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ORIGINAL ARTICLE

Aqueous Leaf Extract of *Clinacanthus nutans* Inhibits Growth and Induces Apoptosis via the Intrinsic and Extrinsic Pathways in MDA-MB-231 Human Breast Cancer Cells

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ABSTRACT

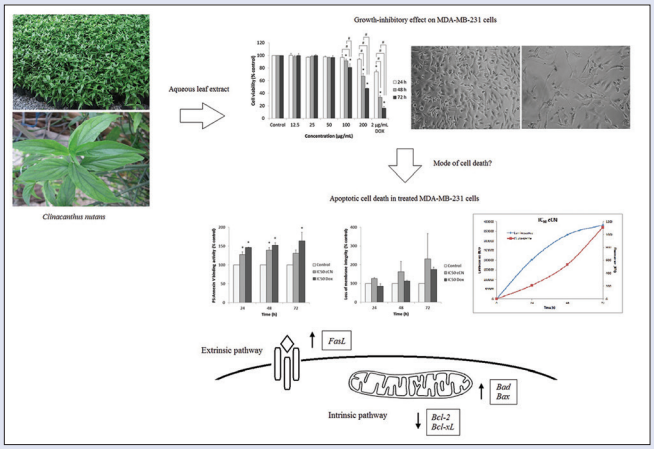
Background: *Clinacanthus nutans* possesses several reported biological activities against different human cancer cells. However, reports on the growth-inhibitory effect of *C. nutans* leaf extract on the aggressive triple-negative breast cancer cells and the mechanisms of induced-cell death in these cells are limited. **Objectives:** The study aimed to assess the anticancer efficacy and associated mechanisms of the crude aqueous extract of *C. nutans* leaves (cCN) in MDA-MB-231 triple-negative human breast cancer cells. **Materials and Methods:** The metabolic viability of the MDA-MB-231 cells following respective treatments with cCN was measured using an adenosine triphosphate luminescent assay. The mode of cell death in MDA-MB-231 cells induced by cCN was examined using a luminescence- and fluorescence-based assay and the mechanisms involved were evaluated by comparative analysis of gene expression by reverse transcription–quantitative polymerase chain reaction. **Results:** Dose- and time-dependent growth inhibition of MDA-MB-231 cells by cCN was observed (IC_{50}: 191.20 µg/mL). cCN also induced apoptotic cell death in the treated cells via the intrinsic and extrinsic apoptosis pathways by affecting the mRNA expression levels of *Bad, Bax, Bcl-2, Bcl-xL*, and *FasL*. **Conclusion:** These results suggest that *C. nutans* can be used as a potential agent in the treatment and prevention of breast cancer.

Key words: Apoptosis, breast cancer, *Clinacanthus nutans*, growth-inhibitory, MDA-MB-231

SUMMARY

- The aqueous leaf extract of *Clinacanthus nutans* (cCN) inhibited growth of MDA-MB-231 cells in a dose-and time-dependent manner
- cCN induced apoptotic cell death in treated MDA-MB-231 cells
- cCN affected the mRNA expression levels of *Bad, Bax, Bcl-2, Bcl-xL*, and FasL genes, suggesting that both intrinsic and extrinsic apoptosis pathways were triggered following treatment.

Abbreviations used: ATP: Adenosine triphosphate; cCN: *Clinacanthus nutans* aqueous leaf extract; Ct: Threshold cycle; Dox: Doxorubicin; IC_{50}: Half-maximal inhibitory concentration; MDA-MB-231: Human breast cancer cell line; mRNA: Messenger ribonucleic acid; MOMP: Mitochondrial outer membrane permeabilization; PS: Phosphatidylserine; RFU: Relative fluorescence units; RLU: Relative luminescence units; RT-qPCR: Reverse transcription–quantitative polymerase chain reaction; SD: Standard deviation.



