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Caffeic Acid Inhibits Proliferation, Migration, and Stemness of DU-145 Prostate Cancer Cells

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Abstract

The issue of toxicity associated with existing cancer drugs necessitates the exploration of the anticancer potentials of natural products such as caffeic acid (CA). Here, we determined the effect of CA on the proliferation, migration, and stem cell-like properties of DU-145 prostate cancer cells. Tetrazolium-based colorimetric assay and flow cytometric analysis showed that CA decreased cell proliferation in a dose- and time-dependent manner without affecting cell cycle progression. CA also inhibited cell migration and repressed epithelial-to-mesenchymal transition by upregulating the expression of *cadherin 1 (CDH1)* and downregulating the expression of *cadherin 2 (CDH2)*. Furthermore, CA reduced the cancer stem cell population from 95% to 63% and 47% at concentrations of 1.25 and 2.5 mg/mL, respectively; and inhibited stem cell-like properties by downregulating the expression of *NANOG* and *octamer-binding transcription factor 4 (OCT4)* genes. These findings suggest that CA could be considered in the development of improved chemotherapy against prostate cancer.

Keywords

caffeic acid, phenolic, prostate cancer, proliferation, migration, stemness

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Introduction

Prostate cancer is the second most diagnosed cancer in males, and the fourth most diagnosed cancer in both sexes according to GLOBOCAN data in 2020.[1] In the same report, about 5.9 to 8.1 per 100 000 men die of prostate cancer globally[1]; with about 90% of such deaths resulting from metastasis.[2]

Metastasis is a complex phenomenon that involves the spread of cancer cells from primary sites to nearby tissues, then to distant sites where they become more aggressive. Metastasis usually begins with the loss of epithelial cell polarity and cell-cell adhesion, and the gain of migratory and invasive properties to become mesenchymal cells in a process known as epithelial-to-mesenchymal transition (EMT).[3] The overexpression of genes such as *vimentin (VIM)*, *cadherin 2 (CDH2)*, *snail family transcriptional repressors (SNAI) 1 and 2*, and *zinc finger E-box binding homeobox 1 (ZEB1)*, and the loss of *cadherin 1 (CDH1)* drive EMT.[4–6] EMT is involved in tumor progression with metastatic expansion and generation of cancer stem cells (CSCs) which can produce new tumors and contribute to drug resistance.[7,8] Thus, EMT and CSCs have generated interest as potential targets in anti-metastasis therapy.[9,10]

The devastating side effects of many anti-cancer drugs, coupled with the intricate nature of human cancers, have led to a growing need to investigate natural sources for highly potent compounds that result in comparatively less toxic adverse effects. Emphases have been placed on flavonoids and other polyphenolic compounds derived from natural sources as potential inducers of apoptotic pathways and suppressors of cancer cell proliferation.[11]

Caffeic acid (3,4-dihydroxycinnamic acid, CA) is a polyphenol found in the bark of *Eucalyptus globulus*[12] and the herb *Dipsacus asperoides*.[13] CA is also found in the freshwater fern *Salvinia molesta*[14] and in the mushroom *Phellinus linteus*.[15] CA has been found to have a variety of biological activities including antioxidant[16] and antihypertensive[17] properties. Studies have shown that CA exhibits growth inhibitory effects on the human HT-1080 fibrosarcoma cell line and cervical cancer

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cells.[18,19] However, the effect of CA on prostate cancer cells remains unstudied. In this research, we investigated the anti-proliferative, anti-migratory, and anti-stemness effects of CA on DU-145 prostate cancer cells.

Materials and Methods

Cell Culture

DU-145 cells, obtained from the American Type Culture Collection, were maintained in Roswell Park Memorial Institute (RPMI) 1640 (Gibco-Life Technologies, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS) (Gibco-Life Technologies, Carlsbad, CA, USA) and 1% penicillin-streptomycin-glutamine (Gibco-Life Technologies, Carlsbad, CA, USA) at 37 °C in a humidified atmosphere containing 5% CO_2.

Cell Viability Assay

DU-145 cells were treated with varying concentrations of CA (Sigma-Aldrich, St Louis, MO, USA) for 24, 48, and 72 h. As a positive control, cisplatin (Sigma-Aldrich, St Louis, MO, USA) was used to treat the cells for the same duration. Then 20 µL of 2.5 mg/mL 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (Sigma-Aldrich, St Louis, MO, USA) was added to cells in each well and incubated for 4 h. Afterwards, 150 µL of acidified isopropanol was added to each well, and absorbance was read with a Varioskan™ LUX multimode microplate reader (Thermo Fisher Scientific, Carlsbad, CA, USA) at 570 nm. From the absorbance values obtained, percent cell viability was determined.

