Article

Human and Experimental Toxicology
Volume 41: 1–10

DOI: 10.1177/09603271221101038
journals.sagepub.com/home/het

The sesquiterpenoid valerenic acid protects neuronal cells from the detrimental effects of the fungicide benomyl on apoptosis and DNA oxidation

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Abstract

Background: Valerenic acid (VA), a sesquiterpenoid of the plant *Valeriana officinalis*, has attracted attention of the research community due to its potential positive role against neurodegenerative diseases induced by chemicals. However, the relevant evidence in the literature is scarce. Therefore, this study aimed to examine the putative protective role of VA on the toxic effects of the fungicide benomyl on SH-SY5Y neural cells.
Methods: Cell viability was determined via the MTT and NRU assays, DNA damage was assessed via comet assay and apoptosis was evaluated through the expression of relevant genes.
Results: According to the results, exposure of the cells to benomyl enhanced viability inhibition and promoted DNA damage and apoptosis since the expression levels of the genes coding for MAPK8, NF-kB, Bax, Caspase-9 and Caspase-3 were increased. Treatment of the cells with VA ameliorated these effects in a concentration dependent manner.
Conclusion: It is concluded that the molecular mechanism through which benomyl exerts its toxic action appears to depend on DNA oxidation and apoptosis induction. Furthermore, VA, a plant-derived compound is a protective antioxidant against pesticide-induced toxicity. Therefore, herbs, extracts and compounds of plant origin could be used as nutritional supplements that back up the beneficial role of medicine in neurodegenerative diseases.

Keywords

Valerenic acid, benomyl, DNA damage, oxidative stress, apoptosis, redox-related mechanism

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Introduction

Pesticides are frequently used in the agricultural sector towards the increase of yield and the enhancement of productivity.[1] Although most pesticides are species-specific, they can cause serious acute and chronic effects on individuals who are exposed to them.[1,2] It is known that the vast majority of pesticides have neurotoxic effects through different molecular mechanisms.[3–8] One of the most common detrimental ways that pesticides affect the nervous system is the manifestation of serious neurodegenerative pathologies such as Parkinson's disease.[3,4] In the molecular level, pesticides cause nerve cell damage via apoptosis and induction of oxidative modifications of macromolecules.[5–8] Therefore, it appears that pesticides exert deleterious effects, and the introduction of novel compounds of plant origin as protective factors for the biological systems seems a promising practice.

The use of herbal extracts as a supplement in the treatment or prevention of various diseases has become popular research objective in recent years.[9–11] There are many studies in the literature that have examined the potential positive impact of herbal products against the harmful effects of pesticides.[9–11] Towards this direction, the beneficial role of numerous plant extracts or herbal products to eliminate or prevent the harmful action of pesticides on neurons have been reported.[12,13] *Valeriana officinalis*, which is called valerian, is a medicinal herb used as sedative, hypnotic and antispasmodic from the ancient times.[14] It has been demonstrated that valerian extracts act through protecting nervous system, thus they could be putatively used as complementary agents in Alzheimer's disease, Parkinson's disease and tardive dyskinesia.[15,16] It has been reported that valerian extracts act through redox dependent mechanisms in the molecular level. Indeed, they seem to improve cerebral ischemia by inhibiting lipid peroxidation[17] and to alleviate redox imbalance and astrocyte dysfunction in rat brain.[18] Apoptosis is another biological process that valerian extracts interfere to as they show a cytoprotective role on an in vitro Parkinson's disease model by reducing apoptotic cell death.[19]

