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Neuroprotective Effects of Rutin and Quercetin Flavonoids in *Glaucium corniculatum* Methanol and Water Extracts

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Abstract: Neurodegenerative diseases (NDs) are characterized by loss of neurons. NDs are associated with development of inflammation. Existing drugs against NDs only delay the progression; however, they do not provide a cure. The studies for the treatment of NDs focused on to discover natural products that have the potential of anti-inhibition and anti-radical properties. The *Papaveraceae* family members are important for the synthesis of pharmaceutically compounds such as flavonoids which act like anti-inflammatory drugs. In this study, methanol and water extracts of *Glaucium corniculatum*, a member of the *Papaveraceae* family, were analysed for flavonoid compounds. The effects of extracts on neuronal PC12 cells viability was determined. The anti-inflammatory effects of extracts were assessed by measuring the levels of IL-6 and IL-10 cytokines on hydrogen peroxide (H_2O_2)-stimulated PC12 cells. As a result of our studies, Rutin and Quercetin flavonoids have been found to be as major. The amount of Rutin was higher in methanol (45 µg/ml) than water (41 µg/ml). Quercetin was better extracted with methanol (12 µg/ml) than water (10 µg/ml). None of the tested extracts were cytotoxic even to PC12 cells. Both extracts showed an anti-inflammatory effect in a dose dependent manner. The water extract showed the maximum anti-inflammatory effect, with IL-6 secretion decreased 79 fold according to the H_2O_2 treated group and IL-10 secretion increased to 87 fold according to the control group. This study is an evidence that the Rutin and Quercetin flavonoids detected in *G. corniculatum* methanol and water extracts have a neuroprotective effect through anti-inflammation.

Keywords: *Glaucium corniculatım,* flavonoid, neurodegenerative diseases, anti-inflammation

1. INTRODUCTION

Neurodegenerative diseases (NDs) such as Alzheimer's disease (AD), Parkinson's disease (PD), amyotrophic lateral sclerosis (ALS) and multiple sclerosis (MS) are defined as disorders with loss and damages of neurons. Approximately 30 million people worldwide are affected by NDs and it is estimated that until 2040, casualties caused by NDs will be the second after cancer. A common link between these diseases is chronic activation of innate immune responses, also known as inflammation [2]. Inflammation is a response that operates against the endogenous or exogenous agents such as infection, injury, and exposure to contaminants to continue the organism's life. The inflammation response that occurs in chronic conditions is caused the extracellular matrix damages and organ dysfunctions [3]. It has been proven that chronic inflammation is a part of the cause of NDs [4, 5]. Mechanisms responsible for the perception,

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conduction and replication of inflammatory processes that cause the production of neurotoxic mediators, such as cytokines and interleukins, are associated with NDs [6, 7]. The evidences of chronic inflammation in NDs are based on the findings on increased levels of proinflammatory cytokines such as interleukin 6 (IL-6) [8] and deceased levels of anti-inflammatory cytokines such as interleukin 10 (IL-10). Understanding the interaction between the inflammation and nervous systems is crucial as a strategy to intervene with chronic NDs.

There are some drugs approved by U.S. Food and Drug Administration (FDA) such as dopamine agonists, cholinesterase inhibitors, anti-inflammatory medications and calcium channel blockers that are used for the treatment and prevention of NDs [9]. The existing drugs against NDs, only delay the progression of the disease by alleviating the symptoms; however, they do not provide a complete cure.

In recent years, the studies for the discovery of novel drugs and biomarkers for the treatment of NDs focused on strategies to discover natural products that have the potential of anti-inflammatory properties. These studies usually focus on drugs used in traditional medicine, most of which are plant extracts. Plant derived bioactive compounds such as steroidal-piperidine-alkaloids, fatty acids, phenols, alkaloids, saponins, terpenes and flavonoids play a major role in the slowing of many NDs. Some plants have traditionally been used for centuries to treat memory impairment. One of these species is *Glaucium corniculatum*, belonging to the family of *Papaveraceae* [10]. The species of the *Papaveraceae* biosynthesize pharmacologically active flavonoids, known to exhibit different pharmacological effects such as anti-inflammatory and neuroprotective [11]. Rutin (3, 3', 4', 5, 7-pentahydroxyflavone-3-rhamnoglucoside) and Quercetin **(**3, 30, 40, 5, 7-pentahydroxyflavone) are bioflavonoids of the flavonol type abundantly present in *Papaveraceae.* Many studies have been carried out to understand the medicinal importance of Rutin and Quercetin. They have been reported to exert numerous biochemical and pharmacological activities, such as the suppression of cellular immune and inflammatory [12] and neuroprotective [13] responses.

