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Antagonistic Effect of Walnut Peel Water-Soluble Extracts on Lead-Induced PC12 Cytotoxicity

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Abstract

As a well-recognized environmental contaminant, lead (Pb) represents a profound threat to human health, with the nervous system being especially vulnerable. Pb exerts its toxic effects through a range of specific mechanisms, such as inducing apoptosis, promoting oxidative stress, disrupting ion channel function, impairing neurotransmitter systems, and causing microglial cell damage within neurons. Previous studies have demonstrated that Walnut Peel Extracts possess substantial antioxidant properties. However, varying extraction methods and solvents yield different bioactive compounds. In our study, we employed varying concentrations of Walnut Peel Water-Soluble Extracts (WPWE) to counteract Pb toxicity. Specifically, we utilized the MTT assay, Hoechst staining, and fluorescence probes to assess cell viability, apoptosis rates, and ROS levels. The results indicate that WPWE can effectively mitigate the toxic effects of Pb on PC-12 cells.

Keywords

Walnut Peel Water-Soluble Extracts, Lead, PC-12, Apoptosis, Reactive Oxygen Species

1. Introduction

The walnut green peel is the thick, green outer layer of the walnut when it is still immature. This peel contains a wealth of biologically active compounds, including naphthoquinones, polyphenols, and flavonoids, which endow it with significant physiological activities such as antioxidant, antitumor, and anti-inflammatory properties [1]. During the walnut harvest, large quantities of walnut green peel are discarded in the fields, leading not only to resource wastage but also to environ-

mental pollution, and subsequently hindering the growth of other flora. Recently, with the advancement of technology and heightened environmental awareness, the multifaceted uses of walnut green peel have gradually been recognized, including their application as a natural hair dye and in the treatment of ulcers, underscoring their importance. Current research suggests that the antioxidant activity of walnut green peel is primarily attributed to its extracts. He *et al.* have shown that walnut green peel extracts exhibit potent scavenging activity against DPPH radicals, ABTS radicals, hydroxyl radicals, and superoxide anions, with this activity being concentration-dependent [2]. The selection of appropriate solvents and extraction methods is critical for obtaining extracts with high antioxidant potential [3]. Results from two in vitro experiments further demonstrate that Walnut Peel water-soluble extracts (WPWE) possess remarkable antioxidant capacity, which is also concentration-dependent [4] [5].

Lead (Pb) is a heavy metal widely used across various industries, but it also stands as a significant environmental pollutant with global implications. Pb can enter the human body through inhalation, ingestion, and dermal exposure, leading to damage in multiple organ systems, with the nervous system being the most critically affected [6] [7]. The neurotoxic effects of Pb are primarily mediated through mechanisms such as apoptosis, oxidative stress, ion channel dysfunction, neurotransmitter imbalances, and microglial cell damage [8]. Given these mechanisms, the neurotoxicity of Pb poses a serious threat to human health. In children, Pb exposure is associated with severe neurological outcomes, including brain development disorders, cognitive deficits, and intellectual decline. Moreover, there is a strong link between lead exposure and the development of Alzheimer's disease in adults [9].

A recent study demonstrated that Juglone-rich oil (JRO) exhibits protective effects against lead acetate-induced reproductive toxicity in rats. Notably, co-administration of JRO effectively restored testicular histopathological features associated with oxidative damage in the Pb exposed group, suggesting that JRO protects against lead-induced reproductive toxicity through antagonizing lead-mediated oxidative stress [10]. Given that WPWE shares similar antioxidant properties with JRO, an intriguing question arises regarding its potential protective role against Pb induced neurotoxicity. However, no studies to date have investigated this potential neuroprotective effect of WPWE against Pb exposure.