Cell Cycle Analysis

After treating cells with 1.25 or 2.5 mg/mL CA for 72 h, the cells were collected, washed with phosphate-buffered saline (PBS), fixed with 70% ice-cold ethanol, and incubated at −20 °C overnight. The fixed cells were then centrifuged at $180 \times g$ for 5 min, and the pellet was rinsed twice with PBS and re-suspended in propidium iodide (PI) (Sigma-Aldrich, St Louis, MO, USA) for 30 min at 4 °C in the dark. Samples were analyzed using a FACScan flow cytometer (BD Biosciences, San Jose, CA, USA) and data were analyzed using BD CellQuest™ Pro Software version 6 (BD Biosciences, San Jose, CA, USA).

Wound Healing Assay

Cells were seeded into 6-well plates at a density of 1.0×10^6 cells/well and incubated for 48 h, after which treatment with 1.25 or 2.5 mg/mL CA was administered. A scratch was created in each well using a 200 µL pipette tip. The cells were incubated at 37 °C in a humidified atmosphere containing 5% CO_2, and images were taken at different time points with an OPTIKA® microscope (OPTIKA, Ponteranica, Italy). The area of the wound was quantified using ImageJ (NIH, Bethesda, MD).

CSC Population Analysis

DU-145 cells were either untreated or treated with 1.25 or 2.5 mg/mL CA for 72 h. The cells were collected and washed in 2% wash buffer (PBS containing 2% FBS) and then re-suspended in 1% wash buffer (PBS containing 1% FBS). The cells were stained with fluorescein isothiocyanate (FITC)-labeled anti-human CD44 antibody (CAT no. 555478. BD Biosciences, San Jose, CA, USA) for 20 min at 4 °C in the dark, washed with 1% wash buffer, and then with 2% wash buffer. The stained cells were analyzed using a FACScan flow cytometer (BD Biosciences, San Jose, CA, USA), and data were analyzed using BD CellQuest™ Pro Software version 6 (BD Biosciences, San Jose, CA, USA).

Reverse Transcription-Quantitative Polymerase Chain Reaction (RT-qPCR)

Cells were seeded into 6-well plates at a density of 2.0×10^5 cells/well and treated with either 1.25 or 2.5 mg/mL CA for 72 h. Total RNA was extracted using the RNeasy® Mini kit (Qiagen, Valencia, CA, USA), following the manufacturer's protocol. The extracted RNA (1 µg) was reverse transcribed, and PCR amplified using a Luna Universal One-Step RT-qPCR kit (New England Biolabs, Ipswich, MA, USA) in a QuantStudio™ RT-PCR System (Thermo Fisher Scientific, Carlsbad, CA, USA). The thermocycling conditions were reverse transcription (55 °C for 10 min), initial denaturation (95 °C for 1 min), 35 cycles of denaturation (95 °C for 15 s), annealing (56 °C for 30 s), elongation (60 °C for 30 s), and final extension (60 °C for 1 min). The housekeeping gene *β-actin* was used as an internal control. The following primer sets were used: ***CDH1*** forward 5′-ATTCTGATTCTGCTGCTCTTG-3′ and reverse 5′-AGTAGTCATAGTCCTGGTCTT-3′; ***CDH2*** forward 5′-GGTGGAGGAGAAGAAGACCAG-3′ and reverse 5′-GGCATCAGGCTCCACAGT-3′; ***NANOG*** forward 5′-GATTTGTGGGCCTGAAGAAA-3′ and reverse 5′-AAGTGGGTTGTTTGCCTTTG-3′; ***OCT4*** forward 5′-GTGGAGGAAGCTGACAACAA-3′ and reverse 5′-CAGGTTTTCTTTCCCTAGCT-3′; ***β-actin*** forward 5′-TTCCTGGGCATGGAGTCCTGTGG-3′ and reverse 5′-CGCCTAGAAGCATTTGCGGTGG-3′. The QuantStudio™ Design & Analysis Software (Life Technologies, Carlsbad, CA, USA) was used to obtain CT values, and the $2^{-\Delta\Delta Cq}$ method[20] to analyze the relative expression of each gene.

