Journal of Nutrition & Food Sciences
ISSN: 2155-9600

Thompson et al., J Nutr Food Sci 2019, 9:1
DOI: 10.4172/2155-9600.1000750

Research Article Open Access

Anti-Cancer Properties of Cinnamon Oil and its Active Component, Trans-Cinnamaldehyde

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Abstract

Bioactive dietary agents (i.e., nutraceuticals) have shown the ability to slow or eliminate cancer growth with marked immediate effects when applied directly to cancer cells. By isolation and inspection of the effective components in these therapeutic agents, with respect to cancer cell inhibition, a clear mechanism of action can contribute to a better understanding of the benefits of these natural products. Here we have examined the effects of three essential oil formulations of nutraceutical agents: Frankincense, Garlic and Cinnamon, and ultimately investigated trans-cinnamaldehyde (TC), the active ingredient in cinnamon oil and a documented anti-inflammatory and anti-cancer agent, on cell viability and migratory potential in *in vitro* models of breast cancer representing different tumorigenic and metastatic potential. Results show that TC exhibits a clear dose-response relationship in inhibiting cancer cell survival and migration in a breast cancer aggression-dependent manner. Our results suggest that TC may be a promising candidate for the development of effective strategies to counter breast cancer *in vivo* by reducing or eliminating its progression.

Keywords: Trans-cinnamaldehyde; Cancer; Breast cancer; Progression; Nutraceutical

Introduction

Breast cancer is second only to lung cancer, as it pertains to cancer-related deaths afflicting women worldwide [1,2]. Highly aggressive or invasive breast cancers in particular contribute significantly to mortality based on their propensity for cancer metastasis. To date, a broad spectrum of treatments employing chemotherapeutics has been established. Several of conventional chemotherapeutic agents originate from plants, e.g, taxanes, vinca alkaloids, podophyllotoxins. However, many are inherently toxic and can't be specifically localized to the cancer site but are systemic in nature and, thus, can cause severe side effects [3,4]. Alternative therapies have shown promise but many are without clinical application due to problematic quality control, safety, and toxicity issues [5,6]. Dietary agents which may supplement therapy and reduce the effective doses of toxic therapies have been proposed as one potential resource [3].

Developing effective alternatives to a solely drug-based treatment for cancer requires that both cancer cells' genotypic and phenotypic characteristics be successfully counteracted, specifically their highly proliferative and migratory characteristics. Ideally, bioactive compounds in the diet should prevent cancer, lower cancer incidence or progression by reducing cancer cell proliferation and reverse their phenotype to a less aggressive one, or render the cancer cells more susceptible to chemotherapeutic treatments to allow for the use of lower but effective concentrations [7,8].

To date, the health benefits of foods as well as other compounds with natural origins utilized as dietary supplements have only received modest clinical support due to little or no effectiveness in clinical trials [7,9]. Targeted chemotherapeutics are usually based on inhibiting specific aberrations and molecular markers. While these treatments have led to improvements for a multitude of diseases, many exhibit limitations in cancer treatment due to the individual genetic differences in patients and redundant signaling pathways that allow cancer cells to alter signaling cascades and circumvent a specific event inhibited by a targeted therapeutic [10-12]. Other limitations are effective tissue targeting, potential for drug-induced toxicities, difficult renal clearance and, from an economic standpoint, health care costs [13,14]. The advantage of nutraceutical agents is that they may target multiple signaling events and thus eliminate redundant signaling, may be more easily degraded and cleared from the body, and even in higher concentrations potentially exhibit less deleterious toxicity as compared to synthetic agents [15].

