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Toxicity of thieves oils to mcf-7 and mda-mb-231 breast cancer cells

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Abstract
Cancer treatment is costly and can be just as harmful as helpful to the patient. As a result, patients often seek alternative treatment options, such as herbal therapy including the use of essential oils. Common essential oils include cinnamon, clove, eucalyptus, lemon, and rosemary; combined, these oils comprise Thieves. To determine if Thieves and the individual oils influence cancer cell viability, MCF-7 and MDA-MB-231 breast cancer cells were exposed to 0.001% to 1.0% oils and their major chemical components for forty-eight hours, followed by determination of toxicity using an MTT cell viability assay. Although all oils led to some cell death, MCF-7 cells were more susceptible to oil treatment than MDA-MB-231 cells. Of the Thieves components, clove and cinnamon were the most toxic, followed by lemon, eucalyptus, and rosemary. These toxicities are most likely due to the major chemical components of the oils, including eugenol, limonene, and cineole.

Keywords: Breast cancer, thieves, essential oil, toxicity

1. Introduction
Cancer is the classification for more than 200 human diseases of uncontrolled cell division, which if not treated properly may lead to death [1]; of these diseases, breast cancer is one of the most detrimental [2, 3]. The substantial rise in the number of cancer cases in recent yearshas been attributed to changes in eating habits, exposure to harmful chemical radiation, and environmental decline [4]. Treatment options often include chemotherapy and/or synthetic drugs. Current cancer treatments often produce a wide range of detrimental side effects to the patient [5-8]. This, in addition to the high cost of treatment and even resistance to current chemotherapeutics, has resulted in an increasing demand for novel treatment options, many of which are found in plants [4, 9-11].
Plants, herbs, and spices used in traditional medicine have become a prime target for identifying compounds with chemopreventive properties [12-16]. Several chemotherapeutics in use have an herbal background such as Taxol, derived from the yew tree [17-19]. An estimated 25% of drugs administered throughout the past 20 years are plant-based [17, 20]. Essential oils are concentrated, hydrophobic liquids possessing aromas produced by aromatic plants [21]. Essential oils, and other plant-derived treatmentsare thought to induce lesser side effects than synthetic drugs, and in some cases, improve quality of life for the cancer patient (reviewed by Gautam *et al*. in) [4]. While there are hundreds of different essential oils, the focus of our study was Thieves, a blend of five essential oils,as well as the chemical components of the individual oils. Thieves has bothantiseptic and antibacterial properties, althoughno reports on its anticancer properties have been published [22]. However, there are several studies based on its individual components.
Two of the oils, rosemary and eucalyptus, contain 1,8-cineole (also known as eucalyptol). Rosemary (*Rosmarinus officinalis*) is known for its antiseptic and antimicrobial properties [22]. Rosemary extracts are often used as preservatives due to their high antioxidant levels [23]. Eucalyptus (*Eucalyptus globulus*) is known for its antibacterial, antiviral, and anti-inflammatory properties [22, 24]]. Several species of eucalyptus leaves contain high levels of essential oils, in addition to being rich in total phenolic compounds that may protect against cancer [25, 26]. Traditionally, eucalyptus leaves were used by native Australians to heal wounds and fungal infections [27, 28].
Two of the other components of Thieves, clove and cinnamon, contain eugenol [29-31]. Eugenol (4-allyl-2-methoxyphenol) may possess antioxidant, anti-inflammatory, and anticancer properties [32]. Accepted as safe by the Food and Drug Administration, common human

exposure to the chemical often occurs in the dental industry and in food and spices [33]. Clove (*Syzygium aromaticum*) possesses the highest antimicrobial, antiseptic, and anti-infectious properties of all essential oils [1, 22]. Cinnamon (*Cinnamomum zeylanicum)* is one of the most powerful antiseptics known, in addition to possessing antibacterial, antiviral, and antifungal properties [22]. Several extracts of cinnamon have been indicated in anti-cancer studies, with beneficial effects including both an inhibition of VEGF as well as more direct inhibition of cell growth in both leukemia and melanoma cell lines [34-36]. As reviewed by Ranasinghe *et al*., these oils may have medicinal potential as they act as anti-oxidants and have very low toxicity in the liver [37].

