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Study of the Cytotoxic Activity of Haarlem Oil on Different Cell Lines and a Higher Organism, *Steinernema feltiae*

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Abstract

Haarlem oil (HO) is a semisynthetic product made by combining terpene oil and sulfur atoms at high temperatures. HO contains organosulfur compounds; these compounds are known to have strong antioxidant properties, such as superoxide dismutase (SOD). This study provides a brief overview of the effects of HO cytotoxicity on several cell lines using several cytotoxicity test methods. The crystal violet (CV) staining assay showed that HO had a strong toxic effect on the A549 cell line. The test results of the trypan blue and celltiter-Glo assay methods showed that HO has a strong cytotoxic effect on HL-60 cells. The results of the MTT and XTT assays indicated that HO produced a fairly strong toxic effect on HL-60 cells and U937 cells. A hoechst staining assay showed that HO was able to increase (induce) apoptotic cell levels and reduce mitotic cell levels after 24 hours of incubation. However, in this study, we were not able to detect any effect of HO on activation and inhibition of the K562 cell line through the NF- κ B pathway. Meanwhile, the live and dead assay showed that HO tends to cause apoptosis. The nematocidal assay showed that HO showed moderate activity against *Steinernema feltiae*.

Keywords: Haarlem oil, cytotoxicity activity, organosulfur compounds, *Steinernema feltiae*

1. Introduction

Haarlem oil (HO) was first introduced in the Netherlands by Thomas Monsieur, a French scientist, as a dietary supplement and stamina aid. In the sixteenth century, a Dutch researcher named Claas Tilly used HO for the first time for the treatment of kidney and urinary stones. HO became increasingly popular in the Netherlands as a health product (**Figure 1**). HO production at that time continued to increase, eventually attracting the attention of scientists because of its efficacy and usefulness in the fields of health and beauty. In the nineteenth century, HO's effects and uses started to be studied pharmacologically. In 1924, HO was widely traded in France as a food supplement. In the 1980s and 1990s, HO again received

the attention of scientists because of its sulfur content. At present, HO is produced semi-synthetically from natural products, including terpene oil and sulfur elements, which are combined at high temperatures. Research shows that, in addition to sulfur compounds, HO also contains iminosugars. Iminosugars are compounds containing nitrogen atom bases in their endocyclic structure. It is known that this nitrogen atom is partially responsible for HO's activity. A study of the literature also shows that HO contains polyunsaturated essential oils; polyunsaturated essential oils are known to have strong antioxidant effects, such as in superoxide dismutase (SOD) [1–3].

HO has been reported to be effective in treating rheumatic diseases, bronchitis, and diseases of the liver and kidneys [2, 3]. Histological and bio-clinical tests on mice showed that the sulfo-terpene in HO stimulated the adrenal cortex by causing mice to secrete adrenocorticotrophic hormone (ACTH) and eliminating corticosteroids in urine [4]. HO also has strong antiseptic properties, which are thought to originate from its terpene content. Other studies have shown that, when applied to bronchial-pulmonary tissue for 15–60 minutes, HO anti-inflammatory action and increased the action of SOD by increasing the levels of thiol (–SH) group in plasma. Antioxidant activity tests have shown that HO is able to increase SOD enzyme activity. Toxicology and posology tests showed no cases of intoxication in patients given HO at a dose of 2500 mg/kg [5]. X-ray analysis of bronchitis patients treated with HO at a dose of 10 mg/kg for 10 days showed improvements [6].

In the body, sulfur is essential because it functions as a regulatory agent in bile glands, as a stimulator in the respiratory system, and as a toxin neutralizer, and also plays a role in the allergic response. In cells, sulfur plays a role in the synthesis of proteins and also contributes to the synthesis of essential amino acids (such as cysteine and methionine), vitamins (thiamine or vitamin B₁, Biotin, and B₆), and coenzyme A (CoA), which plays a role in many metabolic processes. Sulfur compounds also play a major role in the prevention of various cancers and infection by microorganisms such as bacteria and fungi.

HO is a natural product containing unusual sulfur compounds, such as thiosulfinate, polysulphane, 1,2-dithiin, and 1,2-dithiole-3-thione. HO also contains several other isothiocyanates. These compounds are also found in many other natural ingredients such as onions (*Allium* sp.), mustard, and asparagus. Unusual sulfur compounds generally contain reactive sulfur species (RSS), which function as antioxidants and have cytotoxic activities, which give them their anticancer, antibacterial, antifungal, and antisclerodermal effects [7]. This study presents a brief overview of the effects of HO cytotoxicity on several cell lines, using several cytotoxicity test methods such as Crystal Violet (CV), CellTiter-Glo, Trypan Blue, MTT, XTT, NF- κ B pathway, Hoechst staining, and Live and Dead assays. This article also provides brief information about HO activity against *Steinernema feltiae* (*S. feltiae*). *S. feltiae* is a microscopic entomopathogenic nematode. This means that



Figure 1.
Products of Haarlem oil.

it is itself a parasite in other parasites, especially insects and their eggs [8]. *S. felitiae* was selected as a test organism is because this organism is a complex organism and can be used as a model for whole organisms [9, 10].

2. Cytotoxicity studies of Haarlem oil study cytotoxicity by crystal violet staining assay

Crystal violet (CV), or Tris(4-(dimethylamino)phenyl)methylium chloride, is a triarylmethane dye which is used to investigate cell viability responses. CV staining is used mainly in living cell membranes, because this dye is able to bind to proteins and DNA in cells. Cells that die will lose adherence and then disappear from the cell population. Losses from the population of cells reduce the amount of dye staining in a culture, which enables the researcher to count the number of cells in a monolayer culture via absorption of dye by the cells. This test is a simple, fast method of cell viability screening and is useful for acquiring information about relative cell density. CV can also be used to measure the cytotoxicity of a compound [11, 12].

