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# Flusilazole Induced Cytotoxicity and Inhibition of Neuronal Growth in Differentiated SH-SY5Y Neuroblastoma Cells by All-Trans-Retinoic Acid

# All-Trans-Retinoik Asit ile Farklılaştırılmış SH-SY5Y Nöroblastoma Hücrelerinde Flusilazole Bağlı Sitotoksisite ve Nöronal Büyüme İnhibisyonu

**İngilizce Kısa Başlık: Flusilazole Cytotoxicity on Differentiated SH-SY5Y Cells**
**Türkçe Kısa Başlık: Farklılaşmış SH-SY5Y Hücrelerinde Flusilazol Sitotoksisitesi**

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**ABSTRACT**
INTRODUCTION: Flusilazole (FLUS) is a broad-spectrum organosilicon triazole fungicide that is used for protecting economically important cereal and its products and orchard fruits. Considering the exposure route of pesticides, pesticide exposure through food is ineavitable. Furhertly, excessive exposure to pesticides can cause health effects on targets or non-target organisms. The aim of our study was to evaluate the effects of the triazole fungicide FLUS in differentiated SH-SY5Y neuroblastoma cells on cytotoxicity and neurite extention.
METHODS: SH-SY5Y cells were differentiated into mature neurons by 10 μM all-trans retinoic acid (RA) treatment for 7 days. Differentiated SH-SY5Y cells were treated with 50, 100 and 200 μM FLUS for 24 hours. Cell viability assays including crystal violet, neutral red cell viability assays and lactate dehydrogenase (LDH) leakage assay were performed. Additionally, morphological examinations were performed and neurite lengths of the cells were measured for all groups.
RESULTS: FLUS treatment induced cytotoxicity in SH-SY5Y cells differentiated by RA. Remarkable decreases were observed in the percentages of cell viability. Furtherly, neurite length was adversely affected by FLUS treatment at highest concentration.
DISCUSSION AND CONCLUSION: FLUS is widely used fungicide in agriculture for preventing crops from fungal diseases. However intensive usage of these compounds have potential health risks for both human and environment. Accordingly in the present study, it can be concluded that FLUS caused neurotoxicity at higher concentrations by neuronal cell death and had adverse effects on neurite growth in differentiated SH-SY5Y cells. FLUS exposure may cause neuronal degeneration in mammallians.
**Keywords:** Flusilazole, cytotoxicity, SH-SY5Y cell differentiation, neurite growth

**ÖZ**
**GİRİŞ ve AMAÇ:** Flusilazol (FLUS) ekonomik açıdan önemli tahıl ve tahıl ürünlerini ve meyve bahçelerini korumak için kullanılan geniş spektrumlu bir organosilikonlu triazol fungisittir. Pestisitlerin maruziyet yolları dikkate alındığında besin yoluyla pestisit maruziyeti kaçınılmaz olmaktadır. Ayrıca pestisitlere aşırı derecede maruz kalmak hedef ve hedef olmayan organizmalarda sağlık sorunlarına neden olmaktadır. Çalışmamızın amacı, bir triazol fungisit olan FLUS'un farklılaştırılmış SH-SY5Y nöroblastoma hücrelerinde sitotoksisite ve nörit uzaması üzerine etkilerinin değerlendirilmesidir.
**YÖNTEM ve GEREÇLER:** SH-SY5Y hücreleri 10 µM all-trans retinoik asit (RA) ile 7 gün boyunca olgun nöronlara farklılaştırılmıştır. Farklılaştırılmış SH-SY5Y hücreleri 50, 100 ve 200 µM FLUS ile 24 saat boyunca muamele edilmiştir. Kristal viyole, nötral kırmızısı canlılık testleri ile laktat dehidrogenaz (LDH) salım testlerini kapsayan hücre canlılık testleri yapılmıştır. Ek olarak, morfolojik incelemeler yapılmış ve hücrelerin nörit uzunlukları ölçülmüştür.
**BULGULAR:** FLUS uygulaması RA ile farklılaştırılan SH-SY5Y hücrelerinde sitotoksisiteyi indüklemiştir. Hücre canlılık yüzdelerinde dikkate değer düşüşler gözlenmiştir. Ayrıca en yüksek konsantrasyondaki FLUS uygulaması ile nörit uzunlukları olumsuz yönde etkilenmiştir.
**TARTIŞMA ve SONUÇ:** FLUS tarımda ekinleri fungal hastalıklardan koruma amacıyla yaygın olarak kullanılan bir fungisittir. Fakat bu bileşiklerin yoğun kullanımı insan ve çevre sağlığı açısından potansiyel risk oluşturmaktadır. Çalışma sonuçlarına göre, yüksek konsantrasyonlarda FLUS'un farklılaştırılmış SH-SY5Y hücrelerinde nöral hücre ölümüne ve nörit büyümesinde olumsuz etkilere neden olarak nörotoksisiteye yol açtığı sonucu çıkarılabilir. FLUS maruziyeti memelilerde nöronal dejenerasyona neden olabilir.
**Anahtar Kelimeler:** Flusilazol, sitotoksisite, SH-SY5Y hücre farklılaşması, nörit büyümesi

