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Article

Chemical profile, acetylcholinesterase, butyrylcholinesterase, and prolyl oligopeptidase inhibitory activity of *Glaucium corniculatum* subsp. *refractum*

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Papaveraceae is one of the prominent alkaloid-containing families, and plants of the genus *Glaucium* (Papaveraceae) are known for their bioactive alkaloids. *Glaucium* species have been used in traditional medicine in Turkey as an analgesic, narcotic, sedative, and antitussive. In this study, it was planned to evaluate the inhibitory activity of an alkaloidal extract of *Glaucium corniculatum* subsp. *refractum* on acetylcholinesterase (AChE), butyrylcholinesterase (BuChE) and prolyl oligopeptidase (POP), as well as exploring the chemical profile of the plant by using Gas Chromatography-Mass Spectrometry (GC-MS). The AChE, BuChE and POP inhibition activities of the alkaloidal extract of *G. corniculatum* subsp. *refractum* were determined spectrophotometrically. A rapid GC-MS method was used to identify alkaloids that could be responsible for these inhibition activities. In total, eleven alkaloids were identified in the alkaloid extract of the plant by GC-MS. Allocyptopine (52.92%) and protopine (25.38%) were found as the major constituents. The alkaloidal extract of *G. corniculatum* subsp. *refractum* showed potent AChE inhibitory activity (IC_{50}:1.25 µg/mL) and BuChE inhibitory activity (IC_{50}: 7.02 µg/mL). The extract also showed a remarkable inhibitory effect on POP with an IC_{50} value of 123.69 µg/mL. This study presents the first GC-MS investigation and POP inhibitory activity of *G. corniculatum* subsp. *refractum*.

Keywords: *Glaucium corniculatum*. Anticholinesterase. POP inhibitory activity. GC-MS.

INTRODUCTION

Dementia is characterized by the loss of cognitive functioning which affects memory, thinking, recall and behavioral abilities (Ibrahim, Gabr, 2019). Neurodegeneration is the most common cause of dementia and often leads to Alzheimer's disease (AD) (Ibrahim, Gabr, 2019; Prince *et al.*, 2015). AD is a progressive, degenerative, and irreversible illness, which affects approximately 47 million people worldwide (Prince *et al.*, 2015). The major characteristics of AD are amyloid plaques, neurofibrillary tangles, inflammation, and cholinergic abnormalities (Ibrahim, Gabr, 2019; Greig, Lahiri, Sambamurti, 2002). The human brain has two main cholinesterases, namely AChE and BuChE. Acetylcholine (ACh) is a neurotransmitter that is essential for memory and learning abilities which has a crucial role in the peripheral and central nervous systems (Greig, Lahiri, Sambamurti, 2002). The concentration and function of ACh are reduced in patients with AD, and acetylcholinesterase (AChE) inhibitors increase the synaptic levels of ACh (Bajic *et al.*, 2016). Butyrylcholinesterase (BuChE) is another cholinesterase enzyme which is responsible for modulating important neural functions (Bajic *et al.*, 2016; Greig, Lahiri, Sambamurti, 2002; Mesulam *et al.*, 2002; Pinho *et al.*, 2013). The presence of AChE in the brain is more abundant than that of BuChE. However, BuChE also has an important role in cholinergic neurotransmission. Rivastigmine, which inhibits both AChE and BuChE, has

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an important role in the treatment of cognitive deficits related to neurological abnormalities (Bajic *et al*., 2016; Mesulam *et al*., 2002; Pinho *et al*., 2013). The small number of medications commonly used in the treatment of AD has a symptomatic effect rather than a radical cure of the disorder. In this context, it is important to find effective compounds aimed at eliminating the causes of the disease. On the other hand, due to the side effects of the drugs used in AD treatment, especially in the central nervous system and gastrointestinal system, it is a necessity to discover more effective but safer drugs (Ahmad, Ayaz, Murkovic, 2020; Anand, Singh 2013; Ayaz *et al*., 2015).