Specific plant-based agents such as turmeric and its derivative, curcumin, have gained notable traction as a potential nutraceutical agent; however, the prevalence of use, particularly in the western world, is low. In this study, we used formulations of bioactive components from two common edible nutraceuticals (garlic and cinnamon) and one less common agent (frankincense) which have all shown promising suppression of cancer onset and proliferation. These agents were selected based on previous research results as well as experience with select agents at the Virginia-Maryland College of Veterinary Medicine [16,17]. Frankincense oil, distilled from the aromatic resin of the genus *Boswellia* tree, has previously been shown to induce cancer cell-specific death in transitional carcinoma cell lines [15,17], while garlic oil (allyl compound derived from the *Allium* genus) activated carcinogen metabolizing enzymes as well as reduced reactive oxygen species

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Received December 17, 2018; **Accepted** January 14, 2019; **Published** January 22, 2019

Citation: Thompson M, Schmelz EM, Bickford L (2019) Anti-Cancer Properties of Cinnamon Oil and its Active Component, Trans-Cinnamaldehyde. J Nutr Food Sci 9: 750. doi: 10.4172/2155-9600.1000750

[18,19]. Similarly, cinnamon oil (*Cinnamomum* genus) has displayed anti-inflammatory capabilities via inhibition of NF-κb [20,21]. Here, we tested the hypothesis that these oils or their bioactive ingredients can effectively reduce the metastatic potential of the highly metastatic MDA-MB-231 breast cancer cell line. Results may have clinical implications as these agents may be used as dietary supplements alone in preventive efforts, or in combination with conventional drugs for chemotherapeutic treatments.

Materials and Methods

Cell culture and media preparation

All cell lines were grown in a humidified incubator at 37°C with 5% CO_2. The triple-negative MDA-MB-231 cell line (ATCC) was grown in RPMI-1640 (ATCC) supplemented with 10% fetal bovine serum (ATCC). The MCF-10A cell line, a benign line isolated from a patient with fibrocystic disease represents immortalized breast epithelium while the MCF-10AT1 pre-malignancy cell line (from the Karmanos Cancer Institute, Detroit, MI) develop tumors over time [22], both cell lines were grown in DMEM-F12 (Gibco) supplemented with 5% horse serum (Sigma), 20 ng/ml endothelial growth factor, 0.5 mg/ml hydrocortisone, 10 mg/ml insulin and 1% penicillin-streptomycin (Sigma). The advanced MCF-DCIS (ductal carcinoma in situ) cell line (Karmanos Cancer Institute, Detroit, MI) was grown in DMEM-F12 media containing 5% horse serum and 1% penicillin-streptomycin (Sigma). The MCF cell lines were chosen to represent progressive stages of early breast cancer that are potentially responsive to nutritional supplements while more aggressive disease stages require surgical removal and chemotherapy.

Cytotoxicity analyses

The efficacy of the botanical agents, frankincense oil (Böswellness, Burlington, VT), garlic oil and cinnamon oil (Sigma) and the active ingredient trans-cinnamaldehyde (TC) in suppression of cancer cell growth was determined in the breast cancer cell lines at concentrations comparable to those described, between 100-400 μg/mL [23,24]. Albeit high for oral delivery, these concentrations serve as a starting point for a preliminary examination of therapeutic potential. 2000 cells per well were seeded into a flat bottom 96-well plate (Corning) and allowed to grow for 1-3 days as indicated. Then the cells were treated with the indicated concentrations of agents in cell culture media, varying from 10 μg/ml to 300 μg/ml, (n=4-5) samples per treatment concentration. Stock solutions of treatments were generated by dissolving oils in warm media (37°C), vortexing for 1 minute to aid in oil solubilization and administered within 5 minutes to ensure homogeneity [25]. After 24 -72 hours, an alamar blue cell viability assay [26] (Fisher) was conducted and fluorescence intensity was measured using a BioTek Synergy H1 plate reader, where greater fluorescence intensity correlated with an increased number of viable cells. All tests were performed in three biological replicates.