## INTRODUCTION

The use of pesticides in crop culturing enhances the production quality of food and feed, but causes several health problems. Exposure to fungicides may occur primarily via environmental sources or food. Triazole fungicides have common usage in agriculture for preventing fruits, cereals and vegetables from fungal infections, and also it has pharmacological usage for human and animal health. [1, 2] Flusilazole (FLUS) is a broad-spectrum organosilicon triazole fungicide that is used for protecting economically important cereal and its products and orchard fruits. [3, 4] Action mechanism of the triazole fungicides was attributed to inhibition of the lanosterol 14 alpha-demethylase (CYP51) enzyme which plays key role in sterol biosynthesis in fungus. [5] Inhibition of the CYP51 causes depletion of ergosterol synthesis, as a basis element for the fungal cell wall, and fungal cell growth was inhibited. Finally inhibition of the cell growth results for in cause of death for fungal cells. Due to CYP51 enzyme exists in human, inhibition of CYP51 caused by azole fungicides were reported to affected mammalian cells adversely. [6] Previous *in vitro* and *in vivo* studies exerted that triazole fungicides have adverse effects on mammalian steroidogenesis and it may induce developmental toxicity such as craniofacial malformations. [7, 8] Acceptable daily intake (ADI) of FLUS was estimated about 0-0.007 mg/kg bw for humans. [9] However considering the exposure route of pesticides, pesticide exposure through food is ineavitable. Furhertly, excessive exposure to pesticides can cause health effects on targets or non-target organisms. Previously in a study focused on cytotoxicity and oxidative stress of FLUS on dopaminergic PC12 cells. [6] However this study is supposed that it was the first for revealing effects on neurite growth of FLUS and comparing cytotoxicity assays on differentiated SH-SY5Y cells. SH-SY5Y cells (human neuroblastoma cell line) are commonly used in neurotoxicity and neurodegenerative disease models. [10] An *in vitro* cell model SH-SY5Y cells have ability to be

differentiated into mature dopaminergic neuron-like phenotype by retionic acid (RA) induction. [11] RA is known to regulate the cell cycle. [12] Differentiation of the neuronal cells in the experimental studies could serve homogenous neuronal cells. [11] RA-induced differentiation of SH-SY5Y cells was shown to elevate the susceptibility of the cells against neurotoxins and protective agents. [13] Additionally, it is known that differentiated SH-SY5Y cells serve as a good model for studying experimental Parkinson's disease model. [13] In recent years with the increased incidence of neurological diseases, the experimental studies evaluating the relationship the chemicals and diseases have increased remarkably. Considering the exposure to environmental contaminants inavitably, it is important to reveal the potential neurotoxicity effects of the commonly used fungicides. In the present study, it was aimed to evaluate the effects of the triazole fungicide flusilazole in differentiated SH-SY5Y neuroblastoma cells on neurite extention and comparing commonly used three cytotoxicity tests.

## MATERIAL AND METHODS

### Cell Culture Conditions and Differentiation of SH-SY5Y cells

SH-SY5Y human neuroblastoma cell line was purchased from American Type Culture Collection (ATCC® CRL-2266™, ATCC, VA, USA). Cells were cultured with Dulbecco's minimum essentail medium/nutrient mixture F-12 (DMEM/F-12) (Cegrogen Biotech GmbH, Germany) supplemented by 10% fetal bovine serum (Cegrogen Biotech GmbH, Germany) (FBS) and 1% penicillin-streptomycin antibiotic mixture at 37 °C with 5% $CO_2$ in humidified incubator. Culture medium was renewed every 3 days and cells were subcultured with detachment of trypsin EDTA (0.05 %) in DPBS (Cegrogen Biotech GmbH, Germany). SH-SY5H cells were incubated for 48 hours for attachment. Then culture medium was replaced with differentiation medium [Dulbecco's minimum essential medium (DMEM) suplemented with 3% FBS and 10 µM all-trans Retionic acid (RA)] for 7 days until treatment in dark at 37 °C with 5% $CO_2$ in humidified incubator.

