The anti-AChE and anti-proliferative Activities of *Glaucium acutidentatum* and *Glaucium corniculatum* Alkaloid Extracts

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ABSTRACT

In this study, methanol and water extracts of *Glaucium acutidentatum* and *Glaucium corniculatum* were analysed for anti-AChE activity on neuronal PC12 cell and anti-proliferative activity on HT-29 and HeLa cancer cells. In this study, total alkaloid, phenol and flavonoid content were analyzed for the determination of active compounds of *Glaucium acutidentatum* and *Glaucium corniculatum*. Indeed, methanol and water alkaloid extracts of these plants were evaluated for their anti-AChE activities on NGF-differentiated PC12 cells (dPC12) and anti-proliferative activities on HT-29 and HeLa cancer cells. Our data showed that alkaloid was major compound, the different concentrations of plant extracts had 35-90% *in vitro* AChE inhibitory activity and 26-54% cellular AChE inhibitory activity and it is dose dependent manner. Furthermore, *G. acutidentatum* methanolic extracts showed the highest anti-proliferative activity in HT-29 cells (45±5%) Both 1000 μg/ml methanolic extracts showed the maximum anti-proliferative effect in HeLa cells (64±3%). In conclusion, this study has shown that the anti-AChE and anti-proliferative effects of these two plants and the presence of a new AChE inhibitor (AChEi) may be effective in AD and cancer.

INTRODUCTION

In recent years, there has been an increase in the emergence of serious health problems, including Alzheimer's disease (AD) and cancer. AD was first described by Alois Alzheimer (Alzheimer, 1907). It is a progressive neurodegenerative disease characterized by loss of neuron and synapse in various parts of central nervous system (CNS) and associated with a decrease in the cognitive function and self-care deficiencies (Tayeb *et al.,* 2012). It is the most common form of dementia. As noted in World Alzheimer's Report 2015, there are an estimated 46 million people living with dementia worldwide and the number is expected to be increased up to 74.7 million by 2030 and 131 million by 2050. It is estimated that the worldwide cost of dementia is US $ 818 billion in 2015, which represents 1.09% of the global gross domestic product (World Alzheimer Report, 2015). Although there are such negative effects of AD, the exact cause(s) is not well-understood and available therapeutic options are limited. Until now, the major mechanism that results in successful symptomatic treatment of AD is the inhibition of AChE. AChE is one of the major enzyme which involves in cholinergic nerve transmission and hydrolyzes the neurotransmitter ACh into choline and acetate (Weihe *et al*., 1996). In AD, the activity of AChE will be high, resulting in scarcity in the levels of ACh, which ultimately results in the halt of neurotransmission (Syad and Devi, 2014). Furthermore, it has been demonstrated that increased AChE promotes the major neuropathological features of AD (Syad and Devi, 2014).

The existing drugs against AD are AChEi: tacrine, donepezil, rivastigmine and galanthamine, all of which have limited effectiveness and some kind of side effect such as

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dizziness, syncope, bradycardia, atrial arrhythmias, myocardial infarction, angina, seizures, sino-atrial and atrioventricular block (Wollen, 2010; Rowland *et al.,* 2007). For this reason, the determination of novel AChE inhibitory agents with fewer side effects which have preventive and therapeutic potential on the AD has attracted researchers' interest and thus becoming an important field of study. Researchers have focused on naturally-occurring compounds from plants as potential sources of either new or more effective AChEi (Murray *et al.,* 2013).

Although AChE is primarily an important member of the cholinergic system, several isoforms of AChE have been identified in various cell and tissue types. AChE has tasks related to cellular proliferation and differentiation besides the classical ACh hydrolysis function in these tissues and cells (Small *et al.,* 1996), which suggests that AChE is involved in cell proliferation and tumor-genesis. Therefore, the ability to inhibit AChE activity is important for its possible regulatory effects on cancer progression. Some tumor varieties (such as brain, lung, ovarian, breast, hepatocellular, renal and colon cancers) were found, first in the 1980s, to show an over-expression of some cholinesterase activities, especially of acetylcholinesterase (Garaventa *et al.,* 2010). Moreover, studies in recent years have revealed an inverse relationship between cancer and AD (Roe *et al.,* 2005). These studies have identified that patients with AD have a reduced risk of cancer and patients with cancer have a reduced risk of AD. Furthermore, studies have proved that some people have both AD and cancer, but are much less common than expected (White *et al.,* 2013). This inverse relationship indicates that AChEi agents may be effective in treating of both AD and cancer. In addition, the inhibition of AChE is associated with antisense inhibited apoptosis (Xi *et al.,* 2015) and some AChEi suppress the growth of cancer cells and induce mitochondrial pathway of apoptosis in cells (Cheng *et al.,* 2008; Xu *et al.,* 2014). Further, some chemotherapeutic agents, such as Irinotecan, are used in lung cancer treatment and therapy, acting through the inhibition of AChE activity (Dodds *et al.,* 2001). Most of the chemotherapeutic agents in clinical use are non-selective poisons, resulting in toxicity to normal tissues as well as to tumor cell. For this reason, the development of effective anti-cancer agents has been limited (Hyatt *et al.,* 2005). Despite the advances in modern diagnostic and treatment methods for cancer, there is a need to determine new targets for drug treatment (Grassino, 2013).

