ORIGINAL ARTICLE

Regulation of p53 and survivin by *Curcuma longa* extract to caspase-3 dependent apoptosis in triple negative breast cancer cells

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ABSTRACT

Aim Triple negative breast cancer cells (TNBC) are the population of breast cancer cells that are responsible for cancer recurrence and apoptosis resistance. Unfortunately, current therapies have limited efficacy to TNBC population due to apoptosis resistance and chemoresistance. Tumour suppressor p53 and survivin are primary targets for TNBC therapy. Consequently, a search for a natural compound which targets p53 and survivin is needed to further advance TNBC treatment. *Curcuma longa extract* (CL), a natural compound induces apoptosis in several cancer cells by targeting various molecules and possess fewer side effects. However, a possible potential of CL as p53- and survivin modulating agent in TNBC cells has not been investigated.

Methods MDAMB-231 cells were treated with several concentration of CL, after which, viability, p53 gene expression, surviving protein expression, and caspase-3 protein expression were evaluated.

Results After 24-h treatment, CL possessed cytotoxic effect with IC_{50} value of 13 µg/mL. Treatment with 1.625, 3.25, 6.5, and 13 µg/mL of CL resulted in 2.70-25.80% increase in caspase-3 expression levels followed by 94.60 – 21.60% decrease in survivin protein levels. CL induced remarkably p53 gene expression ratio up to 5-fold at 13 µg/mL. Survivin protein levels were inversely proportional to p53 accumulation levels. Low survivin protein levels combined with high levels of p53 accumulation were correlated to higher apoptotic rates.

Conclusion p53 and survivin as molecular targets of CL contribute to caspase-3-dependent apoptosis in TNBC cells and this compound represents an attractive p53- and survivin modulating agent in TNBC.

Keywords: Caspase-3, *Curcuma longa* extract, p53, survivin, TNBC

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INTRODUCTION

Breast cancer is one of the leading causes of morbidity and mortality worldwide with about 20% of all deaths in developed countries (1). Among several cancer treatments, the main treatments that are commonly used to treat patients are surgery, radiotherapy and chemotherapy (2)and biopsy with reassessment of these markers at the time of disease recurrence is strongly recommended. Metastatic breast cancer is generally an incurable disease, with survival that could range from months to several years. Important but modest improvements in overall survival (OS. However, chemotherapy induces chemoresistance, which consequently limits the efficiency of chemo agents due to treatment failure and relapse (3,4). Accumulating evidence suggest that triple negative breast cancer (TNBC) population, a subgroup of cancer cells, is responsible for chemoresistance and cancer relapse, as it has ability to metastatic migration (5). A previous study reported that p53 inactivation and survivin overexpression are associated with chemoresistance and poor prognosis in TNBC (6). p53 is tumour suppressor protein that plays important roles in apoptosis. p53 mutation has been detected in 10-20% of TNBC patients (7). Furthermore, survivin, a member of the family of inhibitors of apoptosis proteins, plays a key role in the regulation of drug resistance and poor prognosis. Survivin is overexpressed in TNBC cells leading to apoptosis resistance (8). Targeting p53 and survivin makes an attractive therapeutic target for the treatment of TNBC. In TNBC population the absence of caspase-3 to inhibit apoptosis was demonstrated by a previous study, which reported that inactivated caspase effectively inhibiting cell death pathway (9, 10). This finding indicated that caspase-3 is also the one of pivotal pro-apoptosis proteins responsible for cancer death, including TNBC (11). Targeting p53, urviving, and caspase-3 in TNBC cells by a therapeutics agent may open up avenues to new therapeutic strategies for TNBC-directed therapy.

The use of natural plants, which contain substances that have medicinal features but are less toxic to healthy tissues and organs, represents a potential approach to decreasing apoptotic resistance (12, 13). *Curcuma longa* (CL) is a medicinal herb that has exhibited cytotoxic activity on several cancer cells (14, 15). A previous study reported that CL extracts might suppress cancer cell proliferation by inducing the G0/G1 cell cycle arrest and trigger cell apoptosis (16, 17). The apoptosis-inducing capabilities of CL were also demonstrated by various studies, which reported CL-induced apoptosis by increasing reactive oxygen species (ROS) levels (18, 19)we found that curcumin, a polyphenolic phytochemical extracted from the plant Curcuma longa, markedly induced the conversion of microtubule-associated protein 1 light chain 3 (LC3. CL induced breast cancer stem cells population through annexin-A2 regulation (20). However, whether CL can inhibit apoptosis resistance in TNBC remains unclear.