### Treatment of Flusilazole

Flusilazole (PESTANAL®, analytical standard, Merck KGaA, Darmstadt, Germany) was dissolved in dimetyl sulfoxide (DMSO, Applichem, Darmstadt, Germany) for preparing 0,1 g/ml stock solution. Study working concentrations were prepared by dilution of stock solution with cell culture medium. Differentiated SH-SY5Y cells were incubated with 0-500 µM flusilazole concentrations and crystal violet cell viability assay was performed for $IC_{50}$ calculation. $IC_{50}$ value was found as 182,42 µM. At high concentrations (500 µM flusilazole) cell viability was found very toxic with 15 % cell viability. Working concentrations for the further analyses was based on calculated $IC_{50}$ value. Low and middle doses were chosen as slight toxic concentrations as lower than $IC_{50}$ and for high dose selection. High dose was based on higher concentration than $IC_{50}$. Chosen flusilazole concentrations for further analyses were 50, 100 and 200 µM.

### Crystal Violet Cell Viability Assay

Differentiated SH-SY5Y cells were seeded at $1\times10^4$ cells/well into 96 well culture plate and incubated for 24 hours for cell attachment. Cells were treated with 0, 50, 100 and 200 µM FLUS at 37 °C and 5% $CO_2$ in a humidified incubator for 24 hours. Cells were performed for crystal violet cell viability assay. [14] Briefly, culture medium with FLUS was discarded and cells were fixed with 4% neutral buffered formalin for 1 hour. Then fixative was removed, cells were stained with 0.1 % crystal violet solution for 30 minutes by shaking at room temperature. Cells were washed with distilled water several times in order to remove excess crystal violet dye. Crystal violet dye in the cells were extracted by 10 % acetic acid solution until dye to be dissolved and absorbance was measured at 595 nm wavelenght at microplate spectrophotometer (BIO-TEK µQuant, BIO-TEK Instruments, Inc, USA). Cell viability was calculated based on 100 % viability of untreated cells.

**Neutral Red Uptake Assay**

Differentiated SH-SY5Y cells were seeded into 96 well culture plate at a density of $1\times10^4$ cells/well and cells were allowed to attach onto surface and grow for 24 hours. After attachment of the cells, cells were treated with FLUS concentrations (0, 50, 100, 200 µM) and incubated at 37 °C and 5% $CO_2$ in a humidified incubator for 24 hours. Neutral red uptake assay was performed for determining the cell viability. Briefly, differentiated SH-SY5Y cells were incubated with 40 µg/ml neutral red dye containing culture media for 4 hours at 37 °C and 5% $CO_2$ in a humidified incubator. Then culture medium was discarded, cells were washed with phosphate buffer saline (PBS) twice and dye in the cells were extracted by neutral red desorb solution (1 % glacial acetic acid, 50 % ethanol in distilled water) for 20-45 minutes on shaker at room temperature. Absorbance was measured at 540 nm wavelenght in an hour at microplate spectrophotometer (BIO-TEK µQuant, BIO-TEK Instruments, Inc, USA). Cell viability was calculated based on 100 % viability of untreated cells.

**Lactate Dehydrogenase (LDH) Leakage Assay**

Lactate dehydrogenase is a stable cytosolic enzyme and it released from cells when cell membrane damage was occured. LDH leakage assay is commonly used for determining cell membrane damage. LDH assay was performed by using commercial kit (Biovision, K313-500, USA). Briefly, differentiated SH-SY5Y cells were seeded at a density of $2\times10^4$ cells/well into 96 well culture plate and incubated at 37°C in a 5% $CO_2$ humidified incubator for 24 hours. Culture medium was discarded, then cells were treated with FLUS concentrations (0, 50, 100 and 200 µM) for 24 hours. At the end of the incubation time, cells were centrifuged at 600 g for 10 minutes by using plate rotor (5810R Centrifuge, Eppendorf AG, Hamburg, Germany). 10 µl sample with 100 µl LDH reaction solution was incubated for 30 minutes in the dark at room temperature. This assay utilizes the enzymatic coupling reaction that, LDH oxidized lactate to generate NADH, WST which is found in the reaction solution generates yellow to amber colour. The generated colour intensity is directly correlated with damaged cell number. Finally, at the end of the incubation time, absorbance was measured at 450 nm wavelenght at microplate spectrophotometer (BIO-TEK µQuant, BIO-TEK Instruments, Inc, USA). Percentage of the cytoxicity was calculated based on 100 % viability of untreated cells.

**Cell Morphology Analysis**

Cell morphology analysis was performed by capturing micrographs of undifferentiated or differentiated of treated cells by inverted microscope (Olympus CKX 41 inverted microscope, CellSence Imaging Software, Olympus, Japan). Each experiment was performed by measuring the neurite lengths of undifferentiated or differentiated neurons by Image J Package with Neuron J. [15, 16]

**Statistical Analysis**

Statistical analyses were performed by using package program of SPSS for Windows. $IC_{50}$ value was calculated by probit analysis. In order to determine the significant differences between FLUS treatment and non-treatment groups, ANOVA test was performed with 95 % confidence interval following Tukey post hoc test. Data were expressed as mean±standart error. All experiments were performed independently triplicate.

