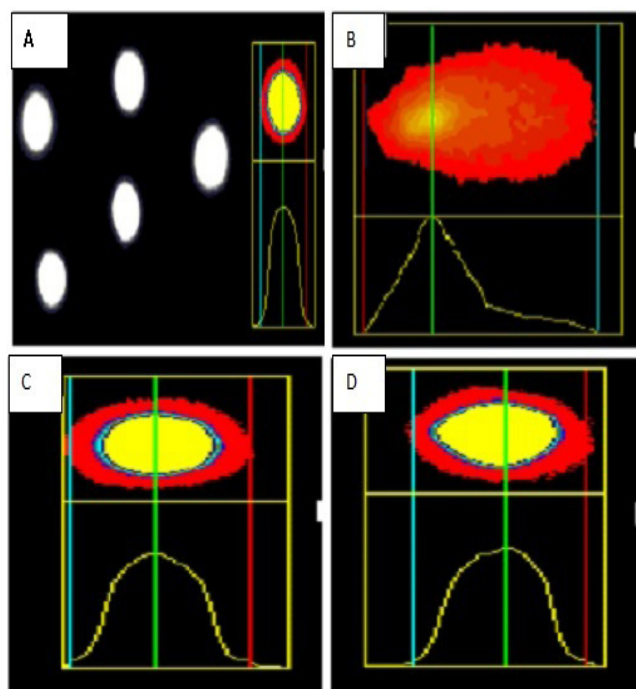
FIGURE 3 - Representative Comet images of dPC12 cells. A: Control (untreated human lymphocytes), B: H_2O_2 treated human lymphocytes C: 500 µg/mL *G. corniculatum* methanol extract pre-treated human lymphocytes D: 500 µg/mL *G. corniculatum* water extract pre-treated human lymphocytes.

Genotoxic and Antigenotoxic Effects of *G. corniculatum* Extracts

The results for the genotoxicity and antigenotoxicity assays conducted using *G. corniculatum* methanol and water extracts and H_2O_2 in alone and combination were presented in Table II and Figure 3 for the comet assay as a measure of DNA damage. In this assay, the tail density was 0.5 in the control group. And it was 0.3-0.8% in the lymphocytes treated with *G. corniculatum* methanol and water extracts (Table II). On the other hand, the tail length, tail moment and % of tail density in the treatments of H_2O_2 revealed a significant increase when compared with control group (* $p < 0.05$).

TABLE I - Effects of H_2O_2 and *G. corniculatum* methanol and water extracts on neurite index and neurite length in dPC12 cells

Treatment	Concentrations	Neurite Index (neurite/cell)	Neurite Length (µm)
Control	-	1.5 ± 0.0	80.3 ± 0.3
***G. corniculatum* methanol**	100 µg/mL	1.3 ± 0.1	79.5 ± 0.7
	250 µg/mL	1.6 ± 1.1	76.2 ± 1.1*
	500 µg/mL	1.7 ± 0.1*	76.0 ± 1.2*
***G. corniculatum* water**	100 µg/mL	1.7 ± 0.1*	62.0 ± 0.2*
	250 µg/mL	1.9 ± 0.2*	63.0 ± 0.1*
	500 µg/mL	2.1 ± 0.1*	66.9 ± 0.2*
H_2O_2	200 µM	1.2 ± 0.1*	50.7 ± 0.4*
***G. corniculatum* methanol + H_2O_2**	100 µg/mL	1.3 ± 0.1	72.3 ± 0.1**
	250 µg/mL	1.3 ± 0.1	76.7 ± 0.9**
	500 µg/mL	1.3 ± 0.1	78.1 ± 0.8**
***G. corniculatum* water + H_2O_2**	100 µg/mL	1.2 ± 0.1	74.8 ± 0.5**
	250 µg/mL	1.3 ± 0.1	76.5 ± 0.2**
	500 µg/mL	1.4 ± 0.0**	77.8 ± 0.4**

Control: untreated dPC12 cells, H_2O_2: 200 µM H_2O_2 treated dPC12 cells for 24 h. * $p < 0.05$ vs. control group. ** $p < 0.05$ vs. H_2O_2-treated group.