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INTRODUCTION

Cancer is a leading cause of global death where approximately 9.6 million deaths were reported in 2018.[1] The most common cancer in women worldwide, including Malaysia, is breast cancer.[2] Early-stage breast cancer can be removed by surgery, but when metastasis occurs, advanced cancer is generally incurable.[3] Other breast cancer treatments include chemotherapy, radiotherapy, and hormone therapy. The triple-negative breast cancer subtypes, in which they lack estrogen receptors, progesterone receptors, and human epidermal growth factor 2 receptors, are generally aggressive and resistant to therapies, making them hard to treat.[4] In chemotherapy, cytotoxic drugs are commonly used to destroy cells that grow and divide at an abnormal rate. However, healthy cells can also be

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affected by most of the chemotherapy drugs, causing undesirable side effects and complications, such as immunosuppressant, breakdown of the nervous system, and in extreme cases, organ damage.[5] Hence, effective anticancer drugs that specifically target the triple-negative cells without affecting normal cells and produce lesser side effects are necessary for breast cancer treatments.

The discovery of new bioactive compounds from natural products that can induce apoptotic cell death in cancer cells remains a popular research area.[6] Plant extracts have been found to promote apoptosis in several types of human cancer cells.[7] Moreover, pytochemical compounds, including phenolics and flavonoids[8] from different plant parts, have been reported to have a protective effect against carcinogens[9] by detoxifying and enhancing the excretion of carcinogens.[10] These compounds can serve as the source of potential anticancer drugs.[11]

Clinacanthus nutans (Burm. f.) Lindau grows natively in Southeast Asia, including Malaysia, and has a wide range of pharmacological activities. These activities include antioxidant, anti-inflammatory, antidiabetic, antimicrobial, and antiviral against Types I and II herpes simplex virus, human papillomavirus, varicella-zoster virus, and dengue virus.[12] Moreover, leaves of *C. nutans* also exhibited cytotoxicity and antiproliferative activity against human gastric cancer,[13] cervical carcinoma (HeLa), Burkitt's lymphoma (Raji), erythroleukemia (K562),[14,15] and melanoma (D24)[16,17] cells.

Although these results suggest that the extracts may contain anticancer compounds and may be used as an adjuvant cancer therapy, there are still knowledge gaps that need to be addressed. Studies on the growth-inhibitory effect of *C. nutans* leaf extract on triple-negative breast cancer cells, which are often resistant to regular therapies, as well as the mechanisms of induced-cell death in these cells are lacking. Therefore, the objectives of this research were to i) assess the metabolic viability of the triple-negative MDA-MB-231 breast cancer cells following treatment with the crude aqueous extract of *C. nutans* leaves (cCN) by measuring the level of adenosine triphosphate (ATP) in living cells using luminescent assay, ii) investigate the mode of cell death induced by the extract in the treated cells by evaluating the phosphatidylserine (PS) exposure on the outer cell membrane leaflet during apoptosis and loss of membrane integrity through measuring the luminescent and fluorescent signals, respectively, and iii) determine the molecular mechanisms involved by gene expression analysis using reverse transcription–quantitative polymerase chain reaction (RT-qPCR).

MATERIALS AND METHODS

Plant materials

C. nutans leaves were freshly collected from Bukit Trig (Trig Hill), Sandakan, Sabah, Malaysia. Identification and authentication of the plant were performed by Julius Kulip from the Institute for Tropical Biology and Conservation, Universiti Malaysia Sabah, and deposited at the Borneensis Herbarium, Universiti Malaysia Sabah (BORH2093). Cleaned leaves were oven-dried at 40°C until a constant weight was obtained. The dried leaves were powdered using an electrical blender.

Crude leaf extraction

Dried powdered leaves were extracted with distilled water (solvent-to-sample ratio of 50:1) on an orbital shaker with a speed of 200 rpm at 22°C for 72 h. Following, the extracted solution was transferred, filtered with Whatman No. 1 filter paper and lyophilized using a freeze dryer (Labconco, USA) until complete dryness to obtain the crude *C. nutans* aqueous dried leaf extract (cCN). The crude cCN was redissolved in sterilized distilled water for subsequent experiments.

Cell culture

MDA-MB-231 cells were cultured in complete high-glucose Dulbecco's Modified Eagle Medium with pyruvate (Gibco, USA), L-glutamine (Gibco), 10% (v/v) heat-inactivated fetal bovine serum (Thermo Fisher Scientific, USA), and 1% (v/v) penicillin/streptomycin (Gibco). The cells were grown in a humidified environment with 5% CO_2 at 37°C.

