THE EFFECTS OF CITRAL ON CASPASE-3 ACTIVATION IN M624 AND HaCaT CELLS

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Abstract

Citral is a terpene compound found in the essential oils of lemon grass and other herbs. This study investigated the protective and cytotoxic effects of citral on HaCaT skin cells and M624 melanoma cells, respectively. Cells were cultured and exposed to varying concentrations of citral for three hours. Cell viability following treatment was analyzed using a clonogenic assay procedure. Results showed that citral has significant toxicity to M624 melanoma cells and little to no toxicity to HaCaT cells. The induction of apoptosis was measured using a caspase-3 colorimetric assay kit. This analysis showed that more caspase-3 protein was activated as the concentration of citral increased. Overall, the results of this study support the use of citral as an anticancer agent.
Introduction

Background Information

In recent years, the use of natural products as innovative anticancer agents has become increasingly popular. Citral is a terpene compound found in the essential oils of herbal plants such as lemon grass (*Cymbopogon citrates*), melissa (*Melissa officinalis*), verbena (*Verbena officinalis*), and other lemon scented plants and herbs (Cahouki et al., 2009). It is ordinarily used as a food additive and as a fragrance material in cosmetics. Citral naturally occurs as a mixture of two isomeric acyclic monoterpenic aldehydes: geranial (*trans*-citral) and neral (*cis*-citral) (Dudai et al., 2005). Its structure is shown below. The main moiety of interest to researchers is the compound’s $\alpha,\beta$-unsaturated aldehyde group (Dudai et al., 2005).

![Citral structure](image)

Figure 1: Citral structure

This study investigates the protective and cytotoxic effects of citral on HaCaT skin cells and M624 melanoma cells, respectively. There have been several previous studies that have analyzed the use of citral as a cytotoxic agent and inducer of apoptosis in various cancer cell lines. All of these studies have shown that citral has promising potential in cancer treatment; however, information about the compound’s effects on a skin cancer cell line was absent.

More information about biological process like apoptosis and necrosis are needed before citral’s effects on a skin cancer cell line are explored either clinically or in the laboratory. In the
majority of applications, the primary role of natural product cancer treatments is to kill unwanted cancer cells. Cell killing mechanisms are generally classified as occurring through either apoptosis or necrosis. Apoptosis is a tightly regulated process of cell suicide that is controlled by both intracellular and extracellular signals (Oleinick et al., 2001). Apoptosis may be thought of as a healthy, controlled self-destruct mechanism for cells when they are damaged beyond repair, or have run out of resources to continue living. Overall, the process limits leakage of the intracellular material to the immediate environment, and thereby prevents neighbor tissue inflammation (Oleinick et al., 2001). In contrast, necrosis results in high levels of cell damage, in which plasma membrane integrity is lost, cells lyse, and tissue inflammation is triggered (Oleinick et al., 2001). In short, the process resembles cellular level collateral damage.

There are two main apoptotic pathways of interest to researchers: the extrinsic, or death receptor pathway, and the intrinsic, or mitochondrial pathway (Elmore, 2007). In short, the intrinsic pathway is generally initialized when a cell no longer has the proper resources to survive. This includes, but is not limited to the absence of certain growth factors, hormones and cytokines, which ordinarily signal the cell to suppress cellular death programs. If these factors aren’t present, apoptosis may be triggered. (Elmore, 2007). Other stimuli that may cause intrinsic activation of apoptosis include, but are not limited to, radiation, toxins, hypoxia, hyperthermia, viral infections, and free radicals (Elmore, 2007). All of these stimuli cause changes in the cell’s inner mitochondrial membrane, and either make the membrane more permeable, or result in the loss or reduction of the mitochondrial transmembrane potential. This ultimately results in the release of normally sequestered pro-apoptotic proteins from the intermembrane space of the mitochondria into the cytosol (Elmore, 2007). The main protein of interest that is released is known as cytochrome c. When cytochrome c is released, it activates a protein known as
procaspase-9, a member of the caspase family of enzymes (Elmore, 2007).

Caspases are a class of protease enzymes. Proteases are enzymes that cleave various cellular targets. Ordinarily, caspases are expressed in an inactive proenzyme form, and are activated when they themselves are cleaved (Elmore, 2007). Once they are activated, they cleave/activate other pro-caspases. This process is known as the caspase cascade (Elmore, 2007). This proteolytic cascade, in which one caspase activates others, amplifies the apoptotic signal and leads to rapid cell death. Once caspases are initially activated, the cell is seemingly committed to death (Elmore, 2007). To date, ten major caspases have been identified and broadly categorized as initiator caspases (caspase-2,-8,-9,-10), effector or executioner caspases (caspase-3,-6,-7), and inflammatory caspases (caspase-1,-4,-5) (Elmore, 2007).

