Unveiling the anticancer effect of citral through inhibition of stemness in estrogen-positive breast cancer

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ABSTRACT

Introduction: Breast cancer remains a significant challenge due to disease recurrence and metastasis, often attributed to the persistence of treatment-resistant breast cancer stem cells (BCSCs). Citral, a compound derived from lemongrass essential oil, has demonstrated cytotoxic effects on various cancers, including breast cancer. This study investigates the anticancer mechanisms of Citral in BCSC-enriched 3D cultured spheroids and evaluates its therapeutic potential for estrogen receptor-positive breast cancer. **Methods**: Using flow cytometry, the $CD44^+CD24^-$ population was analyzed, and real-time PCR was employed to measure the expression of ALDH isoforms and pluripotency genes. Additionally, the Nanostring nCounter® PanCancer Pathway Panel was utilized to identify gene expression changes in cancer-related pathways. **Results**: Citral treatment significantly reduced spheroid size and the $CD44^+CD24^-$ stem-like cell population, accompanied by downregulation of ALDH isoforms and pluripotency genes. Gene expression analysis revealed Citral's modulation of key pathways, including PI3K/Akt signaling, cell cycle control, DNA damage response, and apoptosis. **Conclusion**: These findings underscore Citral's potential as a promising anticancer agent, particularly for targeting estrogen receptor-positive breast cancer cells and BCSCs. Further preclinical and clinical studies are warranted to explore its therapeutic applications.

Key words: Cancer stem cells, Spheroids, Citral, Breast cancer

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INTRODUCTION

Worldwide, cancer continues to be the leading cause of death, with almost 20 million new cases and 9.7 million cancer deaths in 2022[1]. The Global Cancer Observatory 2022 (GLOBOCAN 2024) reported that female breast cancer was ranked second with 2.3 million new cases (11.6%), followed by other cancer types. These data indicate that breast cancer is responsible for one out of every four cancer cases and one out of every six cancer deaths[1]. Despite advancements in treating breast cancer, significant challenges remain, particularly when the disease is detected at advanced stages. Furthermore, disease recurrence and metastasis limit treatment options, leaving patients with an unfavorable prognosis and often resulting in succumbing to the disease. Recurrence is especially challenging in the treatment of estrogen receptor-positive breast cancer, as patients can remain at risk of developing secondary cancer many years after treatment[2]. The ultimate cause of breast cancer recurrence and metastasis remains unknown[3]; however, research has identified the presence of breast cancer stem cells (BCSCs) within tumors as the primary drivers[4,5]. BCSCs are cells that can regenerate and differentiate, similar to normal stem cells, but are highly tumorigenic[6]. First identified by Al-Hajj *et al.*[7],

this unique cell population expresses surface markers, including $CD44^+CD24^-$, aldehyde dehydrogenase (ALDH), and epithelial-specific antigen (ESA) or CD326 (EpCAM)[8,9]. The presence of BCSCs in breast cancer hinders treatment, as these cells are reported to be intrinsically resistant to chemotherapy and radiotherapy[10,11]. Consequently, non-targeted cancer therapies can eliminate the proliferative and more differentiated cells that comprise the tumor mass. However, treatment-resistant BCSCs possess the capacity to regenerate the tumor gradually, which can lead to cancer relapse due to their self-renewal capability. Therefore, it is crucial to identify alternative approaches to cancer therapy that can directly target BCSCs to prevent cancer recurrence. The use of natural products for cancer prevention and treatment is gaining interest in drug discovery, particularly because they offer a rich source of novel therapeutic agents with minimal side effects[12]. Current therapy regimens primarily eliminate fast-growing cancer cells, leaving behind quiescent, drug-resistant BCSCs that can subsequently regrow tumors[13]. Thus, it is essential to identify potential drug candidates that can directly target BCSCs, thereby improving patient survival. Citral (3,7-dimethyl-2,6-octadienal), a naturally occurring essential oil derived from herbal plants

such as lemongrass, has been reported to exhibit anti-cancer effects [14–16]. However, its effect on BCSCs remains unclear. Our previous studies have shown that citral can target drug-resistant breast cancer cells [17], including its ability to inhibit the expression of the cancer stem cell marker ALDH1A3 [18]. Nevertheless, the mechanism behind these effects is still poorly understood, particularly in estrogen receptor-positive breast cancer cells. The organization of cells in a 3D manner provides better insight into the tumorigenesis mechanism of in vitro cancer models, although fully replicating the original tumor microenvironment remains a challenge. The 3D culture system also offers a promising link between traditional 2D cultures and animal models, making it a crucial tool for advancing research in tumor biology [19]. Therefore, this study explored the anticancer effect of citral on BCSCs and demonstrated that citral is a potent anticancer agent capable of reducing the size of generated breast cancer spheroids using a three-dimensional (3D) culture model. This model is believed to be an effective method for enriching BCSCs from breast cancer cell lines, making it a valuable tool for studying cancer stem cells. In the present work, we discovered that citral inhibits the expression of pluripotency genes and ALDH isoforms and reduces the number of $CD44^+CD24^-$ stem-like cells. Citral's mechanism of action on breast cancer spheroids was elucidated through the regulation of various pathways involved in breast cancer, including cell cycle, DNA damage, apoptosis, and stem cell pathways.