Statistical Analysis

Data were analyzed using GraphPad Prism 9.1.2 (GraphPad Software, San Diego, USA). One-way analysis of variance

followed by Dunnett's post-hoc test was used to compare differences between multiple groups. Data are presented as the mean ± standard error of mean (SEM) of 3 independent experiments. Group differences were considered statistically significant when $P < .05$.

Results

Effect of CA on DU-145 Cell Viability

Treatment of DU-145 cells with CA resulted in decreased cell viability in a dose- and time-dependent manner, similar to cisplatin (Figure 1). The IC_{50} values for 24, 48, and 72 h CA treatment were 2.28 ± 1.19, 1.90 ± 0.40, and 1.69 ± 0.11 mg/mL, respectively, whereas that for cisplatin were 8.6 ± 0.58, 3.64 ± 0.30, and 1.30 ± 0.10 µM, respectively.

Effect of CA on Cell Cycle

Flow cytometric analysis revealed that 1.25 mg/mL CA did not influence the cell population in each phase of the cell cycle. Although statistically insignificant, 2.5 mg/mL CA caused cell cycle arrest at both S and G2-M phases, with the number of cells reduced in the G0 phase (Figure 2).

CA Inhibited DU145 Cell Migration and EMT

The effect of CA on migration was determined using a wound-healing assay. Treatment with either 1.25 or 2.5 mg/mL CA inhibited cell migration. There was, however, no significant difference in the rate of wound closure between the 2 concentrations of CA (Figure 3A). CA also upregulated the expression of *CDH1* gene and downregulated the expression of *CDH2* gene (Figure 3B).

CA Inhibited Stem Cell-Like Properties of DU-145 Cells

A significant reduction in stem cell population was observed following treatment. CA at concentrations of 1.25 and 2.5 mg/mL decreased the stem cell population from 95.9% to 60.1% and 46.9%, respectively (Figure 4A). CA also significantly reduced the expression of stemness genes *NANOG* and *OCT4* (Figure 4B).

Discussion

CA is the major phenolic compound in coffee, and it has been studied for its biological activities such as anti-oxidative, anti-inflammatory, and immunomodulatory properties.[21] CA is reported to exhibit growth inhibitory effects on human cervical cancer and fibrosarcoma cell lines.[18,19] However, the impact of CA on prostate cancer cells is not well understood. In this study, the effect of CA on DU-145 prostate cancer cell proliferation, migration, and stemness was assessed. CA decreased the viability of the cells in a dose- and time-dependent manner, consistent with similar studies on breast cancer, fibrosarcoma, and cervical cancer.[18,19,22] Cell cycle analysis also showed that CA did not affect cell cycle progression in the cells. Interestingly, other studies on CA found cell cycle arrest at different phases in different cancers,[21–24] indicating the selectivity of CA in different cancer types. Our results, therefore, suggest that CA exerts its anti-proliferative effect on DU-145 cells without influencing the cell cycle process.

The migration of cancer cells from primary tumors is a crucial step in multi-step metastasis.[25] Potent polyphenols such as curcumin, quercetin, and epigallocatechin gallate (EGCG) have been shown to inhibit migration in different cancer types.[26–28] Here, CA (1.25 and 2.5 mg/mL) inhibited cancer cell migration, suggesting that CA could serve as a potential anti-migratory agent. This finding is similar to that