For several years, natural products have been used as traditional medicine to treat several diseases and disorders. The bioactive compounds from medicinal plants mainly include steroidal-piperidine-alkaloids derivatives and they play a major role in the slowing of many neurodegenerative and pathogenesis disorders such as AD and cancer (Dhivya *et al.,* 2014; Houghton and Howes, 2005; Wszelaki *et al.,* 2010; Rehman, 2006). In addition, many alkaloids with the inhibitory effect of AChE have various effects on neurological diseases such as AD (Murray *et al.,* 2013; Mukherjee *et al.,* 2007; Mehta *et al.,* 2012; Loizzo *et al.,* 2008). Studies on the effectiveness, tolerance and safety of this alkaloid are ongoing (Zhang, 2012). It has also been shown that alkaloids interact with free radicals and transition metals and enhance the activity of antioxidant enzymes, which are cancer markers (Albarracin *et al.,* 2012).

The *Papaveraceae* family polarizes the specialists attention because of its pharmaceutical, ornamental and alimentary valences (Bara *et al.,* 2007; Bara *et al.,* 1985; Bohm and Nixdorf, 1983). The species of the *Papaveraceae* biosynthesize pharmacologically active alkaloids, known to exhibit different pharmacological effects such as anti-tumor, anti-cancer, AChE inhibitory and cytological activities (Sener and Orhan, 2005; Orhan and Sener, 2004). Many species of this family have medicinal and aromatic uses. One of these species is *Glaucium corniculatum*, known to be traditionally used to treat memory impairment (Ahmed *et al.,* 2013) and have a very high potency of inhibiting AChE (Mehta *et al.,* 2012; Orhan and Sener, 2004; Das *et al.,* 2002; Perry *et al.,* 2001; Perry *et al.,* 2000). Moreover, *G. corniculatum* stands out with its richness of alkaloid content (Shafiee *et al.,* 1985; Doncheva *et al.,* 2002; Novak *et al.,* 1972; Phillipson *et al.,* 1981; Al-Wakeel *et al.,* 1995). The other is *G. acutidentatum*. It is endemic to Turkey. Most research to date has focused on the flora of *G. acutidentatum*; there are, however, no published data about the *G. acutidentatum* alkaloid content and its effect on AChE inhibition and cancer. Current studies show that the Glaucium species have AChE inhibitory properties and suggest that the plant is likely to be effective on the AD and cancer. Further, endemic species have attracted the researchers' attention because they may be used for the production of raw materials or preparations containing phytochemicals with health benefits (Exarchou *et al.,* 2002).

The goal of this study was to thoroughly examine the anti-AChE and anti-proliferative activities of methanol and water extracts from *Glaucium acutidentatum* and *Glaucium corniculatum*. In this study, the changes of the metabolics composition in methanol and water extracts of plants were determined and the anti-AChE and anti-proliferative potentials of these plants extracts were also compared with their metabolic contents. This is the first report on the relationship between anti-AChE and anti-proliferative effects of *Glaucium acutidentatum* and *Glaucium corniculatum* alkaloid extracts.

MATERIALS & METHODS

Reagents

High glucose Dulbecco's Modified Eagle's Medium (DMEM), horse serum, foetal bovine serum, L-glutamine, penicillin-streptomycin, acetythiolcholine iodide (ATChI), 5:5-dithiobis-2-nitrobenzoic acid (DTNB), AChE from Electrophus electricus (electric eeI), 3-(4, 5-Dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT), H_2O_2, bovine serum albumin (BSA), Dimethyl sulfoxide (DMSO), sodium bicarbonate, Tris-HCl, NaCl, $MgCl_2$, 1% Triton X-100, bradford were reagents purchased from Sigma, UK. 2,5S Nerve Growth Factor (NGF) (Invitrogen, CA). Paraformaldehyde (PFA) and Collagen type I from rat tail were purchased from Merch.

Plant Material

Crude plants were collected and verification was done by Prof. Dr. Zeki Aytac, Gazi University, Turkey. *G. acutidentatum* HAUSSKN. ET BORNM was collected from southwest of Durulmus village of Sivas on 21.06.2013. *G. corniculatum (L.) RUD.* sub sp. *refractum* (NAB.) CULLEN was collected from Beypazari district in the northwest of Ankara on 9.07.2012. The names of species are according to International Plant Name Index (IPNI).

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. Plant powders (30 gr) were macerated with 30 mL of methanol and water, respectively at room temperature for 6 hours with 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 time of use (Elufioye *et al.*, 2010). The plant extract (1 mg) was dissolved in 1 ml of 2 N HCl and filtered with a 0.2 μm filter (Shamsa *et al.*, 2008).