GC-MS analysis of cinnamon oil

Gas chromatography mass spectrometry (GC-MS) GC-MS analysis was performed using an HP 6890 series split/splitless injector with a 5890 Series II gas chromatograph and a flame ionization detector (FID). Samples (1 μL) were injected into the column with a split ratio of 1:50 using helium as the carrier gas. The oven temperature was kept at 50°C for 2 minutes and was subsequently increased by 20°C/min until the oven reached 250°C where it remained for 2 minutes. Identification of compounds was based on their respective mass spectra and quantified by counting the number of individual light scattered molecular units (LSU). Quantitative analysis of essential oil components, expressed as an area percentage under a respective peak, was determined using peak area normalization. Quantitative analysis of essential oil components, expressed as an area percentage under a respective peak, was determined using peak area normalization.

GC-MS analysis of trans-cinnamaldehyde uptake

MDA-MB-231 cells were seeded at 3,000 cells per well in a 96-well cell culture plate at a volume of 100 μL and grown for 3 days. The cells were then treated with TC in concentrations ranging from 50 μg/ml to 200 μg/ml (n=5 for each concentration) for 24 hours; then the medium was collected and analyzed by GC-MS. Control samples contained TC but no cells.

2D migratory analysis

Cell migration was determined using a wound-healing assay. Briefly, cells were grown to confluency in a Grace BioLabs Secure Seal imaging spacer in 6 well tissue culture-treated plates. Once confluent, a sterile spatula was drawn across the surface of the monolayer once, creating the designed gap. Cells were treated with assay media, devoid of growth serum, or TC in cell culture media at non-toxic concentrations (ranging from 25 μg/ml to 50 μg/ml) to observe effects on migratory capabilities. Images were taken after 24 hours and migration was quantified using Image J.

Three-dimensional (3D) migration analysis

A 3D invasion assay through a collagen matrix was utilized to determine the impact of cinnamaldehyde on the invasive capacity of the MCF cell lines. 50 μl of 2 mg/ml rat tail type I collagen (Corning) in 0.1% glacial acetic acid was layered atop wells in a Corning HTS Transwell 96-well permeable support and allowed to polymerize for 2 hours and kept in complete medium for 4 hours to improve cell survival and adhesion. Then the medium in the support wells was aspirated and MCF cell lines were seeded at 200 cells per well in complete medium atop the polymerized collagen layer and allowed to adhere to the collagen for 4 hours. The media was then replaced by serum-free medium (controls), supplemented with 25 or 50 μg/ml of TC. Cells were incubated for 24 hours. Cells that did not transverse across the permeable support were removed with a sterile cotton swab. Viable cells that had successfully migrated to the bottom of the permeable support were stained with a Cell Trace Calcein red-orange AM stain and counted.

Statistical analysis

All data was analyzed and compared for statistical significance using analysis of variance (ANOVA). p-values <0.05 were considered statistically significant unless otherwise noted, in all analyses. Experimental replicates and sample sizes were determined using a power analysis where a power (1- β) of 0.9 and an alpha (α) of 0.05 were applied. Values were applied in conjunction with the statistical software *JMP Pro 11* to determine sample sizes of statistical relevance.

Results

Cytotoxicity analysis of essential oils

We first compared the botanical extracts in their capacity to reduce cell growth. Both cinnamon oil and garlic oil elicited significant ($p<0.05$) cytotoxic responses in the MDA-MB-231 triple-negative breast cancer cell line after 24 h incubation in a concentration-dependent manner. Even at the lowest tested concentration of 100 μg/ml, cell viability

was reduced by 60 and 70%, by cinnamon and garlic oil, respectively (Figure 1). Frankincense oil did not elicit a comparable reduction of viability even at the highest used concentration. Based on the effective reduction of viability in the MDA-MB-231 cell line, subsequent tests focused solely on the cinnamon oil and garlic oil formulations.

