ORIGINAL ARTICLE

The Potentiality of Citral in Targeting Breast Cancer Multicellular Tumour Spheroids (MTS)

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ABSTRACT

Introduction: As the high incidence of breast cancer has a profound impact on a global scale, there is a critical need to improve the clinical outcome of the patients, including efforts to utilize bioactive natural products as treatment or preventive measures. Citral, the essential oil of lemongrass has been reported to possess cytotoxicity in breast cancer cell line . The aim of present study was to determine the capability of citral in targeting aldehyde dehydrogenase-positive (ALDH+) cells in breast cancer cells. **Methods:** Both MCF-7 and MDA-MB-231 cells were cultured in serum-free media to generate multicellular tumour spheroids for the evaluation of citral as an antiproliferative agent. The cells were treated with identified IC_{50} (50±4.30 μM and 56±3.17 μM of citral, respectively) to investigate the cytotoxicity of citral. Staining using Propidium Iodide (PI) and Hoechst 33342 was carried out to determine cell proliferation and viability. Finally, ALDH+ cells were quantified via ALDEFLUOR assay. Analysis of differences was carried out by analysis of variance (ANOVA) and independent t-test with $p<0.05$ considered statistically significant. **Results:** The size of spheroids in both cancer cell lines were reduced after treatment with the citral. PI and Hoechst 33342 staining also revealed that citral gave rise to a mixture of cells that are normal and undergoing apoptosis and necrosis. ALDEFLUOR assay analysis revealed citral significantly ($p <0.05$) inhibited the population of ALDH+ cells in MCF7 cells. **Conclusion:** It was demonstrated that citral reduced the ALDH+ cell population in MCF7 breast cancer spheroids by inhibiting the ALDH activity.

Keywords: Spheroids, Citral, Aldehyde dehydrogenase, MCF7, MDA-MB-231

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INTRODUCTION

Breast cancer is one of the top five cancers in women worldwide and the most common causes of cancer death (1) . This statistical fact proves that breast cancer has become a major and thus requires an advanced research at the molecular level in order to improve the patients' prognosis and treatment (2). Previous studies have reported that death related to breast cancer was largely associated with the relapse or recurrence of the disease even after successful treatments of lumpectomy and/or mastectomy (3). This may be due to less effective and targeted current treatment strategies which primarily target the population differentiated and fast-growing cells in tumour. As such, the population of cells which not targeted is able to evade these treatments and becomes metastatic and chemo-resistant (4). Hence, these reasons provide a rationale towards a new treatment strategy that specifically targets the treatment-evading cells.

In the search of new strategies to treat or prevent cancer, natural products have been extensively studied for its phytochemicals that greatly exhibit anti-carcinogenic properties (5). Scientific studies also revealed that natural products or phytochemicals are advantageous in cancer treatment by acting directly on specific molecular genes as well as indirectly initiating conjugation in the metabolic pathways (6). Moreover , numerous evidences have shown that natural phytochemicals have the ability to counter the epigenetic changes that are present in cancer progression (7). Therefore, natural-based treatments have been repeatedly proposed as effective adjuvants in combination with the conventional therapy to reduce the possible side effects arising from the conventional treatments (8).

Currently, hormonal drugs that are commercially available for breast cancer treatment, such as tamoxifen

and aromatase inhibitors including exemestane and anastrozole, have been proven to be effective (9). However, despite their effectiveness in treating breast cancer, they come with undesirable side effects which may increase the risk of developing endometrial cancer (10). Besides, over a long-term administration of tamoxifen, breast cancer patients started to acquire resistance against the drug (11). Therefore, researchers turn to natural products that exhibit promising chemo-preventive activity with minimal side effects and less toxicity as shown in several *in vivo* and *in vitro* studies (12,13). One of many studied natural compounds is citral (3,7-dimethyl-2,6-octadienal) which is a key component of essential oils extracted from citrus based plants such as lemon and lemongrass that consists two isomeric aldehydes known as geranial (α-citral) and neral (β-citral) (14). Citral has been commercially used in the pharmaceutical industry to synthesis vitamin A and different types of acetals for aroma and beauty care products (15).