Lastly, lemon (*Citrus limon*) is known for its antiseptic, antibacterial, and immunity promoting properties [22] and is high in limonene [38]. The antibacterial properties of lemon have been extensively studied, including its ability to kill bacteria quickly, even when treated with small doses [39].

Three other chemical components were also studied:β-caryophyllene,β-pinene, and α-pinene. These chemicals were included in this study as they are minor components of several essential oils (Table 1). β-Caryophylleneis attributed to various biological properties including antibiotic, antioxidant, and anti-inflammatory activities [40], and bothα-pinene and β-pinene have been studied for potential anticancer properties [41]. Based on these previous studies, we presumed that treatment of breast cancer cells with the individual essential oils, as well as Thieves blend, would result in cell death. By examining the components, we hoped to identify which volatiles contributed the most to any observed toxicity.

Table 1: Minor components of the essential oils within Thieves. The percent range of each compound contained within the essential oils is stated. indicates that the chemical has not been reported as a component of the essential oil. Data adapted and summarized from [25, 28-31, 38, 42, 43].

	Rosemary	Eucalyptus	Lemon	Clove	Cinnamon
β-Caryophyllene	1-5	-	-	4-17	3
1,8-Cineole	16-55	33-90	-	<1	<1
Eugenol	-	-	-	77-87	77 (20-30)
Limonene	2-4	8	38-73	-	<1
α-Pinene	3-38	4-16	4	<1	<1
β-Pinene	2-8	-	20	-	<1

2. Materials and methods

Individual essential oils were purchased from Puritans Pride (Oakdale, NY): rosemary leaf (*Rosmarinus officinalis*), eucalyptus (*Eucalyptus globulus)*, lemon (*Citrus limon)*, clove bud (*Syzygium aromaticum*), and cinnamon leaf (*Cinnamomum zeylanicum*). Components of the oils(eugenol, 1,8-cineole, limonene, β-caryophyllene, β-pinene, and α-pinene) were purchased from Sigma Aldrich (St. Louis, MO), and pre-made Thievesblend was purchased from Young Living (Lehi, UT). Two Thieves blends were prepared in lab, utilizing individual essential oils in various amounts (Table 2). Stock solutions with concentrations of 1%, 0.1%, 0.01%, and 0.001% essential oil or chemical (v/v) were prepared in dimethyl sulfoxide (DMSO). Immediately preceding each assay, a 1:100 dilution of each stock oil solution was prepared in culture media for final v/v concentrations of 0.00001% to 0.01%.

Table 2: Lab prepared Thieves blends. Blends of essential oils were prepared using pure individual oils. The percentage of each oil within the blend is reported.

	Rosemary	Eucalyptus	Clove	Cinnamon	Lemon
Thieves 1	8.3	12.5	33.3	16.7	29.2
Thieves 2	8.5	12.5	33.5	17.0	28.4

MCF-7 and MDA-MB-231 (231) human breast cancer cells maintained as previously described [44]. Once cells reached 90% confluence, they were plated into a 96-well plate and allowed to adhere for 24 hours prior to oil treatment. Following this period, medium was removed from each well and replaced with 100 µL of control (medium with 1% DMSO) or oil/compound treatment. Treatment was carried out for 48 hours in a humidified incubator at 37ºC with 5% CO_2. Following treatment, MTT (3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide) assay analysis was carried out in the methods of Sargent and Taylor [45]. Plates were analyzed using a BioRadi Mark Microplate Reader at 570 and 595 nm. Cells were treated in triplicate, and a minimum of five replicates of each treatment were performed. Statistical analysis was performed using Univariate Analysis of Variance (ANOVA) with SPSS (IBM SPSS Statistics 21, IBM Corp., Aramonk, NY, USA), with $p<0.05$ indicating significant variation from the controls.

3. Results

Two blends of Thieves oil were prepared (Table 2), in addition to a proprietary purchased blend from Young Living [22]. Both MDA-MB-231 and MCF-7 breast cancer cell lines were treated with the three blends or DMSO control for 48 hours prior to toxicity analysis using MTT (Figures 1A and B). The minimal differences in composition of the prepared Thieves did not affect the overall toxicity, nor did the prepared blends vary from the proprietary blend. Due to a lack of statistical difference between the three blends, results were compiled within each cell type (Figure 1C). After compilation, a statistically significant increase in viability over the cells treated with DMSO control was observed at the lowest concentration of Thieves, whereas statistically significant decreases were observed at higher concentrations (0.001% and 0.01%).

