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Original Research

# Evaluation of the effects of Cyclotrichium niveum on brain acetylcholinesterase activity and oxidative stress in male rats orally exposed to lead acetate

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**Abstract:** Cyclotrichium niveum is an endemic plant for Turkey and it appears to have in vitro antioxidant and acetylcholinesterase inhibition properties. To the best of our knowledge, there has been no study on the in vivo effects of this plant. Therefore, the purpose of this study was to evaluate the effects of C. niveum on lead (Pb)-acetate-induced potential alterations in brain acetylcholinesterase activity, as well as oxidative stress in male rats. The rats were randomly assigned to control, Pb-acetate, C. niveum and Pb-acetate+ C. niveum groups. Pb-acetate was provided in drinking water (500 ppm), and C. niveum was administered via oro-gastric gavage (4 ml/kg) for 30 days. The acetylcholinesterase activity in the brain significantly decreased only in the Pb-acetate group. The malondialdehyde level significantly increased, and the reduced glutathione activity decreased in the Pb-acetate group. The reduced glutathione and glutathione-S-transferase activities of the C. niveum group were higher than the control group. No Pb was detected on a ppb level in the brain tissue of the control and C. niveum groups, while it was detected in the brains of the rats in the Pb-acetate and Pb-acetate+ C. niveum groups (185+8.98 ppb and 206+56.65 ppb, respectively). The data collected in this study suggested that C. niveum may reduce inhibition of brain AChE activity and oxidative stress against Pb-acetate-induced alterations in the brain of male rats.

***Key words:*** Cyclotrichium niveum; Lead acetate; Brain; Acetylcholinesterase; Oxidative stress.

## Introduction

Lead (Pb) is one of the most widely and versatilely used metals in the industry. Most individuals may be exposed to this metal by ingesting contaminated food and water, as well as contaminated materials and dust (1). Once penetrated into the body, Pb has a wide range of destructive effects on many organs, and it induces various physiological and behavioral alterations (2-4). The central nervous system is particularly susceptible to the negative effects of Pb, which has been reported to induce damage in the brain through several direct and indirect pathways such as apoptosis, neurotransmitter dysregulations and oxidative stress (4-6).

Medicinal plants are increasingly important targets as options for discovering new commercial drugs. It is estimated that approximately one-third of the most widely used ways of medication in the world involve natural products or their derivatives (6). In this context, one plant that has attracted interest is Cyclotrichium niveum, which is an endemic species for Turkey and a member of the family Lamiaceae. This plant grows in the Eastern Anatolia Region and its local name is 'dağ nanesi' in Turkish (7). It has a distinct minty smell, and it is used in making soup and herbal teas, while it is also used as food flavoring in Turkey (8, 9). Previous studies on the pharmacological roles of C. niveum revealed that the aqueous and ethanol extracts of this plant possessed in vitro antioxidant, antimicrobial and antispasmodic effects (9-12). Moreover, a study suggested that C. niveum has an in vitro acetylcholinesterase (AChE) inhibitory effect (13).

Many researchers reported that Pb exposure may alter AChE activities (14-16). Moreover, oxidative damage is considered to be a crucial factor in Pb-induced neurotoxicity (16, 17). To the best of our knowledge, there is no study relating the in vivo AChE activity and the antioxidant potential of C. niveum. Therefore, the aim of this study was to evaluate the effects of an aqueous extract of C. niveum on the brain AChE activity, as well as malondialdehyde, reduced glutathione, glutathione reductase, glutathione S-transferase and Pb concentration against Pb-acetate-induced neurotoxicity in rats.

## Materials and Methods

### Animals and chemicals

Twenty-eight adult Wistar albino male rats (320-340 g body weight) were obtained from the Experimental Research Unit of Firat University (Elazig, Turkey). The animals were housed under a standard light/darkness cycle (lights on from 07:00 to 19:00 h), at a regular temperature (21 ± 1 °C) and humidity (55 ± 5%) with free access to food and fresh water. The experimental protocols were approved by the Ethical Committee of Firat University, and the rats were treated in accordance with the national and international laws and policies on the care and use of laboratory animals.