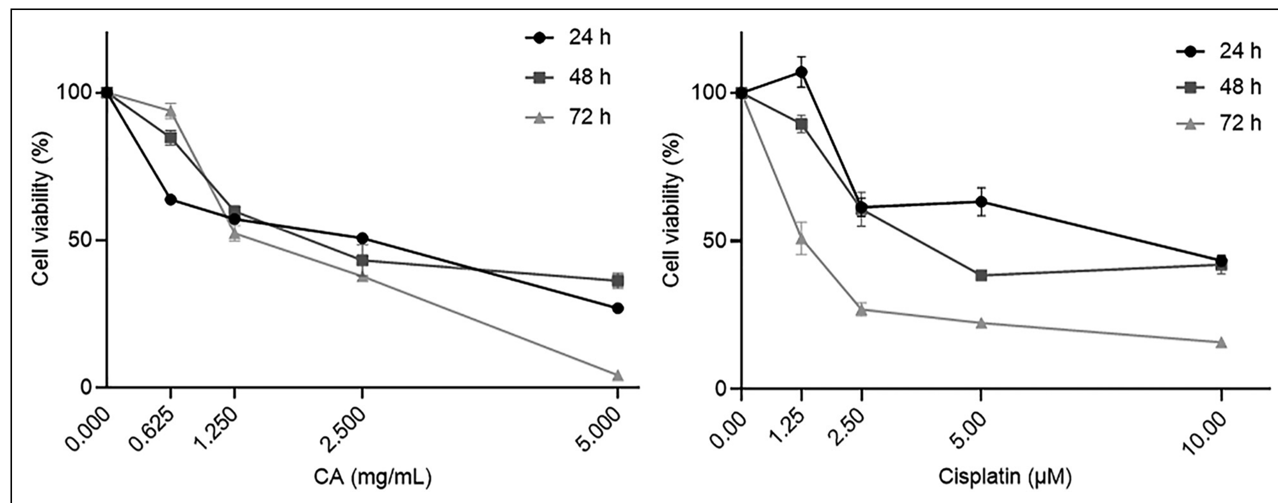

Figure 1. Effect of CA on DU-145 cell viability. DU-145 cells were either untreated or treated with varying concentrations of either CA or cisplatin for 24, 48, and 72 h. Cell viability was determined using the MTT assay. Data are presented as mean ± SEM of 3 independent experiments performed in triplicates.