**RESULTS**

**Morphological Features of Undifferentiated and Differentiated SH-SY5Y cells**

SH-SY5Y cells were differentiated by treatment of 10 µM RA for 7 days. Figure 1 demonstrates morphological features of undifferentiated and differentiated cells. Undifferentiated cells could be distinguished by clustered round cell body shape and short neurites (Figure 1A). Morphologically differentiated SH-SY5Y cells could be characterized by extensive neurites as well as pyramidal cell body shape (Figure 1B). To better characterized the differentiation, neurite lengths of undifferentiated and differentiated SH-SY5Y cells were measured, data were shown in Figure 1C. Undifferentiated cells showed

shorter neurites, however neurites were longer in 7 days RA-induced differentiation for than undiffetentiated cells. Differentiated SH-SY5Y cells were treated with different FLUS concentrations. Figure 2 demostrates the effects of FLUS on RA-induced differentiated SH-SY5Y cells morphologically. Accordingly results showed us that FLUS treatment caused remarkable morphological changes in differentiated SH-SY5Y cells including apoptotic cell death which is charactrerized by round and shrinkage nuclei and decrease in neurite length (Figure 2). Apoptotic cells increased in FLUS treatment groups, and neurite length decreased in response to highest concentration of FLUS which was statistically significant compared to non-treatment group.

**Cell Viability Assay Results**

Cytotoxic effects of FLUS were evaluated by performing different cytotoxicity assays. Crystal violet assay was performed as well as LDH and neutral red cell viability assays. $IC_{50}$ value was found 182,42 µM by crystal violet assay. Study concentrations were based on $IC_{50}$ value. Crystal violet cell viability results were demonstrated in Figure 3. Cell viability decreases were found statistically significant in 100 and 200 µM FLUS treatment groups as compared to control and 50 µM FLUS treatment groups. LDH assay results were shown in Figure 4. According to LDH leakeage assay results, cell death increased in response to increasing FLUS concentrations. At higher concentrations FLUS caused increases in percentage of cell death viability, increases were found statistically significant at 100 and 200 µM FLUS when compared to non-treatment and 50 µM FLUS treatment group. Additionally, according to neutral red cell viability assay, remarkable decreases in cell viability were statistically significant in all treatment groups. Significant decreases were found between non-treatment and FLUS treatment groups, as well as cell viability in 200 µM FLUS treatment group was significantly different from 50 and 100 µM FLUS treatment groups. Neutral red uptake assay results were demonstrated in Figure 5.

**DISCUSSION**

Triazole fungicides have common usage area including agriculture and medicine all over the world. Excessive usage of the fungicides could be cause of generation of harmful residues for the environment and cause risk of human health via consumption of contaminated foods, water. [17] Triazole fungicides inhibit fungal CYP51 in fungal cells and it results for increases in fungal cell wall permeability and finally fungal cell death occurs. [6, 7] Previously published studies conducted with triazole fungicides exerted that harmful effects on nervous system such as triazoles caused neuropathological defects in murine brain as well as neuropathological lesions in peripheral nervous system. [18] Previous studies also reported several adverse effects of azole fungicides such as birth defects, craniofacial malformations and inhibition of steroidogenesis in mammalians. [5, 19, 20] Despite the large scale usage area of human health risks of triazole fungicides, studies about the effects of flusilazole on human health are considerably limited. [6, 7] Due to limited information on the effects of FLUS on differentiated neurons, this study was aimed to elucidate toxicity mechanism of FLUS on neurite outgrowth and comparing cytotoxicity tests by FLUS treatment.

In the field of neuroscience, *in vitro* models resembling functionally neurons are lacking, especially for neurodegenerative diseases including Alzheimer's disease. Although cell lines could be used frequently such as SH-SY5Y cells, these cells are lack of excerting mature neuronal including morphology, ceased cell division as well as expressing specific markers. [21] However, SH-SY5Y cells were proved to be differentiated into mature neuron and differentiated neuron cells could serve homogenous cell types. Present study was set up to investigate the cytotoxic mechanisms of FLUS on differentiated SH-SY5Y cells. Previous studies revealed that SH-SY5Y cells could be differentiated into mature neuron-like cells by RA induction for about 7 days treatment at dark conditions and differentiated cells have changes in morphology characterized by extensive neurites outgrowth. [22, 23] The action

mechanism of RA was characterized by activation of transcription via binding to non-steroid nuclear hormone receptors. Also, RA was shown to induce Wnt signaling pathway, act in regulation of the neurotrophin receptor gene transcription. [13] In this study, morphologically differentiated SH-SY5Y cells had typical neurons with extensive neurites and pyramidal shaped cell body were observed. Otherwise undifferentiated cells were tend to form clusters and had rounded shaped cell body. [11] According to these results, we may suggest that SH-SY5Y cells were differentiated successfully morphologically.