TABLE II - Genotoxic and antigenotoxic effects of *G. corniculatum* extracts on human lymphocytes

Treatment	Concentrations	DNA tail length (µm)	DNA tail moment	DNA tail density (%)
Control	-	12.0 ± 1.2	0.3 ± 0.0	0.5 ± 0.2
G. corniculatum methanol	100 µg/mL	15.0 ± 0.1	0.7 ± 0.1	0.4 ± 0.0
	250 µg/mL	16.3 ± 0.0*	0.8 ±0.0*	0.6 ± 0.1
	500 µg/mL	17.9 ± 0.2*	1.0 ± 0.1*	0.8 ± 0.1*
G. corniculatum water	100 µg/mL	14.6 ± 0.2	0.5 ± 0.1	0.3 ± 0.0
	250 µg/mL	16.0 ± 0.5*	0.7 ± 0.1	0.6 ± 0.1
	500 µg/mL	17.8 ± 0.3*	0.9 ± 0.2*	0.7 ± 0.1
H$_2$O$_2$	50 µM	76.7 ± 1.3*	23.3 ± 1.3*	43.3 ± 1.5*
G. corniculatum methanol + H$_2$O$_2$	100 µg/mL	47.1 ± 0.7**	7.8 ± 0.3**	22.5 ± 0.3**
	250 µg/mL	40.8 ± 0.9**	6.5 ± 0.2**	19.5 ± 0.5**
	500 µg/mL	37.6 ± 1.3**	5.2 ± 0.6**	15.0 ± 0.1**
G. corniculatum water + H$_2$O$_2$	100 µg/mL	50.5 ± 1.0**	8.5 ± 0.2**	24.3 ± 0.3**
	250 µg/mL	40.8 ± 0.9**	6.8 ± 0.3**	20.5 ± 0.2**
	500 µg/mL	37.6 ± 1.3**	6.3 ± 0.5**	17.3 ± 0.2**

Control: untreated dPC12 cells. H$_2$O$_2$: 50 µM H$_2$O$_2$ treated dPC12 cells for 24 h. * $p < 0.05$ vs. control group. ** $p < 0.05$ vs. H$_2$O$_2$-treated group.

Besides, plant extract treatment prior to H$_2$O$_2$ showed a significant reduction in the extent of DNA damage for cells exposed to the three doses (100, 250 and 500 µg/mL) of the extract plus H$_2$O$_2$, compared with the H$_2$O$_2$ treated group alone (** $p < 0.05$) (Table II). However, DNA damage in lymphocytes treated with *G. corniculatum* extracts after H$_2$O$_2$ administration was significantly reduced at all applied concentrations of both extracts compared to the H$_2$O$_2$ treatment group. While the tail density percentage was 43.3% in the 50 µM H$_2$O$_2$ treated group, it was 15.0% in the 500 mg/mL methanol extract-treated group following the H$_2$O$_2$ and 17.3% in the 500 µg/mL water extract-treated group after H$_2$O$_2$. This result showed that *G. corniculatum* methanol extract inhibits DNA damage better than water extract. Data of the Comet assay indicated that *G. corniculatum* extracts did not have genotoxic effects and the extracts protected human lymphocytes from H$_2$O$_2$-induced DNA damage.

QRT-PCR Analysis for AChE mRNA Expression

Changes in AChE gene expression in dPC12 cells treated with *G. corniculatum* extracts (100, 500 and 1000 µg/mL for 24 h) after exposure to H$_2$O$_2$ (100 µM for 24 h) were investigated.

