

# Study Cytotoxicity of Haarlem Oil by Crystal Violet Staining Assay

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## Abstract

Haarlem Oil (HO) is a semisynthetic product made from terpene oil and elemental sulfur by mixing them under high temperature. HO contains organosulfur compounds and these compounds have been known as possessing activities as antioxidant agents. The aim of this study was to determine the cytotoxicity of HO on eight cell line tissue cultures using crystal violet staining assay.

In this assay, the results were reported in percentages of viable cells being established by incubation of the cells with HO and 0.1% solution of detergent nonyl phenoxy polyethoxyl ethanol (NP40). It was then compared with the untreated cells having 100% viability. The result found that HO was the most active against A549 (adeno carcinomic human alveolar basal epithelial) cell line with  $EC_{50}$  values of 6.30 ppm.

**Keywords:** Haarlem oil, organosulfur compounds, nonyl phenoxy polyethoxy lethanol (NP-40), originPro 7.5 software, crystal violet staining assay.

## Introduction

In the western world, Haarlem oil (HO), the first medication manufactured on an industrialized scale in the 17<sup>th</sup> century, was suggested for diagnosing diabetes and skin whitening<sup>1</sup>. HO was first introduced in Holland at 16<sup>th</sup> century and was known as "Dutch Drops". Thomas Monsieur, the France scientist, was the first scientist who discovered the use and pharmacologically effected HO as a supplement (to improve stamina). HO was also used to maintain fitness.

HO was first traded in France as supplement in 1924 and between the 1980's to the 1990s, HO had attracted scientists because of its contents sulphur. It was used as medical drug for health. The study also found that HO was source of aminosugars and iminosugars. These compounds contain a basic nitrogen atom in endocyclic structure and this atom is responsible for biological activity of HO. The literature also showed that HO had been further studied due to the presence of various sulfuric compounds and its potential to be used in medicinal treatment. The study also proved that HO was containing polyunsaturated essential oils and sulfur. This oil had a powerful antioxidant superoxide dismutase (SOD)<sup>2-4</sup>. The *in vitro* cytotoxicity assay with cultured cell is widely used in the testing of compounds or chemicals being rapid, economical and do not require the use of animals<sup>5</sup>.

Crystal violet (CV) is a triphenylmethane dye (4-[(4-dimethyl amino phenyl)-phenyl-methyl]-N,Nimethyl-aniline) and is well known dye that is used in assay that develops a color in response to the viability of cells. CV stain is primarily the membrane of viable cell and this dye quantifies the cell number in monolayer cultures as it functions for the absorbance of the dye taken up by the cells and bonds to the cells. This assay is a simple assay and it is quick for screening cell viability and useful for obtaining the quantitative information about the relative density of the cell. It is also reliable to measure the cytotoxicity of cells<sup>6,7</sup>.

There are two advantages of this assay over other cytotoxicity after the staining observed for morphological changes and it can be stored for a long-term for reference purposes<sup>8</sup>. In this study, we used a CV assay method with 96-well microtitre plates to study the cytotoxicity effects of HO against eight cancer cell lines such as 3T3, CT26, HT29, A549, HUVEC, MCF7, HepG2 and OVCAR cell lines.

## Material and Methods

**Materials:** HO was a gift from Dr. J. Lefevre, ZI La Pelouse-55 190, Void Vacon, France. The CV powder and methanol were purchased from Sigma-Aldrich 96-wells plate was purchased from Greiner bio-one. All used plastic or glass-materials were sterilized prior to use.

**Cell line and culture condition:** All cell lines used in this study were kindly provided by University of Metz, France. Cell cultures assay were performed under the instructions of Dr. Vincent Jamier. A549, CT26, HUVEC and MCF-7 cell lines were grown in RPMI 1640 medium containing fetal bovine serum, FBS (10%) and antibiotics penicillin (100 U mL<sup>-1</sup>) and streptomycin (100 µg mL<sup>-1</sup>). HepG2 cells line were cultured in Dulbecco's modified eagle medium (DMEM) media with 10% FBS and antibiotics penicillin (100 U mL<sup>-1</sup>) and streptomycin (100 µg mL<sup>-1</sup>). NIH 3T3 cell line was cultured in Dulbecco's modified eagle medium (DMEM) media, supplemented, 10% fetal calf serum (FCS), penicillin (20 Unit mL<sup>-1</sup>), streptomycin (20 µg mL<sup>-1</sup>).