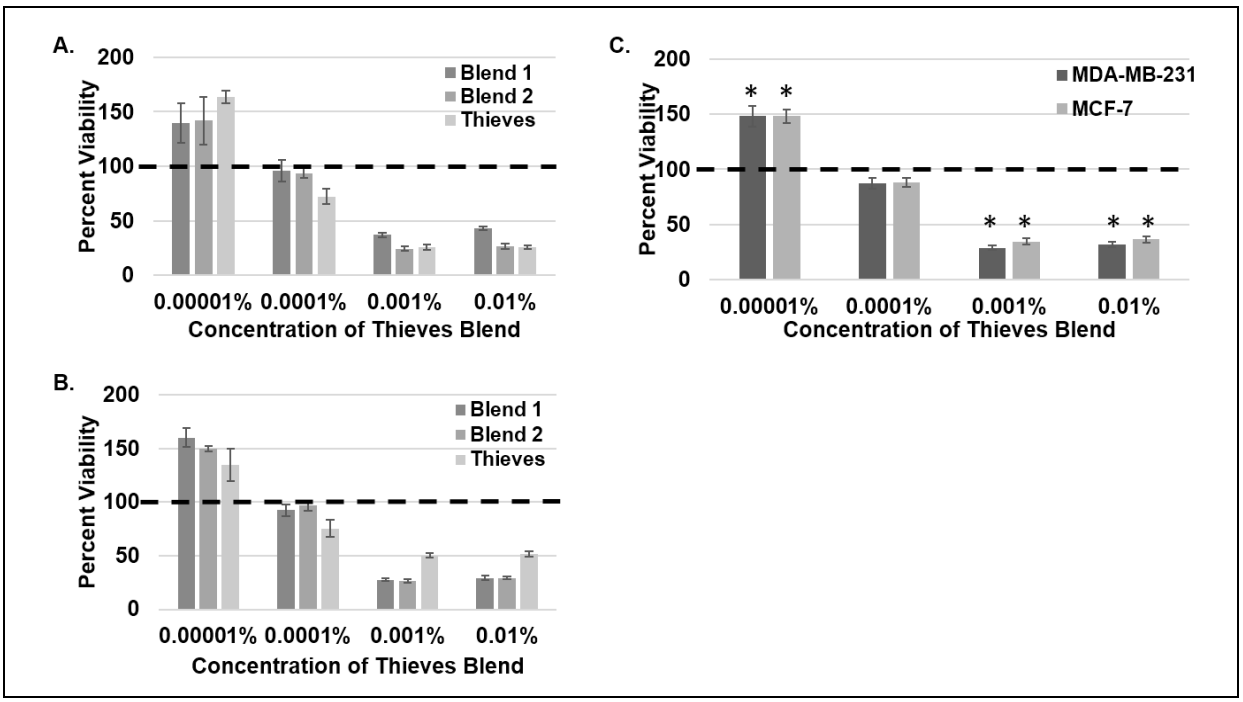

Fig 1: Reduction in cell viability following treatment with Thieves blends. MCF-7 and MDA-MB-231 breast cancer cells were treated with Thieves oil blends in media (0.00001% to 0.01% v/v) for 48 hours prior to viability testing. DMSO control (dotted line) was normalized to 100% viability. Results represent an average of five experiments for each blend performed in triplicate, plus or minus SEM. In (A), MDA-MB-231 cells, and in (B), MCF-7 cell viabilities are shown. In (C), results were compiled and are displayed as overall reduction in viability across fifteen experiments per cell line, as there was no statistical difference between the three blends at each concentration. * represents significant variation in viability for the compiled results ($p<0.05$) compared to the DMSO vehicle control.

Due to these differences in observed viability, we examined the contributions of the individual components within the Thieves oil blend: rosemary, eucalyptus, clove, cinnamon, and lemon. In MDA-MB-231 cells treated with individual essential oils, lemon, clove, and cinnamon led to significant viability reduction in all but the lowest concentration (0.00001% v/v in media, Figure 2A). For rosemary and eucalyptus oils, a statistically significant reduction in cell viability was observed only in the 0.001% and 0.01% dilutions, respectively (Figure 2A). In contrast, MCF-7 cells were more susceptible to oil treatment. A significant reduction in cell viability was observed in all cells treated with at least 0.001% oil (Figure2B).All oils but rosemary also lead to significant reduction in cell viability at the 0.0001% concentration (Figure2B).