As for its medicinal value, based on toxicity studies in *in vivo* and *in vitro* models, citral was found to be non-toxic and non-carcinogenic (16,17). Additionally , an *in vitro* study conducted by Chaoki et al . (18) reported that citral treatment dramatically conferred inhibition of cell proliferation and induced apoptosis in MCF7 cells, which supported a previous study done on HL-60 human leukemia cell line (18,19). These *in vitro* antiproliferative effects of citral on cancer suggested that citral may have promising potential as cancer chemopreventive agent. Previous study reported the ability of citral in inhibiting growth of MDA-MB-231 spheroids at a lower IC_{50} value compared to its monolayer counterpart through the induction of apoptosis in dose dependent manner. Citral was also found to reduce the percentage of ALDH+ cells, which are responsible for the self-renewal capacity of MDA-MB-231 spheroids (20).

Thus, the present study aimed to demonstrate the potential of citral, the key component of essential oils extracted from lemongrass (*Cymbopogon citratus*), in targeting the cells population which is marked by the activity of ALDH in the breast cancer spheroids. ALDH is widely recognised as the signature of breast cancer stem cells and ALDH+ breast cancer cells often evade and become highly resistant against the treatment's regimens. The findings of this work will provide a rationale strategy for successful elimination of cancer, which is reflected to their heterogeneity and cellular plasticity in the presence of various factors in the microenvironment.

MATERIALS AND METHODS

Cell lines and reagents

MDA-MB-231 and MCF7 breast adenocarcinoma cell lines were used in this study to generate breast cancer spheroids as spheroids tumour models. These cell lines were purchased from the American Type Culture Collection (ATCC) and cultured in DMEM and RPMI media respectively, in a monolayer manner prior to experiments.

Identification of citral using Gas Chromatography Mass Spectrometry (GS-MS)

Citral ($C_{10}H_{16}O$) or 3, 7-dimethyl-2, 6-octadienal was purchased from Sigma Aldrich, USA (CAS 5392-40-5). Citral comes in a pale-yellow liquid in a strong lemon odour with the assay percent range of 95% in the essential oils of plants (14). Identification and purity of citral was confirmed using gas chromatography-mass spectrometry (GCMS) by comparing retention time and mass spectra with the libraries of Flavour & Fragrance Natural & Synthetic Compounds (FFNSC1.3) and WILEY229. The column used was RxiTM-5ms fused silica capillary low polarity column (30.0 m length 0.25 mm internal diameter, 0.25 µm thickness). The injection mode of the gas chromatograph was set to split mode with split ratio 10.0. The initial column temperature was 50.0 °C, followed by increases to 300.0 °C at 3.00 °C/min. The gas chromatograph operating conditions were as described in Table I. The relative area of the peaks in the chromatogram was used to estimate the relative quantification of each component. The stock solution of citral (1M) was prepared in absolute of dimethyl sulfoxide (DMSO) and was stored at -20°C until further use.

Generation of breast cancer spheroids

For the generation of breast cancer spheroids, liquid overlay technique (LOT) was utilised in which the wells of the culture plate (Costar Corning, USA) were coated with 0.6 % agarose which were dissolved in distilled water and autoclaved for sterilization before application (21) . Upon attaining 80% confluence, MCF7 and MDA-MB-231 cells were recovered by using 0.25 % trypsin in phosphate buffered saline (PBS) (Sigma, USA). To determine the optimum cell seeding number, optimization was done by seeding the cell lines onto agarose-precoated 6-well plates at a density of 15,000, 20,000 and 25,000 cells/well in serum free media supplemented with growth factors as previously described (22). The generation of spheroids were observed after 5 days of culture by using inverted microscope (Olympus, CKX41, USA). The average size distribution was then measured using ImageJ software for the different cell densities. The suitable cell seeding density was identified and was used for subsequent analysis.

Cytotoxicity assay

As described previously by Pereira et al. (23) cells were seeded at 3000 cells/well in agarose coated 96-well plates and incubated at 37°C with 5 % carbon dioxide (CO_2) for 48 hours for the formation of spheroids . Then, the spheroids were treated with six different concentrations of citral (250, 100, 50, 25, 5 and 0.5

Table I: List of identified compounds of citral determined using Gas chromatography-mass spectrometry (GC-MS)