OVCAR cell line was grown in Dulbecco's modified Eagle's medium (DMEM) containing 3.7 g/l sodium bicarbonate, 4.5 g/L L-glucose and supplemented with L-glutamine (2 mM), 10% fetal calf serum (FCS), penicillin (100 IU/mL), streptomycin (100 µg/mL) and amphotericin B (0.25 µg/ml). While, HT-29 cell line was cultured in RPMI 1640 medium and minimum essential medium (MEM) they were supplemented with 20% FBS, 2% of penicillin/streptomycin

and 1% of amphotericin B. The cells were cultivated at 37°C in a 5% CO<sub>2</sub> atmosphere.

### Crystal Violet Staining Assay

**Preparation of crystal violet staining solution:** The CV staining solution 0.4% was prepared by dissolving 0.4 grams of crystal violet powder in 100 mL of distilled water, then methanol was added. No sterilization procedure is required, store this solution in dark at room temperature. This solution can be used within two months.

**Crystal violet assay:** The assay was performed according to the procedure that was described by Itagaki et al<sup>7</sup> with a few modifications. 3T3, HUVEC, CT26, OVCAR (10<sup>4</sup> cells/well), MCF7, A549, HepG2 (2×10<sup>4</sup> cells/well) and HT29 (3×10<sup>4</sup> cells/well) cell lines were seeded into 96-well plates in 100 µl relevant culture medium with varying concentrations of HO (with a range of concentration from 50 ppm to 3.125 ppm). The cells were then incubated for 72 hours at 37°C under 5% CO<sub>2</sub> in air. The treated (cells were treated with Haarlem oil) and untreated cells (control) were then compared with 0.1% solution of detergent nonyl phenoxy polyethoxyl ethanol (NP40) for 10 minutes and it was stained with 0.4% crystal violet for 30 minutes.

The plate was then washed with water several times, dried and add 200 µL of methanol to each well. The plate was then incubated at room temperature for 20 minutes. Finally, the optical density of each well at 590 nm (OD<sub>590</sub>) was measured with a plate reader<sup>7</sup>.

**Determination of cell viability:** The cytotoxicity of the tested compound (HO and NP40) was expressed as percentages of viable cells and calculated by this formula:

$$\text{Cell viability (\%)} = \frac{(a-b)}{(c-b)} \times 100$$

where *a* is OD<sub>590</sub> values of treated cells with the tested compounds (NP40 and Haarlem oil), *b* is OD<sub>590</sub> values from blank wells, while *c* is OD<sub>590</sub> values of untreated cells which were derived from the controlled wells<sup>9, 11</sup>.

**The EC<sub>50</sub> values:** The EC<sub>50</sub> values were calculated with sigmoidal curve by using the OriginPro 7.5 software.

### Results and Discussion

CV assay is a method that is useful for screening cell viability and it can also be used to quantify the cell number in culture as a function of absorbance of the dye taken up by the cell<sup>8</sup>. This dye binds to protein and DNA from the cells. Cells that undergo cell death lose their adherence and are subsequently lost from the population of cells, reducing the amount of dye staining in a culture.

Recently, the HO is sold as an antioxidant, HO has been further studied due to the presence of various sulfuric compounds and it is potentially used for medicinal

treatment. The mechanism activity of HO is not fully understood. The knowledge about the biological activity of HO, especially with regard to the redox-modulatory behaviour is still very limited<sup>2,3</sup>.