Determination of Total Alkaloids in Plant Extracts

The extracted solution was transferred to a separating funnel, 5 ml of bromocresol green solution and 5 ml of phosphate buffer were added. The mixture was shaken with 1, 2, 3 and 4 ml chloroform. The extracts were collected in a 10mL volumetric flask and then diluted to volume with chloroform. A set of reference standard solutions of boldine (100 to 2000 μg/mL) were prepared in the same manner as described earlier. The absorbance for test and standard solutions were determined against the reagent blank at 470 nm with an UV/Visible spectrophotometer. The total alkaloid content was expressed as mg of AE/g of extract (Shamsa *et al.*, 2008).

Determination of Total Flavonoid Content in Plant Extracts

Total flavonoid content was determined according to the aluminium chloride colorimetric method (Lin and Tang, 2007). Each plant extracts (2 mL, 0.3 mg/mL) in solvent were mixed with 0.1 mL of 10% aluminium chloride hexahydrate, 0.1 mL of 1 M potassium acetate and 2.8 mL of deionized water. After the 40 minutes incubation at the room temperature, the absorbance of the reaction mixture was determined spectrophotometrically at 415 nm. Rutin was chosen as a standard (the concentration range: 5 to 100 μg/mL) and the total flavonoid content was expressed as milligram RE/g of extracts.

Determination of Total Phenolic Content in Plant Extracts

The total phenolic content in the plant extracts was determined spectrophotometrically according to the Folin-Ciocalteu method (Singleton *et al.*, 1999) using galic acid as a standard (the concentration range: 25 to 500 μg/mL). The reaction mixture was prepared by mixing 1 mL of the methanolic solution of the extract, 9 mL of distilled water, 1 mL of Folin-Ciocalteu reagent and 10 mL of 7% sodium carbonate. After the 90 minutes incubation at room temperature, the absorbance was determined spectrophotometrically at 765 nm. The total phenolic content was expressed as GAE in milligram per gram dry extract.

Assay for *in vitro* AChE Inhibitory Activity

Inhibition of *in vitro* AChE activity was assayed as described by Ellman *et al.* (1961) with some modifications. The plant extracts were tested in 100, 250, 500 and 1000 μg/mL concentrations. 20 μL of 0.5 μg/mL AChE in 0.1 M phosphate buffer, pH 8 and 100 μL of the sample dissolved in the DMEM, 50 μL of 0.495 mg/mL 5,5'-Dithiobis-(2-Nitrobenzoic Acid) (DTNB) (which was dissolved with 1 mL of 0.1 M phosphate buffer, pH 8, containing 0.1875 mg/mL sodiumbicarbonate ($NaHCO_3$)) were added to each of 96 wells. The plates were incubated for 10 minutes at room temperature in dark area before the addition of 50 μL of 0.542 mg/mL substrate solution (S-Acetyl thiocholine Iodide) (was dissolved in 1mL distill water) to the reaction mixture. The absorbance of the yellow 5-thio-2-nitrobenzoate anion produced was measured at a wavelength of 412 nm using Epoch Take 3 Plate microplate reader after 10 minutes. Enzyme activity was calculated as a percentage compared to an assay using a buffer without any inhibitor. Galantamine was used as positive control. Percentage enzyme inhibition was calculated by comparing the enzymatic activity with and without inhibitor.

Cell Cultures

PC12 cell line was derived from a transplantable rat pheochromocytoma (Greene and Tischler, 1976). A notable feature of PC12 cells is that they respond to NGF. In response to NGF, PC12 cells are converted from proliferating chromaffin-like cells to nondividing sympathetic-neuron-like cells that extend axons and become electrically excitable (Greene, 1978; Greene and Rein, 1977). Because of these, PC12 cells are well-defined models to work on cellular biology of neurons and widely used to investigate the mechanism comprising neuronal repair, neuronal–substratum interactions, oxidative stress responses, neuroprotective activity, neurotoxicity and pathogenesis of neuronal diseases, including AD (Fedoroff and Richardson, 2001; Ma *et al.*, 2009; Sandhu, 1993; Fath *et al.*, 2002; Sambamurti *et al.*, 1992; Calissano *et al.*, 2009). In addition, PC12 cells were used as model in studies investigating the AChE inhibitory properties of various substances (Das and Barone, 1999; Zhang and Tang, 2000; Wang *et al.*, 2013; Pera *et al.*, 2013).

