

Alpinia Galanga Extract's (Vanillic acid) Protective Effects against Lead Acetate-Induced Toxicity in HEK293T Cells and Caenorhabditis Elegans

Der-An Tsao^{1,*}, Kuei-Ling Huang², Yu-Hsin Wang², Chuang Yi Lin³, Fang-Tsu Liao⁴,
Ching-Hsiao Lee⁴, Cheng-Kuang Wang⁴, Huoy-Rou Chang^{5,*}

¹Department of Medical Laboratory Sciences and Biotechnology, Fooyin University, Kaohsiung, Taiwan

²Kuang Tien General Hospital, Taiwan

³Department of Pathology and Laboratory Medicine, Pingtung Veterans General Hospital, Taiwan

⁴Department of Medical Technology, Jen-teh Junior College of Medicine, Nursing and Management, Taiwan

⁵Department of Biomedical Engineering, I-Shou University, Kaohsiung, Taiwan

*Corresponding author: huoy@isu.edu.tw, sc095@fy.edu.tw

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Abstract Lead is an environmental contaminant that impairs human organ and tissue function. The goal of this study is to employ natural compounds to reduce the damage that lead causes to human organ and tissue function. HEK293T cells were used in this study's research on proliferation, Annexin V, DNA fragmentation, cellular oxidative stress, reverse transcriptional polymerase chain reaction (RT-PCR), and Western blot. *Caenorhabditis elegans* were used in vivo to investigate behavioral and developmental status. The results showed that vanillic acid (*Alpinia galanga* extract) can reduce cell apoptosis and the oxidative stress induced by lead exposure in HEK293T cells. In HEK293T cells exposed to lead, vanillic acid decreased NF- κ B gene and protein expression while increasing β -catenin protein expression. Following lead exposure, vanillic acid improved the *Caenorhabditis elegans* development trend. Vanillic acid can protect cells from lead-induced inflammation, apoptosis, and oxidative responses while also regulating gene transcription factors to improve cell state. It can also enhance the growth performance of lead-exposed *Caenorhabditis elegans*, restoring growth rate and health condition during the developing stage. Both in vivo and in vitro test findings show that vanillic acid has the capacity to protect organisms from the harmful effects of lead exposure.

Keywords: *Alpinia galanga*, vanillic acid, lead acetate, HEK293T cells, *Caenorhabditis elegans*

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1. Introduction

Pollution of heavy metal (lead) in the environment can cause harm to the human body. The long-term accumulation of lead toxicity will induce oxidative stress in human cells. When the antioxidant system responsible for protecting cells in the human body cannot balance the large number of free radicals (ROS) produced, excessive ROS will further promote the production of inflammatory substances in the human body. When the signal transmission pathway inside the cell is affected by excessive ROS, it will lead to the appearance of diseases or tumor cells. The harm of oxidative stress has been proven to be involved in the occurrence of many diseases, including cardiovascular disease, degenerative diseases, progression of inflammatory disorders, and cancer [1,2].

Alpinia galanga (AG), also known as southern ginger,

is a common medicinal plant of the ginger family and is rich in many active compounds. Whether it is the rhizome, leaves, or flowers, *Alpinia galanga* contains many pharmacologically active ingredients and is an important and widely used plant of the ginger family [3]. *Alpinia galanga* (AG) is rich in various active ingredients such as alkaloids, terpenoids, polyphenols, flavonoids, etc. [4]. Because of the presence of these active ingredients, it can be used as a good medicinal plant for treatment. It has pharmacological properties such as antipyretic, anti-inflammatory, antibacterial, antioxidant, reducing cell damage, anti-tumor, and improving gastrointestinal indigestion [5]. It is also used to treat a variety of inflammatory diseases such as bronchitis, heart disease, chronic enteritis, and kidney inflammation [6]. It improves anxiety, poor sleep, and nervous system effects [7].

In the classical Wnt signaling pathway, there is an important key molecule: β -catenin, which can promote cell proliferation, regeneration, and participate in cell

metabolism. Many major disorders, including cancer and non-cancer diseases, are frequently caused by the dysregulation of Wnt/ β -catenin signaling [8]. External environmental stimuli (such as heavy metals) can cause diseases by changing the Wnt signaling pathway [9]. Lead (Pb) often promotes the occurrence of diseases by deregulating the expression of β -catenin [10]. Studies have confirmed that Pb can cause bone diseases, progression of inflammatory disorders, inhibit the production of fat cells, change hippocampal synapses, and affect the developing brain of children by deregulating the expression of β -catenin [9].

NF- κ B is an important transcription factor in cells. Elevated NF- κ B expression levels are also seen in normal cell function metabolism, such as cell proliferation, growth and development, metabolism, cytokine stimulation, or pathogen-induced immune mechanisms [11]. When cells are stimulated by external stress and cause an inflammatory response, the expression of NF- κ B will increase. NF- κ B is an important gene regulator involved in the initiation of inflammatory mechanisms [12,13].

Using HEK293T cells in vitro, the study explored vanillic acid could protect normal cells from the apoptosis and inflammatory response induced by heavy metal lead. In vivo experiments, the study explored that *Caenorhabditis elegans* treated with vanillic acid could improve motor performance and developmental processes induced by heavy metal lead.