The CV test has major advantages over other cytotoxic tests: in a CV test, after staining, changes in cell morphology can be observed and stored for a long time. At present, research on the effects of HO cytotoxicity is still limited. Therefore, this article provides a brief overview of the effects of HO cytotoxicity on several cell lines such as 3T3, CT26, HT29, A549, HUVEC, MCF7, HepG2, and OVCAR cell lines.

At present, HO is sold as a supplement for maintaining stamina and as an antioxidant. HO has been studied previously because of its organosulfur compound content, which may have potential for medical treatments. The mechanism of HO activity against cells is not yet fully understood. Knowledge of the biological activity of HO, especially as related to the behavior of redox-modulators, is also still very limited [1, 12].

In this study, the results of HO cytotoxicity on several cells are expressed in percentages of cell viability, by measuring the Optical Density (OD) of cells at a wavelength of 590 nm (OD_{590}). OD indicates the absorbance of a sample measured at a specific wavelength, and is a common method used to measure concentration, growth conditions, and cell reproduction abilities.

To determine the cytotoxicity of HO, we used several levels of HO concentration, from 13.125 to 50 ppm. In this test, we used a 0.1% cytotoxic agent solution of detergent Nonylphenoxypolyethoxy ethanol (NP40). This detergent acts to break down and open membranes in cells, including the membrane's core. **Figure 1** shows HO's effect on 3T3, CT26, HT29, A549, HUVEC, MCF7, HepG2, and OVCAR cell lines at various concentrations.

The results show that HO has almost the same toxic effect as NP40. In the OVCAR cell line, HO had the highest toxic effect after 72 hours of incubation, while for the HT29 cell line, HO had a very small effect. HO had relatively similar cytotoxic effects on the 3T3, CT26, A549, HUVEC, MCF7, and HepG2 cell lines (**Figure 2**).

EC₅₀ (half maximal effective concentration) is the concentration of a drug, compound, antibody, or toxicant capable of inhibiting growth by 50% after exposure for a certain amount of time. The EC₅₀ value of HO on several cell lines was determined using OriginPro 7.5 software. The EC₅₀ values of HO obtained from the CV staining assay method can be seen in **Table 1**. **Table 1** shows that HO had the most active toxic effect on A549 cell lines (adenocarcinomic human alveolar basal epithelial cells), with an EC₅₀ value of 6.30 ppm. In addition, HO also showed a strong cytotoxic effect on CT26 and HepG2 cell lines, with EC₅₀ values of 6.49 and 6.50, respectively. Meanwhile, HO showed EC₅₀ values of 15.19 and 15.20 ppm, respectively, on the HUVEC and 3T3 cell lines [13].

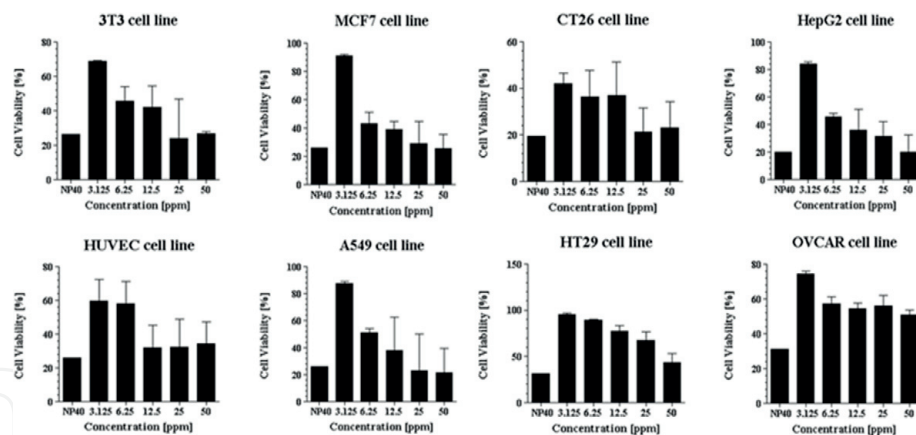


Figure 2. The percentage of cell viabilities of 3T3, CT26, HT29, A549, HUVEC, MCF7, HepG2 and OVCAR cell lines after been exposed to HO for 72 hours by CV staining assay. Data are expressed as the percentage of viability % \pm SD.

Cell line	Characteristics	EC ₅₀ value [ppm]
3T3	Fibroblast cell line	15.19
HUVEC	Human umbilical vein endothelial cells line	15.20
CT26	Colon carcinoma cell line	6.49
HT29	Human colon carcinoma cells line	>50.0
MCF7	Breast cancer cell line	6.50
A549	Adeno carcinomic human alveolar basal epithelial cells line	6.30
HepG2	Perpetual liver tissue cell line	6.50
OVCAR	Human epithelial carcinoma of the ovary cell line	9.07

Table 1. The characteristics and EC₅₀ values of HO on several cell lines were tested with a CV staining assay.

2.1 Study cytotoxicity on HL-60 cell line

The CellTiter-Glo luminescent cell viability assay is a homogeneous method used to determine the number of active cells in cell cultures. This method quantifies cells based on the presence of adenosine triphosphate (ATP) nucleotides in cells. In this method, the presence of ATP is interpreted as an indicator of cell proliferation and an indicator of energy changes in the cell's biological system. ATP is commonly found in living cells because it plays a role in catabolic and anabolic cell processes. The measurement of ATP is fundamental in the study of cells, as the quantities of ATP directly correlate with cell populations [14]. This method uses the enzyme luciferase, which uses ATP to produce luminescence. This luminescence is then measured by the amount of light, or signal, produced, which is strongly correlated to the amount of ATP in the cell population. ATP quantities are highly correlated with the number of living cells [14].