Pb-acetate, acetic acid and other chemicals were obtained from Sigma (Dorset, UK) unless otherwise stated.

### Plant Material and Extraction Procedures

C. niveum plants (Herbarium no: 1383) were collected by Dr. Ahmet Zafer TEL from Nemrut Mountain (Adıyaman, coordinates: 38º1.065' N - 38º43.461' E and altitude: 1383 m) with the permission of the Governorship of Adıyaman, the Provincial Directorate of Food, Agriculture and Livestock (permission number: 44109306/7467-1190).

Extraction was carried out as previously described by Gulcin et al. (9). Briefly, the plants were dried in the shade first. For water extraction, 25 g of air-dried aerial parts of C. niveum was ground into a fine powder in a mill and mixed with 400 ml of boiling water by a magnetic stirrer for 15 min. The extract was then filtered through a colander.

### Experimental design

The animals were randomly divided into four groups as control, Pb-acetate, C. niveum and Pb-acetate+C. niveum (n=7 in each group). The control group rats received vehicle solutions only. C. niveum was administered orally (4 ml/kg) to the animals via orogastric gavage for 30 days. In the Pb-acetate and Pb-acetate+ C. niveum groups, the rats were given a daily dose of 500 ppm of Pb-acetate (dissolved in acetic acid) per liter in drinking water for 30 days (3). The animals were sacrificed at the end of 30 days. The brains were removed from the animals. For biochemical analyses, the brain samples were stored at -20 ºC until the assays were performed.

### Tissue homogenization

The homogenization of the brain samples was performed using a polytron homogenizer (Heidolph RZ 2021, Germany) in a cooled homogenization buffer (0.1 M, pH 7.4 in potassium phosphate buffer; 0.15M KCl, 1mM EDTA, 1mM DTT) at 4 times the total tissue weight (w/v). After homogenization, the homogenates were transferred into Eppendorf tubes and centrifuged at 16,000g for 20 min at 4 ºC (Sigma Centrifuge Model 2–16K, Sigma, St. Louis, MO). After centrifugation, the supernatant fraction was removed and enzyme activities and other parameters were measured in S16 fractions. Three replicate absorbance readings were made for each sample, and the reading was repeated when there was a correlation difference greater than 10% between the values obtained for the same samples.

### Analyses of brain AChE activity

Brain AChE activity was analyzed according to the method described by Ellman and Andres (18) with some modifications (19) for a microplate reader spectrophotometer system (ThermoTM Varioskan Flash, Thermo Scientific, Vantaa, Finland). In the activity assay, acetylcholine iodide (ACTI) was used as the substrate and product formation was determined by color change. For this purpose, the supernatant (10 µl volume) was pipetted into the microplate wells. Thereafter, the final solution was prepared in 0.1 M of buffer (pH 8.0) as 200 µl (0.701 mM ACTI and 0.136 mM 5.5-dithiobis-2-nitro-benzoic acid (DTNB) and transferred into the microplate wells. The change in absorbance was recorded at 412 nm for 1 minute at 25 °C. Specific AChE activity was calculated as nmol/min/mg protein.

The total protein amounts in the tissue homogenates were determined based on the method developed by Bradford (20). After dilution (1:4), 5 µl of the diluted sample and 250 µl of the Bradford solution were added into the microplate wells in this order. The reaction mixture was incubated at room temperature for at least 15 minutes in the dark. Depending on the color change, the absorbance value was measured at a wavelength of 595 nm.

### Determination of brain malondialdehyde (MDA) and reduced GSH levels

MDA, reduced GSH, GR and GST levels were determined using a microplate reader spectrophotometer system (ThermoTM Varioskan Flash, Thermo Scientific, Vantaa, Finland). The brain malondialdehyde levels were measured based on the relative production of the reactive substances of thiobarbituric acid (21). The results are represented as nmol/g wet tissue weight. The reduced GSH activity was measured by the substance's reaction with DTNB, to form a compound that absorbs at 412 nm (22). The level of reduced GSH is expressed as nmol/g wet weight tissue.