GC-MS analysis of cinnamon oil

To identify the active ingredient, cinnamon oil was analyzed by mass spectrometry. As determined by area under the curve (AUC) mass spectra results, cinnamon oil is comprised of ~73% TC, with other miscellaneous components each accounting for less than 10% of the total cinnamon oil formulation (Figure 2).

GC-MS analysis of trans-cinnamaldehyde uptake

To determine if the bioactive component of cinnamon oil can be taken up by the cells, we incubated MDA-MB-231 cells with defined (50 µg-200 µg) TC concentrations, and measured changes in the medium concentrations. For all conditions, the amount of TC in cell culture media incubated with cells was significantly reduced compared to control samples without cells ($p<0.05$). Chromatographic counts of light scattered cinnamaldehyde molecular units for samples incubated with cells were reduced to almost two-thirds that of the control samples in all cases, indicating that cells are rapidly taking up the TC (Figure 3).

Comparison of active ingredients

To determine if the uptake of the bioactive compounds affected the cells' viability, MDA-MB-231 cells were incubated with increasing concentrations of either the oil or their bioactive compound. Both the garlic and the cinnamon oil induced cytotoxic responses. However, the allyl sulfide, even in the pure form derived from garlic oil, did not induce statistically significant ($p<0.05$) toxic responses, with cell viability remaining between 80 and 90% compared to untreated controls, in contrast to the reduction of cell viability induced by its original garlic oil formulation (Figure 4). This was validated using a one-way analysis of variance (ANOVA) ($\alpha=0.05$, $n=5$). The effects of TC were not statistically different from the effects of cinnamon oil with cell viability at 45% at the lowest concentration and 14% at the highest concentration. This suggests that TC is the predominant active ingredient in cinnamon oil that induced cell death in the MDA-MB-231 cell line. As a result, further analyses exclusively focused on TC to determine the impact on cancer cell function.

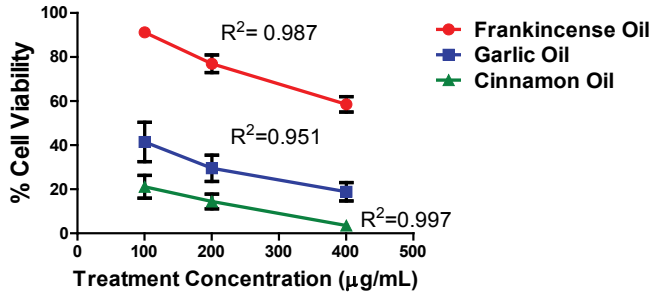

Figure 1: Reduction of viability in MDA-MB-231 TNBC cells post 24 h exposure to select botanical agents. All agents exhibit a linear dose-dependent effect on cells, as depicted by respective high R^2 values.

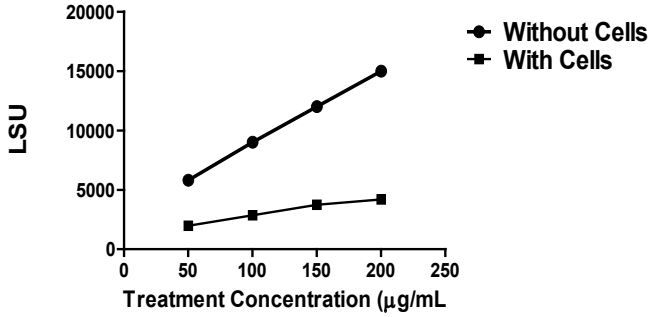

Figure 3: GC-MS analysis of the concentration of TC in solution incubated with and without MDA-MB-231 cells. Results, quantified by light scattered units (LSU), show substantial reductions in the concentration of TC after a 24-hour incubation with cells, suggesting the uptake of the nutraceutical agent, TC.

