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Original Article

Curcuma longa extract inhibits migration by reducing MMP-9 and Rac-1 expression in highly metastatic breast cancer cells

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Abstract

Background and purpose: Highly metastatic breast cancer is a population of cancer cells that has metastasized to other organs in the body leading to apoptosis resistance. It was reported that MDAMB-231 cells contain lower levels of reactive oxygen species associated with metastatic capability. *Curcuma longa* (CL) possesses cytotoxic effects in several cancer cells including metastatic breast cancer cells. This study aimed to investigate the effect of CL-inhibited cell migration in highly metastatic breast cancer MDAMB-231 cells.

Experimental approach: CL was extracted under maceration with methanol. The cytotoxic effect on single and combination treatment of CL was assessed through the MTT assay. Migration analysis was evaluated using scratch wound healing assay, MMP-9 expression by gelatine zymography, Rac-1, and MMP-9 gene expression using Real-Time Quantitative Reverse transcription polymerase chain reaction (qRT-PCR). The apoptosis induction was analyzed through Bax gene expression and Bcl-2 protein expression.

Findings/Results: We found that CL inhibits the growth of MDAMB-231 cells, induces Bax gene expression, and suppresses Bcl-2 expression in a dose-dependent manner. Moreover, cancer cell migration was suppressed by the presence of CL. qRT-PCR and gelatine zymography assay showed that CL downregulates Rac-1 and MMP-9 gene expression.

Conclusion and implications: CL could inhibit the growth and migration of highly metastatic breast cancer cells by reducing the Rac-1 gene expression and regulating apoptosis protein expression.

Keywords: *Curcuma longa*; MDAMB-231; Migration; MMP-9; Rac-1.

INTRODUCTION

Metastatic breast cancer usually exhibits a high mortality rate due to its complexity of physiological aberration with drug resistance, relapse, and genetic mutation phenomena (1). Currently, first-line therapy for metastatic breast cancer still relies on doxorubicin chemotherapy (2). However, a previous study reported that doxorubicin can induce cell cancer migration (3,4). Doxorubicin induces the transforming growth factor β1 (TGF-β1) pathway activation and increases the ability of perivascular TIE2+ macrophage to promote cancer cell invasion and metastasis through epithelial-mesenchymal transition activation (4,5). Thus, the search for new therapeutic compounds based on cancer cell biology with special emphasis on molecular factors involved in migration and metastasis is reasonable (6,7). *Curcuma longa* (CL) has been reported in a wide range of biological activities including anticancer effects in several cancer cells (8,9). However, the anti-metastatic mechanism of CL in single treatment on highly metastatic breast cancer cells remains unclear.

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The mechanism of CL in inhibiting the migration of highly metastatic breast cancer cells involving the Ras-related C3 botulinum toxin substrate 1 (Rac-1) pathway has not yet been studied. Therefore, in this study, we examined the effects of CL as a single-treatment chemotherapeutic agent on inhibiting the expression of matrix metalloproteinase-9 (MMP-9) and Rac-1 associated with optimal anti-metastatic effects in highly metastatic MDAMB-231 breast cancer cells.

MATERIALS AND METHODS

Cell cultures

Highly metastatic breast cancer cell line (murine mammary carcinoma cell line from a BALB/cfC3H mouse; MDAMB-231 cells) were provided from American Type Culture Collection (ATCC). The cells were grown as a monolayer in Dulbecco's modified eagle medium high glucose (DMEM; Catalog #12100046 Gibco; Thermo Fisher Scientific Inc., Waltham, MA, USA) enriched with 10% (v/v) fetal bovine serum (FBS) (Catalog #10099142 Gibco; Thermo Fisher Scientific Inc., Waltham, MA, USA), 100 IU/mL penicillin-100 µg/mL streptomycin (Catalog #10378016 Gibco; Thermo Fisher Scientific Inc., Waltham, MA, USA), and maintained at 37 °C with 5% CO_2 in 100% humidified atmosphere.