This study aimed to investigate the capacity of CL to induce apoptosis and to modulate p53, survivin, as well as caspase-3 expression as a marker of apoptosis pathway activation in TNBC cells.

MATERIAL AND METHODS

Material and study design

This post-test only control group study design was conducted in Stem Cell and Cancer Research (SCCR) Laboratory, Faculty of Medicine, Sultan Agung Islamic University, Semarang from June – October 2021.

The TNBC model used MDAMB-231 cancer cell line purchased from ECACC (Porton Down, Wiltshire, UK). The cells were grown on Dubelco's Modified Eagles Medium (DMEM)-high glucose (Sigma-Aldrich, Louis St, MO). Cells were treated with CL in several concentrations 5-200 µg/mL.

The study was approved by the Ethic Committee of Sultan Agung Islamic University (No. 371/VII/2021/Komisi Bioetik).

Methods

***Curcuma longa* extraction.** *Curcuma Longa* L. was acquired from the Medical Plant and Traditional Medicine Research and Development Center, Tawangmangu, Ministry of Health, Republic of Indonesia at 7.6638°S latitude and 111.1349° E longitude. The dried powder of *Curcuma longa* was extracted with 96% ethanol for 72 hours to ensure the complete extraction. The solvent of crude extract was evaporated under vacuum rotary evaporator at 50. About 90% of the so-

lution was evaporated to get the supersaturated solution. The *Curcuma longa extract* (CL) was subjected to cooling at 4. CL was dissolved in dimethyl sulfoxide (DMSO) (Sigma-Aldrich, Louis St, MO) to prepare a 100.000 μg/mL stock solution and stored in the dark at -20 until use. For each experiment, the CL was freshly prepared from the stock solution at the concertation ranging from 5-200 μg/mL by serially diluting in culture medium. The final DMSO concentration never exceeded 0.1% (v/v).

Cell line and culture condition. MDAMB-231 cancer cell line was purchased from ECACC (Porton Down, Wiltshire, UK) that serves as a model for TNBC. The MDAMB-231 cells were grown in Dubelco's Modified Eagles Medium (DMEM)-high glucose (Sigma-Aldrich, Louis St, MO,), supplemented with 10% Fetal Bovine Serum (FBS) (Gibco, Invitrogen, NY, USA), 1% penicillin (100U/mL)/streptomycin (100 μg/mL) (Gibco, Invitrogen, NY, USA) and 0.25% Amphotericin B (Gibco, NY, USA) under standard conditions at 37 ^{o}C in a humidified incubator with 5% CO_2.

Cytotoxic assay. Cytotoxic activity of CL was measured by 3-(4,5-dimethyl thiazolyl)-2,5-diphenyltetrazolium bromide (MTT) assay using MDAMB-231 cells (21–23). Briefly, $8x10^3$ cells per well were seeded into 96-well plate. After 24 h incubation in a 5% humidified CO2 incubator at 37 °C, the cells were treated with a fresh medium containing different concentrations of CL in triplicates. After 24 h of incubation, 100 μL/well MTT (Sigma Aldrich, Louis St, MO) was added and incubated for 4 h. 100 μL DMSO was added to each well and incubated for 15 minutes with shaking. The absorbance was recorded at 595 nm using a microplate reader (Bio-rad, CA, USA) and IC_{50} value was measured under isobologram analysis (24, 25).

Apoptosis analysis. Apoptosis assay was performed by using Annexin V-FITC/PI using previously described protocol with some modification (26, 27). Briefly, $2x10^5$ cells per well were seeded in 6 well-plates overnight. Cells were then treated with CL at various concentrations for 24 hours. Live cells were stained with FITC-annexin V (BD Biosciences, San Jose, CA) for 15 min in 1x biding buffer and then 5 μL of PI (Himedia) for 15 min at room temperature in the dark. Cells were washed three times with PBS and percent apoptosis cells were acquired using BD Accuri C6 flow cytometer (BD, Singapore) and data were analysed using BD Accuri C6 Plus software (BD Biosciences, CA).