Phenotype- dependence of cytotoxicity

To determine if the responses to TC are dependent on the disease progression, a model for progressive breast cancer was used by examining cells of varying malignancies: MCF10A (non-tumorigenic), MCF10AT1 (pre-malignant) and MCF-DCIS (local carcinoma, not metastatic). More aggressive lines were not used here because of the lack of response of high-grade breast tumors to treatment with natural compounds in relevant concentrations (Figure 5). In all cell lines, TC elicited significant ($p<0.05$) cytotoxic effects over the course of 72 hours. While a distinct dose-dependent reduction of cell viability in

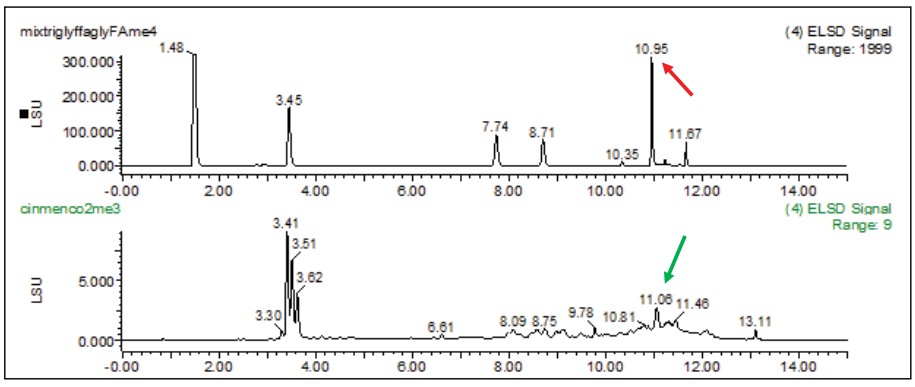

Figure 2: (Top) GC-MS analysis successfully identifies trans-cinnamaldehyde (Red Arrow) through a series of standards at ~ 11 minutes into the analysis. (Bottom) TC is identified again around 11 minutes as ~ 73% of the original cinnamon oil formulation based on the area percentage under the 11.06 min peak (Green Arrow), under the same analysis conditions, suggesting a likely biological significance when present in such high quantities.

the first 24 hours of all treatments was observed in the MCF-10A and MCF10AT1 cell lines, this was less apparent in the carcinoma cell line; however, substantial cell death was seen in all cell lines after 48 and 72 h of treatment. In comparing the level of cytotoxicity at the most relevant concentration of 100 µg/ml, significantly less cytotoxicity was observed in the benign MCF-10A cell line after 24 h compared to the MCF-10AT1 ($p<0.05$) and MCF-DCIS cell lines ($p<0.05$). At later timepoints, the difference in toxic responses between the MCF-10A and MCF-DCIS remained significant ($p<0.05$) (Figure 6). This indicates that the more advanced cancer cells respond with a higher reduction of cell viability despite their described resistance to drug-induced cytotoxicity [27-30].

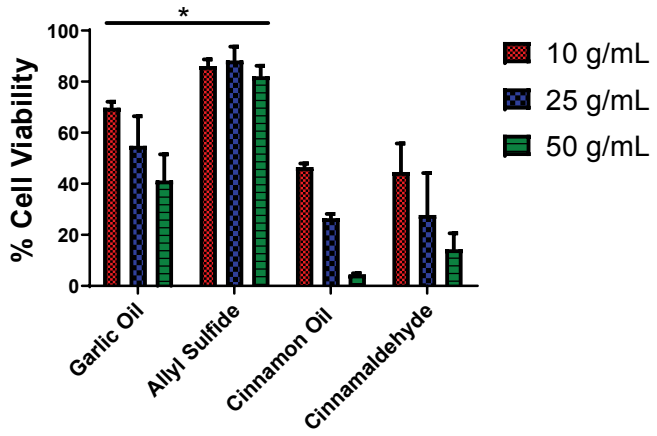

Figure 4: Cytotoxicity of selected botanical agents (garlic and cinnamon oil) and key active ingredients on MDA-MB-231 cells. Asterisks denote that comparison shows the cytotoxic effects of garlic oil and allyl sulfide are statistically different ($p<0.05$) when comparing each respective concentration. The effect of cinnamaldehyde is not statistically different ($p>0.05$) from cinnamon oil. Error bars represent standard deviation.