Extraction procedure

The extractions of CL are explained as follows; the material for this study was in the form of herbs, namely turmeric rhizome simplicia (*Curcuma longa*); the plants were then cleaned with running water until clean, then each herb was dried in a place that was not exposed to direct sunlight, after which it was chopped and powdered (simplicia powder). The simplicia powder was weighed 300 g and then put into the maceration container. One liter of ethanol extraction was added into the maceration container and left for a few hours, then 1 L of 70% ethanol was added until all the sample powder was submerged, then closed tightly. The maceration container was stored in a place protected from direct sunlight for 5 days while stirring frequently. The mixture was then filtered, and the dregs were soaked again with a new solvent. The extraction process was then carried out 4 times with 2 L of ethanol each time. The liquid extract was collected and then concentrated using a Rotavapor device to obtain a thick extract (9,10).

Cytotoxic assay

The cytotoxic activity of CL on MDAMB-231 cells was evaluated using MTT assay according to the previous study by Mosman (11) with slight modification. MDAMB-231 (2×10^3 cells/well) were seeded in a 96-well microplate and allowed to settle overnight before being treated with 0.5-30 µg/mL CL (Sigma-Aldrich, St. Louis, MO, USA) for 24 h. The variations are needed to determine the IC_{50} value, which can be used to determine variations in the concentration of the test solution combination treatments. Untreated cells were considered a negative control. After treatment, 100 µL of MTT (BioVision Inc, SF, USA; 0.5 mg/mL in medium) was added to each well and incubated for a further 4 h at 37 °C with 5% CO_2 in a 100% humidified atmosphere. Afterward, the MTT formazan crystal was dissolved using dimethyl sulfoxide (DMSO) and incubated in the dark condition for 4 h. After solubilizing the purple formazan, absorbance was measured using ELISA plate reader (Bio-Rad, California, USA) at a wavelength of 595 nm. Each treatment was carried out in triplicate and cytotoxic activity was recorded as IC_{50}, which is the concentration necessary to reduce the absorbance of treated cells by 50% compared to the untreated cells (12-14).

ROS level analysis

For all ROS experiments, MDAMB-231 cells (5×10^3) were collected by centrifugation, washed with phosphate-buffered saline (PBS), and incubated with 2.5 µM 2′, 7′-dichlorofluorescein diacetate (DCFDA) in supplemented buffer (10% FBS in PBS) for 30 min in a dark place at 37 °C. Each cell was treated with CL in several concentrations (1/4 IC_{50} (30 µg/mL), 1/8 IC_{50} (15 µg/mL), and 1/16 IC_{50} (7,5 µg/mL) and then incubated for 4 h at 37 °C and CO_2 5%. As reported previously, we used doxorubicin as a positive

control due to its ability to induce ROS levels. Intracellular ROS was determined by flow cytometry (BD Accuri C6 plus BD Biosciences, USA) (15).

Scratch wound healing assay

The migration rate of CL-treated MDAMB-231 cells was assessed by the scratch wound healing assay. The MDAMB-231 cells density of 10×10^4 cells/well were grown in a 24-well microplate and incubated for 24 h at 37 °C and 5% CO_2. After 80% confluent, the medium was discarded and changed to a starvation medium containing 0.5% (v/v) FBS for 24 h. After that, the confluent cells were scraped horizontally with a cell scratcher after 24 h incubation. The debris was removed by washing with PBS and the cells were treated with several concentrations of CL. The cell migration can subsequently be monitored microscopically (×100 magnification), as the cells travel from the intact zones into the scratched region. The migration rate was analyzed using ImageJ (The National Institutes of Health and the Laboratory for Optical and Computational Instrumentation (LOCI), University of Wisconsin, USA), and the percentage of closure after 42 h-treatment with different concentrations of CL was measured and compared to the value obtained at 0 h. An increase in closure percentage suggested cell migration (16).