Peak	Compound	Library	LRI	SI (%)	Composition (%)	Reference
1	*trans*-Rose oxide	FFNSC1.3.lib	1133	83	0.60	Babushok et al. (2011)
2	*cis*-Isocitral	FFNSC1.3.lib	1164	92	0.80	Adams et al. (2017)
3	*trans*-Isocitral	FFNSC1.3.lib	1182	93	1.37	Adams et al. (2017)
4	Neral *	FFNSC1.3.lib	1247	94	43.43	Babushok et al. (2011)
5	Geranial **	WILEY229.lib	1277	95	44.35	Babushok et al. (2011)
6	UNKNOWN	N/A	1285	N/A	1.30	N/A
7	Neryl dimethyl acetal	WILEY229.lib	1318	83	3.25	Yannai et al. (2012)
8	Geranyl dimethyl acetal	WILEY229.lib	1342	87	4.90	Yannai et al. (2012)

* also known as *cis*-citral
** also known as *trans*-citral

Chemical compositions of citral determined using GC-MS analysis were identified based on reference library, Linear Retention Indices (LRI) and Similarity Index (SI). The area under each peak represent the percentage of each compounds identified in citral.

μM) for 72 hours. By applying quantitation of cellular adenosine triphosphate (ATP) as a measure of viability, 50 μL of CellTiter-Glo® 3D reagent (Promega, USA) was added to each well. The plates were then orbitally shaken for 15 minutes at room temperature to facilitate lysis. In addition, the spheroids will be triturated six times to allow complete lysis. Then, the well contents were transferred to a white-bottom 96- well plate before reading on luminescence plate reader. The cell viability was calculated in comparison to the untreated cells, and the 50% cells growth inhibitory concentration (IC_{50}) was estimated by interpolating the generated graph. The experiment was performed independently three times. The IC_{50} values of citral were used for subsequent experiments.

Measurement of breast cancer spheroids after citral treatment

Five days after initial seeding of 20,000 cells/well on agarose-coated 6-well plates, MCF7 and MDA-MB-231 spheroids were treated with 50 μM and 56 μM of citral, respectively, for 72 hours. The size of spheroids formed after treatment were analysed using ImageJ software (24) and the data is presented as mean ± standard deviation (SD).

Cell death analysis

Cell viability of breast cancer spheroids was monitored using double staining of Hoechst 33342 (Biotium, USA) and propidium iodide (PI) (Sigma, USA). Initally, breast cancer spheroids of MCF7 and MDA-MB-231 were generated by culturing 20,000 cells/well in agarose-coated 6-well plate. After 5 days of culture, spheroids were treated with citral at concentration of previously identified IC_{50} values for 72 hours. Then, the spheroids were harvested, washed with PBS and centrifuged at 1500 rpm for 7 minutes. The supernatant was removed prior to the addition of ice-cold ethanol for cell fixation and the fixed cells were incubated in 4°C for 30 minutes. After series of washing and centrifugation steps, Hoechst 33342 was added to the cell suspension and mixed thoroughly prior to incubation at 37°C for 15 minutes. The washing and centrifugation steps were then repeated before PI was added in the cell suspension and mixed thoroughly. Next, the cells were incubated at room temperature for 15 minutes in light-protected area. Finally, the stained cells were washed with PBS and analysed by Olympus CKX41 Zeiss Axio Vert.A1 inverted microscope equipped with ZEN Lite software (Zeiss, German) by colours intensity exhibited by both cell lines.

ALDEFLOUR Assay

Upon citral treatment as previously described, cells were extracted from spheroids of MCF7 and MDA-MB-231 for the quantification of ALDH-positive cell population. In order to detect and quantify the cancer stem cells population, the ALDEFLOUR Stem Cell Identification and Isolation kit (Stem Cell Technologies, USA) was used and analysed via BD LSRFortessa flow cytometer (BD Bioscience, USA). According to the manufacturer's protocol, single cells from the untreated and citral-treated breast cancer spheroids were incubated in ALDEFLOUR assay buffer containing BODIPY-aminoacetaldehyde-diethyl acetate, BAAA-DA and ALDH substrate at 37°C for 30 minutes. For each experiment, a collection of cells from each sample was incubated with the ALDH inhibitor, diethylaminobenzaldehyde (DEAB) as a negative control in this assay. The cells were kept on ice prior to flow cytometry analysis.

Statistical analysis

All experiments were carried out in triplicates and the results were expressed as mean ± SD which were calculated by using IBM SPSS Statistics 23 (SPSS Inc., Chicago, USA) and Graphpad Prism 7 software (Graphpad Prism, California). Statistical analysis of differences was carried out by analysis of variance (ANOVA) and independent t-test. A $p<0.05$ value was considered to indicate statistical significance.