2. Methods

2.1. Extraction Process of *Alpinia Galanga*

Slice the ginger plant of *Alpinia galanga* (Ginger family) and air-dry it in a cool place and then soak it with methanol (MeOH) at room temperature. Filter the plants containing a large amount of methanol and then remove the methanol (MeOH) by vacuum concentration after filtration to obtain *Alpinia galanga* methanol crude extract (without methanol). The crude methanol extract is subjected to column chromatography (Column-Chromatography) on silica gel and separated using five organic solvents of n-hexane, dichloromethane, ethyl acetate, acetone, and methanol with different polarities, thereby enabling purification of various active compounds in the plant. The compounds are separated into single compounds or mixed compounds by thin layer chromatography (Thin layer chromatography; TLC). The last step is to send the purified compounds for NMR spectroscopy for testing.

2.2. Cell Culture

Human Embryonic Kidney 293T (HEK293T) cells were procured from the Food Industry Research and Development Institute. The cell lines were maintained in DMEM/F12 (Gibco BRL, Grand Island, NY) supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin, and 100 μ g/ml streptomycin. Cultivation occurred at 37°C in a humidified atmosphere containing 5% carbon dioxide (CO₂).

2.3. MTT Assay

Cells were cultured in 96-well plates (1x10⁴ cell/ml). After the cells attached (about 24 hours), lead acetate (0.1%), compounds of *Alpinia galanga* (AG1~AG10) (10 μ M), and lead acetate (0.1%) combined with compounds of *Alpinia galanga* (AG1~AG10) (10 μ M) were separately added to the cells and cultured for 24 hours. The DMEM/F12 medium in the well was aspirated, and 10 $\times$ diluted MTT reagent was added. The cells were placed in an incubator for reaction. During this period, the reaction between the MTT reagent and the cells was observed until purple crystals appeared. Then DMSO (100 μ l/well) was added to dissolve the crystals, and a purple liquid was formed. OD was measured at 570 nm. After taking the average of the absorbance values of triplicates for each experimental condition, the cell survival rate was calculated by the absorbance value of the experimental condition/the absorbance value of the control $\times$ 100%.

2.4. Annexin V Apoptosis Assays

Cells were cultured into 6 wells at a density of 3x10⁵ cells/ml and cultured for 24 hours until the cells attached. The wells were added with each experimental condition, including control, lead acetate (0.1%), lead acetate (0.1%) combined with AG5 (10 μ M), and AG5 (10 μ M), and placed in the incubator for 24 hours. The medium in the well was aspirated, and the well was gently rinsed with 1xPBS 200 μ l of Annexin V reagent added to each well. After standing at room temperature and away from light for 30 minutes, the results were observed and recorded using a fluorescent microscope.

2.5. DNA Fragmentation Assay

The cell culturing density is 3x10⁶ cells/ml to a 10 cm dish and placed in an incubator for 24 hours. Add experimental conditions to the well separately: lead acetate (0.1%), lead acetate (0.1%) combined with AG5 (10 μ M), AG5 (10 μ M). Subsequently, the cells were incubated for an additional 18 hours under identical conditions prior to the collection of apoptotic cells. Electrophoresis was performed utilizing a 2% agarose gel at a voltage of 100 mV for a period of 30 minutes.

2.6. Reverse Transcription Polymerase Chain Reaction (RT-PCR)

The cells were cultured in a 10 cm dish (DMEM/F12 medium containing 10% CS, 100 U/ml penicillin-streptomycin) at a density of 5x10⁶ cells/ml, and placed in a (37°C, 5% CO₂) incubator for 24 hours. The experimental conditions separately included control, lead acetate (0.1%), lead acetate (0.1%) combined with AG5 (10 μ M), and AG5 (10 μ M). Total cellular RNA was extracted utilizing TRIzol reagent (Sigma, St. Louis, MO), and the synthesis of complementary DNA (cDNA) was carried out with SuperScript™ III reverse transcriptase (Invitrogen, USA). The polymerase chain reaction (PCR) was executed over thirty amplification cycles, with each cycle consisting of a denaturation phase at 94°C for 15

seconds, an annealing phase at 60°C for 30 seconds, and an extension phase at 72°C for 1 minute. The resulting PCR products were subsequently analyzed under ultraviolet light following electrophoresis on a 2% agarose gel, utilizing 6× loading dye. Taken via a dedicated camera system.

2.7. Western Blot

The cells were cultivated in a 10-cm dish (DMEM/F12 medium containing 10% CS, 100 U/ml Penicillin-streptomycin) at a density of 5×10^6 cells/ml, and placed in a 37°C, 5% CO₂ incubator for 24 hours.

Add experimental conditions: control, lead acetate (0.1%), lead acetate (0.1%) combined with AG5 (10μM), AG5 (10μM). After culturing for 12 hours, use a scraper to scrape off all the cells and collect them into 1.5ml eppendorf tube and centrifuge (12000 rpm/10 min). Aspirate the supernatant into a 1.5ml eppendorf tube and store at -20°C. Cell lysates were subjected to separation via 8% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene fluoride (PVDF) membranes, through a wet blotting technique. The membranes were then blocked with a blocking buffer for a duration of 20 minutes at ambient temperature. Following this, the primary antibody was introduced and allowed to incubate for 24 hours at 4°C. After three washing cycles with TBST (Tris-HCl, pH 7.4, NaCl, and 0.1% Tween 20), each lasting ten minutes, the membranes were incubated for one hour with secondary antibodies conjugated to horseradish peroxidase, specifically goat anti-mouse antibodies. After additional washing, the membranes were evaluated using an enhanced chemiluminescence (ECL) detection method, and densitometric analysis was conducted utilizing Image J software.