Gelatine zymography assay

Gelatine zymography was performed to determine the activity of MMPs in the culture supernatants. The MDAMB-231 cells density of 5×10^5 cells/well were seeded in the 6-well microplate and exposed to CL for 24 h at 37 °C and 5% CO_2. Culture supernatants were then collected and resolved on 8% sodium dodecyl-sulfate polyacrylamide gel electrophoresis (SDS-PAGE) under non-reducing conditions. The gel was supplemented with 0.1% gelatine (Sigma-Aldrich, USA). In each gel run equivalent amounts of protein (150 μg) from culture supernatants were used. Gel was soaked for 30 min in 2% Triton®-X 100 (Merck, USA) solution after electrophoresis at room temperature before incubating in 100 mL of the incubation buffer containing 40 mM tris HCl, pH 8, 10 mM $CaCl_2$, 0.02% NaN_3 for 24 h at 37 °C. Afterward, the gel was stained by Coomassie Brilliant Blue R-250 solution for 2 h and then de-stained (20% methanol, 10% acetic acid, and 70% water) until bands with a dark blue background appeared clear, indicating gelatinolytic activity of MMP-9. The band intensity was calculated by ImageJ (The National Institutes of Health and the Laboratory for Optical and Computational Instrumentation (LOCI), University of Wisconsin, USA) (17-18).

Rac-1, MMP-9, and Bax gene expression analysis

According to the manufacturer's protocol, the genes' expressions were conducted using the real-time quantitative reverse transcription polymerase chain reaction (qRT-PCR). Total RNA from the MDAMB-231 cells was extracted with TRIzol (Invitrogen, Shanghai, China). Briefly, first-stranded cDNA was synthesized with 1 ng of total RNA using Super-Script II (Invitrogen, Massachusetts, USA). SYBR No ROX Green I dye (SMOBIO Technology Inc., Hsinchu, Taiwan) was used for reverse transcription in an RT-PCR instrument (PCR Max Eco 48, Staffordshire, UK), and mRNA levels of the Rac-1, MMP-9, and Bax genes were measured using the respective primers according to gene bank. The thermocycler conditions were as follows: initial step at 95 °C for 10 min, followed by 50 cycles at 95 °C for 15 s, and 60 °C for 1 min. The gene expression was recorded as the cycle threshold (Ct). Data were obtained using Eco Software v5.0 (Illumina Inc, San Diego, CA, USA). All reactions were performed in triplicate, and data analysis used the $2^{-\Delta\Delta Ct}$ method (Livak method) (19).

Immunocytochemistry analysis

The cells (5×10^4 cells/well) were placed on the slide with a coupled bottle (Thermo Fisher, USA) and incubated at 37 °C and 5% CO_2 for 24 h. After that, the cells were treated with CL in several concentrations for 24 h. Then, the culture medium was removed, and the cells were washed with PBS. For fixing the cells, the bottle was incubated with 1 mL of 4% formaldehyde for 20 min. Subsequently, the slides were incubated with 10% hydrogen

peroxide for 30 min to block endogenous peroxidases. Antigen retrieval was performed in a pressure cooker (ARNO, São Paulo, SP, Brazil) at 95 °C with citrate buffer (pH 6.0) for 30 min. After cooling, the slides were covered with bovine serum albumin solution and incubated for 30 min at 4 °C, overnight with B-cell lymphoma 2 (Bcl-2) antibody (Abcam, USA; 1:500). Thus, the cells were washed with PBS for 15 min and incubated with Starr TREK Universal HRP Detection Kit (Biocare Medi-Cal, Concord, CA, USA) for 20 min, and streptavidin-peroxidase complex for 10 min, followed by washing with PBS for 15 min. Next, 0.5% 3,3-diaminobenzidine tetrachloridine (DAB; Sigma Aldrich, USA) was applied for 2 min. The slides were counterstained with hematoxylin and eosin (H&E) for 40 s.

Statistical analysis

All values represent the mean ± SEM of three independent experiments. For statistical comparisons, a one-way analysis of variance (ANOVA) test followed by LSD post hoc was performed using SPSS version 26. P-values < 0.05) were considered statistically significant. The 50% inhibitory concentration (IC_{50}) was calculated by a nonlinear regression curve with the use of Microsoft Office Excel for Windows.