### Measurement of brain glutathione reductase (GR) and glutathione S-transferase (GST) activities

Analysis of GR activity was determined by a modified method (23). The assay solution contained 50 mM of Tris–HCl buffer (pH 8.0), 1 mM of EDTA, 1 mM of GSSG and 0.1 mM of NADPH. One enzyme unit was defined as the amount that oxidizes 1 µmol NADPH per min under the assay conditions. For determining GST activity, firstly 20 mM of 1-chloro-2,4- dinitrobenzene (CDNB) was prepared in 96% ethanol, and this solution was used as the substrate. Reductive glutathione (0.002 M) was used as the cofactor in the reaction (24). 10 µL of the supernatant, 100 µL of the phosphate buffer (0.1 M, pH 6.5) + 100 µL of the GSH mixture and finally 10 µL of CDNB were transferred into the wells of the microplate. After this process, the well plates were placed in the microplate reader system, and the change in absorbance was recorded at 344 nm for 2 min at 25 ºC. Specific GST activity was calculated as nmol/min/mg protein.

### Determination of brain Pb concentrations

The brain Pb levels were measured using a NexION 350 inductively coupled plasma mass spectrometer (ICP-MS, Perkin Elmer, MA, USA) at the Central Research Laboratory of Adiyaman University. The tissue samples were digested in an acid solution using a microwave digestion system. For this purpose, the brain samples (250 mg) were placed into a digestion vessel, 5 ml of nitric acid (65%) was added, and the mixture was carefully shaken. The samples were then heated in a microwave oven in accordance with the digestion procedure described by Rattanachongkiat et al. (25).

The digestion solutions were diluted and analyzed using a NexION 350 series of the ICP-MS. For quality control, duplicate samples were used to measure the precision of the analysis. Detection limits were calculated as three times the standard deviation for the reagent blanks (26). The results of the brain Pb concentrations are presented as ppb.

### Statistical analysis

All results are expressed as mean ± SEM. The comparison of the groups was carried out using one-way analysis of variance (ANOVA) followed by Tukey–HSD test. Statistical significance was accepted as $P < 0.05$.

## Results

### Brain AChE activity

Fig. 1 shows the findings on the brain AChE activity. The AChE activity significantly decreased in comparison to the control group only in the Pb-acetate group (21.07±3.84 nmol/min/mg protein and 13.70±2.48 nmol/min/mg protein, respectively, $P < 0.05$). The AChE activities of the C. niveum (22.15±3.60 nmol/min/mg protein) and Pb-acetate+C. niveum (17.42±1.36 nmol/min/mg protein) groups did not differ significantly from the control group. When compared with the Pb-acetate group, the brain AChE activity was higher in the C. niveum group ($P < 0.05$).

### Findings on the brain MDA and reduced GSH analysis

The brain MDA concentration of the Pb-acetate group was significantly higher than the control group (470±20 nmol/gr and 415±12 nmol/gr, respectively, $P < 0.05$). There was no significant difference in the C. niveum group (386±20 nmol/gr) and Pb-acetate+C. niveum group (394±36 nmol/gr) in comparison to the control group. When compared with the Pb-acetate group, the brain MDA levels were lower in the C. niveum group and the Pb-acetate+C. niveum group ($P < 0.01$ and $P < 0.05$, respectively, Fig. 2). The brain reduced GSH level was significantly lower in the Pb-acetate group than the control group (0.635±0.2 nmol/gr and 1.21±0.2 nmol/gr, respectively, $P < 0.05$). Although the difference was not significant, the brain reduced GSH concentrations were higher in the C. niveum group (1.95±0.5 nmol/gr) and Pb-acetate+C. niveum group (1.74±0.7 nmol/gr) in comparison to the control group. In the C. niveum group and the Pb-acetate+C. niveum group, the brain reduced GSH levels were higher than the Pb-acetate group ($P < 0.01$ and $P < 0.05$, respectively, Figure 3).