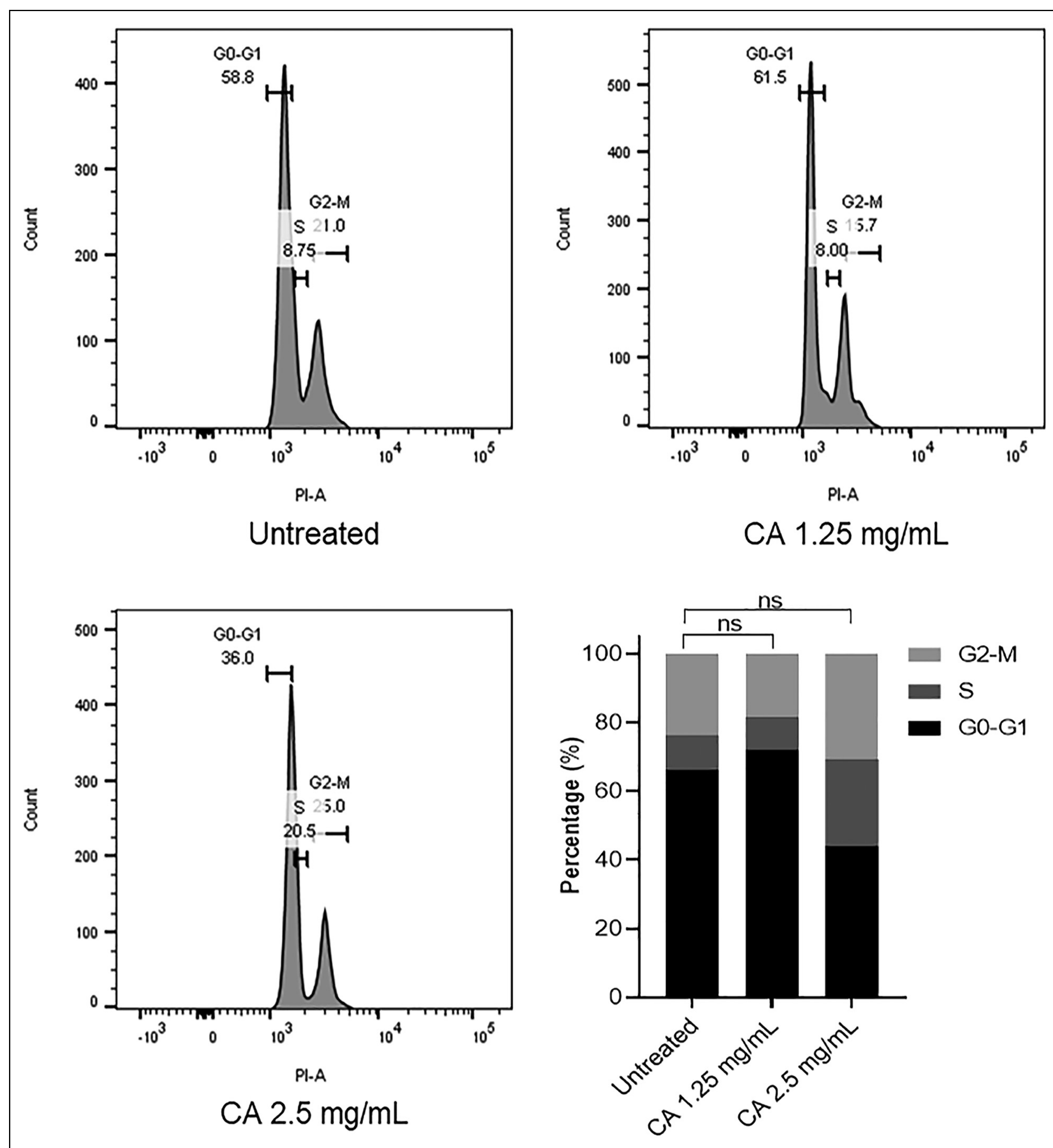

Figure 2. Effect of CA on cell cycle. DU-145 cells were either untreated or treated with 1.25 or 2.5 mg/mL CA and incubated for 72 h before staining with PI followed by flow cytometric analysis. Quantified cell populations are presented as stacked bars. Data are presented as the mean of 3 independent experiments performed in triplicates. S, synthesis; G0, gap 0; G1, gap 1; G2, gap 2; M, mitosis; ns, not significant.

of another study in which CA prevented the migration of breast cancer cells in a dose-dependent manner.[22] Further studies to understand the mechanisms involving the anti-migratory effect of CA would be useful.

EMT plays a central role in the spread of cancer. In anaplastic thyroid carcinoma cells, EGCG inhibits metastasis by upregulating and downregulating EMT markers E-cadherin and vimentin, respectively.[29] Similarly, Amawi *et al* revealed that some polyphenols, including quercetin, curcumin, resveratrol, and hydroxycinnamic acid, regulate these EMT genes in addition to suppressing transcription factors such as ZEB1.[30] In our work, we found that CA upregulates *CDH1* and