In this method, the test compounds are diluted at certain concentrations and incubated for 8, 24, and 48 hours. The amount of ATP is measured at the end of the incubation time. The results showed that, at a concentration of 1:10, HO was able to reduce ATP in HL-60 cells by 50%, while at a concentration of 1:5, HO was unable to reduce ATP, because the cells were lysed or killed due to a decrease in cell glycogenesis. Glycogenesis is crucial for the formation of ATP; and the decrease in glycogenesis results in the halt of glycogen formation, leading to cell death. The

results also showed that HO was able to reduce the ATP in HL-60 cells at all incubation times [15]. This shows that the reduction in ATP by HO occurs very quickly (**Figure 3**).

Trypan Blue assay is a method used to determine the viability of a cell. The basic principle of this method is that normal cells have intact cell membranes that are able to bind to foreign substances, such as the Trypan Blue dye. In abnormal cells, however, the cell membrane does not have the ability to bind foreign substances onto the cell [16]. In this test, a cell suspension is mixed with Trypan Blue dye, then the cell is observed visually and the viability is calculated using a microscope. Observations were made by examining whether these cells would repel or uptake the dye. In this test, viable cells exhibit clear cytoplasm because their cell membranes cannot be penetrated by the dye, while nonviable cells show blue cytoplasm, because damage to the cell membranes allow them to be easily penetrated by foreign substances such as Trypan Blue. The results showed that HO had a significant cytotoxic effect on cells at all treatment durations and concentrations used. These results also showed that HO at high concentrations (i.e., HO: MilliQ water (1:5) and (1:1)) had a strong effect on the percentage of viability of HL-60 cells, with a percent viability of <10% after 8 hours of incubation. If the incubation time is further extended to 24 and 48 hours, the viability decreases to nearly 0% [15]. These results indicate that, out of all the cells tested, HO had the strongest cytotoxic effect on HL-60 cells (**Figure 3**).

The MTT test is a colorimetric, enzyme-based method commonly used to test mitochondrial dehydrogenase activity in cells. This method is frequently used because it is easy, safe, and has a high sensitivity. It is the most commonly used method for testing cell toxicity and viability [17]. The MTT test is used to evaluate the ability of cells to reduce tetrazolium salt or 3-(4, 5-dimethylthiazole-2-yl) 2, 5 diphenyltetrazolium bromide to form insoluble formazan violet crystals. Colored tetrazolium salt, when interacting with cells, will turn purple (formazan). This color is caused by cells undergoing metabolic reduction by the enzyme

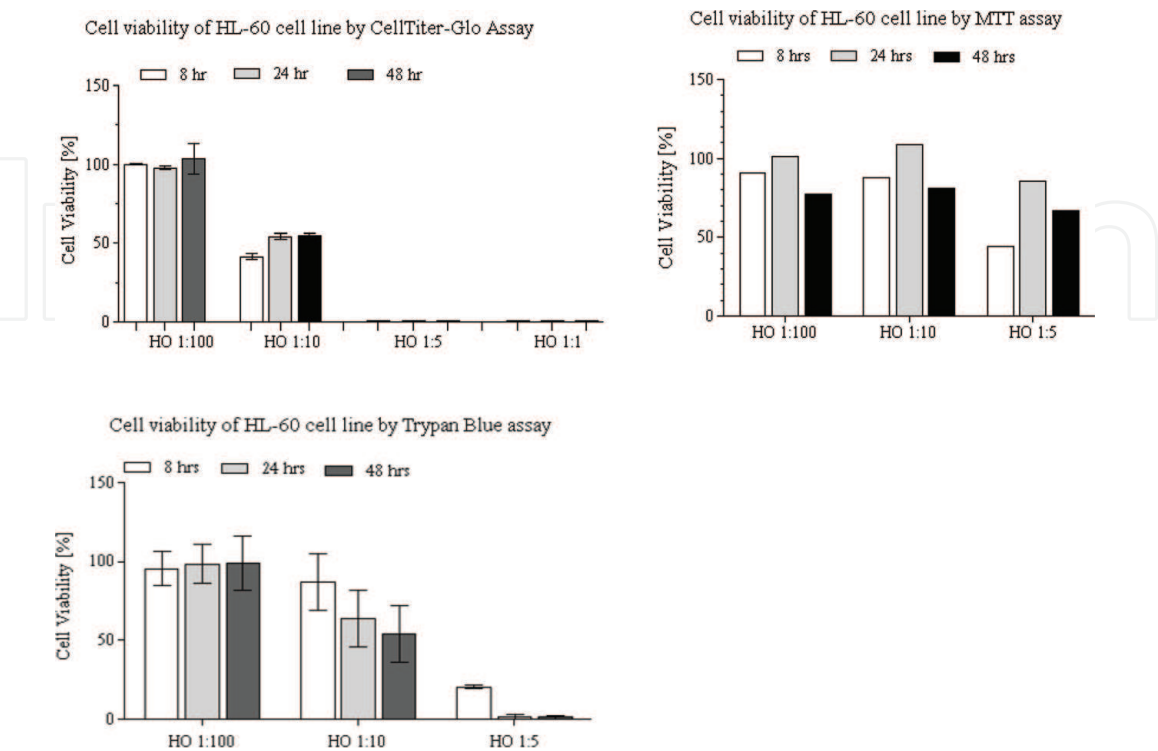


Figure 3. The cytotoxicity effect of HO on HL-60 cell line using several cytotoxicity assays. Data are expressed as the percentage of viability % \pm SD.

dehydrogenase to form NADH or NADPH. It is the absorbance value of this purple color that is measured. This absorbance value is used to determine the cell viability. If the absorbance value observed is smaller than the absorbance value of the control, the cell is undergoing reduction; in other words the cell's ability to proliferate is low. However, on the contrary, if the absorbance produced is higher than the control, the cell's ability to proliferate is very high. If the level of proliferation is too high, however, this can result in cell death, due to potential changes in cell morphology.