2D migratory analysis

A wound-healing assay was performed to determine if TC affects the migratory capacity of the cells. Here we used non-toxic concentrations to discern between reduced cell numbers due to the induction of apoptosis and inhibition of motility. As shown in Figure 7A, treatment with TC significantly reduced the migratory capacity of MCF10AT1 cells. There was no difference in migration distance between MCF10AT and MCF10AT1 cells, but significantly less migration in the tumorigenic MCF-DCIS ($p<0.05$). This confirms earlier reports that the migratory capacity of breast cancer cell lines does not correlate with the tumorigenicity of the cells [31]. In all cell lines, the addition of TC significantly ($p<0.05$) reduced migratory capabilities in a concentration-dependent manner compared to controls of cell culture media alone (Figure 7B).

3D migration analysis

To investigate if TC can affect the invasive capacities of the breast

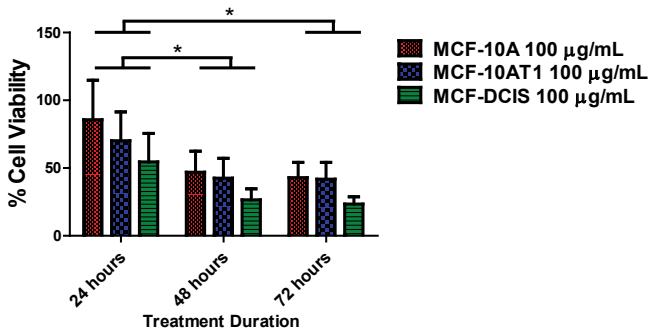

Figure 6: Cytotoxicity analysis comparing all cell lines at a concentration of 100 µg/ml. Significantly less cellular death is observed within 24 hours between all three cell lines ($p<0.05$).

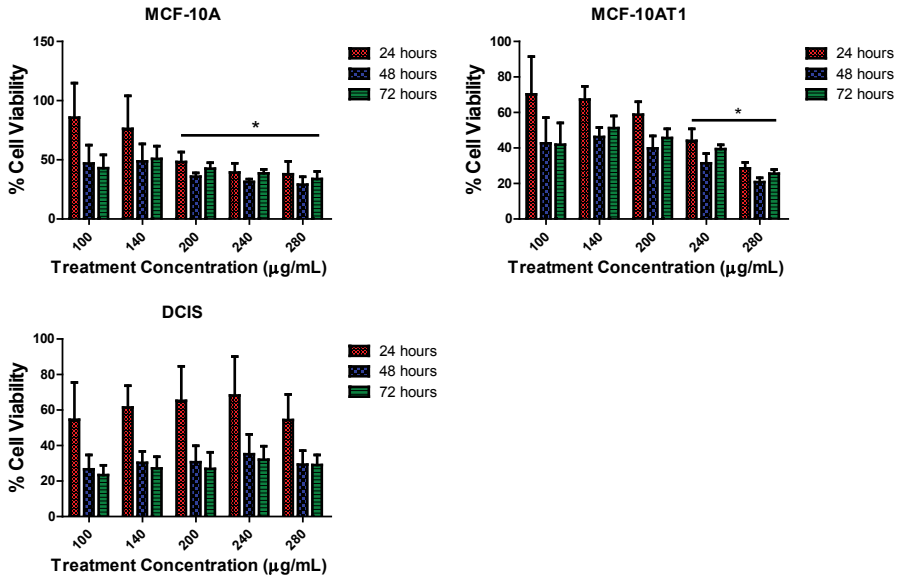

Figure 5: Cytotoxicity of TC in benign (MCF10A) and early breast cancer (MCF-10AT1, DCIS) cells. The initial toxicity to the benign and pre-malignant cells was significantly lower than in the cancer cells. Asterisks denote statistically significant differences ($p<0.05$) across concentrations at their respective time points. Error bars represent SD.