During activation of the intrinsic apoptotic pathway, the cytochrome c released from the mitochondria activates procaspase-9. The resulting activated caspase-9 then activates procaspase-3. Caspase-3 is an executioner caspase which can be activated by caspases 8, 9 and 10 (Elmore, 2007). Caspase-3 is considered to be the most important of the executioner caspases because it specifically activates the enzymes responsible for the remaining processes essential for the completion of apoptosis, including the degradation of DNA, cytoskeletal and nuclear proteins, and the eventual formation of apoptotic bodies that will be phagocytosed by phagocytic cells (Elmore, 2007).

The extrinsic apoptotic pathway involves the activation of transmembrane receptor proteins within the cell membrane known as “death receptors” (Elmore, 2007). Each death receptor has a specific ligand that binds to and activates the receptor. When a ligand binds, cytoplasmic adapter proteins bind to the portion of the receptor on the cytoplasmic side of the membrane. A common example of this is the Fas ligand binding to the Fas receptor protein
(Elmore, 2007). This results in the binding of the adapter protein FADD, which then associates with procaspase-8 via a process known as protein complexation (Elmore, 2007). This process forms what is known as a death-inducing signaling complex (DISC) of proteins, and ultimately results in the autocatalytic activation of procaspase-8 (Elmore, 2007). The resulting caspase-8 then activates caspase-3 in the aforementioned caspase cascade, followed by the remaining apoptotic events.

There are many varieties of cancer that occur when apoptotic self-destruct mechanisms like those discussed are inactivated or inappropriately prevented. When unhealthy, damaged, and/or mutated cells cannot initiate apoptosis, they continue to grow and divide. This may eventually result in cancer. In skin cancer for example, ultraviolet radiation B from the sun penetrates the skin, and may result in multiple breaks and mutations in skin cell DNA (Jhappan et al., 2003). If apoptosis isn’t initiated, skin cancers such as melanoma and carcinoma may result. Skin cancer is the most common malignancy in the United States (Rager et al., 2005). Melanoma is the deadliest variety of skin cancer. Roughly three fourths of all skin cancer deaths are due to melanoma (Hall et al., 1999). Melanoma itself is the sixth leading cause of cancer death in the United States (Rager et al., 2005). There are four main types of melanoma: nodular melanoma, superficial spreading melanoma, lentigo maligna melanoma, and acral lentiginous melanoma (Weyers et al., 1999). Superficial spreading melanoma accounts for roughly 70 percent of all melanoma cases. The disease mainly affects a younger population, as 62 percent of cases are diagnosed before patients reach 65 years of age. The median age at death is 67 years (Rager et al., 2005). Currently, melanoma is ordinarily treated by means of excision surgery or adjuvant therapy. Unfortunately, reliable melanoma prevention strategies have yet to be developed (Rager et al., 2005). In theory, treating a melanoma patient with citral or essential oils
containing citral may be more advantageous than using traditional measures like chemotherapy or surgery. These sorts of methods are sometimes too invasive, or carry too many risks. It may even be more feasible to use these citral solutions in a topical melanoma treatment, perhaps via a cream solution that targets melanoma cells specifically.