RESULTS

Identification of citral using Gas Chromatography Mass Spectrometry (GC-MS)

The constituents of citral were identified based on their mass spectral data using Shimadzu GC-2010 Plus Gas Chromatography (Shimadzu Corporation, Japan) at

Laboratory of Natural Products, Institute of Bioscience, UPM. Based on the GC-MS analysis, the commercially obtained citral is 87.78% pure with the identification of two major component of citral which were neral (43.43%) and geranial (44.35%) with search index (SI) score of 94 and 95, respectively (Figure 1). Additionally, all identified compounds were compared to the linear retention indices (LRI) reported by previous studies (Table 1) (25–27).

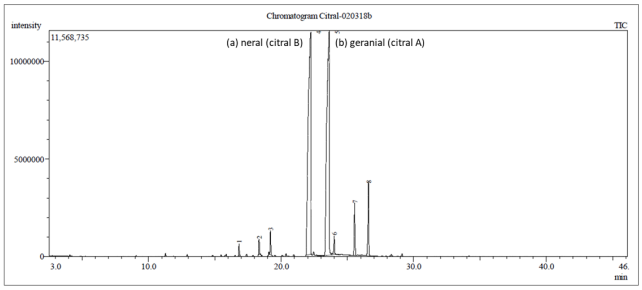

Figure 1: GC-MS chromatogram of citral. Total of eight peaks were identified by gas chromatography-mass spectrometer. The two main peaks indicate the major component of citral which are (a) neral (*cis*-citral) and (b) geranial (*trans*-citral).

Optimization of cell seeding number for breast cancer spheroids generations

In order to obtain reproducible number of spheroids, optimization of cell seeding number was carried out by culturing MCF7 and MDA-MB-231 cell lines with different cell densities which were 15,000, 20,000 and 25,000 cells/well in agarose pre-coated 6-well plates, respectively. The formation of spheroids was observed under the inverted microscope on day 5 of culture. Visually, size of spheroids increased with increasing cell density in both cell lines. However, based on analysis of variance (ANOVA) with Tukey's multiple comparisons test, there was no significant difference between different cell densities for both MCF7 and MDA-MB-231 (Figure 2). Based on this analysis, the cell density of 20,000 cells/well was chosen for subsequent analysis as demonstrated by small error bars presented in statistical testing for both cell lines.

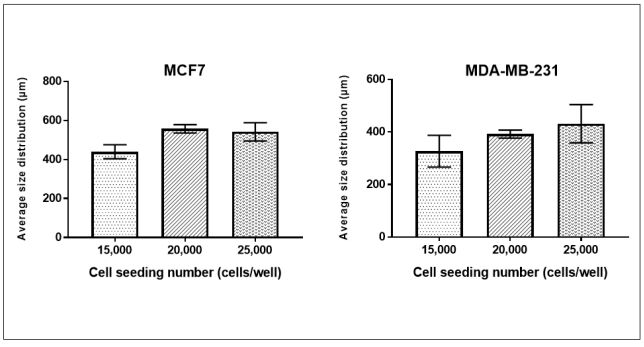

Figure 2: Optimization of cell seeding number. The growth and formation of MCF7 and MDA-MB-231 spheroids in serum free media observed under the inverted microscope at day 5 of culture.Analysis of variance (ANOVA) on the breast cancer spheroids formation measured by their sizes. Data was presented as mean (n=5) ± SD, p<0.05.

of spheroids was examined and measured by using ImageJ software on day 8 of culture. Citral inhibited significantly the average size distribution of MCF7 spheroids in Figure 4 ($p < 0.05$).

Induction of apoptosis by citral in breast cancer spheroids
Induction of apoptosis was determined based on double staining with Hoechst 33342 and propidium iodide that enabled distinct visualization of the viable, necrotic

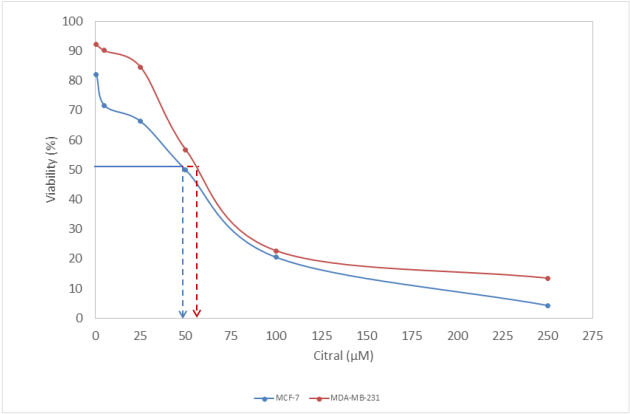

Figure 3: Cytotoxicity of citral on breast cancer spheroids. Cell viability of MCF7 and MDA-MB-231 spheroids after 72 hours of incubation with citral in different concentration ranging between 0.5 and 250 µM. The viability is presented as percentage relative to the untreated cells. The dotted arrows indicate IC_{50} values of each cell lines.