PC12 cells (CRL-1721 ™) were obtained from Gazi University, Biotechnology Laboratory Collection. The cells were grown at 37°C (5% CO_2) in growth medium: Dulbecco's modified Eagle's medium (DMEM) with 4.5% glucose containing 10% horse serum and 10% foetal bovine serum, supplemented with penicillin/streptomycin (100 U/ml)/ (100 μg/ml), L-glutamine (200 mM). The medium steriled and filtered with a 0.2 μm filter. PC12 cells were seeded onto T-75 cm^2 flasks (Corning, NY, USA)

coated with 50 ng/ml rat tail collagen, to achieve 70% confluence. The cells from passages 8–10 were used. The cells were split every other day at a ratio of about 2:3. Neuronal differentiation was initiated by the addition of 100 ng/ml NGF in growth medium. Differentiation medium was refreshed every two days (Jacovina *et al.,* 2001). HeLa (human cervical cancer) (CCL-2™) and HT-29 (human colon cancer) (HTB-38™) cell lines, the most commonly used for testing agents that may have anti-proliferative effect were purchased from ATCC. They were cultured in 75cm^2 flasks in DMEM with 4.5% glucose containing supplemented with 10% fetal bovine serum, penicillin/ streptomycin (100 U/ml)/ (100 µg/ml), L-glutamine (200 mM). The media was sterilized by 0.2 µm filter and the cells were grown at 37 °C in an environment of 5% CO_2. Cell counts were performed by Trypan blue exclusion method using haemocytometer slide (Jafarian *et al.,* 2014; Mahmoudi *et al.,* 2009).

Control of the Morphology and Differentiation of PC12 Cells

To control PC12 cells differentiation, the cells were grown on the Poly-D-Lysine and laminin coated cover slips with differentiation medium. The cover slips were fixed second, fourth and sixth days in 3.7% para-formaldehyde (PFA) for 15–20 minutes at room temperature and subsequently washed in 1xPBS. The cells were viewed using a light microscope (LEICA ICC50 HD, LAS V4.3.0 program). The cells were regarded as differentiated when the neurite length was more than two cell bodies (Ignatius *et al.,* 1985).

Cytotoxicity Assay

In order to determine the toxicity of H_2O_2 and plant extracts on PC12 cells, the cells were plated on to collagen-coated 96-well plates at a density of 1x10^4 cells/well at 37°C in 95% humidified air with 5% CO_2. dPC12 cells were treated with 50, 100 and 250 µM H_2O_2 for 6, 12, 24, 48h and treated with 100, 250, 500 and 1000 µg/ml plant extracts for 4, 10 and 24h which were diluted with differentiation medium. For the control cells, differentiation medium was used instead of the extract solutions.

In order to determine the anti-proliferative activity of plant extracts on two human cancer cell lines (HT-29 and HeLa), the cells were plated on 96-well plates at a density of 1x10^4 cells/well at 37°C in 95% humidified air with 5% CO_2 and treated with 500 and 1000 µg/ml plant extracts for 24h which were diluted with medium.

In vitro cytotoxicity was measured by the method of MTT described by Mosmann (1983) (Mosmann, 1983). This is a colorimetric assay that measures the reduction of yellow (3- (4, 5-dimethylthiazolyl-2) -2, 5-diphenyltetrazolium bromide) (MTT) by mitochondrial succinate dehydrogenase. The MTT enters into the cells and passes into the mitochondria where it is reduced to an insoluble, coloured (dark purple) formazan product measured spectrophotometrically. Since the reduction of MTT can occur only in metabolically active cells, the level of activity is a measure of the viability of the cells. After the treatments of the cells, 20 µl of MTT (5 mg/ml) in distilled H_2O was added to each well. The plates were gently shaken and incubated for 4h at 37°C in 5% CO2 incubator. After the removal of the medium containing MTT, 200 µL DMSO was added to each well. The plates were gently shaken to solubilize the formed formazan. Percent cell survival in the control group was assumed 100. The optical density was measured at 570 nm on a microplate reader. The % cell viability was determined using this formula:

% Cell viability = (Sample absorbance)/(control absorbance) X 100

Control cells were cultured with neither plant extract nor H_2O_2 treatment.

IC50 (50% inhibition of cell growth) value was calculated using a no linear regression log (inhibitor) versus response– variable slope graph.

Determination of Cellular AChE Activity

PC12 cells were plated on to collagen-coated 96-well plates at a density of 1x10^4 cells/well at 37°C in 95% humidified air with 5% CO_2. After the cells were differentiated, they were exposed to 50 and 100 µM H_2O_2 for 6, 12, 24 and 48h. After the H_2O_2 withdrawal, the cells were cultured in differentiation medium containing 1000 µg/ml plant extracts for 24h. To obtain total protein of the application made dPC12 cells, the cultures were washed twice with cold phosphate-buffered saline (PBS), scraped, and then the cell suspension was taken into 1.5 ml tubes, the tubes were centrifuged at 10,000 X g for 5 min at 4°C. PBS was discarded withdrawn. The cell pellets were lysed in solubilisation buffer (pH 7.8) (10 mM Tris-HCl, pH 7.2, 1 M NaCl, 50 mM MgCl$_2$, 1% Triton X-100) (Schwartz *et al.,* 2007). The homogenates were kept on ice for 30 minutes and vortexed briefly every 5 minutes. Following centrifugation of the homogenates (20,000 Xg for 30 min at 4°C), the supernatants were removed on ice. The total protein concentration, present in the extracts, was assayed according to the Bradford method (Bradford, 1977) with bovine serum albumin (BSA) as standard. The remaining protein was stored at -80°C until enzymatic analysis. Relative AChE activity was measured colorimetrically using a modified method of Ellman *et al.* (1961) which was adapted for 96 well plates. Relative AChE activity was measured with an equal amount of total protein. Briefly, equal amount of total protein, 100 µL of solubilisation buffer (pH 7.8) and 50 µL of 0.495 mg/ml DTNB (which was dissolved with 1ml of 0.1M phosphate buffer, pH 8, containing 0.1875 mg/ml NaHCO$_3$) were added to each of 96 wells and the plate was pre-incubated for 10 minutes at room temperature in a dark place. The reaction was started by the addition of 50 µL of 0.542 mg/ml ATCh (was dissolved in 1ml distill water). 10 minutes later, AChE enzyme activity was determined by the absorbance value, measured at a wavelength of 412 nm. The AChE activity was expressed as multiple relative to untreated control and enzyme activity was determined by the formula:

Relatively AChE activity = (Sample absorbance)/(control absorbance) X 100

Statistical Analysis

The statistical analyses were carried out using *t* test with $P< 0.05$ considered to be significant. The differences between the untreated control group and treated group were determined by t-test whereas IC50 (50% inhibition of cell growth) value was calculated using a no linear regression log (inhibitor) versus response–variable slope graph. Pearson's correlation coefficient test was used to assess correlations between the means.

RESULTS AND DISCUSSIONS

Total Alkaloid, Flavonoid and Phenolic Contents

The alkaloid contents were expressed in terms of boldine equivalent as mg of AE/g of extract (the standard curve equation: y=1.3026x–0.0176, R^2=0.9924). The content of flavonoids was expressed in terms of rutin equivalent (the standard curve equation: y=0.024x+0.0556, R^2=0.9916), mg of RE/g of extract. Gallic acid was used as a standard compound and the total phenolic contents were expressed as mg/g gallic acid equivalent using the standard curve equation (y= 0.0318x–0.4032, R^2= 0.9976).

In all plant extracts, alkaloid was found to be the major compound and flavonoid and phenolic contents were less than a substantial degree (Table 1).

This result is in agreement with Shafiee *et al.* (1985) and Novak *et al.* (1971), reporting that the *Glaucium* genus stand out with its richness of alkaloid content.

Table 1: Total alkaloid, flavonoid and phenol contents of G. acutidentatum and G. corniculatum extracts.

Plant Extracts	Total alkaloid content (mg AE/g) of extracts	Total flavonoid content (mg RE/g) of extracts	Total phenolic content (mg GAE/g) of extracts
G. acutidentatum			
Methanol	98.77±0.01[a]	0.70±0.02 [a]	1.84±0.09 [a]
Water	84.79±0.04[b]	1.00±0.02 [a]	1.62±0.05 [a]
G. corniculatum			
Methanol	100.61±0.06[c]	0.80±0.04 [a]	1.63±0.03 [a]
Water	76.65±0.09[d]	1.20±0.04 [a]	1.60±0.06 [a]

Values expressed as mean ± SD. Data with different superscript letters along the same column are statistically different ($P<0.05$).

The total alkaloid content in different extracts were compared according to the statistical calculation and the results showed all the studied alkaloid contents varied according to plant species and extraction solvent. In both plant species, methanol extracts contain more alkaloid compared to water. The methanol extract of *G. corniculatum* was the highest amount of total alkaloid (100.61 mg AE/g) and water extract of *G. corniculatum* had the least content (76.65mg AE/g). It is known that secondary metabolites vary in their behavior in organic solvents because they have different chemical structures. Although both free and salt alkaloids can be dissolved in various solvents, many alkaloids dissolve poorly in water but readily dissolve in organic solvents, such as methanol (Babbar, 2015).

***In vitro* Inhibition of AChE**

Using Ellman's colorimetric assay, all the tested water and methanol extracts of plants demonstrated AChE inhibitory properties in a dose dependent manner. The results show that all of the extracts at 1000 µg/mL displayed high anti-AChE activity for all species (Table 2). The tested extracts were found to have AChE inhibitory properties at varying rates of 35-90% depending on the concentration. *G. corniculatum* methanol extract showed a higher AChE inhibitory effect (90±2%) and *G. acutidentatum* water extract showed a lower AChE inhibitory effect (78±1%) at 1000 µg/mL.

Table 2: The *in vitro* AChE inhibitory activity of the *G. acutidentatum* and *G. corniculatum* extracts.

	AChE Inhibition (%)			
	Extract Concentration (µg/ml)			
Plant Extracts	100	250	500	1000
G. acutidentatum				
Methanol	38±1[a]	47±1[a]	53±2[a]	85±1[a]
Water	35±1[a]	39±2[b]	45±0[b]	78±1[b]
G. corniculatum				
Methanol	59±0[b]	72±2[c]	77±1[c]	90±2[a]
Water	52±1[c]	60±1[d]	68±1[d]	89±3[a]
Galantamine[*]	88±3	88±1	92±2	93±2

Values expressed as mean ± SD. *Positive control. Data with different superscript along the same column are statistically different ($P<0.05$).