METHODS

Analysis of Citral by Gas Chromatography-Mass Spectrometry (GCMS)

To determine the purity and components of citral, also known as 3,7-dimethyl-2,6-octadienal (obtained from Sigma Aldrich; CAS 5392-40-5), a study was conducted using a Shimadzu GCMS QP2010 Ultra gas chromatography-mass spectrometry system from Shimadzu Corporation, Japan. The analysis utilized an Rxi^{TM}-5ms fused silica capillary column with polarity (30.0 m $\times$ 0.25 mm I.D. $\times$ 0.25 μm film thickness) from Restek Corporation, Bellefonte, PA, USA. The chromatographic conditions involved a temperature range from 50.0 °C to 300.0 °C (held for 10 minutes) at a rate of 3 °C/min. The sample injection took place at 250 °C using helium (99.9%) as the carrier gas at a flow rate of 0.8 mL/min, with an injection volume of 1 μL and a split ratio of 10:1. In terms of mass spectrometry operation, the ion source was set at a temperature of 200 °C; electron ionization was performed at 70 eV using a mode covering a mass scan

range of m/z 40–700. Detection of the components in citral was based on comparing the obtained mass spectra with libraries such as NIST11.lib, NIST11s.lib, FFNSC1.3.lib, and WILEY229.LIB, along with cross-references to existing literature sources. Each component was tentatively quantified based on the relative peak areas in the chromatogram.

Cell Culture

The MCF7 breast cancer cell line (human estrogen receptor-positive), sourced from the human cell culture collection of the UPM-MAKNA Cancer Research Laboratory, Institute of Bioscience, Universiti Putra Malaysia, Malaysia, was used in this study. The cells were grown in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS) at 37 °C in a 5% CO_2 incubator (Thermo Fisher Scientific, USA).

Generation of Breast Cancer Spheroids

A 0.6% agarose solution was used to coat cell culture vessels after solidification for 30 minutes at ambient temperature under sterile conditions. To generate breast cancer spheroids, breast cancer cells were cultured in the agarose-coated wells in serum-free DMEM/F-12 medium containing insulin (10 μg/mL), gentamycin (10 μg/mL), human fibroblast growth factor (hFGF) (0.1 μg/mL), hydrocortisone (1 μg/mL), antibiotic-antimycotic (50$\times$), and B27 supplement (50$\times$). Spheroids were grown for 5 days in a CO_2 incubator without disturbing the culture plates and analyzed using an inverted microscope.

CellTiter-Glo® 3D Assay

The cytotoxicity of citral on breast cancer spheroids was assayed using the Promega CellTiter-Glo® 3D reagent (Promega, USA). The CellTiter-Glo® 3D assay is an ATP-based assay that quantifies luminescent signals produced by the cells. This assay incorporates lytic activity to enhance penetration into cell aggregates such as spheroids, allowing full quantification of cell viability [20]. In each well of a 96-well plate coated with agarose, 3,000 cells were plated in 100 μL of spheroid growth medium. Breast cancer spheroids were formed by culturing the cells for 48 hours. These spheroids were then treated with concentrations of citral ranging from 0 μM to 500 μM for 72 hours. After treatment, CellTiter-Glo® 3D solution was added to lyse the spheroids by shaking them for 5 minutes in a microplate reader. The plate was incubated at room temperature for 25 minutes before measuring the luminescence signal using a microplate reader. IC_{50} values for citral on breast cancer spheroids were

determined by creating a dose-response curve using GraphPad Prism 7.0 software. The identified IC_{50} was used for subsequent experiments, and the effects of citral treatment were compared to the untreated group. The IC_{50} is commonly used to measure the drug concentration required to inhibit 50% of a biological process, helping researchers understand the drug's potency in pharmacological research[21]. The CellTiter-Glo® 3D experiments were conducted three times (n=3).

Effects of Breast Cancer Spheroids after Citral Treatment

Following the 72-hour citral treatment, breast cancer spheroids of MCF7 were observed under an inverted microscope. ZEISS ZenPro software was used to capture images of breast cancer spheroids that were both untreated and treated with citral. Subsequently, the size of the spheroids was measured using ImageJ software[22]. The size measurements obtained from both spheroids were recorded, and the results were analyzed by an unpaired t-test using GraphPad Prism 7.0 software.

Immunophenotyping of CD44+/CD24- Breast Cancer Stem Cells

Two surface markers, $CD44^+$ and $CD24^-$, were further assessed to identify the population of breast cancer stem cells (BCSCs). The staining steps for immunophenotyping of $CD44^+CD24^-$ BCSCs were conducted by incubating the cell suspension with conjugated antibodies on ice and protected from light for 30 minutes. Following the manufacturer's recommendations, the conjugated antibodies were diluted in FACS buffer to prepare antibody solutions. The cell suspension was stained concurrently with both antibodies for double staining (CD44-FITC/CD24-PE). Following incubation, the cells were collected by centrifugation at 1000 rpm for 5 minutes. After discarding the supernatant, the cells were washed once with ice-cold FACS buffer. To ensure a uniform, single-cell population, the cells were resuspended in 1 mL of FACS buffer and passed through a 40 μm cell strainer into FACS tubes. Stained cells were kept on ice and in the dark before being analyzed. A BD Fortessa® flow cytometer was utilized to assess the percentage of stained cells.