One of the main ingredients of valerian root extract is valerenic acid (VA), which exerts potent neuroprotective effects. Interestingly, both valerian root extract and VA ameliorated D-galactose-induced decrease in neurogenesis, lipid peroxidation and memory loss in mice.[20] It has also been reported that VA improves the expression of brain-derived neurotrophic factor, which is crucial for neuronal survival, differentiation and maturation on SH-SY5Y cell model in a dose-dependent manner.[21,22] Furthermore, via its beneficial role against apoptosis, it is a promising factor against anxiety, insomnia and other neuronal diseases.[23,24] Neuronal cell death is one of the main mechanisms of the onset of neurodegenerative diseases.[25] Enhanced apoptotic cell death may be associated with neurodegenerative disorders, such as Parkinson's disease and Alzheimer's disease.[26] Many signaling pathways play key roles in the regulation of apoptosis in the neurons. The stress-activated Jun N-terminal kinases (JNK) and p38 mitogen-activated protein kinases (MAPK) are crucial for balancing cell survival or death response following intracellular and extracellular stress stimuli. Upregulation of both enzymes lowers the expression of anti-apoptotic proteins and increases the expression of pro-apoptotic proteins, resulting in apoptotic incidence.[27,28] Activation of caspases is another molecular pathway towards induction, transduction, and amplification of apoptosis.[29] Based on the above, it is indisputable that pesticides act detrimentally on nervous system being responsible for the onset of serious diseases. On the contrary, compounds of plant origin might be complementary agents to medicines and, thus, prevent or alleviate the toxic effects of pesticides. Therefore, the aim of this study was to evaluate the putative beneficial role of VA on toxicity induced by the fungicide benomyl in neuron cells through the molecular mechanism of apoptosis.

Materials and methods

Chemicals

Benomyl (PESTANAL®, analytical standard (CAS Number: 17804-35-2) and valerenic acid (CAS No: 3569-10-6) were purchased from Merck (Germany). SH-SY5Y cells were obtained from American Type Culture Collection (ATCC-HB8065, USA). Dimethyl sulfoxide (DMSO) and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were purchased from Sigma Chemical Co. Ltd. (USA). The chemicals for mRNA isolation and cDNA production were purchased from Roche Diagnostic (Germany). The SensiFast No-Rox Kit was obtained from Bioline (UK). The cell culture mediums and all other supplements were purchased from Multicell Wisent (Quebec, Canada), and sterile plastics were purchased from Corning (Amsterdam, The Netherlands).

Cell viability tests (MTT and NRU tests)

The tested SH-SY5Y cells, i.e., which are a human-derived neuroblastoma cell line and an excellent in vitro model for the study of neuronal function and differentiation, were cultured in DMEM-F12 medium with fetal bovine serum (10% v/v). The cells were incubated at 37°C and 5% CO_2 to ensure growth and viability. The effects of valerenic acid on metabolic activity of SH-SY5Y cells was evaluated using the MTT (3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide) assay. MTT, a yellow-colored tetrazolium, is reduced to purple formazan crystals by metabolically active cells and the magnitude of reduction was measured

with a spectrophotometer (Biotek, Epoch, Vermont, USA) at 590 nm. After incubation for 24 h, the cells were treated with various concentrations (100, 200, 300, 400, 500, 600 and 800 μM) of valerenic acid and were incubated again for 24 h. An MTT solution (5 mg/mL) was added to each well and the cells were further incubated for 3 h at 37°C. DMSO (1%) was used as a solvent control. IC_{50} values were calculated using the equation of a linear regression curve.[30]

The effect of valerenic acid on cell viability after exposure of the cells to benomyl was also evaluated using the neutral red uptake (NRU) test. According to the method, 1×10^5 cells/well in a 96-well plate were incubated for 24 h for attachment. Then, the cells were treated with 6 μM benomyl for 6 h. Afterwards, the medium was discarded and valerenic acid treatment was also performed in concentrations 100, 200 and 400 μM for another 24 h. Valerenic acid was dissolved in cell culture medium. The medium was then replaced with a fresh medium containing 10 μg mL^{-1} neutral red (NR) and the samples were incubated for 3 h at 37°C. Then, the dissolution reagent was added to the cells (50% ethanol, 1% glacial acetic acid) and the absorbance was monitored at 540 nm with a microplate reader (Biotek, Epoch, Vermont, USA). The results were expressed as the percentage of compared control according to the formula below.[31]

Viability % = (corrected mead OD sample/corrected mead OD solvent) × 100

Each concentration was independently assayed three times with three technical replicates.