RESULTS

Cytotoxic activity of CL against MDAMB-231

The cytotoxic effect of CL on MDAMB-231 was analyzed using an MTT assay. Various concentrations of CL were used for a 24-h treatment. In the presence of CL, the growth of MDAMB-231 cells was inhibited in a concentration-dependent manner with IC_{50} of 13 µg/mL value on MDAMB-231 (Fig. 1A).

CL elevated ROS generation on MDAMB-231

To evaluate the potential effect of CL on cell viability loss due to CL-triggered ROS generation, ROS analysis under fluorescence of DCFDA through fluorescence-activated cell sorting (FACS) flow analysis was performed (Fig. 1B). CL increased ROS generation significantly in a concentration-dependent manner (Fig. 1C). These results showed that CL might exert cytotoxic effects through ROS level elevation.

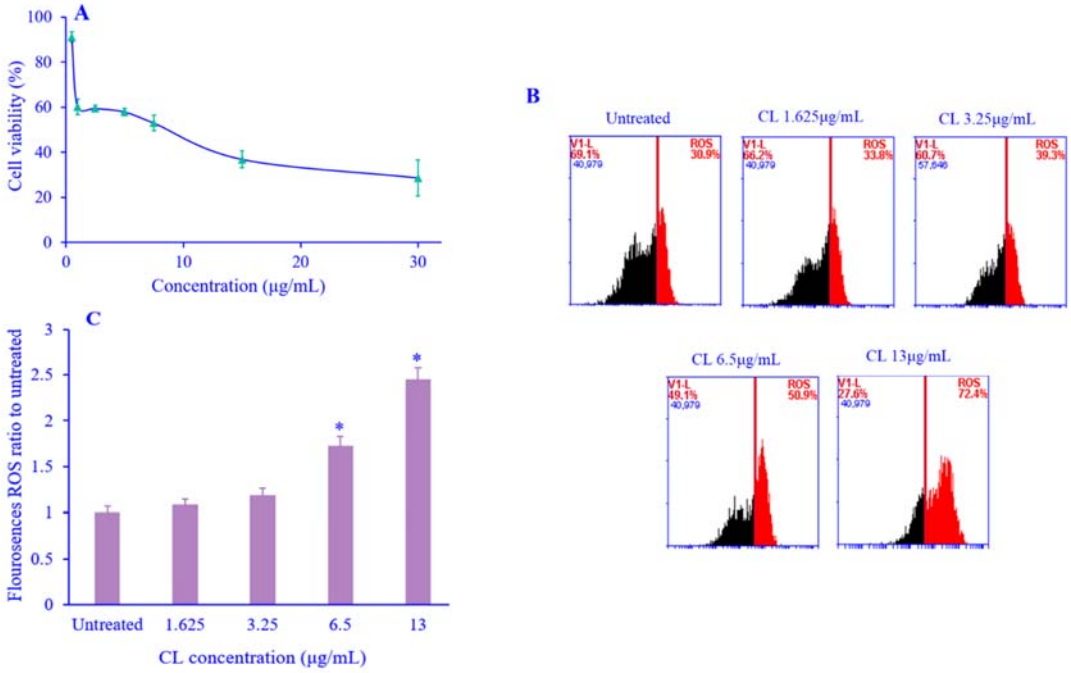

Fig. 1. (A) Cytotoxic activity of various concentrations of CL on MDAMB-231 cells after 24 h; (B) induction of ROS level by CL treatment on MDAMB-231 breast cancer cells, the ROS level of cells exposed to CL individually for 24 h was measured by flow cytometry after staining with 2′, 7′-dichlorofluorescein diacetate; (C) the % level of ROS. Data are presented as mean ± SEM, N = 3. *P < 0.05 Indicates significant differences in comparison with the untreated cells. CL, *Curcuma longa*; ROS, reactive oxygen species.

CL-induced cell apoptosis

Bcl-2 and Bax are proteins that have a role in regulating cell apoptosis. In this study, CL significantly decreased the expression of the anti-apoptotic protein Bcl-2 (Fig. 2A and B), however, CL was also able to increase the expression of the pro-apoptotic gene Bax protein in a concentration-dependent manner (Fig. 2C).