This test uses HL-60 cells, which are commonly used as models to study the differentiation of myeloid cells in humans [18, 19]. HL-60 cells exposed to HO at concentrations of 1:100, 1:10, and 1:5 with incubation times of 8, 24, and 48 hours are presented in **Figure 3**. The results indicate that, at a concentration of 1:5, HO was effective at all incubation times, with the percentage of cell viability between 40 and 65%. This means that only 40–65% of cells were able to proliferate. However, at HO concentrations of 1:100 and 1:5, the results were effectively identical, with the percentage of cell viability ranging from 70 to 85% [15] (**Figure 3**).

2.2 Study cytotoxicity on U-973 cell line

In this section, we tried to determine HO's cytotoxicity on the U-937 cell line using the XTT and Hoechst staining assay methods, as well as testing HO's activation and inhibition of U-937 cells through the NF- κ B pathway. U937 cells are human histiocytic lymphoma cells. The principle of the XTT test is the breakdown of the tetrazolium salt into formazan by succinate-tetrazolium reductase in the mitochondria, involving electron transfer by mitochondrial and non-mitochondrial enzymes. Compared to MTT, the XTT test is faster, more reproducible, and gives more sensitive results. The viable cells in the XTT test were measured based on the activity of mitochondrial enzymes in reducing tetrazolium salt [20]. In this test, the U-937 cell line was treated with HO for 6, 24, 48, and 72 hours. 24 hours after the HO treatment, the cells were then treated with XTT for 3 hours (6 hours treatment) or 4 hours (24, 48, and 72 hours treatment) and measured with a plate reader at a

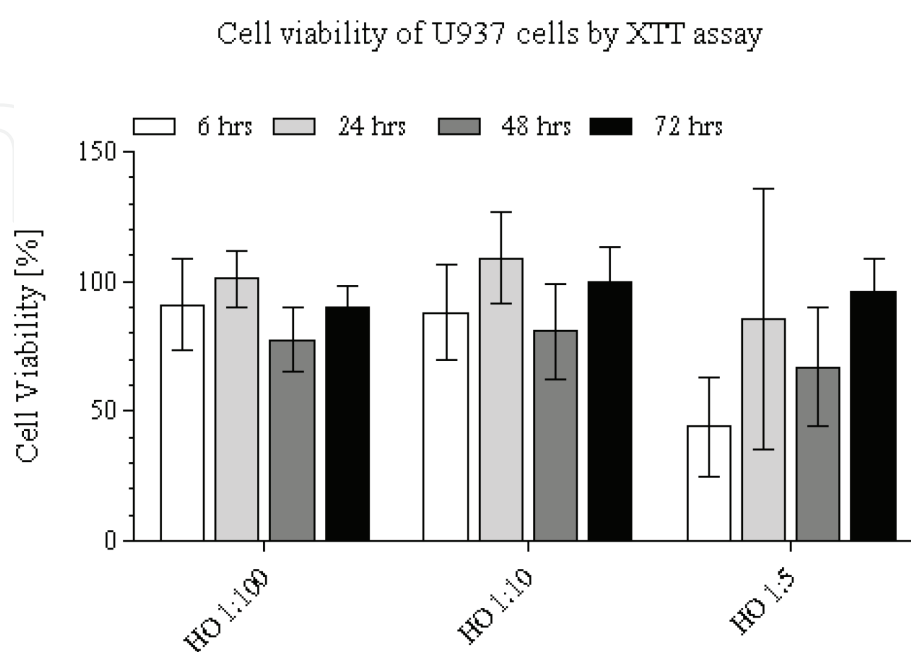


Figure 4.
U937 cell lines were treated with HO using XTT assays. Data are expressed as the percentage of viability % \pm SD.

wavelength of 490 nm. The results showed that at a concentration of 1:5, HO had a cytotoxic effect on the U937 cell line (**Figure 4**).

Apoptosis is a biological mechanism characterized as “programmed cell death.” Apoptosis is used by multicellular organisms to remove cells that are not needed by the body. Apoptosis shows distinctive morphological features, such as plasma membrane blebbing, cell shrinkage, chromatin condensation, and DNA fragmentation. Mitosis is the process of cell division that identically divides genomes into two daughter cells. Mitosis is generally followed by cytokinesis, which divides the cytoplasm and cell membrane. This process produces two identical daughter cells, which have almost the same distribution of organelles and cell components. Mitosis and cytokinesis make up the mitotic phase (M phase) in the cell cycle, where the initial cell is divided into two daughter cells that have the same genetic origin as the initial cell [21]. In testing HO’s effect on U-973 cells, the percentage of apoptotic and mitotic cells was quantified as a fraction. The nuclei of apoptotic cells were observed under a fluorescent microscope using a specific dye, in this case, Hoechst stain 33,342 (Sigma, Bornem, Belgium). The fraction of cells undergoing apoptosis were calculated (at least 300 cells).

Figure 5 shows the ratio of apoptotic and mitotic cells in the U-973 cell line after treatment with HO at a concentration of 1:10. After each treatment, Hoechst staining was performed for 15 minutes and apoptotic and mitotic cells were counted with the fluorescent microscope. The results showed that HO increased the number of apoptotic cells and reduced the number of mitotic cells after 24 hours of incubation (**Figure 5**). The Hoechst staining assay is one method to identify cell apoptosis through cell cycle analysis, mainly sub G1 phase cells.