Comet assay

The oxidative damage of DNA was measured through the comet assay.[32] The cells were seeded into 6-well plate as 5×10^5 cells/well and incubated overnight for attachment. Then, they were exposed to 6 μM benomyl for 6 h to induce toxicity and the treatments of the cells with 50, 100, 200 and 400 μM valerenic acid for 24 h followed. The specific concentration of benomyl (i.e., 6 μM) was used on the basis of a previous study of our group.[33] In particular, this is a concentration that does not induce toxicity in the cells. Furthermore, the concentrations (i.e., 100, 200 and 400 μM) of valerenic acid were chosen because they are lower than the IC30 value. As for the negative control, 1% DMSO was used. After incubation, the cells were detached with trypsin-ethylenediaminetetraacetic acid (EDTA) and washed with phosphate buffered saline. The cells were then mixed with low-melting agarose and layered on microscope slides that were precoated with normal-melting agarose. Following lysis, the cells were treated with alkali in cold fresh electrophoresis buffer (0.3 M NaOH, 1 mM EDTA, pH = 13) and electrophoresis was applied (20 min, 20 V/300 mA). Afterwards, a neutralization process was applied and the evaluation was carried out under fluorescent microscope with ethidium bromide staining (Olympus BX53, Olympus, Tokyo, Japan; Comet Assay IV, Perceptive software (Suffolk, UK). One hundred cells were counted and scored for each concentration and %TDNA and tail intensity were evaluated.[34,35] In our study for each analysis all experimental concentrations were independently assayed three times with three technical replicates

Gene expression analysis

After the cells (10^6 cells/flask) were incubated in 25T flasks overnight for attachment, they were exposed to 6 μM benomyl for 6 h to induce toxicity and subsequently the cells were exposed to 50, 100, 200 and 400 μM valerenic acid for 24 h. As for the negative control, 1% DMSO was used. After exposure, total RNA was isolated with High Pure RNA isolation Kit (Roche Diagnostic, Germany) and cDNA synthesis was performed with A Transcriptor First Strand cDNA Synthesis kit (Roche Diagnostic, Germany). The gene expression levels were evaluated by custom-designed primers and SensiFast No-Rox Kit (Bioline, UK) on a qPCR platform (Light Cycler 480, Roche Diagnostic, Germany) (Table 1). In our study for each analysis all experimental concentrations were independently assayed three times with three technical replicates. B-actin was used as a reference gene and the relative expression was calculated via the $2^{-\Delta\Delta CT}$ method and expressed as fold change as previously described by Livak and Schmittgen.[36]

Statistical analysis

The obtained data was analyzed by one-way ANOVA and post hoc Dunnett's t-test. The results are expressed as mean ± standard deviation (SD). The level of statistical significance was set at $p \leq 0.05$. All analyses were performed using the statistical package SPSS version 20.0 for Windows (SPSS Inc. Chicago, IL, USA).

Results

Cell viability

VA inhibited the viability of SH-SY5Y cancer cells in a concentration dependent manner. The IC_{50} value for VA was calculated as 556.33 μM and for further gene expression analysis the IC_{30} value of VA was calculated as 437.77 μM (Figure 1).

After a 6 h-exposure of the cells to 6 μM benomyl (B6), a 31% cell viability inhibition was observed according to the MTT test and 29% according to the NRU test. A significant decrease in cell viability inhibition values was observed, i.e., 7%, 3% and 1% using both the MTT and NRU tests after

Table 1. Primers used real-time PCR analysis and annealing temperatures (Ta, °C).

Gene	Primer sequences (5′-3′)	Ta (°C)	Reference
Bcl-2	F: TgTggCCCAgATAggCACCCAg R: ACTTCgCCgAgATgTCCAgCCAg	65	37
Bax	F: ACCAAgAAgCTgAgCgAgTATC R: ACAAAgATggTCACggTCTgCC	60	37
CASP9	F: ACCAgAgATTCgCAAACCAg R: TCACCAAATCCTCCAgAACC	57	38
CASP3	F: gCTATTgTAggCggTTgT R: TgTTTCCCTgAggTTTgC	53	37
NF-κB	F: CACTgCTCAggTCCACTgTC R: CTgTCACTATCCCggAgTTCA	61	39
TNF-α	F: CCCAggCAgTCAgATCATCTTC R: AgCTgCCCCTCAgCTTgA	57	40
JNK	F: AACTgTTCCCCgATgTgCT R: TCTCTTgCCTgACTggCTTT	57	39
B-actin	F: AACTACCTTCAACTCCAT R: TgATCTTgATCTTCATTgTg	48	41