CL inhibited cell migration

The ability of MDAMB-231 cell migration was assessed using a scratch wound healing assay. The wound width of the untreated cells narrowed at 24 h and closed completely throughout 42 h (Fig. 3A and B). Treatment with CL was found to significantly inhibit the migration of MDAMB-231 cells in a concentration-dependent manner after 42 h compared to untreated cells. The results indicated the antimigratory potential of CL against MDA-MB-231 cells.

CL inhibited MMP-9 and Rac-1 expression

MMP-9 and Rac 1 are important proteins that regulate the migration process by degrading the matrix. CL significantly decreased the protein and gene expression of MMP-9 (Fig. 4A and B) in a concentration-dependent manner. More importantly, CL also reduced the expression of Rac-1 compared with the untreated cells (Fig. 4C). Overall, CL decreased Rac-1 expression associated with the anti-metastatic activity on highly metastasis breast cancer cells.

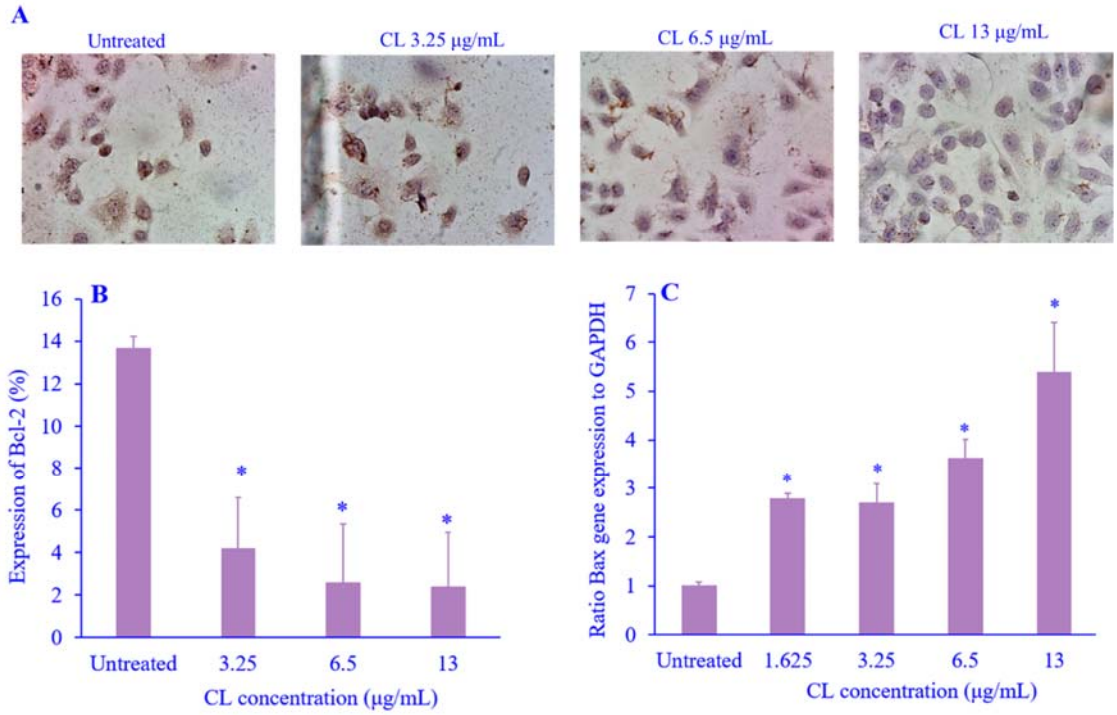

Fig. 2. The effect of CL on the apoptosis regulation. (A) Immunocytochemistry morphology of Bcl-2 expression; (B) quantification of Bcl-2 expression; and (C) percentage of Rac-1 gene expression level of cell migration-related proteins was measured by quantitative real-time polymerase chain reaction. Data are presented as mean ± SEM, N = 3. **P* < 0.05 Indicates significant differences in comparison with the untreated cells. CL, *Curcuma longa*; Bcl-2, B-cell lymphoma 2; Rac-1, Ras-related C3 botulinum toxin substrate 1.