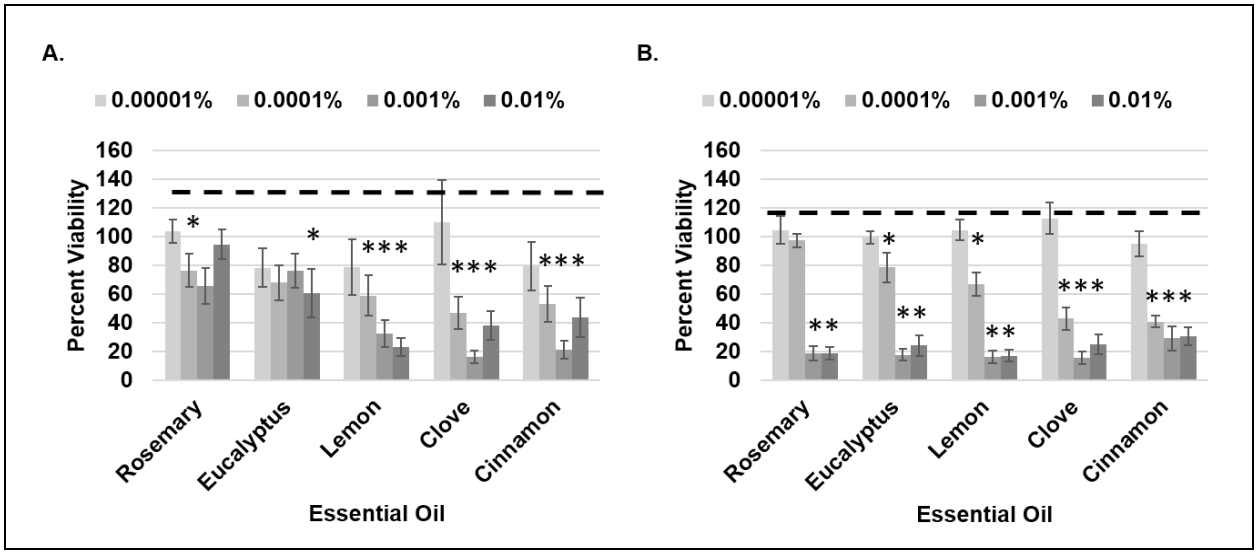

Fig 2: Reduction in viability following treatment with individual essential oils. MDA-MB-231 (A) or MCF-7 (B) breast cancer cells were treated with 0.00001% to 0.01% rosemary, eucalyptus, lemon, clove, or cinnamon essential oils or DMSO vehicle control for 48 hours. DMSO control (dotted line) was normalized to 100% viability. Results represent an average of five experiments performed in triplicate, plus or minus SEM. * represents significant variation in viability ($p<0.05$) compared to the DMSO vehicle control.

As several of the oils contained the same active compounds, we assessed the toxicity of the larger aromatic compounds found within each oil (eugenol, 1,8-cineole, or limonene) as well as several smaller constituents. When MDA-MB-231 cells were treated with the greater components of the oils, statistically significant reductions in viability were observed.

In contrast to the minimal effects of the essential oil mixtures, eugenol, cineole, and limonene all reduced cell viability to less than 40% of the DMSO control at 0.001% and 0.01% v/v (Figure 3A). Additionally, the 0.0001% eugenol treatment also reduced viability below 60% of that of the DMSO control. However, just as large reductions in viability were observed for the essential oils in MCF-7 cells, the volatile compounds also reduced viability (Figure 3B). Larger reductions in viability were observed at the 0.001% and 0.01% concentrations compared to the MDA-MB-231 cells. Conversely, a trend for increased viability was observed at the lower concentrations, similar to what was observed for the lowest concentrations of the Thieves oils. We also examined the toxicity of the lesser components within the oils (Table 1). Significant reduction in cell viability was observed in the 3 more concentrated treatments for both β-caryophyllene and β-pinene in MDA-MB-231 cells (Figure4A).For α-pinene, however, a statistically significant decrease was only seen at 0.01% v/v. A significant increase was also observed at the lowest dilution, 0.00001% v/v. In MCF-7 cells, a similar trend was observed, although the lowest concentration of these compounds had no effect on viability (Figure 4B).