Quantitative Reverse Transcriptase Polymerase Chain Reaction (RT-qPCR)

All primers were designed using the online NCBI Primer-Blast tool available at https://www.ncbi.nlm.n ih.gov/tools/primer-blast/ and were referenced to the NCBI Gene database. The specificity of PCR amplification was ensured based on guidelines for RT-qPCR primer design provided by Integrated DNA Technologies (IDT), Singapore, available online. All primers (**Table 1**) were synthesized by Integrated DNA Technologies (IDT), Singapore. The melting temperature (Tm) of each primer pair was optimized by conventional PCR. Total RNA from breast cancer spheroid cells was isolated using the Total RNA Isolation Kit (Pure Direx, Bio-Helix, Taiwan), and cDNA was prepared using the Tetro^{TM} cDNA Synthesis Kit (Bioline, UK). The synthesized cDNA was prepared before RT-qPCR experiments or stored at -20 °C for long-term preservation.

The qPCR was conducted using a Bio-Rad CFX96 (Bio-Rad, USA) thermocycler, and the analysis was performed using Bio-Rad CFX Manager software version 3.1. The reaction mix was prepared using the SensiFAST^{TM} SYBR® No ROX Kit (Bioline) as recommended by the manufacturer's protocols. The RT-qPCR reaction volume for each sample was 20 μL in 0.2 mL PCR tubes. All reactions were conducted in triplicate (n = 3). All data acquired were analyzed for quantification, melt curve, and gene expression. RT-qPCR was repeated for three independent experiments, and GraphPad Prism 7.0 and Microsoft Excel were utilized for data analysis.

Multiplex Gene Pathway Analysis

The Nanostring nCounter® PanCancer Pathway Panel is a multiplex gene analysis platform that analyzes 770 genes from 13 cancer-associated canonical pathways. In this study, the pathway panel was used to measure the treatment effects of citral on the regulation of cancer pathways in breast cancer spheroids. The nCounter® PanCancer Pathways Panel (Nanostring, USA) was utilized to assess the effects of citral on breast cancer spheroids (n = 3 for each group: untreated and citral-treated MCF7 spheroids) related to cancer-associated canonical pathways. All procedures were conducted following the manufacturer's instructions. The procedure started with hybridizing the Nanostring^{TM} probe with 50 ng of total RNA extracted from each sample at 65 °C overnight (16–24 hours).

Following that, hybridized samples were analyzed, and readout data obtained were processed using the manufacturer's software, nSolver® Analysis Software, to check quality control (QC), mRNA positive control QC, and normalization QC. Genes with >2-fold change in their expression, according to Benjamini &

Table 1: Quantitative Real-Time PCR (RT-qPCR) primers for ALDH isoforms and pluripotency genes

No	Gene	NCBI Gene ID	Name*	5'—3' Sequence	Length	GC %	Tm	Size (bp)
1	ALDH1	NM_000689	f_ALDH1	TGGACCAGTGCAGCAAATCA	20	50	60.18	171
			r_ALDH1	ACGCCATAGCAATTCACCCA	20	50	60.03	
2	ALDH1	NM_000693	f_ALDH1	TGGCACGAATCCAAGAGTGG	20	55	60.32	101
			r_ALDH1	TTGTCCACGTCGGGCTTATC	20	55	60.11	
3	ALDH2	NM_000690	f_ALDH2	TTCTTCAACCAGGGCCAGTG	20	55	60.18	200
			r_ALDH2	TTCCCCGTGTTGATGTAGCC	20	55	60.04	
4	NANOG	NM_024865	f_NANOG	AATACCTCAGCCTCCAGCAGATG	23	52.17	62.08	148
			r_NANOG	TGCGTCACACCATTGCTATTCTTC	24	45.83	61.92	
5	MYC	NM_002467	f_MYC	CATCAGCACAACTACGCAGC	20	55	59.9	120
			r_MYC	GCTGGTGCATTTTCGGTTGT	20	50	59.97	
6	SOX2	NM_003106	f_SOX2	GCCCTGCAGTACAACTCCAT	20	55	60.04	128
			r_SOX2	GACTTGACCACCGAACCCAT	20	55	59.96	
7	OCT3/4	NM_002701	f_OCT3/4	CAAAGCAGAAACCCTCGTGC	20	55	60.04	171
			r_OCT3/4	AACCACACTCGGACCACATC	20	55	59.97	
8	GAPDH	NM_002046	f_GAPDH	GTCATCCCTGAGCTGAACGG	20	60	60.46	187
			r_GAPDH	CCACCTGGTGCTCAGTGTAG	20	60	60.04	

Note: *(f = forward primer, r = reverse primer). **GAPDH was used as a reference control.

Yekutieli[23], with a p-value < 0.05 were considered significant. Differential expression and pathway scoring were performed using a built-in nSolver® advanced analysis module. Gene Ontology and pathway analyses were conducted using the software WebGestalt (https://www.webgestalt.org).

Statistical Analysis

The experiments were performed in triplicate, and the results were expressed as mean ± standard deviation (SD). Data analysis utilized Microsoft Office Excel 2019 and GraphPad Prism 7.0. Statistical significance was defined as a p-value less than 0.05 ($p < 0.05$).