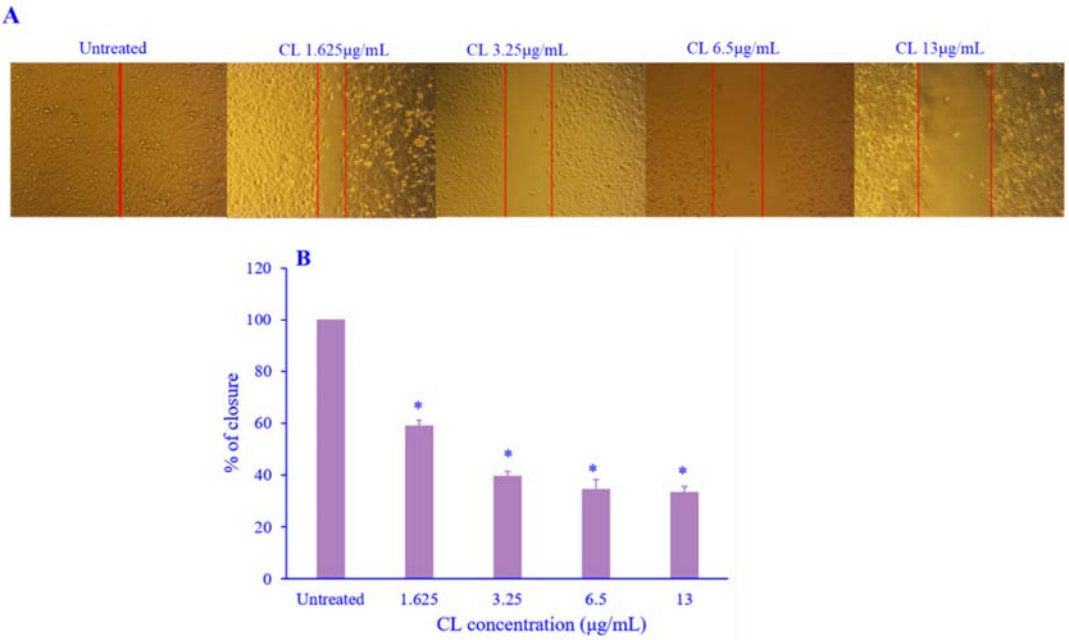

Fig. 3. The effect of CL treatment on the migration ability of MDAMB-231 breast cancer cells. (A) Cell migration was assessed *via* scratch wound healing assay; (B) the migration abilities were quantified by counting the area of closure using ImageJ and the percentage of the treated groups compared to the untreated group. Data are presented as mean ± SD, N = 3. **P* < 0.05 Indicates significant differences in comparison with the untreated cells. CL, *Curcuma longa*.