P53 gene expression. Intracellular p53 protein level was assayed by the qPCR following the procedure provided by the manufacturer. Total RNA was collected from every group of treatment to conduct reverse transcription. P53 primer (Forward primer: 5'TGCGTGTGGAGTATTTGGATG-3' and Reverse primer: 5'TGGTACAGTCA-GAGCCAACCTC-3') was added and the expression of p53 mRNA was detected by qPCR. β-actin was selected as an internal parameter. The qPCR conditions were as follows: initial denaturation at 94°C for 5 minutes, followed by 30 cycles for amplification (94°C for 30 seconds, 56°C for 30 seconds, and 72°C for 1.5 minutes) and 72°C for 5 minutes.

Survivin expression. The TNBC were cultured in 6-well plates and incubated with 1.625, 3.25, 6.5 and 13μg/mL of CL for 24 h. After incubation, cells were lysed in RIPA buffer and protein concentration was measured using Pierce BSA CBB Assay. Aliquots of 10μg total protein were mixed with 2x Laemmli buffer (Biorad) with ratio 1:1, boiled, and separated on 10 % SDS-PAGE gels, transferred to Polyvinylidene Fluoride (PVDF) membranes, then blocked with 5% bovine serum albumin (BSA) (Sigma Aldrich, Louis St, MO) in Phosphate Buffered Saline with Tween (PBST) (Sigma Aldrich, Louis St, MO) for 1 h. Survivin antibodies (Santa Cruz Biotechnology) were applied in blocker overnight at dilutions of 1:1000, after which membranes were washed, incubated with HRP-conjugated secondary antibody (GeneTex Biotechnology), washed again, incubated with ECL reagent and exposed to chemiluminosence. ECL chemiluminescence was captured using the Invitrogen IBright ChemiDoc Imaging System (28).

Caspase-3 protein expression. The TNBCs were cultured in 6-well plates and incubated with 1.625, 3.25, 6.5 and 13μg/mL of CL for 24 h. Following treatment, the cells were isolated by trypsinization and rinsed twice with PBS. Active form of caspase-3 was determined by flow cytometry as previously described (29)a number of studies have shown that apoptosis can be induced in a p53-independent manner as well. In this study, we examined the mechanism of apoptosis in p53-null breast cancer cells in response to the proteasome inhibitor bortezomib. Initially, we determined the p53 status of 4T1 breast carcinoma and 4THMpc

(a highly mestatic derivative of 4T1. Cells were fixed and permeabilized using the Cytofix/Cytoperm TM kit (BD Pharmingen) for 20 min at room temperature. Cells were washed with Perm/WashTM buffer, then stained with phycoerythrin (PE)-conjugated anti-active caspase-3 monoclonal antibodies using 20μL/1×10^6 cells for 60 min at room temperature in the dark. Following incubation with the antibody, cells were washed in Perm/Wash TM buffer, re-suspended in Perm/Wash TM buffer and analysed using BD Accuri C6 flow cytometer (BD Biosciences) (24, 27).

Statistical analysis

All data were presented as mean±standard deviation (SD). Analysis of variance (ANOVA) followed by a Tukey post-hoc was used for multiple comparisons. A $p<0.05$ was considered to be statistically significant.

RESULTS

CL induces morphological alterations of MDAMB-231 cells, the changes in morphology were observed in a concentration-dependent manner (Figure 1A). The typical morphology of TNBC cells were altered starting from the treatment with 100 μg/mL CL and cells appeared shrunken, smaller in size and rounded. CL significantly decreased cell viability in dose-dependent manner with IC50 value of 13 μg/mL on MDAMB-231 (Figure 1B).

To evaluate whether the growth-inhibitory effect observed upon treatment of MDAMB-231 with CL was due to the induction of apoptosis, Annexin V/PI flow cytometry was applied. CL significant increase in the number of cell death was 82.3±0.90% and 98.10±1.31% for 6.5 μg/mL and 13 μg/mL, respectively (Figure 2).

Evaluating the effect of CL treatment on the p53 gene, it was found that after 24-h treatment with CL, the relatively low level of the p53 gene in the untreated MDAMB-231 increased significantly with increasing CL concentration. Particularly, the treatment with 13 μg/mL of CL resulted in 5.0-fold upregulate in p53 gene compared to untreated cells (Figure 3).