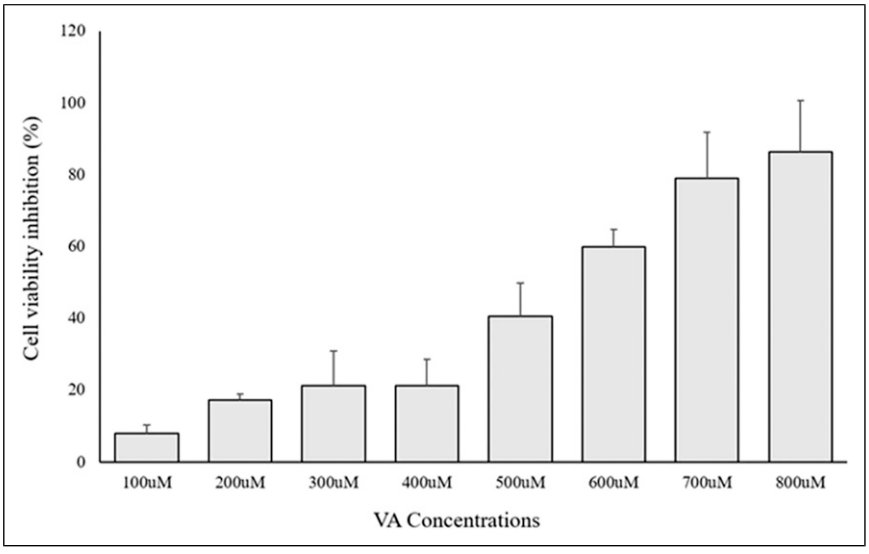

Figure 1. The effect of valerenic acid on the viability of SH-SY5Y cancer cells using the MTT assay.

treatment of the cells with benomyl + VA at concentrations equal to 100 μM (i.e., B6+VA100), 200 μM (i.e., B6+VA200) and 400 μM (i.e., B6+VA400) repsectively (Figure 2).

Oxidative damage of DNA

According to the results obtained from the comet assay, after 6 h of exposure to benomyl, DNA damage was statistically significantly enhanced compared to the control. With the application of VA, DNA damage was found to be significantly reduced in the 100, 200 and 400 μM of VA treatment cell samples compared to the cells exposed to benomyl only. According to these results, treatment of the tested neuroblastoma cells with VA ameliorates the DNA damage caused by their exposure to benomyl (Figure 3).

Expression levels of apoptosis-related genes

In the present study, it was observed that exposure to benomyl caused an increase in the expression of apoptosis-related genes in SH-SY5Y cells. However, these effects were ameliorated in general after treating the cells with benomyl + VA in various concentrations in a concentration dependent manner, indicated by the decreased levels of the expression of some of the aforementioned genes (Figure 4). In particular, the expression levels of the gene coding for

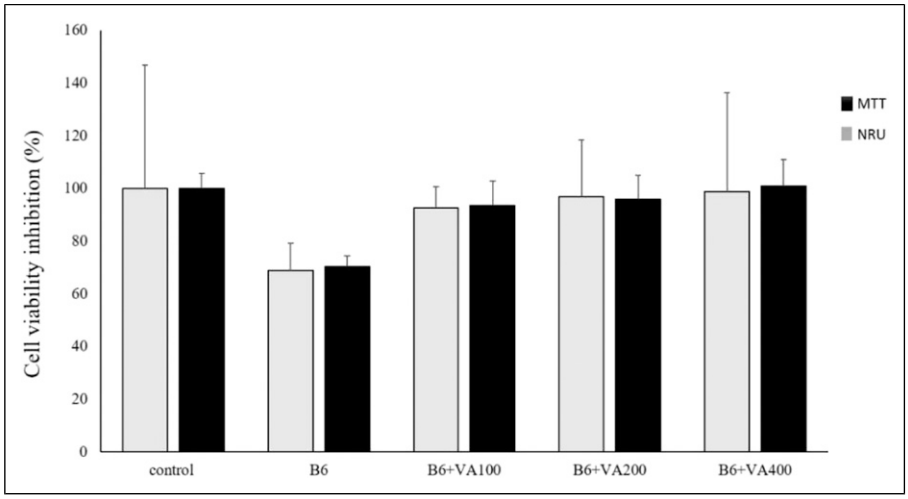

Figure 2. The effects of benomyl and 6 μM benomyl (B6) + valerenic acid at concentrations equal to 100 μM (i.e., B6+VA100), 200 μM (i.e., B6+VA200) and 400 μM (i.e., B6+VA400) on the viability of SH-SY5Y cancer cells using the MTT and NRU assays.