There have been several previous studies conducted that support the use of citral as an inducer of apoptosis. Citral’s apoptotic and antiproliferative effects on MCF-7 human breast cancer cells were tested in a 2009 study by Chaouki et al. In the study, cells were grown in petri plates (in vitro), and exposed to varying amounts of citral for varying amounts of time (24, 48, and 72 hours). Following treatment, a procedure known as an MTT cell proliferation assay was performed. During an MTT assay, treated cells are stained with a solution of a yellow salt known as 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (or MTT), and incubated for approximately four additional hours (Mosmann, 1983). During this period, a ring structure in the compound is cleaved by enzymes in the mitochondria of the cells that survive, converting the MTT to (E,Z)-5-(4,5-dimethylthiazol-2-yl)-1,3-diphenylformazan, or formazan (Mosmann, 1983). This tiny formazan product is purple, crystalline, and insoluble. Following the incubation period, the crystals are dissolved, usually with an acidified alcohol solution. Once this is done, the plates can be read using a plate reader instrument. This machine illuminates the samples using a specific wavelength of light. For an MTT assay, the wavelength used is 550 nm: the wavelength that the formazan product absorbs (Mosmann, 1983). A light detector located on the other side of the wells in the plate measures how much of the initial light is transmitted through the sample. Any light that doesn’t pass through is said to be absorbed by the formazan solution. The absorbance results of the samples treated with the drug of interest can then be compared to the control to express percent toxicity. In the study by Chaouki et al., these absorbance values
were then used to calculate IC\textsubscript{50} concentrations (the concentrations required to eliminate 50% of the cells). Results showed that as the exposure period increased, citral’s IC\textsubscript{50} decreased. Citral’s IC\textsubscript{50} was reported to be 0.266, 0.180, and 0.147 mM at 24, 48 and 72 hours, respectively (Cahouki et al., 2009). Another procedure known as an ethidium bromide and acridine orange double stain was then used to determine whether or not the citral was exerting its effect using an apoptotic pathway or a necrotic pathway. During this procedure, cells are removed from their culture plates and put on a microscope slide. They are stained with a solution of ethidium bromide and acridine orange compounds and viewed under a microscope (Liu et al., 2015). These compounds bind to the DNA within the nucleus. The acridine orange permeates all cells and turns their nuclei green. Ethidium bromide only permeates cells after their cytoplasmic membrane integrity has been lost (like it is during apoptosis related degradation), and stains the nucleus red (Ribble, 2005). Ethidium bromide also dominates over acridine orange (Ribble, 2005). Thus, live cells have a normal green nucleus; early apoptotic cells have bright green nucleus with condensed or fragmented chromatin; late apoptotic cells display condensed and fragmented orange chromatin, and cells that have died from necrosis have a structurally normal orange nucleus (Ribble, 2005). Once the staining process is complete, photographs of the cells are taken and the amount of cells experiencing apoptosis and/or necrosis are counted. In this case, the results of the ethidium bromide and acridine orange double stain indicated that citral exerts its antiproliferative effect through an apoptotic pathway at low doses and through a necrotic pathway at higher concentrations (Cahouki et al., 2009).

The effects of both citral and lemongrass were also tested on ME-180 and HeLa cervical cancer cell lines in a 2013 study by Ghosh. Once again, cells were cultured, and exposed to various concentrations of each solution. After incubation for 24 hours, cell viability was
evaluated and IC$_{50}$ values were calculated using an MTT assay (Ghosh, 2013). In both cell types, the percent cytotoxicity increased with the dose. IC$_{50}$ values for lemongrass oil and citral were found to be 200 $\mu$g/ml and 500 $\mu$g/ml for HeLa cell, and 200 $\mu$g/ml and 300 $\mu$g/ml for ME-180 cells (Ghosh, 2013). As in the study by Chaouki et al., an ethidium bromide and acridine orange double stain was used to visualize condensed chromatin in dead apoptotic cells. HeLa cells showed 95% apoptotic cells in lemongrass oil emulsion and 80% in citral emulsion treatment groups. ME-180 cells showed 98% apoptotic cells in lemongrass oil emulsion and 90% in citral emulsion treatment groups (Ghosh, 2013). Overall, these results showed that both lemongrass oil and citral could be used to induce apoptosis in cervical cancer cells.