Cytotoxicity of citral in MCF7 and MDA-MB-231 spheroids

After 72 hours of treatment with six different concentrations of citral (250, 100, 50, 25, 5 and 0.5 µM), the viability of citral-treated cells was measured by using CellTiter-Glo® 3D assay kit (Promega, USA). Based on Figure 3, citral was cytotoxic against MCF7 and MDA-MB-231 spheroids with IC_{50} values of 50±4.30 µM and 56±3.17 µM, respectively.

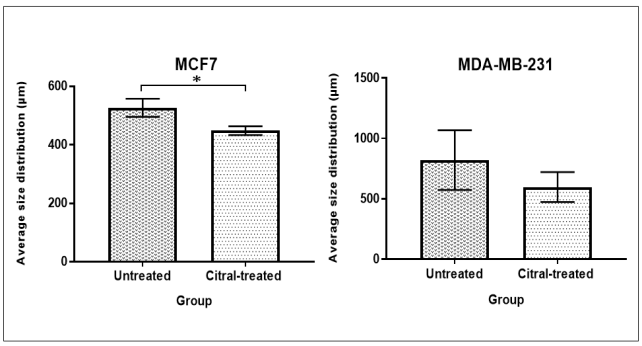

Figure 4: The effect of citral on breast cancer spheroids size. Citral exhibited inhibition on MCF7 and MDA-MB-231 spheroids in number and size at the IC_{50} values of 50 µM and 56 µM, respectively ($p<0.05$).

Antiproliferative effect of citral on spheroids size

To determine the effect of citral on the size of breast cancer spheroids, both cell lines were treated with identified IC_{50} values of citral for 72 hours. The formation

and apoptotic cells. To be specific, viable cells intensely appeared in blue whereas apoptotic cells showed sign of nuclear condensation and appeared red (Figure 5). As shown in Figure 5, treatment of citral in both breast cancer spheroids gave rise to a mixture of cells that are normal and undergoing apoptosis and necrosis .

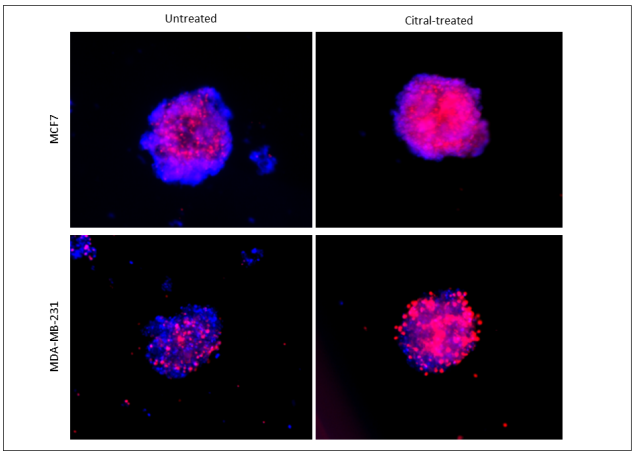

Figure 5: Effect of citral on induction of apoptosis in breast cancer spheroids. The spheroids of MCF7 and MDA-MB-231 were stained with Hoechst 33342 and propidium iodide (PI) and viewed under fluorescence microscope with 100x magnification. The scale bar represents 200 µm in length.

Inhibition of ALDH+ cell population following citral treatment in breast cancer spheroids

In order to determine the population of ALDH+ breast cancer cells after citral treatment, ALDEFLUOR assay was carried out in accordance to the manufacturer's instructions. In details, cells that expressed high level of ALDH were brightly fluorescent. Based on the flow cytometry analysis (Figure 6) citral reduced the population of ALDH+ breast cancer cells in MCF7 from 46.25 % ± 4.46 in the untreated group to 26.30% ± 4.81 (Figure 6(a)). However, the outcome was contradictory in MDA-MB-231 cells in which the percentage of ALDH+ cell after citral treatment was higher (33.50% ± 14.00) than the untreated group (25.10% ± 11.17) (Figure 6(b)). Regardless, the flow cytometry analysis revealed that citral was able to inhibit the activity of ALDH in MCF7 spheroids which may be responsible for the relapse of breast cancer.