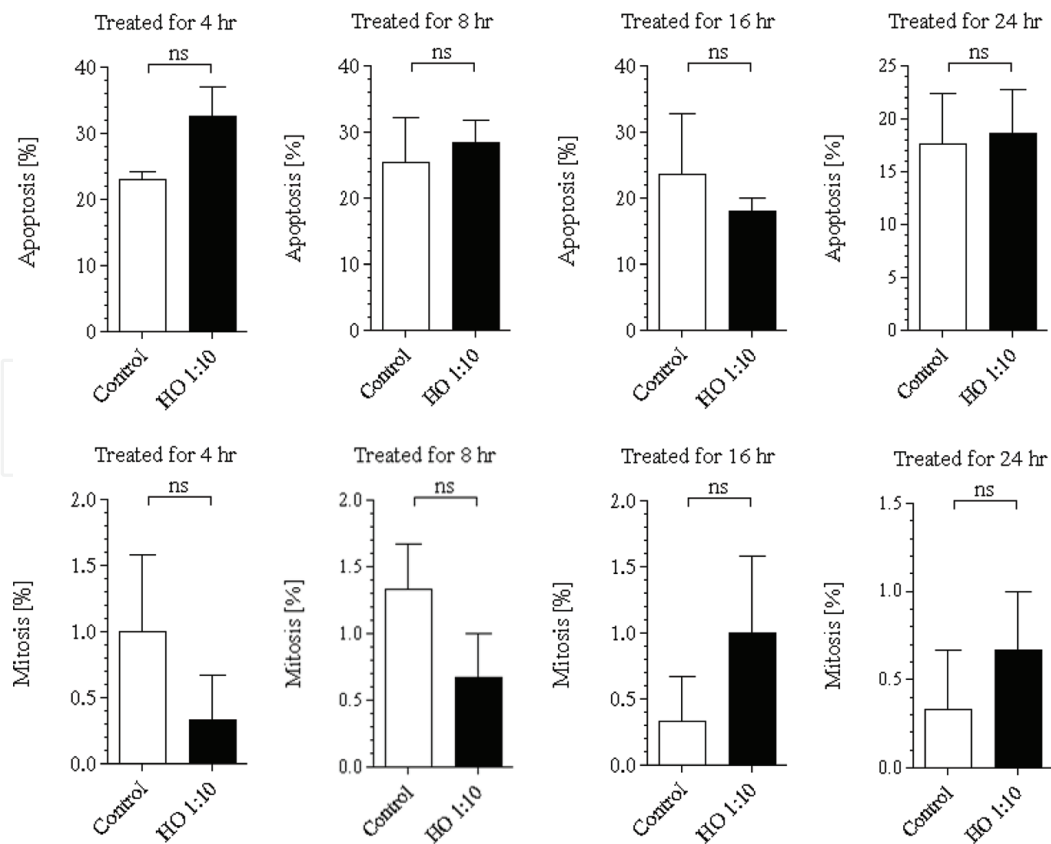


Figure 5. The U-937 cell line was treated for 4, 8, 16, and 24 hours with HO at a concentration of 1:10. After each treatment, Hoechst staining was performed for 15 minutes. Apoptotic and mitotic cells were counted with the fluorescent microscope, $n = 4$. Significances are expressed against the control. Data are presented as viability % \pm SD. Significances: ns = $p \geq 0.05$, * = $p < 0.05$, ** = $p < 0.01$ and *** = $p < 0.001$.

2.3 Study cytotoxicity on K562 cell lines

NF- κ B, or nuclear factor kappa-light-chain enhancer of activated B cells, is a protein complex that controls the DNA transcription process. NF- κ B is an important regulator in determining the fate of a cell, such as apoptosis (programmed cell death), control of cell proliferation, and tumorigenesis. The NF- κ B pathway is activated by cell exposure to lipopolysaccharide (LPS), inflammatory cytokines such as TNF (Tumor Necrosis Factor) or IL-1 (Interleukin-1), growth factors, lymphokines, oxidant-free radicals, inhaled particles, viral infection, or expression of certain viral or bacterial gene products, UV irradiation, B or T cell activation, and other physiological and non- physiological stimuli. The best NF- κ B activators are proinflammatory cytokines IL-1 and TNF, because they cause phosphorylation of κ B on the N-terminus domain side. TNF is an excellent activator for binding to TRADD (TNF-Associated Receptor DEATH Domain Protein) receptors and proteins. TRADD binds TRAF2 (TNF Receptor-Associated Factor-2), which recruits NIK (NF- κ B-Inducible Kinase) [22, 23]. In this test, we used TNF to activate NF- κ B. The activated NF- κ B would then cause the expression of genes that keep cells undergoing proliferation and protect cells from conditions that cause death through apoptosis.

The cell type used in this test was a K562 cell line. K562 cells are human chronic myelogenous leukemia lymphoblastoid cells. The K562 cell line was treated with HO at various concentrations for 2 hours, followed by the addition of 20 ng/ml TNF- α for 6 hours. The experiment was repeated five times. The inhibition of the NF- κ B pathway in the K562 cell line was analyzed based on the percentage of luciferase enzyme activity.