Building on the above, it is evident that lead induces neuronal damage through oxidative stress. Previous studies have shown that walnut green husk extract effectively mitigates oxidative damage and inhibits cell apoptosis [11]. Consequently, we hypothesize that walnut green husk extract may alleviate lead-induced neuronal damage by attenuating oxidative stress. However, different extraction methods often yield varying bioactive compounds, resulting in differences in biological efficacy. This raises the question: can WPWE antagonize lead-induced neurotoxicity? Currently, no definitive reports address this issue. Adrenal pheochromocytoma (PC-12) cells, a differentiated cell line derived from rat adrenal medullary pheochromocy-

toma, exhibit typical neuronal characteristics and maintain stable passaging. As a result, they are widely used as an in vitro model for studying nervous system diseases and cellular toxicity, making them an essential tool in neurophysiological and neuropharmacological toxicology research [12]. This study aims to co-treat PC-12 cells with WPWE and lead acetate to comprehensively assess the antagonistic effects of WPWE on lead acetate-induced toxicity by evaluating cell viability, apoptosis rates, and reactive oxygen species levels. The anticipated outcomes of this study will provide valuable insights for the specific application of WPWE and offer new perspectives for the prevention and control of lead-induced neurotoxicity, with potential implications for practical applications.

2. Materials and Methods

2.1. Lead-Acetate

Referring the text published by Fu L. J. [13] 0.75866 g of lead acetate powder (Tianjin Yongsheng Fine Chemical Co., Ltd., China) was precisely weighed and dissolved in 20 mL of ultrapure water to prepare a 100 mmol/L lead acetate solution. The solution was thoroughly mixed to ensure complete dissolution. Subsequently, the lead acetate solution was sterilized by passing it through a 0.22 μm filter, and the filtered solution was aliquoted into sterile tubes for storage and future use. For subsequent experiments purposes, referring to the papers by Hegg CC and Li N [14] [15], we used above these solutions to prepare different concentrations of lead acetate solutions, specifically 64 μmol/L, 32 μmol/L, 16 μmol/L, 8 μmol/L, 4 μmol/L and 0 μmol/L.

2.2. Walnut Peel Water-Soluble Extracts

A total of 1000 g of walnut peel water-soluble extract (WPWE) powder (Shanxi Haosen Biotechnology Co., Ltd., China) was accurately weighed and dissolved in phosphate-buffered saline (PBS). Based on the results of preliminary experiments, a stock WPWE solution with a concentration of 500 mg/mL was prepared. This stock solution was subsequently diluted with cell culture media (Dulbecco's Modified Eagle Medium) to achieve a series of concentrations for experimental use: 7.8125, 15.625, 32.25, 62.50, 125.00, 250.00 and 500.00 mg/mL.

2.3. Phosphate Buffer Saline

Add the reagents listed in **Table 1** sequentially to a clean glass beaker containing 800 mL of ultrapure water. Stir the solution thoroughly on a magnetic stirrer until all reagents are fully dissolved. Once dissolved, adjust the volume to 1 L with ultrapure water. The cell-specific PBS solution should be autoclaved and allowed to cool before use.

Table 1. PBS preparation.

Components	NaCl	KCl	KH_2PO_4	$Na_2HPO_4{\cdot}12H_2O$	$NaHCO_3$
Mass (g)	8.00	0.40	0.06	0.134	0.35

2.4. Cells Culture

PC-12 cells (Shanghai Yiyan bio-technology Co., Ltd., China) are cultured as a monolayer of adherent cells in glass flasks under the following conditions: 37˚C, saturated humidity with 5% CO_2, with subculturing performed every 3 - 4 days. The culture medium consists of Dulbecco's Modified Eagle Medium (Gibco, USA) supplemented with 10% fetal bovine serum (Gibco, USA) and penicillin-streptomycin (NCM Biotech, USA). When the cells have grown to cover 90% of the bottom of the cell culture flask, subculturing is carried out as follows: in a biosafety cabinet, carefully open the cell culture flask for discarding culture medium. The cells are then washed twice with sterile PBS before adding 1 mL of 0.25% trypsin for digestion, typically for 1 - 2 minutes. Once the cells have detached from the cell culture flask surface, the suspension is centrifuged at 1000 rpm for 10 minutes. After discarding the supernatant, fresh culture medium is added to halt digestion. Gently flush the inner wall of the cell culture flask with culture medium to detach the cells from the surface, forming a cell suspension. Transfer an appropriate amount of the cell suspension to a new culture flask, add a suitable amount of culture medium, mix well, and then place it in the incubator for continued cultivation.