2.8. Caenorhabditis Elegans Growth Assay

In the experiment, pregnant *Caenorhabditis elegans* were broken by bleaching to obtain eggs and then left to grow for one day to form L1 larvae. L1 larvae of the same stage were distributed in 12 wells for culture. Agar with various experimental conditions was configured, experimental condition groups: control, lead acetate (Pb) 10mg/L and 100 mg/L, AG5 100μM, 500μM and 1000μM, Pb 10mg/L combined with AG5 500μM and Pb 10mg/L combined with AG5 1000μM, Pb100mg/L combined with AG5 500μM and Pb100mg/L combined with AG5 1000μM. Count the number of worms and the hatching status of eggs every 24 hours using a counting plate and record them manually: the effects of different experimental conditions (0 hr, 24 hrs, 48 hrs, 72 hrs) and concentrations on *Caenorhabditis elegans*. Using a microscope, observe *Caenorhabditis elegans* over three consecutive days. Record their movement patterns, activity levels, and total number of worms. Count the number of individuals at each developmental stage (L1–L4) and analyze the changes in stage distribution

throughout the observation period.

2.9. Statistical Analysis

The differences between the groups were assessed using a one-way analysis of variance with the statistical analysis system (SAS) software package. All data are presented as means ± standard deviation (SD). A P-value less than 0.05 was indicated statistically significant.

3. Results

3.1. Extraction and Isolation of *Alpinia Galanga*

The collected *Alpinia galanga* plants were sliced and air-dried and then covered with methanol. The crude methanol extract was then concentrated under reduced pressure.

Column chromatography was used for further separation and purification. A suitable organic solvent elution system was selected, including n-hexane, dichloromethane, ethylacetate, acetone and methanol. The five organic solvents were used to purify and separate the components of *Alpinia*

galanga according to their polarity in the flow direction. The compounds were separated into single compounds or mixed compounds by thin layer chromatography (TLC). Compounds AG1~AG10 were extracted from *Alpinia galanga* (Table 1).

Table 1. The ingredients extracted from the *Alpinia galanga* plants (AG1~AG10)

Number	Compound Name	MW	Weight(mg)
AG 1	α -humulene	196	2
AG 2	Zerumbone	192	2
AG 3	zerumbone epoxide	234	2
AG 4	β -sitosterone	412	2
AG 5	vanillic acid	168	2
AG 6	kaempferol-3-O-(3",4"-O-diacetyl)rhannoside	458	2
AG 7	kaempferol-3-O-(4"-O-acetyl)rhannoside	432	2
AG 8	kaempferol-3,4'-O-dimethylether	314	2
AG 9	ferulic acid	194	2
AG 10	β -sitosterol	414	2

3.2. Effects of Lead Exposure at Different Concentrations on the Survival Rate of HEK293T Cells

Different concentrations of lead acetate (0.2%, 0.1%, 0.05%, 0.01%, 0.005%, and 0.001%) were applied to HEK293T cells for 24 hours compared with the untreated control group. The MTT assay results showed that cell viability gradually decreased as the concentration of lead acetate increased. As shown in Figure 1, the IC₅₀ of lead acetate in HEK293T cells was 0.1%.

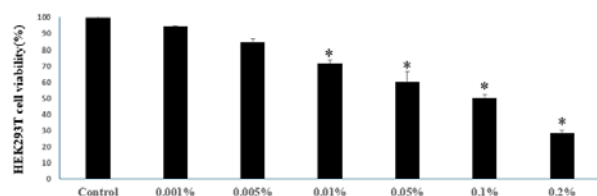


Figure 1. IC50 concentrations of lead exposure determined using the MTT assay and the effects of different lead concentrations on HEK293T cell viability. HEK293T cells were exposed to lead acetate at various concentrations (0.2%, 0.1%, 0.05%, 0.01%, 0.005%, and 0.001%) for 24 hours. The IC50 for the lead acetate concentration was 0.1%. *Indicates statistically significant difference compared to the control group, $P < 0.001$.

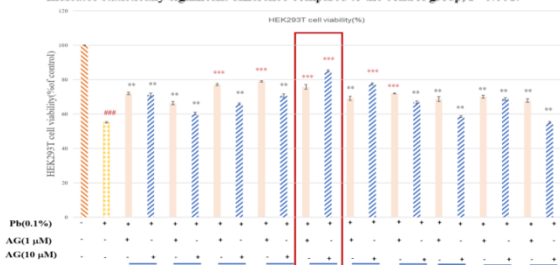


Figure 2. Screening of suitable extracted compounds from Alpinia galanga for optimal survival of lead exposed HEK293T cells using an MTT assay.

Ten compounds extracted from Alpinia galanga were used to treat 0.1% lead exposed HEK293T cells at concentrations of 1 μM and 10 μM for 12 hours. The results are presented as mean $\pm$ standard deviation.

Indicates statistical significance compared to the control group, $P < 0.001$.

** Indicates statistical significance compared to the lead-exposed group, $P < 0.01$.

*** Indicates statistical significance compared to the lead-exposed group, $P < 0.001$.

3.3. Effect of Alpinia Galanga on the Survival Rate of HEK293T Cells Exposed to Lead

The following experiment was conducted using a lead acetate IC50 concentration of 0.1%. The survival of HEK293T cells co-treated with 0.1% lead acetate and ten active Alpinia galanga extracts (AG1–AG10), each at concentrations of 1 or 10 μM , was evaluated. The MTT assay results showed that treatment with 10 μM AG5 (Alpinia galanga extract, vanillic acid) in combination with 0.1% lead acetate significantly increased the cell survival rate (Figure 2).