In this study, we evaluated the cytotoxicity activity of HO on cell lines. The number of the cell lines that was tested for this assay, consisted of eight cancer cell lines. In this study, the cytotoxicity activity of the HO was expressed as percentages of cell viability by measuring the optical density (OD) of the cell at 590 nm (OD<sub>590</sub>). OD, indicating the absorbance of a sample was measured at 590 nm. OD is a common method for estimating the concentration, growth condition and reproducibility of the cells in a liquid.

To determine the cytotoxicity of the HO, we used several variety concentrations of HO. To determine cytotoxicity of HO on the cells, HO was prepared in different concentration (ranging from 13.125 to 50 ppm) dissolved in culture medium and sterilized by membrane filtration (size of membrane was 0.45 µm). In order to test a HO, in this study, we also used a cytotoxic agent of 0.1% solution of detergent nonyl phenoxy polyethoxyl ethanol (NP40). This detergent has a powerful action to break and open all membrane within a cell including the nuclear membrane<sup>10</sup>. Figure 1 shows the effects of HO on 3T3, CT26, HT29, A549, HUVEC, MCF7, HepG2 and OVCAR cell lines at different concentrations.

The results showed that HO had the highest cytotoxicity effect compared to organic solvent such as dimethyl sulfoxide (the data is not shown) but had almost similar effect with cytotoxic agent of detergent nonyl phenoxy polyethoxyl ethanol (NP40). Towards OVCAR cell line, HO had the strongest toxic effect after 72 h was exposed, while on HT29 cell line it had the lowest effect.

The other cell lines such as 3T3, CT26, A549, HUVEC, MCF7 and HepG2 were also tested. The results showed that these cell lines have similar effect as determined by CV staining assay. Khairan (the results not yet published) mentioned that HO shows moderate activity against the Neuro 2 A cell line (a murine neuroblastoma cell line). The morphological structures of the Neuro 2A cells after being treated with HO was changed in morphology and led to bubbling cells when the cells were exposed to HO after 24 hours.

Half maximal effective concentration (EC<sub>50</sub>) refers to the concentration of a drug, antibody or toxicant that induces a response halfway between the baseline and the maximum after a specified exposure time. The EC<sub>50</sub> values of HO on the cells obtained by CV method of cytotoxicity testing by using the OriginPro 7.5 software are listed in table 1.

As shown in table 1, we found that HO was to be the most active against A549 (adeno carcinomic human alveolar basal epithelial) cell line with EC<sub>50</sub> values of 6.30 ppm. Additionally, HO also displayed a strong cytotoxicity

against CT26 and HepG2 cell lines with  $EC_{50}$  values of 6.49 and 6.50 ppm. The  $EC_{50}$  values were fitted with based data concentration and cell viability at  $x$  at  $y_{50}$  using sigmoidal Boltzmann curves-fitting Origin 7.5 software. The sigmoidal curves-fitting was presented in figure 2.

Meanwhile, the HUVEC and 3T3 cell line showed that the  $EC_{50}$  values to these cells were 15.19 and 15.20 ppm respectively. The  $EC_{50}$  to HT29 (Human colon carcinoma) cell line revealed at more than 50 ppm.