DISCUSSION

As an alternative to cancer therapy, natural product-based treatments have been in line with other research not only because it is cost-effective but also less or not toxic. Moreover, since the last decades, natural products have been successful in increasing life span of our society by reducing pain and suffering as well as contributing to the revolutionized medicine (28). The two main components of citral identified using GS-MS revealed neral and geranial as two terpenoid geometric stereoisomers geranial (trans-citral) and neral (cis-citral). Study by Zeng et al. (29) reported geranial was more potent that neral when tested on mice bearing 4T1 xenograft tumors via autophagy mechanisms of tumor growth inhibition in p53-null 4T1 cells. Eventhough the two isomers of citral, cis and trans forms differ only in their conformation, the bioactivities of these two isomers to respond against different subtypes of breast cancer need to be elucidated separately.

3-dimensional (3D) culture model is a very useful approach to study cancer biology. Particularly , the model is more relevant in drug testing and translational study as it resembles the *in vivo* tumour setting better than the conventional monolayer approach (30). However, various conditions or variables need to be carefully chosen in order to obtain the most ideal 3D model. In the present study, the liquid overlay technique (LOT) using agarose-coated plate was chosen to develop 3D spheroids of breast cancer. This technique does not only generate reproducible multicellular tumour spheroids, it is also cost-effective, simple and easy to handle, do not labour intensive and specialized equipment and allows high throughput production of spheroids (31–33).

However, a number of factors need to be considered to ensure accurate and reproducible evaluation of therapeutics efficacy by guaranteeing the homogenous distribution of drug molecules within the microenvironment (21). The cell density of 20,000 cells/well was chosen for subsequent analysis in both cell lines to produce high number spheroids. The same amount of cell density was also utilized by Gong et al. (34) for generating MCTS of MCF7 cells and by Metzger

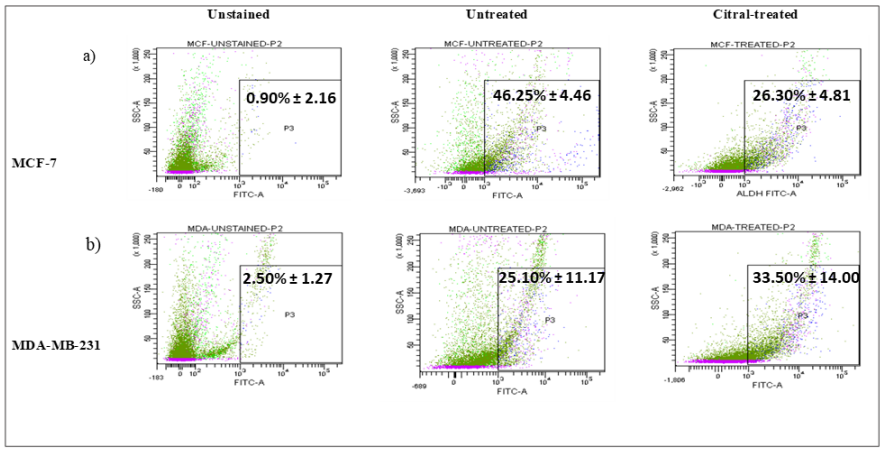

Figure 6: Effect of citral on population of ALDH+ cells in MCF7 and MDA-MB-231 breast cancer spheroids. Flow cytometry analysis using ALDEFLUOR assay was performed to determine the population of ALDH⁺ breast cancer cells after citral treatment of 72 hours with its determined IC50 values for both breast cancer spheroids. The P3 quartile represent the brightly fluorescent ALDH+ cells.

et. al (35) for co-culture spheroids that was developed using LOT.

The optimization showed that the number and diameter of all spheroids increased in a rate proportional to the initial cell density. This is in agreement with Chaouki et al. (18)(14) which discovered that higher initial cell number could lead to an increased number of spheroids with attained average size larger than 500μm. In this study, higher cell seeding density were utilized to obtain higher number of formed spheroids for subsequent analyses.