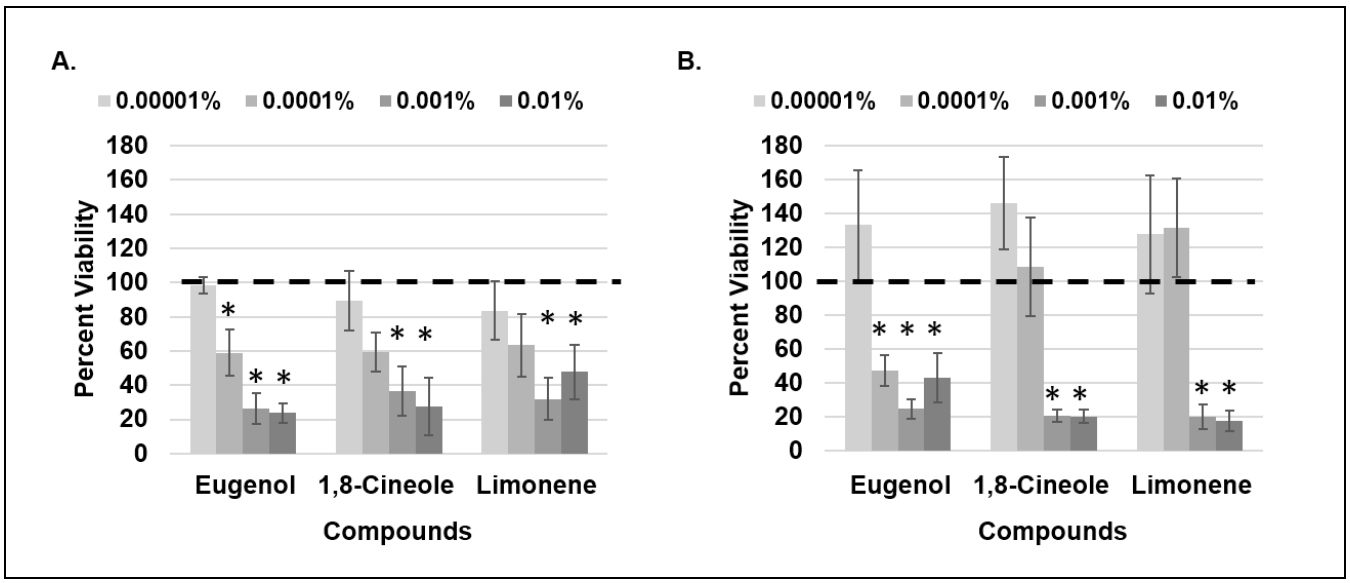

Fig 3: Reduction in viability following treatment with aromatic compounds. MDA-MB-231 (A) or MCF-7 (B) breast cancer cells were treated for 48 hours with DMSO vehicle control or with 0.00001% to 0.01% of the primary volatile compounds within the essential oils studied: eugenol, 1,8-cineole, or limonene. DMSO control (dotted line) was normalized to 100% viability. Results represent an average of five experiments performed in triplicate, plus or minus SEM. * represents significant variation in viability (p<0.05) compared to the DMSO vehicle control.