Glaucium species have a very high potency of inhibiting AChE (Mehta *et al.,* 2012; Orhan and Sener, 2004; Das *et al.,* 2002; Perry *et al.,* 2001; Perry *et al.,* 2000). In a study conducted by Orhan *et al.,* (2004), chloroform: methanol (1:1) extracts of *G. corniculatum* manifested 86.55% AChE inhibition at 1000 µg/ml. On the contrary, not much work has been done on the anti-AChE activity of *G. acutidentatum*. Our results are consistent with the previous studies showing the effect of *G. corniculatum* on AChE inhibition. In addition, it is the first study to show that *G. acutidentatum* is an AChE inhibitor.

In this study, the 1000 µg/mL of *G. corniculatum* extract was found to show an AChE inhibitory activity similar to that of the positive control galanthamine. A study made by Mohsen *et al.,* (2015) determined that 1 mM of donepezil showed 99.01±4.89% AChE inhibitory effect (Mohsen *et al.,* 2015). In a study in which rivastigmine at 40 mg/ml was used as the standard, almost 66% AChE inhibitory activity was identified (Somani *et al.,* 2015). In a study conducted in the same manner, 66% AChE inhibitory activity of 1000 µg/ml of physostigmine (eserine) was determined (Rodrigues *et al.,* 2014). Mandegary *et al.,* (2014) determined that 2 µg/ml of tacrine showed 86.37±3.24% AChE inhibitory effect. According to these results, *G. corniculatum* and *G. acutidentatum* alkaloid extracts showed a higher AChE inhibitory effect than some AChE inhibitor drugs approved for the treatment of AD.

Table 3: The effect of plant extracts on cell viability of PC12.

	Cell Survival (IC$_{50}$) (µg/ml)		
Plant Extracts	**4 hour**	**10 hour**	**24 hour**
G. acutidentatum			
Methanol	1326±4[a]	1080±3[a]	979±4[a]
Water	2775±4[b]	2434±4[b]	1383±7[b]
G. corniculatum			
Methanol	1982±3[c]	1403±1[c]	1203±2[c]
Water	1830±2[d]	1494±3[c]	1166±3[d]

Values expressed as mean ± SD. Data with different superscript along the same column are statistically different (*P*<0.05).

Morphological Control and Differentiation of PC12 Cells

Neurons are morphologically polarized cells and they have extensions reaching from the cell body to the periphery. Extensions are defined as neurites when reaches twice the diameter of the cell body (Van Ooyen, 2005). According to neurite size analyzes performed on an average of 100 cells, baseline neurite densities and differentiation percentages of PC12 cells treated with 100 ng/ml NGF were analyzed on the 2nd, 4th and 6th day of differentiation. In parallel with differentiation times, the increase in neurite lengths of PC12 cells was found to be statistically significant. On the fourth day, neurites started to form and on the 6th day differentiation was observed. 100 ng/ml NGF for 6 days was used in subsequent studies.

Effects of H$_2$O$_2$ on Cell Viability of PC12 Cells

PC12 cell culture was used for the cell viability tests of H$_2$O$_2$. H$_2$O$_2$ did not show any significant cytotoxic effects at concentrations ranging from 50 to 100 µM for 6, 12, 24h, whereas cell survival was significantly decreased at a concentration >100 µM. In addition, high mortality rates were observed after 48h of application (data not shown). Therefore, the 50 and 100 µM concentrations of H$_2$O$_2$ were used for 24h in the subsequent experiments.

Effects on Cell Viability of *Glaucium spp.* Extracts

In our experiments, in general, IC50 values of plant extracts applied to PC12 cells for 4, 10 and 24 hours were found to be high (>1000 µg/ml). The results of cell viability are presented in Table 3. 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 ≤20 µg/mL (Sriwiriyajan *et al.,* 2014). Consequently, all the extracts demonstrated less toxicity on PC12 cells. These results have proven that *Glaucium* extracts do not cause AChE inhibition resulting from cell death in PC12 cells.

Effects on Cellular AChE Activity of *Glaucium spp.* Extracts

We showed that AChE activity did not increase effectively in PC12 cells treated with 50 µM H$_2$O$_2$. However, PC12 cells treated with 100 µM H$_2$O$_2$ for 24 hours showed a significant increase in AChE activity (152±2% AChE activity) when compared to the control (data not shown). Therefore, this concentration was selected to evaluate the potential protective effects of *Glaucium* extract on H$_2$O$_2$ induced AChE increase in PC12 cells.

We investigated whether *Glaucium* extracts could inhibit the increased activity of AChE induced by H$_2$O$_2$ in dPC12 cells. The cells were primarily incubated with 100 µM of H$_2$O$_2$ for 24h and then treated with 1000 µg/ml plant extracts for 24 hour.