Cell viability assay

The viability of MDA-MB-231 cells was assessed using CellTiter-Glo® 2.0 Assay (Promega, USA) as per the manufacturer's protocol. Briefly, MDA-MB-231 cells were seeded at 5×10^3 cells/well on a 96-well white-walled, flat-bottomed plate (Corning, USA) and incubated for 24 h for cell attachment. Then, the cells were treated with different concentrations of cCN (0–200 µg/mL) for 24, 48, and 72 h. Distilled water and doxorubicin (Dox) (0–2 µg/mL) were used as negative (vehicle) and positive controls, respectively. Luminescent signal, which corresponds to the amount of ATP produced by metabolically active cells, was recorded using a GloMax® Discover microplate reader (Promega). The percentage of viable cells was calculated using the following equation:

$$Viable\ cells\ (\%) = \frac{Luminescence\ of\ sample\ (RLU)}{Luminescence\ of\ control\ (RLU)} \times 100$$

The IC_{50} values of cCN and Dox were obtained from the dose–response curve (non-linear regression), generated using GraphPad Prism 7.04 (GraphPad Software, Inc., San Diego, USA).

Following respective treatments, MDA-MB-231 cells were also observed using an Olympus CKX41 (Japan) inverted microscope under a × 10 objective. Images were captured with a Lumenera Infinity-2 digital CCD camera.

Apoptosis and necrosis assay

The type of cell death induced in the cCN-treated MDA-MB-231 cells was detected using RealTime-Glo™ Annexin V Apoptosis and Necrosis Assay (Promega) as per the manufacturer's protocol. Briefly, MDA-MB-231 cells were seeded at 5×10^3 cells/well on a 96-well, white-walled, flat-bottomed plate (Corning) and incubated for 24 h for cell attachment. Then, the cells were treated with cCN and Dox (positive control) at the IC_{50} concentrations as well as distilled water as vehicle control for 24, 48, and 72 h. Luminescent and fluorescent signals, which correspond to phosphatidylserine (PS): Annexin V binding activity (apoptosis) and membrane integrity (necrosis), respectively, were detected using a GloMax® Discover microplate reader (Promega). The apoptotic and necrotic cells percentages were calculated using the following equations:

$$Apoptotic\ cells\ (\%) = \frac{Luminescence\ of\ sample\ (RLU)}{Luminescence\ of\ control\ (RLU)} \times 100$$

$$Necrotic\ cells\ (\%) = \frac{Fluorescence\ of\ sample\ (RFU)}{Fluorescence\ of\ control\ (RFU)} \times 100$$

RNA extraction and reverse transcription–polymerase chain reaction

Total RNA was extracted from the untreated cells and cells treated with the IC_{50} concentrations of cCN and Dox for 48 h using the RNeasy Mini Kit (Qiagen, Germany) as per the manufacturer's protocol. First-strand cDNA was synthesized using the GoScript Reverse Transcription System (Promega, USA). Briefly, a tube containing 10 ng of extracted RNA, 1 µL of 0.5 µg/µL Oligo $(dT)_{15}$ primer, 1 µL of 0.5 µg/µL random primers, and nuclease-free water to make up a final volume of 10 µL

was warmed to 70°C for 5 min and then cooled in an ice bath for 5 min. After brief centrifugation, 4 µL of GoScript 5× reaction buffer, 2 µL of 25 mM $MgCl_2$, 1 µL of PCR nucleotide mixture, 0.5 µL of 2500 U recombinant RNasin ribonuclease inhibitor, 1 µL of 200 U/µL GoScript reverse transcriptase, and 1.5 µL of nuclease-free water were added to the tube. The reaction mixture with a final volume of 20 µL was sequentially incubated in a thermal cycler at 25°C for 5 min, 42°C for 60 min and finally 70°C for 15 min to inactivate the reverse transcriptase.