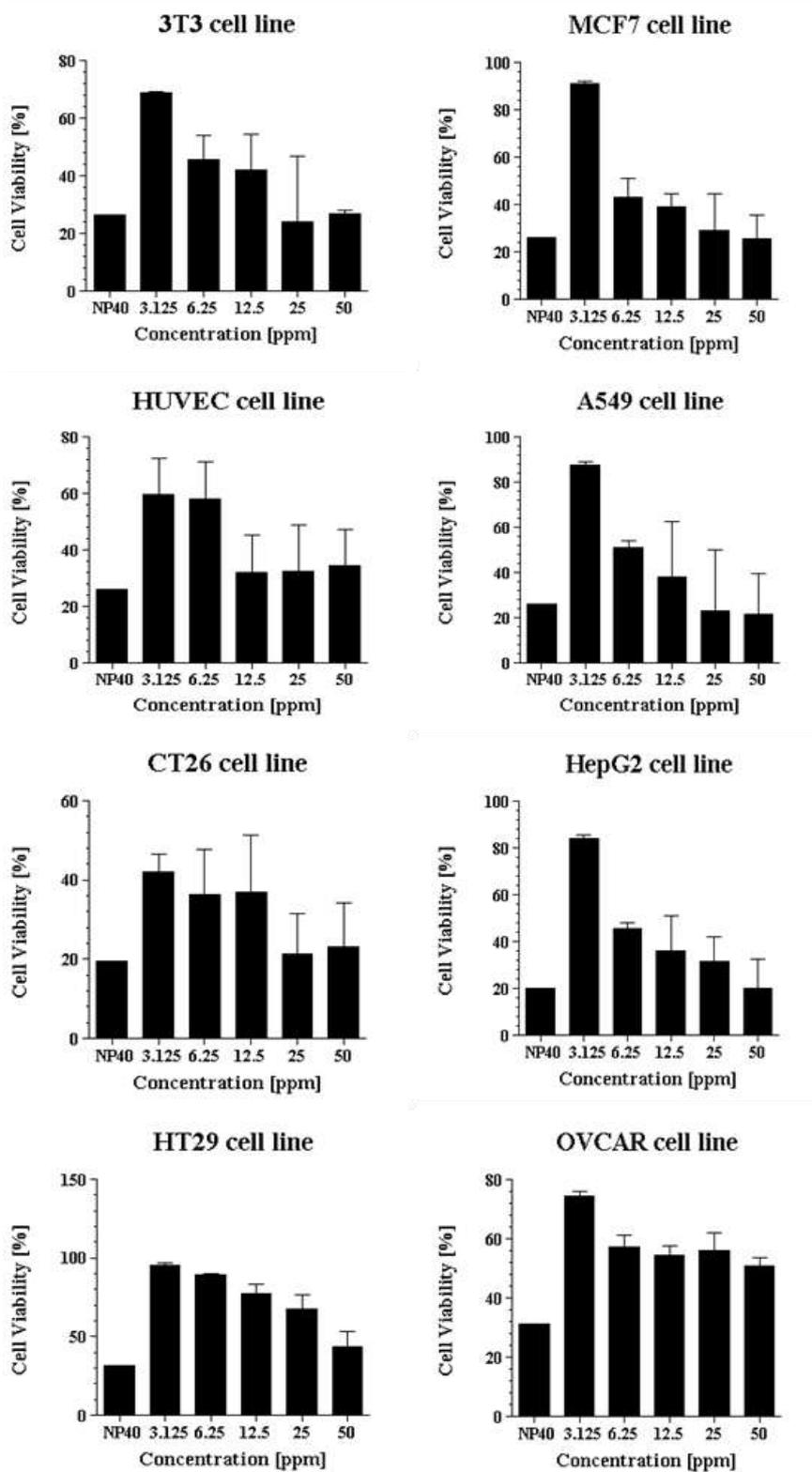


Figure 1: The percentage of cell viabilities 3T3, CT26, HT29, A549, HUVEC, MCF7, HepG2 and OVCAR cell lines after been exposed by HO at 72 h by CV staining assay.