A compound is considered highly cytotoxic if the IC_{50}value is less than 50 μM, mildly cytotoxic if the IC_{50} value is in between 50 to 100 μM, and regarded as inactive with IC_{50} value more than 100 μM (36). Citral also significantly reduced the average size distribution of MCF7 spheroids, but not MDA-MB-231 spheroids. Based on molecular and morphological profiles, various reported studies have classified MDA-MB-231 cell line as the claudin-low, basal-like subtype or commonly known as the triple negative subtype (31)(37). The morphological features of the subtypes in tumors and cell lines accord well, with luminal tumors having better prognosis and luminal cell lines less aggressive than that in triple negative tumors and cell lines (37).

On the other hand, MCF7 cells was classified as the luminal A subtype. This suggests that the anticancer effect of citral is may be subtype-specific in breast cancer. Nevertheless, based on Hoescht 33342/propidium iodide staining, citral treatment gave rise to a mixture of cells that are normal and undergoing apoptosis and necrosis . This result was in line with previous study showing that citral may have a pro-apoptotic effect on the human leukaemia HL-60 cell line (15). Study reported by Sanches et al. (38) also reported the ability of citral to induce apoptotic and necrotic patterns of cell death on melanoma cells via annexin V-FITC/PI staining, and further confirmed by TUNEL assay. Besides, treatment of citral in combination with doxorubicin on human lymphoma Ramos cell line enhanced the expression pro-apototic protein BAK but significantly reduced the expression of anti-apoptotic protein BCL-XL (39) .

Additionally, citral has also been reported to induce the activation of caspase 3, an established marker of apoptosis, in tumour cell lines (40). The staining also indirectly revealed the nature of 3D spheroids which resembles poorly vascularized tumours following an inherent metabolic gradient. In particular, the external, intermediate, and inner layers of the tumour are made up of proliferative cells, quiescent cells and necrotic cells, respectively (21). Therefore, it is not surprising to observe the inner layer or core stained in red, as shown in Figure 5, representing necrotic cells.

Up to this point, citral has shown to be anti-proliferative and pro-apoptotic in breast cancer spheroids, particularly the MCF7 cells. A study by Nigjeh et al. (20) has reported the ability of citral in targeting ALDH+ breast cancer spheroids in MDA-MB-231 cell line, which was contradicted with the findings in the present study. The reason for this is not known but may be due to the different spheroid culture conditions or different subtypes of breast cancer. The former reason might occur during sample preparation in which doublets containing both fluorescent positive and negative cells would be detected as fluorescent positive, leading to increased false positives. As for the latter, it is possible that in MDA-MB-231 spheroids, citral was only successfully able to inhibit the proliferative cells in the outer layer of the spheroids, but not the ALDH+ cells population in the inner core. This phenomenon may explain the increase of ALDH+ cells in MDA-MB-231 spheroids following citral treatment as citral is hypothesised to induce the activation of dormant CSCs in the breast cancer spheroids. Aldehyde dehydrogenase-positive cells (ALDH+) have been reported to exhibit stem-like characteristics including self-renewal and drug resistance which play important roles in breast cancer aggressiveness (41). This would make the ALDH+ cell population appear higher in the treated group, although the cell population in the external layer was the only one decreased. This finding demonstrated the MDA-MB-231 cells exhibit stronger metastatic capacity and aggressiveness compared to the MCF7 cell line. However, a more extensive analysis is required to confirm this scenario. However, the flow cytometry analysis revealed that citral was able to inhibit the activity of ALDH in MCF7 spheroids, which could be responsible for the occurrence of relapse in breast cancer.

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CONCLUSION

In the present study, the effect of citral on MCF7 and MDA-MB-231 breast adenocarcinoma spheroids was determined. Citral was able to significantly inhibit the growth of MCF7 spheroids, induce apoptosis and reduce the ALDH-positive cells population. This suggests that citral may be effective against luminal A subtype of breast cancer represented by MCF7 cells, but not against the triple negative subtype represented by MDA-MB-231 cells. Besides, the use of a 3D culture system yields a more relevant analysis that is comparable to the *in vivo*

setting. However, more extensive studies conducted for citral to be utilised in clinical setting, particularly on its role in inducing apoptosis and its effect on molecular markers aside from ALDH that represent cells that are resistant to therapies. Nevertheless, citral has promising potential as a putative agent against the recurrence of breast cancer.

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