Another study by Xia et al. investigated what specific proteins citral activates in order to induce apoptosis in NB4 leukemia cells. According to the study, citral treatment caused apoptosis in the NB4 cells by activating caspase-3 proteins. This was determined using a technique known as flow cytometry. In short, the researchers treated their cells, lysed them to obtain the cytoplasmic extract, and probed their lysate samples with anti-activated caspase-3 antibodies that fluoresced when hit with a laser within the flow cytometer instrument (Xia et al., 2013). This instrument then allowed the researchers to quantify the amount of activated caspase-3 as the difference in fluorescence relative to a control. As previously discussed, caspases are apoptotic proteins that sequentially activate each other through a cleaving mechanism that continuously amplifies the amount of activated enzymes (Xia et al., 2013). The cascade itself can be activated through either an extrinsic or intrinsic method. In both the intrinsic and extrinsic scenarios, caspase-3 is activated by initiator caspases (Xia et al., 2013). Thus, caspase-3 is thought to be a good indicator of apoptosis because it can be activated by either mechanism (Xia et al., 2013).
A study by Dudai et al. also centered its examination on the protein caspase-3. In this case, the researchers investigated the effects of citral, various citral derivative molecules, and lemongrass oil on multiple leukemic cell lines, including BS 24-1 and RL12 cells from mice and Jurkat, U937 and HL60 cells from humans. The researchers chose to analyze the effects of compounds with similar structures to citral in order to figure out what specific portion of the citral molecule’s structure is crucial for initiating apoptosis. The effects of the various compounds on the leukemic cells were compared to the effects on control spleen cells and thymocytes (Dudai et al., 2005). First, the researchers performed an XTT assay to test the effects of their compounds on cell viability. This assay is analogous to the aforementioned MTT assay, the only difference being the initial salt that is used during staining. Notably, the lemongrass essential oil killed 50-81% of the cells after a four hour exposure, while citral killed 58-90% of cells (Dudai et al., 2005). The calculated IC\textsubscript{50}, or concentration toxic to 50% of the leukemic cells was 47 µg/mL, or 0.309 mM. Normal thymocytes were resistant to the effect of lemongrass, while citral caused 29% death. However, citral had no cytotoxic effect in normal spleen cells (Dudai et al., 2005). Caspase-3 activity was then studied using DEVD-pNA, a colorimetric caspase-3 substrate that contains a short peptide sequence ordinarily cleaved by the enzyme (Dudai et al., 2005). This substrate allows one to quantify caspase-3 activity in a cell sample spectrophotometrically over a set amount of time through the detection of free pNA (at 400 nm) after cleavage (Dudai et al., 2005). In this particular case, the picomoles of pNA cleaved per minute, per microgram of protein used in the assay was the unit of measure for caspase-3 activity. Results showed that caspase-3 was activated in all the aforementioned leukemic cell lines, and not activated in the thymocytes or spleen cells (Dudai et al, 2005). A subsequent experiment then used Ac-DEVD-CHO, a caspase-3 inhibitor in conjunction with the DEVD-
pNA. The Ac-DEVD-CHO completely inhibited any previously observed caspase-3 activity, indicating that caspase-3 was indeed the active enzyme in the treated cells (Dudai et al., 2005). Additionally, these researchers also observed that treatment of leukemic cells with citral derivatives such as citronellal or geranic acid did not lead to the activation of procaspase-3, indicating that the presence of citral’s α,β-unsaturated aldehyde group is crucial for stimulation (Dudai et al., 2005). Based on these results, it seems that citral, and its α,β-unsaturated aldehyde group may serve as core structures for the design of new pro-apoptotic drugs.

Methods Overview and Rationale

The aforementioned studies all support the use of citral as an anticancer agent. However, they all test the compound’s potential apoptotic/antiproliferative capabilities on cancers other than melanoma. Luckily, they do suggest a model to for such a study’s design. That model consists of first growing the cell line of interest, exposing those cells to the compound in various concentrations, and observing the effects through a cell viability assay. Ordinarily, any toxic effects observed are compared to a control cell line, and the magnitude of toxicity is expressed as a percentage of the control. Afterwards, the effects of the compound at the molecular level is studied, usually through protein or DNA analysis. This portion of the analysis is especially important if the cell viability assay shows that the compound of interest is indeed toxic to the cell line. Studying the molecular effects of the compound in this way thus allows one to determine whether the cells that died did so through an apoptotic pathway or a necrotic pathway, depending on the events that occurred.

In order to properly examine citral’s effect on melanoma cells, a similar model was used. The main procedure that was used to analyze citral’s potential toxic effects on both cell lines was the clonogenic assay. A clonogenic assay is similar to the aforementioned MTT assay, and
allows one to find the optimum concentration and time of exposure required for the compound of interest to produce an inhibitory effect on the growth of cell lines. The assay relies on a dye known as crystal violet. In short, the dye is used for staining any remaining surviving cells after treatment with a compound of interest. Following the staining, the plate used to grow the cells is then read with a plate reader. A plate reader is used in this procedure just like it is in the MTT assay, except the wavelength used is 590 nm: the wavelength absorbed by crystal violet. The absorbance values obtained are used to calculate percent toxicity (or lack thereof) as a percent of the control samples.

Both cancerous and non-cancerous cell lines were used to test citral’s potential apoptotic effects. M624 melanoma cells functioned as the cancer cell line of interest. These cells were originally obtained from an African American male in the early 1990s and have been maintained in culture by researchers at Ohio University and Marietta College ever since. HaCaT skin cells functioned as the control cell line. HaCaTs are non-cancerous keratinocytes that were obtained from a Caucasian male in 1988 and have been immortalized ever since. They are commonly used as a control cell line in studies involving the skin because they exhibit normal differentiation and are very resistant to transformation.