RESULTS

Evaluation of Citral by GC-MS

The purity and composition of citral ($C_{10}H_{16}O$) were first assessed by mass spectral GC-MS analysis through the detection of the retention time of each peak. The identification was performed by comparing the compounds' calculated linear retention indices (LRI) to the reported LRI values in the literature (**Table 2**). The mass spectra from the GC-MS analysis were matched against the reference spectra in

Wiley's **Flavors and Fragrances of Natural and Synthetic Compounds** (FFNSC) 1.3 and WILEY229 GC-MS libraries. The GC-MS analysis revealed two major constituents of citral: neral (*cis*-citral) and geranial (*trans*-citral) (**Figure 1**), with percentage compositions of 43.43% and 44.35%, respectively, and an overall purity of 87.7%. Hence, it confirms the suitability of citral for treating breast cancer spheroids.

Effect of Citral on the Proliferation of Breast Cancer Spheroids

After assessing the purity of citral, breast cancer spheroids were exposed to increasing concentrations of the compound, and the viability of cells was assessed after 72 hours. This identified an IC_{50} (concentration of compound required for 50% inhibition of cell viability) value of 61.09 μM ± 6.8. Treatment with citral at this concentration was able to reduce the proliferation of breast cancer spheroids, with a significant size reduction (**Figure 2**).
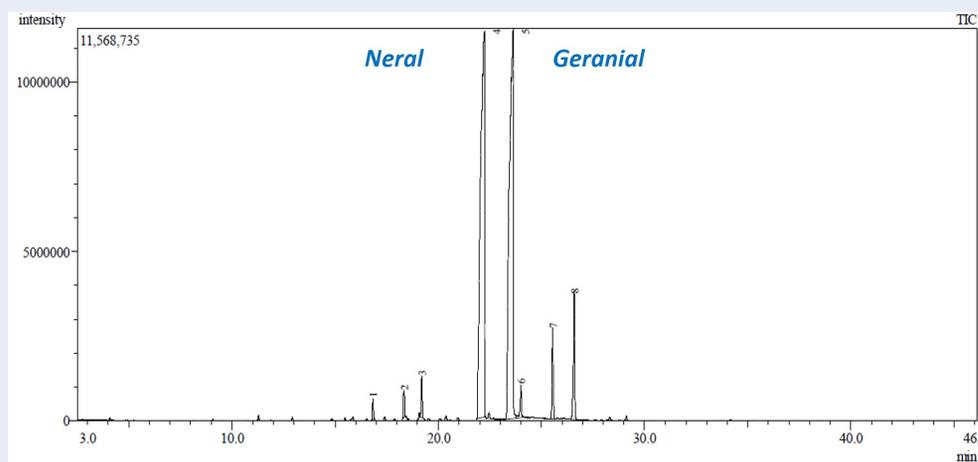
Figure 1: **GC-MS chromatogram of citral**. Gas chromatography-mass spectrometry detected a total of eight peaks. The two peaks represented the primary components of citral, (**a**) Neral (*cis*-citral), and (**b**) Geranial (*trans*-citral).

Figure 2: **Effect of citral on spheroids size**. The IC_{50} value of citral was determined by assessing the viability of spheroids, as shown in the left image. Citral exhibited the ability to inhibit the growth of breast cancer spheroids by significantly decreasing their size. Images of the spheroids were taken using an inverted microscope at $40\times$ magnification and their sizes were measured using ImageJ software. The scale bar in the middle image represents a length of 200 μm. A statistical analysis was performed using an independent t-test (*p < 0.05, n = 3) to compare the sizes of untreated versus citral-treated spheroids. The data is presented as mean $\pm$ standard deviation (SD) in the right image.
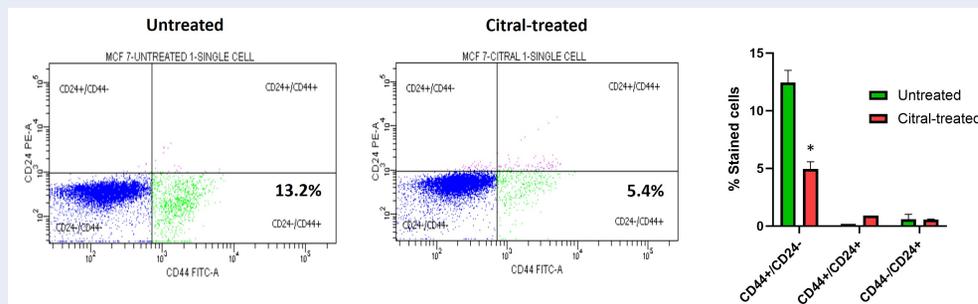

Figure 3: **Immunophenotyping of CD44 and CD24 markers expression in breast cancer spheroids**. In the MCF7 breast cancer spheroids the citral-treated group showed a decreased percentage of $CD44^+CD24^-$ compared to the group (*P < 0.05, t-test n = 3).

Table 2: List of identified compounds in Citral determined by gas chromatography-mass spectrometer (GS-MS) The chemical composition of Citral determined using GC-MS analysis was identified based on reference library, Linear Retention Indices (LRI), and Similarity Index (SI). The LRI obtained from the analysis was cross-checked with available literature for further validation. The area under each peak (Figure 1) represents the percentage of each compound identified in Citral.