The AChE gene expression rate in H$_2$O$_2$ treated cells was increased significantly to 2.1±0.1 compared to the untreated control group (* $p < 0.05$) (Figure 4). In contrast, *G. corniculatum* extracts inhibited H$_2$O$_2$-induced AChE gene expression in a concentration-dependent manner (** $p < 0.05$). The results of methanol and water extract showed similarity. At a concentration of 1000 µg/mL where the highest inhibition was detected, the methanol extract inhibited 0.48 fold of AChE gene expression relative to the H$_2$O$_2$ group. It is noteworthy that the AChE gene expression ratio in the dPC12 cells post-incubated with 1000 µg/mL *G. corniculatum* methanol extract almost returned to the normal levels.
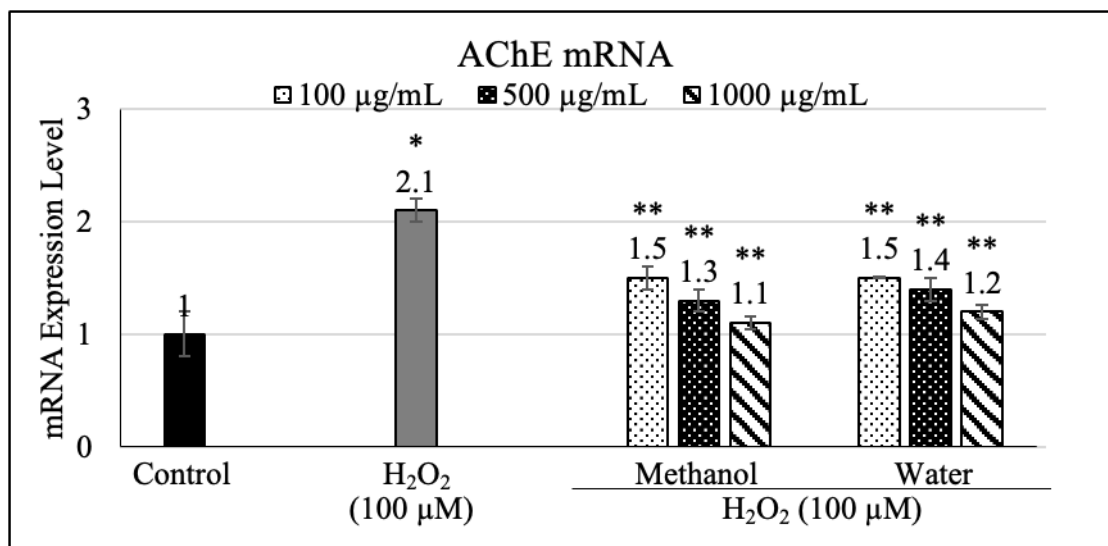
FIGURE 4 - The levels of AChE mRNA expression in dPC12 cells with different concentrations of *G. corniculatum* after H_2O_2. * $p < 0.05$ vs. control group. ** $p < 0.05$ vs. H_2O_2-treated group.

Western Blot Analysis for AChE Protein Expression

The AChE inhibitory effect of *G. corniculatum* methanol extracts was confirmed at the protein level by western blotting. As shown in Figure 5, there was an upregulation of AChE in H_2O_2 treated dPC12 cells compared to untreated cells (* $p< 0.05$). The post-treatment of dPC12 cells with 100-1000 µg/mL of *G. corniculatum* methanol extract resulted in a significant decrease (** $p < 0.05$) in AChE protein level. The inhibition was proportional to the concentration (16%-21% inhibition). However, no significant difference in AChE expression was observed between control and *G. corniculatum* water extract group at the applied concentrations ($p > 0.05$). These results indicated that *G. corniculatum* extracts have AChE inhibitory properties at gene and protein levels.