In this particular case, a protective study was performed on the M624 melanoma cells because if the cells underwent apoptosis and died, it indicated that citral was eliminating the undesirable melanoma cells. In contrast, a toxicity study was performed on the HaCaT cells in order to ensure that citral was not toxic to non-cancerous skin cells. Obviously, killing cancer cells is desirable, but not at the cost of killing non-cancerous cells in the surrounding area as well.

Following cell viability analysis, a caspase-3 colorimetric kit, analogous to the one used
in the study by Dudai et al. was used to examine whether or not citral induced apoptosis in either cell type based on the rationale that caspase-3 activation is a good indicator of apoptosis. As previously described, the caspase-3 protein is activated in both extrinsic and intrinsic apoptotic pathways. Thus, it was assumed that any caspase-3 activation observed using the kit would corresponded to the level of apoptosis experienced by the cells. The colorimetric kit used in this study worked in a similar way as the aforementioned MTT and clonogenic assays where a plate reader is used to measure absorbance of light. As in the study by Dudai et al, the light being absorbed during the reading of the plate is generated by the pNA, a light producing compound known as a chromophore that is activated when an activated caspase cleaves it from the DEVD peptide used in the assay. In this case, the percent increase in the light emitted by the chromophore compared to the light emitted by the control sample (where apoptosis was not induced) was used to quantify the level of apoptosis the cells experienced after they were treated with citral. If no change in caspase-3 activation was observed, it was possible that a necrotic death mechanism was occurring instead.

The cell lines chosen to compare any potential apoptotic or necrotic effects are being used purely to explore the effects of citral in cancerous cells and non-cancerous cells. The same cell lines have been used in similar apoptotic pathway studies for the same purposes (George et al., 2012).

**Preliminary Studies and Results**

The effects of citral on M624 melanoma cells were previously investigated during the summer of 2015. Mainly, this time was used to run preliminary clonogenic assays of M624 melanoma cells. The citral concentrations used during these assays were based off of the calculated IC\textsubscript{50} concentration for the 24 hour cell viability assay in the aforementioned study by
Cahouki et al.: 0.266 mM. The following citral concentrations were used during initial clonogenic assays of M624 cells with an exposure period of 24 hours: 0, 0.25, 0.50, and 0.75 mM. After observing an overwhelmingly toxic effect, the concentrations and exposure time were adjusted to 0, 0.25, 0.375, and 0.5 mM with an exposure period of three hours. These values produced the preliminary results shown in the figure below.

![Graph showing cell viability percentage of M624 cells after a three hour exposure period to different citral concentrations.](image)

**Figure 2: Preliminary clonogenic assay results of M624 cells after a three hour exposure period.**

Results are based on 3 sets of triplicate data. Error bars represent standard deviations.

Moving forward, it was important to test the effects of citral on HaCaT (non-cancerous skin) cells using the same clonogenic assay procedure in order to monitor any differences in citral’s effects on non-cancerous cells. Because this preliminary data showed that cells were indeed dying, it was imperative to analyze whether apoptotic or necrotic mechanisms were occurring using the caspase-3 colorimetric kit. This analysis was necessary in order to confirm that the death occurring was healthy, and not inflammatory in any way. Moving forward, it was hypothesized that citral would have a much stronger effect on cell viability in the M624 cells.
than the control HaCaT cells during the clonogenic assays based on similar phenomena observed in the study by Dudai et al. Based on the expected differences in toxicity, it was also hypothesized that the levels of caspase-3 activation would increase as the concentration of citral increased, and that the activation levels would remain the same for all concentrations during treatment with the HaCaT cells. Theoretically, more apoptotic cell death would occur in the cancerous cells, leading to higher levels of activated caspase-3. In contrast, it was expected that there would be no differences in the levels of cell death in the HaCaT cells, which would lead to little activation of caspase-3 and therefore little differences between the concentrations.
Methods

Reagents and Solutions

DMEM was purchased from Corning Cellgro. Trypsin and FBS were purchased from Thermo Scientific. Penicillin-Streptomycin was purchased from MP Biomedicals. Citral (95% mixture of cis and trans) was purchased from Fischer Scientific (Acros Organics). Crystal Violet was purchased from Ward’s Natural Science Establishment. 10x Phosphate Buffered Saline (PBS) was purchased from Boston BioProducts. The caspase-3 colorimetric kit, containing cell lysis buffer, 2x reaction buffer, DEVD-pNA, DTT, and dilution buffer solutions was purchased from BioVision.