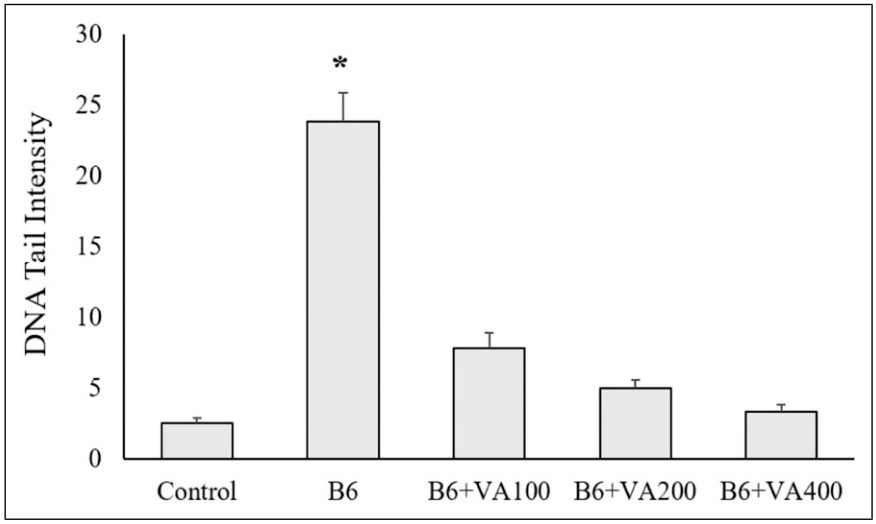

Figure 3. The effects of 6 μM benomyl (B6) and B6 + valerenic acid at concentrations equal to 100 μM (i.e., B6+VA100), 200 μM (i.e., B6+VA200) and 400 μM (i.e., B6+VA400) on DNA damage of the cells. *: Statistically significant compared to the control cells ($p < 0.05$).

MAPK8 (also known as JNK1) which plays an important role at the receptor level in the initiation of apoptosis, were increased approximately 2-fold in the cells exposed to 6 μM benomyl (B6) compared to the control cells (p=0.002), while no effect was observed after treatment of the cells with benomyl +100, 200 and 400 μM valerenic acid (i.e., B6+VA100, B6+VA200 and B6+VA400) in comparison to the control cells (Figure 4(a)). The expression levels of the gene coding for NF-kB that plays an important redox-dependent role in apoptosis were found to be similar to the control group in the B6 cells, while they were increased approximately 2.5 times only in the B6+VA100 cell sample compared to the control cells (p = 0.03) (Figure 4(a)).

The expression levels of the gene coding for the pro-apoptotic Bax protein, which has a pro-apototic role, was found to be approximately 1.8-fold increased in the B6 cells (p = 0.037) and 1.9-fold in the B6+VA100 cells (p = 0.016) compared to the control cells, while it was found to be similar to the control group in the B6+VA200 and B6+VA400 cells, indicating that apoptosis was suppressed significantly in these samples (Figure 4(b)). The expression levels of the gene coding for caspase-9, which is an important initiator in the mitochondrial apoptosis mechanism, was increased approximately 3.7 times in the B6 cells (p=0.001) compared to the control group), whereas in the B6+VA100, B6+VA200 and B6+VA400 cells they were