In vitro cytotoxicity assays could be useful for predicting human toxicity in toxicological studies. Cytotoxicity assays differ in type of assay as well as different results according to test principle. [24] It is important to perform carry out multiple markers to exert reversible or irreversible effects of toxic substances. [25] In the present study, three cytotoxicity assays based on different test principle were performed for differentiated SH-SY5Y cells to evaluate mechanism of toxicity. Crystal violet cell viability assay was performed for evaluating cell viability, this method was selected due to being a reliable and quick method and cyrstal violet specifically binding to DNA and revealing the cell viability. Neutral red uptake assay was performed for evaluating the percentage of the viable cell in terms of lysosomal activity. LDH leakage assay was performed in order to evaluate necrotic cell death.

In order to evaluate the viability of differentiated SH-SY5Y cells, we performed crystal violet cell viability assay which is a basic method for determining viable cells staining with crystal violet dye binding to DNA and proteins. This method was based on staining of viable attached cells because death cells lose their adherence and amount of crystal violet dye is reduced. [26] Results from the crystal violet cell viability assay excerted that, FLUS treatment caused decreases in differentiated SH-SY5Y cells in a dose dependent manner. Previously in a study conducted with 9,5 days old rat embryos in vitro, alterations in hindbrain development as well as cranial nerve abnomalies were reported by triazole treatment and FLUS and other triazole derivatives were found teratogenic. [20] In our study it was shown that FLUS induced cell death at higher concentrations and findings were consistent with previous studies.

LDH release in FLUS treatment groups were significantly increased in higher concentrations of FLUS. LDH is which is an intracellular enzyme found inner compartment of the cell. LDH leakage assay is described a cytotoxity marker revealing cell membrane damage as irreversible cell death. [27] Due to necrotic cells have permeable cell membrane, leakage of the cytoplasmic enzyme LDH into culture media as a result of membrane damage is considered a hallmark of necrotic cell death. [28] In the present study necrotic cell death percentage was performed by LDH leakage assay. In a study Zebra fish liver cell line (ZFL) were treated with triazole fungicides, and effects of triazole fungicides including FLUS were evaluated by LDH leakage assay. That study revealed remarkable increases in LDH leakage at higher concentrations of FLUS. [29] Results of this study were consistent with the previously reported study. Similarly, previous study with another medically used triazole fungicide fluconazole induced also necrotic cell death in L929 cells. [30] In the present study triazole FLUS could adversly affect human neuronal cells. As a result of FLUS treatment membrane damage occured in differentiated SH-SY5Y cells and finally induced necrotic cell death.

Neutral red uptake assay was performed for determining cytotoxicity. Principle of this assay is based on the uptake of neutral red dye (as a water soluble dye) into lysosomes which is energy required process for cells. [31] Neutral red uptake by cells was depend on pH gradients in the process of ATP production. Neutral red dye could be able to enter into the cell because of net charge of dye is zero. However, inside part of the lysosomes have lower pH than the cytoplasm via proton gradient. Due to pH gradient neutral red dye retain positive charged so that dye could retain inside of the lysosomes. In case of cell death or pH gradient changes neutral red dye could not retain in the lysosomes. This assay is reported highly sensitive for cell viability quantification. [32] In case of chemical damage of plasma or lysosomal

membranes, ability of the endocytosis, which requires energy for the process, uptake ability of the neutral red dye decreases. In the present study according to results of neutral red uptake assay, FLUS treatment of SH-SY5Y cells affected cell viability adversely by causing decreases in elevated concentrations of FLUS. These results highlights that FLUS may cause lysosomal damage especially at higher concentrations. All cytotoxicity test results excerted trend of reduction in cell viability. Although some variables, it seemed that the most sensitive test for FLUS on differentiated SH-SH5Y cells was crystal violet.