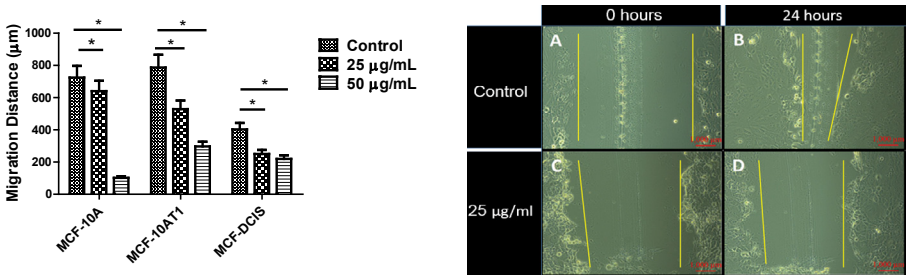

Figure 7: Above: Effects of TC on cancer cell migratory capabilities. The migration of the MCF-10AT1 cells is significantly reduced when comparing controls at t=0 hours (A) and t= 24 hours (B) to cells treated with a low, non-apoptosis inducing concentration (25 µg/ml) of TC at t= 0 hours (C) to t= 24 hours (D). Below: Quantitative analysis of cell migration under exposure to TC. Error bars represent SD ($p<0.05$).

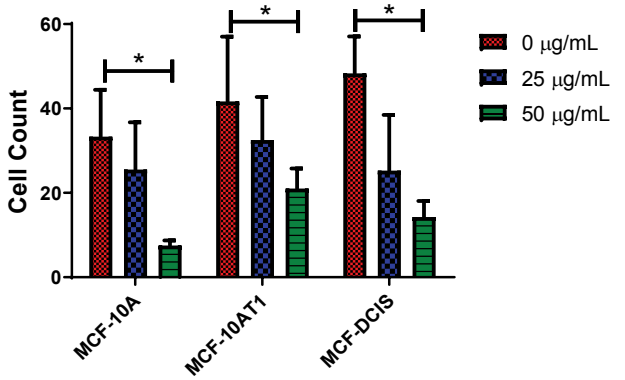

Figure 8: 3D invasion displays a significant reduction in cell migration as TC treatment concentrations increase ($p<0.05$).

cancer cells, their invasion through a collagen layer was evaluated. The invasive capacity of all cell lines in 24 h was very low as expected according to their phenotype; while there was a trend of more aggressive cells (MCF-DCIS) being more invasive, this was highly variable and, thus, not statistically significant ($p>0.05$) However, the invasive capacity was significantly reduced by TC treatment in all cell lines independent of their phenotype ($p<0.05$) (Figure 8).

Discussion

Dietary bioactive compounds are a promising approach to prevent cancer development and progression; however, limitations to a successful cancer suppression might be presented by the aggressiveness of the disease, by the bioavailability of the compounds and the achievement of critical concentrations in the targeted tissue. However, repeated consumption of active components may increase the concentrations over time *in vivo* [24]. Therefore, cell models chosen here represent the potential targets of dietary interventions directed at earlier stages of the disease.

Here we investigated three botanical agents (frankincense, garlic, and cinnamon oil) in their potential to suppress cancer. When applied directly to the highly metastatic MDA-MB-231 triple-negative breast cancer cell line, all three botanical agents show a propensity to inhibit cancer cell survival. Future studies will elucidate the mechanisms through which this process occurs. This effect was shown to be more profound in garlic and cinnamon oils with cinnamon oil producing cancer cell death at clinically desired levels [3]. There was a clear dose-dependent response for cell-death. In comparing the documented active ingredients of two of the essential oils (allyl sulfide for garlic oil and TC for cinnamon oil) allyl sulfide did not exhibit the same cytotoxic effects seen with garlic oil, suggesting it is not solely a key active cytotoxic agent. Conversely, we confirmed TC as the active cytotoxicity-inducing constituent, though it remains possible that alternative compounds within cinnamon oil may play a synergistic role in inducing cell death and that future consideration of cinnamon oil as a whole may be warranted [27]. GC-MS analysis further confirmed that TC was the main and active component in cinnamon oil and quantitatively showed that cinnamon essential oil was predominantly comprised of TC with other individual components accounting for minute amounts.