Real-time quantitative polymerase chain reaction

The expression levels of *Bad, Bcl-2, Bax, Bcl-xL, TNF-α, FasL, TRAIL*, and *FADD* mRNAs were examined using real-time qPCR in a GoTaq qPCR Master Mix System (Promega, USA). Amplification and quantification were performed in an ABI 7500 Real-Time PCR System (Applied Biosystems, USA) in a total volume of 20 µL containing10 µL of 2× GoTaq qPCR Master Mix, 1 µL of 0.5 µM primers [Table 1], 2 µL of 10 ng cDNA, and 6 µL of nuclease-free water. Cycling conditions consisted of a DNA polymerase activation step at 95°C for 2 min, subsequently 40 cycles of denaturation at 95°C for 15 s, and annealing and extension at 58°C for 1 min. *GAPDH* and no-template reaction were used as endogenous and negative controls, respectively. The specificity of amplification was ensured by analyzing the melting curve of each primer pair. qPCR data analysis was performed using the comparative Ct method. The $2^{-\Delta\Delta Ct}$ method as described by Schmittgen and Livak[18] was used to calculate the fold change of gene expression and presented as $\log_2(2^{-\Delta\Delta Ct})$.

Table 1: Primer sequences of apoptosis-associated genes used in the quantitative polymerase chain reaction

Gene	Sequences (5′-3′)	Accession ID
Bad	Forward: CCTCAGGCCTATGCAAAAAG Reverse: AAACCCAAAACTTCCGATGG	NM_032989.2
Bcl-2	Forward: ATTGGGAAGTTTCAAATCAGC Reverse: TGCATTCTTGGACGAGGG	NM_000657.2
Bax	Forward: GCTGGACATTGGACTTCCTC Reverse: CTCAGCCCATCTTCTTCCAG	NM_001291431.1
Bcl-xL	Forward: GGCTGGGATACTTTTGTGGA Reverse: AAGAGTGAGCCCAGCAGAAC	NM_001322240.1
TNF-α	Forward: GACAAGCCTGTAGCCCATG Reverse: TCTCAGCTCCACGCCATT	NM_000594.3
FasL	Forward: CCATGTGAAGAGGGAGAAGC Reverse: AAGACAGTCCCCCTTGAGGT	NM_001302746.1
TRAIL	Forward: TTCACAGTGCTCCTGCAGTC Reverse: TTGGGGTCCCAATAACTGTC	NM_001190943.1
FADD	Forward: AGATGAACCTGGTGGCTGAC Reverse: AGGACGCTTCGGAGGTAGAT	NM_003824.3
GAPDH	Forward: CGACCACTTTGTCAAGCTCA Reverse: CCCCTCTTCAAGGGGTCTAC	NM_001289745.2

Statistical analysis

All experiments were repeated thrice unless stated otherwise. Data were presented as mean ± standard deviation (SD). A statistically significant difference at $P \leq 0.05$ significance level was determined using ANOVA with Tukey's test and *t*-Test.

RESULTS

cCN inhibited growth of MDA-MB-231 cells

The viability of MDA-MB-231 cells after respective treatments was determined by ATP assay using luciferase. As shown in Figure 1a, treatment with cCN at high concentrations, i.e., 100 and 200 µg/mL, for 72 h resulted in a significant ($P \leq 0.05$) decrease in cell viability. Only 47.7% of cells were viable after treatment with the highest dose for the longest exposure time. Besides, viability of MDA-MB-231 cells exposed to 200 µg/mL of cCN significantly ($P \leq 0.05$) reduced after each treatment time. This effect was also observed in cells treated with 2 µg/mL of Dox. The IC_{50} values at 72 h for cCN and Dox were 191.20 and 0.23 µg/mL, respectively. Direct observation using an inverted light microscope showed a reduction in the number of cells across concentrations and treatment times [Figure 1b]. These results suggest that the observed effect was dose and time dependent.