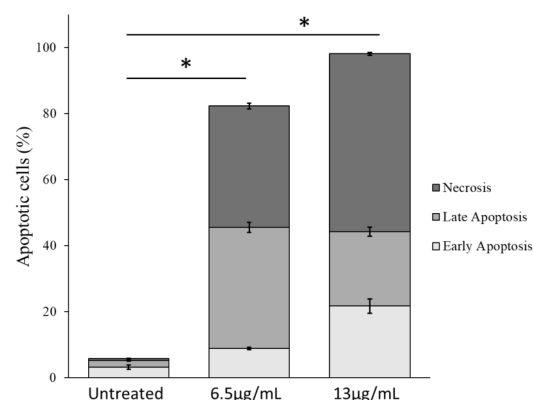

Figure 2. Induction of apoptosis by Curcuma longa (CL) on MDAMB-231. Cells were exposed to CL for 24 h. % cell death was measured by flow cytometry after staining with Annexin V-FITC/PI. The bar represents the mean ± SE value from triplicate independent experiment; *significant p<0.05 control vs each treatment

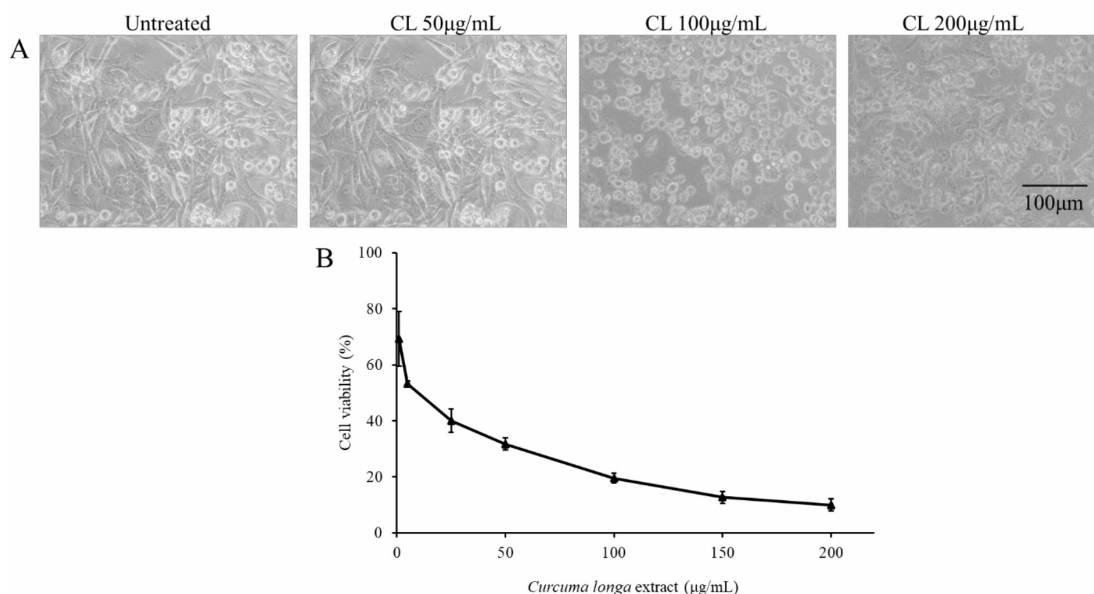

Figure 1. Cytotoxic effect of CL on MDAMB-231. A) Morphology of MDAMB-231 cells after CL treatment for 24 h (magnification 100x); B) MDAMB-231 cells were treated with CL at various concentrations (5-200μg/mL) for 24 h and the viability of cells was evaluated using MTT assay; The results are presented as the mean±SE for n=3

An evaluation of the effect of CL on the expression of antiapoptotic protein of surviving found that after 24-h treatment with CL, the high level of survivin protein in the untreated MDAMB-231 significantly decreased with increasing CL concentration. Especially, the treatment with CL 13 μg/mL resulted in a decrease of survivin protein level up to 21.64% as compared to untreated cells (Figure 4).

CL significantly increased caspase-3 protein expression in a dose-dependent manner and resulted in 4- to 25-fold for 1.625-13 μg/mL CL as compared to untreated cells (Figure 5).