Peak	Compound	Library	LRI	SI (%)	Composition (%)	Reference
1	*trans*-Rose oxide	FFNSC1.3.lib	1133	83	0.60	Babushok, *et al.* 2011[24]
2	*cis*-Isocitral	FFNSC1.3.lib	1164	92	0.80	Adams, 2007[25]
3	*trans*-Isocitral	FFNSC1.3.lib	1182	93	1.37	Adams, 2007[25]
4	Neral *	FFNSC1.3.lib	1247	94	43.43	Babushok *et al.*, 2011[24]
5	Geranial **	WILEY229.lib	1277	95	44.35	Babushok *et al.*, 2011[24]
6	UNKNOWN	N/A***	1285	N/A	1.30	N/A
7	Neryl dimethyl acetal	WILEY229.lib	1318	83	3.25	Yannai, 2012[26]
8	Geranyl dimethyl acetal	WILEY229.lib	1342	87	4.90	Yannai, 2012[26]

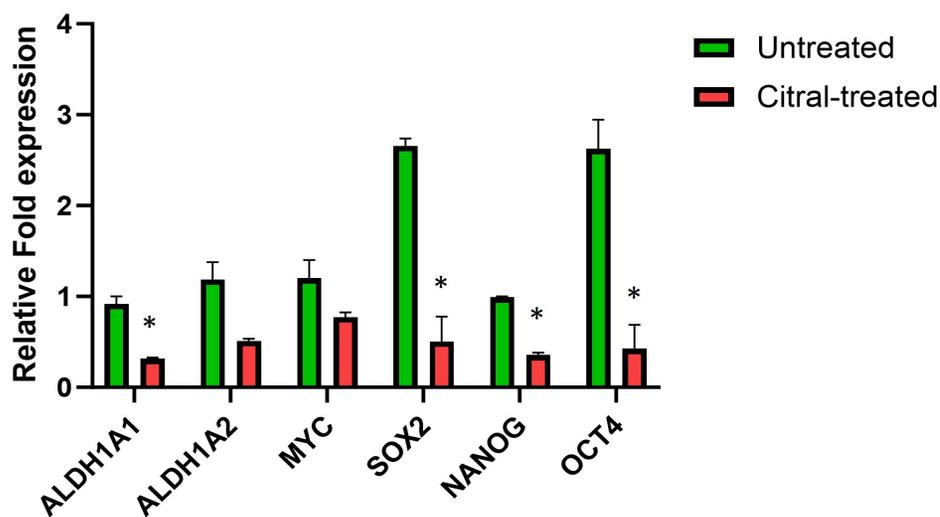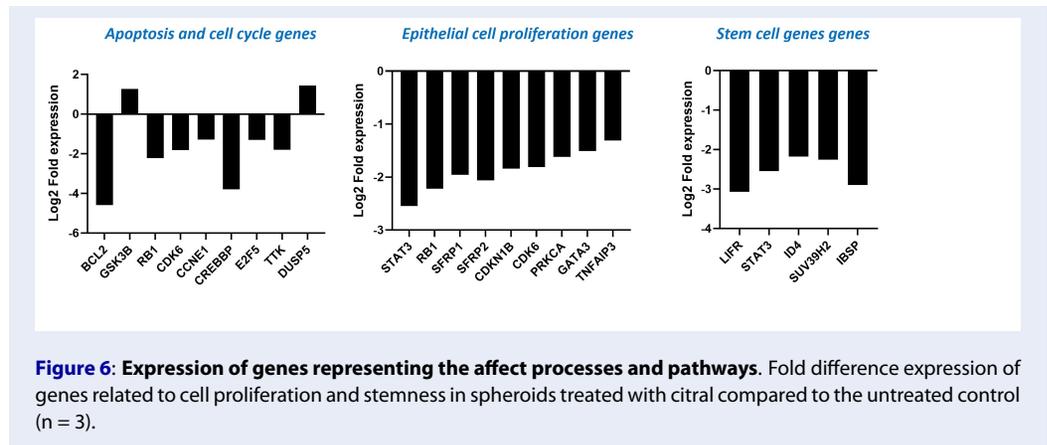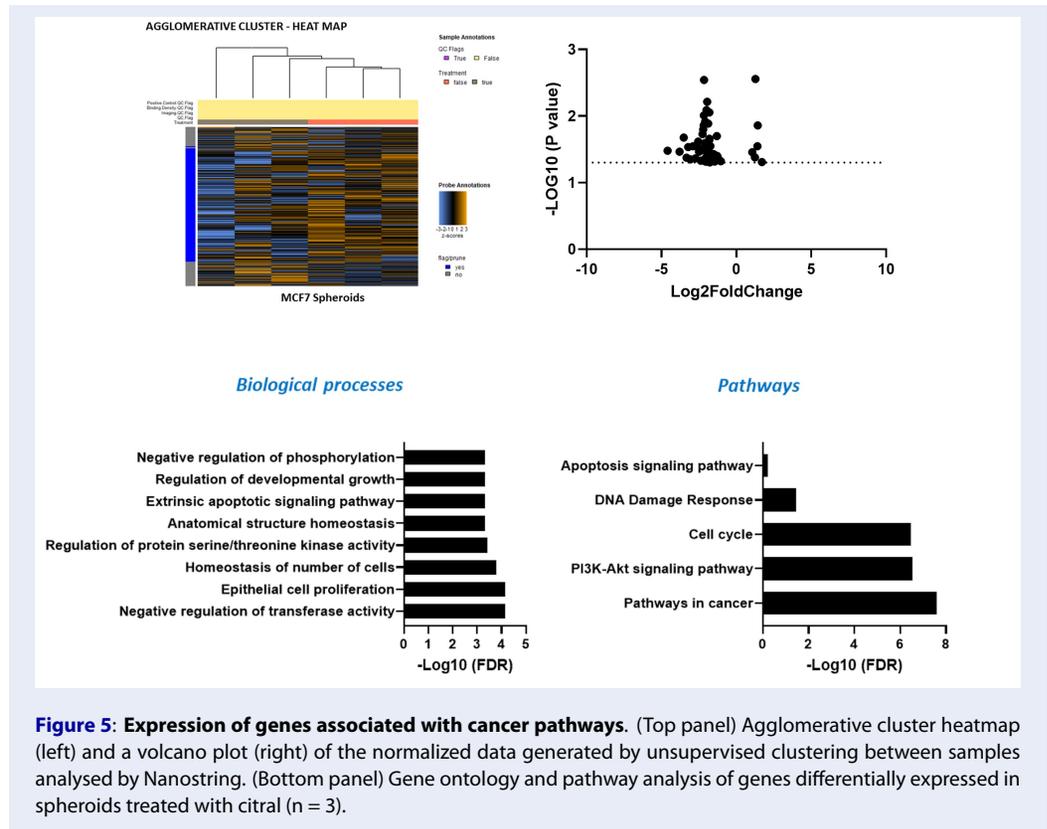* also known as cis-citral, ** also known as trans-citral, *** N/A Non-applicable