Cell Culture

Both types of cells were cultured in Dulbecco’s Modified Eagle Medium (DMEM) supplemented with 10% Fetal Bovine Serum and 1% penicillin/streptomycin. Cells were maintained in an incubator at 37° with 5% humidity.

Citral Solution Preparation

A 0.25 mM standard solution of citral was prepared by dissolving 13.5 µL of citral in 0.3 mL of ethanol. This solution, and complete media (containing the FBS and penicillin/streptomycin) were then used to prepare 0.25, 0.375, and 0.5 mM citral media. This media was then used in both the clonogenic assay and caspase-3 colorimetric assay procedures.

Clonogenic Assay

Cells were grown to ~80% confluency in 100 mm plates. Once confluent, they were harvested with trypsin, and plated onto 12-well cell culture plates. After incubation for 24 hours, the growth media present was swapped out for media containing citral (or new media for the
control samples). The diagram below shows the concentrations assigned to each well in the 12 well plate. Each concentration was tested in triplicate.

<table>
<thead>
<tr>
<th>Concentration of citral (mM)</th>
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<tr>
<td>0</td>
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| ![Diagram of clonogenic assay 12 well plate](image)

Figure 3: Diagram of clonogenic assay 12 well plate.

After 3 hours of exposure, media was removed via vacuum. The remaining cells were then washed with PBS and stained with 0.5% w/v crystal violet. The crystal violet solution was added using plastic Pasteur pipettes, and plates were put on a rotating table for ten minutes. Afterwards, Excess stain was washed away with distilled water. Plates were then read using an Epoch™ plate reader at 590 nm (using the area scanning function). Results were averaged, and standardized against the control to evaluate viability.

*BioVision Caspase-3 Colorimetric Kit*

Twenty four 100 mm plates of cells were grown to ~80% confluency. Six plates were used for each sample. As in the aforementioned clonogenic assay, media was swapped out for media containing citral. After 3 hours, both live and dead cells were lysed. The media, which contained the dead cells, was removed via Pasteur pipet. After centrifugation, the cells were
suspended in PBS. Remaining live cells attached to the plates were washed with PBS and scraped off with the back of a pipette tip. Pelleted cells were then lysed by resuspension in 100 μL of chilled cell lysis buffer on ice for 10 minutes. After centrifugation, 100 μL of lysate was aliquoted for use. A Bradford protein concentration assay was then performed.

**Bradford Protein Concentration Assay**

A standard curve consisted of known concentrations of Bovine Serum Albumin (BSA) at 0.2μg/μL, 0.4μg/μL, 0.6μg/μL, 0.8μg/μL, and 1.0μg/μL. All lysate samples were diluted by a factor of 2 prior to assay. The protein standards, lysate samples, and Bradford reagent (50 mL 95% ethanol, 100 mg Coomassie Brilliant Blue G-250, 100 mL 85% phosphoric acid, diluted to 1L using distilled water, filtered through Whatman filter paper) were added to a 96 well plate in triplicate (200 μL Bradford reagent and 10 μL sample/standard). The absorbance was read at 595 nm. The lysate sample’s protein concentrations (in μg/μL) were calculated using the line equation generated from the absorbancies of the standard curve solutions. An example standard curve graph is shown below. The unknown protein concentrations of the samples are calculated by plugging in their measured absorbancies for Y and solving for X (protein concentration in μg/μL). Dilution was accounted for to obtain the true protein concentration.
**BioVision Caspase-3 Colorimetric Kit**

A volume of lysate containing 50 µg of protein (determined through Bradford protein concentration assay results) for each treatment group was added to a 96 well plate in duplicate. Cell lysis buffer was then added to bring the total amount of solution to 50 µL. Afterwards, 50 µL of freshly made 2x reaction buffer (containing 10 mM DTT) and 5 µL of 4 mM DEVD-pNA substrate (200 µM final conc.) were added to the wells. One additional well was prepared for the 0 mM concentration with all the aforementioned solutions except the DEVD-pNA substrate as a negative control. After incubating the plate at 37°C for 1.5 hours, samples were read at 405 nm with a plate reader. The fold-increase in caspase-3 activity was then determined by standardizing the absorbance results of the samples with those of the uninduced control.