Significant cytotoxic responses ($p<0.05$) were seen in all cell lines after 48 hours and remained after 72 hours, suggesting that the introduction of TC may benefit as a method of inhibiting the proliferation of resistant stages of breast cancer. Furthermore, even at low concentrations, TC was able to inhibit cellular migration. As treatment concentrations were increased, migratory capabilities were further repressed. Interestingly, there was no association of phenotype and migration capacity; this may be cell-type specific since it has been shown that the highly aggressive MCFCA1 cells have a significantly lower migratory distance than MCF10AT1 cells. The loss of directional migration in more aggressive cancer cells can contribute to more random movements and a lower migratory distance [31]. Furthermore, a switch between a preferentially proliferative or migratory phenotype has been reported [32]; this would correspond the non-invasive phenotype of the ductal carcinoma in situ. Thus, while the cancer cells did not exhibit a high migratory rate, they were highly proliferative and, importantly, both events were suppressed by TC, suggesting a potential to suppress the progression of the cells to a metastatic phenotype. It is important to note that 2D assays, while useful, are not necessarily representative of the physiological environment in which these cancers grow and in some cases cancer growth is affected by their physical dissimilarities [27-29].

The efficacy of this therapeutic warrants further consideration with regards to comparable activities to currently used therapeutics. Current therapies, such as the taxane family (docetaxel, paclitaxel, etc.) [33] are commonly administered systemically using nanomolar concentrations [34,35], as such, we may investigate methods of onsite delivery to counter issues that may arise pertaining to bioavailability.

Overall results suggest that TC can not only induce cell death in cancerous cell lines but may play a larger role in inhibiting the migration of drug-resistant cancers, an effect not commonly seen in current chemotherapies. *In vivo*, disseminating breast cancer cells are likely to form spheroids and eventually secondary tumors [36]. Further work is needed to determine the true effectiveness of TC on a more physiologically relevant model of tumor formation and metastasis as well as determining the detailed mechanism of action through TC

metabolism, prior studies suggest TC may play a critical role in cell cycle arrest [37,38].

Conclusion

This study shows that trans-cinnamaldehyde, the active ingredient in cinnamon, can actively reduce cancer cell growth in both benign and tumorigenic cancer cell lines. The application of trans-cinnamaldehyde has also shown a propensity for inhibiting cancer cell migratory and invasive capabilities. This suggests that trans-cinnamaldehyde could potentially be used in the prevention or treatment of early stages of breast cancer and those that do not respond well to conventional therapies, either alone or as part of a combinatorial treatment strategy. Future studies are needed to determine the mechanisms responsible for inhibiting these biological processes in breast cancers as well as applying treatment to more physiologically relevant models. Furthermore, this research warrants the investigation of trans-cinnamaldehyde and/or cinnamon-based agents as potential cancer therapeutics and a closer examination at the potential ability to reverse or halt disease progression.

Acknowledgements

L. Bickford would like to acknowledge start up support provided by the Department of Biomedical Engineering and Mechanics at Virginia Tech. M. Thompson's contribution to this work was further supported by The Cunningham Doctoral Fellowship. M. Thompson would also like to thank Dr. David Kingston and Mehdi Ashraf-Khorassani, Research Assistant in the Virginia Tech Chemistry Department, for guidance and support regarding the analysis of samples using GC-MS as well as thoughtful discussions.

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