Figure 4: **Expression of stem cell markers in breast cancer spheroids treated with citral**. The levels of ALDH isoforms (*ALDH1A1, ALDH1A2*) and pluripotency genes (*MYC, SOX2, NANOG, OCT4*) were normalized based on the GAPDH level using the $2^{-\Delta\Delta Ct}$ method (n = 3).

Figure 5: **Expression of genes associated with cancer pathways**. (Top panel) Agglomerative cluster heatmap (left) and a volcano plot (right) of the normalized data generated by unsupervised clustering between samples analysed by Nanostring. (Bottom panel) Gene ontology and pathway analysis of genes differentially expressed in spheroids treated with citral (n = 3).

Figure 6: **Expression of genes representing the affect processes and pathways**. Fold difference expression of genes related to cell proliferation and stemness in spheroids treated with citral compared to the untreated control (n = 3).

Inhibition of Cancer Stemness in Breast Cancer Spheroids Following Citral Treatment

To study the effect of citral on the BCSC population, the expression of CD44$^+$CD24$^-$ cells was next analyzed. As shown in **Figure 3**, citral reduced the number of CD44$^+$CD24$^-$ breast cancer cells following 72 hours of treatment. The percentage of CD44$^+$CD24$^-$ breast cancer cells in MCF7 spheroids

was significantly decreased from $13.2 \pm 0.75\%$ to $5.4 \pm 0.45\%$ ($p < 0.05$). Furthermore, no statistical difference was measured within the CD44$^+$CD24$^+$ or CD44$^-$CD24$^+$ populations, suggesting the effect of citral may be due to defective proliferation rather than induction of cancer stem cell differentiation. We next evaluated the regulation of stemness genes in the breast cancer spheroids. Treatment with citral significantly downregulated the expression of *ALDH1* and the pluripotency genes *SOX2, NANOG,* and *OCT4*

(**Figure 4**).

Analysis of Gene Expression of Canonical Pathways Regulated by Citral

Given the major effect of citral on MCF-7 breast cancer spheroids, we next evaluated the expression of cancer pathways in this cell line using the Nanostring nCounter® PanCancer Pathway Panel. Two-way hierarchical clustering was conducted on both mRNA (rows) and biological replicates (columns) as a quality control measure to identify outliers, as it groups samples based on the similarity of their gene expression patterns[27].

A total of six samples (3 untreated samples and 3 citral-treated samples) were used for each breast cancer spheroid, and replicates were consistently clustered together between the untreated and citral-treated groups. Treatment with citral resulted in a change in the expression of 52 genes, with 46 genes being downregulated and 6 genes upregulated (**Figure 5**).

Gene ontology analysis highlighted negative regulation of transferase activity, epithelial cell proliferation, and cell homeostasis as the most significant biological processes affected by citral. In addition, the most significant pathways were pathways in cancer, PI3K/Akt signaling, DNA damage, cell cycle, and apoptosis signaling pathways (**Figure 5**). We found a significant downregulation of genes implicated in controlling the cell cycle and proliferation of epithelial cells. The inhibitor of apoptosis, BCL-2, was also downregulated. Importantly, treatment with citral induced downregulation of several additional genes involved in cancer stemness, including *LIFR, STAT3, SUV39H2, IBSP*, and *ID4* (**Figure 6**).