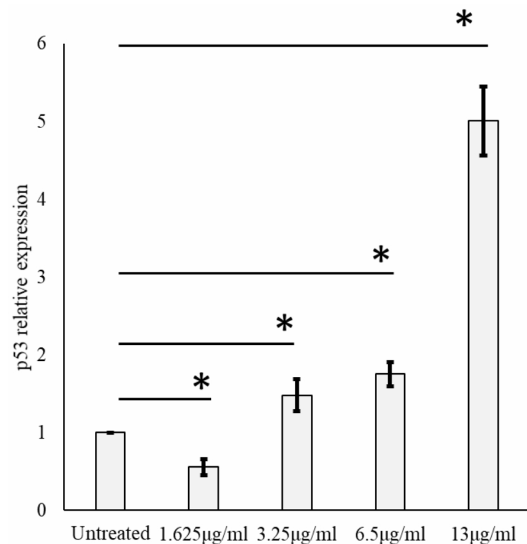

Figure 3. p53 gene expression in MDAMB-231 cells by qPCR after 24 h of *Curcuma longa* (CL) treatment. The bar represents the mean±SE value from triplicate independent experiment; *significant $p<0.05$ control vs each treatment

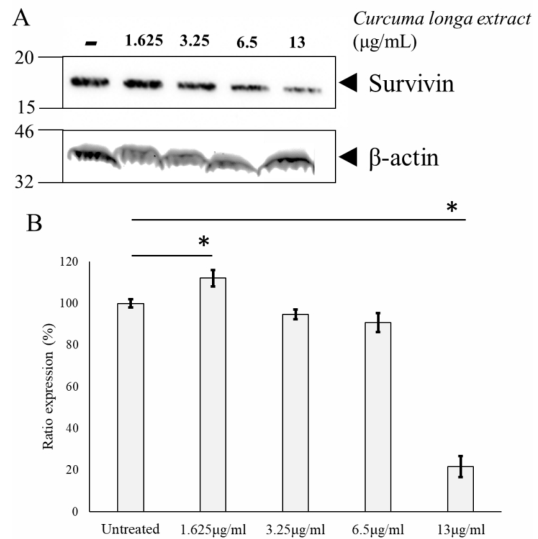

Figure 4. Survivin protein expression level in MDAMB-231 cells by western blot after 24 h of *Curcuma longa* (CL) treatment. A) Representative western blot for survivin detection in the MDAMB-231 cells. B) Quantification analysis of survivin band intensity. The bar represents the mean±SE value from triplicate independent experiment; *significant $p<0.05$ control vs each treatment