Environmental neurotoxins including pesticides, fungicides have been reported to play crucial role in development of neurodegenetive disorders. [33-35] In differentiated neurons, changes in morphology or neurite growth could be sign of neurodegenerative diseases. Up to now, several cell lines including SH-SY5Y were reported to be used to study the effects of toxicants on neurite growth. [36] Differentiated SH-SY5Y cells differentiated by RA induction could serve as a good *in vitro* model for revealing neurotoxicity mechanisms. [37] Differentiated SH-SY5Y cells were reported to have the highest levels of neurite length measurement with 10 µM RA treatment for 3 days. [38] Our study, differentiated SH-SY5Y cells had longer neurites than undifferentiated cells. Neurite growth by RA treatment were measured more than 1,9 fold of undifferentiated cells. Previous *in vitro* studies conducted by organophosphates reported reduction in neurite outgrowth in N2a Mouse neuroblastoma and C6 glioma cells. [39] It was observed that FLUS treatment caused inhibition in neurite lengths of differentiated SH-SY5Y cells.

## CONCLUSION

FLUS is widely used fungicide in agriculture for preventing crops from fungal diseases. However intensive usage of these compounds have potential health risks for both human and environment. Accordingly in the present study it was concluded that FLUS caused neurotoxicity at higher concentrations by neuronal cell death and affect adversly neurite growth in differentiated neurons. This study may serve as preliminary results for further studies. FLUS as a fungicide could cause neuronal degeneration in mammallians. This study needs further analyses to elucidate the mechanism of action.

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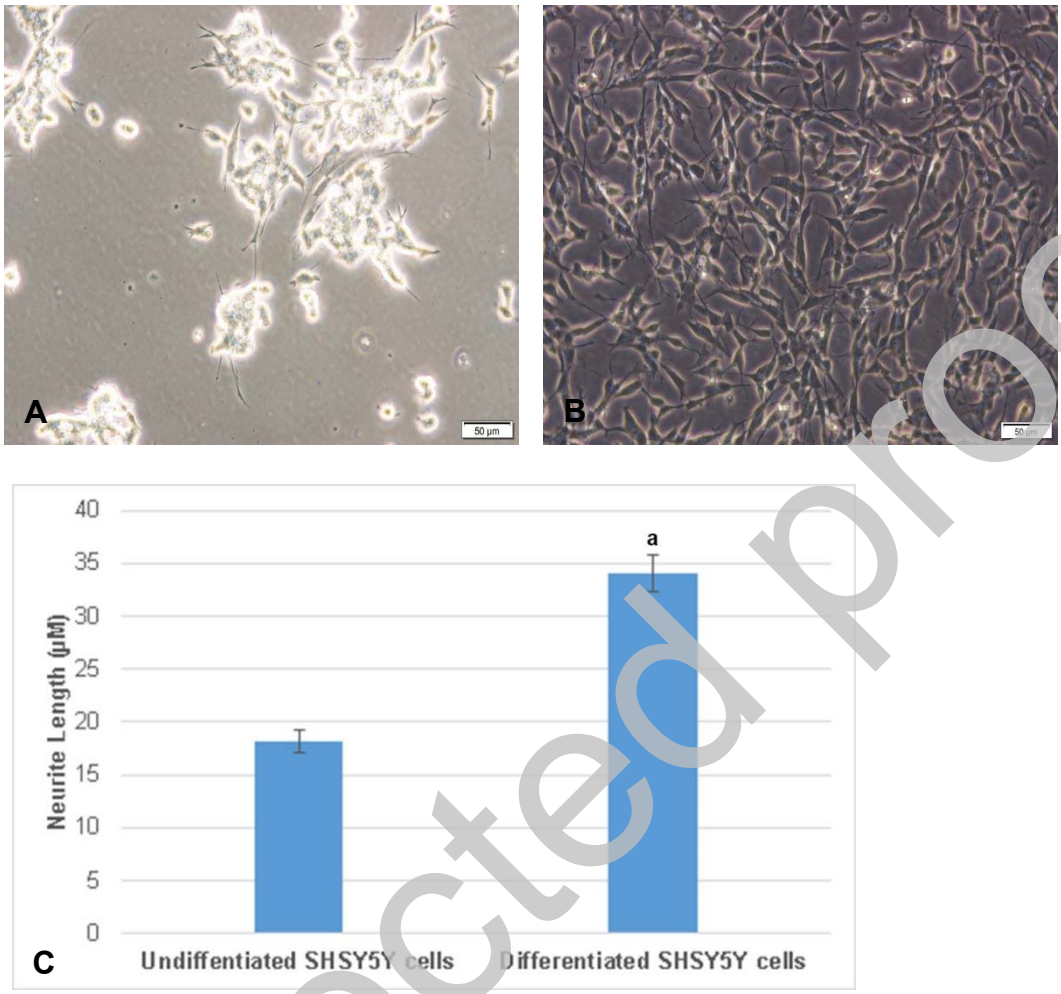


**Figure 1. A)** Undifferentiated SH-SY5Y cells with clustered round shaped cell body and short neurites, **B)** RA-induced differentiated SH-SY5Y cells with pyramidal chaped cell body and extended neurites, **C)** Neurite lengths of undifferentiated and differentiated SH-SY5Y cells.