### Activities of the brain GR and GST

The mean GR activity (EU/mg protein, Fig. 4)

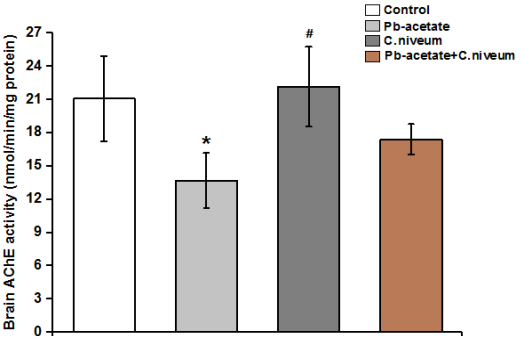


**Figure 1.** Effects of Pb-acetate and C. niveum on the AChE activity in male rat brain. The data represent mean ± SEM (n=7). *: p<0.05 compared to the control group. #: p<0.05 compared to the Pb-acetate group AChE: Acetylcholinesterase, Pb: lead.

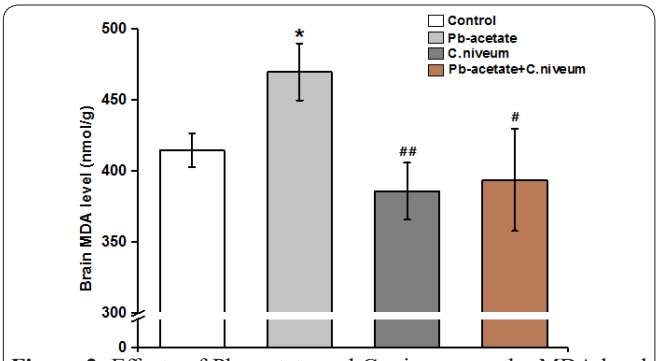


**Figure 2.** Effects of Pb-acetate and C. niveum on the MDA level in male rat brain. The data represent mean ± SEM (n=7). *: p<0.05 compared to the control group. #: p<0.05 and ##: p<0.01 compared to the Pb-acetate group. MDA: Malondialdehyde, Pb: lead.

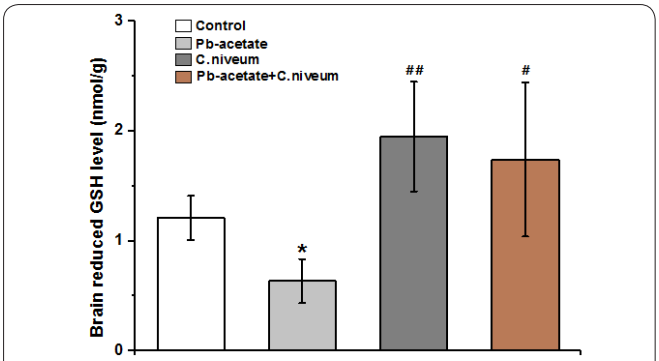


**Figure 3.** Effects of Pb-acetate and C. niveum on the reduced GSH level in male rat brain. The data represent mean ± SEM (n=7). *: p<0.05 compared to the control group, #: p<0.05 and ##: p<0.01 compared to the Pb-acetate group. GSH: Glutathione, Pb: lead.

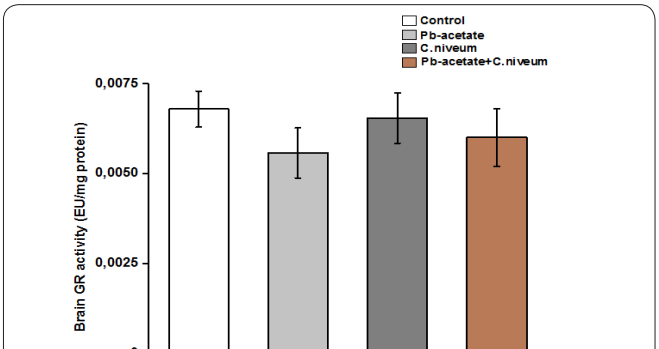


**Figure 4.** Effects of Pb-acetate and C. niveum on the GR activity in male rat brain. The data represent mean ± SEM (n=7). GR: Glutathione reductase, Pb: lead.

significantly declined only in the Pb-acetate group (0.00558±0.0007, P < 0.05). There was no significant difference in the C. niveum group (0.00655±0.0007) and Pb-acetate+C. niveum group (0.00601±0.0008) in comparison to the control group (0.0068±0.0005).