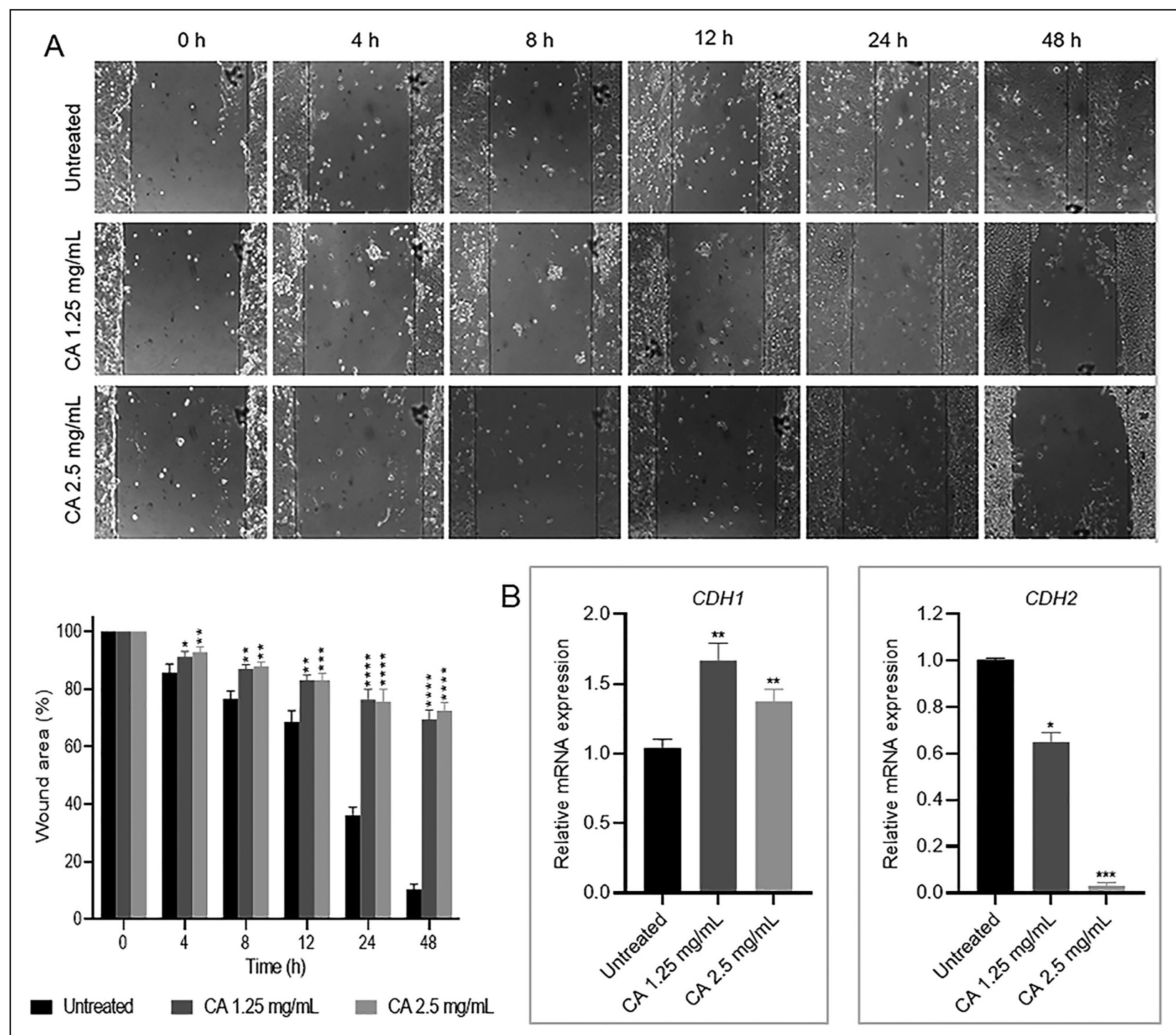

Figure 3. CA inhibited DU-145 cell migration and EMT. (A) Light microscopy images of wound area at different times after treatment with CA. The wound areas at time points are expressed as a percentage of that at 0 h. Original magnification ×100. (B) RT-qPCR of *CDH1* and *CDH2* after treatment with either 1.25 or 2.5 mg/mL CA for 72 h. Data are presented as mean ± SEM of 3 independent experiments performed in triplicates. **P* < .05, ***P* < .01, ****P* < .001, *****P* < .0001 versus untreated.

represses *CDH2*. These genes encode for E-cadherin and N-cadherin, respectively, and the alteration in their expression, as seen in this study, indicates a reversal of EMT.

Studies have shown that EMT leads to an enrichment of CSCs, which tend to self-renew and produce new tumors.[31,32] Quercetin was reported to suppress stem cell renewal by decreasing ALDH1 positive population of breast cancer cells.[33] In another study, resveratrol decreased the expression of CSC-like markers such as *NANOG*, *SOX-2*, *OCT4,* and *ABCG-2* in CD44 + human pancreatic cancer cells.[34] Here, we investigated the effect of CA on stem cell population and gene expression to determine how CA affects EMT-associated DU-145 stem cells. From our results, CA reduced the CD44+ DU-145 stem cell population in a dose-dependent manner and downregulated the expression of CSC markers, *NANOG* and *OCT4*. This is consistent with reports involving malignant human keratinocytes, which revealed that CA reduced stem cell-like properties.[35]

Overall, we have established the potential of CA to inhibit the growth and malignant properties of DU-145 prostate cancer cells by suppressing expression of specific EMT-CSC markers and decreasing the population of prostate CSCs. These findings suggest that CA could be considered in the development of improved chemotherapy against prostate cancer.