3.4. Investigate the Effect of Vanillic Acid on Oxidative Stress in HEK293T Cells Exposed to Lead

HEK293T cells were separately treated with 0.1% lead, 10 μM vanillic acid (AG5), or a combination of 0.1% lead and 10 μM vanillic acid. The CM-H2DCFDA assay results showed that HEK293T cells treated with 10 μM vanillic acid (Figure 3d) exhibited no detectable fluorescence compared with the control group (Figure 3a). Compared with the control group (Figure 3a), HEK293T cells exposed to 0.1% lead (Figure 3b) exhibited a significantly stronger fluorescence signal, indicating that lead exposure induces oxidative stress in the cells.

In HEK293T cells exposed to 0.1% lead, co-treatment with 10 μM vanillic acid markedly reduced fluorescence intensity compared with the 0.1% lead-treated group. These results indicate that vanillic acid attenuated the oxidative stress induced by 0.1% lead in HEK293T cells (Figure 3c).

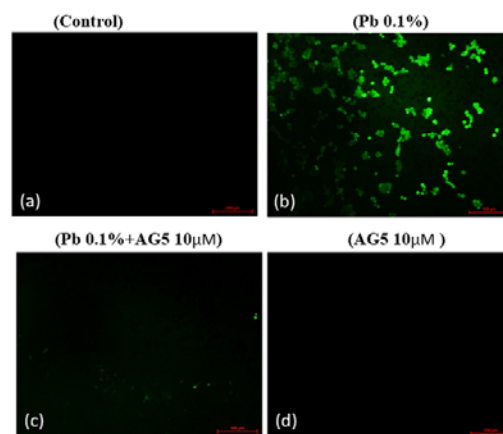


Figure 3. Using CM-H2DCFDA assay to investigate the effect of vanillic acid on oxidative stress in HEK293T cells exposed to lead. CM-H2DCFDA was used to measure oxidative stress in HEK293T cells exposed to lead. (a) Control group (no lead exposure), (b) 0.1% lead exposure, (c) 0.1% lead and AG5 (vanillic acid; 10 μM) combined treatment, and (d) AG5 (10 μM) was added. The oxidative stress response of the groups was observed under a fluorescence microscope at 10X field of view (scale bar 100 μm).

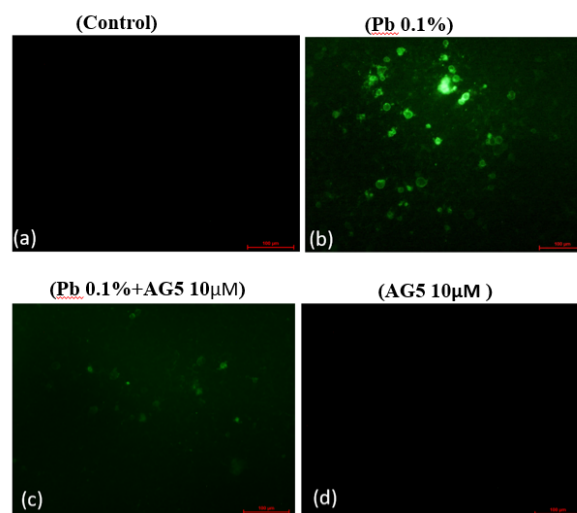


Figure 4. Effect of vanillic acid on early apoptosis in HEK293T cells exposed to lead using the Annexin V assay.

(a) Control, (b) 0.1% lead exposure, (c) 0.1% lead combined with AG5 (vanillic acid) 10 μM treatment, and (d) AG5 (10 μM) treatment. Fluorescence microscopy at 40X magnification (scale bar 100 μm) was used to observe early apoptosis in groups.

3.5. Vanillic Acid Influences Early Apoptosis in Lead-exposed HEK293T Cells

Annexin V assay results showed that HEK293T cells exposed to 10 μM vanillic acid (Figure 4d) exhibited no significant fluorescence compared with the control group (Figure 4a). In contrast, HEK293T cells exposed to 0.1% lead showed obvious fluorescence (Figure 4b), indicating that 0.1% lead induced early apoptosis in HEK293T cells. Treatment with 10 μM vanillic acid combined with 0.1% lead (Figure 4c) significantly reduced fluorescence intensity compared with the 0.1% lead-treated group,

indicating an attenuation of lead-induced apoptosis. These results demonstrate that vanillic acid effectively protects HEK293T cells against apoptosis induced by 0.1% lead.