Prolyl oligopeptidase (POP) inhibitors produce an enhanced performance in cognitive activities and inhibit amyloid accumulation in animal models of AD (Kato *et al*., 1997). POP, also known as prolyl endopeptidase, is a large cytosolic enzyme that belongs to the serine peptidase enzyme family (Polgár, 2002). Impaired serum POP activity has been associated with neuropsychiatric problems including mania, bipolar disorder, and schizophrenia (Polgár, 2002; Rossner *et al*., 2005). In the course of our ongoing studies on the investigation of Turkish medicinal plants as sources of potential enzyme inhibitors, various alkaloid-rich plant species have been analyzed. It was reported in the literature that members of the Papaveraceae family have specific enzyme inhibition activities with structurally diverse alkaloids (Han *et al*., 2016). This family is distributed in the temperate and subtropical regions of the Northern Hemisphere and represented by 42 genera and 775 species in the world (Xu, Deng, 2017). The genus *Glaucium* Mill. belongs to the Papaveraceae family and consists of 28 species found in the Mediterranean region, Southwest Asia and Central Asia (Davis, Mill, Tan, 1988; Kilic, Yildi, Kilic, 2018). Traditionally, *Glaucium* species have been used as antitussive, analgesic, narcotic, sedative, anti-hemorrhoidal substances and in the treatment of skin disorders in Turkey (Baytop, 1984; Erbay, Anıl, Melikoglu, 2018; Hayta, Polat, Selvi, 2014; Sargin, 2015). The isoquinoline alkaloids group is predominant within the Papaveraceae family with significant biological activities. Glaucine (an aporphine-type isoquinoline alkaloid) firstly obtained from *Glaucium flavum* Crantz (Shamma, Slusarchyk, 1964) has been used as a cough suppressant in Eastern Europe. In addition to its antitussive effect, it has been proven to have bronchodilator, anti-inflammatory, analgesic, and antipyretic effects (Constant *et al*., 1983; Cortijo *et al*., 1999; Pinto *et al*., 1998). Besides glaucine, *Glaucium* species include many other biologically active isoquinoline alkaloids such as protopine, stylopine, corydine, and canadine (Chia *et al*., 2006; Shafiee, Ghanbarpour, Akhlaghi, 1985; Vacek *et al*., 2010).

In this study, *Glaucium corniculatum* (L) Rudolph subsp. *refractum* (Nab.) Cullen was collected from Gürun-Sivas in Turkey and analyzed by GC-MS for the determination of its alkaloid profile. The anticholinesterase and POP inhibition potentials of the plant were also investigated. *G. corniculatum* subsp. *refractum* is a perennial wild plant, with large yellowish-orange flowers (Davis, Mill, Tan, 1988). In a previous study that was conducted to examine the alkaloid profile of *G. corniculatum subsp. refractum* of Iranian origin, bulbocapnine, dicentrine and protopine were isolated as the major constituents (Shafiee, Ghanbarpour, Akhlaghi, 1985). Various phytochemical and biological studies that have focused on *Glaucium corniculatum* (Allafchian *et al*., 2018; Kintsurashvili, Vachnadze, 2000; Orhan *et al*., 2004) are available in the literature. However, the literature review in this study revealed no studies the POP inhibition activity and alkaloid profiling of *G. corniculatum* subsp. *refractum* by GC-MS in the literature.

MATERIAL AND METHODS

Chemicals

The standard compounds for GC-MS analysis were previously isolated from several Papaveraceae species, and galantamine for anticholinesterase activity was previously isolated from several Amaryllidaceae species in our laboratory and authenticated by means of spectral analyses (UV, IR, 1D NMR, 2D NMR, and MS). AChE from *Electrophorus electricus* (electric eel), BuChE from equine (*Equus caballus*) serum and POP (recombinant, expressed in *E. coli)* were purchased from Sigma-Aldrich (St. Louis, MO, USA). All chemicals and reagents were

of analytical grade, and they were obtained from either Merck (Darmstadt, Germany) or Sigma-Aldrich.