The results show no detectable effect of HO on the activation and inhibition of the K562 cell line through the NF- κ B pathway. According to Keophiphath,

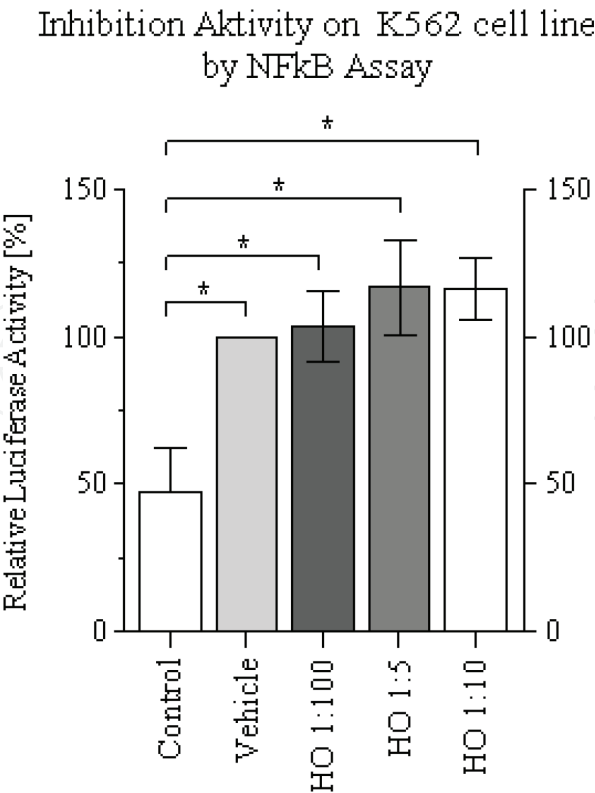


Figure 6.
The transfected K562 cells were treated with HO with concentrations of: 1:100, 1:10, and 1:5 for 2 hours, followed by a TNF- α -treatment for 6 hours, $n = 5$. The vehicle was normalized to a cell viability of 100%. Significances are expressed against the control. Data are presented as viability % \pm SD. Significances: $ns = p \geq 0.05$, $* = p < 0.05$, $** = p < 0.01$ and $*** = p < 0.001$.

a decrease in the synthesis of IL-6 is regulated by the NF- κ B pathway; thus, we suspect that this pathway may be involved, but our results were unable to detect any effect of HO on activation and inhibition of the K562 cell line (Figure 6).

2.4 Study cytotoxicity on Neuro 2a cell line

In this study, testing on the Neuro 2a cell line was carried out using the Live and Dead assay method. In this method, calcein-AM and propidium iodide are used to determine the viability of Neuro 2a cells, a neuroblastoma-derived cell line (Figure 7). There were three reasons for using the Neuro 2a cell line in this study. First, Neuro 2a cells contain GSH at concentrations five times higher than other cell lines, such as PC12 cell line (derived from a transplantable rat pheochromocytoma) [24]. Second, this cell line has expanded to be used as a model to determine the neuronal function of the system. Third, cells from the Neuro 2a cell line are very easy to differentiate using retinoic acid, so that it is structurally close to “real neurons”. In this study, the Neuro 2a cell line was used to determine the effect of HO with the presence or absence of Hydrogen peroxide (H_2O_2). Hydrogen peroxide is used to analyze the oxidative effects of stress on compounds [24].

HO activity shows that HO at a concentration of 1:1 with or without H_2O_2 had a moderate toxic effect on Neuro 2a cells, causing a percent viability of about 6%. The morphology of Neuro 2a cell structure after exposure to HO can be seen in Figure 8. The results show that Neuro 2a cells tend to experience apoptosis (bubbling) following HO exposure at a concentration of 1:5 after 24 hours incubation.

2.5 Activity on *Steinernema feltiae*

Steinernema feltiae (*S. feltiae*) is an entomopathogenic nematode used as a ‘phytoprotectant’ due to its consumption of fly eggs and larvae. Because *S. feltiae* is

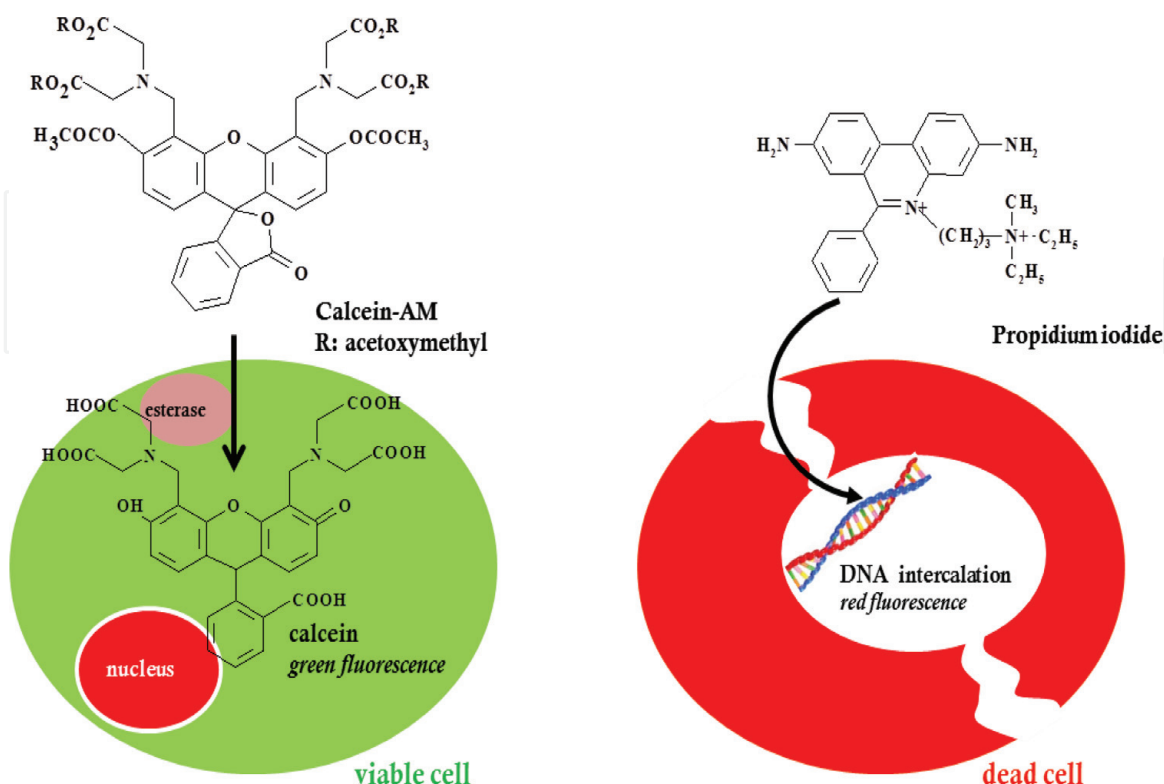


Figure 7.
Schematic representation of a live and dead assay using Calcein-AM and PI staining to determine viable and dead cells [25].