cCN induced apoptosis in MDA-MB-231 cells

To study the type of cell death induced, MDA-MB-231 cells were treated with IC_{50} of cCN, and hallmarks of apoptosis [Figure 2a] and necrosis [Figure 2b] were monitored for 72 h following treatment. The induction of cell death by cCN was compared to that of Dox, an inducer of apoptosis. The intensity of luminescent signal, which was used to measure the amount of PS exposure on the cell membrane outer leaflet during apoptosis, continued to increase within 48 h of treatments with cCN and Dox. However, at 72 h, the luminescent signal was reduced and florescent signal, which corresponded to plasma membrane disintegration, was amplified for cCN, suggesting that the treated cells were entering the late apoptosis stage or secondary necrosis. Besides, treatment with cCN [Figure 2ci] and Dox [Figure 2cii] revealed a time delay between the occurrence of PS: Annexin V binding activity (apoptosis) and plasma membrane disintegration (necrosis), indicating that the treated cells were undergoing apoptosis leading to secondary necrosis. In contrast, control showed a concurrent emergence of luminescent and fluorescent signals, suggesting a non-apoptotic characteristic

cCN regulated apoptotic signaling molecules in MDA-MB-231 cells

The underlying mechanism of cCN-induced apoptotic cell death in MDA-MB-231 cells was elucidated by gene expression analysis using qPCR. Apoptosis can be initiated through two separate pathways, the intrinsic and extrinsic pathways. Some of the major signaling molecules that regulate the intrinsic pathway include the antiapoptotic (*Bcl-2* and *Bcl-xL*) and proapoptotic (*Bad* and *Bax*) molecules. The extrinsic pathway is regulated by death receptors where binding of death receptor ligands, such as *TNF-α*, *FasL*, and *TRAIL* to their respective death receptors will recruit adaptor proteins, such as TRADD and FADD. As shown in Figure 3, treatment with IC_{50} of cCN for 48 h increased the expressions of proapoptotic genes, *Bad* and *Bax*, whereas the expressions of antiapoptotic genes, *Bcl-2* and *Bcl-xL*, were decreased. Besides, cCN increased the expression of *FasL* but produced an opposite