DISCUSSION

For cancer treatment, the presence of cancer stem cells (CSCs) poses a significant therapeutic obstacle. Conventional chemotherapy causes tumor shrinkage at the primary tumor site and simultaneously triggers stress responses to protect a specific subset of cells against the drug activity. Such stimuli induce the generation of chemo-resistant cells that closely align with the phenotype and properties of CSCs[28,29]. Therefore, there is a critical need to find new therapies targeting these resistant cells, which are responsible for cancer recurrence. The preclinical model of 3D spheroid culture is an effective method to enrich for breast cancer stem cells (BCSCs) and derived progenitors, as only CSCs can proliferate in anchorage-independent conditions and the absence

of serum in the medium[30]. Under these conditions, the unique subpopulation of cells enriched in spheroid culture with stem-like properties can self-renew and are highly tumorigenic. In this study, we have utilized the MCF-7 cell line as an *in vitro* model for estrogen receptor-positive breast cancer to test the effectiveness of the naturally abundant compound citral in inhibiting BCSC proliferation as a strategy for new treatments of breast cancer with a high prevalence of recurrence. Ponti *et al.*[31] demonstrated that spheroid culture using the MCF-7 cell line enriched a population of cells with a protein expression signature similar to stem cells and was capable of forming tumors from as few as 1,000 cells when injected into the mammary fat pad of mice.

Citral was found to exert an anticancer effect by reducing spheroid size and significantly decreasing the number of CD44$^+$CD24$^-$ breast cancer stem cells in MCF-7 spheroids. Additionally, it was observed that the expression of several pluripotency genes—*SOX2, OCT4, NANOG*, and *ALDH1* isoforms—was reduced after citral treatment. *OCT4, NANOG,* and *SOX2* are transcription factors that are expressed in both CSCs and embryonic stem cells (ESCs) and are related to pluripotency and stemness[32]. The overexpression of these three transcription factors in cancer cells induces tumorigenicity, tumor metastasis, and recurrence after chemotherapy[33].

Furthermore, overexpression of aldehyde dehydrogenase (ALDH) in breast cancer cells has been shown to demonstrate stem/progenitor properties[8]. Therefore, targeting specific ALDH isoforms may help to eliminate the CSC subpopulation, thereby reducing the likelihood of cancer recurrence. Research conducted by Coyle *et al.*[34] demonstrated that an isoform of the ALDH1 family (ALDH1A1) significantly regulates the proliferation and differentiation of cancer cells via the oxidation of retinol to retinoic acid (RA). Surprisingly, high ALDH1 expression in breast cancer is correlated with a poor prognosis[35].

Therefore, the ability of citral to reduce the expression of pluripotency genes and *ALDH1* genes, including the population of tumor-initiating CD44$^+$CD24$^-$ cells, indicates its potential in specifically targeting the BCSC subpopulation within tumor spheroids.

Additionally, this study aimed to elucidate the mechanism responsible for citral's effect on breast cancer spheroids. Several genes in pathways related to cell cycle regulation and cell growth were significantly downregulated in the spheroids treated with citral, suggesting an effect mediated by cell cycle arrest and reduction of epithelial cell proliferation. Important components involved in cell cycle progression are the

cell cycle cyclin-kinase complexes CDK4/6-cyclin D and the transcription factor E2F5. Cyclin-dependent kinases (CDKs), such as CDK4 and CDK6, are well recognized to govern cell cycle activity and are also strongly linked to the development and progression of tumors[36]. Treatment with citral downregulated *CDK6, CCNE1*, and *E2F5* genes, thus limiting cell proliferation. The formation of cyclin-CDK complexes facilitates control of cell cycle progression by phosphorylating targets such as RB1, which was also found to be downregulated[37].

The expression of GSK3B was instead upregulated. GSK3 limits cell cycle progression by phosphorylation of cyclin D1, thus tagging the protein for degradation. DUSP5, which can negatively regulate members of the proliferation pathways activated by the mitogen-activated protein (MAP) kinase superfamily (MAPK/ERK, SAPK/JNK, p38), was also upregulated and functions as a tumor suppressor[38,39]. In addition, the key inhibitor of apoptosis, BCL2, was found to be downregulated. Low expression of BCL2 can trigger caspase enzymes and promote cell apoptosis[40].

Together with a marked effect on cell proliferation, citral downregulated other pathways associated with the regulation of cancer stemness and recurrence. The LIFR and STAT3 genes were among the most downregulated genes after treatment with citral. The expression of LIFR and STAT3 is correlated with stem cell dormancy and relapse in estrogen receptor-positive breast cancer. Their loss has been demonstrated to reduce the expression of cancer stem cell-associated genes[41]. The same level of downregulation was also found for the ID4 gene, which maintains BCSC self-renewal[42]. Finally, the downregulation of the oncogene SUV39H2 and IBSP has been observed to decrease the metastatic capability of breast cancer cells and, therefore, the incidence of cancer recurrence[43,44].

Despite some study limitations, including that the spheroid model used may not fully replicate the complexity of the tumor microenvironment and cancer stem cell heterogeneity, our data demonstrate that citral may be a good candidate therapeutic compound targeting breast cancer stem cells that can complement other drugs with similar potential. Several BCSC-targeting agents include salinomycin, curcumin, and resveratrol[45,46]. Salinomycin inhibits the Wnt/β-catenin signaling pathway, which is crucial for BCSC maintenance and proliferation. Its mechanism involves disrupting the CSC microenvironment and promoting CSC apoptosis[47]. Meanwhile, curcumin, derived from turmeric, inhibits the proliferation of breast cancer stem cells (BCSCs), suppresses metastasis, and ultimately limits tumor formation[48]. Finally, resveratrol affects BCSCs via multiple signaling pathways, including suppression of Wnt/β-catenin signaling and induction of autophagy[49].