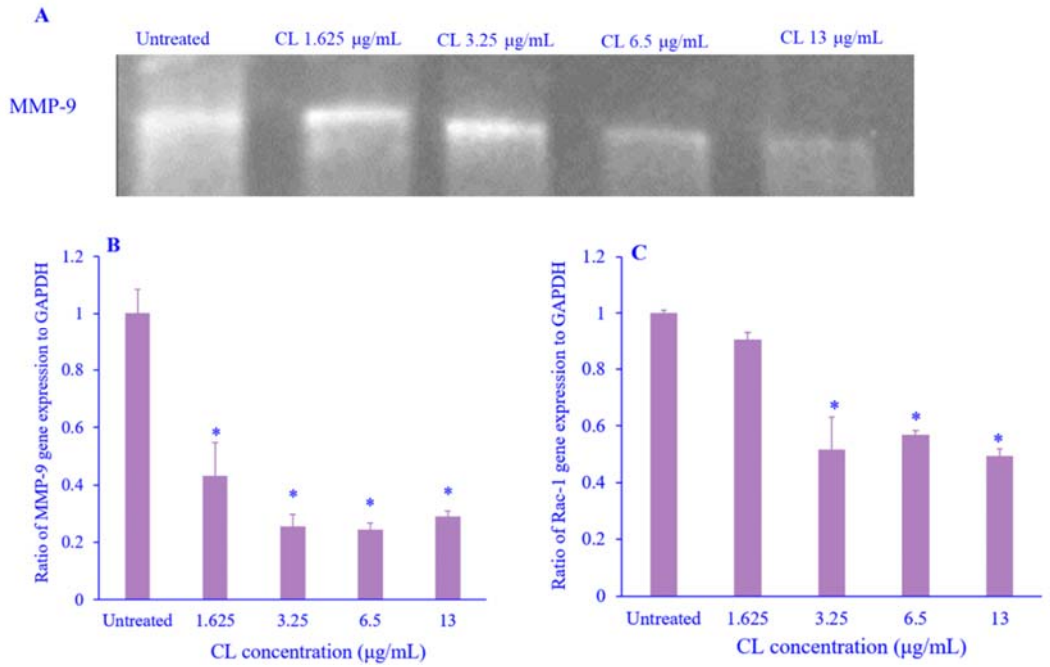

Fig. 4. The activity of MMP-9 and the expression of Rac-1 after CL treatment on MDAMB-231 breast cancer cells. (A) The MMP activity of MMP-9 was visualized as a clear band against the dark blue background of the gelatine substrate in the gel. Molecular weight markers were used to estimate the molecular masses of the pro and active forms of MMP-9. Blots were scanned and intensities of the bands were measured by ImageJ software, normalized with untreated, and plotted; (B) percentage gene expression of MMP-9 activity was measured by qRT-PCR, and (C) Percentage of Rac-1 gene expression level of cell migration-related proteins was measured by qRT-PCR. Data are presented as mean ± SEM, N = 3. **P* < 0.05 Indicates significant differences in comparison with the untreated cells. CL, *Curcuma longa;* MMP-9, matrix metalloproteinase-9; Rac-1, Ras-related C3 botulinum toxin substrate 1; qRT-PCR, quantitative real-time polymerase chain reaction.

DISCUSSION

The formation of metastases is a cascade of closely consecutive molecular and morphological changes, including the reconstruction of the cytoskeleton and extracellular matrix (ECM) (20). MMPs particularly MMP-2 and MMP-9 are responsible for the degradation of several ECM components associated with tumor metastasis and invasiveness (21,22). Furthermore, Rac-1 a member of Rho family GTPases specifically involved in metastasis through the production of sheet-like protrusions known as lamellipodia leading to metastasis (23-25). The inhibition of Rac1-mediated lamellipodia formation blocks the migration and invasion of cancer cells (26). Interestingly, Rac-1 in the interaction with Bcl-2 induced apoptosis through inhibition of S70pBcl-2 (27). This suggests that the inhibition of Rac-1 and MMPs plays an important role in breast cancer metastasis which can also indirectly induce apoptosis in breast cancer cells. CL has cytotoxic effects on some cancer cells, but has no toxic effects on normal cells, indicating the selectivity of CL towards cancer cells.

A previous study by Al-Amin *et al.* stated that chloroform fraction from *Curcuma caesia* contains furanodienone, curcuzederone, (1S, 10S), (4S,5S)-germacrone-1, 4-diepoxide, wenyujinin B, alismoxide, aerugidiol, zedoalactone B, zedoarondiol, isozedoarondiol. The study demonstrated some of the compounds are bioactive compounds and the effect of curcuzederone on MDA-MB-231 cell migration showed significant inhibition in scratch and Transwell migration assays (28).

Another study by Gao *et al.* reporting the effect of petroleum ether extracts of *Curcuma zedoaria* on the proliferation of human triple-negative breast cancer cell line MDA-MB-231 showed that MDA-MB-231 cells were inhibited by petroleum ether extracts of *Curcuma zedoaria*, and the inhibition rate depended on concentrations and time. Petroleum ether extracts of *Curcuma zedoaria* as well as epirubicin significantly arrest the cell cycle at the G0/G1 phase. The level of mRNA expression of proteins E-cadherin and E-cadherin significantly increased, while SDF-1, CCR7, and CXCR4 mRNA expression decreased after being incubated with petroleum ether extracts of *Curcuma zedoaria*. The differences were that the protein CXCR4 mRNA expression level was higher than the vehicle. This study stated that MDA-MB-231 cells' proliferation was inhibited by petroleum ether extracts of *Curcuma zedoaria* (29).