3.6. Vanillic Acid Affects Late Apoptosis in HEK293T Cells Exposed to Lead

HEK293T cells were separately treated with 0.1% lead acetate, 10 μ M vanillic acid (AG5), or a combination of

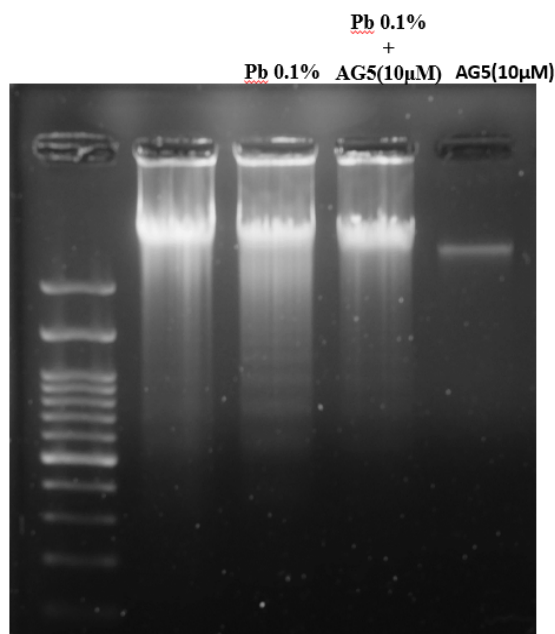


Figure 5. DNA fragmentation assay investigated the effects of vanillic acid on late apoptosis in HEK293T cells exposed to lead.

Four groups were compared: control, lead (0.1%) exposure, lead (0.1%) combined with AG5 (10 μ M), and AG5 (10 μ M). The apoptotic response was observed after lead exposure and in combination with the vanillic acid.

3.7. Investigate the Effect of Vanillic Acid on the Wnt/ β -catenin Pathway in HEK293T Cells Exposed with Lead

In Figure 6, the results showed that the β -catenin mRNA expression level was significantly decreased in HEK293T cells treated with 0.1% Pb, showing a 0.75-fold reduction compared with the control group. In contrast, treatment with 10 μ M vanillic acid in combination with 0.1% Pb significantly increased β -catenin mRNA expression by approximately 1.1-fold compared with the 0.1% Pb-treated group. In Figure 7, compared with the control group, the protein expression of β -catenin was significantly reduced in HEK293T cells treated with 0.1% Pb, with the expression level decreasing by approximately 0.4-fold ($p < 0.05$). However, treatment with 10 μ M vanillic acid in combination with 0.1% Pb significantly increased β -catenin protein expression by approximately 0.95-fold compared with the 0.1% Pb-treated group ($p < 0.05$). These results indicate that lead exposure decreases β -catenin expression, thereby disrupting the Wnt/ β -catenin signaling pathway. Treatment with 10 μ M vanillic acid effectively restored β -catenin expression in

0.1% lead acetate and 10 μ M vanillic acid. As shown in Figure 5, HEK293T cells treated with 0.1% lead acetate exhibited obvious apoptotic fragments. In contrast, cells co-treated with 10 μ M vanillic acid and 0.1% lead acetate showed a marked reduction in apoptotic fragments. These results indicate that vanillic acid attenuated lead-induced apoptosis and reduced the number of late apoptotic HEK293T cells following lead exposure.

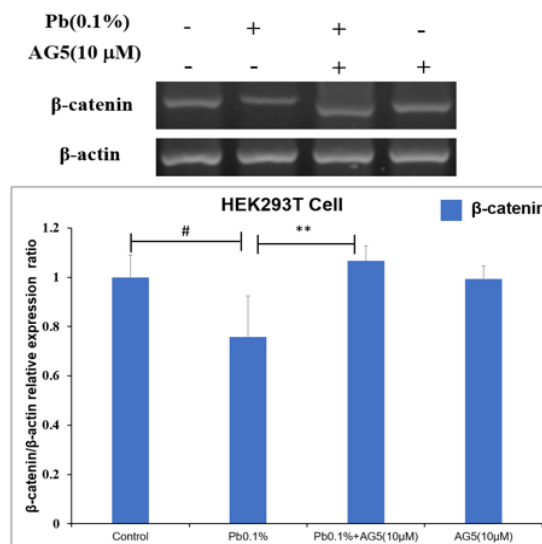


Figure 6. Study on the effect of vanillic acid on mRNA expression of β -catenin in HEK293T cells exposed to lead

HEK293T cells were divided into control, Pb (0.1%), Pb (0.1%) combined with AG5 (10 μ M), and AG5 (10 μ M). The mRNA in the cells was extracted and the expression of β -catenin gene was analyzed by RT-PCR. The band brightness on the electrophoresis graph was analyzed using ImageJ software and the data was statistically analyzed.

Represents a statistical difference between 0.1% lead exposed group and the control group: $P < 0.05$.

** Represents a statistical difference between 0.1% lead exposed group and the Pb (0.1%) combined with AG5 (10 μ M) group: $P < 0.01$.

HEK293T cells exposed to 0.1% Pb, suggesting that vanillic acid may alleviate lead-induced dysregulation of Wnt/ β -catenin signaling.

3.8. Effects of Vanillic Acid on NF- κ B pathway in HEK293T Cells Exposed to Lead

Figure 8 shows that the mRNA expression of NF- κ B increased by 0.6-fold in HEK293T cells exposed to 0.1% lead compared with the control group ($p < 0.001$). In contrast, treatment with 10 μ M vanillic acid in combination with 0.1% lead significantly reduced NF- κ B mRNA expression by 0.4-fold compared with the lead-treated group ($p < 0.01$). These results indicate that lead exposure upregulated NF- κ B mRNA expression in HEK293T cells, whereas co-treatment with vanillic acid significantly attenuated this increase. Figure 9 shows that the protein expression of NF- κ B was significantly increased in HEK293T cells treated with 0.1% lead compared with the control group, reaching 1.95-fold of the control level ($p < 0.001$). However, co-treatment with 10 μ M vanillic acid and 0.1% lead significantly reduced NF-

κ B protein expression compared with the lead-treated group, resulting in an approximately 1.4-fold decrease ($p < 0.01$). These findings suggest that vanillic acid effectively suppresses the lead-induced upregulation of NF- κ B at both the mRNA and protein levels.