Fig. 5 shows the findings on brain GST activity (nmol/min/mg protein). Although the difference was not significant, the brain GST activity was higher in the C. niveum group (35.05±5.40) and lower in the Pb-acetate group (24.43±3.03) in comparison to the control group (29.36±3.47). The value of the GST activity in the Pb-acetate+C. niveum group (30.31±4.87) was on the same level as the control group. When compared with the Pb-acetate group, the brain GST activity was significantly higher in the C. niveum group (P < 0.05).

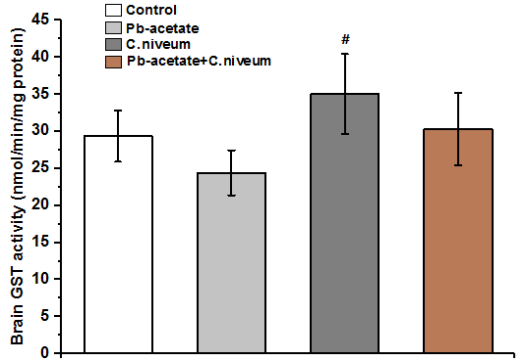


**Figure 5.** Effects of Pb-acetate and C. niveum on the GST activity in male rat brain. The data represent mean ± SEM (n=7). #: p<0.05 compared to the Pb-acetate group GST: Glutathione S-transferase, Pb: lead.

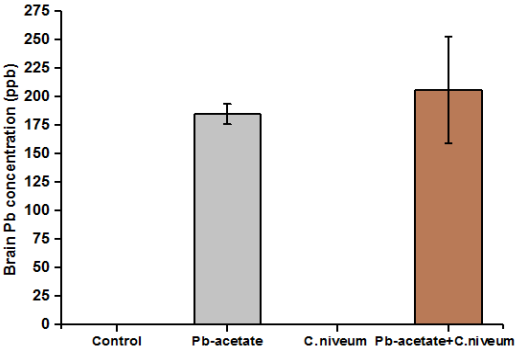


**Figure 6.** Brain Pb concentrations. The data represent mean ± SEM (n=7). Pb: lead.

### Findings on the brain Pb analysis

Pb was not detected on the level of ppb in the brain samples of the control and the C. niveum groups. Pb was detected in the brain samples of the Pb-acetate group (185±8.98) and the Pb-acetate+C. niveum group (206±56.65). However, the Pb concentrations did not significantly differ between the Pb-acetate group and Pb-acetate+C. niveum group (Fig. 6).

## Discussion

This study is the first to evaluate the in vivo AChE activity and antioxidant potential of an aqueous extract of C. niveum in rats exposed to Pb-acetate. In the experiment presented here, we observed that the brain AChE activity significantly decreased only in the Pb-acetate group. There are many in vitro (16) and in vivo (14, 15, 27, 28) studies on the AChE inhibition effects of Pb-acetate. Our results are consistent with these reports. Basha et al. (15) reported that Pb can mimic $Ca^{2+}$ to AChE. Therefore, this interaction may inhibit the accessibility of $Ca^{2+}$, thus leading to decreased enzyme activity. There is only one in vitro study where dichloromethane and ethyl acetate extracts of C. niveum possessed an AChE inhibitory effect, but its essential oil as well as ethanol and aqueous extracts did not exhibit an anti-AChE effect (13). In our experiment, we used an aqueous extract of this plant and observed that AChE activity of the C. niveum group did not significantly differ from the control group. Interestingly, the AChE activity decreased in the Pb-acetate group, but the activity in the Pb-acetate+C. niveum group did not differ from the control group. Therefore, this result indicates that C. niveum may prevent inhibition of brain AChE activity caused by Pb-acetate. We only used an aqueous extract and a single dose of C. niveum. For this reason, determination of the effects of distinct extracts (dichloromethane, ethanol, ethyl acetate) and different doses of this plant is important for assessing potential pharmacological options. There have been a few studies on the essential oil of C. niveum of Turkish origin. Baser et al. (7) noted that the major constituents of this plant are pulegone (32.5–56.4%) and isomenthone (34.2–35.4%). Additionally, Cetinus et al. (10 2007) reported that pulegone (76.84%) is the main component in C. niveum's essential oil. In our previous study, we observed that these components of C. niveum may negatively or positively alter due to altitude differences. The major constituents of our plant samples were defined (29) as pulegone (65.23%), exo-isocamphonone (10.02%), isomenthone (7.23%), and limonene (2.57%).