Plant Material

Aerial parts of *Glaucium corniculatum* (L) Rudolph subsp. *refractum* (Nab.) Cullen were collected from Gürun-Sivas, Turkey in June 2016 and identified by Serdar G. Senol (Department of Biology, Faculty of Sciences, Ege University, Izmir, Turkey). A Voucher Specimen (1574) was deposited in the Herbarium of the Department of Pharmacognosy, Faculty of Pharmacy, Ege University.

Extraction Procedure

Alkaloid extraction was performed using the protocol reported by Bozkurt-Sarikaya *et al.* (2013). The aerial parts of *G. corniculatum* subsp. *refractum* were dried at room temperature and powdered. The plant material (500 mg) was extracted consecutively three times with methanol (5 mL) in an ultrasonic bath for 30 min at room temperature. The solvent was evaporated *in vacuo*, and the residue was taken up in 10 mL 2% sulfuric acid. The neutral compounds were removed with diethyl ether (3 x 10 mL). The acidic aqueous phase was made alkaline with 26% ammonia so that it would reach pH 9.5–10 and extracted with chloroform (3 x 10 mL). The combined chloroform extracts were dried with anhydrous sodium sulfate and filtered, and the organic solvent was evaporated to obtain a crude extract of alkaloids. The obtained extract was used for GC-MS analysis and testing enzyme inhibition activities.

GC-MS Analysis

GC-MS analysis was performed using a Thermo GC-Trace Ultra Ver: 2.0., Thermo MS DSQ II (Thermo Fisher Scientific, San Jose, CA, USA) device operating in the electron impact mode (EI, 70 eV). A TR–5 MS column (30 m x 0.25 mm x 0.25 µm) was used (Bozkurt-Sarikaya *et al.*, 2013). The oven temperature was programmed as: 80 °C for 1 min, 80–230 °C (10 °C min^{-1}), 3-min hold at 230 °C, 230-280 °C (10°C min^{-1}) and 10-min hold at 280 °C. The injector temperature was 250 °C. The helium flowrate was maintained at 0.8 mL min^{-1}. The extract was dissolved in methanol (1 mg/500 µL). All injections were run in the splitless mode.

Anticholinesterase Inhibitory Activity

AChE and BuChE inhibitory activities were determined spectrophotometrically by using a microplate assay modified from Ellman's colorimetric method with a 96-well microplate reader (VersaMax Tunable Microplate reader, Molecular Devices, USA) as previously described (Ellman *et al.*, 1961). The stock solutions of the alkaloid extract of *G. corniculatum* subsp. *refractum* were prepared at concentrations of 600, 60, 6, 0.6, 0.06, 0.006 µg/mL in a phosphate buffer. Subsequently, the respective enzyme (0.25 U/mL) was added and incubated for 30 min at room temperature before the addition of the substrate solution. Final concentrations of 150, 15, 1.5, 0.15, 0.015, 0.0015µg/mL alkaloid extract were tested to calculate the inhibitory concentrations of 50% (IC_{50}, the concentration which inhibits the activity of the enzyme by 50%), and each test was performed in triplicates. The software package GraphPad Prism V5.0 (GraphPad Software, San Diego, CA, USA) was used for calculation. Galantamine was used as the positive control.

POP Inhibitory Activity

A colorimetric method was modified from Yoshimoto *et al.* (1987) and Cahlíková *et al.* (2015) for the POP inhibition activity assay. The enzyme was dissolved in a sodium phosphate buffer (0.2 U/mL). Dilutions were made to obtain concentrations of 1000, 100, 10, 1, 0.1 and 0.001 µg/mL. The diluted test solution, buffer solution and enzyme (POP) were added to the plate. After incubation for 5 min (30 °C), the POP substrate, *Z*-Gly-Pro-*p*-nitroanilide (in 40% 1,4-dioxane), was added. After 30 min of incubation (30 °C), the absorbance was recorded at 405 nm of UV wavelength with a microplate reader (VersaMax Tunable Microplate reader, Molecular Devices, USA). The enzyme inhibition activity was analyzed by measuring the formation of *p*-nitroaniline and calculated as a percentage compared to the blank.