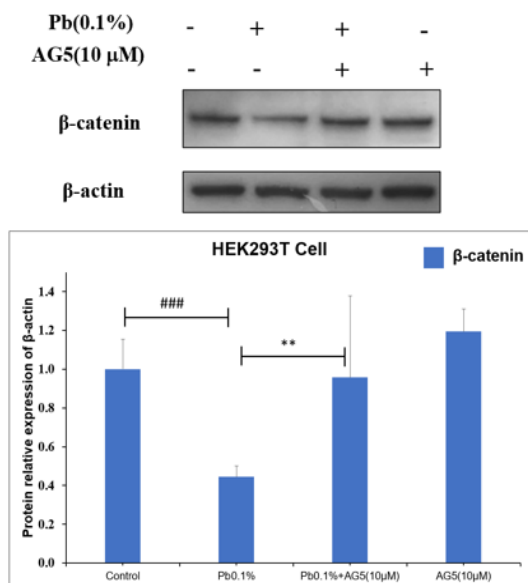


Figure 7. Effect of vanillic Acid on β -catenin protein expression in lead-exposed HEK293T Cells

Four groups were compared: control, lead (0.1%) exposure, lead (0.1%) combined with AG5 (10 μ M), and AG5 (10 μ M). Proteins were extracted from the cells and analyzed by Western blotting to investigate β -catenin protein expression. Band intensities on the electropherograms were analyzed and statistically analyzed using ImageJ software.

Indicates a statistically significant difference between the 0.1% lead exposed group and the control group ($P < 0.001$).

** Indicates a statistically significant difference between the 0.1% lead exposed group and the Pb (0.1%) combined with AG5 (10 μ M) group ($P < 0.01$).

3.9. Effects of Lead Combined with Vanillic Acid on the Developmental Stage Distribution and Motor Behavior of *Caenorhabditis elegans* at Different Developmental Stages, Exposure Times, and Concentrations

The results presented in Figure 10 show that *Caenorhabditis elegans* were divided into nine experimental groups. Compared with the lead-treated group, co-treatment with lead and vanillic acid significantly promoted the growth and developmental rate of *C. elegans* after the L1 larval stage. Locomotor behavior in *C. elegans* has been widely used as an indicator to evaluate the effects of environmental toxicants on body movement and swimming behavior, particularly those affecting the nervous system that regulates locomotion. Assessment of locomotor behavior provides a rapid and convenient approach for the preliminary evaluation of behavioral toxicity induced by heavy metal exposure [14,15].

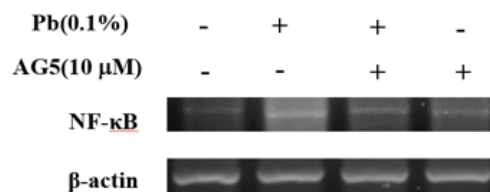
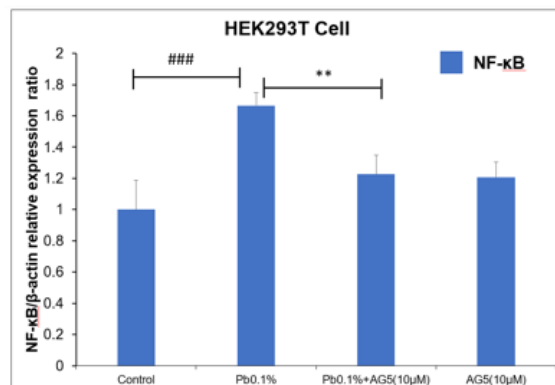


Figure 8. Effect of vanillic Acid on NF- κ B mRNA Expression in Lead-Exposed HEK293T Cells

HEK293T cells were treated with 0.1% Pb, 0.1% Pb + AG5 (10 μ M), and AG5 (10 μ M). Cellular mRNA was extracted and NF- κ B gene expression was analyzed by RT-PCR. Band intensities on the electropherograms were analyzed and statistically analyzed using ImageJ software.

** indicates a statistically significant difference between the lead-exposed group and the lead-compound-treated group ($P < 0.01$). ### indicates a statistically significant difference between the lead-exposed group and the control group ($P < 0.001$).

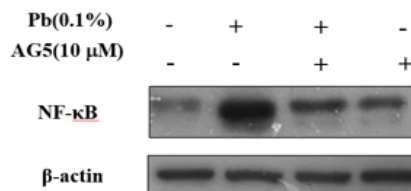
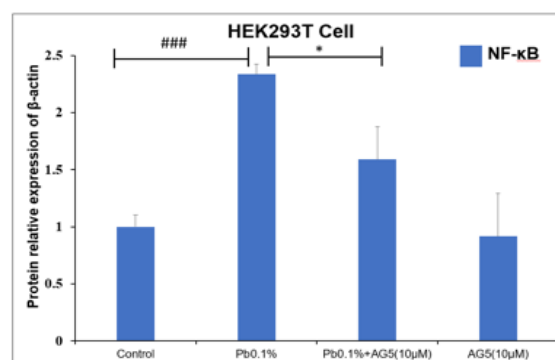


Figure 9. Effect of Vanillic Acid on Protein Expression in the NF- κ B in Lead-Exposed HEK293T Cells

HEK293T cells were treated with 0.1% Pb, 0.1% Pb + AG5 (10 μ M), and AG5 (10 μ M). Proteins were extracted from the cells and analyzed by Western blotting to investigate NF- κ B protein expression. Band intensities on the electropherograms were analyzed and statistically analyzed using ImageJ software.