Regarding the antioxidant activity of C. niveum, we observed that the brain MDA level increased only in the Pb-acetate-treated group. The reduced GSH level and GR activity significantly decreased in the Pb-acetate group. Although the difference was not significant, the GST activities of the C. niveum and Pb-acetate+C. niveum groups were higher than the control group. It is known that chronic exposure to lead acetate leads to an increase in lipid peroxidation and a decrease in reduced GSH (5, 30), GR, and GST activities in the brains of rats (5). Changes in the reduced GSH level, GR and partly GST activities may cause disturbances in the GSH antioxidant system in the brain. Therefore, we suggest that C. niveum has an improved effect on these antioxidant parameters against Pb-acetate-induced alterations. Pb can exclusively bind to the sulfhydryl group and cause a decrease in the GSH antioxidant system in the body (31). In physiological conditions, oxidized glutathione disulfide is reduced to GSH by GR at the expense of reduced nicotinamide adenine dinucleotide phosphate (NADPH) levels, thereby forming a redox cycle (32). The GR redox cycle is responsible for the maintenance of proper GSH concentration in the body (33). GSH is also involved in detoxication of many xenobiotics through the formation of S-conjugates with toxic metabolites in the second phase of biotransformation (34, 35). GSH also forms S-conjugates with products of lipid peroxidation (36, 37). The reaction of S-conjugation may be accelerated by GST (35). With our findings, these reports revealed that lead inactivates GR, GST and other related enzymes, which further depresses the

glutathione antioxidant system in the brain. This effect may also occur in other organs such as the liver (3, 38). In 2015, we reported that lead acetate causes a decrease in glucose 6-phosphate dehydrogenase (G6PD) and 6-phosphogluconate dehydrogenase (6PGD) enzyme activities in the erythrocytes of rats. These enzymes are involved in the oxidative reactions of the pentose phosphate pathway. In particular, the G6PD enzyme is responsible for the production of NADPH, which is very important for the detoxification and synthesis reactions in cells (2). All of these results indicate that lead exposure may cause a multifaceted pathophysiological disorder in the brain and other areas of the body.

We also evaluated the brain Pb levels and determined that the Pb concentration of the Pb-acetate-treated group did not significantly differ from the Pb-acetate+C. niveum group (185±8.98 ppb and 206±56.65 ppb, respectively). However, we did not find Pb on the ppb level in the control and C. niveum groups. Our results indicate that C. niveum has an antioxidant potential, but it has no chelating activity on Pb-acetate in the brain.

In conclusion, this study which firstly examined the in vivo neuroprotective role of an aqueous extract of C. niveum for the first time in a rat model of oral Pb exposure suggests that C. niveum has an ameliorative potential on Pb-acetate-induced inhibition of AChE activity, and this endemic plant exerts an antioxidant effect against Pb-acetate-caused oxidative stress in the brains of male rats. Further studies are needed to confirm C. niveum's mechanism of action and better understand the way it modulates the brain functions.

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**Interest conflict**
The authors declare no conflict of interest.

**Author's contribution**
Each author has made an important scientific contribution to the study and has assisted with the drafting of the manuscript.

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