QRT-PCR Analysis for AChE mRNA Expression

Western blot analysis results supported the qRT-PCR results. This showed that the 1000 µg/mL concentration of methanol extract was the best inhibitor of the H_2O_2-induced AChE stimulation at both the gene and the protein level.
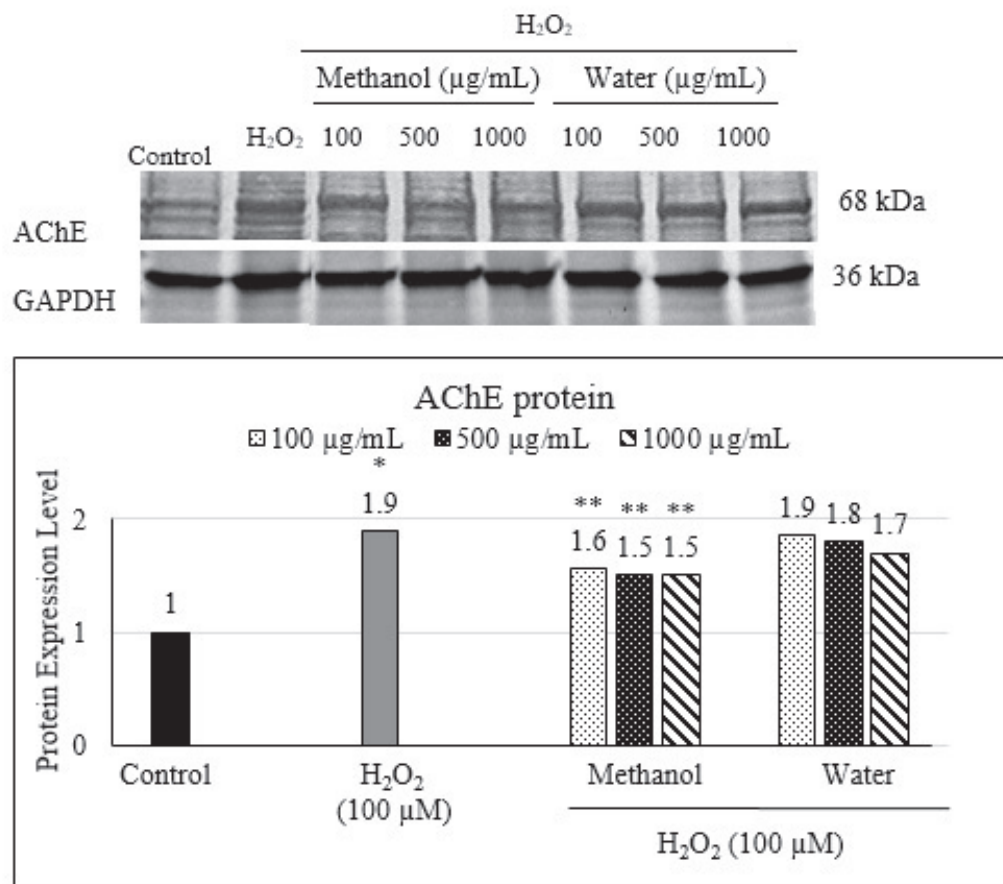
FIGURE 5 - The levels of AChE protein expression in dPC12 cells with different concentrations of *G. corniculatum* after H_2O_2.
* $p < 0.05$ vs. control group, ** $p < 0.05$ vs. H_2O_2-treated group.

DISCUSSION

Accumulated evidence has indicated that oxidative stress-induced dysfunction and disruption at the level of neuronal development is significantly associated with neurodegenerative disorders (Liu *et al*., 2017). Oxidative stress contributes to impaired cholinergic neurotransmission (i.e., upregulation of the AChE), which is a consistent feature of neurodegenerative diseases (Anand, Gill, Mahdi, 2014). Therefore, any effort aimed at developing specific treatments to reduce/prevent oxidative stress, provide AChE inhibition and enhance neuronal survival will be of great significance.

In this work, we assessed the neuroprotective, genotoxicity, antigenotoxicity and AChE inhibitory effects of *G. corniculatum* methanol and water extracts in neurotoxicity stimulated dPC12 cells with H_2O_2,

which is the most commonly used chemical agent in oxidative stress-induced genotoxicity studies (Behravan *et al*., 2011).

In our study, the protective effects of these extracts against H_2O_2 damage of dPC12 cells were demonstrated by MTT analyzes and morphological observations (Figures 1 and 2, Table I). Data from the MTT analysis showed that the cells pretreated with *G. corniculatum* extracts demonstrated significantly increased cell viability and the cell morphology resembled that of the control groups even after the exposure to H_2O_2. According to the results of the MTT analysis, increasing concentrations of the plant extracts showed a preventing effect of H_2O_2-induced cytotoxicity. These data clearly demonstrate that *G. corniculatum* extracts protect dPC12 cells from oxidative stress induced by H_2O_2 and prevent H_2O_2-induced death.