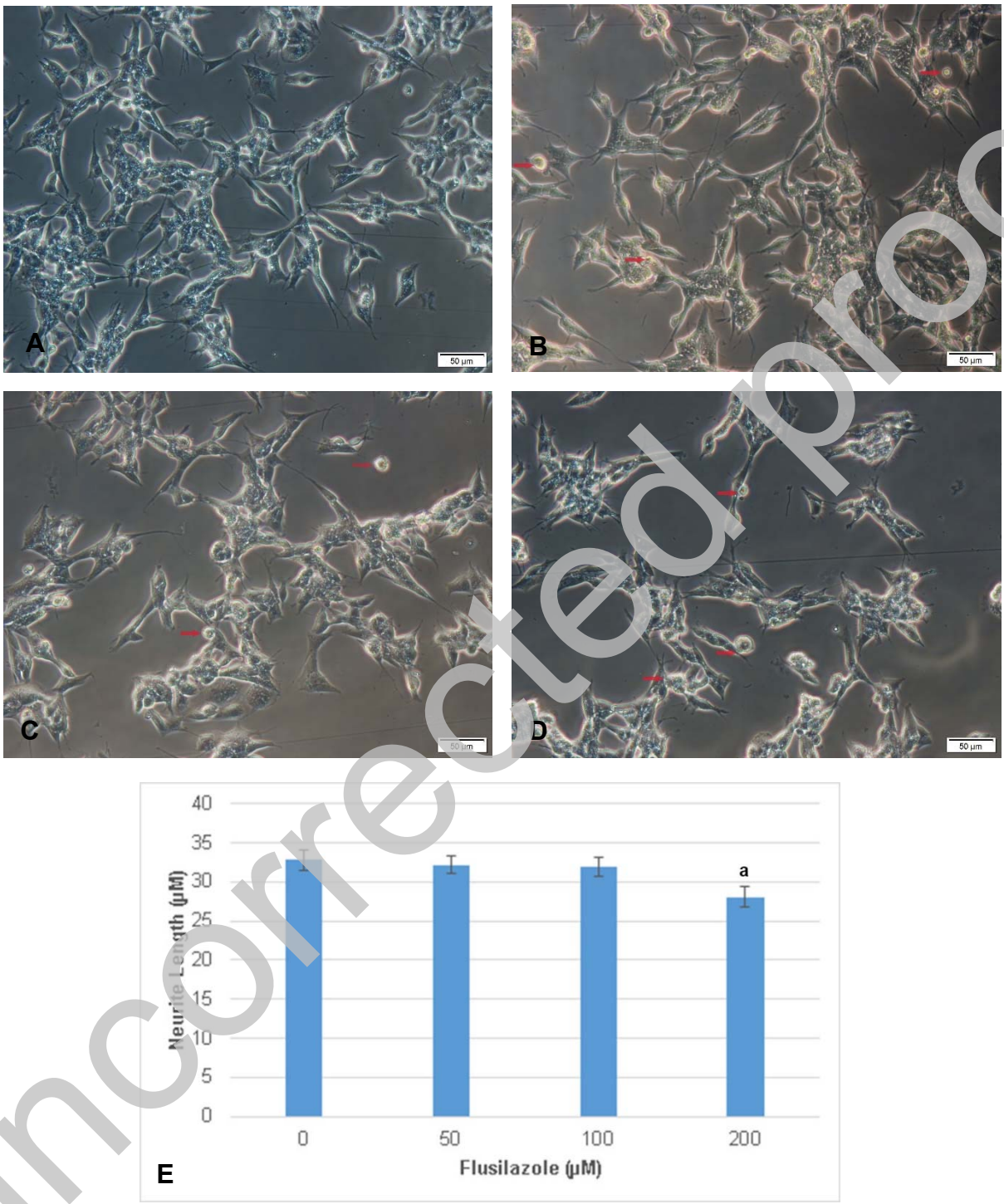


**Figure 2.** Morphological micrographs of differentiated SH-SY5Y cell of flusilazole treatment groups, **A)** Control group, **B)** 50 µM FLUS treatment group, **C)** 100 µM FLUS treatment group, **D)** 200 µM FLUS treatment group, (→) apoptotic cell.