* indicates a statistically significant difference between the lead-exposed group and the lead-compound-treated group ($P < 0.01$). ### indicates a statistically significant difference between the lead-exposed group and the control group ($P < 0.001$).

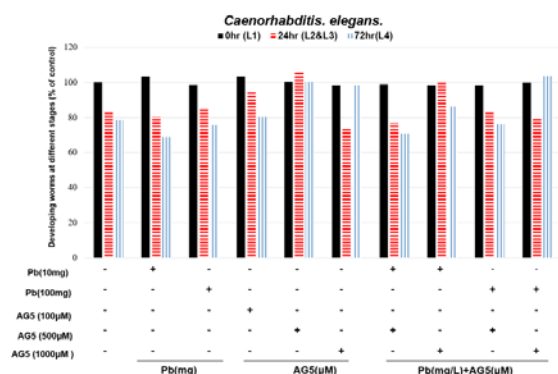


Figure 10. Effects of Lead Combined with *Alpinia officinalis* Extract (AG5) on the Developmental Ratio of *Caenorhabditis elegans* at Different Time and Concentrations. *Caenorhabditis elegans* were treated with different experimental groups: Pb (mg/L), Pb (mg/L) + AG5 (10µM), and AG5 (10µM). The developmental cycle of *Caenorhabditis elegans* typically reaches L4 within 3–4 days. Changes in the developmental ratio of nematodes at different time points and concentrations were recorded over 24 and 72 hours. The results show that compared to the lead alone group and the lead combined with *Alpinia officinalis* extract (AG5) treatment group, the addition of AG5 increased the proportion of nematodes growing and developing beyond the L1 stage.

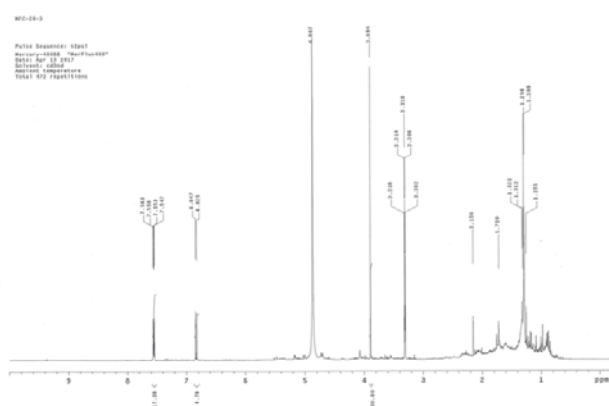


Figure 11. Nuclear Magnetic Resonance (NMR) spectroscopy analysis of the extract of *Alpinia galanga* to identify vanillic acid.

4. Discussion

Lead is a non-biodegradable toxic substance that is widely distributed in the environment. Lead exposure induces multiple toxic mechanisms that damage human organs and tissues, including oxidative stress [16] and the excessive expression of pro-inflammatory factors. *Alpinia galanga* extract's extraction, fractionation, structural identification, and purity information, along with chromatograms/spectral representations (figure11). This study demonstrated that vanillic acid, isolated from *Alpinia galanga*, enhances antioxidant and anti-inflammatory activities against lead-induced toxicity and protects cells from lead-induced damage. As shown in Figure 1, the results indicated that the half-maximal inhibitory concentration (IC_{50}) of lead was 0.1%. An MTT assay was performed to screen ten compounds extracted from *Alpinia galanga* and identify those capable of improving cell viability under 0.1% lead exposure. As shown in Figure 2, among the ten tested compounds, 10 μ M vanillic acid exhibited the strongest protective effect and significantly increased cell viability in cells exposed to 0.1% lead.

Previous studies have demonstrated that the primary mechanism of lead toxicity is the excessive production of reactive oxygen species (ROS), which leads to oxidative stress and subsequent cellular damage [17]. Consistent

with these findings, the present study showed that vanillic acid possesses both antioxidant and anti-inflammatory properties, thereby reducing lead-induced cellular damage.

The CM-H₂DCFDA assay was used to evaluate intracellular oxidative stress. As shown in Figure 3, the lead-exposed group exhibited significantly stronger fluorescence intensity than the control group, indicating that lead induced substantial oxidative stress. In contrast, treatment with vanillic acid markedly reduced the fluorescence intensity in lead-exposed cells, suggesting that vanillic acid effectively attenuated lead-induced oxidative stress.

Apoptosis can be classified into physiological and pathological processes. Physiological apoptosis is a normal biological process that regulates intracellular signaling and plays essential roles in maintaining tissue homeostasis, including preserving tissue integrity and eliminating damaged or unnecessary cells. In contrast, pathological apoptosis is induced by external stimuli or harmful factors, resulting in abnormal cell death that contributes to the development of various diseases [18].

Prolonged oxidative stress induced by lead exposure can cause cellular damage and trigger apoptosis [19]. Apoptosis is generally divided into early and late stages [20]. In the present study, Annexin V and DNA fragmentation assays were performed to evaluate the effects of lead acetate exposure and vanillic acid co-treatment on apoptosis.