2.5. Cells Livability Was Determined by the MTT Assay

Using PC-12 cells in the logarithmic growth phase, a single-cell suspension was prepared and the cell density was adjusted to 1×10^5 cells/mL. Add 100 μL of cell suspension to each well of a 96-well culture plate for cultivation. After an overnight incubation period to allow the PC-12 cells to adhere, the cells were treated with varying concentrations of WPWE (0, 7.8125, 15.625, 32.25, 62.50, 125.00, 250.00, and 500.00 mg/mL) and lead acetate (0, 4, 8, 16, 32, and 64 μmol/L), as well as a combination of 8 μmol/L lead acetate and 62.50 mg/mL WPWE. Each treatment group was replicated three times, with 100 μL of the treatment solution added to each well. After 24 hours, the treatment solutions were discarded, and 100 μL of freshly prepared MTT solution was added to each well. The cells were then incubated for an additional 4 hours, after which the absorbance at 570 nm was measured using a microplate reader. Cell viability (%) was calculated using the following formula: Cell viability (%) = (A_{570} of treatment group/A_{570} of control group) × 100%.

2.6. Apoptosis Was Detected by the Hoechst Staining

Seed 1×10^6 cells per well in a 6-well plate and allow the cells to adhere. The PC-12 cells were then treated for 24 hours with 0 and 8 μmol/L lead acetate, 62.50 mg/mL WPWE, and a combination of 8 μmol/L lead acetate with 62.50 mg/mL WPWE. Following the protocol provided by the apoptosis detection kit (Shanghai Beyotime Biotechnology Co., Ltd.), 100 μL of fixative was added to each well. After 10 minutes, the fixative was discarded, and the wells were washed at least twice with sterile PBS. In a dark environment, 100 μL of Hoechst staining solution was

added to each well and incubated for 5 minutes. Apoptosis was assessed under a fluorescence microscope using an excitation wavelength of 350 nm (UV light). Randomly select fields of view under the microscope, capture photographs, and count 200 cells. The apoptosis rate (%) is calculated using the formula: Apoptosis rate (%) = (number of apoptotic cells/200) × 100%.

2.7. ROS Was Detected by Fluorescent Probe Method

Seed 1×10^6 cells per well in a 6-well plate and allow the cells to adhere. The PC-12 cells were then treated separately for 24 hours with 0 and 8 μmol/L lead acetate, 62.50 mg/mL WPWE, and a combination of 8 μmol/L lead acetate with 62.50 mg/mL WPWE. Following the protocol outlined in the ROS reagent kit (Shanghai Beyotime Biotechnology Co., Ltd.), add 1 mL of DCFH-DA probe, diluted 2000-fold in cell culture medium, to each well and incubate at 37˚C for 20 minutes. In a dark environment, discard the probe and wash the wells at least twice with sterile PBS. Fluorescence was observed using a fluorescence microscope with 488 nm excitation (blue light) to detect green fluorescence, which correlates with ROS levels within the cells. Intense fluorescence indicates elevated ROS levels.

2.8. Statistical Analysis

At least three independent replications were performed for each experiment, and the results of the experimental data were expressed as Mean ± SD. Statistical analyses were performed using SPSS 26.0, with Student's t test (t tests) for comparisons between two groups. One-way Analysis of Variance (ANOVA) for comparisons between multiple groups. And ANOVA with repeated measures for repeated data. The test level $\alpha = 0.05$.