The goal of this study is to thoroughly examine the flavonoid content of methanol and water extracts of *Glaucium corniculatum* by high-performance liquid chromatography (HPLC) method. However, this study involved testing the ability of these extracts to protect differentiated PC12 (dPC12) cells by nerve growth factor (NGF) 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 IL-6 and IL-10 production.

2. MATERIAL and METHODS

2.1. Plant Material

Crube plants were collected and verified by Prof. Dr. Zeki Aytaç (Gazi University, Faculty of Science, Department of Biology). The voucher specimen was kept in the Herbarium of Gazi University, Faculty of Science, Department of Biology. *G. corniculatum (L.) RUD.* subsp. *refractum* (NAB.) CULLEN was collected from Beypazari district in the northwest of Ankara on 9.07.2012.

2.2. Preparation of Plant Extracts

Above-ground tissues of the plant samples were dried, powdered with an electric grinder and stored in laboratories of the Faculty of Science Department of Biology, Gazi University, Turkey. The plant powder (30 gr) were macerated with 300 mL of methanol and water, respectively at the room temperature for 6 hours using soxhlet device (LabHeat). The extracts were filtered by whatman filter paper and evaporated to dryness (45°C) under reduced pressure by rotary evaporator (Heidolph Laborota 4000) and stored in a refrigerator at 4°C until the time of use [14].

2.3. Quantitative HPLC Analysis of Flavonoids in Plant Extracts

Flavonoids in the samples were identified on an Agilent Technologies HPLC 1200 series (Santa Clara, CA, USA) equipped with a quaternary pump, a manual sampler and an ultraviolet/ visible (UV-Vis) detector with a loop size of 20 µl. The reversephase chromatographic analysis was carried out in isocratic conditions using a C-18 reverse phase column (150 mm × 4.6 mm i.d., particle size 5 µm, Agilent Zorbax Eclipse XDB-C18) at 25 °C. Simultaneous monitoring was performed at 254 nm and 330 nm and a flow rate of 1 mL/min. The amount of each phenolic compound was expressed as µg per mg of the extracts [15].

2.4. PC12 Cell Culture

Pheochromocytoma PC12 cells were obtained from Gazi University, Biotechnology Laboratory Collection and they were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 10% horse serum (HS), 1% antibiotic mixture comprising penicilin streptomycin (100 units/mL penicilin-100 µg/mL streptomycin) and 1% (2 mM) L-glutamine in a humidified CO_2 (5%) incubator at 37°C. The medium was changed every other day. The cells were differentiated for 4 days using 100 ng/mL NGF [16]. The differentiation medium was refreshed every two days.

2.5. Cell Viability Assay

In order to determine the toxicity of H_2O_2, the cells were plated at an appropriate density ($1x10^4$ cells/200 µL) in a collagen-coated 96-well plate. After that, the cells were treated with 200 µM H_2O_2 for 12 and 24 h.

To determine the toxicity of *G. corniculatum* extracts, the cells were plated at an appropriate density ($1x10^4$ cells/200 µL) in a collagen-coated 96-well plate at 37°C in 95% humidified air with 5% CO_2. dPC12 cells were treated with 100, 250, 500 and 1000 µg/ml plant extracts which were diluted with differentiation medium for 12 and 24 h. The cell viability was assessed by 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium (MTT) assay. After incubation, to each well 20µl of MTT (5mg/ml) in phosphate buffered saline (PBS) was added and incubated at 37° C for 4h. MTT medium was carefully aspirated from the wells and the formed formazan crystals were solubilized in 200µl of dimethyl sulfoxide (DMSO). Using a micro plate reader the absorbance was measured at 570 nm (Epoch, BioTek) [17]. The % cell viability was determined using the following formula:

% Cell Viability = [Abs (sample) / Abs (control)] x 100 [18].