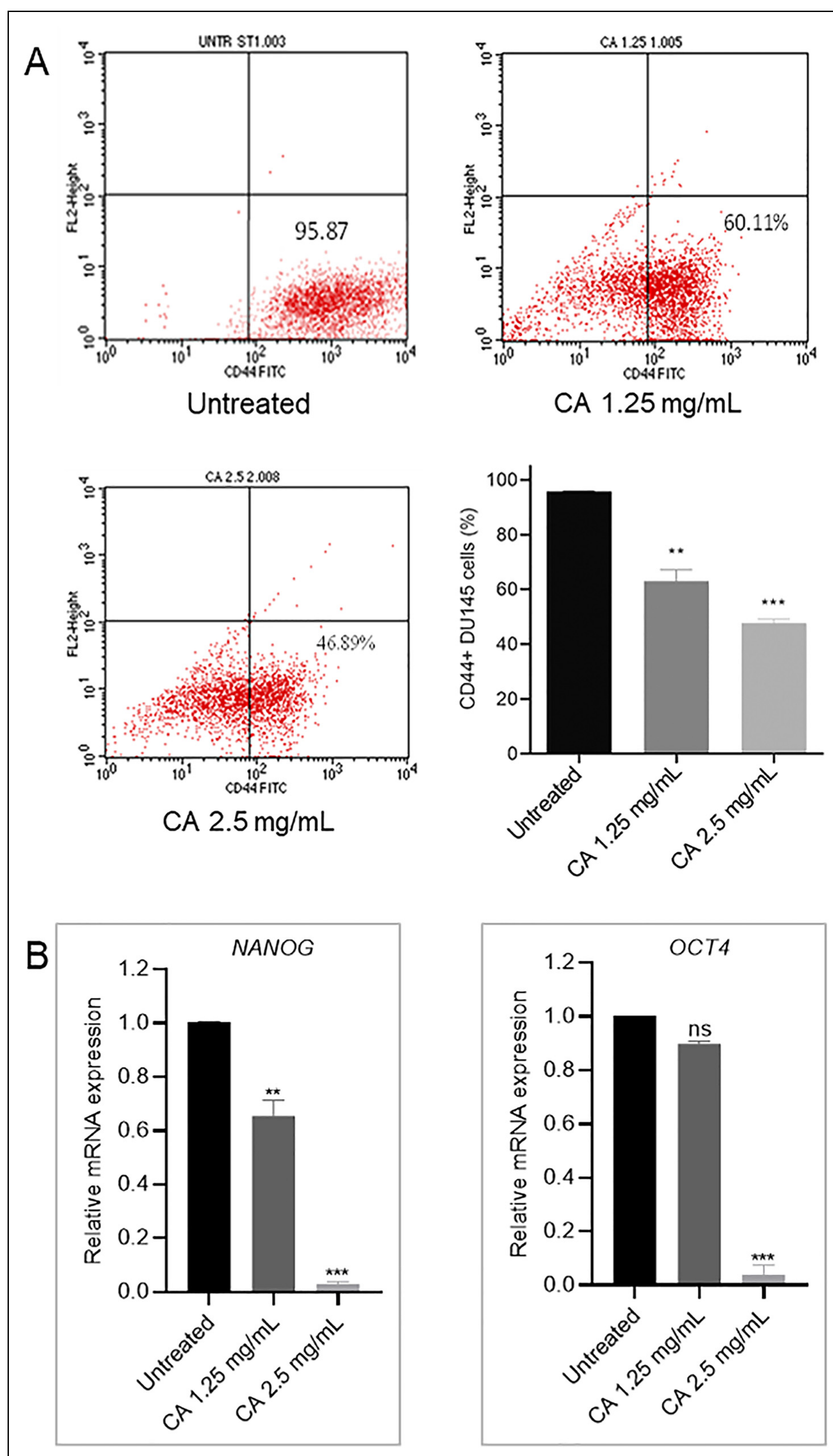

Figure 4. CA inhibited stem cell-like properties of DU-145 cells. Cells were either untreated or treated with 1.25 or 2.5 mg/mL CA, and incubated for 72 h. (A) Staining with FITC-labelled CD44 antibody. Quantified cell populations are presented as a bar graph. (B) RT-qPCR of *NANOG* and *OCT4*. Data are presented as mean ± SEM of 3 independent experiments performed in triplicates. $^{**}P < .01$, $^{***}P < .001$ versus untreated.

Abbreviations

CA	caffeic acid
CDH1	cadherin 1
CDH2	cadherin 2
OCT4	octamer-binding transcription factor 4
EMT	epithelial-to-mesenchymal transition
VIM	vimentin
SNAI1	snail family transcriptional repressor 1
SNAI2	snail family transcriptional repressor 2
ZEB1	zinc finger E-box binding homeobox 1
CSC	cancer stem cell

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Author Contributions

ARA conceived and supervised the work. Experiments, data analysis, and interpretation were performed by PAB, SBB, SMB, and JKAT. ARA and JTQ drafted the manuscript. OQ reviewed and edited the manuscript.

Availability of Data and Materials

All the data supporting the results of this study are available from the corresponding author upon request.

Declaration of Conflicting Interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

Ethical Approval

Ethical Approval is not applicable for this article.

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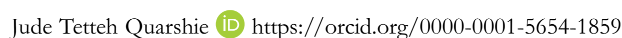
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Statement of Human and Animal Rights

This article does not contain any studies with human or animal subjects.

Statement of Informed Consent

There are no human subjects in this article and informed consent is not applicable.

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