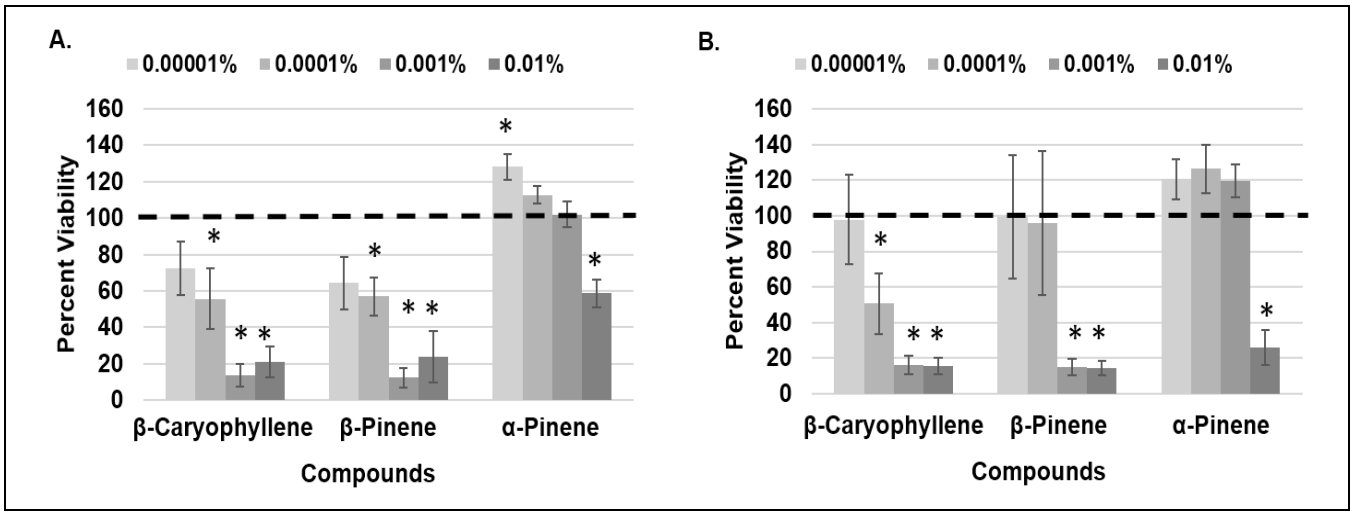

Fig 4: Reduction in viability following treatment with minor oil components. MDA-MB-231 (A) or MCF-7 (B) breast cancer cells were treated for 48 hours with DMSO vehicle control or with 0.00001% to 0.01% of the minor components of the essential studied: β-caryophyllene, β-pinene, or α-pinene. DMSO control (dotted line) was normalized to 100% viability. Results represent an average of five experiments performed in triplicate, plus or minus SEM. * represents significant variation in viability (p<0.05) compared to the DMSO vehicle control.

4. Discussion

In agreement with our hypothesis, we observed that not only did the Thieves essential oils blends cause death in two breast cancer cell lines, but the major components of the individual essential oils did as well. For cells treated with individual oils, as little as 0.001% v/v of lemon, clove, or cinnamon oils caused reduction in viability below 40% of the untreated cells, and this same reduction was mirrored with rosemary and eucalyptus oils in MCF-7 cells. This large death can partially be attributed to the major components of these oils: eugenol, 1,8-cineole, and limonene, although the minor components of β-caryophyllene and β-pinene also caused death at these same amounts.

Clove, cinnamon, and eugenol essential oils show similar

results in a variety of cancer cell lines. Kumar *et al.* [1] observed comparable results in MCF-7 cell lines treated with clove, suggesting clove is an inhibitor of MCF-7 cells in a time- and dose- dependent manner. Additionally, these results, and ours, suggest clove to be an ideal cancer treatment because of its ability to enhance apoptosis and inhibit cell proliferation [1]. Zu *et al.* [46] observed strong cytotoxic activities of cinnamon essential oil in MCF-7 cell lines, and suggest the need for future studies to confirm findings. Vidhya and Devaraj exposed MCF-7 cells to eugenol, and observed inhibited growth and proliferation of the cells through apoptosis, in a dose and time dependent manner [33]. The results of all three, and ours, support the use of clove, cinnamon, and eugenol as potential chemo preventive agents.