2.6. Anti-inflammatory Effect of Extracts

Interleukin-10 (RT IL-10-Life Tech. (KRC0101)) and Interleukin-6 (RT IL-6-Life Tech. (KRC0061)) ELISA kits were used to test the anti-inflammatory effect of the extracts. The cells were seeded in 96-well plates ($1x10^4$ cells per well) and pretreated with 100, 250, 500 µg/ml for 24 h prior to exposure to 200 µM H_2O_2. After 24 h, the cell culture media was collected. The IL-10 and IL-6 levels in cell culture mediums of the samples and control groups were measured using IL-10 and IL-6 commercial kits according to the manufacturer's instruction. In each assay, the duplicates of each sample, standard, and blank control (zero standard) were run on the same microplate. Furthermore, IL-10 and IL-6 levels were expressed in pg/mL of the cell culture medium [19].

2.7. Statiscal Analysis

The analysis of the data was performed by analysis of variance (ANOVA) followed by Tukey's post-test to test for significance, which was set at 5%. The results were presented as mean value ± standard deviation (n=5 for the MTT assays, n=3 for all other analysis).

3. RESULTS and DISCUSSIONS

3.1. Qualitative Flavonoid Analysis

The identification of the individual flavonoids was performed through HPLC, by comparing their masses and λmax with the literature data (Table 1). Rutin and Quercetin were indentified, but Luteolin, Biochanin, Kaemferol, Genistein, Catechin and Apigenin could not be identified (Ni) in methanol extract. Luteolin, Rutin and Quercetin were indentified, but Biochanin, Kaemferol, Genistein, Catechin and Apigenin could not be identified in the water extract. As a result of our studies, Rutin and Quercetin flavonoids were found as major and the other flavonoid contents were insignificant. The amount of the Rutin was higher in methanol (45 μg/ml) than in water (41 μg/ml). Quercetin was also better extracted with methanol (12 μg/ml) than with water (10 μg/ml).

Table 1. Quantitative analysis of flavonoids in *G. corniculatum* methanol and water extracts

Extracts	**Flavonoids (μg/ml)**							
	Luteolin	Biochanin	Rutin	Quercetin	Kaemferol	Genistein	Catechin	Apigenin
Methanol	Ni	Ni	45	12	Ni	Ni	Ni	Ni
Water	0.23	Ni	41	10	Ni	Ni	Ni	Ni

3.2. Effects of H2O2 on viability of dPC12

In our assays, H_2O_2 did not show any significant cytotoxic effects at 100 μM concentrations for 12 and 24h (data not shown). Therefore, the 200 μM concentrations of H_2O_2 were used for 24h in the subsequent experiments.

3.3. Effects of G. corniculatum extracts on viability of dPC12

In our assays, generally, all the extracts were found to have high IC50 values than 1000μg/ml IC50 concentrations (1287±7μg/mL for methanol and 1150±8 μg/mL for water) for 24h. The results of cell viability were presented in Table 2. According to the U.S. NCI plant screening program, a crude extract is generally considered to have *in vitro* cytotoxic activity with an IC50 value ≤20μg/mL [20]. Consequently, all the extracts demonstrated less toxicity on PC12 cells. These results have proven that the extracts do not cause anti-inflammatory effect resulting from cell death in PC12 cells.

Table 2. The effect of *G. corniculatum* methanol and water extracts on cell viability of PC12

	Cell Survival (IC_{50}) (μg/ml)	
Extracts	**12 hour**	**24 hour**
Methanol	1650±5	1287±7
Water	1527±6	1150±8

Values expressed as mean ± SD.

3.4. Anti-inflammatory effects of G. corniculatum extracts

For the determination of anti-inflammatory effects of the plant extracts, IL-6 and IL-10 were measured in dPC12 cells pretreated with 100, 250, 500 μg/ml for 24 h plant extracts prior to exposure to 200 μM H_2O_2 (As shown in Figure 1 and 2).

The results showed that the extracts (100, 250, 500 μg/mL) significantly ($P<0,05$) inhibited IL-6 secretion by H_2O_2-stimulated dPC12 cells and increased anti-inflammatory cytokine IL-10 secretion in a dose-dependent manner.

However, dPC12 cells which were treated with 200 μM H_2O_2 for 24 hours showed a significant increase in the secretion of IL-6 (1106±2 pg/ml) when compared to the control (27±3 pg/ml). Although there was no significant difference between water and methanol extract, the water extract showed the maximum anti-inflammatory effect. The water extract decreased IL-6 secretion 79 fold according to the H_2O_2 treated group for 500 μg/ml for the highest applied plant extract dose.