AChE activity levels were more than 50% higher in H$_2$O$_2$ treated group when compared to the control. AChE activity at dPC12 cells treated with plant extract was significantly decreased when compared to H$_2$O$_2$ treated group. All the plant extracts prevented an increase in AChE activity in H$_2$O$_2$ treated dPC12 cells. Both methanol and water extracts of *G. acutidentatum* had higher AChE inhibitory action compared to *G. corniculatum* extracts. The methanol extract of *G. acutidentatum* showed the most strong inhibitory effects of the activity of AChE, with AChE activity decreased to 71±2%. However, the water extract of *G. acutidentatum* decreased the AChE activity to 81±3%, while methanol and water extracts of *G. corniculatum* decreased to 105±5%, 116±4%, respectively (Fig.1). According to the findings, *in vitro* and cellular AChE inhibitory effect is related to alkaloid due to negligible amounts of total phenolic and flavanoid contents in the extracts. High amounts of alkaloid and AChE inhibitory effect relative to water observed for methanol extracts also support the finding above. These findings indicate that the AChE activity increased in PC12 cells treated with H$_2$O$_2$, and suggest that all extracts that have anti-AChE effects and protective effect against AChE increase caused by H$_2$O$_2$ on PC12 cells.

Many alkaloids have an inhibitory effect of AChE and have various effects on diseases such as AD and cancer (Naaz *et al.,* 2013; Lu *et al.,* 2012). Comparison of the amount of total alkaloids results with its cellular AChE inhibitory activity showed no significant correlation in our study. Although *G.corniculatum* have the highest total amount of alkaloids, it has less cellular AChE inhibitory activity according to *G. acutidentatum*. Indeed, due to different AChE inhibition potentials of different compounds, the AChE inhibition activity of extract strongly dependents on the extraction solvent (Obregon *et al.,* 2005). These differences are due to the content and quality of the alkaloids in the different solvent. These results suggest that synergism between the alkaloids in the extracts and the anti-AChE activity is dependant not only on the concentration but also on the structure of alkaloids.
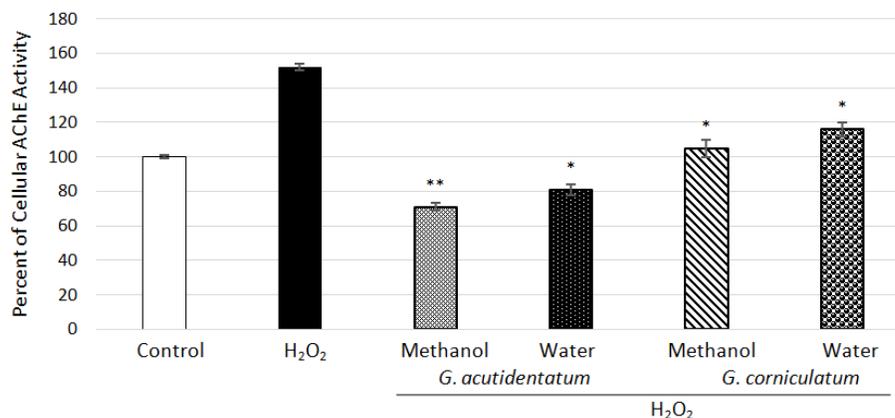
Fig. 1: The effect of H_2O_2, *G. acutidentatum* and *G. corniculatum* alkaloid extracts on AChE activity of dPC12. **Control:** dPC12 cells grown in normal culture conditions Values were expressed as mean ± SD. Statistical significance was taken at a P value < 0.05. [#]$P < 0.05$ v. vehicle control. $*P < 0.05$ v. 100μM H_2O_2 treatment for 24h alone. $**P <0.001$ v. 100 μM H_2O_2 treatment for 24h alone
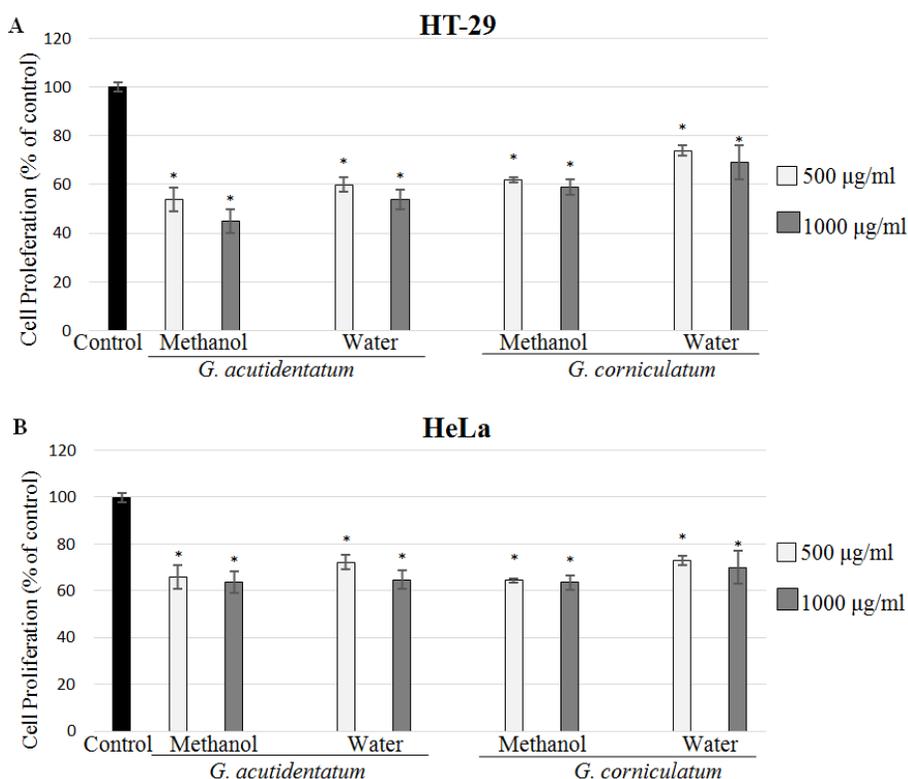
Fig. 2: The anti-proliferative effect of *G. acutidentatum* and *G. corniculatum* alkaloid extracts on HT-29 (A) and HeLa (B) cells. Control: HT 29 and HeLa cells grown in normal culture conditions Values were expressed as mean ± SD. Statistical significance was taken at a P value < 0.05. $*P < 0.05$ v. vehicle control.