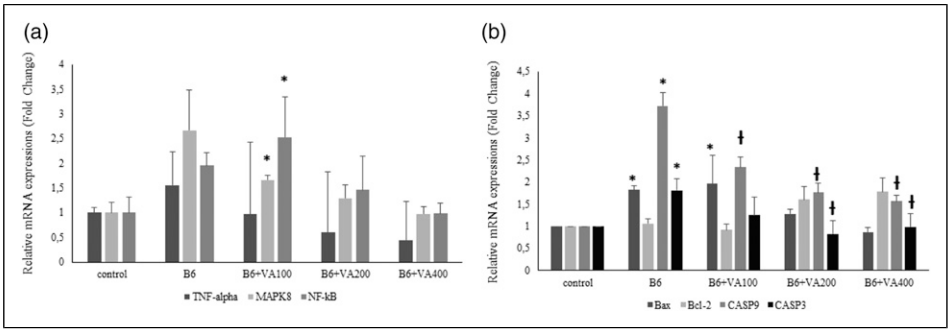

Figure 4. Relative mRNA expression levels (fold change) of apoptosis-related genes after treatment of the cells with 6 μM benomyl (B6) and B6 + valerenic acid at concentrations equal to 100 μM (i.e., B6+VA100), 200 μM (i.e., B6+VA200) and 400 μM (i.e., B6+VA400). (a) TNF-alpha, MAPK8, NF-kB gene expression levels were shown. (b) Bax, Bcl-2, CASP9 and CASP3 gene expression levels were shown. *: Statistically significant compared to the control cells; ł p Statistically significant compared to the B6 cells.

significantly decreased compared to the B6 cells ($p < 0.05$) (Figure 4(b)). The expression levels of the gene coding for caspase-3, which is involved in the last stage of the apoptosis mechanism, were increased approximately 1.8 times in the B6 cells compared to the control cells ($p = 0.028$). Furthermore, caspase-3 gene expression levels in the B6+VA100, B6+VA200 and B6+VA400 cells were significantly decreased compared to the B6 cells ($p < 0.05$) (Figure 4(b)). Finally, no significant effects were observed on the expression levels of the genes coding for TNF-alpha (Figure 4(a)), which is an important initiator of apoptosis ($p > 0.05$) and for Bcl-2 (Figure 4(b)).

Discussion

In this study, SH-SY5Y cells were exposed to the fungicide benomyl as an apoptosis-induction and oxidative stimulus and the potential alleviating effects of VA, an iridoid in valerian plant, in a wide range of concentrations were examined. As a result of benomyl exposure, the cell viability inhibition was enhanced, as was the case for DNA damage increase and the expression of genes coding for molecules related to apoptosis. However, VA acted protectively in a concentration dependent manner regarding the aforementioned measured biomarkers/parameters. In particular, treatment of the cells with VA showed a decrease in cell viability inhibition caused by exposure to benomyl.

Several studies have shown that pesticides and fungicides induce neuronal disorder and oxidative stress.[42–47] Specifically, it has been demonstrated that occupational exposure to pesticides is related to the onset of Parkinson's disease,[3,48–52] whereas other pollutants induce cytotoxicity measured via salivary biomarkers.[53] Furthermore, increasing evidence in the literature indicates that DNA damage and redox-related pathways are involved in the toxicity of such chemicals. To this direction, the pesticide Mancozeb induces oxidative damage of DNA in rats,[43,54] tebuconazole exerts its toxicity through the same processes as well.[44,45,55,56] Regarding benomyl, it constitutes a fungicide with well-known degenerative properties and as such it can be used as a very reliable model of causing such abnormalities in biological systems.[51] Literature confirms such action of benomyl since it has been proposed that it contributes to neurodegeneration through its ability to induces oxidation of biomolecules[52,53] and via causing apoptosis.[57–61]

Apoptosis is one of the most effective cellular pathways against neurogeneration of nerve cells and is activated by various factors, among which is exposure to pesticides. Pesticides trigger the mechanism of apoptosis in nerve cells by causing oxidative stress and DNA damage.[62] Valerenic acid is a compound of *V. officinalis*, which is used as a traditional treatment method in diseases of the nervous system. The ameliorating effects of VA against chemical-induced toxicity has also been scarcely examined. To that end, it has been observed that valerian constituents (i.e., VA, acetoxyvalerenic acid, hydroxyvalerenic acid and methyl valerenate) exert the same effects on GLC4, a human small-cell lung cancer cell line, and COLO 320, a human colorectal cancer cell lines,[63] whereas, it has been shown that VA supresses cell growth and induce apoptosis in glioblastoma cells.[64] In addition, in the same study, it was shown that the mechanism of apoptosis is stimulated in tumor cells through increased expression of pAMPK molecular pathway.[64] Furthermore, Rodriguez-Cruz have reported that in a model of MPTP-induced neurodegeneration as Parkinson's, administration of VA improves inflammatory biomarkers mediating neurodegeneration signs and behavior.[65] Finally, it has been reported that VA inhibits rotenone-induced neurodegeneration by suppressing cellular inflammation and apoptosis.[66]