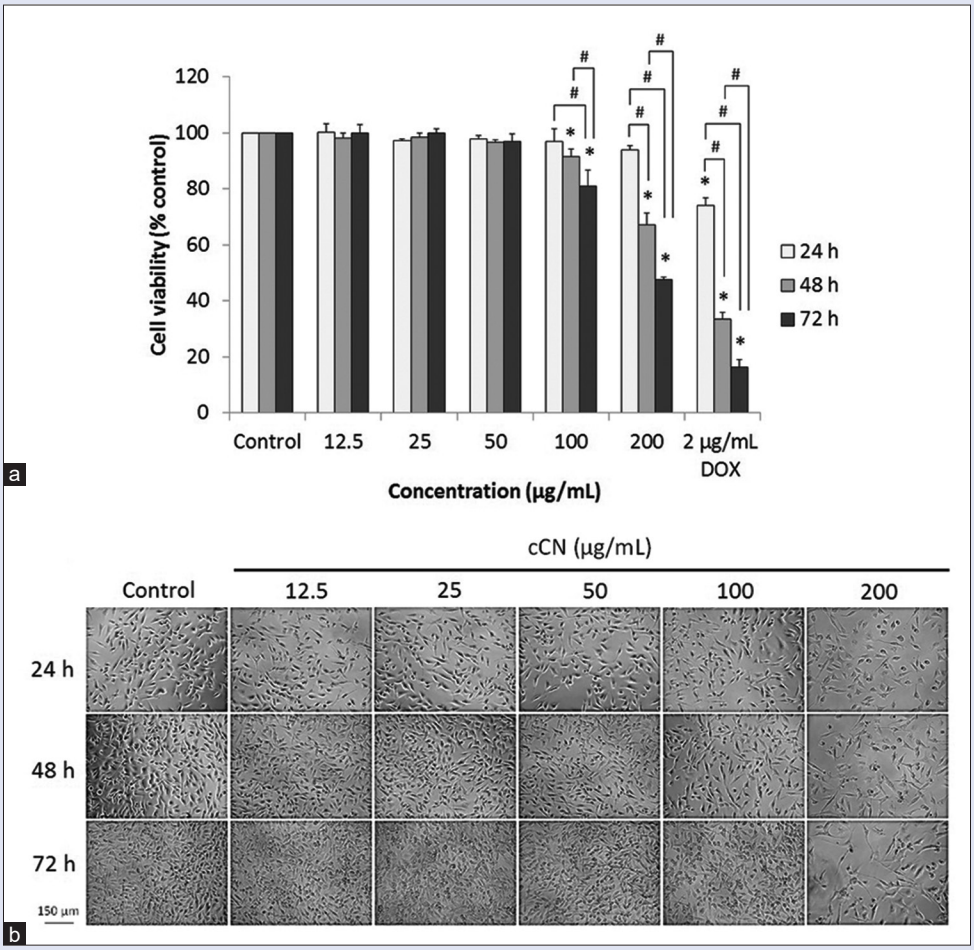

Figure 1: Inhibitory effect of cCN on MDA-MB-231 cells. Viability of MDA-MB-231 cells following treatment with varying concentrations (0-200 µg/mL) of cCN for 24, 48, and 72 h (a). Observation of MDA-MB-231 cells under a light microscope following treatment with indicated concentrations and exposure times (magnification, ×10) (b). Data represent mean ± SD from three independent experiments (n = 3). *$P \leq 0.05$ versus vehicle control; #$P \leq 0.05$ versus treatment times

effect on the other death receptor ligands, i.e., tumor necrosis factor alpha and *TRAIL*. When *FasL* or *TRAIL* binds to their proapoptotic receptors, FADD will be recruited, which allows the death-inducing signaling complex to form. This event will then activates the initiator pro-caspases 8 and 10 and the regulator cellular FADD-like IL-1 β-converting enzyme (FLICE)-inhibitory protein (c-FLIP), eventually leading to apoptotic cell death.[19] However, in this study, cCN decreased the expression of FADD. This might be due to the binding of another signaling molecule, called Daxx, which has been recently reported to be involved in the apoptotic pathway downstream of Fas.[20] These results suggest that cCN induced apoptosis in triple-negative MDA-MB-231 breast cancer cells via the intrinsic and extrinsic pathways.

DISCUSSION

Medicinal plants are still being used to this day as the primary source of medical treatment, especially in developing countries, where assess to modern medicine is limited. Plants contain a wide array of bioactive photochemicals and studies have proven them to be effective in treating a range of diseases. Many plant species are currently being used for the prevention and treatment of cancer, for instance, *Taxus brevifolia*: paclitaxel and *Catharanthus roseus*: vincristine and vinblastine.[21] Thus, drug discovery from natural products is an important area of research and there are still great opportunities to discover potential anticancer agents from medicinal plants.

The main objective of this study was to assess the anticancer efficacy and associated mechanisms of the cCN in MDA-MB-231 triple-negative human breast cancer cells. Dose- and time-dependent growth inhibition of MDA-MB-231 cells by cCN was observed with an IC_{50} value of 191.20 µg/mL at 72 h, suggesting the extract to be moderately active.[22] *C. nutans* contains several biologically active phytochemicals, such as flavonoids, triterpenoids, steroids, phytosterols, and glycosides, which may be responsible for its anticancer activity.[23] The results of this present study are in agreement with the studies by Quah *et al.*[24] and Khiru Nasir *et al.*[25] who reported that the methanol leaf extract of *C. nutans* was able to affect the cell viability of MDA-MB-231 cells, with IC_{50} values of 18.67 and 170 µγ/mL, respectively. The differences in IC_{50} values may be attributed to the type of solvents and extraction methods used.

Apoptosis, a form of programmed cell death, is vital in various processes including normal cell turnover to maintain cell populations in tissues through a homeostatic mechanism.[26] Dysregulation of apoptosis results in uncontrolled cell growth and division, which then leads to the development of different diseases, including cancer.[27] The mechanism of apoptosis generally involves two main pathways: I) the intrinsic pathway, which is mitochondrial mediated and ii) the extrinsic pathway,