3. Result

3.1. Effect of WEPE on the Viability Rate of PC-12 Cells

The effect of WPWE on PC-12 cell viability was evaluated using the MTT assay. PC-12 cells were exposed to various concentrations of WPWE (0, 7.8125, 15.625, 32.25, 62.50, 125.00, 250.00, and 500.00 mg/mL). After a 24-hour treatment period, the WPWE solution was discarded, and MTT solution was added. The cells were then incubated for an additional 4 hours in a cell culture incubator. Absorbance was subsequently measured using a microplate reader to determine cell viability. The results, presented in **Figure 1**, demonstrated that PC-12 cell viability varied with increasing concentrations of WPWE. At a WPWE concentration of 0 mg/mL, cell viability was 100%. At concentrations of 7.8125, 15.625, 32.25, 62.50, 125.00, 250.00, and 500.00 mg/mL, cell viabilities were 65.32%, 64.50%, 76.77%, 83.64%, 70.58%, 65.21%, and 47.55%, respectively. Meanwhile, statistical analysis revealed that the differences between the experimental groups and the control group were significant ($P < 0.05$). Notably, cell viability at several WPWE concentrations did not surpass that of the control group. However, at 62.50 mg/mL, cell viability approached that of the control group, leading us to select 62.50 mg/mL

for subsequent experiments.

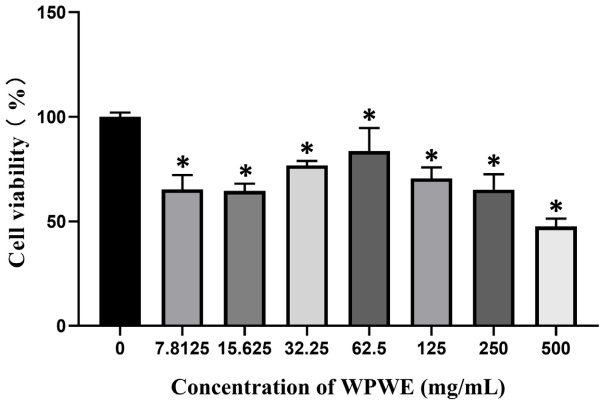

Figure 1. Effect of Walnut Peel water-soluble extracts on the viability rate of PC-12 cells. Note: WEPE: Walnut Peel water-soluble extracts. *$P < 0.05$, versus the control group.

3.2. Effect of Lead Acetate on the Livability Rate of PC-12 Cells

The effect of lead acetate on PC-12 cell viability was assessed using the MTT assay. PC-12 cells were exposed to various concentrations of lead acetate (0, 4, 8, 16, 32, and 64 μmol/L). After a 24-hour incubation, the lead acetate solution was removed, and MTT solution was added. The cells were then incubated for an additional 4 hours. Absorbance was measured using a microplate reader, and cell viability was calculated. As shown in **Figure 2**, increasing concentrations of lead acetate led to a dose-dependent decrease in PC-12 cell viability, from 100% at 0 μmol/L to 79.33%, 75.67%, 67.33%, 65%, and 53.33% at higher concentrations. Meanwhile, statistical analysis revealed that the differences between the experimental groups and the control group were significant ($P < 0.05$). We know that the concentration for cytotoxicity experiments should be chosen based on concentrations where cell viability falls within the range of 60% - 80%, so, based on above results 8 μmol/L lead acetate was selected for subsequent experiments.

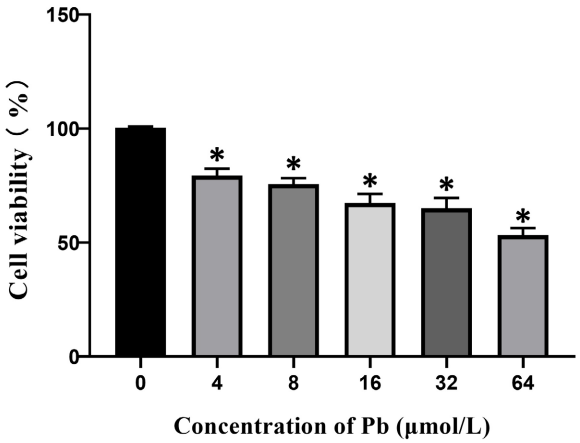

Figure 2. Effect of lead acetate on the viability rate of PC-12 cells. Note: Pb: $(CH_3COO_2)Pb$. *$P < 0.05$, versus the control group.