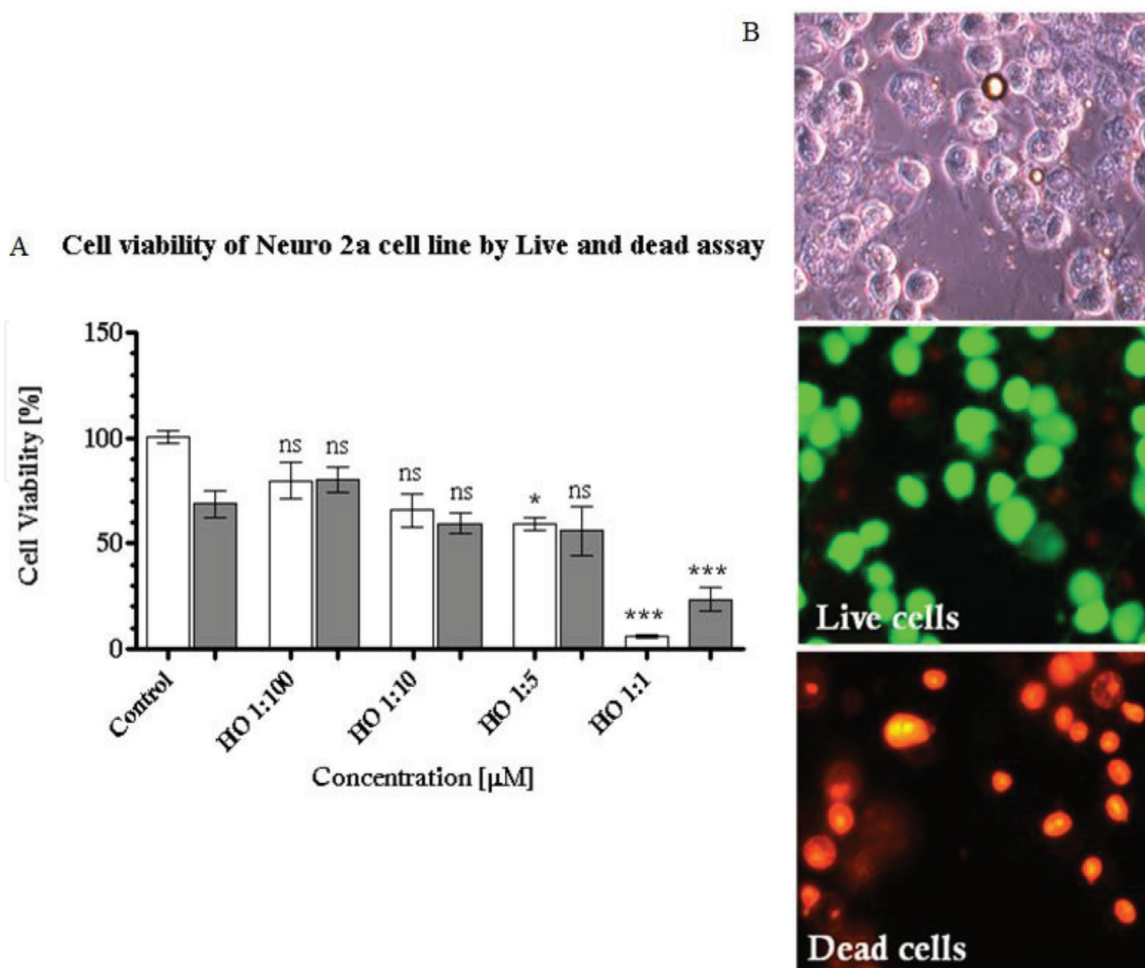


Figure 8.

A) Survival assay of HO on Neuro 2A cells for 24 h on 96-well plate. White bars, the HO was tested in the absence H₂O₂ and gray bars the HO was tested in the presence H₂O₂. The control containing water was normalized to 100% viability, significances are expressed to the control. Data presented as viability % \pm SD. Significances: ns $p \geq 0.05$, * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$. B) Survival assay of Neuro 2A cells upon treatment with HO. Neuronal cells were plated at a density of 10,000 cells/well in 96-well tissue culture plate. After 24 h, the culture medium containing the HO was replaced with 0.2 ml of dye solution (calcein-AM and PI) and cells were observed under the microscope (Magnification 40 \times). Cells treated with HO 1:5.

not a pest but a helpful organism for gardening, these nematodes can be used in normal laboratories without any special security protocols. This nematode is easy to culture, and simple toxicity screens using a normal light microscope are possible [26]. *S. feltiae* is a standard model for related environmental and agricultural vermin and pests. *S. feltiae* is normally used by gardeners as a biological defense against garden pests like flies, bugs, snails, and various other organisms. In the past few years, nematodes have grown in popularity as subjects in laboratory experiments. These small organisms are cheap, easy to handle, and they do not require any specific handling. The fact that they are useful animals in the environment with very short lifespans makes them attractive experimental subjects [27].