In the current study, we explored the potency of CL extract against the highly metastatic MDAMB-231 breast cancer cell. Recently, metastasis has still been highlighted as one of the main problems in cancer cure. Therefore, suppression of cancer migration is urgent and necessary. Cytotoxic activity of CL in several cancer cells and a side effect of chemotherapy-induced metastasis through the activation of the TGF-ß pathway have been reported in several articles (4,30-32). Regardless of the potent cytotoxic activity of CL, the anti-metastatic mechanism of CL to inhibit migration has not yet been investigated.

The hallmark of the current work is that CL exhibited a strong cytotoxic effect in a concentration-dependent manner with the IC_{50} value of 13 µg/mL. Interestingly, we also found that CL induced elevation of ROS levels up to 2.5 folds than untreated cells. ROS recently gained much attention for its role in cancer progression. ROS is physiologically generated from metabolism activities, therefore, over-proliferated cells like cancer cells generate higher levels of ROS (33,34). Cancer cells have mechanisms to shift the redox balance to maintain a high level of ROS such as overexpressing ROS metabolic enzymes (34). On the contrary, highly metastatic cancer cells require a low level of ROS to maintain their metastasis ability (33). The low level of ROS is achieved by keeping the ROS production low and/or enhancing the ROS scavenging system (35). Our study revealed that CL significantly enhanced the ROS levels. Similar to a previous study that curcumin induced ROS levels in MCF-7 cells (36,37). Furthermore, we explored the underlying mechanism involved in the cytotoxic effect of CL. We found that CL significantly induced pro-apoptotic protein Bax gene expression and inhibited anti-apoptotic protein Bcl-2 in a concentration-dependent manner compared with the untreated cells. CL

was also reported to cause A549 lung cancer and Hela cervix cancer cell death through apoptosis induction (38,39). In addition, apoptosis induction by CL was reported due to its ability to regulate p53 signaling (40). Moreover, CL treatment caused cell accumulation in the G2/M arrest by blocking the transition of cells from S to G2 phases, thereby consequently leading to apoptosis (41,42). These findings indicated that the CL treatment inhibited MDAMB-231 cell growth through the regulation of apoptosis protein and elevated ROS levels. Previous studies reported that the apoptosis phenomenon with the increasing doses of CL led to the G2/M phase cell cycle arrest causing ROS-mediated apoptosis in human gall bladder carcinoma and osteosarcoma MG-63 cells (41,43).

One of the interesting aspects of CL is to inhibit migration. In this study, we focused on evaluating the mechanism of CL in inhibiting cell migration that was confirmed through several mechanisms including inducing wound closure, MMP-9 protein expression, and Rac-1 gene expression after cells were treated with CL. The presence of CL significantly reversed the inhibition of wound closure and decreased MMP-9 and Rac-1 gene expression. A previous study has reported inhibited migration and invasion under CL administration by suppressing TGF-β and circulating cancer cells on lung metastatic breast cancer cells (44). MMP-9 activity is associated with cancer occurrence and development, curcumin inhibited MMP-9 activity by regulating the integrin pathway and proteolytic cleavage in human breast cancer cells (4,45).

Our study showed that CL significantly inhibited Rac-1 gene expression. Inhibition of Rac-1 leads to inhibition of migration. These indicated that the anti-metastatic effect of CL may be dependent on Rac-1 and MMP-9 gene expression.

CONCLUSION

To summarize, CL attenuated the metastatic ability of highly metastatic breast cancer cells by reducing MMP-9 and Rac1 expression. Collectively, these findings also indicated that CL as a potentially natural chemotherapeutic agent could be used to reduce cancer migration and prevent cancer recurrence.

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Conflict of interest statement

The authors declared no conflict of interest in this study.

Authors' contributions

All authors contributed equally to this work.

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