The inhibitory concentration of 50% (IC_{50}) was obtained using the software package GraphPad Prism v5.0, and each test was performed in triplicates.

Statistical Analysis

All experimental data were evaluated using at least three independent assays, and the activity results are expressed as IC_{50} (**µg/mL**). All statistical analyses and calculations were carried out using the Microsoft Excel software (Redmond, Washington, USA), Xcalibur software (Thermo Fisher Scientific) and GraphPad Prism v5.0 software (GraphPad Software, San Diego, CA).

RESULTS AND DISCUSSION

GC-MS Analysis

GC–MS is a fast, practical, and reliable method that is frequently used in the characterization of isoquinoline alkaloids without any derivatization (Kreh, Matusch, Witte, 1995). This technique has been proven to be a useful method for the detection of different types of alkaloids especially when identification is required on trace components in complex matrix systems.

This aimed the rapid determination of alkaloids from *G. corniculatum* subsp. *refractum* by GC-MS. A total of eleven structurally different alkaloids were characterized, and eight of them were identified for the first time by GC-MS from *G. corniculatum* subsp. *refractum* of Turkish origin. These alkaloids were revealed to be in the subgroups of isoquinoline alkaloids, namely aporphine (**2**, **9**), protopine (**4**, **5**), tetrahydro berberine (**3**), and benzophenanthridine (**6**, **7**, **11**). It was observed that the plant was very rich in alkaloids. The extraction method was highly selective, and the interference of other non-alkaloidal components was almost negligible. The identification of each compound was carried out by GC-MS without any preliminary derivatization because of their thermal stability under the detection conditions.

The components were identified based on the comparison of their relative retention time, reference spectra from the NIST MS Search 2.0 (National Institute of Standards and Technology, Gaithersburg, MD, USA) or by GC-MS co-chromatography with previously isolated authentic standards. The raw data files were analyzed using the Xcalibur (version 2.0) software and deconvoluted by the use of the AMDIS (automated mass spectral deconvolution and identification system, version 2.65) software supported by NIST (Gaithersburg National Institute of Standards and Technology, MD, United States). The percentage of the total ion current (TIC) for each compound is given in Table I. The proportion of each chemical constituent of the alkaloid extract in relation to the overall amount of total alkaloid content is also given in Table I.

The area of the GC-MS peaks depends not only on the concentration of the related compounds but also on the intensity of their mass spectral fragmentation.

The GC-MS analysis of *G. corniculatum* subsp. *refractum* alkaloidal extract clearly exhibited the presence of isoquinoline-type alkaloids. In total, eleven alkaloids were identified, of which eight of the compounds were glaucine, canadine, protopine, allocryptopine, dihydrosanguinarine, dihydrochelerythrine, dehydroglaucine, and norsanguinarine. Two alkaloids, namely allocryptopine (52.92%) and protopine (25.38%), stood out in proportion in comparison to other alkaloids in this plant (Figure 1). Allocryptopine was the most abundant alkaloid in the alkaloid extract followed by protopine and canadine (9.07%). The results showed the presence of allocryptopine, which exhibited a characteristic molecular ion (M^+) peak at m/z 369 (2%), and the presence of a base peak at 164 (100%). Based on the comparison of the GC peak areas calculated in the total ion current mode, it was the major alkaloid (52.92%) found in the extract. Protopine was detected as the second major alkaloid (25.38%) with a base peak at m/z 190. Allocryptopine and protopine are found as the two major alkaloids in the genus *Glaucium*, as they were previously isolated from many *Glaucium* species (Kintsurashvili, Vachnadze, 2000; Shafiee, Ghanbarpour, Akhlaghi, 1985). Canadine exhibited (9.07%) a molecular ion peak at m/z 339. Canadine methiodide was previously isolated from a quaternary fraction of *Glaucium corniculatum* (Novák, Dolejš, Slavík, 1972), but canadine is reported from *G. corniculatum* subsp. *refractum* in this study for the first time in the