3.3. Effect of Co-Treatment with WEPE and Lead Acetate on the Viability Rate of PC-12 Cells

The experimental procedure was analogous to the previously described methods. PC-12 cells were co-treated with 8 μmol/L lead acetate and 62.50 mg/mL WPWE. As illustrated in Figure 3, lead acetate significantly decreased cell viability, with the viability rate in the 8 μmol/L lead acetate group at 75.67%. In contrast, the cell viability in the 62.50 mg/mL WPWE group was 83.64%. Notably, the cell viability in the WPWE + lead acetate group increased to 85.10%, representing a 9.43% enhancement compared to the lead acetate-only group. This difference was statistically significant ($P < 0.01$), indicating that WPWE partially mitigates the cell death induced by lead acetate and possesses a protective effect against lead acetate-induced cytotoxicity.

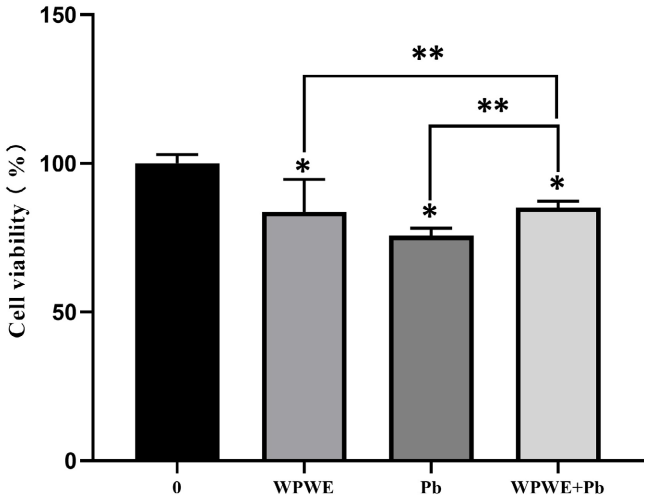

Figure 3. Effect of co-treatment with WEPE and lead acetate on the viability rate of PC-12 cells. *$P < 0.05$, versus the control group, **$P < 0.01$ represents the difference between the two data groups. Note: WEPE: Walnut Peel water-soluble extracts; Pb: $(CH_3COO_2)Pb$.

3.4. Effect of Walnut Peel Water-Soluble Extracts and Lead Acetate on the Apoptosis Rate of PC-12 Cells

PC-12 cells were treated with 8 μmol/L lead acetate and 62.50 mg/mL WPWE in combination, and apoptosis was assessed using the Hoechst staining kit. As shown in Figure 4, in the lead acetate-only group, cells predominantly exhibited a rounded morphology, increased apoptotic cell numbers, and fragmented or intensely stained nuclei, which appeared blue and bright. The apoptosis rates were 72.00% in the lead acetate-only group, 11.67% in the WPWE-only group, 20.00% in the co-treatment group, and 11.00% in the control group. Compared to the control group, the apoptosis rates in both the lead acetate-only and co-treatment groups were significantly higher ($P < 0.05$). However, in the co-treatment group, the apoptosis rate was significantly lower compared to the lead acetate-only group ($P < 0.01$). These results suggest that WPWE effectively mitigates the apoptosis induced by lead acetate exposure.