The effect of HO on *Steinernema feltiae* (*S. feltiae*) worms was assessed based on the nematode movement test after 24 hours of incubation. In this study, dimethyl sulfoxide (DMSO) was used as a solvent. In the activity test, this solvent was used as a control. The movement generated in the movement test was compared to the control. The HO concentrations used in this method ranged from 1:100 to 1:5. Due to the low solubility in water, the HO solution was sonicated or, alternatively, centrifuged, before it was applied to the nematodes. The two different preparations showed no toxic effect on *S. feltiae* (Figure 9). Figure 9 shows that *S. feltiae* showed the most activity when treated with HO at a concentration of 1:5, with a viability

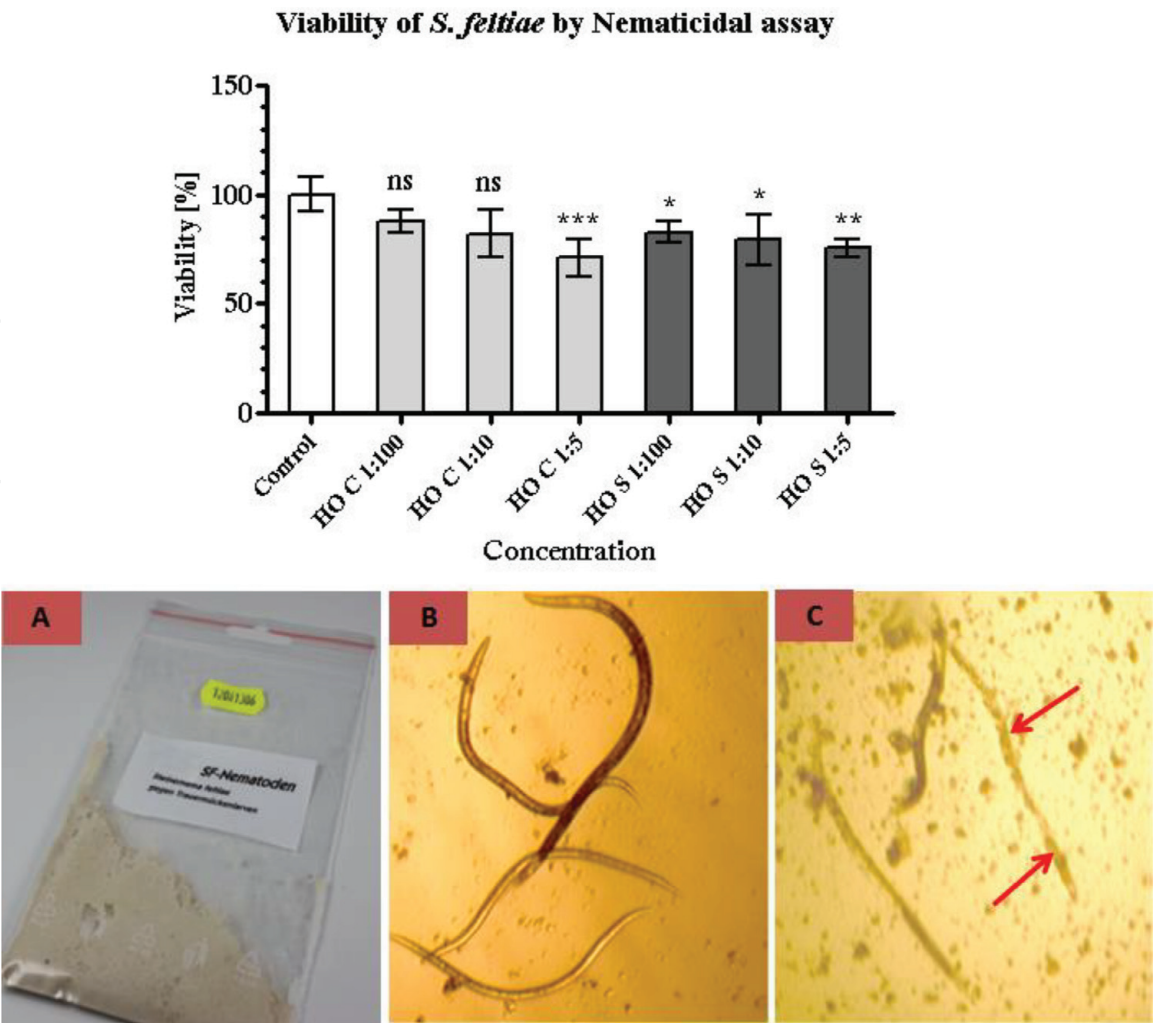


Figure 9.
Effects of HO treatment on *S. feltiae* after incubation for 24 hours. A) A powder cake of *S. feltiae*. B) Live sample of *S. feltiae*. C) Dead sample of *S. feltiae* (red arrow).

percentage of 65%. HO concentrations of 1:100 and 1:10 had no significant effect. This shows that increasing concentration of HO will increase the toxicity towards *S. feltiae*, which increases the organism’s motility.

3. Conclusion

We found that HO has a strong toxic effect on the A549 cell line, with an EC₅₀ value of 6.30 ppm in a CV staining assay. The Trypan Blue and CellTiter-Glo assay showed that HO also has a strong cytotoxic effect on HL-60 cells, especially at concentrations of 1:10 and 1:5. However, the results of the MTT assay showed that HO at a concentration of 1:5 had greater effectiveness than a concentration of 1:10, with a percentage of cell viability between 40 and 65%. The XTT results showed that HO at a concentration of 1:5 had a cytotoxic effect on the U937 cell line. The Hoechst staining assay showed that HO was able to increase (induce) apoptotic cells and reduce mitotic cells after 24 hours of incubation. The results also showed no detectable effect of HO on the activation and inhibition of the K562 cell line through the NF-κB pathway. Meanwhile, after HO exposure at a concentration of 1:5 and 24 hours of incubation, the Neuro 2a cell line tends to activate the apoptotic pathway. The nematicidal test showed that only at a concentration of 1:5 did HO showed significant activity, with a percentage of *S. feltiae* viability of 70%.

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Conflict of interest

None declared.

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