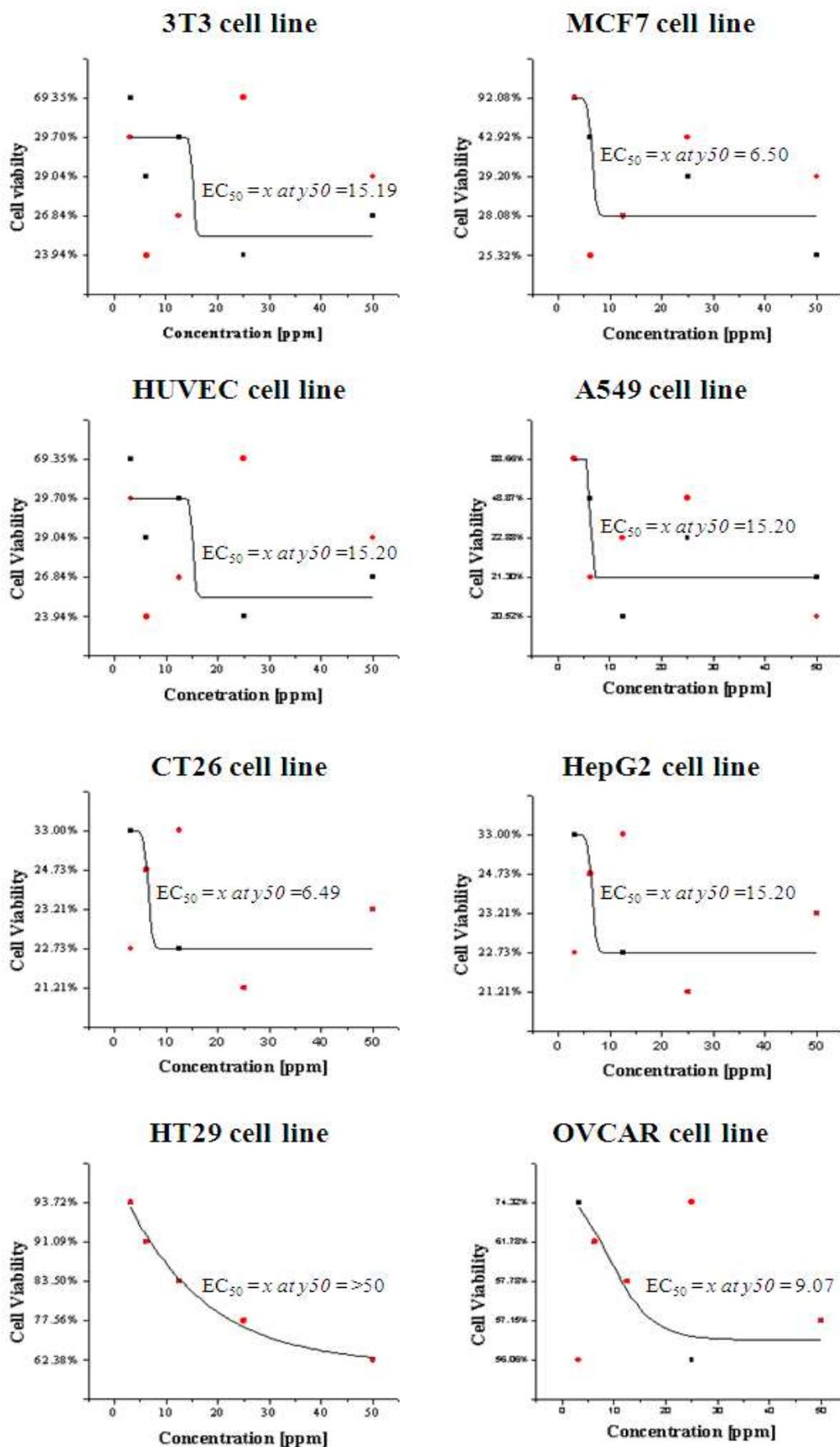


Figure 2: The sigmoidal curve  $EC_{50}$  of HO on several cell lines. The  $EC_{50}$  values were fitted based sigmoidal Boltzmann curves-fitting using Origin 7.5 software.

**Table 1**  
**The characteristics and EC<sub>50</sub> values of cell lines were tested on HO by using CV staining assay.**

Cell line	Characteristics	EC <sub>50</sub> 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
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

### Conclusion

In conclusion, we found that HO had a toxicity effect against 3T3, CT26, HT29, A549, HUVEC, MCF7, HepG2 and OVCAR cell lines. The results also indicated that HO was the most active against A549 (adeno carcinomic human alveolar basal epithelial) cell line with EC<sub>50</sub> values of 6.30 ppm while HT29 (Human colon carcinoma) cell line revealed at more than 50 ppm.

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