![Graph showing the standard curve for the Bradford assay.](image)

**Figure 4: Example standard curve from Bradford assay.**
Results

The initial toxic and protective effects of citral were analyzed via clonogenic assays that used a crystal violet stain. An example of an M624 cell plate stained using this technique is seen in Figure 5. Moving right, less purple stained cells are seen in each column indicating that less cells survived the three hour treatment as the concentration of citral increased.

![Figure 5. Example clonogenic toxicity assay plate: Citral treatment of M624 cells for three hours at 0, 0.25, 0.375 and 0.5 mM.](image)

After the 12 well plates were stained, quantitative results were obtained using a plate reader. The results of this analysis are shown in Figure 6. According to the graph, treatment with citral killed ~20%, ~38% and ~50% of cells at the 0.25, 0.375 and 0.5 mM concentrations, respectively. Far less toxicity was seen in HaCaT cell assay plates (12 well plate picture not shown). According to Figure 6, citral killed ~0.3%, ~4%, and ~7% of cells relative to the control in the aforementioned concentrations. Later, an additional HaCaT cell clonogenic assay with a longer exposure period of eight hours was performed. The results of this assay are displayed in Figure 1 of Appendix A. The increased exposure time seemed to increase citral’s toxicity, as
~22%, ~28% and ~30% of cells died at the 0.25, 0.375 and 0.5 mM concentrations, respectively (relative to the control).

Figure 6. Quantified results of clonogenic toxicity assays. Citral toxicity to HaCaT cells (blue) and M624 cells (orange) after three hours of exposure. Results are based on six sets of triplicate data for each cell type. Error bars represent standard deviations.

Following toxicity analysis, a caspase-3 colorimetric kit was used to determine the levels of caspase-3 activated during cell death at each citral concentration. These results are presented in Figure 7. Assays showed that citral activated caspase-3 in both cell lines compared to the untreated control, as all other observed absorbancies were higher than that of the 0 mM sample. According to the data presented, caspase-3 activation increased in M624 cells in a dose dependent manner, with the exception of the 0.5 mM concentration. Caspase-3 activation also increased steadily in HaCaT cells with concentration, but to lesser extents than in M624 cells relative to the control. The same level of caspase-3 activation was observed after treatment with 0.5 mM citral in both cell lines.
Figure 7. BioVision caspase-3 colorimetric assays. The level of caspase-3 activation is presented for each citral concentration in both M624 and HaCaT cells. Results are based on two sets of duplicate data for each cell type. Error bars represent standard deviations.
Discussion

*Citral’s Toxic Effects to M624 and HaCaT Cells*

Clonogenic assay results showed that citral was more toxic to M624 cells at all the concentrations tested relative to the control. Little to no toxicity was observed in the HaCaT cells. These results support the aforementioned hypothesis that citral would have more detrimental effects on the cancerous cell line rather than the non-cancerous cell line. Additionally, these toxicity results are promising for clinical applications considering the short exposure period of three hours. Following this analysis, an additional assay was performed on HaCaT cells in order to evaluate if a longer exposure time would influence citral’s toxic capability. These results are presented in Appendix A. The increased exposure time seemed to increase citral’s toxicity, however the observed death percentages were still not as high as those of the M624 cells.

It is unclear why citral was more toxic to the M624 cells than the HaCaT cells during the three hour exposure period. Often, cancer is the result of cells failing to initiate apoptosis after they have been damaged. Apoptosis is a very tightly regulated process (Oleinick et al., 2001). Ordinarily, there are antiapoptotic mechanisms in cells that prevent them from undergoing apoptosis under normal circumstances. These mechanisms must be turned off for apoptosis to begin. Thus, it is possible that not all of these factors were turned off when the M624 cells were initially developing, and the melanoma cells continued to grow rather than experiencing apoptosis. Therefore, one possible explanation for the toxicity results could be that the M624 cells were more predisposed to apoptosis than the HaCaT cells (whose anti apoptotic mechanisms were still in place). Perhaps there was only one regulated process committing the cell to apoptosis that needed to be turned off in the M624 cells, while there were many more in
the HaCaT cells.