literature. Glaucine is a bioactive alkaloid, isolated from various species of *Glaucium*, especially from *Glaucium flavum* (Petitto *et al.*, 2010). Glaucine, allocryptopine, protopine and dehydroglaucine were reported before in *G. corniculatum* subsp. *refractum* (Shafiee, Ghanbarpour, Akhlaghi, 1985). However, dihydrosanguinarine, dihydrochelerythrine and norsanguinarine are reported for the first time from *G. corniculatum* subsp. *refractum* in this study. In 2014, a study conducted on *G. corniculatum* by Doncheva *et al.* (2014) showed that the alkaloid contents of samples grown in different climatic conditions could be variable. According to their theory, benzophenanthridine-type alkaloids were not recorded in samples found in the hot climate region (Doncheva *et al.*, 2014; Shafiee, Ghanbarpour, Akhlaghi, 1985). In this study, aporphine, protopine, tetrahydro berberine and benzophenanthridine-type alkaloids were found in *G. corniculatum* subsp. *refractum* collected in Central Anatolia, which is located in the continental climate zone between a temperate Mediterranean climate and a temperate Oceanic climate.

Alkaloids 1, 8 and 10 could not be identified with the available information. The fragmentation peaks at m/z 353 and the base peak at m/z 149 for compound 1 (Doncheva *et al.*, 2014), m/z 351 and the base peak at m/z 148 for compound 8 (Opletal *et al.*, 2014), and m/z 365 and the base peak at m/z 336 for compound 10 are characteristic in isoquinoline-type alkaloids.

TABLE I - Chemical constituents of *G. corniculatum* subsp. *refractum* alkaloidal extract analyzed by GC-MS

No	RT*	Compounds	$[M^+]$	Characteristic ions m/z (Relative intensity, %)	%*
1	24.17	M353	353(33)	352(33), 338(19), 188(29), 164(45), 149(100), 77(17)	2.19
2	24.97	Glaucine	355(87)	354(100), 340(66), 324(47), 297(31), 281(67)	0.68
3	25.58	Canadine	339(36)	338(27), 174(24), 164(55), 149(100), 121(19),77(16)	9.07
4	25.80	Protopine	353(4)	267(9), 190(100), 163(34), 134(17)	25.38
5	25.89	Allocryptopine	369(2)	354(2), 283(9), 267(8), 252(11), 206(24), 164(100), 163(36), 149(54), 148(44), 134(28)	52.92
6	27.20	Dihydrosanguinarine	333(87)	332(100), 318(10), 317(11), 274(6), 260(7), 137(12)	0.84
7	27.46	Dihydrochelerythrine	349(100)	348(96), 334(10), 333(15), 332(19), 318(17), 304(16), 290(22)	1.54
8	27.63	M351	351(34)	322(25),175(32), 148(100), 89(22)	1.32
9	28.98	Dehydroglaucine	353(100)	352(81), 281(42), 253(17), 176(15)	0.38
10	29.84	M365	365(10)	337(20), 336(100), 320(26), 292(15)	4.05
11	30.13	Norsanguinarine	317(100)	316(9), 259(8), 201(23), 174(14), 158(12)	1.63