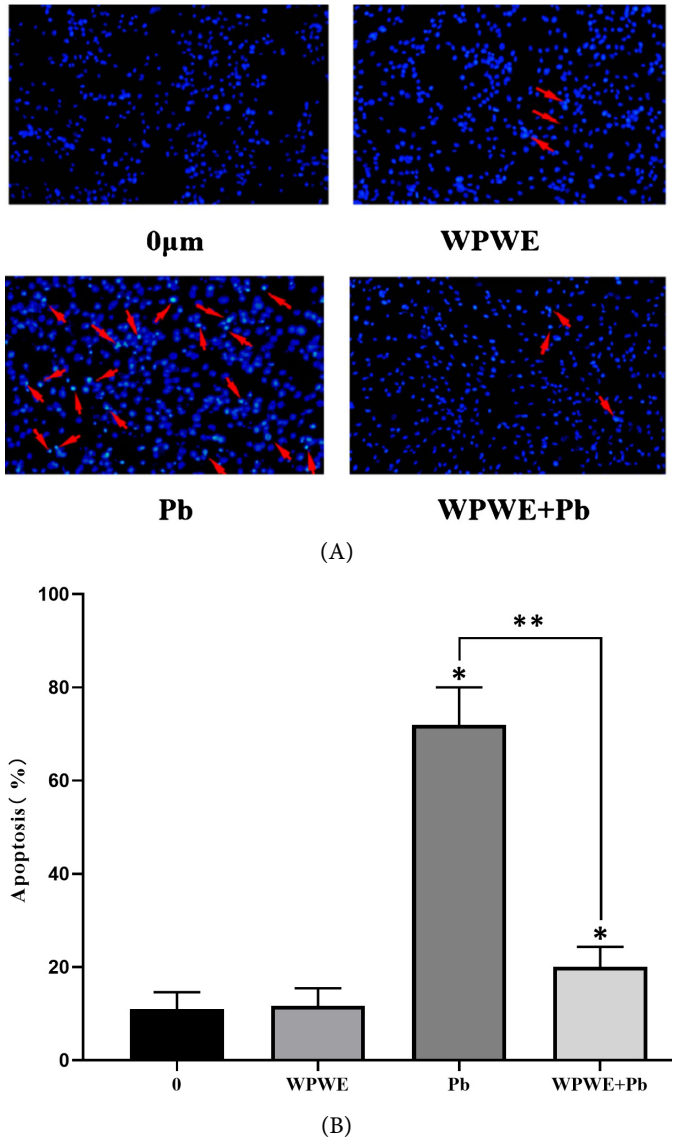

Figure 4. Effect of co-treatment of WEPE and Lead Acetate on the apoptosis of PC-12 cells. (A): Representative of apoptotic morphology (100); (B): Quantitative bar of apoptosis. *P < 0.05, versus the control group, and ** represents a statistically significant difference between the two data groups (P< 0.01). Note: WEPE: Walnut Peel water-soluble extracts; Pb: $(CH_3COO_2)Pb$.

3.5. Effects of Co-Treatment of WEPE and Lead Acetate on the Cellular ROS

To assess reactive oxygen species (ROS) levels, PC-12 cells were treated with 8 μmol/L lead acetate and 62.50 mg/mL WPWE. Following the ROS detection kit instructions, 1 mL of medium containing 10 μmol/L DCFH-DA probe was added to each well, and the cells were incubated at 37˚C for 20 minutes. As shown in Figure 5, treatment with lead acetate significantly elevated ROS levels in the cells. In contrast, co-treatment with WPWE and lead acetate effectively reduced ROS levels.