Further studies could investigate the effect of citral in combination with other compounds and the long-term toxicity of citral compared to other agents. While citral is natural and generally considered safe, its full safety profile in cancer therapy remains to be thoroughly investigated.

To further validate the exact mechanism of citral, some suggestions are proposed for future investigation. Several studies have suggested improved methods for enriching breast cancer stem cells *in vitro*. The current spheroid culture can be improved by creating a hypoxic environment to further enrich the BCSC population and by isolating BCSCs using fluorescence-activated cell sorting. In addition, the introduction of other tumor microenvironment factors to the *in vitro* culture can be achieved by co-culturing breast cancer stem cells with stromal cells such as fibroblasts and macrophages to better recapitulate the tumor niche. Finally, high-throughput molecular biology techniques such as next-generation sequencing and proteomics could be applied to further investigate the anticancer potential of citral.

CONCLUSION

In conclusion, our data point to a significant inhibitory effect on cell proliferation and stem cell pathways. Therefore, the ability of citral to limit BCSC proliferation is of great importance for developing new therapeutic strategies for both the treatment and prevention of breast cancer. Citral was found to exert its anticancer mechanism, as determined by the reduction of spheroid size, induction of apoptosis, and a decrease in the percentage of ALDH$^+$ and CD44$^+$CD24$^-$ breast cancer stem cell populations. Furthermore, the current study showed that citral was able to inhibit the expression of pluripotency and ALDH isoforms that regulate the stemness property of BCSCs. These *in vitro* findings provide a rationale for further preclinical and clinical evaluation of citral for anti-BCSC therapy. Although the exact underlying mechanism of citral in targeting BCSCs was not conclusively determined using the Nanostring^{TM} platform, the outcome has provided important insights, especially into cell proliferation and stem cell pathways. However, although the pathways affected by citral require further validation by Western blotting and real-time PCR, the outcomes of this study have shown that citral exhibits compelling potential in targeting BCSCs.

ABBREVIATIONS

ALDH - Aldehyde Dehydrogenase, **BCL-2** - B-Cell Lymphoma 2 (an anti-apoptotic protein), **BCSCs** - Breast Cancer Stem Cells, **CCNE1** - Cyclin E1, **CD44+CD24-** - A surface marker phenotype associated with breast cancer stem cells, **CDK** - Cyclin-Dependent Kinase, **cDNA** - Complementary DNA, **CSCs** - Cancer Stem Cells, **DMEM/F-12** - Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12, **DNA** - Deoxyribonucleic Acid, **DUSP5** - Dual Specificity Phosphatase 5, **E2F5** - E2F Transcription Factor 5, **EpCAM** - Epithelial Cell Adhesion Molecule (CD326), **ESA** - Epithelial-Specific Antigen, **ESCs** - Embryonic Stem Cells, **FACS** - Fluorescence-Activated Cell Sorting, **FBS** - Fetal Bovine Serum, **GC-MS** - Gas Chromatography-Mass Spectrometry, **GSK3B** - Glycogen Synthase Kinase 3 Beta, **hFGF** - Human Fibroblast Growth Factor, **IBSP** - Integrin-Binding Sialoprotein, **IC$_{50}$** - Half Maximal Inhibitory Concentration, **ID4** - Inhibitor of DNA Binding 4, **LIFR** - Leukemia Inhibitory Factor Receptor, **MAPK/ERK** - Mitogen-Activated Protein Kinase/Extracellular Signal-Regulated Kinase, **mRNA** - Messenger RNA, **NANOG** - A key pluripotency transcription factor, **nSolver®** - Software for analyzing Nanostring data, **OCT4** - Octamer-Binding Transcription Factor 4, **PI3K/Akt** - Phosphoinositide 3-Kinase/Protein Kinase B**QC** - Quality Control, **RA** - Retinoic Acid, **RB1** - Retinoblastoma 1 (a tumor suppressor gene), **RNA** - Ribonucleic Acid, **RT-qPCR** - Reverse Transcription Quantitative Polymerase Chain Reaction, **SAPK/JNK** - Stress-Activated Protein Kinase/c-Jun N-Terminal Kinase, **SOX2** - SRY-Box Transcription Factor 2, **STAT3** - Signal Transducer and Activator of Transcription 3, **SUV39H2** - Suppressor of Variegation 3-9 Homolog 2, **Tm** - Melting Temperature, **WebGestalt** - WEB-based GEne SeT AnaLysis Toolkit, **Wnt/β-catenin** - A signaling pathway involved in stem cell maintenance and cancer progression

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AUTHOR'S CONTRIBUTIONS

Muhammad Ehsan Fitri Rusli: Conducting experiments & writing. Rozita Rosli: Conceptualization, Writing - Review & Editing, Supervision. Cinzia Allegrucci: Conceptualization, Writing - Review & Editing. Norazalina Saad: Conceptualization, Funding acquisition, Writing-review & editing, Supervision, Project administration. All authors read and approved the final manuscript.

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AVAILABILITY OF DATA AND MATERIALS

Data and materials used and/or analyzed during the current study are available from the corresponding author on reasonable request.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

Not applicable.

CONSENT FOR PUBLICATION

Not applicable.

COMPETING INTERESTS

The authors declare that they have no competing interests.

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