*RT: Retention time, M: Molecular weight, m/z: Mass-to-charge ratio

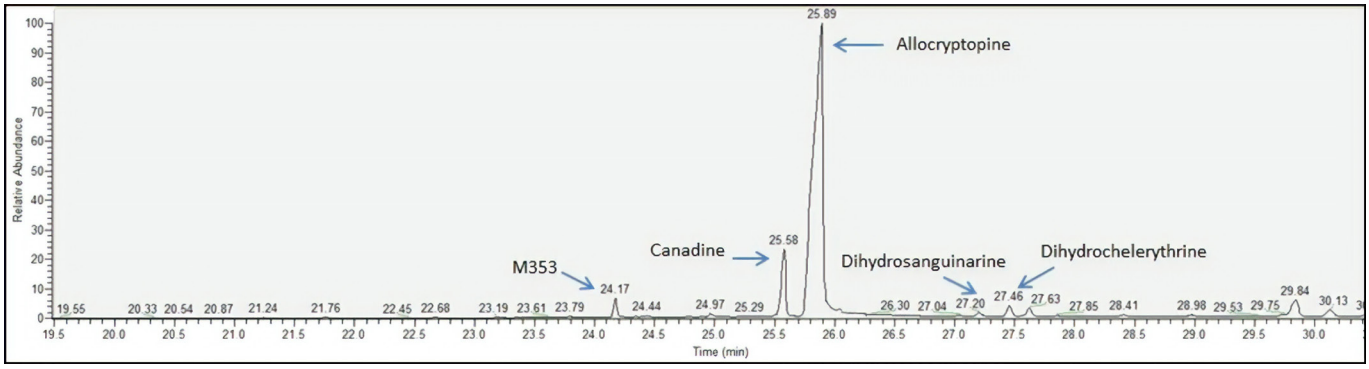

FIGURE 1 - A partial GC-MS chromatogram of *Glaucium corniculatum* subsp. *refractum* with retention times of the main compounds.

Anticholinesterase and POP Inhibitory Activity

Acetylcholinesterase inhibitors block the action of the enzyme cholinesterase, which is responsible for the hydrolysis of acetylcholine (Bajic *et al.*, 2016; Greig, Lahiri, Sambamurti, 2002; Mesulam *et al.*, 2002; Pinho *et al.*, 2013). As a part of our ongoing investigations on anticholinesterase-active medicinal plants, we present herein the AChE and BuChE inhibition activities of *G. corniculatum* subsp. *refractum* using galantamine as the positive control. The results revealed that the alkaloidal extract of *G. corniculatum* subsp. *refractum* showed potent AChE inhibition activity with an IC_{50} value of 1.25 µg/mL and BuChE inhibition activity with an IC_{50} value of 7.02 µg/mL. The inhibitory activities of the plant extract were examined for AChE and BuChE. Allocryptopine, protopine and canadine were the alkaloids with the highest proportion in the *G. corniculatum* subsp. *refractum* extract. Considering previous biological activity studies, allocryptopine, protopine and canadine might have been responsible for the AChE inhibition activity of the alkaloidal extract. These compounds were found to be a potent AChE inhibitor in the literature (Chlebek *et al.*, 2011; Vacek *et al.*, 2010).

Another enzyme associated with neurodegenerative disorders is POP (Polgár, 2002). It is reported for the first time in this study that *G. corniculatum* subsp. *refractum* has a potential for POP inhibition activity. Some of the alkaloids we identified by the GC-MS technique have been previously proven to have effects against POP activity. Canadine and dihydrosanguinarine have moderate activity against the POP enzyme (Cahlíková *et al.*, 2015). The *G. corniculatum* subsp. *refractum* extract also possessed the ability to inhibit POP in a dose-dependent manner with an IC_{50} value of 123.69 µg/mL. Z-pro-prolinal, a synthetic POP inhibitor, was used as the positive control, and it exhibited an IC_{50} value of 0.045 µg/mL.

The anticholinesterase and POP inhibition activity results of the alkaloidal extract of *G. corniculatum* subsp. *refractum* are shown in Table II.

In conclusion, the cholinesterase and POP inhibition activities of *G. corniculatum* subsp. *refractum* were evaluated, and the results showed that the plant exerted significant AChE, BuChE and POP inhibition effects. In light of the data reported in the literature, it is evident that the alkaloidal constituents of the plant were responsible for the significant biological activity results. The chemical profiling of the alkaloidal extract using GC-MS confirmed that the plant was a source of isoquinoline-type alkaloids with diverse chemical structures. Further studies are needed to confirm these activities, including the isolation of the detected alkaloids and evaluation of their enzyme inhibition activity potential.

TABLE II - Enzyme inhibition activities of *Glaucium corniculatum* subsp *refractum*

Sample	AChE IC_{50} (µg/mL)	BuChE IC_{50} (µg/mL)	POP IC_{50} (µg/mL)
Glaucium corniculatum subsp. *refractum* extract	1.25	7.02	123.69
Z-pro-prolinal	-	-	0.045
Galantamine	0.043	0.711	-

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