In our study, IL-10 levels were found to be 21±3 pg/ml in the control group (untreated dPC12 cells). The secretion of IL-10 in *G. corniculatum* methanol extract treated dPC12 cells was found as 1173±3, 1401±5 pg/ml 1810±4 pg/ml and in *G. corniculatum* water extract treated dPC12 cells as 1224±5 1472±3 1845±4 pg/ml, respectively 100, 250 and 500 μg/ml. Treatment with *G. corniculatum* water extract showed the maximum anti-inflammatory effect with increased IL-10 by 87 fold according to the control group for 500 μg/ml, for the highest applied plant extract dose.

Our results suggested that *G. corniculatum* methanol and water extracts exhibited an anti-inflammatory effect against H_2O_2-induced inflammation of dPC12 cells via increasing anti-inflammatory cytokine IL-10 and decreasing pro-inflammatory cytokine IL-6 secretions.

Linear regression analysis proved that the amount of flavonoid and anti-inflammatory activity showed a negative correlation with extract type. Therefore, the inhibition of the inflammation by the extracts may be partially due to the amount of their flavonoid content but might be attributed to the diversification of flavonoid types.

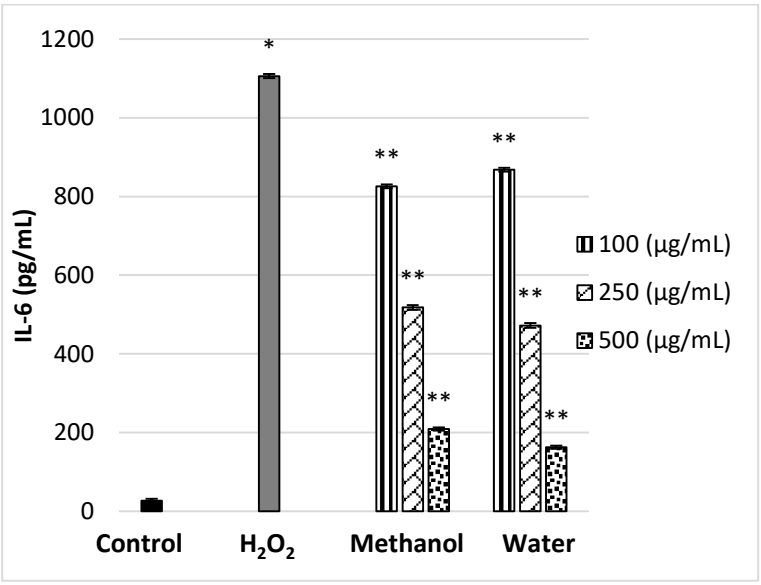

Figure 1. Dose-dependent inhibition of H_2O_2 mediated production of IL-6 by *G. corniculatum* methanol and water extracts in dPC12 cells. $^{*}p<0,05$; statistically different compared to negative control (untreated cells). $^{**}p<0,05$; statistically different compared to H_2O_2 treated group.

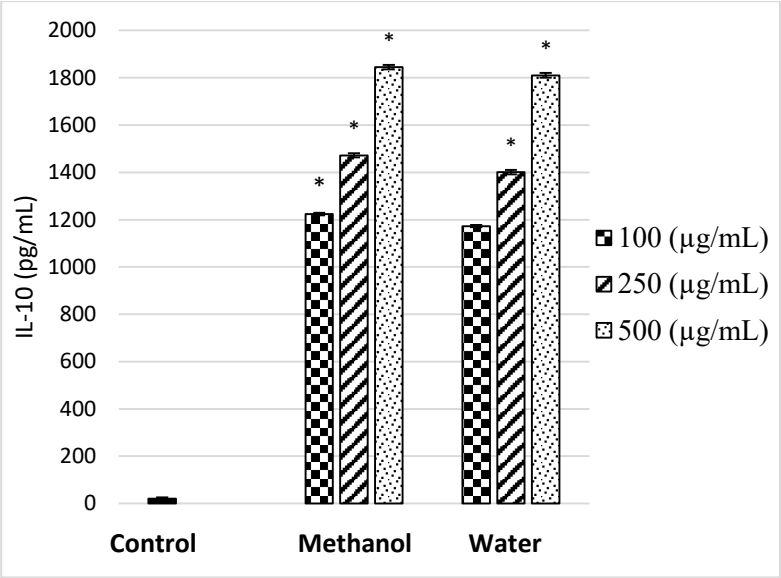

Figure 1. Dose-dependent inhibition of IL-10 TNF-α by *G. corniculatum* methanol and water extracts in PC12 cells. *p<0,05; statistically different compared to the control (untreated cells).