Neurite growth in cultured neurons is considered a symptom of neurodegenerative potential. Since the damaged brain does not have a reconstructive capacity, neurotrophic compounds that can promote neurite growth appear to be an important step in achieving neuronal survival (More *et al*., 2012). The provision of neurite development by natural compounds such as plant extracts is important in reducing the potential side effects (Elufioye, Berida, Habtemariam, 2017). Therefore, the discovery of the natural compounds that support neurite development is of strategic importance. In our study, morphological findings showed that water and methanol extracts of *G. corniculatum* increased the number of neurites per cell significantly (except for *G. corniculatum* 100 µg/mL methanol extract) compared to the control group in dPC12 cells. This result showed that these extracts may be effective in the formation of new neurites. Also, it was determined that the extracts applied before the treatment with H_2O_2 did not significantly affect the number of neurites per cell (neurite index), but significantly preserved neurite lengths compared to H_2O_2 administration. Taken together, these results suggest that *G. corniculatum* extracts can be used both as a natural stimulator for neurite development and as a natural neuroprotector against morphological defects caused by H_2O_2.

Scientific evidence for the safety of the herbal products also requires regulatory clearance for wide acceptance. For this, the toxicity of herbal products should be investigated (Moreira *et al*., 2014). Therefore, we evaluated the genotoxicity and antigenotoxicity of these extracts. Anderson *et al*., (1994) reported that <5% damaged DNA represents undamaged cells. It can be said that these *G. corniculatum* extracts do not show genotoxic activity since they cause tail density between 0.3-0.8% according to the control. Also, *G. corniculatum* extracts showed an antigenotoxic effect, inhibiting the genotoxicity induced by the H_2O_2. *G. corniculatum* methanol extract showed a more effective antigenotoxic effect than water extract. There is no information about genotoxic and antigenotoxic effects of *G. corniculatum* extracts in the literature. Therefore, these results reported for the first time that the antitoxic properties of these

extracts are of great pharmacological importance and may be effective for neurodegenerative diseases.

A significant correlation has been found between the neuroprotective effect of plant extracts and their antigenotoxic effects. Since neuronal cells are partially differentiated cells, most of the DNA damage in these cells are repaired by base excision repair and repair with replication is not possible in these cells (Şen *et al*., 2017). Therefore, preventing DNA damage with antigenotoxic agents in neurons, it will support DNA repair mechanisms and prevent neurodegeneration. The fact that these extracts of *G. corniculatum* did not cause genotoxic damage in DNA and had high inhibitory ability against DNA damage induced by H_2O_2 emphasized their neuroprotective properties. In the results we obtained previously, we also showed that *G. corniculatum* methanol and water extracts have neuroprotective effects due to their anti-inflammatory properties (Kocanci, Hamamcioglu, Aslim, 2017b). Neuroprotective effects have also been demonstrated in members of the same genus: It has been shown that the methanolic extract of *G. grandiflorum* has DNA protection activity against the destructive effects of hydroxyl radicals (Ozsoy *et al*., 2018). Furthermore, it has also been found that *G. acutidentatum* methanol and water extracts have neuroprotective activity due to their anti-mutagenic and anti-genotoxic effect (Hamamcioglu, Kocanci, Aslim, 2018).

AChE inhibitory property is an important marker for the discovery of active substances on neurodegenerative diseases since today AChE inhibition is the most effective strategy in the treatment of Alzheimer's disease (AD) which is a very common progressive neurodegenerative disorder (Anand, Gill, Mahdi, 2014). Several studies have shown that AChE expression increases in both gene and protein levels in various cell types exposed to oxidative stress (Yang, He, Zhang, 2002). It has been reported that overexpression of AChE increases neurodegeneration and neural death. On the other hand, it has also been suggested that oxidative stress-induced neurodegeneration can be eliminated by decreasing AChE expression (Jin *et al*., 2004). There are also studies showing that excess acetylcholine causes proximal effects in the cell: It has been determined that cholinesterase activity has