Although we did not observe as much of an effect with rosemary and eucalyptus, especially within MDA-MB-231 cells, evidence does suggest that these, too, could have anticancer potential. Yesil-Celiktas *et al*. [23] observed similar results to ours when using rosemary oils, and suggest the use of rosemary oils as a treatment option for both chemotherapy-resistant cancers and as a part of anti-cancer diets. Various derivatives of eucalyptus also show anticancer potential. Ashour [27] showed cytotoxic activity of eucalyptus oils in MCF-7 cells, in addition to antibacterial and antifungal properties. Likewise, Vuong *et al.* [26] described potent anti-cancer activity of eucalyptus extracts against a variety of cancer cell lines, with the strongest cytotoxic effects observed in breast and pancreatic cell lines. Althoughboth Wu *et al*. and Schmidt *et al*. failed to show a cytotoxic effect of 1,8-cineole in several cancercelllines [47, 48], other studies have indicated that 1,8-cineole is, in fact, cytotoxic in both cancerous cell lines and *in vivo*. In an assessment of several malignant bone, skin, and colon cell lines, both Sampath *et al*. and Murata *et al*. demonstrated activation of ROS-mediated apoptotic pathways after cells were exposed to 1,8-cineole [49, 50]. However, these studies did not use pure 1,8-cineole and rather plant extracts where 1,8-cineole was a major component. Based on work by Setzer *et al*., we know that although alone 1,8-cineole may show minimal cytotoxicity in MCF-7 cells, combining it with other minimally toxic compounds can result in a synergistic effect and greatly reduce viability [51, 52].These results contradict the majority of our results;thus future studies should continue to test for anti-cancer properties of 1,8-cineole.

Components of lemon essential oilhave been studied extensively for their role as antibacterial agents. However, minimal studies have tested for anti-cancer properties. Zu *et al*. included lemon in a study of 10 essential oils tested for anti-cancer properties [46]. Lemon essential oil resulted in viability reductions similar to those observed in our study, but limonene was not examined. These results suggest more studies should include both lemon and limonene to test for anti-cancer properties.

While one might assume that the primary cytotoxic effects of the essential oils is due to the volatile ompounds comprising a majority of the oils, we have also identified reduced viability in the presence of the lesser components β-caryophyllene, α-pinene, and β-pinene. Legault and Pichette [40] suggested β-caryophyllene increases membrane permeability, thus allowing for a greater effect of chemotherapeutics. This is a common mechanism of action for essential oils as a whole due to the hydrophobicity of the components, leading to their effectiveness as antimicrobials. Essential oils have been shown to interfere with bacterial membrane structure (reviewed in [53, 54]) they also can exhibit cytotoxicity through membrane disruption in cancerous cells [55]. Although this may be one mechanism by which it contributes to cell death, it does not explain the toxicity of the compound by itself. Like β-caryophyllene, it has been suggested that both α- and β-pinenework synergistically with other oil compounds to induce death in MCF-7 cells [41]. In fact, Wright *et al*. reported in 2007 that the cytotoxicity of several essential oil components could be maximized through the addition of other oil components [52]. This was shown in particular with the addition of hexanal to several components such as β-caryophyllene and β-pinene. Thus, these minor components may help facilitate the cytotoxic activity observed with essential oil treatment.

Our study utilized common representatives of both hormone-dependent and hormone-independent breast cancers: MCF-7 and MDA-MB-231 cell lines [56]. MCF-7 cells are hormone dependent and tend to mound up during growth, whereas MDA-MB-231 cells are not hormone dependent, but are known to metastasize during growth [57-59]. Despite both being cancerous, they exhibited different responses in the presence of rosemary and eucalyptus, as well as α-pinene. Additionally, we consistently observed different results for our lowest concentrations within both cell lines, where increases in viability were observed. This was true for all three Thieves blends, and trends for increases were observed with eugenol, cineole, and limonene. These differences in responsiveness are not unknown in cancerous cells, as several endocrine system and estrogen receptor modulators have been shown to have opposing effects that are concentration dependent [60-64]. Thus, dependent upon receptor availability and concentration of the component, each cell type may differentially respond to the oil mixture.

To the best of our knowledge, this is the first study to test for anticancer effects using blended Thieves oil, and further examines the efficacy of the contributing essential oils and their major and minor components. Based on our results, Thieves reduces viability in a dose-dependent manner, largely in part from the contributions of eugenol, 1,8-cineole, and limonene within the five essential oils. Future work examining the mechanistic actions of these essential oils and their components may elucidate the specific pathways through which their toxic effects are induced.

5. Conclusions

MCF-7 cells were more susceptible to oil treatment than MDA-MB-231 cells. However, cell death was observed with all essential oils, with the greatest death in the cells exposed to the highest concentrations of each oil. Of the Thieves components, clove and cinnamon were the most toxic, followed by lemon, eucalyptus, and rosemary. These toxicities are most likely due to the major chemical components of the oils, including eugenol, limonene, and 1,8-cineole.

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