Additionally, the variance in toxicity results could be due to elevated levels of lipid rafts in the M624 cells. Lipid rafts are dynamic microdomains of the cell membrane that contain both lipids and proteins. Their major components include cholesterol, sphingolipids, and caveolin membrane proteins (George et al., 2012). These rafts play an important role in the regulation of many cellular signaling pathways, including apoptosis. They essentially provide the signaling platform necessary for the activation of apoptosis, and assist in recruiting the necessary death receptor proteins for extrinsic apoptotic pathways (George et al., 2012). A previous study by Li et al. stained human prostate epithelial cells, human prostate cancer cells, human breast epithelial cells, and various human breast cancer cell lines with filipin (0.05 mg/ml) and cholera toxin B subunit (CTXB)-Alexa 568 (0.04 µg/ml) to probe for plasma membrane cholesterol and GM1 (Li et al., 2006). Interestingly, results showed that the cancer cell lines showed stronger cholesterol and GM1 staining than the normal cell lines, as well as greater co-localization of cholesterol and GM1 (Li et al., 2006). Overall, these data suggest that the cancer cell lines contain elevated levels of rafts/caveolae, probably as a result of cholesterol accumulation, and that they are more susceptible to apoptosis (Li et al., 2006). It could be possible that the M624 cells used in this study also contain elevated levels of lipid rafts rendering them more susceptible to apoptosis. If this is true, the citral could potentially be interacting with these microdomains in a much more significant way in the cancerous cells rather than the non-cancerous HaCaT cells.

*Activation of Caspase-3 and Associated Apoptotic Mechanisms*

Results of the caspase-3 colorimetric assay showed that caspase-3 was indeed activated by treatment with citral in both cell lines. Thus, it is very likely that the death seen in both cell lines was apoptotic as caspase-3 activation is a trademark indicator of both intrinsic and extrinsic
apoptotic pathways.

Perhaps the most notable result of the assays was the two fold increase in caspase-3 activation relative to the control in the 0.375 mM M624 cell sample. This was somewhat unexpected because this was not the concentration where the most toxicity was observed per the M624 clonogenic assay results. Rather, a mere 50% increase in caspase-3 activation was observed in the most toxic 0.5 mM sample relative to the control. The notion that citral induces apoptosis at lower concentrations and necrosis at higher concentrations discussed in the aforementioned study by Cahouki et al. may help explain these results. In this case, the drop in caspase-3 activation observed in the M624 cells may be due to the higher concentration of citral causing more necrosis than apoptosis. Indeed, it is possible that there is a tipping point where increasing the concentration of citral drives cells more M624 cells towards necrotic death pathways than apoptotic ones.

Conversely, results of the HaCaT cells clonogenic assays showed that the little death that occurred in the HaCaT cells was likely apoptotic as the level of caspase-3 activation increased with the concentration of citral. These results are encouraging for clinical applications as they imply that no excess inflammatory death would occur in cells surrounding an area of the skin where melanoma was present during potential citral treatments.

Although these results support the notion that citral induces apoptosis, it is still unclear if apoptosis is activated by an extrinsic or intrinsic mechanism. The aforementioned study by Dudai et al. claims that the compound’s α,β-unsaturated aldehyde group is essential for it to have any toxic effects. Perhaps this means that this portion of the molecule is interacting with a specific receptor protein on the surface of the cell membrane. Activation of this receptor might lead to the eventual activation of the caspase cascade as part of the extrinsic pathway.
Alternatively, the sheer presence of so much of an excess organic toxin could be influencing the cell’s inner mitochondrial membrane, and mitochondrial transmembrane potential, eventually leading to the release of normally sequestered pro-apoptotic proteins like cytochrome c in an intrinsic pathway.

**Future Experiments and Conclusions**

Overall, the results of these assays suggest that the cell death that occurred in the clonogenic assays was indeed apoptotic due to caspase-3’s important role in the caspase cascade, and in both the intrinsic and extrinsic apoptotic pathways. Moving forward, further confirmation that apoptosis occurred is necessary using other analyses such as an ethidium bromide and acridine orange double stain to evaluate DNA condensation/cleavage, or western blotting/ELISA techniques to investigate other proteins being activated.

Additionally, the citral concentrations used in this study that produced the most noticeable toxicity were relatively high compared to the results of aforementioned studies by Cahouki et al. and Dudai et al. Perhaps future experiments could be conducted to see if lower concentrations of citral (<0.200 mM) could be used in conjunction with other types of cancer therapies like photodynamic therapy or chemotherapy. In this case, the citral could theoretically be used to coerce the cells to undergo apoptosis, while the additional therapy delivers a more robust attack. Using citral as a pretreatment could render the other therapy more efficient. Ultimately, this study, and future analyses like these could provide more insight into citral’s potential role as an anticancer agent.
References


Figure 1: Clonogenic assay of HaCaT cells after an eight hour exposure period. Results are based on one set of triplicate data.