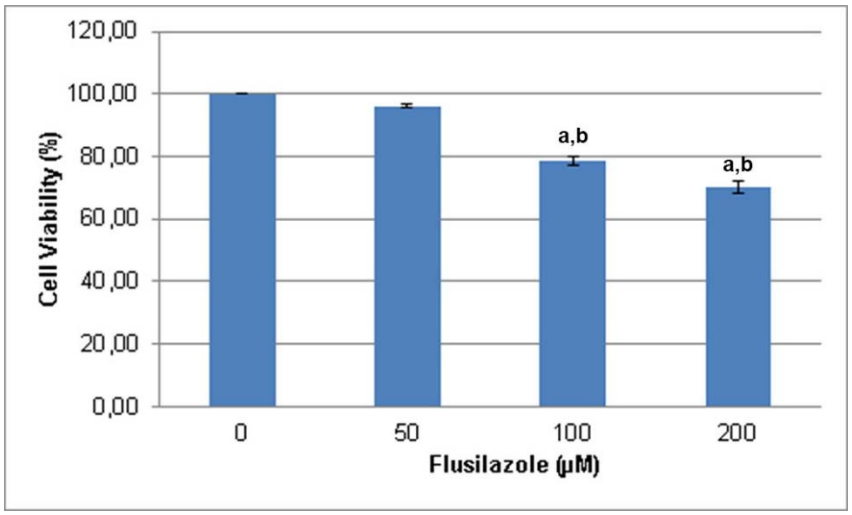


**Figure 3.** Crystal violet cell viability assay results (%). [a]Significantly different from control group, [b]Significantly different from 50 µM FLUS group.

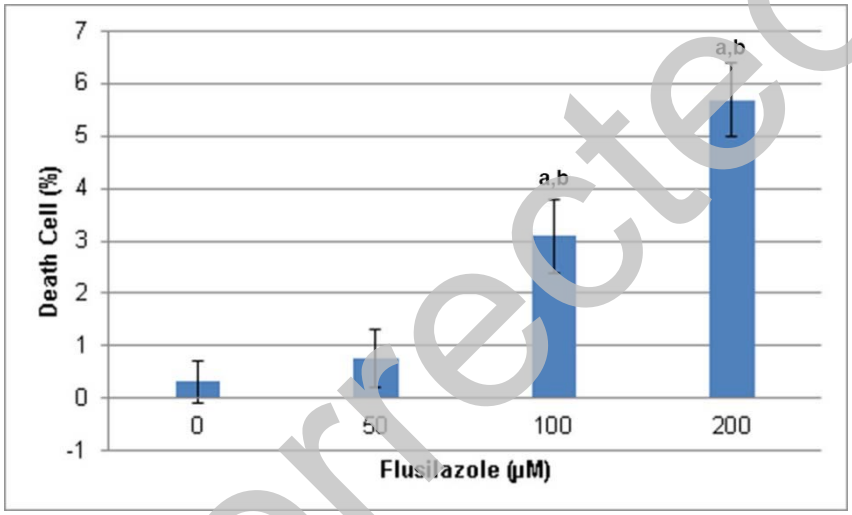


**Figure 4.** LDH leakage assay results, cell death (%). [a]Significantly different from control group, [b]Significantly different from 50 µM FLUS group.

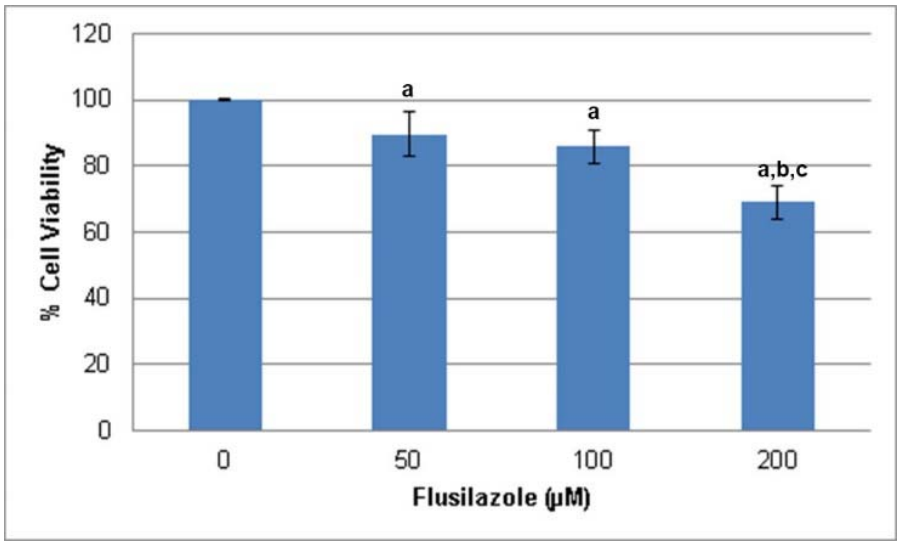


**Figure 5.** Neutral Red cell viability assay results, [a]Significantly different from control group, [b]Significantly different from 50 µM Flusilazole group. [c]Significantly different from 100 µM FLUS group.