As shown in Figure 3b, cells exposed to lead acetate exhibited markedly increased Annexin V fluorescence intensity, indicating the induction of early apoptosis. In contrast, co-treatment with vanillic acid significantly reduced Annexin V fluorescence intensity (Figure 3c), suggesting that vanillic acid effectively attenuated lead acetate-induced early apoptosis.

Late apoptosis was further evaluated using the DNA fragmentation assay. As shown in Figure 4, lead acetate exposure caused pronounced DNA fragmentation, indicating that lead-induced nuclease activation promoted the progression of late-stage apoptosis. Notably, co-treatment with vanillic acid significantly reduced DNA fragmentation, demonstrating its protective effect against lead-induced late apoptosis.

Taken together, the results of the Annexin V and DNA fragmentation assays demonstrated that lead acetate induced apoptosis during both the early and late stages. Furthermore, co-treatment with vanillic acid significantly alleviated lead acetate-induced apoptotic cell death, indicating that vanillic acid exerts a protective effect against lead-induced apoptosis.

The Wnt/ β -catenin signaling system plays a vital role in regulating cell growth, tissue homeostasis, and disease progression. It is a highly conserved pathway involved in various biological processes, including cell proliferation, embryonic development, tissue regeneration, and nervous system activation [8]. Figures 6 and 7 demonstrate that β -catenin mRNA and protein expression levels were decreased in the lead-exposed group compared with the control group. However, co-treatment with vanillic acid and lead significantly increased β -catenin mRNA and protein expression levels compared with the lead-exposed group. These findings suggest that vanillic acid supplementation can alleviate the lead-induced

suppression of β -catenin expression.

The Wnt/ β -catenin and NF- κ B signaling pathways independently regulate the expression of target genes involved in cell proliferation, survival, and differentiation. In addition to these shared functions, NF- κ B serves as a key regulator of inflammatory responses, whereas Wnt/ β -catenin signaling plays essential roles in development and tissue regeneration [21].

NF- κ B coordinates inflammatory and immune responses, as well as cell differentiation, proliferation, and survival. Dysregulation of NF- κ B has been linked to several inflammatory diseases, including rheumatoid arthritis, multiple sclerosis, asthma, and inflammatory bowel disease [21].

NF- κ B regulates cancer development by controlling cell proliferation, apoptosis, inflammation, and tumor immunity [22,23]. Lead induces inflammatory responses by activating the NF- κ B signaling pathway through inhibition of the Wnt/ β -catenin signaling pathway [10].

Lead exposure increases oxidative stress [24], leading to the generation of pro-inflammatory mediators and the activation of NF- κ B signaling [25].

As shown in Figures 8 and 9, lead exposure significantly increased both NF- κ B mRNA and protein expression compared with the control group. In contrast, co-treatment with vanillic acid significantly reduced NF- κ B mRNA and protein expression compared with the lead exposure group. These findings suggest that vanillic acid may attenuate lead-induced inflammation by suppressing NF- κ B expression.

Caenorhabditis elegans is a multicellular organism with a fully sequenced genome, and its genes share approximately 60-80% homology with human genes. Furthermore, *Caenorhabditis elegans* are simpler to culture and require fewer days of experimental work than mammalian models like mice. These advantages make it a popular research subject. Studies have shown that lead exposure can lead to growth retardation in children, causing damage to the nervous system and altered gene regulation [26]. In addition to exploring the mechanisms of gene transcription in cells, *Caenorhabditis elegans* have also been used in numerous studies investigating the effects of environmental heavy metal toxicity on genes in organisms [27,28]. Therefore, this study used *Caenorhabditis elegans* as the multicellular organism model in vivo. In Figure 10, the results showed that lead combined with vanillic acid treatment, the developmental proportions of the nematodes at different stages can be improved over time. Table 1 showed that lead combined with vanillic acid treatment can improve the movement behavior of *Caenorhabditis elegans* caused by lead exposure and increase its swimming speed.

The study had shown that a single exposure to lead does not necessarily cause significant developmental effects in *Caenorhabditis elegans*, but it will gradually reduce the developmental rate of *Caenorhabditis elegans* in subsequent generations [29]. In recent years, some articles have suggested that *Caenorhabditis elegans* can improve their stress resistance and prolong their lifespan through treatment with vanillic acid [30].

5. Conclusion

Based on the above results, lead exposure can promote the occurrence of oxidative stress and promote cell apoptosis. The transmission of signals within cells will be affected by lead exposure, reducing the expression of β -catenin, affecting cell proliferation, and significantly increasing the expression of the inflammatory factor NF- κ B, causing cell damage. However, the combination of vanillic acid (AG5), a compound extracted from *Alpinia galanga*, with lead treatment can significantly improve the effects of lead. The combination of vanillic acid and lead can not only significantly reduce the occurrence of oxidative stress but also reduce cell apoptosis and improve cell survival rate. In cell signaling, vanillic acid can regulate and enhance the gene expression of β -catenin and significantly reduce the activation of NF- κ B, an inflammatory factor, to protect cells from cell damage caused by lead. Additionally, vanillic acid restored the growth rate and overall health status of *Caenorhabditis elegans* during development, thereby improving the growth performance of lead-exposed nematodes. Results from both vitro and in vivo experiments suggest that vanillic acid exerts a protective effect against lead-induced toxicity and helps mitigate the adverse effects of lead exposure.

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Conflict of Interest

The authors declare no conflict of interest.

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