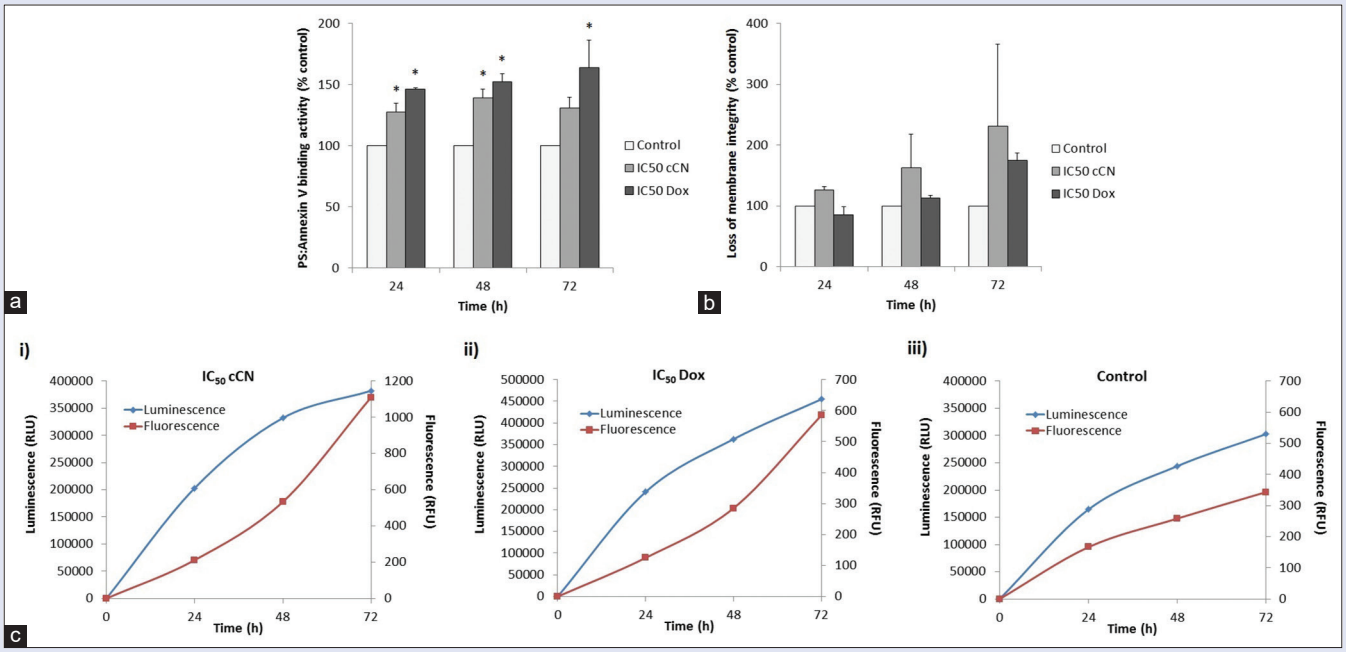

Figure 2: Cell death mechanism of MDA-MB-231 cells treated with IC_{50} concentrations of cCN and Dox for 24, 48, and 72 h. Data were expressed as a percentage relative to the vehicle control. PS: Annexin V binding activity of MDA-MB-231 cells, indicative of apoptosis (a). Loss of membrane integrity of MDA-MB-231 cells, indicative of secondary necrosis (b). Kinetic profiles of PS exposure (luminescence) and loss of plasma membrane integrity (fluorescence) of MDA-MB-231 cells (c) treated with IC_{50} concentrations of cCN (i), Dox (ii) and vehicle control (iii) for 24, 48, and 72 h. Data represent mean ± SD from three independent experiments ($n = 3$). *$P \leq 0.05$ versus vehicle control

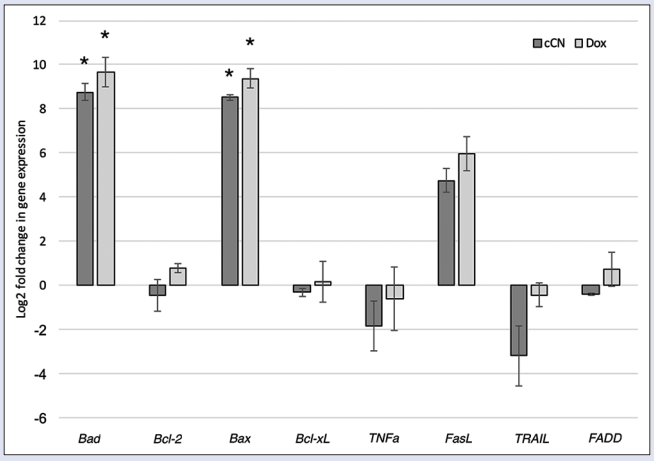

Figure 3: Effects of cCN and Dox on the mRNA expression of intrinsic and extrinsic signaling molecules in MDA-MD-231 cells. Cells were treated with IC_{50} concentrations of cCN and Dox for 48 h. The mRNA expression of apoptosis-related genes was analyzed by qPCR using SYBR Green method. Target gene expression was normalized to *GAPDH* mRNA expression, and the results were expressed as log2 ($2^{-\Delta\Delta Ct}$) fold change relative to control (untreated cells). Data represent mean ± SD from two independent experiments ($n = 2$). *$P \leq 0.05$ versus control