4. DISCUSSION

Glaucium-derived products have been used in traditional medicine in the treatment various inflammatory diseases. However, only limited numbers of studies have been conducted to elucidate the action mechanisms of these products on inflammatory diseases [21-23].

Oxidative stress due to the imbalance between ROS and antioxidant defenses will lead to the activation of survival pathway that involves in inflammation. H_2O_2 was thought to be the major precursor of reactive free radicals [24]. H_2O_2 activated macrophages and stimulated the production of pro-inflammatory cytokines such as IL-1, IL-6, TNF-α, leukotrienes, and nitric oxide (NO) [25]. It is known that NDs are associated with neuronal loss, triggered by neurodegenerative agents leading to oxidative stress and the development of inflammation. The levels of IL-6 are normally low and tightly regulated in a cell, but elevated levels of IL-6 have also been associated with various neurological disorders, including AD, PD, ALS and HD [26-28].

The anti-inflammatory effects of *G. corniculatum* methanol and water extracts were assessed here by measuring the expression and the release of pro-inflammatory cytokine IL-6 and the anti-inflammatory cytokine IL-10. However, in this article, the effects of *G. corniculatum* flavonoids in the suppression of inflammation and the preservation of neurons were investigated. In recent years, various herbal extracts and phytochemicals have been found to have anti-inflammatory properties. For instance, several flavonoids, such as Rutin and Quercetin were found to block IL-6 production [29, 30]. It has been found that flavonoids activate the endogenous antioxidant status in neuronal cells hence protecting them from undergoing neurodegeneration [31]. Polyphenols such as Quercetin and Rutin have distinct features in upregulating the production of intracellular antioxidant enzymes induced in PC-12 cells [32-34]. However, it has been reported that Quercetin and Rutin flavonoids produced anti-inflammatory effects by inhibiting proinflammatory cytokines [36, 36]. Hu et al. demonstrated that Rutin ameliorates inflammasome activation, leading to suppressed inflammation in the kidney of rats [37]. Similarly, Javed et al. reported the activity of Rutin in preventing cognitive impairments in rats [38].

The results show that *G. corniculatum* methanol and water extracts inhibit the H_2O_2 induced IL-6 production and increased the production of IL-10 in dPC12 cells. Previous studies

have shown an antagonist effect of IL-10 on secretion of pro-inflammatory cytokines, suggesting that *G. corniculatum* extract mediated inhibition of the H_2O_2 induced IL-6 secretion may pass through the induction of IL-10 production. These outcomes were consistent with the results of anti-inflammatory properties; however, the amount of flavonoids showed a negative correlation with extract type. Therefore, the results suggest that anti-inflammatory effect may be related to flavovoid diversity rather than flavonoid amounts. The ability of *G. corniculatum* extract to modulate the pro-inflammatory and anti-inflammatory cytokines suggests that it may be an alternative agent for the treatment and / or prevention of inflammatory diseases and NDs.

5. CONCLUSSION

The amount of flavonoid content of methanol and water extracts of *G. corniculatum* was successfully screened. Rutin and Quercetin flavonoids are major flavonoids and *G. corniculatum* methanol extract has the highest flavonoid amount.

All of the *Glaucium* extracts used in this study provide anti-inflammatory effect without damaging dPC12 cells. These results bring attention to the inhibitory effects of *G. corniculatum* on inflammation and can explain why this plant is considered as a traditional medicinal plant. According to these results, *G. corniculatum* extracts may have a favourable pharmacological profile in the treatment of NDs and inflammation. Moreover, this study proves that the Rutin and Quercetin flavonoids detected in *G. corniculatum* methanol and water extracts have a neuroprotective effect through anti-inflammation.

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Conflict of Interests

Authors declare that there is no conflict of interests.

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