correlation with apoptotic cell count, comet assay tail length, long-tailed nucleus number and thus genotoxic effect (Zeljezic, *et al*., 2008). Therefore, AChE inhibition may be a marker of cytotoxic and genotoxic inhibition. In our study, it was determined that *G. corniculatum* methanol and water extracts inhibited the increase in AChE expression caused by H_2O_2 at both gene and protein levels. Furthermore, the same genus members have been shown to have *in vitro* AChE inhibitory effect: Extracts of *G. acutidentatum* (Hamamcioglu, Kocanci, Aslim, 2018) and *Glaucium grandiflorum* var. *Grandiflorum* (Ozsoy *et al.,* 2018) and alkaloids of *Glaucium flavum* (Puzyrevská, 2017). Although there are studies showing the *in vitro* and cellular AChE inhibitory properties of *G. corniculatum* extracts (Orhan *et al*., 2004; Kocanci, Hamamcioglu, Aslim, 2017a), these extracts' AChE inhibitory properties at gene and protein levels are shown for the first time in our study. *In vitro* and cellular levels of AChE showed that *G. corniculatum* methanol extract was more effective than water extract (Kocanci, Hamamcioglu, Aslim, 2017a). These previous results are in parallel with findings obtained from our study. 1000 μg/mL of *G. corniculatum* methanol extract showed the most effective inhibition on AChE gene and protein expression (90% in gene expression, 21% inhibition in protein expression), in this study. When a general evaluation of the effects of the extracts on gene and protein expressions was made, it was found that the inhibitory effects at the gene level were higher than the effect on the protein level. It has been reported that gene expression in cells first occurs by the expression of mRNA expression, then protein expression (Schwanhäusser *et al*., 2011). Accordingly, this may be the reason why the inhibition effect of protein expression is lower than gene expression in our study.

In various studies, neuroprotective, antigenotoxic and AChE inhibitory effects of plant extracts obtained with different solvents were shown to be different (Załuski, Kuźniewski, 2016; Iloki-Assanga *et al*., 2015). It is thought that this may be due to the differences in the extracts' content. Flavonoids are one of the herbal compounds that may be responsible for these effects. In order to determine whether the effects detected in our study were related to the content, we compared the results of this study with the results of *G. corniculatum*

flavonoid obtained from the previous HPLC analysis. In a previous study, while luteolin, rutin and quercetin flavonoids were detected in both extracts, the methanolic extract contained higher rutin and quercetin than the water extract. Also, in the water extract, luteolin was detected, this substance was not present in the methanol extract (Kocanci, Hamamcioglu, and Aslim, 2017b). Although in this study there was no significant difference between the level of neuroprotection of methanol and water extracts, the methanol extract was more effective than the water in terms of the results of antigenotoxic and AChE inhibition activities. These results suggest that rutin and quercetin flavonoids may be responsible for these effects. Our findings support the evidence that rutin and quercetin flavonoids increase the survival rate of neurons, increase neurite growth, provide significant protection against oxidative stress, and are effective in antigenotoxicity and AChE inhibition (Magalingam, Radhakrishnan, Haleagrahara, 2015; Tangsaengvit *et al*., 2013). Thus, it can be mentioned that *G. corniculatum* methanol extract is a good source for compounds which exhibit neuroprotective antigenotoxic and AChE inhibitory activity.

To sum up, our results clearly indicated that *G. corniculatum* extracts exhibited a neuroprotective ability against H_2O_2-induced neurodegeneration. Furthermore, the presence of antigenotoxic properties of extracts has shown that it can safely be used in the treatment or prevention of neurodegenerative diseases. Another important finding was the remarkable AChE inhibitory activity of these extracts at gene and protein level, which is of significant value in the pharmacological investigation for the treatment of AD. Our study provided the first experimental evidence indicating that *G. corniculatum* extracts also contribute to the neuroprotection mechanisms through regulation of DNA damage in addition to AChE inhibitory activity. Also, it was determined that these effects could be related to rutin and quercetin flavonoids of *G. corniculatum*. In future studies, this molecular mechanism needs to be validated *in vivo* as a positive result, if confirmed, would provide essential information towards the development of new approaches for effective agents against neurodegenerative diseases.

CONCLUSION

The results showed that *G. corniculatum* methanol and water extracts exhibited a neuronal developmental effect and prevented H_2O_2-induced DNA damage in dPC12 cells and demonstrated neuroprotective activity. The results suggested that neuroprotective effects on neurite development and cell viability may be related to antigenotoxic and acetylcholinesterase inhibiting activity and may also be related to rutin and quercetin type flavonoids of *G. corniculatum*. The neuroprotective effect of *G. corniculatum* found in our study, in addition to its widely described biological activities, suggests a potential application in neurodegenerative diseases.

CONFLICT OF INTEREST

The authors have declared that there is no conflict of interest

ACKNOWLEDGMENTS

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