Several studies have demonstrated that various herbal extracts have positive effects on toxin-induced neurodegeneration, as well as against pesticide-induced toxicity.[67–69] Indeed, ginseng aqueous extract ameliorates hepatorenal toxicity caused by an insecticide in rats.[70] Moreover, N-acetylcysteine, a precursor molecule of reduced GSH, the most important endogenous antioxidant

molecule, acts protectively against penconazole-induced neurodegenerative and neuroinflammatory disorders in rats,[71] lupeol protects against mancozeb-induced genotoxicity in human lymphocytes[72] and ascorbic acid supplementation is effective against cytotoxicity and DNA fragmentation induced by triphenyltin on human liver carcinoma cells.[73] In addition, manganese supresses oxidative stress, inflammation and caspase-3 activation in rats exposed to chlorpyrifos[74] and VA protects against benomyl-induced oxidative stress and apoptosis in neural cells.[75] On this basis, it appears that plant-derived compounds, antioxidant molecules and minerals could be important agents against neurodegenerative pathologies caused by chemicals, such as pesticides and fungicides.[76]

To our knowledge, this is the first study to report the ameliorative effects of VA on neurotoxicity dut to pesticide exposure. However, there are a few studies examining the neuroprotective effects of *V. officinalis* against xenobiotics.[77–79] Indeed, it has been shown the therapeutic potential of *V. officinalis* against rotenone toxicity in both C6 glioma cells and rats in vivo.[77] Administration of *V. officinalis* root powder in rats improved cortical spreading depression (CSD) levels, which expressed neuronal excitation potential against rotenone administration.[77] Moreover, climbing and mobility of *Drosophila melanogaster* were impaired by rotenone administration, while *V. officinalis* exerted ameliorative effects and restored the levels of antioxidant enzymes, such as superoxide dismutase and catalase.[80] In another study, the effects of different isolates of *Valeriana amurensis* were evaluated on an Alzheimer's mice model. It was reported that isolates restored the levels of apoptosis markers, namely Bcl-2 to Bax ratio and ERK, thus, reducing apoptosis reduced in neurons.[81] The ameliorative effects of valeric acid isolated from *V. officianilis* on rotenone induced Parkinson's mice model have also been evaluated. It has been found that administration of valeric acid prevents inflammation, oxidative stress induction and α-synuclein expressions, whereas it protects the dopaminergic neuron loss via supressin autophagy mechanism.[82] In consistency with the aforementioned studies, we report herein the ameliorative effects of VA on apoptotic markers after pesticide admnistration in neurons. In our study, we investigated VA ameliorative effects on fungucide benomyl toxicity in SH_SY5Y cells. DNA damage and apoptosis mechanisms were evaluated. However, a limitation of our study is that we could not include the effect of VA on the mechanism of apoptosis and its relationship with parameters such as inflammation and ROS induction.

Conclusion

As a conclusion, VA which is a plant-derived compound ameliorates the detrimental action of the fungicide benomyl on neuronal cells. Although benomyl increased cell viability inhibition and induced oxidative modification of cellular DNA and apoptosis, VA acted protectively through reversal of the aforementioned effects. It can be deduced that the biological mechanism according to which benomyl exerts its toxicity is related to the induction of DNA oxidation and apoptosis and compounds of plant origin with strong antioxidant potential might be of high importance towards amelioration of the detrimental effects of pesticides and other chemicals.

Acknowledgements

We would like to thank Prof. Dr Gül Özhan and Prof. Dr Sibel Özden for their valuable support.

Declaration of conflicting interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

Funding

The author(s) disclosed receipt of the following financial support for the research, authorship, and/or publication of this article: This study was supported by Istanbul University Scientific Research Projects Fund (Project No. TSA-2017-27370).

Data availability

All data generated or analyzed during this study are included in this article.

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