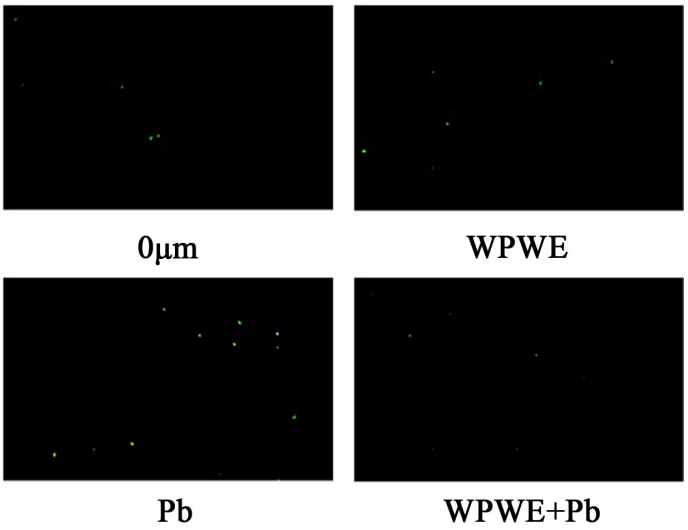

Figure 5. Effect of WEPE and Lead Acetate on the Cellular ROS levels. Note: WEPE: Walnut Peel water-soluble extracts; Pb: $(CH_3COO_2)Pb$.

4. Discussion

Walnut green peel, a traditional Chinese medicine, is recognized for its therapeutic properties, including clearing heat, detoxifying, expelling wind, alleviating pain, and treating dysentery. Recent toxicological research has demonstrated its efficacy in managing conditions such as vitiligo and stubborn tinea, and it has shown significant anti-tumor effects [16]. Chauhan A. further highlighted that walnut green peel extracts offer neuroprotective benefits [11]. Despite various extraction methods and solvents employed to obtain active compounds from walnut green peel for diverse research applications, there remains a paucity of toxicological studies. Our experiments revealed that Walnut Peel Water-Soluble Extracts (WEPE) exhibited some cytotoxicity toward PC-12 cells. Specifically, the viability of PC-12 cells treated with WEPE at different concentrations did not surpass that of the control group. Notably, at a concentration of 62.50 mg/mL, PC-12 cell viability was significantly higher compared to other concentrations and approached that of the control group. Statistical analysis confirmed that these findings were statistically significant relative to the control group.

Lead is a potent neurotoxin that can cause severe damage to the nervous system. Studies, including those by Wang X. N. have demonstrated that lead exposure diminishes PC-12 cell viability, increases apoptosis, and exacerbates autophagy [17]. Additionally, another research indicates that lead elevates reactive oxygen species (ROS) levels in PC-12 cells [18], undoubtedly these researches confirming its detrimental effects on nerve cells. Previous investigations have shown that lead, a heavy metal, causes severe damage to the human nervous system, and existing chelation therapies have proven inadequate in alleviating neurological symptoms [13]. Furthermore, chelation therapy is not recommended for chronic lead poisoning or lead poisoning in children [19]. In contrast, Chauhan A. reported that walnut green peel extracts offer neuroprotective benefits [11]. Our experimental

results support this observation, showing that walnut green peel extracts effectively counteracted lead acetate-induced nerve cell damage. Specifically, the cell viability rate in the co-treatment group increased by 9.43% compared to the lead acetate-only group. The apoptosis rate in the co-treatment group was 20.00%, compared to 72.00% in the lead acetate-only group, and ROS fluorescence intensity was reduced in the co-treatment group, whereas it was elevated in the lead acetate-only group.

5. Conclusion

We observed that the PC-12 cell viability in all WEPE concentration groups were not higher than that of the control group. Notably, at a WEPE concentration of 62.50 mg/mL, the PC-12 cell survival rate closely matched that of the control group, with the difference being statistically significant. Further investigation demonstrated that WEPE effectively mitigates lead acetate-induced neurocyte damage. Specifically, in comparison to the lead acetate-only group, co-treatment group resulted in increased cell viability, decreased apoptosis rates, and reduced ROS levels, indicating that WEPE provides substantial protection against lead-induced nerve cell damage. For the treatment of lead-induced neuronal damage, our research offers novel therapeutic strategies. Additionally, our study highlights the potential economic value of walnut green peel, presenting new opportunities for income generation and environmental protection.

Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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