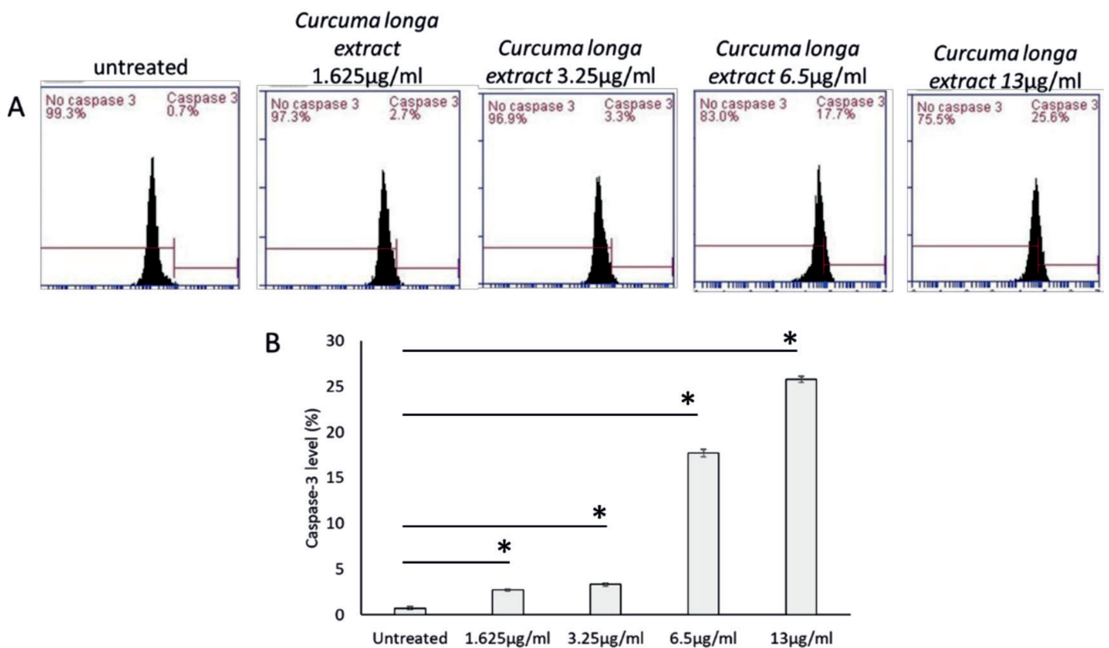

Figure 5. Induction of caspase-3 protein expression by *Curcuma longa* (CL) on MDAMB-231. A) Histogram of caspase-3 flow cytometry analysis; Cells were exposed to CL for 24 h. Percentage of caspase-3 level was measured by flow cytometry after incubation with antibody caspase-3. B) Quantification of caspase-3 level after treated with several concentration of CL for 24 h. The bar represents the mean±SE value from triplicate independent experiment; *significant $p<0.05$ control vs each treatment

DISCUSSION

TNBC cells have a specific characterisation including cancer progression, cancer relapse, metastatic and drug resistance due to their ability to self-renew and differentiate into heterogeneous lineages of cancer cells (5, 6). Apoptosis resistance appears to be the biggest problem for cancer treatment specially to eliminate TNBC (30). It is now becoming apparent that many drugs can kill tumour cells by activating common apoptotic pathways (3). Significant majority of the TNBCs tested lacked caspase-3 expression and p53 mutations. Patients with relapsed TNBC have p53 mutations suggesting a deficient p53 signalling pathway. In addition, caspase-3 is a member of the cysteine protease family, which plays a central role in the execution of apoptosis (9). A previous study reported that inhibited caspase-3 protein expression appears to be one of the primary means used by TNBC to block the apoptosis pathway, that makes TNBC difficult to eliminate using most common chemotherapy (26).

In our study, CL possess strong cytotoxic activity through apoptosis induction in MDAMB-231 cell line. Our data showed that CL induced apoptosis in a dose-dependent manner up to 11.47% for 13µg/mL and it is in line with the previous study, reported that curcumin as the main secondary metabolite from *Curcuma longa* extract induced apoptosis of BCSCs by increasing p53 protein levels, many apoptosis proteins such as caspase-3 and reducing antiapoptotic protein such as Bcl-2 (31, 32). Curcumin is also reported to cause leukemic cells death through apoptosis induction and ROS elevation (33). This evidence could mean an advantage of CL over other chemotherapeutics that need functional several proteins apoptotic to provoke its cytotoxic effect.

Survivin has been found to be upregulated in TNBC but rarely in normal cells (34). Therefore, it may provide a potential target for selective therapy in TNBC. Survivin mediates the suppression of apoptosis by direct inhibition of caspase-3. In our study the induction of caspase-3 expression through inhibition of the survivin expression seems to be one of the molecular pathways leading to CL apoptotic effect in MDAMB-231 cells. In agreement with our data, different researchers showed that *Curcuma longa* extract can dose-dependently induce downregulation of survivin and trigger caspase-3 activation and apoptosis in different kinds of cancer cells (31,35).

In this study, we also found that p53 protein was highly expressed in all treatment groups in doses-dependent manner, which indicated that CL can robustly induce apoptosis. These findings agreed with those reported by a previous study, which found that inhibited p53 gene expression correlated with apoptosis resistance of TNBC (36)the role of early life experiences in future disease risk is gaining attention. The Barker hypothesis proposes fetal origins of adult disease, with consistent evidence demonstrating the deleterious consequences of birth weight outside the normal range. In this study, we investigate the role of birth weight in CVD risk prediction. Methods and Results—The Women's Health Initiative (WHI. Therefore, the emergence of high apoptotic resistance in TNBC may be due to the low level of caspase-3 and p53 gene expression. However, we found that CL treatment could increase caspase-3 and p53 gene expression and decrease survivin protein expression level in all treatment groups, indicating that CL treatment can induce apoptosis in TNBC. Therefore, CL could be introduced as a new TNBC-sensitizing agent to chemotherapeutic drugs due to its effect on decreasing survivin protein expression and induction of apoptosis in TNBC cells.

Based on our study, we conclude that CL as a traditional medicine may enhance apoptosis in TNBC by increasing the expression of caspase-3 as pro-apoptotic protein, increasing p53 tumour suppressor gene, and decreasing survivin as an anti-apoptotic protein. These findings also suggest that the potential anticancer activities of CL should be further developed for cancer treatment in the future.

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TRANSPARENCY DECLARATION

Conflict of interest: None to declare.

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