which is death receptor mediated. Each of the pathways is regulated by specific signaling molecules. For instance, the intrinsic pathway is modulated by Bcl-2 proteins: proapoptotic effector proteins (*Bax* and *Bak*), which are essential for mitochondrial outer membrane permeabilization (MOMP) for the induction of apoptosis; the anti-apoptotic *Bcl-2* proteins (e.g., *Bcl-2*, *Bcl-xL*, and *Mcl1*), which inhibit MOMP; and the BH3-only proteins (e.g., Bid, Bim, Bad, and Noxa), which trigger the proapoptotic effectors or neutralize the antiapoptotic proteins or both.[28] Meanwhile, the extrinsic pathway is modulated by death receptors, i.e., Fas, TRAIL-R, and TNFR where binding to their respective legends will recruit a caspase-activation platform, inducing apoptosis.[28] Thus, apoptosis induction is a key target in cancer therapy, and understanding the mechanisms involved will help enhance the efficacy of the therapy. The discovery of anticancer agents with the ability to induce apoptosis with minimal side effects, for example, inflammation, has gained increasing interest during the last decade.

As an initial step to elucidate the mechanism of cell death responsible for the reduction of MDA-MB-231 cell viability following treatment with cCN, an apoptosis and necrosis assay was used to evaluate the type of cell death induced. During the early apoptosis, the PS is translocated to the outer leaflet on the plasma membrane phospholipid bilayer where it is exposed to a luciferase-tagged Annexin V fusion protein. Binding of the protein to the PS generates a luminescent signal, indicating that an increase in cellular apoptosis increases the intensity of the luminescent signal. The assay also uses a DNA-binding dye that penetrates the damaged cell membrane during necrosis and produces a fluorescent signal upon DNA release.[29] In this study, a time-dependent intensification of luminescence within 48 h of cCN exposure followed by a delayed increase in fluorescent signal in treated MDA-MB-231 cells was observed. This kinetic profile has been described by Kupcho *et al.*,[30] as a characteristic of an apoptotic phenotype.

To investigate which pathways are involved in the induction of apoptosis in the treated MDA-MB-231 cells by cCN, the expression of apoptosis-related genes was assessed by qPCR. cCN affected the mRNA expressions of *Bax, Bad*, *Bcl-2,* and *Bcl-xL* genes, which are associated with the apoptosis intrinsic signaling pathway as well as *FasL*, a gene related to the extrinsic pathway. These results suggest that cCN induced apoptosis in triple-negative MDA-MB-231 breast cancer cells via both intrinsic and extrinsic pathways. Despite the different types of *C. nutans*

extract used, the results from the current study are in accordance with previous findings. For instance, a study by Ng *et al.*[31] found that the hexane extracts of *C. nutans* leaves and stem induced apoptosis via both extrinsic and intrinsic pathways in liver cancer (HepG2), non-small cell lung cancer (A549) and nasopharyngeal cancer (CNE1). In another study, Wang *et al.*[32] discovered that the ethyl acetate fraction of *C. nutans* ethanolic leaf extract activated the extrinsic and intrinsic signaling pathways in HCT116 human colorectal cancer cells.

CONCLUSION

The cCN exhibited growth-inhibitory effect on the MDA-MB-231 triple-negative breast cancer cell line. The extract also induced apoptosis in the treated cells by triggering both intrinsic and extrinsic apoptotic pathways. This was determined by the increased mRNA expression of *Bad*, *Bax*, and *FADD* and decreased levels of *Bcl-2* and *Bcl-xL*. The results from this study suggest that *C. nutans* holds a therapeutic potential in the intervention of breast cancer.

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Conflicts of interest

There are no conflicts of interest.

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