Anti-proliferative Effect of *Glaucium spp.* Extracts

The anti-proliferative activity of the plant alkaloid extracts on two types of cancer cell lines were expressed by the percentage of cell viability (Figure 2). Based on the report by Fouche *et al.* (2008) the anti-proliferative activities of the extracts were categorized according to the percent decrease in cell viability into four groups: inactive (1-20%), weakly active (20-50%), moderately active (50-70%) or very active (70-100%)**.** Methanol extract of *G. acutidentatum* were moderately active against HT-29 cells at 1000 μg/ml with 55±5% decrease in cell viability and the others were weakly active (Fig 2A). Both methanol and water

extracts showed maximal anti-proliferative effect at 1000 μg/ml dose in HeLa cells, with cell viability decreased to 64±3% of the control cells (Fig.2B). Hence, on the overall, plant extracts showed a higher anti-proliferative effect in HT-29 cells than HeLa cells. When compared to the extract types, methanol extracts showed more anti-proliferative activity than water extracts in HT-29 and HeLa cells. The same signals may produce different responses in different cell types. This 'cell specificity' is due to the specific receptors and intracellular signalling molecules that are active in different cell types. There are some possible mechanisms for the formation of different cell-specific outcomes against the

same stimulus. One of this, the signal activation level and the effector processing mechanism could be cell specific (Miller-Jensen *et al.,* 2007). The fact that plant extracts have a higher anti-proliferative effect in HT-29 than HeLa cells may be associated with cell-type specificity. These outcomes were consistent with the results of the cellular AChE inhibition and showed that *G. acutidentatum* and *G. corniculatum* extracts had anti-AChE and anti-proliferative activity. Although many results show that AChE activities are increased in cancer patients, conflicting results have been found (Montenegro *et al.,* 2006; Zhang *et al.,* 2002; Martínez-López de Castro *et al.,* 2008; Jin *et al.,* 2004). There are studies that show that AChE activity decreases in lung cancer, which may contribute to lung cancer growth. Zhao et al. demonstrated that the expression of AChE was significantly decreased in the cancer tissues of 69.2% of hepatocellular carcinoma (HCC) patients, and the low expression level of AChE in HCC was correlated with tumor aggressiveness, the increased risk of postoperative recurrence, low survival rate, and poor prognosis (Zha *et al.,* 2011). For this reason, the functions of AChE in different cell types need to be further investigated.

Linear regression analysis proved that cellular anti-AChE activity and anti-proliferative activity showed negative correlation with total alkaloid amount. However, there was a strong relationship between the cellular anti-AChE and anti-proliferative activity ($r = 0.960$, $r^2 = 0.922$). Therefore, the inhibition of cancer cell proliferation and AChE by the extracts may be partial due to the amount of their alkaloid content but might be attributed to the diversification of alkaloid types. Further, these results demonstrate that determination of *in vitro* AChE inhibition is not sufficient alone for the exploration of AChEi, for there was a negative correlation between the *in vitro* and cellular AChE inhibitory activity.

CONCLUSION

We successfully demonstrated the anti-AChE and anti-proliferative activities of methanol and water extracts of *G. acutidentatum and G. corniculatum.*

All of the *Glaucium* alkaloid extracts used in our study provide AChE inhibition without damaging the PC12 cells. These results bring attention to the inhibitory effects of the *Glaucium* species on AChE and can explain why this plant is considered as a traditional medicinal plant. In addition, the alkaloid extracts of *G. acutidentatum* and *G. corniculatum* have potential anti-proliferative activity against HT-29 and HeLa cells. According to these results, *Glaucium* alkaloid extracts may have a favourable pharmacological profile in the treatment of AD and cancer. This study promotes greater awareness about the therapeutic use of AChEi in carcinogenetic diseases.

Conflict of Interests: There are no conflicts of interest.

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